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SYNTHESIS OF AUXINS, GIBBERELLINS AND CYTOKININS BY *AZOTOBACTER VINELANDII* AND *AZOTOBACTER BEIJERINCKII* RELATED TO EFFECTS PRODUCED ON TOMATO PLANTS

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SUMMARY

Culture supernatants of *Azotobacter vinelandii* and *Azotobacter beijerinckii* contain auxins, at least three gibberellin-like substances and three cytokinin-like substances. Treating roots of tomato seedlings with these cultures accelerates plant growth and increases yield of fruit, effects probably caused by activity of the plant hormones. Amounts of hormones produced in these cultures are similar to those produced by *Azotobacter chroococcum* and *Azotobacter paspali*.

INTRODUCTION

Plant growth regulators of the auxin and gibberellin type are produced in culture supernatants of *Azotobacter chroococcum* [5 8], *Azotobacter vinelandii* [17] and *Azotobacter paspali* [2]. Cytokinins are also produced by *Azotobacter chroococcum* [10] and *Azotobacter paspali* [2].

Responses in plant growth following treatment of seeds or seedling roots with cultures of *A. chroococcum* and *A. paspali* are probably caused by the gibberellins but cytokinins or auxins may also be involved.

A. vinelandii and *A. beijerinckii* used as inoculants for early vegetables grown in the sub-tropical area of the Mediterranean in Spain also improved growth probably by producing growth regulators [1]. This paper reports experiments to verify presence and activity of growth regulating substances in cultures of *A. vinelandii* and *A. beijerinckii*.

MATERIAL AND METHODS

Cultures

Eleven strains of Azotobacter were isolated from the rhizospheres of tomato plants (cultivar Marglobe) growing in a sand: farmyard manure mixture as used in the sub-tropical area of Spain [22]. The Azotobacter were identified by the method of Callao *et al.*[9] as *A. vinelandii* and *A. beijerinckii*. Strains of two species A_4 (*A. vinelandii*) and A_5 (*A. beijerinckii*) were selected for study because of their effectiveness as bacterial inoculants [1]. These strains were grown for 14 days in 70 ml medium [5] in 250 ml flasks on a rotary shaker incubated at 28°C.

Effect of Azotobacter on tomato growth

Roots of seedling tomato (cultivar Marglobe) were dipped in cultures of the Azotobacter strains A_4 and A_5 before transplanting the seedlings to pots of a mixture of sand:peat:farmyard manure (1:1:1 v/v). Control seedlings were dipped in diluted culture medium. Plants were fed every two weeks with 5 ml/pot of nutrient solution [15]. One series of plants were grown for 60 days and dry weights of shoots then obtained and another series was grown until fruit had formed. Numbers of flowers and fruits and weight of fruit were recorded.

Establishment of Azotobacter in the tomato rhizosphere

Rhizosphere soil was sampled at 14 day intervals and *A. vinelandii* or *A. beijerinckii* counted on the nitrogen-deficient medium described by Brown *et al.*[6] Numbers were related to 1 g dry rhizosphere soil.

Extraction of plant growth regulators

Cultures of *A. vinelandii* or *A. beijerinckii* were centrifuged at 2000 × *g* for

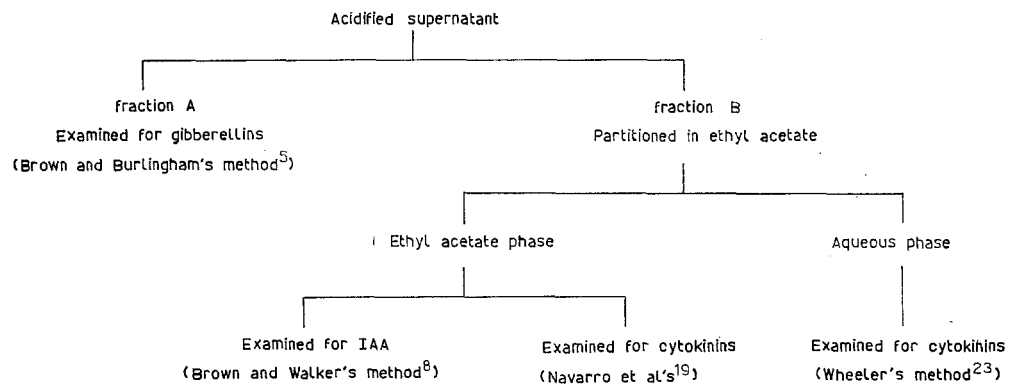

Fig. 1. Diagram showing methods used for extraction of plant growth regulating substances from bacterial cultures.

40 min. Each supernatant fluid was acidified to pH 3 and divided into two equal fractions, A and B. These fractions were examined as in figure 1.

Both organic and aqueous phases after partitioning with ethyl acetate were examined for cytokinins because in model experiments with kinetin it was found that most of the kinetin partitioned in the organic phase [14 19], with a small proportion only partitioning in the aqueous phase.

Paper partition chromatography

The different extracts were examined by descending paper chromatography using as solvent system freshly mixed isopropanol:ammonia:water (10:1:1) by volume. After development chromatograms were examined for fluorescense under UV light (wavelength, 350 nm) before and after spraying with chromogenic reagent (5% conc. H_2SO_4 in methanol). R_f values ranged from 0.3 to 0.4 for IAA (Merck AG Darmstad. Germany) 0.5 to 0.7 for GA_3 (Sigma Chemical Co., St Louis, Missouri, U.S.A.) and 0.7 to 0.8 for kinetin (Sigma) used as standard authentic substances.

Chromatograms portion not treated with chromogenic reagent were dried for at least 7 days to remove solvents and 10 equal strips representing the sequence of R_f values 0.1 to 1.0 used in bioassays.

Bioassays

The yeast bioassay described by Barea *et al.*[3], was used to screen the extracts for presence of growth regulators. Specific bioassays were then used.

(i) IAA. The portion of chromatogram corresponding in position to the R_f value for authentic IAA was used for the bioassay in which length increase of wheat coleoptile segments was measured [8 20].

(ii) Gibberellins. Extension of lettuce hypocotyls [13] and extension of cucumber hypocotyls [4] were used as bioassays.

(iii) Cytokinins. Expansion of radish cotyledons [18] and measurement of optical density of chlorophyll retained in the excised three first leaves of oat [23] were used as bioassays.

To minimize interference by gibberellins and auxins in cytokinin bioassays strips of chromatograms representing the sequence of R_f values were heated at 115°C for 20 min; this destroyed the gibberellins and auxins, but not the cytokinins. These strips were then used in the respective bioassays and responses compared with those from untreated strips.

Amounts of growth regulators were calculated from dose response curves obtained with authentic substances and given as μg equivalents per ml of culture supernatant.

RESULTS

Effects on tomato growth of treatment with A. vinelandii *or* A. beijerinckii

Treating roots of seedling tomatoes with cultures of Azotobacter strains A_4 and A_5 significantly improved plant growth, and fruits formed two weeks earlier than those on control plants.

TABLE 1

Effects of Azotobacter inoculation on growth of tomato plants

Topics	Pots inoculated with *A. vinelandii*	*A. beijerinckii*	Control	LSD (5%)
Stem length (mm)				
after 15 days	22	24	19	3.1
after 30 days	107	109*	97	14.2
Shoots dry weight (g)				
after 60 days	2.53 *	2.46 *	1.60	0.71
n° of flowers/pot	31 *	32 *	25	5.2
n° of fruits/pot	8 **	8 **	5	1.5
Weight of fruits (g/pot)	39.40 ***	37.10 ***	27.37	6.85

* Significance at 5% level; ** at 2% level; *** at 1% level.

Table 1 shows that dry weight of shoots from plants grown 60 days was significantly increased by inoculation and these plants formed more flowers and heavier fruits.

Establishment of A. vinelandii *or* A. beijerinckii *in tomato rhizosphere*

Table 2 shows counts of *A. vinelandii* or *A. beijerinckii* recovered from rhizospheres of tomato. The inocula decreased in number rapidly but some cells were still present after 14 weeks.

TABLE 2

Number of *A. vinelandii* and *A. beijrinckii* in tomato rhizosphere. (no.s/g dry rhizosphere soil)

Azotobacter spp.	Weeks						
inoculated	2	4	6	8	10	12	14
A. vinelandii	3700	2300	1200	1600	950	400	80
A. beijerinckii	14000	7500	1500	2300	1050	500	80

Production of plant growth regulators by A. vinelandii *and* A. beijerinckii

(i) Yeast bioassay. Tables 3 and 4 show the optical density and number of cells in cultures of *Saccharomyces cerevisieae* incubated with eluated of each R_f value from the different chromatograms.

Eluates from the heated chromatogram of fraction B organic phase, between R_f 0 and 0.4 and 0.6 to 0.7 caused increased O. D. and cell number, and eluated from the heated chromatogram of fraction B aqueous phase, between R_f 0 and 0.2, 0.3 and 0.5, 0.6 and 0.7, and 0.8 and 1.0, also caused increases, all indicating cytokinin activity. This was confirmed by specific bioassays.

(ii) Auxins. In the wheat coleoptile bioassay, substances with R_f 0.3 to 0.4 eluted from chromatograms of extracts from both cultures possessed auxin activity equivalent to 0.3 to 0.4 μg IAA per ml culture.

TABLE 3

Response of *Saccharomyces cerevisieae* to eluates of each R_f value from chromatograms of different extracts of *Azotobacter vinelandii*

	Fraction A		Fraction B					
			Ethyl acetate phase				Aqueous phase	
					(Heated chromatograms)			
R_f values	Optical density	Cell no. $\times 10^6$/ml	Optical density	Cell no. $\times 10^6$/ml	Optical density	Cell no. $\times 10^6$/ml	Optical density	Cell no. $\times 10^6$/ml
0–0.1	0.62 *	17.0	0.64	17.6	0.64	17.5	0.62	16.1
0.1–0.2	0.55	12.4	0.63 *	17.1	0.63 *	17.5	0.66 *	18.0
0.2–0.3	0.60	16.1	0.62 *	17.1	0.62	17.1	0.58	14.0
0.3–0.4	0.61 *	16.3	0.62 ×	17.2	0.61 *	17.0	0.66 *	18.1
0.4–0.5	0.58	13.8	0.58	13.6	0.60	15.8	0.64	18.1
0.5–0.6	0.62 °	17.1	0.60 °	16.2	0.60	15.9	0.62	17.0
0.6–0.7	0.58	13.7	0.58 *	13.6	0.61 *	16.7	0.66 *	18.2
0.7–0.8	0.62 °	17.5	0.61 °	16.9	0.59	15.9	0.57	13.5
0.8–0.9	0.57	13.5	0.57	13.5	0.58	13.5	0.64	17.6
0.9–1.0	0.57	13.6	0.57	13.4	0.57	13.8	0.64 *	17.6
Control	0.55	12.3	0.55	12.3	0.55	13.1	0.57	14.0

* Indicates cytokinin activity.
× Indicates auxin activity.
° Indicates gibberellin activity.

TABLE 4

Response of *Saccharomyces cerevisieae* to eluates of each R_f value from chromatograms of different extracts of *Azotobacter beijerinckii*

	Fraction A		Fraction B					
			Ethyl acetate phase				Aqueous phase	
					(Heated chromatograms)			
R_f values	Optical density	Cell no. × 10^6/ml	Optical density	Cell no. × 10^6/ml	Optical density	Cell no. × 10^6/ml	Optical density	Cell no. × 10^6/ml
0–0.1	0.62	16.8	0.64	17.5	0.54	11.5	0.62	17.1
0.1–0.2	0.64 *	17.5	0.64 *	17.6	0.65 *	18.0	0.64 *	17.9
0.2–0.3	0.60	14.7	0.61	15.3	0.60	16.1	0.61	16.8
0.3–0.4	0.61	17.1	0.62	17.5	0.61 *	16.4	0.56	12.8
0.4–0.5	0.61 *×	17.2	0.62 *×	17.9	0.58	14.3	0.61	16.3
0.5–0.6	0.60	16.8	0.61	16.2	0.52	11.2	0.63 *	17.4
0.6–0.7	0.62 °	17.2	0.63 °	17.5	0.64	17.5	0.58	14.0
0.7–0.8	0.64 *	17.4	0.64 *	17.5	0.64 *	17.9	0.64	17.7
0.8–0.9	0.60	16.1	0.60	16.1	0.60	16.2	0.63 *	17.3
0.9–1.0	0.58	14.3	0.59	14.4	0.62 *	17.0	0.58	14.1
Control	0.55	12.4	0.56	12.8	0.55	12.3	0.55	12.4

* Indicates cytokinin activity.
× Indicates auxin activity.
° Indicates gibberellin activity.

(iii) Gibberellins. *Lettuce hypocotyls bioassay*: Figures 2 and 3 show the activity of eluates from chromatograms of fraction A on lettuce hypocotyl extension.

Significant increases were produced by substances extracted from cultures of *A. vinelandii* with R_f values between 0.4 and 1.0, giving a peak of activity at R_f 0.55 (corresponding in position to authentic GA_3). This was equivalent to 0.05 μg per ml culture supernatant fluid.

Significant hypocotyl extension was produced by substances extracted from cultures of *A. beijerinckii* with R_f values between 0.2 and 0.3, and 0.5 and 0.7 giving a peak of activity at R_f 0.55. This was equivalent to 0.04 μg GA_3 per ml culture supernatant fluid. Substances with R_f 0.9 to 1.0 significantly decreased hypocotyl growth.

Cucumber hypocotyl bioassay: Figures 2 and 3 also show the effects

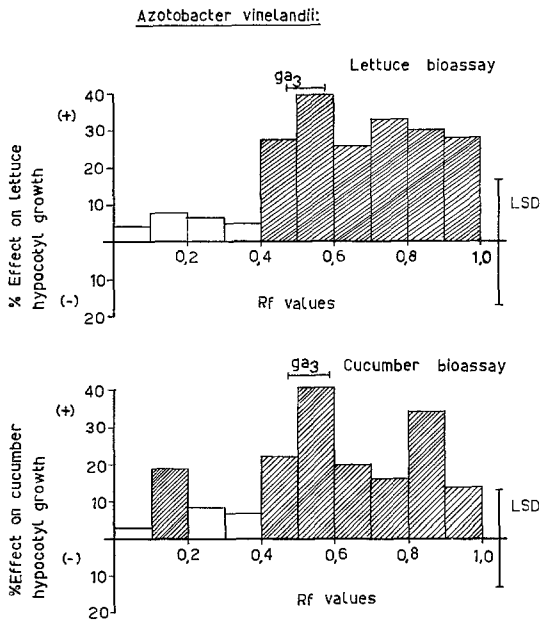

Fig. 2. Effects on extension of lettuce and cucumber hypocotyls by components of fraction A of the supernatant fluid of *Azotobacter vinelandii* cultures separated by chromatography. Shaded portion represents activity significant at 5% level. Horizontal line at the top of the figure represents position of authentic GA_3.

of eluates from chromatograms of fraction A on cucumber hypocotyl extension. Results are similar to those obtained in the lettuce bioassay. Eluates from cultures of *A. vinelandii* also show activity at R_f 0.1 to 0.2, and those from cultures of *A. beijerinckii* activity at R_f 0 to 0.1 and 0.2 to 0.3.

The supernatant fluid fraction was calculate to contain 0.06 μg (*A. vinelandii*) and 0.05 μg (*A. beijerinckii*) of GA_3 equivalent per ml.

(iv) Cytokinins. Heated portions of chromatograms from extracts of fraction B organic and aqueous phases were used.

Chlorophyll retention test: Table 5 show results from tests based on determining the optical density of chlorophyll retained in three excised first leaves of oat. *A. vinelandii* and *A. beijerinckii* gave similar results with peaks of activity shown by eluates with R_f

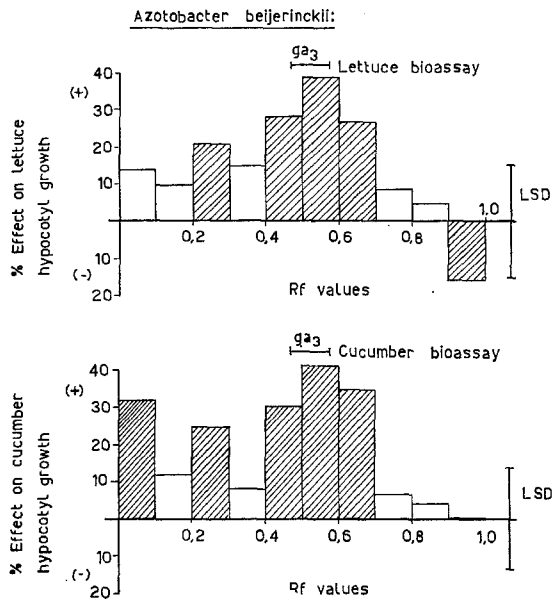

Fig. 3. Effects on extension of lettuce and cucumber hypocotyls by components of fraction A of the supernatant fluid of *Azotobacter beijerinckii* cultures separated by chromatography. Conventions as in Fig. 2.

TABLE 5

Cytokinin bioassay by measuring optical density of chlorophyll retained in three excised first leaves of oat

	Heated chromatograms from			
	Azotobacter vinelandii		*Azotobacter beijerinckii*	
R_f values	Ethyl acetate phase	Aqueous phase	Ethyl acetate phase	Aqueous phase
0–0.1	0.13 *	0.12 *	0.10	0.12 *
0.1–0.2	0.12	0.10	0.13 *	0.10
0.2–0.3	0.12	0.11	0.10	0.12
0.3–0.4	0.13 *	0.12 *	0.10	0.12 *
0.4–0.5	0.11	0.12	0.11	0.12
0.5–0.6	0.13 *	0.13 *	0.11	0.14 *
0.6–0.7	0.12	0.12	0.11	0.12
0.7–0.8	0.12 *	0.12	0.12 *	0.12
0.8–0.9	0.11	0.12 *	0.10	0.11
0.9–1.0	0.10	0.10	0.13 *	0.12 *
Control	0.10	0.10	0.10	0.10

* Indicates cytokinin activity.

Authentic kinetin: 0.1 μg = O.D. 0.12 1.0 μg = O.D. 0.40 10.0 μg = O.D. 0.68

TABLE 6

Cytokinin bioassay using excised radish cotyledons. (Mean fresh weight (mg) of cotyledon (5 reps))

	Heated chromatograms from			
	Azotobacter vinelandii		*Azotobacter beijerinckii*	
R_f values	Ethyl acetate phase	Aqueous phase	Ethyl acetate phase	Aqueous phase
0–0.1	17.40	16.20 *	12.00	15.70
0.1–0.2	16.10 *	11.30	11.20	16.30 *
0.2–0.3	13.60	11.60	12.10	12.00
0.3–0.4	13.00 *	14.40 *	15.70 *	14.75
0.4–0.5	11.60	12.00	12.40	13.00 *
0.5–0.6	13.10	12.50	12.20	12.20
0.6–0.7	13.00 *	16.20 *	17.00 *	11.80
0.7–0.8	12.10	11.20	12.60	15.00 *
0.8–0.9	11.20	17.20	12.80 *	12.60
0.9–1.0	12.00	13.00 *	12.10	12.00
Control	12.10	12.10	12.10	12.10

* Indicates cytokinin activity.

Authentic kinetin: 0.02 μg = 13.87 mg
0.20 μg = 18.08 mg
2.00 μg = 22.35 mg.

values 0 to 0.2, and 0.5 to 0.8, this latter corresponding in position to zeatin. *A. vinelandii* also gave activity at R_f 0.2 to 0.4 and *A. beijerinckii* activity at 0.9 to 1.0.

Radish cotyledons expansion test: Table 6 shows that this test gave similar responses to those obtained from the oat leaf bioassay.

The original culture supernatant was calculated to contain 0.05 μg kinetin equivalent per ml. The cytokinins produced by the two cultures of Azotobacter partitioned equally in the organic and aqueous phases.

DISCUSSION

Culture supernatants of *A. vinelandii* and *A. beijerinckii* contain at least three gibberellin-like substances, indolyl-3-acetic acid and at least three substances possessing cytokinin activity. All behave in bioassays similarly to the substances found in cultures of *A. chroococcum* and *A. paspali*, and were produced in similar quantities.

Like inocula of *A. chroococcum* [7], inocula of *A. vinelandii* and *A. beijerinckii* survive in rhizospheres of treated plants until harvest, but do decrease in number. Despite this decline, the effect on tomato growth continues until fruit set. Unlike *A. chroococum* [16] *A. vinelandii* and *A. beijerinckii* increase the number and yield of fruit It may be that because conditions in the greenhouse experiments resembled those of the subtropical area of Spain from where the bacteria were originally isolated, that activity of these cultures was particularly favoured.

Similarity of plant growth production by *A. chroococcum*, *A. vinelandii*, *A. beijerinckii*, and *A. paspali* supports the evidence of a close affinity between the species as suggested several authors [11 12 21] These authors showed that those species had closely related DNA base composition and should be grouped together being the only species to bear the generic name *Azotobacter*.

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