FRANKIA STRAINS FOR IMPROVING GROWTH, BIOMASS AND NITROGEN FIXATION IN *CASUARINA EQUISETIFOLIA* SEEDLINGS

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KARTHIKEYAN A. 2016. *Frankia* strains for improving growth, biomass and nitrogen fixation in *Casuarina equisetifolia* seedlings. *Casuarina equisetifolia* fixes atmospheric nitrogen (N) to produce root nodules where actinomycete fixes atmospheric N for all metabolic activities of plant. Two strains of *Frankia* (CeFr1, CeFr2) were isolated, cultured in artificial propionic acid medium and analysed for nitrogenase activity by acetylene reduction assay to ascertain their capabilities in N fixation. Seedlings of *C. equisetifolia* were inoculated with *Frankia* strains in the nursery and their growth performances were assessed. Seedlings inoculated with *Frankia* strains showed improved growth, biomass and tissue N content over control seedlings. Nitrogenase activity of *Frankia* strains were significantly ($p < 0.05$) and negatively correlated with tissue N content. Inoculated seedlings were then planted on a farmland without adding fertilisers and assessed after 2 years. Seedlings inoculated with CeFr1 and CeFr2 showed significantly improved growth in height (9.5 and 8.4 m respectively) compared with control. Seedlings inoculated with *Frankia* strains achieved > 95% survival and had improved biomass and N fixation.

Keywords: Nitrogenase, inoculation, cultured, height growth, farm forestry

INTRODUCTION

Casuarina equisetifolia is a nitrogen (N)-fixing tree that grows naturally in Australia, Indonesia, Philippines, Malaysia, Thailand and some Pacific islands. It is used for paper production and scaffolding for building construction (Pinyopusarerk & Williams 2000). This tree is also used in agroforestry system together with vegetable and pulse crops. It grows up to 50 m high with 50 cm girth. Farmers in India harvest *C. equisetifolia* after 3.5 to 4 years. Annual pulpwood production of *C. equiseitifolia* is 10 million tonnes which amount to USD300,000 (Karthikeyan et al. 2009). Currently, poles of *C. equisetifolia* cost USD100–120 tonne^{-1} in India and they are widely used for fuel, shelterbelts, windbreaks and rehabilitating mine spoils and nutrient-poor areas (Diagne et al. 2013). The tree is important in protecting coastal environment against storms and tsunami and has become a main bioshield component in many coastal afforestation programmes (Karthikeyan et al. 2009). This tree has good nutrient turnover through litter decomposition (Uma et al. 2014).

Casuarina equisetifolia fixes atmospheric nitrogen through symbiotic relationship with *Frankia*, a soil bacterium of actinomycete group (Benson & Silvester 1993). Nitrogen is generally considered as one of the major limiting nutrients for plant growth. As part of the symbiotic relationship with *Frankia*, this tree converts fixed ammonium ion into nitrogen oxides and amino acids to form proteins and other molecules (e. g. alkaloids). In return for the fixed N, this tree supplies carbon to the symbiotic bacteria (Santi et al. 2013). Symbiotic association of *Frankia* with *C. equisetifolia* fixes N at an estimated rate of 362 kg ha^{-1} year^{-1} (Shantharam & Mattoo 1997). Generally *Frankia* is being inoculated by farmers into seedlings of *C. equisetifolia* through application of crushed root nodules collected from its mature trees. This practice is often unsuccessful as root nodules may contain dead or inactive *Frankia* that fail to nodulate seedlings.

In India, farmers of Pondicherry and Tamilnadu also apply diammonium phosphate fertiliser as N supplement after planting of *C. equisetifolia*. This increases the cost in planting operations besides creating environmental problems (Karthikeyan et al. 2013). Hence, there is a need to develop a low cost and effective protocol for N fertilisation for *C. equisetifolia*.

As *Frankia* is a symbiotic N-fixing bacterium, the possible ways of its use under nursery and in farmland conditions were explored in this study. It was hypothesised that the capability of *Frankia* to fix N and its influence on biomass improvement of C. *equisetifolia* under field conditions would vary with different strains of bacteria. To examine this hypothesis, *Frankia* strains were evaluated based on their N-fixation capabilities, nitrogenase activities and their influences in nursery and field experiments of *C. equisetifolia* seedlings. The results of this study showed the beneficial effects of *Frankia* which will be helpful to reduce the use of chemical fertilisers and minimise cost in cultivation of *C. equisetifolia*.

MATERIALS AND METHODS

Study area

This nursery experiment was conducted at the Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore, Tamilnadu, India. Field trials were carried out at a farmland in Karaikal, Pondicherry located about 316 km away from the Institute.

Materials

Seeds of *C. equisetifolia* were collected from the seed bank of IFGTB and sown in nursery beds containing pure sand with sufficient water spray. After germination, 10-day-old seedlings of uniform height (5 cm) were transplanted to polythene bags (10 cm × 14 cm) containing sterile sand and red soil (1:1 v/v). Root nodules of *C. equisetifolia* were collected from field-grown trees as source of *Frankia*. The location and the characteristics of collected nodules are given in Table 1. Nodules were collected and transported in an ice box and kept at -4 °C until use. Nodules were surface sterilised with 30% hydrogen peroxide and kept at room temperature for 30–40 min. Under aseptic conditions the nodules were rinsed in sterile water and 0.2 g of nodule was ground manually using sterile mortar and pestle. The nodule solution obtained was centrifuged at 1000 g for 20 min and the supernatant was filtered through Whatman No. 1 filter paper. The suspension was then placed on propionic acid medium (P medium) and incubated at 25 °C for 3–4 weeks. One litre of P medium contained 10 g calcium chloride dehydrate, 20 g magnesium sulphate, 0.46 g propionic acid, 0.15 g boric acid, 0.15 g zinc sulphate heptahydrate, 0.45 g manganese sulphate monohydrate, 0.004 g copper sulphatehydrate, 0.028 g sodium molybdate dehydrate, 0.009 g calcium chloride hexahydrate, 0.04 g biotin, 100 g dipotassium phosphate, 67 g sodium dihydrogen phosphate, 0.1 g ethylene diamine tetraacetic acid ferric sodium salt and 8 g agar (Shipton & Burgraff 1983). The pH of the medium was adjusted to 6.8. After 25 days of incubation, *Frankia* growth was observed as fluffy white cloudy colonies in P medium plates. These colonies were transferred into P medium broth for scaling up the inoculum. The two strains of *Frankia* were named CeFr1 and CeFr2. Morphometric characters of *Frankia* strains were also measured using calibrated ocular scale.

Analysis of nitrogenase activity

Nitrogenase activity of *Frankia* was determined in 25-day-old cultures in N-free P medium broth using acetylene reduction technique (Hardy et al. 1968). Thirty mililitres of culture were placed in 130-mL sterilised vials and sealed with rubber

Table 1 Locations where *Frankia* strains were isolated from *C. equisetifolia* root nodules and characteristics of nodules

Frankia strains	Location	Soil type	Source of root nodules	Colour of root nodules	Root nodules diameter (cm)
CeFr1	Cuddalore (11.8° N, 79.8°E), Tamilnadu	Sandy clay loam	Coastal plantations	Brown to pale yellow	1.5 ± 0.002
CeFr2	Nagapatinam (10.8° N, 79.8° E), Tamilnadu	Sandy clay loam	Coastal plantations	Pink to light brown	0.9 ± 0.001

± standard error of mean

stoppers. The 10% air space in each vial was replaced by pure acetylene and allowed to stand for one hour incubation at room temperature (24–25 °C). To measure ethylene production, about 0.5 mL of the gas was removed from each vial and injected into a gas chromatography equipped with flame ionisation detector and 2 m × 2.1 mm stainless steel column packed with Porapak Q on 80/100 mesh. Oven temperature was set at 70 °C, injector temperature 50 °C and detector temperature 120 °C. Flow rate of the N carrier gas was adjusted to 30 mL min^{-1}. Blanks comprised air from bottles to which no inoculum was added. Peaks of ethylene were compared with ethylene standard (purity 99.9%). Nanomoles of ethylene produced per time unit were standardised to total cell protein. Protein concentration of the cells was determined according to Lowry et al. (1951) using bovine serum albumin as standard. Specific nitrogenase activity was expressed as nanomoles of ethylene mg^{-1} protein $hour^{-1}$. The rate of N fixation was calculated using the formula:

$$\text{Nitrogenase activity} = P \times 0.0006 \times V_1 \times V_2 \times W$$

where P = peak area count, V_1 = volume of gas injected into vial incubation $time^{-1}$, V_2 = volume of gas injected into gas chromatography and W = total weight of protein in sample (mg).

Inoculation of *Frankia* strains

Cultured *Frankia* strains CeFr1 and CeFr2 in P medium broth were inoculated in the root zone of *C. equisetifolia* seedlings grown in polybags at 10 mL $seedling^{-1}$ with 15 replicates. Inoculated seedlings and controls were placed in the shade house (mean temperature 24 °C and mean relative humidity 65.6%) and watered regularly. These seedlings were maintained for 3 months in the research nursery of IFGTB after which they were harvested for analysis and field planting.

Analyses of growth, biomass and tissue N content

A set of five replicates of *C. equisetifolia* seedlings were harvested and analysed in terms of shoot length, root length, number of lateral roots, collar diameter, dry weights of shoot and root, number of nodules and nodule biomass. Shoot and root dry weights were determined after oven drying at 50 °C until constant weight. Total N content was estimated in root and shoot samples using autoanalyser to determine the influence of N in inoculated *Frankia* strains on N content of *C. equisetifolia* seedlings. Dried plant sample (0.25 g) was digested with 3 g of catalyst mixture (potassium sulphate and cupric sulphate 5:1) and 10 mL of sulphuric acid in Kjeldahl digestion system at 420 °C for 1 hour. Digested sample was diluted with 10 mL distilled water and distilled. The distillate was titrated against 0.1 N hydrochloric acid.

Field planting and analysis of soil nutrients

The remaining seedlings from the nursery were taken for field planting to test the performance of inoculated *Frankia* strains seedlings. Seedlings were planted at a farmland located at the coastal area of Karaikal, Pondicherry. Inoculated and control seedlings were planted at spacing of 1.5 m^2 in three plots in a randomised block design. Growth and survival of the seedlings were monitored for 2 years. Major soil nutrients (N, phosphorus and potassium), soil pH and electrical conductivity of the soil were analysed before and after planting of *C. equisetifolia* according to Jackson (1973).

Statistical analysis

Each measured variable in the nursery and field experiments was statistically analysed using Duncan's multiple range test. Relationship between nitrogenase activity of *Frankia* and tissue N content was described by performing simple linear regression (SPSS version 17, 2008).

RESULTS

Microscopic structures of *Frankia* strains

Microscopic structures of *Frankia* strains showed branched and septate hyphae and round or oval vesicles. The morphometrics of *Frankia* strains showed branched septate hyphae in both strains CeFr1 and CeFr2. The vesicles, which are the N-fixing sites of *Frankia*, were round in the strain CeFr1 and oval in CeFr2. Hyphal width was smaller in CeFr2 (1.3 µm) than CeFr1 (1.7 µm).

Similarly vesicle dimension was smaller (2.2 µm) in CeFr2 than CeFr1 (2.8 µm) (Table 2).

Nitrogenase activity

After 25 days incubation in liquid culture, CeFr1 showed that nitrogenase activity was 45.4 µmol ethylene mg^{-1} protein $hour^{-1}$ while CeFr2 had 33.2 µmol ethylene mg^{-1} protein $hour^{-1}$ (Table 3).

Growth and biomass of *C. equisetifolia* seedlings

After 20 days of inoculation with *Frankia*, *C. equisetifolia* seedlings had clubbed roots and root nodules whereas control seedlings did not form any root nodules. Inoculated seedlings showed significantly increased shoot length, root length, collar diameter and biomass compared with control seedlings (Table 4). Seedlings had heavier nodules when inoculated with CeFr1 than with CeFr2. However, the latter had more number of root nodules compared with the former. Root nodules developed in *C. equisetifolia* seedlings inoculated with CeFr1 weighed to 42.2 mg $seedling^{-1}$ and number root nodules obtained was 10.6 $seedling^{-1}$. Seedlings inoculated with CeFr2 had 16.2 root nodules $seedling^{-1}$ and nodule biomass of 35.6 mg $seedling^{-1}$. Root:shoot dry weight ratios of inoculated seedlings were significantly lower than control (Table 4).

Tissue N content and relationship with nitrogenase activity

Significant difference in total (shoot + root) N content was evident between control and inoculated seedlings. CeFr1- and CeFr2-inoculated seedlings showed significantly higher total N content compared with control (2.9, 1.7

Table 2 Morphometrics of *Frankia* strains

Strain	Hyphal width (µm, 40×)	Hyphal shape	Vesicle dimension (µm, 40×)	Vesicle shape
CeFr1	1.7 ± 0.012	Branched and septate	2.8 ± 0.007	Round
CeFr2	1.3 ± 0.010	Branched and septate	2.2 ± 0.009	Oval

Results are means of five replicates, ± standard error of mean

Table 3 Mean values of nitrogenase activity of *Frankia* strains by acetylene reduction assay

Strain	Nitrogenase activity (µmol ethylene mg^{-1} protein $hour^{-1}$)
CeFr1	45.43 ± 1.26
CeFr2	33.20 ± 1.32

Results are means of five replicates, ± standard error of mean

Table 4 Growth biomass and tissue nitrogen (N) content of *C. equisetifolia* seedlings inoculated with *Frankia strains* at 90 days under nursery conditions

Strain	Collar diameter (cm $plant^{-1}$)	Shoot length (cm $plant^{-1}$)	Root length (cm $plant^{-1}$)	No. of lateral roots ($plant^{-1}$)	Shoot dry weight (g $plant^{-1}$)	Root dry weight (g $plant^{-1}$)	Root/ shoot dry weight ratio	No. of nodules ($plant^{-1}$)	Nodule biomass (mg $plant^{-1}$)	Tissue N content (mg g^{-1})
CeFr1	1.58 c	111.3 c	22.8 b	11.5 b	5.86 c	3.76 c	0.64 b	10.6 b	42.2 b	2.92 c
CeFr2	1.47 b	98.8b	18.6 b	9.3 b	4.65 b	2.89 b	0.62 b	16.2 a	35.6 a	1.70 b
Control	0.71 a	58.6a	10.10 a	3.58 a	2.1 a	1.65 a	0.78 a	-	-	0.89 a

Results are means of 15 replicates, means followed by the same letters are not significantly different at $p < 0.05$

and 0.9 mg g^{-1} dry weight respectively) (Table 4). Significant negative correlation ($p < 0.05$) was found between nitrogenase activity of the two *Frankia* strains and tissue N contents of the seedlings (Figures 1 and 2).

Field performance and soil nutrients

Inoculation of *Frankia* significantly ($p < 0.05$) increased growth and survival of inoculated *C. equisetifolia* seedlings compared with control (Table 5). After 2 years, survival rate reached 98.3% in CeFr1-inoculated seedlings and 94.6% in CeFr2-inoculated seedlings. Soil nutrients, particularly soil N, improved after planting of inoculated *C. equisetifolia* seedlings (Table 6).

DISCUSSION

Inoculation of *Frankia* cultures showed early root nodule formation in *C. equisetifolia* seedlings. *Frankia* infection always takes place through intracellular penetration of hyphae via root hair of host plants (Franche & Bogsuz 2012). Hence, inoculation of *Frankia* should be executed after root initiation in the host seedlings for successful infection. After incubation for 20 days in the nursery, nodulation occurred in *C. equisetifolia*

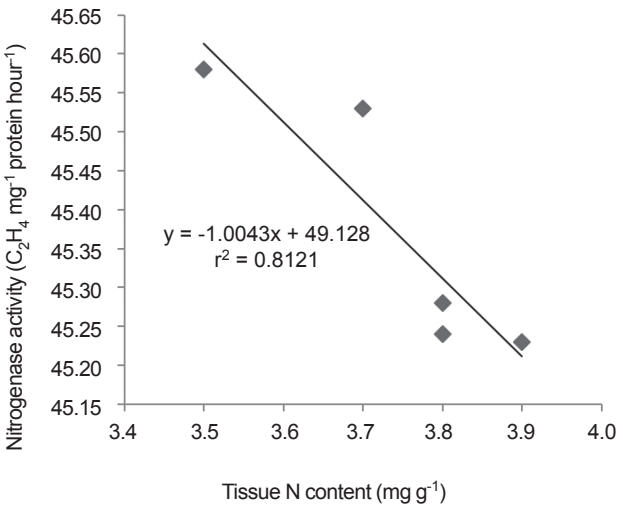

Figure 1 Relationship between nitrogenase activity of *Frankia* strains CeFr1 and tissue nitrogen (N) content in *Casuarina equisetifolia* seedlings; significant at $p < 0.05$

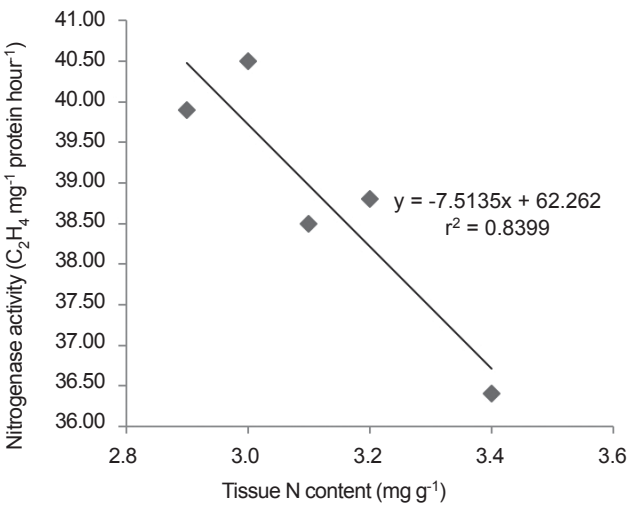

Figure 2 Relationship between nitrogenase activity of *Frankia* strain CeFr2 and tissue nitrogen (N) content in *Casuarina equisetifolia* seedlings; significant at $p < 0.05$

Table 5 Field performance of *C. equisetifolia* seedlings inoculated with *Frankia* strains after 2 years

Frankia strain	Height (m)	Stem girth (cm)	Survival (%)
CeFr1	9.5 b	12.8 b	98.3 c
CeFr2	8.4 b	11.6 b	94.6 b
Control	3.2 a	5.6 a	57.3 a

Results are means of 10 replicates, means followed by the same letters are not significantly different at $p < 0.05$

Table 6 Soil properties before and two years after planting

	PH	Electrical conductivity (mS)	Nitrogen (mg kg^{-1})	Phosphorus (mg kg^{-1})	Potassium (mg kg^{-1})
Before planting	6.0 ± 0.14	0.08 ± 0.02	1.30 ± 0.02	1.98 ± 0.13	4.0 ± 0.06
After planting	6.8 ± 0.12	0.19 ± 0.01	3.63 ± 0.03	2.40 ± 0.16	5.3 ± 0.08

Results are means value of five replicates, ± standard error of mean

seedlings. However, there are reports of shorter incubation times with *Frankia*, e.g. Vergnaud et al. (1985) obtained axenic nodulation in *Alnus glutinosa* within 10 days. This showed that *Frankia* activity in nodulation may differ in different host plants. It was also reported that *Frankia* helped N transfer through nodulation in *C. cunninghamiana* seedlings (He et al. 2005). Higher values of nodulation biomass and nodule numbers were found in *Frankia*-inoculated seedlings of *C. equisetifolia* compared with control, reflecting high symbiotic N fixation in host. This will improve photosynthetic activity in the host as energy in the form of ATP is supplied to *Frankia* (Arnone & Gordon 1990). Inoculation of *Frankia* strains improved growth, biomass and N uptake of *C. equisetifolia* seedlings and this confirmed findings by Muthukumar and Udaiyan (2010). In this study, it was further observed that *Frankia* strain CeFr1 induced better growth of *C. equiestifolia* than strain CeFr2. Strains of *Frankia* tend to vary in their ability to improve growth of host plants (Rojas et al. (1992).

Nitrogenase activity of *Frankia* also varied between strains based on the acetylene reduction. These results are in accordance with results reported by Dillon and Baker (1982) and Rojas et al. (1992) who found variations in nitrogenase activity associated with alders. Significant negative correlation was found between tissue N content of *C. equisetifolia* and nitrogenase activity of *Frankia* strains. However, compared with CeFr2, strain CeFr1 showed higher nitrogenase activity and produced higher N content in *C. equisetifolia*. Higher nitrogenase activity helps improve N fixation (Zhang et al. 2012). Increased growth and biomass of casuarinas due to inoculation of *Frankia* was strongly correlated with improved accumulation of N fixation by *Frankia* (Karthikeyan et al. 2011, 2013). Increased biomass in *C. equisetifolia* seedlings could be the result of increased N inflow rates through *Frankia*. Increased tissue N content of inoculated seedlings compared with control plants showed the influence of *Frankia* in N fixation (Franche et al. 2009). The nursery experiments supported the positive response of *C. equisetifolia* to *Frankia* inoculation that strengthens the plants survival in field.

Frankia-inoculated *C. equisetifolia* seedlings showed increased growth and successful survival in the farmland without application of any chemical fertilisers. *Frankia*-inoculated *C. equisetifolia* seedlings also had higher survival rate and rapid growth in the field compared with control seedlings. *Frankia* inoculation in the form of crushed root nodules together with azospirillum, phosphobacterium and arbuscular mycorrihzal fungi improved total biomass in *C. equisetifolia* in farmland (Rajendran & Devaraj

2004). These field studies emphasised the importance of beneficial microbes such as *Frankia* as alternative to chemical fertilisers. Extensive use of chemical fertilisers may cause environmental problems in farmland. Chemical fertilisers either directly or indirectly affect beneficial soil microbes which are important to plant growth (Sapp et al. 2015). *Casuarina equisetifolia* seedlings inoculated with *Frankia* showed healthy and improved growth due to N fixation. It has been reported that *Alnus cordata* inoculated with *Frankia* had increased growth and survival in field conditions due to the uptake of N (Lumini et al. 1994). *Frankia*-inoculated *C. equisetifolia* seedlings showed improved nutrient status in farm soil. Results from this study strongly supported the inoculation of cultured *Frankia* into seedlings of *C. equisetifolia* for enhancement of growth, biomass and nutrient uptake in farmlands.

CONCLUSIONS

Inoculation of *Frankia* at seedling stage of *C. equisetifolia* is a prerequisite for growth and nutrient improvement in farmlands. Although *Frankia* is efficient in biological N fixation, the amount of N fixed vary between strains. Hence, necessary attention is to be given in selection of *Frankia* strains for inoculation. Application of *Frankia* for growth improvement of *C. equisetifolia* could limit the use of chemical fertilisers in farm forestry.

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REFERENCES

Arnone JA & 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 Phytologist* 116: 55–66.

Benson DR & Silvester WB. 1993. Biology of *Frankia* strains, actionomycete symbionts of actinorhizal plants. *Microbiological Reviews* 57: 293–319.

Diagne N, Karthikeyan A, Ngom M et al. 2013. Use of *Frankia* and actinorhizal plants for degraded lands reclamation. *BioMed Research International* doi. org/10.1155/2013/948258.

Dillon JT & Baker D. 1982. Variations in nitrogenase activity among pure-cultured *Frankia* strains tested on actinorhizal plants as an indicator of symbiotic compatibility. *New Phytologist* 92: 215–219.

Franche C, Lindstorm K & Elmerich C. 2009. Nitrogen fixing bacteria associated with leguminous and non leguminous plants. *Plant and Soil* 321: 35–59.

Franche C & Bogsuz D. 2012. Signaling and communication in the actinorhizal symbiosis. Pp 73–92 in Perotto S & Baluska F (eds) *Signaling and Communication in Plant Symbiosis*. Springer, Berlin.

Hardy RWF, Holsten RD, Jackson EK & Burns RC. 1968. The acetylene–ethylene assay for N_2 fixation: laboratory and field evaluation. *Plant Physiology* 43: 1185–1207.

He X, Critchley C, Ng H & Bledsoe B. 2005. Nodulated N_2 fixing *Casuarina cunninghamiana* is the sink for net N transfer from non N_2 fixing *Eucalyptus maculata* via an ectomycorrhizal fungus *Pisolithus* sp. using $^{15}NH_4^+$ or $^{15}NO_3^-$ supplied as ammonium nitrate. *New Phytologist* 167: 897–912.

Jackson ML. 1973. *Soil Chemical Analysis* Prentice Hall, New Delhi.

Karthikeyan A, Deeparaj B & Nepolean P. 2009. Reforestation in bauxite mine spoils with *Casuarina equisetifolia* Frost. and beneficial microbes. *Forests, Trees and Livelihoods* 19:153–165.

Karthikeyan A, Savio MDM & Nepolean P. 2011. Growth response of *Casuarina junghuhniana* to indigenous *Frankia,* arbuscular mycorhizal fungi and phosphobacterium under nursery conditions. Pp 131–136 in Zhong C et al. (eds) *Improving Smallholder Livelihood Through Improved Casuarina Productivity: Proceedings of the 4th International Casuarina Workshop.* 21–25 March 2010, Haikou.

Karthikeyan A, Chandrasekaran K, Geetha M & Kalaiselvi R. 2013. Growth response of *Casuarina equisetifolia* Forst. rooted stem cuttings to *Frankia* in nursery and field conditions. *Journal of Biosciences* 38: 741–747

Lowry OH, Rosebrough NJ, Fam AL & Randall RJ. 1951. Protein measurement with the folin phenol reagent. *The Journal of Biological Chemistry* 193: 265–275.

Lumini E, Bosco M, Puppi G et al. 1994. Field performance of *Alnus cordata* Loisel (India alba) inoculated with *Frankia* and VA mycorrhizal strains in mine spoil afforestation plots. *Soil Biology and Biochemistry* 26: 659–661.

Muthukumar T & Udaiyan K. 2010. Growth response and nutrient utilization of *Casuarina equisetifolia* seedlings inoculated with bioinoculants under tropical nursery conditions. *New Forests* 40: 101–118.

Pinyopusarerk K & Williams ER. 2000. Range-wide provenance variation in growth and morphological characteristics of *Casuarina equisetifolia* grown in Northern Australia. *Forest Ecology and Management* 134: 219–232.

Rajendran K & Devaraj P. 2004. Biomass and nutrient distribution and their return of *Casuarina equisetifolia* inoculated with biofertilizers in farm land. *Biomass and BioEnergy* 26: 235–249.

Rojas NS, Perry DA, Lr CY & Friedman J. 1992. Influence of actinomycetes on *Frankia* infection, nitrogenase activity and seedling growth in red alder. *Soil Biology and Biochemistry* 24: 1043–1049.

Sapp M, Harrison M, Hanry U, Charlton A & Thivaites R. 2015. Comparing the effect of digestate and chemical fertilizer on soil bacteria. *Applied Soil Ecology* 86: 1–9.

Santi C, Bogsuz D & Franchie C. 2013. Biological nitrogen fixation in non legume plants. *Annals of Botany* 48: 1–25.

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

Shipton WA & Burgraff AJP. 1983. Aspects of the cultural behaviour of *Frankia* and possible ecological implication. *Canadian Journal of Botany* 61:2783–2792.

Uma M, Saravanan TS & Rajendran K. 2014. Growth, litterfall and litter decomposition of *Casuarina equisetifolia* in a semi arid zone. *Journal of Tropical Forest Science* 26: 125–133.

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

Zhang X, Shen A, Wang Q & Chen Y. 2012. Identification and nitrogen fixation effects of symbiotic *Frankia* inoculated from *Casuarina* spp. in Zhejiang, China. *African Journal of Biotechnology* 11: 4022–4029.