Screening of Basidiomycetes and Ascomycetes for Plant Growth Regulating Substances. Introduction of the Gibberellic Acid Induced *de-novo* Synthesis of Hydrolytic Enzymes in Embryoless Seeds of *Triticum aestivum* as Test System

Ralf Hautzel and Heidrun Anke

Universität Kaiserslautern, Abteilung für Biotechnologie, Paul-Ehrlich-Straße 23, D-6750 Kaiserslautern, Bundesrepublik Deutschland

Z. Naturforsch. **45c,** 1093–1098 (1990); received July 19, 1990

Galiella rufa, Hypholoma species, α-Amylase, Plant Growth Regulator, Galiellalactone, 3,5-Dichloro-4-methoxybenzyl Alcohol

A new test system for the detection of plant growth regulating activities was successfully employed. In a screening for inhibitors of the gibberellic acid controlled synthesis of hydrolytic enzymes in embryoless wheat seeds *(Triticum aestivum)* 160 cultures of ascomycetes and basidiomycetes were tested. In the extracts of two cultures inhibitory activities were detected. From fermentations of a *Hypholoma*-species (basidiomycetes) 3,5-dichloro-4-methoxybenzyl alcohol was isolated as the active principle.

Galiellalactone and two other new phytotoxins were isolated from cultures of the ascomycete *Galiella rufa*. At concentrations of 50 μg/ml all four compounds inhibited the *de-novo* synthesis of α-amylases, proteases, and phosphatases. Further investigations on the mode of action revealed, that all four metabolites interfere with early steps of the biosynthetic pathways induced by gibberellic acids. *In vivo*, the germination of the seeds of several plants was inhibited by these compounds.

Introduction

The aleurone layer of wheat seeds is a secretory tissue surrounding the endosperm. During germination it serves two major functions. It is the source of enzymes mobilizing the endosperm nutrient reserves as well as the source of mineral ions for the developing embryo. The synthesis and secretion of hydrolytic enzymes respond to the release of gibberellins from the embryo. Chen and Jones [1] have shown that up to 70% of the proteins newly synthesized during the incubation of isolated barley aleurone layers in the presence of gibberellins are α-amylases. Among the other proteins are hydrolases like α-glucosidases, β-glucanases, phosphatases and proteases. Since inhibitors of this hormone-controlled mobilization of endosperm reserves could be a target for specific germination inhibitors, 160 cultures of higher fungi were screened for the production of inhibitors of the gibberellic acid-controlled synthesis of hydrolytic enzymes in embryoless halves of wheat seeds *(Triticum aestivum)*. Using the screening system described in the experimental section, inhibitors of transcription, translation and secretion as well as inhibitors of hydrolytic enzymes like α-amylase, gliadin proteases, α-glucosidase or acid phosphatase can be found. In addition gibberellin antagonists can be detected.

In the following we wish to describe the screening system, the fermentation, isolation and biological properties of inhibitors found with this testsystem. Three inhibitors were isolated from cultures of the ascomycete *Galiella rufa* A 75-86 and 3,5-dichloro-4-methoxybenzyl alcohol (DCMB) from cultures of *Hypholoma* sp. 86130.

Reprint requests to Prof. Dr. Heidrun Anke.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/1100–1093 $ 01.30/0

Experimental

Screening

Embryoless halves of wheat seeds were surface sterilized by treatment with 2% sodium hypochloride for 10 min and then rinsed with distilled water. Groups of 6 seed halves were incubated in 25 ml Erlenmeyer flasks containing 8 ml sodium acetate buffer (10^{-3} M, pH 4.8) and gibberellic acid GA 3 (10^{-6} M). To prevent microbial growth streptomycin sulfate (10 μg/ml) was added. Incubations were carried out for 36 h on a rotary shaker (120 rpm/30 °C). α-Amylase activity was assayed in the supernatant after centrifugation for 5 min at 10,000 × *g*. The inhibitory activity of the crude

fungal extracts was estimated by comparison with the α-amylase activity of two hormone-induced standards. Control 1 contained no inhibitor (100% *de novo* synthesis of enzymes); control 2 contained 20 µg/ml cycloheximide (0% enzyme synthesis).

Enzyme assays

α-Amylase activity was measured as described by Jones and Varner [2]. The proteolytic activity of the incubation medium was estimated by the ninhydrin method of Moore and Stein [3] with gliadin as substrate. The activity of acid phosphatase was measured according to Jones [4]. The total protein concentration of the medium was determined by the method of Bradford [5].

Gel electrophoresis

SDS gel electrophoresis was performed according to the method of Laemmli [6]. The separated proteins were stained with Coomassie Blue R 250. Native gels were prepared as described by Davis [7]. After electrophoresis the native gels were incubated for 30 min in a starch solution (1% w/v in sodium acetate buffer; 20 mM, pH 6.9), washed twice with distilled water and stained for α-amylase activity with Lugol solution for 4–6 min.

Seed germination assay

The inhibition of seed germination was tested as described previously [8].

Test for mutagenicity

Mutagenicity was tested according to the method of Ames and coworkers [9]. Mutants of *Salmonella typhimurium* strain TA 98 and TA 100 were used for the spot test, with and without rat liver microsomes.

Test for cytotoxicity and antimicrobial activity

The antimicrobial spectrum and the cytotoxic activities against cells of the Ehrlich ascites carcinoma (H. Probst, University of Tübingen), HeLa-S3 (ATCC CCL 2.2) and L-1210 cells (ATCC CCL 219) were measured as described previously [10–12].

Fermentation

Mycelial cultures of *Galiella rufa* (A 75-86) and *Hypholoma* sp. 86130 were derived from fruiting bodies collected in the Smoky Mountains, U.S.A. *Hypholoma* sp. 86130 was kindly provided by T. Anke, Kaiserslautern. The strains are deposited in the collection of the Lehrbereich Biotechnologie, University of Kaiserslautern, F.R.G. They were maintained on agar slants with YMG medium (yeast extract 0.4%, glucose 0.4%, malt extract 1%, pH 5.5). For submerged cultivation 250 ml of YMG medium were inoculated with mycelium from one agar slant and incubated for 10 days on a rotary shaker (22 °C, 120 rpm). These cultures were used to inoculate 20 l of the same medium in a Biolafitte fermentation apparatus. Silicone antifoam was added initially. Fermentations were carried out at 22 °C, 120 rpm and an aeration rate of 3 l/min. The production of the inhibitors in cultures of *Galiella rufa* was calculated by the absorption coefficients at the UV maxima.

Results and Discussion

Identification of the inhibitor from Hypholoma sp. 86130

The inhibitor from cultures of *Hypholoma* sp. 86130 proved to be identical with 3,5-dichloro-4-methoxybenzyl alcohol (DCMB) described by Pfefferle *et al.* [13] from cultures of a *Stropharia* species. The genera *Hypholoma* and *Stropharia* belong to the same family (Strophariaceae).

Isolation of the inhibitors from cultures of Galiella rufa

A typical fermentation diagram of *G. rufa* in YMG medium is depicted in Fig. 1. After 13 days the metabolites A 75-I, A 75-II and A 75-III were isolated from the culture broth (18 l) and the mycelium by extraction with ethyl acetate and acetone respectively. The purification scheme is shown in Fig. 2. The UV maxima and the R_f values of the isolated compounds are given in Table I. The elucidation of the structures will be published elsewhere [14]. A 75-I, galiellalactone, is a new fungal metabolite with a carbon skeleton not previously found in nature [15]. The structure is shown in Fig. 3.

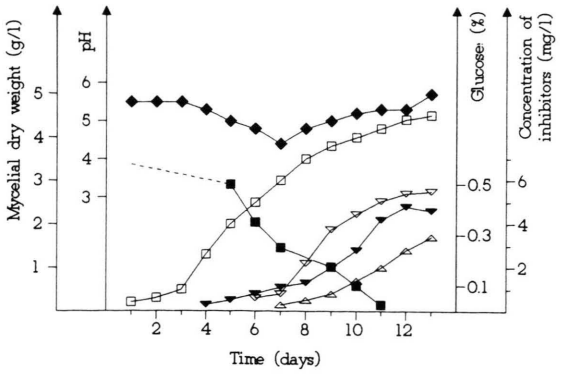

Fig. 1. Fermentation of *Galiella rufa*. —□— dry weight, —■— glucose, —◆— pH, —▼— galiellalactone, —▽— A 75-II, —△— A 75-III.

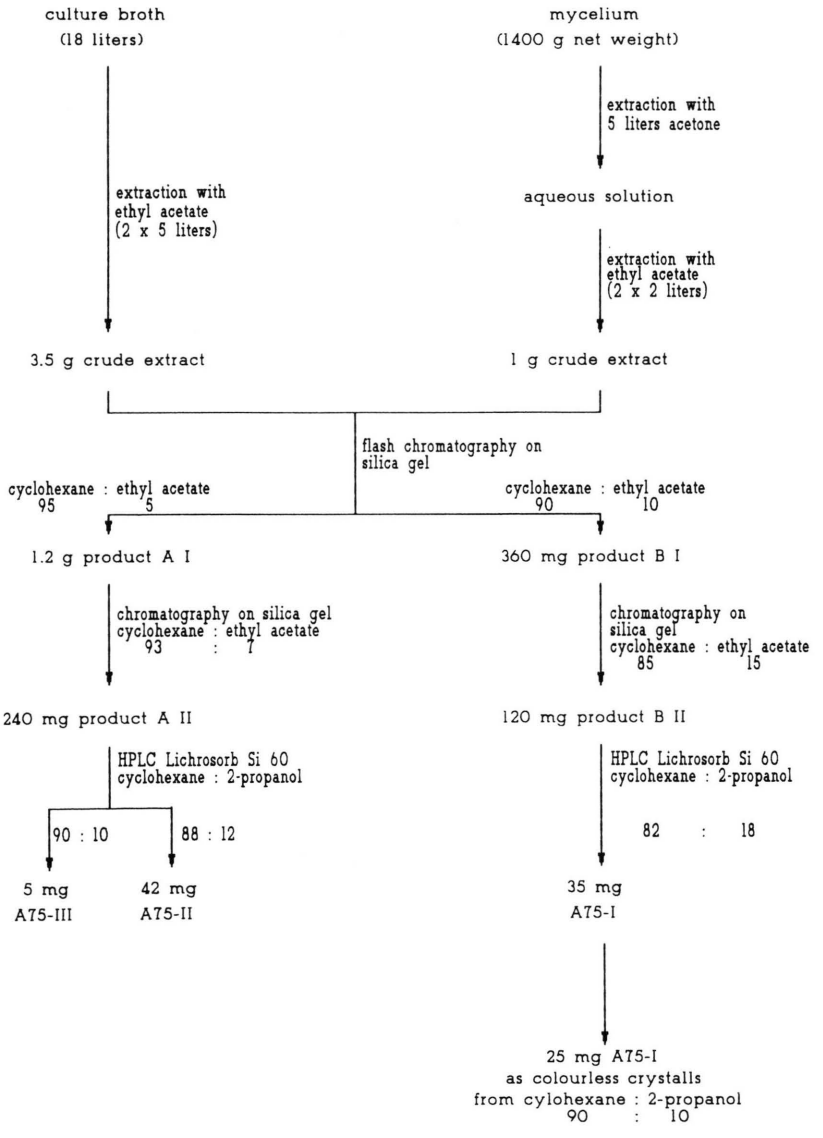

Fig. 2. Isolation of galiellalactone, A 75-II and A 75-III from the culture broth and the mycelia from *Galiella rufa*.

Table I. UV-maxima and chromatographic behaviour of the metabolites isolated from cultures of *Galiella rufa*.

Compound	UV-max. [nm]	$E^{1\%}_{1cm}$	R_f*
Galielalactone	219	350	0.46
A 75-II	250	380	0.80
A 75-III	(247), 340	(310), 1830	0.85

* Silica gel; cyclohexane-isopropanol (9:1).

Biological properties

DCMB, galiellalactone, A 75-II and III decreased the synthesis of GA_3-induced proteins in embryoless halves of wheat seeds. As shown in Fig. 4. A 75-II was the most active inhibitor. At 100 µg/ml no *de novo* synthesis of α-amylase and other proteins was observed. The compounds had no inhibitory effect on α-amylases, proteases and phosphatases at concentrations up to 100 µg/ml.

Based on their isoelectric points Gale and Spencer [16] classified α-amylases in two groups, in high pI and low pI isoenzymes. Native gel electrophoresis and staining for α-amylase activity showed that the four fungal metabolites reduced the synthesis of both groups of α-amylase isoenzymes. The proteins of the incubation medium obtained by SDS-gel electrophoresis after addition of the inhibitors are given in Fig. 5. The protein patterns obtained in the presence of A 75-II and DCMB are similar or identical to those obtained in the presence of cordycepin (transcription inhibitor) and cycloheximide (inhibitor of protein synthesis). Galiellalactone and A 75-III are less effective. Compared to the gibberellin-induced control the same protein bands are present but in greatly reduced concentrations. Addition of the inhibitors at different time intervals revealed that all four compounds interfere at the level of transcription or an earlier stage similar to cordycepin as can be deduced from the data given in Table II. Compet-

Table II. Effect of galiellalactone, A 75-II, A 75-III and DCMB on the GA_3 induced *de novo* synthesis of α-amylase: Addition of the inhibitors at different times.

Compound		Addition of inhibitors and gibberellic acid (10^{-6} M) Simultaneously	Inhibitor 24 h after GA
		Inhibition of α-amylase activity (%) compared to the control with GA_3	
Cycloheximide	(20 µg/ml)	100	100
Cordycepin	(1 mM)	100	0
Galiellalactone	(100 µg/ml)	75	0
A 75-II	(100 µg/ml)	100	0
A 75-III	(100 µg/ml)	83	0
DCMB	(100 µg/ml)	100	0

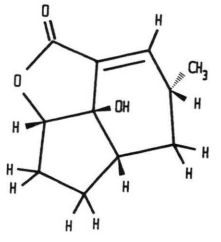

Fig. 3. Galiellalactone ($C_{11}H_{14}O_3$).

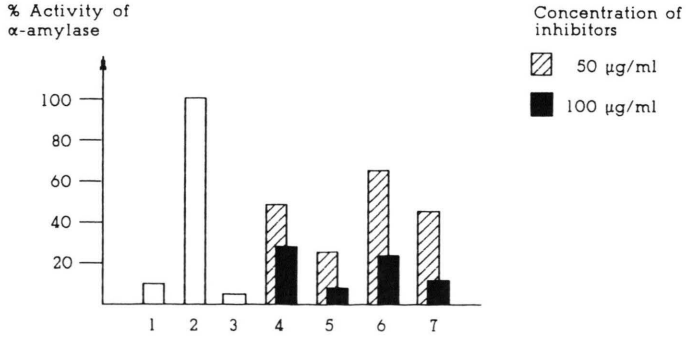

Fig. 4. Inhibition of the gibberellic acid (GA_3) induced *de novo* synthesis of α-amylase in embryoless halves of wheat seeds by galiellalactone, A 75-II, A 75-III and DCMB.
1) Control; 2) control + GA_3 (10^{-6} M); 3) control + GA_3 (10^{-6} M) + cycloheximide (20 µg/ml); 4) galiellalactone + GA_3 (10^{-6} M); 5) A 75-II + GA_3 (10^{-6} M); 6) A 75-III + GA_3 (10^{-6} M); 7) DCMB + GA_3 (10^{-6} M).

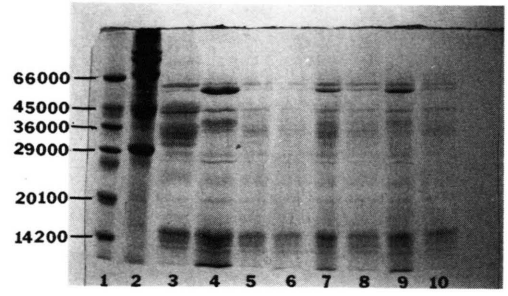

Fig. 5. SDS-gel electrophoresis of the proteins of the incubation medium.
1 + 2) Molecular weight standards; 3) control; 4) control + GA_3 (10^{-6} M); 5) control + GA_3 (10^{-6} M) + cycloheximide (20 µg/ml); 6) control + Ga_3 (10^{-6} M) + cordycepin (1 mM); 7) galiellalactone (100 µg/ml) + GA_3 (10^{-6} M); 8) A 75-II (100 µg/ml) + GA_3 (10 M); 9) A 75-III (100 µg/ml) + GA_3 (10^{-6} M); 10) DCMB (100 µg/ml) + GA_3 (10^{-6} M).

itive interactions with postulated gibberellin receptors could be excluded since the addition of excess gibberellic acid (100-fold) had no effect on the inhibitory action of A 75-I, A 75-II and A 75-III. Non-competitive interactions with gibberellin receptors, however, cannot be excluded.

In agreement with the effect on the hormone-induced enzyme synthesis, an inhibition of seed germination of several plants was observed (Table III).

Further investigations showed that all inhibitors exhibit only weak antimicrobial and cytotoxic activities. At 50 µg/ml none of the tested bacteria *e.g. Bacillus brevis, B. subtilis, E. coli, Mycobacterium phlei, Staphylococcus aureus, Proteus vulgaris and Acinetobacter calcoaceticus* were affected. Only at concentrations of 100 µg/ml or higher inhibition of bacterial growth was observed. In the test for mutagenicity no induction of revertants of *S. typhimurium* strain TA 98 and TA 100 could be observed with 100 µg/disc. With bovine erythrocytes no hemolytic activity was detected at 100 µg/ml. Galiellalactone was the only compound with moderate cytotoxic activities towards mammalian cells. The results of the cytotoxicity tests are given in Table IV.

Table IV. Cytotoxic activities of galiellalactone, A 75-II, A 75-III, and DCMB towards ECA-cells, L 1210 cells and Hela S-3 cells.

Compound	IC_{50} (µg/ml) ECA	L 1210	Hela S-3
DCMB	>100	>100	>100
Galiellalactone	25	15	25
A 75-II	50	>100	100
A 75-III	50	>100	100

The results obtained so far clearly show, that the new test system is suitable for the detection of new plant growth inhibitors and can be successfully applied in the search for new biologically active compounds in microbial cultures.

Acknowledgements

This work was supported by grants from the BASF AG and the Ministry for Science and Technology (BMFT A 0319215). We thank the Kultusministerium Rheinland-Pfalz for special support. The expert technical assistance of A. Helfer, B. Meisenheimer and A. Wehrle is gratefully acknowledged.

Table III. Effect of galiellalactone, A 75-II, A 75-III and DCMB in the seed germination assay. Six seed were placed on a filter disk (13 mm ∅) bearing the inhibitors in 200 µl H_2O and incubated in ahumid chamber at 20 °C.

Seed	Inhibition of seed germination MIC (µg/paper disc) A 75-I	A 75-II	A 75-III	DCMB
Poa annua	30	30	100	30
Lolium tetraflorum	100	30	100	50
Lepidium sativum	30	>100	>100	70
Lactuca capitata	70	30	100	30
Brassica napus	>100	>100	>100	120
Setaria italica	>100	100	>100	100
Amaranthus retroflexus	30	30	>100	70
Linum perenne	30	>100	>100	>100

[1] R. M. Chen and R. L. Jones, Planta **119,** 193 (1974).
[2] R. L. Jones and J. E. Varner, Planta **72,** 155 (1966).
[3] S. Moore and W. H. Stein, J. Biol. Chem. **176,** 367 (1948).
[4] K. C. Jones, Plant Physiol. **44,** 1695 (1969).
[5] M. M. Bradford, Anal. Biochem. **72,** 248 (1976).
[6] U. K. Laemmli, Nature **227,** 680 (1979).
[7] B. J. Davis, Ann. N. A. Acad. Sci. **121,** 404 (1964).
[8] H. J. Noll, Dissertation University Kaiserslautern (1987).
[9] B. N. Ames, J. McCann, and E. Yamasaki, Mutation Res. **31,** 347 (1975).
[10] T. Anke, F. Oberwinkler, W. Steglich, and G. Schramm, J. Antibiotics **30,** 806 (1977).
[11] J. Kupka, T. Anke, F. Oberwinkler, G. Schramm, and W. Steglich, J. Antibiotics **32,** 130 (1979).
[12] K. Leonhardt, T. Anke, E. Hillen-Maske, and W. Steglich, Z. Naturforsch. **42c,** 420 (1987).
[13] W. Pfefferle, H. Anke, M. Bross, and W. Steglich, Agric. Biol. Chem. **54,** 1381 (1990).
[14] H. Anke, R. Hautzel, W. S. Sheldrick, I. Casser, and W. Steglich, Abstracts of the 7th Internat. Congress on Pesticide Chemistry, 1990 in print and W. Steglich *et al.* manuscript in preparation.
[15] J. Berdy, Bioactive Natural Products Database, Szensor Management Consulting Company, Budapest (1990).
[16] M. D. Gale and D. M. Spencer, Biochem. Genet. **15,** 47 (1977).