REVIEW ARTICLE

Isolation of AM Fungi from Rhizospheric Soil and Assessing Their Potential in Revegetating the Mining Wasteland of Gujarat using Inoculated Trees Saplings

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Abstract

Fluorspar (CaF_2), is a common mineral, occurring in green, purple, yellow, or colorless crystals, which is used in metallurgy and making a variety of chemicals used in refrigeration. Kadipani in Gujarat has large deposits of this ore. Open cast mining by GMDC has resulted in disturbed ecology. Earlier the efforts have been made by the forest department to grow tree species, in order to restore the forest ecosystem, but the plants grew slowly and with stunted morphology and scanty survival. The obligate fungal symbionts known as arbuscular mycorrhizal fungi (AMF) present in plants play a key factor supporting plant growth in stressed conditions. Therefore, AMF strains were first isolated from mining area and were used to raise the saplings first in polybags. Then after 75 days these were used for revegetating the mine burdens or nearby waste land. The paper presents a pioneering efforts made to isolate and identify, the AMF from Fluorspar mines in Gujarat and then *Glomus fasciculatum* was incorporated in polybags filled with normal garden soil. Plants were also raised by single spore inoculation method. Preliminary experiments revealed that the inoculated AMF strains promoted seedling growth of *Leuceana leucocephala* and *Millettia pinnata*. The inoculation of AMF spores enhanced the growth of both the plants, and such application can be used in sustainable restoration of the mining areas. Review discusses the structure of AM spores and mechanisms involved to equip them to survive in stressed situations and as symbionts help the plants to perform better.

Keywords: Fluorspar, Mining waste land, Arbuscular Mycorrhizae, *Glomus fasciculatum*, Revegetation.

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Introduction

It is found that plants first started establishing across terrestrial environments about 430 million years ago, these were accompanied by a symbiotic fungi, now known as arbuscular mycorrhizal fungi (AMF) (Wilkinson, 1998). The diversity of AMF present under ground shows a major factor contributing to the sustenance of plant wealth, diversity as well as ecosystem functioning (van der Heijden *et al*., 1998). Mining leads to depletion of forest cover which are main reservoir of terrestrial carbon. Forests play a vital role in global carbon budget (Nandy and Kushwaha, 221). Declaration on Forests put forward a global commitment of restoring 350 million hectares of deforested and degraded lands until 2030 (Jacobs *et al*., 2015). Sustainable Development Goals - Target 15 deals with ecological aspects (UN, 2015). Although restoration projects achieved limited success or failed misrebly (Thomas *et al*., 2015), further efforts are needed in this direction (Asmelash *et al*., 2016).

This group of fungi (*Endomycorrhizae*, AMF), from the fungal phylum, Glomeromycota- is ubiqutus, which can be traced in 95% of all vascular plant families, excluding Brassicaceae, Cactacea, and families of aquatic plants.Although known for a long time, major studies were conducted by Nicolson (1959), Mosse (1962), Gerdemann and Nicolson (1963), Gerdemann (1968), Phillips and Hayman (1970), Schenck and Perez (1990) in last few decades have helped us to understand these eukaryotes better. AMF as natural biofertilizers, are capable of providing the host with nutrients, soil moisture and safe protection from numerous pathogens, in exchange for photosynthetic products (Bagyaraj and Balakrishna 2012). A significant change in total chlorophyll including a, b content (Arya and Buch, 2013). The direct re-introduced AMF can help in bioremediation and increased soil fertility. In order to reduce the use of chemical fertilizers by incentivizing states, the Union government of India has introduced a new scheme called- PM Promotion of Alternate Nutrients for Agriculture Management (PM PRANAM) Yojana, which intends to reduce the subsidy burden on chemical fertilizers, of ₹2.25 lakh crore in 2022-2023. These fungal entities can rebuild the soil of the disturbed area. Earlier, Sustainable Development Model on natural forests and coal mines was found to be feasible and created a synergy with 30% of forest area exploited for coal mining and pay compensation to the employers, governments and society (Djuwita, 2013).

Heavy metals reach to the soil through activities of mining, foundries, smelters, combustion and pesticides (Nagajyoti *et al*., 2010). Copper is still used in vineyards to prevention against fungal attack (Ruyters *et al.,* 2013). Mining cause destruction of soil substrata and negatively impacts by changing the chemistry of soil. Some metals are beneficial in plant growth but lethal in higher concentration (Singh *et al*., 2015). Plant genome encode

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a number of transporters specific with a particular metal component, their expression, and in cellular localization to manage these in whole plant system (Colangelo and Guerinot 2006; Hwang *et al*., 2016). Topsoil removal and accumulation prior to mining destroys active mycelial network of AMF, breaks symbiosis, reduces soil inoculum potential, and AMF species composition (Gould and Liberta, 1981; Waaland and Allen, 1987). Schramm (1966) noted the rarity of endomycorrhizae-forming plant species on naturally recolonized black mine wastes in Pennsylvania. Other scientists have described the endomycorrhizal flora of various spoils supporting vegetation (Draft and Hacskaylo,1976; Khan 1978). The symbiotic function is critical for supplying plants with phosphrus and minerals, in exchange of organic energy substrates (Barrow *et al*., 1977; Cavagnaro *et al*., 2005). The more effective strains of AMF may benefit re-establishment of native vegetation in disturbed habitats, as long as host-specificity is not a barrier (Thorne *et al*., 2013). Non metallic and shimmery, Fluorspar is extensively used as a raw material for manufacturing hydrofluoric acid, refrigerant gases and flux in metallurgical industries. Gujarat Mineral Development Corporation's (GMDC) Fluorspar open cast mine is located at Kadipani, Gujarat. The review describes the potential of AM fungus *Glomus* on growth performance of two tree spp. *Leuceana leucocephala* (Lam.) de Wit. and *Millettia pinnata* (L.) Panigrahi, in mining area.

Association of Mycorrhiza with Roots

The fungal spores initiate their cycle in plants by entering through mycorrhizal hyphe into roots. The release of a plant hormone strigolactone helping AM Fungal spore to germinate is depicted in Fig.1. The availability of low-phosphorous, and secreted strigolactone ensures the development of hyphae (Fig. 2b). The fungi penetrate the plant roots form network, and "arbuscules" which are highly branched structures for nutrient exchange with the plant (Fig. 2a). A higher tolerance and immobilization of harmful chemicals including the heavy metals in plants by endomycorrhizeis investigated (Chanmugathas and Bollag, 1988; Dehn and Schuepp, 1989).

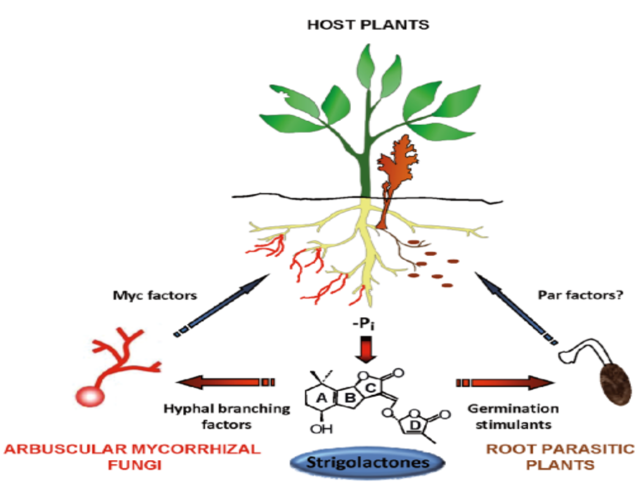

Fig. 1: Production of Strigolactones useful for Symbiotic association of Plants (Source: Lopez-Raez *et al*., 2011).

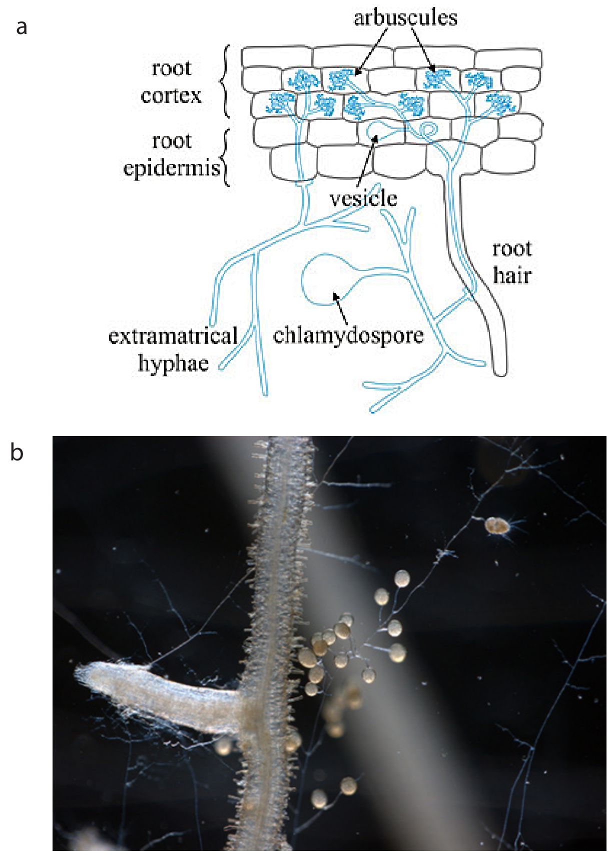

Fig. 2: (a) Figure and caption from 21st Century Guide book to Fungi. Cambridge University Press. (b) Microphotograph showing several AM spores attached to the hyphae on the exterior of a plant root. (Source: Moore *et al*., 2011),

Smart Strategies

Different fungi are pursuing their own agendas and appear to be very much in favor of conciliation and equitable distribution of information and resources (Perry 1998). Organic matter stimulatesthe microbial activity (Alexander, 1977), better establishment of plants was found on lead-zinc chat tailingswith its incorporation (Norland, 1991).

The symbiotic relationship by inoculating fresh fungal and bacterial strains with plant saplings can assist in restoring areas affected by mining or desertification as the symbionts provide better uptake of water, nutrients particularly P by its extensive mycelial network. Availability of P promotes root growth. These also provide the plants capability of drought resistance and transplantation shock. In maize roots infected by *Glomus intraradices*, the expression of two functional aquaporin genes, GintAQPF1 and GintAQPF1, was significantly enhanced in arbuscule-enriched cortical cells and extraradical mycelia under drought stress (Li *et al*., 2013), suggesting that the aquaporin genes from the AMF are involved in water transport via mycorrhizal hyphae to the plant parts (Wu *et al*., 2013).

The first report of AMF enhancing production of caftaric and feruloyl tartaric acids was made in basil by Scagel and Lee (2012). Biological control of diseases by AMF is another plus point. It was observed thatpresence of *G. fasciculatum* reduced the infection in cowpea caused by *Fusarium oxysporum* (Devi

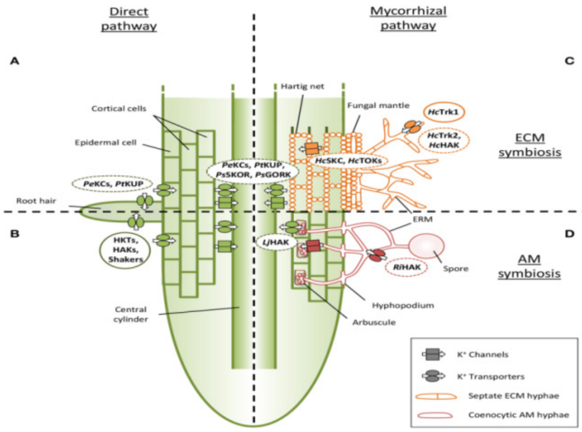

Fig. 3: Diagrammatic sketch of root showing the direct and mycorrhizal pathway of transfer of nutrients. Note the Hartig net formation in Ecto and the presence of arbuscules and vesicles in the cortex by AMF. (Source:Garcia and Zimmermann, 2014).

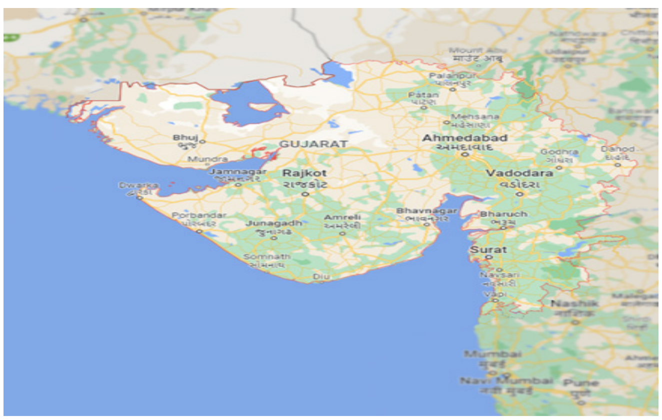

Fig. 4: Location of Kadipani mines in Gujarat, shown with an arrow.

and Goswami, 1992) and *Gigaspora margarita* controlled severity in pigeon pea *caused by F. udum* (Siddiqui and Mahmood, 1995). Fig. 3 clearly depicts unlike absorption from root hairs in normal case presence of cortical network of hyphae in ecto and endomycorrhizae help in transfer of nutrients. The new arbuscules are produced time to time where transfer of nutrients from fungal hyphae is facilitated. Through passive mechanisms sequestration of trace elements occurs in fungal cell wall and vacuoles.

AM Fungi as Metal Hyperaccumulator

In nature, some plants hyperaccumulate heavy metals, for example, *Viola calaminaria* may contain over 1% dry weight of zinc and *Alyssum bertolinii* nickel. These species are respectively called calamine and serpentine flora. Amino acids like cysteine, histidine glutamic acids, and glycine form heavy metal complexes (Homer *et al.,* 1997). The transportation occurs through xylem. Hyper-accumulator plants increase the availability of metals by releasing chelating phytosiderophores (Lombi *et al.,* 2011). AMF especially *Glomus intraradices,* colonized *Festuca* and *Agropyron* species have shown higher heavy metal content (Giasson *et al.,* 2006). They synthesize cysteine-rich metal binding proteins called metallothioneins (Kotrba *et al.,*

a

b

Fig. 5 Pictures of Flourspar mining areas (Source: Chokshi and Arya, 2012). (a) Figure showing the culver point of the mining area (b) The debris left in the mining area. Total number of spores found in different places of Kadipani mine is shown in Table 1. These sites were 1000 m apart from cental point of start.

1999). The mycorrhizal colonization can have an impact on heavy metal assimilation by plants (Bradley *et al.*, 1981), in lettuce roots (Jamal *et al.* 2002), grasses (Brejda*et al.* 1993) Species of *Glomus* and *Gigaspora* are the two genera found associated with metal rich soils (Chaudhry *et al.*, 1998). Detoxification of plants is caused due to 1.) Uptake of harmful chemical, heavy metals etc. by fungal structures, 2.) Selective function of plasma membrane, 3.) Activation of transporters and openings present in plasma membrane helping in translocation from one side to other. 4.) Chelation in cytosol including use of metallothioneins 5.) Active transport by different pathway,6.) Transportation through hyphae, 7.) Sequestration in plant vacuoles. (Cabra *et al.*, 2015).

Occurrence of AM Fungi in Disturbed Soil

When soil moisture is adequate and growth hormones present, the spore germination can commence. Arya *et al.* (2013) conducted a survey of degraded forest areas having grasses in Godhra and Baria divisions, of Vadodara. Rhizospheric soil samples collected from 22 different places reveiled that 220/100 g spores were present in *Heteropogon contortus* as compared to that of *Themeda triandra* 165/100 g. In the Kalitalai soil of grass *Chloris barbata*, number of spores were 150 than in Rampara (110). The morphological characters of isolated fungal spores were used to visualize the specific identifying features of these fungi. *Manual for Identification* by Schenck and Perez (1990) was used for confirmation of AM fungi. Identification of AM

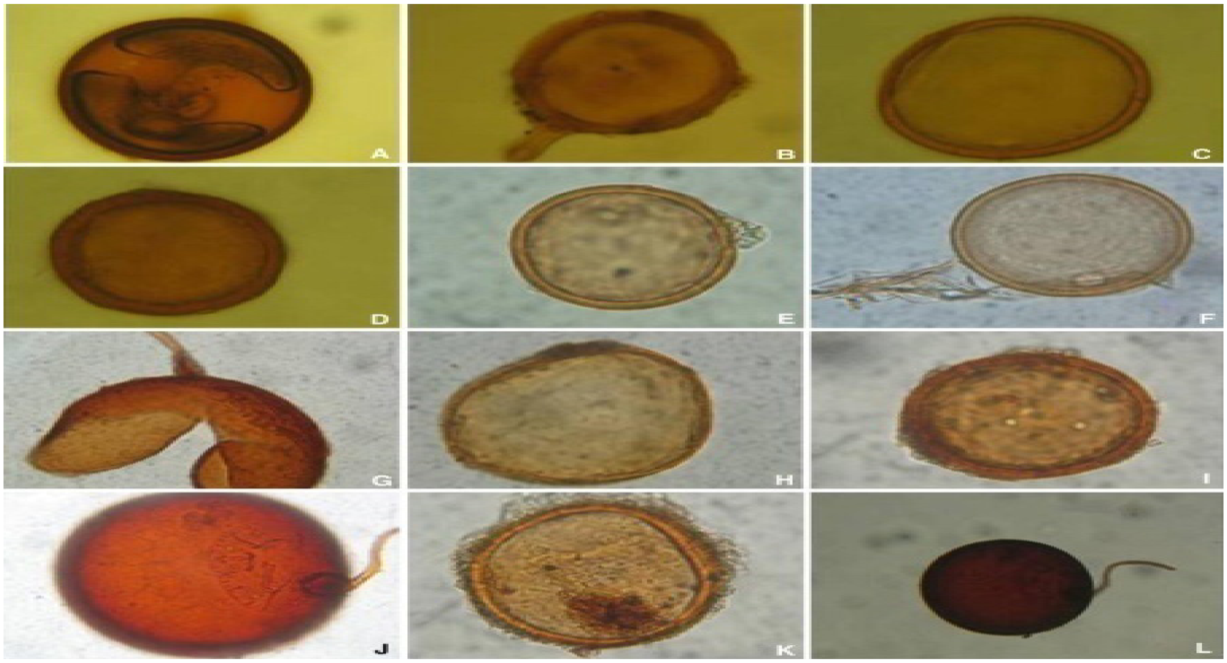

Fig. 6 A *Glomus melanosporum*, **B** *G. mosseae*, **C** *G. glomerulatum*, **D** *G. claroides*, **E** *G. citricola*, **F** *G. macrocarp*, **G** *Gigaspora candida*, **H** *G. etunicatum*, **I** *G. geosporum*, (**Source:** Arya *et al.,* 2013).

Table1: Number of AM spores in non-rhizosphericsoil and characteristics of mining and dumping soils (Source: Choksi and Arya, 2012)

Sample	*No/100g soil*	*pH*	*EC*	*Moisture*	*Color*	*Ca g/kg*	*F g/kg*
Dumping site	70	7.8	01	0.6	India spice	--	--
Mining site 1	55	7.9	01	1.0	Bison	0.241	BD
Mining site 2	13	7.2	03	1.8	Blondine wood	0.16	BD
Mining site 3	15	8.1	01	0.7	Dog wood pig	0.12	0.016
Mining site 4	8.0	8.0	01	0.9	New coca natal	0.16	0.033

Table 2: Characteristics of mining soils: Elements reported (Source: Choksi and Arya, 2012)

%Content	*In Fluorspar Mined Soil*
CaF_2	17–18%
$BaSO_4$	0.5–0.6%
SiO_2	44–46%
Al_2O_3	7–8%
MgO	0.4–0.5%
Fe_2O_3	15–17%

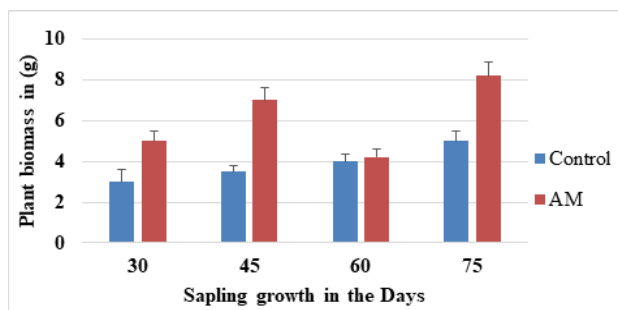

Fig. 7: Plant biomass (in g) of *Millettia pinnata* under the control and Arbuscular Mycorrhiza (AM) treatment (Based on data of Choksi and Arya, 2012).

spores resulted in prevalence of different species belonging to three genera *i.e. Glomus, Gigaspora*, and *Acaulospora* (Arya *et al*., 2013). Arya *et al*., (2010) studied their role in control of soil borne pathogens. Helping other plants by root association was reported from Germany (Wholleben, 2015).

Mycorrhizal Studies on Kadi Pani Flourspar Mines

GMDC's Fluorspar mines (Fig. 4, 5) are located at Kadipani, Ambadungar. Post - Kadipani, in Chhota Udepur, Gujarat, North Lat: 21.96399' East Long: 74.02604. Physicochemical studies conducted by Choksi and Arya (2012) showed following results results (Tables 1 and 2). Wet sieving and decanting method was followed (Gerdemann and Nicolson, 1963). Staining procedures of plant root samples included clearing of roots by boiling in water, and using Trypane blue for staining (Phillips and Hayman, 1970). The arbuscules, vesicles and aseptate hyphae was located to confirm the presence of AM in roots. The analysis of 100g of rhizosphericsoil of 21 angiospermic plants of mining and surrounding areas (Table 3) showed the presence of *Alysicarpus vaginalis* (143), *Indigofera linifolia* (118), *Acacia nilotica* sub sp.

Table 3: Presence of AM spores per 100g rhizospheric soil and pH along different plants (Source: Choksi and Arya, 2012)

S. No.	*Plant*	*No. of AM Spores*	*pH*
1.	*Acacia nilotica* sp. *indica* (Benth) Brenan	89	7.90
2.	*Aerva lanata* (L.) Juss.	21	7.82
3.	*Ailanthus excelsa*Roxb.	30	7.90
4.	*Alysicarpus vaginalis* DCProder	143	7.70
5.	*Argemone maxicana* L.	56	7.95
6.	*Bothrichloa pertusa* (L.)A. Camus	60	7.90
7.	*Blumi aalata* DC	32	7.91
8.	*Boreria alata* Meyer	6	7.92
9.	*Calotropis procera*Ait.	28	8.28
10.	*Cassia tora* L.	62	7.46
11.	*Echinops echinatus* R.	39	7.95
12.	*Echinochloa colonum* (L.) Link.	20	7.55
13.	*Euphorbia hirta* L.	36	7.44
14.	*Flocourta montan*Grah.	5	7.91
15.	*Indigofera linifolia* Retz.	118	7.32
16.	*Lindenbergia urtifolia*Lehm.	45	8.10
17.	*Oldenlandia umbellate* L.	39	7.23
18.	*Prosopis chilensis* L.	78	8.50
19.	*Tridax procumbens* L.	45	7.54
20.	*Vernonia cinera* Less.	69	7.90
21.	*Xanthium strumarium* L.	20	7.78

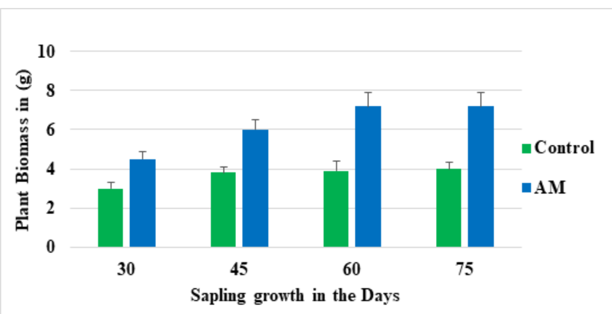

Fig. 8: Plant biomass (in g) of *Leuceana leucocephala* under the control and Arbuscular Mycorrhiza (AM) treatment (Based on data of Choksi and Arya, 2012).

indica (89), *Boreriaalata* (6), and *Flocourtamontan*had least number of spores 5 (Chokshi and Arya, 2012).The dominance of different species of *Glomus* and *Gigaspora* was observed (Fig. 6).The pH and soil characteristics were also analyzed. *Gigaspora* prefers acidic soil and is characterized by absence of vesiclesin roots where hypha attaches to spore with a globular structure. Since these fungi do not form vesicles hence name was changed from VAM to AM fungi. The wall caricaturists are defining structure of AM species, size of spores also counts. Fig. 6 depicts some isolated AM spores from mining and the disturbed soils. For incorporation of AM in plants, funnel method (Arya *et al.*, 2013) was used. The 25 plants each of *Leuceana leucocephala* and *Millettia pinnata* in all 50 inoculated saplings each of treated and non-treated plants were raised in nursery. *In vivo* plantation was done after 75 days in mining site. Results of biomass produced by sapling are presented in two histograms (Fig. 7, 8). It can be clearly seen that growth in two tree sapling was more in AM inoculated plants as the plants grew well. The growth rate showed the significant increase in plant biomass, but comparatively the biomass was more in *Millettia pinnata* after 75 days, it was almost double than control set of plants. There was no difference in growth and survival of two set of saplings one raised by single spore and another with direct inoculation in polybags. Other scientists have tried mixed consortia of AM spores. Bagyaraj and Balakrishna (2012) recorded increase in biomass of tomato and *Capsicum*. Similarly, Jasper *et al.* (1988) found better results in AM inoculated plants grown in iron ore mines. Role of specific genes in alleviation of harmful compounds and heavy metals is shown by Shi *et al.* (2019).

Conclusion and Future Perspective

It can be clearly seen that the soil was basic of the Kadi pani mining area and AM spores per 100g of non-rhizospheric soil ranged from 8 to 70. While in rhizospheric soil it varied from 5 to 143/100g soil. The presence of AM spore was more in rhizosphere as compared to non-rhizospheric soil. Mycorrhizospheric spores were there but their increased number may be helping plants more to survive in stressed environment. Isolation was done, spores were identified and growth of two tree sapling was analyzed after incorporating *G. fasciculatum*. The growth rate showed that the increase in biomass was regular in all the treatments but comparatively the biomass was more in *Millettia* *pinnata* after 75 days. The incorporation of AM spores on large area is cumbersome as spore number and type differs. The experiments with use of polybag inoculation, or preparation of seed balls with AM spores should be tried to observe the success rate. Significant increase in biomass of two plants *Millettia pinnata* and *Leuceana leucocephala* confirmed this assumption. More such studies are needed to ascertain the strains suitable for revegetation of such waste and disturbed land.

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