Acta Œcologica
Œcol. Plant., 1984, Vol. 5 (19), n° 3, p. 231-239.

The *Colletia spinosissima-Frankia* symbiosis

D. Gauthier (1), L. Frioni (2), H. G. Diem (1), Y. Dommergues (1)

(1) *ORSTOM/CNRS B. P. 1386, Dakar, Senegal.*
(2) *Facultad de Agronomia, Universidad de Rio Cuarto, 5800 Rio Cuarto, Cordoba, Argentina.*

SUMMARY

Colletia spinosissima, an actinorhizal Rhamnalian species native to South America, is known for its beneficial effect on soil fertility (improvement of the soil nitrogen status).

The symbiotic endophyte, *Frankia*, was isolated (strain ORS060501) and shown to be infective and effective. Similar to other *Frankia* strains, strain ORS060501 fixed nitrogen *in vitro*. This strain nodulated other Rhamnales *(Hippophaë rhamnoides, Elaeagnus angustifolia)* but failed to nodulate the non-Rhamnales tested, suggesting that strains of *Frankia* associated with Rhamnales form a cross-inoculation group. This concept, however, should be applied with flexibility since some strains isolated from non-Rhamnales (such as strains ORS020602, HFPCcI1, and HFPCcI2) are able to nodulate Rhamnales.

The *Colletia-Frankia* symbiosis is characterized by a N_2-fixing potential which is comparable with other actinorhizal systems.

KEY-WORDS: *Argentina* - Colletia spinosissima - Frankia - *Actinorhizae* - *Rhamnale* - *Nitrogen fixation.*

RÉSUMÉ

Colletia spinosissima est une Rhamnale actinorhizienne originaire d'Amérique du Sud. Cette plante est connue pour son effet améliorant la fertilité des sols (accroissement de leur teneur en azote).

On a isolé l'endophyte symbiotique (souche de *Frankia* ORS060501) et on a montré que cette souche était infective et effective. De même que les autres souches de *Frankia*, la souche ORS060501 fixe l'azote *in vitro*. Cette souche a nodulé d'autres Rhamnales *(Hippophaë rhamnoides, Elaeagnus angustifolia)* mais n'a pu noduler les autres non-Rhamnales testées, ce qui suggère que les souches de *Frankia* associées aux Rhamnales forment un groupe d'inoculation croisée. Cependant ce concept ne doit pas être appliqué avec rigueur car certaines souches isolées de non-Rhamnales (telles que les souches ORS020602, HFPCcI1 et HFPCcI2) peuvent noduler les Rhamnales.

Les potentialités fixatrices d'azote de la symbiose *Colletia spinosissima-Frankia* sont comparables à celles des autres systèmes actinorhiziens connus.

MOTS-CLÉS : *Argentine* - Colletia spinosissima - Frankia - *Actinorhize* - *Rhamnale* - *Fixation d'azote.*

I. — INTRODUCTION

The family Rhamnaceae comprises six genera, *Ceanothus*, *Colletia*, *Discaria*, *Kentrothamnus*, *Trevoa*, *Talguenea* which have been reported to bear actinorhizal nodules (BOND, 1976; MEDAN & TORTOSA, 1976, 1981; BOND & BECKING, 1982). The genus *Colletia* itself comprises 17 shrubby species confined to South America (BOND & BECKING, 1982). Two species of *Colletia* are well known to be native to

Acta Œcologica/Œcologia Plantarum, 0243-7651/84/03/231/9/$ 2.90/

Argentina: *C. spinosissima* Gmel. (syn.: *C. spinosa* Lam), *C. paradoxa* (Spreng.) Escalente (syn.: *C. cruciata*) (DIMITRI, 1959). These species differ according to the shape of their stems and thorns, which are cylindrical in *C. spinosissima* and markedly flattened in *C. paradoxa* (DIMITRI, 1959).

According to Pr. A. H. MERZARI from the faculty of Agronomy of Buenos Aires quoted by MIGUEL *et al.* (1978), « The beneficial effects of *Colletia* in Argentina lands have long been recognized by Araucan Indians ». Since these effects probably result from the ability of *Colletia* to fix nitrogen and consequently improve the soil nitrogen status, we attempted to evaluate the N_2-fixing potential of the *Colletia-Frankia* system. This investigation required the isolation of the specific *Frankia* strain and a preliminary study of its ability to fix N_2 *in vitro*.

II. — MATERIALS AND METHODS

Plant cultivation and obtention of nodules for further isolation of Frankia.

Seeds, nodules and rhizosphere soil of *Colletia spinosissima* were collected on plants growing in a stony soil close to Embalse Dam on the Rio Tercero, Argentina (plate I, fig. 1), a location with a low rainfall (600 mm per annum, with rain occurring mainly in spring and summer).

Plate I. FIG. 1. — A bush of spontaneously growing *Colletia spinosissima* at flowering stage, Embalse Dam, Rio Tercero, Argentina.

To obtain fast and regular germination, the seeds were pretreated with concentrated sulfuric acid for 3 min, then washed with water until all traces of acid were removed. The seeds were sown in sterile nitrogen-deficient soil placed in pots in a glasshouse at ORSTOM research station, Dakar, Senegal. Young seedlings were inoculated with a suspension of dried crushed nodules plus rhizo-

sphere soil. The pots were irrigated with distilled sterile water and supplied with nitrogen-free HOAGLAND's solution (HOAGLAND & ARNON, 1950) every week. When the *Colletia* seedlings were 6 months old, some of them were dug out and the fresh nodules were excised and used for isolation of *Frankia* as indicated below; the remaining plants were clipped to get cuttings used for inoculation experiments.

Isolation of Frankia *from* Colletia *nodules.*

Nodule lobes were superficially sterilized in a 3 % aqueous solution of osmium tetroxide for 4 min, were then thoroughly rinsed in sterile water and chopped into small pieces (LALONDE *et al.*, 1981). These pieces were transferred onto a 1.5 % water agar layer in a 10 cm Petri dish, on which 3 ml of QMOD (LALONDE & CALVERT, 1979) agar was poured to form the top layer (DIEM & DOMMERGUES, 1983).

The Petri dishes were incubated at 25-28° C for one month in a saturated atmosphere. Typical colonies of *Frankia* emerging from nodule pieces were separately transferred into 10 vials containing 4 ml of QMOD medium. When colonies had grown to 1 mm diameter, they were gently broken into fragments using a magnetic stirrer and subcultured in liquid QMOD medium. The strain thus obtained was designated ORS060501 according to Lechevalier's proposed nomenclature rule (LECHEVALIER, 1983).

Frankia *strains of collection.*

Four strains were kindly provided by Dr. D. BAKER: CpI1, isolated from *Comptonia peregrina* (CALLAHAM *et al.*, 1978); MgI5, isolated from *Myrica gale*; AvcI1, isolated from *Alnus viridis*; PtI1 isolated from *Purshia tridentata* (BAKER, 1982).

Other strains used had been previously isolated in our laboratory: ORS140101 (syn.: H13), isolated from *Hippophaë rhamnoides* (GAUTHIER *et al.*, 1981 *a*); ORS020602 (syn.: D11) isolated from *Casuarina equisetifolia* (DIEM *et al.*, 1982 *a*); ORS021001 (syn.: Cj1-82) isolated from *Casuarina junghuhniana* (DIEM *et al.*, 1983).

Plants tested.

The following actinorhizal plants were tested in our study: *Purshia tridentata* (seeds kindly provided by D. BAKER), *Elaeagnus angustifolia* (seeds from Versepuy Seed Company, Le Puy, France) *Hippophaë rhamnoides* (seeds harvested by A. GUILLERMIN in the vicinity of Grenoble, France), *Casuarina equisetifolia* (seeds provided by CNRF/ISRA, Dakar, Senegal) and *Allocasuarina torulosa* (seeds provided by the Botanical Garden, Canberra, Australia).

Nitrogenase activity in vitro.

A culture of ORS060501 grown on QMOD medium was centrifuged and homogenized using a magnetic stirrer. The suspension thus obtained was used to inoculate 145 ml vials containing 25 ml of the following nitrogen-free medium (NFM) expressed as g/l: KH_2PO_4: 1.0; $MgSO_4$, 7 H_2O: 0.1; $CaCl_2$, 2 H_2O: 0.01; $FeSO_4$: 0.01; sodium succinate: 1.2; glucose: 1.0; $NaMoO_4$, 2 H_2O: 0.005; $MnSO_4$, H_2O: 0.025, $ZnSO_4$, 7 H_2O: 0.007, $CuSO_4$, 5 H_2O: 0.00125, $CoSO_4$, 7 H_2O: 0.0014, H_3BO_3: 0.0003, pH 6.8. The inoculated vials were incubated under either 80 % Ar-20 % O_2 or 80 % N_2-20 % O_2 gas mixtures. After a 15-day incubation, C_2H_2 was added (10 %) and acetylene reduction activity (ARA) was measured daily by the method of POSTGATE (1972) for 3 days and expressed as nmoles of C_2H_4 produced per hour and per mg of dry weight.

Plant infection tests.

Colletia spinosissima was grown in test tubes with an aluminium foil cap (GIBSON, 1963). Cuttings were inserted through the aluminium cap so that their base, previously dipped into a commercial mixture of phytohormones (1), remained immersed. The nutrient medium in each tube was made of two phases: one solid phase made of an agar slant containing 1/4 strength nitrogen-free HOAG-

(1) ROOTONE F., Compagnie française de Produits Industriels, 117, quai du Docteur-Dervaut, F-92601 Asnières.

LAND solution according to KNOWLTON *et al.* (1980) supplemented with (in grams per litre) activated charcoal Merck ref. 2186: 20 g; $CaCO_3$: 1 g; agar: 16 g and one liquid phase which was a 1/4 strength HOAGLAND solution. The liquid phase was complemented with nitrogen ($(NH_4)_2SO_4$: 17 mg l^{-1}) during the first 5 weeks to allow a satisfactory plant growth, then no more nitrogen was added. From then on inoculation was performed by adding a few drops of a suspension of the different strains of *Frankia* to each tube.

A similar device was used for growing the other plants except that they were obtained from seeds instead of cuttings.

Estimation of the N_2-fixing potential.

Nodules of each root system were incubated in 7 ml vials under a 90 % air-10 % C_2H_2 gas mixture. ARA was measured after 0.5 h and 1 h incubation and expressed as μmoles of C_2H_4 produced per hour and per g of dry nodule. Total nitrogen content of the whole plantlets was analysed using the microKjeldahl method.

Electron microscopy

Nodules for study under the electron microscope were fixed in 2.5 % glutaraldehyde in 0.025 M cacodylate buffer, post-fixed in 1 % osmic acid and then embedded in epon 812. Ultrathin sections were cut from the blocks on an ultramicrotome, stained with uranyl acetate-lead citrate and examined in a Siemens Elmiskop 101.

III. — RESULTS AND DISCUSSION

1. *Morphological characteristics of strain ORS060501.*

When grown on QMOD medium strain ORS060501 exhibited the three usual structures of *Frankia*, i. e.: hyphae, sporangia and vesicles. This strain was morphologically very similar to strain ORS020602 (syn.: D11) except that it produced no pigment. Strain ORS060501 produced vesicles (but did not fix N_2) in some media containing combined nitrogen, such as yeast extract and casamino-acids (that are contained in QMOD medium). Such behavior was similar to that of strain ORS020602, but contrasted with that of strains CpI1 and ORS021001 which form vesicles only in conditions of N_2 fixation, *i. e.*, in nitrogen-free media (TJEPKEMA *et al.*, 1980; GAUTHIER *et al.*, 1983). From these observations, one can infer that the *Frankia* strains known up to now fall into two categories: those which form only N_2-fixing vesicles (*e. g.* CpI1; ORS021001) and those which form vesicles without necessarily fixing N_2.

2. *N_2 fixation* in vitro.

Table I shows that in nitrogen-free medium (NFM), strain ORS060501 could grow only when the gas mixture contained N_2; in other words the strain appeared to grow with N_2 as the sole nitrogen source. Table I also shows that N_2 fixation by the strain ORS060501 occurred at high pO_2 (normal atmosphere). These results confirm the fact that *Frankia* has a highly efficient protection system against O_2, a conclusion already drawn by TJEPKEMA *et al.* (1981) and GAUTHIER *et al.*(1981 *b*), with other *Frankia* strains, respectively strain CpI1 and strain ORS020602.

3. *Infectivity of strain ORS060501 and potential N_2 fixation in plants.*

Strain ORS060501 was not only infective but effective since it profusely nodulated and sustained the growth of the cuttings of *Colletia spinosissima* in the nitrogen-free nutrient medium (plate 2, fig. 1 and 2).

The N_2-fixing potential of the *Colletia-Frankia* symbiosis was evaluated in terms of acetylene reduction activity expressed per hour and per g (dry weight) of plant

TABLE I. — *Nitrogenase activity and growth of* Frankia *strain ORS060501 under two gas mixtures after 15-day incubation in nitrogen-free medium (NFM).*

Gas mixture	*Frankia* biomass (1) (mg dry weight vial^{-1})	ARA (1) (nmoles C_2H_4 h^{-1} mg^{-1} dry weight)
80 % N_2: 20 % O_2	2.6	165
80 % Ar: 20 % O_2	0.3	7

(1) Mean value of 10 replications.
Frankia inoculum was 0.17 mg dry weight vial^{-1}.
ARA = acetylene reduction activity.

TABLE II. — *Effect of inoculation with* Frankia *strain ORS060501 on dry weight, nitrogen content and acetylene reduction activity (ARA) of 3-month-old hydroponically grown* Colletia spinosissima.

Treatments	Dry weight (mg plant^{-1})	N %	Total N (mg plant^{-1})	ARA (1)
Control	56	0.8	0.45	0
Inoculated	113	1.5	1.69	0.68

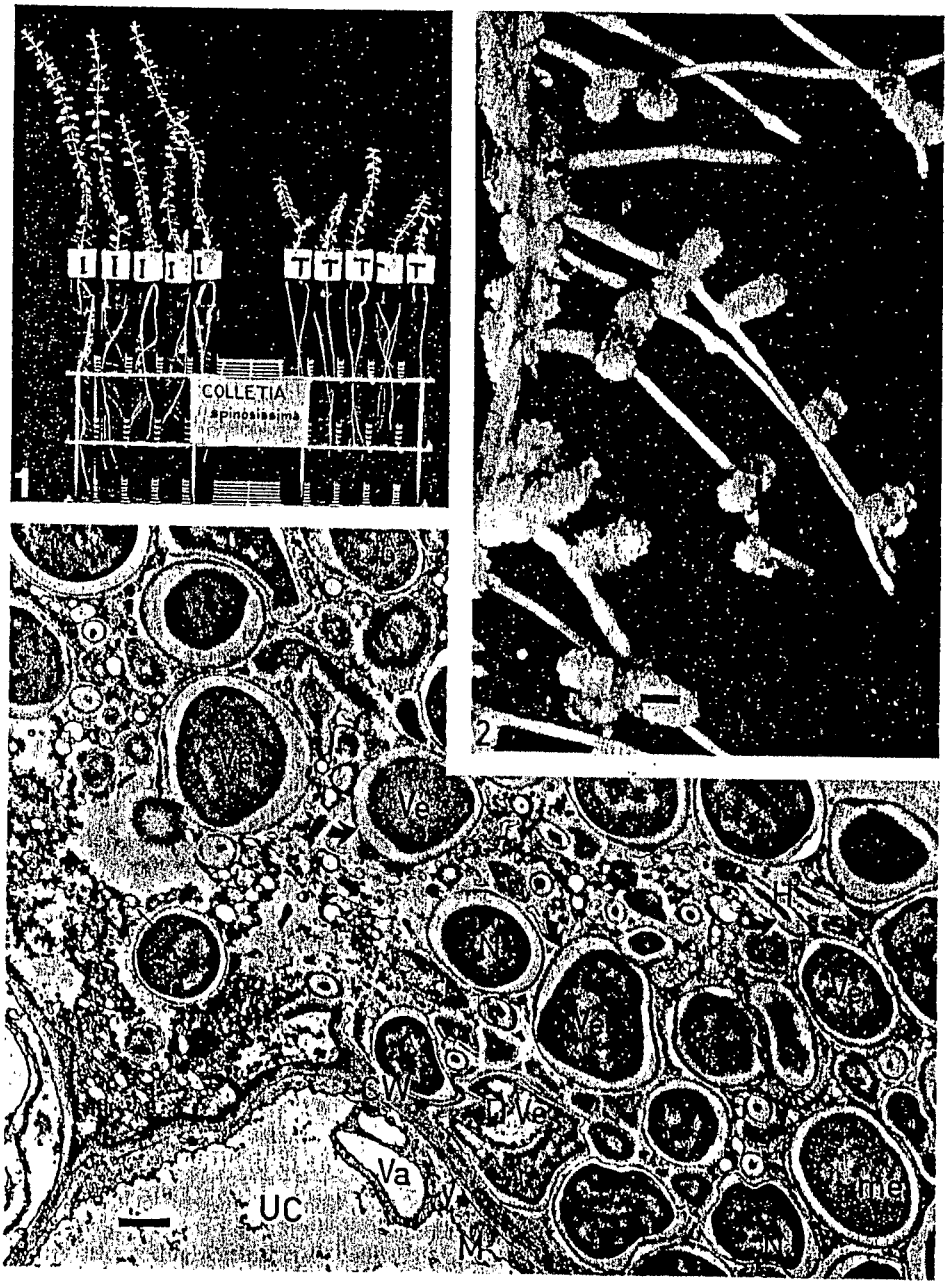
(1) Acetylene reduction activity expressed as μmole C_2H_4 h^{-1} plant^{-1}.

TABLE III. — *Nodulation response of six actinorhizal plants inoculated with four strains of* Frankia.

Host of isolation and related strain	Test host					
	Colletia spinosissima	*Hippophaë rhamnoides*	*Elaeagnus angustifolia*	*Purshia tridentata*	*Casuarina equisetifolia*	*Allocasuarina torulosa*
Colletia spinosissima (ORS060501)	*E* (12)	*E* (8)	*E* (10)	—	—	—
Hippophaë rhamnoides (OR140101; syn.: H13)	*E* (6)	*E* (50)	*E* (*nd*)	—	—	—
Casuarina equisetifolia (ORS020602; syn.: D11)	*E* (2)	*E* (66)	*E* (*nd*)	—	—	—
Casuarina junghuhniana (ORS021001; syn.: Cj1-82)	—	—	—	—	*E* (38)	—

Remarks: E, effective nodulation; —, no nodules. Between parentheses: SARA as μmoles C_2H_4 h^{-1} g^{-1} nodule dry weight; *nd:* SARA non determined.
The following strains were unable to nodulate *C. spinosissima:* CpI1, MgI5, AvcI1, PtI1.

tissue (ARAP), or acetylene reduction activity expressed per hour per gram (dry weight) of nodule (SARA), or amount of total N fixed evaluated by the difference between inoculated and uninoculated *Colletia* (ΔN). The related figures were 6 (ARAP), 12 (SARA) and 1.24 (ΔN) (table II and III).



These figures are comparable to those obtained with other actinorhizal N_2-fixing systems. For example, SARA for *Casuarina equisetifolia* measured in comparable conditions was 24-30 μmoles C_2H_4 h^{-1} g^{-1} nodule (dry weight) (DIEM *et al.*, 1982 *b*; DIEM *et al.*, 1983). Studying the SARA of *Colletia paradoxa in situ*, BOND & BECKING (1982) found that the related SARA was 8.5, which is a figure very close to ours.

The nodules of *C. spinosissima* contained typical spherical septate vesicles 2-3 μm in diameter. Our micrograph of *Colletia spinosissima* nodules (plate 2, fig. 3) is remarkably similar to that of *Colletia paradoxa* published by BOND & BECKING (1982).

4. *Cross-inoculation studies.*

Table III shows that the *Frankia* strain isolated from *Colletia spinosissima* (ORS060501) could effectively nodulate the host of isolation and two other actinorhizal Rhamnales tested, namely *Hippophaë rhamnoides* and *Elaeagnus angustifolia*, a result which could be expected in spite of the fact that strain ORS060501 was isolated from nodules and the rhizosphere of a plant growing far away from the distribution area of *Hippophaë* and *Elaeagnus*. A strain isolated from *Hippophaë rhamnoides* (ORS140101) was able to effectively nodulate the three Rhamnales, which suggests that *Frankia* isolated from Rhamnales could form a homogeneous cross-inoculation group. This conclusion was confirmed by the other cross-inoculation studies reported in table III (last line and remarks) which show that a number of *Frankia* strains isolated from non-Rhamnales, namely a species from Casuarinales (ORS021001), two from Myricales (CpI1 and MgI1), a species from Fagales (AvcI1) and one from Rosales (PtI1) cannot nodulate *Colletia spinosissima*.

One should be aware of the fact that the concept of a Rhamnales cross-inoculation group is flexible, since anomalies are already known, such as that related to strain ORS020602 (syn.: D11). This last strain isolated from nodules of *Casuarina equisetifolia*, which is unable to nodulate its own host of isolation, can nodulate *Hippophaë rhamnoides* (GAUTHIER *et al.*, 1981 *a*) and is shown here to also nodulate *Colletia spinosissima* and *Elaeagnus angustifolia*. Thus we are led to consider that strain ORS020602 belongs to the Rhamnales cross-inoculation group defined above. The suggested explanation for this surprising result is that nodules of *Casuarina* probably contain not only infective strains (such as ORS021001) but also strains

---

Plate II. FIG. 1. — Seven-week-old plants of *C. spinosissima* (*I*) showing nodules on the roots 18 days after inoculation with strain ORS060501. Uninoculated plants of *C. spinosissima* at the same age do not bear any nodules on the roots. Leaves of inoculated plants (*I*) are green whereas those of uninoculated ones (*T*) are yellow.

FIG. 2. — Nodules formed on secondary roots of 7-week-old hydroponically grown *C. spinosissima* inoculated with strain ORS060501. Bar represents 1 mm.

FIG. 3. — Transmission electron micrograph showing a partial view of an infected cell adjacent to a portion of an uninfected cell (*UC*). The infected cell contains numerous endophytic spherical vesicles (*Ve*) and hyphae (*H*) both surrounded by a capsule (large arrows). In addition to nuclear material (*N*), some vesicles show the presence of septa (small arrows) and mesosomes (*Me*). Near the host cell wall (*Cw*), a degenerating vesicle (*DVe*) has partially shrunk, but still contains septa. Note the presence of mitochondria (*M*) and vacuoles (*Va*) within the cytoplasm (*Cy*) of the uninfected cell. Bar represents 1 μm.

failing to infect the host of isolation (such as ORS020602). Both types of isolates were recently obtained by ZANG ZHONGZE *et al.* (1984) from nodules of *Casuarina cunninghamiana:* one strain of the first type (HFPCcI3) nodulated *Casuarina* whereas two strains of the second type (HFPCcI1 and HFPCcI2) failed to nodulate *Casuarina*, but effectively nodulated two Rhamnales, namely *Elaeagnus* and *Hippophaë*.

CONCLUSION

1. The cross-inoculation studies reported here suggest that the strains of *Frankia* associated with Rhamnales form a cross-inoculation group. However, the existence of some strains isolated from non-Rhamnales, but able to nodulate Rhamnales (such as strain ORS020602 and strain HFPCcI1 and HFPCcI2) implies that there is a continuum between the typical Rhamnalian strains (such as strain ORS060501 isolated from *C. spinosissima*) and some strains from the Casuarinaceae. This situation is reminiscent of that encountered with the continuum of *Rhizobium* strains which extend from the typical *R. japonicum* strains, to the cowpea miscellany (BROMFIELD & ROUGHLEY, 1980).

2. The *Colletia-Frankia* symbiosis is characterized by a N_2-fixing potential which is comparable with other actinorhizal systems. One factor limiting N_2-fixation in the field is probably the absence of the specific endophyte since nodules are not always present (RUNDEL & NEEL, 1978). This situation can easily be improved since we have now isolated the specific *Frankia* strain. Of course, other limiting factors can be involved, which should be investigated in the field.

ACKNOWLEDGEMENTS

We acknowledge the fine assistance of Pr. MATTEI, University of Dakar, for TEM studies. We thank Dr. D. BAKER, for providing four *Frankia* strains and seeds of *Purshia tridentata*, and Aïnina SAMB for technical assistance.

REFERENCES

BAKER D., 1982. — A cumulative listing of isolated *Frankiae*, the symbiotic, nitrogen-fixing actinomycetes. In: *The Actinomycetes*, **17**, 35-42.

BOND G., 1976. — The results of the IBP survey of root-nodule formation in non-leguminous angiosperms. In: *Symbiotic nitrogen fixation in plants* (P. S. NUTMAN, Ed.). Cambridge University Press, 443-474

BOND G. & BECKING J. H., 1982. — Root nodules in the genus *Colletia*, *New Phytol.*, **90**, 57-65.

BROMFIELD E. S. P. & ROUGHLEY R. J., 1980. — Characterisation of rhizobia isolated from nodules on locally-adapted *Glycine max* grown in Nigeria. *Ann. Appl. Biol.*, **95**, 185-190.

CALLAHAM D., DEL TREDICI P. & TORREY J. G., 1978. — Isolation and cultivation *in vitro* of the actinomycete causing root nodulation in *Comptonia*. *Science*, **199**, 4331, 899-902.

DIEM H. G. & DOMMERGUES Y., 1983. — The isolation of *Frankia* from nodules of *Casuarina*. *Can. J. Bot.*, **61**, 2822-2825.

DIEM H. G., GAUTHIER D. & DOMMERGUES Y. R., 1982 *a*. — Isolation of *Frankia* from nodules of *Casuarina equisetifolia*. *Can. J. Microbiol.*, **28**, 526-530.

DIEM H. G., GAUTHIER D. & DOMMERGUES Y. R., 1982 *b*. — Isolement et culture *in vitro* d'une souche infective de *Frankia* isolée de nodules de *Casuarina*. *C. R. Acad. Sci. Paris*, Sér. III, **295**, 759-763.

DIEM G. H., GAUTHIER D. & DOMMERGUES Y. R., 1983. — An effective strain of *Frankia* from *Casuarina* sp. *Can. J. Bot.*, **61**, 2815-2821.

DIMITRI M. J., 1959. — Rhamnaceas. In: *Enciclopedia Argentina de Agricultura y Jardineria*, (L. PARODI, Ed.). Vol. 1, 573-574.

GAUTHIER D., DIEM H. G. & DOMMERGUES Y. R., 1981 *a*. — Infectivité et effectivité de souches de *Frankia* isolées de nodules de *Casuarina equisetifolia* et d'*Hippophaë rhamnoides*. *C. R. Acad. Sci. Paris*, Sér. III, 293, 489-491.

GAUTHIER D., DIEM H. G. & DOMMERGUES Y. R., 1981 *b*. — *In vitro* nitrogen fixation by two actinomycete strains isolated from *Casuarina* nodules. *Appl. Environ. Microbiol.*, 41, 306-308.

GAUTHIER D. L., DIEM H. G. & DOMMERGUES Y. R., 1983. — Tropical and Subtropical Actinorhizal Plants. In: *Proc. Symposium on Nitrogen Fixing Trees for The Tropics*. Rio de Janeiro, September 19-24, 1983 (in press).

GIBSON A. H., 1963. — Physical environment and symbiotic nitrogen fixation. The effect of root temperature on recently nodulated *Trifolium subterraneum* plants. *Aust. J. Biol. Sci.*, 28. 28-42,

HOAGLAND D. R. & ARNON D. I., 1950. — The water culture method for growing plants without soil. *Calif. Agric. Expt. Sta. Circ.*, 347.

KNOWLTON S., BERRY A. & TORREY J. G., 1980. — Evidence that associated soil bacteria may influence root hair infection of actinorhizal plants by *Frankia*. *Can. J. Microbiol.*, 26 (8), 971-977.

LALONDE M. & CALVERT H. E., 1979. — Production of *Frankia* hyphae and spores as an infective inoculant for *Alnus* species. In: *Symbiotic Nitrogen Fixation in the Management of Temperate Forests* (J. C. GORDON, C. T. WHEELER & D. A. PERRY Eds). Forest Research Laboratory, Corvallis, 95-110.

LALONDE M., CALVERT H. E. & PINE S., 1981. — Isolation and use of *Frankia* strains in actinorhizae formation. In: *Current Perspectives in Nitrogen Fixation*. (A. H. GIBSON & W. E. NEWTON Eds). Australian Academy of Science, Canberra, pp. 296-312.

LECHEVALIER M. P., 1983. — Cataloging *Frankia* strains. *Can. J. Bot.*, 61, 2964-2967.

MEDAN D. & TORTOSA R. D., 1976. — Nodulos radicales en *Discaria* y *Colletia* (Ramnaceas). *Bol. Soc. Argentina Bot.*, 17, 323-336.

MEDAN D., TORTOSA R. D., 1981. — Nodulos actinomicorricicos en especies Argentinas de los generos *Kentrothamnus*, *Trevoa* (Rhamnaceae) y *Coriaria* (Coriariaceae). *Bol. Soc. Argentina de Bot.*, 20, 71-81.

MIGUEL D., CANIZO A., COSTA A. & RODRIGUEZ-BARRUECO C., 1978. — Some aspects of the *Alnus*-type root nodule. In: Limitations and potentials for biological nitrogen fixation in the tropics (J. DOBEREINER, R. H. BURRIS & A. HOLLAENDER, Eds). Plenum Press, New York and London, 121-133.

POSTGATE J. R., 1972. — The acetylene reduction test for nitrogen fixation, p. 343-356. In: J. R. NORRIS & D. W. RIBBONS Eds, *Methods in microbiology*, Vol. 2 B. Academic Press, Inc., London.

RUNDEL W. R. & NEEL J. W., 1978. — Nitrogen fixation by *Trevoa trinervis* (Rhamnaceae) in the Chilean matorral. *Flora*, 167, 127-132.

TJEPKEMA J. D., ORMEROD W. & TORREY J. G., 1980. — Vesicle formation and acetylene reduction activity in *Frankia* sp. CpI1 culture in defined nutrient media. *Nature*, 287, 633-635.

TJEPKEMA J. D., ORMEROD W. & TORREY J. G., 1981 — Factors affecting vesicle formation and acetylene reduction (nitrogenase activity) in *Frankia* sp. CpI1. *Can. J. Microbiol.*, 27, 815-823.

ZHANG ZHONGZE, LOPEZ M. F. & TORREY J. G., 1984. — A comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of *Casuarina* species. *Plant Soil* 78, 79-90.