Symbiosis, 13 (1992) 167–179
Balaban, Philadelphia/Rehovot

Differential Effects of *Azospirillum*, Auxin and Combined Nitrogen on the Growth of the Roots of Wheat

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Received October 13, 1991; Accepted January 26, 1991

Abstract

Using a simple assay system where germinated wheat seeds are grown for 6–10 d in a hydroponic system in test tubes, it is shown that incubation with *Azospirillum* drastically enhances the formation of lateral rots, but causes at best a slight increase in the dry weight of roots and in the formation of root hairs/lateral roots. In contrast to *Azospirillum*, auxin (IAA) has virtually no effect on the formation of lateral roots. IAA increases the dry weight only slightly similar to *Azospirillum*. Nitrite, either added directly or excreted by *Azospirillum* in nitrate respiration, similarly causes a drastic increase in the formation of lateral roots. It is suggested that the growth promotion effect on wheat roots by *Azospirillum* is due to the formation of nitrite in the assay system used. In these tests, nitrite cannot be substituted by nitrate, ammonia or sulfite. The addition of low amounts of ascorbate together with nitrate results in a slight further increase in the formation of lateral roots. Nitrite as well as auxins are unlikely factors involved in the establishment of the specificity of the *Azospirillum*-plant association.

Keywords: *Azospirillum*, plant-bacteria association, auxin and root morphology, nitrate respiration, nitrite as growth factor

1. Introduction

Bacteria of the genus *Azospirillum* live in association with roots of plants, particularly grasses, and were repeatedly reported to stimulate the growth of cereals (Döbereiner and Pedrosa, 1987). The growth stimulatory substances have not been elucidated unambiguously. Roots of tropical grasses can contain *Azospirillum* in larger quantities, and *Azospirillum* in an N_2-fixing bacterium. However, inspite of the high number, the total mass of the bacteria does not make up more than maximally 0.1% of the total dry weight of the roots. *Azospirillum* does not significantly excrete NH_4^+, glutamine or other N-compounds when growing actively in suspension culture under N_2-fixing conditions (Bothe et al, 1981). Although the subject is still somewhat controversial, most investigators agree that N_2-fixation is unlikely the factor for the improved growth of roots of cereals. Better candidates for such promoting factors could be phytohormones (Tien et al., 1979). As phytohormones act on the roots in minute amounts, the concentrations of phytohormone producing bacteria in the roots of cereals could well be sufficient. *Azospirillum* produces indole acetic acid (IAA, auxin; Tien et al., 1979; Reynders and Vlassak, 1979). This latter capability is, however, widespread among bacteria. The production of IAA by *Azospirillum* occurs only in the stationary growth phase in suspension cultures and is largely (or even strictly) dependent on tryptophan (Zimmer and Bothe, 1988). Other phytohormones like gibberellins or cytokinines are not excreted by *Azospirillum* or at least not in such quantities which are required for plant growth promotion (Zimmer and Bothe, 1988).

This laboratory recently forwarded an alternative explanation for the root growth promotion by *Azospirillum* (Zimmer et al., 1988). The bacterium produces nitrite during nitrate respiration in large quantities (Nelson and Knowles, 1978; Bothe et al., 1981), and nitrite was found to exert phytohormonal effects in several bioassays. In the cells, nitrite may interact with ascorbic acid to form a phytohormonal active compound. In the course of the same project a simple assay system was designed which allows to monitor the effects of bacteria or chemicals on the development of roots of plants (Zimmer et al., 1988; Bothe and Zimmer, 1988). This system was used in the present study to investigate the differential effects of *Azospirillum*, auxin and combined nitrogen on the growth of roots of wheat.

2. Materials and Methods

The bacterial strain used, *Azospirillum brasilense* Sp 7 (ATCC 29145) was grown in 100 ml flasks placed in a water bath (60 strokes/min) at 30°C for at

least 24 hr in the medium described earlier (Zimmer and Bothe, 1988) with 2.0 g/l KNO_3 as the N-source. The simple assay system to test the stimulatory effects of bacteria or chemicals on the root growth had been introduced earlier (Zimmer and Bothe, 1988; Zimmer et al., 1988). For this test, a surface-sterilized (Neuer et al., 1985) grain of spring wheat (*Triticum aestivum* variety Ralle) was allowed to germinate for 1–2 d until the first three seminal roots (see Langer, 1979) became visible. The germinated seed was then put into an Eppendorf plastic cup the tip and lid of which had been cut off. The cup was placed in a 25 ml test tube on top of 10 ml liquid wheat medium containing the different addenda as indicated in the legends of the figure. All handlings were performed under sterile conditions. The plants in the test tube were incubated in a growth chamber at 23°C for 6–10 d with a light/dark cycle of 12.5 hr light (approximately 100 $\mu E \times s^{-1} \times m^{-2}$ at the surface of the test tubes/11.5 hr dark. The outside of the test tubes was covered with aluminium foil in the height of the nutrient solution to exclude effects of light on the root development. When the first 4–6 seminal and nodular roots (Langer, 1979) were at least 6 cm long in all tubes of an experiment, the number of lateral roots which had developed were counted visually (usually after 7 d). All the points in the figures of this publication represent the means of the data obtained from five different test tubes (= plants). In some experiments, it was attempted to determine the amount of root hairs of wheat by the method described for *Pisum sativum* by Röhm and Werner (1987). For this, excised roots were rapidly frozen with liquid nitrogen on a metal plate, and root hairs could be scratched off the roots with a painter's brush. The isolated root hairs were collected for determining either their dry weight or their protein content. The dry weight was measured by the standard technique using self-made aluminium cups. Protein was determined by the Bradford method.

In the experiments with *Azospirillum*, 10 μl of a 1 d old culture with an optical density of about 1.4 at 560 nm was injected into the test tube. Nitrite was determined colourimetrically with the naphthylamine/sulphanilic acid reagent (Nicholas and Nason, 1957) and nitrate by nitration of salicylic acid (Cataldo et al., 1975). The concentration of indole acetic acid in the medium was determined by its absorbance at 280 nm after separation by HPLC as described previously (Zimmer et al., 1988).

3. Results

The simple assay system introduced in the preceding publications (Zimmer et al., 1988; Zimmer and Bothe, 1988) was used for all the experiments of this investigation. When germinated wheat seeds were incubated with *Azospirillum*

under strictly controlled conditions (light, temperature) in a growth chamber, the bacteria caused a drastic increase in the lateral root formation which was consistently observed in all of the many experiments (Fig. 1). The dry weight

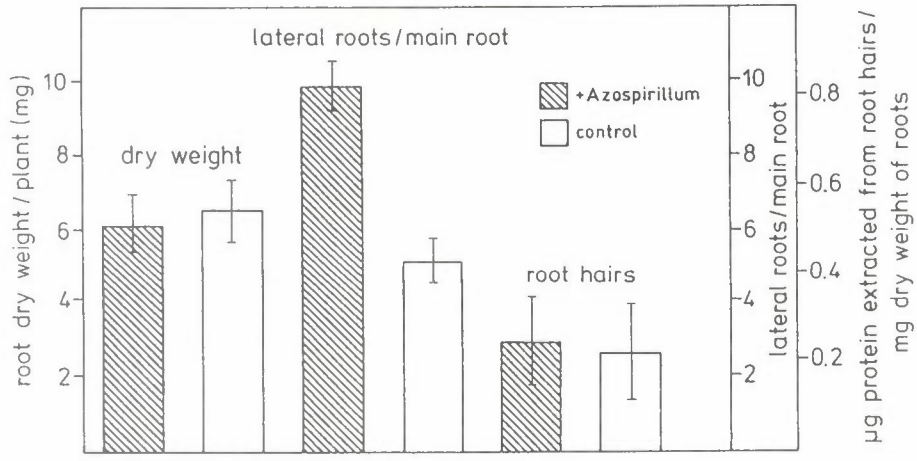

Figure 1. Growth of wheat in the presence of *Azospirillum*. Effects on dry weight, lateral root and root hair formation. Germinated wheat seedlings in the excised Eppendorf cups were placed on top of the nutrient solution and incubated with *Azospirillum*. The medium was exchanged after 6 d (without a further inoculation with *Azospirillum*). Lateral root formation was determined after 7 d and dry weight and root hairs after 10 d. For other experimental details see Materials and Methods.

of the roots was slightly enhanced (Fig. 1) but the effect was not statistically sound and was variable to some extent from experiment to experiment. In addition, as judged by eye, the number of root hairs increased by incubating the plants in the test tubes with *Azospirillum*. In the case of *Pisum sativum*, root hairs could be scratched off the roots with a painter's brush after freezing the roots in liquid nitrogen on a metal plate (Röhm and Werner, 1987). Using the same technique with wheat, the overall number of root hairs was, however, too small to give reliable differences between inoculated and non-inoculated material (Fig. 1). Thus, a good method to quantify the increase in root hair formation was not available. The effect of *Azospirillum* on the increase in lateral root formation could readily be counted and was always discernible, either when the test tubes were exposed to high or low light intensities, when the experiments were performed at 10, 20 or 30°C or when the test tubes were placed into a growth chamber or on a window bench. Experiments performed on the window bench often gave also increases in the root dry weight and resulted in the formation of longer seminal and nodular roots. The test tubes on the window bench were, of course, exposed to large fluctuations in light

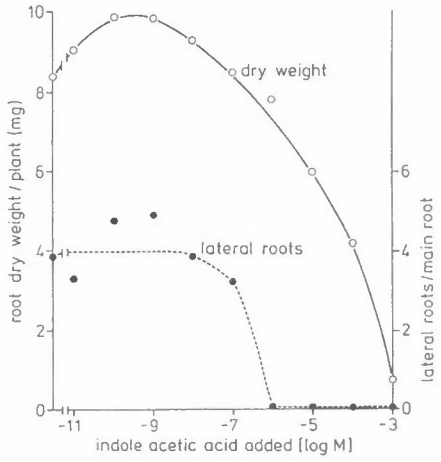

Figure 2. Growth of wheat in the presence of indoleacetic acid (auxin). The plants were grown for 10 d and the medium was exchanged after 6 d. Lateral root formation was counted after 7 d. Each point in the figure is the means of the data from 5 test tubes.

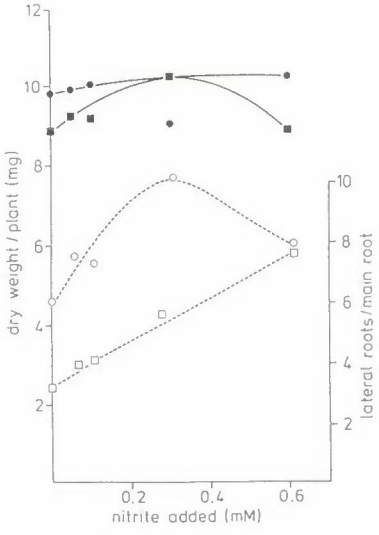

Figure 3. Growth of wheat in the presence of nitrite: Formation of lateral roots and effects on dry weight. The plants were grown for 10 d without an exchange of the medium.

□ - - - □ lateral root formation

■——■ dry weight

for plants grown in test tubes with additional 3.5 mM nitrate:

○ - - - - ○ lateral roots

●——● dry weights

intensities and in the temperature regime; therefore such increases in the dry weight were not consistently observed. When the germinated wheat seedlings were incubated with indole acetic acid (IAA) instead of *Azospirillum* in growth chamber experiments, the dry weight increased maximally by 20% (Fig. 2) and an enhancement of the root hair formation became visible. There was, however, virtually no effect on the induction of lateral roots. These experiments with IAA gave a typical dose response curve with higher amounts being strictly inhibitory (Fig. 2). The plants used up IAA completely within the first 6 d as determined by HPLC (not documented).

It had previously been shown that nitrite can exert phytohormonal effects similar to IAA in a number of assays like the straight growth test with *Avena* coleoptiles, the production of C_2H_4 by pea epicotyl segments and the test with wheat root segments in which the increase of wet weight was determined (Zimmer et al., 1988). In the test tube assay with the germinated wheat seeds used in this study, nitrite largely stimulated the formation of lateral roots (Fig. 3). When the test tubes contained 3.5 mM KNO_3 additionally, nitrite also induced the formation of lateral roots with an optimum at 0.4 mM NO_2^-. There was no statistically distinct effect of nitrite on the dry weight (Fig. 3). Root hair number did not change per single lateral root, but due to the drastic enhancement of the lateral root formation, the overall number of root hairs per plant might have increased.

The wheat seedlings used up 0.6 mM nitrite in the test tubes almost completely within 3 d (not documented). The effect of nitrite on the formation of lateral roots could be best seen after 6–7 d (Fig. 4). The stimulation was still detectable up to 10 d after the start of the experiments, although the controls without nitrite then had also formed more lateral roots (Fig. 4). Later stages of the plant development could not be examined due to the limitations of the experimental system.

In suspension cultures, *Azospirillum* excreted up to 3 mM nitrite formed by nitrate respiration (Neuer et al., 1985). Therefore the effects of *Azospirillum* and of nitrite on the root morphology could be related to each other. In the test tubes of the current experiments, the number of bacteria increased from 2×10^6 to $1–5 \times 10^7$ cells/ml within the first 2 d after inoculation and then gradually decreased either due to death or to penetration into the roots (Fig. 5). The bacteria actively reduced nitrate to nitrite, and after 2–3 d the concentration of nitrite in the medium decreased also. This could have been due either to nitrite assimilation by the plant cells or due to the action of the bacteria which had been shown to reduce NO_2^- to N_2 and partly to N_2O and NO (Danneberg et al., 1989; Voßwinkel et al., 1991). The four graphs selected for Fig. 5 and numerous others showed that the extent of nitrite excretion was somewhat

variable from experiment to experiment. Likewise, the effect of *Azospirillum* on the formation of lateral roots, though reproducible and consistently observed, showed also variations in its extent which can be explained by the different concentrations of nitrite excreted by the bacteria.

In the stimulation of lateral root formation, nitrite could not be substituted by sulfite (Fig. 6). It had been pointed out that nitrite alone can hardly exert phytohormonal effects (Zimmer et al., 1988). These authors showed that the effects of nitrite were enhanced by the addition of ascorbate in some assays like the C_2H_4-formation by pea epicotyl sections. It was, therefore, postulated that nitrite interacts with ascorbate in the plant cells to a component which in turn functions as phytohormone (Zimmer et al., 1988). The addition of low amounts of ascorbate to the tubes incubated with nitrite slightly increased the formation of lateral roots and did not cause a change in the dry weight (Fig. 7). Higher amounts of ascorbate were, however, inactive. The effect of low concentrations of ascorbate was observed in many experiments, but the stimulation never exceeded 10%. Thus, with respect to ascorbate, the present experiments gave not so clear-cut results as the tests on the C_2H_4-formation by pea epicotyl sections (Zimmer et al., 1988).

The addition of NH_4Cl (at concentrations of 2 mM and above) to the

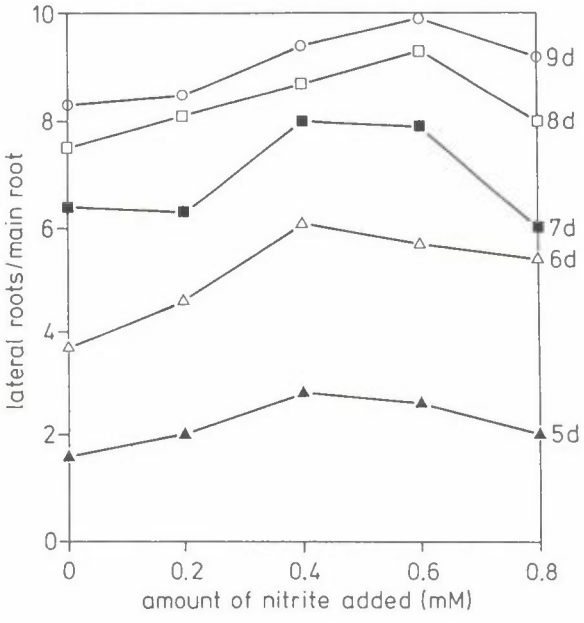

Figure 4. The stimulation of lateral root hair formation by nitrite in dependence of time. Lateral roots were counted each day between the 5th and the 9th day of growth.

nutrient solution caused a drastic decrease in the formations of lateral roots and of dry weight (Fig. 8). Nitrite could virtually not reverse the effect (Fig. 8). This decrease was probably not due to NH_4^+ itself, but was caused by H^+-excretion accompanied with NH_4^+-utilization. The wheat roots utilized NH_4^+ with a rate of approximately 1 μmol/ml × d in the test tubes (not documented). Roots are known to utilize NH_4^+ by a H^+/NH_4^+ antiport system (Nye 1981). Indeed, the pH dropped to pH 4.5 at the higher NH_4^+-concentration range, whereas it stayed constant at 7.5 in the tubes without NH_4^+ during the course of the experiment (Fig. 8). Thus, it cannot be concluded from this type of experiment that NH_4^+ counteracted with NO_2^-. A six-fold increase of the phosphate buffer concentration in the medium caused a general retardation of growth of the wheat seedlings which did not permit to perform the experiments with NH_4^+ and NO_2^- in such highly buffered solutions.

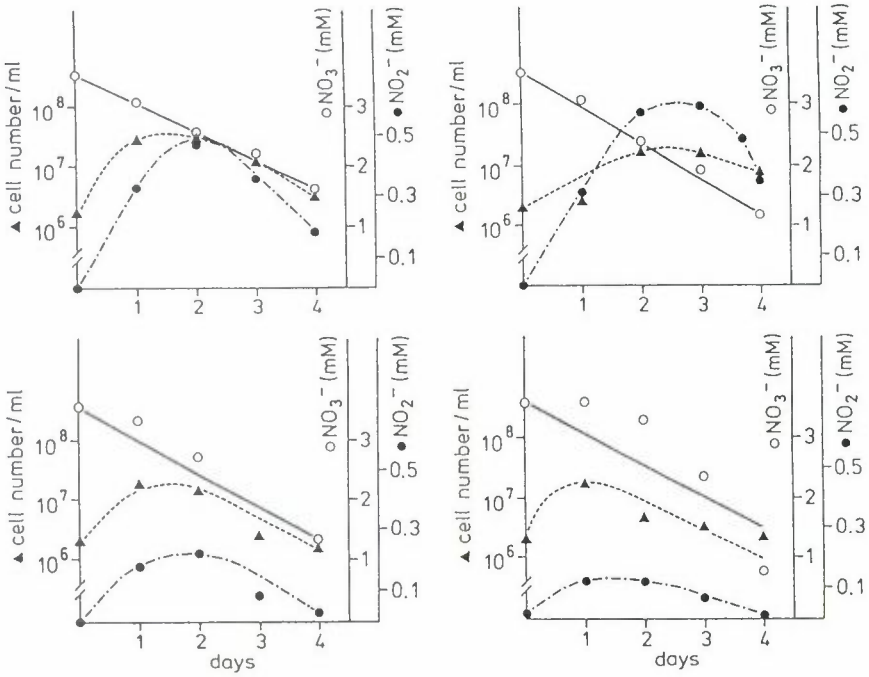

Figure 5. Cell number, nitrate and nitrite content in the medium of the test tubes in which a plant and *Azospirillum* had been grown for 4 d. For the daily determinations, an aliquot of the medium was removed from the tube by a Pasteur pipette under sterile conditions.

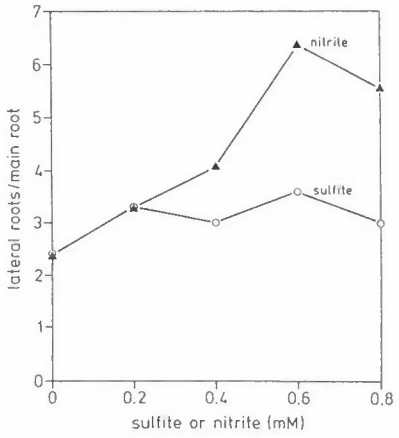

Figure 6. The stimulation of lateral root formation in wheat: Effect of nitrite and sulfite. Lateral root formation was counted after 7 d of growth.

4. Discussion

The present study showed that *Azospirillum* and auxin (IAA) affect the root morphology of wheat in a different way. Auxin slightly increased the dry weight at low concentrations, had no effect on the formation of lateral roots and apparently increased the density of root hairs as judged visually. As many other bacteria (see Sembdner et al., 1980), *Azospirillum* excreted auxin when the media contained tryptophan. The positive effects of *Azospirillum* on root growth can hardly be explained by the excretion of auxin alone. Basic text books on plant physiology teach that the auxin concentration in roots (but not in shoots) is optimal and that an addition of auxin generally causes a retardation rather than a promotion of root growth.

In the present study, the effects of *Azospirillum* on the wheat root morphology can better be correlated with those caused by nitrite. Both had only slight positive effects on the dry weight, caused a drastic increase on the ratio of lateral roots and had no significant effect on the formation of root hairs/lateral root. However, as the number of lateral roots was enhanced considerably, the total number of root hairs per plant seemingly increased in a parallel way. In the present experiments the effects of nitrite were only slightly enhanced by ascorbate and only in the low concentration range of this vitamin in contrast to the situation in the C_2H_4-formation assay by pea epicotyl sections. Such findings do not argue against an involvement of another compound like

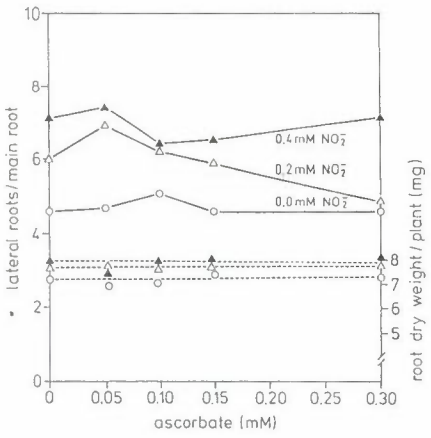

Figure 7. The effect of different concentrations of ascorbic acid on lateral root and on dry weight formation in wheat.
▲ addition 0.4 mM NO_2^- in the tubes
△ addition 0.2 mM NO_2^- in the tubes
o no nitrite
straight lines: lateral root formation, dashed lines: dry weights (determined after 7 d of growth).

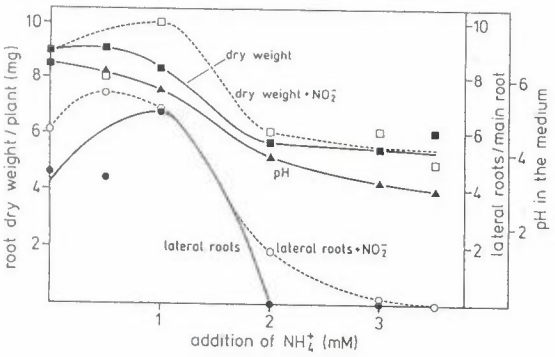

Figure 8. Growth of germinated wheat in the presence of NH_4^+: Lateral root formations and changes in pH. The plants were grown in the presence of different NH_4Cl amounts varying from 0.0–3.5 mM. To ensure an equal amount of combined nitrogen in all the tubes (= 3.5 mM), the different concentrations of NH_4Cl were supplemented with KNO_3. The Cl-concentration in each tube was also made up to 3.5 mM by adding KCl. The buffer was 1 mM K-phosphate pH 6.7. Where indicated, the nitrite content was 0.4 mM. Determinations were performed after 7 d of growth.

ascorbate besides nitrite in either systems. The optimal concentration of a phytohormone is variable for one morphogenetic response to the other, and the amount of ascorbate may have been almost optimal in wheat roots but not so in pea epicotyl sections.

The experiments reported here indicated that combined nitrogen did not limit root growth in the first 10–12 d of wheat development. The dry weight remained more or less constant by the addition of any form of combined nitrogen. By the action of either *Azospirillum* or nitrite, the root material available for synthesis was shifted from the formation of few thick seminal and nodular roots to the generation of many lateral roots. Such changes in total root morphology should be paralleled with an increase of the root surface area. An induction of the formation of branched and lateral roots by bacteria had earlier been described for other plants (Purnell, 1960; Malajczuk and Bowen, 1974) and was also reported to be caused by *Azospirillum* (Umali-Garcia et al., 1980). The effects of nitrite described here appear to be new. It had, however, been published for *Azospirillum* that mutants defective in nitrate reduction are inferior to wild-type strains in establishing plant-bacteria associations (Baldani et al., 1986) and are less effective in plant growth promotion (Boddey et al., 1986).

An increase of the surface area by the formation of additional lateral roots could enable the plants to better exploit the nutrients available, as suggested by Lin et al. (1983). *Azospirillum* could thereby enhance crop productivity or, more importantly, could ensure stable plant growth even in a year where the climate conditions are adverse. Another positive effect on plant health could be caused by the colonization of the roots by *Azospirillum* which could suppress infection by phytopathogenic organisms. To our knowledge, such an aspect has not yet been investigated in much detail for *Azospirillum*. Another unresolved question is the bacteria-plant specificity. *Azospirillum* preferentially lives in association with tropical grasses, whereas it does not occur abundantly in soils of the temperate zone where genera like *Pseudomonas, Bacillus, Enterobacter* and others dominate. Factors like auxin or nitrite can hardly be involved in establishing the association in the tropics, because, as said, many bacteria excrete auxin and nitrite. Thus components like lectin binding, chemotaxis and/or others (Reinhold et al., 1985; Gafny et al., 1986; Myers and Hubbell, 1987; Eyers et al., 1988) are more likely involved in the formation of the association between *Azospirillum* and cereals in the tropics.

Acknowledgements

This work was kindly supported by a grant from the BMFT (project no. 0318961 B9 through KFA Jülich).

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