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N_2 Fixation by *Azospirillum brasilense* and Its Incorporation into Host *Setaria italica*

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Growth and nitrogen fixation were followed during the life cycle of *Setaria italica* (foxtail millet) inoculated with *Azospirillum brasilense* in controlled-environment growth chambers. The plants were fertilized at seeding with a limiting amount of combined nitrogen and maintained with an N-free mineral solution. During maturation of the plants, substantial nitrogenase activity, measured by acetylene reduction, developed in the rhizosphere, with total fixation estimated to be equivalent to 20% of the N in the inoculated plants. The peak of this activity coincided with depletion of soluble nitrogen from the system, which in turn was reflected by a sharp decrease in the nitrate reductase activity of the leaves. *A. brasilense* was found in association with the root populations at 8×10^7 cells per gram of dry weight. An increase in shoot growth occurred at this time, but no significant increase in total plant nitrogen could be demonstrated. $^{15}N_2$ enrichment experiments confirmed that fixation was occurring, but only about 5% of the nitrogen fixed by *A. brasilense* was incorporated into the plants within 3 weeks. There was thus no evidence of direct bacterium-to-plant transport of fixed nitrogen, but rather a slow transfer suggesting the gradual death of bacteria and subsequent mineralization of their nitrogen, at least under growth-room conditions.

Organisms of the genus *Azospirillum* have been associated with roots of various grasses (9). A significant benefit in growth and yield of *Setaria italica* (foxtail millet) from root inoculation with *Azospirillum brasilense* has been obtained previously in the greenhouse and in the field (2, 5–7). Significant increases in dry weights of panicles and whole shoots were obtained in inoculated plants fertilized with suboptimal NH_4NO_3 levels. The increases in nitrogen content were presumed to be due in part to N_2 fixation (5).

Acetylene reduction had been shown, with the highest rates in intact inoculated plants observed at the booting stage at temperatures of 32°C. However, the contribution of any of this newly fixed N_2 had not been directly demonstrated. In the present work, we have used nitrogen balances, acetylene reduction, and $^{15}N_2$ to assess the direct benefit to the inoculated plant from newly fixed nitrogen.

MATERIALS AND METHODS

Organisms and growth conditions. *A. brasilense* (8) strain Cd ATCC 29729 was grown in nutrient agar (Difco Laboratories, Detroit, Mich.) at 30°C. Cultures (24 h old) were washed from the agar with 0.05 M potassium phosphate buffer (pH 6.8). A suspension containing approximately 10^8 CFU/ml was used as inoculum.

S. italica (foxtail millet, German millet) seeds were purchased from the Old Seed Co., Madison, Wis. Plants (one per container) were grown in 2-liter plastic bottles (9-cm diameter 25 cm high) wrapped in aluminum foil, with a 1-cm hole drilled in the bottom to allow for water drainage. This was loosely plugged with glass wool to reduce gas exchange and thereby lower O_2 concentration to more physiological levels. The bottles were covered with a metal screw cap with a 1-cm hole in the center for stem emergence. A Plasticine seal was later placed around the stem.

Each bottle was filled with 200 g of sterilized vermiculite (Terra-lite; Horticultural Products, W.R. Grace and Co., Cambridge, Mass.) containing no minerals available to the plants. This was supplemented with 1% (wt/wt) $CaCO_3$. The pH of this medium remained close to 6.8 throughout plant development.

Each pot was watered at seeding with 200 ml of Hoagland plant nutrient solution containing 42.0 mg of combined nitrogen (45% NH_4^+—N, 55% NO_3^-—N). Either an N-free plant nutrient solution or deionized water was used for subsequent watering. Root moisture was maintained near field capacity throughout plant growth by daily drip irrigation with water or N-free medium and by placing a tin foil cup at the bottom of the bottle to retain moisture. The bottles were covered with foil to avoid algal development. After

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seedling emergence, each pot was inoculated with 1 ml of a culture of *A. brasilense* Cd applied to the top of the pot and watered in. One hundred plants were grown under controlled environmental conditions in a growth chamber with a photoperiod of 14 h at a bench-level light intensity of 800 microeinsteins per m^2 per s at 31 ± 1°C and 25 ± 1°C during the dark period. High relative humidity was maintained by constantly dripping water on the floor of the growth chamber.

In a smaller pilot experiment, only 9 mg of combined N was initially present in both test and control pots. Test pots were inoculated as above, and autoclaved inoculum was added to controls. Cross-inoculation was avoided by covering the vermiculite with sterilized waxed sand and by catching the drainage water in a dish of $KMnO_4$ solution as described by Nur et al. (7). The results (see below) guided our main studies. For example, initial nitrogen was increased to give healthier plants. Also, attempts to maintain axenic conditions were discontinued, both because they proved cumbersome with large plant populations and because we recognized that they were highly artificial. Although the microbial populations in our growth medium may differ from "typical" soils, still the competition for and interchange of nutrients should be more representative of natural field conditions than axenic cultures would be.

Measurements. Plant growth and enzyme activities were followed during plant ontogeny, with data for each assay representing six plants per point. Shoot and washed root dry weights were obtained by drying the plants at 80°C for 48 h in a forced-air oven. Percent nitrogen in the dry material was measured with a protein-nitrogen apparatus made by LECO Corp., St. Joseph, Mich. A wheat standard of 2.84% was used.

Nitrate reductase activity in leaf extracts was determined by a modification of the method of Jaworski (4). Four fresh leaves from each plant were dipped in 40 ml of incubation medium in 60-ml serum bottles. The incubation medium contained 0.025 M KNO_3, 0.1 M potassium phosphate buffer (pH 6.9), 0.5% (vol/vol) isobutyl alcohol, and 0.01 mg of chloramphenicol per ml. A vacuum was pulled for 30 s to infiltrate the tissue. The samples were incubated in the dark for 30 min at room temperature, and 2-ml samples were transferred to test tubes. Nitrite content was determined colorimetrically by the addition of 0.5 ml of 1% (wt/vol) sulfanilamide in 3 N HCl and 0.5 ml of 0.02% (wt/vol) aqueous *N*-1-naphthylethylenediamine hydrochloride. Absorbancy at 540 nm was measured after 20 min against $NaNO_2$ standards. Specific nitrate reductase activities were expressed as μmol of NO_2^- produced per g of leaf dry weight per h.

To determine the bacterial population in association with the roots, the roots were freed of excess vermiculite by gentle shaking in water. Each plant's root system was then shaken (250 strokes per min) in 100 ml of 0.05 M sterile potassium phosphate buffer with 0.1% agar (for a more homogeneous suspension) for 30 min. The roots were then crushed with mortar and pestle. Tenfold serial dilutions were made in the same buffer. Samples were inoculated into nutrient broth with 0.1% yeast extract for the estimation of total bacterial counts and into an N-free malate broth with yeast extract for the estimation of nitrogen-fixing *A. brasilense* by the most probable number method.

For acetylene reduction assays, the root systems were tightly sealed with a rubber stopper at the bottom of the bottles and with Plasticine surrounding the stems. C_2H_2 was injected to a final concentration of 20% (vol/vol), and the C_2H_4 produced was determined after 3, 6, and 24 h by gas chromatography with a flame ionization detector (1). Ethylene production was linear with time during the first 24 h.

To determine $^{15}N_2$ fixation and incorporation, enclosed root systems were evacuated and filled with argon 15 times. O_2 to a final concentration of 4% and $^{15}N_2$ to a final concentration of 30% were added. Nitrogen $^{15}N_2$ (gas) (99 atom % ^{15}N; lot no. 1061-F) was purchased from Merck Sharp and Dohme Canada, Ltd., Montreal. Samples for ^{15}N determination were withdrawn into evacuated tubes after allowing 1 h for equilibration. Contamination with $^{14}N_2$ was avoided by flushing needles and tubes with argon. The enclosed systems were incubated for 72 h. A parallel set of plants was assayed for acetylene reduction under similar conditions (4% O_2, 20% C_2H_2 in argon). Acetylene concentrations in the system diminished by 0% after 24 h, 5% after 48 h, and 42.5% after 72 h. After 72 h, the ^{15}N-treated systems were opened, and plants were allowed to grow to maturity under a normal atmosphere. The plant material (roots, shoots, and panicles) was dried and digested in a micro-Kjeldhal apparatus, and the NH_3 was steam distilled into 20% H_2SO_4. ^{15}N-enrichment of the NH_4^+ was determined with an MAT-250 mass spectrometer (Finnegan-MAT Corp., Sunnyvale, Calif.).

RESULTS AND DISCUSSION

The effect of *A. brasilense* inoculation on nitrogen fixation and yield of *S. italica*, as compared with controls treated with a killed culture, was tested in a pilot experiment. The results obtained corroborated those reported by Kapulnik et al. (5) with the same plant and bacterium. There was a significant increase in root and shoot dry weight as a result of inoculation and an apparent gain of about 5 mg of N above the initial amount in inoculated plants. Also, acetylene reduction activity was detected at the booting stage, and *A. brasilense* was recovered from the rhizosphere only in inoculated plants.

In subsequent experiments, plant growth, nitrogen-fixing activity, and more extensive nitrogen balance measurements were made with inoculated systems containing 42 mg of combined N.

The results of one such experiment are shown in Fig. 1. Four other experiments showed similar trends. Plant growth slowed at the booting stage 27 to 33 days after planting (Fig. 1A), at which time acetylene reduction activity sharply increased to a maximum and began to decrease (Fig. 1D). In this period, leaf nitrate reductase specific activity decreased to almost zero (Fig. 1B), suggesting depletion of NO_3^- in the pots, Nitrate N content of the vermiculite medium between day 23 and maturity was less than 3 mg

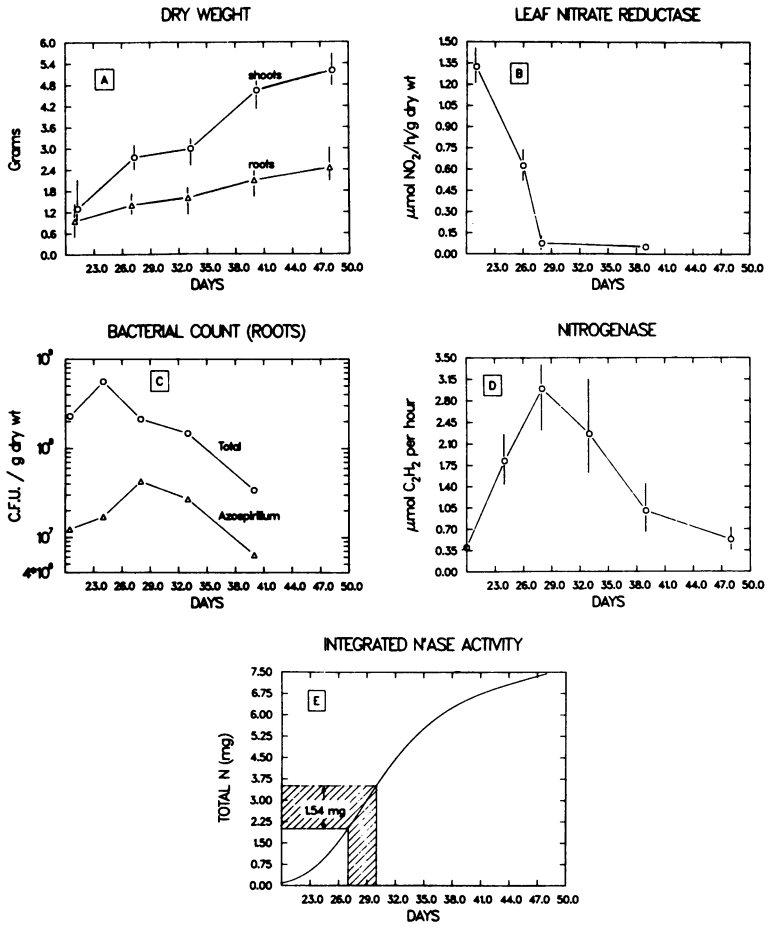

FIG. 1. Growth, bacterial, and nitrogen-related parameters during the growth cycle of *S. italica* inoculated with *A. brasilense* in a controlled environment growth room. Results represent the means of 6 to 10 plants at each sampling: (A) shoot and root dry weight; (B) leaf nitrate reductase activity; (C) total bacterial and *A. brasilense* root populations (means of replicates); (D) nitrogenase activity measured as acetylene reduction; (E) cumulative N fixed in system, based on integrating curve D and assuming 4:1 ratio of acetylene reduction to N_2 reduction. The amount of fixation expected during the 72-h $^{15}N_2$ exposure is indicated.

of N per pot. The numbers of *A. brasilense* cells associated with the roots (Fig. 1C) represented about 10% of the total bacterial population and paralleled acetylene reduction activity. The contribution of *A. brasilense* to the microbial rhizosphere biomass may be even larger than 10% because azospirilla are relatively large and also may cluster in microcolonies or aggregates that are counted as one organism by the most probable number method (3, 8).

The total nitrogen fixed by the bacteria over the growth period was approximated at 7 mg of N or 17% of the plants' total N budget. We integrated the nitrogenase (C_2H_2 reduction) activity (Fig. 1E) and assumed that 4 mol of C_2H_2 was equivalent to 1 mol of N_2 reduced plus 1 mol of H_2 evolved.

After the peak of nitrogenase activity, there was consistently renewed growth, particularly of the shoot (Fig. 1A). Although the total N content of the plants appeared to rise slightly during the period studied, any increase beyond the initial 42 mg provided was within experimental error. Thus the N balance did not show the transfer of all or most of the N_2 fixed to the plant, although it did not preclude some assimilation.

Pulsing of the rhizosphere with $^{15}N_2$ and subsequent analysis of the plant confirmed that a measurable, but very small, portion of bacterially fixed N was incorporated into the plants. The 72-h labeling experiments were done at both the booting (28 days) and panicle emergence stages (33 days), with parallel acetylene reduction as-

TABLE 1. Incorporation of newly fixed ^{15}N into *S. italica* inoculated with *A. brasilense* Cd[a]

Days after pulse	^{15}N atom % excess (pulse)	Plant part sampled	^{15}N atom % excess (sample)	mg of N in plant part	mg of ^{15}N in plant part	% of plant N derived from 3-day pulse	N incorporated/ N fixed[b]
21	70.3	Root	0.1021	14.4	0.0207	0.14	0.054
		Shoot	0.0301	28.9	0.0118	0.10	
		Panicle	0.0515	6.3	0.0039	0.06	
14	71.3	Root	0.0421	12.9	0.0073	0.06	0.021
		Shoot	0.0150	27.9	0.0063	0.02	
		Panicle	0.0188	2.6	0.0007	0.03	
7	69.0	Root	0.0351	11.4	0.0057	0.04	0.017
		Shoot	0.0094	28.1	0.0026	0.01	
		Panicle	0.0210	5.4	0.0016	0.03	

[a] All values represent averages of three plants.
[b] The average N fixed during the 3-day pulse was estimated at 1.5 mg, on the basis of acetylene reduction data for the rhizosphere.

says to confirm that N_2 fixation was occurring (Table 1). The plants were then grown an additional 7to 21 days (maturity). At that time, all parts—roots, shoots, and panicles—were found to be significantly enriched in ^{15}N (Table 1), but the level of ^{15}N incorporation accounted for only microgram quantities of nitrogen.

These values are probably underestimates because of ^{15}N leakage during the 72-h test period, but this error factor of 1.5 to 1.8 does not materially affect the conclusion. Based on C_2H_2 reduction measurements, the expected N_2 fixation for the pulse period should be about 1.5 mg of N. Of this, only 1% is incorporated into the plant after 7 days, 2% is incorporated after 14 days, and 5% is incorporated after 21 days.

We conclude that *A. brasilense* in the rhizosphere fixed N_2, based on both C_2H_2 reduction and ^{15}N enrichment. However, the transfer of nitrogen from *A. brasilense* Cd to *S. italica* is very slow and indirect, presumably requiring bacterial decomposition and mineralization. Possible secondary uptake by other microorganisms (both in our system and in a natural rhizosphere) may contribute to this delay. These too, of course, must ultimately contribute to increased steady-state soil nitrogen. Hence, early generations of field-grown plants may gain little nitrogen from the associative bacterial growth, although the contribution of long-term inoculation should be significant. This is in contrast to the directly coupled symbiotic system where the transfer is rapid.

Our results do not preclude ecological factors, operative under some field conditions, which accelerate nitrogen transfer in associative fixation systems. Plant growth stimulation has been repeatedly observed in these associations (6). This may be due to such processes or to hormonally active secretions of azospirilla or to increased effectiveness of root ion uptake in their presence (6a).

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