INFLUENCE OF *AZOSPIRILLUM SPP.* ON THE NITROGEN SUPPLY OF A GRAMINEOUS HOST

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Proefschrift

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STELLINGEN[1]

1. Hedendaagse akkerbouw heeft een hoog energie verbruik. Om een voldoende voedselproduktie te garanderen dient de mens óf nieuwe en goedkope energiebronnen te vinden óf de energiebehoefte van de akkerbouw te verlagen.

2. Acetyleen-reductie als een indirecte maat voor stikstoffixatie is bruikbaar voor de bepaling van nitrogenase activiteit maar is niet bruikbaar om de stikstofwinst bij fixatie over een lange periode te berekenen.

3. Bij de ^{15}N isotoop-verdunningsmethode moet men rekening houden met de isotopenfractionering van de plant zelf en heeft daarom een betrouwbare, niet stikstof-fixerende plant ter controle nodig.

4. Grasachtigen hebben tijdens de evolutie geen nauwe band met stikstof-fixerende procaryoten ontwikkeld zoals de symbiose van *Rhizobium* met Leguminosen omdat grasachtigen een zeer dichte vegetatie vormen waar binnen de rhizosfeer een zeer efficiënte stikstof-turnover plaatsvindt.

5. De bestaande rhizosfeer-associatie tussen *Azospirillum* en grasachtigen moet eerder worden beschouwd als een parasitair systeem dan als een symbiotisch systeem.

6. Onderzoek op het gebied van de plant-bacterie associatie vereist voldoende kennis van de algemene microbiologie, de genetica, de plantenfysiologie en de bodemkunde.

7. Indien in Nederland gemiddeld 60 kg N per hectare aan minerale stikstof door luchtverontreiniging in de bodem terecht komt, dan is onderzoek op het gebied van biologische stikstof-fixatie 'water naar de zee dragen'.

8. Ook voor een microbioloog is het zinvol om een suikerbiet van haver te kunnen onderscheiden.

9. Een kenmerk van rhizosfeer bacteriën is de productie van phytohormonen waarmee zij de ontwikkeling van de gastheer wortel stimuleren.

10. Biologische stikstoffixatie is altijd verbonden met een hoog verbruik van metabolische energie, omdat gefixeerde NH_4^+ uit de cel lekt en dan door een aktieve ammoniumtransportketen in de cel terug moet worden gebracht.

11. Het steunen van de Europese landbouw met behulp van produktsubsidie is op lange termijn de duurste en inefficiëntste methode.

12. Veel milieu-activisten zijn niet bereid te erkennen dat de mens nèt als vroeger de zuurstof producerende bacterie of de dinosaurus, een absoluut noodzakelijk deel van het milieu uitmaakt. Het enige verschil is dat de mens pech heeft dit te begrijpen.

[1] Stellingen behorende bij het proefschrift "Influence of *Azospirillum brasilense* spp. on the nitrogen supply of a gramineous host" van Christian Christiansen-Weniger.

Wageningen, 2 oktober 1991.

CONTENTS

CHAPTER 1

INTRODUCTON

History

Azospirillum spp. was first described by Beijerinck (1922, 1925) as a Gram-negative motile rod. The organism, isolated from a dutch soil, was named first *Azotobacter spirillum* and later *Spirillum lipoferum*. Beijerinck could not prove unequivocally that this bacterium could fix nitrogen. Only 40 years later Becking demonstrated clearly that this microorganism was able to utilize $^{15}N_2$ under O_2 limited conditions in the presence of yeast extract (Becking 1963). After other similar bacteria were isolated, Tarand et al. (1978) suggested to combine these organisms under the genus name *Azospirillum*. The genus *Azospirillum* is described in literature as an obligate aerobic soil bacterium with a temperature optimum of 35°-37°C (Tarand et al. 1984). The high temperature optimum may explain, why *Azospirillum* is found in tropical or subtropical soils with cell numbers being hundred to thousand times higher than in soils of a temperate region (Baldani et al. 1983, De Coninck et al. 1988, Jagnow 1981, Magalhaes et al. 1979). Up to now four species of *Azospirillum* are known. *Azospirillum lipoferum* and *Azospirillum brasilense* are the best studied strains. They are reported to be Gram-negative to Gram-variable rods of vibroid shape with a single polar flagellum when grown on liquid media. Lateral flagella appear on solid media. Both strains develop small red colonies on congo-red containing medium. Characteristics differentiating both strains are the ability of *A. lipoferum* to use glucose as sole carbon source and its requirement of biotine for growth. Other than *A. brasilense*, *A. lipoferum* forms pleomorphic structures under alcaline conditions (Hartmann et al. 1985, Tarand et al. 1978). The characteristics of these two strains together with *Azospirillum amazonense* (Magalhaes et al. 1983) are given in Table 1. A fourth strain, *Azospirillum halopraeferans*, a salt tolerant strain of high temperature preference (40°C), has been described recently (Hartmann 1988, Reinhold et al. 1987).

Interest in *Azospirillum* has clearly increased after the report of Döbereiner and Day (1976) that the organism could colonize the rhizosphere of forage grass *Digitaria decumbens*, resulting in increased rhizosphere nitrogen fixation. Since then several other grasses, among them important crops as rice, corn, sorghum and wheat were found to be potential hosts for *Azospirillum* spp. (Hegazi et al. 1979, Vlassak and Bohool 1983, Lakshmi Kumari 1976, Lamm and Neyra 1981, Neyra and Döbereiner 1977, Staphorst and Strijdom 1978, Wong and Stenberg 1979).

Physiology of *Azospirillum*

Nitrogen fixation

The nitrogenase protein complex was isolated, purified and analyzed from *A. brasilense*, *A. lipoferum* and *A. amazonense* (Ludden et al. 1978, Okon et al. 1977, Song et al. 1985). The nitrogenase of *A. brasilense* is a three component system with a Mo-Fe protein (dinitrogenase), a Fe protein (dinitrogenase reductase) and an activating enzyme. Each component isolated by its own does not reduce N_2 (Ludden et al., 1978). Activation of the Fe protein requires manganese and ATP. Purified Mo-Fe protein of *A. amazonense* is composed of two subunits of 55000 and 50000 daltons. Nitrogenase activity of *A. amazonense* is independent of the availability of an activation enzyme or of manganese (Song et al. 1985). Similarities in structure and function of the *Azospirillum* nitrogenase with the nitrogenase of N_2-fixing *Rhodospirillum rubrum* was reported by Hartmann et al. (1986), Kanemoto and Ludden (1984) and Nair et al. (1983). Yet, the genetic control and expression of *Azospirillum* nitrogenase is likely similar to those of other diazotrophic bacteria (Bozouklian et al. 1986, Pedrosa and Yates 1984, Singh and Klingmüller 1988, Sing et al. 1989).

Two physiological characteristics of the nitrogenase activity of *Azospirillum* are of prime importance for its performance in the rhizosphere of plants.

1) The nitrogenase of *Azospirillum* is affected by free oxygen. Nitrogenase proteins are irreversibly inacivated by any O_2. Because of the poor protection of its nitrogenase against the toxic effect of O_2, pure cultures of *Azospirillum* have a narrow activity peak at a low oxygen tension of approximately 0.1-0.5 kPa O_2. No activity can be measured at oxygen tensions > 3 kPa and under anaerobic conditions (Hartmann 1988). Differences in nitrogenase oxygen sensitivity have been reported for *A. lipoferum*, *A. brasilense* and *A. amazonense* (Hartmann 1988). A shortterm protection of the nitrogenase enzymes against a temporal oxygen stress, comparable to that of *Azotobacter chroococcum* (Drozd and Postgate 1970) was reported by Hartmann et al. (1988).

2) Mineral nitrogen, NH_4^+ or NO_3^-, represses the nitrogenase activity of *Azospirillum* (Day and Döbereiner 1976, Hartmann et al. 1986, Nelson and Knowles 1987). N_2-fixation in all *Azospirillum* species is completely inhibited at NH_4Cl concentration > 1 mM. Reason for this is a strict feedback regulation of the nif gene transcription, which is thought to prevent the bacterium to continue the energy devouring N_2-reduction if other sources of nitrogen are available. Glutamine synthetase, which has a responsible function in bacterial nitrogen assimilation, was shown to be involved in these regulatory processes (Bani et al. 1980, Gauthier and Elmerich 1977).

N-metabolism

Azospirillum is able to grow aerobically on NH_4^+, NO_3^- or NO_2^- as sole nitrogen sources (Döbereiner 1983, Döbereiner and De Polli 1980). The keyenzymes for nitrogen assimilation in *Azospirillum* are glutamine synthetase and glutamate synthetase. Nitrogen assimilation via glutamate dehydrogenase, which is common in procaryotes growing under non nitrogen limited conditions, was found in all *Azospirillum* strains when grown on glutamate (Hartmann et al. 1988), but seems to play a minor role (Westby et al. 1987).

Table 1: Differences between *Azospirillum* spp. (from Döbereiner 1983; Okon 1982)

	A. amazonense	*A. lipoferum*	*A. brasilense*
Growth on medium with pH above 6.8	very poor	good	good
Colony type on potato agar	white flat raised margin	pink raised	pink raised
Tolerance to O_2 for nitrogenase activity	very low	low	low
Dissimilation of			
NO_3 --> NO_2	± [a]	+	+
NO_2 --> NO_2O	-	±	±
Cell width (nm, N_2 grown)	0.68 ± 0.08	1.0 to 1.5[b]	0.9 ± 0.03
Polar flagellum	+	+	+
Lateral flagella on nutrient agar	-	+	+
Polymorph cells in alcaline media	-	+	-
Biotin requirement	-	+	-
Use of sucrose	+	-	-
Generation time for N_2 dependent growth at optimal pO_2	10 h	5-6 h	6 h
DNA base comp. (mol % G + C)	67-68	69-70	69-70

[a] Explanation of signs: + positive in more than 90% of the strains
± positive in less than 50% of the strains
- negative

[b] Cells of *A. lipoferum* may become even wider and much longer in older alcaline cultures

The existence of an active ammonia uptake mechanism across the cell membrane by NH_4^+ carrier proteins was reported by Hartmann and Kleiner (1982) using ^{14}C labelled methylamine. NH_4^+ transport across cell membranes was found in several free living N_2-fixing bacteria, such as *Azotobacter*, *Klebsiella* and *Clostridium* (Kleiner 1981, 1984). Kleiner (1981, 1984) postulated that intracellularly formed NH_4^+ does diffuse through cell membranes along with the pH gradient, but that membrane bound proteins with high affinity for ammonium will immediately carry back this released NH_4^+. This mechanism is thought to be the reason why *Azospirillum* does not release fixed nitrogen to its environment (Hartmann et al. 1988).

Nitrate reduction

Assimilatory as well as dissimilatory nitrate reduction occurs in *Azospirillum* (Eskew et al. 1977, Magalhaes et al. 1983, Neyra and Van Berkum 1977). Under low oxygen tensions (PO_2 < 3 kPa) nitrate appeared to be rapidly reduced to NO_2^- (Nelson and Knowles 1978). Neyra et al. (1977) found nitrate reductase as well as nitrite reductase in nearly all examined strains of *A. lipoferum*, while several strains of *A. brasilense* lack the nitrite reductase system and accumulate nitrite under a low oxygen partial pressure O_2 or at anaerobic conditions (Magalhaes et al. 1978). In *A. amazonense* nitrite reductase activity was not found either (Okon 1982).

Scott et al. (1979) demonstrated that under anaerobic conditions nitrate as well as nitrite can function as electron acceptor which allows for anaerobic nitrogen fixation (Neyra et al. 1977). Denitrification was demonstrated to occur in *Azospirillum* when associated with plants (Bothe et al. 1983). This means that N_2 fixation and denitrification are parallel active. At low oxygen concentrations and in alkaline soils both processes may result in a loss of plant available nitrogen (Neurer et al. 1985).

C-metabolism

Azospirillum is a heterotrophic organism, which depends on a carbon metabolism via the citric-acid passway. Various organic acids, such as malic, succinic, lactic and pyruvic acid can be used by *A. brasilense*, *A. lipoferum* and *A. amozonense*. *A. brasilense* and *A. lipoferum* grow on glycerole in contrast to *A. amazonense*. All three strains grow on fructose, galactose and L-arabinose. Glucose is used by *A. lipoferum* and *A. amazonense*, but not by *A. brasilense*. Sucrose, D-ribose and xylose are growth subsrates for *A. amazonense*. None of the strains grow on manitol as sole carbon and energy source (Tarand et al. 1984, Martinez-Drets et al. 1985).

Auxine production

Azospirillum produces the auxine indole-acetic-acid (IAA) a plant growth substance, when DL-tryptophan as an essential precursor is present (Reijnders and Vlassak 1979, Tien et al. 1979). The metabolic pathways of IAA biosynthesis (Schneider and Wightman 1974) in *Azospirillum* are not clear. The discovery of different aromatic amino acid transferases makes it likely that a multiple pathway is followed via tryptamine and indol-pyruvic acid. (Ruckdäschel et al. 1988). The hypothesis that beside IAA other plant growth substances, such as gibberilin or

cytocinin, are produced by *Azospirillum* as well (Tien et al. 1979) could not be substantiated. Recently, Zimmer and Bothe (1988) reported the production of a yet unspecified substance with a stimulating effect on the development of wheat roots.

Isolation of Azospirillum

Methods for isolation and purification of *Azospirillum* spp. were described by Döbereiner and Day (1976), and then further modified by Okon et al. (1977), Albrecht and Okon (1980) and Bashan and Levanony (1985). In all these methods azospirilli are first enriched in semisolid (0.2% agar) nitrogen free mineral medium with 0.5% DL malic acid ,socalled NfB medium (Albrecht and Okon 1980). The nitrogen fixing microaerophilic *Azospirillum* with a considerable aerotaxis and a good motility will form a growth band typically 0.5 to 1.5 cm beneath the agar surface. This band is commonly referred to as subsurface pellicle. Isolation is performed on NfB plates, supplemented with congo red (4 g/l). *Azospirillum* grows out to typical small purple colonies (Rodriquez Careres 1982).

Azospirillum-plant interaction

Localisation of Azospirillum

The different species of *Azospirillum* show a distinct host-plant-specification. C_4 plants, such as maize and sorghum, are predominantly colonized by *A. lipoferum*, whereas C_3 plants, such as wheat, rice, oat and barley, are mostly host to *A. brasilense* strains lacking nitrate reductase activity (Baldani and Döbereiner 1980, Rocha et al. 1981). A specific chemotactical response of *Azospirillum* strains to various amino acids, sugars and organic acids is reported as well (Barak et al. 1983, Heinrich and Hess 1985, Reinhold et al. 1985). Significant differences in this chemotaxis are observed between *Azospirillum* strains isolated from C_4 and C_3 plants (Reinhold et al. 1985).

The nature of the association between *Azospirillum* and its host plant is still a matter of debate. Scanning electron micrographs showed *Azospirillum* settling with high densities on the root surface, closely bound with fibrillar material (Whallon et al. 1985, Murty and Ladha 1987). By using immuno-gold labelling, Bashan and Levanony (1988) demonstrated that *Azospirillum* also colonizes the intercellular spaces of the root cortex. However, whether *Azospirillum* is able to colonize inner plant tissues as was postulated by Patriquin and Döbereiner (1978), or even to infect plant cells as suggested by Tilak and Rao (1987), remains to be proven.

Influence of Azospirillum on the host plant

Nitrogen fixation of *Azospirillum* attached to roots was measured both in excised root samples and in undisturbed growing plants. (Albrecht et al. 1981, Charylulu et al. 1985, Watanabe and Lin 1984). A correlaton was found between the number of *Azospirillum* cells isolated from roots and the amount of rhizosphere nitrogenase activity (Bülow and Döbereiner 1975). Enhancement of

plant growth, nitrogen content and plant yield following *Azospirillum* inoculation have often been reported (Baldani et al. 1983, Baldani et al. 1987, Boddey et al. 1986, Kapulnik et al. 1981, Kapulnik et al. 1987, Pereira et al. 1988, Sarig et al. 1984, Schank et al. 1981). The effects reported for *Azospirillum* inoculation appear to be dependent on the type of the host plant, the *Azospirillum* strain used, and the environmental conditions of the experiment. For example, data on the stimulation of dry matter production as a response to *Azospirillum* inoculation vary from no significant response (Albrecht 1977, Barber et al. 1976) up to a stimulation of 80% (Nur et al. 1980). Increase in plant nitrogen content were reported in some cases to be 120% to 150% in *Azospirillum* inoculated grasses as compared to the non-inoculated controls (Cohen et al. 1980, Kapulnik et al. 1981). Whether these positive effects on plant growth are only due to the nitrogenase activity of the associated *Azospirillum* is doubtful. Isotope experiments with ^{15}N labelled nitrogen, directly by $^{15}N_2$ enriched atmospheres as well as indirectly using the ^{15}N-isotope dilution technique (Boddey et al. 1983) showed that very small amounts of the fixed nitrogen are transferred from the bacterium into host plant material (De Polli et al. 1977, Ito et al. 1980, Nayak et al. 1986, Owens 1977). Besides nitrogen fixation, other physiological characteristics of *Azospirillum* may be involved in plant growth stimulating effects of *Azospirillum*. Nitrate reductase activity supports the nitrate assimilation of the host plant (Ferreira et al. 1987, Scott et al. 1979). ^{15}N-isotope dilution experiments with nitrate reductase negative mutants of *A. brasilense* indicated that the nitrate reductase activity of root associated *Azospirillum* enhance the nitrogen uptake of wheat (Baldani et al. 1986, Boddey et al. 1986). These results were confirmed by Ferreira et al. (1987), who showed that the total nitrogen incorporation by wheat was significantly higher in plants inoculated with wild type strains of *A. brasilense* than in plants inoculated with nitrate reductase negative mutants.

Indole-acetic acid produced by *Azospirillum* stimulate root growth and root development, which result in a enhanced nutrient and water uptake of the host plant (Barton et al. 1985, Reinders and Vlassak 1979, Umalia-Garcia 1980). The increased uptake of, in particular, nitrate, phosphate and potassium by the plant was postulated by Okon (1982) as a "sponge effect" of an *Azospirillum* inoculation. Effects of associated *Azospirillum* on root elongation, branching and root hair differentiation were reported to be comparable to those of a pure IAA addition (Harari et al. 1988, Kapulnik et al. 1985, Patriquin et al. 1983). Mutants lacking the capacity to produce auxin were not able to increase root elongation or lateral root formation (Barbieri 1986). Mutants with an increased production of auxin did not further affect root development of the host plant (Harari et al. 1988).

Outline of this thesis

Most ecological, physiological and genetical studies have been performed with *Azospirillum* strains of tropical origin. For example, the best studied *A. brasilense* strain is strain Sp 7 (ATCC 29145), an isolate from a brazilian soil (Day and Döbereiner 1976). In view of a possible application in temperate climates and realising that bacteria adapt to prevailing climatic conditions, azospirilli were isolated for this study from wheat, maize and a forage grass (*Poa pratense*),

grown under temperate conditions.

Much of the present information on the association of *Azospirillum* and plants is based on either uncontrolled field experiments or on experiments in artificial systems without soil or with removed roots. In order to obtain unambiguous information on the ecology and activity of *Azospirillum* in the rhizosphere, experimental approaches are needed which allow for studies with optimal control of experimental conditions of plant and soils. The objectives of this study were the investigate the behaviour of *Azospirillum* in the rhizosphere of wheat in relation to mineral nitrogen fertilization and O_2-concentrations in soil. As nitrogen fixation of *Azospirillum* is strictly dependent on free oxygen, mineral nitrogen and the soil temperature, nitrogenase activity was determined in a controlled root environment system. To exclude competition between introduced *Azospirillum* and the native microbial rhizosphere population, the root part of the host plant was kept gnotobiotic over the entire period of plant development.

The growth system that was developed to allow for experiments under these controlled conditions is described in the chapters 2 and 4. In these chapters results are presented on the reaction of root associated *A. brasilense* on changing environmental conditions and on the effect of *A. brasilense* on plant development and on nitrogen uptake. The influence of the plant itself on the association of *A. brasilense* through the production of specific root exudates is described in the chapters 3 and 4. The influence of plant-phytohormones on *A. brasilense* growth and nitrogenase activity is given in chapter 5.

In order to determine the establishment and the survival of introduced *A. brasilense* in natural soils, it was necessary to provide a physiological marker to *A. brasilense*, which allowed a selective reisolation. A double antibiotic resistance was chosen as a marker which was introduced to the bacterium by a transposon (Tn5) insertion into the genome. Together with the ability of *A. brasilense* to grow on N-free medium and to form red colonies in the presence of congo red, reisolation of the targed *A. brasilense* cells at a low detection limits was possible (Chapter 6).

In order to stimulate the transfer of fixed nitrogen from *A. brasilense* to its host we isolated mutant strains, which lack its ammonia transport system across cell membranes and which allow fixed nitrogen to leak out of their cells. A physiological description of such mutants as well as their introduction to wheat is reported in chapter 7.

The present study was aimed to answer the following questions:

- Does *Azospirillum* under temperate soil conditions establish cell densities in the plant rhizosphere which are high enough to cause a significant effect on the host plant?

- What is the role of competitive bacteria in the association between *Azospirillum* and the host plant?

- Does the plant play an active role in the colonization and activity of *Azospirillum*?

- Does *Azospirillum* fix atmospheric nitrogen under the conditions provided by an active growing root?

- How much of the fixed nitrogen is transferred to the host plant?

- Does mutant *Azospirillum*, which releases substantial amounts of fixed nitrogen to its environment, enhance the nitrogen supply of the host?

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CHAPTER 2

NITROGEN FIXATION OF *AZOSPIRILLUM BRASILENSE* IN SOIL AND RHIZOSPHERE UNDER CONTROLLED ENVIRONMENTAL CONDITIONS [1]

Summary

Acetylene reduction activity (ARA) of *Azospirillum brasilense*, either free living in soils or associated to wheat roots, was determined in a sterilized root environment at controlled levels of oxygen tension and with different concentrations of mineral nitrogen. The nitrogenase activity in an unplanted inoculated soil remained low at approximately 40 nmol C_2H_4 . h^{-1} per 2 kg fresh soil, increasing to 300 nmol C_2H_4 . h^{-1} when malic acid was added as carbon source via a dialyse tubing system. Nitrogen fixation of *A.brasilense* in the rhizosphere of an actively growing plant was much less sensitive to the repressing influence of free oxygen as compared with free living bacteria. An optimum nitrogenase activity was observed at 10 kPa O_2 with relatively high activities remaining even at an oxygen concentration of 20 kPa. Nitrate as well as ammonia caused a repression of nitrogenase activity which was less pronounced in the presence, than in the absence of plants. Highest survival rates of inoculated *A.brasilense* and highest rates of acetylene reduction were found in plants treated with *Azospirilli* immediately after seedling emergence. Plants inoculated during a later stage of growth showed a lower bacterial density in the rhizosphere and as a conseqence also a lower nitrogen fixing potential. Subseqent inoculations with *A.brasilense* during plant development did neither increase root colonisation nor stimulate associated acetylene reduction. Using the ^{15}N dilution method, the response of an *A.brasilense* inoculation to plant nitrogen was calculated to be 0.067 mg fixed nitrogen per plant, *i.e.* 3.3 % of the nitrogen in the root and 1.6 % in the plant shoot were found to be of atmospheric origin. This ^{15}N dilution was comparable to that in plants inoculated with a non N_2-fixing *Pseudomonas fluorescens.*

Introduction

Nitrogen fixing *Azospirillum* species are known to establish succesfully in the rhizosphere of gramineous plants (Tilak and Rao 1978; Whallon et al. 1985). Nitrogen fixation by root associated *Azospirillum* spp. has been reported both

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[1] Accepted by *Biol. Fertil. Soils*

from field and from *in vitro* experiments, but the results were not consistent (Bothe et al. 1983; De Polli et al. 1982, Watanabe and Lin 1984). This has been attributed to the fact that bacterial nitrogenase is strongly affected by the environment, in particular by the presence of oxygen and mineral nitrogen (Balandreau and Dommergues 1973; Day and Döbereiner 1976; Okon et al. 1977). The nitrogenase activity of *A.brasilense* reaches its maximum at 1.7 kPa O_2 and little or no activity remains at oxygen tensions of 3 kPa O_2 and above, whereas no nitrogenase activity was found under anaerobic conditions. Both NO_3^- and NH_4^+ strongly influence nitrogen fixation by *A.brasilense* as well (Day and Döbereiner 1976; Nelson and Knowles 1978). Nitrogenase activity is completely inhibited after the addition of 1 mM NH_4^+ to a N_2-fixing *Azospirillum* culture (Hartmann et al. 1985). 10 mM NO_3^- represses the nitrogenase activity of *Azospirillum* by more than 90% (Magalhaes et al. 1978).

Biological fixation of nitrogen in a plant-soil system is strictly dependent on soil oxygen tension and the concentration of mineral nitrogen (Alexander et al. 1987; Alexander and Zuberer 1988). To determine the nitrogen fixation of an intact *Azospirillum*-plant association, the oxygen tension as well as the concentration of available mineral nitrogen in the rhizosphere have to be controlled during the entire period of determination. This is very important since the relative contribution of nitrogenase activity of root associated *Azospirillum* to the nitrogen supply of the host plant is as yet a matter of debate (Kapulnik et al. 1981, Kapulnik and Okon 1983, Mayak et al. 1986, Schank et al. 1981). In this paper, we describe a system which allows an accurate measurement of *in situ* nitrogenase activity of an *Azospirillum* rhizosphere population under controlled conditions of oxygen tension, nitrogen concentration and temperature. In addition, the amount of fixed nitrogen which is translocated to the host plant can also be quantified. In this plant-soil system roots of wheat could be kept gnotobiotic over the entire period of growth to exclude competition and cross-reaction with bacteria other then the introduced *A.brasilense* cells.

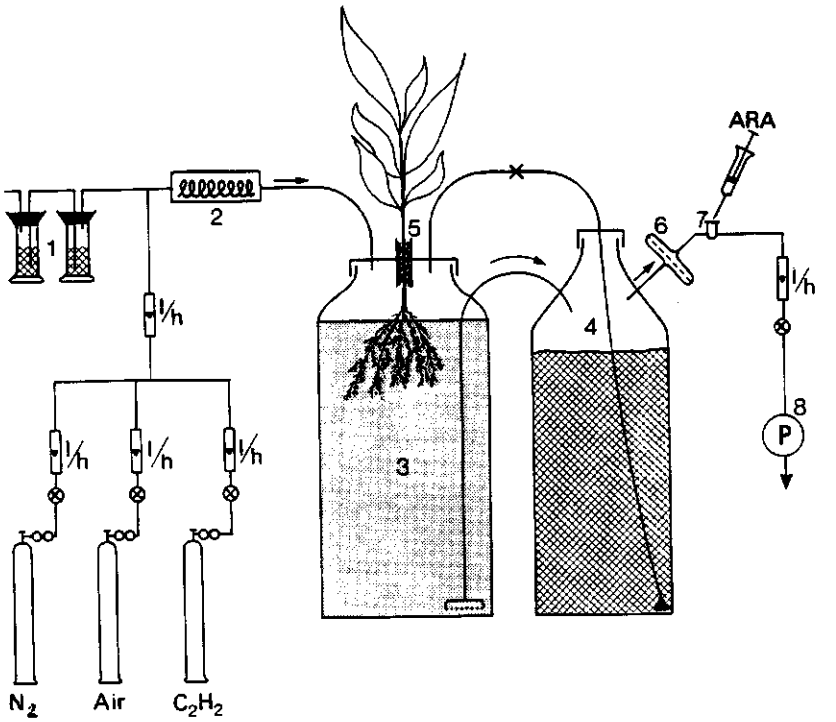
Materials and methods

Growth system

The plant-soil system is described in Figure 1. Each 2 l growth container was filled with 2 kg of either a carefully washed sterile sea- sand or sterile soil. For rhizosphere experiments wheat plants were grown in this system on a washed sand being sealed airtight around the stem with a paraffine/lanoline seal (5% / 95%) to keep the root compartment sterile. Sterilised Hoagland nutrient solution (Hoagland and Broyer 1936) was supplied to the plants by flooding and subsequent draining of the growth container. Aeration was carried out with a aspiration pump via membrane filter (0.2 µm). The oxygen content of the flushing air was controlled by a continuous flow panel. In an attempt to imitate the organic carbon exudation by the root, a dialyse tubing system (2 cm diameter, 30 cm length, 1884 cm^2 surface) was placed into the soil or seasand of an unplanted growth system. A 0.5% solution of malic acid was circulated continuously at a flow rate of 3 $ml.min^{-1}$ through the tubing.

Soils and plants

The soil was an unfertilised loamy sand (0.3 mg kg^{-1} Nt, 3.5% OM, 15% moisture, pH 5,3), sterilized with 4 Mrad γ radiation. Sterilized water was added daily and adjusted to constant weight.



1: Communicating tubes **2: Cotton filter** **3: Growth container**
4: Nutrient solution **5: Lanoline seal** **6: Membrane filter**
7: Sampling port

Figure 1: Functional design of a plant growth system for *in situ* determination of rhizosphere acetylene reduction under controlled environmental conditions.

The sea sand (river sediment) used was carefully washed with demineralized water to eliminate residual mineral nitrogen and heat sterilized at 120°C. Spring wheat, *Triticum turgidum* vr. "Carasinho" (obtained from the Institute for Plant Breeding SVP, Wageningen) was used as plant material. This cultivar develops an effective association with *A.brasilense* (Christiansen- Weniger, unpublished results). Seeds were surface sterilised by shaking in a 1.5% solution of Na-hypochloride during 15 min. and pregerminated on trypton-soya agar to check for

sterility. Surface sterile seedlings were transferred after 8 days to the growth containers and sealed immediately with the lanoline-parafine seal. The plants were grown under a regime of 12 hours day at 20°C and 12 hours night at 15°C. Relative humidity was kept at 70%. The nutrient solution was changed every fifth day.

Bacteria

The bacterium used in all experiments was *Azospirillum brasilense* Wa3, a strain isolated from the rhizosphere of a field grown with summer wheat. A *Pseudomonas fluorescens*, strain R2f, isolated from the rhizosphere of grassland (Van Elsas et al. 1988) served as a control organism . The bacteria were grown overnight on Luria-broth medium, washed and resuspended in 0.8% NaCl solution. For inoculation 30 ml of this cell suspension was injected to each pot. The total number of inoculated bacteria was approximately 3.10^8 cells per pot, which was equal to a final density of $1,5.10^5$ cells per gram fresh soil or sand. Inoculation with *A.brasilense* was done at different days after planting (Table 1). Counting of root associated *A.brasilense* was performed as follows: Carefully washed roots were macerated in homogenising tubes, diluted in ten fold steps and plated out on a solid congo-red containing NFB minimal medium (Bashan and Levanony 1985).

Table 1: Different inoculation treatments of wheat with *Azospirillum brasilense*. Acetylene reduction assay and plant harvest took place at day 49.

	Inoculation with $5*10^8$ CfU[a] $plant^{-1}$			
	Day 1	Day 15	Day 30	Day 45
Treatment I	+	+	+	-
Treatment II	+	-	-	-
Treatment II	-	-	+	-
Treatment IV	-	-	-	+

[a] Cell-forming units

Biochemical assays

Acetylene reduction assay (ARA) of pure bacterial culture was performed in a semisolid (0.2% agar) nitrogen free minimal NFB medium (Okon et al. 1977a) with 10% v/v acetylene in the head space. The total bacterial protein was analysed after cell lysis in 1 N NaOH at 60°C according to Lowry et al. (1951) using bovine-serum-albumine as standard. Auxin formation was tested by incubating *A.brasilense* for 48 hours in a batch of NFB medium containing 10 mM NH_4Cl and

100 mg/l DL-tryptophan. Produced Indole-acetic-acid (IAA) was determined in the supernatant after centrifugation by Salkofski color reaction (Tang and Bonner 1946). Nitrate-reductase activity was qualitatively determined in a semisolid (0.2% agar) NFB medium with 8 mM NH_4NO_3 by the method of Nocholas and Nason (1957).

Acetylene reduction assay

For ARA the pots were transferred to a water bath with a constant temperature of 23°C. The air outlet was connected to an autosampling gaschromatograph (Varian 1700), equipped with porapack T column and a FID detector. Prior to ARA the Hoagland nutrient solution was removed from the system by flooding and subseqent draining of each pot with a solution of 2 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.4 mM Ca $(HPO_4)_2$ and trace elements according to Hoagland. Before assay the entire system was continuously flushed for 30 min with a gasmixture of air and at a flow rate of 2.5 $l.h^{-1}$ pot^{-1}. The oxygen tension was chosen dependent on the experiment in a range from zero to 20 kPa O_2 and acetylene was added to a final concentration of 10% v/v. Ethylene formed as a product of nitrogenase activity was determined for each pot separately after approximately 3 hours of incubation. An ethylene standard was given to uninoculated and unplanted pots. It was ensured that no ethylene was formed by the plants themselves.

In order to determine the influence of mineral nitrogen on acetylene reduction each pot was flooded and subseqently drained with a nutrient solution as described above, containing either 1) no mineral nitrogen, 2) 2.5 mM or 5 mM KNO_3 or 3) 2.5 mM or 5 mM NH_4Cl. This exchange of nutrients were carried out 12 hours before and a second time immediately before acetylene reducton assay.

Nitrogenase activity of a pure culture of *A.brasilense* under different conditions of oxygen and mineral nitrogen was determined using an overnight culture grown on Luria broth, washed and resuspended in either nitrogen free or KNO_3 or NH_4Cl containing NFB medium. Four ml of this cell suspension were injected into airtight 50 ml serum bottles with pure in the headspace. Air was added in different amounts to obtain the final concentration of oxygen desired. Of the headspace 10 % was replaced with acetylene and formed ethylene was determined after intense shaking at 30°C. ARA started after five hours of incubation to enable the bacteria to form an active nitrogenase system.

^{15}N dilution technique

In order to determine to which extent the plant benefits from bacterial nitrogen fixation a ^{15}N dilution measurement was carried out. Plants were grown until the end of grainfilling (70 days). Nutrition was by a half strength Hoagland nutrient solution with $K^{15}NO_3$ concentration of 0.95 atom% excess ^{15}N . Inoculation took place soon after planting with either *Azospirillum brasilense* Wa3 (5.10^8 CFU per pot) or with *Pseudomonas fluorescens* R2f ($8,7.10^8$ CFU per pot). Uninoculated plants were taken as controls. All treatments were carried out with four replications. In situ nitrogenase activity was measured prior to harvest at 23°C and at 12 kPa O_2 in the growth system. Plants were divided after harvest in roots, stems with leaves and pannicle for separate determination of dry weight, N

content by Kjeldahl analysis (Bremner and Shaw 1958) and $^{14}N/^{15}N$ ratio by mass spectrometry (Finnigan MAT 250) according to Bremner (1965). Plant-nitrogen, originating from the atmosphere was calculated with the equation (Boddey et al. 1983):

$$\% \text{ N from fixation} = 1 - \frac{^{15}\text{N\% in the plant}}{^{15}\text{N\% in the control}} \times 100$$

Statistical analysis

All experiments were carried out in a randomised block design. Data were statistically assayed by analyses of variance, using the statistical program Genstat 5, release 1.3 (Genstat 5 Committee, 1987). Standard errors of differences were calculated by Student t test. All significant differences reported are at a level of P = 0.05 at least.

Results

In the sea sand inoculated with *A.brasilense* the nitrogenase activity at a constant oxygen tension of 2 kPa O_2 increased constantly during 7 days up to a rate of 60 nmol $C_2H_4.h^{-1}.pot^{-1}$, whereas nitrogenase activity in soil reached its maximum 4 days after inoculation (Fig. 2). In both environments the final nitrogenase activities reached a low level of appprox. 30-60 nmol $C_2H_4.pot^{-1}.h^{-1}$ after 7 days. A constant supply of additional carbon as malic acid via a dialyse tubing system resulted in a strong increase of nitrogenase activity within 3 days after inoculation with final activities up to six-fold the values measured in the same environment without a carbon addition. There was no significant difference between soil and sea sand when malic acid were added.

Batch-grown *A.brasilense* as well as bacteria attached to a dialyse system were strongly influenced by free oxygen with a narrow nitrogenase activity peak at 1.7 kPa O_2 (Fig.3). At oxygen tensions above 5 kPa little activity was observed. However, in association with an active plant root, N_2-fixation increased with the oxygen tension up to 15 kPa O_2. In plants where inoculation took place in an early stage of plant growth, the nitrogenase activity in the rhizosphere reached a maximum of approximately 600 nmol $C_2H_4.pot^{-1}.h^{-1}$ at more than 10 kPa O_2 and it decreased only slightly at higher oxygen tensions. Inoculation in a later phase of plant development resulted in much lower levels of nitrogen fixation and furthermore in a higher sensitivity of the nitrogenase to higher O_2 concentrations. When *A.brasilense* was added only a few days before harvest the maximum nitrogenase activity was relatively low with 200 nmol C_2H_4 $pot^{-1}.h^{-1}$ with nearly no activity at an oxygen level of 20 kPa (Fig. 3). Based on these data all following acetylene reduction assays were done at 12 kPa O_2 for a rhizosphere association and at 1.7 kPa O_2 for plant-free *A.brasilense* culture .

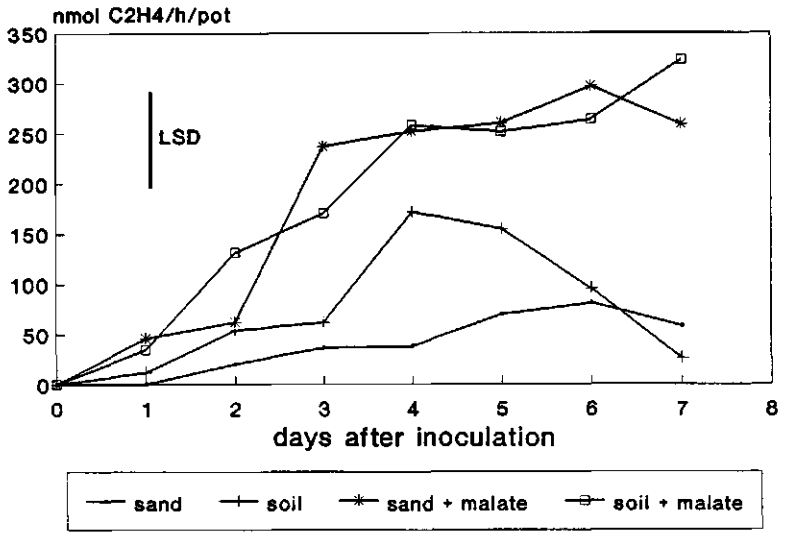


Figure 2: Acetylene reduction activity of *Azospirillum brasilense* in a sterile environment at a constant oxygen tension of 2 kPa and at 23°C. Inoculation took place in a soil and a sea sand with and without a carbon (malate) addition via a dialyse tubing system.

Mineral nitrogen, ammonia as well as nitrate reduced nitrogen fixation. The nitrogenase activity of freliving *A.brasilense* is completely repressed by a NH_4^+ concentration above 2.5 mM and it is strongly reduced by 2.5 mM NO_3^- (Table 2). Similar values were found for the dialyse tubing system. When associated with 2actively growing roots nitrogenase activity of introduced *A.brasilense* is less sensitive to the presence of mineral nitrogen. When plants were treated with 2.5 mM NH_4^+, up to 39% of the activity measured under nitrogen free conditions still remained. Even at NH_4^+ concentrations of 5 mM nitrogenase activity was detectable in the rhizosphere. Relatively high values for nitrogen fixation (40% to 80% of the initial activity) were still present at 2.5 or 5 mM NO_3^-.

The final nitrogenase activities as well as the total number of *A.brasilense* cells on the roots were highest in plants inoculated immediately after planting (Table 3). Additional inoculations did not cause a significant increase of neither the number of surviving bacteria nor the nitrogenase activity. Lower numbers of root associates and considerably lower nitrogen fixation rates were found when inoculation took place at a later stage of plant development. The specific nitrogenase activities, calculated as nmol $C_2H_4 \cdot h^{-1}$ per 10^4 cells were in the range of 2.7 to 3.3 without significant differences between the inoculation treatments.

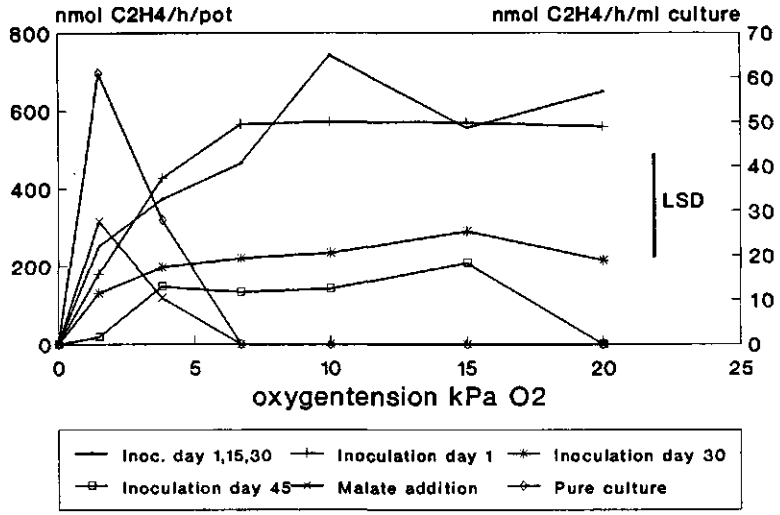


Figure 3: Effect of increasing oxygen tension on the nitrogenase activity of *Azospirillum brasilense*, inoculated to wheat at different stages of plant development (Table 1) or attached to a dialyse tubing system with a constant malate addition. Acetylene reduction assay took place at day 49 after planting. The influence of oxygen on the nitrogen fixation of batch grown *A.brasilense* is given as control (nmol C_2H_4 h^{-1} ml^{-1} bacterial culture).

In the ^{15}N dilution experiment a *Pseudomonas fluorescens* strain (R2f) was used as a non-nitrogen-fixing control bacterium. *P.fluorescens* R2f produces, similarly to *A.brasilense*, auxin (IAA) when grown on DL-typtophan containing medium and it also shows nitrate-reductase activity. No N_2-fixation was detected when incubated on a semisolid (0.2% agar) nitrogen free-minimal medium (Table 4).

In comparison to uninoculated control plants, *A.brasilense* as well as *P. fluorescens* caused an increase of root and shoot biomass although not statistically significant (Table 5).

Table 2: Acetylene reduction activity (ARA) in wheat roots inoculated with *Azospirillum brasilense* at different stages of plant development (Table 1) or attached to a dialyse tubing system with a constant malate addition. ARA was measured at day 49 after planting and at different concentrations of NO_3 and NH_4. Pure culture grown *A. brasilense* is given as control.

	(nmol C_2H_4 h^{-1} $plant^{-1}$)				
	No N added	KNO_3		NH_4Cl	
		2,5 mM	5 mM	2,5 mM	5 mM
Inoc. day [a] 1,15,30	556 (100)[d]	377 (67.8)	223 (40.1)	216 (38.9)	62 (11.2)
Inoculation day 1	569 (100)	469 (82.4)	395 (69.4)	222 (39.0)	54 (9.5)
Inoculation day 30	291 (100)	212 (72.9)	118 (40.6)	111 (38.1)	29 (10.0)
Inoculation day 45	208 (100)	85 (40.9)	20 (9.6)	61 (29.3)	13 (6.3)
Malate [b] addition	325 (100)	14 (4.3)	6 (1.9)	8 (2.5)	8 (2.5)
Pure [c] Culture	52.6 (100)	4.6 (8.7)	2.8 (5.3)	0 (0)	0 (0)
SED	76	88	77.5	79.8	93.4

a) Treatments as given in Table 1. Values are means of two replicates.
b) Values are means of six replicates.
c) Activity is measured as C_2H_4 h^{-1} ml^{-1} culture, values are means of two replicates.
d) Data in parantheses represent percentage of activity compared to the values obtained with no mineral nitrogen added.
SED: standard error of difference

Table 3. Acetylene reduction activity (ARA), total number of root attached bacteria and the specific nitrogenase activity at wheat roots inoculated with *Azospirillum brasilense* at different stages of plant development (Table 1). ARA and harvest took place at day 49 after planting.

	Acetylene reduction	Total bacteria	Specific activity
	nmol C_2H_4 h^{-1} $plant^{-1}$	log CfU[a] $plant^{-1}$	nmol C_2H_4 h^{-1} 10^4 CfU
Inoc. day 1, 15, 30	556	6.3	3.1
Inoculation day 1	569	6.28	3.0
Inoculation day 30	241	5.90	3.3
Inoculation day 45	213	5.90	2.7
SED	76	0.17	1.34

[a] Cell-forming units
SED: standard error of differences
Values are means of two replicates

Table 4: Auxin (IAA) production, nitrogen fixation and nitrate reductase activity of *Azospirillum brasilense* and *Pseudomonas fluorescens*.

	IAA production	Nitrogen fixation	Nitrate reductase
	(μg IAA ml^{-1} 24 h^{-1})	(nmol C_2H_4 min^{-1} mg^{-1} protein)	
Azospirillum	5.2	14.4	+
Pseudomonas	1.35	0	+

ARA was observed in the *A.brasilense* inoculated pots but not in the controls and in *P.fluorescens* treatments. When $K^{15}NO_3$ (0.95 atom % excess ^{15}N) as the sole nitrogen source was applied, ^{15}N dilution occurred in roots and shoots of all plants including the non-inoculated controls. ^{15}N dilution was in general higher in roots than in plant shoot material. In comparison to the control plants the inoculation with *A.brasilense* as well as with *P.fluorescens* caused a significant decrease of the $^{15}N/^{14}N$ ratio in both roots and shoots. Lowest ^{15}N contents were found in a treatment of a combined *Azospirilli* and *Pseudomonads* inoculation.

Table 5. Dry weight, nitrogen content, ^{15}N dilution and acetylen reduction (ARA) of wheat plants inoculated with *Azospirillum brasilense* or *Pseudomonas fluorescens*. $K^{15}NO_3$ as sole plant nitrogen source was labelled with 0.95 atom % excess ^{15}N.

	Dry weight (mg)		Nitrogen content (%N)		^{15}N dilution (Atom% ^{15}N		ARA ($nmolC_2H_4$ h^1plant^{-1})
	Root	Top	Root	Top	Root	Top	
Control	187	159	0.39	1.81	0.860	0.937	0
AZO	242	182	0.31	1.48	0.832	0.924	173
PS	432	183	0.30	1.54	0.836	0.907	0
AZO+PS	429	182	0.23	1.22	0.801	0.879	129
SED	148	46	0.07	0.25	0.01*	0.007*	94.6

Control : Uninoculated plants
AZO : *A. brasilense*
PS : *P. fluorescens*
SDS : Standard error of differences (* significant P<0.05)
Values are means of four replicates

When using these ^{15}N dilution rates to calculate the amount of plant nitrogen that comes from a source other than mineral fertilization, both *A.brasilense* and *P.fluorescens* seemed to add extra nitrogen to plants on top of the NO_3^- uptake (Table 6). The nitrogen gain due to an *A.brasilense* inoculation was highest in the plant root (3.25% of the nitrogen), but with only a part of this nitrogen transferred to the upper plant part. The total nitrogen support by *A.brasilense* of 0.067 mg N per plant was half as high as compared to the *P.fluorescens* inoculation treatment

(0.128 mg N per plant). When *A.brasilense* and *P.fluorescens* were inoculated together, the nitrogen gain for each plant part was in the range of the additional effect calculated for each single inoculation (Table 6).

Discussion

The increase of nitrogenase activity found in an unplanted soil or sea sand when malic acid is offered constantly via a dialyse tubing system indicates that in a natural environment the availability of a carbon source will be an important limiting factor for bacterial activity. This may also explain the activity peak occurring in the soil a few days after inoculation (Fig.2), which is contributing to the release of easily available organic carbon in the beginning of the experiment, possibly enhanced by preparation and gamma radiation of the soil. This allows an active bacterial population, which comes to starvation when this source of substrate is consumed. This confirms, that the activity of root associated *Azospirillum*, as well as of other rhizosphere bacteria, is dependent on root carbon exudation (Beck and Gilmour 1983; Newmann 1985).

Table 6: Relative nitrogen gain in root, stem and leaves and panicle of wheat plants due to bacterial inoculation as calculated by ^{15}N dilution technique.

	Nitrogen gain					
	(% N of plant N)			(mg N)		
	AZO	PS	AZO+PS	AZO	PS	AZO+PS
Panicle	1.17	2.89	5.70	0.009	0.026	0.035
Stem,leaves	1.56	3.43	6.35	0.030	0.066	0.102
Root	3.25	2.83	6.88	0.028	0.037	0.068
Total plant	1.88	3.12	6.39	0.067	0.128	0.205

AZO : *Azospirillum brasilense*
PS : *Pseudomonas fluorescens*
Values are means of four replicates

It has been suggested before, that this dependency on root exudation is the reason for the variation in rhizosphere nitrogen fixation found in different varieties of the same plant species (Kortzky et al. 1988).

The results reported here demonstrate that the nitrogenase enzyme system of root colonizing *A.brasilense* is protected against inhibitory environmental influences such as the presence of oxygen and mineral nitrogen. In particular, the negative effect of oxygen on nitrogenase activity was highly reduced in the root. This is in contrast with results found in comparable experiments for N_2-fixation in the rhizosphere of various grasses in natural environments (Alexander et al. 1987; Zuberer and Alexander 1988; Döbereiner et al. 1973).

In experiments with excised rice roots the associative nitrogen fixation had an optimum value at a very low oxygen tension of 0.25 kPa and decreased to zero when the oxygen concentration was more than 2.5 kPa O_2 (Van Berkum and Sloger 1982). The reason why the nitrogenase activity of associated *A.brasilense* in our experiments showed relatively high activities at O_2 concentrations of 10 kPa O_2 or more might be, that *A.brasilense* was inoculated to the plant under strictly monoxenic conditions, excluding the competition with other rhizosphere bacteria. This enabled introduced *A.brasilense* to colonize the inner rhizosphere and to settle inside root niches, thus being protected against the environmental influences mentioned. Such niches may be close to the root surface, in the mucigel layer or in the intercellular spaces of the cortex. That *Azospirillum sp.* are actually able to infect and to establish inside the root was demonstrated earlier with immuno-gold labelling and with electron microscopy by Okon et al. (1983), Murty and Ladha (1987) and Bashan and Levanony (1988).

Nitrogenase repression by ammonia and nitrate is less pronounced in the rhizosphere as well, possibly affected by root nitrogen uptake and a reduced diffusion of mineral nitrogen to places of bacterial colonisation. Another mechanism involved could be the removement of toxic nitrite from the bacterial environment through the plant's nitrite reductase system (Ferreira et al.1987). As *A.brasilense* forms nitrite by dissimilatory nitrate reduction (Scott et al. 1979), an accumulation of this metabolite would inhibit bacterial nitrogen fixation (Döbereiner and De Polli 1980; Magalhaes et al. 1978).

The total number of surviving root bacteria and the final nitrogenase activities at different inoculation treatments should give information about the pattern of root colonization. It is obvious, that an *A.brasilense* inoculation several times during plant growth neither caused an increase of bacterial density on the root nor of nitrogenase activity as compared to plants inoculated only once early at planting. This may indicate, that when applied to young plants *A.brasilense* develops along the root immediately occupying all potential attachment places in the rhizosphere. Cells, added after this first inoculation event may not find suitable niches to create a higher degree of root colonization. Lower numbers of rhizosphere bacteria and significantly reduced nitrogenase activities were found in plants, to which *A.brasilense* was inoculated only in a late phase of plant development at day 30 or day 45 after planting. This reduction of bacterial density and nitrogen fixation at late inoculated plants may be due to senescence and lignification of the root. This would prevent the bacteria to penetrate into the cortex tissue. Such a colonization behaviour may explain the observation, that *A.brasilense*, when applied to the plant in relatively low cell numbers (log 6 CFU per plant) by seed coating, is able to

create an equal rhizosphere population as when applied to a young plantlet by seedling inoculation with a bacterial suspension of a 100 times higher cell density (log 8 CFU per plant) (Christiansen-Weniger, in preparation).

Non-N_2-fixing *P.fluorescens* caused a decrease of the $^{15}N/^{14}N$ ratio in root and shoot equal or even higher than nitrogen fixing *A.brasilense* does (Tables 5 and 6). It was suggested, that this effect might be caused by the ability of some *Pseudomonas* strains to scavenge traces of combined nitrogen as NH_3 or O from the atmosphere and to simulate in this way biological nitrogen fixation (Hurek et al. 1988; Zolk and Ottow 1975).

The positive effect of an inoculation with *A.brasilense* as well as with *P. fluorescens* on root and shoot dry matter (Table 5) was certainly due to the auxin production to which both bacterial strains are capable (Table 3, Harari et al. 1988; Malik and Bilal 1988). The reduction of N% in plant tissue confirms this.

The released amount of fixed nitrogen from an *A.brasilense* rhizosphere population to the host plant as calculated by ^{15}N dilution technique was very small (Table 6). The present results make it doubtful whether atmospheric nitrogen fixation or other mechanisms are responsible for the dilution of ^{15}N in the plantissue. The relatively low level of ^{15}N dilution in the plant shoot further showed that gained nitrogen was not translocated directly inside the plant, but stayed for the major part in the root, probably as bacterial substance. This unequal distribution of rhizosphere fixed nitrogen between root and upper plant parts was also reported in earlier papers by Najak et al.(1986) and Kapulnik et al.(1987). It is an indication that root associated nitrogen fixing bacteria do not transfer nitrogen directly to their environment but only after dying off. It also confirms the idea, that the known diazotrophic bacteria do not significantly contribute to the nitrogen supply of wheat (Ito et al. 1980; Eskew et al. 1981).

In conclusion, plants may provide suitable niches for *Azospirillum* which protect them against environmental factors such as high oxygen tension and mineral nutrient concentrations. These niches are occupied by *Azospirillum* immediately after inoculation of young developing roots and are less accessible for the bacteria at later development stages. This may provide a basis for succesful inoculation procedures of crops. Yet, the nitrogen supplying capacity of *Azospirillum* to wheat is very low, *i.e.* almost negligible.

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CHAPTER 3

EVALUATION OF NITROGEN FIXATION IN SORGHUM CULTIVARS INOCULATED WITH DIFFERENT STRAINS OF *AZOSPIRILLUM* SPP. [1]

Summary

Three cultivars of sorghum were grown in closed 7 l pots with the aerial part under free sunlight. Washed sand mixed with 1% organic matter labelled with ^{15}N was used and the applied N-fertilizer was labelled with equal N^{15} % excess. Four strains of *Azospirillum* spp., isolated from sorghum roots were used as inoculants. The strains were resistant against certain antibiotics, so that their identification was possible upon reisolation. At different plant growth stages C_2H_2 reduction was assayed after sealing the pots around the stems and total N and the N^{15} dilution were measured at harvest. To study the establishment of the inoculated strains, bacterial numbers in the root system were counted by the MPN method. The results and their meaning for understanding the function of a bacterial root-association are discussed.

Introduction

Until now the functioning of *Gramineae-Azospirillum* associations is not very well understood, but it has been reported in several papers that both the strain of the bacteria and the genotype of the plant have influence on the whole system. High variations between the acetylene reducing activity of sorghum cultivars were demonstrated, ranging from almost zero to more than 5 µmol C_2H_4 plant.h^{-h} (Wani et al. 1983). Also differences in the effectivity of associated *Azospirillum* strains were observed. One of the possible reasons for this was suggested to be the ability of some bacteria to infect and establish in the inner root tissue (Baldani et al. 1983, Magalhaes et al. 1979).

In the present paper we report interactions of different plant cultivars with *Azospirillum* strains isolated from within roots or from the root surface in sorghum grown in pots with sand and ^{15}N labelled organic matter.

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[1] In: *Azospirillum* III, 1985 (Klingmüller, ed). Springer Verlag, Berlin

Materials and methods

A pot experiment with a randomized complete block design was planted, including 4 replications, 3 sorghum cultivars and 5 inoculation treatments. The cultivars used were CMSXS 136 and CMSXS 114, both tolerant to aluminium toxicity, and BT 007b, which is known to be aluminium sensitive. The parameter of aluminium tolerance was chosen because one aluminium tolerant cultivar was shown to contain a higher organic acid content in the root (mainly malic and trans-aconitic) than that of an aluminium sensitive cultivar (Cambrai et al. 1983). The plants were grown in plastic pots containing 7 kg of washed sand mixed with 1% organic matter (dry, finely-ground, ^{15}N labelled grass with 0.83% N). Nutrients were added to each pot as follows: 53 ppm P, 66 ppm K and 1 ml kg^{-1} of trace element solution (Franco et al. 1978). Mineral nitrogen was added every 2 days (20 mg N per addition) as ^{15}N labelled KNO_3. Both the organic matter and the potassium nitrate had the same ^{15}N label of 0.2855 atom % excess. The experiment was performed outdoors until panicle emergence and was then transferred to a temperature controlled greenhouse. For acetylene reduction assays the pots were closed with lids and 10% v/v acetylene was injected through a septum in the container bottom as described by Wani et al. (1984). Gas samples were taken after 2.5 and 6 hours and analysed for C_2H_4 by gas chromatography. To eliminate residual acetylene, which initially was found to persist in the sealed pots for several days, the pots were blown through with air after assay. The plants were harvested at an early stage of maturity and divided into root, stem plus leaves, and panicle for subsequent drying and analysis for total nitrogen and ^{15}N enrichment (Boddey et al. 1983).

Bacteria were grown 24 hours at 30°C in liquid NFb medium (Badani and Döbereiner 1980) supplemented with 0.5 $g.l^{-1}$ NH_4Cl. One ml of this suspension was added directly to 7 days old seedlings and control pots were inoculated in the same way with an autoclaved bacterial culture. For inoculation four strains of *Azospirillum* were used:

Strain	Origin
S 82 A lipoferum	Surface sterilized sorghum roots (1% chloramine T, 30 min)
S 19 A. lipoferum	Surface sterilized sorghum roots (1% chloramine T, 30 min)
S 65 A. lipoferum	Washed sorghum roots
Sp 7 A. brasilense	ATCC 29145 from rhizosphere soil of *Digitaria decumbens*

Immediately after harvest MPN counts (McCrady) of the bacterial root population were made on semi-solid NFb medium containing 5 g malate and 2.5 g glucose l^{-1} (Döbereiner, 1980).

Results and discussion

After the stage of panicle emergence, acetylene reduction activity was measured every week. As demonstrated in Fig. 1 the activity over time showed similar patterns for all three cultivars.

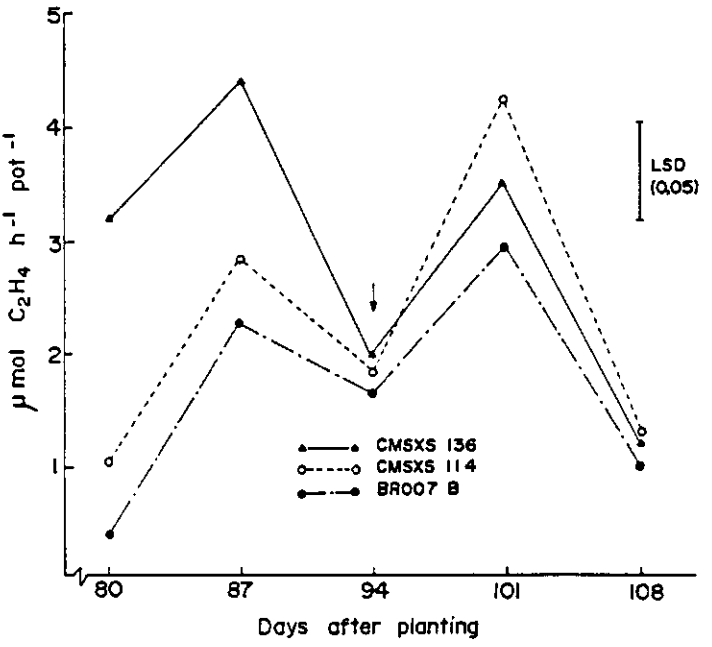

Figure 1: Acetylene reduction activity of three sorghum cultivars measured over 5 weeks. The arrow indicates the mid grain filing stage.

It seems that the main activity develops only in the late phase of plant growth in particular after flowering. This agrees with other work on cereal associations (Bülow and Döbereiner 1983, Wani et al. 1983, Watanabe et al. 1979), although the reason for this is not yet known. Possibly during flowering and grain filling production and translocation of organic acids in the plant increase so that at this time sufficient carbon sources are available for bacterial metabolism. The depression of nitrogenase activity during grain filling (arrow) could be explained by the competition for photosynthates between the panicle and the root-associated bacteria.

The cultivar BR 007b, which is sensitive to aluminium toxicity showed over all assays a significantly lower acetylene reducing activity than the aluminium tolerant cultivars. This seems to confirm expected differences in the organic acid content in the root systems (Cambraia et al. 1983).

The effect of bacterial inoculation on the acetylene reduction activity is shown in Table 1. Significant differences in activity were observed between plants inoculated with strain S 82 isolated from inside the roots and S 65 isolated from the root surface or Sp 7 isolated from rhizosphere soil.

Table 1: Effect of the inoculation treatments on acetylene reduction activity, N content and ^{15}N dilution in the plant.Means of the three cultivars and 4 replicates. The values for ARA are means of 5 seperate assays.

	ARA μmol C_2H_4 $h^{-1}pot^{-1}$	Total N mg $plant^{-1}$	N-content %	Top N^{15} % excess	% N from fixation
S 82	3061 a	518 a	1.17 a	0.2797 a	2.03 b
S 19	2245 abc	499 ab	1,10 ab	0,2799 a	1,96 b
S 65	1397 c	467 b	1,07 ab	0,2788 ab	2,35 ab
SP 7	1967 bc	467 b	1,02 b	0,2777 b	2,73 a
Control	2554 ab	465 b	1,06 b	0,2784 ab	2,49 ab
F value	**	*	*	*	*

** P = 0.01
* P = 0.05
n.s. and values with the same letter are not significantly different

That the inoculated control treatment also developed high acetylene reducing activity must be attributed to the fact that the growth medium was not sterilized and contamination with native *Azospirillum* occurred. This is confirmed by the MPN counts, which showed high numbers of *Azospirillum* in the roots (mean 2.5 x 10^6 cells per g fresh root) of all treatments including the controls without significant differences between cultivars or inoculation treatments.

Total N content and % N data in the plant are shown in Tables 1 and 2. Unfortunately the three sorghum cultivars were of different phenotypic growth habits and therefore there is a large difference between their dry matter production and the periods in which they are actively taking up nutrients. Comparison between cultivars to assess nitrogen fixing activity from differences in N accumulation is therefore difficult.

A comparison of the inoculation treatments shows that the strains S 82 and S 19, both isolated from within roots of sorghum, caused the highest values of total N and N percent in the plant. Similar observations were made in a wheat experiment where the total N accumulation in the plant tops correlated significantly with the numbers of *Azospirillum* in chloramine T treated roots, but

not with the *Azospirillum* numbers in the unsterilized roots (Baldani et al. 1983).

To quantify the contribution of nitrogen fixation to the plant N assimilation the ^{15}N enrichment was determined. As all the available nitrogen in the growth medium had the same ^{15}N enrichment, any diluation of the ^{15}N label in the plant should be caused by an input from atmospheric nitrogen. These results are reported in Tables 1 and 2. Amongst the cultivars, BR 007b showed the highest value for % ^{15}N content and therefore the lowest contribution of nitrogen from nitrogen fixation. This difference is significant and confirms the estimates made with the acetylene reduction method (Fig. 1). The relatively low proportion of fixed nitrogen in the plant tops (2 to 2.6% of total N) may be caused by the fact that most nitrogen fixing activity in the roots starts only in a late phase of plant growth, when most nitrogen has already been taken up from the growth medium.

Table 2: Dry matter production, N content and ^{15}N dilution of the three sorghum cultivars. Means of all inoculation treatments and 4 replicates.

	Top			Root	Top	
	dry matter g	N total mg	N content	N-content	N^{15} % excess	% N from fixation
CMSXS 136	39.9 b	487.2	1.22 a	0.40 b	0.788 b	2.35 a
CMSXS 114	48.0 a	471.3	0.98 c	0.51 a	0.2781 b	2,59 a
BR 007b	45.6 b	498.7	1.09 b	0.36 b	0.2800 a	1.96 b
F-value test	**	n.s.	**	**	**	**

** P = 0.01
* P = 0.05
n.s. and values with the same letter are not significantly different.

The effects of the inoculation treatments on ^{15}N dilution are more difficult to explain than those of the cultivars. Strains S 82 and S 19, which caused the highest nitrogen content in the plant, had the lowest value for ^{15}N dilution. On the other hand, strain Sp7, although it has only a small effect on plant nitrogen shows in all inoculation treatments the highest contribution of nitrogen derived from nitrogen fixation. The explanation for these apparently contradictory results must be that the associated bacteria, besides their ability to fix nitrogen interact with the plant uptake of mineral nitrogen from the soil. This could be a) an indirect effect, where bacterial produced plant growth substances caused an increase in the root system as described by Tien et al. (1979) and Okon (1982) or b) a direct participation of the associated bacteria in the nitrate-reducing pathway of the plant as suggested by Döbereiner (1983).

Table 3: Mean ^{15}N dilution and percent nitrogen from fixation in panicle, stem plus leaves, and root. The label of the mineral nitrogen source was 0.2855 atom % excess

	% N^{15} excess	% N from fixation
Panicle	0.2845	0.35
Stem, leaves	0.2735	4.20
Root	0.2115	25.90

The mean ^{15}N dilution values of roots, stem plus leaves, and panicle are shown separately in Table 3. The ^{15}N enrichment in the roots indicates that approximately 26% of the nitrogen in the roots was derived from associated biological nitrogen fixation, as opposed to 4% in the leaves and stem and virtually no contribution to the panicle. It seems probable therefore that the nitrogenase activity is used initially only for bacterial growth in the rhizosphere and very little fixed N is translocated to the shoot immediately. Only in the late phase of plant growth nitrogen from dead bacterial tissue is translocated in some form to the leaves and stems of the plant.

Conclusion

Between sorghum and *Azospirillum* spp. exists a N_2-fixing association of relatively high acetylene reduction activity. The acetylene reduction data indicate that the cultivars resistant to aluminium toxicity had higher associated N_2-fixing activity than a aluminium sensitive cultivar which may be related to the higher organic acid content in the roots of such cultivars (Cambraia et al. 1983). Some nitrogen from fixation was translocated to the shoot of the plant.

Between the different inoculation treatments the strains isolated from within roots had a significantly greater effect on plant nitrogen than strains isolated from washed roots or rhizosphere soil. It was demonstrated that the associated *Azospirillum* besides having the ability to fix nitrogen also influenced the assimilation of nitrate by the plant.

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CHAPTER 4

ASSOCIATIVE N_2-FIXATION AND ROOT EXUDATION OF ORGANIC ACIDS FROM WHEAT CULTIVARS OF DIFFERENT ALUMINIUM TOLERANCE [1]

Summary

Three wheat cultivars with different tolerance against free aluminium were grown monoxenically in association with *Azospirillum brasilense*. *In situ* nitrogen fixation, measured with the acetylene reduction assay, was higher by the aluminium-tolerant cultivars than by the sensitive cultivar. The transfer of fixed nitrogen to the host plant, determined by the ^{15}N dilution technique, was also significantly higher in the aluminium-resistant wheat plants. Total accumulation of fixed nitrogen in the host plants due to *A. brasilense* inoculation was low, approximately 3% of the total nitrogen in the root and only 1% of the nitrogen in the shoot.

The quantity and quality of exudates released in liquid nutrient solution were analysed separately for two of the wheat cultivars, one aluminium-tolerant and one aluminium-sensitive. After 29 days of growth the aluminium-tolerant plants exudated significantly higher total amounts of carbon than aluminium-sensitive plants. No differences between the two cultivars existed in the carbon exudation rate per gram dry root.

Much higher concentrations of low molecular dicarbonic acids, *i.e.* succinic, malic and oxalic acid, were found in the exudates of aluminium-tolerant plants. Dicarbonic acids are potential chelating compounds for positively charged metals, such as aluminium and they may play an important role in protecting the plant against aluminium incorporation. They are also very suitable substrates for *Azospirillum* spp. It is therefore suggested that these factors may cause the higher associative nitrogen fixation rates which was found in the aluminium-tolerant wheat cultivars.

Introduction

Bacteria of the genus *Azospirillum* colonize the roots of various plants (Döbereiner and Day, 1976, Neyra and Döbereiner, 1977). In particular under tropical and subtropical conditions high densities of the bacterium are found in the rhizosphere of maize, rice, sorghum, wheat and forage grasses (Lakshmi-Kurami et al., 1976, Staphorst and Strijdom, 1978, Wong and Stenberg, 1979). In temperate soils much lower cell numbers are generally found in the rhizosphere

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of grasses (De Coninck et al., 1988, Jagnow, 1981). Because of its close binding to host plant roots and its ability to fix atmospheric nitrogen *Azospirillum* has been suggested as a potential biofertilizer (Kapulnik et al., 1981b). Whether *Azospirillum* is actually contributing to the nitrogen supply of the host plant is still under discussion. Inoculation experiments show widespread results, from no or even a negative effect (Owens, 1977, Schank et al., 1980) to a plant nitrogen increase of more than 120 % (Cohen et al., 1980, Kapulnik et al., 1981 a). Significant differences in response to *Azospirillum* inoculation are reported even between cultivars of the same plant species (Christiansen-Weniger et al., 1985, Nayak et al., 1986, Wani et al., 1984).

The host plant itself will have a strong influence on the nitrogenase activity of root associated-bacteria through the supply of a suitable carbon source via root exudation (Beck and Gilmour, 1982). Besides the total amount of excreted carbon the availability of specific organic compounds in the exudates is of influence on nitrogen fixation in the rhizosphere (Krotzky et al., 1988, Matsumoto et al., 1979).

In this paper we report on the nitrogen fixation activity by *A. brasilense* in the rhizosphere of different spring wheat cultivars. Phenotypically, these wheat cultivars differed in their tolerance against free aluminium. This characteristic was chosen since it has been shown that plant tolerance to increased concentrations of dissolved aluminium was caused by enhanced exudation of low molecular carbonic or dicarbonic acids (Cambraria et al., 1983, Foy et al., 1978). As such organic compounds form large chelating complexes with positively charged metal ions they are thought to prevent aluminium from diffusion through root membranes (Suhayda and Haug, 1986). Several of these low molecular organic acids are known to form a suitable carbon substrate for *A. bralilense* (Tarand et al., 1984).

Since we were primarily interested in the effects of *A. brasilense* on N_2-fixation, N-uptake and response to root exudates in comparison with a native bacterial rhizosphere population, we compared *A. brasilense* inoculation and plant treatment with an unspecific rhizosphere microflora.

Material and methods

Plants and growth condition

We used three spring wheat cultivars: *Triticum aestivum*, var. *Carasinho* (recommended as highly aluminium tolerant), var. *Allas 50* (aluminium tolerant) and var. *Buck Bolivar* (aluminium sensitive). The cultivars were obtained from the Foundation for Agricultural Plant Breeding SVP, Wageningen. Seeds were surface-sterilized by gentle shaking in a 1,5% hypochloride solution during 90 minutes, and pregerminated on trypton soya agar to check for sterility. After eight days uncontaminated seedlings were transferred to plant growth containers. Plants were grown with 12 hours day at 20°C and 12 hours night at 15°C at a relative humidity of 70%.

To determine the potential rhizosphere nitrogen fixation plants were grown in a closed root environment on 2 kg carefully washed and heat-sterilized (120°C, 30

min) sea sand. Plants were sealed airtight with a parafine/lanoline seal (5%/95%). Sterilized Hoagland nutrient solution (Hoagland and Broyer, 1936) was added every fifth day by flooding and subsequent drainage of the growth container. Roots were aerated by flushing the root environment with air via a sterile cotton filter.

To determine root carbon exudation seedling roots were transferred to Erlenmeijer flasks containing 500 ml of a sterile half strength Hoagland nutrient solution as described by Kraffczyk et al. (1984). Plants were sealed with a parafine/lanoline seal around the stem to prevent contamination. Aeration was continuous via membrane filters (0.2 μm). Samples of the nutrient solution were taken with a syringe through a silicone septum.

Bacteria

The bacterium used was *Azospirillum brasilense* strain Wa3, a strain isolated from the rhizosphere of a greenhouse-grown spring wheat (Christiansen-Weniger, 1988). Bacteria were grown overnight on Luria broth (tryptone, 10 g l^{-1}, yeast extract, 5 g l^{-1}, NaCl, 5 g l^{-1}), washed once and resuspended in 0.8% NaCl solution. For inoculation 25 ml of the cell suspension was injected into each growth container, which yielded a final cell density of approximately $2.6x10^8$ cells per plant.

Control plants were treated with a rhizosphere soil suspension which was obtained by shaking roots with adhering soil in a 1% K-pyrophosphate solution during 10 min. The soil suspension was allowed to settle for 20 min, after which 20 ml of the supernatant was injected into the growth container of each control plant.

Acetylene reduction assay

Acetylene reduction assay (ARA) was carried out on day 30, 43, 50 and 59 after planting. The growth containers were transferred to a water bath with a constant temperature of 23°C. All pots were flushed during one hour prior to each assay with a gas mixture of 87% v/v N_2, 3% v/v O_2 and 10% v/v C_2H_2 followed by airtight closure. Gas samples were taken after 15 min and again after three hours. C_2H_4 production was determined with a gas chromatograph (Varian 1700, Porapak T column, FID detector). An ethylene standard was added to uninoculated and unplanted growth containers. After each assay the growth containers were carefully flushed with air to remove residual toxic ethylene.

^{15}N dilution assay

The incorporation of fixed nitrogen into host plant material was determined by a ^{15}N dilution assay. The nitrate of the Hoagland nutrient solution as sole mineral nitrogen source was labelled with $K^{15}NO_3$ to a final concentration of 1.16 atom % excess. Plants were harvested 60 days after planting, and roots, stems with leaves and the panicle were separately analysed. Plant material was dried at 80°C until constant weight and subsequently macerated. Total nitrogen was determined by the Kjeldahl assay (Bremner and Shaw, 1958). The $^{14}N/^{15}N$ ratio was determined by mass spectrometry (Finnigan MAT250) according to Bremner (1965). The relative nitrogen gain due to *A. brasilense* inoculation was calculated with the equation (Boddey et al., 1983):

$$\% \text{ N from fixation} = 1 - \frac{^{15}\text{N\% in the plant}}{^{15}\text{N\% in the control}} \times 100$$

Root exudation

Plants were grown on liquid nutrient solution and 1 ml samples were taken at day 23 and 29 after planting by syringe. Of each sample 0.5 ml was directly injected into a total organic carbon analyser (TOC, Beckmann 9815 A) to determine the solved organic carbon concentration. Anorganic carbon, which could eventually interfere with the total organic carbon analysis was not detected in the nutrient solution. Potassium biphthalate was used as organic carbon standard. At day 29 plants were harvested and root and shoot dry matter were determined separately. For assays of the root exudate composition the residual nutrient solution was collected after harvest and subsequently freeze-dried and stored at -12°C. These samples were dissolved in a 2-ml NaOH solution (pH 11.73) with dodecandioic acid added to each sample as internal standard. Residual root cells were removed from the samples by ten minutes centrifugating at 8000 rpm. One ml of the supernatant was transferred to reaction tubes and the solved organic compounds were methylated during 30 min at 60°C with an acidic methanol solution (40 ml methanol in 60 ml 37% HCl) as described by Drozd (1981). Methylated organic acids were separated from salts by a two-fold chloroform extraction and subsequently concentrated on ice under dried air. One µl of each solution was then injected on-column into a gas chromatograph (Carlo Erba HRGC). Separation was performed by a WCOT fused silica capilary column (25m, 0.32 mm ID) coated with CP Sil 5 CB. Detection was by flame ionisation; the injector block temperature was 70°C and the detector block temperature 260°C. The column temperature was isotherm for 4 minutes at 70°C, then increasing by 10°C per minute to 210°C and kept constant at this temperature up to 25 minutes. Carrier gas was purified He with an inlet pressure of 0.5 bar. A water dissolved mixture of various dicarbonic acids were used as standard and for peak identification.

Statistical analysis

All experiments were carried out in a complete factorial design with four replications for each treatment. Analysis of variance was by the statistical program Genstat 5, release 1.3 (Genstat 5 Committee 1987). Standard errors of differences were calculated and the Student T test was performed. All significant differences reported are at a level of $P = 0.05$ at least.

Results

Nitrogen fixation

The acetylene reduction activity of plants inoculated with *Azospirillum brasilense* was not significantly different from that of controls treated with a rhizosphere suspension. The ^{15}N dilution, however, which is inversely related to the accumulation of fixed nitrogen in the host plant material, was highest in roots and shoots of the *A. brasilense* treatments. *A. brasilense* caused an almost twofold increase in root dry matter production in comparison with control plants, whereas the root nitrogen content was significantly decreased. *A. brasilense* inoculation did not have a strong effect on shoot dry matter and shoot nitrogen content (Table 1).

Acetylene reduction activity of the two aluminium-tolerant cultivars *Allas 50* and *Carasinho* was higher than that of the aluminium-sensitive *Buck Bolivar* (Fig. 1).

Table 1. Dry matter production, nitrogen content, ^{15}N dilution and acetylene reduction activity (ARA) of wheat plants inoculated with *Azospirillum brasilense*. Control plants were treated with a rhizosphere-soil suspension. Plant nitrogen source was labelled with 1.16 atom % excess ^{15}N.

	Dry matter (g)		Nitrogen content (%N)		^{15}N dilution (atom %^{15}N)		ARA (nmol C_2H_4 h^{-1} pl^{-1})
	Shoot	Root	Shoot	Root	Shoot	Root	
Control	3.23	1.68	0.99	0.33	1.131	1.023	130
Azospir.	3.44	2.93	0.98	0.21	1.121	0.989	102
SED	0.25	0.35*	0.05	0.03*	0.005	0.012*	94.6

Values are means of three wheat cultivars and four replicates.
ARA values are means of four separate assays.
SED: Standard error of difference (* $P < 0.05$)

These differences were statistically significant when calculated as means over all four assays. Besides a higher rhizosphere acetylene reduction both aluminium-tolerant cultivars accumulated more nitrogen from atmospheric source than the aluminium-sensitive cultivar (Tables 2 and 3). In all three cultivars most of the fixed atmospheric nitrogen was recovered in the roots: a maximum of 5.7 % of the root nitrogen being of atmospheric origin. Only a small proportion of the fixed nitrogen was transferred to the upper plant parts: 0.6 - 1.2 % of the nitrogen in

the shoots originated from fixation (Table 2). It is obvious that more of the fixed nitrogen was incorporated in the late-developing panicle than in stem and leaves (Table 3).

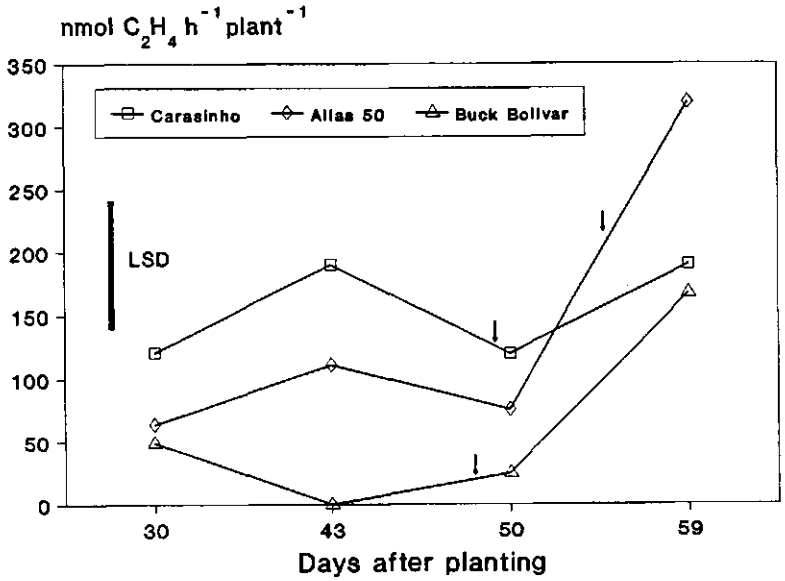

Figure 1. Acetylene reduction activity of the wheat cultivars *Carasinho*, *Allas 50* and *Buck Bolivar* during plant growth, after monoxenical association with *Azospirillum brasilense*. Arrows indicate the begin of panicle emergence. LSD: Least significant difference.

The total fixed nitrogen accumulation by the aluminium-tolerant varieties *Carasinho* and *Allas 50* was 825 µg and 524 µg N respectively; this is two to three time higher than the aluminium-sensitive *Buck Bolivar* (211 µg fixed N). Most of the fixed nitrogen remained in the roots of both aluminium-tolerant cultivars whereas the aluminium-sensitive *Buck Bolivar* transferred most of the fixed nitrogen to its panicle (Table 3).

Root carbon exudation

In the 500 ml nutrient solution the concentration of exudated total organic carbon increased from approximately 20 mg l^{-1} C per plant at day 17 to 80 mg l^{-1} C at day 29 (Fig. 2). At day 23 as well as day 29 the aluminium-tolerant cultivar *Carasinho* accumulated higher amounts of exudated carbon than the aluminium sensitive

cultivar *Buck Bolivar* (Table 4). Root dry matter production of *Buck Bolivar* was significantly lower which is in agreement with the results obtained from plants grown on sand (Tables 2 and 4). With 115 mg and 126 mg exudated carbon per gram dry root the relative amounts of carbon exudation were similar at both cultivars (Table 4).

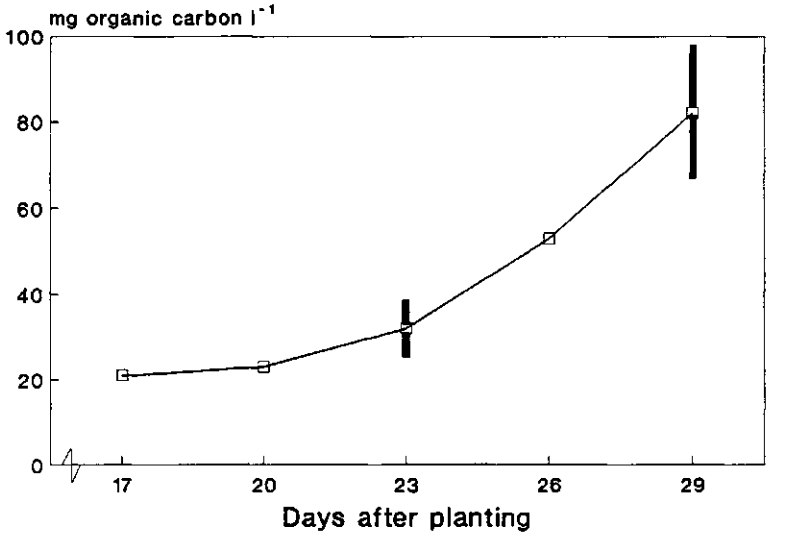

Figure 2. Cumulative total organic carbon excretion by wheat roots grown on sterile liquid nutrient solution. Values on day 17, 20 and 26 are means of three random samples; values on day 23 and 29 are means of two cultivars and four replicates. Bars indicate the standard error of difference.

The methylation method resulted in a proper gas chromatographic separation of the exudated organic acids. Although sample preparation and further purification was poor, without any ion exchange step, the twofold chloroform extraction allowed a sufficient elimination of mineral salts and other diluted inorganic compounds. Typical gaschromatograms of methylised exudates from both cultivars are presented in Fig. 3. Oxalate, malate and succinate developed narrow, well separated peaks. A fourth peak, present in both cultivars, remained unidentified. Several by-products of the methylation method occurred but did not interfere with relevant organic acid peaks. The internal standard signal appeared late (16.9 min) without overlaying other peaks.

Table 2. Dry matter production, nitrogen content, nitrogen from fixation as calculated by the ^{15}N dilution technique and acetylene reduction activity (ARA) of three wheat cultivars inoculated with *Azospirillum brasilense*.

	Dry matter (g)		Nitrogen content (%)		% N from fixation (%)		ARA (nmol C_2H_4 h^{-1} pl^{-1})
	Shoot	Root	Shoot	Root	Shoot	Root	
Carasinho	4.1	3.9	0.78	0.21	1.24	5.65	155
Allas 50	3.5	2.9	0.92	0.24	0.91	3.22	139
Buck Bolivar	2.9	1.9	1.06	0.27	0.56	0.74	59
SED	0.3*	0.4*	0.06*	0.04	0.56	1.52*	36.6*

Values are means of four replicates.
ARA values are means of four seperate assays.
SED: Standard error of difference (* $P < 0.05$)

Table 3. Atmospherically fixed nitrogen in roots, stems and leaves and in the panicle of three wheat cultivars inoculated with *Azospirillum brasilense* as calculated by the ^{15}N dilution technique.

	Nitrogen from N_2 fixation (μg fixed N $plant^{-1}$)			
	Root	Stem and leaves	Panicle	Total plant
Carasinho	428 (5.63)[1]	191 (1.01)	209 (1.65)	829 (2.13)
Allas 50	237 (3.22)	87 (0.57)	200 (1.33)	524 (1.37)
Buck Bolivar	42 (0.74)	14 (0.12)	188 (0.99)	211 (0.58)
SED	104*	81*	148	217*

[1]Values in parantheses give the percentage of plant nitrogen resulting from nitrogen fixation.
Values are means of four replicates.
SED: Standard error of difference (* $P < 0.05$)

Malate, succinate and oxalate were released in much higher amounts by the aluminium-tolerant cultivar *Carasinho* than by the aluminium-sensitive cultivar *Buck Bolivar*. Succinic and malic acid were released by *Carasinho* up to a concentration of 218 µg and 566 µg per gram dry root, respectively; only small amounts were released by *Buck Bolivar* (Table 5).

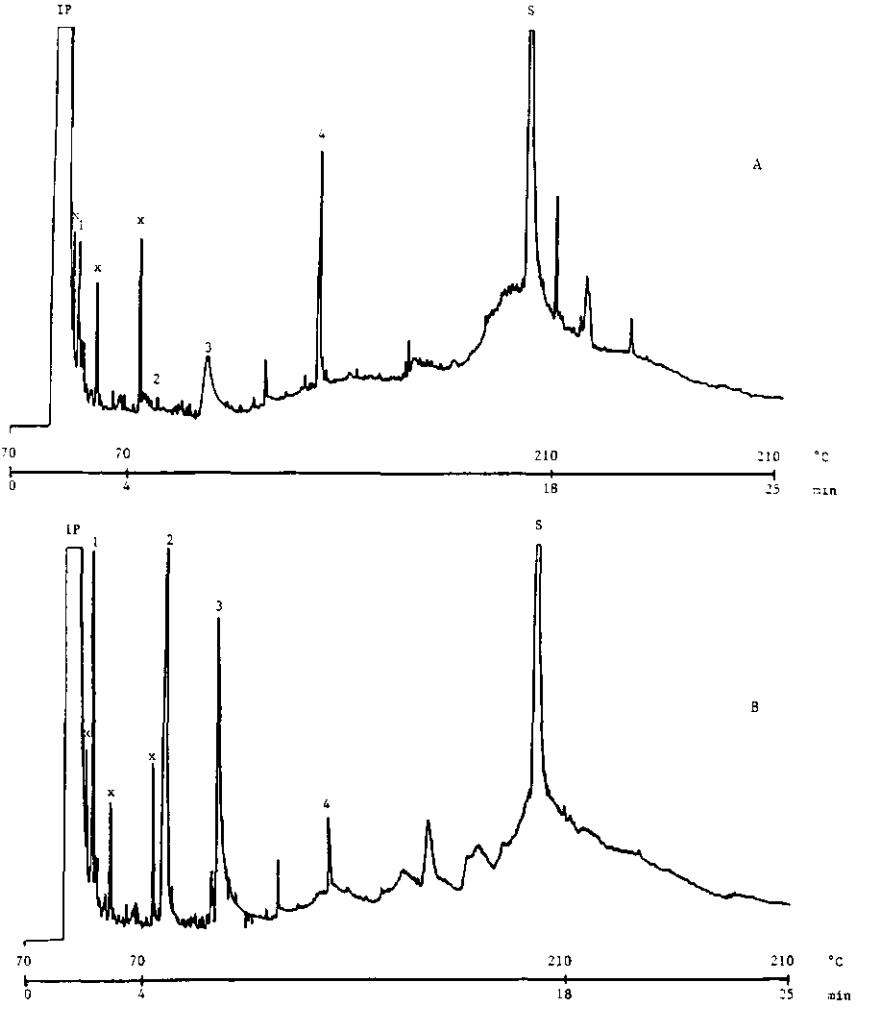

Fig. 3. Gas chromatographs of root-exudated organic acids of *T. aestivum* cv. *Buck Bolivar* (A) and *Carasinho* (B) after methylation and chloroform extraction according to Drozd (1981). The following peaks were identified: oxalic (1), succinic (2), malic (3) and dodecandioic (S,internal standard) acid and byproducts (X) due to methylation. Peak (4) could not be identified.

Discussion

The rate of acetylene reduction activity by *Azospirilum brasilense* is considerable when associated with wheat roots (Fig. 1). Since the bacterial nitrogenase activity is strongly affected by oxygen and mineral nitrogen concentration (Hartmann et al., 1985, Nelson and Knowles, 1978), which are both difficult to keep constant in an actively growing root, the actual acetylene reduction rate cannot be extrapolated to quantitative nitrogen fixation. However, when assuming constant bacterial acetylene reduction during the full period of plant development, the total amount of fixed nitrogen can be calculated from the average activities given in Table 2: 1.04 mg and 0.43 mg fixed N per plant for the aluminium-resistant cultivars *Carasinho* and *Allas 50*, respectively, and 0.26 fixed N per plant for the aluminium-sensitive cultivar *Buck Bolivar*.

Table 4 Total organic carbon exudation and root dry matter production of the wheat cultivars *Carasinho* (aluminium-tolerant) and *Buck Bolivar* (aluminium-sensitive) grown on liquid nutrient solution.

	Dissolved organic C (mg l^{-1})		Exudated organic C (mg $plant^{-1}$)		Root dry matter (mg)	C exudation per g root (mg g^{-1})
	Day 23	Day 29	Day 23	Day 29	Day 29	
Carasinho	36.5	102.3	16.5	35.4	317	115
Buck Bolivar	24.1	69.7	11.4	27.0	239	126
SED	5.3*	16.2*	2.3*	5.9	31*	27

Values are means of four replicates.
SED: Standard error of difference (* $P < 0.05$)

The acetylene reduction activity by control plants was similar to the activity of plants treated with *A. brasilense* (Table 1). This may have been caused by the fact that the natural rhizosphere microflora obviously contained a considerable number of nitrogen fixing organisms. As growth containers allowed light transmission, blue-green algae may have been active as well. This acetylene reduction activity of control plants creates a problem in calculating biological nitrogen fixation via the ^{15}N dilution technique. ^{15}N dilution was used in other studies to quantify the associative nitrogen fixation in grasses (Boddey et al., 1983, Giller et al., 1986, Rennie, 1980).

Table 5 Root exudation of low molecular dicarbonic acids by wheat cultivars *Carasinho* (aluminium-tolerant) and *Buck Bolivar* (aluminium-sensitive).

	Acid produced (μg plant^{-1})			Acid produced (μg g root^{-1})		
	oxalic	malic	succinic	oxalic	malic	succinic
Carasinho	67	211	80	192	566	218
Buck Bolivar	36	27	9	162	117	42
SED	12*	56*	25*	63	118*	69*

Values are means of four replicates
SED: Standard error of difference (* $P < 0.05$)

The technique is based on the availability of a non nitrogen fixing control, against which the dilution of an ^{15}N enriched nitrogen source is calculated. Problems in using such controls are discussed by Chalk (1985) and Wittey (1983). Another problem associated with the ^{15}N dilution method is the occurrence of isotope fractionation in plants (Kohl and Shearer, 1980). Mariotti et al. (1982) reported a substantial nitrogen isotope fractionation in young pearl millet, which diminished as plants matured. These uncertainties in the ^{15}N dilution technique make it difficult to discuss the results of our experiments in terms of an absolute amount of associative fixed nitrogen; the reported ^{15}N dilution rates can only be considered as a relative response to *A. brasilense* inoculation in comparison with the unspecifically treated control.

Although the controls as well as the *A. brasilense* treatments showed a considerable acetylene reduction activity *A. brasilense* significantly increased ^{15}N dilution in the roots (Table 1). As *Azospirillum* is demonstrated to colonize closely attached to the root surface or even inside intercellular spaces of the root cortex (Bashan and Levanony, 1988, Murty and Ladha, 1987), most of the ^{15}N dilution might be derived from bacterial substances which remain in and on the roots after washing. This was confirmed by the higher concentrations of fixed nitrogen found in *A. brasilense* inoculated roots than in the shoot material (Table 2). This phenomenon has often been observed when associative nitrogen fixation was assayed in the rhizosphere of grasses during a short period within a single plant generation (Christiansen-Weniger et al., 1985, Nayak et al., 1986, Okon et al., 1983). Malik et al. (1987) found that 32% of the nitrogen in roots of *Lepochloa fusca* was from atmospheric origin against only 6% of the shoot nitrogen. It was hypothesized that root-associated diazotrophs fix atmospheric nitrogen but translocate only little of this fixed nitrogen from the cells to the host plants. Higher fixed nitrogen concentrations were observed in the late developing pannicle than in stem and leaves (Table 3). This also indicates that associated *Azospirillum* does not provide fixed nitrogen to the

host plant at a constant level throughout the growing period but mainly in a late phase of plant development, probably after dying-off. Our results confirm that rhizosphere nitrogen fixation does not directly affect plant nitrogen supply but rather that it has a long-term effect on soil nitrogen level (Eskew et al., 1981, Rao et al., 1987).

A. brasilense inoculation considerably increased root dry matter production (Table 1). This effect on root development has been reported before; it has been suggested to be due to the production of plant growth substances (Reijnders and Vlassak, 1979, Tien et al., 1979). In particular indole-acetic-acid (IAA), which is formed by *Azospirillum* spp., affects root branching and causes root hair deformation (Harari et al., 1988, Patriquin et al., 1983). The decrease in root nitrogen content associated with an enhanced dry matter production, is probably caused by nitrogen dilution in the plant material.

Both aluminium-tolerant wheat cultivars *Carasinho* and *Allas 50* developed a more effective association with N_2-fixing *A. brasilense* than the aluminium-sensitive cultivar *Buck Bolivar*. This may be due to the fact that both aluminium-tolerant cultivars showed a higher root mass production (Table 2) and thus offered more space for bacterial rhizosphere colonization. However, this can not explain the much higher incorporation rates of fixed nitrogen into root and shoot nitrogen of the tolerant cultivars (Fig. 3). Besides, root mass and associative N_2 fixation are not necessarily correlated, as shown by Krotzky et al. (1986, 1988). When grown on liquid nutrition *Carasinho* exudated about 30% more total organic carbon than *Buck Bolivar*. Although *Carasinho* developed more root biomass, the relative carbon exudation per gram dry root was similar to that of *Buck Bolivar* (Table 4). Barber and Lynch (1977) and Bennet and Lynch (1981) suggested that the specific composition of the root exudates rather than the total amount is determinative for the proliferation of diazotrophs in the rhizosphere of wheat. Organic acids, such as oxalate, succinate and malate, were present in much higher amounts in the root exudates of *Carasinho* than in those of *Buck Bolivar*. In particular the exudation rates of succinic and malic acids, which are known to be suitable carbon sources for *A. brasilense* (Tarand et al., 1984) were more than five times higher in the roots of the aluminium-tolerant cultivar *Carasinho* (Table 5). These observations are consistent with results of Krotzky et al. (1980), who showed that a sorghum cultivar with a high associative nitrogen fixation activity released more malic, fumaric and succinic acid than a less active cultivar. Rennie (1980) reported that the addition of sugars and organic acids to *A. brasilense* inoculated maize strongly promoted the incorporation of atmosphere-derived nitrogen in the plant. Succinate and malate stimulated dinitrogen fixation more than sucrose. These results confirm that plants are able to stimulate the nitrogenase activity of root-associated *A. brasilense* by its own exudation behaviour. Our results point to a close interaction between the release of specific organic acids such as malate and succinate by aluminium-tolerant plants and its nitrogen fixation activity by root-associated *A. brasilense*. Our evidence has its limitations, because rhizosphere nitrogen fixation and root carbon exudation are determined in different growth systems. Root carbon exudation is influenced by the substrate on which the plant is grown (Martin, 1977) as well as by the presence of a bacterial rhizosphere population (Barber and Martin, 1976, Kraffczyk et al., 1984). Yet we decided to use the experimental set-up as described in Materials and methods to allow accumulation of exudated organic carbon and to prevent root-released substances from bacterial

decomposition.

Whether a general link exists between plant aluminium resistance and its ability to form a potential association with *A. brasilense* is difficult to conclude. Döbereiner (pers. comm.) suggested that aluminium tolerance of grasses can be used for the selection of plant genotypes with a high affinity to rhizosphere nitrogenase activity. In a previous experiment with sorghum cultivars of different resistance against free aluminium we also observed that aluminium-tolerant cultivars had significantly higher associative N_2 fixation rates than an aluminium-sensitive cultivar (Christiansen-Weniger et al., 1985).

The potential contribution of *A. brasilense* to the total nitrogen supply of wheat is small and will not be of any significance to arable cropping as long as the fixed nitrogen cannot efficiently be released from the bacteria to the root environment.

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CHAPTER 5

AN INFLUENCE OF PLANT GROWTH SUBSTANCES ON GROWTH AND NIGROGENASE ACTIVITY FROM *AZOSPIRILLUM BRASILENSE* [1]

Summary

Plant growth substances, such as auxins, cytokinin, gibberillin and abciscin were tested for their effect on growth and nitrogenase activity from batch culture grown *Azospirillum brasilense*. The present results indicate that the auxin indol-3-acetic acid (IAA) and the cytokinin 6-benzyl-aminopurine (BAP) are of a stimulating influence on the bacterial growth. Treatments with gibberillin (GA) and IAA at a final concentration of 2.5 mg/l are highly increasing the nitrogenase activity, whereas GA at a low concentration (0.025 mg/l) represses N_2-fixation. Comparing IAA and 2,4 dichlorophenoxy-acetic-acid (2,4 D), both auxins of plant regulating activity with 3,5 dichlorophenoxy-acetic-acid (3,5 D), which is not a plant growth substance, IAA and 2,4 D are positively affecting the nitrogen fixation, whereas 3,5 D does not. When tested for their effect on the NH_4^+-nitrogenase repression only GA showed to have a slight derepressing effect in the lowest concentration tested. This effect became stronger and statistical more significant, when the GA addition was combined with an abscisic acid (ABA) or IAA treatment. ABA itself did not show any effect.

In conclusion, this paper will demonstrate, that nitrogen fixation of the rhizosphere bacteria *Azospirillum* could be influenced by plant produced growth substances.

Introduction

Involved in the discussion over the association between grammineous host and *Azospirillum* spp. there are several works, in which the development of the nitrogenase activity in the root is observed over a longer period (Christiansen-Weniger et al. 1985, Wani et al. 1984, Watanabe and Lin 1984). It is obvious, that higher activities often occur only in a late phase of plant-growth, shortly before flowering or even later. This could be affected by different factors, for example by a decrease of nitrogenase repressing NH_4^+ in the rhizosphere, or in the same way, by a change of the O_2 tension. The knowledge here about is incomplete, because until now it is not possible to measure rhizosphere acetylene reduction

[1] In: *Azospirillum* IV, 1988 (Klingmüller W., ed.). Springer Verlag, Berlin.

under controlled soil conditions. But even if the partial O_2 pressure in the root environment was hold at a constant level, nitrogenase activity increases drastically during panicle emergence (Christiansen-Weniger, in preparation). Facing this, it may be hypothesized that the host plant could influence the activity of the associated bacteria by means of its own hormonal regulatory system. To prove this some natural and artificial plant growth substances were tested for their effect on growth, nitrogenase activity and NH_4^+ nitrogenase repression of *Azospirillum brasilense*.

Material and methods

The bacteria used in all experiments was *Azospirillum brasilense* (Wa 5), a strain isolated from the rhizosphere of a greenhouse-grown summer wheat. For cell division studies the bacteria were grown in a batch on Nutrient Broth (Oxoid), at 28°C measuring the optical density at 450 nm. The plant growth substances were added at the start of the experiment by sterile filtration to a final concentration of 2.5 mg/l, 4 replicates each.

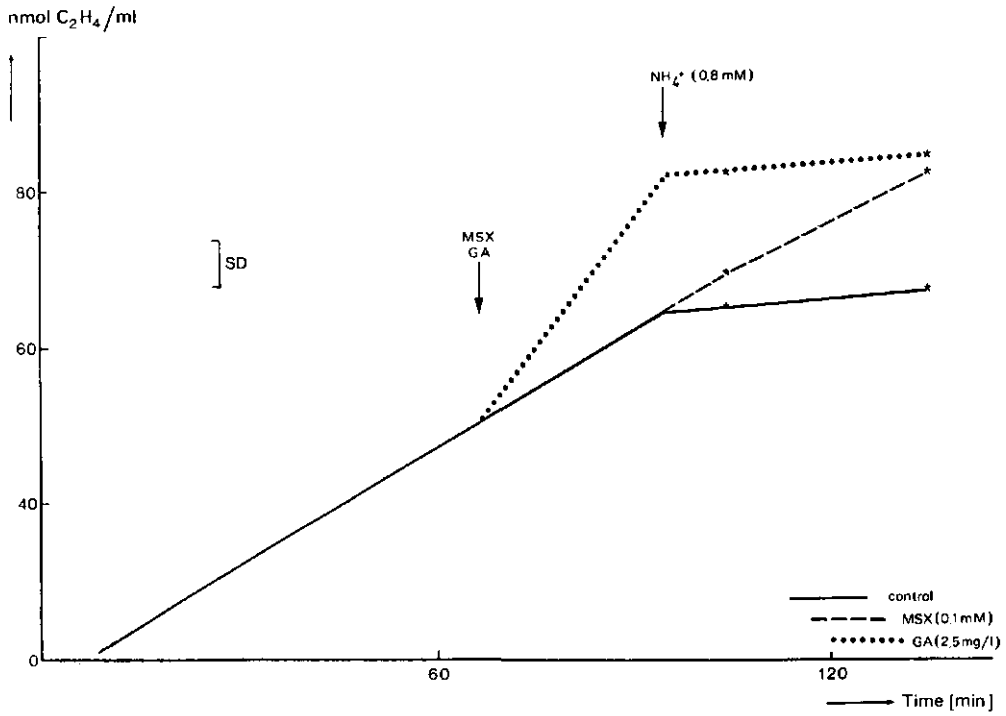

Figure 1. Acetylene reduction of an *Azospirillum brasilense* culture at 1.5% O_2 treated with MSX (0.1 mM) and GA (2.5 mg/l).

To determine the influence of the plant growth substances on the nitrogen fixation and further on the NH_4^+ nitrogenase repression the method described by Hartmann et al. (1985) was used. *Azospirillum* was grown in a batch with a controlled atmosphere of 88% Argon, 10% C_2H_2 and 2% O_2 until N_2 fixation occurred. The experiment was started by distributing 4 ml samples of this batch to anaerobic 39 ml serum bottles, adding 1,5% O_2 and 10% C_2H_2 to the headspace and shaking intensively at 28°C. After 60 minutes the plant growth substances were injected. To induce nitrogenase repression, the culture was treated after 90 minutes with 0.8 mM NH_4Cl. An example for the development of acetylene reduction in such a system is given in Figure 1.
Because of the relatively large experimental error involved in acetylene reduction assay all treatments were done with 6 replicates each. Following substances were used:

Auxins : Indole-3-acetic acid (IAA), 2,4 dichlorphenoxy-acetic-acid (2,4 D), 3,5 dichlorphenoxy-acetic-acid (3,5 D)[2]

Cytokinin : 6-benzyl-aminopurine (BAP)

Gibberillin: gibberillic acid (GA)

Abscisin : abscisic acid (ABA)

The acidity of the stock solution was set upon pH 7 with HCl or NaOH.

Results and discussion

The influence of IAA, BAP and GA on growth of *Azospirillum brasilense* is demonstrated in Figure 2. IAA and BAP showed both an increasing effect on bacterial growth. GA depressed cell division slightly but within the experimental error. ABA (2.5 mg/l) did not influence the growth of the bacterial culture.

The influence of plant growth substances on the nitrogenase activity are given in Table 1. From the substances tested, IAA and GA at a final concentration of 2.5 mg/l had an increasing effect on nitrogen fixation. GA at a low concentration of 0.025 mg/l inhibited nitrogenase activity. ABA and BAP did not show an effect on the N_2 fixation of *A. brasilense*.

To prove whether the measured influence on nitrogen fixation is due to the regulatory ability of plant growth substances, IAA was tested against other auxines such as 2,4 D and 3,5 D. 3,5 D is of nearly the same chemical structure as 2,4 D (Fig. 3), but without a comparable function the plant physiology (E. Knecht, pers. communication). As demonstrated in Table 2 IAA and 2,4 D induced a high increase of nitrogenase activity, whereas 3,5 D did not.

Testing the influence on the NH_4^+ nitrogenase repression, only GA in a low final concentration of 0.025 mg/l was found to have a slight nitrogenase derepressing

[2]A gift from Dr. E. Knecht, Department of Plant Physiology, Wageningen

ability (Fig. 4). This effect was stimulated and became statistical more significant when the GA treatment was combined with an addition of 2.5 mg/l ABA (Fig. 4).

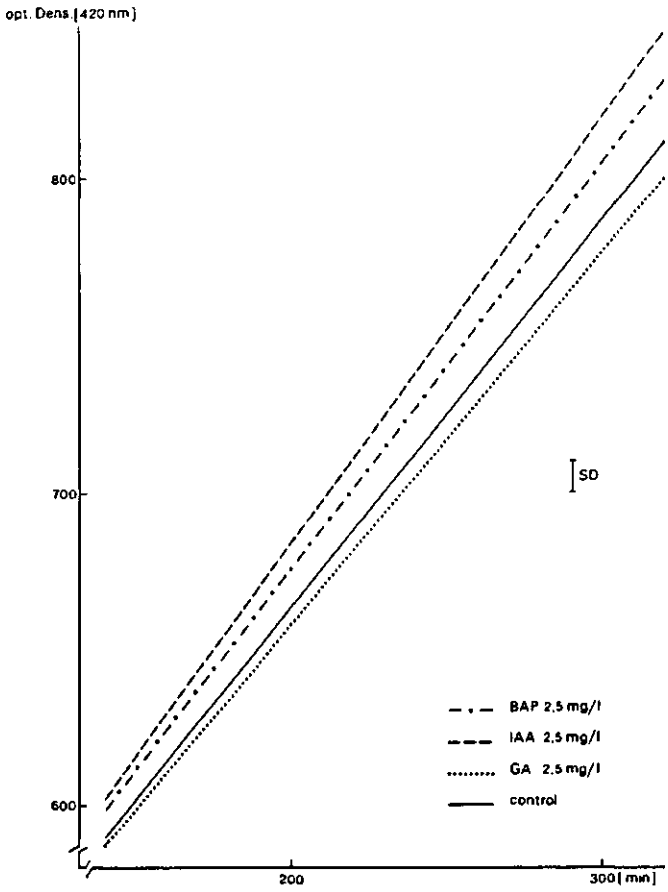

Figure 2. Influence of IAA, BAP and GA on growth of *Azospirillum brasilense*

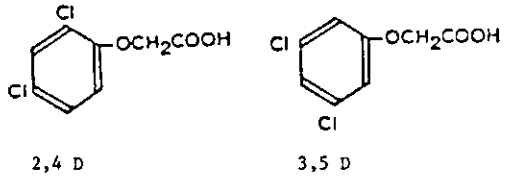

Figure 3. 2,4 and 3,5 dichlorphenoxy-acetic-acic

Table 1. Acetylene reduction of *Azospirillum brasilense* treated with plant growth substances at different final concentrations (nmol C_2H_4/h per ml bacterial suspension)

	0 mg/l	0.025 mg/l	0.25 mg/l	2.5 mg/l	25 mg/l
IAA	93	89	131	216	195
	b	b	ab	a	a
GA	93	71	115	126	107
	bc	c	ab	a	ab
BAP	93	73	82	92	84
	a	a	a	a	a
ABA	93	96	76	94	102
	a	a	a	a	a

Means with the same letter are not significantly different (p = 0.05)

In the same way, GA showed a higher nitrogenase derepressing activity together with IAA, 2.5 mg/l (Fig. 5). This derepression of nitrogen fixation affected by GA and ABA (IAA) may explain the fact that acetylene reduction often occurres in *Azospirillum* inoculated roots even when the plants are treated with nitrogen ferilizer.

Table 2. Acetylene reduction of *Azospirillum brasilense* treated with IAA, 2.4 D and 3.5 D at a final concentration of 2.5 mg/l (nmol C_2H_2/h per ml bacterial suspension)

Control	IAA	2.4 D	3.5 D
93	233	276	51
b	a	a	b

Means with the same letter are not significantly different (P = 0.05)

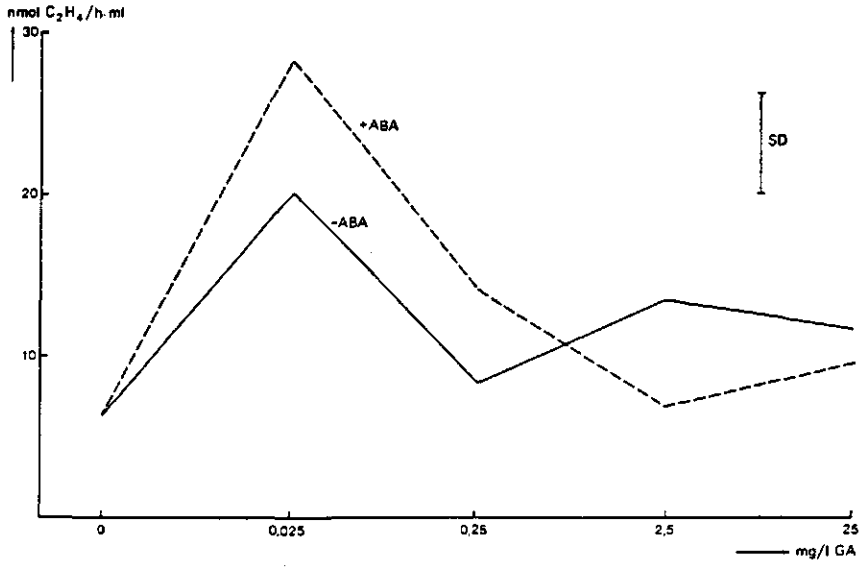

Figure 4 : Effect of GA on NH_4^+ (0.8 mM) repressed nitrogenase from *Azospirillum brasilense* with and without ABA, (2.5 mg/l).

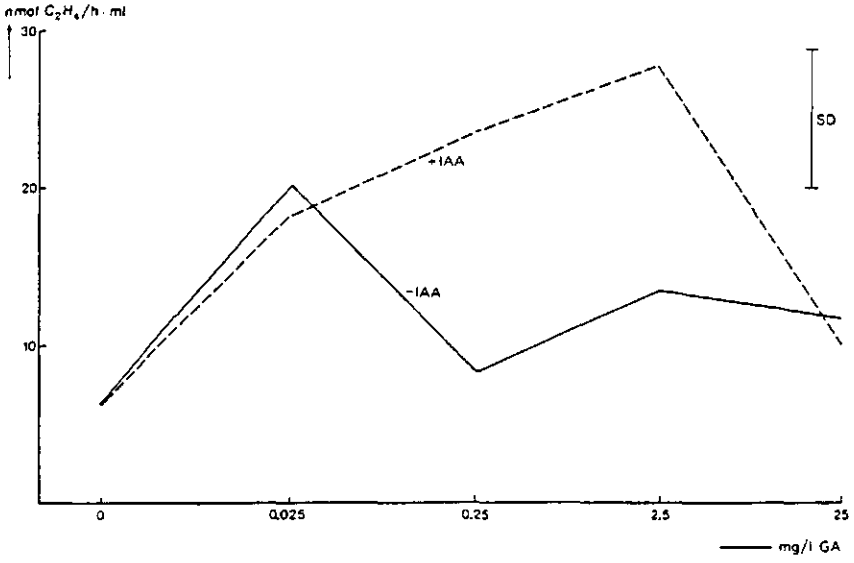

Figure 5: Effect of GA on NH_4^+ (0,8 mM) repressed nitrogenase from *Azospirillum brasilense* with and without IAA (2,5 mg/l).

How the plant growth substances interact with the nitrogenase regulation of *Azospirillum* is not clear. There are several indicationshints, that the here described influences are really hormonal affected, comparable with the manner in which plant growth substances are regulating the physiology of plants: So, for example that 2,5 D increases nitrogenase activity, but not the almost identical 3.5 D. GA in a very low concentration shows a significant effect on nitrogen fixation, that disappears in treatments with higher concentrations. The additive crossreaction between some growth substances as described in Figures 4 and 5 will lead to the same conclusion. But at least nearly nothing is known about the physiological way in which plant growth substances are affecting *A. Brasilense*. It is obvious that auxins are shown to be produced by *A. Brasilense* itself (Tien et al. 1979), which may indicate, that it is involved in bacterial physiology. To prove this, further investigations are necessary.

For the ecology of *A. Brasilense* root interactions the here presented results may give a new and very interesting aspect. In particular the positive effect on nitrogenase and nitrogenase repression due to an IAA and GA treatment will be of great importance. Both plant growth substances are known to be involved in the regulation of root development. They could either be produced in the root itself or transferred there from the shoot (Bridges et al. 1973, Scott 1972, Elliott and Greenwood 1974). The same is true for ABA (Hartung and Behl 1974, Pillet 1976). Cytokinin, which is shown here to have a positive influence on bacterial growth, also occurs in the root and it is suggested to be produced there (Wareing et al. 1977). For the transport of plant growth substances inside the root the cortical tissue is likely to play an important role (Shaw and Wilkins 1974). Moreover, there are several works which indicate that the root tip contains a high concentration of auxins (Bernet and Pillet 1976, Pillet 1976). So, the root areas which are demonstrated in electron micrographs to be predestinately colonized by *Azospirillum* (Murty and Ladha 1987) are places of a potential availability of plant growth substances. It is evident that Gramineous plants in theory, are able to stimulate the nitrogen fixation activity of a root associated *A. Brasilense* with help of its growth substances. Whether this really happens *in vivo* will be very difficult to prove because of the strict effect plant growth substances always have on the plant itself.

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CHAPTER 6

DYNAMICS OF A TRANSPOSON Tn5 MUTANT OF *AZOSPIRILLUM BRASILENSE* IN SOIL AND RHIZOSPHERE OF SPRING WHEAT [1]

Summary

Azospirillum brasilense was marked by insertion of transposon Tn5 into its genome. The Tn5 insertion did not interfere with physiological characteristics as nitrogen fixation, auxine production and nitrate reduction nor with the growth rate of the bacterium. The detection limit of the technique was as low as approx. 25 cells per gram dry soil.

Upon introduction of the Tn5 marked *A. brasilense* to an unplanted, sterilized soil the number of cells remained constant over a period of 100 days at approximately 10^7 cells per gram dry soil. In a non-sterilized soil the population decreased during the same period from 10^4 to 10^3 cells per gram soil. Within 45 days after introduction, *A. brasilense*::Tn5 was found in roots and rhizosphere soil of a sterile-grown spring wheat in amounts of approximately 10^6 cells per gram dry root and 10^6 cells per gram dry soil, respectively. The number in a similar non-sterile grown plant was low at approximately 10^3 cells per gram dry root and 10^4 cells per gram dry rhizosphere soil. In all cases the number of *A. brasilense*::Tn5 cells was 10-100 times higher in the soil fraction close to the roots than in the root-free soil. *A. brasilense*::Tn5 could not be isolated from inner root tissue after root surface sterilization.

Significant differences in later root colonization were not found when *A. brasilense*::Tn5 was applied to the plant either by seedling inoculation with a cell suspension or by bacterial seed coating. *A. brasilense*::Tn5, when introduced locally by seed coating, was able to develop with the elongating root. In plants grown under non-sterile conditions, the colonization of the developing root system appeared to be much lower than in sterile-grown plants. Competition between *A. brasilense*::Tn5 and a *Pseudomonas fluorescens* strain as a competing introduced rhizosphere bacterium was not found to have an effect.

Introduction

Azospirillum spp. are known to influence growth and nutrient uptake of the host plant (O'Hara et al., 1987; Warembourg et al., 1987). Increases in root dry matter production, nitrogen content and plant yield due to inoculation by *Azospirillum* have been reported by Baldani et al. (1983), Schank et al. (1981), Watanabe and

[1] Submitted to *Soil Biol. Biochem.*

Lin (1984) and others. The mechanisms of this growth-stimulating activity of *Azospirillum* are still under debate. Improved N-supply by N_2-fixation, the production of plant growth-stimulating substances (Tien et al., 1979; Harari et al., 1988) and bacterial nitrate reductase activity (Scott et al., 1979) have been mentioned in this respect. Several attempts have been made to make use of these potentially beneficial characteristics of *Azospirillum* by field inoculation of crops (Kapulnik et al., 1981; Reijnders and Vlassak, 1982).

Irrespective of the mechanisms involved, a vital factor for successful application is that *Azospirillum* is able to establish in soils and in the rhizosphere in sufficient numbers, and that the introduced cells are able to compete with the native bacterial population in a natural environment. Such survival studies require a bacterial marker which does not interfere with essential physiological characteristics. Spontaneously drug-resistant *Azospirillum spp* have been used before to trace introduced *Azospirillum* in a natural environment (Nayak et al., 1986). In our experiments, we marked *Azospirillum brasilense* by a transposon (Tn5), inserted into its genome. This marked *A. brasilense*::Tn5 was used to study its potential survival capacities either under sterile or under non-sterile conditions. The suitability of Tn5 transposon mutants for such studies has been shown successfully by van Elsas et al. (1986) and Fredrickson et al. (1989). The main objective of our work was to quantify the dynamics of introduced *A. brasilense*::Tn5 in a wheat-root environment.

Materials and methods

Bacterial strains

As wild type *Azospirillum brasilense* we used strain Wa5, originating from the rhizosphere of greenhouse-grown spring wheat (Christiansen-Weniger, 1988). Tn5 transposon insertion into *A. brasilense* was carried out as described by Singh and Klingmüller (1986). The Tn5 transposon contains genes coding for resistance against kanamycin (Kn) and streptomycin (Sm). The bacteria were screened for spontanous resistance against rifampicin (50 $\mu g\ ml^{-1}$). Rifampicin-resistant *A. brasilense* was mated overnight at 37°C on a nitrocellulose filter in co-culture with *Escherichia coli*, containing the transposon Tn5 on the suicide plasmid pGS9 (Selvaraj and Iyer, 1983; kindly provided by W.Klingmüller, Bayreuth). Mated bacteria were resuspended in 0.8% NaCl solution and plated out on selective Luria broth agar (50 μg rifampicin ml^{-1}, 80 μg kanamycin ml^{-1}). Transconjugant *A. brasilense* were picked up as small white rifampicin- and kanamycin-resistant colonies. Selected Tn5 mutant *A. brasilense* were tested for their growth, nitrogenase activity, auxin production and nitrate reductase activity to see whether the transposon insertion had damaged these key characteristics of the bacterium.

Pseudomonas fluorescens strain R2f was used for competition studies. This strain, an isolate from the rhizosphere of grass, contained the plasmid RP4 (Van Elsas et al., 1988). The bacterium was resistant against kanamycin and tetracyclin.

Biochemical assays

Bacterial growth (OD 540) was determined in a 50 ml-batch (30°C, 350 rpm) of minimal NFB medium (Okon et al.,1977), supplemented with 10 mM NH_4Cl.

Acetylene reduction was measured under oxygen-limited conditions in a semi-solid (0.2% agar) NFB medium with 10% v/v acetylene added to the head space.

Total bacterial protein was analysed after cell lysis in 1N NaOH at 60°C by the procedure of Lowry et al. (1951) using bovine serum albumine as standard.

Auxin formation was tested by incubating *A. brasilense* for 48 hours in a batch of NFB minimal medium containing 10 mM NH_4Cl and 100 mg DL-tryptophan per litre (30°C, 350 rpm). Produced indole-acetic-acid (IAA) in the supernatant was determined by the Salkofski color reaction after centrifugation (Tang and Bonner, 1946).

Nitrate reductase activity was determined in semi-solid (0.2% agar) NFB medium containing 8 mM NH_4NO_3 (Nicholas and Nason, 1957).

Soils and plants

The soil used was a loamy sand (0.3 mg N kg^{-1}, 3.5% organic matter, 15% moisture). 70 mg P, 98 mg K, 50 mg N per kg soil and trace elements according to the Hoagland nutrient solution (Hoagland and Broyer, 1936) were added. The soil was sterilized by exposing soil samples to 4 megarad γ-radiation. For incubation studies 2 liter pots with 1 kg fresh soil were used, covered with plastic lids to avoid contamination. Sterilized water was added daily to adjust the moisture content.

To be able to follow root colonization over different distances from the point of inoculation, plants were grown for two weeks in narrow soil columns (5 cm diameter and 50 cm length), both under axenic and natural conditions. As the columns could be opened without disturbing the root environment separation of root and soil samples over the entire length of the column was possible.

The spring wheat used was *Triticum aestivum* var. *"Ralle"* (received from the Foundation for Agricultural Plant Breeding SVP, Wageningen). Seeds were surface-sterilized with 1.5% Na-hypochlorite (90 min) and pregerminated on tryptone soya agar (TSA, Oxoid) to check for sterility. Seeds with a bacterial coating were pregerminated in long tubes on sterilized or non-sterilized soil. After 7 days the seedlings were transferred to the growing pots, three plants each. One plant was transferred to each soil column. Plants were sealed around the stem with a cotton plug. Growth conditions were: 12 hours day at 20°C and 12 hours night at 12°C with a relative humidity of 70%.

Bacterial inoculations

Bacteria were grown in a batch (350 rpm) at 37°C in Luria Broth (LB) until the end of the logarithmic growth phase. The culture was centrifuged and resuspended in a 0.85% NaCl solution. Final cell density was 1.4×10^8 CFU (colony forming units) ml^{-1}. Five mls of the cell suspension was injected in the centre of each pot in case of unplanted soils and for seedling inoculation. The final bacterial density was 7.9×10^5 CFU per gram dry soil. Controls were treated with an autoclaved bacterial suspension. For bacterial coating seeds were treated with a solution of sterile gum arabic (40%), supplemented with a bacterial culture to a final cell density of 1.3×10^7 CFU ml^{-1}. Surface-sterilized, as well as untreated

seeds, were dipped into this mixture and transferred to germination tubes. The average number of bacteria on the seeds was 2 x 10^6 CFU per seed. Treatments were carried out with three replicate plants each; in column experiments with two replicate columns each.

Sampling and determination of bacterial numbers

Soil samples were taken from the 2 l pots with sampling tubes of 1 cm diameter and 10 cm length at 3 cm distance from the point of inoculation; material was collected over the full depth of the pot. Samples were shaken intensively in 95 ml 1% Na-pyrophosphate solution; 15 g gravel was added to disrupt soil aggregates. The suspensions were plated out after subsequent tenfold dilution steps.

To determine the numbers of root colonizing bacteria, root samples were divided into three different fractions. The first fraction existed of rhizosphere soil, which remained on the roots after shaking and which was released by careful washing in 95 ml 1% Na-pyrophosphate solution. The second fraction was the washed root itself, and the third fraction was formed by the roots after surface-sterilization with 1% chloramine T solution (15 min, Patriquin and Döbereiner, 1978). For counting bacteria, root material was macerated in homogenizing tubes. Bacteria were not only determined in these rhizosphere fractions but also in root-free bulk soil. Since an accurate separation of replicate plant roots was impossible in pot experiments (roots were intertwining) an average sample of all three replicate plants was taken.

Soils and roots were dried at 80°C for dry matter analysis.

The bacterial suspensions were plated on the following media:

- for total bacteria: tryptone soya agar, TSA (Oxoid);

- for *A. brasilense*::Tn5: NFB minimal malate medium with kanamycin (50 mg l^{-1}), rifampicin (50 mg l^{-1}) and kongo-red added to a final concentration of 4 g l^{-1} (Bashan and Levanony, 1985);

- for *P. fluorescens R2f*: Kings B medium (20 g l^{-1} proteose peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4$, 15 ml glycerol, pH 7.3) with kanamycin 50 mg l^{-1} and tetracycline 30 mg l^{-1}.

All media were supplemented with cycloheximide (60 mg l^{-1}) and benomyl (30 mg l^{-1}) to suppress fungal growth.

Double-resistant *A. brasilense* or *P. fluorescens* spp. were not detected in the natural soils as evidented by plating on selective Kings B and NFB media.

Results and discussion

Neither the occurrence of a spontaneous rifampicin-resistance nor the transposon Tn5 mutagenesis caused a serious decrease in the growth rate of *Azospirillum brasilense* (Fig. 1). Key characteristics, such as N_2-fixation, auxin production and nitrate reductase activity were not significantly affected (Table 1).

When plated out on congo-red containing NFB medium, *A. brasilense* formed typical small red colonies. Together with the double resistance against kanamycin and rifampicin a powerful marker combination is formed, which allows efficient detection and a detection limit as low as 25 cells per gram dry soil. As encystation of *A. brasilense* is reported (Papen and Werner, 1982; Sadasivan and Neyra, 1985) and colony development from cysts on minimal agar plates is not

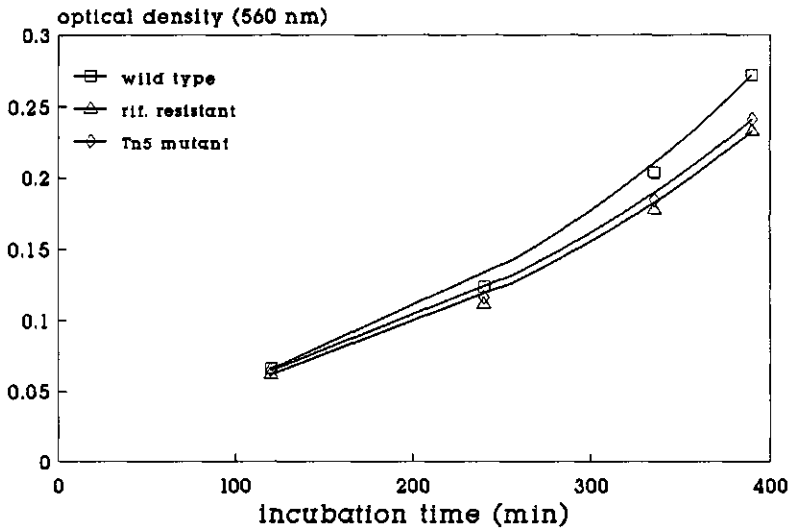

Figure 1. Growth of *Azospirillum brasilense* wild type, a rifampicin-resistant strain and a Tn5 mutant.

well understood, the given CFU values may include an underestimation of real *A. brasilense* populations.

Upon introduction into a sterilized, unplanted soil the number of *A. brasilense*::Tn5 cells increased within 20 days after incubation from approximately 10^6 CFU per gram soil to 10^8 CFU and remained at that level until day 100 (Fig. 2). These numbers were similar to those observed for *Rhizobium leguminosarum* biovar *trifolii* and *Pseudomonas fluorescens R2f* in the same soil (van Elsas et al., 1989; Postma et al., 1990). The observed dynamics of an introduced *A. brasilense::Tn5* population in a sterilized soil were similar to data reported by Steinberg et al. (1989); total cell numbers, however, were approximately 100 times lower in our study. This may be attributed to the fact that in the study of Steinberg et al. (1989) bacterial dynamics were studied in small microcosms of 8-10 g soil to which the bacteria were introduced by spreading over the complete samples, followed by intensive mixing. In our experiments a relatively small amount of

inoculation suspension (5 ml) was added to a large amount of soil (1 kg) so that the bacteria may have been clustered through the lack of any vector for bacterial movement over larger distances, so that full exploitation of the available substrate was not possible (Postma and Van Veen, 1989).

The survival of *A. brasilense*::Tn5 in a non-sterilized soil was low. 10^3 CFU were counted on day 6 and this number decreased slowly to 10^2 CFU on day 100 (Fig. 2). These results correspond with those of Harris et al. (1989) for the survival of introduced *A. brasilense* under field conditions. It is also shown that genetically modified *A. brasilense*::Tn5 survives for more than three months in a natural environment, which may have consequences for the assessment of the risks involved in the use of genetically modified microorganisms in soil (Van Elsas et al., 1991a).

Table 1. Nitrogen fixation, auxin production and nitrate reductase activity of *Azospirillum brasilense* wild type, a rifampicin-resistant strain and a Tn5 mutant.

	Nitrogen fixation	Auxin production	Nitrate reductase activity
	(nmol C_2H_4 min^{-1} mg $protein^{-1}$)	(μg IAA ml^{-1} 48 h^{-1})	
A.brasilense wildtype	14.43	43.3	+
Rifampicin-resistant strain	8.18	45.5	+
Tn5 mutant strain	11.1	44.3	+

When introduced to wheat plants by coating surface-sterilized seeds, *A. brasilense*::Tn5 was observed in the root-free soil fraction with cell numbers above the detection limit only after 14 days, once they slightly increased in sterilized soils (Fig. 3 and 4). On roots grown under non-sterile conditions, the number of *A. brasilense*::Tn5 cells decreased from 10^4 CFU per gram root on day 7 after planting to 10^3 CFU on day 35. In the rhizosphere soil, the number of *A. brasilense*::Tn5 remained stable over the entire period of plant growth at 10^4 CFU per gram dry soil as compared to only 10^2 in the root-free soil fraction (Fig. 3). With sterile-grown plants the number of *A. brasilense*::Tn5 increased in the root-free soil within 35 days to 10^5 CFU per gram soil. The amounts of *A. brasilense*::Tn5 in the root and in rhizosphere soil were stable at 10^5 CFU per gram dry root or 10^6 CFU per gram rhizosphere soil, respectively (Fig. 4).

Table 2. Number of *Azospirillum brasilense::Tn5* cells from soil (log CFU g^{-1} dry soil) and roots (log CFU g^{-1} dry root) of sterile-grown plants (35 days after inoculation).

	Seedling inoculation	Coating of surface-sterilised seed	Coating of untreated seed
Root-free soil	4.94	4.69	4.72
Rhizosphere soil	5.90	5.43	5.85
Washed root	4.38	5.19	4.41
Surface-sterile root	[0][1]	[0]	[0]

[1] arithmetic zero, below detection limit (1.4 log CFU g^{-1}dry root)

Table 3. Number of *Azospirillum brasilense::Tn5* cells from soil(log CFU g^{-1} dry soil) and roots (log CFU g^{-1} dry root)of nonsterile grown plants (35 days after inoculation).

		Seedling inoculation	Coating of surface-sterilised seed	Coating of untreated seed
Root-free soil	TP[1]	7.61	7.13	7.26
	AZO[2]	2.06 (0.01)[3]	2.88 (0.25)	1.82 (<0.01)
Rhizosphere soil	TP	8.11	8.08	8.18
	AZO	4.50 (0.25)	4.7 (0.42)	3.71 (0.03)
Washed root	TP	7.34	7.02	7.38
	AZO	3.57 (0.17)	3.62 (0.40)	2.04 (<0.01)
Surface-sterile root	TP	5.23	5.33	5.62
	AZO	[0][4]	[0]	[0]

[1] TP : total bacterial population
[2] AZO : *A. brasilense*::Tn5
[3] relative densities of *A. brasilense*::Tn5 are given in parenthesis as % of the total number of bacteria
[4] arithmetic zero, below detection limit (1.4 log CFU g^{-1}dry root)

In sterile as well as in non-sterile grown plants, the cell densities of *A. brasilense*::Tn5 were 10-100 times higher in the rhizosphere soil than in corresponding root-free soil (Tables 2 and 3). Large numbers of *A. brasilense*::Tn5 (10^4 CFU to 10^5 CFU per gram root, Tables 2 and 3) were also found on the roots after careful washing. *Azospirillum* was mentioned to be bound to roots by fibrillar material (Whallon et al., 1985; Murty and Ladha, 1987), as well as to colonize inside intercellular spaces of the root cortex (Bashan and Levanony, 1988). This means that the plant root forms a suitable colonization niche for introduced *A. brasilense*. Moreover, *Azospirillum* is chemotactically attracted by organic acids, sugars and amino acids exuded by roots (Barak et al., 1983; Heinrich and Hess, 1985; Reinhold et al., 1985). Active migration of *A. brasilense* in soils towards an attractant is reported by Bashan (1986). This binding to roots, however, is not an essential process for the survival of introduced *Azospirillum::Tn5,* as can be concluded from the large numbers of bacteria in the adhering soil. Furthermore, no introduced *A. brasilense*::Tn5 was reisolated from surface-sterilized roots, although 10^5 total bacterial CFU, which is approximately 1% of the total root population, were counted inside the roots after surface-sterilization with 1% chloramine T (Table 3). This contradicts the hypothesis of Patriquin and Döbereiner (1978) that *Azospirillum* settles at high cell densities inside the xylem system of the host plant.

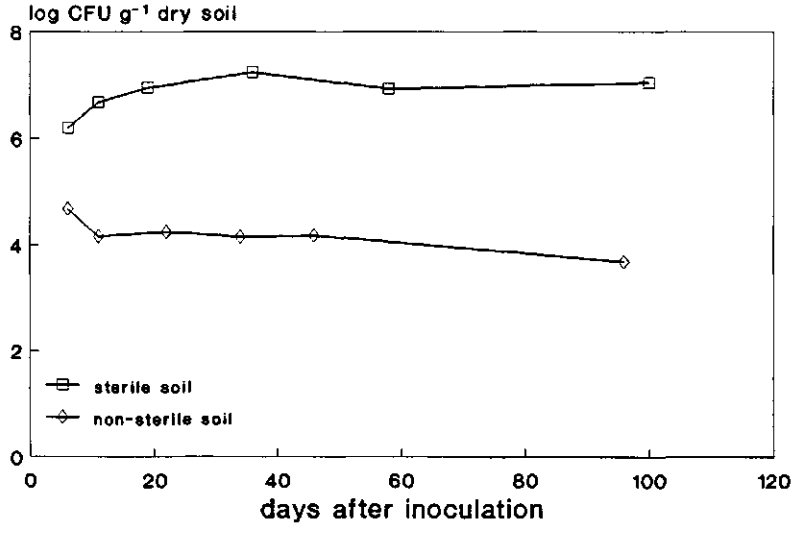

Figure 2. Survival of *Azospirillum brasilense::Tn5* in a sterilized and in a non-sterilized soil; inoculation by 5 ml bacterial suspension (1,4 x 10^8 CFU ml^{-1}) to 1 kg fresh soil.

Table 4. Number of *Azospirillum brasilense::Tn5* cells from soil (log CFU g^{-1} dry soil) and roots (log CFU g^{-1} dry root) of sterile-grown plants inoculated together with *Pseudomonas fluorescens R2f* (35 days after inoculation).

		No PS	PS, seedling inoculation	PS, late inoculation (day 15)
		A. brasilense introduced by seedling inoculation		
Root-free soil	PS[1]		6.92	5.24
	AZO[2]	4.94	4.97	4.63
Rhizosphere soil	PS		7.58	6.01
	AZO	5.9	6.42	5.98
Washed root	PS		6.26	4.98
	AZO	4.38	4.94	4.23
		A. brasilense introduced by seed coating		
Root-free soil	PS		6.52	5.76
	AZO	4.69	5.08	5.25
Rhizosphere soil	PS		8.04	6.48
	AZO	6.43	5.90	6.14
Washed root	PS		6.71	4.83
	AZO	5.19	4.90	4.55

[1] PS : *P. fluorescens R2f*
[2] AZO : *A. brasilense*::Tn5

Among the different application methods the highest number of *A. brasilense*::Tn5 cells was observed in the root-free soil following seedling inoculation. Seed coating (using surface-sterilized seeds) gave better *A. brasilense*::Tn5 rhizosphere and root colonization (Tables 2 and 3), although the initial numbers of introduced *A. brasilense*::Tn5 cells were approximately 100 times lower in case of seed coating than upon seedling inoculation. Coating of non-surface-sterilized seeds caused lower numbers of *A. brasilense*::Tn5 cells

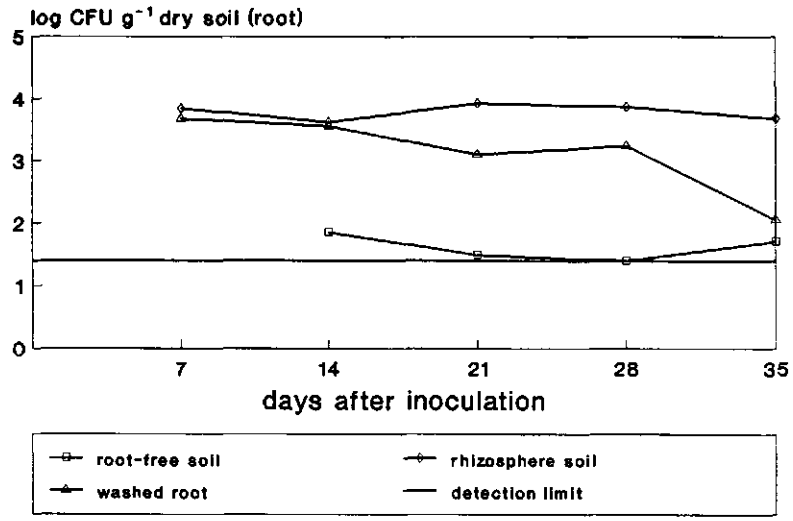

Figure 3. Survival of *Azospirillum brasilense::Tn5* in the rhizosphere of non-sterile grown summer wheat; inoculation by bacterial seed coating (2 x 10^6 CFU $seed^{-1}$).

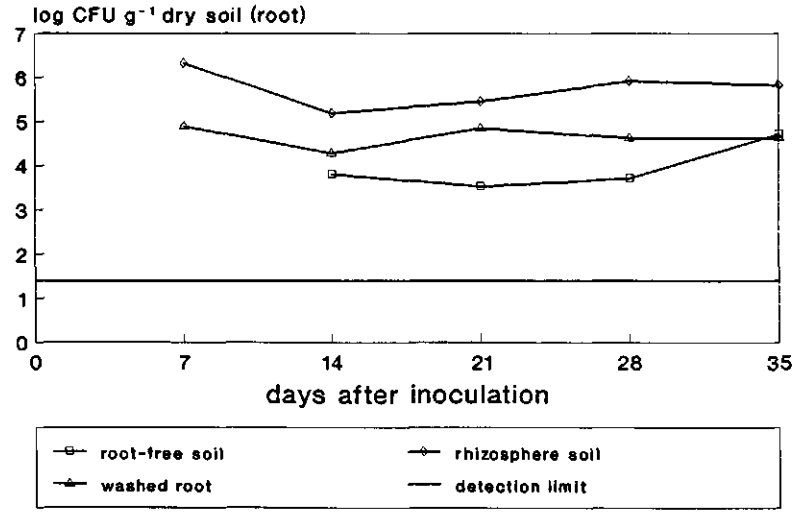

Figure 4. Survival of *Azospirillum brasilense::Tn5* in the rhizosphere of sterile-grown summer wheat; inoculation by bacterial seed coating (2 x 10^6 CFU $seed^{-1}$).

in all three root fractions than coating of surface-sterilized seeds (Table 2). As the percentage of A. brasilense*::Tn5* relative to the total bacteria population was very low (maximum 0.042% of total number of bacteria, Table 3), competition with the natural flora might be an important aspect. However, *Pseudomonas fluorescens R2f*, when introduced together with *A. brasilense*::Tn5 as a second rhizosphere bacterium, did not affect the root colonization of *A. brasilense*::Tn5. Again, no differences in later rhizosphere colonization occurred between seedling inoculation and coating of surface-sterilized seeds (Table 4). This indicates that *P. fluorescens R2f* and *A. brasilense*::Tn5 may occupy different niches on the host root. Yet, when *P. fluorescens R2f* was inoculated in a later stage of plant growth (day 15) smaller amounts of soil and root colonisation were observed (Table 4). Van Elsas et al. (1989, 1991b) reported recently a plasmid loss of RP4 from *P. fluorescrens R2f* when bacteria were introduced to a loamy sand. This led to an underestimation of the counted bacterial population.

A. brasilense::Tn5 when introduced by seed coating grew along the developing root and established a population over the total rhizosphere (Fig. 5). Under axenic conditions the numbers of *A. brasilense*::Tn5 at distances 25 to 37.5 cm from the seed were of the same order of as the numbers close to the seeds. This was true for all three rhizosphere fractions (Fig. 5). With plants grown under non-sterile conditions the number of *A. brasilense*::Tn5 decreased with the distance from the place of inoculation, but root and rhizosphere were still colonized. At a distance of 33 to 50 cm from the seed, introduced *A. brasilense*::Tn5 could not be detected in the root-free soils (Fig. 6).

When treated with *A. brasilense*::Tn5 plants growing in sterilized soil developed a significantly higher root biomass than plants in non-sterilized soil; the phenotype of a sterile-grown root showed increased branching and higher lateral root development than roots in a natural environment (Fig. 7). This may be explained by the fact that associated *Azospirillum* stimulates root growth and morphology, probably due to the production of plant growth substances, such as indole-acetic-acid (IAA) (Barbieri et al., 1986; Harari et al.,1988).

Thus, although roots provided a suitable niche for *Azospirillum*::Tn5, and movement together with the developing root system is possible, competition, predation and other interactions with the soil fauna and flora prevent the bacterium to fully develop over the root system. This clearly also affects the influence of *Azospirillum*::Tn5 on root development.

Our experiments demonstrate the establishment of *Azospirillum*::Tn5 in the soil and in the wheat rhizosphere; cell densities, however, were negligible low. This makes it unlikely that under temperate field conditions *Azospirillum* inoclation will have a considerable effect on the host plant. The Tn5 transposon insertion in combination with congo red staining and growth on nitrogen free medium was found to be a reliable marker for tracing introduced *Azospirilli* in natural environments.

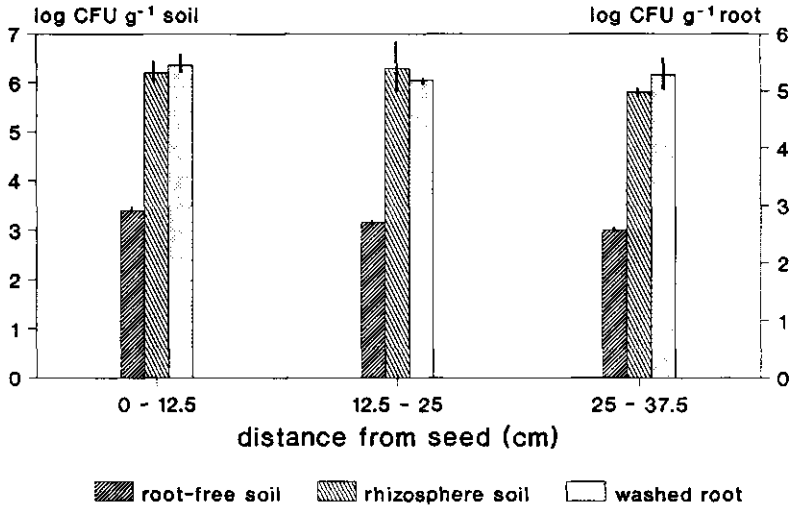

Figure 5. Survival of *Azospirillum brasilense::Tn5* in the rhizosphere of sterile-grown summer wheat at different distances from the point of inoculation; inoculation by bacterial seed coating (2 x 10^6 CFU $seed^{-1}$); harvest 14 days after planting. Small bars indicate the standard deviation of two replicate columns.

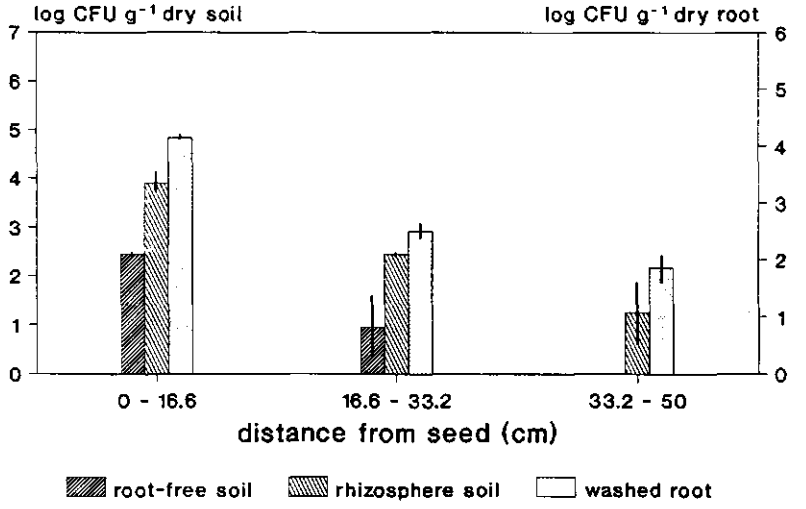

Figure 6. Survival of *Azospirillum brasilense::Tn5* in the rhizosphere of non-sterile grown summer wheat at different distances from the point of inoculation; inoculation by bacterial seed coating (2 x 10^6 CFU $seed^{-1}$); harvest 14 days after planting. Small bars indicate the standard deviation of two replicate columns.

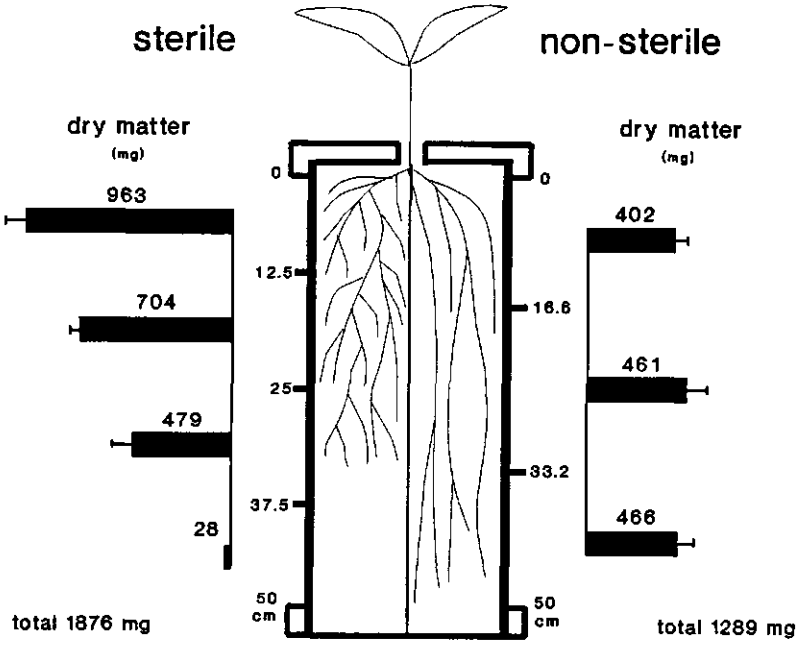

Figure 7. Root dry matter and root morphology of *Azospirillum brasilense::Tn5* inoculated summer wheat grown on sterilized and non-sterilized soil; inoculation by bacterial seed coating (2×10^6 CFU seed^{-1}); harvest 14 days after planting. Small bars indicate the standard deviation of two replicate columns.

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CHAPTER 7

NH_4^+-EXCRETING MUTANTS OF *AZOSPIRILLUM BRASILENSE* ENHANCE THE NITROGEN SUPPLY OF A WHEAT HOST [1]

Summary

Spontaneous ethylenediamine resistant mutants of *Azospirillum brasilense* were selected for their excretion of NH_4^+. Two mutant strains showed no repression of their nitrogenase enzyme system in the presence of high (20 mM) concentrations of NH_4^+. The nitrogenase activity on nitrogen-free minimal medium was two to three times higher than that of the wild type. The mutant strains excreted substantial amounts of ammonia when grown either under oxygen limitation (1 kPa O_2) or aerobically on nitrate or glutamate. The mutants grew well on glutamate as sole nitrogen source but only poorly on NH_4Cl. As the mutant strains failed to accumulate [^{14}C]methylamine, there are probably mutations in their ammonia transport system across cell membranes. Nitrate reductase was active at wild type and at mutants. For both mutant strains we demonstrated a nitrite ammonification.

Upon introduction, wild type *A. brasilense* as well as the mutant strains established in the rhizosphere of axenically grown wheat to levels of $> 10^7$ g^{-1} root. Comparable effects on root and shoot development were observed. The acetylene reduction activity in the rhizosphere was highest in the treatments with the mutant strains. When plants were grown on a nitrogen-free nutritional medium both mutant strains caused a significant increase of root and shoot dry matter as compared to wild type treated plants or to non-inoculated controls. Total plant nitrogen accumulation was positively affected as well. When exposed to a [^{15}N]$_2$-enriched atmosphere, both mutant *A. brasilense* strains incorporated significantly higher amounts of ^{15}N inside root and shoot material than the wild type. Nitrogen balance and ^{15}N enrichment studies both indicated that NH_4^+-excreting *A. brasilense* potentially supports the nitrogen supply of the host plants.

Introduction

Azospirillum spp. is known to colonize the rhizosphere of graminaceous plants where it develops considerable rates of N_2 fixation (Charylulu et al. 1985, Nur et al. 1985, Watanabe and Lin 1983). However, the amount of nitrogen fixed by *Azospirillum* which is transferred to its host plant appears to be small (Boddey et al. 1986, kapulnik et al. 1985, Kucey 1988). Investigations with the ^{15}N isotope

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dilution technique indicate that most of the fixed nitrogen remains below ground, probably still bound to bacterial cells, and contributes only very little to the upper plant parts (Christiansen-Weniger et al. 1985, Nayak et al. 1986, Okon et al. 1983). In experiments with wheat monoxenically associated with *Azospirillum brasilense* only 1 to 2 percent of the shoot nitrogen originated from atmospherical sources (Christiansen-Weniger, in preparation). The total nitrogen gain from nitrogen fixation of root-associated *A. brasilense* was calculated to be 2 to 3 kg N $ha^{-1}year^{-1}$; by comparison, in intensive arable farming chemical fertilizer input is up to 250 kg N ha^{-1} $year^{-1}$.

The reason for this limited nitrogen supply by associative nitrogen fixation is probably that *Azospirillum*, similar to other free-living diazotrophs, but contrary to symbiotically living rhizobia, does not release fixed nitrogen to its environment. Kleiner (1981, 1984) postulated that intracellularly formed NH_4^+ does diffuse through cell membranes along with the pH gradient but that membrane-bound proteins with high affinity for ammonia will immediately carry this released NH_4^+ back. Hartmann and Kleiner (1982) demonstrated that such an ammonia transport system is active in the cell membranes of *A. brasilense*. In order to stimulate the release of fixed nitrogen from *Azospirillum* cells we tried to find *A. brasilense* mutants which lack this ammonia transport system. This was done by selection for resistance against ethylenediamine, an inhibitor of bacterial glutamine synthetase. Ethylenediamine-resistant mutants of *A. brasilense* were selected earlier, yielding mutant strains unrepressed for nitrogenase activity at high concentrations of NH_4Cl (Fischer et al. 1986). Recently, Pedrosa and co-workers have reported ethylenediamine resistant *A. brasilense* strains which released substantial amounts of ammonia to their environment (Machado et al. 1990). However, it was not reported whether these mutants were active in the rhizosphere of plants and whether they supplied fixed nitrogen to host plants.

In this paper we will describe two NH_4^+-excreting *A. brasilense* strains and report on their behaviour in the rhizosphere of wheat and on their effect on growth and nitrogen uptake of the plant. Besides determining root colonization and rhizosphere acetylene reduction, we quantified the supply of fixed nitrogen to the host plants using the ^{15}N enrichment technique. This technique was earlier demonstrated to be a suitable method for determination of heterotrophic nitrogen fixation in grasses (Eskew et al. 1981, Ito et al. 1980). Its advantage over the more widely used ^{15}N dilution technique (Boddey et al. 1983, Giller et al. 1986, Owens 1977, Rennie 1980) is that it allows a direct tracing of fixed nitrogen inside plant material as compared to the ^{15}N dilution technique which can be considered only as an indirect assay for the N supply of plants by N_2 fixation activity (Wittey 1983).

Materials and methods

Bacterial strains

As wild type we used *Azospirillum brasilense* strain Wa5, which originated from the rhizosphere of a greenhouse-grown summer wheat (Christiansen-Weniger 1988). *A. brasilense* Wa5 was grown overnight on Luria broth (LB), centrifuged and washed twice with a 0.85% NaCl solution. 100 µl of the final cell suspension (approx. 10^7 cells) was plated out on a nitrogen-free minimal malate medium

(NFB, Okon et al. 1977), supplemented with ethylenediamine to a final concentration of 0.05% (Fischer et al. 1986). Colonies resistant to ethylenediamine were picked up and cells were added to a semisolid (0.2% agar) glycerol minimal medium (15 ml 87% glycerol, 0.3 g K_2HPO_4, 0.3 g KH_2PO_4, 0.2 g $MgSO_4$, 0.1 g NaCl, 0.02 g $CaCl_2$, 0.5 g $FeSO_4$ per liter demineralized water, trace elements according to Day and Döbereiner (1976); pH 6.8). This glycerol medium was chosen since it allows *Azospirillum* to grow without affecting the pH of its medium. When *Azospirillum* is grown on malic acid or succinic acid as carbon source the medium becomes strictly alcaline, which will result in loss of released ammonia. Bacterial NH_4^+ excretion was tested in media containing either no nitrogen source or a) 8 mM KNO_3, or b) 3 mM glutamate. Selection was made on the basis of NH_4^+ accumulation in each medium within three days. NH_4^+-excreting strains were further tested for acetylene reduction in semi solid (0.2% agar) NFB medium in the presence of 10 mM NH_4Cl.

Bacterial assays

Bacterial growth rates were determined at 30°C in a 100 ml batch of minimal NFB medium (350 rpm), supplemented with either 3 mM NH_4Cl or 3 mM glutamate.

Bacterial acetylene reduction was measured in airtight vials containing semi solid (0.2% agar) glycerol minimal medium with 10% (v/v) acetylene added to the headspace. The acetylene reduction activity was determined both in N-free medium and in media containing different concentrations of ammonia, nitrate and glumatate. The production of ethylene was assayed by gas chromatography (Varian 1700, Porapack T Column, FID-detector).

Total bacterial protein was analysed after a cell lysis in 1 N NaOH at 60°C by the procedure of Lowry et al. (1951) using bovine serum albumine as standard.

In order to determine the excretion rate of NH_4^+ bacteria were grown overnight on 40 ml LB, washed once with 0.85% NaCl and resuspended in 100 ml glycerol minimal medium containing either 8 mM nitrate or 3 mM glutamate as sole nitrogen source or no nitrogen at all. The cell suspension was transferred to closed 1-l serum bottles and incubated with intense shaking at 30°C. To reduce the oxygen concentration the headspace air was replaced by pure N_2 and air was added to obtain a final O_2 concentration of 1 kPa. Sampling for NH_4^+ started after 4 hours to allow bacteria to develop an active nitrogenase system. Excreted NH_4^+ was determined in the supernatant after centrifugation of the cell suspension by a modified Berthelot color reaction (Krom 1980). As glutamate interfered with the color development, NH_4^+ assay in glutamate containing medium was performed using Nesslers reagens (Krug et al. 1979). The pH of the medium was determined prior to each assay and at the end of the incubation time.

Transport of ammonia through cell membranes was determined by growing bacteria in a [^{14}C]methylamine enriched NFB minimal medium (Hartmann and Kleiner 1982, Kleiner 1982). Overnight grown *A. brasilense* was centrifuged, washed twice with 0.85% NaCl solution and resuspended in 100 ml of a NFB medium, containing 0.3 mM NH_4Cl and [^{14}C]methylamine to a final concentration of 1.6 µM. The ^{14}C activity was 34 kBq ml^{-1}. 1-ml samples were removed at 10-min intervals and centrifuged. Bacterial cells were washed once with 0.85% NaCl, resuspended in 1 N NaOH and lysed 1 hour at 60°C. Intracellular radioactivity was

measured by liquid scintillation counting (Packard Tri Carb 4530).

Bacterial nitrate reductase activity was assayed by growing bacteria in semi solid (0.2 % agar) NFB medium supplemented with 8 mM NH_4NO_3. Formed nitrite was determined colorimetrically after 48 hours according to Neyra and Van Berkum (1977).

Nitrite ammonification was measured in a semi solid (0.2 % agar) glycerole minimal medium supplemented with 3 mM $NaNO_2$. Formed NH_4^+ was determined after 24 hours by a modified Berthelot color reaction (Krom 1980).

Plants and growth conditions

As host plant *Triticum aestivum* var. *'Carasinho'* (received from the Foundation for Agricultural Plant Breeding SVP, Wageningen) was used, an aluminium tolerant cultivar known to develop an effective rhizosphere association with *A. brasilense* Wa5 (Christiansen-Weniger, in preparation). Seeds were surface-sterilised by soaking in 1.5% Na-hypochlorite for 90 min. Plants were grown in glass tubes (20 cm length, 3 cm diameter), two plants each, on 2.5 g sterilised perlite supplemented with 15 ml of a sterile nitrogen free nutrient solution (Fahreus 1957). Seeds were covered with a thin layer of sterile gravel to enable developing roots to penetrate the perlite.

Bacterial inoculation was performed by seed-coating with a mixture of sterile gum arabic (40%) and a bacterial suspension of 1.3×10^7 cfu (cell forming units) ml^{-1}. The final bacterial density on each seed was approx. 2×10^6 cfu. Control plants were treated with a sterile gum arabic solution only.

To trace the transfer of fixed nitrogen to host plant material plants were exposed to a $[^{15}N]_2$-enriched atmosphere. The tubes were sealed airtight with a rubber seal 7 days after planting (DAP), after which 10 ml of the headspace was replaced with a 96.5%-enriched $[^{15}N]_2$ gas (VEB Technische Gase, Leibzig), yielding a final ^{15}N concentration of 7.302 atom % excess, which was maintained over the entire period of plant growth. Flushing of the headspace and subsequent $[^{15}N]_2$ injection was repeated at 14, 20 and 25 DAP.

Plants were grown 31 days for 12 hours light at 20°C and for 12 hours dark at 12°C with a relative humidity of 70%.

Plant assays

The acetylene reduction activity was determined at 30 DAP by replacing 10% (v/v) of the tube headspace with acetylene. Then, plants were incubated for 24 hours at light conditions and at a constant temperature of 25°C. The production of ethylene was determined by gas chromatography as mentioned before. An ethylene standard was given to unplanted tubes. It was ensured that no ethylene was formed by plants themselves.

Plants were harvested at 31 DAP and roots and shoots were sampled separately. Plant dry matter was measured after drying at 80°C for 48 hours. Nitrogen content was measured using an Automatic Nitrogen Analyzer (Carlo Erba NA 1500) and the ^{15}N enrichment was determined by mass spectroscopy (VG Sira 10). Atom % ^{15}N values from unexposed plants were used as controls to determine the gain of nitrogen in the plant from atmospherically fixed nitrogen.

Bacterial root colonization was determined by carefully macerating 250 mg fresh root in homogenizing tubes which was suspended in 10 ml 0.85% NaCl. Ten

fold dilutions were made in 0.85% NaCl and 100 µl of appropriate dilution steps were plated out on trypton soya agar (TSA, Oxoid).

Statistical analysis

Bacterial acetylene reduction was measured with three replicate cultures each determination. Inoculation experiments were carried out in a complete factorial design with four replicates per treatment. Bacterial counting in plant roots was done with two replicates. The standard error of difference (SED) was calculated by analyses of variance (Genstat 5 Committee, 1987) and the Student T test was performed. All significant differences reported are at a level of $P = 0.05$ or $P = 0.01$ at least.

Results

Bacterial strains

Spontaneous ethylenediamine resistance among bacteria occurred at a frequency of approximately 2.10^{-5} per cell. Out of 120 colonies tested five appeared to accumulate substantial amounts of NH_4^+ in all three media. From these two mutant strains did not repress their nitrogenase system in the presence of 10 mM NH_4Cl. Nitrogenase activity (C_2H_2 reduction) of both mutant strains was two to three times higher on nitrogen-free medium and on 3 mM glutamate than that of the wild type. The nitrogenase system of strain C5 was repressed at 10 mM NH_4Cl by approximately 70% and by 90% at 20 mM NH_4Cl. At strain C3 the nitrogenase was completely unrepressed even at 20 mM NH_4Cl. Neither of the bacterial strains tested showed any nitrogenase activity at 8 mM KNO_3 (Table 1).

Table 1. Acetylene reduction activity by *Azospirillum brasilense* (Wa5) wild type and NH_4^+-excreting mutants C3 and C5 grown on different nitrogen sources

Strain	Acetylene reduction (nmol C_2H_4 h^{-1} mg^{-1} protein)				
	NF[a]	NH_4Cl		KNO_3	Glutamate
		10 mM	20 mM	8 mM	3 mM
Wild type	51	0	0	0	32
Mutant C3	183	186	172	0	162
Mutant C5	101	29	11	0	88

[a] NF, nitrogen-free medium

Nitrate reductase was active in wild type as well as in NH_4^+-excreting mutant *Azospirillum brasilense* Wa5. Both mutant strains showed a nitrite ammonification (Table 2).

Table 2. Nitrate reductase activity and nitrite ammonification by *Azospirillum brasilense* (Wa5) wild type and NH_4^+-excreting mutants C3 and C5

Strain	Nitrate reduction[a] (μM NO_2 48 h^{-1})	Nitrite ammonification[b] (μM NH_4^+ 24 h^{-1})
Wild type	3.1	11
Mutant C3	1.7	293
Mutant C5	1.9	403

[a]Nitrate reduction was measured as nitrite accumulation in a semisolid (0,2% agar) malate minimal medium (37) supplemented with 8 mM KNO_3.
[b]Nitrite ammonification was measured as NH_4^+ accumulation in a semisolid (0,2% agar) glycerol minimal medium supplemented with 3 mM $NaNO_2$.

Table 3. Ammonium excretion by *Azospirillum brasilense* (Wa5) wild type and NH_4^+-excreting mutants C3 and C5 grown on different nitrogen sources and at different oxygen tensions

Strain	NH_4^+ excretion (ng NH_4^+ h^{-1} mg^{-1}protein)					
	1 kPa O_2			20 kPa O_2		
	NF[a]	NO_3^- 8 mM	Glutamate 3 mM	NF	NO_3^- 8 mM	Glutamate 3 mM
Wild type	1.2	0.5	96	0	1.8	7
Mutant C3	156	146	322	15	149	155
Mutant C5	118	117	340	12	104	123

[a] NF, nitrogen-free medium

During incubation on glycerol minimal medium the pH of the growth medium remained stable at pH 6.8 (Fig. 1). *A. brasilense* Wa5 released only very little NH_4^+ to its environment when grown on N-free medium or on nitrate. Only when grown on 3 mM glutamate some NH_4^+ was excreted. Both mutant strains C3 and C5 released high amounts of ammonia when grown at 1 kPa O_2 on N-free medium, on nitrate and on glutamate. NH_4^+ excretion was inhibited when the mutant strains grew aerobically on N-free medium or on glutamate, but was not inhibited at aerobic growth on nitrate. In all cases the mutant strain C3 released the highest amounts of NH_4^+ (Table 3).

On 3 mM NH_4Cl the growth of both mutant strains was slower than that of *A. brasilense* Wa5 wild type. Mutant strain C3 produced only little protein on medium with ammonia as the sole nitrogen source. However, all three bacterial strains showed similar growth rates on 3 mM glutamate (Fig. 2).

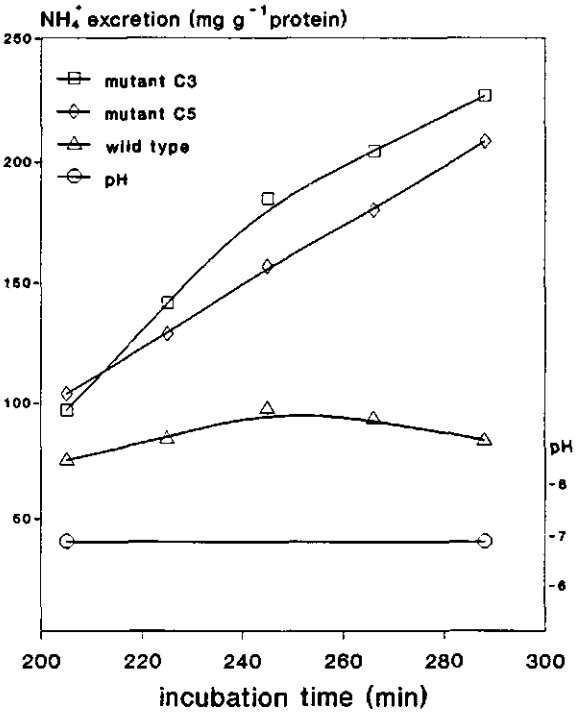

Figure 1. NH_4^+ release by *Azospirillum brasilense* (Wa5) wild type and NH_4^+-excreting mutants C3 and C5 grown in nitrogen-free glycerol minimal medium at 1 kPa O_2 oxygen tension and at 30°C. pH of the medium was measured before and after incubation.

When grown on [^{14}C]methylamine enriched NFB medium *A. brasilense* Wa5 wild type accumulated a considerable amount of radioactivity inside its cells. Both mutants failed in their [^{14}C]methylamine uptake during 140 min of incubation (Fig. 3).

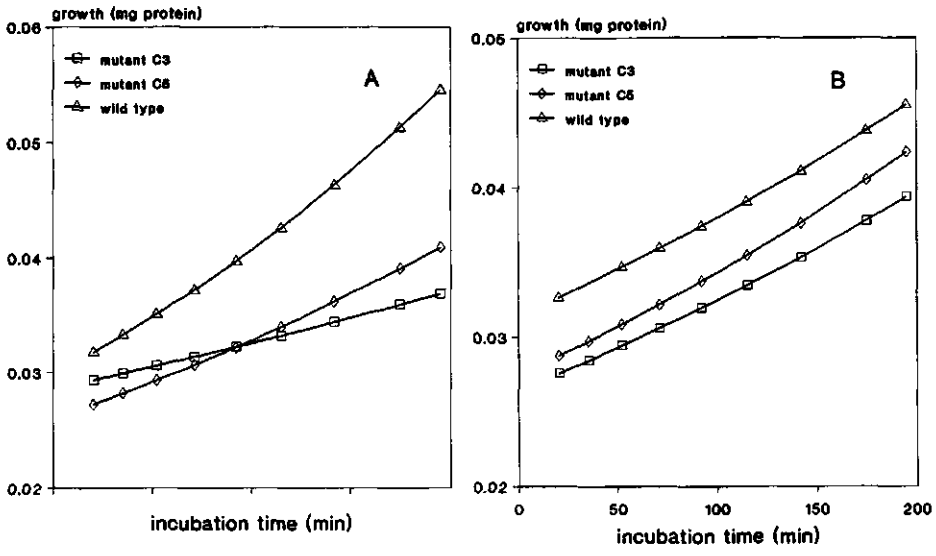

Figure 2. Growth of *Azospirillum brasilense* (Wa5) wild type and NH_4^+-excreting mutants C3 and C5 in malate minimal medium (Okon et al. 1977) supplemented with (A) 3 mM NH_4Cl and (B) 3 mM glutamate (30°C).

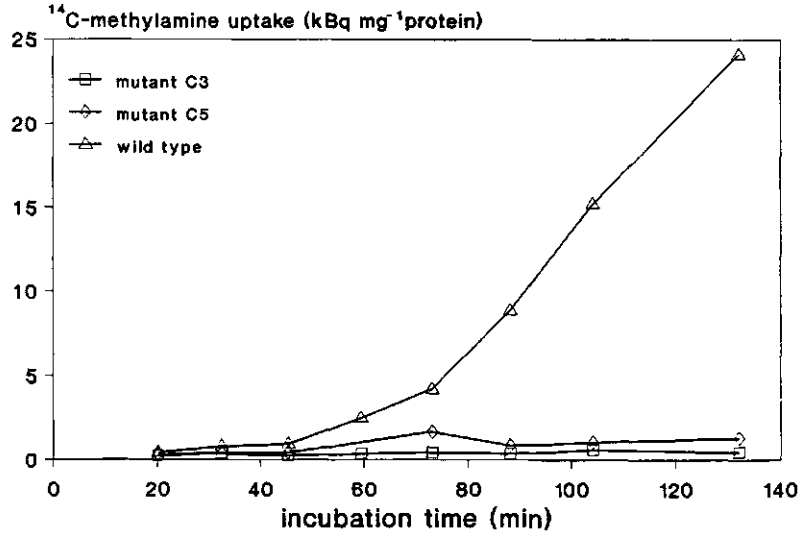

Figure 3. [^{14}C]metylamine uptake by *Azospirillum brasilense* (Wa5) wild type and NH_4^+ excreting mutants C3 and C5 grown in malate minimal medium (Okon et al. 1977) on 0.3 mM NH_4Cl and 1.6 μM [^{14}C]methylamine (34 kBq/ml) at 30°C.

Plant bacteria interaction

When introduced to plants by a seed-coating both the wild type and the mutants C3 and C5 of *A. brasilense* Wa5 established high cell numbers in the rhizosphere (Table 4). All bacterial strains strongly influenced root growth and morphology. The bacteria caused a decrease of the root elongation and the plant developed an increased number of short roots at the root base; root branching was enhanced as well.

Table 4. Root-colonisation and nitrogen gained from atmospheric source as calculated by ^{15}N enrichment in wheat. Plants were grown axenically on a nitrogen free nutrition (Fahreus 1957) exposed to an atmosphere of 7,302 atom % $[^{15}N]_2$ excess. Inoculation was by coating seeds with *Azospirillum brasilense* (Wa5) wild type or NH_4^+-excreting mutants C3 and C5 (2×10^6 CfU per plant). Non-inoculated plants were taken as controls. Harvest was 31 days after planting

Strain	Bacteria in roots (log CfU[a] g^{-1} dry root)	Nitrogen gain (ng N)		
		Root	Shoot	Total plant
Control[b]	ND[c]	79	289	368
Wild type	8.4	107	368	474
Mutant C3	7.6	172	662	835
Mutant C5	7.2	151	545	695
SED[d]	0.7	16**	96*	96**

[a] CfU, cell-forming units
[b] Control, non-inoculated plants
[c] ND, not determined
[d] SED, standard error of difference (* $P<0.05$, ** $P<0.01$)
Values of root-bacteria are means of two replicates
Values of the nitrogen gain are means of four replicates

The nitrogenase activity (C_2H_2 reduction) in the rhizosphere was highest in the prsence of mutant strains C3 and C5 (Table 5). The absolute values of the acetylene activity reduction appeared to be lower in comparison to earlier observations in a similar system (Christiansen-Weniger in preparation).

The average nitrogen content of the wheat seeds was 0.71 mg N per seed, so two seeds introduced a total of 1.42 mg N to each tube. At harvest the non-inoculated controls and plants inoculated with wild type *A. brasilense* Wa5 neither differed in their dry matter production nor in their nitrogen uptake. Dry matter and total nitrogen in root and shoot was significantly increased in plants inoculated with strains C3 and C5, although the relative nitrogen concentration was not affected by any bacterial inoculation (Table 6).

Table 5. Acetylene reduction activity (ARA), ^{15}N content and total ^{15}N accumulation in wheat. Plants were grown axenically on a nitrogen-free nutrition (Fahreus 1957), exposed to an atmosphere of 7,302 atom % $[^{15}N]_2$ excess. Inoculation was by coating seeds with *Azospirillum brasilense* (Wa5) wild type or NH_4^+-excreting mutants C3 and C5. Non-inoculated plants were taken as controls. Harvest was 31 days after planting.

Strain	ARA (nmol C_2C_4 24 h^{-1} $plant^{-1}$)	$^{15}N^c$ content (atom % excess) 10^{-3}		Total ^{15}N (ng ^{15}N)	
		Root	Shoot	Root	Shoot
Control[a]	0	2.00	1.76	5.73	21.1
Wild type	1.09	2.67	2.33	7.77	26.8
Mutant C3	1.83	2.67	3.00	12.6	48.4
Mutant C5	2.96	2.44	2.83	11.0	39.8
SED[b]	0.81*	0.35	0.52	1.16**	7.04*

[a] Control, non-inoculated plants
[b] SED, standard error of difference (* P<0.05, ** P<0.01)
Values are means of four replicates
[c] ^{15}N values are relative to unexposed wheat plants

When exposed to a ^{15}N-enriched atmosphere all plants including non-inoculated controls incorporated ^{15}N above the natural level, which could be attributed to ^{15}N adsorption, isotope exchange processes or contamination of the added $[^{15}N]_2$ gas. The relative ^{15}N content in the plant material was higher for plants inoculated with bacteria than in the controls although these differences were not statistically significant ($P = 0.05$). The total ^{15}N accumulation was more than twice as high in plants inoculated with NH_4^+-excreting mutants C3 and C5 than in controls. Total ^{15}N incorporation in plants inoculated with the *A. brasilense* Wa5 wild type was slightly increased as well

(Table 5). On the basis of these data we calculated that the potential nitrogen supply which was gained from atmospherically fixed nitrogen was 474 ng nitrogen for plants inoculated with *A. brasilense* Wa5 wild type, whereas strains C3 and C5 supplied 695 to 835 ng N to the host plants (Table 4).

Table 6. Dry-matter production, nitrogen content and total nitrogen accumulation in wheat plants grown axenically on a nitrogen free nutrition (Fahreus 1957). Inoculation was by coating seeds with *Azospirillum brasilense* (Wa5) wild type or NH_4^+-excreting mutants C3 and C5. Non-inoculated plants were taken as controls. Harvest was 31 days after planting

Strain	Dry weight (mg)		N content (%N)		Total N (µg)	
	Root	Shoot	Root	shoot	Root	Shoot
Control[a]	22.0	34.3	1.30	3.64	290	1240
Wild type	21.7	35.0	1.35	3.32	290	1170
Mutant C3	35.7	46.0	1.36	3.49	480	1600
Mutant C5	32.9	39.3	1.39	3.59	460	1400
SED[b]	3.1**	2.8*	0.05	0.16	40**	90*

[a] Control, non-inoculated plants
[b] SED, standard error of difference (* P<0.05, ** P<0.01)
Values are means of four replicates

Discussion

Both mutant *Azospirillum brasilense* strains C3 and C5 fail in the incorporation of [^{14}C]methylamine, which makes it most likely that they lack the necessary enzyme systems to transport ammonia across the cell membranes. Also, the NH_4^+ repression of the nitrogenase enzyme system appears to be affected in both mutant strains. This fits the hypothesis that the biosynthesis of nitrogenase and of the ammonia carrier proteins are both controlled by the same regulation processes (Kleiner et al. 1981). Since the mutant strains C3 and C5 are able to grow well on glutamate but only poorly on NH_4Cl, it is likely that the mutation occur somewhere in the ammonia assimilation chain. Ammonia assimilation is controlled by the availability of NH_4^+ itself and the regulation mechanisms are reported to be commonly present in various diazotrophs

including *Azospirillum* (Alef and Kleiner 1982, Kleinschmidt and Kleiner 1981, Streicher et al. 1974). The detailed mechanisms are not yet clear. Merrick (1982) offered a model for bacterial regulation of enzymes by NH_4^+ in which products of regulatory genes such as *ntr*A, *ntr*B and *ntr*C, repress or activate, respectively, a promoter region for the controlled operons. In this model *ntr*C codes for an enzym which can either function under influence of the *ntr*A gene product as an activator or together with the *ntr*B gene product as a repressor for the transcription of the NH_4^+-controlled genes (Espin et al 1982, McFarland et al 1981). Bani et al. (1980) and Gauthier and Elmerich (1977) provided evidence that a similar regulation mechanism is active in *Azospirillum*. Since in our mutant *A. brasilense* Wa5 strains the *nif* (nitrogen fixation genes) expression was not the sole function affected, we suggest that the mutation is unlikely to occur at the regulatory *nif* AL sites, which is the promoter region for the *nif* operon (Gussin et al. 1986, Pedrosa and Yates 1984) but rather at *ntr*-like genes.

The wild type *A. brasilense* Wa5 released some ammonia only when grown under oxygen limitation on glutamate, which fits in with earlier work by Hartmann et al. (1988). Both mutant strains excreted high amounts of ammonia when grown on nitrate, although bacteria did not develop nitrogenase activity. This, together with the fact that the ammonia excretion under aerobic conditions was similar to that under oxygen limitation makes it likely that the excreted NH_4^+ originated from the nitrate source. Thus evidence is given that the mutant strains have retained their ability to take up and further reduce NO_3^- even if the NH_4^+ uptake is inhibited. This is confirmed by the observation that both mutant strains showed nitrate reductase activity as well as a nitrite ammonification.

Upon introduction to wheat roots, the numbers of rhizosphere-colonizing *A. brasilense* cells, wild type as well as mutant strains, were approximately 100 times higher than in earlier inoculation experiments with axenically grown summer wheat (Christiansen-Weniger, in preparation). These relatively high cell densities in roots might explain the profound influence of all three bacterial strains on root development and morphology. This is probably due to the bacterial production of the plant growth substance indole-acetic acid (Harari et al. 1988, Patriquin et al. 1983).

Since the plants were grown on a nitrogen-free medium, the availability of mineral nitrogen will be the limiting factor for plant growth. Plants inoculated with NH_4^+-excreting mutants of *A. brasilense* showed significantly higher dry mass production than either the controls or plants inoculated with the wild type. As the numbers of rhizosphere-colonizing bacteria were similar for all three strains these effects must be attributed to an additional supply of nitrogen rather than to bacterially produced plant growth substances. This is confirmed by the higher accumulation of total nitrogen in root and shoot material after inoculation with strains C3 and C5 as compared to the controls and to wild type treatments. The relative nitrogen concentration in the plants was similar for all four treatments (including the uninoculated controls). This is explained by the fact that in a nitrogen-limited plant growth system an additional input of nitrogen will lead to a proportional increase of plant dry matter and so to a dilution of incorporated nitrogen.

The sole mineral nitrogen input in our system was by introducing wheat seeds of an average of 1.42 mg N per tube. Thus, the nitrogen accumulation in plants on top of the applied amount was only 0.04 mg N for the wild type-treated plants and 0.66 mg N and 0.44 mg N for the plants inoculated with strain C3 and strain C5, respectively.

The wild type *A. brasilense* Wa5 did neither enhance plant dry matter production nor its total nitrogen accumulation, although it settled with high numbers inside the rhizosphere and was actively fixing nitrogen. This strengthens our hypothesis that

rhizosphere associated *Azospirillum* does not contribute fixed nitrogen directly to its host, but only in a late stage of plant development in form of postmortal decomposed bacterial substance.

Both NH_4^+ excreting mutant *A. brasilense* Wa5 strains caused a significantly higher accumulation of ^{15}N excess in roots and shoots than the wild type strain. This additional nitrogen supply can only be attributed to biological nitrogen fixation. The total nitrogen gain as calculated on the basis of these ^{15}N enrichment values appeared to be much lower than that calculated by the N balance study. Therefore, the mutant *A. brasilense* Wa5 strains transferred nitrogen to the system which came from other origins than from N_2 fixation only. Possibly, recently fixed ^{15}N nitrogen is exchanged for intracellularly present bacterial ^{14}N, which is excreted unproportionally. This phenomemon has often been mentioned as the reason for artifacts observed when N uptake of plants was studied using ^{15}N-enriched fertilizer (Hart et al. 1986).

We demonstrated that NH_4^+-excreting mutants of *A. brasilense* were capable of establishing in the rhizosphere of a wheat host to the same extent as the wild type strain, and of supplying higher amounts of nitrogen to the plant. However, much more research will be necessary to quantify the nitrogen benefits of such strains in plants grown over a longer period and under more natural conditions, including their competitive ability in the plant rhizosphere with the natural microbial population.

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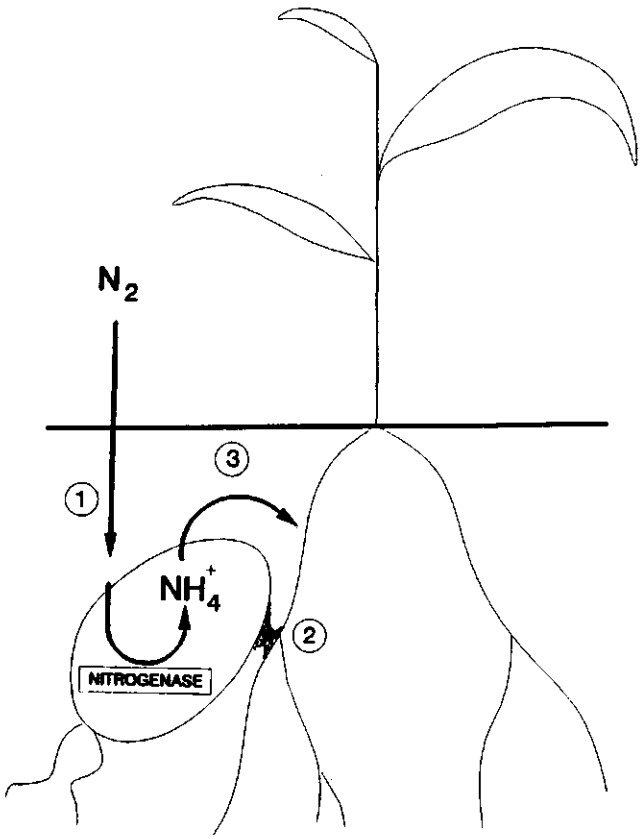
CONCLUDING REMARKS

An association between diazotrophic bacteria and gramineous plant in which the plant benefits from the N_2-fixation of the related bacteria has to meet the following requirements.



1. Associated bacteria have to fix nitrogen under the environmental conditons provided in the field.
2. The bacteria have to live in effective numbers in close attachment to the host or even to settle inside plant tissue.
3. The bacteria must release fixed nitrogen to their host.

In the present work we studied whether the rhizosphere association between *Azospirillum* spp. and spring wheat met these prerequisites

We demonstrated that *Azospirillum*, when associated gnotobiotically with a wheat host, developed considerable rates of acetylene reduction activity (ARA). As the bacterial nitrogenase activity in the plant-rhizosphere was less sensitive to free oxygen and to the presence of mineral nitrogen than in a pure bacterial culture we evidenced that *Azospirillum* settles, well protected, in niches close to the root. The host plant itself is able to stimulate the nitrogenase activity in its rhizosphere by exudation of particular organic compounds. This supports the idea that a host plant cultivar could be selected which will develop a more effective rhizosphere association with *Azospirillum*. As we suggested a possible link between plant aluminium tolerance and its rhizosphere nitrogen fixation activity this physiological characteristic may form a suitable and simply to handle selection parameter.

Although ARA is only an indirect measure for bacterial nitrogen fixation it allows for the calculation of the potential N_2-fixation of a rhizosphere-attached *Azospirillum* population. ARA values of approximately 550 nmol h^{-1} $plant^{-1}$ as a maximum is equal to 2,57 µg N fixed per hour and plant. When assuming that this nitrogen fixation activity continues during the entire growth period this yields approx. 100 kg N ha^{-1} $year^{-1}$ in an intensive wheat cropping system of about 450 plants m^{-2}. This, however, is only true in a gnotobiotically wheat-*Azospirillum* association. When grown under natural conditions the number of root colonizing *Azospirillum* cells decreased drastically. Competition with native rhizosphere bacteria lead to a *Azospirillum* cell density in rhizosphere soil and in the root itself which were 10 to 100 times lower than under axenical conditions. The relative cell density of introduced *Azospirillum* in the rhizosphere was only approximately 0.04% of the total bacterial rhizosphere population. This low cell frequency makes it unlikely that under temperate field conditions *Azospirillum* will have a considerable effect on a host plant.

In all experiments we demonstrated that a rhizosphere *Azospirillum* population, even if it shows high rates of ARA, does not transfer large amounts of fixed nitrogen to its host. This fits well with results found in various earlier inoculation studies with *Azospirillum*. This phenomenon must be attributed to the strict regulation of nitrogen uptake and nitrogen fixation in *Azospirillum*. Carrier proteins, bound to bacterial cell membranes prevent mineral nitrogen to leak out of the bacterial cell. Mutant *Azospirillum* strains with a deletion in the ammonia uptake mechanism releases more fixed nitrogen to its environment. In association with wheat such mutant strains supported the nitrogen supply of the host to a much higher extent than did the *Azospirillum* wildtype.

In conclusion, under strictly axenical conditions we succeeded to create an association between wheat and mutant *Azospirillum* in which the bacteria settles with high cell densities in the rhizosphere, fix nitrogen and deliver fixed nitrogen to the host plant. However, whether such an association will function under natural soil conditions has to be proven yet. It is most likely, that ammonia excreting mutant *Azospirillum* strains will be highly disadvantated against other competing rhizosphere bacteria, which will cause a suppression of it's potential root colonization. Therefore, further investigations have to look for possible ways in which introduced NH_4^+-excreting mutant *Azospirillum* can compete with the occurring bacterial wild population and which allows them to establish sufficient cell numbers in the rhizosphere or inside the root itself. This could either by a suitable manner of

bacterial application. We demonstrated that when introduced to the plant by a bacterial seed-coating *Azospirillum brasilense* grows along with the developing root and forms a considerable population all over the root.

Another way to get introduced *Azospirillum* established inside the hosts root system is by treating plants with the auxine 2,4 dichlor-phenoxy-acetic-acid (2,4 D). In recent experiments we showed that under influence of 2,4 D wheat roots form nodule like roottumors, in which *Azospirillum* develop high amounts of nitrogenase activity. Whether the induction of such root structurs will be a practicable way to introduce NH_4^+-excreting mutant *Azospirillum* inside gramineous plants must be the topic of future research.

SUMMARY

The main objectives of this study were to identify factors that control the behaviour of *Azospirillum* in the rhizosphere of a gramineous plant in order to be able to optimize the association between the bacteria and the host plants in terms of nitrogen supply to the host.

Plant produced growth substances such as the auxines indole-acetic-acid (IAA) and 2,4-dichlorphenoxy-acetic-acid (2,4 D) or gibberilic acid enhance the acetylene reduction activity of a pure *A. brasilense* culture. IAA and the cytocinine 6-benzyl-aminopurine also stimulated bacterial growth. It should be pointed out, that *Azospirillum* also produces IAA itself, which is often mentioned to be the reason of its plant-growth stimulating activity.

When associated with living roots, the nitrogen fixation (acetylene reduction) activity of *Azospirillum brasilense* is much less sensitive to the repressive influence of free oxygen and mineral nitrogen, i.e. NO_3^- and NH_4^+, than in the absence of an active growing root. Potential acetylene reduction rates varied from 10 to 550 nmol C_2H_4 h^{-1} $plant^{-1}$ depending on environmental conditions.

In order to be able to determine the fate of introduced *Azospirillum* in a soil and in the root environment *A. brasilense* strains were marked by a transposon (Tn5) insertion into its genome so that reisolation upon double resistance against kanamycin and rifampicin was possible. *A. brasilense*::Tn5 established in the rhizosphere of an axenically grown spring wheat to cell numbers as high as 10^6 cells per gram dry rhizosphere soil and 10^6 cells per gram dry root, respectively. In the rhizosphere of a non-sterile grown plant the number of *A. brasilense*::Tn5 was much lower, i.e. approximately 10^4 cells per gram dry rhizosphere soil and 10^3 cells per gram dry root. The number of *A. brasilense*::Tn5 cells was 10 to 100 times higher in the soils closely attached to the roots than in root-free soils. *A. brasilense* could not be reisolated from inner root-tissue after a root-surface sterilization with 1% chloramine T. When introduced to plants in an early stage of plant growth either by seedling inoculation or by a seed-coating, *A. brasilense* was able to develop with the growing root and to establish a strong population all over the root.

Most intensive root colonization of introduced *A. brasilense* and highest acetylene reduction rates were observed when plants were treated with *Azospirillum* cells immediately after seedling emergence as compared to the colonization of roots after inoculation at a later stage of growth. Subsequent inoculations during plant development after an initial addition did neither stimulate root colonization nor acetylene reduction activity.

When comparing wheat and sorghum cultivars with different levels of aluminium tolerance a larger rhizosphere acetylene reduction activity was observed when *Azospirillum* was introduced to roots of aluminium-tolerant cultivars than to roots of Al-sensitive cultivars. The amount of fixed nitrogen, transferred from *Azospirillum* to the host as calculated by the ^{15}N dilution technique was also significantly higher in

case of Al-tolerant cultivars. Aluminium-tolerant plants appeared to exudate significantly larger amounts of total organic carbon than Al-sensitive plants. Not only the quantity but also the quality of the exudates differed in the sense, that higher concentrations of low molecular dicarbonic acids such as succinic, malic and oxalic acid were observed at root-exudates of aluminium-tolerant wheat plants. These organic acids are known to be preferable carbon substrates for *Azospirillum* spp, what might explain the more intensive colonization and higher nitrogen fixation capacity in the rhizosphere of Al-tolerant plants.

Although *Azospirillum* develops considerable activities in the rhizosphere of host plants the transfer of fixed nitrogen to the host as determined with the ^{15}N-dilution technique appeared to be rather low. Only approximately 3% of the root nitrogen and approximately 2% of the shoot nitrogen was calculated to be derived from the N_2-fixation activity of the *Azospirillum* cells.

In order to enhance the transfer of nitrogen to the host *A. brasilense* was selected on ethylenediamine, yielding mutant strains which lack their ammonia transport system across cell membranes and which excrete substantial amounts of NH_4^+ to the environment. Two of these mutant strains fixed nitrogen in the presence of high concentrations (20 mM) of NH_4^+. Nitrogenase activity of the NH_4^+-excreting mutants was two to three times as high as that of the wild type. The mutant strains colonized the roots of axenically grown wheat to high cell numbers and developed rhizosphere acetylene reduction activities comparable to that of the wild type. Both mutant strains caused a significant increase of dry matter production and of total plant N-accumulation as compared to wild type treated plants or to non-inoculated controls. When exposed to a $^{15}[N]_2$ enriched atmosphere the *A. brasilense* mutant strains transfered higher amounts of ^{15}N to their hosts than the wild type did. ^{15}N-enrichment and nitrogen balance studies both indicated that NH_4^+-excreting *A.brasilense* support the nitrogen supply of a wheat host.

SAMENVATTING

Hoofddoel van dit onderzoek was het gedrag van *Azospirillum* spp. in de rhizosfeer van een grasachtige plant te bestuderen, teneinde om in staat te zijn de associatie tussen bacterium en de gastheer-plant in termen van stikstofvoorziening te verbeteren.

Door de plant geproduceerde groeihormonen als onder andere de auxines indole-azijnzuur (IAA) en 2,4-dichloorfenoxy-azijnzuur (2,4 D) of gibberiline zuur versterken de acetyleenreductieactiviteit van een *Azospirillum brasilense* cultuur. IAA en het cyutocinine 6-benzyl-aminopurine bevorderen de bacteriegroei. *Azospirillum* produceert zelf IAA, wat meestal genoemd wordt als de oorzaak voor zijn plantengroei-stimulerende werking.

Wanneer geassocieerd met levende wortels is de stikstoffixatie (acetyleenreductie) van *A. brasilense* veel minder gevoelig voor de repressieve invloed van vrije zuurstof en minerale stikstof (NO_3^- en NH_4^+) dan in de afwezigheid van een aktief groeiende wortel. Potentiële acetyleenreductie varieerde van 10 tot 550 nmol C_2H_4 h^{-1} $plant^{-1}$, afhankelijk van milieu-omstandigheden.

Om het verblijf van geïntroduceerde *Azospirillum* in de bodem en in het wortelmilieu te bestuderen, zijn *Azospirillum* stammen door een transposon (Tn5) insertie in hun genoom gemarkeerd, zodat het mogelijk was deze stammen vanwege hun dubbele resistentie tegen kanamycin en rifampicin te herisoleren. *A. brasilense*::Tn5 koloniseerde de rhizosfeer van een axenisch groeiend zomertarwe met celdichtheden van 10^6 cellen per gram droge rhizosfeer grond en 10^6 cellen per gram droge wortel. In de rhizosfeer van een niet steriel gegroeide plant waren de aantallen van *A. brasilense*::Tn5 veel lager, circa 10^4 cellen per gram droge rhizosfeer grond en 10^3 cellen per gram droge wortel. De aantallen van *A. brasilense*::Tn5 cellen waren 10 tot 100 keer hoger in de nauw aan de wortel gehechte grond dan in wortel vrije grond. *A. brasilense*::Tn5 kon niet hergeïsoleerd worden uit inwendig wortelweefsel na een worteloppervlak sterilisatie met 1% chloramin T.

Wanneer *A. brasilense* aan planten in een vroeg stadium van plantengroei geïntroduceerd wordt of door middel van kiemplant inoculatie of door een zaadcoating, dan was het in staat met de groeiende wortel mee te ontwikkelen en een sterke populatie over de hele wortel te handhaven. De sterkste wortelkolonisatie van geïntroduceerde *A. brasilense* en de hoogste acetyleenreductie activiteit worden gevonden, wanneer planten met *A. brasilense* direct na de kiemplantontwikkeling worden behandeld, in tegenstelling tot de kolonisatie van wortels na inoculatie in een later stadium van groei. Verschillende inoculaties tijdens plantontwikkeling na een eerste toevoeging stimuleert noch wortelcolonizatie noch acetyleenreductie-activiteit.

De vergelijking van tarwe en sorghum cultivars met verschillende aliminiumtolerantie leverde een hogere rhizosfeer acetyleenreductie-activiteit voor aluminiumtolerante cultivars op dan voor aluminium gevoelige cultivars. De hoeveelheid gefixeerde stikstof die van *Azospirillum* naar de gastheer wordt overgedragen (berekend door de ^{15}N-verdunningen-techniek) was significant hoger

voor aluminiumtolerante cultivars. Aluminiumtolerante planten scheiden veel hogere hoeveelheden van totale organische stikstof uit dan aluminiumgevoelige planten. Niet alleen de kwantiteit als ook de kwaliteit van de exudaten waren verschillend. Hogere concentraties van laag moleculaire dicarbonzuren zoals succinic-, malic- en oxalic-zuur worden in wortelexudaten van aluminiumtolerante planten gevonden. Deze organische zuren staan bekend als voornamelijke koolstofbron van *Azospirillum* spp. Dit verklaart mogelijk de sterke acetyleenreductie-activiteit en de verhoogde overdracht van gefixeerde stikstof in de rhizosfeer van aluminiumtolerante planten.

Ofschoon *Azospirillum* duidelijke N_2-fixatie-activiteiten in de rhizosfeer van de gastheerplant ontwikkelt, blijkt de overdracht van gefixeerde stikstof aan de gastheer heel laag te zijn. Gemeten met de ^{15}N-verdunningstechniek was circa 3% van de wortelstikstof en circa 2% van de stikstof in de bovengrondse gedeelten afkomstig van N_2 fixatie van *Azospirillum*.

Om de overdracht van stikstof naar de gastheerplant te versterken, wordt mutant *A. brasilense* op ethyleendiamine geselecteerd. Dit leverde stammen op, die hun ammoniumtransportsysteem in hun celmembranen verloren hadden en die duidelijke hoeveelheden van NH_4^+ uitscheidden. Twee van deze mutantenstammen fixeerden stikstof in aanwezigheid van hoge concentraties (20 mM) NH_4Cl. Nitrogenase activiteit van de NH_4^+ uitscheidende stammen was twee tot drie keer zo hoog als dat van het wild type. De mutantenstammen koloniseerden de wortel van tarwe en ontwikkelden rhizosfeer acetyleenreductie-activiteit vergelijkbaar met het wild type. Beide mutantestammen veroorzaakten een significante verhoging van droge stofproduktie en van totale stikstofaccumulatie in de plant, vergeleken met wild type behandelde planten of met niet inoculerende controleplanten. Wanneer blootgesteld aan een $^{15}N_2$-verrijkte atmosfeer, droegen de mutantenstammen hogere hoeveelheden van ^{15}N aan hun gastheer over dan het wild type. ^{15}N-verrijking- en stikstofbalans-studies geven beide aan dat NH_4^+ uitscheidende *A. brasilense* stammen de stikstofbehoefte van een tarwegastheer ondersteunen.

Curriculum vitae

Christian Walter Christiansen-Weniger was born on 18 january 1958 in Eckernförde, Germany. He visited the 'Jungmann Gymnasium' in Eckernförde where he graduated in 1978. From 1978 to 1979 he joined the military service in Germany. From 1979 to 1981 he studied agricultural sciences at the 'Georg-August University' in Göttingen, Germany and from 1981 at the 'Christian-Albrechts University' in Kiel, Germany, where he graduated in march 1986 at the faculty of plant breeding. From september 1984 to july 1985 he stayed at the EMBRAPA Institute for Soil Biology in Rio de Janairo, Brazil to study applied microbiological techniques. From may 1986 he worked at the former ITAL Research Institute and from January 1990 at the Institute for Soil Fertility, both in Wageningen, The Netherands.