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An alternative explanation for plant growth promotion by bacteria of the genus *Azospirillum*

W. Zimmer, K. Roeben, and H. Bothe*
Botanisches Institut der Universität Köln, Gyrhofstrasse 15, D-5000 Köln 41, Federal Republic of Germany

Abstract. Experiments were performed to identify the substances that are excreted by the soil bacterium *Azospirillum brasilense* Sp7 and that were reported to stimulate the formation of lateral roots and of root hairs of grasses. *Azospirillum* forms indole-3 acetic acid (IAA), but only in the late stationary growth phase or when tryptophan is present in the medium, but not in continuous cultures or in the logarithmic growth phase of batch cultures. Formation of IAA by *Azospirillum* requires aerobic conditions. Nitrite can replace IAA in several phytohormone assays, and is even more active than IAA in a test with wheat root segments in which the increase of wet weight is determined. Higher amounts of nitrite are necessary for activity in other classical auxin assays. Nitrite shows 40–60% of the activity of IAA in the straight-growth test of *Avena* coleoptiles and in the formation of C_2H_4 by pea epicotyl segments. Like IAA, nitrite is inactive in promoting C_2H_4 formation by ripe apple tissues. Since nitrite alone can hardly exert phytohormonal effects, it is postulated that nitrite reacts with a substance in the cells and that a product formed by this reaction functions as auxin. Such a substance could be ascorbate. Exogenously added ascorbate enhances the rate of nitrite-dependent C_2H_4 formation by pea epicotyl sections and the nitrite-dependent increase in the wet weight of wheat root segments. Nitrite is formed by nitrate respiration of *Azospirillum*. The findings that nitrite can have phytohormonal effects offers an alternative explanation of the promotion of the growth of roots and the enhancement of mineral uptake of grasses by *Azospirillum*. Indole-acetic acid completely and nitrite partly substitute for an inoculation with *Azospirillum* in an assay where the increase of the dry weight of intact wheat roots is determined after an incubation for 10 d. Nitrite and IAA are, therefore, possibly the only factors causing an enhancement of the growth of roots of grasses.

Key words: *Azospirillum* – Plant growth promoter – Indoleacetic acid – Nitrate respiration – Nitrite (phytohormonal effect)

Introduction

Soil bacteria of the genus *Azospirillum* live in association with grasses and other plants. Compared with other bacteria, their relative number in soils of the temperature zone is small, but they are reported to occur abundantly under tropical conditions. Cereals like corn, rice, wheat, sugar cane and *Sorghum* can contain 10^4–10^7 cells/g dry weight of roots (Döbereiner and Pedrosa 1987). They seem to colonize the root hairs, the root cap, the root cortex, the protoxylem vessels or a zone a few millimeters away from the surface of the roots. Their exact location is difficult to establish and remains controversial (see Skinner and Uomala 1986; Klingmüller 1983, 1985). *Azospirillum* can live from the carbon of the plant cell-wall material. The bacterium can utilize xylan (Halsall et al. 1985) and L-arabinose (Novick and Tyler 1982).

Azospirillum has been widely discussed as a biofertilizer. As gramineous plants are difficult to manipulate by modern biotechnology, bacteria like *Azospirillum* forming associations with plants could offer an alternative chance to enhance crop productivity or to improve plant health. *Azospirillum* performs N_2 fixation and could supply the

* To whom correspondence should be addressed

Abbreviations: HPLC = high-performance liquid chromatography; IAA = indole-3-acetic acid

plants with nitrogen from this reaction. Several authors demonstrated by use of the ^{15}N isotope that nitrogen fixed by *Azospirillum* can be transferred to plants (Döbereiner and Pedrosa 1987). Most authors, however, agree that the numbers of *Azospirillum* are too small even in tropical soils to contribute significantly to the nitrogen demand of plants (Okon 1985; Elmerich 1984).

In addition to beneficial effects by N_2 fixation, *Azospirillum* could increase crop productivity by an excretion of phytohormones. *Azospirillum* forms auxins (particularly indole-3 acetic acid, IAA) and was also reported to excrete gibberellins and three cytokinin-like substances to the culture medium (Tien et al. 1979). These phytohormones were found to increase the formation of root hairs and of additional lateral roots (Tien et al. 1979; Umali-Garcia et al. 1980; Kapulnik et al. 1983). Such morphological changes could enhance water and mineral uptake by the plant and thus plant productivity (Lin et al. 1983).

This laboratory recently studied aspects of denitrification and nitrogen fixation of *Azospirillum* both in the free-living state (Zimmer et al. 1984; Penteado Stephan et al. 1984) and in association with wheat (Neuer et al. 1985; Danneberg et al. 1986). We also confirmed that the formation of IAA by *Azospirillum* is dependent on tryptophan in the medium (Zimmer and Bothe 1988). In a survey of the plant growth promoters excreted by *Azospirillum*, this laboratory noted that nitrite can cause phytohormonal effects. The experimental evidence for this is reported in the present communication. The relative importance of the different plant growth promoters excreted by *Azospirillum* is also evaluated.

Materials and methods

Organism and growth. All the experiments were performed with *Azospirillum brasilense* Sp7 (ATCC 29143). The cells were grown in 100-ml flasks in a shaking water bath (60 strokes·min^{-1}) at 30° C for at least 24 h in the medium described earlier (Zimmer and Bothe 1988). The medium contained either 2.0 g·l^{-1} KNO_3 or 0.265 g·l^{-1} NH_4Cl. When grown anaerobically, the 100-ml flasks were continuously flushed with N_2.

Separation of indole derivates by high-performance liquid chromatography (HPLC) and quantitative determinations. The culture broth was made cell-free by centrifugation (35000·*g*, 10 min) and 25 µl of it was directly injected into a Waters (Königstein, FRG) HPLC apparatus. Ethyl-acetate extraction and evaporation (see Tien et al. 1979; de Francesco et al. 1985) were avoided to minimize losses of indole derivates. Analysis by HPLC of the indole derivates was performed by isocratic reversed phase on a Waters Novapak C_{18} column (0.8 cm i.d., 10 cm long, 5 µm particle size) equilibrated with methanol/1% H_3PO_4=40/60 (v/v) at room temperature. The flow rate was 1 ml·min^{-1}, the injection volume 25 µl and the detection at 278 nm. Quantitation of the substances was performed by integration of the peak areas using a Heyden chromatochart system (Heyden and Son Comp., Rheine, FRG) and IAA (Merck, Darmstadt, FRG) as standard.

Auxin tests

(i) Increase in the wet weight of wheat root segments. Wheat root segments respond to the addition of auxin by an increase of the wet weight (Libbert 1957). Spring wheat grains (*Triticum aestivum* cv. Ralle) in a Petri dish were watered with 10 ml H_2O for 24 h at 4° C. The grains were then placed onto two layers of Whatman filter paper moistened with 6 ml H_2O and incubated for 72 h at 26° C in the dark for germination. Twelve wheat root segments of 5 mm length, cut from the zone 2 mm to 7 mm from the tip, were placed in a Petri dish containing one layer of filter paper moistened with 2.5 ml of the solution to be assayed for auxin content. The Petri dishes were incubated for 24 h at 26° C in the dark, and the wet weight of the root segments was determined afterwards. The test allowed IAA to be measured between 10^{-12}–10^{-5} mg IAA·ml^{-1} with an optimal response of the increase of the wet weight at 10^{-8} mg·ml^{-1}.

(ii) Elongation of Avena coleoptiles (straight growth test, Bentley 1962). Oat (*Avena sativa* cv. Nova) was grown in greenhouse soil for 4 d at 25° C in the dark. The length of the coleoptiles was then 40–50 mm. Twenty coleoptile segments, each 10 mm long and taken 3 mm from the tip, were transferred into 25-ml Erlenmeyer flasks with 10 ml of the test solution containing 1% sucrose, 10 mM K_2HPO_4, 5 mM Na-citrate pH 7.0 and the sample to be assayed for auxin content. The lengths of the coleoptile segments were measured after an incubation for 48 h in the dark. The segments responded to IAA concentrations between 10^{-6}–10^{-1} mg·ml^{-1} with the optimum at 10^{-3} mg·ml^{-1}.

(iii) Formation of C_2H_4 by pea epicotyl segments (Lieberman and Kunishi 1975). Production of this gas is largely stimulated by auxins. As C_2H_4 can easily be quantified with high sensitivity by gas chromatography, this assay was used routinely for auxin determinations. Pea (*Pisum sativum* cv. Kleine Gärtnerin) was grown in greenhouse soil for 4 d in the dark at 26° C. Stem segments (10 mm long) were taken from the subhook region. Twelve segments were transferred onto two layers of filter paper moistened with 4 ml H_2O and incubated for 24 h at 26° C to reduce the blank rate of the C_2H_4 formation. The segments were then rinsed with water and transferred to 7.2-ml Fernbach flasks containing in 1 ml: 0.1 mM K-phosphate buffer pH 7.0 and the sample to be assayed. The Fernbach flasks were sealed with gas-tight rubber stoppers and incubated for 4 h in a shaking water bath at 30° C. All manipulations were performed in the dark or in dim green light. The C_2H_4 formed was determined in a Varian (Walnut Creek, Calif., USA) model 940 gas chromatograph equipped with a flame-ionization detector, using a Porapak R column, N_2 as the carrier gas and a column temperature of 50° C. The test responded to 10^{-4}–10^{0} mg IAA·ml^{-1} with an optimum of $9 \cdot 10^{-3}$ mg·ml^{-1}.

(iv) Formation of C_2H_4 by ripe apple tissue (Lieberman et al. 1966). Cylinders (8 mm long, 9 mm diameter) were cut out of apples (*Malus sylvestris* cv. Golden Delicious) and had an average wet weight of 0.4 g. Two of these cylinders were transferred into a 7.2-ml Fernbach flask containing in 2 ml:0.4 M sucrose and the sample to be assayed. The flasks

were sealed and incubated for 1 h at 30° C in a shaking water bath. The C_2H_4 formed was determined by gas chromatography.

The system for assaying the stimulatory effect of Azospirillum or nitrite on the growth of wheat roots (Zimmer and Bothe 1988). A surface-sterilized grain of spring wheat (*Triticum aestivum* cv. Ralle) was put into an Eppendorf plastic cup the tip of which had been cut off. The cup was transferred into a-25 ml test tube and remained on top of 10 ml liquid wheat medium containing 3.5 mM KNO_3. The surface-sterilisation method for the grains and the wheat medium were as described by Neuer et al. (1985). In the experiments with *Azospirillum*, 10 µl of a 1-d-old culture with an optical density of 1.43 at 560 nm were injected into the test tube. All manipulations were performed under sterile conditions. The samples were incubated at 23° C for 10 d (14 h daylight, 10 h darkness per day), and the wheat medium and the additions were exchanged after 7 d (but no new inoculation with *Azospirillum*). The dry weight of the excised roots was determined after 10 d. The test responded to 10^{-8}–10^{-1} mg IAA·ml^{-1} with an optimum at $1.8 \cdot 10^{-5}$ mg·ml^{-1}.

Other procedures. Nitrite was determined colourimetrically with the naphthylamine/sulphanilic acid reagent (Nicholas and Nason 1957). Spectra were recorded by a Perkin-Elmer 550 spectrophotometer (Perkin-Elmer, Überlingen, FRG).

Results

Comparison of the absorption spectra and chromatographic behavior of nitrite and IAA. This investigation was started by purifying the substance(s) that was (were) excreted by *Azospirillum* and that caused the formation of additional root hairs and lateral roots of cereals. The culture broth was made *Azospirillum*-free by centrifugation and directly subjected to HPLC analysis without the use of the ethyl-acetate extraction performed by others (Tien et al. 1979) to avoid loss of phytohormones. Under the specific conditions employed (see *Material and methods*), an unknown compound had a retention time of 162 s. For comparison, indole derivates had the following retention times under these conditions: indoleacetic acid 306, tryptophan 132, indole-3 lactate 234, indole-3 acetaldehyde 271, indole-3 pyruvate 520 and indole-3 propionate 540 s (Fig. 1). A whole series of other related substances also did not cochromatograph with the unknown compound (data not shown). This compound was then purified extensively (by ethanol extraction, separation on silica gel and chromatography on Sephadex G10, see Zimmer 1988) and showed a broad absorption maximum around 350 nm at pH 7.0. Under mild acid conditions (pH 6.2), it exhibited a distinct spectrum with maxima at 340, 356 and 377 nm and shoulders at 290, 344 and 385 nm (Fig. 2). This spectrum showed some resemblance to that of IAA with the absorption maxima shifted to longer wavelengths by some 80 nm. When excited at the wavelength 350 nm, the substance gave an emission spectrum with a maximum at 380 nm.

Admittedly, these data led us to believe for a while that the compound could be an IAA derivative. The following findings, however, prove that the compound is nitrite:

(i) In the HPLC analysis under the conditions employed, nitrite and the compound have the same retention time of 162 s.

(ii) A solution of 10 mM $NaNO_2$ or KNO_2 shows the same absorption spectrum as the compound formed by *Azospirillum* at pH 6.2 (Fig. 2) and at pH 7.0 (data not shown). The fluorescence emission spectra were also identical (not documented).

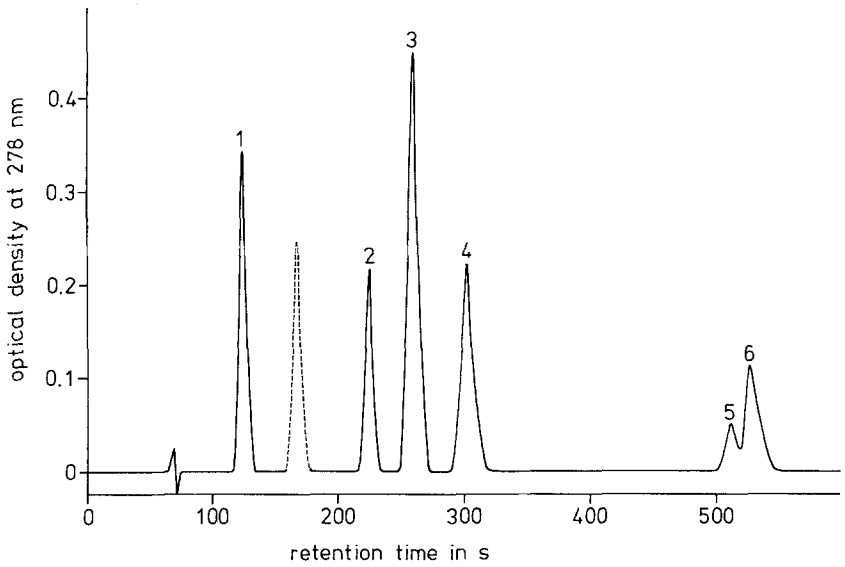

Fig. 1. Separation by HPLC of indole derivates excreted by *Azospirillum*. The procedure is described under *Material and methods*. 1 = tryptophan, 2 = indole-3 lactate, 3 = indole-3 acetaldehyde, 4 = indole-3 acetate, 5 = indole-3 pyruvate, 6 = indole-3 propionate, *dashed line* = unknown substance = nitrite

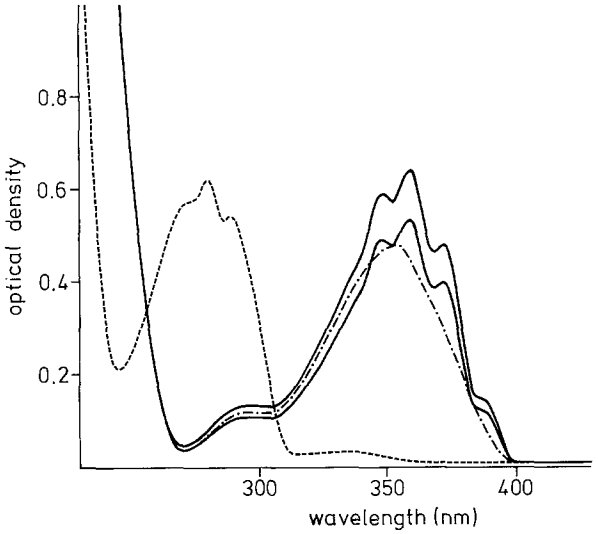

Fig. 2. Absorption spectra of IAA, nitrite and the purified substance excreted by *Azospirillum*. To record the spectra, the substances were dissolved in 80% ethanol/20% 1 mM K-phosphate buffer. ---, Indoleacetic acid (0.1 mM, pH 7.0); -·-·-, purified substance (pH 7.0); —, *upper line*: purified substance (pH 6.2); —, *lower line*: KNO_2 or $NaNO_2$ (10 mM, pH 6.2)

The spectrum is to be attributed to NO_2^- and not to NO, NO_2, N_2O_4 or other compounds formed in minute amounts from the high concentration of 10 mM NO_2^- (Pestemer et al. 1951).
(iii) Both ^{13}C-nuclear magnetic resonance and mass spectrometry indicated that the purified compound did not contain carbon.
(iv) When *Azospirillum* cultures were grown with ^{14}C-malate, the centrifuged culture broth contained labelled carbon. However, radioactivity was completely lost with the purification of the compound (Zimmer 1988). The compound was formed when *Azospirillum* had been grown with nitrate and not with ammonia as the nitrogen source.

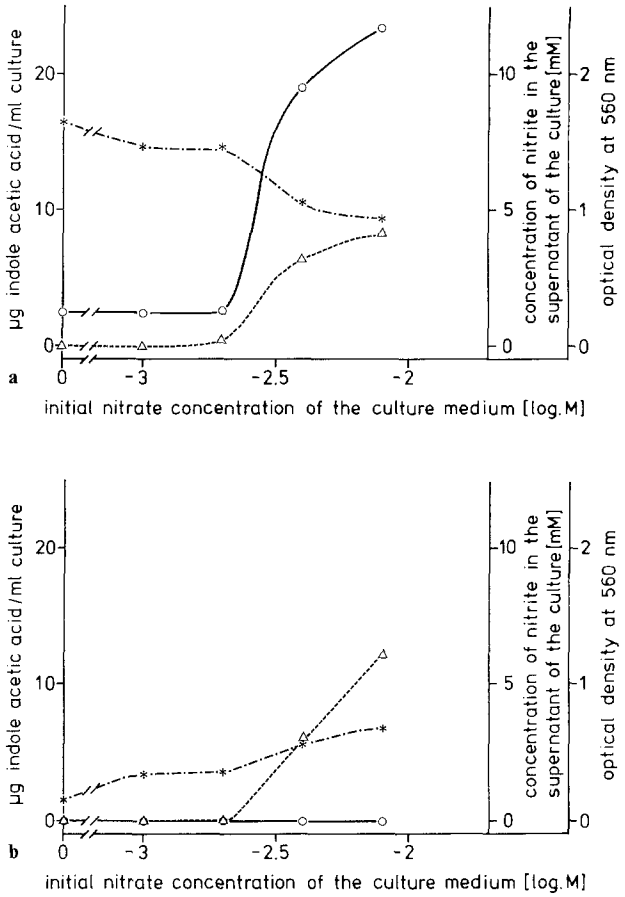

Fig. 3a, b. Formation of nitrite and indole-3 acetic acid by *Azospirillum* as a function of nitrate in the culture medium. **a** Aerobic culture; **b** anaerobic culture. The bacteria were grown for 48 h in 100-ml flasks in a shaking-water bath at 30° C in the NH_4^+-medium described under *Material and methods*. The medium was supplemented with varying amounts of nitrate as indicated by the abscissa. The aerobic culture was gassed with synthetic air and the anaerobic culture with N_2 (10 ml gas·min^{-1}, each). o—o, Excretion of IAA; △—△, formation of nitrite; *—*, growth of the culture after 48 h measured by the optical density at 560 nm

The formation of nitrite and indoleacetic acid by Azospirillum brasilense Sp7. In the preceeding investigation (Zimmer and Bothe 1988), the concentration of IAA excreted into the medium of 4- to 5-d-old cultures grown with nitrate (with $8.3 \cdot 10^8$ cells·ml^{-1}) was determined as 15 µM. Indole-3-acetic acid was formed only in the late stationary growth phase. The excretion of IAA by *Azospirillum* was enhanced 6- to 8-fold and started much earlier when the medium was supplemented with tryptophan. These data agree well with those recently communicated by others (De Francesco et al. 1985; Jasmin et al. 1987). Figure 3a shows the formation of IAA by different *Azospirillum* batches grown aerobically with varying concentrations of nitrate. The excretion of IAA was independent of nitrate up to an initial concentration of $5 \cdot 10^{-3}$ M in the medium. Higher levels of nitrate resulted in a large increase in the formation of IAA that paralleled the excretion of nitrite into the medium. Dense cell populations ($O.D._{560nm} \sim 1.0$) utilize nitrate as a respiratory electron acceptor and convert it to nitrite even when aerated. Oxygen is normally the preferred electron acceptor, but the supply of O_2 is apparently not sufficient for the demand of a dense *Azospirillum* population (Bothe et al. 1981). When nitrate is in excess, nitrate respiration stops with nitrite; otherwise nitrite is reduced further to N_2 and N_2O. The formation of more than 3 mM nitrite is toxic to the cells as indi-

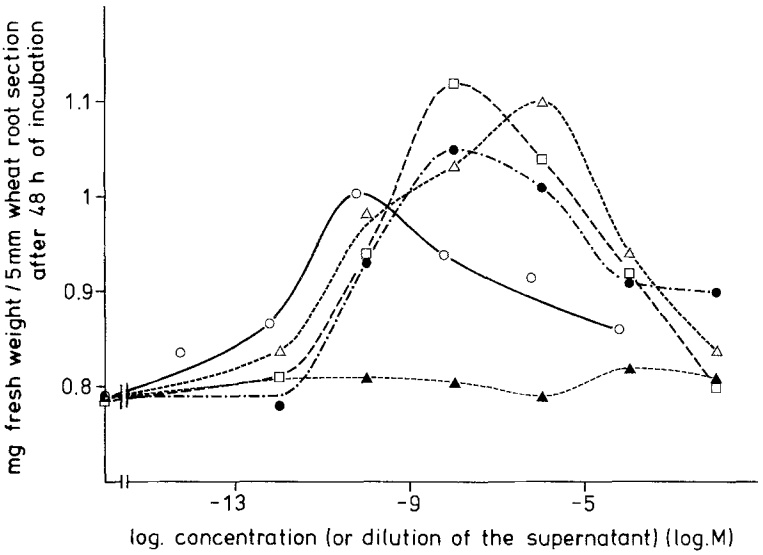

Fig. 4. Increase of the wet weight of wheat root segments induced by IAA, nitrite, nitrite plus ascorbate, and supernatant freed from *Azospirillum* by centrifugation. The assay system is based on the method of Libbert (1957) and is described under *Material and methods*. Increase in wet weight caused by: ○—○, IAA (M concentrations given on the *abscissa*); ●–·–·–●, KNO_2 (M); □–––□, KNO_2 (in M, different concentrations given on the *abscissa*) plus 0.1 mM Na-ascorbate; △-----△, by the supernatant of nitrate-grown cells. The supernatant was obtained by growing *Azospirillum* in the nitrate medium for 1 d and by centrifuging (35000 × *g*, 10 min). The supernatant contained 6.3 mM nitrite as determined colourimetrically (see *Material and methods*). Different dilutions of this supernatant (see *abscissa*) were assayed; ▲-----▲, by the supernatant of NH_4^+-grown cells, performed in the same way

cated by the decrease in the optical density of the culture (Fig. 3a). A proportion of the population may die and release tryptophan into the medium, and tryptophan may stimulate IAA formation by the bacteria still surviving in the culture.

As described earlier (Nelson and Knowles 1978; Bothe et al. 1981), *Azospirillum* grows with nitrate as the respiratory electron acceptor when O_2 is not available. However, anaerobic cultures reached only 20–25% of the optical density of cells grown with O_2 and excrete much more nitrite (compare Fig. 3a and 3b). Anaerobic cultures do not form any IAA (Fig. 3b). In IAA biosynthesis of plants, the conversion of indole-3 acetaldehyde to IAA is O_2-dependent (Sembdner et al. 1980), which might also apply to the biosynthesis of this phytohormone by *Azospirillum*.

Auxin causes increases of the wet weight of root segments (Libbert 1957) and the formation of additional lateral roots (Blakely et al. 1986). A typical dose-response curve was obtained when IAA was added to excised wheat roots (Fig. 4). The medium freed from *Azospirillum* by centrifugation was even more active than IAA in increasing the wet weight of the segments (Fig. 4). Nitrite was also more effective than IAA in this assay. The medium was only stimulatory when the cells had been grown with nitrate and not with ammonium as the nitrogen source (Fig. 4). The concentration of nitrite in the supernatant from nitrate-grown cells was 6.3 mM in this assay. Nitrite was apparently the factor causing the increase in the wet weight of wheat-root segments.

In the coleoptile-elongation test, the medium freed from *Azospirillum* by centrifugation showed 40–50% of the activity of IAA (Fig. 5). Nitrite at an optimal concentration of 10^{-4} M could fully replace the activity of the medium (Fig. 5), indicating once more that a major stimulatory compound excreted by *Azospirillum* was nitrite.

A simple assay suitable for routine analysis is the auxin-stimulated formation of C_2H_4 by pea epicotyl sections. In this assay also, nitrite could partly replace indoleacetic acid (Table 1; Fig. 6). The maximal stimulation was at 2.0 mM nitrite, which gave approx. 60% of the activity of optimal IAA concentrations in this assay.

Auxin activities are exerted not only by IAA but also by several other compounds. However, a distinct relationship exists between the chemical constitution of these compounds and their biological activity (see Stoddart and Venis 1980). Nitrite alone can hardly fulfil the requirement of an auxin. Nitrite could, however, interact with a compound in the cell, and the product of this reaction could act as an auxin. To test this idea, several compounds listed in Table 1 were assayed for their capability to enhance NO_2^--dependent C_2H_4 formation. Among these, ascorbate significantly stimulated C_2H_4 evolution in addition to nitrite (Table 1). Ascorbate alone was inactive. Optimal concentrations of Na-ascorbate (0.2 mM) plus nitrite (2.0 mM) reached 80% of the activity of the control with IAA (Fig. 6). All other compounds tested which included tryptophan, tryptamine, glutamine, tyrosine, threonine, urea, formaldehyde, glutathi-

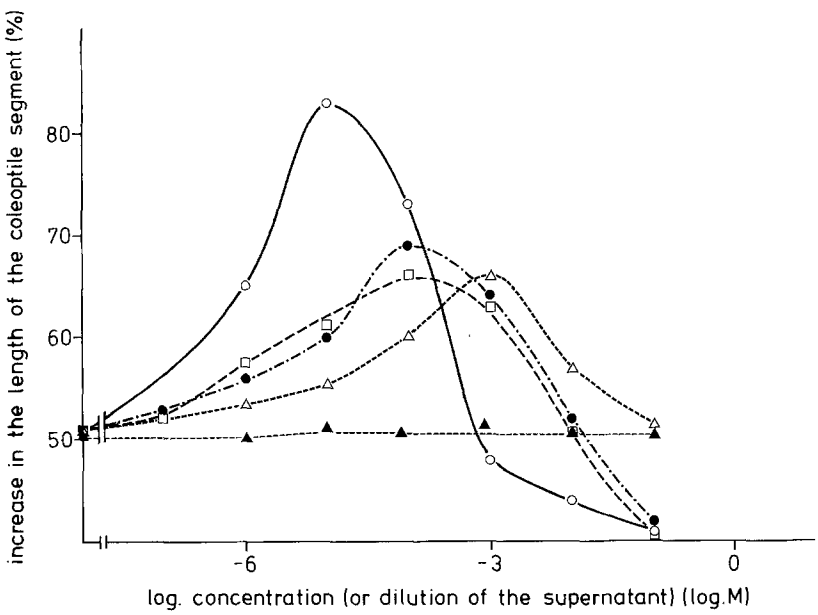

Fig. 5. The elongation of *Avena* coleoptile segments as a function of IAA, nitrite, nitrite plus ascorbate, or supernatant freed from *Azospirillum* cultures. Stimulation of straight growth by: o——o, IAA; ●-·-·-●, nitrite, □——□, nitrite plus 0.1 mM Na-ascorbate; △-----△, supernatant of nitrate-grown *Azospirillum* (prepared as described in Fig. 4); ▲-----▲, supernatant of NH_4^+-grown cells

Table 1. Auxin-stimulated C_2H_4 formation by pea epicotyl sections. Others substances tested that were virtually without effect: proline, asparagine, aspartic acid, glutamic acid, methionine, anthranilic acid, glycine, alanine, glycylglycine, imidazole, pyruvate, nicotinic acid, guanine. Rates are given in pmol C_2H_4 formed per 12 pea segments·4h

Assay conditions			C_2H_4 formed
Buffer			17.5
Indoleacetic acid (0.05 mM)			225.9
Nitrite (2.0 mM)			135.0
5′ OH-Tryptophan	(0.1 mM)		18.8
5′ OH-Tryptophan	(0.1 mM)	+ Nitrite (2.0 mM)	118.5
5′ OH-Tryptamine	(0.1 mM)		17.5
5′ OH-Tryptamine	(0.1 mM)	+ Nitrite (2.0 mM)	114.8
Glutamine	(0.1 mM)		16.7
Glutamine	(0.1 mM)	+ Nitrite (2.0 mM)	82.8
Tyrosine	(0.1 mM)		23.9
Tyrosine	(0.1 mM)	+ Nitrite (2.0 mM)	114.8
Threonine	(0.1 mM)		18.5
Threonine	(0.1 mM)	+ Nitrite (2.0 mM)	139.7
Urea	(0.1 mM)		18.9
Urea	(0.1 mM)	+ Nitrite (2.0 mM)	109.5
Formaldehyde	(0.1 mM)		19.0
Formaldehyde	(0.1 mM)	+ Nitrite (2.0 mM)	110.5
Glutathione	(0.1 mM)		18.4
Glutathione	(0.1 mM)	+ Nitrite (2.0 mM)	139.1
Na-ascorbate	(0.1 mM)		17.4
Na-ascorbate	(0.1 mM)	+ Nitrite (2.0 mM)	148.8
Na-ascorbate	(0.2 mM)		17.7
Na-ascorbate	(0.2 mM)	+ Nitrite (0.2 mM)	182.8

one (Table 1), proline, asparagine, aspartic acid, glutamic acid, methionine, anthranilic acid, glycine, alanine, glycylglycine, imidazole, pyruvate, nicotinic acid and guanine (data not shown) were ineffective.

Sodium ascorbate also enhanced nitrite-dependent activity in the assay with wheat root segments (Fig. 4). The results were, however, not statistically different in the *Avena* coleoptile-elongating test (Fig. 5).

Ripe apple tissues also produce C_2H_4. This gas formation is independent of auxin but is stimulated by methionine (Lieberman et al. 1966). The data of Table 2 confirm these statements. Nitrite did not stimulate in this assay irrespective of the concentration used (Table 2).

In the test system described under *Material and methods*, changes in the root morphology could even be seen macroscopically. Plants inoculated with *Azospirillum* had longer roots and had formed additional root hairs and lateral roots. The same was also true when seeds were incubated with nitrite (data not shown). The dry-weight determinations of the roots excised after 10 d gave statistically sound data (Table 3). The addition of *Azospirillum* resulted in a significant increase in dry weight. Nitrite (10^{-4} M) or IAA (10^{-7} M) also stimulated in this assay, but nitrite could reach only 30% of the enhancement obtained by inoculating with *Azospirillum*. When the experiments were performed with both nitrite and ascorbate, the data were not statistically different from those of the assays with nitrite alone. In this assay, IAA reached even more than 100% of the activity obtained by inoculating with *Azospirillum* (Table 3).

Discussion

The processes contributing to the plant growth responses for the *Azospirillum*-grass associations

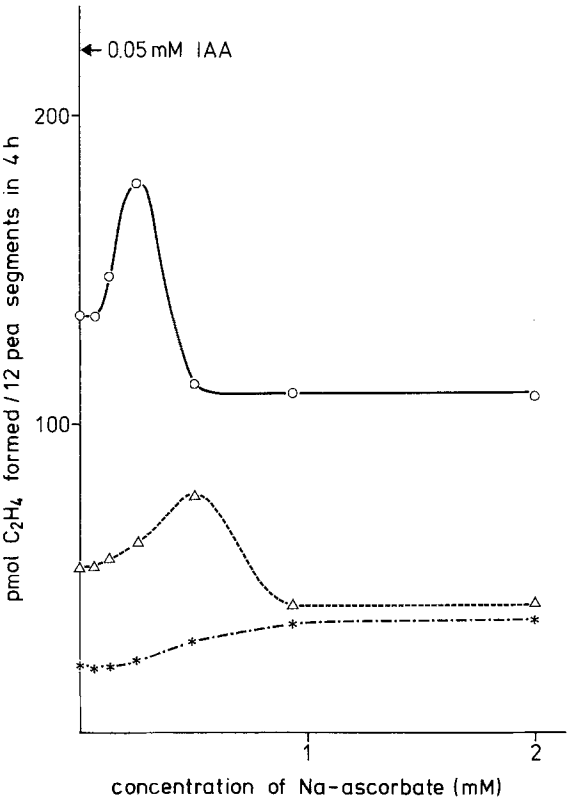

Fig. 6. The stimulation of C_2H_4 formation by pea epicotyl segments in the presence of nitrite and Na-ascorbate. The assay is based on the method of Lieberman and Kunishi (1975) and is described under *Material and methods*. The concentrations of ascorbate were varied in this assay.
–·–·–, Experiment performed with 0.5 mM KNO_2; △-----△, experiment performed with 1.0 mM KNO_2; ○——○, experiment performed with 2.0 mM KNO_2; ←, control with IAA

Table 2. Ethylene formation by apple tissue. The assay is based on the method described by Lieberman et al. (1966) and is described under *Material and methods*. Rates are given in mmol C_2H_4 formed per 0.8 g fresh weight of apple tissue in 1 h

Conditions (supplements to the assay)		C_2H_4 formed
Control with H_2O		10.5
Indoleacetic acid	(0.5 mM)	11.0
Nitrite	(0.2 mM)	7.2
Nitrite	(0.5 mM)	10.2
Methionine	(0.1 mM)	15.4

Table 3. Stimulatory effects of *Azospirillum* and nitrite on the root growth of intact wheat plants after 10 d of incubation

Growth conditions[a]	Root DW (mg·plant^{-1})	P^b
Culture medium	6.30	–
Culture medium + *Azospirillum*	8.55	98.9%
Culture medium + Nitrite (0.1 mM)	7.02	93.9%
Culture medium + Nitrite (0.1 mM)	6.18	55.4%
Culture medium + Nitrite (10.0 mM)	4.60	97.3%
Culture medium + Nitrite (0.1 mM) + Na ascorbate (0.1 mM)	6.95	92.4%
Culture medium + Indoleacetic acid (0.1 μM)	9.94	99.9%

[a] The experimental conditions are described under *Material and methods*
[b] The probability (P) was calculated from the Student-t-distribution (Mudra 1958) using six different experiments. The control (incubation of wheat with culture medium alone) was used as the reference in the Student-t-test. A probability of more than 90% is taken as a statistically sound difference

have not been elucidated conclusively (van Berkum and Bohlool 1980; Döbereiner and Pedrosa 1987). Several authors have stated that phytohormones (Tien et al. 1979; Kapulnik et al. 1985), particularly auxins (Patriquin et al. 1983; Jain and Patriquin 1985; Kolb and Martin 1985) are the major interacting factors. It is now established that *Azospirillum* forms IAA in the stationary growth phase and that IAA production is further stimulated by the addition of tryptophan to the medium (Reynders and Vlassak 1979; de Francesco et al. 1985; Zimmer and Bothe 1988). Indoleacetic acid causes the branching of wheat root hairs (Jain and Patriquin 1985). Several experimental findings, however, indicate that IAA is not the determining factor in the expression of this association:

(i) Auxins are not formed in the logarithmic growth phase of batch cultures (de Francesco et al. 1985; Zimmer and Bothe 1988) or in continuous cultures (Zimmer 1988) in the absence of tryptophan. As suggested above, auxin formation in the stationary growth phase might be connected to a tryptophan release from dead bacterial cells. Such a statement is corroborated by the finding that the auxin content drastically increases when part of the cell population is killed by high amounts of nitrite formed in nitrate respiration (Fig. 3a). It remains to be shown whether *Azospirillum* in the roots of grasses is supplied with tryptophan by the plants or is in a physiological state comparable to the stationary growth phase of batch cultures.

(ii) Auxin formation from tryptophan requires O_2 (compare Fig. 3a and 3b), and the concentration of O_2 in roots may not be sufficient for this conversion to proceed.

(iii) Many microorganisms, particularly epiphytic bacteria, are known to produce IAA (Sembdner et al. 1980). If auxins were the chemical basis, the association could hardly be specific for *Azospirillum* and grasses.

A similar conclusion that auxin cannot be the only factor was drawn from experiments with an

IAA-overproducing mutant of *Azospirillum* isolated by Hartmann et al. (1983). This strain did not affect elongation of wheat roots above the effect obtained with the wild strain of *Azospirillum* (Kapulnik et al. 1985). In a search for other factors, this laboratory recently could not confirm an earlier report (Tien et al. 1979) that *Azospirillum* excretes gibberellins and cytokinins into the medium in concentrations that could affect plant growth (Zimmer and Bothe 1988). *Azospirillum* may, however, produce these phytohormones in minute amounts detectable only by the immunoassay technique. By the use of this technique, Kolb and Martin (1985) found only traces of cytokinins and abscisic acid in moderate amounts. Gibberellins and cytokinins are, therefore, unlikely to be important in establishing the association.

The central message presented here is that nitrite shows phytohormonal effects. The mechanisms by which nitrite acts appear to be different in the assay with wheat root segments and in the other biological tests tried here. In the test with wheat root segments where the increase of wet weight was determined, nitrite was even more active than IAA. The optimum was at the low concentration of 10^{-8} M nitrite which was rapidly formed by nitrate respiration of *Azospirillum* even under aerobic conditions. When media are acidified, nitrite becomes toxic by the formation of nitrous acid, whereas nitrite can be assimilated by plants at neutral pH values (Mevius 1958). Despite the fact that the assays were performed at pH 7.0, it was as if the plants tried to dilute-out nitrite by increasing the wet weight of roots to prevent any destruction. This may be achieved by the influx of H_2O causing cell elongation and/or volume increase. At this point, any suggestion about the reaction mechanism of nitrite in this specific test is speculative.

In the other biological tests, nitrite was 40–60% as active as IAA, and the optimal concentration varied with the test used: 0.1 mM in the *Avena* coleoptile elongation test (Fig. 5), 2 mM in C_2H_4 formation by pea epicotyl sections (Fig. 6, Table 1), and 0.1 mM in the assay in which the dry weight of intact wheat roots was determined (Table 3). Such concentration dependences were, however, also observed with IAA (Figs. 5, 6; Table 3). In all cases the application of higher amounts of nitrite resulted in inhibitions. The fairly high concentration of nitrite required for the optima could be indicative of a compound like NO, N_2O or N_2O_4, formed from nitrite by disproportionation (Jones 1973), being the active species in these phytohormone assays. However, the Porter-Thimann charge-separation model (see Stoddart and Venis 1980; Bearder 1980) predicts that a compound functioning as auxin must have a negative charge (in most cases a carboxyl group) and a weak positive charge, and that both charges should be separated from each other by some 0.5 nm. Nitrite and the other compounds just mentioned can hardly fulfil these requirements, because all of them are too small and the weak positive charge is too close to the negative one in each of the molecules. Therefore, nitrite might interact with a substance in the cells, and the reaction product could cause the phytohormonal effects. Such a substance in the cells could be ascorbate, because supplementing the assays with ascorbate in addition to nitrite resulted in 80% of the activity of the auxin controls in C_2H_4 formation by pea epicotyls and also enhanced the nitrite-dependent increase of wet weight of wheat root segments. The concentration of ascorbate in plant cells is about 1–2 mM (Barnes 1972; Mehlhorn et al. 1986; Osswald et al. 1987), so it is high enough to interact with the nitrite formed in nitrate respiration of *Azospirillum*. Ascorbate is believed to function as an antioxidant to detoxify SO_2 or ozone (Osswald et al. 1987). Ascorbate or a degradation product of ascorbate may interact with nitrite or a disproportionation product of nitrite in a similar way. It is difficult to determine exactly the chemical species that is active in the auxin assays, because nitrite is known to react with many organic compounds (Azhar et al. 1986).

The finding that nitrite can act like a phytohormone was unexpected to us and also new in the literature to our knowledge. Interactions between nitrogen metabolism and auxin function have, however, been described (Wort 1961). Application of IAA to plants can cause a change in nitrate reductase activity and a considerable accumulation of nitrate. Evaluating the different mechanisms that could be responsible for the expression of the association between *Azospirillum* and plants, nitrite formation by respiration may be more important than phytohormone production. Such a conclusion can also be reached from observations that strains predominate within roots that do not reduce nitrite to the gaseous nitrogen compounds N_2 or N_2O (Baldani and Döbereiner 1980) and that a nitrate-reductase-negative mutant of *A. brasilense* Sp 245 does not compete well with other soil microorganisms in colonizing the wheat rhizosphere in contrast to the parent strain (Baldani et al. 1986). Döbereiner's group, therefore, suggested that strains dissimilating nitrate to nitrite have a competitive advantage (Döbereiner and

Pedrosa 1987, p. 93). Opposite conclusions could be reached from experiments by Umali-Garcia et al. (1980) in which the presence of 5 mM $Ca(NO_3)_2$ suppressed the effects of bacterial inoculation on root hair formation and lateral root morphogenesis. In the latter experiments, the concentration of nitrite formed by *Azospirillum* was possibly so high that it caused this inhibition, which was also observed with high amounts of nitrite in all the assays tried in the present investigation. Nitrite formation can hardly be the reason for the specificity of the association between *Azospirillum* and grasses, however. A nitrate-respiration capability is relatively rare among bacteria but is distributed among systematically unrelated groups (Ingraham 1981). It remains to be shown whether other nitrate-respiring bacteria are inferior to *Azospirillum* in establishing an association with grasses. Further experiments have to show whether *Azospirillum* performs nitrate respiration and excretes nitrite in the roots of grasses.

It cannot be ruled out that other, still unidentified factors (phytohormones) are involved in the formation of the intimate association between *Azospirillum* and grasses. However, IAA could completely and nitrite partly substitute for an inoculation with *Azospirillum* in the experiments of Table 3 in which the increase of wet weight of intact wheat roots was investigated. Thus nitrite and auxins may be the only plant growth-promoting substances excreted by *Azospirillum*, but then the question about the specificity of the association remains intriguing.

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