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Abundance of *Frankia* from *Gymnostoma* spp. in the rhizosphere of *Alphitonia neocaledonica*, a non-nodulated *Rhamnaceae* endemic to New Caledonia

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Abstract – The capacity of different soils of New Caledonia to induce nodulation in *Gymnostoma poissonianum* was studied. The soils were sampled under five *Gymnostoma* species, *Alphitonia neocaledonica* (a non-nodulated endemic Rhamnaceae) and *Pinus caribea* (an introduced species) growing in various ecological conditions. Using *G. poissonianum* as trap-host, we observed a higher abundance of *Frankia* from *Gymnostoma* spp. in the rhizosphere of *A. neocaledonica* as compared with bare soils and *P. caribea* rhizosphere. The nodulating capacity of *A. neocaledonica* rhizosphere was almost similar to that of the five *Gymnostoma* species (symbiotic host) studied in the same stations. In comparison, bare soils or rhizosphere of *P. caribea* had poor nodulating capacities. We isolated fourteen *Frankia* strains from nodules of *G. poissonianum* after baiting with the rhizospheric soils of five *Gymnostoma* and *A. neocaledonica*. Using the PCR/RFLP method, we confirmed the similarity with those already described. *Frankia* was abundant in the rhizosphere of *A. neocaledonica* in all the sites studied. One explanation could be a positive tropism of *Frankia* towards species belonging to families having nodulated species, which is the case of *A. neocaledonica* endemic in New Caledonia. We can suppose that the non-nodulated plants belonging to these families can excrete some chemical substances able to attract *Frankia* and to induce its proliferation. © 2000 Éditions scientifiques et médicales Elsevier SAS

***Frankia* / *Gymnostoma* / *Alphitonia neocaledonica* / *Pinus caribea* / nodulating capacity / riparian soils / maquis / paraforest maquis / trapping / endemic / New Caledonia / nitrogen fixation / PCR-RFLP**

1. INTRODUCTION

In spite of its reduced area (19 000 km^2), New Caledonia harbours species belonging of three actinorhizal families: Casuarinaceae, Rhamnaceae and Myricaceae. Actinorhizal plants species belonging to eight families and 25 genera of non-leguminous form root nodules with the nitrogen fixing actinomycete *Frankia* [1]. The Rhamnaceae are represented by six genera (*Colubrina*, *Emmenospora*, *Gouania*, *Rhamnela*, *Ventilago*, *Alphitonia*) with ten non-nodulated species, among which seven are endemic. The only endemic Myricaceae (*Canacomyrica monticola*) is also non-nodulated. On the contrary, the endemic Casuarinaceae species are abundant since eight of the eighteen *Gymnostoma* species have been described as belonging exclusively to the island flora. *Gymnostoma* appears to be well nodulated in nature [10]. Five species (*G. leucodon*, *G. webbianum*, *G. chamaecyparis*, *G. deplancheanum* and *G. intermedium*) are frequently associated with the endemic non-nodulated *Alphitonia neocaledonica* (Rhamnaceae) in various ecological conditions such as riparian, maquis and paraforest maquis soils. These ultramafic soils are characterized by a very low level of P, K and Ca, a high level of Ni and Mn, a slow mineralization of organic matter, and a poor level of available N. *A. neocaledonica* is a fast-growing pioneer plant able to grow up to 2 to 3 m high in poor land or 25–30 m in

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humid forests. A previous study did not show any definite difference in the foliar analysis of *A. neocaledonica* and *Gymnostoma* species [10]. There are numerous reports of *Frankia* occurrence in soils lacking nodulated host plants [5, 9, 23–25] but only in temperate and non-endemic conditions. The aim of this study was to compare the populations of *Frankia* from *Gymnostoma* estimated in the rhizosphere of *A. neocaledonica* (an endemic Rhamnaceae) with those of the host plants (*Gymnostoma* spp.) and of *Pinus caribea* (an introduced species).

2. MATERIALS AND METHODS

2.1. Soils

The soils used to trap *Frankia* were sampled under various plants in three types of New Caledonian sites:
– Riparian sites. Soils under *G. leucodon* and *A. neocaledonica* in Rivière des Pirogues and under *G. webbianum* and *A. neocaledonica* in Rivière Bleue.
– Maquis sites. Soils under *G. chamaecyparis* and *A. neocaledonica* in Monts de la Tontouta and under *G. deplancheanum*, *A. neocaledonica* and *Pinus caribea* in Plaine des lacs.
– Paraforest maquis site. Soils under *G. intermedium* and *A. neocaledonica* in Monts Dzumac.

Each soil was divided in two fractions:
– Rhizospheric soil: RS (1 kg).
– Rhizospheric soil (1 kg) mixed with crushed root (100 g without nodules): RS+CR. This fraction is designated as the rhizosphere of the plant studied.

At each station, we also sampled soil in zones without plants of the actinorhizal families as a control (without plant soil: WPS).

To compare the nodulating capacity of the rhizosphere to the crushed nodules of the five *Gymnostoma* species studied, we used soils of each station sterilized by autoclaving at 120 °C twice at 24-h interval and inoculated with 50 g fresh nodule weight for 1 kg soil. Nodules were disinfected for 10 to 20 min in 30 % (w/v) H_2O_2. Controls were sterilized soils.

2.2. Plant material

The absence of specificity in the *Frankia-Gymnostoma* symbiosis is well documented [8, 13]. The seeds of *G. poissonianum* are easy to harvest and to germinate unlike other *Gymnostoma* species, so we used this species, adaptable to all the soils studied, as trap-host. Seeds were sterilized by incubation in concentrated H_2SO_4 for 2 min. They were rinsed with sterile distilled water and germinated in sterile sand in a shade house. After 1 month, the seedlings were transferred into pots containing 1 kg of the different fractions of the soils studied and watered every 12 h. Experiments were conducted in triplicate pots containing four plants each. Due to the very poor growth of *G. poissonianum*, the number of nodulated plants and the total number of nodules were measured after 11 months.

2.3. Infectivity and effectivity

Infectivity was estimated by the total number of nodules for each treatment, and effectivity was determined through the acetylene reduction activity method (ARA) on six nodules. Ethylene production was determined on a Carlo Erba GC6000 gas chromatograph. Results were expressed as $\mu M\ C_2H_4 \cdot h^{-1} \cdot g^{-1}$ nodule dry weight.

2.4. Isolation of *Frankia* strains

Nodules were harvested from the roots of *G. poissonianum*. For each treatment done with the rhizosphere of the various plants studied, fifty nodule lobes were individually rinsed under tap water, disinfected for 10 to 20 min in 30 % (w/v) H_2O_2 and 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) [13], for 1 to 3 months. This method allows to ascertain that each strain originates from one single original lobe [7]. The isolated strains, identified microscopically by the presence of the *Frankia* characteristic morphological structures, including hyphae, vesicles, sporangia and spores, were then subsequently cultured in the CM medium.

2.5. DNA extraction from cultures

Extraction of DNA from 1-month-old *Frankia* strains was performed according to Brenner et al. [3] and Simonet et al. [21] as already successfully used with *Frankia* strains from *Gymnostoma* spp. [8].

2.6. PCR amplification

For *Frankia* strains belonging to the Elaeagnaceae-group [8, 13, 14], amplification of the 16S/23S Inter-Transcribed Spacer (ITS) rRNA fragment was performed using primers FGPL2054' (5'-CCG-GGT-TTC-CCC-ATT-CGG-3') (beginning of 23S, universal) [17] and FGPS989e (5-GGGGTCCTTA-GGGGCT-3') (end of 16S, Eleagnaceae-specific) [2]. PCR were run on a Perkin-Elmer 2400 GeneAmp PCR System.

2.7. RFLP

Restriction analyses of the PCR products were performed with three enzymes: *Hae*III, *Msp*I and *Cfo*I. Digestion was performed on 10 µL PCR products at 37 °C for 2 h. Electrophoresis was carried out on 3 % agarose gels (Sigma) at 140 mV for 1 h.

Table I. Localities, soils and plants studied.

Localities	Soils	Plants studied
Riparian sites		
Rivière des Pirogues	Recent fluvial alluvium	*Gymnostoma leucodon, Alphitonia neocaledonica*
Rivière Bleue	Ultramafic alluvium	*G. webbianum, A. neocaledonica*
Maquis sites		
Plaine des lacs	Oxisol	*G. deplancheanum, A. neocaledonica, Pinus caribea*
Monts de la Tontouta	Hypermagnesian inceptisol	*G. chamaecyparis, A. neocaledonica*
Paraforest Maquis sites		
Monts Dzumac	Colluvial acid	*G. intermedium, A. neocaledonica*

3. RESULTS AND DISCUSSION

The location of the study sites is shown *table I*. In all sites, *Gymnostoma* spp. were the dominant species.

The methodology used did not allow the quantification of populations of *Frankia* in the soils studied but allowed the comparison of the potential *Frankia* reservoir in the different part of the soils under different plants. As mentioned by Smolander [22], this method underestimates the real nodulating capacity of the soils studied.

3.1. Nodulating capacity of riparian soils

The number of nodulated plants (one to two) and the total number of nodules (seven to eleven) obtained on *G. poissonianum* grown on bare soil (WPS) were very low (*table II*). The fact that *Frankia* was present in bare soil could be explained by its capacity to survive in the absence of actinorhizal plants [19, 22] probably due to his saprophytic ability and capacity to sporulate. In this dormant form, *Frankia* can remain infectious [15].

In the case of *G. leucodon* and *G. webbianum* soils, the number of nodulated plants and the total number of nodules (measured by trapping *Frankia* with *G. poissonianum*) increased when the trapping was done on rhizospheric soil (RS) and on the rhizosphere (rhizospheric soil mixed with crushed roots: RS+CR). This result could be explained by the well-known rhizosphere effect and by the extranodular growth ability of *Frankia* [6].

The inoculation of *G. poissonianum* by crushed nodules allowed a slightly better result revealing a high level of *Frankia* infective cells in the rhizosphere of the *Gymnostoma* species growing on riparian soils.

In the case of *A. neocaledonica*, an increase in the number of nodulated plants and the total number of nodules when rhizospheric soil or rhizosphere were used for trapping compared with bare soil was also

Table II. Estimation of *Frankia* population from riparian soils sampled under various plants using *Gymnostoma poissonianum* as trap-host (twelve repetitions). nd, no data. WPS, Without plant soil; RS, rhizospheric soil; CR, crushed root (root without nodules); CN, crushed nodules.

Localities and species	Number of nodulated plants (twelve plants tested)	Total number of nodules	ARA[(1)]
Rivière des Pirogues			
Gymnostoma leucodon			
WPS	2	10	31–68
RS	6	25	37–61
Rhizosphere: (RS+CR)	9	47	nd
CN	10	75	28–70
Alphitonia neocaledonica			
WPS	2	11	27–42
RS	5	21	30–60
Rhizosphere: (RS+CR)	4	32	41–59
Rivière bleue			
Gymnostoma webbianum			
WPS	2	11	42–90
RS	5	30	nd
Rhizosphere: (RS+CR)	8	45	25–72
CN	12	81	41–59
Alphitonia neocaledonica			
WPS	1	7	15–58
RS	3	25	31–70
Rhizosphere: (RS+CR)	5	31	27–81

(1) ARA as µmoles $C_2H_4 \cdot h^{-1} \cdot g^{-1}$ nodules dry weight (extreme values).

Table III. Estimation of *Frankia* population from paraforest maquis and maquis soils sampled under various plants using *G. poissonianum* as trap-host (twelve repetitions). nd, no data. WPS, Without plant soil; RS, rhizospheric soil; CR, crushed root (root without nodules); CN, crushed nodules.

Localities and species	Number of nodulated plants (twelve plants tested)	Total number of nodules	ARA[(1)]
Monts Dzumac			
Gymnostoma intermedium			
WPS	2	11	nd
RS	5	40	70–95
Rhizosphere: (RS+CR)	8	65	35–110
CN	11	100	42–105
Alphitonia neocaledonica			
WPS	1	6	58–75
RS	3	25	47–81
Rhizosphere: (RS+CR)	5	41	31–92
Monts de la Tontouta			
Gymnostoma chamaecyparis			
WPS	1	nd	nd
RS	4	19	32–98
Rhizosphere: (RS+CR)	7	41	51–120
CN	10	71	38–101
Alphitonia neocaledonica			
WPS	2	nd	nd
RS	4	23	49–108
Rhizosphere: (RS+CR)	5	31	37–99
Plaines des lacs			
Gymnostoma deplancheanum			
WPS	2	17	18–61
RS	5	35	31–72
Rhizosphere: (RS+CR)	9	70	nd
CN	12	101	25–89
Alphitonia neocaledonica			
WPS	2	20	22–57
RS	4	30	31–70
Rhizosphere: (RS+CR)	6	61	37–82
Pinus caribea			
WPS	1	8	18–49
RS	2	10	27–57
Rhizosphere: (RS+CR)	1	7	18–70

(1) ARA as μmoles $C_2H_4 \cdot h^{-1} \cdot g^{-1}$ nodules dry weight (extreme values).

noted. This result showed a significant high density of *Frankia* in soils under *A. neocaledonica* although it is a non-nodulated Rhamnaceae. The fact that *A. neocaledonica* is a Rhamnaceae could explain this phenomenon already described for *Betula pendulata* (non-nodulated Betulaceae) and *Alnus* (nodulated Betulaceae) by Smolander [22].

The effectiveness of all the nodules obtained were equivalent, suggesting that the *Frankia* population of the riparian soils under *Gymnostoma* and *A. neocaledonica* were similar.

All the *G. poissonianum* growing on sterilized soils from these two sites died after a few months and did not nodulate.

3.2. Nodulating capacity of paraforest maquis and maquis soils

In the case of bare soils (WPS) of paraforest maquis and maquis sites, the number of nodulated plants and the total number of nodules obtained were also very low (*table III*) confirming a low level of *Frankia* population in non-rhizospheric soil.

For the three *Gymnostoma* species studied in these sites, the number of nodulated plants and the total number of nodules increased in the rhizospheric soils (RS to RS+CR) as already observed with riparian soils.

The result of inoculation with crushed nodules is only slightly better.

As already observed in riparian soils, the effectiveness (ARA) of the nodules obtained after inoculation by different fractions of soils and crushed nodules were similar.

For *A. neocaledonica* soils in these three sites, the number of nodulated plants and the total number of nodules also increased with the rhizospheric soil (RS to RS+CR) as already observed in riparian soils.

Table IV. Isolation of *Frankia* strains trapped by *Gymnostoma poissonianum* on rhizospheres sampled under five *Gymnostoma* species, *Alphitonia neocaledonica* and *Pinus caribea*. Isolations were done on fifty nodule lobes. TA, *Frankia* trapped on rhizosphere of *A. neocaledonica*; TG, *Frankia* trapped on rhizosphere of *Gymnostoma* spp.

Localities and rhizospheres tested (RS+CR)	Number of nodules obtained on *G. poissonianum*	Number of *Frankia* strains isolated and ITS group
Rivière des Pirogues		
Gymnostoma leucodon	47	2 TGl.1 (ITS.2), TGl.2 (ITS.3)
Alphitonia neocaledonica	32	1 TA.1 (ITS.2)
Rivière Bleue		
G. webbianum	45	1 TGw.1 (ITS.3)
A. neocaledonica	31	1 TA.2 (ITS.3)
Monts Dzumac		
G. intermedium	65	1 TGi.1 (ITS.3)
A. neocaledonica	41	2 TA.3 (ITS.2), TA.4 (ITS.2)
Monts de la Tontouta		
G. chamaecyparis	41	1 TGc.1 (ITS.4)
A. neocaledonica	31	1 TA.5 (ITS.4)
Plaine des lacs		
G. deplancheanum	70	3 TGd.1 (ITS.1), TGd.2 (ITS.2), TGd.3 (ITS.2)
A. neocaledonica	61	1 TA.6 (ITS.3)
Pinus caribea	7	0
Total *Gymnostoma* spp.	268	8
Total *Alphitonia neocaledonica*	196	6
Total *Pinus caribea*	7	0

All the *G. poissonianum* growing on sterilized soils from these three sites died after a few months and did not nodulate.

3.3. Comparison between *Frankia* populations in the rhizosphere (RS+CR fraction) sampled under *Gymnostoma* spp., *A. neocaledonica* and *Pinus caribea*

In the site of Plaine des lacs, a third species was present: *P. caribea*.

We did not observe an increase in the *Frankia* population in the rhizosphere sampled under *P. caribea* as compared with the five *Gymnostoma* studied and *A. neocaledonica* (*table IV*). The number of nodules obtained after trapping *Frankia* on rhizosphere (RS+CR) of *A. neocaledonica* was 73 % of that observed in *Gymnostoma* species and was only 2.6 % for *P. caribea*. In the case of *A. neocaledonica*, the simple rhizosphere effect is not sufficient to explain the *Frankia* abundance of the rhizosphere noted in all the stations studied. One explanation could be the natural attraction of *Frankia* to species belonging to families comprising nodulated species, which is the case of *A. neocaledonica*. It can be hypothesized that this non-nodulated Rhamnaceae can secrete in its rhizosphere substances that could act in several ways, such as (i) by activating *Frankia* spore germination, (ii) by activating *Frankia* hyphal growth and (iii) by attracting distantly growing saprophytic *Frankia* hyphae.

Proliferation of *Frankia* in the rhizosphere of various actinorhizal plants such as *Alnus* [18], *Elaeagnus* [12] and *Casuarina* [6] has already been described.

3.4. Isolation and study of *Frankia* strains

3.4.1. Isolation of Frankia *strains*

Strains were isolated from nodules obtained after trapping *Frankia* by *G. poissonianum* from the rhizosphere (fractions RS+CR) of the plants studied.

By dissecting 530 nodule lobes, we obtained fourteen isolates originating from rhizospheres of the five *Gymnostoma* species (eight isolates) and *A. neocaledonica* (six isolates) in the five sites studied (*table IV*). This corresponded to about a 2.6 % success among the *G. poissonianum* nodule lobes collected. In a first study with nodules of eight *Gymnostoma* spp. harvested in the field, we obtained about 6 % success [8]. Whereas the first strain of *Frankia* was isolated

20 years ago [4], the isolation step is still a major bottleneck in the availability of pure cultures, and strong variability in the success ratios is observed depending on the host plants and the methods used. Using different methods and media, Rosbrook and Reddell [20] reported isolation rates of 50 % for *Casuarina equisetifolia*, 17 % for *C. glauca* and 3 % for *C. cunninghamiana*.

We observed that isolation was more successful on nitrogen free medium (12/14 successful isolations). This could be due to the early selection of nitrogen fixers and non-proliferation of contaminants, as well as a better compatibility between culture medium and *Frankia* strains.

The strains isolated from *G. poissonianum* exhibited all the characteristic morphological structures of the genus *Frankia* including hyphae, sporangia, spores and vesicles. Vesicles were very numerous in the NFM medium and were still present after ammonium complementation. All the strains presented an orange to blood-red pigmentation. Sporulation was often very abundant. These characteristics were close to those of the 128 strains already isolated from the eight endemic *Gymnostoma* species from New Caledonia [8].

3.4.2. *Characterization of* Frankia *strains*

The DNA extraction protocol yielded DNA that was pure enough for amplification with primer FGPS989e, which is Elaeagnaceae-group specific. The amplified fragments were about 1 000 bp long.

Discriminant patterns were observed when PCR products were digested with *Hae*III and *Msp*I. By combining the three profiles thus obtained, four ITS groups were discriminated among the isolates (*table IV*). These groups were the same (ITS groups 1, 2, 3 and 4) as those already described in *Frankia* of *Gymnostoma* [8]. As in *Frankia* strains isolated using nodules harvested in natural stands of *Gymnostoma* spp., the ITS groups 2 and 3 were dominant (6/14 and 5/14) and groups 1 and 4 were minor (1/14 and 2/14). These fourteen strains were also infective and effective on all the *Gymnostoma* species tested (data not shown). These results suggested that the *Frankia* isolated from nodules harvested in the field [8] and *Frankia* trapped on rhizospheres of *Gymnostoma* spp. and *A. neocaledonica* in this study could be the same strains.

4. CONCLUSIONS

The main results of this study were:

– The ability of the bare soils of all five sites studied to nodulate *G. poissonianum* indicated the general ubiquity of *Frankia* in New Caledonian soils in the absence of actinorhizal plants. This result was confirmed by trapping on thirteen bare soils sampled in different sites of the island (data not shown). This phenomenon is well known in soils lacking nodulated host plants [16, 19, 22]. In this study, we did not isolate *Frankia* after trapping on bare soils. Interesting further research would be to investigate if the *Frankia* present in the bare soils and those of the rhizosphere of various plants are the same.

– The *Frankia* from *Gymnostoma* isolated through trapping on various rhizospheres of plants of New Caledonia fit into groups already described for strains isolated from nodules harvested in natural stands of *Gymnostoma* spp. [8].

In the case of the five *Gymnostoma* species, the abundance of *Frankia* in the rhizosphere could be explained by the continuous supply of *Frankia* through nodule decay, the rhizosphere effect, the extranodular growth ability of *Frankia* [6] and the ability of *Frankia* to resist extreme environmental conditions [11].

– The nodulating capacities of the rhizosphere (RS+CR fractions) of *A. neocaledonica* were almost equal to those of the five *Gymnostoma* species studied in the same sites (*tables II, III*). In comparison, bare soils or rhizosphere of *P. caribea* had poor nodulating capacities.

We can formulate the hypothesis that the *Frankia* from *Gymnostoma* could use *A. neocaledonica* as an alternative non-symbiotic host in the absence of *Gymnostoma* spp. after momentous destruction of its host by fire or human deforestation. Thus *A. neocaledonica* could constitute a reservoir of *Frankia* for a future colonization of the site by *Gymnostoma* species.

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