Nodulation and Nitrogen Fixation in Rooted Stem Cuttings of *Casuarina junghuhniana* Miq. by *Frankia* Inoculation

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Abstract

Casuarina junghuhniana Miq. fixes atmospheric nitrogen (N) through the symbiotic relationship with *Frankia*, a soil actinomycete group. The roots of *C. junghuhniana* produce root nodules where the bacteria fix atmospheric N, which is an essential nutrient for all plant metabolic activities. High-yielding and genetically superior trees of *C. junghuhniana* are selected and propagated vegetatively for commercial use. Yet, as the vegetative propagation uses inert material (vermiculite) for rooting, there is no chance for *Frankia* association that results absence of root nodules in rooted stem cuttings. Therefore, after planting of these stocks, there is a necessity to apply chemical fertilizers for N supply that increase the planting cost. To reduce the chemical fertilizers costs and to establish the N fixation in vegetatively propagated rooted stem cuttings of *C. junghuhniana*, the isolated actinomycete *Frankia* from root nodules of *C. junghuhniana* was cultured in artificial liquid P media and applied in this study. Application of the *Frankia* inoculum at the rate of 5 ml during the root initiation stage resulted in the development of an average of 12 nodules, weighing 43-mg/rooted stem cuttings of *C. junghuhniana* after 25 days. The rooted stem cuttings of *C. junghuhniana* were also on increase in shoot and root growth, number of lateral roots, shoot biomass, root biomass and tissue N content due to inoculation of *Frankia*. In this study, the acetylene reduction assay on *Frankia* liquid culture was also made and found the release of 150.69 nmol of C_2H_4/mg of protein/h in gas chromatography. This study supports the inoculation of *Frankia* in rooted stem cuttings of *C. junghuhniana* for biological N fixation so as to reduce the chemical fertilizers.

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Frankia · Nitrogen · *Casuarina junghuhniana* · Rooted stem cuttings · Root nodules

Introduction

Actinorhizal plants usually form root nodules in association with the nitrogen (N)-fixing actinomycete *Frankia* that helps them to survive even in nutrient-poor soils by N fixation. *Frankia* is an actinomycetes organism, which interacts with the roots of appropriate host plants to form N-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 (Echhab et al. 2007). *Casuarina junghuhniana* Miq. belongs to Casuarinaceae, and on account of its high economic value, farmers are interested in planting this tree as an agroforestry crop in the states of Tamil Nadu and Pondicherry (India). It is useful to wind break, soil improvement, an ornamental live fencing and building construction material (Jayaraj 2010). *Frankia* is associated with *C. junghuhniana* for N fixation, and it has been estimated that *Frankia* fixes atmospheric N up to 362 kg N/ha/yr, which is an essential nutrient for all plant metabolic activities and growth (Shantharam and Mattoo 1997).

In *Frankia* inoculum, generally farmers used to collect the root nodules from mature Casuarina trees and then crush and add at the time of planting in new sites along with seedlings/cuttings of *Casuarinas*. This practice is often unsuccessful if the crushed root nodules contain dead or inactive *Frankia*. Further, for pulp and paper production, high-yielding and genetically superior trees of *C. junghuhniana* are selected and multiplied by rooted stem cuttings through farmers of Tamil Nadu and Pondicherry. But the rooted stem cuttings are being propagated in an inert material (vermiculite) so that there is no chance for *Frankia* association. Though inoculation of *Frankia* is essential in rooted stem cuttings of *C. junghuhniana*, there is no report found on nodulation of rooted stem cuttings in *C. junghuhniana*. Hence, there is an urgent need to find an alternate solution for the use of these chemical fertilizers for the rooted stem cuttings of *C. junghuhniana* during plantation. We attempted to study the effect of inoculation of cultured *Frankia* strain in rooted stem cuttings of *C. junghuhniana* on growth, biomass and nodulation, which 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. junghuhniana* rooted stem cuttings.

Materials and Methods

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), Tamil Nadu, 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 and 21 °C, respectively.

Isolation and Multiplication of *Frankia*

The *Frankia* used in this study was isolated from *C. junghuhniana* root nodules collected from the coastal area, and the location and characteristics of collected nodules are described 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 shaker for 40 min. Under

Table 1 Source of *Frankia*

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

aseptic conditions, the nodules were rinsed with sterile water and 0.2 g of nodule was ground manually in sterile mortar and pestle. Then, the nodule solutions were centrifuged at 1,000 rpm for 20 min, and the supernatant was filtered through Whatman's No.1 filter paper. The suspension was then plated in P media and incubated at 25 °C for 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 30 days of incubation, the *Frankia* growth was observed as fluffy white colonies on P media plates. These colonies were transferred to P media broth for mass multiplication.

Analysis of Nitrogenase Activity

The nitrogenase activity of *Frankia* was determined in 21-day-old culture in N-free P media broth by using the acetylene reduction technique (Hardy et al. 1968) to confirm the presence of nitrogenase in the *Frankia* strain which is essential to break down the triple bond of N. 30 ml of culture is placed in 130 ml capacity of sterilized vials and sealed with rubber stoppers. About 10 % of the airspace in each vial was replaced by pure acetylene and allowed to stand for 1-h incubation at room temperature. About 0.5 ml of the gas was removed from each vial and injected into a gas chromatography (GC: Model: Nucon 910980) equipped with a flame ionization detector and a 2 m × 2.1 mm stainless steel column packed with Porapak Q on 80–100 mesh. The oven temperature was adjusted to 70 °C; injector temperature 50 °C; detector temperature 120 °C. The N carrier gas flow rate was adjusted to 30 ml/min to measure ethylene production. Blanks comprised air from bottles to which no acetylene was added. Peaks of ethylene were compared with ethylene standard (purity 99.9 %) injected into the GC to calculate concentrations. The nanomoles of ethylene produced per time unit was standardized to total cell protein. The protein concentration of the cells was determined as described by Lowry et al. (1951) with bovine serum albumin as the standard. The specific activity of nitrogenase was expressed as nanomoles of ethylene produced per mg of protein per hour. The rate of N fixation was calculated using the formula:

$$\text{Nitrogenase activity} = \frac{\text{Peak area count} \times 0.0006 \times \text{volume of gas injected into vial}}{\text{Incubation time} \times \text{volume of gas injected into GC} \times \text{total mg of protein in the sample}}$$

Collection and Propagation of *C. junghuhniana* Stem Cuttings

The stem cuttings were collected from the clones Cj 18 at model nursery, IFGTB, and treated with 0.1 % carbendazim fungicide and 2,000 ppm of indole butyric acid (IBA). After the treatment with IBA, the cuttings were placed in 100-cc root trainers that contain the inert media vermiculite. The rooted cuttings were thereafter placed in polytunnels made of polythene sheets (32–35 °C and 60–65 % RH) for 30 days. After 25 days, the cuttings showed initiation of 2–3 lateral roots with 1–1.5 cm length. In this stage, the rooted stem cuttings were transferred to shade house and watered regularly.

Inoculation of *Frankia* in *C. junghuhniana* Rooted Stem Cuttings

The cultured *Frankia*-strained P media broth was inoculated in the root zone of rooted stem cuttings of *C. junghuhniana* clone Cj 18 at the rate of 5-ml^{-1} cutting and maintained 15 replicates. Root trainers containing inoculated cuttings and uninoculated controls were placed in the shade house and watered regularly. The initiation of nodules and nodule numbers in each rooted stem cuttings was assessed. These planting stocks were maintained for 3 months in the model nursery of IFGTB and harvested for analysis of growth and biomass. The dry weights of *Frankia* inoculated these planting stocks were determined after oven drying at 50 °C to a constant weight.

Analyses of Growth, Biomass and Tissue N Content

The growth of *Frankia*-inoculated rooted stem cuttings and uninoculated cuttings was analysed in terms of shoot height, 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. junghuhniana.* The dried plant sample (0.25 gm) was digested with 3 gm of catalyst mixture: (potassium sulphate and cupric sulphate in the ratio of 5:1) and 10 ml of H_2SO_4 in Kjeldahl 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.

Statistical Analysis

Each measured variable in the nursery experiment was subjected to analysis of variance, and means were separated using Duncan's multiple range test (SPSS version 10).

Results

Morphological Characteristics of *Frankia* Isolate

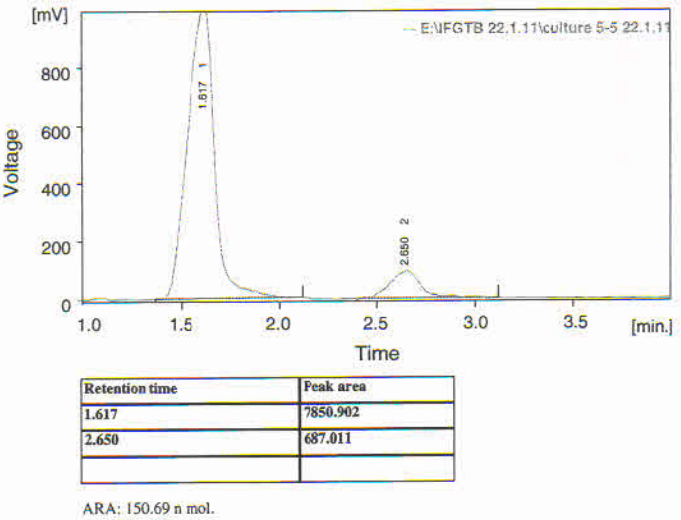
Under optimum conditions (28–32 °C), the growth of the isolate that formed white fluffy colonies on the P media plates was examined under light microscope. It showed branched and septate hyphae and round vesicles. The morphometrics of *Frankia* is shown in Table 2.

Nitrogenase Activity

The nitrogenase activity of *Frankia* was measured at various intervals. The experiment was repeated three times, and the mean value of the isolate was calculated. The observation of ability to reduce acetylene in vitro supports the fact that actinomycete was isolated in this experiment which is able to fix N in the *Frankia*-inoculated

Table 2 Morphometric of *Frankia*

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



Retention time	Peak area
1.617	7850.902
2.650	687.011

ARA: 150.69 n mol.

Fig. 1 GC analysis for nitrogenase activity of *Frankia*

rooted stem cuttings. The *Frankia* showed the nitrogenase activity at 25-day-old liquid culture that results an amount of 150.69 nmol of ethylene produced per mg of protein per hour (Fig. 1).

Growth and Biomass of *C. junghuhniana* Rooted Stem Cuttings

Nodulation of *Frankia* was observed in 25 days after inoculation in the rooted stem cuttings of *C. junghuhniana*. The initial infections at 20 days showed clubbed roots in the rooted stem cuttings, and the nodule development occurred at 25 days. The rooted stem cuttings of the inoculated with *Frankia* strain showed significantly increased growth in shoot height, root length, collar diameter and biomass than 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 12 root nodules per cutting were obtained. The R/S ratio was significantly lower in *Frankia*-inoculated rooted stem cuttings than in the uninoculated control (Table 3). The new finding in the present study is the successful nodulation establishment in the *C. junghuhniana* rooted stem cuttings in inert media without using soil.

Tissue N Content

Significant differences in total N content in comparison with uninoculated controls were observed. The total N content was found 5.64 mg/g dry weight in the rooted stem cuttings, whereas the uninoculated control rooted stem cuttings showed a mean value of 0.41 mg/g dry weight (Fig. 2).

Discussion

The results of this study have clearly shown that *Frankia* can improve the plant growth through increased uptake of N. *Frankia* results in positive effect on the rooted stem cuttings of

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

Clone No.	Treatments	Collar diameter (cm $plant^{-1}$)	Shoot length (cm $plant^{-1}$)	Root length (cm $plant^{-1}$)	No. of lateral roots/ plant	Shoot dry weight (mg / $plant^{-1}$)	Root dry weight (mg $plant^{-1}$)	R/S ratio	Nodulation time	No. of nodules	Nodule biomass (mg^{-1}nodule)
Cj 18	*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	43
	Control	0.542 a	5.9 a	4.8 *a*	1.8 a	0.288 a	0.199 a	0.690 a	–	–	–

Data were mean of 15 replicates; means followed by same letters are not significantly different at $p < 0.05$ according to Duncan's multiple range test

C. junghuhniana growth through improvement in 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 N due to *Frankia* (Reddell et al. 1988). This study further supports the positive response of *C. junghuhniana* rooted stem cuttings in the nursery to *Frankia* application and strengthens the *Frankia* dependency of *C. junghuhniana* 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), the *Frankia* effects on the plant growth promotion have been demonstrated in sterile soil substrates. However, the growth-promoting effect of *Frankia* on *C. junghuhniana* 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* is not possible to transmit with the seed either within or on its surface (Torrey 1983).

Inoculation experiments of this kind in nursery conditions are essential for *C. junghuhniana* 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. junghuhniana*. However, Vergnaud et al. (1985) have obtained axenic nodulation in *Alnus glutinosa* within 10 days. This also has shown that there is a difference in nodulation behaviour between *Alnus* and *C. junghuhniana*. Nodulation biomass and nodule number increased the rooted stem cuttings of *C. junghuhniana* raised in inert media. This reflects that the symbiotic N fixation depends on host photosynthesis (Arnone and Gordon 1990), where the ATP is supplemented to *Frankia*. The increased biomass in the rooted stem cuttings of both the clones could be the result of increased nutrient inflow rates through *Frankia*. The nitrogenase activity of *Frankia* in this study has shown that the *Frankia* culture contains more vesicles. Because vesicles of

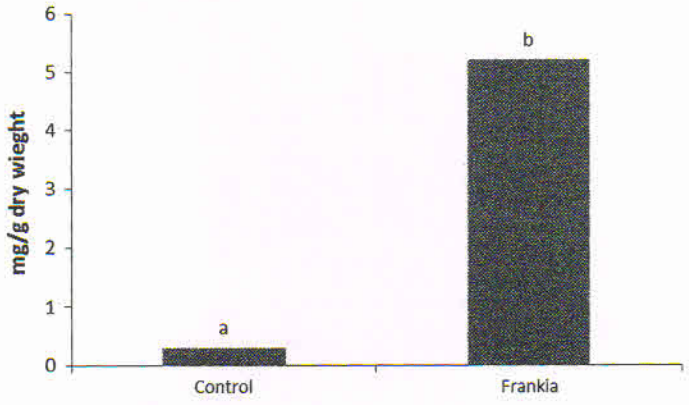


Fig. 2 Total N content in the rooted stem cuttings of *C. junghuhniana* inoculated with *Frankia*. Means followed by same letters are not significantly different at $p < 0.05$ according to Duncan's multiple range test

Frankia have been considered as the sites of nitrogenase for many years (Gauthier et al. 1981; Fontaine et al. 1984). The nitrogenase activity of *Frankia* also corroborates supply of Mg-ATP from the *Frankia*-inoculated *C. junghuhniana* cuttings that give energy for N fixation (Huss-Dannel and Hablin 1988). Increased tissue N content of *Frankia*-inoculated rooted stem cuttings of *C. junghuhniana* raised in inert media than the uninoculated control plants showed more influence of *Frankia* in N fixation.

Conclusion

The results from this study support the inoculation of cultured *Frankia* to the rooted stem cuttings of *C. junghuhniana* for enhancement of growth, biomass and nutrient uptake. It is essential to introduce potential *Frankia* in the rooted stem cuttings of *C. junghuhniana* as they propagated in inert media. This method of inoculation of *Frankia* in the rooted stem cuttings of C. *junghuhniana* will be beneficial for early establishment in the field without additional chemical fertilizers even in low-fertile lands.

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