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NITROGEN FIXATION AND DENITRIFICATION BY A WHEAT-AZOSPIRILLUM ASSOCIATION

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Abstract

An association between wheat and Azospirillum brasilense Sp.7 has been studied for N_2-fixation (C_2H_2-reduction) and denitrification activity. The association was grown in semi-solid agar medium for a week in air and then assayed under microaerobic conditions. When the amount of nitrate was less than 1 mM in the medium, the association commenced to perform C_2H_2-reduction 3-5 h after the removal of air. C_2H_4-formation stopped above 1 mM nitrate in the medium, and the association evolved N_2O by denitrification. C_2H_2-reduction and N_2O-formation were strictly dependent on the presence of both Azospirillum and wheat plants in the assays. The addition of carbohydrates to the medium did not enhance each of the activities indicating that the bacteria must have lived from carbon sources of the plants. C_2H_2-reduction and N_2O-formation were marginal at a temperature prevailing in the temperate climate zone and maximal at a temperature comparable to those of tropical regions. Some C_2H_2-reduction but no N_2O-formation activities were observed when the assays were performed in air.

Introduction

The association between roots of grasses and Azospirillum species has attracted special attention in the past few years. N_2-fixing Azospirillum was suggested to provide fixed nitrogen to crop plants which could increase crop productivity or reduce the requirements for fixed nitrogen fertilizers or both. Results of greenhouse or field experiments have not yet been conclusive. The majority of the reports, however, gave positive indications (for reviews see 1,2). In addition to supplying the plants with fixed

nitrogen, Azospirillum may augment plant growth by excreting phytohormones such as auxins, cytokinins and gibberellins (3-5) or by stimulating the uptake of NO_3^-, K^+, $H_2PO_4^-$ or other mineral ions into plants (6). The issue may be disturbed by the fact that Azospirillum can also perform denitrification (7-9). Recent experiments have shown that Azospirillum can even grow with nitrate as the terminal respiratory electron acceptor in batch culture(10). When the O_2-concentration is low in the culture, nitrate is converted to nitrite and partly to N_2O and N_2. Up till-date, denitrification has only been studied in pure cultures of Azospirillum and not in the grass-Azospirillum association. It is conceivable that crop plants may lose combined nitrogen by the denitrification capability of Azospirillum when the levels of nitrate are high and those of O_2 are low in soils.

In an attempt to understand the interactions between crop plants and Azospirillum, we have started to do experiments under defined laboratory conditions using wheat and the type strain, Azospirillum brasilense Sp.7. The present communication will indicate that the Azospirillum-wheat association either performs N_2-fixation (C_2H_2-reduction) or denitrification, depending on the amount of oxygen and nitrate available

Materials and Methods

For the experiments with the wheat-Azospirillum association, Azospirillum brasilense Sp.7 was grown as batch culture in an autoclaved medium containing in g/l: $MgSO_4$. 7 H_2O, 0.2; NaCl, 0.1; $CaCl_2$.2 H_2O, 0.02; $NaMoO_4$. 2 H_2O, 0.02; $MnSO_4$. H_2O, 0.01; KH_2PO_4, 0.25; $FeSO_4$. 7 H_2O, 0.0069; ethylenediaminetetraacetic acid, 0.0093; DL-malate, 5. $FeSO_4$-ethylenediaminetetraacetic acid and phosphates were autoclaved separately and added to the medium after cooling. The pH was adjusted to 6.9 prior to autoclavation. After growth for 24 h at 30°C, 1 ml of the bacterial suspension containing approximately 1.3 x 10^9 cells was used as the inoculum.

Wheat (Triticum aestivum L. Ralle) seeds were disinfected by treatment with 70 % ethanol for 10 min, followed by treatment with

0.1 % $HgCl_2$ dissolved in 0.05 N HCl for 10 min and by washing 5 x with sterilized destilled water. The seeds were placed on sterile and wet filter papers in Petri dishes and germinated for 2-3 d in a growth chamber with light/dark cycles (12.5 h in the light at 33°C, 11.5 h in the dark at 23°C).

The experiments were performed in 1 l flasks containing 100 ml sterilized medium with 0.8 % agar (Merck) and the following salts in g/l: $CaCl_2 . H_2O$, 0.14; $MgSO_4 . 7 H_2O$, 0.17; $MnSO_4 . H_2O$, 0.01; KH_2PO_4, 0.14; $Na_2HPO_4 . 12 H_2O$, 0.21; $FeSO_4 . 7 H_2O$; 0.04 and KNO_3 as indicated in the legends of the figures and the tables. 25 germinated wheat seeds and 1 ml of the bacterial suspensions were given onto the agar surface of the flasks under sterile conditions. The flasks were sealed with sterile cotton wool and incubated for 7 d in the growth chamber at light/dark cycles (12.5 h in the light at 33°C and 11.5 h in the dark at 23°C). The cotton wool was then replaced by sterilized rubber stoppers, and the flasks were then repeatedly evacuated and gassed with argon to remove the air. The O_2-content in the gas phase was approximately 0.2 % at the beginning of the experiment. After injection of 5 ml C_2H_2, the flasks were incubated for 24 h in the growth chamber under the conditions just mentioned. C_2H_4-formation was determined by gas chromatography using a flame ionization detector and a Porapak R column, and N_2O and N_2 in a gas chromatograph fitted with a thermal conductivity detector and a Porapak Q column with He as the carrier gas.

For the experiments of fig. 1 and 2 (pH curves for NO_2^- or N_2O utilization by Azospirillum in liquid culture), Azospirillum brasilense Sp. 7 was grown in continuous culture with nitrite as terminal respiratory electron acceptor. Details of these growth conditions will be published elsewhere(M. P. Stephan, W. Zimmer, H. Bothe, in preparation). Azospirillum taken from the fermenter was centrifuged (10 min at 12 000 x g at room temperature) to concentrate the cells 2 - 3 fold and to remove NO_2^-. The pellet was then suspended in the growth medium in which combined nitrogen (NO_3^- or NO_2^-) was omitted. The cells were assayed in 7.2 ml Fernbach flasks containing in a final volume of 3 ml: 100 mM TES or

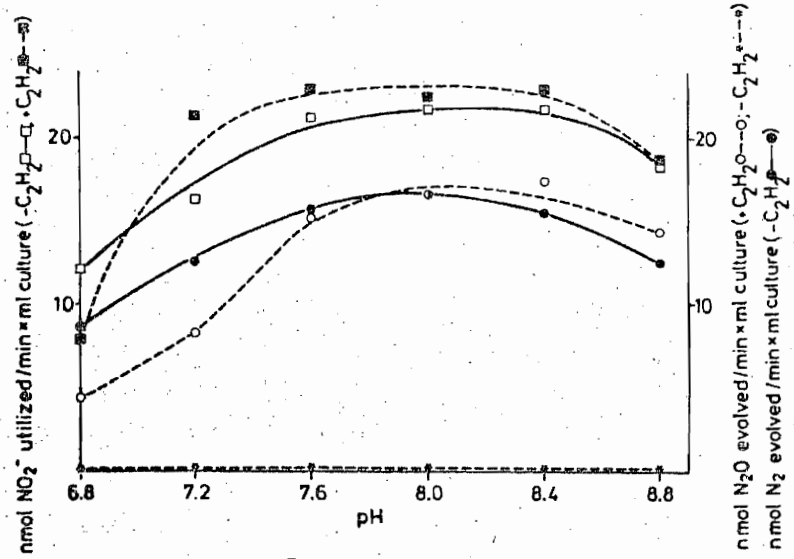

Fig. 1: Dissimilatory NO_2^--reduction and N_2-formation as well as N_2O-formation (in the presence of C_2H_2) by a batch culture of Azospirillum

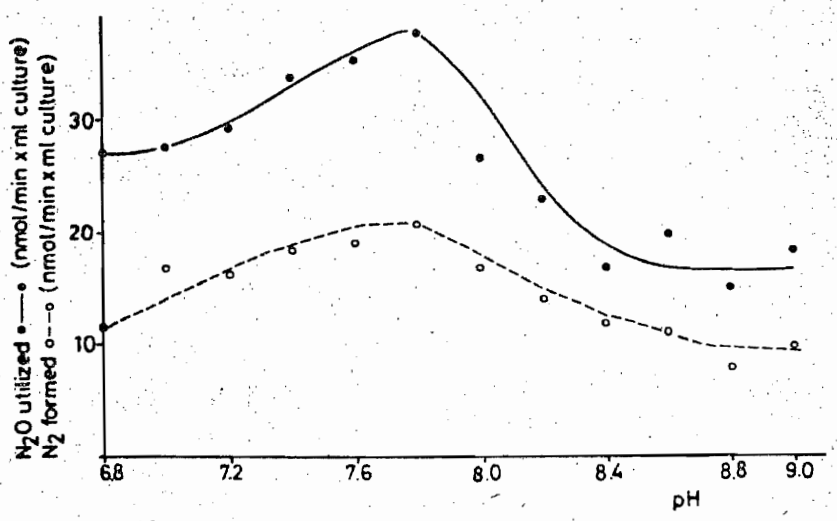

Fig. 2: Dissimilatory N_2O-utilization and N_2-formation by a batch culture of Azospirillum

Tricine-buffer, pH as indicated in fig. 1 and 2, 6 mM $NaNO_2$ or 9 mM N_2O (in the gas phase), 2.2 ml cells (optical density at 560_{nm}=0.43) and 7 mM C_2H_2 when indicated. The reaction was performed at 30°C for 4 h under argon in a shaking water bath, and gas utilization or formation was determined by gas chromatography.

Results

Azospirillum brasilense Sp. 7 grows in the absence of O_2 using NO_3^- as terminal respiratory electron acceptor (10). The present communication shows that the bacterium also utilizes NO_2^- (fig.1) or N_2O (fig.2) when neither NO_3^- nor O_2 are available. In respiratory NO_2^--reduction, the stoichiometry between NO_2^--disappearance and N_2-formation is approximately 2:1, and neither free N_2O (fig. 1) nor ammonia (not documented) are formed. Nitrite is, however, quantitatively converted to N_2O in the presence of C_2H_2 in the assay vessels (fig. 1). C_2H_2 specifically blocks N_2O-reductase among the enzymes of dissimilatory nitrate reduction (see 11). The stoichiometry between N_2-formation (in the absence of C_2H_2) and N_2O-production (in the presence of C_2H_2) is approximately 1:1. NO_2^--utilization and N_2-formation as well as N_2O-production (in the presence of C_2H_2) are independent of the H^+-concentration in the assays between 7.6 and 8.4 (fig. 1). Fig. 2 shows the utilization of N_2O as respiratory electron acceptor by Azospirillum. N_2O is mainly converted to N_2. As the stoichiometry between N_2O-consumption and N_2-production does not approach to unity, N_2 may partly be reduced further to ammonia which is, however, not excreted by the bacterium under these assay conditions. N_2O-utilization and N_2-formation show an optimum at pH 7.8.

The knowledge of these experiments with liquid cultures of Azospirillum served as the background for the tests with the wheat-Azospirillum association. For these experiments, germinated wheat seeds and Azospirillum were placed onto the surface of a semisolid agar medium and incubated for a week in a growth chamber under light/dark cycles. After this, the C_2H_2-reduction and N_2O-formation activities of the association were assayed for 24 h un-

Table 1: Assay conditions for C_2H_4- and N_2O-formations by the wheat-*Azospirillum* association

Assay condition	C_2H_2-reduction		N_2O-formation	
	$-KNO_3$	$+KNO_3$	$-KNO_3$	$+KNO_3$
1. complete (=+ *Azospirillum*, + wheat)	5.8	0.4	0.0	9.1
2. - *Azospirillum*	0.04	0.04	0.0	0.0
3. - wheat	0.02	0.02	0.0	0.0
4. wheat, where shoots were excised at the inoculation	0.9	0.3	0.0	4.5
5. complete + malate (1 mM)	7.8	1.4	0.0	7.4
6. complete + arabinose (1 mM)	4.8	0.2	0.0	8.3
7. complete + sucrose (1 mM)	6.3	1.4	0.0	8.0
8. complete + glucose (1 mM)	4.1	0.2	0.0	7.5

25 germinated wheat seeds and 1 ml *Azospirillum* culture containing approximately 1.3×10^9 cells were inoculated onto the surface of a semisolid agar/mineral salt medium in 1 l flasks and incubated for a week in a growth chamber (see Materials and Methods). C_2H_2-reduction and N_2O-formation activities were then assayed for a day where the gas phase consisted of argon supplemented with 0.5 % C_2H_2 and 1 % O_2. Rates are given in µmol C_2H_4 or N_2O formed / d x flask and standard deviations are 1-2 µmol/ d x flask for all measurements.

der a gas phase consisting of argon supplemented with 0.2 % O_2 and 0.5 % C_2H_2 (see Materials and Methods). Significant C_2H_2-reductions and N_2O-formations were reproducibly observed in the presence of both plants and Azospirillum in the flasks. Table 1 indicates that no C_2H_2-reduction and no N_2O-formation were detectable when either plants or Azospirillum were omitted. The addition of nitrate to the medium drastically reduced the daily C_2H_4-formation activity. On the other hand, N_2O-formation was only observed with associations grown in the presence of nitrate and assayed with C_2H_2 in the gas phase. C_2H_2-reduction and N_2O-formation were dependent on intact plants, because the activities decreased when the shoots were excised at the beginning of the incubation. C_2H_4-formation and N_2O-production did not occur at the expense of malate from the culture medium in which Azospirillum had

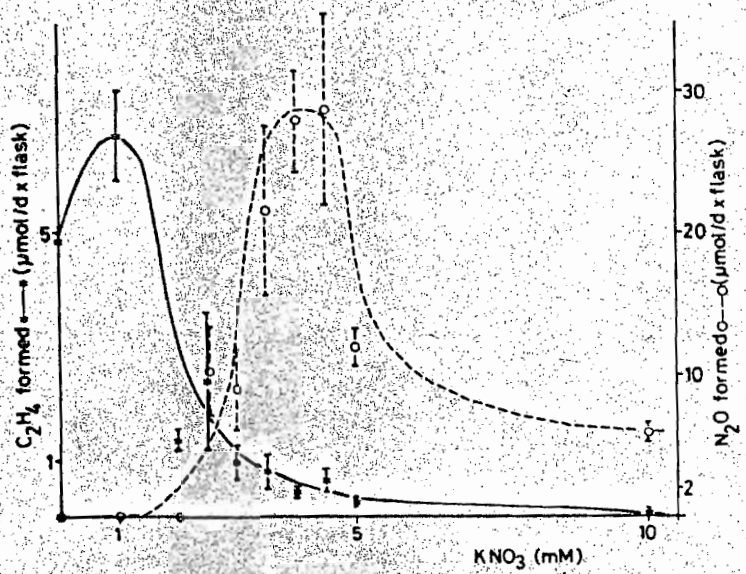

Fig. 3: N_2O- formation and C_2H_2-reduction by the wheat - Azospirillum association : Dependence on nitrate in the medium

been grown prior to inoculation. This statement can be taken from the finding that controls without wheat plants did not show any C_2H_2-reduction or N_2O-formation. The addition of malate, arabinose, sucrose or glucose did not enhance C_2H_4- or N_2O-formations (table 1). Wheat plants obviously supply enough carbohydrates to support both activities in Azospirillum. On the average, the rates of N_2O-formation were 1.5 - 3 fold higher than those of C_2H_4-production.

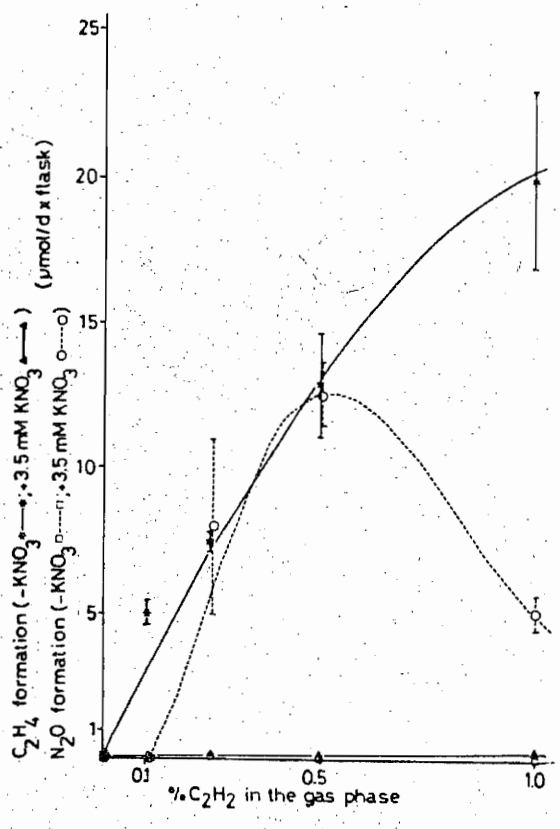

Fig. 4: N_2O-formation and C_2H_2-reduction of the wheat -Azospirillum association: Dependence on C_2H_2 in the gas phase of the vessels

The activities of N_2O-formation and C_2H_2-reduction of the wheat-Azospirillum association were dependent on the amount of nitrate in the agar medium (fig. 3). C_2H_2-reduction was drastically inhibited by the addition of more than 1 mM KNO_3 to the association. In contrast, N_2O-formation required the presence of more than 1 mM KNO_3 during growth of the association, and was optimal between 3-5 mM with higher concentrations being inhibitory (fig. 3). This decrease was due to unspecific effects, because the wheat plants showed impaired growth at such high KNO_3-concentrations. As expected, C_2H_2 was required for both C_2H_4- and N_2O-formations (fig. 4). C_2H_4-formation was therefore due to the plant- bacteria association and not to abiotic effects. The reactions were saturated at about 1 % C_2H_2 in the gas phase for C_2H_4-formation and at 0.5 % C_2H_2 for N_2O-formation. Higher concentrations than 0.5 % C_2H_2 in the gas phase decreased N_2O-formation for some unknown reason.

Fig. 5a and fig. 5b give the kinetics for C_2H_2-reduction and N_2O-formation, respectively. When the wheat - Azospirillum association, grown in the growth chamber for a week, was incubated under argon plus C_2H_2, both C_2H_2-reduction and N_2O-production commenced after a lag phase of 3 - 5 h and then proceeded linearly during the next 20 h. Microscopic examinations showed that the motile bacteria had moved from the agar surface to the roots. As already judged by examination with the eye, Azospirillum had multiplied in the neighbourhood of the roots during growth of the association for a week. We have, however, not been able to quantify growth of the bacteria, because it was difficult to separate them from the agar.

N_2O-formation strictly required the removal of air (table 2). C_2H_2-reduction per day and association was also considerably enhanced by replacing the air by argon. The O_2-content in the gas phase was low then (approximately 0.2 %). However, it must be kept in mind that part of the O_2 could probably not be removed from the roots sticking in the agar and that the plant leaves produced O_2 photosynthetically during the assay. Remarkably, some C_2H_4-formation was also detected under aerobic assay conditions (table 2).

Both C_2H_2-reduction and N_2O-formation activities were much dependent on the growth temperature of the association in the chamber (table 3). Activities were only marginal when the association was grown at 23° in the light and 16° C in the dark (conditions comparable to the temperate climate zone) and not as usual at 33°in the light and 23° in the dark (conditions of tropical regions).

Table 2: C_2H_2-reduction and N_2O-production by the wheat-Azospirillum association under aerobic and microaerobic conditions

assay condition	C_2H_2-reduction		N_2O-formation	
	$-KNO_3$	$+KNO_3$	$-KNO_3$	$+KNO_3$
under argon- 0.2 % O_2	6.9	0.9	0.0	12.6
in air	0.4	0.2	0.0	0.0

Data are given in µmol C_2H_4 or N_2O formed/d x flask. The experimental conditions are the same as in table 1.

Table 3: C_2H_2-reduction and N_2O-formation by the wheat-Azospirillum association: Comparison of the activities when the association was grown at higher and at lower temperature

assay condition	C_2H_2-reduction		N_2O-formation	
	$-KNO_3$	$+KNO_3$	$-KNO_3$	$+KNO_3$
1. 12.5h at 33° in the light 11.5h at 23° in the dark	4.9	0.8	0.0	21.6
2. 12.5h at 23° in the light 11.5h at 16° in the dark	0.3	0.1	0.0	2.2

Data are given in µmol C_2H_4 or N_2O formed/d and flask. The experimental conditions are the same as in table 1

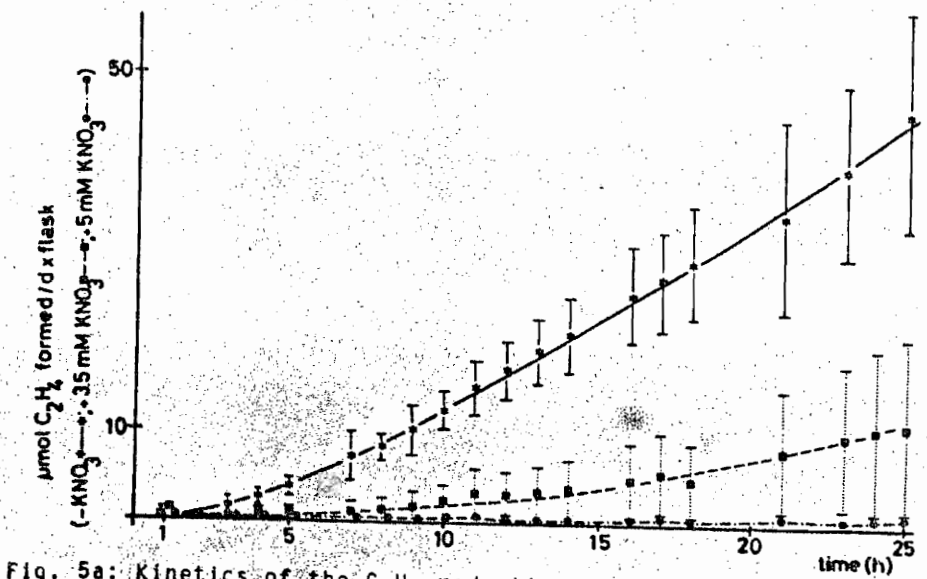

Fig. 5a: Kinetics of the C_2H_2-reduction by the wheat-Azospirillum association

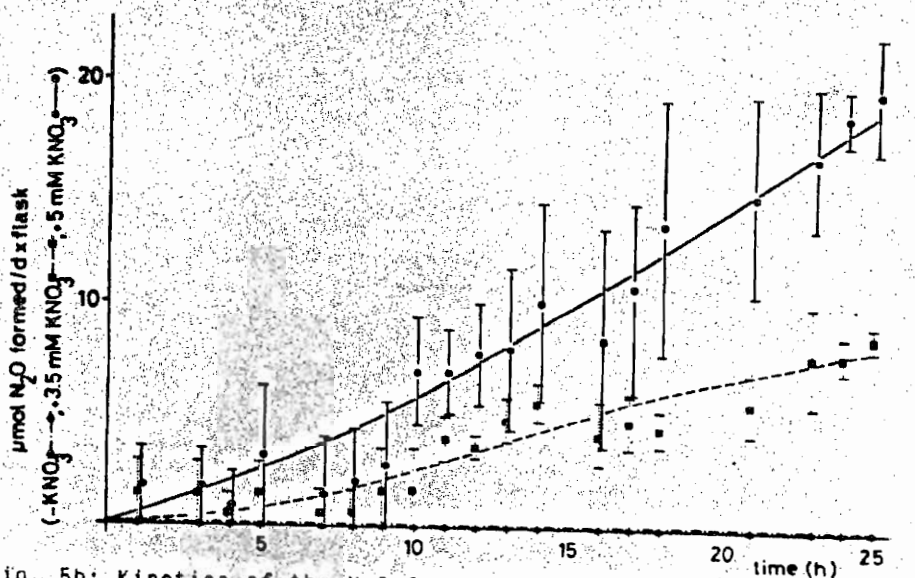

Fig. 5b: Kinetics of the N_2O-formation by the wheat-Azospirillum association

Discussion

The present communication shows that the wheat-Azospirillum association performs either C_2H_2-reduction or N_2O-formation, depending on the growth conditions in the semisolid agar medium. C_2H_2-reduction by the bacteria proceeds only at concentrations of nitrate smaller than 1 mM in the cultures. All bacteria preferentially meet their nitrogen requirements by assimilatory nitrate reduction and not by N_2-fixation, and Azospirillum is no exception to the general rule (10,12). In the present investigation, the addition of carbohydrates did not stimulate C_2H_2-reduction which means that the bacteria must have lived from the carbon compounds of the wheat plants. C_2H_2-reduction activity of the association was greatly enhanced by removing the air out off the flasks. It was pointed out by several authors that long incubation times at low O_2-concentrations lead to an overestimation of the nitrogenase activity occurring in situ(1,2). In the present investigation, C_2H_2-reduction commenced as early as 3-5 h after the removal of air. Some activity was also observed when the assays were performed in air. Little is known about the O_2-concentrations prevailing at the root surface or in the root cortex. It is conceivable that compartments in the roots exist where the O_2-levels are low due to respiration of the plant cells and where the bacteria find optimal conditions even in natural environments.

C_2H_2-reduction activity in the present investigation did not show diurnal fluctuations and was not lowered during the dark period as observed by others(13). Such differences in the results are probably due to different experimental conditions. In the present communication, it was extremely important to keep the conditions (number of plants and bacteria, incubation time, temperature etc.) to get reproducible results. Such experiments also require more repetitions than physiological or biochemical tests for statistically sound data. All our data are averages of at least 10 sets of experiments for every figure and table.

Remarkably, C_2H_2-reduction was greatly enhanced when the association was grown at higher temperature. Rather little activi ty was observed at conditions prevailing in temperate climate

soils. Thus temperature appears to be particularly important for maximal nitrogenase activity of the association between wheat and Azospirillum brasilense Sp.7. Azospirillum strains are particularly abundant in tropical soils. The contributions of Azospirillum to the nitrogen economy in temperate soils are presumably modest, although positive evidence has been forwarded also for the association grown in cold-climate soils (13,14,15).

The present communication also shows that the wheat-Azospirillum association performs denitrification provided the NO_3^--concentration in the medium is high and the O_2-level in the gas phase is low. All denitrifying bacteria, including Azospirillum, preferentially utilize O_2 as respiratory electron acceptor. In the absence of O_2 or at low O_2-concentrations, Azospirillum brasilense Sp.7 dissimilates NO_3^- to N_2 via NO_2^- and N_2O. In the present investigation, the experiments were performed in the presence of C_2H_2, because nitrogenase and denitrification activities can be tested simultaneously in one and the same vessel and because N_2O-formation is more easily measured than N_2-production. When artificial plant-Azospirillum associations are to be constructed for applications, Azospirillum strains are available which have nitrogenase and which convert nitrate to nitrite but not to gaseous N_2 or N_2O (8). It is conceivable that naturally occurring strains living in associations with grasses perform denitrification to a significant extent when the NO_3^--content is high and the O_2-level is low in soils (e.g. under waterlogged conditions).

In the present communication, the experiments were done with semisolid agar and in a mineral medium in order to simplify and to get well-defined experimental conditions. Growth of the wheat plants was not significantly enhanced by N_2-fixation or reduced by denitrification of the bacteria. Such enhancement or reduction was not expected to occur, since young wheat plants are sufficiently supplied with nitrogen from the protein reserves of the seeds. Further experiments have to show whether N_2-fixation can increase plant growth at later stages of the plant development and whether denitrification does the opposite.

References

1. Döbereiner,J. and De-Polli,H. 1980.In: Nitrogen fixation, Stewart,W.D.P. and Gallon,J.R. (eds.), pp. 301-333, Academic Press,London.

2. Van Berkum,P. and Bohool,B.B. 1980. Microbiol. Rev. 44,491-517

3. Umali-Garcia,M.,Hubbell,D.H., Gaskin,M.H. and Dazzo,F.B. 1980. Appl. Environm. Microbiol. 39,219-226

4. Martin,P. and Glatzle A. 1982.Experientia Suppl. 42, 108-120

5. Hartmann,A., Singh,M. and Klingmüller,W. 1983.Can. J. Microbiol. in press

6. Lin,W., Okon Y. and Hardy, R.W.F. 1983.Appl. Environm. Microbiol. 45, 1775-1779

7. Neyra,C.A. and van Berkum,P. 1977. Can.J.Microbiol. 23, 306-310

8. Magalhaes,L.M.S., Neyra,C.A. and Döbereiner,J. 1978. Arch. Microbiol. 117, 247-252

9. Scott,D.B.,Scott, C.A. and Döbereiner,J. 1979. Arch. Microbiol. 121,141-151

10. Bothe,H., Klein,B.,Stephan,M.P. and Döbereiner,J. 1981. Arch. Microbiol. 130, 96-100

11. Payne,W.J. 1981. In: Denitrification, nitrification and atmospheric nitrous oxide, Delwiche,C.C. (ed.),pp. 85-103,Wiley & Sons,New York

12. Bothe,H., Barbosa,G. and Döbereiner,J. 1983.Z. Naturforsch. in press.

13. De-Polli,H.,Boyer,C.D. and Neyra,C.A. 1982. Plant Physiol. 70, 1609-1613

14. Haahtela,L., Wartiovaara,T. and Sundman,V. 1981. Appl. Environm. Microbiol. 41, 203-206

15. Haahtela,L., Kari,K. and Sundman,V. 1983. Appl. Environm. Microbiol. 45, 563-570