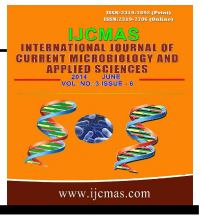
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 6 (2014) pp. 527-539**
http://www.ijcmas.com



Original Research Article

Isolation and identification of some Arbuscular Mycorrhiza (AM) fungi for phytoremediation in soil contaminated with paper mill effluent

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K e y w o r d s

Arbuscular Mycorrhiza, Trace metals, Phytoremediation, *Glomus*, Paper mill effluents.

A B S T R A C T

Arbuscular mycorrhizal fungi (AM) are known to enhance plant tolerance to a variety of stresses including nutrients,drought,metal toxicity,salinity and pathogens all of which may affect plants success in a contaminated or polluted soil. A study was undertaken to access the influence of paper mill effluents on mycorrhizal colonization and mycorrhizal spore count and regression analyses revealed that the mycorrhizal colonization and mycorrhizal spore count are significantly and positively correlated with various physio-chemical properties in the polluted soil. *Glomus* was the most dominant isolated mycorrhizal genus of which three dominant species *Glomus fasciculatum Glomus macrocarpum*, *Glomus mosseae* have been identified. The study helps us to develop a protocol by studying the association of arbuscular mycorrhizal fungi in plants growing in poluted soil and the potential use of these AM fungi as a future bioremediation agent for rehabilitation of the polluted site contaminated with various trace metals.

Introduction

Frank (1885) first gave the name Mycorrhiza to describe the essential structure and functioning of the peculiar associations between the roots and ectomycorrhizal fungi. The genera Gigaspora and *Scutellospora* produce only arbuscules and inter and intracellular hyphae, where as *Glomus*, *Entrophosphora*, *Acaulospora* and *Sclerocystis* produce vesicles in addition to arbuscules. Arbuscualr mycorrhizal (AM) fungi are ubiquitous obligate mycobionts forming symbiosis with the terrstrial plant communities (Barea and Jeffries 1995).The mycosymbionts are widespread among both cultivated and wild plants (Harley and Smith,1983).So far more than 170 species of AM fungi have been recoreded and described (http://invam.caf.wvu.edu/) and many more still awaits discovery.The role of mycorrhizae in plant development pertains to mineral nutrition especially the uptake

of phosphate (Moose 1972).This effect has been attributed to (i)an increase in the absorbing surface and the exploitation of a larger soil volume by the extra radical mycelium,(ii) the small hyphal diameter leading to an increased P absorbing surface area and compared to non-mycorrhizal roots,higher P influx rates per surface unit,(iii) the formation of polyphosphates (Poly P) by mycorrhizal fungi and thus low internal P concentrations, and (iv)the production of organic acids and phosphatases,which catalyze the release of P from organic complexes (Bagyaraj,1984;Entry *et al*., 2002., Fomina *et al*., 2005).The mycorrhizal infection can also increase the uptake of Pb and Mn from soil solutions conataining low concentrations of these metals (Heggo *et al*.1990, Malcova and Gryndler,2003).

AM fungi can increase the rate of plant survival, reduce plant stress and increase plant nutrients acquisition, increase carbon and nitrogen deposition into soil (Almas *et al*., 2004). The metal tolerant AM isolates decrease the metal absorption capacity and filter the intake of metal ions in plants (Martina and Vosatka 2005). The various metal tolerant mycorrhizal fungi have which are found to be evolved as a trace metal-tolerance and thus that they may play important role in the phytoremediation of the site (Liao *et al*., 2003; Gohre and Paszkowski, 2006; Orłowska *et al*., 2011; Zarei *et al*., 2010).

In recent years several studies have shown the harmful effects of metals on microbial diversity and activity in soil (Citterio *et al*., 2005 and Glassman and Casper 2012).High concentrations of trace metals in soil have an adverse effect on microorganisms.The accumulation of metals in soils at high concentrtaions can be due to anthropogenic activities such as application of sewage sludge.Addition of sludge increases the amount of trace metals in soil considerably,causing changes in soil properties which could be toxic to both plants and micrrorganisms.Mycorrhizal fungi that provide a direct link between soil and roots may be of great importance to plants growing in soils contaminated with trace metals (Leyval *et al*.,1997).

AM are known to enhance plant tolerance to a variety of stresses including nutrients,drought,metal toxicity,salinity and pathogens all of which may affect plants success in a contaminated or polluted soil (Olexa *et al.,* 2000).*Glomus* was found to be most dominnat trace metal tolerant AM fungi isolated from Contaminated sludegea s reported earlier by various workers (Joner,2003,Malcova, 2003).

The pulp and paper mill which has been categorized as one of the twenty most polluting industries in India discharge huge quantities of coloured and waste water (effluent) into the environment and are responsible for soil pollution consequently the hazardous chemicals enter into surface or ground water and poison the soil or crops.There has been continuous decrease of plant diversity due to trace metal soil toxicity by dumping of paper mill effluents through the neighbouring area. Thus,it is very much essential to develop the restoration protocol in the contaminated habitats by use of benificial rhizosphere fungi like AM fungi that are tolerant to various stresses as they are able to decrease the trace metal absorption capacity and thus considerable increase soil nutrients to the host plant.

Materials and Methods

Location of the study area

The study was conducted at the polluted site inside the campus of Hindustan Paper Corporation Limited, HPC,Assam,India where the solid sludge and effluent have been dumped. The study area was located at an altitude of 116mMSL between 24052`N and 92036`E longitides.

Collection of soil Sample

From the polluted soil,the rhizospheric soil samples were randomly selected and then mixed together to obtain a composoil representative sample.The soil samplings were done from Januar 2012 to October 2013 in three seasons,i.e., winter (November to Februar),summer (March to June) and Monsoon or rainy (July to October).The soil samples were brought to the laboratory in sterile condition and stored in a refrigerator at 4^0C until they were processed.

Collection of root samples

Fine roots from plants of the same species were randomly collected and mixed properly and a composoil root sample was obtained. Trypan blue method was followed for the determination of the intensity of root colonization as described by Phillips and Hayman (1970).

Isolation of Mycorrhizal spores

Spore extraction from the soil was carried out using the Wet Sieving and Decanting Technique by Gerdemann and Nicolson (1963).The isolated spores were mounted on glass slide using Polyvinyl Alcohol-Lactic acid Glycerol (PVLG) and observed under compound microscope (100-1000X).Spores were identified according to the manual of identification of VAM fungi by Schenek and Perez (1990).The INVAM worksheet was used for diagnosing the spores.Additional spores not included in the manual were identified as per the description given in the INVAM websoil (http://invam.caf.wvu.edu/).

Soil Physico-chemical analysis

The physical chracteristics of soil i.e., Moisture content,soil pH and soil temperature were estimated for the collected polluted soil samples.

The chemical chracteristic i.e., N,P,K,Organic C,Mg,Ca etc of the polluted soil samples were estimated using the technique of Jackson (1985).Concentration of trace metalss i.e.,Cu, Ni and Zn were determined by Atomic Absorption Spectrophotometer (VARIAN Spectra AA 220).

Results and Discussion

The phsio-chemical properties of soil were estimated and the maximum moisture content, soil pH, and soil temperature were observed in the rainy seasons followed by summer and winter.The pH was found to be more alkaline in winter seasons in comparison to summer seasons.(Table 1).The all the chemical constituents soil N,K,Organic C (%), Copper (Cu) Nickel (Ni) and Zinc (Zn) were estimated maximum in rainy seasons followed by summer and winter seasons except soil Phosphorus(P),Magnesium(mg) and Calcium (Ca) recorded high values in winter followed by summer and rainy saesons (Table 2).The maximum number of mycorrhizal spore count and percentage of root infection were observed in the rainy seasons followed by summer.In winter seasons, lees number of mycorrhizal spore count and low percentage of root infection were reported (Table 3).

Liner regression analyses were calculated to find out the influence of various edaphic factors on mycorrhizal colonization and mycorrhizal spore population. The results of regression analysis showed a positive and significant correlation coefficient (R) values between mycorrhizal spore population with soil moisture content ($r = 0.99$; $P < 0.01$; Fig.1(a)),soil temperature ($r = 0.96$; $P < 0.01$; Fig. 1(c)),Nitrogen ($r = 0.81$;$P < 0.01$;Fig. 1(d));potassium ($r = 0.88$; $P < 0.01$; Fig. 1(f));Organic carbon ($r = 0.95$; $P < 0.01$; Fig. 1(g)); Copper ($r = 0.97$;$P < 0.01$; Fig. 1(j));Zinc ($r = 0.97$;$P < 0.01$; Fig. 1(k)) and Nickel ($r = 0.97$;$P < 0.01$; Fig. 1(l)).

While the regression values were estimated negative and significant in parameters like soil pH ($r = 0.92$; $P < 0.01$; Fig. 1(b)); Phosphorus ($r = 0.87$; $P < 0.01$; Fig. 1(e)); Magnesium ($r = 0.93$; $P < 0.01$; Fig. 1(h)) and Calcium ($r = 0.74$; $P < 0.01$; Fig. 1(i)).

The result of regression analysis showed a positive and significant correlation coefficient (R) values between mycorrhizal colonization with soil moisture content ($r = 0.98$; $P < 0.01$; Fig.2(a)); soil temperature ($r = 0.98$; $P < 0.01$; Fig. 2(c));Nitrogen ($r = 0.84$; $P < 0.01$; Fig. 2(d));potassium ($r = 0.90$; $P < 0.01$; Fig. 2(k));Organic carbon ($r = 0.95$; $P < 0.01$; Fig. 2(g)); Copper ($r = 0.97$; $P < 0.01$; Fig. 2(g));

Zinc ($r = 0.90$; $P < 0.01$; Fig. 2(k)) and Nickel ($r = 0.97$;$P < 0.01$; Fig. 2(l)). While the regression values were estimated negative and significant in parameters like soil pH ($r = 0.93$; $P < 0.01$; Fig. 2(b)); Phosphorus ($r = 0.87$; $P < 0.01$; Fig. 2(e)); Magnesium ($r = 0.93$; $P < 0.01$; Fig. 2(h)) and Calcium ($r = 0.69$; $P < 0.01$; Fig. 2(i)).

The major keys for identification of isolated *Glomus* spores (Walker, 1983; Mukerji, 1996)

The genus includes both sporocarpic and non-sporocarpic species. The spores are formed at the end of a hypha which may be constricted at the pointof attachment to the spore, have parallel side walls, or become markedly occluded at the point of attachment to the spores. The spore wall can have one to many layers, without ornamentation.

A. *Glomus fasciculatum* (Thaxter sensu Gerd.) Gerd. and Trappe

Chlamydospores formed in loose aggregation in sporocarp; 80-120 μm when globose, smooth or rough from adherent debris. Spore wall is 4.2 μm thick, hyaline to yellow, the thicker walls often minutely perforated with thick inward projections, hyphal walls occluded at maturity (Figure 1(A)).

B. *Glomus macrocarpum* Tul. and Tul.

The spores are red brown to dark brown (honey colored), 120-400 μm in width, subtending hypha not inserted, spore without a plug but may be much occluded by lateral walls, spores borne singly, multilayered with cross channels in walls with lignified in growth from outer side of the wall(Figure 1(B))

C. *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe

Spores rarely filled with hyphae, sporocarp containing 1-10 spores, diameter of subtending hypha at widest part 18-50 μm, outer surface of inner wall is not ornamented; thin, hyaline outer wall may not be obvious.

Table.1 Seasonal variation in the physical properties of polluted soil

Sampling Period (Seasons)	Physical parameters		
	Moisture Content (%)	pH	Soil Temperature (C^0)
Winter,2012	5.4±0.05	6.9±0.03	18.2±0.04
Summer,2012	18.2±0.03	5.8±0.06	22.3±0.02
Rainy,2012	34.2±0.08	5.1±0.05	26.8±0.03
Winter,2013	5.2±0.02	7.1±0.08	17.9±0.04
Summer,2013	16.8±0.05	5.6±0.03	20.8±0.06
Rainy,2013	31.5±0.02	4.9±0.04	27.4±0.05

Data are represented in mean ±SEM

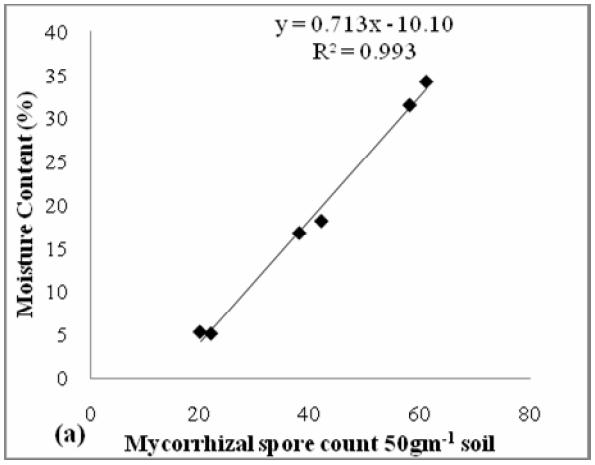
Table.2 Seasonal variation in the chemical properties of polluted soil

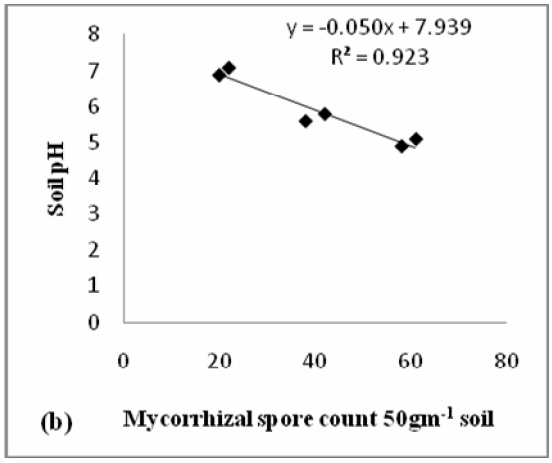
Sampling periods (Seasons)	Chemical parameters								
	N (mg/g)	P (mg/g)	K (mg/g)	Organic C%	Mg (mg/g)	Ca (mg/g)	Cu (ppm)	Ni (ppm)	Zn (ppm)
Winter,2012	.284±0.06	.0045±0.03	.17±0.02	1.08±0.05	2.01±0.06	5.48±0.06	0.18±0.02	0.04±0.05	.242±0.04
Summer,2012	.315±0.03	.0025±0.02	.23±0.04	1.85±0.03	1.34±0.05	4.76±0.03	0.44±0.03	0.24±0.02	.296±0.02
Rainy,2012	.514±0.07	.0018±0.04	.42±0.03	2.31±0.02	1.03±0.04	4.23±0.02	0.78±0.06	0.41±0.03	.362±0.03
Winter,2013	.272±0.02	.0052±0.05	.18±0.05	1.02±0.01	2.13±0.03	4.89±0.04	0.21±0.04	0.07±0.02	.218±0.05
Summer,2013	.288±0.05	.0028±0.03	.26±0.06	1.89±0.05	1.41±0.02	4.32±0.05	0.46±0.03	0.21±0.05	.273±0.04
Rainy,2013	.523±0.08	.0021±0.02	.47±0.04	2.41±0.06	1.08±0.05	4.12±0.04	0.82±0.02	0.38±0.06	.323±0.06

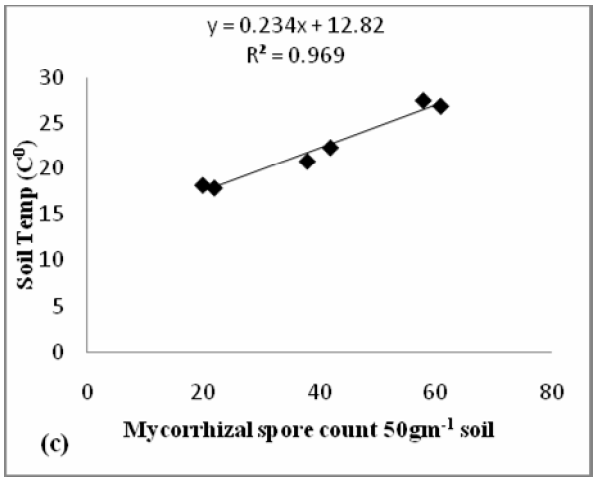
Table.3 Seasonal variation in the Mycorrhizal spore population and Mycorrhizal root colonization (%) in $50gm^{-1}$ soil of polluted soil

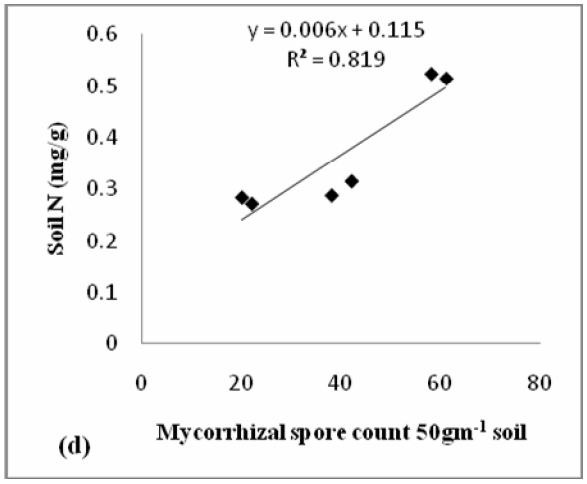
Sampling periods (Seasons)	Mycorrhizal Spore Population($50gm^{-1}$)	Mycorrhizal colonization (%)
Winter,2012	20±0.3	17±0.6
Summer,2012	42±0.8	32±0.4
Rainy,2012	61±0.6	44±0.5
Winter,2013	22±0.5	16±0.2
Summer,2013	38±0.2	28±0.3
Rainy,2013	58±0.4	46±0.6

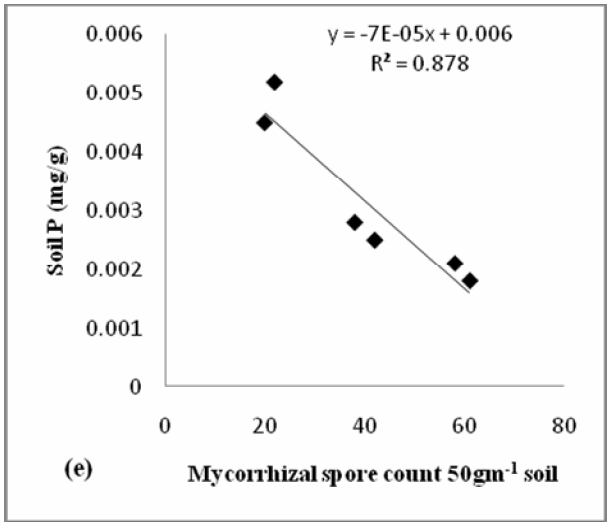
Data are represented in mean ±SEM

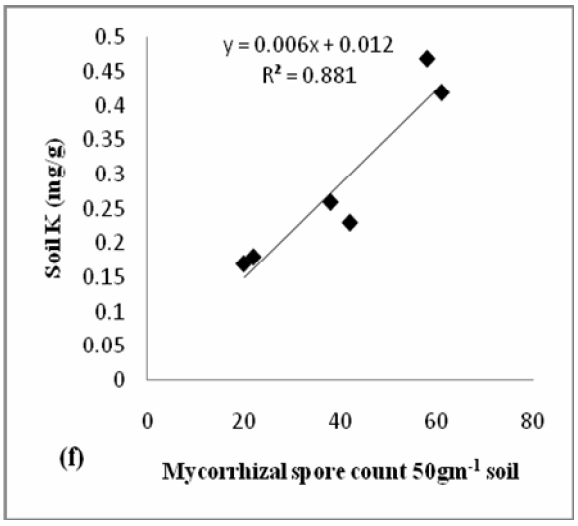

y = 0.713x - 10.10
R² = 0.993
Moisture Content (%)
Mycorrhizal spore count 50gm-1 soil
(a)


y = -0.050x + 7.939
R² = 0.923
Soil pH
Mycorrhizal spore count 50gm-1 soil
(b)


y = 0.234x + 12.82
R² = 0.969
Soil Temp (C0)
Mycorrhizal spore count 50gm-1 soil
(c)


y = 0.006x + 0.115
R² = 0.819
Soil N (mg/g)
Mycorrhizal spore count 50gm-1 soil
(d)


y = -7E-05x + 0.006
R² = 0.878
Soil P (mg/g)
Mycorrhizal spore count 50gm-1 soil
(e)


y = 0.006x + 0.012
R² = 0.881
Soil K (mg/g)
Mycorrhizal spore count 50gm-1 soil
(f)

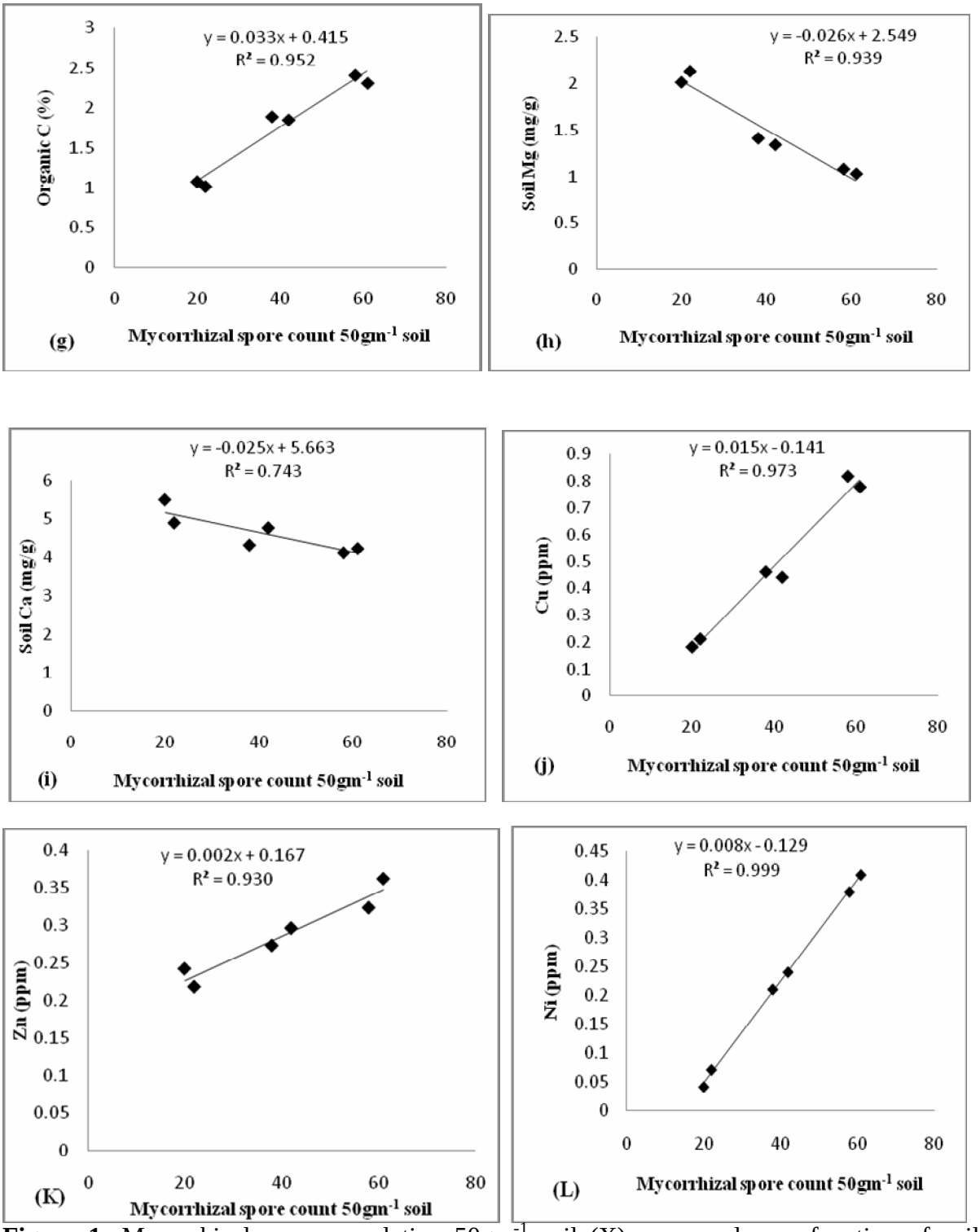
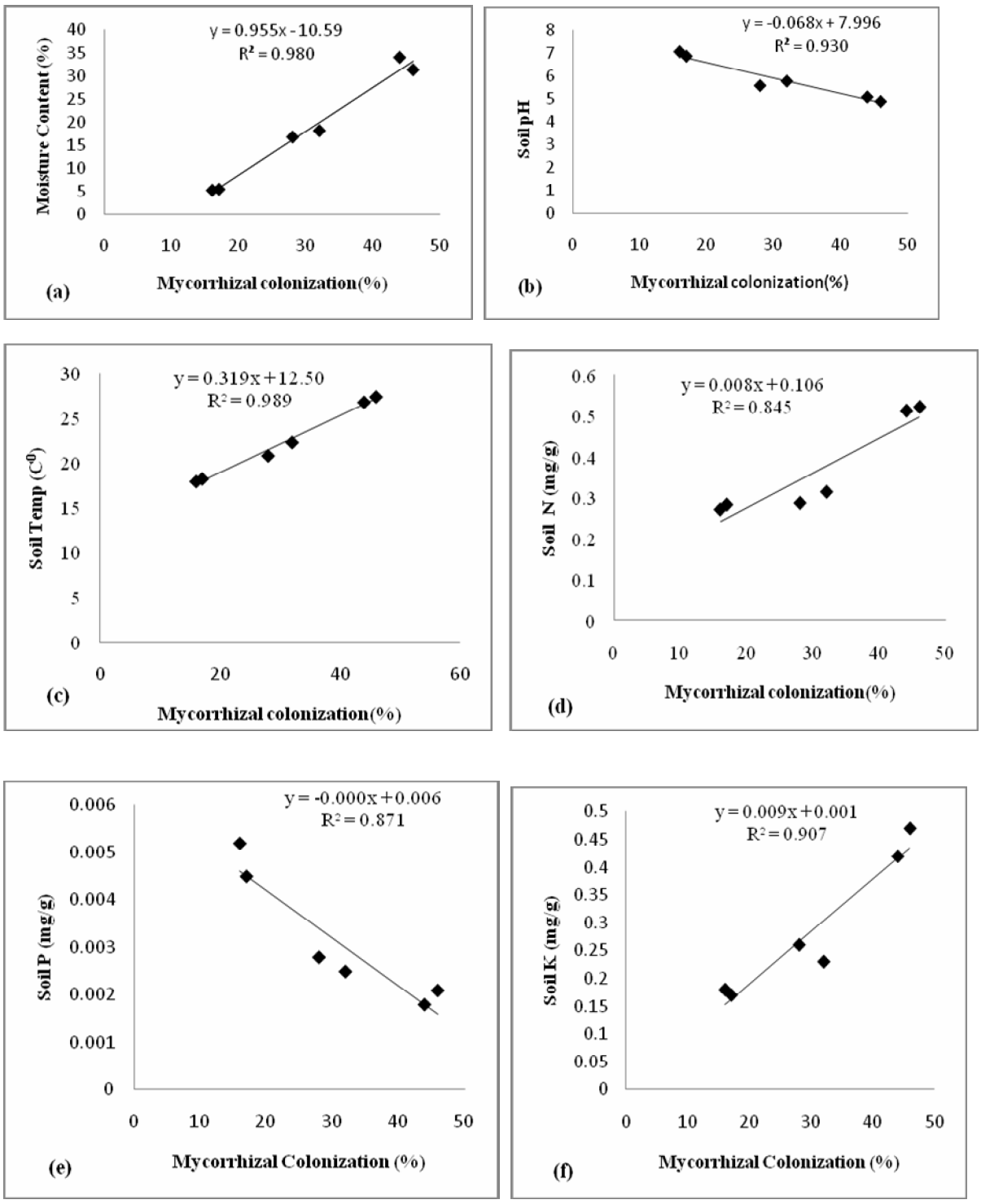


Figure 1: Mycorrhizal spore population $50gm^{-1}$ soil (X) expressed as a function of soil physio-chemical factors (Y) in the polluted soil.Regression is drawn only for statistically significant relationship ($p < 0.01$). (MC=Moisture Content; Soil temp(C^0),soil pH,Nitrogen (N), Potassium (K), Phosphorus (K),Organic Carbon (%),Calcium (Ca),Copper (Cu), Zinc (Zn) and Nickel (Ni)).


y = 0.955x - 10.59
R² = 0.980
Moisture Content (%)
Mycorrhizal colonization(%)
(a)
y = -0.068x + 7.996
R² = 0.930
Soil pH
Mycorrhizal colonization(%)
(b)
y = 0.319x + 12.50
R² = 0.989
Soil Temp (C⁰)
Mycorrhizal colonization(%)
(c)
y = 0.008x + 0.106
R² = 0.845
Soil N (mg/g)
Mycorrhizal colonization(%)
(d)
y = -0.000x + 0.006
R² = 0.871
Soil P (mg/g)
Mycorrhizal Colonization (%)
(e)
y = 0.009x + 0.001
R² = 0.907
Soil K (mg/g)
Mycorrhizal Colonization (%)
(f)

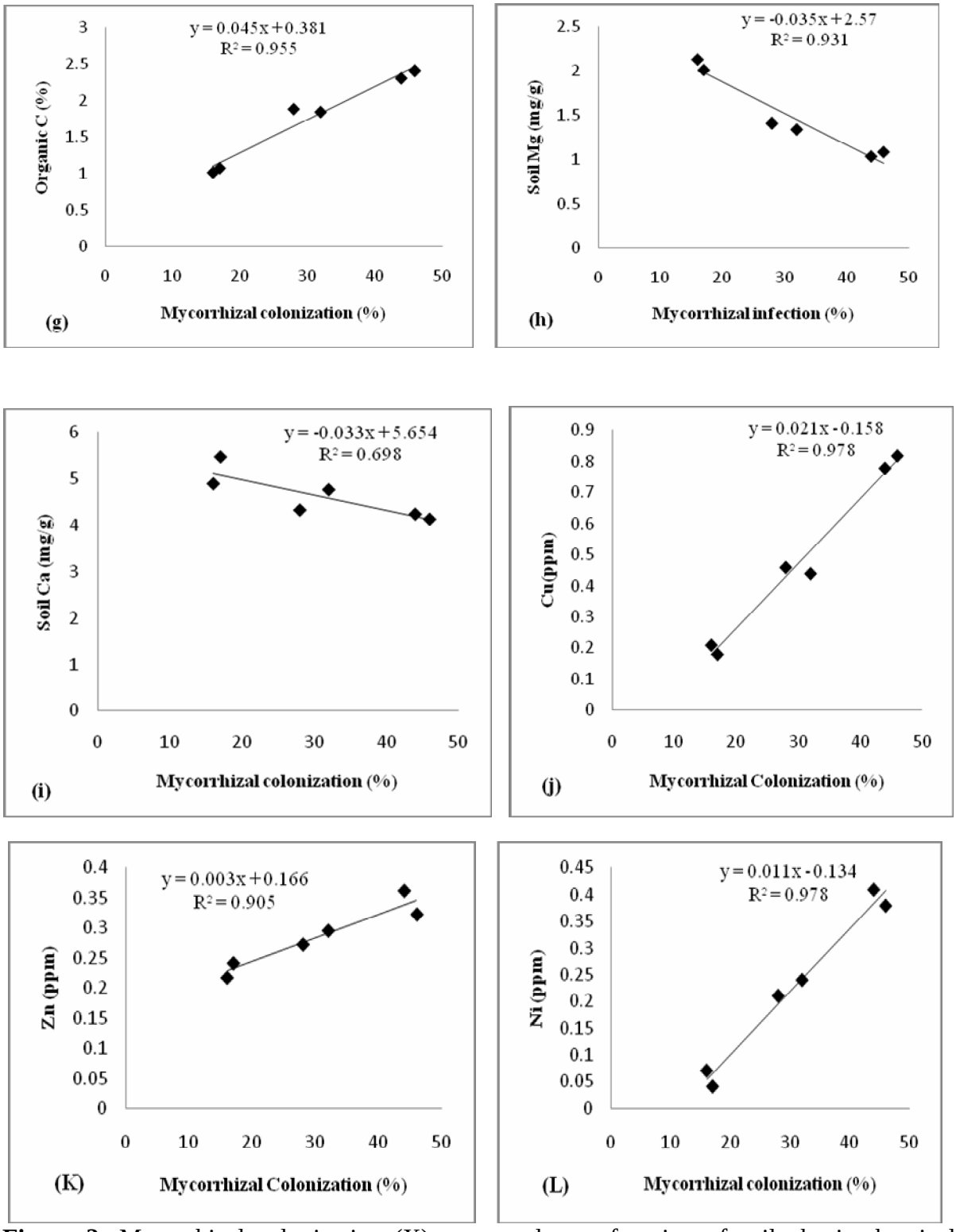
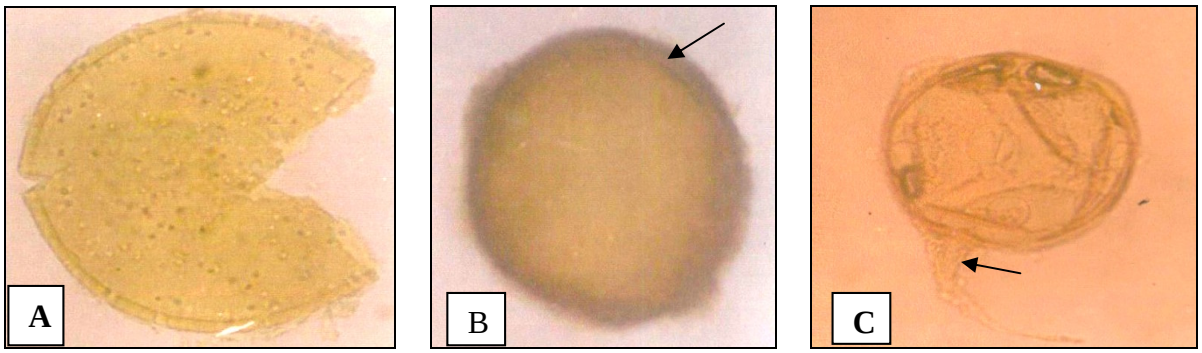


Figure 2: Mycorrhizal colonization (X) expressed as a function of soil physio-chemical factors (Y) in the polluted soil.Regression is drawn only for statistically significant relationship ($p < 0.01$). MC=Moisture Content; Soil temp(C^0),Nitrogen (N), Phosphorous (P),Organic Carbon (%),Calcium (Ca),Magnesium (Mg),Copper (Cu),Nickel Zinc (Zn) and Nickel (Ni)).

Figure.3 Some of the major dominant *Glomus* spores (A-C) isolated from polluted soil
A: Single spore of *Glomus fasciculatum* (100X) showing thicke spore wall.
B: Single spore of *Glomus macrocarpum* (100X) showing multilayer spore wall.
C: Single spore of *Glomus mosseae* (100X) showing funnel shaped hyphal attachment.



Spores are more than 100 μm in size, subtending hypha is generally funnel-shaped with cup-shaped septum (Figure 1(C)).

The present experimental findings revealed the relationship of mycorrhizal spore population and mycorrhizal colonization with various physio-chemical properties of soil polluted with trace metals. The positive and significant regression analysis of mycorrhizal spore population and mycorrhizal root infection is observed with the various physio-chemical properties of soil. The presence of trace metals in the polluted soil may be responsible for less percentage of root colonization in the soil. The high alkalinity, pH and higher soil temperaure in the polluted soil is also responsible for decrease in the number of mycorrhizal spores and root infection Schenck and Smith (1982).

AM spore population decreased with increased amount of trace metals in the soil (Val *et al*., 1999; Hayes *et al*., 2003). The mycorrhizal root colonization of AMF fungi were found decreased by the higher levels of trace metals in the soil. Our results also supports the findings of (Shah *et al.,* (2010); Biro *et al.,* (2005); Mathur *et al.*,(2007)).AM vesicles are arbuscules can accumulate various trace metals and can reduce a series of changes in plant physiology, nutrient availability and microbial composition that may determine the outcome of a phytoremediation attempt in the metal-stressed environment.

Among the isolated genera of AM fungi, *Glomus* was the most dominant AM genus isolated during the present investigation. The dominance of *Glomus* sp in the polluted soil is due to its higher metal tolerance capacity as reported earlier by various workers (Martina and Vosatka 2005; Schwartz *et al*., 2006; Chen *et al.*, 2007; Zaefarian *et al.*, 2010; Carrasco *et al.*, 2011). The various metal tolerant mycorrhizal fungi which are found to be evolved as a trace metal-tolerance and thus they can play a very important role in the phytoremediation of the polluted environment.

The above findings suggest that the higher concentrations of physical and chemical properties of the soil are due to the dumping of solid wastes and effluent by the paper mill. The reported indigenous AM isolates are significantly correlated

with various physic-chemical properties of soil which are able to colonize the plant roots in polluted soil contaminated with various trace metals and higher concentrations of chemical constituents of the soil. So, these AM isolates are adapted to higher concentrations of trace metal and can be used as a combination for inoculation of the plant species growing in the paper mill polluted soil contaminated with trace metals for future bioremediation programmes.

Acknowledgement

The authors are grateful to the Department of Life Science, Microbiology Laboratory, Assam University (Silchar), India for providing the laboratory facilities.

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