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 (GA3) 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 GA3; the other two were not identified. The average concentration of gibberellin/ml. culture was 0.03 pg. GA3 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
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(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 (0.05-0.01 μg.) of commercially produced gibberellin GA3, the plants responded in the same way as after treatments with 14-day cultures of Azotobacter chroococcum strain A6. Treatment of seedling roots with 0.5 μg. IAA, the concentration per ml. in Azotobacter cultures (Burlingham, 1964), had no effect on plant development, and adding 0.5 μg. IAA with GA3 had no greater effect on growth than GA3 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 A6, was grown for 14 days in 100 ml. medium containing (g./l.): sucrose, 5.0; K2HPO4, 0.8; MgSO4·7H2O, 0.2; FeSO4·7H2O, 0.04; Na2MoO4, 0.005; CaCO3, 2.0; pH 7.0; in 500 ml. flasks on a rotary shaker incubated at 26°C.

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°C. 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°C for 18 hr. The hydrolysate was then acidified to pH 3.0, activated charcoal added and after adsorption extracted as described.
Growth substances produced by Azotobacter

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 GA 3 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 RF 0.53 and IAA by yellow fluorescence at RF 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 RF 0.05 and IAA at RF 0.55.

With solvent systems A and B the RF 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 RF 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 RF 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

\[
100 \left( \frac{(B - A) \text{ treated} - (B - A) \text{ control}}{(B - A) \text{ control}} \right).
\]

A series of plants was also treated with 1·0, 0·1, 0·01 and 0·001 μg. GA 3 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*ₚ 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 GA₃.

**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*ₚ 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*ₚ 0 and *R*ₚ values 0·02 to 0·06 and 0·1 to 0·16. With solvent system B the extracts fluoresced only at *R*ₚ 0. There was no fluorescence corresponding to the positions of IAA or GA₃. 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*ₚ values 0·1 to 1·0. In the different tests growth responses were registered by substances with *R*ₚ values 0·0 to 0·2, 0·4 to 0·7 and 0·7 to 1·0, showing peaks of activity at *R*ₚ 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*ₚ values between 0·5 and 0·7 corresponded in position to authentic GA₃. Calculated from the response curve of peas to standard amounts of GA₃, the original supernatant fluid fraction contained from 0·009 to 0·1 μg GA₃ equivalent per ml.; the amount differed between batches of cultures, but most contained 0·03 μg GA₃ equivalent/ml. In three of the ten experiments there was also a peak of activity at 30 to 50% at *R*ₚ 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 \( \mu g \) GA3 equivalent/ml.

**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 \( \mu g \) 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.

**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.
Growth substances produced by *Azotobacter*

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

<table>
<thead>
<tr>
<th>Position ($R_F$) of supernatant fluid fraction on chromatogram</th>
<th>Truss development shortened (−) or lengthened (+) (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>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</td>
<td>1st truss  2nd truss</td>
</tr>
<tr>
<td>−3 +1 +5 +1 −3 −3 +2 +1 −4 −4</td>
<td>0 0 0 0 0 0 0 0 0 0</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>$R_F$ value of substance (solvent system B)</th>
<th>No. of leaves formed</th>
<th>Accelerated growth Height</th>
<th>Leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-0.1</td>
<td>2</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>0-1-0.2</td>
<td>2</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>0-9-1.0</td>
<td>2</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>0-1-0.2</td>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0-3-0.4</td>
<td>4</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>0-4-0.5</td>
<td>4</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>0-9-1.0</td>
<td>6</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>0-1-0.2</td>
<td>6</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

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 RF 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 (RF position)

<table>
<thead>
<tr>
<th>RF in solvent A</th>
<th>RF in solvent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 to 0.2</td>
<td>0 to 0.1</td>
</tr>
<tr>
<td>0.5 to 0.6</td>
<td>0 to 0.1</td>
</tr>
<tr>
<td>0.8 to 0.9</td>
<td>0 to 0.1, 0.5 to 0.6, 0.7 to 0.8</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isopropanol + ammonia + water</td>
<td>Benzene + acetic acid + water</td>
</tr>
<tr>
<td>Gibberellin</td>
<td>RF value</td>
<td>RF value</td>
</tr>
<tr>
<td>A1</td>
<td>0.55</td>
<td>0.05</td>
</tr>
<tr>
<td>A4</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>A5</td>
<td>0.61</td>
<td>0.51</td>
</tr>
<tr>
<td>A7</td>
<td>0.70</td>
<td>0.65</td>
</tr>
<tr>
<td>A8</td>
<td>0.42</td>
<td>0.0</td>
</tr>
<tr>
<td>A9</td>
<td>0.72</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Material eluted at RF 0.5 to 0.6 in solvent system A developed at RF 0 to 0.1 in solvent system B, that is, in the position of GA3 in both solvent systems and so was probably GA3 or a very closely related substance such as GA1 which also developed in the same positions in both solvent systems (Brian, Grove & MacMillan, 1960). Material eluted at RF 0.1 to 0.2 in solvent system A developed in the position of GA3 in solvent system B, and again was probably closely related to this compound. The material at RF 0.8 to 0.9 in solvent system A separated into three components when developed in solvent system B, one with the RF value of GA1 or GA3, 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 RF 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 GA1 or GA3; the other two were not identified.
DISCUSSION

Of the three gibberellin-like substances detected in the present work in cultures of Azotobacter chroococcum strain 36, the one with the same $R_F$ value as GA1 or GA3 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. GA3 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.

Thanks are due to Mrs A. Shepherd-Smith for excellent technical assistance.

REFERENCES


