Contribution of *Azospirillum brasilense* Cd to growth of tomato seedlings is not through nitrogen fixation

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A wild type and a site-directed mutant strain (Nif⁻) of *Azospirillum brasilense* Cd, totally deficient in N_2-fixation capability, were used to evaluate if improvement in tomato seedling growth is due to the N_2-fixation process. The mutant (Nif⁻) exhibited several physiological, ecological, and antigenic features similar to the parental strain. When inoculated onto tomato seeds, the significant positive effects of the *A. brasilense* Nif⁻ on seedling growth under greenhouse conditions in artificial medium were similar to that of the wild-type strain. It is concluded that the contribution of *A. brasilense* Cd to the improvement of tomato seedling growth is not through N_2-fixation.

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Un type sauvage et une souche de mutant Nif⁻ d'*Azospirillum brasilense* Cd, totalement inapte à la fixation d'azote, furent utilisés afin d'évaluer si l'augmentation de croissance de plantules de tomate est due au processus de fixation d'azote. Le mutant Nif⁻ a montré plusieurs caractéristiques physiologiques, écologiques et antigéniques similaires à la souche parentale. Lorsqu'inoculé à des graines de tomate, les effets significatifs positifs d'*A. brasilense* Nif⁻ sur la croissance des plantules en conditions de serre sur milieu artificiel furent similaires à ceux des souches de type sauvage. En conclusion, la contribution d'*A. brasilense* Cd à la meilleure croissance des plants de tomato n'est pas due à la fixation de l'azote.

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Introduction

During the last decade, interest in the *Azospirillum* genus has been increasing because of its reported ability to contribute to the yield of various cereals (4, 30). The mechanism(s) involved in this plant–bacterial interaction is unknown, although studies have suggested N_2-fixation (3, 10, 13, 17, 20, 21, 28, 29, 36), hormonal effects (12, 23, 40), improvements of root development, minerals and water uptake (4, 5, 27, 31), and stimulation of nitrate assimilation by the plants (16). Since species of *Azospirillum* are known to fix N_2 (39), great attention has been paid to the contribution of fixed nitrogen to the nitrogen content of plants. N_2-fixation by *Azospirillum* was considered to be the main factor operating between *Azospirillum* and monocotyledonous plants (for review, see 33). However, a few studies have presented circumstantial evidence showing that the amount of fixed N_2 incorporated into the plants is relatively small (22, 32).

Several chemically induced mutants defective in N_2-fixation (Nif⁻) ability have been isolated and characterized (19, 34). The *nif* structural genes of two species of *Azospirillum* were cloned (15, 35) and a site-directed transposon mutagenesis technique was applied to *Azospirillum* (37, 38, 41). Among the available Nif mutants, the most suitable mutant for plant inoculation experiments is the site-directed (cartridge mutagenized) Nif⁻ mutant (37) that is described in the present study in detail. Until now, no evidence for a direct contribution to plant growth by any of the Nif⁻ mutants has been presented.

The aim of the present study was to evaluate the contribution of the N_2-fixation process to the growth of tomato (*Lycopersicum esculentum* Mill.) seedlings by inoculation (9) of seedlings with a known beneficial strain, *A. brasilense* Cd, in comparison with its isogeneic N_2-fixation deficient (Nif⁻) mutant.

Materials and methods

Bacteria

Azospirillum brasilense Cd (ATCC 29710), *A. brasilense* Nif⁻ (29710-10b), *A. brasilense* Sp-7 (ATCC 29145), and *Escherichia coli* (HB 101) were used in this study. Bacteria were grown in nutrient broth (Difco) for 16 h (logarithmic phase of growth) at 30°C in Erlenmeyer flasks equipped with shallow grooves to increase aeration of the culture in a rotary shaker (250 rpm). Cells were harvested by centrifugation at 7000 × *g* for 10 min at 4 ± 1°C and washed three times in sterile water and their number initially adjusted to 10^9 cfu/mL (1.05 A_{540} units) and then decimally diluted to the required inoculation level.

Construction of A. brasilense *Nif⁻ mutant*

The Nif⁻ mutant (29710-10b) was obtained from the parental strain *A. brasilense* Cd (rif-resistant) by a site-directed replacement of the nitrogenase structural genes *nif* DK by a kanamycin-resistant cartridge (37, 38). The procedure used was as follows. The plasmid pMS205 (resistant to kanamycin, tetracycline, and ampicillin) was constructed as described in Fig. 1. The plasmid pMS175-2 containing the *nif* HDK genes of *A. brasilense* (38) in pSUP202 was digested with PstI

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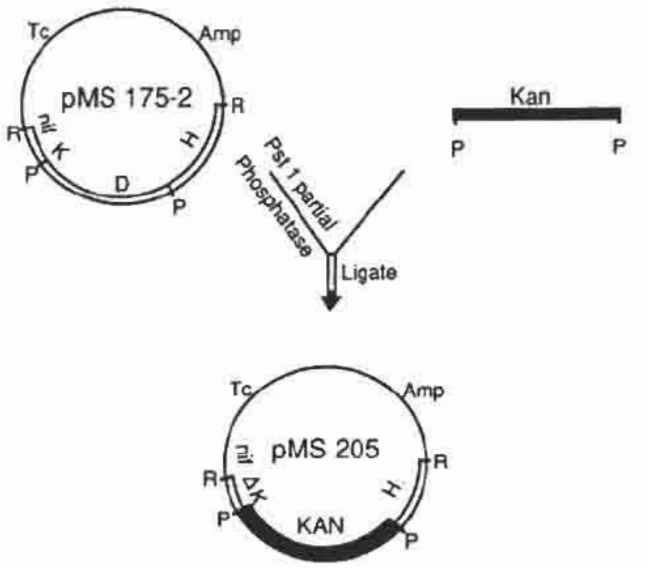

FIG. 1. Construction of plasmid pMS205 resistant to kanamycin, tetracycline, and ampicillin that was transferred to *A. brasilense* Cd to construct the site-directed Nif⁻ strain.

and ligated to a PstI fragment (coding for resistance to kanamycin) of Tn903. The recombinant plasmid pMS205 contained a deletion of *nif* DK that was replaced by the kanamycin-resistant cartridge from Tn903. This Kan-DNA fragment is incapable of transposition that prevents both the reversion of the Nif phenotype and the induction of further mutations (38). When pMS205 was transferred to *A. brasilense* Cd by conjugation with HB101 (pMS205), the mutant *nif* genes (with Kan in the map) are exchanged for the wild-type genes; the latter are lost because the vector pSUP202 cannot replicate in *Azospirillum*.

Physiological characteristics of A. brasilense *Cd and* A. brasilense *Cd Nif⁻*

The following strain characteristics were evaluated. N_2-fixation activity was measured by the acetylene reduction assay (1); growth on minimal medium and utilization of ammonium and nitrate was as described by Albrecht and Okon (1); growth on N-free medium was measured according to Bashan and Levanony (7); plasmid pattern was as described by Singh and Klingmüller (38); motility was tested in cultures at logarithmic phase of growth under light microscope; chemotaxis was tested in an open channel system according to Barak et al. (2); motility in quartz sand towards wheat seedlings was measured according to Bashan (6); red pigmentation and colony morphology was as described by Eskew et al. (14); aggregation was evaluated by measuring the rate of decrease in absorbance at 540 nm of cultures in stationary phase of growth; and attachment to quartz sand particles was measured according to Bashan and Levanony (8).

Enzyme-linked immunosorbent assay (ELISA)

Whole cells of *A. brasilense* Cd were used to elicit antibodies in white rabbits by immunization with multiple intradermal injections as described elsewhere (25). These polyclonal antibodies, after purification, showed high specificity towards *A. brasilense* Cd with negligible cross reactions to other rhizosphere bacteria as well as towards several other *Azospirillum* species (24, 25). Indirect and competition ELISA were performed as previously described (25).

Plants, growth conditions and inoculation

Tomato (*Lycopersicum esculentum* L.) seedlings 'Naama' and 'Faculta-121' (fresh market cultivars for field and greenhouse cultivation, respectively) were used. Plants were grown in an air-conditioned greenhouse at 26 ± 2°C in the following mixture in commercial seedling trays: peat (Klasmann, Federal Republic of Germany), vermiculite (Agrical, Habonim, Israel) (1:1, v/v) supplemented with 12.5 μg NH_4NO_3 per seedling (this nitrogen level permits nitrogen fixation by *A. brasilense* Cd (18)); 50 μg $Ca(H_2PO_4)_2 \cdot H_2O$; 50 μg KCl and micronelements per seedling. Each tray contained 100 trapezoidal compartments (7 × 3 × 3.2 cm, 25 mL substrate for each seedling grown in a separate compartment). The trays were mounted 30 cm above the greenhouse table surface to avoid possible transfer of bacteria through the free drainage water holes from inoculated to noninoculated trays and to avoid root crossover into other compartments. The trays were covered with a very fine white antiaphids and mites net that did not cause etiolation (0.2 × 0.2 mm pore size; Sdeh Nizan, Israel) for the whole growth period. Water irrigation (15 mL per compartment, twice a day) was given to plants through the net. Alternatively, 15-day old seedlings were transferred from the tray to 4-L pots containing the same "soil" mixture, fertilized, and grown for an additional 30 days as previously described (9).

Azospirillum brasilense Cd and *A. brasilense* Cd Nif⁻ were cultured as described, and the number of bacteria were adjusted in sterile nonchlorinated single deionized water to 10^6 cfu/mL. (Bashan (5) demonstrated a marked effect on wheat seedling growth by this bacterial concentration.) Seeds were suspended in the bacterial suspension and subjected to light vacuum (water pump) for 30–60 min. The vacuum was released abruptly, allowing the bacteria to penetrate into seed cavities. The seeds were imbibed for an additional 12–16 h in the bacterial suspension at ambient temperature to ensure colonization of the germinating seedlings and then sown in the trays. A second inoculation was performed 10–13 days after sowing by applying 5 mL bacterial suspension in water at the same concentration to each seedling. Changes in root colonization were monitored by the improved selection technique (7) on N-free BL medium (for *A. brasilense* Cd) and on nutrient agar (Difco) supplemented with 15 $\mu g \cdot mL^{-1}$ tetracycline and 30 $\mu g \cdot mL^{-1}$ kanamycin (for *A. brasilense* Cd Nif⁻).

Plant parameter measurements

The following plant parameters were quantified: seedling height (30 and 34 days after sowing ('Faculta-121')); leaf dry weight in both cultivars (34 days after sowing (forced draught oven 60°C, 24 h)); stem circumference and the number of leaves ('Naama'; 45 days after sowing).

Experimental design and statistical analysis

All experiments were conducted in a randomized fashion design in five replicates. Each replicate consisted of five plants or the leaves of six individual seedlings (dry weight). ELISA values are means of six microtiter wells for each of the bacterial concentrations. All experiments were repeated twice. The results given are the means of the two experiments. Significance is given by $P \leq 0.05$ (dry weight, stem circumference, and number of leaves) and $P \leq 0.001$ (height).

Results and discussion

Many N_2-fixing *Azospirillum* strains are known as plant beneficial bacteria especially to cereals (30, 33). Several explanations for the mode of action of these bacteria towards plants have been proposed. Contribution of N_2-fixation to plant growth was evaluated by numerous authors using indirect methods, i.e., mainly acetylene reduction and ^{15}N-isotope dilution techniques. Although greater N_2-fixation activities were detected in inoculated plants than in noninoculated control plants, no evidence was provided for the contribution of N_2-fixation in the overall events that finally resulted in an increase in several plant growth parameters. It was also suggested from ^{15}N and acetylene reduction data that the increase of N in field-grown wheat plants was not due to N_2-fixation (11, 18). Furthermore, contribution of negligible amounts of fixed N_2 to plants were recorded in wheat and *Setaria* (22, 32). Other free-living N_2-fixing bacteria, such as *Pseudomonas putida*, has also been shown to affect rapeseed seedling growth by means other than N_2-fixation (26).

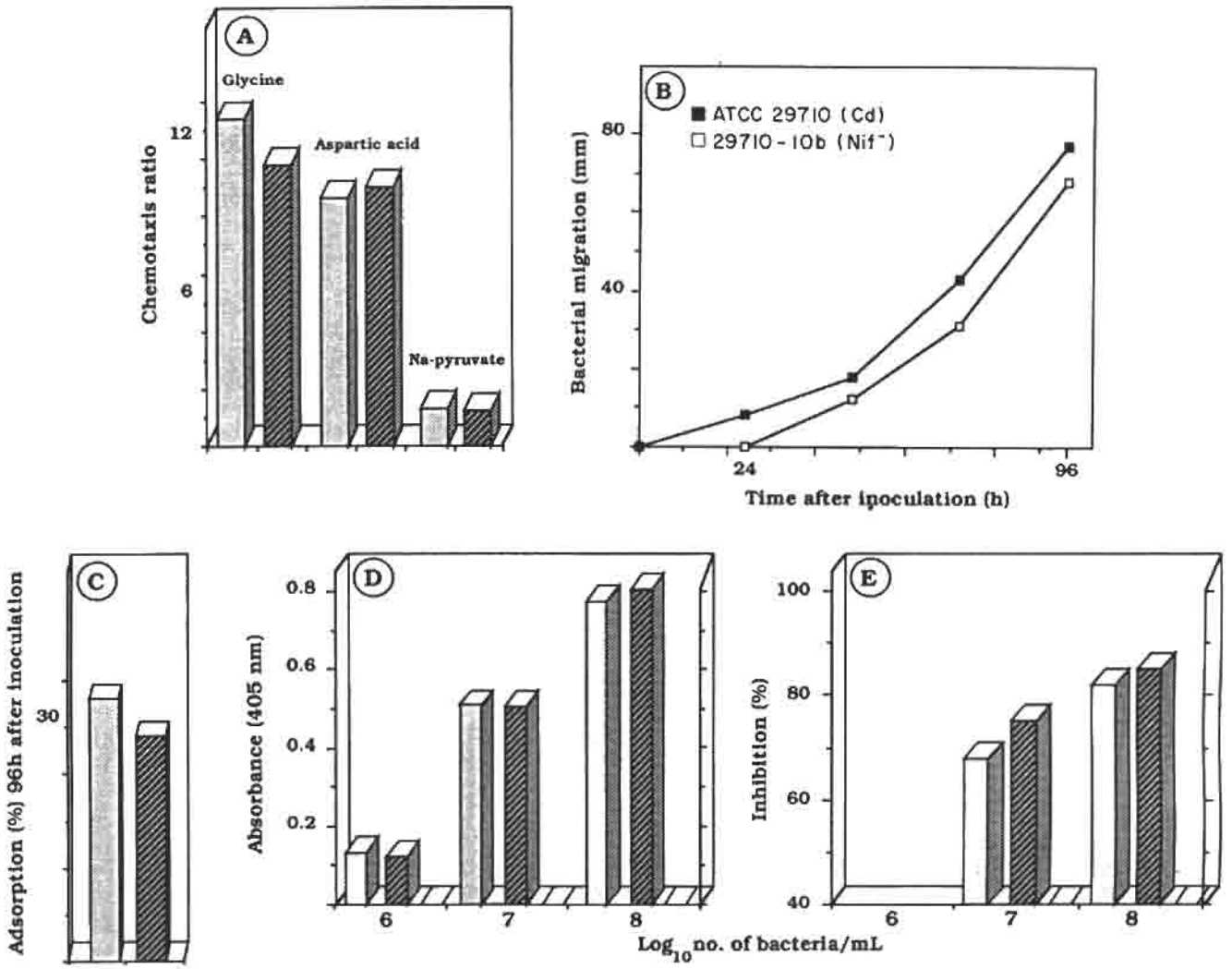

FIG. 2. Chemotaxis of *Azospirillum brasilense* strains (A) in open-channel system toward the attractants glycine and aspartic acid and towards the nonattractant Na-pyruvate; (B) self-motility in quartz sand towards germinating wheat seedlings; (C) attachment to quartz sand; (D) detection by indirect ELISA; and (E) detection by competition ELISA. Stippled bar, ATCC 29710 (Cd); diagonal bar, 29710-10b (Nif⁻); open bar, ATCC 29145 (Sp-7).

The present study evaluated the contribution of N_2-fixation to the growth of tomato seedlings. Tomato plants have been shown to be more sensitive to *Azospirillum* inoculation than cereals (9). The Nif⁻ strain was found to be similar with its parental strain in its basic physiological and ecological features and differed from the wild-type strain only by its complete inability to fix N_2 both in culture medium and when inoculated onto tomato seedlings (Table 1, and Figs. 2A–2C). When the Nif⁻ strain was exposed to highly specific antibodies produced against the parental strain (24, 25), these antibodies did not differentiate between the two strains, indicating no changes in the external antigens between them. Further verification of these results was done by exposing *A. brasilense* Sp-7 (which has similar DNA homology to strain Cd) to these antibodies. No recognition occurred between the antibodies and Sp-7 strain utilizing two different ELISA procedures (Figs. 2D-2E).

The most marked effects of *Azospirillum brasilense* on tomato plant development are the measurable effects on plant growth (9). When inoculated on tomato seedlings, both the wild-type strain (Cd) and its Nif⁻ mutant significantly increased the height of tomato seedlings (up to 25% increase). The difference between response of seedlings towards both strains was not statistically significant, indicating similarity in plant response. Although the magnitude of plant response slightly decreased with time, it was still statistically significantly different from noninoculated plants (Fig. 3A). A similar decrease in plant response towards inoculation with plant age was previously demonstrated for wheat plants (5). When the two strains were evaluated for their effect on dry weight of leaves in two tomato cultivars, the dry weight of leaves over noninoculated plants was significantly increased by both strains, although the increase by the wild type was smaller than of the Nif⁻ mutant (Fig. 3B). In addition, both strains increased stem circumference and the number of leaves of 45-day-old plants over noninoculated plants (Figs. 3C, 3D). No significant statistical difference was found between the effects of both strains. Analysis of tomato root colonization revealed a similar pattern in root colonization, although the wild-type strain (Cd) colonized the roots in slightly higher numbers (Fig. 3E). Thus, these indicate similarities in plant response between the N_2-fixing and the Nif⁻ strains.

Although the mechanism of stimulation of tomato growth by

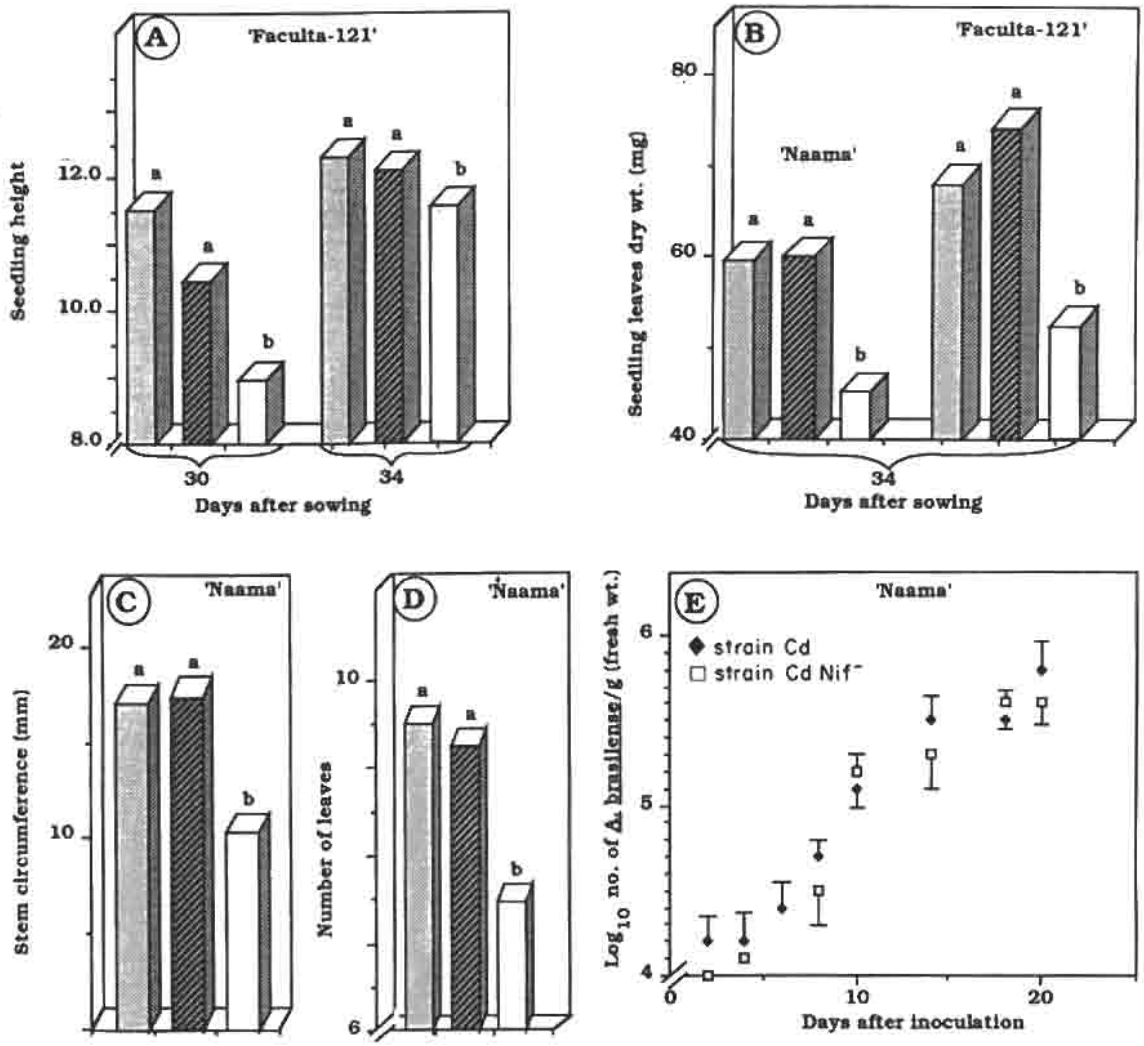

FIG. 3. Effect of inoculation of *A. brasilense* Cd and *A. brasilense* Cd Nif⁻ on (A) tomato seeding height; (B) dry weight of leaves; (C) stem circumference; (D) number of leaves; and (E) changes in population size of *A. brasilense* Cd and *A. brasilense* Cd Nif⁻ in tomato roots following inoculation at sowing. Stippled bar, strain Cd; diagonal bar, strain Cd Nif⁻; open bar, noninoculated plants. In each subfigure, the group of columns representing cultivars or date followed by a different letter differs significantly at $P \leq 0.001$ (A) and $P \leq 0.05$ (B–D). Bars (E) represent SE. Results described in Figs. 3A and 3B were obtained from seedlings grown in peat–vermiculite trays and those of Figs. 3C and 3D were obtained from plants grown for 45 days in 4-L pots containing the same mixture. Root colonization was measured in seedlings grown in quartz sand in trays.

TABLE 1. Characteristics of *Azospirillum brasilense* Cd and *A. brasilense* Cd Nif⁻

Characteristic	*A. brasilense* Cd	*A. brasilense* Cd Nif⁻
Acetylene reduction in culture (μmol C_2H_4/(mg protein · min))	55	0
Acetylene reduction in plants (μmol C_2H_4/(plant · h))	2.1	0
Plasmid pattern	Similar in the two strains	
Nitrogenase structural genes	*nif* HDK present	*nif* DK deleted
Motility	Both strains are highly motile	
Growth on minimal media	+	+
Growth on N-free medium (BL)	+	−
Utilization of NH_4^+ and NO_3^- as sole nitrogen source	+	+
Pink pigmentation	+	+
Colony morphology	Dry with protruding ridges for both strains	
High aggregation at stationary phase of growth	+	+
Absorbance at 540 nm for 10^9 cfu/mL	1.05±0.03	1.05±0.03

Azospirillum has not been identified, these results suggest that the contribution of *A. brasilense* Cd to growth of tomato seedlings in not through the N_2-fixation process.

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