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Development and evaluation of mycorrhiza for rhizosphere bioremediation

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ABSTRACT
Objectives: Development and evaluation of mycorrhiza at the laboratory scale for further use in rhizosphere bioremediation.
Methodology and results: Vesicular arbuscular mycorrhiza (VAM) inoculum was developed at the laboratory scale in designed perforated trays for a period of 2.5 months to prepare mycorrhizal soil for further use in rhizosphere bioremediation of organic contaminants. The physico-chemical parameters (pH, EC, moisture content, %OC, %N, %P and K) of the soil were determined during the development of mycorrhizal inoculum. Bacterial and fungal counts were assessed during the third, sixth and ninth week of the process along with the microbial diversity present in the developed inoculum. The mycorrhizae were characterized during inoculum preparation and in the final product measuring the percentage root colonization and spore density. The developed mycorrhizal inoculum was found to contain high percentage of organic carbon and nitrogen while the bacterial and fungal count increased.
Conclusion and application of findings: Mycorrhiza inoculum developed in the present study was found to have symbiotic association of bacteria and fungi in the rhizosphere; therefore it would significantly contribute in establishing an effective mycorrhizosphere that can provide the environment for the survival and growth of microorganisms as well as increase in organic carbon which further helps in enhancement of degradation of organic contaminants in the soil during rhizosphere bioremediation.

Key words: Soil based inoculum, sorghum, mycorrhizosphere, phytoremediation, VAM.

INTRODUCTION
Mycorrhiza is mutualistic association between plant roots and fungus. The most common mycorrhizal association is the vesicular- arbuscular type, which produces fungal structures like vesicles and arbuscules in the cortex region of the roots. Vesicular arbuscular mycorrhiza (VAM) lives in symbiosis with about 80% of land plant species. VAM belong to the order Glomales (Zygomycetes) that is further divided into families and genera on the basis of its distinctive spore formation (Quilambo, 2003). VAM associations with plants are important in natural and managed ecosystems due to their nutritional and non-nutritional benefits to their symbiotic partners. VAM induces changes in the host root exudation pattern, which alters the microbial equilibrium in the mycorrhizosphere by influencing host root colonization. It's known to improve plant growth and health by improving

mineral nutrition and increasing resistance or tolerance to biotic and abiotic stresses. They also have an ability to alter plant productivity as VAM can act as biofertilizers, bioprotectants or biodegraders (Khan, 2006).

Highly polluted sites are usually devoid of beneficial soil microorganisms that plants naturally rely on. Naturally occurring allies like VAM could be used to enhance plant survival on difficult sites (Turnau *et al*., 2002). Phytoremediation is an emerging technique that uses plants to clean up by establishment of vegetation in soils contaminated with hazardous organic and inorganic compounds. It is an aesthetically pleasing mechanism that can reduce remedial costs, restore habitats, and clean up contamination in situ rather than transporting the problem to another site (Zynda, 2001). The potential role of VAM in phytoremediation of soils and water contaminated with heavy metal has been reported (Gaur & Adholeya, 2004).

The mechanisms through which plants remove metals from soil are either by phytoextraction, phytostabilization or phytovolatillization; whereas remediation of organics such as PAHs, pesticides and radio nucleotides is achieved by rhizosphere bioremediation or phytodegradation. Rhizosphere bioremediation has been found to increase soil organic carbon, bacteria, and mycorrhizal fungi, all factors that encourage degradation of organics in soil (Schnoor, 1997). Mycorrhizosphere is generally used to describe a sphere of influence encompassing plant roots, and includes the root symbiotic mycorrhizal fungi and soil in the immediate vicinity of the root. Different compartments of the mycorrhizosphere are believed to be specialized despite their continuity. The mycorrhzospheric microbial community (including non – symbiotic bacteria and fungi) may act in concert with mycorrhizal fungi to degrade organic contaminants (Ghosh *et al*., 2005). Many persistent organic chemicals like atrazine, hexachlorocyclohexane (HCH), aromatic hydrocarbons (BTEX) and polycyclic aromatic hydrocarbons (PAHs) are known to be degraded in the mycorrhizosphere directly or indirectly (Joner & Leyval, 2001; Volante *et al*., 2005; Huang *et al*., 2006; Sainz *et al*., 2006).

Vesicular arbuscular mycorrhiza (VAM) has been reported to modify the quality and abundance of rhizosphere microflora and alter overall rhizosphere microbial activity, which affects bioremediation in the contaminated soil (Khan, 2006). Mycorrhiza not only provide the plants with water and mineral compounds and help to improve the structure of soil, but have also been shown to act as filters, blocking toxic compounds within their mycelium resulting into reduced toxicity to the plants. Moreover, they influence the physiology of their host plants making them less vulnerable to pathogens, soil pollution, salinity, drought and a number of other environmental stress factors. Mycorrhiza fungal strains have shown improved tolerance to toxic compounds, and hence could increase the success of phytoremediation (Turnau *et al*., 2002).

The objective of this study was to develop mycorrhizal inoculum at the laboratory scale which can be applied to the soil for better survival and growth of plants in contaminated soils during the process of rhizosphere bioremediation of hazardous organic compounds.

MATERIALS AND METHODS

Soil collection and characterization: Soil was collected from a depth of about 0 - 15 cm along the banks of Surya River, Palghar (located 100 km from Mumbai). The soil used in this study is referred to as alluvial soil. Stones and plant tissues were carefully removed from the soil prior to drying under laboratory conditions. The soil was screened through 2 mm stainless steel sieve, and stored in a plastic bag at room temperature until use. The soil collected was characterized for its physicochemical parameters (Table 1). After 20 min of vigorously mixing the samples at 1: 2.5 in deionized water, the pH and electrical conductivity (EC) were measured using digital meters [Deluxe water and soil analysis kit, Model 191E] with a combination pH electrode (TL42) and a 1 cm platinum conductivity cell (Type CD10 electrode) (APHA, 1998). Total nitrogen, phosphorus and potassium were determined according to the standard methods of the

American Public Health Association (1998). Cation exchange capacity was determined after extraction with ammonium acetate at pH 7.0 and the organic carbon was determined by using Walkley–Black method (Jackson, 1973).

Mycorrhizal inoculum preparation: Soil based mycorrhizal inoculum was developed in the laboratory with the help of starter inoculum and using sorghum, as a host plant. The soil collected and characterized was used for the preparation of the mycorrhizal inoculum. A starter culture of mycorrhizal fungi (VAM) was obtained from the Division of Microbiology, IARI, New Delhi. Seeds of sorghum were obtained from the Ratanshi Agro-Hortitech (Byculla, Mumbai). The seeds were surface-sterilized by immersing in 3% (v/v) of formaldehyde for 5 minutes and washed with distilled water several times before use (Peralta *et al*., 2001).

Plastic trays of 65' X 45' X 45' dimensions, perforated at the base were used for better drainage of water and aeration during the experiment, which was conducted for about two and a half months. A mixture of 3:1 soil – sand was used for growing plants and the perforated trays were kept in five replicates arranged side by side. The tray was first layered with 2-3 inches of coarse sand to avoid water logging. About 500 gram of starter culture of mycorrhiza was thoroughly mixed with the 5 kg of potting mixture, which was then put on the coarse sand layer. Fifty sorghum seeds were sown on the layer in each tray. Sorghum seeds germinated within three to four days and were maintained to grow further for 2 ½ month. The plants were watered every day and 25 ml Hoagland solution without P content was provided in the 3rd, 6th and 9th week. The trays with plants were placed in a greenhouse with 12/12 hrs light/dark cycle at 27 – 28 ^{0}C during the day and 24-26 ^{0}C at night. The average relative humidity was 65-69%. Care was taken during watering to prevent cross contamination of the trays with plants by crawling insects or splashing water. After two and half month's incubation, water was withheld and roots were allowed to dry and decompose for at least two weeks. The plant shoots were removed and discarded. The medium and roots from the pots were taken onto a clean tray. Dried roots were chopped and mixed in the same soil. This "soil based inoculum" was packed in polythene bags and stored in a cool dry place.

Mycorrhizal inoculum characterization: Composite soil samples were taken from the five replicates for analysis and the roots of sorghum grass were also sampled. The physico-chemical parameters of soil during inoculum preparation and of the developed inoculum (as an end product) were characterized as described above. The total viable bacterial and fungal counts along with the dominant microorganisms present in the soil were also assessed as described by Kumar (2004). The mycorrhizal inoculum was characterized for its root colonization and spore counts. The collected root samples from the rhizosphere of sorghum grass were freed from adhering soil, gently washed and then cut into 1 cm segments. The percentage colonization was determined by trypan blue method (Phillips & Hayman, 1970). The spore counts were taken by the wet-sieving and decanting method (Gerdeman & Nichalson, 1963). The distinct morphological types of mycorrhizal spores extracted from the root zone soil were observed under the stereomicroscope to isolate them individually. The spores were then mounted on a glass slide with a drop of lactoglycerol as mounting fluid, covered with a cover slip and observed under the microscope at 10X and 45X magnifications.

RESULTS AND DISCUSSION

The endomycorrhizal inocula, especially VAM, can be produced by several methods. These include spore inoculum, infected root inoculum, soil based inoculum, peat based inoculum or carrier material (Bagyaraj *et al*., 2002). In the present research work, mycorrhizal soil based inoculum was prepared in designed perforated trays for a period of two and a half months (10 weeks) for rhizosphere bioremediation. Sorghum was used as the host plant and made to grow in the perforated trays for improved drainage of excess water and aeration for development of mycorrhizal inoculum in bulk.

The physico-chemical and mycorrhizal characterization of soil was performed. The pH of the soil remained in the rage of 7.2 to 7.6 while the electrical conductivity (EC) was found to increase over the period of the experiment (table 2). Mycorrhizal colonization is low in anaerobic soil and it is encouraged under low irrigated but well aerated soil; therefore the moisture content of the soil was maintained at 38 to 52. The collected soil was observed to be low in organic carbon content (0.84%), which however increased over the duration of the experiment and ranged from 0.96 to 1.80%. Nitrogen content in the soil fell between 0.17 to 0.42% while phosphorus content did not vary significantly, and was on average 0.03%. Increase in carbon and nitrogen levels as

observed in the third, sixth and ninth week may be contributing to the better growth and health of the plants.

Table1: Physico-chemical characteristics of the soil samples obtained from the site, during the inoculum development (in ranges) and of the developed mycorrhizal inoculum.

Soil parameters	Soil samples collected from site[a]	During inoculum[b] development	Mycorrhizal inoculum[c]
pH	7.6	7.2-7.6	7.3
Electric conductivity (mMohs)	0.18	0.34-0.52	0.46
Moisture content (%)	28	38-52	38.4
Organic carbon (%)	0.84	0.96-1.80	1.38
Total Nitrogen (%)	0.14	0.17-0.42	0.24
Phosphorus (%)	0.058	0.01-0.04	0.03
Potassium (mg/kg)	22	16-27	23
VAM colonization (%)	-	15-69	67
Spore count (per 100gm soil)	-	33-575	322

a - Values are average of the three replicate samples; b - Values are given in ranges are average of the five replicate samples. c - Values are average of five replicate samples.

Table 2: Physico- chemical parameters of soil during mycorrhizal development**.**

Week	pH	EC (mMohs)	OC (%)	Total N (%)	P (%)	K (mg/kg)	Moisture %
1	7.2	0.34	0.96	0.21	0.03	22	52.7
2	7.5	0.36	1.32	0.23	0.02	23	48.4
3	7.6	0.38	1.20	0.17	0.03	23	39.4
4	7.5	0.40	1.80	0.26	0.04	27	47.7
5	7.3	0.38	1.50	0.36	0.01	21	51.3
6	7.3	0.46	1.02	0.29	0.02	19	45.1
7	7.5	0.46	1.38	0.42	0.03	16	52.4
8	7.5	0.47	1.46	0.36	0.02	24	38.3
9	7.4	0.50	1.69	0.19	0.02	18	49.5
10	7.4	0.52	1.52	0.29	0.03	22	50.9

***Values are average of the five replicate samples obtained during the development of inoculum

Mycorrhizal characterization was done by evaluating the percentage VAM colonization and spore count every week during the 10 week period of plant growth. The results of VAM characterization are illustrated in figure 1. The roots of the host plants became colonized by the 15th day. The percentage root colonization by VAM escalated from 15 to 59% from the 3rd to the 7th week. The colonization was distinctly high in the 7th week and further increased from 62 to 69% by the end of the experiment. Upon observation of slides, vesicles and hyphae were found to be highly stained (fig 2a and b). Intercellular hyphae forming intercellular vesicles were seen in both cortical and middle hypodermal region. Mycorrhizal spores of different sizes were also scrutinized (fig 2 c and d). Spores are the most important phase of the life cycle of VAM; apart from being a vegetative propagule, its morphological characteristics aid identification and categorization of VAM into separate genera (Quilambo, 2003). In this study, the spore count increased with time (figure 1). Among the recovered spores, Glomus was observed to be the predominant genera in the produced inoculum which may be credited to the soil's indigenous and introduced inoculums' (starting inoculum) mycorrhizal flora.

Mycorrhiza has been known to interact and modify the microbial communities and their abundance in the soil (Smits, 2005). Therefore in the current study

biological parameters of the soil along with physico-chemical parameters were also estimated. The microbial population of bacteria and fungi were assessed at three intervals (Table 3). The bacterial count marginally increased along the period but fungal count varied from 1.6 X 10^4 to 1.8 X 10^4 to 1.4 X 10^4 when sampled in the 3rd, 6th and 9th week, respectively. VAM, being symbiotic fungi, forms various associations with the existing microflora in the rhizosphere of the roots it colonizes. Hence, the microbial diversity of the developing mycorrhizosphere was also explored. The bacterial species present in the developed inoculum included *Pseudomonas, Bacillus, Rhizobium., Azobactor,* and *Arthrobactor* species; whereas *Azospirullum, Penicillum., Asperigillus* and *Rhizopus* species comprised the main fungal species present.

Table 3: Microbial count during development of mycorrhizal soil.

Microbial Count	**Sampling time**		
	3rd Week	6th week	10th Week
Viable Bacterial Count	$3.9X10^6$	$5.1X10^6$	$6.6X10^6$
Fungal count	$1.6X10^4$	$1.8X10^4$	$1.4X10^4$

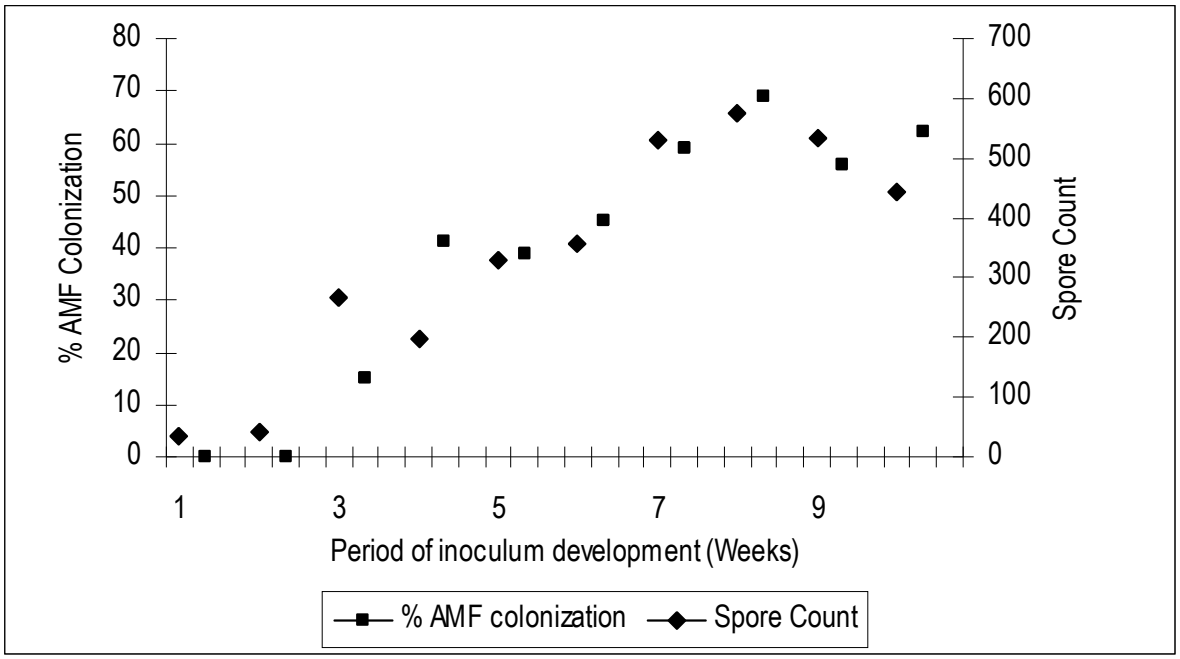

Figure 1: Mycorrhizal Characterization of soil during mycorrhizal development.

In previous studies, several beneficial bacteria, e.g. *Pseudomonas, Arthrobactor, Acaligens, Corynebacterium, Flavobacterium, Achromobacter, Micrococcus* and *Mycobacterium* were reported to be associated with the roots of plants in their mycorrhizosphere (Ghosh *et al*., 2005). The physico-chemical and mycorrhizal characteristics of the final product developed were also determined (table 1). The colonization of VAM on the sorghum plant roots was found to be as high as 67%. The spore count were determined to be 322 per 100 gm of soil when the final inoculum was prepared, air dried to the lower moisture content (38.4%) to be packed in polythene bags for storage.

In the process of phytoremediation, plants help establish association with mycorrhizal fungi to metabolize the organic pollutants in the rhizosphere. Moreover this mechanism is also supported by secreting plant exudates, e.g. short chain organic acids, phenolics, and small concentrations of high molecular weight compounds (enzymes and proteins) to stimulate bacterial transformations (enzyme induction); by building up of organic carbon to increase microbial mineralization rates (substrate enhancement) or by

providing habitat for increased microbial populations and activity.

Researchers have examined five plant enzyme systems in sediments and soils. These are dehalogenase enzymes that are important in dechlorination reactions of chlorinated hydrocarbons; nitroreductase which is needed in the first step for degradation of nitroaromatics, laccase which serves to break aromatic ring structures in organic contaminants; whereas peroxidase and nitrilase are important in oxidation reactions. This release of exudates and enzymes that stimulate microbial activity, biochemical transformations and enhancement of mineralization in the rhizosphere (the root-soil interface) has been attributed to mycorrhizal fungi and the microbial consortia (Schnoor, 1997).

Rhizosphere bioremediation capitalizes on the innate qualities of the vegetation and the potential for mineralization of organic pollutants in the rhizosphere. Therefore, researchers have been focusing on rhizosphere as a zone of enhanced biodegradation and on the plant-microbe interactions that occur there (Olson *et al*., 2003). Research work done by many scientists has either used spores of specific mycorrhiza for inoculation ranging from 200 to 500 spores or mycorrhizal inoculum which also varied from 5-10% of the soil (Joner & Leyval, 2001; Joner & Leyval, 2003; Liu *et al*., 2004; Huang *et al*., 2006).

The mycorrhizal inoculum developed in the present research work was further effectively used by the authors in green house experiments conducted using ryegrass for the rhizosphere bioremediation of anthracene (Korade & Fulekar, 2008).Thus mycorrhiza inoculum developed in the current study was found to have symbiotic association of bacteria and fungi along the root zone in the rhizosphere; thereby contributing significantly to the establishment of effective mycorrhizosphere that can provide the environment for the survival and growth of microorganisms as well as increase in organic carbon which further helps to enhance degradation of organic contaminants in the soil.

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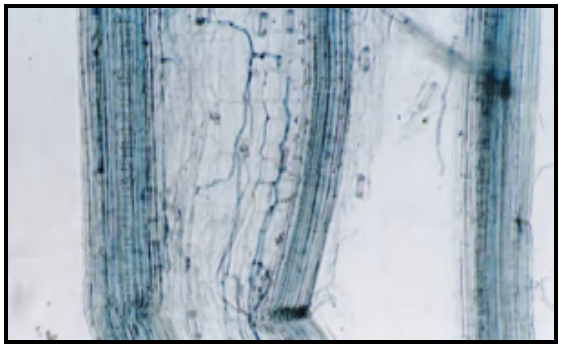
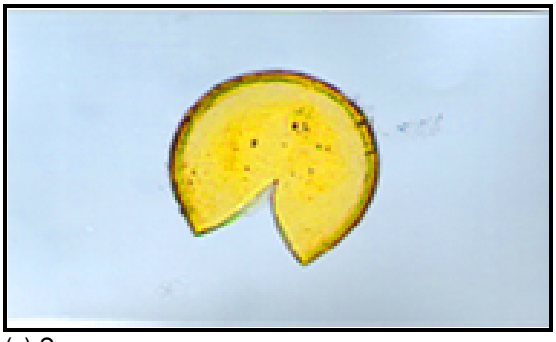
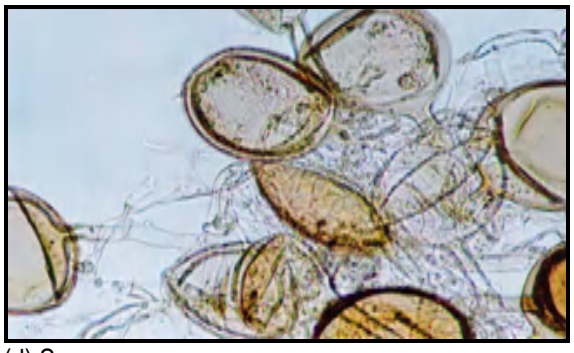
(a) Vesicles and hyphae



(b) Hyphae (Root colonization)



(c) Spore



(d) Spores

Figure 2: Various structures in the Life cycle of Mycorrhiza obtained during the development of the mycorrhizal inoculum in the laboratory.

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