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Production of Plant Growth Substances by *Azotobacter chroococcum*

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SUMMARY

Cultures of *Azotobacter chroococcum* strain A6 were grown for 14 days in a nitrogen-deficient mineral medium, the supernatant fluid and bacteria extracted and examined by paper partition chromatography with two solvent systems which separate authentic gibberellin (GA 3) and indolyl-3-acetic acid (IAA). Gibberellin-like substances were not detected on the chromatograms examined under ultraviolet (u.v.) radiation, but were detected when chromatograms were cut into ten equal strips representing a sequence of R_F values and the eluates tested in dwarf pea and lettuce hypocotyl bioassays. Certain eluates applied to the roots of tomato seedlings also altered the later growth of stems, leaves and flowers. The *Azotobacter* cultures contained three gibberellin-like substances, of which probably the dominant was one with an R_F value similar to that of GA 3; the other two were not identified. The average concentration of gibberellin/ml. culture was 0.03 μ g. GA 3 equivalent. The gibberellins in *Azotobacter* cultures probably cause the reported effects on plant development and yield when seeds or roots are inoculated with *Azotobacter*. Plant growth may also be affected by synthesis of further gibberellins in the root zone when the *Azotobacter* inoculum colonizes developing roots.

INTRODUCTION

Azotobacter chroococcum has long been used in the Soviet Union to inoculate seeds or roots of crop plants, and increases in yields from this practice have been reported (Mishustin & Naumova, 1962). Recent pot trials and field trials outside the Soviet Union have also shown that frequently plant growth was altered and sometimes yield increased. Jackson, Brown & Burlingham (1964) found that inoculation with *Azotobacter* accelerated the stem and leaf growth of tomato and shortened the time between bud appearance and petal fall. Rovira (1965) found that the onset of flowering of wheat was hastened, and Denarié & Blachère (1966) that growth of potato haulms and stems of tomato was accelerated by some, but not all, strains of *Azotobacter*. These responses suggest that *Azotobacter* probably influences the development of plants by producing growth-regulating substances.

Burger & Bukatsch (1958) and Brakel & Hilger (1965) showed that *Azotobacter* produced indolyl-3-acetic acid (IAA) when tryptophan was added to the medium; Vancura & Macura (1960), Burlingham (1964) and Hennequin & Blachère (1966) found small amounts of IAA in old cultures grown without added tryptophan. Vancura

(1961) and Burlingham (1964) also detected gibberellin-like substances in old cultures, but Hennequin & Blachère (1966) did not detect these in cultures of six strains of *Azotobacter*, including three supplied by Vancura and one by Burlingham.

Brown, Jackson & Burlingham (1968) have found that after treating tomato seeds or seedling roots with small amounts (0.5–0.01 $\mu\text{g.}$) of commercially produced gibberellin GA 3, the plants responded in the same way as after treatments with 14-day cultures of *Azotobacter chroococcum* strain A 6. Treatment of seedling roots with 0.5 $\mu\text{g.}$ IAA, the concentration per ml. in *Azotobacter* cultures (Burlingham, 1964), had no effect on plant development, and adding 0.5 $\mu\text{g.}$ IAA with GA 3 had no greater effect on growth than GA 3 alone. These results indicated that the active substance in *Azotobacter* culture was a gibberellin.

The amounts of growth substances in cultures of *Azotobacter* were determined in the present work by using paper partition chromatography and bioassay techniques. Particular attention was paid to gibberellins; their activity was tested on dwarf peas, lettuce hypocotyls and tomato seedling roots. The first two bioassays are specific for the gibberellins and there is a linear relationship between log dose and log plant response (Brian & Hemming, 1955; Frankland & Wareing, 1960). Indolyl-3-acetic acid had no effect in any of these bioassays.

METHODS

Cultures. *Azotobacter chroococcum*, strain A 6, was grown for 14 days in 100 ml. medium containing (g./l.): sucrose, 5.0; K_2HPO_4 , 0.8; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04; Na_2MoO_4 , 0.005; CaCO_3 , 2.0; pH 7.0; in 500 ml. flasks on a rotary shaker incubated at 26°.

Extraction procedures. Cultures were centrifuged at 3000 rev./min. for 20 min. and supernatant fluid and sedimented bacteria extracted as follows. Analar chemicals were used unless otherwise stated.

Supernatant fluid. After acidifying with N-HCl to pH 2.8–3.0, 50 ml. of supernatant liquid was shaken with 0.5 g. activated charcoal (British Drug Houses Ltd.) for 2 hr. The charcoal was separated by centrifugation, extracted with aqueous acetone (16 ml. 95% (v/v) acetone in water), the acetone evaporated in a current of air at room temperature and the moist residue shaken twice with 1.5 times its volume of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness in cold air; the residue was dissolved in methanol (0.5 ml.) and examined by paper partition chromatography.

Sedimented bacteria. The deposit was suspended for 24 hr in aqueous acetone (10 ml. 70% (v/v) acetone in water), which was then evaporated in cold air and the extract made to known volume, acidified to pH 2.8–3.0 with N-HCl and shaken with activated charcoal. The charcoal was extracted as described above.

The bacterial fraction was also treated to release 'bound' gibberellin (McComb, 1961). After washing with distilled water, the centrifuged deposit was frozen and the bacteria disrupted in a Hughes press at -20° . The disrupted material was thawed, the volume made to 10 ml., 0.08 g. crude ficin (Koch-Light Laboratories Ltd.) and 0.12 mg. L-cysteine added, and the mixture incubated at 35° for 18 hr. The hydrolysate was then acidified to pH 3.0, activated charcoal added and after adsorption extracted as described.

Uninoculated culture medium without added sucrose was also extracted as described for the supernatant fluid fraction.

Paper partition chromatography. The different extracts were examined on Whatman no. 1 chromatography paper with two solvent systems which separate GA₃ and IAA. Control additions of 25 µg. authentic GA₃ and IAA were developed at the same time.

With solvent system A (freshly mixed isopropanol + ammonia solution sp.gr. 0.880 + water; 10 + 1 + 1 by vol.) chromatograms were equilibrated in solvent-saturated air for 1 to 2 hr and then developed with solvent descending for 16 hr until the front was about 33 cm from the origin. The chromatograms were dried in air. Strips with authentic GA₃ and IAA and one spot of extract were cut and dipped in 5% (v/v) conc. sulphuric acid in methanol, dried in hot air and exposed for 15 min. to u.v. radiation (wavelength 350 mµ). GA₃ was identified by green fluorescence at R_F 0.53 and IAA by yellow fluorescence at R_F 0.36. IAA was also identified as a pink spot when chromatogram strips were dipped in 0.05 M-FeCl₃ in 3% (v/v) conc. sulphuric acid in methanol mixture.

With solvent system B (benzene + acetic acid + water; 4 + 2 + 1 by vol.) chromatograms were equilibrated overnight with the lower phase of the solvent mixture in the bottom of the tank and then developed with the upper phase of the mixture as descending solvent for 2 hr until the front was about 25 cm. from the origin. Strips from dried chromatograms were dipped in chromogenic reagents and examined under u.v. radiation. GA₃ fluoresced at R_F 0.05 and IAA at R_F 0.55.

With solvent systems A and B the R_F values differed slightly for each development, but GA₃ and IAA were always in the same position relative to each other and never overlapped.

Chromatogram portions not treated with chromogenic reagents were dried for at least 7 days to remove solvents, cut into 10 equal strips representing the sequence of R_F values 0.1–1.0 and the strips eluted separately for bioassays.

Bioassays

Elongation of dwarf pea internodes. Chromatogram strips representing the sequence of 10 R_F values were eluted with acetone, the acetone evaporated in cold air and the residues dissolved in methanol (0.5 ml.).

Seeds of *Pisum sativum* cultivar Meteor, were germinated for 2 days on wet filter paper in Petri dishes incubated at 25°, before planting, 5 per pot, in Eff Soil-Less Compost (Eff Products Ltd., Bracknell, Berks). After 16 days in the glasshouse, plants were selected for uniformity and the distances between third and fifth nodes (interval A) measured. A minimum of ten replicates were each treated with 10 µl. of methanol extract, which was placed on the leaf subtending the third node. Distances between third and sixth nodes (interval B) were measured after 5 days. Control plants were treated with 10 µl. methanol extract of a portion of chromatogram over which only solvents had run. The increment in growth due to treatment was expressed as a percentage calculated from the equation

$$\frac{100 \{(B - A) \text{ treated} - (B - A) \text{ control}\}}{(B - A) \text{ control}}$$

A series of plants was also treated with 1.0, 0.1, 0.01 and 0.001 µg. GA₃ in methanol, as above.

Elongation of lettuce hypocotyls. Seeds of lettuce, *Lactuca sativa* cultivar Tom Thumb, were germinated for 2 days in continuous light on wet filter paper in Petri dishes incubated at room temperature (about 18°). Chromatogram strips representing the sequence of R_F values were placed on filter paper moistened with 6 ml. distilled water in 9 cm. diameter Petri dishes. Ten seedlings with roots about 5 mm. long were placed on each chromatogram strip. The seedling hypocotyls were measured after 3 days at room temperature (about 18°) in continuous light and percentage increases in growth over controls calculated. Control seedlings were placed on a portion of chromatogram over which only solvents had run. Seedlings were also placed on filter paper impregnated with 0.1, 0.25, 1.0 or 2.5 μg . GA 3.

Tomato bioassay. Tomato seeds, *Lycopersicum esculentum* cultivar Money Maker, were germinated in potting compost. When the cotyledons had expanded, seedlings were transplanted and their roots treated with 0.1 ml. of aqueous extract of test substance. Plants were graded at weekly intervals by measuring stem height and leaf length, and for flower and fruit development, as described by Brown, Jackson & Burlingham (1968). The following extracts were tested by this method. (1) Supernatant fluid fraction; the residue from ethyl acetate extract taken up in 1.0 ml. distilled water. (2) Sterile culture medium; the residue from ethyl acetate extract taken up in 1.0 ml. distilled water. (3) Acetone eluates from chromatogram strips representing a sequence of 10 R_F values; the residues were taken up in 1.0 ml. distilled water. (4) Untreated whole *Azotobacter* culture and sterile culture medium free from sucrose were also tested.

RESULTS

Detection of growth substances on chromatograms

The supernatant fluid and bacterial fractions from 50 ml. *Azotobacter* culture contained substances separated by paper chromatography with solvent system A which fluoresced under u.v. radiation at R_F 0 and R_F values 0.02 to 0.06 and 0.1 to 0.16. With solvent system B the extracts fluoresced only at R_F 0. There was no fluorescence corresponding to the positions of IAA or GA 3. Extracted culture medium did not fluoresce after separation in either solvent system.

Detection of gibberellin-like substances by pea bioassay

Supernatant fluid fraction. The supernatant fluid fractions of ten different batches of *Azotobacter* culture were extracted and separated by paper chromatography with solvent A. Figure 1 is a histogram of the growth effects on peas produced in one such experiment by the sequence of ten eluted strips corresponding to R_F values 0.1 to 1.0. In the different tests growth responses were registered by substances with R_F values 0 to 0.2, 0.4 to 0.7 and 0.7 to 1.0, showing peaks of activity at R_F values 0.15, 0.55 and 0.85. The magnitudes of the peaks ranged from (%): 12 to 68, 16 to 74 and 14 to 42, respectively. Substances with R_F values between 0.5 and 0.7 corresponded in position to authentic GA 3. Calculated from the response curve of peas to standard amounts of GA 3, the original supernatant fluid fraction contained from 0.009 to 0.1 μg . GA 3 equivalent per ml.; the amount differed between batches of cultures, but most contained 0.03 μg . GA 3 equivalent/ml. In three of the ten experiments there was also a peak of activity of 30 to 50% at R_F 0.3 to 0.4. This substance did not correspond in position on chromatograms to authentic IAA.

Eluates from chromatograms of the supernatant fluid fraction developed with

solvent system B were also tested on peas. Figure 2 shows that significant growth responses were registered by substances with R_F values 0 to 0.4, 0.5 to 0.6 and 0.7 to 0.9. Substances with R_F 0 to 0.1 corresponded in position to authentic GA₃ and in different tests gave growth increases from 10% to 30%. Calculated from the response curve of peas to standard amounts of GA₃, the original supernatant fluid fraction contained on average 0.02 μg . GA₃ equivalent/ml.

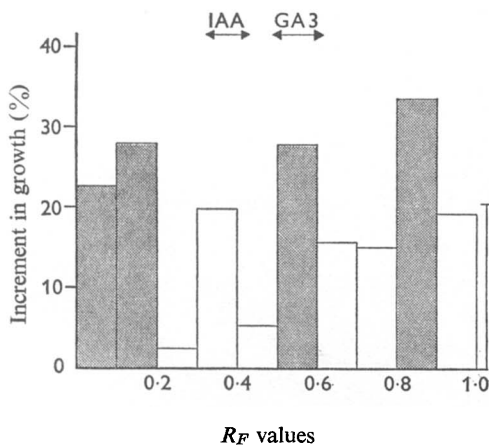


Fig. 1

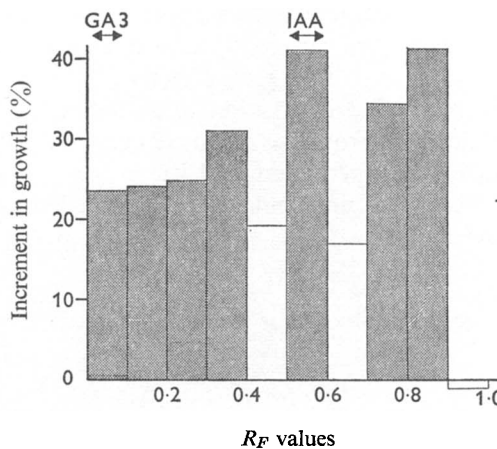


Fig. 2

Fig. 1. Effects on growth of dwarf pea (*Pisum sativum* cultivar Meteor) internodes by components of the supernatant fluid from *Azotobacter chroococcum* cultures separated by chromatography in solvent A (isopropanol + ammonia + water). Vertical line represents least significant difference, $P = 0.05$. Shaded portion represents activity significant at 5% level. Horizontal lines at top of figure represent positions of authentic gibberellin GA₃ and indolyl-3-acetic acid (IAA).

Fig. 2. Effects on growth of dwarf pea (*Pisum sativum* cultivar Meteor) internodes by components of the supernatant fluid from *Azotobacter chroococcum* cultures separated by chromatography in solvent B (benzene + acetic acid + water). Conventions as in Fig. 1.

Bacterial fraction. Figure 3 shows the effects on peas of eluates from chromatograms of acetone-extracted bacteria developed with solvent system A. Substances with growth-promoting activity occurred at R_F values 0 to 0.7 and 0.8 to 0.9; that at R_F 0.4 to 0.6 corresponded in position to authentic GA₃. Substances with R_F values 0 to 0.3 and 0.8 to 0.9 were probably the same as those in the supernatant fluid fraction which developed in the same positions. Calculated from the response curve of peas to standard amounts of GA₃, the bacteria from 1 ml. of original culture contained on average 0.01 μg . GA₃ equivalent.

Eluates from crushed and hydrolysed bacteria also produced growth responses of peas at R_F values 0.1 to 0.2, 0.5 to 0.6 (corresponding to authentic GA₃) and 0.8 to 0.9, but the crushed bacteria yielded no more gibberellin than was contained in acetone extracts. The *Azotobacter* organisms did not contain 'bound' gibberellins.

Uninoculated culture medium gave no growth response.

Lettuce bioassay

Supernatant fluid fraction. Eluted material from the supernatant fluid fraction separated by paper chromatography with solvent system A was tested by lettuce bioassay. Figure 4 shows that substances with R_F values 0 to 0.2 and 0.3 to 0.6 (corresponding in position to authentic GA 3), significantly increased growth of lettuce hypocotyls by 14% and 12%, respectively. Substances with R_F values 0.8 to 0.9 that promoted extension of dwarf peas stems were inactive on lettuce hypocotyl. Eluates from chromatograms run in solvent system B gave a growth response at R_F 0 to 0.2 with a peak of activity of 27% at R_F 0.15. Substances with R_F 0 to 0.1 corresponded in position to authentic GA 3 and gave a growth increase of 10%. Calculated from the response curve of lettuce hypocotyls to standard amounts of GA 3, the supernatant fluid fraction contained on average 0.01 μg . GA 3 equivalent/ml.

Extracted uninoculated culture medium produced no growth responses of lettuce hypocotyls.

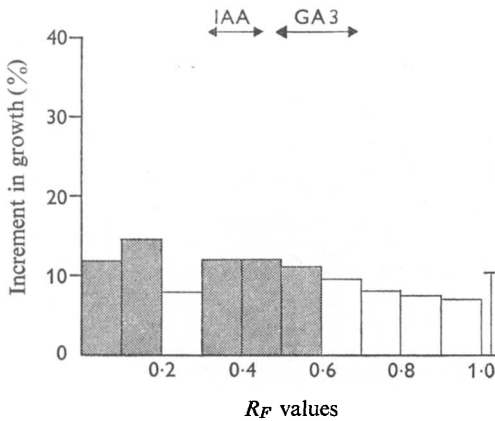


Fig. 3

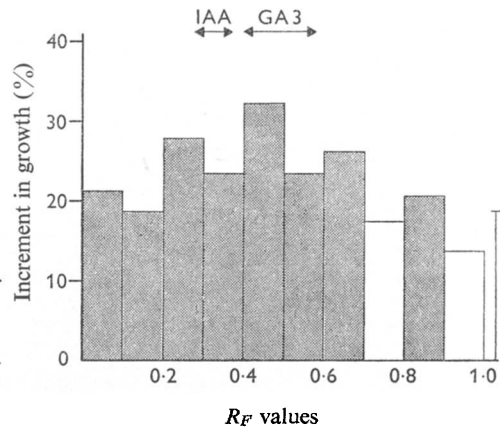


Fig. 4

Fig. 3. Effects on growth of dwarf pea (*Pisum sativum* cultivar Meteor) internodes by components of acetone-extracted *Azotobacter chroococcum*, and separated by chromatography in solvent A (isopropanol+ammonia+water). Conventions as in Fig. 1.

Fig. 4. Effects on growth of lettuce (*Lactuca sativa* cultivar Tom Thumb) hypocotyls by components of the supernatant fluid of *Azotobacter chroococcum* cultures separated by chromatography in solvent A (isopropanol+ammonia+water). Conventions as in Fig. 1.

Tomato bioassay

Whole Azotobacter culture and medium. Tomato seedling roots were treated with whole *Azotobacter* culture or with uninoculated medium. The *Azotobacter* culture significantly increased stem length and leaf size until five true leaves were formed; it also shortened the time between appearance of flower buds and petal fall, on the first truss by 4 days, and on the second by 6 days.

Supernatant fluid fraction. The supernatant fluid fraction from the *Azotobacter* culture behaved like the whole culture in increasing growth of tomato stems and leaves and shortening the time of development of the first truss by 1 day and of the second truss by 5 days.

Extracted chromatogram strips representing a sequence of ten R_F values. After eluting chromatogram strips of the supernatant fluid fraction with acetone, the dry residues were dissolved in distilled water and applied to tomato seedling roots. Material with R_F values 0.4 to 0.6 and 0.7 to 0.8 (solvent system A) significantly accelerated growth of stems and leaves until six true leaves had formed. From then until flower buds were showing, significant responses were caused only by substances with R_F values 0.4 to 0.6. Table 1 shows that substances with R_F values 0 to 0.1, 0.4 to 0.6 and 0.8 to 1.0 shortened, and those with R_F 0.1 to 0.4 and 0.6 to 0.8 lengthened, the time between flower bud appearance and petal fall of the first truss. Only substances with R_F values 0.4 to 0.6 shortened the time of development of the second truss. It was calculated that eluted substances with R_F values 0.4 to 0.6 contained 0.02 μg . GA₃ equivalent/ml.

Table 1. *Effect on truss development in *Lycopersicum esculentum* cultivar Money Maker of growth substances in *Azotobacter* culture supernatant fluid separated on chromatograms*

	Position (R_F) of supernatant fluid fraction on chromatogram									
	0-0.1	0.1-0.2	0.2-0.3	0.3-0.4	0.4-0.5	0.5-0.6	0.6-0.7	0.7-0.8	0.8-0.9	0.9-1.0
	Truss development shortened (-) or lengthened (+) (days)									
1st truss	-3	+1	+5	+1	-3	-3	+2	+1	-4	-4
2nd truss	0	0	0	0	-4	-3	0	0	0	0

Table 2. *Effects of substances in *Azotobacter* supernatant fluid on stem and leaf growth of *Lycopersicum esculentum* cultivar Money Maker at different stages of development*

R_F value of substance (solvent system B)	No. of leaves formed	Accelerated growth	
		Height	Leaf
0-0.1	2	+	-
0.1-0.2		+	-
0.9-1.0		+	+
0.1-0.2	4	+	+
0.3-0.4		+	-
0.4-0.5		+	-
0.9-1.0		+	-
0.1-0.2	6	+	-
0.3-0.4		+	-

Several substances in eluates from chromatograms developed with solvent system B significantly affected stem and leaf growth of tomato at different stages of plant development. Table 2 shows that substances with R_F values 0.1 to 0.2 accelerated stem growth until six true leaves had formed. The substance corresponding in position to authentic GA₃ (R_F 0.05) accelerated internode growth of young plants only. When developed with solvent system A, substances in the same position as GA₃ were active to truss development. This difference might be caused by phenolic acids interfering in the tomato bioassay because these acids also developed R_F values 0 to 0.1 in solvent system B.

Identification of gibberellin-like substances in the supernatant fluid fraction of Azotobacter cultures

Substances with R_F values 0.1 to 0.2, 0.5 to 0.6 and 0.8 to 0.9 separated with solvent system A were eluted and then developed on paper chromatograms with solvent system B. After development the chromatograms were cut into ten equal strips and each eluted and tested on dwarf peas. Table 3 shows the position of substances that significantly increased growth.

Table 3. *Substances in Azotobacter supernatant fluid which affected the extension of dwarf pea (Pisum sativum cultivar Meteor) internodes (R_F position)*

R_F in solvent A	R_F in solvent B
0.1 to 0.2	0 to 0.1
0.5 to 0.6	0 to 0.1
0.8 to 0.9	0 to 0.1, 0.5 to 0.6, 0.7 to 0.8

Table 4. R_F values of different gibberellins in two solvent systems

Gibberellin	Solvent system	
	A	B
	Isopropanol + ammonia + water	Benzene + acetic acid + water
	R_F value	
A 1	0.55	0.05
A 4	0.67	0.67
A 5	0.61	0.51
A 7	0.70	0.65
A 8	0.42	0.0
A 9	0.72	0.90

Material eluted at R_F 0.5 to 0.6 in solvent system A developed at R_F 0 to 0.1 in solvent system B, that is, in the position of GA 3 in both solvent systems and so was probably GA 3 or a very closely related substance such as GA 1 which also developed in the same positions in both solvent systems (Brian, Grove & MacMillan, 1960). Material eluted at R_F 0.1 to 0.2 in solvent system A developed in the position of GA 3 in solvent system B, and again was probably closely related to this compound. The material at R_F 0.8 to 0.9 in solvent system A separated into three components when developed in solvent system B, one with the R_F value of GA 1 or GA 3, and two unidentified gibberellin-like substances. Cavell, MacMillan, Pryce & Sheppard (1967) identified 17 gibberellins but only six were available for tests in the present work. These latter were separated by paper chromatography with the solvent systems A and B and compared with the unknown gibberellin-like substances from *Azotobacter*. Table 4 shows the R_F values of the authentic gibberellins. The unidentified active components in *Azotobacter* supernatant fluid fraction could not be related to the authentic compounds. Thus *Azotobacter* produced three growth-promoting substances which were gibberellin-like in character in that they induced shoot elongation of dwarf mutant plants whose growth responses are thought to be specific to the gibberellins (Phinney & West, 1960). One substance resembled GA 1 or GA 3; the other two were not identified.

DISCUSSION

Of the three gibberellin-like substances detected in the present work in cultures of *Azotobacter chroococcum* strain A6, the one with the same R_F value as GA 1 or GA 3 was probably the most important. Although the amount was too small to detect by fluorescence on paper chromatograms, bioassays readily detected it and suggested that the concentration in 14-day cultures ranged between 0.01 and 0.1 μg . GA 3 equivalent/ml. This amount of gibberellin-like substance was seemingly enough, when an inoculum of *Azotobacter* was added to seeds or roots, to alter the later development of tomato plants, possibly because it was taken up by the seedlings at a critical stage of development, when vegetative and reproductive primordia were differentiating. However, not all the gibberellin taken up by the seedlings may have come from the initial inoculum, for gibberellins may have continued to be synthesized for a short period when the roots were being colonized by the *Azotobacter* inoculum which moved from the seed to the germinating root and multiplied (Jackson & Brown, 1966). Only 14-day *Azotobacter* cultures grown in a nitrogen-deficient mineral medium have so far been studied; it has yet to be determined whether the conditions of cultivation affect the production of gibberellins by *Azotobacter*.

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