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The phytohormonal interactions between *Azospirillum* and wheat

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Abstract

A simple model system was designed to detect positive effects of *Azospirillum* on the root growth of cereals. Cultures of *A. brasilense* Sp7 and *A. lipoferum* Sp59 did not excrete gibberellins and cytokinins in the logarithmic and in the early stationary growth phase. Indoleacetic acid (IAA) was formed, however, only in the stationary phase of the cultures. The addition of D,L-tryptophan to the medium enhanced the formation of IAA. A further, still unidentified substance was produced by *Azospirillum* under denitrifying conditions in the logarithmic growth phase. The substance was almost twice as active as IAA in increasing the wet weight of wheat root segments. It is suggested that this unidentified substance is the major stimulus affecting the growth of cereals.

Introduction

There is currently much interest in soil bacteria of the genus *Azospirillum*. *Azospirillum* is not a major constituent of soils of the temperate zone where the genera *Pseudomonas* and *Enterobacter* are more dominant (Lindberg and Granhall, 1984; Kleeberger *et al.*, 1983). For tropical soils, however, numbers as high as 10^4–10^7 *Azospirillum* cells g^{-1} dry weight of roots have been determined. *Azospirillum* lives in association with grasses (wheat, corn, rice, sugar cane, *Sorghum*). It seems to colonize the inner cortex of the roots, the root cap and/or a zone a few mm away from the root surface (Döbereiner, 1983b; Elmerich, 1984; Okon, 1985; Whallon *et al.*, 1985).

Azospirillum is thought to improve the nitrogen supply to cereals by means of N_2-fixation and thereby to enhance crop productivity. However, *Azospirillum* can perform N_2-fixation only under microaerobic conditions. A direct transfer of products of N_2-fixation (NH_4^+, glutamate, glutamine or other compounds) from *Azospirillum* to plants has never been demonstrated. *Azospirillum* could have a shorter life span than the cereals, and could perhaps decompose before the grain-filling stage and so improve crop productivity by its mineralization. However, most investigators agree that the number of *Azospirillum* cells in roots, though considerable, is too small to enhance crop yield significantly by means of N_2-fixation and mineralization (Zimmer *et al.*, 1987).

Azospirillum also deserves special attention because of its denitrification properties (Bothe *et al.*, 1981; Nelson and Knowles, 1978). When oxygen is limiting in cultures, *Azospirillum* can utilize either nitrate, nitrite or nitrous oxide as terminal respiratory electron acceptors. Experimental conditions were established for both batch and continuous cultures where the bacterium anaerobically reduced nitrate to nitrite, nitrous oxide or molecular nitrogen, nitrite to nitrous oxide or molecular nitrogen, and nitrous oxide to molecular nitrogen. Growth yield measurement indicated that the energy yields are comparable between O_2 and N_2O as respiratory electron acceptors. They are 1/3 lower with nitrite and 2/3 lower with nitrate than with O_2 (Danneberg *et al.*, 1985; Zimmer *et al.*, 1987). Model experiments with wheat indicated that *Azospirillum* performs either nitrogen fixation

* Dedicated to Professor E.-G. Niemann, Hannover, on the occasion of his 60th birthday.

(C_2H_2-reduction) or denitrification (N_2O-formation) depending on the assay conditions (Neuer *et al.*, 1985). *Azospirillum* may contribute significantly to NO_x production in soils particularly when the concentration of nitrate is high and that of O_2 is limiting.

Azospirillum also produces phytohormones and this production may enable *Azospirillum* to enhance plant growth even when occuring in small quantities in roots. Tien *et al.*, (1979) showed that *Azospirillum* produced indoleacetic acid (IAA) and indolelactic acid when the medium contained tryptophan. The authors also stated that the bacterium produced small amounts of gibberellins and three cytokinin-like compounds. These compounds remained to be identified. Tien *et al.*, (1979) showed that the phytohormones produced by *Azospirillum* stimulated the formation of additional root hairs and of lateral roots with pearl millet (*Pennisetum americanum*). Hartmann *et al.*, (1983) isolated mutants of *A. brasilense* Cd which formed thirty times more IAA than the wild strain. Some mutants also produced IAA independently of tryptophan in the medium. The effect of *Azospirillum* on the formation of root hairs and lateral roots is due not only to IAA but, probably, to still unidentified phytohormones. Such a conclusion follows from the experiments of Kapulnik *et al.*, (1985) who showed that the overproducing mutants of *A. brasilense* Cd had the same effects on root hair and lateral root formation as the wild strain.

In the current study, cultures of the two type strains, *A. brasilense* Sp7 and *A. lipoferum* Sp59, were assayed for phytohormone production. The medium in which *Azospirillum* had lived was tested for gibberellin, cytokinin and indoleacetic acid content using classical plant assays for these phytohormones. In addition, a simple assay was designed to detect stimulatory effects of bacteria on the root growth of cereals.

Materials and methods

Organisms and growth

The two *Azospirillum* strains used, *Azospirillum brasilense* Sp7 (ATCC 29145) and *A. lipoferum* Sp59 (ATCC 29707), were grown in 100 ml and 1 l flasks in a shaking water bath (60 strokes min^{-1}) at 30°C for 24 h under batch culture conditions and in a medium containing (gl^{-1} water): K_2HPO_4, 0.78; KH_2PO_4, 0.61; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.00625; EDTA (= Titriplex III), 0.0093; $Na_2MoO_4 \cdot 2H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.01; malate (adjusted to pH 7.0 with KOH), 5.0; KNO_3, 2.0. In the cultures grown with NH_4^+ as the nitrogen source, KNO_3 was replaced by 0.265 $g\,l^{-1}$ NH_4Cl. The *A. lipoferum* cultures were supplemented with a sterile solution of biotin (10^{-5} $g\,l^{-1}$). The cultures had an optical density of 1.0–1.5 at 560 nm after 24 h of growth under sterile conditions.

Assay to test the stimulatory effects of Azospirillum *on root growth*

A surface sterilized (see Neuer *et al.*, 1985) grain of spring wheat (variety Ralle) was placed in an Eppendorf plastic tube of which the tip had been excised. The tube was placed in a 25 ml test tube on top of 10 ml of the wheat medium described by Neuer *et al.*, (1985) containing 3.5 m*M* $NaNO_3$, but no agar in this case. In the experiments with *Azospirillum* (see Fig. 1), the test tubes were supplemented with 10 μl of a 1-day-old culture of *A. brasilense* Sp7 with an optical density of 1.43 at 560 nm. All manipulations were performed under sterile conditions. After incubating at 23°C for 10 d (14 h in the light, 10 h in the dark per day), the dry weight of the excised roots was determined.

Phytohormone tests

Assay for cytokinins. The *Amaranthus* test used is based on the cytokinin-induced formation of betacyanins in the cotyledons and hypocotyls of this plant incubated in the dark in the presence of tyrosine, as described by Biddington and Thomas (1973). Seeds of *Amaranthus caudatus* (C. Walz Seed Co., Stuttgart-Feuerbach) were sown in 10 cm × 2 cm Petri dishes on a double layer of filter paper moistened with 5 ml distilled H_2O and incubated for 4 d in the dark at 25°C. The seed coats and the roots were then removed, and the upper part of each germinated plant, consisting of the hypocotyl plus the cotyledons (10 pieces), were placed on a double layer of filter paper in a 25 ml Erlenmeyer flask. The filter paper was moistened

with 2 ml of a solution containing 13.3 m*M* potassium phosphate buffer pH 8.0, 2 mg tyrosine and the cytokinin solution to be assayed. The Erlenmeyer flasks were incubated at 25°C for 18 h in the dark and the *Amaranthus* samples were then removed and transferred to 3 ml of distilled water. The samples were frozen and re-thawed twice, and the quantity of betacyanin released was determined by calculating the differences between the absorbances at 542 nm and 620 nm which correlate with the cytokinin concentration. The detection limit was 0.5 ng cytokinin ml^{-1} *Azospirillum* culture.

Assay for auxins. The cell elongation of wheat root segments was shown to be dependent on the presence of indoleacetic acid and can be taken as a test for determining the amount of auxin in a solution (Libbert, 1957). Spring wheat grains (var. Ralle) were watered with 10 ml H_2O for 24 h at 4°C in a Petri dish. For germination, they were placed on two layers of filter paper moistened with 6 ml H_2O in a Petri dish and incubated for 72 h at 26°C in the dark. Twelve wheat root segments of 5 mm length, taken from the zone 2–7 mm away from the tip, were transferred to a Petri dish containing one layer of filter paper moistened with 2.5 ml of the solution to be assayed for auxin content. The segments were incubated for 24 h at 26°C in the dark, and the wet weight of the root segments was then determined. This test is a direct assay of the effect of auxins on the growth of roots with a detection limit of 1 pg indoleacetic acid ml^{-1} of culture and gives statistically sound data.

Assay for gibberellins. The method described by Jones and Varner (1967) was used as the bioassay for gibberellins. Barley seeds were cut in halves transversely. The embryo was removed and the halves were sterilized with an 1% NaOCl solution for 5 min under vacuum, followed by three washings in sterile distilled water. The sterilized halves were incubated for 4 d in sterile Petri dishes with 4 ml H_2O at 4°C in the dark. Ten half seeds for each vessel were transferred to autoclaved 7.2 ml Fernbach flasks each containing 2 ml 1 m*M* sodium acetate buffer pH 4.8, 10 m*M* $CaCl_2$ and the gibberellin solution to be tested. After incubation of this mixture for 48 h at 30°C in a shaking water bath, the medium was decanted into centrifuge tubes, and the Fernbach flasks were washed with 2 ml distilled water. The combined solution (medium and washing) was centrifuged (10 min, 10,000 g). The assay for α-amylase activity in the supernatant could be performed in the same way as by Jones and Varner (1967), but it was easier to adopt the procedure described in the Sigma diagnostic leaflet no. 576. In this, 0.6 ml of the Sigma reaction mixture containing mainly *p*-nitrophenyl-D-maltoheptaoside as substrate and 0.2 ml of the supernatant were mixed. After 1 min, the formation of *p*-nitrophenol was monitored by reading the absorbance changes at 420 nm. This gibberellin-induced release of α-amylase allows a determination of 25 pg gibberellin ml^{-1} of culture as the detection limit.

Separation of the auxins by HPLC

The separation was performed by the use of a Novapak C_{18} (0.8 × 10 cm) reversed phase column with 5 μm particle size using a Waters model 510 HPLC. The solvent was methanol/1% H_3PO_4 in water = 40/60(v/v), the flow rate was 1 ml min^{-1} and the injection volume was 25 μl. The peak areas (u.v.-detector at 278 nm) were calculated by using an arbitrary unit for the unidentified substance (see Results). One peak area unit corresponded to 1/4096 optical density unit at 278 nm × 1/20 sec. The unidentified substance had a retention time of 162 sec and IAA had 306 sec under these conditions. IAA could be quantified by standards.

Results

A new test to assay the effects of Azospirillum on root growth

Tien *et al.*, (1979) reported that the root system of pearl millet responded to inoculation of *A. brasilense* Sp13t SR2 by forming additional root hairs and lateral roots. The same effect can also be seen in a simple and convenient test system described in detail under Materials and methods. Figure 1 indicates that 10-d-old wheat plants had formed much longer roots, and more root hairs and lateral roots after the incubation with *Azospirillum* in a medium containing nitrate. These assays were performed with either *A. brasilense* Sp7 or *A.*

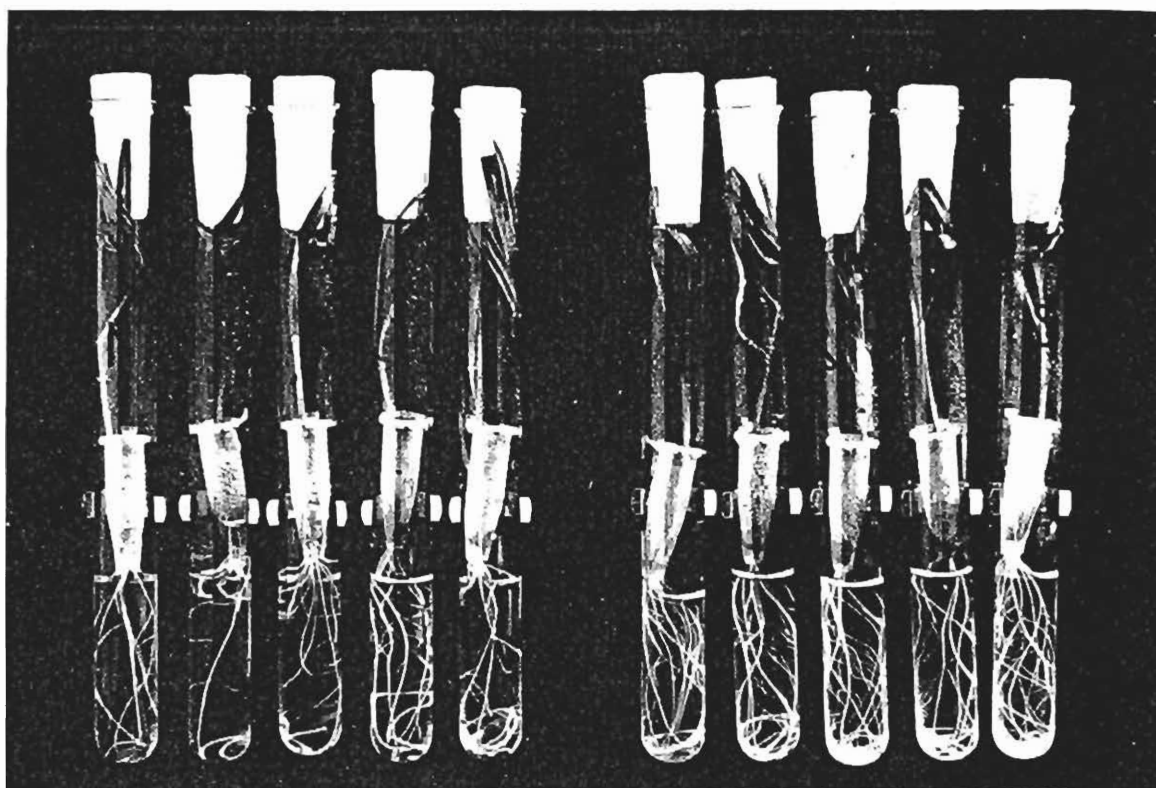

Fig. 1. The stimulatory effect of *Azospirillum* on the growth of wheat roots.
A wheat plant was grown for 10 d in the presence of *A. brasilense* Sp7 (5 test tubes at the right side of the photo). The controls without *A. brasilense* are the 5 tubes at the left side. For experimental details see Materials and methods.

Table 1. Test for cytokinins in the supernatant of *Azospirillum*

Assay conditions	Absorbance difference $E_{542}-E_{620}$
1. control without cytokinin	0.024
2. control with	
a) 0.2 ng kinetin assay	0.036
b) 200 ng kinetin assay	0.060
c) 2000 ng kinetin assay	0.123
3. supernatant of *A. brasilense* Sp7	
a) of an 1-day-old culture	0.023
b) of a 3-days-old culture	0.024
4. supernatant of *A. lipoferum* Sp59	
a) of an 1-day-old culture	0.024
b) of a 3-days-old culture	0.022

The test for cytokinin was performed by measuring the production of betacyanin in seedlings of *Amaranthus caudatus*. The standard was kinetin (6-furfurylaminopurine, Sigma no. K 2751). Both *Azospirillum* cultures had O.D.$_{560\,nm}$ = 1.3 after 1 d and 1.5 after 3 d. The cultures were centrifuged, and 0.2 ml of the supernatant was assayed for cytokinin content. The method would have allowed detection of 100 pg cytokinin 0.2 ml^{-1}. Thus the cultures contained less than 0.5 ng cytokinin ml^{-1}.

lipoferum Sp59 with the same results. The dry weight of roots excised afterwards gave 8.55 ± 1.74 mg for 5 plants incubated in the presence of *Azospirillum brasilense* for 10 d, and 6.30 ± 0.74 mg for 5 plants grown without the bacteria for the same time. Using the Student *t*-test, the confidence in the difference of these data was 98.9%.

Assays for cytokinins and gibberellins

The *Amaranthus* assay was chosen for determining the amount of cytokinins by *Azospirillum*. Cytokinin induces the formation of betacyanin in the cotyledons and hypocotyls of this plant. Betacyanin can easily be extracted and measured quantitatively with high sensitivity. Table 1 indicates that betacyanin formation by the plants was, indeed, dependent on the addition of low amounts of kinetin (6-furfurylaminopurine) as a control.

Table 2. Test for gibberellins in the supernatant of *Azospirillum*

Assay conditions	$\Delta E_{420\,nm}$/min
1. control without gibberellins	0.000
2. control with 10 pg GA_3/assay	0.015
3. control with 1000 pg GA_3/assay	0.273
4. supernatant of *A. brasilense* Sp7	
of an 1-day-old culture	0.000
of a 3-days-old culture	0.000
5. supernatant of *A. lipoferum* Sp59	
of an 1-day-old culture	0.000
of a 3-days-old culture	0.000

The test for gibberellins was performed with 10 sterilized endosperm halves of barley seeds. Both *Azospirillum* cultures were grown aerobically with nitrate as the nitrogen source and had an $O.D._{560\,nm}$ of 1.3 after 1 d and of 1.5 after 3 d. The cultures were centrifuged, and 0.2 ml of the supernatant was assayed for gibberellin content. The method would have allowed to detect 5 pg gibberellin 0.2 ml^{-1}. Thus the cultures contained less than 25 pg gibberellin ml^{-1}.

There were, however, no indications for the release of cytokinins into the medium by *Azospirillum brasilense* Sp7 and *A. lipoferum* Sp59 when either 1- or 3-d-old cultures were assayed (Table 1).

The production of α-amylase in barley endosperm is a sensitive bioassay for gibberellins. This test has the advantage of correlating the concentration of gibberellin with the activity of an enzyme which is released specifically upon the addition of this phytohormone. The test is also insensitive to solvents (Jones and Varner, 1967). Table 2 indicates that the addition of GA_3 as a control, indeed resulted in the formation of high α-amylase activity. In contrast, the medium of either 1- or 3-d-old *Azospirillum* cultures did not contain detectable levels of gibberellins.

Auxin formation by Azospirillum

Azospirillum brasilense Sp7 had a generation time of 5.5 h under the growth conditions of the present study and the stationary phase was reached within 1 d (Fig. 2). An excretion of IAA was not detectable until 3 d after inoculation. The maximal production was 15 μM IAA after 4–5 d in this culture which then had an optical density of 1.5 at

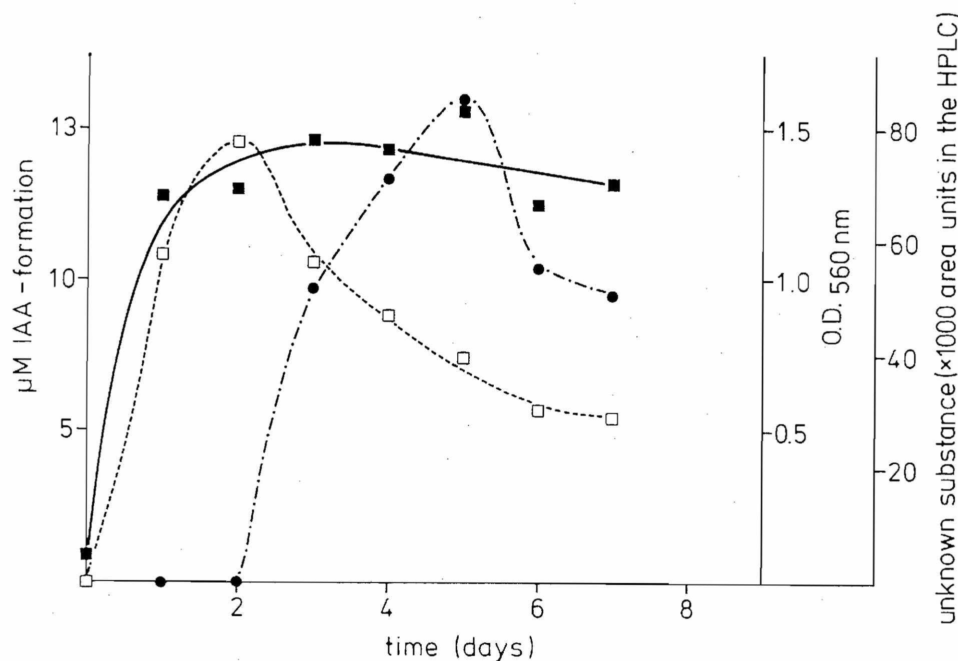

Fig. 2. The formation of IAA and of the unidentified substance by *A. brasilense* Sp7 under batch culture conditions.
■——■ growth of the culture ($O.D._{560\,nm}$); ●-·-·-● IAA-formation; □- - - -□ formation of the unknown substance. The experimental details are described under Materials and methods.

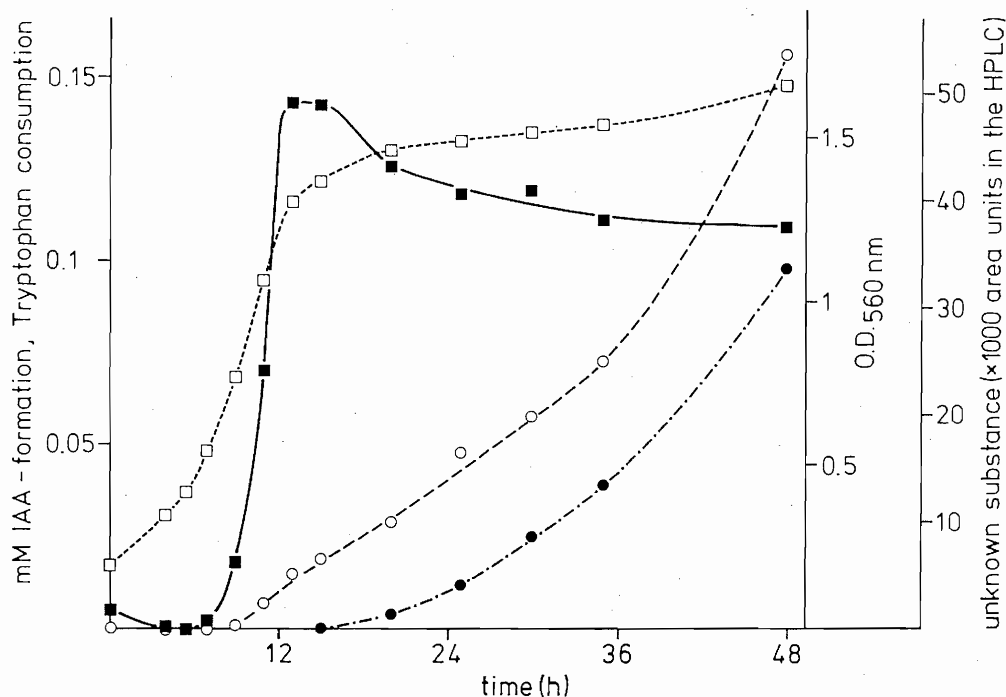

Fig. 3. The formation of IAA and of the unidentified substance by *A. brasilense* Sp7 under batch culture conditions grown in the presence of tryptophan. □----□ growth of the culture; ●-·-·-● IAA-formation; ■——■ formation of the unknown substance. ○---○ consumption of tryptophan
The *Azospirillum* medium was supplemented with D,L tryptophan (100 mg l^{-1}) for this experiment.

560 nm (corresponding to *ca.* 8.3 × 10^8 cells ml^{-1}). After that time the amount of IAA in the medium decreased indicating that the cells may then have re-utilized this phytohormone in the late stationary phase when nutrients became severely limiting. Figure 2 also indicates that the cultures excreted a still unidentified substance which was effective as a phytohormone (see below). The production of this compound paralleled growth of the culture and its concentration decreased significantly with the onset of the stationary phase.

The addition of D,L-tryptophan (100 mg l^{-1}) to the medium did not significantly enhance the growth rate of the cultures and did not extend the length of the logarithmic phase (Fig. 3). However, the cells produced more IAA, and this production paralleled the consumption of the amino acid. After 2 d, the medium contained 0.1 m*M* IAA and thus 6–8 times more than was produced maximally by a culture grown without tryptophan. The formation of the unidentified compound was independent of the presence of tryptophan in the medium, as can be seen from a comparison of Figs 2 and 3. The extent of its excretion was reduced by increasing the shaking rate of the culture vessel (not documented). The unidentified compound was produced when the cells were grown on nitrate as the nitrogen source and could not be detected in NH_4^+ grown cultures. In contrast, IAA was produced independently of the N-source in the medium (not documented). The experiments on auxin production and the formation of the unidentified compound gave the same results with *A. lipoferum* Sp59 as with *A. brasilense* Sp7.

Excised roots of wheat seedlings respond positively to the addition of exogenous IAA by an increase in weight (Libbert, 1957) and by the formation of additional lateral roots (Blakely *et al.*, 1986). The addition of IAA largely enhanced the wet weight of wheat root segments under the con-

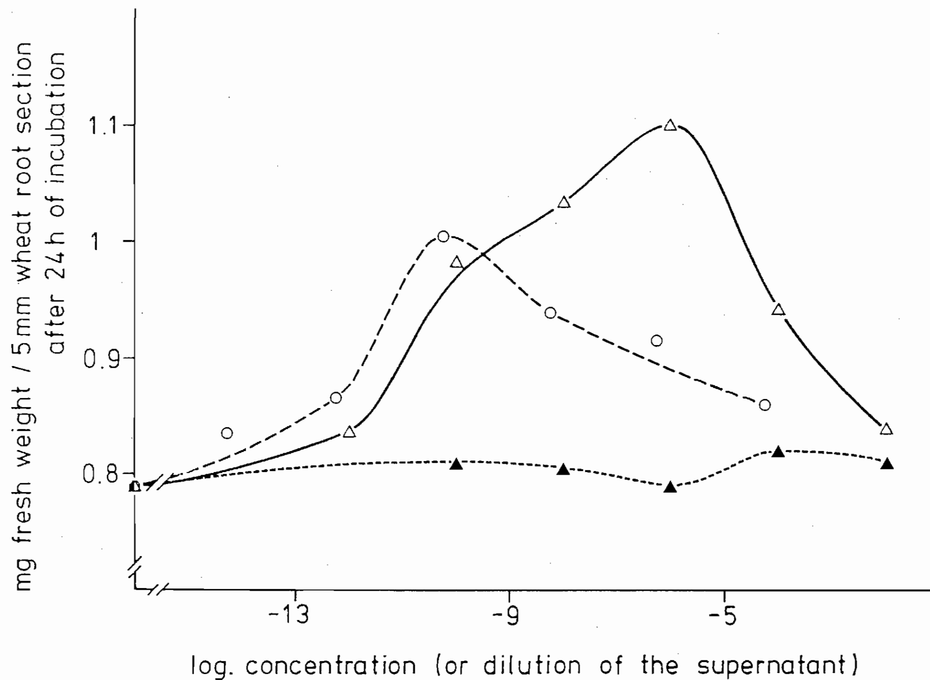

Fig. 4. Effects of IAA and of the unknown substance on wheat root segments.
○– – –○ increase of the wet weight by IAA (IAA concentration in *M*); △——△ increase by the supernatant (obtained by growing *A. brasilense* Sp7 for 1 d in the *Azospirillum* medium and in the presence of NO_3^- and by removing the cells by centrifugation); ▲- - - -▲ supernatant of a culture grown in the presence of NH_4^+.

ditions employed in the present study. A curve with a pronounced optimum and with higher concentrations being inhibitory is typical for a phytohormonal response, and this was also obtained in Fig. 4 by using either IAA or the unidentified substance. Remarkably, the unidentified compound was almost twice as active as IAA in this assay. It was formed only when the cells were grown with nitrate as the nitrogen source in the medium. Supernatant from NH_4^+-grown cells completely failed to stimulate the growth of wheat root segments (Fig. 4).

Discussion

The simple test designed in the present study to detect stimulatory effects of *Azospirillum* on the growth of wheat roots gives impressive results. Profound differences between plants inoculated with and without bacteria can be seen even by eye after a week. Thus this test confirms the reports about positive effects of *Azospirillum* on the growth of plants published by others (Döbereiner, 1983; Elmerich, 1984; Kapulnik *et al.*, 1985; Okon, 1985). This rapid test may also replace more complicated assays described earlier (Tien *et al.*, 1979; Thomas-Bauzon *et al.*, 1982; Neuer *et al.*, 1985). It might also be suitable for the assay of many kinds of associations between plants and bacteria.

The present study shows that *A. brasilense* Sp7 and *A. lipoferum* Sp59 do not produce gibberellins and cytokinins in the logarithmic and early stationary growth phase. The bioassays used here allowed the detection of 0.5 ng cytokinin ml^{-1} and 25 pg gibberellin ml^{-1} culture. It could well be that *Azospirillum* had produced the phytohormones in concentrations under these detection limits. The radioimmunoassay for phytohormones as a more sensitive assay (Weiler, 1984) was not tried in the present study, because very low production might be insufficient to have a stimulatory effect on the root growth of cereals even when the population of bacteria in the roots is dense. In the late stationary

phase or when the cultures are dying, *Azospirillum* and any other bacterium may produce cytokinin as a degradation product of DNA or RNA and may possibly also form gibberellins. This was not investigated in this study, because such conditions can hardly be controlled in any association between bacteria and cereals.

The results of this communication are contradictory to those of Tien *et al.*, (1979) who reported the excretion of gibberellins and cytokinins by *Azospirillum*. Several facts may explain this discrepancy. Tien *et al.*, (1979) used a different strain which grew very slowly. The stationary phase of their culture was not reached before 10 d (see Fig. 7 in Tien *et al.*, 1979), and the cell density was only 1/3 of that obtained with the cultures used here. The formation of phytohormones by *Azospirillum* may be strain specific and growth-phase dependent as noted for auxins (Hartmann *et al.*, 1983). Tien *et al.*, (1979) did not identify the gibberellin-like substance. As the bioassay for gibberellins, they chose the increase of the growth of lettuce hypocotyls which was described to be gibberellin specific (Frankland and Warening, 1960). However, elongations of hypocotyls are often also stimulated by auxins. In the present study, therefore, the gibberellin dependent release of α-amylase activity in barley endosperms was chosen as specific assay where gibberellin cannot be substituted by other phytohormones. Tien *et al.*, (1979) also did not identify the cytokinins, and almost all *n*-butanol fractions were stimulatory in the chlorophyll retention assay which they chose for assaying these plant growth substances. The tests used in the present study allowed us to detect 1/2000 of the gibberellin and 1/2 of the cytokinin found in the medium of *A. brasilense* Sp13t SR2 by Tien *et al.*, (1979). Since we could not find any cytokinin and gibberellin in our cultures, we did not attempt to purify these compounds. The tests in the present study were performed directly with the medium in which *Azospirillum* had lived. Control experiments indicated that the salts of the medium did not interfere with the bioassays.

The present communication confirms that *A. brasilense* Sp7 produces IAA. Significant amounts are, however, only formed in the late stationary growth phase. Within a detection limit of 1 pg ml^{-1} culture, there was no evidence for the phytohormone production in the logarithmic phase (Fig. 2) nor in continuous cultures (unpublished results). The present communication confirms earlier reports that *Azospirillum* forms IAA in larger quantities when the medium is supplemented with tryptophan (Tien *et al.*, 1979; Reynders and Vlassak, 1979; Hartmann *et al.*, 1983). We suspect that the production of IAA in the cultures of the late stationary growth phase comes from dying cells releasing tryptophan. This may stimulate auxin production by the still living cells in the population.

The stimulus involved in the formation of additional root hairs and lateral roots needs to be identified. Auxin produced by *Azospirillum* may particularly cause the formation of lateral roots as observed for several plants (see Blakely *et al.*, 1986 and references therein). However, this laboratory attributes more importance to the still unidentified substance which drastically increases the wet weight of wheat roots (Fig. 4). This is concluded from the findings that *Azospirillum* could exert its effects on the wet weight increase of roots when the medium contained nitrate but not ammonia as the only N-source in the assay system used in Fig. 1 (not documented). *Azospirillum* excretes this compound only when grown on nitrate, and oxygen suppresses its formation. Denitrifying conditions apparently favour its formation. A publication describing its characteristics is in preparation.

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