Critical Reviews in Plant Sciences, 16(1):1-30 (1997)

Stem Nodulation in Legumes: Diversity, Mechanisms, and Unusual Characteristics

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ABSTRACT: Rhizobia can establish a nitrogen-fixing symbiosis with plants of the Leguminosae family. They elicit on their host plant the formation of new organs, called nodules, which develop on the roots. A few aquatic legumes, however, can form nodules on their stem at dormant root primordia. The stem-nodulating legumes described so far are all members of the genera *Aeschynomene, Sesbania, Neptunia,* and *Discolobium.* Their rhizobial symbionts belong to four genera already described: *Rhizobium, Bradyrhizobium, Sinorhizobium,* and *Azorhizobium.* This review summarizes our current knowledge on most aspects of stem nodulation in legumes, the infection process and nodule development, the characterization and unusual features of the associated bacteria, and the molecular genetics of nodulation. Potential use as green manure in lowland rice of these stem-nodulating legumes, giving them agronomical importance, is also discussed.

KEY WORDS: stem-nodulated legumes, *Sesbania, Aeschynomene, Azorhizobium,* photosynthetic *Bradyrhizobium,* nitrogen fixation.

I. INTRODUCTION

The symbiosis between leguminous plants and soil bacteria of the Rhizobiaceae leads to the formation of nitrogen-fixing nodules, generally exclusively appearing on the roots. A few legume species, however, form nodules not only on their roots, but also at stem-located root primordia. The first example of this phenomenon was first reported in 1928 in *Aeschynomene aspera* L. by Hagerup,[82] and subsequently in 1936 in *A. paniculata* by von Suessenguth and Beyerle.[168] In addition, stem nodulation has been reported in *Neptunia oleracea*[136] and later in five other *Aeschynomene* species (for references, see Table 1).

In 1981, the interest in stem-nodulated legumes and their rhizobia was renewed when Dreyfus and Dommergues[44] reported the discovery of profuse stem nodulation in the fast-growing sahelian annual legume *Sesbania rostrata.* Since then, spontaneous aerial nodulation has also been reported in four other species of *Sesbania,* including *S. punctata,* native in Madagascar and closely related to *S. rostrata,* and in 16 new species of *Aeschynomene* (Table 1). More recently, N_2-fixing nodules have also been found on the stems of the Brazilian legume *Discolobium pulchellum.*[105] All known stem-nodulated legumes belong to only four genera: *Aeschynomene* (22 species), *Discolobium* (1 species), *Neptunia* (1 species), and *Sesbania*

0735-2689/97/$.50

TABLE 1
Geographical Distribution and Grouping of Stem-Nodulating Legumes

Legume	Geographic distribution	Plant group[a]	Ref.
Aeschynomene			
Afraspera	Africa	I	2
Nilotica	Africa	I	2
Aspera	Africa, S. Asia	II	82
Ciliata	Africa, S. America	II	2
Cristata	Africa, Madagascar	II	98
Denticulata	S. America	II	53
Evenia	S. America	II	12
Indica	Pantropical	II	11, 175
Paniculata	S. America	II	168
Pratensis	S. America	II	53
Rudis	S. America	II	53
Scabra	S. America	II	53
Schimperi	Africa	II	2
Sensitiva	Pantropical	II	2, 53
Tambacoundensis	Africa	II	2
Uniflora	Africa	II	98
Villosa	S. America	II	12
Fluminensis	S. America	II	106
Virginica	N. America	II	55
Crassicaulis	Africa	III	2
Elaphroxylon	Africa	III	87
Pfundii	Africa	III	2
Sesbania			
Rostrata	Africa	I	48
Punctata	Madagascar	I	48a
Speciosa	Asia, Africa	II	98
Pubescens	Africa	II	48a
Javanica	S.E. Asia	III	98
Neptunia			
Oleacera	Pantropical	III	136
Discolobium			
Pulchellum	S. America	III	105

[a] Legumes are grouped according to their ability to nodulate on the stem. I, nodulation all along the stem; II, nodulation mostly on the submerged part of the stem but aerial nodulation possible; III, nodulation restricted to the lower and submerged part of the stem.

(5 species) (Table 1). These generally belong to the subfamilies Papilionoideae (*Aeschynomene, Discolobium,* and *Sesbania*) and Mimosoideae (*Neptunia*).[8] *Aeschynomene* and *Discolobium* belong to the same tribe, the Aeschynomeneae.[105] These legumes are mostly annual or perennial herbs or shrubs, and a few of them, such as *A. elaphroxylon* and *D. pulchellum,* are small trees. They share the ability to grow in waterlogged soils, swamps, or riverbanks of tropical regions of Africa, South or Central America, and Asia, with the exception of *A. virginica,*[55] which grows in North America. The adaptation of these plants to flooded habitats has sparked a substantial interest in stem-nodulated legumes, particularly *S. rostrata* and *A. afraspera*[14,47] because these plants can be used

as green manures in lowland rice-growing tropical regions of the world. The symbiosis between *S. rostrata* and its microsymbiont, *Azorhizobium caulinodans*, has been discussed by de Bruijn,[32] and the potential agronomic applications of stem-nodulated legumes used as green manure for rice has been reviewed recently by Ladha et al.[98]

During recent years, the characterization of bacteria able to induce N_2-fixing nodules on different genera and species of legumes has progressed considerably. Rhizobial strains isolated from stem nodules have been shown to be quite diverse and to belong to four genera: *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Azorhizobium*.[38,48,105,174] Moreover, rhizobial molecular genetics and molecular mechanisms leading to nodule formation have been investigated in considerable detail (see Reference 41). The *Sesbania rostrata-A. caulinodans* symbiosis has indeed become one of the most studied models in plant-bacteria interactions.

The aim of this article is to review the current information on most aspects of stem nodulation, that is, the infection and nodulation processes, the characterization and unusual characteristics of the bacteria responsible for these processes, and the molecular genetics of the early steps of the plant-microbe interactions.

II. CLASSIFICATION OF STEM-NODULATING LEGUMES

The most distinctive characteristic of stem-nodulating aquatic legumes is the presence of predetermined nodulation sites on the stem.[46] The formation of these sites is totally independent of rhizobial infection. Nodulation sites correspond to preformed dormant root primordia located on the stem.[158] Anatomical studies have showed that these dormant primordia exhibit a typical root structure, a fact confirmed by the ability of these primordia to develop into adventitious roots when stems are immersed in water.[50,53] Depending on the host plant, the primordia can be distributed over the whole length of the stem or restricted to its lower part. They remain either hidden under the stem cortex, often forming an epidermal dome, or they slightly pierce this structure, showing a protruding dormant apex that forms a circular cavity in the stem epidermis.

Only protruding or scarcely piercing primordia are accessible to rhizobial infection, as bacteria enter the root primordium by intercellular invasion (see Section V). Therefore, the nature and the localization of the stem nodulation site are important factors for host sensitivity to rhizobial infection, and have been used for a distinctive classification of stem-nodulating legumes.[4,46,98] Three groups of plants can be distinguished (Table 1).

Group I comprises aquatic legumes that readily nodulate all along the stem. It includes *S. rostrata* and *S. punctata*, with nodulation sites distributed in three or four vertical rows all along the stem,[44,48,50] and *Aeschynomene afraspera* and *A. nilotica*, with their primordia covering the whole stem. In this group, the root primordium always protrudes through the cortex (epidermis), allowing easy aerial bacterial infection.

Group II, considered as intermediate, comprises some *Aeschynomene* species with primordia less developed than those of group 1. The root primordia, often located next to lenticels, scarcely penetrate the epidermis. Three typical species of this group are *A. indica*, *A. sensitiva*, and *A. scabra*.[2] Their root primordia are still accessible to rhizobial infection, and these species nodulate mostly on the submerged part of the stem, but also on the aerial stem, though less readily than *S. rostrata* and *A. afraspera*. Two species of *Sesbania*, *S. speciosa*[98] and *S. pubescens*,[48a] could also belong to this group because nodulation can be induced on aerial stem portions under high humidity conditions.

In group III, the root primordia remain embedded in the stem cortical tissues as long as the dormancy of the root primordia is not broken by waterlogging. Nodules are never found on aerial stems. Two typical species of this group are *A. elaphroxylon* and *A. crassicaulis*, where stem nodulation is strictly restricted to the lower and submerged part of the stem. *Discolobium pulchellum* belongs to this group as well, because stem and root nodulation require permanent submergence in water.[105] A similar situation occurs in *Neptunia oleracea*,[136] where the primordia, located in the vicinity of the stem nodes, remain embedded in the stem cortex. In this case, flooding is also a prerequisite for induction of the root primordia into typical adventitious roots susceptible to form nodules at their base. In a closely related species, *N. plena*, nodules are also located on adventitious roots arising from the stem,[85,144] and therefore the authors considered these nodules as root nodules and suggested that only nodules that had vascular connections to the stem (submerged or unsubmerged) should be considered as stem nodules.

Adventitious roots can also be induced from root primordia at the base of the stem in other legumes. Nodules formed on these adventitious roots are found around the lower stem portion of some cultivars of both *Arachis hypogaea*[119] and *Vicia faba*,[64] as well as in *Cassia tora* and the tree species *Parkinsonia aculeata*.[177] Waterlogging has been reported to induce nodules on submerged stem portions of *Glycine max*[54] and *Sesbania sesban*.[98] Interestingly, aerial nodulation has also been observed at the base of trunks of the actinorhizal nonlegume tree *Casuarina cunninghamiana*, growing in humidity-saturated areas of Reunion Island.[127]

In conclusion, the unambiguous aerial presence of stem-located root primordia accessible to rhizobial infection could be the key to the classification of stem-nodulating legumes.[46,98] In these conditions, legumes belonging to groups I and II (*Sesbania* and *Aeschynomene*) would be the only species unequivocally classified as true stem-nodulating legumes. The aquatic legumes of group III (*Aeschynomene*, *Discolobium*, and *Neptunia*) could be an intermediate evolutionary stage between true stem-nodulating legumes and other plants that induce typical adventitious roots and should definitely not be considered as stem-nodulating legumes.

III. DIVERSITY AND SPECIFICITY OF STEM-NODULATING RHIZOBIA

The classification of rhizobia, formerly based on the ability to nodulate a particular leguminous host species, is in constant evolution. Since 1988, two new bacterial genera, *Sinorhizobium* and *Azorhizobium*, and several new species have been added to the two existing genera, *Rhizobium* and *Bradyrhizobium*.[38,48,180] This recent classification is based on comparative studies that not only include host specificity, but also universally approved techniques in microbial taxonomy such as auxanographic tests, DNA:DNA and DNA:rRNA hybridizations, whole-cell protein SDS-PAGE, and 16S rRNA sequencing.[78]

Bacteria associated with *D. pulchellum* and *N. oleracea* have not yet been taxonomically characterized. Their only known characteristics are that rhizobia from *N. oleracea* are fast growers,[46] and those from *D. pulchellum* either belong to fast- or slow-growing rhizobia.[105]

In contrast, considerable progress has been made recently in the characterization of stem-nodulating rhizobia associated with both *Sesbania* and *Aeschynomene* species. Most stem-nodulating rhizobia appear to be host specific. For example, no cross-inoculation between rhizobia-nodulating *S. rostrata* and *Aeschynomene* sp. has been shown.[2,46] In this section, we examine the

actual knowledge on the host specificity and characterization of rhizobia from *Sesbania* and *Aeschynomene* species.

A. *Azorhizobium* and *Sinorhizobium* Nodulate *Sesbania rostrata*

The genus *Azorhizobium* was first characterized by a complete taxonomical study of strains isolated from both stem and root nodules of *S. rostrata* growing in different regions of Senegal, by carrying out a numerical analysis of phenotypic features, whole-cell protein SDS-PAGE analysis, and DNA-DNA and DNA-ribosomal RNA hybridizations. Most strains isolated from stem nodules appeared phenotypically and genotypically very distantly related to both *Bradyrhizobium* and *Rhizobium*. They were classified as a new separate genus, *Azorhizobium*, with a single species, *A. caulinodans*, whose closest relative is the chemoautotrophic hydrogen-oxidizing bacteria *Xanthobacter autotrophicus*.[48] A second genomic species has been identified,[132] but has not been named yet because phenotypic and additional genotypic tests remain to be carried out.

A. caulinodans strains have a narrow and specific host range, mainly restricted to the nodulation of *Sesbania* species.[18a] However, they fix nitrogen only in stem or root nodules of *S. rostrata* and *S. punctata*, and thus appear highly specific to these two closely related stem-nodulated *Sesbania*.[18,48a] *A. caulinodans* has been shown to nodulate also *Phaseolus vulgaris* and *Leucaena leucocephala*.[169]

Recently, a collection of root isolates from *S. rostrata* and various other *Sesbania* species growing in Senegal have also been characterized by polyphasic taxonomy, including 16S-rRNA gene sequencing. Two main genotypically and phenotypically distinct groups of *Sesbania* strains were found on the *R. meliloti-R. fredii* rRNA branch, and reclassified within the emended genus *Sinorhizobium*[26] as two new species, *S. teranga* and *S. saheli*.[38] *S. teranga* also comprises strains isolated from *Acacia* species, exhibiting a completely different host range.[107] Like *A. caulinodans*, most *Sinorhizobium* isolates from *Sesbania* exhibit a very narrow host range restricted to the *Sesbania* species. However, they form effective nodules on most *Sesbania* species.[18]

Most *Sinorhizobium* strains isolated from root nodules of *S. rostrata* were first described to be root specific and unable to induce stem nodules.[48] Recently, several reports showed that these strains, although all isolated from root nodules, were also able to form stem nodules, provided the roots were not already nodulated.[18b,156] However, in the natural habit of *S. rostrata*, although 50 to 60% of the root nodules contained *Sinorhizobium* strains, these only formed 10% of the stem nodules; the remaining 90% contained *Azorhizobium* strains.[133] Therefore, *Sinorhizobium* strains may not be considered as typical stem-nodulating bacteria (see Section IV.C).

B. The "Photosynthetic" and Non-"Photosynthetic" *Bradyrhizobium* sp. of *Aeschynomene*

Strains isolated from different *Aeschynomene* species were originally shown to share characteristics of both fast- and slow-growing rhizobia,[2,149] which first suggested that they belonged to a particular taxonomic group of rhizobia.

Recently, a number of rhizobia from *Aeschynomene* have attracted attention because of their unusual ability to produce the photosynthetic pigment bacteriochlorophyll *a* (Bchl *a*) and their photosynthesis activity during heterotrophic growth[55] (see Section IV). The first such "photosynthetic" rhizobium described was strain BTAi1 isolated

by Eaglesham and Szalay[53] from stem nodules of *A. indica*. Only on the basis of the presence of Bchl *a*, the strain BTAi1 has been initially assigned to a new genus, *Photorhizobium thompsonianum*,[55] but Young et al.[179] showed that this strain was very closely related to *Bradyrhizobium japonicum* and *Rhodopseudomonas palustris*, suggesting that BTAi1 should be named *B.* sp. *(Aeschynomene indica)*. Since then, a large number of photosynthetic and nonphotosynthetic rhizobia have been isolated from stem or root nodules of several *Aeschynomene* species and recent progress has been made in the taxonomical characterization of both BChl-synthesizing and nonphotosynthetic rhizobia.

Lorquin et al.[104] first characterized 83 photosynthetic and 43 nonphotosynthetic strains isolated from 11 *Aeschynomene* species. Using a taxonomic analysis based on comparative SDS-Polyacrylamide gel electrophoresis of whole-cell proteins patterns, we confirmed that both photosynthetic and nonphotosynthetic isolates belong to the *Bradyrhizobium* genus.[118] Most of the nonphotosynthetic strains belong to a large cluster that includes the *B. japonicum*-type strain. The photosynthetic strains are mainly grouped in three large, new *Bradyrhizobium* clusters, except the strain BTAi1, which forms a separate small cluster with one photosynthetic strain isolated in Senegal from *A. indica*. Using cellular fatty acid methyl ester analysis and 16S-rRNA sequencing, So et al.[141] have showed that 35 photosynthetic isolates from stem nodules of nine *Aeschynomene* species form a separate subcluster in the *Bradyrhi-zobium* cluster. These results are consistent with those of Wong et al.[174] Indeed, by 16S rRNA gene sequencing, they showed that five photosynthetic strains isolated from four *Aeschynomene* species (*A. afraspera, A. aspera, A. indica,* and *A. nilotica*), growing in different geographical regions, formed a phylogenetically homogeneous *Bradyrhizobium* subcluster. Phenotypic data obtained by Ladha and So[99] are in contradiction with previous results because they concluded that *Aeschynomene* photosynthetic rhizobia formed an homogenous cluster, separate from the genera *Bradyrhizobium, Rhizobium,* and *Azorhizobium*. Such contradictory conclusions raise the necessity to use a polyphasic approach for complete characterization of the large *Bradyrhizobium* genus.

A common feature to all *Aeschynomene* rhizobia is their ability to nodulate both stem and roots of the host species.[2] Three cross-inoculation groups were identified by Alazard[2] among different *Aeschynomene* species.

Nonstem-nodulated *Aeschynomene* species (*A. americana, A. falcata,* and *A. histrix*), and species whose stem nodulation is restricted to the submerged lower part of the stem (*A. elaphroxylon, A. crassicaulis,* and *A. pfundii*), belong to cross-inoculation group 1. Plants of this group are nodulated by nonspecific bradyrhizobia that also form effective nodules on the roots of *Macroptilium atropurpureum* and *Acacia albida*, which are two test host plants for the *Bradyrhizobium* spp. of the cowpea-miscellany group. None of these strains are photosynthetic. Only a few of them also form nodules on cross-inoculation group 2.

Cross-inoculation group 2 (*A. afraspera* and *A. nilotica*) is more specific, and its bradyrhizobia comprise both nonphotosynthetic and photosynthetic strains. Only the photosynthetic strains, unable to nodulate the cowpea-miscellany group 1, can form nodules on cross-inoculation groups 2 and 3. Species such as *A. fluminensis, A. schimperi, A. uniflora,* and *A. villosa* can also be included in this group. They are nodulated by both photosynthetic and nonphotosynthetic bradyrhizobia.[99,106]

Cross-inoculation group 3 (*A. ciliata, A denticulata, A. evenia, A. indica, A. pratensis A. rudis, A. scabra, A. sensitiva,* and *A tambacoundensis*) is nodulated by highly specific bradyrhizobia, mostly photosynthetic

unable to nodulate *Aeschynomene* species of cross-inoculation group 1 and 2. In *A. indica* and *A. sensitiva*, naturally growing in Senegal, 95% of the strains isolated from stem nodules were identified as photosynthetic strains.[104] However, 50% of the nonphotosynthetic strains were isolated by van Berkum et al.[162] from root nodules of *A. indica* grown in soils of different geographical origins.

It is of great significance that photosynthetic strains are exclusively found in groups 2 and 3, which correspond to the true stem-nodulated legumes (see Section II). Unlike nonphotosynthetic isolates, photosynthetic strains are highly host specific, as they never form nodules on nonstem-nodulated *Aeschynomene* or species belonging to cross-inoculation group 1. Thus, the specificity of nodulation in true stem-nodulated *Aeschynomene* species could have concurrently evolved with the rhizobial photosynthesis.

IV. THE UNUSUAL PROPERTIES OF STEM-NODULATING BACTERIA

Compared with other rhizobia, stem-nodulating bacteria have to face a wide variety of conditions under which they must be able to survive and grow, outside and inside the plant stem-nodule. Three important factors notably differ from what has been found in the rhizosphere or in root nodules: the fully aerobic oxygen tension encountered at the stem level, the lack of combined nitrogen to survive on stems or leaves, and the presence of light and plant photosynthesis in the nodule cortex. These different environmental conditions might explain how *Azorhizobium caulinodans* strains of *Sesbania rostrata* and the photosynthetic *Bradyrhizobium* sp. *Aeschynomene* display free-living nitrogen fixation or photosynthesis, which are unique properties among all other symbiotic rhizobia.

A. Free-Living Nitrogen Fixation and Growth on N_2

Most rhizobia only reduce atmospheric N_2 to ammonia during symbiosis when differentiated into bacteroids within the infected plant cells of the nodules. However, a limited number of rhizobia strains, mainly belonging to the genus *Bradyrhizobium*, have been shown to express nitrogenase activity *ex planta* when they were grown in pure culture under very low oxygen tension.[71] Despite their nitrogenase activity in pure culture, none of these strains were able to use the fixed N for their metabolism and still required the addition of combined N for growth. In 1982, we first reported that *Azorhizobium caulinodans* strain ORS571 was not only able to fix nitrogen *ex planta*, but also had the ability, at that time unique among rhizobia, to grow in the free-living state with N_2 as the sole N source.[45,56] Thus, strain ORS571 appears as intermediate between the symbiotic and the free-living N_2-fixing bacteria such as *Klebsiella pneumoniae* and *Azotobacter vinelandii*. Since then, strain ORS571 has been studied as a model for physiological and genetic studies on the nitrogen fixation process and its regulation in rhizobia (see References 31, 32, and 60). The prerequisites for N_2 fixation are a low dissolved O_2 tension (DOT) and the absence of combined nitrogen. In nitrogen-free batches or continuous cultures, nitrogen fixation and growth of strain ORS571 is optimal at a relatively high O_2 concentration (DOT = 3 to 4% or 9 to 15 m*M* dissolved O_2) and temperature (37°C).[20,65,94] For growth in a defined medium, the vitamins biotin, pantothenate, and nicotinic acid are essential.[57] Interestingly, under nitrogen-fixing conditions, strain ORS571 requires about ten times more nicotinic acid for derepression of nitrogenase (0.3 m*M*) than when grown in the presence of ammonia. The dependence of N_2-fixing *Azorhizobium caulinodans* for

nicotinic acid anabolism and catabolism has been investigated in detail.[22,92,93,109,128] First, *A. caulinodans* is auxotrophic for NAD^+ biosynthesis and thus requires nicotinic acid only at micromolar concentrations as an anabolic substrate to synthesize pyridine nucleotides.[92] Second, nicotinate catabolism is not necessary for both the induction of nitrogenase activity[22,92] and growth on N_2,[129] and thus *A. caulinodans* is a true diazotroph. However, higher concentrations of nicotinate indirectly stimulate free-living N_2 fixation by increasing nitrogen pools in culture.[22,92,93]

The nitrogenase has been purified from free-living N_2-fixing cells of ORS571 and has been found to consist of two protein components, a Mo-Fe-protein and a Fe-protein, resembling those observed in other diazotrophs.[94] In the presence of ammonium or glutamine, nitrogenase activity is reversibly inactivated with a "switch on/off" mechanism already described for photosynthetic bacteria such as *Rhodospirillum rubrum* and not demonstrated in other rhizobia. This "switch on/off" control also occurs in the presence of glutamine, but not with ammonia, in a suspension of bacteroids from ORS571 directly extracted from nodules of *S. rostrata*.[94] This mechanism of inactivation has not yet been elucidated, but could involve reversible mono-ADP-ribosylation of the Fe-protein, as described for *R. rubrum* (for a review, see Reference 108) and *Azospirillum brasilense*.[182]

Apart from *Azorhizobium caulinodans*, selected other rhizobial strains are also able to grow on N_2 as the sole N source. With the exception of *Rhizobium leguminosarum* bv. *trifolii* strain 0403,[161] these strains all belong to the photosynthetic *Bradyrhizobium* isolated from stem nodules of different *Aeschynomene* species. Strains ORS310 and ORS322 isolated from stem nodules of *Aeschynomene indica* and *A. afraspera*, respectively, were first shown to exhibit significant growth on N_2, but at a much lower O_2 concentration (DOT = 0.5%) than for strain ORS571.[6] Since then, we found that this property is a widespread feature in photosynthetic *Bradyrhizobium*. Thus, growth on N_2 in culture could be a general characteristic of true aerial rhizobia.

B. Photosynthesis in *Bradyrhizobium*

Strain BTAi1, isolated from stem nodules of *A. indica*, was the first photosynthetic rhizobia described.[55] When grown aerobically under a light-dark cycle (16 h/8 h), strain BTAi1 can synthesize photosynthetic pigments, including both Bchl *a* and carotenoids, and forms photosynthetic reaction centers like those of the purple nonsulfur photosynthetic bacteria, the Rhodospirillaceae.[58] Light-induced CO_2 and light-decreased O_2 uptakes gave evidence of the photosynthetic activity of this strain.[84] Since its photosynthetic apparatus is operative, strain BTAi1 can thus be considered as a "photosynthetic rhizobium". However, unlike purple photosynthetic bacteria that grow photoautotrophically under anaerobic conditions (Pfennig, 1978), the Bchl-containing *Bradyrhizobium* strain BTAi1, (1) like all Rhizobiaceae, is strictly aerobic and (2) performs heterotrophic photosynthesis, as it is unable to grow without an organic carbon source even in the light.[84] Heterotrophic photosynthesis and bacteriochlorophyll synthesis have also been reported in several strict aerobes such as the marine bacteria *Erythrobacter longus* Och101,[80,81,137] *E. sibericus*,[181] *Roseobacter litoralis*, and *R. denitrificans*, formerly *Erythrobacter* sp. Och114,[138] the facultative methylotrophic bacteria assigned to *Methylobacterium* species,[79,90,117,135,160] and *Porphyrobacter neustonensis*.[63]

Several reports have investigated the conditions for Bchl *a* formation in strain

BTAi1.[58,171–173] They have showed that a photoperiod is required for maximal Bchl *a* synthesis. Bchl *a* is formed efficiently in the dark during the light/dark period, following a short light initiation period, but prolonged light exposure and/or high light intensity inhibits pigment accumulation. That could explain the requirement for an intermittent light. During photoperiod conditions, Bchl *a* synthesis is also regulated by oxygen. The cellular bacteriochlorophyll content in BTAi1 is maximum at atmospheric pO_2, and Bchl *a* accumulation is limited by sub- or supraatmospheric oxygen tensions.[173] As in other photosynthetic bacteria, Bchl molecules in strain BTAi1 are bound to proteins that form Bchl-protein complexes. Indeed, membrane preparations from BTAi1 cells have been showed to contain structural and functional components of the photosynthetic apparatus similar to that of many species of purple bacteria. It consists of 80 molecules of light-harvesting complexes per molecule of photochemical reaction center.[58]

Since the discovery of strain BTAi1, a large number of rhizobia isolated from stem nodules of several *Aeschynomene* species have been reported to contain bacteriochlorophyll and light-induced carotenoids, and thus can also be considered as photosynthetic rhizobia.[97,99,104,118,141,162,174] The carotenoid pigments of 83 photosynthetic strains, including strain BTAi1, have been characterized by thin-layer chromatography and total pigment spectra.[104,104a] Spirilloxanthin, which is a common carotenoid in Rhodospirillaceae and Chromatiaceae, was found to be present in all the strains. Some orange-colored strains contained canthaxanthin, which represented 85% of the total carotenoid content of these strains.

Cytological examination of *A. indica* mature stem-nodules revealed the presence of an endophyte coccoid form with an elaborated internal membrane system of "chromatophore-like structures" resembling that of purple photosynthetic bacteria.[61] These endophytes were found to contain a pigment spectroscopically identical to Bchl *a* and to exhibit fluorescence emission and excitation spectra similar to that of the purple photosynthetic bacteria *Rhodospirillum rubrum*. These observations suggested that coccoid endophytes may be photosynthetic. Indeed, bacteroids of strain BTAi1 in mature stem nodules also contain intracytoplasmic membrane-bound vesicles, continuous to each other, quite similar to the intracytoplasmic membrane system of the Rhodospirillaceae and to that of aerobic photosynthetic bacteria.[58] Late log phase cells of BTAi1 culture also form similar vesicles. The photosynthetic pigment synthesis and the presence of light-harvesting complexes is concomitant with membrane-bound vesicle formation.[58,116a]

C. Epiphytic Ecology

The presence of large populations of *S. rostrata*-nodulating rhizobia on leaves and flowers of the host plant were first reported by Adebayo et al.[6] In a recent ecological comparison of the occurrence and distribution of *Azorhizobium* and *Sinorhizobium* in Senegal, *Azorhizobium* was found to be present at higher densities than *Sinorhizobium* on both the stems and leaves of *S. rostrata*, whereas *Sinorhizobium* was more abundant in the rhizosphere of the host plant,[133] indicating that *Azorhizobium* is much more adapted to an epiphytic habitat than *Sinorhizobium*, being more typical of soil bacteria. This adaptation to epiphytic conditions could explain why 90% of the naturally occurring stem nodules of *S. rostrata* were occupied by azorhizobia, while they occupied only 39 to 48% of the root nodules. The same situation might occur with the photosynthetic *Bradyrhizobium* found in great numbers on the leaves of *Aeschynomene* species.[6a]

The adaptation of stem-nodulating rhizobia to the epiphytic habitat of legumes give them an atypical aerial ecological niche among other rhizobia. Therefore, they should be considered as aerial or epiphytic rhizobia, and not as typical soil bacteria, as the other microsymbionts of legumes.

V. INFECTION PROCESS AND STEM NODULE DEVELOPMENT

In root-nodulated legumes, there are two main types of nodules: indeterminate and determinate (for reviews, see References 21, 83, 91, 120, and 124). Indeterminate nodules generally develop on temperate legumes and are characterized by the formation of infection threads through root hairs and by a cylindrical shape due to the persistent apical activity of the nodule meristem.[49,103,153,166] Determinate nodules occur on most tropical legumes. The rhizobia generally infect their host plant intercellularly by direct "crack entry" without formation of infection threads.[7,24,25,85,123] In this case, the nodule meristem activity is transient and the nodule displays the round shape typical of determinate nodules.[124]

A. Nodule Organogenesis in *Sesbania* and *Aeschynomene*

In *S. rostrata,* formation of stem and root nodules has been investigated in detail.[50,51,121,158] Stem and root nodule organogenesis share a number of similarities. Nodulation sites are predetermined and restricted to the cavities formed, either at the base of the stem-located primordia or at the base of lateral roots emerging from the tap root. One day after inoculation, the only observed difference is the absence of root hair induction at the stem nodulation site, compared with that observed at the base of the lateral roots.[121] In both cases, however, the azorhizobia penetrate by direct intercellular infection ("crack entry" of determinate nodules) between the basal cells of the primordium or the lateral root, where they multiply very actively, forming wide intercellular spaces filled with bacteria. Simultaneously, dedifferentiation of cortical cells starts to form the nodule primordium. The large intercellular spaces then extend toward the cortex progressively forming narrow, branched, intercellular infection threads that spread into the meristematic cells induced in the cortex. Azorhizobia are then released from the infection threads into the cell cytoplasm and are surrounded by the peribacteroid membrane. At this early stage of nodule development (4 to 5 d after inoculation), the central infected zone starts to develop the red color characteristic of leghemoglobin and the azorhizobia begin to fix N_2 symbiotically while all stages of symbiont differentiation can still be observed in the same section of a growing nodule.[121] Interestingly, these last features are typical of indeterminate nodules. In *S. rostrata,* however, the meristematic activity, that normally continues for many weeks in indeterminate nodules, abruptly comes to an end after 1 or 2 weeks. The nodules of *S. rostrata* then display the round shape of determinate legumes typical of most tropical legumes (Figure 1A). Differentiation of *Sesbania* stem and root nodules thus appears as intermediate between indeterminate and determinate types of nodule development.

Sesbania stem nodules, and not the dark-developed root nodules, show a green cortex containing chloroplasts closely surrounding the nitrogen-fixing cells (see Reference 32). Proteins involved with O_2 evolution in photosystem II have been shown to be present in the inner and midcortex of the stem nodule.[86] It is suggested that the higher level of intercellular glycoprotein observed in the cortex of aerial nodules could prevent oxygen damage to the nitrogen-fixing zone.[86]

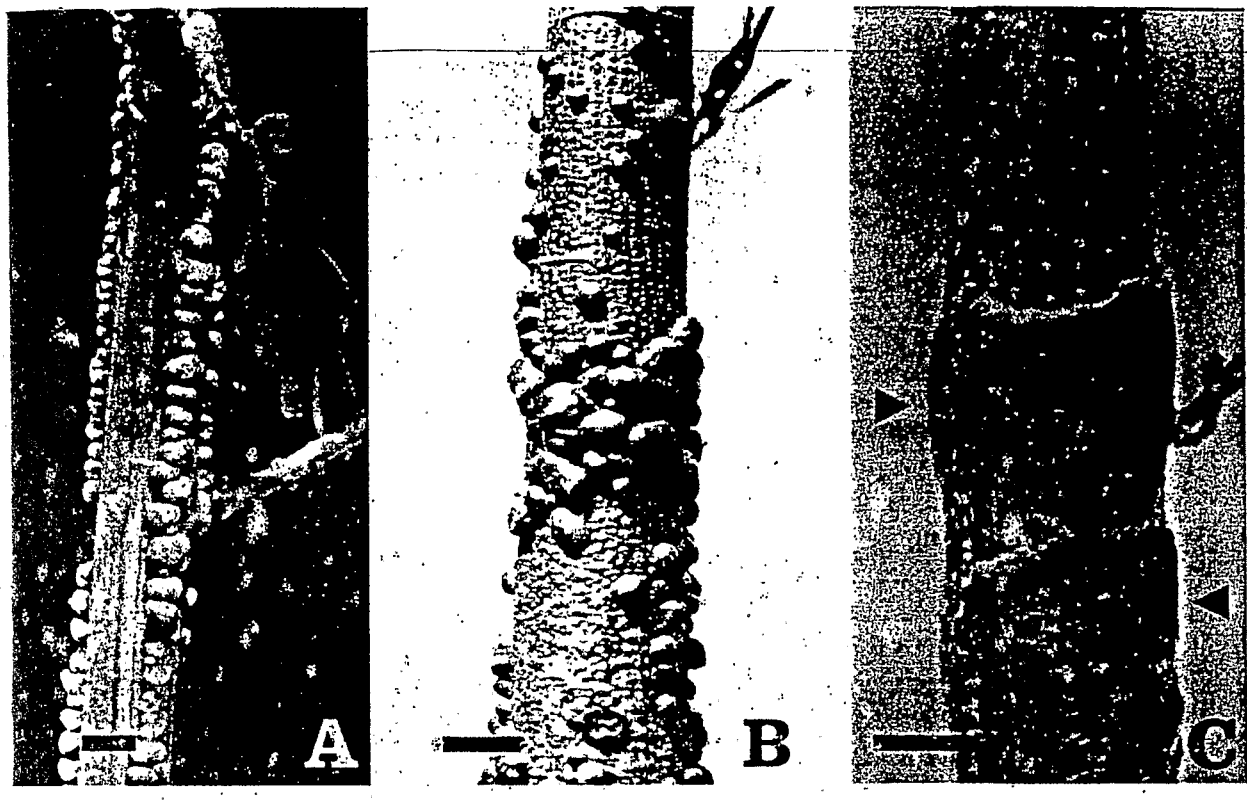


FIGURE 1. Nitrogen-fixing nodules on the stem of *Sesbania rostrata* (A), *Aeschynomene afraspera* (B), and *Aeschynomene sensitiva* (C). The unusual collar nodules around the stem of *A. sensitiva* are indicated by arrows. Bars, 1 cm.

In *Aeschynomene*, stem and root nodules are also very similar in morphology and structure and belong to the typical aeschynomenoid determinate nodule type.[147] Green photosynthetic nodules also characterize stem nodulation. They appear within 8 to 12 d after stem inoculation in *A. denticulata, A. evenia, A. indica, A. pratensis, A. rudis,* and *A. scabra.*[53] They are different from the spherical nodules of *S. rostrata* and form hemispherical or ovoidal swellings under the epidermis, often not easily detachable from the stem (Figure 1B). Among the other *Aeschynomene*, the perennial species *A. sensitiva* bears unusual stem nodules that form a collar around the stem (Figure 1C). These stem collar nodules are probably induced by a continuous meristematic activity in the stem cortex.[104]

Stem infection occurs at the base of root primordia in *A. indica*[167] and *A. afraspera*[5] by direct "crack entry". In *A. afraspera*,[5] invasion of the bacteria progresses intercellularly toward the cortical tissue between cells without formation of infection threads. The bacteria then penetrate into a few inner cortical cells by invagination of the cell wall and actively multiply in these first invaded cells, which enlarge and progressively collapse, forming intercellular infection strand-like structures. Infection then progresses from cell to cell by internal invagination and host cell wall dissolution. Invaded host cells cease to collapse and divide repeatedly to form the central N_2-fixing tissue characterized by the absence of uninfected interstitial cells.

A similar process has been described for root and stem nodules of *A. indica*.[11,167,176] *A. fluminensis* could differ from the usual aeschynomenoid-type because occasional infection threads have been recently reported in root nodules of this species.[106]

Mature nodules of *A. indica* were found to contain two distinct types of bacteroids, the rod-shaped and the spherical form, in the intracellular symbiosomes.[61,62,151] To date, it is not clearly confirmed whether these two types of endophytes correspond to the nonphotosynthetic and photosynthetic forms of the same symbiotic bacteria, respectively (see Section VII). The same observation has been made recently in stem collar nodules of *A. sensitiva*.[52a]

B. Stem Nodulation in *Discolobium* and *Neptunia*

Little information is available on the infection process and nodule development in *D. pulchellum*. However, the formation of infection threads has been observed in both stem and root nodules that only differed by the vasculary connections of the stem nodule to the submerged stem.[105]

The infection process in *N. oleracea* is close to that of *S. rostrata* and proceeds via intercellular infection followed by the formation of large bacteria-filled intercellular spaces from which infection threads spread into the nodule meristematic cells.[136] The vascular bundles of nodules, initiated at the base of adventitious roots arising from the stem, are connected to the vasculature of these roots and not to that of the stem, confirming that they are not true stem nodules.[85] Mature stem nodules of *N. oleracea* belong to the indeterminate type. They are elongated as the result of an apical meristem, measure up to 12 mm long, and never contain chloroplasts in their cortex.

It thus appears that true stem-nodulating legumes can be nodulated via different infection processes and exhibit different nodule developmental patterns. None of them can be considered as stem specific because stem nodulation does not sensibly differ from root nodulation. This may be due to the fact that aerial nodules originate from root primordia resembling normal lateral roots. Morphologically, the so-called stem nodules are root nodules present on the stem. The main differences between stem and root nodules result from the aerial stem environment, which could involve the adaptation of the microsymbionts more than that of the host plant itself.

VI. MOLECULAR GENETICS OF STEM NODULATION

Rhizobia interact with leguminous plants to form nitrogen-fixing nodules through a process that requires the induction and expression of specific genes in both plant (nodulin genes) and microsymbiont (nodulation and fixation genes). Molecular mechanisms in the early steps of the interaction have been partly elucidated, as infection, nodulation and host specificity have been demonstrated to be largely controlled by signal exchanges between the two symbionts. Legumes secrete phenolic inducing compounds (mainly flavonoids) perceived by the bacterium, which in turn responds by producing specific signal molecules, the Nod factors, that trigger the nodule developmental program (reviewed in References 40 and 59). Nodulation signal molecules isolated from various rhizobial species have a very similar lipooligosaccharide core, with species-specific modifications, some of which have been correlated with the determination of host specificity (see References 39, 41, 139, and 142). These lipochitooligosaccharides acting as mediators in plant morphogenesis may represent a new

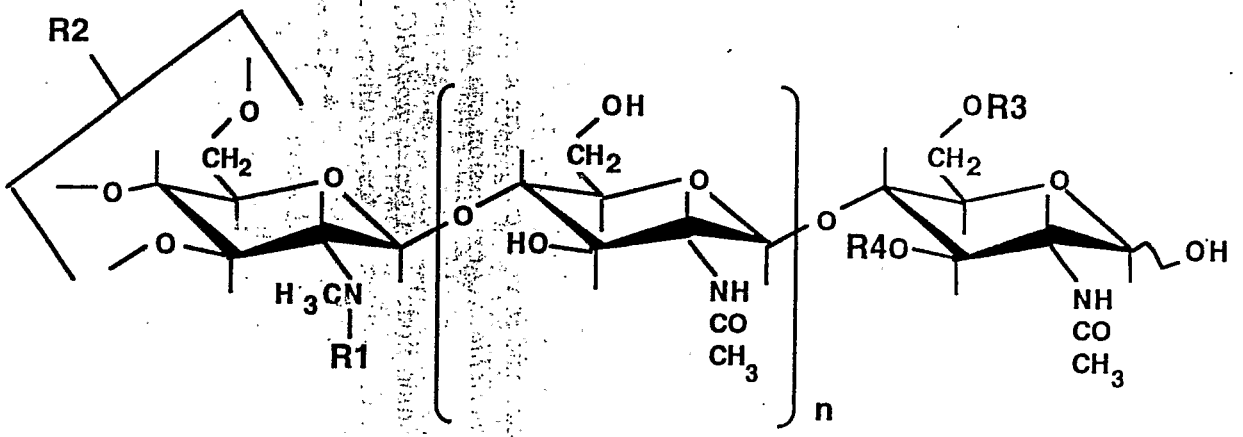
class of plant growth regulators (see Reference 41) and have thus been actively studied since their discovery.[101]

The symbiosis between aerial rhizobia and true stem-nodulating legumes represents an interesting system for studying the molecular basis of bacterium-plant interactions, particularly in two promising directions: (1) the molecular mechanisms of recognition, infection, and nodule development in stem nodulation and (2) the evolutionary origin of nodulation genes of the diazotrophic *Azorhizobium* or photosynthetic *Bradyrhizobium*. As the molecular analysis has almost exclusively dealt with the *A. caulinodans*-*S. rostrata* interaction, we mainly present results on the molecular genetics of nodulation of this symbiosis, with references to the *Bradyrhizobium*-*Aeschynomene* symbiosis when available.

A. Nodulation Factors: Characterization and Biosynthesis

Nod factors have been identified from a genetically modified derivative of the stem-nodulating *A. caulinodans* strain ORS571 (NodARc). They are chitin tetramers or pentamers, carrying a vaccenoyl or a stearoyl acyl chain and bearing a *N*-methyl and *O*-carbamoyl substitution at the nonreducing end. An unusual modification, a D-arabinose, has been found on the reducing end in 30% of the molecules[113] (Figure 2). At concentrations varying from 10^{-6} to 10^{-9} *M*, NodARc induces morphological changes in the host plant, such as the formation of deformed root hairs, cellular divisions, and rare nodule-like structures, that have not been demonstrated to be complete nodular structures, as shown for *Rhizobium meliloti* and *Bradyrhizobium japonicum* signaling compounds.[148,157] Recently, *Sinorhizobium saheli* strain ORS611, *S. teranga* strain ORS604,[104a] and *A. caulinodans* wild-type strain ORS571[115a] have been found to synthesize a population of Nod factors harboring both an arabinosyl group and a fucosyl substitution at the reducing end of the molecules (Figure 2), thus suggesting a strong selection pressure in favor of these substitutions for *Sesbania* symbionts. This illustrates the fact that Nod factor structure is not determined by the taxonomic position of rhizobia, but seems to result from host plant adaptation.[111]

A set of *Azorhizobium* genes involved in Nod factor biosynthesis, and previously identified as nodulation genes, has been characterized. First, *A. caulinodans* harbors a single functional *nodD* gene that is constitutively expressed and regulates other *nod* gene expression in conjunction with the host plant liquiritigenin or the related flavanone naringenin.[72,116] As in other rhizobia, the structural *nod* genes can be divided into two classes: (1) the common genes identified in all the rhizobia studied so far and (2) the specific genes present only in certain species and in various combinations. The biochemical functions of several structural *A. caulinodans nod* gene products have been proposed from a mutant and *in vitro* enzymatic studies and in *Escherichia coli* expression and/or sequence comparisons to known enzymes. The common nodulation genes, *nodABC*, determine the synthesis of the lipooligosaccharide core: *nodC* codes for a *N*-acetyl-glucosaminyltransferase responsible for the oligomerization of the chitooligosaccharide backbone,[70,115] *NodB* removes the *N*-acetyl moiety from the nonreducing end, and *nodA* codes for the *N*-acylation of the nonreducing end.[115] The specific *nodSU* and *nodZ* genes code for modifications of the Nod factor core: *nodS* is a *S*-adenosyl-L-methionine-dependent methyltransferase that *N*-methylates chitooligosaccharides deacetylated at the nonreducing end by NodB,[66,68,115] *nodU* is involved in 6-*O*-carbamoylation of Nod factors, and *nodZ* and/or downstream genes may be involved in 6-*O*-arabinosylation and 3-*O*-



Rhizobial species	n	R1	R2	R3/R4	Reference
A. caulinodans (modified strain)	2,3	C18:1, C18:0	Cb (O-6), H	D-Ara, H / H	Mergaert et al., 93
S. saheli, S. teranga, and *A. caulinodans* (wild-type strain)	2,3	C18:1, C18:0, C16:0	Cb	D-Ara , H/ Fuc, H *	J. Lorquin, unpublished P. Mergaert, unpublished

* *In S. saheli* and *S. teranga* C-3 and C-6 respectively bear either one fucose or one D-arabinose, the exact location of these groups being yet unknown. Cb = Carbamoyl, D-Ara = D-Arabinose, Fuc = Fucose.

FIGURE 2. Structure of Nod factors produced by *Sesbania* symbionts.

fucosylation.[83a] It is worth noting that the homologous counterpart of the azorhizobial *nodZ* gene in *B. japonicum* controls the 6-*O*-fucosylation of Nod factors.[145] A new symbiotic gene, *nolK*, shows homology to NAD/NADP-binding sugar epimerase/dehydrogenases and may also play a role in the glycosylation of NodARc.[75] Differences in Nod factor structures observed between the wild-type strain ORS571 and ORS571 (pRG70), the modified strain originally used for Nod factor chemical analysis, are presumably caused by gene dosage effects, and this provides an indication for a role of the *nolK* locus, absent in pRG70, in fucosylation.[83a]

The regulatory and common genes, absolutely required for NodARc synthesis, are essential for root as well as stem nodulation. Insertion mutants in the *nodS*, *nodU*, and *nodZ* genes show only a delayed nodulation phenotype,[66,68] indicating that these nodulation genes, involved in the specific modification of the core molecule, are apparently not essential for *S. rostrata* nodulation. A *nodS*, but not a *nodU* mutation results in a Nod$^-$ phenotype on both *Leucaena* and *Phaseolus*, indicating that the azorhizobial *nodS* is required for the nodulation of these legumes, a situation similar to that observed in *R. tropici* and *Rhizobium* sp. NGR234.[169] It is surprising that mutations in genes presumably specifying the arabinosylation and fucosylation of the molecule (*nodZ* or genes downstream), which is characteristic of *Sesbania* symbionts, do not have a drastic effect on host specificity. Because *A. caulinodans* is able to infect a variety of *Sesbania* species, some specific genes could mainly play a role in some of these host plant nodulations. Indeed, a *nolK* mutant has been shown to be affected in the root nodulation of *S. formosa*.[75] Also, possible functional reiterations of *nod* genes should not be excluded, so it appears that determinants for host specificity in the *Sesbania*-rhizobia symbiosis remain to be fully elucidated.

As described above, developmental patterns of root and stem nodulation are quite similar and the molecular mechanisms governing stem nodulation generally are those that control root nodulation as well. In support of that, the recently demonstrated ability of *Sinorhizobia* to nodulate both the stem and root of *S. rostrata* tends to exclude the existence of specific determinants involved in stem vs. root nodulation. However, it was recently shown that, unlike most *Sinorhizobium* strains, *Azorhizobium* strains were able to nodulate the stem of already root-nodulated *S. rostrata*.[18b,156] Such a property may be critical for epiphytic bacteria in the field where the root has been nodulated previously to the stem. We suggest that this is a stem-nodulation adaptation function common to naturally occurring stem-nodule rhizobia, such as azorhizobia. Introduction of the *nodD* gene of *A. caulinodans* diminished the sensitivity of *S. teranga* to previous root nodulation, suggesting that *nod* gene expression and/or Nod factor biosynthesis are involved in this phenomenon.[156] Therefore, because of their dual nodulation topology, the stem-nodulated legumes remain of interest as experimental systems for the analysis of the systemic suppression of nodulation.

Bradyrhizobium strains isolated from *Acacia albida*,[52] which also nodulate *Aeschynomene afraspera* and *A. elaphroxylon*,[18b] produce a family of Nod factors consisting of a lipo-pentasaccharide chain with a *N*-methyl and one or two carbamoyls at the reducing end and an *O*-methyl-fucose, sulfated or not sulfated, at the nonreducing end.[58a] These structures are close to those produced by *Rhizobium* sp. NGR234 and *B. japonicum*, which, however, do not nodulate *Aeschynomene* species. The structural requirements for *Aeschynomene* nodulation are still completely unknown. Quantitative variation in Nod factors production seems to influence host range[130,139] and may perhaps partly explain the host specificity diversity

of rhizobia and bradyrhizobia synthesizing similar Nod factors.

B. Organization and Phylogeny of *nod* Genes

Three *nod* loci have been described that are dispersed over the *Azorhizobium* strain ORS571 chromosome.[72,73,75,165] One locus contains the regulatory *nodD* gene, the second contains the common and specific genes *nodABCSUIJZorf9* organized in one operon, and the last locus contains the gene *nolK*, the exact function of which remains unknown. The *nod* sequences are characterized by a GC content of approximately 55%,[66,73] in contrast with the higher overall GC content of the chromosome (67%).[48] Moreover, the three loci are flanked by repetitive elements that are reiterated in the ORS571 genome, some of which have homology to insertion elements.[67,74] As *A. caulinodans* is taxonomically more related to the diazotrophic *Xanthobacter* than to any other rhizobia,[48] *Azorhizobium* could either have evolved from a diazotrophic to a symbiotic bacteria or be a primitive form of rhizobia. The above observations suggest that *A. caulinodans* acquired *nod* genes by horizontal transfer. The two *nod* boxes preceding the two inducible *nod* loci display about 50% homology to each other, suggesting that the loci were acquired independently from different strains.[74] It was recently shown that *nod* gene transfer in *R. loti*, which also possesses chromosomal nodulation genes, occurs in the field after a few years.[152] Lateral gene transfer between rhizobia has already been suggested because the phylogeny of 16SRNA of rhizobia in general does not relate to their host specificity,[125] nor to the phylogeny of NodD protein.[178,180] A good correlation was found between phylogenetic trees constructed from *nodC* sequences and host plant leghemoglobin, suggesting a host legume-rhizobium nodulation function coevolution.[159] Although there is a significant homology between the ORS571 *nod* genes and their counterparts in *Rhizobium* and *Bradyrhizobium*, the *Azorhizobium* sequences are the most divergent described so far,[72,73,159] suggesting that the genes had been acquired long ago. Interestingly, two of the repeated elements associated to the *Azorhizobium* nodulation genes show homology with genetic elements of *R. meliloti* and *R. fredii*,[74] two species of the *Sinorhizobium* branch.[38] *S. teranga* and *S. saheli* isolated from *Sesbania* exhibit the same narrow host range, produce very similar Nod factors, and live in the same tropical area. It would thus be very interesting to compare their *nod* genes to the azorhizobial one's and evaluate whether horizontal transfer could have occurred between these two distantly related genera.

C. Plant Gene Expression during Nodule Development

Plant genes that are specifically expressed during symbiosis are called nodulin genes. In early studies, we demonstrated developmentally regulated, nodule-specific plant gene expression during symbiosis. At least 26 polypeptides are *de novo* synthesized or stimulated in both root and stem nodules.[37] A few early nodulin (*Enod*) genes, expressed during the early steps of symbiosis (infection and nodule formation), have now been identified in *S. rostrata: Enod2*[36,150] and five partial cDNA clones corresponding to novel nodulin genes.[76]

Enod2 is a well-characterized early nodulin gene, found highly conserved in a variety of legumes,[77] that is activated even in the absence of infecting rhizobia in the nodule inner cortex (parenchyma layer), a tissue that is postulated to act as a barrier to limit

O_2 diffusion toward the nitrogen fixation zone.[163] The *Enod2* gene encodes a proline-rich protein strongly resembling (hydroxy) proline-rich (glyco)proteins identified in the cell walls of plants (see References 77 and 120). Dehio and de Bruijn[36] cloned and sequenced the *Enod2* gene of *S. rostra* (*SrEnod2*) and showed differential expression of this gene in root vs. stem nodules. Van de Wiel et al.[163,164] suggested that the transient expression of *Enod2* in root nodules could be the result of early degradation of the nodule parenchyma, while the stable *Enod2* expression in stem nodules could reflect a continuous need for an effective O_2 barrier in these aerial nodules.[33,120] Using *SrEnod2* as a probe, Dehio and de Bruijn[36] showed that *SrEnod2* gene expression is specifically induced in the roots of *S. rostrata* plantlets treated with different types of cytokinins and in stems of *S. rostrata* via *Agrobacterium*-mediated transformation, suggesting that *Enod2* expression is linked to cytokinin action. Rhizobial Nod factors were suggested to act by modifying the auxin/cytokinin balance because cytokinin compounds blocking auxin transport and Nod factors both induce cortical cell divisions in roots.[41] Until now, no obvious link has been found between Nod factor signaling and *Enod2* expression.[34]

More recently, Goormachtig et al.[76] used the differential display technique to isolate five partial cDNA clones corresponding to novel genes, the expression of which is specifically induced or enhanced in stem-located root primordia after rhizobial infection. The expression of some of these genes depends on Nod factor signaling. DNA sequences of these clones revealed significant homology with either hydroxyproline-rich glycoproteins genes, class III chitinase genes, chalcone reductase genes, or the soybean nodulin cDNA GmN#93.[76] The roles of these proteins are as yet unknown, but have been speculated to be implicated in the hypersensitive response in nodulation, nodule development, or Nod factor activity.[114] It has been suggested that chitinases are involved in the autoregulation of nodulation (see Reference 112), as such enzymes can hydrolyze Nod factors, thus altering their biological activity.[146] The active concentration of Nod factors could thus result from both Nod factor production and degradation. *A. caulinodans* and *S. teranga*, whose ability to induce subsequent nodulation on *S. rostrata* is very different, were reported to induce different root nodule-specific plant gene expression.[37] It would thus be interesting to identify the function of nodulins whose expression specifically depends on infection by *A. caulinodans* or *S. teranga*.

D. *Sesbania rostrata* Mutants

Following ethyl methane sulfonate (EMS) mutagenesis of *S. rostrata* seeds, we isolated a pleiotropic mutant that lacked stem-nodulation sites and exhibited a modified root morphology and growth.[143,155] This mutant forms nodules on roots exclusively. Cytological studies of the mutant stem could not reveal any primordium hidden in the cortical tissue of the stem.[143] Classical genetic studies of this mutant indicate that the mutation is dominant and that the lack of stem-located root primordia is controlled by one main gene. However, the existence of phenotypes with very few primordia suggested that this character could be controlled by one main and several minor genes.[100a] The mutation could not be complemented by reciprocal wild-type/mutant-type cross-graftings.[143] Analysis of *in vitro* translation products by 2D-PAGE evidenced differential gene expression between mutant and wild-type phenotypes.[38a]

Recently, Joshua and Ramani[89] reported the isolation, via γ-ray mutagenesis, of a *S.*

rostrata mutant with an extended vegetative phase. Such a mutant could be of great importance for agronomic applications.

VII. POTENTIAL OF STEM-NODULATED LEGUMES AND THEIR BACTERIAL SYMBIONTS IN RICE PRODUCTION

A. *Sesbania* and *Aeschynomene* as Green Manure in Lowland Rice

Most aspects of the agronomic applications of stem nodulation have already been reviewed by several authors[14,47,98,131] and therefore are not developed in detail in this review. The high nitrogen-fixing potential of stem-nodulating legumes such as *S. rostrata* and *Aeschynomene afraspera* as rice green manure has been demonstrated to significantly increase lowland rice yield and contribute to soil fertility and sustainability in rain-fed areas. Tolerance to soil flooding and fast growth rates also make these species extremely valuable legumes in these areas.[3,13,122] *S. rostrata* and *A. afraspera* have the potential to supply wetland soils with considerable amounts of N after green manuring.[43] Up to 200 kg of N per hectare may be accumulated in 6 to 8 weeks of growth, with a major portion of the N derived from biological nitrogen fixation (BNF), when grown on suitable soils under appropriate day length.[13,17,69,98] The estimated equivalence of mineral fertilizer (amount of split applied urea N required to obtain equivalent yield) of *S. rostrata* and *A. afraspera* green manure range from 80 to 100 kg/ha. At these current input levels, the green manure N-use efficiency (kilograms of rice grain yield increase per kilogram of N added) with *S. rostrata* and *A. afraspera* is higher than that of mineral fertilizer N.[15,42,98] Moreover, rice grain yield due to green manuring can be more than doubled, as reported from Asia and Africa.[17,23,98,122]

However, most of the use of these green manures has largely been limited to research and demonstration trials,[17] and the main question remains whether stem-nodulating legume green manure technology can be adopted by small farmers and used on a large-scale in rice-farming systems. Several key agronomic and economical constraints responsible for the limited farm-level adoption of green manure technology have been identified.[9,16,17,122,140,170] In most Asian countries, however, the use of green manures could remain limited, as a majority of small farmers can now buy chemical fertilizers for their rice despite the traditional use of legumes as green manure.

The situation is different in Africa, where most small farmers cannot buy chemical fertilizers and therefore could widely benefit from the use of stem-nodulating legumes. African farmers have little experience with green manure use,[134] and lowland systems are usually not mechanized, making adoption of stem-nodulating green manure technology difficult in many cases. Nevertheless, as a result of extensive efforts recently made by a large number of small farmer organizations in West Africa, the use of *S. rostrata* could rapidly develop on a large scale in the traditional rice-based systems of the region.

B. Rice Stem-Nodulating Rhizobia Endophytic Associations

In the protective nodule environment, nitrogen fixed by rhizobia directly benefits the host plant. In contrast, associations between rice and free-living diazotrophs are much less efficient, as there is an intensive competition in rhizosphere colonization and nitrogen fixed by associative bacteria can be rapidly lost, mainly by denitrification in the rhizosphere. The interest in extension of symbiotic or at least endophytic nitrogen

fixation to rice is now renewed by the considerable progress made in elucidating the molecular basis of symbiotic plant microbe interactions. Furthermore, it was recently discovered that abundant populations of endophytic nitrogen-fixing bacteria within the roots and aerial tissues of sugarcane probably contribute to the important biological nitrogen fixation of this gramineae.[19] It was thus suggested that improvement and/or modification of existing endophytic associations of nitrogen-fixing bacteria with rice plants would be a reasonable way to increase the BNF contribution to wetland rice.[19] Another promising approach could be the selection of nitrogen-fixing rhizobia able to colonize rice tissues. Because of their "primitive" features (N_2 free-living fixation, O_2 tolerance, photosynthesis, and stem and root invasion by crack entry) and their aquatic environment, stem-nodulating bacteria appear as the best candidates for such studies. Indeed, they have already been tested for their ability to invade and induce nodule-like structures on cereals (see References 35 and 100).

The first interaction of stem-nodulating rhizobia with rice was reported by Ladha et al.,[96] who observed, after the use of *S. rostrata* as green manure in paddy fields, that the rice rhizosphere harbored large populations of *Azorhizobium caulinodans* ORS571. High nitrogenase activity in rice rhizospheres inoculated with ORS571, comparable to that obtained with the rhizospheric free-living bacteria *Azospirillum lipoferum*, was also detected. Nitrogen fixation by *Azorhizobium caulinodans* in paranodules induced on wheat roots has also been reported.[27,154] A high-frequency induction of nodule-like structures on rice roots by rhizobia of *S. cannabina* was also claimed,[88,102] but these reports were controversial.[35] These last authors concluded that only modified lateral root meristem develops on healthy rice plants, which are invaded by populations of endorhizosphere-colonizing bacteria, without the host exhibiting obvious symptoms of disease. Recently, Cocking[30] confirmed previous observations that two stem-nodulating strains, *A. caulinodans* ORS571 and ORS310, a photosynthetic *Bradyrhizobium* strain from *Aeschynomene indica*,[4] both isolated in Senegal, were able to invade the rice roots and induce the formation of short, thickened lateral roots resembling nodular structures without any previous enzyme or hormone treatment.[29,30] The bacteria enter the root system of rice through the root epidermis by "crack entry", where the lateral roots emerge. The formation of pockets of intercellular rhizobia and the progressive invasion of rhizobia into meristematic cells were also observed.[10,28,29] Such first steps in the infection process resemble those already described for *S. rostrata* and *A. indica* (see Section V).

The multiplication of stem-nodulating rhizobia in the rice rhizosphere could be interpreted as a survival strategy for bacteria living in aquatic environments where rice and stem-nodulated legumes can thrive together. More interesting is the induction of nodule-like structures on rice roots, which appears as the first step of a bacterial infection, but not as a symbiosis so far. Future and independent research will have to confirm these observations, determine the molecular mechanisms leading to these structures, and improve such rice-endophytic rhizobia associations.

VIII. CONCLUDING REMARKS

The natural habitat of stem-nodulated legumes is restricted to tropical waterlogged or very humid soils. Such an aquatic environment has led to an evolutionary adaptation of both partners of this peculiar symbiosis, the leguminous plant and the rhizobia.

The aquatic legumes are unusual hosts because they produce primordia all along their stems, which present typical root struc-

tures and can develop either into adventitious roots when immersed in water or into stem nodules after infection by rhizobia. Therefore, it is not surprising that stem nodules, originating from stem-located root tissues, resemble the typical root nodule organization and structure. The main difference between stem and root nodules results from the presence of chlorophyll-containing chloroplasts in the green stem nodule cortex. Thus, stem nodules of *Sesbania* and *Aeschynomene* species might fix significant CO_2 and supplement their energy supply by their own photosynthetic activity.

Such an aerial and photosynthetic environment, however, may require a greater adaptation of the microsymbionts than that of the host plant itself. Indeed, the stem-nodulating rhizobia appear unique among all other known rhizobia, the *Azorhizobium* from *S. rostrata* being diazotrophic and the *Bradyrhizobium* from *Aeschynomene* being capable of heterotrophic photosynthesis. Phylogenetic investigations of these two bacterial genera have shown that (1) the *Azorhizobium* are related to the autotrophic bacteria *Xanthobacter*, able to grow chemoautotrophically in the presence of H_2 and CO_2, and (2) the *Bradyrhizobium* are closely related to the photosynthetic purple nonsulfur bacteria, which grow photoautotrophically under anaerobic conditions. Interestingly, both *Xanthobacter* and photosynthetic bacteria are rather common in nitrogen- and carbon-deficient tropical freshwaters, and may be considered as the ancestors of the true stem-nodulating rhizobia. Autotrophic photosynthesis and CO_2 autotrophy may have become less important for the survival of these primitive rhizobia by virtue of the carbon sources provided for growth and reproduction through symbiosis. Therefore, these properties may have been lost during the evolution from free-living life to symbiosis. However, the remaining genetic information for heterotrophic photosynthesis and diazotrophy could still be considered as a selective advantage, making the rhizobia less dependent on the host plant. In symbiosis, bacterial photosynthesis could allow more efficient interaction by diminishing the need of carbon for the microsymbiont. In the soil or on the plant surface, nitrogen fixation and growth at the expense of the fixed nitrogen at relatively high oxygen concentration, as well as bacterial photosynthesis, could sustain a better growth and survival of the bacteria and give a competitive advantage for stem nodulation.

The fact that rhizobia do not form a discrete clade but are phylogenetically intermixed with nonsymbiotic bacteria such as *Agrobacterium* or *Rhodopseudomonas*[110] may be the consequence of nodulation gene transfer as a result of plant-bacteria coevolution. The knowledge of the minimal information needed to transfer nodulation ability to *Xanthobacter* or *Rhodopseudomonas* could allow the creation of true photosynthetic or highly O_2-tolerant diazotrophic rhizobia. Such qualified rhizobia could be of great interest not only in symbiosis with legumes, but also in endophytic associations with major crops such as rice.

ACKNOWLEDGMENTS

We thank all our co-workers who gave us preprints and information about their unpublished work. We are indebted to Dr. Marcelle Holsters and Scott Wettlaufer for critical reading of the manuscript.

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