TROPICAL AND SUBTROPICAL ACTINORHIZAL PLANTS

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ABSTRACT - Most tropical countries face two major problems: increasing overexploitation of wood reserves for timber or fuel wood and diminishing soil fertility for agricultural production. The present consensus is that N_2-fixing plants can help prevent the forthcoming wood and food crisis. Together with the vast group of N_2-fixing Leguminoseae, actinorhizal plants (AP) can play a major role in wood production and land regeneration. AP comprise eight families of dicotyledonous plants all of which are represented in the tropics. In this paper the most promising tropical AP are briefly presented and the emphasis is put mainly on Casuarinaceae. This family forms a group of four genera (*Casuarina, Allocasuarina, Gymnostoma* and genus C) with 82 species, including many trees that can yield wood of good quality. The *Frankia* strains that have been isolated from Casuarinaceae grow slowly and are able to reduce acetylene *in vitro* under air, which indicates that the protection against O_2 is much more efficient in *Frankia* than in rhizobia. Only the species belonging to the *Casuarina* genus could be nodulated by strain ORS 021001, isolated from *Casuarina junghuhniana*, suggesting the existence of cross-inoculation groups within the family Casuarinaceae. N_2 fixation of *Casuarina equisetifolia*, grown in 1 m^2 microplots at the ORSTOM experimental station of Dakar, was estimated by comparing total N in non-inoculated plants and plants inoculated with *Frankia* strain ORS 021001. Extrapolating to 1 hectare, N_2 fixation was 131 kg for a 6.5-month period. Using the N fertilizer equivalence method, inoculation appeared to be equivalent to the application of 288 kg of N fertilizer per hectare.

Index terms: *Frankia*, nodules, N_2-fixing trees.

PLANTAS ACTINORRÍZICAS TROPICAIS E SUBTROPICAIS

RESUMO - A maioria dos países tropicais enfrenta dois grandes problemas: o aumento constante da exploração das reservas florestais para extração de madeira ou lenha e a diminuição da fertilidade do solo para produção agrícola. O conceito geral é o de que plantas fixadoras de N_2 podem evitar a propalada crise de madeira e alimentos. Juntas com um grande número de leguminosas fixadoras de N_2, plantas actinorrízicas podem ter um papel importante na produção de madeira e recuperação de solos. As plantas actinorrízicas compreendem oito famílias de dicotiledôneas, todas elas representadas nos trópicos. Neste trabalho, as mais promissoras plantas actinorrízicas tropicais são brevemente apresentadas e a ênfase é dada principalmente à família Casuarinaceae. Esta família compreende quatro gêneros (*Casuarina, Allocasuarina, Gymnostoma* e gênero C), com 82 espécies, incluindo muitas árvores que podem produzir madeira de boa qualidade. As estirpes de *Frankia* que têm sido isoladas das Casuarinaceae crescem lentamente e são capazes de reduzir acetileno *in vitro* sob ar, o que indica uma capacidade de proteção contra o O_2 muito mais eficiente do que no *Rhizobium*. Apenas as espécies do gênero *Casuarina* podem ser noduladas pela estirpe ORS 021001, isolada de *Casuarina junghuhniana*, o

que sugere a existência de grupos de inoculação dentro da família Casuarinaceae. A fixação de N_2 de *Casuarina equisetifolia*, crescida em microparcelas de 1 m^2, na estação experimental de ORSTM, em Dakar, foi estimada através da comparação do N total em plantas não-inoculadas e plantas inoculadas com a estirpe ORS 021001. Extrapolando para um hectare, a fixação de N_2 foi de 131 kg/N em um período de 6,5 meses. Usando o método de equivalência com adubo nitrogenado, a inoculação parece ser equivalente à aplicação de 288 kg de N/ha^{-1}.

Termos para indexação: *Frankia*, nódulos, árvores fixadoras de N_2.

INTRODUCTION

Compared with the vast group of N_2-fixing Leguminoseae, the actinorrhizal non-legumes form a much less numerous body of N_2-fixing species. In spite of this numeric inferiority, actinorrhizal plants (AP) are of considerable fundamental and practical significance for at least three reasons:

1. The ability of AP is not restricted to one family but expands to eight families, indicating that the plants ability to be symbiotically associated with a N_2-fixing endophyte is more widely spread when the endophyte is an actinomycete.

2. Most AP are good and sometimes agressive colonizers, capable of regenerating poor soils or disturbed sites, the surface of which is rapidly increasing in many tropical countries.

3. Tropical AP, especially *Casuarina*, produce not only timber but also firewood and charcoal; they provide shelter to the cattle and are used for amenity purposes; in many instances they contribute most effectively to the protection and regeneration of endangered or impoverished soils. With the exception of *Casuarina* and *Myrica*, the AP have been considered as restricted to temperate and cold areas but now that more scientists have become interested in tropical AP, it is probable that a number of new species and genera will be discovered as bearing actinorrhizal nodules. Opportunities for the discovery of new AP exist especially in countries where no serious surveys have been undertaken, tropical Africa and Madagascar for instance. Some families like the Rhamnaceae are most promising as reservoirs of AP (Bond & Wheeler 1980).

Table 1 is a tentative list of AP described up to now in tropical and subtropical areas including tropical highlands. Most of the information is retrieved from the last major reviews (Silvester 1977, Bond & Wheeler 1980, Akkermans & Roelofsen 1980, Becking 1982), updated with more recent unpublished and published data. In the first of this presentation we shall deal with some of the best known tropical and subtropical AP (Casuarinaceae excepted). The second part will be devoted to the most important family of Casuarinaceae.

TROPICAL AND SUBTROPICAL AP, CASUARINACEAE EXCEPTED

In this section we shall only present the genera and species of known AP which appear to be most promising or about which unpublished information has been obtained since Becking's 1982 review.

TABLE 1. Genera and distribution of tropical and subtropical species of actinorrhizal plants.

Family	Genus	Main known species	Distribution
Casuarinaceae	*Casuarina*		Tropical and subtropical
	Allocasuarina (see text)		Australia; Indo-Pacific areas
	Gymnostoma		from India to Polynesia
Myricaceae	*Myrica*	*M. javanica*	Mountains of Indonesia and Philippines
		M. cacuminis	Mountains of Cuba
		M. punctata	id
		M. shaferi	id
Betulaceae	*Alnus*	*A. jorullensis*	South America
		A. nepalensis	Nepal
		A. japonica	Mountains, Asia
		A. maritima	id
Elaeagnaceae	*Eleagnus*	*E. latifolia*	Indonesia
		E. conferta	id
		E. philippensis	Philippines
Rhamnaceae	*Ceanothus*	*C. prostratus*	Pacific North American Coast to Mexico
	Discaria	*D. americana*	South America
		D. serratifolia	Argentina
		D. trinervis	
		D. nana	
	Colettia	*C. paradoxa*	South America
		C. infausta	
		C. spinosissima	
		C. armata (= spinosa)	
	Trevoa	*T. trinervis*	Chile
	Talguenea		Chile
	Kentrothamus		Chile
Coriariaceae	*Coriaria*	*C. japonica*	Mountains of Asia
		C. nepalensis	id
		C. sinica	China (Hunan)
Rosaceae	*Rubus*	*R. ellipticus*	Continental Asia, Sri Lanka, Luzon, Indonesia
	Cowania	*C. mexicana*	California, Mexico
	Dryas	*Dryas* sp.	Mountains of Asia
	Cercocarpus	*C. ledifolius*	California
Datiscaceae	*Datisca*	*D. cannabina*	Mediterranean area to Central Asia
		D. glomerata	SW and NW Mexico

Mainly according to Bond & Wheeler (1980) and Becking (1982).

Myrica

The *Myrica* genus is famous because *Frankia* was first isolated from a *Myrica, Myrica asplenifolia* (= *Comptonia peregrina*). In their last review Bond & Wheeler (1980) list 25 nodulated species. Among these species, *M. javanica* has been reported to naturally thrive in the highlands of Indonesia and the Philippines (Aspiras et al. 1981, Becking 1966). Three other species have been recently described as bearing nodules: *M. cacuminis, M. punctata* and *M. shaferi,* which grow in higher regions in Cuba (Merca-

do 1978). According to J. Bosser (personal communication), there are four endemic species of *Myrica* in Madagascar, the two main representatives of the genus being *M. spatulata*, which colonizes the coastal sand of the East of Madagascar, and *M. phillyreifolia* (vernacular names: tsilaky, tahisihady) which grows in marshes and along the rivers in the forests in the center of the Island. In the Comoro Islands another species, *M. dentulata*, is known to grow on higher lands at the border of the forest. The nodulation of these species has not yet been looked for, but taking into account their colonization ability and the fact that nodulation is a generic character in all AP investigated so far, one can predict without much risk of error that the *Myrica* from Madagascar and the Comoro Islands belong to the AP group. It is interesting to note that *M. spatulata* grows at sea level, that is in a hot and humid climate, whereas other *Myrica* species (Akkermans & Dijk 1981) tend to be restricted to higher, thus colder areas.

Alnus

Whereas the genus *Alnus* with 34 nodulated species (Bond & Wheeler 1980) is well represented in temperate regions, fewer species occur in the tropics. *Alnus jorullensis*, which is native to Columbia, is considered a valuable wood in South America. Other tropical species of *Alnus* are worth being mentioned, namely *A. nepalensis* (higher lands of S.E. Asia), *A. japonica* and *A. maritima* that have been successfully introduced in the highlands of Indonesia and the Philippines (Becking 1982).

Elaeagnus

The list of 17 nodulated species established by Bond & Wheeler (1980) comprises two tropical species: *E. latifolia* and *E. conferta*, which reportedly occur in Java (Becking 1979). Another tropical species was found to be nodulated, *E. philippensis* (Aspiras et al. 1981).

Colletia

The genus *Colletia* (Rhamnaceae) comprises 17 shrubby species occurring in South America. Among them at least four are known to be nodulated: *Colletia infausta* (Bond & Becking 1982), *C. paradoxa* (= *C. cruciata*) (Bond 1976, Medan & Tortosa 1976), *C. spinosa* (= *C. armata*) (Bond & Becking 1982) and *C. spinosissima* (Medan & Tortosa 1976).

In collaboration with Dr. L. Frioni from the University of Rio Cuarto, Argentina, we have initiated a study of this last species which occurs in Brazil, Chile, Peru and Argentina. Seeds were obtained from individual plants growing under semi-arid conditions (rainfall: 600 mm) in a very poor, stony soil close to the Embalse dam on Rio Tercero. The endophyte, *Frankia* strain ORS 060501, was isolated and shown to be infective and effective. *In vitro* this strain forms vesicles and sporangia. When derepressed in a N-free medium it reduces acetylene under air like strains of *Frankia* isolated from other hosts and described by Gauthier et al. (1981a) and Tjepkema et al. (1980). But in contrast with the latter strains, ORS 060501 was not able to grow on N_2 as the sole nitrogen source on usual media. Like nodules of most AP, nodules of *C. spinosissima* contain vesicles (diameter 2-3 μm) with septae (Fig. 1b), which have already been described by Bond & Becking (1982).

Coriaria

Three out of the 15 species of *Coriaria* are known to be nodulated: *C. japonica* (Kataoka 1930), *C. nepalensis* (Canizo & Rodriguez-Barrueco 1978) and *C. sinica* (Watanabe, Hunan Soil and Fertilizer Institute, personal communications 1980). The two former species are found at higher elevations in Asia.
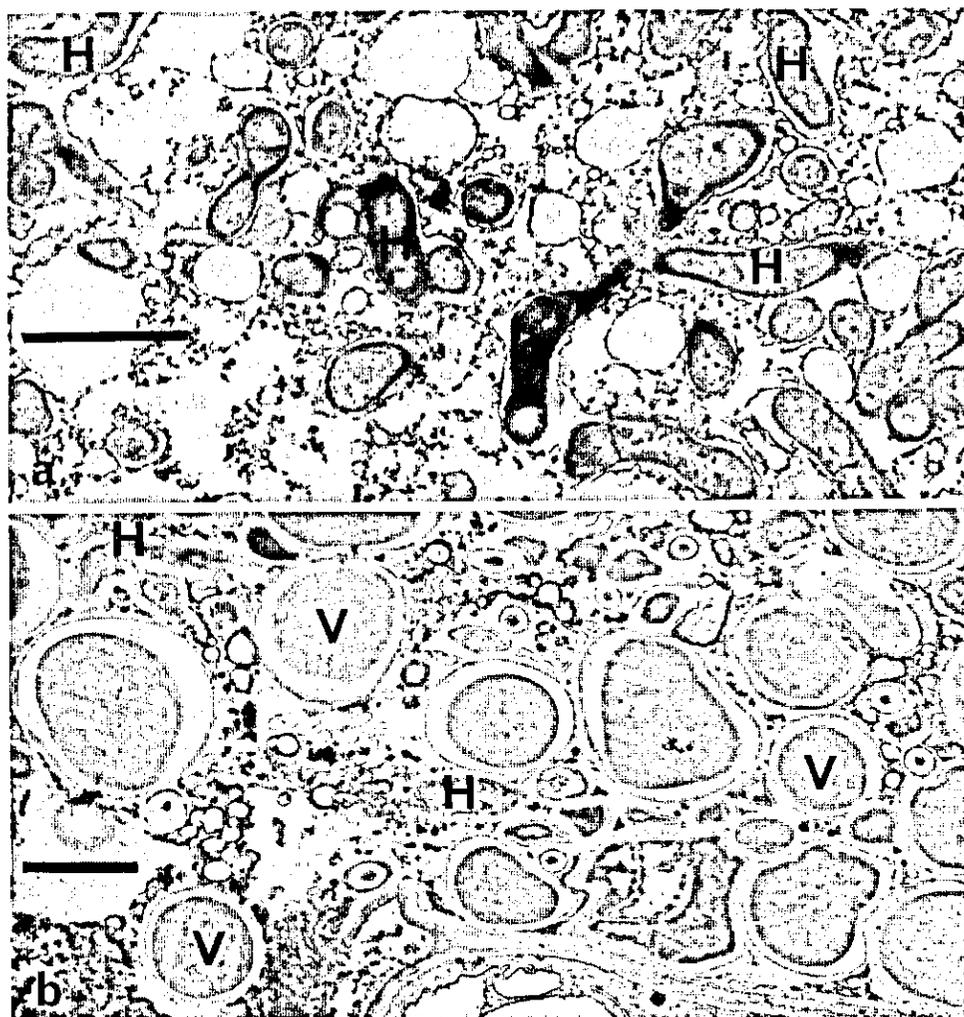
FIG. 1. Transmission electron micrographies of nodules of *Casuarina equisetifolia* (a) and *Colletia spinosissima* (b). Whereas typical vesicles are present in nodules of *Colletia spinosissima,* they are absent in nodules of *Casuarina equisetifolia.* H: *hyphae·* V: vesicles. Bar = 2 μm.

The third one, *C. sinica,* is a deciduous bush, widely grown in Hunan, China, as a source of green manure and also for feeding silkworms. *Coriaria sinica* grows fast so that the stems can be harvested 4-5 times each year giving 10 T of fresh biomass per ha.

Rubus

Only one species of *Rubus, R. ellipticus,* has been shown to nodulate. Nodulated *R. ellipticus* occurs in continental Asia, Sri Lanka, the Philippines and Java. As underlined by Becking (1982), the discovery of a nodulating *Rubus, R. ellipticus,* caused some surprise when it was reported for the first time (Becking 1979) because in this genus nodulation is apparently far from being a generic character. The fruit of *R. ellipticus* is comparable to the fruit of other species of *Rubus* (raspberries and blackberries). Hybridization of *R. ellipticus* with other *Rubus* would be worth of attempt (Becking 1982).

Datisca

Two species of the genus *Datisca* are known to be nodulated: *Datisca cannabina* and *Datisca glomerata* (Becking 1982). Recently, the endophyte of *Datisca cannabina* was isolated and grown *in vitro* (Fanzia Hafeez, personal communication).

CASUARINACEAE

. Taxonomy and distribution of Casuarinaceae

With the exception of *Alnus*, most of AP dealt within the previous section are shrubs or small trees. By contrast, most species of the Casuarinaceae are trees. According to the upcoming National Academy of Sciences publication, *"Casuarina:* Tree resources for the future", the family Casuarinaceae forms a group of 82 species native mostly to Australia, but also to Indo-Pacific areas from India to Polynesia. Johnson (1982) recognizes four genera:

— genus *Casuarina* (*sensu stricto*): 16 species occurring mostly in Australia, also in South east Asia to the Pacific Islands. Trees often quite large. Best known or promising species: *C. cristata, C. cunninghamiana, C. equisetifolia, C. junghuhniana* (= *C. montana*), *C. glauca, C. obesa, C. oligodon* and *C. rumphiana.*

— genus *Allocasuarina:* 45 species, endemic to Australia. Trees to small shrubs. Best known or promising species: *A. campestris, A. decaisneana, A. distyla, A. fraseriana, A. huegeliana, A. littoralis* (= *Casuarina suberosa*), *A. luehmannii, A. torulosa* and *A. verticillata* (= *Casuarina stricta*).

— genus *Gymnostoma:* 19 species, native to Sumatra, New Caledonia, Fiji. Trees or tall shrubs. Example: *G. deplancheana*, native to New Caledonia.

— genus C: two species, native to islands from Palawan to West Irian. Trees.

In vitro studies on *Frankia* from *Casuarina*

Isolation of *Frankia* from nodules of *Casuarina*. The endophyte of *Casuarina* is more difficult to isolate than the *Frankia* from other AP probably because the nodules of *Casuarina* contain a limited number of structures able to grow out of the nodule when a piece is placed onto the nutrient medium. Another reason for the failure of many isolation attempts has probably been the very slow growth of *Frankia* from *Casuarina* creating a high risk of overgrowth by contaminants. Finally, the exact nutrient requirements of *Frankia* strains from *Casuarina* are still not yet known. Diem & Dommergues (1983) described a method of isolation that led to the isolation of strains of *Frankia* which were both infective and effective: ORS 021001 (syn.: Cj1-82) from *C. junghuhniana*, ORS 020606 (syn.: CeD1-82) and ORS 020607 (syn.: CeF1-82) from *C. equisetifolia.*

Previously the same authors (Diem et al. 1982a) working in Senegal had used another method of isolation (serial dilution of a suspension of crushed nodules) which had given two strains, ORS 020602 (syn.: D11) and ORS 020604 (syn.: G2), which were unable to infect the host plant, *Casuarina equisetifolia*, but could effectively nodulate *Hippophaë rhamnoides* (Gauthier et al. 1981b), an AP that does not occur in Senegal and is not taxonomically related to the Casuarinaceae. By contrast, strain ORS 021001 (syn.: Cj1-82), which effectively nodulated *Casuarina equisetifolia* (Table 2), did not nodulate *Hippophaë rhamnoides*. One explanation for these surprising results is that the procedure used for isolating strain ORS 020602 favored the growth of *Frankia* mutants that would appear in the *Casuarina* nodules and then in nodule suspensions. The fact that *Hippophaë rhamnoides* also nodulated when inoculated by crushed nodules of *Casuarina equisetifolia* (Gauthier et al. 1981b) confirms this hypothesis.

TABLE 2. Nodulation of different species of *Casuarina* by strain ORS 021001.

Species	Nodulation
Casuarina cristata	+
Casuarina cunninghamiana	+
Casuarina equisetifolia	+
Casuarina glauca	+
Casuarina junghuhniana	+
Casuarina obesa	+
Allocasuarina decaisneana	o
Allocasuarina distyla	o
Allocasuarina fraseriana	o
Allocasuariana huegeliana	o
Allocasuariana littoralis	o
Allocasuariana verticillata	o
Allocasuariana torulosa	o
Gymnospoma deplancheana	o

Morphology. When grown *in vitro Frankia* from *Casuarina* always forms spore-producing sporangia characteristic of the *Frankia* genus. Some strains (e.g. ORS 020602 or ORS 020604) readily form vesicles on QMOD medium, a complete medium (Lalonde & Calvert 1979), whereas other strains (e.g. ORS 021001) form vesicles only on some media (see 3.2.3). Up to now we found two types of *Frankia* strains:

1. Strains type ORS 020602, producing a pigment (blood-red to pink), forming vesicles on many media, and only typical sporangia.

2. Strains type ORS 021001, producing no pigment, forming vesicles only on specific media and producing not only typical sporangia, but also toruloid, thick-walled structures described by Diem et al. (1983) as sporangia-like-structures (SLS).

In vitro N_2 fixation. It is now established that some strains of *Frankia* are able to exhibit a significant acetylene reduction activity *in vitro* and can grow with N_2 as the sole nitrogen source (Tjepkema et al. 1980, Gauthier et al. 1981a).

Using strain ORS 020602, one of us (DG) showed that the biosynthesis of nitrogenase was maximum when PO_2 in the atmosphere above the culture was 10kPa whereas the nitrogenase activity was apparently not affected by O_2 tension (Fig. 2). Nitrogen sources in the nutrient medium directly affect the morphology of *Frankia*. Thus NH_4 Cl 20 mM hinders the production of vesicles and subsequently N_2 fixation. By contrast, aspartate and glutamate (7mM) allow vesicle formation and N_2 fixation (DG, unpublished). Thus the question arose as to whether ammonium originating from nitrogenase activity was assimilated through the GS-GOGAT pathway in *Frankia* as it is in other nitrogen-fixing prokaryots. A first attempt to answer this question was to follow the derepression of nitrogenase biosynthesis in *Frankia* in the presence of ammonium with or without MSX (methionine sulfoximine), known to inhibit GS and GOGAT (Gordon & Brill 1974). When MSX was added, nitrogenase could be synthesized even in the presence of ammonium, which supports the hypothesis that GS could play a role in the regulation of nitrogenase biosynthesis in *Frankia* (Gauthier 1983). Preliminary results confirm the presence of GS, GOGAT, and alanine dehydrogenase in *Frankia* growing in nitrogen-free medium (NFM)
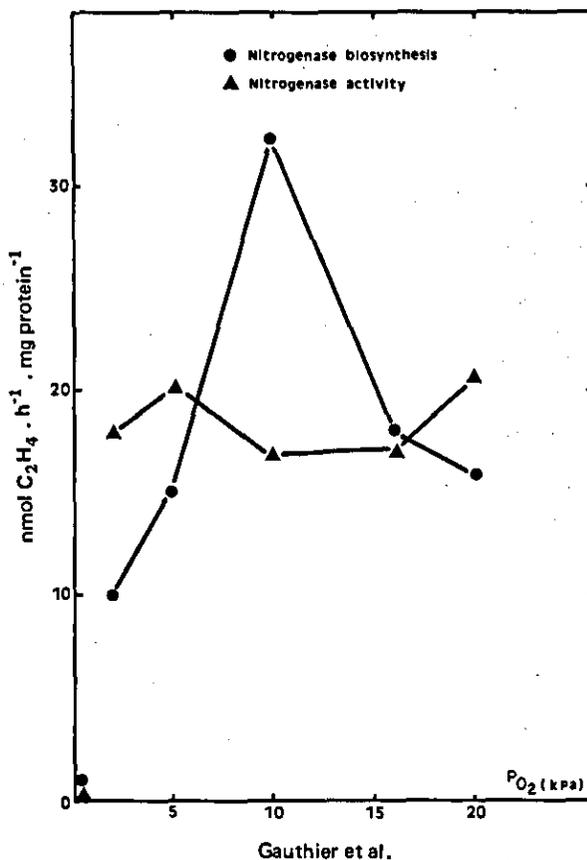
FIG. 2. Effect of PO_2 on nitrogenase activity and nitrogenase biosynthesis in *Frankia* strain ORS 020602.

Nitrogenase biosynthesis: a 10-day-old culture of *Frankia* obtained in QMOD medium (Lalonde an Calvert 1979), thus not exhibiting any nitrogenase activity, was centrifuged and resuspended in a nitrogen-free medium (NFM) (Gauthier et al. 1981) and incubated under different $Ar - O_2 - C_2H_2$ gas mixtures. Specific acetylene reduction activity calculated from daily measurements was expressed as nmoles C_2H_4 produced per h and per mg of protein.

Nitrogenase activity: a derepressed culture of *Frankia* (i.e. exhibiting acetylene reduction activity) was resuspended in the NFM medium complemented with chloramphenicol (100 μg/ml) to inhibit the neosynthesis of proteins. Nitrogenase activity remained constant whatever $Ar - O_2$ ratio was applied.

(DG, unpublished). N_2 fixation occurred only when vesicles were present in the culture (Gauthier et al. 1981c). In some media vesicles could appear without expressing any nitrogenase activity. Such a situation is reminiscent of that of preheterocysts of cyanobacteria (Gauthier 1983). When strain ORS 021001, an infective strain, was used instead of strain ORS 020602, factors governing the vesicle formation appeared to significantly differ. Thus some nitrogenous compounds (e.g. yeast extract) that did not affect the vesicle production by strain ORS 020602 inhibited the process in strain ORS 021001.

Proliferation of *Frankia* outside the nodules and in the rhizosphere. Recently Diem et al. (1982c) presented photographs showing clearly that *Frankia* could thrive in the vicinity of nodules and in the rhizosphere of hydroponically grown *Casuarina equisetifolia*. The *Frankia* hyphae apparently escaped from some nodules and proliferated at their surface producing typical vesicles and sporangia. The ability of *Frankia* to grow and sporulate outside the nodule probably contributes actively to the dissemination of this actinomycete in the soils.

Nodule initiation and development

Infection of *Casuarina cunninghamiana* roots occurs through root hairs (Callaham et al. 1979), a mode of penetration known also to occur in *Alnus, Myrica* and *Comptonia*. The infection through root hairs was confirmed later in *Casuarina equisetifolia* by Diem et al. (1982b) who used a pure *Frankia* strain (ORS 021001). These authors assumed that infection might be initiated not only by hyphae but by granule-like cells entrapped in the fold of curled root hair tips. One important observation was that only young and still elongating root hairs are able to curl and be infected by *Frankia*, a point already mentioned by Bauer (1981) and Hubbell (1981) in their study of legume infection by rhizobia.

The infective encapsulated and often branched hyphae invade the root cortical cells which subsequently swell, leading to the formation of the nodule (Kant & Narayana 1977, Callaham et al. 1979, Diem et al. 1982b). A single infection is sufficient to initiate a nodule, but generally several root hair infections lead to a single nodule if they are close enough to each other, a fact that opens the possibility of multiple infection in one nodule. Nodules are visible to the naked eye within 14 days after inoculation when environmental conditions are favorable.

Whereas nodules *Casuarina sensu stricto* form nodule roots that may be more than 1 cm long, nodules of *Allocasuarina torulosa* and *A. verticillata* lack nodule roots (Coyne 1981). With the exception of Gardner's report (1976), recently aptly criticized by Torrey (1982), up to now no typical vesicles have been observed within *Casuarina* nodules (Fig. 1a), which raises the question of the site of N_2 fixation in these nodules. The absence of vesicles in the nodules is all the more surprising in that vesicles often occur in large numbers in a pure culture (cf. 3.2.3).

Specificity

Preliminary investigations reported by Coyne at the International *Casuarina* Workshop, held at Canberra in 1981, provide evidence of inter-generic specificity in host-endophyte relations within the Casuarinaceae family. Coyne used six species belonging to two genera: *Casuarina sensu stricto* (*C. cristata, C. cunninghamiana, C. glauca*) and *Allocasuarina* (*A. verticillata, A. torulosa, A. littoralis*). Seedlings of the six species were inoculated with crushed nodules of the same species and from each of the other species. According to the response to inoculation the different species could be classified into two main groups: one comprising the three species of the genus *Casuarina* which behaved similarly, the other group

comprising the species of the *Allocasuarina* genus, which could be divided into two subgroups: (1) *A. verticullata* and *A. torulosa* and (2) *A. littoralis.*

Using a pure strain of *Frankia* (ORS 021001) isolated from *Casuarina junghuhniana* to inoculate different species of *Casuarina* and *Allocasuarina,* we found (Table 2) that only the species belonging to the *Casuarina* genus could be nodulated, but none of the species of the *Allocasuarina* genus could be. Experiments under way showed that, inoculated with crushed nodules of *Casuarina equisetifolia* instead of a pure culture of *Frankia,* a low percentage of *Allocasuarina torulosa* (ca. 20%) and *A. verticillata* (ca. 30%) could form 2-3 nodules per plant. Thus the host spectrum of the inoculum was apparently wider when crushed nodules were used instead of a pure strain. This result might be attributed to the fact that the crushed nodules contain more than one strain of *Frankia* (contaminant strains or mutants) or strains with a host spectrum wider than that of strain ORS 021001.

Need for inoculation

Even within their natural distribution area the different species of native Casuarinaceae are not always regularly nodulated. Thus a survey of 77 sites in Victoria, Australia, revealed that nodulation occurred in less than 30% of these sites (Lawrie 1982). This may indicate absence of the endophyte or inhibition of nodulation. Outside their natural distribution area, Casuarinaceae are seldom nodulated. In N-deficient soils, the absence of the competent strain of *Frankia* is the main cause of the failure to establish *Casuarina* (Torrey 1982, Gauthier et al. 1981c). In such situations inoculation is required. Up to now, inoculants have been prepared from suspensions of crushed or dried nodules (Torrey 1982). The first inoculation trial with a pure culture of *Frankia* is described in paragr. 3.6.4.

Nitrogen fixation

Acetylene reduction activity. To the best of our knowledge nitrogenase has not yet been isolated from any *Frankia* strain isolated from *Casuarina* nodules. Highest specific acetylene reduction activities (SARA) of *Casuarina* nodules collected in the field or obtained in the laboratory range from 10 to $100\ \mu$moles C_2H_2 reduced per g (dry weight) of nodules per h (e.g. Becking 1975, Dommergues 1982, Bond & Becking 1982). Since *Casuarina* nodules are perennial and the outer layer of lobes alone is active, older nodules tend to exhibit a lower SARA. Thus one of us (DG) found that the SARA of 4-month-old nodules was 50-90 μmoles C_2H_2 per g (dry wt) per h whereas that of 13-month-old nodules was only 10. Consequently the field evaluation of N_2 fixation based on the estimation of nodule weight and SARA is far from being reliable.

Hemoglobin. For many years the dogma was that hemoglobin did not occur in AP nodules. Only one author (Devenport 1960) had reported the presence of an insoluble hemoglobin in nodules of AP and especially of *Casuarina.* Recently Appleby et al. (1983) and Tjepkema (1984) could confirm the presence of hemoglobin in *Casuarina* nodules by studying the absorption spectra obtained through slices of nodules and by extracting "soluble" hemoglobin from *Casuarina cunninghamiana* nodules using new isolation procedures.

Early field evaluations of N_2 fixation. By comparing the soil and plant N contents of a 13-year-old plantation of *Casuarina equisetifolia* growing in sand dunes of the Senegalese Coast with the soil N content of an adjacent plot devoid of vegetation, we calculated that the mean yearly N_2 fixation was ca 58 kg N/ha (Dommergues 1963). Estimations based on N evaluation of soil and forest floor N suggested

that *Allocasuarina littoralis* growing on a coastal low-fertility sandstone soil in Australia could fix 200 kg N_2/ha/year (in: Sylvester 1977).

Evaluation of N_2 fixation in microplots at the ORSTOM station, Dakar (Tables 3 and 4). The experiment was conducted in 1982-1983 at the ORSTOM station in Dakar, Senegal, on 12 microplots (1 m^2 each, 1 m deep). The soil used was a typical N-deficient sandy soil of the Cap-Vert peninsula (pH 7.0), homogenized and fumigated with methyl bromide. One-month-old seedlings of *Casuarina equisetifolia* were planted into 5 x 25 cm polyethylene pouches filled up with a mixture of vermiculite and sterile soil (1:5). Inoculated seedlings were obtained by dipping the roots in a suspension of a 3-month-old culture of *Frankia* ORS 021001. Plants were raised in the pouches for 3.5 months and then transplanted into the 1 m^2 microplots with four plants per microplot. At transplantation time the seedlings were 4.5 months old; the height of inoculated seedlings was ca. 30 cm and that of uninoculated ones was ca. 25 cm. The mean N content of inoculated seedlings was 30 mg/plant.

Three treatments, each with four replications, were used as follows:

– Treatment 1. Seedlings inoculated with *Frankia* ORS 021001. At planting time microplots were fertilized with PK (17 g K_2HPO_4/m^2) and $(NH_4)_2SO_4$ (2 g N/m^2);

– Treatment 2. Uninoculated seedlings. Same PK and N fertilization as treatment 1;

– Treatment 3. Uninoculated seedlings. Same PK fertilization as in treatments 1 and 2. Higher $(NH_4)_2SO_4$ fertilization (10 g N/m^2).

When plants were eight months old, they exhibited deficiency symptoms that stopped when 1 1 of Hewitt nitrogen-free nutrient solution was applied to each 1 m^2 microplot every 2 weeks. Throughout their growth the plants were carefully irrigated to avoid any moisture stress. Plants were harvested 6.5 months after transplantation to the microplots. Three out of 16 plants submitted to treatment 2 and also three out of 16 plants submitted to treatment 3 had been contaminated, the mean nodule weight of those plants being only 4 g (dry wt) per plant compared with 17 g (dry wt) for inoculated plants from treatment 1. All contaminated plants were discarded so that calculations were based on 13 plants

TABLE 3. Influence of inoculation with *Frankia* (strain ORS 021001) on height and dry weight of *Casuarina equisetifolia*. Microplot experiment at the ORSTOM station, Dakar, Senegal.

Treatments			Height (cm)	Dry weight (g/plant)			Total	Nodule dry weight
N^o.	Inoc.[1]	$(NH_4)_2SO_4$ (g N/m^2)		Cladodes	Branchlets (diam. < 4 mm)	Branches (diam. > 4 mm)		
1	+	2	216 a	260 a	76 a	188 a	524 a	17
2	o	2	170 b	151 b	43 b	100 b	294 b	0
3	o	10	192 b	204 b	65 a	140 b	409 b	0

[1] + : seedlings inoculated with strain ORS 021001
o : uninoculated seedlings
All microplots received a first application of K_2HPO_4 at the rate of 17 g/m^2, then 1 l/m^2 of nitrogen-free Hewitt solution every two weeks, when trees were eight months old. Trees harvested when eleven months old. Figures followed by same letter do not differ significantly, P = 0.05 (Student test).

TABLE 4. Influence of inoculation with *Frankia* (strain ORS 021001) on N content of *Casuarina equisetifolia*. Microplot experiment at the ORSTOM station, Dakar, Senegal.

N°	Treatments		Cladodes		Branchlets (diam. < 4 mm)		Branches (diam. > 4 mm)		Total N content (g/plant)
	Inoc.	(NH₄)₂SO₄ (g.N/m²)	N%	Total N (g/plant)	N%	Total N (g/plant)	N%	Total N (g/plant)	
1	+	2	1.30 a	3.59 a	0.54 a	0.42 a	0.40 a	0.78 a	4.78 a
2	o	2	0.86 b	1.31 b	0.36 b	0.16 b	0.30 b	0.30 b	1.77 a
3	o	10	1.00 c	2.06 c	0.39 b	0.25 b	0.29 b	0.40 b	2.71 c

For experimental details see Table 3. Figures followed by same letter to not differ significantly, P = 0.05 (Student test).

instead of 16 in treatments 2 and 3. Shoots were divided into three fractions, cladodes, branchlets (diameter < 4 mm) and branches (diameter > 4 mm), dried to a constant weight at 60°-70°C, weighed and analysed for total N.

Tables 3 and 4 show that inoculating *Casuarina* resulted in an increase of 27% in height, 78% in weight, and 159% in total N compared with uninoculated plants with the same N application ($2 g N/m^2$). The effect of the higher N application (treatment 3) was much less marked since the increases in height, weight and N content were only 13%, 39% and 48% respectively, indicating that inoculation was three times as efficient as application of 10 g of N per m^2.

Estimation of N_2 fixation during the 6.5 month period between transplantation and harvest by the difference method. Assume that n is the mean total N content of the plants in g per tree at transplantation time, N_1 and N_2 the mean total N content of the inoculated (treatment 1) and uninoculated plants (treatment 2) in g per tree at harvest time, i.e. 6.5 months later, the mean N_2 fixation (f) expressed in g per tree during this period is given by:

$$f = N_1 - N_2 - n$$

Extrapolating to 1 ha with 40,000 trees the mean N_2 fixation F expressed in kg per ha during the 6.5 month period is:

$$F = (N_1 - N_2 - n) \times 40$$

Since N_1, N_2 and n were respectively 4.78, 1.77 and 0.03 g per tree,

$$F = 119 \text{ kg } N_2 \text{ fixed/ha for the 6.5 month period.}$$

The nitrogen content of the nodules was 1.8% and their mean dry weight was 17 g per tree. In other words, each nodulated tree had 0.3 g N in its nodules, that is, 12 kg N/ha. This amount of N should probably be added to the figure of N_2 fixation presented above, so that N_2 fixation was at least 119 + 12 = 131 kg/ha.

Extrapolation of this figure to an older stand with 4,000 trees per hectare should be made taking into account the influence of tree density (4,000 instead of 40,000 trees per ha) and the fact that N_2 fixation increases with age up to a maximum (ca 2-5 years), then decreases.

Estimation of N_2 fixation during the 6.5-month period by the N fertilizer equivalence method. We have already defined n and N_2. If N_3 is the mean total N content of the uninoculated trees with higher N application (treatment 3) at harvest time, the mean yield (y) increase expressed in g N per tree attributable to the higher application of N($10 - 2 = 8 g/m^2$ or 80 kg/ha) is:

$$y = N_3 - N_2 - n$$

Extrapolating to 1 ha with 40,000 trees, the mean yield increase expressed in kg N/ha is:

$$y = (N_3 - N_2 - n) \times 40$$

Since N_3, N_2 and n were 2.71, 1.77 and 0.03 g per tree,

$$y = 0.91 \times 40 = 36.4$$

Comparing F and y suggests that inoculation was approximately equivalent to the application of $80 \times \dfrac{131}{36.4} = 288$ kg N-fertilizer per ha, which indicates that the use of N-fertilizers would be unrealistic.

Association with mycorrhizal fungi and proteoid roots

Like many plants, AP are susceptible to infection by endomycorrhizal fungi (Rose & Trappe 1980). Casuarinaceae are no exception, since Rose (1980) found different species of vesicular-arbuscular mycorrhizal fungi (*Gigaspora* sp., *Glomus* sp) associated with *Casuarina equisetifolia* and *C. cunninghamiana* growing in sandy soils of Florida and Japan. Diem et al. (1981) also reported mycorrhizal infection of *C. equisetifolia* growing in sand dunes of Senegal. An experimental study carried out by Diem & Gauthier (1982) in a sterile N-deficient soil showed that 6-month-old seedlings of *C. equisetifolia* inoculated with *Glomus mosseae* and *Frankia* (as crushed nodules) contained twice as much nitrogen (96 mg/plant) as seedlings that had been inoculated with *Frankia* alone (51 mg/plant). The effect of the double inoculation on the dry weight of the plants was also beneficial since seedlings inoculated with *Frankia* and *G. mosseae* weighed 7.69 g, whereas seedlings inoculated with *Frankia* alone weighed only 4.23 g.

When associated with endomycorrhizal vesicular-arbuscular fungi, roots of *Gymnostoma deplancheana* bear structures called "mycorrhizal nodules" by Huguenin (thesis, University of Rouen 1969). These so-called nodules are interpreted by one of us (HGD) as short roots with a swollen cortex subsequently invaded by mycorrhizal fungi. The distribution of these structures along the root axis is reminiscent of proteoid roots or *Podocarpus* nodules.

Ectomycorrhizae can also appear on the root system of Casuarinaceae. Bamber et al. (1980) experimentally obtained ectomycorrhizae on the roots of *Allocasuarina distyla* by inoculating them with *Pisolithus tinctorius*.

Endo or ectomycorrhizae are known to markedly improve the absorbing capacity of the roots for P and some non-mobile micronutrients such as Zn or Cu, thus indirectly enhancing the N_2-fixing capacity of the host plant. In other terms, mycorrhizae confer to Casuarinaceae already infected with *Frankia* the ability to thrive under conditions of extreme deficiency of P, N and micronutrients.

Finally one should note that typical proteoid roots have also been reported on the root systems of different species of *Casuarina* (Diem et al. 1981). These structures which occur as tightly packed rows of rootlets probably increase the ability of the host plant to absorb different nutrients, especially P, thus contributing to the adaptation of Casuarinaceae to the very poorest of soils.

For all their practical potential, ecto or endomycorrhizae (and probably also proteoid roots) are not a panacea, always restoring the N_2-fixing activity of AP located in deficient soils. The deficiency may even be so marked that moderate applications of P or micronutrients are unavoidable.

CONCLUSION

Most tropical countries face two major problems: increasing overexploitation of wood reserves for timber or fuelwood and diminishing soil fertility for agricultural production. The present consensus is that N_2-fixing trees can help prevent the forthcoming wood and food crisis and emphasis has been put on these plants for the last few years. Programs have been initiated which are focused on legume trees, but generally few investigations have been envisioned in the field of actinorrhizal plants (AP). In spite of the fact that many tropical AP have been described up to now, a number of species have not yet been discovered. Systematic surveys of tropical AP are thus urgently needed. The example of the discovery of new AP, *Kentrothamnus* and *Talguena*, by Chilean colleagues (Medan & Tortosa 1981) and Cowania Mexicana, by Righetti & Munns (1980), is encouraging. Even the best known genera (e.g. *Casuarina*) are far from being thoroughly studied and their exact potentialities are not yet properly delineated.

Tropical AP comprise trees that can yield wood of good quality (e.g. *Casuarina* sp or *Alnus* sp), but many species are shrubs or small bushes, which raises the question of their value for wood production. One should keep in mind that even small AP can play a major role in wood production by increasing the nitrogen input into forest ecosystems. This ability is well illustrated by *Coriaria* sp. growing as an understory in different forest types, indirectly enhancing wood production of non-N_2-fixing trees by providing them with an extra input of nitrogen.

Tropical AP might play a prominent role in land reclamation by covering bare soil of disturbed areas, fixing dunes, preventing wind and water erosion and markedly improving the soil nitrogen status. AP can also contribute directly to agricultural production by providing organic nitrogen, e.g.,*Coriaria sinica* (see supra) or *Casuarina oligodon*.

We have seen that the N_2-fixing potential of an important woody AP, *Casuarina equisetifolia*, is higher than anticipated, suggesting that the growth of some AP might not be limited by their N_2-fixing potential but by unfavorable limiting factors, such as deficiencies in nutrients,toxicity of some elements (e.g., salts) or moisture stress. Thus,future investigations should aim at obtaining AP which would be more tolerant to different environmental stresses. The recent advances in the field of *in vitro* techniques should facilitate the exploitation of the genetic variations among individual AP thus producing clones of plants genetically more tolerant to major environmental stresses.

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