Use of revegetated coal mine spoil as source of arbuscular mycorrhizal inoculum for nursery inoculations

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The present investigation examines the potential use of revegetated coal mine spoil as a source of arbuscular mycorrhizal inoculum for inoculating nursery seedlings. Rhizosphere soils of five tree species were used as sources of mycorrhizal inoculum. Soils contained seven spore-forming species of AM fungi. The substrate used in the pot experiment was a mixture of unsterilized coal mine spoil (without any mycorrhizal propagule) and autoclaved sandy loam soil. *C. siamea* and *D. indica* were used as the test plants. Measurements were made of shoot and root biomass, P uptake, per cent mycorrhizal infection and spore population of AM fungi. Growth measured as shoot and root dry weight was significantly higher in seedlings inoculated with soil inoculum from under *D. sissoo*, *C. siamea*, *D. indica* and *A. indica*. *A. scrobiculata* was found to be the best fungus in terms of root colonization ability and effectiveness to promote P uptake and growth in plants. A consistently poor growth response of the seedlings to soil inoculum from under *E.* hybrid was due to the ineffective association formed by *G. geosporum*. Whilst spores of *S. calospora* were not present in the rhizosphere soils of *D. indica*, they were formed in *C. siamea* pots inoculated with the same soil. This indicates that *S. calospora* also persisted in the soil in the form of propagules other than the spores. The results of this study justify the use of revegetated coal mine spoil as an effective and economical source of endomycorrhizal inoculum for inoculating nursery seedlings.

REVEGETATION and reclamation of mine spoils has been problematic because of the poor physical conditions, extremes of temperature and pH, low levels of organic and inorganic nutrients, toxic levels of heavy metals and lack of beneficial micro-organisms. Spreading top soils, a source of some beneficial organisms before revegetation may be helpful in establishing vegetation on mine spoils[1], but this has met with limited use because of the high costs of transportation of top soils. Reclamation of mined lands, therefore, requires innovative approaches that reduce the cost and increase the chances of success of plant establishment and survival[2]. Plant establishment on mine spoils can be facilitated by endomycorrhizas formed by arbuscular mycorrhizal (AM) fungi[3], as they are particularly effective in making positionally unavailable nutrients available through greater exploration of soil volume[4]. Introducing mycorrhizal fungi in freshly stockpiled overburden spoil would require planting of nursery seedlings inoculated with propagules of AM fungi. Use of this method will be most effective if plants are inoculated with AM fungi which are known to form mycorrhizal associations on mine site[5]. Differences in AM endophytes in their ability to colonize roots and improve growth[6] and P uptake[7] in plants have been observed. Native AM fungi have been found to be more effective than introduced fungi in improving the plant growth[8,9]. Studies on growth responses of different host species to AM fungi in coal waste[10] have concluded that research on value of endomycorrhizas to survival and growth of plants should be concentrated on testing a variety of endophytes which have persisted on the bituminous coal mine spoil, in order to find an ecologically adapted AM endophyte.

AM fungi are obligate symbionts and artificial medium for their independent growth has not been identified yet. Plants must be inoculated with inoculum produced on living roots in open pot cultures. Production of large amounts of mycorrhizal inoculum in pots for large-scale nursery applications is not economically and practically feasible. Rhizosphere soils of different tree species colonizing old mine spoils at the mine site, may prove to be valuable sources of inoculum of ecologically adapted strains of AM fungi[2]. In order to use revegetated mine spoil successfully, it is necessary to test its effectiveness as a source of mycorrhizal inoculum on different plant species. Moreover, an understanding of how individual fungi colonize roots, survive and effect P uptake from mixed populations on revegetated mine spoils will also be valuable in assessing the contributions of AM fungi under nursery conditions[11].

The aim of this study was to examine the potential use of revegetated coal mine spoil from under different tree species as a source of endomycorrhizal inoculum for inoculating seedlings of two nitrogen-fixing tree species, *Cassia siamea* Lamk. and *Derris indica* (Lam.) Benett. suitable for restoring degraded tropical areas.

The substrate used for the pot experiment was a mixture of unsterilized coal mine spoil obtained from the freshly stockpiled overburden at Jayant open cast mine site (E 82°36′40″–82°41′15″ and N 24°6′46″–24°11′5″) of Northern Coalfields Ltd, Singrauli, India and autoclaved sandy-loam soil (2 : 1 v/v). Bioassay test using corn (*Zea mays* L.) plants showed that the coal mine spoil had no mycorrhizal propagules. The substrate soil had the following chemical properties: pH, 6.2; OC, 1.33%; EC, 0.27 dsm^{-1}; P, 2.2 $mg\ kg^{-1}$; K, 46.2 $mg\ kg^{-1}$. Five kg of air-dried soil was transferred to each 20 cm wide and 14 cm high clay pots, watered thoroughly and allowed to drain for 1 week. *C. siamea* and *D. indica* seeds were surface-sterilized in 10% solution of sodium hypochlorite for 2 min, soaked in sterile water for 24 h and sown in pots containing sterilized sandy-loam soil.

Rhizosphere soils (20 cm below soil surface) of five tree species, *Dalbergia sissoo* Roxb., *Cassia indica* A. Juss. and *Eucalyptus* hybrid growing on a 5-year-old reclaimed overburden at Jayant coal mine site, were used as sources of mycorrhizal inoculum. Soil samples were kept at room temperature[12] for one month before use. Rhizosphere soils were subjected to wet sieving and decanting[13] and spores were collected and counted on grids drawn on filter paper. Spores were identified to species using current taxonomic guide[14] and original species descriptions. Spore wall characteristics were examined at ×1000 magnification using stains and Melzer's reagent. Spellings of scientific names of mycorrhizal species are those suggested by Almeida[15]. Seven spore-forming AM fungi, *Acaulospora scrobiculata* Trappe, *Glomus geosporum* (Nicol. & Gerd.) Walker, *Glomus aggregatum* Schenck & Smith emend. Koske, *Glomus micraggregatum* Koske, Gemma & Olexia, *Scutellospora calospora* (Nicol. & Gerd.) Walker & Sanders, and undescribed species of *Acaulospora* (AY) and *Gigaspora* (GiB) were present in the rhizosphere of different tree species (Table 1).

Forty grams of soil inoculum, containing resting spores, infected root fragments and mycelia was placed about 6 cm below the soil surface in each mycorrhizal pot. Thirty-day-old uniform seedlings of *C. siamea* and *D. indica* were planted in mycorrhizal and non-mycorrhizal pots at the rate of one seedling per pot. For each host species, there were five treatments and each treatment was replicated three times. Pots were kept in the greenhouse in a randomized block design and watered daily as necessary. The temperature during the experiment ranged from 20 to 35°C and the photoperiod was 13 h.

Seedlings were harvested 180 days after transplanting. Dry weight of shoot and root was determined after drying samples at 70°C for 96 h. Phosphorus content was determined after digesting dried plant parts in tri-acid mixture containing $HNO_3 : H_2SO_4 : HCLO_9$ (10 : 1 : 3) and analysing by molybdo-phosphate method[16]. Roots were examined at ×100–400 magnifications for the presence of infection after clearing with 10% KOH, acidifying in dil.HCl and staining in 0.01% acid fuchsin in lacto-phenol[17]. Darkly pigmented roots were immersed in an alkaline solution of H_2O_2 until bleached[18]. Fifty 1 cm root segments, randomly collected from each plant species, were scored for the presence or absence of infection using slide method for assessing percentage mycorrhizal infection[19]. Spores were identified to species and counted by the method described above. Data were analysed using one-way analysis of variance.

Shoot and root dry weight and tissue P concentration in *C. siamea* plants were significantly increased in mycorrhizal pots inoculated with soils from under *D. sissoo*, *C. siamea*, *D. indica* and *A. indica* relative to uninoculated control. Plants inoculated with *E.* hybrid soils showed no significant difference in shoot and root dry weight and tissue P concentration relative to uninoculated control. Among mycorrhizal treatments, significantly higher shoot and root dry weight and shoot P uptake was observed in pots inoculated with *D. indica* soils. Arbuscular mycorrhizal infection was significantly higher in plants inoculated with the rhizosphere soils of *D. indica* (Table 3). Spores of *S. calospora* were present in pots inoculated with *C. siamea* and *D. indica* soils. Maximum number of spores (27) was observed in the pots inoculated with rhizosphere soils of *D. sissoo*. Lowest percentage of mycorrhizal infection (48) was observed in pots inoculated with *E.* hybrid soils.

Mycorrhizal inoculation with rhizosphere soils of *D. sissoo*, *C. siamea*, *D. indica* and *A. indica* significantly improved shoot and root dry weight and tissue P concentration of *D. indica* over uninoculated control. Shoot and root dry weight and tissue P concentration in plants inoculated with *E.* hybrid soils showed no significant difference relative to control. Among mycorrhizal treatments, significantly higher increase in root dry weight and shoot and root P concentration was observed in pots inoculated with *A. indica* soils. There was no significant difference in per cent mycorrhizal infection in plants inoculated with soil inoculum from under *D. sissoo*, *C. siamea*, *D. indica* and *A. indica* (Table 5). Maximum number of total spores (100) was observed in pots inoculated with the rhizosphere soils of *D. sissoo*. Lowest mycorrhizal infection (67.6%) and total

Table 1. Mean number of spores of AM fungi in the rhizosphere soils of five tree species colonizing coal mine spoil at Jayant

Host species	No. of spores of AMF species/100 g dry soil							
	ASCB[a]	*A.* sp. (AY)	*Gi.* sp. (GiB)	LGSP	CCLS	LMAG	LAGR	Total
D. sissoo	224	156	2	4	–	–	–	386
C. siamea	48	–	–	7	6	12	–	73
D. indica	42	–	–	6	–	–	4	52
A. indica	58	–	–	–	–	–	–	58
E. hybrid	–	–	–	8	–	–	12	20

[a]Mycorrhizal species code as suggested by Perez and Schenck[29]. *A.* sp. (AY) ad *Gi.* sp. (GiB) are undescribed species of *Acaulospora* and *Gigaspora*, respectively.

spore population (6) were observed in pots inoculated with soil inoculum from under *E.* hybrid.

In general, rhizosphere soils of *D. sissoo*, *C. siamea*, *D. indica* and *A. indica* tested as AM inoculants on *C. siamea* and *D. indica* produced more than 40% increase in shoot dry matter production over uninoculated control. There was no relationship between per cent mycorrhizal infection and spore numbers. Spores or sporocarp of mycorrhizal species, *G. aggregatum*, *G. micraggregatum*, *Acaulospora* sp. (AY) and *Gigaspora* sp. (GiB) present in rhizosphere soils of different host species from revegetated mine spoil were not observed in mycorrhizal pots.

The study shows that whilst rhizosphere soils of *D. sissoo*, *C. siamea*, *D. indica* and *A. indica* acted as an effective source of arbuscular mycorrhizal inoculum, soils taken from under *E.* hybrid were not effective in enhancing plant growth. The AM fungal species, *A. scrobiculata*, not only effectively colonized root but also consistently increased P uptake and shoot dry matter production in both *C. siamea* and *D. indica*. Studies on competitive interactions between arbuscular mycorrhizal species have suggested that the fungal species that colonize root first, may be at an advantage over late colonizers[20]. This may account for the widespread occurrence of *A. scrobiculata* in revegetated coal mine spoil, as is evident by its presence in the rhizosphere soils of all the tree species, except in *E.* hybrid. Significantly higher increase in growth and P uptake in *C. siamea* inoculated with *D. indica* soils was an effect of higher root colonization due to the presence of both *A. scrobiculata* and *S. calospora*, as was evident by the presence of spores of the two mycorrhizal species in pots. Previous studies have also revealed that inoculations with mixed inoculum, containing more than one AM endophyte, resulted in higher root colonization and increased P uptake and growth in plants[7,21,22]. Recently, increased growth in plants inoculated with dual inocula of AM fungi has been reported to be due to the increased transfer of P to the shoot from the root[7]. No significant change in shoot biomass and P uptake in plants inoculated with *E.* hybrid soils, relative to uninoculated control, was possible due to the constant balance maintained in carbon or P demand between the source (host) and the sink (fungus). *G. geosporum* associated with plants inoculated with *E.* hybrid soils was not effective in increasing P uptake and shoot biomass.

From this study, it is evident that whilst *C. siamea* support growth and reproduction of both *A. scrobiculata* and *S. calospora*, *D. indica* favours growth of *A. scrobiculata* only. This supports the works showing that host plants can be selective in the growth and reproduction of certain AM fungal species under a particular set of environmental conditions[22,24]. Apparent host specificity may, however, occur if host susceptibility does not coincide with the propagule infectivity[5]. Spores of *S. calospora* were not present in *D. indica* soils used as inoculum, but were formed in mycorrhizal pots of *C. siamea* inoculated with the same soil. This indicates

Table 2. Dry weight and phosphorus concentration of *Cassia siamea* inoculated with soil inoculum from under five tree species

Soil inoculum source	Shoot dry wt (mg/plant)	Root dry wt (mg/plant)	Shoot P conc. (%)	Root P conc. (%)
D. sissoo	1.63^{b}	0.71^{b}	0.21^{b}	0.15^{b}
C. siamea	1.19^{b}	0.51^{bc}	0.15^{b}	0.15^{a}
D. indica	2.23^{a}	1.03^{a}	0.5^{a}	0.14^{a}
A. indica	1.0^{b}	0.3^{c}	0.15^{b}	0.14^{a}
E. hybrid	0.34^{c}	0.08^{d}	0.07^{c}	0.06^{b}
Control	0.59^{c}	0.18^{d}	0.04^{c}	0.09^{b}

In each column, the mean values superscribed with the same letter do not differ significantly ($P = 0.05$).

Table 3. Comparison of percentage infection and spore numbers in *C. siamea* inoculated with soil inoculum from under five tree species

Soil inoculum source	Mycorrhizal infection (%)	Spore number of AMF species/100 g dry soil			
		ASCB	LGSP	CCLS	Total
D. sissoo	75^{b}	27	–	–	27^{a}
C. siamea	76^{b}	8	–	3	11^{c}
D. indica	85^{a}	11	–	9	20^{b}
A. indica	58^{c}	21	–	–	21^{b}
E. hybrid	48^{d}	–	3	–	3^{d}

In each column, the mean values superscribed with the same letter do not differ significantly ($P = 0.05$); – = absent.

Table 4. Dry weight and phosphorus concentration of *Derris indica* inoculated with soil inoculum from under five tree species

Soil inoculum source	Shoot dry wt (mg/plant)	Root dry wt (mg/plant)	Shoot P conc. (%)	Root P conc. (%)
D. sissoo	2.53[a]	2.06[b]	0.11[b]	0.09[b]
C. siamea	2.36[a]	2.03[b]	0.10[b]	0.10[b]
D. indica	2.71[a]	2.15[b]	0.10[b]	0.10[b]
A. indica	2.86[a]	3.10[a]	0.16[a]	0.15[a]
E. hybrid	1.42[b]	1.53[c]	0.07[c]	0.07[c]
Control	1.38[b]	1.05[c]	0.08[c]	0.06[c]

In each column, the mean values superscribed with the same letter do not differ significantly ($P = 0.05$).

Table 5. Comparison of percentage infection and spore in *D. indica* introduced with soil inoculum from under five tree species

Soil inoculum source	Mycorrhizal infection (%)	Spore number of AMF species/100 g dry soil			
		ASCB	LGSP	CCLS	Total
D. sissoo	83.3[a]	100	–	–	100[a]
C. siamea	88.9[a]	35	–	–	35[b]
D. indica	84.6[a]	36	–	–	36[b]
A. indica	79.6[a]	16	–	–	16[c]
E. hybrid	67.6[b]	–	6	–	6[d]

In each column, the mean values superscribed with the same letter do not differ significantly ($P = 0.05$); – = absent.

that *S. calospora* also persisted in the form of propagules other than resting spores. Previous studies have shown that the network of intra- and extra-radical hyphae or intra-radical spores act as important sources of AM propagules in dry soils[12,20,25]. The variable amount of mycorrhizal spores present in the soils used as inoculum source had little effect on the percentage infection of roots at the final harvest. Gazey *et al.*[26] also found that the amount of *A. laevis* inoculum added had little effect on the proportion of roots colonized, once maximum percentage of root length colonization was reached. Therefore, it appears that the increasing number of infections with increasing inoculum density usually results from an increasing probability of infection by individual propagules of mycorrhizal species, rather than from increased energy. Maximum number of spores of *A. scrobiculata* were consistently produced in pots inoculated with rhizosphere soils of *D. sissoo*, which incidentally had the highest amount of spore inoculum. This supports the work of Gazey *et al.*[26] who also found that the number of spores produced in *A. laevis* differs with the inoculum quantity. Since the root system of *C. siamea* is relatively coarse with fewer root hairs than *D. indica*, it is plausible that a comparatively lower population of *A. scrobiculata* in *C. siamea* pots relative to *D. indica* pots was due to the differences in the root morphology[27].

Unsterilized coal mine soil was used as a substrate in the present investigation, as it provided 'natural conditions' for the growth of ecologically adapted strains of AM fungi. Evidence of ecological adaptation in mycorrhizal fungi has been provided by several workers[3,28]. The results of this study justify the use of revegetated coal mine spoil as an effective and economical source of endomycorrhizal inoculum from within the production system, but suggest the need to evaluate the specific effects of rhizosphere soils from revegetated overburden as sources of AM inoculum on individual host species prior to their utilization in large-scale nursery inoculations.

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ACKNOWLEDGEMENTS. Financial assistance by the Department of Science and Technology, New Delhi is gratefully acknowledged. I also thank the staff at the Northern Coalfields Ltd., Singrauli for their kind help in sample collection.

Received 8 November 1995; revised accepted 23 February 1996

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