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RESEARCH PAPER

EFFECTS OF ARBUSCULAR MYCORRHIZAL FUNGI, *PHOSPHOBACTERIUM* AND *AZOSPIRILLUM SP*. ON THE SUCCESSFUL ESTABLISHMENT OF *EUCALYPTUS CAMALDULENSIS* DEHN. IN BAUXITE MINE SPOILS

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ABSTRACT

Eucalyptus camaldulensis Dehn seedlings were inoculated with the cultured arbuscular mycorrhizal (AM) fungi *Glomus aggregatum* Schenck & Smith emend. Koske, and the beneficial microbes *Phosphobacterium* and *Azospirillum sp*. individually and in combinations in the nursery . Mined out wastes of bauxite, called bauxite mine spoils, were collected and used as a potting medium to grow *E. camaldulensis* seedlings for three months in the nursery. The AM fungal and other beneficial microbial inoculations improved the seedling quality in terms of total biomass and growth. The seedlings were transplanted on to bauxite mine spoils and the growth and survival of seedlings were monitored for two years. In these field conditions, the inoculated seedlings of *E. camaldulensis* showed 95% survival and significantly higher growth than the control seedlings. From this study it was deduced that *E. camaldulensis* inoculated with beneficial microbes is a suitable tree species for the rehabilitation of bauxite mine spoils, and is cost effective and environment friendly

Key words: afforestation, India, site amelioration, nursery work, minespoils, bio inoculants

INTRODUCTION

Over the past two decades large scale planting of *Eucalyptus spp*. as a fast growing tree species has occurred in India as part of reforestation and to create adequate supplies of fuel and timber for rural communities. *Eucalyptus spp*. were first introduced in India in 1843 in the Nilgiri Hills as an experiment to find high yielding species for fuel and timber (Penfold and Willis, 1961) and it soon become a favoured species. Among *Eucalyptus spp., E. camaldulensis* Dehn. has been found to be very productive and has become a popular choice for afforestation due to its fast growth and short rotation. This is a species that produces quality pulp wood for paper and newsprint making. In the State of

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Tamilnadu, India, *E. camaldulensis* has been planted in an area of 35,000 ha by Tamilnadu Forest Plantation Corporation Ltd. The main yield of *E. camaldulensis* on a rotation of seven year is about 20 tonnes per ha. *E. camaldulensis* has been used for the afforestation of degraded areas like mine spoils.

In India, some 700,000 hectares of land is under mining, and mining activities on these lands have created wide spread environmental damage that has severely affected and not only scarred the landscape but also reduced the social well-being of inhabitants living nearby (Prasad, 1988), including health hazards with high incidences of asthma and stomach ailments due to the mining dust and contamination of water, as well as being extremely unsightly. Mining also has led to the spreading of millions of tons of waste rock and detritus, called 'mine spoils', dumped over hundreds of thousands of hectares. Reclamation of these mine spoils is essential to overcome the health hazards and ecological problems.

This reclamation work is difficult because the mine spoil has low water–holding capacity, lacks organic matter and is deficient in nitrogen (N) and phosphorus (P). Conventional reclamation techniques of mine spoils – grading, re-soiling, and fertilizing - are not always economically feasible. Planting of trees directly on mine spoils is an excellent alternative method for reclamation. This method helps to stabilize the mine spoils so that the mined out wastes are sealed. Generally, the topsoil is stripped off and stored until the site is ready for restoration, which may be after many years. Studies on reclaimed bauxite mine spoil surfaced with topsoil in Australia suggest that the recovery of biological and microbial activity is a good indicator of reclamation success (Jasper *et al*., 1998; Jeffries *et al*., 2003). But the microbial populations in stored mine spoils are low when compared to undisturbed sites (Miller and Cameron, 1976). Further, the carbon content of mine spoils is significantly lower due to lack of microbes (Abdul-Kareen and McRae, 1984). These characters lead to reduced soil quality, slow nutrient cycling and lower availability of nutrients. Moreover, the process of opencast bauxite mining scars the landscape and destroys microbial communities. The degraded environment left in the aftermath of open cast mining often cannot support biomass development and the spoils remain uncolonised. A recent study by Dupponnois *et al.* (2007) showed that inoculated arbuscular micorrhizal (AM) fungi on *Acacia holosericea* seedlings improved their growth significantly over control seedlings over 30 months on degraded soils. Therefore a study on reclaiming bauxite mine spoils with *E. camaldulensis* and beneficial arbuscular mycorrhizal fungi (AM) and the nitrogen fixing bacteria *Azospirillum*, and Phosphobacterium (PSB) was started.

MATERIALS AND METHODS

Study site

The study was centred on the bauxite mine spoils reclaimed at Yercaud Hill, Salem District, Tamil Nadu, India (11° 48′ to 11° 50′ and 78° 13′ to

78º 14 E′) at an elevation of 1640 m a.s.l where the Madras Aluminium Company (MALCo) has acquired about 190 ha of land for open cast mining. The site receives 1500mm average rainfall and has a wide diversity of plant species around the bauxite mined areas. The bauxite mine spoil was collected from the mined area and used for nursery experiments at the Institute of Forest Genetics and Tree Breeding, Coimbatore, India. The physical and chemical properties of tis bauxite mine spoil were assessed according to Jackson (1973) and showed:

- a pH 6.0 ($\pm$ 1.24),
- electrical conductivity 0.08 mS ($\pm$ 0.02),
- nitrogen 0.30 mg Kg^{-1} ($\pm$ 0.068),
- phosphorus 1.30 mg Kg^{-1} ($\pm$ 0.68), and
- potassium 4.0 mg Kg^{-1} ($\pm$ 1.56).

The figures in parentheses are the Standard Errors of the Means.

E. camaldulensis

The seeds of *E. camaldulensis* were procured from the Seed Technology Division of Institute of Forest Genetics and Tree breeding, Coimbatore. The *E. camaldulensis* seeds were directly sown in nursery beds of pure sand and irrigated with water sprays. Ten day old seedlings were transplanted to polythene bags (14 × 27 cm) containing sieved bauxite mine spoil.

Isolation and Culture of AM fungi:

In areas adjacent to the MALCo bauxite mines, *Syzgium cumini* L. was the dominant species. The AM fungus *Glomus aggregatum* Schenck & Smith emend. Koske was isolated from the rhizosphere of *S. cumini* by the method of Gerdemann and Nicolson (1963) and identified using the Schenck and Perez manual (1990). Freshly collected *G. aggregatum* spores were then multiplied and maintained in a sterile soil media (alfisoil:sand) with *Sorghum bicolor* (L.) Moench (as a host) under laboratory conditions at 19–23ºC and 40–45% relative humidity (RH) for six months in clay pots.

Isolation of Azospirillum sp and Phosphobacterium (PSB)

The *PSB* medium composition per litre of distilled water was of 10 g sucrose, 5.0 g Ca_3 (PO_4), 0.27g NH_4 NO_3, 0.2g KCl, 0.1g $MgSO_4$. $7H_2O$, 0.1g Yeast extract, 1.0mg $MnSO_4.6H_2O$, 1.0mg $FeSO_4.7H_2O$, and 15g agar. The plates were incubated at 27°C for 3-5 days. The colonies in PSB medium formed a clear zone on the medium and were easily counted.

A Congo red medium was used for *Azospirillum* as proposed by Rodriguez-Caceres (1982) The medium had the following composition in g^{-1} l of distilled water: KH_2 PO_4, 5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; Yeast extract, 0.5; $FeCl_3.6H_2O$, 0.2; NaCl, 0.1; Yeast extract, 0.5; Fe $Cl_{3.}$ $6H_2O$, 0.015; DL malic acid, 5.0; KOH, 4.8; agar, 20; 15 ml of 1: 400 solution of autoclaved Congo red was added to the medium prior to plating. In this medium *Azospirillum sp*. was readily distinguished from other free-living organism as it absorbs Congo red and forms scarlet colonies. The plates were inoculated for 72 hr at 37ºC for 3–5 days.

Inoculation of AM fungi and other microbes

Glomus aggregatum

The AM fungal inoculum of *G. aggregatum* along with soil from pot cultures of *S. bicolor* comprising mycorrhizal roots, soil hyphae and spores was used for inoculations. 10 g of inoculum at the rate of 1367 propagules/g of inoculum (Karthikeyan, 1997) was placed 5 cm below the soil surface of each polythene bag of seedlings. Thereafter the seedlings were maintained under nursery conditions for 2 months and watered regularly.

Azospirillum sp and PSB

15 ml of inoculum of *Azospirillum sp* and *PSB* cultured in broth cultures were applied to each seedling of *E. camaldulensis* individually and in combinations with AM fungi.

Nursery experimental layout

5 treatments , including a control, were replicated 5 times, using 5 seedlings per treatment per replicate – i.e 125 seedlings in all.

- Control
- AM fungi
- PSB
- *Azospirillum sp*
- AM + PSB +*Azospirillum sp*.

The seedlings were arranged in a randomized block (RDB) design under green house conditions at 31.6ºC ($\pm$ 2.8), 72% ($\pm$ 5.2) RH and watered regularly to maintain turgidity. Other seedlings were given the same treatments in preparation for planting in the field in a separate experiment.

Harvest and assessment of seedlings

Sixty days after transplantation the seedlings were harvested with their entire root system intact. The root length, shoot length, no. of branches and collar diameter of each seedlings of *E. camaldulensis* were measured.

The Field experiment

Seedlings from the 5 treatments of *E. camaldulensis* were transplanted into bauxite mine spoils in 1ft deep pits at an espacement of 1.5 × 1.5 m using a similar randomised block design as in the nursery experiment. The growth and survival of all the seedlings were monitored regularly at monthly intervals collecting data on shoot height, collar diameter and number of leaves. 10g of leaves were collected from the transplanted seedlings of *E. camaldulensis* of each treatment and oven dried at 70ºC for 48 hours in order to estimate leaf biomass at the end of 2 years.

Statistical analyses

Each measured variable in the nursery and field experiments was subjected to analyses of variance and differences between means subjected to Duncan's Multiple Range Test (SPSS. Ver. 10.)

RESULTS

Nursery experiments

The seedlings of *E. camaldulensis* showed that the triple bio inoculants inoculated (AM + PSB + *Azospirillum sp*.) seedlings significantly improved growth and biomass over the control and other combined bio inoculants (Table. 1).

Field experiments: Survival performance

AM alone inoculated seedlings and AM + PSB + *Azospirillum* inoculated seedlings showed 90–95 % survival. The uninoculated control seedlings showed only 40% survival at the age of two years after planting.

Height, collar diameter and number of branches

In *E.camaldulensis* the bio-inoculated seedlings (AM + PSB + *Azospirillum sp*.) already showed improved growth and collar diameter three months after planting. At the age of two years after planting the AM + PSB + *Azospirillum sp*. inoculated seedlings increased the growth five fold over control and also increased the collar diameter and number of branches to a similar extent (Tables 2 a & b).

TABLE 1

Growth response of *Eucalyptus camaldulensis* to inoculations of AM fungal and other beneficial microbes inoculation under nursery conditions (mean of 5 replicates)

Treatments	Root length (cm)	Shoot length (cm)	Collar diameter (mm)	Branches/ Plant	Root Dry weight (g/plant)	Shoot Dry weight (g/plant)
T_1	5.6 a	5.0 a	0.35 a	1 a	0.007 a	0.012 a
T_2	6.3 a	7.2 a	0.38 a	1 a	0.009 a	0.012 a
T_3	5.9 a	6.2 a	0.35 a	1 a	0.0096 a	0.010 a
T_4	5.3 a	6.1 a	0.35 a	1 a	0.0076 a	0.011 a
T_5	7.8 b	9.6 b	0.55 b	2 a	0.017 b	0.02 b

T_1: Control; T_2 AM; T_3 :PSB; T_4: Azospirillum; T_5: AM +PSB + Azospirillum
Means followed by same letter are not significantly different ($p<0.05$) according to Duncan's Multiple Range Test.

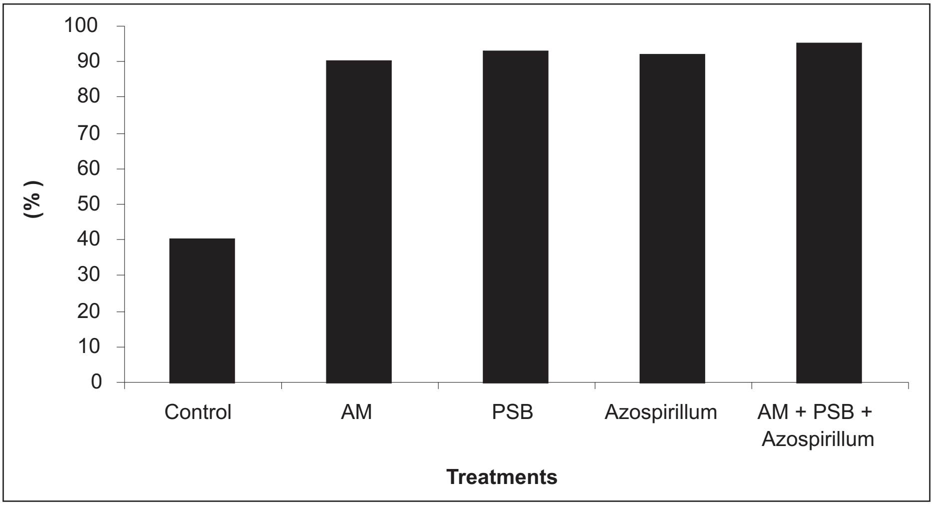

Figure 1. Per cent survival of *E. camaldulensis* at bauxite mine spoils

Leaf Biomass

Significantly increased leaf biomass was showed in AM + PSB + *Azospirillum sp*. inoculated seedlings over the other treatments and control after 2 years (Figure 2).

DISCUSSION

The excessive use of chemical fertilizer and pesticides has generated many environmental problems, some of which may be avoided by the use of bio

TABLE 2

Growth performances of *E. camaldulensis* seedlings inoculated with AM and beneficial microbes at bauxite mine spoils (mean of five replicates)

a. Three months after planting:

Treatments	Height (cm)	Collar diameter (mm)	No. of branches/plant
T_1	26.2 a	2.35 a	0.6 a
T_2	75.5 bc	5.46 b	2.33 b
T_3	64.0 b	4.64 b	4.22 b
T_4	77.3 bc	5.46 b	2.72 b
T_5	84.7 c	6.62 c	5.66 c

b. Two years after planting:

Treatments	Height (cm)	Collar diameter (mm)	No. of branches/plant
T_1	45.5 a	8.51 a	7.66 a
T_2	179 b	25.9 b	16.33 b
T_3	158.8 b	21.27 b	16.6 b
T_4	188.4 c	25.03 b	15.22 b
T_5	250.2 d	35.5 c	25.2 c

T_1: Control; T_2 :AM; T_3: PSB; T_4: Azospirillum; T_5: AM + PSB + Azospirillum
Means followed by same letter are not significantly ($p<0.05$) according to Duncan's Multiple Range Test

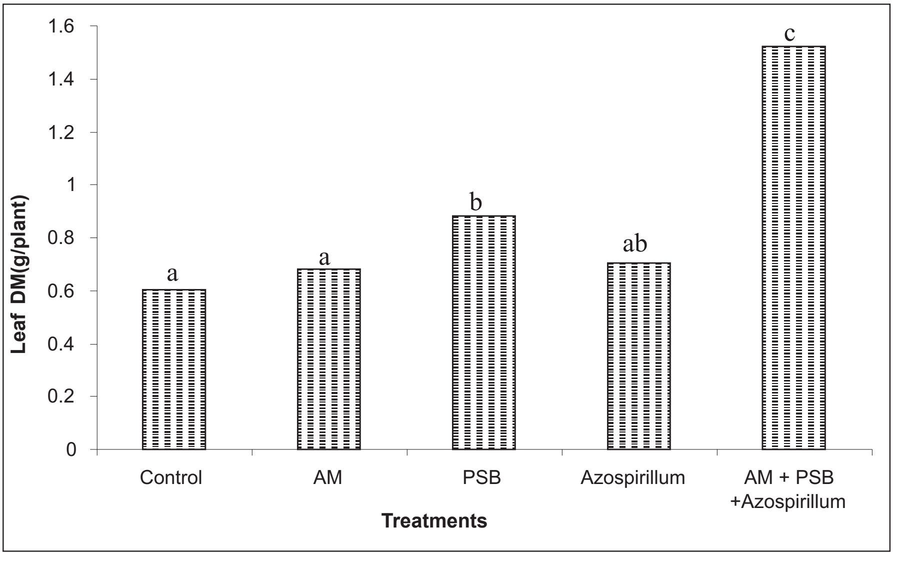

Figure 2. Leaf Biomass of *E. camaldulensis* at bauxite mine spoils (2 years after planting)
Values of Bars (mean of 5 replicates) having same letter(s) do not differ significantly (Duncan's Multiple Range Test, $p<0.05$).

inoculants and bio-pesticides that are natural, beneficial and eco-friendly. Increased phosphate absorption – increasing shoot and root growth and leaf and root hormonal changes and effects on stomatal conductivity – may be ascribed to bio inoculants particularly AM fungi (Auge *et al*., 1992). AM + PSB + *Azospirillum sp*. inoculated seedlings showed significantly improved the growth and biomass in *E. camaldulensis* in the present study. Muthukumar et al. (2001) found similar results with *Azadirachta indica* under nursery conditions. The AM colonization may alter the host root physiology, which may in turn influence the microbial populations. The flow of carbon from shoot to root may be increased by AM colonization which may alter the carbon availability for *Azospirillum sp*. and PSB in the rhizosphere (Muthukumar and Udaiyan, 2000). Multiple inoculations AM + PSB + *Azsopirillum* may interact with each other to improve soil nutrient availability (Garbaye 1994). In a recent study multiple inoculants of AM + PSB + *Azospirillum* stimulated seedling growth and nutrient uptake of *Dendrocalamus strictus* due to the activities of bio-inoculants in improving the bio-availability of the major nutrients (Muthukumar and Udaiyan 2006). Multi microbial interactions including AM fungi, and plant growth promoting rhizobacteria (PGPR) have been investigated in tree species (Muthukumar *et al*., 2001; Muthukumar and Udaiyan, 2006). Interactions between AM fungi and *Azospirillum* sp. may also enhance mycorrhizal formation (Volpin and Kapulnik, 1994). In the present study the growth and survival performance of bio-inoculated seedlings was significantly higher than uninoculated controls.

Most field studies of AM fungi inoculated seedlings reported in the literature have been on disturbed sites. Field inoculation of *Prosopis juliflora* with AM and *Rhizobium sp* in a study conducted on semi arid wasteland significantly increased plant biomass and soil nutrient after six years of growth (Bhatia *et al*., 1998). Similar results were observed in this study. The significant growth enhancement of *E. camaldulensis* with AM + *Azospirillum* or AM + PSB may be due to increased nitrogen fixation and phosphorus uptake (Jeffries *et al*., 2003; Muthukumar and Udaiyan, 2006). These effects may improve nutrient utilization efficiency of planted seedlings that gives better plant survival at bauxite mine spoils.

CONCLUSION

The results from this study supports the general conclusion that the introduction of plants to mine spoils with microbial symbionts is a beneficial bio-technological tool to aid the recovery of degraded ecosystems. The AM fungi and the other beneficial microbes have the potential to increase the efficiency of the shoot and root systems by providing the seedlings with essential levels of P and N for growth. Therefore seedlings of *E. camaldulensis* should be inoculated with symbiotic microorganisms before planting to restore mine spoils. This will be more effective than chemical inputs.

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