Eur. J. Soil Biol., 1999, **35** (1), 9-15 © 1999 Éditions scientifiques et médicales Elsevier SAS. All rights reserved
S1164556399001053/FLA

Occurrence of both *Casuarina*-infective and *Elaeagnus*-infective *Frankia* strains within actinorhizae of *Casuarina collina*, endemic to New Caledonia

Daniel Gauthier[a]*, Tanguy Jaffré[b], Yves Prin[a]

[a] *Laboratoire des symbioses tropicales et méditerranéennes (Cirad/ENSA-M/Inra/IRD), BP 5035, 34032 Montpellier cedex, France.*

[b] *Laboratoire de botanique, Centre IRD de Nouméa, BP A5 Nouméa cedex, New Caledonia.*

* *Corresponding author: (fax: +33 467593802)*

Received March 1, 1999; accepted September 7, 1999.

Abstract – New Caledonia is characterized by an exceptional concentration of endemic *Casuarinaceae* species including eight *Gymnostoma* species and *Casuarina collina*. *Frankia* strains isolated from nodules from *C. collina* have been studied through a range of techniques including molecular typing, infectivity/effectivity and host spectrum. All these isolates had characteristics similar to those of atypical *Frankia* from *Casuarina* previously described. This work evidenced several original features within these 26 isolates: (i) they are localized inside the nodule; (ii) they fit into the *Elaeagnus*-infective *Frankia* group; (iii) they belonged to four ITS groups already described for *Frankia* from *Gymnostoma*. In fact, it is highly probable that atypical *Frankia* from *C. collina* and *Frankia* from *Gymnostoma* are the same. We can formulate the hypothesis that the *Frankia* from *Gymnostoma* could use *C. collina* as an alternative host in absence of *Gymnostoma* spp. or when the ecological conditions are favourable to *C. collina*. © 1999 Éditions scientifiques et médicales Elsevier SAS

***Casuarinaceae* / infectivity / effectivity / nitrogen fixation / ITS 16S/23S / co-infection**

Résumé – Isolement et caractérisation de *Frankia* de *Casuarina collina* endémique à la Nouvelle-Calédonie. La Nouvelle-Calédonie possède une forte concentration en *Casuarinaceae* endémiques. On y dénombre huit espèces de *Gymnostoma* et une espèce de *Casuarina* : *C. collina*. Vingt-six souches de *Frankia* ont été isolées de nodules de *C. collina* et étudiées par diverses techniques incluant l'infectivité, l'effectivité, le spectre d'hôte et la PCR/RFLP de l'ITS 16S-23S. Tous ces *Frankia* présentent les mêmes caractéristiques que celles décrites chez les souches atypiques de *Casuarina* spp. Ce travail a mis en évidence, chez les *Frankia* de *C. collina*, les caractéristiques originales suivantes : (i) ils sont localisés à l'intérieur des nodules ; (ii) ils appartiennent au groupe des *Frankia* infectifs sur *Elaeagnus* ; (iii) ils se regroupent dans quatre groupes ITS déjà décrits chez *Gymnostoma*. En fait, il est très probable que les souches atypiques de *C. collina* et les *Frankia* de *Gymnostoma* soient les mêmes. On peut formuler l'hypothèse que les *Frankia* de *Gymnostoma* pourraient utiliser *C. collina* comme alternative en absence de leur hôte naturel ou quand les conditions écologiques sont favorables à *C. collina*. © 1999 Éditions scientifiques et médicales Elsevier SAS

***Casuarinaceae* / infectivité / effectivité / spectre d'hôte / fixation d'azote / ITS 16S-23S / co-infection**

1. INTRODUCTION

In spite of a reduced area (19 000 km^2), New Caledonia is characterized by the abundance of the endemic *Casuarinaceae* species since eight out of the eighteen *Gymnostoma* species and one *Casuarina* species have been described as belonging exclusively to the island flora [10]. These species are actinorhizal plants able to fix atmospheric nitrogen through a symbiotic association with the actinomycete *Frankia*.

The eight species of *Gymnostoma* are growing on soils originating from ultramafic rocks, with the exception of *G. nodiflorum* growing on alluvial soils which are of volcano-sedimentary origin. Ultramafic soils are

characterized by a very low level of P, K and Ca, and high levels of Ni and Mn. These soils present a slow mineralization of organic matter and poor level of available nitrogen.

Casuarina collina is the most common *Casuarinaceae* in New Caledonia. Natural stands frequently are riparian formations, but the species is abundant in secondary formations on a wide range of soils. In natural stands, it also occurs on veins of serpentinite included within metamorphic rock formations or on calcareous soils. As a secondary formation, *C. collina* can overrun disturbed lands thanks to its rapid growth together with its fire resistance and its ability to sucker. In this kind of conditions, it becomes rapidly the dominant species. For these reasons, *C. collina* has a high potential for rehabilitation of degraded lands in New Caledonia where nickel mining generates large areas of sterile and toxic lands.

The aims of this study were: (i) to isolate *Frankia* strains from *C. collina*; (ii) to characterize these strains from the symbiotic to the molecular aspect; and (iii) to test the behaviour of these strains on other endemic *Casuarinaceae.*

2. MATERIALS AND METHODS

2.1. Nodules harvest

Nodules of *C. collina* were harvested in their natural stands, mainly in the Province Sud, within a radius of about 100 km around Nouméa where all the *Gymnostoma* species are represented (*table I*).

2.2. *Frankia* strains isolation

Nodules were directly harvested from the roots of *C. collina*. Each lobe was individually rinsed under tap water, peeled and disinfected for 10 to 20 min in 30 % (wt/v) H_2O_2. They were rinsed with sterile distilled water before being cut into small fragments. All the fragments from one individual lobe were incubated at 30 °C, in one tube containing 5 mL nitrogen-free medium (NFM) or complex medium (CM) [12], for 1 to 3 months. This method allows to ascertain that each strain originates from one single original lobe and originates from the inside part of the lobe. The isolated strains, identified microscopically by the presence of the *Frankia* characteristic structures (hyphae, vesicles, sporangia and spores), were then subsequently cultured in CM medium.

2.3. *Frankia* strains used in this study

The *Frankia* strains used in this study were: (i) CJ-1-82, a typical *Frankia* strain isolated from *Casuarina equisetifolia* (i.e. able to nodulate the host of origin) [5]; (ii) D11, an atypical *Frankia* strain isolated from *C. equisetifolia* (i.e. strain unable to nodulate the host of origin but nodulating species from *Eleagnaceae* and *Rhamnaceae* families) [7, 8]; (iii) Gd03, a *Frankia* strain isolated from *Gymnostoma deplancheanum* (Gauthier, unpubl.).

2.4. Plant material

Seeds were sterilized by incubation in concentrated H_2SO_4 for 2 min. The seeds were rinsed out with sterile distilled water and germinated in sterile sand, in a shade house. We tested *C. collina*, *E. angustifolia* and *G. deplancheanum*. After 1 month, the seedlings were transferred into pots containing Tontouta sand and watered every 12 h. Two-month-old plants were fertilized with 1 $g \cdot L^{-1}$ standard Welgro nutrient solution (Ulvir limited, Barcelona, Spain).

2.5. Soil

The soil used in this study was the Tontouta soil from New Caledonia. It is a river sand derived from ultramafic rocks, with low P, K and Ca and high Ni levels. The C/N ratio is about 15 with a low organic decomposition and a poor N availability. All species studied in this study are able to grow on it. The soil has been sterilized by autoclaving at 120 °C for 2 h twice at a 24-h interval.

2.6. DNA extraction

2.6.1. From cultures

Extraction of DNA from 1-month-old *Frankia* strains was performed according to Brenner et al. [3] and Simonet et al. [17].

2.6.2. From nodules

Nodules lobes were disinfected with 30 % w/v H_2O_2 for 5 min, rinsed with sterile distilled water and kept at –20 °C. One nodule lobe was crushed in 500 mL TCP buffer (100 mM Tris-HCl, pH 8, 1.4 M NaCl, 20 mM EDTA, pH 8, 2 % w/v CTAB (Sigma, St Louis, MO, USA) and 3 % w/v PVPP (Sigma)). The mixture was incubated at 65 °C for 1 h and centrifuged at 3 000 × *g* for 5 min (20 °C). The supernatant was chloroform extracted and ethanol precipitated. The DNA pellet was dissolved in 10 mL TE buffer (pH 7.5) [12].

2.7. PCR amplification

Amplification of the 16S/23S inter-transcribed spacer (ITS) rRNA fragments were performed using primers

FGPL2054' (5'-CCGGGTTTCCCCATTCGG-3') (beginning of 23S, universal) [15] and FGPS989e (5'-GGGGTCCTTAGGGGCT-3') (end of 16S, *Eleagnus*-specific) [2] or FGPS989ac (5'-GGGGTCCG-TAAGGGTC-3') (end of 16S, *Casuarina*-specific) [2] as described by Simonet et al. [18] and Nazaret et al. [13]. PCR were run on Perkin-Elmer 2400 GeneAmp PCR Systems.

2.8. RFLP

Amplicons were compared after digestion with two restriction enzymes, *Hae*III and *Msp*I (Eurogentec). For both enzymes, digestion was performed on 10 µL PCR products at 37 °C for 2 h. Electrophoreses were made on 3 % agarose (Sigma) at 140 mV for 1 h.

2.9. Cross-inoculation studies

C. collina, *G. deplancheanum* and *E. angustifolia* were grown as already described, on the sandy soil of Tontouta. They were inoculated 4 months after germination with 10 mg (packed-cell volume) [14] of a 1-month-old *Frankia* culture or by 100 mg crushed nodules per plant. Each treatment was repeated four times and two non-inoculated controls were used for each plant species. Due to its very poor growth, *G. deplancheanum* was measured 11 months after inoculation. The two other species were measured 6 months after inoculation. Infectivity was qualitatively estimated by presence/absence of nodules for each plant, and effectivity was determined through the acetylene reduction method on the entire root system under standard procedures. Ethylene production was determined on a Carlo Erba GC6000 gas chromatograph. Results were expressed as µM $C_2H_4 \cdot h^{-1} \cdot g^{-1}$ nodule dry weight.

2.10. Co-inoculation study

For this study, we used two *Frankia* strains: (i) CJ-1-82 as a typical *Frankia* from *C. equisetifolia* and (ii) Cc01 as an atypical *Frankia* strain from *C. collina* belonging to the predominant ITS group 3. Inoculation was performed by delivering to each plant 10 mg inoculum consisting of pure strain or of an equal volume (packed-cell volume) mixture of the two selected strains. Ten inoculated plants and two uninoculated controls were used for each treatment. Eleven months after inoculation, nodules were harvested and used for isolation, tests of nodulation and DNA extraction for specific amplification.

3. RESULTS AND DISCUSSION

3.1. *Frankia* strains isolation

The isolation method we used (one nodule lobe peeled and disinfected per tube, liquid medium) had two main advantages: (i) it allowed to quickly and easily eliminate the contaminated lobes and (ii) it made sure that the isolate originates from the inside part of one lobe.

Strains were successfully isolated and in vitro propagated from eleven different natural stands of *C. collina* (*table I*). *Frankia* hyphae grow out nodule pieces, preferentially in the NFM medium (18 out of 26), within 2–3 months. All the isolated strains provided enough biomass to be efficiently subcultured. It has to be noted that the isolation seemed to be more successful on nitrogen-free medium (69 % of the successful isolations). This could be due to the early selection of nitrogen fixers (and non-proliferation of contaminants) as well as a better compatibility culture medium/*Frankia* strains. The percentage of successful isolation was about 8 %.

Table I. Origin of the *Frankia* isolated from nodules of *Casuarina collina*.

Frankia isolates from *C. collina*	Geographical origin	*Gymnostoma* species in the area
Cc01 to Cc06	Col de Yaté	*G. deplancheanum* *G. poissonianum*
Cc07 to Cc09	Prony	*G. deplancheanum*
Cc10 to Cc12	Plum	–
Cc13	Rivière des Pirogues	*G. leucodon*
Cc14	Rivière bleue	*G. webbianum*
Cc15 and Cc16	Ciu	*G. nodiflorum*
Cc17	Goro	–
Cc18 and Cc19	Piste du Humboldt	*G. chamaecyparis* *G. leucodon*
Cc20	Rivière Tontouta	*G. chamaecyparis* *G. leucodon* *G. poissonianum*
Cc21 and Cc22	Dzumac	*G. intermedium* *G. poissonianum*
Cc23 to Cc26	Nèpoui	*G. chamaecyparis*

Microscopic observations allowed to assess that the isolates belonged to the genus *Frankia* as they presented its characteristic structures including hyphae, sporangia, spores and particularly vesicles. Vesicles were very numerous in the NFM medium with a decrease of their number in presence of NH_4Cl. All the isolates were pigmented from orange to blood-red.

All the characteristics of these 26 strains are close to those of atypical *Frankia* strains isolated from nodules of *Casuarina* (i.e. strains unable to nodulate the host of origin but nodulating species from *Eleagnaceae* and *Rhamnaceae* families) [7, 8, 19].

Table II. Host spectrum of crushed nodules and *Frankia* strains from *Casuarina* spp. and *Gymnostoma* spp.

Inoculum	Plants tested[1]		
	Gymnostoma deplancheanum	*Casuarina collina*	*Elaeagnus angustifolia*
Crushed nodules			
Gymnostoma deplancheanum	I: 100 E: 76–212	NI	I: 75 E: 62–65
Casuarina collina	I: 75 E: 81–135	I: 100 E: 80–158	I: 75 E: 21–81
Frankia strains			
CJ-1-82 (typical *Frankia* from *C. equisetifolia*)	NI	I: 100 E: 10–22	NI
D11 (atypical *Frankia* from *C. equisetifolia*)	I: 75 E: 5–15	NI	I: 100 E: 8–17
Gd.03. (*Frankia* from *G. deplancheanum*)	I: 75 E: 15–37	NI	I: 100 E: 18–31
Twenty-six atypical *Frankia* from *Casuarina collina*			
ITS group 1 (1 strain)	I: 75 E: 8–12	NI	I: 100 E: 15–36
ITS group 2 (5 strains)	I: 75–100 E: 10–25	NI	I: 75–100 E: 26–37
ITS group 3 (16 strains)	I: 75–100 E: 15–25	NI	I: 75–100 E: 12–16
ITS group 4 (4 strains)	I: 75–100 E: 6–11	NI	I: 75–100 E: 8–27

[1] Four repetitions for each species.
I, Infective (% of nodulation, extreme values); E, effective (mmoles of $C_2H_4 \cdot h^{-1} \cdot g^{-1}$ nodules dry weight, extreme values); NI, non-infective.

In spite of repeated efforts, we were unable to isolate a typical (i.e. able to nodulate the host of origin) strain of *Frankia* from *C. collina*. For this reason, we used the strain CJ-1-82 [5] as a typical *Frankia* strain in this study.

3.2. Molecular characterization of the rRNA operon of the isolates

3.2.1. PCR amplification

The DNA extraction protocol yielded DNA that was pure enough to obtain amplification with primer FGPS989e, which is *Elaeagnus*-specific, and no amplification was obtained with primer FGPS989ac, which is *Casuarina*-specific.

A DNA fragment of about 1 000 bp corresponding to the 16S-23S ITS was obtained for all the 26 DNA from *Frankia* isolates. These results confirm that these strains belong to the *Elaeagnus*-infective *Frankia* group.

3.2.2. RFLP

The two restriction enzymes, *Hae*III and *Msp*I, were selected on the number of fragments they generated in our amplicons. Each enzyme gave three profiles which together allowed to discriminate four different groups among the isolates (*table II*). The groups were named 1, 2, 3 and 4. ITS group 3 (sixteen strains) was predominant while ITS group 1 was in minority (one strain). ITS groups 2 and 4 had respectively five and four strains. These four ITS groups were identical with those of *Frankia* isolated from *Gymnostoma* spp. (Gauthier, unpubl.) (*figure 1*).

3.3. Cross-inoculation tests

Nodulation tests were conducted by inoculating each of the host plant species with crushed nodules or with

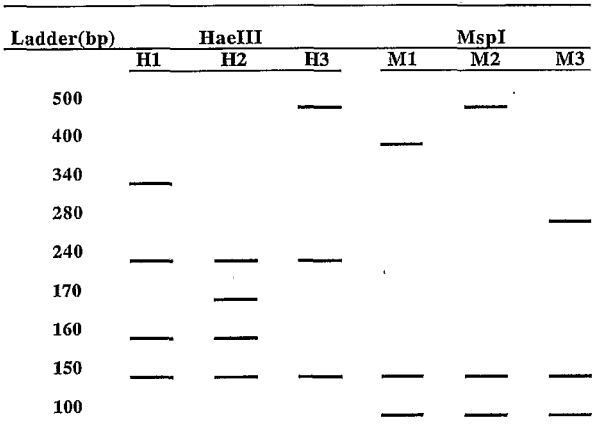

Figure 1. PCR/RFLP patterns of the 16S-23S ITS after digestion with *Hae*III (H) and *Msp*I (M). ITS group 1, H1, M1; ITS group 2, H1, M2; ITS group 3, H2, M3; ITS group 4, H3, M1.

different *Frankia* strains. Uninoculated controls did not nodulate (*table II*).

Crushed nodules from *C. collina* nodulated the three plant species while crushed nodules from *G. deplancheanum* nodulated only *G. deplancheanum* and *E. angustifolia.*

The 26 strains isolated from *C. collina* share in common, with strains D11 (atypical *Frankia* from *Casuarina*) and Gd03 (*Frankia* from *G. deplancheanum*), the same host spectrum: they were able to nodulate *G. deplancheanum* and *E. angustifolia* but not *C. collina*. The four ITS groups were found to be infective and effective on *G. deplancheanum* and *E. angustifolia*. They were non-infective on *C. collina.* No significative differences were found both in infectivity and effectivity between the different groups. The strain CJ-1-82 can nodulate only *C. collina*. It has to be noted that the ARA values of the nodules obtained after inoculation with any of the *Frankia* strains were constantly lower than those obtained after inoculation with crushed nodules.

The host spectrum of crushed nodules (peeled and disinfected) from *C. collina* seems to be the addition of both host spectrum from strains CJ-1-82 (typical *Frankia* from *Casuarina*) and D11 (atypical *Frankia* from *Casuarina*). This result suggests the presence of both typical and atypical *Frankia* within the nodules of *C. collina*.

These data permitted to assess that the 26 atypical *Frankia* strains isolated from *C. collina* belonged to the *Elaeagnus*-infective *Frankia* group like the others atypical *Frankia* isolated from *Casuarina* spp. [7, 8, 19], the *Frankia* from *Elaeagnaceae* or *Rhamnaceae* [4, 9, 13] and the *Frankia* from *Gymnostoma* ([12]; Gauthier, unpubl.).

3.4. DNA characterization of isolated and non-isolated (in nodule) *Frankia*

The PCR amplifications were performed by using two sets of primers (FGPL2054'/FGPS989e, *Elaeagnus*-specific and FGPL2054'/FGPS989ac, *Casuarina*-specific) targeting the 16S-23S ITS.

The PCR results of the different DNA origin/specific primer combinations confirmed those of the cross-inoculation study except for the DNA from nodule from *C. collina* (*table III*).

It was obvious that the strain CJ-1-82 belonged to the *Casuarina*-infective *Frankia* group while the strains D11, Gd03 and the 26 atypical *Frankia* from *C. collina* belonged to *Elaeagnus*-infective *Frankia* group. The DNA extracted from nodule from *Gymnostoma deplancheanum* amplified only with *Elaeagnus*-specific primer. This data was in conformity with the host spectrum of the crushed nodules from *G. deplancheanum* (able to nodulate *Gymnostoma* and *Elaeagnus* but not *Casuarina*).

Table III. DNA characterization of isolated and non-isolated (in nodule) *Frankia.*

DNA	PCR[1]	
	Elaeagnus specific primer (FGPS989e)	*Casuarina* specific primer (FGPS989ac)
Nodules[2]		
Casuarina collina	–	+
Gymnostoma deplancheanum	+	–
Frankia strains		
CJ-1-82 (typical *Frankia* from *C.equisetifolia*)	–	+
D11 (atypical *Frankia* from *C.equisetifolia*)	+	–
Gd03 (*Frankia* from *G. deplancheanum*)	+	–
Twenty-six atypical *Frankia* from *C.collina*	+	–

[1] ITS 16S-23S; (–), no amplification; (+), positive amplification.
[2] Nodule lobes peeled and disinfected.

The DNA extracted from nodules from *C. collina* amplified only with *Casuarina*-specific primer. This result was not in agreement with the host spectrum of the crushed nodule from *C. collina* (able to nodulate *Casuarina*, *Gymnostoma* and *Elaeagnus*) suggesting the presence of both typical and atypical *Frankia* inside the natural nodule of this species. Such a conflicting situation could be explained by a very low level of atypical *Frankia* inside the nodule of *C. collina* which could preserve both the infectibility and the high isolability of this type of *Frankia* but was insufficient to be detected by PCR.

3.5. Co-infection with atypical and typical *Frankia*

For this study, we used two *Frankia* strains: (i) CJ-1-82 as a typical *Frankia* from *C. equisetifolia* and (ii) Cc01 as an atypical *Frankia* strain from *C. collina* belonging to the predominant ITS group 3. Each plant was inoculated using the two *Frankia* strains alone or in mixture (50–50 %). Uninoculated controls did not nodulate (*table IV*).

In the case of *C. collina,* the nodulation was obtained after inoculation by CJ-1-82 or by the mixture 'CJ-1-82 + Cc01'. Cc01 alone was not able to nodulate.

Using nodules from *C. collina* obtained after inoculation with CJ-1-82, re-isolation of this strain was successfully performed (2/50) and no atypical *Frankia* strain was isolated. The DNA extracted from these nodules amplified only with the *Casuarina*-specific

Table IV. Co-infection with atypical and typical *Frankia*.

Plants tested[1]	Inoculum[2]	Nodulation[3]	Characterization of nodules obtained				
			Host specificity[4]	PCR[5]		Isolation[6]	
				Elaeagnus specific primer (FGPS989e)	*Casuarina* specific primer (FGPS989ac)		
Casuarina collina	CJ-1-82	I,E	Cas^+Gym^-	–	+	(+) CJ-1-82	(2/50)
	Cc01	NI					
	CJ-1-82 + Cc01	I,E	Cas^+Gym^+	–	+	(+) CJ-1-82	(1/50)
						(+) Cc01	(8/50)
Gymnostoma deplancheanum	CJ-1-82	NI					
	Cc01	I,E	Cas^-Gym^+	+	–	(+) Cc01	(6/50)
	CJ-1-82 + Cc01	I,E	Cas^-Gym^+	+	–	(+) Cc01	(5/50)
						(–) CJ-1-82	(0/50)

[1] Ten plants for each test. [2] CJ-1-82, typical *Frankia* from *Casuarina equisetifolia*; Cc01, atypical *Frankia* from *C. collina* (ITS group 3). [3] I, Infective (estimated as % of nodulation); E, effective (estimated as ARA); NI, non-infective. [4] (+), Crushed nodule infective and effective on the tested species: *Cas* (*Casuarina collina*) and *Gym* (*Gymnostoma deplancheanum*); (–), crushed nodule non-infective. [5] ITS 16S-23S: (–), no amplification; (+), positive amplification. [6] Isolation from fifty nodule lobes obtained after inoculation or co-inoculation. (+), Successful isolation; (–), no isolates obtained.

primer. Using these nodules peeled and crushed as inoculum, it was possible to nodulate *C. collina* but not *E. angustifolia* nor *G. deplancheanum*. These data demonstrated that the atypical *Frankia* could not appear spontaneously and was not a putative mutation of the typical *Frankia*.

Using nodules from *C. collina* obtained after inoculation by the mixture 'CJ-1-82 + Cc01', re-isolation was performed for CJ-1-82 (1/50) and for Cc01 (8/50). These results illustrates the high isolability of the atypical *Frankia* and the difficulty of isolating typical *Frankia* even with the strain CJ-1-82, cultivated in vitro since 1982 [5]. The DNA extracted from these nodules amplified only with the *Casuarina*-specific primer (as the natural nodules from *C. collina*). Using these nodules peeled and crushed as inoculum, it was possible to nodulate *C. collina*, *G. deplancheanum* and *E. angustifolia* (like the natural nodules from *C. collina*). These data demonstrated the occurrence of both typical and atypical *Frankia* inside the nodule of *C. collina* in contradiction with PCR results which failed to detect the atypical *Frankia*.

In the case of *G. deplancheanum*, nodulation was obtained after inoculation with Cc01 alone or with the mixture 'CJ-1-82 + Cc01'. Inoculation by CJ-1-82 alone did not induce nodulation.

Using nodules from *G. deplancheanum* obtained after inoculation with Cc01, re-isolation of this strain was successfully performed (6/50). The DNA extracted from these nodules amplified only with the *Elaeagnus*-specific primer. Using these nodules peeled and crushed as inoculum, it was possible to nodulate *G. deplancheanum* and *E. angustifolia* but not *C. collina* (like natural nodules from *G. deplancheanum*).

Using nodules from *G. deplancheanum* obtained after inoculation with the mixture 'CJ-1-82 + Cc01', the re-isolation was possible for Cc01 (5/50) but not for CJ-1-82 (0/50). The DNA extracted from these nodules amplified only with the *Elaeagnus*-specific primer. Using these nodules peeled and crushed as inoculum, it was possible to nodulate *G. deplancheanum* and *E. angustifolia* but not *C. collina* (like natural nodules from *G. deplancheanum*). These data suggested that typical *Frankia* (CJ-1-82) would not participate to nodulation of *G. deplancheanum*.

Using the mixture 'CJ-1-82 + Cc01' as inoculum, we established that co-infection could occur with *C. collina* but not with *G. deplancheanum*.

There are two known pathways by which *Frankia* can infects actinorhizal plants:

– within plants belonging to the *Casuarinaceae* family (like in *Betulaceae*) infection occurs through the 'root hair infection' (RHI) process. This process of infection was described in the case of: (i) *Casuarina equisetifolia* [6]; (ii) *Gymnostoma papuanum* [16]; and (iii) *Alnus rubra* [1].

– within plants belonging to *Elaeagnaceae* family, infection occurs through an intercellular penetration (IP) process as described in the case of *Elaeagnus angustifolia* [11].

The *Casuarinaceae* family can establish a symbiotic association with *Frankia* belonging both to the *Casuarina*-infective group (i.e. typical *Frankia*) [6] and to the *Elaeagnus*-infective group (i.e. atypical *Frankia* and *Frankia* from *Gymnostoma*) [8, 12].

The infection process in *Gymnostoma* spp. is the RHI [16] in spite the fact that the *Frankia* of *Gymnostoma* belongs to the group of the *Elaeagnus*-infective *Frankia*.

From other papers [6, 16], we can assess that nodulation of *C. collina* by strain CJ-1-82 like nodulation of *G. deplancheanum* by Cc01 strain probably occurs via

RHI. Although strains CJ-1-82 and Cc01 use the same infection process (RHI), the result of their co-inoculation (CJ-1-82 + Cc01) seems to be different on *C. collina* (co-infection) and on *G. deplancheanum* (infected only by Cc01). These results suggest that the infection process could be under the control of the plant.

An interesting subject of research would be to investigate the means used by Cc01 to infect *Elaeagnus angustifolia* (RHI or IP) indicating which partner controls the mode of entry of *Frankia* in the root. This kind of work could also help to understand how the co-infection by both kind of strains can occur in *C. collina*.

The present study of *Frankia* isolated from *C. collina* allowed to demonstrate that (i) atypical *Frankia* are inside nodules from *C. collina*, (ii) they belong to the *Elaeagnus*-infective *Frankia* group, (iii) their host spectrum and their ITS groups (determined by PCR/RFLP) are the same as those of *Frankia* from *Gymnostoma*.

In New Caledonia, *C. collina* is the only endemic species from the genus *Casuarina* in a territory which includes eight species from the *Gymnostoma* genus. We can formulate the hypothesis that the *Frankia* from *Gymnostoma* could use *C. collina* as an alternative host in absence of *Gymnostoma* spp. or when the ecological conditions are more favourable to *C. collina*.

Acknowledgements

The authors are indebted to B. Dreyfus and E. Giraud for helpful discussions.

REFERENCES

[1] Berry A., McIntyre L., McCully M., Fine structure of root hair infection leading to nodulation in the *Frankia-Alnus* symbiosis, Can. J. Bot. 64 (1986) 292–305.

[2] Bosco M., Fernandez M.P., Simonet P., Materassi R., Normand P., Evidence that some *Frankia* sp. strains are able to cross boundaries between *Alnus* and *Elaeagnus* host specificity groups, Appl. Environ. Microbiol. 58 (1992) 1569–1576.

[3] Brenner D.J., McWorter A.C., Leete Knutson J.K., Steigerwalt A.G., *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds, J. Clin. Microbiol. 15 (1982) 1133–1140.

[4] Clawson M.L., Caru M., Benson D.R., Diversity of *Frankia* strains in root nodules of plants from families *Elaeagnaceae* and *Rhamnaceae*. Appl. Environ. Microbiol. 64 (1998) 3539–3543.

[5] Diem H.G., Gauthier D.L., Dommergues Y.D., Isolation of *Frankia* from nodules of *Casuarina equisetifolia*, Can. J. Microbiol. 28 (1982) 526–530.

[6] Diem H.G., Gauthier D.L., Dommergues Y.D., An effective strain of *Frankia* from *Casuarina* sp., Can. J. Bot. 61 (1983) 2815–2821.

[7] Gauthier D.L., Diem H.G., Dommergues Y.D., *In vitro* nitrogen fixation by two actinomycete strains isolated from *Casuarina* nodules, Appl. Environ. Microbiol. 41 (1981) 306–308.

[8] Gauthier D.L., Diem H.G., Dommergues Y.D., Infectivité et effectivité des souches de *Frankia* isolées de nodules de *Casuarina equisetifolia* et de *Hippophae rhamnoides*, C. R. Acad. Sci. 293 (1981) 489–491.

[9] Gauthier D.L., Frioni L., Diem H.G., Dommergues Y.D., The *colletia spinosissima-Frankia* symbiosis, Acta Oecol. Plant. 5 (1984) 231–239.

[10] Jaffré T., Gauthier D., Rigault F., McCoy S., Les *Casuarinaceae* endémiques. Bois et Forêts des Tropiques, Dossier Nouvelle Calédonie 242 (1994) 31–42.

[11] Miller I., Baker D., The initiation, development and structure of root nodules in *Elaeagnus angustifolia* L., (*Elaeagnaceae*), Protoplasma 128 (1985) 107–119.

[12] Navarro E., Nalin R., Gauthier D., Normand P., The nodular microsymbionts of *Gymnostoma* spp. are *Elaeagnus*-infective *Frankia* strains, Appl. Environ. Microbiol. 63 (1997) 1610–1616.

[13] Nazaret S., Cournoyer B., Normand P., Simonet P., Phylogenetic relationships among *Frankia* genomic species determined by use of amplified 16S rDNA sequences, J. Bacteriol. 173 (1991) 4072–4078.

[14] Nittayajarn A., Baker D., Methods for the quantification of *Frankia* cell biomass, Plant Soil 118 (1989) 199–204.

[15] Ponsonnet C., Nesme X., Identification of *Agrobacterium* strains by PCR-RFLP analysis of pTi and chromosomal regions, Arch. Microbiol. 161 (1994) 300–309.

[16] Racette S., Torrey J., The isolation, culture and infectivity of a *Frankia* strain from *Gymnostoma papuanum* (*Casuarinaceae*), Plant Soil 118 (1989) 165–170.

[17] Simonet P., Capellano A., Navarro E., Bardin R., Moiroud A., An improved method for lysis of *Frankia* with Achromopeptidase allows detection of new plasmids, Can. J. Microbiol. 30 (1984) 1292–1295.

[18] Simonet P., Grosjean M.C., Misra A.K., Nazaret S., Cournoyer B., Normand P., *Frankia genuus* specific characterization by polymerase chain reaction, Appl. Environ. Microbiol. 57 (1991) 3278–3286.

[19] Zhang Z., Lopez M.F., Torrey J.G., A comparison of cultural characteristics and infectivity of *Frankia* isolates from roots nodules of *Casuarina* species, Plant Soil 78 (1984) 79–90.