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THE RELATIVE CONTENT OF GIBBERELLIN IN SEEDLINGS OF GYNOECIOUS AND MONOECIOUS CUCUMBER (*CUCUMIS SATIVUS*)*

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Abstract—Gibberellin A_1 was isolated and identified from etiolated monoecious cucumber seedlings using paper, TLC and G–LC and bioassayed by utilizing dwarf pea, cucumber epicotyl, dwarf corn d_1 and d_5 plants. The gynoecious line, in which external application of GA_3 accelerated staminate flower formation, contained less GA_1. GA_1 is possibly required for staminate flower formation in the monoecious, but not in the gynoecious line.

INTRODUCTION

SEVERAL years ago Peterson[1] reported the development of a new cucumber breeding line, Michigan State University 713-5, homozygous for gynoecious sex expression in normal greenhouse and field environments. MSU 713-5 was derived from a cross between a gynoecious segregate found in the Korean cultivar, Shogoin (PI 220860) and monoecious Wisconsin SMR 18, followed by four back crosses of predominantly female segregates to the monoecious parent. Two generations of inbreeding were then accomplished by self-pollination of predominantly female plants and three more by selfing gynoecious plants after induction of staminate flowers with GA_3 following procedures outlined by Peterson and Anhder.[2] This inbred line is of interest in the development of commercial F_1 hybrids and is also valuable for experimental studies on the genetics, physiology, and molecular biology of sex expression. Foliar sprays of gibberellin A_3 (GA_3) were found effective for induction of staminate flowers on gynoecious genotypes that produce exclusively pistillate flowers. However, gibberellin A_7 (GA_7) was the most active of the original nine gibberellins for vegetative growth.[3,4] This effect of exogenous GA_3 on staminate flower induction suggested the idea of identifying the gibberellins present in this gynoecious species and an almost isogenic monoecious species and of comparing their gibberellin levels at various growth stages. Such information would indicate whether or not gibberellin plays a role in the regulation of cucumber sex expression.

RESULTS

As shown in Fig. 1 the monoecious line (MSU-736) contained much more gibberellin than did the gynoecious line (MSU 713-5). The active compound of the monoecious line

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[1] C. E. PETERSON, *Quart. Bull. Mich. Ext. Sta.* **43**, 40 (1960).
[2] C. E. PETERSON and L. D. ANHDER, *Science* **131**, 1673 (1960).
[3] S. H. WITTWER and M. J. BUKOVAC, *Naturwiss.* **13**, 305 (1962).
[4] L. M. PIKE and C. E. PETERSON, *Euphytica* **18**, 106 (1969).

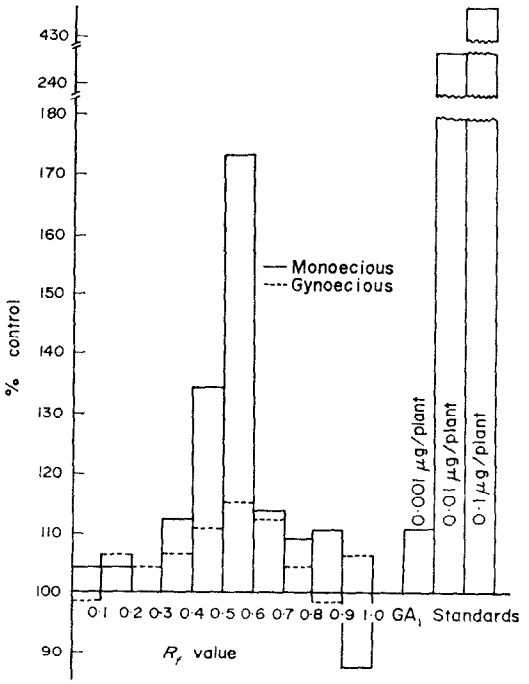

FIG. 1. GIBBERELLIN ACTIVITIES IN THE DWARF PEA BIOASSAY EXHIBITED BY ELUATES OF THE TEN ZONES OF THE PAPER CHROMATOGRAMS (SOLVENT SYSTEM: ISOPROPANOL–AMMONIUM HYDROXIDE–WATER, 10:1:1, BY VOL.) FROM THE EXTRACTS OF 6-day-old GYNOECIOUS AND MONOECIOUS CUCUMBER SEEDLINGS.

appears to be GA_1 or GA_3 for two reasons: first, its R_f value on the paper chromatogram was similar to that of GA_1 or GA_3 and secondly the results from the dwarf pea bioassay of the partially purified extracts at different growth stages of the monoecious line indicated the presence of GA_1 and/or GA_3 in the paper chromatogram zone between 0·5 and 0·6 R_f values. The gibberellins were extracted from this active zone with ethyl alcohol for further tests. The total endogenous gibberellin content in these extracts of the monoecious variety (Table 1) increased up to 6 days following germination and remained at almost the same level for an additional 12 days. However, the free form of gibberellin decreased after reaching a similar peak at 6 days. The free gibberellins were produced to a lesser degree under the same conditions in the gynoecious line (Table 1). The amount of the bound form of the gibberellin, which was obtained by hydrolysis of phosphate buffer extracts of the plant material with ficin and β-glucosidase, was higher in the monoecious than in the gynoecious line (Table 1).

The TLC purification of the ethyl acetate extracts of the monoecious line from the R_f 0·5–0·6 zone of the above paper chromatograms gave gibberellins which migrated to a R_f value of 0·57 on the TLC (Fig. 2) developed with benzene–*n*-butanol–acetic acid, 70:25:5, by vol. One spot exhibited a blue fluorescence after being sprayed with a solution of 5% sulfuric acid in 95% ethanol. The substance at this zone showed biological activity in the dwarf pea and dwarf corn d_1 and d_5 bioassays, but not in the cucumber hypocotyl elongation test. The bioassay results as given in Table 2 suggest strongly the presence of GA_1 or GA_3.

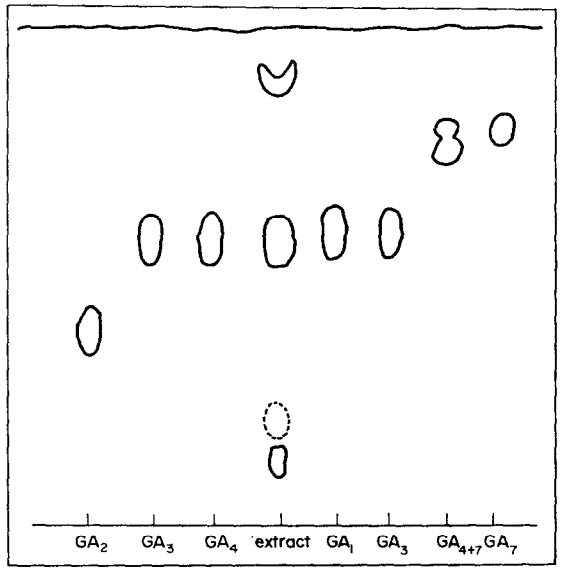
GA2
GA3
GA4
extract
GA1
GA3
GA4+7
GA7

FIG. 2.

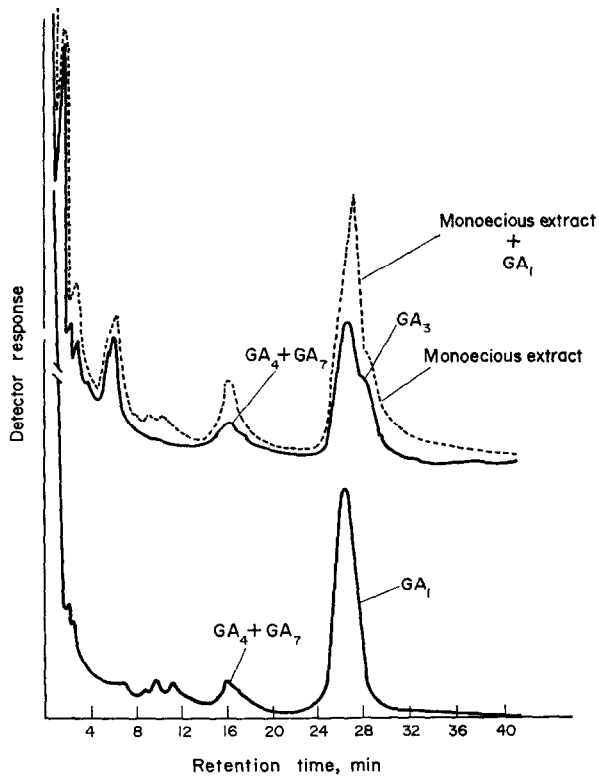
Monoecious extract
+
GA1
GA3
GA4+GA7
Monoecious extract
GA1
GA4+GA7
Detector response
4
8
12
16
20
24
28
32
36
40
Retention time, min

FIG. 3.

TABLE 1. GIBBERELLIN* ACTIVITY OF ETHYL ACETATE EXTRACTS FROM DIFFERENT GROWTH STAGES OF MONOECIOUS (LINE MSU-736) AND GYNOECIOUS (LINE MSU 713-5) CUCUMBER SEEDLINGS AS DETERMINED BY DWARF PEA BIOASSAY

	Monoecious				Gynoecious			
Days after germination	Fr. wt. (g)	Free GA (μg/kg)	Bound GA (μg/kg)	Total GA (μg/kg)	Fr. wt. (g)	Free GA (μg/kg)	Bound GA (μg/kg)	Total (μg/kg)
0	15·7	64·5	37·8	102·3	14·9	57·7	44·1	101·8
1	17·6	49·0	106·2	155·2	16·8	48·1	71·3	119·4
2	22·6	52·5	208·1	260·6	21·8	55·3	131·6	186·9
6	28·9	625·0	101·7	726·7	27·1	77·6	141·3	218·9
10	35·5	370·0	408·0	778·0	32·9	112·9	211·7	324·6
14	37·8	232·5	517·0	749·5	35·3	188·7	306·5	495·2
18	38·1	261·1	462·7	723·8	37·6	211·2	321·3	532·5

* The active compounds were those obtained from the R_f 0·5–0·6 zones of the paper chromatogram developed with isopropanol–ammonium hydroxide–water, 10:1:1, by vol. (see Fig. 1).

TABLE 2. GIBBERELLIN ACTIVITY OF ELUATES FROM PAPER CHROMATOGRAMS OF MONOECIOUS (LINE MSU-736) CUCUMBER SEEDLINGS AND OF SEVERAL GIBBERELLIN STANDARDS IN VARIOUS BIOLOGICAL ASSAYS*

Substance	Dwarf peas	Cucumber hypocotyl	Dwarf corn d_1	Dwarf corn d_5
Sample† 1 (R_f 0·5–0·6)	173·4	101·8	122·6	121·9
Sample‡ 2 (R_f 0·53–0·58)	261·5	102·5	137·3	131·8
Gibberellin§ A_1	237·1	98·2	147·1	134·5
Gibberellin§ A_3	245·3	107·1	132·6	122·6
Gibberellin§ A_7	287·6	235·8	124·9	121·9
Gibberellin§ $A_4 + A_7$	228·3	219·4	122·8	120·6

* Values expressed as % of control.

† Sample 1 obtained by developing the substance obtained from a paper chromatogram having an R_f value of 0·5–0·6 with isopropanol–ammonium hydroxide–water (10:1:1, by vol.).

‡ Sample 2 obtained by purifying Sample 1 on a Silica Gel F_{254} TLC with benzene–*n*-butanol–acetic acid (70:25:5, by vol.).

§ Applied at a concentration of 0·01 μg of gibberellin per plant.

Gibberellin A_1 was identified by chromatographing its methyl ester on a thin-layer plate with benzene–acetic acid–water, 8:3:5, by vol. The methyl gibberellate migrated to a spot having an R_f value of 0·32 which is in agreement with the R_f value of an authentic methyl ester of GA_1. The identity of the methyl ester of GA_1 was further confirmed by GLC. Trace amounts of gibberellin A_3, A_4 and A_7 were also found to be present (Fig. 3).

DISCUSSION

Sex expression and vegetative growth of cucumbers are regulated by indole-3-acetic acid (IAA) and other auxin compounds.[5] In the gynoecious line, reversion from pistillate to

[5] F. LAIBACH and F. KRIBBEN, *Ber. Deut. Botan. Ges.* **63**, 119 (1950).

staminate flowers was induced by GA_3 at 10^{-3} M concentration.[6] The total gibberellin content, which may include other gibberellin-like substances, increased in the monoecious cucumber seedlings between 0 and 6 days in complete darkness whereas there was only a slight increase for the same growth period in the gynoecious line.[7,8]

Gibberellins A_4, A_7 and A_9 are the most active among the original nine gibberellins in promoting hypocotyl elongation of cucumber seedlings.[3,9] Externally applied GA_3 enhanced male sex expression, but not cell elongation or expansion of vegetative growth in the cucumber plant. GA_3 was observed by Hayashi *et al.*,[10] as the main gibberellin in the cortical tissue of parthenocarpic apple fruits, while GA_4 and GA_7 were identified in the immature apple seeds.[11] One explanation for these results is that one form of gibberellin may be converted enzymatically to another. The action of GA_3 or GA_1 in the cucumber tissues is different from that of GA_4, GA_7, and GA_9. In the early stages of the germinating cucumber seedlings, the GA_1 level in both varieties was not high, but after 6–18 days the total gibberellin content in the monoecious line was considerably increased; there was a peak of free gibberellin A_1 6 days after germination. On the other hand the total gibberellin content in the gynoecious line increased gradually although the rate of increase was lower than that of the monoecious line.

EXPERIMENTAL

10 g (~500–510 seeds) of each variety (monoecious line MSU-736 and gynoecious line MSU 713-5) of cucumber, *Cucumis sativus* L., were placed in a plastic pot, covered with cheese cloth, and soaked in running tap water. After 8 hr the water was drained off; the pots, still covered with cheese cloth, were placed in plastic pans of 5 cm depth containing water to a depth of 1 cm into which the cheese cloth extended. The seeds were germinated and grown in the dark at 25° and were harvested at different growth stages over a period of 0–18 days.

The harvested seedlings for GA determinations were homogenized with a hot solution of 80% EtOH in a Waring blender. The homogenate was filtered and the residue was re-extracted twice with hot EtOH. The extracts were combined, evaporated to dryness, and 5% $NaHCO_3$ added. The $NaHCO_3$ was then extracted with ethyl acetate. The aqueous layer, containing most of the active acidic gibberellins, was adjusted to pH 3·0 and again extracted with ethyl acetate. The ethyl acetate layer was washed 3 × H_2O, dried (Na_2SO_4), and evaporated to dryness. The gummy residue was purified further by descending paper chromatography on Whatman 3 MM paper using as solvent iso PrOH–NH_4OH–H_2O, 10:1:1, by vol. The active compounds were eluted from the paper with 80% EtOH and then used for TLC and bioassays.

TLC was restricted to the active zone having R_f values of 0·5–0·6 in the above described paper chromatography system. The EtOH eluate was concentrated to a small volume *in vacuo* and was spotted on a Brinkman precoated TLC plate of silica gel F_{254} having a thickness of 250 nm. The chromatogram was developed first with CCl_4–HOAc–H_2O, 8:3:5, by vol. non-aqueous phase plus 20% ethyl acetate. This solvent system removed many interfering compounds since the active compounds do not migrate. The residue at the origin was eluted with 80% EtOH and rechromatographed using benzene–*n*-BuOH–HOAc, 70:25:5, by vol. as solvent.

The TLC plate was divided into five sections and examined by fluorescence with and without a solution of 5% H_2SO_4 in 95% EtOH. The portion of the chromatogram which exhibited biological activity and a bluish-yellow band under a fluorescent lamp (R_f 0·57) was eluted with 80% EtOH. The eluate was concentrated to dryness *in vacuo* and divided into three parts. Part 1 was esterified with CH_2N_2,[12] the 2nd part was treated with

[6] WM. D. MITCHELL and S. H. WITTWER, *Science* **136**, 880 (1962).
[7] F. HAYASHI, H. M. SELL and C. E. PETERSON, *Seventh International Congress of Biochemistry*, Abstract J-180, Tokyo (1967).
[8] D. ATSMON, A. LANG and E. N. LIGHT, *Plant Physiol.* **43**, 806–810 (1968).
[9] P. W. BRIAN and H. G. HEMMING, *Nature* **189**, 74 (1961).
[10] F. HAYASHI, R. NAITO, J. M. BUKOVAC and H. M. SELL, *Plant Physiol.* **43**, 448 (1968).
[11] F. G. DENNIS, JR. and J. P. NITSCH, *Nature* **211**, 781 (1966).
[12] F. ARNDT, in *Organic Synthesis*, (edited by A. H. BLATT), Collective Vol. II. p. 165. John Wiley, New York (1943).

trimethylchlorosilane and hexamethyldisilazane in dry pyridine solution,[13] and the third part was used for bioassays. Part 1 was subjected to TLC using as the solvent system benzene–HOAc–H_2O, 8:3:5, by vol. and gave an R_f value of 0·32. The gibberellins in both parts 1 and 2 were determined on a F and M Model 400 GLC equipped with a H_2 flame ionization detector and a pyrex glass U column 1 m in length and 4·5 mm dia. packed with 3·0% OV-1 (Methyl silicone polymer obtained from Applied Science Laboratory) on a 70–80 mesh chromasorb (obtained from Analabs, Inc.) at a column temperature of 200°.[14] Nine authentic gibberellin esters (GA_3 to GA_9) with a concentration of 2 μg/5 μl as free-acid were determined chromatographically in the same manner as those from parts 1 and 2. The GLC results obtained with the gibberellins from the extracts were then compared with the peaks obtained with the authentic gibberellin samples.

The dried residues from both the paper chromatograms and the TLC (part 3) were prepared for bioassays by dissolving in 1 ml 0·1% aq. Tween 80 and 1 ml EtOH; a series of further dilutions in a 50% alcoholic 0·05% Tween-80 solution was made when necessary.

The first bioassay for the quantitative detection of GA_1, GA_3, GA_4, GA_5, and GA_7 is a method devised by Hayashi and Rappaport.[15] For this assay 300 g of dwarf pea seeds (Morse's Progress No. 9 from Ferry Morse Seed Co.) were soaked in running tap water for 8 hr and then planted in moist vermiculite and grown in the dark at 25° for 4 days. Seedlings which measured 2·7–3·0 cm from the cotyledonary node to the highest visible node were transferred to a H_2O culture and irradiated with red light (2000 ergs/in^2) 45 cm above the seedlings. 12 hr later 10 μl of each sample were applied to the apex of each seedling and they were kept under red light at 25° and 65–70% R.H. for 5 days. The response to the treatment was determined by measuring the distance from the cotyledonary node to the highest visible node.

In the second bioassay, GA_1, GA_3, GA_4 and GA_7 were detected by Phinney's method[16] by using d_1 and d_5 dwarf corn (*Zea mays* L). 6–8 times as many plants as required for the bioassay were planted in vermiculite and grown in a greenhouse at 24° for 4 days. All tall, backward or mishapen plants and any dwarfs grown within 2 cm radial distance of each other were removed. 10–50 μl of the sample were applied to each plant. After 7 days the response of the treatments was evaluated by measuring the length of the leaf base of the first and second leaf to the nearest mm.

The third bioassay[17] employed cucumber seedlings (*Cucumis sativus* L.; monoecious line MSU-736) to detect GA_1, GA_3, GA_4, GA_7 and GA_9. In this method the seeds were soaked in running tap water for 8 hr planted in vermiculite, and grown in the greenhouse for 5 days at 24°. 10 μl of each of the gibberellin extracts were then applied to the apical part of the first true leaf. The biological activity was a measure of the elongation of the hypocotyl in millimeters 7 days after treatment.

Bound gibberellins were detected by hydrolyzing the plant sample with the proteolytic enzyme ficin[18] (obtained from the Pierce Chemical Co.) and also with crystalline β-glucosidase (emulsin purchased from Sigma Chemical Co.). Seedlings of the monoecious and gynoecious lines were harvested at different growth stages and homogenized with 150 ml of cold 0·1 M phosphate buffer at pH 6·2 and the homogenate was divided into three parts. Part 1 was treated with 20 mg ficin and 2 ml of toluene; part 2 was subjected to 5 mg of β-glucosidase at pH 5·3; part 3 was used as a control without enzyme. The three solutions were incubated at 30° for 24 hr and the enzyme activity was stopped by the addition of 450 ml EtOH to the slurry in each flask. The solutions were filtered and the filtrates concentrated to dryness *in vacuo* at 30° and 16 mm pressure. The gibberellins were extracted from each of the three solutions as previously described.

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[13] C. C. SWEELEY, R. BENTLEY, M. MAKITA and W. W. WELLS, *J. Am. Chem. Soc.* **85**, 2497 (1963).
[14] NOBUO IKEKAWA, TSUNEO KAGAWA and YUSUKE SUMIKI, *Proc. Japan Acad.* **39**, 506 (1963).
[15] F. HAYASHI, S. BLUMENTHAL-GOLDSCHMIDT and L. RAPPAPORT, *Plant Physiol.* **37**, 774 (1962).
[16] B. O. PHINNEY, *Proc. Natl. Acad. Sci. U.S.* **42**, 185 (1956).
[17] S. H. WITTWER and M. J. BUKOVAC, *Am. J. Botany* **49**, 524 (1961).
[18] A. J. MCCOMB, *Nature* **192**, 575 (1961).