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Acetylene reduction in gnotobiotic cultures with rhizosphere bacteria and wheat

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Summary Spring-wheat plant seedlings were inoculated with various isolates of nitrogen-fixing rhizosphere bacteria, including *Azospirillum brasilense*, in gnotobiotic sand cultures. Bacteria which had lost their acetylene reduction activity (ARA) during purification did not regain it in the presence of the plant. Bacteria with stable ARA were stimulated to low ARA (maximum 5.6 nmol C_2H_4 $plant^{-1}$ h^{-1}) by young (22–32-day) wheat seedlings.

Introduction

Most attempts to establish diazotrophic rhizocoenosis in wheat under temperate conditions have been unsuccessful so far. Nitrogenase activity is generally low in intact systems, except under very wet conditions[4,6,13,14,16,19]. Nitrogen fixation in wheat under temperate conditions has been estimated at 1.5–3 kg N ha^{-1} $year^{-1}$, as recalculated from acetylene reduction values[13], and attempts to increase nitrogen fixation by inoculation have not resulted in agronomically significant nitrogen fixation[10,12,13]. Nitrogenase activity in cereals, such as wheat, may thus be generally low under temperate conditions, but specialized systems with high-potential nitrogen fixation have also been described[21,22,23].

Inoculations under warm climatic conditions in Israel[1,7,9] and Brazil[3] and under temperate conditions in Belgium[24] have resulted in significant yield increases. The effects may, however, be attributed in part to production of plant-growth regulators by the bacteria[5].

Acetylene reduction, induced by the presence of plants, has been found in various greenhouse-grown grains and forage grasses in Sweden[14]. We have isolated nitrogen-fixing bacteria in connection with these experiments, as well as from the rhizosphere of field-grown wheat in Central Sweden[15]. The prime obejctive of the present investigation was to as certain whether plants support acetylene reduction by the bacteria under gnotobiotic conditions.

Materials and methods

Plant material

In most experiments we used spring wheat (*Triticum aestivum*), cv. Drabant, and, in one experiment, rye (*Secale cereale*), cv. Östgöta gråråg. The seeds were sterilized by the following sequence of treatments: 1 minute in 70% ethanol, 6 minutes in 0.1% $HgCl_2$ + 0.1% Tween 80, 1 hour in running tap-water, 1 minute in 70% ethanol, and finally 5 washings in sterile distilled water. The sterilized seeds were pregerminated on nutrient agar to check for contamination.

Gnotobiotic sand cultures

Plants were grown in large test-tubes (length 48 cm, dia. 4.3 cm) with sterilized sand, 90–175 g dry weight $tube^{-1}$, plugged with sterile cotton. For simplicity, smaller test-tubes with 5–8 g sand, without plants, served as controls. For comparison, results obtained from the small test-tubes have been scaled up to the level of the large test-tubes. Unless otherwise stated, the sand was moistened to 90% of WHC (water-holding capacity). One sterilized seed was planted in each test-tube.

Plants were grown in the greenhouse in ambient light (supplemented with fluorescent lamps for 16 h) and ambient temperature (using radiators, providing a minimum temperature of 15°C). In experiment P5, a growth chamber with a day/night cycle of 16 h, 25°C/8 h, 15°C was used.

Plant nutrients

Experiments P1–P3. All nutrients were added to the sand at the start of the experiments. The trace-element solution of Baldani and Döbereiner[2] was used at 1 $\mu l\,g^{-1}$ sand. In experiment P1 and P2, other nutrients were provided by the bacterial medium containing the inoculum, while in experiment P3 we also added 109 $\mu g\,K_2SO_4\,g^{-1}$ sand and 361 μg superphosphate g^{-1} sand, dissolved in 10 m*M* phosphate buffer, pH 7.0, resulting in a pH of 6.3. In experiments P1 and P2, 20 $\mu g\,N\,g^{-1}$ sand, and in experiment P3, 10 $\mu g\,N\,g^{-1}$ sand, was added in the form of $Ca(NO_3)_2\,4H_2O$.

Experiments P4–P5. Total amounts of trace elements, P and K were added as above. Nitrogen was added as a mixture of $Ca(NO_3)_2$ and $(NH_4)_2HPO_4$. Contrary to the other experiments, nutrients were added in portions during the experiments:

In experiment P4, 39% was added on planting, 16% after 11–12 days, and 7.5% after 18, 25, 32, 39, 46 and 53 days. The proportion NH_4^+-N: NO_3^--N was 2:3, and three levels of inorganic nitrogen were used; 10, 20 and 40 $\mu g\,N\,g^{-1}$ sand. The experiment was performed at two moisture levels, 40% and 80% of WHC.

In experiment P5, the proportion NH_4^+-N:NO_3^--N was changed to 3:2, and 10 $\mu g\,N\,g^{-1}$ sand was added. The nutrients were divided into six equal portions, which were added on planting and on days 15, 28, 35, 49 and 58. The experiment was performed at 80% WHC.

Bacteria

Various strains, isolated from the roots of cereals in Central Sweden, were used as bacterial inoculum: the unidentified strains DG 7-6 and DM 1-1, from greenhouse-grown spring wheat (cv. Drabant), and KM 1-2 and KM 2 from greenhouse-grown winter rye (cv. Kungs II)[14]. The isolates initially showed acetylene reduction activity (ARA) but lost it during purification[15]. The isolates were included to investigate whether ARA could be induced by the presence of plants. The other local isolates used, obtained from the roots of field-grown spring wheat, retained ARA during purification[15]. These were: the unidentified isolates A1, A2 and A3, *Bacillus polymyxa* strains B1 and B2, and *Enterobacter agglomerans* strain C1. We also used isolates D1 and E1, D1 being necessary for good growth and nitrogenase activity in E1, probably by supplying vitamin B_{12}.

The local strains were compared with *Azospirillum brasilense*, strain USA 10, in all experiments. Two other strains of *A. brasilense* were used in experiment P2: USA 15 and a local isolate, strain 2250-10, from soil under wheat (Lindberg, unpublished). These three

strains were chosen since, when tested in pure culture, they were the only ones among 11 tested strains of *Azospirillum brasilense* that grew under nitrogen-fixing conditions at relatively low temperatures (24°C).

Bacteria were grown for 2–3 days in batch cultures at 24°C on low-N semisolid media (GLU or MAL) containing glucose or malic acid as carbon source[15]. In experiment P1-P2, the batch cultures, including remaining bacterial nutrients, were used as inoculum. In experiments P3-P5, the inoculum was centrifuged and washed twice in phosphate buffer (0.05 *M*; pH 7.0). Washed, autoclaved inoculum, sterile medium or sterile buffer was added to controls. Large test-tubes containing plants were inoculated with 5.0 ml added to the sand, and small test-tubes and penicillin bottles (see below) with 0.25 ml. The inoculum contained 2×10^8 cells ml^{-1}.

Bacterial experiment (B1)

Isolate A2, Cl and *A. brasilense* USA 10 were grown under different O_2 conditions in 10-ml penicillin bottles, containing i) bacterial medium, ii) plant nutrients except nitrogen, and carbon source, or iii) plant nutrients, carbon source and sand.

Acetylene reduction activity (ARA)

Large test-tubes were enclosed in gastight plastic bags[14] or sealed with rubber stoppers. When required, the test-tubes were evacuated and refilled with 1000 ml N_2, after which air was added to initial concentrations of 2 or 5% O_2. One percent CO_2 was added in all plant experiments to compensate for consumption by photosynthesis. Small test-tubes and penicillin bottles were sealed with rubber stoppers and occasionally flushed with N_2 through syringe needles. Air to the required O_2 concentration was then added.

To measure acetylene reduction activity, 10 percent of acetylene (C_2H_2) was added, while vials without C_2H_2 served as controls (ethylene was never detected). Ethylene production was measured after 24 hours, using a Packard 428 gas chromatograph, as described by Lindberg and Granhall[14].

Other analyses

Using a glass electrode, pH was measured in sand: water (1:2). Dry weight was determined after drying at 85°C.

Results

Experiments P1 and P2

The experiments aimed at investigating whether the local isolates which had lost their ARA during purification[15] could regain it in the presence of plants. ARA was tested 4–6 times under 0, 2 and 5% O_2. Uninoculated plants and the four local isolates never showed ARA, while the Azospirillum strain USA 10 showed ARA varying between 0–287 nmol C_2H_4 $vial^{-1}$ h^{-1} in experiment P1, and the three Azospirillum strains tested in experiment P2 showed ARA varying between 0–24 nmol C_2H_4 $vial^{-1}$ h^{-1}. In neither of the experiments was any consistent effect noted of the presence of plants or of the different O_2 conditions during ARA assays. Addition of malic acid (68 mg $vial^{-1}$) to the sand during experiment P2 resulted in transiently higher ARA, giving a maximum of 62 nmol C_2H_4 $vial^{-1}$ h^{-1}. At the end of experiment P1, plant vials inoculated with Azospirillum were divided into

sand and root samples and immediately tested individually for ARA at 2% O_2. Activity was only present in the sand fraction.

Inoculation of wheat plants stimulated primarily shoot weight (Table 1), which resulted in total yield increases of 11–24%, even when bacteria had not shown ARA. Addition of autoclaved USA 10 resulted in the production of extra shoot weight. USA 10 had no effect on tillering (which occurred after 47 days in controls), while tillering was earlier (23–33 days) in plants inoculated with local isolates.

Table 1. Spring-wheat yield after 55 days' growth and the effect of inoculation (experiment P1)

Inoculum	Dry weight (mg plant^{-1})		Total yield (%)
	Shoot	Root	
Uninoculated	55 ± 7	18 ± 1	100
Live DG 7-6	68 ± 8	18 ± 2	117
Live DM 1-1	68 ± 8	21 ± 2	121
Live USA 10	61 ± 4	21 ± 1	111
Autoclaved USA 10	72 ± 5	19 ± 1	124

Note: Sterile medium was added to the uninoculated plants. Mean ± SE. n = 9.

For rye plants (Table 2), the effect of inoculation on yield was variable. The tendency was that live Azospirillum lowered the yield. As in experiment P1, addition of dead bacteria produced some extra yield, compared with live inoculum.

Table 2. Rye plant yield after 70 days' growth and the effect of inoculation (experiment P2)

Inoculum	Dry weight (mg plant^{-1})		Total yield (%)
	Shoot	Root	
Uninoculated	48 ± 4	29 ± 3	100
KM 1-2 live	47 ± 6	30 ± 4	101
KM 1-2 dead	55 ± 8	25 ± 3	105
KM 2 live	55 ± 9	28 ± 5	108
KM 2 dead	59 ± 10	33 ± 8	120
USA 10 live	38 ± 6	29 ± 5	87
USA 10 dead	nd[1]	nd	nd
USA 15 live	24 ± 6	22 ± 5	60
USA 15 dead	67 ± 17	42 ± 13	142
2250-10 live	35 ± 10	26 ± 5	80
2250-10 dead	48 ± 7	27 ± 6	98

Note: Sterile medium was added to the uninoculated plants.
Mean ± SE. n = 3.
[1] nd = not determined.

Experiment P3

In experiment P3, we inoculated wheat plants with two ARA-positive local isolates (A2 and C1) or *A. brasilense* USA 10. To reduce background ARA, the inoculum was washed free from nutrients. ARA was tested six times during 100 days' growth, both anaerobically and under 2% O_2, but ARA was never detected in any of the isolates. However, addition of malic acid (208 mg $vial^{-1}$; USA 10) or glucose (415 mg $vial^{-1}$; A2 and C1) occasionally resulted in ARA in USA 10 (maximum 163 nmol C_2H_4 $vial^{-1}$ h^{-1}) and in C1 (maximum 24 nmol C_2H_4 $vial^{-1}$ h^{-1}), while addition of yeast extract (3.5 mg $vial^{-1}$) never had any effect. Tubes with isolate A2 never showed ARA in any of the treatments. The different inoculations did not affect yield significantly.

Experiment B1

Using the same isolates as in experiment P3, we investigated the effect of plant nutrients and different O_2-concentrations on ARA. The bacteria were grown in sand and liquid culture in the presence of readily available carbon (Table 3). In stagnant-liquid batch culture with bacterial medium, 20% O_2 in the gas phase was optimal for *A. brasilense* USA 10, while anaerobic conditions were optimal for the local isolates. The plant nutrients supplemented with carbohydrates could not support ARA in any of the isolates. Mixing the plant nutrients with sand, however, allowed nitrogenase activity in USA 10 and C1, but not in A2. This is consistent with experiment P3, where A2 never showed any ARA.

Experiments P4 and P5

To avoid repression of ARA by inorganic nitrogen and to minimize the risk that plant nutrients may have a negative effect on the bacteria, plant nutrients were added in portions. In experiment P4, wheat was grown together with the local isolates B1, C1 or *A. brasilense* USA 10 at two moisture levels and three N levels. ARA was tested on four occasions during 60 days' growth. The various inocula, moisture or nitrogen additions on any of the occasions were not found to have any significant effect on ARA. In pooling the results of all treatments for each sampling occasion, differences were found between different sampling occasions, with a peak in mean ARA at 32 days (Table 4). The pH in the sand varied between 7.8 and 8.6 during the experiment, with no significant differences between treatments. The development of above-ground parts was largely unaffected, with the exception that plants inoculated with USA 10, as in experiment P1, showed later tillering, compared with other inocula. Maximum above-ground yield was 608 mg dry weight $plant^{-1}$.

Table 3. ARA of bacterial isolates under different O_2 and nutrient conditions (experiment B1)

Isolate	O_2 (%)	ARA (nmol C_2H_4 $vial^{-1}$ h^{-1})		
		Bacterial medium[1]	Plant nutrients + carbohydrates[2]	Plant nutrients + carbohydrates + sand[3]
A2	0	148 ± 6.8	0	0
	2	30 ± 4.8	0	0
	5	17 ± 2.3	0	0
	20	11 ± 3.5	0	0
C1	0	1365 ± 14	0	431 ± 75
	2	774 ± 18	0	251 ± 18
	5	738 ± 57	0	178 ± 20
	20	243 ± 27	0	215 ± 128
USA 10	0	0	0	5.7 ± 2.0
	2	14 ± 2.8	0	41 ± 6.2
	5	14 ± 3.6	0	29 ± 3.6
	20	170 ± 21	0	0.1 ± 0.1

Note: The bacteria were grown for three days at 24°C under the respective O_2 concentration before ARA assay. Mean ± SE. n = 5.

[1] Five ml GLU or MAL medium without agar $vial^{-1}$.

[2] Five ml $vial^{-1}$ of plant nutrients as in experiment P3 (except nitrogen), and 1% glucose (A2, C1) or 0.5% malic acid (USA 10).

[3] Plant nutrients (1.8 ml $vial^{-1}$), carbohydrates and 6.5 g sand $vial^{-1}$.

Table 4. ARA on different sampling occasions after inoculation of spring wheat with three ARA-positive bacteria (experiment P4)

Day	ARA (nmol C_2H_4 $vial^{-1}$ h^{-1})		
	Range	Mean	SE
18	0–1.4	0.3	0.1
32	0.1–1.9	0.9	0.2
46	0.1–1.2	0.4	0.1
60	0–0.9	0.2	0.1

Note: The inoculum was washed free from nutrients. ARA was measured at ambient O_2. The results for all treatments (three inocula; two moisture levels; three nitrogen levels) on each sampling occasion are pooled. n = 9 – 14.

In experiment P5, N addition was altered to maintain a lower pH during plant growth. The pH in the sand, 6.3 on planting, was maintained at 5.2–5.8 during the experiment. As before, we used *A. brasilense* USA 10 as a reference, and compared it with six local strains. ARA was measured three times during 65 days' growth (Table 5). ARA was consistently higher with plants than without, and was highest on days 22–24. There was no significant difference between the various inocula. Shoot yield varied between treatments, indicating an effect of the different bacteria (Table 6). The highest yield (isolate B2) was 51% greater than the lowest (D1 + E1).

Table 5. ARA in seven different bacterial isolates (including one *Azospirillum brasilense*) and the effects of spring wheat (experiment P5)

Treatment	ARA (nmol C_2H_4 $vial^{-1}$ h^{-1}) on different days after inoculation		
	22–24	42–43	64–65
With plant	2.9–5.6	1.6–2.4	0.0–0.2
Without plant	0.0–0.4	0.0–0.3	0.0–0.2

Note: The inoculum was washed free from nutrients. ARA was measured at ambient O_2. Values are ranges of the mean for each isolate. n = 6 for tubes with plants and 3 for tubes without plants for each isolate.

Table 6. Wheat plant development after 67 days' growth and the effect of inoculation (experiment P5)

Inoculum	Dry weight of shoot (mg $plant^{-1}$)
Uninoculated	260 ± 12
A1	225 ± 26
A3	252 ± 8
B1	212 ± 14
B2	271 ± 1
C1	204 ± 7
D1 + E1	180 ± 16
USA 10	215 ± 13

Note: The inoculum was washed free from nutrients. Sterile buffer was added to uninoculated plants. Mean ± SE. n = 6.

Discussion

The local isolates from wheat and rye which had lost their ARA could not be stimulated to reduce acetylene in the presence of plants. Possible reasons for the loss of activity have been discussed by Lindberg and Granhall[15]. It is likely that the ARA observed in Azospirillum in experiments P1-P2 was supported by the bacterial nutrients added together with the inoculum, since the plants did not stimulate ARA. With Azospirillum, ARA activity could be observed after 55 days (P1) and even after 79 days if extra malic acid was added to the sand (P2).

When local isolates with stable ARA were used, initial tests with wheat plants did not result in ARA unless a readily available carbon source was added. In such cases, activity was only occasionally detected (P3).

The above experiments did not indicate any significant contribution of the plant to bacterial ARA. Plant nutrition and oxygen concentrations during ARA tests were varied, but never resulted in clearly enhanced ARA. An experiment (B1) intended to yield more information about the influence of specific factors on the bacteria showed

that the expression of ARA was rather unsensitive to O_2 in the gas phase (Table 3). Experiment B1 also showed that plant nutrients, supplemented with a suitable carbohydrate, could not support bacterial ARA in liquid culture, while, for unknown reasons, ARA was obtained in two isolates out of three when sand was present (Table 4).

ARA-positive bacteria were tested together with wheat at different moisture and inorganic nitrogen levels. ARA was not appreciably affected by the treatments or by different inocula, but plant influence on all bacteria was noted (experiment P4 and P5). ARA was highest during early vegetative growth (22–32 days). However, in other diazotrophic rhizocoenoses, nitrogenase activity is generally low during early vegetative growth, reaching a maximum during the reproductive stages[18]. The maximum ARA induced by inoculation (5.6 nmol C_2H_4 plant^{-1} h^{-1}) in our experiments is much lower than the maximum values reported for other gnotobiotically grown plants inoculated with Azospirillum, for example 2350 nmol C_2H_4 plant^{-1} h^{-1} for *Setaria italica*[8]. Acetylene reduction values, expressed per plant, for gnotobiotically grown wheat are, to our knowledge, not available for comparison. For wheat grown in soil, Lethbridge *et al.*[13] reported 67 and 500 nmol C_2H_4 plant^{-1} h^{-1} for low- and high-activity plants, respectively. Klucas *et al.*[11] found less than 1.0 nmol C_2H_4 plant^{-1} h^{-1} produced in wheat grown in pots. In experiments P4 and P5, multiple additions of small amounts of nutrients resulted in better plant growth, which might be the reason for the detection of low ARA. Another possibility is that high concentrations of plant nutrients had a direct negative influence on the bacteria, as indicated by experiment B1 (Table 4).

The prime limiting factor in our plant experiments, as in a majority of systems studied[17,20], was probably the supply of carbon and energy to the bacteria, since additions of carbohydrates, but not yeast extract, mostly resulted in ARA. Lethbridge and Davidson[12], using the ^{15}N isotope dilution technique and acetylene reduction, could not demonstrate nitrogen fixation in the rhizosphere of wheat unless carbohydrates were added.

Inoculation affected plant development – primarily shoot yield and time of tillering – but the effects were variable, depending on the experimental conditions, and did not correlate to ARA. Positive effects are usually ascribed to plant-growth regulators produced by the bacteria[5]. Although increases in yield are common upon inoculation[18], negative effects have been found for pearl millet grown in test-tubes and inoculated with *A. brasilense*[25], for the root production of greenhouse-grown wheat inoculted with *A. brasilense*[24,26] and for wheat inoculated with *Azotobacter beijerinckii* or a natural soil microflora in

presence of added carbohydrates[12]. As suggested by Lethbridge and Davidson[12], the decrease in yield on inoculation may be due to competition for O_2 and inorganic nitrogen between the plant and the bacteria.

In summary, these results do not present evidence for the establishment of a specific N_2-fixing association between any of the investigated bacteria and wheat plants in sand culture. For shorter periods, however, several nitrogen-fixing bacteria, including Azospirillum, may be stimulated by young wheat plants to low acetylene reduction. It should be noted that we tested only one plant cultivar, while – at any rate in the comparison of yields – the response to bacterial inoculation may vary considerably between different cultivars[1].

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