Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Successful nodulation of *Casuarina* by *Frankia* in axenic conditions

H. Echbab[1], M. Arahou[1], M. Ducousso[2], S. Nourissier-Mountou[2], R. Duponnois[2], H. Lahlou[1] and Y. Prin[2]

1 Laboratoire de Botanique, Département de Biologie, Faculté des Sciences, Université Mohammed V-Agdal, Rabat, Morocco
2 LSTM, UMR 113 Agro-M, CIRAD, INRA, IRD, UM2, TA10 J, Montpellier Cedex, France

Keywords
actinorhizal plant, *Casuarina*, *Frankia*, *in vitro* plant cultivation, nitrogen-fixing nodule, nodulation pathway.

Correspondence
Yves Prin, LSTM, UMR 113 Agro-M/CIRAD/INRA/IRD/UM2, TA10 J, F-34398, Montpellier Cedex 5, France.
E-mail: yves.prin@cirad.fr

2006/1641: received 24 November 2006, revised 23 February 2007 and accepted 16 March 2007

doi:10.1111/j.1365-2672.2007.03425.x

Abstract

Aims: In order to depict the fine interactions that lead to nodulation, absolute microbiological control of the symbiotic partners is required, i.e. the ability to obtain *in vitro* axenic nodulation, a condition that has never been fulfilled with the *Casuarina–Frankia* symbiosis. The effects of culture conditions on plant growth and nodule formation by *Casuarina cunninghamiana* were investigated.
Methods and Results: Axenic (capped tubes with different substrates), and nonaxenic cultures (Gibson tubes, pot cultures) were tested. In axenic conditions, *C. cunninghamiana*, inoculated with *Frankia*, had poor growth and did not form nodules at 6 weeks. Plants cultivated in Gibson tubes reached the four axillary shoots stage within 6 weeks and formed nodules 4 weeks after inoculation. Sand-pot cultures allowed us to relate the plant development stage at inoculation with nodulation.
Conclusions: The sterile replacement of the cap by a plastic bag increased plant growth and enabled nodule formation 6 weeks after inoculation.The new system of plant culture allows the axenic nodule formation 6 weeks after inoculation. Nodulation behaviour is related to plant development and confinement.
Significance and Impact of the Study: This axenic plant nodulation system is of major interest in analysing the roles of *Frankia* genes in nodulation pathways.

Introduction

Frankia is a filamentous soil bacteria, which interacts with the roots of appropriate host plants (collectively called actinorhizal plants) to form nitrogen-fixing nodules, also called actinorhizae (Benson and Silvester 1993). Actinorhizal plants include *Casuarinaceae*, which is a major family of trees that have been disseminated throughout the tropics owing to their ability to grow in adverse conditions. The *Casuarinaceae* comprises four genera: *Casuarina*, *Allocasuarina*, *Gymnostoma* and *Ceuthostoma* (Torrey and Berg 1988). The genus *Casuarina* is by far most widely used as a windbreak and soil stabilizer, particularly with the species *Casuarina equisetifolia*, *Casuarina cunninghamiana* and *Casuarina glauca*. In Australia, the native area of most of the *Casuarinaceae*, the occurrence of nodulation is far from regular (Reddell *et al.* 1986). Of the five species belonging to the genus *Casuarina* examined by the authors of this article (representing a total of 246 individuals), *C. equisetifolia*, *C. cunninghamiana* and *C. glauca* were the most frequently nodulated. The first species was the only one that systematically nodulated. Interestingly, the authors confirmed the field observations through baiting experiments using surface soils. *Casuarina cunninghamiana*, which was used in the present study, displayed more than 80% nodulation in baiting experiments with Australian surface soils (Reddell *et al.* 1986). However, nodulation is far more difficult in laboratory conditions, even after inoculation with a compatible *Frankia* strain. Since the first isolation of *Frankia* from *Casuarina* sp. (Diem and Dommergues 1983), numerous nodulation tests involving pure *Frankia* strains and *Casuarina* have been conducted, although never in strict axeny. Diem *et al.* (1983) made successful actinorhizae formation tests with the strain Cj1 in semi-axenic conditions, using a modified Petri dish with the aerial part of the plant

outside the Petri dish. Sougoufara (1990) also successfully developed semi-axenic nodulation tests using Gibson's tubes and maintaining the aerial part of the plant outside the tube. Diouf *et al.* (1995) successfully nodulated transgenic hairy roots of composite *C. glauca* using Gibson tubes with the shoot growing outside. So far, we have not found any report of actinorhizae formation by the genus *Casuarina* after inoculation with a pure *Frankia* strain in axenic conditions (Table 1). Axenic nodulation would be essential:

1. In the fulfilment of Koch's postulate with new bacterial isolates. As Torrey (1990) recalls, 'under a strict interpretation of fulfilling Koch's postulates in the demonstration of the causal organism for *Frankia*-induced nodulation, the re-infection of the root by the cultured organism should be performed with only *Frankia* in the association'. This means that, theoretically, in the absence of axenic nodulation systems, the isolates from *Casuarina* nodules should not be named *Frankia*. A reliable taxonomy of the genus *Frankia* needs strict experimental conditions.

2. In the fine study of the interactions between *Frankia* and its host plant, allowing the recognition of both symbiotic partners and leading to infection and nodulation processes. Mansour and Torrey (1991) failed to nodulate *C. glauca* seedlings (although they obtained root hair deformation) in axenic conditions (using a modified Fahraeus-type slide apparatus). However, they obtained nodulation with the same partners in nonaxenic conditions. Even in nonaxenic conditions, the latter authors barely reached 35% of nodulated plants.

Actinorhizae formation is thought to be the result of a cellular differentiation process mediated by molecular signal exchanges, which involve the expression of specific genes in both *Frankia* and the host plant, as it has been described in the rhizobium/legume interactions (e.g. Broughton *et al.* 2000; Perret *et al.* 2000). Two pathways for root infection have been described in compatible *Frankia* interactions: intercellular penetration or root hair infection (e.g. Berry and Sunell 1990; Franche *et al.* 1998; Wall *et al.* 2002). This last process, found in *Casuarina* (Torrey 1976), starts with root hair curling, which is induced by an undefined *Frankia* signal. Following *Frankia* penetration of the root hair cell, a series of cell divisions occurs in the hypodermis and cortex of the root, resulting in the formation of a prenodule; the actual actinorhizae lobe originates in the pericycle (Callaham *et al.* 1979). As with legumes, it has been suggested that flavonoids play a fundamental role in actinorhizal plant–*Frankia* interactions (Prin and Rougier 1987; Van Ghelue *et al.* 1997; Laplaze *et al.* 1999). The recent sequencing and annotation of three *Frankia* genomes in the United States and France will probably exacerbate the need for reliable axenic nodulation systems to test the roles of symbiotic genes (Normand *et al.* 2007).

The aim of this research was to obtain axenic nodulation of *C. cunninghamiana* as quickly as possible after seed sterilization, with a nodulation percentage as close to

Table 1 Nodulation data from *in vitro* inoculation experiments on the genus *Casuarina*. Data before 1982 (when the first pure strain of *Frankia* was isolated from *Casuarina*), and experiments in open systems (pots, hydroponics or water cultures) were not taken into account

Plant species*	*Frankia* strain	Nodulation system†	Nodulation	Reference
C. equ.	ORS021001 (Cj1)	PPD (NA)	+	Diem *et al.* 1982
C. equ.	ORS021001 (Cj1)	PPD (NA)	+	Diem *et al.* 1983
C. equ.	ORS021001 (Cj1)	GT (NA)	+	Diem *et al.* 1983
C. equ.‡	CFN 022901 (Dec)‡	GT (NA)	+	Sougoufara 1990
C. gl.‡	ORS 022602 (TA)‡	GT (NA)	+	Sougoufara 1990
C. cunn.‡	CFN 022302 (URU2)‡	GT (NA)	+	Sougoufara 1990
C. cunn.‡	HFP022801 (ALI1)‡	GT (NA)	+	Sougoufara 1990
C. gl.	HFP020804 (CgI4)	CFS (A)	–	Mansour and Torrey 1991
C. gl.	HFP020804 (CgI4)	FS (NA)	+	Mansour and Torrey 1991
C. gl.	CFN020201 (Thr)	GT (NA)	+	Diouf *et al.* 1995
C. gl. GM	CFN020201 (Thr)	GT (NA)	+	Diouf *et al.* 1995
C. gl.	CFN020201 (Thr)	GT (NA)	+	Franche *et al.* 1999
C. gl. GM	CFN020201 (Thr)	GT (NA)	+	Franche *et al.* 1999
C. gl.	CFN020201 (Thr)	GT (NA)	+	Smouni *et al.* 2002
C. gl. GM	CFN020201 (Thr)	GT (NA)	+	Smouni *et al.* 2002
C. gl. GM	CFN020201 (Thr)	GT (NA)	+	Santi *et al.* 2003
C. gl.	CFN020201 (Thr)	PPD (NA)	+	Sy *et al.* 2003

**C. equ.*, *Casuarina equisetifolia*; *C. gl.*, *Casuarina glauca*; *C. cunn.*, *Casuarina cunninghamiana*; GM, genetically modified.

†PPD, pierced Petri dish; GT, Gibson tube; CFS, closed Farhaeus slide; FS, Farhaeus slide; A, axenic; NA, nonaxenic.

‡All combinations of plant species and *Frankia* strains were tested by this author, all successful in nodulation.

100% as possible, and to identify the parameters that need to be taken into account to allow for this rate of nodulation. The seedlings were subjected to various growing conditions and different levels of microbiological control. Seedling growth and development were analysed at the time of inoculation and nodulation.

Materials and methods

Preparation of the *Frankia* culture for plant inoculation

Frankia strain ORS021001 (synonym Cj1), originally isolated from a *Casuarina junghuniana* × *C. equisetifolia* hybrid (Diem and Dommergues 1983), was chosen. It was reported as infective and effective on the genus *Casuarina* from laboratory to field conditions (Sougoufara *et al.* 1989). The strain was subcultured three times in a BAP medium (Murry *et al.* 1984), for 8–10 days to ensure that the *Frankia* culture was made up of purely young vegetative hyphae. The *Frankia* culture was placed in sterile 10-ml tubes and centrifuged (10 000 ***g*** at 4°C for 10 min). After being washed three times in the same medium, the pellet was disrupted into small hyphal fragments by passages through a 0·8-mm diameter needle and used for inoculation. *Frankia* growth was estimated using the Lowry protein assay, as previously described by Arahou *et al.* (1998).

Plant material and germination

Seeds of *C. cunninghamiana* were collected from the outskirts of Rabat (Morocco). For germination, they were soaked in sterile water for 24 h before being surface-sterilized using 30% H_2O_2 for 40 min. The seeds were then kept in sterile distilled water for 80 min before being rinsed three times with sterile distilled water. Surface-sterilized seeds were placed in Petri dishes on water agar (7 g l^{-1}, w/v) for germination, and incubated in a growth chamber at 27°C.

Plant cultivation and inoculation

For the five following experiments, plants were placed in a growth chamber with a 14-h day (26°C), a 10-h night (20°C) and 70–80% relative humidity throughout the experimental period. Cool-white fluorescent tubes provided light in the chamber.

Experiment 1

This experiment aimed at testing a standard nodulation protocol on *C. cunninghamiana* that was successfully used to nodulate axenically *Alnus glutinosa* (Vergnaud *et al.* 1985). This protocol (called 'PTS' for paper tube system) was compared with the classical 'semi-axenic' Gibson tube ('GT') system used on *Casuarina* sp. by Sougoufara (1990) and several other authors. Briefly, in PTS, 1-week-old sterile plants were aseptically placed into 25 × 250-mm glass tubes (Kimble, Owen, IL, USA) containing 15 ml of a diluted one-quarter strength Hoagland and Arnon (1938) nutrient solution supplemented with N [as $(NH_4)_2SO_4$, 17 mg l^{-1}]. Their roots were inserted between the fold of a 30 × 120-mm paper support (cut in the paper foil of a CYG growth pouch; Mega International, Minneapolis, USA) and the inside of the tubes, which were then sealed by a transparent plastic cap (KimCap; Kimble). In GT, after seed germination, each sterile plant was placed in a 25 × 250-mm glass tube (Kimble) containing a 1·6% slanted agar medium, supplemented with 2% active charcoal and 15 ml of a diluted one-quarter-strength Hoagland and Arnon (1938) nutrient solution (Sougoufara 1990). The tubes were capped by an aluminium foil pierced with a hole; the rootlets were introduced into the tube to ensure contact with the slanted agar medium, while the shoot remained outside. To maintain the nutrient solution level in the tubes, sterile distilled water was added to compensate for evaporation. In both systems, the roots stayed in aeroponic conditions until they reached the liquid HA, which took 4–6 weeks. In GT, very few secondary roots penetrated the slanted agar. When the seedlings were 4-weeks old, in both PTS and GT, the nutrient solution was replaced by the same solution but without N. Two weeks later, inoculation was performed by adding 1 ml of a suspension of homogenized *Frankia* culture to each tube, adjusted to 20 μg protein ml^{-1}. It was used to sprinkle the whole root system abundantly. There were 10 replicates of each of the four treatments, namely PTS and GT with both inoculated and control treatments.

Experiment 2

This experiment was designed to check whether or not the difference in the response between PTS and GT could be attributed to the paper used. Two additional axenic tube systems, using the same tubes, volumes and nutrient solution, as described in experiment 1, were tested simultaneously with the PTS. In these two systems, the paper was replaced by either 1·6% agar + liquid nutrient solution ('ATS' for agar tube system), or sand ('STS' for sand tube system), with nutrient solution. To promote plant growth, the nutrient solution was supplemented with N [as $(NH_4)_2 SO_4$, 17 mg l^{-1}]. The 60 tubes [three systems, two inoculation treatments (*Frankia*- and noninoculated), 10 replicates], were capped with KimCap (Kimble). When the seedlings were 4-weeks old, the nutrient solution was replaced by the same solution without N. Two weeks later, inoculation was performed by adding 1 ml of a

homogenized suspension of *Frankia* culture (adjusted to 20 μg protein ml^{-1}), to each tube.

Experiment 3

This experiment was designed to test if the developmental stage of the plant (i.e. the number of axillary shoots) at inoculation could influence the nodulation rates. In order to ensure that the conditions were as close as possible to natural nodulation conditions, this experiment was designed in nonaxenic conditions using sand-filled pots. After being sterilized, the seeds were germinated in a sterile bed of sand. One-week-old seedlings were transplanted into plastic pots (700-ml capacity) containing sterilized sand, with three seedlings per pot and four pots per treatment, irrigated with a diluted one-quarter strength Hoagland and Arnon (1938) nutrient solution, supplemented with N, as previously described. At different times of growth (14, 21, 28, 35 and 45 days after pot transplantation), each seedling was inoculated with 1 ml (20 μg protein ml^{-1}) of a homogenized *Frankia* suspension and irrigated with the nutrient solution without N. The roots were examined for nodulation 4 weeks after inoculation.

Experiment 4

This experiment was set up to test the effect of an increase in gas exchange at the shoot level. The seedlings were cultivated in PTS as described earlier, until plants formed five axillary shoots, which required 6 months of growth. After this period, Sunbags were used to replace the Kimcaps for half of the tubes used. The former are sterile transparent plastic bags (4-l capacity), with a filter window to allow sterile gas exchange with the external atmosphere (Sigma, l'Isle d'Abeau, France). At this time, the plants were inoculated with *Frankia*. This system is called 'BTS' (bag tube system).

Experiment 5

In the last experiment, the plants were transferred to PTS or BTS directly after the germination stage in order to reduce the time required for axenic nodulation. When they had developed four axillary shoots, i.e. after 8 weeks of growth in BTS, all plants in PTS and BTS tubes were inoculated with *Frankia*, as described previously.

Measurements of growth parameters

The growth parameters measured were either: nondestructive, namely, plant-shoot height, root length, number of axillary shoots; or destructive, i.e. shoot dry weight, root dry weight, number of actinorhizae per plantand chlorophyll content. In order to determine the chlorophyll content, chlorophyll was extracted by placing about 20 mg of fresh apical tissue from each plant in 4 ml of *N*,*N*-dimethylformamide (Hiscox and Israelstam 1979). After 24 h at 4°C in the dark, all chlorophyll pigments had dissolved and the optical density of the solution was measured at 652 nm, according to the Bruinsma (1963) method.

Sterility controls

To assess the sterility of our axenic nodulation system, some root fragments from each tube were axenically excised from plants cultivated in axenic conditions and were individually macerated in 500 μl of sterile distilled water into 1·5-ml microtubes. The macerate was plated on nutrient agar (Difco), and the Petri dishes were incubated at 25°C for 1 week.

Statistics

The data set was analysed using the Statistical Analysis System computer program (SAS Institute 1985), and significant differences between treatments were compared using Newman and Keul's multiple-range test.

Results

Experiment 1

The effect of growing conditions on root nodulation and plant growth is illustrated in Table 2. Four weeks after

Table 2 Comparison of culture effects in GT (nonaxenic conditions) and in PTS (axenic conditions) on nodulation and growth of *Casuarina cunninghamiana* seedlings in *Frankia*-inoculated and noninoculated plants. Plants were inoculated 6 weeks after germination and harvested 3 months later

	Shoot dry weight (mg)	Root dry weight (mg)	Number of nodulated plants	Number of nodules per plant
PTS control	5·5^{a}	4·1^{a}	0	0
PTS-inoculated plants	5·3^{a}	4·3^{a}	0	0
GT control	14·2^{b}	7·2^{b}	0	0
GT-inoculated plants	26·5^{c}	12·2^{c}	8	4·5

PTS, paper tube system; GT, Gibson tube.
For each parameter measured, values with the same letter are not significantly different according to the *t*-test ($P < 0·05$; $n = 10$).

Table 3 Effect of culture substrate in capped tubes on nodulation and growth of *Casuarina cunninghamiana* 3 months after inoculation with *Frankia*

	Number of nodules per plant	Number of shoot ramifications per plant	Shoot height (cm per plant)	Root length (cm per plant)
STS control	0	1·5[a]	2·1[a]	12·9[a]
STS-inoculated plants	0	1·7[a]	2·2[a]	13·2[a]
PTS control	0	3[b]	2·8[b]	19·8[b]
PTS-inoculated plants	0	3·1[b]	3[b]	20[b]
ATS control	0	2·9[b]	2·7[b]	20·2[b]
ATS-inoculated plants	0	3[b]	2·7[b]	20·4[b]

STS, sand tube system; PTS, paper tube system; ATS, agar tube system.
For each parameter measured, values with the same letter are not significantly different according to the *t*-test ($P < 0{\cdot}05$; $n = 10$).

inoculation, plants inoculated with *Frankia* in GT formed actinorhizae at a rate of 80%. In PTS, *Frankia* was unable to form actinorhizae, and no response to inoculation was obvious in plant growth. In GT, both shoot and root dry weights, measured 3 months after inoculation, suggested that the inoculated plants had significantly better growth compared with the noninoculated plants. Plant growth in PTS was dramatically lower than that in GT: 2·5 and 4·5 times lower than the control and the inoculated GT, respectively. Six weeks after transfer to the tubes (i.e. at the time of inoculation with *Frankia*), plants cultivated in GT had developed four axillary shoots, whereas plants cultivated in PTS had developed only one axillary shoot.

Experiment 2

The effects of the three different substrates used, filter paper (PTS), agar (ATS) and sand (STS), on the nodulation and growth of seedlings of *C. cunninghamiana* are presented in Table 3. No nodulation occurred in any of the three substrates. Axillary shoot number, shoot height and root length were significantly less developed in plants cultivated in sand than in plants cultivated in agar or filter paper. In the three substrates tested, seedlings had formed only one axillary shoot at the time of inoculation, 6 weeks after transfer. As in experiments 1 and 2, plants of the same age displayed variable phenological stages (i.e. axillary shoots number), at inoculation. A third experiment was designed to test the effect of the phenological stage at inoculation on nodulation.

Experiment 3

The influence of plant development on root nodulation, evaluated either as the number of shoot ramifications or shoot height, of seedlings grown in potted sand, are presented in Fig. 1. Young seedlings of *C. cunninghamiana* were inoculated at different ages corresponding to different stages of shoot development. The presence or absence of root nodules was examined 4 weeks after inoculation. The results indicated that the formation of nodules could only take place if *Frankia* was added to the seedlings that were at least 28-days old, which corresponds to a minimum of three axillary shoots. When the plants had one or two axillary shoots at the time of inoculation, *Frankia* did not result in root nodulation.

Experiment 4

In order to achieve nodulation in axenic conditions, seedlings were grown in plugged tubes until the five axillary shoots had formed, a stage that meant that more than 80% of the plants nodulated in experiment 3. In plugged tube conditions, it took 6 months of growth to reach the five axillary shoots stage. After this period, the plugs for half of the tubes used were replaced by plastic bags before being inoculated by *Frankia*. The seedlings in BTS displayed active growth and started to form actinorhizae 6 weeks after inoculation, while seedlings in PTS grew slowly and did not form actinorhizae (Table 4).

Experiment 5

The same results were obtained when cultures were started directly in PTS or BTS at the time of seed germination. In this experiment, after 8 weeks of growth, plants in BTS had developed four axillary shoots and had extensive root growth (Table 5). In contrast, seedling growth was insignificant (axillary shoots number = 1) when plants were maintained in PTS. Six weeks after inoculation, the ability of *Frankia* to induce nodules in BTS was confirmed. In PTS, however, plants remained stunted and unable to form actinorhizae (Table 5) throughout the experiment which ended 12 weeks after inoculation.

The effectiveness of actinorhizae in axenic conditions was proven by the sustained growth and chlorophyll

Journal compilation © 2007 The Society for Applied Microbiology, Journal of Applied Microbiology **103** (2007) 1728–1737

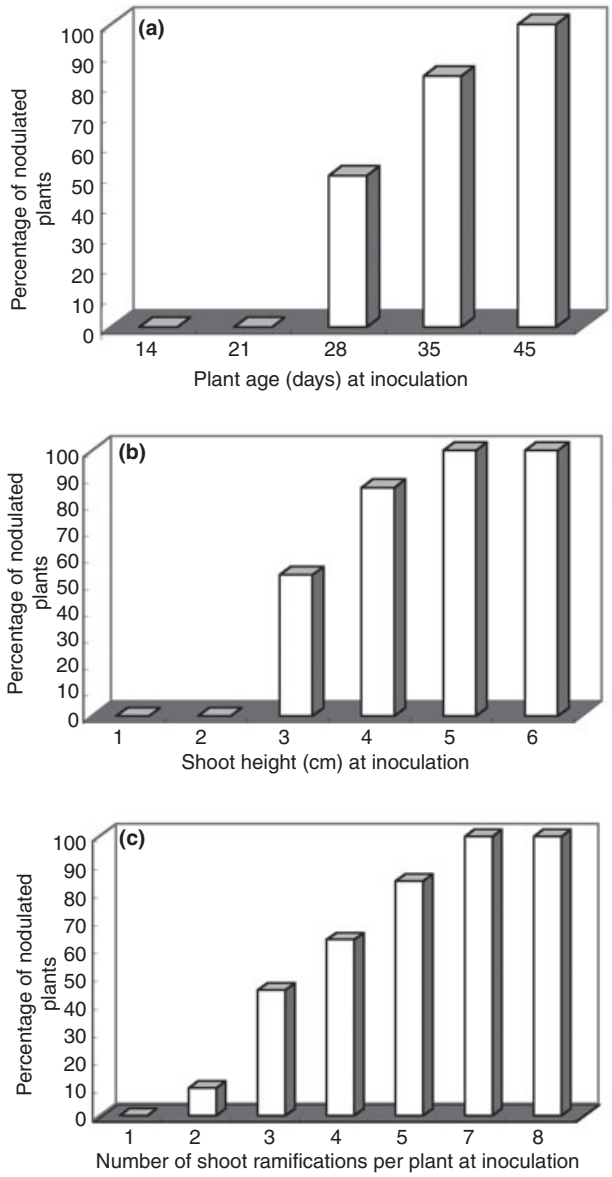

Figure 1 Relationship between plant age at inoculation (a), shoot height at inoculation (b) and number of shoot ramifications at inoculation (c), and the percentage of nodulated *Casuarina cunninghamiana* plants, 4 weeks after inoculation. Seedlings were transplanted in pots just after their germination.

content of seedling shoots (Tables 4 and 5). Indeed, the chlorophyll concentration in nodulated seedlings was more than twice that of non-nodulated seedlings.

Sterility controls

After incubation for 1 week at 25°C, no contaminants were detected from nodules or roots on Petri dishes, which confirms the strict axeny of nodulation in BTS.

Discussion

It has been repeatedly reported that spontaneous nodulation of the genera *Casuarina* and *Allocasuarina* is unlikely outside their natural habitat. This may be attributed to the fact that *Frankia* is not transmitted with the seed – either within it or on its surface (Torrey 1983). Moreover, inoculation experiments conducted in hydroponic conditions on *Casuarina* sp., which bring together the root system and a pure compatible strain, frequently led to an incomplete response with less than 50% nodulation among the test plants (Torrey and Racette 1989). Mastering the nodulation of *C. cunninghamiana* is clearly far from easy and we generally assume (depending on the number of replicates) that 80% of the nodulated plants can be considered as a maximum rate, similar to that occurring in the plant's native area.

Our results show that *Frankia* can nodulate and increase the growth of *C. cunninghamiana* when grown in classical GT, with the shoot outside the tube. Different studies (Table 1) have shown the possibility of obtaining nodules when *Casuarina* is cultivated in GT (Diem *et al.* 1983; Sougoufara 1990; Smouni *et al.* 2002; Santi *et al.* 2003). Petri dish systems in which a hole is pierced in the lids to allow the aerial parts of the plant to be outside, have also been successfully used to nodulate *C. equisetifolia* (Diem *et al.* 1982, 1983) and *C. glauca* (Sy *et al.* 2003) in 'semi-axenic' conditions. The PTS, which, as we mentioned earlier, was found to allow the successful axenic nodulation of *A. glutinosa* (Vergnaud *et al.* 1985), failed to induce the nodulation of *Casuarina*. Further research is required to determine whether *A. glutinosa* would tolerate the confinement and hyperhydricity of PTS better than *C. cunninghamiana*. One can hypothesize that PTS hyperhydricity would limit evapotranspiration and growth of *C. cunninghamiana*, as it is well known that a good rate of evapotranspiration may be necessary for good plant development (George 1996). Limited plant growth would not allow nodulation and symbiotic nitrogen fixation.

As we were unable to obtain nodulation in PTS, we used two other substrates, STS and ATS, to test: (i) the putative inhibitory effect of the paper in PTS; or (ii) the promoting effect of an abrasive substrate, like sand. In fact, ATS reproduced the GT system exactly, although it also enclosed the shoot in the tube. No inoculation effect was observed on plant growth nodulation or on actinorhizae formation (the latter which was not obtained). In water culture, in nonaxenic conditions, Zhang and Torrey (1985) failed to obtain nodulation between *Allocasuarina littoralis* and *Frankia* strain AllI1. However, with the same strain and nutrient solution, nodulation occurred systematically in sand culture.

Table 4 Actinorhizae formation in axenic conditions: *Casuarina cunninghamiana* seedlings were grown in PTS until the formation of five ramifications per plant. At this stage, plastic caps were replaced by plastic bags and half of the tubes were inoculated with *Frankia*. Harvest occurred after 3 months of growth

	Number of nodules per plant	Chlorophyll content (mg per 10 g)	Root dry weight (mg per plant)	Shoot dry weight (mg per plant)
PTS control	0	3·6[a]	6·25[a]	12·45[a]
PTS-inoculated plants	0	4[a]	7·5[a]	14·17[a]
BTS control	0	6·5[a]	10·85[b]	26·58[b]
BTS-inoculated plants	4	9·4[b]	18·36[c]	40·41[c]

PTS, paper tube system; BTS, bag tube system.
For each parameter measured, values with the same letter are not significantly different according to the *t*-test ($P < 0{\cdot}05$; $n = 10$).

Table 5 Actinorhizae formation, chlorophyll content and growth of *Casuarina cunninghamiana* seedlings cultivated in BTS or PTS 3 months after inoculation

	Number of nodules per plant	Chlorophyll content (mg per 10 g)	Root dry weight (mg per plant)	Shoot dry weight (mg per plant)
PTS control	0	2·2[a]	3·5[a]	4·5[a]
PTS-inoculated plants	0	3[a]	4·3[a]	5·3[a]
BTS control	0	4·95[b]	9·45[b]	13·75[b]
BTS-inoculated plants	3·6	7[b]	13·2[c]	21·1[c]

PTS, paper tube system; BTS, bag tube system.
For each parameter measured, values with the same letter are not significantly different according to the *t*-test ($P < 0{\cdot}05$; $n = 10$).

At the time of our study, we identified several factors that could inhibit nodulation in closed systems *vs* open systems: plant phenology (i.e. plant shoot and axillary shoot development) at inoculation. Closed systems seriously limited plant development, which may be attributed to limited gas exchanges. It is important to remember that in GT, the tubes are capped with an aluminium foil, pierced with two holes – one for the plant (maintaining the crown but not strictly joined) and the other for adding nutrient solution that evaporated regularly. These holes allow the passage of gases. The periodic refilling of liquid media with sterile water may have diluted inhibitory molecules (and gases) within the tube. Being outside the GT, the shoot parts are not submitted to putative inhibitory gases. A physical effect of the tube size could also have been a cause, but this seems unlikely as plant shoots and roots were not in physical contact with the bottom of the tube or the cap. From the GT experiments, we knew that nodulation was not inhibited by the fact that roots were in physical contact with the bottom of the tubes.

In order to discriminate between these factors, we designed experiment 3 to test the effect of plant age and development (phenology) at inoculation on nodulation. Results from the first two experiments demonstrated that nodulation was good in GT with well-developed plants with four axillary shoots. Whereas, in PTS, SDS or ATS, plants with one axillary shoot and their cotyledons did not nodulate. In addition, there was no nodulation in plants of less than 28 days at inoculation, or plants with an average shoot height of less than 3 cm, or those with an axillary shoot number below 2. Moreover, plants with an axillary shoot number below 2 did not nodulate even 6 months after inoculation (data not shown), as if a putative plant defence reaction was induced by a single early inoculation and was still effective 6 months later. Furthermore, in PTS, we observed the development of *Frankia* colonies around the root systems dipping in the nutrient solution (data not shown). Microscopic observations of these colonies revealed the presence of typical vesicles and sporangia. These observations show the development and survival of *Frankia* around the root system a long time after inoculation. In natural conditions, the rhizosphere of young seedlings would be colonized by a great diversity of competing micro-organisms, not just one strain of *Frankia*; one would also expect that *Frankia* will be much more diluted than in our inoculum. Thus, the progress of rhizosphere colonization by *Frankia* will probably be much slower on the young root surface and the plant defence reaction much more attenuated than in our experimental conditions. Another hypothesis is that the plant's defence reaction, induced early by one *Frankia* strain, would have only been broken by other bacterial strains (*Frankia* or other). At the time of inoculation, plant growth parameters like shoot height or axillary

shoot number can be quite reliable predictors of the maximum rate of nodulation. When plants more than 4-cm high or with more than five axillary shoots are inoculated, 100% of the plants should form actinorhizae 4 weeks later. The plant age is probably less reliable because individual variations may occur within these growth parameters. Four weeks after inoculation, we observed the first nodules on 50% of the plants. We never observed less than three nodules per plant. With *A. glutinosa* inoculated in PTS at 3 weeks (two cotyledons + three leaves), Vergnaud *et al.* (1985) obtained axenic nodulation of 100% plants within 10 days. This was consistent with the results published by Knowlton and Dawson (1983) on *Alnus rubra*. These data show that there is a major difference in growth and nodulation behaviour between *Alnus* and *Casuarina*.

Given the results obtained in experiment 4, it seems clear that the plant development stage at inoculation is not the only important parameter for nodulation in axenic conditions. Combined with experiment 5, this data showed that the use of BTS acted at two levels – they increase plant growth and allow nodulation. Moreover, we observed that these two actions were independent from each other as plant growth was seriously enhanced in controls that were not inoculated with BTS. Evidently in BTS, nodulation dramatically increased plant growth compared with the control. Therefore, it would be interesting to examine the possible mechanisms involved in these independent responses. Truly, the 4-l Sunbags represent a higher atmospheric volume than closed tubes. Additionally, their sterile gas exchange window allows for atmosphere renewal at an unknown rate. Therefore, BTS would allow for better evapotranspiration and development of the drought-adapted foliage of *C. cunninghamiana*. Given the improved growth and corresponding higher nitrogen requirements, plants would form actinorhizae much faster than in PTS.

Another important factor that has to be considered is the problem of putative gas accumulation in PTS. Of the several gases that have been involved in establishing symbioses, the effects of exogenous and endogenous ethylene are probably the most well documented. Ethylene is a phytohormone whose role on nodulation and mycorrhization has recently been reviewed by Guinel and Geil (2002). Ethylene has been shown to have had a strong negative effect on infection thread growth, and thus infection and nodulation in various legumes (Spaink 1997). In actinorhizal symbioses, ethylene was involved in the nodulation process of *Discaria trinervis* (Valverde and Wall 2005). The processes described in plants seem very diverse which makes it difficult to generalize (Guinel and Geil 2002). Similarly, several strategies have been described on the rhizobial side in order to lower ethylene levels produced by the legume partner and increase nodulation (Ma *et al.* 2002). In arbuscular mycorrhizal symbioses, exogenous ethylene has also been shown to play an inhibitory role in arbuscular mycorrhizae (AM) formation (Geil *et al.* 2001). From controversial data (possibly reflecting a diversity of plant mechanisms), it appears that AM colonization of roots either enhances or reduces host ethylene production (Guinel and Geil 2002). It would probably be worth testing the effect of antagonists of ethylene action, like silver, on nodulation in PTS inoculated at the four axillary shoots stage.

The relationship between nodule formation and atmospheric gases, like CO_2, has also been investigated in different studies. The effect of CO_2 concentration on nitrogen fixation in the *Rhizobium* – leguminous symbiosis has been examined since the 1950s. Most studies (Mulder and van Veen, 1960; Finn and Brun 1982; Yamakawa *et al.* 1997) described an increase in nitrogen fixation, nodule and plant biomass with increasing CO_2 concentrations. Several other gases (like oxygen and hydrogen) are known to interfere with nodulation and/or nitrogen fixation. However, their impact on early infection is generally poorly documented.

This research demonstrates the potential for obtaining axenic nodulation of *C. cunninghamiana* at a rate of 70%, 12 weeks after inoculation. Nodulation was shown to be dependent on two main factors: the stage of plant development at inoculation and the atmospheric volume of the axenic system.

Acknowledgements

This research received financial support from the Programme de Recherche en Agronomie pour le Développement (PRAD 00/19). H.E. received a grant from CIRAD. The authors wish to thank Dr F.C. Guinel for numerous helpful recommendations with the manuscript.

References

Arahou, M., Diem, H.G. and Sasson, A. (1998) Influence of iron depletion on growth and production of catechol siderophores by different *Frankia* strains. *World J Microbiol Biotechnol* **14**, 31–36.

Benson, D.R. and Silvester, W.B. (1993) Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiol Rev* **57**, 293–319.

Berry, A.M. and Sunell, L.A. (1990) The infection process and nodule development. In *The Biology of Frankia and Actinorhizal Plants* ed. Schwintzer, C.R. and Tjepkema, J.D. pp. 61–81. New York: Academic Press.

Broughton, W.J., Jabbouri, S. and Perret, X. (2000) Keys to symbiotic harmony. *J Bacteriol* **182**, 5641–5652.

Bruinsma, J. (1963) The quantitative analysis of chlorophylls a and b in plant extracts. *Photochem Photobiol* **2**, 241–249.

Callaham, D., Newcomb, W., Torrey, J.C. and Peterson, R.L. (1979) Root hair infection in actinomycete-induced root nodule initiation in *Casuarina*, *Myrica* and *Comptonia*. *Bot Gaz* **140**, 1–9.

Diem, H.G. and Dommergues, Y.R. (1983) The isolation of *Frankia* from nodules of *Casuarina*. *Can J Bot* **61**, 2822–2825.

Diem, H.G., Gauthier, D. and Dommergues, Y.R. (1982) Isolement et culture *in vitro* d'une souche infective et effective de *Frankia* isolée de nodules de *Casuarina* sp. *Comptes Rendus Acad Sci Ser III-Sci Vie-Life Sci* **295**, 759–763.

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

Diouf, D., Gherbi, H., Prin, Y., Franche, C., Duhoux, E. and Bogusz, D. (1995) Hairy root nodulation of *Casuarina glauca*: a system for the study of symbiotic gene expression in an actinorhizal tree. *Mol Plant-Microbe Interact* **8**, 532–537.

Finn, G.A. and Brun, W.A. (1982) Effect of atmospheric CO_2 enrichment on growth, non-structural carbohydrate content, and root nodule activity in soybean. *Plant Physiol* **69**, 327–331.

Franche, C., Laplaze, L., Duhoux, E. and Bogusz, D. (1998) Actinorhizal symbioses: recent advances in plant molecular and genetic transformation studies. *Crit Rev Plant Sci* **17**, 1–28.

Franche, C., Bogusz, D., Smouni, A., Diouf, D., Gherbi, H. and Duhoux, E. (1999) Genetic transformation of *Casuarina glauca*. In *Biotechnology in Agriculture and Forestry, Vol 44: Transgenic Trees* ed. Bajaj, Y.P.S. pp. 15–28. Berlin Heidelberg: Springer-Verlag.

Geil, R.D., Peterson, R.L. and Guinel, F.C. (2001) Morphological alterations of pea (*Pisum sativum* cv. Sparkle) arbuscular mycorrhizas as a result of exogenous ethylene treatment. *Mycorrhiza* **11**, 137–143.

George, E.F. (1996) *Plant Propagation by Tissue Culture, Part 2: In Practice*. Edington: Exegetics Ltd.

Guinel, F.C. and Geil, R.D. (2002) A model for the development of the rhizobial and arbuscular mycorrhizal symbioses in legumes and its use to understand the roles of ethylene in the establishment of these two symbioses. *Can J Bot* **80**, 695–720.

Hiscox, J.D. and Israelstam, G.F. (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. *Can J Bot* **57**, 1332–1334.

Hoagland, D.R. and Arnon, D.I. (1938) The water culture method for growing plants without soil. *Cal Agricult Exp Station Cir* **347**, 1–39.

Knowlton, S. and Dawson, J.O. (1983) Effects of *Pseudomonas cepacia* and cultural factors on the nodulation of *Alnus rubra* roots by *Frankia*. *Can J Bot* **61**, 2877–2883.

Laplaze, L., Gherbi, H., Frutz, T., Pawlowski, K., Franche, C., Macheix, J.J., Auguy, F., Bogusz, D., *et al.* (1999) Flavan-containing cells delimit *Frankia*-infected compartments in *Casuarina glauca* nodules. *Plant Physiol* **121**, 113–122.

Ma, W., Penrose, D.M. and Glick, B.R. (2002) Strategies used by rhizobia to lower plant ethylene levels and increase nodulation. *Can J Microbiol* **48**, 947–954.

Mansour, S.R. and Torrey, J.G. (1991) *Frankia* spores of strain HFPCgI4 as inoculum for seedlings of *Casuarina glauca*. *Can J Bot* **69**, 1251–1256.

Mulder, E.G. and van Veen, W.L. (1960) Effect of pH and organic compounds on nitrogen fixation by red clover. *Plant Soil* **13**, 91–113.

Murry, M.A., Fontaine, M.S. and Torrey, J.G. (1984) Growth kinetics and nitrogenase induction in *Frankia* sp HFPArI3 grown in batch culture. *Plant Soil* **78**, 61–78.

Normand, P., Lapierre, P., Tisa, L.S., Gogarten, J.P., Alloisio, N., Bagnarol, E., Bassi, C.A., Berry, A.M., *et al.* (2007) Genome characteristics of facultatively symbiotic *Frankia* sp. strains reflect host range and host plant biogeography. *Genome Res* **17**, 7–15.

Perret, X., Staehelin, C. and Broughton, W.J. (2000) Molecular basis of symbiotic promiscuity. *Microbiol Mol Biol Rev* **64**, 180–201.

Prin, Y. and Rougier, M. (1987) Pre-infection events in the establishment of *Alnus-Frankia* symbiosis: study of the root hair deformation step. *Plant Physiol* **6**, 99–106.

Reddell, P., Bowen, G.D. and Robson, A.D. (1986) Nodulation of Casuarinaceae in relation to host species and soil properties. *Aust J Bot* **34**, 435–444.

Santi, C., Svistoonov, S., Constans, L., Auguy, F., Duhoux, E., Bogusz, D. and Franche, C. (2003) Choosing a reporter for gene expression studies in transgenic actinorhizal plants of the Casuarinaceae family. *Plant Soil* **254**, 229–237.

SAS Institute, Inc. (1985) *SAS User's Guide: Statistics, 5*. Cary, NC: SAS Institute Inc.

Smouni, A., Laplaze, L., Bogusz, D., Germanche, F., Auguy, F., Duhoux, E. and Franche, C. (2002) The 35S promoter is not constitutively expressed in the transgenic tropical actinorhizal tree *Casuarina glauca*. *Funct Plant Biol* **29**, 649–656.

Sougoufara, B. (1990) La fixation de N2 par les casuarinas: amélioration par sélection clonale et quantification par différentes méthodes. *Thèse*, Université Henri Poincaré, Nancy 1, France.

Sougoufara, B., Diem, H.G. and Dommergues, Y.R. (1989) Response of field-grown *Casuarina equisetifolia* to inoculation with *Frankia* strain ORS021001 entrapped in alginate beads. *Plant Soil* **118**, 133–137.

Spaink, H.P. (1997) Ethylene as a regulator of *Rhizobium* infection. *Trends Plant Sci* **2**, 203–204.

Sy, M.O., Sagna, M., Diedhiou, S., Franche, C., Auguy, F. and Duhoux, E. (2003) Devising an *in vitro* nodulation system for two actinorhizal plants: *Allocasuarina verticillata* (Lam.) L. Johnson and *Casuarina glauca* Sieb. ex Spreng (Casuarinaceae). *In Vitro* Cell Dev Biol-Plant **39**, 545–550.

Torrey, J.G. (1976) Initiation and development of root nodules of *Casuarina* (Casuarinaceae). *Am J Bot* **63**, 335–344.

Torrey, J.G. (1983) Root development and root nodulation in *Casuarina*. In *Casuarina Ecology Management and Utilization* ed. Midgley, S.J., Turnbull, J.W. and Johnston, R.D. pp. 180–192. Canberra: Australia CSIRO Publishing.

Torrey, J.G. (1990) Cross-inoculation groups within *Frankia* and host-endosymbiont associations. In *The Biology of Frankia and Actinorhizal Plants* ed. Schwintzer, C.R. and Tjepkema, J.D. pp. 83–106. New York: Academic Press.

Torrey, J.G. and Berg, R.H. (1988) Some morphological features for generic characterization among the Casuarinaceae. *Am J Bot* **75**, 864–874.

Torrey, J.G. and Racette, S. (1989) Specificity among the Casuarinaceae in root nodulation by *Frankia*. *Plant Soil* **118**, 157–164.

Valverde, C. and Wall, L.G. (2005) Ethylene modulates the susceptibility of the root for nodulation in actinorhizal *Discaria trinervis*. *Physiol Plant* **124**, 121–131.

Van Ghelue, M., Lovaas, E., Ringo, E. and Solheim, B. (1997) Early interactions between *Alnus glutinosa* and *Frankia* strain ArI3: production and specificity of root hair deformation factor(s). *Physiol Plant* **99**, 579–587.

Vergnaud, L., Chaboud, A., Prin, Y. and Rougier, M. (1985) Pre-infection events in the establishment of *Alnus-Frankia* symbiosis: development of a spot inoculation technique. *Plant Soil* **87**, 67–78.

Wall, L.G., Valverde, C. and Huss-Danell, K. (2002) Regulation of nodulation in the absence of N_2 is different in actinorhizal plants with different infection pathways. *J Exp Bot* **54**, 1253–1258.

Yamakawa, T., Tanaka, S. and Ishizuka, J. (1997) Effect of CO_2-free air treatment on nitrogen fixation of soybeans inoculated with *Bradyrhizobium japonicum* and *Rhizobium fredii*. *Soil Sci Plant Nutr* **43**, 819–826.

Zhang, Z. and Torrey, J.G. (1985) Studies of an effective strain of *Frankia* from *Allocasuarina lehmanniana* of the Casuarinaceae. *Plant Soil* **87**, 1–16.

