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NOTES

Epiphytic Occurrence of *Azorhizobium caulinodans* and Other Rhizobia on Host and Nonhost Legumes

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A large population of *Azorhizobium caulinodans* was present on *Sesbania rostrata*; up to 5×10^{-5} cm^{-2} were found on leaves and fewer were found on flowers. Although *A. caulinodans* was also present on the leaves of *Sesbania aculeata* (nonhost), the populations were much smaller than that observed on *S. rostrata*. The population of *S. aculeata* rhizobia on host leaves was less than 30 cm^{-2}, and their presence on host flowers was sporadic. *Aeschynomene afraspera* and *Aeschynomene aspera* rhizobia, which are profusely stem nodulating, were found on the leaves of host and nonhost plants and on the flowers of host plants, but, *Aeschynomene pratensis* and *Aeschynomene sensitiva* rhizobia were not found on the leaves and flowers of host plants.

Stem nodulation in *Sesbania rostrata* and its potential as a green manure crop were reported a few years ago (2). The organism responsible for stem nodulation was classified as a new species: *Azorhizobium caulinodans* (4). Among the rhizobia, *Azorhizobium* spp. are unique and interesting because they can grow and fix nitrogen under free-living conditions without added combined nitrogen (3).

Recently, Ladha et al. (7) showed that azorhizobia released from stem nodules can survive and grow in flooded soils and in the rhizosphere of wetland rice. The occurrence and growth of rhizobia in the rhizosphere of host and nonhost plants have already been recognized (1, 10). Little is known, however, about the epiphytic occurrence and growth of rhizobia on the aerial parts of host and nonhost plants. Epiphytic survival may be particularly important for legumes such as *S. rostrata* and *Aeschynomene afraspera*, which produce profuse nodules on the aerial parts of the stem. Although it has been reported that azorhizobia were found in the seeds of host plants (8), the source of this contamination was not clear. Epiphytic survival may explain the spontaneous nodulation on the stem commonly observed in the field and the contamination of seeds with azorhizobia.

In this study we examined the presence of *A. caulinodans* and other stem nodule bacteria as epiphytes on host and nonhost leguminous plants grown in the field.

A field plot (200 by 200 m) was used in the 1987 dry season (DS) and 1988 wet seasons (WS) at the International Rice Research Institute Farm. The field was divided into two equal parts. One part was planted with *S. rostrata*, a root- and stem-nodulating species (2), and the other was planted with root-nodulating *S. aculeata* (11). Seeds of both plants were treated with concentrated sulfuric acid for 30 min and rinsed thoroughly with water to favor germination and were broadcast on moist soil at 30 kg/ha. After 2 weeks of growth, the plots were flooded, and the flooding was maintained until harvest.

In the 1988 WS, *S. rostrata* and *S. aculeata* were grown on both upland and wetland fields at the International Rice Research Institute. At the time of sampling, the plants were not flowering.

In another field, several *Sesbania* and *Aeschynomene* spp. were grown in three rows of 4 m, with 60-cm row-to-row spacing. Four stem nodulating *Aeschynomene* spp. (*Aeschynomene afraspera*, *Aeschynomene aspera*, *Aeschynomene pratensis*, and *Aeschynomene sensitiva*) were used during the 1988 WS.

During the experiments, rhizobia were not inoculated but *S. rostrata*, *Aeschynomene afraspera*, and *Aeschynomene aspera* profusely produced nodules on the aerial parts of the stem, while *Aeschynomene pratensis* and *Aeschynomene sensitiva* had scant nodulation on the submerged part of the stem. The *Sesbania* species used were photoperiod sensitive: *S. rostrata* and *S. aculeata* flowered 33 to 37 days after being sown during the short-day period (September to March) and 80 to 132 days after being sown during the long-day period (April to August).

Duplicate composite samples of 10 g each of fresh leaves and flowers were collected from the uppermost parts of several plants per species. Each sample was placed into a sterile 250-ml Erlenmeyer flask, with 1 g of glass beads and 90 ml of sterile 0.02 M phosphate–0.85% saline buffer (pH 7.0). Each flask was shaken on a rotary shaker for 2 h at 120 rpm at room temperature. The supernatant was decanted and serially diluted 10 times with 0.02 M phosphate–0.85% saline buffer to a 10^{-10} dilution. The dilutions were used for most-probable-number (MPN) counting of rhizobia and plate counting of total aerobic, heterotrophic bacteria on 0.1% tryptic soy agar with 200 mg of cycloheximide per liter.

The method described by Ladha et al. (7) was slightly modified for MPN counting. Seeds of *S. rostrata* and *S. aculeata* were first treated with concentrated sulfuric acid for 30 min and then sterilized with 70% (vol/vol) ethanol for 5 min, with 5.25% Saniclor (commercial solution of NaOCl) for 10 min, and with 0.1% acidic $HgCl_2$ solution for 3 min. The seeds were rinsed with sterile distilled water several times and then soaked in sterile distilled water for 12 h. They were transferred to 1% water agar and germinated in the dark at 30°C for 16 to 24 h. Contaminant-free seedlings were

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TABLE 1. Epiphytic occurrence of rhizobia and azorhizobia on host and nonhost plants

Season and symbiont-host combination	Plant species and part	Level of rhizobia on following day after planting:					
		63		90		180	
		Rhizobia/g[a]	Rhizobia/cm^2[b]	Rhizobia/g	Rhizobia/cm^2	Rhizobia/g	Rhizobia/cm^2
1987 DS							
Azorhizobium-S. rostrata	*S. rostrata* leaves	7.6 (73%)[c]	5.7	7.1	5.7	6.2	4.8
Rhizobium-S. aculeata	*S. rostrata* leaves	7.1[d]	5.2	6.4[d]	4.9	4.9[d]	3.5
Azorhizobium-S. rostrata	*S. rostrata* flowers	1.3 (16%)	–[e]	6.3	–	5.1	–
Rhizobium-S. aculeata	*S. rostrata* flowers	ND[f]	–	ND	–	ND	–
Rhizobium-S. aculeata	*S. aculeata* leaves	2.5	1.5	1.8	0.7	1.7	0.6
Azorhizobium-S. rostrata	*S. aculeata* leaves	2.5[d]	1.5	1.1	0.1	3.3	2.2
Rhizobium-S. aculeata	*S. aculeata* flowers	3.3	–	1.6	–	1.0	–
Azorhizobium-S. rostrata	*S. aculeata* flowers	ND	–	4.1	–	0.3	–
1988 WS							
Azorhizobium-S. rostrata	*S. rostrata* leaves	4.8	3.5	6.9 (72%)	5.6	3.2 (37%)	1.9
Rhizobium-S. aculeata	*S. rostrata* leaves	4.8[d]	3.5	3.5[d]	2.1	1.4[d]	0.1
Azorhizobium-S. rostrata	*S. rostrata* flowers	NF[g]	–	2.8 (35%)	ND	1.2 (16%)	–
Rhizobium-S. aculeata	*S. rostrata* flowers	NF	–	ND	ND	1.2[d]	–
Rhizobium-S. aculeata	*S. aculeata* leaves	ND	ND	1.6[d]	0.3	1.4[d]	0.1
Azorhizobium-S. rostrata	*S. aculeata* leaves	ND	ND	3.2	1.9	1.6[d]	0.3
Rhizobium-S. aculeata	*S. aculeata* flowers	NF	–	ND	–	ND	ND
Azorhizobium-S. rostrata	*S. aculeata* flowers	NF	–	ND	–	ND	ND

[a] Log_{10} MPN rhizobia per gram (dry weight) of leaves and flowers.
[b] Log_{10} MPN rhizobia per square centimeter of leaf surface area.
[c] Numbers in parentheses show the rhizobia as a percentage of total aerobic heterotrophs.
[d] Ineffective nodules.
[e] –, Surface area of flower not determined.
[f] ND, Not detected.
[g] NF, No flower.

transferred into tubes (25 by 220 mm) containing a nitrogen-free Jensen (5) agar slant. The tubes were closed with silicone plugs and placed in a growth cabinet (Koito Kogyo Co., Tokyo, Japan) at 30°C and 25 klx for 14 h and 25°C in the dark for 10 h. This light-dark regimen continued for 10 days, after which the tubes containing plants that showed uniform growth were used to inoculate the dilutions. First, 15 ml of Jensen liquid medium was added, followed by 1 ml of each dilution (four tubes per dilution). A few tubes were set without inoculation. After another 2 weeks of growth, nodulation was checked and the MPN was calculated by using Vincent's table (12). After another 4 weeks of growth under the same conditions before inoculation, rhizobia from effective nodules that had formed on *S. rostrata* were isolated on yeast extract-lactate agar (3). The nodules on *S. rostrata* and *S. aculeata* were examined for the reisolated bacteria by using the same method of growing plants.

Aeschynomene species for MPN counting and inoculation were grown in the same way, except that nodulation took longer than for *Sesbania* species.

In both seasons, a large population of *A. caulinodans* was found on the leaves of the homologous host, *S. rostrata*, throughout the experiments. The maximum value reached 5 $\times 10^5$ cm of leaf surface^{-1} or 4 $\times 10^7$ g of dry leaves^{-1} and constituted 37 to 70% of the total epiphytic bacteria (Table 1). The populations were lower in the WS than in the DS. Azorhizobia were also found on the leaves of the heterologous host, *S. aculeata*, although to a lesser extent than on the host leaves. Two periodic countings of *S. rostrata* flowers in the WS and three countings in the DS showed that *A. caulinodans* was present and that it constituted 16 to 35% of the total epiphytic bacteria on the flowers. The *A. caulinodans* populations were larger in the DS than in the WS. *A. caulinodans* was detected on the flowers of *S. aculeata* in the WS only.

S. aculeata rhizobia were found on the leaves of host plants in both seasons, but the populations were quite small (less than 30 MPN · cm^{-2}) and the nodules were ineffective in the WS. On the flowers, *S. aculeata* rhizobia were found only in the DS. Although the leaves of *S. rostrata* also harbored a large number of rhizobia nodulating on *S. aculeata*, the number of rhizobia declined with time and only ineffective rhizobia were found. It is therefore unlikely that this large number reflected large populations of *S. aculeata* rhizobia on the leaves of *S. rostrata*. Populations on *S. rostrata* flowers were counted five times, and *S. aculeata* rhizobia were found only once.

Large populations (2 $\times 10^4$ cm of surface leaf^{-2} or 5 $\times 10^5$ g of dry leaves^{-1}) of *A. caulinodans* were found on the leaves of host plants grown in both lowland and upland fields. However, there was no significant difference in the counts between the two sets of conditions. *S. aculeata* rhizobia were not found on the leaves of host plants grown in upland fields. Even on the leaves of host plants grown in lowland fields, the population was less than 2 MPN · cm^{-2}.

Leaves of *Aeschynomene afraspera* and *Aeschynomene aspera* harbored low densities (10 MPN · cm^{-2}) of their homologous rhizobia. Although the leaves of *Aeschynomene sensitiva* and *Aeschynomene pratensis* did not contain their homologous rhizobia, they harbored a low density (20 MPN · cm^{-2}) of heterologous rhizobia (*Aeschynomene afraspera* and *Aeschynomene aspera* rhizobia). Flowers of

Aeschynomene pratensis and *Aeschynomene sensitiva* harbored neither homologous nor heterologous rhizobia.

These studies showed that *A. caulinodans* organisms are present as epiphytic bacteria on the leaves and flowers of the host, *S. rostrata*, and, to a lesser extent, on the nonhost, *S. aculeata*. The population of *A. caulinodans* on the host leaves was similar to that of N_2-fixing bacteria found on the leaf surfaces of cocoa and citrus plants by Ruinen (9).

The results that (i) the large populations of *A. caulinodans* on the leaves and flowers were maintained throughout the growth of the host and (ii) the population constituted a major fraction of the total epiphytic bacteria rule out the possibility that *Azorhizobium* spp. are simply incidental inhabitants.

Surveys of *Aeschynomene* spp. showed that epiphytic occurrence was observed only for rhizobia of species that produce aerial stem nodules profusely. The ability of *S. aculeata* rhizobia to grow or survive epiphytically on its host leaves may be lower than that of *A. caulinodans*, because their number was much smaller.

Recently, Ladha et al. (8) found a high frequency of azorhizobia on seeds of *S. rostrata*. A pair of stem nodules are often observed on the first node of the stem, while the first leaf is developing. It is likely that these stem nodules are formed by azorhizobia borne on seeds. Mild sterilization of seeds with soft coats is often not effective in removing *A. caulinodans* from the seeds. However, the drastic method of sterilization used in this study was effective. Ladha et al. (8) assumed that the organisms on the seeds could originated from senescence of the nodules. The present study, however, suggests that the presence of rhizobia on the seeds could be due to epiphytic occurrence of the rhizobia on leaves and particularly on flowers.

These results strongly suggest that as a general rule, rhizobia that can form nodules on the aerial stem are epiphytic bacteria. Tuzimura and Watanabe (10) suggested that the rhizospheric growth on the host and nonhost plants is an ecological adaptation for root nodule bacteria. Recently, Ladha et al. (7) also observed the colonization of a large population of *A. caulinodans* in the rhizosphere of wetland rice grown in an *S. rostrata*-rice rotation. In addition to the growth in the rhizosphere, epiphytic growth may be an ecological adaptation for stem nodule bacteria. Because colony types of epiphytic bacteria are slightly different from those of rhizospheric bacteria (6), different taxonomic positions for azorhizobia and common rhizobia may be understandable.

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