Growth response of *Casuarina equisetifolia* Forst. rooted stem cuttings to *Frankia* in nursery and field conditions

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Casuarina equisetifolia Forst. is a tree crop that provides fuel wood, land reclamation, dune stabilization, and scaffolding for construction, shelter belts, and pulp and paper production. *C. equisetifolia* fixes atmospheric nitrogen through a symbiotic relationship with *Frankia*, a soil bacterium of the actinobacteria group. The roots of *C. equisetifolia* produce root nodules where the bacteria fix atmospheric nitrogen, which is an essential nutrient for all plant metabolic activities. However, rooted stem cuttings of elite clones of *C. equisetifolia* by vegetative propagation is being planted by the farmers of Pondicherry as costeffective method. As the vegetative propagation method uses inert material (vermiculite) for rooting there is no chance for *Frankia* association. Therefore after planting of these stocks the farmers are applying 150 kg of di-ammonium phosphate (DAP)/acre/year. To overcome this fertilizer usage, the *Frankia*-inoculated rooted stem cuttings were propagated under nursery conditions and transplanted in the nutrient-deficient soils of Karaikal, Pondicherry (India), in this study. Under nursery experiments the growth and biomass of *C. equisetifolia* rooted stem cuttings inoculated with *Frankia* showed 3 times higher growth and biomass than uninoculated control. These stocks were transplanted and monitored for their growth and survival for 1 year in the nutrient-deficient farm land. The results showed that the rooted stem cuttings of *C. equisetifolia* significantly improved growth in height (8.8 m), stem girth (9.6 cm) and tissue nitrogen content (3.3 mg g^{-1}) than uninoculated controls. The soil nutrient status was also improved due to inoculation of *Frankia*.

[Karthikeyan A, Chandrasekaran K, Geetha M and Kalaiselvi R 2013 Growth esponse of *Casuarina equisetifolia* Forst. rooted stem cuttings to *Frankia* in nursery and field conditions. *J. Biosci.* **38** 741–747] **DOI** 10.1007/s12038-013-9362-3

1. Introduction

Actinorhizal plants usually form root nodules in association with the nitrogen-fixing actionmycete *Frankia*, which helps them to survive even in nutrient-poor soils by nitrogen (N) fixation. *Frankia* is a filamentous soil bacteria that interacts with the roots of appropriate host 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 has been disseminated throughout the tropics owing to their ability to grow in adverse conditions (Echhab *et al*. 2007). *Casuarina equisetifolia* Forst. belongs to Casuarinaceae and due to its high economic value, farmers are interested in planting this tree as an agroforestry crop in Tamil Nadu and Pondicherry (India). It is useful as wind break, an ornamental plant, soil improvement, live fencing and building material (Nicodemus 2009). *Frankia* is associated with *C. equisetifolia* for N fixation and it has been estimated that *Frankia* fixes atmospheric nitrogen up to 362 kg N/ha/year, which is an essential nutrient for all plant metabolic activities and growth (Shantharam and Mattoo 1997).

To inoculate *Frankia* generally farmers used to collect the root nodules from mature trees of *C equisetifolia* and then crush and add them at the time of planting in new sites along with seedlings/cuttings of *C. equisetifolia*. This practice is often unsuccessful if the crushed root nodules contain dead or inactive *Frankia*. Further, for pulp and paper production high-yielding genetically superior trees of *C. equisetifolia* are selected and multiplied by rooted stem cuttings by the farmers of Tamil Nadu and Pondicherry . But the rooted stem cuttings are being propagated in an inert material (vermiculite), and so there is no chance for *Frankia* association.

Keywords. *Casuarina equisetifolia* rooted stem cuttings; *Frankia*; nitrogen; root nodules

Published online: 1 October 2013

Although inoculation of *Frankia* is essential in rooted stem cuttings of *C. equisetifolia* there is no report found on nodulation of rooted stem cuttings in *C. equisetifolia.* Further, farmers of Pondicherry and Tamil Nadu (India) apply 150 kg of DAP/year/ha after planting of *C. equisetifolia* seedlings or rooted stem cuttings in their farmlands. Hence, there is an urgent need to find an alternate solution for use of these chemical fertilizers for the rooted stem cuttings of *C. equisetifolia*. The effect of inoculation of cultured *Frankia* strain in rooted stem cuttings of *C. equisetifolia* on growth, biomass and nodulation was studied, and this could reduce the use of chemical fertilizers. Further, it is intended to decide the effect of *Frankia* on the efficiency of N uptake of *C. equsetifolia* rooted stem cuttings.

2. Materials and methods

2.1 *Location of the study*

The study was conducted at the Model Nursery of Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore (11°01′ N and 96°93′ E; altitude 410 m a.s.l.), India. The climate is monsoonal, with an annual precipitation of 640 mm and a dry season between January and April. The maximum and minimum monthly temperatures are 31°C and 21°C, respectively.

2.2 *Isolation and multiplication of Frankia*

The *Frankia* used in this study was isolated from *C. equisetifolia* root nodules collected from coastal area, and the location and characteristics of collected nodules are shown in the table 1.

The nodules were collected in ice box and stored in frozen condition at −4°C. Afterwards, the nodules were surface-sterilized with 30% H_2O_2 and kept in a shaker for 30–40 min. Under aseptic conditions the nodules were rinsed in sterile water and 0.2 g of nodule was ground manually in sterile mortar and pestle. Then the nodule solutions were centrifuged at 1000 rpm for 20 min and the supernatant was filtered through Whatman No.1 filter paper. The suspension was then plated in P medium and incubated at 25°C for 3–4 weeks. One litre of P medium was prepared as follows (Shipton and Burgraff 1983): 10 g $CaCl_2$,$2H_2O$, 20 g $MgSO_4$, 0.46 g propionic acid, 0.15 g H_3BO_3, 0.15 g $ZnSO_4$.7H2O, 0.45 g $MnSO_4.H_2O$, 0.004 g $CuSO_4.5H_2O$, 0.028 g $Na_2MoO_4.2H_2O$, 0.009 g $CaCl_2.6H_2O$, 0.04 g Biotin, 100 g K_2HPO_4, 67 g $NaH_2PO.2H_2O$, 0.1 g FeNa EDTA, and 8.g agar. The pH of the medium was adjusted to 6.8. After 21–30 days of incubation the *Frankia* growth was observed as fluffy white cloudy colonies in P media plates. These colonies were transferred in to P medium broth for mass multiplication. Under microscopic observations the *Frankia* showed pin-headed vesicles and septate hyphae (figure 1). The morphometric characters of the *Frankia* was also assessed and presented in table 2. The identification of *Frankia* was also confirmed at molecular level by16s RNA sequence and named as strain Ce Co1 (Karthikeyan *et al*. 2012).

2.3 *Collection and propagation of C. equisetifolia stem cuttings*

The stem cuttings of single elite Clone No. CE 100 were collected from the Casuarina germplasm bank at Model Nursery, IFGTB, Coimbatore, India, where 250 clones of Casuarina have been housed for research and breeding purpose. The stem cuttings were collected from the terminal ends of the twigs of the CE 100 during the month of November 2011 and preserved in ice box (4°C). Uniform sized stem cuttings with a length of 5 cm (±0.4.3) and a girth of 0.3 cm (±0.052) were taken for this study. The basal ends of the cuttings have been crossed cut to optimize the Indole Butyric Acid (IBA) uptake for better rooting. The stem girth was measured at the basal end where root initiation (root collar) will actually take place. The stem cuttings were treated with 0.1% carbendazim fungicide for 3 min and after 2000 ppm of IBA (40 mg of IBA + 20 g of talcum powder) at the basal end of the cuttings for 0.5 min by dip method. After the treatment with IBA the cuttings were placed in 100 cc root trainers containing the inert media vermiculite. The rooted cuttings were thereafter placed in polytunnels made of polythene sheets (180 cm × 90 cm) and maintained with temperature of 32–35°C and 60–65% relative humidity for 25 days. After 25 days the cuttings showed initiation of 2 to 3 lateral roots of 1 cm to 1.5 cm length. At this stage the rooted stem cuttings were transferred to shade house and watered regularly.

2.4 *Inoculation of Frankia in C. equisetifolia rooted stem cuttings*

An amount 0.1 mL of the cultured *Frankia* strain Ce Co1 in P medium broth was inoculated in conical flasks with 250 mL capacity. These liquid cultures were incubated at 32°C in

Table 1. Source of *Frankia*

Place	Soil type	Source of nodules	Nodules colour	Nodules diameter
Cuddalore (TN) Coastal zone	Sandy clayloam	Coastal plantations of *Casuarina equisetifolia*	Brown	1–1.5 cm

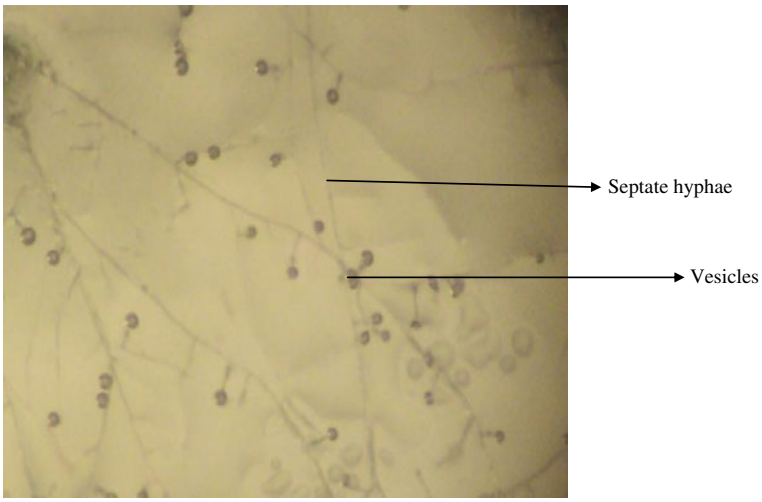

Figure 1. Microscopic structures of *Frankia.*

the Orbitol Shaker (Gyromax 703 R, USA) at 20 rpm for 25 days. Later the cultures were homogenized in the centrifuge at the rate of 1000 rpm for 20 min. Thereafter the cultures of *Frankia* were inoculated in the root zone of rooted stem cuttings of *C. equisetifolia* at the rate of 5 mL^{-1} cutting that contained 2.2 µg protein mL^{-1}. The inoculated rooted stem cuttings were maintained in the nursery with 15 replicates including uninoculated controls in the shade house and watered regularly. The initiation of root nodules and nodule numbers in each rooted stem cuttings were assessed. These planting stocks of *C. equisetifolia* were maintained for 3 months in the model nursery of IFGTB and harvested for analysis of growth and biomass. The dry weight of *Frankia* inoculated these planting stocks was determined after oven drying at 50°C to a constant weight.

2.5 *Analyses of growth, biomass and tissue nitrogen content*

A set of (5 replicates) of *C. equisetifolia* rooted stem cuttings were taken for harvest and analysis The growth of *Frankia*-inoculated rooted stem cuttings and uninoculated cuttings were analysed in terms of shoot length, root length, number of lateral roots, collar diameter, dry weights of shoot, root, number of nodules and nodule biomass. The dry weights were determined after oven drying at 50°C to a constant weight. The total N content was estimated in root and shoot sample using KELPLUS auto analyser to determine the N fixation by inoculation of *Frankia* in the rooted stem cuttings of *C. equisetifolia* The dried plant sample (0.25 gm) was digested with 3 gm of catalyst mixture (potassium sulphate and cupric sulphate in ratio of 5:1) and 10 mL of H_2SO_4 in Kjeldhal digestion system (KELFLOW) at 420°C for 1 h. Then the digested sample was diluted with 10 mL of distilled water before distillation. After distillation, the collected distillate was titrated against 0.1 N hydrochloric acid.

2.6 *Field planting*

The other 10 replicates were taken for field planting to test the effect of inoculated *Frankia* strain in field conditions. The rooted stem cuttings of *C. equisetifolia* were planted in a farm land located at coastal area of Karaikal, Pondicherry (India). The planting stocks were planted at an espacement of 1.5×1.5 met. The uninoculated control rooted stem cuttings were also planted in the same area in randomized block design. The growth of the rooted stem cuttings were monitored for 1 year under field conditions.

2.7 *Analysis of soil nutrients*

The major soil nutrients (N, P and K), soil pH and electrical conductivity of the soil were analysed before and after

Table 2. Morphometrics of *Frankia*

Hyphal width (in µm @ 40×)	Vesicle dimension (in µm @ 40×)	Sporangia shape	No. of days grown in media
1–1.5	2–3	Circular	25 days

planting of *C. equisetifolia* inoculated with *Frankia* according Jackson (1973).

2.8 *Statistical analysis*

Each measured variable in the nursery and field experiments were statistically analysed using Duncan's multiple range test (SPSS ver. 16).

3. Results

3.1 *Morphological characteristics of Frankia isolate*

Under optimal conditions (28–32°C), the growth of the isolate formed white fluffy colonies on the P media plates. They were examined under a light microscope and showed branched and septate hyphae and round vesicles. The morphometrics of *Frankia* are shown in table 2.

3.2 *Growth and biomass of C. equisetifolia rooted stem cuttings*

Nodulation of *Frankia* was observed 30 days after inoculation in the rooted stem cuttings of *C. equisetifolia*. The initial infection at 20 days showed clubbed roots in the rooted stem cuttings, and the nodule development occurred at 30 days. The rooted stem cuttings inoculated with *Frankia* strain showed significantly increased growth in shoot length, root length, collar diameter and biomass as compared with the uninoculated control seedlings. The rooted stem cuttings showed higher nodule biomass than the uninoculated control. *Frankia*-inoculated cuttings showed dense root nodules in the root region, whereas the uninoculated cuttings showed absence of nodules. The root nodules developed in the rooted stem cutting weighed up to 43 mg and the number of root nodules obtained was 12 per cutting. The R/S ratio was showed significantly lower in *Frankia*-inoculated rooted stem cuttings than the uninoculated control (table 3). A new finding was also derived from this study – successful nodulation establishment in the *C.equisetifolia* rooted stem cuttings in inert media without using soil (figure 2).

3.3 *Tissue N content*

Significant differences in nitrogen concentration of *Frankia*-inoculated rooted stem cuttings of *C. equisetifolia* in comparison with uninoculated controls were observed. The total N concentration was found 3.30 mg/g dry weight in the rooted stem cuttings, whereas the uninoculated control rooted stem cuttings showed a mean value of 1.32 mg/g dry weight (figure 3).

Table 3. Growth and bio mass of *C. equisetifolia* rooted stem cuttings to *Frankia* inoculation at 90 days under nursery conditions

Treatments	Collar diameter (cm plant^{-1})	Shoot length (cm plant^{-1})	Root length (cm plant^{-1})	No. of lateral roots plant^{-1})	Shoot dry weight (mg plant^{-1})	Root dry weight (mg plant^{-1})	R/S ratio	Nodulation time	No. of nodules	Nodule biomass (mg nodule^{-1})
Frankia	1.871 b	18.89 b	14.3 b	15.1 b	0.905 b	0.557 b	0.615 b	30 days	12.12(±1.1)	43(±1.3)
Control	0.542 a	5.9 a	4.8 a	1.8 a	0.288 a	0.199 a	0.690 a	00	00	00

Data was mean of 15 replicates; means followed by same letters are not significantly different at $p<0.05$ according to Duncan's Multiple Range Test. ± Standard error of the mean.

Control Formation of root nodules

Figure 2. Establishment root nodules in the rooted stem cuttings of *C. equisetifolia* inoculated with *Frankia.*

3.4 *Soil nutrients*

The soil nutrients, particularly soil N, was highly increased after planting of *C. equisetifolia* rooted stem cuttings inoculated with *Frankia*. The electrical conductivity and soil pH was also changed after planting (table 4).

3.5 *Field performance*

Frankia inoculated rooted stem cuttings of *C. equisetifolia* significantly ($p<0.05$) increased their growth in terms of height and stem girth as compared with uninoculated controls. Three-fold increase of growth was observed due to inoculation of *Frankia* in the rooted stem cuttings of *C. equisetifolia* under field conditions (figure 4).

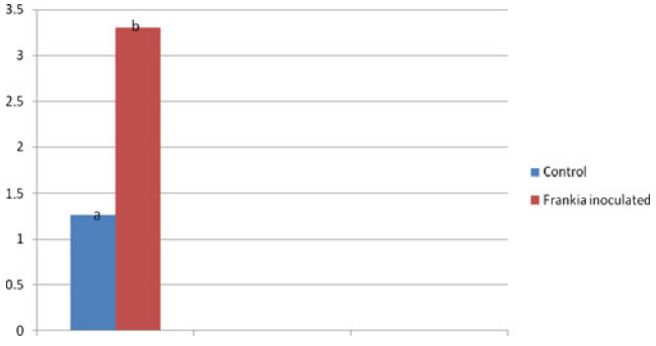

Figure 3. Tissue N content (mg g^{-1}). Means followed by same letters are not significantly different at $p<0.05$ according to Duncan's Multiple Range Test.

4. Discussion

The results of this study have clearly shown that *Frankia* can improve plant growth through increased uptake of N. *Frankia* results in a positive effect on the rooted stem cuttings of *C. equisetifolia* growth through improvement of growth and biomass. Earlier studies also reported that the increase in growth and biomass of casuarinas due to inoculation of *Frankia* might be strongly correlated with improved accumulation of nitrogen due to *Frankia* (Reddell *et al.* 1988). This study further supports the positive response of *C. equisetifolia* rooted stem cuttings in the nursery to *Frankia* application and strengthens the *Frankia* dependency of *C. equisetifolia* in low fertility. Similar results were reported for *Frankia* (nodule suspension) inoculation employed in *C. equisetifolia* seedlings (Muthukumar and Udaiyan 2010). In several studies (Lesueur and Duponnois 2005; Yamanka *et al.* 2003) *Frankia* effects on plant growth promotion has been demonstrated in sterile soil substrates. However, the growth promoting effect of *Frankia* on *C. equisetifolia* rooted stem cuttings in inert media has not been reported. It has been repeatedly reported that spontaneous nodulation of the genera *Casuarina* is unlikely outside their natural habitat. This may be attributed to the fact that *Frankia* cannot transmit with the seed either within or on

Table 4. Soil nutrient status of the planting site before and after planting of *C. equisetifolia* rooted stem cuttings inoculated with *Frankia*

	pH	E.C mS	N mg kg^{-1}	P mg kg^{-1}	K mg kg^{-1}
Before planting	6.0±1.24	0.08±0.02	0.30±0.025	1.30±0.68	4.0±1.56
After planting	6.8±1.32	0.19±0.01	3.63±1.03	2.4±0.83	5.3±1.45

its surface (Torrey 1983). Inoculation experiments of this kind in nursery conditions is essential for *C. equisetifolia* rooted stem cuttings, which bring together the root system and nodulation as they propagated in inert media. In this study nodulation occurs in 30 days in the rooted stem cuttings of *C. equisetifolia*; however, Vergnaud *et al.* (1985) obtained axenic nodulation in *Alnus glutinosa* within 10 days. This also showed that there is a difference in nodulation behaviour between *Alnus* species and *C. equisetifolia.* Nodulation biomass and nodule number were increased the rooted stem cuttings of *C. equisetifolia* raised in inert medium. This reflects the symbiotic nitrogen fixation is dependent on host photosynthesis (Arnone and Gordon 1990), which affords energy in the form of ATP to *Frankia* in root nodules. The increased biomass in the rooted stem cuttings of both the clones could be the result of increased nutrient inflow rates through *Frankia*. The increased tissue N content of *Frankia*-inoculated rooted stem cuttings of *C. equisetfolia* raised in inert media as compared with the uninoculated control plants showed the influence of *Frankia* in N fixation. The field performance of *Frankia*-inoculated rooted stem cuttings showed increased growth, which may be due to increased uptake of N through *Frankia*. Earlier studies also showed that the growth of *Alnus cordata* inoculated with *Frankia* had increased growth and survival in field conditions due to the uptake of N (Lumini *et al.* 1994). Soil nutrients also improved due to the activity of *Frankia*, which supports the inoculation

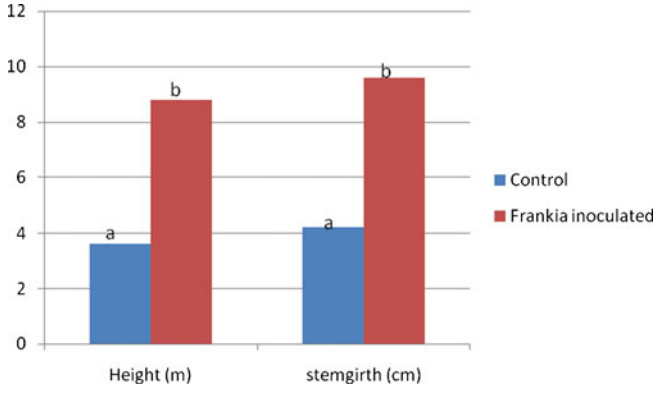

Figure 4. Growth performance of *C. equisetifolia* rooted stem cuttings inoculated with *Frankia* under field conditions after one year of planting. Means followed by same letters are not significantly different at $p<0.05$ according to Duncan's Multiple Range Test.

of *C. equisetifolia* with cultured *Frankia* for enhancement of growth, biomass, nutrient uptake and soil fertilization. It is essential to introduce potential *Frankia* in the rooted stem cuttings of *C. equisetifolia* as they propagated in inert media (vermiculite). The N fertilization through biological nitrogen fixation by *Frankia i*mproved significantly the growth and biomass in field conditions as compared with uninoculated *C. equisetifolia*. These results are also confirmed the earlier work on Alders inoculated with *Frankia* for reclamation of oil stand sites where *Frankia*-inoculated Alders produced significant biomass in the reclaimed oil stands in Canada (Lefroncois *et al* .2010). From this study it was deduced that inoculation of *Frankia* in *C. equisetifolia* would be beneficial for early establishment in the field without additional chemical fertilizers.

Acknowledgements

The authors thank the Department of Biotechnology, New Delhi, for financial support for a research project (Project No. BT/PR10464/AGR/21/239/2008).

References

Arnone JA and Gordon JC 1990 Effect of nodulation nitrogen fixation and CO_2 enrichment on the physiology, growth and dry mass allocation of seedlings of *Alnus rubra* Bong. *New Phytol.* **116** 55–66

Benson DR and Silvester WB 1993 Biology of Frankia strain, actionomycete symbionts of actinorhizal plants. *Microbiol. Rev.* **57** 293–319

Echhab H, Anatous M, Ducousto M, Nourrssion –Mountour S, Dupponnis SR and Lahlour H 2007 Successful nodulation of Casuarina by *Frankia* in axenic conditions. *J. Appl. Microbiol.* **103** 1728–1737

Jackson ML 1973 *Soil chemical analysis* (New Delhi: Prentice Hall)

Karthikeyan A, Chandrasekaran K and Geetha M 2012 Frankia sp CeCo1 16S Ribosomal RNA gene partial sequence. NCBI, USA, Accession No. JQ 412181

Lefrancois E, Quoreshi A, Khasa D, Fung M, Whyte L G, Roy S and Greer C.W 2010 Field performance of alder – Frankia symbionts for the reclamation of oil stands sites. *Appl. Soil. Ecol.* **46** 183–191

Lesueur O and Duponnois R 2005 Relations between rhizobial nodulation and root colonization of *Acacia crassicarpa*

provinces by an arbuscular mycorrhizal fungus *Glomus intrradices* Schenk and Smith or an ectomycorrhizal fungus *Pisolithus tinctorius* coke & couch. *Ann. For. Sci.* **62** 467–474

Lumini E, Bosco M, Puppi G, Isopi R, Fralergrani M, Bureti E and Faviki F 1994 Field performance of *Alnus cordata* Loisel (India alba) inoculated with *Frankia* and VA mycorrhizal strains in mine spoil afforestation plots. *Soil. Biol. Biochem.* **26** 659–661

Muthukumar T and Udaiyan, K 2010 Growth response and nutrient utilization of *Casuarina equisetifolia* seedlings inoculated with bio inoculants under tropical nursery conditions. *New. For.* **40** 101–118

Nicodemus A 2009 *Casuarina a guide for cultivation* (ICFRE Publication)

Reddell, P, Rosbrook PA, Bowen GO and Gnale D 1988 Growth response in *Casuarina cunnighamiana* plantings inoculation with *Frankia* . *Plant Soil* **108** 76–86

Shantharam S and Mattoo AK 1997 Enhancing biological nitrogen fixation: an appraisal of amount and alternative technologies for N in put into plants. *Plant Soil* **194** 205–216

Shipton WA and Burgraff AJP 1983 Aspects of the cultural behaviour of *Frankia* and possible ecological implication. *Can. J. Bot*. **61** 2783–2792

Torrey JG 1983 Root development and root nodulation in casuarinas; in *Casuarina ecology management and utilization* (ed) SJ Midgley, JW Turnbul and RP Johnstorn (Canberra: Australia CSIRO publishing) pp 180–192

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

Yamanka, T, Li CY, Bormann BT and Okabe H 2003 Tripartite association in an alder: effects of *Frankia* and *Alpova diplophloeus* on the growth, nitrogen fixation and mineral acquisition of *Alnus tenuifolia*. *Plant Soil* **254** 179–186