

Brassinosteroids

Chemistry, Bioactivity, and Applications

Horace G. Cutler, EDITOR
U.S. Department of Agriculture

Takao Yokota, EDITOR
Teikyo University (Utsunomiya, Japan)

Günter Adam, EDITOR
Institute of Plant Biochemistry (Halle/Saale, Germany)

Developed from a symposium sponsored
by the Division of Agrochemicals
at the 200th National Meeting
of the American Chemical Society,
Washington, D.C.,
August 26–31, 1990,
and an international workshop sponsored
by the Federation of European Chemical Societies
at the Institute of Plant Biochemistry,
Halle/Saale, Germany,
October 29–November 2, 1990





Library of Congress Cataloging-in-Publication Data

Brassinosteroids: chemistry, bioactivity, and applications / Horace G. Cutler, editor, Takao Yokota, editor, Günter Adam, editor.

p. cm.—(ACS symposium series; 474)

“Developed from a symposium sponsored by the Division of Agrochemicals at the 200th National Meeting of the American Chemical Society, Washington, D.C., August 26–31, 1990, and an international workshop sponsored by the Federation of European Chemical Societies at the Institute for Plant Biochemistry, Halle/Saale, Germany, October 29–November 2, 1990.”

Includes bibliographical references and index.


ISBN 0–8412–2126–X

1. Brassinosteroids—Congresses.

I. Cutler, Horace G., 1932– . II. Yokota, Takao, 1942– . III. Adam, Günter. IV. American Chemical Society. Division of Agrochemicals. V. Federation of European Chemical Societies. VI. Series.

QK898.B85B73 1991
581.19'27—dc20

91–31405
CIP

The paper used in this publication meets the minimum requirements of American National Standard for Information Sciences—Permanence of Paper for Printed Library Materials, ANSI Z39.48–1984. 

Copyright © 1991

American Chemical Society

All Rights Reserved. The appearance of the code at the bottom of the first page of each chapter in this volume indicates the copyright owner's consent that reprographic copies of the chapter may be made for personal or internal use or for the personal or internal use of specific clients. This consent is given on the condition, however, that the copier pay the stated per-copy fee through the Copyright Clearance Center, Inc., 27 Congress Street, Salem, MA 01970, for copying beyond that permitted by Sections 107 or 108 of the U.S. Copyright Law. This consent does not extend to copying or transmission by any means—graphic or electronic—for any other purpose, such as for general distribution, for advertising or promotional purposes, for creating a new collective work, for resale, or for information storage and retrieval systems. The copying fee for each chapter is indicated in the code at the bottom of the first page of the chapter.

The citation of trade names and/or names of manufacturers in this publication is not to be construed as an endorsement or as approval by ACS of the commercial products or services referenced herein; nor should the mere reference herein to any drawing, specification, chemical process, or other data be regarded as a license or as a conveyance of any right or permission to the holder, reader, or any other person or corporation, to manufacture, reproduce, use, or sell any patented invention or copyrighted work that may in any way be related thereto. Registered names, trademarks, etc., used in this publication, even without specific indication thereof, are not to be considered unprotected by law.

PRINTED IN THE UNITED STATES OF AMERICA

American Chemical Society
Library

1155 16th St., N.W.
Washington, D.C. 20036

ACS Symposium Series

M. Joan Comstock, *Series Editor*

1991 ACS Books Advisory Board

V. Dean Adams
Tennessee Technological
University

Paul S. Anderson
Merck Sharp & Dohme
Research Laboratories

Alexis T. Bell
University of California—Berkeley

Malcolm H. Chisholm
Indiana University

Natalie Foster
Lehigh University

Dennis W. Hess
Lehigh University

Mary A. Kaiser
E. I. du Pont de Nemours and
Company

Gretchen S. Kohl
Dow-Corning Corporation

Michael R. Ladisch
Purdue University

Bonnie Lawlor
Institute for Scientific Information

John L. Massingill
Dow Chemical Company

Robert McGorin
Kraft General Foods

Julius J. Menn
Plant Sciences Institute,
U.S. Department of Agriculture

Marshall Phillips
Office of Agricultural Biotechnology,
U.S. Department of Agriculture

Daniel M. Quinn
University of Iowa

A. Truman Schwartz
Macalaster College

Stephen A. Szabo
Conoco Inc.

Robert A. Weiss
University of Connecticut

Foreword

THE ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that, in order to save time, the papers are not typeset, but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the editors with the assistance of the Advisory Board and are selected to maintain the integrity of the symposia. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation. However, verbatim reproductions of previously published papers are not accepted.

Preface

BRASSINOSTEROIDS, a new group of biologically active natural products, are under consideration as another class of phytohormones. Their discovery and development mark a major milepost in the field of plant growth regulators, and have had a profound effect on plant physiology and agronomy. These natural products are also of interest to other disciplines, such as mycology, entomology, horticulture, and (perhaps in the future) even mammalian physiology. The potential economic effects these environmentally benign compounds may have on grain crop yields are immense.

The original discovery of brassinolide, a natural plant-growth promoter extracted from bee-collected pollen, was made at the USDA Agricultural Research Center in Beltsville, Maryland. Although the USDA terminated brassinosteroid research, others around the world have carried on the work. This volume addresses the history, biochemistry, physiology, practical applications, production, synthesis, and entomological effects of these compounds.

As the product of significant international effort, this book offers a compilation of the current work in the field. To date, it is the only comprehensive review of the subject. Without the generous support of the Division of Agrochemicals of the American Chemical Society and the substantial help of Maureen Rouhi, the book would have been impossible. We also thank BASF AG, Hoechst AG, Firmenich AG, Mandaus AG, Schering AG, Shell Agrar AG, and Stickstoffwerke AG Piesteritz which generously supported the Halle workshop. Without the effort of all these people, this volume would not have been possible.

HORACE G. CUTLER
Richard B. Russell Research Center
Agricultural Research Service
U.S. Department of Agriculture
Athens, GA 30613

GÜNTER ADAM
Institute of Plant Biochemistry
Weinberg 3
Halle/Saale, O-4050, Germany

TAKAO YOKOTA
Department of Biosciences
Teikyo University
Utsunomiya 320, Japan

July 22, 1991

Chapter 1

U.S. Department of Agriculture Brassins Project: 1970–1980

G. L. Steffens

Beltsville Agricultural Research Center, Agricultural Research Service, U.S.
Department of Agriculture, Beltsville, MD 20705

The discovery of a new naturally occurring group of plant growth promoting substances, termed brassins, from various sources of pollen was reported in 1970 (*Nature* 1970, 225:1065). In 1974 a cooperative effort for identifying the active component(s) of brassins was initiated among U.S.D.A. scientists at the Northern Regional Research Center (NRRC), Peoria, IL; the Eastern Regional Research Center (ERRC), Philadelphia, PA; and the Beltsville Agricultural Research Center (BARC), Beltsville, MD. To obtain enough crude pollen extract for identification, about 227 kg of bee-collected rape (*Brassica napus* L.) pollen was processed via a pilot plant-size solvent extraction procedure at ERRC and partially purified at BARC (*Ind. Eng. Chem. Prod. Res. Dev.* 1978, 17:351). A few crystals (4 mg) were obtained at NRRC and subjected to x-ray crystallographic analysis to determine structure (*Nature* 1979, 281:216). This biologically active plant growth promoter, brassinolide [$2\alpha, 3\alpha, 22\alpha, 23\alpha$ -tetrahydroxy-24 α -methyl- β -homo-7-oxa-5 α -cholestan-6-one], was found to be a steroidal lactone with an empirical formula of $C_{28}H_{48}O_6$ (MW = 480). The concentration of brassinolide in rape pollen is estimated to be about 100 parts per billion. Syntheses of brassinosteroids, biologically active analogues of brassinolide, were first conducted at BARC (*J. Organic Chem.* 1979, 44:5002).

The structure of the unique biologically active plant growth promoter, brassinolide, from pollen of the rape plant (*Brassica napus* L.) was published in 1979 (1). That

publication was the culmination of more than 10 years work, at first by a few USDA scientists at Beltsville, MD and later by a multi-disciplinary team at three USDA locations: The Northern Regional Research Center (NRRC), Peoria, IL; The Eastern Regional Research Center (ERRC), Philadelphia, PA; and The Beltsville Agricultural Research Center (BARC), Beltsville, MD. This Chapter presents an historical perspective of the research effort that led to the isolation and identification of brassinolide and to the first synthesis of brassinosteroids, the group of compounds that are structural isomers of brassinolide (2). General aspects of the project, along with details of the chemistry of natural and synthetic brassinosteroids have recently been published (3). Additional information on biological and chemical properties of brassinolides will be presented in the Chapters that follow.

Prior to 1970 - BARC, Beltsville, MD

Early in his career, J.W. Mitchell (ret.) was interested in the possibility of finding plant hormones and growth substances in extracts of pollen (4) and immature seeds (5). The rationale for this was the fact that such plant tissues have the capacity for rapid growth which most likely is stimulated or triggered by specific hormones or growth substances. In order to determine if plant growth inhibiting or promoting substances reside in these or other plant tissues, active components needed to be extracted, at least partially separated from impurities, and then subjected to some type of biological assay system. Mitchell sought out and developed plant bioassay systems for the purpose of evaluating and documenting specific responses to known and newly discovered plant hormones and growth substances (6).

1970 to 1974 - BARC

Mitchell's search for biological activity in pollen led in 1970 to the first published report on the biologically active fraction, termed brassins, from an ethyl ether extract of pollen of the rape plant (*Brassica napus* L.) (7). The partially purified biologically active extract from rape pollen will be referred to as "brassins" in this Chapter. The extract was partially purified via a thin layer silica gel chromatographic procedure. Biologically active fractions were detected and monitored via the "bean 2nd internode bioassay" (6). Fractions from the chromatogram were mixed with a carrier (fractionated lanolin) and applied, with the aid of a dissecting microscope, to 1 mm long 2nd internodes of bean plants (*Phaseolus vulgaris* L., cv. Pinto). Plants were grown for four days under 700 foot-candles of fluorescent light (9 hr. photoperiod) in a room maintained at 20°-23°C and then the internodes were measured. The initial report indicated

that"brassins induced very marked elongation of both second and third internodes of the intact plants when applied at the rate of 10 μg per plant. For example, second internodes grew an average of 155 mm during four days immediately after treatment, while controls treated with the fractionated lanolin alone grew only 12 mm." (7). Histological studies showed that the response to brassins was different from the response induced by gibberellic acid. Reported chemical and physical properties of brassins suggested that it was a "family" of new plant hormones that appeared to have glyceride structures.

Further studies of pollen extracts of a number of different plant types for brassins-like activity showed that some induced the same type activity whereas others did not, and some were inactive (8, 9). Because rape pollen was a rich source of brassin-like activity, and because it could be relatively easily obtained, it was chosen for further detailed chemical and biological studies.

Along with the bioassay studies, an active program to investigate the physiological properties of brassins was being pursued. Worley and Mitchell (10) showed that brassins accelerated growth of the treated internodes, as well as internodes above and below the treated area, in beans. These responses involved both cell elongation and cell division which caused marked changes in the vascular anatomy of leaves above the site of brassins application. Studies by Worley and Krizek showed that woody plants also responded to brassins treatment much the same as bean plants (11) and that light influenced the way bean internodes responded to brassins (12). More controversial was the report by Mitchell and Gregory (13) that brassins increased the overall growth of plants. These findings were the basis for postulating that brassins may function by providing hormonal control at fertilization for accelerating and enhancing overall plant growth and development - the "alpha hormone" function. Criticism (14) was also directed at the apparent lack of detailed chemical and physical information on the active component(s) of brassins.

Although there was no formal response to the published criticism, much effort was devoted to answering some of the questions posed and to evaluating the effects of brassins on intact plants under both greenhouse and field conditions. Unpublished data [Mitchell, J.W., USDA, (ret.) personal communication, 1973] obtained from several cooperative experiments in different parts of the country suggested that field-grown cereal and vegetable crops responded to brassins treatment by producing larger seed yields and/or larger plants. Many detailed greenhouse and growth chamber studies were also conducted at Beltsville based on the concept that brassins stimulated plants to express, more fully, their growth potential. This then allowed brassins-treated plants to attain full development sooner, provided the environment was suitable (15).

Because of the need for larger amounts of brassins, one phase of the chemistry program during this period centered on a convenient, practical, and reproducible method for their production from dried rape pollen. The preparative thin- and thick-layer chromatographic method reported by Mitchell et al. (7) was quite time-consuming. Mandava et al. (16) successfully developed a more rapid procedure for the production of brassins using column chromatography. The procedure was based on three steps - 1. the extraction of dried rape pollen with ethyl ether to yield a crude extract, 2. the chromatographic clean-up of the crude extract on a silica gel column with benzene-methanol-acetic acid (45:8:4,v:v:v) to yield a biologically active extract, and 3. the further purification of the active extract via preparative silica gel thin layer chromatography with diisopropyl ether-acetic acid (95:5,v:v) to yield brassins. The other phase of the chemistry program continued to be directed towards determining the chemical identity of the active component(s) of brassins. At this point Mandava and Mitchell (17) speculated that brassins were composed of fatty acids linked by an ester bond to glucose at carbon 1 in the β -form.

Prior to his retirement from the USDA in 1974, Mitchell sought increased support to establish field trials to evaluate the effects of brassins for enhancing yields of several crop plants. Because the purification, isolation, and identification of the active component(s) were proving to be so very difficult, Mitchell also sought additional support for a concerted research effort in these areas. The ARS administration considered both aspects to be of importance and plans for a multi-disciplined, multi-location project were made.

1974 to 1980 - NRRC, Peoria, IL; ERRC, Philadelphia, PA; BARC, Beltsville, MD

In 1974 plans were submitted by the NRRC, ERRC, and BARC outlining the extent and type of input that would be available from each location for extracting large amounts of brassins from rape pollen and for the chemical isolation and identification studies. The overall program was coordinated at Beltsville (G.L. Steffens with assistance from M. Jacobson for chemical aspects). The pilot plant-size extraction of rape pollen was conducted at ERRC (M.F. Kozempel); the large scale chromatographic clean-up of the crude extract for the production of active brassins was done at BARC (N. Mandava); and the isolation, purification and identification of the active component(s) of brassins were undertaken at NRRC (M.D. Grove) and BARC (N. Mandava). All bean 2nd internode assays required to follow biological activity during all stages of the project were conducted at BARC (J.F. Worley and D.W. Spaulding). Field studies in the U.S. and Brazil were also coordinated through BARC

(G.L. Steffens). A number of other scientists made major contribution to the program, as will be shown. It was a multi-phase project and each phase will be discussed separately although several phases were usually being conducted simultaneously.

Purification, Isolation, and Identification of Active Component(s) of Brassins. As indicated, the brassins complex was found to contain glucosyl esters of fatty acids (17) which were thought to be the active brassins constituents. Starting with this information, glucosyl esters from rape pollen were purified and esters of these types were synthesized (18, 19, 20). However, neither the esters purified from pollen nor those synthesized proved to have brassins-like biological activity in the bean 2nd internode test. It was therefore concluded that components other than glucosyl esters, most likely present in very small amounts, were responsible for the biological activity of brassins.

Because of the apparent low concentration of the active component(s) in the brassins complex, it was necessary to plan for the extraction of a large quantity of rape pollen. Honeybee-collected rape pollen was available from Canada since pollen is used in specialty-type natural food products, for example baked goods. Arrangements were made (not without some difficulty) to obtain a large lot (over 1/4 ton) of rape pollen from Canada for a pilot plant-scale extraction at ERRC. Earlier work by Mandava et al. (16) was the basis for the pilot plant extraction procedure that was developed and used (21). Contributors to the project, in addition to M. Kozempel and N. Mandava, were H. Kenney of ERRC; J.F. Worley, D. Matthees, J.D. Warthen, Jr., M. Jacobson and G.L. Steffens of BARC; and M.D. Grove, NRRC.

Pilot Plant Extraction - ERRC. An outline of the pilot plant extraction steps is shown in Figures 1 and 2. Batches of pollen (5 batches of 45.5 kg each, for a total of 227 kg pollen) were extracted first with deionized water in a 227 L kettle to remove simple sugars. The aqueous slurry was filtered via a Sparkler filter and the filter cake continuously washed with water until the filtrate was clear and colorless. The washed pollen (filter cake) was then freeze-dried, moved back to the extraction kettle and extracted with 114 L of 2-propanol. The slurry was pumped to a Sparkler filter, allowed to soak, and the 2-propanol drained off. The pollen cake was extracted in this manner 7 more times and then it was recycled to the extraction kettle where it was again extracted with 114 L of 2-propanol. After filtration, the pollen was washed twice with 2-propanol and then recycled to the extraction kettle for a final 2-propanol extraction (a total of eleven 2-propanol extractions). The 2-propanol was evaporated in a glass evaporator at <40°C to yield ca. 7.6 L of concentrate

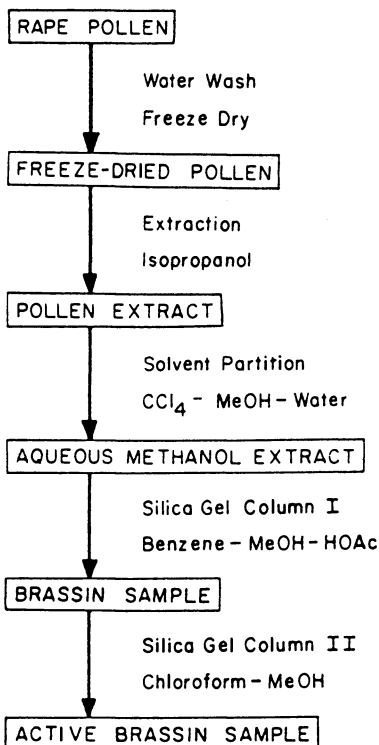


Figure 1. Flow diagram for the isolation of brassins from rape pollen. (Reproduced from reference 21. Copyright 1978 American Chemical Society.)

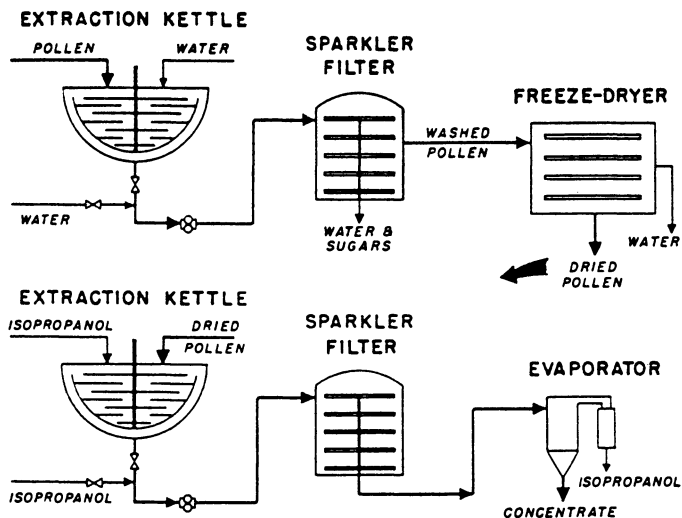


Figure 2. Diagram showing the pilot plant extraction process. (Reproduced from reference 21. Copyright 1978 American Chemical Society.)

per 45.5 kg pollen (38 L per 227 kg pollen). Suspended solids were removed using a large centrifuge and the liquid was further concentrated under vacuum which reduced the 38 L to 16 kg of a brown syrupy concentrate from the 227 kg of pollen.

Solvent Partition and Column Chromatography - BARC.

The 16 kg of brown syrupy concentrate were partitioned between methanol and carbon tetrachloride in about eighty batches. Usually 200 g of the concentrate were dissolved in 1000 ml of carbon tetrachloride and placed in a separatory funnel, 100 ml of water were added, the funnel thoroughly shaken and, on standing, a two phase system developed. A set of five separatory funnels, each containing 400 ml of carbon tetrachloride, 200 ml methanol, and 26 ml of water, was arranged in a series. The lower carbon tetrachloride phase from the original separatory funnel was transferred to the first funnel of the set of five. After shaking and allowing the layers to separate, the lower phase was transferred to the next funnel and this procedure was repeated through all 5 funnels (6 transfers). After the final transfer, the aqueous methanol layers from all the funnels were combined and the solvent evaporated. The 16 kg of brown syrupy concentrate from the 227 kg of pollen was reduced to ca. 7.5 kg of dark brown gum.

Chromatographic columns (85 X 9 cm) were packed with ca. 1500 g silica gel in benzene-MeOH-acetic acid (90:16:8,v:v:v) to a bed length of ca. 50 cm. 100 g portions of the biologically active dark brown gum were mixed with 200 g of silica gel in 2-propanol, and, after the 2-propanol was evaporated, the brown powder was applied to the prepared column. The column was then eluted with benzene-MeOH-acetic acid (90:16:8,v:v:v) and the eluate monitored via spectrophotometry. Active fractions that correlated with a maximum % transmittance at 400 nm appeared after the first 3 L of solvent eluted. Thereafter, 1 L fractions were collected and those showing activity were combined. After solvent evaporation, about 450 g of active material from 181 kg of pollen were obtained (ca. 564 g active material per 227 kg pollen, equivalent).

A second column chromatography clean-up step similar to the first was then conducted on the active material from the first column. Sixty g batches of the active residue were applied and the column successively eluted with increasing concentrations of methanol in chloroform. Bioassay monitoring showed that essentially all the activity was eluted from the column when the methanol concentration reached 10-20%. The second chromatography step reduced the 450 g of active material to ca. 200 g of active brassins per 181 kg pollen (ca. 252 g active brassins per 227 kg pollen, equivalent).

Active Component Identification - NRRC and BARC. Active brassins from the second silica gel column was distributed to chemists at all three USDA locations where additional purification steps and various procedures for the identification of the biologically active brassins component(s). Data on these highly purified samples were obtained by various spectrometric methods including electron impact, chemical ionization, and field desorption mass spectrometry. Grove at NRRC subjected the highly active brassins sample to successive chromatographic separation on silica gel (chloroform-methanol, 9:1,v:v; toluene-absolute ethanol, 9:1,v:v), on C₁₈ Hi-flosil (methanol-water, 6:4,v:v), and by HPLC on μ -Bondapak C₁₈ (methanol-water, 65:35,v:v). Fractionation was guided by bioassay results. As the active brassins component became concentrated, responses in the bean 2nd internode assay progressed from internode elongation to swelling and curvature, to internode splitting (Figure 3). Using these procedures Grove obtained ca. 4 mg of crystals from methanol, which represented ca. 40 kg of pollen (23 mg crystals per 227 kg pollen equivalent or about 100 parts per billion). The structure of the active component (Figure 4), termed brassinolide, was completely established by single crystal X-ray procedure.

Information obtained from the various sources showed that the active brassinolide molecule [$2\alpha,3\alpha,22(R),23(R)$ -tetrahydroxy- $24(S)$ -methyl-B-homo-7-oxa- 5α -cholestan-6-one or $2\alpha,3\alpha,22\alpha,23\alpha$ -tetrahydroxy- 24α -methyl- β -homo-7-oxa- 5α -cholestan-6-one] is a polyhydroxylated steroidal lactone with an empirical formula of C₂₈H₄₈O₆ and a molecular weight of 480. The presence of a lactone moiety in the B ring of a naturally occurring plant steroid was relatively unusual. Additional details on the chemical and physical properties can be found in the 1979 paper by Grove et al. (1). Contributors to this paper, in addition to M.D. Grove, were, G.F. Spencer and W.K. Rohwedder of NRRC; N. Mandava, J.F. Worley, J.D. Warthen and G.L. Steffens of BARC; J.L. Flippen-Anderson, Crystallographer, The Naval Research Laboratory, Washington, DC.; and J.C. Cook, Jr., University of Illinois, Urbana, who provided important spectral information. It should be noted that D.W. Spaulding had conducted about 9000 bean 2nd internode assays at BARC by the time brassinolide was identified.

Synthesis of Brassinolide Analogues - BARC. After the structure of brassinolide was established, it only took a short time for brassinolide analogues to be synthesized by Thompson et. al. (2). Two highly physiologically active brassinosteroids, structural isomers of brassinolide, were synthesized from ergosterol. The synthesis of compounds with brassins activity and the comparative X-ray analyses again confirmed the structure of the steroidal nucleus of brassinolide. The synthesis of these brassinosteroids also provided a method for the preparation of large enough

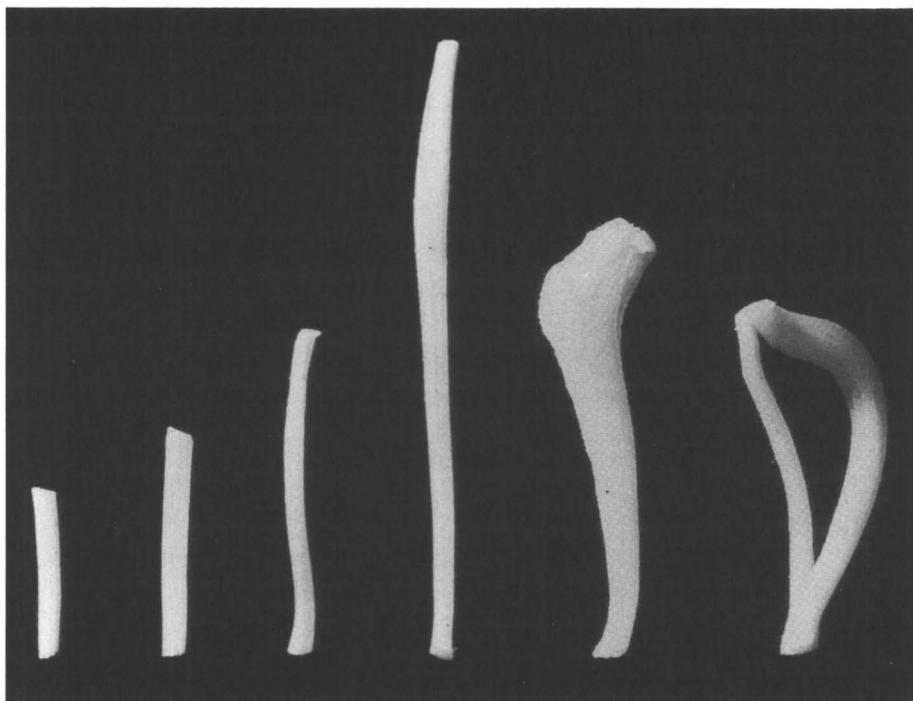


Figure 3. Responses of bean internode segments in the "bean 2nd internode assay" to increases in concentration (left to right) of the active component of brassins nearing final purification. (Reproduced with permission from reference 22. Copyright 1981 Butterworth-Heinemann.)

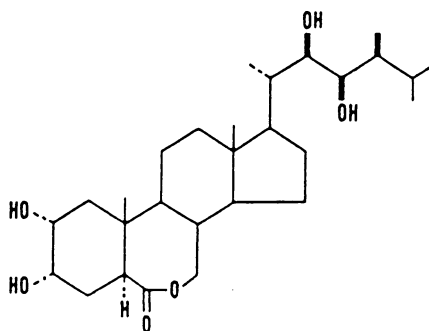


Figure 4. Chemical structure of brassinolide.

quantities of active brassinosteroids for greenhouse and field evaluations. Additionally, brassinolide itself and its 22 β ,23 β -isomer were synthesized from a C-24 epimeric 60:40 mixture of 22-dehydrocampesterol (24 α -methyl) and brassicasterol (24 β -methyl) obtained from oysters (22). Synthesis of brassinolide via C₂₂ steroids had been reported earlier by two laboratories (23,24).

1974 and 1975 Field Evaluations of Brassins. Starting in 1974, brassins were prepared from rape pollen as outlined by Mandava et al. (16) for use in field experiments conducted in the U.S. and Brazil. Earlier work suggested that treatment of seeds with brassins prior to planting resulted in increases in plant size and/or seed yield under greenhouse (13) and field conditions [Mitchell, J.W., USDA (ret.) personal communication, 1973; see also (15)]. The earlier report (13) suggested that brassins may stimulate the growth of smaller, slow growing plants to a greater degree than those growing more vigorously. Therefore, an objective of these field experiments was to determine if plants from brassins-treated small seed grew to the same size and produced as much seed as plants grown from large seed. Thus, prior to planting, seeds were separated into average, small and large sizes. In order to obtain field data as rapidly as possible, cooperative field experiments were established in Brazil during the U.S. winter of 1974-75 using soybeans as the test crop. Additional experiments were established in the U.S. during the 1975 growing season using soybeans and barley as test crops. These field experiments were coordinated at BARC (G.L. Steffens) with statistical design and data evaluation directed by E.J. Koch, BARC.

1974-75 Soybean Field Experiment - Brazil. Cooperative experiments were established in Nov. and Dec. 1974 by H.C. Minor, Soybean Ecologist, EMBRAPA/USAID/WISCONSIN, near Porto Alegre, Brazil (30°S latitude). They consisted of two plantings, each with 15 treatments replicated 6 times - 3 seed sizes (average, small and large) and each seed size treated with five levels of brassins (untreated, solvent only, 150 ppm, 300 ppm and 450 ppm). Soybean seeds were divided by size at BARC, treated by soaking for 1 minute in dichloromethane solutions containing the brassins, dried, and shipped to Brazil. In brief, no significant differences were found, or the differences that were found followed no pattern related to brassins treatment for most of the 22 variables evaluated, which included seed yield and plant size (Steffens, G.L., USDA, unpublished data).

1975 Soybean Field Experiments - Seven U.S. Locations. Field experiments at seven locations in the U. S. were established to determine if increased seed yields could be obtained by treating soybean with brassins prior to

planting. Seeds of eight cultivars, representing eight soybean maturity groups, were again divided into average, small and large sizes at BARC and each was treated by soaking for 1 minute in dichloromethane solutions containing brassins at 0, 200 or 400 ppm (9 treatments X 6 replicates). Two cultivars were planted at each of the seven locations (14 plantings). When average data over locations were considered, brassins application dosage had no statistically significant effect on seed yield or weight per 100 seed. The application of 400 ppm brassins to seed prior to planting significantly increased protein of seed from the resulting plants (about 0.5 %) compared to that of seed from untreated plants or plants treated with 200 ppm brassins. However, the differences found were considered minimal and of no major economic importance (Steffens, G.L., USDA, unpublished data). The experiments were conducted at Brookings, SD (C.D. Dybing and A.D. Lunden); Urbana, IL (R. Johnson, and R.L. Cooper); Beltsville, MD (R.C. Leffel and G.L. Steffens); Portageville, MO (G. Shannon and V.D. Luedders); Raleigh, NC (C.A. Brim); Stoneville, MS (E.E. Hartwig) and Gainesville, FL (K. Hinson).

1975 Barley Field Experiments - Five U.S. Locations. The barley experiments were similar to the U.S. soybean field experiments. Barley seeds of 4 cultivars were divided into 3 seed sizes at BARC (average, small and large) and treated by soaking in dichloromethane solutions containing brassins at 0, 200 or 400 ppm. Two cultivars were grown at each of 5 locations (10 plantings of 9 treatments X 6 replicates). When values were averaged over all locations no significant differences due to brassins treatment were observed for seed yield or seed protein content. Examination of data from the ten individual plantings showed that significant differences in barley yield due to brassins application were observed in two of the ten. As with the soybean experiments, the differences found due to brassins treatment were relatively small and not considered to be of economic importance (Steffens, G.L., USDA, unpublished data). The locations were Aberdeen and Tetonia, ID (D.M. Wesenberg and H.C. McKay); Bozeman and Moccasin, MT (E.A. Hockett and G.D. Kushnak); and Brookings, SD (P.B. Price).

Summary of 1974-1975 Field Experiments. From these rather large and detailed soybean and barley field studies, it was apparent that biological activity of brassins was not transferred into economic yield increases via the technique used to treat the seed in these experiments.

Intact Plant Studies with Brassins - BARC. Because yields were not increased in the 1974-75 field studies by the brassins treatment procedure used, application techniques

other than seed treatment were investigated. Greenhouse and preliminary field experiments at BARC using various application techniques showed that brassins caused growth responses in many instances. Responses were most pronounced when preparations were applied as sprays to very young seedlings in greenhouse experiments. Significant increases in the yield, for example, of radishes, some leafy vegetables, and potatoes were also obtained in preliminary field experiments when applications of brassins were made to young seedlings or, in the case of potatoes, to "eyes" prior to planting [Meudt, W.J. USDA, (ret.) and Gregory, L.E., USDA, (ret.), unpublished data].

After synthetic brassinosteroids became available in 1979, field studies were initiated with active analogues (25, 26, and see also 27). In a 1979 study, aqueous solutions of 0.01 ppm brassinosteroid were applied as sprays to seedlings of lettuce (two varieties), radishes, peppers, tomatoes, beans, and corn grown in small field plots. Plants were treated as the seedlings broke ground and spray applications were repeated three more times at 2-day intervals. At maturity, the brassinosteroid treatments significantly increased ($P < 0.01$) the average size of lettuce heads by 25-32% (mean of three crops). The yield of radishes was increased by about 20% (significant at $P < 0.05$). Increases of 9% in the fruit weight of peppers and of 6% in the weight of beans per plant were obtained (not significant at the 5% level). Significant differences were obtained, however, when the data were analyzed as a frequency distribution of size of harvestable fruits. Fruit lots from treated plants contained a higher percentage of larger-sized fruits than lots from untreated plants. Fruit size variations within treatments were apparently too great to allow significance when the data were subjected to evaluation by analyses of variance procedures. No differences were observed for yields of tomatoes and corn. The 1979 field results obtained with the synthetic brassinosteroid analogue were similar to those obtained in 1977 with brassins extracted from rape pollen [Meudt, W.J. USDA, (ret.), unpublished data].

Bioassay Studies of Brassins - BARC. As indicated by Mandava (3), bioassay systems have been rather extensively used to study brassins physiology. In addition to the bean 2nd internode bioassay, the bean first internode curvature bioassay (28) and the mung bean epicotyl bioassay (29) were found to be useful by Meudt and Gregory. In many cases, details of early brassins studies using these and other assay systems were not published because the chemical nature of the active component(s) of brassins was unknown at the time. However, types of responses obtained with the synthetic brassinosteroids were essentially the same as those obtained with brassins (29, 30, 31, 32, 33).

Bioassay information obtained with synthetic

brassinosteroids also began to be obtained at BARC shortly after they were synthesized and some of these findings were summarized by Meudt (27). Early studies with brassins (34), as well as later studies with synthetic brassinosteroids, showed that, in the presence of auxin, their application enhanced stem segment-growth of etiolated beans (*Phaseolus vulgaris* L. cv. Bush Burpee Stringless). However, when applied alone to tissues low in auxin, as in this bioassay (28), they caused very little cell enlargement. It was found that brassinosteroids could increase the sensitivity of the bean tissues to auxin 10- to 50-fold and therefore were considered to be auxin synergists. Using the bean first internode curvature bioassay he developed, Meudt observed that brassins stimulated gravity-induced curvature of the bean sections. He also found that younger tissues were more responsive than older tissues and that brassins stimulated ethylene production in the bean plumular hook closing bioassay. The brassins response was, however, different from the hook closing response caused by ethylene [Meudt, W.J., USDA, (ret.) personal communication, 1990].

Using the mung bean bioassay, Gregory et al. (35) found that brassins enhanced epicotyl growth of cuttings and the response was even more marked with crystalline brassinolide - at 2×10^{-7} M brassinolide enhanced epicotyl growth ten fold. It caused epinasty of the epicotyl at 2×10^{-8} M and higher, but caused no hypocotyl elongation. Additional information can be found in references (3) and (29).

J.H. Yopp, Southern Illinois University, Carbondale, became interested in the brassins project in its early stages (36) and he conducted cooperative brassins studies with both Mitchell and Mandava. Yopp et al. (37, 38) evaluated brassins responses in a number of auxin, gibberellin, and cytokinin bioassays and showed that brassins responded similarly to some of the known hormones in certain systems but not in others. These studies led to more cooperative investigation of brassinolide and other brassinosteroids (39, 40).

Interest in the brassinosteroids grew as a result of these bioassay studies and much cooperative work has been conducted with scientists in various parts of the world using brassinosteroid samples synthesized by M.J. Thompson, USDA, (ret.) (3, 31).

Epilogue

During the decade since publication in 1979 of the structure of brassinolide (1), and the synthesis of the first brassinosteroid (2), also in 1979, events related to the "brassins project" have taken some interesting turns. At this point (October 1990), there seems to be little interest in developing brassinosteroids for agricultural

usage in the U. S. Later chapters in this book will show that interest in the chemistry and physiology of brassinosteroids, as well as in their potential agricultural uses, has been especially strong in Japan. There continues to be some interest in the physiology of the brassinosteroids in the U.S. but there seems to be considerably more interest in Japan, in several European countries, and in Australia and China.

As to the USDA scientists who had a major involvement in the joint project, the careers of some individuals were advanced because of having been involved in the project whereas in other cases, scientific careers were simply interrupted without much reward. Several of the key scientists died shortly after the program was complete (J.F. Worley, BARC and M.D. Grove, NRRC; and also H. Kenney, ERRC). A number of individuals have retired or left the USDA (J.W. Mitchell, L.E. Gregory, W.J. Meudt, M.J. Thompson and M. Jacobson of BARC retired and N.B. Mandava took a position outside USDA). None of the remaining scientists are in any way now connected with brassinosteroid research. The information obtained with the funds and scientific effort expended by the U. S. Department of Agriculture on the "brassins project" is, for the most part, being further expanded by scientists in other countries of the world.

The use of a company or product name does not constitute an endorsement by USDA nor imply approval to the exclusion of other suitable products.

Literature Cited

1. Grove, M.D.; Spencer, G.F.; Rohwedder, W.K.; Mandava, N.; Worley, J.F.; Warthen, J.D.; Steffens, G.L.; Flippen-Anderson, J.L.; Cook, J.C., Jr. *Nature* 1979, 281:216-217.
2. Thompson, M.J.; Mandava, N.; Flippen-Anderson, J.L.; Worley, J.F.; Dutky, S.R.; Robbins, W.E., Lusby; W. *J. Org. Chem.* 1979, 44:5002-5004.
3. Mandava, N.B. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 1988, 39:23-52.
4. Mitchell, J.W.; Whitehead, M.R. *Bot. Gaz.* 1941, 102:770-791.
5. Mitchell, J.W.; Shaggs, D.P.; Anderson, W.P. *Science* 1951, 114:159-161.
6. Mitchell, J.W.; Livingston, G.A. *Methods of Studying Plant Hormones and Growth Regulating Substances*. U.S. Dept. Agric., Agric. Handb. No. 336. Washington, DC, GPO, 1968, p. 26-28.
7. Mitchell, J.W.; Mandava, N.; Worley, J.F.; Plimmer, J.R.; Smith, M.V. *Nature* 1970, 225:1065-1066.
8. Mitchell, J.W.; Mandava, N.; Worley, J.F.; Drowne, M.E. *J. Agri. Food Chem.* 1971, 19:391-393.

9. Mandava, N.; Mitchell, J.W. *Indian Agric.* 1971, 15:19-31.
10. Worley, J.F.; Mitchell, J.W. *J. Am. Soc. Hort. Sci.* 1971, 96:270-273.
11. Worley, J.F.; Krizek, D.T. *HortScience* 1972, 7:480-481.
12. Krizek, D.T.; Worley, J.F. *Bot. Gaz.* 1973, 134:147-150.
13. Mitchell, J.W.; Gregory, L.E. *Nature-New Biol.* 1972, 239:253-254.
14. Milborrow, B.V.; Pryce, R.J. *Nature* 1973, 243:46.
15. Gregory, L.E. *Am. J. Bot.* 1981, 68:586-588.
16. Mandava, N.; Sidwell, B.A.; Mitchell, J.W.; Worley, J.F. *Ind. Eng. Chem. Prod. Res. Dev.* 1973, 12:138-139.
17. Mandava, N.; Mitchell, J.W. *Chem. Ind.* 1972, 930-931.
18. Pfeffer, P.E.; Moore, G.G.; Hoagland, P.D.; Rothman, E.S. In *Synthetic Methods for Carbohydrates*; El Khadem, H.S., Ed.; ACS Series 39. Am. Chem. Soc., Washington, DC, 1977, pp. 155-178.
19. Grove, M.D.; Spencer, G.F.; Pfeffer, P.E.; Mandava, N.; Warthen, J.D., Jr.; Worley, J.F. *Phytochem.* 1978, 17:1187-1189.
20. Mandava, N.; Chandra, G.R. In *Symposium on the Pharmacological Effects of Lipids*; Chapt. 13, AOAC Monograph No. 5, 1978, pp. 133-144.
21. Mandava, N.; Kozempel, M.; Worley, J.F.; Matthees, D.; Warthen, J.D.; Jr.; Jacobson, M.; Steffens, G.L.; Kenney, H.; Grove, M.D. *Ind. Eng. Chem. Prod. Res. Dev.* 1978, 17:351-354.
22. Thompson, M.J.; Mandava, N.B.; Meudt, W. J.; Lusby, W.R.; Spaulding, D.W. *Steroids* 1981, 38:567-580.
23. Fung, S.; Siddal, J.B. *J. Am. Chem. Soc.* 1980, 102:6581.
24. Ishiguro, M.; Takatsuto, S.; Morisaki, M.; Ikekawa, N. *J. Chem. Soc. Chem. Comm.* 1980, 962.
25. Meudt, W.J.; Thompson, M.J.; Bennett, H.W. *Proc. Plant Growth Reg. Soc. Am.* 1983, 10:312-318.
26. Meudt, W.J.; Thompson, M.J., Mandava, N.B.; Worley, J.F. *Method of Promoting Plant Growth*; Can. Patent No. 1173659, 1984. Assigned to U.S.A., 11 pp.
27. Meudt, W.J. In *Ecology and Metabolism of Plant Lipids*; Fuller, G., Nes, W.D., Eds.; ACS Series 325. Am. Chem. Soc., Washington, DC, 1987, pp.53-75.
28. Meudt, W.J.; Bennett, H.W. *Physiol. Plant.* 1978, 44:422-428.
29. Gregory, L.E.; Mandava, N.B. *Physiol. Plant.* 1982, 54:239-243.
30. Meudt, W.J.; Worley, J.F., Gregory, L.E.; Mandava, N.; Buta, J.G.; Steffens, G.L. *10th Int. Conf. on Plant Growth Substances*, Univ. Wisc., Madison. 1979, Abs. 502, p 25.
31. Thompson, M.J.; Meudt, W.J.; Mandava, N.B.; Dutky, S.R.; Lusby, W.R.; Spaulding, D.W. *Steroids* 1982, 39:89-105.
32. Cohen, J.D.; Meudt, W.J. *Plant Physiol.* 1983, 72:691-694.

33. Buta, J.G.; Meudt, W.J. *10th Int. Conf. on Plant Growth Substances*, Univ. Wisc., Madison. 1979, Abs. 551, p. 49.
34. Steffens, G.L.; Buta, J.G.; Gregory, L.E.; Mandava, N.B.; Meudt, W.J.; Worley, J.F. In *Advances in Pesticide Science*, Geissbuhler, H., Ed.; IUPAC, Zurich; Pergamon Press, Oxford. 1978, Part 2; pp 343-346.
35. Gregory, L.E.; Mandava, N.B.; Cina, D.K. *10th Int. Conf. on Plant Growth Substances*, Univ. Wisc., Madison. 1979, Abs. 552. p. 49.
36. Yopp, J.H.; Holst, R.W. *Proc. So. Agric. Workers Conf.* 1973, 17:217.
37. Yopp, J.H.; Ladd, D.; Jaques, D.; Mandava, N. *10th Int. Conf. on Plant Growth Substances*, Univ. Wisc., Madison. 1979, Abs. 504, p. 25.
38. Yopp, J.H.; Mandava, N.B.; Thompson, M.J.; Sasse, J.M. *Proc. Plant Growth Reg. Soc. Am.* 1981, 8:138-145.
39. Yopp, J.H.; Mandava, N.B.; Sasse, J.M. *Physiol. Plant.* 1981, 53:445-452.
40. Mandava, N.B.; Sasse, J.M.; Yopp, J.H. *Physiol. Plant.* 1981, 53:453-461.

RECEIVED July 22, 1991

Chapter 2

Brassinosteroids in Leaves of *Distylium racemosum* Sieb. et Zucc.

The Beginning of Brassinosteroid Research in Japan

Hiroshi Abe¹ and Shingo Marumo²

¹Department of Plant Protection, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan

²Department of Agricultural Chemistry, Nagoya University, Chikusa, Nagoya 464, Japan

The first preliminary report of brassinosteroids in Japan was released in 1968 under the title "The Presence of Novel Plant Growth Regulators in Leaves of *Distylium racemosum* Sieb. et Zucc." two years before the first report on the development of brassins, the crude brassinolide, by Mitchell *et al.* in *Nature*(1). From 430 kg of leaves 751 μ g of *Distylium* factor A₁, 50 μ g of A₂ and 236 μ g of B were isolated and all showed much higher activity than indole-3-acetic acid [IAA] on a rice-lamina inclination bioassay. However, the very minute amounts precluded any chemical studies at that time. Just after the structure of brassinolide was announced by the USDA group in 1979, (22*S*, 23*S*)-24-homobrassinolide and brassinolide were synthesized by Japanese chemists, and their biological activity was examined by the rice lamina inclination (or joint) test. Both compounds showed activity levels ten thousand times stronger than the activity of IAA, demonstrating that this bioassay was exclusively specific for brassinosteroids and was not contaminated by other plant hormones. *Distylium* factors were reinvestigated, and A₁ was identified as a mixture of brassinone and castasterone and B as a mixture of brassinolide and 28-norbrassinolide.

Distylium racemosum Sieb. et Zucc., known as "Isunoki" in Japanese, is an evergreen tree belonging to the family *Hamamelidaceae*. It is well known that several varieties of aphids form insect galls on the young, fresh leaves or buds of this tree. The aphid, *Neothoracaphis yanonis*, attacks the small, young leaves on the tree. The attacked leaf soon begins to swell and to form a quite small gall (gall diameter, 1-2mm) in Spring. In May, the insect gall on the leaf rapidly grows and enlarges (gall diameter

about 1 cm). *Distylium* factor research was began in an effort to identify the substance which induces the abnormal growth, swelling and expansion observed during insect gall formation. Prof. Munakata of Nagoya University found an active substance in the neutral fraction of methanol extracts of the insect galls when bioassayed by means of a rice-lamina inclination (or joint) test [LJT], which had been established as an auxin bioassay in 1965 by Prof. Maeda of Nagoya University(2).

In 1966, we carried out further investigations for the purification, isolation and elucidation of the chemical properties of the active substance.

Distylium Factors: Novel Plant Growth Regulators in Fresh Leaves of *Distylium racemosum* Sieb. et Zucc.

A preliminary experiment revealed the presence of the active substance not only in the insect galls on the leaves but also in fresh healthy leaves of *Distylium racemosum*. Fresh healthy leaves (430 kg) of the plant, therefore, were collected in the suburbs of Anjo city at the end of May, 1966. Methanol extracts of the material were separated by conventional techniques to give an ether-soluble neutral substance having fairly strong activity in the LJT. The neutral substance was chromatographed on a silicic acid column (*n*-hexane-EtOAc-EtOH), from which *Distylium* factor A fraction, including A₁ and A₂, was eluted with 80% EtOAc-*n*-hexane, and then *Distylium* factor B with 100% EtOAc. Each fraction was furthermore purified repeatedly by Sephadex LH-20 gel filtration column chromatography(EtOH), Silica gel TLC (Kieselgel H, EtOAc), and alumina column chromatography (EtOAc-EtOH) affording three kinds of active substances. All three were detected on a thin-layer chromatogram as a purple spot under ultraviolet light upon heating with 0.5% vanillin-H₂SO₄.

The amount of *Distylium* factors A₁, A₂, and B, named after their botanical source, was 751, 50 and 236 μ g, respectively. The *R_F* values of *Distylium* factors on silica gel TLC developed with EtOAc were: A₁, 0.35; A₂, 0.55; B, 0.13. The values of PPC developed with isoPrOH/NH₄OH/H₂O (8:1:1, v/v) were: A₁, A₂, B, 0.9-1.0. These factors had clearly different *R_F* values from the known phytohormones, were negative for Ehrlich and Salkowsky reagents, and were detectable as a purple spot under ultraviolet light after spraying with H₂SO₄ containing 0.5% vanillin, suggesting that they are non-indolyl compounds. The amounts isolated were too small to technically perform their structural determination by means of spectroscopic analysis at that time, so it was decided to halt the studies at this point.

Biological activities of *Distylium* factors were investigated using several known bioassays for plant hormones. *Distylium* factors B and A₁ were found to exhibit much higher activity than IAA in the LJT; that is, the same inclinations of lamina joint sections were observed when the factors were applied at concentrations of one hundredth and one thirtieth, respectively. These factors also displayed stronger elongation than IAA in the

Avena first-internode test, the activity of B was about 10 times higher, whereas they showed lower activity in the *Avena* coleoptile section test at a concentration of 0.01 ppm, and were negative for the *Avena* curvature test at a concentration of 5 ppm. The letter is a classical bioassay for auxin detection. The cytokinin activity of B was also tested and recognized in the radish leaf expansion test and tobacco pith callus test. The activity of B was nearly equal to that of kinetin at a concentration of 0.1 ppm. GA activity was also examined, but was not detected in either the rice seedling or the dwarf maize seedling elongation tests. As described above, the results of tests clearly indicated that *Distylium* factors were new types of plant growth regulators which combined some of the attributes of auxin activity with those of cytokinin activity and which had a broad spectrum of plant physiological activity.

The universality of distribution of *Distylium* factors A and B in plants was tested using methanol extracts of fourteen plant species. Plants surveyed were *Disanthus cercidifolia* (leaves), *Hamamelis japonica* (leaves), *Corylopsis glabrescens* (leaves), *Pittosporum tobira* (leaves), *Camellia sasanqua* (leaves), *Thea sinensis* (leaves), *Cocculus trilobus* (leaves), *Viburnum awabuki* (leaves), *Phaseolus vulgaris* (shoots), *Pisum sativum* (seeds), *Vicia faba* (seeds), *Dolichos lablab* (seeds), *Glycine max* (seeds) and *Arachis hypogaea* (seeds). After partition of the neutral substances, separated from methanol extracts, between CH_3CN and *n*-hexane, the CH_3CN -soluble portions were charged onto an Al_2O_3 column. *Distylium* factor A was eluted with 20% EtOH in EtOAc and then *Distylium* factor B with 40% EtOH in EtOAc. Both fractions were applied to the LJT. *Distylium* factors A and B were confirmed to be present not only in *Distylium racemosum*, but were found to be distributed widely across a variety of higher plants surveyed.

As mentioned above, the research into *Distylium* factors has provided the following significant information.

Distylium factors:

- (1) are detectable as a purple spot under UV light after spraying 0.5% vanillin-sulfuric acid and then heating;
- (2) shows several hundred times higher activity than IAA in LJT;
- (3) possesses a broad spectrum of biological activities compared with the known phytohormones; and
- (4) are distributed widely in the plant kingdom.

We reported these results in *Agric. Biol. Chem.* in 1968 (3).

Purification and Isolation of *Corn* Factor from Corn Germ Oil

In relation to *Distylium* factors research in *Distylium racemosum* leaves, Prof. Munakata has further studied the provisionally named " *Corn* factor " from corn germ oil(4). He has examined many plant oils in an effort to obtain a better source of novel plant growth regulators, including coconut oil, corn germ oil, cotton seed oil, linseed oil, olive oil, peanut oil, poppy oil, rape oil, sesame oil, soya oil, and turpentine oil, and found that corn germ oil showed a most remarkable activity in the LJT. Based

on the purification procedure for *Distylium* factor, an active substance named *Corn* factor was extracted and separated from the neutral fraction of the oil and finally isolated in the form of needle crystals with a yield of 2 mg. The activity of *Corn* factor was about 2500 times higher than that of IAA in the LJT, but was similar to IAA in the *Avena* first internode test. Further studies on the chemical structure of *Corn* factor could not be conducted because of insufficient yield.

The Rice Lamina Inclination as a Specific Bioassay for Brassinosteroids

The isolation and structural determination of brassinolide [BR] was reported in Nature by Dr. N. B. Mandava and co-workers at the USDA in 1979(5), and was subsequently announced in *Chem. & Eng. News*(6). The steroidal skeleton of the chemical structure of BR prompted us to examine the effect on the LJT. (22*S*,23*S*)-homobrassinolide was synthesized by Mori in 1980(7) at our request just after the structural determination of BR. BR itself was also produced by Ikekawa and his co-workers the same year(8). Wada, a co-worker in our group, tested the activity of BR and homobrassinolide on rice lamina inclination and found that both compounds dramatically induced the inclination of laminae (see Table I) as expected(9). These findings strongly suggested

Table I. Effect of brassinolide, homobrassinolide^a and IAA on the lamina inclination of rice seedlings

Angle degrees between laminae and sheaths (\pm standard error)			
Conc (μ g/ml)	IAA	Brassinolide	Homobrassinolide
0(control)	91 \pm 6.6		
50	124 \pm 8.6		
10	90 \pm 1.7		
5	101 \pm 12.4		
1	85 \pm 9.0		
0.5		161 \pm 9.1	175 \pm 8.3
0.1		169 \pm 11.6	159 \pm 9.7
0.05		158 \pm 8.5	159 \pm 9.8
0.01		156 \pm 9.9	155 \pm 12.9
0.005		163 \pm 7.2	151 \pm 11.0
0.001		150 \pm 16.2	114 \pm 12.7
0.0005		143 \pm 11.1	123 \pm 6.6
0.0001		116 \pm 9.8	112 \pm 17.1

^a This compound is a stereoisomeric mixture of 2 α ,3 α ,22*R*,23*R*- and 2 α ,3 α ,22*S*,23*S*-tetrahydroxy-24*S*-ethyl-B-homo-7-oxa-5 α -cholestan-6-one.

that *Distylium* factors might be brassinosteroids, and we thus began to reinvestigate those plant growth promoting steroids distributed in various plant sources using LJT. At the same time, many Japanese chemists started to search for naturally occurring brassinosteroids in plants.

After brassinosteroids were identified in Chinese cabbage shoots(10,11), followed by green tea leaves(12,13), we tried to identify *Distylium* factors A and B in the fresh healthy leaves of *Distylium racemosum*, which were again collected in the suburbs of Anjo city in 1982. (See Figure 1.)

Identification of *Distylium* Factors A₁ and B as Brassinosteroids

The ethyl acetate-soluble neutral fraction partitioned from fresh Isunoki leaves (16 kg) was purified by successive chromatographic separations on silica gel (*n*-hexane-EtOAc-EtOH), Amberlite XAD-2 (H₂O-EtOH), and on silica gel for preparative HPLC (1 x 100cm, CH₂Cl₂-EtOH) to give an active fraction. The active fraction was further purified by repeated HPLC on Fuji ODS-Q3 (2 x 25cm, CH₃CN:H₂O=60:40) and on Develosil ODS-5 (4.6 x 250mm, CH₃CN:H₂O=50:50) to afford the two active substances. The polar fraction appeared to contain a substance chromatographically similar to brassinosteroid-lactone itself, while the less polar fraction contained a brassinosteroid-ketone-like substance. Final identification of these active substances was carried out by GC-MS analysis after conversion to the bismethaneboronate derivative, which was established by Dr. Ikekawa and his co-workers as a microanalytical method for brassinosteroids(14). Four brassinosteroids were identified from the Isunoki leaves, namely brassinolide and norbrassinolide from the polar fraction corresponding to *Distylium* factor B, and their biosynthetic precursors, castasterone and brassinone from the less polar fraction corresponding to *Distylium* factor A₁. *Distylium* factor A₂ has yet to be identified, but is probably a 2-deoxy-type brassinosteroid, which may be the biosynthetic precursor of brassinosteroid-ketone, castasterone and/or brassinone. Thus, the *Distylium* factors were found to be brassinosteroids(15).

The amount of endogenous BS obtained by the GC/MS method was estimated to be approximately 156ng of norbrassinolide, 23ng of brassinolide, 16ng of brassinone, 133ng of castasterone per one kilogram fresh weight leaves. However, this quantification data is considered to be an underestimate due to losses encountered during sample purification, but it still remains quite a small quantity.

A Modified Method of the Rice Lamina Joint Test for Brassinosteroids

The LJT bioassay was developed for an auxin bioassay by Dr. Maeda of Nagoya University in 1965(2). As mentioned above, the LJT is extremely sensitive and specific to BS. We established a

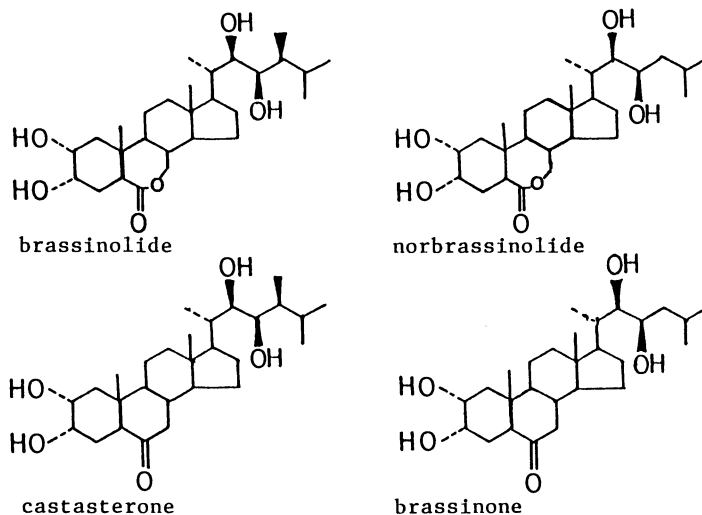


Figure 1. Brassinosteroids identified from *Distylium racemosum* leaves.

micro-quantitative analytical method for BS bioassay and examined the sensitivity of various rice cultivars to determine suitable experimental conditions. We selected the Italian cultivar Arborio as one of the most suitable for this brassinosteroid bioassay system(16).

After soaking rice seeds in a 0.25% aqueous solution of Benlate (fungicide) for 48hr, the seeds were cultivated at 28-29 °C in darkness on a plastic net floating on distilled water in a plastic tray. Lamina joint sections were excised from etiolated 6-7-day-seedlings, which consisted of the second leaf lamina (0.5-1cm long), the second lamina joint, and the second leaf sheath (0.5-1cm long). These sections were floated on distilled water for 24hr. Sections, uniformly bent to an angle of about 30 degrees between the lamina and the sheath were then selected and seven to ten of the sections were incubated in one ml of 2.5 mM aqueous potassium maleate buffer solution containing the test sample. After incubation for 48hr under the same conditions, the magnitude of the angle induced between the lamina and the sheath was measured. Every operation was carried out under red light wavelengths, except for the final measurement step of the angle. This modified procedure is now the standard method for BR detection in Japan.

Conclusion

The beginning of brassinosteroid research in Japan was initiated with *Distylium* factor research. *Distylium* factors were discovered in *Distylium racemosum* leaves, when bioassayed, using

LJT and were found to be brassinosteroids as expected. The amount of four brassinosteroids was estimated to be lower than about 150ng in one kilogram of fresh leaves of *Distylium racemosum*. The small amounts obtained make it easy to see that the quantity of crude *Distylium* factors purified in 1968 had been too small to allow elucidation of the chemical structure. The LJT for purification of *Distylium* factor and *Corn* factor was shown to be the most suitable bioassay method for identifying naturally-occurring brassinosteroid. It is well-known that successively new analogues of brassinolide were isolated and identified from many kinds of plants using this bioassay. Brassinolide is the first steroid compound with plant growth-promoting activity to be discovered in plants. The unique chemical structure and plant-physiological activity of brassinolide and its analogues has attracted the special interests of organic chemists and plant physiologists.

Literature Cited

1. Mitchell, J. W.; Mandava, N. ; Worley, J. F.; and Plimmer, J. R. *Nature* 1970, 225, 1065.
2. Maeda, E. *Physiol. Plant* 1965, 18, 813.
3. Marumo, S.; Hattori, H.; Abe, H.; Nonoyama, Y. and Munakata, K. *Agric. Biol. Chem.* 1968, 32, 528.
4. Munakata, K.; Kato, N. and Ikeda, M. "*Plant Growth Substances 1973*," Hirokawa Publishing Co. Inc., Tokyo, 1974, pp.39-43.
5. Grove, M. D.; Spencer, W. K.; Rohwedder, W. K.; Mandava, N.; Worley, J. F.; Warthen, J. D.; Steffens, G. L.; Flippen-Anderson, J. L. and Cook, J. C. Jr. *Nature* 1979, 281, 218.
6. *Chem. & Eng News* 1979, No. 5, 20.
7. Mori, K. *Agric. Biol. Chem.* 1980, 44, 1211.
8. Ishiguro, M.; Takatsuto, S.; Morisaki, M. and Ikekawa, N. *J. Chem. Soc. Chem. Commun.* 1980, 962.
9. Wada, K.; Marumo, S.; Ikekawa, N.; Morisaki, M. and Mori, K. *Plant Cell Physiol.* 1981, 22, 323.
10. Abe, H.; Morishita, T.; Uchiyama, M.; Marumo, S.; Munakata, K.; Takatsuto, S. and Ikekawa, N. *Agric. Biol. Chem.* 1982, 46, 2609.
11. Abe, H.; Morishita, T.; Uchiyama, M.; Takatsuto, S. and Ikekawa, N. *Agric. Biol. Chem.* 1984, 48, 2171.
12. Morishita, T.; Abe, H.; Uchiyama, M.; Marumo, S.; Takatsuto, S. and Ikekawa, N. *Phytochemistry* 1983, 22, 1051.
13. Abe, H.; Morishita, T.; Uchiyama, M.; Takatsuto, S.; Ikekawa, N.; Ikeda, M.; Sassa, T.; Kitsuwa, T. and Marumo, S. *Experientia* 1983, 39, 351.
14. Takatsuto, S.; Ying, B.; Morisaki, M. and Ikekawa, N. *J. Chromatogr.* 1982, 239, 233.
15. Ikekawa, N.; Takatsuto, S.; Kitsuwa, T.; Saito, H.; Morishita, T. and Abe, H. *J. Chromatogr.* 1984, 290, 289.
16. Wada, K.; Marumo, S.; Abe, H.; Morishita, T.; Nakamura, K.; Uchiyama, M. and Mori, K. *Agric. Biol. Chem.* 1984, 48, 719.

RECEIVED May 8, 1991

Chapter 3

Natural Occurrences of Brassinosteroids

Seong-Ki Kim

Biotechnology Laboratory, Korea Research Institute of Chemical
Technology, P.O. Box 9, Daedeog-Danji, Taejon 305-606,
Republic of Korea

Brassinosteroids are steroidal plant hormones which promote plant growth. To date the presence of over sixty kinds of brassinosteroids has been verified from various plant sources and thirty one of them fully characterized. In this chapter, the distribution and structural characteristics of the naturally-occurring brassinosteroids are discussed.

Distribution of Brassinosteroids in the Plant Kingdom

In 1970, Mitchell and colleagues reported that a lipoidal extract obtained from rape (*Brassica napus* L.) pollen elicited strong elongation activity in the bean second-internode bioassay (1). The activity was distinguished from other known plant hormones in that the lipoidal extract promoted not only cell elongation, but also cell division. The activity was termed 'brassins' activity and extracts showing the same type of activity were also obtained from pollens of other plants (see ref. 2). Thus they proposed that new types of lipoidal plant hormone coined the 'brassins' were contained in pollen.

From 40 kg of rape pollen, 4 mg of an active compound was eventually isolated and shown to be the novel plant growth substance brassinolide (1) (3). The structure, as determined by X-ray crystallography, was [(2 α , 3 α , 22R, 23R)-tetrahydroxy-24 α -methyl-B-homo-7-oxa-5 α -cholestane-6-one]. This structure was unique in possessing a 24 α -methyl, a 7-oxalactonic B ring, and vicinal hydroxyls on the A ring (C2 α and C3 α) plus a side chain (C22R and C23R).

Brassinolide and its analogs were immediately synthesized chemically (4-8) and evaluated in several bioassays. Wada *et al.* reported (9) that brassinolide and its synthetic analog, homobrassinolide, was very active at 0.0005 μ g and 0.005 μ g/ml in the rice lamina inclination assay, which was originally developed for detecting indole-3-acetic acid (10). Later, Yokota *et al.* isolated 95 μ g of the second naturally-occurring brassinosteroid, castasterone (3) from 40 kg insect galls of chestnut (*Castanea crenata*) using the rice lamina inclination assay for monitoring the

purification steps (11). $^1\text{H-NMR}$ revealed that castasterone contained a 6-ketonic B ring instead of a 7-oxalactonic B ring in brassinolide. Castasterone was, therefore, considered to be a biosynthetic precursor of brassinolide.

Generally, immature seeds have been demonstrated to be one of the richest sources of plant hormones. Thus, Yokota *et al.* examined brassinosteroids in immature seeds of *Dolichos lablab* (12-15). As a result, two C_{28} brassinosteroids with a 24-exomethylene [dolicholide (2) and dolichosterone (4)]; two C_{29} brassinosteroids with a 24(*E*)-ethylidene [homodolicholide (10) and homodolichosterone (11)]; and two 6-deoxodihydro brassinosteroids [6-deoxodihydrocastasterone (5) and 6-deoxodihydrodolichosterone (6)] were unequivocally identified and later the presence of brassinolide and castasterone were determined. The content of the major brassinosteroid, dolicholide, was 200 $\mu\text{g}/\text{kg}$, which paralleled the concentration of brassinolide in rape pollens.

Microanalytical techniques, consisting of methaneboronation of the vicinal hydroxyls (bismethaneboronate or methaneboronate-TMS-ether) have been developed by Takatsuto *et al.* for the GC/MS or GC/SIM of brassinosteroids (16). Consequently brassinone (15), 24-ethylbrassinone (12) and 28-norbrassinolide (14) were detected by GC/MS without isolation from Chinese cabbage (*Brassica campestris*), green tea (*Thea sinensis*) and chestnut insect galls (*Distilium racemosum*) (17-19). The presence of brassinolide and castasterone in the tissues of these plants has been also detected.

A combination of the rice lamina inclination assay and GC/MS or GC/SIM analyses have allowed for identification of the brassinosteroids in various plant tissues: These include brassinolide, 6-deoxodihydrocastasterone, brassinone from the insect galls of *Castanea crenata* (18,20); castasterone and 6-deoxodihydrocastasterone from the stems, leaves and flowers of *Castanea crenata* (20); brassinolide, castasterone, typhasterol (7), and teasterone (8) from leaves of *Thea sinensis* (21-23); castasterone and brassinone from the fruit of *Pharbitis purpurea* (Japanese morning glory) (24); brassinolide, castasterone and 24-epibrassinolide(29) from immature seed and/or pollen of *Vicia faba* (broad bean) (25, 26); brassinolide and castasterone from the pollen of *Alnus glutinosa* (European alder) (27).

Among the plant sources investigated, immature seeds of *Phaseolus vulgaris* have been vigorously examined and it was found that immature seeds of *P. vulgaris* contained brassinolide, castasterone, dolicholide, dolichosterone, 6-deoxodihydrocastasterone, 6-deoxodihydrodolichosterone and 6-deoxodihydrohomodolichosterone (13) (28, 29). In addition, a number of unidentified brassinosteroids have been also detected by HPLC and GC/MS analysis (29). From large scale experiments with seeds (136 kg), over sixty kinds of brassinosteroids have been found (30, 31). Among them thirteen free brassinosteroids, 2-epicastasterone (16), 3-epicastasterone (17), 2,3-diepicasterone (18), 3,24-diepicasterone (19), 1 β -hydroxycastasterone (21), 3-epi-1 α -hydroxycastasterone (22), 3-epi-6-deoxodihydrocastasterone (20), 25-methyldolichosterone (23), 2-epi-25-methyldolichosterone (24), 2,3-diepi-25-methyldolichosterone (25), 2-deoxy-25-methyldolichosterone (26), 3-epi-2-deoxy-25-methyldolichosterone (27) and 6-deoxodihydro-25-methyldolichosterone (28), and two brassinosteroid conjugates, 23-O- β -D-glucopyranosyl-25-methyldolicho-

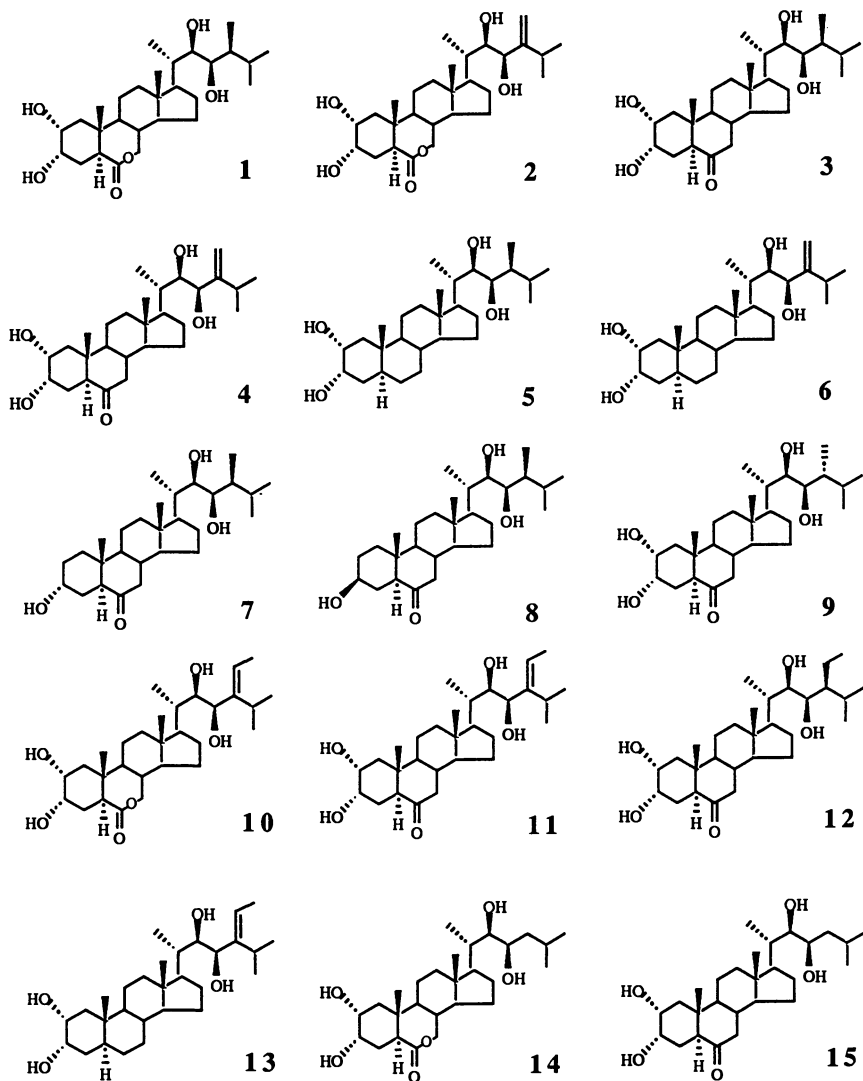


Figure 1. Structures of free brassinosteroids from plants. 1; brassinolide, 2; dolicholide, 3; castasterone, 4; dolichoesterone, 5; 6-deoxodihydrocastasterone, 6; 6-deoxodihydrodolichoesterone, 7; typhasterol, 8; teasterone, 9; 24-epicastasterone, 10; homodolicholide 11; homodolichoesterone, 12; 24-ethylbrassinone, 13; 6-deoxodihydrohomodolichoesterone, 14; 28-norbrassinolide, 15; brassinone, 16; 2-epiacasterone, 17; 3-epicastasterone, 18; 2,3-diepicasterone, 19; 3,24-diepicasterone, 20; 3-epi-6-deoxodihydrocastasterone, 21; 1β -hydroxycastasterone, 22; 3-epi-1- α -hydroxycastasterone, 23; 25-methyldolichoesterone, 24; 2-epi-25-methyldolichoesterone, 25; 2,3-diepi-25-methyldolichoesterone, 26; 2-deoxy-25-methyldolichoesterone, 27; 3-epi-2-deoxy-25-methyldolichoesterone, 28; 6-deoxodihydro-25-methyldolichoesterone, 29; 24-epibrassinolide.

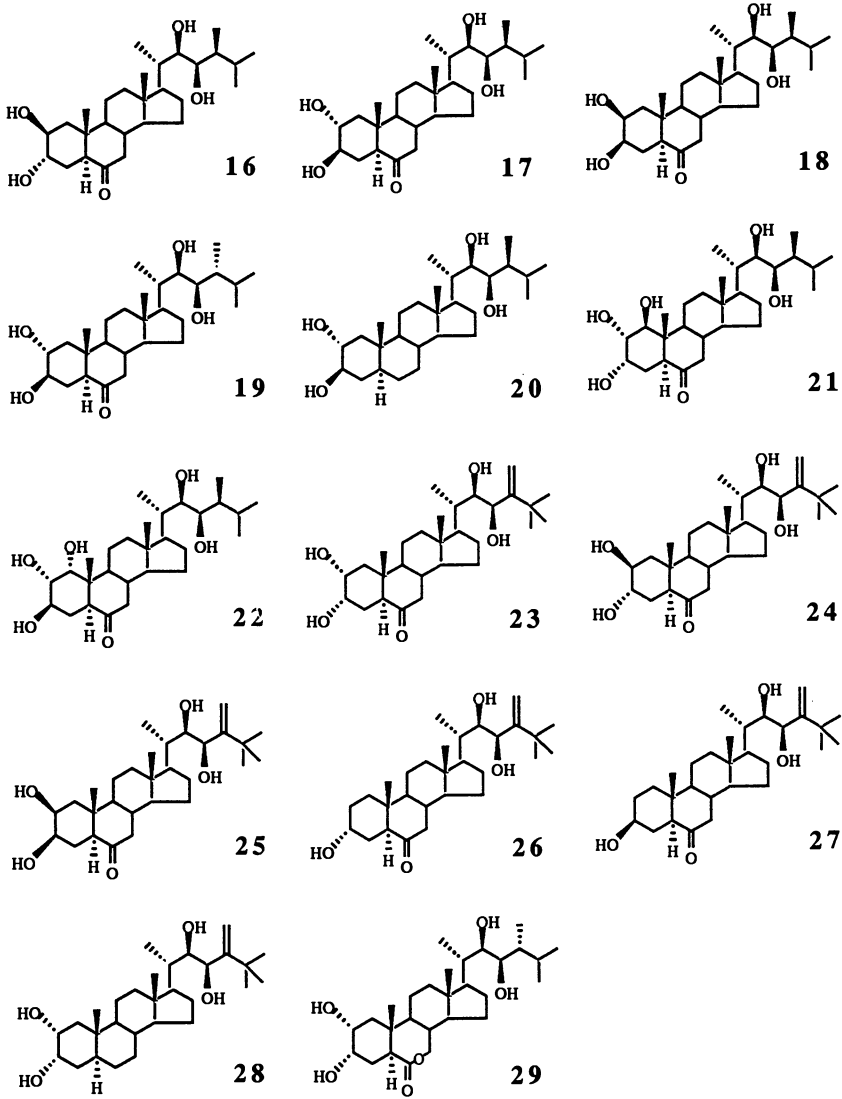


Figure 1. Continued.

sterone (30) and 23-O- β -D-glucopyranosyl-2-epi-25-methyl-dolichosterone (31) were isolated in pure state (30-34). Their structures have been characterized by 400 MHz $^1\text{H-NMR}$. Besides these new brassinosteroids, typhasterol and teasterone were also found. Much information concerning the structural characteristics of other as yet uncharacterized brassinosteroids has also been accumulated using GC/MS analyses.

The presence of brassinosteroids in monocots has also been demonstrated. Typhasterol (2-deoxycasterone) from cattail (*Typha latifolia*) pollen (35), castasterone and dolichosterone from rice shoots (*Oryza sativa*) (36) and castasterone, typhasterol and teasterone from corn (*Zea mays*) pollen (37) were identified.

The occurrence of brassinosteroids in gymnosperms has been reported from conifers. Yokota *et al.* isolated typhasterol (2-deoxycasterone) from pollen of Japanese black pine (*Pinus thunbergii*) (38) and identified castasterone and typhasterol from shoots of sitka spruce (*Picea sitchensis*) (39). Kim *et al.* also identified castasterone and brassinolide from cambial scrapings of Scots pine (*Pinus silverstris*), using a modified dwarf rice lamina inclination assay which showed a synergistic response of brassinosteroids with indole-3-acetic acid (40).

The brassinosteroids, 24-epicastasterone (9) and 24-ethylbrassinone have been identified in lower plant such as the green alga, *Hydrodictyon reticulatum* (41). Brassinosteroids from the green algae would be interesting for analysing the biosynthetic relationship between brassinosteroids and major sterols related to C24 alkylation.

In summary, twenty nine free brassinosteroids and two brassinosteroid conjugates have been fully characterized from thirteen angiosperms including 10 dicots and three monocots, three gymnosperms, and a green alga. These findings, combined with Mitchell and Mandava's preliminary bioassay data for 'brassin' activity (2), suggest an ubiquitous distribution of brassinosteroids throughout the plant kingdom.

Although plant roots have not yet been investigated, it is likely that all plant parts contain brassinosteroids. It would appear that reproductive tissues, such as pollen and immature seed, have a relatively higher content ($\times 2-3$) of brassinosteroids than vegetative tissues, suggesting that brassinosteroids may be connected to the physiology of reproductive growth. In this respect, it is worth noting that insect galls of *Castanea crenata* and *Distilium racemosum* contain higher amounts of brassinosteroids than normal tissues, suggesting a possible role for the brassinosteroids in the abnormal growth of plant tissues.

Among all naturally-occurring brassinosteroids, brassinolide and castasterone are considered to be the most important brassinosteroids because of their wide distribution as well as their potent biological activity.

The Structural Characteristics of Naturally-occurring Brassinosteroids

All the naturally-occurring brassinosteroids are known to be derivatives of 5 α -cholestane. Diverse structural variations thus come from the kinds and orientation of functionalities on the skeleton.

With respect to B ring oxidation stage, brassinosteroids are grouped into 7-oxalactonic (1, 2, 10, 14, 29), 6-ketonic (3, 4, 7-9, 11, 12, 15-19, 21-27) and non-oxidized brassinosteroids (5, 6, 13, 20, 28). In general, 7-oxalactonic brassinosteroids have greater activity than do the 6-ketonic and non-oxidized congeners. The 6-ketonic brassinosteroids are active (sometimes as active as 7-oxalactonic brassinosteroids), but non-oxidized brassinosteroids reveal almost no activity in the bean internode assay and very weak activity in the rice lamina inclination assay (29,42). It has been considered that the 6-ketonic brassinosteroids can be converted to lactonic brassinosteroids by Baeyer-Villiger-type oxidation, as seen in the chemical synthesis of lactonic brassinosteroids. Recently Yokota *et al.* demonstrated that ³H-labelled castasterone (3, 6-ketonic brassinosteroid) was converted to ³H-labelled brassinolide (1, 7-oxalactonic brassinosteroid) in crown gall cultures (43). Non-oxidized brassinosteroids have also been considered to be precursors of 6-ketonic brassinosteroids. However, because of the very low biological activity of non-oxidized brassinosteroids, these do not appear to be converted into biologically active 6-ketonic congeners (42).

Brassinosteroids are grouped into C₂₇, C₂₈, and C₂₉ steroids like the typical phytosterols. These classifications result basically from different alkyl substitutions in the side chain, that is no substituent in C₂₇ steroids (14, 15), a methyl (1, 3, 5, 7-9, 16-22, 29) or an exomethylene (2, 4, 6) at C24 in C₂₈ steroids, and an ethyl at C24 (12) or an ethylidene at C24 (10, 11, 13) or an exomethylene at C24 with a methyl at C25 (23-28) in C₂₉ steroids. The structure-activity relationship reveals that alkylation in the side chain is necessary for biological activity. More especially, the presence of a saturated alkyl (a methyl or an ethyl) at C24 and a methyl at C25 makes brassinosteroids biologically more active. Thus, 25-methylbrassinolide bearing both 24-methyl and 25-methyl functions has been chemically synthesized and it is more potent than brassinolide which, until now, had been considered to be the most potent brassinosteroid (44).

Most of the brassinosteroids carry an *S*-oriented alkyl (a methyl or an ethyl) at C24. Three exceptions are 24-epicastasterone (9), 3, 24-diepicasterone (19) and 24-epibrassinolide (29).

Brassinosteroids are polyhydroxysteroids. Because all brassinosteroids carry vicinal hydroxyls at C22_R and C23_R, variations in the A ring structure may be found. Twenty three brassinosteroids carry other vicinal hydroxyls at C2 and C3 (1-6, 9-20, 23-25, 28, 29). These steroids can be thus classified as tetrahydroxysteroids. Concerning the orientation of the vicinal hydroxyls, only the 2 α , 3 α were discovered in those brassinosteroids characterized earlier. However, brassinosteroids carrying 2 β -3 α - [2-epicastasterone (16), 2-epi-25-methyldolichosterone (24)], 2 α , 3 β - [3-epicastasterone (17), 3-epi-6-deoxodihydrocastasterone (20) and 3,24-diepicasterone (19)] and 2 β , 3 β - [2,3-diepi-castasterone (18) and 2,3-diepi-25-methyldolichosterone (25)] vicinal hydroxyls have also been identified in the immature seed of *P. vulgaris*. With respect to orientation of the vicinal hydroxyls at C2 and C3, all kinds of combination have occurred. Decreasing order of activity 2 α , 3 α > 2 α ,3 β > 2 β ,3 α > 2 β ,3 β shown by structure-activity relationship suggests that the α -oriented hydroxyl at C2 is essential for strong biological activity (30).

Four trihydroxysteroids carry an isolated hydroxyl at C3. Typhasterol and 2-deoxy-25-methyldolichosterone carry a 3α -hydroxyl, but teasterone and 3-epi-25-methyldolichosterone carry a 3β -hydroxyl instead. These trihydroxysteroids are considered to be precursors of the tetrahydroxybrassinosteroids. The two remaining known steroids carry the fifth hydroxyl at C1 α (3-epi-1 α -hydroxycastasterone) and C1 β (1 β -hydroxycastasterone). These pentahydroxysteroids are probably catabolites of the tetrahydroxysteroids because the introduction of the fifth hydroxyl has no effect in bioassays (30).

There are two conjugates that are glycopyranosyl ethers of 25-methyldolichosterone and its 2-epimer (Fig. 2). It is interesting that glucosylation only occurs with the hydroxyl at C23. The biological activity of these conjugates most likely depends on the activity of their aglycones (34).

Structural characteristics of unknown brassinosteroids in immature seed of *P. vulgaris* have been elucidated by GC/MS analyses. They are either stereoisomers of known brassinosteroids, or brassinosteroids that have been oxidized during metabolism [23-keto-brassinosteroids (34), brassinosteroids bearing either carbonyl (32) or hydroxyl (33)], or brassinosteroids bearing an extra carbon atom [CO (35) or COO (36)] on the A ring (Fig. 3). However, their complete structures remain to be characterized (30).

It seems probable that when all the brassinosteroids have been elucidated, and their specific biological roles assigned, a complex picture will emerge much as it has for the gibberellins. However, each of them may be useful for select agrochemical use.

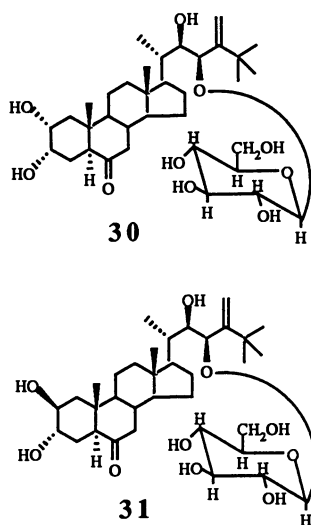


Figure 2. Structures of brassinosteroid conjugates from *P. vulgaris* seed.

30; 23-O- β -D-glucopyranosyl-25-methyldolichosterone, 31; 23-O- β -D-glucopyranosyl-2-epi-25-methyldolichosterone.

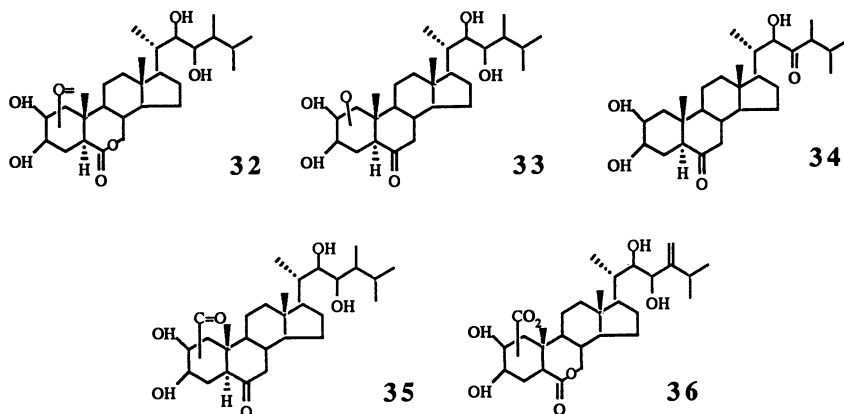


Figure 3. Possible structures of representative unknown brassinosteroids in *P. vulgaris* seed.

Literature Cited

1. Mitchell, J. W.; Mandava, N.; Worley, J. F.; Plimmer, J. R. Smith, M. V. *Nature* **1970**, *225*, 1065.
2. Mandava, N. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **1988**, *39*, 23.
3. Grove, M. D.; Spencer, G. F.; Rohwedder, W. K.; Mandava, N.; Worley, J. F.; Warthen, J. D. Jr.; Steffens, G. L.; Flippen-Anderson, J. L.; Cook, J. C. Jr. *Nature* **1979**, *281*, 216.
4. Ishiguro, M.; Takatsuto, S.; Morisaki, M.; Ikekawa, N. *J. Chem. Soc., Chem. Commun.* **1982**, *239*, 233.
5. Fung, S.; Siddall, J. B. *J. Amer. Chem. Soc.* **1980**, *102*, 6580.
6. Thompson, M. J.; Mandava, N.; Flippen-Anderson, J. L.; Worley, J. F.; Dutky, S. R.; Robbins, W. E.; Lusby, W. *J. Org. Chem.* **1979**, *44*, 5002.
7. Mori, K. *Agric. Biol. Chem.* **1980**, *44*, 1211.
8. Takatsuto, S.; Ying, B.; Morisaki, M.; Ikekawa, N. *Chem. Pharm. Bull.* **1981**, *29*, 903.
9. Wada, K.; Marumo, S.; Ikekawa, N.; Morisaki, M.; Mori, K. *Plant and Cell Physiol.* **1981**, *22*, 323.
10. Maeda, E. *Physiol. Plant.* **1965**, *18*, 813.
11. Yokota, T.; Arima, M.; Takahashi, N. *Tetrahedron Lett.* **1982**, *23*, 1275.

12. Yokota, T.; Baba, J.; Takahashi, N. *Tetrahedron Lett.* **1982**, *23*, 4965.
13. Baba, J.; Yokota, T.; Takahashi, N. *Agric. Biol. Chem.* **1983**, *47*, 659.
14. Yokota, T.; Baba, J.; Takahashi, N. *Agric. Biol. Chem.* **1983**, *47*, 1409.
15. Yokota, T.; Baba, J.; Koba, S.; Takahashi, N. *Agric. Biol. Chem.* **1984**, *48*, 2529.
16. Takatsuto, S.; Ying, B.; Morisaki, M.; Ikekawa, N. *J. Chromatogr.* **1982**, *239*, 233.
17. Abe, H.; Morishita, M.; Uchiyama, M.; Marumo, S.; Munakata, K.; Takatsuto, S.; Ikekawa, N. *Agric. Biol. Chem.* **1982**, *46*, 2609.
18. Ikeda, M.; Takatsuto, S.; Sassa, T.; Ikekawa, N. Nukina, M. *Agric. Biol. Chem.* **1983**, *47*, 655.
19. Abe, H.; Morishita, T.; Uchiyama, M.; Takatsuto, S.; Ikekawa, N.; Ikeda, M.; Sassa, T.; Kitsuwa, T.; Marumo, S. *Experientia* **1983**, *39*, 351.
20. Arima, M.; Yokota, T.; Takahashi, N. *Phytochemistry* **1984**, *23*, 1587.
21. Morishita, T.; Abe, H.; Uchiyama, M.; Marumo, S.; Takatsuto, S.; Ikekawa, N. *Phytochemistry* **1983**, *22*, 1051.
22. Abe, H.; Morishita, T.; Uchiyama, M.; Takatsuto, S.; Ikekawa, N. *Agric. Biol. Chem.* **1984**, *48*, 2171.
23. Ikekawa, N.; Takatsuto, S.; Kitsuwa, T.; Saito, H.; Morishita, T.; Abe, H. *J. Chromatogr.* **1984**, *290*, 289.
24. Suzuki, Y.; Yamaguchi, I.; Takahashi, N. *Agric. Biol. Chem.* **1985**, *49*, 49.
25. Park, K.-H.; Yokota, T.; Sakurai, A.; Takahashi, N. *Agric. Biol. Chem.* **1987**, *51*, 3081.
26. Ikekawa, N.; Nishiyama, F.; Fujimoto, Y. *Chem. Pharm. Bull.* **1988**, *36*, 405.
27. Plattner, R. D.; Taylor, S. L.; Grove, M. D. *J. Nat. Prod.* **1986**, *49*, 540.
28. Yokota, T.; Morita, M.; Takahashi, N. *Agric. Biol. Chem.* **1983**, *47*, 2149.
29. Yokota, T.; Koba, S.; Kim, S.-K.; Takatsuto, S.; Ikekawa, N.; Sakakibara, M.; Okada, K.; Mori, K.; Takahashi, N. *Agric. Biol. Chem.* **1987**, *51*, 1625.
30. Kim, S.-K. In *Study on Bio-organic Chemistry of Naturally-occurring Brassinosteroids* (in Japanese) Ph. D. thesis, The Univ. of Tokyo, Tokyo, Japan **1988**.
31. Yokota, T.; Kim, S.-K.; Takahashi, N. In *13th Int. Conf. on Plant Growth Substances*. Abstr. No 168, Calgary, Canada **1988**
32. Kim, S.-K.; Yokota, T.; Takahashi, N. *Agric. Biol. Chem.* **1987**, *51*, 2303.
33. Yokota, T.; Kim, S.-K.; Kosaka, Y.; Ogino, Y.; Takahashi, N. In *Conjugated Plant Hormone, Structure, Metabolism and Function*; Schreiber, K.; Schutte, H. E.; Sembdner, G. Ed; VEG Deutcher Verlag der Wissenchaften; **1987** pp286-296.
34. Kim, S.-K.; Yokota, T.; Ogino, Y.; Takahashi, N. In *13th Int. Conf. on Plant Growth Substances*. Abstr. No 401, Calgary, Canada **1988**
35. Schneider, J. A.; Yoshihara, K.; Nakanishi, K.; Kato, N. *Tetrahedron Lett.* **1983**, *24*, 3859.
36. Abe, H.; Nakamura, K.; Morishita, T.; Uchiyama, T.; Takatsuto, S.; Ikekawa, N. *Agric. Biol. Chem.* **1984**, *48*, 1103.
37. Suzuki, Y.; Yamaguchi, I.; Yokota, T.; Takahashi, N. *Agric. Biol. Chem.* **1986**, *50*, 3133.

38. Yokota, T.; Arima, M.; Takahashi, N.; Takatsuto, S.; Ikekawa, N.; Takematsu, T. *Agric. Biol. Chem.* **1983**, *47*, 2419.
39. Yokota, T.; Amima, M.; Takahashi, N.; Crozier, A. *Phytochemistry* **1985**, *24*, 1333.
40. Kim, S.-K.; Abe, H.; Little, C. H. A.; Pharis, R. P. *Plant Physiol.* **1990**, *94*, 1709.
41. Yokota, T.; Kim, S.-K.; Fukui, Y.; Takahashi, N.; Takeuchi, Y.; Takematsu, T. *Phytochemistry* **1987**, *26*, 503.
42. Tokota, T.; Takahashi, N. In *Plant Growth Substances* 1985; Bopp, M. Ed; Springer-Verlag, Berlin Heidelberg New York, **1986**, pp129-138
43. Yokota, T.; Ogino, Y.; Takahashi, N.; Saimoto, H.; Fujioka, S.; Sakurai, A. *Agric. Biol. Chem.* **1990**, *54*, 1107.
44. Mori, K.; Takeuchi, T. *Liebigs Ann. Chem.* **1988**, 815.

RECEIVED June 17, 1991

Chapter 4

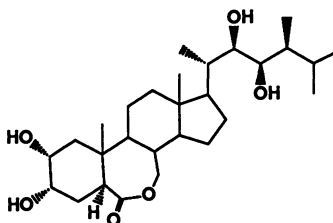
Synthesis of Brassinolide

T. C. McMorris, J. R. Donaubauer, M. H. Silveira, and T. F. Molinski

Department of Chemistry (0506), University of California—San Diego,
La Jolla, CA 92093–0506

Several reported syntheses of brassinolide, (22R,23R,24S)-2 α ,3 α ,22,23-tetrahydroxy-24-methyl-B-homo-7-oxa-5 α -cholestan-6-one, involve a C-22 aldehyde derived from stigmasterol, which is used for construction of the sidechain with chiral centers at C-22, C-23 and C-24. In our synthesis, (20 S)-6 β -methoxy-3 α ,5-cyclo-5 α -pregnane-20-carboxaldehyde was reacted with the anion of 2,3-dimethylbutenolide at $-78\text{ }^{\circ}\text{C}$ to give a lactone which was readily converted to the desired sidechain. Functionalization of the steroid nucleus completed the synthesis. We have investigated an alternative approach starting from 22,23-bisnor-5-cholenic acid-3 β -ol acetate, in which functionalization of the nucleus is first carried out leading to the intermediate 6-ethylenedioxy-2 α ,3 α -isopropylidenedioxy-bisnor-5 α -cholanal. The aldehyde can be used in aldol condensations with the lithium salt of 2,3-dimethylbutenolide or of 3-isopropylbutenolide, giving analogs of brassinolide with the natural 22R,23R stereochemistry.

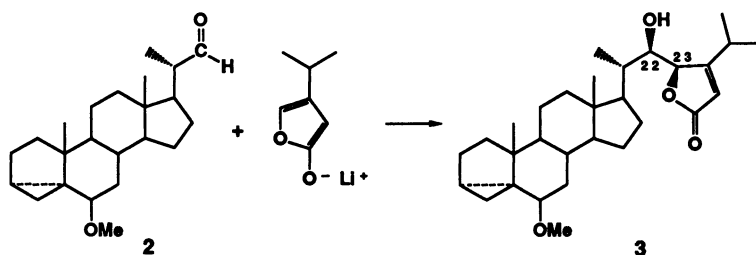
The identification of brassinolide (1) by workers at the USDA laboratories in Beltsville, Maryland in 1979 (1) was a most significant event because it established steroids as a new class of plant hormones. We were particularly interested in the structure of brassinolide as a result of our studies with somewhat



1

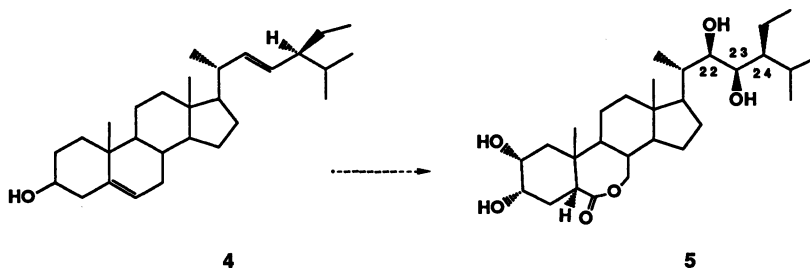
similar steroids which function as hormones, or perhaps more accurately, pheromones, in the sexual reproductive process of the aquatic fungus *Achlya* (2). They include male-activating steroids antheridiol and deoxyantheridiol as well as female-activating steroids dehydro-oogoniol and oogoniol (3).

We have developed a synthesis of antheridiol in which the key step is an aldol condensation of a C-22 aldehyde with the anion derived from 3-isopropylbut-2-enolide (4) which gives directly the sidechain of antheridiol as illustrated by structures 2 and 3. In this reaction, chiral centers are created at C-22 and C-23. The stereochemistry at C-22 in the major product is that predicted by the Cram rule (i.e. R) and careful study of the reaction showed that the stereochemistry at C-23 is determined by the temperature at which the aldol reaction is carried out. If the temperature is maintained below -70°C , the major product has the R configuration at C-23. Thus, this method could be used to construct the sidechain of brassinolide with correct stereochemistry at C-22 and C-23.



Different Synthetic Routes to Brassinolide

Before discussing our synthetic studies, mention should be made of different approaches employed in the synthesis of brassinolide by other investigators. The most practical method would be to start with a readily available steroid having the same carbon skeleton as brassinolide, and then introduce the required functional groups in rings A and B and the sidechain. In the case of 28-homobrassinolide (5), the ideal starting compound would be the abundant sterol stigmasterol (4).



Literature methods have been available for some time for functionalization of rings A and B to give the $2\alpha,3\alpha$ -dihydroxy-B-homo-7-oxa-6-one structure. Stigmasterol possesses a $24\text{-}\alpha$ ethyl group and a Δ^{22} double bond which, in principle, can be hydroxylated, leading to the 22R,23R,24S product. Unfortunately, the standard method of *cis* hydroxylation, reaction of the alkene with osmium tetroxide, gives almost exclusively the isomeric 22S,23S product (5-12). Less direct ways involving epoxidation of the Δ^{22} double bond of a stigmasterol derivative, followed by *trans* ring opening of the resulting epoxides by HBr-acetic acid then an inversion reaction at the carbon-bearing bromine, by

acetoxy ion, gave a mixture of the 22R,23R and 22S,23S isomers in low yield (13,14).

The required starting compound for the synthesis of brassinolide by hydroxylation of a Δ^{22} double bond is 22-dehydrocampesterol, which is not readily available (7). It can be isolated as a mixture with the C-24 epimer, brassicasterol, from oysters (*Crassostrea virginica*), but the mixture is difficult to separate. It is more readily prepared by synthesis (6). For the synthesis of 24-epibrassinolide, brassicasterol, which can be obtained from rapeseed oil, is an obvious starting compound (15). Ergosterol, which is readily available, has also been employed (16).

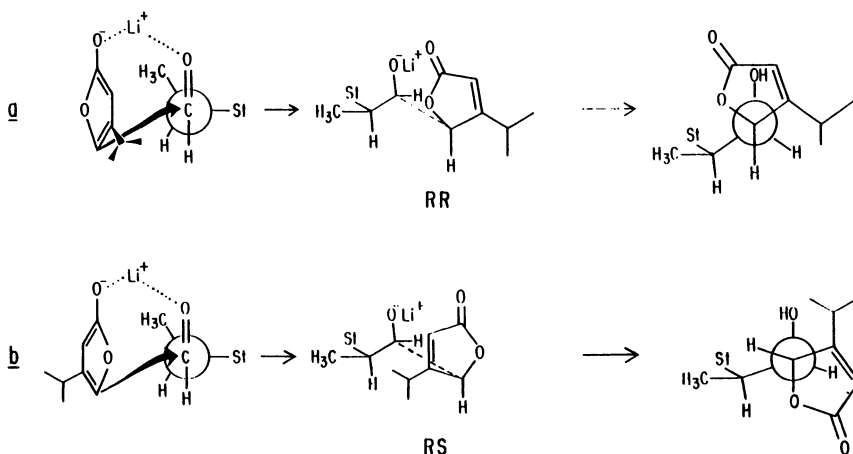
A more widely used approach to brassinolide is reaction of a C-22 aldehyde, derived from stigmasterol, with a carbanion containing a double bond (or potential double bond). In nearly all cases, epoxidation of the double bond was employed to introduce one or both oxygen functions (17-24). A synthesis of the brassinolide sidechain starting from pregnenolone has also been reported (25).

Synthesis of Brassinolide

Our first synthesis of brassinolide started from stigmasterol which was converted to (20S)-6 β -methoxy-3 α ,5-cyclo-5 α -pregnane-20-carboxaldehyde (2). This pathway involved formation of the mesylate with methanesulfonyl chloride in pyridine and tetrahydrofuran, treatment of the mesylate with potassium acetate in methanol, and ozonolysis of the i-sterol with reductive work-up.

The aldehyde was then used in an aldol reaction with the anion from 3-isopropylbut-2-enolide. [The lactone was prepared in the following way: bromination of 3-methyl-2-butanone under kinetic conditions (-15 °C) afforded the 1-bromo derivative. The bromine was displaced by acetate on refluxing a solution in acetone with anhydrous KOAc. Reaction of the resulting keto-acetate with the anion from triethylphosphonoacetate afforded the desired butenolide in 55% yield.] The anion was generated in tetrahydrofuran from the butenolide and lithium diisopropylamide and was cooled to -78 °C before addition of the aldehyde. The temperature was maintained below -70 °C for 5h and the reaction was quenched with ammonium chloride at this temperature. Under these conditions (kinetic) the 22R,23R intermediate (3) was obtained in 65% yield (26).

The preferred stereochemistry at C-22 (R) is predicted by the Cram or Felkin-Anh models for the transition state (27). The stereochemistry at C-23 appears to be determined by the approach of the anion as shown in Scheme I. It is

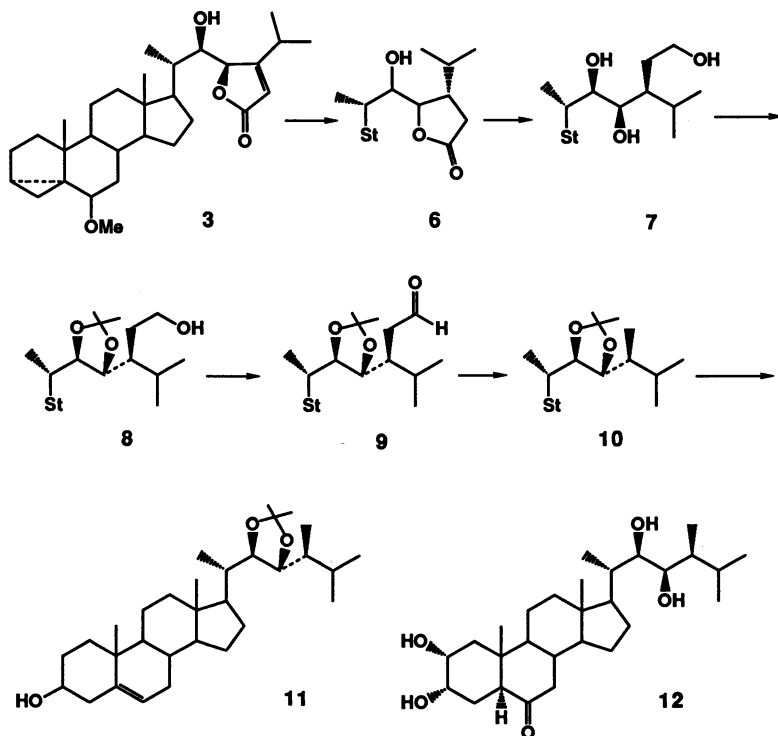


Scheme I

seen that path A involves fewer steric interactions and is thus favored over path B, which results in the predominance of the 23R stereochemistry. When the aldol reaction mixture was allowed to warm up to 0°C before quenching, the major product was the 22R,23S isomer. Apparently, the reversible nature of the aldol reaction led to the more stable product at higher temperature.

Catalytic hydrogenation of the intermediate butenolide (3) (Pt/activated carbon, freshly distilled dioxane) gave a 78:22 mixture in high yield. Fortunately, the major product (6) was subsequently found to have the desired stereochemistry at C-24, i.e. S. This result could not be easily predicted.

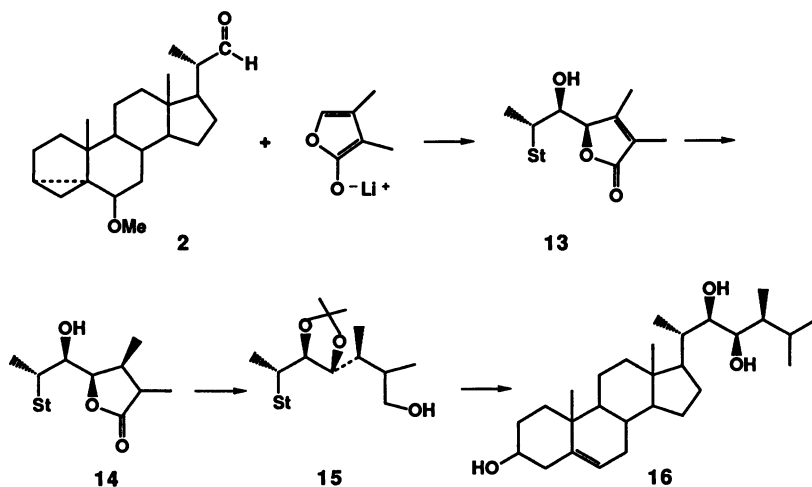
The remaining steps in the construction of the sidechain were high yielding and relatively standard reactions. Reduction of the lactone with LiAlH₄ afforded the trihydroxy derivative (7) in 90% yield, and this compound was heated with 2,2-dimethoxypropane (55-60 °C) and pyridinium p-toluenesulfonate to form the 22,23-acetonide and a mixed ketal at C-29. The crude product was refluxed in methanol for 1h to give the C-29 hydroxy compound (8) in an overall yield of 92%. Oxidation of the primary alcohol with pyridinium dichromate gave the corresponding aldehyde (9) (94%) which was decarbonylated with tris (triphenylphosphine) rhodium chloride to give the 24S methyl compound in 78% yield. The overall yield for the sidechain synthesis starting from the aldehyde was 32%.



Regeneration of the 3 β -hydroxy- Δ^5 -ene (11) in the usual way (p-TsOH, acetone-water, 2h, reflux) followed by mesylation and treatment with diborane-tetrahydrofuran then hydrogen peroxide and sodium bicarbonate gave the 3-mesyloxy-6-hydroxy compound. Oxidation of the alcohol and treatment of the

resulting ketone with lithium bromide in dimethylformamide at 150 °C, (1.5h) afforded the Δ^2 -ene together with some of the Δ^3 -isomer. Cis hydroxylation of the Δ^2 -ene with osmium tetraoxide (catalytic amount) and N-methyl morpholine-N-oxide and removal of the acetonide protecting group (acetic acid, 60 °C) gave castasterone (12). Brassinolide was obtained by forming the 2 α ,3 α -diacetate (in the intermediate containing the acetonide at C-22,C-23) then oxidation with 3,5-dinitroperoxybenzoic acid. The protecting groups were removed by treatment with potassium carbonate in methanol followed by heating the crude product in acetic acid. The overall yield of brassinolide from the intermediate with the correct sidechain was 15%.

The key aldol reaction for constructing the sidechain has also been used with other butenolides. 2,3-Dimethylbut-2-enolide gave with the aldehyde (2), 22R,23R hydroxy butenolide (13) in 74% yield together with small amounts of the 22R,23S isomer (6%) and 22S,23S isomer (10%). Catalytic hydrogenation of the butenolide gave the saturated lactone 14 (77%), which was found to have the 24S configuration. Reduction of the lactone with LiAlH₄ gave a triol which was converted to the acetonide (15). The free primary hydroxyl was then removed by treatment with methane sulfonyl chloride in pyridine followed by reaction of the mesylate with LiAlH₄. The overall yield for the four steps from the saturated lactone was 70%.

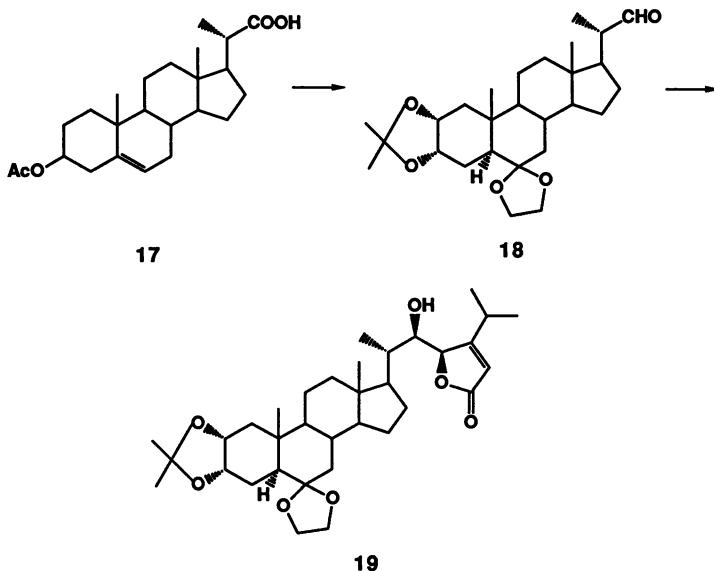


It should be noted that catalytic hydrogenation of 22R,23R butenolides from the aldol reactions gave products which had mainly the 24S stereochemistry. Initially, we had reasoned that if hydrogenation occurred at the face of the butenolide ring nearest the C-22 hydroxyl, the first butenolide would yield the 24S product while the second would yield the 24R isomer. The only explanation we can give for the fact that both yield mainly the 24S isomer is that the favored conformation of the sidechain is different in the two compounds so that the more accessible face for hydrogenation for **3** is opposite to that for **13**.

Subsequent to our use of butenolide anions in aldol reactions to construct sidechains of steroids, two groups have reported a similar method for synthesis of brassinolide or its analogs. Kametani and co-workers employed the dianion of 3-isopropyltetronic acid which was reacted with a 20-keto steroid. The aldol product was dehydrated and the product hydrogenated to furnish the brassinolide sidechain (28,29). Zhou and Tian have used the reaction of a C-22 aldehyde derived from

hydroxycholeic acid with the anion from 3-methylbutenolide. Hydrogenation and methylation of the aldol product then led to the brassinolide sidechain (30).

We have felt for some time that our method of synthesizing brassinolide (and homobrassinolide) might be improved by carrying out modification of the steroid nucleus before constructing the sidechain. One advantage would be that hydrogenation could be done without any risk of affecting the nucleus. (This is not the case when an *i*-sterol is hydrogenated.)



The target intermediate for the new synthesis is 2 α ,3 α -isopropylidene-dioxy-6-ethylenedioxy-bisnor-5 α -cholanal (18), which we have prepared starting from commercially available 3 β -acetoxy-bisnor-cholenic acid (17). This compound is readily obtained from stigmasterol. Functionalization of rings A and B was carried out using the same reactions as mentioned above for our first synthesis. The aldehyde (18) has also been synthesized in a slightly different way from stigmasterol by other workers (6, 30). Aldol condensation of the aldehyde and the anion from 3-isopropylbut-2-enolide under kinetic conditions gave a 60% yield of the 22R,23R butenolide (19). The structure has been confirmed by X-ray crystallographic analysis of the corresponding 2 α ,3 α -dihydroxy-6-ketone. Completion of the synthesis requires a few steps similar to those that have been used in our previous synthesis of brassinolide.

In conclusion, several groups including ours find that stigmasterol is the most suitable starting material for preparation of brassinolide and its analogs possessing the 22R,23R,24S stereochemistry. Many steps are required for the conversion, but the majority of these are high-yielding, and further refinements in the less satisfactory steps are certainly possible.

Literature Cited

1. Grove, M.D.; Spencer, G.F.; Rohwedder, W.K.; Mandava, N.; Worley, J.F.; Warthen, J.D., Jr.; Steffens, G.L.; Flippen-Anderson, J.L.; Cook, J.C., Jr. *Nature* **1979**, *281*, 216.
2. McMorris, T.C. *Phil. Trans. R. Soc. Lond. B* **1978**, *284*, 459.

3. Moon, S.; Stuhmiller, L.M.; Chadha, R.K.; McMorris, T.C. *Tetrahedron* **1990**, *46*, 2287.
4. McMorris, T.C.; Seshadri, R.; Arunachalam, T. *J. Org. Chem.* **1974**, *39*, 669.
5. Anastasia, M.; Allen, P.; Brasca, M.G.; Ciuffreda, P.; Fiecchi, A. *Gazz. Chim. Ital.* **1984**, *114*, 159.
6. Mori, K.; Sakakibara, M.; Ichikawa, Y.; Ueda, H.; Okada, K.; Umemura, T.; Yabuta, G.; Kuwahara, S.; Kondo, M. *Tetrahedron* **1982**, *38*, 2099.
7. Thompson, M.J.; Mandava, N.B.; Meudt, W.J.; Lusby, W.R.; Spaulding, D.W. *Steroids* **1981**, *38*, 567.
8. Wada, K.; Marumo, S. *Agric. Biol. Chem.* **1981**, *45*, 2579.
9. Anastasia, M.; Ciuffreda, P.; Del Puppo, M.; Fiecchi, A. *J. Chem. Soc. Perkin Trans. 1* **1983**, 383.
10. Takatsuto, S.; Ikekawa, N. *Chem. Pharm. Bull.*, **1982**, *30*, 4181.
11. Mitra, R.B.; Harza, B.G.; Kampoor, V.M. *Indian J. Chem.* **1984**, *23B*, 106.
12. Thompson, M.J.; Meudt, W.J.; Mandava, N.B.; Dutky, S.R.; Lusby, W.R.; Spaulding, D.W. *Steroids* **1982**, *39*, 2894.
13. Takatsuto, S.; Ikekawa, N. *J. Chem. Soc. Perkin Trans 1* **1984**, 439.
14. Sakakibara, M.; Mori, K. *Agric. Biol. Chem.* **1982**, *46*, 2769.
15. Takatsuto, S.; Ikekawa, N. *Chem. Pharm. Bull.* **1984**, *32*, 2001.
16. Thompson, M.J.; Mandava, N.; Flippen-Anderson, J.L.; Worley, J.F.; Dutky, S.R.; Robbins, W.E.; Lusby, W. *J. Org. Chem.* **1979**, *44*, 5002.
17. Fung, S.; Siddall, J.B. *J. Am. Chem. Soc.* **1980**, *102*, 6581.
18. Ishiguro, M.; Takatsuto, S.; Morisaki, M.; Ikekawa, N. *Chem. Commun.* **1980**, *20*, 962.
19. Mori, K.; Sakakibara, M.; Okada, K. *Tetrahedron* **1984**, *40*, 1767.
20. Sakakibara, M.; Mori, K. *Agric. Biol. Chem.* **1983**, *47*, 663.
21. Takatsuto, S.; Yazawa, N.; Ishiguro, M.; Morisaki, M.; Ikekawa, N. *J. Chem. Soc. Perkin Trans 1* **1984**, 139.
22. Takatsuto, S.; Ikekawa, N. *J. Chem. Soc. Perkin Trans 1* **1983**, 2133.
23. Takatsuto, S.; Yazawa, N.; Ikekawa, N. *Phytochemistry* **1984**, *23*, 525.
24. Hayami, H.; Sato, M.; Kanemoto, S.; Morizawa, Y.; Oshima, K.; Nozaki, H. *J. Am. Chem. Soc.* **1983**, *105*, 4491.
25. Takahashi, T.; Ootake, A.; Yamada, H.; Tsuji, J. *Tetrahedron Letters* **1985**, *26*, 69.
26. Donaubauer, J.R.; Greaves, A.M.; McMorris, T.C. *J. Org. Chem.* **1984**, *49*, 2833.
27. Bartlett, P.A. *Tetrahedron*, **1980**, *36*, 2.
28. Kametani, T.; Katoh, T.; Fujio, J.; Nogiwa, I.; Tsubuki, M.; Honda, T. *J. Org. Chem.* **1988**, *53*, 1982.
29. Kametani, T.; Kigawa, M.; Tsubuki, M.; Honda, T. *J. Chem. Soc. Perkin Trans 1* **1988**, 1503.
30. Zhou, W-S.; Tian, W-S. *Tetrahedron* **1987**, *43*, 3705.

RECEIVED March 12, 1991

Chapter 5

Synthesis and Some Practical Aspects of Brassinosteroids

V. A. Khripach, V. N. Zhabinskii, and R. P. Litvinovskaya

Institute of Bioorganic Chemistry, Byelorussian SSR Academy of Sciences,
220045, Minsk, Zhodinskaya, 5/2 Union of Soviet Socialist Republics

This brief review covers the period of the last ten years in the development of brassinosteroid chemistry in our laboratory. Our interest has been focused on the synthesis of natural brassinosteroids (brassinolide, homobrassinolide, epibrassinolide, norbrassinolide, etc.), their analogues and intermediates starting from stigmaterol, ergosterol, or pregnenolone. Some aspects of biological activity are discussed.

The isolation and structural elucidation of brassinolide (1), a novel polyhydroxysteroidal lactone with strong plant growth-promoting activity, was the beginning of intensive study of a new class of phytohormones, termed brassinosteroids (BS). Since then, a number of related compounds have been identified and isolated from various plants (2,3). The ability of BS to activate at very low concentrations the metabolic and growth processes was established and consequently to increase total plant productivity. These properties, along with specific structural features and extremely low content of BS in natural sources, made their synthesis especially interesting. The efforts of scientific groups in different countries (USA, Japan, Italy, and some others) led to the elaboration of many schemes for BS synthesis (2,3, and Lakhvich, F., Khripach, V., Zhabinskii, V. *Uspekhi Khim.*, in press.). In extending our studies in natural and transformed steroids, BS synthesis was the subject in our laboratory for a number of years. During this period, different synthetic approaches starting with accessible natural steroids have been developed, and a lot of natural BS and their analogues have been synthesized and biologically tested in laboratory and field conditions.

Synthesis of BS from stigmasterol. For our purpose commercially available stigmasterol 1 was attractive as a starting material because its 3 β -hydroxy-5-ene functionality permitted the necessary transformations of the cyclic part and the side chain. The conversion of 1 into natural BS was achieved using two different strategies: a) with the retention of the carbon skeleton for the synthesis of C₂₉ derivatives; and b) via the C-22 aldehyde for the construction of new side chains, especially for BS of the C₂₇ and C₂₈ series. The transformation of 1 into 22S,23S-homobrassinosteroids without replacement of the native side chain is shown in Figure 1. The tosylate of 1 was solvolysed to the corresponding 3,5-cyclo-6-alcohol which upon oxidation gave cycloketone 2 (4), the key intermediate in BS synthesis from stigmasterol. The cycloketone 2 was treated at room temperature with hydrobromic acid to regioselectively give the bromoketone 3. Its dehydrobromination in boiling DMF in the presence of lithium carbonate followed by hydroxylation of the resultant dienoketone with osmium tetroxide gave (5) the tetrahydroxyketone 4, the 22S,23S isomer of the natural hormone ethylbrassinone. Finally, 22S,23S-homobrassinolide 5 was synthesized from 4 via successive steps by protecting the hydroxyl groups, Baeyer-Villiger oxidation, saponification and re-lactonization. Another synthesis of 5 was also accomplished (Figure 2) (6-9). It eliminated the necessity of protecting and deprotecting the hydroxyl groups and gave a relatively good yield of

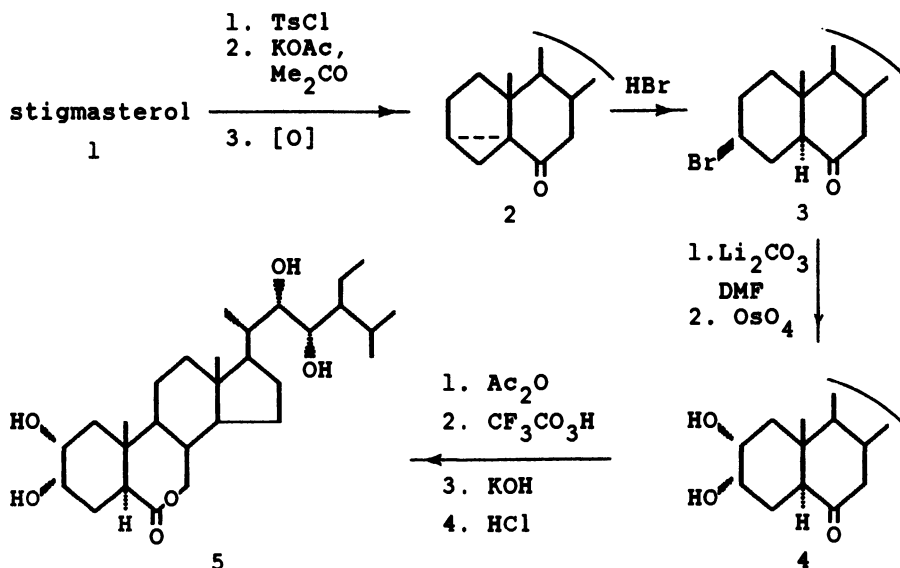


Figure 1. Synthesis of 22S,23S-homobrassinolide

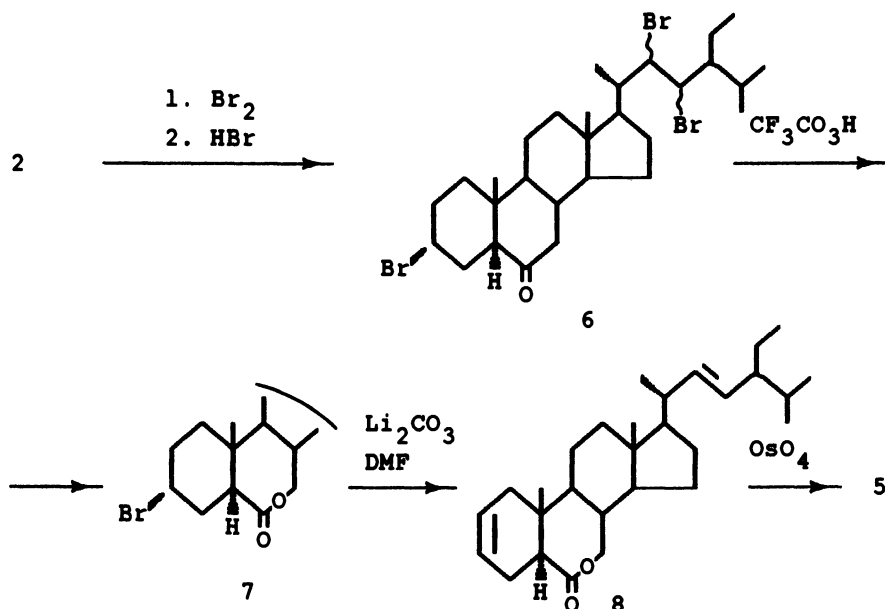


Figure 2. Alternative synthesis of 22S,23S-homobrassinolide

the desired product. The key step to this approach was the regioselective addition of bromine and hydrogen bromide to the double bond and cyclopropane of 2, respectively. Subsequent Baeyer-Villiger oxidation of the tribromoketone 6 and debromination with simultaneous dehydrobromination of 7 under the action of lithium carbonate in boiling DMF led to the dienolactone 8. The oxidation of the latter with osmium tetroxide afforded the tetrahydroxylactone 5. Unfortunately, the direct hydroxylation of 22-ene precursors leads, preferentially, to the 22S,23S configuration of hydroxyl functions. That is why for the synthesis of BS, possessing natural configurations of substituents in the side chain, we used an approach (10,11) based on the transformation of 22R,23R-epoxides which are the major isomers in the epoxidation of 22-olefines with MCPBA. As shown in Figure 3, the epoxidation of 3 and further dehydrobromination gave the unsaturated epoxide 9. Trans-opening of the epoxide ring with HBr and acetylation followed by nucleophilic substitution of bromine in the bromoacetate intermediate gave the diacetate 10. It was further transformed into homocastasterone 11 and homobrassinolide 12 in the usual way. The same method has been also employed for the synthesis of 22S,23S-homobrassinosteroids (9,12).

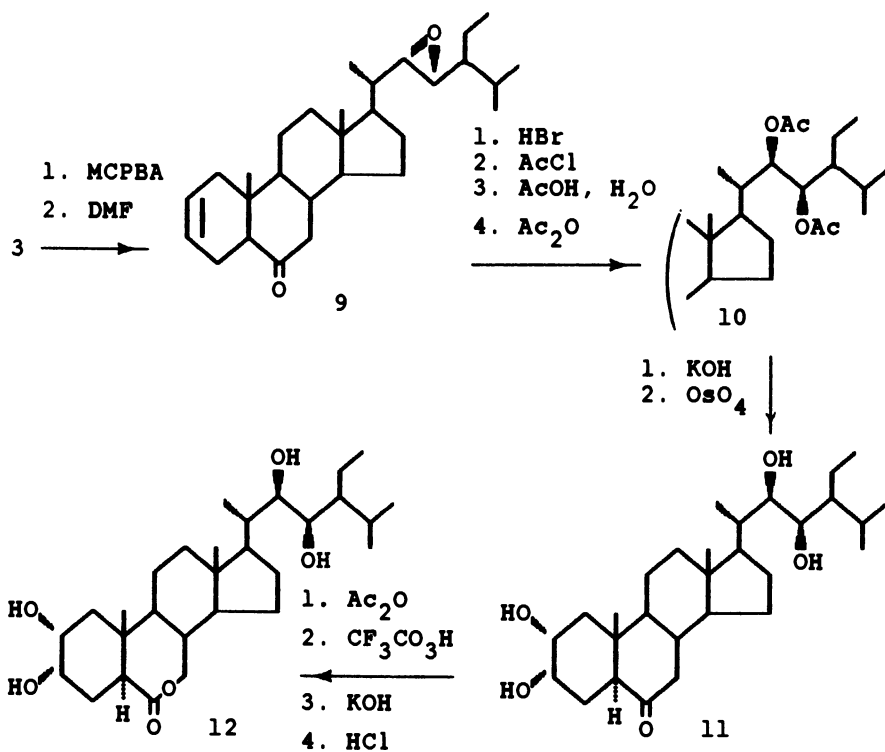
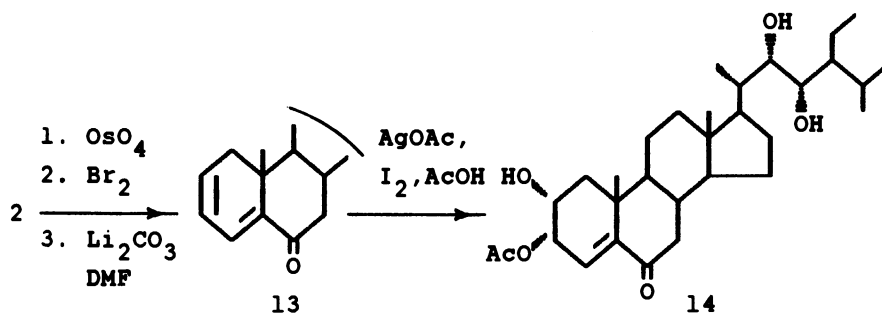


Figure 3. Synthesis of homobrassinolide

Figure 4. Synthesis of Δ^4 -analogue of 22S,23S-homobrassinolide

Some BS-analogues with additional double bond in the A-cycle were synthesized using the α -cis-hydroxylation method developed by us earlier (13-16). As shown in Figure 4, the oxidation of 2 with osmium tetroxide followed by bromination and debromination led to dienoketone 13 which was subjected to hydroxylation under the action of iodine and silver acetate in aqueous acetic acid to give the unsaturated BS analogue 14.

Synthesis of BS via 22-aldehydes. A large number of BS possessing both natural and unnatural side chains (brassinolide, norbrassinolide, homobrassinolide and their isomers) have been synthesized employing the reaction of sulfone derivatives 16 with the steroidal 22-aldehyde 15, as shown in Figure 5 (18-20). Sulfones 16 were obtained from isovaleric acid by successive steps of α -alkylation, LAH-reduction, tosylation of resulting alcohols, nucleophilic substitution of tosylates by sulfide anion and MCPBA-oxidation of the corresponding sulfides. Reaction of the aldehyde 15, obtained from stigmasterol, by ozonolysis of the 22-double bond, with the carbanion derived from 16 and further acetylation gave β -acetoxy sulfone 17. Its reduction with Na-amalgam gave β -hydroxy sulfone 18. Its reduction with Na-amalgam and subsequent hydroxylation gave β -hydroxy sulfone 19.

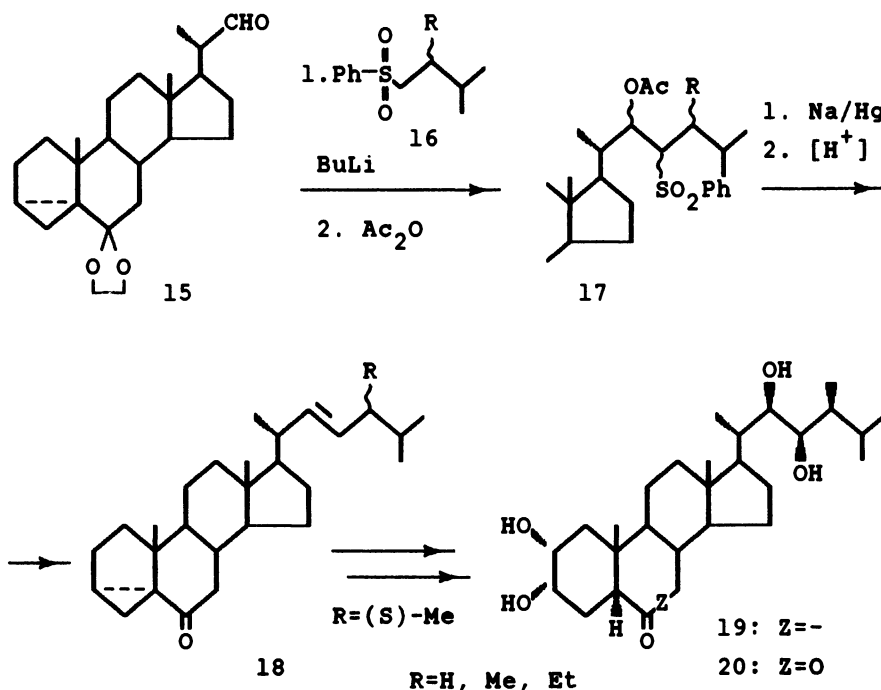


Figure 5. Synthesis of brassinosteroids via sulfones

followed by deprotection gave an olefinic product 18 with a complete carbon skeleton. The construction of the 22,23-dihydroxy functionality starting from 18 and subsequent transformation of the diols into aim products were achieved as discussed above for homobrassinolide. It should be pointed out that this method in the case of brassinolide synthesis gives with 7.8% a higher total yield than the approach proposed earlier by K.Mori et al. (21) with 1.3% starting from stigmasterol. Along with the products of the normal 20 β -series some 20 α -methyl analogues of BS have been synthesized in a similar manner.

A number of methods for the side chain construction in BS synthesis utilizes a reaction of steroidal 22-aldehydes with various vinyl carbanions. The main shortcomings of these approaches are the formation of 22 β -alcohols along with desired 22 α -isomers and relatively low yields of the latter. It was found (22) that employing the carbanion bearing trimethylsilyl substituent at the anionic center led to a substantial increase of 22 α -alcohol formation. Thus, the reaction of vinylsilane 21 with aldehyde 15 gave a 10:1 mixture of alcohol 22 and its 22 β -isomer in 97% total yield (Figure 6). Epoxidation of 22 followed by the nucleophilic cleavage of the Si-C-bond afforded the epoxide 24 as a major product. Otherwise, the removal of the trimethylsilyl group followed by epoxidation of the resultant alkene 25 led to brassinosteroid intermediate 26. The further synthetic path from 26 to brassinolide is known (23,24).

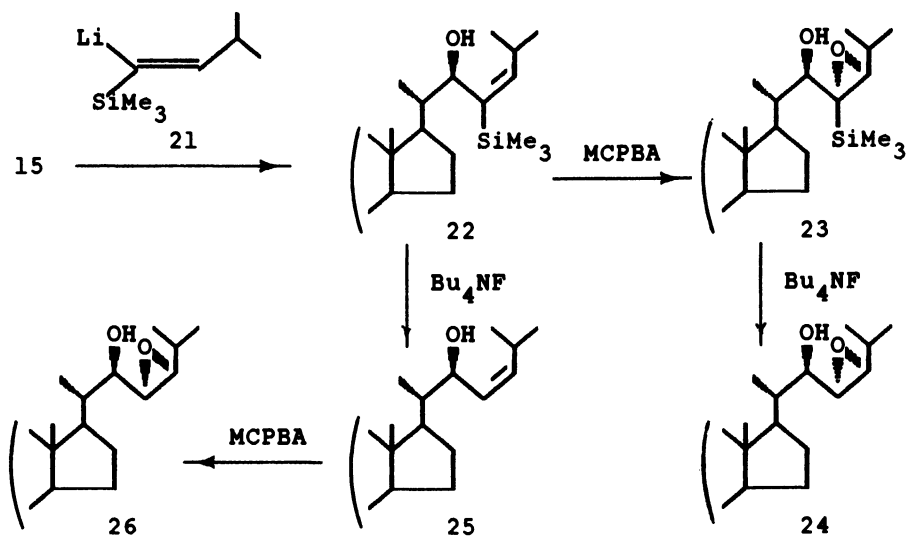


Figure 6. Synthesis of BS-intermediates via vinylsilanes

Synthesis of BS from ergosterol. Among natural BS one of the most promising for practical application in agriculture is epibrassinolide. This is because of its high biological activity and the relative ease of employing synthetic schemes based on the use and modification of the intact side chain of easily available ergosterol. As shown in Figure 7, following the procedure of Barton et al. (25) the first step of our approach (26) to epibrassinosteroids from ergosterol 27 was the reduction of the diene system in the B-cycle by the action of Li in diethylamine. The isomer 29 was converted

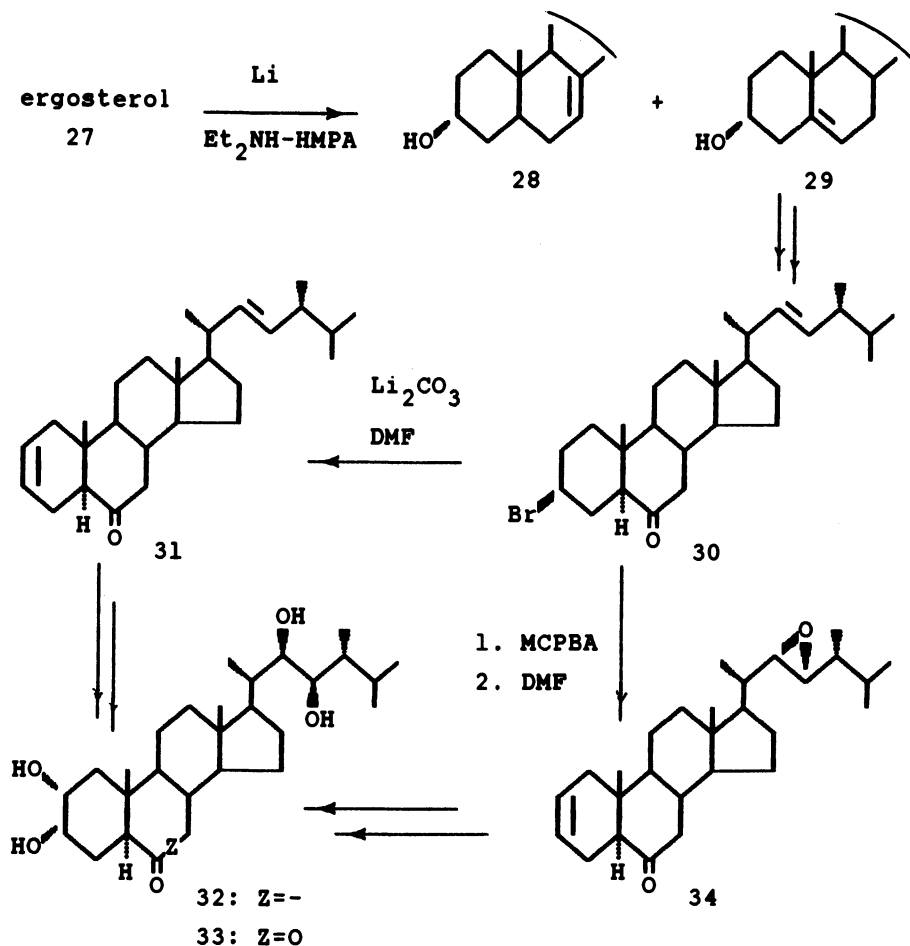


Figure 7. Synthesis of epibrassinolide

without isolation into the 3-bromo derivative 30 which was further transformed into the dienoketone 31. The latter was hydroxylated with osmium tetroxide to give 24-epicastasterone 32 and its 22S,23S-isomer. 24-Epicastasterone 32 was transformed, after chromatographic separation, into 24-epibrassinolide 33 in the usual way. An alternative synthesis of 33 was performed via epoxyenone 34. Synthesis of brassicasterol 29 free from its 5-dihydroergosterol is shown in Figure 8. Dienoketone 35, obtained from ergosterol 27 in two steps, was reduced with lithium in liquid ammonia in the presence of NH_4Cl to give 29.

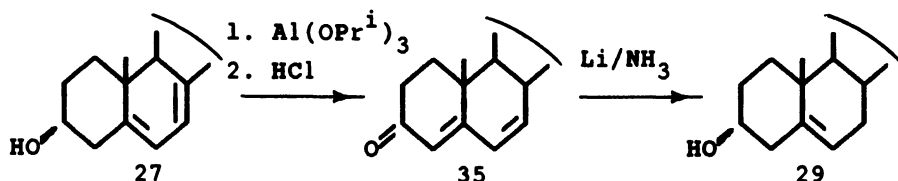


Figure 8. Synthesis of brassicasterol

Syntheses via heterocyclic intermediates. During the last few years we have been interested in developing new approaches in the construction of steroid polyfunctional side chains using heterocyclic intermediates which contain the desired functionality in a latent form. Especially attractive in this respect seems to be the use of isoxazoles and 2-isoxazolines - the adducts of 1,3-dipolar cycloaddition reactions of nitrile oxides with unsaturated compounds (27). For the construction of the side chain having a brassinolide-like carbon skeleton we studied the cycloaddition (28-30) of the steroidal acetylenes 36 and olefines 40, prepared from 3β -acetoxy-pregn-5-en-20-one to iso-butyronitrile oxide, as shown in Figure 9. Both reactions proceeded with high regio- and stereoselectivity (in the case of 40 the major product 41 had R configuration at C-22) to give the cycloadducts in good yields. The oxidative dehydration of 41 under the action of thionyl chloride in DMF afforded (31) the isoxazole 38. The latter was also prepared from 37 under acidic condition. The catalytic hydrogenation of the isoxazole 38 over Raney nickel gave the enaminketone with the natural (20β -methyl) configuration at C-20 (28,29) which was further transformed into 39 in the usual way (26). Unsaturated ketones 39 are known intermediates in brassinolide synthesis (32,33). Two

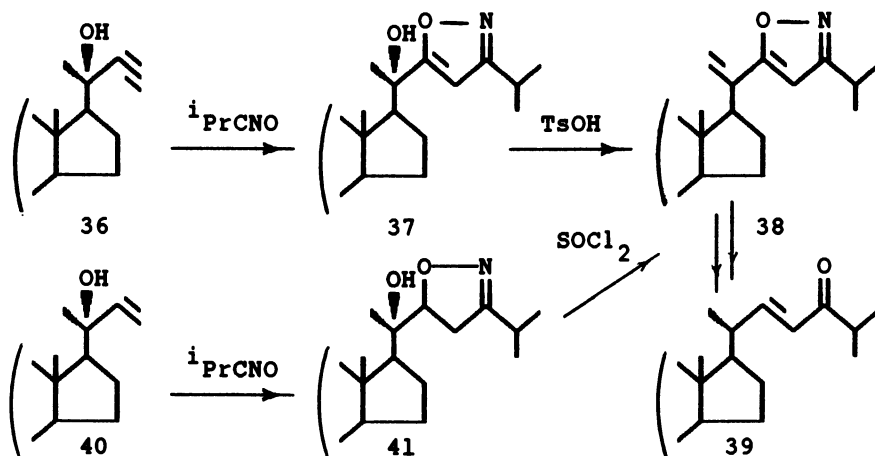


Figure 9. Synthesis of 22-en-24-ones via isoxazoles

other routes for transformation of isoxazolines 43 into 22-en-24-ones 39 were then elaborated by us as shown in Figure 10. The first one included the cleavage of the heterocycle under strong basic conditions (action of DMSO-anion) followed by hydrolysis of the resultant enoxime 44 (34). In the second route the isoxazolines 43 were hydrogenated over Raney nickel in acidic medium (35,36) to afford the β -ketols 45 which were then dehydrated by TsOH in refluxing benzene to give the eneketone 39. Using the isoxazolines 43 we realized a highly efficient synthesis of unsaturated ketones 49 and alcohols 50, well known intermediates of brassinolide (37,38). The reaction sequence involved the reductive

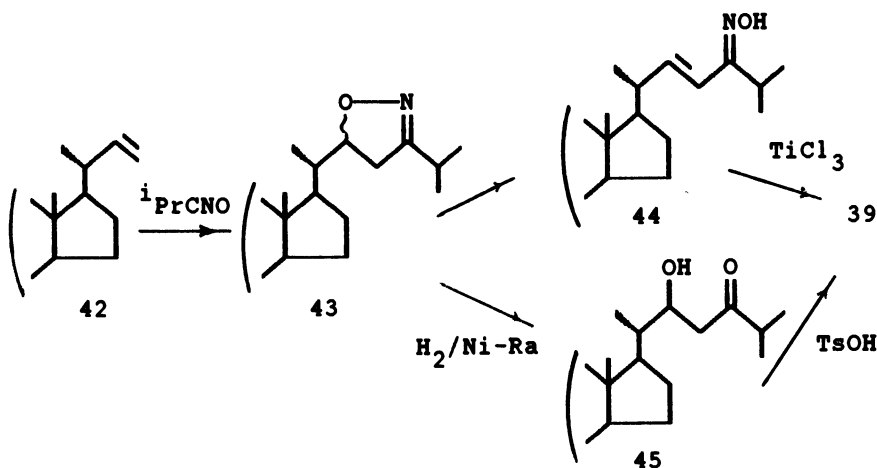


Figure 10. Synthesis of 22-en-24-ones via isoxazolines

cleavage of isoxazolines 43 giving ketols 46 as shown in Figure 11. Their interaction with methyl lithium produced 22,23-diols 47, which were oxidized into 22-keto derivatives 48 with Jones reagent (Khripach, V.A., Litvinovskaya, R.P., Baranovsky, A.V., Akhrem, A.A. Dokl. AN SSSR, in press.). The dehydration of alcohols 48 with thionyl chloride in pyridine gave eneketone 49. Its reduction with complex hydride proceeded stereoselectively to give the allylic alcohol 50. Further transformation of 50 to brassinolide is known (38).

Practical application. Along with the elaboration of syntheses of BS we studied their biological activities. At first the action of BS was compared with those of auxins and gibberellins using the appropriate laboratory tests. But these results did not reflect the real value of BS as means for increasing plant productivity. Later, we synthesized a sufficient quantity of BS to carry out large scale field testing in different regions of the USSR. A significant increase in seed productivity was noted for lucerne following BS treatment. The plants were treated with aqueous solutions of 24-epibrassinolide and homobrassinolide. Doses of BS were 50 mg in 500 liters per hectare. Yield increases were 16% for homobrassinolide and up to 26% for 24-epibrassinolide (Figure 12). Similar results have been obtained for peas. The plants were sprayed with solutions of 24-epibrassinolide twice - at the 8-9 leaf stage and at the budding stage. The average increase of crop yield was 2800 kg/ha. An example of the action of brassinosteroids on barley is shown in Figure 13. In this case, 24-epibrassinolide was compared with brassinolide. Brassinolide was more active at doses of 10 mg/ha, but less active at doses of 50 and 100

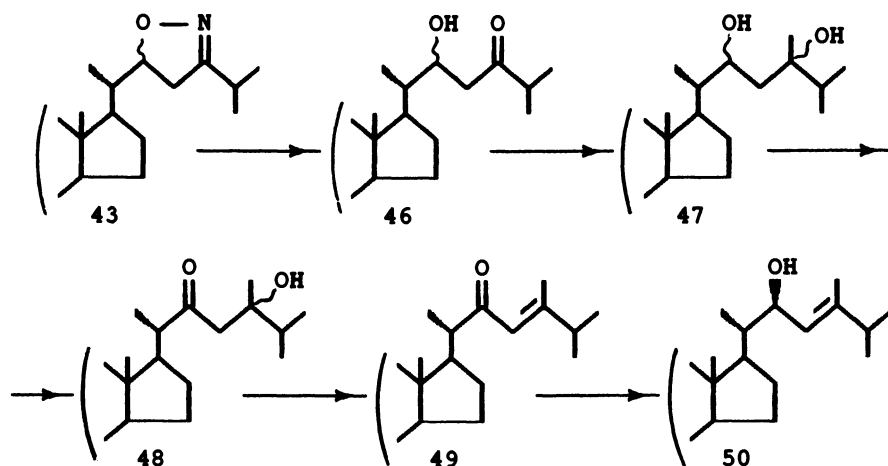


Figure 11. Synthesis of key intermediates of brassinolide

mg/ha. Crop yield increases were up to 25%. Good results have been obtained for potatoes. Depending on the phase of treatment, the increased crop yield amounted to 11-34%. Additionally, data on increased plant productivity were obtained for buckwheat, soy-bean, rye, wheat, hay and maize. It should be noted that treatment with brassinosteroids did not give pronounced results every time.

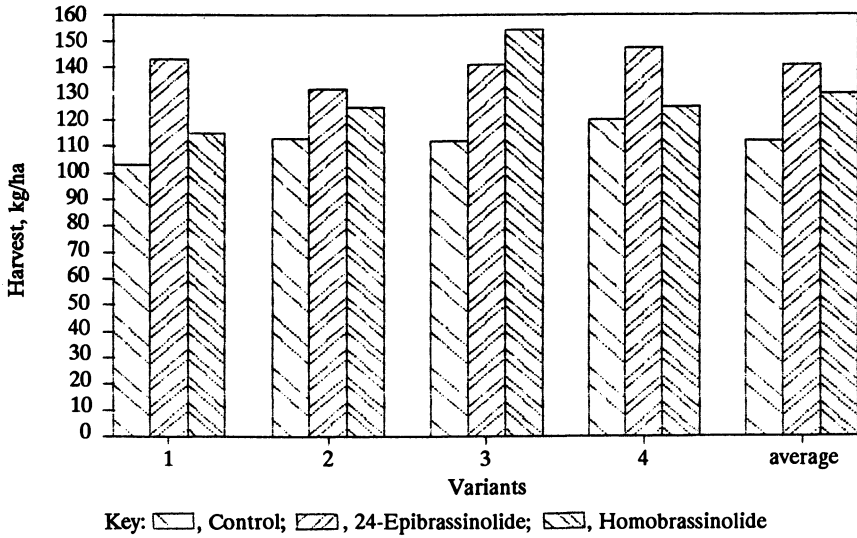


Figure 12. Action of BS on crop yield of lucerne

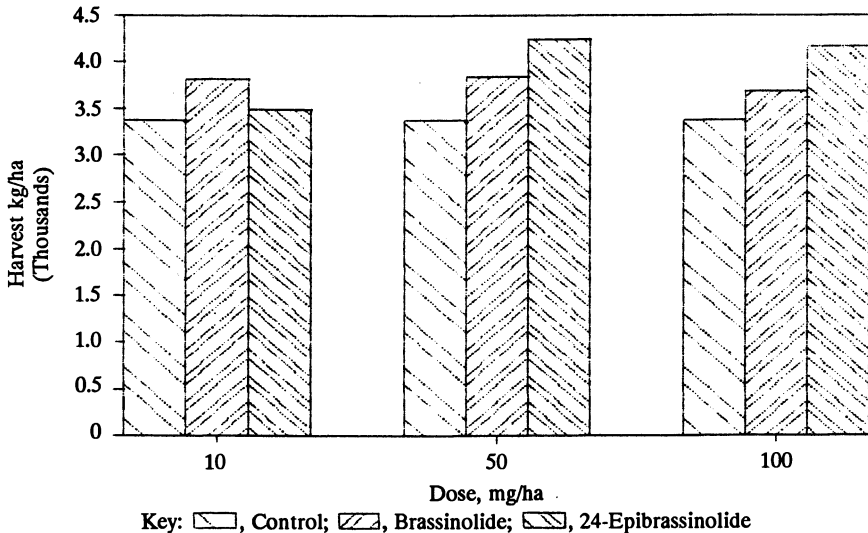


Figure 13. Action of BS on crop yield of barley

Literature Cited

1. Grove, M.D.; Spencer, G.F.; Rohwedder, W.K.; Mandava, N.; Worley, J.F.; Warthen, J.D.; Steffens, G.L.; Flippen-Anderson, J.L.; Cook, J.C. *Nature* (London). 1979, 281, 216-217.
2. Lakhvich, F.A.; Khripach, V.A.; Zhabinskii, N.V. *Vesti AN BSSR, ser.khim.navuk.* 1990, 99-116.
3. Adam, G.; Marquardt, V. *Phytochemistry.* 1986, 25, 1787-1799.
4. Pat.1162816 (USSR).
5. Akhrem, A.A.; Lakhvich, F.A.; Khripach, V.A.; Zhabinskii, V.N.; Kovganko, N.V. *Dokl. AN SSSR.* 1984, 275, 1089-1091.
6. Akhrem, A.A.; Lakhvich, V.A.; Khripach, V.A.; Kovganko, N.V. *Dokl. AN SSSR.* 1983, 269, 366-368.
7. Akhrem, A.A.; Lakhvich, F.A.; Khripach, V.A.; Kovganko, N.V.; Zhabinskii, V.N. *Dokl. AN SSSR,* 1985, 283, 130-133.
8. Akhrem, A.A.; Lakhvich, F.A.; Khripach, V.A.; Zhabinskii, V.N.; Kovganko, N.V. *Zhurn.Org.Khim.* 1984, 20, 2140-2143.
9. Akhrem, A.A.; Lakhvich, F.A.; Khripach, V.A.; Zhabinskii, V.N.; Zhurn.Org.Khim. 1987, 23, 762-770.
10. Pat.1270154 (USSR).
11. Pat.1270155 (USSR).
12. Khripach, V.; Lakhvich, F.; Kovganko, N.; Zhabinskii, V. 3 Int.Conf.on Chem.and Biotechn. of Biol.Active Nat.Prod.(Sofia). 1985, 5, 259-263.
13. Akhrem, A.A.; Lakhvich, F.A.; Khripach, V.A.; Kovganko, N.V. *Dokl. AN. SSSR.* 1981, 257, 1133-1135.
14. Akhrem, A.A.; Lakhvich, F.A.; Khripach, V.A.; Kovganko, N.V. *Zhurn.Org.Khim.* 1983, 19, 1249-1256.
15. Akhrem, A.A.; Lakhvich, F.A.; Khripach, V.A.; Kovganko, N.V.; Zhabinskii, V.N. *Zhurn.Org.Khim.* 1989, 25, 1661-1665
16. Akhrem, A.A.; Galitsky, N.M.; Yasnitsky, G.A.; Gritsuk, V.I.; Lakhvich, F.A.; Khripach, V.A.; Kovganko, N.V. *Zhurn.Org.Khim.* 1990, 26, 771-775.
17. Akhrem, A.A.; Lakhvich, F.A.; Khripach, V.A.; Kovganko, N.V. *Vesti AN BSSR, ser.khim.navuk.* 1983, 65-70.
18. Khripach, V.; Zhabinskii, V.; Olkhovick, V. 5 Int.Conf. on Chem and Biotechn.of Biol.Active Nat.Prod.(Varna). 1989, 3, 230-234.
19. Khripach, V.A.; Zhabinskii, V.N.; Olkhovick, V.K.; Akhrem, A.A. *Vesti AN BSSR, ser.khim.navuk.* 1989, 69-73.
20. Khripach, V.A.; Zhabinskii, V.N.; Olkhovick, V.K.; Lakhvich, F.A. *Zhurn.Org.Khim.*, 1990, 26, 2200-2206.
21. Sakakibara, M.; Okada, K.; Ichikawa, Y.; Mori, K. *Heterocycles.* 1982, 17, 301-304.
22. Khripach, V.A.; Zhabinskii, V.N.; Olkhovick, V.K. *Tetr.Letters.* 1990, 37, 4937-4940.
23. Ishiguro, M.; Takatsuto, M.; Morisaki, M.; Ikekawa, N. *J.Chem.Soc., Chem.Commun.* 1980, 962-964.
24. Mori, K.; Sakakibara, M.; Okada, K. *Tetrahedron.* 1984, 40, 1767-1781.
25. Barton, D.H.R.; Lusinchi, X.; Magdzinski, L.; Ramirez, J.S. *J. Chem. Soc. Chem. Commun.* 1984, 1236-1238.

26. Akhrem, A.A.; Khripach, V.A.; Zhabinskii, V.N.; Olkhovick, V.K. *Vesti AN BSSR, ser. Khim. nauk.* 1989, 69-73.
27. Lakhvich, F.A.; Koroleva, E.V.; Akhrem, A.A. *Khim. Heterocycl. Soed.* 1989, 435-453.
28. Akhrem, A.A.; Khripach, V.A.; Lakhvich, F.A.; Zavadskaya, M.I.; Drachenova, O.A.; Zorina, I.A. *Dokl. AN SSSR.* 1987, 297, 364-367.
29. Akhrem, A.A.; Khripach, V.A.; Lakhvich, F.A.; Zavadskaya, M.I.; Drachenova, O.A.; Zorina, I.A. *Zhurn. Org. Khim.* 1989, 25, 2120-2128.
30. Akhrem, A.A.; Khripach, V.A.; Litvinovskaya, R.P.; Baranovsky, A.V.; Zavadskaya, M.I.; Kharitonovich, A.N.; Borisov, E.V.; Lakhvich, F.A. *Zhurn. Org. Khim.* 1989, 25, 1901-1908
31. Khripach, V.A.; Litvinovskaya, R.P.; Baranovsky, A.V. *Khim. Heterocycl. Soed.* 1990, 852-853.
32. Huang, Y.; Shi, L.; Li, S. *Synthesis.* 1988, 975-977.
33. Shan, Z.-W.; Zhou, W.-S.; *J. Chem. Soc., Perkin Trans. I.* 1990, 1765-1767.
34. Khripach, V.A.; Litvinovskaya, R.P.; Baranovsky, A.V.; Ermolenko, E.A. *Khim. Heterocycl. Soed.* 1990, 1389-1393.
35. Akhrem, A.A.; Lakhvich, F.A.; Khripach, V.A.; Klebanovich, I.B. *Dokl. AN SSSR.* 1979, 244, 615-617.
36. Khripach, V.; Litvinovskaya, R.; Baranovsky, A. 5 *Conf. on Chem. and Biotechn. of Biol. Act. Nat. Prod. (Varna).* 1989, 3, 227-229.
37. Takahashi, T.; Ootake, A.; Yamada, H.; Tsuji, J. *Tetr. Letters,* 1985, 26, 69-72.
38. Fung, S.; Siddall, J.B. *J. Am. Chem. Soc.* 1980, 102, 6580-6581.

RECEIVED July 22, 1991

Chapter 6

Types of Brassinosteroids and Their Bioassays

L. Kohout¹, M. Strnad², and M. Kaminek³

¹Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6, Czechoslovakia

²Institute of Experimental Botany, Czechoslovak Academy of Sciences, 772 00 Olomouc, Czechoslovakia

³Institute of Experimental Botany, Czechoslovak Academy of Sciences, 166 30 Prague, Czechoslovakia

Some structure-activity relationships, synthesis of new brassinosteroids, and new sensitive bioassays for brassinosteroids are presented.

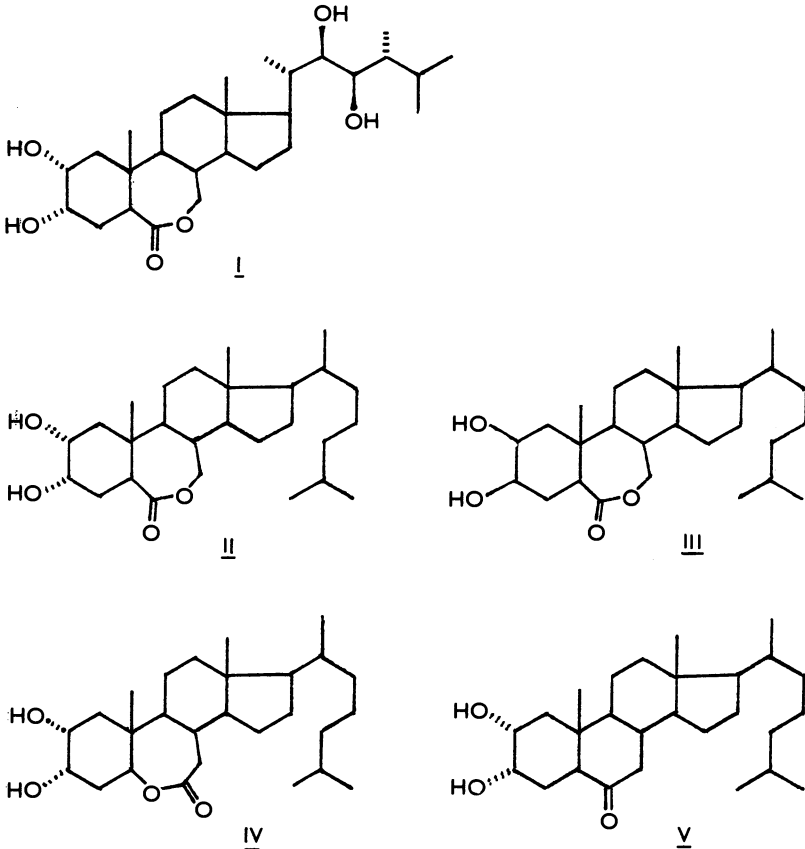
More than ten years ago the active principle of brassins - brassinolide (BR) - was isolated and its structure determined (1). BR is very interesting from a botanical point of view, because of its activity in promoting plant growth. BR is also very interesting for chemists for several reasons: a) BR is a naturally occurring steroid compound with a seven membered ring B, b) isolation of BR from natural material is very difficult, mainly because of its low content (0.1 ppm in rape pollen), and c) because BR is very active in promotion of plant growth, its isolation from natural sources is very difficult and, in addition, its synthesis is not very simple and synthetic yields are very low, it is important to synthesize BR analogues - brassinosteroids (BRst) - which would have an acceptably high specific activity. Furthermore, it would be helpful if the synthetic route were easier for the congeners compared to the tedious steps necessary to produce BR.

Here, we shall describe the synthesis of some new BRst, our findings in structure-activity relationships and a more sensitive bioassay for BRst.

Because BR was not available at the beginning of our studies and because 24-epibrassinolide I (24-epiBR) ((22R, 23R, 24R)-2 α , 3 α , 22, 23-tetrahydroxy-24-methyl-7-oxa-B-homo-5 α -cholestan-6-one) has almost the same activity as BR, we prepared this compound as a standard possessing high specific activity. For the structure-activity relationship studies it was necessary to have also a standard with low specific activity for comparison and we chose a cholestane analogue of BR, compound II (2 α , 3 α -dihydroxy-7-oxa-B-homo-

5 α -cholestan-6-one), an analogue with the same structural features in ring A and B as BR but possessing the cholestane side chain. This compound (II) has a very low activity both in the second internode bio-assay (2) and in the rice lamina inclination test (3).

In the course of the synthesis (4) of this cholestane type of BRst (II) we prepared also compounds with 2 β ,3 β -diol group (III), the 6-oxa-7-keto isomer of II (IV) and, of course, the 6-keto derivative V, which is an analogue of castasterone.



Some structure-activity relationships have been reported in the literature (5, 6):

- two cis hydroxyl groups are necessary in the side chain in positions 22 and 23 in the molecule for high activity in the bean second internode bioassay (that means that compound I would be much more active than II)
- compounds with a lactone group in the B-ring are much more active than compounds with a 6-keto group only (II would be more active than V)

- compounds with a "normal" lactone group, for example 6-keto-7-oxa-, are more active than compounds with an "isomeric" lactone group, for example 7-keto-6-oxa (II more than IV)

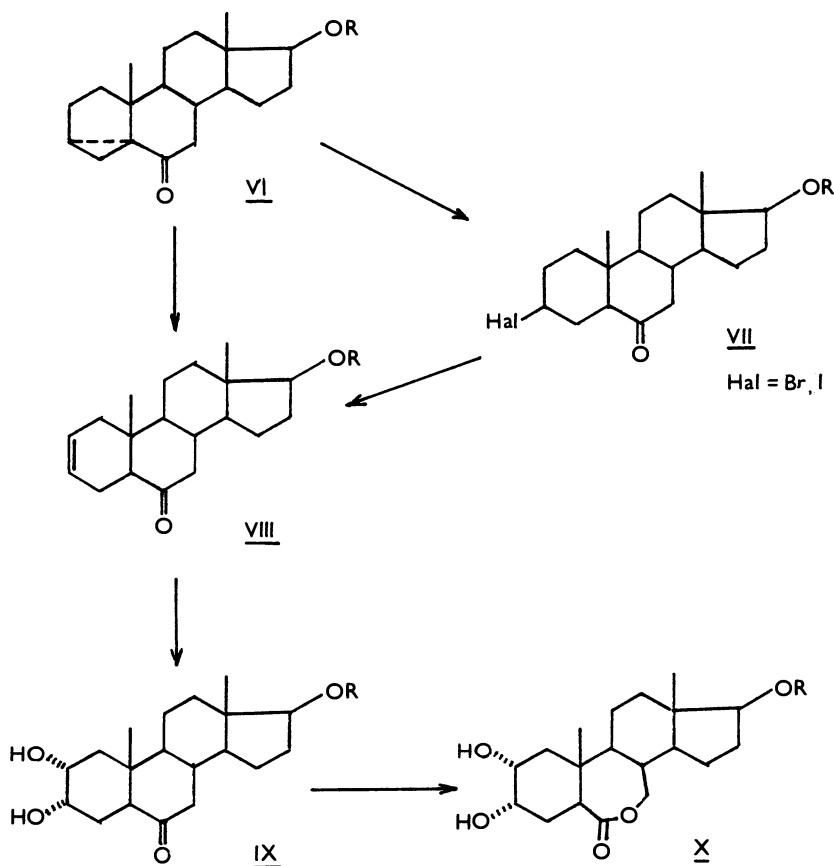
- compounds with 2 α ,3 α -diol group exhibit higher activity than compounds with 2 β ,3 β -diol group (II more than III).

Although the differences between activities of the cholestane analogues were small, agreement with these data was found (Table I).

Table I
Bean Second Internode Bioassay

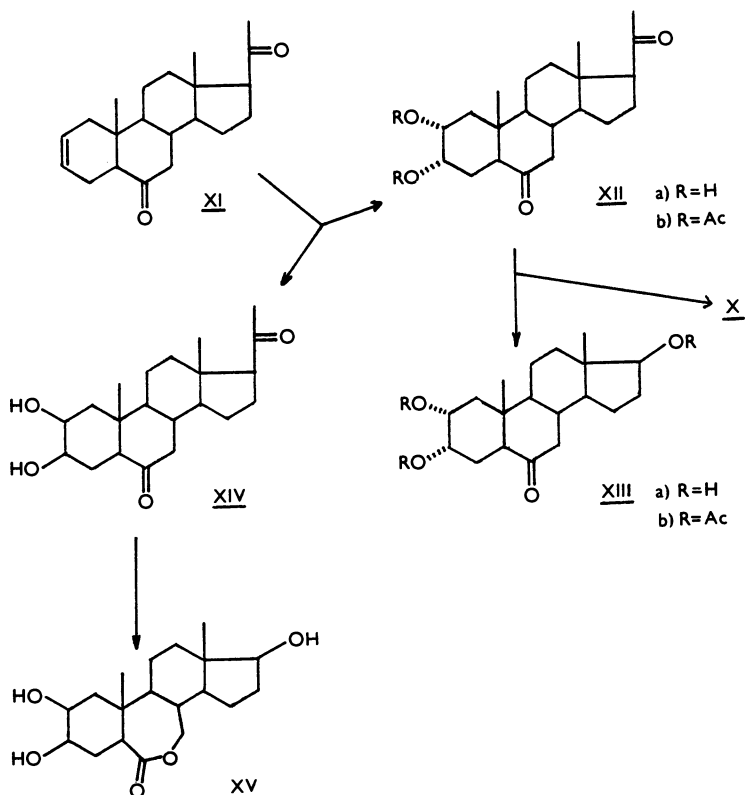
<u>Compound No.</u>	<u>E (mm)</u>
I	32
II	14
V	9.5
IV	8
III	7

E means elongation of the second internode to control



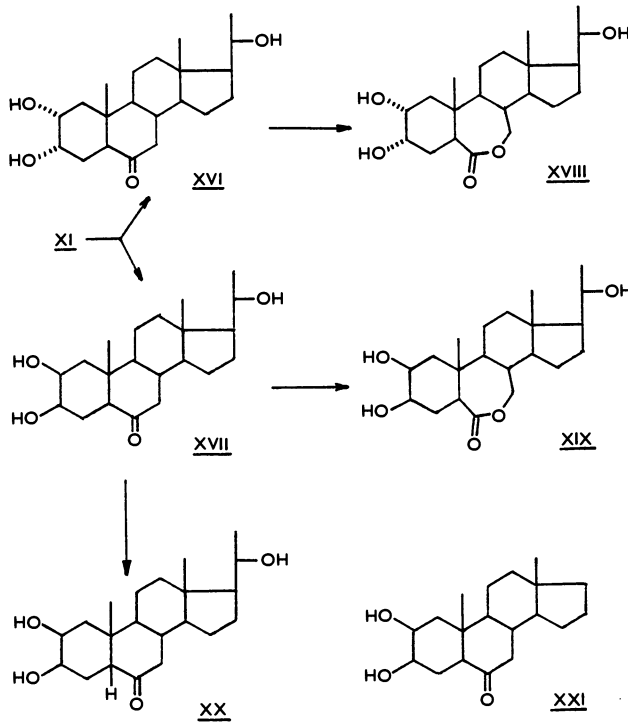
Next we devoted our attention to the androstane type of BRst. For their preparation we used two reaction pathways (7): the first used androstane compound VI as a starting material. Olefin VIII was prepared either through halogeno derivative VII or directly from cyclopropano derivative VI. Olefin VIII was then converted to diol IX and this diol with trifluoroperacetic acid afforded the androstane analogue of BR, compound X (R=H). This compound has the same structural features in the A and B ring as BR but instead of a side chain there is only one hydroxyl group which is in position 17 β .

The second method uses the pregnane derivative XI as a starting material. This compound, on hydroxylation with osmium tetroxide, afforded the diol XIIIa. This diol was then converted to the androstane analogue X. The same approach was independently published also by Kondo and Mori (3). As a by-product of hydroxylation of olefin XI with osmium tetroxide, we obtained 2 β ,3 β -diol XIV and, from this, the lactone XV. However, when we treated XII (a corresponding diacetate, respectively) with trifluoroperacetic acid we found that oxidation of the side chain surprisingly proceeded much faster than oxidation of the B ring. Thus we obtained not only compound X but also an intermediate, compound XIIIb, and on hydrolysis compound XIIIa, that is the androstane analogue of castasterone.



In a similar way we also obtained from olefin XI the pregnane analogues XVI and XVIII.

All but one of these compounds exhibited very low activity in the bean second internode bioassay. If 24-epiBR elongates the second internode by about 32 mm,

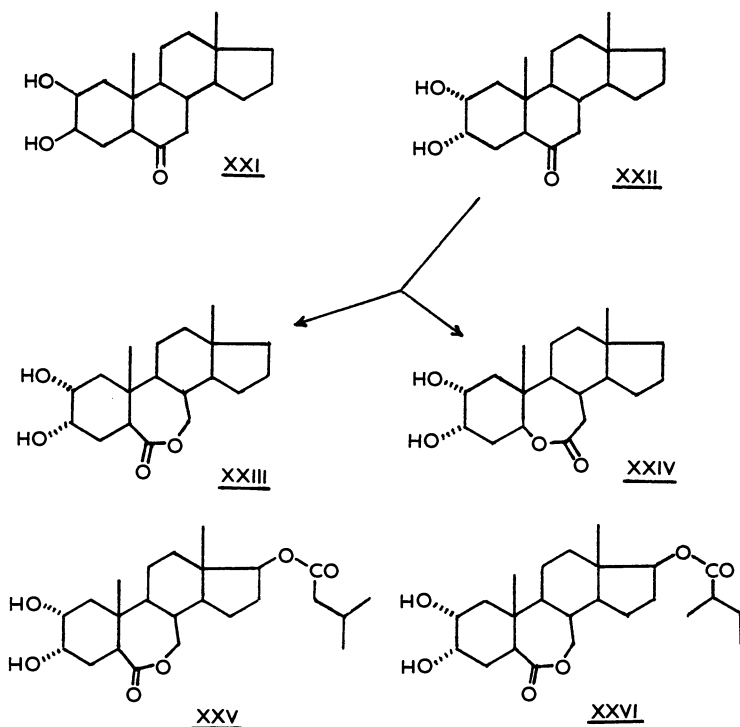


compound XIIIa elongates it by about 26 mm. This was the first example that a 6-keto analogue is more active than the corresponding lactone.

Later, we found more contradictions to structure-activity relationships as reported in the literature (5, 6). For example compound XX with 5 β -H was more active (12 mm) than compound XVII (10 mm). Both these compounds, and also compound XIX, were more active than compound XVIII. We also discovered that compound XXI which was without any substituent in position 17 exhibited quite high activity (17.5 mm). This compound is very special from another point of view also: it exhibits two peaks of almost equal activity at different application concentrations (they differ by about three orders of magnitude: 10^{-11} and 10^{-8} M in their application rate). All these compounds do not exhibit typical BR activity, i.e. swelling and splitting of the second internode; they only stimulate elongation of the bean second internode.

Therefore, we also synthesized a similar compound (XXII) with a $2\alpha,3\alpha$ -diol group which is even more similar to BR and should theoretically be more active than XXI. In the course of this synthesis we also prepared compounds XXIII and XXIV. Unfortunately, all prepared compounds exhibited very low biological activity. The most desired compound (XXII) was practically inactive. This is another example in which a compound with a $2\beta,3\beta$ -diol group (XXI) may be more active than the isomeric compound with a $2\alpha,3\alpha$ -diol group.

In addition, it is interesting that lactones XXIII and XXIV also exhibited two maxima in the bioassay, similar to compound XXI. While the activity of XXIII was very low (3 to 4 mm), the activity of XXIV was about 2.5 times higher. Hence, in this case the isomeric lactone XXIV (6-oxa-7-ketone) is again more active than the normal lactone XXIII (7-oxa-6-ketone).



In the next synthetic series we prepared compounds with two oxygen atoms in the side chain - however not as a diol but as an ester group.

Surprisingly, we found that compounds with 3-methylbutanoate group (XXV) as well as with a 2-methylbutanoate group (XXVI) in position 17β exhibited very high activity

in the bean second internode bioassay. 2-Methyl-butanoate XXVI (at 10^{-7} M applied per plant) thickened the second internode in a way similar to BR (24-epiBR, respectively), and 3-methyl-butanoate (XXV) even induced splitting of the second internode, that is they exhibited a typical BR effect.

From all that has been mentioned here it follows that there are many possible modifications in the structure of BRst which can influence activity of the molecule and, up to now, we are not able to accurately predict that activity. Even such structural features as two hydroxyl groups in positions 22 and 23 in the side chain are not necessary for a compound to exhibit either low or high biological activity.

Sensitive Bioassays for BRst

Since the discovery of the new plant growth promoter, BR, much effort has been made to evaluate its biological activity using a number of bioassay systems that are used for auxins, gibberellins and cytokinins. During the course of our investigation of structure-activity relationships of BRst, we used and increased the sensitivity of two different bean internode bioassays (4, 8, 9). These bioassays are based either on the enhancement of auxin-induced curvature of plant organs or on stimulation of organ elongation. On the basis of the results of Meudt and Thompson (10), indicating that BRst sensitize plant tissue respond to auxin, one can assume that bioassays based on auxin-specific organ curvature also respond to BRst. These bioassays are, however, usually less convenient for large scale testing. On the other hand, tests based on stimulation of stem elongation are generally less sensitive than the curvature tests (Table II).

Table II
Sensitivity of Different Bioassays for BRst

Bioassay	Detection limit (M)	Reference
Bean first internode curvature	3×10^{-10}	(11)
Sensitized bean first internode curvature	3×10^{-13}	(9)
Bean second internode elongation	2×10^{-11}	(11)
Sensitized bean second internode elongation	1×10^{-14}	(this c.) ^a
Rice lamina inclination test	1×10^{-13}	(12)
Radish seedling elongation	8×10^{-9}	(13)
Tomato hypocotyl elongation	4×10^{-9}	(13)
Wheat leaf-unrolling	1×10^{-12}	(14)
Pea inhibition	1×10^{-14}	(this c.) ^a

^a this c. = in this communication

Differences between two groups of tests can be demonstrated by the first and the second bean internode bioassays which are based on the curvature and elongation of internodes, respectively. In this chapter we would like to report: A) how the sensitivities of these two bioassays can be increased, B) to compare the activities of several synthetic BRst in the two tests, and C) to describe a new bioassay for BRst based on growth inhibition.

The First Internode Bioassay (15) is based on the curvature of the first internode section after insertion of a paper disc containing auxin to one side of the morphologically basal part of the internode. When it is used for the testing of BRst, the paper discs containing tested BRst are applied to the internode 1 h prior to auxin application. BRst stimulate the lag-phase of auxin action and its activity is expressed as a difference in internode curvature between BRst + IAA tested sections and controls treated with auxin alone.

Our improvement of this test is based on the incubation of internode sections in the inversed position (9). Under these conditions the substances undergoing test are applied to young auxin-sensitive apical internode tissues and auxin is translocated basipetally toward the zone of curvature (Figure 1). Using this simple improvement the sensitivity of the test to IAA was increased 1,000 times; as little as 10 fmoles of IAA can be detected.

High sensitivity to auxin was closely related to the elevated sensitivity to 24-epiBR (I). As shown in Figure 2, the dependence of curvature of internode sections on the amount of 24-epiBR applied is expressed by a two peak curve. When evaluated on the first peak basis, inversion of sections caused an increase in the sensitivity of bioassay by factor 100. The unusual two-peak-response is probably a result of interactions of the two growth regulators applied successively to the internode sections, that is, 24-epiBR and IAA. The first peak was always recorded when equimolar quantities of the two regulators were used.

The Bean Second Internode Bioassay (16) was adopted for BRst testing by Thompson et al. (11). Our improvement of this test is based on the following modifications (Figure 3): A. Bean seedlings were grown in Hoagland's solution diluted 1:10 from which nitrate was omitted. This resulted in shortening of the second internode in controls and enhancement of the relative effect of 24-epiBR on their elongation. Inspired by the experiments of Artheca (17) who found synergistic action of Ca^{2+} with BRst and IAA on ethylene production in bean plants, we tested the effect of Ca^{2+} concentration in nutrient solution on the sensitivity of the test under consideration. A concentration of 3 mmol.l⁻¹ was found optimal. The level of Mn^{2+}

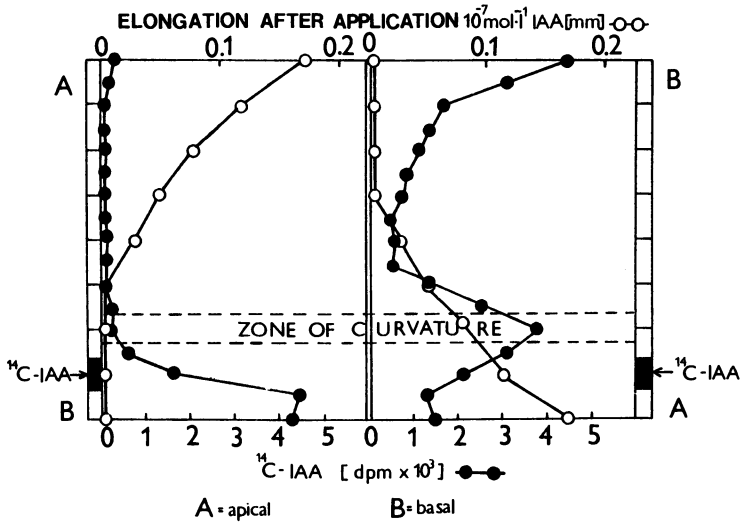


Figure 1. The effect of inversion of bean first internode sections on distribution of ^{14}C -IAA and its accumulation in auxin-sensitive zone. (Reproduced with permission from reference 9. Copyright 1985.)

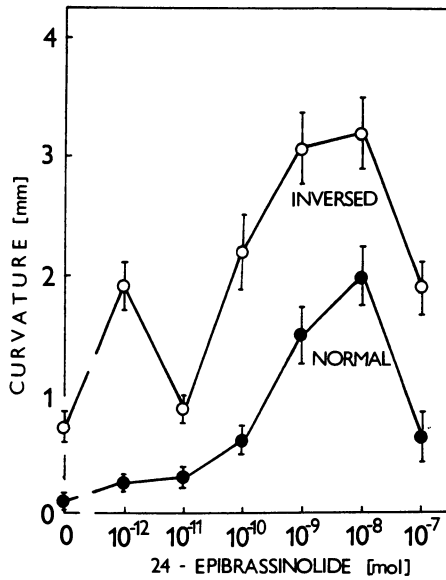


Figure 2. The effect of inversion of bean stem sections on their response to 24-epiBR. (Reproduced with permission from reference 9. Copyright 1985.)

in the nutrient solution produced a similar effect.

B. Substances under study were applied in 5 microliter drops of fractionated lanolin to the sear left after the removal of bract from the base of the second internode. A crucial condition for sensitivity in the bioassay is the length of the second internode which has to be about 2 mm long at the time of BRst application.

The second internode test is not specific to BRst. As shown in Figure 4, gibberellic acid (GA_3), IAA and even trans-zeatin are also active in this bioassay. However only BRst are able to induce internode splitting as reported by (11).

The sensitized second internode elongation bioassay is about 100 times more sensitive to 24-epiBR compared to the sensitized first internode test (Figure 5) when the first peak of activity in the later is taken into account. For this reason and because of low labour input and high reliability, the second internode bioassay can be recommended for large-scale testing of BRst preparations free of other plant growth regulators. The first internode test can be used for testing samples that are auxin free and for characterizing the specificity of their hormonal action. It is also an excellent bioassay for testing auxin activity. With respect to the double phasic response of internode curvature and also, in some cases, of internode elongation to BRst, the estimation of their biological activities must be based on testing of broad range doses of substances under the test.

Different Activities of Several Brassinosteroids in Two Bean Internode Tests. It is a well-known phenomenon that structurally closely related plant growth regulators exhibit different orders of activity in different bioassays. This makes it difficult to define the criteria of specificity of their hormonal action. The same situation exists in the case of BRst, and it can be documented by the comparison of different BRst in the two bioassays.

In addition to 24-epiBR (I), the following compounds were used as standards: II, X and (22R,23R,24R)-2 α ,3 α ,22,23-tetrahydroxy-24-methyl-5 α -cholestan-6-one (XXVII). We found that the activities of the compounds paralleled the published data (2, 3, 8, 18) and corroborate the suitability of the plant material used in the bioassay. It means that elimination of the 7-oxa function (XXVII), replacement of the side chain by an hydroxyl group (X), and removal of the 22 and 23 hydroxyl group (II) did not change the order of activity.

The substances shown in Figure 6 are new compounds synthesized previously (10). The most significant difference in bioassay activities was obtained in the case of 2 α ,3 α -dihydroxy-7-oxa-6-oxo-23,24-dinor-B-homo-5 α -cholanic acid (XXVIII) when compared with 24-epiBR. The side chain of this BRst is shorter and terminated at the position 22 with a carboxyl group. This modification makes compound XXVIII 10

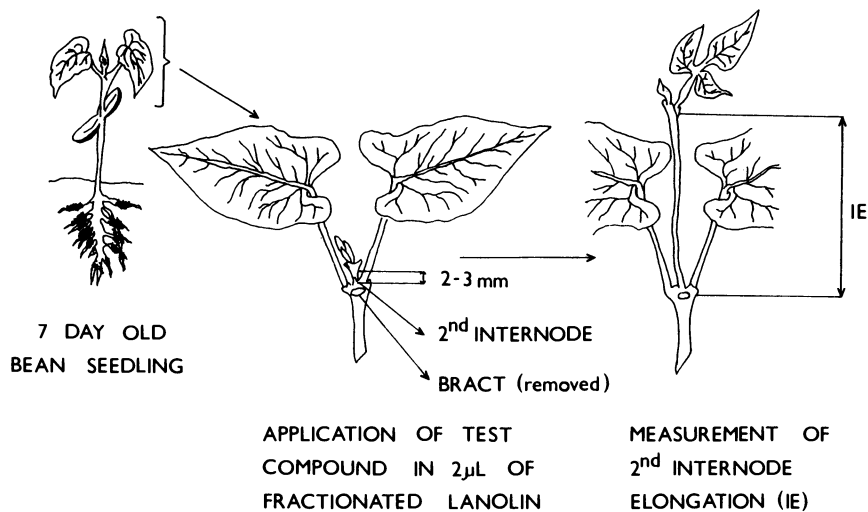


Figure 3. Scheme of the bean second internode bioassay.

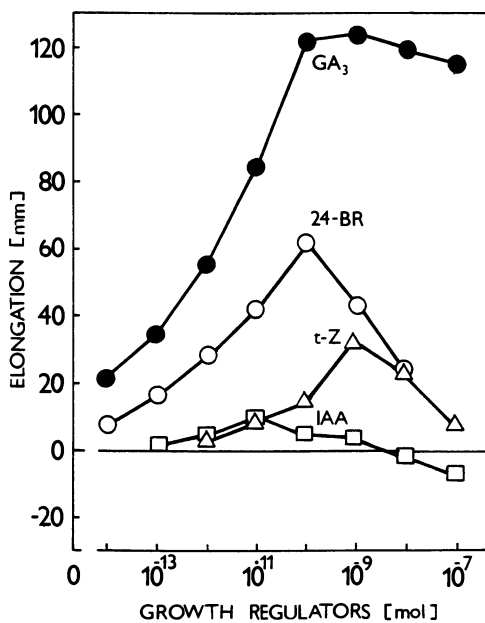


Figure 4. Effect of 24-epiBR, IAA, GA₃, and trans-zeatin on growth of the bean second internode.

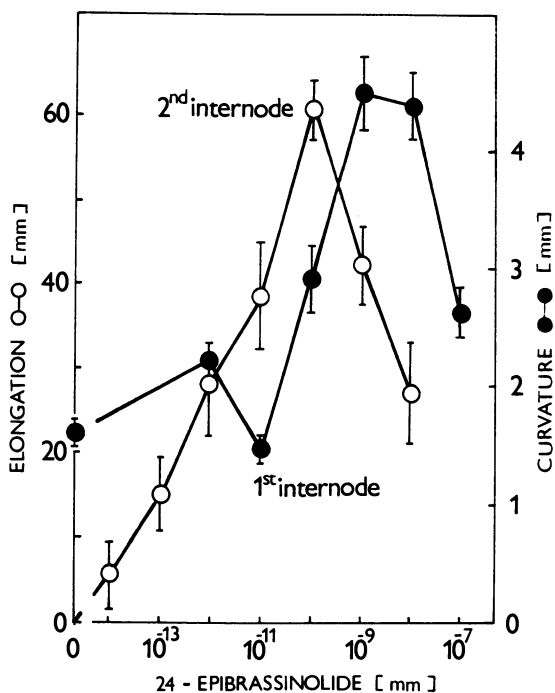


Figure 5. Effect of 24-epiBR on curvature (●—●) and elongation (o—o) of the bean internodes.

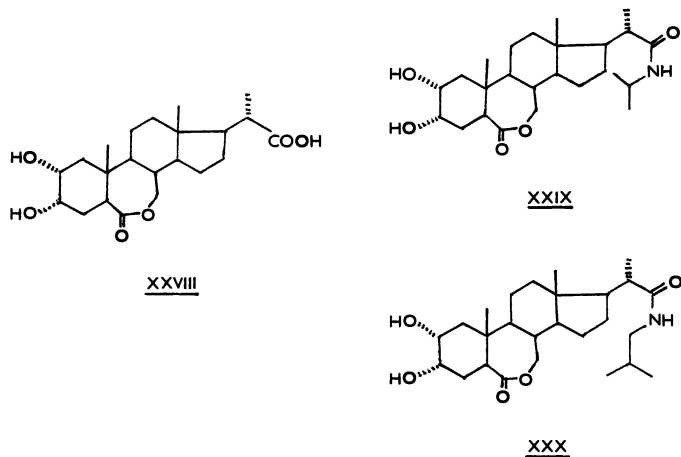


Figure 6. BRst: XXVIII and two different amides (XXIX and XXX).

times more active than 24-epiBR in the first internode curvature test (Figure 7) while in the second internode elongation test its activity is much lesser (Figure 8). Compounds XXIX and XXX with an amide group in the side chain exhibit relatively high activity in the first internode curvature test while their effect on internode elongation is much less significant. Extension of the side chain of the amide derivative by one carbon increased the activity in the second internode bioassay while decreased it in the first internode curvature test.

New Bioassay for Brassinosteroids - Pea Inhibition Test. The response of plant tissue to BRst varies with the concentration of BRst applied. In most cases, an optimum and lower BR concentration induces elongation and curvature. Supraoptimal concentrations do not inhibit growth, but split the bean second internode as the result of a unique biological response to cell division and cell elongation (11). It was shown that in all bioassay systems BRst themselves only promote growth and BRst per se are never inhibitory.

There are, however, other ways of regulating growth by BRst. For example BRst inhibit the growth of etiolated seedlings at high concentrations. This inhibition is probably caused by ethylene production which is mediated by BRst. Thus, in some cases the inhibition of growth, in particular the elongation of etiolated pea stems by high concentrations of BRst can be completely accounted for by BRst-stimulated ethylene production. The same effect was also observed after application of auxin (Figure 9). The effects of ethylene involve alteration of the normal planes of cell growth. Radial swelling or abnormal radial expansion of stems, such as that seen in the response of etiolated pea seedlings, results from inhibited elongation, increased radial expansion and probably also it accounts for leaf epinasty. These effects caused by ethylene which was induced by 24-epiBR application, were used as the basis for the development of a new bioassay for BRst (Figure 10). This test is very sensitive, because as little as 10 fmol of 24-epiBR may be detected. The elongation of the stems was linearly dependent on the logarithm of 24-epiBR concentration over four orders of magnitude (Figure 9).

On investigating the basis of the enhanced sensitivity of the present test, we found that several factors are involved.

First is the application of substances under study in fractionated lanolin as 5 microliter droplets to sear left after bract removal. A critical condition for sensitivity in the bioassay is the length of the stem which has to be approximately 5-10 mm long and must be at the early stage of the logarithmic phase of growth. Application in lanolin is important because it appears that BRst must be in continual contact with the plant. When BRst are removed, or applied in water, ethanol or another solution, the concentration of

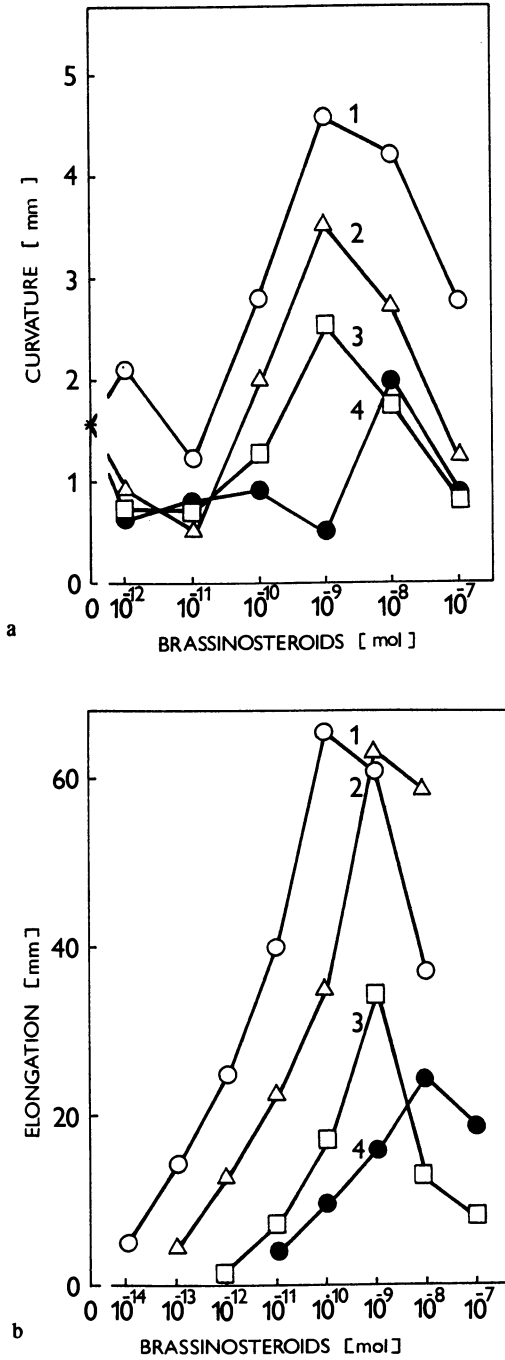


Figure 7. Effect of BRst on curvature (a) and elongation (b) of the bean internodes.

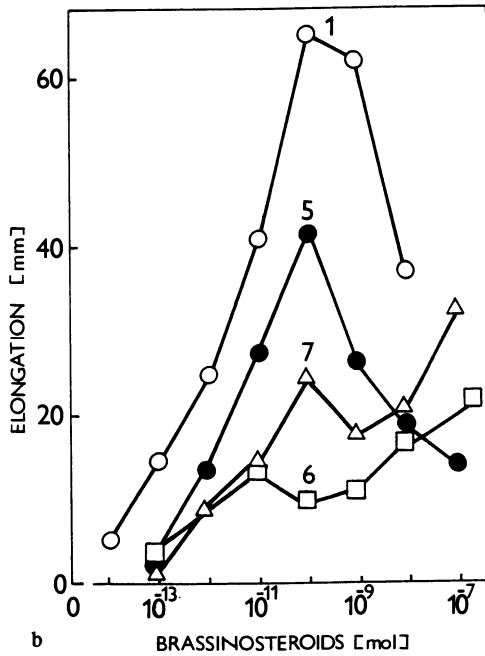
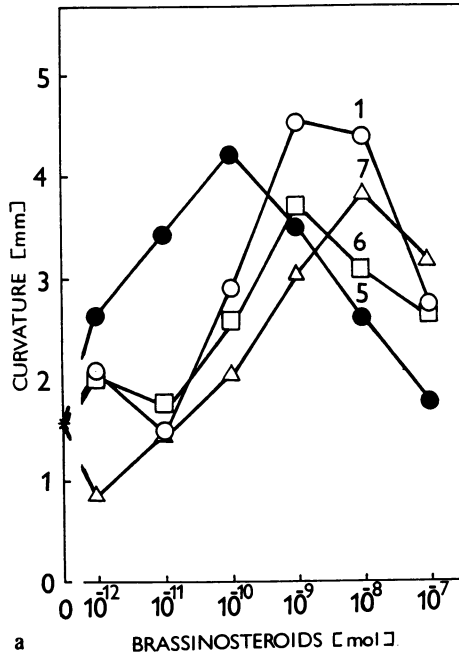


Figure 8. Effects of BRst on curvature (a) and elongation (b) of the bean internodes.

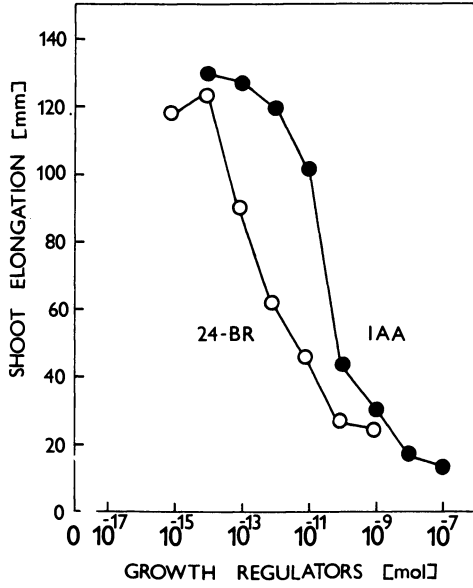


Figure 9. Effect of various IAA and 24-epiBR concentrations on growth of etiolated pea seedlings.

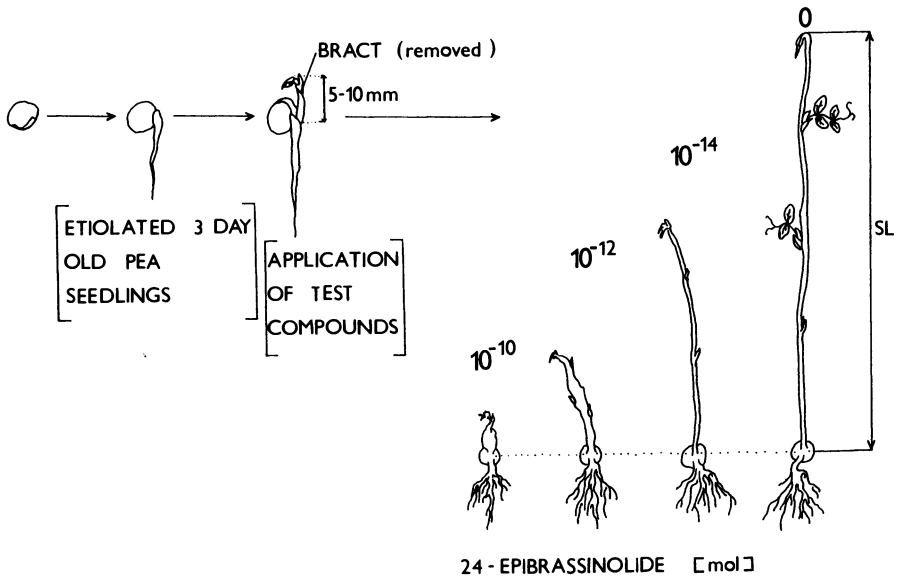


Figure 10. Scheme of the pea inhibition test.

ethylene probably rapidly decreases and plants start to elongate. Moreover, as the etiolated tissue greens it becomes less sensitive to BRst. Therefore, it is quite important that plants must be kept either in dark or under a green light (540 nm) and all operations must be carried out also under these conditions.

The last factor influencing the sensitivity of the test is cultivar of the pea plants. Comparing about 20 different cultivars we found that in many cultivars the growth of pea stems does not continually decreased with the increase of 24-epiBR concentration and the growth response is often biphasic (Figure 11). Cultivar "Amino" was found suitable for assays - it grows fast and the elongation response to applied BRst is not multiphasic.

Factors influencing the sensitivity and specificity of this test are under further investigation and will be described in detail elsewhere.

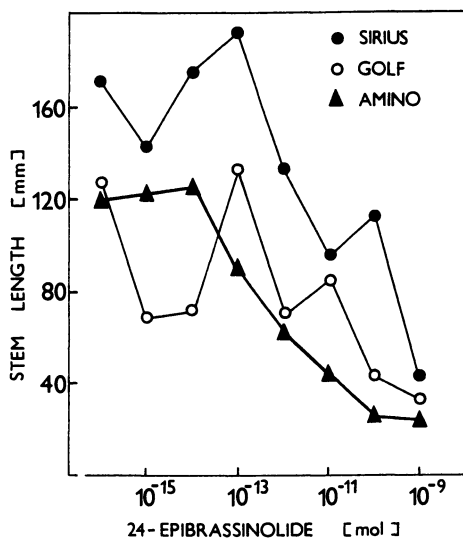


Figure 11. Sensitivity of different pea cultivars to 24-epiBR.

References

1. Grove, M.D.; Spencer, G.F.; Rohwedder, W.K.; Mandava, N.; Worley, J.F.; Warthen, J.D.jr.; Steffens, G.L.; Flippen-Anderson, J.L.; Cook, J.C.jr. *Nature* 1979, 281, 216.
2. Thompson, M.J.; Meudt, W.J.; Mandava, N.B.; Dutky, S.R.; Lusby, W.R.; Spaulding, D.W. *Steroids* 1982, 39, 89.
3. Kondo, M.; Mori, K. *Agric. Biol. Chem.* 1983, 47, 97.
4. Kohout, L.; Strnad, M. *Coll. Czech. Chem. Commun.* 1986, 51, 447.
5. Meudt, W.J.; Thompson, M.J.; Mandava, N.B. *Plant Physiol.* 1982, 69 (4th Suppl.), 12.

6. Takatsuto, S.; Ikekawa, N.; Morishita, T.; Abe, H. *Chem. Pharm. Bull.* 1987, 35, 211.
7. Kohout, L.; Velgova, H.; Strnad, M.; Kaminek, M. *Coll. Czech. Chem. Commun.* 1987, 52, 476.
8. Cerny, V.; Strnad, M.; Kaminek, M. *Coll. Czech. Chem. Commun.* 1986, 51, 687.
9. Strnad, M.; Kaminek, M. *Biol. Plant.* 1985, 27, 209.
10. Meudt, W.J.; Thompson, M.J.; Malcolm, J. *Proc. Natl. Growth Regul., Soc. Am.* 1984, 10, 306.
11. Thompson, M.J.; Mandava, N.B.; Meudt, W.J.; Lusby, W. R.; Spaulding, D.W. *Steroids* 1981, 38, 567.
12. Wada, K.; Marumo, S.; Abe, H.; Morishita T.; Nakamura, K.; Uchyama, M.; Mori, K. *Agr. Biol. Chem.* 1984, 48, 719.
13. Takatsuto, S.; Yazawa, N.; Ikekawa, N.; Takematsu, T.; Takeuchi, Y.; Koguchi, M. *Phytochem.* 1983, 22, 2437.
14. Wada, K.; Kondo, H.; Marumo, S. *Agric. Biol. Chem.* 1985, 49, 2249.
15. Meudt, W.J.; Bennett, H.W. *Physiol. Plant.* 1978, 44, 422.
16. Mitchell, J.W.; Livingstone, G.A. *Methods of Studying Plant Hormones and Growth Regulating Substances; Agricultural Handbook; No. 336; US Government Printing Office; Washington, D.C.* 1968, p. 26.
17. Artheca, N.B. *Physiol. Plant.* 1984, 62, 102.
18. Akhrem, A.A.; Lakhvich, F.A.; Khripach, V.A.; Kovganko, N.B. *Dokl. Akad. Nauk SSSR, Ser. Khim.* 1983, 269, 266.

RECEIVED July 16, 1991

Chapter 7

Aspects of Synthesis and Bioactivity of Brassinosteroids

Günter Adam¹, V. Marquardt¹, H. M. Vorbrodt¹, C. Hörhold²,
W. Andreas³, and J. Gartz⁴

¹Institute of Plant Biochemistry, Weinberg 3, Halle/Saale,
O-4050, Germany

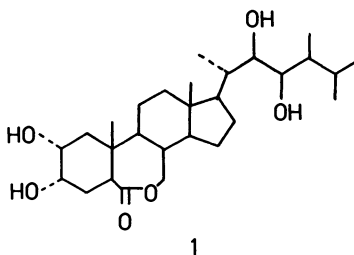
²Institute of Microbiology and Experimental Therapy, Beutenbergstrasse
11, Jena, O-6900, Germany

³Applied Research Division, Stickstoffwerke AG Piesteritz, Cunnersdorf,
O-7101, Germany

⁴Institute of Biotechnology, Leipzig, O-7050, Germany

The synthesis and bioactivity of new types of brassinosteroid analogs as well as original biological effects of brassinosteroids on radish and higher fungi are presented. Our chemical work involved pathways to the following three groups of structural analogs: 2,3-*seco* brassinosteroids, 5,6-*seco* compounds and analogs with a spirostan side chain moiety. Contrary to the 5,6-*seco* series some prepared 2,3-*seco* compounds were shown to be quite active in the rice lamina inclination test, for example the 2,3-*seco* tetrahydroxy lactone 11 showing 80% of the response of (22S,23S)-homobrassinolide 3. A similarly high bioactivity was obtained with the spirostan analogs 23 and 29. In first microbial transformations from (22S,23S)-homocastasterone 18 and 3 the corresponding 12 β -hydroxylated brassinosteroids 33 and 34, respectively, were obtained by fermentation with *Cunninghamella echinulata*. In biological experiments with radish cotyledons brassinosteroids induced a marked increase of invertase activity probably due to an activation of the *de novo* synthesis of this enzyme. Furthermore, not only was strong growth promotion observed in mycelial cultures of *Psilocybe cubensis* and *Gymnopilus purpuratus* for the first time, but also stimulating effects of brassinosteroids have been found on higher fungi, suggesting an additional physiological role of these compounds in lower plants.

Since the discovery of brassinolide 1 in 1979 (1) brassinosteroid research has been a field of intense activity. With respect to further chemical work, efforts were directed to the discovery of new



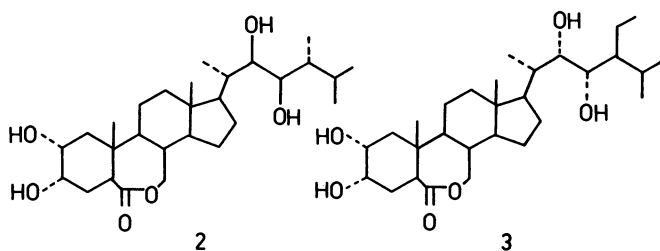
naturally occurring brassinosteroids as well as to the synthesis of native members and structural analogs for further biological investigations (2,3). Extensive studies on the physiology and molecular mechanisms of this new group of steroidal phytohormones were conducted in many laboratories and investigations are under way to check their practical application as ecologically safe bioregulators and stressmodulators both in agriculture and horticulture (4,5). An essential link within these interdisciplinary research activities are the structure-activity relationships of brassinosteroids which are under further development and need additional investigations. In this contribution our recent results on the synthesis and bioactivity of new types of brassinosteroid analogs having an opened ring A and B of the steroidal skeleton, respectively, as well as the preparation of spirostan analogs will be discussed. Also the first biotransformations of the brassinosteroids are presented. These original biological studies involve investigations on the influence of brassinosteroids on growth and invertase activity in radish cotyledons and growth promoting effects observed for the first time on *Basidiomycetes*.

Synthesis of New Brassinosteroid Analogs

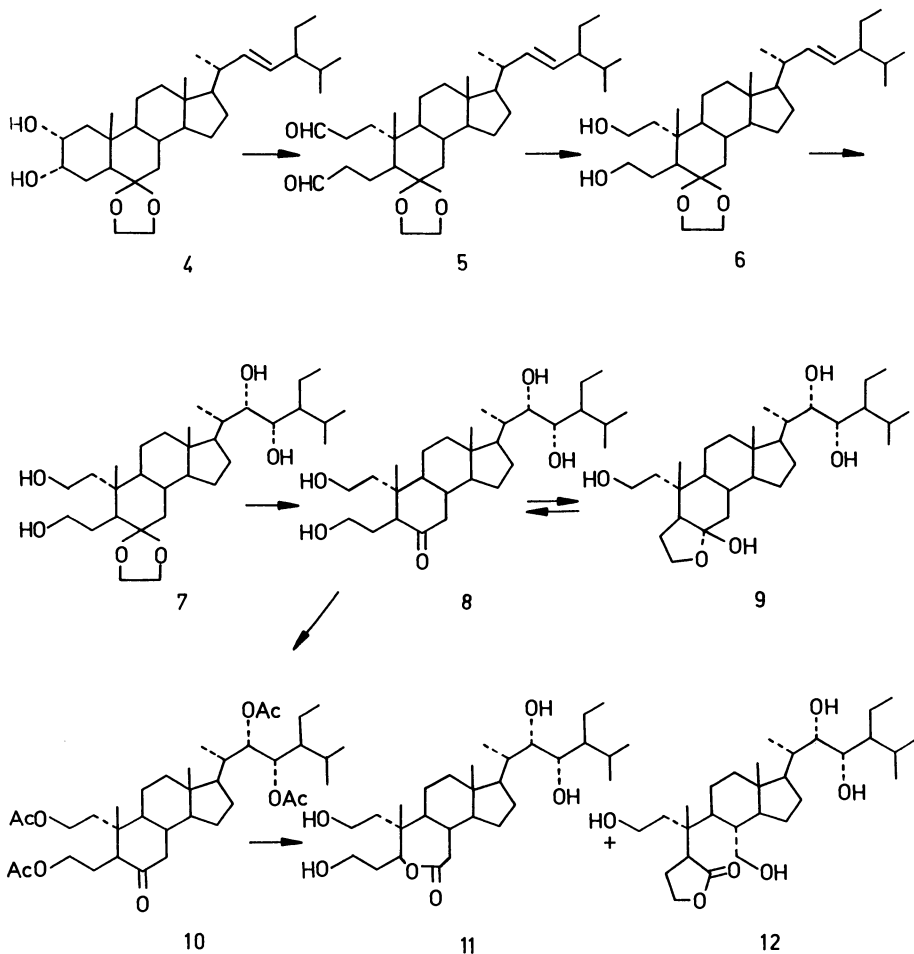
Of special interest for practical applications, as well as for future receptor studies, are the structure-activity relationships of the brassinosteroids. As postulated (6) the structural requirements for a high activity are the following: (22R,23R)-vicinal diol moiety; (24S)-methyl- or ethyl group; 7-oxa-lactone or 6-oxo functionality; 3 α -hydroxy group, 2 α ,3 α -vicinal diol or 3 α ,4 α -vicinal diol and A/B-trans-fused ring junction.

However, some side chain epimers like 24-epibrassinolide 2, trisepi-brassinolide and (22S,23S)-homobrassinolide 3 show remarkable activity and are therefore used widely for biological experiments. Furthermore several structural analogs with a shortened side chain moiety are active in specific test systems (7). Thus, structure-activity relationships of brassinosteroids are a field of continual development and require further examination.

For an interaction to occur with a putative brassinosteroid receptor, both stereospecifically arranged vicinal diol functions in the ring A, as well as in the side chain, can be assumed as important. In our synthetic program for modified brassinosteroids, we were especially interested in new types of analogs with the ring A- and B-seco structure to study the influence of the geometry of this molecular adaptation on biological activity.

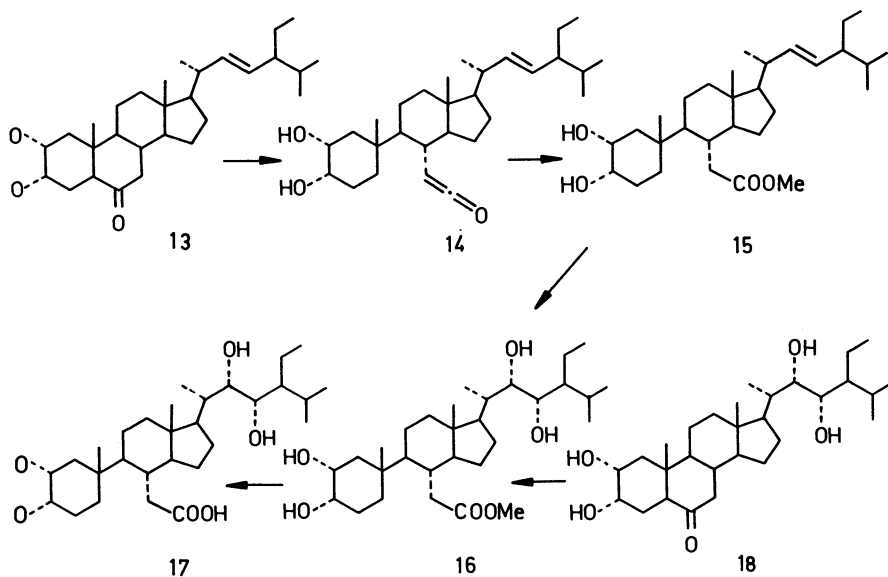


2,3-*seco* Brassinosteroids. Our synthesis of brassinosteroid analogs with the 2,3-*seco* structure involves, as a key step, the periodate cleavage of the acetal protected 2 α ,3 α -dihydroxy-6-ketone **4**, which is readily available from stigmasterol (**8**). The unstable product, dialdehyde **5**, was immediately reduced with NaBH₄ to give the diol **6**. Hydroxylation of the diacetate of **6** with OsO₄/NMMNO, and deacetylation, yielded the tetrahydroxy compound **7**. The deprotected 6-ketone **8** was found to be in an equilibrium with its hemiacetal **9**. Baeyer-



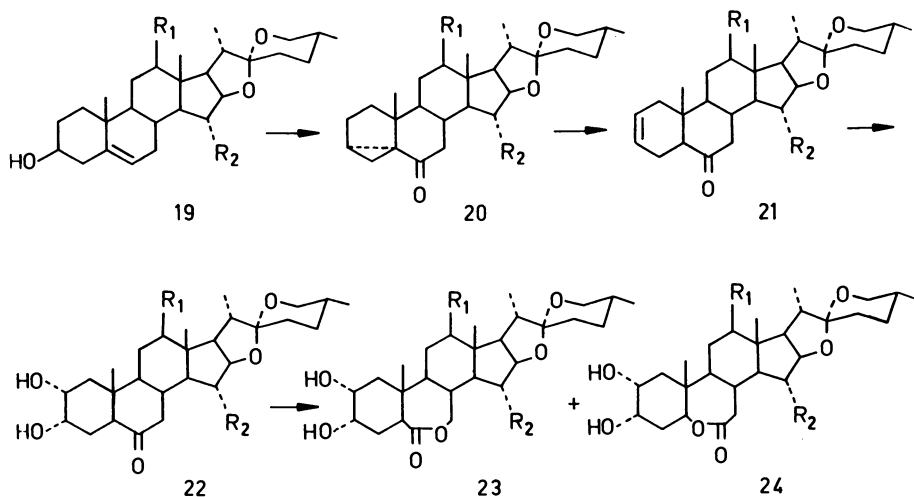
Villiger oxidation of the tetraacetate 10 with $\text{CF}_3\text{CO}_2\text{H}$, and saponification, afforded a 3:1 ratio of the 2,3-*seco*-6-oxalactone 11 and the 5-membered lactone 12, the latter arising from an intramolecular transesterification of the corresponding 7-oxa-lactone (9).

5,6-*seco* Brassionosteroids. For preparation of 5,6-*seco* analogs, the 2 α ,3 α -dihydroxy-6-ketone 13 was irradiated in methanol to afford, via a Norrish type I mechanism, the ketene 14, which reacted spontaneously with the solvent to give the corresponding 5,6-*seco* ester 15. Hydroxylation of the Δ^{22} -double bond to 16 followed by saponification led to the tetrahydroxylated 5,6-*seco* carboxylic acid 17, which could be obtained alternatively by irradiation of (22S,23S)-homocastasterone 18 via 16 (10).

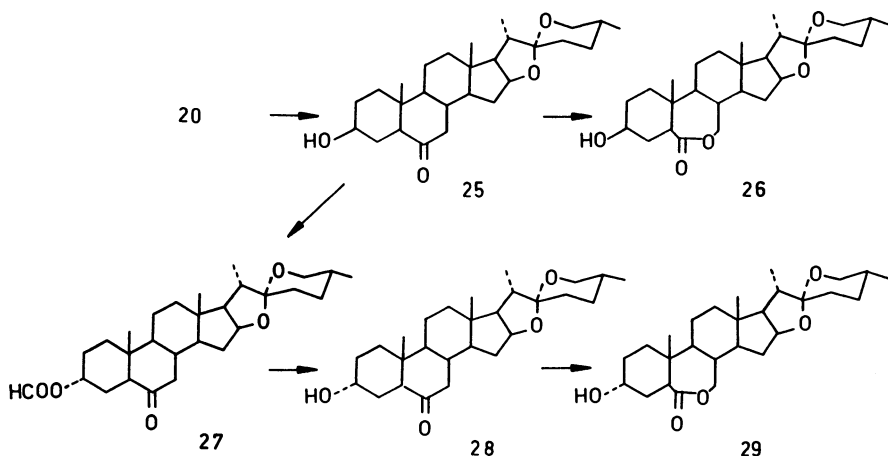


Spirostan Analogs. In our program for the synthesis of modified brassinosteroids, we further developed a reaction sequence leading to new types of analogs with the spirostan side chain moiety. Diosgenin 19, ($R_1=R_2=H$) and other spirostans, for example isochiapagenin 19, ($R_1=OH, R_2=H$) or bahamgenin 19, ($R_1=R_2=OH$), are interesting starting compounds, which are readily available by extraction of plant sources like *Dioscorea* species or *Solanum bahamense*. Such spirostans possess the suitable prerequisites for an easy introduction of the A/B ring functionalities of brassinolide 1, show the presence of oxygen functionalities in the position C-22 and exhibit (in the case of isochiapagenin and bahamgenin, for example) additional functionalities in the C/D rings, which are of special interest for structure-activity considerations.

The reaction sequence used for the introduction of the A/B ring structural features is given in the structural diagrams. The synthesis was carried out by starting with diosgenin 19, ($R_1=R_2=H$) via tosylation, followed by isomerization with KHCO_3 /acetone and oxida-

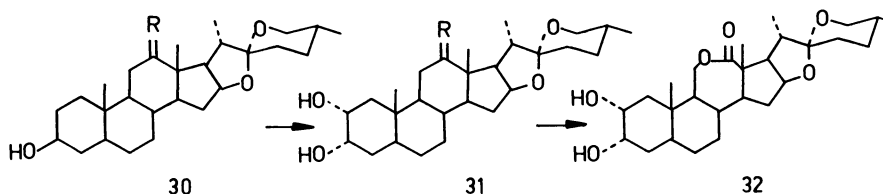


tion with the CrO₃/pyridinium complex to afford 3 α ,5-cyclo-6-ketone 20. For the isomerization of 20 to the Δ^2 -6-ketone 21 the best conditions were found to be refluxing with pyridinium hydrobromide in DMF after attempting different procedures. Hydroxylation with OsO₄/NMMNO to the 2 α ,3 α -diol 22 and Baeyer-Villiger oxidation of its diacetate with MCPBA afforded the desired 7-oxa-6-oxo lactone 23 besides approximately 15% of the isomeric 6-oxa-7-oxo lactone 24. The corresponding spirostan analogs with hydroxy functions in rings C and D were synthesized starting with isochiapagenin 19, (R₁=OH, R₂=H,) and bahamgenin 19, (R₁=R₂=OH) via their 12 β -acetyl and 12 β ,15 α -diacetyl derivatives, respectively; the latter were both obtained by partial hydrolysis of the corresponding completely acetylated steroids with LiOH. The introduction of the lactone functionality involved the same steps as described for the diosgenin analog (11).



The synthesis of typhasterol and teasterone analogs possessing the spirostan side chain was achieved starting with 3 α ,5-cyclo-6-ketone 20. Reaction with sulfuric acid/ acetic acid and hydrolysis afforded the teasterone counterpart 25. Mesylation of 25, followed by refluxing with Li₂CO₃ in DMF yielded, under rearrangement, the 3 α -formiate 27 and also in equal amounts the Δ^2 -6-ketone 21. Hydrolysis of 27 gave the typhasterol analog 28. Baeyer-Villiger oxidation of both 6-ketones 25 and 28 afforded the corresponding 6-oxo-7-oxa lactones 26 and 29, respectively.

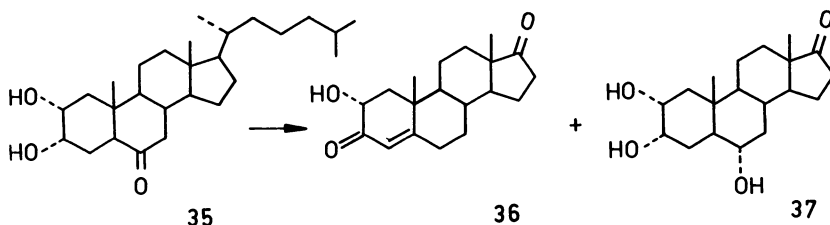
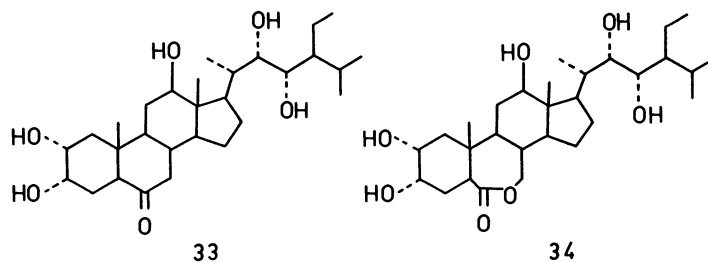
Furthermore, starting with hecogenin 30, (R = O), compounds with a lactone function in the C ring and without functionality in the B ring were synthesized. Introduction of the vicinal diol moiety in the A-ring via mesylation, elimination and hydroxylation with OsO₄/NMMNO to the 2 α ,3 α -dihydroxy-12-ketone 31, followed by Baeyer-Villiger oxidation with MCPBA yielded the 12-oxa-12 α -oxo compound 32 and also equal amounts of the 12-oxo-12 α -oxa isomer. Similarly, starting with tigogenin 30, (R = H₂), a 2 α ,3 α -dihydroxylated compound 31, without functions in the rings B, C and D, was synthesized (Marquardt, V.; Adam, G.; Alonso, E.; Coll, F., unpublished).



Microbial Transformation of Brassinosteroids. Until now, information concerning the microbial transformations of brassinosteroids has not been published. Such biotransformations could open novel pathways to additionally functionalized members for structure-activity investigations as well as provide information about possible metabolic processes of such compounds. Furthermore, the intermediate metabolites may have practical application.

From qualitative screening with a series of fungi and bacteria, we selected the fungus *Cunninghamella echinulata* as a primary model for more detailed studies. For other steroids, the hydroxylating potential of this microorganism has been reported as leading to 6 β -, 11 α - and 11 β -functionalized compounds (12, 13). In our case the biotransformation of (22S,23S)-homocastasterone 18 and (22S,23S)-homobrasinolide 3 was carried out in a submerged culture of the fungus at 28 °C using malt-yeast extract as the medium. Chloroform extraction and SiO₂ chromatography afforded, in both cases, more polar main biotransformation products. Their structures were elucidated by spectroscopy, especially NMR, as 12 β -hydroxylated brassinosteroids 33 and 34, respectively, representing the first brassinosteroids functionalized in that position (14).

In other model experiments, we studied the biotransformation of 2 α ,3 α -dihydroxy-5 α -cholestan-6-one 35, prepared from cholesterol, having the structural functions of castasterone only in the A/B ring moiety. In that case, the fermentation with *Mycobacterium vaccae* led, via side chain degradation accompanied by typical transforma-



tions in rings A/B, to both 17-ketones **36** and **37** yielded an insight into the possible metabolic sequences of the castasterone type possessing the 2 α ,3 α -dihydroxy-6-oxo structural feature (Vorbrodt, H.M.; Adam, G.; Porzel, A.; Hörhold, C.; Dähnhardt, S.; Böhme, K.H. *Steroids*, in press).

Biological Aspects

Structure-Activity Relationships of Synthesized Analogs. In our studies on structure-activity relationships, the highly sensitive and specific rice lamina inclination test (15) was used as the assay for our synthesized substances (Table I). (22S,23S)-homobrassinolide **3** and 24-epibrassinolide **2** were used as standards.

Table I. Biological Activity of Selected Brassinosteroid Analogs in the Rice Lamina Inclination Test

Compound N°	Degree of bending relative to control at a concentration of					(ppm)
	10	5	1	0,5	0,1	
2	106	105	93	83		
3	102		99	95	85	
8	78	72	38		4	
11	109		38	25		
12	77		33		14	
16	50		22			
17	37		25			
22 (R ₁ =R ₂ =H)	11		1			
23 (R ₁ =R ₂ =H)	76	47				
23 (R ₁ =OH, R ₂ =H)	21		10		20	
28	35		32		25	
29	84	56				
34	96	80			75	

In comparing both seco series, we noted that in general the 2,3-seco compounds were shown to be more active than the 5,6-seco derivatives. The observed response of the 2,3-seco-tetrahydroxyketone 8 and of the 5-membered lactone 12 attained approximately 80% of the response of (22S,23S)-homobrassinolide 3 at 10 ppm, although activity decreased rapidly at lower concentrations. Interestingly, even the 2,3-seco analog 11, possessing the 6-oxa-7-oxo lactone functionality, a structural feature which normally deletes brassinosteroid activity, gave nearly the same response as the standards 2 and 3. These results suggest that in spite of ring opening between C-2/C-3 the hydroxy functionality in compounds 8, 11 and 12 is capable of markedly contribute to bioactivity.

Compared to these results the response of the 5,6-seco analogs 16 and 17 was shown to be considerably lower to those of the 2,3-seco compounds. This reflects a more substantial change in the conformation of the molecular structure in these compounds, probably caused by rotation around the C-9/C-10 bond.

Also, in the synthesized spirostan series, especially for the diosgenin analog 23 and the corresponding 3 α -hydroxy compound 29, the observed responses in the rice lamina inclination test were distinct. The found activity for both analogs was determined to be nearly 80% of the value for the standard 24-epibrassinolide 2 at 10 ppm. Similarly, as established for native brassinosteroids, activity decreased to approximately 10% in going from the 6-oxo-7-oxa lactone to the 6-keto type as shown for compounds 22 and 23. Introduction of a 12 β -hydroxy group, as in 23 (R₁= OH, R₂= H) suppressed the response to 25% of the parent compound. All other synthesized spirostan analogs showed no, or negligible activity (Creuzburg, D.; Vorbrodt, H.M.; Marquardt, V.; Adam, G., unpublished).

These results lead to the conclusion that for brassinosteroid activity the spirostan side chain moiety is able to substitute, at least partially, for the 22,23-vicinal diol function.

The 12 β -hydroxylated (22S,23S)-homobrassinolide 34, obtained from 3 by microbial transformation showed, at concentrations of 10 ppm and 5ppm, the same response as the parent (22S,23S)-homobrassinolide 3. However, on going to 1ppm its activity decreased rapidly.

Brassinosteroid-Induced Stimulation of Growth and Invertase Activity in Radish Cotyledons. Brassinosteroids act as powerful plant growth promoters stimulating the elongation of young stem tissues in a series of test systems. Among the more than 20 applied bioassays the bioassay using *Raphanus sativus* (16) is useful as a quite sensitive whole plant assay. The occurrence of endogenous brassinosteroids in *Raphanus sativus* was established only very recently, where castasterone and brassinolide 1 have been identified from seeds by GC-MS analysis (17). Comparative quantification experiments with seeds and germinated seeds carried out by GC-SIM technique showed a remarkable shift in the ratio of both constituents from 0.4:1 to 1:1.1 in favour of the formation of brassinolide 1 during germination. This supports the suggestion that castasterone represents a biogenetic precursor of brassinolide 1.

In biological experiments with *Raphanus sativus* (var. Remo) we could also establish the growth stimulating activity of brassinosteroids on isolated radish cotyledons. Furthermore, we have found that this effect is accompanied by a distinct activation of invertase (Andreas, W.; Vorbrodt, H.M.; Adam, G., unpublished).

In these studies, a modified radish cotyledon test used as a standard bioassay for cytokinins (18), was employed. Treatment with brassinolide 1 and (22S,23S)-homobrassinolide 3 resulted in a large fresh-weight gain of radish cotyledons. With brassinolide 1, this effect is markedly distinct at $2 \cdot 10^{-10}$ M leading to a weight increase of 60% with a concomitant increase at higher concentrations. At $2 \cdot 10^{-5}$ M brassinolide 1 the weight increase amounts to 166%. As expected, (22S,23S)-homobrassinolide 3 showed a weaker growth promoting effect with an observed fresh weight increase of 48 and 120% at concentrations $2 \cdot 10^{-6}$ and $2 \cdot 10^{-4}$ M, respectively (Figure 1). A similar stimulating effect connected with H^+ -extrusion in isolated cotyledons has been reported for 24-epibrassinolide 2 (19).

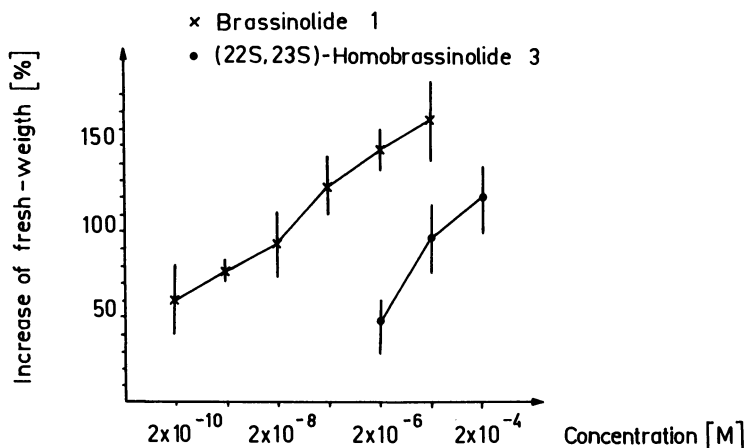


Figure 1 Effect of brassinosteroids on the growth of radish cotyledons

The radish cotyledon test was originally developed as a standard bioassay for kinetin (18) and the lowest threshold for activity is $5 \cdot 10^{-8}$ M. In our hands using $2 \cdot 10^{-5}$ M kinetin as the control caused a fresh weight increase of 117% indicating an activity of about the same order as (22S,23S)-homobrassinolide 3. The observed data show that the fresh-weight gain of radish cotyledons can be used also as a bioassay for brassinosteroids. The sensitivity is very high lying in the same order of magnitude as the mung bean epicotyl bioassay which is used for brassinosteroids (20). In promoting expansion of cucumber cotyledons, brassinolide 1 was found to be half as effective as kinetin. However, in three other cytokinin bioassays brassinolide 1 was ineffective.

It has been assumed (21) that the fresh-weight gain of radish cotyledons by kinetin is due to the activation of *de novo* synthesis of the hydrolytic enzyme invertase. Seeking a similar influence of brassinosteroids on this enzyme we also found, that brassinolide 1 and (22S,23S)-homobrassinolide 3 treatment resulted in an increased activity of the soluble invertase even to a remarkably higher extent than kinetin in the case of brassinolide 1 (Figure 2). This increase

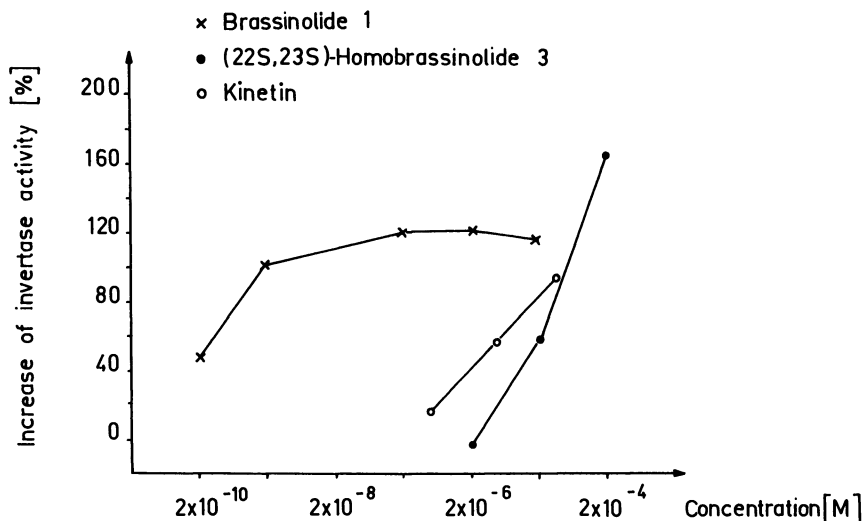


Figure 2 Promotion of the invertase activity by brassinosteroids compared to kinetin

in enzyme activity appears after a lag-phase between 12 and 24 hours and is connected with increased protein production. Thus, our results suggest that brassinosteroids seem to stimulate the *de novo* synthesis of invertase which is considered to represent an important enzyme in plant growth processes (22).

Growth Stimulation on Higher Fungi. In many publications the growth stimulating and antistress effects of brassinosteroids on higher plants have been described indicating promising results for increasing crop yields and fruit quality as well as overcoming unfavourable environmental factors. However, until now no studies about the biological effects of brassinosteroids on fungi have been published. For our first studies with higher fungi we selected the *Basidiomycete*, *Psilocybe cubensis* (Earle) Singer, as a suitable species. This subtropical fungus is well characterized by high growth stability under different cultivation conditions (23). The cultivation of the mycelium was carried out on malt agar and on a horse dung/rice grain mixture. Using (22S,23S)-homobrassinolide 3, a strong promoting effect on mycelial growth and fruiting has been observed (24). Thus, in experiments with agar using 10^{-2} ppm (22S,23S)-homobrassinolide 3 a 2 to 3 times faster mycelial growth was established relative to control. In the dung/grain substrate, addition of 3 caused a fruiting of the mycelium in 3 - 3.5 weeks, whereas in the control the sporocarps were formed within 4 to 5 weeks. Furthermore, brassinosteroid treatment increased the number of fruiting bodies in the first flush and caused a distinctly higher dry-mass than observed in the controls (Table II). The normally observed formation of incomplete fruit bodies was almost suppressed by brassinosteroid. Comparable effects were found for *Psilocybe cubensis* with brassinolide 1 and 24-epibrassinolide 2 as well as with (22S,23S)-homobrassinolide 3 using the second fungal species *Gymnopilus purpuratus* (Gartz, J.; Adam, G.; Vorbrod, H.M.; Marquardt, V., unpublished). The observed strong growth promoting effects in mycelial cultures of

Table II Growth-promoting Effect of (22S,23S)-Homobrassinolide 3 in Mycelial Cultures of *Psilocybe cubensis* (Earle) Singer

	Cultivation with 10 ⁻² ppm brassinosteroid	Control
Biomass (dry-mass)	3.4 - 3.7 g	2.3 - 2.7 g
First sporocarps produced (weeks)	3 - 3.5	4 - 4.5
Number of fruiting bodies (first flush)	4 - 7	1 - 2

both fungi suggest that brassinosteroids probably play a physiological role in higher fungi also although the natural occurrence hitherto has not been reported. In addition, the practical use of brassinosteroids in fungi of commercial value to produce fruiting bodies from mycelium, *in vitro*, is suggested from our findings. Thus, we are pursuing further studies in this direction.

Literature cited

- Grove, M.D.; Spencer, G.F.; Rohwedder, W.K.; Mandava, N.B.; Worley, J.F.; Warthen, J.D.; Steffens, G.L.; Flippen-Anderson, J.L.; Cook, J.C. *Nature* 1979, 281, 216.
- Adam, G.; Marquardt, V. *Phytochemistry*, 1986, 25, 1787.
- Khripach, V.A. *Pure Appl. Chem.* 1990, 62, 1319.
- Meudt, W.J. In "Ecology and metabolism of plant lipids", Fuller, G.; Ness, W.D., Eds., *ACS Symposium Ser.* 1987, 325, 53.
- Hamada, K. *FFTC Book Ser.* 1986, 34, 188.
- Takatsuto, S.; Ikekawa, N.; Morishita, T.; Abe, H. *Chem. Pharm. Bull.* 1987, 35, 211.
- Cerny, V.; Strnad, M.; Kaminek, M. *Collect. Czech. Chem. Comm.* 1986, 51, 687.
- Mori, K.; Sakakibara, M.; Ichikawa, Y.; Ueda, H.; Okada, K.; Umemura, T.; Yabuta, G.; Kuwahara, S.; Kondo, M.; Minobe, M.; Sogabe, A. *Tetrahedron* 1982, 38, 2099.
- Kien, N.T. *Dissertation*, Halle 1990.
- Adam, G.; Vorbrodtt, H.M.; Marquardt, V.; Kien, N.T.; Porzel, A. *Proc. 5th Conf. Chem. Biotechnol. Biol. Active Nat. Prod.* Varna 1989, 3, 159.
- Marquardt, V.; Adam, G.; Coll, F.; Alonso, E. *German (East)* 1988, DD 273 638.
- Mahato, S.B.; Banerjees, S.; Podder, S. *Phytochemistry* 1989, 28, 7.
- Mahato, S.B.; Mukherjee, A. *Phytochemistry* 1984, 23, 2131.
- Adam, G.; Vorbrodtt, H.M.; Hörhold, C.; Böhme, K.H.; Dähnhardt, S.; Porzel, A. *German (East)* 1989, WP C12P 3 326 921.
- Wada, K.; Marumo, S.; Abe, H.; Morishita, T.; Nakamura, K.; Uchiyama, M.; Mori, K. *Agric. Biol. Chem.* 1984, 48, 719.
- Takatsuto, S.; Yazawa, N.; Ikekawa, N.; Takematsu, T.; Takeuchi, Y.; Koguchi, M. *Phytochemistry* 1983, 22, 2437.
- Schmidt, J.; Yokota, T.; Adam, G.; Takahashi, N. *Phytochemistry* 1991, 30, 364.
- Letham, D.S. In "Plant Growth Substances 1967", Wightman, F., Ed., Runge Press Ltd., Ottawa, Canada, 1968, 19.

19. De Michelis, M.; Lado, P. *Physiol. Plant.* 1986, *68*, 603.
20. Gregory, L.E.; Mandava, N.B. *Physiol. Plant.* 1982, *54*, 239.
21. Howart, H.F.; Witham, F.H. *Plant Physiol.* 1983, *73*, 304.
22. Morris, D.A.; Arthur, E.D. *Physiol. Plant.* 1985, *65*, 257.
23. Gartz, J. *J. Basic Microbiol.* 1989, *23*, 347.
24. Gartz, J.; Adam, G.; Vorbrodt, H.M. *Naturwissenschaften* 1990, *77*, 388.

RECEIVED March 12, 1991

Chapter 8

Metabolism and Biosynthesis of Brassinosteroids

Takao Yokota¹, Y. Ogino², H. Suzuki², N. Takahashi², Hiroshi Saimoto³,
Shozo Fujioka⁴, and Akira Sakurai⁴

¹Department of Biosciences, Teikyo University, Utsunomiya 320, Japan

²Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku,
Tokyo 113, Japan

³Somar Corporation, Soka-shi, Saitama 340, Japan

⁴Institute of Physical and Chemical Research, Wako-shi, Saitama 351-01,
Japan

Castasterone was found to be a biosynthetic precursor of brassinolide using *Catharanthus roseus* crown gall cells. However, castasterone was not converted to brassinolide in tissues of mung bean and rice, indicating that growth-promoting activity of castasterone observed in these tissues was exerted by castasterone itself.

Brassinosteroids are distributed in a wide range of plants including dicots, monocots, gymnosperms and algae (1-3). Recently, fern was found to contain brassinosteroids (4). And Park and his coworkers found that *Catharanthus roseus* crown gall cells produce brassinosteroids (5). Such wide occurrence of brassinosteroids in the plant kingdom and their unique biological activities suggest that brassinosteroids are the 6th plant hormone. At present as many as 24 natural brassinosteroids are known (Figure 1), although some of them have not yet been published. In addition, we have got further evidence that many unknown compounds are present in plant tissues (6). However, it is likely that not all brassinosteroids are important because many of these are distributed in a limited number of plant species and some are biologically inactive by-products (6). Among them, brassinolide has a strongest biological activity and has been found in more than one-third of plant species so far examined. Castasterone was isolated as the second brassinosteroid and elicits high biological activity also. Distribution of castasterone is the widest among brassinosteroids and is found in most of the plant extracts so far examined. Some HPLC analytical data for the occurrence of brassinolide and castasterone in shoot of *Pisum sativum* (Yokota et. al. *Gibberellin Symposium Tokyo 1989*, Springer-Verlag, in press), young stem of *Phaseolus vulgaris* (7) and seeds of *Psophocarpus tetragonolobus*, a tropical bean plant (Yokota et. al, unpublished data) are shown in Figure 2. Therefore, these two brassinosteroids seem to be the most important from physiological aspects and also from biosynthetic aspects.

Figure 3 shows hypothetical biosynthetic pathway of brassinolide. Namely campesterol or its analogs will be converted to teasterone via several steps of oxidation, then successively oxidized to typhasterol, castasterone and brassinolide. In parentheses, biological activities in the rice lamina inclination assay are shown. The

activity increases as oxidation proceeds, suggesting that the biosynthetic pathway shown here might be plausible. However, another explanation might be possible that the strength of biological activity is dependent on the structural similarity to brassinolide.

This chapter deals with metabolism of castasterone and brassinolide and discusses the question as to whether brassinolide is synthesized from castasterone. Furthermore the question as to whether the biological activity of castasterone is mediated through conversion to brassinolide is also discussed.

Tracers used in this study were 24,28-tritiated castasterone and brassinolide, which were prepared by tritium catalytic reduction of dolichosterone and dolicholide, respectively (7). Figure 4 shows a reverse phase HPLC profile of tritiated products of dolicholide, in which brassinolide was a major component but, in addition, its 24-epimer was also present in the product. Tritiated brassinolide was separated from its 24 epimer by this HPLC technique. In the case of castasterone, the product was likewise purified by HPLC to separate tritiated castasterone from its 24-epimer.

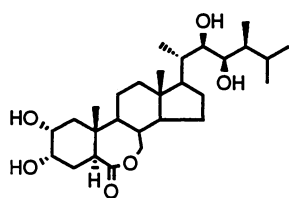
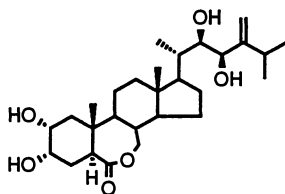
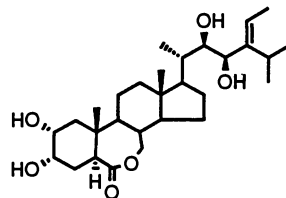
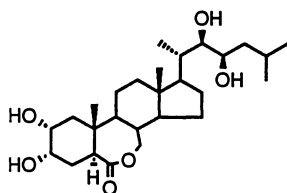
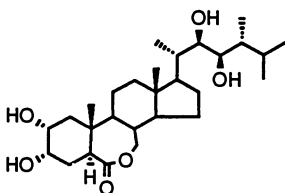
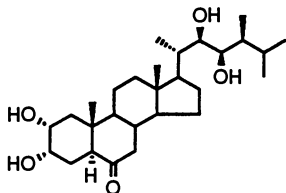
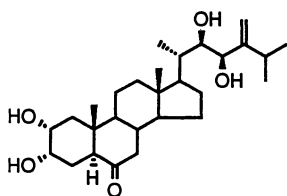
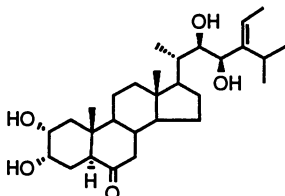
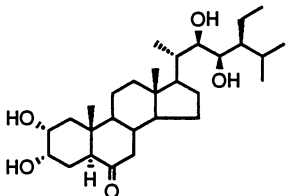
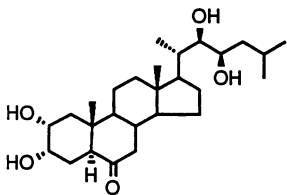
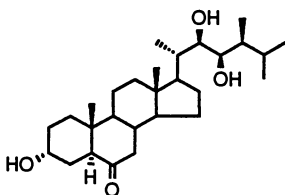
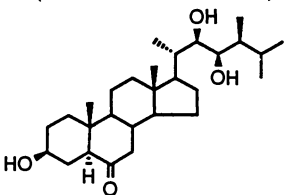
Plant tissues used for metabolism were: (1) crown gall cells of *Catharanthus roseus* in which castasterone and brassinolide are endogenous (5), (2) cutting of mung bean seedling and (3) rice seedling and explants in which castasterone and dolichosterone are endogenous (8). It is well known that brassinosteroids promote elongation of mung bean seedling (9, 10) and bending of rice lamina (11).

Metabolism of Castasterone in Crown Gall Cells of *Catharanthus roseus*.

The extract of cells of *Catharanthus roseus* were partially purified by reverse phase HPLC and were analyzed by GC-MS, disclosing that the major brassinosteroids in the cells are brassinolide and castasterone (5). Change of the contents of brassinolide and castasterone during cell growth was pursued by GC-SIM using D₆ brassinolide and D₆ castasterone as internal standards (12). As shown in Figure 5, brassinolide and castasterone rapidly increased after the 9th day. The maximum content of castasterone was obtained on the 12th day and was about 200ng per flask, while the maximum content of brassinolide was obtained on the 15th day and was about 400 ng per flask. Cell weight reached a maximum on the 12th day, then declined. The fact that castasterone was accumulated 3 days before brassinolide suggests that castasterone is a precursor of brassinolide.

Tritiated castasterone was fed to 12-day-old crown gall cells and incubated for 2 days. Cells and medium were separated and were extracted with methanol. The extracts were partitioned between chloroform and water. About 30 % of the radioactivity was present in the cell, of which 20% was chloroform-soluble and 10% was water-soluble. On the other hand, 70% of the radioactivity was in the medium, of which 30% was chloroform-soluble and about 40% was water-soluble. These fractions were partially purified, then analyzed by ODS HPLC. In the chromatogram of the chloroform-soluble fraction obtained from cells, a distinct radioactive peak of brassinolide was observed, the incorporation rate being 5% (Figure 6). Also, in the case of the medium, a radioactive peak of brassinolide is observed, the incorporation being 3.5%. The identity was further substantiated by isotope dilution method in which the putative radioactive brassinolide was mixed with cold brassinolide and repeatedly crystallized to constant specific activity. In the chromatogram of water-soluble fractions, radioactive peaks due to brassinolide and castasterone, as well as polar metabolites which were eluted with little retention were observed. From this analysis, it is most likely that polar metabolites were the first produced, but, because of their instability, it released aglycone during storage in refrigerator.

Thus it was found that *Catharanthus* crown gall cells convert castasterone to brassinolide as well as conjugates of castasterone and brassinolide. Since brassinosteroids have been reported to affect growth of cultured cells (13-15), endogenous castasterone and brassinolide in *Catharanthus* might have their own roles

**1** Brassinolide**2** Dolicholide**3** 28-Homodolicholide**4** 28-Norbrassinolide**5** 24-Epibrassinolide**6** Castasterone**7** Dolichosterone**8** 28-Homodolichosterone**9** (24*S*)-24-Ethylbrassinone
(28-Homocasterone)**10** Brassinone
(28-Norcastasterone)**11** Typhasterol**12** Teasterone**Figure 1. Structures of Natural Brassinosteroids**

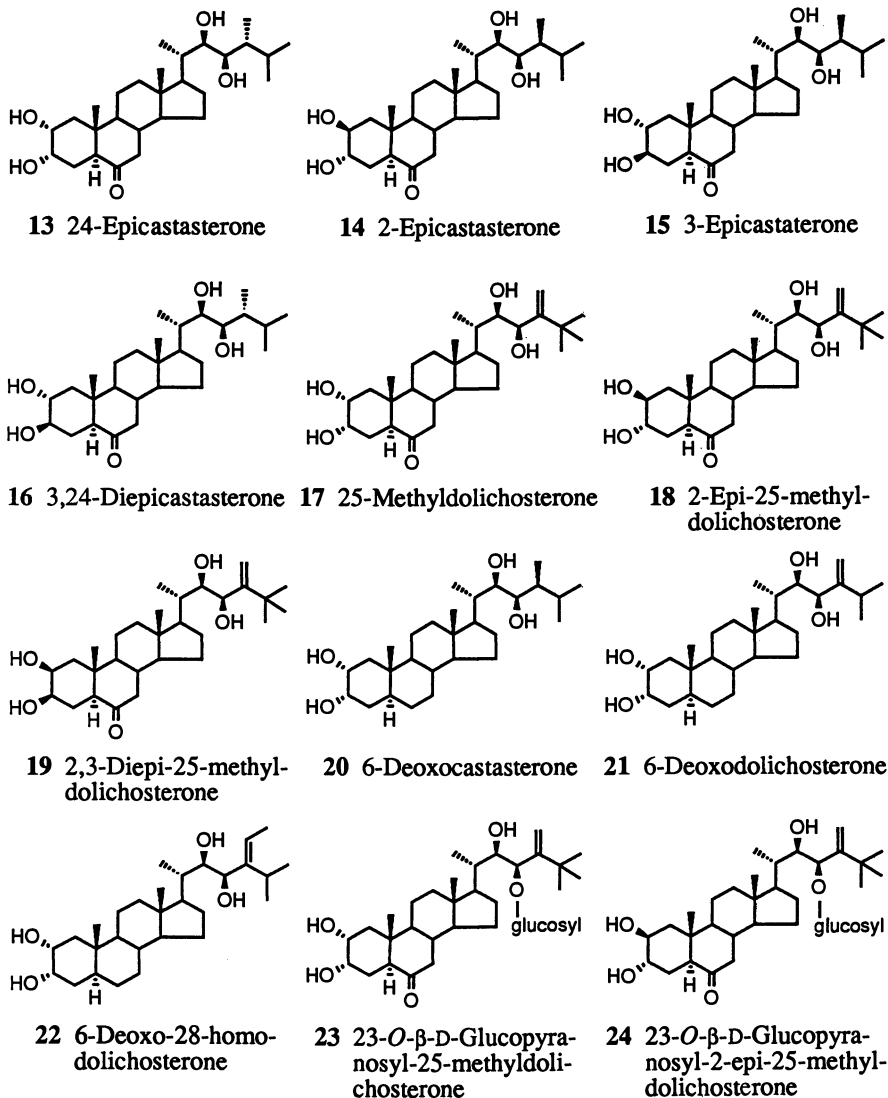


Figure 1. Continued.

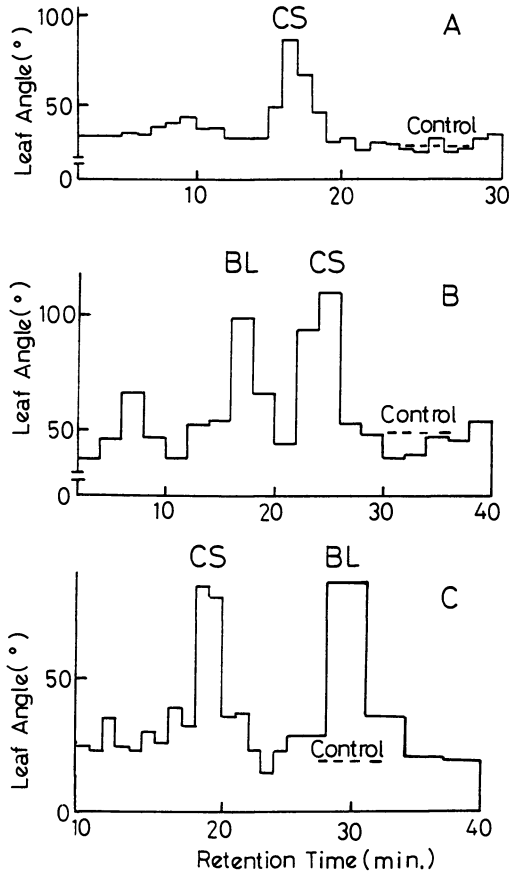


Figure 2. HPLC Profiles of Brassinosteroids Contained in Shoots of *Pisum sativum* (A), Young Stems of *Phaseolus vulgaris* (B) and Immature Seeds of *Psophocarpus tetragonolobus* (C)

Biological activity was determined by the rice lamina inclination assay. HPLC support and mobile phase: A, C₁₈ silica and 45% acetonitrile; B, C₁₈ silica and 45% acetonitrile; C, silica (Aquasil) and chloroform-methanol (95:5, 1% water). Abbreviations: CS, castasterone; BL, brassinolide.

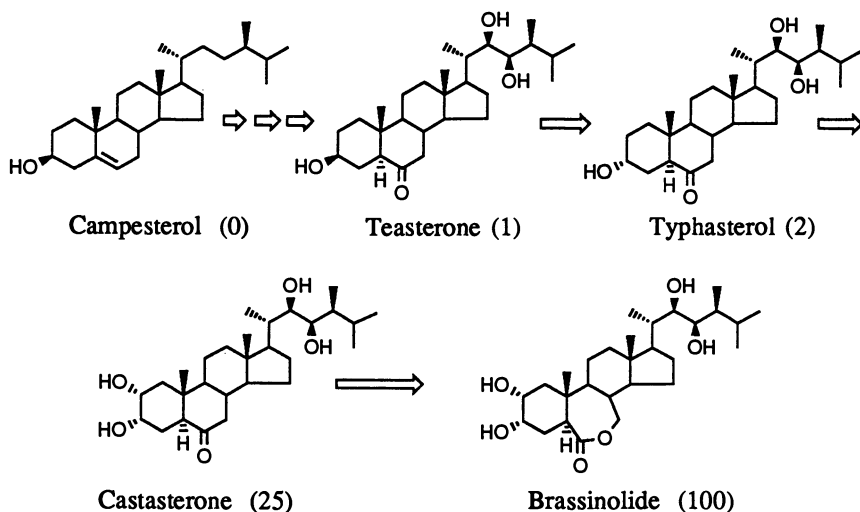


Figure 3. Hypothetical Biosynthetic Pathway of Brassinolide
Relative biological activities in the rice lamina inclination assay are given in parentheses.

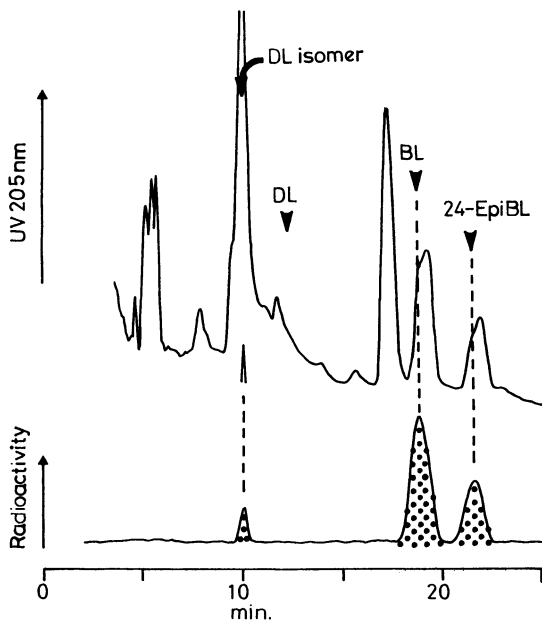


Figure 4. Reverse Phase (C₁₈ Silica) HPLC Profile of Tritiated Product of Dolicholide
Mobile phase, 45% acetonitrile.
Abbreviation: DL, dolicholide.

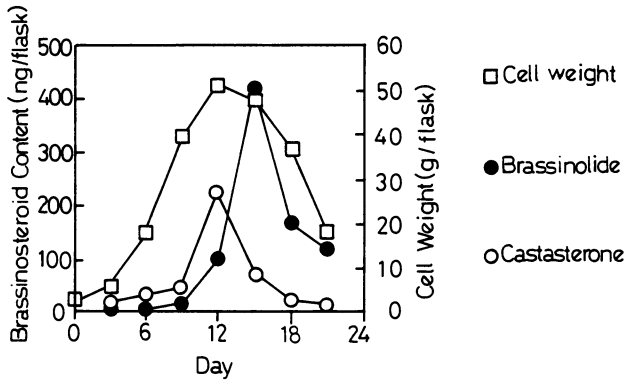


Figure 5. Change of Contents of Brassinolide and Castasterone during Growth of *Catharanthus roseus* Crown Gall Cells
Cells were grown in 150 ml Murashige-Skoog liquid medium at 27°C using 500 ml conical flasks.

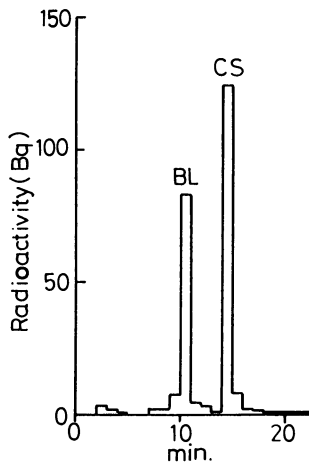


Figure 6. Reverse Phase (C₁₈ Silica) HPLC Analysis of Chloroform-Soluble Fraction Obtained from *Catharanthus roseus* Crown Gall Cells Fed with Tritiated Castasterone
Mobile phase, 45% acetonitrile.

in cell growth. The conversion of castasterone to brassinolide also seems important because the conversion apparently amplifies the biological activity.

Metabolism of Castasterone and Brassinolide in Mung Bean Cuttings

Radioactive brassinolide and castasterone (each 0.1 ppm) were continuously administered to mung bean cuttings for 72 hr. Brassinolide induced elongation more than castasterone. During 72 hr., absorption rates of brassinolide and castasterone were both increased. After 72 hr., most of the absorbed radioactivity was retained in hypocotyls while only a minor part of the radioactivity was found in epicotyls. The radioactivity present in hypocotyls was separated into chloroform-soluble and water-soluble fractions prior to analyzing by TLC and HPLC. In the case of tritiated castasterone feeding, the radioactivity in the chloroform soluble fraction moved to the castasterone region on TLC. The castasterone-like compound obtained from 72 hr. incubation was identified as castasterone by ODS HPLC. However, any conversion of castasterone to brassinolide was not observed. On the other hand, the radioactivity in water-soluble fraction remained at the origin in the TLC analysis. In the HPLC analysis, most of the radioactivity was rapidly eluted with little retention. This fraction was hydrolyzed with pectolyase which is a mixture of strong hydrolases, but a major part of water-soluble metabolite remained unchanged. Therefore, the major components in the water-soluble fraction seem to be non-glycosidic.

In the case of tritiated brassinolide feeding, quite similar chromatographic profiles were obtained. Namely, in the TLC analysis, the radioactivity in the aqueous phase remained at the origin and the radioactivity of the chloroform phase moved to the brassinolide zone. HPLC analysis showed that the chloroform fraction contained unchanged brassinolide, while the aqueous phase contained a fast-moving polar metabolite. In contrast with the tritiated castasterone feeding this metabolite seemed to be glycosidic, because it was easily hydrolyzed by pectolyase to release brassinolide. In order to isolate the brassinolide glycoside, a large amount of cold brassinolide was fed to a number of bean cuttings. Extensive purification resulted in the isolation of the brassinolide glycoside. This was hydrolyzed with 1N hydrochloric acid to yield brassinolide and glucose. Glucose was rigorously identified by GC while brassinolide was unequivocally identified by mass spectroscopy using GC-MS. In the positive FAB mass spectrum, a cation due to M^+ plus sodium was observed at m/z 665, indicating that the metabolite was composed of one mole each of glucose and brassinolide. In the 400MHz proton NMR spectrum of the brassinolide glucoside, all the protons attached to carbinol carbons were assigned by modern pulse techniques. After acetylation, proton NMR was again determined and we found that C-23 proton and anomeric proton do not suffer acetylation shift, indicating that C-23 hydroxyls are glucosylated. Thus the metabolite of brassinolide in mung bean was determined as 23-O- β -D-glucopyranosylbrassinolide (25). Interestingly, naturally-occurring conjugates of brassinosteroids (23 and 24) isolated from *Phaseolus vulgaris* seed also have 23-O-glucoside structures (16). Therefore, 23-O-glucosylation seems to be important in the metabolism of brassinosteroids.

It is now evident that castasterone is not converted to brassinolide, indicating, in mung bean, that the biological activity of castasterone is exerted by castasterone itself instead of a conversion to brassinolide. The major polar metabolite derived from brassinolide is its 23-O-glucoside while, in contrast, that derived from castasterone is non-glycosidic.

Metabolism of Castasterone and Brassinolide in Rice Seedlings and Etiolated Leaf Explants

In the first experiment, radioactive brassinolide and castasterone were fed to roots of

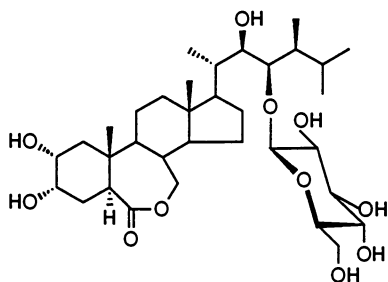
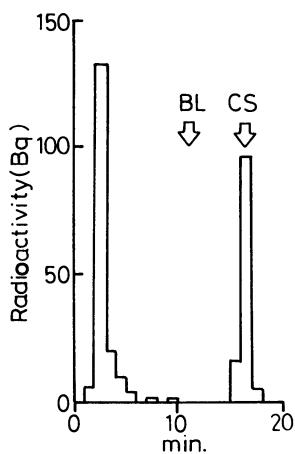
25 23-O- β -D-Glucopyranosylbrassinolide

Figure 7. Reverse Phase (C₁₈ Silica) HPLC Analysis of Whole Extract Obtained from Etiolated Rice Leaf Explants Fed with Tritiated Castasterone for 72 hr. Mobile phase, 45% acetonitrile.

three-leaf stage rice seedlings (16). The seedlings were separated into roots, shoots and seeds prior to extracting with methanol. Since it was found that most radioactivity was localized in the roots, further analyses were restricted to extracts from the roots. The methanol extract was partitioned between water and chloroform and then partially purified on C18 cartridges and silica cartridges prior to HPLC analysis. In the case of feeding of tritiated castasterone, polar metabolites were detected in both the chloroform and aqueous fractions. However, no trace of brassinolide was found. After enzymic hydrolysis by pectolyase, the polar metabolites did not release castasterone or its congeners, suggesting that the metabolites are non-glycosidic. In the case of feeding of tritiated brassinolide, polar metabolites were formed and were also found to be non-glycosidic as in the castasterone feeding.

The rice lamina inclination assay is very sensitive to brassinosteroids (11). In the second set of studies, we examined the metabolism of radioactive castasterone in the rice lamina assay (Yokota, T. et. al., unpublished data). The fate of tritiated castasterone was monitored for 72 hr. During incubation, again brassinolide was not detected. However, polar metabolites accumulated and the amount increased during 72 hr. (Figure 7). The polar metabolites seemed not to be changed after hydrolysis using either enzyme, hydrochloric acid or sodium hydroxide.

Thus castasterone is not converted to brassinolide in either rice seedlings or etiolated leaf explants, but it is metabolized to seemingly non-glycosidic compounds. Therefore it might be postulated that castasterone seems to be biologically active by itself in rice.

Concluding Remarks

From the present work, two important conclusions are drawn. First, from the experiment using *Catharanthus* crown gall cells, castasterone can be assigned a sole precursor of brassinolide. Secondly, in mung bean and rice, castasterone was converted to non-glycosidic polar metabolites but was not converted to brassinolide. Namely, castasterone exerts its biological activity even when not converted to brassinolide. In other words, castasterone seems to be biologically active in its own right.

Acknowledgments We are grateful to Brassinolide Research Association in Tokyo for financial support and to the Ministry of Education for research funds.

Literature Cited

1. Yokota, T.; Takahashi, N. In *Plant Growth Substances 1985*; Bopp, M. Ed.; Springer-Verlag, Berlin Heidelberg New York, 1986, pp 129-138.
2. Adam, G.; Marquart, V. *Phytochemistry*, **1986**, *25*, 1787
3. Mandava, N. B. *Ann. Rev. Plant Physiol. Plant. Mol. Biol.* **1988**, *39*, 23.
4. Takatsuto, S.; Abe, H.; Gamoh, K. *Agric. Biol. Chem.* **1990**, *54*, 1057.
5. Park, K.-H.; Saimoto, H.; Nakagawa, S.; Sakurai, A.; Yokota, T.; Takahashi, N.; Syono, K. *Agric. Biol. Chem.* **1989**, *53*, 805.
6. Yokota, T.; Koba, S.; Kim, S.-K.; Takatsuto, S.; Ikekawa, N.; Sakakibara, M.; Okada, K.; Mori, K.; Takahashi, N. *Agric. Biol. Chem.* **1987**, *51*, 1625.
7. Yokota, T.; Watanabe, S.; Ogino, Y.; Yamaguchi, I.; Takahashi, N. *J. Plant Growth Regul.* **1990**, *9*, 151.
8. Abe, H.; Nakamura, K.; Morishita, T.; Uchiyama, T.; Takatsuto, S.; Ikekawa, N. *Agric. Biol. Chem.* **1984**, *48*, 1103.
9. Gregory, L. E.; Mandava, N. B. *Physiol. Plant.*, **1982**, *54*, 239.
10. Arteca, R. N.; Tsai, D.-S.; Schlaghauffer, C.; Mandava, N. B. *Physiol. Plant.* **1983**, *59*, 539.

11. Wada, K.; Marumo, S.; Abe, H.; Morishita, T.; Nakamura, K.; Uchiyama, M.; Mori, K. *Agric. Biol. Chem.* **1984**, *48*, 719.
12. Yokota, T.; Ogino, Y.; Takahashi, N.; Saimoto, H.; Fujioka, S.; Sakurai, A. *Agric. Biol. Chem.* **1990**, *54*, 1107.
13. Sala, C.; Sala, F. *Plant Cell Reports* **1985**, *4*, 144
14. Bellincampi, D.; Morpurgo, G. *Plant Sci.* **1987**, *51*, 83
15. Roth, P. S.; Bach, T. J.; Thompson, M. J. *Plant Sci.* **1989**, *59*, 63.
16. Yokota, T.; Kim, S.-K.; Kosaka, Y.; Ogino, Y.; Takahashi, N. In *Conjugated Plant Hormones, Structure, Metabolism and Function*; Schreiber, K.; Schütte, H. E.; Sembdner, G. Ed; VEG Deutscher Verlag der Wissenschaften; 1987, pp 288-296.

RECEIVED May 16, 1991

Chapter 9

Production of Brassinosteroids in Plant-Cell Cultures

Akira Sakurai¹, Shozo Fujioka¹, and Hiroshi Saimoto²

¹Institute of Physical and Chemical Research, Wako-shi, Saitama 351-01, Japan

²Somar Corporation, Soka-shi, Saitama 340, Japan

During the course of our investigation on production of plant hormones in plant cell cultures, a strain of crown gall cells of *Catharanthus roseus* was found to produce remarkable amounts of brassinosteroid-like active substances, which were purified and identified as brassinolide and castasterone by GC-MS. The contents in the cells were much greater than those of the intact plant tissues. Then, we investigated the production of brassinosteroids in other kinds of plant cells such as crown gall cells or normal cells from *C. roseus*, tobacco and rice using the bioassay of rice lamina inclination test. Crown gall cells containing a high level of endogenous IAA or normal cells grown in media containing auxins were found to produce varying amounts of brassinosteroid-like active substances.

Brassinosteroids occur widely in higher plants and act as an endogenous plant hormone in plant growth regulation (1). Their biological activity resembles those of the other plant hormones, auxins, cytokinins and gibberellins (2, 3). The effects of brassinosteroids on plant cell cultures have been reported, such as promotion of growth of calluses of various plants in combination with auxin, that is similar to that obtained with cytokinins (4). Sala and Sala showed that brassinosteroids induced cell enlargement of cultured carrot cells but no cell division (5). Recently, Roth *et al* reported that brassinosteroids were effective inhibitors of the growth of crown gall cells from tobacco (6). These results suggest that plant cells produce brassinosteroids in cultures, to regulate growth and proliferation, as endogenous regulators. However, the presence of brassinosteroids in plant cell cultures has not yet been established. We have been studying endogenous plant hormones in plant cell cultures. During the course of our examinations, we found that a strain of crown gall cells from *Catharanthus roseus* G. Don (*Vinca rosea* L.) produced remarkable amounts of brassinosteroid-like active substances, which were detected by the rice lamina inclination test. This finding provides the possibility that plant cell cultures could not only be used for industrial production, but may also be useful tools for elucidating biosynthetic pathways and for physiological studies of brassinosteroids.

Identification of Brassinosteroids Produced by Crown Gall Cells of *Catharanthus roseus*

Two kinds of crown gall cells were generated from *Catharanthus roseus* through transformation by two different strains of *Agrobacterium tumefaciens* carrying different Ti-plasmids. As shown in Table I, V208 cells produce nopaline as the characteristic amino acid of crown gall cells, and V277 cells produce the amino acid octopine. Each cell line was grown as suspension culture in Murashige-Skoog medium at 27°C by shaking at 100 rpm in the dark. Both cells showed the same growth as unorganized cell aggregates under this condition.

Table I. Crown Gall Cells of *Catharanthus roseus* (*Vinca rosea* L.)

<i>Cell Line</i>	<i>Induced Strain</i>	<i>Ti-Plasmid</i>	<i>Opine Type</i>
V208	<i>A. tumefaciens</i> A208	pTi-T37	nopaline
V277	<i>A. tumefaciens</i> A277	pTi-B6 806	octopine

Detection of brassinosteroid-like active substances. The cells were harvested in several growth stages and extracted with methanol. After the removal of methanol, the extract was subjected to solvent fractionation by usual method to give an ethyl acetate soluble neutral fraction (NE fraction). The content of brassinosteroid-like active substances in the fraction was examined by rice lamina inclination test using a cultivar "Koshihikari", which is the most sensitive bioassay for brassinosteroids (7). The results are shown in Figure 1. The cell extract of V208 was found to produce remarkable amounts of brassinosteroid-like active substances at the early stationary phase of growth. The culture filtrates were also extracted with ethyl acetate, and the biological activity of the NE fractions was examined. As shown in Figure 1, the contents in the filtrates were much less than that of the cell extracts. Brassinosteroid-like active substances in the cell cultures were accumulated in the cells. On the other hand, V277 extract showed a little activity in every growth stage. The difference in the production between V208 and V277 cells will be discussed later. Identification of brassinosteroids in V208 cells was then carried out.

Identification of the active principles. V208 cells from 13-day-old cultures were collected and about 1 kg of the cells were extracted with methanol. The NE fraction of the extract, obtained by solvent fractionation, was purified successively with silica gel, Sephadex LH-20 and carbon column chromatography. The active fraction was finally purified by HPLC on an ODS column. As shown in Figure 2, the biological activity was detected in the two fractions designated A and B. Retention times of the fractions corresponded to those of brassinolide and castasterone, respectively. Identification was accomplished by GC-MS analysis of the methane-boronate derivatives of these fractions. The retention times in GC and the full mass spectra of the bismethaneboronate derivatives of fraction A and B were identical with those of brassinolide and castasterone, respectively. Thus, structures of brassinosteroids produced by V208 cells were unambiguously identified as brassinolide and castasterone as shown in Figure 3 (8). The total content of brassinosteroids in the cells was estimated to be 30-40 µg/kg fresh weight from the biological activity.

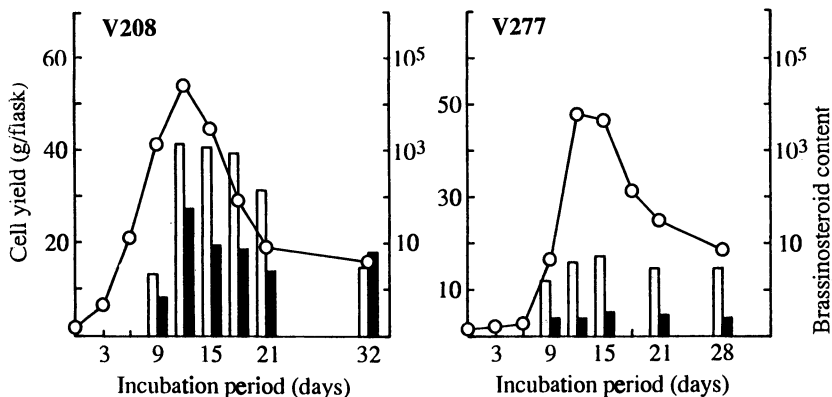


Figure 1. Growth profile and brassinosteroid contents of *C. roseus* crown gall cells. Open circle: yields of cells; open column: brassinosteroid content in the cells; closed column: brassinosteroid content in the culture filtrate.

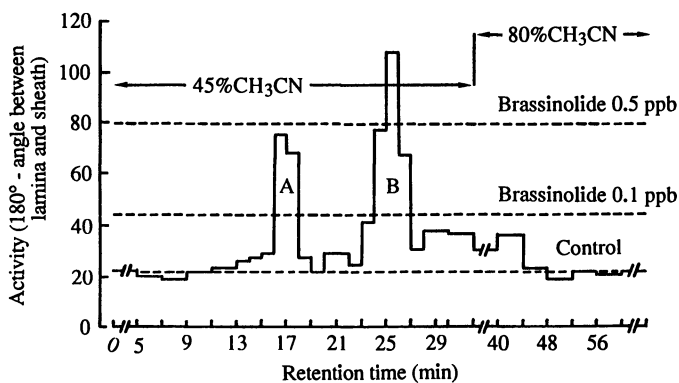


Figure 2. Distribution of biological activity in HPLC on an ODS column. Biological activity is expressed as the response in the rice lamina inclination test.

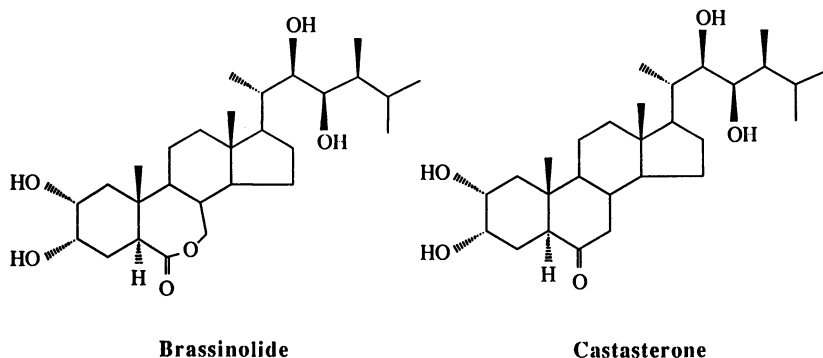


Figure 3. Structures of brassinosteroids in *C. roseus* crown gall cells of V208.

Production of Brassinosteroid-like Active Substances by Plant Cells

Cell lines and culture conditions. We further studied on the production of brassinosteroid-like active substances in plant cells which were available in our laboratory listed in Table II. Apart from the crown gall cells of V208 and V277, normal cells of Vn were derived from normal tissues of *Catharanthus roseus*, and grown in medium containing 2,4-D. The three kinds of crown gall cells from *Nicotiana tabacum* (tobacco) referred to as 3n-series were generated through transformation by the same Ti-plasmid as in the case of V208. Of the transformants, three different morphological clones of 3n-3 (shoot-forming teratoma), 3n'-6 (compact unorganized callus) and 3n'-3R (friable unorganized callus) were obtained (9). Each cell line was grown as callus of different morphology on agar medium. CG is an octopine-type crown gall cells obtained from tobacco through transformation by *A. tumefaciens* of different strain from that of V277 (10). BY-2 was derived from normal tissues of tobacco and grown in the medium containing 2,4-D. The line 2b-13, generated by habituation of BY-2, was grown in the medium without addition of any plant hormones (10). The two cell lines of Oc208 and Oc are from *Oryza sativa* (rice). Oc208 was obtained through transformation of rice cells by introducing the same Ti-plasmid as that of V208 and grown without any plant hormones in the medium. Oc was derived from normal rice tissues and grown in medium containing 2,4-D. Each of these lines was cultured under the conditions shown in Table II and harvested in several growth stages.

Examination on the production of brassinosteroid-like active substances. The cells were extracted with methanol, and the NE fractions of the extracts were subjected to bioassay using the rice lamina inclination test by the same

Table II. Cultured Cells Examined for Brassinosteroid Production

Cell Line	Origin	Characteristics	Culture Condition
V208	<i>Catharanthus</i>	crown gall cell	MS ^a , suspension ^c
V277	<i>roseus</i>	crown gall cell	MS, suspension
Vn		normal cell	MS+2,4-D (0.5 ppm), suspension
3n-3	<i>Nicotiana</i>	crown gall cell	MS, agar ^d
3n'-6	<i>tabacum</i>	crown gall cell	MS, agar
3n'-3R		crown gall cell	MS, agar
CG		crown gall cell	LS ^b , suspension
BY-2		normal cell	LS+2,4-D (0.5 ppm), suspension
2b-13		habituated cell	LS, suspension
Oc208	<i>Oryza sativa</i>	crown gall cell	MS, suspension
Oc		normal cell	MS+2,4-D (1.0 ppm), suspension

^a Murashige-Skoog medium supplement with 3% sucrose.

^b Linsmaier-Skoog medium supplement with 3% sucrose.

^c Cultured by reciprocal shaking at 100 rpm in the dark at 27°C.

^d Cultured in the dark at 27°C

Table III. Contents of Brassinosteroid-like Active Substances and Auxin

<i>Plant Material</i>	<i>Cell Line</i>	<i>Activity of Brassinosteroid</i>	<i>Endogenous Auxin IAA (ng/g f.w.)</i>	<i>Auxin added to Medium 2,4-D (ppm)</i>
<i>Catharanthus roseus</i>				
Cultured Cells	V208	++++	680	-
	V277	+	36	-
	Vn	+++	21	0.5
Shoots and Leaves		+		
<i>Nicotiana tabacum</i>				
Cultured Cells	3n-3	+	1.4	-
	3n'-6	+	1.5	-
	3n'-3R	+	3.6	-
	CG	++	630	-
	BY-2	++	2.4	0.5
	2b-13	-	<1	-
Shoots and Leaves		+		
<i>Oryza sativa</i>				
Cultured Cells	Oc208	++	10	-
	Oc	+++	2.3	1.0
Shoots and Leaves		+		

Activity of brassinosteroid is expressed as, -: not detected, +: <0.1, ++: 0.1-1, +++: 1-10, ++++: 10-100 ng/g f.w. equivalent to brassinolide.

procedure as described before. Some representative results are shown in Figure 4 and 5. As shown in Figure 4, octopine-type crown gall cells of tobacco, CG, produced more amounts of the active substances than nopaline-type cells of tobacco, 3n-3. The other cell lines of 3n-series with different morphologies gave similar results to 3n-3, even though they are the same type of crown gall cells as V208. Figure 5 showed that normal cells from *C. roseus*, and tobacco, produced varying amounts of the active substances.

The results of all cell lines examined are summarized in Table III, together with the contents in the mother plants of shoots and leaves. The mother plants of *C. roseus*, tobacco and rice were grown in a glass house under natural light. Shoots and leaves approximately 10 to 20 cm in length were harvested and extracted with methanol. The biological activity of the NE fractions of the extracts was examined in the same manner as those of the cell extracts.

From the results so far obtained, certain crown gall cells and normal cells from *C. roseus*, tobacco and rice were found to produce brassinosteroid-like active substances, and their amounts were greater than that of their mother plant tissues. Since normal cells produced the active substances and the production by crown gall cells did not depend on their tumor types, T-DNA transformed to the cell genome is not directly involved in the production. Therefore, the production of brassinosteroid-like active substances was found to depend on neither the original plant nor the Ti-plasmid of crown gall cells.

Effects of Plant Hormones on Production of Brassinosteroids in Cell Cultures

As described in the first section, two crown gall cell lines of V208 and V277 from *C. roseus* showed remarkable difference in the production of brassinosteroids. That is, nopaline-type cells of V208 produced greater amounts of brassinosteroids than octopine-type cells of V277. It seems probable, therefore, that there must be some differences in levels of endogenous plant hormones between these cell lines of different tumor types, even though both cells grow in a similar manner as unorganized cell aggregates. Consequently, the endogenous levels of cytokinins and IAA in V208 and V277 cells were analyzed (11).

Analysis of endogenous cytokinins. The cells, cultured in the same condition as described before, were harvested in several growth stages and extracted with 80% methanol. The extracts were purified successively with Cellulose P-1, HPLC on a Hitachi gel 3011C and on an ODS column. Two fractions containing ribosylzeatin and zeatin were obtained, and each fraction was subjected to a combined gas chromatography-selected ion monitoring mass spectrometry (GC-SIM) analysis after derivatization to trimethylsilyl ethers. Quantification of zeatin and ribosylzeatin was made by using internal standards of *d*₅-*trans*-zeatin and *d*₅-*trans*-ribosylzeatin which were added before purification. The results are shown in Figure 6.

The main cytokinins in crown gall cells of *C. roseus* have been shown to be *trans*-ribosylzeatin (12). Our results also showed that levels of *trans*-ribosylzeatin were much higher at the early growth stage than that of *trans*-zeatin in both types of cells. The levels of *trans*-ribosylzeatin in V277 cells was higher than that in V208 cells. *trans*-Zeatin was at a higher level in 9-day-old cells of V208 than those of V277 cells. However, the levels of the cytokinins fluctuated during the growth stages. No distinct differences between V208 and V277 cells were observed in the endogenous levels of cytokinins.

Analysis of endogenous IAA. The extracts of cells with methanol were subjected to solvent fractionation using methylene chloride to give methylene chloride soluble acidic fractions. The fractions were purified by HPLC on an ODS column and finally by HPLC on a Nucleosil 5N(CH₃)₂ column with a fluorescent detector. Quantification was made by using an internal standard of indole-3-propionic acid which was added before purification. The results are shown in Figure 7.

Levels of IAA in V208 cells were much higher than that in V277 cells in every growth stage. Content of IAA in V208 cells reached 20-fold much more than that of V277 cells at the maximum stage. Because a great difference in the endogenous levels of IAA was found between V208 and V277 cells, we compared levels of auxins in the culture of cell lines examined in the second section. Endogenous levels of IAA in Vn, BY-2, Oc208 and Oc were analyzed by the same methods as described above, and those in 3n-series cells (9), CG (13) and 2b-13 (10) are based on reported data. As shown in Table III, crown gall cells of V208, CG and Oc208, were highly productive of brassinosteroid-like active substances, contained relatively high levels of endogenous IAA. Moreover, normal cells of Vn, BY-2 and Oc, though they contained low levels of endogenous IAA, and were cultured in the presence of 2,4-D, produced varied amounts of brassinosteroid-like active substances. On the other hand, little production of brassinosteroid-like substances was detected in the culture of crown gall cells of 3n-series and habituated cells of 2b-13 which contained low levels of IAA.

Effects of exogenous auxins on the production of brassinosteroids. As far as these results are concerned, production of brassinosteroid-like active sub-

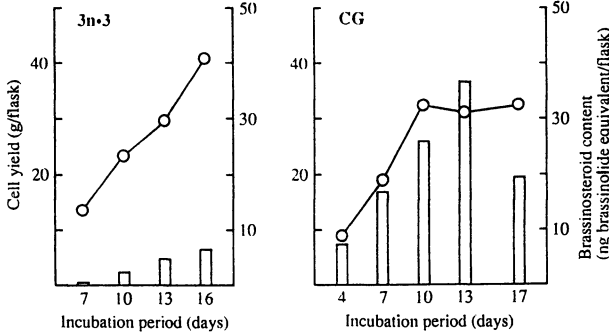


Figure 4. Growth profile and brassinosteroid contents of tobacco crown gall cells. Open circle: yields of cells; open column: brassinosteroid content in the cells.

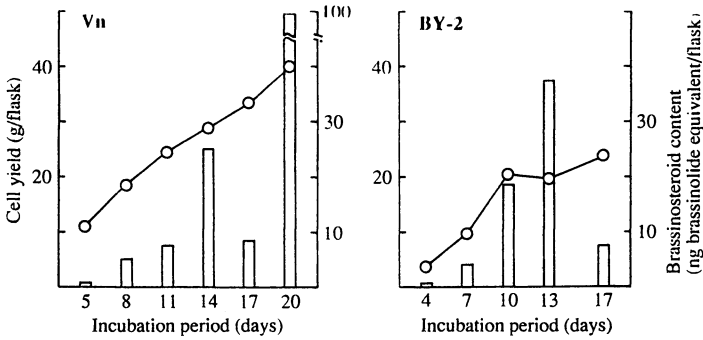


Figure 5. Growth profile and brassinosteroid contents of normal cells of tobacco and *C. roseus*. Open circle: yield of cells; open column: brassinosteroid content in the cells.

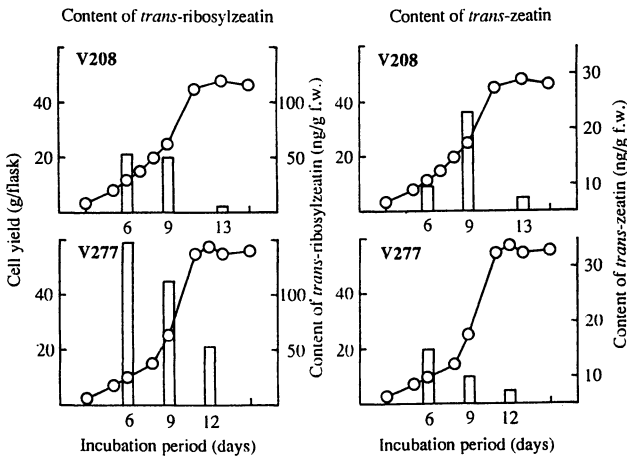


Figure 6. Contents of cytokinins in *C. roseus* crown gall cells. Open circle: yield of cells; open column: cytokinin content in the cells.

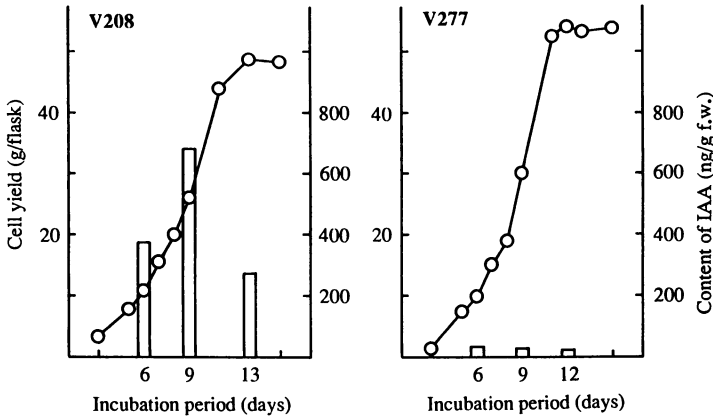


Figure 7. Contents of IAA in *C. roseus* crown gall cells. Open circle: yield of cells; open column: IAA content in the cells.

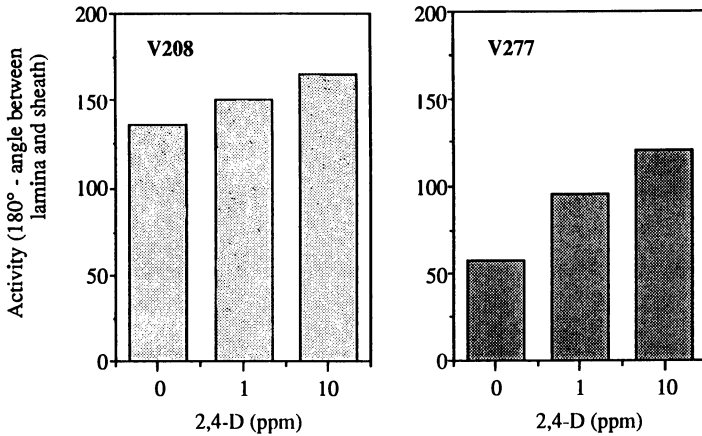


Figure 8. Effects of 2,4-D on production of brassinosteroids in the cultures of *C. roseus* crown gall cells. Brassinosteroid contents in the cells are expressed as the response in the rice lamina inclination test.

stances in plant cells may be related to auxin levels in the cultures. The bioassay using rice lamina inclination tests used in this study is known to respond to auxins (14). The response of rice lamina inclination to brassinolide was examined in the presence of various doses of 2,4-D. Response of 2,4-D at a single dose of 100 μ g was almost equivalent to that of brassinolide at a single dose of 2 ng. In the presence of both chemicals, the response of rice lamina inclination was additive, but no synergistic effects were detected. This observation indicates that 2,4-D in the cultures does not affect the bioassay for brassinosteroids.

Therefore, we examined the effect of 2,4-D added to the medium on production of brassinosteroids using V208 and V277 cells. However, addition of 10 ppm of 2,4-D to the culture medium at the start of the culture process caused growth inhibition of the crown gall cells. Therefore, 2,4-D was added to the medium when cultures were 10-days old. After culturing another 4 days, the cells were harvested and extracted with methanol. Brassinosteroid activity of the NE fractions of the cell extracts was examined by the rice lamina inclination test. The results are shown in Figure 8.

The octopine-type cells, V277, low in endogenous IAA and less prolific in brassinosteroid production, were enhanced in the production of brassinosteroid-like active substances by addition of 2,4-D at the concentration of 1 to 10 ppm. Even in V208 cells containing high level of endogenous IAA, the production was also increased by the addition of 2,4-D. The effects of other auxins such as IAA and NAA were examined by the same procedure. The production of brassinosteroid-like active substances was increased by the addition of IAA or NAA. Among them, 2,4-D appeared to be the most effective. In the results so far obtained, the production of brassinosteroid-like active substances in plant cell cultures seems to be related to auxin level; that is, high auxin level in the culture promotes the production of brassinosteroids.

Acknowledgments

We express our thanks to Prof. K. Syono of Department of Pure and Applied Sciences, The University of Tokyo for his collaboration to supply us cell lines from *Catharanthus roseus* and rice, to Prof. T. Yokota of Department of Bioscience, Teikyo University for his collaboration in identification of the brassinosteroids. We also wish to thank Dr. K-H. Park, Mrs S. Nakagawa, Miss M. C. Cifuentes-Barreto, Miss M. Ohtsuka and Miss M. Yamamoto for their collaboration in these studies at our laboratories.

Literature Cited

- (1) Yokota, T.; Takahashi, N. In *Plant Growth Substances 1985*; Bopp, M. Ed.; Springer-Verlag: Berlin, Heidelberg, Germany, 1986, pp 129-138
- (2) Yopp, J. H.; Mandava, N. B.; Sasse, J. M. *Physiol. Plant.*, **1981**, *53*, 445-452
- (3) Mandava, N. B.; Sasse, J. M.; Yopp, J. H. *Physiol. Plant.*, **1981**, *53*, 453-461
- (4) Takematsu, T.; Takeuchi, Y.; Koguchi, M. *Shokucho*, **1985**, *18*, 2-15 (in Japanese)
- (5) Sala, C.; Sala, F. *Plant Cell Reports*, **1985**, *18*, 144-147
- (6) Roth, P. S.; Bach, T. J.; Thompson, M. J. *Plant Science*, **1989**, *59*, 63-70
- (7) Wada, K.; Marumo, S.; Ikekawa, N.; Morisaki, M.; Mori, K. *Plant Cell Physiol.*, **1981**, *22*, 323-325

- (8) Park, K-H.; Saimoto, H.; Nakagawa, S; Sakurai, A.; Yokota, T.; Takahashi, N.; Syono, K. *Agric. Biol. Chem.*, **1989**, *53*, 805-811
- (9) Nakagawa, S.; Tjokrokusumo, D. S.; Sakurai, A.; Yamaguchi, I.; Takahashi, N.; Syono, K. *Plant Cell Physiol.*, **1987**, *28*, 485-493
- (10) Nakajima, H.; Yokota, T. Matsumoto, T.; Noguchi, M.; Takahashi, N. *Plant Cell Physiol.*, **1979**, *20*, 1489-1499
- (11) Saimoto, H.; Nakagawa, S.; Kobayashi, M.; Fujioka, S.; Cifuentes-Barreto, M. C.; Sakurai, A.; Syono, K. *Plant Cell Physiol.*, **1990**, *31*, 365-370
- (12) Miller, C. O. *Proc. Natl. Acad. Sci. USA.*, **1974**, *71*, 334-338
- (13) Nakajima, H.; Yokota, T.; Takahashi, N.; Matsumoto, T.; Noguchi, M. *Plant Cell Physiol.*, **1981**, *22*, 1405-1410
- (14) Maeda, E. *Physiol. Plant.*, **1965**, *18*, 813-827

RECEIVED April 1, 1991

Chapter 10

Microanalysis of Naturally Occurring Brassinosteroids

Suguru Takatsuto

Department of Chemistry, Joetsu University of Education, Joetsu-shi,
Niigata 943, Japan

During the past decade, studies on brassinosteroids have greatly widened our knowledge of the new steroidal plant hormones. This review summarizes the studies on brassinosteroids from the view points of bioassays and microanalyses. Highly sensitive and specific bioassays, being employed to isolate brassinosteroids from plant sources, are (i) bean-second internode assay, (ii) rice-lamina inclination test, and (iii) wheat leaf unrolling test. Microanalyses for brassinosteroids have been developed using (i) gas chromatography/mass spectrometry, (ii) high-performance liquid chromatography, and (iii) radioimmunoassay. The bioassays and the microanalyses have greatly contributed to the studies on identification of many natural brassinosteroids and also to their distribution in plant kingdom.

Brassinolide (BL) and/or its related steroidal compounds are known collectively as brassinosteroids (BRs). BL, present at 0.1 mg/kg in rape (*Brassica napus* L.) pollen, was the first BR isolated in 1979 in a crystalline form as a new plant growth promoter and its structure was determined by spectroscopic analysis and X-ray diffraction to be (22*R*,23*R*, 24*S*)-2 α ,3 α ,22,23-tetrahydroxy-24-methyl-*B*-homo-7-oxa-5 α -cholestan-6-one (1). In 1982, another new BL-related compound termed castasterone (CS), was isolated in pure form from the insect galls of chestnut (*Castanea crenata*) and its structure was determined by spectroscopic methods to be (22*R*,23*R*,24*S*)-2 α ,3 α ,22, 23-tetrahydroxy-24-methyl-5 α -cholestan-6-one (2). CS differs from BL only in B-ring functionality. Rape pollen was later shown to contain CS (3). After the discovery of BL, chemical synthesis of BL and its related compounds, physiological study of them, and study of the structure-activity relationship were intensively and extensively carried out by many scientists. After these investigations, BL, 28-homoBL, and 24-epiBL, have been tested for many years for their practical applications in agriculture fields. These BRs have been shown to possess the following characteristics: promotion of germination and plant growth, raising of ripening, thickening promotion, recovery from stresses under various conditions unfavorable for plants, and effects on flowering or its differentiation. Several excellent reviews on these subjects are available (3-11).

In isolating these BRs from plant sources, bioassays sensitive and specific to BRs are indispensable. The bean-second internode assay was used to isolate BL from the rape pollen, and rice-lamina inclination test was used to isolate CS from the insect galls. After these works, the latter bioassay has been widely employed in Japan to successfully isolate BRs from a number of plant sources, because of its simplicity, high sensitivity, and specificity for BRs. It has now been believed that BRs are ubiquitously distributed in higher plants (phanerogams). Some lower plants (cryptogams) have been investigated for the presence of BRs and positive results have been obtained. These data strongly suggest that BRs occur widely in plant kingdom, like other known plant hormones, and that BRs play some physiological functions in plant growth and development.

Microanalytical methods for BRs have also been developed: (i) gas chromatography/ mass spectrometry (GC/MS) analysis of BRs as bismethaneboronate (BMB) derivatives (12) or methaneboronate-trimethylsilyl (MB-TMS) derivatives (13), (ii) high performance liquid chromatography (HPLC) analysis of BRs as bisboronate derivatives having a fluorophore or an electrophore (14), (iii) Radioimmunoassay (RIA) for BRs (15). Among these microanalytical methods, the GC/MS analysis has greatly contributed to the study on identification and characterization of a number of natural BRs.

In this review, I summarize the BRs research from the following points: (i) distribution of BRs in plant kingdom, (ii) bioassays for BRs, (iii) microanalytical methods of BRs.

Distribution of Brassinosteroids in Plant Kingdom

Since the discovery of BL and CS, intensive and extensive studies on isolation of new BRs from plant sources and on screening of BRs in plant kingdom have been made mainly by Japanese scientists. In these studies, a sensitive and specific bioassay, rice-lamina inclination test (16), and a GC/MS analysis (12,13) have been very effective and useful. Until now, twenty four BRs have been isolated and their structures have been chemically characterized (5). The BRs are found to be distributed in angiosperms (dicots and monocots), gymnosperms, and alga. In addition, Abe *et al.*, have examined the pollens of 8 species of higher plants and they have identified BRs in these pollens (17). From these results and the bioassay data of *Mandava* (3), it is most plausible that BRs are widely distributed in higher plants (phanerogams), as in the case of other known plant hormones. Some lower plants (cryptogams) have been investigated for the presence of BRs. Green alga, *Hydrodictyon reticulatum* has been reported to contain BRs (18), and in fern, *Equisetum arvense* L., specific biological activity for BRs has been obtained by the rice-lamina inclination test and BRs have been tentatively identified by HPLC analysis (19). We have recently noticed the presence of BRs-like bioactive substances in a highly purified fraction from *Chrorella pyrenoidosa*, which was obtained by the rice assay (Takatsuto, S.; Abe, H., unpublished data). These data strongly suggest that BRs also occur in lower plants, and that BRs play some physiological functions in plant growth and development. It is now believed that BRs are ubiquitously distributed in the plant kingdom.

As far as the amount of BRs content in plant tissues is concerned, pollens are the richest sources of BRs, immature seeds have also high content of BRs, while shoots and leaves have lower levels (3,4,7). Roots have not yet been examined. Other interesting tissue is insect gall. The galls of *Castanea crenata* and *Distylium racemosum* have higher levels of BRs than the normal tissues (20,21). Another high BRs content tissue is the crown gall (nopaline type) cells of *Catharanthus roseus* (22). The crown gall cells have higher contents of BL and CS than the normal cells.

In the same plant tissues the young growing tissues are likely to have higher contents of BRs than old tissues. In *Dolichos lablab* immature seeds, BRs content is

higher at a younger stage of the seed (4). In the pollens of green tea (*Thea sinensis*) and lily (*Lilium longiflorum*), the bioactivity by rice-lamina inclination test increased as pollens grew mature and it reached the maximum value just before anthesis and after the anthesis the activity decreased (23). The research is interesting both because BRs occur in pollens at a high level, compared with other tissues and because the result suggests the possibility that BRs should play an important role in the regenerative growth regulation.

Among the plants thus far investigated, CS occurs most frequently and BL is the next one. Therefore, these two BRs are believed to be important. In most plants, several kinds of BRs are found. In this respect, it is interesting that more than 30 BRs including unknown compounds (partial structures being determined by GC/MS analysis) occur in immature seeds of *Phaseolus vulgaris* (24). Based on the data, it is likely that the number of natural BRs will increase in the future.

Structural features of natural BRs are summarized as follows (3-5, 7). All natural BRs contain a steroid nucleus (most of them with the oxygen function in the B-ring) with a side chain at C-17. Other common features for all BRs, in addition to β -oriented angular C-18 and C-19 methyl groups, are (a) α -orientation at C-5 (A/B ring junction); (b) α -oriented hydroxyl groups at C-22 and C-23 (side chain); and (c) α -oriented hydroxyl groups (*cis*-geometry) at C-2 and C-3 in ring A of the steroid nucleus (exceptions: typhasterol contains only one hydroxyl group at C-3 in the α position and teasterone contains a β -hydroxyl at C-3 only; both compounds lack hydroxyl at C-2). BRs generally differ in functional groups at C-24 (steroidal side chain): CH₃, BL and CS; =CH₂, dolicholide (DL) and dolichosterone (DS); =CH-CH₃, homoDL and homoDS; and C₂H₅, 28-homoCS. BRs lacking the substituent at C-24 have also been isolated. They are 28-norBL and 28-norCS.

The structural relationship between phytosterols and BRs has been proposed from the biosynthetic points of view. All naturally occurring BRs possess carbon skeletons identical to those of common phytosterols (e.g., campesterol, 24-methylene-cholesterol, isofucoesterol, sitosterol, and cholesterol). Thus, BRs may be speculatively regarded as the enzymatic oxidation products of phytosterols with the corresponding carbon skeletons, as is the case of the biosynthesis of other steroid hormones (e.g., ecdysteroids (25) and 1,25-dihydroxyvitamin D₃ (26)). Although BL has recently been proved to be biosynthesized from CS in crown gall cells of *Catharanthus roseus* (27), a major part of the biosynthesis of BRs is remained to be investigated. Experiments using radio-labeled precursors are required to clarify the biosynthesis of BRs in a suitable plant system.

Bioassays for Brassinosteroids

Since the isolation of BL, BL and its related compounds have been tested by a number of bioassays originally designed for known plant hormones. BRs have been shown to have a broad spectrum of biological activities (3,4,8). Structure-activity relationship of BRs has also been clarified by bean second-internode bioassay, bean first-internode bioassay, raphanus test, tomato test, and rice-lamina inclination test (28-31).

Development of bioassays for the isolation of bioactive compounds from natural sources has played an important role in recent natural products chemistry. For the isolation and purification of BRs from plant sources, highly sensitive and specific bioassays are indispensable, because of the very low concentration of BRs in plants. The following three bioassays have been employed for the BRs purification procedure to guide the fractionation.

(1) Bean Second-Internode Bioassay

The bean second-internode bioassay was used for the isolation of BL from the pollen of rape (*Brassica napus* L.) (1). The procedure of the bioassay is described in

the literature (32). Plants were grown at 25-27°C with light 7.5 kilolux in 12-h periods. The test compound was dispersed in lanolin, and the mixture was applied to the second internode of each 6-day-old bean seedling (*Phaseolus vulgaris* L., pinto variety). The internode was no more than 2-mm-long to ensure the optimum effect. Control plants were treated with lanolin only. After four days the increase in internode length of the treated plants compared with the controls was recorded. Test plants showed not only elongation but also curvature, swelling, and splitting of the internodes, depending on the amount of BL. Both natural and synthetic BL gave elongation with curvature and swelling at 0.01 µg and splitting of the internodes at 0.1 µg. Synthetic isomers of BL and ketone type BRs were also found to be active but at a higher concentration than natural BL. Although in this bioassay gibberellins cause only elongation of the treated and upper internodes, BRs characteristically evoke both cell elongation and cell division resulting in elongation, swelling, curvature, and splitting of the second internode. Auxins and cytokinins are not detected by this bioassay.

(2) Rice Lamina Inclination Test

Rice lamina inclination test, originally developed as a bioassay for auxins, has been found to be a highly sensitive and specific bioassay for BRs (16). The bioassay has been used to guide the fractionation during the purification procedure of the plant extracts, successfully resulting in isolation and identification of a number of BRs with both lactone and ketone groups in the B-ring. 2-Deoxy compounds (typhasterol and teasterone) have also been isolated by this test (33-35). The rice test is routinely employed at the purification steps mainly by Japanese scientists. This test has also been developed as a microquantitative assay for BL and CS in the concentration range of 5×10^{-5} to 5×10^{-3} µg/ml (36).

Etiolated seedlings of the rice cultivars Arborio J-1 and Nihonbare were grown for seven days, and uniform seedlings were then selected. Leaf segments, which consisted of the second leaf lamina (0.7 cm long) and the second lamina joint and sheath (0.7 cm long), were excised. These segments were floated on distilled water for 24 h, after which uniformly bent segments were selected. Eight of these segments were incubated in 1 ml of 2.5 mM aqueous dipotassium maleate solution containing the BRs. After the segments had incubated for 48 h at 29°C in darkness, the magnitude of the angle induced between the leaf and sheath was measured.

In the rice test with both cultivars, a linear correlation was obtained between 5×10^{-3} and 5×10^{-5} µg/ml for BL and CS. The induced angles leveled off at higher concentrations. Indole-3-acetic acid (IAA) was tested and was found to produce only a weak effect, five orders of magnitude less than BL. Cytokinins were inactive and actually counteracted the effect of BL. Abscisic acid (ABA) also counteracted the effect of BL. This assay is thus highly specific for BRs and is also the most sensitive, concentrations as low as 0.05 ng/ml of BL being readily detected.

Koshihikari cultivar has also been used as a highly sensitive plant in this test (20). Dipotassium maleate is not necessarily indispensable for the bioassay, and without this, the detection limit of BRs was found to be the same as its addition in test solution.

(3) Wheat Leaf Unrolling Test

Wheat leaf unrolling test has been developed by Wada *et al.* (37), in which BRs showed strong activity. Wheat seedlings (*Triticum aestivum* L. cv. Norin No. 61) were grown in darkness at 26°C for six days, and leaf segments (1.5 cm long) were excised from the region 1.5-3.0 cm from the leaf tip under a dim green safe light. Ten of these segments were incubated in 1 ml of 2.5 mM dipotassium maleate solution containing the test sample for 24 h at 30°C in the dark. The unrolling of the leaf segment was determined by measuring their width with calipers.

BL and CS dramatically stimulated wheat leaf unrolling, their activity being dose dependent. At 0.5 ng/ml both compounds markedly stimulated unrolling, and at 0.01 $\mu\text{g/ml}$ or higher, BL produced complete unrolling of the leaf segments to about 3.6 cm. This assay is about one-tenth as sensitive as the rice-lamina inclination test, but it is simpler to carry out. GA_3 produced only slight unrolling at 0.1 to 10 $\mu\text{g/ml}$, as did the cytokinin 6-(3-methyl-2-butenyl)aminopurine. However, zeatin, 6-(4-hydroxy-3-methyl-2-butenyl)-aminopurine, caused complete unrolling at 1 $\mu\text{g/ml}$ and had a measurable effect at 0.001 $\mu\text{g/ml}$. ABA, IAA, and indole acetonitrile inhibited unrolling of leaf segments.

There have been some reports of the isolation of BRs from plant sources, employing the wheat leaf unrolling test as a bioassay; BL was isolated in pure form from the stems of *Solidago altissima* L. (38), CS was identified by the GC/MS analysis from the highly purified fraction of the immature seeds of corn (39). Similarly, BL, CS, and 28-homoCS were identified in the immature seeds of wheat (*Triticum aestivum* L.) (40). These reports suggest that isolation and purification of BRs from plants are successfully guided by the wheat leaf unrolling test.

Highly sensitive and specific bioassays are indispensable for the studies of isolation of BRs from plant sources. The above-described three bioassays have greatly contributed to the study of BRs and will continue to do so in the future.

Microanalytical Methods of Brassinosteroids

As reported in the structural determination of BL, CS, DL, and typhasterol, MS is an essential technique for BRs isolated in pure form. However, in most cases, isolation of BRs in pure form is time-consuming and tedious work because of their very low concentration in plant materials. BRs are highly polar and involatile compounds. Therefore, conversion of BRs into volatile derivatives in gas phase makes it easy to characterize BRs in a partially purified bioactive fraction by GC/MS or GC/selected ion monitoring (SIM), which are analytical techniques most frequently used in natural products chemistry. The desired derivatives of BRs are BMBs or MB-TMSs. Another convenient and useful technique is HPLC. HPLC has now been routinely and effectively employed in the purification of natural BRs. Microanalysis of BRs by HPLC has recently been developed, which involves transformation of BRs into derivatives with a fluorophore or an electrophore by use of pre-labeling reagents. Immunoassay techniques to analyze plant hormones have recently advanced and are readily accessible by plant physiologists. RIA for BRs has also been developed. In this section, microanalytical methods of BRs using GC/MS (SIM), HPLC, and RIA are described.

(1) GC/MS(SIM) Microanalysis

An analytical method of BRs by GC/MS(SIM) was developed by Takatsuto *et al.* (12,13,21). BRs are converted into a volatile derivative by use of the presence of two pairs of vicinal hydroxyl groups to prepare the BMB derivative of BRs. The derivative is suitable for gas phase analysis and also for analysis of fragmentation ions in electron impact (EI)-mass spectra. The BMBs of BL, 28-homoBL, 28-norBL and their corresponding 6-keto analogues, were well separated on GC using packed and capillary columns (capillary column showed better resolution) and gave sharp peaks. In EI mass spectrum of BL BMB, a weak molecular ion ($M^+ = 528$) was obtained and stronger peaks at m/z 457 ($\text{C}_{23}\text{-C}_{24}$ fission), 374 ($\text{C}_{20}\text{-C}_{22}$ fission), 345 ($\text{C}_{17}\text{-C}_{20}$ fission), and 332 (B-ring fission). The base peak was at m/z 155 ($\text{C}_{20}\text{-C}_{22}$ fission). In the case of EI mass spectra of BMB derivatives of 6-keto BRs, intensive molecular ions and strong ions resulting from the similar fissions of the side chain were obtained. Characteristic fragmentation patterns of BMB derivatives of BRs in EI/MS are summarized in our previous review (41). In the case of 2-deoxyBRs (e.g., teasterone, typhasterol), the side-chain was first methaneboronated and then the remaining 3-hy-

droxyl group was trimethylsilylated (13). The MB-TMS derivatives were also equally suitable for GC/MS analysis.

The GC/MS method has successfully been applied to identify traces of natural BRs from plant sources. When a sufficient amount of purified BRs was obtained from plants, full mass spectrum is taken by EI-MS. Our recent example is the identification of BL, CS, and 28-norCS in the pollen of sunflower (*Helianthus annuus* L.) (42). Full mass spectra of the BMB derivatives of these BRs were obtained and rigorous identification of them was made in this study.

In the cases of much lower content or crude samples, GC/SIM was found to be effective in detecting BRs. In the chemical ionization (CI) mass spectra the M+1 ions were the base peaks. The presence of these peaks could be used to detect BRs. By the technique of GC/SIM, BL (detected as its BMB, m/z 529) and CS (BMB, m/z 513) were found in extensively purified fractions from extracts of the immature seeds and sheaths of the Chinese cabbage, *Brassica campestris* var. *pekinensis* (43), the leaves of green tea, *Thea sinensis* (44), and chestnut (*Castanea crenata*) galls (45). The GC-SIM method was capable of detecting BRs at the picogram level. Using the GC/SIM method, rigorous identification was also attained by GC/high resolution (HR)-SIM technique. The technique was applied to the identification of BRs in the pollens of broad bean (*Vicia faba* L.) (46) and buckwheat (*Fagopyrum esculentum* Moench) (47). A highly purified bioactive fraction from buckwheat pollen was derivatized with methanboronic acid and the resulting BMB derivatives were analyzed by the GC/HR-SIM method using a capillary column in the EI-MS mode. Under our GC/MS conditions, authentic BMB derivatives of CS and BL were eluted at 13.56 and 15.16 min, respectively. The SIM results of the derivatized samples obtained from the active fraction are presented in Fig. 1. Monitoring of the molecular ion of CS BMB at m/z 512.3842 and that of BL BMB at m/z 528.3791 exhibited sharp peaks with the same retention times as those of authentic BMBs, thereby establishing rigorously the presence of BL and CS in buckwheat pollen.

Deuterio-labelled BRs have been synthesized (48). Quantitative analysis of natural BRs by the GC/MS method employing the deuteriated BRs has been carried out (49). In order to understand the growth retardation mechanism of *S*-uniconazole, the shoots of *Pisum sativum* L. treated with *S*- and *R*-uniconazoles were analyzed in terms of the levels of the endogenous GAs, BRs, and phytoosterols. Only referring to BRs, it is of interest to examine whether uniconazoles modify the biosynthesis of BRs. BRs contained in the shoots of *P. sativum* L. were extracted, purified, and analyzed by the GC/MS. GC/MS analysis of the active fraction led to the identification of CS: m/z (rel. int.) 512 (M^+ , 54%), 155 (100%). GC/SIM quantitation using an internal standard (d_6 -CS) revealed that the content of CS in the control plants was 0.9 ng/g fr. wt. and, after treatment with *S*- and *R*-uniconazoles, reduced to 54% and 34% of the controls, respectively. The result suggests that the altered metabolism of BRs is likely to be involved in the action mechanism of *S*-uniconazole.

An alternative highly sensitive technique by GC/MS is that of tandem MS or MS/MS. In this technique, the first mass filter is used to select the ion of interest from all the other ions produced from the matrix. This ion is usually the molecular ion in the EI mode or the protonated molecule (MH^+) in the CI mode. These selected ions then undergo collisionally activated dissociation to produce daughter ions which are then separated by a second mass filter and analyzed. The GC/MS/MS has been applied to identification of BRs in the pollen of European alder, *Alnus glutinosa* (L.) Gaertn. (50). A crude bioactive fraction obtained from the pollen was derivatized with methanboronic acid and the resulting derivatives were analyzed by the GC/MS/MS in the CI mode, because the protonated molecular ions of BRs BMBs are produced as base peaks in the CI mode. BL and CS have been identified as their BMB derivatives in European alder pollen.

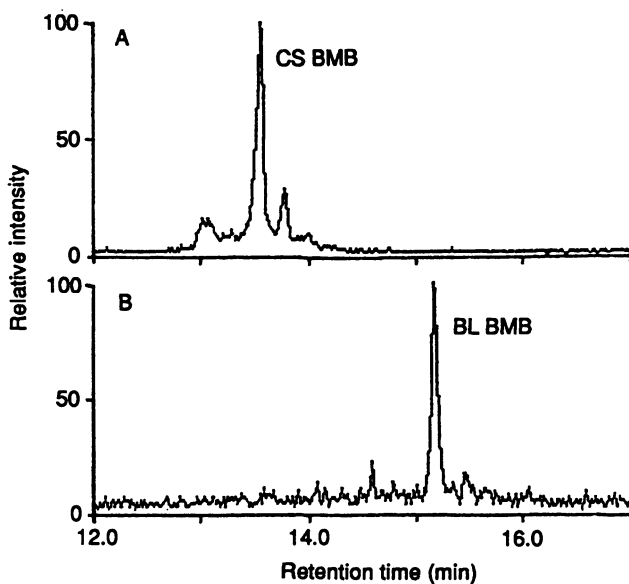


Fig. 1 GC/HR-SIM of the BMBs of CS and BL obtained from buckwheat pollen.
A: Detection of CS BMB by monitoring the molecular ion at m/z 512.3842.
B: Detection of BL BMB by monitoring the molecular ion at m/z 528.3791.

The GC/MS(SIM) microanalytical method for BRs has most frequently been employed in the analytical studies of trace levels of BRs in plants and it has greatly contributed to the studies on identification of many natural BRs and also to their distribution in plant kingdom.

(2) HPLC Analysis

Although HPLC has been employed effectively during the purification procedures of natural BRs (1,51), microanalytical method using HPLC has not been developed until recent years, because BRs themselves do not have an appropriate UV-active chromophore in the molecules. Analysis of BRs by HPLC using pre-labeling reagents has recently been developed by Takatsuto and Gamoh (14). As in the case of GC-MS analysis of BRs, the HPLC method was based on boronation of BRs with boronic acid derivatives having a fluorophore or an electrophore in the molecule. The pre-labeling boronic acid reagents were 9-phenanthreneboronic acid (52), 1-cyanoisindole-2-m-phenylboronic acid (53), dansylaminophenylboronic acid (54) and ferroceneboronic acid (55). The former three are fluorescence labeling reagents and the last one is an electrochemical one. The derivatized BRs were effectively separated by reversed-phase column using a mixture of acetonitrile and water as an eluent, and they were monitored by fluorimetric or electrochemical detector with detection limit of *ca.* 20-50 pg, depending on the pre-labeling reagents. Among the reagents, dansylaminophenylboronic acid is the most effective one because BRs derivatives derived from the reagent can be detected at longer wavelength (*Ex.* 345 nm/*Em.* 515 nm) than those from the other fluorescence reagents. Therefore, the chromatogram obtained from the derivatives is less interfered with the matrix than that from the other derivatives. The HPLC method is very convenient, highly sensitive and specific one. In some cases (14), a crude BRs fraction obtained from preparative thin-layer chromatography was sufficient to detect known BRs, because of its high sensitivity and specificity.

In order to demonstrate the usefulness and effectiveness of the HPLC method, BRs contained in buckwheat pollen were investigated (47). A highly bioactive fraction was obtained by extraction of the pollen, solvent partitionings, four successive chromatographic purification procedures including charcoal chromatography and reversed-phase preparative thin-layer chromatography. Aliquots of the active fraction were derivatized with 9-phenanthreneboronic acid and dansylaminophenylboronic acid, respectively. The resulting respective bisboronate derivatives were analyzed by reversed-phase HPLC with a fluorimetric detection of *Ex.* 305 nm/*Em.* 375 nm and of *Ex.* 345 nm/*Em.* 515 nm, respectively. The chromatograms are presented in Figs. 2. The sharp peaks with retention times of 11.17 and 15.62 min (Fig. 2A) were identified as those of the bis-9-phenanthreneboronates of BL and CS, respectively, by co-chromatography using authentic samples. In the case of the dansylaminophenylboronates, the peaks with retention times of 10.03 and 13.58 min (Fig. 2B) were identified as those of the BL and CS derivatives, respectively, by co-chromatography using the authentic samples. These HPLC data clearly indicate the presence of BL and CS in buckwheat pollen.

The HPLC analytical method seems to be convenient for the identification and quantitation of trace levels of known BRs in plants, because of its high sensitivity and specificity.

(3) Radioimmunoassay

RIAs for BRs have been developed by two groups. In the RIA by Horgan *et al.* (56), synthetic 24-epiBL was the hapten that was noncovalently bound to fetal calf serum. Hybridoma clones were generated from CAF₁ mice and employed in an enzyme-linked immunosorbent assay (ELISA) for examination of BL distribution in *Brassica napus* tissues. Pollen-producing tissues gave the highest ELISA values, extracts of pistils also gave high values, roots gave moderately high values, and leaves

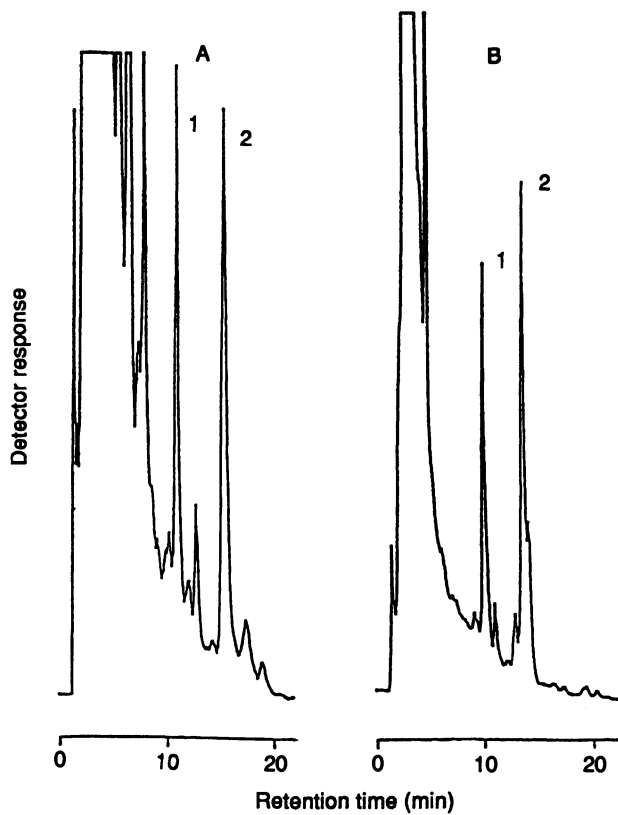


Fig. 2 Chromatograms of the 9-phenanthreneboronates (A) and the dansylaminophenylboronates (B) of BL and CS obtained from buckwheat pollen. Peaks: 1 = BL; 2 = CS.

and petals gave low values. It should be noted that the assay did not exhibit complete specificity for BL or 24-epiBL, although the latter gave the most positive ELISA values. Natural BL gave values 25% lower than the antigen whereas ecdysone, a naturally occurring plant steroid and sitosterol (a very common phytosterol), gave values 50% lower, ergosterol and stigmasterol reacted considerably less effectively. Therefore, the RIA by Horgan *et al.* appears to be quite unreliable and impractical.

Recently, Yokota *et al.* have reported the more reliable and useful RIA for BRs and they have applied it to analysis of BRs in *Phaseolus vulgaris* L. (15). They compared the detectability of BRs in *P. vulgaris* L. by employing the RIA, bioassay (rice-lamina inclination test), and the GC/SIM methods. Antiserum against CS was produced by immunizing a rabbit with CS-carboxymethylamine oxime conjugated with bovine serum albumin. In the RIA, the antiserum recognized a range of naturally occurring BRs with varying specificities. Detection limits of CS and BL were approximately 0.3 pmol, which are superior to those of the rice bioassay and the GC/SIM. Cross-reactivities of 27 compounds, including the natural and synthetic BRs, and specificities of the antiserum in the recognition of various functional groups were investigated. Although cross-reactivities of BRs with a 24-methylene group were quite low, it is of interest that functional groups for high biological activity obtained by the study of structure-activity relationship (28-31) roughly hold true in the functional group recognition.

This RIA system was successfully used for analyzing endogenous BRs in seeds and stems of *P. vulgaris* L. Fig. 3 shows the distribution of BRs after reversed-phase HPLC of shoot and seed extracts of *P. vulgaris* L. as determined by bioassay and RIA. The bioassay revealed two distinct peaks of biological activity (Fig. 3A), which were assignable to CS and BL, on the basis of the HPLC retention times. GC/MS of the combined fractions 23-26 after methaneboronation confirmed the presence of CS. BL could not be identified by GC/MS for the combined fractions 17,18 because of the low amount. The RIA for the same extracts (Fig. 3B) showed a prominent peak of CS as well as several peaks, one of which was ascribed to BL. The estimates of CS in the stem as determined by RIA and bioassay, as well as those of BL, are in good agreement. The estimates were 2.5-fold lower than that determined by GC/SIM using a deuterated internal standard because of the loss during purification procedures. Immature seeds have been shown to contain more than 30 BRs (24). Analysis of the extract by bioassay after reversed-phase HPLC (Fig. 3C) could not distinguish and quantify BRs, except for CS. In contrast, RIA could make visible several sharp peaks of BRs (Fig. 3D). One of the reasons for this is that the antiserum does not recognize biologically active BRs with the 24-methylene group. The peaks ascribable to CS, typhasterol, and teasterone were nearly completely isolated, enabling us to quantify them. The estimates of CS obtained by bioassay and RIA were in good agreement. Thus, it was revealed that stems are quite different from seeds in terms of species and quantity of the endogenous BRs. In addition, the parallelism between dosages of plant extracts and immunoreactivity was investigated because of its importance for quantitative estimation. Rather crude fractions obtained after silica gel chromatography of a seed extract were subjected to RIA at two different concentrations. The results indicated that the estimates were nearly proportional to the dosages, suggesting that RIA might be useful for rough estimates of BRs in even crude extracts, such that interfering compounds are not present in the extract.

The RIA for BRs developed by Yokota *et al.* (15) is highly sensitive and specific, and it will be one of the useful microanalytical methods.

Conclusions

Studies on BRs by many scientists more than ten years have greatly widened our knowledge of the chemistry and plant physiology of BRs, and also have paved the way

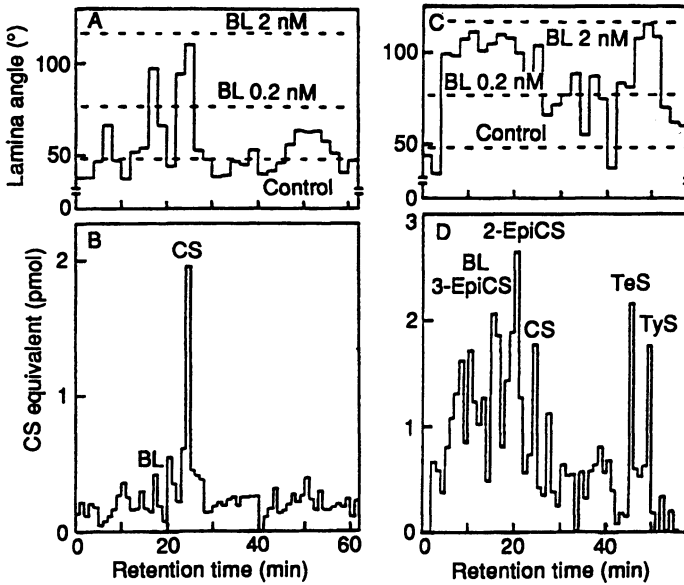


Fig. 3 Reversed-phase HPLC of the shoot and seed extracts of *P. vulgaris* as monitored by rice-lamina inclination test [(A) stem; (C) seed] and RIA with anti-CS antiserum [(B) stem; (D) seed]. Dosages in fresh-weight tissue equivalents: 150 g in A; 2 g in B; 30 g in C; and 0.5 g in D. TeS : teasterone; TyS : typhasterol.

to the possibility of their practical applications in agriculture. With respect to microanalysis of BRs, highly sensitive and specific bioassays and analytical methods including GC/MS (SIM), HPLC, and RIA have been developed. Combination of the bioassays and the microanalyses has successfully led to demonstration of ubiquitous distribution of BRs in the plant kingdom and identification of more than 30 compounds from plant sources. In particular, the microanalyses will contribute to the more detailed study on the physiological mechanism of BRs, because they are indispensable to identification and quantitation of endogenous BRs which are involved in plant growth and development.

Literature Cited

- 1) Grove, M. D.; Spencer, G. F.; Rohwedder, W. K.; Mandava, N.; Worley, J. F.; Warthen Jr., J. D.; Steffens, G. L.; Flippen-Anderson, J. L.; Cook Jr., J. C. *Nature*, **1979**, *281*, 216.
- 2) Yokota, T.; Arima, M.; Takahashi, N., *Tetrahedron Lett.*, **1982**, *23*, 1275.
- 3) Mandava, N. B., *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, **1988**, *39*, 23.
- 4) Yokota, T.; Takahashi, N., In *Plant Growth Substances 1985*, Bopp, M., Ed.; Springer-Verlag: Berlin Heidelberg, **1986**, pp. 129-138.
- 5) Yokota, T., *Chemical Regulation of Plants*, **1987**, *22*, 10 (in Japanese).
- 6) Mori, K., *J. Synth. Org. Chem. Japan*, **1985**, *43*, 849 (in Japanese).
- 7) Adam, G.; Marquardt, *Phytochemistry*, **1986**, *25*, 1787.
- 8) Takematsu, T.; Takeuchi, Y.; Koguchi, M., *Chemical Regulation of Plants*, **1983**, *18*, 38 (in Japanese).
- 9) Fujita, F., *Chemistry and Biology*, **1985**, *23*, 717 (in Japanese).
- 10) Kamuro, Y.; Takatsuto, S., *Chemical Regulation of Plants*, **1988**, *23*, 142 (in Japanese).
- 11) Takatsuto, S.; Futatsuya, F.; *J. Japan Oil Chem. Soc.*, **1990**, *39*, 227 (in Japanese).
- 12) Takatsuto, S.; Ying, B.; Morisaki, M.; Ikekawa, N., *J. Chromatogr.*, **1982**, *239*, 233.
- 13) Takatsuto, S.; Ikekawa, N., *Chem. Pharm. Bull.*, **1986**, *34*, 3435.
- 14) Takatsuto, S.; Gamoh, K., *Chemical Regulation of Plants*, **1990**, *25*, 114 (in Japanese).
- 15) Yokota, T.; Watanabe, S.; Ogino, Y.; Yamaguchi, I.; Takahashi, N., *J. Plant Growth Regul.*, **1990**, *9*, 151.
- 16) Wada, K.; Marumo, S.; Ikekawa, N.; Morisaki, M.; Mori, K., *Plant Cell Physiol.*, **1981**, *22*, 323.
- 17) Abe, H.; Tsunoda, A.; Furuhashi, A., In *Kagaku Kenkyuho Hojokin (Siken Kenkyu 1) Kenkyu Seika Houkokusho*, Takahashi, N., Ed.; **1987**, pp. 17-21.
- 18) Yokota, T.; Kim, S.-K.; Fukui, Y.; Takahashi, N.; Takeuchi, Y.; Takematsu, T., *Phytochemistry*, **1987**, *26*, 503.
- 19) Takatsuto, S.; Abe, H.; Gamoh, K., *Agric. Biol. Chem.*, **1990**, *54*, 1057.
- 20) Arima, M.; Yokota, T.; Takahashi, N., *Phytochemistry*, **1984**, *23*, 1587.
- 21) Ikekawa, N.; Takatsuto, S.; Kitsuwa, T.; Saito, H.; Morishita, T.; Abe, H., *J. Chromatogr.*, **1984**, *290*, 289.
- 22) Park, K.-H.; Saimoto, H.; Nakagawa, S.; Sakurai, A.; Yokota, T.; Takahashi, N.; Shono, K., *Agric. Biol. Chem.*, **1989**, *53*, 805.
- 23) Abe, H., Abstracts of Symposium on Brassinosteroids, American Chemical Society Brassinosteroids Conference, 27-31 August, 1990, Washington DC, Abstract 131.
- 24) Yokota, T.; Koba, S.; Kim, S. K.; Takatsuto, S.; Ikekawa, N.; Sakakibara, M.; Okada, K.; Mori, K.; Takahashi, N., *Agric. Biol. Chem.*, **1987**, *51*, 1625.

- 25) Thompson, M. J.; Svoboda, J. A.; Feldlaufer, M. F., In *Analysis of Sterols and Other Biologically Significant Steroids*, Nes, W. D.; Parish, E. J., Ed.; Academic Press, San Diego, California, 1989, pp. 81-105.
- 26) DeLuca, H. F., In *Biochemistry of Steroid Hormones*, Makin, H. L. J., Ed.; Blackwell Scientific Publications, Oxford, 1984, pp. 71-116.
- 27) Yokota, T.; Ogino, Y.; Takahashi, N.; Saimoto, H.; Fujioka, S.; Sakurai, A., *Agric. Biol. Chem.*, 1990, 54, 1107.
- 28) Thompson, M. J.; Meudt, W. J.; Mandava, N. B.; Dutky, S. R.; Lusby, W. R.; Spaulding, D. W., *Steroids*, 1982, 39, 89.
- 29) Takatsuto, S.; Yazawa, N.; Ikekawa, N.; Takematsu, T.; Takeuchi, Y.; Koguchi, M., *Phytochemistry*, 1983, 22, 2437.
- 30) Takatsuto, S.; Yazawa, N.; Ikekawa, N.; Morishita, T.; Abe, H., *Phytochemistry*, 1983, 22, 1393.
- 31) Takatsuto, S.; Ikekawa, N.; Morishita, T.; Abe, H., *Chem. Pharm. Bull.*, 1987, 35, 211.
- 32) Thompson, M. J.; Mandava, N. B.; Meudt, W. J.; Lusby, W. R.; Spaulding, D. W., *Steroids*, 1981, 38, 567.
- 33) Schneider, J. A.; Yoshihara, K.; Nakanishi, K.; Kato, N., *Tetrahedron Lett.*, 1983, 24, 3859.
- 34) Yokota, T.; Arima, M.; Takahashi, N.; Takatsuto, S.; Ikekawa, N.; Takematsu, T., *Agric. Biol. Chem.*, 1983, 47, 2419.
- 35) Abe, H.; Morishita, T.; Uchiyama, M.; Takatsuto, S.; Ikekawa, N., *Agric. Biol. Chem.*, 1984, 48, 2171.
- 36) Wada, K.; Marumo, S.; Abe, H.; Morishita, T.; Nakamura, K.; Uchiyama, M.; Mori, K., *Agric. Biol. Chem.*, 1984, 48, 719.
- 37) Wada, K.; Kondo, H.; Marumo, S., *Agric. Biol. Chem.*, 1985, 49, 2249.
- 38) Tada, E.; Uchiyama, M.; Funayama, S., *Japan Patent*, 1-117899, (1989).
- 39) Ikeda, M.; Sassa, T.; Tulloch, A. P., *Nippon Nogeikagaku Kaishi*, 1988, 62, 376 (in Japanese).
- 40) Ikeda M.; Sassa, T.; Tulloch, A.P.; Hoffmann, L. L., *Nippon Nogeikagaku Kaishi*, 1988, 62, 1607 (in Japanese).
- 41) Ikekawa, N.; Takatsuto, S., *Mass Spectroscopy*, 1984, 32, 55.
- 42) Takatsuto, S.; Yokota, T.; Omote, K.; Gamoh, K.; Takahashi, N., *Agric. Biol. Chem.*, 1989, 53, 2177.
- 43) Abe, H.; Morishita, T.; Uchiyama, M.; Marumo, S.; Munakata, K.; Takatsuto, S.; Ikekawa, N.; *Agric. Biol. Chem.*, 1982, 46, 2609.
- 44) Morishita, T.; Abe, H.; Uchiyama, M.; Marumo, S.; Takatsuto, S.; Ikekawa, N., *Phytochemistry*, 1983, 22, 1051.
- 45) Ikeda, M.; Takatsuto, S.; Sassa, T.; Ikekawa, N.; Nukina, M., *Agric. Biol. Chem.*, 1983, 47, 655.
- 46) Ikekawa, N.; Nishiyama, F.; Fujimoto, Y., *Chem. Pharm. Bull.*, 1988, 36, 405.
- 47) Takatsuto, S.; Omote, K.; Gamoh, K.; Ishibashi, M., *Agric. Biol. Chem.*, 1990, 54, 757.
- 48) Takatsuto, S.; Ikekawa, N., *Chem. Pharm. Bull.*, 1986, 34, 4045.
- 49) Yokota, T.; Nakamura, Y.; Takahashi, N.; Nonaka, M.; Sekimoto, H.; Oshio, H.; Takatsuto, S., In *Gibberellin Symposium Tokyo 1989*, Takahashi, N.; Phinney, B. O.; MacMillan, J., Ed.; Springer-Verlag: Berlin Heidelberg, 1990, pp. 339-349.
- 50) Plattner, R. D.; Taylor, S. L.; Grove, M. D., *J. Natural Products*, 1986, 49, 540.
- 51) Yokota, T.; Baba, J.; Koba, S.; Takahashi, N., *Agric. Biol. Chem.*, 1984, 48, 2529.
- 52) Gamoh, K.; Omote, K.; Okamoto, N.; Takatsuto, S., *J. Chromatogr.*, 1989, 469, 424.
- 53) Gamoh, K.; Takatsuto, S., *Anal. Chim. Acta*, 1989, 222, 201.

- 54) Gamoh, K.; Okamoto, N.; Takatsuto, S.; Tejima, I., *Anal. Chim. Acta*, **1990**, *228*, 101.
- 55) Gamoh, K.; Sawamoto, H.; Takatsuto, S.; Watabe, Y.; Arimoto, H., *J. Chromatogr.*, **1990**, *515*, 227.
- 56) Horgan, P. A.; Nakagawa, C. H.; Irvin, R. T., *Can. J. Biochem. Cell Biol.*, **1984**, *62*, 715.

RECEIVED May 1, 1991

Chapter 11

Molecular Analysis of Brassinolide Action in Plant Growth and Development

S. D. Clouse and D. Zurek

Department of Biology and Molecular Biology Institute, San Diego State
University, San Diego, CA 92182

Brassinolide, a plant steroid lactone, can substitute for auxin in a soybean epicotyl elongation assay. We have shown by two dimensional gel analysis of *in vitro* translated mRNA, that sub-micromolar concentrations of brassinolide alter the pattern of gene expression in elongating soybean stem sections, both in the presence or absence of added auxin. Northern blot analysis, using cDNAs corresponding to auxin-regulated genes as probes, has shown that the molecular mechanism of brassinolide-induced elongation is likely to differ from that of auxin-induced elongation in this system. We have also found that nanomolar concentrations of brassinolide can enhance xylem differentiation in cultured explants of Jerusalem artichoke tubers. These molecular data provide further evidence that brassinolide is a true plant growth regulator.

Recent developments in plant molecular biology have provided powerful tools of analysis for examining important problems in classical plant physiology at new levels of molecular detail and at deeper levels of insight into complex regulatory mechanisms. One critical area of plant biology which will be clarified by this molecular approach is the control of plant differentiation and development by phytohormones. Brassinolide (BR), a plant steroid lactone, has pronounced effects on the growth and development of a wide variety of crop plants. As described elsewhere in this volume, BR represents the most biologically active compound of a group of related natural products termed brassinosteroids. BR acts at very low concentrations (10^{-9} M) and shows strong synergistic interactions with auxin and additive interactions with gibberellins in many test systems. Over the past decade, the molecular analysis of auxin, gibberellic acid, abscisic acid and ethylene has begun in earnest in a number of laboratories and substantial progress has been made in identifying genes that are transcriptionally regulated by plant hormones (*1*). Despite the obvious growth-promoting effects of BR on plants and its demonstrated interactions with other growth regulators, little is

known about the molecular mechanisms of BR action. Molecular studies on brassinolide may have been slowed by the lack of readily available BR and radioactive BR, and by lack of a model system with properties suitable for recombinant DNA analysis of BR effects on plant growth.

Our laboratory has recently begun a molecular analysis of brassinolide action in plant growth and development in collaboration with Dr. Trevor McMorris, Department of Chemistry, University of California, San Diego (UCSD) and Dr. Michael Baker, UCSD School of Medicine. Our broad, long-term goals are; (1) to examine the effects of BR on gene transcription and mRNA translation in elongating, dividing and differentiating plant cells, (2) to identify and characterize a BR receptor, and (3) to produce large enough quantities of BR, BR analogs and radioactive BR to achieve 1 and 2. Some of the questions we wish to address are (a) what are the number and nature of genes that respond to BR in systems such as elongating soybean stem sections and differentiating tuber explants of Jerusalem artichoke in culture? (b) what is the molecular basis for the observed synergism between BR and auxin and where in the signal perception and transduction pathways of the two compounds does the overlap occur? (c) is the mechanism of BR action similar to animal steroid hormones in terms of binding to a zinc-fingered protein followed by transcriptional activation of specific genes? (d) what functional groups of BR are necessary for optimal activity and can a 'super' BR analog be synthesized that has more potent growth effects than the natural compound?

In order to establish a molecular data base for BR and begin to address questions (a) and (b) above, we have examined whether BR affects the transcription of auxin-induced genes in elongating soybean epicotyl sections using available auxin-induced soybean sequences as probes. More generally, we have shown changes in protein synthesis caused by BR in soybean epicotyls and hypocotyls by *in vitro* translation of mRNA (+ or - BR) followed by 2-D gel electrophoresis.

Regulation of Gene Expression by Auxins

Since one of our objectives is to examine the effects of BR on auxin-inducible gene expression, a brief review of studies on auxin-regulated gene expression follows. The application of recombinant DNA techniques has provided the necessary tools to demonstrate convincingly that plant hormones can cause selective and rapid changes in the levels of specific mRNAs. The molecular mechanisms of this hormonal gene regulation and its causal relationship to physiological processes has not, as yet, been clarified. The role of auxins in the regulation of gene expression has been the subject of four recent reviews (1-4). Initial studies utilized elongating tissue sections and compared *in vitro* translation products on 2-D PAGE for tissues plus or minus added auxin. Several hundred peptides appeared on the gels but only 1 or 2 % changed in response to auxin (1). Zurfluh and Guilfoyle found that ten polypeptides (25 -35 kDa) of soybean hypocotyls increased after treatment of the hypocotyls for one hour with 2,4-D. One of the ten increased after only 15 minutes (5). Several research groups have reported construction of cDNAs to mRNAs that are increased rapidly after various auxin treatments. Walker and Key (6) demonstrated a direct, rapid (15-30 min.) and selective effect of auxin on the expression of a small number of mRNAs in

soybean tissue undergoing both cell elongation and cell division in response to auxin. A direct causal relationship between mRNA induction and cell elongation could not be established, however. Two of the cDNA clones (pJCW1 and pJCW2) were characterized and later used to isolate the corresponding genomic clones from soybean (7). Sequence data indicated the genes coded for hydrophilic proteins, showed several regions of high homology and belonged to two related multigene families. Two conserved sequences of 9 and 11 base pairs occurred at similar distances upstream of the transcription start site in each gene. It was speculated these sequences might be involved in transcriptional regulation but a functional promoter assay for auxin-induced sequences has yet to be employed.

Hagen, et al. (8), utilized differential hybridization to isolate four auxin-responsive clones from a cDNA library constructed from 2,4-D treated soybean seedlings. The four clones (designated pGH1, pGH2, pGH3 and pGH4) were induced within 15-30 min., increased 2 to 16 fold after 30 minute treatment with 2,4-D and gave similar results in tissue excised from elongating and non-elongating soybean hypocotyls (indicating perhaps a role in cell division as well as cell elongation). Further studies (9) using run-on transcription in nuclei isolated from soybean plumules (plus or minus auxin treatment) showed all four clones were transcriptionally activated 10 to 100 fold by auxin with increased rates observed within 5 minutes of 2,4-D addition. Protein synthesis was not required for the induction as demonstrated by lack of inhibition by cycloheximide. These results showed that in this system, auxin-regulated gene expression is at least partly under rapid transcriptional control and suggests that these transcriptional events are close to the primary site of auxin action. McClure and Guilfoyle (10), isolated three additional cDNA clones, termed Small Auxin Up Regulated (SAUR) sequences, which hybridized to small (550 nucleotides) auxin-induced mRNAs in elongating soybean hypocotyls. The induction was specific to auxins and was extremely rapid. The mRNAs could be detected on Northern blots of total RNA within 2 to 5 minutes after addition of 50 μ M 2,4-D. The activation of two of the mRNAs was not affected by cycloheximide, whereas one was, indicating different mechanisms of induction. The sequence and organization of the genomic clones corresponding to these cDNAs has recently been published (11). Theologis, et al. (12), isolated cDNAs to three IAA-inducible mRNAs in pea epicotyl tissue. The induction was again rapid (15 min.), substantial (50-100 fold), and specific to auxins. The induction was independent of protein synthesis and was abolished by the RNA polymerase II inhibitor alpha-amanitin. Finally, van der Zaal, et al. (13), found that treating hormone-starved cell suspension cultures of tobacco with 2,4-D caused accumulation of seven mRNAs as early as 15-30 minutes after auxin addition. Run-on transcription in isolated nuclei showed some of the mRNAs were increased by enhanced transcription rates and four of the seven were induced independently of cycloheximide. The auxin-regulated genes from soybean which have been cloned to date, are summarized in Table I.

Effects of Brassinolide on Elongating Soybean Stem Sections

A logical place to begin our molecular studies of BR action was to use the soybean stem elongation assays developed over the last decade by many researchers, as

Table I. Soybean Auxin-Regulated Genes

Clone ^a	Size	Induction	Earliest	Max.
pGH1	1700	3x	15 min.	4 hr
pGH3	2400	33x	15 min.	2 hr
pGH4	1100	6x	15 min.	2 hr
SAUR 6	560	25x	2 min.	1 hr
SAUR 10A	530	26x	2 min.	1 hr
SAUR 15	530	50x	2 min.	1 hr
JCW1	1164	3x	30 min.	-
JCW2	999	8x	15 min.	-

^aGH clones are described in reference 8; SAUR clones in ref. 10; JCW clones in ref. 6.

described above. Since we had on hand four auxin-inducible sequences from soybean (pGH1-4, provided by Dr. Gretchen Hagen) and we knew that BR caused elongation of mung bean epicotyls without exogenous auxin (14), our first experiment was simply to substitute BR for 2,4-D in the soybean auxin elongation system developed by Zurfluh and Guilfoyle (5) and Hagen and Guilfoyle (8). While the early soybean work focused on hypocotyls, our experiments utilized epicotyls from light grown soybean seedlings because of reports that BR affects epicotyl elongation but not hypocotyl elongation in mung bean and soybean (15) and then, only in the light. However, recently we have found that BR also stimulates elongation of soybean hypocotyl sections in the absence of added auxin. Epicotyl sections (1.5 cm) directly below the plumules (elongating epicotyl sections) were excised from 10 day old, light grown soybean seedlings and preincubated 2 hr to deplete endogenous auxin as described (5). 1.5 cm sections were also taken from directly above the cotyledons (basal epicotyl sections) and treated similarly. After preincubation, the media was discarded and replaced with either fresh media alone or media containing 2.5×10^{-8} M BR. Sections were incubated at 28⁰ C with shaking under constant fluorescent light for 17 hr. Figure 1 shows that BR alone, without added auxin, caused a dramatic increase in the length of epicotyl sections from the elongating but not the basal region of soybean epicotyls. This mimics the effect of auxin alone on this system (8). We also found, using the same assay system that BR causes elongation of *Arabidopsis thaliana* peduncles (1 cm sections taken directly below the terminal inflorescence). This is significant in view of the importance of *A. thaliana* as a model system for plant molecular biology (16).

Many view the role of BR as a sensitizer of tissue to endogenous auxin (15). Although we incubated the sections for 2 hr without auxin, this is almost certainly not

sufficient to deplete all endogenous auxin in the epicotyl sections and, therefore, BR may be acting to enhance endogenous auxin activity in the epicotyl tissue. To test this idea further, we are examining the effect of BR on the expression of auxin-inducible genes in soybean. We have isolated total RNA from light grown soybean epicotyl sections using assay conditions exactly as described (5). Treatments were; control (no hormones), 2,4-D alone, BR alone, and BR plus 2,4-D. The RNA was analyzed by Northern blot analysis in 1.2% agarose denaturing gels (17). Eight independent auxin-inducible sequences are available: cDNA clones pGH1, pGH3, and pGH2/4 (2 and 4 turned out to be from the same gene (18)), and 5 oligonucleotides synthesized from the published sequence of 5 independent auxin regulated genes (Table 1). We have already shown that BR causes elongation in soybean epicotyls. Therefore, we are poised to perform a whole series of straightforward molecular bioassays based on RNA isolation and Northern blot analysis using the 8 auxin-up sequences as probes. By applying BR at different concentrations and in different combinations (and order of addition) with auxin and then testing for the effect on expression of these 8 genes, we should be able to determine if the same pathway is being affected and if the response is synergistic or additive. For example, suppose BR is a transcriptional activator of the gene encoding an auxin receptor (via a zinc-fingered protein as in vertebrates?). Trewavas (19) has proposed that sensitivity of a tissue (perhaps determined by the number of receptors) is more critical in determining response to a hormone than the hormonal concentration. If BR causes a rapid increase in an inducible auxin receptor, the synergism of BR and auxin observed in many systems could be explained. It would also explain the required preincubation of tissue with BR in order to obtain synergism with auxin (20). If this were the case in soybean, we should observe all 8 genes being induced by BR (after a suitable lag and assuming all 8 genes are in the same signal perception and transduction pathway). Since BR causes elongation in this system without added auxin, if BR treatment alone does not cause up-regulation of the genes it would work against the hypothesis that BR causes increased sensitivity to endogenous auxin.

Figure 2 shows our initial results from these studies using as probe pGH1. Clearly, even though BR alone causes soybean epicotyl elongation, it does not cause the rapid induction of mRNA corresponding to the auxin-inducible pGH1. After 17 hours, there is a clear difference in GH1 expression between control and BR-treated tissue. However, comparison of the later timepoint with the 2 hour blot indicates that this difference in expression may be due to differential mRNA stability brought about by BR, rather than induction of the GH1 gene. Stabilization of mRNAs by steroid hormones is a common phenomenon in animal systems (Michael Baker, personal communication). These results are not consistent with the hypothesis that BR is simply sensitizing the tissue to auxin or merely increasing the endogenous levels of auxin in the epicotyl tissue. BR appears to cause elongation by a mechanism that is different, although very likely related to and intertwined with that of auxin. Northern blots would also be useful in studying the effects of auxin antagonists such as TIBA on BR-regulated expression of the 8 genes as well as with inhibitors of elongation such as cytokinins. For example, Walker et al. (21) found that while kinetin inhibited auxin-induced hypocotyl elongation, the auxin-induced expression of pJCW1 and pJCW2

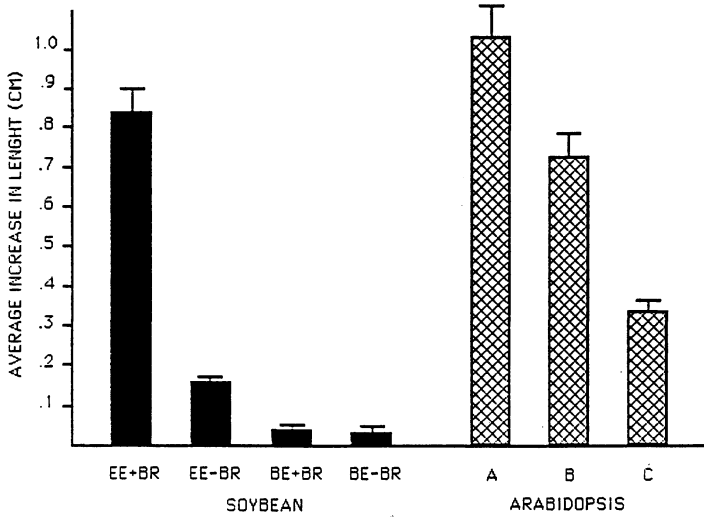
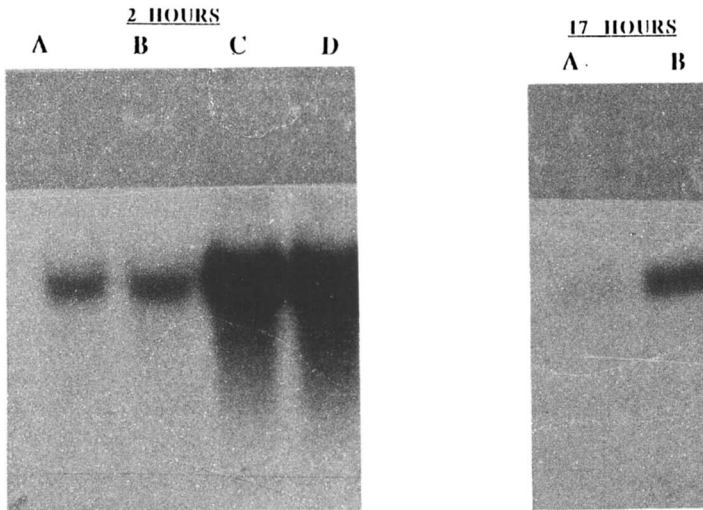


Figure 1. Effect of Brassinolide on Elongation of Soybean and *Arabidopsis thaliana* stem sections. Epicotyl sections (1.5 cm, soybean) or peduncle sections (1 cm, *Arabidopsis*) were excised and treated as described in the text. EE + BR = 25 nM BR, elongating epicotyl; EE - BR = elongating epicotyl control; BE + BR = 25 nM BR, basal epicotyl; BE - BR = basal epicotyl control. A = 250 nM BR, peduncle; B = 25 nM BR, peduncle; C = control peduncle.



PROBE: GH1
 A = CONTROL, B = 10^{-7} M BRASSINOLIDE
 C = 5×10^{-5} M AUXIN, D = 10^{-7} M BR + 5×10^{-5} M AUXIN

Figure 2. Northern Blot Analysis of 1.5 cm Soybean Elongating Epicotyl Sections. RNA was isolated 2 or 17 hours after addition of BR and electrophoresed through a 1.2% denaturing agarose gel (17). After transfer to a nylon membrane (Zeta Probe, Bio-Rad) blots were hybridized with 10^6 cpm/ml 32 P labeled pGH1.

was not affected. The effect of kinetin on BR induced elongation and gene expression will be of interest.

Brassinolide Affects Gene Expression in Elongating Stem Sections

As noted above, all plant hormones studied to date affect gene expression in some way. To determine if BR also follows this pattern, we used the same RNA isolated for Northern blot analysis of soybean epicotyls and hypocotyls for *in vitro* translation and examination by 2-D gel electrophoresis. The pattern of protein synthesis induced by auxin in this system is known (5) and we were thus able to compare BR and auxin effects. Figure 3 shows that BR alone, causes quantitative changes in gene expression in excised soybean hypocotyl sections. Poly-A+ RNA was isolated from auxin-depleted hypocotyl sections two hours after treatment with buffer or 0.34 μM brassinolide. The isolated RNA was translated *in vitro* with wheat germ extract and radiolabeled methionine and subjected to 2-D gel analysis as described by O'Farrell (22). Proteins induced by BR are indicated by numbers, while those down-regulated by BR treatment are marked with letters. Proteins 6, 7 and 8 are strongly induced by BR. We were also interested to determine if BR in the presence of auxin could induce specific peptides that were different from those induced by auxin alone. The results shown in Figure 4 confirm that this is the case. A summary of peptides induced by brassinolide under various conditions is presented in Table II. We have also found that BR alters the pattern of gene expression in elongating peduncles of *Arabidopsis thaliana* by a similar analysis (data not shown). Our findings that BR affects the levels of a variety of specific gene products lends further credibility to the argument that BR is a true plant growth regulator.

Effect of BR on *H. tuberosus* xylem differentiation.

Another system we are exploring in our laboratory is the auxin and cytokinin induced differentiation of tuber explants of Jerusalem artichoke (*Helianthus tuberosus*) into xylem elements. We are interested in genes that are transcriptionally activated when tuber explants of *H. tuberosus* are transferred from a control (non-differentiating) to a xylogenic medium. We chose the culture system of Markland and Haddon (23) for *H. tuberosus* because the higher yield of tracheary elements (36%) and the shorter culture period is more practical for molecular investigations than liquid culture systems (24). The system has also been used effectively for biochemical studies (25). Figure 5 shows cells that have been cultured on control or xylogenic medium. The distinct helical patterns of secondary cell wall differentiation in the tracheid elements (panel B) is clearly visible. Normally, xylem differentiation in this system requires 3 to 4 days with very few vascular elements appearing in the first 24 hr after transfer from preculture to xylem-inducing medium. To our surprise, when BR (at 6.8×10^{-9} M) was added to the xylem-inducing medium, a tenfold increase in xylem differentiation was observed after 24 hr. This was a very extensive experiment involving microscopically counting thousands of cells. The entire experiment was then repeated with nearly identical results (experiment 1; 3.4 % xylem (+BR), 0.35% xylem (-BR): experiment 2; 3.47% xylem (+BR), 0.56% xylem (-BR)). The experiment was

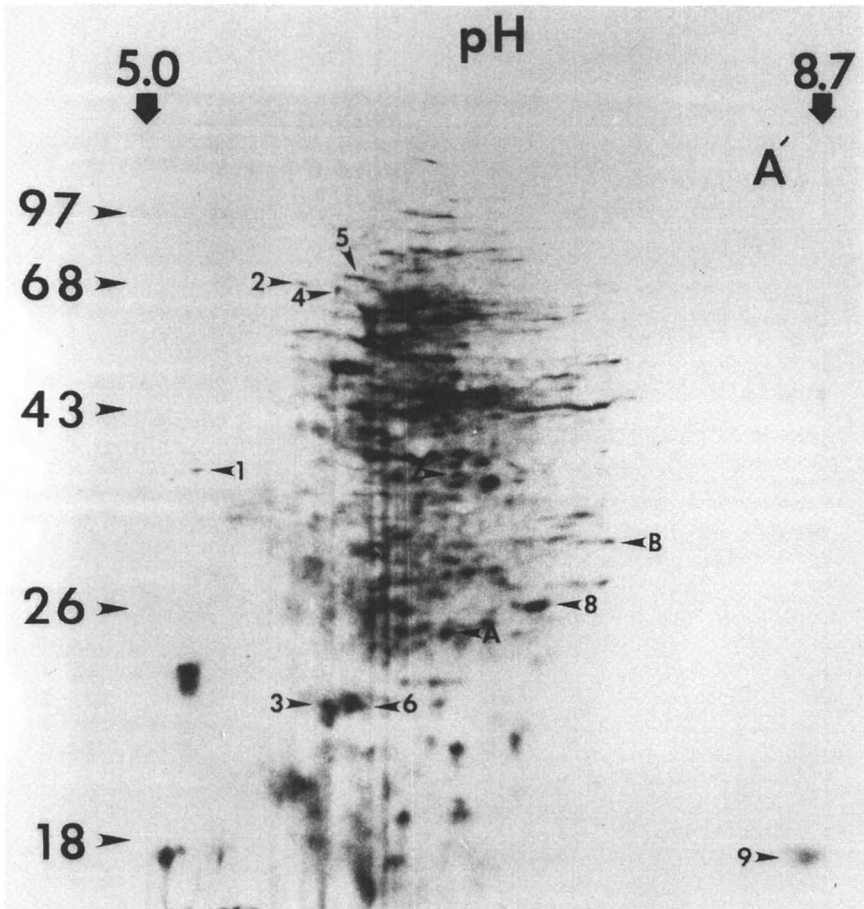


Figure 3. 2-D Gel Electrophoresis of *in vitro* Translated Soybean Hypocotyl mRNA. Numbers to the left indicate migration of molecular weight markers (kilodaltons). The separating gel was 12% polyacrylamide. Numbered arrows indicate polypeptides that are up-regulated by BR while lettered arrows show polypeptides that are down-regulated in response to BR. Other experimental details are described in the text. A = hypocotyl sections auxin-depleted for 2 hours followed by buffer treatment for 2 hours; A' = as in A with 340 nM BR replacing buffer treatment.

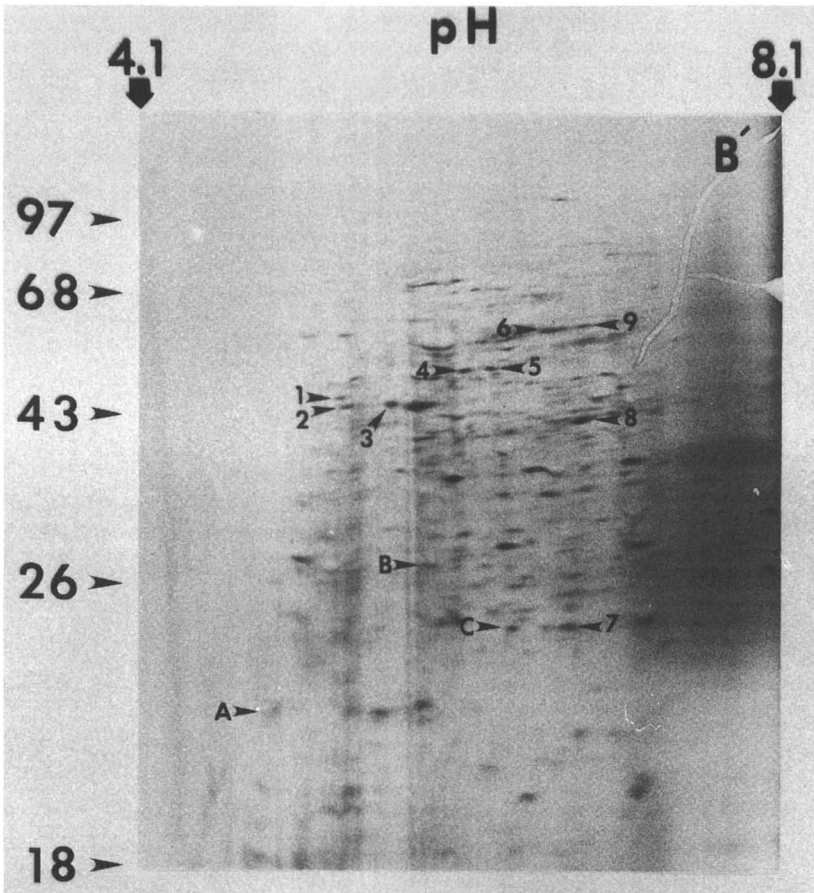


Figure 4. 2-D Gel Electrophoresis of in vitro Translated Soybean Epicotyl mRNA. Experimental conditions are as described in Figure 3. B = epicotyl sections auxin depleted for 2 hours followed by incubation with 5×10^{-5} M 2,4-D for 2 hours; B' = epicotyl sections auxin depleted as in B followed by incubation with 3.4×10^{-7} M BR for 30 minutes with a further treatment of both 3.4×10^{-7} M BR and 5×10^{-5} M 2,4-D for 2 hours.

repeated two more times with slightly more variable results but, as shown in Figure 6, there is a significant increase in both the proportion of xylem elements and total cell number, indicating BR is influencing both cell division and some process in the differentiation pathway. To our knowledge, this is the first example of BR being tested in such a model system of differentiation.

Table II. Specific Polypeptides Induced by Brassinolide in Elongating Soybean Stem Sections

Treatment	pI	MW	#Induced
1. Soy Epicotyl, 2 hr 340 nM BR, 50 μ M 2,4-D	5.0-6.0	15-25 kD	2
		25-43 kD	1
	6.0-7.0	15-25 kD	1
		25-43 kD	2
		43-68 kD	4
2. Soy Epicotyl, 17 hr 100 nM BR	5.0-6.0	15-25 kD	1
		25-43 kD	2
		43-68 kD	5
	6.0-7.0	15-25 kD	1
		25-43 kD	1
		43-68 kD	1
3. Soy Hypocotyl, 2 hr 340 nM BR	5.0-6.0	15-25 kD	4
		25-43 kD	5
		43-68 kD	2
	6.0-7.0	15-25 kD	4
		25-43 kD	6
		43-68 kD	4
	7.0-8.0	25-43 kD	2
	8.0-9.0	15-25 kD	4
	25-43 kD	2	

Current and Future Research Objectives

Cloning of BR-induced genes in soybean by subtractive hybridization. Now that we have shown a change in the pattern of gene expression in soybean stems, our immediate goal is to clone genes that are regulated by brassinolide in this system. Based on the results from our 2D gel analysis we chose 17 hour epicotyl mRNA as the optimal initial experimental condition for generating a cDNA library. We have constructed the cDNA library in a modified phage insertion vector termed Lambda Zap II (Stratagene). This vector combines the efficiency of a phage vector with the ease of analysis (sequencing, mini-preps) of a plasmid vector. Lambda Zap II contains a 3 kb



Figure 5. Differentiation of *Helianthus tuberosus* Explants in Culture. Explants were cultured as described by Markland and Haddon (23). A, control medium; B, xylogenic medium. Explants were collected after 4 days of incubation, stained with Safferin O and photographed under phase contrast microscopy.

Publication Date: November 4, 1991 | doi: 10.1021/bk-1991-0474.ch011

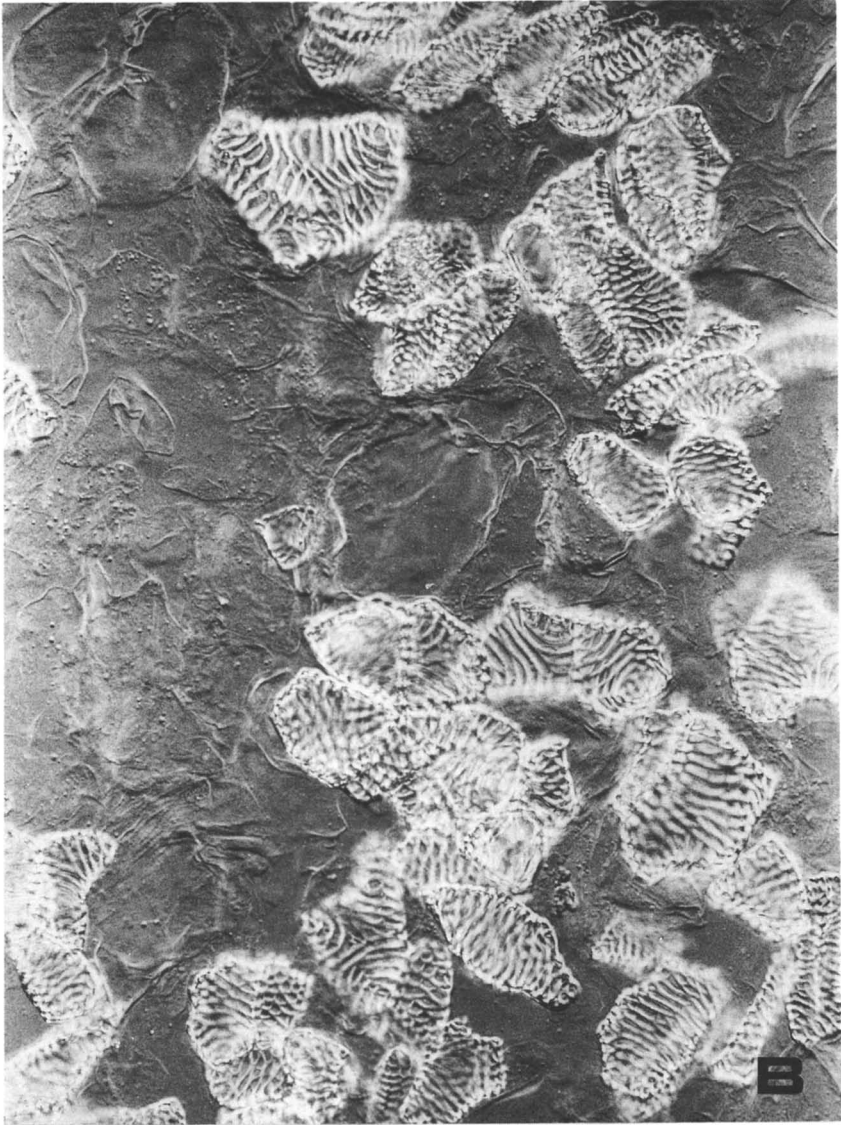


Figure 5. Continued.

plasmid (excisable *in vivo* with helper phage) within a lambda phage for rapid subcloning of inserts. cDNAs are flanked on either side by T7 and T3 promoters for *in vitro* RNA transcription. Several molecular techniques have become available which monitor changes in gene expression in two related tissue types (e.g. control and hormone-induced). The two most applicable for our purpose are +/- screening (26) and subtractive hybridization (27). Both procedures use probes derived from populations of mRNAs rather than gene-specific oligonucleotides, antibodies, or heterologous probes and therefore can identify regulated genes of unknown function. The technique of +/- screening works well when an mRNA is abundant in one cell type (at least 0.1% of the mRNA population) and rare or absent in another (26). Because of a high background of common clones, +/- screening cannot detect differential transcripts below 0.1% abundance. To detect rare transcripts (0.01% or less of total mRNA) a "subtractive hybridization" method is used. In this procedure labeled first strand cDNA is prepared from mRNA of one tissue and hybridized to excess mRNA from the second tissue. Sequences common to both tissues hybridize leaving the sequences unique to tissue one single-stranded. Single strands are separated from double strands and the single-stranded hot probe used to screen a cDNA library derived from tissue one. The reduction in probe complexity results in decreased background so longer exposure times can be used. This leads to the increased sensitivity. Figure 7 summarizes the subtractive hybridization procedure we are currently using. Subtractive hybridization has been used successfully to identify rare, differentially expressed transcripts in auxin-treated soybean (10), T cell lymphocytes (28-30), rat tumors (31), SV-40 transformed mouse cells (32), human tumor cells (33), and developing fungi (34). Cloning cDNAs for BR-responsive genes in soybean will give us probes for monitoring the kinetics of gene induction by BR under different circumstances, will allow *in situ* hybridization probes to be made and will allow us to isolate genomic clones for future analysis of BR-regulated promoter activity in electroporated soybean protoplasts (35) and transgenic plants.

Xylem Differentiation in Jerusalem Artichoke. As mentioned above, we have noticed a clear effect of BR on enhanced xylem differentiation in culture. We will isolate mRNA from differentiating tuber explants at various time-points (with or without BR treatment) and perform *in vitro* translation and 2-D gel analysis to determine the overall patterns of gene expression. To isolate genes that are transcriptionally activated by BR treatment we will construct a cDNA library from xylogenic cultures + BR at 24 hr. Rather than subtractive hybridization, we will use an elegant differential screening technique (which directly identifies transcriptionally activated genes) first described by Somssich et al. (36). This procedure involves differential screening of a cDNA library using labeled run-off transcripts derived from nuclei of treated (+) and untreated (-) cells. We already have on hand transcriptionally active nuclei isolated from xylogenic *H. tuberosus* explants treated with or without BR. Cloning of these cDNAs will provide probes for kinetic studies of expression during xylogenesis and *in situ* hybridization studies in developing plants to test for xylem specificity.

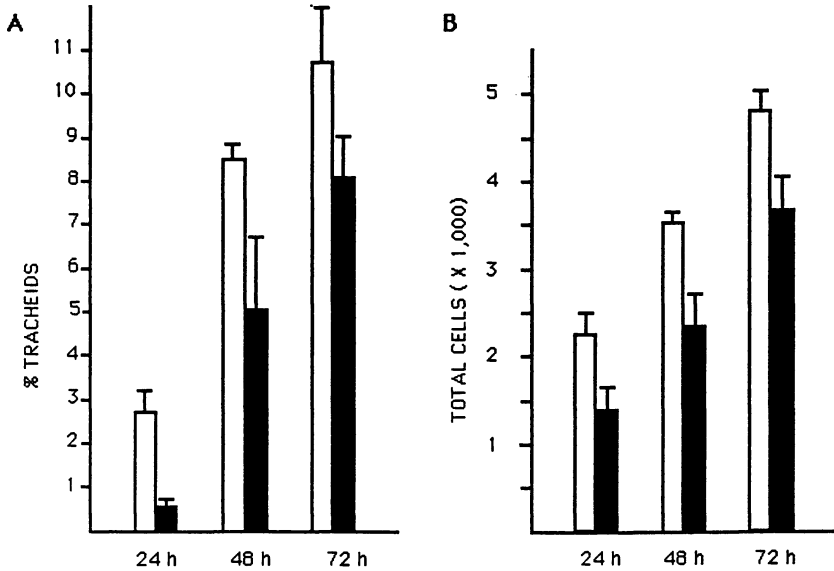


Figure 6. Effect of Brassinolide on Xylem Differentiation in *Helianthus tuberosus*. Explants were harvested 24, 48 or 72 hours after incubation on xylogenic medium (23) with (open bars) or without (closed bars) 68 nM BR.

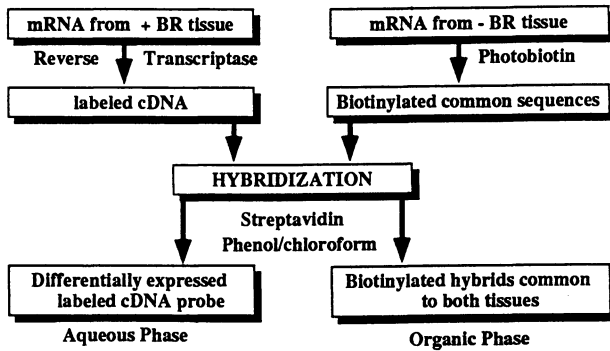


Figure 7. Probe Generation for Subtractive Hybridization.

Effects of BR on expression of xylem-specific promoter/GUS fusions in transgenic tobacco. Since we have demonstrated an effect of BR on xylem differentiation, we will perform a straightforward test to determine if BR modulates promoter activity of two translational fusions of bean promoters known to be expressed in developing xylem in transgenic tobacco. Both constructs were kindly provided by Dr. C.J. Lamb of the Salk Institute. The first consists of the bean phenylalanine ammonia lyase (PAL) promoter fused to a bacterial beta-glucuronidase (GUS) reporter gene (37). PAL is the first enzyme in the phenylpropanoid pathway leading to a multitude of products including lignin-specific monomers. Lignin is universally associated with vascular tissue in higher plants (38) and is deposited on the secondary walls of tracheary elements late in the xylogenic process (39). If BR affects the differentiation of xylem in tobacco at some point in the pathway, we would expect to see increased GUS activity (eventually) in BR treated plants. The second construct consists of the promoter from a bean cell wall glycine rich protein (GRP) fused to GUS (40). GRP is co-localized with the annular and helical secondary thickenings in cell walls of xylem elements indicating a close relationship between GRP and lignin. The construct has been shown to be specifically expressed in protoxylem tracheary elements in the developing transgenic tobacco. We are not suggesting that BR will regulate these promoters directly; there may be a complicated set of intermediate steps between BR perception and promoter activation, but these experiments will be easy to perform, rapid and may lay the groundwork for some very interesting future work.

Brassinolide Receptor Studies. The insect moulting hormone ecdysone is structurally very similar to brassinolide. In fact, Lehmann et al. (41) have shown that brassinosteroids bind to the ecdysone receptor from the blowfly *Calliphora vicina* and Richter et al. (42) have demonstrated that brassinosteroids delay the imaginal molt when fed to the last larval instar of the cockroach, *Periplaneta americana*. Based on these results, it is possible that the BR receptor has structural homology with the insect ecdysone receptor. We have obtained a full-length cDNA clone corresponding to the *Drosophila melanogaster* ecdysone receptor from Dr. David Hogness (Stanford University) and are currently screening our soybean cDNA library and an *Arabidopsis thaliana* genomic library in hopes of finding a structural analog to this receptor in plants.

Conclusion

While a great deal is known about the physiology and biochemistry of plant hormones, little is currently understood about the primary sites of action and the molecular mechanisms of hormone action. Therefore, the information gained from the experiments described here is relevant, particularly since no work of this kind has been reported for brassinolide. Any attempt to alter developmental genes by recombinant DNA techniques requires detailed knowledge of gene structure and function. Recently, McClure and Guilfoyle (43) used antisense RNA probes to monitor the rapid redistribution of auxin-regulated RNAs during gravitropism and provided a clear correlation between expression of genes under auxin control and a morphogenetic phenomenon traditionally known as an auxin response. The molecular

analysis of cell elongation and cell division in response to auxin is also proceeding rapidly. The interaction of BR with auxin may provide more information on this system, as well as on the mechanism of auxin-independent elongation controlled by BR. There are few reports to date, however, on a molecular study of the role of auxin or BR on cell differentiation. In the case of the Jerusalem artichoke we have a system involving a highly specialized developmental pathway which offers unique opportunities to examine the molecular events of differentiation over time in response to defined signals such as BR addition.

The field of plant molecular biology is rapidly accelerating towards the goal of understanding the molecular mechanisms which underlie inducible gene responses. The observation that auxin may stimulate transcription within five minutes (9) may indicate that the signal transduction pathway between recognition of signal and gene activation contains relatively few steps. Much current work in the field is centered on the role of protein phosphorylation, plant protein kinases, and calmodulin in modulating signal transduction, and on the purification and cloning of *trans*-acting protein factors. Example is being drawn from animal transcription studies and common patterns are being found. We are now in a position to add steroid hormones to the list and determine whether plants share some of the same molecular mechanisms for activation of genes by steroids as vertebrates. Besides being useful for fundamental studies of plant growth and development, BR has been shown to have dramatic effects on crop yields. Any increase in knowledge about the mechanisms of BR action thus has potential applications to agriculture.

Acknowledgements

We thank Dr. Trevor McMorris for providing us with highly purified samples of synthetic brassinolide, Dr. Gretchen Hagen for the clone pGH1 and Dr. Michael Baker for many helpful discussions.

Literature Cited

1. Guilfoyle, T. 1986. Auxin-regulated gene expression in higher plants. *Crit. Rev. Plant Sci.* 4 : 247-276.
2. Hagen, G. 1987. The control of gene expression by auxin. In, *Plant Hormones and Their Role in Plant Growth and Development*, ed. P.J. Davies, pp. 149-163. Boston: Martinus Nijhoff.
3. Key, J.L., Kroner, P., Walker, J., Hong, J.C., Ulrich, T.H., Ainley, W.M., Gantt, J.S., Nagao, R.T. 1986. Auxin-regulated gene expression. *Phil. Trans. R. Soc. Lond.* B 314:427-440.
4. Theologis, A. 1986. Rapid gene regulation by auxin. *Ann. Rev. Plant Physiol.* 37:407-438.
5. Zurfluh, L.L., Guilfoyle, T.J. 1982. Auxin-induced changes in the population of translatable messenger RNA in elongating sections of soybean hypocotyl. *Plant Physiol.* 69:332-337.

6. Walker, J.C., Key, J.L. 1982. Isolation of cloned cDNAs to auxin-responsive poly (A) +RNAs of elongating soybean hypocotyl. *Proc. Natl. Acad. Sci. USA* 79:7185-7189.
7. Ainley, W.M., Walker, J.C., Nagao, R.T., Key, J.L. 1988. Sequence and characterization of two auxin-regulated genes from soybean. *J. of Biol. Chem.* 263:10658-10666.
8. Hagen, G., Kleinschmidt, A., Guilfoyle, T. 1984. Auxin-regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. *Planta* 162:147-153.
9. Hagen, G., Guilfoyle, T.J. 1985. Rapid induction of selective transcription by auxins. *Mol. and Cell Biol.* 5:1197-1203.
10. McClure, B.A., Guilfoyle, T. 1987. Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. *Plant Mol. Biol.* 9:611-623.
11. McClure, B.A., Hagen, G., Brown, C.S., Gee, M.A., Guilfoyle, T.J. 1989. Transcription, organization, and sequence of an auxin-regulated gene cluster in soybean. *Plant Cell* 1:229-239.
12. Theologis, A., Huynh, T.V., Davis, R.W. 1985. Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. *J. Mol. Biol.* 183:53-68.
13. Van der Zaal, E.J., Memelink, J., Mennes, A.M., Quint, A., Libbenga, K.R. 1987. Auxin-induced mRNA species in tobacco cell cultures. *Plant Mol Biol.* 10:145-157.
14. Gregory, L.E., Mandava, N.B. 1982. The activity and interaction of brassinolide and gibberellic acid in mung bean epicotyles. *Physiol. Plant.* 54: 239-243.
15. Mandava, N.B. 1988. Plant growth-promoting brassinosteroids. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 39; 23-52.
16. Meyerowitz, E.M. 1987. *Arabidopsis thaliana*. *Ann. Rev. Genet.* 21:93-111.
17. Asubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. 1988. *Current Protocols in Molecular Biology*. New York: Wiley-Interscience.
18. Hagen, G., Uhrhammer, N., Guilfoyle, T.J. 1988. Regulation of expression of an auxin-induced soybean sequence by cadmium. *J. Biol. Chem.* 263:6442-6446.
19. Trewavas, A.J. 1982. Growth substance sensitivity: The limiting factor in plant development? *Physiol. Plant.* 55:60-72.
20. Katsumi, M. 1985. Interaction of a brassinosteroid with IAA and Ga₃ in the elongation of cucumber hypocotyl sections. *Plant Cell Physiol.* 26: 615-625.
21. Walker, J.C., Legocka, J., Edelman, L., Key, J.L. 1985. An analysis of growth regulator interactions and gene expression during auxin-induced cell elongation using cloned complementary DNAs to auxin-responsive messenger RNAs. *Plant Physiol.* 77:847-850.
22. O'Farrel, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 10:4007-4021.
23. Markland, W., Haddon, L. 1982. An improved technique for the culture of Jerusalem Artichoke (*Helianthus tuberosus* L.) explants for use in study of xylem differentiation. *Plant Cell Reports* 1:229-231.

24. Minocha, S.C., Halperin, W. 1974. Hormones and metabolites which control tracheid differentiation, with or without concomitant effects on growth, in cultured tuber tissue of *Helianthus tuberosus* L. *Planta* 116:319-331.
25. Koritsas, V.M. 1988. Effect of ethylene and ethylene precursors on protein phosphorylation and xylogenesis in tuber explants of *Helianthus tuberosus* (L.). *J. Exp. Bot.* 39:375-386.
26. Cochran, B.H., Zumstein, P., Zullo, J., Rollins, B., Mercola, M., Stiles, C.D. 1987. Differential Colony hybridization: Molecular cloning from a zero data base. *Meth. in Enzym.* 147:64-85.
27. Sargent, T.D. 1987. Isolation of differentially expressed genes. *Meth. in Enzym.* 152:423-432.
28. Jongstra, J., Schall, T.J., Dyer, B.J., Clayberger, C., Jorgensen, J., Davis, M.M., Krensky, A.M. 1987. The isolation and sequence of a novel gene from a human functional T cell line. *J. Exp. Med* 165:601-614.
29. Littman, D.R., Thomas, Y., Maddon, P.J., Chess, L., Axel, R. 1985. The isolation and sequence of the gene encoding T8: a molecule defining functional classes of T lymphocytes. *Cell* 40:237-246.
30. Raschke, W.C. 1987. Cloned murine T200 (Ly-5) cDNA reveals multiple transcripts within B- and T-lymphocyte lineages. *Proc. Natl. Acad. Sci. USA* 84:161-165.
31. Dear, T.N., Ramshaw, I.A., Kefford, R.F. 1988. Differential expression of a novel gene, WDNM1, in nonmetastatic rat mammary adenocarcinoma cells. *Cancer Research* 48:5203-5209.
32. Scott, M.R., Westphal, K., Rigby, P.W. 1983. Activation of mouse genes in transformed cells. *Cell* 34:557-567.
33. Anisowicz, A., Bardwell, L., Sager, R. 1987. Constitutive overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. *Proc. Natl. Acad. Sci. USA* 84:7188-7192.
34. Timberlake, W.E. 1980. Developmental gene regulation in *Aspergillus nidulans*. *Devel.Biol.* 78:497-510.
35. Dron, M., Clouse, S.D., Dixon, R.A., Lawton, M.A., Lamb, C.J. 1988. Glutathione and fungal elicitor regulation of a plant defense gene promoter in electroporated protoplasts. *Proc. Natl. Acad. Sci.* 85:6738-6742.
36. Somssich, I.E., Bollman, J., Hahlbrock, K., Kombrink, E., Schulz, W. 1989. Differential early activation of defense-related genes in elicitor treated parsley cells. *Plant Mol. Biol.* 12:227-234.
37. Liang, X., Dron, M., Lamb, C.J. 1989. Developmental and environmental regulation of a phenylalanine ammonia-lyase-beta-glucuronidase gene fusion in transgenic tobacco plants. *Proc. Natl. Acad. Sci.* 86: 9284-9288.
38. Grisebach, H. 1981. Lignins. In, *The Biochemistry of Plants*, eds. P.K. Stumph, E.E. Conn pp457-459.
39. Esau, K. 1965. *Vascular Differentiation in Plants*. New York: Holt, Rinehart & Winston.
40. Keller, B., Schmid, J., Lamb, C.J. 1989. Vascular expression of a bean cell wall glycine-rich protein-beta-glucuronidase gene fusion in transgenic tobacco. *EMBO Jour.* 8: 1309-1314.

41. Lehman, M., Vorbodt, H.M., Adam, G., Koolman, J. 1988. Antiecdysteroid activity of brassinosteroids. *Experientia* 44:355-356.
42. Richter, K., Adam, G., Vorbodt, H.M. 1987. *J. Appl. Ent.* 103:532.
43. McClure, B.A., Guilfoyle, T. 1989. Rapid redistribution of auxin-regulated RNAs during gravitropism. *Science* 243:91-93.

RECEIVED March 26, 1991

Chapter 12

Effect of Brassinosteroids on Protein Synthesis and Plant-Cell Ultrastructure under Stress Conditions

O. N. Kulaeva¹, E. A. Burkhanova¹, A. B. Fedina¹, V. A. Khokhlova¹,
G. A. Bokebayeva¹, H. M. Vorbrott², and Günter Adam²

¹Institute of Plant Physiology, USSR Academy of Sciences,
ul.Botanicheskaja 35, Moscow, Union of Soviet Socialist Republics, 127276

²Institute of Plant Biochemistry, Weinberg 3, Halle/Saale O-4050,
Germany

The mechanisms for the brassinosteroid antistress effect in plants were investigated. In wheat leaves under heat shock (40°C) 22S,23S-homobrassinolide and 24-epibrassinolide activated total protein synthesis and induced *de novo* polypeptide synthesis at normal and high temperatures. Molecular weight determinations showed that some of the proteins induced by brassinosteroids at normal temperature corresponded to heat shock proteins. 22S,23S-homobrassinolide also stimulated formation of heat shock granules in the cytoplasm and increased thermotolerance of total protein synthesis under heat shock. 24-epibrassinolide produced a protective effect on leaf cell ultrastructure in leaves placed under saline stress (0.5 M NaCl) and prevented nuclei and chloroplast degradation.

Brassinosteroids (BSs) represent a new group of plant hormones that possess a broad spectrum of physiological activities (1,2). A most intriguing property of BSs is their capacity to increase stress resistance in plants, but the mechanism of such an antistress activity still remains unknown (1). As cell stress resistance is usually associated with stress protein synthesis (3,4) our aim was to study the BS effect on protein synthesis and ultrastructure of wheat leaf cells at normal temperature and under heat shock conditions. We have also studied the influence of BSs on mesophyll cell ultrastructure under saline stress.

Effect of 22S,23S-Homobrassinolide
on Total Protein Synthesis in Wheat Leaves.

The effect of 22S,23S-homobrassinolide (HBR) on protein synthesis was studied in the first leaves of 14-day-old wheat plants cv. Saratovskaja 29. The leaf segments were preincubated in water or HBR solution for 18 h at 23°C and then incubated for 2.5 h at various temperatures (Figure 1). During the final 2 h, the segments were incubated in the presence of ^{35}S -methionine. Uptake of labeled methionine by leaf segments and its incorporation into protein was analyzed. The percentage of label incorporated into protein from its total accumulation in leaf segments was calculated. This calculation allowed us to compare protein synthesis activities in leaf segments under various conditions (5).

As Figure 1C shows, HBR at very low concentrations (10^{-8}M and 10^{-6}M) promoted protein synthesis in leaves under normal and high temperatures and decreased protein synthesis sensitivity to heat shock. In the presence of HBR (10^{-6}M) the rate of protein synthesis at 43°C was similar to that obtained at 23°C. However, in untreated leaves protein synthesis decreased 2.5 fold at 40°C as compared to control samples (Figure 1C).

HBR also induced also some changes in ^{35}S -methionine uptake by leaf segments, thus indicating that HBR affected cell membranes, especially at 35°C and 45°C. Another BR, 24-epibrassinolide (EB), exerted the same effect on ^{35}S -methionine accumulation in leaf segments and its incorporation into protein (data not presented).

Hence, physiologically active BSs (HBR and EB) activated protein synthesis in wheat leaves, increased stress resistance of the protein synthesizing system and modified cell membrane properties.

Effect of HBR and EB on the set of proteins synthesized
in leaves under normal and heat shock temperatures.

Polypeptides synthesized at control temperature (23°C) or under heat shock conditions (2.5 h, 40°C) in wheat leaf segments treated with HBR (10^{-8}M , 10^{-6}M) or EB (10^{-8}M , 10^{-6}M) or incubated in water for 18 h, were analysed by one-dimensional SDS-PAAGE (6). Data obtained (Figure 2) showed that HBR and EB activated protein synthesis and altered a set of polypeptides synthesized both at normal temperature and under heat shock.

At normal temperature, HBR activated the protein synthesis in leaves at 10^{-8}M and induced (or strongly

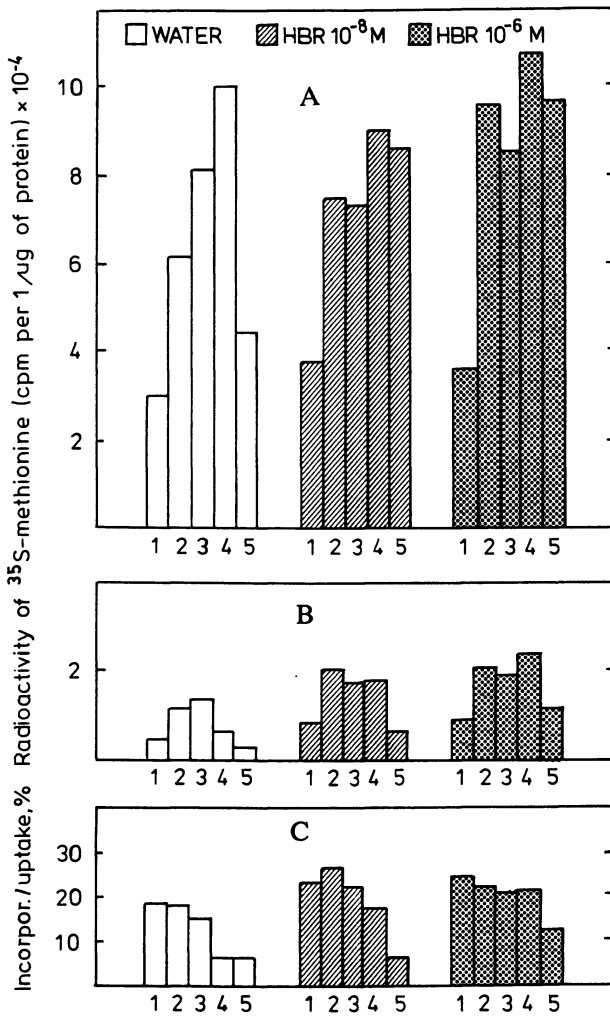


Figure 1. Uptake of ³⁵S-methionine and its incorporation into proteins in wheat leaves as a function of temperature.

A) Uptake of ³⁵S-methionine by leaf segments; B) Incorporation of ³⁵S-methionine into total protein; C) ratio of ³⁵S-methionine incorporation into protein to its uptake, (per cent). 1-23°C; 2-35°C; 3-40°C; 4-43°C; 5-45°C

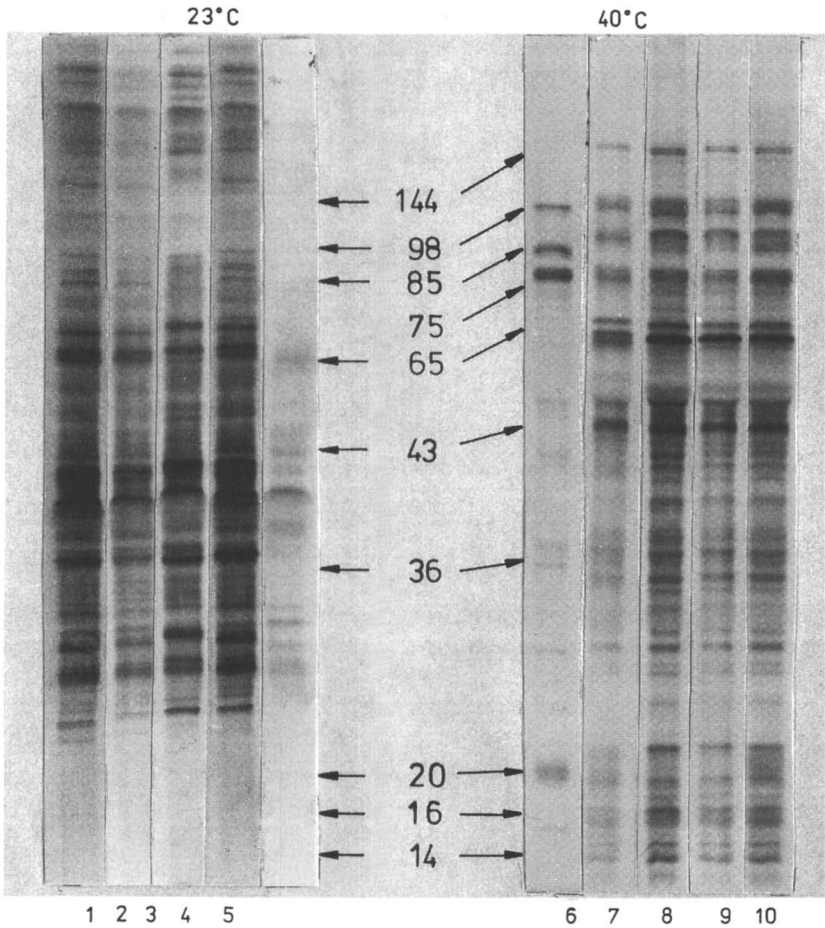


Figure 2. Fluorographs of ^{35}S -methionine-labeled leaf proteins fractionated by SDS-PAAG electrophoresis (7.5 - 15 % gradient). The equal amounts of protein were applied to each trace. The numbers on the middle indicate the molecular weights of proteins (kDa) synthesis of which was activated or induced by BRs. Lanes: 1,6-Water; 2,7-HBR, 10^{-8} M; 3,8-HBR, 10^{-6} M; 4,9-EB, 10^{-8} M; 5,10-EB, 10^{-6} M.

activated) the production of a number of polypeptides. While 10^{-8} M EB at 23°C was much less effective in the activation of protein synthesis than HBR, although at 10^{-6} M it was as active as HBR. They both exerted similar effects on the set of polypeptides synthesized at 23°C . One-dimensional electrophoresis revealed the activation of the synthesis of 11 polypeptide groups namely 144, 98, 94, 85, 65, 43, 36-30, 20, 16, 15, 14 kDa.

In control leaves placed under heat shock conditions (2.5 h, 40°C) the production of the majority of proteins diminished and the synthesis of heat shock proteins (HSPs) was revealed. The data concerning HSPs in roots and coleoptiles of 3-day-old wheat seedlings (7) and in aerial parts of 6-day-old etiolated wheat seedlings (8) were published. They indicated the non-identical HSP patterns in various organs (7) or wheat varieties (8). Moreover the duration of heat shock also affected the HSP pattern (7). Our experiments differed from (7,8) in the duration of heat shock and were performed with the green leaves of 14-day-old wheat seedlings (cv. Saratovskaja 29). Thus the complete identity of HSP patterns in our and (7,8) experiments was hardly expected but a certain similarity was revealed. Under heat shock conditions (40°C) HBR exerted a maximal effect on protein synthesis at 10^{-6} M (Figure 2,8). At 40°C , BSs induced the synthesis of the same polypeptides as those observed at normal temperature (144, 98, 94, 85, 65, 43, 36-30, 20, 16, 15, 14 kDa). Among these polypeptides, 65 kDa protein synthesis dominated. It will be interesting to check whether this protein corresponds to late HSPs of 63-64 kDa revealed in wheat seedlings (7). Molecular weights of some BS-induced proteins were identical to HSPs thus indicating the possibility of BSs participation in the regulation of HSP synthesis. At the same time, BSs slightly diminished the synthesis of the typical HSPs. It is important that pretreatment of leaves with BSs preserved (at 40°C) the synthesis of many polypeptides typical for normal temperature. The production of these polypeptides was inhibited at 40°C in the control leaves.

BSs-induced changes in the sets of proteins synthesized in leaves were analysed in more detail by two-dimensional SDS-PAAGE (9). Two-dimensional SDS-PAAGE fluorographs of polypeptides synthesized in HBR-treated leaves at 23°C and 40°C are shown in Figure 3 and Figure 4, respectively. At 23°C the production of 180 polypeptides in control leaves (Figure 3 A) and of 225 polypeptides in HBR-treated ones (Figure 3 B) were observed. HBR had strongly activated label incorporation into the 46-65 kDa protein family subdivided to more than 20 isoforms. HBR also activated the synthesis of some polypeptides located near the RUBISCO large subunit (Figure 3 B, 1S). The frame 1 shows the polypeptides whose synthesis was predominantly activated by HBR. The

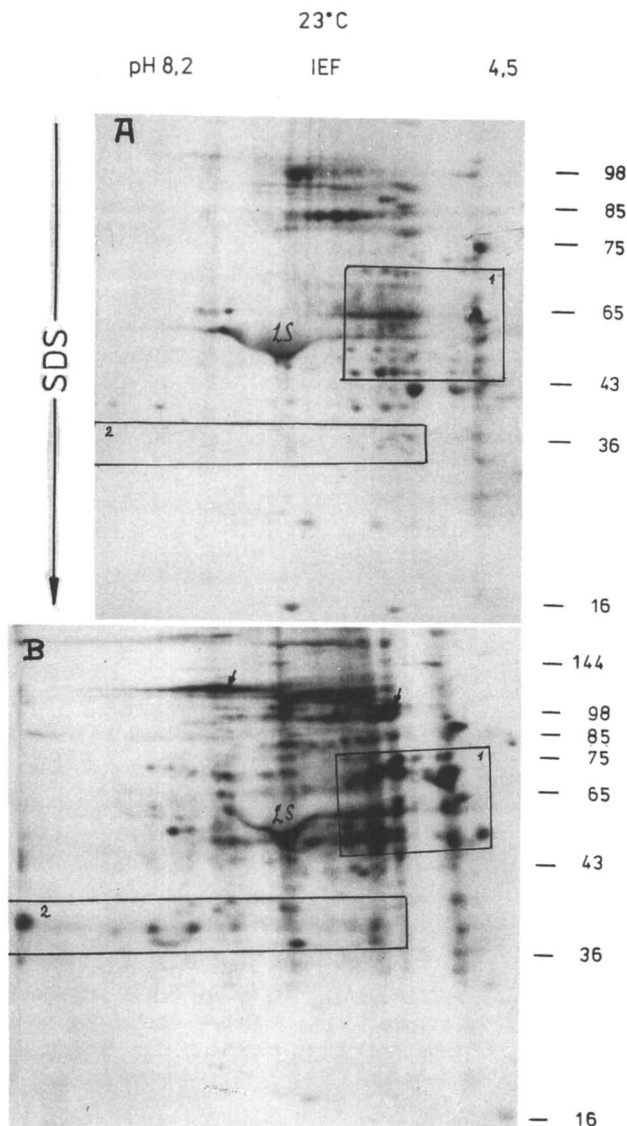


Figure 3. Fluorographs of ^{35}S -methionine-labeled leaf proteins fractionated by two-dimensional gel electrophoresis. Equal amounts of proteins were applied. The numbers on the right indicate the molecular weights of proteins (kDa). A-water; B-HBR, 10^{-8} M. Framed spots indicate polypeptides the synthesis of which was highly activated by HBR. Arrows indicate the polypeptides whose synthesis was activated by heat shock and HBR. LS-large subunit of RUBISCO.

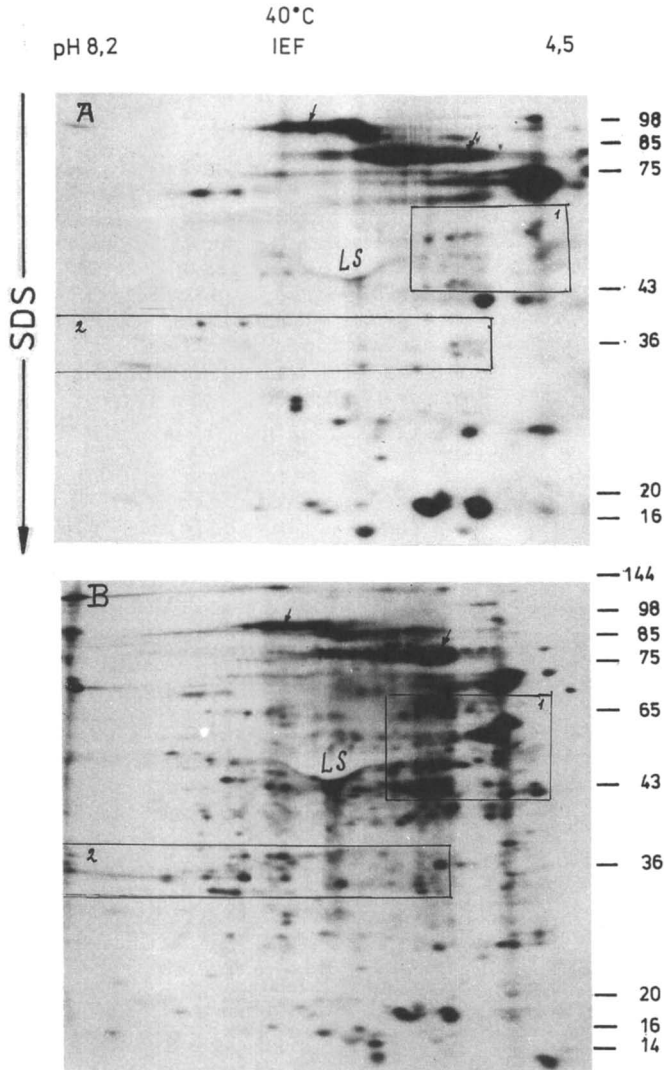


Figure 4. Fluorographs of ^{35}S -methionine-labeled leaf proteins fractionated by two-dimensional gel electrophoresis. Equal amounts of proteins were applied. The numbers on the right indicate the molecular weights of proteins (kDa). A-water; B-HBR, 10^{-6} M. Framed spots indicate polypeptides the synthesis of which was highly activated by HBR. The temperature used was 40°C . Other details as described in Figure 3.

protein fluorographs, after two-dimensional gel electrophoresis, revealed some new spots after HBR treatment that corresponded to polypeptides 144 and 36-30 kDa, the latter are indicated by frame 2 in Figure 3 B. HBR also activated the formation of polypeptides 85 and 98 kDa which are shown by arrows on Figure 3 B. They corresponded to HSPs (Figure 3 B, Figure 4 B)

The preincubation of leaves in HBR solution also resulted in protein synthesis activation under heat shock and to an increase in the number of spots in the corresponding fluorographs. It indicated that HBR induced (or activated) synthesis of a great number of polypeptides under heat shock. The production of 144 polypeptides in control leaves and of 237 in leaves incubated in HBR solution were observed at 40°C (Figure 4). HBR treatment slightly diminished HSP synthesis at 40°C and simultaneously induced synthesis of a number of polypeptides, induced by BSs at normal temperature. Among these proteins, polypeptides of the 65-46 kDa family dominated (Figure 4 B) at 40°C. Also, HBR strongly activated the synthesis of polypeptides located on the fluorographs near the large subunit of RUBISCO. Synthesis of these polypeptides was inhibited at 40°C in untreated leaves (Figure 4 A). These data indicated that BSs had a protective effect on the formation of polypeptides typical for normal temperature conditions. Two-dimensional SDS-PAAGE has revealed at least two protein groups, synthesis of which was induced or activated by both heat shock and HBR. These polypeptides were 98 and 85 kDa (arrows in Figure 4 B). The HBR effects on protein synthesis at normal and stress temperatures observed in wheat leaves of cv.Saratovskaja 29 were revealed also in our experiments with cv.Opal (data not presented).

EB exerted similar effects on protein synthesis in wheat leaves as HBR (Figure 5). EB was less active in the stimulation of polypeptide synthesis in wheat leaf segments at 23°C than HBR and the number of isoforms induced (or activated) by EB was fewer (data are not presented). Nevertheless EB was as efficient as HBR in the alteration of protein set synthesized in leaves at 42°C (Figure 5 B).

Therefore, BSs can induce significant changes in the activity of protein synthesis in wheat leaves. In addition, BSs increased the thermoresistance of protein synthesis under heat shock and protected the synthesis of a great number of polypeptides which were typical for leaves at normal temperatures. A characteristic feature of BSs action is the induction or activation of a number of polypeptides (65-46, 36-30 kDa), synthesis. Some of these polypeptides were dominant at normal, and especially at high temperatures. It is known that heat shock strongly inhibits protein synthesis typical for normal temperatures but it did not affect BSs-induced

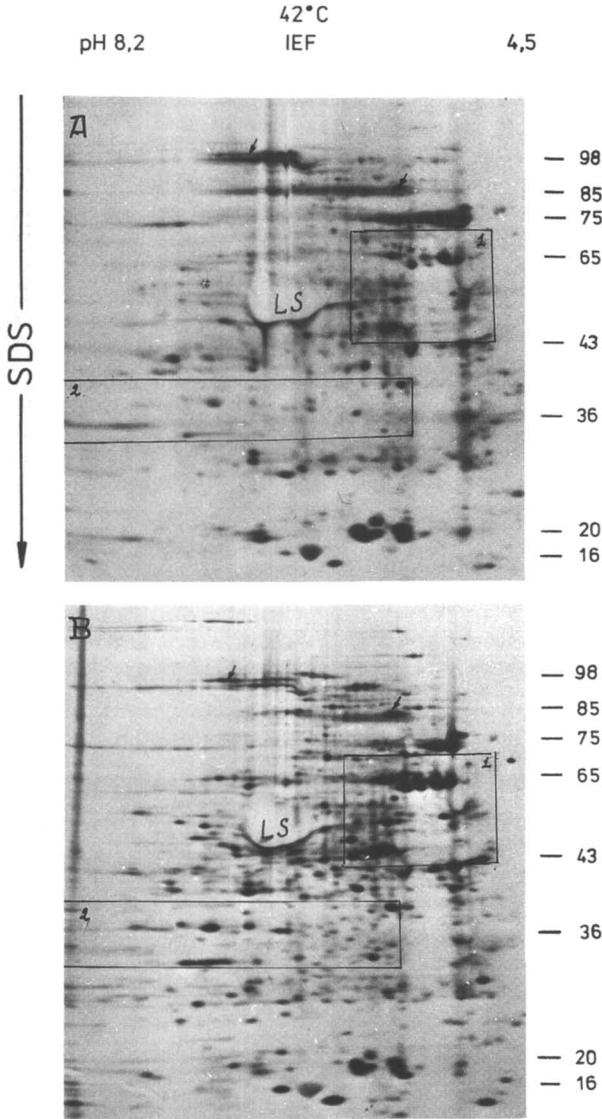


Figure 5. The effect of EB on the composition of polypeptides synthesized at 42°C in wheat leaves. A-water; B-EB, 10⁻⁶M. Other details as described in Figure 3.

polypeptide synthesis. This fact supported the concept of a protective role of these proteins under stress conditions.

Effect of HBR on Heat Shock Granule Formation in Wheat Leaf Cells.

Heat shock is known to exert an effect on plant cell ultrastructure. The most typical change is represented by the formation of cytoplasmic heat shock granules (HSG). These structures have been observed under heat shock conditions in many plant cells (10-12). HSGs are RNP-particles containing temporary (during heat shock) non-translated mRNAs for proteins usually synthesized at normal temperature (13). Also, they contain two general groups of plant HSPs: high-molecular proteins and mainly low-molecular proteins (10). It is suggested that HSPs localized in granules protect mRNAs from heat shock-induced degradation. Because of BS-activated synthesis of a number of polypeptides with molecular weights similar to HSPs, we have investigated the effect of HBR on HSG formation. Figure 6 A shows the ultrastructure of mesophyll cells in wheat leaf segments under heat shock conditions. Electron-dense HSGs aggregated in small clusters and were well distinguished. An average number of HSGs per cluster was 17 and the average cluster area was 0.211 mkm^2 . In leaf segments preincubated in HBR solution (10^{-6} M , 18 h) and then subjected to heat shock (40°C 2.5 h), HSG formation was also observed in the cytoplasm (Figure 6 B). Morphometric analysis has shown that HBR stimulated the HSG formation. The average number of granules in clusters increased up to 23 and the average cluster size extended up to 0.287 mkm^2 . Therefore HBR stimulated both processes by 40 per cent. These results are consonant with the HBR effect on protein synthesis under heat shock conditions.

Protective Effect of EB on Barley Leaf Cell Ultrastructure Under Saline Stress.

The EB effects on cell ultrastructure were studied with the first leaf of 10-day-old barley seedlings (cv. Viner). Leaf segments were preincubated in EB solution (10^{-6} M), or water, for 2 h and were then incubated in 0.5 M NaCl solution during 24 h in the presence or absence of EB. Cells of control leaves had the nuclei of nucleonema type with diffused and condensed chromatin (Figure 7 A). Chloroplasts revealed a well developed membrane system of

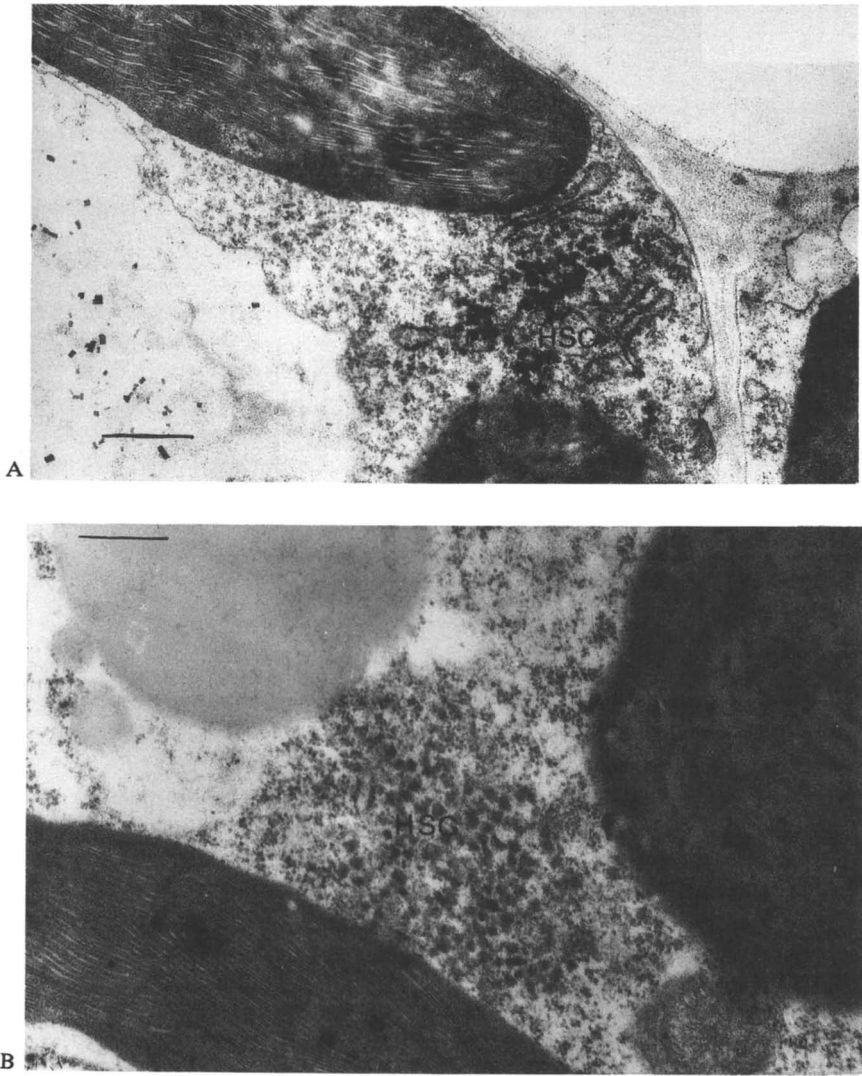


Figure 6. The effect of HBR on the fine structure of HSGs in mesophyll cells of wheat leaves under heat shock (40°, 2 h).A-HSGs in control leaf cells after incubation in water; B-HSGs in the cells of 10^{-6} M HBR-treated leaves. Scale bars, 3 mkm.

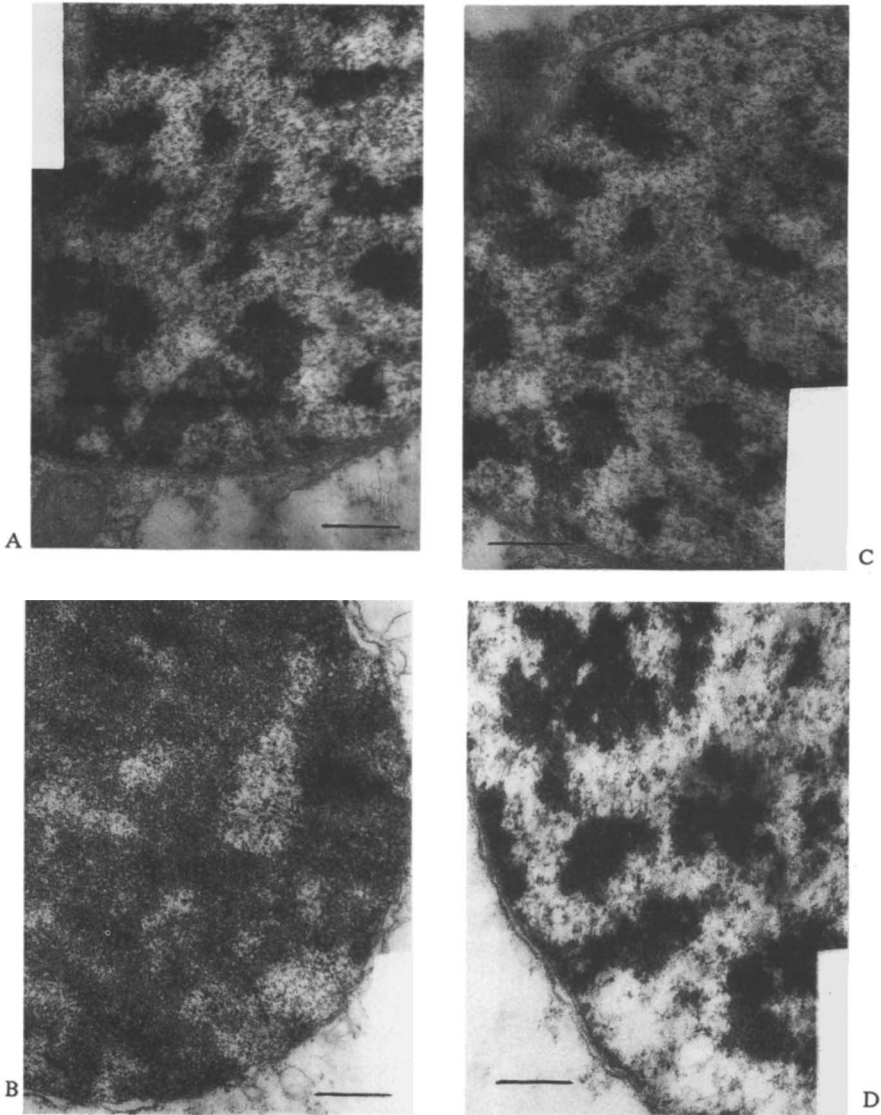


Figure 7. Protective effect of 10^{-8} M EB on nuclear ultrastructure in barley leaf mesophyll cells under saline stress (0.5 M NaCl). Leaf segments were incubated in: A-water, 26 h; B-water, 2 h, +0.5 M NaCl, 24 h; C-EB 10^{-8} M, 26 h; D-EB, 10^{-8} M, 2h, + (EB, 10^{-8} M + 0.5M NaCl), 24 h. Scale bars, 5 μ m.

lamellae (Figure 8 A). 0.5 M NaCl induced disturbance of the ultrastructure of the nuclei and chloroplasts including strong chromatin condensation (Figure 7 B), and disorganization of the chloroplast membrane system (Figure 8 B). EB had little or no effect on the leaf cell ultrastructure under normal conditions (Figures 7 C, 8 C) and significantly reduced damage induced by saline stress (Figures 7 D, 8 D). Hence EB exerted a protective effect on barley leaf cell ultrastructure under saline stress conditions.

Conclusions.

BSs protected cereal leaf cells from heat shock or saline stress. Leaf pretreatment with BSs decreased cell ultrastructure degradation from heat shock and high salt conditions. BSs increased HSG formation which is supposed to protect preformed mRNA in plant cells during heating. BSs enhanced heat shock resistance of the leaf protein-synthesizing system. The effect of BSs on RNA and protein synthesis was shown earlier (14). We observed protein synthesis activation in wheat leaves by BSs in normal and under stress conditions. Two-dimensional SDS-PAAGE of ³⁵S-methionine labeled proteins demonstrated BS-induced changes in the set of polypeptides synthesized in leaves and in the rate of their synthesis.

BSs induced (or strongly activated) synthesis of a number of polypeptides. Synthesis of some of them was dominant in leaves under normal and heat shock conditions. These results permit us to conclude that BSs induced the expression of a number of genes in wheat leaves. At what level this regulation occurs is still unknown. The changes in gene expression were demonstrated in our experiments after prolonged period of BS action (18 h). It is necessary to find out when these alterations occur in cells and whether they relate to the previously described changes of membrane structure/function (15,16). BS effect on ³⁵S-membranes was also demonstrated in our experiments by ³⁵S-methionine uptake by leaf segments. Also, BSs significantly changed the set of protein isoforms, as a possible result of their effect on gene expression and (or) posttranslational protein modification.

It is important that BS-induced changes in the synthesized polypeptide set were observed not only at normal temperature but also under heat shock conditions. It is known that in the cells of all organisms, from bacteria to man, the synthesis of proteins typical for normal temperature is inhibited by high temperature and synthesis of HSPs is induced (3). Some evidence confirm the probable protective role of HSPs under stress conditions (3,12). The synthesis of BS-induced polypeptides under heat shock gives support to the assumption that BS-induced proteins may also fulfil a protective role.

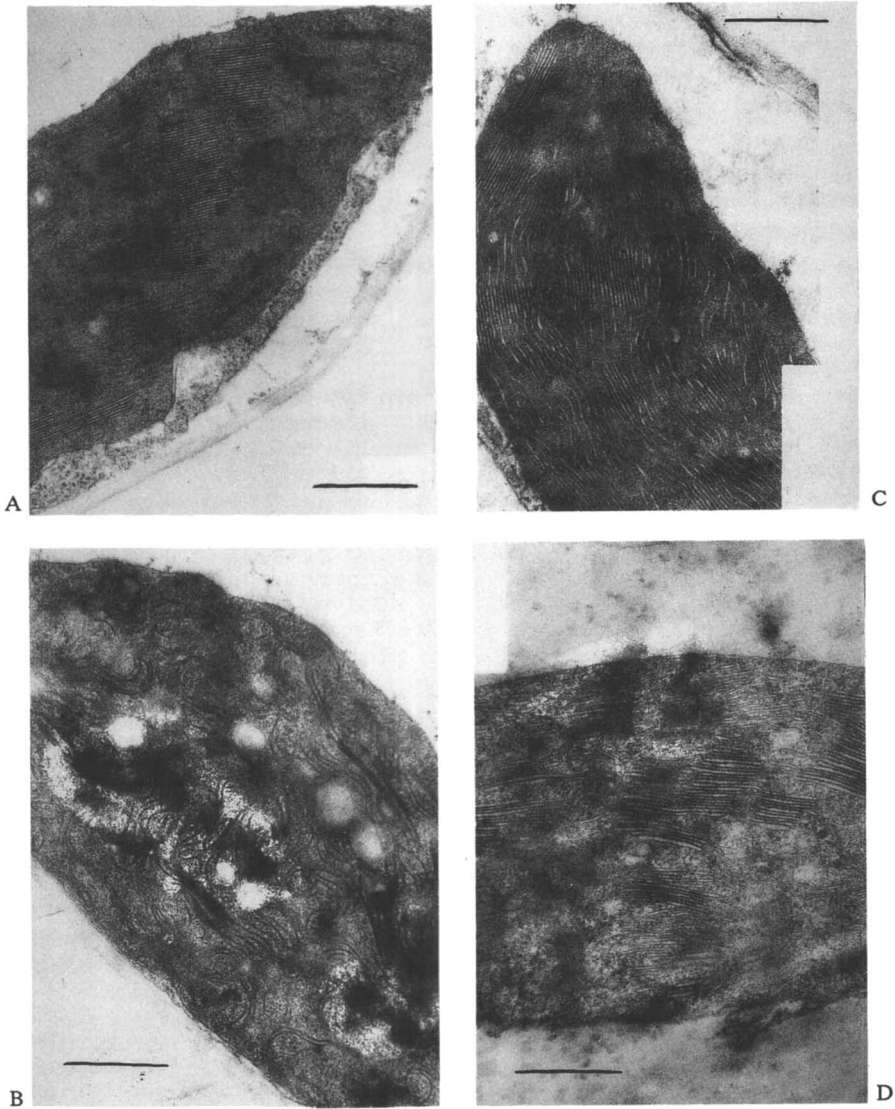


Figure 8. Protective effect of EB on chloroplast ultrastructure in the barley leaf mesophyll cells under saline stress (0.5 M NaCl). Leaf segments were incubated in: **A**-water, 26 h; **B**-water, 2 h, + 0.5 M NaCl, 24 h; **C**-EB 10^{-8} M, 26 h; **D**-EB 10^{-8} M, 2h, + (EB 10^{-8} M + 0.5M NaCl), 24 h. Scale bars, 5 μ m.

Literature Cited.

1. Mandava, N.B. *Ann. Rev. Plant Physiol.* 1988, vol.39, pp.23-52.
2. Adam, G.; Marquart, V. *Phytochemistry* 1986, vol. 25, pp.1787-1799.
3. Shlesinger, M. *J. Biol. Chem.* 1990, vol.265, pp.12111-12114.
4. Fedina, A.B.; Khadeeva, N.V.; Dridze, I.L.; Maisurjan, A.N.; Shikunova, N.I.; Kulaeva, O.N. *Physiol. Rast.* 1987, vol.34, pp.127-134.
5. Kulaeva, O.N.; Burkhanova, E.A.; Fedina, A.B.; Danilova, N.V.; Adam, G.; Vorbrodt, H.M.; Khripach, V.A. *Dokl. Akad. Nauk. S.S.S.R.* 1989, vol.305, pp.1277-1279.
6. Laemmli, U.K. *Nature* 1970, vol.227, pp.680-685.
7. Necchi, A.; Pogna, N.E.; Mapelli, S. *Plant Physiol.* 1987, vol.84, pp.1378-1384.
8. Zivy, M. *Theor. Appl. Genet.* 1987, vol.74, pp.209-213.
9. O'Farrell, P.H. *J. Biol. Chem.* 1975, vol.250, pp.4007-4021.
10. Neumann, D.; zur Nieden, U.; Manteuffel, G.; Walter, K.D.; Scharf, K.-D.; Nover, L. *Eur. J. Cell Biol.* 1987, vol. 43, pp.71-81.
11. Khokhlova, V.A.; Porfirova, S.A.; Fedina, A.B.; Burkhanova, E.A. *Physiol. Rast.* 1987, vol.34, pp.869-878.
12. Neumann, D.; Nover, L.; Parthier, B. et al. *Biol. Zentralblatt.* 1989, B.108, S.1-156.
13. Nover, L.; Scharf, K.-D.; Neumann, D. *Mol. Cell Biol.* 1989, vol.9, pp.1298-1308.
14. Kalinich, G.F.; Mandava, N.B.; Todhunter, G.A. *J. Plant Physiol.* 1985, vol. 120, pp.207-214.
15. Katsumi, M. *Int. Workshop Brassinosteroids-Chemistry, Bioactivity Application; Inst. Plant Biochem.: Halle/S., F.R.G., 1990; Vol.1, p.29.*
16. Dahse, I.; Waelmer, S.M.; Grammatikopoulos, G.; Nicolopoulos, D. *Int. Workshop Brassinosteroids-Chemistry, Bioactivity Application; Inst. Plant Biochem.: Halle/S., F.R.G., 1990; Vol.1, p.34*

RECEIVED May 29, 1991

Chapter 13

The Case for Brassinosteroids as Endogenous Plant Hormones

Jenneth M. Sasse

School of Botany, University of Melbourne, Parkville, Victoria,
Australia 3052

Brassinosteroids occur widely across the plant kingdom and act at concentrations comparable to those of the recognised hormones in bioassays and whole plants. They have a range of effects which can be distinguished from those of the recognised hormones, and are especially promotive of young vegetative growth. They can move in the plant and they interact with, and are modulated by, the effects of the recognised hormones. They also interact with environmental signals such as light, gravity and temperature, and can stimulate the synthesis of particular proteins. Evidence is accumulating that the brassinosteroids do constitute a new family of plant hormones but there are great gaps in our knowledge and many areas in the physiology and biochemistry of brassinosteroids need further research.

Physiologists have long wondered whether there are steroid hormones in plants comparable in activity to those in mammals; is there evidence that the brassinosteroids fill that role? In animals, a hormone is a "chemical messenger": there is a localized site of synthesis, transport to a target tissue, control of physiological response via concentration. The term was carried into plant physiology by analogy, and used initially for auxin physiology. We now realize the analogy was simplistic, particularly about one site of synthesis and transport, but Davies' (*1*) definition of plant hormones, while accurate, is very wide

"...they are natural compounds in plants with the ability to affect physiological processes at concentrations below those where either nutrients or vitamins would affect these processes."

There are many naturally occurring compounds that have plant growth regulatory effects in whole plants or bioassays, and brassinosteroids are certainly potent - but should they have plant hormone status?

The generally recognised families of endogenous plant hormones comprise:

auxins:	indole-3-acetic acid 4-chloroindole-3-acetic acid phenylacetic acid
gibberellins:	>80 diterpene acids
cytokinins:	>20 purine derivatives
abscisic acid:	sesquiterpene acid
ethylene:	hydrocarbon.

Other candidates are the polyamine and jasmonic acid families, and all have precursors, metabolites, conjugates, and synthetic analogues. Their biosynthesis and metabolism, which provide additional control points for their function, are studied intensively.

The recognised families are regarded as ubiquitous in the plant kingdom; have multiple effects; can modulate each other's effects; move around the plant, free or in a conjugated form; and interact with environmental signals such as light, water availability, gravity and temperature. Hormone signals trigger the synthesis of particular proteins, and the search for hormone receptors, their distribution, and the transmission and amplification of the signal are currently of great interest. Using these criteria, do brassinosteroids behave like plant hormones?

Ubiquity

The family, whose biosynthesis and metabolism are being investigated (2,3), now consists of over 30 members (4). They have been detected in various parts of plants, such as pollen, leaves, flowers, seeds, shoots, galls, and stems. Bioassay evidence, particularly in angiosperms, suggests they are ubiquitous, and there is firm chemical evidence from at least 22 plants, ranging from an alga and *Equisetum* through gymnosperms to both monocots and dicots (5,6,7,8).

Physiological activity of brassinosteroids

Specificity. Using the first member of the family, brassinolide (BR), an extensive survey of its effects in 17 bioassays, which varied in their responses to gibberellins, auxins and cytokinins, showed that BR did not behave exclusively as any one of those hormones. In some supposedly specific bioassays BR was as effective, or more so, as the hormone the assay was supposed to detect (9,10). This also applies to the rice lamina inclination assay (11), which is now frequently used.

Thus it is no longer possible to assume standard bioassays are specific, and brassinolide cannot be classified as belonging to any of the known groups of plant hormones. Indeed, one reviewer has remarked " .. it could be considered to belong to all of them!" (12). The "brassin response" of swelling and splitting in

the second internode of bean requires a high concentration of BR (13), and at lower concentrations elongation only is induced, like the response to gibberellin. However, it is possible to follow isolation procedures with a carefully chosen assay if one bears in mind the relative activities of the hormones, and their polarities and pKa's.

There is a sequential response to plant hormones in elongating tissue which was first described by Wright (14) in coleoptiles and first leaves of wheat. If his experiment with coleoptiles is repeated, BR fits in neatly between gibberellin and auxin. This also occurs in pea tissue, so BR is again behaving in a comparable manner to the recognised hormones, in contrast to the general promoter, fusicoccin, which promotes all but the most mature tissue (15). It cannot be concluded from such experiments that putative receptors are not present in the tissue that does not respond to exogenous hormones. Many factors could prevent a response - for example, optimal endogenous levels of the hormone, the presence of inhibitors or lack of effectors, physical or chemical constraints on the amplification or expression of the signal. Other endogenous hormones can also contribute to a response induced by an exogenous hormone; clearly, knowledge of endogenous levels in particular tissues would contribute greatly to our understanding, as would receptor mapping.

Modulation of and by other hormones. The most BR-responsive part of the pea stem is the transition from the hook to laterally enlarging and elongating tissue (15). This is a very plastic zone of the stem, and the different morphological effect of BR can be shown clearly with segments from this zone. BR not only promotes elongation, but it maintains a narrow basal diameter and the morphology can be changed dramatically by low concentrations of the other regulators as well. The experimental system can also illustrate the additive effect of gibberellin and BR, and the interactions between BR and auxin or zeatin or abscisic acid or ethylene (16,17,18).

BR also interacts with auxin in bending responses (19,20,21,22), and BR is required simultaneously or as a pre-treatment for the effects to occur. Other workers have reported interactions also, so BR can modulate the effects of the recognised hormones, and be modulated by them. Thus, if these effects are reflected endogenously, BR may be involved in the control of the overall form of the plant - the particular morphology of the etiolated state would match the properties of this regulator, but a causal role is not yet proven.

It is also tempting to call BR a botanical "juvenile hormone", prolonging the elongating phase of growth, while auxin and particularly zeatin contribute to lateral enlargement in the transition from hook to mature stem. Zhao et al.(23) considered BR retarded maturation and senescence, while the work of Suge (24), where an inflorescence reverted to a vegetative shoot, is also suggestive, as are isolated reports of BR promoting shoot formation in tissue culture (25,26). In whole plants, Braun and Wild (27) showed a promotive effect on photosynthetic capacity and biomass production in the primary developmental stages, which led to accelerated growth of the whole plant. They also noted increased synthesis of

reducing sugars. The USDA group (28) did greenhouse and field studies, and noted the acceleration of growth of barley and lettuce and there are now many claims for increased yields of other crops, and a recent presentation described potent promotive effects in a fungus (29).

Pleiotropic effects. Other properties have been reported for BR, e.g. increased membrane permeability (30), reduction of phenotypic variation (31) and improvement of germination rates in aged seeds (32,33). There are also claims that BRs enhance resistance to various stresses, such as cold, fungal infection, herbicide injury and salt (34). They are protective in heat shock (35) and recently, several authors reported enhanced resistance to chilling after BR treatment (36,37,38). Fruit set can also be improved (39) and there are several reports of more complete grain filling (e.g., 36,37). So the possibilities for agricultural application are of great interest, and stability in the field, and mode of administration are under investigation (39,40).

In reproductive growth, BR has been shown to promote the growth of pollen tubes *in vitro*, at a concentration an order of magnitude below those of auxin or gibberellin (41). Abe (42) has recently reported correlative changes in the levels of BRs in lily pollen as it matures, with increases in two of the more active BRs. Taken together with the response of pollen tubes *in vitro* (41), his data suggest active BRs are present in mature pollen in adequate amounts to contribute to the control of pollen tube extension *in vivo*.

The effect of homoBR on flowering tissues was to produce bisexual and pistillate flowers on a staminate inflorescence. Also, sepals were deformed (24), and one would suspect some of these effects were due to induced ethylene biosynthesis, as the dosage of the brassinosteroid used was very high. Excess hormone levels are known to induce the biosynthesis of ethylene; BR can also do this, and it interacts with auxin and cytokinin in the induction (43). BR can also affect endogenous auxin and abscisic acid levels in treated tissue (21,44,45). Thus BR does have multiple and modulatory effects.

Movement in the plant

Treatment of the roots of tomato and radish plants with BR led to elongation of the petioles and hypocotyls, and treatment of the bases of mung bean hypocotyl cuttings led to the elongation of the epicotyls (46,47,48). In cucumber hypocotyl segments, washing with water reduced the effect of BR treatment (49), and the uptake of BR has been studied in detail in maize roots, where it accumulated independently of energy supply and 30% was irreversibly bound (50).

Such behaviour is analogous to that of the recognised hormones, and the easy uptake and movement of BR, or a conjugate or metabolite, to "target tissues" is established. Recognition events could still be occurring in other tissues, and hormones as messengers are also important at different levels of organization in the plant (51,52,53). BR has been cited (51) as a possible gametophytic hormone which "might exert an enabling effect at the highest level of plant organization, the

whole plant/community boundary". While this is speculative, at lower levels of interaction with the environment, BR does act like a hormone.

Interaction with environmental signals

BR enhances the gravitropic response and recovery from gravitropic stimulation (22). BR also interacts with light effects. Light quality affected BR-induced internode elongation in beans (54), and there was evidence for a role for BR in the mobilization of photosynthate to particular parts of the plant (55). BR can overcome the light-induced inhibition of elongation of etiolated plants, and there is evidence for phytochrome involvement (56). Xu and Zhao (57) observed marked differences in the effects of BR on peroxidase activities between light- and dark-grown tissue. Exogenous BR also modifies the response to heat shock, preserving protein synthesis (35) and the response to chilling has been described above.

Synthesis of particular proteins

Intact protein synthesis is necessary for BR-induced effects (58), again consistent with the behaviour of the recognised hormones. Also levels of particular proteins can be affected by BR treatment (27). Treatment of wheat leaves with homoBR led to altered gene expression, with the synthesis of some new proteins, while not affecting others. There were further alterations at high temperature, and changing the concentration of the brassinosteroid also altered the particular profile of the proteins synthesized (35). Recently, Clouse et al. (59) reported *in vitro* synthesis of new polypeptides from RNA induced by BR treatment of soybean tissue, as well as some down-regulation of others. The data of Xu and Zhao (57) also suggest some down-regulation, in their case of peroxidase synthesis, with some differences between isozymes. By analogy with steroids in mammals (60), BR bound to a receptor protein might enhance gene expression by increasing the rate of transcription initiation, as well as inducing *de novo* synthesis.

Conclusions

By the criteria listed, the evidence is consistent with brassinosteroids being a new family of endogenous plant hormones. However, when Jacobs' rules (61) for determining the involvement of a hormone in a process (Presence, Excision, Substitution, Isolation, Generality, Specificity), are considered:-

- P:** endogenous levels in pollen are known, but levels in responsive vegetative tissue are not
- E:** there is synthesis in pollen (42) and crown gall cells (62)
- S:** it is assumed endogenous BR is depleted when isolated tissue sections are treated with BR

- I:** single cells respond but more subcellular studies need to be done - there are some recent data on BR effects on enzyme activity (30,57,63) and molecular biological approaches have begun (35,59)
- G:** young vegetative tissues are sensitive, for example, bean, pea, mung bean, Azuki bean, wheat, maize, sunflower, cucumber, radish, tomato, birch, elm, crabapple, soybean, *Arabidopsis*
- S:** the effect of BR is distinct from those of gibberellin and auxin, and there are structure/activity variations within the brassinosteroids,

our information is incomplete, as it is for the recognised hormones also. Several areas in the physiology and biochemistry of BRs still need study, and many questions can be asked:

what are BRs' effects on anatomy and the physical parameters of growth;

are BRs synthesized in tissues other than pollen?

what are the endogenous levels in growing vegetative tissue?

does BR help maintain the etiolated state?

does BR modulate the phytochrome response?

what is BR's role in pollen?

how does BR accelerate growth, particularly protein synthesis?

do BRs' synergisms reflect increased receptor numbers?

where is BR's putative receptor?

does it behave like an animal steroid receptor?

or is it membrane-bound, requiring a second messenger system?

Even with these and other unanswered questions, the case for the brassinosteroids as endogenous hormones is strong, and I suggest that brassinosteroids have an independent role in the early stages of vegetative growth. Their promotive effects in other tissues may be accelerative of an already determined program for growth, or the effects of other hormones. The few reported inhibitory effects may reflect supraoptimal levels, probably mediated via ethylene, while the results with pollen (41,42) suggest the effects of brassinosteroids must be included when the role of endogenous plant hormones in plant growth is considered.

Literature Cited

1. Davies, P.J. In *Plant Hormones and their Role in Plant Growth and Development* Davies, P.J., Ed.; Martinus Nijhoff: Dordrecht, 1987; pp 12-23.
2. Yokota, T.; Ogino, Y., Takahashi, N., Saimoto, H., Fujioka, S., Sakurai, A. *Agric. Biol. Chem.* 1990, 54, 1107-08.
3. Yokota, T.; Ogino, Y., Suzuki, H., Takahashi, N., Saimoto, H., Fujioka, S., Sakurai, A. *Abstract 133, Agrochemical Section, 200th Annual Meeting, Amer. Chem. Soc.*, Washington, D.C., 1990.
4. Yokota, T.; Kim, S-K., Takahashi, N. *Abstract 168, 13th Inter. Conf. Plant Growth Subst.*, Calgary, Canada, 1988.
5. Takatsuto, S.; Yokota, T., Omote, K., Gamoh, K., Takahashi, N. *Agric. Biol. Chem.* 1989, 53, 2177-80.
6. Takatsuto, S.; Abe, H., Gamoh, K. *Agric. Biol. Chem.* 1990, 54, 1057-9.
7. Takatsuto, S.; Omote, K., Gamoh, K., Ishibashi, M. *Agric. Biol. Chem.* 1990, 54, 757-62.
8. Mandava, N.B. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1988, 39, 23-52.
9. Yopp, J.H.; Mandava, N.B., Sasse, J.M. *Physiol. Plant.* 1981, 53, 445-52.
10. Mandava, N.B.; Sasse, J.M., Yopp, J.H. *Physiol. Plant.* 1981, 53, 453-61.
11. Wada, k.; Marumo, S., Abe, H., Morishita, T., Nakamura, K., Uchiyama M., Mori, K. *Agric. Biol. Chem.* 1984, 719-26.
12. Geuns, J.M.C. *Biochem. Soc. Trans.* , 1983, 11, 543-48.
13. Grove, M.D.; Spencer, G.F., Rohwedder, W.K., Mandava, N.B., Worley, J.F., Warthen, J.D., Steffens, G.L., Flippen-Anderson, J.L., Cook, J.C. *Nature (London)*, 1979, 281, 216-17.
14. Wright, S.T.C. In *Biochemistry and Physiology of Plant Growth Substances*; Wightman, F., Setterfield, G. Eds.; The Runge Press: Ottawa, 1968; pp 521-42.
15. Sasse, J.M. *Physiol. Plant.*, 1985, 63, 303-08.
16. Sasse, J.M. *Pro. Plant Growth Reg. Soc. Amer.* 1987, 14, 30-39.
17. Sasse, J.M. *Pro. Plant Growth Reg. Soc. Amer.* 1989, 16, 82-87.
18. Sasse, J.M. *Physiol. Plant.* 1990, 80, 401-08.
19. Yopp, J.H.; Mandava, N.B., Thompson, M.J., Sasse, J.M. *Pro. Plant Growth Reg. Soc. Amer.* 1981, 8, 138-45.
20. Takeno, K.; Pharis, R. *Plant Cell Physiol.* 1982, 23, 1275-81.
21. Cohen, J.D.; Meudt, W.J. *Plant Physiol.* 1983, 72, 691-94.
22. Meudt, W.J. *Plant Physiol.* 1987, 83, 195-98.
23. Zhao, Y.; Luo, W., Wang, Y., Xu, R. *Zhiwu Shengli Xuebao* 1987, 13, 129-35.
24. Suge, H. *Plant Cell Physiol.* 1986, 27, 199-205.
25. Hirai, Y.; Sasaki, H., Katsura, N. Patent. *Chem. Abstr.*, 1988, 109, 21620m.
26. Roth, P.S., Bach, T.J., Thompson, M.J. *Plant Science* 1989, 59, 63-70.
27. Braun, P.; Wild, A. *J. Plant Physiol.* 1984, 116, 189-96.
28. Meudt, W.J.; Thompson, M.J., Bennett, H.W. *Pro. Plant Growth Reg. Soc. Amer.* 1983, 10, 312-18.
29. Adam, G. *Abstract 132, Agrochemical Section, 200th Annual Meeting, Amer. Chem. Soc.*, Washington, D.C., 1990.

30. Katsumi, M.; Tsuda, A., Sakurai, H. *Pro. Plant Growth Reg. Soc. Amer.* **1987**, *14*, 25-20.
31. Gregory, L.E. *Am. J. Bot.* **1981**, *68*, 586-88.
32. Yamaguchi, T.; Wakizuka, T., Hirai, K., Fujii, S., Fujita, A. *Pro. Plant Growth Reg. Soc. Am.* **1987**, *14*, 26-27.
33. Yamaguchi, T.; Hirai, K., Tsukamoto, Y., Takahashi, K. *Abstract 122, Agrochemical Section, 200th Annual Meeting, Amer. Chem. Soc.*, Washington, D.C., 1990.
34. Hamada, K.; Nishi, S., Uezono, T., Fujiwara, S., Nakazawa, Y. *Abstract 12th Internat. Conf. on Plant Growth Subst.* Heidelberg, West Germany, 1985.
35. Kulaeva, O.N.; Burkhanova, E.A., Fedina, A.B., Danilova, N.V., Adam, G., Vorbrodt, H.M., Khrpach, V.A. *Dokl. Akad. Nauk S.S.S.R.* **1989**, *305*, 1277-79.
36. Fujii, S.; Hirai, K., Saka, H. *Abstract 79, Agrochemical Section, 200th Annual Meeting, Amer. Chem. Soc.*, Washington, D.C., 1990.
37. He, R.Y.; Wang, X.S., Wang, G.J. *Abstract 100, Agrochemical Section, 200th Annual Meeting, Amer. Chem. Soc.*, Washington, D.C., 1990.
38. Katsumi, M. *Abstract 120, Agrochemical Section, 200th Annual Meeting, Amer. Chem. Soc.*, Washington, D.C., 1990.
39. Sugiyama, K.; Kuraishi, S. *Acta Hort.* **1989**, *239*, 345-48.
40. Kamuro, Y.; Takatsuto, S. *Abstract 78, Agrochemical Section, 200th Annual Meeting, Amer. Chem. Soc.*, Washington, D.C., 1990.
41. Hewitt, F.R.; Hough, T., O'Neill, P., Sasse, J.M., Williams, E.G., Rowan, K.S. *Aust. J. Plant Physiol.* **1985**, *12*, 201-11.
42. Abe, H. *Abstract 131, Agrochemical Section, 200th Annual Meeting, Amer. Chem. Soc.*, Washington, D.C., 1990.
43. Arteca, R.N., Bachman, J.M., Mandava, N.B. *J. Plant Physiol.* **1988**, *133*, 430-35.
44. Eun, J-S.; Kuraishi, S., Sakurai, N. *Plant Cell Physiol.* **1989**, *30*, 807-10.
45. Kuraishi, S.; Sakurai, N., Eun, J-S., Kojima, K., Sumi, T. *Abstract 121, Agrochemical Section, 200th Annual Meeting, Amer. Chem. Soc.*, Washington, D.C., 1990.
46. Gregory, L.E.; Mandava, N.B. *Physiol. Plant.* **1982**, *54*, 239-43.
47. Takatsuto, S.; Yazawa, N., Ikekawa, N., Takematsu, T., Takeuchi, Y., Koguchi, M. *Phytochem.* **1983**, *22*, 2437-41.
48. Takatsuto, S.; Yazawa, N., Ikekawa, N. *Phytochem.* **1984**, *23*, 525-28.
49. Katsumi, M. *Plant Cell Physiol.* **1985**, *26*, 615-25.
50. Allevi, P.; Anastasia, M., Cerana, R., Ciuffreda, P. *Phytochem.* **1988**, *27*, 1309-13.
51. Barlow, P. In *Hormone Action in Plant Development*; Hoad, G.V., Lenton, J.R., Jackson, M.B., Atkin, R.K., Eds. Butterworth: U.K., 1987, pp 39-51.
52. Barlow, P. *Environ. Exp. Bot.* **1989**, *29*, 1-5.
53. Zucchini, F. *Israel J. Bot.* **1988**, *37*, 131-44.
54. Krizek, D.; Mandava, N.B. *Physiol. Plant.* **1983**, *57*, 317-23.
55. Krizek, D.; Mandava, N.B. *Physiol. Plant.* **1983**, *57*, 324-29.
56. Kamuro, Y.; Inada, K. *Pro. Plant Growth Reg. Soc. Amer.* **1987**, *14*, 221-23.

57. Xu, R.; Zhao, Y. *Zhiwu Shengli Xuebao* **1989**, *15*, 263-67.
58. Cerana, R.; Bonetti, A., Marrè, M.T., Romani, G., Lado, P., Marrè, E. *Physiol. Plant.* **1983**, *59*, 23-27.
59. Clouse, S.; Zurek, D., Hall, A. *Abstract 139, Agrochemical Section, 200th Annual Meeting, Amer. Chem. Soc.*, Washington, D.C., 1990.
60. Schena, M. *Experientia* **1989**, *45*, 972-83.
61. Jacobs, W.P. *Dev. Biol.* **1959**, *1*, 527-33.
62. Sakurai, A.; Fujioka, S., Saimoto, H. *Abstract 138, Agrochemical Section, 200th Annual Meeting, Amer. Chem. Soc.*, Washington, D.C., 1990.
63. Mai, Y.; Lin, J., Zeng, X., Pan, R. *Zhiwu Shenglixue Tongxun* **1989**, (2), 50-52.

RECEIVED March 12, 1991

Chapter 14

Brassinosteroid-Induced Changes of Plasmalemma Energization and Transport and of Assimilate Uptake by Plant Tissues

I. Dahse¹, U. Petzold², C. M. Willmer³, and E. Grimm²

¹Lehrstuhl fuer Biophysik, Friedrich Schiller Universität Jena, Philosophenweg 14a, Jena, O-6900, Germany

²Sektion Biowissenschaften, WB Pflanzenphysiologie, Martin Luther Universität Halle-Wittenberg, Am Kirchtor 1, Halle/Saale, O-4020, Germany

³Department of Biological and Molecular Sciences, University of Stirling, Stirling FK9 4LA, United Kingdom

Several steroids, among them 22S,23S-homobrasinolide (SSHB) and 24-epibrassinolide (24-E), were examined with respect to their effects on plasmalemma energization and assimilate uptake by measuring the electrical potential difference (PD) across the plasmalemma and medium acidification, stomatal opening and uptake of radioactive α -aminoisobutyric acid and sucrose using different plant material. All steroids tested cause, at least temporarily, hyperpolarization of the PD. No correlation of the ability to hyperpolarize the plasmalemma with known growth effects was found. The results are compatible with a steroid effect on the membrane-embedded moiety of the plasmalemma H^+ -ATPase. This does not seem to be the specific effect of brassinosteroids. However, both brassinosteroids promote solute uptake and inhibit stomatal opening.

The uptake of nutrients into plant cells as well as their distribution between organs and tissues is a prerequisite for growth, and it is generally accepted that transport demands the energization of the plasma membrane (plasmalemma), particularly in cases where transport is accomplished against the (electro-)chemical gradient of the solute in question. In plants the proton motive force

across the plasmalemma ($pmf = 59\Delta pH - \Delta\psi$; $\Delta\psi$ - electrical potential difference PD; pmf and $\Delta\psi$ in mV) may, in principle, be harnessed as the driving force for solute uptake even when its electrical component seems to be of higher significance. There is overwhelming evidence that the plasmalemma proton pump builds up the PD and maintains the proton gradient. Phytoeffectors which interfere with this process may, therefore, affect growth. The discovery of brassinosteroids as plant growth substances poses the inverse issue: Are their effects on growth due to a specific interaction with the plasmalemma proton pump as might be suggested by analogy to the fungal phytotoxin, fusaric acid (FC), or to the steroidal glycoside, ouabain, which is a powerful inhibitor of the Na,K-ATPase in several animal cells? A clear answer is obscured by the possibility of various interactions of steroids with membrane proteins and/or their lipid environment and finally by the failure to find specific receptors - a problem which is well known for other plant hormones.

In order to delimit the possible mode(s) of action of brassinosteroids, we report here on a series of different experiments with different plants which should indicate changes of membrane energization and transport as induced by phytoeffectors. The following well-characterized test systems were used: for measuring the PD: leaves of *Egeria densa* (1); for recording medium acidification: leaves of *Egeria* and *Vicia faba* (2); for measurement of stomatal opening: epidermal strips of *Commelina communis* (3); for assaying ATPase activity: guard and mesophyll cell protoplasts of *Commelina* (4); for sugar and amino acid uptake: leaves of *Egeria* and *Vicia* and conducting tissue of *Cyclamen persicum* (5). As a result it was shown once again that relations in more complex systems like tissues or organs cannot be deduced from results obtained from transport at the cellular level.

Material and Methods

Plant Material. Growing conditions and treatment of plant material has been described elsewhere: for the aquatic weed, *Egeria densa* PLANCH., in (6), *Vicia faba* L. in (2), *Commelina communis* L. in (7) and *Cyclamen persicum* MILL. in (5).

Electrophysiological Measurements. Membrane potentials of *Egeria* leaf cells were measured using standard microelectrode technique (8). An isolated leaf was mounted in a plexiglass chamber which was continuously perfused by a standard medium (1.0 mM KCl, 1.0 mM Ca(NO₃)₂, 0.25 mM MgSO₄, 1.0 mM Na₂HPO₄/NaH₂PO₄ buffer, pH 5.7). Experiments were performed in the light (50 W m⁻² at the leaf level) as well as in darkness. Steroids were added at the point when the PD reached a stationary magnitude for at least 10 min. All phytoeffectors were added from methanolic stock solutions.

Recording of Medium pH. pH measurements in the medium were made out in unbuffered solution (10 or 20 ml) with 10 *Egeria* leaves (30-40 mg fresh weight) or 20 *Vicia* leaf discs (200 mg fresh weight). The CO₂-free aerated solution contained 1 mM CaCl₂ and 1 mM KCl (*Egeria*) or 0.5 mM CaCl₂, 0.5 mM MgCl₂ and 250 mM mannitol as osmoticum (*Vicia*) plus the steroids.

Uptake of α -Aminoisobutyric Acid (AIB) and Sucrose. Leaves of *Egeria* or *Vicia* were incubated with radioactively labelled solutes as described (2). The uptake rate was calculated from the initial specific activity in the external medium. The radioactivity in the methanolic extracts was recorded by liquid scintillation spectrometry.

Sucrose Absorption by Vascular Bundles. Sucrose uptake into 2-4 mm long pieces of vascular bundles isolated from the petiole of *Cyclamen persicum* was described in detail in (5). Briefly, the tissue material was preincubated (30 min) with brassinosteroids or related compounds, which were also present during the following incubation with 1 mM ¹⁴C-labelled sucrose (30 min). All solutions contained 1 mM CaSO₄, 1 mM KCl and 10 mM morpholinoethanesulfonic acid and were adjusted with NaOH to pH 5.0. After a 5-min-washing procedure and extraction in methanol the uptake rate for sucrose was determined analogously to the experiments with leaves.

Results and Discussion

Brassinosteroids - like other steroids - are lipophilic or amphiphilic; some of them are mesogens. These properties cause them to accumulate in membranes or at membrane surfaces. Therefore, detailed insight into their role as plant growth substances might be obtained by studying their interference with membrane functions. Applied externally, the first membrane they meet is the plasmalemma. The functioning of the plasmalemma is crucial for transport in plants. Hereafter, some results on the plasmalemma are reported which reflect short-term effects of the substances applied.

Effect of Different Steroids on the Electrical Cell Potential of *Egeria* leaves. *Egeria* leaves maintain a high membrane potential of about - 230 mV in the light due to enhanced proton pumping by the plasmalemma ATPase, whereas the PD in darkness is considerably lower (1). Therefore, a stimulation of proton extrusion, for example, by the addition of phytoeffectors, is electrophysiologically easier to detect in the dark. In contrast, inhibition of proton pumping indicated by a depolarization of the plasmalemma should be better seen under illumination. Consequently, the steroids tested were applied in the light and in the dark. In the light, SSHB and 2 α -3 α -dihydroxy-5 α -stigmast-22-en-6-one (DHS) were similar to each other and similar to FC in causing hyper-

polarization of the PD whereas the common plant sterol, stigmasterol, was less effective. In the dark, these three steroids showed comparably large hyperpolarizations (8). Surprisingly, 24-E which has been found to be highly growth-promotive in growth tests (9) did not usually affect the PD of *Egeria* leaf cells in the light and hyperpolarized the plasmalemma only sometimes in darkness. The cardiac glycoside, ouabain, was ambiguous in causing PD changes in the light, whereas in the dark, it caused transient hyperpolarization resting on a depolarized state of the cell.

Effect of Steroids on Proton Extrusion. Photosynthesizing leaf tissues were capable of extruding protons into the external medium owing to the activity of the plasmalemma ATPase. Applying steroids in such experiments, different results were obtained with *Egeria* and *Vicia*. Using *Egeria* leaves, SSHB, DHS and stigmasterol caused strong medium acidification in the light and in darkness (8). However, in accordance with its failure to hyperpolarize the plasmalemma, 24-E, like ouabain, did not acidify the medium in any case.

In contrast to *Egeria* leaves, with *Vicia* leaf slices, medium acidification was enhanced not only in the presence of SSHB but also by 24-E (Figure 1). The addition of the plasma membrane ATPase inhibitor erythroisine B did strongly reduce brassinosteroid-induced H^+ -extrusion indicating that the stimulating effect of brassinosteroids is mediated by the ATP-driven proton pump.

Stomatal Opening as Influenced by Steroids. Owing to the fact that guard cells of stomata are highly specialized in membrane transport, they were included in this investigation. In particular, it is known that the plasmalemma proton pump delivers the driving force for the strong

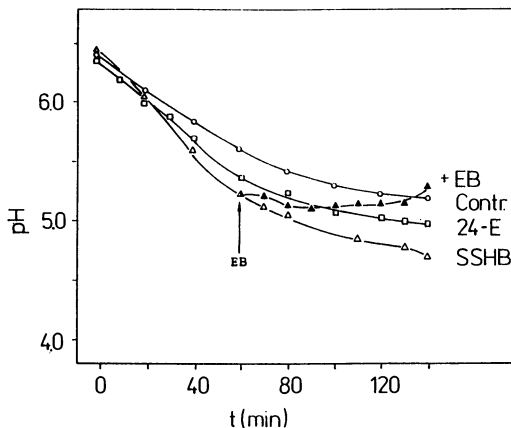


Figure 1. Enhancement of light-induced H^+ extrusion by 24-epibrassinolide (24-E; 10^{-7} M) and 22S,23S-homobrassinolide (SSHB; 10^{-7} M) and its inhibition by erythroisine B (EB; $5 \cdot 10^{-5}$ M) in *Vicia* leaf discs.

ion influx required for stomatal opening. Therefore, stomatal movement is expected to provide evidence for a modified pump activity complementary to that of measurements of the membrane potential or of medium acidification.

Following this strategy, we measured the effect of steroids on stomatal opening in epidermal strips of *Commelina*. As a result, SSHB (not shown) and 24-E as well as ouabain significantly inhibited stomatal opening (Figure 2) whereas DHS slightly stimulated it (not shown). Stigmasterol did not impair stomatal movement.

Using guard or mesophyll cell protoplasts of *Commelina*, in an assay according to (4), activities of solubilized plasmalemma and tonoplast ATPase were found unchanged after steroid treatment.

Effects of Brassinosteroid on Solute Uptake. According to the results obtained with *Egeria* and *Vicia* leaf tissues (2) the stimulating effect of brassinosteroids on the proton pump should increase the proton gradient-driven influx of organic solutes. Therefore, uptake of AIB and sucrose into leaf tissues of *Egeria* and *Vicia* as well as into conducting tissue of *Cyclamen* was measured

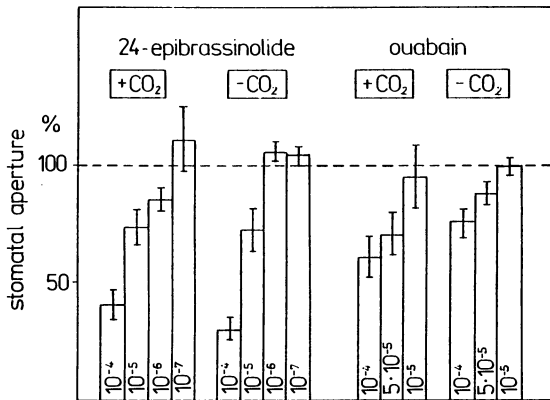


Figure 2. Effect of 24-epibrassinolide and ouabain on stomatal opening of *Commelina* epidermal strips in the light in the absence or presence of carbon dioxide in the air. Aperture of the control: 11.6 μm (without CO₂); 6.3 μm (with CO₂).

using radioactive tracers. For comparison FC was used as a known activator of H⁺-ATPase. The results in Figure 3 and Table I demonstrate the promotive effect of SSHB and FC on the uptake of AIB and sucrose into the leaves of *Egeria* and *Vicia*. Additional autoradiographic studies using *Vicia* leaf discs of mature, photosynthate exporting leaves (not shown) indicated that within two minutes of incubation, the minor vein network concentrates C¹⁴-labelled sucrose. This observation is consistent with the involvement of phloem loading. Therefore, the increased

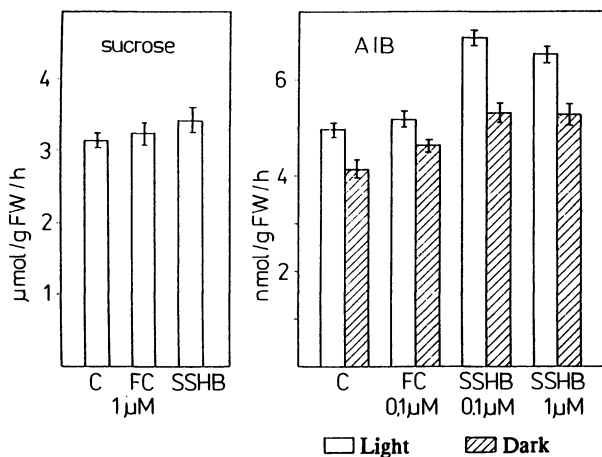


Figure 3. Effect of SSHB and fusicoccin (FC) on substrate uptake into leaf cells of *Egeria* in the light and in the dark. Concentration of external solutes: AIB, 0.5 mM; sucrose, 5 mM. C - control.

sucrose uptake into the leaf discs appeared to result not only from effects of FC and SSHB on sugar entry into the mesophyll cells (as in *Egeria*) but also on the processes of phloem loading.

Table I. Effect of FC and SSHB (in M) on Uptake ($\mu\text{M/gFW/2h}$) of Sucrose (5 mM in the Incubation Medium) in *Vicia* Leaf Discs in the Light

Control	FC	SSHB
-	10^{-6}	10^{-6}
4.61 (± 0.21)	4.53 (± 1.3)	5.29 (± 0.13)
4.72 (± 1.2)	5.20 (± 0.08)	

Actually, the data from Table II indicates a slight, but distinctive stimulation of sucrose uptake into isolated vascular bundles of *Cyclamen* by brassinolide, 24-E and DHS, whereas SSHB and stigmasterol were found to be without any significant effect. Nevertheless, this short-term increase in the range between 15 and 24% for brassinosteroids is less effective than the fusicoccin-mediated stimulation of sucrose absorption (33-35%). Again, this data suggests that brassinosteroid-stimulated substrate uptake may be mediated through the modifying of proton gradient-maintaining pump at the plasma membrane.

Involvement of Plasmalemma Energization. For SSHB and DHS the membrane potential measurements in *Egeria* leaf

Table II. Effect of Brassinosteroids and Related Compounds on Uptake (nmol/g FW/30 min) of Sucrose (1 mM in the Incubation Medium) into Isolated Conducting Tissue of *Cyclamen*

Treatment	uptake rate	stimulation (%)	significancy
Control	342 ± 32	-	-
BR, 10 ⁻⁶	397 ± 48	16	P < 5%
BR, 10 ⁻⁷	423 ± 88	24	P < 5%
SSHB, 10 ⁻⁶	344 ± 39		n.s.
SSHB, 10 ⁻⁷	372 ± 70		n.s.
24-E, 10 ⁻⁶	379 ± 49		n.s.
24-E, 10 ⁻⁷	402 ± 33	18	P < 1%
stigmasterol, 10 ⁻⁶	396 ± 61		n.s.
stigmasterol, 10 ⁻⁷	394 ± 54		n.s.
DHS, 10 ⁻⁶	393 ± 29	15	P < 5%
DHS, 10 ⁻⁷	398 ± 52	16	P < 5%
FC, 10 ⁻⁶	463 ± 41	35	P < 1%
FC, 10 ⁻⁷	454 ± 59	33	P < 1%

cells revealed the electrogenic nature of the steroid-enhanced H⁺-extrusion. Unexpectedly, this could not be found for 24-E although good correlation of hyperpolarization and medium acidification has been reported for this brassinosteroid in Azuki bean (*Vigna angulare*) epicotyls (9). These findings and the brassinosteroid-induced H⁺-extrusion by *Vicia* leaf slices, which was inhibitable by erythrosine B (Figure 1), suggest that plasmalemma energization displays differing sensitivity to brassinosteroids in different plants. The strong steroid-induced hyperpolarization of green *Egeria* cells in darkness indicates a stimulation of the proton pump rather than an increased resistance of the plasmalemma. This view is additionally supported by the sensitivity of H⁺-extrusion to erythrosine B. In contrast to our results with *Egeria*, where ouabain and 24-E failed to induce proton pumping, it has been reported that in root tissues of maize all steroids tested enhanced proton extrusion, whereas only for brassinosteroids or related derivatives has growth promotion been found (10). This indicates a stimulating effect on the plasmalemma ATPase independently of growth. Unfortunately, the comparability of these results is limited in view of the fact that phytohormone-induced proton extrusion is known to be different in roots in comparison to the shoot.

Similarly, stomatal opening in epidermal strips of *Commelina* was affected by the steroids tested here in a different manner (Figure 2) although membrane energization and hyperpolarization of guard cells are a prerequisite of opening. Surprisingly, both brassinosteroids, SSHB and 24-E, (like ouabain) inhibited opening in parallel, whereas the physiologically inactive (regarding growth) DHS stimulated it.

The lack of effect of steroids on the ATPase activity in the assay with *Commelina* protoplasts is no proof against an interaction with this membrane protein as the experiment has been performed in the presence of a detergent (Triton X-100), that is, under conditions where the protein is not capable of vectorial transport. This result is, rather, a hint for an interaction with the membrane-embedded moiety of the proton pump.

Sucrose absorption by the conducting tissue of the *Cyclamen* petiole has been shown to be a carrier-mediated step accomplished by cotransport with protons (5). Stimulated sucrose absorption in the presence of brassinosteroids as observed in this investigation may, therefore, be attributed to a higher proton pump activity of the plasmalemma ATPase.

Concluding Remarks

An important question, raised by the results presented on brassinosteroid effects, is how stimulation of proton pump cooperates with the processes of photosynthesis and partitioning of photoassimilates as a whole. From this investigation it is too early to draw conclusions concerning the consistency of the data obtained from different transport steps. Our aim was to find out the mode of action of brassinosteroids on membrane transport using for comparison other steroids; therefore, well-characterized test systems were initially used. Even if this strategy is accepted, its drawback will become evident: the data collected from different species and organs provides only indirect evidence for the interaction of steroids with membranes and is not strictly comparable. From all information known to us, two unexpected facts emerge: 1) We could not correlate the potency of steroids in causing hyperpolarization and medium acidification with structural requirements. 2) The lack of correlation between the steroid effects on membrane energization obtained in different systems is a hint to their differing sensitivity.

Nevertheless, it was clearly demonstrated that brassinosteroids, like the other steroids investigated here, interact with the plasmalemma thereby showing short-term effects on the membrane potential and/or medium acidification. In some cases these effects correlate with stomatal movement and solute uptake into leaves or conducting tissue. The results are compatible with an effect of these substances on the membrane-embedded moiety of the plasmalemma H^+ -ATPase yielding a modified proton pump rate. They support, therefore, the "Annulus Hypothesis" according to which sterol effects are caused by direct lipid-protein binding.

Acknowledgments

The authors would like to thank Drs G. Adam, V. Marquardt and H.M. Vorbrodt for the generous gift of SSHB, 24-E,

DHS and stigmasterol and Dr G. Grammatikopoulos for performing the ATPase assay.

Literature Cited

1. Dahse, I., Linsel, G., Müller, E., Dawczynski, M., Opfermann, J. *Biochem. Physiol. Pflanzen* 1987, **182**, 117-128.
2. Petzold, U., Dahse, I., Müller, E. *Biochem. Physiol. Pflanzen* 1985, **180**, 655-666.
3. Dahse, I., Willmer, C.M., Meidner, H. *J. Exp. Bot.* 1990, **41**, 1109-1113.
4. Fricker, M., Willmer, C.M. *J. Exp. Bot.* 1987, **38**, 642-648.
5. Grimm, E., Bernhardt, G., Rothe, K., Jacob, F. *Planta* 1990, **182**, 480-485.
6. Petzold, U. *Biochem. Physiol. Pflanzen* 1981, **176**, 60-70.
7. Birkenhead, K. & Willmer, C.M. *J. Exp. Bot.* 1984, **35**, 1260-1264.
8. Dahse, I., Sack, H., Bernstein, M., Petzold, U., Müller, E., Vorbrod, H.M., Adam, G. *Plant Physiol.* 1990, **93**, 1268-1271.
9. Cerana, R., Bonetti, A., Marrè, M.T., Lado, P., Marrè, E. *Physiol. Plant.* 1983, **59**, 23-27.
10. Cerana, R., Lado, P., Anastasia, M., Ciuffreda, P., Allevi, P. *Z. Pflanzenphysiol.* 1984, **114**, 221-225.

RECEIVED May 1, 1991

Chapter 15

Brassinosteroids Specifically Inhibit Growth of Tobacco Tumor Cells

Thomas J. Bach¹, Petra S. Roth¹, and Malcolm J. Thompson²

¹Universität Karlsruhe, Botanisches Institut II, Karlsruhe 1, D-7500, Germany

²Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705

Auxin- and cytokinin-autotrophic tobacco cells, generated through transformation by *Agrobacterium tumefaciens*, were used for studying the effect of two synthetic brassinolides (2 α ,3 α ,22 β ,23 β -tetrahydroxy-24 β -methyl-*B*-homo-7-oxa-5 α -cholestan-6-one, **BR-1**, and 2 α ,3 α ,22 α ,23 α -tetrahydroxy-24 β -methyl-*B*-homo-7-oxa-5 α -cholestan-6-one, **BR-2**). Both artificial brassinosteroids have been shown active in brassinolide-specific tests using intact plants or plant organs. Both in callus and suspension cultures BR-1 and BR-2 significantly inhibited cell growth, with BR-1 being slightly more effective at concentrations below 10⁻⁸ M. This specific growth response, becoming significant below a concentration of 10⁻⁹ M, cannot be easily explained solely by membrane effects but rather suggest the presence of specific hormone receptors mediating gene activation/inactivation similar to the situation in animal cells. This inhibitory effect was much less prominent, if visible at all, in hormone-auxotroph cell lines that have also been tested, e.g. from wild-type tobacco, carrot, and *Petunia*. BR-1 treated tobacco tumor cells produce less cytokinin and auxin than the controls. From this and other data a model is derived that might provide an explanation of how BR interferes with the regulation of the cell cycle in transformed cells. Ecdysone, having some structural similarities to brassinolide, also exerted some growth-inhibitory effect, although at concentrations about three magnitudes higher. The preliminary data available, using radiolabeled ponasterone that usually serves as a substrate for the kinetic analysis of insect ecdysteroid receptors, indicates some specific binding to protein in homogenates from *LA6* cells. Since growth inhibition can be monitored that easily, tobacco tumor cells might be useful in the screening for other natural products having BR activity.

In 1970, Mitchell et al. (1) isolated from pollen of rape a lipoidal complex termed "brassins" that has growth-promoting effects on plants. The active compound was eventually identified as a steroid derivative, which was given the name brassinolide (2). In the same year, two highly physiologically active brassinosteroids (a generic term for all compounds structurally related to brassinolide), 22*S*,23*S*,24*S* (tri-*epi*)-brassinolide (further referred to as BR-1) and 22*R*,23*R*,24*S* (*epi*)-brassinolide (BR-2) were synthesized (3, 4). Ever since various synthetic routes have been developed to synthesize BR and its isomers (see elsewhere in this volume).

Brassinosteroids are reported to stimulate overall plant growth and development, especially under stress conditions, to enhance auxin-induced growth as well as auxin-induced ethylene production (5, 6). Brassinosteroids interact with most of the phytohormones, such as cytokinins and gibberellins, and in particular with auxin.

In this article we refer to the effect of brassinosteroids on plant cell cultures, in particular on plant tumor cells.

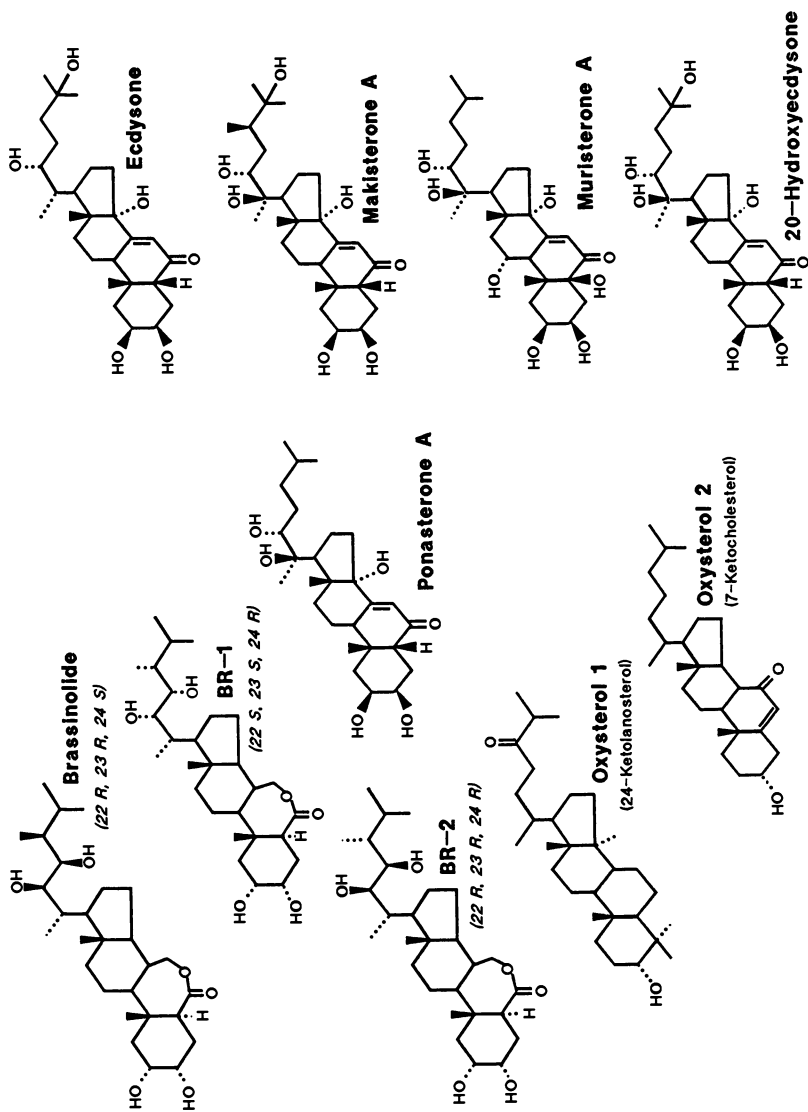
Transformed tobacco cultures are hormone-autotroph. The integration of the T-DNA of the plasmid from *Agrobacterium tumefaciens* into the plant genome provides the genes required for the synthesis of auxin and cytokinins. The amounts produced maintain the cells in a juvenile, undifferentiated stage and support rapid growth. This specific condition offers the possibility to singularize the effect of an additional growth regulator.

Like all steroids, brassinosteroids derive from a single common precursor: mevalonic acid. Some phytohormones are synthesized totally or in part via the isoprenoid pathway, such as abscisic acid, gibberellins and cytokinins. The importance of this biosynthetic pathway in processes involved with cell cycle regulation and tumorigenesis in mammals is well documented. Having in mind the similarities between certain regulatory systems in plants and animals the question arose whether brassinosteroids as putative plant steroid hormones would show a specific effect on plant tumor cells.

Methods

Transformed tobacco cells (strain *LA6*) were kindly provided by Professor Jeff Schell, MPI Cologne. Culture maintenance conditions as well as application of brassinosteroid to culture-media were described previously (7).

Ethylene evolution. Five days after the addition of brassinosteroid to the culture media, aliquots (5 ml) of the suspension cultures were transferred to sterile plastic tubes and tightly closed. The tubes were placed on a gyrotory shaker (140 rpm) for 12 and 24 hours and kept in the dark. Then samples were withdrawn with a syringe and injected into a gas chromatograph (Hewlett Packard 5880A; column 1m x 3mm packed with Al₂O₃, 60/80 mesh; carrier N₂, combusive gas



Scheme 1: Structures of steroids used in this study. The stereochemistry of natural brassinolide compared to the synthetic analogs (BR-1 and BR-2) is indicated.

mixture O₂ and H₂; gas flow rate N₂,H₂ 20/24 ml/min; temperatures: column isothermal 80°C, oven 250°C).

Endogenous phytohormone levels

IPA: Transformed tobacco callus cultures were cultured on BR-containing medium for 3 weeks, harvested and weighed. Plant material was frozen in liquid nitrogen and then extracted according to the manual of the manufacturer's kit (see below).

The calli were homogenized using a mortar and pestle (previously chilled with liquid N₂). Then they were extracted with 10 ml per g original fresh weight 80% MeOH containing 10 mg per liter BHT (2,6-Di-*tert*-butyl-4-methylphenol) at 4°C (cold chamber) for 1h under continuous stirring. At this stage an internal standard was added. The sample was pelleted and decanted. The extraction was repeated twice, the extracts were combined and water was added to a final concentration of 70% MeOH. Lipids and pigments were removed by passage of the extract through reverse-phase C₁₈ cartridges (Pharmacia). MeOH was removed by rotary evaporation followed by a stream of nitrogen. Immunological quantification of IPA (isopentenyl adenosine) was performed with commercially available assay kits (Phytodetek-IPA, Idetek, Denmark), according to the manufacturer's manual.

IAA: Indole-3-acetic acid content of treated calli was determined by HPLC. The frozen calli (liquid N₂) were homogenized with 4 ml per 500 mg original fresh weight cold acetone/water (80:20, v/v) in a chilled mortar, then in a Waring Blendor. The homogenate was centrifuged for 15' at 5000 x g, resuspended in fresh acetone (80%) and centrifuged again. The supernatants were combined. Acetone was removed with a rotary evaporator, the aqueous residue was frozen at -20°C, thawed and centrifuged again (30', 5000 x g). The supernatant was removed by aspiration, the remaining material brought to pH 3 with 1 N HCl and extracted with ether. The ether was removed and the resulting residue was dissolved in MeOH. HPLC was performed by using a reverse-phase C₁₈ cartridge (Nucleosil, Merck), using two elution solutions (A: 20% MeOH in 0.1 acetic acid; B: 100% MeOH; gradient: from 20% B to 98% B in 20'). The standard was pure IAA in MeOH. Fractions of the samples co-eluting with the standard were collected and spectrophotometrically identified.

Ecdysteroids: All ecdysteroids used in this work were a kind gift of Prof. Dr. Jan Koolmann, University of Marburg. They were ecdysone, 20-hydroxyecdysone, makisterone and muristerone for the growth experiments, as well as radiolabeled ponasterone A for the binding assay. Preparation of stock solutions, sterilization and addition to media was exactly the same as for brassinosteroids.

Binding assay: 20 g of freshly harvested, three-week-old transformed tobacco calli were homogenized in a mortar with 25 ml of tris-buffer (10 mM Tris, 1.5 mM EDTA; pH 7.4 at 20°C), then in a glass homogenizer (0.37mm gap, 200 rpm) and finally passed through 9 layers of gauze. The filtrate was centrifuged for 10' at 2°C and 2000 rpm (Beckmann, model J-21, rotor JA 20). The pellets were combined and resuspended in a small amount of tris-buffer, homogenized in a

second glass homogenizer (0.1mm gap, 500 rpm) and centrifuged again. All steps were performed in a cold chamber (2-4°C).

The procedure of the DCC-assay followed the protocol of Lehmann & Koolman (8, 9).

Composition of the DCC-suspension: 2.7% (w/v) Norit A (charcoal), 0.27 % (w/v) Dextran T 40, 0.15 % (w/v) gelatine, 10 mM Tris/HCl, 1.5 mM EDTA, 7 mM DTT, pH 7.4 at 20°C.

Assay of total binding: 133 μ l of [3 H]-PoA (equivalent to 80 nM, in MeOH; 5 μ Ci/ml) was dried under a stream of nitrogen and dissolved in 45 μ l of buffer.

Unspecific binding: 133 μ l of (3 H)-PoA (equivalent to 80 nM, in MeOH; 5 μ Ci/ml) plus 30 μ l unlabeled PoA (equivalent to 24 μ M, in MeOH) were dried under N₂ and also dissolved in 45 μ l of buffer.

To each assay 405 μ l cell homogenate were added and then incubated for 9 hours (+ 4°C). Afterwards aliquots were withdrawn (100 μ l each), and 100 μ l of DCC-suspension added and well shaken for 30'. Particles of charcoal were removed in a short centrifugation step (2'). 100 μ l of the supernatants were pipetted into scintillation vials, 4 ml cocktail were added and the samples were counted.

The specific binding is given by: Total binding minus unspecific binding.

Results

Growth Inhibition. Both brassinosteroids (BR-1, tri-epi-brassinolide, and BR-2, epi-brassinolide) dramatically inhibit growth of transformed tobacco cultures, with BR-1 being the more active compound at low ($<10^{-8}$ M) concentrations (Figure 1).

This strikingly differs from the well known growth-promoting effect that brassinosteroids produce on many plants and plant segments (5, 6). Callus and suspension cultures behaved more or less the same way (Figures 1 and 2), although cells kept in suspension appear to be even more sensitive, possibly due to a facilitated uptake of BR. Growth inhibition appears to be more pronounced when comparing fresh and dry weight of BR-treated calli (Table I). This leads us to conclude that one mechanism underlying the growth inhibition might be the interference with processes governing water uptake in tumor cells.

The transformed tobacco cells react in a very sensitive and specific way on brassinosteroid-treatment. Various non-transformed, hormone-heterotrophic callus cultures such as wild-type tobacco (strain *SRI*), carrot and *Petunia* showed rather undefined or weak reaction upon the application of BR (7).

Ethylene Production. The production of ethylene is reduced in brassinosteroid-treated suspension cultures (Table II). In various other systems BR has been reported to stimulate ethylene formation (see 6 and literature cited therein).

Effect of Further Growth Regulators. Some other growth regulators were tested for their effect on transformed tobacco callus cultures. Triacantanol, a long-chain alcohol with growth-promoting activities in several plant species (10) and even in single-cell algae (11, 12), as well as its putative natural antagonist

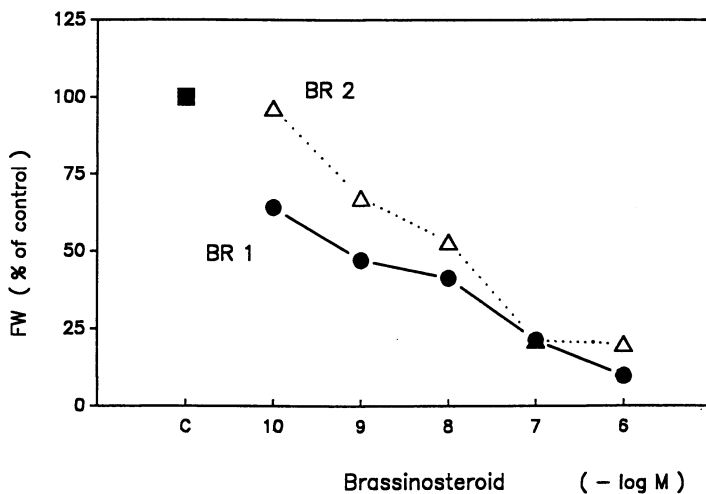


Figure 1: Comparison of the effect of two synthetic analogs (BR 1 and BR 2) of brassinolide on the growth (measured as increase of fresh weight - fw - in % of the untreated control) of transformed *Nicotiana tabacum* callus-cultures. Culture period: 3 weeks. CV <5%

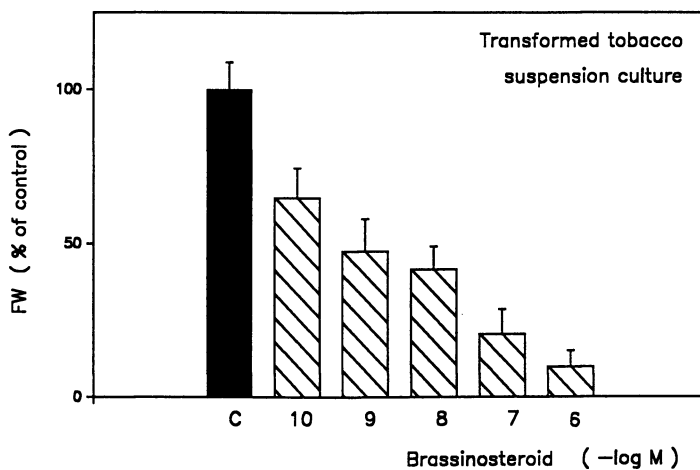


Figure 2: Growth of transformed *Nicotiana tabacum* suspension-cultures (in % of the untreated control) in presence of brassinosteroid BR 1. Culture period: 7 days. Concentration range: 10^{-10} to 10^{-6} M. CV >5%

heptacosanol, did not exert any significant effect at the concentrations tested (10^{-5} M and 10^{-4} M; data not shown).

Oxysterols are reported to act as potent inhibitors of growth in mammalian cell systems because of their interference with sterol biosynthesis (13). Upon superficial examination, brassinosteroids could be regarded as just highly oxygenated sterols. Therefore, two oxysterols (24-ketolanosterol and 7-

Table I: Water content of transformed tobacco callus-cultures grown on brassinosteroid-containing medium for 3 weeks. The brassinosteroid employed (BR-1) is a synthetic 22*S*,23*S*,24*S*-analog of the naturally occurring brassinolide. The concentration of BR-1 was varied from 10^{-10} M to 10^{-6} M. CV < 5%

Concentration of brassinosteroid [M]	Water content [% of fresh weight]
Control	97.1 ± 0.2
10^{-10}	95.9 ± 0.2
10^{-9}	95.1 ± 0.3
10^{-8}	94.8 ± 0.2
10^{-7}	94.1 ± 0.1
10^{-6}	92.7 ± 0.2

ketocholesterol, kindly provided by Dr. Ed Parish, Auburn University) were applied to tobacco calli at concentrations from 10^{-9} M to 10^{-5} M (Figure 3). At the lower concentrations there was no effect at all; 10^{-6} M and 10^{-5} M slightly inhibited callus growth. The data suggests that the growth-inhibiting effect of brassinosteroids on tobacco tumor cells cannot be solely ascribed to an unspecific oxygenation of the sterol backbone, but is rather a function of specific interaction with as yet unidentified genes/factors being involved in the cell cycle regulation of tumor cells. Similarly, mevinolin, a natural antibiotic and highly specific inhibitor of mevalonate biosynthesis and efficient growth regulator in intact plants (*14*) blocked growth of *LA6* cells only at concentrations higher than 1 μ M, about ten times higher than was shown to be required with a hormone-auxotroph cell culture of *Silybum marianum* (data not shown).

Table II: Ethylene production by brassinosteroid (BR-1)-treated, transformed tobacco suspension cultures

BR-1 [M]	Ethylene [nl/g fresh weight]	
	12h	24h
Control	158	548
10^{-10} M	130	325
10^{-8} M	139	371
10^{-7} M	130	258
10^{-6} M	124	380

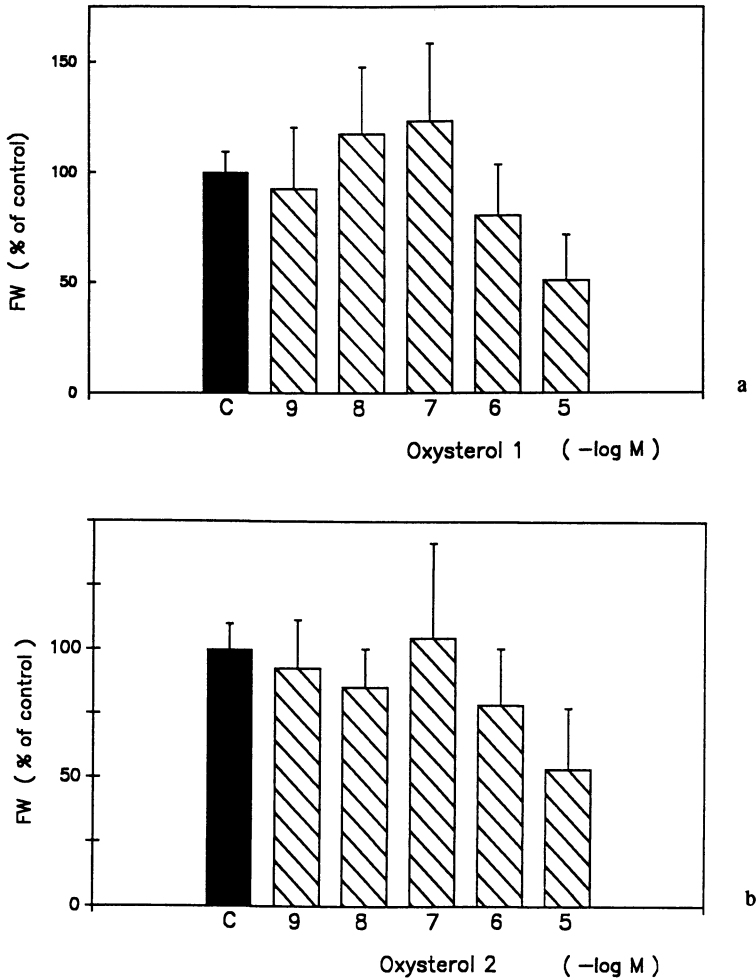


Figure 3a,b: Growth of transformed *Nicotiana tabacum* callus-cultures in the presence of oxysterols. a) 24-ketolanosterol; b) 7-ketocholesterol. Concentrations of oxysterol were varied from 10^{-9} to 10^{-5} M.

Effect of BR on Endogenous Cytokinin and Auxin Content. We have previously shown that addition of exogenous IAA (10^{-5} M and 10^{-4} M) also reduced growth of LA6 cells (7, 10). Simultaneous addition of IAA (10^{-5} M) and of BR-1 to the cultures inhibited growth of cells to a lesser degree than did the application of any of the compounds alone. Intermediate levels of BR-1 (10^{-9} and 10^{-8} M) in combination with high auxin (10^{-4} M) even stimulated FW/DW increase considerably, thus revealing some yet to be defined interactions between the two phytohormones. Therefore, we were interested to measure the auxin content of BR-1 treated LA6 calli. Extractable, endogenous IAA was dramatically reduced in brassinosteroid-treated transformed tobacco calli (Table III).

Table III: Content of IAA in BR-1 treated, transformed tobacco cells.
IAA was determined by the aid of HPLC. Age of cultures: 3 weeks

BR-1 [M]	nmoles IAA/g fresh weight
0 (control)	93.7
10^{-10}	15.9
10^{-8}	19.0
10^{-7}	27.6
10^{-6}	13.7

The content of cytokinin (IPA) was also drastically reduced (Figure 4); the reduction was dramatic and tended to be dose-dependent. Therefore, the growth-inhibiting effect of BR might in part be correlated to lowered amounts of endogenous auxin and cytokinin that fall below the levels required for sustaining growth and/or cell division. The formerly well balanced inner equilibrium of these two phytohormones in the transformed cells appears to be severely disturbed by the addition of BR. The striking growth-inhibiting effect of BR on tobacco tumor cells, in contrast to its much less pronounced effect on normal plant tissues (7) prompts the speculation of whether BR is closely involved in processes governing tumorigenesis itself.

Effect of Ecdysteroids: Brassinosteroids are structurally related to the insect moulting hormones, the ecdysteroids. Thus the question was raised whether

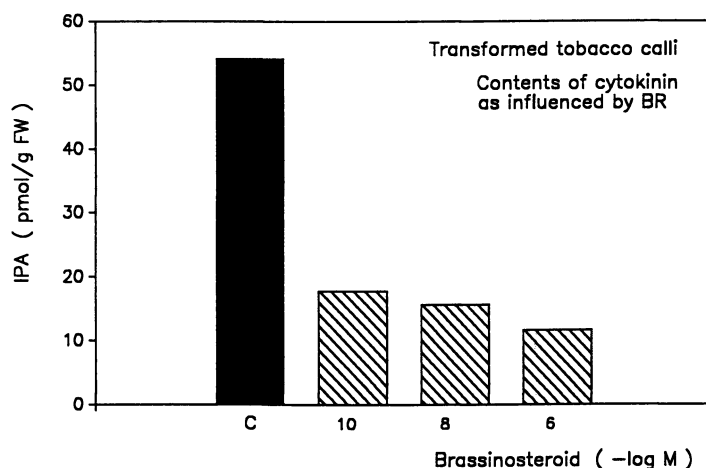


Figure 4: Effect of brassinosteroid (BR-1) on endogenous cytokinin in tobacco LA6 cells. Cytokinin (isopentenyladenosin, IPA) was determined by the aid of an ELISA test.

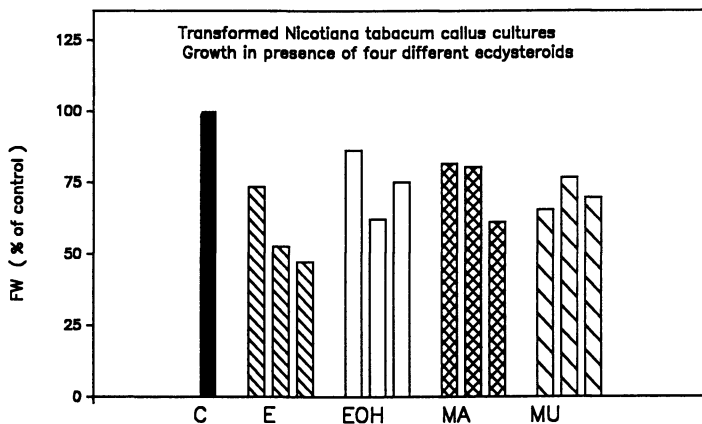


Figure 5: Effect of 4 different ecdysteroids on the fresh weight increase of *LA6* calli. E: Ecdysone; OH-E: β -Hydroxy-ecdysone; MA: Makisterone; MU: Muristerone. Concentration range: 10^{-8} to 10^{-6} M. Age of cultures: 3 weeks. CV < 10%

there might exist a functional, evolutionary relationship (cf. ref. 16, and elsewhere in this volume). Various ecdysteroids were tested upon their effect on transformed tobacco callus cultures. It has been shown that brassinosteroids display an antiecdysteroid activity in insects (16). The concentrations employed varied from 10^{-9} M to 10^{-5} M. The ecdysteroids inhibited growth of treated cultures, to a much lesser extent than BR (Figure 5). Only the effect of ecdysone was clearly dose-dependent.

Due to their structural similarity, ecdysteroids might compete with brassinosteroids for the same receptor site. With the aim of looking at a possible interaction of both BR-1 and ecdysone, the compounds were combined covering a wide range of concentrations (Table IV). The effect of BR-1 was largely

Table IV: Comparison of the effects of ecdyson and BR-1 on growth of transformed tobacco callus-cultures. The concentrations of hormones were varied as indicated. Culture period: 3 weeks. Each value represents the mean of 5 replicates. CV < 5%

Brassinosteroid [M]	Ecdysone [M]			
	0	10^{-8}	10^{-7}	10^{-6}
0	100	73.5	52.6	47.2
10^{-10}	69.3	70.2	76.3	63.9
10^{-9}	68.2	37.5	55.0	52.0
10^{-8}	48.3	79.1	50.5	57.5
10^{-7}	37.1	48.1	24.9	30.6
10^{-6}	21.7	18.4	4.7	4.7

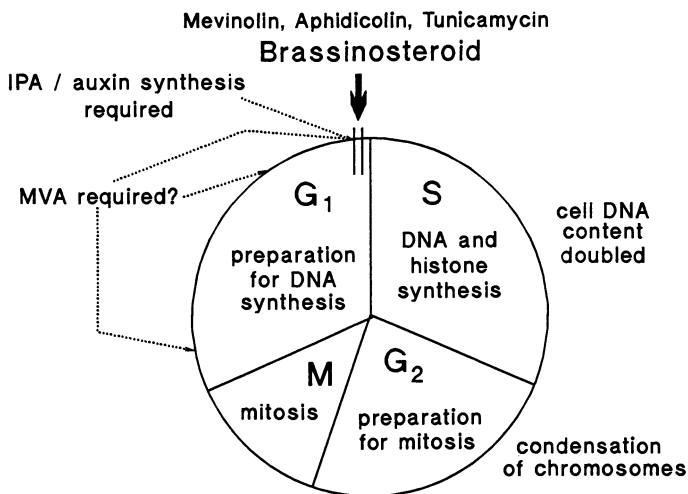
predominant, with a slight but not significant tendency of ecdysone at medium concentrations to counteract the BR-1 effect.

Nevertheless, an initial study was done using radiolabeled ponasterone A, which usually serves as a substrate for the analysis of ecdysteroid-receptors in insect preparations. Indeed, in homogenates of the transformed *LA6* cells a specific binding of 23% was observed. This is not high when compared to specific binding in insect cell-free preparations. Possibly the structural differences between ponasterone (a plant metabolite!) and brassinosteroid is already too extreme to exactly fit this steroid into the putative binding site of a receptor protein. Even then this preliminary observation encourages to further efforts not only to identify the brassinosteroid receptor, but also to gaining some more evidence for the close relationship between the two hormone groups in plants and animals.

Discussion

In our studies, brassinosteroid(s) exerted a strong inhibitory effect on growth of transformed, hormone-autotroph tobacco cells, much more than on wild-type tobacco cultures and especially on other hormone-auxotrophic cultures of *Petunia hybrida* and of *Daucus carota* (7). Therefore, the tumor cells were subjected to a deliberate disturbance of their endogenous auxin balance by addition of considerably high dosages of IAA (7, 15). In fact, additional auxin reduced the BR elicited inhibition, and in two cases remarkably sparked growth. These results suggested that BRs interfere with the endogenous levels of auxin. Indeed, as is shown in this contribution, BR treatment resulted in a clear decrease of extractable IAA. Together with the finding that a parallel cytokinin content also appears to be drastically reduced; this observation leads us to suggest that BR might arrest the tumor cells in the cell cycle. In transformed cells there is a high level of endogenously produced auxin and cytokinin, the synthesis of which is ensured by a set of genes derived from the bacterial Ti-plasmid. These are stably integrated into the plant genome (cf. refs. 7, 17, and literature cited therein) and are involved in tumorigenesis, e.g. by forcing the cells to move on within the cell cycle and to divide perpetually. From studies on the importance of mevalonate derivatives on the transition from the late G1-phase to the S-phase (DNA synthesis) using mammalian cell lines, it can be concluded that in plant tumor cells similar processes take place (discussed in ref. 17). It is tempting to localize the action of BR somewhere to the same point within the cell cycle, as seems to be true with mevinolin as an inhibitor of mevalonate biosynthesis and with other xenobiotics such as aphidicolin, an inhibitor of DNA polymerase, or with compounds interfering with protein glycosylation (see Scheme 2). To finally prove this working hypothesis further studies are needed.

That the effect of BR is not simply due to the "oxidized" sterol structure is supported by our observation that oxysterols, which are strong inhibitors in mammalian cell cultures, were nearly inactive in our system. Again this points to



Scheme 2: A working hypothesis of how brassinosteroids might interfere with the cell cycle of tobacco tumor cells (see ref. 17 for a discussion of the effects of compounds other than brassinosteroid). Putative control points, where synthesis of mevalonate appears to be mandatory, are indicated.

the highly specific action of brassinosteroids on these undifferentiated cells. Based on the striking structural similarity between BR and the insect ecdysteroids, Meudt (5) suggested similar functions for brassinosteroids in plants as have steroidal hormones in animals, functioning as primary chemical messengers during early events of growth. Recently it has been demonstrated that BR (or its biosynthetic precursor castasterone) can compete with ecdysteroid for the binding site of ecdysteroid receptors of *Calliphora vicina* (16, see also elsewhere in this volume), thereby raising the question of whether this interaction did play a role in the co-evolution of plant and insect hormone systems. In our system only ecdysone, but not other ecdysteroids tested, exerted some inhibitory action. Although the numbers obtained for specific binding of radiolabeled ponasterone, a few hundred cpm, are not high, the data might indicate a further clue to the existence of BR-specific receptors in plant cells (see also Clouse et al., elsewhere in this volume). The availability of tritium-labeled BR (but only with an extremely high specific activity) could enable interested research groups with identifying the putative steroid hormone receptor(s) in plant cells. In any case the high specificity of the BR-response in our system, especially in view of the much less pronounced effect of further growth regulators tested (if visible at all) excludes unspecific membrane effects, that is, disturbances in membrane architecture, as might hold true with any lipophilic compound added in excess. It seems plausible to assume that we have observed a specific and exclusively hormonal action of BR on plant tumor cells. Thus, such cells might provide a further sensitive test system for BR-related growth regulators.

Literature Cited

1. Mitchell, J.W.; Mandava, N.B.; Worley, J.F.; Plimmer, J.R.; Smith, M.V. *Nature (London)* 1970 **225**, 1065-1067.
2. Grove, M.D.; Spencer, G.F.; Rohwedder, W.K.; Mandava, N.B.; Worley, J.F.; Warthen Jr., J.D.; Steffens, G.L.; Flippen-Anderson, J.L.; Cook Jr., J. *Nature (London)* 1979 **281**, 216-217.
3. Thompson, M.J.; Mandava, N.B.; Flippen-Anderson, J.L.; Worley, J.F.; Dutkey, S.R.; Robbins, W.E. *J. Org. Chem.* 1979 **44**, 5002-5004.
4. Thompson, M.J.; Mandava, N.B.; Meudt, W.J.; Lusby, W.R.; Spaulding, W.D. *Steroids* 1981 **38**, 567-580.
5. Meudt, W.J. in "Ecology and Metabolism of Plant Lipids"; Fuller, G.; Nes, W.D.; eds.; ACS Symposium Series No. 325, American Chemical Society: Washington, DC, 1987, pp. 53-75.
6. Mandava, N.B. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1988 **38**, 23-52.
7. Roth, P.S.; Bach, T.J.; Thompson, M.J. *Plant Sci.* 1989 **59**, 63-70.
8. Lehmann, M.; Koolman, J. *Mol. Cell. Endocrinol.* 1988 **57**, 239-249.
9. Lehmann, M.; Koolman, J. *Eur. J. Biochem.* 1989 **181**, 577-582.
10. Ries, S.K.; Wert, V. *Planta* 1977 **135**, 77-82.
11. Houtz, R.L.; Ries, S.K.; Tolbert, N.E. *Plant Physiol.* 1985 **79**, 357-364.
12. Houtz, R.L.; Ries, S.K.; Tolbert, N.E. *Plant Physiol.* 1985 **79**, 365-370.
13. Kandutsch, A.A.; Chen, H.W.; Heininger, H.-J. *Science* 1978 **201**, 498-501.
14. Bach, T.J.; Lichtenthaler, H.K. in "Ecology and Metabolism of Plant Lipids"; Fuller, G.; Nes, W.D.; eds.; ACS Symposium Series No. 325, American Chemical Society: Washington, DC, 1987, pp. 109-139.
15. Roth, P.S.; Bach, T.J.; Thompson, M.J. in "Braunschweig Symposium on Applied Plant Molecular Biology", Proceedings; Galling, G. ed.; Publisher: Zentralstelle für Weiterbildung der Technischen Universität Braunschweig, F.R.G. 1989, pp.205-208.
16. Lehmann, M.; Vorbrodtt, H.-M.; Adam, G.; Koolman, J. *Experientia* 1988 **44**, 355-356.
17. Bach, T.J. *Plant Physiol. Biochem.* 1987 **25**, 163-178.

RECEIVED April 1, 1991

Chapter 16

Stimulation of Growth Induced by Brassinosteroid and Conditioning Factors in Plant-Cell Cultures

Daniela Bellincampi¹ and Giorgio Morpurgo²

¹Dipartimento di Biologia Vegetale, Università degli Studi "La Sapienza",
Rome, Italy

²Istituto di Biologia Cellulare, Università di Perugia, Perugia, Italy

The multiplication of plant cells in culture is strongly dependent on cell density. To obtain division of single cells in culture conditioning factors (CF) produced by cells themselves must be present in the medium. We have demonstrated that different classes of CF are produced by cells. We have previously isolated and partially characterized, one class of CF that stimulate growth in diluted carrot cell suspensions. The effect of CF on the increase of plating efficiency can be mimicked by 24-epibrassinolide (BR). However BR, unlike CF, induces cell enlargement and its action is synergistic with CF. Experiments on cross-conditioning between different species have demonstrated that interspecific conditioning is more effective than the intraspecific one.

When some years ago we started researching plant cell cultures, our aim was not to study the factors inducing cellular proliferation, but to utilize plant cells as a tool to study the mechanism of differentiation.

In order to study differentiation processes, it was necessary to apply methodologies on single-cell cloning to select and culture single cells individually.

We soon discovered that the research we had planned was impossible or at least extremely difficult because of the extremely low plating efficiency of plant cells. Even using carrot cells, one of the best species for this kind of research, at rather high cell densities (2000–1000 cell units/mL) the plating efficiency was lower than 1% (1).

The multiplication of isolated plant cells in culture needs the presence of elusive conditioning factors (CF) secreted into the medium by cells themselves (2,3,4,5).

The inability of cells to grow at low population densities could be caused by an excessive diffusion of conditioning metabolites, which don't reach the levels required for survival in the medium (6).

Several techniques for recovering colonies from single cells or cells plated at suboptimal density require that the medium be conditioned. The conditioning was obtained by growing single cells in a reduced volume of culture medium, i.e., microdrop or microchamber culture (7,8) or by exposure of the single cells to the nurse effect of high-density cell suspensions (9,10,11). Attempts to isolate and identify conditioning factors have been less successful.

Kao and Michayluk (12) obtained a good growth with *Vicia hajstana* at an initial population density of 1–2 cells/mL when the mineral salt medium was enriched with organic acids, sugar, sugar alcohols and indefinite supplements such as coconut water and casamino acids. They assumed that an excessive diffusion of metabolic intermediates into the culture medium was responsible for the inability of plant cells to grow at very low densities. Sargent and King (13) found that cultures of soybean root cells conditioned the medium in which they were growing; the conditioning lessened the apparent ammonium requirement for rapid growth but, although the amount of NH_4 in the medium or in the cells is an important factor in conditioning, its presence alone is not sufficient to induce the process. The authors suggested that amides such as glutamine and its derivatives could be involved in the conditioning processes. These observations, however, could be applied only to a limited number of species, while the conditioning process is a well-known general phenomenon.

Street and his group (5,14) have demonstrated in sycamore cells that the medium, conditioned by dense, stationary-phase cultures separated by a dialysis membrane, promoted growth of cells at suboptimal densities. Although a medium containing gibberellic acid and amino acids (corresponding to those detectable in a conditioned medium), reduced the minimum cell density required for the growth from 10×10^3 cells/mL to 2×10^3 , these compounds were less effective than the conditioned medium that reduced the initial cell density to 1×10^3 cells/mL. The failure of the conditioned medium to promote growth at lower cell densities and the evidence of beneficial effects obtained by exposing a conditioned, low-density culture to the atmosphere of an actively growing culture suggested to them that some volatile product(s) must be required to initiate single-cell division. Subsequent work (15) has shown, both in experiments with low-density suspensions and low-density platings of cultured sycamore cells, that carbon dioxide was essential to the initiation of growth. They demonstrated that the cells were able to fix supplied carbon dioxide into organic and amino acids, but attempts to replace the carbon dioxide growth-promoting effect by these metabolites have not been successful. The authors noticed that this CO_2 growth promotion could be further increased by simultaneous application of ethylene (5); however, after their failure to reproduce the promotive effect of the volatile(s)

released from the actively growing culture, they concluded that other unknown volatiles might be involved in the conditioning processes.

Undiscouraged by the failure of the previous attempts to isolate and determine the nature of the CFs, we shifted our interests into a new attempt to identify these compounds.

In order to detect the effect of conditioning on growth, the stimulation of multiplication on carrot cell low-density cultures was utilized as bioassay. Our initial approach has been simply to repeat, with minor modification, the experiments of Street (5) and we confirmed that a CF is a molecule of low molecular weight that can diffuse through a dialysis membrane.

We have isolated a crude CF preparation produced by carrot cells after dialysis against distilled water (16). At variance with the results of Street on the volatile nature of conditioning factors, we have demonstrated that CF(s) isolated from carrot cultures could be lyophilized and remain indefinitely active if stored at -20°C . This finding is obviously useful, because it allows fractionation of the crude preparation to find an active fraction and possible identification of the chemical structure.

We demonstrated that CF is strongly water soluble (Table 1), thus it is unlikely that CF(s) is (are) the more lipophilic factor(s) responsible for the promotion of callus formation from barley anther cultures (17). CF is resistant to boiling and to acidic and alkaline pH treatment (Table 2).

From Sephadex G-10 and G-15 gel filtration, the molecular weight of CF was calculated as about 700 daltons (16). To obtain further information about the chemical nature of CF, the active CF fraction, isolated after Sephadex G-10 gel filtration, was treated with different enzymes. Enzymes proteinase K, cellulase, α and β amylase did not reduce the CF activity (data not shown).

Our results regarding chemical characterization of CF(s) were subsequently confirmed by other authors studying other biological systems. Schröder et al. (18) isolated and determined some properties of viability factors (VFs) from tobacco cells when using colony growth at suboptimal density as bioassay. These VFs shared many chemical properties with CF(s). Birnberg et al. (19) isolated and partially characterized conditioning factor(s) from a medium conditioned by *Zea mays* L. protoplasts (CMF). CMF was very hydrophylic, stable after boiling, and had an apparent molecular weight of ca. 1200 daltons.

The CF biological effect on growth stimulation was detected also in dispersed cell culture, in which the contact between cells is avoided by moving liquid culture. It is known that in suspension culture the growth rate is different, with respect to the solid culture one, because cells are subjected to different factors influencing growth, such as metabolite diffusion into the medium or aeration. CF stimulated cell multiplication of diluted cell suspensions without an evident effect on cell enlargement (Table 3).

Table 1. Increase of plating efficiency by CF and CF after water/diethyl ether repartition. Ten milliliters of agar culture medium were inoculated with 1×10^3 cell units/ml and were incubated for 20 days at 25 °C. Mean value from three experiments and S.E. are reported. (Reproduced with permission from reference 16. Copyright 1987 Elsevier.)

Control	CF	CF after water/ether repartition	
		Aqueous phase	etheric phase
0.25±0.10	2.60±0.30	1.90±0.25	0.21±0.08

Table 2. Stability of CF under different experimental conditions. Ten milliliters of agar culture medium with or without CF were inoculated with 1000 cell units/ml and were incubated for 20 days at 25 °C. Mean value from three experiments and S.E. are reported. (Reproduced with permission from reference 16. Copyright 1987 Elsevier.)

Treatment	Plating efficiency %		
	Control	CF	Increase
After dialysis	0.20±0.22	1.16±0.05	5.80
Lyophilized	0.25±0.07	1.10±0.12	4.40
pH 4 (1 day)	0.25±0.05	1.06±0.13	4.24
pH 11 (1 day)	0.25±0.05	1.14±0.19	4.56
25°C (1 day)	0.30±0.06	1.74±0.18	5.80
4°C (1 day)	0.30±0.06	1.34±0.14	4.46
100°C (15 min)	0.30± 0.06	1.70±0.09	5.66

Table 3. Effect of CF on cell suspension cultures. 1000 cell units/ml were inoculated in each 2 ml sample. After 10 days of growth, the packed cell volume was determined. (Reproduced with permission from reference 16. Copyright 1987 Elsevier.)

	Cell number $\times 10^5$	Packed cell volume ml	Mean cell volume
Control	4.90±0.30	0.15±0.01	1.00±0.05
CF	11.60±0.37	0.37±0.02	1.03±0.09

One or More Conditioning Factors?

One important question is the following: Is the CF sufficient to stimulate the multiplication of single cells in culture? Some observations convinced us that the factor we have isolated is necessary but is not sufficient to ensure the cellular multiplication of a single cell in culture.

The crucial observation is the following: If we plate carrot cells at increasing dilutions (i.e., 1000, 500, 250 cell units/mL) in the presence and absence of CF (Table 4) the plating efficiency decreases, even if in the presence of CF it remains higher than in control cultures. This effect was evident regardless of the concentration of the added CF (20). Also in experiments carried out on low-density-plating carrot cells with CFs produced from different species (20), the plating efficiency is smaller at lower cell density. These results on positive effect of heterologous CFs extended earlier findings on interspecificity of conditioning factors activity (21). One possible interpretation of the failure to obtain with CF(s) high plating efficiency at lower cell density is that CF(s) is necessary to ensure the multiplication of the cells but it is not sufficient. Evidently the cells receive, by the other cells present in the medium, a second signal that is necessary for cell division.

When the cells are too dilute, they can no longer communicate with each other and the plating efficiency dramatically drops. Therefore, a second conditioning factor, not present in our extracts, must exist so that we did not obtain single-cell division.

Is Brassinolide the Second Conditioning Factor?

One possibility that we have taken into account is that brassinosteroids, a widely distributed new class of plant hormones with positive effects on growth in different biological systems (22,23,24), could be active in low-density cell growth. If brassinosteroids are produced by carrot cells in culture, they could be the postulated second CF essential for cell proliferation.

CF preparation was isolated after dialysis against water; the low water solubility of brassinosteroids suggested that it was unlikely that these compounds were present at significant concentration in our aqueous preparation.

Very few papers have been published on the activity of brassinosteroids in plant cell cultures (25,26). It was therefore of interest to investigate one of these compounds in our system. We have tested 24-epibrassinolide (BR), a synthetic epimer of brassinolide, with our carrot cell system.

On low-density-plated carrot cells BR induced an improvement of plating efficiency (Table 5) and an enlargement of cells (27). In improving plating efficiency at low cell density, BR has shown a conditioning factor-like effect. Enlargement of cells was strongly evident in liquid culture but BR did not show a conditioning-like effect because it was unable to

stimulate cell division (Table 6). These results confirmed the data obtained by Sala and Sala on carrot cells in culture (25).

One possible explanation of this different effect on growth in relation to different culture conditions could be that in solid medium when cells are induced to form colonies, the effect on cell multiplication could be determined not by BR but by other cell metabolites (i.e., conditioning factors); their production could be stimulated by BR and their accumulation around the colonies could be the factor(s) responsible for growth.

In solid medium, the conditioning factors produced by the colonies appear to diffuse in a concentration gradient that is higher in proximity to the colony. This phenomenon is not present in a shaken liquid medium where the rapid diffusion and dilution of these compounds could cause the consequent lack of any effect on multiplication.

The BR effect on growth was shown with other species. In *Nicotiana plumbaginifolia* L. we have obtained similar results for cell enlargement in liquid cell suspensions, no effect on multiplication, but stimulation of plating efficiency at the BR concentration of 1 μM .

To verify the possible interaction between BR and CF on growth, we have tested both compounds, at the optimal concentration, on carrot cells plated at different dilutions (Table 7). The compounds acted in a synergistic manner therefore demonstrating that the mechanism of action is different. It has been seen however that, even if BR improves the plating efficiency at all cell densities, it does not prevent its decrease with cell dilution; moreover the phenomenon remains unaltered when BR and CF were added together to culture medium. In conclusion, BR is not the second conditioning factor necessary for growth of single cells.

We may wonder why BR increases the plating efficiency and why its effect is additive to the CF one. We think that, the increase of plating efficiency could be dependent on the increase in the volume of the cells. It is likely that the enlarged BR-treated cells, could excrete a larger amount of the conditioning factors. The synergistic effect could be determined by hyperproduction and accumulation near the colonies of auto-genous CF. These experiments on liquid cell cultures, which demonstrate that there is no direct effect on cell multiplication, are in agreement with the explanation here reported.

The Second Factor Is Also Water Soluble and Dialyzable. Following the failure of previous attempts to overcome the decrease in the plating efficiency caused by cell dilution, we have examined the effect of the direct contact of the callus with the cells seeded in solid medium.

In this kind of experiments, we used the following technique: A nurse callus was placed in a glass ring closed at the bottom, with a dialysis membrane. The glass ring was placed on the surface of a layer of solid medium in which diluted cells were previously dispersed (Figure 1). After 20 days of growth, the colonies were counted in the area under and outside the conditioning ring (Table 8). By using this technique, we noticed that two different kinds of conditioning effects are present: One

Table 4. Plating efficiency of carrot cells as influenced by CF treatment and cell density. (Reproduced with permission from reference 20. Copyright 1989 Elsevier.)

Cell units/ml	Control	CF
1000	3.15±0.35	6.65±0.58
500	0.86±0.12	2.87±0.38
250	0.40±0.11	1.15±0.31

Table 5. Effect of BR on cell growth. Four milliliters of culture medium containing 1% agar were inoculated with 1000 cell units/ml, were poured into 5 cm Petri dishes and were incubated 20 days at 25 °C. Mean value from five experiments and S.E. are reported.

BR (10^{-6} M)	Plating efficiency %
0	1.2±0.3
0.01	1.5±0.35
0.1	2.8±0.25
1.0	5.9±0.21
10.0	5.7±0.25

Table 6. Effect of BR on cell growth in liquid condition. 1000 cell units/ml were inoculated in each 2 ml samples. After 10 days of growth, the packed cell volume and the mean cell volume were determined. (Reproduced with permission from reference 27. Copyright 1988 Elsevier.)

BR 10^{-6} M	Cell number $\times 10^5$	Packed cell volume ml	Mean cell volume
0	4.60±0.43	0.148±0.012	1.00±0.04
0.1	4.09±0.11	0.227±0.016	1.77±0.08
1.0	4.72±0.28	0.310±0.026	2.09±0.08

Table 7. Effect of CF and BR on plating efficiency at different cell densities. Aliquots (0.2 ml) of CF corresponding to 2 ml of the original volume and BR (1×10^{-6} M) were tested. (Reproduced with permission from reference 20. Copyright 1989 Elsevier.)

Cell units/ml	Control	CF	BR	CF+BR
1000	1.20±0.15	2.98±0.95	3.65±0.57	9.87±0.62
500	0.97±0.26	2.06±0.53	3.48±0.42	8.15±1.81
250	0.20±0.35	0.70±0.13	2.26±0.56	4.45±0.52

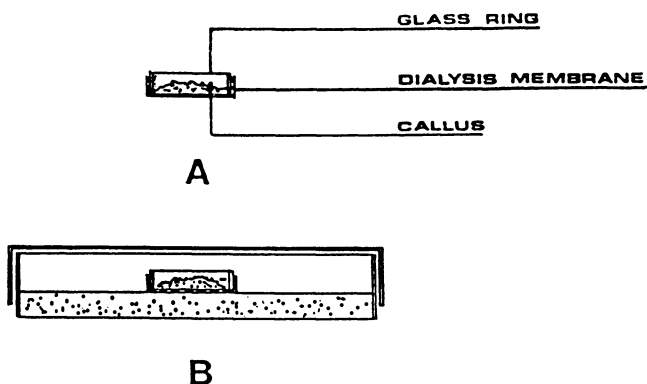


Figure 1. Callus conditioning method. A: a piece of callus, of about 100 mg fresh weight, is placed into a glass ring (22 mm diameter) previously sealed on the bottom by dialysis membrane. B: the ring is placed on the surface of the inoculated agar medium.

Table 8. Plating efficiency of carrot cells at different cell densities in the presence of conditioning carrot callus. After 20 days of growth, the glass rings containing callus were removed and the plating efficiency, under and outside the rings, were determined. (Reproduced with permission from reference 20. Copyright 1989 Elsevier.)

Cell units/ml	Plating efficiency%		
	Control	Under nurse callus	Outside nurse callus
1000	5.05±0.87	22.75±1.15	6.58±0.65
500	0.98±0.16	20.90±1.00	3.37±0.70
250	0.21±0.38	19.48±1.06	1.03±0.28
125	0.17±0.10	26.05±2.59	0.56±0.19

that is much more evident under the ring and the other that is spread all over. The plating efficiency in the area under the conditioning ring was always extremely high and, more important, absolutely independent from the cell dilution of the plated cells. Outside the ring, on the contrary, the plating efficiency stimulated by the nurse cells decreases in the usual way with cell dilution.

By using induction by nurse cells, the independence of the plating efficiency by cell concentration was strictly limited to the area in direct contact with the nurse cells.

The most likely explanation is that callus excretes a second factor that cooperates with CF in stimulating cell proliferation. We may ask why this factor, which is dialyzable, is not present in our CF preparation and why it does not migrate into the medium. It is unlikely that this factor is a volatile compound, which has been suggested as CF in *Acer pseudoplatanus* L. cultures (5). The effect of a volatile, in fact, should spread over the whole dish; although in our experiments, it was strictly limited in the area under the nurse cells.

There are, therefore, different explanations:

- The second factor migrates very slowly
- It spontaneously decays very rapidly after its production
- It is immediately and actively destroyed by the cells that it stimulates.

We have demonstrated that the first and second hypothesis are probably false. In another experiment, the rings containing nurse callus have been separated with agar blocks of different thickness (0.25 mm, 0.5 mm, 0.75 mm) from the layer in which the cells were seeded. However the conditioning was successful and independent of the cell concentration, a fact demonstrating that the second factor diffused without degradation along the blocks of agar (data not shown). Our hypothesis is that this second conditioning factor is rapidly degraded by cells that it stimulates.

Some Results Utilizing Interspecific Cross-Conditioning. It is well known that the phenomenon of conditioning is not species specific. This fact was ascertained first by Benbadis (21) and successively confirmed by other authors (11,28,29).

In general, few attempts have been made to examine if the characteristics of the interspecific conditioning are the same as the intraspecific one. We have experimented on cross-conditioning to measure the efficiency of interspecific and intraspecific conditioning.

In this work (Bellincampi, D. and Morpurgo, G., unpublished data), we have used, with the nurse technique previously described, three different species (carrots, tomatoes, and potatoes) plated at low density and conditioned by nurse cells of the same or of nine different species.

The results of these experiments are the following:

- Under the ring, the plating efficiency was in all cases, whether by intra- or interspecific conditioning, very high and was independent of the density of the plated cells.
- In the area outside the ring, in the vast majority of cases, the interspecific conditioning was more efficient than the intraspecific one.
- The plating efficiency outside the ring was, with intraspecific conditioning, strongly dependent on dilution of the plated cells, while the situation was more variable with interspecific conditioning. In some cases of interspecific cross-conditioning, the plating efficiency was independent of the cell density of the plated cells in the area outside the ring. In these last cases, the independence of plating efficiency on cell density was not related to the potency of the conditioning effect.

The most likely explanation of these results is that in these cases the second conditioning factor may migrate outside the limits of conditioning rings without being degraded. One possibility is that the various species produce different varieties of CFs that are able to stimulate the cellular proliferation. The heterospecific factors, according to the hypothesis, should be degraded at lower rates by interspecific plated cells.

Although the final structures of CFs are unresolved, our results support the working hypothesis that at least two interacting factors must be involved in the conditioning processes and that various plant species can produce different CFs with interspecific activity.

We conclude that the conditioning seems to be a very complex and not yet well known phenomenon in which hormones, such as brassinolide, may have a synergistic effect with conditioning factors.

Literature Cited

1. Bellincampi, D.; Babudri, N.; Morpurgo, G. *Plant Cell Reports* 1985; 4,155.
2. Muir, W. H.; Hildebrandt, A. C.; Riker, A. J. *Science* 1954; 119,877.
3. Torrey, J. G. *Proc. Acad. Sci. USA* 1957; 43,887.
4. Reinert, J. *Nature* 1963; 200,90.
5. Street, H. E. in: *Plant Tissue and Cell Culture* Blackwell Scientific Publications Melbourne 1973 p:191.
6. Ham, R. G. *Proc Acad:Sci: USA* 1965: 53,288.
7. Eigel, L.; Knoop, H. V. J. *Plant Physiol.* 1989; 134,577.
8. Jones, R. B.; Hildebrandt, A. C.; Riker, A. J.; Wu, J. H. *Am. Journal of Botany* 1960; 47,468.
9. Horsch, R. B.; Jones, L. E. *In Vitro* 1980;16(2),103.

10. Mottley, J.; Sybenga, J. *Plant Cell Rept.* 1988;7,193.
11. Cella, R.; Galun, E. *Plant Science Letters* 1980;19,243
12. Kao, K. N.; Michayluk, M. R. *Planta* 1975; 126,105.
13. Sargent, P.A.; King, J. *Can. J. Botany* 1974; 52,1747.
14. Stuart, R.; Street, H. E. *Journal of Exp. Botany* 1971; 22,96.
15. Gathercole, R. W. E.; Mansfield, K. J.; Street, H. E. *Physiol. Plant.* 1976; 37,213.
16. Bellincampi, D.; Morpurgo, G. *Plant Science* 1987; 51,83.
17. Köhler, F.; Wenzel, G. *J. Plant Physiol.* 1985; 121,181.
18. Schöeder, R.; Gärtner, F.; Steinbrenner, B.; Knoop, B.; Beiderbeck, R. *J. Plant. Physiol.* 1989; 135,422.
19. Birnberg, P. R.; Somers, D. A.; Brenner, M. L. *J. Plant Physiol.* 1988; 132,316.
20. Bellincampi, D.; Morpurgo, G. *Plant Science* 1989; 65,125.
21. Benbadis, A. in: *Les Cultures Des Tissus De Plantes* Paris, 1968 p.121.
22. Mitchell, J. W.; Mandava, N.; Worley, J. F.; Plimmer, J.R.; Smith, M. V. *Nature* 1970; 225,1065.
23. Adam, G.; Marquardt, V. *Phytochemistry* 1986; 25, 1987.
24. Thompson, M. J.; Muedt, W. J.; Mandava, B. N.; Sampson, R. D.; Lusby, W. R.; Spaulding, D. W. *Steroids* 1981; 39,89.
25. Sala, C.; Sala, F. *Plant Cell Reports* 1985; 4,144.
26. Roth, P. S.; Each, T. J.; Thompson M. J. *Plant Science* 1989; 59,63.
27. Bellincampi, D.; Morpurgo, G. *Plant Science* 1988; 54,153.
28. Vardi, A.; Raveh, D. *Z. Pflanzenphysiol. Bd.* 1976; 78,350.
29. Hane, B.; Lorz, H.; Hane, G. *Plant Cell Reports* 1990; 8,590.

RECEIVED August 1, 1991

Chapter 17

Rice–Lamina Inclination, Endogenous Levels in Plant Tissues and Accumulation during Pollen Development of Brassinosteroids

Hiroshi Abe

Department of Plant Protection, Tokyo University of Agriculture and
Technology, Fuchu, Tokyo 183, Japan

Search for brassinosteroid [BS] by using the bioassay system of rice-lamina joint test [LJT] demonstrated that BS was present in all parts of plants. BS content in vegetative tissue was extremely low compared with those of known phytohormones, but that in plant male gametophyte (pollen) tissue was exceptionally high. Enrichment of the reproductive tissue with BS, especially typhasterol is enigmatic. Change in the levels of the BS activity was observed during maturation of pollen. Through the measurement of the rate of rice lamina-inclination, a certain difference was observed between brassinolide and IAA.

Since the discovery of brassinolide, ten years ago, research on brassinolide and related steroids has attained a remarkable degree of development. So far, twenty-three brassinosteroids [BS] have been isolated and identified from twenty-two plant species. Furthermore, many unknown brassinolide-like active substances have been detected, so that the number of identified ones is likely to increase in the future. The background to successful isolation of new BS from various plant sources is due to the use of a rice-lamina inclination test for screening that is sensitive and specific for brassinosteroids.

In this paper, I will describe:

- (1) Search for naturally occurring brassinosteroid and endogenous levels in plants.
- (2) Brassinosteroid accumulation during development of plant male gametophyte.
- (3) Brassinosteroid-induced rice-lamina inclination response.

1. Search for Naturally Occurring Brassinosteroid and Endogenous Levels in Plants.

We are able to readily search for naturally occurring brassinosteroid using rice-lamina joint test [LJT] (1) as the bioassay method and GC/MS as the microanalytical method, which was established by Ikekawa and his co-workers as a microanalytical method for BS (2). Plant material was extracted three times in methanol or 80% methanol-H₂O. After the extract was evaporated under reduced pressure, the resulting aqueous residue was extracted with EtOAc at pH3 and the EtOAc fraction was partitioned with Na₂CO₃-NaHCO₃ buffer at pH10 to get neutral substances.

The neutral EtOAc-soluble substances after evaporation was then purified on a small aluminum oxide (10g, Merck Aluminum oxide 90, II-III, 70-230mesh) which was used to build a small column in a glass syringe barrel (i.d. 1.8cm, length 3.5cm). The sample was dissolved in 10ml of EtOAc-*n*-hexane (4:1, v/v) and loaded onto the column, which was washed with an additional 40ml of the same solvent system. After washing, 50ml of EtOH-EtOAc (4:1, v/v), which elutes the BS, was passed under pressure through the column. The BS eluent was dissolved in 80% methanol-H₂O (20ml) and passed under pressure at 2-3ml/min through the first C₁₈-reversed-phase Bond Elut cartridge column (size 6cc, Analytichem International). The column then was washed with an additional 20ml of 80% methanol-H₂O. An additional 24ml of water was added to the combined eluates of 80% methanol and the resultant 50% methanol solution was passed through second C₁₈-Bond Elut column.

The second column was then washed with the same solvent of 20ml. Subsequent elution of the column with 20ml of 100% MeOH eluted BS retained on the column and was taken to dryness under reduced pressure, from which aliquots could be taken for thin-layer chromatography (Kieselgel 60F₂₅₄, 0.25mm thickness, Merck). Thin-layer chromatogram, which was developed with EtOAc/EtOH (22:3, v/v) solvent system and development distances of 15cm, was divided into fifteen equal zones and after elution with a suitable solvent, each eluate was taken for bioassay. BS standards are separated on the TLC developed with the same solvent system and the *R_F* values are: norbrassinolide, 0.22; brassinolide, 0.24; homobrassinolide, 0.27 (lactone group); brassinone, 0.32; castasterone, 0.35; ethylbrassinone, 0.38 (ketone group); typhasterol, 0.51; teasterone, 0.50 (2-deoxyketone group). They appear in the range of *R_F* 0.2 to 0.5 and it is readily distinguished by the *R_F* values between the lactone group and the ketone group, and also between the ketone group and the 2-deoxyketone group. Thus, TLC should be favorable for making preliminary estimation of endogenous BS in plants. However, TLC is no longer distinguishable from BS with the structural variation within the same group. The problem could be solved by the use of HPLC. The crude BS fraction after purification on the Bond Elut column was then chromatographed on a reversed-phase C₁₈ Develosil HPLC column (i.d. 4.6mm x 25cm) eluting with CH₃CN-H₂O

(75:25, v/v) or $\text{CH}_3\text{CN-H}_2\text{O}$ (45:55, v/v). Each fraction was then taken to dryness, from which an aliquot was bioassayed by LJT. The active fractions were combined and analyzed by GC/MS as the bismethaneboronate and/or methaneboronate trimethylsilyl ether derivatives.

The LJT bioassay, coupled with the microanalytical techniques using GC/MS, led to the identification of new congeners of brassinosteroid with structural variations from diverse plant species and plant tissues, such as some stereoisomers of hydroxyl- and alkyl-groups in the A-ring and side chain, as shown in Table I. The amount was less than one microgram per kilogram fresh weight, indicating that the amount was quite small. Furthermore, many unknown BS-like active substances have been detected, so that the number of identified BS is likely to increase in the future. Thus, search for BS has demonstrated that BS is present in all parts of vegetative tissues of plants, that the amounts in the tissues were less than one μg in one kilogram fresh weight, so it was extremely low as compared with those of known phytohormones, and also that endogenous BS differs among plant species and plant tissues.

2. Brassinosteroid Accumulation during Development and Growth of Plant Male Gametophyte.

Pollen is shown to be a rich source of plant growth substances. When bioassayed by the LJT, an extract from the plant male gametophyte (pollen) were found to contain an exceptionally high content of BS. The 80% acetonitrile-water-soluble fraction obtained from a methanol-chloroform extract of *Lilium longiflorum* pollen was washed with *n*-hexane, and separated into its neutral and acidic fractions. As described above, the neutral fraction was purified on an aluminum adsorption column and C_{18} Bond Elut cartridge column. The active fraction was subjected to reversed-phase C_{18} Develosil HPLC, using the acetonitrile-aqueous mobile phase. By HPLC, three fractions with quite high biological activity were separated. Each fraction was analyzed by GC/MS after converting to the methaneboronate and/or methaneboronate trimethylsilyl ether by the method mentioned above. The main BS found in lily pollen were typhasterol (2-deoxycastasterone), castasterone and brassinolide. We also identified endogenous BS from the pollen of several kinds of plants, that is, typhasterol, teasterone, castasterone and brassinolide in *Citrus unshiu* (orange); typhasterol and teasterone in *Typha latifolia* (cat tail); typhasterol in *Tulipa gesneriana* (tulip); castasterone in *Thea sinensis* (green tea). The amount of typhasterol, which is a main BS in pollen of orange, tulip, lily, cat tail, was one hundred μg or more in one kilogram fresh weight of lily pollen. Thus, the plant reproductive tissue, especially plant pollen, was confirmed to be one of the richest sources of BS compared with that of plant vegetative tissue. This fact suggests that BS possesses a physiological function in the

Table I. Amounts of Brassinosteroids in Higher Plants

Brassinosteroid	Isunoki (ng/kg)	Green tea (ng/kg)	Cabbage (ng/kg)	Rice plant (ng/kg)	Mung bean (ng/kg)
I	156	-	1.3	-	-
II	23	4.6	9.4	trace	id.
III	-	-	trace	-	-
IV	16	2.0	0.78	-	-
V	133	110.0	1.6	13.6	id.
VI	-	trace	0.13	-	-
VII	-	-	-	8.4	-
VIII	-	id.	-	-	-
IX	-	id.	-	-	-

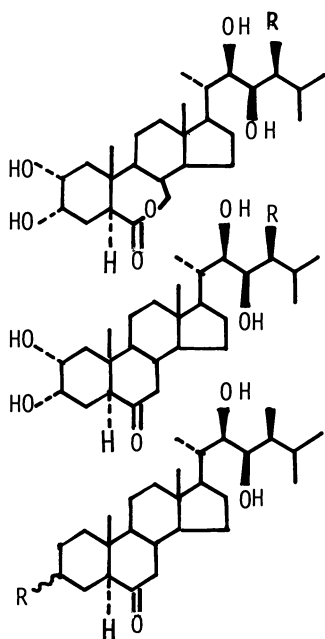
id. : identified but not quantified.

Isunoki : Leaves (3); Green tea : Leaves (4)

Cabbage : Immature seeds and sheaths (5)

Rice plant : Shoots (6)

Quantification data presented are underestimates due to losses encountered during sample purification.



Norbrassinolide (R=H, I)

Brassinolide (R=CH₃, II)

Homobrassinolide (R=CH₂CH₃, III)

Brassinone (R=H, IV)

Castasterone (R=CH₃, V)

Ethylbrassinone (R=CH₂CH₃, VI)

Dolichosterone (R=CH₂, VII)

Typhasterol (R=α-OH, VIII)

Teasterone (R=β-OH, IX)

regulation of reproductive growth and also pollen is a very useful material for studying the biosynthesis of BS.

Liliaceous plant exhibit a close relationship between the flower bud length and the developmental stage of pollen (7). Pollen of *Lilium longiflorum* cv. Georgia was grouped and collected into six classes, among developmental stages from an immature uninucleate pollen after meiosis, to a mature binucleate pollen after anthesis, based on the flower bud length. Using 500 of the male regenerative tissues from each stage, the BS fraction was separated and purified by a simple and rapid method, which consists of 5 steps, namely, methanol-chloroform extraction, acetonitrile-hexane partition, ethyl acetate-buffer partition (in order to separate neutral substances only), alumina column chromatography and C₁₈ Bond Elut cartridge column chromatography. An aliquot, equivalent to 0.5 male tissues, was taken for the LJT. Stage V, just before anthesis, had the greatest BS activity (Figure 1). The same change in the levels of BS activity was observed during the development of green tea (*Thea sinensis*) pollen. The active fraction of Stages I, V and VI was further purified by reversed-phase C₁₈ Develosil HPLC, to distinguish between two activities by typhasterol and by castasterone and/or brassinolide. An aliquot, equivalent to 10 male tissues, was applied to the bioassay. BS activity in the Stage I was due to a trace amount of typhasterol only, and that noted in Stages V and VI, composed of the combined activity of typhasterol and castasterone/brassinolide. The activity of both fractions was almost the same. As the relative activity of typhasterol is one tenth lower than that of castasterone, and one twentieth that of brassinolide in this bioassay system, typhasterol was detected at a very high concentration in the endogenous levels. The respective amounts of BS in the stages I, V and VI were estimated, by bioassay, to be approximately 22, 235 and 186 μg , respectively, in one kilogram fresh weight of the male reproductive tissue as shown in Table II. We also analyzed phytosterol in the male tissues of Stages I and VI. The main sterols in both stages were β -sitosterol and campesterol, but the ratio of campesterol to β -sitosterol was about 1:1 at Stage I, and was about 1:3 at Stage VI. The endogenous BS were found to change in the levels of quantity and quality during maturation of pollen. Enrichment of the lily's reproductive tissue with typhasterol is enigmatic but is useful for establishing the biosynthetic pathways and metabolic fates of typhasterol, castasterone and brassinolide in plants and also the relationship between endogenous BS levels and their physiological significance in plant growth regulation, especially in reproductive growth regulation.

3. Brassinosteroid-induced Rice-lamina Inclination Response

A rice-lamina joint is a very important tissue in regulating the inclination of the leaf blade. Brassinolide was ten thousand times higher in activity on lamina inclination than IAA. The reason

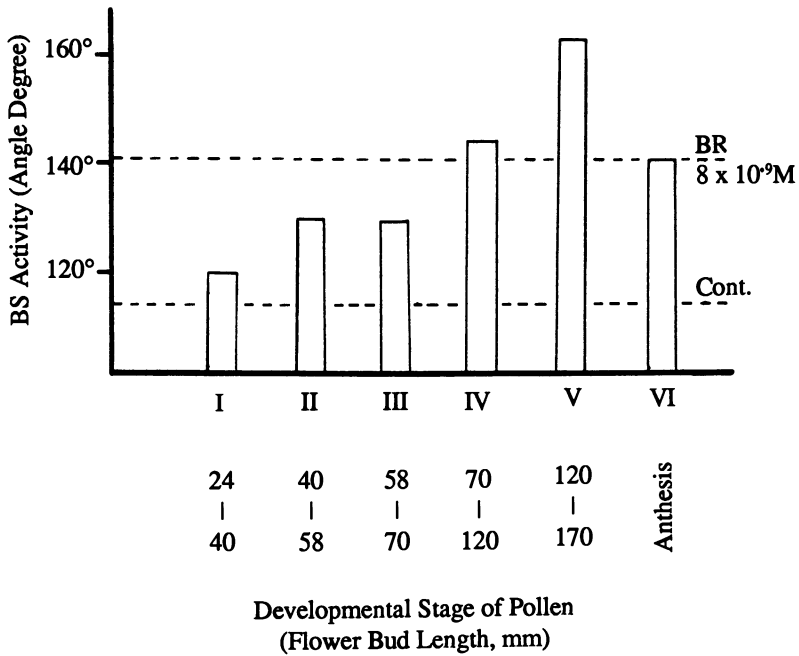


Figure 1. Change in the level of brassinosteroid activity during the development of pollen of *L. longiflorum* c.v. Georgia. Equivalent to 0.5 male tissues was taken for the LJT. BR: brassinolide.

Table II. Amount of Brassinosteroid in the Male Reproductive Tissue of *Lilium longiflorum* cv. Georgia

Developmental stage of pollen	Brassinosteroid Amount	
	Typhasterol (μ g/kg)	Castasterone-Brassinolide (μ g/kg)
Stage I	22	N.D
Stage V	210	25
Stage VI	180	6

Stages I,V and VI show developmental stages of an immature uninucleate pollen just after the meiosis, a mature binucleate pollen just before and after the anthesis, respectively. Fresh weight of each stage is 85.0, 178.4, and 95.5 mg in one male tissue, respectively. The BS amount was estimated from the biological activity in LJT of each active fraction after HPLC.

for the high specific response of rice-lamina joint cells to BS is still obscure. A rice-lamina joint section is composed of 2nd leaf blade, 2nd leaf sheath, 3rd leaf, lamina joint, auricle (meaning leaf ear), and ligule (meaning leaf tongue). The inclination at the lamina joint has been known to result from more expansion of the adaxial cells than that of the dorsal cells. On the other hand, a rice cultivar without the ligule, named Muzozetsu in Japanese, did not respond to brassinolide at the high concentration of 10^{-7} M, so, the ligule, being in contact with the adaxial cells, was found to be a physiologically important tissue in the lamina inclination. In order to select a suitable rice cultivar for micro-quantitative bioassay, we examined the sensitivity of about sixty types of rice cultivars. Eighteen cultivars were judged sensitive enough. Arborio (Italian cultivar), Kinmaze and Nihonbare (Japonica ones) were sensitive to BS and useful cultivars. Arborio, especially, grew the tallest, about 14cm, and it was easy to cut segments from these seedlings. In general, we have used the Arborio cultivar in screening tests of naturally-occurring BS and in micro-quantitative bioassay of BS. The correlation between the induced angles of the lamina joint and the concentration of brassinolide and castasterone was determined using Arborio and Nihonbare; a linear correlation was obtained between 5×10^{-8} and 5×10^{-5} μ g/ml. When measured every 90 minutes, angles of lamina joints induced by 6-ketone-type BS, that is, brassinone, castasterone and ethylbrassinone, and their corresponding lactones, norbrassinolide, brassinolide and homobrassinolide, increased linearly during the initial 24hr incubation, then gradually leveled off. The 6-ketone compounds showed no time lapse (lag phase) for their activity, when compared with their lactones. These results suggest that these 6-ketone compounds themselves affect the lamina joints without being converted to their respective lactones (δ).

Through the measurement of the rate of lamina-inclination, it is possible to observe a certain difference between brassinolide and IAA. An excised lamina-joint segment with the whole of the 2nd leaf blade was used in this experiment. The segment was floated on distilled water for 18 hours after cutting, and after was transferred to potassium maleate buffer solution, then pre-incubated for 2 hours, it was then charged to the test solution of BS and IAA for 4 to 5 hours under the conditions described above. The degree angle was measured at 15 minute intervals. The rate of lamina inclination was determined by the following equation;

$$\text{the rate} = (\text{An increased degree angle for 15 min. in test solution}) \div (\text{An increased degree angle for 15 min. in buffer solution for 2 hours}) \times 100.$$

The rate of lamina inclination induced by BS exhibits its maximum value after 120-180 minutes from the beginning of the treatment. The maximum rate induced by IAA in comparison to that by brassinolide occurs within 30 minutes after treatment begins. We must find out the physiological reason for encountering such difference in the rate of the lamina inclination between brassinolide and IAA.

Conclusion

1. BS were present in all parts of vegetative and reproductive tissues at a extremely low concentration, and endogenous BS differed from plant species and plant tissues.
2. Plant mature-pollen was rich in the BS level and endogenous BS was actively produced and accumulated in pollen during the growth and development. The role of brassinosteroid in pollen is unknown, but may be important in fertilization.
3. Rice-lamina joint inclination response was highly sensitive and specific for BS. The expansion of adaxial cells at lamina joints is considered to be significant physiological functions of BS, but the mechanism is still obscure.

Literature Cited

1. Maeda, E. *Physiol. Plant* 1965, 18, 813.
2. Takatsuto, S.; Ying, B.; Morisaki, M.; Ikekawa, N. *J. Chromatogr.* 1982, 239, 233.
3. Ikekawa, N.; Takatsuto, S.; Kitsuwa, T.; Saito, H.; Morishita, T.; Abe, H. *J. Chromatogr.* 1984, 290, 289.
4. Abe, H.; Morishita, T.; Uchiyama, M.; Takatsuto, S.; Ikekawa, N. *Agric. Biol. Chem.* 1984, 48, 2171.
5. Abe, H.; Morishita, T.; Uchiyama, M.; Marumo, S.; Munakata, K.; Takatsuto, S.; Ikekawa, N. *Agric. Biol. Chem.* 1982, 46, 2609.
6. Abe, H.; Nakamura, K.; Morishita, T.; Uchiyama, M.; Takatsuto, S.; Ikekawa, N. *Agric. Biol. Chem.* 1984, 48, 1103.
7. Tanaka, I.; Ito, M. *Plant Sci. Lett.* 1980, 17, 729.
8. Wada, K.; Marumo, S.; Abe, H.; Morishita, T.; Nakamura, K.; Uchiyama, M.; Mori, K. *Agric. Biol. Chem.* 1984, 43, 719.

RECEIVED May 1, 1991

Chapter 18

Influence of Brassinosteroids on Organ Relations and Enzyme Activities of Sugar-Beet Plants

Günther Schilling, Christiane Schiller, and Steffan Otto

Institut fuer Pflanzenernährung und Düngung, Martin Luther Universität
Halle-Wittenberg, Adam-Kuckhoffstrasse 17b, Halle/Saale, O-4020,
Germany

Sugar beet plants (variety Ponemo) were exposed to different levels of drought stress in pots or in Mitscherlich vessels. The water supply amounted to 80 % (=normal), 40-50 % (=mild stress) or 25-30 % (=high stress) of the maximum substrate water capacity. Plants were treated with 100 and 1000 mg/ha of (22 S, 23 S)-homobrassinolide (SSHB), at each stress level. The biomass of control plants was reduced in mild stress by 8 %, in high stress by 32 % in comparison with normally grown plants. Treatments with SSHB completely compensated for the effect of mild stress. Taproot mass invariably increased under stress conditions by 25 to 30 % when brassinosteroids were applied. Sucrose content, however, increased only in cases of high stress. Increases in biomass correlated with increases in acid invertase activity (EC 3.2.1.26) in young leaves which became larger and provided more assimilates to the plant.

Increased formation of taproot mass by application of SSHB correlated with increase in activity of acid invertases in these organs. The increased hydrolysis of sucrose may have generated more material for cell growth at the expense of sucrose accumulation. Increase in sugar accumulation elicited by brassinosteroids in high stress was related to changes in sucrose synthase activity (EC 2.4.1.3).

It was a very important discovery when, in 1979, a novel plant growth-promoting steroidal lactone, termed brassinolide, was found in rape pollen (1). After this observation, structurally similar brassinosteroids were discovered, and today we have congeners which are produced synthetically (2, 3). The function of these compounds is not clear, especially when the whole plant is considered. Therefore, the objective of this paper is to present information on the effects of SSHB (Figure 1) in sugar beet plants, and to discuss relations found between morphological effects and activities of some enzymes, following SSHB treatment.

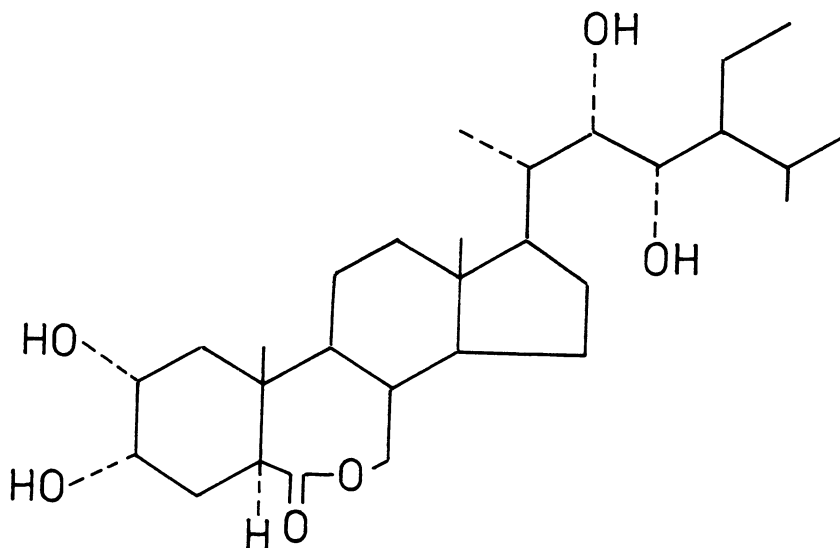


Figure 1 Chemical Structure of SSHB

Morphological effects of brassinosteroids.

Effects of brassinosteroids in short-time experiments.

Some years ago we began our experiments with radish plants in small plastic vessels. The pots were 45 mm high and 90 mm long. The substrate was quartz sand supplemented with a general nutrient solution. In each vessel 5 plants were cultivated. After the first true leaves had appeared an aqueous homobrassinolide solution was applied by spraying the leaves. Four weeks later, the plants were harvested, dried, measured and weighed. After certain experiments showed a small effect from homobrassinolide applications on root-, epicotyl- and total biomass, in the case of low water saturation, we carried out similar trials with sugar-beet plantlets (Table I).

Table I. Effect of SSHB applications to sugar-beet seedlings (cv. Ponemo)

Conditions: quartz sand with nutrient solution, 6 plants per vessel, SSHB applied during emergence of first foliar leaf, 6 to 7 week growth period, average of 3 experiments. SSHB dosage based on 100,000 plants ha⁻¹.

<i>Treatment (dose in mg/ha)</i>	<i>Leaf Dry Mass in mg/vessel</i>	<i>Root Dry Mass in mg/vessel</i>	<i>Length of the Longest Leaf in cm</i>
--	---------------------------------------	---------------------------------------	---

1. 90 % saturation of water capacity:

Control	767 (100)	168 (100)	9,5 (100)
SSHB(100)	(104)	(83)	(104)

2. 55 % saturation of water capacity:

Control	592 (100)	208 (100)	7,1 (100)
SSHB(100)	(111)	(119) ^{x)}	(112)
SSHB(1000)	(105)	(133) ^{x)}	(109)

x) significant in comparison with control
(t-test; $\alpha \leq 0,05$)

Under high water saturation no effects were visible, but in the case of 55 % saturation of water capacity, root dry matter was increased following homobrassinolide application. In contrast, the leaves and total biomass were not significantly different. The length of the longest leaf was greater, but not significantly so. Therefore, we concluded that SSHB increased the root growth only in the case of low water supply: an interesting fact and a new finding. However, this gives rise to the question as to whether the same observations would be made in the case of a longer vegetation period.

Effects of homobrassinosteroids on the yield of sugar-beet plants under drought stress. In order to investigate this problem we carried out experiments with Mitscherlich vessels using quartz sand as a substrate. Mitscherlich vessels were 200 mm high and had a capacity of 6 kg substrate. Two sugar-beet plants were grown in each pot and 6 replicates were prepared. Under drought stress the dry mass decreased considerably (Table II).

Table II. Effect of SSHB applications to sugar-beet seedlings grown in Mitscherlich Vessels

Conditions: Planted 10 May 1989, SSHB and 24-E (24-epibrassinolide) applied 12 June once (6 leaves), plants harvested 12 October (6 replicates), sucrose content determined polarimetrically

<i>Treatment (dose in mg/ha)</i>	<i>Dry Mass in g/vessel</i>			<i>Sucrose Content in °S</i>	<i>Sucrose Yield in g/vessel</i>
	<i>Total</i>	<i>Leaf</i>	<i>Root</i>		
<u>1. 75-80 % of maximum water capacity:</u>					
Control	65 (100)	31 (100)	28 (100)	14,7	23 (100)
SSHB(100)	(103)	(97)	(113)	14,3	(108)
SSHB(1000)	(110)	(107) ^{x)}	(111)	14,4	(112)
<u>2. 45-50 % of maximum water capacity (mild stress):</u>					
Control	60 (100)	28 (100)	25 (100)	15,1	21 (100)
SSHB(100)	(114) ^{x)}	(100)	(127) ^{x)}	15,0	(99)
SSHB(1000)	(116) ^{x)}	(98)	(130) ^{x)}	14,7	(105)
24-E(100)	(113) ^{x)}	(98)	(133) ^{x)}	14,7	(101)
<u>3. 25-30 % of maximum water capacity (high stress):</u>					
Control	45 (100)	22 (100)	16 (100)	10,2	7 (100)
SSHB(100)	(99)	(92)	(106)	11,3	(120)
SSHB(1000)	(104)	(93)	(125) ^{x)}	12,9 ^{x)}	(165) ^{x)}
24-E(100)	(101)	(94)	(111)	11,4	(125) ^{x)}

^{x)} significant in comparison with control (t test; $\alpha \leq 0,05$)

But the application of 100 or 1000 mg SSHB per ha compensated for this effect completely when the plants suffered only from mild stress. In all stress treatments homobrassinolide increased taproot mass. This result corresponded with the trend observed in the experiments with young plants.

The contents of sucrose - measured in degrees S - were not changed by mild drought stress or by homobrassinolide application. The sucrose content is referenced against fresh mass.

Because the percentage of dry mass increased as a consequence of mild drought stress and homobrassinolide application, we did not find an increase in sugar yield.

Therefore, the additional dry mass must have been composed of organic compounds other than sugar. In the case of high drought stress, application of homobrassinolide increased both the dry mass of tap-roots and the sugar contents in the fresh mass. Therefore, the sugar yield per vessel increased considerably. Similar effects are observed with (22 S, 23 S)-24-epibrassinolide. Hence, SSHB affects yields only in case of drought stress which means that the compound is able to reduce the damage caused by drought stress.

Table III: Effect of SSHB on sugar-beet total biomass and leaf acid invertase activity

Conditions: see Table II; Assay Procedures: The activity of acid invertase (free and bound) was determined in plant material that was frozen in liquid nitrogen, freeze-dried, pulverized, homogenized with mercaptoethanol, centrifuged at 25,000g for 30 min (4°C); solution and residue were dialyzed (48 h, 4°C) and then incubated with sucrose (pH 4.7, 1 h, 30°C) and glucose was analyzed electrochemically.

Treatment (dose in mg/ha)	Total Dry Mass in g/vessel	Activity of Acid Invertase in μmol , sucrose hydrolyzed 100 mg^{-1} dry mass h^{-1}	
		Young Leaves	Old Leaves

1. 75-80 % of maximum water capacity:

Control	65 (100)	22 (100)	8 (100)
SSHB(100)	(103)	(74)	(80)
SSHB(1000)	(110)	(89)	(85)

2. 45-50 % of maximum water capacity (mild stress):

Control	60 (100)	21 (100)	10 (100)
SSHB(100)	(114) ^{x)}	(133) ^{x)}	(70)
SSHB(1000)	(116) ^{x)}	(154) ^{x)}	(69)

3. 25-30 % of maximum water capacity (high stress):

Control	45 (100)	20 (100)	8 (100)
SSHB(100)	(99)	(109)	(96)
SSHB(1000)	(104)	(94)	(101)

^{x)} significant in comparison with control (t-test; $\alpha \leq 0,05$)

Analysis of effects

On the basis of these results the question arises as to how homobrassinolide produces its effects. A systematic analysis of each response follows.

Effects of brassinosteroids on the activity of acid invertases in leaves. The first question to be answered was why does SSHB increase the total biomass under mild stress conditions? It is known that rate of cell growth correlates with specific activity of acid invertase (4) and the role of acid invertase in the leaves of sugar-beet plants has been described (5). Table III shows total mass (from Table II) in relation to the activity of acid invertases.

An increase in total dry mass always corresponded with a rise in invertase activity in young leaves. The older leaves do not act in such a manner and there was no rise in invertase activity.

The next question to be considered was what relation exists between the total biomass of plants and the activity of acid invertases in young leaves? It seemed possible that this question could be answered by measuring these parameters shortly after homobrassinolide application. The results of experiments with young plants are given in Table IV.

The data show that SSHB increased the root dry matter in the same way it did in the other experiments. Simultaneously, we observed an increase in leaf length and leaf area which corresponded with an increase in invertase activity. The higher enzyme activity appeared to be the key to the whole problem. It is well known that acid invertase splits the disaccharide sucrose so that the two monosaccharides formed from it can become building stones for additional leaf substances. That is, glucose is recycled through the Embden-Meyerhof-Parnas pathway. Likewise, fructose is readily available for conversion into fructose 1,6-diphosphate. Subsequently, more leaf area arises which is able to produce more assimilates. The consequence is an increased yield. Thus, photosynthetic efficiency increases considerably.

Effects of brassinosteroids on the activity of acid invertase in tap-root. The next question to be considered was whether such a relationship may also be valid for increases in the amount of tap-root dry matter after application of SSHB. Table V shows these data for the roots. There is a close correlation between increases in root dry matter and the activity in acid invertase after application of SSHB. This may be

Table IV. Effect of SSHB on sugar-beet growth and acid invertase measured a short time after application

Conditions: see Table I, 6 weeks growth period, average of 5 experiments, mild stress; Assay Procedures: Activity of acid invertase, see Table III conducted 1 week after application

Treatment (dose in mg/ha)	Dry Mass in mg/vessel Total	Leaf	Root	Length of Leaves (average) in cm	Activity of Acid Invertase of Leaves in $\mu\text{mol sucrose hydrolyzed}$ $100 \text{ mg}^{-1} \text{ dry mass h}^{-1}$ (1 week after application)
Control	1039 (100)	743 (100)	286 (100)	8 (100)	44 (100)
SSHB(100)	(97)	(90) ^x	(114) ^x	(109) ^x	(118)
Control	1029 (100)	807 (100)	222 (100)	8 (100)	- -
SSHB(1000)	(103)	(78) ^x	(126) ^x	(105) ^x	(165) ^x

^x) significant in comparison with control (t-test; $\alpha \leq 0,05$)

Table V. Effect of SSHB on sugar-beet tap-root growth and activity of acid invertase and sucrose synthase

Conditions: see Table II; Assay Procedures: Activity of acid invertase, see Table III conducted at harvest time; Activity of sucrose synthase - conducted at harvest time on freeze-dried plant material that was mixed with the extraction solvent bull serum albumin and buffer (HEPES, EDTA, dithiothreite, pH 7.5 at 4°C), incubated with fructose and ¹⁴C UDP glucose (1 h, 30 °C), separated via thin layer chromatography and the ¹⁴C sucrose assayed by scanning. r = correlation coefficient

Treatment (dose in mg/ha)	Dry Mass of Roots in g/vessel (see Table II.)	Enzyme Activity in Tap-Roots at Time of Harvesting Activity of Sucrose Synthase: μmol sucrose hydrolyzed 100 mg ⁻¹ dry mass h ⁻¹	Activity of Acid Invertase: μmol sucrose hydrolyzed 100 mg ⁻¹ dry mass h ⁻¹	r in relation to dry mass =0,86 (significant)	r in relation to dry mass =0,68 (significant)
1. 45-50 % of maximum water capacity (mild stress):					
Control	25 (100)	3 (100)		r in relation to dry mass =0,86 (significant)	17,9 (100)
SSHB(100)	(127) x)	(137)			-
SSHB(1000)	(130) x)	(191)			(68)
2. 25-30 % of maximum water capacity (high stress):					
Control	16 (100)	4 (100)		r in relation to dry mass =0,68 (significant)	19,1 (100)
SSHB(100)	(106)	(116)			-
SSHB(1000)	(125) x)	(114)			(68)

x) significant in comparison with control (t-test; α ≤ 0,05)

interpreted as a higher production of dry matter from SSHB treatment because of increased invertase activity which produced the root mass increase. Thus, tap-roots provide a situation similar to the one for young leaves and the possible explanation for the effects noted appears to be the same, theoretically.

Effects of brassinosteroids on the activity of sucrose synthase in tap-roots. Finally, we had to consider the effect of homobrassinolide application on sugar content and sugar yield. It is inferred from Table II that both characteristics were increased only in the case of high drought stress which may be explained by increased activity of sucrose synthase as a consequence of SSHB application. Sucrose synthase is a key enzyme in the root storage of sugar-beet plants and this enzyme is able to synthesize sucrose (6).

In Table V, data indeed show a higher enzyme activity of sucrose synthase after SSHB application under high drought stress than under mild stress because 68 % of $19.1 \mu\text{mol sucrose } 100 \text{ mg}^{-1} \text{ dry material h}^{-1}$ constitute more than 68 % of $17,9 \mu\text{mol sucrose } 100 \text{ mg}^{-1} \text{ dry material h}^{-1}$. Nevertheless the effect is very small, and the experiments have to be confirmed.

Discussion and Conclusion

We recognize that the effects of SSHB application on plant growth and composition depend on environmental conditions, so that:

- there was no effect under wet conditions,
- there was an increase of tap-root mass without an increase of sucrose yield under mild drought stress,
- there was an increase in sucrose content and sucrose yield under high stress.

We presume that the effects of mild drought stress are connected with a higher concentration of endogenous auxins. It is well known that SSHB can increase the sensitivity of tissues to auxins (7, 8). In other experiments we found that auxin (2,4-D) increased the invertase activity in leaves of sugar beet plants, which were similar to results obtained with SSHB application. It is obvious, however, that SSHB can produce other effects as well. It may not have a direct effect on acid invertase activity, but rather, it may enhance acid secretion. Brassinosteroids are similar in action to auxin in that they stimulate growth, H^+ extrusion and

Table VI. Effect of SSHB on sugar-beet growth and sugar yield in field trials at Seehausen (near Leipzig) and at Halle

Conditions: SSHB applied at 12 leaf stage (once) at 300 l ha⁻¹ with 4 replicates. The corrected sugar yield - a measure for the extractable white sugar yield - was calculated from the corrected sugar content. Determining values for this were the sucrose content and the quality - governing constituents, the alkali ions potassium and sodium as well as the aminonitrogen in which amino acids and amides are summarized.

Year	Treatment (dose in mg/ha)	Yield of Tap-Roots in dt/ha	Sucrose Content %S	Corrected Sugar Yield in dt/ha
1989 (Seehausen)	Control	425	16,9	60,4
	SSHB(1000)	447	17,3 ^x	65,2
1990 (Halle)	Control	329	15,8	44,3
	SSHB(1000)	352	15,8	46,8

^x) significant in comparison with control (t-test; $\alpha \leq 0,05$)

hyperpolarization of the transmembrane electric potential in root segments of maize and Azuki bean (9, 10). We have, however, not investigated the effect of homobrassinolide on sucrose phosphate synthase (EC 2.4.1.14) and neutral invertase (EC 3.2.1.26) although these enzymes are also of importance in taproot for sucrose storage and growth possibly (6, 11). Further studies on transport, distribution and metabolism of brassinosteroids using labelled molecules are necessary.

Finally, it is important to know whether laboratory demonstrated effects can be repeated in field trials. Table VI shows that field experiments with SSHB confirm many of the results we have obtained in greenhouse studies. Consequently brassinosteroids may become of interest as bioregulators that reduce drought stress in sugar-beet plants.

Acknowledgement

The authors wish to thank Prof. G. Adam of the Institute of Plant Biochemistry, Halle, for the samples of (22S, 23S)-homobrassinolide and (22S, 23S)-24-epibrassinolide.

Literature cited

1. Grove, M.D.; Spencer, F.G.; Rohwedder, W. K., Mandava, N.B.; Worley, J.F.; et al. *Nature* 1979, 281, 216
2. Mandava, N.B. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 1988, 39, 23
3. Adam, G.; Marquardt, V. *Phytochemistry* 1986, 25, 1787
4. Morris, D.A.; Arthur, E. D. *Plant Growth Reg.* 1984, 2, 327
5. Giaquinta, R.T. *Plant Physiol.* 1978, 61, 380
6. Fieuw, S.; Willenbrink, J.J. *Plant Physiol.* 1987, 131, 153

7. Yokota, T.; Takahashi, N. In *Plant Growth Substances*; Bopp, M. Ed.; Springer-Verlag: Berlin, Heidelberg, New York, Tokyo; Germany, USA, Japan, 1986, pp. 129-138.
8. Cohen, J.D.; Meudt, W.J. *Plant Physiol.* 1983, 72, 691
9. Romani, G.; Marré, M.T.; Bonetti, A.; Cerana, R.; Lado, P.; Marré, E. *Physiol. Plant.* 1983, 59, 528
10. Cerana, R.; Bonetti, A.; Marré, M.T.; Romani, G.; Lado, P.; Marré, E. *Physiol. Plant.* 1983, 59, 23
11. Masuda, H.; Takahashi, T.; Sugawara, S. *Agric. Biol. Chem.* 1987, 51, 2309

RECEIVED May 29, 1991

Chapter 19

Effects of Brassinolide on Growth and Chilling Resistance of Maize Seedlings

Ruo-yun He, Guan-jie Wang, and Xue-shu Wang

Department of Basic Science, Shenyang Agricultural University, Shenyang 110161, China

Maize seedlings were cultured in plastic pots containing perlite or on filter paper and treated with brassinolide (BR) in the dark or in the light. The results showed that at 10^0 ppm BR promoted the elongation of coleoptiles and mesocotyls but retarded the growth of leaves and roots, whether in the dark or in the light. The mesocotyls of etiolated seedlings showed either twining or transverse geotropism when treated by higher concentrations of BR. At 10^{-3} – 10^{-1} ppm, BR improved the greening of etiolated leaves at different temperatures, especially at lower temperature in light. BR also promoted the growth recovery of maize seedlings following chilling treatment. The physiological effect of BR seems to be that of both auxin and cytokinin, and BR as a steroid may act on biological membrane systems.

It has been reported that brassinolide (BR) has some actions similar to auxin and cytokinin (*I*). In order to compare the physiological effects of BR in these respects and to determine if this is so, we studied three aspects as follows:

1. Characteristic responses to brassinolide in maize seedling organs
2. Effect of brassinolide on greening of etiolated maize seedlings under light
3. Effect of brassinolide on growth recovery of maize seedlings after chilling

Our experiments were carried out mainly with maize (*Zea mays* L.) hybrids 171 × 330A and Danyu 13, provided by Shenyang Agricultural University and Shenyang Seed Company. Brassinolide ($2\alpha,3\alpha,22R,23R$ -tetrahydroxy-24S-methyl-B-homo-oxa-5 α -cholestan-6-one) (Figure 1), a natural type synthesized by Fugisawa Pharmaceutical Co., Ltd., was

provided by the National Federation of Agricultural Co-operative Association (Zen-Non) of Japan. It was dissolved in 85% alcohol to make a 200-ppm solution and then diluted into various concentrations with water.

Maize seedlings were cultured in plastic pots containing perlite, or on filter paper, and were treated with various concentrations of brassinolide that ranged from 10^{-6} – 10^0 ppm in the dark (etiolated seedlings) or in the light (green seedlings). Details of experimental methods are as follows.

Characteristic Responses of Maize Seedling Organs to Brassinolide

Maize seeds were soaked and germinated at 25 °C. When the radicles were about 1 cm, the seedlings were treated with various concentrations of brassinolide that ranged from 10^{-6} – 10^0 ppm in the light and in the dark for 4 days. Following treatment, the lengths and dry weights of various plant parts were measured for both green and etiolated seedlings. The following conclusions were drawn from the data obtained (Figure 2):

- Concentrations of brassinolide from 10^{-6} ppm to 10^{-2} ppm had no significant effects on the growth of coleoptile, mesocotyl, and the first true leaf. (In fact, the mesocotyl of etiolated seedling was slightly promoted and the first true leaf was slightly retarded.) At 10^{-2} ppm, brassinolide showed a tendency to promote the growth of the second leaf in the light and in the dark. These results paralleled those obtained for barley (2) and wheat (3, 4), which indicated that brassins and brassinosteroid promoted the elongation of true leaves in monocotyledons.
- At 10^0 ppm, brassinolide promoted the elongation of coleoptiles and mesocotyls but retarded the growths of true leaves and roots, whether in the light or in the dark.
- At 10^0 ppm, brassinolide increased the dry weight of seedling shoots (including mesocotyls) and decreased the dry weight of roots; thus, the root/top ratio decreased remarkably. We suggest that the organic matter stored in the endosperm was distributed mainly to coleoptiles and mesocotyls as seeds germinated and developed into seedlings, because brassinolide at 10^0 ppm inhibited leaves from growing.
- The effects of brassinolide were similar in the light and in the dark, but the coleoptile and mesocotyl were elongated more and leaf growth was retarded more in the dark than in the light by brassinolide at 10^0 ppm. These results were in sharp contrast to those experimental results obtained with *Avena* coleoptiles (5) and with soybean and mung bean tissues (1, 6).

Etiolated maize seedling mesocotyls elicited two responses to higher concentrations of brassinolide (10^{-1} – 10^0 ppm). The first response was transverse geotropism and the second was twining (Figure 3). The seedlings in light culture treated with brassinolide at 10^0 ppm had the same

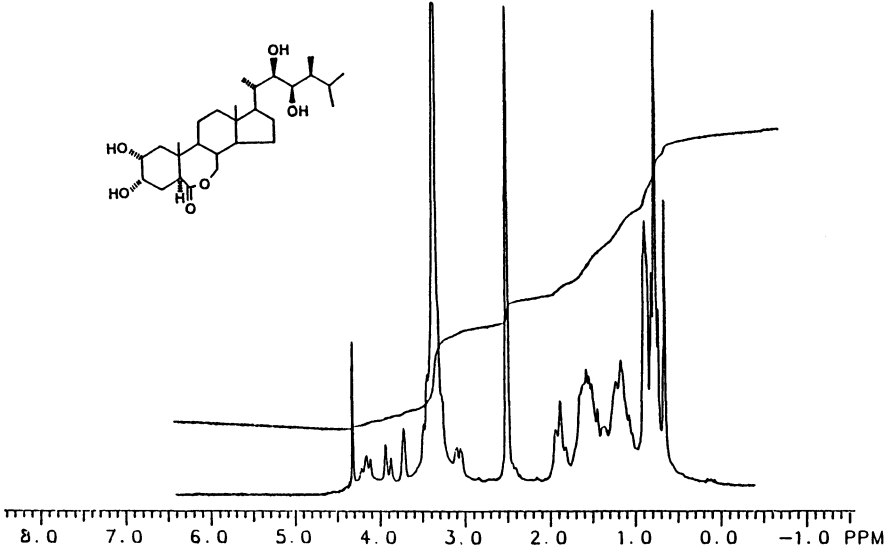


Figure 1. Brassinolide and its NMR spectrogram (D₂O) (He et al.)

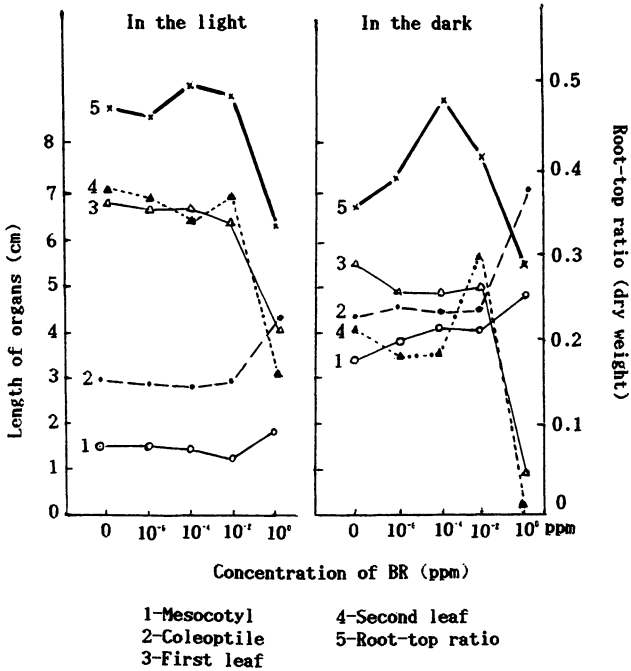


Figure 2. Effects of brassinolide on the growth of organs in maize seedlings.

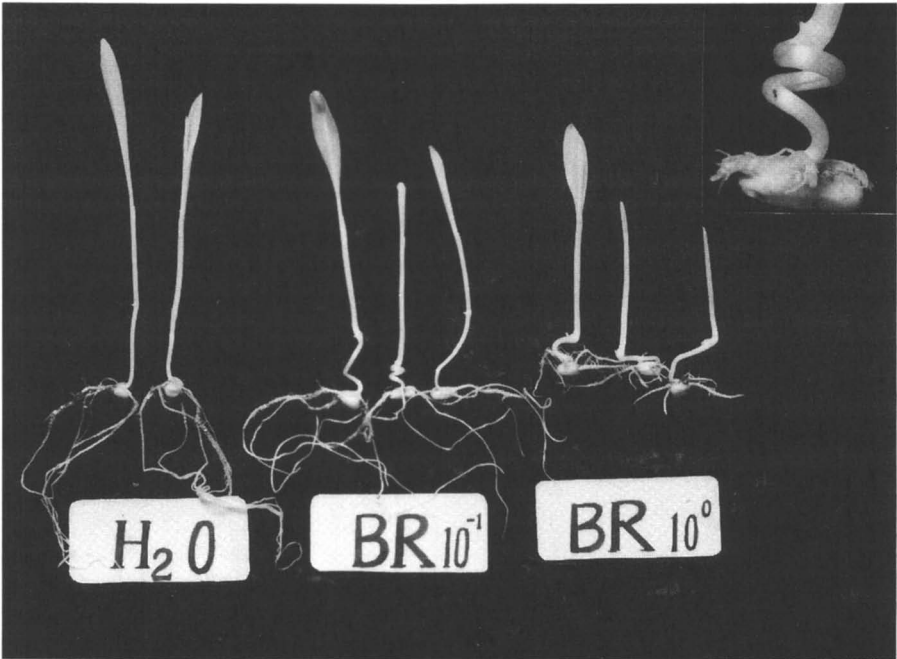


Figure 3. The characteristic responses of mesocotyls of etiolated maize seedlings to high concentrations of BR.

transverse geotropism, but no twining growth response was observed. However, with brassinolide treatment at 10^{-1} ppm and exposure to light, mesocotyls were not twined, nor was transverse geotropism noted.

Transverse geotropism in stems is a characteristic response to ethylene. It has been reported that treatments with epibrassinolide at concentrations of 1–10 ppm accelerated ethylene production in etiolated mung bean hypocotyl segments by increasing ACC (1-aminocyclopropane-1-carboxylic acid) (7). Thus it is possible that the transverse geotropism and even the twining growth response evoked by brassinolide at 10^{-1} – 10^0 ppm were related to the production of ethylene.

We cultured maize seedlings with various concentrations (200–4000 ppm) of Ethrel (2-chloroethylphosphoric acid) in the dark to ascertain if ethylene induced the same responses in corn. The results showed that mesocotyls had neither a transverse geotropism response nor a twining response. Earlier, we stated that both transverse geotropism and twining growth were the characteristic responses of maize mesocotyls to high concentrations of brassinolide. We now suggest that the transverse geotropism and the twining response are specific phenomena of brassinolide treatment and are not induced by ethylene in corn. In addition, the degree of mesocotyl twining response induced by brassinolide is different in different maize hybrids and is concentration dependent. For example, hybrid 171 × 330A is more sensitive than hybrid Danyu 13, with twining mesocotyls of 87.5% and 52.6%, respectively, upon treatment with 10^0 ppm of brassinolide, and the probability of twining was about 25% for hybrid 117 × 330A when treated with 10^{-1} ppm of brassinolide.

Dong Jiao-wang (Beijing Agricultural University, in Proceedings of the 2nd National Plant Growth Substance Seminar, Shanghai, China, 1987; unpublished data) found that the primary roots of rice had greater twining when the brassinolide concentration increased from 10^{-3} ppm to 10^{-1} ppm, but IAA, GA, and ABA (1–10 ppm) did not cause the rice roots to twine. Wang Yu-qin (Shanghai Plant Physiology Institute, in Proceedings of a National Plant Growth Substance Seminar, Nanjing, China, 1989; unpublished data) also found that rice primary roots twined with epibrassinolide treatment at 1 ppm. GA, auxin, and cytokinin did not induce twining; with four BR derivatives, however, the twining response was seen in primary roots of rice. We also found that young roots of wheat cultured in brassinolide also had twining.

All the data presented so far indicate that the twining effect in roots or mesocotyls is a characteristic response to brassinolide or brassinosteroids. The mechanism whereby twining is induced by brassinolide and the significance of this phenomenon in bioassays have yet to be elucidated.

Effect of Brassinolide on Greening of Etiolated Maize Seedlings under Light

It has been reported (8–12) that brassinolide increases the chlorophyll content of several crops in the field, but the effect of brassinolide on the

synthesis of chlorophyll in etiolated leaves has not yet been elucidated. Therefore, we studied the effect of brassinolide on the greening of etiolated maize leaves at different temperatures in light and compared the response of maize with the response of cucumber.

Etiolated seedlings were grown in brassinolide solution and in water as control. The first true leaves (sometimes also the second leaves) were detached and greened in the light, and then the chlorophyll content was determined. The results are shown in Tables I and II.

Table I. Effect of BR on Greening of Etiolated Maize Leaves Exposed to Light (Hybrid Danyu 13; 2 klux; 30 °C; 12 h)

Treatment	First Leaf ^a		Second Leaf ^b	
	Chl (mg/gfw)	%	Chl (mg/gfw)	%
Water (CK)	0.0702	100	0.1797	100
BR 10 ⁻² ppm	0.1244	177.2	0.3061	170.3
BR 10 ⁻¹ ppm	0.1348	192	0.4253	236.7
BR 10 ⁰ ppm	0.0334	47	0.2278	126.8

^aApproaching senescence

^bNot yet unfolding

Table II. Effects of BR on Greening of Etiolated Maize Leaves Exposed to Light at Various Temperatures (Hybrid 171 × 330A; 5 klux)

Treatment	30 °C, 24 h		18 °C, 24 h		15 °C, 48 h	
	Chl (mg/gfw)	%	Chl (mg/gfw)	%	Chl (mg/gfw)	%
Water (CK)	0.472	100	0.151	100	0.060	100
BR 10 ⁻³ ppm	0.598	126.7	0.379	251	0.077	124
BR 10 ⁻¹ ppm	0.641	135.8	0.356	236	0.130	209

NOTE: Figures are average values from three experiments.

From data in Tables I and II, the following conclusions can be made:

- Brassinolide concentrations ranging from 10⁻³ ppm to 10⁻¹ ppm improved the greening of etiolated leaves, although the degree of greening was different for the two hybrids.
- Maize is a hot-weather crop. Table II shows that low temperature depressed the synthesis of chlorophyll in etiolated leaves exposed to light, and brassinolide relaxed the depression of chlorophyll synthesis, especially at low temperature. For example, the chlorophyll content of the control at 18 °C was 68% less than that of control at 30 °C. However, the chlorophyll content of brassinolide-treated leaves at 18 °C was only 36–44.5% less than that of brassinolide-treated leaves at 30 °C. A

temperature of 15 °C is the critical point of growth in thermophilic plants. Chlorophyll content of the control at 15 °C was 94% less than that of the the control at 30 °C. For plants treated with BR (10^{-1} ppm), the chlorophyll content at 15 °C was 90% less than at 30 °C.

The greening of etiolated cucumber cotyledons exposed to light was also accelerated by brassinolide. We found that the effect of brassinolide on accelerating chlorophyll synthesis in etiolated cucumber cotyledons was similar to that of cytokinin, which deleted the lag phase induced by light during greening(He et al., Shenyang Agricultural University, unpublished data; 13, 14). Otherwise, the accumulation of chlorophyll, especially at low temperature, was promoted by brassinolide, and it is possible that brassinolide protects the biological membrane system and protects chlorophyll from photooxidation (15). We (He et al., unpublished data) studied the activity of superoxide dismutase (SOD) in etiolated and greened cucumber cotyledons that were cultured with brassinolide and benzyladenine (BA) for 6 days. The results showed that BR and BA promoted the activity of SOD in both etiolated and greened cotyledons (Table III).

Table III. Effects of BR and BA on SOD Activity in Etiolated and Greened Cucumber Cotyledons

Treatment	<i>Etiolated cotyledon^a</i>		<i>Greened cotyledon^b</i>		
	SOD (units/gdw)	%	SOD (units/gdw)	%	Chl ^c (mg/gdw)
Water (CK)	708.83	100	1100.22	100	1.166
BR 10^{-3} ppm	892.17	125.9	1652.97	150.2	2.676
BR 10^{-1} ppm	923.00	130.2	2366.19	215.1	3.420
BA 10^{-4} ppm	747.82	105.5	1543.22	140.3	1.580
BA 10^{-2} ppm	789.26	111.3	1767.52	160.7	2.727

^aDetached and cultured for 6 days in the dark

^bDetached and cultured for 12 h in the light and 5.5 days in the dark

^cMeasured after greening in the light for 12 h

SOD played an important role in protecting the stability of the membrane system because it scavenged $O_2^{\cdot-}$ radicals (superoxide free radicals). The effect of SOD is to protect chlorophyll from photooxidation at low temperature (16), thus it causes accumulation of chlorophyll in leaves under light.

Effect of Brassinolide on Growth Recovery of Maize Seedlings after Chilling Stress

Organs of maize seedlings are very sensitive to chilling stress during germination and the early growth stage. Chilling temperature not only affects

organ growth at that time but also prevents growth recovery after rewarming, which is unfavourable to the development of roots and the emergence of seedlings in early spring. It was reported (17–19) that brassinolide induced resistance to chilling in rice, cucumber, and corn. We have examined the effect of brassinolide on the growth recovery of maize seedlings following chilling treatment to confirm the role of BR in chilling resistance.

Maize (*Zea mays* L. hybrid 171 × 330A) seeds were soaked in BR, calcium chloride (CaCl_2), and water at 25 °C for 24 h and cultured in the same solutions for 2 days. Water treatment was used as control. The germinating seeds were subjected to chilling (0–3 °C) in the dark when their coleoptiles were about 1 cm and radicles were 2 cm in length. The seedlings were rewarmed at 25 °C after chilling for 2, 4, 6, 8, and 10 days and cultured at the same temperature for 2 days. The lengths of organs were measured before and after culture. The daily growth rate (cm/day) was used as an index of growth recovery. The growth recoveries in three organs of maize seedlings were as follows:

Coleoptile. The effect of brassinolide on the average daily growth rate of coleoptiles following seedlings chilled for different days is shown in Figure 4A, which shows that the growth rate decreased as the number of days of chilling increased. The growth of controls (water treatment) ceased after 8 days of chilling, which indicated irreversible damage. Both 10^{-3} and 10^{-1} ppm concentrations of brassinolide unequivocally promoted the growth recovery of coleoptile. The effect of CaCl_2 on growth recovery was approximately equal to that of BR for 2 days of chilling. For over 2 days of chilling, however, the growth recovery of coleoptiles with CaCl_2 treatment decreased sharply and finally looked like that of the controls (water treatment).

Mesocotyl. The effect of brassinolide on the average daily growth rate of mesocotyls following chilling for different days is shown in Figure 4B. The growth trend for mesocotyls paralleled that for coleoptiles (Figure 4A), but a higher concentration (10^{-1} ppm) of BR was more favourable to the growth recovery of mesocotyls.

Radicle. Maize radicles were very sensitive to chilling, and the growth of radicles was nearly zero after more than 2 days of chilling. The different effects of the agents appeared only for 2 days of chilling. Figure 5 shows the effect of agents (BR 10^{-3} and 10^{-1} ppm and CaCl_2 0.25%) on the daily growth rate of maize radicles chilled for 2 days. From Figure 5 we conclude that both BR and CaCl_2 , especially CaCl_2 , have obvious effects on the growth recovery of maize radicles. On the first day of rewarming, the BR 10^{-3} -ppm treatment yielded values close to that of the CaCl_2 treatment, but on the second day, the growth rate was lower than that of the first day. This result shows that the ability for growth recovery was lower. The control and BR 10^{-1} -ppm treatment exhibited the same phenomenon. However, the daily growth rate on the second day of rewarming was higher than that of the first day with CaCl_2 treatment. It appeared that growth recovery by CaCl_2 treatment was higher than that by BR treatment.

Brassinolide obviously affected the growth recovery of maize seedlings

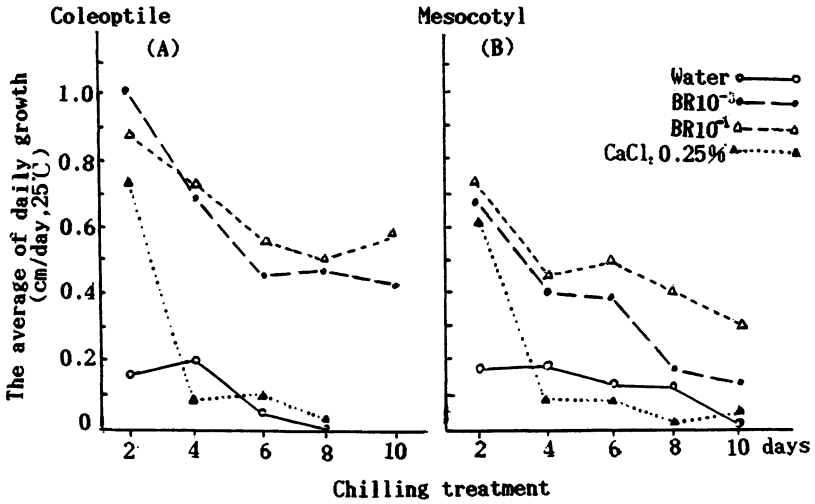


Figure 4. The effect of BR on the growth recovery of maize organs after chilling.

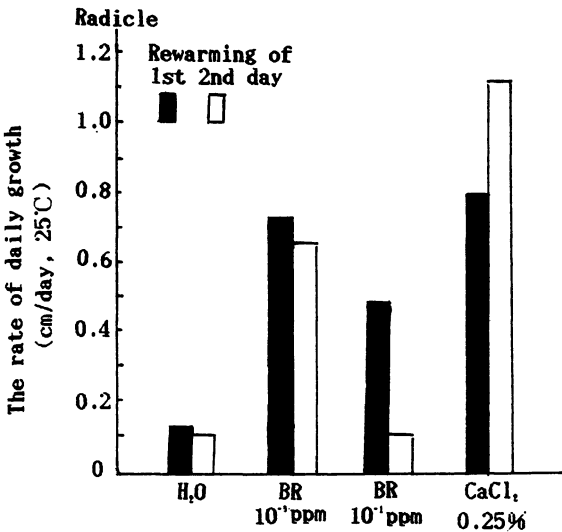


Figure 5. The effect of BR on the growth recovery of maize radicles on the 1st and 2nd days of rewarming after chilling for 2 days.

after chilling. The damage induced by chilling stress to thermophilic crops is generally realized to be brought about by imbalance of the endogenous growth-regulating substances (20, 21). The effect of brassinolide on the growth recovery of seedlings following chilling was most probably due to the role that brassinolide plays in adjusting or compensating the equilibrium of plant growth regulators in the plant (22, 23). In other words, exogenous brassinolide acted as a growth regulator and most probably as a steroid. The latter favoured growth recovery after chilling probably through a protective effect brought about by a change in the membrane system.

Summary

The three experiments with brassinolide showed that the physiological effect of brassinolide seems to be that of both an auxin and cytokinin. The twining growth effect, however, is the characteristic response evoked by brassinosteroids, and it was thought that the twining movement of the tendril and the twiner (climber) reported for auxin, or abscisic acid (ABA), or ethylene earlier is doubtful. In other words, brassinolide as a steroid may act on membrane systems.

Literature Cited

1. Mandava, N. B. *Ann. Rev. Plant Physiol. Plant Mol.* **1988**, 39:23–52.
2. Gregory, L. E. *Am. J. Bot.* **1981**, 68:585–588.
3. Braun, P.; Wild, A. In *Advances in Photosynthesis Research* Proc. 6th Congress on Photosynthesis; Sybesma, C., Ed.; Nijhoff: The Hague, 1984; 3:461–464.
4. Braun, P.; Wild, A. *J. Plant Physiol.* **1984**, 116:189–196.
5. Yokota, T.; Takahashi, N. In *Plant Growth Substances*; 1985. Bopp, M., Ed.; Springer: Berlin, 1986; pp 129–138.
6. Gregory, L. E.; Mandava, N. B. *Physiol. Plant.* **1982**, 54:239–243.
7. Wu, Y. M.; Bao, Y. W.; Liu, Y. *Acta Phytophysiologica Sinica* **1987**, 13(1):107–111.
8. Chen, F. Y.; Bin, Y. Q.; He, R. Y. *Lioning Agric. Sciences (China)* **1988**, 5:37–41.
9. Yu, S., et al. *ZHIWU ZAZHI (Plants)* **1990**, 17(5):24–25.
10. Hao, J. J.; Xuan, Y. S.; He, R. Y. *J. Shenyang Agric. University* **1990**, 21(1):43–47.
11. Zhang, X. M., et al. *Acta Agri. Universitatis Henanensis* **1987**, 21(1):1–7.
12. Luo, B. S. *Plant Physiol. Commun.* **1986**, 2:14–17.
13. Beevers, L., et al. *Planta* **1970**, 90:256.
14. Fletcher, R. A., et al. *Can. J. Bot.* **1971**, 49:2197.
15. Hasselt, P. R. V. *Acta Bot. Neerl.* **1972**, 21:539–548.
16. Wang, Y. R., et al. *Acta Phytophysiologica Sinica* **1986**, 12(3):244–251.
17. Fujita, F. *Farming and Technique (Japan)* **1988**, 43(1):19–24.
18. Zhou, A. Q.; Luo, B. S.; Ren, X. B. *J. Hwazhong Agric. University* **1987**, 6(1):8–13.
19. Luo, B. S. *Plant Physiol. Commun.* **1986**, 1:11–14.

20. Luo, Z. R. *Plant Physiol. Commun.* **1989**, 3:1–5.
21. Guo, Q.; Pan, R. C. *Acta Phytophysiologica Sinica* **1984**, 10(4):295–303.
22. Eun J. S.; Kuraishi, S.; Sakurai, N. *Plant Cell Physiol.* **1989**, 30(6):807–810.
23. Luo, B. S.; Yu, D. Q.; Zhou, D. Y. *Plant Physiol. Commun.* **1988**, 5:31–34.

RECEIVED June 14, 1991

Chapter 20

Brassinosteroids and Root Development

J. G. Roddick and M. Guan¹

Department of Biological Sciences, University of Exeter, Exeter,
United Kingdom

Relatively little is known about the effects of brassinosteroids (BS) on root growth and development and nothing at all concerning their occurrence in roots. Results are variable, but BS mainly inhibit root development in cuttings and seedlings, and at low concentrations (10 μM). Simultaneous, but variable, effects may occur on the shoot, but cultured excised root studies suggest that root inhibition is a direct effect. Promotion of root growth is reported for cuttings, normal and stressed seedlings, and segments, but some of these conclusions are open to question. In segments, BS show opposite effects to IAA on proton extrusion, Δ pH and membrane potential. BS appear to be transportable in cultured roots and this system may be particularly useful for further studies of BS effects on roots.

Following the discovery of 'brassin' in 1970 (1), the isolation and chemical characterization of the active ingredient brassinolide (BR) in 1979 (2) and the subsequent identification of a number of other naturally-occurring brassinosteroids (BS) (see 3), considerable research effort has been directed towards determining the taxonomic and morphological distribution and biological activity of these compounds in plants.

Studies of the occurrence of BS in the plant kingdom have shown them to be present in virtually all higher plants examined so far from a wide range of families (3,4), and also in the unicellular green alga *Hydrodictyon reticulatum* (5). Considering this trend, it is not beyond the bounds of possibility that BS may prove to be universal constituents of (at least higher) plants. Within the plant itself, BS have been detected in almost every organ, albeit at variable (but low) levels, as summarized in Table I.

¹Current address: Diagnostic Biotechnology (Pte) Ltd., 65 Science Park Drive, Singapore Science Park, Singapore 0511

Table I. Occurrence and Levels of BS in Higher Plant Tissues/Organs

Tissue/Organ	Conc. ($\mu\text{g}/\text{kg}$)	Ref.
Pollen	5 - 190	<u>6</u>
Seeds	0.2 - 5	<u>7</u> - <u>9</u>
Fruits	Trace - 1	<u>10</u> - <u>12</u>
Flower buds	3 - 15	<u>9</u> , <u>13</u>
Shoots	0.008 - 27	<u>12</u> - <u>14</u>
Leaves	0.0005 - 30	<u>10</u> , <u>13</u> , <u>15</u>
Galls	0.01 - 26	<u>10</u> , <u>12</u>
Cultured crown-gall tissue	30 - 40	<u>16</u>

Notable by its absence from this list is any reference to roots or other subterranean structures. The importance of roots as absorptive, anchorage and storage organs needs no elaboration here and yet all analyses of plant material for BS so far conducted have been confined to aerial tissues. Amazingly, this oversight has persisted for more than a decade.

Arguably, the most eminent biological effect of BS is their ability to enhance shoot elongation and growth in a variety of systems: excised segments, cuttings, seedlings and plants (17,18). Other important developmental effects of BS include stimulation of pollen tube growth (19), sensitizing tissues to gravistimulation (20), induction of epinasty (21) and accelerating flower production and altering the flower sex ratio (22). The potential importance of such effects is apparent from the fact that application of BS to the shoots of a number of crop plants can produce greater or earlier crop yields (4,17,18,23).

Once again, however, roots have been sadly neglected in studies into the biological activity of BS, although not totally so. A relatively small number of published reports exist in this field which collectively indicate that BS are capable of altering developmental processes in roots at low concentrations. Unfortunately, some of these reports are based on incidental observations and/or are not sufficiently quantified. The consequent uncertainties are further compounded by an element of variability between some reports. As a result, no clear picture has yet emerged of the developmental significance of BS in roots and we are left with a very unbalanced knowledge of the distribution and possible importance of BS in the plant body as a whole. At this stage therefore, there exists a need to bring together the various scattered reports of BS effects on root growth and development in order to assess critically the present status of these compounds in this important organ and to identify directions in which future work in this field might proceed.

Despite the small number of studies on the influence of BS on roots, all of the most important experimental root systems, that is, adventitious cutting roots, intact seedling/plant roots, excised root segments and excised cultured roots have already been employed.

Information relating to these different systems is reviewed in the four major sections of this article.

Adventitious roots

Probably the earliest investigation of the effects of BR on adventitious root formation is described in a two-part report published in 1981 by Yopp *et al.* (24) and Mandava *et al.* (25). The former study used mung bean (*Phaseolus aureus* cv. Berken) cuttings in which adventitious root development normally responds well to auxin. While the number of adventitious roots emerging from the hypocotyl was unaffected by 0.1 μM BR, it was more than halved by 1 μM BR and totally suppressed by 10 μM BR. The lengths of adventitious roots were not recorded, but shoot length was stimulated by increasing concentrations of BR. This inhibitory effect of BR on rooting is of considerable interest, contrasting as it does with the promotory effect of IAA in this system (24), whereas in azuki bean (*Vigna angularis* cv. Takara) epicotyls, BR and IAA show similar growth-enhancing effects (24,26). These findings have been interpreted as demonstrating that BR inhibits root emergence but not root initial formation (4); however, this conclusion is probably not warranted in the absence of histological examination of the hypocotyl. Dihydroconiferyl alcohol and chlorogenic acid, which synergize some gibberellin and auxin responses respectively, had no effect on BR inhibition of rooting in mung bean (*P. aureus*) hypocotyls (27).

The second part of the study (25) essentially confirmed inhibition of adventitious rooting by BR in other species *viz.* bean and cucumber, although the observations were incidental and the effects not quantified. In etiolated bean (*Phaseolus vulgaris* cv. Executive) hypocotyls originally used to study hook opening, treatment with 10 μM BR suppressed adventitious root development. Etiolated cucumber (*Cucumis sativus* cv. Long Green) hypocotyls lacking cotyledons normally used for elongation experiments also failed to produce adventitious roots when treated with 10 μM BR. In cotyledon-bearing hypocotyls, roots never formed at the base in the presence of BR, although some did appear approximately 1 cm from the basal end. Although gibberellins strongly inhibit adventitious root development in a number of plant systems (28), the authors (25) caution that this BR effect cannot necessarily be regarded as 'gibberellin-like' as cytokinins are also capable of interfering with rooting (29).

Recent work of a more quantitative nature (30,31) provides further support for an inhibitory effect of BR on adventitious root formation. In hypocotyl and epicotyl cuttings from mung bean (*P. aureus*), both the number of roots produced and the mean root length were markedly reduced by 24-epibrassinolide (epiBR) (30). Rooting was significantly and consistently impaired by 0.1 μM BR with maximal effects at 1.0 μM . These effects on root number and elongation compounded to produce significant reductions (approx 50%) in total root length for each type of cutting at 0.01 μM (Table II).

Table II. Approx. Concentration (μM) of EpiBR producing 50% Inhibition of Adventitious Root Growth in Cuttings

Species (Type)	Growth parameter		
	Number of Roots	Mean Root Length	Total Root Length
Mung Bean (hypocotyl)	0.01	0.1	0.01
Mung bean (epicotyl)	0.05	0.07	0.01
Tomato	0.1	0.7	0.04

SOURCE: Adapted from ref. 30, 31.

In tomato (*Lycopersicon esculentum* cv. Best of All) cuttings, epiBR again caused a general inhibition of rooting, although individual parameters responded somewhat differently from mung bean (31). With increasing concentrations up to 1 μM , the number of roots produced declined, but at 10 μM there was an upturn in the graph and cuttings produced a greater number of roots (roughly equivalent to that in controls) higher up on the hypocotyl. However, mean root length progressively decreased with increasing epiBR concentration with the result that total root length per cutting showed an essentially inverse relationship with BS strength. The sensitivity of adventitious roots of tomato to epiBR appeared less than that of mung bean roots (Table II).

In both mung bean and tomato cuttings, increasing levels of epiBR not only progressively reduced rooting but simultaneously stimulated shoot elongation, especially epicotyl elongation. Such differential growth effects are in keeping with similar earlier observations in mung bean (24) and cucumber (25). The former authors (24) concluded that this effect was not a result of preferential nutrient diversion to aerial organs. The reasons for it still remain unclear, but it is tempting to speculate that root development might be more sensitive to BS than shoot tissues such that levels of BS promotory to shoots might be inhibitory to roots, a situation which is known for auxins. Nevertheless, these data do not rule out the possibility that effects on root development may be somehow mediated via effects on shoot tissues (but see below).

In contrast to the above, Vorbrodt *et al.* (32) reported that the development of roots from cloned, excised shoots of *Matricaria chamomilla* cultured on agar-solidified medium was increased by between 2-6 times by treatment with 0.001-10 ppm (2nM-20 μM) (22S, 23S)-homobrassinolide (homoBR) either incorporated into the agar medium or applied by dipping into a powder or solution. Low concentrations of epiBR have also been found to enhance rooting in excised soybean (*Glycine max*) hypocotyls under certain conditions (33). Hypocotyls from seedlings raised in continuous light or darkness were not responsive to the BS, whereas long-day-grown (16h) tissues responded maximally in terms of both root number and length to 0.0001 ppm (0.2 nM) for 8h. The effect of 0.001 ppm (2 nM) for 4h was also

significantly greater than the control. Where roots increased in number, they were present over a greater length of the hypocotyl which the authors suggest might have resulted from a more extensive distribution of epiBR in the hypocotyl. No inhibitory effects of epiBR were apparent even at the highest concentration (200 nM).

Seedling roots

The response of seedling roots to exogenous BR also figures in the early communication of Yopp *et al.* (24). In dark-grown cress (*Lepidium sativum* cv. Curley) seedlings, root growth was inhibited by IAA at 0.1 μM or greater, but little affected either way by up to 10 μM BR.

Possibly as a result of this 'neutral' effect, no major quantitative study of the effects of BS on seedling roots appeared for a number of years. A few sporadic mentions of root effects were reported but, because of their qualitative nature, these made no significant contribution to the knowledge base. For example, spraying a 1 μM solution of an unidentified BS on to hydroponically-grown wheat (*Triticum aestivum* cv. Kolibri) seedlings promoted photosynthesis, soluble reducing sugars, and growth of the shoot and root, but none of the data presented applied to the root effect (34). In his recent review, Mandava (4) cites unpublished work by Gregory claiming that BR "induces both root and leaf growth in lettuce" (*Lactuca sativa*), but again no supporting experimental data are available. Seedlings of radish (*Raphanus sativus* cv. Tokinashi) and tomato (*L. esculentum* cv. Giant Cherry) with their roots immersed in BS solutions for short periods have been used in bioassays, but only shoot growth was measured (35).

In a more quantitative study, Luo *et al.* (36) also noted enhancement of photosynthesis in wheat (*T. aestivum*) by BR as well as of primary root formation in seedlings. Root (and shoot) elongation was not stimulated by BR but, if anything, slightly inhibited by higher concentrations. Promotion of shoot growth but inhibition of root growth was recorded in radish (*R. sativus*) seedlings sprayed with ≥ 0.1 ppm (0.2 μM) BR with the effect being less pronounced under long days than short days (37). EpiBR was likewise found to promote shoot growth but reduce root growth (as measured by dry weight) in hydroponically-treated tomato seedlings (31). Seedling roots did not appear as sensitive as cutting roots being hardly affected by 0.01 μM epiBR, although root weight was reduced by approximately 40% by 1 μM . This differential sensitivity may be a result of different rates of uptake by, and transport within, intact and excised organs. More recent work by one of us (JGR), currently being prepared for publication, has confirmed the ability of low levels of epiBR to inhibit root development in hydroponically-grown seedlings of other species e.g. mung bean (*P. aureus*), wheat (*T. aestivum* cv. Sir Galahad) and maize (*Zea mays* cv. Santos) (Table III). In the first two species, main root number and extension growth were unaffected by the BS, but lateral root number and

Table III. Approx. Concentration (μM) of EpiBR producing 50% Inhibition of Seedling Root Growth

Species	Growth parameter			
	Main Root Number	Main Root Length	Lateral Root Number	Total Lateral Root Length
Wheat	N.I. ^a	N.I. ^a	1	0.01
Mung Bean	N.I. ^a	N.I. ^a	10	0.1
Maize	10	>10? ^b	N.I. ^a	7

^a No inhibition with up to 10 μM

^b Inhibition < 50% at maximum tested concentration (10 μM).

elongation were depressed, although the magnitude of the effects varied. Wheat also showed considerable morphological distortion of the root. Maize, on the other hand, differed in its response and sensitivity in that the growth of the main root was affected as was lateral length (but not lateral number), but some effects were only observed at, or approaching, the highest concentration (10 μM).

EpiBR had no effect on shoot growth in wheat or maize but in the mung bean seedlings, concentrations of 0.1 μM or greater consistently inhibited epicotyl extension growth. This contrasts with the promotion of elongation in mung bean cuttings (24, 30) and in tomato seedlings (31), but the reasons for this are not yet known.

Of considerable interest as well as potential importance, is the very recent claim (38) that inhibitory effects of salt stress (500 ppm NaCl) on root growth in rice (*Oryza sativa* cv. Nihonbare) seedlings could be essentially nullified by pre-soaking seeds in homoBR solutions for 48h. All tested concentrations from 10^{-6} - 10^{-1} ppm (2 pM - 200 nM) were equally effective. Unfortunately, data from control seedlings raised in the absence of NaCl are not included and so the extent of recovery of root growth attributed to homoBR cannot be properly gauged.

The same authors (38) also studied the effect of epiBR on root growth in rice (*O. sativa* cv. Aepung) seedlings the seeds of which had previously been stored at 5°C for different lengths of time *viz.* 3 or 5 years. In the latter, root elongation was approximately 25% of that in the former, but could be partially restored (to about 55-65%) by the presence of low concentrations of epiBR (10^{-12} - 10^{-6} ppm, 0.002 fM - 2 pM). Enhancement of root growth by epiBR in the 3-year-old treatment was much less and possibly insignificant. It would have been useful to know how growth and responses to epiBR in 3- and 5-year-old treatments compared with those in fresh seeds, but this treatment was not included. Incidentally, an effect of epiBR at 10^{-12} ppm (0.002 fM) must rank as one of (if not the) lowest active concentrations of BS recorded.

Root segments

The fact that BS and auxins exert quite different effects on root development in cuttings and seedlings (24) was very much in the minds of Bonetti, Romani, Cerana and co-workers when they embarked on a programme of investigations into the effects and mechanisms of BS on root segments. As background to this work also was their earlier finding (26) that epiBR (like IAA) increases acidification of the walls of azuki bean epicotyl cells probably through a stimulation of electrogenic proton extrusion.

Working with 5 mm apical and sub-apical segments of maize (*Z. mays* cv. DeKalb XL 640) roots, treatment with 3 μM epiBR increased extension growth by more than twice that in controls (39, 40). This effect was accompanied by a reduction in medium pH, the magnitude of which was related to $[\text{K}^+]$ in the medium. This was interpreted as resulting from an antiport exchange of K^+ and H^+ via an electrogenic proton pump. Further evidence for H^+ extrusion was a hyperpolarization of the membrane potential and inhibition of the BS effects by the protonophore (*p*-trifluoromethoxy)-carbonylcyanide-phenylhydrazone (FCCP). These findings (summarized in Table IV) further demonstrated that BR and IAA effects on roots also differ at the cellular/biochemical level, the latter causing inhibition of root segment elongation, increase in pH and depolarization of the membrane potential (39, 40).

Table IV. Effect of EpiBR on Growth of Maize Root Segments, Medium pH, Proton Extrusion and Membrane Potential

Treatment	Mean % Increase in Length ^a	ΔpH^a	Proton Extrusion ^a ($\mu\text{mol/g}$ FW)	Membrane Potential ^b (mV)
Control	5.3	-0.53	2.04	-105
1 μM BR	-	-0.92	3.27	-
3 μM BR	12.8	-0.86	3.15	-125
10 μM BR	-	-0.84	3.11	-124

^a Sub-apical 5 mm segments incubated in Na^+ -MES buffer for 4h.

SOURCE: Adapted from ref. 39.

^b Apical 2.5 cm segments in Na^+ -MES buffer after 30 min.

SOURCE: Adapted from ref. 40.

A comparative study (41) of the effects of epiBR, twelve BS analogues and three sterols (stigmasterol, ergosterol and cholesterol) on growth and proton extrusion in sub-apical maize (*Z. mays* cv. DeKalb XL 72) root segments revealed five analogues to be virtually as active as epiBR on root growth, the other seven of reduced activity and the sterols inactive. These data confirmed that the structural requirements for BS activity in roots were the same as those for activity in shoots (42). Of interest however was that all the BS tested, whether of high or low growth activity, as well as the

growth-inactive sterols altered the external pH to the same extent. Cholesterol also proved to be as active as epiBR in stimulating K^+ uptake into the segments and dark CO_2 fixation.

Further investigations (43) confirmed the importance of K^+ (but not Na^+ or Cl^-) in epiBR and cholesterol enhancement of H^+ extrusion and related this to an increased rate of K^+ influx (as measured by ^{86}Rb) and proton pumping. In addition, growth stimulation in root segments by BS was shown not to be a result of an increase in the concentration of osmotically-active cell solutes.

Although possible reasons were offered for the finding that sterols alter pH and H^+ extrusion but not growth (43), this discrepancy casts some doubt on the involvement of acidification processes in BS-induced growth. These doubts may also be compounded by closer examination of the data for growth in root segments which are expressed on a percentage basis. The minimum and maximum % increases in growth in control segments were 4.9% and 9% respectively while the corresponding values for the 3 μM epiBR treatments were 13.8% and 16% (40, 41). In 5 mm segments, these values represent growth increments of only 0.25 mm and 0.45 mm in controls, and 0.69 mm and 0.8 mm in BS treatments. No details were given of how extension growth in root segments was measured and although the values may be statistically acceptable, the smallness of the changes renders the biological significance of these data uncertain. In similar experiments with apical and sub-apical root segments from wheat (*T. aestivum* cv. Sir Galahad), mung bean (*P. aureus*) and maize (*Z. mays* cv. Santos) we were unable to detect any significant BS-induced changes in linear dimensions (Table V). Although we did not subject segments to the elaborate pretreatments in $CaSO_4$ solutions used in the above work, we nevertheless obtained elongation equivalent to, or in excess of, that detailed above. Despite these

Table V. Effect of EpiBR on Elongation of Excised Root Segments^a

Species (Type of segment ^b)	Initial Length (mm)	Final Length (mm)				
		BR Conc. (μM)				
		0	0.01	0.1	1	10
Wheat (A)	5.10	6.44	6.47	6.54	6.73	6.43
Wheat (SA)	5.10	5.93	5.78	5.50	5.52	5.47
Maize (SA)	5.02	5.44	5.59	5.52	5.52	5.39
Mung Bean (SA)	4.85	5.41	5.34	5.31	5.28	5.22

^a Segments incubated in the dark at 25°C for 5h, except wheat (7h). Lengths measured using a photographic enlarger (x 5). Values are means of 10 replicates. S.E. range was $\pm 0.06 - \pm 0.15$.

^b A = apical, SA = subapical from 3 mm below apex.

reservations, root segments may offer a convenient method for studying the uptake, transport and possible binding characteristics of BS. This was the rationale underlying work by Allevi et al. (44) in

which the movement of deuterated epiBR into maize (*Z. mays* cv. DeKalb XL 72) root segments was followed by multiple selected ion monitoring. The BS quickly accumulated in root tissues by non-energy-dependent processes possibly involving adsorption to cell membranes. However, a comparison of the uptake and release patterns of epiBR in living and frozen tissues revealed that in the former there is a greater element of irreversible binding of BS. How this is achieved still awaits clarification.

Cultured excised roots

Convenient as root segments may be for some work, their usefulness is diminished by the fact that they represent only a fraction of the root system thus making it difficult to extrapolate to the whole root. Adventitious and seedling roots do not present such problems, but their physical association with shoot tissues can make for difficulties in determining whether roots respond directly to BS or indirectly via primary effects on the shoot.

One approach which may circumvent both these methodological problems is to grow excised roots in isolation in a sterile liquid culture medium. Using tomato roots (*L. esculentum* cv. Best of All) grown in this way, we have established that roots are directly influenced by BS (31). Concentrations of epiBR between 1 pM and 1 nM did not affect root growth, but 10 nM significantly inhibited elongation of the main root axis and the number of lateral roots produced. In both these parameters and also in total extension of laterals, growth was totally suppressed by 1 μ M epiBr. These findings (Figure 1) suggest that root inhibition by BS in tomato seedlings and cuttings (31), mung bean cuttings (24) and cucumber cuttings (25) where effects on shoot tissues simultaneously occurred is probably mainly due to a direct effect on the root system, although interactive effects deriving from the shoot cannot be totally ruled out.

Excised tomato roots grown in sterile culture appeared to be more sensitive to epiBR than their intact counterparts in seedlings, which might be explained in a number of ways. For example, greater access of BS into excised roots via the cut surface, lack of microbial degradation/transformation of test compounds in sterile conditions, altered hormonal/nutrient status of cultured roots as a result of excision, etc. In this respect, the generally greater responsiveness to auxin of excised plant parts compared with intact structures should be borne in mind. Also, because cultured excised roots responded in essentially the same way as adventitious and seedling roots, they could be useful in offering a more sensitive and controlled system for investigating BS effects on roots.

To date, most experimental work demonstrating effects of BS on root development has involved exogenous presentation of the test steroids to the root (or rooting) medium. Without doubt, this bears no resemblance to the *in vivo* situation where roots would respond (if at all) to endogenous BS either synthesized within the root or transported from the shoot. As a first step towards assessing the

responsiveness of roots to endogenously-supplied BS, we have presented epiBR to the basal or apical ends of cultured excised tomato roots and measured subsequent growth in the corresponding distal regions of the root. This work is still of a preliminary nature, but early results indicate that main axis extension growth and lateral root development can be markedly inhibited by BS applied at some other part of the root (Figure 2). With external transport along the root essentially prevented by a vaseline barrier, our data suggest first - that roots are able to respond to BS supplied endogenously, and second - that BS are transportable both acropetally and basipetally in root systems. It is not yet known if epiBR is transported in this or a derivatized (e.g. glucosylated) form.

Whether BS are actually transported within roots or between roots and shoots (or within shoots) in intact plants has yet to be established. BS are obviously and effectively transported into cuttings via the transpiration stream, but this is not evidence of transport *in vivo*. Also effects on root (and shoot) growth following a seed soak in BS solutions (38) or spray application of BS to the shoot (34) cannot be regarded as unequivocal proof of BS transport to the roots or a response by this organ to endogenously-supplied BS. Obviously, much remains to be done in this potentially important area of BS physiology and, once again, cultured excised roots might offer a valuable, complementary approach to some of the more intractable problems.

Discussion and conclusions

Because of the relatively small amount of work done in this field and the variability which exists between some reports, it is difficult to arrive at a consensus regarding the effects of BS on roots. Nevertheless, there does appear to be a certain weight of evidence that low concentrations of exogenously- (and possibly endogenously-) supplied BS can cause serious impairment of root development. This is a particularly interesting phenomenon because it stands in complete contrast to the powerful promotory effects of BS on shoot growth and raises interesting questions not only about the mechanism of action of BS (which still remains unresolved), but also about whether the mechanism (or consequences) might differ in different parts of the plant.

Of possible relevance in this respect are the observations that BS and auxin appear to produce opposite effects in root systems whereas they usually act in a similar (but not identical) way on the shoot and commonly show important interactive effects. The most strikingly different effects of these two compounds in roots relate to adventitious root formation in cuttings which is inhibited by BS (24, 30, 31) and promoted by auxin (24), and proton secretion and membrane potential in root segments which are enhanced and hyperpolarized respectively by BS and inhibited and depolarized respectively by auxin (39, 40). These data could suggest either that BS act independently of auxin in roots or that they antagonize

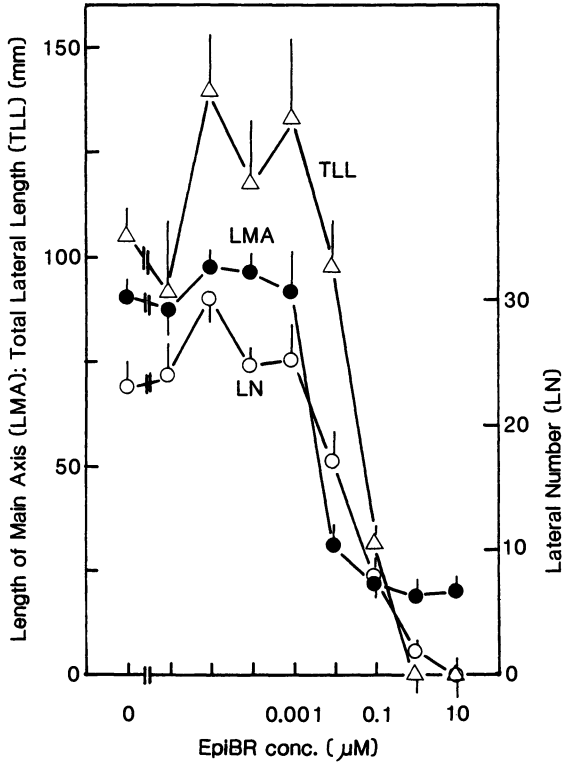


Figure 1. Effect of epiBR on growth of cultured excised tomato roots after 7 days. Inocula were 10 mm with no laterals. Points represent the mean of 10 replicates and vertical bars the S.E. (Adapted from ref. 31).

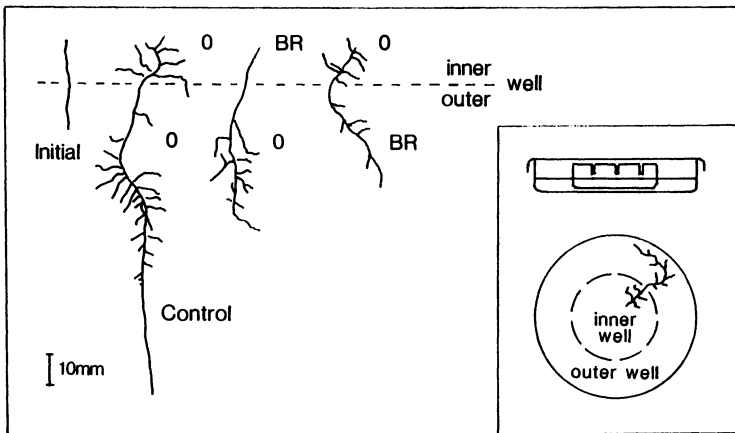


Figure 2. Tracings of typical cultured excised tomato roots treated at the apical or basal end with 10 μM epiBR for 7 days. The two-well treatment/culture vessel is shown inset.

endogenous auxin (4, 39). On the other hand, BS are capable of stimulating ethylene synthesis in shoot tissues and can synergize with auxin in this respect (45-47). It is also well known that root growth can be suppressed by ethylene (48, 49) and thus the possibility that ethylene release by root tissue may be highly sensitive to BS stimulation might be worthy of exploration.

Testing a wide range of epiBR concentrations (10^{-12} - 10^{-5} M) on cultured excised tomato roots (31) did not identify a clear optimum concentration suggesting that the inhibitory effects on roots are possibly not due to greater sensitivity of root cells than shoot cells to BS. On the other hand, Zhao (Shanghai Institute of Plant Physiology, personal communication, 1990) found lower concentrations of BS (e.g. 0.1 μ M) stimulated root growth in wheat (*T. aestivum*) while higher concentrations were inhibitory, as did also Luo *et al.* (36). Also, the BS dose-response curve for root growth in rice seedlings from 5 year old seeds (38) could be taken as some evidence that BS produce an optimal effect at very low concentrations. Whether these results reflect species specific responses is not yet known.

In order to rationalize some of the conflicting reports of BS on roots, further careful attention needs to be given to the mode, level and time of exposure to BS, all of which are inter-related in a complex manner through events such as uptake, transport and compartmentation. Exogenous application is informative, but highly artificial, and it would be preferable to adopt alternative systems in which BS arrive at target cells by an endogenous route. The use of excised root cultures could prove particularly valuable here although such a system is highly reliant on another major unknown relating to BS, *viz.* transport within (or between) organs. The increasing availability of radioisotopically-labelled BS will hopefully help clarify this situation soon. Ideally, the level of, and exposure times to, BS should be kept to a minimum, but this may not be realistic if problems exist in the uptake or transportation of these compounds. In fact the apparent lack of effect of BS on cress seedling roots in an early study (24) could possibly be due to the treatment time being only 17h. While inhibitory effects of BS on rooting and root growth can be criticized on the basis of the lengths of time of exposure (e.g. up to several days), it should be noted that significant enhancement of growth in some shoot tissues requires similar treatment times (50).

At the same time, claims that BS stimulate root growth or development cannot be totally dismissed, although some reservations must attach to certain reports because of the smallness of the effects (39-41) or the absence of controls (38). Even so, the possibility that BS may exert quite different effects on roots exposed to stress conditions (see 38) compared with non-stressed organs could have particularly important practical implications (cf 4). It seems unlikely that the enhancement of rooting in soybean hypocotyls (33) but inhibition of this process in mung bean hypocotyls (24, 30) can be attributed to the species difference, although

too few species have been studied so far to rule this out as an important determinant of response. More likely in this case (but as yet inexplicable), is that the BS effect was somehow conditioned by the lighting regime, since seedlings raised in long (16h) days produced responsive hypocotyls while those raised in constant light or darkness did not.

That root tissues are highly (and for reasons not yet understood, variably) responsive to applied BS is now beyond question. Although much remains to be explained regarding the nature, specificity and mechanisms of these effects, of probably greater importance at this stage is determining whether or not BS are actually present in root tissues and, if so, whether this is due to biosynthesis within the root or transport from elsewhere. Until such information becomes available, it will not be possible to place most of the findings described here into any meaningful physiological context.

Acknowledgments

We thank Professor N. Ikekawa for gifts of epiBR and Anna Rijnenberg for technical assistance.

Literature cited

1. Mitchell, J.W.; Mandava, N.; Worley, J.F.; Plimmer, J.R.; Smith, M.V. *Nature* **1970**, 225, 1065.
2. Grove, M.D.; Spencer, G.F.; Rohwedder, W.K.; Mandava, N.; Worley, J.F.; Warthen, J.D. Jr.; Steffens, G.L.; Flippen-Anderson, J.L.; Cook, J.C. *Nature* **1979**, 281, 216.
3. Adam, G.; Marquardt, V. *Phytochemistry* **1986**, 25, 1787.
4. Mandava, N.B. *Ann. Rev. Plant Physiol.* **1988**, 39, 23.
5. Yokota, T.; Kim, S.K.; Fukui, Y.; Takahashi, N.; Takeuchi, Y.; Takematsu, T. *Phytochemistry* **1987**, 26, 503.
6. Ikekawa, N.; Nishiyama, F.; Fujimoto, Y. *Chem. Pharm. Bull.* **1988**, 36, 405.
7. Yokota, T.; Baba, J.; Koba, S.; Takahashi, N. *Agric. Biol. Chem.* **1984**, 48, 2529.
8. Yokota, T.; Baba, J.; Takahashi, N. *Tetrahedron Letters* **1982**, 23, 4965.
9. Suzuki, Y.; Yamaguchi, I.; Takahashi, N. *Agric. Biol. Chem.* **1985**, 49, 49.
10. Abe, H.; Morishita, T.; Uchiyama, M.; Takatsuto, S.; Ikekawa, N.; Ikeda, M.; Sassa, T.; Kitsuya, T.; Marumo, S. *Experientia* **1983**, 39, 351.
11. Ikekawa, N.; Takatsuto, S. *Mass Spectroscopy (Japan)* **1984**, 32, 55.
12. Ikekawa, N.; Takatsuto, S.; Kitsuya, T.; Saito, H.; Morishita, T.; Abe, H. *J. Chromatog.* **1984**, 290, 289.
13. Arima, M.; Yokota, T.; Takahashi, N. *Phytochemistry* **1984**, 23, 1587.
14. Abe, H.; Nakamura, K.; Morishita, T.; Uchiyama, M.; Takatsuto, S.; Ikekawa, N. *Agric. Biol. Chem.* **1984**, 48, 1103.

15. Morishita, T.; Abe, H.; Uchiyama, M.; Marumo, S.; Takatsuto, S.; Ikekawa, N. Phytochemistry **1983**, 22, 1051.
16. Park, K-H.; Saimoto, H.; Nakagawa, S.; Sakurai, A.; Yokota, T.; Takahashi, N.; Syōno, K. Agric. Biol. Chem. **1989**, 53, 805.
17. Mandava, N.B.; Thompson, M.J. In Isopentenoids in Plants. Biochemistry and Function; Nes, W.D.; Fuller, G.; Tsai, L-S., Eds.; Marcel Dekker: New York, **1984**, pp. 401-431.
18. Meudt, W.J. In Ecology and Metabolism of Plant Lipids; Fuller, G.; Nes, W.D., Eds.; ACS Symposium Series 325, American Chemical Society: Washington D.C., **1987**, pp. 53-75.
19. Hewitt, F.R.; Hough, T.; O'Neill, P.; Sasse, J.M.; Williams, E.G.; Rowan, K.S. Aust. J. Plant Physiol. **1985**, 12, 201.
20. Meudt, W.J. Plant Physiol. **1987**, 83, 195.
21. Schlagnhauser, C.D.; Arteca, R.N. Plant Physiol. **1985**, 78, 300.
22. Suge, H. Plant Cell Physiol. **1986**, 27, 199.
23. Meudt, W.J.; Thompson, M.J.; Bennett, H.W. 10th Proc. Plant Growth Regul. Soc. Am. **1983**, pp. 312-318.
24. Yopp, J.H.; Mandava, N.B.; Sasse, J.M. Physiol. Plant. **1981**, 53, 445.
25. Mandava, N.B.; Sasse, J.M.; Yopp, J.H. Physiol. Plant. **1981**, 53, 453.
26. Cerana, R.; Bonetti, A.; Marrè, M.T.; Romani, G.; Lado, P.; Marrè, E. Physiol. Plant. **1983**, 59, 23.
27. Yopp, J.H.; Mandava, N.B.; Thompson, M.J.; Sasse, J.M. 8th Proc. Plant Growth Regul. Soc. Am. **1982**, pp. 238-245.
28. Goodwin, P.B. In Phytohormones and Related Compounds; Letham, D.S.; Goodwin, P.B.; Higgins, T.J.V., Eds.; Elsevier/North Holland: Amsterdam, **1978**, Vol. II; pp. 31-144.
29. Humphries, E.C. Physiol. Plant. **1960**, 13, 659.
30. Guan, M.; Roddick, J.G. Physiol. Plant. **1988**, 73, 426.
31. Guan, M.; Roddick, J.G. Physiol. Plant. **1988**, 74, 720.
32. Vorbrodt, H.M.; Donath, P.; Adam, G.; Luckner, M.; Neumann, D.; Beinbauer, K. DDR Patent **1989**, No. 271445.
33. Sathiyamoorthy, P.; Nakamura, S. Plant Growth Regulation **1990**, 9, 73.
34. Braun, P.; Wild, A. J. Plant Physiol. **1984**, 116, 189.
35. Takatsuto, S.; Yazawa, N.; Ikekawa, N.; Takematsu, T.; Takeuchi, Y.; Koguchi, M. Phytochemistry **1983**, 22, 2437.
36. Luo, B-S.; Kumura, A.; Ishii, R.; Wada, Y. Japan Jour. Crop Sci. **1986**, 55, 291.
37. Kamuro, Y.; Inada, K. 14th Proc. Plant Growth Regul. Soc. Am. **1987**, pp. 221-224.
38. Takematsu, T.; Takeuchi, Y. Proc. Japan Acad. **1989**, 65, 149.
39. Romani, G.; Marrè, M.T.; Bonetti, A.; Cerana, R.; Lado, P.; Marrè, E. Physiol. Plant. **1983**, 59, 528.
40. Bonetti, A.; Cerana, R.; Lado, P.; Marrè, E.; Marrè, M.T.; Romani, G. In Pollen: Biology and Implications for Plant Breeding; Mulcahy, D.L.; Ottaviano, E., Eds.; Elsevier: New York, **1983**, pp. 9-14.

41. Cerana, R.; Lado, P.; Anastasia, M.; Ciuffreda, P.; Allevi, P. Zeit. Pflanzenphysiol. **1984**, 114, 221.
42. Thompson, M.J.; Meudt, W.J.; Mandava, N.B.; Dutky, S.R.; Lusby, W.R.; Spaulding, D.W. Steroids **1982**, 39, 89.
43. Cerana, R.; Spelta, M.; Bonetti, A.; Lado, P. Plant Science **1985**, 38, 99.
44. Allevi, P.; Anastasia, M.; Cerana, R.; Ciuffreda, P.; Lado, P. Phytochemistry **1988**, 27, 1309.
45. Arteca, R.N.; De-Sheng, T.; Schlagnhauser, C.; Mandava, N.B. Physiol. Plant. **1983**, 59, 539.
46. Schlagnhauser, C.; Arteca, R.N.; Yopp, J.H. Physiol. Plant. **1984**, 61, 555.
47. Schlagnhauser, C.; Arteca, R.N. Physiol. Plant. **1985**, 65, 151.
48. Lieberman, M. Ann. Rev. Plant Physiol. **1979**, 30, 533.
49. Feldman, L.J. Ann. Rev. Plant Physiol. **1984**, 35, 223.
50. Gregory, L.E.; Mandava, N.B. Physiol. Plant. **1982**, 54, 239.

RECEIVED May 13, 1991

Chapter 21

Physiological Modes of Brassinolide Action in Cucumber Hypocotyl Growth

Masayuki Katsumi

Biology Department, International Christian University, Osawa, Mitaka-shi,
Tokyo 181, Japan

To show that the modes of brassinolide (BR) action in the control of seedling growth are unique and different from those of auxin (IAA) and gibberellin (GA₄), experimental results obtained with hypocotyls of light-grown cucumber seedlings are described. The stimulating effect of BR on the elongation of hypocotyl sections is characteristic in terms of its very low effective concentration range, pH independence, the modes of interactions with other hormones, the effects of inhibitors, etc. The most interesting action of BR is to reinforce seedlings against cold stress.

Since the first isolation and identification of brassinolide (BR) from rape pollen by Grove et al in 1978 (1), a wide occurrence in the plant kingdom of related steroidal compounds which are now collectively called brassinosteroids, as well as BR itself, has been confirmed (2).

Brassinosteroids have been demonstrated to elicit various pronounced effects on plant growth and development (3), and may now be considered as comprising an entirely new class of plant hormones in terms of their physiological modes of action and chemical structures as well. Brassinosteroids stimulate the growth of seedlings and the elongation of stem segments (4-9), the expansion of etiolated cotyledons (6), the enlargement of cultured cells (10), the growth of callus (11), pollen tube elongation (12), lamina joint bending (13, 14), epinasty (15), leaf senescence (6), proton secretion (16-20), ethylene formation (21), photosynthetic activity (22) and both nucleic acid and protein syntheses (23). Thus, they show activities similar to those of auxin, gibberellin and cytokinin. However, their physiological modes of action are unique and distinctly different from those of the other hormones, as will be explained later.

In this Chapter, results of experiments on the analysis of the modes of brassinolide (BR) action in growth stimulation are described, particularly, as compared with those of auxin (IAA) and gibberellin (GA₄). The experiments were carried out in most cases by using hypocotyl sections of light-grown cucumber (*Cucumis sativus* L. cv. Aonagajibai and cv. Spacemaster) seedlings which have been studied in detail for their physiological responses to auxin and gibberellin (24).

Effects of BR on Intact Seedlings

Hypocotyl Elongation. Although BR applied to the apex has been reported to have no effect on the hypocotyl growth of intact mung bean seedlings (3), it can elicit a distinct growth response in cucumber seedlings. BR is almost a hundred times as active as IAA which has also been shown to have the same effect in the same material (26). The activity of BR at the dosage levels of 10 - 100 ng/plant is approximately the

same as that of GA₄ which is the native physiological gibberellin of cucumber and which very actively stimulates hypocotyl elongation (27), but at higher dosages GA₄ is much more active than BR. Hypocotyls responding to a high dosage of BR are slightly twisted and partly thickened, which resemble those responding to higher dosages of IAA. Such abnormal morphology due to BR treatment has also been reported for other plant materials (3). This morphological effect of BR may be due to ethylene formation induced by BR as is known for IAA. In this case BR acts similarly to IAA.

Cotyledon Expansion. Cotyledon expansion is markedly stimulated by GA₄, and by BR as well, but to a lesser extent: the activity is about 1/100 that of GA₄. On the other hand, IAA has practically no effect. Dark-grown cucumber cotyledons have also been shown to expand when subjected to BR treatment (6). Since expansion of cotyledons and leaves of intact seedlings is a normal effect of gibberellin, in this case BR acts like gibberellin.

Adventitious Root Formation. IAA stimulates adventitious root formation in cucumber hypocotyl cuttings (28). On the other hand, BR has either no effect or is slightly inhibitory, while it stimulates hypocotyl elongation. Gibberellin is usually inhibitory. However, it has been reported that in mung bean cuttings, the formation of root primordia was not inhibited (29).

Growth under Chilling Stress. In recent years, brassinosteroids have been evaluated for practical use in agriculture, especially in Japan and China. Of great interest is the effect of BR in reinforcing the resistance of plants against various external stresses such as high concentrations of salts, low temperatures, pathogens, agricultural chemicals, etc. (30).

When cucumber seedlings, germinated and grown in the light at 25°C for 4 days, are subjected to cold treatment at 5°C for 3 days, the growth rate of the seedlings as measured by dry weight increase is markedly reduced during the cold period, and the recovery of the growth rate after transferring back to 25°C is very small for at least several days. However, when seeds are soaked with BR solution during imbibition, the growth of seedlings exposed to cold temperature is significantly greater than in controls (without BR treatment) (Figure 1A). Since BR has practically no effect on the growth of seedlings which have not been exposed to cold temperature, this growth stimulation effect of BR can be attributed to its reinforcement of seedlings against chilling stress.

Another parameter of the chilling effect is the decrease in chlorophyll content which is more sensitive than the reduction of dry weight increase. In seedlings continuously grown at a constant 25°C the chlorophyll content in the cotyledons increases as growth proceeds. On the other hand, in seedlings subjected to cold temperature, the content decreases distinctly, even after transfer back to 25°C. With BR treatment, however, the chlorophyll level before cold treatment is retained, and it increases after transfer back to 25°C (Figure 1B). These results indicate that the growth stimulating effect of BR is more significant under conditions where normal growth is reduced, and suggest that BR may act to help seedlings maintain their normal cell activities under unfavorable temperature conditions.

Hypocotyl Sections

The Activity of BR. Five mm long hypocotyl sections excised below the cotyledonary node respond to both IAA and gibberellin, with the younger hypocotyl tissue being more sensitive to gibberellin, while older tissue is more sensitive to IAA (31). BR also stimulates the elongation of hypocotyl sections, and it is almost 100 times as active as IAA. The lowest concentration eliciting activity is 0.1nM (Figure 2). Younger tissue is much more sensitive to BR (8).

The activity of BR is not pH dependent except at a very acidic pH of 3.5, while that

American Chemical Society

Library

1155 16th St., N.W.

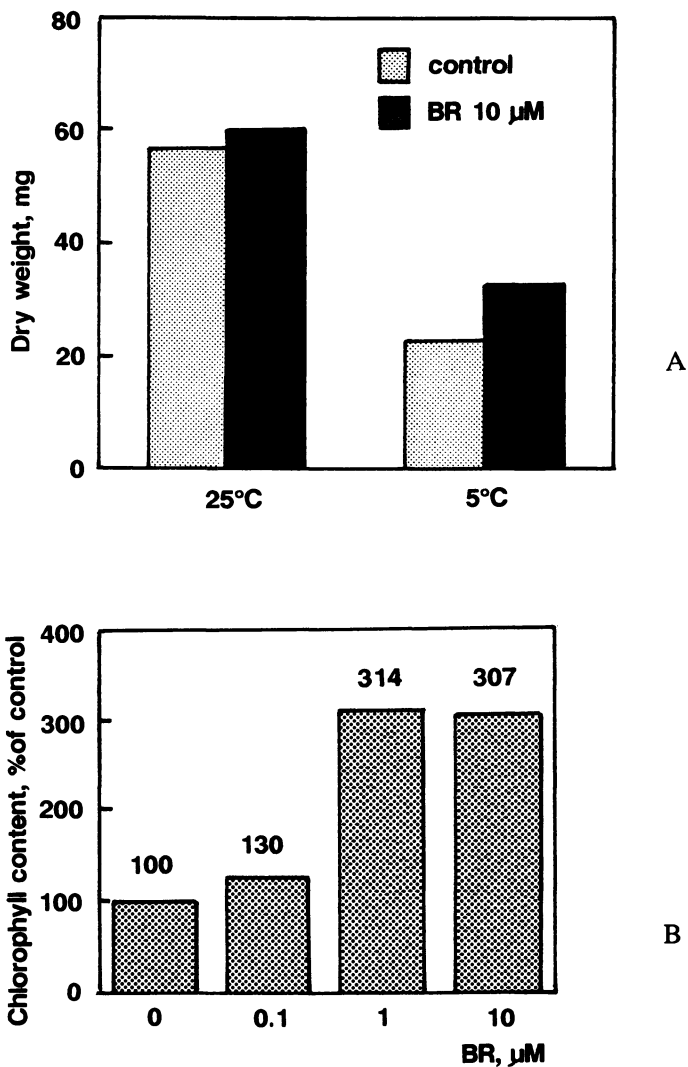


Figure 1. Effect of brassinolide on the growth (A) and cotyledon-chlorophyll content (B) of cucumber seedlings subjected to chilling stress, measured 5 days after chilling treatment. (Ochi, N. and Katsumi, M. unpublished)

of IAA is significantly affected by pH change. The activity declines at a pH lower than 7.0. Since IAA is a weak electrolyte and is transported into the cytosol through the plasma membrane in its dissociated form, the activity of IAA must be pH-dependent. On the other hand, BR is lipophilic and is a non-electrolyte. Therefore, its activity may not be affected by the pH of the medium.

BR-induced elongation also differs from that induced by IAA in that the former is not affected but rather enhanced in the presence of 50mM sucrose in the light, while the latter is inhibited. A similar sucrose-enhancement has been shown for gibberellin-induced elongation (24). In this case the mode of BR action is similar to that of gibberellin.

Time-Course Response. The chronological effect of BR is characteristic. As shown in Figure 3, BR also shows a biphasic response pattern similar to that of IAA (32). The first phase is presumably ascribed to acid growth. The unique pattern of BR-induced response is that the second phase lags behind that of the IAA-induced second phase by 6 h. This may indicate that the action of BR is not immediate as far as elongation is concerned.

Effects of Inhibitors. BR-induced elongation of sections is inhibited in the presence of *p*-chlorophenoxyisobutyric acid (PCIB), an antiauxin (8). The same effect has also been demonstrated in other plant materials (5,14). Kinetin is also an inhibitor of IAA-induced elongation (33), and inhibits BR-induced elongation (8). These facts suggest that the presence of endogenous auxin is necessary for BR-induced elongation. The fact that BR is more active in younger tissue may reflect a higher endogenous level of auxin in this tissue. As to whether or not BR affects the endogenous level of auxin is not clear and conflicting results have been reported (34,35).

N,N'-Dicyclohexylcarbodiimide (DCCD), an inhibitor of membrane bound ATPase, has been shown to strongly inhibit IAA-induced elongation of cucumber hypocotyl sections, while it has no effect on GA-induced elongation (36). DCCD markedly inhibits BR-induced elongation (8), suggesting that BR acts differently from GA, but similarly to IAA in this particular case.

Interaction with Auxin and Gibberellin.

Simultaneous Interaction. In the literature, the interaction of BR with auxin has been reported to be additive or synergistic depending on experimental systems and conditions (3). In cucumber hypocotyl sections, the interaction is definitely synergistic (8). The synergism is especially significant when the concentration of one of the two is suboptimal. The interaction at their optimal concentrations is synergistic only during the early period of incubation and becomes additive finally. A synthetic auxin, 2,4-D also interacts with BR similarly (8).

On the other hand, the interaction of BR with GA is entirely additive (8). This has been confirmed in other plant materials (3).

Interaction of BR with IAA in a Curvature Test. Cucumber hypocotyl sections with the epidermis of the one side of the square pillar peeled, respond to IAA and low pH very quickly by curvature (32). Curvature toward the non-peeled side (+) occurs within a few minutes and this represents acid growth, while curvature toward the peeled side (-) in response to IAA occurs within 30 minutes, and the latter reflects epidermis-dependent growth (Figure 4). The maximum point of plus curvature indicates that the growth rates of both peeled and non-peeled sides have equilibrated, and the point where a given curve crosses the line zero line of curvature is the point at which negative curvature starts. As shown in Figure 4, BR alone induced a slightly positive curvature but has no further effect until 6 h, corresponding to the lag period of

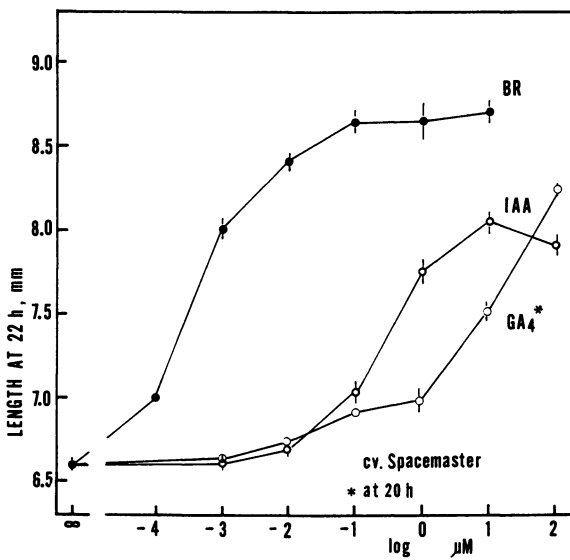


Figure 2. Comparison of the activities of brassinolide, IAA and GA₄ in the stimulation of hypocotyl sections during 24 hr treatment.

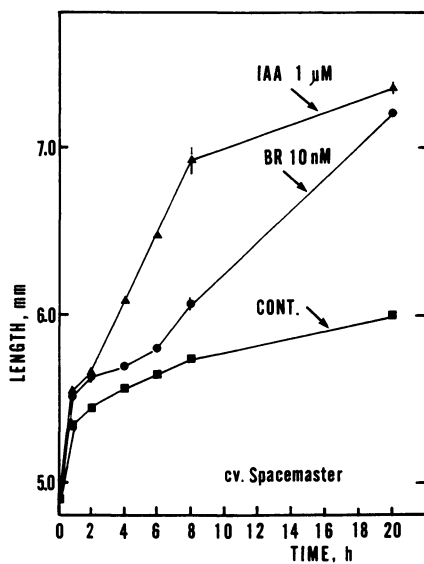


Figure 3. Time-course responses of hypocotyl sections to brassinolide and IAA.

the time course curve (Figure 3). In this figure, the curve starts to bend toward the negative side at 6 h. In fact, if incubation is continued beyond 6 h, BR-induced negative curvature is observed.

Simultaneous application of BR and IAA results clearly and drastically in synergism. The turning point of BR+IAA is much earlier than that with IAA alone, and the degree of curvature is also larger for BR+IAA.

Sequential Interaction. Synergism between two hormones is sometimes more clearly observed in sequential treatments of the two hormones. Thus, GA pretreatment always results in synergistic enhancement of IAA-induced elongation of hypocotyl sections (37). Sequential interaction between BR and IAA is very similar to that between GA and IAA (8). Sections pretreated with BR for 2 h respond to IAA synergistically. The reverse order treatment is rather inhibitory as is the case for GA. Therefore, both BR and GA seem to sensitize hypocotyl sections to a later response by IAA. These facts suggest that cells which have already been initiated to elongation by IAA are not the target cells of BR/GA.

Although BR and GA behave very similarly in their relationship to IAA, the mechanisms of how the two hormones act are probably different from each other. First, the GA-pretreatment effect can be reduced by a high concentration of mannitol, while the BR pretreatment effect is not much affected by mannitol. Second, the GA-pretreatment effect is diminished by washing pretreated sections before IAA treatment, while the BR pretreatment effect is not (8). Third, the interaction of BR and GA in their pretreatment effects is simply additive (8).

Proton Secretion.

Auxin is known to stimulate proton secretion from the cytosol to the cell wall matrix. BR also does the same in cucumber hypocotyl sections as has been reported for other plant materials (16-20). When sections with the epidermis peeled off are incubated in a weakly alkaline buffer, the pH of the buffer drops considerably, indicating that protons are secreted from the tissue to the medium. BR at 10 nM and IAA at 10 μ M are almost equally effective (Figure 5). The interaction of BR and IAA is rather inhibitory at the early period of incubation. However, proton secretion continues longer in the presence of both BR and IAA, and finally exceeds those by BR or IAA alone. BR behaves similarly to IAA in this effect.

Conclusion

The experimental results on BR behavior described above are summarized in Table I in comparison with those of IAA and GA. BR elicits physiological effects that are very similar to those of other plant hormones, in particular, auxin and GA. However, the behavior of BR is unique and different from those of the others. The facts that BR modifies plant growth by itself or in association with other hormones, that its effective concentration is very low (10nM), that it has different modes of action, and that it occurs widely in the plant kingdom strongly indicate that BR is a new plant hormone which is probably essential for plant growth and development.

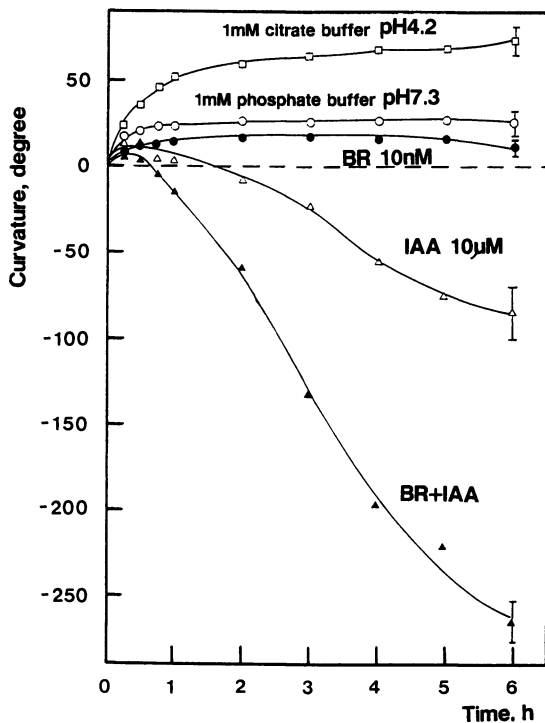


Figure 4. Time-course responses of one-side peeled hypocotyl sections to brassinolide, IAA, IAA+brassinolide and acidic pH.

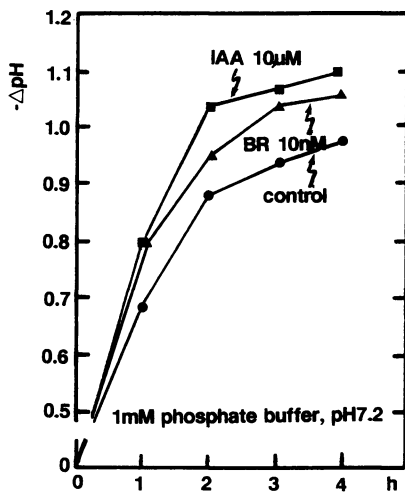


Figure 5. Effects of Brassinolide and IAA on proton secretion from peeled hypocotyl sections.

Table I. Comparison of the Modes of Actions of BR, IAA and GA4

	BR	IAA	GA4
<i>Hypocotyl Sections</i>			
Effective conc. range	0.1nM - 10nM	100nM - 100µM	>100nM
Optimal concentration	100nM	10µM	>100µM
Tissue age-Response	young > old	old > young	young > old
Interaction of BR with	-----	synergistic	additive
Interaction of IAA with	synergistic	-----	synergistic
Pretreatment for IAA	synergistic	-----	synergistic
Pretreatment for BR/GA	-----	slightly inhibitory	-----
Antiauxin	inhibitory	antagonistic	inhibitory
Kinetin	inhibitory	inhibitory	inhibitory
Sucrose (50mM) in the light	promotive	inhibitory	promotive
DCCD	inhibitory	inhibitory	no effect
<i>Intact Seedlings</i>			
Hypocotyl growth	promotive	promotive	highly promotive
Cotyledon expansion	promotive	slightly inhibitory	highly promotive
Advent. root formation	inhibitory	promotive	inhibitory

Acknowledgment

The author wishes to thank ZEN-NOH Agricultural Technical Center, Kanagawa for their kind supply of brassinolide and for providing us with a research grant.

Literature Cited

1. Grove, M.D.; Spencer, G. F.; Rohwedder, W.K.; Mandava, N.B.; Worley, J.F.; Warthen, J.D., Jr.; Steffens, G.L.; Flippen-Anderson, J.L.; Cook, J., Jr. *Nature* **1979**, *281*, 216.
2. Yokota, T.; Takahashi, N. In *Plant Growth Substance 1985*; Bopp, M., Ed.; Springer-Verlag: Berlin/Heidelberg, **1986**; pp.124-38.
3. Mandava, N.B. *Ann. Rev. Plant Physiol.* **1989**, *39*, 23.
4. Meudt, W.J. In *Ecology and Metabolism of Plant Lipids*; Fuller, G.; Nes, W.D., Ed.; ACS Symposium Ser. No. 325; ACS: Washington, DC, **1987**, pp.53-75.
5. Yopp, J.H.; Mandava, N.B.; Sasse, J.M. *Physiol. Plant.* **1981**, *53*, 445.
6. Mandava, N.B.; Sasse, J.M.; Yopp, J.H. *Physiol. Plant.* **1981**, *53*, 453.
7. Gregory, L.E.; Mandava, N.B. *Physiol. Plant.* **1982**, *54*, 239.
8. Katsumi, M. *Plant Cell Physiol.* **1985**, *26*, 615.
9. Sasse, J.M. *Physiol. Plant.* **1985**, *63*, 303.
10. Sala, C.; Sala, F. *Plant Cell Rep.* **1985**, *41*, 144.
11. Takematsu, T.; Takeuchi, Y.; Koguchi, M. *Chem. Regul. Plants*, **1983**, *18*, 2. (in Japanese)
12. Hewitt, F.R.; Hough, T.; O'Neill, P.; Sasse, J.M.; Williams, E.G.; Rowan, K.S. *Aust. J. Plant Physiol.* **1985**, *12*, 201.
13. Wada, K.; Marumo, S.; Ikekawa, N.; Morisaki, M.; Mori, K. *Plant Cell Physiol.* **1981**, *22*, 323.
14. Takeno, K.; Pharis, R.P. *Plant Cell Physiol.* **1982**, *23*, 1275.
15. Schlagnhauser, C.D.; Arteca, R.N. *Plant Physiol.* **1985**, *78*, 300.
16. Cerana, R.; Bonetti, A.; Marre, M.T.; Romani, G.; Lado, P.; Marre, E. *Physiol. Plant.* **1983**, *59*, 23.
17. Romani, G.; Marre, M.T.; Bonetti, A.; Cerana, R.; Lado, P.; Marre, E. *Physiol. Plant.* **1983**, *59*, 528.
18. Cerana, R.; Colombo, R.; Lado, P. *Physiol. Veg.* **1983**, *21*, 875.
19. Cerana, R.; Lado, P.; Anastasia, M.; Ciuffreda, P.; Allevi, P. *J. Plant Physiol.* **1984**, *114*, 221.
20. Cerana, R.; Spelta, M.; Bonetti, A.; Lado, P. *Plant Sci.* **1985**, *38*, 99.
21. Mandava, N.B. *Physiol. Plant.* **1983**, *59*, 539.
22. Braun, P.; Wild, A. *J. Plant Physiol.* **1984**, *116*, 189.
23. Kaeinich, J.F.; Mandava, N.B.; Todhunter, J.A. *J. Plant Physiol.* **1985**, *120*, 207.
24. Katsumi, M.; Kazama, H. *Bot. Mag. Tokyo* **1978**, *Special Issue 1*, 141.
25. Kazama, M.; Katsumi, M. *Plant Cell Physiol.* **1973**, *14*, 449.
26. Katsumi, M.; Purves, W.K.; Phinney, B.O. *Physiol. Plant.* **1964**, *18*, 550.
27. Nakayama, M.; Yamane, H.; Yamaguchi, I.; Murofushi, N.; Takahashi, N.; Katsumi, M. *J. Plant Growth Regul.* **1989**, *8*, 237.
28. Katsumi, M.; Chiba, Y.; Fukuyama, M. *Physiol. Plant.* **1969**, *22*, 993.
29. Gregory, L.E.; Mandava, N.B. *Physiol. Plant.* **1982**, *54*, 239.
30. Takematsu, T.; Takeuchi, Y.; Choi, C - D. *Shokuchō* **1986**, *20*, 2. (in Japanese)
31. Kazama, H.; Katsumi, M. *Plant Cell Physiol.* **1973**, *14*, 449.
32. Kazama, H.; Katsumi, M. *Plant Cell Physiol.* **1976**, *17*, 467.
33. Katsumi, M.; Kazama, H. *Plant Cell Physiol.* **1978**, *19*, 107.
34. Cohen, J.D.; Meudt, W.J. *Plant Physiol.* **1983**, *72*, 691.
35. Eun, J.-S.; Kuraishi, S.; Sakurai, N. *Plant Cell Physiol.* **1989**, *30*, 807.
36. Katsumi, M. *Plant Cell Physiol.* **1976**, *17*, 139.
37. Kazama, H.; Katsumi, M. *Plant Cell Physiol.* **1974**, *15*, 307.

RECEIVED May 14, 1991

Chapter 22

Brassinolide-Induced Elongation

Jenneth M. Sasse

School of Botany, University of Melbourne, Parkville, Victoria,
Australia 3052

The promotive effects of brassinosteroids on the elongation of vegetative tissue have been observed in many species, but only a few have been studied in detail. The most responsive zone in stem tissue from etiolated dwarf pea is the transition from hook to elongating and laterally enlarging stem. BR affects the morphology of segments from this zone, promoting elongation and maintaining a narrow basal diameter, in contrast to the effects of other regulators. Treatment with the recognised plant hormones affects the elongation induced by brassinolide; gibberellin has an additive effect and zeatin an inhibitory one. With auxin, there is a synergism where brassinolide allows auxin to induce elongation when it is ineffective alone. Exogenous auxin affects the kinetics of the response to brassinolide, but the synergism cannot be described by a sensitivity parameter estimator program. However, the reported synergism in cucumber can be attributed to an increase in the amplitude of the response to auxin. This is also the parameter affected when brassinolide-induced elongation in pea is inhibited by abscisic acid. Ethephon can overcome brassinolide-induced elongation, while colchicine and specific inhibitors of protein and nucleic acid synthesis are also potent inhibitors. They are not competitive inhibitors, and elongation continues in the presence of an inhibitor of DNA synthesis. The data suggest mRNA synthesis and/or maintenance is essential for brassinolide-induced elongation and in aged pea segments several protein bands are affected by brassinolide in the early stages of elongation. Brassinolide-induced elongation can be markedly retarded by an inhibitor of cellulose biosynthesis, contrasting with reported auxin effects. Also, diagnostic concentrations of the anti-auxin, *p*-chlorophenoxybutyric acid, do not affect BR-induced elongation, so it is proposed that BR does not depend on auxin as a mediator in the promotion of elongation in younger tissue.

In the first recognition of brassinosteroid-type activity (1), a marked promotion of elongation was observed in an assay then used for gibberellins, and this was confirmed with pure brassinolide (BR) (2). Promotion of elongation has now been observed in many species, and it is probable that BR's effect on the elongation of young vegetative tissue is general. The work of Sala and Sala (3) showed that BR can impose an elongated morphology on auxin-starved single cells of carrot in culture, but in a whole plant, final height and form are likely to be the result of many influences, such as hormones, nutrient and water status, and environmental effects, with complex interrelationships between them.

However, it is possible to study their effects in stem tissue that is still plastic, and it may be possible in future to use such data to model extension growth in vivo. If members of the brassinosteroid family can be shown to be present at appropriate levels in responsive tissue, together with their as yet putative receptors, the potent promotive effects of these compounds on elongation will need to be included in any such models.

The most brassinolide-responsive zone in greening dwarf pea tissue is in the transition from hook to laterally enlarging and elongating stem (4). BR affects the morphology of segments from this zone, promoting elongation and maintaining a narrow basal diameter, in contrast to the effects of the recognised plant hormones (5). The segment itself is unusual in its slight relief of tension, and its lack of specific responses to plant growth regulators when split longitudinally (6). The data suggest the limiting effect of the epidermal layers and the differing sensitivities to auxin in the epidermis and cortex (7) have not yet developed in this segment from the upper stem.

Effects of other plant hormones

BR-induced elongation in the segment can be markedly inhibited by cytokinin (5), abscisic acid (ABA) (8) and ethephon (6). There was no evidence suggesting competitive inhibition by these regulators but analysis and comparison of data sets using the parameter estimator program (PEST) of Weyers et al. (9) showed that the ABA effect was not just subtractive. The difference between the data sets could be accounted for by a change in the difference between the minimum and maximum response rates (the parameter R_{AMP}), suggesting that the response capacity of the tissue to BR was reduced. With ethephon, there was a complex interaction where only partial recovery occurred with increasing concentrations of BR, while the effect of increasing concentrations of ethephon on BR-induced elongation was antagonistic (6). The inhibition of BR-induced elongation by the cytokinin, zeatin, is accompanied by a synergistic enhancement by BR of the lateral enlargement of stem segments induced by cytokinin. Lateral enlargement induced by IAA can also be synergistically enhanced by BR (5). Thus, if BR plays an endogenous role in the control of elongation and the morphology of the intact plant, the recognised hormones could modulate the effects of BR, and it in turn could enhance their effects. Segments incorporating both GA_1 - and BR-sensitive tissue show an additive effect for these two regulators (5,8), confirming the effects observed with

pea tip segments (10), and PEST analysis showed the only difference between data sets was in the values for the minimum rates of response (8).

Synergism with auxin. Hook sections from etiolated dwarf peas, which do not respond to exogenous IAA alone, show a synergistic response with combinations of BR and IAA (11), and this can be confirmed with the larger stem segments that incorporate more mature stem tissue as well (5). Other auxins also show this response, and, as the auxin concentration increases, elongation can increase synergistically then decrease (6) suggesting that the hormones reach supraoptimal levels, perhaps inducing ethylene biosynthesis in the tissue. Also, if the efflux of endogenous auxin is reduced by treating the segments with phytohormones without intrinsic auxin activity, there is a significant increase in elongation (6). Thus, if BR is supplied, IAA can enhance elongation in conditions where it is ineffective alone.

A synergism between IAA and BR was also described by Katsumi (12) in green cucumber hypocotyl segments, and analysis by the PEST program showed the difference between data sets for IAA alone and IAA with fixed concentrations of BR can be accounted for by a change in the parameter R_{AMP} , suggesting that the response capacity of the tissue to IAA is enhanced by BR (8). Possible explanations for this effect could be increased numbers of receptors for IAA, amplification of the IAA-induced signal, or its transmission, increased transcription or translation rates for IAA-induced protein synthesis, decreased turnover of mRNA or proteins, increased rates of delivery of cell wall components, etc. Much more research is needed to examine these possibilities.

Changes in the other parameters calculated by PEST might also account for a synergistic response, e.g., a steeper dose/response curve (increasing p values) or a shift of the concentration for 50% relative response ($[H]_{50}$) to lower values. In the pea stem segments, very slight alterations in the concentration of the exogenous auxin can affect the kinetics of the BR-induced elongation response markedly. With very low concentrations of auxin (0.05 μ M 4-chloroindole-3-acetic acid or 0.01 μ M IAA) and a concentration range of BR, a significant change in the parameter $[H]_{50}$ was observed, but it was not in the direction of a positive synergism, as the value of $[H]_{50}$ increased (Figure 1). If physico-chemical meaning can be ascribed to the parameters calculated by PEST (9), very low levels of auxin might affect the binding of BR and its dissociation to and from a receptor - investigation of such a possibility must wait on the isolation and characterization of such BR-receptors. High concentrations of auxin gave data sets whose dose/response curves were not suitable for analysis by PEST, and the clear synergisms seen in the pea stem segment with conventional experimental protocols could not be detected by the PEST program.

It has been proposed using data from bending responses (13,14,15) that responses induced or accelerated by BR require the presence of some auxin, either exogenously or endogenously; if this applied to extension growth, the characteristics of sustained auxin-induced growth should be observed after BR treatment, e.g., antiauxins should reduce the BR-induced elongation. Diagnostic

concentrations (where the compound is not toxic to the tissue (16,17)) of the auxin antagonist, p-chloroisobutyric acid (PCIB), do not affect BR-induced elongation in the pea stem segment (6). This is in contrast with the data of Katsumi (12), but there, more mature, auxin-sensitive green tissue was used, and BR may have been accelerating the auxin response. The results of Katsumi's study of the order and timing of application of the hormones is consistent with this idea. Also, sustained auxin-induced elongation is remarkably insensitive to inhibition of cellulose biosynthesis by treatment with 2,4-dichlorobenzonitrile (18), but BR-induced elongation is quickly reduced by very low concentrations of this inhibitor, suggesting cellulose supply rapidly becomes rate-limiting for extension (6). So, taken together with the work of Sala and Sala (3), data from studies with the upper stem segment from pea suggest BR has its own role in elongation, but the mechanism of its effect is as yet unknown.

Extension of responsive cells

Essential to BR-induced elongation is the maintenance of axial growth, rather than isodiametric expansion. Elongation in stem cells correlates with the orientation of arrays of cellulose microfibrils which are in turn correlated with microtubules within the cytoplasm, and hormones such as gibberellin and ethylene are known to affect the orientation of such arrays (19,20). That the correlation between the microfibrils and microtubules may not be causal has been discussed recently, and is receiving some experimental support (21,22), but it is clear that treatment of plant cells with antimitotic drugs that disrupt microtubule polymerization causes isodiametric expansion instead of continuing elongation. BR-induced elongation is no exception (13), and with the lowest effective concentrations of colchicine in the pea stem section there is no evidence for competitive inhibition (Figure 2), suggesting no direct effect of BR on microtubule polymerization. However, the orientation of the microtubule array could still be affected by BR in a manner similar to gibberellin (23).

Cell wall synthesis requires, as well as cellulose, hemicellulose, pectin, lignin precursors and proteins. Lignification and crosslinking of cell wall components limit extension growth, and retardation of these processes (e.g., if peroxidase levels were lower, or lignin precursor concentrations were reduced) could permit continued elongation. Some evidence exists for peroxidase reduction after BR-treatment (24), but detailed studies on the effects of BR on lignification or the crosslinking of extensin have not yet been done. Results with the inhibitors *trans*-cinnamic acid and 2,2'-bipyridyl need very careful interpretation, as these compounds are not really specific inhibitors.

It is known that protein synthesis is necessary for BR-induced effects in root tissue (25), and BR-treatment increases nucleic acid and protein synthesis in bean stem (26). In the pea stem segment, kinetic studies with selected protein and nucleic acid synthesis inhibitors showed no evidence for competitive inhibition in polypeptide chain elongation, post-transcriptional polyA addition to heterogeneous RNA, DNA-dependent RNA synthesis, or of the DNA-dependent RNA polymerase

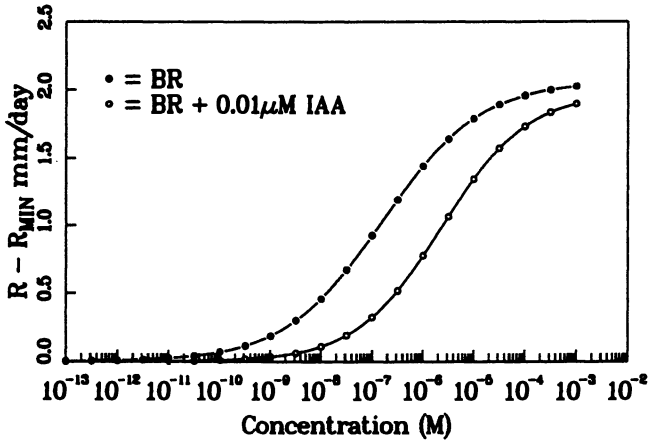


Figure 1. Effect of 0.01 μM IAA on the BR-induced elongation of pea stem segments. Lines are curves of best fit as calculated by PEST, and comparison gave a model where a change in $\ln[H]_{50}$ accounted for the difference between the data sets.

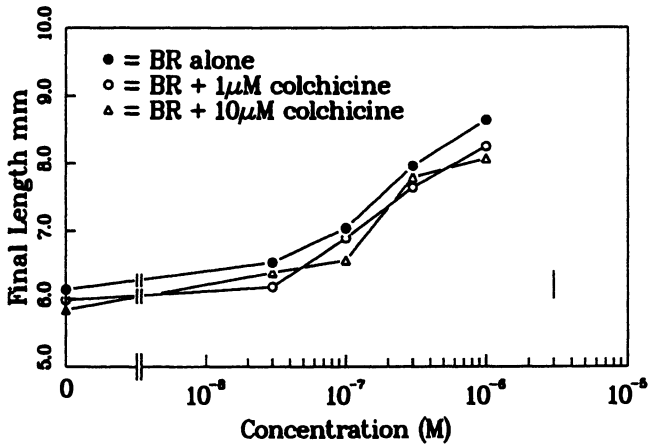


Figure 2. Effect of 1 and 10 μM colchicine on BR-induced elongation of pea stem segments, 14 segments per treatment, 29.5h growth. BR and colchicine effects significant at $p < 0.01$, interaction NS, bar is LSD $P = 0.05$.

II (Figures 3a-e). In all these experiments, BR-induced elongation was reduced markedly by low concentrations of the inhibitors (27) and the data suggested mRNA synthesis, and/or its maintenance, was essential for BR-induced elongation. However, the elongation was unaffected by a high concentration of an inhibitor of DNA synthesis, suggesting that growth in this part of the stem remains predominantly expansionary (28) in the presence of BR.

Specific gene expression

Kulaeva et al. (29) have demonstrated modified gene expression in leaf tissue after administration of BR, with several new proteins being induced, and evidence for concentration effects. Also, Clouse et al (30) have reported new polypeptides, and up and down regulation in auxin-sensitive soybean stem segments treated with BR.

After aging for 24h to reduce endogenous hormone levels, the pea stem segment will still elongate in response to BR, or the gibberellin GA₁, or fusicoccin (all at 1μM), or IAA (10μM) or 2% sucrose (Figure 4). Profiles of the proteins synthesized in the first hours after such treatment show some differences to control after the addition of BR, and time effects (Table I), with similarities to protein profiles after the other elongation-promoting treatments, and sucrose lanes have distinct differences to control or BR. Such experimental tissue is starved tissue, which survives by controlling an autophagic process (31), and it is not known whether the induction of particular hydrolytic enzymes is increased in such a state. There are certainly some clear differences to control when sucrose is provided.

Using all these sources of information, we propose to select particular bands for isolation and further analysis using N-terminal sequencing. Perhaps this will provide us with some indication of the function of early-synthesized protein, and its relationship to the marked elongation induced by BR.

Table I. Protein Profiles from Aged Pea Stem Segments on 1-Dimensional SDS-PAGE Gels

Regime (h)		Comparison with Cont.	~KD	Present in 2% sucrose
BR	+AA			
7	2	2 bands ↑↑	43	Yes
			38	Yes
3	2	1 new or ↑↑	60	Yes
1.5	2	1 new or ↑	47	Yes
2	1	3 new or ↑	28	Yes
			30	Yes
			40	Yes
		3 or more ↓		No, yes, ?
2	2	1 new or ↑	47	Yes

"↑" The intensity of the band is increased.

"↓" The intensity of the band is decreased.

"Yes" The band occurs in the corresponding lane.

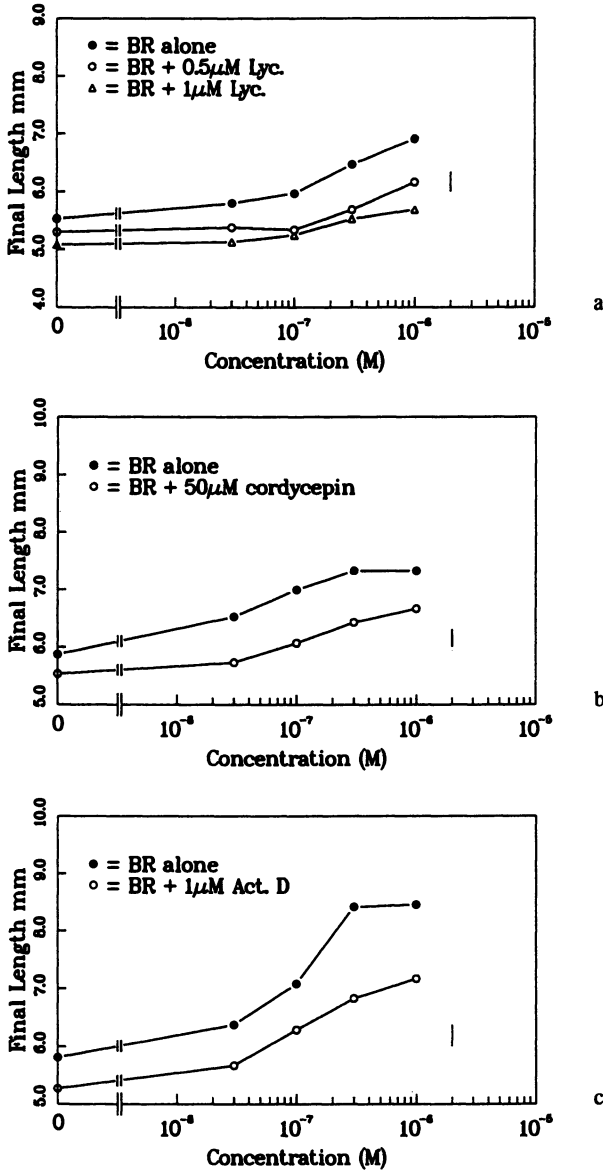


Figure 3. Effect of inhibitors of protein and nucleic acid synthesis on BR-induced elongation of pea stem segments, 15-16 segments per treatment, growth 29-31h. a. Lycoricidinol and BR effects significant at $P < 0.01$, interaction significant at $P < 0.05$. b. Cordycepin, brassinolide and interaction effects significant at $P < 0.01$. c. Actinomycin D and BR effects significant at $P < 0.01$, interaction NS. d. α -Amanitin and BR effects significant at $P < 0.01$, interaction NS. e. BR effect significant at $P < 0.01$, 5-fluorodeoxyuridine and interaction effects NS. Bars are LSDs $P = 0.05$.

Continued on next page

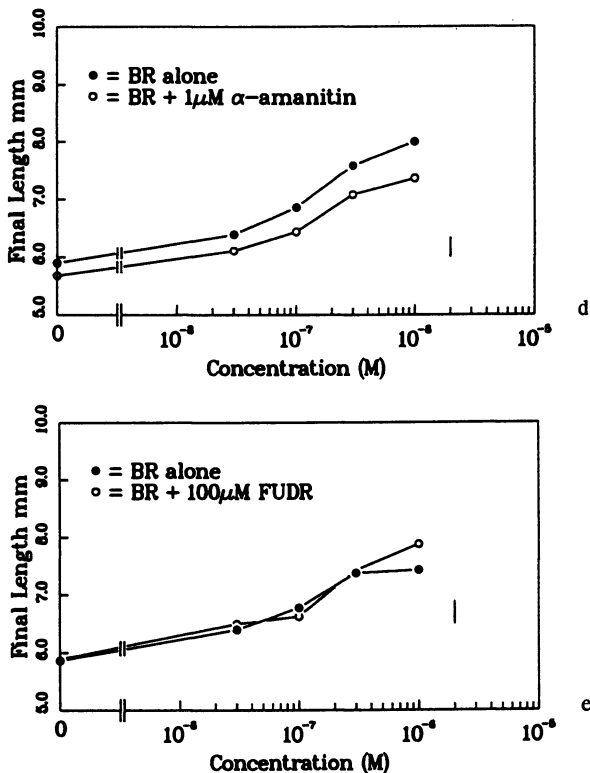


Figure 3. Continued.

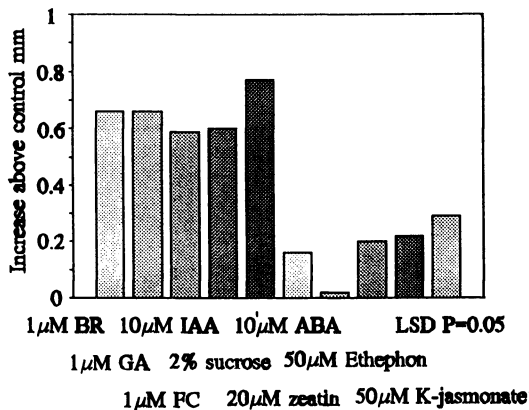


Figure 4. Effects of plant growth regulators on pea stem segments aged for 25h, then grown in hormone solutions for 24h, 12 segments per treatment. Initial value 5.27mm, control length 5.32mm.

Literature Cited

1. Mitchell J.W.; Mandava, N.B., Worley, J.F., Plimmer, J.R., Smith, M.V. *Nature (London)* **1970**, *225*, 1065-66.
2. Grove, M.D.; Spencer, G.F., Rohwedder, W.K., Mandava, N.B., Worley, J.F., Warthen, J.D., Steffens, G.L., Flippen-Anderson, J.L. Cook, J.C. *Nature (London)* **1979**, *281*, 216-17.
3. Sala, C.; Sala, F. *Plant Cell Rep.* **1985**, *4*, 144-47.
4. Sasse, J.M. *1985. Physiol. Plant.* **1985**, *63*, 303-08.
5. Sasse, J.M. *Pro. Plant Growth Reg. Soc. Amer.* **1987**, *14*, 30-39.
6. Sasse, J.M. *Physiol. Plant.* **1990**, *80*, 401-08.
7. Pearce, D.; Penny, D. *Plant Sci. Lett.* **1983**, *30*, 347-53.
8. Sasse, J.M. *Pro. Plant Growth Reg. Soc. Amer.* **1989**, *16*, 82-87.
9. Weyers, J.D.B.; Paterson, N.W. and A'Brook, R. *Plant Cell Environ.* **1987**, *10*, 1-10.
10. Mandava, N.B.; Sasse, J.M. and Yopp, J.H. *Physiol. Plant.* **1981**, *53*, 453-61.
11. Yopp, J.H.; Mandava, N.B. and Sasse, J.M. *Physiol. Plant.* **1981**, *53*, 445-52.
12. Katsumi, M. *Plant Cell Physiol.* **1985**, *26*, 615-25.
13. Yopp, J.H.; Mandava, N.B., Thompson, M.J. and Sasse, J.M. *Pro. Plant Growth Reg. Soc. Amer.* **1981**, *8*, 138-45.
14. Takeno, K.; Pharis, R. *1982. Plant Cell Physiol.* **1982**, *23*, 1275-81.
15. Cohen, J.D.; Meudt, W.J. *Plant Physiol.* **1983**, *72*, 691-94.
16. Burstrom, H. *1950. Physiol. Plant.* **1950**, *3*, 277-92.
17. Cleland, R.; McCombs, N. *Science* **1965**, *150*, 497-98.
18. Brummell, D.A.; Hall, J.L. *Physiol. Plant.* **1985**, *63*, 406-12.
19. Hardham, A.R. In *The Cytoskeleton in Plant Growth and Development*; Lloyd, C.W. Ed.; Academic Press: London, 1982; pp 377-403.
20. Lloyd, C.W. *Annu. Rev. Plant Physiol. Plant Molec. Biol.* **1987**, *38*, 119-39.
21. Preston, R.D. *Planta* **1988**, *174*, 67-74.
22. Emons A.M.C.; Wolters-Arts, A.M.C., Traas, J.A., Derksen J. *Acta Bot. Neerl.* **1990**, *39*, 19-27.
23. Katsumi, M.; Mita, T. *Abstract P69, 12th Internat. Conf. Plant Growth Subst. Heidelberg*, August, 1985.
24. Xu, R.; Zhao, Y. *Zhiwu Shengli Xuebao* **1989**, *15*, 263-67.
25. Cerana, R.; Bonetti, A., Marrè, M.T., Romani, G., Lado, P., Marrè, E. *Physiol. Plant.* **1983**, *59*, 23-27.
26. Kalinich, J.F.; Mandava, N.B., Todhunter, J.A. *J. Plant Physiol.* **1985**, *120*, 207-214; **1986**, *125*, 345-53.
27. Sasse, J.M. *Abstract P04, 12th Internat. Conf. Plant Growth Subst. Heidelberg*, 1985.
28. Adamson, D.; Low, V.H.K., Adamson, H. In *Biochemistry and Physiology of Plant Growth Substances*; Wightman F., Setterfield, G. Eds.; The Runge Press: Ottawa, 1968; pp 505-520.28.
29. Kulaeva, O.N.; Burkhanova, E.A. Fedina, A.B., Danilova, N.V., Adam, G., Vorbrod, H.M., Kripach, V. A. *Dokl. Akad. Nauk SSSR* **1989**, *305*, 1277-79.

30. Clouse, S.; Zurek, D., Hall, A. *Abstract 139, Agrochemical Section, 200th Annual Meeting, Amer. Chem. Soc.* Washington, D.C. 1990.
31. Douce, R.; Bligny, R., Dorne, A-J., Roby, C. 1988. In *Plant Membranes - Structure, Assembly and Function*; Harwood, J.L., Walton, T.J., Eds.; The Biochemical Society: London, 1988; pp 189-99.

RECEIVED March 12, 1991

Chapter 23

Antiecdysteroid Effects of Brassinosteroids in Insects

Klaus Richter¹ and Jan Koolman²

¹Sächsische Akademie der Wissenschaften zu Leipzig, Forschungsgruppe
Jena, PB 322, Jena, O-6900, Germany

²Physiologisch-Chemisches Institut, Philipps Universität Marburg,
Deutschhausstrasse 1, Marburg, W-3550, Germany

Brassinosteroids show striking structural similarities with ecdysteroids, the moulting hormones of insects and other arthropods. When ingested by insects with their food, brassinosteroids are resorbed and were found to affect insect development. In cockroaches the duration of a larval stage was increased. The effect was dependent on the dose and type of brassinosteroid applied. In ecdysteroid bioassays with Dipteran tissues, brassinosteroids exhibited a significant antagonistic activity. This effect was dose-dependent and reversible. Brassinosteroids were also found to have an influence on the electrical activity of insect neurons. When tested in a radioreceptor assay, brassinosteroids showed affinity for the ecdysteroid binding site of the ecdysteroid receptor. These observations indicate that brassinosteroids interfere with the hormone system of ecdysteroids directly at the site of their action. Brassinosteroids may have both ecdysteroid agonistic and antagonistic activities. They represent the first true antiecdysteroids observed. Due to the fact that they are natural products, brassinosteroids are potential candidates for a safer insect pest control.

Brassinosteroids are a family of hormones found in plants in which they are potent growth-promoters (reviews: 1, 2). Ecdysteroids on the other hand are a group of hormones occurring in all phyla of protostomian animals. In arthropods they are involved in the control of growth, morphogenesis, and reproduction (reviews: 3, 4). While brassinosteroids have been detected in plants only, ecdyster-

Dedication: This paper is dedicated to Malcolm J. Thompson, Beltsville, Maryland USA, on the occasion of his retirement. We appreciate his most significant contributions to the chemistry of ecdysteroids and brassinosteroids.

oids were found to occur both in animals (5) as well as in plants (6). Their significance in plants, however, is unknown as yet.

Chemical differences

Brassinosteroids and ecdysteroids show similarities in their chemical structures (Figure 1). Molecules belonging to both groups are poly-hydroxylated steroids with carbonyl function at C-6. Presently, only C₂₈ and C₂₉ brassinosteroids are found in plants, and thus differ from the animal ecdysteroids which contain mostly the 27 carbon skeleton of cholesterol. Interestingly, C₂₇ ecdysteroids are often the major ecdysteroids of plants, but overall there are also a number of C₂₈ and C₂₉ ecdysteroids in plants. Additional differences are:

- * the configuration of the junction between rings A and B:
In ecdysteroids this is usually *cis* (with a 5 β -H), in brassinosteroids *trans* (with a 5 α -H).
- * the existence of a seven membered ring B in some brassinosteroids: In one group of brassinosteroids (typical representative: brassinolide) ring B is extended by an additional oxygen atom due to the introduction of a lactone structure. The other group of brassinosteroids (representative: castasterone) has a six membered ring B resembling ecdysteroids.
- * the stereochemistry of hydroxyls at C2 and C3:
The hydroxyls on ring A have an α - or β -position in brassinosteroids and a β -position in ecdysteroids (with the exception of a few 3-epiecdysteroids).
- * the number and position of hydroxyl groups at ring D and in the side-chain: Brassinosteroids do not have a hydroxyl group in ring D.

The similarity of ecdysteroids and brassinosteroids may cause some difficulties in their definition. Ecdysteroids were initially defined as "all compounds structurally related to ecdysone" (3). This definition would include brassinosteroids. Recently an attempt has been made by Lafont and Horn to redefine ecdysteroids (6). According to their definition one may "distinguish between true ecdysteroids as compounds where the steroid bears a *cis*-fused A/B ring junction, a 7-ene-6-one chromophore and a 14 α -OH (irrespective of activity in a moulting hormone assay); and ecdysteroid-related compounds which do not fulfil all the above criteria (either they lack the 14 α -OH, or the 7-ene, or they present one additional 4- or 14-ene double bond, etc.)" (6). The latter group would include brassinosteroids. Unfortunately, comparable definitions of brassinosteroids are not available so far.

Molecular modelling of ecdysteroids (7) and brassinosteroids is still in its infancy. A comparison of the three dimensional (3D-) structures of the two biologically most active representatives of brassinosteroids and ecdysteroids, i.e. of brassinolide and 20-hydroxyecdysone, suggests that functional groups relevant for bioactivity are found at similar positions in both molecules in spite of the structural differences mentioned above. More detailed comparative studies of the 3D-structures of ecdysteroids and brassinosteroids are eagerly awaited.

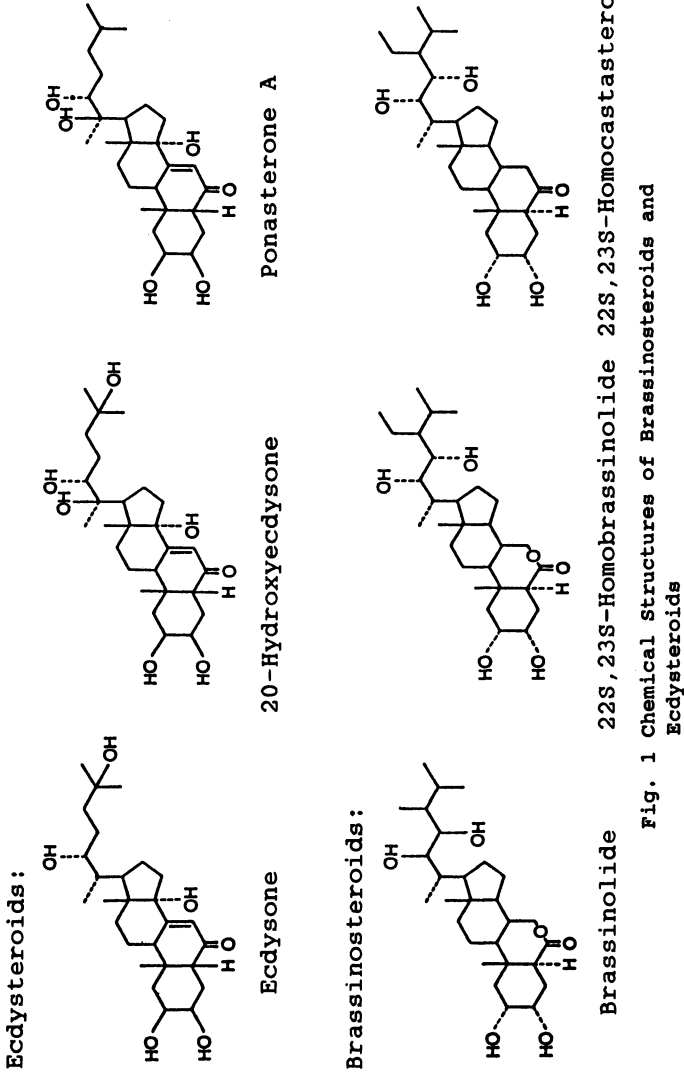


Fig. 1 Chemical Structures of Brassinosteroids and Ecdysteroids

In view of the similarities in chemical structures of ecdysteroids and brassinosteroids, we wondered whether there is a biological relationship between these two hormone systems. Three aspects of this question will be discussed in this chapter:

- * Effects of brassinosteroids in arthropods
- * Effects of ecdysteroids in plants
- * Evolution of the two different hormone systems

Effects of brassinosteroids in arthropods

The concentration of brassinosteroids in plants generally is very low. Observed concentrations of brassinolide range between 10^{-11} and 2×10^{-7} mol/kg (1). The circulating concentrations of ecdysteroids in arthropods lie between 10^{-8} and 10^{-6} mol/kg (8). Assuming a similar biological activity for both groups of steroids it is rather unlikely that insects and other arthropods could be affected by natural brassinosteroids upon direct contact with plants or by ingestion of plant material with the food. The situation may be quite different in agriculture if brassinosteroids are applied artificially to cultivated plants to improve yield and quality of plant products. Such field applications of brassinosteroids would call for a careful analysis of pharmacological and toxicological effects on arthropods and other organisms to which ecdysteroids are essential. While there is no doubt about the hormonal role of ecdysteroids in arthropods (*Chelicerata*, *Crustacea*, *Insecta*), more and more indications accumulate that ecdysteroids may also have hormonal roles in other groups of invertebrates, such as nematodes and molluscs (for a discussion of these aspects see 9). These organisms should be included into ecological considerations when brassinosteroids are applied in agriculture.

Before the effects of brassinosteroids on arthropod systems are reported and discussed we should like to point out that the limited number of observations does not allow a generalisation. Arthropod systems and insect systems in particular are well known for their diversity because of the adaptation of insects to ecological conditions. Many differences have been observed among species and strains with regard to the efficiency of compounds applied in experiments. Adaptability to environmental conditions is highly developed in insects. Nevertheless we feel obliged to come to some conclusions and will regard them as hypothetical as long as they have not been tested on more arthropod species.

Synthetic brassinosteroids or plant extracts containing brassinosteroids have been found to affect insects at different levels:

1. on intact animals when supplied with the food,
2. on isolated tissues under *in vitro* conditions,
3. on particular insect neurons, and
4. on protein molecules.

1. Effects of brassinosteroids on moulting of the cockroach, *Periplaneta americana*. Cockroaches present a well established model for the study of postembryonic development of hemimetabolous insects. These insects are particularly suitable in feeding experiments

because they are easily available and polyphagous (10, 11). The length of the last larval instar of *Periplaneta americana* ranges between 20 and 40 days, indicating some individual variation. However the mean length of the instar among animals from the same batch is constant: Under the experimental conditions employed in our laboratory 50 % have moulted after 30.3 (± 0.7 ; $n = 267$) days. To test the effects of brassinosteroids on moulting initially experiments were performed with crude extracts of rape blossom (*Brassica napus*) (12). Freeze dried blossom was extracted step-wise with ethanol and water. The extracts were used to impregnate food (rat standard food; pulverised) which was dried and then fed to cockroaches beginning on the 11th day of the last larval instar. While food treated with water extract did not have any effect on the length of the instar, food containing ethanolic extract caused a retardation of moulting by about 10 days. This was an increase of the length of the instar by 33 % (Figure 2).

To show that the effects observed with extracts from rape blossom were due to the presence of brassinosteroids synthetic compounds (i.e. pure, defined substances) were applied to the cockroach food. Enteral intake of 22S,23S-homobrassinolide (highest dose: 50mg/4g food/10 larvae) resulted in a delay of the following (imaginal) moult on average by about 11 days (Figure 2). Lower doses had virtually no effect. In analogous feeding experiments 22S,23S-homocastasterone had no effect on the duration of the instar, even at the highest dosages (Figure 2). This is in contrast to the effects of 22S,23S-homocastasterone observed in *Calliphora* (see below).

It is concluded that brassinosteroids applied to insects with food may be resorbed and may have effects. Apparently the effects depend on the species as well as the dose and type of brassinosteroid applied. Selective metabolism of brassinosteroids in the gut cannot be excluded. The overall effect of an active brassinosteroid is a lengthening of the larval stage by a delay of moulting.

2. Effects of brassinosteroids on growth and differentiation of imaginal discs of flies. The development of holometabolous insects is characterised by a series of juvenile stages (larval instars), followed by a puparium stage after which the adult emerges. Adult structures begin to grow in the final phase of the last larval instar from small dormant cell nests, called 'imaginal discs' (13). Imaginal discs are induced by ecdysteroids to develop into organs and structures for the adult insect (legs, wings, genitals, etc.). The process of growth and differentiation of these discs can be studied *in vitro*. Following addition of 20-hydroxyecdysone, the active moulting hormone of insect larvae, or other even more potent ecdysteroids such as ponasterone A, imaginal discs evaginate within a few hours (14). This process can be evaluated quantitatively by morphological as well as by biochemical parameters (Figure 3).

Effects of brassinosteroids on imaginal discs isolated from two different fly species (*Phormia terraenovae*, 15; *Calliphora vicina*, 16, 17) were observed. As expected, the imaginal discs of *Phormia* and *Calliphora* larvae evaginated in response to 20-hydroxyecdysone.

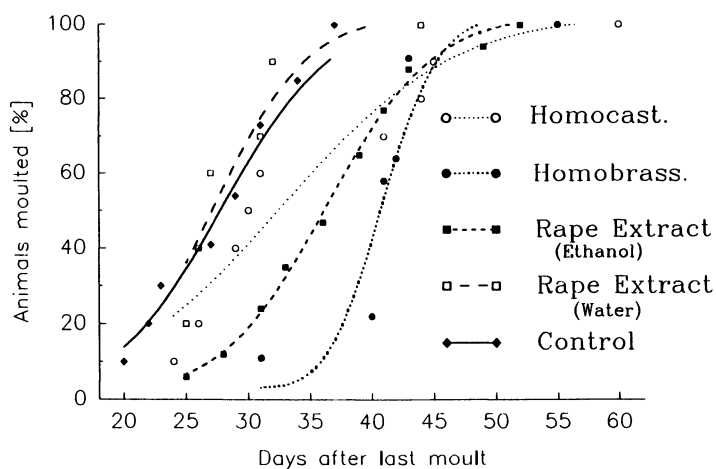
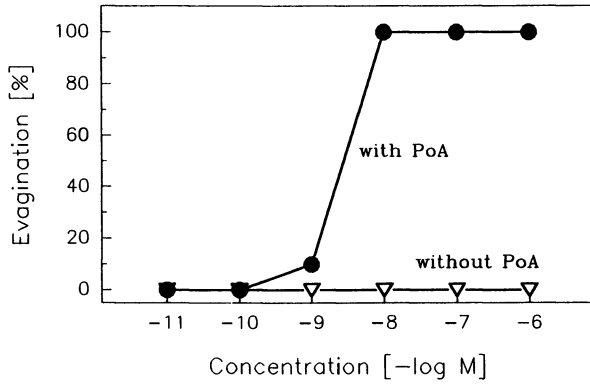


Fig. 2 Effect of Brassinosteroids on Moulting of Cockroaches

The length of the last larval instar of *Periplaneta americana* is given in days. The percentage of individuals who passed the imaginal moult has been recorded. Untreated larvae from the same batch served as controls. 22S,23S-homobrassinolide (Homobrass) and 22S,23S-homocastasterone (Homocast) were applied by oral uptake (in concentrations of 50mg/4g food/10 larvae); rape extract was applied in a concentration corresponding to 6g freeze-dried blossoms/4g food/10 larvae. (after 12). The controls are given as SEM (n = 40)

A: Morphogenesis



B: Protein Synthesis

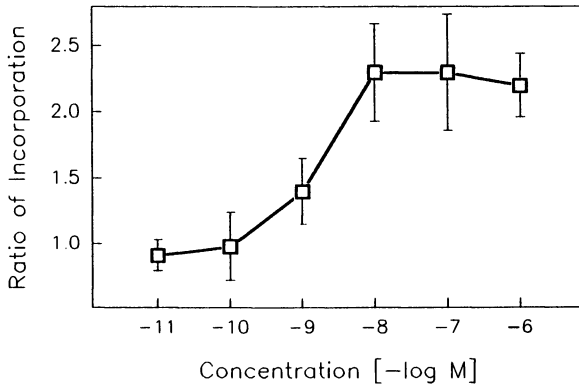


Fig. 3 Effects of Ponasterone A on Imaginal Discs of *Calliphora vicina*

A: Effects on morphogenesis expressed as % evaginated leg discs,

B: Effects on protein biosynthesis detected by incorporation of radiolabeled amino acids and expressed relative to control discs (no ecdysteroid present). (from 16)

The effective concentration to cause a 50 % response (EC50) of *Calliphora* discs was about 5×10^{-8} M. This EC50 lies in the range of the physiological concentration of the hormone (18) and correlates well with the affinity of ecdysteroid receptors for the hormone (K_D 3×10^{-8} M; 19). None of the brassinosteroids tested on imaginal discs of *Calliphora* (in concentrations up to 10^{-5} M) showed any effect similar to 20-hydroxyecdysone (Blattmann and Koolman, unpublished). In *Phormia*, however, homodolicholide and castasterone showed a slight promoting (i.e. agonistic) effect on the evagination of imaginal discs at 10^{-4} M which was near a saturating concentration (15).

To test a possible interference of brassinosteroids with 20-hydroxyecdysone in the induction of evagination (i.e. to test an antagonistic effect), the imaginal discs of *Phormia* and *Calliphora* were incubated in the presence of 20-hydroxyecdysone at a concentration causing half-maximal response (15, 16). The concomitant presence of certain brassinosteroids inhibited the response to 20-hydroxyecdysone. However, effective concentrations of brassinosteroids had to be higher by a factor of 100. In *Phormia* castasterone and 22S,23S-homobrassinolide were found to be most active and five other brassinosteroids also showed some inhibitory activity: 22S,23S-iso-homobrassinolide, homodolicholide, dolicholide, homobrassinolide and brassinolide (decreasing inhibitory activities; 15). In *Calliphora* likewise 22S,23S-homocastasterone was found to have inhibitory activity. Its antagonistic activity (expressed as EC50) was 5×10^{-6} M as compared with an EC50 agonistic activity of 20-hydroxyecdysone at 5×10^{-8} M. In all cases the inhibitory effects of the brassinosteroids could be reversed by increased concentrations of the ecdysteroid (Table).

We conclude that brassinosteroids may have a slight (if any) agonistic activity in a bioassay for ecdysteroids. More importantly, they reveal a significant antagonistic activity. This antagonistic effect is dose dependent and reversible.

3. Effect of brassinosteroids on the spike activity of insect neurons. Moulting in insects is controlled by ecdysteroids (20). The activity of moulting glands that synthesise and secrete ecdysteroids is under control of the brain by humoral and, in some species, by neuronal stimuli of thoracic ganglia (21). Humoral regulation of the molting gland is mediated by the neuropeptide PTH (prothoracicotropic hormone; 22) which is released from the retrocerebral glandular complex. This neurohemal complex is connected with the brain by the *nervi corporis cardiaci*. In larvae of the tobacco hornworm, *Manduca sexta*, transport of the neurohormone PTH has been shown to be mediated by the *nervus corporis cardiaci II* (NCCII; 23). The spike activity of this nerve reflects the rate of transportation of neurosecretory material from the site of its synthesis in the brain to the site of its release in the *corpora cardiaca*.

The spike activity of NCCII itself is inhibited by the presence of 20-hydroxyecdysone. The inhibitory effect reflects a feedback mechanism of ecdysteroids on the system controlling their synthesis

Table. Antagonistic Effects of 22S,23S-Homocastasterone and 20-Hydroxyecdysone on Evagination of Leg Imaginal Discs of *Calliphora vicina* (from 16)

20-Hydroxy- ecdysone	22S,23S-Homocastasterone			
	0 nM	500 nM	5,000 nM	50,000 nM
0 nM	0	0	0	0
5 nM	0	0	0	0
50 nM	62	50	37	11
500 nM	90	80	80	30
5,000 nM	100	100	100	100
50,000 nM	100	100	100	100
	Evagination in [%]			

as was observed in assays with isolated brains of cockroach larvae (24). The addition of brassinosteroids to this test system revealed dose-dependent effects on the efferent spike activity of NCCII comparable to those of 20-hydroxyecdysone. A decrease of spike activity by 50 % could be elicited by a 3-fold higher concentration of 22S,23S-homocastasterone and a 10-fold higher concentration of 22S,23S-homobrassinolide only (Figure 4; Richter, 1990; in press). It is noteworthy that the brassinosteroids tested acted on NCCII like ecdysteroid agonists.

We conclude that brassinosteroids may have an influence on the activity of insect neurons. The assay system revealed an ecdysteroid-like (agonistic) activity of brassinosteroids.

4. Effects of brassinosteroids on ecdysteroid receptors. Ecdysteroid receptors are key molecules mediating the hormonal signal to the site of their action in the cell nucleus of target tissues. Purified ecdysteroid receptors reveal all characteristics of hormone receptors that depend on lipophilic signals: they have a limited capacity for their ligand and show a high affinity as well as selectivity for their ligand (25). We have used an ecdysteroid receptor preparation purified from *Calliphora vicina* larvae (19) to test possible effects of brassinosteroids on the hormone receptor complex. In competition experiments the two brassinosteroids 22S,23S-homocastasterone and 22S,23S-homobrassinolide were found to compete with radiolabeled ponasterone A, which is a ligand able to bind to the ecdysteroid receptor with high affinity (K_D 1×10^{-9} M; 19) (Figure 5). The affinity of 22S,23S-homocastasterone revealed a

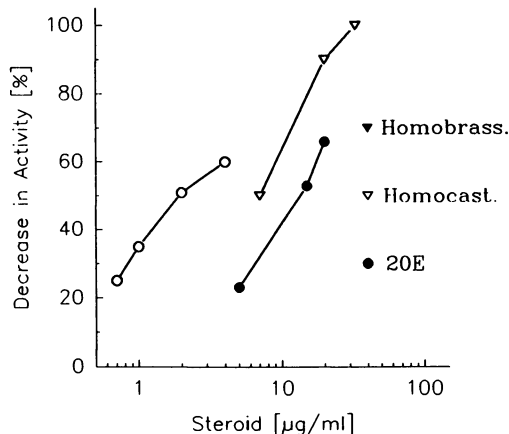


Fig. 4 Neurodepressing Effect of 20-Hydroxyecdysone and Two Synthetic Brassinosteroids on Spike Activity of the Nervus Corporis Cardiaci II of *Periplaneta americana* under *in vitro* Conditions (after Richter, 1990, *in press*)

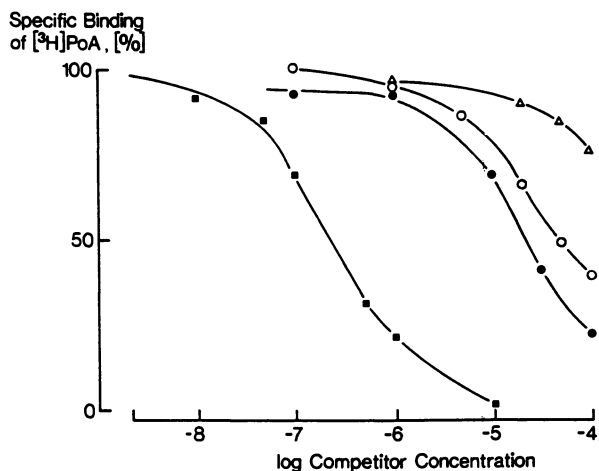


Fig. 5 Competition of Ecdysteroids and Brassinosteroids for the Ligand-Binding Site of the Ecdysteroid Receptor A purified preparation of ecdysteroid receptors from nuclei of third instar larvae of *Calliphora vicina* was incubated with 8 nM tritiated ponasterone A in the presence of increasing concentrations of unlabeled 20-hydroxyecdysone (○), ecdysone (●), 22S,23S-homocastasterone (△), 22S,23S-homobrassinolide (□). After equilibrium had been achieved, specific binding of tritiated ponasterone A was assayed. The amount of tritiated ponasterone A specifically bound in the absence of competitor was defined as 100% (from 26).

K_D of about 5×10^{-6} M. Binding of the two brassinosteroids was reversible.

We conclude from this observation that the effect of brassinosteroids on insects may be explained by their competition with ecdysteroids at the binding site of the hormone receptors. In *Calliphora* brassinosteroids appear to have an antagonistic effect on ecdysteroid systems rather than an agonistic effect. Thus they are the first true antiecdysteroids observed (26).

These experiments with enriched ecdysteroid receptor preparations demonstrate the potential of some brassinosteroids to interact with ecdysteroids at the target site of the hormone. However, this does not exclude other sites of interference with ecdysteroids. Theoretically, brassinosteroids may also affect the hormone system at the site of transportation in the haemolymph, at the sites of enzymatic conversion of ecdysteroids or at the excretory system.

The endocrine system of insects is a potential target for so-called 'third generation pesticides' (27). Compounds which specifically interfere with the insect hormone system would have several advantages over existing pesticides:

- * an improved specificity - the pesticides should only harm insects,
- * a shorter persistence in the environment - the pesticides should be identical or similar to natural, biodegradable substances,
- * a higher activity - the pesticides should interact with signal systems and not with the major pathways of metabolism,
- * an invulnerability to insect resistance - the pesticides should interfere with processes that should not easily be circumvented by evolutionary changes.

With a number of juvenile hormone analogues it could be shown that the concept of insect control by interaction with their hormone system is valid (28, 29). In view of the slow progress with compounds affecting the hormone system of ecdysteroids (30) the introduction of brassinosteroids may give some new impetus to the search for ecdysteroid-based insect growth regulators.

Effects of ecdysteroids in plants. The discovery that insect hormones occur in plants (31, 32) was a real surprise for endocrinologists. In the meantime more than 100 different phytoecdysteroids have been detected (6). Some of them are identical with insect ecdysteroids, some are unique to plant systems. According to Lafont and Horn (6) phytoecdysteroids have been found in most embryophytes, mainly ferns, gymnosperms and angiosperms. Their occurrence in thallophytes has not been reported. The concentration of ecdysteroids in plants may reach or even surpass 1 % of dry weight. Thus ecdysteroids represent the most widespread and quantitatively most abundant family of steroidal animal hormones.

The variability of structures among phytoecdysteroids led to the discrimination between 'true ecdysteroids' and 'ecdysteroid-related compounds' (6; see above). It should be pointed out that these definitions are based on chemical structures and do not discrimi-

nate between hormonally active and inactive compounds. Brassinosteroids in fact would fall into the category of ecdysteroid-related compounds (or *vice versa*). In view of the diversity of ecdysteroids in plants it is most likely that a large array of substances with intermediate structures between ecdysteroids and brassinosteroids occurs in plants. Ajugasterone B, amarasterone A, dolichosterone, and makisterone C are representatives of this group of steroids.

What is the biological significance of 'true' ecdysteroids in plants? Although this question has been asked repeatedly, no firm answer can be given as yet. A few studies indicate that the concentration of ecdysteroids in plants varies with the season (33, 34) and that their composition depends on the physiological state of the plant (35). Unfortunately no evidence is available so far for an endogenous function of ecdysteroids in plants, although some significance is seen in their role as allelochemicals against insects. The occurrence of ecdysteroids in plants would provide a means of protection against 'non-adapted' phytophagous insects; this defence of course had been overcome by 'adapted' insects who during evolution invented means to protect themselves against phytoecdysteroids. This complex aspect of coevolution of plants and insects has been discussed in more detail recently (6, 30).

Evolution of the two different hormone systems. Peter Karlson has postulated that the steroid hormones of animals, i.e. the ecdysteroids, oestrogens, androgens, mineralo- and glucocorticoids, and the seco-steroids derived from vitamin D, evolved from a common progenitor steroid which had the structure of a hydroxylated cholesterol to render it sufficiently water soluble (36). It is evident that the steroid hormones share a high degree of structural similarity. Signal substances with steroid structure are also known from algae: oogoniol and antheridiol. With the inclusion of brassinosteroids one now may assume that the use of steroidal molecules for signal transmission was invented by evolution before plant and animals diverged from protists (protozoa and unicellular algae). This speaks for the conservative maintenance of the early in the evolution introduced and proven principle of steroidogenic regulation. Why nature behaves so conservative is an often debated question (36, 37).

The evolution of hormones can only be seen in the context of the evolution of their entire hormone systems (38, 39, 40). It is too early to speculate whether and how the totally different hormonal systems of ecdysteroids in animals and brassinosteroids in plants derived from a common progenitor system of signal transmission.

Acknowledgements

We thank Professor Adam and his coworkers, Halle, for generous support with synthetic brassinosteroids. We are indebted to Dr. Del Bradbrook for critical reading of the manuscript. The work reported here was supported by grants from the Sächsische Akademie der Wissenschaften (to K.R.), Deutsche Forschungsgemeinschaft and European Community (to J.K.).

Literature cited.

- (1) Adam, G.; Marquardt, V. *Phytochemistry* 1986, 25, 1787.
- (2) Mandava, N.B. *Ann. Rev. Plant Physiol., Plant Mol. Biol.*, 1988, 39, 23.
- (3) *Ecdysone*; Koolman J., Ed., Georg Thieme-Verlag: Stuttgart and New York, Germany and USA, 1989.
- (4) Koolman, J. *Zool. Sci.* 1990, 7, 563.
- (5) Rees, H.H. In *Ecdysone*; Koolman, J., Ed.; Georg Thieme-Verlag: Stuttgart and New York, Germany and USA, 1989, pp 28-38.
- (6) Lafont, R.; Horn, D.H.S. In *Ecdysone*; Koolman, J., Ed.; Georg Thieme-Verlag: Stuttgart and New York, Germany and USA, 1989, pp 39-64.
- (7) Zander, J.; Koolman, J. *Invert. Reprod. Dev.* 1990, 18, 133.
- (8) Steel, C.G.H.; Vafopoulou, X. In *Ecdysone*; Koolman, J., Ed.; Georg Thieme-Verlag: Stuttgart and New York, Germany and USA, 1989, pp 221-231.
- (9) Franke, S.; Käuser, G. In *Ecdysone*; Koolman, J., Ed.; Georg Thieme-Verlag: Stuttgart and New York, Germany and USA, 1989, pp 296-307.
- (10) Richter, K.; Birkenbell, H. *J. Insect Physiol.* 1987, 33, 933.
- (11) *Cockroaches as Models for Neurobiology: Applications in Biomedical Research*; Huber, J.; Masler, E.P.; Rao, B.R., Eds., CRC Press, Boca Raton, U.S.A., 1990, Vol. I/II.
- (12) Richter, K.; Adam, G.; Vorbrodrt, H.-M. *J. appl. Entomol.*, 1987, 103, 532.
- (13) Fristrom, J.W.; Natzle, J.; Doctor, J.; Fristrom, D. In *Metamorphosis*; Balls, M.; Bownes, M., Eds.; The Eighth Symposium of the British Society for Developmental Biology, Clarendon Press, Oxford U.K., 1985, pp. 162-180.
- (14) Chihara, C.J.; Petri, W.H.; Fristrom, J.W.; King, D.S. *J. Insect Physiol.* 1972, 18, 1115.
- (15) Hetru, C; Roussel, J.-P.; Mori, K.; Nakatani, Y. *C.R.Acad. Sci. Paris*, 1986, 302, 417.
- (16) Blattmann, M. Diplomarbeit in Humanbiologie, University of Marburg, 1989.
- (17) Blattmann, M.; Käuser, G.; Koolman, J. *Invert. Reprod. Dev.* 1990, 18, 105.
- (18) Koolman, J.; Walter J. In *Metamorphosis*; Balls, M.; Bownes, M., Eds.; The Eighth Symposium of the British Society for Developmental Biology, Clarendon Press, Oxford U.K., 1985, pp. 198-220.
- (19) Lehmann, M.; Koolman, J. *Mol. Cell. Endocrinol.* 1988, 57, 239.
- (20) Sehnal, F. In *Ecdysone*; Koolman, J., Ed.; Georg Thieme-Verlag: Stuttgart and New York, Germany and USA, 1989, pp 271-278.
- (21) Richter, K.; Käuser, G.; Bidmon, H.-J. In *Ecdysone*; Koolman, J., Ed.; Georg Thieme-Verlag: Stuttgart and New York, Germany and USA, 1989, pp 319-326.
- (22) Ishizaki, H. In *Ecdysone*; Koolman, J., Ed.; Georg Thieme-Verlag: Stuttgart and New York, Germany and USA, 1989, pp 204-210.
- (23) O'Brien, M.A.; Katahira, E.J.; Flanagan, T.R.; Arnold, L.W.; Haughton, G.; Bollenbacher, W.E. *J. Neurosci.* 1988, 8, 3247.

- (24) Richter, G.; Gersch, M. *Zool. Jb. Physiol.* 1981, 85, 412.
- (25) Bidmon, H.-J.; Sliter, T.J. *Inv. Reprod. Dev.* 1990, 18, 13.
- (26) Lehmann, M.; Vorbrod, H.-M.; Adam, G.; Koolman, J. *Experientia*, 1988, 44, 355.
- (27) Williams, C. *Sci. Am.* 1967, 217, 13.
- (28) Sehnaal, F. In *Endocrinology of Insects*; Downer, R.G.H.; Laufner, H., Eds.; Alan Liss, New York, USA, 1983, Vol. I; pp 657-672.
- (29) Bowers, W.S. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*; Kerkut, G.A.; Gilbert, L.I. Eds.; Endocrinology I; Pergamon Press, Oxford, U.K. 1985, Vol. 7, pp. 551-564.
- (30) Richter, K. *Biol. Rundsch.* 1989, 27, 73.
- (31) Nakanishi, K.; Koreeda, M.; Sasaki, S.; Chang, M.L.; Hsu, H.Y.; *Chem. Commun.*, 1966, 915.
- (32) Galbraith, M.N.; Horn, D.H.S. *Chem. Commun.*, 1966, 905.
- (33) Hikino, H.; Okuyama, T.; Jin, H.; Takemoto, T. *Chem. Pharm. Bull.* 1973, 21, 2292.
- (34) Yen, K.-Y.; Yang, L.-L.; Okuyama, T.; Hikino, H.; Takemoto, T. *Chem. Pharm. Bull.* 1974, 22, 805.
- (35) Kholodova, Y.D. *Biokhim. Zhivotn. Chel.* 1987, 11, 27.
- (36) Karlson, P. *Hoppe Seyler's Z. Physiol. Chem.* 1983, 364, 1067.
- (37) Richter, K. *Biol. Rundsch.* 1987, 25, 93.
- (38) *The Evolution of Hormonal Systems*; Gersch, M.; Karlson, P., Eds.; Nova Acta Leopoldina NF 255, Halle, Germany, 1984; Vol. 56.
- (39) Bückmann, D. In *Ecdysone*; Koolman, J., Ed.; Georg Thieme-Verlag: Stuttgart and New York, Germany and USA, 1989, pp 20-26.
- (40) Käuser, G. In *Ecdysone*; Koolman, J., Ed.; Georg Thieme-Verlag: Stuttgart and New York, Germany and USA, 1989, pp 327-336.

RECEIVED June 11, 1991

Chapter 24

Application of 24-Epibrassinolide in Agriculture

Nobuo Ikekawa¹ and Yu-Ju Zhao²

¹Iwaki Meisei University, Iwaki, Fukushima 970, Japan

²Shanghai Institute of Plant Physiology, Academia Sinica, Shanghai 200032, China

Large scale field trials over 6 years have demonstrated significant effect of 24-epibrassinolide, a newly developed alternative to brassinolide, on the production of crop and vegetables. More than 10 % increase in the yield of wheat, corn, tobacco, watermelon, and cucumber was achieved.

Since the structure of a new plant growth substance, brassinolide, was announced by the USDA group in 1979 (1), much effort has been devoted to the synthesis of this interesting steroidal lactone and to the evaluation of its biological activities (2). A number of brassinosteroid analogs have been synthesized in order to elucidate the structure-activity relationship and for use as a standard in analysis of the natural brassinosteroids (3). As a consequence, the structural requirements for biological activity and wide distribution of brassinosteroids in the plant kingdom are quite apparent today (4). Also, extensive research work on the physiological role of brassinosteroids has included the application of analogs in crop production.

The first chemical synthesis of brassinolide was achieved by us in 1980 (5), but its multi-step synthesis process indicated costly preparation for agricultural use. The situation did not change, even after the discovery of many different synthetic routes were discovered. Thus, only a few brassinosteroids candidates have been tested in the field during the past several years.

Since the first promising preliminary results were announced by the USDA group, it has taken ten years to obtain firm evidence to show the utility of brassinosteroids in increasing crop yields and biomass of vegetables, cereal grains and other products. Brassinosteroids may well be the only the practical substances available for use in increasing the crop yields. Our results concerning this topic will now be described.

24-Epibrassinolide

We developed several methods for construction of the side chain and the A/B ring of brassinosteroids, and synthesized more than fifty analogs (6). In order to obtain the structure-activity relationship, those analogs were subjected to bioassay in the rice-lamina inclination (7), *Raphanus*, and tomato tests (8). In general, the structural

requirements necessary for growth-promoting activity of the brassinosteroids in the *Raphanus* and tomato tests are more stringent than for the rice-lamina inclination test. The important structural requirements are 22*R*,23*R*-vicinal diol, 7-oxalactone and 2 α ,3 α -vicinal diol functions. It should, however, be pointed out that the biological activities of the brassinosteroids vary and to some extent depend upon the plant species.

From among the synthetic analogs of brassinolide, we selected 24-epibrassinolide (epibrassinolide hereafter in this text) as a potential candidate in practical use in agriculture because of its biological activity and easy preparation from brassicasterol (1) (9). We previously noted, by gas chromatographic analysis (10), that brassicasterol is a 10-20% component of the sterol fraction of rapeseed oil. This sterol can be obtained in quantity after purification by repeated recrystallization from the sterol mixture (Figure 1) with an appropriate solvent, making it very desirable for use as the starting material. Treatment of its 3-*O*-mesylate with sodium carbonate gave the isoform, which was oxidized with chromic acid, thereby affording the 3,5-cyclo-6-one (2). The compound (2) was derived to the 2,22-dien-6-one (3) by the acid catalyzed isomerization. Treatment of the diene with a catalytic amount of osmium tetroxide, in the presence of *N*-methylmorpholine *N*-oxide, gave a separable mixture of the 2 α ,3 α ,22*R*,23*R*-tetrol (4) and the 2 α ,3 α ,22*S*,23*S*-tetrol (5) in a ratio of 3:5. Baeyer-Villiger oxidation of each compound respectively gave epibrassinolide (6) and its 22*S*,23*S*-epimer (7). The overall yield from brassicasterol was ca. 20% (Scheme 1) (9).

Recently, by the ligand-accelerated osmium catalyzed asymmetric hydroxylation using a cinchona alkaloid derivative, the 22*R*,23*R*-epimer was obtained as the major product of the oxidation (11). Thus, epibrassinolide may be ideal for practical use, and, therefore, is one of the most desirable brassinosteroids because of the ease with which it can be synthesized. This compound showed about one tenth the activity of brassinolide in *Raphanus* and tomato bioassay (8). In field trials, the activity of epibrassinolide was about the same as of brassinolide. Epibrassinolide is a natural sterol. This was confirmed by GC-MS analysis, which identified epibrassinolide, along with co-existing brassinolide, brassinone, and castasterone, from the bee pollen of the broad bean *Vicia faba* obtained in China (12).

Application of Epibrassinolide in Japan

Some preliminary tests of brassinosteroids by the USDA Beltsville group suggested that brassinosteroids can be used for increasing food production (13). This early work led to extensive studies on effects of brassinosteroids on plant growth and application in agriculture which were carried out in Japan, due to the availability of brassinosteroids (14). A significant amount of epibrassinolide was provided *vide supra* for the field tests. The samples of brassinolide and 22*S*,23*S*-homobrassinolide were also supplied for field tests. The basic biological studies on brassinosteroids were started in Japan in 1980 and field tests for practical use have been undertaken since 1983. The results of these studies have been reviewed in Japanese articles (15).

Extensive field trials with epibrassinolide were conducted by Takematsu's group over a several-year period. The results of the effect of epibrassinolide on growth and yield of several important crops such as wheat, rice, rapeseed and soybean were summarized in their recent paper (16).

In the case of wheat, weight increase was analyzed in detail. When a solution of epibrassinolide was sprayed at flowering time, the panicle weight increased by 20-30% on the thirty-fifth day after treatment. The number of seeds per panicle also increased up to 30%. Grain yield increased in the upper, middle and lower portions as well on the panicle of the plants. The greatest increase was observed in the middle portion as shown in Table I (16,17).

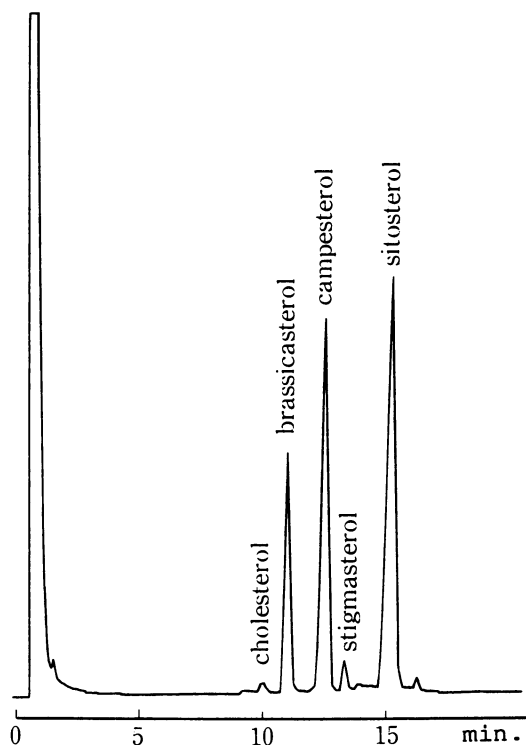
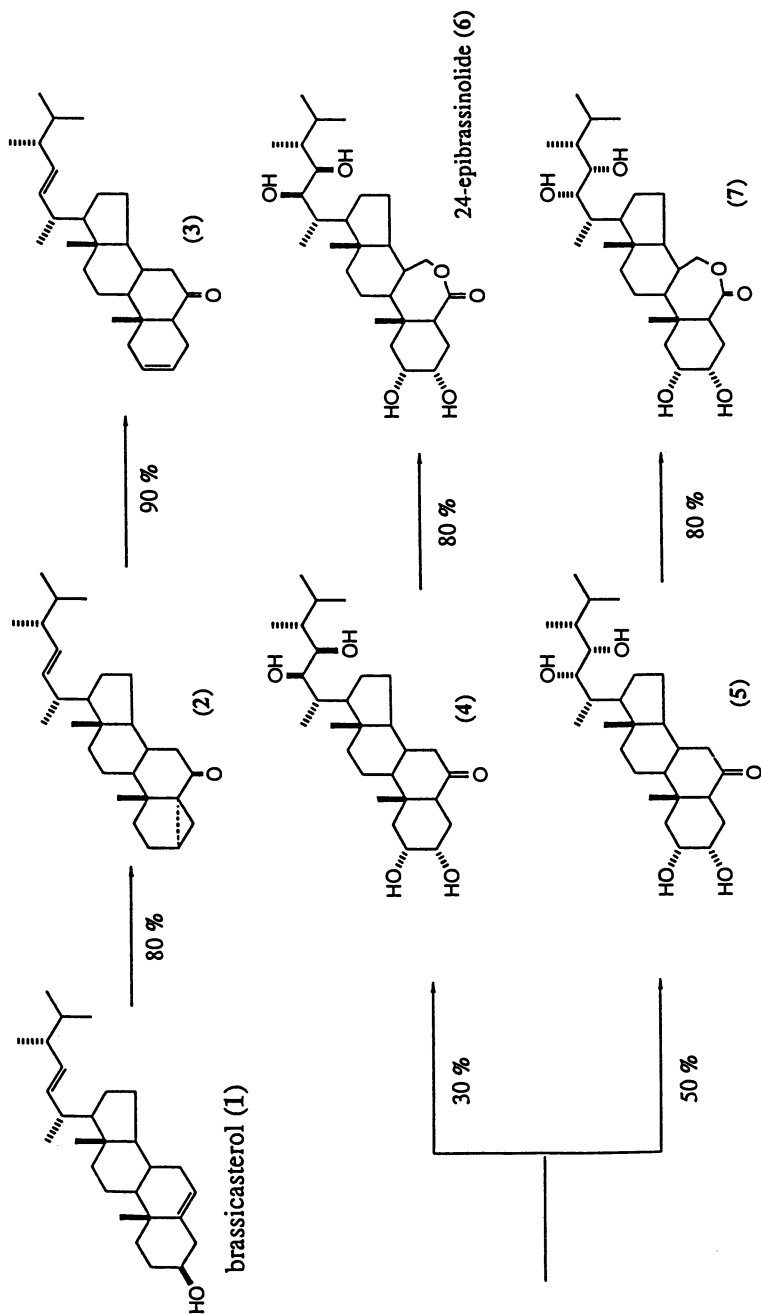


Figure 1. GC Analysis of Rapeseed Oil Sterols
column silica capillary, 30 m X 0.53 mm i.d.,
J & W DB-1, 295°C, He

Table I. Effect of Epibrassinolide Applied at Flowering on Yield of
Wheat

Concentration (ppm)	Weight of Ear (g)	Number of Seeds/Ear	Weight of Spikelet (mg)		
			Lower	Middle	Upper
control	1.08±0.08	22±3	63±4	80±4	41±3
0.001	1.37±0.10	27±2	63±4	80±4	43±2
0.010	1.40±0.05	28±3	76±3	103±3	45±1
0.100	1.38±0.12	29±2	77±2	102±2	45±3
1.000	1.34±0.08	26±2	70±3	93±5	40±2



Scheme 1

The effect of epibrassinolide on the uptake of sucrose into grains of wheat panicles was also investigated. Thus, a panicle collected one day after flowering was cultured in water supplemented with [^{14}C]sucrose and epibrassinolide. After four days, the grain weight and sucrose content of each spikelet were measured. As shown in Figure 2, increases in grain weight and an acceleration of the incorporation of sucrose were observed when compared with the control. The effects were more significant in the upper portion (the 3rd- and 4th grain) than the lower portion (the 1st- and 2nd grain) (18).

Application of epibrassinolide at flowering increased the yield of rice by 11 %. A 10-20 % increase was also observed for the weights of rape seed and soybean (16). Similar promising results were obtained from tests with corn, potatoes, sweet potatoes, spinach, *etc* (15). Occasionally, tests with wheat showed varying results depending on the location.

Long-term tests for practical agricultural application on various plants started in 1985 at many experimental stations, and have so far demonstrated fairly good results generally, as mentioned above.

The effects of brassinosteroids were compared with those of other plant growth substances and the following characteristic features should be pointed out (15):

- 1) Brassinosteroids are active at extremely low concentration, generally 0.1-0.001 ppm solution, which is one hundred time lower range than for other plant growth regulators.
- 2) Brassinosteroids stimulate root growth.
- 3) Brassinosteroids causes no deformity of plant.
- 4) The effect of brassinosteroids on plant growth is particularly strong under adverse growth conditions (*i.e.* suboptimal temperature, salinity), thus, brassinosteroids may be called "stress hormones".
- 5) Brassinosteroids have low toxicity *vide post*.

Agricultural Application of Epibrassinolide in China

Collaborative studies between Japan and China on the practical application of epibrassinolide in agriculture were launched in 1985. The epibrassinolide material was supplied to by us at Tokyo Institute of Technology. A series of preliminary greenhouse tests were carried out at the Shanghai Institute of Plant Physiology. Small and large scale field trials on cereals and vegetables were conducted by the latter Institute. These early studies showed that epibrassinolide accelerated the growth of cereals (wheat and corn), vegetables (watermelon, cucumber and grape), and tobacco. Yields were also improved when epibrassinolide was applied at low concentration at specific growth stages of these plants. Follow up field trials have been pursued at many stations in Shanghai, Henan and Zhejiang Provinces over a five years period. The results of these trials are summarized as follows.

a) Effect on Wheat. Epibrassinolide was sprayed on wheat at its flowering or filling stage in an aqueous solution. Following treatment, the carbohydrates in the flag leaf were analyzed. Table II shows that the carbohydrate content in the flag leaf was decreased, in comparison to the control. This may suggest that carbohydrate transport from leaf to ear is facilitated by epibrassinolide.

When a solution of 0.01-0.05 ppm of epibrassinolide, usually in 50 l per 6.66 a (1 mu in Chinese), was sprayed, the yield of wheat was significantly increased. The ear weight as well as the grain-setting were increased when epibrassinolide was applied at a concentration of 0.01 ppm, as shown in Table III. The results were obtained at two locations in Henan Province in 1986 and 1987. These percentage of grain-set, especially the setting of less viable flowers in the upper part of the ear was increased compared with control. The number of caryopses per spike and the weight of 1000

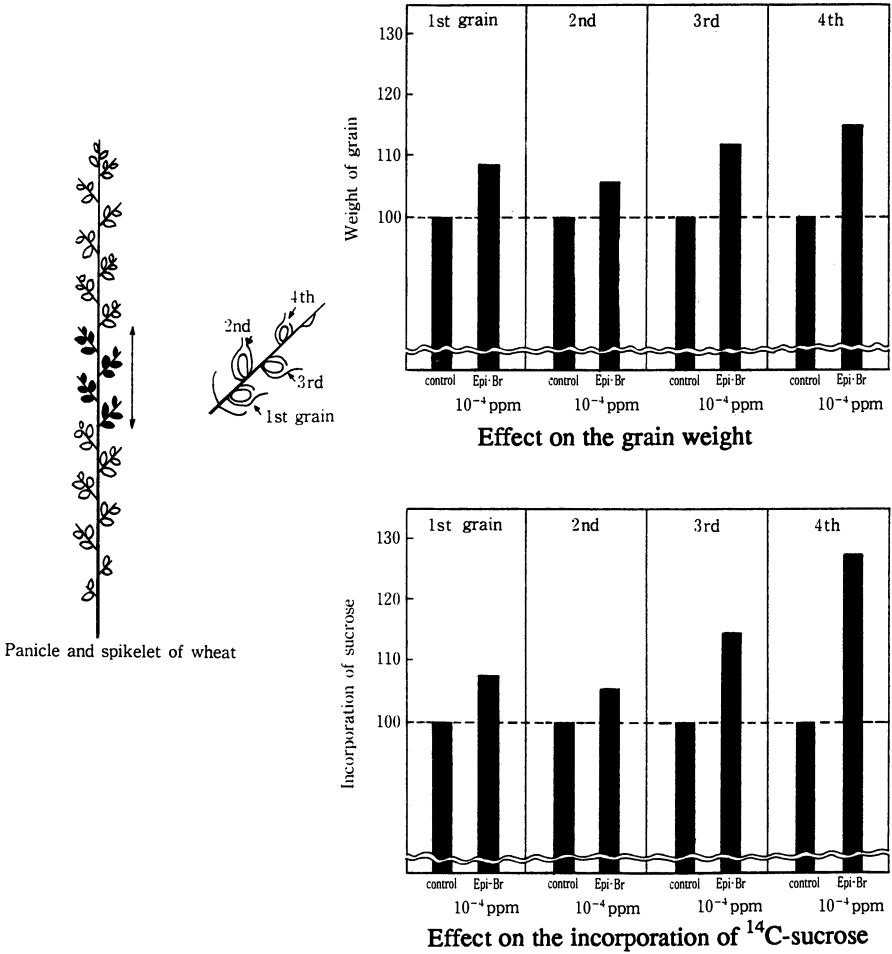


Figure 2. Effect of Epibrassinolide on the Incorporation of ¹⁴C-Sucrose into the Grain

Table II. Effect of Epibrassinolide on Content of Soluble Carbohydrates in Flag Leaf (mg/g)

Test date	A		B		C	
	Control	Epibr	Control	Epibr	Control	Epibr
before spray (April 28)	43.17		39.78		49.50	
spray time (May 5)	80.68		71.67		51.50	
1 week after spray (May 12)	67.73	63.89	55.71	51.28	44.55	41.70
2 weeks after spray (May 19)	55.21	51.19	46.50	40.90	31.70	25.80

Soil with 19 % water, 0.07 % N, 0.154 % P₂O₅ and 1.2 % organic matter was added to a bucket (15 kg soil per bucket). Wheat (Zhengzhou 831) was planted in the bucket (15 plants for each bucket). Epibrassinolide (0.01 ppm) was sprayed. Three different treatments: A. Fertilizing the bucket with 6 g urea. B. Fertilizing the bucket with 4 g urea. C. No urea

Table III. Results of Field Test with Epibrassinolide

Year	Site	Conc. kg/a	Yield (%)	Increase 1000 Grain	Weight (g) of Caryopses/Ear	No. of Caryopses	Fruiting Spikelet	Sterile Spikelet
1986	1	Control	46.49	—	36.20	27.83	15.23	4.40
		0.01 ppm	53.56	15.27	36.00	31.81	16.23	3.71
	2	Control	41.34	—	41.30	31.00	15.50	3.30
		0.01 ppm	45.50	10.06	41.50	33.50	15.40	3.20
1987	1	Control	50.50	—	29.60	31.00	16.30	3.00
		0.01 ppm	56.80	11.10	30.12	34.50	17.30	2.50
	2	Control	66.05	—	42.30	31.20	16.40	4.50
		0.01 ppm	73.53	12.00	45.20	34.30	17.10	2.00

caryopses were also increased. In contrast, infertile spikelets, especially those in the upper part of the ears were reduced significantly. The average increase in crop yield at the two stations was 13%, and these data were obtained from field application over a total area of about 35.6 ha in Henan Province. It should be mentioned that a similar increase in yield was also observed in Shanghai [12.84% (1987) and 9.31 % (1988)], and in Zhejiang [9.95% (1987)], where the climate and the wheat species are different from those in Henan. An overall increase of 18.07% was attained when 0.05 ppm solutions were used in Zhejiang. Figure 3 shows statistical data obtained on wheat at all the test sites in China from 1986 to 1988. Most of the locations demonstrated significant increases ranging from 5 to 15%.

The disease resistance of wheat was also enhanced by treatment with epibrassinolide, *e.g.*, in Henan Province. Leaf wilt of wheat is one of the most harmful effects induced by environmental stress during the period from the filling stage to the ripening stage. Epibrassinolide simultaneously reduced the incidence of this phenomenon and the accumulation of free ammonia and putrescine which are regarded as an indicator of this phenomenon as shown in Table IV.

The effects of epibrassinolide on wheat production in large field tests are summarized in Table V. The total area tested during 1985-1990 was 3,333 ha (50,000 mu). It should be emphasized that the results accumulated from repeated experiments over a period of 6 years from a vast testing area were quite constant and reliable, and the agricultural significance of epibrassinolide is readily confirmed from these data.

b) Effect on Corn. The effects of epibrassinolide on corn were also tested, since corn is an important food and industrial material in China. The corn cultivars used in this study was Danyu 13, which is a high-yield species, although it produces a very serious kernel abortion in the tips of the ear. It was planted on the farm at Henan Agricultural University on May 29 and harvested on September 19, 1987. A completely randomized design was used with six treatments and three replications. It is apparent from Table VI that epibrassinolide increased crop yield, while kernel abortion at the tips of the ear was reduced.

Hamada reported (19) that epibrassinolide sprayed on the ear and silk of corn plants significantly increased crop yield, however, better results were obtained, as shown in Table 6, by spraying prior to the emergence of the tassel rather than after the appearance of the silk in our tests. The improved yield of corn seems to be attributable to the increase of the weight of 1000 caryopses and the number of grains per ear.

The statistical data for corn obtained in all the sites of China from 1986 to 1988 are shown in Figure 4. The increased yields in most tests were about 10-20%. The test consisted of a single spray of 0.01 ppm solution, 50 l per 6.66 a and 1 g of epibrassinolide was sprayed on 120 ha. The total field area tested during 1985-1990 was 2,300 ha (35,000 mu).

c) Effect on Tobacco and Other Plants. Epibrassinolide promoted the growth of tobacco plants when sprayed on leaves. It promoted the growth of leaves and root systems, which are crucial for the synthesis of nicotine, resulting in the improvement of both the quality and quantity of leaves. As shown in Table VII, root weight, leaf area, and nicotine content were increased by treatment with epibrassinolide. Tobacco is an essential economic crop and an export material in China, so that the practical use of epibrassinolide on tobacco will be of a great economic benefit.

Watermelon and cucumber are favorite fruits in Shanghai. Epibrassinolide had a great effect on these plants. The growth of watermelon vines was significantly promoted when epibrassinolide was sprayed on the leaves during the seedling stage and at flowering. Node position of the first pistillate flower along the main vine was also markedly reduced compared with the control. Epibrassinolide promoted fruit setting, thereby increasing the yield of watermelon, 10-20%. Higher concentrations gave a

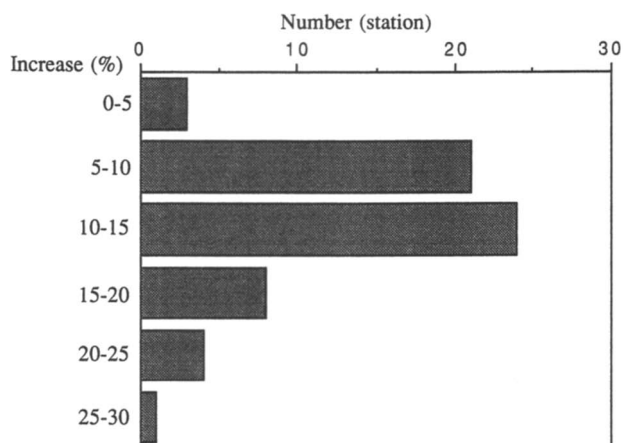


Figure 3. Effect of Epibrassinolide on the Yield of Wheat in China from 1986 to 1988

Table IV. Effect of Epibrassinolide on Content of Free Ammonia and Putrescine and Incidence of Leaf Wilt

Test time		Control	Epibrassinolide
May 25	Incidence of Leaf Wilt (%)	46.15	26.09
May 25	Free Ammonia ($\mu\text{g/g}$)	1314.21	1060.89
May 25	Putrescine ($\mu\text{g/g}$)	734.16	357.22
May 30	Putrescine ($\mu\text{g/g}$)	1290.42	468.37

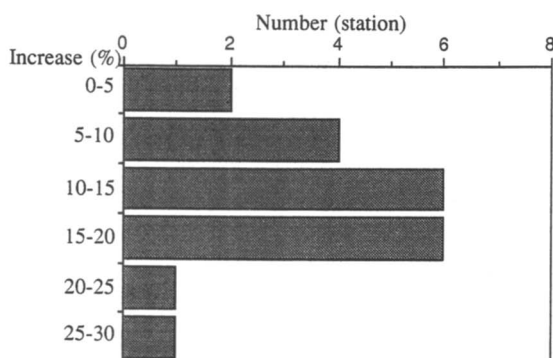
The wheat treated with epibrassinolide (0.01 ppm) on May 5 (Table 2, A) was analyzed on the indicated day.

Table V. Effect of Epibrassinolide on Wheat Yield in China

Year	Field Area	Yield Increase (Average %)
1985-1987	266 ha	>10 %
1988	666 ha	6-15 %
1989	400 ha	11 %
1990	2000 ha	11 %

Table VI. Effect of Epibrassinolide on Abortion of Ear of Corn

Treatment	Abortion Ratio (%)	Yield kg/a	Yield Increase (%)
Control	18.8	2,022	---
Spray once on the ear 3 days after silking	14.7	2,220	9.8
Spray twice on the ear 3 and 10 days after silking	13.7	2,299	13.7
Spray 3 times on the ear 3, 10 and 17 days after silking	12.5	2,323	14.9
Spray 3 times on the whole plant 3, 10 and 17 days after silking	6.5	2,360	16.7
Spray once on the whole plant before the emergence of tassel	4.1	2,393	18.4

**Figure 4. Effect of Epibrassinolide on the Yield of Corn in China from 1986 to 1988****Table VII. Effect of Epibrassinolide on Root Weight, Leaf Area, and Nicotine Content of Leaves in Tobacco**

Treatment	Root Weight (g)	Leaf Area per Plant (cm ²)	Nicotine Content (%)
Control	14.0	6,750	1.4
0.01 ppm	26.8	8,140	2.5
0.05 ppm	20.6	7,398	2.0
1.00 ppm	16.3	6,810	2.1

Tobacco cultivar: NC 89. Epibrassinolide was sprayed on the plant 20, 35 and 50 days after transplant

better result, and occasionally the yield increases reached more than 40% when 0.1 ppm solutions were applied. The yield of cucumber was also markedly increased (10-20%) by the application of epibrassinolide.

When epibrassinolide was applied on grape plants at the flowering stage, the number of grapes per cluster increased significantly and the total yield increased by 66.7 % (0.01 ppm) and 29.9 % (0.1 ppm). This effect may result from the prevention of abscission of the fruit, especially under stress conditions.

Epibrassinolide has been shown to be highly desirable for application to increase crop yields. Farmers in China acknowledge the practical use of epibrassinolide, especially since it is naturally derived and is an effective plant growth-promoting substance. Until now, the practical use of epibrassinolide on wheat and corn has been expand to an area of more than 2,664 ha (40,000 mu) per year.

The effects observed in China were much more remarkable than in Japan. These discrepancies can be attributed to some extent to the differences of the agricultural conditions between the two countries, *e.g.*, the kinds of soils, plant species, cultural practices, temperature difference between day and night time, and so on.

d) Toxicity. The toxicity of epibrassinolide was investigated by researchers at Nippon Kayaku Co. The acute toxicity, LD₅₀, was more than 1,000 mg/kg for the mouse by oral administration, and more than 2,000 mg/kg for the rat both orally and dermally. Fish toxicity, TML48, was more than 10 ppm for carp. The Ames test for mutagenicity was negative and 0.01% solution caused no eye irritation in the rabbit. Therefore, the toxicity is extremely low.

Conclusion

The significant effects of epibrassinolide on plant growth and food production have been observed over the last several years. But, depending on the cultural conditions and method of application, the results were not always remarkable in Japan, compared to those obtained in China. Further studies on improvements in the formulation, the application method, and the application stage or time for the each plant should be investigated. It is anticipated that there will be great benefits for humans by this particularly important substance especially in food production.

Acknowledgments

We extend our sincere appreciation to Professor Emeritus T. Takematsu, Utsunomiya University, and Professor Y. G. Shen, Director of the Shanghai Institute of Plant Physiology, Academia Sinica, for their kind encouragement and stimulating discussions throughout this work. Thanks are also due to Professors K. Kakinuma and Y. Fujimoto, Tokyo Institute of Technology, for their helpful advice.

References

- (1) Grove, M. D.; Spencer, G. F.; Rohwedder, W. K.; Mandava, N. B.; Worley, J. F. *Nature* **1979**, *281*, 216.
- (2) (a) Adam, G.; Marquardt, V. *Phytochemistry* **1986**, *25*, 1787. (b) Yokota, T.; Takahashi, N. In *Plant Growth Substances*; Bopp, M., Ed.; Springer-Verlag: Berlin/Heidelberg, 1986; pp 129. (c) Mandava, N. B. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **1988**, *39*, 23.
- (3) Ikekawa, N. *Trends in Anal. Chem.* **1990**, *9*, 337.
- (4) Ikekawa, N. In *Physiology and Biochemistry of Sterols*; Patterson, G. W. ; Nes, W. D., Eds.; Am. Oil Chem. Soc.: Chapter 12, Brassinosteroids, 1991.

- (5) Ishiguro, M.; Takatsuto, S.; Morisaki, M.; Ikekawa, N. *J. Chem. Soc. Chem. Commun.* **1981**, 962.
- (6) (a) Takatsuto, S.; Ikekawa, N. *J. Chem. Soc. Trans. Perkin I* **1983**, 2133. (b) Takatsuto, S.; Yazawa, N.; Ishiguro, M.; Morisaki, M.; Ikekawa, N. *ibid.* **1984**, 139. (c) Takatsuto, S.; Ikekawa, N. *ibid.* **1984**, 439.
- (7) (a) Takatsuto, S.; Yazawa, N.; Ikekawa, N.; Morishita, T.; Abe, H. *Phytochemistry* **1983**, *22*, 1393. (b) Takatsuto, S.; Ikekawa, N.; Morishita, T.; Abe, H. *Chem. Pharm. Bull.* **1987**, *35*, 211.
- (8) (a) Takatsuto, S.; Yazawa, N.; Ikekawa, N.; Takematsu, T.; Takeuchi, Y.; Koguchi, M. *Phytochemistry* **1983**, *22*, 2437. (b) Takatsuto, S.; Yazawa, N.; Ikekawa, N. *ibid.* **1984**, *23*, 525.
- (9) Takatsuto, S.; Ikekawa, N. *Chem. Pharm. Bull.* **1984**, *32*, 2001.
- (10) Tsuda, K.; Sakai, K.; Ikekawa, N. *Chem. Pharm. Bull.* **1961**, *9*, 835.
- (11) Sharpless, K. B.; Ikekawa, N.; Kim, B. M. unpublished data.
- (12) Ikekawa, N.; Nishiyama, F.; Fujimoto, Y. *Chem. Pharm. Bull.* **1988**, *36*, 405.
- (13) Maugh II, T. H. *Science* **1981**, *212*, 33.
- (14) Marumo, S. In *Proceedings of the 14th Annual Plant Growth Regulator Society of America Meeting*, Honolulu, August 2-6, 1987, pp 174.
- (15) (a) Takematsu, T.; Takeuchi, Y.; Koguchi, M. *Chem. Regul. Plants* **1983**, *18*, 38. (b) Fujita, F. *Kagaku to Seibutsu* **1985**, *23*, 717. (c) Takematsu, T.; Takeuchi, Y.; Choi, C. *Shokuchō* **1986**, *20*, 2. (d) Kamuro, Y.; Takatsuto, S. *Chem. Regul. Plants* **1988**, *23*, 142. (e) Takatsuto, S.; Futatsuya, F. *J. Japan Oil Chem. Soc. (Yukagaku)* **1990**, *39*, 227.
- (16) Takematsu, T.; Takeuchi, Y. *Proc. Japan Acad.* **1989**, *65*, Ser. B, 149.
- (17) Takematsu, T.; Takeuchi, Y.; Ikekawa, N.; Choi, C.; Futatsuya, F.; Shida, A. In *Ann. Meeting of Chem. Regulation Plant*, 1986 Abstract pp 44.
- (18) Shida, A.; Igawa, T.; Seguchi, K.; Futatsuya, F.; Kumura, A.; Takematsu, T.; Ikekawa, N.; Takatsuto, S. *ibid.* 1989, Abstract pp 47.
- (19) Hamada, K. In *Conf. Proc. Int. Seminar Plant Growth Regul.*, Tokyo, 1986 October 15, Abstract pp 1985.

RECEIVED May 1, 1991

Chapter 25

Capability for and Problems of Practical Uses of Brassinosteroids

Yasuo Kamuro¹ and Suguru Takatsuto²

¹BAL Planning Company, Ltd., 2-15-16 Hanaike, Ichinomiya-shi, Aichi 491, Japan

²Department of Chemistry, Joetsu University of Education, Joetsu-shi, Niigata 943, Japan

Brassinolide is known as the most potent compound for inducing plant growth promoting activity in various bioassay systems. Many researchers have attempted to demonstrate brassinosteroid application for agricultural use. However, test results have been disappointing in many cases under field conditions. Here, we discuss the capability and problems surrounding the potential practical uses of brassinosteroids.

Since the isolation and characterization of brassinolide (BR) in 1979 (1), many BR analogs have been synthesized and their physiological activities have been tested in many different bioassay systems (2,3). As a result, it was shown that natural BR dramatically increased growth and consequently plant growth promoting activities of BR have been considered for its potential agricultural use (3-6). Accordingly, BR has been tested widely under practical cultivation conditions. In many field trials, test results proved BR to be ineffective and disappointing. We presume that there are many reasons why field trial results were not effective.

Instability of Brassinolide

One of the most interesting effects of BR application is that of increasing yields of seeds and fruits, and it is well known that the best timing for treatment of BR is during the period of anthesis. In greenhouse tests, plants were selected uniformly so that the treatment timing could be strictly controlled, and good results were obtained. Under field conditions, however, the growing stages of plants are not uniform, and moreover, the flowering period of a community generally lasts from one to a few weeks. Accordingly, the prolonged activity of the compound is a very important factor. So, the instability of BR might be one of the reasons why the results of field trials were not effective as compared with those test results obtained in bioassay and greenhouse conditions. BR could be inactivated in the plant body within one to two days after treatment and there are many test results to support this contention.

We treated with BR at the time when the 1st flower in the flower cluster of tomato plant came into bloom. BR showed distinctly clear effects only in the 1st flow-

er and no effects were observed in flowers that bloomed after the day of treatment. These results indicate that the effect of BR disappears rapidly and the data suggests that BR is not useful for use in practical cultivation, because it would be necessary to frequently apply BR during the flowering period.

It is well known that BR accelerates elongation in young plant. We measured the daily increase in the length of mung bean epicotyls treated with BR. The increase in plant length per day was statistically significant, as compared with that of non-treated plants, up to the 1st or 2nd day, but after the 3rd day, no difference was observed between them. In another test, young soybean plants were treated with BR when the 1st true leaves were young. At 2 weeks after treatment, the fresh weight of the 1st, the 2nd and the 3rd true leaves of BR-treated plants were compared with those of non-treated plants, respectively. As a result, only the 1st true leaf showed significant differences. In many experiments, BR only showed promoting effects for a short period following spray treatment.

BR fed to rice seedlings was rapidly converted into acidic, water-soluble compounds. Radioactivity was found in both a chloroform-soluble fraction and a water-soluble fraction in the ratio 51: 49, after a 5 hr feeding period (7).

These results indicate that BR could be rapidly metabolized in plants. Accordingly, we presumed that for practical use these bioactive compounds had to retain their chemical integrity in order to be practically useful. So, we prepared a number of brassinosteroids with modified structure and screened their biological activities. Our first target compounds for use as long-lasting biologicals which retained activities were compounds which would be gradually transformed into an active form in the plant. We also wanted compounds with appropriate lipophilicity to increase their permeability into plant cells.

Table I shows the activity of BR-analogs in lamina joint inclination test (L. J. test) and the growth of radish plants cultivated under field conditions. Compound No.

Table I. Biological Activities of BR and Its Analogs

Compound	L. J. growth rate (%) (conc. 0.01 ppm)	Radish growth rate (%) (conc. 0.001 ppm)	
		Day after treatment	
		10	25
No. 1	218.0	124.8	108.6
No. 2	190.4	115.2	109.5
No. 3	212.8	122.6	108.9
No. 4	97.1	106.7	109.8
No. 5	92.3	104.8	110.4
No. 6	109.7	102.3	112.1
No. 7	98.3	97.0	110.2
No. 8	103.6	100.4	109.0
No. 9	111.5	96.6	109.1
Cont.	100.0 (68.2°)	100.0 (371 gr)	100.0 (1170 gr)

No. 1; Brassinolide

No. 2; Castasterone

No. 3; 28-Homobrassinolide

No. 4; 28-Homobrassinolide tetraacetate

No. 5; 22*R*,23*R*-Epoxy-28-homobrassinolide 2,3-diacetate

No. 6; 22*R*,23*R*-Epoxy-28-homobrassinolide 2,3-acetonide

No. 7; 22*S*,23*S*-Epoxy-28-homobrassinolide 2,3-acetonide

No. 8; 22*R*,23*R*/22*S*,23*S*-28-homocastasterone 2,3-acetonide

No. 9; 28-Homobrassinolide bisacetone

1 (BR) showed the highest activity in L. J. test. Compound No. 2 (castasterone) and compound No. 3 (28-homoBR) were also active in L. J. test, but compounds No. 4-9 were completely inactive in bioassays. On the other hand, test results obtained under field conditions were quite different from those of the bioassay. Early-maturing radish plants were sprayed with the test compounds 18 days after sowing. The treatment concentration was 0.001 ppm sprayed in water at the rate of 400 liters per acre. At 10 and 25 days after treatment, 15 plants were randomly harvested from each plot and the total fresh weight was measured. At 10 days after treatment, compounds No. 1-3 showed clear growth promoting effects and compounds No. 4-9 showed no effects. At 25 days after treatment, however, compounds No. 4-9 showed growth promotion that was superior to BR during extended cultivation under field conditions.

Table II shows the fruit setting effect of compound No. 1 (BR) and compound No. 6 in tomato. Tomato is one of the most important crops during winter, in Japan. Fruit set is not good in the winter because of low temperatures. So, a fruit setting agent, a phenoxy compound, is widely used. Both compound No. 1 (BR) and No. 6 (0.1 ppm) were sprayed on each flower cluster at the time when the 1st flower in the

Table II. Fruit Setting Effect of Compounds No. 1 (BR) and No. 6 on Tomato

Compound	Flowering day after treatment and average fruit weight (gr)				
	0-1	2-5	6-10	11-15	16-20
No. 1 (BR)	56.2	≈ 0	≈ 0	≈ 0	≈ 0
No. 6	≈ 0	38.8	47.2	46.5	22.6

cluster came into bloom. Each fruit weight was investigated 3 weeks after blooming, respectively. The fruits were classified into 5 groups and the average fruit weight of each group was recorded (Table II). As a result, BR induced a fruit setting effect only in the 1st flower. On the other hand, compound No. 6 was ineffective in the 1st flower, but appeared to be the best fruit setting agent in flowers that bloomed 6-15 days after treatment.

These results suggest that BR was inactivated within a few days but that some BR-analogs could be changed into active compounds within several days following treatment. It could be expected that many more useful compounds could be discovered by modifying the chemical structure of BR for practical horticultural and agronomic use. From this point of view, we suggest that the evaluation methods used to check continuous activity of these compounds are very important. That is, in bioassay systems, the biological activity is generally measured within a few hours or days following treatment. Under practical conditions, crops are cultivated over a period of month.

Environmental Conditions; Temperature

The effects of BR were influenced by environmental conditions, especially by temperature. The growth rate (%) against control generally tended to greater under lower than under optimum temperatures. In many field trials, BR had no significant growth effects under optimum cultivation conditions. This trend existed with respect to plant height, fruit set, grain ripening and so on.

Table III shows the fruit setting effect of BR on tomato under two sets of cultivation; greenhouse conditions during winter and suitable field conditions during summer. BR (0.1 ppm) was sprayed to the flowers and fruit weight was investigated 30 days after the treatment. BR demonstrated a better growth rate (%) against the control in winter than in summer.

Table III. Fruit Setting Effect on BR on Tomato in Different Season

Season	Fruit weight (%) against control	
	0.1 ppm BR	Control
Winter	256.2	100.0 (57.5 gr)
Summer	119.8	100.0 (144.7 gr)

Table IV. Effect of BR on Dry Weight of Leaf and Root in Young Rice Plant under Different Temperature

Conc. of BR (ppm)	D. W. rate (%) against control			
	Leaf		Root	
	15-18°C	25-35°C	15-18°C	25-35°C
1.0	115.2	95.3	114.7	90.4
0.1	111.4	90.2	113.2	96.8
0	100.0 (3.6 gr)	100.0 (5.7 gr)	100.0 (1.7 gr)	100.0 (2.6 gr/5 plants)

Rice plants at 5th true leaf stage were sprayed with 1.0 or 0.1 ppm of BR, and grown under different temperatures; 15°C night-18°C day, or 25°C night-35°C day. The dry weight of leaves and roots were investigated one month following treatment. The growth promoting effects of BR were obvious under low temperature, but not under optimum environmental conditions at 25-35°C (Table IV). Hence, the inductive effects of BR may depend on temperature conditions. In this respect, BR seems to be useful under adverse environmental conditions.

Environmental Conditions; Day-Length and Light-Conditions

In many tests, the positive effects of BR have been clearly demonstrated under white light, green light or weak red light (3,8-12), whereas little or no BR effects have been noted in complete darkness (3,7-11). In our tests, rice plants also showed a different response to BR in the light than in the dark. Roots of young rice plants were dipped in a solution of 0.1 ppm BR and subjected to white light or to complete darkness for 3 days. As a result, the response of the lamina joint inclination was seen only in the light, but not in the dark.

In practical cultivation, the growth promoting effects of BR were also influenced by day-length and light-conditions. BR-analogs (see Table I) were tested on ice seedlings under long-day or short-day conditions. Seeds of rice were dipped for 15 hr in a water solution of 0.1 or 0.01 ppm BR, and sowed in plots containing soil. Seedlings were grown under natural long-day (mid-summer) or natural short-day (mid-winter) conditions. In both tests, the temperature was kept at 20°C. The dry weight per 100 plants of each plot was investigated when plants were at the 6th true leaf stage. The test results are shown in Table V. Under long-day conditions, the effects were distinct, but were not clear under short-day conditions.

Other test results are shown in Table VI. An early-maturing radish variety planted in pots (10 cm diameter and 10 cm depth) was grown in a temperature controlled room under long-day (16 hr day-length) or short-day (8 hr day-length) conditions. Light sources were Mitsubishi-BOC lamps with a photosynthetic photon flux $320 \mu\text{mol m}^{-2} \text{s}^{-1}$ (20 klux) at the plant top level. The wavelength was 400-700 nm. The temperature was kept at 25°C. A water solution (2 ml/plant) of 0.1 ppm BR was sprayed at the 4th true leaf stage. The treatments were applied twice; the 2nd time was

Table V. Growth Promoting Effects of BR Analogs in Rice Seedlings under Long-Day or Short-Day Conditions

Compound	D. W. rate (%) against control			
	Long-day		Short-day	
	0.1 ppm	0.001 ppm	0.1 ppm	0.001 ppm
No. 1	104.2	104.9	97.6	98.4
No. 2	102.1	109.3	102.8	107.4
No. 3	108.4	112.4	98.5	102.3
No. 4	109.5	113.6	95.6	99.0
No. 5	107.6	115.8	100.9	104.5
No. 6	111.9	117.3	94.3	95.6
No. 7	106.4	108.5	94.7	96.2
No. 8	111.4	107.2	93.4	96.0
No. 9	104.0	107.2	103.3	104.6
Cont.	100.0	100.0	100.0	100.0
	(3.21 gr/100 plants)		(2.37 gr/100 plants)	

Table VI. Growth Effects of BR in Radish Plant under Long-Day or Short-Day Conditions

Condition	Conc. (ppm)	F. W. rate (%) against control		
		Leaf	Root	Total
Long-day	0.1	111.7	110.0	111.1
	0	100.0	100.0	100.0
		(161.5 gr)	(93.4 gr)	(254.9 gr/10 plants)
Short-day	0.1	109.7	70.0	97.0
	0	100.0	100.0	100.0
		(102.0 gr)	(47.3 gr)	(149.3 gr/10 plants)

carried out 7 days after the first treatment. The fresh weight of leaves and roots per 10 plants was measured 11 days after the first treatment. As shown in Table VI, the effects

of BR were fairly different between the two conditions. Under long-day conditions, the growth promoting effects of BR on leaves and roots were observed, and the total plant weight clearly increased. On the other hand, even the growth inhibiting effects in roots were recognized under short-day conditions, and total plant weight showed no difference compared with controls. These test results suggest that the growth effects of BR might be dependent upon the photoperiod. Depending upon the conditions, BR might elicit the different effects on plant species depending upon whether long-day plants or short-day plants are used.

We also tested the effects of light quality on BR induced growth. Cuttings of mung bean seedlings were irradiated by white light, or various monochromatic lights, in the presence or in the absence of BR. In the absence of BR, monochromatic red light elicited growth retardation. BR distinctly eliminated the inhibitory effects of light. Under complete darkness or under far-red light, BR affected the growth very little. The spectral dependence of growth disappeared in the presence of BR. BR seems to overcome the growth inhibition of light (Kamuro, Y., *Plant Growth Regul.*, in press). These results suggest that BR-induced growth effects might be related to the phytochrome action.

It has been demonstrated that the physiological function of BR is mediated through endogenous plant hormones (3). It has been also suggested that plant hormones were related to the photomorphogenic events caused by phytochrome (13).

These instances and our test results indicate the needs to clarify the relationship between phytochrome and BR action. In order to establish the practical uses of brassinosteroids, the interaction between BR and other plant hormones under field conditions also should be investigated further.

Formulation

It is well known that formulation and additives in the treatment solutions generally influence the effects of BR. It is very interesting that water or ethanol solution of BR is hardly translocated into plant tissue, even in the presence of a surfactant, and the addition of chemicals to avoid the quick evaporation of BR solution from plant surfaces was essential to induce the BR effect (14). Another test result has also demonstrated that only 2-10% of ¹⁴C-BR applied to the leaf of rice plant was incorporated into plant tissue, and 90-98% of BR remained on plant surface (15).

It is not yet clear if the variabilities of BR under field conditions are due to chemical instability or a lack of absorption into plant tissue. We have little information about absorption and translocation of BR in plants. However, these results suggest that formulation could be one of the important factors for getting practical field results.

The mode of action of brassinosteroids seems to be very characteristic and different from other plant hormones. They are regarded as a new class of plant hormones and they are expected to be effective as plant growth regulators in crop production; however, many problems remain to be resolved.

Literature Cited

- 1) Grove, M. D.; Spencer, G. F.; Rohwedder, W. K.; Mandava, N.; Worley, J. F.; Warthen, J. D.; Steffens, Jr., G. L.; Flippen-Anderson, J. L.; Cook, Jr., J. C.; *Nature*, **1979**, *281*, 216.
- 2) Adam, G.; Marquardt, V.; *Phytochemistry*, **1986**, *25*, 1787.
- 3) Mandava, N. B.; *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, **1988**, *39*, 23.
- 4) Fujita, F.; *Kagaku-to-seibutu*, **1985**, *23*, 717 (in Japanese).
- 5) Yokota, T.; Takahashi, N.; In *Plant Growth Substances 1985*; Boop, M. Ed.; Springer-Verlag, Berlin/Heidelberg: **1986**; 129.
- 6) Takematsu, T.; Takeuchi, Y.; *Proc. Japan Acad.*, **1989**, *65* (Ser. B), 149.
- 7) Yokota, T.; Kim, S.-K.; Kosaka, Y.; Ogino, Y.; Takahashi, N.; *Proc. Int. Symp. Conjugated Plant Hormones Structure, Metabolism and Function*; Gera, GDR, Nov. 3-7, **1986**, pp. 288.
- 8) Krizek, D. T.; Mandava, N. B.; *Physiol. Plant.*, **1983**, *57*, 317.
- 9) Krizek, D. T.; Mandava, N. B.; *Physiol. Plant.*, **1983**, *57*, 324.
- 10) Krizek, D. T.; Worley, J. F.; *Physiol. Plant.*, **1981**, *51*, 259.
- 11) Mandava, N. B.; Sasse, J. M.; Yopp, J. H.; *Physiol. Plant.*, **1981**, *53*, 453.
- 12) Yopp, J. H.; Mandava, N. B.; Sasse, J. M.; *Physiol. Plant.*, **1981**, *53*, 445.
- 13) De Greef, J. A.; Frederico, H.; In *Encyclopedia of Plant Physiol., N. S.*, Shropshire, W. Jr.; Mohr, H. Ed.; Springer-Verlag, Berlin/Heidelberg/New York, **1983**, *16A*, 401.
- 14) Sugiyama, K.; Kuraiishi, S.; *Acta Hort.*, **1989**, *239*, 345.
- 15) Uchiyama, M.; Ishizaka, M.; Aburatani, M.; Takeuchi, I.; *Proc. the 11th Ann. Meeting of Pest. Sci. Soc. Japan*, **1986**, pp. 104 (in Japanese).

RECEIVED May 1, 1991

Chapter 26

Effects of Brassinolide on Conditioning and Germination of Witchweed (*Striga asiatica*) Seeds

Y. Takeuchi¹, A. D. Worsham², and A. E. Awad³

¹Weed Control Research Institute, Utsunomiya University, Utsunomiya 321, Japan

²Crop Science Department, North Carolina State University, Raleigh, NC 27695-7620

³International Institute of Tropical Agriculture, Ibadan 5320, Nigeria

Brassinolide applied at an early stage of conditioning, shortened the conditioning period required before witchweed seeds would germinate after exposure to a germination stimulant. Although *dl*-strigol, natural stimulants from sorghum (*Sorghum bicolor* L. Moench) root exudate, kinetin, ethephon and gibberellin A₃ applied after conditioning work as germination stimulants, they inhibit both conditioning and germination when applied during the conditioning period. Brassinolide eliminated the inhibitory effects on conditioning and subsequent germination caused by these chemicals applied during the conditioning period. The combination of brassinolide with either kinetin or ethephon induced higher germination of the seeds during the conditioning period than kinetin or ethephon alone. Brassinolide applied after conditioning increased the rate of seed germination induced by the stimulants. Brassinolide eliminated the inhibitory effects of seed germination caused by indoleacetic acid and light.

Witchweed is a very troublesome root parasite of many warm-season grass crops including corn (*Zea mays* L.), sorghum and sugarcane (*Saccharum officinarum* L.) (4). Reviews of seed germination of parasitic angiosperms have recently been published (33, 39, 40). In nature in soil, witchweed seeds will not germinate unless they are exposed to natural stimulants released from roots of host and many non-host plants (4, 15, 17, 38-40). Sorgoleone and its analogues (5) from sorghum, a host species and *dl*-strigol (6, 7) from cotton (*Gossypium hirsutum* L.), a non-host were isolated and their structures identified. Some analogues of *dl*-strigol, (15, 23), cytokinins (15, 37, 42), abscisic acid (ABA) (21), ethylene (10) and ethephon (9, 10) (an ethylene-producing compound) were also found to induce the germination of witchweed seeds. Gibberellin A₃ (GA₃) induced the germination of scarified seeds, but did not induce the germination of non-scarified seeds while indoleacetic acid (IAA) inhibited seed germination (11).

Witchweed seeds must be kept in a moist environment at 20 to 30°C for 1-3 weeks before the stimulants can initiate germination (4, 15, 39, 40). This process is defined

as conditioning by Hsiao *et al.* (15). It has been proposed that conditioning may (a) promote synthesis of a germination stimulant (3), (b) cause leaching of germination inhibitors from the seeds (2, 3, 8, 29, 35, 36), or (c) increase the permeability of a structure within the seeds, apparently the aleurone layer (35, 39). After completing the conditioning process(es), seeds can respond to exposure to germination stimulants (4, 15–18, 39). Stimulants such as *dl*-strigol, cytokinins, ethylene, ethephon and GA₃, applied during the early stage of conditioning inhibited the conditioning process which in turn inhibited subsequent germination of the seed (15–18, 20, 21). ABA and IAA showed no apparent effect on the conditioning process (21). Zeatin induced 3 to 20% germination of seeds during conditioning. However germination of the remaining seeds was inhibited when exposed to the usual stimulants (21). A higher concentration of stimulants (15, 17, 21, 31) and a longer exposure time (15, 17, 21) were required to initiate germination if seeds were conditioned in the presence of certain stimulants compared to seeds conditioned in water (15, 17, 21, 31). The effects of many compounds have been tested on the conditioning of the seeds, but no promotive compound has been reported to date.

Many studies have been conducted to determine the effectiveness of brassinosteroids as growth regulators in agricultural production systems (1, 12, 13, 30, 32, 41). However, the promotive effects of brassinosteroids on germination of weed have not been reported. This paper describes studies on the effects of brassinolide alone or in combination with various plant growth regulators on conditioning and germination of witchweed seeds (34).

Materials and Methods

Seeds. Witchweed seeds were collected from plants in experimental field of the USDA Methods Development Laboratory, Whiteville, N.C., USA and stored for three years at room temperature. Viability of these seeds was more than 95% according to a tetrazolium chloride viability test.

Chemicals. *dl*-Strigol was obtained in 1974 from Dr. C. J. Sih of the University of Wisconsin and stored at 4°C. Brassinolide was obtained from Dr. Ikegawa of Tokyo Institute of Technology in 1982 and stored at 4°C.

Natural stimulants were obtained as follows: Five sorghum seeds ('Dekalb 64') were placed in sand in 1000 ml plastic cups. Each cup had five 1 mm-holes in the bottom and was placed in another cup of the same size. The seedlings were grown in a greenhouse and 200 ml of distilled water were added during the incubation. After 14 days, 15 ml of solution containing root exudates from sorghum had leaked into the outer cup. The solution was filtered and used as the natural stimulant.

Seed Conditioning and Germination. Five hundred witchweed seeds were placed in a 9 cm-Petri dish and immersed in 5 ml of conditioning solution at 30°C for 3 to 15 days. After the conditioning period, the seeds were rinsed with distilled water and the wash water was removed. Groups of 50 seeds each were transferred to a 6-cm Petri dish containing a sheet of filter paper wetted with 2 ml of the terminal treatment solution. The dishes were placed in the dark at 30°C for 24 h. After the terminal treatment, germination of the seeds was determined under a 10x binocular microscope. Seeds were considered to have germinated if the radicle had emerged from the seed coat. Standard errors of the means were computed for all treatments which had three replications each and data were presented as the means of three separated experiments.

In studying the effects of chemicals on germination, solution containing the chemicals were used as the terminal treatments for 24 h after seeds were conditioned in water for either 7 or 14 days. In studying the effects of chemicals on the conditioning of seeds and their subsequent germination, solutions containing the chemicals were used during conditioning and then stimulants (*dl*-strigol or natural stimulants) were applied as the terminal treatments. Percent germination was recorded after 24 h.

Results and Discussion

Effects of Brassinolide and Combinations of Brassinolide with Various Plant Growth Regulators on Germination of the Seeds Conditioned in Water. Brassinolide at 10^{-15} to 10^{-5} M did not induce the germination of seeds conditioned in water for 7 or 14 days. However treatment with 10^{-7} M *dl*-strigol and undiluted natural stimulants (sorghum root exudate) resulted in approximately 60% germination of seeds conditioned in water for 7 days and more than 90% germination of seeds conditioned in water for 14 days (Table I).

Table I. Germination of Witchweed Seeds Conditioned in Water at 30°C for 7 and 14 Days Followed by 24-h Terminal Treatment with Brassinolide, *dl*-Strigol, and Natural Stimulants at 30°C in the Dark

Terminal treatment	Germination (%)	
	Period of conditioning	
	7 days	14 days
Water	0	0
Brassinolide 10 ⁻¹⁵ M	0	0
Brassinolide 10 ⁻¹³ M	0	0
Brassinolide 10 ⁻¹¹ M	0	0
Brassinolide 10 ⁻⁹ M	0	0
Brassinolide 10 ⁻⁷ M	0	0
Brassinolide 10 ⁻⁵ M	0	0
<i>dl</i> -Strigol 10 ⁻⁷ M	63±4	94±3
Natural stimulants diluted 4 x	28±2	85±5
Natural stimulants diluted 2 x	43±3	90±4
undiluted natural stimulants	63±4	94±3

Two- and 4-fold-diluted natural stimulants, 10^{-10} M strigol, 6×10^{-4} and 2×10^{-4} M ethephon, and 10^{-4} M kinetin induced 28 to 43%, 54%, 61 and 30%, and 38% germination, respectively. When brassinolide was used with these plant growth regulators, it greatly increased seed germination, especially at 10^{-7} M (Table II). IAA used as a terminal treatment at 10^{-3} M did not induce germination. When used in combination

Table II. Germination of Witchweed Seeds Conditioned in Water at 30°C for 7 Days Followed by 24-h Terminal Treatments with Combinations of Brassinolide (BR) with Natural Stimulants (STM), *dl*-Strigol (ST), Ethephon (ET), Kinetin, or Gibberellin (GA₃) at 30°C in the Dark

Terminal treatment	Germination (%)	Terminal treatment	Germination (%)
Water	0	ST 10 ⁻¹⁰ M	54±5
BR 10 ⁻⁹ M	0	ST 10 ⁻¹⁰ M + BR 10 ⁻⁹ M	80±3
BR 10 ⁻⁷ M	0	ST 10 ⁻¹⁰ M + BR 10 ⁻⁷ M	83±6
STM diluted 4 x	28±4	ET 2 x 10 ⁻⁴ M	30±4
STM diluted 4 x + BR 10 ⁻⁹ M	75±6	ET 2 x 10 ⁻⁴ M + BR 10 ⁻⁹ M	45±2
STM diluted 4 x + BR 10 ⁻⁷ M	90±5	ET 2 x 10 ⁻⁴ M + BR 10 ⁻⁷ M	63±5
STM diluted 2 x	43±7	ET 6 x 10 ⁻⁴ M	61±2
STM diluted 2 x + BR 10 ⁻⁹ M	80±4	ET 6 x 10 ⁻⁴ M + BR 10 ⁻⁹ M	65±3
STM diluted 2 x + BR 10 ⁻⁷ M	91±5	ET 6 x 10 ⁻⁴ M + BR 10 ⁻⁷ M	84±5
GA ₃ 10 ⁻⁴ M	0	Kinetin 6 x 10 ⁻⁴ M	38±3
GA ₃ 10 ⁻⁴ M + BR 10 ⁻⁹ M	0	Kinetin 2 x 10 ⁻⁴ M + BR 10 ⁻⁹ M	43±4
GA ₃ 10 ⁻⁴ M + BR 10 ⁻⁷ M	0	Kinetin 2 x 10 ⁻⁴ M + BR 10 ⁻⁹ M	83±6

with $10^{-7}M$ *dl*-strigol or natural stimulants, IAA had inhibitory effects on germination induced by *dl*-strigol or natural stimulants. When used with *dl*-strigol and IAA or with natural stimulants and IAA, brassinolide eliminated the inhibitory effect of IAA on germination (Table III). These results indicated that brassinolide does not act as a stimulant for the germination of conditioned seeds, but that it enhances the effect of stimulants on germination. Brassinolide may increase the sensitivity of the seeds to stimulants by promoting some physiological processes, e.g. activation of endogenous growth substances (23).

Table III. Germination of Witchweed Seeds Conditioned in Water at 30°C for 7 Days Followed by 24-h Terminal Treatments with Combinations of Brassinolide (BR) and Indoleacetic Acid (IAA) with *dl*-Strigol (ST), or with Natural Stimulants (STM) at 30°C in the Dark

<i>Terminal treatment</i>	<i>Germination (%)</i>
Water	0
BR $10^{-7}M$	0
IAA $10^{-3}M$	0
ST $10^{-7}M$	63±4
ST $10^{-7}M$ + IAA $10^{-3}M$	43±3
ST $10^{-7}M$ + BR $10^{-7}M$	92±3
ST $10^{-7}M$ + IAA $10^{-3}M$ + BR $10^{-7}M$	88±4
STM diluted 2 x	43±7
STM diluted 2 x + IAA $10^{-3}M$	22±4
STM diluted 2 x + BR $10^{-3}M$	91±5
STM diluted 2 x + IAA $10^{-3}M$ + BR $10^{-7}M$	76±6

Effect of Brassinolide on *dl*-Strigol-Induced Germination of Witchweed Seeds Inhibited by Light. Light has been shown to inhibit the germination of witchweed seeds (11, 28, 39, 40). In our experiment, the inhibitory effect of light was also evident. When seeds were conditioned in water for 7 days and then exposed to $10^{-7}M$ *dl*-strigol, seeds germinated 31%±5 in the light and 63%±4 in the dark. However, seeds treated with the combination of $10^{-7}M$ brassinolide and $10^{-7}M$ *dl*-strigol germinated 94%±4 in the light and 93%±3 in the dark (Table IV). These results indicated that brassinolide greatly reduced the inhibitory effect of light on seed germination. The effect of light on the germination of witchweed seeds is believed to be under phytochrome control (39). Other brassinolide-elicited growth responses may be under similar phytochrome control (25–27, 41).

Table IV. Germination of Witchweed Seeds Conditioned in Water at 30°C for 7 Days Followed by 24-h Terminal Treatments with Brassinolide, *dl*-Strigol (ST) with Brassinolide (BR) at 30°C in the Dark or in the Light

<i>Terminal treatment</i>	<i>Germination (%)</i>	
	<i>dark</i>	<i>light</i>
Water	0	0
ST $10^{-7}M$	63±4	31±5
ST $10^{-7}M$ + BR $10^{-7}M$	93±3	94±4

Effects of Brassinolide and Combinations of Brassinolide with Various Plant Growth Regulators on Seed Conditioning and Subsequent Germination. Generally, when witchweed seeds are conditioned in water, germination is increased by longer periods of conditioning and by higher stimulant concentrations in the terminal treatment (17). In our experiments, seeds conditioned in water for 3, 5

and 7 days germinated 0, 0 and 58%, respectively, with natural stimulant solutions as terminal treatments. On the other hand, seeds conditioned in 10^{-7}M brassinolide for 3, 5 and 7 days germinated 22, 58 and 92%, respectively, in terminal natural stimulants solution. These results indicated that brassinolide promoted the rate of the conditioning process(es) and subsequently shortened the conditioning period required (Figure 1).

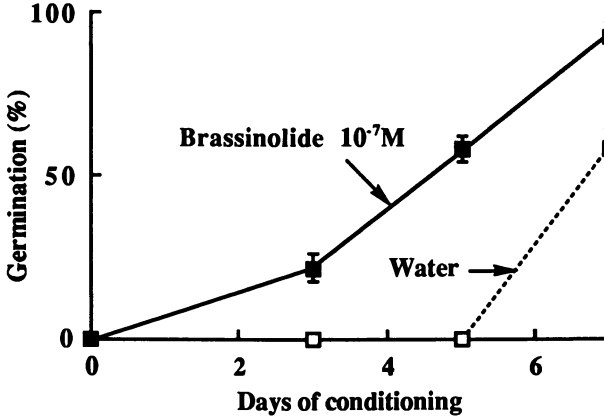


Figure 1. Germination of Witchweed Seeds Conditioned in Water and Brassinolide (10^{-7}M) for 3-7 Days before Terminal Treatment with Natural Stimulants. Bars Represent Standard Errors of Means for 3 Replications over 3 Experiments.

Hsiao *et al.* (17) showed that stimulants, which promote germination of the conditioned seeds, inhibit the conditioning process when applied during an early stage of conditioning. In our experiments, 10^{-10}M *dl*-strigol, natural stimulants, and $2 \times 10^{-4}\text{M}$ ethephon each promoted the germination of conditioned seeds (Table II). However, they inhibited conditioning of seeds and subsequently germination was lower. During conditioning in 10^{-5}M or 10^{-4}M kinetin for 7 days, seeds germinated 25% and 20%, respectively, while the remaining seeds failed to germinate with natural stimulant solution as a terminal treatment. IAA at 10^{-4}M or 10^{-4}M ABA had no apparent effects on conditioning (Table V). When 10^{-9}M brassinolide or 10^{-7}M brassinolide was used with 10^{-4}M GA_3 during conditioning, brassinolide eliminated the inhibitory effects of GA_3 on conditioning and subsequent germination (Table VI). Seeds conditioned in $2 \times 10^{-4}\text{M}$ ethephon did not germinate during conditioning, but germinated 15% with natural stimulants as a terminal treatment. When seeds were conditioned in the combinations of $2 \times 10^{-4}\text{M}$ ethephon with 10^{-9}M brassinolide or with 10^{-7}M brassinolide, they germinated 20 and 43%, respectively during conditioning (Table VI). The rate of germination increased to 25 and 70%, respectively, with natural stimulants. Twenty % of the seeds germinated when conditioned in 10^{-5}M kinetin, while the remaining seeds did not germinate upon treatment with natural stimulants. When seeds were conditioned in the combinations of 10^{-5}M kinetin with 10^{-9}M brassinolide or with 10^{-7}M brassinolide, they germinated 44 and 72%, respectively, during conditioning, and the rate of the germination increased to 52 and 85%, respectively, after terminal treatment with natural stimulants (Table VI). These results indicated that brassinolide eliminated the inhibitory effects of GA_3 , ethephon and kinetin on conditioning and subsequent germination. Since brassinolide shortened the conditioning period, stimulants such as ethephon and kinetin acted to promote germination at earlier stages of conditioning.

Table V. Germination of Witchweed Seeds Conditioned in Various Plant Growth Regulators at 30°C for 7 Days with and without Terminal Treatments of Natural Stimulants (4-fold Diluted) from Sorghum at 30°C

Conditioning media		Germination (%)	
		After terminal treatment	Without terminal treatment
Water		28±4	0
<i>dl</i> -Strigol	10 ⁻⁸ M	11±2	0
Natural stimulants		0	0
Gibberellin A ₃	10 ⁻⁴ M	15±3	0
Ethephon	2x10 ⁻⁴ M	15±4	0
Kinetin	10 ⁻⁵ M	20±5	20±5
Kinetin	10 ⁻⁴ M	25±3	25±3
IAA	10 ⁻⁴ M	30±3	0
Abscisic acid	10 ⁻⁴ M	24±4	0
Brassinolide	10 ⁻¹⁵ M	51±5	0
Brassinolide	10 ⁻⁷ M	92±3	0

Table VI. Percent Germination of Witchweed Seeds Conditioned in Combinations of Brassinolide with Gibberellin A₃ (GA₃), Ethephon, or Kinetin at 30°C for 7 Days Followed by 24-h Terminal Treatment with Natural Stimulants (4-fold Diluted) from Sorghum at 30°C

Conditioning media		Brassinolide*					
		0		10 ⁻⁹ M		10 ⁻⁷ M	
		with	without	with	without	with	without
Water		28±4	(0)	75±6	(0)	92±5	(0)
GA ₃	10 ⁻⁴ M	15±3	(0)	45±2	(0)	65±4	(0)
Ethephon	2x10 ⁻⁴ M	15±4	(0)	25±3	(20±5)	70±6	(43±4)
Kinetin	10 ⁻⁵ M	20±5	(20±5)	52±5	(44±3)	85±3	(72±6)

*With or without terminal treatment of natural stimulants.

Hsiao (21) reported that when *dl*-strigol was applied at an early stage of conditioning, it inhibited subsequent germination and longer conditioning times were required for seeds to overcome this inhibition of the conditioning process and subsequent germination. In our experiments, *dl*-strigol at 10⁻¹² to 10⁻¹⁰M also caused a great reduction in germination when used in the conditioning solution (Table VII). Seeds conditioned in 10⁻¹²M and 10⁻¹⁰M *dl*-strigol required 13 to 15 days and more than 15 days, respectively, to overcome the inhibition of conditioning. However, seeds conditioned for 7 days in 10⁻⁷M brassinolide alone and in combinations of brassinolide with 10⁻¹² or 10⁻¹⁰M *dl*-strigol, germinated more than 90% with the terminal treatment of 10⁻⁷M *dl*-strigol. Consequently the inhibitory effects of *dl*-strigol addition during conditioning was eliminated by including brassinolide during this period (Table VII). Brassinolide was also effective in overcoming the inhibitory effects of natural stimulants on conditioning (data not shown).

Summary

It was suggested (17) that *dl*-strigol and natural stimulants inhibit the conditioning process when applied during an early stage of conditioning, but stimulate germination when applied at a later stage. Hsiao (17) also suggested that whether these germination regulators stimulate germination or inhibit seed conditioning and their subsequent germination depends upon mainly the stage of conditioning at which the compounds are applied. We suggest that brassinolide may have promoted conditioning through increasing permeability of some membrane of the seeds or perhaps changing the

Table VII. Percent Germination of Witchweed Seeds Conditioned in the Combination of *dl*-Strigol (ST) with Brassinolide (BR) at 30°C for 7-15 Days Followed by 24-h Terminal Treatments with *dl*-Strigol (10⁻⁷M) at 30°C

Conditioning media	Days of conditioning			
	7	9	13	15
Water	62±4	80±2	93±5	94±4
ST 10 ⁻¹² M	48±2	75±5	87±3	95±3
ST 10 ⁻¹⁰ M	37±2	48±1	61±6	75±5
BR 10 ⁻⁷ M	92±3	96±3	95±3	95±3
ST 10 ⁻¹² M + BR 10 ⁻⁷ M	93±4	93±4	94±3	96±3
ST 10 ⁻¹⁰ M + BR 10 ⁻⁷ M	93±3	93±3	94±4	96±4

balance of endogenous promoters and inhibitors in seeds. It was demonstrated that steroids, including brassinolide, showed stimulative effects on cell permeability (14, 22, 24).

A significant practical application of the findings reported herein could be that the addition of brassinolide to seed germination stimulants such as *dl*-strigol or its analogues (19, 23) applied to soil to cause "suicidal germination" might greatly increase effectiveness of this weed control approach. Lower concentrations of germination stimulants should be effective in promoting germination if combined with brassinolide; also the problem of exposure of seeds to a soil-applied stimulant before the conditioning process was completed, which would normally result in lower seed germination, might be overcome if brassinolide were also present.

The mechanism of action of brassinolide is still unknown; therefore studies should be conducted further to understand the physiological effects of brassinolide on conditioning and germination of witchweed seeds.

Acknowledgments

The authors thank Dr. R. E. Eplee, USDA, APHIS, Whiteville Methods Development Center, Whiteville, NC for the witchweed seeds used in this research and other helpful suggestions.

Literature Cited

1. Adam, G.; Marquardt, V. *Phytochemistry* **1986**, *25*, 1787-1789.
2. Bharathalakshmi. *Studies on the Root Parasite - Striga asiatica (L.) Kuntze*, Ph.D. Thesis, Bangalore University, India, 1982.
3. Brown, R. *Nature (London)* **1965**, *157*, 64.
4. Brown, R. *Handb. Pflanzenphysiol.* **1965**, *15*, 925-932.
5. Chang, M.; Netzly, D. H.; Butler, L. G.; Lynn, D. G. *J. Am. Chem. Soc.* **1986**, *108*, 7858-7860.
6. Cook, C. E.; Whichard, L. P.; Turner, B.; Wall, M. E.; Egley, G. H. *Science*, **1966**, *154*, 1189-1190.
7. Cook, C. E.; Wall, M. E.; Egley, G. H.; Coggon, P.; Luhan, P. A.; McPhail, A. *T. J. Am. Chem. Soc.* **1972**, *94*, 6198-6199.
8. Edward, W. G. H.; Hiron, R. P.; Mallet, A. I. Z. *Pflanzenphysiol.* **1976**, *80*, 105.
9. Egley, G. H.; Dale, J. E. *Proc. South. Weed Sci. Soc.* **1969**, *22*, 379.
10. Egley, G. H.; Dale, J. E. *Weed Sci.* **1970**, *18*, 586-589.
11. Egley, G. H. *Ann. Bot.* **1972**, *36*, 755-770.
12. Gregory, L. E. *Am. J. Bot.* **1981**, *68*, 586-588.
13. Grove, M. D.; Spencer, F. G.; Rohwedder, W. K.; Mandava, N. B.; Worley, J. F. *Nature* **1979**, *281*, 216-217.
14. Heffman E. *Lipids* **1971**, *6*, 128-132.

15. Hsiao, A. I.; Worsham, A. D.; Moreland, D.E. In *Proceedings of the Second International Symposium on Parasitic Weeds*; Musselman, L. J.; Worsham, A. D.; Eplee, R. E., Ed.; North Carolina State University, Raleigh, NC, 1979, pp. 193-201.
16. Hsiao, A. I.; Worsham, A. D.; Moreland, D. E. *Weed Sci.* **1981**, *29*, 98-100.
17. Hsiao, A. I.; Worsham, A. D.; Moreland, D. E. *Weed Sci.* **1981**, *29*, 101-104.
18. Hsiao, A. I.; Worsham, A. D.; Moreland, D. E. *Z. Pflanzenphysiol.* **1981**, *104*, 1-8.
19. Hsiao, A. I.; Worsham, A. D.; Moreland, D. E. *Weed Sci.* **1983**, *31*, 763-765.
20. Hsiao, A. I.; Worsham, A. D.; Moreland, D. E. *Weed Res.* **1987**, *27*, 321-328.
21. Hsiao, A. I.; Worsham, A. D.; Moreland, D. E. *Ann. Bot.* **1988**, *62*, 17-24.
22. Johnson, A.; Roseberry, W. G.; Parker, C. *Weed Res.* **1976**, *16*, 223-227.
23. Katsumi, M.; Tsuda, A.; Sakurai, H. *Proc. Plant Growth Regul. Soc. Am.* **1987**, *14*, 215-220.
24. Kirkman, M. *Biochem. Physiol. Pflanzen.* **1971**, *176*, 524-534.
25. Krizek, D. T.; Mandava, N. B. *Physiol. Plant* **1982**, *57*, 317-323.
26. Krizek, D. T.; Mandava, N. B. *Physiol. Plant* **1982**, *57*, 324-329.
27. Krizek, D. T.; Worley, J. F. *Bot. Gaz.* **1973**, *13*, 147-50.
28. Kumar, L. S. S.; Solomon, S. *Curr. Sci.* **1940**, *9*, 541.
29. Kust, C. A. *Weeds* **1966**, *14*, 327-329.
30. Mandava, N. B. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **1988**, *39*, 23-52.
31. Pavlista, A. D.; Worsham, A. D.; Moreland, D. E. In *Proceedings of the Second International Symposium on Parasitic Weeds*; Musselman, L. J.; Worsham, A. D.; Eplee, R. E., Ed.; North Carolina State University, Raleigh, NC, 1979, pp. 228-237.
32. Takematsu, T.; Takeuchi, Y.; Koguchi, M. *Chem. Regul. Plants* **1983**, *18*, 2-15 (in Japanese).
33. Sahai, A.; Shivanna, K. R. *Seed Sci. Tech.* **1982**, *10*, 565-583.
34. Takeuchi, Y.; Worsham, A. D.; Awad, A. E. *Proc. 12th Asian-Pacific Weed Sci. Soc. Conf.* **1989**, 149-158.
35. Vallance, K. B. *Ann. Bot.* **1951**, *15*, 109-128.
36. Williams, C. N. *Nature (London)* **1959**, *184*, 1577-1578.
37. Worsham, A. D.; Moreland, D. E.; Klingman, G. C. *Science* **1959**, *130*, 1654-1656.
38. Worsham, A. D.; Moreland, D. E.; Klingman, G. C. *J. Expt. Bot.* **1964**, *15*, 556-567.
39. Worsham, A. D. In *Biology and control of parasitic weeds. I. Striga.*; Musselman, L. J., Ed.; CRC Press, Boca Raton, FL, 1987, pp. 45-61.
40. Worsham, A. D.; Egley, G. H. In *Witchweed Research and Control in the United States*; Sand P. F.; Eplee, R. E.; Westbrooks, R. G., Ed.; Monog. Ser., Weed Sci. Soc. Am., No. 5, Weed Science Society of America, Champaign, IL, 1990, pp. 11-26.
41. Yokota, T.; Takahashi, N. In *Plant growth substances*; Bopp, M., Ed.; Springer-Verlag, Berlin/Heidelberg, 1986, pp. 128-139.
42. Yoshikawa, F.; Worsham, A. D.; Moreland, D. E.; Eplee, R. E. *Weed Sci.* **1978**, *26*, 119-122.

RECEIVED May 2, 1991

Chapter 27

Growth-Regulating Action of Brassinolide in Rice Plants

S. Fujii¹, K. Hirai¹, and H. Saka²

¹Shiraoka Research Station of Biological Science, Nissan Chemical Industries, Ltd., Shiraoka, Minamisaitama, Saitama 349-02, Japan

²Hokkaido National Agricultural Experimental Station, Sapporo, Hokkaido 062, Japan

Brassinolide increased the grain weight and the percentage of ripened grains in rice plants. Brassinolide treatment promoted assimilation of ¹⁴C in leaf blades and successive translocation of the radioactive assimilate into panicles. Accumulation of starch in panicles was enhanced by brassinolide treatment. The effect of brassinolide on the ripening of rice plants may be attributed to the enhancement of the synthesis and translocation of photosynthetic products. It was found that brassinolide treatment altered the levels of endogenous indole acetic acid and abscisic acid in panicles.

In wheat, the ear weights of both tillers and the main stem were increased, in comparison with untreated controls by brassinolide (BR) sprays (1). In corn, BR treatment had significant influence on the length of the unfertile tip portion of the ear and on the number of vacant kernels. The yield increased compared with untreated controls (2). Little is known of the effect of BR on the ripening of rice plants, and the mode of action of BR on the ripening of rice has not been studied. To increase crop yield, it is necessary to improve the photosynthetic efficiency and promote the translocation of photosynthetic products in the panicles. Therefore, we assessed the ripening of rice, the translocation of photosynthetic products, and plant hormone levels in panicles.

Increase of Grain Weight and Percentage of Ripened Grains

Rice (*Oryza sativa* L. cv. Nipponbare) seeds were directly sown in pots and grown in a greenhouse before heading. BR was sprayed with 30 ml per pot at concentrations of 10^{-3} and 10^{-2} ppm 10 days before

heading and heading time, respectively. After flowering, rice plants were grown in a greenhouse or a phytotron (20°C/13°C). Under greenhouse conditions (Table IA), BR increased the grain weight and the percentage of ripened grains of rice plants at 3 weeks after heading and the percentage of ripened grains at 6 weeks. Moreover, under phytotron conditions (Table IB), BR greatly increased both the grain weight and the percentage of ripened grains at 3 and 6 weeks after heading. BR exerts its effectiveness most especially under environmental conditions of stress. And BR increased the weight of grains located in all rachis-branches, but in particular, the weight of those grains located in the lower branches.

Table I. Effect of Brassinolide on Weight of Grain in Rice Plants (cv. Nipponbare) under (A) Greenhouse and (B) Phytotron (20°C/13°C) Conditions

(A)				
BR (ppm)	3 Weeks after Heading		6 Weeks after Heading	
	Weight of 1000 grains(g)	Percentage of ripened grains(%)	Weight of 1000 grains(g)	Percentage of ripened grains(%)
0	18.0	50.3	23.3	75.3
10 ⁻³	19.2	61.2	23.6	82.6
10 ⁻²	19.5	63.8	23.2	81.0

(B)				
BR (ppm)	3 Weeks after Heading		6 Weeks after Heading	
	Weight of 1000 grains(g)	Percentage of ripened grains(%)	Weight of 1000 grains(g)	Percentage of ripened grains(%)
0	11.7	15.4	21.6	62.2
10 ⁻³	12.7	25.3	22.7	75.8
10 ⁻²	12.8	24.5	22.9	75.3

Translocation of ¹⁴C incorporated in Rice Plants.

Rice plants were grown in a greenhouse and treated with BR as described above. At 3 days after heading, rice plants were placed in an artificially controlled growth chamber. From small vessels, which were placed in the growth chamber, ¹⁴CO₂ was released by acidifying the NaH¹⁴CO₃ (17.5 MBq) with HClO₃. Rice plants were fed with ¹⁴CO₂ for 1 hour. All operations of the assimilation procedure were performed under light (light intensity 550–660 micro mols m⁻²s⁻¹) at 25°C. After feeding, rice plants were grown in the same growth chamber. The photoperiod was 12h and temperature was maintained at 25°C. Amounts of the ¹⁴C in each plant part were determined at regular intervals. In

untreated controls, 63% of the ^{14}C incorporated into each rice plant was present in the leaf blades 1 hour after feeding. Subsequently, the ^{14}C in the leaf blades rapidly decreased and translocated into the culms and the grains. Most of the ^{14}C accumulated into the culms from 1 to 7 days after feeding. In rice plants treated with BR, amounts of ^{14}C in leaf blades were present at higher levels (126%) relative to untreated controls at 1 hour, suggesting that a high photosynthetic rate was induced by BR in the leaf blade. The ^{14}C rapidly decreased in leaf blades in a similar manner to untreated controls. From 3 days after assimilation, amounts of ^{14}C in grains greatly increased. The increases of ^{14}C in the grain at 3, 7, and 21 days were 119.5, 220.8, and 443.3 Bq compared with untreated controls (88.0, 140.2, 389.3 Bq) respectively. The accumulation of ^{14}C in culms was suppressed (65%). These results show that the translocation of photosynthetic products in panicles is probably promoted by treatment with BR.

Distribution of Starch and Sucrose.

Little starch was made in the leaf blades during the ripening of rice. Most of the starch accumulated in the panicles. However, rice plants stored starch in the leaf sheaths and the culms to some extent. Starch content in the leaf sheaths (Figure 1) and the culms under greenhouse conditions was appreciably less than that under phytotron conditions. In the case of treatment with BR, starch content in the leaf sheaths (Figure 1) and the culms was suppressed under both conditions while total starch contents in the panicles increased (Figure 2). So it is proposed that the decrease of the starch content in the leaf sheaths and the culms may be correlated to the ripening of the rice panicles, that is, the increase of grain weight and the percentage of ripened grains. In addition, sucrose content in panicles increased by BR treatment under both conditions. It may be concluded that BR enhances the accumulation of starch and sucrose in the panicles. The reconstitution of the starch is probably promoted in the panicles, not in leaf sheaths or culms.

Plant Hormone Levels in the Panicles.

Untreated rice plants and those treated with BR (10^{-3} ppm) were grown in a greenhouse or a phytotron (22°C/17°C) after flowering. The weight of grains significantly increased at the milk-ripe stage (10 to 15 days after heading) by treatment with BR under both conditions. Levels of endogenous IAA and ABA in grains at 10 and 15 days after heading were determined by HPLC and ELISA, respectively. Ethylene from panicles was determined by GC. In grains obtained from greenhouse experiments at 10 and 15 days after heading, BR treatment increased the level of IAA but, in contrast, decreased the level of ABA (Table II). In grains obtained from phytotron experiments, BR treatment decreased both the levels of IAA and ABA at 10 days after heading, while it

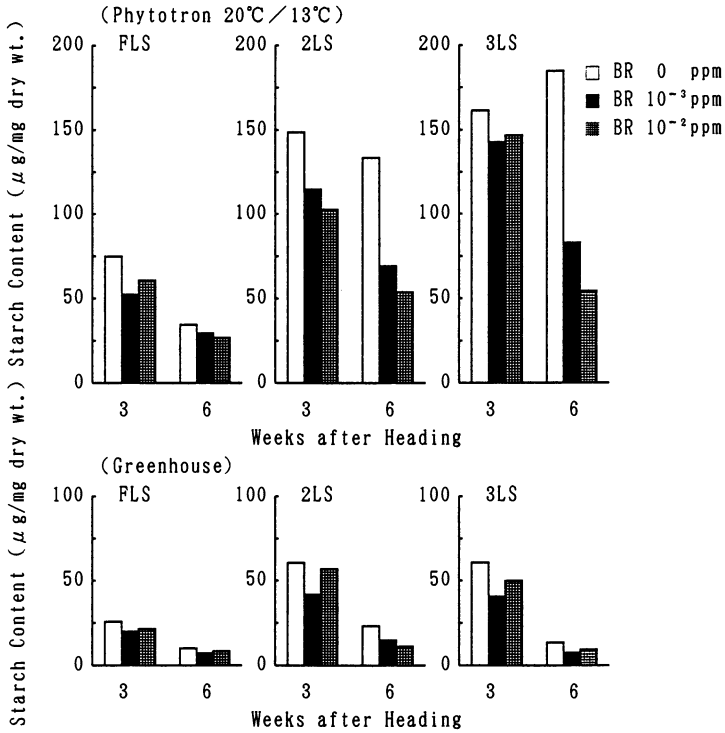


Figure 1. Effect of Brassinolide on Starch Content in Leaf Sheath of Rice Plants. FLS:flag leaf sheath, 2LS:2nd leaf sheath, 3LS:3rd leaf sheath.

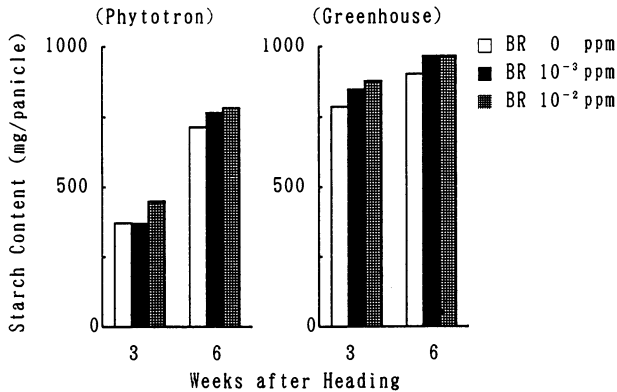


Figure 2. Effect of Brassinolide on Total Starch Content in Panicles of Rice Plants.

increased both the levels of IAA and ABA at 15 days after heading (Table II). Ethylene evolution from panicles at the milk-ripe stage increased by BR treatment (Table III) under greenhouse conditions. It is difficult, from these results, to ascertain the relation of the change in these plant hormone levels induced by BR to grain development. However, endogenous levels of IAA and ABA in panicles were much higher than those in vegetative tissue (3-4), suggesting that these

Table II. Effect of Brassinolide on IAA and ABA Levels in Grains under Greenhouse and Phytotron (22°C/17°C) Conditions

BR (ppm)	Greenhouse				Phytotron(22°C/17°C)			
	Days after heading				Days after heading			
	10		15		10		15	
	IAA (ng/g fwt)	ABA (ng/g fwt)	IAA (ng/g fwt)	ABA (ng/g fwt)	IAA (ng/g fwt)	ABA (ng/g fwt)	IAA (ng/g fwt)	ABA (ng/g fwt)
0	670	155	1360	350	530	151	1140	213
10 ⁻³	840	125	1535	165	450	113	1610	270

Table III. Effect of Brassinolide on Ethylene Evolution from Panicles under Greenhouse Conditions

BR (ppm)	Ethylene evolution (nl/g fwt/24h)	
	Days after heading	
	15	20
0	9.7	3.9
10 ⁻³	12.5	6.4

plant hormones may regulate the development of seeds. Accordingly, the changes of the balance of these levels induced by BR may be associated with promotion of the translocation of photosynthetic products in panicles and also accumulation of the starch in grains. Much more information is needed to establish unequivocally a relationship between the changes of the plant hormones brought about by BR and the promotion of grain growth. Also, investigation for the contents of BR and other hormones in grains during the ripening period and the enzyme activities governing carbohydrate metabolism may be needed.

Acknowledgment. We wish to thank Dr. S. Watanabe, Institute of Agrobiological Resources, for helpful discussions about the endogenous plant hormone analyses.

Literature Cited

(1) Luo, B.; Kumura, A.; Ishii, R.; Wada, Y. *Japan. J. Crop Sci.* **1986**, *55*, 291.

- (2) Hamada, K.; Nishi, S.; Uezono, T.; Fujiwara, S.; Nakazawa, Y. *Abstract of papers of the 12th International Conference on Plant Growth Substances (Heidelberg) 1985*, 43.
- (3) Kobayashi, M.; Sakurai, A.; Saka, H.; Takahashi, N. *Agric. Biol. Chem.* **1989**, *53*, 1089.
- (4) Suzuki, Y.; Kurogouchi, S.; Murofushi, N.; Ota, Y.; Takahashi, N. *Agric. Biol. Chem.* **1981**, *22*, 1085.

RECEIVED May 13, 1991

Chapter 28

Effect of Brassinolide on Levels of Indoleacetic Acid and Absciscic Acid in Squash Hypocotyls

Possible Application for Preventing Fruit Abortion

S. Kuraishi¹, N. Sakurai¹, J.-S. Eun², and K. Sugiyama³

¹Department of Environmental Studies, Faculty of Integrated Arts and Sciences, Hiroshima University, Naka-ku, Hiroshima 730, Japan

²Department of Horticulture, College of Agriculture, Jeonbug National University, Jeonju 560-756, Republic of Korea

³Shizuoka Prefectural Experiment Station, Shimizu, Shizuoka 424, Japan

Brassinolide stimulated an increase in fresh weight of segments of hypocotyls of etiolated squash (*Cucurbita maxima* Duch.). Brassinolide-treated segments contained a slightly higher level of IAA than water-treated segments and also showed a tendency towards decreased level of ABA.

Flowers of 'Morita' navel oranges were treated with brassinolide solution at anthesis. Fruit set of trees treated with 0.1 and 0.01 ppm brassinolide increased by ca. 5 and 2.5 times respectively, when surveyed 50 days after treatment. At harvest there were no differences in quality between treated and untreated fruits. Since brassinolide-treated squash segments only showed slight changes in hormone content, brassinolide may have a direct action on stimulating fruit set without marked changes in the levels of other endogenous phytohormones.

The brassinosteroid found in rape pollen (1), showed marked stimulation of growth of segments from several kinds of plants (2-5). Since auxin stimulates, and ABA retards the growth of segments, brassinosteroid-induced change in growth may occur as a result of changes in levels of these hormones in segments. First, to examine this possibility, experiments were performed to measure levels of IAA, ABA and ethylene in brassinolide-treated segments of squash (*Cucurbita maxima* Duch.) hypocotyls.

Citrus cultivation in Japan accounts for 60% of the total fruit production. Nearly 70% of the citrus production is of satsuma mandarin. Navel oranges show poor fruit set in the south western coastal areas of Japan, because of cool conditions and, for this reason, are sometimes grown in glasshouses or under plastic.

Recently brassinolide was found to retard the abscission of leaf explants of Calamondin (6). Thus, the present experiments were also included to observe the effects of brassinolide on the fruit set of 'Morita' navel oranges (*Citrus sinensis*), a cultivar derived from Washington navel.

Growth Stimulation of Squash Hypocotyl Segments after the Treatment with Brassinolide

Seeds of *Cucurbita maxima* Duch. cv. Houkou-aokawa-amaguri were soaked for 14 h in tap water, and then germinated for 2 days in the dark on two layers of moistened filter paper. The germinated seeds were placed on stainless-steel mesh in a plastic box that contained 2 liters of 1/5 strength Hoagland solution, as previously described (7), and cultured hydroponically for two days. When etiolated hypocotyls attained a length of 70 - 80 mm, the top 5 mm were excised and the 10-mm segments immediately below this region were harvested.

Segment Growth after the Treatment with Brassinolide. Ten, 10-mm segments of hypocotyls were floated on a solution of 0.0002 μM to 0.2 μM brassinolide with and without 0.1 or 1.0 μM IAA and their growth was observed after 3, 5 and 8 h of incubation (Figure 1). Low concentrations of brassinolide, even as low as 0.002 μM , significantly stimulated an increase in the fresh weight of segments after 8 h incubation. Simultaneous application of IAA in brassinolide solution only caused additive effect on the increase in fresh weight, suggesting that brassinolide-induced stem growth is not due to changes in IAA content in the segments. Since 0.2

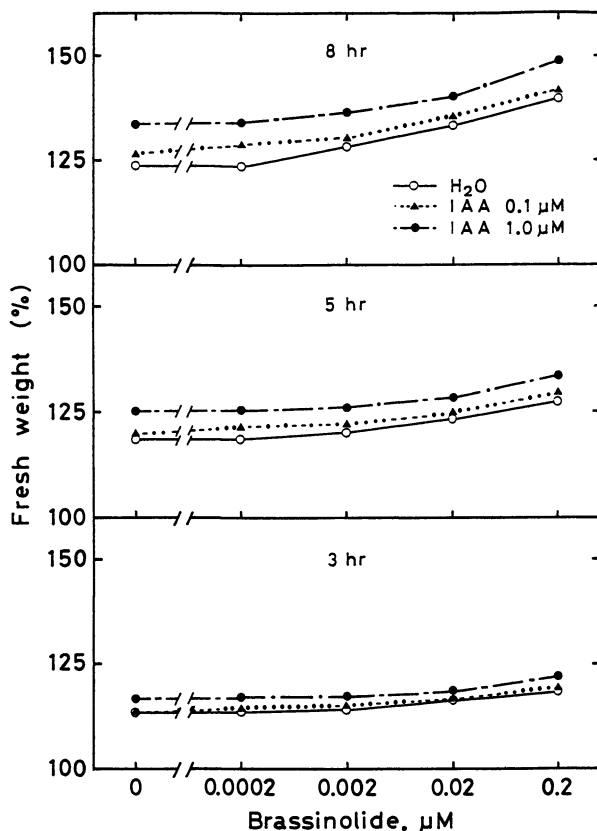


Figure 1. Growth stimulation of squash hypocotyl segments floated on IAA and brassinolide in the presence of mannitol solution. (Reproduced with permission from reference 8. Copyright 1989 The Japanese Society of Plant Physiologists.)

Table I. Number of Epidermal Cells along the Etiolated Squash Hypocotyl Segments

Treatment	Number of cells
Initial	300.0± 3.6
Water	300.8± 7.6
IAA 10 μ M	304.6± 6.8
Brassinolide 0.2 μ M	305.6±10.9

10-mm sections were incubated for 8 h.

μ M brassinolide gave an almost maximum increase in fresh weight, that concentration was used for the following experiments.

Total cell numbers of hypocotyl segments after treatments were observed under the microscope to see whether the brassinolide-induced growth stimulation was due to cell elongation or cell division. As shown in Table I, the cell number of epidermal cells was not changed during 8 h incubation, suggesting that brassinolide-induced hypocotyl elongation is caused by cell expansion and not by cell division.

Changes in Osmoticum during Brassinolide-induced Stem Elongation.

Cell elongation is caused by either changes in osmoticum in the vacuole or by cell wall extension. It has long been known that IAA-induced primary stem elongation has no relation to the increase in osmoticum in vacuoles. Ten, 10-mm hypocotyl sections were incubated in 0 to 0.30 M mannitol solution for 8 h and their fresh weight increase was observed. As shown in Figure 2, fresh weight of hypocotyls decreased when higher concentrations of mannitol were given to the incubation medium. Even in the presence or absence of IAA and brassinolide, fresh weight increase was stopped by a concentration of 0.2 M mannitol solution. If growth due to brassinolide is caused by an increase in osmoticum concentration, the increase of fresh weight should be observed even at the higher concentration of mannitol solution. From these

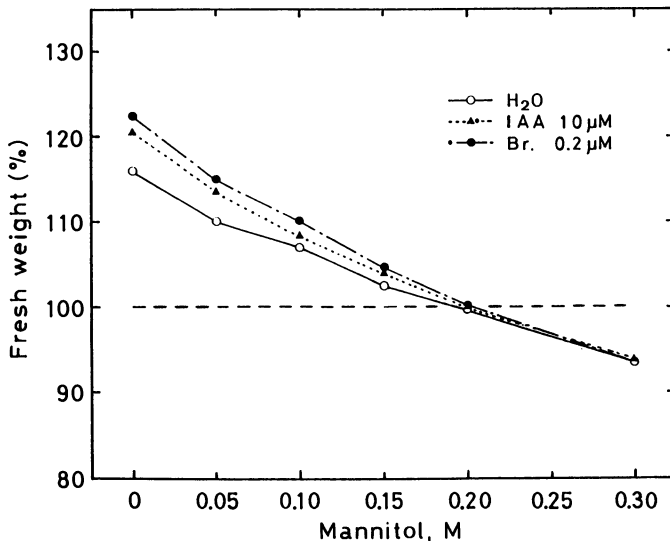


Figure 2. Growth of hypocotyl sections after treatment with brassinolide in the presence of mannitol solution. (Reproduced with permission from reference 8. Copyright 1989 The Japanese Society of Plant Physiologists.)

experiments brassinolide-caused cell growth is thought to be caused by the cell wall extension, although further evidence to prove this point unequivocally is needed.

Levels of IAA and ABA during Treatment with Brassinolide

Ten squash hypocotyl segments were floated on a solution of 0.2 μM brassinolide and the evolution of ethylene was measured. Exactly one hundred and fifty, 10-mm segments of etiolated squash segments were treated with 0.2 μM brassinolide. Although segment growth was observed after 3 h treatment, there was no significant increase in ethylene evolution in brassinolide-treated tissues compared with water-treated tissues (8). Production of ethane also did not show any marked change during the incubation period. The brassinolide-treated segments showed a slight, but significant increase in endogenous IAA content both at 3 and 5 h incubation (Table II). On the other hand, water-treated segments tended to show a decrease in IAA content. Furthermore, brassinolide-treated segments had lower endogenous ABA levels at 5 h incubation, when compared with either initial or 5 h water treated segments. The results indicated that high endogenous level of IAA may have some effects on the brassinolide-induced effects. However, the increased IAA level is not high and no synergistic elongation between IAA and brassinosteroids was observed (Figure 1). Furthermore, using the cucumber epicotyl elongation assay, Katsumi (3) showed no interaction between brassinosteroid and PCIB, a competitive inhibitor of IAA. Thus, brassinolide-induced physiological effects would be caused by direct effect as well as by the intervention of changes in other hormone levels in the tissue. Further evidence is needed to draw final conclusions.

Table II. Levels of IAA and ABA in Brassinolide-treated Segments of Squash Hypocotyls

Treatment	period (h)	IAA contents (pmole/ g fresh weight)
Initial		25.8 \pm 1.8
Water	3	22.5 \pm 2.7
0.2 μM Brassinolide	3	31.7 \pm 2.5
Water	5	12.2 \pm 1.8
0.2 μM Brassinolide	5	27.0 \pm 3.1

Treatment	period (h)	ABA contents (pmole/ g fresh weight)
Initial		201.7 \pm 37.2
Water	3	105.0 \pm 20.6
0.2 μM Brassinolide	3	85.0 \pm 3.5
Water	5	136.0 \pm 29.0
0.2 μM Brassinolide	5	75.3 \pm 11.2

Levels of IAA were analyzed by HPLC with a fluorometric detector and indole-propionic acid as an internal standard. Levels of ABA were analyzed by GLC with an ECD and [^{14}C]ABA as an internal standard. Means of results of 3 experiments and standard errors are given ($n=3$). Reproduced and revised with permission from ref. 8.

Brassinolide-induced Inhibition of Leaf Abortion

Iwahori (6) recently showed the inhibition of leaf abortion of explants of Calamondin (*Citrus madurensis* Lour), when brassinolide was fed through the petiole. Mature leaves of 2-4 months old were cut at the leaf blade a distance of 5 mm from the

laminar abscission zone. Brassinolide solutions were placed in Petri-dishes which were covered with solid plastic sheets containing 10 small holes. Leaf explants were placed so that the petiole was passed through the hole into the solution. Trifluoperazine (TFP), an inhibitor of the calmoduline-calcium complex, accelerated abscission, and IAA, when added to TFP, markedly retarded the abscission caused by TFP. When 0.21 μM brassinolide was added to TFP, the abscission induced by TFP was also retarded. However, brassinolide was less effective than IAA, although brassinolide alone retarded the abscission more strongly than IAA alone, indicating that brassinolide inhibits leaf abscission by means of a different mechanism from IAA.

Growth Retardation of Leaves of *Cucumis melo* after the Treatment with Brassinolide

Seedlings of *Cucumis melo* were grown under a plastic for 30 days. When seedlings were treated only once with brassinolide solution at 1 μM so that buds, young and mature leaves were completely wet, severe growth retardation was observed only at the young growing leaves. Although buds were also simultaneously treated with solution, they did not show any retarded growth even after a few days when they were at the actively growing phase, suggesting that brassinolide has an effect only at a limited period of the growth stage. The evidence also suggests that brassinosteroids may be inactivated at least within a few days and that no further retardation of young leaves could be observed therefore, even when they attained the active growth stage. Thus it is necessary to apply brassinolide within a very limited physiological stage or repeat treatment for successful application in agricultural use.

Application of Brassinolide for Prevention of Young Citrus Fruits

Each 10 year-old tree of 'Morita' navel orange was grown in a hexagonal concrete pot in the field at Shizuoka Prefectural Experiment Station, Shimizu, Shizuoka. Until the previous summer, trees were grown under one of the following three conditions:-

1. grown in the open,
2. grown under a plastic film cover,
3. grown in a plastic house.

Thereafter, all the trees were grown in the open.

Brassinolide solution was sprayed until flowers and fruits were completely wet. Treatments were done on the day of anthesis and also 2 months after anthesis when "June drop" had ceased. During June drop non-treated plants showed ca. 98% abortion (Table III). Flower numbers and initial fruit set were computed on the day of treatment.

Rates of fruit set at anthesis treatment were measured 25 and 50 days after treatments and those after cessation of "June drop" were measured at 28 days after the treatment. Fruits were harvested at maturity and the number of fruit, fresh weight, sugar content and titratable acidity were determined.

Preliminary experiments revealed that water or ethanol solutions of brassinolide were hardly incorporated into plant tissue, even in the presence of a surfactant. Thus, addition of chemicals to avoid quick evaporation of brassinolide solution from plant surfaces is prerequisite to induce a brassinolide effect. Thus a new brassinolide solution for agricultural use was devised.

The brassinolide solution was prepared as follows:-

- (a) stock solution: 30 mg brassinolide was dissolved in a solution composed of 3 g dimethylformamide, 3 g polyethyleneglycol (average molecular weight = 1,000) and 48 g ethanol.
- (b) dilution solution was composed of 45 g dimethylformamide, 45 g polyethyleneglycol and 720 ml ethanol.

The dilution solution was added to the stock solution, then the final dilution (x10) was done with water. When further dilution was necessary, a further addition of polyethyleneglycol was made. Surfactant, such as tween 20, was added at 0.1% in the spray mixture.

Table III shows the % fruit set of the trees treated at anthesis. The control fruit set 25 days after anthesis was ca. 10% and on 50 days was ca. 1.6%, showing that

Table III. Percentage Fruit Set on June 1 and June 26, 1987 after Brassinolide Treatment at Anthesis (May 7, 1987)

Brassinolide conc. (ppm)	Previous tree condition	Percentage fruit set (%)	
		June 1	June 26
0	In the open	9.4	1.8
	Under plastic cover	10.3	1.3
	In plastic house	9.5	1.6
Mean±S.E.		9.7±0.3	1.6±0.2
0.01	In the open	15.9	3.2
	Under plastic cover	18.6	3.9
	In plastic house	25.8	4.8
Mean±S.E.		20.1±3.6	4.0±0.6
0.1	In the open	14.8	14.8
	Under plastic cover	17.2	2.5
	In plastic house	67.3	7.6
Mean±S.E.		31.9±19.5	8.5±4.4

NOTE: Reproduced with permission from reference 9. Copyright 1989 International Society for Horticulture Science.

continuous fruit abortion took place during the period. Differences for trees kept in different environmental conditions in previous years were not seen in control sprayed with the solvent system alone.

A marked increase of fruit set in trees treated with brassinolide was observed. A count at 25 days after anthesis revealed ca. 2 to 3 times increase and that at 50 days showed ca. 2.5 to 5 times increase in fruit set on trees treated with brassinolide. Solutions of 0.1 ppm brassinolide were more effective than 0.01 ppm.

The previous environmental conditions of trees also resulted in conspicuous differences in fruit set with brassinolide. As shown at 50 days after treatment, trees grown in the open and in the plastic house showed ca. 8 and 2 times increase over the controls respectively, suggesting that trees previously exposed to a cold environment react more strongly to brassinolide.

The experiments after the cessation of "June drop" were also counted. However, after that period, only 5% of abortion was seen even in the control trees. Thus, it was difficult to show a stimulating effect of brassinolide in this treatment.

Fruit quality was also observed at the time of harvest. However, no difference between solvent and brassinolide treatment was found (Table IV).

IAA inhibited leaf abortion of *Coleus* when given at a high concentration (10). Although brassinolide was found to increase the IAA level in squash segments, such a slight increase may not cause much effect on fruit abortion in navel orange.

Table IV. Yield and Quality of Navel Orange after Treatment with Brassinolide

Brassinolide conc.(ppm)	Yield (kg)	Number	Weight (g)	Sugar conc.	Titrateable acidity (%)
0	13.9±1.0	67.3± 3.6	206± 6	12.0±0.2	1.51±0.06
0.01	13.6±5.3	76.7±28.9	176± 3	12.4±0.5	1.48±0.09
0.1	17.1±4.5	84.7±23.1	205±10	12.3±0.2	1.60±0.08

NOTE: Reproduced with permission from reference 9. Copyright 1989 International Society for Horticulture Science.

Problems with Respect to the Use of Brassinolide in Agriculture.

Many experiments were performed with brassinosteroid for agricultural application. However, most of the results could not be repeated. The reasons for a lack of uniform success may be considered as follows:

Difficulty in Translocating Brassinolide through Plant Tissue. The navel orange experiments revealed that brassinolide was only effective when polyethyleneglycol was added to the spray solution to prevent quick evaporation. Brassinosteroid is active at very low concentrations, such as 0.1 ppm. When such a low concentration was sprayed in solution on the plants with surfactant, the solution dried up immediately and did not be penetrated into the plant tissue. Thus, addition of chemicals to avoid quick evaporation is necessary for a successful treatment.

Easy Decomposition and Difficulty of Movement into Active Site. As shown in *Cucumis melo* experiments, only actively growing leaf tissue could respond to brassinolide. The results suggest that brassinolide might be easily decomposed in the tissue and therefore, is not retained for long period in the active form. Young apple fruits were sprayed with brassinolide and showed marked prevention of fruit drop only when the solution was sprayed once every two weeks for a 3 month period (private communication from Dr. Kiyoshi Yokota of Iwate University). Continuous application of brassinolide would be prerequisite for preventing fruit drop of apples.

Explants of Calamondin clearly showed a marked retardation of leaf and fruit abortion (δ). Repeated application of brassinolide to intact plants of Calamondin failed to prevent either leaf or fruit abortion (private communication from Dr. Iwahori of Kagoshima University). This suggests that brassinosteroids hardly move into plant abscission zones. Thus, it would be necessary for practical use to find tissues to which brassinosteroids are easily translocated. In contrast it would be almost impossible to use brassinosteroids on young cereal ears to prevent fruit abortion, because they are covered with leaf sheaths.

Brassinosteroid had an effect of preventing fruit abortion when plants were kept in previously stressed conditions. As shown in Table III, marked effect was observed when plant environments were drastically changed. Brassinosteroids appear to exert their influence on stressed plants.

Many trials for increasing fruit set of mandarin orange using brassinosteroids revealed no positive effect (unpublished data). Mandarin oranges used were grown in most of southern Japan, showing they were kept under favorable environmental conditions. Thus it would be plausible to apply brassinosteroids to the plants kept under unfavorable environmental conditions.

Literature Cited

- (1) Grove, M. D.; Spencer, G. F.; Rohwedder, W. K.; Mandava, N. B.; Worley, J. F.; Warthen Jr., J. D.; Steffens, G. L.; Flippen-Anderson, J. L.; Cook Jr., J. C. *Nature* **1979**, *281*, 216.
- (2) Gregory, L. E.; Mandava, N. B. *Physiol. Plantarum* **1982**, *54*, 239.
- (3) Katsumi, M. *Plant Cell Physiol.* **1985**, *26*, 615.
- (4) Mandava, N. B.; Sasse, J. M.; Yopp, J. H. *Physiol. Plantarum* **1981**, *53*, 453.
- (5) Yopp, J. H.; Mandava, N. B.; Sasse, J. M. *Physiol. Plantarum* **1981**, *53*, 445.
- (6) Iwahori, S.; Tominaga, S.; Higuchi, S. *Plant Growth Reg.* **1990**, *9*, 119.
- (7) Sakurai, N.; Akiyama, M.; Kuraishi, S. *Plant Cell Physiol.* **1985**, *26*, 15.
- (8) Eun, J.-S.; Kuraishi, S.; Sakurai, S. *Plant Cell Physiol* **1989**, *30*, 807.
- (9) Sugiyama, K.; Kuraishi, S. *Acta Horti* **1989**, *239*, 345.
- (10) Scott, T. K.; Jacobs, W. P. *Science* **1963**, *13*, 589.

RECEIVED May 16, 1991

Chapter 29

Brassinosteroids

U.S. Department of Agriculture Contributions and Environmental Protection Agency Registration Requirements

N. Bhushan Mandava

SRS International, 1625 K Street, N.W., Suite 975, Washington, DC 20006

The discovery of brassinolide by scientists at USDA led to the development of a new group of plant growth substances, termed brassinosteroids, which have the potential for increasing crop yields and biomass. The chapter summarizes the USDA's contributions to brassinosteroids, which include isolation and identification of brassinolide, synthesis of brassinolide and its analogs, and limited biological and field studies on brassinosteroids. An overview of the current data requirements for the registration of brassinosteroids as biochemical pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act is presented.

USDA Contributions to Brassinosteroids

The success story of brassinolide and its analogs (collectively known as brassinosteroids), which is the subject of this chapter, came to realization as a result of a team effort by several chemists, biochemists, chemical engineers, plant physiologists, and plant pathologists within and outside the U.S. Department of Agriculture (USDA). The information presented in this chapter has been generated collectively by a group of scientists whose names are identified by George Steffens in this volume.

Historical Background. This chapter summarizes the USDA's contributions to brassinolide research, a field that has attracted very much attention since the discovery of brassinolide from rape pollen in 1979. For several decades, plant scientists have been investigating the reproductive process in plants, but without much success. In vertebrates, it is known that sex hormones are involved in the reproductive process. Such a process is poorly understood in plants. In the late 1930s, USDA scientists initiated pollen research to gain some insight into the plant reproductive system. We consider this initiative to be the beginning of brassinolide research.

The early USDA efforts to isolate hormones from corn pollen were not very fruitful. The USDA finally terminated the pollen research project in 1944 due to lack of (a) suitable plant bioassay systems to detect the hormones present in pollen and (b) proper analytical methods to isolate and identify the pollen hormones.

In subsequent years, John W. Mitchell developed two bioassays, popularly known as the bean first internode and the bean second internode bioassays. The first internode test was successfully used for screening several commercial chemicals, including such popular pesticidal chemicals as 2,4-D, 2,4,5-T, and Amo 1618. The bean second internode bioassay did not find immediate application, although gibberellins, including gibberellic acid (GA_3) showed elongating properties in this test.

In the 1960s the Agricultural Research Service of the USDA was reorganized, and it established a pioneering laboratory for plant growth substances under the leadership of Mitchell. This pioneering status allowed this laboratory to conduct a broad range of fundamental investigations. The pollen research was reinitiated with the availability of several kinds of pollen. Mitchell tested the crude ether extracts of these pollens in the bean second internode test; the treated internodes showed unusual morphological changes, including elongation and swelling. This kind of response has not been observed previously with other growth substances, including gibberellins. Mitchell screened about 50 kinds of pollen, more than half of which elicited this unusual growth response in the bean second internode bioassay (1).

Discovery of Brassinolide. Being thoroughly convinced that the pollen growth factors were different from the known hormones, Mitchell collaborated with Bhushan Mandava to determine the factors responsible for biological activity.

Rape pollen, in early work, was extracted with ether, and the extract was chromatographed to give an active fraction, termed brassins after the genus *Brassica* (2). When tested in the bioassay, this fraction elicited a very unusual morphological response, termed brassin activity, which was a combination of elongation, curvature, and swelling in the treated internode. Histopathological examination of these treated sections showed a dual response of cell elongation and cell division. On the contrary, GA_3 -treated sections showed only cell elongation (Figure 1).

Chemical examination of brassins showed that it was a complex lipid mixture consisting of several triglycerides, glucosyl esters of fatty acids, and sterols, among other numerous lipids (3). When subjected to repeated HPLC purification, this fraction gave a very small amount of the active material, which elicited a new response, called "splitting response" of the treated internode (Figure 2). Subsequently, it was shown that all the biological responses were due to the same active constituent but the variation in morphological responses (i.e., elongation, swelling, curvature, and splitting) was due to the concentration levels of the active ingredient in the

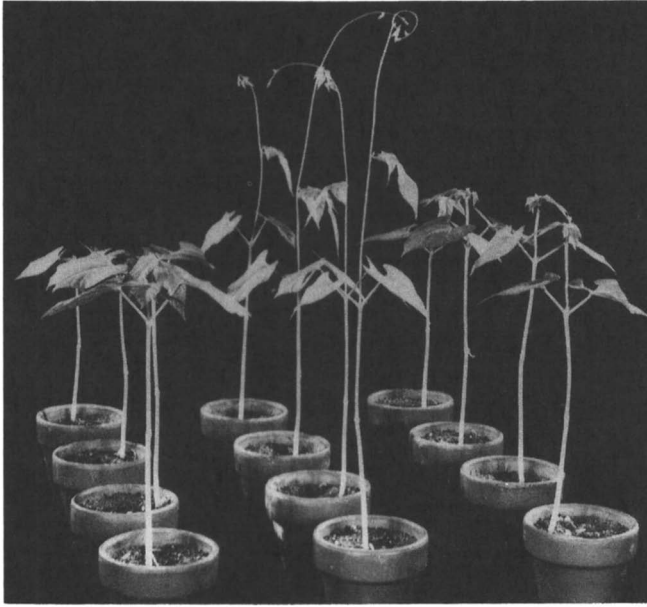


Figure 1. Bean second internode bioassay after 4 days: controls (left) and bean plants treated with gibberellic acid at 10 μg (center) and with pollen extracts at 10 μg (right). (Reproduced from reference 1. Courtesy of Marcel Dekker, Inc.)



Figure 2. Splitting of bean second internode after treatment with 0.1 μg of brassinolide.

crude extract and partially purified fractions; this observation was later confirmed after identification and synthesis of the active ingredient.

Recognizing that the active ingredient was present in such minute quantities, we initiated a crash program to determine the chemical identity of the active ingredient. In this crash program, the USDA group (a) procured 1000 lb of rape pollen from Canada (all the batch shipments of pollen had the designated biological activity); (b) devised a suitable method for pilot-plant extraction of pollen; (c) extracted 500 lb of pollen with isopropanol in 50-lb batches to give an extract (5 lb); (d) partitioned the 5-lb isopropanol extract between a mixture of carbon tetrachloride, methanol, and water to remove inactive materials; (e) sorted the resulting 500 g of the aqueous methanol extract on large chromatographic silica columns; (f) reduced the material to 10 g by repeated open-column chromatography with silica gel and alumina as adsorbents followed by preparative HPLC; and (g) obtained 10 mg of the final product after repeated purification by reverse-phase HPLC.

The biological activity was monitored throughout the isolation stages, i.e., from solvent extraction to final HPLC purification. The 10 mg of material was further purified to give 8 mg of crystalline material. On the basis of the yield of the crystalline product, the active ingredient was shown to be present at a level of 200 ppb in pollen.

The spectroscopic information on the crystalline product indicated that it was a steroid molecule that contained four hydroxyl groups, an oxygen function, and a carbonyl function. We also found that the compound contained a side chain (same number of carbons as in cholesterol) and oxygen and carbonyl functions due to a lactone in the steroid skeleton. The exact locations of the hydroxyl groups and the lactone in the steroid molecule were revealed by X-ray crystallography, which showed the structure **1** (Figure 3). This compound was named *brassinolide*, derived from a combination of words, "brassin" (after the genus *Brassica*) and "olide" for lactone (**4**).

Synthesis of Brassinosteroids. USDA scientists were the first to report the synthesis of various brassinolide analogs, some of which were later found to be present in plants. This work led to, in addition to identifying new brassinosteroids from plants, establishing the structure–activity relationship of brassinosteroids. The reasons for undertaking the synthesis were (a) to ascertain that brassinolide was indeed the active ingredient of the pollen, (b) to determine structure–activity relationships, and (c) to provide quantities of the active ingredient(s) sufficient for biological studies and field work.

Inspection of the brassinolide structure revealed that the molecule is unique, because it contains 13 asymmetric centers, not found in any other steroid. With these many asymmetric centers, theoretically 2^{13} (= 8192) stereoisomers can be derived when one undertakes the total synthesis of brassinolide, but nature produces only one isomer. Chemists in recent years, however, have designed synthetic schemes in such a way that the desired isomer can be obtained very selectively or specifically. Because

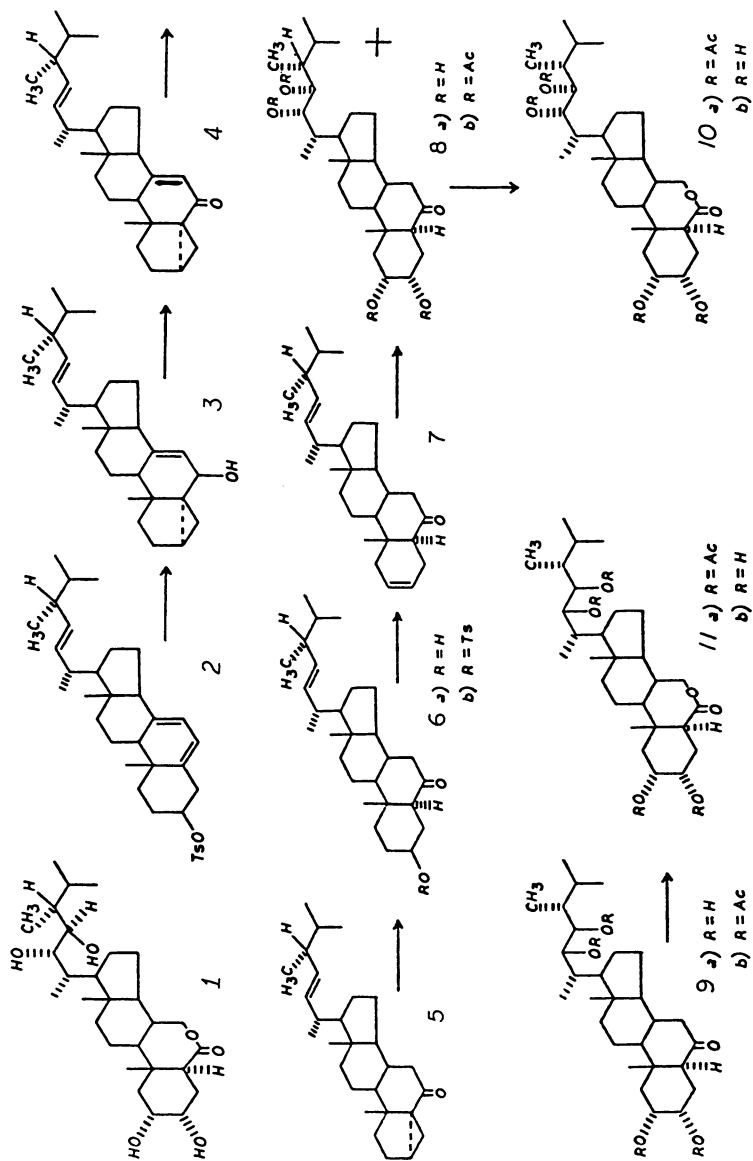


Figure 3. General synthetic scheme for brassinosteroids. (Reproduced from reference 1. Courtesy of Marcel Dekker, Inc.)

one of the objectives of our synthetic program was to make brassinolide or the other active analog(s) economically (we learned that making brassinolide available from pollen is prohibitively expensive), we devised a general synthetic scheme (Figure 3) for partial synthesis of brassinolide and its analogs (termed "brassinosteroids") from commercially available sterols such as cholesterol, stigmasterol, and ergosterol (5, 6). Later several of the synthesized analogs were found to be naturally occurring. For the synthesis of brassinolide, we could not find a suitable sterol from plant sources. For brassinolide synthesis, therefore, we used as starting material a sterol from a marine source (oysters).

Early in our synthetic program for brassinolide and its analogs, we learned that there were certain structural requirements in the steroid molecule for eliciting the biological activity. These requirements include the following (1, 5–7):

- steroid skeleton with angular methyls at C-18 and C-19
- side chain at C-17 similar to the side chain in cholesterol
- α -orientation at C-5 (A/B ring junction)
- α -orientation of hydroxyl groups (*cis* geometry) at C-2 and C-3 of ring A
- hydroxyl groups at C-22 and C-23
- oxygen function at C-6 in ring B

Our synthetic work on brassinosteroids showed that the stereochemistry in the steroid molecule is very important to retain the biological activity. We found that, of the 13 asymmetric centers in the molecule, only 3 asymmetric centers (at C-22, C-23, and C-24) can be altered. On the basis of this information, we prepared brassinolide and all the analogs (2³) by using the general synthetic scheme (Figure 3). Additionally, we prepared compounds having different substituents (H, CH₃, and C₂H₅) at C-24 (1, 7).

Biochemical and Physiological Responses. Brassinosteroids have been reported to elicit several plant physiological functions, including the following (7):

- promoting plant growth and development of different crop plants
- increasing crop yields
- increasing biomass production
- improving crop quality and enhancing early crop maturation
- overcoming plant stress effects due to nutrients and fertilizers, salt tolerance, and cold tolerance
- increasing resistance to pesticide injury (acting as safeners)

These findings (especially the first four findings) were made by USDA scientists. Other groups, notably Japanese scientists, substantiated the USDA findings (7).

Physiological Responses. Brassinosteroids promote plant growth by promoting cell elongation and cell division. In the presence of other plant hormones, when tested in systems specifically designed for them, brassinosteroids show the following effects, which are reversible (7–10):

1. Brassinosteroids (BRs) interact strongly with auxins. In some test systems, the response is synergistic. BRs, alone and in combination with auxins, induce the synthesis of ethylene.
2. BRs and gibberellin (GA) show independent responses. Their responses are additive.
3. BR and cytokinin responses vary. They show neither synergism nor additive relationship.
4. BRs interact with abscisic acid (ABA).

Biochemical Effects. In addition to physiological responses of cell elongation and cell division, the following biochemical changes are observed as a result of BR application to plants (1, 7, 11, 12):

1. BRs regulate nucleic acid and protein metabolism, because they increase the synthesis of nucleic acids and cellular proteins and the activity of RNA and DNA polymerase.
2. BRs increase the levels of soluble proteins and reducing sugars.
3. BRs do not affect the peroxidase and polyphenol oxidase activities (this is in contrast with auxin action), a fact indicating the synthesis of specific proteins by BRs.
4. BRs increase the activity of ATPase (an enzyme responsible for acid secretion and changes in membrane levels).
5. BRs increase CO₂ fixation (an indication of cytoplasmic malate synthesis via phosphoenol pyruvate carboxylase, PEP carboxylase; this activity results in the accumulation of fraction-1 protein) and also in vivo CO₂ fixation by ribulose biphosphate action

EPA Registration Requirements

Until the discovery of brassinolide by USDA scientists in 1979, it was thought that only five groups (indole auxins, gibberellins, cytokinins, abscisic acid, and ethylene) of hormones were responsible for regulating plant growth and development. Following this discovery, a number of compounds similar to brassinolide both in structure and physiological activity were isolated from different parts of plants. On the basis of published

reports (1, 7), brassinosteroids occur not only in higher plants but also in lower plants. Furthermore, it is now accepted that they are ubiquitous in nature and that they comprise a recognized sixth group of plant hormones.

Commercialization of Brassinosteroids. Brassinosteroids are compounds of significant importance at the national level because of their potential use in agricultural applications for food, feed, and fuel production. These compounds can be used in increasing crop efficiency, not only to increase crop yields but also to maintain crops under stress conditions, such as the lack of soil moisture under drought conditions and the lack of sufficient nutrients for plant growth. They also have potential application for crops grown in semiarid and arid lands, in which land fertility and rainfall are nonexistent or minimal. They may be useful for crops raised under extreme stress conditions, because they are reported to overcome the different types of stress exerted by temperature, water, and salt. These compounds may become commercially viable if it can be demonstrated that can promote early maturation and increase the production of seasonal crops and fruits. Further, their application to crops may overcome pesticide injury and thus they may act as safeners. Combined with genetic engineering for susceptibility, tolerance, and disease resistance, they may also play an important role in practical agriculture.

We know that brassinolide and the other brassinosteroids are novel plant growth regulators that contribute either to crop yield increases or to promotion of crop quality. How do we successfully exploit them for their use in commercial applications to agricultural productivity?

Natural Products as Biorational Pesticides. In the United States and other countries, plant growth regulators need to be registered before they are applied to crops on a commercial scale. In the United States, plant growth regulators are regarded as pesticides, which are covered by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). The U.S. Environmental Protection Agency (EPA) administers FIFRA, under which all pesticides need to be registered before they are marketed. Currently, there are over 600 pesticide active ingredients and 45,000 products derived from the active ingredients. Under the 1988 FIFRA amendments, the EPA has a mandate to reregister all the pesticides that are currently marketed. It is believed that about 200 active ingredients and 20,000 products will be canceled in the reregistration program because of the stringent data requirements for continued registration.

Consistent with the EPA's mission to protect human health and the environment, the agency has from time to time adopted policies and procedures designed to promote technologies and practices either considered beneficial to human health or the environment or considered to pose less risk to the aforesaid than other alternatives, even though such other alternatives may not pose a risk of unreasonable adverse effects per se. That is, EPA has acted in various ways to consciously promote and encourage

environmentally sound industrial and agricultural practices. The agency has undertaken these activities in the public interest.

As one such activity, EPA's Office of Pesticide Programs (OPP) created the category of "Biorational Pesticides" (13) to promote the development and use of such agents by simplifying the data requirements for such agents. The agency's stated reason for doing so are as follows:

“. . . the Agency expects that many biorational pesticides pose lower potential risks than conventional pesticides. Therefore, these pesticides are subject to a different set of data requirements as specified in SS 158.165.”

In this proposed classification, some of the relevant factors that distinguish biorational pesticides from conventional pesticides include:

“. . . their unique non-toxic mode of action, low use volume, target species specificity, and natural occurrence.”

Biochemical Pesticides. The characteristics noted above were not specified as being all of the attributes that a biorational pesticide might exhibit, nor was a biorational pesticide required to exhibit all of the cited characteristics. Proposed SS 158.165(a) (3) noted that EPA would make case-by-case decisions on what was or was not a biochemical-type biorational pesticide. This provision was included so that “chemicals which are substantially similar to biochemicals” but might not “technically meet the two criteria established for defining biochemical pest control agents” might be none-the-less classified as biochemical pesticides.

The two criteria given in the proposed Section 158.165 regulation were

“(a) The chemical must exhibit a mode of action other than direct toxicity in the target pest (e.g., growth regulation, mating disruption, attraction). Pesticides such as strychnine, rotenone, nicotine, pyrethrin, which exhibit direct toxicity, are not considered biochemical pest control agents; and, (b) The chemical must be naturally occurring, or if the chemical is synthesized by man, then it must be structurally identical to a naturally occurring chemical . . .”

Part 158 in Title 40 of the Code of Federal Regulations was finalized in 1984 (14). In the final rule, the term “Biorational Pesticide” was dropped and the term “Biochemical and Microbial Pesticides” was adopted to describe this class of pest control agents (Figure 4). The general characteristics of biochemical and microbial pesticides remained essentially unchanged.

“Biochemical and microbial pesticides are generally distinguished from conventional chemical pesticides by their unique modes of action, low use volume, target species

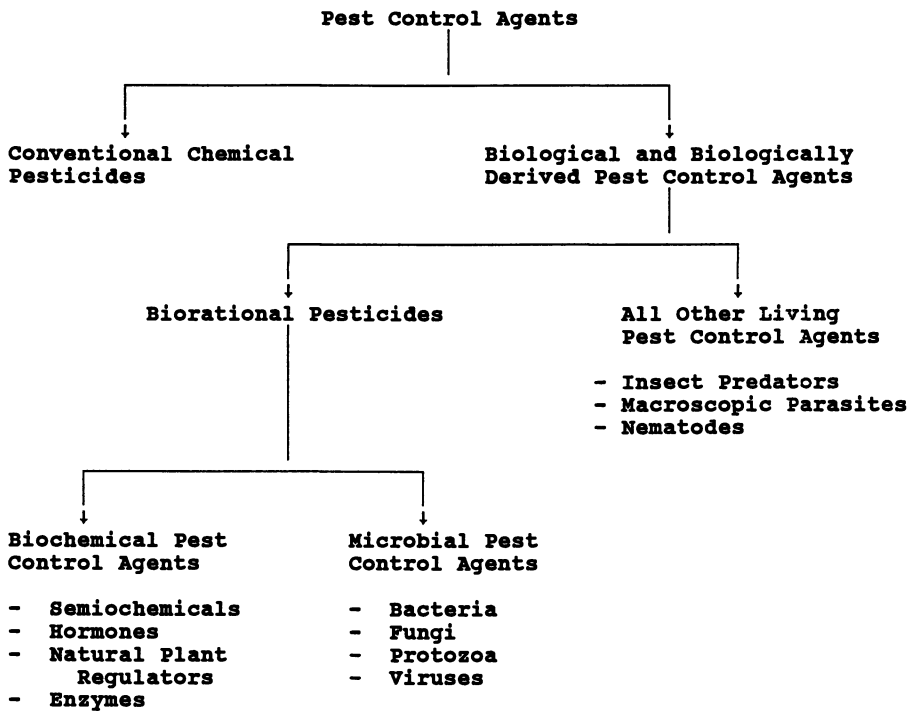


Figure 4. Relationship between conventional pesticides, biological control agents, and biorational pesticides. (Reproduced from reference 1. Courtesy of Marcel Dekker, Inc.)

specificity, or natural occurrence. In addition, microbial pesticides are living entities capable of survival, growth, reproduction, and infection. Biochemical and microbial pesticides are subject to a different set of data requirements, as specified in SS 158.165 and 158.170, respectively.”

The proposed criteria for a biochemical pesticide were, however, changed in the final regulation. In the current 158.165 regulation a biochemical pesticide is defined simply as follows:

“Biochemical pesticides include, but are not limited to, products such as semiochemicals (e.g., insect pheromones), hormones (e.g., insect juvenile growth hormones), natural plant and insect regulators, and enzymes. When necessary the Agency will evaluate products on an individual basis to determine whether they are biochemical or conventional chemical pesticide.”

Thus, under the current regulation, the exact nature of the natural product being considered as a potential biochemical pesticide is less relevant to its classification than is the question of the degree to which it exhibits the characteristics that distinguish biochemical pesticides from conventional chemical pesticides. Further, consistent with the regulatory history in this area, the overall effect of the distinguishing characteristics on potential risk would appear to be a significant factor in classifying a substance as a biochemical pesticide.

These characteristics, although not all inclusive, are given in the current regulation as essentially (a) unique mode of action, (b) low use volume, (c) target species specificity, or (d) natural occurrence.

Brassinosteroids as Biochemical Pesticides. Applicants wishing to register brassinolide and the other brassinosteroids with EPA should request the agency to classify brassinosteroids as biochemical pesticides. The basis for such a request is that brassinosteroids possess the characteristics (*viz.*, unique mode of action, low volume use, target species specificity, and natural occurrence) required for categorizing them as biochemical pesticides. The applicants need to assert that brassinolide (and other brassinosteroids) possesses qualities to varying degrees that contribute to an overall very low potential risk associated with use as a plant regulator and that make classification of brassinolide (and other brassinosteroids) as a biochemical pesticide the most appropriate regulatory decision in this matter. We suggest that the applicants provide information on how brassinolide (and other brassinosteroids) fits these characteristics in a point by point manner. Also, the applicants are required to provide documentation for the stated assertions with respect to the characteristics of brassinolide (and other brassinosteroids). Furthermore, the applicants need to demonstrate that introduction

of brassinolide (and other brassinosteroids) into U.S. agriculture is environmentally beneficial in those crops in which it is efficacious.

Brassinosteroids as Safer Pesticides. As stated above, for classification of chemicals as biochemical pesticides, the EPA considers the following criteria: (a) natural occurrence, (b) low use volume, (c) unique mode of action, and (d) target species specificity. Brassinosteroids would meet the criteria set by the EPA for biochemical pesticides because of the following reasons:

1. Brassinosteroids are ubiquitous in nature. Brassinolide was first isolated from the pollen of rape (*Brassica napus* L.) plant and has plant-growth-promoting activity on various plants. Following the brassinolide discovery, other (about 20) brassinosteroids including brassinolide were found mostly from various higher plants.
2. Brassinosteroids are the most potent of the all the plant hormones in eliciting plant-regulating activity. On the basis of limited information, use volume of brassinosteroids will not exceed 20 g/acre.
3. On the basis of literature data, brassinosteroids undoubtedly exhibit a unique mode of action and target species specificity for both plants and animals.

Brassinosteroids, therefore, would qualify for classification as biochemical pesticides. Once they are classified as biochemical pesticides, the data requirements for U.S. registration would be significantly reduced (because of a tier approach), especially in the areas of toxicology, ecology and wildlife, and environmental fate. Because of their low use volume, brassinosteroids are unlikely to leave any residues in crops, and they would qualify for exemption from the requirement of a tolerance. If that were the case, what applicants seeking U.S. registrations need to do is to develop data on product chemistry and acute toxicology, as well as use profiles for their products, which will be dealt with on a case-by-case basis.

In conclusion, we suggest that the applicants approach the EPA for classifying brassinosteroids as biochemical pesticides. They save time as well as the costs for developing the required data for registration of the products derived from brassinosteroids.

The EPA in recent years has been encouraging the agrochemical industry to develop safer pesticides that, among others, would minimize the risks associated with pesticides and also reduce environmental contamination. Brassinosteroids as a group will have a promise for safer pesticides because they would fit into the agency's definition of safer pesticides.

Literature Cited

1. Mandava, N. B.; Thompson, M. J. In *Isopentenoids in Plants: Biochemistry and Function*; Nes, W. D., Fuller, G., Tsai, T. S., Eds.; Marcel Dekker: New York, NY, 1984; pp 401–431.
2. Mitchell, J. W.; Mandava, N. B.; Worley, J. F.; Plimmer, J. R.; Smith, M. V. *Nature* **1970**, *225*, 1065–1066.
3. Grove, M. D.; Spencer, G. F.; Pfeffer, P. E.; Mandava, N.; Warthen, J. D., Jr.; Worley, J. F. *Phytochemistry* **1978**, *17*, 1187–1189.
4. Grove, M. D.; Spencer, G. F.; Rohwedder, W. K.; Mandava, N. B.; Warthen, J. D., Jr.; Worley, J. F.; Steffens, G. L.; Flippen-Anderson, J. L.; Cook, J. C., Jr. *Nature* **1979**, *281*, 216–217.
5. Thompson, M. J.; Mandava, N. B.; Flippen-Anderson, J. L.; Worley, J. F.; Dutky, S. R.; Robinson, W. E. *J. Org. Chem.* **1979**, *44*, 5002–5004.
6. Thompson, M. J.; Mandava, N. B.; Meudt, W. J.; Lusby, W. R.; Spaulding, D. W. *Steroids* **1981**, *38*, 567–580.
7. Mandava, N. B. *Ann. Rev. Plant Physiol. Mol. Biol.* **1988**, *39*, 23–52.
8. Yopp, J. H.; Mandava, N. B.; Sasse, J. M. *Physiol. Plant.* **1981**, *53*, 445–452.
9. Mandava, N. B.; Sasse, J. M.; Yopp, J. H. *Physiol. Plant.* **1981**, *53*, 453–461.
10. Gregory, L. E.; Mandava, N. B. *Physiol. Plant.* **1982**, *54*, 239–243.
11. Kalinich, J. F.; Mandava, N. B.; Todhunter, J. A. *J. Plant Physiol.* **1985**, *120*, 207–214.
12. Mandava, N. B.; Thompson, M. J.; Yopp, J. H. *J. Plant. Physiol.* **1987**, *128*, 63–68.
13. Proposed Section 158.65, 47 FR 531292, November 24, 1982.
14. 49 FR 42881, October 24, 1984.

RECEIVED June 7, 1991

Chapter 30

Brassinosteroids through the Looking Glass

An Appraisal

Horace G. Cutler

Richard B. Russell Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Athens, GA 30613

The brassinosteroids are a unique class of biologically active natural products that possess high specific activity. Their low application rates on crops make them environmentally safe and those used on a large scale (brassinolide and 24-epibrassinolide) are non-toxic. At the physiological level they elicit many changes and may represent a new class of hormones in plants. Limited fungal experiments demonstrate that brassinolide and 24-epibrassinolide increase mycelial growth and reduce the time to produce fruiting sporocarps. Brassinosteroids affect moulting in insects and structurally resemble the ecdysteroids. Applications of 24-epibrassinolide in China over a six-year period have given a significant increase (~ 15%) in wheat yields and horticultural crops. The economic aspects of the brassinosteroids may have worldwide effects.

Historical Perspective. From a distinctly personal viewpoint, the unfolding of the brassinosteroids research was sharply divided into three segments. Upon examination they may, in part, answer the riddle as to why the later development and practical applications for these potentially important compounds passed from being virtually the sole domain of researchers in the United States to others.

The first incident occurred at an evening meeting of the Potomac Division of the Plant Physiology Society at the National Arboretum in the winter of early 1963 when J. W. Mitchell presented a time-lapse movie showing the effects of an extract from rape pollen (*Brassica napus* L.) on the extension of bean second internodes. A great deal of excitement was generated, but it was clear that because of the physical limitations a lifetime's work lay ahead. In those days, sophisticated nuclear magnetic resonance spectrometry and mass spectrometry were things of the future, though infra-red spectroscopy was available and fairly advanced. Additionally, one of the major problems was the isolation of sufficient quantities of pure compound to make an unequivocal identification, though the quest appeared to be one worthy of major support.

Later, in the mid 1970's, a concerted effort was mounted by the USDA, ARS, laboratories in Maryland. The pitfalls to be encountered were calculated well

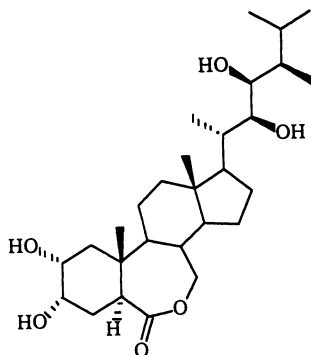


Figure 1. Brassinolide

in advance. For example, there were sincere doubts about obtaining sufficient yields of the active material(s) from rape pollen. The chance that the bioactive natural products might be known gibberellins were gravely considered, relative to the investment. Indeed, it was even suggested that a new gibberellin might be discovered. But, the possibility also existed that a whole new class of natural products might be discovered and if that were to be the case the potential practical application might have enormous benefits for agriculture. This proved to be correct when, in 1979, the structure for brassinolide, (22R,23R)-2 α -3 α ,22,23-tetrahydroxy-24S-methyl- β -homo-7-oxa-5 α -cholestan-6-one (Figure 1) was finally reported (1), marking the end of an almost twenty-five year effort to isolate and identify one of the major biologically active components in rape pollen.

The third part of the trilogy took place in a hotel room in 1980. It was late at night and a colleague had invited me for a nightcap after a long, strenuous day. Having poured myself a libation, I was about to put it to my lips when, out of curiosity, I asked, "And what are we going to do with brassinolide and the brassinosteroids?" To which came the reply, "Nothing!" The glass travelled through my right hand, but I managed to grasp it before it slipped through my fingers. "What!" I exclaimed. Again came the reply, "Nothing. We'll let someone else develop them." To which I retorted, "Do you think that wise?" The answer, "Absolutely." It seemed the better part of discretion to terminate the topic at that point. But, it was reasonable to assume that such an interesting class of compounds would become the focus for development as practical plant growth regulators, by others.

Armed with this tripartite piece of historical information we now discuss the brassinosteroids along three major lines. That is, their physiology in plants and fungi, their economic potential and the future, and their speculative practical use.

Physiology of the brassinosteroids in plants and fungi.

Hormonal properties on plants. The occurrence and distribution of the brassinosteroids in assorted plant tissues has been comprehensively discussed (2). They occur in immature seeds, leaves, stems, shoots, pollen, and fruit. Furthermore, they have been found in insect galls and the corresponding healthy tissues. Although the chemical species remained the same in both sets, only brassinolide occurred in galls, but not in healthy tissues. Again, these compounds

are found throughout the plant kingdom including the algae, gymnosperms, and monocotyledonous and dicotyledonous plants. While they have yet to be unambiguously shown to be present in bacteria and fungi there may be some limited evidence to support their occurrence in fungi. The fact that thirty-one brassinosteroids and two glucoside conjugates have been fully characterized to date and another thirty have been verified (3) indicates, in the evolutionary sense, that this class of natural products has co-evolved well.

While it may be necessary to gather more evidence before an absolute assignment for the brassinosteroids as phytohormones can be made, the information obtained from basic plant physiology experiments points to the conclusion that brassinosteroids, and specifically brassinolide, may represent a new class of plant hormones, in addition to the indoles, ethylene, gibberellins, abscisic acid and the cytokinins. It was obvious very early in the isolation of brassinolide, that the major active component was present in very small quantities in rape pollen but that exceedingly small amounts elicited profound responses in bioassays. The figure given for the occurrence for brassinolide in rape pollen is in the order of magnitude of 100 parts per billion (4) and the effects of 10 μg of crude extract, probably containing a mixture of brassinosteroids, when applied to bean second internodes, were huge increases in length. After four days, internodes had extended to 155 mm *versus* 12 mm for controls (5). Even allowing for at least two highly variable factors, the relative purity of the extract and the possible synergistic action of other brassinosteroids or endogenous plant growth regulators, the response is indeed hormonal based on the quantities involved and the concomitant response.

There is further evidence to support the claim that brassinosteroids are hormonal in their action (6). These are the effects of brassinolide on gravitropism (7), effects in conjunction with light quality (8), effects on photosynthate partitioning (9), probable effects on phytochrome (10), substitution for indole-3-acetic acid in soybean epicotyls (11), enhancement of xylem differentiation (11), stimulation of membrane permeability in cucumber hypocotyls (12), and stimulation of ATPase activity (12). Taken objectively, many of these specific physiological and biochemical functions which are attributed to brassinolide, and by inference to the brassinosteroids in general, have been attributed to the other plant hormones, especially indole-3-acetic acid and the gibberellins (6, 13).

Hormonal properties on fungi. While many observations lead to the inevitable conclusion that the physiology of plants presents a wealth of opportunities to conduct research with the brassinosteroids, there are other implications in the plant kingdom. Consider the reference to the possibility that brassinosteroids may occur in fungi, albeit tenuous at present (14). The research reported by Adam, et al. (15) demonstrates that brassinolide (Figure 1), 24-epibrassinolide (Figure 2), and 22S,23S-homobrassinolide (Figure 3), induced 2-3 times more rapid mycelial growth and greatly reduced the time in which fruiting sporocarps were produced in the fungus *Psilocybe cubensis* (an hallucinogenic genus) and *Gymnopilus purpuratus*. This implies two possible physiological roles for the brassinosteroids in fungi. Either the brassinosteroids mimic the chemical characteristic of an initiator chemical template or the brassinosteroids, in some form, are indeed the

fruiting elicitors. Considering the former, there then exists the possibility that the brassinosteroids mimic a sterol, or steroid initiator which is responsible for the completion, both sexually and architecturally, of the fruiting body. If the latter is established, then it would seem that the brassinosteroids are, at least in the lower plants, sexual hormones. One must also consider that if the brassinosteroids occur in algae then one might also expect to find them in lichens which consist of an intimate association of a fungus with an alga. Since there are approximately eighteen thousand species of lichens that have been classified (16) the probability of discovering the occurrence of a brassinosteroid in a lichen is good. Furthermore, because the alga is embedded in the fungus there exists the opportunity for the transfer of nuclear material between the two organisms. That is, the specific genetic mechanisms encoding for the production of brassinosteroids have passed from the alga to the fungus and, more importantly, *vice versa*, which means that the genetic information has transferred from a fungus previously associated with a producer alga to a non-producer alga during the evolutionary process.

Occurrence in pollen. That brassinosteroids occur in pollen suggests that either they are there by an accident of nature, and nature tends to the conservation and not the useless dissipation of energy, or they have some active role, perhaps in the generative act. It is possible, therefore, that the brassinosteroids may be sex hormones, or at least may aid as a growth promoter for pollen tubes during their lengthy passage from the stigma, through the style to the ovary, to deliver the nuclei for fertilization of the egg.

Because brassinolide, and probably other brassinosteroids, occurs in pollen, other aspects which may affect plant, insect, and animal physiology will have to be considered in the future. For example, what about the effects of brassinosteroid laced nectar and pollen to foraging birds and insects? It will be recalled that the collection of sufficient rape pollen to isolate brassinolide came not from manual collection, but from pollen collected by honeybees (4). That fact leads to at least three thoughts.

First, pollen is prized by health food aficionados and, while the brassinosteroids have not been detected in all pollen, what is the effect on the metabolism of those who ingest pollens that do contain these materials? While this may appear to be an irrelevant question, it is of interest to note that bees collecting nectar and pollen from foxglove (*Digitalis* sp.) may produce honey that contains digitalis in sufficient quantity to be fatal to those with heart problems. Honey derived from the nectar of azaleas and rhododendrons is also toxic. Second, what is the effect on the bee brood that feeds on brassinosteroid laden pollen during the developmental stage, assuming that these natural products possess steroidal properties? Third, what is the effect on the larvae of wax-moth (*Galleria mellonella* L.) in hives that are filled with brassinosteroid containing pollen? The work published by Richter and Koolman (17) indicates that the brassinosteroids bear a remarkable structural similarity to the ecdysteroids which control moulting in insects and, furthermore, the brassinosteroids were demonstrated to influence

electric activity of neurons. Thus, if the period for the larval stage of *Galleria* is extended, then the damage to bee honeycomb and brood becomes greater.

Potential research topics. Because of their steroidal structure, the brassinosteroids offer many potential avenues for research both in phytochemistry and zoochemistry. If, for example, the presence of the brassinosteroids is relatively ubiquitous in plants, is it then possible that they play a role in host parasite relationships? It is of note that many plant parasitic and intestinal nematodes must obtain their cholesterol precursors from their respective hosts (18), a paradigm of the former being the plant gall inducing nematode *Ditylenchus dipsaci* and of the latter the somewhat large intestinal nematode *Ascaris lumbricoides*. Certain phytopathogenic fungi also have a sterol requirement for completion of their life cycle (19), as do certain insects (20).

It remains to be seen what effect will be demonstrated by the brassinosteroids on the mammalian system, if any. If one accepts the thesis that the animal steroids had their genesis in a common phytosterol ancestor and that certain mechanisms evolved in the animal system for steroid production, the question then arises as to what responses are elicited in the mammalian system when it is challenged with select brassinosteroids. Another way of asking the question is, do the brassinosteroids have any medicinal value? Until now, limited quantities have precluded such examinations, but because synthesis is becoming more efficient the day cannot be far off for pharmaceutical evaluations if, in fact, they have not been tested already.

At the basic level in plant physiology, the brassinosteroids will most probably be an impetus to the isolation of other endogenous growth regulators. Over forty years ago, it was inconceivable that plant hormones other than indole-3-acetic acid, gibberellic acid, and ethylene would be discovered. But because the sum total of these potent natural products could not answer the complex questions posed by experimental and practical plant physiology, the hunt continued for other hormones. For example, the discovery of the cytokinin, kinetin, was a marvelous piece of sleuthing and when the structure was finally elucidated it was obvious in retrospect (it nearly always is) that anyone with a strong background in nucleic acid chemistry could have arrived at the same molecular discovery by synthetic trial and error. Furthermore, because kinetin was found in stale fish sperm, the same discovery could have been made by a zoological-biochemist testing natural products involved in reproductive chemistry. The genius lay in the way that the problem, one in tissue culture, developed and was solved. The same reasoning holds true for the discovery of abscisic acid, which was isolated from dormant buds and cotton bolls. Until the 1950's it was presumed, and taught as gospel, that indole-3-acetic acid controlled, by apical dominance, the dormancy of axillary buds in plants. Hence, the basic physiological work of Darwin, Boysen-Jensen, Went, and Thimann which led to the hormonal properties and influence of indole-3-acetic acid in plants already contained the seeds of discovery for abscisic acid albeit somewhat hidden and obscure at the time. If the papers contained in this volume are carefully reviewed, it is seen that the brassinosteroids by no means answer the enigma of hormonally regulated plant growth and development, or that of microorganisms,

even when all the information is dovetailed with the known plant growth regulators. Hence, something is still missing and implies further interactions with other co-factors and, perhaps as yet, undiscovered hormones. But as a footnote, those remaining discoveries will depend upon the correct choice of bioassays, as was the case with the brassinosteroids.

Economic potential.

Field trial results. The primary question asked by industry and agribusiness is, "Does there appear to be a practical use for the brassinosteroids; can the product be sold for agricultural and other uses?" From the data gathered by the Japanese, the answer is an emphatic yes! In 1987, the Plant Growth Regulator Society of America and the Japan Society for the Chemical Regulation of Plants met jointly for the first time in Hawaii, and part of the program dealt with the brassinosteroids as plant growth regulators for practical use. Shingo Marumo presented work showing that ears of corn (*Zea mays* L.) treated with brassinolide filled completely to the uppermost kernel (2). The magnitude of that discovery brought home the applied use in a single crop. That disclosure caused many to keep alert for pieces of information and, from time to time, ideas concerning brassinolide were discussed and fragments of data were revealed. One of the most astonishing was the private disclosure from China that wheat (*Triticum aestivum* L.) yields had been increased ~ 10% for two successive years. This seemed to be a significant figure and one that, if it were true, might have a major impact on international wheat markets. When Nobuo Ikekawa and Yu-Ju Zhao presented their work at the Brassinosteroid Conference (21), the full extent of the potential effect of the brassinosteroids on agricultural crops was clear. The presentation fleshed out the effects of 24-epibrassinolide on wheat in Japan and China using concentrations ranging from 0.1 to 0.001 ppm, over a six year period. These experiments were based on such a large scale that 3,333 hectares were treated. The results were remarkably consistent. The average increase in wheat yields was up to 15%, with some as high as 20-25%. Results with corn (*Zea mays* L.) showed a 10-15% increase at one set of locations and an equal increase of 15-20% at a number of other locations. Tobacco also responded to 24-epibrassinolide treatment. At 0.01 ppm root weight almost doubled, leaf area per plant increased by one-seventh, and nicotine content almost doubled. As an important footnote, results were included from trials with watermelon and cucumber; the former had increased yields of 10-20%, though 0.1 ppm 24-epibrassinolide gave 40% increases, while the latter improved by 10-20%. Numbers of grapes per cluster also increased ~ 66% with 0.01 ppm treatment and ~ 29% with 0.1 ppm. However, the caveat was extended that the effects were more pronounced in China than in Japan. American researchers appear to have obtained erratic results with brassinolide in field evaluations (4).

It would appear that the projected use for 24-epibrassinolide is greater in China because of the consistent responses obtained, the land mass, and the population density, so that while the market may be limited in Japan because of sporadic responses, the market in China may be exceedingly large. Though

obviously, any of the large wheat growing areas of the world, especially America, Australia, and Russia, would be a large market.

Perhaps the most reassuring part of Ikekawa's report was the toxicological data. The LD₅₀ was 1 g/kg (oral) for the mouse, 2 g/kg (oral and dermal) for the rat, and the Ames test for mutagenicity was negative.

It is worth considering that the apparent erratic behavior for 24-epibrassinolide in Japan has parallel in another agricultural chemical, gibberellic acid A₃. Treatment of rice to increase seed germination and later during the life cycle to advance maturity and increase yields has been shown to critically depend on two factors. In seed treatment with Release™, which consists of formulated GA₃, semi-dwarf rice can be planted deeper than normal and there follows good germination so that the seedlings, which are now taller than control plants, can compete very favorably against weeds. Obviously, the semi-dwarf varieties are GA deficient and the addition of GA₃, which is probably metabolized to other "essential" GA's, compensates and this explains the biochemical response. More difficult to rationally explain is the critical timing for Ryzup™ treatment, which is a formulation of GA₃, and which must be applied at the 2-3 leaf stage to induce earlier maturity and increased production. The physiological explanation for application at this particular time during the life cycle has yet to be elucidated and, in addition, the cultivar and cultural practice play a vital role in the response (discussions with A. Croveti and R. Carlson). But whether the response in the case of 24-epibrassinolide and other brassinosteroids, depends upon formulation, nutrient levels, daylength, cultivar, stage of physiological development, or temperature, remains to be seen. A critical analysis of all the data from Japan and China may give the necessary clues, but again, the success of this venture requires funds, considerable time, and serious financial investment.

Plant protectants. Another use for the brassinosteroids has been as plant protectants from both pesticide and environmental adversity. Rice treated with brassinolide was more tolerant to simazine [6-chloro-N,N'-diethyl-1,3,5-triazine-2,4-diamine] and there was a marked decrease in herbicide injury from simetryn [N,N'-diethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine], butachlor [2-chloro-2',6'-diethyl-N-(butoxy methyl) acetanilide], and pretilachlor [2-chloro-N-(2,6-diethylphenyl)-N-(2-propoxyethyl) acetamide] (22). Also, brassinolide treated rice plants were less susceptible to salt injury (22). Chinese cabbage was also shown to be more resistant to plant pathogens following brassinolide treatment (22) and it is tempting to speculate whether or not these plant metabolites interfered with the sterol pathways. In turn, this prompts the thought that suitably rearranged brassinosteroids, either by biotransformation or synthesis, might yield some sterol biosynthesis inhibitors thereby producing some environmentally acceptable fungicides.

Insect control. Another practical use for the brassinosteroids appears to be in the area of insect control. Richter and Koolman (17) have presented data to show that these compounds possess antiecdysteroid activity. The duration of the larval stage was delayed in the cockroach, *Periplaneta americana*, by approximately ten days.

Some specificity was demonstrated in that 22S,23S-homobrassinolide induced the delay, whereas 22S,23S-homocastasterone (Figure 4) had no effect. Once again, this pioneer work leads to several possibilities including the potential biological activity of brassinosteroid derivatives, and it is certain that as a result of this research other laboratories will be evaluating this class of compounds in entomological systems.

Speculative practical uses.

Reproductive inhibitors in plants. If the brassinosteroids regulate some stage of the reproductive cycle in plants, and other species, the opportunity exists for the production of brassinosteroid mimics that will substitute in the biosynthetic pathway effectively shutting down a directed process. Such a series of compounds could have a major effect, especially in certain horticultural crops where the elimination of the flowering sequence would ensure continuous production of other tissues such as leaves, bulbs, and other storage organs. A projection of this concept is the control of certain seed bearing weeds, where cessation of the flowering cycle eliminates proceeding generations. While the thought that one brassinosteroid derivative would control reproduction and flowering in a large number of diverse species is open to some debate, because natural products possess the feature of being relatively target specific, it is important to realize that plant growth regulators, which are natural products, appear to be an exception to this rule because they are ubiquitous. For example, indole-3-acetic acid, the gibberellins, cytokinins, and abscisic acid are found throughout the entire plant kingdom, including microorganisms, and each may elicit a series of similar responses in different species.

Fruiting body hormones in fungi. From the data presented by Adam, et al. (15), a good deal of practical work remains to be done with the higher fungi and the brassinosteroids. The fact that 22S,23S-homobrassinolide and 24-epibrassinolide treatments increased the number of fruiting bodies in *Psilocybe cubensis* and *Gymnopilus pupuratus* implies that these and other brassinosteroids may have practical use for the induction of fruiting bodies from the mycelial, or filamentous stage. Simply stated, the edible fungi fall into two main categories, the Ascomycetes and Basidiomycetes. Examples of the former are the morels, helvels and pezizas. The morels, including *Morchella conica*, *M. hybrida*, and *M. vulgaris*, are prized by mushroom hunters throughout the world who keep the whereabouts of their finds a closely guarded secret. Some of the Basidiomycetes of economic and Epicurean importance are *Boletus appendiculatus*, *B. auranticus*, *B. badius*, *B. bovis*, *B. castaneus*, *B. chrysenteron*, *B. cynescens*, *B. edulis*, *B. elegans*, *B. erythropus*, *B. granulatus*, *B. luridus*, *B. luteus*, *B. queletu*, *B. scaber*, *B. subtomentosus*, and *B. viscidus* not to mention the humble field mushroom *Agaricus campestris bisporus*. All these await commercial development, except *A. campestris*. There is, of course, an interesting mycological conundrum posed in the

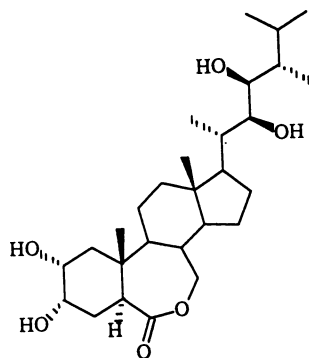


Figure 2. 24-Epibrassinolide

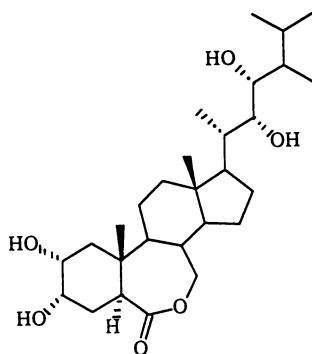


Figure 3. 22S,23S-Homobrassinolide

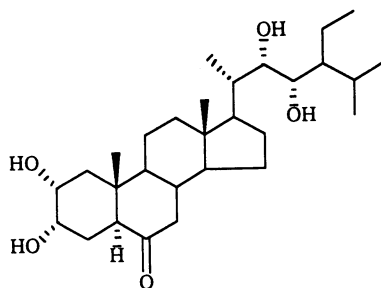


Figure 4. 22S,23S-Homocasterone

form of a question. What happens if those Fungi Imperfecti that reproduce by filamentous division are treated with brassinosteroids?

Sources of brassinosteroids for further testing.

Synthetic. The ultimate question concerning the use of brassinosteroids from a practical aspect hinges on the availability of sufficient quantities of these compounds for commercial use. In recent years the synthesis of brassinolide has been achieved using the natural sterols and their degraded products as starting materials and these have included stigmasterol, brassicasterol, pregnenolone, and dinorcholenic acid (2). The synthetic work of McMorris et al., (23) indicated that stigmasterol is the most convenient generator for brassinolide and those congeners that have a 22R, 23R, 24S stereochemistry. Furthermore, while the syntheses are complex the yields are high, for the most part. Thus it can be said that synthesis is a practical route to obtaining the brassinosteroids and the field trials conducted by Ikekawa show that sufficient material may be made available to treat significant acreage.

Biological sources. While the synthesis of brassinosteroids appears to be a logical route to obtaining sufficient quantities of these metabolites, there is one serious drawback and that is that the satellite natural products, which may also have practical application, are not produced. This is best illustrated by the development of abscisic acid over a thirty-five year period. Historically, the observations that a potent plant growth inhibitor was present in the dormant buds of *Acer pseudoplatanus* and cotton bolls, in extremely small quantities, were made in the early 1950's. The structure was eventually proved by Ohkuma, et al., in 1965 (24) and the same year Cornforth synthesized (\pm)-abscisic acid (25). The yields from plant sources were low and the synthetic was a mixture of isomers. It was in 1977, over twenty years after the initial observations, that (+)-cis abscisic acid was found, in pure optically active form, in the fungus *Cercospora rosicola* (26), and, in 1982, in *C. cruenta* in relatively large amounts (27). Then, in 1987 it was reported that the metabolite, again as the (+)-isomer, had been found in blue light (360 nm) irradiated *Botrytis cinerea* at a titre of 93 mg/L in liquid culture (28). More importantly, this discovery led to the isolation and identification of satellite metabolites, many of which exhibit biological activity. The serendipity lay in the accession of the right organism and, additionally, the use of blue light.

Of course, the presence of brassinosteroids has yet to be proved in microorganisms. But as we have briefly discussed, the fact that they have been found in algae and because of the association of the algae with fungi in the lichens there exists the possibility that the intergeneric transfer of the genetic encoding for brassinosteroid production may already have taken place. That the brassinosteroids are found among diverse members of the plant kingdom further suggests that during the course of evolution the genes governing their biosynthesis have survived intact in the passage from one genus, or species, to another.

It is highly probable that work is presently afoot to splice the brassinosteroid eliciting genetic information from higher plants into microorganisms, perhaps

Escherichia coli, and that we shall see the fermentation production of these metabolites within the decade. Cell culture production has been outlined (29) and awaits suitable induction to establish higher yields.

Conclusion.

There is little doubt that the brassinosteroids can have a major effect on world grain markets and on the increased production of horticultural crops utilizing the same land area. This means reduced energy costs and that, coupled to the apparently environmentally safe aspects of these compounds, makes them ideal candidates for industrial development. But a number of critical problems remain and it seems that there will be limited fundamental examination with the brassinosteroids unless they are used practically. Frankly stated, funds generated from the sales of marketable brassinosteroids will drive all the other studies. While this is a critical time in the development of the brassinosteroids, their future promises to be interesting politically, scientifically, and practically. To quote an old aphorism, to the victor will go the laurels.

REFERENCES

1. Grove, M. D.; Spencer, G. F.; Rohwedder, W. K.; Mandava, N.; Worley, J. F.; Warthen, J. D., Jr; Steffens, G. L.; Flippen-Anderson, J. L.; Cook, J. C., Jr. *Nature* 1979; **281**, 216.
2. Marumo, S. *Proc. 14th Ann. Meeting Plant Growth Reg. Soc. America* 1987, **14**, 174.
3. Kim, S-K. (This volume).
4. Steffens, G. L. (This volume).
5. Mitchell, J. W.; Mandava, N.; Worley, J. F.; Plimmer, J. R.; Smith, M. V. *Nature* 1970, **225**, 1965.
6. Sasse, J. M. (This volume).
7. Meudt, W. J. In "Ecology and Metabolism of Plant Lipids"; Fuller, G.; Nes, W. D.; eds; *ACS Symposium Series No. 325*, American Chemical Society; Washington, DC 1987; p. 53.
8. Krizek, D.; Mandava, N. B. *Physiol. Plant* 1983, **57**, 317.
9. Krizek, D.; Mandava, N. B. *Physiol. Plant* 1983, **57**, 324.
10. Kamuro, Y.; Inada, K. *Proc. 14th Ann. Meeting Plant Growth Reg. Soc. America* 1987, **14**, 221.
11. Clouse, S. D.; Zurek, D. (This volume).
12. Katsumi, M.; Tsuda, A.; Sakurai, H. *Proc. 14th Ann. Meeting Plant Growth Reg. Soc. America*, 1987, **14**, 215.
13. Sasse, J. M. (This volume).
14. Ohishi, K. Discussions and communications.
15. Adam, G.; Marquardt, V.; Vorbrodt, H. M.; Hörhold, C.; Andreas, W.; Gartz, J. (This volume).

16. Kimball, J. W. In *Biology*; Addison-Wesley Publishing Co., Chicago, IL 2978; p. 659.
17. Richter, K.; Koolman, J. (This volume).
18. Cole, R. J.; Dutky, S. R. *J. Nematology* 1969, 1, 72.
19. Nes, W. D. In "Ecology and Metabolism of Plant Lipids"; Fuller, G.; Nes, W. D.; eds; *ACS Symposium Series No. 325*, American Chemical Society; Washington, DC 1987; p. 304.
20. Svoboda, J. A.; Thompson, M. J. In "Ecology an Metabolism of Plant Lipids"; Fuller, G.; Nes, W. D.; eds; *ACS Symposium Series No. 325*, American Chemical Society; Washington, DC 1987; p. 176.
21. Ikekawa, N.; Zhao, Y-J. (This volume).
22. Hamada, K.; Nishi, S.; Uezono, T.; Fujita, S.; Nakazawa, Y. *Abstracts of the 12th IPGSA* (Heidelberg) 1985; p. 43.
23. McMorris, T. C.; Donaubauer, J. R.; Silveira, M. H.; Molinski, T. F. (This volume).
24. Ohkuma, K.; Addicott, F. T.; Smith, O. E.; Thiessen, W. E. *Tetrahedron Lett.* 1965, 29, 2529.
25. Cornforth, J. W.; Milborrow, B. V.; Ryback, G. *Nature* 1965, 206, 715.
26. Assante, G.; Merlini, L.; Nasini, G. *Experientia* 1977, 33, 1556.
27. Oritani, T.; Ichimura, M.; Yamashita, K. *Agric. Biol. Chem.* 1982, 46, 1959.
28. Marumo, S.; Kohno, E.; Natsume, M.; Kanoh, K. *Proc. 14th Ann. Meeting Plant Growth Reg. Soc. America* 1987, 14, 146.
29. Sakurai, A.; Fujioka, S.; Saimoto, H. (This volume).

RECEIVED July 8, 1991

Author Index

- Abe, Hiroshi, 18,200
Adam, Günter, 74,141
Andreas, W., 74
Awad, A. E., 298
Bach, Thomas J., 176
Bellincampi, Daniela, 189
Bokebayeva, G. A., 141
Burkhanova, E. A., 141
Clouse, S. D., 122
Cutler, Horace G., 334
Dahse, I., 167
Donaubauer, J. R., 36
Eun, J.-S., 312
Fedina, A. B., 141
Fujii, S., 306
Fujioka, Shozo, 86,97
Gartz, J., 74
Grimm, E., 167
Guan, M., 231
He, Ruo-yun, 220
Hirai, K., 306
Hörhold, C., 74
Ikekawa, Nobuo, 280
Kaminek, M., 56
Kamuro, Yasuo, 292
Katsumi, Masayuki, 246
Khokhlova, V. A., 141
Khripach, V. A., 43
Kim, Seong-Ki, 26
Kohout, L., 56
Koolman, Jan, 265
Kulaeva, O. N., 141
Kuraishi, S., 312
Litvinovskaya, R. P., 43
Mandava, N. Bhushan, 320
Marquardt, V., 74
Marumo, Shingo, 18
McMorris, T. C., 36
Molinski, T. F., 36
Morpurgo, Giorgio, 189
Ogino, Y., 86
Otto, Steffan, 208
Petzold, U., 167
Richter, Klaus, 265
Roddick, J. G., 231
Roth, Petra S., 176
Saimoto, Hiroshi, 86,97
Saka, H., 306
Sakurai, Akira, 86,97
Sakurai, N., 312
Sasse, Jenneth M., 158,255
Schiller, Christiane, 208
Schilling, Günther, 208
Silveira, M. H., 36
Steffens, G. L., 2
Strnad, M., 56
Sugiyama, K., 312
Suzuki, H., 86
Takahashi, N., 86
Takatsuto, Suguru, 107,292
Takeuchi, Y., 298
Thompson, Malcolm J., 176
Vorbrott, H. M., 74,141
Wang, Guan-jie, 220
Wang, Xue-shu, 220
Willmer, C. M., 167
Worsham, A. D., 298
Yokota, Takao, 86
Zhabinskii, V. N., 43
Zhao, Yu-Ju, 280
Zurek, D., 122

Affiliation Index

- Academia Sinica, 280
BAL Planning Company, Ltd., 292
Byelorussian SSR Academy of Sciences, 43
Czechoslovak Academy of Sciences, 56
Friedrich Schiller Universität Jena, 167
Hiroshima University, 312
Hokkaido National Agricultural Experimental Station, 306
Institute of Biotechnology, 74

- Institute of Microbiology and Experimental Therapy, 74
 Institute of Physical and Chemical Research, 86,97
 Institute of Plant Biochemistry, 74,141
 International Christian University, 246
 International Institute of Tropical Agriculture, 298
 Iwaki Meisei University, 280
 Jeonbug National University, 312
 Korea University of Education, 107,292
 Korea Research Institute of Chemical Technology, 26
 Martin Luther Universität Halle-Wittenberg, 167,208
 Nagoya University, 18
 Nissan Chemical Industries, Ltd., 306
 North Carolina State University, 298
 Philipps Universität Marburg, 265
 Sächsische Akademie der Wissenschaften zu Leipzig, 265
 San Diego State University, 122
 Shenyang Agricultural University, 220
 Shizuoka Prefectural Experiment Station, 312
 Somar Corporation, 86,97
 SRS International, 320
 Stickstoffwerke AG Piesteritz, 74
 Teikyo University, 86
 Tokyo University of Agriculture and Technology, 18,200
 U.S. Department of Agriculture, 2,176,334
 USSR Academy of Sciences, 141
 Universität Karlsruhe, 176
 Università degli Studi "La Sapienza", 189
 Università di Perugia, 189
 University of California—San Diego, 36
 University of Exeter, 231
 University of Melbourne, 158,255
 University of Stirling, 167
 University of Tokyo, 86
 Utsunomiya University, 298

Subject Index

A

- Abscisic acid (ABA)
 levels in rice plants, 308,310r
 levels in squash hypocotyls, 315r,316
 Active component of brassins
 identification, 9,10f
 pilot plant extraction, 6,7f,8
 solvent partition and column chromatography, 8
 Adventitious root(s)
 development, 233,234r,235
 formation, 247
 Agriculture, application of
 24-epibrassinolide, 280–290
 22-Aldehydes, synthesis of
 brassinosteroids, 47–50f
 α hormone function, description for
 brassins, 4
 Androstane type of brassinosteroids,
 synthesis, 59–60
 Antheridiol, synthesis, 37
 Arthropods, effect of brassinosteroids,
 268–275

- Assimilated uptake by plant tissues,
 brassinosteroid-induced changes,
 168–174
 Auxins, regulation of gene expression,
 123–124,125r

B

- Barley crop yields, effect of
 brassinosteroids, 52,53f
 Barley field experiments, description for
 brassin evaluation, 12
 Barley leaf cell ultrastructure,
 protective effect of brassinosteroids
 under saline stress, 150,152f,153,154f
 Bean first-internode bioassay, 321
 Bean second-internode bioassay
 activities of compounds, 65,68,69–70f
 application, 321
 brassin purification, 3–4
 brassinosteroids, description, 109–110
 development, 321
 scheme, 63,65,66f

- Institute of Microbiology and Experimental Therapy, 74
 Institute of Physical and Chemical Research, 86,97
 Institute of Plant Biochemistry, 74,141
 International Christian University, 246
 International Institute of Tropical Agriculture, 298
 Iwaki Meisei University, 280
 Jeonbug National University, 312
 Korea University of Education, 107,292
 Korea Research Institute of Chemical Technology, 26
 Martin Luther Universität Halle-Wittenberg, 167,208
 Nagoya University, 18
 Nissan Chemical Industries, Ltd., 306
 North Carolina State University, 298
 Philipps Universität Marburg, 265
 Sächsische Akademie der Wissenschaften zu Leipzig, 265
 San Diego State University, 122
 Shenyang Agricultural University, 220
 Shizuoka Prefectural Experiment Station, 312
 Somar Corporation, 86,97
 SRS International, 320
 Stickstoffwerke AG Piesteritz, 74
 Teikyo University, 86
 Tokyo University of Agriculture and Technology, 18,200
 U.S. Department of Agriculture, 2,176,334
 USSR Academy of Sciences, 141
 Universität Karlsruhe, 176
 Università degli Studi "La Sapienza", 189
 Università di Perugia, 189
 University of California—San Diego, 36
 University of Exeter, 231
 University of Melbourne, 158,255
 University of Stirling, 167
 University of Tokyo, 86
 Utsunomiya University, 298

Subject Index

A

- Abscisic acid (ABA)
 levels in rice plants, 308,310r
 levels in squash hypocotyls, 315r,316
 Active component of brassins
 identification, 9,10f
 pilot plant extraction, 6,7f,8
 solvent partition and column chromatography, 8
 Adventitious root(s)
 development, 233,234r,235
 formation, 247
 Agriculture, application of
 24-epibrassinolide, 280–290
 22-Aldehydes, synthesis of
 brassinosteroids, 47–50f
 α hormone function, description for
 brassins, 4
 Androstane type of brassinosteroids,
 synthesis, 59–60
 Antheridiol, synthesis, 37
 Arthropods, effect of brassinosteroids,
 268–275

- Assimilated uptake by plant tissues,
 brassinosteroid-induced changes,
 168–174
 Auxins, regulation of gene expression,
 123–124,125r

B

- Barley crop yields, effect of
 brassinosteroids, 52,53f
 Barley field experiments, description for
 brassin evaluation, 12
 Barley leaf cell ultrastructure,
 protective effect of brassinosteroids
 under saline stress, 150,152f,153,154f
 Bean first-internode bioassay, 321
 Bean second-internode bioassay
 activities of compounds, 65,68,69–70f
 application, 321
 brassin purification, 3–4
 brassinosteroids, description, 109–110
 development, 321
 scheme, 63,65,66f

- Bean second-internode bioassay—*Continued*
 sensitivity, 65,67f
 specificity, 65,66f
- Bioassays for brassinosteroids
 bean first-internode bioassay, 63,64f
 bean internode tests, activities of
 compounds, 65,68,69–70f
 bean second-internode bioassay,
 63,65–70,109–110
 pea inhibition test, 68,71–72f
 rice lamina inclination test, 110
 sensitivity, 62t,63
 structures of compounds used in bean
 internode tests, 65,67f
 wheat leaf unrolling test, 110–111
- Bioassay studies of brassins, 13
- Biochemical effects, brassinosteroids, 326
- Biochemical pesticides
 characteristics, 328,330
 EPA definition, 328,329f,330
 EPA registration requirements as
 brassinosteroids, 330–331
- Biological sources, examples, 343–344
- Biorational pesticides, natural products,
 327–328
- Brassicasterol
 synthesis, 50f
 synthesis of 24-epibrassinolide, 38
- Brassin(s)
 1974–1975 soybean field experiment in
 Brazil, 11
 1974 and 1975 field evaluations, 11–12
 1975 barley and soybean field experiments
 in U.S. locations, 11–12
 α hormone function, 4
 bioassay studies, 13–14
 brassinolide analogs, synthesis, 9,11
 chemical and biological studies, 4–5
 definition, 3
 discovery, 26
 future interest and research, 14–15
 intact plant studies, 4,12–13
 physiological property studies, 4
 production method from dried rape pollen, 5
 purification, 3–4
 purification, isolation, and identi-
 fication of active component, 6–10
- Brassinolide
 activity, 26
 biological activities, 122,293t,294
- Brassinolide—*Continued*
 chemical synthesis, 280
 citrus fruit set, 316,317t
 cloning of induced genes in soybean by
 subtractive hybridization, 131,134,135f
 corn, 306
 discovery, 107,209,321,322f,323,324f
 decomposition and movement into
 active site, 318
 elongating soybean stem sections,
 125–126,127f,128
 expression of xylem-specific promoter/
 β -glucuronidase fusions in transgenic
 tobacco, 136
 field trial results, 339
 formulation, effect on activity, 297
 fruit setting, 294t
 gene expression in elongating stem sections,
 128,129–130f,131t
 growth-regulating action in rice plants,
 306–310
 growth retardation of *Cucumis*
melo leaves, 316
Helianthus tuberosus xylem differen-
 tiation, 128,131,132–133f,135
 hormone levels in squash hypocotyls
 experimental procedure, 313
 growth stimulation of segments after
 treatment, 313f,314t,315
 levels of IAA and ABA during treatment,
 315t,316
 osmoticum changes during brassinolide-
 induced elongation, 314f,315
 hypothetical biosynthetic pathway, 86,91f
 identification, 9,10f,22,23f,36
 induced elongation
 ABA, 256
 colchicine, 258,259f
 IAA, 256–257,259f
 inhibitors of protein and nucleic acid
 synthesis, 258,260,261–262f
 plant hormones, 256–258,259f
 responsive cells, 258,259f,260,261–262f
 specific gene expression, 260t,262f
 synergism with auxin, 257–258,259f
 zeatin, 356
 instability, 292,293–294t
 interaction with environmental signals, 162
 isolation, 177
 key intermediates, synthesis, 51,52f

Brassinolide—Continued

- light conditions, effect on activity, 295,296*t*,297
- maize seedling(s), growth and chilling resistance
 - experimental materials, 220–221
 - greening of etiolated maize seedlings under light, 224,225–226*t*
 - growth of organs in maize seedlings, 221,222*f*
 - growth recovery after chilling stress, 226–227,228*f*,229
- maize seedling organs, characteristic responses, 221,222*f*,223*f*,224
- superoxide dismutase activity, 226*t*
- temperature, 225*t*,226
- metabolism
 - crown gall cells of *Catharanthus roseus*, 87,92*f*,93
 - mung bean cuttings, 93
 - rice seedlings and etiolated leaf explants, 93,94*f*,95
- modulation of and by hormones, 160–161
- molecular analysis, 122–123
- movement in plants, 161–162
- navel orange fruit, 316,317–318*t*
- NMR spectrum, 221,222*f*
- physiological action, 246–253
- pleiotropic effects, 161
- problems with use in agriculture, 318
- promotion of elongation, 256
- protein synthesis, 162
- publication of structure, 2–3
- receptor studies, 136
- second conditioning factor
 - cell growth, 193–194,195*t*
 - plating efficiency, 193–194,195*t*
 - plating efficiency vs. cell densities, 194,196*t*
- specificity, 159
- structure, 9,10*f*,26,28*f*,36,75,178
- synthesis, 37–41,323,324*f*
- temperature, effect on activity, 294,295*t*
- translocation through plant tissue, 318
- wheat, 306
- witchweed seeds, conditioning and germination
 - chemical preparation, 299
 - combination with other stimulants, 300–301*t*

Brassinolide—Continued

- witchweed seeds, conditioning and germination—*Continued*
 - conditioning in water and brassinolide and treatment with stimulants, 301,302*f*,303–304*t*
 - seed conditioning and germination procedure, 299
 - seeds conditioned in water, 300–301*t*
 - dl*-strigol-induced germination of witchweed seeds inhibited by light, 301*t*
 - xylem differentiation in Jerusalem artichoke, 134
- Brassinolide analogs
 - characteristics, 107
 - synthesis, 9,11
- Brassinone
 - identification, 22,23*f*,27
 - structure, 28*f*
- Brassinosteroid(s)
 - accumulation during development and growth of plant male gametophyte, 202,204,205*f*,206*t*
 - acid invertase activity in tap root, 213,215*t*,216
 - adventitious root development, 233,234*t*,235
 - antistress activity, 141
 - arthropods, 268–275
 - barley crop yield, 52,53*f*
 - bioassays, 62–72,109–111
 - biochemical effects, 326
 - biological effects, 232
 - biological sources, 343–344
 - chemical differences from ecdysteroids, 266
 - classifications, 31
 - commercialization, 327
 - cultured excised root development, 239–240,241*f*
 - derivation from mevalonic acid, 177
 - description, 26,265
 - distribution in plant kingdom, 26–30,32,108–109
 - ecdysteroid receptors, 273,274*f*,275
 - economic potential, 339–341
 - EPA registration requirements, 326–331
 - evolution, 276
 - field trial results, 339–340
 - fruiting body hormones in fungi, 341,343
 - growth and differentiation of imaginal disks of flies, 269,271*f*,272,273*t*

Brassinosteroid(s)—Continued

- historical perspective, 334–335
- hormonal properties in plants, 335–337
- identification
 - crown gall cells of *Catharanthus roseus*, 98*t*,99*f*
 - Distylium* factors A₁ and B, 22,23*f*
- identification and structure, 27–30
- increasing crop yields, 280
- insect control, 340
- lamina inclination, 204
- leaf acid invertase activity in sugar beets, 212*t*,213,214*t*
- lucerne crop yield, 52,53*f*
- microanalytical methods, 111–115
- molecular modeling, 266
- molting of cockroach, 268–269,270*f*
- morphological effects, 209,210–211*t*,212
- occurrence, 265
 - higher plant tissues and organs, 231,232*t*
 - nature, 86,108
 - plant kingdom, 231,246
 - pollen, 337–338
- physiological action, 246–253
- physiological functions, 325,326
- physiology in plants and fungi, 335–339
- plant cell cultures, 97
- plant growth activity, laboratory vs. field results, 217*t*,218
- plant growth and composition, effect of environmental conditions, 216,218
- plant growth and development, 177,246
- plant protectants, 340
- production in cell cultures, effect of plant hormones, 102,103–104*f*,105
- protein synthesis and plant cell ultrastructure under stress conditions, 142–154*f*
- registration as biochemical pesticide, data requirements, 331
- reproductive inhibitors in plants, 341
- research efforts, 74–75
- research topics, 338–339
- rice lamina inclination response, 204,206–207
- specific bioassay, 21*t*,22
- test, use for screening, 200
- root development, 232
- root development activity, comparison with that of auxin, 240–241

Brassinosteroid(s)—Continued

- root development activity, effect of concentration, 242
 - root development activity, effect of treatment time, 242
 - root segment development, 237–238*t*,239
 - seedling root development, 235,236*t*
 - spike activity of insect neurons, 272–273,274*f*
 - structural requirements for activity, 57,58*t*,280–281,323,325
 - structure, 57,335*f*
 - sucrose synthase activity in tap roots, 216
 - sugar beet plants, yield under drought stress, 210,211*t*,212
 - sugar beet seedlings in short-time experiments, 209,210*t*
 - synthesis, 56,323,325
 - 22-aldehydes, 47–48*f*
 - androstane type, 59–60
 - diol compounds, 61
 - ergosterol, 49–50*f*
 - esters, 61–62
 - heterocyclic intermediates, 50,51–52*f*
 - lactones, 61
 - pregnane type, 60
 - stigmaterol, 44–46*f*,47
 - synthetic scheme, 323,324*f*
 - synthetic sources, 343
 - tobacco tumor cell growth, inhibition, 177–187
 - USDA contributions, 320–326
 - uses, speculative, 341,343
 - See also* Naturally occurring brassinosteroids
- Brassinosteroid analogs**
- growth stimulation
 - higher fungi, 83,84*t*
 - radish cotyledons, 81,82–83*f*
 - structure–activity relationships, 80*t*,81
 - synthesis of new compounds, 75–80
- Brassinosteroid(s) as endogenous plant hormones**
- action as hormone, 162–163
 - future research, 163
 - interaction with environmental signals, 162
 - modulation of and by hormones, 160–161
 - movement in plants, 161–162
 - pleiotropic effects, 161
 - protein synthesis, role in activity, 162

Brassinosteroid(s) as endogenous plant hormones—*Continued*
 specificity, 159–160
 ubiquity, 159

Brassinosteroid-induced changes
 electrical cell potential of *Egeria* leaves, 169–170
 electrophysiological measurement procedure, 168
 growth enhancement, 172–174
 plant material preparation, 168
 proton extrusion, 170*f*
 medium pH measurements, 169
 solute uptake, 171,172*f,t*,173*t*
 stomatal opening, 170,171*f*
 sucrose absorption by vascular bundles, 169
 uptake of α -aminoisobutyric acid and sucrose, 169

Brassinosteroidlike active substance
 production by plant cells
 cell lines and culture conditions, 100*t*
 growth profile and content
 normal cells, 100–101,103*f*
 tobacco crown gall cells, 100–101,103*f*

Brassins project, *See* U.S. Department of Agriculture brassins project

C

Carbon-14 incorporation in rice plants, translocation, 307–308

Castasterone
 discovery, 107
 identification in *Distylium racemosum* leaves, 22,23*f*
 metabolism
 crown gall cells of *Catharanthus roseus*, 87,92*f*,93
 mung bean cuttings, 93
 rice seedlings and etiolated leaf explants, 93,94*f*,95
 structure, 26–27,28*f*

Catharanthus roseus crown gall cells, *See* Crown gall cells of *Catharanthus roseus*

Chilling resistance of maize seedlings, brassinolide, 226–227,228*f*,229

Citrus fruits
 effect of brassinolide on fruit quality, 317,318*t*
 effect of brassinolide on fruit set, 316,317*t*

Cockroach, effect of brassinosteroids on molting, 268–269,270*f*

Coleoptile, effect of brassinolide on growth rate, 227,228*f*

Conditioning factors in plant cell cultures
 biological effect on growth stimulation, 191,192*t*

brassinolide as second conditioning factor, 193–198

interspecific cross conditioning, 197–198

need for more than one factor, 193,195*t*

solubility in water, 191,192*t*

stability in boiling water and during pH treatment, 191,192*t*

studies, 190–191

water solubility and dialyzability of second factor, 194,196*f,t*,197

Conditioning of witchweed seeds, effect of brassinolide, 299–304

Corn

brassinolide, 306

24-epibrassinolide, effect on yield, 287,289*t,f*

Corn factor, purification and isolation from corn germ oil, 20–21

Cotyledon expansion, effect of brassinolide, 247

Crop yields, effect of brassinosteroids, 52,53*f*

Crown gall cells of *Catharanthus roseus*
 active principles, 98,99*f*

biological activity distribution in HPLC, 98,99*f*

brassinosteroid structures, 98,99*f*

cell types, 98*t*

detection of brassinosteroidlike active substances, 98

Cucumber hypocotyl growth, physiological modes of brassinolide action, 246–253

Cucumis melo, effect of brassinolide on growth retardation of leaves, 316

Cultured excise roots, effect of brassinosteroids on development, 239–240,241*f*

D

- 2,4-D, effect on brassinosteroid production, 104f,105
- Day length, effect on brassinolide activity, 295,296t,297
- 22-Dehydrocampesterol, synthesis of brassinolide, 38
- 2 α ,3 α -Dihydroxy-5 α -cholestan-6-one, biotransformation, 79–80
- 2 α ,3 α -Dihydroxy-7-oxa-*B*-homo-5 α -cholestan-6-one, 56–57,58t
- Discovery of brassinolide, 321–324f
- Distylium* factors
biological activities, 19–20
isolation and identification, 19,22,23f
 R_f values, 19
universality of distribution, 20
- Distylium racemosum* Sieb. at Zucc.
aphid attack on leaves, 18–19
description, 18
Distylium factors, 19–20
identification of brassinosteroids, 22
- Dolicholide
identification, 27
reversed-phase HPLC profile of tritiated products, 87,91f
structure, 28f
- E
- Ecdysteroid(s)
biological significance in plants, 276
chemical structures, 266,267f
definition, 266
description, 265
evolution, 276
molecular modeling, 266
occurrence, 265–266
preparation, 179
specific inhibition of tobacco tumor cell growth, 184,185f,t,186
- Ecdysteroid receptors, effect of brassinosteroids, 273,274f,275
- Electrical cell potential of *Egeria* leaves, effect of steroids, 169
- Elongation, brassinolide-induced. *See* Brassinosteroid-induced changes
- Endogenous cytokinins, analysis, 102,103f

- Endogenous indoleacetic acid, analysis, 102,104f
- Endogenous plant hormones, 159–163
- Environmental Protection Agency (EPA)
brassinosteroid registration requirements
biochemical pesticides, 328–331
commercialization, 327
hormones, 326–327
natural products as biorational pesticides, 327–328
(22*R*,23*R*,24*S*)-Epibrassinolide, structure, 177
- 24-Epibrassinolide
activity, 56–57,58t,281
agricultural application
China, 284,286–290
Japan, 281,282t,284,285f
application at flowering, effect on wheat yield, 281,282t
barley leaf cell ultrastructure under saline stress, 150,152f,153,154f
corn, 287,289t,f
field trial results, 284,286t,287,339–340
free ammonia and putrescine content and leaf wilt incidence, 287,288t
identification, 27
proteins synthesized under normal and heat shock temperatures, 142,144f
root development, 233–243
soluble carbohydrate content in flag leaf, 284,286t
[¹⁴C]sucrose incorporation
into grain, 284,285f
tobacco, 287,289t,290
wheat, 281,282t,284,286t,287,288f,t
structure, 29f,57
synthesis, 38,49f,50,281,283
toxicity, 290
- Ergosterol
brassinosteroids, 49–52f
24-epibrassinolide, 38
- Ethylene, effect of brassinolide on evolution from rice plant panicles, 310t
- Etiolated bean and cucumber, effect of brassinosteroids on adventitious root development, 233
- Etiolated leaf extracts, metabolism of castasterone and brassinolide, 93,94f,95
- Exogenous auxins, effect on brassinosteroid production, 102,104f,105

F

- First-internode bioassay
 - description, 63
 - sensitivity improvements, 63,64f
- Flies, effect of brassinosteroids on
 - growth and differentiation of imaginal disks, 269,271f,272,273t
- Formulation, effect on brassinolide activity, 297
- Fruiting body hormones in fungi, brassinosteroids, 341,343
- Fungi, brassinosteroid-induced stimulation of growth, 83,84t

G

- GC/MS microanalysis, description for brassinosteroids, 111–112,113f
- Gene expression
 - effect of brassinolide in elongating stem sections, 128,129–130f,131t
 - regulation by auxins, 123–124,125t
- Germination of witchweed seeds, effect of brassinolide, 299–304
- Gibberellin A₃, effect on witchweed seed germination, 298–304
- 23-O-β-D-Glucopyranosylbrassinolide, structure, 93–94
- Green alga, occurrence of brassinosteroids, 30
- Greening of etiolated maize seedlings under light, effect of brassinolide, 224,225–226t
- Growth of maize seedlings, effect of brassinolide, 220–226
- Growth-regulating action of brassinolide in rice plants
 - experimental procedure, 306–307
 - grain weight under greenhouse and phytotron conditions, 307t
 - percentage of ripened grains, 307t
 - plant hormone levels in panicles, 308,310t
 - starch and sucrose distribution, 308,309f
 - translocation of ¹⁴C incorporation, 307–308
- Growth under chilling stress, effect of brassinolide, 247,248f
- Gymnosperms, occurrence of brassinosteroids, 30

H

- Heat shock granule formation in wheat leaf cells, effect of brassinosteroids, 150,151f
- Heat shock temperatures, effect of brassinosteroids on protein synthesis, 142,144–150
- Helianthus tuberosus* xylem differentiation, effect of brassinolide, 128,131,132–133f,135f
- Heterocyclic intermediates, synthesis of brassinosteroids, 50,51–52f
- High-performance liquid chromatographic (HPLC) analysis, description for brassinosteroids, 114,115f
- History of brassinosteroids, 320–321
- (22S,23S)-Homobrassinolide
 - acid invertase activity in tap root, 213,215t,216
 - activity, 26
 - alternative synthesis, 44,45f
 - biotransformation, 79
 - chemical structure, 209f
 - environmental conditions, effect on plant growth and composition activity, 216,218
 - heat shock granule formation in wheat leaf cells, 150,151f
 - laboratory vs. field results, comparison for plant growth activity, 217t,218
 - leaf acid invertase activity in sugar beets, 212t,213,214t
 - proteins synthesized under normal and heat shock temperatures, 142,144f
 - root development, 234–243
 - sucrose synthase activity in tap roots, 216
 - sugar beet plants, yield under drought stress, 210,211t,212
 - sugar beet seedlings in short-time experiments, 209,210t
 - synthesis, 44,45,46f
 - total protein synthesis in wheat leaves, 142,143f
- 28-Homobrassinolide, synthesis, 37
- (22S,23S)-Homobrassinolide Δ⁴ analog, synthesis, 46f,47
- (22S,23S)-Homocastasterone, 79
- Hormonal properties, brassinosteroids, 335–337
- 1β-Hydroxycastasterone, structure, 29f
- 20-Hydroxyecdysone, structure, 178

Hypocotyl elongation, effect of
brassinolide, 246–247

Hypocotyl sections of cucumber
brassinolide activity, 247,249,250f
inhibitors, effect on brassinolide
activity, 249
time–course response, 249,250f

I

Imaginal disks of flies
brassinosteroids, effect on growth and
differentiation, 269,271f,272,273t
definition, 269

Indoleacetic acid (IAA)
brassinolide, effect on levels in rice
plants, 308,310t
brassinolide, effect on levels in
squash hypocotyls, 312–316
rice lamina inclination test,
204,206–207
witchweed seed germination,
300,301t

Insect control, use of brassinosteroids,
340–341,342f

Insect neurons, effect of brassinosteroids
on spike activity, 272–273,274f

Instability of brassinolide, 292–294t

Intact plant studies with brassins,
description, 12–13

Isoxazoles, synthesis of 22-en-24-ones,
50,51f

Isoxazolines, synthesis of 22-en-24-ones,
50,51f

J

Japan, application of epibrassinolide,
281,282t,284,285f

Jerusalem artichoke, effect of
brassinolide on xylem
differentiation, 134

K

6-Ketonic brassinosteroids, structural
characteristics, 31

L

Leaf abortion, brassinolide-induced
inhibition, 316

Light conditions, effect on brassinolide
activity, 295,296t,297

Lucerne crop yields, effect of
brassinosteroids, 52,53f

M

Maize
root segment development, effect of
brassinosteroids, 237–238t,239
seedling root development, effect of
brassinosteroids, 235,236t

Makisterone, structure, 178

Matricaria chamomilla, effect on
brassinosteroids on adventitious root
development, 234–235

Mesocotyl, effect of brassinolide on
growth rate, 227,228f

Microanalytical methods, brassinosteroids
GC/MS microanalysis, 111–112,113f,114
HPLC analysis, 114,115f
radioimmunoassay, 114,116,117f

Microbial transformation of
brassinosteroids, 79

Microtubular polymerization, effect of
brassinolide, 258

Molecular analysis of brassinolide action
in plant growth and development
brassinolide receptor studies, 136
cloning of brassinolide-induced genes in
soybean by subtractive
hybridization, 131,134,135f
elongating soybean stem sections,
125–126,127f,128
expression of xylem-specific promoter/
 β -glucuronidase fusions in
transgenic tobacco, 136
gene expression in elongating stem
sections, 128,129–130f,131t
Helianthus tuberosus xylem differentiation,
128,131,132–133f,135f
regulation of gene expression by auxins,
123–124,125t
xylem differentiation in Jerusalem
artichoke, 134

Molting of cockroach, effect of
brassinosteroids, 268–269,270f

Monocots, occurrence of brassinosteroids, 30

Mung bean
adventitious root development, 233,234t
root segment development, 238t,239
seedling root development, 235,236f

Mung bean cuttings, metabolism of
castasterone and brassinolide, 93

Muristerone, structure, 178

N

Naturally occurring brassinosteroids
bioassays, 109–111
distribution, 109
endogenous levels in plants, 202,203t
HPLC profiles, 86,90f
identification and structures, 26–30,32
microanalytical methods, 111–115
search using rice lamina inclination
test, 201–202
structural characteristics, 30–32,33f,109
structures, 86,88–89f

Navel oranges
fruit quality, 317,318t
fruit set, 316,317t

Neurons, insect, effect of brassinosteroids
on spike activity, 272–273,274f

Nonoxidized brassinosteroids, structural
characteristics, 31

Norbrassinolide, identification in
Distylium racemosum leaves, 22,23f

P

Pea inhibition test
ethylene, effect on growth, 68,71f
light, effect on sensitivity, 72
pea plant cultivar, effect on
sensitivity, 72f
stem length, effect on sensitivity, 68,72

Phaseolus vulgaris seed, possible structures
of unknown brassinosteroids, 32,33f

Physiological activity of brassinosteroids
modulation of and by hormones, 160–161
pleiotropic effects, 161
specificity, 159–160

Physiological modes of brassinolide action
in cucumber hypocotyl growth
adventitious root formation in intact
seedlings, 247
auxin and gibberellin, simultaneous
interaction, 249
cotyledon expansion in intact seedlings, 247
gibberellin and IAA, sequential
interaction, 251
growth under chilling stress in intact
seedlings, 247,248f
hypocotyl elongation in intact seedlings,
246–247
hypocotyl sections, brassinolide activity,
247,249,250f
inhibitors, effect on activity in
hypocotyl sections, 249
interaction with IAA in curvature test,
249,251,252f
modes of action, comparison with those of
IAA and gibberellin, 251,253t
protein secretion, 251,252f
time–course response in hypocotyl
sections, 249,250f

Physiological responses, brassino-
steroids, 326

Phytohormones, study approach for plant
differentiation and development, 122

Plant(s), effect of ecdysteroids, 275–276

Plant cell(s)
prerequisites for growth, 167
production of brassinosteroidlike
active substances, 100–101t,103f

Plant cell cultures
conditioning factors for multiplication,
189–190
differentiation mechanism, 189
plating efficiency, 189
stimulation of growth induced by
brassinosteroids and conditioning
factors, 189–198

Plant cell ultrastructure, effect of
brassinosteroids under stress
conditions, 150–154

Plant differentiation and development by
phytohormones, study approach, 122

Plant growth and development, molecular
analysis of brassinolide action, 122–136

Plant growth promoting substances,
research interest prior to 1970, 3

- Plant hormone(s)
 definition, 158
 effect of brassinolide on levels in panicles, 308,310*t*
- Plant hormone effect on brassinosteroid production in cell cultures
 effect of exogenous auxins, 102,104*f*,105
 endogenous cytokinin analysis, 102,103*f*
 endogenous IAA analysis, 102,104*f*
- Plant kingdom, distribution of brassinosteroids, 26–30,32
- Plant male gametophyte, brassinosteroid accumulation during development and growth, 202,204,205*f*,206*t*
- Plant protectants, use of brassinosteroids, 340
- Plasmalemma, definition, 167
- Plasmalemma energization and transport, brassinosteroid-induced changes, 168–174
- Plasmalemma proton pump, interference by phytoeffectors, 168
- Pleiotropic effects
 brassinolide, 161
 brassinosteroids, 161
- Pollen, occurrence in brassinosteroids, 337–338
- Ponasterone A, structure, 178
- Pregnane type of brassinosteroids, 60
- Pregnenolone, synthesis of brassinolide, 38
- Protein synthesis, effect of brassinosteroids under stress conditions, 142–150
- Proton extrusion, effect of steroids, 170*f*
- Proton secretion in cucumber, effect of brassinolide, 251,252*f*
- R**
- Radicle, effect of brassinolide on growth rate, 227,228*f*,229
- Radioimmunoassay, description for brassinosteroids, 114,116,117*f*
- Radish, effect of brassinosteroids on seedling root development, 235
- Radish cotyledons, brassinosteroid-induced stimulation of growth and invertase activity, 81,82–83*f*
- Rape pollen, use for studies of brassin activity, 4
- Rapeseed oil sterols, GC analysis, 281,282*f*
- Recombinant DNA techniques, studies of auxin-regulated gene expression, 123
- Reproductive inhibitors in plants, brassinosteroids, 341
- Rice, effect of brassinosteroids on seedling root development, 236
- Rice lamina inclination test
 brassinosteroid accumulation during development and growth of plant male gametophyte, 202,204,205*f*,206*t*
 brassinosteroid response, 204,206–207
 description for brassinosteroids, 110
 rate of inclination determination, 207
 search for naturally occurring brassinolide and endogenous levels in plants, 201
 specific bioassay for brassinosteroids, 21*t*,22
See also Rice lamina joint test
- Rice lamina joint test
 modified method for brassinosteroid detection, 22–23
See also Rice lamina inclination test
- Rice plants, growth-regulating action of brassinolide, 306–310
- Rice seedlings, metabolism of castasterone and brassinolide, 93,94*f*,95
- Root development, effect of brassinosteroids, 231–243
- Root segments, effect of brassinosteroids on development, 237–238*t*,239
- S**
- Secobrassinosteroid(s), synthesis, 76–77
- Seedling roots, effect of brassinosteroids on development, 235
- Solute uptake, effect of brassinosteroid, 171,172*f*,*t*,173*t*
- Sorgoleone, effect on witchweed seed germination, 298–304
- Soybean auxin-regulated genes, list, 124,125*t*
- Soybean field experiments, description for brassin evaluation, 11–12
- Soybean stem sections
 elongation, 125–126,127*f*,128
 gene expression in elongation, 128,129–130*f*,131*t*
- Specific gene expression, effect of brassinolide, 260*t*,262*f*

Specific inhibition of tobacco tumor cell growth by brassinosteroids
 binding assay procedure, 179–180
 cytokinin and auxin content, endogenous, 183,184*f*
 ecdysteroid(s)
 effect, 184,185*f*,186
 preparation, 179
 ethylene evolution procedure, 177
 ethylene production, 180,182*t*
 experimental materials, 177
 growth inhibition, 180,181*f*,182*t*
 growth regulators, 180–182,183*f*
 hypothesis of mechanism, 186–187
 IAA level determination, 179
 isopentenyladenosine level determination, 179
 total binding assay procedure, 180
 unspecific binding determination procedure, 180

Specificity
 brassinolide, 159
 brassinosteroids, 159–160

Spike activity of insect neurons, 272–273,274*f*

Spirostan analogs of brassinosteroids, synthesis, 77–79

Squash hypocotyls, effect of brassinolide on hormone levels, 312–317

Starch, effect of brassinolide on distribution in rice plants, 308,309*f*

Stigmasterol
 synthesis of brassinosteroids, 44–46*f*,47
 synthesis of 28-homobrassinolide, 37

Stomatal opening, effect of steroids, 170,171*f*
dl-Strigol, effect on witchweed seed germination, 298–304

Structural characteristics, naturally occurring brassinosteroids, 30–32,33*f*

Subtractive hybridization, cloning of brassinolide-induced genes in soybean, 131,134,135*f*

Sucrose, effect of brassinolide on distribution in rice plants, 308

Sugar beet plants, effect of brassinosteroids on organ relations and enzyme activities, 209–218

Sulfones, synthesis of brassinosteroids, 47*f*,48

Synthesis of brassinolide, 37–41

Synthetic sources, examples, 343

T

Temperature, effect on brassinolide activity, 294,295*t*

Third-generation pesticides, advantages, 275

Tobacco, effect of 24-epibrassinolide on yield, 287,289*t*,290

Tobacco tumor cells, specific inhibition of growth by brassinosteroids, 177–187

Tomato
 adventitious root development, 234*t*
 cultured excise root development, 239–240,241*f*
 seedling root development, 235

Toxicity, 24-epibrassinolide, 290

Transformed tobacco cultures, hormone autotrophicity, 177

Transgenic tobacco, expression of xylem-specific promoter/ β -glucuronidase fusions, 136

Transverse geotropism, response of maize seedlings to brassinolide, 221,223*f*,224

Triepibrassinolide, structure, 177

Twining, response of maize seedlings to brassinolide, 221,223*f*,224

U

Ultrastructure, plant cell, *See* Plant cell ultrastructure

U.S. Department of Agriculture (USDA) brassins project, 3–15

U.S. Department of Agriculture (USDA) contributions to brassinosteroids biochemical and physiological responses, 325–326
 discovery of brassinolide, 321,322*f*,323,324*f*
 historical background, 320–321
 synthesis of brassinosteroids, 323,325

V

Vinylsilanes, synthesis of brassinosteroid intermediates, 48*f*

W

Wheat

effect of brassinolide, 306
root segment development,
238*t*,239
seedling root development,
235,236*t*
yield, 281–288

Wheat leaf unrolling test, description for
brassinosteroids, 110–111

Witchweed, description, 298

Witchweed seeds

conditioning and germination, 298–304
germination conditions and stimulants, 298

X

Xylem differentiation

Helianthus tuberosus, 128,131,132–133*f*,135*f*
Jerusalem artichoke, 134

Xylem-specific promoter/ β -glucuronidase
fusions in transgenic tobacco, 136

Production: Margaret J. Brown
Indexing: Deborah H. Steiner
Acquisition: A. Maureen Rouhi
Cover design: Amy Meyer Phifer

Printed and bound by Maple Press, York, PA