EFFECT OF MICROBIAL INOCULATION ON QUALITY SEEDLING PRODUCTION OF *CASUARINA EQUISETIFOLIA*

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RAJENDRAN, K., SUGAVANAM, V. & DEVARAJ, P. 2003. Effect of microbial inoculation on quality seedling production of *Casuarina equisetifolia*. Pot culture experiments were conducted to select suitable microbial combinations to improve quality seedling production of *Casuarina equisetifolia*. Seedlings of 5 cm length were transplanted to polythene bags with a potting mixture of unsterilised substrate and inoculated individually and in combinations with *Azospirillum*, phosphobacterium, a VAM fungus and *Frankia*. Control plants were not inoculated. Root length, shoot length and basal diameter were recorded at bimonthly intervals up to six months. Root and shoot weight, nodule number and nodule weight were recorded after six months. Soil samples and root bits were also taken for enumeration of microbial population. Results show that the total seedling length and biomass were significantly increased in all the treatments compared with the control plants. The inoculation of the seedlings with the combination *Azospirillum* + phosphobacterium + VAM + *Frankia* produced the maximum growth, biomass and quality.

Key words: *C. equisetifolia* - nursery - biofertilisers - *Azospirillum* - phosphobacterium - VAM fungus - *Frankia*

RAJENDRAN, K., SUGAVANAM, V. & DEVARAJ, P. 2003. Pengaruh penginokulatan mikrob terhadap pengeluaran anak benih *Casuarina equisetifolia* yang berkualiti. Ujian kultur pasu dijalankan untuk memilih kombinasi mikrob yang sesuai bagi meningkatkan pengeluaran anak benih *Casuarina equisetifolia* yang berkualiti. Anak benih yang panjangnya 5 cm dipindah tanam ke beg politin berisi campuran pasu yang terdiri daripada substrat yang tidak disteril dan diinokulat setiap satu dan juga dicampur dengan *Azospirillum*, fosfobakteria, kulat VAM dan *Frankia*. Pokok kawalan tidak diinokulat. Panjang akar, panjang pucuk dan diameter basal dicatatkan selang dua kali sebulan sehinggalah enam bulan. Berat akar dan pucuk, bilangan nodul dan berat nodul dicatatkan selepas enam bulan. Contoh tanih dan bit akar juga diambil bagi mengira populasi mikrob. Keputusan menunjukkan bahawa jumlah panjang anak benih dan biojisim bertambah dalam semua rawatan berbanding tumbuhan yang kawalan. Penginokulatan anak benih dengan kombinasi *Azospirillum* + fosfobakteria + VAM + *Frankia* menghasilkan pertumbuhan, biojisim dan kualiti maksimum.

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Introduction

The beneficial contribution of rhizosphere microorganisms to plant development is to supply the essential nutrients for growth. Nitrogen and phosphorus are the two major plant nutrients in the soil; thus microorganisms associated with nitrogen fixation and phosphorus mobilisation are of primary importance. Soil actinomycetes of the genus *Frankia* under favorable conditions are capable of fixing atmospheric nitrogen in association with *Casuarina* spp. at rates comparable to those of effectively nodulated legumes (Torrey 1978). Reddell (1990) found that the *Frankia* inoculated seedlings of *Casuarina equisetifolia* had higher survival rate and grew more rapidly than uninoculated seedlings. Domergues (1976) also drew attention to a tripartite association between plant, *Frankia* and mycorrhizal fungi in *Casuarina* spp. and suggested that the plant is optimally capable of utilising nutrients even in the poor soil. Nitrogen fixing bacteria of the genus *Azospirillum* promoted tree growth (Wong & Stenberg 1979), and also increased root biomass, root surface area, root diameter, density and length of root hairs (Okon & Kapulink 1986).

The efficiency of nutrient uptake by plants is generally attributed to the activity of mycorrhizae. *Casuarina equisetifolia* seedlings inoculated with the VAM fungus, *Glomus fasciculatum,* increased shoot and root biomass (Vasanthakrishna *et al.* 1995). Sidhu *et al.* (1990) reported that *Glomus fasciculatum* and *Scutellospora calospora* colonised the roots of *C. equisetifolia.* These VAM fungi can be successfully used especially when this species is planted in degraded soils. Barrow *et al.* (1977) reported that when phosphate is added to soil, it slowly becomes firmly bound and less available to plants. Firmly held phosphate is made available to plants by VAM fungi. *Casuarina equisetifolia* seedlings inoculated with a combination of *Frankia* and VAM fungi increased growth and biomass (Ramirez *et al.* 1992).

There is little information available on *Frankia* and *Azospirillum* interaction and their effect on growth and biomass of *Casuarina* spp. (Swaminath & Vadiraj 1988). The growth of *C. cunninghamiana* seedlings was stimulated when inoculated with *Azospirillum brasilense.* Increase in whole plant dry weight was due to a significant increase in both shoot and root biomass which corresponded with a higher total nutrient content of the plants inoculated with *Azospirillum* (Rodriguez-Barrueco *et al.* 1991).

The soil used for the production of planting stock in the nurseries of the Institute of Forest Genetics and Tree Breeding, Coimbatore, and the Coimbatore Forest Division in Tamil Nadu, India, is very low in nutrient content and beneficial microbial population. Even when the soil is mixed with farmyard manure (FYM), the quality of seedlings is very poor due to insufficiency of desired microorganisms (many of the microorganisms are host specific) and the rate of mineralization and nitrogen fixation is very low. This problem can be overcome by providing suitable microorganisms to improve the growth and nutrient uptake in *Casuarina* seedlings. Hence the present study was undertaken to find out the compatibility of different microbial inoculants and their augmentation effect on the production of quality seedlings.

Materials and methods

Soil

The nursery soil was found to be sandy clay loam (clay 24.4%: silt 16.8%: sand 58.8%) with a pH of 7.9. The soil showed low availability of organic carbon (0.98%), total N (0.39%), P (0.06%), K (0.3%), Ca (0.13%) and Mg (0.03%). The microbial population of nursery soil was determined using serial dilution plate counting technique and was found to be very low in actinomycetes, *Azospirillum* and phosphobacterium (1. 8×10^3, 0. 95×10^4 and 2×10^2 cells g^{-1} soil respectively). The soil contained low VAM spore density of 20 spores / 100 g of soil.

Seeds

Seeds were collected from plus trees of *Casuarina equisetifolia* located at Vaithikovil of Pudukkottai district, Tamil Nadu, India. Matured cones were collected and the seeds were separated and graded; only big seeds were used for raising seedlings.

Microbial inoculants

VAM fungus

The VAM fungus, *Glomus fasciculatum,* was isolated and recorded as the dominant species in the rhizosphere soil of 4-y-old *C. equisetifolia* plantation at Pudukkottai, Tamil Nadu, India. It was multiplied in pot culture in the sterilised mixture of sand: soil (1:1 v/v) and maintained in the roots of *Sorghum vulgare* as the host plant. The inoculum contained extra matrical hyphae, chlamydospores and infected root segments. Inoculum potentials were determined by the most probable number (Porter 1979) and approximately 12 500 infective propagules (10 g of soil) were added in the root zones of each seedling.

Azospirillum and phosphobacterium

Peat based culture of *Azospirillum* and phosphobacterium with a population load of 10^{-9} and 10^{-8} cells g^{-1} respectively were obtained from the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Frankia nodule collection and preparation of inoculum

Frankia nodules were collected from the plus trees of *C. equisetifolia.* Nodules were washed and surface sterilised with 30% hydrogen peroxide for 20 min followed by 70% alcohol for 1 min and then rinsed three times in distilled water, ground with a pestle and mortar and diluted with 2% sucrose solution (Reddell *et al.* 1988).

Experimental design

Experiment was conducted at the silviculture nursery of the Institute of Forest Genetics and Tree Breeding, Coimbatore, Tamil Nadu, India. The experiment was set up in a completely randomised block design with 16 treatments and 3 replicates. Each replication comprised 45 seedlings.

Potting media and transplanting of seedlings

Healthy and uniform sized (about 5 cm height) seedlings were transplanted to 13 × 26 cm size polythene bags with a potting mixture of unsterilised sand: red soil: farmyard manure (2:3:1).

Application of biofertilizers

Ten days after transplanting, 10 g each of *Azospirillum*, phosphobacterium and VAM fungus and 10 ml of *Frankia* nodule suspension (treatments) were applied to polythene bags at 5 cm depth near the root zone by making holes. Water was sprayed once a day.

Treatments

T1 – *Azospirillum*
T2 – Phosphobacterium
T3 – Vesicular arbuscular mycorrhizae (VAM)
T4 – *Frankia*
T5 – *Azospirillum* + phosphobacterium
T6 – *Azospirillum* + VAM
T7 – *Azospirillum* + *Frankia*
T8 – Phosphobacterium + VAM
T9 – Phosphobacterium + *Frankia*
T10 – VAM + *Frankia*
T11 – *Azospirillum* + phosphobacterium + VAM
T12 – *Azospirillum* + phosphobacterium + *Frankia*
T13 – *Azospirillum* + VAM + *Frankia*
T14 – Phosphobacterium + VAM + *Frankia*
T15 – *Azospirillum* + phosphobacterium + VAM + *Frankia*
T16 – Control (uninoculated)

Harvesting and measurements

Shoot length and basal diameter were recorded at bimonthly intervals up to six months. After six months in each treatment, a total of 12 seedlings with average height and basal diameter were selected and carefully uprooted for the estimation

of root and shoot fresh weight, nodular number and nodular weight. VAM spore density in each soil sample was estimated by a modified wet sieving and decanting technique as described by Gerdemann and Nicolson (1963). Mycorrhizal root infection was assessed following the procedure of Phillips and Hayman (1970).

Nitrogen content in the seedlings was analyzed colorimetrically using Kjeldahl auto analyser 1030 (Jackson 1973). Phosphorus content was estimated colorimetrically using spectrophotometer by the Bray P_2 method. Potassium content was determined using flame photometer (Jackson 1973). Calcium and magnesium contents were determined by the method described by Jackson (1973).

Microbial population

The percentage of nodulated seedlings by *Frankia* was assessed six months after inoculation. Twelve seedlings were examined and the number of nodule bearing seedlings was counted. The percentage of nodules bearing seedlings was calculated by the following formula:

$$\text{Percentage of nodulating seedlings} = \frac{\text{number of seedlings with nodules}}{\text{total number of seedlings observed}} \times 100$$

Quantitative estimation of the microorganisms

The dilution plate counting method was employed for the enumeration of microbial population in the soil samples. Appropriate dilutions were done with Pikovskaya's medium for phosphobacteria (Sundara Rao & Sinha 1963). N-free semi-solid malate medium for *Azospirillum* (Dobereiner *et al.* 1976) was used for the respective organism. An aliquot of 1 ml of the respective dilution was spread in sterile petriplates of 90-mm diameter and dispensed with the respective medium. Plates were rotated gently thrice in clockwise and anticlockwise directions to ensure uniform distribution of the soil suspension. The plates were incubated at 28 °C. The colonies were counted on the third day for phosphobacteria and *Azospirillum* using colony counter and expressed as the population colony-forming unit (cfu) per gram of soil.

Assessment of mycorrhizal infection

Mycorrhizal root infection was assessed following the procedure of Phillips and Hayman (1970). The root segments were placed in a 2.5% aqueous solution of KOH (w/v) and boiled in a water bath at 90 °C for 15 min. The roots were rinsed with several changes of water and lightened in H_2O_2 (3 ml of 20% NH_4OH in 30 ml H_2O_2) for 10–45 min. They were again thoroughly rinsed with water several times and acidified by soaking in 40-50 ml of 1% HCl for 3 min. Acidified roots were stained in an acidic glycerol solution (500 ml glycerol, 450 ml H_2O, 50 ml 1%

HCl) containing 0.05% trypan blue. The trypan blue solution was poured off and the roots were washed in acidic glycerol at room temperature to remove excess stain. The stained roots were mounted in a glass slide and the percentage of infection was calculated:

$$\text{Percentage of root colonisation} = \frac{\text{number of root bits showing VAM infection}}{\text{total number of root bits examined}} \times 100$$

Seedling quality index

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

$$\text{Seedling quality index (SQI)} = \frac{\text{total weight (g/ plant)}}{\frac{\text{height (cm)}}{\text{root collar diameter (mm)}} + \frac{\text{shoot weight (g/plant)}}{\text{root weight(g/plant)}}}$$

Statistical analysis

The data were statistically analysed by analysis of variance (ANOVA) and treatment means were separated using Duncan's multiple range test ($p<0.05$) (Duncan 1955).

Results

Shoot length

Significant increases in shoot length were recorded in *Casuarina equisetifolia* seedlings inoculated with the different microbial inoulants compared to the uninoculated control at 60, 120 and 180 days after inoculation (Table 1). From the analysis of growth data the combined inoculation of *Azospirillum* + phosphobacterium + VAM + *Frankia* (T15) was found to be the most effective combination in increasing the growth of seedlings at all the stages.

Among all the treatments, the inoculation with *Azospirillum* + phosphobacterium + VAM + *Frankia* (T15) showed maximum shoot length (62.2% increase over the control) after 180 days . Among the individual inoculations, *Frankia* (T4) showed higher shoot length and was statistically on par with VAM (T3) inoculated seedlings. Among the double inoculations, VAM + *Frankia* (T10) was superior to the others. *Azospirillum* + VAM + *Frankia* (T13) registered the highest shoot length among the triple inoculations and this result was statistically similar with phosphobacterium + VAM + *Frankia* (T14), and *Azospirillum* + phosphobacterium + *Frankia* (T12) inoculated seedlings at 180 days after inoculation.

Basal diameter

The differences among periods, treatments and their interaction were highly significant (Table 1). The combined inoculation of *Azospirillum* + phosphobacterium + VAM + *Frankia* (T15) gave significantly better growth than other treatments at 60, 120 and 180 days after inoculation, registering 34.2, 81.7 and 63.5% increases over the control respectively. VAM (T3) inoculated seedlings showed superior growth over other single treatments at 60 and 180 days after inoculation. Among the double inoculations, phosphobacterium + VAM (T8) registered the highest basal diameter but was on par with *Azospirillum* + *Frankia* (T7), followed by phosphobacterium + *Frankia* (T9), and VAM + *Frankia* (T10), at 60 days after inoculation. Phosphobacterium + VAM + *Frankia* (T14) registered the highest basal diameter among the triple inoculations at 60 and 120 days after inoculation. At 120 days after inoculation the treatment phosphobacterium + VAM + *Frankia* (T14) showed the second highest basal diameter. Inoculated seedlings after 180 days showed significant differences among the treatments. The combined application of *Azospirillum* + phosphobacterium + VAM + *Frankia* (T15) registered the highest diameter increase of 63.5% over the control. It was followed by VAM + *Frankia* (T10) inoculated seedlings which was on par with *Azospirillum* + VAM + *Frankia* (T13).

Table 1 Effect of microbial inoculants on shoot length (cm) and basal diameter (mm) of *Casuarina equisetifolia* at 60, 120 and 180 days after inoculation

	60 days		120 days		180 days	
Treatment	Shoot length	Basal diameter	Shoot length	Basal diameter	Shoot length	Basal diameter
T1	37.57 d	0.35 a	66.92 cde	2.81 b	75.42 b	4.35 c
T2	35.43 ab	0.37 bc	61.50 b	3.01 bc	71.65 a	4.07 b
T3	37.57 d	0.41 e	63.30 bc	3.11 cd	81.91 de	4.53 e
T4	36.03 bc	0.37 cd	63.67 bc	3.38 e	84.83 e	4.46 de
T5	37.73 d	0.39 d	63.59 bc	3.25 cde	80.55 cd	4.13 b
T6	35.70 bc	0.35 ab	63.33 bc	3.32 d	84.71 e	4.41 cd
T7	37.80 d	0.41 e	64.02 bc	3.69 f	77.51 bc	4.43 cd
T8	36.20 bc	0.42 e	62.20 b	3.80 fg	84.08 e	4.87 f
T9	37.73 d	0.38 cd	64.49 bcd	3.65 f	85.21 e	4.98 g
T10	39.17 e	0.37 cd	66.98 cde	3.73 fg	94.62 g	5.95 k
T11	36.37 c	0.36 ab	62.56 b	3.72 fg	89.59 f	5.11 h
T12	40.53 f	0.39 d	68.31 de	3.82 fg	95.33 g	5.68 i
T13	40.47 f	0.41 e	68.68 e	3.94 fgh	96.88 g	5.92 k
T14	40.80 fg	0.42 e	69.46 e	3.98 gh	95.75 g	5.76 j
T15	41.37 g	0.47 f	74.94 f	4.18 h	111.24 h	6.23 l
T16	34.80 a	0.35 ab	55.94 a	2.30 a	68.59 a	3.81 a

Means followed by common letter(s) in the same column are not significantly different at 5% level by DMRT.

Treatments: T1 *Azospirillum*; T2 Phosphobacterium; T3 Vesicular arbuscular mycorrhizae (VAM); T4 *Frankia*; T5 *Azospirillum* + phosphobacterium; T6 *Azospirillum* + VAM; T7 *Azospirillum* + *Frankia*; T8 Phosphobacterium + VAM; T9 Phosphobacterium + *Frankia*; T10 VAM + *Frankia*; T11 *Azospirillum* + phosphobacterium + VAM; T12 *Azospirillum* + phosphobacterium + *Frankia*; T13 *Azospirillum* + VAM + *Frankia*; T14 Phosphobacterium + VAM + *Frankia*; T15 *Azospirillum* + phosphobacterium + VAM + *Frankia*; and T16 Control.

Shoot biomass

The data pertaining to dry matter accumulation of shoot, root and root nodules and total biomass are presented in Table 2. Significant responses were observed among the treatments evaluated at 180 days after inoculation. The largest significant biomass in the shoot was recorded in seedlings inoculated with *Azospirillum* + phosphobacterium + VAM + *Frankia* (T15). It recorded 113.4% increase over the control. It was followed by inoculation with *Azospirillum* + VAM + *Frankia* (T13). Among the single and double inoculations, VAM (T3) and VAM + *Frankia* (T10) were the most effective in producing shoot biomass.

Table 2 Effect of microbial inoculants on the biomass of *Casuarina equisetifolia* seedlings (180 days after inoculation)

Treatment	Shoot dry weight (gram/plant)	Root dry weight (gram/plant)	Nodular weight (gram/plant)	Total dry weight (gram/plant)
T1	7.60 c	3.71 bc	0.000 a	11.31 cd
T2	7.97 d	2.54 a	0.000 a	10.51b
T3	8.82 f	2.88 a	0.000 a	11.70 d
T4	8.26 e	3.36 b	0.059 b	11.70 d
T5	7.28 b	3.75 bc	0.000 a	11.03 bc
T6	8.69 f	4.22 d	0.000 a	12.91 e
T7	7.45 bc	3.82 c	0.067 bc	11.34 cd
T8	8.09 de	2.57 a	0.000 a	10.66 b
T9	8.69 f	2.68 a	0.090 bc	11.45 cd
T10	11.12 i	3.58 bc	0.064 bc	14.76 g
T11	9.09 g	4.28 de	0.056 b	13.43 f
T12	9.36 h	4.28 de	0.077bc	13.72 f
T13	12.51 j	4.65 e	0.088 bc	17.25 i
T14	11.12 i	4.21 d	0.077 bc	15.41h
T15	14.62 k	5.61 f	0.096 c	20.32 j
T16	6.85 a	2.60 a	0.000 a	9.45 a

Means followed by common letter(s) in the same column are not significantly different at 5% level by DMRT.

Treatments: T1 *Azospirillum*; T2 Phosphobacterium; T3 Vesicular arbuscular mycorrhizae (VAM); T4 *Frankia*; T5 *Azospirillum* + phosphobacterium; T6 *Azospirillum* + VAM; T7 *Azospirillum* + *Frankia*; T8 Phosphobacterium + VAM; T9 Phosphobacterium + *Frankia*; T10 VAM + *Frankia*; T11 *Azospirillum* + phosphobacterium + VAM; T12 *Azospirillum* + phosphobacterium + *Frankia*; T13 *Azospirillum* + VAM + *Frankia*; T14 Phosphobacterium + VAM + *Frankia*; T15 *Azospirillum* + phosphobacterium + VAM + *Frankia*; and T16 Control.

Root and nodular biomass

Root and nodular biomass showed significant differences among the treatments. Inoculation of *Azospirillum* (T1) alone and in combination with other inoculants was found to significantly increase root biomass when compared to other treatments. Root biomass was the highest in *Azospirillum* + phosphobacterium + VAM + *Frankia* (T15), followed by *Azospirillum* + VAM + *Frankia* (T13). The latter was also statistically on par with *Azospirillum* + phosphobacterium + *Frankia* (T12) and *Azospirillum* + phosphobacterium + VAM (T11). Among the single and double

inoculations, *Azospirillum* treated seedlings, especially *Azospirillum* + VAM (T6), showed better root biomass than other treatments. Nodular biomass in seedlings inoculated with *Frankia* in combination with other inoculants was significantly higher than that of seedlings inoculated with *Frankia* alone, the highest value being given by *Azospirillum* + phosphobacterium + VAM + *Frankia* (T15) (Table 2).

Total biomass of seedling

Seedling biomass was maximum in the treatment comprising *Azospirillum* + phosphobacterium + VAM + *Frankia* (T15) and it was 115.0% more than that of the control. Seedlings inoculated with *Azospirillum* + VAM + *Frankia* (T13) recorded 82.5%, the next highest biomass, followed by phosphobacterium + VAM + *Frankia* (T14), which gave 63.1% more than the control (Table 2).

Seedling quality index

The best quality seedlings were obtained in treatment (T15), *Azospirillum* + phosphobacterium + VAM + *Frankia*. *Azospirillum* + VAM + *Frankia* (T13) recorded the next highest seedling quality index, followed by phosphobacterium + VAM + *Frankia* (T14). Among the single inoculations, *Frankia* (T4) and VAM (T3), and among the double inoculations, VAM + *Frankia* (T10) had the highest seedling quality index (Figure 1).

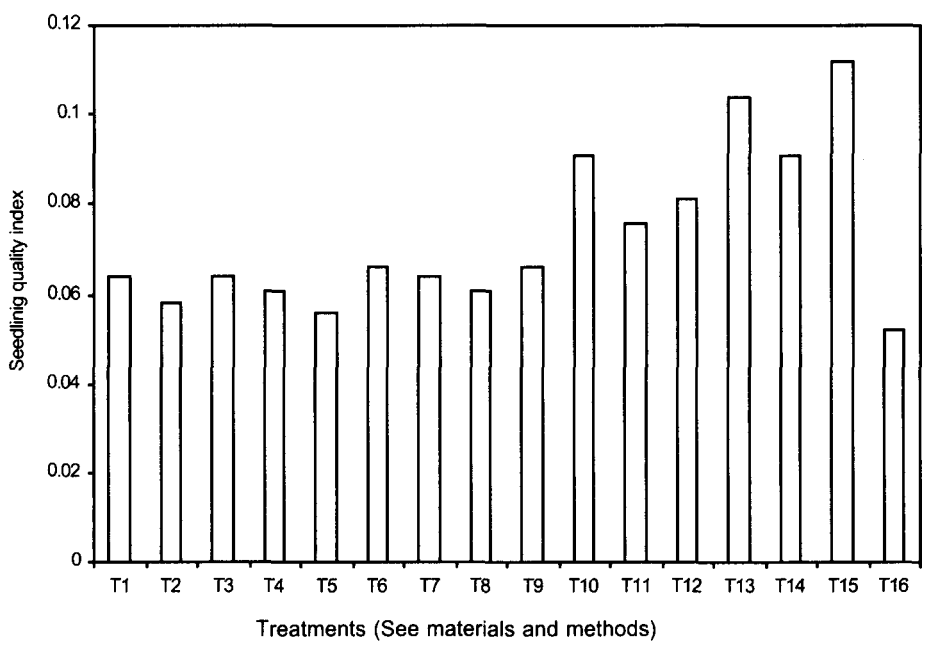

Figure 1 Seedling quality index of *Casuarina equisetifolia* treated with microbial inoculants (180 days after inoculation)

Nutrient content

Nitrogen

The total nitrogen contents of inoculated *C. equisetifolia* seedlings showed significant increases over the control (Table 3). The highest nitrogen content was observed in seedlings inoculated with *Azospirillum* + phosphobacterium + VAM + *Frankia* (T15) followed by the triple inoculations of *Azospirillum* + VAM + *Frankia* (T13) and phosphobacterium + VAM + *Frankia* (T14). The above treatments produced 319.0, 251.0 and 203.0% more than the control respectively. Statistically there was no significant difference between *Azospirillum* + phosphobacterium + VAM (T11), and *Azospirillum* + phosphobacterium + *Frankia* (T12). Among the single and double inoculations, *Azospirillum* + VAM (T6) and VAM + *Frankia* (T10) recorded higher nitrogen contents when compared to other treatments.

Table 3 Nutrient uptake (mg/seedling on oven dry weight basis) of inoculated *Casuarina equisetifolia* seedlings (180 days after inoculation)

Treatment	N	P	K	Ca	Mg
T1	151 c	8.0 b	123 b	124 bc	33 bc
T2	125 b	8.5 bc	115 b	114 b	30 b
T3	160 cde	12.5 ef	154 d	147 e	46 f
T4	176 fg	11.0 de	123 b	130 cd	34 bc
T5	160 cde	10.5 cde	122 b	123 bc	35 cd
T6	192 h	13.9 f	167 b	161 f	52 g
T7	166 def	12.1 ef	115 b	125 bcd	35 c
T8	152 cd	9.5 bcd	143 cd	136 cde	43 ef
T9	189 gh	10.4 cde	140 c	139 de	40 de
T10	291 j	20.6 h	208 f	262 h	65 h
T11	216 i	17.0 g	170 e	168 f	54 g
T12	217 i	13.5 f	172 e	197 g	50 g
T13	351 k	23.5 i	250 g	317 i	80 i
T14	303 j	20.8 h	209 f	274 h	63 h
T15	419 l	29.9 j	305 h	389 j	97 j
T16	100 a	5.4 a	82 a	81 a	22 a

Means followed by common letter(s) in the same column are not significantly different at 5% level by DMRT.

Treatments: T1 *Azospirillum*; T2 Phosphobacterium; T3 Vesicular arbuscular mycorrhizae (VAM); T4 *Frankia*; T5 *Azospirillum* + phosphobacterium; T6 *Azospirillum* + VAM; T7 *Azospirillum* + *Frankia;* T8 Phosphobacterium + VAM; T9 Phosphobacterium + *Frankia*; T10 VAM + *Frankia*; T11 *Azospirillum* + phosphobacterium + VAM; T12 *Azospirillum* + phosphobacterium + *Frankia*; T13 *Azospirillum* + VAM + *Frankia*; T14 Phosphobacterium + VAM + *Frankia*; T15 *Azospirillum* + phosphobacterium + VAM + *Frankia;* and T16 Control.

Phosphorus

The phosphorus content was highest in the seedlings treated with *Azospirillum* + phosphobacterium + VAM + *Frankia* (T15), followed by *Azospirillum* + VAM + *Frankia* (T13) and VAM + *Frankia* (T10) registering 453.7, 335.1 and 281.5% more than the control plants respectively. Among the single inoculations, VAM (T3) had more phosphorous content than the rest (Table 3).

Potassium, calcium and magnesium

K, Ca, and Mg contents in the seedlings showed the highest values in the combination of *Azospirillum* + phosphobacterium + VAM + *Frankia* (T15), followed by *Azospirillum* + VAM + *Frankia* (T13), and VAM + *Frankia* (T10). Among single inoculations, plants inoculated with VAM fungi (T3) contained more K, Ca and Mg than other single treatments (Table 3).

Microbial population

Maximum *Azospirillum* population was enumerated in the rhizosphere soil of *C. equisetifolia* seedlings inoculated with *Azospirillum* (T1) followed by the combined applications of *Azospirillum* with other microorganisms.

Maximum phosphobacterium population was enumerated in the rhizosphere soil of seedlings inoculated with phosphobacterium (T2), the treatment which was statistically on par with phosphobacterium coinoculated with VAM + *Frankia* (T14), followed by *Azospirillum* + phosphobacterium + VAM + *Frankia* (T15).

The highest VAM infection was produced in T14, followed by T15, which was statistically on par with *Azospirillum* + VAM + *Frankia* (T13), VAM + *Frankia* (T10) and VAM (T3). The lowest VAM infection was calculated in the control plants and was statistically on par with that in *Azospirillum* (T1) and *Azospirillum* + phosphobacterium (T5).

Treatments with *Frankia* and *Frankia* in combination with VAM showed markedly better *Frankia* infection than other treatments. The percentage of nodulated seedlings was highest in *Azospirillum* + VAM + *Frankia* (T13) and it was statistically on par with the values given by phosphobacterium + VAM + *Frankia* (T14) and VAM + *Frankia* (T10). Among individual inoculations *Frankia* (T4) inoculated seedlings had the best nodulation. The lowest percentage of nodulated seedlings was the control (TI6) and it was statistically on par with the values given by *Azospirillum* inoculated seedlings (TI) and *Azospirillum* + VAM (T6) (Table 4).

Table 4 Microbial population (cfu/g of dry soil), and VAM and *Frankia* infection of inoculated *Casuarina equisetifolia* seedlings (180 days after inoculation)

Treatment	*Azospirillum* ($\times 10^6$)	Phosphobacterium ($\times 10^4$)	VAM infection percentage	*Frankia* infection percentage
T1	3.8 f	1.3 b	24.63 a	3.77 ab
T2	0.1 ab	4.2 def	32.33 c	7.57 bc
T3	0.8 b	1.5 b	83.63 fg	15.33 de
T4	0.4 ab	1.3 b	34.97 c	82.60 g
T5	2.2 cd	2.6 c	26.90 ab	12.33 cd
T6	3.2 e	0.5 a	63.60 e	4.67 ab
T7	2.8 de	1.6 b	31.40 bc	76.00 f
T8	0.5 ab	2.6 c	59.00 e	12.43 cd
T9	0.4 ab	4.5 ef	34.33 c	79.90 fg
T10	0.4 ab	2.2 c	82.67 fg	92.23 hi
T11	0.1 a	3.8 d	63.00 e	21.13 e
T12	2.6 cde	4.6 f	46.83 d	86.17 gh
T13	2.7 be	1.6 b	80.53 f	93.90 i
T14	2.0 c	4.0 de	86.63 g	91.43 hi
T15	2.8 de	3.9 de	83.83 fg	85.63 gh
T16	1.0 a	1.35 b	21.77 a	0.53 a

Means followed by common letter(s) in the same column are not significantly different at 5% level by DMRT.

Treatments: T1 *Azospirillum*; T2 Phosphobacterium; T3 Vesicular arbuscular mycorrhizae (VAM); T4 *Frankia*; T5 *Azospirillum* + phosphobacterium; T6 *Azospirillum* + VAM; T7 *Azospirillum* + *Frankia*; T8 Phosphobacterium + VAM; T9 Phosphobacterium + *Frankia*; T10 VAM + *Frankia*; T11 *Azospirillum* + phosphobacterium + VAM; T12 *Azospirillum* + phosphobacterium + *Frankia*; T13 *Azospirillum* + VAM + *Frankia*; T14 Phosphobacterium + VAM + *Frankia*; T15 *Azospirillum* + phosphobacterium + VAM + *Frankia*; and T16 Control.

Discussion

Biologically active products, more appropriately called microbial inoculants, containing active strains of selective microorganisms like *Azospirillum*, phosphobacterium, VAM fungi and *Frankia* alone or in combination, help plant growth by different mechanisms among which are biological nitrogen fixation and solubilisation of insoluble phosphate fertilizer. In the present study, the height, diameter and dry matter of *Casuarina equisetifolia* seedlings were significantly improved by such microbial inoculation. The increase of growth may be attributed to improved uptake of N, P, K, Ca and Mg. Good nodulation, increased microbial population and high VAM infection also support the growth of the seedlings.

Azospirillum inoculated seedlings showed better growth and root biomass when compared to the control. Rodriguez-Barrueco *et al.* (1991) observed that *A. brasilense* increased the growth of *C. cunninghamiana* seedlings by 90% over uninoculated control. This may be due to increased root biomass and accumulation of nitrogen (Wong & Stenberg 1979), and the production of gibberellin and cytokinin-like substances (Tien *et al.* 1979), which promote the growth of the seedlings.

In the present study phosphobacteria inoculated *C. equisetifolia* seedlings showed improved growth and nutrient uptake in relation to the uninoculated control plants. Similar observations were made by Mohammad and Ram Prasad (1988) in *Eucalyptus camaldulensis* and Young (1990) in *Leucaena leucocephala.* This may be

due to conversion of insoluble phosphorus to soluble form thus making it available for uptake by plants. Phosphobacteria also produce auxins and gibberellin, which may have favourable effect on plant growth (Somani *et al.* 1990).

Abbott and Robson (1984) showed that VAM enhanced plant growth as a result of improved mineral nutrient of the host plant. In the present study VAM inoculated seedlings improved growth and nutrient content in relation to the uninoculated control plants. Vasanthakrishna *et al.* (1995) also observed similar findings in *C. equisetifolia* seedlings. This can be attributed to the increased absorbing surface area due to extensive external network of mycelium produced by the VAM fungi in association with the host root system (Howeler *et al.* 1981). Stribley (1987) inferred that P is the most important nutrient involved and other nutrients such as N, K, Ca, and Mg are translocated along with VAM hyphae.

Frankia inoculated seedlings showed better growth, nodulation and nutrient concentration. Similar results were reported by Reddell (1990) indicating that the artificial application of nodule crush increased dry matter yield of *Casuarina.* Mwanza (1990) obtained increased overall growth, nodulation, shoot and root dry weight and nitrogen content by inoculation of *Frankia* in *C. equisetifolia.* This may be due to fixation of atmospheric nitrogen by *Frankia* in the host plant. Inoculation of *Frankia* contributed to a greater amount of N uptake for the demand of the tree to put forth growth compared to the control as reported by Mansour and Baker (1994). Gauthier *et al.* (1985) calculated from his experiment that about 40-60 kg N_2 would be fixed per hectare per year at a normal density of 10 000 trees per hectare.

Casuarina equisetifolia seedlings inoculated with *Frankia* and VAM had more biomass with increased nodular numbers (Ramirez *et al.* 1992). In the present study also, double inoculation VAM + *Frankia* influenced the growth and biomass, and triple inoculations boosted better growth and diameter than single and double inoculations. *Azospirillum* + VAM + *Frankia* combination resulted in significantly higher growth and biomass.

Among all the treatments, the combined inoculation of *Azospirillum,* phosphobacteria, VAM and *Frankia* produced excellent growth, biomass and tissue nutrient concentration. The increase in height, diameter and dry matter of the *C. equisetifolia* seedlings after co-inoculation with all the inoculants might be caused by the improved accumulation of nitrogen due to *Frankia* (Reddell *et al.* 1985) and *Azospirillum* (Gunjal & Patil 1992), and phosphorus by VAM fungi (Young *et al.* 1988) and phosphobacteria (Kuccy 1987).

It is inferred that under appropriate management practices, the use of efficient microbial inoculants leads to increased growth and biomass of *C. equisetifolia.* The present study clearly shows that the combined application of *Azospirillum* + phosphobacterium + VAM + *Frankia* plays a significant role in improving the growth response and nutrient uptake of *C. equisetifolia* seedlings, thereby producing good quality planting stock. These treated seedlings may perform better in nutrient impoverished soil.

References

ABBOTT, L. K & ROBSON, A. D. 1984. Colonisation of the root system of subterranean clover by three species of VAM fungi. *New Phytology* 96: 275–281.

BARROW, N., MALAJCZUK, J. N. & SHAW, T. C. 1977. A direct test to the ability of VAM to help plants take up fixed soil phosphate. *New Phytologist* 78: 269–276.

DICKSON, A., LEAT, A. L. & HOSNER, J. L. 1960. *Forest Chronicle* 36: 237–241.

DOBEREINER, J., MARRIEL, J. E. & NERY, J. 1976. Ecological distribution of *Spirillum lipoferum* Beijerinick. *Canadian Journal of Microbiology* 22: 1461–1473.

DOMMERGUES, Y. 1976. Mycorrhizes et fixation d'azote. *Ann Edafolia Agrobiologia* 35: 1039–1056.

DUNCAN, D. B. 1955. Multiple range and multiple f-tests. *Biometrics* 11: 1–42.

GAUTHIER, D., DIEM, H. G. & DOMMERGUES, Y. R. 1985. Assessment of N_2 fixation by *Casuarina equisetifolia* inoculated with *Frankia* ORS.021001 using ^{15}N methods. *Soil Biology and Biochemistry* 17: 375–379.

GERDEMANN, J. M. & NICOLSON, H. 1963. Spores of mycorrhizal endogne species extracted from soil by wet sieving and decanting. *Transactions of the British Mycological Society* 46: 235–244.

GUNJAL, S. S. & PATIL, P. L. 1992. Mycorrhizal control of wilt in *Casuarina*. *Agroforestry Today* 4: 14–15.

HOWELER, R. H., EDWARDS, D. G. & ASHER, C. J. 1981. Application of the flowering solution culture techniques to studies involving mycorrhizae. *Plant and Soil* 59: 179–183.

JACKSON, M. L. 1973. *Soil Chemical Analysis*. Printice Hall of India (Pvt) Ltd., New Delhi.

KUCCY, R. M. N. 1987. Increased phosphorus uptake by wheat and field beans inoculated with a phosphorus solubilizing *Penicillium bilaji* strain and with vesicular arbuscular mycorrhizal fungi. *Applied Environmental Microbiology* 52: 2699–2703.

MANSUR, R. S. & BAKER, D. D. 1994. Selection trials for effective N_2 fixing *Casuarina, Frankia* combinations in Egypt. *Soil Biology and Biochemistry* 26: 655–658.

MOHAMMAD, G. & RAMPRASAD. 1988. Influence of microbial fertiliser on biomass accumulation in polypotted *Eucalyptus camaldulensis* seedlings. *Journal of Tropical Forestry* 4: 74–77.

MWANZA, E. J. M. 1990. *Response of Casuarina to Frankia Inoculation in Saline Unsterile Sand Vermiculite Medium*. Research Note. No. 4. Kenya Forestry Research Institute. 14 pp.

OKON, Y. & KAPULINK, Y. 1986. Development and function of *Azospirillum* inoculated roots. *Plant and Soil* 21: 43–50.

PHILIPS, J. M. & HAYMAN, D. S. 1970. Improved procedures for clearing roots and staining parasite and vesicular arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* 55: 158–161.

PORTER, W. M. 1979. The most probable number method for enumerating infective propagules of vesicular-arbuscular mycorrhizal fungi in soil. *Australian Journal of Soil Research* 17: 515–518.

RAMIREZ, S. H., VALDES RAMIRES, M. & CRUZ-CISNEROS, R. 1992. Inoculation and irrigation with waste water of *Casuarina* in nurseries. *Turrialba* 42: 476–481.

REDDELL, P. 1990. Increasing productivity in plantations of *Casuarina* by inoculation with *Frankia*. Pp. 133–140 in El-Lakany, M., Turnbull, J. W. & Brewbaker, J. L. (Eds.) *Advances in Casuarina Research*. Desert Development Centre, AUC, Cairo.

REDDELL, P., BOWEN, G. D. & ROBSON, A. D. 1985. The effects of soil temperature on plant growth, nodulation and N fixation in *Casuarina cunninghamiana*. *New Phytologist* 101: 441–450.

REDDELL, P., ROSBROOK, P. A., BOWEN, G. D. & GWAZE, D. 1988. Growth responses in *Casuarina cunninghamiana* plantings to inoculation with *Frankia*. *Plant and Soil* 108: 79–86.

RODRIGUEZ-BARRUECO, C., CERVANTES, E., SUBBA RAO, N. S. & RODRIGUEZ CACERES, E. 1991. Growth promoting effect of *Azospirillum brasilense* on *Casuarina cunninghamiana* Mig. *Plant and Soil* 135: 121–124.

SIDHU, O. P., BEHL, H. M., GUPTA, M. L. & JANARDHAN, K. K. 1990. Occurrence of vesicular arbuscular mycorrhizae in *Casuarina equisetifolia*. *Current Science* 59: 422 –423.

SOMANI, L. L., BHANDARI, S. C, SEXENA, S. N. & GULATI, I. J. 1990. Pp. 271–294 in Somani, L. L., Bhandari, S. C., Sexena, S. N. & Vyas, K. K. (Eds.) *Phosphomicroorgansims – Biofertilizers*.

STRIBLEY, D. P. 1987. Mineral nutrition. Pp. 193–211 in Safir, G. R. (Ed.) *Ecophysiology of VA Mycorrhizal Plants*. CRC Press, Boca Raton, Florida.

SUNDARA RAO, W. V. B. & SINHA, M. K. 1963. Phosphate dissolving organisms in the soil and rhizosphere. *Indian Journal of Agricultural Science* 33: 272–278.

SWAMINATH, M. H. & VADIRAJ, B. A. 1988. Nursery studies on the influence of *Azospirillum* biofertilizers on the growth and dry matter of forestry species. *Myforest 24*: 289–294.

TIEN, T. M., GASKIN, M. H. & HUBBELL, D. H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). *Applied Environmental Microbiology* 33: 1016–1024.

TORREY, J. G. 1978. Nitrogen fixation by actinomycete nodulated angiosperms. *Bioscience* 28: 586–592.

VASANTHAKRISHNA, M., BAGYARAJ, D. J. & NIRMALNATH, P. J. 1995. Selection of efficient VAM fungi for *Casuarina equisetifolia* second screening. *New Forest* 121: 157–162.

WONG, P. P. & STERNBERG, N. E. 1979. Characterization of *Azospirillum* isolates from nitrogen fixing roots of harvested sorghum plants. *Applied Environmental Microbiology* 38: 1189–1191.

YOUNG, C. C. 1990. Effects of phosphorus solubilizing bacteria and VAM fungi on the growth of tree species in subtropical-tropical soils. *Soil Science and Plant Nutrition* 36: 225–231.

YOUNG, C. C., JUANG, T. C. & CHAO, C. C. 1988. Effect of *Rhizobium* and VAM inoculation on nodulation and soybean yield in subtropical fields. *Biology and Fertility of Soils* 6: 165–169.