Journal of Tropical Forest Science 35(2): 203–210 (2023)
https://doi.org/10.26525/jtfs2023.35.2.203
ISSN: 0128-1283, eISSN: 2521-9847

INFLUENCE OF *CASUARINA EQUISETIFOLIA* WITH *FRANKIA* AS HOST PLANT ON GROWTH AND NUTRIENT IMPROVEMENT OF *SANTALUM ALBUM*

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Submitted August 2022; accepted October 2022

Santalum album is a commercially important tree for its aromatic heart wood and oil. Establishing *S. album* seedlings in field is difficult due to its semi parasitic nature. It requires a host to thrive and survive. In this study the *C. equisetifolia* was selected as host along with nitrogen fixing actinobacteria *Frankia* for *S. album* in nursery to understand their relationship. Both *S. album* and *C. equisetifolia* seedlings were propagated in nursery and the cultured *Frankia* in P agar medium was inoculated in *C. equisetifolia* seedlings. The *S. album* seedlings were grown in soil along with *C. equisetifolia* (+/- *Frankia*). After 120 days, the seedling of *S. album* was harvested and found improvement in growth and nutrient uptake. *S. album* seedlings with the host of *C. equisetifolia* along with *Frankia* showed significantly improved root length (38.3 cm seedling^{-1}), shoot length (45.2 cm seedling^{-1}), root collar diameter (3.2 cm seedling-^{1}), 14.2 haustorial attachment seedling^{-1}, shoot biomass (1.96 g seedling^{-1}), root biomass (0.72 g seedling^{-1}) and showed lowest root/shoot ratio (0.36). The nutrient contents particularly N was significantly found in *S. album* seedlings associated with *C. equisetifolia* + *Frankia*. The result showed that essential nutrient nitrogen transfer was mediated through the nitrogen fixing bacteria to *C. equisetifolia* and absorbed by *S. album*. In conclusion, the combination of *C. equisetifolia* + *Frankia* provided suitable host for the growth and biomass improvement in *S. album* seedlings.

Keywords: Actinobacteria, biomass, nursery, nitrogen, aromatic tree

INTRODUTION

Santalum album is a medium sized semi parasitic evergreen tree known for its fragrant heart wood and oil. It is mostly found in dry deciduous forests and naturally distributed from Indonesia in the east to Chile in the west and Hawaiian archipelagos in the north to New Zealand in the south. *S. album* is classified as sensible species by International Union for the Conservation of Nature. India is one of the countries with more natural populations of *S. album* particularly in the states of Karnataka, Tamil Nadu and Kerala. *S. album* is a slow growing tree and takes 10 years to form the heart wood. This tree can grow up to 1800 m above mean sea level and in various types of soils. *S. album* is exported for the aromatic and medicinal properties of its wood (Dhanya et al. 2010). The price of *S. album* wood oil ranged from USD1750 to USD2500 kg^{-1}. The heart wood cost from USD35 to USD50 kg^{-1} (Thomson 2020). *S. album* wood oil is widely used in folk medicine and religious practices in India. Due to high demand of these economically high valuable properties, more than 1500 plantations were raised in India (Doddabasawa & Chittapur 2021). However, establishment of *S. album* is difficult due to its semi parasitic nature as is depends on host plant for nutrient uptake particularly nitrogen and phosphorus through a structure called haustorium naturally found in roots (Lu et al. 2013, Rocha et al. 2015). Thus it requires a suitable host for a better survival condition and growth in the field. Earlier studies proved the successful plantations of *S. album* was strongly related with nitrogen fixing host species (Li 2003). The nitrogen fixing species such as *Cajanus cajan* shrub and *Crotalaria juncea* herb were used in previous studies and found to promote the growth of *S. album* (Annapurna et al. 2006).

Other nitrogen fixing host species examined were *Casuarina equisetifolia* (Rocha et al 2014), *Acacia* tree, *Sesbania* shrub (Radomiljac et al. 1999) and *Dalbergia odorifera* medium sized tree (Lu et al. 2014). Host plants generally helped in the uptake of minerals, nutrients and water of *S. album* (Hiremath 2004). Earlier study found that the significant influence of the host plant *C. equisetifolia* on carbon assimilation, water and nutrient absorption in the field grown sandalwood tree (Rocha et al. 2014). In the current study, the *C. equisetifolia* was selected as host plant along with nitrogen fixing actinobacteria, *Frankia* for *S. album* growth improvement at the nursery. The microbial association was very essential to meet the initial nutrient problems in *S. album* as the seedlings struggled to thrive at its early stage in nursery environment. Hence, the *C. equisetifolia* + *Frankia* was used as host to *S. album*. This study served to provide a clearer understanding of growth performance, nutrient transfer and biomass improvement of *S. album* seedlings along with the *C. equisetifolia* as the host plant. This study may provide management strategies for *S. album* propagation in nurseries and successful establishment in the field.

MATERIALS AND METHODS

Propagation of *S. album* seedlings

A total of 500 g *S. album* seeds were soaked in 0.05 mg of gibberellic acid (GA_3) dissolved in 100 ml of sterile distilled water for 24 hrs. The soaked seeds were then sown in sterile germination bed containing pure sand under a shade house with the temperature of 28 °C and relative humidity of 65%. After 21 days, 80% germination was observed.

Propagation of *C. equisetifolia* seedlings

A total of 10 g *C. equisetifolia* seeds were sown directly in germination bed containing pure sand and covered with rice straw for protection. The germination bed was regularly sprayed with water and received 32% germination after 10 days.

Culture of *Frankia*

Fresh root nodules of *C. equisetifolia* were collected at coastal area of Pondicherry, India (11.94° N, 79.80° E; 3.1 m above sea level) as the suitable isolation source for active *Frankia* (Karthikeyan et al. 2013). The collected nodules were transported in air-tight container to the laboratory and stored at -4 °C. The nodules were surface sterilised with 30% (w/w) H_2O_2 for 30–40 minutes and rinsed 10 times with sterile distilled water. Isolation of *Frankia* from the nodule suspension was carried out by grinding of 0.1 g fresh weight of the sterilized nodules in 50 ml of sterile distilled water with a sterile mortar under aseptic conditions. The suspension of nodules was centrifuged at 1000 rpm for 20 min and the supernatant was filtered. The suspension containing *Frankia* spores was poured on P agar medium (10 g of $CaCl_2·2H_2O$, 20 g of $MgSO_4$, 0.46 g of propionic acid, 0.15 g of H_3BO_3, 0.15 g of $ZnSO_4·7H_2O$, 0.45 g of $MnSO_4·H_2O$, 0.004 g of $CuSO_4·5H_2O$, 0.028 g of $Na_2MoO_4·2H_2O$, 0.009 g of $CaCl_2·6H_2O$, 0.04 g of Biotin, 100 g of K_2HPO_4, 67g of $NaH_2PO·2H_2O$, 0.1 g of FeNa EDTA, 8 g of agar in one litre of distilled water) (Karthikeyan et al. 2013). The *Frankia* spores were identified by 16 S rRNA gene sequence and deposited in NCBI with accession No. JQ412181 (Karthikeyan et al. 2013).

Nursery experiments on *S. album* along with *C. equisetifolia* seedlings

The 20 days old *S. album* seedlings and *C. equisetifolia* with uniform length of 15 cm were transplanted to polythene bags (v.4.15l; size 4 × 27 cm) containing red soil + sand (1:1). The cultured *Frankia* in P agar medium (Karthikeyan et al. 2013) was inoculated at the rate of 10 ml/seedling (protein content 2.2 µg m L^{-1}) in the seedlings of *C. equisetfolia* along with *S. album*. Three treatments on the seedling such as *S. album* only, *S. album* + *C. equisetifolia* (- *Frankia*) and *S. album* + *C. equisetifolia* (+ *Frankia*) were designed for the nursery conditions. The seedlings were maintained in randomised block design with 10 replicates per treatment.

Harvest and data collection

After 120 days of treatment, the seedlings of *S. album* and *C. equisetifolia* were harvested and measured in terms of root length, shoot length, root collar diameter, haustorial attachment in *S. album* and root nodules of *C. equisetifolia*. The shoot and root biomass of *S. album* seedlings were measured after drying in oven at 50 °C for 48 hours. Simultaneously, the growth and biomass of the host *C. equisetifolia* was also measured.

Seedling quality index

Seedling quality index of *S. album* was calculated using the formula (Dickson et al. 1960):

$$\text{Seedling Quality index} = \frac{\text{seedling dry weight (g)}}{[\text{seedling height (cm)} / \text{root collar diameter (mm)} + \text{shoot dry weight (g)} / \text{root dry weight (g)}]}$$

Tissue nutrient analysis

A mixture of *S. album* (5 g) & *C. equisetifolia* (5 g) root and shoot samples were digested with potassium sulphate and copper sulphate (5:1) catalyst and triple acid containing nitric acid, sulphuric acid and perchloric acid (9:3:1) in a digestion system at 400 °C for one hour. The samples were then analysed for tissue nutrient content such as nitrogen, phosphorus and potassium. The total nitrogen was determined on a kjeltec auto analyser, phosphorus was analysed by vandomolybdate phosphoric yellow method and potassium content was determined by flame photometer (Jackson 1973).

Statistical analysis

All data were statistically analysed using Duncan's multiple range tests in Statistical Package for the Social Sciences, USA (Ver. 17). The mean data with standard error was also calculated.

RESULTS

Nursery experiments

The seedlings of *S. album* provided with *C. equisetifolia* as host showed improved growth performance as compared to the seedlings of *S. album* without *C. equisetifolia.* The seedlings of *S. album* showed root length (30.2 cm seedling^{-1}), shoot length (32.3 cm seedling^{-1}), root collar diameter (2.8 cm seedling^{-1}) and had 4.7 haustorial attachments seedling^{-1}. The growth and biomass also significantly improved and received low root/shoot ratio than the *S. album* seedlings without host species (Table 1). However, the host species of *C. equisetifolia* along with *Frankia* showed significantly (p = 0.05) improved root length (38.3 cm seedling^{-1}), shoot length (45.2 cm seedling^{-1}), root collar diameter (3.2 cm seedling^{-1}), 14.2 haustorial attachment (seedling^{-1}), shoot biomass (1.96 g seedling^{-1}), root biomass (0.72 g seedling^{-1}) and had lowest shoot to root ratio (0.36). The haustorial attachments were also prominent with *C. equisetifolia* as observed in this study (Figure 1a & Figure 1b). The host *C. equisetifolia* showed increased growth and had higher biomass with *Frankia* inoculated seedlings (Table 2).

Seedling quality index

Seedling quality index showed significantly (p = 0.05) higher in *S. album* seedlings associated

Table 1 Growth response of *S. album* seedlings associated with *C. equisetifolia* (+/- *Frankia*)

Treatment	Root length (cm) seedling^{-1}	Shoot length (cm) seedling^{-1}	Root collar diameter (cm) seedling^{-1}	No. of root nodules seedling^{-1}	No. of haustoria seedling^{-1}	Biomass (g) seedling^{-1}		Shoot to root ratio seedling^{-1}
						Root	Shoot	
S. album	23.5 a (±1.2)	18.4 a (±0.8)	0.91 a (±0.08)	0	0	0.31 a (±0.007)	0.45 a (±0.004)	0.68 a (±0.005)
S. album + C. equisetifolia (-*Frankia*)	30.2 b (±1.1)	32.3 b (±1.5)	2.8 b (±0.8)	0	4.7 a (±0.8)	0.48 b (±0.004)	1.08 b (±0.003)	0.44 b (±0.004)
S. album + C. equisetifolia (+ *Frankia*)	38.3 c (±1.3)	45.2 c (±1.2)	3.2 bc (±0.6)	8.6 (±0.9)	14.2 b (±0.5)	0.72 c (±0.005)	1.96 c (±0.005)	0.36 c (±0.002)

(±) = standard error of mean; mean of 10 replicates; means in a column followed by the same letter(s) were not significantly different according to Duncan's multiple range test (p < 0.05)

Table 2 Growth and biomass *C. equisetifolia* seedlings (+/- *Frankia*) used as host for *S. album*

	Collar diameter (cm) seedling^{-1}	Shoot length (cm) seedling^{-1}	Root length diameter (cm) seedling^{-1}	Biomass (g) seedling^{-1}		Shoot to root ratio seedling^{-1}
				Shoot	Root	
- *Frankia*	0.85 (±0.005)	65.40 (±2.6)	18.80 (±1.8)	4.2 (±0.02)	2.6 (±0.01)	1.6 (±0.003)
+ *Frankia*	1.10 (±0.0.03)	88.64 (±2.6)	31.26 (±1.5)	6.3 (±0.01)	3.8 (±0.01)	0.6 (±0.002)

(±) = standard error of mean; mean of 5 replicates

Figure 1a *S. album* seedling associated with *C. equisetifolia*

Figure 1b Haustorial attachment of *S. album* with *C. equisetifolia* + *Frankia* (Figure 2). Low seedling quality index was observed in the *S. album* seedlings without host species (Figure 2).

Tissue nutrient contents

The presence of essential plant nutrients such as nitrogen, phosphorus and potassium were analysed in the *S. album* seedlings. The *S. album* seedlings without host species showed low nutrient content while the seedlings associated with *C. equisetifolia* (*-Frankia*) showed higher nutrient content. However, high amount of nutrient contents particularly nitrogen was found significantly ($p < 0.05$) in *S. album* seedlings associated with *C. equisetifolia* + *Frankia* (Figure 3). In the host *C. equisetifolia*, higher tissue nutrient contents particularly nitrogen in the *Frankia* (+) inoculated seedlings were found as compared to the non-inoculated *Frankia* (-) seedlings. The other nutrients such as phosphorus and potassium also showed higher amount in the *Frankia* (+) inoculated seedlings than *Frankia* (-). Low shoot to root ratio was also observed in *Frankia* (+) inoculated seedlings.

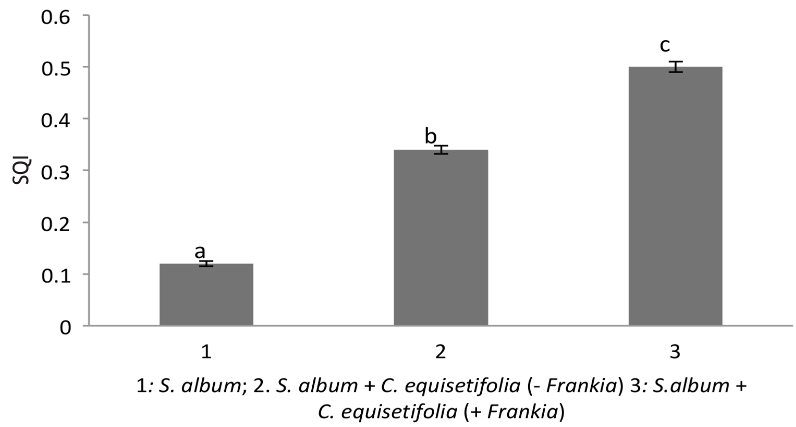

Figure 2 Seedling quality index (SQI) of *S. album*
The same letter(s) are not significantly different according to Duncan's multiple range test ($P < 0.05$); mean of 10 replicates

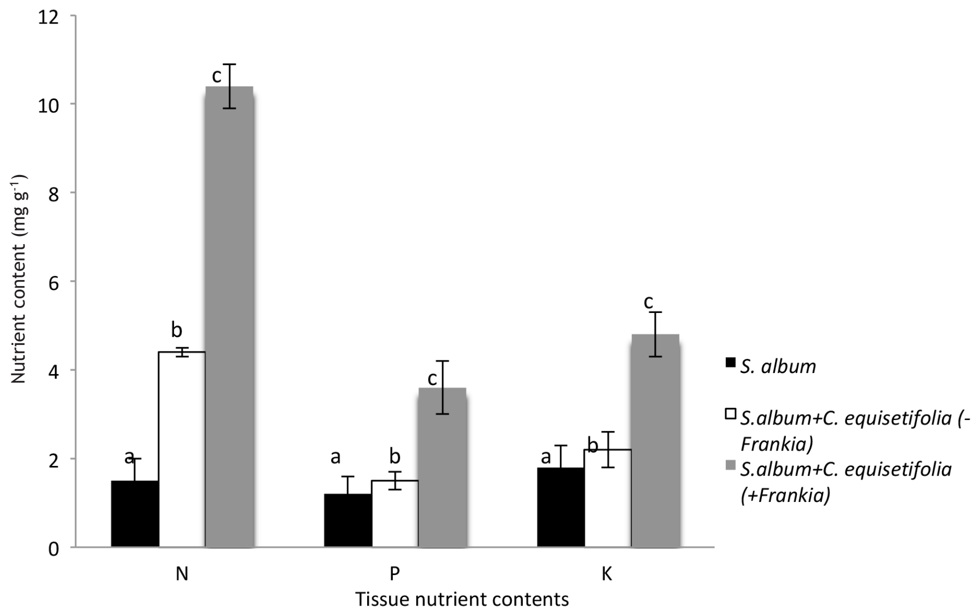

Figure 3 Tissue nutrient contents in *S. album*
The same letter(s) are not significantly different according to Duncan's multiple range test ($P < 0.05$); mean of 5 replicates

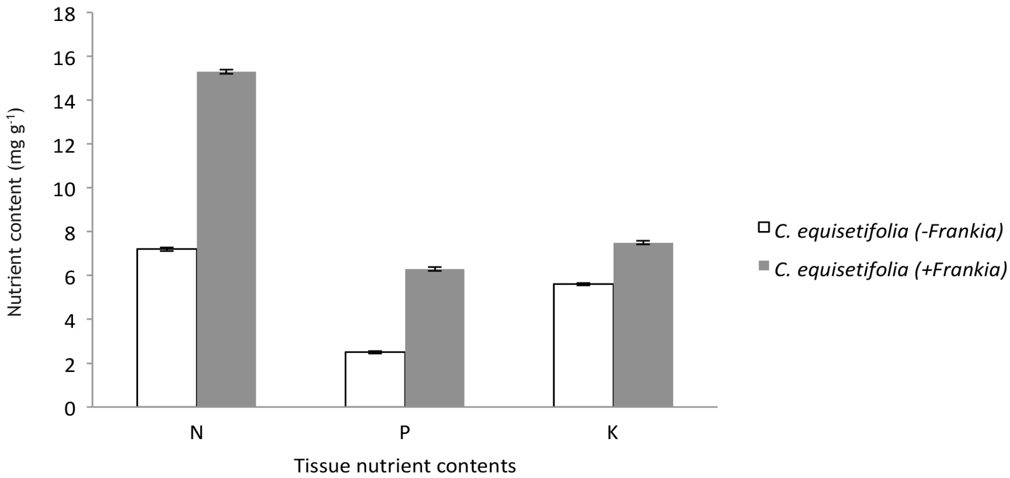

Figure 4 Tissue nutrient contents in the host mean of 5 replicates

DISCUSSION

Santalum album naturally required a host for its survival through parasitism (Radomiljac et al. 1999). It depended on host species for nutrients particularly nitrogenous content through their special absorbing structure in roots called haustorium (Bell & Adams 2011). This study used the nitrogen fixing host *C. equisetifolia* along with *Frankia* which a nitrogen fixing actinobacteria to improve the growth and biomass of *S. album* seedlings. Nitrogen fixing host species were reported to assist *S. album* in growth improvement (Lu et al. 2014) as compared to the non-nitrogen fixing plants. However, the nitrogen fixing bacteria used along with the host species for *S. album* had not yet been reported. In this study, *S. album* seedlings showed improved growth, biomass and nutrient contents in presence of the host (*C. equisetifolia*). In absence of host, the growth of *S. album* seedlings did not improve significantly due to the absence of haustoria. The high number of haustoria formation of the host resulted in high biomass production and growth improvement in *S. album* seedlings (Lu et al. 2014). The *S. album* haustoria attached itself to the xylem of the host and nutrients in the sap particularly nitrogen was transferred to *S. album* seedlings, which resulted in the growth improvement of the *S. album* seedlings (Pageau et al. 2003). Furthermore, the nitrogen transfer was mediated through the nitrogen fixing bacteria on *C. equisetifolia* and absorbed by *S. album* as confirmed in the earlier study conducted in *Dalbergia odorifera* as host for *S. album* (Lu et al. 2013). The current study showed that the host *C. equisetifolia* had more nitrogen content due to the inoculation with *Frankia* which increased the nitrogen in *C. equisetifolia* through nitrogen fixation from the atmosphere (Karthikeyan 2016). Thus nitrogen transfer from the host to *S. album* resulted in more content of nitrogen in *S. album.* The other nutrients such as phosphorous and potassium also found to increase in *S. album* due to the absorption of the excess nutrients from the host through the haustoria. Phosphorous transferred from the host was also possible through the haustoria which was confirmed through studies on *S. album* with different hosts (Rocha et al. 2015). Potassium transfer was also found more in *S. album* seedlings due to the presence of *Frankia.* The observation was reported by Haro and Benito (2019) indicating that potassium transfer was possible through endophytes. Knowing that *Frankia* is an endophyte, it may transfer potassium nutrient from *C. equisetifolia* absorbed from the soil. A recent review on potassium nutrient also indicated that the possible ability of microorganisms to transfer potassium to plants (Soumare et al. 2022).

Seedling quality index is an essential value, necessary for the analysis of various physiological and morphological attributes (Ritche 1984). In the current study, high value of seedling quality index found in *S. album* seedlings associated with *C. equisetifolia* + *Frankia* indicated stronger stem and proportional shoot dry weight. This character was very much necessary for the successful establishment of *S. album* seedlings in field. The findings supported the findings of the earlier study in *Pterocarpus santalinus* seedlings (Karthikeyan & Arunprasad 2021). Low shoot to root ratio in *S. album* seedlings associated with *C. equisetifolia* + *Frankia* resulted by increasing above ground production and reduction of below ground production due to sufficient nutrient acquisition from the host through nutrient transfer from the beneficial microbes like *Frankia* (Smith & Smith 2012, Karthikeyan 2016).

CONCLUSIONS

S. album is grown as commercial crop in private lands of India in present days. The establishment of *S. album* seedlings to a host species is very much essential for their successful survival in the fields. In the current study, the host *C. equisetifolia* + *Frankia,* a nitrogen fixing actinobacteria were used for the growth improvement of *S. album.* As *C. equisetifolia* is a nitrogen fixing tree with symbiotic association of *Frankia,* it significantly promoted the growth and biomass of *S. album* via the nitrogen transfer process. Hence, the present study concluded that the combination of *C. equisetifolia* + *Frankia* can be suggested as host for *S. album* seedlings to promote growth and biomass improvement.

ACKNOWLEDGEMENT

The authors thanked the Indian Council of Forestry Research and Education, Dehra Dun, India for providing necessary facilities for the study. The study was funded by compensatory afforestation fund management and planning authority, Ministry of Environment, Forest & Climate change, Government of India under the scheme 'Strengthening forestry research for ecological sustainability and productivity enhancement' and Project No. AICRP-21 (Biofertilisers)

REFERENCES

Annapurna D, Rathore TS & Joshi G. 2006. Modern nursery practices in the production of quality seedlings of Indian sandal wood (*Santalum album* L) stage of host requirement and screening of primary host species. *Journal of Sustainable Forestry* 22: 33–55. doi: 10.1300/J0p1v22n03.03

Bell TL & Adaks MA. 2011. Attack on all fronts: Functional relationships between aerial and root parasitic plants and their woody hosts and consequences for ecosystems. *Tree Physiology* 31: 3–15. doi: 10.1093/treephys/tpq108

Dhanya B, Syam V & Seema P. 2010. Sandal (*Santalum album* L.) conservation in Southern India. A review of policies and their impacts. *Journal of Tropical Agriculture* 48: 1–10.

Dickson A, Leaf AL & Hosner JF. 1960. Quality appraisal of white spruce and white pine seedling stock in forest nurseries. *Forest Chronicles* 36: 10–13. doi: 10.5558/tfc36010-1

Doddabasawa & Chittapur BMJ. 2021. Sandalwood plantations—points to ponder. *Current Science* 120: 1184–1193. doi: 10.18520/v120/i7/1184-1193

Haro R & Benito B. 2019. The role of soil fungi in K^+ plant nutrition. *International Journal of Molecular Sciences* 20:(3).3169 doi: 10.3390/ijms20133169

Hiremath VS. 2004. Influences of soil moisture regimes and stage of host introduction on seedling growth of sandal provenances. MSc thesis, Kerala Agricultural University, Thrissur, India.

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

Karthikeyan A & Arunprasad T. 2021. Growth responses of *Pterocarpus santalinus* L.f. seedlings to native microbial symbionts (Arbuscular mycorrhizal fungi and *Rhizobium aegyptiacum*) under nursery conditions. *Journal of Forestry Research* 32: 225–231. doi: 10.1007/s11676-019-01072-y

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: 741–749. doi:10.1007/s12038-013-9362-3

Karthikeyan A. 2016. *Frankia* strains for improving growth, biomass and nitrogen fixation in *Casuarina equisetifolia* seedlings. *Journal of Tropical Forest Science* 28: 235–242

Li YL. 2003. *Sandalwood: Introduction and Research.* Science Press, Beijing.

Lu JK, Kang LH, Sprent J I, Xu DP & He XH. 2013. Two-way transfer of nitrogen between *Dalbergia odorifera* and its hemiparasite *Santalum album* in enhanced when the host is effectively nodulated and fixing nitrogen. *Tree Physiology* 33: 464–474. doi.org/10.1093/treephys/tpt024

Lu JK, Xu DP, Kang LH & He XH. 2014. Host species dependent physiological characteristics of hemiparasite *Santalum album* in association with N_2 fixing hosts native to Southern China. *Tree Physiology* 34: 1006–1017. doi:10.1093/treephys/tpu073

Pageau KJ, Simier P, Le Bizea B, Robins RJ & Fer A. 2003. Characterization of nitrogen relationships between *Sorghum bicolour* and the root hemiparasitic angiosperm *Striga hermonthica* (Del.) Benth. using K^{15} NO_3 as isotopic tracer. *Journal of Experimental Botany* 54: 789–799. doi: 10.1093/jxb/erg081

Radomiljac AM, McComb JA & Pate JS. 1999. Intermediate host influences on the root hemi-parasite *Santalum album* L. biomass partitioning. *Forest Ecology and Management* 113: 143–153. doi: 10.1016/s0378-1127(98)00421-6

Ritchie JA. 1984. Assessing seedling quality. Pp 243–259 in Mary LD & Landis JD (eds) *Forest nursery manual: Production of bare root seedlings.* Martinus Nijhoff/Dr W. Junk Publishers, The Hague.

Rocha D, Ashokan PK, Santhoshkumar AV, Anoop EV & Sureshkumar P. 2014. Influence of host plant on the physiological attributes of field grown sandal tree (*Santalum album*). *Journal of Tropical Forest Science* 26: 166–172.

Rocha D, Ashokan PK, Santhoshkumar AV, Anoop EV & Sureshkumar P. 2015. Anatomy and functional status of haustoria in field grown Sandalwood tree (*Santalum album* L). *Forest Research* 4: 3. 1000148. doi. 10.4172/2168-9776.1000148

Smith SE & Smith FA. 2012. Fresh perspectives in the roles of arbuscular mycorrhizal fungi in plant nutrition and growth. *Mycologia* 104: 1–13. doi: 10.3852/11-229

Soumare A, Sarr D & Diedhiou AG. 2022. Potassium sources, microorganisms and plant nutrition–challenges and future directions: A review. *Pedosphere* doi: 10.1016/j.pedsph.2022.06.025

Thomson LAJ. 2020. Looking ahead—global sandal wood production and markets in 2040, and implications for pacific island produces. *Australian Forestry* 83: 245–254. doi: 10.1080/00049158.2020.1841441