Studies on rehabilitation of red mud and fly ash ponds using arbuscular mycorrhizal fungi

A thesis submitted in fulfillment of the requirement for the award of the degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

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CERTIFICATE

Certified that the thesis **"Studies on rehabilitation of red mud and fly ash ponds using arbuscular mycorrhizal fungi"** which is submitted by Mr. A. Giridhar Babu, in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out by him under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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I hereby declare that the work which is being presented in this thesis **"Studies on rehabilitation of red mud and fly ash ponds using arbuscular mycorrhizal fungi"** submitted by me for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Dr. M Sudhakara Reddy, Professor and Head, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

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Abbreviations

bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	2’-deoxynucleoside-5’-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
g	Gram
hr	Hours
IPTG	Isopropyl-β-thiogalactoside
kb	Kilo base
L	Litre
mg	Milligram
ml	Milliliter
PCR	Polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
Tris	Tris-(hydroxymethyl-) aminomethane
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
µg	Microgram
µl	Microlitre
mS cm^{-1}	Microsiemens per cm

Chapter 1

Introduction

Chapter 1

Introduction

Pollution of biosphere with toxic metals due to industrialization and other human activities poses major consequent problems for the human population. Migration of these toxic contaminants into non-contaminated sites as a dust or leachate through the soil contributes towards contamination of our ecosystems. Although industrialization has been accepted as a hallmark of civilization, the industrial wastes, which contain toxic materials, have been adversely affecting the biosphere. Among such wastes, red mud (bauxite residue) and fly ash are the effluents generating from alumina refinery and thermal power stations respectively.

Red mud (bauxite residue)

Red mud (RM) is a waste by-product produced from its ore bauxite, in alumina refinery industry by Bayer process. Bauxite ore is the only economic source of alumina production worldwide today (Hind *et al.*, 1998). Typical composition of industrially used bauxite is: Al_2O_3 (40-60%), combined H_2O (12-30%), Fe_2O_3 (7-30%), SiO_2 free and combined (1-15%), TiO_2 (3-4%), F, P_2O_5, V_2O_5 and others (0.05-0.2%) (Josnamayee *et al.*, 1998).

Bayer process is generally followed by two main steps for production of aluminium throughout the world today, in the first step the crushed bauxite is washed with concentrated caustic sodium hydroxide (NaOH) solution at elevated

temperatures up to 270 °C (digestion). Under these conditions, the majority of the aluminium containing molecules in the ore is dissolved into aluminium hydroxide (Stubbs, 1997); the rest being an insoluble residue is called red mud which is removed by settling: filtration processes (clarification). After solids separation (red mud), aluminium trihydroxide (gibbsite; $Al(OH)_3$) is precipitated by cooling the solution and seeding with gibbsite, essentially reversing the initial dissolution process (Grjotheim and Welch, 1998). In the second step, the gibbsite is removed and washed prior to calcinations, where the gibbsite is converted to alumina (Al_2O_3) to yield aluminium metal by Hall and Herroult method (Kirke, 1982).

The output of red mud can be summarized as follows: Red mud is mainly composed of iron oxides, quartz, sodium aluminosilicates, calcium carbonate, aluminate, NaOH as Na_2CO_3, sodalite, ferrite and titanium dioxide (generally present at trace levels) etc. The use of caustic soda (NaOH) at elevated temperature in the Bayer process results the bauxite residue extremely saline, sodic and alkaline with a pH from 9 to 13 (Wong, 1990). Although the introduction of Bayer's process to the industries is an increasing demand for manufacturing alumina, but accumulation of red mud at increasing rate is a great problem throughout the world. Depending on the quality of the raw material processed, 1-2.5 tons of red mud is generated per ton of alumina produced. In addition to generation in huge quantities, it occupies large area of land. World production of this waste has been estimated at 30 million Mg per year (Menzies *et al.,* 2004) and is disposed on land in large residue disposal areas (tailings dams), either as wet slurry or de-watered and dry-stacked (TAA, 2000). In India, the slurry is pumped (Fig 1.1) out to nearby estate dug into ponds where it is left for sun

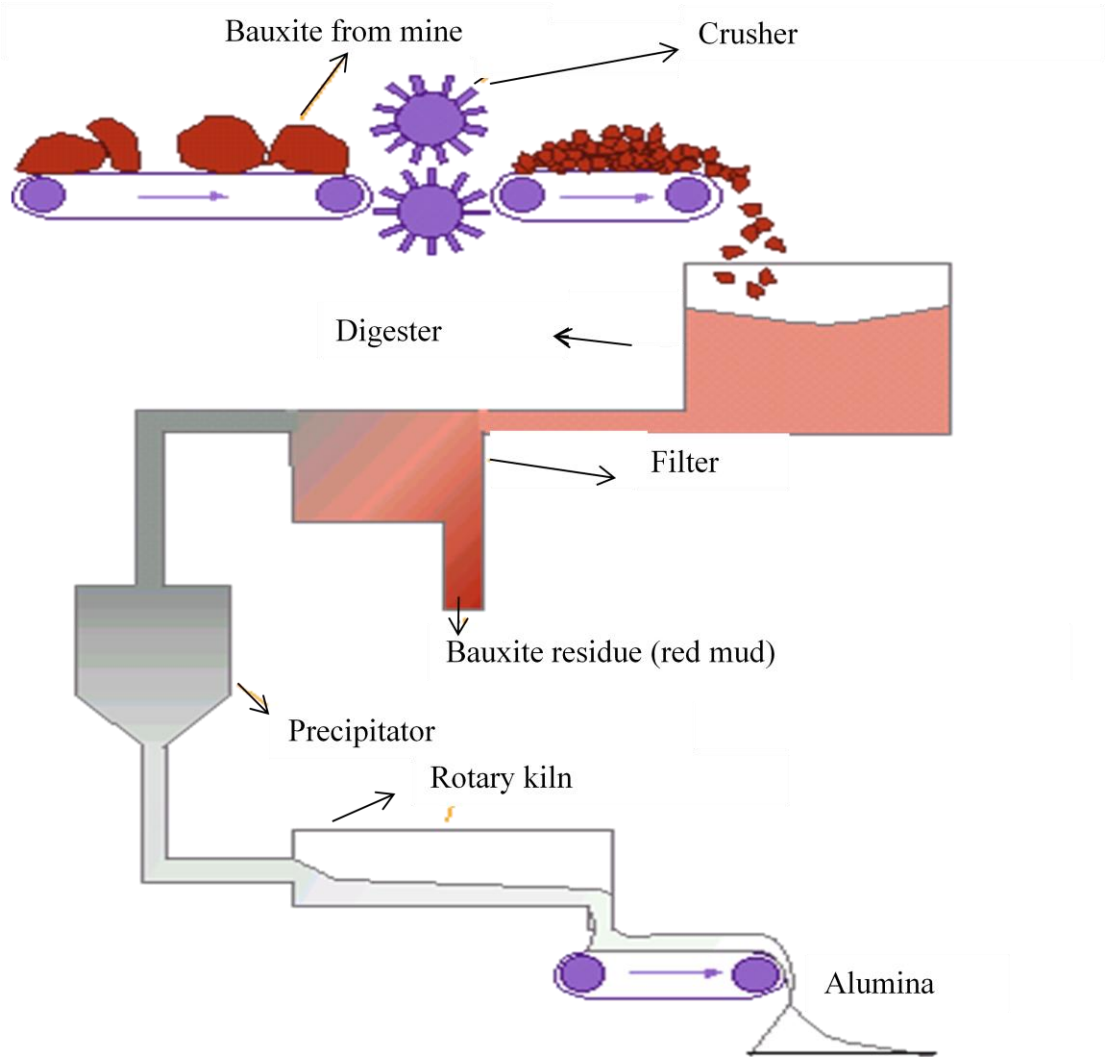

Fig 1. 1: Schematic diagram of red mud (bauxite residue) generation during alumina production (Source: http://www.scribd.com/doc/53710411/2/Production-of-Aluminium).

drying (Thakur and Das, 1994). Disposal of any solid waste is associated with space/ real estate near industry, cost of disposal and pollution, which are now crucial factors.

Obviously, these three problems are also associated with red mud disposal and the problem has become more acute with increasing amount of red mud, shortage of real estate around the industry and environmental awareness of the society. Disposal of such large quantity of waste is expensive and adds up to 1–2% of the alumina price due to its volume and alkalinity. In addition to this, environmental impacts that have occurred near red mud impoundments include leakage of alkaline leachate into the groundwater, erosion of red mud and discharge into nearby surface waters and air pollution due to the generation of residual NaOH as Na2CO3 dust. Observations by the scientists and technologists in the middle of the twentieth century concluded that red mud is a pollutant and has a detrimental effect on environment and human health (Thakur and Das, 1994; Fortin and Karam, 1998). Moreover, they felt deeply that the good amount of metal value is thrown as a waste for which mining and transport cost have been paid. Hence, the scientists and technologists directed their efforts in possible utilization and reclamation of red mud.

Attempts have been made to recover minerals present in bauxite residue such as iron for the steel industry or to utilize the residue as a raw material for road building. However, bauxite does not offer unique or beneficial properties as a raw material for these applications (Hamdy and Williams, 2001). Current technologies used to utilize the bauxite refinery residue are generally too expensive and they may

generate additional risks for the operatives and produce secondary wastes (TAA 2000).

Certain microorganisms are able to proliferate in extreme ecosystems (strongly acidic pH, highly oxidizing conditions, high concentration of metal ions in solution, high alkalinity) and have immense metabolic capacity to allow passage of metals into solution catalyzed by microbial and mainly bacterial activity. Based on this, the potential use of bauxite residue for recovery of aluminum by chemical or biological leaching (*Aspergillus* and *Penicillium*) has been reported (Ward and Koch, 1996: Ghorbani *et al*., 2008). Edwards *et al.* (1999) suggested using bauxite residue as a lime substitute for treating acid soils as well as acid mine drainage sites and also to enhance mineral deficient soils. Anand *et al.* (1996) used *Bacillus polymyxa* for the removal of calcium and iron from bauxite ore.

Establishment of vegetation cover on mine residues (tailings) can fulfill the objectives of stabilization, pollution control, visual improvement and removal of threats to human beings (Wong, 2003). These issues will only be successfully managed if self-sustaining vegetation is established. But, revegetation of RM has been demonstrated to be difficult because of the high alkalinity, salinity, sodicity, soluble Al content, low nutrient availability and poor soil permeability (Chen et al., 2009; Courtney et al., 2008; Fortin and Karam, 1998; Wehr et al., 2006). Consequently, the chemical and physical properties of the refinery residues must be treated prior to revegetation, if the refinery residues are to form part of the plant growth medium (Wehr et al., 2006). Many studies have developed partial solutions to these problems such as neutralization of red mud by acid, copperas (FeSO4), gypsum

and seawater additions to correct pH and sodicity (Eastham et al., 2006; Eastman and Mullins, 2004; Jones et al., 2010; Kopittke et al., 2004; Stewart, 1995; Wehr et al., 2006; Wong and Ho, 1994); adjust alkalinity and salinity; and fertilizers which are applied at high rates alone or with organic amendments to increase plant available nutrients (Bell et al., 1997; Courtney and Timpson, 2004; Eastham et al., 2006; Gherardi and Rengel, 2003; Jasper et al., 2000; Wong and Ho, 1991).

Although these manipulations have been successful to varying degrees but high alkalinity, saline-sodic, low soil permeability capacity and the poor nutrient retention of residue still remain major constraints to long term self sustaining vegetation. Such unfavorable conditions for plant growth require applying methods, such as microbial inoculation for improving nutrient balance, microbial activity and soil quality. Banning et al., (2011) emphasized that for successful rehabilitation of bauxite residue, microbial driven organic matter turnover and mineral nutrient cycling must be developed which help in providing nutrients to plants.

Attempts were made to remediate the residue deposits and mines with and without neutralization, by establishing vegetation cover using microbes that are able to survive in the adverse conditions. In a study by Hamdy and Williams (2001), bauxite residue was treated with hay and yard waste which supported growth of organisms such as *Lactobacillus*, *Micrococcus*, *Staphylococcus* and *Pseudomonas* and led to reduction in pH from 13 to 7. In a recent study by Krishna and Reddy (2005 and 2008), the potential use of *Aspergillus tubingensis* and red mud bacterial isolates in different red mud amendments improved the growth of plants as well as physico-chemical properties of red mud amendments was reported. They concluded

Fig 1.2: Disposal of red mud in the form of wet slurry to nearby artificial impoundments at NALCO, Damanjodi, Orissa, India.

Fig 1.3 (a & b): Red mud ponds are left open for drying, leaving cracks after being dried at NALCO, Damanjodi, Orissa, India.

that the addition of such materials is likely to improve the ease of rehabilitation/revegetation. Thus, establishment of revegetation for stabilization and pollution control using microorganisms is probably the most realistic approach to the reclamation of the land. Microbial remediation is accomplished with the help of diverse group of microorganisms, particularly indigenous AM fungi associated with plants growing in soil. Moreover, diversity of AM fungi associated with plants growing in red mud might provide the clue for the selection of effective AM fungi for rehabilitation of red mud. On the other hand, use of the stress adapted AM fungi alone or combination with other microbes may help in the establishment of vegetation on red mud.

Fly ash

Fly ash (FA) is the by-product from combustion of pulverized coal and industrial waste incinerators. Coal based FA is produced mainly by coal-fired plants. In this process, coal is allowed for burning in a coal-fired boiler (fire box); the resultant fine-grained, powdery particulate material carried off in the flue gas is usually collected by the means of electrostatic precipitators, bag houses or mechanical collection devices such as cyclones (Fig 1.3). In this coal combustion process over 70-75% of total coal utilization by-product (FA, bottom ash, slag, and flue gas desulfurization products) is FA (Haynes, 2009).

Fly ash is a complex heterogeneous material consisting both amorphous and crystalline phases (Mattigod *et al.*, 1990). Generally, FA is highly saline and alkaline with the pH range from 8.5-12.5 due to presence of hydroxides and carbonate salts of Ca and Mg (Carlson and Adriano, 1993; Misra and Shukla, 1986; Singh and Yunus, 2000). However, pH of FA may vary from 4.5 to 12.0 and it depends largely on sulphur content of parent coal. Fly ash contains several elements like Si, Ca, Mg, Na, K, Cd, Pb, Co, Cu, Fe, Mn, Mo, Ni, Zn, B, F, Ca and Al (Gupta *et al.*, 2002) and is considered as a ferro-alumino silicate mineral with Si, Al, Fe, Ca, Na and K as predominating elements (Adriano *et al.*, 1980).

The FA components and resulting properties are largely dependent upon the composition and property of the parent coal, the combustion type and finally handling. The concentration of various elements also varies according to particle size (Davison *et al.*, 1974; Khan and Khan, 1996; Page *et al.*, 1979). Fly ash contains most of the elements required for plant growth and metabolism, with the exception of

nitrogen and available phosphorus, as well as most toxic non-essential elements for plants (El-Mogazi *et al.,* 1988; Plank and Martens, 1974, 1975; Singh and Yunus, 2000). The major coal combustion residue (CCR) producing countries are USA, China, and India. Overall, world FA production is now reported to have reached 360 million tonnes (MT). In India alone, the annual production of FA is about 120 MT by 82 power plants and expected to rise to 150-170 MT per year by the end of 2012 (MOEF, 2007).

Present strategy for disposal of FA is by wet and dry methods. In dry disposal, the FA is dumped in landfills and FA basins. In wet methods, the FA is mixed with water and pumped out as slurry into artificial dams, lagoons or settling ponds which are termed as ash ponds. Over the time water is allowed to drain away from these ash ponds. In India the most common practice is wet disposal method (Fig 1.4). Both methods used for disposal of FA are dumping it in landfills or open land. The potentially deleterious effects of this practice on the environment include wind and water erosion and leaching of substances (e.g. salts, heavy metals) into the groundwater. In addition to this, discharge of rain water and run off from the ash mound areas into surface water bodies can also be a source of water pollution (Sushil and Batra, 2006**)** as well as reduction in plant establishment and growth (Wong and Wong, 1990). Fly ash particulates, when released into the air can cause irritation and inflammation to eyes, skin, throat and upper respiratory tract of humans. Toxic and/or mutagenic constituents of these particles are considered to be metals, polycyclicaromatic hydrocarbons and silica (Borm, 1997; Smith *et al.*, 2006). In addition, some metals, such as Se, Cd, Cu and Zn can also be ingested by humans in

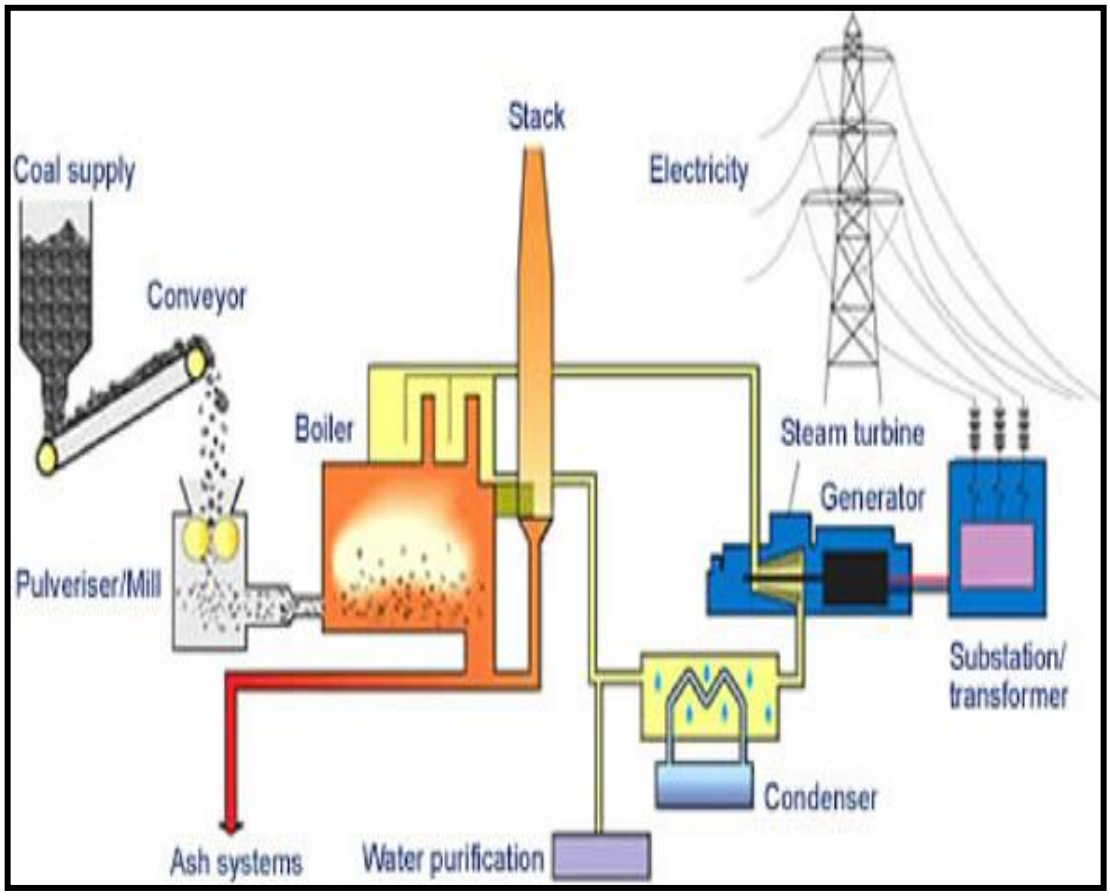

Fig 1.3: Schematic diagram of coal combustion and generation of fly ash and electricity (World Coal Association, 2006).

in larger than recommended concentrations by consumption of fish from waters close to poorly managed ash dams (Kirby *et al.*, 2001) or from vegetables growing on ash (Tsadilas *et al.*, 2006).

Another special exposure pathway to humans is drinking contaminated ground waters, exemplified by heavy metal enriched well waters, as what has afflicted a large number of people residing around the industries. Considerable amount of work has been carried out all over the world on the utilization and remediation of FA. Major uses are in the making of bricks, cements and concrete, wood substitute product, soil stabilizing, road base/embankments, consolidation of ground and land reclamation (Asokan et al., 2005; Jala and Goyal, 2006). The use of FA as a soil amendment in agriculture has been demonstrated but the amount of FA utilization is very small because of the toxic effects of metals on plant growth (Basu et al., 2008; Martens, 1971). Gaikwad and Bhardawj (1995) have demonstrated the application of FA in metal extraction and as a conditioner in waste water treatment.

In recent years, the biohydrometallurgical approach on municipal solid waste FA by *Aspergillus* species has been intensively studied (Castro et al., 2000; Jain and Sharma, 2004; Mulligan et al., 2004; Ren et al., 2009; Wu and Ting, 2006; Yang et al., 2009). The bioleaching of industrial waste materials for detoxification has been considered more economical and environment friendly as compared to traditional metal extraction methods with high energy cost and increase in pollution load of environment (Jain and Sharma, 2004). However, despite positive uses of FA, the rate of its production clearly outweighs its utility as a by-product. This is because the remaining FA material and its disposal practices involve holding ponds, lagoons, landfills and slag heaps, all of which can be regarded as unsightly, environmentally undesirable and/or a non-productive for use of land resources, as well as posing an

on-going financial burden through their long-term maintenance (Iyer and Scott, 2000).

The revegetation establishment on FA ponds stabilizes the FA against wind and water erosion, provide shelter and habitat for wildlife and give aesthetically pleasing landscape (Selvam and Mahadevan, 2002). However, FA possesses very unfavorable physico-chemical properties for plant growth due to deficiency of essential nutrients (absence of N and less available P), degraded microbial community, high soluble salt concentrations of trace elements and the presence of compacted cement layers on ash disposal sites (Enkhtuya *et al.*, 2005; Selvam and Mahadevan, 2002; Wu *et al.*, 2009). Many efforts have been made to revegetate these ponds by adding different amendments such as inorganic, organic and bio- fertilizers to promote the growth of several plant species. In a study by Gupta *et al* (2007), application of various amendments like press mud, garden soil and saw dust to fly ash improved the growth of *Cicer arietinum* L. varieties (var. CSG-8962 and var. C-235). The vermiculite or sewage sludge compost ameliorated lagoon ash, with or without nitrogen fixing bacterial inoculation of two legume species, *Acacia auriculiformis* and *Leucaena leucocephala* showed significant suppressions in biomass and plant nutrient content in comparison to an agricultural soil (Cheung *et al*., 2000).

Fig 1.4: Disposal of fly ash in the form of wet slurry to nearby artificial impoundment at NALCO, Damanjodi, Orissa, India.

Mycorrhizal symbiosis can stimulate plant growth on nutrient poor and polluted sites, and can reduce stress caused by low water holding capacity, unfavorable soil structure, low pH, high salinity, toxic metals etc. (Sylvia and Williams, 1992). Enkhtuya et al. (2005) and Wu et al. (2009) reported the effects of selected isolates of AM fungi on the plant growth, nutrient uptake and aggregation of FA. Selvam and Mahadevan (2002) isolated 15 arbuscular mycorrhizal (AM) fungal species naturally associated with plant roots in abandoned lignite FA pond. Thus, functional activity and survival of AM fungi associated with plants growing in fly ash is indicative of its adaptation to fly ash. Using these effective AM fungi as an inoculum may be more beneficial to plants growing in fly ash than the non-adaptive AM fungal species and can improve or speed up the rate of restoration process.

Role of arbuscular mycorrhizal (AM) fungi on disturbed ecosystem

Studies of primary successional ecosystems have suggested that microorganisms play a critical role to establish early ecosystem development, due to functional abilities such as nitrogen fixation, organic matter turnover, mycorrhizal symbiosis and potential facilitation of plant establishment (Hodkinson *et al.,* 2002; Walker *et al.*, 2003). Among soil microorganisms, arbuscular mycorrhizal (AM) fungi are obligate symbiotic mycorrhizal fungi associated with plants and provide a direct link between soil and plant roots. There are growing evidences that AM fungi can play a relevant role in plant establishment and improvement of soil properties in stress (alkaline, heavy metal and saline-sodic) environments (Ileana *et al.,* 2007; Mendoza *et al.*, 2005; Ortega-Larrocea *et al.*, 2010; Vivas *et al.*, 2005; Zarei *et al.*, 2008a).

Thus, the tolerance and diversity of AM fungi in heavy metal contaminated soil is indicative of AM fungal adaptation to stress conditions (Colpaert and Vandenkoornhuyse, 2001; Joner *et al.*, 2000; Leyval *et al.*, 1995). However the tolerance strategies of AM fungi to adverse conditions may include the existence of adapted propagules able to maintain an infective inoculum. The spontaneous selection of infective and effective AM fungi is a long process in contaminated sites (Takács *et al*., 2005). It has also been demonstrated that the application inoculation techniques with adapted AM fungal strains in restoration and bioremediation studies is more effective than applying non-adapted strains (Vivas *et al.*, 2003). Besides, it is also evidence that AM fungi alter the soil microbial communities in rhizosphere directly or indirectly through changing the root exudation patterns (Barea *et al.*, 2005) and enhancing the soil enzyme activities (Wang *et al.*, 2006). Isolation and selection of such species could open the door for applying these fungi as biotechnological tools to overcome the limitation in such a habitat. Moreover, it is necessary to understand the ecological role of AM fungi associated with plants growing in such a stress conditions.

On the other hand, it is known that soil microorganisms influence the development of the AM symbiosis (Jeffries and Dodd, 1996). The saprobe fungi are important and common components of the rhizosphere. These fungi obtain greater nutritional benefits from organic and inorganic compounds released from living roots together with sloughed cells (Dix and Webster, 1995). They are also able to degrade toxic substances (Madrid *et al.*, 2005; Wainwright, 1992). Some authors confirm the existence of synergistic effects of saprobe fungi on plant root colonization by AM

fungi and on the effectiveness of AM fungi in plant resistance to heavy metals in soils (Fracchia *et al.,* 2000; Medina *at al.,* 2006; Srinath *et al.,* 2003; Yang *et al.,* 2009). In addition to this, some bacteria are known to facilitate mycorrhizal formation by affecting spore germination or root colonization (Fitter and Garbaye, 1994).

Motivation for the study

There is an increasing need for the industries producing FA and RM, to adopt a more environment friendly approach, release less harmful products and to establish vegetation on red mud and FA ponds. Today, throughout the world mining industries needs to incorporate rehabilitation as part of their mining operations. Arbuscular mycorrhizal fungi are efficient candidates for rehabilitation of disturbed ecosystem; especially, the application of inoculum techniques with RM and FA adapted AM fungi may increase the rate of rehabilitation process. AM fungi also interact with other soil microorganisms and improve plant growth and soil microbial community structure. Thus, the investigation into their synergetic role on plant growth is also required because the advantage in application of native AM fungi may not show deterrent effects on indigenous microbial community and produce some clues for speed up the process.

To our knowledge, no research publication is available on the role of AM fungi on rehabilitation of red mud, and less than five research publications in the last 30 years based on microbial remediation have been published. Very few reports are available on AM fungi in rehabilitation of FA. Arbuscular mycorrhizal fungal diversity of both these sites has not been estimated entirely. There is a need to screen the highly adapted AM fungi, which can help in plant mineral nutrition and can be used for further rehabilitation of the FA and RM ponds. The diversity analysis will

impart information regarding the different population and community structure of the AM fungi and also about their role in biogeochemical cycle.

Much work needs to be done to exploit the AM fungi inhabiting in the extreme conditions. This indigenous AM fungi can be used as inoculum to rehabilitate the waste sites for sustainable environment. Keeping these facts in view, in the present study the diversity AM fungi associated with plants growing adjacent to red mud and plants growing in fly ash ponds was investigated. The application of these AM fungal inoculum was also studied to evaluate their role on the growth of plants grown in different red mud amendments as well as in fly ash.

Objectives

- To study the diversity of AM fungi in red mud, fly ash ponds and surrounding areas
- To study the influence AM fungi on the growth and survival of plants in red mud and fly ash amended soils
- Studies on evaluation of AM fungi in rehabilitation of red mud and fly ash ponds

On the other hand, red mud bacterial isolates and *A. tubingensis*, a phosphate solubilizer were also used as an individual and combination with AM fungi to study the effect of these microbes on the growth of bermudagrass grown in red mud and red mud amended soils. In case of fly ash experiment, *A. tubingensis* was used with combination of AM fungi to evaluate the growth of bamboo plants.

Chapter 2

Review of literature

Chapter 2

Review of literature

Red mud (bauxite residue)

Red mud (RM), as the name suggests, is brick red in color and has slimy appearance due to presence of high content of iron oxide and caustic soda. The average particle size is about <10 µm but few particles having size greater than 20 µm can also be found (Mohapatra *et al.*, 2000). About 35% by weight of solids have size less than 5 µm and 80% less than 8 µm. The sand, silt, clay of red mud is 3-5, 79-80, 14-15% respectively. Red mud is highly alkaline (pH >11), saline-sodic, thixotropic and posses high surface area in the range of 13-16 m^2 /g with a true density of 3.30 g/cc (Paramguru *et al.*, 2005). The free moisture content of the RM is highly variable (30-60%) depending upon the disposal method.

The leaching chemistry of bauxite suggests that the physico-chemical properties of RM depend primarily on the bauxite used and to a lesser extent, the manner in which the bauxite is processed by the Bayer's process (Paramguru *et al.*, 2005). The important parameters that have significance in its further handling, disposal, and use are 1) moisture content 2) rheology 3) surface area 4) particle size 5) mineralogy 6) contents of valuable metals 7) presence of rare earth metals and 8) presence of toxic substances etc. (Paramguru *et al.*, 2005). Composition of RM samples from some randomly selected locations is given in Table 1.

RM disposal and environmental concern

Disposal of waste material means transport, dig out the land and deposit. Reports on this issue in the open literature seem scarce and important objectives remain the same, i.e. 1) reduced mud volume 2) reduced caustic content 3) reduced environment impact 4) improved reclamation potential and 5) improved rehabilitation possibilities (Paramguru *et al.*, 2005). However, we can find some general information about the disposal of RM as well as the common practice in the USA, India, China, Japan, Spain, and Greece. In a research by Hind *et al.* (1999), it was reported that conventional disposal methods have revolved around the construction of clay-lined dams or dykes, into which the RM slurry is simply pumped and allowed to dry naturally.

The design and construction of such residue impoundments has varied considerably over the years (Salopeka and Strazisar, 1993) but the disposal practices are generally dependent upon the nature of the immediate environment. However, these conventional disposal areas were simple and inexpensive, the potential impact on the surrounding groundwater and environment and difficulties associated with surface rehabilitation, forced significant changes in disposal practices (Cooling and Glenister, 1992). This led to the construction of doubly sealed impoundments, incorporating a polymeric membrane as well as clay lining and drained lakes, having a drainage network incorporated in the lining material. Drained disposal systems have been found to reduce the threat of the residue to the environment, while also increasing storage capacity as a result of better residue consolidation (Colembera and Want, 1982; Hudson, 1987).

Table 2.1: Composition of RM generated in plants of different parts of the world (Mohapatra *et al.,* 2000)

	Major constituents, weight (%)				
	Fe_2O_3	**Al_2O_3**	**TiO_2**	**SiO_2**	**Na_2O_3**
India					
Al. Corporation	20.26	19.6	28.0	6.74	8.09
MALCO	45.17	27.0	5.12	5.7	3.64
HINDALCO	35.46	2.0	17.2	5.0	4.85
BALCO	33.8	15.58	22.5	6.84	5.2
NALCO	52.39	14.73	3.30	8.44	4.0
USA					
ALCOA Mobile	30.40	16.2	10.11	11.14	6.8
REYNOLD Arkansas	55.6	12.15	4.5	4.5	2.0
Sherwon	50.54	11.13	Traces	2.56	1.5-5.0
FRG Baudart	38.75	20.0	5.5	13	9.0
Hungarian	38.45	15.2	4.6	10.15	8.12
Jamaican	50.9	14.6	6.87	3.4	3.18
Surinam	24.81	19.0	12.15	11.9	9.29
Taiwan	41.3	20.21	2.9	17.93	8.16
Australia	40.5	27.7	3.5	19.9	3.8

As an alternative, improved dewatering techniques have been facilitated semi-dry disposal methods by separating fine mud from the sand fractions and dewatering to a solid concentration of 50-60% before transporting by pipelines (Cooling and Glenister, 1992; Nguyen and Boger, 1998). The current residue management in Western Australia is dry stacking disposal method. Due to higher hydraulic conductivity (Wong and Ho, 1993) of red sand and thus ease of caustic removal by washing results less of an environmental threat than RM and can be safely disposed off. In contrast, RM has a very low hydraulic conductivity and is saturated with caustic (Wong and Ho, 1994). After separation from red sand, the RM is de-watered and dry stacked in RM disposal areas (RDA) (Hudson, 1987). In a dry stacking method, RM involves discharging and spreading in layers and allowing each layer to dry before adding another layer (Chandler, 1987; Pohland and Tielens, 1987; Robinsky, 1987).

This method greatly reduces the volume of RM and gravity drain at the bottom of the red mud disposal areas (RDAs) enables continuous recovery of caustic, which in turn reduces the risk of caustic leakage and subsequent impacts on the environment. Nonetheless, RM desiccates in the field which eventually hardened to form the crack beside formation of white soda flakes on the outer surface of the dried RM and which is blown off by the winds and create environmental hazards (Fig 2.1). However, this method associated with utilization of huge amount of area and runoff alkaline water at rainy season is some extent to environmental risk. Despite of this, presently, worldwide less than 10% of RM disposal are still directly pumping it into

Fig 2.1: Deposition of soda flakes, which appears white on the surface of red mud pond at NALCO, Damanjodi, Orissa, India.

the sea in a planned manner because of scarcity of land. But, some previous studies demonstrated that a "dead zone" is established in the center of the RM deposit at the sea bottom where only RM resistant organisms survive near this zone (Maria *et al.*, 2002).

RM remediation

The contamination of surrounding environment is a global environmental issue, which can be solved by utilization and neutralization for environmental benefits. Although extensive uses for RM have been investigated and many have already been pilot tested, there are limited examples of commercial scale reuse of RM. There is an "inherent" safety in keeping RM stored, as the risk of misuse or environmental damage is low (Tuazon and Corder, 2008). However, investigations into RM reuse are continued; if RM could be reused then it would create a valuable synergy between the Gladstone alumina refineries and other reuse stakeholders (Corder, 2005).

RM utilization

Several attempts have been employed for utilization of RM throughout the world. For example, RM can be used in the heap leaching of gold ores (Browner, 1995) in the removal of sulfur compounds from kerosene oil (Singh *et al.*, 1993), in the hydrogenation of anthracene (Alvarez *et al.*, 1995; Llano *et al.*, 1994), coals and aromatic compounds (Eamsiri *et al.*, 1992), in the metal fixation of contaminated soils (Friesl *et al.*, 2004; Friesl *et al.*, 2006; Lombi *et al.*, 2002 a and b; Gray *et al.*, 2005), in the extraction of iron and titanium oxides (Parek and Goldberger, 1976), in the synthesis of inorganic polymeric materials (Dimas *et al.*, 2009) and as a pigment in anticorrosive marine paints (Skoulikidis *et al*, 1992).

In India, it is reported that 2.5 million tons of RM was utilized in the cement industry whereas in China, about 10% of RM used for metal extraction or utilized as a raw material for brick production (Agarwal and Sashikanth, 2008). Alcoa alumina processing plant at Kwinana, Western Australia has been using bauxite residue bricks to build homes in the South-West of Western Australia (Source :West Australian news, 1 February 2002). However, the Health Department rejected the building after tests registered radioactivity readings which bordered on the maximum acceptable radiation exposure levels for 19 hours a day (Source: Australian Fluoridation News Jan-Feb 2002 Edition). In Japan, plant scale tests were initiated in 2003 to confirm the possibility of using residual bauxite (i.e. dry "RM") in cement production. The tests confirmed that residue added cement, as well as mortar and concrete made from this cement meet the Japanese Industrial Standards (Japan Aluminum Organization, 2004).

Red mud has been successfully used as an amendment on sandy, acidic soils both in agriculture and mine spoils due to its neutralizing capacity and increased water holding capacity (Fortin *et al.*, 2000; Komnitsas *et al.*, 2004; Paradis *et al.*, 2007; Summers *et al.*, 2001). Summers *et al.* (1996) reported phosphorus retention and leachates from sandy soil amended with bauxite residue. Application of RM to sandy soil increased the yield and phosphorus response in subterranean clover (Summers *et al.*, 2001). Ward and Summers (1993) modified the sandy soil with the fine residue from bauxite refining to retain phosphorus and increase plant yield. The rehabilitation program of mined out bauxite areas with RM and vice versa have been studied in Australia and Greece (Boufounos, 2006; Paffenhoefer, 1972). The authors

discussed the details of attempted vegetation on the pond surfaces. These ponds were built in such a way as to minimize the impact on environment by vegetation of the pond surfaces when they are filled and out of use. In a study of Anderson *et al.* (2007), as an on-site amendment of residue capping sands, altered residue fines to increase water holding capacity, reduced overall pH and sodicity, while improved the medium's ability to retain nutrients and increased essential plant nutrients.

RM neutralization and vegetation establishment

In response to these negative effects, a number of alternative options have therefore been investigated which are regarded as less intrusive and more cost effective. One technology that has received a considerable amount of attention is addition of various amendments (Gray *et al.*, 2006). This method relies on the addition of an amendment to a soil to increase the proportion of the total soil metal burden within the soil solid phase, either by precipitation or increased metal sorption, thereby decreasing metal solubility (Basta and McGowen, 2004; Oste *et al.*, 2002).

Wehr *et al.* (2006) found the rationale approach to neutralize RM with seawater, thereby lowering the alkalinity of the RM to pH 9 by precipitation of Mg-, Ca- and Al-hydroxide, and carbonate minerals (Menzies *et al.*, 2004). Furthermore, it was observed that on drying, shrank and a stable blocky structure of RM developed. This would allow leaching of the salinity and alkalinity of RM by rainfall and permit penetration of roots into RM along the cracks. Further, amelioration of the surface layer (upper 0.5 m) of seawater neutralized RM with gypsum, followed by drying, ripping and/or tillage to break the RM into smaller aggregates, because soft ''pasty'' RM cannot be penetrated by roots. The structured substrate of RM is then covered

(without mixing) by soil, salt and alkali tolerant plants were transplanted and allowed to derive the most of their nutrients and water from the capping layer. Roots also grow into the RM for water after some time; presumably once the soil capping has dried out during the dry season which result growth retardants of plants (Wehr *et al.*, 2006).

Published research on rehabilitation of RM focused on two approaches: 1) amelioration of the surface layer of the residue by adding amendments and 2) vegetation establishment. Vegetation, most commonly a grass species, is planted into the amended surface layer (Wong and Ho 1993). Amelioration of the surface layer has been widely attempted using gypsum (Wong *et al.*, 1991), manure/compost (Fortin *et al.*, 1998), sewage sludge (Courtney *et al.*, 2004), soil (Lewis *et al*, 1995) and combinations thereof. Capping of the residue with more benign material has been evaluated. For instance, Wong *et al.* (1991) placed 20 cm of sandy soil onto RM at Kwinana in Western Australia, while Mohan *et al.* (1997) tried marine dredging sediment as capping material for RM at Point Comfort, Texas.

At Alcoa World Alumina, Australia, filled red mud disposal areas (RDA) are topped with red sand and amended with gypsum and sewage sludge (Glenister, 1992). This form of surface treatment has been shown to improve the structure of residue and decrease its alkalinity and sodicity to levels that allow the establishment of alkaline tolerant plants (Fuller *et al.*, 1982; Glenister 1987; Ho 1987; Ward 1986; Wong 1990; Wong and Ho 1993). Although surface remediation helps to reduce erosion problem and may allow productive use of land, it does not alter the alkaline, saline and sodic nature of RM deeper in profile. Continuous leaching of caustic and

sodium from the RM profile requires diligent maintenance of RDA linings to prevent ground water pollution. Refineries are thus faced with long term environmental responsibilities and liabilities that may extend beyond residue production. Any method of rehabilitating the residue throughout its profile would be desirable (Valarie, 1999).

A field experiment was conducted to evaluate the different forms of inorganic fertilizer, to assess their effectiveness as alternatives to poultry manure (organic fertilizer) for supplying nutrients to dust control crops (Eastham *et al.*, 2006). The results of this study concluded that inorganic fertilizer could provide a suitable, cost effective alternative to poultry manure for growth and nutrient uptake of dust control crops. They have also reported that DAP fertilizer blend could be used as an effective replacement for poultry manure for growing dust control crops on bauxite residue sand. The laboratory studies of Regional Research Laboratory (RRL), Bhuvneshwar (1996), provided adequate growth of bermudagrass when sufficient gypsum was added to lower down the pH to 8.3 for RM but amount of gypsum is added equivalent to 34 tons/hectare which is an expensive amelioration process. It has been found that addition of paper pulp waste to RM did not enhance the growth of bermudagrass unless it is treated with gypsum. It has also been found that fly ash and coarse texture RM can be mixed for treatment in the ratio 20:80. After reducing the pH to 7-8 and after adding NPK fertilizer it becomes suitable for growth of Rhodes grass. Further, many attempts have been made with glucose and potassium acid phosphate in RM for the reduction of alkalinity.

Courtney and Timpson (2004) examined the use of gypsum and thermally dried sewage sludge as amendments for establishing the *Trifolium pretense* and *Holcus lanatus* on a RM with 10% process sand mix at the Aughinish Alumina Ltd., Bayer Plant, Ireland followed by two-year field investigation in 1999. Gypsum was applied to plots at rates of 0 and 3% (w/w) with both treatments receiving thermally dried sludge at 35 t/ha. After one year, plants calcium levels were adequate, but there were deficiencies for nitrogen, manganese, potassium and magnesium. Sodium levels were not adequate in year one and levels declined along with nitrogen, calcium, manganese, magnesium, phosphorous and potassium further in year two. As levels were already deficient in year one, the further decrease suggest severe nutrient shortage in the residue substrate. They have suggested long-term success of revegetation of bauxite residue, even after added gypsum and organic amendment, the deficiencies of nutrients in the substrate must be overcome.

Courtney and Timpson (2005) investigated the use of mixing coarse fraction residue with fine fraction residue, at two different application rates (10% and 25%), with and without the use of gypsum as an ameliorant, for re-vegetation of the residue with *Trifolium pratense*. The thermally dried sewage sludge was applied to all treatments at rate of 35t/ha and mixed in manually to a depth of 20 cm. Trials were conducted on the residue disposal area at the Aughinish Alumina Limited refinery, Ireland. Optimum plant growth was observed in treatments that had also received gypsum amendment, with higher plant biomass, Mn nutrition and lower Al and Fe concentration. However, use of process sand at the higher application rate (25%) promoted lower levels of soluble Al and Fe and exchangeable Na in the substrate and,

consequently, lower plant uptake of Na. Results indicate that co-disposal of the coarse fraction sand at 25% w/w with fine fraction residue can improve the substrate and, therefore, plant uptake and growth. They suggested that further monitoring is recommended to determine the effect of the absence of gypsum and other nutrient sources on plant growth.

Courtney *et al.* (2008) conducted a study in 2005 to evaluate the status of three revegetated sites by the comparison of species diversity, substrate conditions, and plant uptake characteristics of BRDA at the Aughinish Alumina Ltd. Refinery, Limerick, southwest, Ireland. These sites had been revegetated in the period 1997-2001. The site 1 and 3 which had been revegetated in 1997 and 1999 respectively with amendment of process sand (25%) and spent mushroom compost (80 tonnes/ha), whereas revegetated in site 2 with 3% gypsum (w/w), 25% process sand (w/w) and spent mushroom compost at 80 t/ha in 1999. When compared initial seeded with a mixture 6 species like *Agrostis stolonifera*, *Fescue longifolia*, *Holcus lanatus*, *Lolium perenne*, *Trifolium repens*, and *Trifolium pretense* at rates of 100 kg/ha, there were 47 species belonging to 38 genera and 15 families growing three sites recorded with greatest diversity in 1997 site. Among the 15 families, Asteraceae and Poaceae were the dominant families with 14 and 9 species, respectively. Moreover, the limited variation in the residue properties observed in three treatments and indicated that the diversity of plants in threes sites were most influenced by succession not by substrate conditions. Dominant species in the 1999 treatments, *Holcus lanatus* and *Trifolium pratense*, were analyzed for elemental composition. The foliar nitrogen, phosphorus, potassium, and calcium were adequate and sodium levels were low. Manganese and

magnesium levels were also reported as low. Chen *et al.* (2010 a and b) demonstrated a laboratory experiment by adding significant proportions of P as DAP to bauxite residual sand and results suggested that added P could remain labile or moderately labile for plants use during the rehabilitation of bauxite-processing residue sand (BRS) disposal areas. There was limited capacity of BRS for fixing P in more recalcitrant forms (e.g., Ca-P and residual-P). Concentrations of most P pools in BRS increased with DAP application rate and the impact of the pH treatment on P availability varied with type of P pools and DAP rate.

Jones *et al.* (2010) investigated how the addition of organic wastes (biosolids and poultry manure), in presence or absence of added residue mud, would affect the properties of the residue sand and its suitability for revegetation. In this regard, samples of freshly deposited residue sand was treated with phosphogypsum (2% v/v) and incubated to displace and remove accumulated soluble salts in solution. After that, a laboratory experiment was set up in which the two organic wastes were applied at 0 or equivalent to 6% (w/v) in combination with residue mud added at rates of 0%, 10% and 20% (v/v). Samples were incubated for 8 weeks, after which, chemical, physical and microbial properties of the residue sand were measured along with seed germination. The results generated from this study are encouraged for the improvement of physical and microbial properties of residue sand for subsequent revegetation. For example, addition of residue mud to sand induced aggregation and produced the beginnings of soil structural formation and this effect was magnified in the presence of poultry manure. In addition, increased residue mud addition, in association with either of the organic wastes increased the size and activity of the

microbial community by the effect of aggregation; which results protecting organic matter from rapid decomposition plus aggregation providing a suitable microenvironment for microbial growth proliferation and *Lepidium sativum* seed germination. The combination of both poultry manure and residue mud had the most positive effects on both physical, microbial properties as well as seed germination.

Restoring biological fertility

Vegetation establishment on amended RM by various plants that are able to survive in adverse conditions have been studied (Banning *et al.,* 2010; Courtney and Timpson 2005; Eastham *et al.*, 2006; Wehr *et al.*, 2006; Wong and Ho 1993). Further, restoration of this biological component of soil fertility should be an important aim of RM rehabilitation. Sustainable plant establishment on bauxite processing residues requires development of functional soils which includes microbially driven organic matter turnover and mineral nutrient cycling for the long term provision of plant nutrients (Banning *et al.*, 2010). These can be achieved only by two aspects; firstly, ameliorate the top layer with amendments to optimize the survival of beneficial microorganisms, soil animals and seeds, secondly, inoculation with key symbiotic microorganisms (Wong and Ho 1993, 1994).

Hamdy and Williums (2001) isolated low levels of injured bacterial cells from RM using various added nutrients and/or hay. A total of 150 isolates grew from less than 10 to more than 10^9 cells g^{-1} bauxite residue and formed organic acids that lowered the pH from 13 to about 7.0. The isolated species were identified as *Bacillus, Lactobacillus, Leuconostoc, Micrococcus, Staphylococcus, Pseudomonas, Flavobacterium and Enterobacter*. The effect of these isolates have demonstrated by

using scanning electron micrographs that bauxite residue particles were highly dispersed with microcolonial structures. Furthermore, the treated bauxite residue supported growth of several plants and earthworms that survived for over 300 days. In a test plot, bioremediation on a residue deposit at Alcoa Point Comfort, TX, the bermudagrass hay used was effective mulch material and encouraged water filtration, leading to establishment and growth of salt tolerant vegetative species.

Valerie (1999) investigated the microbiology of the red mud. She isolated the bacteria from RM sample and characterized these indigenous bacteria for their ability to produce acidic substances in alkaline glucose medium. After series of pot trials it was found that these acid producing bacteria reduce the alkalinity of glucose amended RM which ascertained the possibility of using these bacteria to reduce the alkalinity of RM. This study also showed that the properties of RM influence the types of bacteria present. Different types of bacteria having different degrees of tolerance at high alkalinity and salinity were isolated from RM and can be used to ameliorate the RM in presence of added nutrients to encourage their growth. It was revealed that for pH reduction under the chosen conditions, oxygen was necessary and also that the pH reduction was related to bacterial growth.

In a study of Krishna and Reddy, 2005, inoculation of *A. tubingensis* a P-solubilizer fungi to red mud amended with soil increased the growth, nutrient uptake of maize plants and reduced the pH of soils. In another study, Krishna (2008) isolated 10 bacterial isolates from red mud which were closely related to *Planococcus sp., Bacillus sp., Psedomonas sp., Kocuria sp., Micrococcus sp., Agromyces sp. and Salinococcus sp.* These isolates mainly belong to Phylum- Firmicutes, Actinobacteria

and Proteobacteria. The potential use of indigenous bacterial isolates and exogenous phosphate solubilizer, *Aspergillus tubingensis* on the growth effect of bermudagrass was studied in gypsum, topsoil, sludge, fly ash amended with RM and RM alone treatments. The results showed that the inoculation of indigenous red mud bacterial isolates and *A. tubingensis* significantly increased the growth of bermudagrass in all different amended RM treatments. The interesting observation of study is the inoculation of bacterial isolates promoted the growth of bermudagrass even in non amended RM and no growth was observed in non-inoculated treatment. Among the different amendments, sludge amended treatments supported the maximum growth followed by gypsum treatment. The physico-chemical and bio-chemical properties were improved along with metabolic diversity of bacteria on Biolog plates in all inoculated treatments.

The development of microbial diversity and function and effect of organic matter addition (composted manure) in residue sand embankments of Alcoa's fine residue storage ponds have been investigated by Banning *et al*. (2010). The residue sand embankments underwent rehabilitation with native vegetation overtime, allowing study of a 3-year chronosequence using space-for-time substitution. In their study, although the size of the microbial biomass appeared to be limited by the low organic matter content of the residue sand, a decline in microbial metabolic quotient indicated a potential alleviation of microbial stress with rehabilitation age. Despite the low microbial biomass, the ability of the residue sand microbial community to function with respect to the metabolism of added amino acids developed rapidly. The

diversity of the bacterial and fungal community also developed rapidly and was similar to or higher than, the coastal sand analog in 0.5-year-old rehabilitation.

Fly ash (FA)

Combustion of various types of coals like bituminous sub-bituminous coal and lignite generates FA, bottom ash, boiler slag and flue gas desulfurization (FGD) materials, which are commonly known as coal combustion residues (CCRs) (Haynes, 2009). Fly ash consisting of small particles that are carried off in boiler by the flow of exhaust gases and are collected from the stack gases using electrostatic precipitators (ESP), flue gas desulfurization (FGD) systems and bag houses or mechanical collection devices such as cyclones. About 70-75% of the CCR is ESP-FA. Fly ash components and resulting properties are largely dependent upon the composition and property of the parent coal, the combustion type and finally storage and handling (Adriano *et al.,* 1980; Carlson and Adriano, 1993; Page *et al.,* 1979). The concentration of various elements also varies according to particle size (Page *et al.,* 1979; Khan *et al.,* 1996). Therefore, ash produced by burning of anthracite, bituminous and lignite coal has different compositions (Table 2).

Fly ash, physically comprised of fine particles having size from 0.01 to 100 µm (average diameter of <10 µm) (Davison *et al.,* 1974). Fly ash particles are hollow, empty spheres (cenospheres) filled with smaller amorphous particles and crystals (plerospheres). The cenosphere fraction constitutes as much as 1% of the total mass and becomes airborne easily (Hodgson and Holliday, 1966). The specific gravity of FA ranges from 2.1 to 2.6 g cm^{-3} and mean particle density for nonmagnetic and magnetic particles is 2.7 and 3.4 g cm^{-3} respectively (Natusch and Wallace, 1974). Bulk density of FA varies from 1 to 1.8 g cm^{-3} while moisture retention ranges from 6.1% at 15 bar to 13.4% at 1/3 bar (Natusch and Wallace, 1974). Chemically, FA is

heterogeneous in nature on account of being composed of large number of trace and heavy metals in variable proportions. About 95-99% of FA consist oxides of Si, Al, Fe and Ca, about 0.5 to 3.5% consists Na, P, K and S and the remainder is composed of trace elements (Kumar *et al.*, 1998). In general, the pH of FA ranges from 4.5 to 12.0 depends largely on the presence of S content in parent coal (Plank and Martens, 1974).

FA disposal

Present disposal of FA allover countries, follows wet and dry methods. In dry disposal, the FA is dumped in landfills and FA basins. In wet methods, the FA is mixed with water and piped as a slurry form into open artificial dams, lagoons or settling ponds is termed ash pond, and over time the water is allowed to drain away (Haynes *et al.*, 2009). In India, most common disposal practice is wet disposal method. Sushil and Batra, (2006) studied the wet and dry disposal strategies of three main FA generated plants in India. In wet disposal method, the surface of the pond ash is sprinkled with the water treatment plant sludge. This has some advantages of controlling the fugitive erosion of fine ash particles, enhancing the fertility of ash for growth of vegetation and ground cover and thirdly by providing a disposal option for the WTP (water treatment plant) sludge. Asia's first 100% dry FA producing plant has extraction system with transit ash storage silos and final storage in the form of ash mounds. Once the ash mound has reached the prescribed height, it is sprayed with polymer layer and vegetation is grown on top of it. In addition, the power plants have the flexible system for collecting graded dry FA from different fields of ESP and the

Fig 2.2: Disposal of fly ash in the form of wet slurry to nearby artificial impoundments at NALCO, Damanjodi, Orissa, India.

facilities to directly fill the FA into open land or trucks. As per the guidelines on utilization of FA, pond ash and bottom ash is supplied free of cost to all types of users on ‘‘as is where is’’ basis.

However, the discharge water from the pond is collected and discharged in a river or drain prior to sludge water treatment is source water pollution. Moreover, lining of ash ponds is not being followed in proper practice, which results seep in and degrades soil and endangers the environment. Therefore, ground water contamination by seepage from ash ponds may be more compared to leaching from landfills and ash mounds (Theis *et al.,* 1978). In addition to this, discharge of rain water and run off from the ash mound areas into surface water bodies can also be a source of water pollution (Sushil and Batra, 2006). Although dry disposal method saves the use of land and water, while transportation of dry ash can also source of air pollution. The coal industry in the United States spends millions of dollars on lining FA dumping grounds.

FA utilization

Many attempts have been established on utilization of FA in various applications because of stagnating use of FA in dumping and its impact on environment. As a result in the recent past, many applications have been made for FA. Major uses are in: cements, bricks, road base/embankments, building tiles, timber substitute products, consolidation of ground, land and mine filling reclamation, metal extraction, creation of cenospheres, wastewater treatment and most recently in agriculture (Asokan *et al.*,

Table 2.2: Typical concentrations of major and trace elements in fly ash

Major elements	**Units (%)**	**Trace elements**	**Units (mg kg-1)**
Al	5.0–25	As	2.0–70
Ca	0.8–30	B	2.0–5000
Fe	1.0–35	Sb	0.8–25
Si	2.0–3.0	Cd	0.1–100
Mg	0.04–5.0	Co	1.0–100
Na	0.04–7.9	Cr	3.0–900
K	0.15–6.0	Cu	10.0–2000
S	0.03–5.0	Hg	0.01–12
P	0.01–3.0	Mn	30.0–3000
N	0.01–1.0	Mo	1.0–250
		Ni	10.0–3000
		Pb	3.0–500
		Se	0.2–50
		Zn	10.0–1000
		Ba	0.01–1.0
		La	17–100
		Rb	30–200
		Cs	1.5–20

Source: Page *et al.* (1979), Bilski *et al.* (1995) and Asokan *et al.* (2005).

2005; Brain *et al.*, 2003). However, the average utilization of FA has been estimated and produced amount is slightly more than 30% (Asokan *et al.,* 2005; Haynes, 2009; Pandey *et al.,* 2009) and there are large differences in utilization of ash between countries. During 2005, the percentage utilization of FA was 100% in Italy, Denmark and Netherlands, 50-80% in USA and Germany and 45% in China. In India, FA utilization has increased from 15% (Sinha and Basu, 1998) to 38% in 2005 (Basu *et al.*, 2009). In view of above the significant amount of FA is still being disposed off in landfills by dry and wet disposal methods. Thus, the production and utilization of FA not at all proportional and it is an everlasting problem to environment until develop a suitable reclamation technology.

Although as mentioned earlier about beneficial effects of FA in agriculture, there are some possible concerns, which need to be consider under control, such as the release of toxic elements into ground water, decreased germination rates of some crops due to high levels of FA application including uptake of heavy metal toxic elements by the plants (Ferreira *et al.*, 2002). It is also reported that the extent use of FA increased the uptake of heavy metals, toxic elements and reduced synthesis of chlorophyll by plants when FA was applied to the soil (Yanusa *et al.*, 2009). These elements were found to be absorbed by plants grown on such soils (treated with FA) and could enter into the food chain (Ferreira *et al.*, 2002).

Vegetation establishment on FA

Many attempts of vegetation establishment on FA have been made by applying amendments. In a part of phytoremediation programme, plants of *Leucaena leucocephala* were grown in 100% soil, 100% FA and FA amended with 50% press

mud for 80 days. FA amended with press mud enhanced plant shoot, root and number of root nodules of *L. leucocephala*. The physiological responses such as chlorophyll, protein, *in vivo* nitrate reductase activity also enhanced compared to 100% fly ash treated plants. The accumulation patterns of all the estimated metals were different in both leaf and root. Uptake of Fe, Zn, Mn and Cu in leaves and roots from 100% fly-ash was significantly ($p < 0.01$) higher than uptake in those from amended ash and soil (Gupta *et al.*, 1999).

Cheung *et al.* (2000) evaluated the potential use of two legume species, *Acacia auriculiformis* and *L. leucocephala* for growth on ameliorated lagoon ash with or without nitrogen fixing bacteria. Amendments of 30% (w/w) vermiculite or with sewage sludge compost were added to improve the chemical and physical limitations of lagoon ash. The results showed significant suppressions in biomass and plant nutrient content with ameliorated lagoon ash in comparison to an agricultural soil. Nodulation was inhibited in ameliorated lagoon ash but not in agricultural soil. Both species showed potential to establish on amended lagoon ash, with *A. auriculiformis* being the best adapted. Sinha and Gupta (2005) showed that plants of *Sesbania cannabina* Ritz grown on different amendments of FA had a high accumulation of metals (Fe, Mn, Zn, Cu, Pb and Ni). The different amendments of FA with garden soil (GS) showed decreased metal accumulation with an increase in FA application ratio from 10% to 50% FA. The level of malondialdehyde, antioxidants (ascorbic acid, free proline cysteine, and non-protein thiol), chlorophyll, and carotenoid contents increased with an increase in the FA amendment ratio from 10% to 50% FA for all the exposure periods as compared to GS. Thus, the plants may be used for

phytoremediation of metals from FA contaminated sites and *S. cannabina* is suitable species for plantation on FA landfills. Yunusa *et al.* (2009) suggested that plant dry weights, rather than pigment concentrations and/or instantaneous rates of photosynthesis, are more consistent for assessing subsequent growth in plants supplied with FA. Rai *et al.* (2004) conducted a revegetation trail on FA to evaluate growth of legume species, *Prosopis juliflora* L. The FA was ameliorated with combination of various organic amendments, blue-green algal biofertilizer and *Rhizobium* inoculation. Significant enhancements in plant biomass, photosynthetic pigments, protein content and *in vivo* nitrate reductase activity were found in the plants grown on ameliorated FA in comparison to the plants growing in unamended FA or garden soil. Plants accumulated higher amounts of Fe, Mn, Cu, Zn and Cr in various FA amendments than in garden soil.

Juwarkar and Jumbhulkar (2008) studied the revegetation practice on FA ponds, applying biological interventions, organic amendment and selection of suitable plant species along with specialized nitrogen fixing strains of biofertilizers on 10 ha area of FA dump. The results showed that the improvement of physical properties of FA such as maximum water holding capacity from 40.0 to 62.42% while porosity improved from 56.78 to 58.45%. The nitrogen content was increased by 4.5 times due to addition of nitrogen fixing strains of *Bradyrhizobium* and *Azotobacter* species, while phosphate content was increased by 10.0 times due to addition of AM fungi. Due to biofertilizer inoculation different microbial groups such as Rhizobium, *Azotobacter* and AM spores, which were practically absent in FA improved to 7.1×10^7, 9.2×10^7 CFU/g and 35 AM spores/10 g of FA, respectively. Amendment of FA with Farm

Yard Manure (FYM) and biofertilizers helped in profuse root development showing 15 times higher growth in *Dendrocalamus strictus* plant as compared to the control.

Arbuscular mycorrhizae may make a substantial contribution to successful crop establishment in soils overlying areas of coal FA. Effects of two arbuscular mycorrhizal (AM) fungi, *Glomus mosseae* and *G. versiforme* on the growth and nutrient uptake of maize grown in different depths of soil layer overlying coal FA was studied by Bi *et al.* (2003). Colonization by both AM fungi increased plant growth compared with non-mycorrhizal controls. *G. mosseae* gave higher yields of maize than *G. versiforme* at the same depths of soil. Inoculation with arbuscular mycorrhizal (AM) fungi along with different amendments can be promising approach for rehabilitation of FA. Wu *et al.* (2009) studied the effect of *Glomus mosseae* and *Rhizobium* on *Medicago sativa* grown on mixture of coal wastes and sands (CS), coal wastes and FA (CF), and FA (FA). The results showed that dual inoculation in CS and CF substrates elicited a synergistic effect on growth and yield parameters of plant. In a study by Hrynkiewicz *et al.* (2009) concluded that inoculation with mycorrhizae promoting bacterial strains (*Sphingomonas* sp. 23L) might be a suitable approach to support mycorrhiza formation with autochtonous site-adapted ectomycorrhizal fungi (*Geopora* sp.) in FA and thereby to improve re-vegetation of FA landfills with willows clones (*Salix viminalis* and *S. viminalis* x *Caprea*).

Mycorrhizae

Many soil microorganisms originate in the soil or are closely associated with soil environments and have substantial impact on mankind. Soil microorganisms inhabiting the rhizosphere environment interact with plant roots and mediate nutrient

availability, for example those forming useful symbiotic association with roots and contribute to plant nutrition. Among them, mycorrhizal fungi occur in the soil of most ecosystems, including polluted soils by acquiring mainly phosphate, micronutrients and water and delivering a proportion to their hosts and enhance the nutritional state of their hosts (Smith and Read, 1997). There are several types of mycorrhizae mainly characterized on basis of morphology, host preference and mode of infection. The most widespread and researched groups are ectendomycorrhiza, ectomycorrhizae, arbuscular mycorrhizae, orchidaceous mycorrhizae and ericaceous mycorrhizae.

Arbuscular mycorrhizal fungi (AMF)

Amongst the mycorrhizal fungi, arbuscular mycorrhizal (AM) fungi are universal, ubiquitous and obligate and form symbiotic relationships with roots of 80% to 90% land plants in natural ecosystems. Arbuscular mycorrhizal fungi belong to the fungal phylum Glomeromycota (Schüßler *et al.*, 2001). The phylum Glomeromycota is divided into four orders, eight families and ten genera. The genera which include most of the described species are *Acaulospora*, *Gigaspora*, *Glomus* and *Scutellospora* (Schüßler, 2005). Among soil microorganisms, AM fungi provide a direct link between soil and roots and play relevant roles for establishment, survival of plant species, maintain plant diversity and improved soil properties in stressed environments (Koide and Dickie, 2002; Vivas *et al.*, 2005; Zarei *et al.*, 2008a; Ortega- Larrocea *et al.*, 2010).

AM fungi in alkaline, saline-sodic and heavy metal contaminated soils

Red mud is highly alkaline and saline sodic in nature due to presence of caustic soda (NaOH) and metal hydroxides. Alkaline–saline soils are soils with a pH >9 that

contain large amounts of soluble salts or soluble Na^+ and have a high electrolytic conductivity (Fitzpatrick *et al.*, 2001). Hence, somehow it shows resemblances with RM. Several studies have reported that the plants colonized by AM fungi in alkaline conditions (Ileana *et al.*, 2007; Oliveira *et al.*, 2005), saline-alkaline soil (Juniper and Abbott, 1993; Wang *et al.*, 2004), salt marshes (Carvalho *et al.*, 2004), and saline-sodic soils (Escudero and Mendoza, 2005; Mendoza *et al.*, 2005). Adverse environmental conditions can negatively affect the infectivity and survival of AM fungal propagules from one period of root growth to the next (Juniper and Abbott, 1993). Previous contributions of AM fungi suggest that it could survive in roots of some species and improve tolerance to alkaline and saline-sodic soils (Escudero and Mendoza, 2005; Mendoza *et al.*, 2005; Oliveira *et al.*, 2005). Improved salt tolerance of mycorrhizal plants can be mainly related to enhance mineral nutrition, particularly N or P (Graham,1986), and changes in physiological processes such as increased carbon dioxide exchangeable rate, transpiration, stomatal conductance and water efficiency (Ruiz-Lozanoand Azcon, 2000).

AM fungi have been reported in plants, growing on heavy metal contaminated sites indicating that these fungi have evolved a heavy metal tolerance and play an important role in the reclamation and recovery of potentially disturbed environments. Thus, the tolerance and diversity of AM fungi in heavy metal contaminated soil are indicative of AM fungal adaptation to heavy metals (Colpaert and Vandenkoornhuyse 2001; Joner *et al.*, 2000; Leyval *et al.*, 1995). However, spontaneous selection of infective and effective AM fungi is a long process in FA ponds. It has also been demonstrated that the use of adapted AM fungal strains in restoration and

bioremediation studies, is more effective than applying non-adapted strains (Enkhtuya *et al.,* 2000; Vivas *et al.,* 2003) Hence, it is necessary to understand the ecological role of AM fungi associated with plants growing in these sites and use of the native AM fungi for soil remediation purposes by vegetation of mining sites or barren land or restoration of disturbed ecosystems (Chen *et al.*, 2007) which is beneficial in environment management.

AM fungal diversity analysis

The diversity of AM fungi has been studied based on spore morphology and molecular methods. The taxonomic identification of AM fungal species is based upon morphological characteristics of their spores (size, color and spore wall characteristics) or spore bearing characters and currently about 200 morphospecies are distinguished based on spore walls (Redecker, 2006; http://www.amf-phylogeny.com). The way the spore is formed on the hypha (‘‘mode of spore formation’’) has been important to circumscribe genera and families; the number of spore walls and their layered structure as well as ornamentation are used to distinguish species (Morton, 1988). Walker (1983) established the murographs concept to describe and compare the layered structure of the spore walls more easily. Morton (1995) and Sturmer and Morton (1997 and 1999) included considerations of the spore development to group these wall components hierarchically into complexes linked by ontogeny.

Although these characters are specific, the task of identifying spores based on their morphology is notoriously difficult and similar spore types can be formed by fungi that are distantly related (Redecker *et al*., 200). Merryweather and Fitter (1998)

suggested that there is always no correlation in spore formation and root colonization and thus the AMF community that is interacting with rhizospheric microorganisms and plants cannot be reflected by evaluation of AM fungal communities based only on spores. However, Morton and Redecker (2001) has reported several lineages of AM fungi that do not stain at all within the roots or only very weakly using the standard dyes. Field-collected spores are often parasitized or degraded and therefore unidentifiable which is another limitation of morphological identification (Bever *at al*., 2001). This problem can be circumvented by obtaining fresh spores of all developmental stages using "AM fungal trap cultures" i.e. the soil samples from the field site are brought into contact with suitable plant hosts of AM fungi under controlled conditions in order to propagate the species occurring at the field site (Redecker *et al*., 2003). However, the kind of AM fungi detected, is influenced by the plant species used in the trap cultures (Jansa *et al*., 2002).

Moreover, because the morphological characters are scarce in some AMF, even the identification of healthy spores sometimes may pose problems. For example, *Glomus* and *Paraglomus* are distantly related but cannot be distinguished by spore morphology as it is more or less same (Morton and Redecker, 2001). Even after the separation of the genus from *Paraglomus*, the genus *Glomus* is still polyphyletic, because according to molecular criteria, it comprises two independent lineages (Schwarzott *et al*., 2001). The morphological identification of dimorphic AM fungal species (e.g. *Archaeospora leptoticha*, *Glomus dimorphicum*) can also cause further confusions. However, potential limitations of morphological approach are widely acknowledged and accepted (Reddy *et al.*, 2005).

Molecular approach

Molecular approach is an alternative approach that avoids the limitations of cultivation and morphology and facilitates the direct analysis of fungal nucleotide sequences within the spores, roots and soil. Molecular methods combined with the polymerase chain reaction (PCR) have been used in studies of the diversity and phylogeny of AM fungi (Clap *et al.*, 2002; Rani *et al.*, 2004; Sanders *et al.*, 1996). Significant successes have also been achieved in field studies based on molecular markers and provided numerous new insights into not only identification but also in the dynamics of AM fungal communities and have presented evidence for host preference and host specificity (Helgason *et al.*, 2002). The molecular studies have also given the effects of season and plant host development stage (Husband *et al.*, 2002), as well as an influence of agriculture and ecosystem type (Hijri *et al.*, 2006) on AM fungal communities. These studies have also shown that while some AM fungal species appear to be rather limited in their distribution, others are generalists and surprisingly widespread (Opik *et al.*, 2006).

Presently, AM fungal molecular identification and phylogeny has been established by sequence analysis of the nuclear ribosomal encoded DNA (rDNA) (Redecker, 2006). The rRNA genes (28-26S, 18S, 5.8S, and 5S) are present in multiple copies and repeated several hundred to a few thousand times in the genome of one or a few chromosomes (Rooney and Ward, 2005). Despite limitations of the 18S rRNA gene, it has been the most widely used, exploiting both the conserved and the variable regions contained within it (Helgason *et al.*, 1999; Lee *et al.*, 2008; Sykorova *et al.*, 2007; Wubet *et al.*, 2006). In addition, internal transcribed spacer

(ITS) regions are located between the 18S rRNA and 28S rRNA genes, and incorporating the 5.8S rRNA gene, have also been targeted (e.g. Gardes and Bruns, 1993; Larena *et al.*, 1999; White *et al.*, 1990). Some studies also used a part of the large subunit (LSU) rRNA gene (Gollotte*et al.*, 2004; Mummey and Rillig, 2008; Rodríguez-Echeverría 2009; Rosendahl *et al.*, 2004; Wu *et al.*, 2007). Studies on restriction site variation in the ribosomal DNA (rDNA) in populations have shown that coding regions are conserved (18S and 28S), while spacer regions (ITS) are variable (Alvarez and Wendel, 2003). Non-coding rDNA spacer regions, such as the ITS, benefit from a fast rate of evolution, resulting in greater sequence variation between closely related species compared with the more conserved coding regions of the rRNA gene cluster. Thus, internal transcribed spacer (ITS) region of the rDNA also allows species composition analysis (Wubet *et al.*, 2003).

Different authors have developed AM fungal-specific primers targeting different regions of the small subunit of the rDNA (18S). Simon *et al.* (1992) designed the first primer VANS1 to be specific for AM fungi and targeted to the 5' end of the SSU, and does not amplify all glomeromycotan lineages (Clapp *et al.*, 1995). Moreover, its targeted region is not variable enough to distinguish taxa and amplification problems appeared when it was used for field root samples (Clapp *et al.*, 1999). Helgason *et al.* (1998), in the meanwhile, designed the AM1 primer, which in combination with the universal primer NS31 amplifies the variable central region of the SSU. This primer combination is also widely used in field studies (Opik *et al.*, 2006). However, it does not amplify Archaeosporaceae and Paraglomeraceae and neither *Glomus* group B. Other authors constructed primers targeting the LSU of

rDNA (Gollotte *et al.*, 2004; Kjoller and Rosendahl, 2000). Similar to the AM1/NS31 primer pair, these primers also amplify only a subset of the Glomeromycotan taxa.

Redecker, (2000) and Redecker *et al.* (2003) designed a different primer sets GLOM1310/ITS4i (specific for *Glomus* group A), GLOMBS1670/ITS4i (specific for *Gloum mosseae* sub group of within *Glomus* group A) LETC1677/ITS4i (specific for *Glomus* group B), ACAU1661/ITS4i (*Acaulosporaceae*), ARCH1311AB/ITS4i (*Archaeosporaceae*), PARA1313/ITS4i (*Paraglomus*) NS5/GIGA5.8R or NS7/GIGA5.8R or GIGA1313/GIGA5.8R (*Gigasporaceae*) for the partial SSU and ITS regions allowing to detect seven genera of the Glomeromycota including *Archaeosporaceae* and *Paraglomeraceae*, which is the largest possible portion of taxon diversity recognized so far. Regarding this, polymerase chain reaction (PCR) has performed in a nested procedure for the amplification of rDNA region of AM fungi. The first round of amplification nested procedure has performed using universal eukaryote primers NS5 and ITS4 to reduce the complicity of genomic DNA from target DNA (White *et al.*, 1990).

Different AM fungal taxa may be present in a single root sample and many different variants of the same gene can be present in a single spore, so the PCR products obtained by the primer combinations mentioned above have to be cloned and sequenced. For screening of the clones, different techniques such as restriction fragment length polymorphism (RFLP; Helgason *et al.*, 1999), single strand conformation polymorphism (SSCP; Kjoller and Rosendahl, 2000) and denaturing gradient gel electrophoresis (DGGE; de Souza *et al.*, 2004; Santos *et al.*, 2006) may be used. An effective approach of finger printing of AM fungal community in large

numbers of samples without the requisite of cloning and sequencing appears to be t-RFLP (terminal restriction fragment length polymorphism; Lekberg *et al.,* 2007; Mummey and Rillig, 2006). However, it is impossible to detect the non-specific (non-AM fungi) PCR amplicons of used primers, by this approach.

Diversity of AM fungi in alkaline, saline-sodic, heavy metal contaminated sites

Although AM fungal symbiosis is widespread and providing a direct physical link between soil and plant roots, the symbiotic functions of AM fungal species are not equivalent and vary according to the specific AM fungal isolates, host plants, soil properties and seasons (e.g. Hempel *et al.,* 2009; Hildebrandt *et al.,* 1999; Karthikeyan and Selvaraj, 2009; Kelly *et al.*, 2005; Redon *et al.,* 2008, 2009). Therefore, the identification of specific phylotypes of AM fungi and their relationship with soil properties are crucial and provide an important step to understand the ecology of AM fungi at heavy metal (HM) contaminated ecosystems (Gaur and Adholeya, 2004).

Several surveys on the effect of alkalinity, saline-sodicity and heavy metals on diversity and abundance of AM fungi have been conducted in alkaline, saline-sodic and heavy metal polluted areas around the globe (e.g. Bedini *et al,* 2010; Chao and Wang, 1990; del Val *et al.*, 1999; Oleivera *et al.*, 2006; Regvar *et al.*, 2003; Sambandan *et al.*, 1992; Vallino *et al.*, 2006). Colonization and AM fungal distribution in heavy metal soils is not only highly correlated with soil parameters, such as soil nutrient concentration and pH, (del Val *et al.*, 1999), but also depends upon the heavy metal content, salinity gradient in the soil and seasonal variation. Because, increasing heavy metal contamination, salinity gradient between the sites as

well as seasons studied were followed by a decrease in AM fungal spore numbers, mycorrhizal colonization parameters and the number of AM fungal sequence types colonizing the roots (Karthikeyan and Selvaraj, 2009; Wilde *et al.*, 2009; Zarei *et al.*, 2008a; Zarei *et al.*, 2010).

However, AM fungal propagules never disappeared completely even in soils with the highest rates of heavy metals and only spore numbers were affected by HM concentrations than root colonization. The variations of AM fungal propagules were better related to available than to total concentration of metals. Spore numbers were positively correlated with mycorrhizal colonization parameters, particularly with arbuscules abundance (Zarei *et al.*, 2008b). It also found that the number of AM fungal spores did not significantly decrease with soil salinity and reported a relatively high spore number (mean of 100 per 10 g soil) (Evelin *et al.*, 2009). The higher fungal spore density in saline soils may be due to the fact that sporulation is stimulated under salt stress (Tressner and Hayes, 1971) which means that AM fungi may produce spores at low root-colonization levels in severe saline conditions (Aliasgharzadeh *et al.*, 2001).

Chapter 3

Materials and methods

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Sample collection

Red mud (RM) and fly ash (FA) samples were collected randomly from different locations of the red mud and fly ash sedimentation ponds (GPS location 18° 46' 26" N 082° 53' 37" E) respectively of National Aluminum Company Ltd. (NALCO), Damanjodi (18° 49′ 12″ N, 82° 43′ 12″ E), Orissa (India). Samples were collected from 0-40 cm of depth by sterilized stainless steel implements and sterile bags. Red mud samples were collected after removal of upper soda flakes. All the respective samples were pooled and mixed thoroughly, and transported to laboratory at 4 °C. Samples were air-dried, crushed and sieved to < 2 mm and some portions of samples were maintained at 4°C for further biochemical analysis. The remaining part of red mud and fly ash samples were processed and analyzed for physico-chemical characteristics.

Collection and isolation of arbuscular mycorrhizal fungi

Root samples along with the RM flooded rhizosphere soil samples were collected from plants growing adjoining to RM pond due to absence of vegetation. In case of samples regarding fly ash, the roots and the adhered rhizosphere fly ash samples were collected from plants, naturally growing in the pond. The plants were identified as far as possible from vegetative material. The samples were collected from 0-40 cm depth

and three replicates were collected for each plant species. All the respective samples (soil and roots) of each plant species were mixed thoroughly and preserved with proper labeling at 4 °C until processing. Some amount of composite root samples were stored in formyl-acetic acid [FAA: aquatic solution of 6.0% formaldehyde, 2.3% glacial acetic acid and 45.8% ethanol (all v/v)] for root percent colonization analyses.

AM fungal colonization

The percentage of root length colonized by AM fungi was calculated by the gridline intersect method (Giovannetti and Mosse, 1980) after staining with trypan blue (Phillips and Hayman, 1970).

1. The roots sample collected from the plants growing adjoin to the RM pond were washed in 0.1% 'Teepol' followed by distilled water to remove surface adhered RM. Some amount of washed samples of roots were chopped to 1cm and simmered in 10% KOH at 90 °C for 1-2 h.
2. The roots were rinsed 3-4 times in tap water and acidified by immersing them in 2% hydrochloric acid for 5 min.
3. The acid was drained off, 0.05% trypan blue in lactophenol (Appendix I) was added and boiled again in stain for three min.
4. Drained off the stain, added lactophenol and left for 30 min to destain the tissues.
5. The roots were examined by mounting in glycerol.
6. Stained root samples were examined microscopically between 10 and 20x magnification to assess the percent root length colonization of AM fungi using the gridline intersects

$$\% \text{ root length colonization of AM fungi} = \frac{\text{no. of horizontal and vertical intersects with roots}}{\text{no .of root pieces infected with AM fungi}} \times 100$$

AM fungal spore isolation and enumeration

The spores were isolated by Wet Sieving and Decanting Technique (Gerdeman and Nicolson, 1963) from the rhizospheric samples.

1. Around one third of containers were filled with rhizosphere soils, mixed well with water until all soil aggregates were dispersed and even suspensions were made. It was kept undisturbed for 6 h.
2. The suspensions were decanted through 4 sieves of different sizes arranged in the order of 710 µm, 250µm, 105 µm and lastly 50 µm sieves along with continuous washing with a jet of water under the tap.
3. The washings of 50 µm sieve were collected in one 1litre beaker.

Inoculum production of arbuscular mycorrhizal fungi (trap culture)

In the field samples, low spore number, parasitization of spores, age and environmental alteration of spores (e.g. discoloration) will hinder accurate identification (Bever *et al*., 2001). Moreover, the spore populations recovered at field site may not reflect the vegetative fungal population in the natural community and therefore trap culture may be used to possibly recover more fungal species than detected by the direct analysis of spores from field soils. By this approach, enough AM fungal inoculum can be obtained for the micro-field experiments and maintenance of AM fungal adaptation. Hence, the AM fungal populations collected from the field soils were multiplied in trap cultivation under greenhouse condition using trap plants (maize, wheat and sorghum). The five seeds of each plant species

were sown in 1kg capacity pots containing unsterilized red mud and heat sterilized (121 ^{o}C for 3h) sandy loam soil in the ratio of 40:60 (v/v) and sub samples of spores and roots pieces collected from adjoin to red mud pond. Whereas, in case of fly ash the five seeds of each plant species were sown in pots containing 100% unsterilized fly ash with sub samples of spores and root pieces obtained from rhizospheric fly ash samples collected from fly ash pond. For each of the trap plants, five replicates were maintained. The seeds of these three trap plants were surface sterilized with 0.5% $NaOCl_2$/0.01% $HgCl_2$ and subsequently washed several times with distilled water before sowing. Plants were allowed to grow for two successive cycles of three months each. The pots were watered with tap water and maintained under green house conditions. No organic/manuring amendment was added to pots while multiplying the AM fungi. After the experimental period, the inoculum for RM experiments was prepared by mixing spores and chopped roots produced from trap plants (colonized by AM fungi in RM) in the density of 90-100 spores/10 g of sterile vermiculite. However for FA experiments, the inoculum was prepared by mixing spores and chopped roots of trap plants (colonized by AM fungi in FA) in the density of 90-100 spores/10 g of fly ash. The subsamples of spores were allowed for morphological identification.

Inoculum production of *Aspergillus tubingensis*

The phosphate solubilizing fungus *A. tubingensis*, isolated from the rhizosphere soil of eucalyptus plantation in Punjab, India (Reddy *et al.*, 2002) was used as co-inoculant. Fungal inoculum was prepared by growing it on Pikovskaya's agar (Pikovskaya, 1948; Appendix I) plates at 28 °C up to formation of dense spores.

Spores were scraped and mixed with sterile vermiculite with inoculum size of $1.7x10^8$ spores/g vermiculite.

Inoculum production of red mud bacterial isolates

Indigenous bacterial consortium isolated from red mud pond of NALCO (Table 4.6) (Pankaj Krishna, 2008) was also used as co-inoculant with AM fungi in this study. Bacterial isolates were grown in RM amended nutrient broth, Horikoshi and tryptic soya broth at pH 10.0, (Appendix I) and flasks were incubated at 37°C with constant shaking at 120 rpm till the absorbance (A_{600}) reached to 1.0. Bacterial consortium was concentrated by centrifugation and mixed in sterile soilrite. Enumeration of the colony forming unit was done by serial dilution method using same media used for bacterial culture growth. The inoculum was produced around 300 Log CFU/50 g soilrite.

Table 4.6: Red mud isolates used in this study and their closest relative species inferred from 16S rRNA gene sequences of existing database

S. No	Red Mud Isolates	Identified names
1	RM9P	*Planococcus maitriensis*
2	RM10E	*Bacillus cereus*
3	RM10D	*Micobacterium hominis*
4	RM11R	*Salinicoccus roseus*
5	RM12W	*Pseudomonas pseudoalcaligenes*
6	RM13Y	*Kocuria palustris*
7	RM1	*Kocuria polaris*
8	RM1A	*Bacillus* sp.
9	RM6	*Bacillus litoralis*
10	RM8	*Agromyces mediolanus*

AM fungal identification

Identification of AM fungal spores based on spore morphology

Isolated spore samples from the adjoining areas of RM pond, FA pond and pot cultures were identified according to their morphological characteristics including shape, size, color, distinct wall layer, attached hyphae and surface orientation of spores. Spores were examined microscopically and identified according to the taxonomic system proposed by Morton (1988) and Walker (1992). Original descriptions were consulted (Schenck and Perez, 1990) and spore morphology was compared with an internet-published reference culture database established by Morton (http://invam.caf.wvu.edu/Myc_Info/ Taxonomy/ species.htm). Spores were also compared with freshly formed AMF spores from trap cultures originating from the same field site. Spores were observed and photographed with either a stereomicroscope (Olympus SZX12), using combined through- and reflected-light illumination provided by fibre optics or on a compound microscope (Olympus AX70) using Nomarski contrast.

The spores were mounted in water (for stereomicroscopy only) or in either polyvinyl-lacto-glycerol (PVLG) or PVLG + Melzer's reagent (1:1 v:v) (Appendix I). At least 20 spores of each species were used for identification. Only apparently viable spores were used for the identification with the exception of damaged spores recovered from the field soil. In this case, identification could be performed only to the genus level based on the spores from the field soil. Some spore specimens could not be identified to species as only a few spores were isolated, or the spores lacked distinguishable, fine taxonomic characters. These spores were statistic in spore

density (spores in 100 g soil) and not statistic in species richness (species numbers in each soil sample).

AM fungal molecular identification

DNA extraction from roots

Fine root samples collected from plants growing in adjoining area of red mud and fly ash pond were pooled separately and ground to powder in liquid nitrogen with sterile ceramic pestle and mortar. Total DNA was extracted using DNeasy Pant Mini Kit following the manufacturer's instructions (Qiagen, Hilden, Germany).

Electrophoresis of DNA on non-denaturing agarose gels

DNA samples were loaded on a 0.7% (w/v) agarose gels prepared in 0.5x TBE buffer pH 8.0 (Appendix I) using a 6x loading dye (Appendix I). Ethidium bromide (EtBr) (0.5 µg/ml) was added to stain the gel prior to pouring. DNA was then migrated at 60 volts for 45-55 min and visualized on a U.V. transilluminator (312 nm).

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was performed in a nested procedure for the amplification of rDNA region of AM fungi as described by Redecker (2000) to eliminate nonspecific amplification from plant roots with minor modification. The first round of amplification was performed using universal primers NS5 and ITS4 (White *et al.*, 1990). To estimate the quantity of amplified product, amplicons were diluted in TE buffer (1:10, 1:50 and 1:100) and 5 µl of each dilution was run on a 1.5% (w/v) agarose gel. In the second round PCR, different AM fungal genera

specific primers, ARCH1311, ACAU1660, LETC1670, GLOM1310 and PARA1313 (Table 3) were used in combination with ITS4i in separate reactions. The reverse primers GLOM5.8 and GIGA5.8R were used only in combination with NS5 primer (Redecker, 2000; Redecker *et al.*, 2003) (Fig 3).

The PCR reaction was carried out in a final volume of 25 µl containing 1x PCR reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 0.5 µM of each primer and 0.15 units of *Taq* DNA polymerase (Larova, Teltow, Germany). The PCR program was carried out as follows: initial denaturation at 95 °C for 4 min, followed by 25 cycles of 1 min at 95 °C, 1 min at 51 °C annealing temperature (annealing temperature for second round PCR at 61 °C), 1 min at 72 °C and a final extension of 72 °C for 8 min. Amplifications were performed using a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA). Amplified products were visualized on a 1.2% agarose gel.

Purification of PCR product

The PCR products were run on a 0.8 % (w/v) agarose gel electrophoresis prior to cloning. The target bands were excised and purified with QIAquick PCR purification Kit (Qiagen, USA) following the instruction of the manufacturer.

Nucleic acid quantification

The concentration of extracted DNA in suspension was estimated by spectrophotometric measurement at A_{260}. For double-stranded DNA suspensions, an OD of 1.0 at a wavelength of 260 nm and using a cuvette with 1 cm light path, is equal to a concentration of 50 µg/mL. The quality of the DNA was evaluated by

measurement of the A_{260} and A_{280} and the A_{230}/A_{260} ratios. Ideally, the A_{260}/A_{280} ratio should be 1.8-2.0. Ratios less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

Ethidium bromide fluorescent DNA quantification (visual method)

DNA was migrated electrophoretically in an agarose gel containing ethidium bromide (0.5µg/ml) with reference to a known linear double stranded DNA concentration (20 ng /µl) of lambda phage (MBI Fermentas, USA) by comparing the intensity of fluorescence.

Table 3: Oligonucleotide primers used for PCR reaction in this work

Primer	**Sequence**	**Target**
ACAU1661	5'-TGAGACTCTCGGATCGGG-3'	Acaulosporaceae
ARCH1311	5'-TGCTAAATAGCCAGGCTGY-3'	Archaeosporaceae
GIGA5.8R	5'-ACTGACCCTCAAGCAKGTG-3'	Gigasporaceae
GLOM1310	5'-AGCTAGGYCTAACATTGTTA-3'	*Glomus* group A
GLOM5.8R	5'-TCCGTTGTTGAAAGTGATC-3'	Archaeosporaceae/ *Paraglomus*
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	ITS regions of fungi
ITS4i	5'-TTGATATGCTTAAGTTCAGCG-3'	ITS regions of fungi
LETC1670	5'-GATCGGCGATCGGTGAGT-3'	*Glomus* group B
NS5	5'-AACTTAAAGGAATTGACGGAAG-3'	General fungi
PARA1313	5'-CTAAATAGCCAGGCTGTTCTC-3'	*Paraglomus*

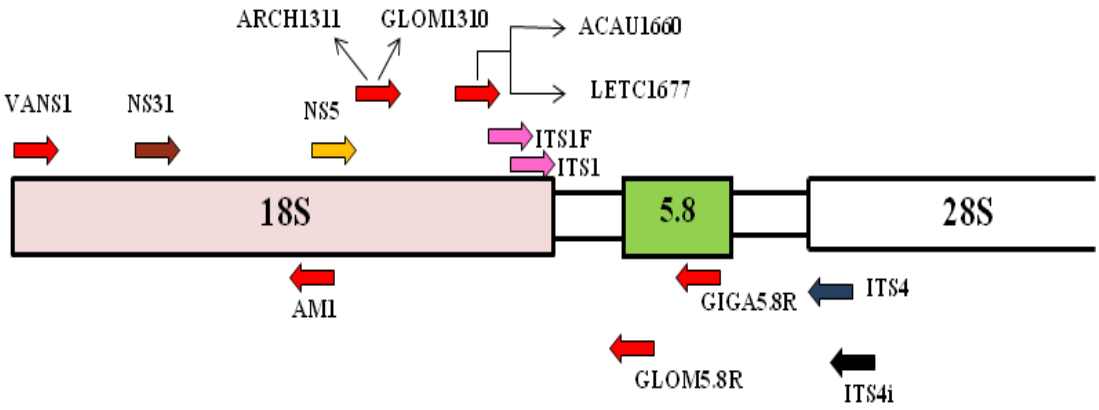


Fig 3: Schematic diagram of rDNA structure and different primer sites for study of AMF community. Locations with red color indicated AMF-specific primers, in white universal primers. Diagram is not to scale.

Ligation

Purified PCR products were cloned using PGEM -T Easy Vectors Kit (Promega Cat no. A1360) and ligation was carried out according to manufacturer's instruction.

Ligation reaction components were added as following

PGEM –T vector	1µl
10x ligation buffer	5 µl
T4 ligase	1 µl
Purified DNA	x µl
Nuclease free milli Q water	x µl

The sample was incubated at 4°C overnight.

To stop the reaction, the samples were kept at -20 °C. Positive and negative controls were also used to access the performance of the PGEM -T Easy Vector system. For the positive control, a control insert DNA supplied with the kit was used while for the negative control no insert was added.

Transformation of DH5α cells by heat shock method

1. Freshly grown *E.coli* DH5α cells were taken from glycerol stock after streaked on Luria Agar (LA) plate incubated at 37 °C overnight.
2. Single colonies were isolated and inoculated in 25 ml of Luria Broth (LB) (Appendix) and incubated overnight at 37 °C.
3. 200 µl of the overnight grown culture was inoculated in 25 ml LB and incubated in a shaker at 180 rpm for two and half hour and the culture was centrifuged at 5000 rpm for 10 min at 4 °C in autoclaved 30 ml Oak Ridge tubes.
4. The supernatant was discarded. 10 ml of filter sterilized 0.1M $CaCl_2$ was added and the tubes were incubated in ice for 15 min.
5. The cells were again centrifuged and the supernatant was discarded. 1 ml of 0.1M $CaCl_2$ was added, and the tubes were incubated in ice for two and half hour to make competent cells.
6. To 100 µl of competent cells, 5 µl of ligated product was added, mixed gently and kept in ice for 30 min for binding of the plasmid to the cells.
7. Then the cells were given a heat shock treatment.
8. With the help of floater, tubes were kept for heat shock treatment at 42 °C in a circulating water bath without shaking for exactly 2 min.

9. The cells were rapidly transferred into ice and kept for 2-3 min.

10. One ml of LB was added to each tube and the tubes were incubated for 1 h at 37 °C to allow the bacteria to recover and to express amp^r gene of the transformed cells.

11. 100 µl of the transformed cells were spreaded on LA-Amp.-X-gal-IPTG (Appendix I) plates.

12. The plates were incubated at 37 °C for overnight for appearance of colonies.

Screening of recombinant (positive clones) bacterial cells

Transformed plasmid with or without insert DNA into the ($LacZ^-$) DH5α bacterial cells were plated on LA-Amp.-X-gal-IPTG plates (Appendix I) for screen of positive clones containing recombinant plasmid. If the insert was present in the multiple cloning site (MCS) in the pGEM-Teasy vector's LacZα gene, non functional β-galactosidase is produced, which results transformed bacterial colony and appears white. White colonies were picked and grown in 2 ml LB containing ampicillin (50 µm/ml) for plasmid isolation and simultaneously respective clones were patched on LA-Amp. plates.

Bacterial plasmid DNA isolation

The plasmid DNAs of the recombinant bacterial cells were isolated to confirm the presence of the insert. The insert specific primers were used for PCR reaction to know whether the insert is present in the plasmid or not.

1. The recombinant colonies were inoculated in 2 ml of LB containing ampicillin and incubated overnight at 37 °C.

2. 1.5 ml of the culture was taken in a fresh microfuge tube and centrifuged the cells at 8000 rpm for 1min. The supernatant was discarded.

3. 100 µl of ice cold Solution 1 (Appendix I) was added, vortexed and kept at room temperature for 5 min.

4. 200 µl of freshly prepared Solution II (Appendix I) was added to the cells, mixed the contents by inverting the tubes 10-12 times vigorously to ensure that the pellet was dispersed, and kept for 2 min at room temperature.

5. 150 µl of ice cold Solution III (Appendix I) was added and the tubes were inverted slowly 10-12 times and kept in ice for 5-10 min.

6. 400 µl of phenol: chloroform: isoamylalcohol (25:24:1) was added to the pellet and inverted the tubes for 2 min and centrifuged at 12,000 rpm for 10 min.

7. The upper aqueous layer was carefully transferred to a fresh tube and added equal volume of chilled isopropanol.

8. After proper mixing, the tubes were kept at room temperature for 5 min and centrifuged at 12,000 rpm for 10 min for precipitation of DNA.

9. The supernatant was discarded and the tubes were kept in inverted position to allow all of the fluid to drain away.

10. Pellet was washed with 400 µl of 70% ethanol and centrifuged at 12,000 rpm for 10 min.

11. The pellet was air-dried and dissolved in 50 µl of TE buffer (pH 8.0).

Restriction digestion analysis by electrophoresis

The restriction fragment length polymorphism (RFLP) analysis of amplicons was performed using *Hinf*I and *Mbo*I enzymes.

In 1.5 ml tubes added following reaction components for 20 µl final reaction volume

Deionized H_2O	7 µl
10x restriction enzyme buffer	2 µl
PCR product (50 ng/µl)	10 µl
Restriction enzyme (5 units/µl)	1 µl

The tubes were spun down at slow speed for 5–10 sec and were incubated at the appropriate temperature for 2 h. To stop the reaction, 5 µl of gel loading buffer was added, mixed by vortexing and ran on ethidium bromide – stained 2% agarose gel to determine the distinct RFLP patterns of clones.

Sequence analysis

The inserts with different RFLP patterns in the plasmids were sequenced by chain termination method (Sanger *et al.*, 1977) using Applied Biosystems automatic sequencer (DNA sequencing facility, Department of biochemistry, South campus, Delhi University, New Delhi, India). The sequencing reactions were performed with M13 forward and reverse primers (Promega, Madiason, WI, USA).

Sequence similarities were determined using the BLAST sequence similarity search tool provided by GenBank of NCBI (Altschul *et al.*, 1997). Sequences of AM fungi are deposited in the NCBI database for accession numbers. The sequences

along with reference sequences were aligned with MAFFT programme (http://mafft.cbrc.jp/alignment) with some manual adjustment. The phylogenetic analysis was carried out by neighbor-joining method using MEGA software (Tamura *et al.,* 2007) with Kimura 2-parameter model. Bootstrap values of 1000 were used to assess the relative support for each clade.

Micro-field nursery trails to rehabilitate the red mud (bauxite residue) pond

A micro-fiend experiment was conducted at NALCO nursery, Damanjodi, Orissa by inoculation of RM adapted AM fungi with different amendments. The amendments like Gypsum, topsoil, sludge and fly ash were used as amendments to change the physico-chemical properties of RM. The RM, FA and gypsum were brought from NALCO, whereas sludge and top soil were collected locally. *Aspergillus tubingensis*, a phosphate solubilizer (Krishna *et al.*, 2005) and indigenous bacteria isolated from of red mud (Krishna, 2008) was also used with combination of AM fungi to know the synergetic effect on amelioration of the RM soil. Moreover, to explore the effect of the use of these microbes on AM fungal colonization, the growth and survival of the plants in red mud ponds were also studied. Because of the unproductivity of the RM; topsoil, sewage sludge, gypsum and FA were amended to RM and inoculated with AM fungi, consortium of RM bacterial isolates (Krishna, 2009), *A. tubingensis* and combination of AM fungi + bacterial isolates + *A. tubingensis* (consortium). The spore inoculum (100 g/plot having 1.7 x 10^8 spores/g) of *A. tubingensis*, the indigenous bacterial consortium (~350 Log CFU/plot) and AM fungal inoculum (50 g/plot having 90-100 spores/10 g of soil) was mixed in sterile vermiculite to prepare the inoculum. A native alkali-tolerant bermudagrass was

planted to check the performance of chemical and biological amendments of RM for the revegetation.

Experimental design

Randomized block design was prepared and the treatment plots were designed in such a way that every chemical amendment had five treatments such as RM amended with gypsum contained control (without any biological inoculation), AM fungi, bacterial consortium (BC), *A. tubingensis* and consortia of AM fungi + *A.tubingensis* + BC inoculation. Same pattern was followed for sewage sludge, topsoil, FA and without any amendment of RM. The plot size of each treatment was 72 x 72 x 30 cm^3. All the amendments were added at 10% to RM and were mixed in the range of 15 cm from the upper layer to reduce the dilution of amendments. An alkali-tolerant bermudagrass (*Cynodon dactylon)* maintained at NALCO nursery was collected washed with water prior to planting. The indigenous AM mycorrhizal colonization was determined prior to planting. About 125 healthy seedlings having similar length (5 cm) were planted to each plot. While planting, AM fungal inoculum (50 g/plot having 90-100 spores/10 g of soil) was applied near the roots. The grass was irrigated without adding any fertilizer. The grass was replanted after 2 months of initial plantation in unamended RM due to failure of the growth.

Micro field nursery trails to rehabilitate the fly ash pond

The nursery experiment was conducted at NALCO nursery, Damanjodi, Orissa, India. Fly ash was used as substrate to grow the bamboo plants. The experimental plots were prepared as randomized blocks design with three replicates for each treatment and size of each treatment plot was 60×60×40 cm^3. The experiment

consisted of four treatments (1) fly ash, (2) fly ash and AM fungi, (3) fly ash and *A. tubingensis*, and (4) fly ash with AM fungi and *A. tubingensis*. Three replicates were maintained for each treatment. In each treatment, nine bamboo plants (2 months old) obtained from the NALCO nursery were planted and inoculated with AM fungi (inoculum size 50 gm/plant with 90–100 spores/10 g) and 5 g/plant (1.7×10^8 spores) of *A. tubingensis* fungal spores. The AM fungal inoculum consisted of spores and roots from a pot culture of trap plants. Control plants received 30 ml of AM fungal and *A. tubingensis* inoculum filtrate (< 20 µM) to provide similar microflora. Plants were watered to maintain soil moisture at 60% – 70% of the water holding capacity by adding tap water up to one month without adding any fertilizer.

Harvest

After six and twelve months of experiment, non-destructive sampling was followed for both red mud and fly ash micro-field experiments. Plant and soil samples were harvested and some selective chemical and biochemical properties of soil were analyzed in both experiments.

AM fungal colonization

To determine the AM fungal colonization in red mud treatments, the bermudagrass grown in red mud treatments were dug out (about 30 plants) randomly, after clipping of aerial portion of grass from each plot, using a stainless steel auger and fine roots were collected carefully. The dug out plants were replanted with grass again. In case of estimation of AM fungal colonization in roots of bamboo plants grown in fly ash treatments, the root samples were collected randomly from the each bamboo plant of every treatment plot using stainless steel auger. The colonization

was estimated at 6 and 12 months of both red mud and fly ash treatments by Phillips and Hayman (1970) method. The percentage of root length colonized by AM fungi was calculated by the gridline intersect method (Giovannetti and Mosse, 1980).

Plant analysis

The above ground biomass of bermudagrass grown in red mud treatments was harvested both at 6 and 12 months by clipping to a height of 3 cm to determine the biomass. The grass was washed with de-ionized water to remove the soil particles and dried at 70 °C for 48 h or till the constant weight was obtained. Following biomass determination, sub-samples were digested with nitric acid and perchloric acid (3:1) as described in Page *et al.* (1982) for mineral nutrients such as K, Ca, Mg, Na and heavy metals viz., Al, Fe, Zn and Cu by ICP-MS (Page *et al.*, 1982). In case of fly ash treatments, the bamboo plants height were measured and number of branches for each plant of all treatments was counted. To determine the uptake of minerals and metals by bamboo plants in different fly ash treatments, three randomly collected shoots from each plot were used. The shoot tissues were washed with de-ionized water and dried samples were digested (Page *et al.*, 1982). These samples were analyzed for mineral nutrients such as K, Ca Na, Mg, and heavy metals viz., Al, Fe, Zn, and Cu by inductively coupled plasma mass spectroscopy. Total P content in the bermudagrass and shoot tissues of bamboo plants was determined by colorimetric method as describes in Kitson and Mellon (1944).

Soil analysis

After six months of experiment, soil chemical characteristics such as pH, electrical conductivity, organic carbon, available phosphorus, total nitrogen;

enzymatic assays such as acid phosphatase alkaline phosphatase and urease of treatments were determined in all red mud and fly ash treatments. Soil samples were collected separately for each treatment with Dutch Auger. In red mud treatments, the soil samples were collected randomly in the depth of 0 to 10 cm in each plot of different treatments and three replicates were collected for each plot. In case from fly ash treatments, the sampling of fly ash experimental plots was as follows; 9 to 12 single samples consisting of 0.5 dm^3 of soil from each treatment. Each single sample was composed by two sub-samples, collected in opposite positions under the each plant canopy, 15 to 20 cm from the stem and 0 to 20 cm deep. Single samples were pooled to form one compound sample of 0.5 dm^3 of soil per replicate of each treatment and kept in sealed plastic bags at 4 ºC until brought to laboratory.

Subsamples of different red mud and fly ash treatments rhizosphere soil were analyzed separately for AM fungal spore isolation, chemical and bio-chemical properties for each treatment. The pH and electrical conductivity (EC) of the soil was checked by the method of International Society of Soil Science (1930). The total organic carbon (Walkley and Black, 1934) and total nitrogen (Total Organic Carbon Analyzer) were estimated. Available P was estimated by Olsen *et al.* (1954) and Bray and Kurtz (1945) methods according to the pH of samples. Urease activity was determined by the method of McGarity and Myers (1967) and acid and alkaline phosphatase by Tabatabai and Bremner (1969).

Soil chemical analysis

Prior to analysis, the soil samples were air-dried and sieved through a 2 mm mesh sieve for physical analysis. Further, the 2 mm-sieved soil was crushed to pass through 0.2 mm sieve for chemical analysis.

Determination of pH

a) 20 g of air dried soil samples were weighed and taken in a 100 ml beaker.

b) Added 50 ml of distilled water and thoroughly stirred for 2-3 min using a glass rod.

c) Further, it was kept in shaking condition (120 rpm) for 2 h.

d) Suspension was allowed to settle down for 30 min.

e) Mean while, pH meter was switched on and checked with two buffer solutions of known pH viz. one acidic and other alkaline with the help of standardization knob.

f) The pH of sample was measured by immersing the electrode in supernatant solution and recorded when the reading was stabilized (usually after 30 sec).

g) The electrode was rinsed with distilled water and carefully wiped with filter paper for every sample.

Determination of Total Soluble Salts (Electrical Conductivity mS cm^{-1})

a) Samples were mixed with water and stirred as described previously.

b) In the meanwhile, switched on the EC meter and allowed it to warm for 10 min.

c) 0.01 M KCl solution (EC = 1.413 mS cm^{-1}) was prepared followed by manufacturer instructions for calibrate the meter.

d) Dipped the electrode in the supernatant solution and recorded reading displayed.

The pH and electric conductivity (EC) was determined in 1: 2.5 (w/v) water extract using Deluxe Water and Soil Analysis Kit (Model 191 E).

Total Organic Carbon (Walkley and Black, 1934)

a) 1 g soil sample was weighed and transferred it into a 150 ml conical flask.

b) Added 10 ml of 1N $K_2Cr_2O_7$ solution into flask and mixed well.

c) The conical flask was kept on teflon /asbestos sheet prior to added 20 ml of concentrated H_2SO_4 from the sides of the flask. Swirled the flask during addition.

d) The flask was allowed to stand for 30 min to complete the reaction and there after 70 ml of water was added.

e) The blank was prepared by adding all reagents except soil.

f) The flasks were swirled thoroughly and allowed to settle the soil particles overnight.

g) The supernatant was decanted and read the color intensity using red filter at 660 nm.

Standard curve

Weighed 0, 10, 20, 30, 40 and 50 mg of anhydrous sucrose crystals into a 100 ml volumetric flask and repeated the same procedure.

Calculation

The quantities of sucrose (0, 10, 20, 30, 40 and 50 mg) were multiplied by 0.4207 and found the quantities (%) of carbon present. Because 10 mg sucrose contained 4.207 mg C and if suppose 4.207 mg C was found in 1000 mg of soil, it meant

100 mg soil contained = (4.207 x 100)/ 1000 mg carbon;

Organic carbon (%) = Colorimetric reading x 0.0042.

Because organic matter contains 58% carbon so,

Organic matter = Organic carbon (%) x 100 / 58 or

Organic matter = Organic carbon (%) x 1.724 (Van Bemmelen factor).

Available phosphorus suitable for acidic soil (Bray and Kurtz, 1945)

a) Weighed 2.5 g of soil sample into a 150 ml conical flask.

b) Added 50 ml of extractant P-1 into flask and kept on reciprocating shaker for 5 min (soil- to- solution ratio 1:20).

c) Sample was filtered through Whatman No. 1 filter paper quickly and collected the filtrate.

d) Transferred 5 ml of filtrate into 50 ml volumetric flask and diluted to about 20 ml with distilled water.

e) Added 8 ml of freshly prepared ascorbic acid (0.042 g) solution and made final volume of 50 ml with distilled water.

f) Samples were kept for 10 min for color formation. Thereafter measured the color intensity at 882 nm.

g) Blank was prepared with the 2 ml of extracting solution in place of soil and followed as above.

Standard curve

Measured 0, 2, 5, 10, 15 and 20 ml of working (1mg/L standard) P solution (KH_2PO_4) in 6 different 50 ml volumetric flasks and added 5 ml of extractant solution. Thereafter 8 ml of ascorbic acid solution was added into all flasks and made final volume to 50 ml with distilled water. The phosphorus concentration of these solutions was 0.04, 0.1, 0.2, 0.3 and 0.4 µg ml^{-1} respectively.

Calculation

$$\text{Available phosphorus (mg/kg)} = \frac{Q \times V}{A \times S}$$

Q = quantity of phosphorus in µg read on X-axis against a sample reading; V = volume of extracting reagent (ml); A = volume of aliquot used (ml); S = weight of soil sample taken (g)

Available phosphorus suitable for alkaline soils (*Olsen et al., 1954*)

a) 2.5 g soil sample was weighed into 100 ml Erlenmeyer flask contained 50 ml of extracting solution.

b) Kept flask on the reciprocating shaker for 30 min (soil to solution ration 1:20). The rate of shaking was constant for all samples.

c) Thereafter, filtered through Whatman No. 42 filter paper. Flask was shaken immediately before pouring suspension into funnel.

d) Transferred 10 ml of filtrate into 50 ml volumetric flask.

e) Added 1.0 ml 2.5 M H_2SO_4 to lower the pH to 8.5 followed by dilution of 15.5 ml distilled water.

f) Added 8 ml of freshly prepared ascorbic acid (0.042 g) solution and made final volume of 50 ml with distilled water.

g) Prepared blank same as described above using 10 ml extracting solution in place of soil extract

h) Samples were kept for 10 min and measured the color intensity at 882 nm.

Standard curve

Measured 0, 2, 5, 10, 15 and 20 ml of working (1mg/L standard) P solution (KH_2PO_4) in 6 different 50 ml volumetric flasks. Their after, 8 ml of ascorbic acid solution was added into all flasks and made final volume to 50 ml with distilled water. The phosphorus concentration of these solutions was 0.04, 0.1, 0.2, 0.3 and 0.4 µg ml^{-1} respectively.

Determination of elements by Inductively Coupled Plasma mass spectroscopy (ICP-MS) (Page *et al*., 1982)

a) Weighed 1 g of dried above ground grass /shoot material into a 100 ml conical flask.

b) Added 15 ml of concentrated nitric acid and kept for 1 h, and then added 5 ml of perchloric acid (3:1 ratio).

c) Kept on a hot plate in acid-proof digestion chamber having fume exhaust system and heated at about 100 °C for first one h and then raised the temperature to about 200 °C.

d) The digestion process was continued until the contents become colorless and white dense fumes appeared.

e) Reduced the acid content to about 2-3 ml by continuous heating at the same temperature.

f) The sample was removed from the hot plate, cooled and treated with diluted HCl.

g) The solution was filtered through Whatman No. 42 filter paper and the final volume was made to 20 ml with diluted HCl.

h) The concentration of mineral elements in the filtrate was measured using Inductively Coupled Plasma mass spectroscopy.

i) The calibration curve was prepared for each element by recording absorbance of a series of standard solutions of increasing concentrations.

Calculation

$$\text{Mineral ion (mg/g)} = \frac{C \times 20}{W}$$

C = concentration in the sample obtained on X-axis against the reading

W = weight of the mycelium taken

Total N estimation

Samples were mixed with water in 1:5 ratios. The water soluble extract was subjected to total N analysis by Total Organic Carbon (TOC) analyzer (Thermo-Hyper TOC, Thermo scientific, USA) coupled with Total Nitrogen Module. Soil sample is combusted to nitrogen monoxide and nitrogen dioxide. The nitrogen species are then reacted with ozone to form an excited state of nitrogen dioxide. Upon

returning to ground state, light energy is emitted. Then, total nitrogen (TN) is measured using a chemiluminescence detector.

Enzyme assays

Determination of acid phosphatase activity (Tabatabai and Bremner, 1969)

a) 2 g soil sample was taken in a sterile 30 ml screw cap tubes followed by addition of 4 ml of sterile modified universal buffer (Appendix I) solution.

b) 1 ml of filter sterilized 0.115 M disodium *p*-nitrophenyl phosphate solution was added and contents were incubated at 30 °C in a water bath for 2 h in the dark.

c) After incubation, 5 ml of 0.5N NaOH was added to stop the reaction and filtered through Whatman No. 1 filter paper.

d) The filtrate was transferred to glass cuvette and measured the yellow color intensity with UV-VIS spectrophotometer (Hitachi U-2001) at 410 nm.

e) To perform controls, followed the procedure described for the assay but made the addition of 1 ml p-nitrophenyl phosphate after the addition of 0.5N NaOH (i.e. immediately before filtration).

Standard curve

Phosphatase activity was indicated as the amount of *p*-nitrophenol released in the filtrate from the *p*-nitrophenyl phosphate substrate per gram of soil sample. Measured *p*- nitrophenol content with reference to a calibration graph plotted from the results obtained by standards containing 0, 10, 20, 30, 40 and 50 µg of *p*-nitrophenol.

Calculation

Phosphatase activity (μM PNP/g/h) = Concentration of PNP (in μM)/ Wt of soil.

Determination of alkaline phosphatase activity

Alkaline phosphatase activity was determined same as described in the previous section except that the pH of the modified universal buffer (MUB) was set at pH 9.0

Determination of urease activity (McGarity and Myers, 1967)

a) 10 g soil sample was placed in 100 ml volumetric flask followed by addition of 1 ml toluene to prevent microbial activity.

b) Thereafter, 10 ml buffer (pH 8.0) and 5 ml of 10% urea solution were added.

c) The flasks were shaken and incubated at 37 °C for 3 h.

d) To prepare control, 10 ml urea solution was replaced by 10 ml distilled water.

e) After incubation, the volume of each flask was made up to 100 ml by adding distilled water.

f) The flasks were then thoroughly shaken and filtered through a Whatman No. 5 filter paper.

g) The NH_3 released as a result of the urease activities was measured by the indophenol blue method. Half a milliliter of filtrate was placed in a 25 ml volumetric flask and 5 ml distilled water was added.

h) The mixture was treated with 2 ml phenolate solution and 1.5 ml NaHClO, and the volume of the flask was made up to 25 ml by adding distilled water.

i) The extinction of the blue color that developed as a result of the urease activities was read spectrophotometrically at 630 nm.

$$\text{Enzyme activity } (\mu M/g/h) = \frac{\text{Ammonia concentration} \times \text{Vol of enzyme used} \times \text{Dilution factor}}{\text{Enzyme reaction period x Wt of sample}}$$

Soil microbial analysis

Soil samples (1g) collected from different treatments in sterile containers were suspended in 1:10 ratio (w/v) of single sterile distilled water and disrupted by vortexing for 1–2 min. The resulting suspensions were serially (1/10) diluted in a sterile water.

The survival and biomass of *A. tubingensis* in different treatments was estimated from colony forming units (CFU) growing on Pikovskaya medium. Total alkaliphilic cultivable bacteria were also estimated by growing on alkaline nutrient, Horikoshi and tryptic soya agar plates (Appendix I) having pH 10.5. Plates were incubated for 2 days at 37°C. Three replicates per dilution were maintained.

Statistical analysis

Three replicates were used for each treatment plot in nursery experiment. The data was completely randomized in this experiment and were subjected to one-way analysis of variance (ANOVA). The significant differences among the means were compared with Tukey's test at $P< 0.05$ and Pearson correlation was followed where ever it necessary, using Graphpad Prism version 5.0 software.

Chapter 4

Results

Chapter 4

Results

4.1. Diversity of arbuscular mycorrhizal (AM) fungi

Disposal of red mud and fly ash at NALCO, Damanjodi follows wet slurry methods and directly pump these materials into lagoons nearby alumina refinery. The natural vegetation on these sites has been disappearing due to unfavorable conditions of these substances for plant growth. In this study, plants growing very poorly in red mud flooded area adjoin to the red mud pond were chosen and collected the rhizosphere soil along with adhered roots samples for AM fungal diversity analysis. In the case of AM fungal diversity study from fly ash pond, very few plants growing in fly ash pond were selected.

4.1.2. Diversity of AM fungi in adjoining areas of the red mud pond

Red mud flooded rhizosphere soil along with root samples representing two plant species such as *Acacia pennata* (Mimosaceae) and *Lantana camara* (Verbenaceae) growing in red mud flooded area of the RM pond was collected. From these samples, spore abundance and colonization of AM fungi were observed in the rhizosphere soil and root samples of both plant species, respectively. The number of spores retrieved from red mud flooded soil samples was very low with samples having fewer than 32 spores per 100 g of soil (Table 4.1) Spore number was significantly varied from 12 to 32 per 100 g rhizosphere soil of *Acacia pennata* and *Lantana camara* respectively (Table 4.1). The degree of root length colonization by AM fungi was determined

microscopically after typan blue staining. The lowest mycorrhizal colonization rates were found in root samples and it was ranged from 26% to 39% root length of two species of *Acacia pennata* and *Lantana camara* respectively (Table 4.1). Mean values indicate that the degree of mycorrhization of plant roots coming from two different species were significantly different (Table 4.1).

Table 4.1: Spore number in RM flooded rhizosphere soil and percent root length colonization on the roots of *Acacia pennata* and *Lanana camera* growing adjoining to the RM pond

Plant species	No. of spores in 100g of RM flooded soil	% of AM fungal root colonization
Acacia pennata	12	26
Lantana camara	24*	39*

Values with the asterisks are significant at $P < 0.05$.

The spore samples of trap cultures and also from pot culture were pooled, mounted on a single slide, and the presence or absence of different descriptions and unknown species were determined. The all identified species of arbuscular mycorrhizal fungi belonged to the genus of *Glomus* and the identified species were *Glomus delhiense*, *G. heterogama*, *G. mosseae*, *G. pubescens*, and *G. clarum*. One species, *Glomus* sp. 6 could not be matched with species descriptions in INVAM and Schenck and Perez (1990). The morphological characters of some identified arbuscular mycorrhizal fungi were illustrated in Figures 4.1-4.1.5.

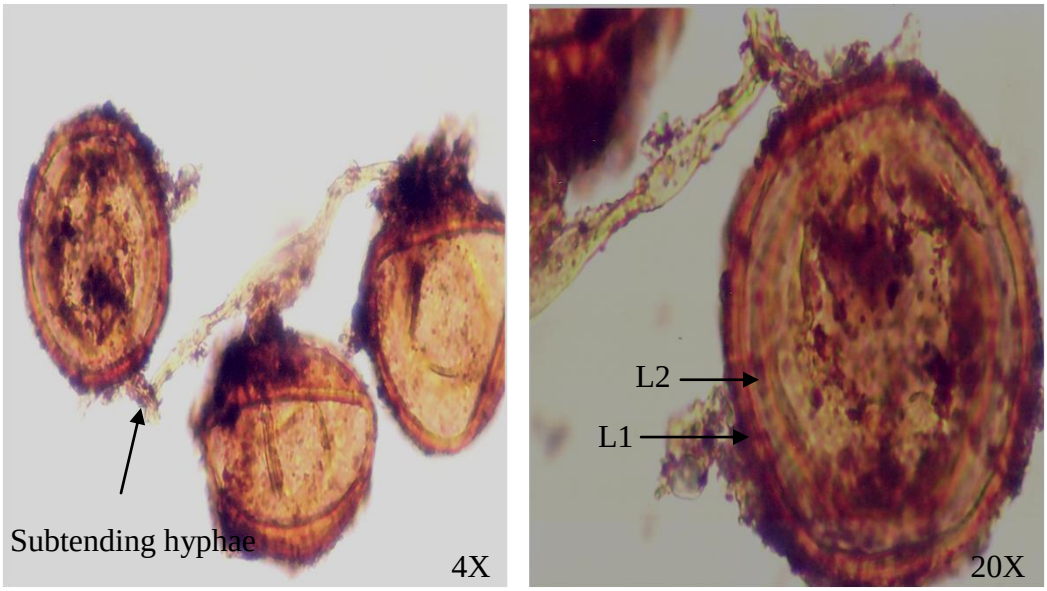


Fig 4.1 *Glomus delhiense*

Glomus delhiense

- Spores formed singly on subtending hyphae
- Shape globose 100-125 µm
- Color yellow brown
- Spore size 50 µm to 150 µm
- Spore wall double; outer layer (L1) 5-7 µm, yellowish brown, laminate and slightly roughened; inner layer (L2) 5 µm, hyaline

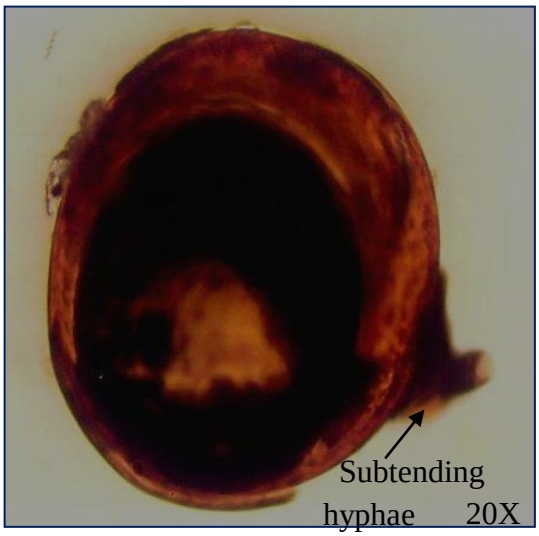


Fig 4.1.1 *Glomus heterogama*

Glomus heterogama

- Spores formed singly
- Spores predominantly globose to subglobose
- Spore walls 2-6 µm thick, yellow to brown
- Spore contents globular, yellow to light brown. Walls of the spore extending into the hyphal attachment
- Spores are light brown to dark brown.
- Spore size is 50 µm to 150 µm

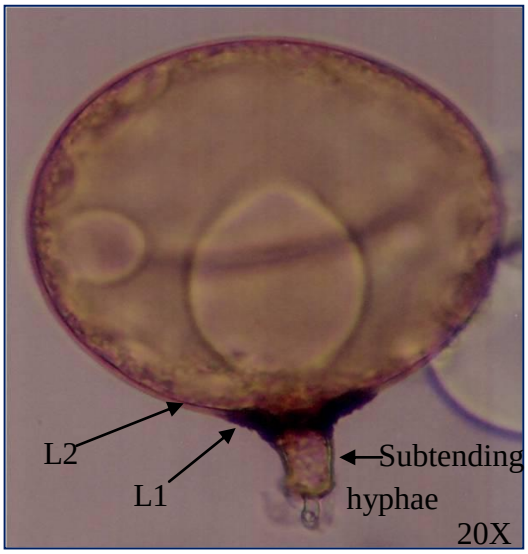


Fig 4.1.2 *Glomus mosseae*

Glomus mosseae

- Spores formed singly on subtending hyphae
- Shape globose
- Color yellow brown
- Spore size 50 µm to 150 µm
- Spore wall double; outer layer (L1) 5-7 µm, yellowish brown, laminate and slightly roughened; inner layer (L2) 5 µm, hyaline

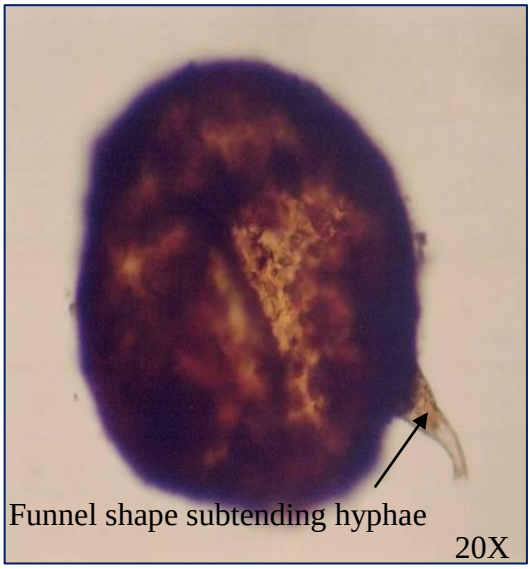


Fig 4.1.3 *Glomus pubescens*

Glomus pubescens

- Spores formed singly on funnel shaped subtending hyphae
- Hyphal attachments less than 2 µm diam, the attached hyphae hyaline thin walled and very inconspicuous
- Shape sub-globose to globose; smooth, filled with oil globles of variable size
- Spore wall 2-5 µm thick, nearly hyaline to high yellow, the opening occluded at maturity by wall thickening
- Color yellow brown to red yellow
- Spore size is 50 µm to 150 µm

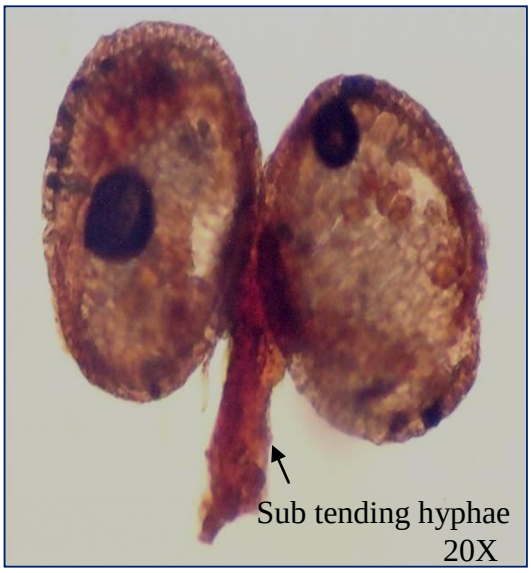


Fig 4.1.4 *Glomus clarum*

Glomus clarum

- Spores formed singly or in small clusters in the soil, also commonly formed with in the root, rupturing the cortex with age
- Spores hyaline, globose to subglobose, 55-190 µm in diam
- Spore contents hyaline, consisting of globules of variable size
- Spore walls complex, hyaline, becoming yellowish to light brown with age. Walls 5-20 µm in diam

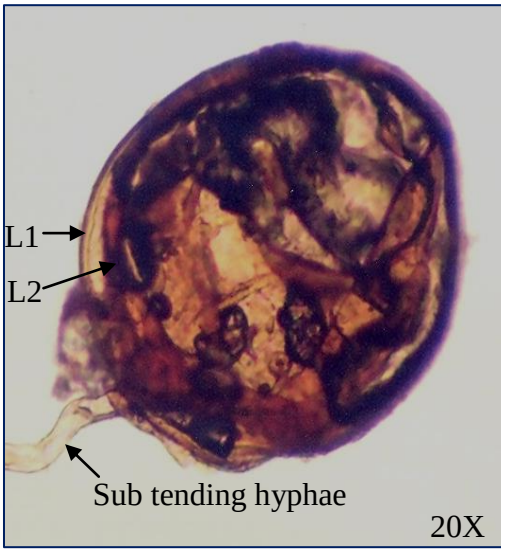


Fig 4.1.5 *Glomus* sp. 6

***Glomus* sp. 6**

- Spores are singly on subtending hyphae
- Color yellow to yellow brown
- Spore shape sub-globose to globose and ovoid
- Spore size of vary from 50µm to 170µm.
- Cell wall composed of two layers (L1 and L2)
- Cell wall thickness 5µm diam

4.1.3 Diversity of AM fungi associated with plants growing in fly ash pond

Roots and fly ash rhizosphere soil of seven actively growing plant species were collected from different location of fly ash pond and estimated percentage of AM fugal colonization and spore density (Table 4.1.1). AM fungal colonization was observed in root samples of all plants growing in fly ash pond. Average percent colonization of AM fungi was very low and varied from 14 to 33%. Maximum colonization was observed in *Jatropha gossypifolia* while the minimum in *Cynodon dactylon* (Table 4.1.1). The number of AM fungal spores also very low and ranged from 0 to 4 spores/100g in fly ash rhizosphere soil (Table 4.1.1). Spores did not formed in rhizosphere soil samples of *Calotropis gigantean* and *Cynodon dactylon*. Spores isolated from fly ash pond and trap cultures were identified based on their different morphological characters. Eight different AM fungal morphotypes belonging to two genera viz., *Glomus* and *Scutellospora* were recorded and identified. The genus *Glomus* was noticed as dominant in rhizosphere soil of fly ash pond. Among the species identified, 6 species were of *Glomus* (*Glomus etunicatum, G. heterogama, G. maculosum, G. magnicaule*, *G. multicaule* and *G. rosea*) and 2 species of *Scutellospora* (*Scutellospora heterogama* and *S. nigra*). The morphological characters of some identified arbuscular mycorrhizal fungi were illustrated in Figures 4.1.6 – 4.1.13.

Table 4.1.1 Spore number in fly ash rhizosphere soil and percent root length colonization in roots of plants growing edges of fly ah pond

Name of the plant	AM fungi colonization (%)	Number of spores/100 gm
Acacia pennata	27b	4a
Calotropis gigantea	19c	0
Cassia occidentalis	26b	3a
Cynodon dactylon	14d	0
Jatropha gossypifolia	33a	3a
Lantana camara	31a	3a

Values sharing a common letters within the column are not significant at $P<0.05$**.**

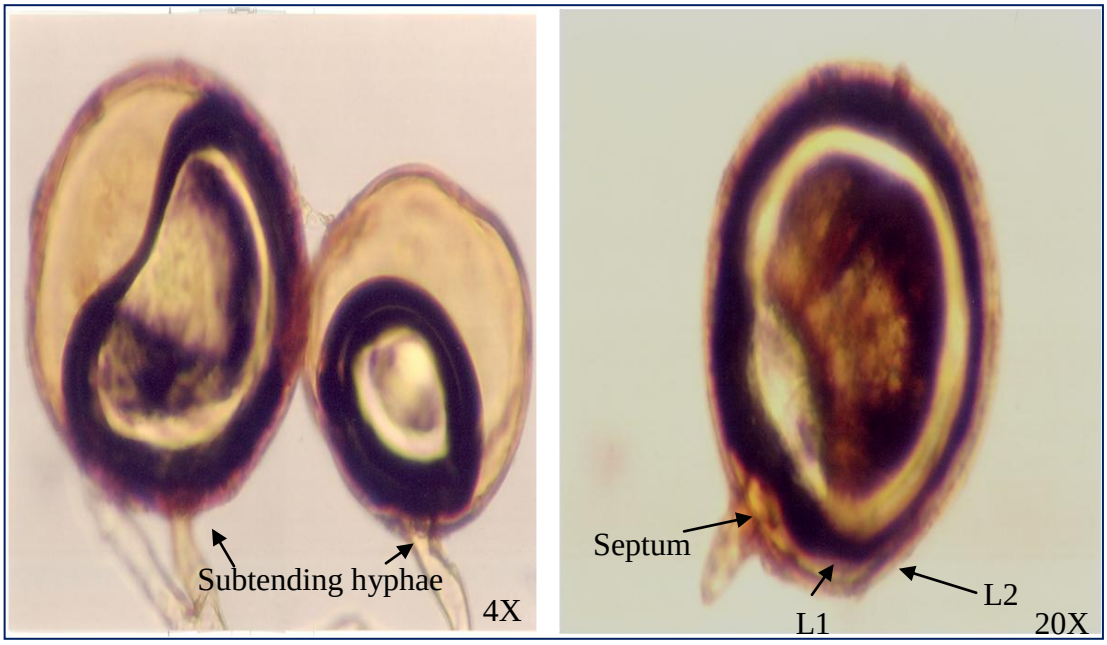


Fig 4.1.6 *Glomus etunicatum*

Glomus etunicatum

- Color pale yellow to yellow
- Size 60 to 160 µm
- Shape sub-globose and globose
- Subtending hyphae is cylindrical to slightly flare
- Spore wall consist of two layers (L1and L2) and continuous with the two layers of subtending hyphae, and wall thickness is 5 µm

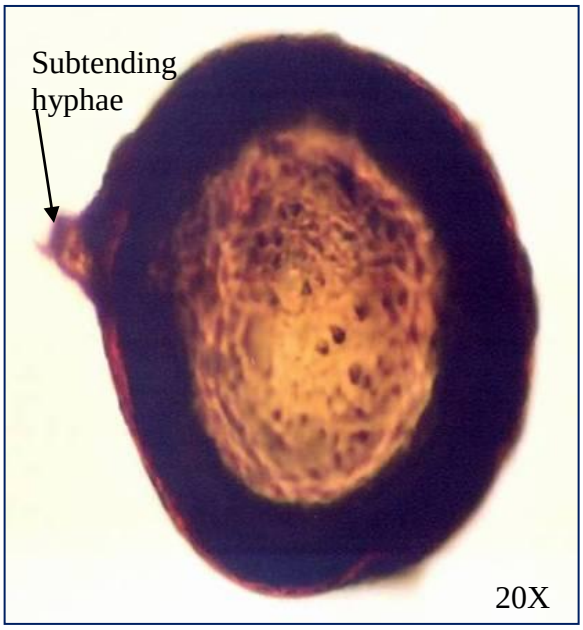


Fig 4.1.7 *Glomus heterogama*

Glomus heterogama

- Spores formed singly
- Spores predominantly globose to subglobose
- Spore walls 2-6 µm thick, yellow to brown
- Spore contents globular, yellow to light brown. Walls of the spore extending into the hyphal attachment
- Spores are light brown to dark brown
- Spore size is 50 µm to 150 µm

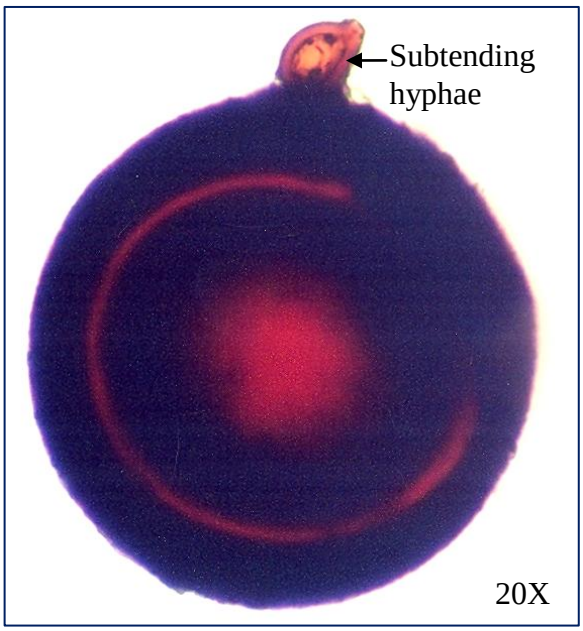


Fig 4.1.8 *Glomus maculosum*

Glomus maculosum

- Spores formed singly or in clusters
- Spores globose to subglobose in shape; brown to dark brown in color
- Spore walls 3-7 µm thick, light brown to reddish brown in color
- Subtending hyphae is straight to sharply recurved, parallel-sided, or funnel shaped, sometimes constricted at the spore base
- Spore contents globular, yellow to light brown. Walls of the spore extending into the hyphal attachment
- Spores are 150 to 225 µm in size

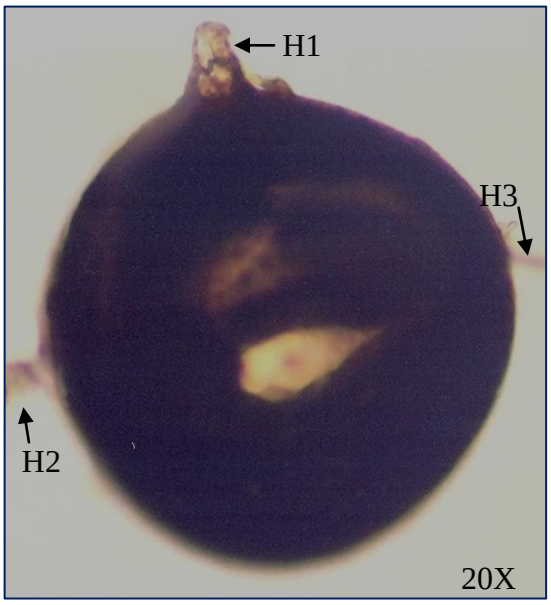


Fig 4.1.9 *Glomus multicaule*

Glomus multicaule

- Spores formed singly or in compact clusters of 3-5 spores.
- Spore shape ellipsoid, broadly ellipsoidal and sub-globose
- Multiple attached subtending hyphae (H1, H2 and H3)
- More than one subtending hyphae and it varies from 1-4 in many specimens
- Hyphae rarely positioned adjacent to each other. Subtending hypha attached at different ends of the spore. Light brown spores with 2 wall years
- Very thick ornamented spore wall

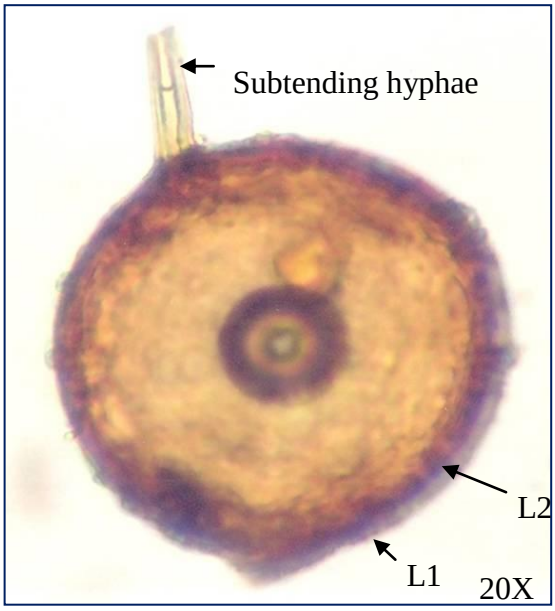


Fig 4.1.10 *Glomus magnicaule*

Glomus magnicaule

- Spores formed singly in soil
- Spore shape is globose to sub-globose; 140-220 µm
- Subtending hyphae is light to yellow to light brown in color; 2-3 layers and smooth. Septum was found in the subtending hyphae
- Spore wall is thick and light brown to dark brown in color
- Spore contents globular, yellow to light brown

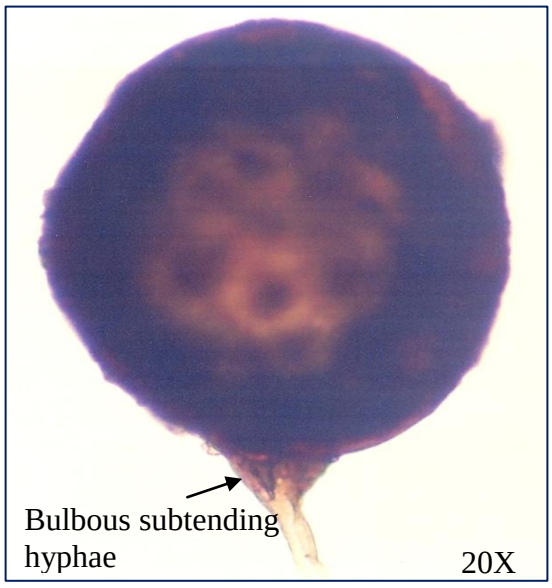


Fig 4.1.11 *Gigaspora rosea*

Gigaspora rosea

- Azygospores formed singly in soil
- Spores 235-300 x 205-185 µm in size, subglobose to ellipsoid, yellowish brown to dark brown
- A thin outer wall tightly covered the inner wall; the inner wall was 2-4 µm thick.
- Suspensors like cells, 25-37 µm diam., gave rise to a slender hyphae that projects to the spore
- Spores were yellowish brown with thin outer wall, tightly covering an

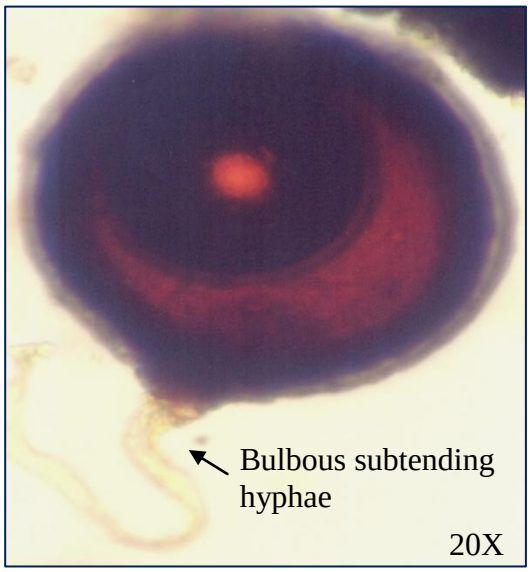
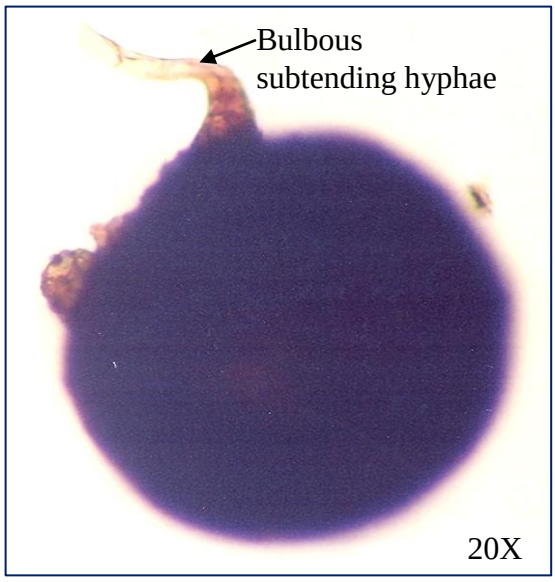


Fig 4.1.12 *Scutellospora heterogama*

Scutellospora heterogama

- Spores are dark orange to brown. Immature spores are white to cream with a rose tint colour
- Subglobose to oblong in shape; 120-200 µm, mean = 159 µm
- Spore walls are three layers of equal thickness
- Subtending hyphae 24-28 µm in thickness
- Germination shield is light yellowish brown to darker orange-brown



Scutellospora nigra

- Spores are light brown to dark brown
- Spores are globose to subglobose in shape; 130-190 µm
- Spore walls are 3 layers of equal thickness
- Subtending hyphae 24-32 µm in thickness
- Germination shield is pale yellow to light brown in color

Fig 4.1.13 *Scutellospora nigra*

4.1.4 Molecular identification of AM fungi

The root samples collected from adjoining site of red mud and fly ash ponds were allowed for DNA extraction and PCR amplification for identification of functional AM fungi associated with plant roots growing in fly ash pond and red mud adjoining to red mud pond. The amplification of DNA isolated from roots using NS5 and ITS4 primers were not visualized on agarose gel due to their low copy number. In second round of PCR, the 18S; partial, ITS1- 5.8S- ITS2 and 28S region of the rDNA from AM fungi were amplified using genera specific primers. When the PCR products of this first reaction were diluted and used as templates for the second reaction of nested PCR with the genera specific primer, amplified products of approximate 1000bp were generated from the mycorrhizal root samples.

4.1.5 Molecular diversity of AM fungi associated with plants growing in red mud

The nested PCR with the Glomeromycota genera specific primers GLOM/ITS4i and ARCH/ITS4i primer pairs only amplified the target DNA with expected size (Fig 4.15). No amplification was observed with LECT/ITS4i, PARA/ITS4i, NS5/GIGA5.8 and NS5/GLOM 5.8 primers. The PCR products of each primer pair set were cloned in pTZ57R/T vector and transformed into *E.coli DH5*α cells to separate the complex patterns of AM fungi. A total 75 clones of each GLOM/ITS4i and ARCH/ITS4i primer set were picked randomly. Plasmid DNA was isolated from these selected clones and amplified again with respective nested PCR primer sets. The amplified DNA fragments of different clones were screened for restriction enzymes *Hinf*I and *Mbo*I simultaneously to identify possible variations in different clones. After digestion with restriction enzymes, total of 32 different clones amplified with

GLOM/ITS4i primer set and 17 clones from ARCH/ITS4i primer set had shown distinct band patterns. The clones with different banding patterns by restriction endonucleases were then sequenced in both directions using Applied Biosystems automatic DNA sequencer.

The different cloned sequences were subjected for the similarity analysis in the GenBank DNA database using BLASTN (NCBI), which revealed that only five distinct cloned sequences named RMC3, RMP20, RMP23, RM32 and RM44 were belonged to phylum Glomeromycota related species belong to the genus *Glomus*. Amplification with these two sets of primers also produced the sequences of Ascomycetes and Basidiomycetes related species (data not showed). RFLP pattern of these AM fungal sequences had shown only with the enzyme *Hinf*1 and no variation in banding pattern was observed with *Mbo*1enzyme.

The RFLP analysis of AM fungal sequences digested with H*inf*1 on gel revealed that in lane 1-5 are showed entirely different restriction pattern of RMC3, RMP20, RMP23, RM32 and RM44 (Fig 4.1.15). Sequenced analysis showed that AM fungal isolates had 91% to 99% similarities with the AM fungal sequences of NCBI database (Table 4.17). The sequences of RMC3 (92%) and RMP20 (91%) were similar to *Glomus indicum* clones, ITS14 and ITS10 (99% query coverage) respectively. RM23 had shown 98% similarity with uncultured *Glomus* clone PPO-ui_GLOM_18 with 100% query coverage while RM32 had 99% similarity with uncultured glomeraceous AM fungus clone Glom3524.1 (100% query coverage). RM44 had 93% similarity with *Glomus etunicatum* clone pHS112-36 with 83% query coverage (Table 4.1.3).

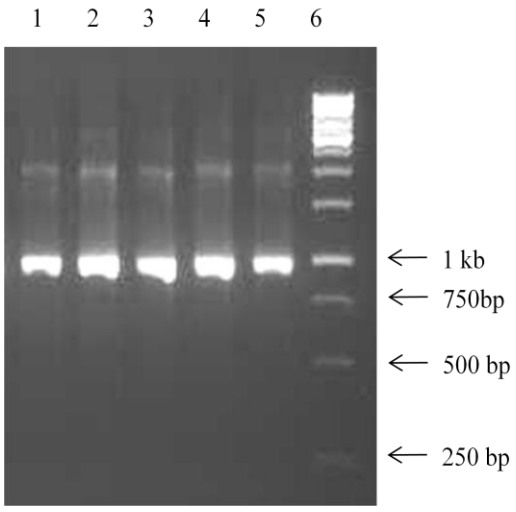


Fig 4.1.17: PCR amplification of RMC3, RMP20, RMP23, RM32 and RM44 clones generated by AM fungal GLOM1310/ITS4i primers. Lane 1-5: RMC3, RMP20, RMP23, RM32 and RM44 and Lane 6: 1kb ladder marker (Fermentas).

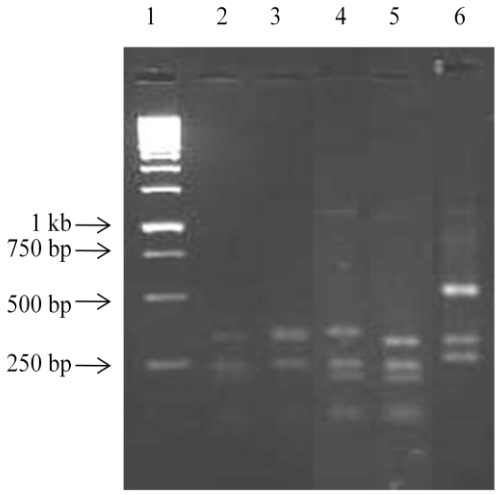


Fig 4.1.18: RFLP banding pattern of RMC3, RMP20, RMP23, RM32 and RM44 clones generated by AM fungal GLOM1310/ITS4i primers after digestion with *Hinf*I restriction enzyme. Lane 2-6: RMC3, RMP20, RMP23, RM32 and RM44 and Lane 1: 1kb ladder marker (Fermentas).

Identified AM fungal sequences were submitted in the NCBI database under the accession numbers HQ917526 to HQ917530.

Phylogenetic analysis of AM fungal 18S-ITS1-5.8S-ITS2 rDNA regions (sequences) included with BLAST sequences revealed that these sequences were clustered into 4 groups with high bootstrap values. The sequences of RMC3 and RMP20 clustered in group 1 and were associated with *Glomus indicum* and unidentified species of *Glomus*. The group 2, which were belong to RMP23, genus *Glomus mosseae* and uncultured *Glomus* sp. (Fig 4.1.16). The sequence RM32 clustered in group3 showing similarity with reference sequence of uncultured *Glomus* and *Glomus intraradices* and group 4 clustered RM44, *Glomus etunicatum*, *Glomus* sp. and *Glomus leteum* (Fig 4.1.16). These results showed that only limited numbers of AM fungi are associated with the plants growing in red mud pond and all the sequences were belonged to genus *Glomus* (Fig 4.1.16).

Table 4.1.3 Arbuscular mycorrhizal fungi associated with roots of plants growing in red mud flooded sites adjoining of red mud pond and their affiliation to the rDNA sequences of NCBI database

AM fungal sequences (Accession No.)	**Closest relative in database**	**% identity to closest related**
RMC3 (HQ917526)	*Glomus indicum* clone ITS14 ; GU059549	92
RM20 (HQ917527)	*Glomus indicum* clone ITS10; GU059545	91
RM23 (HQ917528)	Uncultured *Glomus* clone PPO-ui_GLOM_18; AM992824	98
RM32 (HQ917529)	Uncultured glomeraceous clone Glom3524.1; AY744278	99
RM44 (HQ917530)	*Glomus etunicatum* strain CA-OT-126-3-2, clone pHS112-36; N547623	99

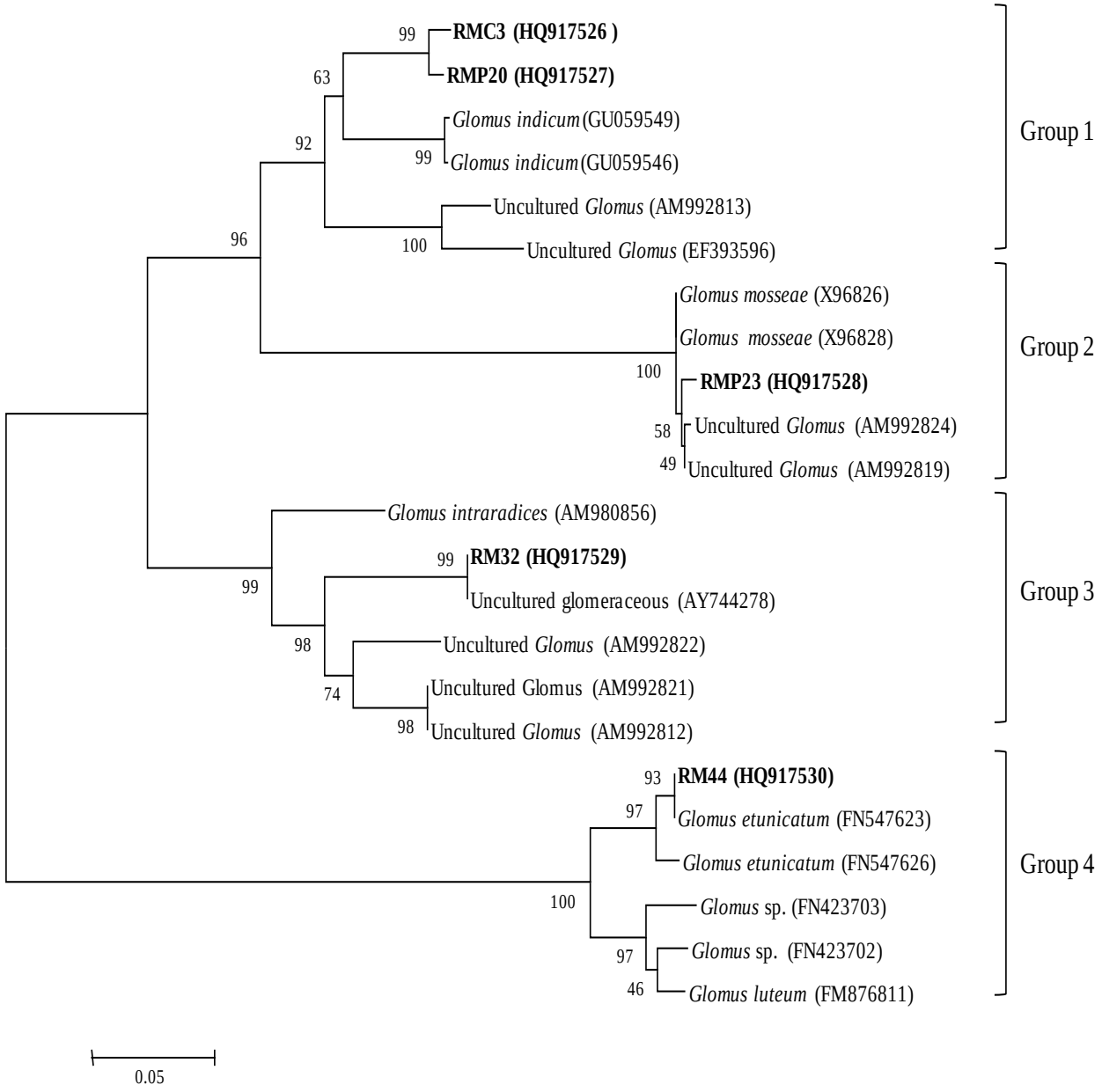


Fig 4.1.16 Neighbor-joining phylogenetic analysis of AM fungal partial 18S-ITS1-5.8S-ITS2 rDNA region sequences obtained from plant root samples of RM pond soil. Sequences obtained in the study are shown in *boldface.* Numerical values on branches are the bootstrap values. The scale represents substitution per site.

4.1.6 Molecular diversity of AM fungi associated with plants growing in fly ash pond

Amplification with subsequent nested PCR primers GLOM/ITS4i and ARCH/ITS4i were amplified the target DNA with expected size but no amplification was observed with LECT/ITS4i, PARA/ITS4i, NS5/GIGA5.8 and NS5/GLOM 5.8 primers with expected size. After cloning of amplified products, a total of 44 RFLP types for GLOM and 27 clones for ARCH primer were detected from randomly selected 150 transformed clones. RFLP pattern of these AM fungal sequences had also shown only with the enzyme *Hinf*1 and no variation in banding pattern was observed with *Mbo*1enzyme. Sequence similarities were determined using the BLAST analysis of NCBI. BLAST results of 18S-ITS1-5.8S-ITS2 rDNA region sequences indicated that among 44 cloned sequences, 7 clones (FA8, FA14, FA19, FA23, FA29, FA31 and FArc1) showed similarity to Glomeromycota and the other sequences either to Ascomycetes or Basidiomycetes (Fig 4. 1.4). The RFLP pattern of these AM fungal sequences digested with H*inf*1 on gel revealed that in lane 2-8 are showed entirely different restriction pattern of FA8, FA14, FA19, FA23, FA29, FA31 and FArc1 (Fig 4.1.18).

Of these 7 sequences, 6 (FA8, FA14, FA19, FA 23, FA29 and FA31) sequences showed homology with the genus *Glomus* and one to *Archaeospora* (FArc1) (Table 4. 1.4). FA8 showed 99% similarity with uncultured *glomus* clone PPO-ui_GLOM_25 (100% query coverage) and FA14 showed 93% similarity with uncultured glomeraceous AM fungus clone Glom3538.2 (51% query coverage) (Table 4. 1.4). The sequence FA19 had shown 91% similarity with the uncultured clone PPO-

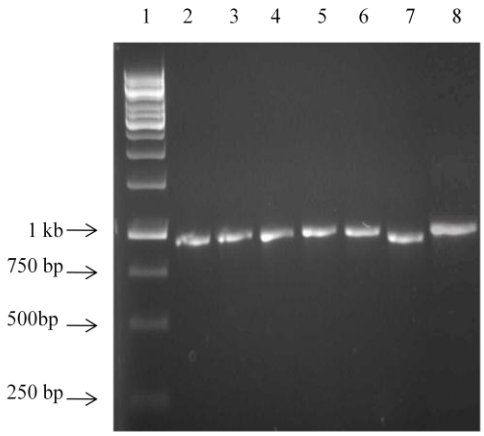


Fig 4.1.17 PCR amplification product of FA8, FA14, FA19, FA 23, FA29 FA31 and FArc1clones by Glom1310/ITS4i and ARCH1311/ITS4i primer sets, respectively. Lane 2-8 FA8, FA14, FA19, FA 23, FA29 FA31 and FArc1 and Lane 1: 1kb ladder marker (Fermentas).

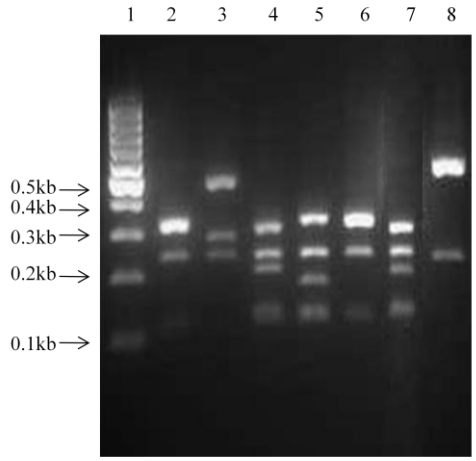


Fig 4.1.18 RFLP banding pattern of FA8, FA14, FA19, FA 23, FA29 FA31 and FArc1clones generated by Glom1310/ITS4i and ARCH1311/ITS4i primer sets, respectively after digestion with *Hinf*I restriction enzyme. Lane 2-8 FA8, FA14, FA19, FA 23, FA29 FA31 and FArc1 and Lane 1: 100 bp ladder marker (Fermentas).

Table 4. 1.4 Arbuscular mycorrhizal fungi associated with roots of plants growing in fly ash pond and their affiliation to the rDNA sequences of NCBI database

AM fungal sequences (Accession No.)	**Closest relative in database**	**% identity to closest related**
FA8 (HM159456)	Uncultured *glomus* clone PPO-ui_GLOM_25 (AM992831)	100
FA14 (HM159457)	Glom 3538.2 (AY744279)	93
FA19 (HM159458)	Uncultured *Glomus* clone clone PPO-ui_GLOM_16 (AM992822)	91
FA23 (HM159459)	*Glomus intraradices* (FJ009595)	97
FA29 (HM159460)	Uncultured glomeraceous AM fungus clone Glom3524.1 (AY744278)	94
FA31 (HM159461)	*Glomus indicum* clone ITS24 (GU059547)	92
FArc1 (HM159462)	Uncultured *Archaeospora* clone PPO-ui_ARCH_3 (AM992859)	95

ui_GLOM_16 (100% query coverage) (Table 4.1.4). FA 23 was 93% similar to Glomus intraradices clone 05 with 100% query coverage while FA 29, 94% similar with uncultured glomeraceous AM fungus clone Glom3524.1 (100% query coverage). FA 31 had shown 92% similarity and 99% query coverage with Glomus indicum clone ITS24 (Table 4. 1.4). The sequence of FArc1 had 95% similarity with uncultured *Archaeospora* clone PPO-ui_ARCH_3 (95% query coverage) (Table 4.1.4). Sequences of AM fungi were deposited at NCBI database under the accession numbers HM159456 to HM159462 (Appendix II).

Phylogenetic analysis based on 18S-ITS1-5.8S-ITS2 rDNA region revealed that these sequences were clustered into 4 groups; 2 groups (Group I and II) belonging to genus *Glomus*, group III showing no similarity with any reference sequence and group IV clustering with *Archaeospora* (Fig. 4.1.19). The sequences of FA19, FA23 and FA29 were clustered with *Glomus irregular*, *Glomus intraradices* and some unidentified species of *Glomus* in group I. The sequences of FA31 and FA8 were clustered in group II and were associated with group of unidentified species of *Glomus* (Fig. 4.1.19). The sequence of FA14 formed separate group (group III) without any known sequences in the database. Group IV included sequence of FArc1; it was distantly associated with genus *Archaeospora* group and an unidentified group of *Archaeospora* (Fig. 4.1.19).unidentified species of *Glomus* (Fig. 4.1.19). The sequence of FA14 formed separate group (group III) without any known sequences in the database. Group IV included sequence of FArc1; it was distantly associated with genus *Archaeospora* group and an unidentified group of *Archaeospora* (Fig. 4.1.19).

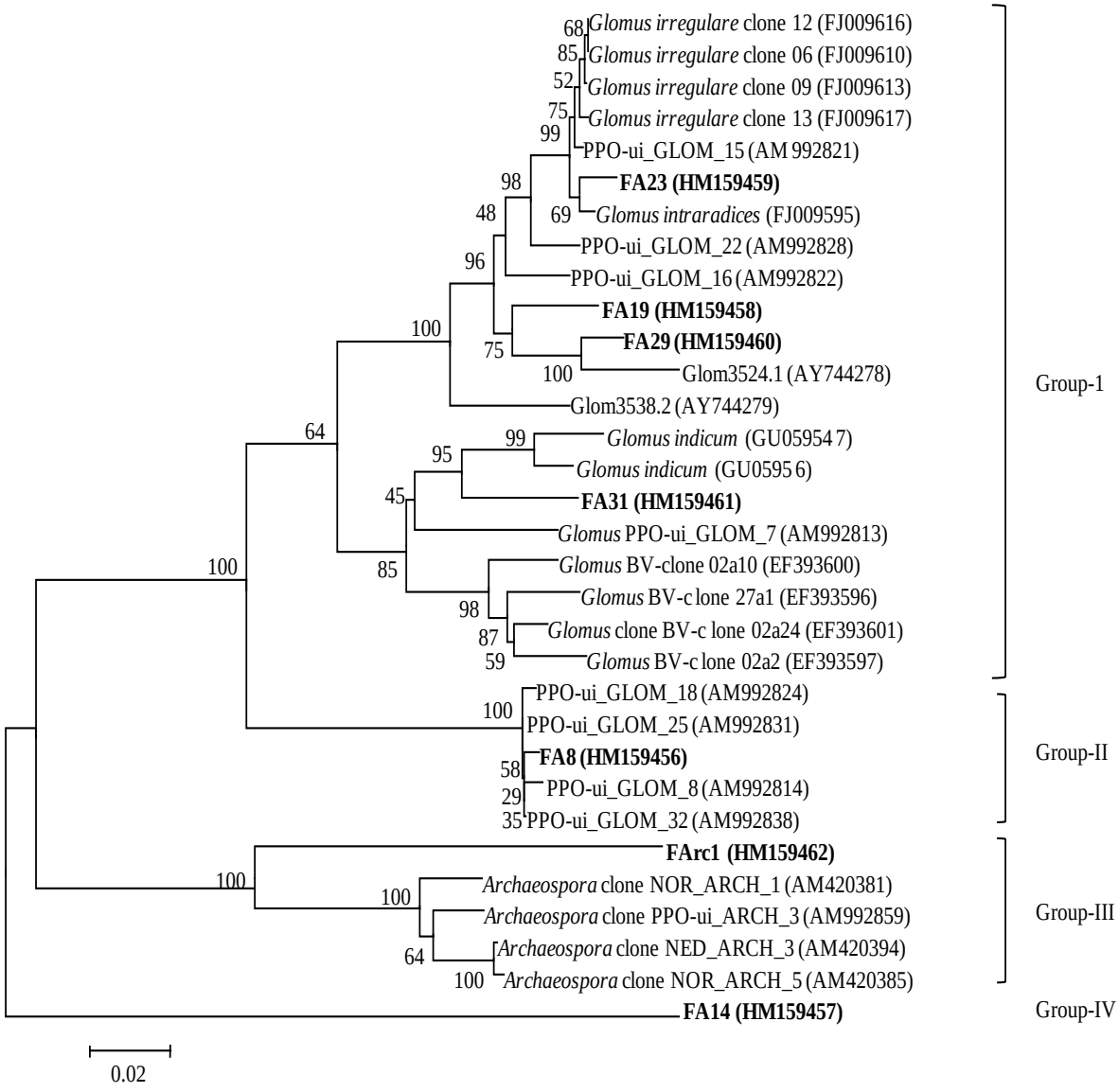


Fig. 4..1.19: Neighbor-joining phylogenetic analysis of AM fungal partial 18S-ITS1-5.8S-ITS2 rDNA region sequences obtained from plant root samples of fly ash pond. Sequences obtained in the study are shown in *boldface.* Numerical values on branches

are the bootstrap values as percentage of bootstrap replication from 1000 replicate analysis. The scale represents substitution per site.

Salient features

- Root colonization and diversity of arbuscular mycorrhizal (AM) fungi were analyzed in plants growing in red mud flooded sites and fly ash pond. AM fungal colonization and spores were noticed in both sites but very low colonization and low spore number. However, presence of colonization and spores were indication of certain adaptation to these adverse conditions.
- Six species could be separated morphologically from red mud flooded soil whereas eight from fly ash pond. Phylogenetic analyses after PCR amplification of the ITS region followed by RFLP and sequencing revealed five different AM fungal sequence types from red mud as well as seven from fly ash pond.
- Phylogenetic analysis showed that these sequences from both sites cluster into 4 discrete groups separately, belonging to the genus *Glomus* for red mud soil, and *Glomus* and *Archaeospora* for fly ash. Further, isolated and identified based on the spore morphology and molecular characterization revealed that the most of the species have been identified in salt marshes, fly ash and heavy metal contaminated soils.

4.2 Rehabilitation of red mud in micro-field studies

The chemical characteristics of the red mud (RM) samples were analyzed which were collected randomly from different locations of the red mud pond of National Aluminum Company (NALCO), Damanjodi, Orissa, India. Red mud had very stable chemical composition, characterized by the dominance of iron, aluminum and sodium. The pH of the bauxite residue was very high and never recorded below 10.3 of all the collected samples. While the organic carbon, available phosphorus and total nitrogen were very low and major constituents of red mud are iron and aluminum (Table 4.2). Sub optimal level of nutrients along with high sodium, pH and other heavy metals are considered to be major constraints for RM reclamation/revegetation. An attempt was made to improve the physico-chemical characters of red mud, for promotion of plant growth and microbial activity, by using different amendments like gypsum, top soil, sludge and fly ash prior to microbial inoculation.

Application of different amendments (10% w/w) such as fly ash, gypsum, sludge and topsoil to red mud was studied. Fly ash, gypsum, sludge and topsoil amended with RM improved the physico-chemical characteristics of red mud mixtures. The addition of gypsum to red mud reduced the pH from 11.45 to 10.22 (Table 4.2). Higher pH reduction was observed in gypsum amendment than the other amendments. Addition of other ameliorants decreased the pH to about 10.57 to 10.41 (Table 4.2). Maximum percentage of organic carbon was recorded in sludge amended (1.05%) red mud followed by fly ash (0.71%) and top soil (0.64%) as compared to RM alone (Table 4.2). Available phosphorus content was higher in sludge (1.48 mg/kg) and fly ash (1.03 mg/kg) amended

red mud than other amendments (Table 4.2). Total nitrogen was highest in sludge followed by top soil and fly ash amended RM (Table 4.2).

Micro-filed experiment in nursery of NALCO was conducted using these amendments along with different microbes such as RM adapted AM fungi, indigenous RM bacterial consortia, exogenous *A. tubingensis* alone and combination of these microbes. As shown in table 5.2, there were total 25 different treatments used in the present study. Red mud (RM), red mud amended with AM fungi (RM+AM), *A. tubingensis* (RM+At), bacterial consortia (RM+BC) and combination of AM+At+BC (RM+consortium); Sludge (S): sludge amended with RM, AM (RM+S+AM), At (RM+S+At), BC (RM+S+BC) and combination of AM+At+BC (RM+S+consortium); Top soil (TS): top soil amended with RM, AM (RM+TS+AM), At (RM+TS+At), BC (RM+TS+BC) and AM+At+BC (RM+TS+consortium) combination; Gypsum: gypsum amended with RM, AM (RM+G+AM), At (RM+G+At) BC (RM+G+BC) and combination of AM+At+BC (RM+G+ consortium); Fly ash: fly ash amended with RM, AM (RM+FA+AM), At(RM+FA+At), BC (RM+FA+BC) and AM+At+BC combination (RM+FA+consortium) (Table 5.2). An alkali tolerant, bermudagrass collected near from aluminum refinery area in Damanjodi (maintaining by NALCO Company) was planted in each treatment to study the performance and survival of this grass. Experiment was undertaken for observation and data were analyzed at six and twelve months after plantation.

4.2.1 Bermudagrass growth

Although the vegetation establishment on RM residue is a beneficial part of their environmental management, plant species selection is an important factor in determining

Table 4.2: Chemical properties of RM and its amendments

Parameters	**RM**	**RM+G**	**RM+TS**	**RM+S**	**RM+FA**
pH	11.45±0.11	10.22±0.09	10.47±0.06	10.57±0.06	10.41±0.41
EC (mS/cm)	1.86±0.01	2.09±0.04	0.92±0.01	1.23±0.02	1.35±0.01
Organic carbon (%)	0.34±0.01	0.47±0.02	0.64±0.00	1.05±0.02	0.71±0.02
Available P (mg/kg)	0.26±0.03	0.88±0.06	0.47±0.06	1.48±0.06	1.03±0.02
Total N (mg/kg)	4.00±0.01	9.00±0.00	19.00±7.00	26.0±0.00	11.0±7.00

RM, red mud; RM+G, red mud with gypsum; RM+TS, red mud with top soil; RM+S, red mud with sludge and RM+FA, red mud with fly ash.

Table 4.2.1: Schematic representations of different red mud treatments of micro-field experiment

Red mud (RM)	**RM+ Gypsum (RM+G)**	**RM+ Top soil (RM+TS)**	**RM+ Sludge (RM+S)**	**RM+ Fly ash (RM+FA)**
RM+ AM fungi (AM)	**RM+ G+ AM**	**RM+ TS+ AM**	**RM+ S+ AM**	**RM+ FA+ AM**
RM + *A. tubingensis* (At)	**RM+G+At**	**RM+TS+At**	**RM+S+At**	**RM+FA+At**
RM+ RM bacterial consortia (BC)	**RM+ G+ BC**	**RM+ TS+ BC**	**RM+ S+ BC**	**RM+ FA+ BC**
RM + AM+At+BC (Consortium)	**RM+ G + Consortium**	**RM+ TS + Consortium**	**RM+ S + Consortium**	**RM+ FA + Consortium**

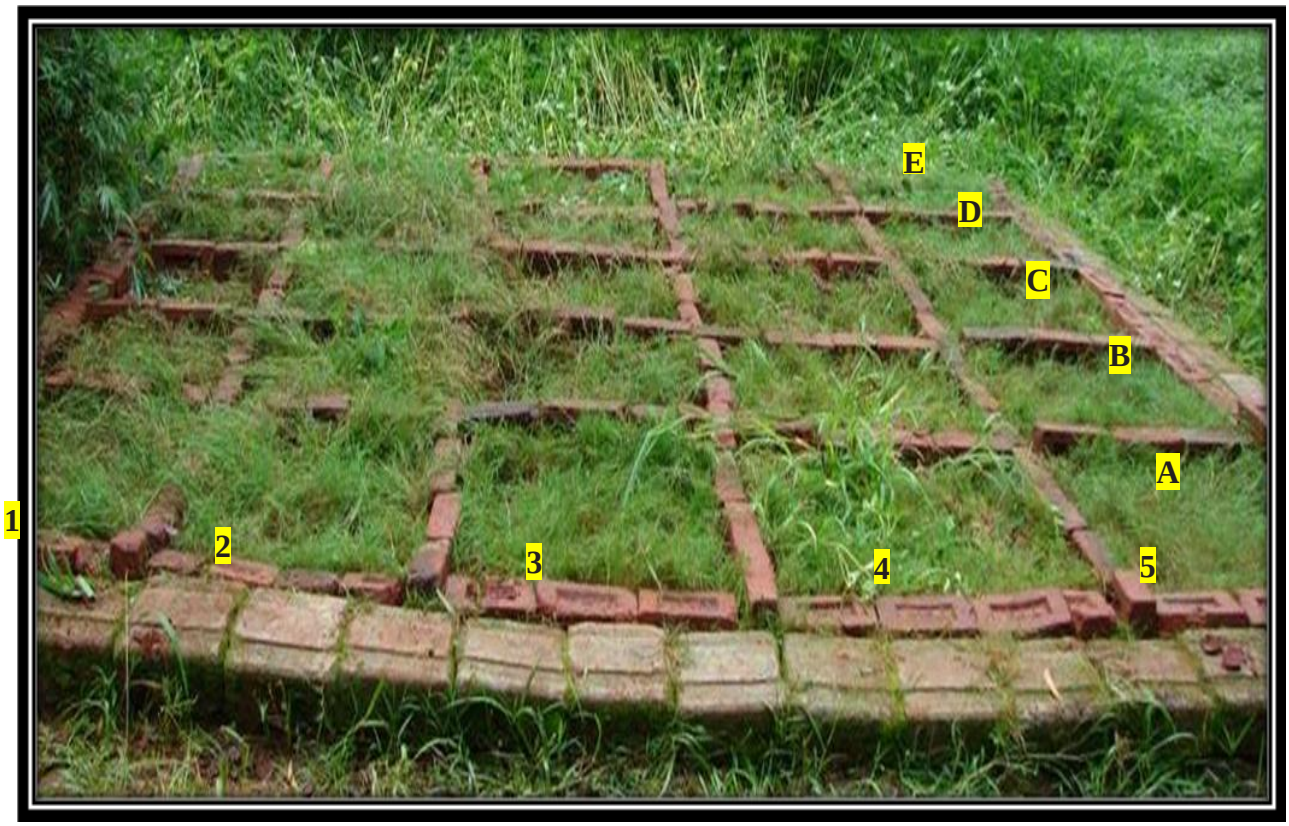


Fig 4.2: Effect of different microbial inoculation for rehabilitation of red mud amended treatments after 12 months of experiment. Column 1, 2, 3, 4 and 5 is RM, RM+S, RM+TS, RM+G and RM+FA respectively. Row A, B, C, D and E is consortium, AM, BC, At and without microbes respectively.

the success of revegetation of residue storage areas. Plant species selected should grow at high levels of salinity and sodicity. Bermudagrass was chosen as a species for rehabilitation of amended RM as the presence of alkali-tolerant grass can result in greatly increased dry matter production. At each harvest time (6 and 12 months), there were significant differences in growth response between plants treated with each amendment, non-amendment, control and microbial inoculated plants. Addition of different amendments to RM significantly increased the growth of bermudagrass compared to RM alone. Microbial inoculated plants growth was characterized by increased biomass in comparison to controls (Table 4.2&3; Fig 4.2.1a &b). After 6 months, red mud inoculated with consortium resulted in high biomass of grass compared to other treatments. Bacterial consortia and At inoculated treatments significantly increased grass biomass when compared to AM and no significant difference of biomass was observed in these two treatments. In gypsum amended treatment, the maximum biomass was obtained from consortium followed by AM, BC and At treatments. No significant difference of biomass was observed in At and BC. In case of topsoil amendment, the maximum biomass of grass was observed in consortium followed by BC, AM and At inoculated treatments.

The maximum biomass of grass was obtained with inoculation of consortium followed by AM, BC and At in sludge amended treatment. No significant difference of biomass production was observed in AM and BC inoculated treatments. This same trend was also observed in case of gypsum amended treatment but vary in biomass production. In fly ash amended treatment, inoculated treatments had shown maximum biomass production in the order of consortium>BC>At>AM (Table 4.2.2; Fig 4.2.1a). Overall biomass production was more in gypsum amended treatments followed by sludge treatments. In

case of inoculated treatments the maximum biomass was observed in gypsum amended consortium followed by AM inoculation.

After 12 months of harvest, it was found that the biomass of all treatments was gradually enhanced from 6 months to 12 months of growth and it was higher in inoculated treatments compared to controls. In RM treatment, the enhanced rate of biomass was fallowed as; BC<AM<consortium. In this treatment, inoculation with At showed similarity in biomass production with control treatment. The maximum biomass production was observed in consortium followed by AM, BC and At inoculated to gypsum amended treatment. In topsoil amended treatment, consortium inoculated treatment had maximum biomass compared to other treatments. Inoculation with AM and BC did not show significant difference of biomass production and both treatments were found to be more camped to At. In sludge amended treatment, the biomass production was maximum in consortium followed by AM, BC and At. In case of fly ash amended treatment, the maximum biomass production was observed in consortium followed by AM. Interestingly, at the end of experiment, consortium followed by AM inoculated all treatments had shown maximum biomass production, especially in gypsum amended treatment (Table 4.2.3; Fig 4.2.1b).

4.2.2 AM fungal colonization

Mycorrhizal colonization was observed in AM inoculated (AM and consortium inoculated treatments) and non-AM fungal propagules inoculated (At, BC inoculated and control treatments) treatments. The non-AM fungal inoculated plants were colonized by native AM fungi but, colonization was much lower than AM propagules inoculated treatments and the range of colonization was 4.2% to 37%. The % root colonization in all

treatments was significantly greater in other microbial (At and BC) inoculated treatments when compared to their respective controls. Further, treatments inoculated with AM fungal propagules had shown significantly higher percentage of mycorrhizal root infection compared to other inoculated treatments and it was varied with different amendments (Table 4.2.2; Fig 4.2.2a).

In RM treatment, the maximum % root colonization was observed in consortium (19.3%) followed by AM (16.7%), BC (7.5%) and At (5.5%) inoculated treatments. In case of gypsum amended treatment, the maximum % root colonization was observed in AM (59%) followed by consortium (47.8%), BC (37.4%) and At (30.1%). In topsoil amended treatments highest % root colonization was observed in consortium (27%) followed by AM (24.3%) compared to BC and At. Inoculation of sludge treated red mud with AM fungi followed by consortium showed significantly higher % root colonization (39.3% and 35.1% respectively) compared to other At and BC. The % root colonization in plants of BC and At (18.5%) treatments, BC inoculated treatment (25.3%) showed more % root colonization than At. In case of fly ash amended treatment, the higher % root colonization was found in consortium (32.3%) followed by AM (25%) compared to other treatments. Overall the highest % root colonization was observed in gypsum followed by sludge amended AM inoculated treatments (Table 4.2.2; Fig 4.2.2a).

After 12 months of growth, AM fungal % root colonization was gradually increased in all treatments from 6 months to 12 months and the maximum colonization was observed in AM propagules inoculated treatment, compared to other non-AM fungal propagules inoculated treatments. As compared to all control treatments, At and BC inoculated treatments significantly increased % root colonization and maximum was in BC. In RM

treatment, the colonization was followed as; consortium >AM>BC and At (23.5%, 17.5%, 13.3% and 12.5% respectively). In gypsum amended treatment, the maximum colonization was observed in consortium (81.3%) followed by AM (73.7%). In top soil amended treatment, AM (49%) and consortium (46%) inoculated treatments had shown higher % root colonization. In sludge amended treatment, the higher colonization was observed in AM (66.3%) followed by consortium (55%). In fly ash amended treatment also the higher colonization was observed in AM (56.3%) followed by consortium (49.4%), BC (38.9%) inoculated treatments (Table 5.4; Fig 3b). At the end of the experiment, the maximum colonization was observed in AM followed by consortium inoculated gypsum (Table 4.2.3; Fig 4.2.2b).

4.2.3 Microbial load and their survival in different treatments

The soil microbial population structure is one of the important markers to indicate the situation of the soil microbial community and stability of the soil biological system. The presence of unit phosphate solubilizing fungal colonies and colony forming units of alkaliphilic bacteria in each treatment were determined.

4.2.4 Unit P-solubilizing fungal colonies (UFC)

The presence of UFC count from different treatments was determined; no unit P-solubilizing fungal colonies were formed in the treatments that were not inoculated with *A. tubingensis* spores at 6 months of observation. After 6 months, the UFC count was significantly decreased in all *A. tubingensis* spore (At and consortium) inoculated treatments when compared with initial number. However, significant rise of UFC count was observed in the consortium ($0.07x10^4 – 13x10^4$) inoculated treatments compared to

A. tubingensis ($0.04x10^4$ – $12.4x10^4$) and it was further found to be increased at 12 months of observation (Table 5.3).

After 12 months, except RM treatment, all *A. tubingensis* ($0.23 x10^4$ – $21x10^4$) inoculated treatments significantly increased UFC count compared to all consortiums ($0.19x10^4$–$19x10^4$). The highest number of UFC was counted in sludge followed by gypsum amended treatments. After 12 months, UFC were observed in bacterial consortia inoculated sludge amended red mud treatment and it was 133 UFC/g (Table 5.4).

4.2.5 Alkali-tolerant bacterial count

After 6 months, the alkali-tolerant bacteria from all the treatments were counted. Number of bacterial colony counts was significantly higher in inoculated treatments compared to controls at 6 months. Inoculation of BC and consortiums significantly increased the number of alkali-tolerant bacteria counts in all treatments compared to AM and At, both at 6 and 12 months. As compared with At, AM fungi inoculated treatments significantly increased the number of bacterial counts in gypsum, top soil and fly ash amended compared at 6 months. In case of BC and consortium inoculated treatments, consortium inoculated gypsum, fly ash amended and RM treatments had highest number of alkali-tolerant bacterial counts. Overall highest number of alkali-tolerant bacterial count at 6 months was observed in sludge (BC>consortium) followed by gypsum (consortium>BC) inoculated treatments (Table 5.3). After 12 months, bacterial consortia and consortium inoculated treatments had higher number of count compared to AM and At. In a comparison between BC and consortium, top soil and fly ash amended with BC treatments had mores number than the consortium. The highest alkaliphilic bacterial

population was recorded in bacterial consortia and consortium inoculated sludge followed by gypsum amended treatments at 12 months (Table 5.4).

Table 4.2.2: Effect of different microbial inoculations on the growth of bermudagrass, AM fungal % root colonization and microbial count (P-solubilizing fungi and alkali-tolerant bacteria) in different red mud treatments after 6 months

Treatments	**Grass dry biomass (gm/plot)**	**AM fungal colonization (%)**	***A. tubingensis* (x 10^4cfu/gm soil)**	**Bacterial (x10^4 cfu/gm soil)**
		Red mud		
C	4.6±0.3p	4.2±0.2o	Nil	365±37q
AM	6.8±0.2o	16.7±0.5jk	NiI	612±35o
At	7.7±0.2no	5.5±0.4no	0. 04x10^4± 32i	750±46mn
BC	8.0±0.2no	7.5±0.2n	NI	1671±120f
Cons.	9.1±0.1mn	19.3±0.2i	0. 07x10^4±45h	2011±154d
		Red mud with gypsum		
C	14.1±0.1j	16.5±0.4k	NiI	805±37m
AM	34.3±0.2b	59.3±1.7a	NiI	1475±99g
At	26.0±0.8d	30.1±0.9f	11x10^4±64d	1156±134jk
BC	26.3±0.5d	37.4±1.1c	Nil	1980±121d
Cons.	37.7±0.6a	47.8±0.7b	14x10^4±57a	2264±89c
		Red mud with top soil		
C	9.0±0.6mn	9.5±0.4m	Nil	519±23p
AM	11.7±0.2l	24.3±0.5h	Nil	1119±211k
At	10.2±0.2lm	16.0±0.4k	0.5x10^4±26f	907±431
BC	14.8±0.4ij	16.9±1.1k	Nil	1819±197f
Cons.	16.2±0.9hi	27.7±1.0g	0.59x10^4±76e	1415±76h
		Red mud with sludge		
C	18.3±0.3fg	12.8±0.2l	Nil	1186±28j
AM	25.0±0.8d	39.3±0.5c	Nil	1479±76g
At	20.9±0.7e	18.5±0.4ij	12.4x10^4±57c	1801±86e
BC	25.6±0.9d	25.3±1.5g	Nil	3219±216a
Cons.	29.7±0.3c	35.1±0.8d	13x10^4±75b	2973±199b
		Red mud with fly ash		
C	10.9±0.1l	10.2±0.2m	Nil	488±54p
AM	11.8±0.3kl	25.0±0.8g	Nil	867±99l
At	13.6±0.4jk	16.5±0.4k	0.34x10^4±34g	717±216n
BC	17.0±0.7gh	17.4±1.7ijk	Nil	1167±125j
Cons.	20.2±0.7ef	32.3±1.5e	0.47x10^4±54f	1300±176i

Values sharing a common letter within the column are not significant at P<0.05.

Table 4.2.3: Effect of different microbial inoculations on the growth of bermudagrass, AM fungal % root colonization and microbial count (P-solubilizing fungi and alkali-tolerant bacteria) in different red mud treatments after 12 months

Treatments	**Grass dry biomass (gm/plot)**	**AM fungal colonization (%)**	***A. tubingensis* (x 10^4cfu/gm soil)**	**Bacterial (x10^4 cfu/gm soil)**
		Red mud		
C	57.2±1.0o	6.3 ± 0.2o	Nil	334±18p
AM	73.2±0.3l	17.5 ± 0.4m	NI	932±12k
At	60.1±0.8o	12.5 ± 0.4n	0. 12x10^4 ± 24j	827±17m
BC	66.2±0.3n	13.3 ± 0.5n	NI	1827±76d
Cons.	85.1±0.9j	23.5 ± 1.2k	0. 17x10^4±32i	2062±111b
		Red mud with gypsum		
C	87.4±1.6ij	27.3±0.5ij	NI	486±6o
AM	174.7±2.3b	73.7±0.9b	NI	1669±45f
At	112.7±0.3g	35.0±0.8g	18x10^4±41c	1370±66gh
BC	142.7±0.5e	42.0±1.0f	Nil	2106±15b
Cons.	237.5±0.7a	81.3±2.4a	16x10^4±56d	2088±28b
		Red mud with top soil		
C	67.1±1.5n	18.3±0.5lm	Nil	689±11n
AM	77.3±0.4k	49.0±0.8e	Nil	1038±67j
At	71.5±0.4lm	25.0±0.8jk	2.7x10^4±48e	1242±21i
BC	78.7±0.4k	31.7±0.5gh	Nil	1940±87c
Cons.	115.1±0.6g	46.0±2.0e	2 x10^4±65f	1433.5±76a
		Red mud with sludge		
C	84.5±1.6j	30.7±0.8hi	Nil	1288±32i
AM	144.8±3.8d	66.3±0.5c	Nil	2065±65b
At	100.8±0.4h	33.1±0.7gh	21x10^4±81.2a	1754±45e
BC	120.0±0.7f	39.3±0.5f	33±0.3k	3042±132a
Cons.	170.0±0.2c	55.0±1.5d	19x10^4±92b	3030±122a
		Red mud with fly ash		
C	69.1±1.0mn	22.0±0.8kl	Nil	854±31lm
AM	89.5±0.4i	56.3±1.2d	Nil	904±7kl
At	79.1±0.5k	33.7±1.2gh	0.23x10^4±51g	841±22m
BC	80.5±0.4k	38.9±1.0f	Nil	1362±65h
Cons.	112.8±1.6g	49.4±1.0e	0.19x10^4±97h	1284±17i

Values sharing a common letter within the column are not significant at P<0.05.

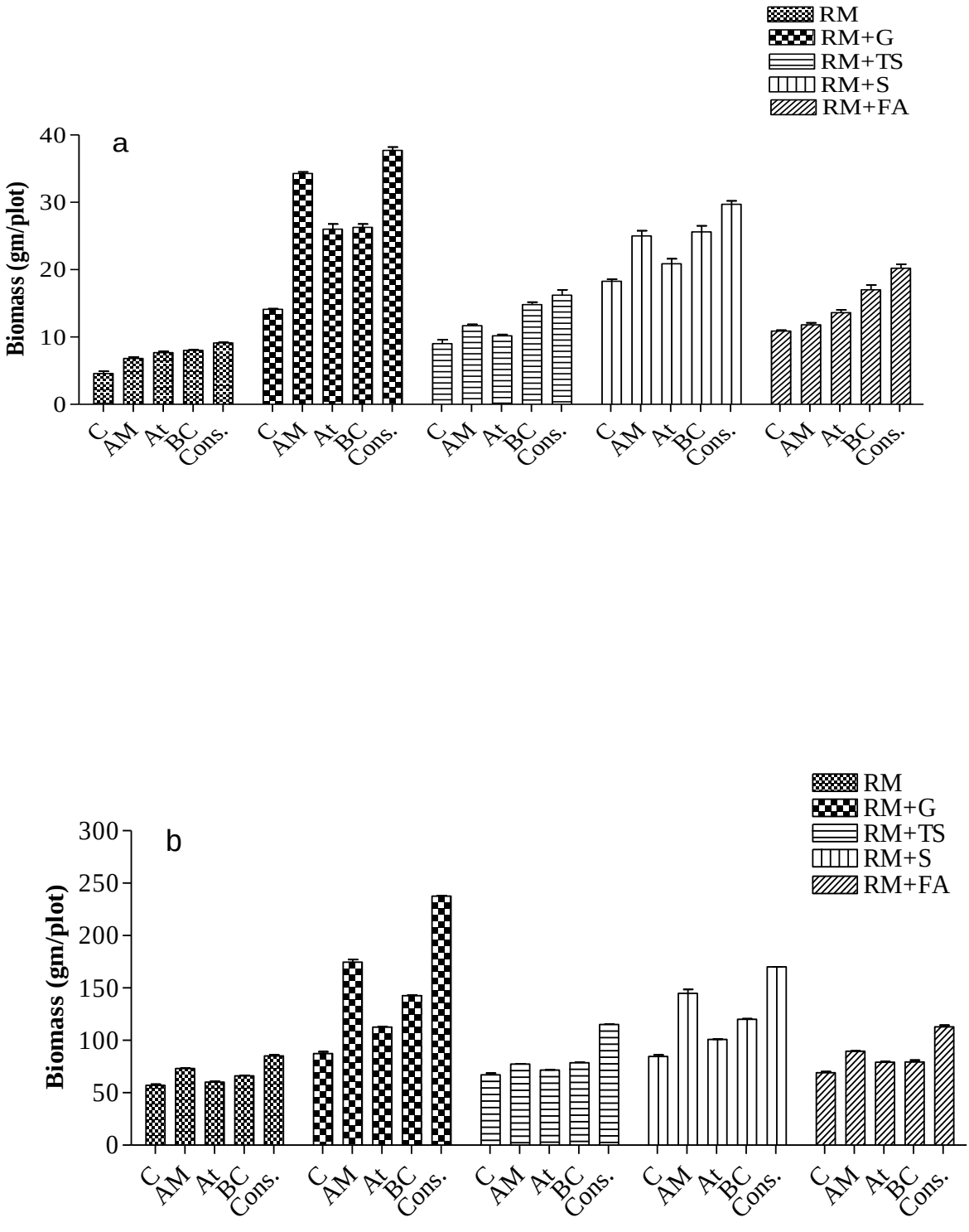


Fig 4.2.1: Biomass yield of bermudagrass in different microbial inoculated treatments after 6 (a) and 12 (b) months. Mean ±SD (n=3).

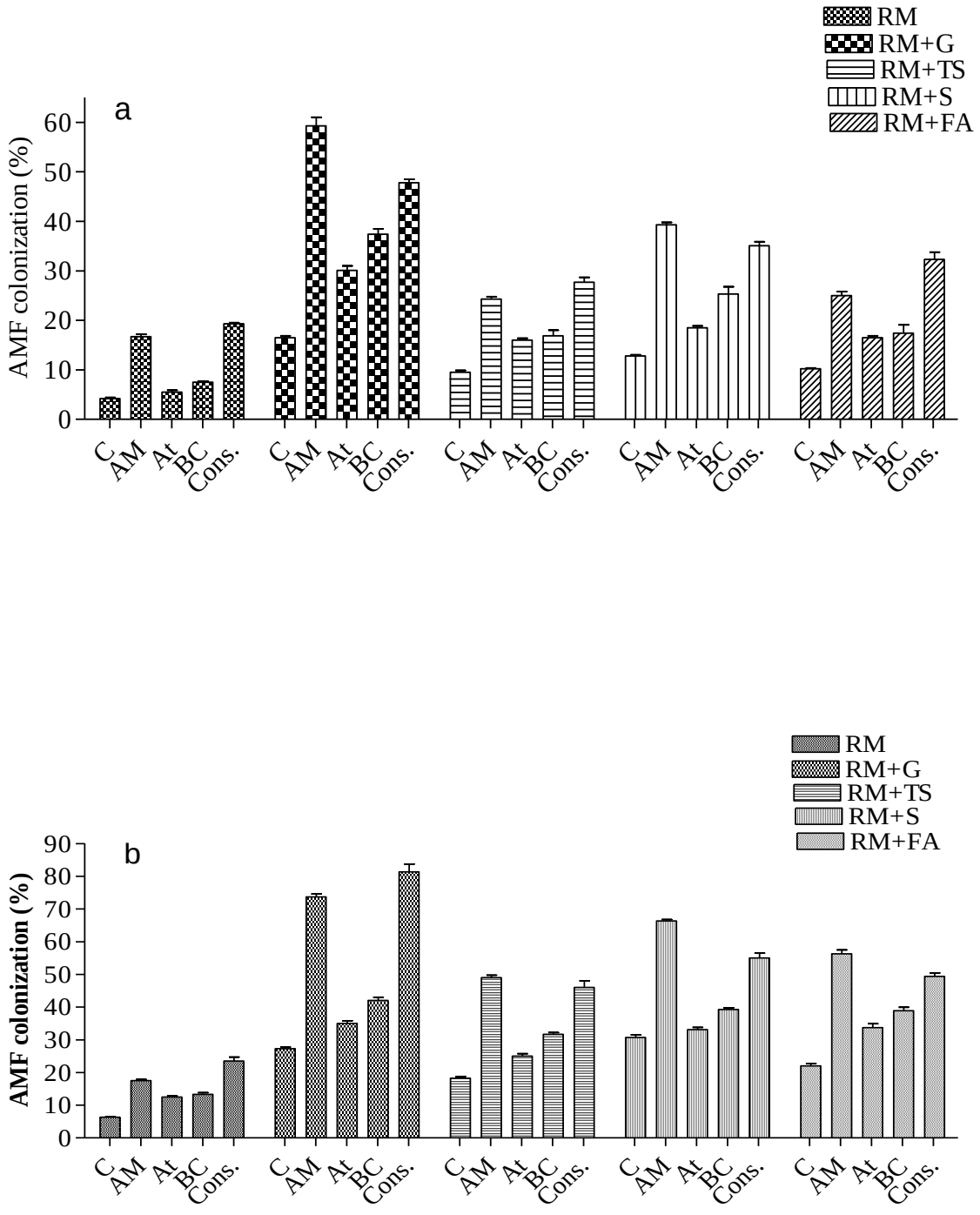


Fig 4.2.2: The root colonization of AM fungi (%) in different red mud amended treatments inoculated with microbes 6 (a) and 12 (b) months. Mean ±SD (n=3).

4.2.6 Plant metals

Red mud is not only highly alkaline and saline-sodic material also contain high amount of Fe, Al and Na. Thus, accumulation of Fe, Al and Na were estimated based on their relative abundance in red mud and their potential toxicity.

Iron (Fe)

After 6 months of plants growth, various levels of iron accumulation in grass were observed in all the treatments. In RM treatment, microbial inoculation decreased the uptake of iron compared to those of control. The maximum reduction in Fe accumulation was observed in consortium followed by BC, At and AM. Red mud amended with gypsum significantly reduced the accumulation of iron compared to all other RM amended and RM alone treatments. Further, inoculated treatments significantly reduced the levels Fe compared to their respective controls. Consortium inoculated treatment showed maximum reduction of iron uptake of grass than other inoculated treatments. When compared amongst the AM, At and BC inoculated treatments, inoculation of AM significantly decreased accumulation of iron in grass. In top soil amended treatment, BC inoculated treatment had shown significant increase of Fe accumulation in grass compared to all those of other treatments. Application of sludge amendment also fixed the Fe availability to grass as observed in gypsum amendment. Maximum reduction of iron accumulation was observed in AM followed by At inoculated treatments. In sludge amended treatment, all microbial inoculated treatments significantly lowered the Fe accumulation of grass except BC and reduced order was followed as; consortium<At<AM. As observed in top soil amended treatment, BC inoculated treatment increased Fe uptake of grass compared to control. In case of fly ash amended

treatment, inoculation of At and BC had shown increased content of Fe compared to control. Inoculation of AM and consortium treatments were significantly decreased Fe uptake of grass and it was high in consortium (Table 2.2.4; Fig 4.2.3a).

Overall, gypsum followed by sludge amended treatments fixed the Fe availability to grass and increased Fe availability in top soil and fly ash amendments. The maximum reduction of Fe accumulation was observed in gypsum amended consortium followed by AM, BC and AM inoculated treatments.

After 12 months, accumulation of iron in the grass was found to be reduced in all treatments with various degrees compared to 6 months. In RM treatment, Fe accumulation was reduced in all microbial inoculated treatments compared to their respective controls. The maximum reduction was observed in consortium followed by AM inoculated treatments. Iron accumulation of grass was reduced to minimum level in gypsum amended red mud than other treatments and this accumulation was further decreased in microbial inoculated treatments. Inoculation of consortium followed by At, AM and BC significantly reduced compared to control. In top soil amended treatment, inoculation of At followed by AM significantly reduced Fe uptake compared to control. In sludge amended treatment, consortium followed by BC inoculated treatments significantly increased Fe uptake but, AM, and At inoculated treatments significantly reduced Fe accumulation when compared to control treatment. The maximum reduction was observed in AM fungi followed by At. Iron accumulation was highly reduced in fly ash amended treatments compared to 6 months. The maximum reduction of Fe accumulation was observed in gypsum (consortium) followed by sludge (At) amended treatments (Table 2.2.5; Fig 4.2.3b).

Aluminum (Al)

In RM treatment, BC inoculated treatment had shown increased accumulation of Al in grass compared to control treatment. In At followed by consortium treatments, Al uptake of grass was found to be reduced compared to BC and control treatments. Microbial inoculated gypsum amended treatments reduced Al uptake of grass compared to control treatment. The lowest accumulation with no significant differences was observed in AM, BC and consortium compared to At. In top soil amended treatments, consortium followed by AM inoculated treatments reduced Al accumulation in grass tissue compared to At and BC inoculated and control treatment. Maximum Al accumulation was observed in BC than At. In sludge amended treatment, reduced Al uptake in grass was observed in AM followed by At compared to control treatment. Fly ash amended treatments inoculated with microbial inoculates also reduced Al accumulation of grass compared to their control treatment and higher reduction was recorded in AM, BC followed by At and consortium. Overall, highest accumulation of Al in plants was observed in top soil and fly ash amended treatments, and content of Al accumulation was high compared to Fe in these amended treatments only (Table 4.2.4; Fig 4.2.4a).

After 12 months of growth, accumulation of Al was gradually reduced from 6 months to 12 months of growth. Microbial inoculated RM treatments significantly reduced Al uptake of grass compared to their respective controls. The maximum reduction was observed in AM and consortium than At and BC. As compared with At, BC was shown higher Al upate. In case of gypsum amended treatment, the maximum reduction was observed in the following order; AM<BC<At<consortium. In top soil

amended treatment, inoculation of AM fungi followed by consortium significantly reduced grass Al uptake but, BC inoculated treatments significantly increased the uptake of Al compared to control. Inoculation of consortium and BC to sludge amended treatments significantly increased Al accumulation in grass compared to other inoculated and control treatments. However, inoculation with AM followed by At significantly reduced grass Al uptake than the control treatment. In fly ash amended treatments, consortium followed by AM inoculated treatment significantly reduced Al accumulation compared to other treatments. Overall, uptake of aluminum by grass was greatly reduced from 6 months to 12 months of growth. Inoculation with BC and consortium to top soil, BC and consortium to sludge and At and BC to fly ash amended treatments significantly increased accumulation of Al in grass compared to other inoculated and control treatments (Table 4.2.5; Fig 4.2.4b).

Sodium (Na)

After six months of growth, sodium uptake in grass was found to be reduced. In RM treatment, microbial inoculated treatments increased Na uptake of grass compared to control. Inoculation of AM and consortium significantly increased Na uptake in grass compared to At and BC. Amongst all the treatments, BC inoculated treatment showed less Na uptake. Interestingly, microbial inoculated gypsum amended treatments did not show any significant difference in Na uptake of grass. In top soil amended treatment, AM and BC inoculated treatments showed higher Na uptake of grass compared to all other inoculated and control treatments. Microbial inoculated sludge amended treatments had shown increased Na content in grass compared to control treatments and maximum was observed in AM. No significant difference was observed in BC and consortium, and both

were significantly increased Na uptake compared to At. All microbial inoculated fly ash amendments also increased Na uptake compared to the control and it was maximum in AM and consortium (both insignificant) followed by BC treatments. Overall, gypsum amended treatments had lowered the uptake of Na in grass compared to all other RM amended treatments (Table 4.2.4; Fig 4.2.5a).

After 12 months of growth, Na levels in grass were increased in most of treatments from 6 months to 12 months and it was further increased by inoculated treatments compared to their respective controls. In red mud treatment, microbial inoculated treatments significantly increased Na uptake of grass compared to their control. The highest Na uptake was observed in consortium amongst the other inoculated treatments. In gypsum amended treatments, consortium inoculated treatment had high Na uptake of grass compared to those all other treatments. AM and BC inoculated treatments had no significant difference in Na uptake of grass and found to be higher than At and control. In At and control treatments also difference in Na uptake was not significant. In top soil amended treatment, higher content of Na in grass was observed in microbial inoculated treatments and maximum was in AM and consortium compared to At and BC. Sodium content of grass grown in gypsum amended and microbial inoculated treatments found to be significantly increased compared to control and it was in the following order: BC>consortium>AM>At. In fly ash amended treatment, grass Na uptake was higher in microbial inoculated treatments than control treatments. There was no significant difference amongst the AM, BC and consortium treatments but these treatments showed significantly more Na uptake than At inoculated treatment. Overall, as compared to all

other treatments, very low level of Na uptake was observed in gypsum amended treatments (Table 4.2.5; Fig 4.2.5b).

Phosphorus uptake (P)

Microbial inoculated treatments significantly increased grass P content compared to their respective controls at 6 months of harvest. Inoculation of consortium increased P uptake of grass compared to other inoculated treatments. In gypsum amended treatment, the high P content in grass was observed in consortium followed by AM inoculated treatments. In case of top soil amended treatment, inoculation of At and consortium treatments significantly increased P content of grass compared to AM and BC treatments and these treatments did not show significant difference in P uptake of grass. As compared with BC, AM inoculated treatment had high P uptake of grass. In sludge amended treatment, consortium followed by AM. Inoculation of microbial inoculants to fly ash amended treatment significantly increased in order of consortium >AM>BC>At. Overall at 6 months the maximum P uptake was observed in gypsum amended treatment (Table 4.2.4; Fig 4.2.6a).

After 12 months of growth, it was observed that the uptake of P was gradually increased in all control treatments compared to 6 months. In addition to this, microbial inoculated treatments further increased P uptake compared to their respective controls except in At inoculated top soil amended treatment. In red mud amended treatment, increased P uptake was observed in At and consortium treatments compared to other treatments. In case of AM treatment, increase P uptake was observed compared to BC. In gypsum amended treatment, more P uptake was observed in AM followed by consortium treatments. In case of top soil treatment, more P uptake was observed in consortium

followed by AM and BC inoculated treatments. The P uptake of grass was higher in fly ash amended consortium inoculated treatments compared to other treatments. Inoculation with BC significantly increased P uptake compared to AM and At. After 12 months, the maximum P uptake in grass was observed in gypsum amended (AM inoculated) treatment (Table 4.2.5; Fig 4.2.6b).

Calcium (Ca)

After 6 months of growth, inoculation of microbial treatments increased Ca content of grass compared to their respective control treatments. In red mud treatment, the maximum Ca uptake was observed in BC and consortium inoculated treatment compared to other treatments. In gypsum treatment, BC inoculated treatment showed higher level of Ca in grass compared to other inoculated treatments. *Aspergillus tubingensis* and consortium inoculated treatments did not show any significant difference in Ca uptake of grass but, showed increase of Ca uptake compared to AM inoculated treatment. In top soil amended treatment, it was found that calcium content was higher in At compared to other treatments. In sludge amended treatment, BC followed by AM inoculation showed more Ca uptake than other inoculated treatments. Inoculation with consortium had shown significantly higher Ca uptake of grass than At. In case of fly ash amended treatment, more Ca uptake was estimated in AM and At followed by BC and consortium inoculated treatments. Overall, gypsum amended treatment and further microbial inoculated treatments had shown maximum increases of Ca uptake in grass than other amended and control treatments respectively (Table 4.2.4; Fig 4.2.7a).

After 12 months, calcium uptake was increased compared to 6 months in all treatments except in fly ash and red mud amended treatments. In red mud treatment,

increased Na uptake of grass was observed in all inoculated treatments compared to control and the high Ca uptake was in AM and BC compared to consortium. In gypsum amended treatment, inoculation of consortium followed by AM and BC treatments significantly increased Ca uptake in grass compared to control. In tops soil amended treatment, microbial inoculated treatments increased Ca uptake of grass compared to their control and maximum was observed in consortium followed by BC. In sludge amended treatment, inoculated treatments increased Ca uptake compared to control and high Ca uptake was observed in consortium followed by AM. In fly ash amended treatment also inoculated treatments increased Ca uptake of grass than control. The maximum Ca uptake was observed in consortium followed by AM. Overall, gypsums amended all treatments had shown high amount of Ca in grass compared to all other treatments (Table 4.2.5; Fig 4.2.7b).

Magnesium (Mg)

After 6 months of growth, microbial inoculated treatments significantly increased Mg uptake compared to their respective control treatments except in BC + gypsum, At + fly ash amended and At + RM alone treatments. These three treatments did not show significant difference in Mg uptake of grass compared to their control treatments. In gypsum amended treatment, highest level of Mg was observed in AM compared to other treatments. In sludge amended treatment, Mg uptake was more in consortium followed by AM compared to At and BC inoculated treatments. In case of fly ash amended treatment, the higher Mg uptake was observed in consortium inoculated treatments compared to AM and BC treatments. Uptake of Mg was very low in gypsum amended treatment compared to all other treatments after 6 months (Table 4.2.4; Fig 4.2.8a).

Magnesium uptake was gradually increased in most of the treatments from 6 months to 12 months. Microbial inoculated treatments increased Mg uptake of grass compared to their respective controls except in At and BC inoculated RM and topsoil amended BC treatments. In RM treatment, the maximum Mg was observed in consortium followed by AM. In gypsum amended treatment also showed high magnesium content in consortium followed by AM inoculated treatments. In tops soil amended treatment, the high Mg content in grass was recorded in AM compared to At and consortium inoculated treatments. In sludge amended treatment, the maximum Mg uptake was observed in consortium followed by At>AM>BC inoculated treatments. Fly ash amended treatment also showed the maximum Mg uptake of grass in consortium treatments compared to other treatments. Overall, it was observed that the maximum Mg uptake of grass was stimulated by sludge when compared to all control treatments. However, irrespective of amendments, microbial inoculated treatments increased Mg uptake of grass in all treatments (Table 4.2.5; Fig 4.2.8b).

Potassium (K)

After 6 months of growth, microbial inoculated treatments significantly increased K uptake compared to their respective control treatments. In red mud treatment, potassium uptake was more in consortium followed by BC, AM and At. In case of gypsum amended treatment, the maximum K uptake was observed in AM and consortium inoculated treatments compared to BC. In sludge amended treatment, the maximum K uptake was observed in consortium followed by BC and AM inoculated treatments. In case of fly amended treatment, it was more in AM followed by consortium, At and BC inoculated treatments. Overall, amongst all the control treatments, potassium uptake was more in

gypsum amended treatment. In case of microbial inoculated treatments, maximum K uptake was observed in consortium inoculated sludge amended treatment (Table 4.2.4; Fig 4.2.9a).

Potassium uptake gradually increased from 6 months to 12 months of growth except in gypsum amended control and consortium inoculated red mud treatments. However, inoculation of microbes significantly increased K uptake compared to their respective control treatments except in At inoculated top soil amended treatment. In gypsum amended treatment, inoculation of AM followed by BC had shown maximum K uptake compared to At and consortium. Consortium inoculated treatment showed significantly increased K uptake compared to At. In case of top soil amended treatment, the maximum K uptake was observed in consortium followed by AM and BC inoculated treatments. The sludge amended consortium treatment had more K uptake of grass compared to other inoculated treatments. Inoculation of At treatment had shown significantly more K uptake compared to other treatments. In fly ash amended treatment, the higher K uptake was observed in consortium followed by AM compared to other treatments. After 12 months, overall K uptake was more in sludge amended treatment compared to all control treatments (Table 4.2.5; Fig 4.2.9b).

Table 4.2.4: Influence of microbial inoculations on uptake of different nutrients and heavy metals (mg/kg) in aerial portion of bermudagrass in different red mud treatments after 12 months

Treatments	Fe	Al	Na	P	Ca	Mg	K
Red mud							
C	2652±8a	2187±4a	225±4e	319±8n	869±5l	250±2f	785±5k
AM	1752±3g	1580±7d	364±5c	419±8l	992±8fg	308±5e	1031±4g
At	1998±5d	1628±4c	264±5d	489±2k	796±3m	262±7f	1088±6g
BC	2169±5b	1858±4b	378±2c	388±4m	994±4fg	256±4f	1029±15g
Cons.	1049±6n	1577±6d	464±6a	478±3k	965±2gh	395±5c	1116±13e
Red mud with gypsum							
C	1358±4i	1063±5f	146±2f	688±2g	1154±8e	205±8h	829±3j
AM	1127±6l	879±3i	218±3e	1068±6a	1329±7b	271±1f	1366±4a
At	1019±4o	678±6c	158±6f	842±5ef	1138±6e	221±11g	901±1i
BC	1151±7l	787±6j	227±6e	952±4c	1262±7c	219±3g	1279±7b
Cons.	717±1g	559±3n	278±4d	1009±4b	1507±4a	286±6f	1160±17d
Red mud with top soil							
C	1972±6d	776±5j	242±8e	237±5p	810±5m	219±3g	826±4j
AM	1188±5k	669±6l	343±4c	364±6m	930±6ij	351±3d	934±4h
At	1080±3m	764±2j	280±5d	214±3p	919±3jk	256±6f	830±5j
BC	1240±3j	899±3i	272±3d	284±4o	951±4hi	228±5g	922±9h
Cons.	2169±4b	1267±4e	393±5c	392±3m	994±3fg	252±6f	1033±14g
Red mud with sludge							
C	1483±5h	891±6i	255±1e	376±3m	760±4n	287±9f	940±3h
AM	776±4r	633±4m	396±6c	887±3d	1129±11e	395±3c	1112±2e
At	1020±10o	842±3i	297±3d	549±3j	896±5kl	349±4d	1256±4c
BC	1818±4f	923±4h	452±3a	636±4n	1015±4f	478±8b	1109±2e
Cons.	2083±5c	1019±5g	415±3b	868±1de	1228±4d	558±6a	1359±14a
Red mud with fly ash							
C	1852±6e	861±1i	238±3e	374±5m	559±3o	229±6h	842±7j
AM	1089±4m	747±5k	366±6c	537±2j	1003±3f	332±8d	1068±7f
At	1193±0k	845±4i	286±2d	549±3j	881±3l	333±5d	1030±4g
BC	931±4q	817±5i	360±9c	599±2i	918±7jk	349±5d	1033±3g
Cons.	972±2p	678±2l	377±5c	837±6f	1122±2e	475±7b	1112±11e

Values sharing a common letter within the column are not significant at $P<0.05$.

Table 4.2.5: Influence of microbial inoculations on uptake of different nutrients and heavy metals (mg/kg) in aerial portion of bermudagrass in different red mud treatments after 12 months

Treatments	Fe	Al	Na	P	Ca	Mg	K
Red mud							
C	2652±8a	2187±4a	225±4e	319±8n	869±5l	250±2f	785±5k
AM	1752±3g	1580±7d	364±5c	419±8l	992±8fg	308±5e	1031±4g
At	1998±5d	1628±4c	264±5d	489±2k	796±3m	262±7f	1088±6g
BC	2169±5b	1858±4b	378±2c	388±4m	994±4fg	256±4f	1029±15g
Cons.	1049±6n	1577±6d	464±6a	478±3k	965±2gh	395±5c	1116±13e
Red mud with gypsum							
C	1358±4i	1063±5f	146±2f	688±2g	1154±8e	205±8h	829±3j
AM	1127±6l	879±3i	218±3e	1068±6a	1329±7b	271±1f	1366±4a
At	1019±4o	678±6c	158±6f	842±5ef	1138±6e	221±11g	901±1i
BC	1151±7l	787±6j	227±6e	952±4c	1262±7c	219±3g	1279±7b
Cons.	717±1g	559±3n	278±4d	1009±4b	1507±4a	286±6f	1160±17d
Red mud with top soil							
C	1972±6d	776±5j	242±8e	237±5p	810±5m	219±3g	826±4j
AM	1188±5k	669±6l	343±4c	364±6m	930±6ij	351±3d	934±4h
At	1080±3m	764±2j	280±5d	214±3p	919±3jk	256±6f	830±5j
BC	1240±3j	899±3i	272±3d	284±4o	951±4hi	228±5g	922±9h
Cons.	2169±4b	1267±4e	393±5c	392±3m	994±3fg	252±6f	1033±14g
Red mud with sludge							
C	1483±5h	891±6i	255±1e	376±3m	760±4n	287±9f	940±3h
AM	776±4r	633±4m	396±6c	887±3d	1129±11e	395±3c	1112±2e
At	1020±10o	842±3i	297±3d	549±3j	896±5kl	349±4d	1256±4c
BC	1818±4f	923±4h	452±3a	636±4n	1015±4f	478±8b	1109±2e
Cons.	2083±5c	1019±5g	415±3b	868±1de	1228±4d	558±6a	1359±14a
Red mud with fly ash							
C	1852±6e	861±1i	238±3e	374±5m	559±3o	229±6h	842±7j
AM	1089±4m	747±5k	366±6c	537±2j	1003±3f	332±8d	1068±7f
At	1193±0k	845±4i	286±2d	549±3j	881±3l	333±5d	1030±4g
BC	931±4q	817±5i	360±9c	599±2i	918±7jk	349±5d	1033±3g
Cons.	972±2p	678±2l	377±5c	837±6f	1122±2e	475±7b	1112±11e

Values sharing a common letter within the column are not significant at $P<0.05$.

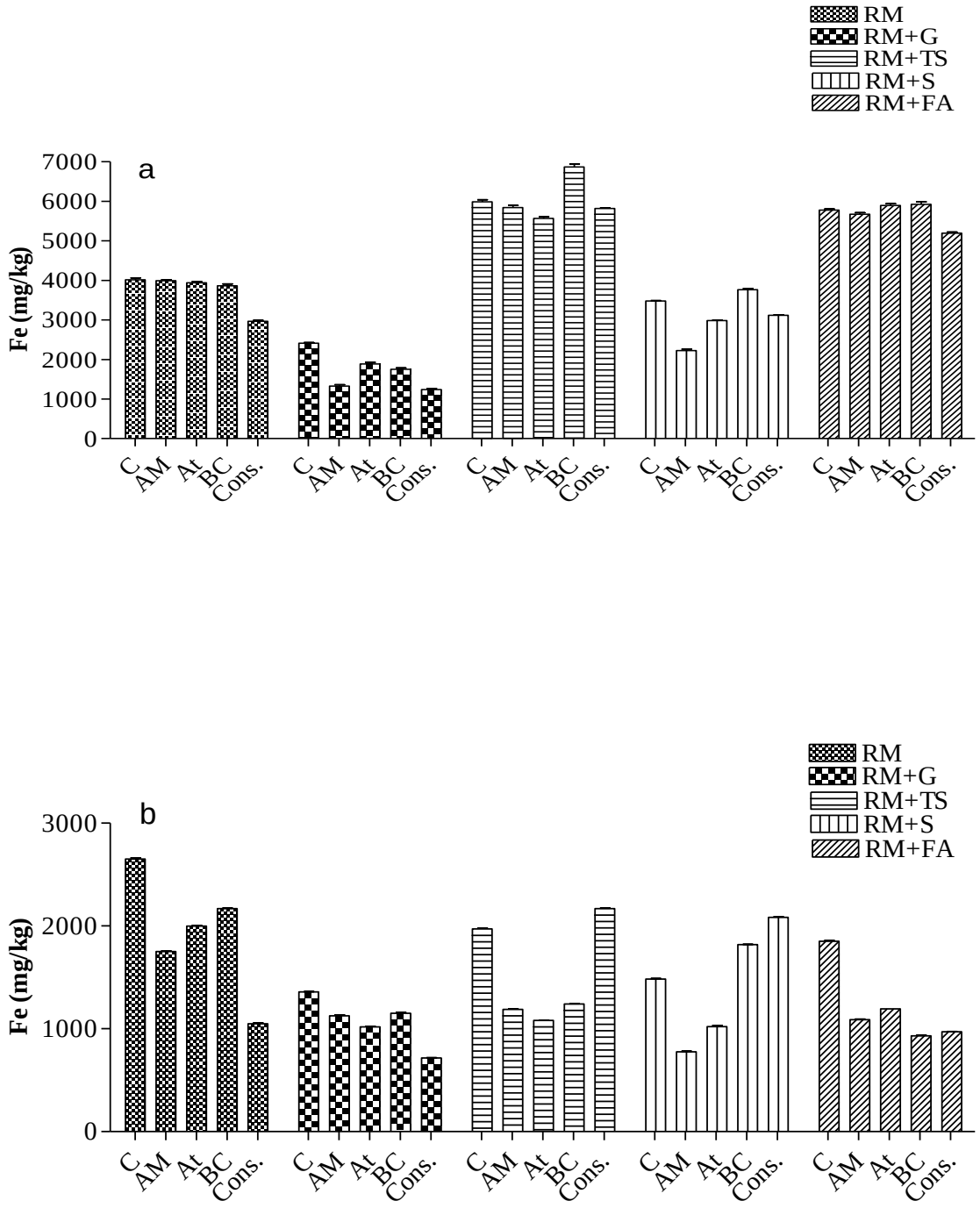


Fig 4.2.3: Effect of different microbial inoculated treatments on Fe accumulation in bermudagrass after 6 (a) and 12 (b) months. Mean ±SD (n=3).

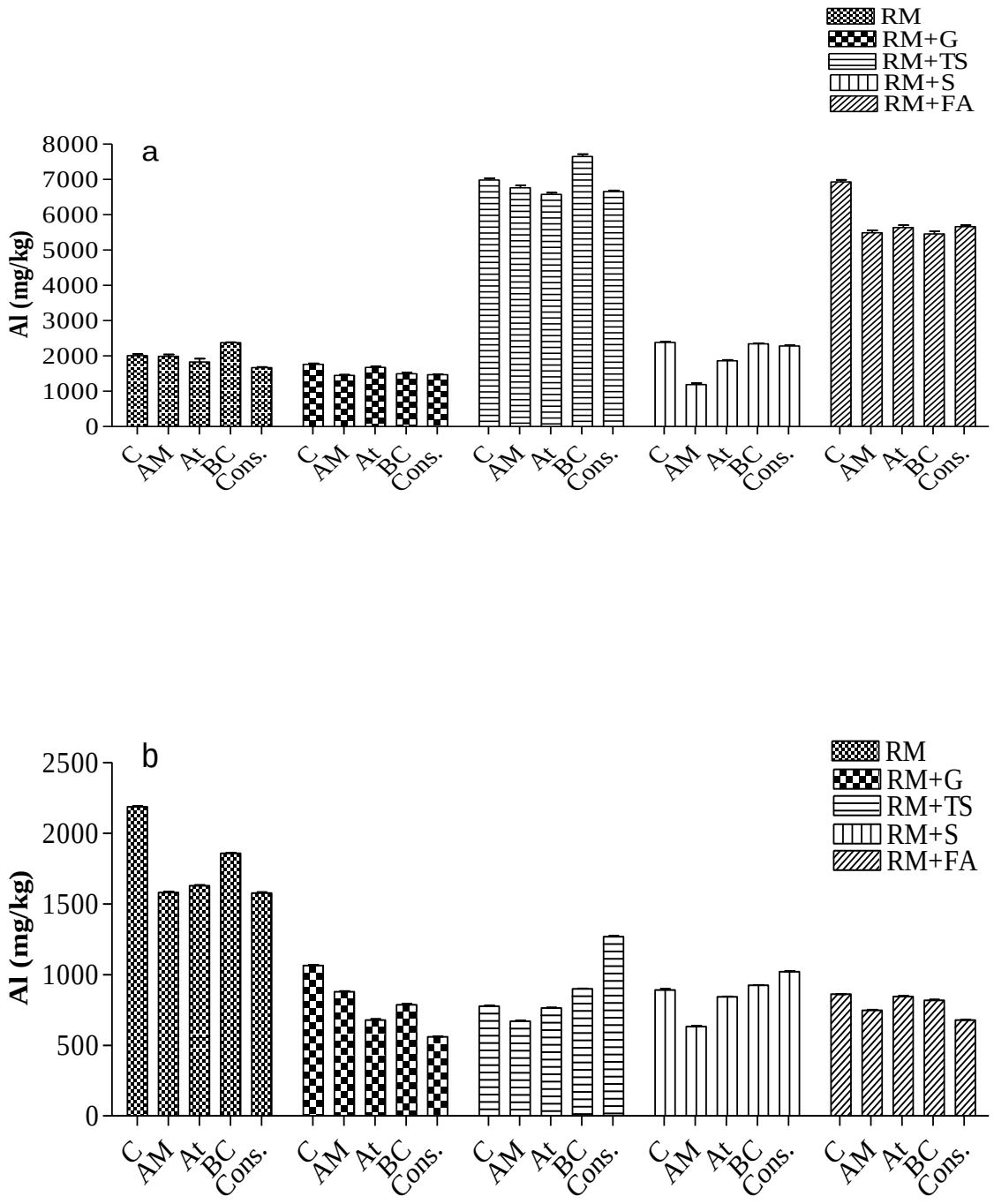


Fig 4.2.4: Effect of different microbial inoculated treatments on Al accumulation in bermudagrass after 6 (a) and 12 (b) months. Mean ±SD (n=3).

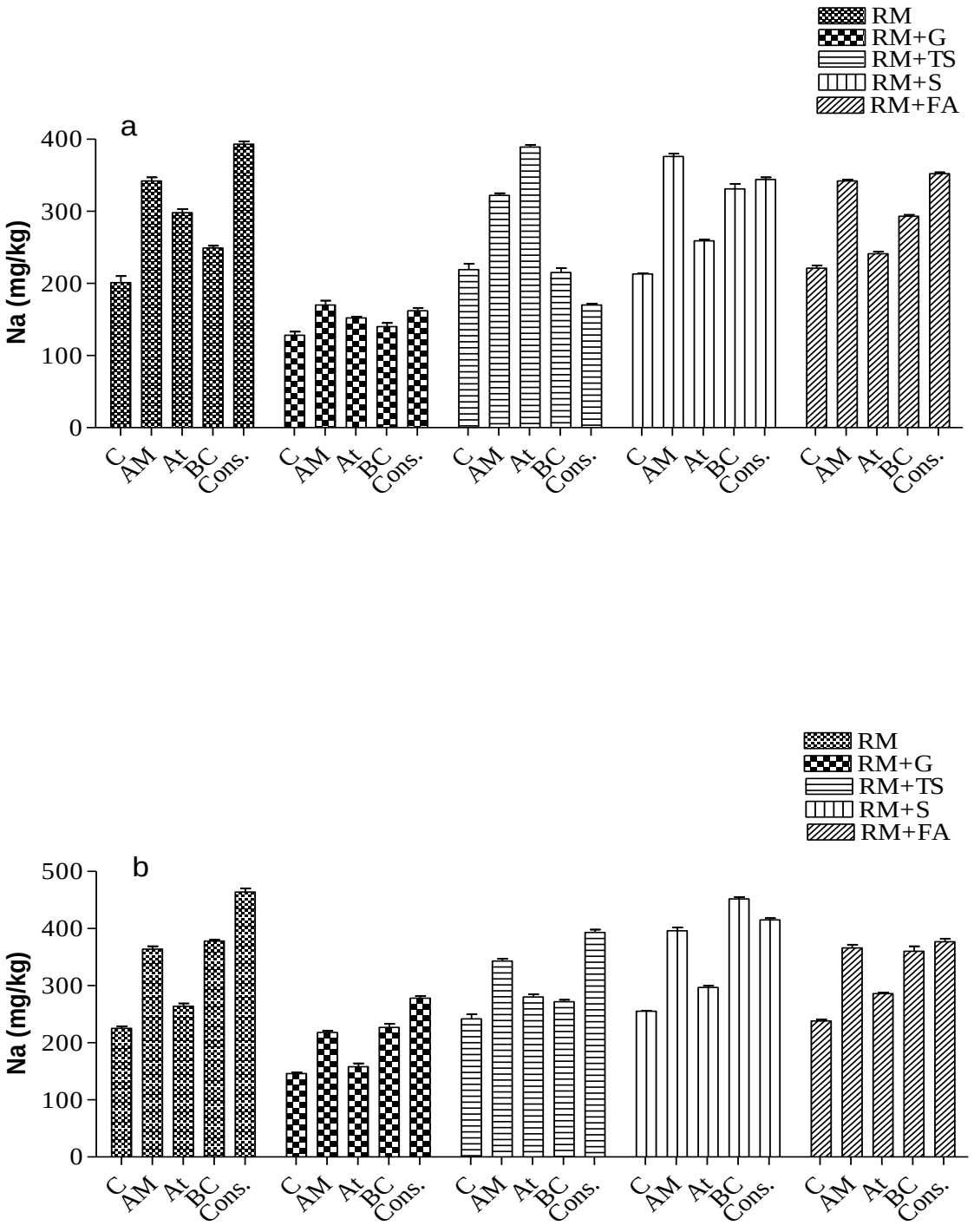


Fig 4.2.5: Effect of different microbial inoculated treatments on Na accumulation of bermudagrass after 6 (a) and 12 (b) months. Mean ±SD (n=3).

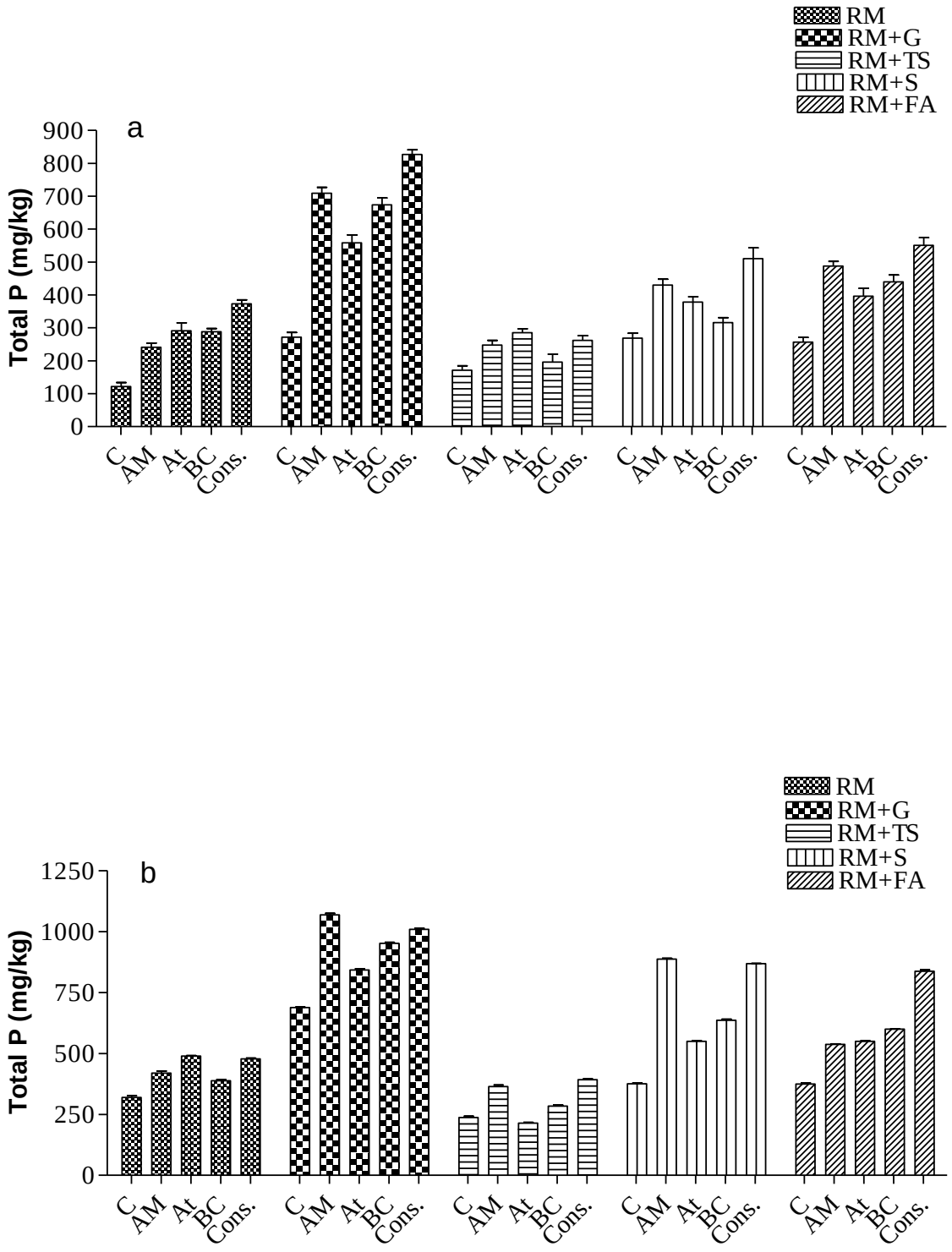


Fig 4.2.6: Effect of different microbial inoculated treatments on total P uptake of bermudagrass after 6 (a) and 12 (b) months. Mean ±SD (n=3).

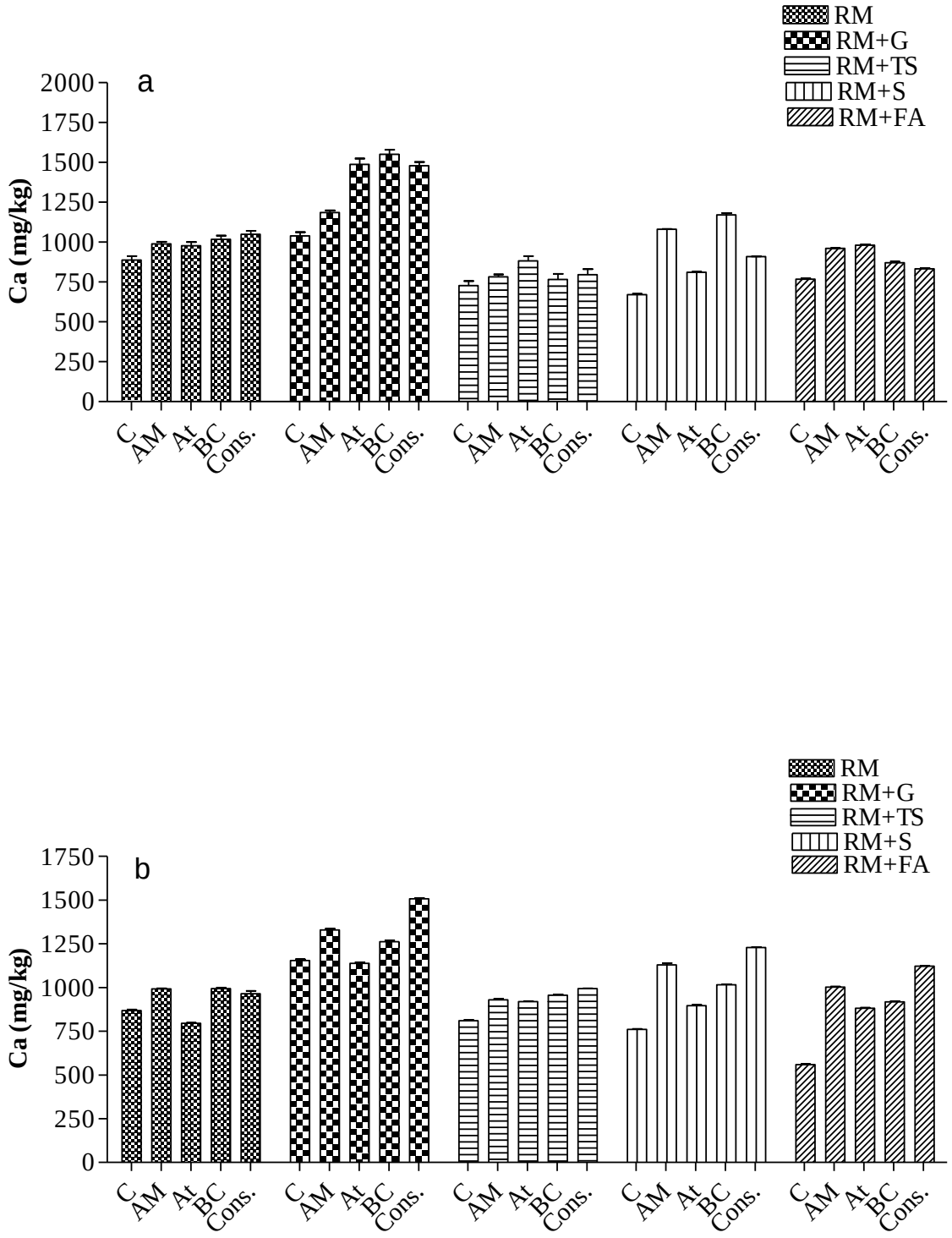


Fig 4.2.7: Effect of different microbial inoculated treatments on Ca uptake of bermudagrass after 6 (a) and 12 (b) months. Mean ±SD (n=3).

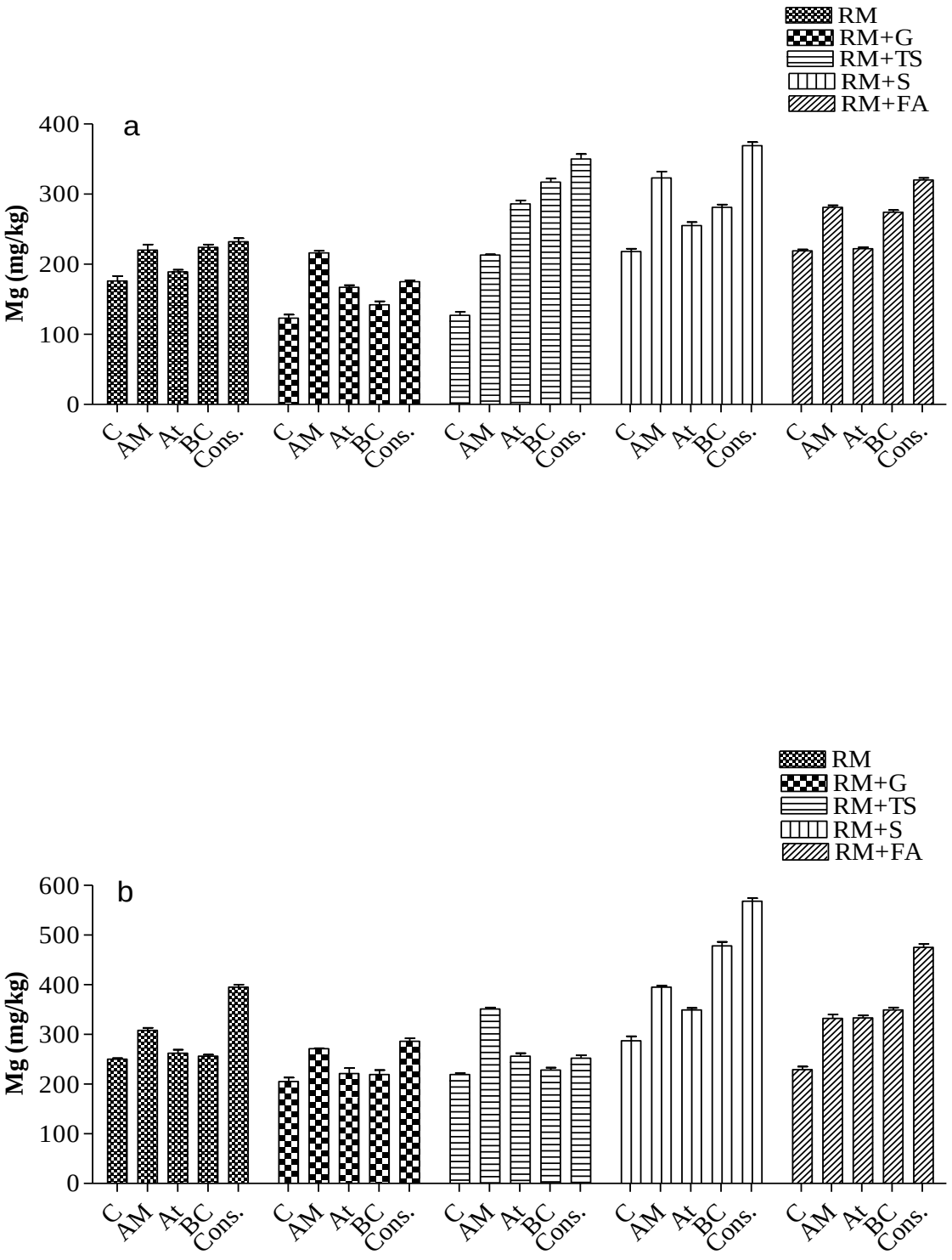


Fig 4.2.8: Effect of different microbial inoculated treatments on Mg uptake of bermudagrass after 6 (a) and 12 (b) months. Mean ±SD (n=3).

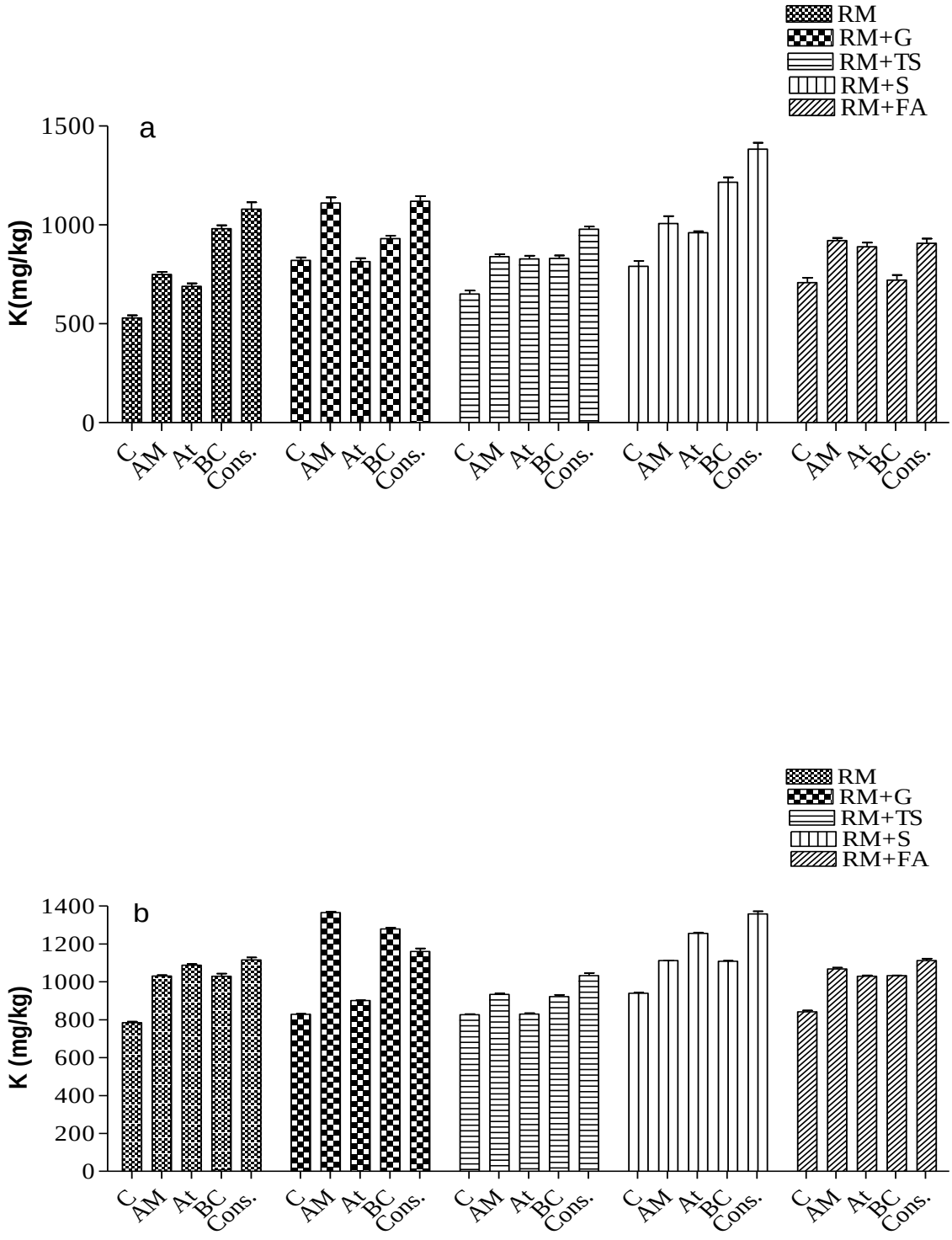


Fig 4.2.9: Effect of different microbial inoculated treatments on K uptake of bermudagrass after 6 (a) and 12 (b) months. Mean ±SD (n=3).

4.2.7 Chemical analysis of experimented soil

Red mud is notoriously hard to revegetate because of its mainly high alkaline, saline-sodic nature. Hence, establishment of revegetation on red mud only possible when pH of red mud can be reduced. Application of different ameliorates such as gypsum, topsoil, sludge and fly ash improved the properties of RM. After 6 months of plantation, the pH of the all the red mud mended treatments were significantly reduced compared to their initial values.

In RM treatment, microbial inoculated treatments At, BC and consortium significantly reduced the pH compared to other treatments. Among them, consortium inoculated treatment had shown more pH reduction compared to others. In the gypsum amended control treatment, there was a reduction of pH of 2 units compared to RM control treatment. The pH of gypsum amended treatments reduced in all microbial inoculated treatments compared to their respective controls. Inoculation of consortium to gypsum amended treatment reduced the pH to lowest compared to all other microbial inoculated treatments. The pH of top soil amended control treatment significantly reduced one unit compared to initial value of pH. Further, inoculation of consortium followed by At significantly reduced compared to other treatments (Table 4.2.6; Fig 4.2.10a). The initial pH of sludge amended treatment was 10.57, which was significantly reduced 8.82 after 6 months of plants growth in control treatment. Moreover, inoculation of different microbial treatments further decreased the pH compared to control treatments. The maximum reduction of pH was observed in consortium when compared to all other microbial inoculated treatments. In fly ash amended treatment, the pH was significantly reduced 1.5 units in control treatment compared to initial pH (10.41).

Inoculation of microbial treatments significantly reduced the pH compared to their respective control treatments. The maximum pH reduction was observed in consortium inoculated treatments (Table 4.2.6; Fig 4.2.10a).

After 12 months of plantation, the pH was further reduced in all treatments when compared to 6 months of observation. In red mud treatment, all inoculated treatments significantly decreased the pH compared to their respective controls. Amongst all the microbial inoculated treatments, inoculation of At and consortium significantly decreased the pH compared to AM and BC inoculated treatments. All gypsum amended microbial inoculated treatments significantly reduced the pH than the control treatment and no significant variation in pH was recorded in this treatments. In top soil amended treatment, AM and At inoculated treatments had shown reduced level of pH compared to other treatments. In case of sludge, microbial inoculants significantly decreased the pH compared to control treatment. The higher pH reduction was observed in consortium compared to all other treatments. After 12 months of observation, overall the maximum pH reduction was observed in gypsum amended treatments (Table 4.2.7; Fig 4.2.10b).

Electrical conductivity (EC)

Soil electrical conductivity (EC) is a common measure of water soluble salts (salinity) in soil and correlates with soil properties that affect soil productivity, including soil texture, cation exchange capacity (CEC), drainage conditions, organic matter level, and subsoil characteristics.

After 6 months of grass growth, the EC of all the treatments significantly reduced compared to initial EC. Inoculation of different microbial treatments had shown significant reduction of EC in all treatments compared to controls except in all sludge and

AM inoculated FA amended treatments. In RM inoculated with At and BC treatment, the reduction in EC was minimum i.e. 45.3%. There was no significant difference in electric conductivity of AM and consortium inoculated RM, however these values were 31% lower than control. The highest EC was observed gypsum treated RM compared to other treatments. However, inoculated treatments decreased the EC levels. The maximum reduction in EC was 20% which was observed in consortium inoculated treatment followed by AM (12%) whereas in At and BC the reduction was only around 7%. In case of topsoil the value of EC increased in the AM inoculated treatment whereas in all other treatments it reduced when compared to control. Maximum reduction i.e. 20% was observed in At inoculated treatment followed by 11% reduction in BC and 2% in consortium. In contrast to other amendments, in case of sludge, the values of EC increased as compared to control in all treatments. The increase in electrical conductivity in AM inoculated treatment was 51% whereas in BC and At it was around 28% than control treatments. The highest reduction in EC was observed in fly ash as compared to the initial values. The reduction of EC was 33% in case of AM, BC and consortium inoculated treatments whereas it was 22% in At compared to control. Overall, the maximum EC was observed in gypsum amended treatment. In sludge amended treatments, inoculated treatments significantly increased EC that the control treatments (Table 4.2.6; Fig 4.2.11a).

After 12 months, EC of all the treatments further decreased except in top soil, fly ash amended treatments and AM inoculated RM. In gypsum amended treatment there was no significant difference in EC amongst the different inoculations as compared to control except consortium. In topsoil with BC, there was 19% increase in value of EC, whereas

in At and consortium, it was 23 and 16% decrease respectively than control. In case of sludge, the microbial inoculated treatments caused 11.9% increase in EC compared to control. Amongst the other treatments, the variation of increase in EC was high after 12 months (as compared to six months) in fly ash amended treatment. In At inoculated treatment, the increase in EC was almost double than control, while in other inoculations the increase was 26.7%. Overall, the EC was significantly higher in gypsum treatments when compared to other treatments. This was in contrast to the pH, as maximum pH reduction was noted in gypsum amended red mud treatment (Table 4.2.7; Fig 4.2.11b).

Organic carbon (%)

After 6 months of plantation, organic carbon was increased in all treatments compared to initial values. Inoculations of different microbial treatments increased the organic carbon with various degrees compared to their respective control treatments. In red mud treatment, the maximum organic carbon was observed in consortium followed by AM and BC. In gypsum amended treatment, the maximum organic carbon was observed in consortium inoculated treatment compared to all other inoculated treatments. Arbuscular mycorrhizal fungi inoculated treatment significantly increased organic carbon when compared to At and BC inoculated treatments and At treatment had shown higher organic carbon than BC. Inoculation of consortium to top soil amended treatment had shown maximum organic carbon compared to all other inoculated treatments (Table 5.9; Fig 5.13a). However, AM fungal and BC inoculated treatments significantly increased the organic carbon compared to At inoculated treatment and high organic carbon was in BC than AM. In sludge amended treatment also the maximum organic carbon was determined in consortium compared to other inoculated treatments. Inoculation of AM

and BC significantly increased the organic carbon when compared to At. Slight significant difference in organic carbon was observed in AM and BC. In fly ash amended consortium treatment there was more organic carbon when compared to all other inoculated treatments. *Aspergillus tubingensis* treatments had shown slight increase of organic carbon than AM and BC. Overall, the maximum organic carbon was observed in sludge followed by gypsum amended treatments (Table 4.2.8; Fig 4.2.12a).

After 12 months, organic carbon was significantly changed with various levels in all treatments from 6 months. In red mud treatment, organic carbon gradually increased from 6 months to 12 months and this was higher in inoculated treatments. In gypsum amended treatments, organic carbon decreased in all treatments compared to 6 months. However, inoculated treatments especially consortium had shown higher amount of organic carbon when compared to other inoculated and control treatments. In case of top soil amended treatment except At inoculated treatment, organic carbon was found to be decreased from 6 months to 12 months. However, organic carbon was increased by inoculated treatments compared to control. The maximum organic carbon was observed in consortium inoculated treatments compared to other treatments (Table 4.2.9; Fig 4.2.11b).

The maximum organic carbon was observed in sludge amended treatments compared to all other red mud amended or red mud alone treatments. Consortium inoculated treatment had shown maximum organic carbon compared to AM, At and BC inoculated treatments. Overall, organic carbon was recorded highest in sludge followed by gypsum amended treatment. Further, inoculation of consortium followed by AM and BC significantly increased organic carbon in all the treatments (Table 4.2.9; Fig 4.2.11b).

Available Phosphorous (P)

After 6 months of plantation, available P of all treatments was significantly increased with various degrees when compared to initial values. Further microbial inoculated treatments significantly increased the available P compared to respective controls except in RM treatment. In RM treatment, maximum available P was estimated in BC inoculated treatment followed by At and consortium. Inoculation of consortium to gypsum amended treatment had shown increased amount of available P and it was followed by BC, At and AM fungal inoculated treatments. Available P was higher in At inoculated treatment followed by consortium top soil amended treatments. In sludge amended treatment, there was no significant difference in available P amongst the AM, At and BC inoculated treatments and maximum available P was observed in these treatments compared to consortium. In fly ash amended treatment, the highest available P was recorded in At and consortium followed by AM and BC. Overall, maximum available P was observed in gypsum followed by sludge amended treatments (Table 4.2.8; Fig 4.2.13a).

After 12 months, it was observed that available P was found to be gradually increased in all treatments from 6 months. In RM treatment, the maximum available P was observed in consortium followed by BC than the other treatments. Gypsum amended consortium treatment had shown significant increase of available P compared to all those of inoculated treatments. In top soil amended treatment, consortium and AM fungal inoculated treatments significantly increased available P compared to At and BC treatments. Amongst all other microbial treatments, inoculation of consortium to sludge amended treatment had shown maximum available P. Available P was maximum in consortium followed by BC, AM and At inoculated treatments. Overall, maximum

available P was observed in gypsum followed by sludge amended treatments. Further, microbial inoculation treatments significantly increased compared to their control treatments (Table 4.2.9; Fig 4.2.13b).

Total nitrogen (N)

Total N significantly increased, after 6 months of grass growth, in all the treatments compared to their initial values. Microbial inoculation increased the amount of total N with various levels compared to their respective control treatments. In the RM treatment, total N was higher in consortium followed by BC, AM and At. The higher total N in gypsum amended treatment was observed in consortium followed by AM, BC and At inoculated treatments. The higher total N was observed in consortium inoculated top soil amended treatment followed by AM treatments. Inoculation of BC and consortium to sludge amended treatment had shown higher amount of total N compared to AM and At, and AM treatment had more total N than At. In fly ash amended treatment, high total N was observed in consortium followed by AM. The maximum total N was observed in sludge followed by gypsum amended treatments (Table 4.2.8; Fig 4.2.14a).

After 12 months, inoculation with different microbes significantly increased the total N in all treatments. In the RM treatment, total N was significantly increased in consortium followed by BC inoculated treatments. Inoculation of microbial treatments increased total N in the following order in gypsum amendment; consortium>BC>AM>At. As compared to all other microbial inoculated top soil amended treatments, consortium inoculation showed maximum total N. Bacterial consortia inoculated treatment also significantly increased the total N compared to At and AM fungal inoculated treatments. Lowest amount of total N was observed in AM fungal

inoculated treatment. After 12 months of plants growth, the maximum total N was observed in sludge followed by gypsum amended treatments (Table 4.2.9; Fig 4.2.14b).

Soil enzyme activity

Soil enzymes are bio-indicators and its activity indicates the microbial activity and also provides the information of processes in soil such as decomposition and nutrient cycling. In this study, phosphatases (acid and alkaline) and urease activity were determined. The addition of different amendments significantly increased enzymatic activities in rhizospheric soil.

Acid phosphatase activity

After 6 month of plants growth, acid phosphatase enzyme activity was found to be increased with various degrees in all microbial inoculated treatments compared to their respective control treatments. Amongst all the microbial inoculated treatments, consortium inoculated treatments significantly increased the acid phosphatase activity compared to other inoculated treatments. In RM treatment, bacterial consortia inoculation significantly enhanced the acid phosphatase activity compared to AM fungi and At inoculants. Inoculation of At had increased the acid phosphatase activity compared to AM inoculated treatment. The maximum acid phosphatase activity was observed in gypsum amended treatments when compared to other treatments. As compared to inoculated treatments, acid phosphatase activity was high in AM followed by BC and At (Table 4.2.8; Fig 4.2.15a).

After 12 months, acid phosphatase activity changed with various degrees in all treatments. In RM treatment, acid phosphatase activity gradually increased from 6 months. The maximum acid phosphatase activity was observed in At followed by

consortium, AM and At inoculated treatments. In case of gypsum amended, treatment acid phosphatase activity was reduced from 6 months to 12 months except in At inoculated treatment. However, inoculation with microbes significantly increased the acid phosphatase activity in all treatments compared to their respective control treatments. Inoculation of At enhanced acid phosphatase activity compared to inoculation of AM, BC and consortium (Table 4.2.9; Fig 4.2.15b).

The maximum acid phosphatase activity was observed in consortium followed by AM>At>BC inoculated treatment at 12 months in top soil treatment. Sludge amended treatments gradually increased the acid phosphatase activity from 6 months to 12 months. Bacterial consortia inoculated treatment enhanced the acid phosphatase activity when compared to AM inoculated treatment. In fly ash amended treatment, it was observed that the acid phosphatase activity gradually increased from 6 months and consortium inoculated treatment had higher acid phosphatase activity than the other inoculated treatment. AM fungal inoculation significantly increased the acid phosphatase activity compared to BC and At inoculated treatments (Table 4.2.9; Fig 4.2.15b).

Alkaline phosphatase

After 6 months of observation, alkaline phosphatase activity was found to be higher than acid phosphatase activity except in At inoculated gypsum amended treatment at 12 months. Microbial inoculated treatments had shown higher alkaline phosphatase activity compared to their respective controls. In RM treatment, alkaline phosphatase activity was higher in consortium followed by BC>At>AM fungal inoculated treatments. Alkaline phosphatase activity in gypsum amended treatment was higher in At and BC inoculated treatments compared to AM. In top soil amended treatment, alkaline phosphatase activity

was found to similar in all inoculated treatments. In sludge amended treatment, consortium inoculated treatment had shown higher alkaline phosphatase activity compared to other inoculated treatments. In case of fly ash amended treatment, the high activity of alkaline phosphatase was observed in At and consortium than other AM and BC inoculated fly ash amended treatments (Table 4.2.8; Fig 4.2.16a).

In gypsum amended treatment, alkaline phosphatase activity was highest compared to other red mud amended and red mud alone treatments at 12 months. In sludge amended treatment, higher alkaline phosphatase activity was observed in consortium followed by AM>At>BC inoculated treatments. In case of fly ash amended treatment, At inoculated treatments had shown higher alkaline phosphatase activity compared to other inoculated treatments. Bacterial inoculated treatments significantly increased alkaline phosphatase activity when compared to AM fungi and consortium inoculated treatments (Table 4.2.9; Fig 4.2.16b).

Urease activity

After 6 months of plantation, microbial inoculated treatments had shown highest urease activity compared to their respective controls. In RM treatment, BC inoculated treatment showed more urease activity compared to other treatments. Arbuscular mycorrhizal fungi and At inoculated treatments did not show significance difference in urease activity. In gypsum amended treatments, consortium inoculated treatment significantly increased urease activity compared to all other inoculated treatments. Significant increase of urease activity was recorded in consortium inoculated top soil amended treatment compared to all other treatments. In sludge amended treatments, the consortium inoculated treatments had higher urease activity compared with AM and BC,

which were shown high activity than At. Urease activity did not differ in AM and BC inoculated treatments. Overall, maximum urease activity was observed in gypsum and sludge amended treatments (Table 4.2.8; Fig 4.2.17a).

After 12 months, urease activity was reduced with various degrees in all the treatments except in BC and At inoculated top soil and fly ash amended treatments. Microbial inoculated treatments significantly increased urease activity compared to their respective control treatments. In RM treatment, higher urease activity was recorded in BC followed by consortium inoculated treatments compared to AM and At. Inoculation with AM fungi followed by consortium, At and BC significantly increased the urease activity to gypsum amendment. In tops soil amended treatment, the highest urease activity was observed in AM and BC inoculated treatments, which was further followed by At and consortium. In sludge amended treatment, the higher urease activity was observed in consortium followed by BC, AM and At inoculated treatments. In case of fly ash amended treatment the higher urease activity was observed in BC inoculated treatment than the other inoculated treatments. Sludge with microbial inoculated treatments had show the maximum urease activity compared to all other treatments (Table 4.2.9; Fig 4.2.17b).

Table 4.2.6: Effect of microbial inoculations on reduction of pH and electric conductivity of different red mud treatments after 6 months

Treatments	pH	EC (mS/cm)
Red mud		
C	9.81±0.01a	0.97±0.01e
AM	9.82±0.02a	0.64±0.02f
At	9.61±0.02b	0.53±0.02g
BC	9.60±0.02b	0.53±0.01g
Cons.	9.46±0.07c	0.66±0.01f
Red mud with gypsum		
C	7.81±0.3k	1.87±0.01a
AM	7.54±0.03l	1.65±0.01c
At	7.52±0.01l	1.78±0.01b
BC	7.58±0.00l	1.74±0.00b
Cons.	7.43±0.02m	1.49±0.10d
Red mud with top soil		
C	9.48±0.13c	0.38±0.01hi
AM	9.33±0.02c	0.43±0.02h
At	9.21±0.02d	0.30±0.00jkl
BC	9.46±0.04c	0.34±0.01ijk
Cons.	9.13±0.04e	0.37±0.03hij
Red mud with sludge		
C	8.82±0.03g	0.43±0.01h
AM	8.70±0.05h	0.65±0.00f
At	8.55±0.00i	0.52±0.00g
BC	8.57±0.02i	0.55±0.02g
Cons.	8.13±0.12j	0.92±0.03e
Red mud with fly ash		
C	8.92±0.02f	0.36±0.02hij
AM	8.70±0.01h	0.21±0.00m
At	8.70±0.04h	0.28±0.02klm
BC	8.73±0.05h	0.23±0.01lm
Cons.	8.83±0.02g	0.24±0.01lm

Values sharing a common letter within the column are not significant at $P<0.05$.

Table 4.2.7: Effect of microbial treatments on reduction of pH and electric conductivity of different amendments of red mud after 12 months

Treatments	pH	EC (mS/cm)
	Red mud	
C	9.24±0.08a	0.49±0.02ef
AM	8.79±0.06b	0.67±0.02d
At	8.69±0.04bc	0.54±0.02e
BC	8.80±0.09b	0.53±0.03e
Cons.	8.62±0.02bc	0.44±0.01f
	Red mud with gypsum	
C	7.70±0.06j	1.71±0.02a
AM	7.47±0.12k	1.63±0.02ab
At	7.40±0.21k	1.64±0.03ab
BC	7.51±0.02k	1.64±0.03ab
Cons.	7.49±0.01k	1.60±0.01b
	Red mud with top soil	
C	8.66±0.04c	0.70±0.01d
AM	8.45±0.06d	0.71±0.02d
At	8.37±0.02de	0.54±0.01e
BC	8.62±0.02c	0.83±0.02c
Cons.	8.61±0.01c	0.69±0.01d
	Red mud with sludge	
C	8.82±0.03g	0.43±0.01h
AM	8.70±0.05h	0.65±0.00f
At	8.55±0.00i	0.52±0.00g
BC	8.57±0.02i	0.55±0.02g
Cons.	8.13±0.12j	0.92±0.03e
	Red mud with fly ash	
C	8.92±0.02f	0.36±0.02hij
AM	8.70±0.01h	0.21±0.00m
At	8.70±0.04h	0.28±0.02klm
BC	8.73±0.05h	0.23±0.01lm
Cons.	8.83±0.02g	0.24±0.01lm

Values sharing a common letter within the column are not significant at $P<0.05$.

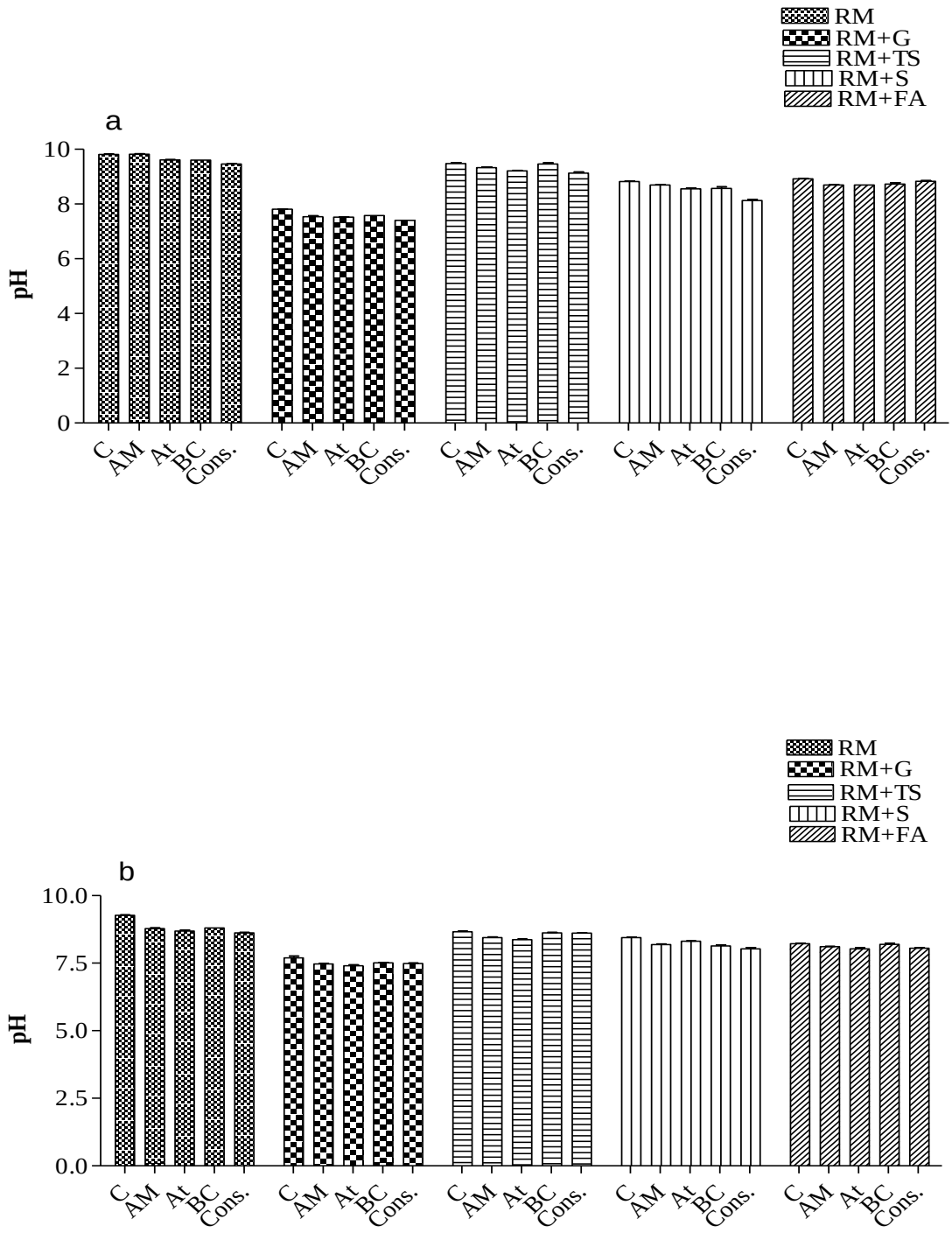


Fig 4.2.10: Influence of different microbial treatments on rhizosphere soil pH changes in different red mud treatments after 6 (a) and 12 (b) months. Mean ±SD (n=3).

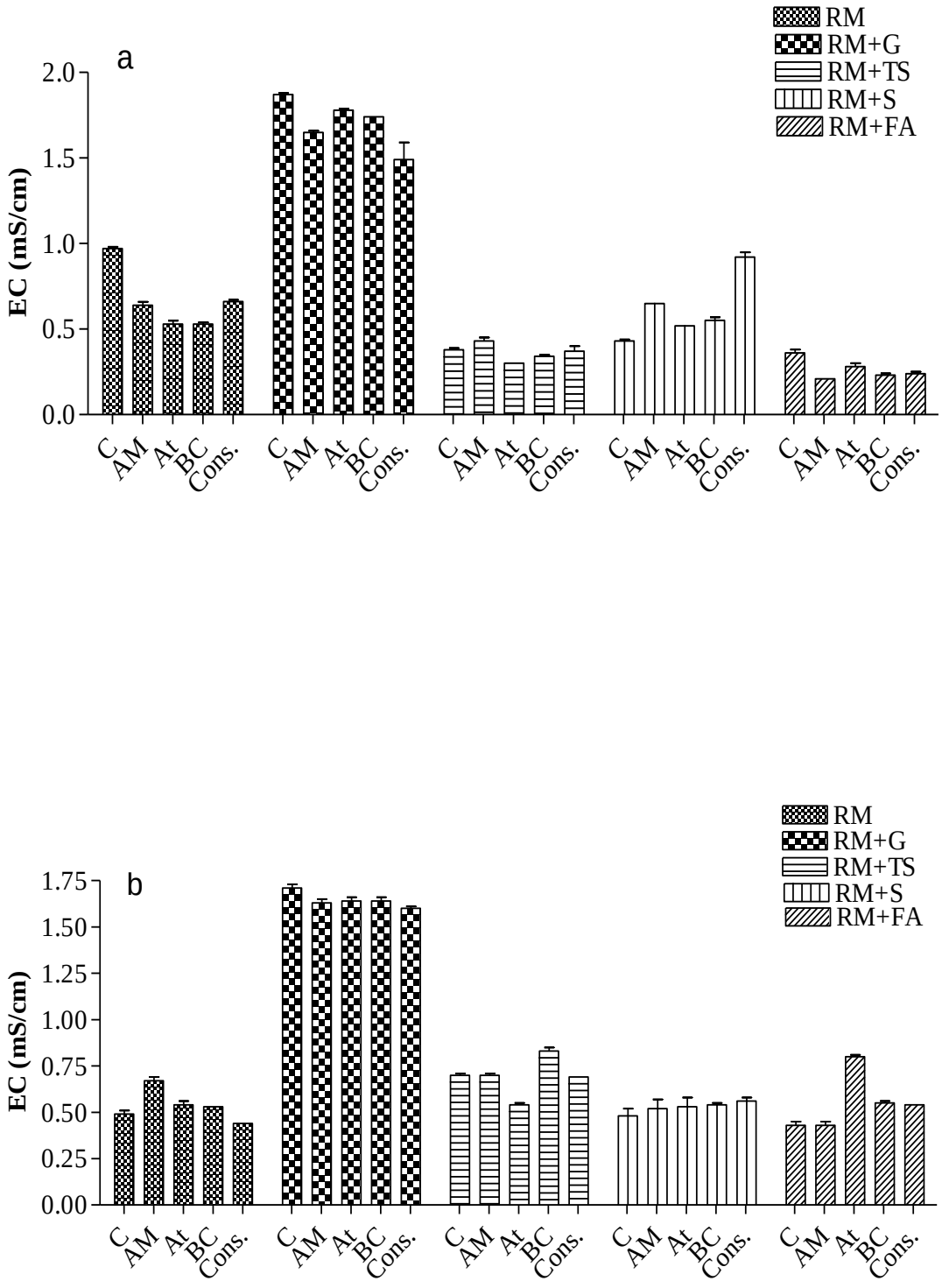


Fig 4.2.11: Effect of different microbial inoculated treatments on changes of rhizosphere soil EC after 6 (a) and 12 (b) months. Mean ±SD (n=3).

Table 4.2.8: Effect of microbial inoculation on chemical and biochemical properties of different red mud treatments after 6 months

Treatments	Organic carbon (%)	Available P (mg/kg)	Total N (mg/kg)	Acid P.ase (µM/g/hr)	Alkaline P.ase (µM/g/hr)	Urease (µM/g/hr)
Red mud						
C	0.38±0.02l	0.53±0.02l	85±1p	142±2m	168±3g	14.6±1.0i
AM	0.63±0.0jk	0.57±0.03l	125±3o	178±3l	206±1h	26.8±0.5h
At	0.51±0.01kl	0.84±0.03jkl	135±2o	246±1j	257±3f	25.9±0.7h
BC	0.69±0.01jk	0.94±0.04ijk	159±4n	222±0k	275±0e	42.6±0.3f
Cons.	0.74±0.03ij	0.70±0.03jkl	205±7m	255±5i	290±1d	35.3±0.5g
Red mud with gypsum						
C	0.91±0.01h	2.82±0.10e	459±6j	348±2f	368±0c	53.8±0.5c
AM	1.83±0.09d	3.43±0.05d	692±1d	410±1d	423±2b	76.5±0.9b
At	1.23±0.05f	3.80±0.04c	492±6i	433±2c	493±3a	65±1.4c
BC	1.89±0.08c	4.17±0.20b	673±0e	446±11b	490±1a	75.3±0.8b
Cons.	2.14±0.04b	4.92±0.32a	717±4c	459±3a	425±1b	83.4±1.2a
Red mud with top soil						
C	0.74±0.03ij	0.61±0.02kl	329±5l	226±0k	291±1d	33.3±0.7g
AM	1.08±0.09fg	0.77±0.03jkl	571±2g	247±0j	355±1c	55±0.8d
At	0.81±0.03hi	1.21±0.14i	459±3j	245±0j	364±2c	58.3±0.7d
BC	1.96±0.10c	0.85±0.05jkl	527±2h	231±1j	384±3c	57.3±0.5d
Cons.	2.13±0.09b	0.99±0.03ij	634±1f	268±7h	362±0c	66.2±1.5c
Red mud with sludge						
C	1.22±0.05f	2.45±0.11fg	500±1i	265±4h	315±2d	47.8±0.5e
AM	1.81±0.09cd	4.20±0.17b	773±6b	325±4g	365±1c	72.7±0.3b
At	1.41±0.04e	4.39±0.05b	666±3e	339±3g	384±3c	67.6±0.5c
BC	1.93±0.01c	4.47±0.17b	817±7a	328±6g	366±1c	73.4±1b
Cons.	2.61±0.17a	3.24±0.18d	829±3a	365±3e	420±1b	85.8±0.6a
Red mud with fly ash						
C	0.82±0.04hi	2.00±0.08h	438±1k	217±1k	256±1f	24.3±1.6h
AM	0.99±0.09h	2.57±0.05efg	492±3i	241±2j	315±3d	40.8±1.3f
At	1.02±0.01gh	2.75±0.03ef	459±2j	236±3j	385±2c	44.9±1ef
BC	0.99±0.06h	2.26±0.13gh	493±7i	230±0j	317±4d	48±0.7e
Cons.	1.77±0.13c	2.64±0.23ef	539±6h	272±6h	372±1c	44.6±1.0ef

Values sharing a common letter within the column are not significant at $P<0.05$.

Table 4.2.9 Effect of microbial inoculation on chemical and biochemical properties of different red mud treatments after 12 months

Treatments	Organic carbon (%)	Available P (mg/kg)	Total N (mg/kg)	Acid P.ase (µM/g/hr)	Alkaline P.ase (µM/g/hr)	Urease (µM/g/hr)
Red mud						
C	0.51±0.03h	0.86±0.01n	57±1o	172±1m	274±2k	8.1±0.3n
AM	1.08±0.03f	1.31±0.06m	100±1mn	233±5j	386±5i	19.5±0.1k
At	0.74±0.01g	1.40±0.02lm	81±1no	370±0e	428±2i	16.6±0.3k
BC	1.15±0.09f	1.32±0.05m	120±4lm	228±1j	385±5i	29.8±0.3j
Cons.	1.25±0.08f	1.70±0.05l	146±2l	265±4i	414±6i	33.5±0.4i
Red mud with gypsum						
C	0.60±0.00gh	4.47±0.04f	390±1hi	294±3g	416±1i	47.9±0.5f
AM	1.29±0.05f	7.93±0.09c	485±2ef	395±3c	515±4f	67.6±0.6a
At	0.96±0.04f	8.27±0.04c	425±1gh	490±1a	499±2g	59.0±0.3c
BC	1.62±0.02d	8.86±0.03b	539±4d	359±1e	475±4h	56±1.5d
Cons.	2.2±0.08b	9.46±0.03a	664±6c	433±6b	556±7d	62.4±0.8b
Red mud with top soil						
C	0.57±0.02k	1.39±0.04lm	268±0k	210±0l	315±1j	28.9±0.7j
AM	0.89±0.01f	2.00±0.04k	343±4j	261±3i	401±1i	69.4±0.7a
At	1.00±0.05f	1.71±0.04l	425±1gh	238±1j	382±3i	40.1±0.4g
BC	0.89±0.08f	1.70±0.02l	425±4gh	221±4k	391±8i	68.3±0.9a
Cons.	1.88±0.26de	2.22±0.04k	519±4de	277±4h	419±10i	49.9±0.4e
Red mud with sludge						
C	1.09±0.07f	2.86±0.07j	353±1ij	362±2e	417±2i	28.9±0.6j
AM	1.91±0.17b	5.84±0.05e	690±1c	413±1c	592±1b	41.3±0.8g
At	1.40±0.02e	4.07±0.02g	538±3d	361±2e	577±0c	36.5±0.4h
BC	2.04±0.04b	5.57±0.37e	742±8b	435±1b	536±5e	59.2±0.2c
Cons.	2.86±0.09a	6.84±0.12d	818±12a	492±3a	769±9a	68.3±0.9a
Red mud with fly ash						
C	0.80±0.01f	2.12±0.08k	323±2j	335±2f	415±1i	13.8±0.6m
AM	1.2±0.04f	3.70±0.05h	448±1fg	382±2d	472±1h	30.5±0.7j
At	1.21±0.01f	3.25±0.00i	477±2f	367±0e	550±1d	28.8±0.8j
BC	1.24±0.06f	4.38±0.05f	526±8d	368±1e	501±14g	37.6±1.1h
Cons.	1.79±0.17c	4.55±0.05f	664±9c	397±3c	479±7h	30.7±0.5j

Values sharing a common letter within the column are not significant at $P<0.05$.

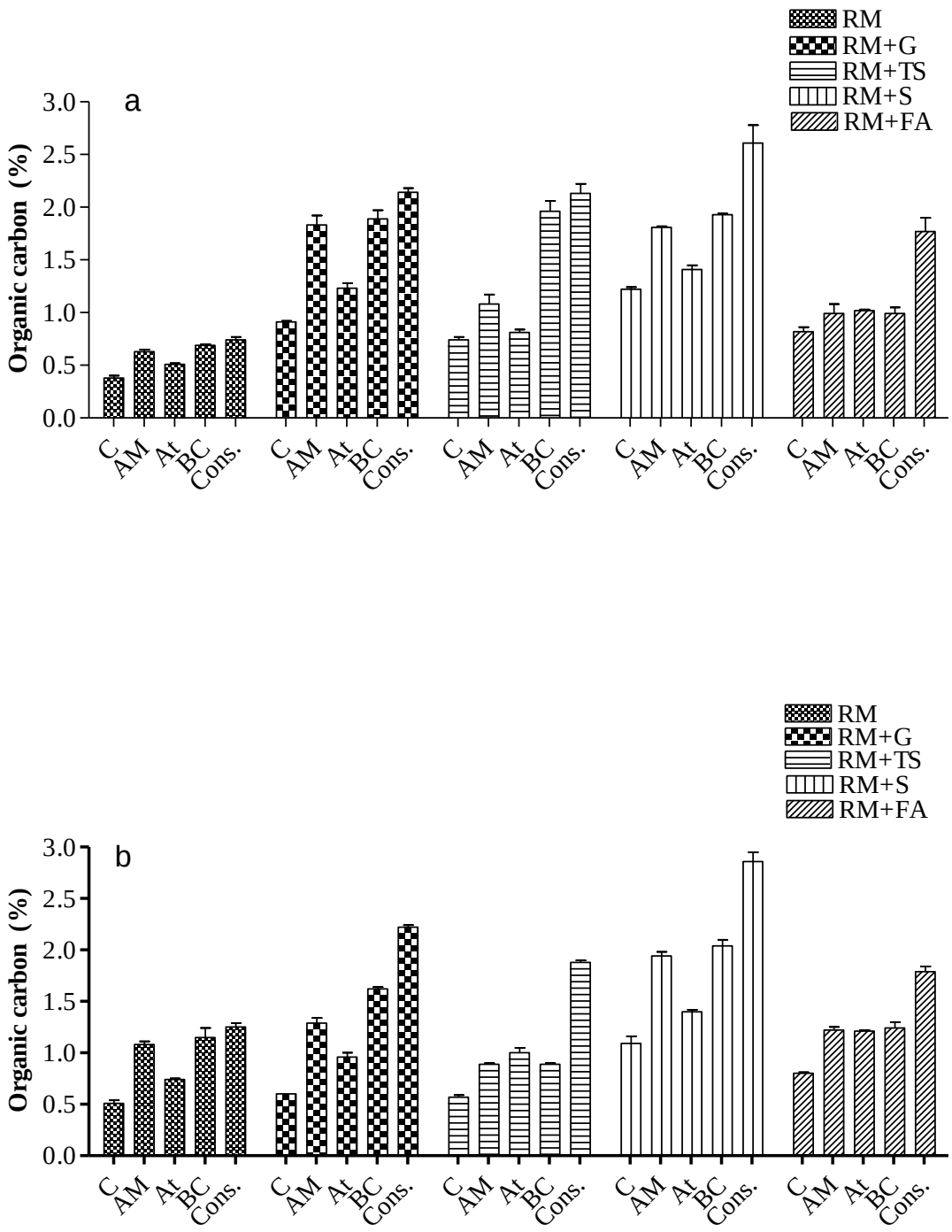


Fig 4.2.12: Organic carbon (%) content in rhizosphere soils of different red mud treatments inoculated with different microbes after 6 (a) and 12 (b) months. Mean ±SD (n=3).

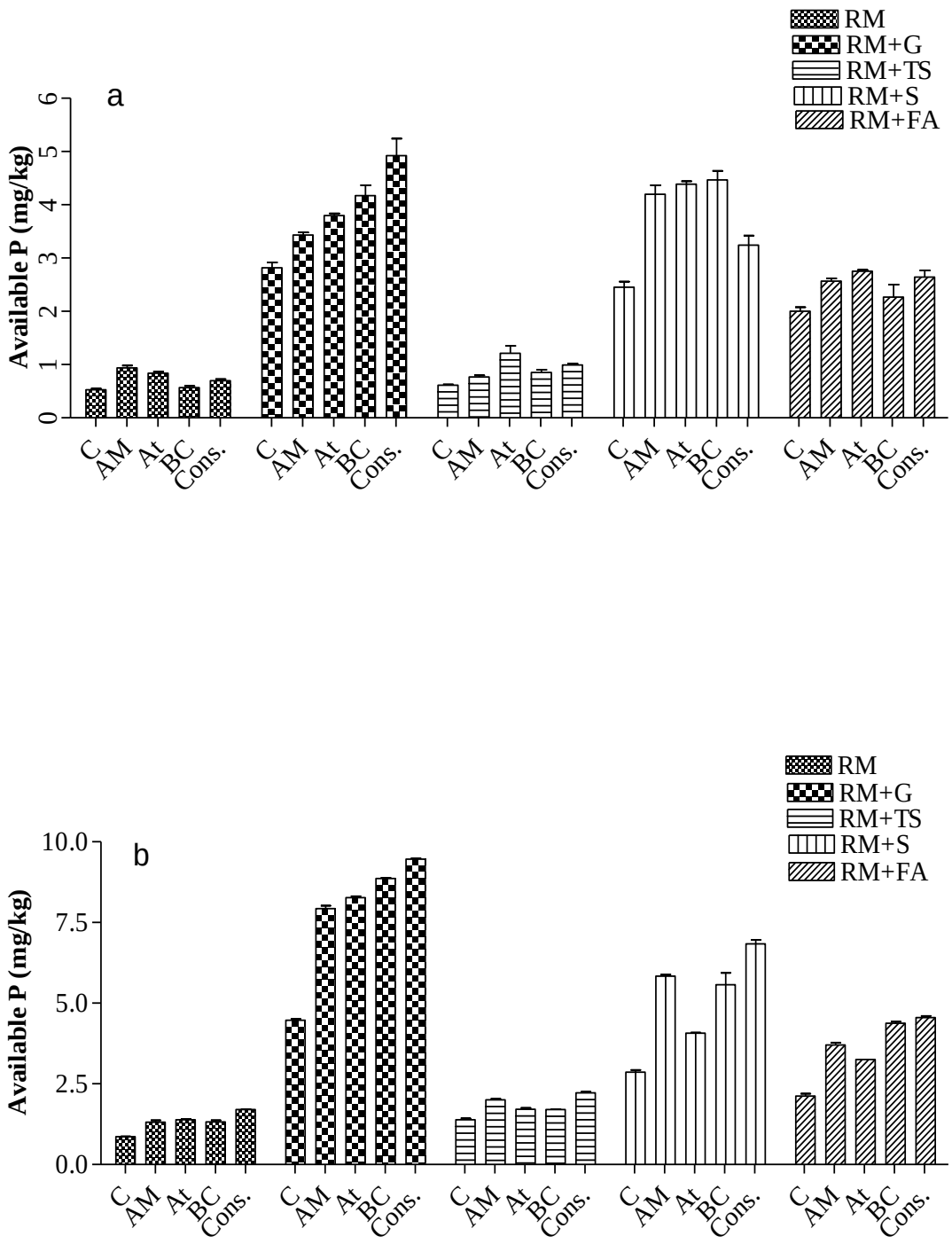


Fig 4.2.13: Available P content in rhizosphere soils of different red mud treatments inoculated with different microbes after 6 (a) and 12 (b) months. Mean ±SD (n=3).

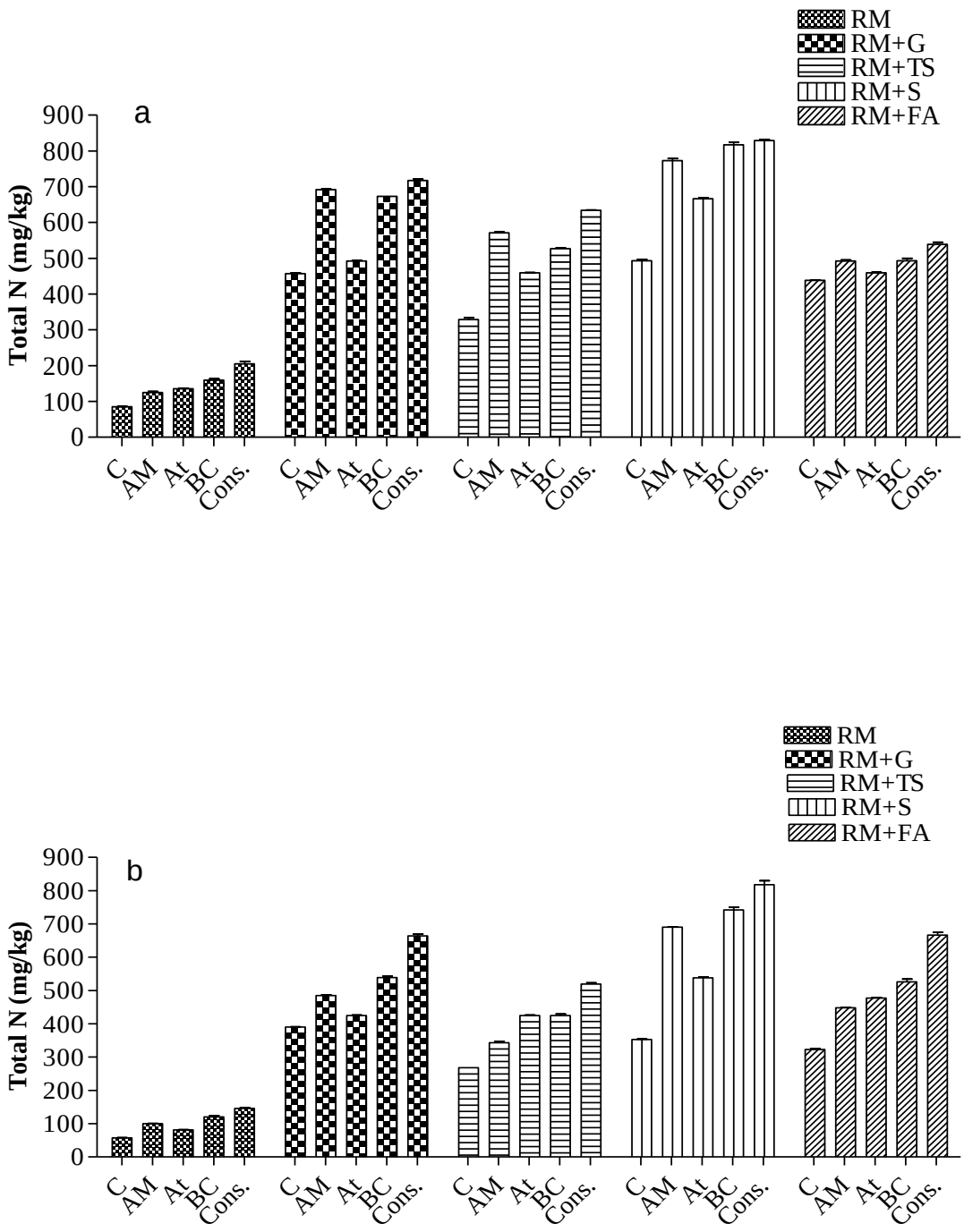


Fig 4.2.14: Total N content in rhizosphere soils of different red mud treatments inoculated with different microbes after 6 (a) and 12 (b) months. Mean ±SD (n=3).

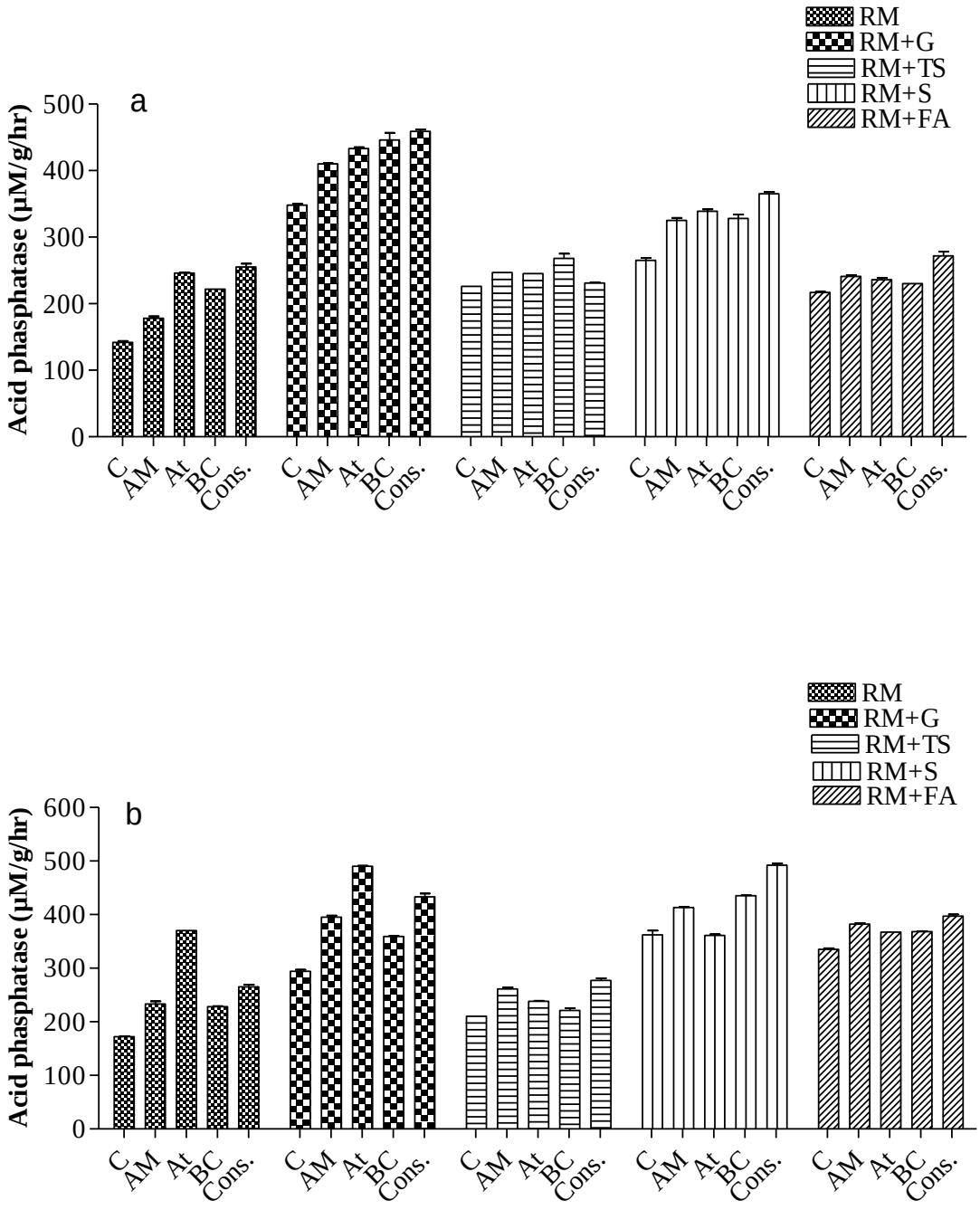


Fig 4.2.15: Influence of different microbial treatments on acid phosphatase activity in rhizosphere soils of different red mud treatments after 6 (a) and 12 (b) months. Mean ±SD (n=3).

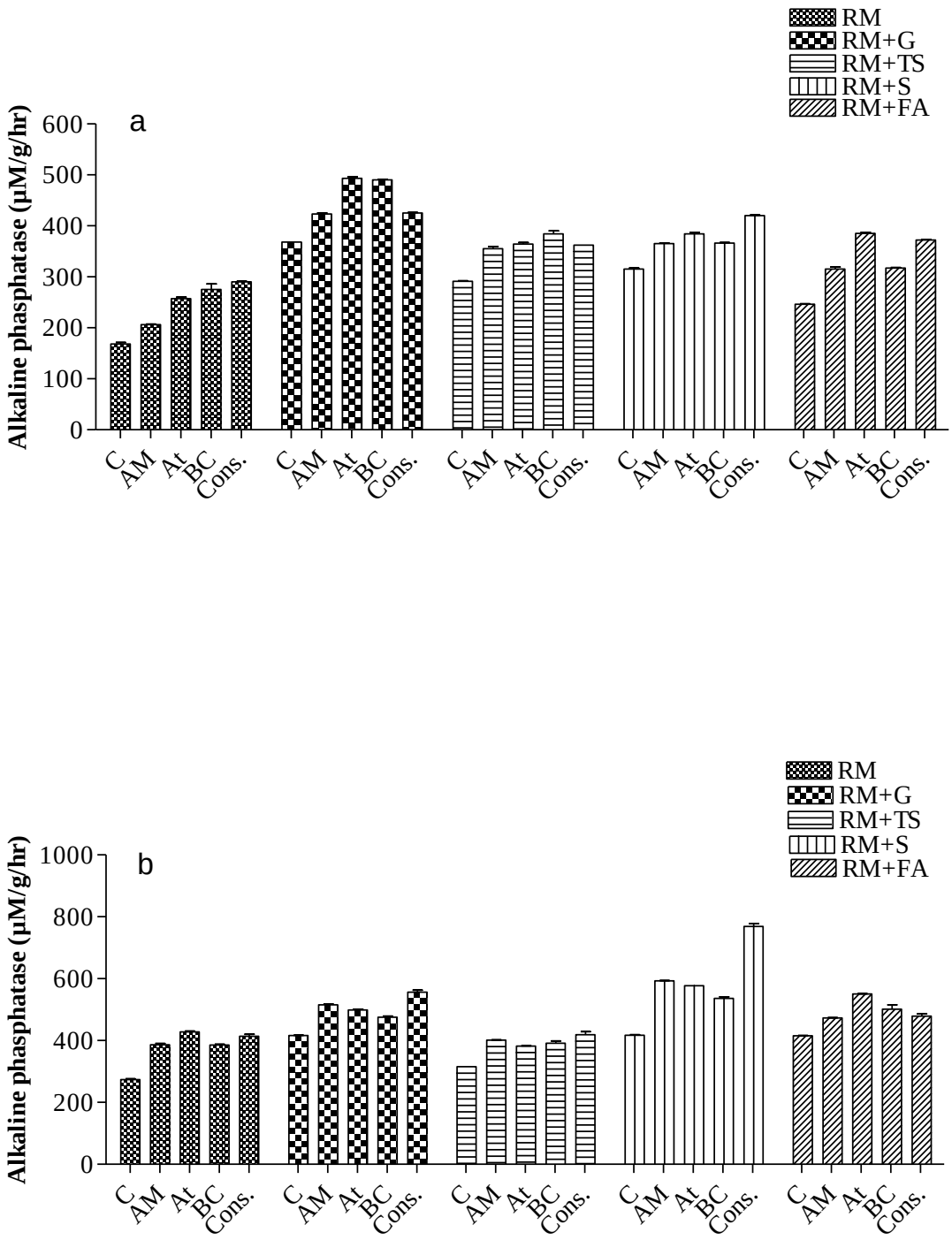


Fig 4.2.16: Influence of different microbial treatments on alkaline phosphatase activity in rhizosphere soils of different red mud treatments after 6 (a) and 12 (b) months. Mean ±SD (n=3).

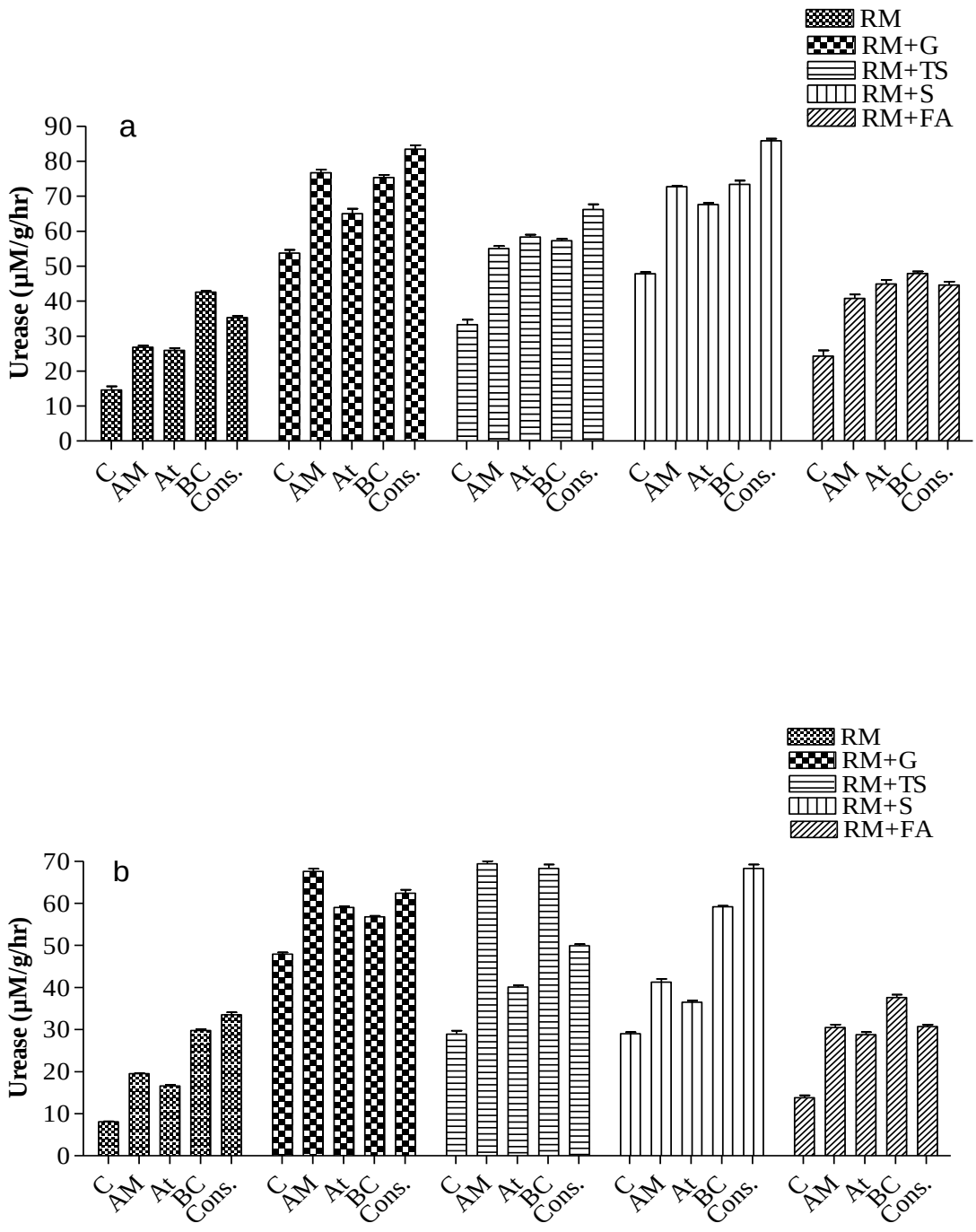


Fig 4.2.17: Influence of different microbial treatments on urease activity in rhizosphere soils of different red mud treatments after 6 (a) and 12 (b) months. Mean ±SD (n=3).

Salient findings

- Application of different amendments improved the properties of red mud with various degrees. pH of red mud decreased rapidly when gypsum was added at initial time while organic carbon, available P and total N was increased highly in sludge amended red mud. After 6 months of plantation, different amendments added to red mud promoted the growth of bermudagrass with various proportions compared to red mud alone. In this study, red mud did not support the bermudagrass growth in red mud alone treatment in initial stages and the growth was initiated after 2 months when re-transplanted.

- Among all the amended treatments, gypsum followed by sludge had shown maximum growth of bermudagrass. Apart from the positive effect of these amendments, inoculation of different microbial inoculants further increased the growth of bermudagrass and the maximum growth was recorded in the gypsum followed by sludge amended inoculated treatments. After 6 months, the highest growth was observed in all the consortium inoculated treatments than their individual treatments especially in the gypsum treatment. In this treatment, consortium followed by AM fungi had shown a higher growth of bermudagrass than those of all other inoculated treatments. After 12 months, growth of bermudagrass markedly increased and it was highest in all consortium followed by AM fungal inoculated treatments especially in gypsum followed by sludge amended treatments.

- Inoculation of different microbes increased uptake of nutrients in all treatments with various degrees and the maximum nutrient uptake of bermudagrass was observed in gypsum and sludge amended treatments both at 6 and 12 months.

- After 6 months, P uptake of grass was highest in consortium followed by AM fungal inoculated treatments. The same observation was noticed in many other nutrients uptake of bermudagrass after 12 months. Gypsum amended treatments significantly decreased the availability of Fe Al and Na uptake of bermudagrass after 6 and 12 months. Further, microbial inoculated treatments decreased the levels of Fe and Al accumulation of bermudagrass.

- After 6 months, the % root AM fungal colonization was observed in all inoculated and non-inoculated treatments. However, the maximum AM fungal colonization was observed in AM fungal propagules inoculated treatments followed by those of all other individual inoculated treatments. Interestingly, bacterial consortia followed by *A. tubingensis* inoculated treatments promoted the native AM fungal root colonization efficiency in all the treatments. In this study, overall the maximum AM fungal colonization was observed in gypsum amended AM fungal inoculated treatment both at 6 and 12 months of grass growth.

- After 6 months, the highest P-solubilizing fungal count was observed in the entire consortium followed by *A. tubingensis* inoculated treatments and maximum count was observed in sludge amended treatment. The highest population of alkali-tolerant bacteria was counted in bacterial consortia inoculated sludge amended treatment at 6 months of grass growth. After 12 months, it was found highest in consortium inoculated topsoil, sludge and bacterial consortia inoculated sludge amended treatment.

- Among all the treatments, gypsum amended red mud showed higher reduction of pH. Further, gypsum amended microbial inculcated treatments reduced the pH and highest reduction was observed in consortium inoculated treatment at 6 months.
- Maximum increase of organic carbon was observed in sludge amended consortium inoculated treatment while the maximum increase of available P was observed in gypsum amended and consortium inoculated treatment after 6 and 12 months. After 6 months, the maximum amount of total N was measured in sludge amended bacterial consortia and consortium inoculated treatment.
- Highest acid phosphatase activity was observed in gypsum amended consortium inoculated treatment at 6 months but, it was found to be highest in gypsum amended A. *tubingensis* and sludge amended consortium inoculated treatments at 12 months. Alkaline phosphatase activity was recorded highest in gypsum amended *A. tubingensis* and bacterial consortium inoculated treatments at 6 months while it was highest in consortium inoculated sludge amended treatments after 12 months. After 6 months, the highest urease activity was observed in gypsum and sludge amended consortium inoculated treatment. After 12 months, it was found highest in AM fungal inoculated gypsum amended treatment.

4.3 Rehabilitation of fly ash in micro-field studies

The main source of chemical elements in fly ash is coal, which contains almost all the naturally occurring elements with higher levels. Chemical analysis of the fly ash collected randomly from different sites of fly ash pond of NALCO, Damanjodi, Orissa, India was shown in Table 6.1. Fly ash exhibited neutral to slight alkaline range of pH (7.35) while the electrical conductivity was 0.30 mS/cm. Organic carbon measured was 0.3%, available P 2.3 mg/kg, total N 85 mg/kg and high content of Al and Fe (Table 4.3).

The most eco-friendly alternative for abandoned fly ash disposal sites is to stabilize them through revegetation. Establishment of vegetation on fly ash pond serves a variety of functions including stabilization of ash against wind and water erosion, reduction of leaching through water loss as evapotranspiration, provision of shelter and habitat for wildlife and bringing about a more aesthetically pleasing landscape. However, chemical, physical and microbial factors can limit plant establishment and growth in fly ash deposits. Among the chemical limitations are an initial pH and high soluble salt concentrations, phytotoxic levels of trace elements and nutrient deficiencies (e.g. N and P). Physical impediments include restriction of root growth due to natural compaction of fine ash particles and/ or formation of solid cemented layers due to the pozzalanic nature of some ashes. Microbial factors may

Table 4.3: Chemical properties of fly ash collected from NALCO, Damanjodi, India

Parameters	Units
pH	7.35
*EC (mS/cm)	0.30
Organic Carbon (%)	0.30
Olsen P (mg/kg)	2.30
Total N (mg/kg)	85.0
Elemental analysis (mg/kg)	
Al_2O_3	281,500
Fe_2O_3	51,500
P_2O_5	4,500
SO_3	13,500
CaO	8,900
MgO	3,800
Na_2O	3,700
MnO	500
K_2O	800
ZnO	100
CuO	150

*EC: electrical conductivity.

include a general lack of microbial activity and attendant low nutrient turnover and a lack of inoculum of symbiotic microorganisms.

Plant species selection is an important factor in determining the success of rehabilitation of fly ash ponds and selected species should grow in an enriched level of trace elements, and often a highly adverse environment. Therefore, one promising option for the revegetation on these sites is the cultivation of energy crops like *Dendrocalamus strictus* (bamboo). On account of extensive underground root and rhizome system (having significant capacity to bind the top soil), accumulation of leaf mulch, abundant litter fall; bamboo serves as an efficient agent in rehabilitation of degraded land, preventing soil erosion, conserving moisture.

The nursery experiment was conducted at NALCO nursery, Damanjodi, Orissa, India in a micro-field level. Flay ash adapted AM fungi isolated from fly ash and the phosphate solubilizing fungus *A. tubingensis* was used as co-inoculants to bamboo plants in this study. Treatments were as followed as: 1) control (fly ash without inoculation), 2) AM fungi 3) *A. tubingensis* and 3) AM fungi + *A. tubingensis*. For each treatment, three replicates were prepared. After 6 and 12 months, the data was collected for various growth and other parameters.

4.3.1 Plant growth

Plant growth was considered as height as well as number of branches per plant. Inoculation of AM fungi, *A. tubingensis* and combination of these two were significantly increased the bamboo plants height and number of branches compared to control treatments both at 6 and 12 months (Table 4.3.1; Fig 4.3&4.3.1). The maximum height and number of branches were significantly increased in dual

inoculation followed by the individual inoculations, AM fungi and *A. tubingensis* both at 6 and 12 months of the study (Table 4.3.1; Fig 4.3&4.3.1).

4.3.2 AM fungal colonization and spore density

After 6 months of growth, AM fungal colonization was observed in all inoculated and control treatments. *Aspergillus tubingensis* inoculated treatment increased the indigenous AM fungal colonization compared to control treatment (Table 4.3.1; Fig 4.3&4.3.2) The highest AM fungal colonization was observed in AM inoculated treatment followed by co-inoculation. It was observed that the percent root colonization of all treatments significantly increased after 12 months compared to 6 months and the maximum colonization of AM fungi was observed due to dual inoculation followed by individual inoculation of AM fungi. After 6 months, spore density was found to be very low in rhizosphere soil of all treatments ((Table 4.3.1; Fig 4.3&4.3.3). However, the highest number of spores was observed in rhizospheric soil inoculated with AM fungi followed by dual inoculation compared to both *A. tubingensis* and control treatments. The maximum spore count was significantly increased in all treatments from 6 months to 12 months of plants growth in all treatments. The higher density of spores was observed in dual inoculation followed by AM fungal individual treatment (Table 4.3.1; Fig 4.3&4.3.3)

Table 4.3.1: Growth parameters, AM fungal colonization and spore numbers in fly ash of bamboo plants inoculated with AM fungi (AMF), *A. tubingensis* (At) and dual inoculum of AMF +At

Treatment	**Height (cm/plant)**	**Branches/plant**	**Colonization (%)**	**Spores/100 g fly ash**
		6 months		
Control	27.6±2.0c	18±1.0c	4.0±0.6c	0
AMF	58.1±2.1a	29±1.5ab	52.0±3.0a	20±1.5a
At	40.1±1.5b	22±1.5bc	17.5±1.5b	3±1.0b
AMF+At	66.5±2.0a	34±2.0a	46.5±1.5a	16±1.5a
		12 months		
Control	136.9±0.0c	37±2.5c	17.5±1.5c	5±2.0c
AMF	226.9±3.3a	49±2.0ab	68.5±1.5a	17±1.5b
At	181.0±8.2b	42±2.0bc	26.0±1.0c	7±0.5bc
AMF+At	247.8±3.1a	57±1.0a	55.0±4.0b	29±2.5a

AMF, AM fungi and At, A. *tubingensis.* Values sharing a common letter within the column and time periods are not significant at $P<0.05$.

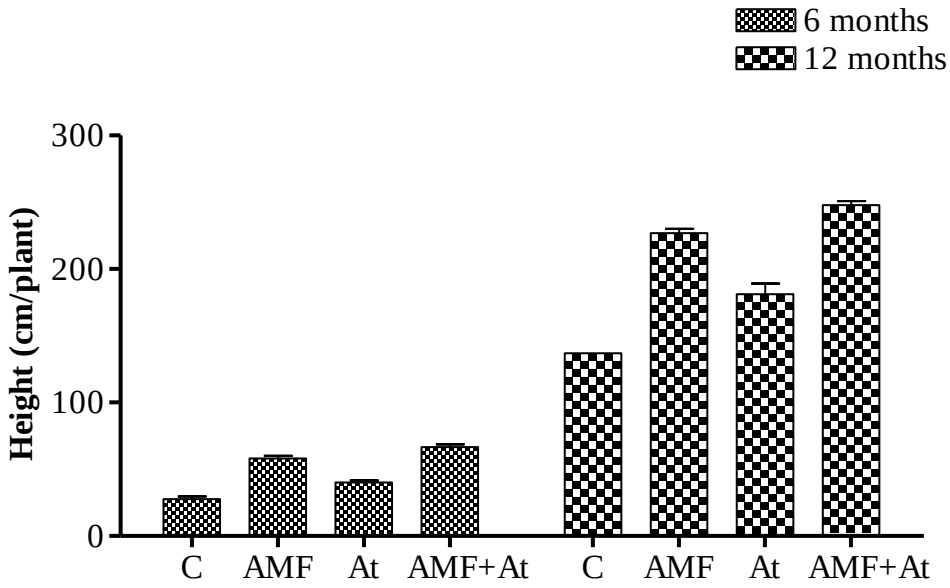


Fig 4.3: Influence of microbial inoculations on the growth (height) of bamboo plants grown in different treatments after 6 and 12 months. Mean ±SD (n=3).

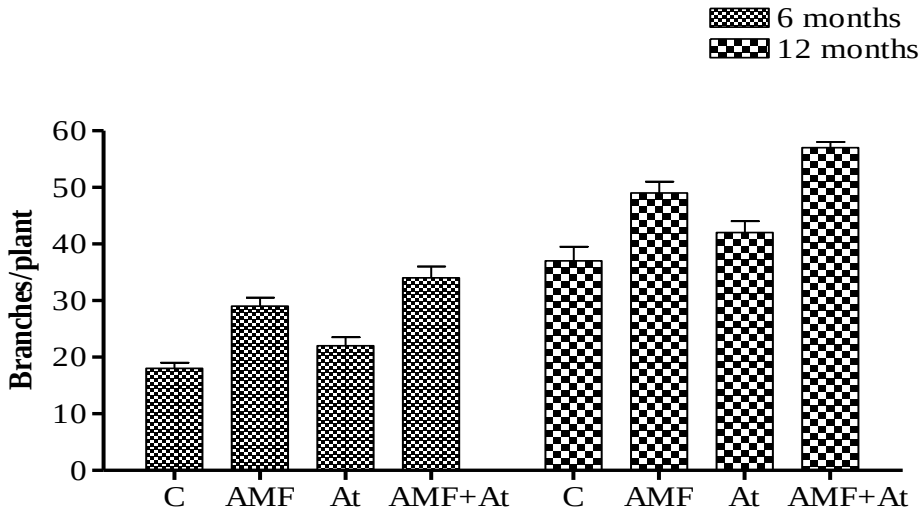


Fig 4.3.1: Influence of microbial inoculations on the growth (branches/plant) of bamboo plants grown in different treatments after 6 and 12 months. Mean ±SD (n=3).

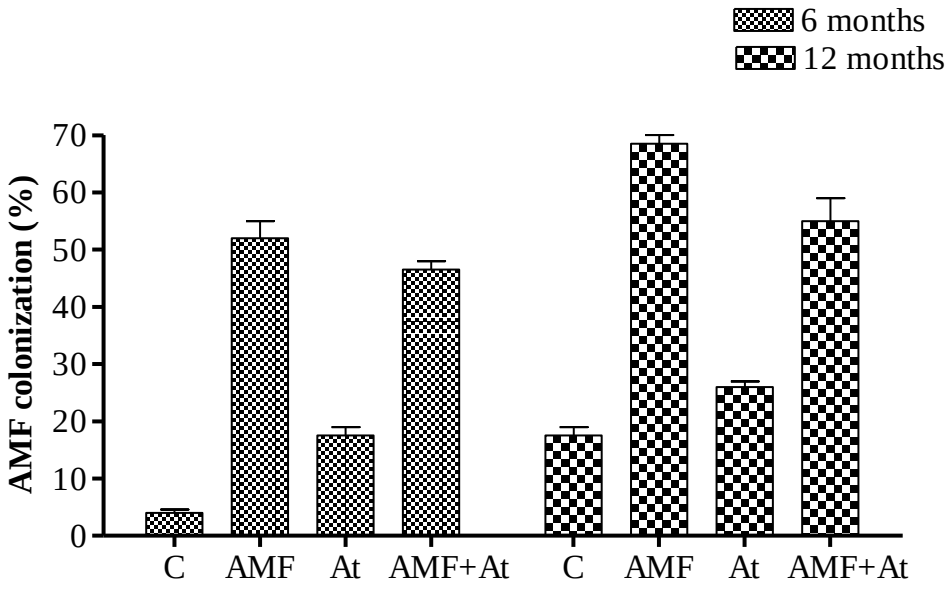


Fig 4.3.2: AM fungal colonization in roots of bamboo plats after 6 and 12 months. Mean ±SD (n=3).

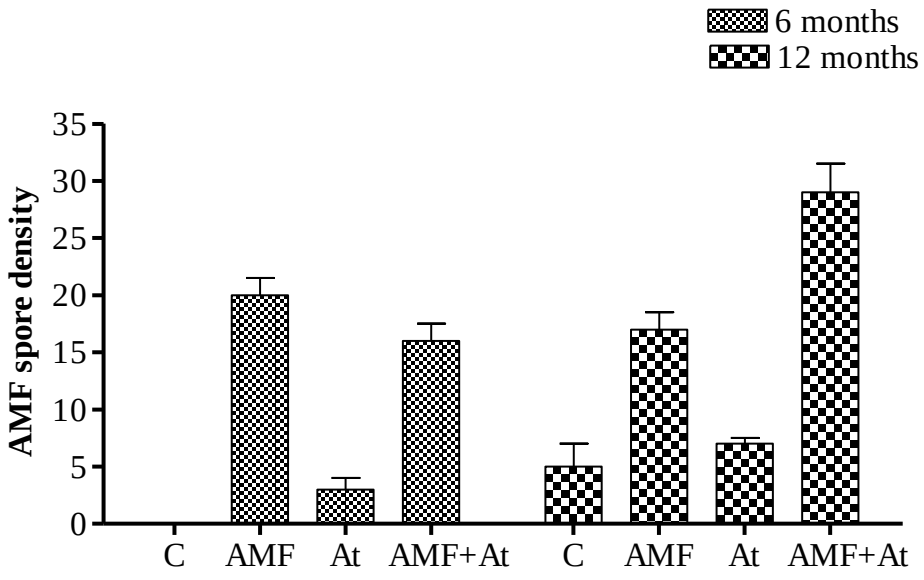


Fig 4.3.3: AM fungal spore density in rhizosphere fly ash of different treatments after 6 and 12 months. Mean ±SD (n=3).

4.3.3 Plant nutrient uptake

Inoculation of individual and combination treatments significantly increased the shoot P uptake of bamboo plants compared to control after 6 and 12 months of plantation (Table 4.3.2; Fig 4.3.4a). The highest P uptake was observed in co-inoculation followed by single AM fungi and *A. tubingensis* treatments at 6 and 12 months. After 12 months, there were no significant differences in P uptake of shoot between single AM fungal and dual inoculated treatments. Potassium (K) uptake of bamboo plants was increased by all individual and dual inoculated treatments compared to control at 6 and 12 months of growth (Table 4.3.2; Fig 4.3.4b). The highest K uptake of shoot was observed in inoculated with combination of AM fungi and *A. tubingensis* inoculation followed by individual treatments both at 6 months. Inoculation of bamboo plats with AM fungi, *A. tubingensis* and combination significantly increased the Ca uptake compared to control treatment at both 6 and 12 moths (Table 4.3.2; Fig 4.3.4c). Further, the highest Ca uptake of shoot was recorded in dual inoculation followed by AM fungi and *A. tubingensis* treatments. Single inoculations and combination increased the Mg uptake of shoot compared to control treatment at 6 and 12 months growth (Table 4.3.2; Fig 4.3.4d). The highest Mg uptake was observed in dual inoculation followed by AM fungal and *A. tubingensis* treatments.

Overall, dual inoculation significantly increased the P (150%), K (67%), Ca (106%) and Mg (180%) in shoot tissues compared to control treatments at 12 months after plantation (Table 4.3.2; Fig 4.3.4 a, b, c and d). In case of Na uptake of shoots, inoculated treatments had show higher compared to control and the maximum was

observed in dual inoculation followed by AM and *A. tubingensis* at 6 months (Table 4.3.2; Fig 4.3.5a). The uptake of Na levels decreased at 12 months compared to 6 months. Inoculated plants with AM fungi, *A. tubingensis* and combination significantly reduced the accumulation of Fe, Al and Cu in shoot tissue of bamboo plants compared to control (Table 4.3.2; Fig 4.3.5b, c and d). Further, the accumulation of these metals gradually decreased from 6 months to 12 months in all treatments. The maximum decrease of iron accumulation was observed in dual inoculation followed by individual, *A. tubingensis* and AM fungi ((Table 4.3.2; Fig 4.3.5b). After 6 and 12 months, the reduced levels of Al in shoots were higher in dual inoculation followed by AM fungi and *A. tubingensis* (Table 4.3.2; Fig 4.3.5c). The maximum reduction of Cu was observed in AM fungi followed by *A. tubingensis* and dual inoculation after 12 months (Table 4.3.2; Fig 4.3.5d). The Zn levels were significantly increased in shoot tissues of bamboo plants inoculated with individual and combination and the increased order was in dual inoculation followed by *A. tubingensis* and AM fungi at 6 and 12 months (Table 4.3.2; Fig 4.3.6).

Table 4.3.2: Uptake of different nutrients and heavy metals in tissues of bamboo plants inoculated with AM fungi (AMF), *A. tubingensis* (At) and dual inoculum of AMF + At

	mg/kg								
Treatments	**P**	**K**	**Ca**	**Mg**	**Na**	**Fe**	**Al**	**Zn**	**Cu**
				6 months					
Control	135±6d	94±0.4d	135±1.0d	158±0.8d	90±3.6d	3375±6.4a	9326±2.1a	96±0.8d	63±1.2a
AMF	348±1b	124±1.8c	209±1.7b	264±2.1b	136±2.2b	2880±2.9b	6453±10.0b	111±1.1c	51±1.5b
At	269±8c	166±3.3b	169±2.0c	187±1.8c	116±1.0c	2618±10.6c	7861±9.7c	169±0.6b	34±0.1c
AMF+At	419±2a	191±1.0a	287±1.9a	297±1.2a	161±1.7a	1917±7.0d	5733±8.3d	178±0.3a	31±2.4c
				12 months					
Control	257±0c	864±0.8c	562±2.1d	159±0.1c	62±0.5d	953±3.0a	2635±5.2a	87±0.8d	32±0.0d
AMF	645±0a	1328±1.2a	974±1.7b	380±0.5ab	72±0.5c	598±1.0b	1315±3.1c	98±1.0c	18±0.1c
At	569±9b	1269±4.9b	755±1.9c	334±0.6b	82±0.6b	359±0.9c	1424±1.8b	123±1.1b	23±0.4b
AMF+At	653±2a	1444±3.5a	1162±1.9a	457±0.3a	93±0.3a	360±1.4c	1245±11.9d	143±0.3a	26±0.7a

Values sharing a common letter within the column and time periods are not significant at <0.05.

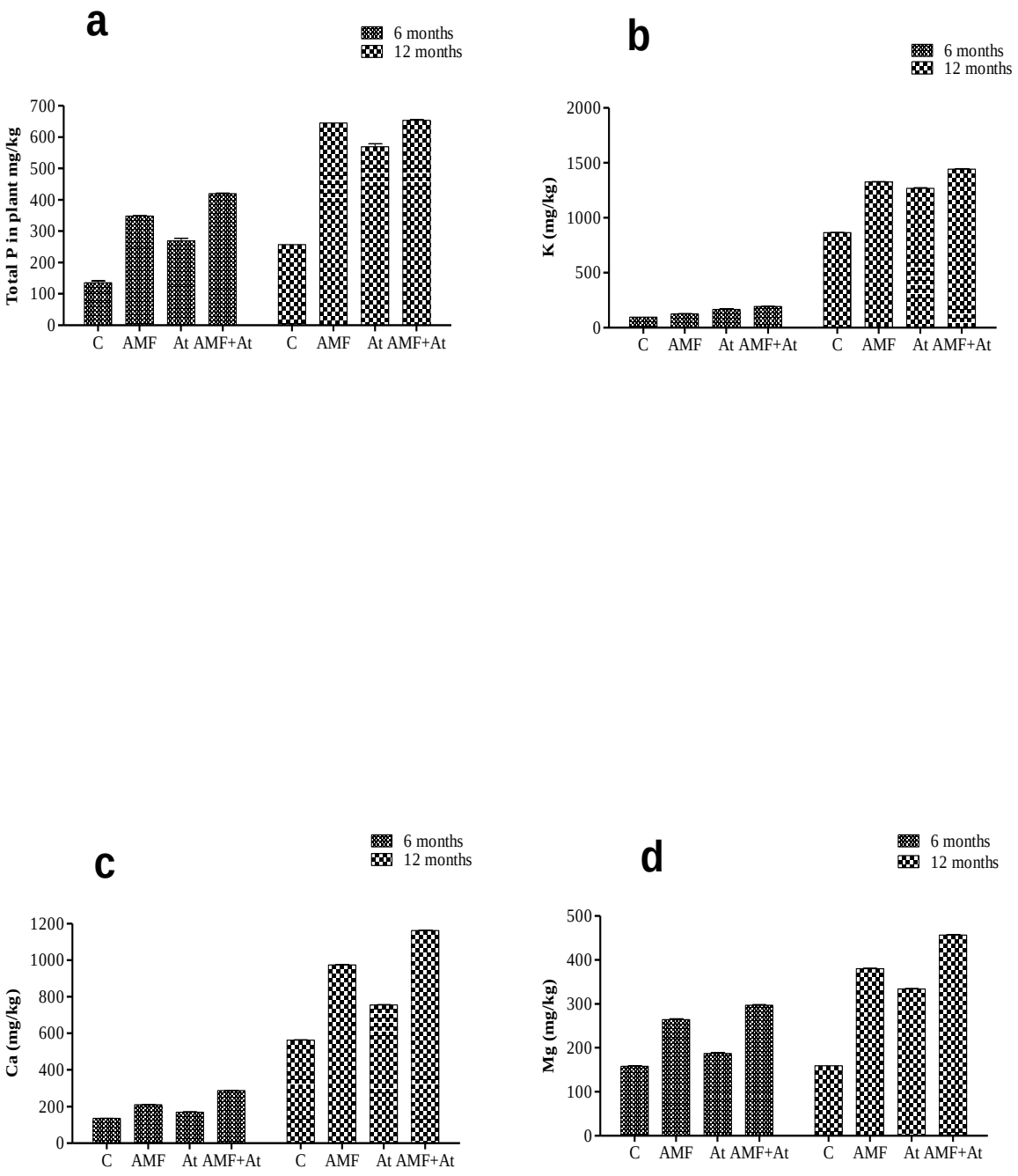


Fig 4.3.4: Mineral (a; total P, b; K, c; Ca and d; Mg) uptake pattern in shoot of bamboo plants grown in different treatments after 6 and 12 months. Mean ±SD (n=3).

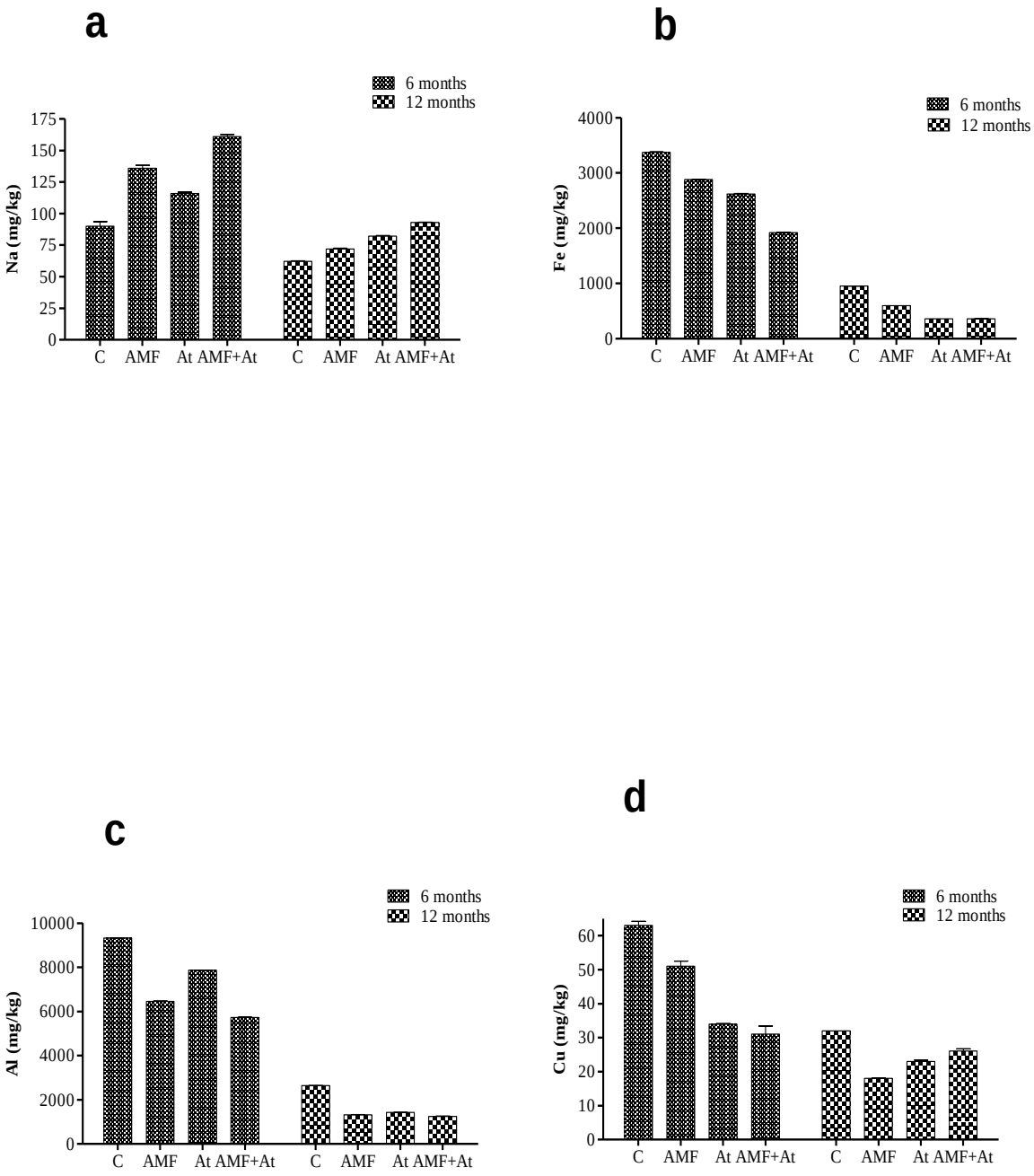


Fig 4.3.5: Metal (a; Na, b; Fe, c; Al and d; Cu) accumulation pattern in shoot of bamboo plants grown in different treatments after 6 and 12 months. Fig Mean ±SD (n=3).

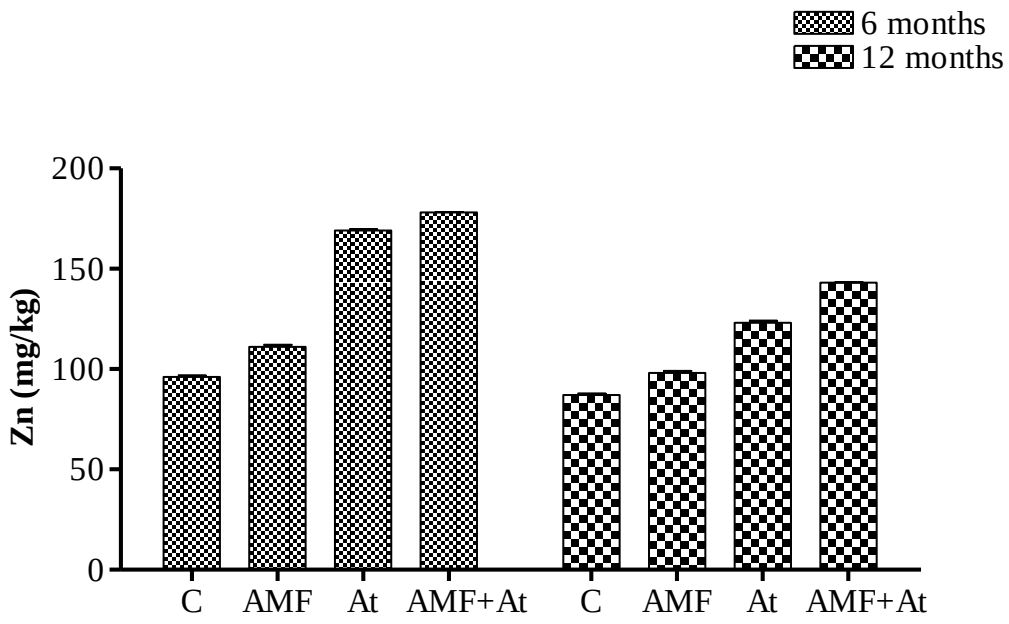


Fig 4.3.6: Zinc accumulation pattern in shoot of bamboo plants grown in different treatments after 6 and 12 months. Mean ±SD (n=3).

4.3.4 Chemical properties of FA

Over the period of 6 and 12 months of plantation, fly ash chemical properties were significantly improved in all inoculated treatments compared to control treatment. The pH of fly ash decreased from slight alkaline to moderate acid range in all treatments compared to initial pH and it was further decreased in 12 months (Table 4.3.3; Fig 4.3.7a). After 6 months, there were no significant differences in pH reduction between all treatments. The higher reduction in pH was observed in *A.*

tubingensis alone followed by dual inoculation, AM fungi alone and control treatment after 12 months. However, the slight decrease in pH reduction was noticed in the dual inoculation, control when compared to *A. tubingensis* and AM fungi respectively. After 6 months, electrical conductivity (EC) was increased in all treatments compared to beginning of experiment. Further, inoculated treatments significantly increased the EC when compared to control both at 6 and 12 months 4.3.3; Fig 4.3.7b). The highest increase of EC was observed in dual and AM fungal inoculated treatments compared to *A. tubingensis* at 6 months. Inoculated treatments significantly increased the EC compared to control and the highest was observed in dual inoculation followed by *A. tubingensis* and AM fungi alone treatments. After 6 months of plants growth, organic carbon levels in all treatments were increased compared with initial levels in fly ash and further it was increased by inoculated treatments 4.3.3; Fig 4.3.7c).

The high levels of organic carbon was observed in dual inoculation followed AM fungi and *A. tubingensis*. Inoculation of plants with AM fungi, *A. tubingensis* and combination increased the available P compared to initial levels in fly ash and it was decreased in case of control treatment (4.3.3; Fig 4.3.7d). After 6 months, the higher levels of available P was observed in individual treatments, AM fungi and *A. tubingensis* compared to dual inoculation (4.3.3; Fig 4.3.7d). The available P was three times higher in dual inoculation after 12 months compared to control treatment. After 6 months of plantation, the N content in rhizosphere fly ash increased in all inoculated treatments and it was further increased by inoculated treatments compared to control (4.3.3; Fig 4.3.8a). The maximum N content was observed in dual inoculation followed by single, AM fungal and *A.tubingensis* treatments. After 12

months also the same trend was followed in inoculated treatments but N content was reduced from 6 months to 12 months of plants growth (4.3.3; Fig 4.3.8a).

Acid phosphatase and urease activities were increased in inoculated treatments compared to control. At 6 months, the highest acid phosphatase activity was observed in *A. tubingensis* followed by AM fungal and dual inoculated treatments (4.3.3; Fig 4.3.8b). After 12 months, the activity was gradually increased in all treatments but, it was highest in dual inoculation and *A. tubingensis* than the AM fungal inoculated treatment. Urease enzyme activity was higher in dual inoculation followed by *A. tubingensis* and AM fungi. After 12 months, urease activity was increased in all treatments and the maximum was observed in dual inoculation followed by AM fungi and *A.tubingensis* (4.3.3; Fig 4.3.8d).

Table 4.3.3: Chemical and biochemical properties of fly ash inoculated with AM fungi (AMF), *A. tubingensis* (At) and dual inoculum (AM+At)

Parameters	**Control**	**AMF**	**At**	**AMF+At**
		6 months		
pH	6.81±0.05a	6.74±0.13a	6.62±0.01a	6.60±0.08a
EC (mS/cm)	0.35±0.00b	0.47±0.00a	0.36±0.01b	0.50±0.01a
Organic carbon (%)	0.5±0.0d	1.4±0.1a	1.0±0.1c	1.7±0.1a
Available P (mg/kg)	1.2±0.0c	3.2±0.1a	3.6±0.1a	2.5±0.2b
N (mg/kg)	203±9.9d	280±0.0b	224±0.0c	420±0.0a
Acid Phospatase (µM/g/1hr)	212±3.8c	252±0.7b	297±0.4a	189±0.1d
Urease (µM/g/hr)	12.2±0.6d	23.0±0.1a	28.6±0.5b	31.9±0.4ca
		12 months		
pH	6.20±0.14 a	5.92±0.03ab	5.67±0.04bc	5.59±0.01c
EC(mS/cm)	0.16±0.01b	0.24±0.02b	0.40±0.02a	0.47±0.00a
Organic carbon (%)	0.6±0.0d	1.4±0.0c	1.8±0.0b	2.1±0.0a
Available P (mg/kg)	1.6±0.1c	4.0±0.1b	4.3±0.0b	4.7±0.1a
N (mg/kg)	134±0.0d	238±0.0b	210±0.0bc	357±9.9a
Acid Phosphatase (µM/g/1hr)	218±4.0c	428±5.3b	507±3.5a	541±9.9a
Urease (µM/g/hr)	19.6±1.7d	44.7±1.4b	36.0±0.7c	56.6±1.3a

Values sharing a common letter within the column and time periods are not significant at <0.05.

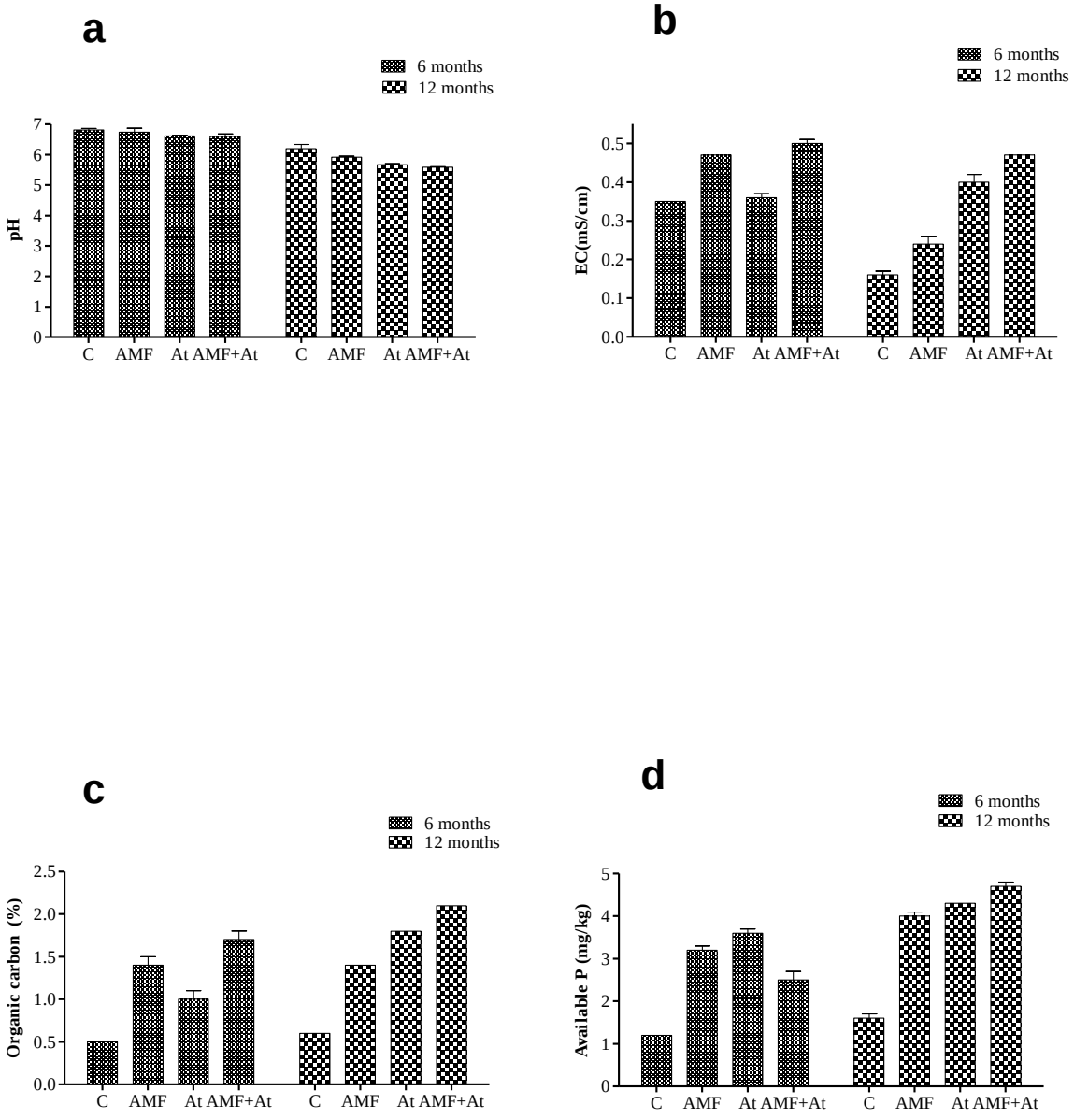


Fig 4.3.7: Influence of microbial inoculations on pH (a), EC (b), organic carbon (c) and available P (d) content in rhizosphere fly ash of different treatments after 6 and 12 months. Mean ±SD (n=3).

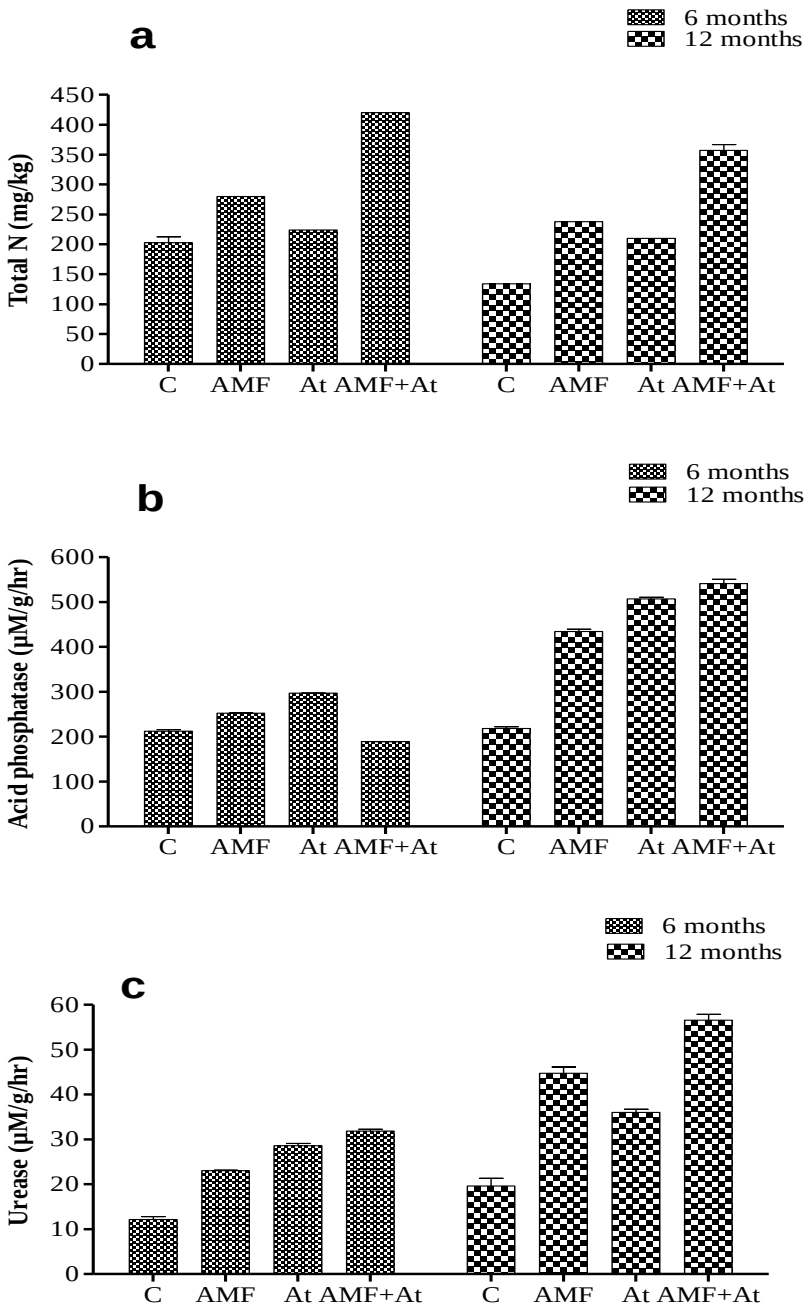


Fig 4.3.8: Influence of microbial inoculations on total N (a), acid phosphatase (b) and urease (c) activity in rhizosphere fly ash of different treatments after 6 and 12 months. Mean ±SD (n=3).

Salient findings

- The individual inoculation of AM fungi, *A. tubingensis* and combination of these two fungi increased the growth (height and number of branches) of bamboo plants compared to control treatment. The maximum growth was significantly increased in dual inoculation compared to individual inoculum both at 6 and 12 months.

- The % root AM fungal colonization was observed in all treatments and it was significantly increased after 12 months compared to 6 months. The colonization of native AM fungi increased due to inoculation of *A. tubingensis*. After 12 months, the maximum colonization was observed in AM inoculated treatments whereas maximum spore count was observed in dual inoculated plants.

- Dual inoculation significantly increased the P (150%), K (67%), Ca (106%), and Mg (180%) in shoot tissues compared to control treatments at 12 months after plantation. Sodium levels increased in inoculated plants compared to control. The uptake of Al, Cu, and Fe were significantly reduced due to inoculation compared to non-inoculated plants. The content of these metals were significantly lesser in dual inoculated treatment than individual inoculation. The Zn levels were significantly increased with dual inoculum compared to control as well as individual inoculations.

- The physico-chemical properties of fly ash significantly improved in all inoculated treatments compared to control treatment both at 6 and 12 months. The organic carbon and available P increased significantly after 6 months as well as 12 months of plantation and it was higher in case of dual inoculation. After 6 months of plantation, the N content in fly ash increased significantly in inoculated treatments as compared to control, the maximum was observed in dual inoculated treatment. Acid phosphatase and urease enzyme activities also increased in all inoculated treatments and the maximum enzyme activities were observed in dual inoculated treatments than other treatments.

Chapter 5

Discussion

Chapter 5

Discussion

7.0 Diversity of AM fungi associated with plants growing in red mud flooded site and fly ash pond

The low rate of AM fungal colonization and the presence of very few spores in rhizosphere of plants grown on red mud flooded sites and fly ash were attributed to adverse conditions. The soil structure and composition not only affect the spore population but also the biological activity of endophytes. Variation in spore density and colonization of AM fungi associated with different host plant species may depend on a variety of mechanisms, including variation in host species and their phenology, mycorrhizal dependency, host plant-mediated alteration of the soil micro-environment, or other unknown host plant traits (Eom *et al.*, 2000; Lorgio *et al.*, 1999)**.**

Arbuscular mycorrhizal fungal morphological identification might be a good tool to describe AM communities in the soil but it might present limitations due to the differential sporulation by certain AM species in response to specific AM fungi-plant interactions or certain environmental factors (Ortega-Larrocea *et al*., 2010). This might affect the presence of AM fungal spores in the rhizospheric substrates studied. Molecular studies suggest that the spores not found in the surrounding plant soil even show presence of AM fungal colonizing root tissue (Sanders, 2004). In order to rule out this possibility, molecular identification was attempted using genomic DNA

obtained from AM fungal colonized root tissues and used a nested PCR technique with eukaryotic pair primers NS5-ITS4 and genera specific primers at the first and second PCR reaction respectively (Redeker, 2003, Redeker *et al*., 2003). Although primers were chosen in this work as an attempt to amplify preferentially AMF ITS rDNA sequences, we did not succeed in obtaining all groups of (genus) of Glomeromycota in this study except *Glomus* from red mud pond sites, *Glomus* and *Archaeospora* from fly ash pond. This is probably due to the low density of these groups in these hostile conditions which failed to detect AM fungal sequences.

The identification of AM fungi using spore morphology and molecular approaches revealed that maximum species belong to the genus *Glomus*. This can be due to the high sporulation rates of this genus (Daniell *et al.,* 2001) and other factors such as seasonal variations. However, our results and reports of other investigations congruently indicate that *Glomus* species are predominant not only in agricultural sites (Daniell *et al.,* 2001), tropical forests (Wubet *et al.,* 2003) but also in phosphate contaminated (Renker *et al.,* 2005), heavy metal polluted soils (Vallino *et al.,* 2006; Whitfield *et al.,* 2004) and fly ash (Bedini *et al.,* 2010; Selvam and Mahadevan, 2002). In this investigation, among the species indentified from roots by molecular characterization only one sequence, *Glomus mosseae* was identified as a spore from red mud site. The species identified in colonized roots in fly ash based on molecular characterization were not similar to those identified by spore characterization. This can be attributed to the fact that spore production might not have occurred in AM fungi associated with roots or some of these species may rely on vegetative strategies

for colonization and dispersal (Clapp *et al.,* 1995) or may forms spores in different seasons (Pringle and Bever, 2002).

This is the first report on AM fungal molecular diversity in red mud flooded sites and fly ash pond of electrostatic precipitator. The molecular diversity of AM fungi had been reported from the bottom ash dump by Bedini *et al*. (2010). The primers Glom1310 and ARCH1311 used in this study also amplified the sequences of Ascomycota and to a smaller extent Basidiomycota related species (data not shown) due to nonspecific amplification of these primers. A similar observation was also reported by other researchers (Appoloni *et al.,* 2008; Redecker, 2000). Our results show that non-mycorrhizal fungi can be present at a relatively high level in roots and should be taken into account in studies on plant-soil-microorganism interactions. The role of AM fungal diversity within roots and its relation to plant functions also needs to be highlighted.

Phylogenetic analysis of red mud and fly ash related sequences revealed that the sequences clustered into 4 discrete clades, and only a small portion of molecular clade was assigned to the known species, leaving other sequences unidentified. Pennisi (2008) emphasized on the growing number of unidentified fungal sequences without corresponding morphospecies records in the International Nucleotide Sequence Database Collaboration (INSDC) stating that the fungal diversity is more than the current estimate. The results of phylogenetic analysis support the hypothesis of Öpik *et al*. (2006) that AM fungi may exhibit different distribution patterns, and a number of AM fungi related to *G. intraradices* may have global distribution whereas *G. etunticatum, G. irregulare* and *G. mosseae* in saline and heavy metal contaminated

soils (Wilde., *et al* 2009). In the present study, sequences of RMC3, RMP20 and FA31 were identified recently and named as a *G. indicum* which has been isolated from coastal sands (Błaszkowski *et al*., 2010). The AM fungi identified through spore morphology such as *G. etunicatum*, *G. mosseae* and *S. heterogama* reported in this investigation have also been found in alkaline, salt marshes, alkaline anthropogenic sediment and heavy metal contaminated soils (Gonazalez-Chavez *et al.,* 2002; Karthikeyan and Selvaraj, 2009; Oliveira *et al*., 2005; Wilde *et al*., 2009; Zarei *et al.*, 2010), indicating their adaptability to different stress conditions.

7.1 Rehabilitation of red mud in micro-field studies

Establishment of vegetation on residues produced from the bauxite refining process is a beneficial part of their environment stabilisation. Rehabilitation involves the stabilisation of surface materials through appropriate landscape reconstruction, establishment of soil organic matter, nutrient availability and the establishment of long-term sustainable vegetation community (Sharma, *et al*., 2000; Singh *et al*, 2002). However, bauxite residue rehabilitation represents a significant challenge as this material is highly alkaline, saline, sodic, has poor water retention and containing virtually no organic matter and has numerous nutrient deficiencies (Estham and Morald, 2006; Gherardi and Rengel, 2001). Till date, rehabilitation performance has been variable and very little information available on the effect of microorganisms on the vegetation cover. Remediation of contaminated sites may be facilitated by selection of tolerant plant species as well as soil microbes. Soil microorganisms are important in the recovery of disturbed and potentially toxic environments because they produce plant growth stimulating substances such as hormones and vitamins, immobilize heavy metals in the soil, bind soil particles into stable aggregates which improve soil structure, reduce erosion potential and can contribute to nutrient availability to plants (Gadd, G.M., 1993.; Shetty *et al*., 1994).

Plants can be used in the remediation of soils contaminated with heavy metals. In fact, plants have mechanisms for accumulation, tolerance or alleviation of high levels of heavy metals in contaminated soil (Khan *et al*., 2000). In the present study, we have selected an alkali-tolerant bermudagrass for vegetation cover because of its multi metal tolerance, adaptability to local conditions and its fibrous root production.

The fibrous roots would provide a larger surface for colonization by soil microorganisms than a tap-root.

The microbes such as, AM fungi (isolated from red mud flooded sites adjoining area of RM pond), red mud bacterial consortium (isolated from red mud) and *A. tubingensis* (exogenous P-solubilizer) were used as inoculants. The inoculum of AM fungi was produced in pot culture in presence of RM to make AM fungi tolerance to these conditions. Oliveira *et al*. (2010) reported that AM fungi quickly lose their symbiotic efficacy when cultivated without edaphic stresses of the environment from where they are originally isolated. They also recommended that the inoculum should be produced with original edaphic stresses especially for AM isolates from extreme environments.

In order to promote plant establishment in contaminated sites, it is essential to improve the physical and chemical properties of the substrate (Chen *et al*., 2007). We have amended gypsum, fly ash, sewage sludge and top soil to RM to improve the properties of the RM prior to plantation of bermudagrass. After plantation bermudagrass was failed to grow initially in red mud without any amendment, but growth was improved in other red mud amended treatments, which might be due to the inherent hostile properties of RM (Chen *et al*., 2010a; Courtney *et al*., 2009; Pagano *et al.*, 2002). Fortin and Karam (1998) and Pagano *et al.* (2002) reported the failure of vegetation in unamended RM. Bermudagrass replanted after 2 months showed survival throughout the experiment which might be due to leaching of some of the toxic components of the RM.

Bermudagrass growth

After 6 months of initial plantation, inoculation of different microbes increased the growth of bermudagrass with various levels in different amended and red mud alone treatments compared to their respective controls. Further, the maximum growth was increased by microbial inoculants, *A. tubingensis* and bacterial consortium when combined with AM fungi compared to those of all individual inoculated treatments and this trend was gradually increased to 12 months. In case of individual inoculated red mud and red mud amended treatments, each one showed different specificity on the growth of bermudagrass with various levels. For example, inoculation of BC to red mud alone, top soil and fly ash, and AM to gypsum amended treatments had shown maximum growth of bermudagrass, and in sludge amended treatment no significant difference in growth was observed between AM and BC. Increased growth may be due to better uptake of nutrients and production of plant growth promoting substances in the rhizosphere by the effect of microbes. This indicates the specific functional abilities of each one of these inoculate to red mud and red mud amendments. It can also be concluded that inoculation of plants with microbes significantly increased tolerance of bermudagrass in RM of all concentrations used in this study.

As compared with 6 months of growth, the growth was increased rapidly from 6 months to 12 months. It may be attributed that the leach out of the salts from surface layers of residue mud under the influence of rainfall because during the time of this study had received a 1451 mm rainfall. In this harvest (after 12 months), consortium followed by AM fungal inoculated treatments significantly increased the growth of

bermudagrass compared to all other individual microbial treatments except in topsoil amended treatment. In this treatment no significant difference in growth promotion by AM and bacterial consortium inoculated treatments were observed. These findings suggest that AM fungal colonization and their functional symbiotic activity was exhibited very low due to inherent effect of red mud toxic components (high pH, sodicity, salinity and high content of Fe and Al) at the early stage plant growth.

Among the different amendments, the gypsum followed by sludge amended treatments had shown overall the higher biomass production. In case of inoculated treatments of these amendments, the maximum growth was observed in consortium followed by AM. Addition of gypsum or sludge might have increased the nutrition level and lowered the pH of the residue which might have promoted the maximum growth of grass. Courtney and Timpson (2005) reported the plant growth in treatments that had received gypsum amendment, with higher plant biomass, Mn nutrition and lower Al and Fe concentration. The increase of biomass due to sewage sludge application is a true reflection of its effect on the physical and chemical properties, as well as the nutritional status of the soil. This may result from decrease of soil pH and to the solubilization of nutrients and enhanced nutrient availability or the stimulation of biodegradation through increased the populations and activities of soil microorganisms.

Overall, if we consider the growth of bermudagrass in all treatments, individual inoculants and as well as its combination with AM fungi had shown increased growth of bermudagrass compared to those of controls. This indicated that AM fungi, *A. tubingensis* and bacterial consortium are able to survive and improve the plant growth

development under such red mud and red mud amended conditions. A relevant effect of the inoculated microorganisms was the improvement of AM fungal colonization. This effect of the inoculants may have contributed to the improved nutrition and development of AM fungal inoculated plants in this soil. Studies also reported that free-living microbial inoculants could also stimulate mycorrhizal colonization (Vosátka and Gryndler, 1999). Conversely, mycorrhizae formation can affect the microbial population in the rhizosphere directly or indirectly through changes in root exudation (composition and quantity) patterns, or through fungal exudates. This is the so-called 'mycorrhizosphere effect' (Linderman, 1992). AM fungi increased the nutrient levels to plants directly from soil to roots through its hyphal structure and reduce the detrimental effects of soil associated plant stresses such as lack of nutrients, organic matter, high salinity or high pH (Entry *et al*., 2002; Oliveira *et al*. 2010).

Plant mineral nutrients

In the present study, application of different amendments such as gypsum, topsoil, sludge and fly ash to red mud improved the properties of red mud which results in plant growth compared to red mud alone. Inoculation of different red mud amended soils with microbial inoculants further enhanced the growth of bermudagrass compared to their respective controls. It indicates that application of different amendments facilitated the nutrient availability compared to red mud alone. Courtney and Timpson (2004) also showed that gypsum and sewage sludge amendment enhanced the nutrient status of *Lolium perenne* and *Holcus lantanus* plants grown in alkaline bauxite processing residue. The concentrations of P in

bermudagrass were significantly higher in gypsum amendment compared to other treatments. The enhanced P levels in the gypsum treatment are likely to be the reduction of the pH of the soil. The P levels were significantly increased by the inoculation of microbial inoculants. The high soluble P content in gypsum also explains the higher tissue P contents in gypsum amended treatments as suggested by Wong and Ho (1993). The P concentrations are lower than critical values of 0.15 % for bermudagrass (Jones *et al*., 1991). However inoculation of microbial inoculants increased the P levels in all the amendments.

The higher uptake of nutrients in microbial inoculated plants could be explained by the production of plant growth stimulating substances such as hormones and vitamins, immobilize heavy metals in the soil, binding of soil particles into stable aggregates which improve soil structure, reduction of erosion potential and contribution in nutrient availability to plants (Gadd, 1993; Shetty *et al*., 1994). Gypsum addition markedly increased Ca content than all other amendments. Inoculation with microbial inoculated treatments increased Ca uptake of bermudagrass compared to their respective control treatment. It indicates that applied amount (10%) of gypsum was not adequate for releasing of exchangeable Ca inions on surface of red mud soil. However calcium levels were not adequate (0.5%) for bermudagrass (Jones *et al*., 1991). The Mg levels were increased significantly in all the amendments inoculated with microbes compared to their respective controls, but the levels are not adequate. Bermudagrass grown in gypsum amended treatments had lower content of magnesium compared to other treatments that had not received gypsum. Low level magnesium and potassium in vegetation growing in red mud have

been attributed to high levels of entertained sodium and from refining process and Ca from gypsum amendment (Eastham *et al*., 2006) and these might be the reason why low amount of magnesium in gypsum amended treatment. However microbial inoculated treatments had significantly increased magnesium content in bermudagrass.

Potassium concentrations were significantly increased due to *A. tubingensis* inoculation especially in sludge and fly ash amended treatments. The K levels were adequate only in these treatments (1%), whereas in other treatments these levels were marginally lower for bermudagrass (Jones *et al*., 1991). Exchangeable forms of potassium are considered the primary source of K for plant uptake (Knudsen *et al.*, 1982) and could be hampered by Na in red mud residue and Ca in gypsum. Gypsum, top soil, sludge and fly ash and microbial inoculated treatments had significantly higher concentration of K compared to red mud alone treatment. This indicates that the exchange of K is complex in red mud.

Availability of major cations is a concern in revegetating bauxite residues due to excessive levels of exchangeable sodium. Calcium and potassium levels in the amended residue are within the satisfactory limits reported for mine soils (Monterroso *et al.,* 1999). Available magnesium levels recorded for all treatments are within the slight to severe deficiency range reported by Monterroso *et al.* (1999) for mine soils. In the present study, Mg concentration was significantly increased in all treatments but a steep rise was recorded in gypsum and topsoil with bacteria and *A. tubingensis*. The results showed that microbes carry out the mobilization of Mg more efficiently. Sodium concentration was lesser than 3% in all bacterial and fungal treatments

combined with different amendments, except that of red mud amended with sludge and *A. tubingensis,* where sodium concentration was reported to be 3.8%. Lower concentration of sodium is necessary for the adequate growth of plants. Overall, the nutrient uptake levels of bermudagrass were inadequate to marginal ranges in all treatments. However, the maximum growth of bermudagrass from 6 months to 12 months was evident for its adequacy. In addition to this, however, levels of these minerals cited as low, adequate, deficient or higher will vary between plants, different geographical regions and soil types.

Fe, Al and Na accumulation

Bioavailability of metals released from mineral deposits is very complex and dependent on many interrelated chemical, biological, and environmental processes. These processes may vary over time and among micro-organisms, plants, and animals. In soil and surface water, the mining method, presence or absence of sulfide minerals, quantity of water, acid-buffering capacity, presence of organic matter and iron and manganese oxide minerals, element speciation, and concentrations of other constituents in water may impact dissolved and bioavailable metal and metalloid contents (Adriano, 1992).

The lowest Fe and Al content was observed in gypsum and microbial inoculated treatments. This is attributed to the reduction in pH following gypsum addition causing precipitation of aluminate as aluminium hydroxide thereby reducing soluble Fe and Al content in the substrate (Wong and Ho, 1993) and further reduction of pH by microbial inoculation due to secretion of organic acids. The decrease in soluble iron levels can be attributed to an improvement in substrate conditions. The relatively

insoluble ferric ion (Fe^{3+}) is associated with well-drained soils, compared to ferrous iron (Fe^{2+}) associated with waterlogged soils (Troeh and Thompson, 1993). Though the levels of Fe are more than adequate for bermudagrass (4-350 ppm), it was suggested that high iron values are not considered to be a problem for the growth (Jones *et al*., 1991).

In general, aluminum levels in red mud are closely correlated with pH (Fuller *et al.*, 1982), with solubility increasing above pH 9.2. In the present study, aluminum concentration of grass grown in top soil and fly ash amended treatments was found to be very high irrespective of its pH. After 6 months, the pH of top soil and fly ash was in the range of 9.12 to 9.48 and 8.92 to 8.63 respectively, even Fe and Al accumulation was very high compared to other treatments. On the other hand, the red mud pH range was 9.42 to 9.81 and accumulation Fe and Al was very low compared to top soil and fly ash amended treatments. It indicates that the compactness of red mud resulted in low availability of Fe and Al to plants. The pH directly does not affect the aluminum concentration of different treatments. Moreover, addition of top soil and fly ash to red mud could be tend to increase the coarse- textured sand material which could make availability (dilution effect) of Fe and Al to bermudagrass. Topsoil used in study is mined out soil of bauxite ore, generally rich in Fe and Al, and fly ash generally consisted of high content of Fe, Al and other trace elements. Thus, the concentration and availability of potentially toxic elements in amendments used are representing a risk of further soil contamination. However, this problem can be reduced by microbial inoculations because microbial inoculated

treatments significantly increased the growth by reducing or increasing the metal uptake of bermudagrass with various mechanisms.

In this study, inoculation of microbial treatments had shown different variations on the accumulation of Fe and Al in bermudagrass compared to control treatments at 6 and 12 months. This suggest that the amendments associated with inoculants can somewhat reduce Fe and Al toxicity through their interaction with plant growth. The decreased Fe and Al toxicity is evidenced by an increased biomass production, a more reliable growth index. Although microbial inoculants proved to be effective for reducing metal accumulation by the plants, it may still be useful for enhancing nutrients mobilization from soil particles. In this study, biomass of bermudagrass was gradually increased irrespective of microbial inoculants in all red mud amended and red mud alone treatments at both harvests. Nevertheless, the interactive microbial effects on bermudagrass were more relevant when applied microorganisms were involved. This is the first evidence of the effectiveness of red mud bacterial isolates and *A. tubingensis* singly or associated with AM fungus, in increasing bermudagrass growth and mineral uptake, which represents a positive microbial effect on plants grown under red mud stress environments. Thus, the highest growth in inoculated plants, as a result of symbiotic associations, must be taken into account in future revegetation of degraded soils.

After 12 months, accumulation of Fe and Al drastically decreased in all treatments over the time period (after 12 months) compared to 6 months; it might be fixation of these metals or leach out by rainfall. Sodium is one of the major components in the red mud to make salinity and sodicity but uptake of sodium levels

in bermudagrass at time of first harvest had significantly dropped in all treatments and it was very low compared to high levels in bermudagrass reported by Wong and Ho, (1993). Further, gypsum amendment decreased the sodium uptake of bermudagrass. Decreased levels of Na are attributed to the high content of Ca element in gypsum. This might be due to leaching of Na following gypsum amendment (Wong and Ho 1993). However, microbial inoculated treatments of gypsum and other treatments increased sodium uptake of bermudagrass.

Therefore, the application of the most effective microbial groups is recommended for a better plant establishment. One important aspect for successful application of microbial inoculants is the appropriate selection of effective isolates. Our results showed that the survival and development of bermudagrass in red mud and red mud amended soils are highly dependent upon the activity of microbial populations. The improvement of bermudagrass establishment is achieved by microbial inoculants, particularly those adapted to local conditions. Such microbial management can improve the biochemical soil properties in degraded areas. In general, few mechanisms have been involved in alleviation of metal tolerance to plants. The ability of *Aspergillus* fungi to withstand stress induced by toxic metal in their environment may be connected with their ability to immobilize and bind the toxic metals (extracellular metal sequestration and precipitation, metal binding to the fungal cell walls, intracellular chelating by metallothionieins or phytochelatins, sequestration in vacuoles) (Gadd, 1993; Perotto and Martino, 2001).

In addition, the increase of AM colonization due to *A. tubingensis* and bacterial consortia might also have enhanced the plant mineral nutrition and plant growth with

a resulting dilution effect of the metal in the host plant. The external fungal hyphae exploit a larger volume of nutrient deficient resources in the soil that are otherwise unavailable for uptake by roots alone (Wang *et al*., 2005); this may be indirectly, by enhancing the plant mineral nutrition and increasing plant growth with a resulting dilution effect of the metal in the host plant or directly, by binding of the metal to the fungal mycelium and immobilization in the rhizosphere or the roots (Chen *et al*., 2001). Arbuscular mycorrhizal fungi may also play a role in protection of roots from heavy metal toxicity by mediating interactions between metals and plant roots (Wang e*t al*., 2011).

Soil at harvest

Reclamation of red mud ponds is difficult because of high alkalinity, sodicity, salinity, high concentration of soluble ions, which are toxic and competitively inhibit the uptake of nutrients in plants and microbes (Thakur and Das, 1994). The high alkalinity also reduces the water availability to the plants. These properties when coupled with lack of nutrients and anoxic conditions can prevent the vegetation. Hence, remediation of bauxite residue must be in such a way so that it can promote the plant growth and improve the nutritional status.

Red mud neutralization by microbial means has been investigated by a small number of researchers despite early results showing significant promise (Hamdy and Williams, 2001; Krishna *et al*., 2005; Vachon *et al*. 1994). In the present study, addition of different amendments to RM has improved the residue properties. Furthermore, microbial inoculated treatments enhanced the growth of bermudagrass by lowering the pH, increasing the organic carbon, available P, total N and the soil

enzyme activities such as acid phosphatase, alkaline phosphatase and urease compared to unamended RM. All treatment groups showed a gradual decrease of pH from 6 months to 12 months period. Since gypsum was used as a conditioning agent for red mud, addition of gypsum to red mud caused a maximum reduction in pH than other amendments. In red mud, the main alkaline anions buffering the solution are HCO_3^-/CO_3^{2-}, $Al(OH)_4^-$ and OH^-. Gypsum addition effectively lower the pH which is related to the ability of gypsum to dissolve and release Ca^{2+} into the solution to react with OH^-, $Al(OH)_4^-$ and CO_3^{2-} (Oster *et al*., 1999). Xendis *et al*. (2005) observed that gypsum solubility limited the extent of the pH reduction and only upon activation with H_2SO_4 did the desired pH reductions takes place. This limitation might have occurred due to the precipitation of $CaCO_3$ on gypsum particles as has been observed by Kopittke *et al*. (2004).

In the present study, reduction of alkalinity was achieved up to pH 7.67 in red mud amended gypsum control treatment of micro-field experiment after 6 months and it was stable for 6 months to 12 months. Further, the pH values were reduced by microbial inoculants. It was high in consortium inoculated treatments (pH 7.45). Gherardi and Rengel, (2003) in their four years study on leaching and precipitation, it was observed that pH reduced from 10 to 8.8. Plants growth may be favored at this pH. Conversely, in another study, within 5 years of planting, the pH of the red mud under vegetation had decreased from 10.5 to 9.5 (Hinz, 1982). In a study of Courtney *et al*., (2009), long-term growth of vegetation on weathered red mud at field level that had received gypsum and organic matter, the pH decreased from 10.9 to 8.09.

Similarly, application of sludge also reduced the pH but not to the extent of gypsum. The decrease of pH in sludge amended treatment was caused by the generation of organic acids from the mineralization of organic substrates. Moreover, the nitrification process taking place at this later period might also be responsible for the decrease in pH (Lai *et al*., 1998). However, though the exact mechanism of neutralization reaction is not fully understood, it is assumed to be a combination of organic acids released by the microbes and root exudates and the diffusion of the respiratory gases into the environment (Hamdy and Williams, 2001; Krishna *et al*., 2005). The neutralization of RM using microorganisms is significant because it is continuously controlled by a biological entity rather than the application of acid, the pH is buffered by microbes as long as they are provided with nutrients and microbes improves drainage nutrient exchange and chances for establishing a plant cover (Grafe *et al*., 2009).

The Electrical Conductivity (EC) of all treatments showed a decline with various levels in all treatments compared to initial EC, indicating that the ion and salt contents in the soil mixtures were might be diminished by biological uptake and/or leaching. For some inoculated treatments, the EC was increased compared to their respective controls due to the release of ions and salt from treatment soils. Electrical conductivity of all the treatments was below 0.9 at 6 months and it was reduced to 0.83 after 12 months except in gypsum amended red mud. The gypsum amended sample, in addition to Ca and SO_4, contained Si and small amounts of Na, Fe, K, etc. The high electrical conductivity of gypsum amended red mud might be due to presence of high concentration of Ca in treatment, which is exchanged with the

sodium ion of the red mud (Xenidis *et al.,* 2005). Exchangeable calcium is the major reserve of soil calcium available to plant roots (Haby *et al.,* 1990) as it acts as a supply of calcium over a longer period.

The importance of application of organic carbon to mine residues to improve nutrient availability and soil physical properties is well established (Courteny *et al*., 2009). Organic carbon levels in red mud are low with reported values ranging from trace amounts to 0.3% (Wong and Ho, 1993). In this study, organic carbon in red mud used was 0.34% and it was increased after 6 months of plants growth in all treatments. It was high in inoculated treatments particularly in all consortium treatments after 12 months. In a study of Courteny *et al*., (2009), long-term growth of vegetation on weathered red mud at field level that had received gypsum and organic matter, the organic carbon levels were greater than 0.07 g/kg (Gherardi and Rengel, 2001). In our study, organic carbon values were recorded in the range of 0.38 to 2.93% in all treatments after 12 months. It is much greater than 0.58– 1.88% range reported by Ye *et al.* (2002) for sparsely and densely vegetated tailings.

Nitrogen is usually deficient in mine soils, which limits vegetation establishment and sustained productivity (Courtney *et al*., 2009). Nitrogen contents of unamended bauxite residues are low with levels ranging from trace to 0.02% as reported by Wong and Ho, (1993) and Krishna *et al*, (2005). Values found in the current study show a significant increase in total nitrogen levels compared to initial levels after 6 months. In red mud amended with sludge and consortia, nitrogen was about 818 mg/kg after 12 months. Generally, inoculated treatments increased total nitrogen levels compared to their respective controls.

Phosphorus nutrition is limited in red mud due to large amounts of sesquioxides, and thus had a very high phosphorus retention capacity (Snars *et al.,* 2004). Analysis with acidic extracting reagent (pH 4.8) showed high values of greater than 150 mg/kg phosphorus as described by Courtney *et al*., (2008). Similarly, Meecham and Bell (1977) found that available P levels of 8.2 mg/kg phosphorus with an 8 fold increase in acid extract. Available P was gradually increased in all treatments from 6 months to 12 months and it was high in inoculated treatments than control. The maximum P was recorded in gypsum amended consortium inoculated treatment and it was 4.94 mg/kg at 6 months. After 12 months, it was increased to 9.46 mg/kg. These results indicate that although phosphorus reserves may be high, available amounts are low. This might be due to high pH values of the red mud. The results from present investigation suggest that available P increasing by a biological entity rather than the application of acid. The maximum P in gypsum amended treatment might be due to the reduction of pH and fixation of Fe and Al.

Although revegetation of bauxite mine sites is relatively straightforward, the bauxite residue (red mud) is difficult to revegetate. The refinery residue is characterized by high pH (pH >10), high electrical conductivity (EC > 30 mS/cm), and high exchangeable sodium percentage (>70%) (Hinz, 1982; Meecham and Bell, 1977; Wong and Ho, 1993). On the other hand, concentrations of plant nutrients such as calcium, magnesium, manganese, nitrogen and phosphorus are low (Hinz, 1982; Meecham and Bell, 1977) and the fine texture obstructs penetration of plant roots (Meecham and Bell, 1977). Consequently, the chemical and physical limitations of the red mud must be addressed prior to revegetation if the refinery residues are to

form part of the plant growth medium. Inclusion of gypsum ($CaSO_4$. $2H_2O$) and other materials (sewage sludge, fly ash, chicken manure) is used to improve the physical characteristics and reduce nutrient deficiencies (Fuller *et al.,* 1982; Ward, 1986; Wong and Ho, 1994, 1993; Wehr *et al.,* 2006).

Amelioration of the surface layer has been widely attempted using gypsum (Wong and Ho 1991), manure/ compost, sewage sludge (Wong and Ho 1991; Courtney *et al*., 2003; Courtney and Timpson 2004), soil (Wong and Ho 1991), and combinations thereof. Vegetation survival was only good at the highest application rates of these substrates. Most researchers attributed the positive effect of high addition rates for improvement of the physical structure (bulk density, porosity) of red mud, thereby improving drainage and leaching of salts from red mud (Wehr *et al.,* 2006). However, there are no indications that vegetation would have survived over longer timescales or with minimal input, or that roots penetrated into the unamended red mud. Furthermore, the quantities suggested by these studies are economically unlikely to be viable (Wehr *et al.,* 2006).

Courtney et *al.* (2003) demonstrated that the establishment of clover species on red mud and process sand can be achieved without the addition of gypsum and that thermally dried sewage sludge is a beneficial organic amendment. A period of leaching is however required to effectively lower pH and sodium levels. Revegetation is only achievable when sodium and pH levels are sufficiently low. Such threshold levels have yet to be established. But, microbial remediation is not studied so far in red mud. As soil microorganisms play a crucial role in increasing soil fertility and accelerating revegetation process through their activities in disturbed soils (Visser *et*

al., 1983). Hence, use of microbes in combination with chemical amendment in the current study, has helped in sufficient reduction of pH. Other parameters such as levels of the soil nutrients (organic C, total N and available P) were improved and promoted the growth and activities of different microbial groups, thus showed both the nutrient pools and the stability of the soil ecosystem in the revegetated sites which gradually reestablished by inoculated microbes.

Soil enzymes

The biological and biochemical properties that are most useful for detecting the deterioration of soil quality are those that are most closely related to nutrient cycles, including soil respiration, microbial biomass, nitrogen mineralization capacity and the activities of soil enzymes (Visser and Parkinson, 1992). In particular, soil enzymes are important in catalyzing several functional reactions necessary for the life processes of microorganisms in soils and the stabilization of soil structure, the decomposition of organic wastes, organic matter formation and nutrient cycling (Dick *et al.,* 2000). These enzymes are constantly being synthesized, accumulated, inactivated and/or decomposed in the soil, hence playing an important role in nutrients cycling (Tabatabai, 1994; Dick, 1997).

Phosphatases are a broad group of enzymes and in ecosystems, these enzymes are believed to play critical roles in P cycles (Speir *et al.,* 1980) as evidence shows that they are correlated to P stress and plant growth. Apart from being good indicators of soil fertility, phosphatase enzymes play key roles in the soil system (Dick *et al.,* 2000; Dick and Tabatabai, 1992; Eivazi and Tabatabai, 1977). Urease is one of the enzymes involved in environmental nitrogen transformations and can be used to

indicate part of the N cycling in soil (Park and Hausinger, 1995). Phosphatase and urease were therefore selected to represent the phosphorus and nitrogen cycle respectively in the micro-ecosystem of the amended soil. Some features of soil microorganisms, such as the microbial biomass size, respiration rate, and enzyme activity are often used as bioindicators of the ecotoxicity of heavy metals.

In the present study, the activities of acid, alkaline phosphatase and urease enzymes were higher in inoculated treatments compared to controls. As a result of their functions, it indicates the presence of microorganisms and serves as indicators of red mud amended soils. In this study, alkaline phosphatase activity found to be higher than the acid phosphatase activity in all treatments and it was further increased by microbial inoculated treatments. It has been suggested that the rates of synthesis, release and stability of acid and alkaline phosphatases by soil microorganisms are dependent on soil pH, so alkaline phosphatase activity is induced in high pH soils (Acosta-Martínez and Tabatabai, 2000; Acosta-Martínez *et al*. 2003; Deng and Tabatabai, 1997) but only from microorganisms, not from plants (Böhme and Böhme, 2006). In this study, the variation between acid and alkaline phosphatase activities was very low especially in gypsum and sludge treatments compared to other treatments. The observed differences are related to different origin of these enzymes in soils (Matinizadeh (2008). For example plant roots are major source of acid phosphatase in soil (Dinkelaker and Marsclmer, 1992; Speir and Cowling, 1991) while soil microorganisms (including bacteria, fungi and fauna) are major source of alkaline phosphatase in soil (Findenegg and Neiemans, 1993; Tarafdar, 1995). Hence, higher growth of bermudagrass in these amended treatments could be evidence for the

source of high production of acid phosphatase and it was more in inoculated treatments. It was attributed that the synergetic affect of amendment, microbes and bermudagrass resulted in high release of acid phosphatase and its activity. Acid and alkaline phosphatase activities were more in gypsum amended treatments followed by sludge amended treatments even in presence of high organic matter in sludge at 6 months.

After 12 months, maximum acid and alkaline phosphatase activity were observed in sludge treatments. It might be lower pH over the time period and more availability of organic matter in sludge. In fact, the activity of acid and alkaline phosphatases was found to correlate with organic matter in various studies (Aon and Colaneri, 2001; Jordan and Kremer, 1994). But, in the present study, organic carbon is not directly correlated with the phosphatase activity in most of the treatments. Another factor, which might be playing vital role in various treatments of red mud, is the soil pH that influences the rate of synthesis, release and stability of this enzyme (Martínez and Tabatabai, 1997). Urease is one of the enzymes involved in environmental nitrogen transformations and can be used to indicate part of the N cycling in soil. Urease activity was recorded in very low concentration as compared to other enzymes. No urea was added in due course of field experiment, which is probable reason for the lower activity of the urease enzyme in various treatments. The amendments such as top soil, sludge and irrigated water used to various treatments might contain some amount of urea, which is hydrolyzed by microbial, and plant urease in the present experiment. After 12 months, the activity of acid phosphatase and urease was

decreased in some treatments compared to 6 months. However, microbial inoculated treatments had shown increased activity compared to their respective control.

The activities of these enzymes in soils undergo complex biochemical processes consisting of integrated and ecologically connected synthetic processes (Khaziyev and Gulke, 1991). In this regard, all red mud treatments contain a group of enzymes that determine soil metabolic processes (McLaren, 1975) which, in turn, depend on its physical, chemical, microbiological and biochemical properties. In practice, the biochemical reactions are brought about largely through the catalytic contribution of enzymes and variable substrates that serve as energy sources for microorganisms (Kiss *et al.,* 1978). In the present study, activities of soil enzymes of various treatments of red mud can be used to give preliminary indication of some of the physical and chemical properties of soil for soil management strategies.

In general, red mud contains low amounts of organic carbon and nitrogen and low levels of phosphatase and urease enzyme activity (Krishna, 2008). In the present study, gypsum and sludge amendments with microbial inoculants had shown similar urease activity. But after 12 months it was more in sludge amended inoculated treatments compared to gypsum treatment. It indicates that the urease enzyme activity was significantly correlated not only with organic carbon, but also with pH of red mud amended soils. It was also reported that the urease activity in alkaline soils was significantly correlated not only with organic carbon and $CaCO_3$, but also with pH (Rao and Ghai, 1985).

Microbial analysis and effects of red mud its amendments on microbial population

Soil microorganisms are very sensitive to environmental change (Turco *et al*., 1994), and significant changes of the microbial community can occur following disturbance, both in terms of total biomass and species composition (Harris *et al*., 1991, 1993). Measures of the microbial community following the initiation of reclamation efforts could be used as an indicator of restoration progress (Harris *et al*., 1991) and may give insights into potential ways to accelerate a restoration process. Generally, heavy metal contamination might lead to two kinds of effects on the microorganisms: one, due to heavy metal toxicities, sensitive populations would be inhibited, the kinds and amounts of microbial populations would be reduced gradually or become extinct; secondly less sensitive or resistant population that had adapted to the contaminated condition would grow, increase and become the dominant population in soil microbial community (Frostegard *et al*., 1996; Wang, 2002); as a result, heavy metal contamination would cause the changes of the soil microbial population and community. Some results proved that different kinds of microbes had different responses to the heavy metal contamination, in which the sensitive order commonly might be actinomycetes>bacteria>fungi (Sun *et al*., 2000).

P-solubilizing fungi

After 6 months of grass growth, P-solubilizing population was observed in *A. tubingensis* and consortium inoculated treatments and it was more in consortium inoculated treatment especially sludge amended treatments. This might be due to the cumulative effect of the inoculated AM fungi and bacteria consortia and the native

populations and organic matter in the soil at initial period. After 12 months, P-solubilizing population was drastically increased from 6 months to 12 months, might be due to reduction of pH and metal (Fe and Al) stress. After 12 months, the high P-solubilizing fungi were observed in all *A. tubingensis* inoculated treatments and it was higher in sludge amended treatments. The increase in soil P-solubilizing fungal populations with *A. tubingensis* inoculum was due to the addition of an inoculum that had an additive effect on the total fungal population. In contrast to this, the low proportion of P-solubilizing fungal count in all consortium (except in red mud alone) treatments might be due to higher proportion of soil microbial population and development of competition between them for nutrients after 12 months lead to low P-solubilizing fungal count.

Alkali-tolerant bacteria

In the present study, the alkaliphilic bacterial load was reduced in all treatments after 6 months when compared with initial bacterial population loaded to different treatments. The reduced bacterial population in all treatments could be due to high alkalinity, saline and sodic, Fe and Al content of red mud. Addition of different amendments with microbial inoculants and the growth of bermudagrass after six months have facilitated the increase in bacterial abundance. Though, there was clear trend in individual treatments, AM fungi, *A. tubingensis*, bacterial consortia and combination had triggered the increase of alkaliphilic bacteria compared to those of controls. After 12 months, microbial population was gradually increased in all inoculated treatments except in gypsum amended consortium, top soil amended AM, sludge amended *A. tubingensis* and fly ash amended consortium inoculated

treatments. This indicates, each microbial inoculant and amendment was differentially affected the alkaliphilic bacterial count. This result supported the idea that synergetic effect of soil microbes (native and/or introduced) and amendments are specific and which result of produced qualitative and quantitative changes in the composition of root exudates. The maximum population was observed in sludge and gypsum amended treatments. The inhibition of microbial and enzyme activities in red mud and increase of these activities reflects the addition of amendments, irrespective of microbial inoculants.

In most cases, the microbial and enzyme activities differed between the different red mud amended treatments. These differences can be explained by more favorable soil biophysical environment of gypsum and sludge compared with other amended treatments. The differences between gypsum, sludge and other substrates were quite larger. With regard to microbial and enzyme activities investigated in this study it has to be stated that, there is no obvious inhibition of microbial activities by the use of sludge and gypsum in red mud soils. It can be assumed that, the substances improve physical properties of the treated soils, thus promoting microbial and enzyme activities

In the current study, microbial population of P-solubilizing fungi and alkaliphilic bacterial consortia counts were reduced in all the treatments compared to initial load at 6 months. pH, electrical conductivity, organic carbon and nitrogen might have direct impact on the structure and abundance of the microbial community. It was reported that the extremes of pH can restrict microbial community directly by imposing stress on colonizing microorganisms, as well as indirectly through the

regulation of dissolved organic matter availability (Cookson *et al*., 2007). Microbial growth and activity in soil is often limited by the availability of carbon (Lynch and Whipps, 1990) and nitrogen (Wardle, 1992) and it may respond to a varying supply of substrates either by physiological adaptation or by changes in the community composition. In addition to plant net primary production and input of organic matter through root exudation, root biomass and litterfall (Banning *et al*., 2010), even small additions of organic substrates (in the form of sludge, fly ash, gypsum and topsoil) may trigger a shift in the composition of the microbial community and an accompanying change in the relative abundance of specific hydrolytic ecto-enzymes (Pinhassi *et al.*, 1999). Although it has been observed that P-solubilizing fungi and alkali-tolerant bacterial count has affected by red mud, it is evident that fungi and bacteria were not affected by red mud when inoculated in red mud added with different amendments. This phenomenon may be attributed to the fact that the fungi and bacteria are relatively affected by other soil condition, such as organic matter content in sludge and reduced pH and stabilization of metals, improved by sludge and gypsum amendments.

Inoculation of bacterial consortium and *A. tubingensis* in gypsum and sludge amended soils had shown its high population after 6 and 12 months of grass growth. A rapid establishment of an active soil microorganisms community in connection with a favorable humus and nutrient balance is of great importance for mine soils ecosystem functioning. In this study, the rapid increase of microbial population in red mud and its amended soils than the control is an evident of microbial community structure development. It was also reported in a study of Banning *et al*. (2010) that

the rapid increase of microbial community within residue sand may have a role in facilitating early establishment of the native vegetation. Organic waste materials like sewage sludge, coal sludge, composted sewage sludge and compost seem to be able to stimulate and increase microbial biomass and activity as well as enzyme activities in soils (Emmerling *et al*. 1996; Perucci 1990, 1992). The input of organic matter and of nutrients as a source for microorganisms as well as an inoculation with microorganisms may cause this stimulation and increase in sludge amended treatment.

AM fungal colonization

After 6 months of growth, mycorrhizal colonization was observed in all treatments including non-AM inoculated treatments. However, inoculation with AM fungal treatments showed higher colonization and was observed to be various degrees with roots of bermudagrass grown in different treatments. AM fungi colonizing the roots of bermudagrass grown in this stress condition are an indication of its effectiveness (tolerance) to red mud and its amended soils. It was reported that the AMF may have developed tolerance to heavy metals under long-term stress; thus, they are able to survive in polluted soil (del Val *et al*., 1999). The inoculated AM fungi were collected from red mud flooded sites and were maintained in red mud to adapt these fungi to red mud condition. However, the percentage of root colonization of bermudagrass in red mud treatments was significantly lower than the other amended treatments. This may be the effect of alkalinity, salinity and sodicity as well as high content of Fe and Al of red mud. It was also reported that in individual studies of AM fungi in alkaline, saline-sodic and heavy metal contaminated soil have shown

a reduction of AM fungal colonization and sporulation. Red mud is a multi chemical characteristic waste material having high alkalinity, salinity, sodicity and rich in Fe and Al content.

On the other hand, mycorrhizal colonization was higher in all inoculated treatments than the non-inoculated treatments, indicating that mycorrhizal inoculation can increase the root colonization percentage. As compared to red mud treatment, amended treatments had shown increased AM fungal colonization especially in gypsum amended treatment. This may be due to the reduction of pH by gypsum which result maximum colonization of AM fungi. Presence of AM fungal colonization in non-inoculated grass roots of all treatments might be due to pre-existed indigenous AM fungal colonization in roots and propagules present in amended soils used in the experiment. Higher mycorrhizal colonization in the AM inoculated treatments than in the non-inoculated treatment was due to the presence of higher amount of RM adapted AM fungal inoculum. Further, the colonization was gradually increased from 6 months to 12 months. This may have developed tolerance after a long time exposure to red mud stress or pH reduction or leaching of metals into soil along with plant growth. Generally natural mycorrhizal potential in the soil samples in terms of both the number of AMF spores and the AM colonization levels was very low in field soil (del Val, 1998).

Effect of *A. tubingensis* and red mud bacterial isolates on AM colonization.

Interestingly, the native AM fungal colonization was enhanced by *A. tubingensis* and BC inoculated treatments compared to control treatments and it was achieved further more in BC inoculated treatments. This is attributed that *A. tubingensis* and

red mud bacterial isolates stimulated the AM fungal root infection rate of grass. Soil microorganisms may interact with AM fungi and increases colonization of AM fungi with plants roots has been reported in many studies (Artursson *et al*., 2006; Mansfeld-Giese *et al.* 2002; Medina *et al.*, 2006; Tian *et al*., 2002; Vosátka and Gryndler, 1999). The significant increase of mycorrhizal colonization by bacterial consortium inoculation may be due to phytohormone production by these microorganisms, which in turn may stimulate mycorrhizal colonization. It has also been reported that beyond the phosphate solubilisation, many P-solubilizing microorganisms increase the mycorrhizal root colonization by producing specific metabolites such as vitamins, amino acids and hormones (Barea *et al*., 2005). The enhancement of mycorrhizal root colonization by addition of amendments and inoculation with *A. tubingensis* and/or red mud bacterial consortium may be attributed to effects on root morphology or on the physiology of the fungal symbionts, or could be due to the creation of more acidic conditions by an increasing population of native as well as the introduced combination of *A. tubingensis* and red mud bacterial isolates. This result suggests that, there was a synergistic effect of triple inoculation with AM fungi, *A. tubingensis* and bacterial consortium.

Role of AM fungi on the growth, nutrient uptake and metal accumulation

Symbiosis offers some protection against metal toxicity. Most reports note a positive effect of AM inoculation on the growth of plants in alkaline, saline-sodic and metal-contaminated soils (Ileana *et al*., 2008; Oliveira *et al*., 2010; Vivas *et al*., 2005; Zarei *et al*., 2010). This protective benefit may be related to the adsorptive or binding capability for metals of the relatively large fungal biomass associated with the host

plant roots, which may physically minimize or exclude the entry of metals into host plant (Cairney and Meharg, 2000).

In the present investigation, individual inoculations of AM fungi in red mud and red mud amended treatments such as top soil and fly ash treatments were not reflect the effect of AM fungi on the growth, nutrient uptake of bermudagrass at initial stage (after 6 months of observation). However, inoculation of AM fungi with combination of *A. tubingensis* and bacterial consortia significantly increased the growth with high root colonization, nutrient uptake and reduced the Fe and Al accumulation of bermudagrass when compared with individual inculcations, AM fungi, *A. tubingensis* and bacterial consortia. This study suggested that, the efficiency of AM fungal colonization with roots was adversity affected by highly alkaline, saline-sodic and high content of Fe and Al of this red mud and amended soils. This result suggests that *A. tubingensis* + AM fungi + bacterial consortia tripartite offer some protection against alkaline, saline-sodic and metal toxicity. However, inoculation of gypsum and sludge amended red mud with AM fungi alone had shown higher colonization efficiency with bermudagrass and enhanced the growth compared to individual inoculation of *A. tubingensis* and bacterial consortia and it was further enhanced to 12 months of observation.

This observation suggests that among the amendments used in this study, gypsum and sludge can act as supportive medium for spore germination and colonization efficiency with plant roots at initial stage. After 12 months, AM fungal colonization was significantly increased in all treatments which directly shown as an increased growth of bermudagrass in all AM inoculated treatments. In this study,

apart from AM fungal inoculum, colonization of native AM fungi in non-inoculated treatments was significantly increased by *A. tubingensis* and bacterial consortia and it was high in gypsum and sludge treatments (25-42%). From this observation, it can also be suggested that the native AM fungal colonization can also play a significant role in effecting physiological status of host plant. Overall, the present study (Table 2) indicates that, although the individual microbial inoculations increased the bermudagrass growth, nutrient uptake, and lowered the metal accumulation with various degrees, combination with AM fungi further enhance the growth, nutrient uptake and reduce the stress of red mud stress to bermudagrass in red mud amended or red mud treatments. The efficiency can be further improved if inoculation will be done with either gypsum or sludge amendments.

7.2 Rehabilitation of fly ash in micro-field studies

Establishment of vegetation on fly ash pond serves a variety of functions including stabilization of ash against wind and water erosion, reduction of heavy metal leaching, provision of shelter and habitat for wildlife and bringing about a more aesthetically pleasing landscape (Haynes, 2009). However, physical, chemical and microbial factors can affect the vegetation establishment in fly ash deposits. Among the chemical limitations are an initial pH and bioavailable heavy metal concentrations and their toxicity to plants and soil microbes, and nutrient deficiencies (e.g. N and P). Physical impediments include restriction of root growth due to natural compaction of fine ash particles and/ or formation of solid cemented layers due to the pozzalanic nature of some ashes (Haynes, 2009). In addition to this, microbial factors such as general lack of microbial activity and attendant low nutrient turnover and a lack of inoculum of symbiotic microorganisms (Haynes, 2009). Thus, inoculation of suitable microorganisms may play significant role on vegetation establishment and many studies have also been proved in many contaminated soils.

Plant growth and AM fungal colonization

Inoculation of AM fungi significantly increased the growth of bamboo plants in fly ash compared to non-inoculated treatment. Co-inoculation of *A. tubingensis* increased the mycorrhizal colonization compared to individual inoculation of AM fungi after 12 months of plantation. Many P solubilizing microorganisms increase the mycorrhizal root colonization by producing specific metabolites such as vitamins, amino acids and hormones apart from P solubilization (Barea *et al*., 2005). The colonization of AM fungi in non-AM inoculated plants may be due to either the

presence of propagules in substrate (fly ash) or in bamboo seedlings which were grown previously in the soil. The slow rate of indigenous AM fungal colonization and the limited number of species growing on fly ash reflects the adverse growth conditions of fly ash deposits. We have observed 6 different plant species growing in fly ash colonized with 8 different species of AM fungi and most of the AM fungi belong to *Glomus* species. These species are also been reported as dominant AM fungi in extreme environments by other researchers (Bedini *et al*., 2010; Zarei *et al*., 2008a).

Plant mineral nutrition

Fly ash contains many essential plant nutrients and their availability to the plant may be problematic as reported by different authors (Pandey *et al*., 1994; Singh *et al*., 1997). However, inoculation of AM fungi supports the growth and nutrient uptake of plants and aggregation of fly ash (Enkhtuya *et al*., 2005; Wu *et al*., 2009). In the present study, all individual inoculation as well as its combination significantly increased the growth of bamboo plants compared to non-inoculated plants. Moreover, inoculation of *A. tubingensis* along with the AM fungi significantly increased height and number of branches of bamboo plants compared to individual inoculations. In the present study, higher accumulation of Al, Cu and Fe in shoots of bamboo plants, suggesting that the bioavailability of these metals to plants. In general, heavy metal bioavailability in soil not only depends on pH, also on its concentration and speciation (physical and chemical forms) of metals, organic carbon (both particulate and dissolved fractions), clay content and other factors, especially in artificially contaminated soil (Adriano, 1986; Wu *et al*., 2006). The uptake of nutrients such as

P, K, Ca, Mg, and Na were significantly increased and the contents of Al, Cu, and Fe were reduced in the shoot tissues compared to individual inoculation. This may be due to the synergetic effect of *A. tubingensis* with AM fungi. Some experimental results confirm the existence of synergetic effect of saprobe fungi on plant root colonization by AM fungi (Fracchia *et al*., 2000). Arriagada *et al*. (2004) reported that saprobe fungi absorb heavy metal from the contaminated site and increase the AM colonization of plants when inoculated in combination. It is also known that AM fungi may reduce the uptake of Al, Fe, and Mn that may be present in toxic levels in some soils (Ning, 2000). Alleviation of heavy metal phytotoxicity by AM fungi has been indicated in several studies (e.g. Chen *et al*., 2007; Arriagada *et al*., 2004). The AM fungi may enhance plant P nutrition and increase the plant growth by diluting metal effect in host plant or by binding of the metal to the fungal mycelium and immobilize them in rhizosphere or roots (Chen *et al*., 2001).

Chemical changes of fly ash

Fly ash contains considerable amounts of essential and nonessential elements such as Al, Fe, and Cu and many other elements which are toxic at elevated concentrations (Basu *et al*., 2009). In this study, pH of the fly ash decreased in all treatments from slight alkaline to moderate acidic range. The pH reduction in fly ash may be due to presence of high content of sulfur in fly ash while leaching of base ions (Juwarkar and Jambhulkar, 2008). Further, inoculated treatments reduced the pH of rhizospheric fly ash with various degrees which might be secretion of root exudates from plants as well as acid production of microbes (Barea *et al*., 2005). Since C and N in the fly ash are likely to be oxidized to gaseous constituents during combustion,

they usually are present in trace amounts and P in fly ash is relatively unavailable to plants (Bradshaw and Chadwick, 1980). In this study, organic carbon in rhizospheric fly ash was significantly increased in all inoculated treatments. These increases might be attributed to better root developments and it root exudates induced by microbial inoculations. Higher concentrations of P in fly ash reported in this study might be due to solubilization of insoluble P present in the fly ash by both mycorrhizal and phosphate solubilizing *A. tubingensis*. The AM fungi interact with other rhizosphere microorganisms (Jeffries *et al*., 2003) and affect rhizodeposition and thus the quantity and quality of organic C delivered to the soil via fungal hyphae (Barea *et al*., 2005).

Soil enzymes are important in catalyzing several reactions necessary for life processes of microorganisms in soils and the stabilization of soil structure, the decomposition of organic wastes, organic matter formation, and nutrient cycling (Dick *et al*., 2000). Among the soil enzymes, phosphatases (acid and alkaline) and urease play an important role in P, N, and C mineralization. In this study, acid phosphatase and urease activity in inoculated treatments were increased in all treatments compared to control treatment. The increase of enzymatic activities in soils is involved in an increase in the availability of nutrients to the plants, which in turn have a positive influence on soil fertility (García *et al*., 1997). However, after 12 months, the decrease in total N in all treatments might be due to increase in microbial and plant metabolic activity which results in continuous utilization of total N for its metabolisms and physiological activities.

Summary

Summary

Red mud (bauxite residue) is waste produced during aluminum extraction from bauxite ore with concentrated sodium hydroxide at elevated temperature in the Bayer process (Evans, 1993). The major components in the red mud are iron oxide, silica, un-reacted alumina and residual NaOH as Na_2CO_3 as well as alkali bound in the form of sodalite, ferrite etc. The residual alkali content makes the red mud alkaline with a pH range from 9 to 13. At present, red mud is either directly disposed onto the land or creating huge ponds. Reclamation of red mud ponds are difficult because of high pH, high concentration of soluble ions such as sodium and carbonate which are toxic and competitively inhibit the uptake of nutrients in plants and microbes. Many studies, using different chemical amendments of red mud, have been conducted to rehabilitate the red mud. However, using microorganisms for rehabilitation and vegetation of red mud is very scarce. In this study, we have evaluated the AM fungal colonization efficiency and diversity of AM fungi associated plants growing in red mud affected soils.

This is the first report of AM fungal diversity analysis of red mud affected soil. Arbuscular mycorrhizae fungal colonization and spores were noticed with very low colonization and spore number. Morphologically six species were indentified whereas five different AM fungal sequence were obtained after PCR amplification of the ITS region followed by RFLP and sequencing. Phylogenetic analysis of these five sequences clusters into 4 discrete groups separately, belonging to the genus *Glomus*. The isolated AM fungal species were multiplied in red mud amended soil for adaptation and were used as a mass inoculum in micro-field experiment. In addition

to this, indigenous red mud bacterial isolates and exogenous *Aspergillus tubingensis* with combination of different amendments (fly ash, topsoil, and gypsum, and sewage sludge) were also applied to evaluate the effect of these microbes on the growth of bermudagrass.

An alkali-tolerant bermudagrass which was native of NALCO, Damanjodi, Orissa was transplanted and the growth and survival of was studied. Results after six months from initial plantation revealed that the biomass of grass in different microbial inoculated treatments with different amendments was compared; it showed that biomass of bermudagrass was very less in the unamended red mud and was high in gypsum amended red mud inoculated with consortium. In all the treatments of gypsum, the percentage of AM fungal colonization was significantly higher in AM fungal inoculation followed by consortium inoculated treatments compared to other amended treatments. The maximum colonization was observed in AM inoculated followed by consortium inoculated gypsum amended RM treatment.

All microbial inoculated treatments increased nutrient uptake of bermudagrass with various degrees compared to their respective controls, irrespective of amendments. Moreover, each microbial inoculated treatment had specific for particular parameter both at 6 and 12 months. However, in case of the total P of grass grown in gypsum amended inoculated with consortium had maximum followed by AM inoculated treatment. The P content of grass in all the amended treatments increased as compared to unamended red mud. The inoculation of different microbial treatments increased nutrient uptake of grass with various degrees compared their control treatments. In all the amendments of red mud, the availability of Fe, Al and

Na decreased as compared to top soil amended red mud. However, this availability was low in treatments involving gypsum. The maximum pH reduction was observed in all the treatments involving gypsum, whereas the EC was significantly high when compared to other amendments. This indicates that gypsum reduced the pH through release of Ca ions which dominates Na exchangeable sites resulted in reduce the soluble Fe and Al. The organic carbon was found to be highest in sludge followed by gypsum amended consortium inoculated treatments.

The available P was highest in consortium followed by bacterial consortia inoculated gypsum, AM fungal, *A. tubingensis* and bacterial consortia inoculated sludge amended treatments. The total N was very low in RM treatments, whereas it increased on addition of different amendments. It was highest, when compared to other amendments, in sludge amended inoculated with bacterial consortia and consortium followed by AM fungi inoculated treatment. The alkaline phosphatase activity was more than acid phosphatase activity in all the treatments, whereas it was higher in *A. tubingensis* and bacterial consortia inoculated gypsum followed by AM fungal and consortium, and in consortium inoculated sludge amended treatment. The urease enzyme activity was found to be high in the consortium inoculated gypsum and sludge amended treatments followed by AM and bacterial treatments.

The results of one year data showed that the growth of bermudagrass significantly increased as compared to six month growth in all treatments. As observed at 6 months, consortia inoculated treatments had the maximum growth of bermudagrass compared to other inoculated treatments. Interestingly, AM fungal inoculated treatments significantly increased the growth of grass as compared to other

individual (*A. tubingensis* and bacterial consortia) inoculated treatments. However, consortium inoculated gypsum amended treatment showed highest biomass followed by AM inoculated treatment. When compared to six months, significant increase in colonization was observed in all the treatments after one year. Gypsum followed by sludge being inoculated with AM fungal propagules showed maximum colonization as compared to their respective non inoculated controls. The maximum percent colonization was observed in gypsum amended consortium inoculated treatment followed by AM inoculated treatment. All microbial inoculated treatments increased nutrient uptake of bermudagrass with various degrees compared to their controls, irrespective of amendments. However, inoculation of AM fungi followed by consortium to gypsum and sludge amended treatments significantly increased the P uptake of grass compared to other treatments.

The concentration of Fe and Al in grass was markedly reduced in all the treatments as compared to values of the six months. Further, almost all of microbial inoculated treatments decreased the accumulation of Fe and Al in grass with various degrees compared to their controls. The pH of all treatments further decreased from 6 months to 12 months. The maximum pH reduction was observed in gypsum amended all microbial inoculated treatments whereas highest EC was observed in this treatment. The highest organic carbon and total N was increased by consortium inoculated sludge amended treatment. In gypsum amended treatment, the increase in available P in all the treatments was many times higher than the values after six months. This increase was observed maximum in consortium inoculated treatment. Enzyme activities such as acid, alkaline phosphatase and urease were increased

almost all microbial inoculated treatments compared to their controls treatments. Alkaline phosphatase activity was shown higher than the acid phosphatase activity. The highest acid phosphatase activity was measured in consortium followed by bacterial consortium inoculated sludge as well as *A. tubingensis* inculcated gypsum amended treatments. The maximum alkaline phosphatase activity was noted in sludge amended inoculated with consortium followed by AM fungal treatment. In case of urease enzyme activity, it was increased by consortium inoculated treatments of sludge followed by gypsum amended treatments.

Overall present study showed that, inoculation of different microbes significantly increased the bermudagrass grass in various amended treatments. Amongst all the amendments, gypsum followed by sludge amended treatments promotes higher growth of bermudagrass as well as enhanced the microbial activity compared to other amendments which results increased growth of bermudagrass than the their controls. After 6 months, inoculation of microbial consortium significantly increased the bermudagrass growth in all treatments followed by various microbial treatments at 6 months. In case of gypsum amended treatment, AM fungal inoculated treatments significantly increased the growth of bermudagrass compared to *A. tubingensis* and bacterial consortia treatments. The experiment provided evidence for the potential use of bermudagrass in combination of all microbes as a consortium or single inoculation of red mud tolerant AM fungi with gypsum or sludge amendments for ecological restoration of bauxite residue sites.

Establishment of vegetation on fly ash pond serves a variety of functions including stabilization of ash against wind and water erosion, reduction of heavy

metal leaching, provision of shelter and habitat for wildlife and bringing about a more aesthetically pleasing landscape (Haynes, 2009). In this regard, fly ash adapted arbuscular mycorrhizal fungi were isolated from rhizosphere soil and root of the plant growing in fly ash pond. Prior to this, the AM fungal colonization and diversity was studied. AM fungal colonization associated with plants growing in fly ash pond and spore number in rhizosphere fly ash samples was very low. Overall, eight species were identified based on spore morphology. The PCR amplification of the ITS region followed by RFLP and sequencing revealed that the seven different sequences were belongs to AM fungi. Phylogenetic analysis showed that these sequences cluster into 4 discrete groups separately, belonging to the genera *Glomus* and *Archaeospora*. These AM fungi were multiplied in fly ash for inoculum production and used as individual and combination of *A. tubingensis* a phosphate solubilizer. Results after 6 and 12 months revealed that the inoculation of AM fungi, *A. tubingensis* and combination of these two fungi increased the growth of bamboo plants compared to control treatment. Plant height as well as number of branches/plant was significantly increased in dual inoculation than the individual inoculum both at 6 and 12 months of the study. AM fungal colonization was observed in all treatments and the colonization of indigenous AM fungi increased due to inoculation of *A. tubingensis* inoculated plants. It was observed that the percent root colonization of all treatment plants gradually increased to 12 months. Maximum spore count was observed in dual inoculated plants after 12 months.

Dual inoculation significantly increased the P, K, Ca, and Mg in shoot tissues compared to control treatments at 12 months after plantation. Individual inoculation

also increased the levels of these minerals compared to control plants. Sodium levels increased in inoculated treatments however, the uptake of Na levels decreased at 12 months compared to 6 months. The uptake of Al, Cu, and Fe were significantly reduced in plant shoots due to inoculation. The Al content was reduced up to 50% in inoculated plants compared to control plants at 12 months. The Fe content was significantly reduced (about 60%) in inoculated plants compared to control plants at the end of 12 months. The content of these metals were significantly lesser in dual inoculated treatment than individual inoculation. The Zn levels were significantly increased (above 64%) in tissues of bamboo plants inoculated with dual inoculum compared to control as well as individual inoculations.

The chemical properties of fly ash improved in all inoculated treatments both at 6 and 12 months. In case of dual inoculation, the organic carbon and available P increased significantly after 6 months as well as 12 months of plantation. The available P was 3 times higher in dual inoculation treatment after 12 months compared to control treatment. After 6 months of plantation, the N content in fly ash increased in inoculated treatments as compared to control, the maximum being observed in dual inoculation treatment. Acid phosphatase and urease enzyme activities also increased in inoculated treatments compared to control. The maximum enzyme activities were observed in dual inoculated treatments than other treatments. Therefore, Inoculation of fly ash adapted AM fungi along with *A. tubingensis* might be a promising strategy to promote the vegetation in fly ash ponds.

Conclusions

Conclusions

This is the first report of AM fungal diversity analysis of red mud flooded site and fly ash pond (by molecular techniques). The plants growing in both red mud flooded site and fly ash pond had shown very poor AM fungal colonization and spore numbers in rhizosphere soils. Isolated AM fungal species were identified and which most were of belonged to genus *Glomus.*

Amelioration of red mud with different amendments such as fly ash, gypsum, sludge and top soil improved the properties of red mud which results improvement of the growth of bermudagrass especially in gypsum and sludge amendments. Inoculation of different microbial treatments such as AM fungi, *A. tubingensis*, bacterial consortia and its combination further significantly increased the growth of bermudagrass in all treatments than their control treatments. However, at the end of the experiment, the higher growth of bermudagrass was observed in all consortium inoculated treatments and it was highest in inoculation of gypsum with consortium followed by AM fungi. Inoculation of consortium followed by AM fungi decreased the accumulation Al and Fe to shoots of bermudagrass in gypsum amended treatments.

Application of different amendments improved the chemical properties of red mud. The maximum pH reduction was found in gypsum amendment of red mud. Red mud amended with gypsum and consortium treatments rhizosphere soil had shown maximal organic carbon and total N. The available P was highest in gypsum amended consortium inoculated treatment. Increased growth may be due to better uptake of nutrients and production of growth promoting substances in the rhizosphere especially in gypsum and sludge amended treatments.

Overall, the present study showed that consortium or red mud adapted AM fungi played an important role in bermudagrass growth, increasing the nutrient status reducing the metal transfer into shoot and improved soil properties. The results of this red mud micro-field study suggest that the combination of AM fungi, *A. tubingensis* and bacterial consortia together or red mud adapted AM fungi alone with gypsum or sludge amendments increased the growth of bermudagrass than the other amendments or individual treatments. Thus, the sustainable bermudagrass establishment on different amendments of red mud with microbial inoculations is direct evidence in development of functional soil, which included reduced pH, increased organic carbon, available P, total N and establishment of active microbial community. These preliminary micro-field experimental results suggest that the rehabilitation of red mud can be achieved by combination of microbes with gypsum or sludge amendments. These results also give a support to further establishment of revegetation on red mud disposal ponds by chemical amended microbial inoculations. To best of our knowledge it the first report of amelioration of red mud using different biological means in micro-field trial.

Vegetation establishment on fly ash produced from the coal combustion process is a beneficial part of their environmental management. The effectiveness of fly ash adapted arbuscular mycorrhizal (AM) fungi and phosphate solubilizing fungus *Aspergillus tubingensis* alone and combination on the growth, nutrient and metal uptake of *Dendrocalamus strictus (Roxb.) Nees.*, (bamboo) grown in fly ash was studied. Co-inoculation of these fungi significantly increased the height, number of branches, nutrient uptake (P, K, Ca, Mg and Na) and reduced the metal (Al, Fe and

Cu) concentration in shoot tissues compared to their individual treatments after 6 and 12 months. The chemical and biochemical properties of fly ash were improved compared to those of individual inoculation and control. The results reveal that combination of fly ash adapted AM fungi and *A. tubingensis* elicited a synergetic effect which results increased plant growth and uptake of nutrients with reducing metal translocation. Overall, this study suggests that dual inoculum of mycorrhizal fungi and phosphate solubilizing fungi contributes in sustainable maintenance of plant health and chemical and biological properties of fly ash.

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Appendix I

Alkaline nutrient agar

Ingredients	Quantity (g/L)
Casein Hydrosylates	15.0
Peptone	5.0
NaCl	5.0
Agar	20.0

pH adjusted with 10N NaOH to 10.5 before autoclaved at 15 lbs pressure (121 °C) for 15 min.

Alkaline tryptic soya agar

Ingredients	Quantity (g/L)
Casein enzyme hydrosylates	17.0
Papaic digest of soyabean meal	3.0
Sodium chloride	5.0
Dipotassium phosphate	2.5
Dextrose	2.5
Agar	20.0

pH adjusted with 10N NaOH to 10.5 before autoclaved at 15 lbs pressure (121 °C) for 15 min.

Alkaliphilic Horikoshi agar medium

Ingredients	Quantity (g/L)
Sucrose	10.0
Yeast extract	5.5
Polypeptone	5.5
K_2HPO_4	1.1
$Mg_2SO_4.7H_2O$	0.2
Na_2CO_3	10.0
Agar	20.0

Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Pikovskaya's agar

Ingredients	Quantity (g/L)
Agar	15.0
Ammonium sulphate	0.5
Calcium phosphate	5.0
Dextrose	10.0
Ferrous sulphate	0.0001
Magnesium sulphate	0.1
Manganese sulphate	0.0001
Potassium chloride	0.2
Yeast extract	0.5

Suspended 31.3 g in 1000 ml distilled water. Boiled to dissolve the medium completely and sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Luria-Bertani (LB) medium

Ingredients	**Quantity (g/L)**
Nacl	10.0
Beef extract	5.0
Tryptone	10.0
Agar	10.0

pH 7 adjusted with 5N NaOH and sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min. (Added filter sterilized ampicillin 50 µg/ml to prepare LB - Ampicillin plates).

LB/amp+ agar plates

Prepared LB broth as above. Added agar (18 g/L), autoclaved, and cool to 50 °C. To this, added 50 µg/ml ampicillin and poured plates.

IPTG stock solution (0.1M)

To 1.2 g IPTG, added water to 50 ml final volume. Filter sterilized and store at 4°C.

X-Gal (2ml)

100 mg of 5-bromo-4-chloro-3-indolyl-D-galactoside dissolved in 2 ml N, N´-dimethylformamide. Covered with aluminum foil and stored at 20 °C.

LB plates with ampicillin/IPTG/X-Gal

Made the LB plates with ampicillin as above; 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal was spread over the surface of an LB ampicillin plates and allowed to absorb for 30 minutes at 37 °C prior to use.

Melzer's reagent

Potassium iodide	2.50–3.75%
Iodine	0.75–1.25%

Remained of the solution being 50% water and 50% chloral hydrate.

Modified Universal buffer (5x)

Tris (hydroxyl methyl) amino methane	3.025 g
Maleic acid	2.90 g
Citric acid	3.50 g
Boric acid	1.57 g
NaOH (1N)	122 ml
Water	up to 250 ml
pH	5.5

Phosphate buffer

Stock solution A
2 M monobasic sodium phosphate, monohydrate (276 g/L)
Stock solution B
2 M dibasic sodium phosphate (284 g/L).

Mixed an appropriate volume (ml) of A and B as shown in the table below and diluted to a total volume of 200 ml, a 1 M phosphate buffer of the required pH at room temperature.

A (ml)	**B (ml)**	**pH**
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	84.0	7.5

Carbonate and bicarbonate buffer

Stock solution A
0.l M Sodium carbonate. 10.599 g anhydrous Na_2CO_3 were dissolved in water and diluted to 1 L.
Stock solution B
0.l M Sodium bicarbonate. 8.4 g $NaHCO_3$ were dissolved in water and diluted to 1 L.
Solution A and B was mixed in following way:

A (ml)	**B (ml)**	**pH**
5	5	9.90
6	4	10.14
7	3	10.28
8	2	10.53
9	1	10.83

TBE buffer (10x)

Tris-HCl	0.09 M (pH 8)
Boric acid	0.9 M
EDTA	0.02 M (pH 8)

Trypan blue solution

Trypan blue	0.5 g
Glycerol	500 ml
H20	450 ml
Hcl 1%	50 ml

PVLG mounting solution: Polyvinylâlcohol, high viscosity (24-32 cP), 1.66 g; H_2O, 1 0 ml; lactic acid, 10 ml; glycerol, 1.0 ml. Dissolved PVA in water; stir vigorously while adding lactic acid and glycerol; gentle heating rnay be necessary.

1 N $K_2Cr_2O_7$: 49.04 g of potassium dichromate per liter of solution.

Olsen's P-extracting solution

0.5 M $NaHCO_3$ extracting solution: 84 g of sodium bicarbonate was added in distilled water and the volume was made up to 2L. The pH was adjusted to 9 with 1M or 1N NaOH.

Bray's P-1 extracting solution
Dissolved 1.110 g of AR grade ammonium fluoride in one liter of 0.025N HCl.

Molybdate-tartarate - Reagent A
i) Dissolved 12 g of ammonium molybdate in about 250 ml distilled water.
ii) Dissolved 0.291 g of antimony potassium tartarate in 100 ml of distilled water. Added the two solutions to 1L of 5N H_2SO_4. Mixed thoroughly and made the volume to 2L with distilled water.

Reagent B (freshly prepared)
1.058g of ascorbic acid dissolved in 200 ml of reagent A.

Sulphuric acid (2.5 M): 140 ml of concentrated H_2SO_4 diluted to 1L.

Plasmid extraction solution I (10x)

Tris-HCl	25 mM (pH 8.0)
Glucose	50 mM
Na_2EDTA	10mM

Plasmid extraction solution II

NaOH	5M
SDS	10%

Plasmid extraction solution III

5.0 M K-acetate (pH 4.5)

Agarose gel loading dye (6x)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.0%

Ligation reaction of amplicon in pTZ57R/T

Plasmid pTZ57R/T (55ng/µl)	3 µl
Amplicon (75ng/µl)	x µl
Buffer (10x)	3 µl
T4 Ligase	1 µl
H_2O	x µl
Total volume 15 µl	

Primers

M13 forward primer	5''-GTAAAACGACGGCCAGT-3'
M13 reverse primer	5'-CAGGAAACAGCTATGAC-3'

Appendix II

Uncultured Glomus clone RMC3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
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2011
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 AUTHORS   Babu,G.A. and Reddy,S.M.
 TITLE     Diverity of arbuscular mycorrhizal fungi associated with plants
           growing on red mud flooded sites adjoining red mud pond and their
           role on the growth of bermuda grass grown in red mud
 JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 957)
 AUTHORS   Babu,G.A. and Reddy,S.M.
 TITLE     Direct Submission
 JOURNAL   Submitted (21-JAN-2011) Department of Biotechnology,Thapar
           University, M. Sudhakara Reddy, Bhadson Road, Patiala, Punjab
           147004, India
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Uncultured Glomus clone RMP20 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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REFERENCE   1  (bases 1 to 964)
 AUTHORS   Babu,G.A. and Reddy,S.M.
 TITLE     Diverity of arbuscular mycorrhizal fungi associated with plants
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           role on the growth of bermuda grass grown in red mud
 JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 964)
 AUTHORS   Babu,G.A. and Reddy,S.M.
 TITLE     Direct Submission
 JOURNAL   Submitted (21-JAN-2011) Department of Biotechnology,Thapar
           University, M. Sudhakara Reddy, Bhadson Road, Patiala, Punjab
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Uncultured Glomus clone RMP23 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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 TITLE     Diverity of arbuscular mycorrhizal fungi associated with plants
           growing on red mud flooded sites adjoining red mud pond and their
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 JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 993)
 AUTHORS   Babu,G.A. and Reddy,S.M.
 TITLE     Direct Submission
 JOURNAL   Submitted (21-JAN-2011) Department of Biotechnology,Thapar
           University, M. Sudhakara Reddy, Bhadson Road, Patiala, Punjab
           147004, India
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Uncultured Glomus clone RM32 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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 TITLE     Diverity of arbuscular mycorrhizal fungi associated with plants
           growing on red mud flooded sites adjoining red mud pond and their
           role on the growth of bermuda grass grown in red mud
 JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 975)
 AUTHORS   Babu,G.A. and Reddy,S.M.
 TITLE     Direct Submission
 JOURNAL   Submitted (21-JAN-2011) Department of Biotechnology,Thapar
           University, M. Sudhakara Reddy, Bhadson Road, Patiala, Punjab
           147004, India
FEATURES             Location/Qualifiers
    source          1..975
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                    /db_xref="taxon:231055"
                    /clone="RM32"
                    /country="India: NALCO alumina refinery, Damanjodi,
                    Orissa"
                    /lat_lon="18.7739 N 82.8936 E"
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    misc_feature    331..571
                    /product="internal transcribed spacer 1"
    rRNA            572..731
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    misc_feature    732..917
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    rRNA            918..>975
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    121 ctacactgat gaagtcatcg agttcatttc ctttatcgga agatatgggt aatcttttga
    181 aacttcatcg tgctggggat agagctttgc aattattgct cttgaacgag gaatccctag
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    361 aaacctgggt tttaaaaagt tggtcaaact tggtcattta gaggaagtat aactcgtaac
    421 aaggtttccg tatgtgaacc tgcgggaagg atcattacca attttagcgt acctaacctt
    481 ttttgggtta tggtctcgcg aaaaaactgt atttaaaacc ccactcttta atataataaa
    541 tgaatctatt tatataataa taaataaaga tcactttcaa caacggatct cttggctctc
    601 gcatcgatga agaacgtagc taagtgcgat aagtaatgtg aattgcagaa ttccgtgaat
    661 catcaaattt ttgaacgcaa attgcacttc ctggcatccc ggggagtatg cctgtttgag
    721 ggtcattata attaaaaatc ggtgtgttgc accggaggtg ggttatcttg atctaatttt
    781 aggttaagag gcttaaaatt gatatctttt gcgcattttt tagacgtaca taaaattttt
    841 ttttattcgc ctattttaat gccaaaatcc tattagatgc gaccttattt tttttatatg
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    961 acttaagcat atcaa
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Uncultured Glomus clone RM44 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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DEFINITION  Uncultured Glomus clone RM44 18S ribosomal RNA gene, partial
            sequence; 5.8S ribosomal RNA gene, complete sequence; and 28S
            ribosomal RNA gene, partial sequence.
ACCESSION   HQ917530
VERSION     HQ917530
KEYWORDS    .
SOURCE      uncultured Glomus
 ORGANISM  uncultured Glomus
           Eukaryota; Fungi; Glomeromycota; Glomeromycetes; Glomerales;
           Glomeraceae; environmental samples.
REFERENCE   1  (bases 1 to 1009)
 AUTHORS   Babu,G.A. and Reddy,S.M.
 TITLE     Diverity of arbuscular mycorrhizal fungi associated with plants
           growing on red mud flooded sites adjoining red mud pond and their
           role on the growth of bermuda grass grown in red mud
 JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1009)
 AUTHORS   Babu,G.A. and Reddy,S.M.
 TITLE     Direct Submission
 JOURNAL   Submitted (21-JAN-2011) Department of Biotechnology,Thapar
           University, M. Sudhakara Reddy, Bhadson Road, Patiala, Punjab
           147004, India
FEATURES             Location/Qualifiers
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    rRNA            586..736
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    misc_feature    737..976
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    rRNA            977..>1009
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    121 ctacactgat gaagtcaacg agtttatatc ctttatcgga agatatgggt aatcttttga
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    241 taagcgtaag tcatcagctt gcgttgatta cgtccctgcc ctttgtacac accgcccgtc
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    421 aaggtttccg taggtgaacc tgcggaagga tcattataaa attttcatat attaaaattc
    481 atttaatata taaaattata tacaaaatgt attcaaaacc cacactcctt ataaccataa
    541 aaaaatttat attattattt ataatataaa aaaaaaaaca actttcaaca acggatctct
    601 tggctctcgc atcgatgaag aacgcagcga attgcgataa gtaatgtgaa ttgcagaatt
    661 acgtgaatca tcgaatcttt gaacgcatat tgcactctct ggtaatccgg ggagtatgcc
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    841 gaccgaatgg agcaattaaa caaattctct cgttaggcgg attctcatca agcaattacg
    901 atttttggc cgactcggca ctttttaaaa gcgcttggcc gagatcgtga gtttttaac
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Uncultured Glomus clone FA8 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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LOCUS       HM159456              993 bp    DNA     linear   ENV 31-AUG-2010
DEFINITION  Uncultured Glomus clone FA8 18S ribosomal RNA gene, partial
            sequence; internal transcribed spacer 1, 5.8S ribosomal RNA
            gene,  and internal transcribed spacer 2, complete sequence; and
            28S ribosomal RNA gene, partial sequence.
ACCESSION   HM159456
VERSION     HM159456.1  GI:304561118
KEYWORDS    ENV.
SOURCE      uncultured Glomus
  ORGANISM  uncultured Glomus
            Eukaryota; Fungi; Glomeromycota; Glomeromycetes; Glomerales;
            Glomeraceae; environmental samples.
REFERENCE   1  (bases 1 to 993)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE      Molecular diversity of arbuscular mycorrhizal fungi associated
with
            plants growing in fly ash pond
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 993)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE     Direct Submission
  JOURNAL      Submitted  (28-APR-2010)  Biotechnology,  Thapar  University,
Bhadson
            Road, Patiala, Punjab 147004, India
FEATURES             Location/Qualifiers
     source          1..993
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                     /clone="FA8"
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     misc_RNA        458..566
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     rRNA            567..723
                     /product="5.8S ribosomal RNA"
     misc_RNA        724..947
                     /product="internal transcribed spacer 2"
     rRNA            948..>993
                     /product="28S ribosomal RNA"
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      121 ctacactgat gaagtcaacg agttcatatc ctttatcgaa agatatgggt aatcttttga
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Uncultured Glomus clone FA14 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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LOCUS       HM159457              979 bp    DNA     linear   ENV 31-AUG-2010
DEFINITION  Uncultured Glomus clone FA14 18S ribosomal RNA gene, partial
            sequence; internal transcribed spacer 1, 5.8S ribosomal RNA
            gene, and internal transcribed spacer 2, complete sequence; and
            28S ribosomal RNA gene, partial sequence.
ACCESSION   HM159457
VERSION     HM159457.1  GI:304561119
KEYWORDS    ENV.
SOURCE      uncultured Glomus
  ORGANISM  uncultured Glomus
            Eukaryota; Fungi; Glomeromycota; Glomeromycetes; Glomerales;
            Glomeraceae; environmental samples.
REFERENCE   1  (bases 1 to 979)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE      Molecular diversity of arbuscular mycorrhizal fungi associated
with
            plants growing in fly ash pond
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 979)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE     Direct Submission
  JOURNAL      Submitted  (28-APR-2010)  Biotechnology,  Thapar  University,
Bhadson
            Road, Patiala, Punjab 147004, India
FEATURES             Location/Qualifiers
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                     /environmental_sample
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     misc_RNA        457..586
                     /product="internal transcribed spacer 1"
     rRNA            587..712
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     misc_RNA        713..894
                     /product="internal transcribed spacer 2"
     rRNA            895..>979
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      121 ctacactgat gaagtcatcg agttcatttc ctttatcgga agatatgggt aatcttttga
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Uncultured Glomus clone FA19 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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LOCUS       HM159458               959 bp    DNA     linear   ENV 31-AUG-2010
DEFINITION  Uncultured Glomus clone FA19 18S ribosomal RNA gene, partial
            sequence; internal transcribed spacer 1, 5.8S ribosomal RNA
            gene, and internal transcribed spacer 2, complete sequence; and
            28S ribosomal RNA gene, partial sequence.
ACCESSION   HM159458
VERSION     HM159458.1  GI:304561120
KEYWORDS    ENV.
SOURCE      uncultured Glomus
  ORGANISM  uncultured Glomus
            Eukaryota; Fungi; Glomeromycota; Glomeromycetes; Glomerales;
            Glomeraceae; environmental samples.
REFERENCE   1  (bases 1 to 959)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE     Molecular diversity of arbuscular mycorrhizal fungi associated
with
            plants growing in fly ash pond
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 959)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (28-APR-2010) Biotechnology, Thapar University,
Bhadson
            Road, Patiala, Punjab 147004, India
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     rRNA            567..717
                     /product="5.8S ribosomal RNA"
     misc_RNA        718..913
                     /product="internal transcribed spacer 2"
     rRNA            914..>959
                     /product="28S ribosomal RNA"
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      241 taagcacaag tcatcagctt gtgatgatta cgtccctgcc ctttgtacac accgcccgtc
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Uncultured Glomus clone FA23 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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DEFINITION  Uncultured Glomus clone FA23 18S ribosomal RNA gene, partial
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            gene, and internal transcribed spacer 2, complete sequence; and
            28S ribosomal RNA gene, partial sequence.
ACCESSION   HM159459
VERSION     HM159459.1  GI:304561121
KEYWORDS    ENV.
SOURCE      uncultured Glomus
  ORGANISM  uncultured Glomus
            Eukaryota; Fungi; Glomeromycota; Glomeromycetes; Glomerales;
            Glomeraceae; environmental samples.
REFERENCE   1  (bases 1 to 974)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE     Molecular diversity of arbuscular mycorrhizal fungi associated
with
            plants growing in fly ash pond
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 974)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (28-APR-2010) Biotechnology, Thapar University,
Bhadson
            Road, Patiala, Punjab 147004, India
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     rRNA            564..720
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     misc_RNA        721..928
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     rRNA            929..>974
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Uncultured Glomus clone FA29 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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DEFINITION  Uncultured Glomus clone FA29 18S ribosomal RNA gene, partial
            sequence; internal transcribed spacer 1, 5.8S ribosomal RNA
            gene, and internal transcribed spacer 2, complete sequence; and
            28S ribosomal RNA gene, partial sequence.
ACCESSION   HM159460
VERSION     HM159460.1  GI:304561122
KEYWORDS    ENV.
SOURCE      uncultured Glomus
  ORGANISM  uncultured Glomus
            Eukaryota; Fungi; Glomeromycota; Glomeromycetes; Glomerales;
            Glomeraceae; environmental samples.
REFERENCE   1  (bases 1 to 974)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE     Molecular diversity of arbuscular mycorrhizal fungi associated
with
            plants growing in fly ash pond
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 974)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (28-APR-2010) Biotechnology, Thapar University,
Bhadson
            Road, Patiala, Punjab 147004, India
FEATURES             Location/Qualifiers
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     rRNA            574..732
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     misc_RNA        733..928
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     rRNA            929..>974
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```

Uncultured Glomus clone FA31 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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LOCUS       HM159461               957 bp    DNA     linear   ENV 31-AUG-2010
DEFINITION  Uncultured Glomus clone FA31 18S ribosomal RNA gene, partial
            sequence; internal transcribed spacer 1, 5.8S ribosomal RNA
            gene, and internal transcribed spacer 2, complete sequence; and
            28Sribosomal RNA gene, partial sequence.
ACCESSION   HM159461
VERSION     HM159461.1  GI:304561123
KEYWORDS    ENV.
SOURCE      uncultured Glomus
  ORGANISM  uncultured Glomus
            Eukaryota; Fungi; Glomeromycota; Glomeromycetes; Glomerales;
            Glomeraceae; environmental samples.
REFERENCE   1  (bases 1 to 957)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE      Molecular diversity of arbuscular mycorrhizal fungi associated
with
            plants growing in fly ash pond
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 957)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE     Direct Submission
  JOURNAL      Submitted  (28-APR-2010)  Biotechnology,  Thapar  University,
Bhadson
            Road, Patiala, Punjab 147004, India
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /isolation_source="plant growing in fly ash pond"
                     /db_xref="taxon:231055"
                     /clone="FA31"
                     /environmental_sample
     rRNA            <1..456
                     /product="18S ribosomal RNA"
     misc_RNA        457..560
                     /product="internal transcribed spacer 1"
     rRNA            561..715
                     /product="5.8S ribosomal RNA"
     misc_RNA        716..911
                     /product="internal transcribed spacer 2"
     rRNA            912..>957
                     /product="28S ribosomal RNA"
ORIGIN
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      61 tggaagtttg aggcaataac aggtctgtga tgcccttaga tgttctgggc cgcacgcgcg
     121 ctacactgat gaagtcaacg agtttatatc ctttatcgga agatatgggt aatcttttga
     181 aacttcatcg tgctggggat agagcattgc aattattgct cttaaacgag gaatccctag
     241 taagcacaag tcaccagctt gtgctgatta cgtccctgcc ctttgtacac accgcccgtc
     301 gctactaccg attgaatggc ttagtgaggc cctcggatcg atattcatga actggcaaca
     361 gtttttgttt gttgagaagt tggtcaaact tggtcattta gaggaagtaa aagtcgtaac
     421 aaggtttccg taggtgaacc tgcggaagga tcattaaaaa tatagcaaac caagcgttag
     481 cgaggttttg cgatcaaaat tgtatttaaa acccaattct ttgaatcaaa tatattatgt
     541 attttaataa ataaaagatc actttcaaca acggatctct tggctctcgc atcgatgaag
     601 aacgtagcga agtgcgataa gtaatgtgaa ttgcagaatt ccgtgaatca tcgaatcttt
     661 gaacgcaaat tgcactttct ggtattccgg agagtatgcc tgtttgaggg tcagtgtaat
     721 aaataaatcg tggttgttac caccttcgt ggtgacgctt cggaattgag tcgtcttatc
     781 ttcggttaag tgacttaaaa tttttatacg atttcgaaac gtatttaatg tattatgtac
     841 gttcacgaat atcgaatgct tattaggtgc ggtcattttc atgaattcgc gtctataatt
     901 ttttttttt ttgacctcaa atcaggtaag agtacccgct gaacttaagc atatcaa
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```

Uncultured Archaeospora clone FArc1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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LOCUS       HM159462               981 bp    DNA     linear   ENV 31-AUG-2010
DEFINITION  Uncultured Archaeospora clone FArc1 18S ribosomal RNA gene,
            partial  sequence; internal transcribed spacer 1, 5.8S ribosomal
            RNA gene, and internal transcribed spacer 2, complete sequence;
            and 28Sribosomal RNA gene, partial sequence.
ACCESSION   HM159462
VERSION     HM159462.1  GI:304561124
KEYWORDS    ENV.
SOURCE      uncultured Archaeospora
  ORGANISM  uncultured Archaeospora
            Eukaryota;      Fungi;      Glomeromycota;      Glomeromycetes;
Archaeosporales;
            Archaeosporaceae; environmental samples.
REFERENCE   1  (bases 1 to 981)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE      Molecular diversity of arbuscular mycorrhizal fungi associated
with
            plants growing in fly ash pond
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 981)
  AUTHORS   Reddy,S.M. and Babau,G.A.
  TITLE     Direct Submission
  JOURNAL      Submitted  (28-APR-2010)  Biotechnology,  Thapar  University,
Bhadson
            Road, Patiala, Punjab 147004, India
FEATURES             Location/Qualifiers
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                     /clone="FArc1"
                     /environmental_sample
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     misc_RNA        454..580
                     /product="internal transcribed spacer 1"
     rRNA            581..736
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     misc_RNA        737..922
                     /product="internal transcribed spacer 2"
     rRNA            923..>981
                     /product="28S ribosomal RNA"
ORIGIN
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       61 gaagtttgag gcaataacag gtctgtgatg cccttagatg ttctgggccg cacgcgcgct
      121 acactgacag agccaacgag ttcttttcct tggccggaag gtctgggtaa tcttgttaaa
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      361 caccttgagc cgggaagttc gacaaactcg gtcatttaga ggaagtaaaa gtcgtaacaa
      421 ggtttccgta ggtgaacctg cggaaggatc attaaaagag ggtcgtaaga cctattttta
      481 aaacctacca ccacttatca tctatgggtg agatggaggg ggtatggttt tttaaaatcc
      541 aattattaca aaatatatcg tctaacgacg aaacaaaaaa atatctaaaa actttcaacg
      601 atggatctct tggctctcgc atcgatgaag aacgcagcga attgcgataa gtagtatgaa
      661 ttgcaaattc tgtgaatcat cgaatctttg aacgtatatt gcgctctctg gtattccgga
      721 gagcatgcct gtttgagggt aattaattaa atctttgacc ttttttggtc aagggatatg
      781 ggcattccgt gtttagagcg gtggcctaaa atgtatggca tggtcaaaac aaatattttt
      841 tgacattacc tcaaatcagg taggatagtc tcatcaagtt gagatgtgta ataatattta
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      961 cgctgaactt aagcatatca a
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