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The *Sesbania* Root Symbionts *Sinorhizobium saheli* and *S. teranga* bv. sesbaniae Can Form Stem Nodules on *Sesbania rostrata*, although They Are Less Adapted to Stem Nodulation than *Azorhizobium caulinodans*

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Sesbania **species can establish symbiotic interactions with rhizobia from two taxonomically distant genera, including the *Sesbania rostrata* stem-nodulating *Azorhizobium* sp. and *Azorhizobium caulinodans* and the newly described *Sinorhizobium saheli* and *Sinorhizobium teranga* bv. sesbaniae, isolated from the roots of various *Sesbania* species. A collection of strains from both groups were analyzed for their symbiotic properties with different *Sesbania* species. *S. saheli* and *S. teranga* bv. sesbaniae strains were found to effectively stem nodulate *Sesbania rostrata*, showing that stem nodulation is not restricted to *Azorhizobium*. Sinorhizobia and azorhizobia, however, exhibited clear differences in other aspects of symbiosis. Unlike *Azorhizobium*, *S. teranga* bv. sesbaniae and *S. saheli* did not induce effective stem nodules on plants previously inoculated on the roots, although stem nodulation was arrested at different stages. For *Sesbania rostrata* root nodulation, *Sinorhizobium* appeared more sensitive than *Azorhizobium* to the presence of combined nitrogen. *S. saheli* and *S. teranga* bv. sesbaniae were effective symbionts with all *Sesbania* species tested, while *Azorhizobium* strains fixed nitrogen only in symbiosis with *Sesbania rostrata*. In a simple screening test, *S. saheli* and *S. teranga* bv. sesbaniae were incapable of asymbiotic nitrogenase activity. Thus, *Azorhizobium* can easily be distinguished from *Sinorhizobium* among *Sesbania* symbionts on the basis of symbiotic and free-living nitrogen fixation. The ability of *Azorhizobium* to overcome the systemic plant control appears to be a stem adaptation function. This last property, together with its host-specific symbiotic nitrogen fixation, makes *Azorhizobium* highly specialized for stem nodulation of the aquatic legume *Sesbania rostrata*.**

Biological nitrogen fixation by legumes is the result of a symbiotic relationship between the host plant and rhizobia. Rhizobia elicit the formation of specialized organs called nodules on their legume host. In nodules, which are usually formed on the roots, rhizobia can reduce N_2 to ammonia, which is then used by the plant as a source of nitrogen. This biological N_2 fixation is exploited in agriculture by using legume crops as an alternative to the application of nitrogen fertilizer.

One of the most exciting recent advances in the agricultural use of biological N_2 fixation is the development of an annual tropical African legume shrub, *Sesbania rostrata*, as a green manure crop for lowland rice. *Sesbania rostrata* grows rapidly in the wet season, producing high levels of biomass even in flooded conditions, and exhibits high rates of nitrogen accumulation, making this species one of the most valuable legumes (17, 21). The genus *Sesbania* includes about 50 species, which are widespread throughout tropical and subtropical areas. Most species are annual or biannual herbs or shrubs (*Sesbania rostrata*, *Sesbania pubescens*, and *Sesbania pachycarpa*), but a few are small trees (*Sesbania grandiflora*, *Sesbania sesban*, and *Sesbania javanica*). Many exhibit potential for use in paddy fields, intercropping, agroforestry, and food production (1).

Except for *Sesbania rostrata*, very little work has been done with rhizobia that nodulate these agriculturally important legumes. *Sesbania rostrata* establishes a highly specific interaction with the genus *Azorhizobium*, which contains only one named species, *Azorhizobium caulinodans* (7, 10), which is phylogenetically separated from other rhizobia (10). This symbiosis exhibits several unusual features (for reviews, see references 2 and 4). Like other rhizobia, *A. caulinodans* induces effective nodules on roots, but it also nodulates the stems of *Sesbania rostrata* at the sites of dormant root primordia (11, 30). Compared to root nodulation, stem nodulation and related N_2 fixation are less inhibited by combined nitrogen (7, 19). *A. caulinodans* also has the unique capacity among rhizobia to fix N_2 in the free-living state and to utilize fixed nitrogen for growth (9).

Sesbania rostrata and other *Sesbania* species also can enter into symbiosis with other rhizobia (10, 25), including the newly described species *Sinorhizobium saheli* and *Sinorhizobium teranga* (5). The latter species has been subdivided into bv. sesbaniae (*Sesbania*-nodulating strains) and bv. acaciae (*Acacia*-nodulating strains) (18). *S. saheli* and *S. teranga* belong to the group containing *Rhizobium meliloti* and *Rhizobium fredii*, which have recently been placed in the genus *Sinorhizobium*, which is phylogenetically distant from *Azorhizobium* (5). The ability of such distantly related bacteria to establish interactions with *Sesbania* species raises the questions of whether sinorhizobia exhibit the unusual characteristics of azorhizobia, i.e., stem nodulation and free-living N_2 fixation, and whether they have similar or specific symbiotic properties. More knowledge of both symbioses may improve the use of *Sesbania* as a green manure. Furthermore, *S. rostrata*, with its dual nodulation topology, offers a unique system for investigating which

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TABLE 1. Wild-type strains used in this study

Strain	Original host plant	Geographical origin	Reference or source
Sinorhizobium saheli			
ORS600	*Sesbania pachycarpa*	Senegal	7
ORS609T	*Sesbania cannabina*	Senegal	7
ORS611	*Sesbania grandiflora*	Senegal	7
Sinorhizobium teranga bv. sesbaniae[a]			
ORS8	*Sesbania rostrata*[b]	Senegal	7
ORS15	*Sesbania* sp.	Senegal	7
ORS19	*Sesbania cannabina*	Senegal	7
ORS22	*Sesbania rostrata*[b]	Senegal	7
ORS51	*Sesbania rostrata*[b]	Senegal	7
ORS52	*Sesbania rostrata*[b]	Senegal	7
ORS53	*Sesbania rostrata*[b]	Senegal	7
ORS604	*Sesbania aculeata*	Senegal	7
ORS613	*Sesbania sesban*	Senegal	7
ORS1013	*Acacia senegal*[c]	Senegal	7
Azorhizobium caulinodans			
ORS571T	*Sesbania rostrata*[d]	Senegal	6
ORS590	*Sesbania rostrata*[d]	Senegal	6
ORS591	*Sesbania rostrata*[d]	Madagascar	6
ORS592	*Sesbania rostrata*[d]	Madagascar	6
Azorhizobium sp.			
ORS56	*Sesbania rostrata*[b]	Senegal	This study
ORS314	*Sesbania rostrata*[d]	Senegal	This study
ORS470	*Sesbania rostrata*[d]	Senegal	This study
ORS478	*Sesbania rostrata*[d]	Senegal	This study
ORS484	*Sesbania rostrata*[d]	Senegal	This study
ORS494	*Sesbania rostrata*[d]	Senegal	This study
ORS500	*Sesbania rostrata*[d]	Senegal	This study
ORS552	*Sesbania rostrata*[d]	Senegal	This study
ORS599	*Sesbania rostrata*[d]	Senegal	This study
SG05[e]	*Sesbania rostrata*[d]	Senegal	29

[a] In *S. teranga*, two biovars, bv. sesbaniae (*Sesbania*-nodulating strains) and bv. acaciae (*Acacia*-nodulating strains) have been distinguished on the basis of host specificity (18).

[b] Strains isolated from root nodules.

[c] ORS1013 originates from *A. senegal* but was shown to be a *Sesbania*-nodulating strain (20).

[d] Strains isolated from stem nodules of *Sesbania rostrata.*

[e] SG05 was shown to belong to a genomic species separate from *A. caulinodans* (25).

bacterial properties are essential for stem nodulation, a property contributing to its considerable N_2 fixing ability (17. To evaluate and compare their symbiotic properties towards *Sesbania* species, a collection of *S. saheli*, *S. teranga* bv. sesbaniae, *A. caulinodans*, and *Azorhizobium* sp. was investigated for stem and root nodulation of *Sesbania rostrata* and for root nodulation of various other *Sesbania* species, as well as for symbiotic and free-living N_2 fixation.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Wild-type strains used in this study are described in Table 1. *Azorhizobium* sp. strains were isolated from naturally occurring *Sesbania rostrata* stem nodules harvested from different regions in Senegal, as already described (10). The taxonomic position of these isolates was assessed by auxanographic tests and whole-cell-protein electrophoretic analysis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), which indicated that they are different strains (14). ORS571*nifA*$^-$, an ineffective mutant of *A. caulinodans* ORS571, has been previously described (15). *Sinorhizobium* strains were grown on yeast extract-mannitol agar (5). Strains of *Azorhizobium* were maintained on solid lactate medium (9), and liquid cultures were grown in solid lactate medium supplemented with nicotinic acid (20 mg/liter). All strains were grown at 37°C. Strains of *S. saheli* and *S. teranga* bv. sesbaniae but not of *Azorhizobium* were resistant to nalidixic acid (200 μg/ml) and erythromycin (100 μg/ml). Strains of *Azorhizobium* and *S. saheli* but not *S. teranga* bv. sesbaniae were resistant to carbenicillin (100 μg/ml).

Nodulation tests. Seeds were surface sterilized by immersion in concentrated sulfuric acid for 30 to 60 min and washed and soaked in water for 24 h. Surface-sterilized seeds were germinated at 30°C for 24 to 48 h and then transferred to Gibson tubes containing nitrogen-free Jensen slant agar (32) and Jensen liquid medium (with or without nitrogen as mentioned below) for root and/or stem nodulation trials; six to eight plants were tested for each strain. The plants were grown under continuous light (20 W/m^2) at 28°C. Sterile Jensen liquid medium was added when necessary.

For root nodulation tests, nitrogen-free liquid Jensen medium was used as plant growth medium, except where stated otherwise, and 3- to 4-day-old seedlings were inoculated by addition of a few drops of exponential-phase liquid culture.

For stem nodulation tests, tubes containing nitrogen-free Jensen slant agar were filled with Jensen liquid medium supplemented with 3 mM NH_4NO_3 before seedling transfer. After 3 weeks, the liquid medium was removed completely and replaced by nitrogen-free Jensen liquid medium. Simultaneously, stems were spray inoculated with exponential-phase liquid cultures. For stem nodulation of previously root-inoculated plants, plants were prepared as for stem nodulation tests. Eighteen days after seedling transfer, liquid medium was completely removed and replaced with nitrogen-free Jensen liquid medium. Simultaneously, roots were inoculated with *A. caulinodans* ORS571. Two days after root inoculation, stems were inoculated as described above. Alternatively, plants were prepared as for root nodulation tests and roots of 4-day-old seedlings were inoculated with *A. caulinodans* ORS571. Next, stem inoculation was performed on 3-week-old plants as mentioned above. In all cases, plants were harvested 3 weeks after stem inoculation and examined for nodules. Effectiveness was estimated by measuring the fresh weights of aerial parts and by visual observation of plant vigor and foliage color of 45- to 60-day-old plants.

Microscopy. Stem nodules of *Sesbania rostrata* inoculated with *S. teranga* ORS604 were collected on days 2, 7, and 14 after inoculation. Nodules were fixed for 1 h in 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer (pH 7.2) and postfixed for 1 h in 1% osmium tetroxide in the same buffer. Fixed samples were then rapidly washed with distilled water and dehydrated through graded ethanol solutions and propylene oxide before being embedded in Spurrs. Spurrs-embedded samples were cut on a Reichert Ultracut ultramicrotome. Semithin sections (0.95 μm) were laid on a slide and stained by the basic fuchsin-methylene blue method (22) before observation by bright-field microscopy.

Asymbiotic nitrogenase activity assays. Rubber-capped tubes (15 ml) containing 6 ml of semisolid LO medium (9) were inoculated by a deep prick method with a fresh culture of bacteria and left for 30 h at 30°C. C_2H_2 was then injected to a 2% final volume in the gas phase. After 6 days, samples of gas were analyzed for C_2H_4 production with a Varian Aerograph model 1400 gas chromatograph with a flame ionization detector.

RESULTS

***S. saheli* and *S. teranga* bv. sesbaniae can nodulate *Sesbania rostrata* on the stem.** Among *Sesbania* species, *Sesbania rostrata* is unique in developing stem nodules at dormant root primordia present in rows along the stem when it is inoculated with *A. caulinodans* (8). The *Sesbania rostrata* stem nodulation ability of rhizobia isolated from *Sesbania* roots and identified as *S. saheli* or *S. teranga* bv. sesbaniae was evaluated. Test strains included both *A. caulinodans* and *Azorhizobium* sp. (Table 1). All the *Sinorhizobium* strains tested were able to nodulate stems of root-uninoculated *Sesbania rostrata* plants grown in nitrogen-free conditions (partially presented in Table 2 and Fig. 1). Bacteria were reisolated from the nodules induced by several different strains of *S. saheli* and *S. teranga* and were shown to correspond to the inoculant strains on the basis of antibiotic resistance (see Materials and Methods), growth on different media, and colony morphology (5, 10). Between strains of the two genera, no significant differences in nodule number, fresh weights (Table 1), or the kinetics of nodule development were observed (data not shown). The cortex of nodules induced by *S. saheli* and *S. teranga* was dark red, indicating the presence of functional leghemoglobin. Two-month-old plants nodulated by either *Sinorhizobium* or *Azorhizobium* strains looked similarly green and healthy, and their aerial parts were of equivalent sizes and fresh weights (data not

TABLE 2. Stem nodulation of *Sesbania rostrata* by *S. saheli*, *S. teranga*, *A. caulinodans*, and *Azorhizobium* sp.[a]

Strain	No. of stem nodules/plant			Stem nodule fresh wt (mg/plant)		
	Without previous root inoculation	After root inoculation by:		Without previous root inoculation	After root inoculation by:	
		Treatment A	Treatment B		Treatment A	Treatment B
S. saheli						
ORS600	10.6[c]	7.4[c]	4.1[c]	109.6[c]	7.0[d]	6.6[d]
ORS609	2.1[c]	4.9[c]	9.5[d]	28.7[c]	4.4[d]	9.8[d]
ORS611	4.2[c]	2.9[c]	9.1[d]	77.4[c]	2.1[d]	11.8[d]
S. teranga bv. sesbaniae						
ORS15	8.3[c]	ND	ND	75.9[c]	ND	ND
ORS19	9.6[c]	1.5[d]	3.9[d]	128.3[c]	1.8[d]	1.8[d]
ORS22	12.1[c]	0.4[d]	3.5[d]	166.8[c]	0.4[d]	6.5[d]
ORS51	16.6[c]	0.0[d]	0.0[d]	69.5[c]	0.0[d]	0.0[d]
ORS52	ND	ND	0.1[d]	ND	ND	0.0[d]
ORS53	10.2[c]	0.0[d]	3.6[d]	63.4[c]	0.0[d]	10.1[d]
ORS604	11.4[c]	0.5[d]	3.4[d]	117.5[c]	0.1[d]	4.4[d]
ORS613	13.5[c]	0.0[d]	1.9[d]	128.8[c]	0.0[d]	0.8[d]
ORS1013	12.3[c]	0.0[d]	4.5[d]	126.3[c]	0.0[d]	4.2[d]
A. caulinodans						
ORS571	8.5[c]	19.4[d]	20.4[d]	117.6[c]	32.6[d]	48.9[d]
ORS590	21.9[c]	ND	ND	156.9[c]	ND	ND
ORS591	14.8[c]	14.5[c]	12.2[c]	83.9[c]	11.9[d]	14.2[d]
ORS592	14.2[c]	17.2[c]	15.9[c]	138.5[c]	23.9[d]	38.1[d]
Azorhizobium sp.						
ORS314	11.8[c]	ND	17.6[c]	119.5[c]	ND	38.6[d]
ORS470	10.1[c]	9.1[c]	12.7[c]	107.2[c]	7.1[d]	20.6[d]
ORS552	13.4[c]	15.6[c]	20.0[c]	135.4[c]	22.4[d]	46.2[d]
ORS599	11.4[c]	10.0[c]	19.4[d]	121.4[c]	14.0[d]	46.6[d]

[a] Stem inoculation was performed on 3-week-old plants, and nodulation ability was observed 3 weeks after stem inoculation, as described in Materials and Methods. Only easily detachable nodular structures were considered nodules, and their numbers and fresh weights were determined. In treatment A, stem inoculation was performed 2 days after root inoculation of 18-day-old plants with ORS571. In treatment B, stem inoculation was performed 20 days after root inoculation of 4-day-old seedlings with ORS571. All numbers are means from eight replications. Stem nodule numbers and stem nodule fresh weights on the same line and followed by different letters are significantly different (Fisher test, $P \leq 0.05$). Independent experiments gave similar results. ND, not determined.

shown). Thus, sinorhizobia and azorhizobia were equally effective.

The organization of young and mature stem nodules induced by *S. teranga* ORS604 was studied. From light microscopy of semithin nodule sections, the infection process was intercellular (crack entry) (Fig. 2B) and revealed the presence of large infection pockets extending inward towards cortical plant cells (Fig. 2B and C). Narrow infection threads originating from the infection pockets grew towards meristematic cells induced in the plant cortex (Fig. 2C). Developing *Sesbania rostrata* stem nodules contained an actively dividing nodule meristem located at the periphery (Fig. 2A and B), an intermediary zone, and a central region corresponding to the N_2-fixing zone (Fig. 2B), an organization similar to that of indeterminate nodules of other plants (31). The central tissue contained both infected and uninfected cells (Fig. 2D). When meristematic activity ceased, 2 weeks after inoculation, mature nodules possessed a large central mass of N_2-fixing cells surrounded by peripheral tissues (data not shown), a histological organization typical of determinate nodules. The peripheral tissues comprised an outer layer of periderm, nodule parenchyma cells, and vascular bundles (data not shown).

Previous root inoculation inhibits stem nodulation by *S. saheli* and *S. teranga* bv. sesbaniae. Stem nodulation requires the development of root primordia on the stem. In the field, root nodulation usually occurs before stem nodulation. We thus studied the effect of root inoculation on subsequent stem nodulation. For this purpose, *S. saheli*, *S. teranga* bv. sesbaniae, *A. caulinodans*, or *Azorhizobium* sp. strains were inoculated on *Sesbania rostrata* stems, either 2 days after root inoculation of 18-day-old plants (treatment A) or 20 days after root inoculation of 4-day-old seedlings (treatment B), with *A. caulinodans* ORS571.

Previous root inoculation had no effect on the formation or the number of stem nodules induced by *Azorhizobium* strains (Table 2). In all cases, 100% of the plants were nodulated and nearly all the nodules fixed N_2, as estimated by the dark-red color of the nodule interior (data not shown). However, a significant reduction of the nodule size (fresh weight) was observed (Table 2).

In the case of *S. teranga* bv. sesbaniae, root inoculation strongly inhibited subsequent stem nodule development (Table 2). When stem inoculation was performed 2 days after root inoculation, most plants formed only swellings at nodulation sites (Fig. 1). Some developed very small nodule-like structures, whose interior was green, indicating that they were ineffective. In treatment B, the number and the sizes of nodule-like structures increased and, rarely, some plants developed nodules harboring the dark-red color characteristic of functional leghemoglobin. *S. saheli* showed little inhibition of stem nodulation (Table 2). However, almost all stem nodules were ineffective, as estimated by their interior color, and had not developed completely. To evaluate the role of symbiotic N_2 fixation in this phenomenon, previous root inoculation was performed with *A. caulinodans* ORS571*nifA*$^-$, an ineffective mutant. ORS571*nifA*$^-$ also inhibited stem nodulation by *S.*

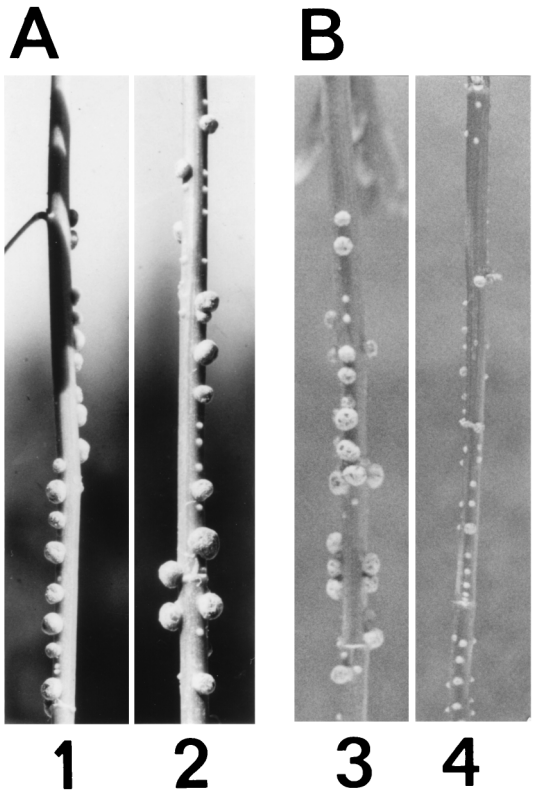

FIG. 1. Stem nodulation of *Sesbania rostrata* by *Azorhizobium* and *Sinorhizobium* strains under different conditions. (A) Three-week-old stem nodules formed on non-root-nodulated plants. Lanes: 1, *Azorhizobium* sp. strain ORS314; 2, *S. teranga* bv. sesbaniae ORS22. (B) Nodules or swellings formed on root-nodulated plants, 3 weeks after stem inoculation (treatment B) (Table 2). Lanes: 3, *A. caulinodans* ORS592; 4, *S. teranga* bv. sesbaniae ORS15.

teranga bv. sesbaniae ORS19 and ORS51 (data not shown). Cultivation of plants in Jensen medium supplemented with 3 mM NH_4NO_3 did not inhibit the induction of stem nodule formation by *S. teranga* bv. sesbaniae ORS51 (data not shown).

Root nodulation of *Sesbania rostrata*. All 11 *Azorhizobium* and 13 *Sinorhizobium* strains tested formed effective root nodules on *Sesbania rostrata*. Both partially and highly effective strains were found in both genera (data not shown). When comparing the two genera in nodulation kinetics over a 15-day period, no differences were observed (Fig. 3). However, 3 mM NH_4NO_3 completely abolished nodulation by sinorhizobia while it caused only a two- to threefold reduction in the number of nodules after inoculation with azorhizobia (Fig. 3).

Depending on the strains, variations in the morphologies and locations of 3- to 5-week-old root nodules were observed. Nodules induced by *Azorhizobium* and *S. saheli* were usually small (1 to 2 mm), dark green, and distributed along the length of the root (Fig. 4). By contrast, nodules induced by *S. teranga* strains were often yellow-green, big, and multilobed (1 to 5 mm) and occurred mostly on upper roots (Fig. 4). Interestingly, a correlation between the sensitivity of stem nodulation to previous root nodulation and the positions and morphologies of root nodules was noted.

Host-specific nitrogen fixation of *Azorhizobium*. A collection of 3 *S. saheli*, 10 *S. teranga*, 4 *A. caulinodans*, and 7 *Azorhizobium* sp. strains was compared for their nodulation and effectiveness on *Sesbania grandiflora* and *Sesbania pubescens*. No difference in nodulation kinetics between sinorhizobia and azorhizobia was observed with *Sesbania pubescens* (data not shown). In contrast, nodulation of *Sesbania grandiflora* by *Azorhizobium* was delayed (about 6 days) and the number of nodules was dramatically reduced when compared to that for *Sinorhizobium* (Fig. 5). Nodules induced on both *Sesbania pubescens* and *Sesbania grandiflora* by all azorhizobia were ineffective as estimated by fresh weight and visual observation of aerial parts (partially shown in Table 3) and the green color of the nodule interior. Bacteria could be reisolated from nodules, indicating that azorhizobia were Inf^+ and Fix^-. By contrast, almost all sinorhizobia tested were effective on both plants (Table 3). To confirm that *Azorhizobium* and *Sinorhizobium* strains differ in their symbiotic nitrogen-fixing abilities, many different *Sesbania* species, mostly originating from Africa or Asia, were inoculated with a collection of azorhizobia and sinorhizobia. The five *Azorhizobium* strains tested induced ineffective nodules on all plants, whereas the three *S. saheli* and the five *S. teranga* strains were effective on almost all plants (Table 3).

Lack of free-living-nitrogen fixation by *S. saheli* and *S. teranga* bv. sesbaniae. Using a simple assay (Materials and Methods), we screened *S. saheli* and *S. teranga* isolates for their ability to fix N_2 under free-living conditions. *A. caulinodans* ORS571 and ORS592 and *Azorhizobium* sp. strains ORS56 and ORS314 served as proficient controls. Only *Azorhizobium* strains exhibited significant nitrogenase activity in pure culture (data not shown).

DISCUSSION

Two taxonomically distant genera, *Azorhizobium* and *Sinorhizobium*, contain strains able to nodulate *Sesbania* species, among them *A. caulinodans*, *Azorhizobium* sp., *S. saheli*, and *S. teranga* bv. sesbaniae. Rhizobia from both groups nodulate stems of *Sesbania rostrata* and roots of all *Sesbania* species tested, but they exhibit clear differences in other aspects of symbiosis.

Previously, nodulation on the *Sesbania rostrata* stem was thought to be restricted to *Azorhizobium* strains, since most nonazorhizobial strains isolated from *Sesbania* were reported to be root specific (10), including the recently described *Rhizobium* sp. strain SIN-1 (24). Rinaudo et al. (25) characterized nonazorhizobial strains able to form stem nodules on *Sesbania rostrata* as very poorly effective or ineffective. Recently, the ability of some *S. teranga* strains to stem nodulate under particular conditions was demonstrated (29). In this work, we showed that, whatever their original host plant, all *S. saheli* and *S. teranga* bv. sesbaniae strains tested nodulate stems as efficiently as do *Azorhizobium* strains, when roots were not previously inoculated (Table 2; Fig. 1). Infection by *S. teranga* bv. sesbaniae ORS604 occurs through intercellular proliferation and formation of intercellular infection pockets extending as intercellular infection threads towards the meristematic zone (Fig. 2). This form of infection is similar to the organogenesis of both *Sesbania rostrata* stem (11, 30) and root (22) nodules after inoculation with *A. caulinodans*. Moreover, early steps of stem nodule development closely resemble those of root nodules initiated by *A. caulinodans* (22) and display characteristics of both indeterminate and determinate nodules.

Unlike *Azorhizobium*, the ability of *Sinorhizobium* strains to form nodules on stems is highly dependent on the nodulation status of the plant. Indeed, whereas stem nodulation by azorhizobia is relatively unaffected by previous root inoculation, stem nodulation by *S. teranga* bv. sesbaniae is strongly inhibited, with most of the strains able to develop only swellings or

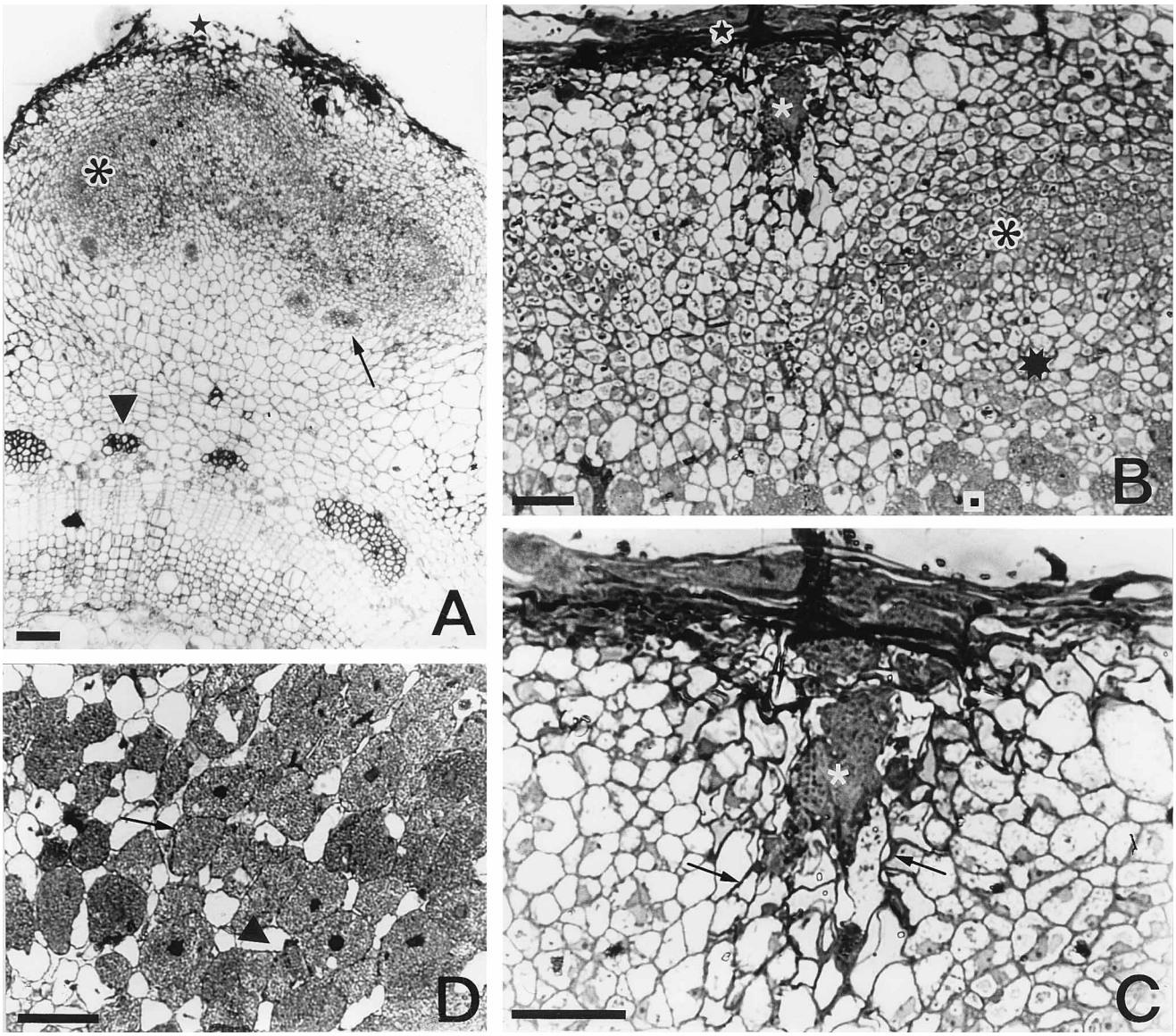

FIG. 2. Light microscopy of semithin sections (width, 0.95 μm) of stem nodules induced by *S. teranga* bv. sesbaniae ORS604 on non-root-nodulated *Sesbania rostrata*. Nodules were fixed 48 h (A) and 7 days (B, C, and D) after inoculation. Bars, 100 μm (A) and 50 μm (B, C, and D). (A) Typical basket structure of the meristem (asterisk) positioned below the original site of infection (star). The arrow indicates the nodule's vascular bundles, and the arrowhead shows the stem's vascular bundles. (B) Section of a developing nodule showing different histological zones from the outside towards the central tissue: the infection zone (five-rayed black star) with the presence of large infection pockets (five-armed white asterisk) extending inwards, the actively dividing nodule meristem (six-armed black asterisk) located at the periphery, the intermediate zone (eight-rayed black star), and the central nitrogen-fixing zone (black square) with the invaded cells. (C) Magnification of panel B showing the spread of infection threads (arrows) originating from the intercellular infection pockets (asterisk) filled with rhizobia. (D) Magnification of panel B showing the central nitrogen-fixing zone comprising both invaded (arrow) and noninvaded cells (arrowhead).

pseudonodules. Stem nodulation by *S. saheli* strains is less sensitive to prior root inoculation, but the nodules formed are generally ineffective. Stem nodulation by *Azorhizobium* under these conditions led only to smaller nodules, probably as a result of the presence of N_2-fixing root nodules. Indeed, Moudiongui et al. (20) observed that combined nitrogen has no significant effect on stem nodule number but inhibits the development of stem nodules, with a strong effect on nodule size and effectiveness. It was recently reported that root nodulation of *Sesbania rostrata* suppresses stem nodulation by *S. teranga* ORS51 and ORS52 but not *A. caulinodans* ORS571 (29). Here, the use of a collection of azorhizobia and sinorhizobia shows that insensitivity to root nodulation is specific to the *Azorhizobium* genus among Senegalese isolates and furthermore indicates that the sensitivity to previous root inoculation increases from *S. saheli* to *S. teranga* bv. sesbaniae. In the field, root inoculation usually occurs prior to stem infection. Despite their ability to nodulate stems, *S. saheli* and *S. teranga* bv. sesbaniae form only about 10% of the naturally occurring stem nodules on *Sesbania rostrata* (26). The density of *Azorhizobium* on stems and leaves of *Sesbania rostrata* was far higher than the density of *Sinorhizobium* (26). We conclude that both the relative insensitivity to root inoculation and the epiphytic survival of azorhizobia may explain their greater competitiveness for stem nodulation. Furthermore, the ability to infect root-nodulated plants may be a stem adaptation function com-

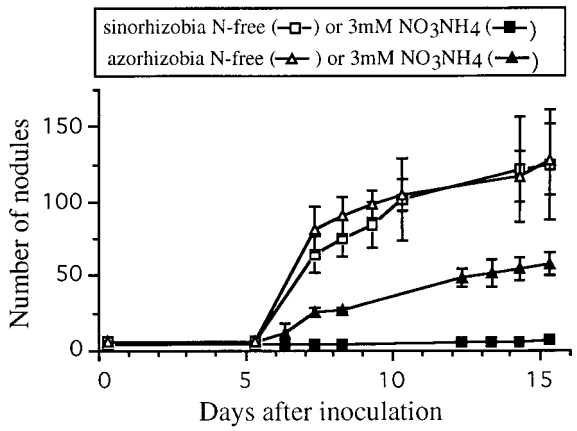

FIG. 3. Nodulation kinetics with *Sinorhizobium* and *Azorhizobium* strains on *Sesbania rostrata* roots. The numbers of nodules correspond to the means of the total sums of nodules present on plants inoculated with sinorhizobia or azorhizobia. Eight plants were tested for each strain. Thirteen *Sinorhizobium* strains (*S. saheli* ORS600, ORS609, and ORS611 and *S. teranga* bv. sesbaniae ORS8, ORS15, ORS19, ORS22, ORS51, ORS52, ORS53, ORS604, ORS613, and ORS1013) and 12 *Azorhizobium* strains (*Azorhizobium* sp. strains ORS56, ORS314, ORS470, ORS484, ORS494, ORS500, ORS552, and ORS599 and *A. caulinodans* ORS571, ORS590, ORS591, and ORS592) were tested when plants were grown in Jensen medium free of nitrogen. Six *Sinorhizobium* strains (*S. saheli* ORS600 and ORS611 and *S. teranga* bv. sesbaniae ORS19, ORS22, ORS51, ORS52, and ORS604) and four *Azorhizobium* strains (*Azorhizobium* sp. strains ORS314 and ORS599 and *A. caulinodans* ORS571 and ORS590) were tested when plants were grown in Jensen liquid medium supplemented with 3 mM NO_3NH_4. Standard deviations are given as error bars.

mon to epiphytic rhizobia, such as *Azorhizobium*. Stem nodulation may be crucial for these aquatic legumes, since, in waterlogged soils, root N_2 fixation is much lower and may be replaced by stem N_2 fixation (6).

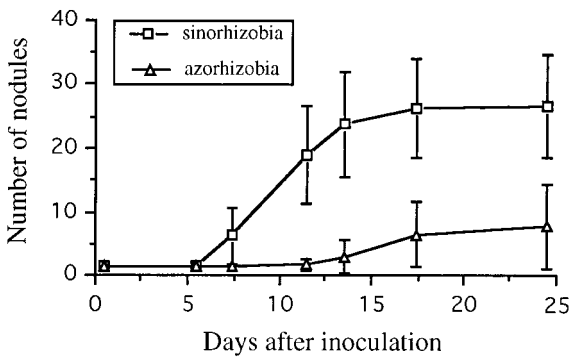

FIG. 5. Nodulation kinetics with *Sinorhizobium* and *Azorhizobium* strains on *Sesbania grandiflora* roots. The numbers of nodules correspond to the means of the total sums of nodules present on plants inoculated with sinorhizobia (13 strains, namely, *S. saheli* ORS600, ORS609, and ORS611 and *S. teranga* bv. sesbaniae ORS8, ORS15, ORS19, ORS22, ORS51, ORS52, ORS53, ORS604, ORS613, and ORS1013, with eight plants for each strain) or with azorhizobia (12 strains, namely, *Azorhizobium* sp. strains ORS56, ORS314, ORS470, ORS484, ORS494, ORS500, ORS552, and ORS599 and *A. caulinodans* ORS571, ORS590, ORS591, and ORS592, with eight plants for each strain). Standard deviations are given as error bars.

Using an ineffective *A. caulinodans* ORS571 derivative as a root inoculum, we showed that N_2 fixation in root nodules does not itself inhibit stem nodulation by *S. teranga* bv. sesbaniae. The control of the number of nodules formed on the overall plant, known as autoregulation of nodulation or systemic suppression of nodulation, has been described for several legumes by the split-root system (see reference 3). Our discovery of natural strains exhibiting different degrees of sensitivity towards the plant systemic control makes the *Sesbania rostrata*-

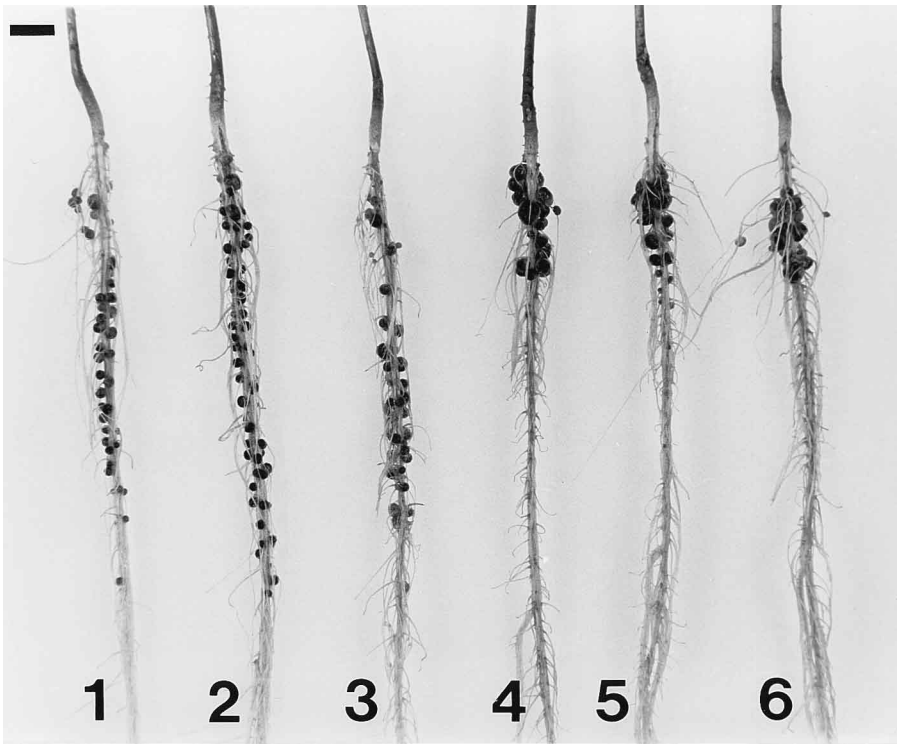

FIG. 4. Morphologies and distribution of *Sesbania rostrata* root nodules induced by *S. saheli*, *S. teranga*, and *A. caulinodans* strains. Lanes: 1, *A. caulinodans* ORS571; 2, *A. caulinodans* ORS590; 3, *S. saheli* ORS611; 4, *S. teranga* bv. sesbaniae ORS19; 5, *S. teranga* bv. sesbaniae ORS22; 6, *S. teranga* bv. sesbaniae ORS604. Bar, 1 cm.

TABLE 3. Root symbiotic N_2 fixation specificity of *Azorhizobium* and *Sinorhizobium* strains among *Sesbania* species

Strain (country of origin)	Symbiotic effectiveness[a] of rhizobial strain:												
	A. caulinodans				*Azorhizobium* sp.	*S. saheli*			*S. teranga* bv. sesbaniae				
	ORS571	ORS590	ORS591	ORS592	ORS489	ORS600	ORS609	ORS611	ORS51	ORS52	ORS53	ORS604	ORS613
Sesbania pubescens (Senegal)	I	I	I	I	I	E	E	E	E	E	E	E	E
Sesbania grandiflora (Thailand)	I	I	I	I	I	E	E	E	E	E	E	E	E
Sesbania aculeata (Ethiopia)	I	I	I	I	I	E	E	I	E	E	E	E	E
Sesbania aculeata (India)	I	I	I	I	I	E	E	E	E	E	E	E	E
Sesbania cannabina (Vietnam)	I	I	I	I	I	E	E	E	E	E	E	E	E
Sesbania pachycarpa (Senegal)	I	I	I	I	I	E	E	E	E	E	E	E	E
Sesbania formosa (India)	I	I	I	I	I	E	E	E	E	E	E	E	E
Sesbania aegyptiaca (Egypt)	I	I	I	I	I	E	E	E	E	E	E	E	E
Sesbania sesban (Kenya)	I	I	I	I	I	E	E	E	E	E	E	E	E
Sesbania sericea (West Indies)	I	I	I	I	I	E	E	E	E	E	E	E	E
Sesbania emerus (East Africa)	I	I	I	I	I	E	E	E	E	E	E	E	E
Sesbania alba (Kenya)	I	I	I	I	I	E	E	E	E	E	E	E	E
Sesbania tetraptera (Madagascar)	I	I	I	I	I	E	E	E	E	E	E	E	E

[a] I, ineffective nodulation; E, effective nodulation, as estimated by plant visualization and determination of aerial part fresh weight 45 days after inoculation.

Azorhizobium or -*Sinorhizobium* system unique for studying the mechanisms involved in the autoregulation of nodulation and in natural stem nodulation.

The greater sensitivity of *S. teranga* bv. sesbaniae strains towards plant nodulation controls may be responsible for the particular distribution and morphology of nodules on *Sesbania rostrata* roots. *S. teranga* develops a few big multilobed nodules, while *Azorhizobium* and *S. saheli* induce many smaller, round nodules along the length of the root (Fig. 4). Root nodule development has been shown to occur through two successive steps, the first one showing indeterminate characteristics and the last one showing determinate characteristics (22). *S. teranga* bv. sesbaniae might prolong the first indeterminate stage, while *Azorhizobium* might quickly progress to the final determinate step. If true, nodule morphogenesis might not be solely under host plant control (16). The supernodulation ability of *Azorhizobium* may contribute to the low percentage, around 50 to 60%, of root nodules occupied by fast-growing rhizobia, which are present in higher densities than azorhizobia in the *Sesbania rostrata* rhizosphere (26).

When compared to that by azorhizobia, nodulation by sinorhizobia was found to be much more sensitive to the presence of fixed nitrogen (3 mM) in the plant culture medium (Fig. 3). The inhibitory effect of nitrogen on root nodulation, also observed in the *Sesbania rostrata-A. caulinodans* system (19), is still poorly understood (27, 28). Ammonium has been shown to regulate the expression of the *nodABC* genes in *Sinorhizobium meliloti* (12), a bacterium closely related to *S. teranga* and *S. saheli* (5). The greater sensitivity of sinorhizobia to nitrogen inhibition of nodule formation could thus reflect a greater influence of nitrogen in nodulation gene regulation compared to that of azorhizobia.

Originally, the new genus *Azorhizobium* and species *caulinodans* were assigned to *Sesbania* symbionts exhibiting three properties: the ability to stem nodulate *Sesbania rostrata*, the ability to fix atmospheric N_2, and the ability to grow while fixing N_2 (10, 13). Our results show that stem nodulation is not restricted to *Sesbania*-azorhizobium symbiosis, although *Azorhizobium* is best adapted to aerial nodulation. In contrast, free-living N_2 fixation remains uniquely typical of *Azorhizobium* among *Sesbania* isolates (10, 25), since none of the *Sinorhizobium* strains exhibits asymbiotic nitrogenase activity (data not shown). Furthermore, azorhizobia fix N_2 only in symbiosis with *Sesbania rostrata* while sinorhizobia are effective with all the *Sesbania* species tested (Table 3). Ex planta and in planta N_2 fixation activity tests, easy to perform under laboratory conditions, might be used to distinguish *Azorhizobium* strains from other *Sesbania* isolates in agro-ecological studies, together with API Systems auxanographic tests (5). This is the first description of two rhizobium genera exhibiting different symbiotic properties and nodulating the same legume.

Azorhizobium is phylogenetically more related to *Xanthobacter* spp. and *Aquabacter* spp., both of which include nonsymbiotic aquatic bacteria, than to other rhizobia (10, 23). *Azorhizobium* is also found in high densities in African temporary ponds, where the aquatic legume *Sesbania rostrata* grows (6). Several other *Sesbania* species, nodulated by the soil-prevalent *Sinorhizobium* strains, thrive on submerged soils near these ponds. In these natural conditions, *Azorhizobium* might have inherited *nod* genes from *Sinorhizobium*, simultaneously evolving into an epiphytic and symbiotic bacterium highly specific to the stem-nodulating legume *Sesbania rostrata*. *Sinorhizobium nod* gene sequencing would support or refute such a hypothesis.

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