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Correlation between organic acid exudation and metal uptake by ectomycorrhizal fungi grown on pond ash in vitro

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Abstract Experiments were conducted to investigate the effect of coal ash on organic acid exudation and subsequent metal uptake by ectomycorrhizal fungi. Four isolates of ectomycorrhizal fungi namely, *Pisolithus tinctorius* (EM-1293 and EM-1299), *Scleroderma verucosum* (EM-1283) and *Scleroderma cepa* (EM-1233) were grown on pond ash moistened with Modified Melin-Norkans medium in vitro. Exudation of formic acid, malic acid and succinic acid by these fungi were detected by HPLC. Mycelial accumulation of Al, As, Cd, Cr, Ni and Pb by these fungi was assayed by atomic absorption spectrophotometer. Relationship between organic acid exudation and metal uptake was determined using classical multivariate linear regression model. Correlation between organic acid exudation and metal uptake could be substantiated when several metals are considered collectively. The finding supports the widespread role of low molecular weight organic acid as a function of tolerance, when exposed to metals in vitro.

Keywords Fly ash · Mycorrhiza · Organic acid · Heavy metal

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Introduction

The environmental and health effects of traditional technologies used in thermal power plants have always been harmful. Coal based thermal power plants generate over 70% of the total electric power in India. Ash content in Indian coal is high (40–50%); therefore the problem is enormous (Ray et al. 2005a, b).

Some of the coal ash produced fuses at the operating temperature (1,150°C) and crystallizes into granules called bottom ash, and the rest of the ash produced along with the flue gases is entrapped into the bag filters of electrostatic precipitators (ESP) known as ESP ash. Ash collected from the hoppers and the bottom of the furnaces is removed in the form of slurry through pipelines and disposed off into pond (allocated land) known as pond ash. This prevailing wet disposal method causes ecological damage. Besides requiring large tracts of land for disposal, air pollution results in the form of fugitive dust emission.

The problem of these fugitive dust emissions can be solved by developing green cover extensively on and around disposal sites that will further prevent contamination of ground water and soil otherwise caused by the leaching of the metals.

Inoculations of forest tree seedlings with ecologically adapted ectomycorrhizal fungi could be the best option for the future. Several ectomycorrhizal fungi can protect their host plants against the toxicity of heavy metals in soil (Colpaert and Vanassche 1993). The role of low molecular weight organic

acids as metal chelators and their role as detoxification agents have been widely discussed (Jones 1998). Of several low molecular weight organic acids released by plant roots, bacteria and fungi, oxalate, citrate and malate are the strongest chelators of the trivalent metals such as Al^{3+} and Fe^{3+} (Jones 1998). Production of organic acids is a well-known phenomenon in ectomycorrhizal fungi. Carboxylic acids such as citric acid and malic acid are potential ligands for heavy metals and so could play a role in tolerance and detoxification (Rauser 1999; Clemens 2001). However a strong evidence for function in tolerance, such as clear correlation between amount of acids produced and exposure to metals has not been produced to support a widespread role. The main objectives of the present study were to look at the influence of pond ash on organic acid exudation and correlate with the subsequent metal accumulation by ectomycorrhizal fungi. We selected six metals namely Al, As, Cd, Cr, Ni and Pb based on their relative abundance in the ash and their potential toxicity. Accumulation of these six metals in the mycelia of these fungi was assayed by atomic absorption spectrophotometer. Exudation of three organic acids namely formic acid, malic acid and succinic acid by the selected ectomycorrhizal fungi were investigated by high performance liquid chromatography (HPLC). Finally, relationship between organic acid exudation and metal uptake was determined using classical multivariate linear regression model, using ordinary least square (OLS) method.

Materials and methods

Selection of isolates

The isolates were procured from the Centre for Mycorrhizal Culture Collection (CMCC), The Energy and Resources Institute (TERI), New Delhi, India. The isolates showing high metal tolerance and metal uptake ability were selected for the present study (Ray et al. 2005a, b). These included *Pisolithus tinctorius* (Mich. Ex Pers) Coker and Couch, (EM-1293) (*Eucalyptus tereticornis*); *P. tinctorius* (EM-1299) (*Eucalyptus tereticornis*), *Scleroderma cepa* Pers., (EM-1233) (*Eucalyptus camaldulensis*); and *Scleroderma verucosum* (Bull.) Pers., (EM-1283) (*Shorea robusta*) isolated from their respective hosts. For the experiment, these isolates were maintained on Modified Melin-Norkrans (MMN) Agar (Marx 1969).

Preparation of ash gradients

Pond ash was collected from Super thermal power station, Korba, Chattisgarh, Central India. The elemental composition of ashes were analyzed and was reported elsewhere (Ray et al. 2005a).The ash was autoclaved under dry conditions (121°C, 1 h) twice at an interval of 24 h. Ash was then poured into tissue culture petridishes at the rate of 15 g (25%), 30 g (50%), and 45 g (75%) w/v respectively containing 60 ml of MMN with 0.3% phytagel (Sigma chemical Co.) as gelling agent. Change of gelling agent instead of agar was done to facilitate easy harvest of fungal colonies by deionising the solid media with sodium citrate buffer (Doner and Becard 1991). Control plates without any ash amendments were also prepared. All the petridishes were prepared in triplicate for all the treatments.

Inoculation

The media impregnated with coal ash were inoculated with three circular mycelial agar disc of 7 mm diameter placed equidistantly from each other on a petridish. The colonies were cut from the edges of active mycelial colony growing on MMN agar. The inoculated plates were incubated in a B.O.D incubator and maintained at 24 ± 1°C in dark till the edge of the adjacent colony touched each other.

Measurement of growth and analysis of metals

After growth, the entire media along with the fungal colony in each of the petridishes were carefully scooped off gently under laminar hood and put in glass jars containing 60 ml of sodium citrate buffer (Doner and Becard 1991). These jars were kept in incubator shaker at 180 rpm at room temperature for over night. This deionization process dissolves the phytagel and subsequently removes ash particles loosely adhered to the mycelial surface (Ray et al. 2005a, b). Following deionization, fungal dry weight was determined after overnight drying in triplicate. Dried fungal colonies were digested with 5 ml of HNO_3 and 1 ml of HF in closed Teflon vessels at 170°C (Kalra et al. 1989), using MARS5 (microwave

accelerated reaction system 5), CEM Corp, for metal analysis. The digested samples were analyzed for Al, As, Cd, Cr, Ni and Pb contents within the mycelia. The analysis was carried out by TJA Solutions AAS, Model SOLAAR M5 Series, graphite furnace equipped with FS 95 auto sampler (TJA Solutions, St Andrews Road, Cambridge, UK).

Analysis of organic acids

Deionized media were first filtered by passing through Millipore Filter paper No. 1, to remove ash particles. The clear filtrate was further filtered by passing through 0.22 μm Millipore filter (Millipore Corporation, BedFord, MA) using Swinnex mini filter assembly (Millipore Corporation, BedFord, MA). Altogether three organic acids (formic acid, malic acid and succinic acid) were detected, separated and quantified using C-18 column, 250 × 4.6 mm, SS Wakosil HG, SGE. The mobile phase constituted of 20 mM phosphoric acid adjusted to pH 2.5 with sodium dihydrogen phosphate. The flow rate of the mobile phase was adjusted to 1 ml/min. The column temperature was set at 30°C. The organic acids were detected at 210 nm using variable wavelength UV detector. HPLC was done in Agilent 1100 Series, Agilent Technologies, Deutschland.

The data obtained in triplicate were subjected to one-way ANOVA (Analysis of Variance) with 1% confidence limit to determine the significant difference in results between different treatments. Relationship between organic acid exudation and metal uptake was determined using classical multivariate linear regression model, using OLS.

Results

Measurement of growth

Growth in terms of colony dry weight of different ectomycorrhizal isolates tested in pond ash in vitro is presented in the Figs. 1–4.

In *S. cepa* (EM-1233) (Fig. 1) optimum growth was recorded at 25% ash concentrations and least at control. Growth decreased with increase in ash concentrations. In *S. verucosum* (EM-1283) (Fig. 2) optimum growth was recorded at 50% and 75% ash

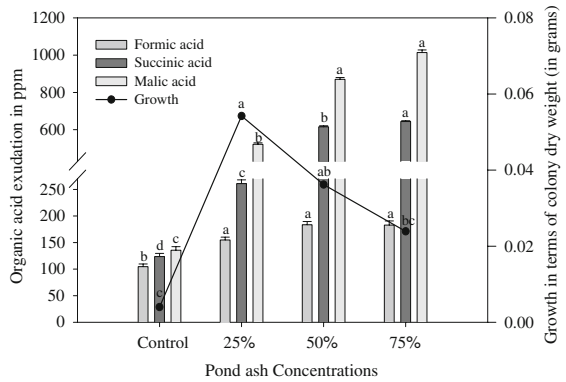

Fig. 1 Organic acid exudation (in ppm) and subsequent growth in terms of colony dry weight (in grams) by *S. cepa* [EM-1233] in vitro. Bars represent acid exudation and lines represent growth. For growth and each acid type, values with different letter indicate significant difference between treatments according to Duncan's multiple range test at $P \leq 0.01$

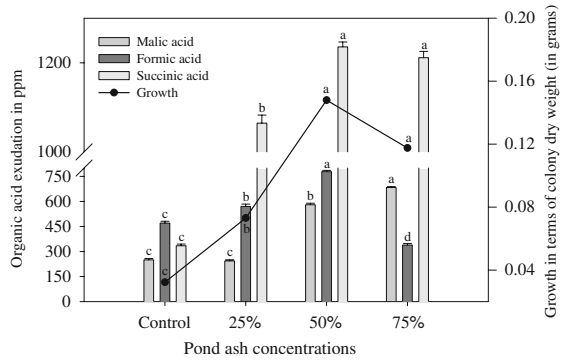

Fig. 2 Organic acid exudation (in ppm) and subsequent growth in terms of colony dry weight (in grams) by *S. verucosum* [EM-1283] in vitro. Bars represent acid exudation and lines represent growth. For growth and each acid type, values with different letter indicate significant difference between treatments according to Duncan's multiple range test at $P \leq 0.01$

concentrations respectively and least at control. In *P. tinctorius* (EM-1299) (Fig. 3) optimum growth was recorded at 75% ash concentrations and least at control and 25% ash concentrations. In this isolate, growth increased with increase in ash concentrations. In *P. tinctorius* (EM-1293) (Fig. 4), optimum growth was recorded at control and 50% ash concentrations and least at 25% and 75% ash concentrations. In this isolate, no linear trend in terms of growth was observed with respect to the treatments.

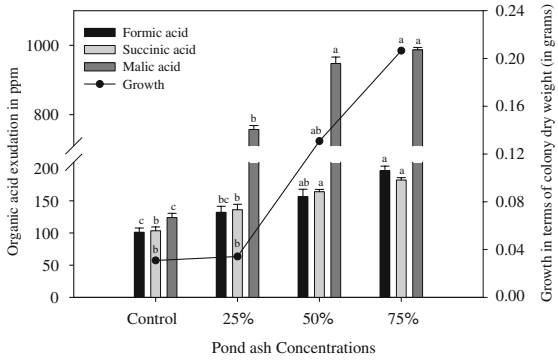

Fig. 3 Organic acid exudation (in ppm) and subsequent growth in terms of colony dry weight (in grams) by *P. tinctorius* [EM-1299] in vitro. Bars represent acid exudation and lines represent growth. For growth and each acid type, values with different letter indicate significant difference between treatments according to Duncan's multiple range test at $P \leq 0.01$

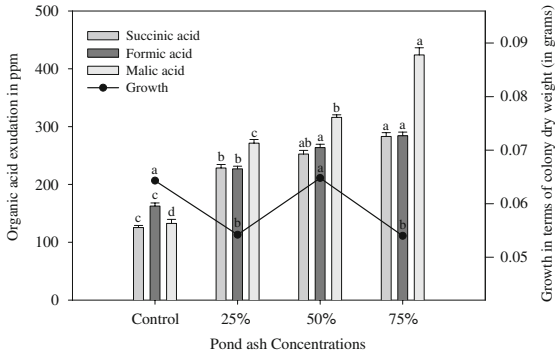

Fig. 4 Organic acid exudation (in ppm) and subsequent growth in terms of colony dry weight (in grams) by *S. verucosum* [EM-1293] in vitro. Bars represent acid exudation and lines represent growth. For growth and each acid type, values with different letter indicate significant difference between treatments according to Duncan's multiple range test at $P \leq 0.01$

Analysis of metal uptake

In general, uptake of all the metals increased with increase in ash amendments in all the isolates. However the increase was not always statistically significant with respect to the increase in ash amendment (Table 1).

Maximum uptake of Al (6246.58 ppm; 75% ash) and Pb (87.48 ppm; 75% ash) was recorded in *P. tinctorius* (EM-1293); As (227.10 ppm; 75% ash), Cd (7.38 ppm; 75% ash) and Cr (43.92 ppm; 75% ash) in *S. cepa* (EM-1233) and that of Ni (16.70 ppm, 75% ash) was recorded in *P. tinctorius* (EM-1299). Minimum uptake of Al (709.53 ppm, 25% ash), As (12.09 ppm; 25% ash) and Cd (0.49 ppm; 25% ash) was recorded in *S. verucosum* (EM-1283), Cr (2.73 ppm; 25% ash) in *P. tinctorius* (EM-1293) and that of Pb (18.57 ppm, 25% ash) and Ni (2.26 ppm, 25% ash) in *S. cepa* (EM-1233) except control.

Organic acid exudation and analysis of estimated regression equation (OLS)

Exudations of three organic acids and subsequent growth of the ectomycorrhizal fungi are presented in the Fig. 1–4. Estimated regression equation between organic acid exudation and metal uptake by different isolates of ectomycorrhizal fungi is presented in the Table 2.

Scleroderma cepa (EM-1233)

Exudation of all the three acids increased with the increase in ash amendments. Increase in formic acid was not statistically significant whereas the increase was highly significant in case of succinic acid and malic acid (Fig. 1).

In *S. cepa* (EM-1233), formic acid was found to have negative correlation to Al uptake. Malic acid was found to have positive correlation with Cr and Cd negative correlation with As when considered simultaneously. No regression equation could be framed between succinic acid exudation and subsequent metal having $\bar{r}^2$ value ≥ 0.50.

Scleroderma verucosum (EM-1283)

Exudation of malic acid and succinic acid increased with increase in ash amendment (Fig. 2). Exudation of formic acid increased up to 50% and then decreased at 75% ash amendment.

In *S. verucosum* (EM-1283), succinic acid exudation was found to have positive correlation with Cr and negative correlation with Cd and Pb. Formic acid exudation was positively correlated to both Al and Cd. When Al, Cr, Cd, Pb and Ni uptake was considered, exudation of malic acid was found to be positively correlated to Al, Cr and Ni and negatively correlated to Pb and Cd.

Table 1 Metal uptake (in ppm) by ectomycorrhizal fungi grown on pond ash in vitro

Isolates	Ash %	Al	As	Pb	Cd	Cr	Ni
Scleroderma cepa [EM-1233]	25	736.03 a	113.25 b	18.57 bc	1.76 b	17.27 b	2.26 b
	50	812.00 a	143.50 b	28.67ab	2.98 b	21.53 b	7.42 a
	75	913.68 a	227.10 a	29.96 a	7.38 a	43.92 a	7.96 a
	Control	328.78 b	24.59 c	8.33 c	0.95 b	4.50 c	0.87 b
LSD (0.01)		268.04	40.64	10.59	3.26	9.34	2.83
Scleroderma verucosum [EM-1283]	25	709.53 a	12.09 b	27.45 b	0.49 b	16.25 a	3.34 bc
	50	841.19 a	17.43 b	76.05 a	0.70 b	16.72 a	6.57 b
	75	932.93 a	49.79 a	76.53 a	1.77 a	19.63 a	12.36 a
	Control	237.59 b	7.29 c	12.68 c	0.33 b	5.21 b	1.54 c
LSD (0.01)		213.38	10.39	8.40	0.70	6.77	3.28
Pisolithus tinctorius [EM-1299]	25	2230.38 a	50.25 a	24.35 b	0.73 b	14.15 c	4.62 b
	50	2422.35 a	56.91 a	31.79 ab	1.71 b	19.04 b	5.70 b
	75	2585.63 a	58.57 a	38.28 a	4.01 a	26.26 a	16.70 a
	Control	398.72 b	13.96 b	12.47 c	0.28 b	3.55 d	1.62 b
L.S.D (0.01)		428.74	21.07	10.44	1.79	4.24	4.36
Pisolithus tinctorius [EM-1293]	25	1107.81 b	22.33 c	47.05 c	2.00 b	13.09 c	4.84 b
	50	1459.16 b	69.89 b	65.54 b	2.22 b	32.17 b	7.68 a
	75	6246.58 a	95.59 a	87.48 a	2.78 a	39.48 a	8.29 a
	Control	498.53 c	14.91 c	9.37 b	0.55 c	7.30 b	1.51 c
L.S.D (0.01)		484.12	8.93	14.78	0.42	4.15	1.44

Values with different letter within a column for each isolate indicate significant difference between treatments according to Duncan's multiple range test at $P \leq 0.01$

Table 2 Estimated regression equation (OLS) between organic acid exudation and metal uptake by different isolates of ectomycorrhizal fungi grown on pond ash in vitro

Isolates	Acid types	Equation	$\bar{r}^2$
Scleroderma cepa [EM-1233]	Formic acid	657.68 − 0.64Al	0.67
	Malic acid	26.39Cr + 95.72Cd − 6.98As + 545.39	0.56
Scleroderma verucosum [EM-1283]	Formic acid	0.29Al + 39.45Cd + 341.16	0.89
	Malic acid	0.64Al + 14.41Cr + 31.54Ni − 9.1Pb − 272.46Cd + 18.23	0.90
	Succinic acid	2.51Cr − 11.04Cd − 1.0Pb + 72.46	0.97
Pisolithus tinctorius [EM-1299]	Formic acid	0.04Al − 0.76As − 41.27Cd − 131.00Pb + 6.75Cr + 62.79	0.81
	Malic acid	0.39Al − 19.04Pb − 17.15Cr + 228.23	0.75
	Succinic acid	0.04Al − 0.84As − 39.19Cd − 1.29Pb + 6.24Cr + 65.33	0.81
Pisolithus tinctorius [EM-1293]	Formic acid	0.004Al + 14.16Cd + 3.56Ni − 0.35As + 40.54	0.98
	Malic acid	0.01Al − 0.74As + 17.46Cd + 8.85Ni + 23.59	0.98
	Succinic acid	0.003Al − 0.27As + 29.79Cd + 25.79	0.97

Estimated coefficients in the regression equations were found to be statistically significant at $P \leq 0.01$

Pisolithus tinctorius (EM-1299)

Exudation of all the three acids increased with increase in ash amendments (Fig. 3). Exudation of malic acid was comparatively higher than other two acids where as increase in succinic acid exudation was statistically very significant with respect to increase in ash amendments.

In *P. tinctorius* (EM-1299), malic acid exudation was found to have positive correlation with Al uptake

and negative correlation with Pb and Cr uptake. Regression estimates showed that when Al, As, Cd, Cr and Pb were considered together, formic acid and succinic acid exudation were positively correlated with Al and Cr and negatively correlated with As, Cd and Pb.

Pisolithus tinctorius (EM-1293)

Exudation of all the three acids increased within ash amendment levels (Fig. 4). Exudation of malic acid was comparatively higher and the increase in exudation was statistically very significant with respect to increase in ash amendments.

Succinic acid was positively correlated with Al and Cd and negatively correlated with As. Formic acid ($\bar{r}^2 = 0.98$) and malic acid ($\bar{r}^2 = 0.98$) exudation was very well explained when Al, As, Cd and Ni uptake was considered simultaneously. Formic acid and malic acid exudation was positively correlated to Cd, Ni and Al and negatively correlated to As.

Discussions

Increasing evidence suggests that exudation of organic acids (Jones 1998) plays a major role in Al tolerance of higher plants. In fungi, metal tolerance in some cases has been linked to extracellular chelation by organic compounds (Gadd 1993). Since mycorrhizal fungi exude a range of organic acids (Lapeyrie et al. 1987) or produce slime capable of binding metals, it is possible that organic compounds released by mycorrhizal fungi are responsible for the amelioration of metal toxicity in mycorrhizal plants. Stimulation of oxalic acid production by Al and Cu in mycorrhizal and non-mycorrhizal roots has been reported, especially with certain ectomycorrhizal fungi (Jonnarth et al. 2000). Jongbloed et al. (1992) found that *Lactarius rufus* Fr. and *Lactarius hepaticus* Plowr. ap Boud. were very sensitive to Al at concentrations as low as 30 mM in solution, whereas *Laccaria bicolor* was comparatively Al tolerant. There are several such other reports on organic acid exudation by ectomycorrhizal fungi when exposed to a predetermined level of different heavy metals individually. But an approach to investigate the effect of a natural substrate like fly ash on organic acid exudation has not been done so far to the best of our knowledge.

In the present study it has been found that, mostly, organic acid exudation increases with increase in ash amendment levels. The regression was done to further correlate organic acid exudation with the individual metal uptake singly and collectively to get the best possible equation. Any consistent relationship of increased exudation at elevated levels of a particular metal concentration was not found. This was in concordance to the review of Hall (2002), who has mentioned that a strong evidence for such function in tolerance, such as clear correlation between amount of acids produced and exposure to metals has not been reported to support a widespread role. Jonnarth et al. (2000), has also reported such inconsistency for several low molecular weight organic acids except oxalic acid. Our results also indicate that organic acid exudation pattern cannot be singly attributed to certain metals when grown on natural substrates like fly ash.

The production of Al-binding organic acids has the potential to reduce the impacts of Al on ion accumulation (Huang et al. 1992). In the present study, Al affected the correlation between organic acid exudation with other accumulated metals. For example, in *P. tinctorius* (EM-1290), Al uptake was positively correlated to all of the organic acid exudation in bottom ash but negatively correlated to the same acids in case of ESP ash. The presence of Al in the equation also substantially changed the relationship of the other metals with respect to the organic acid exudation profile.

Metal uptake varied significantly with ash amendment level for most of the elements and all the isolates. This phenomenon sufficiently explains the exudation pattern of different organic acids with change in the amendment level of coal ash.

Besides it was also evident that organic acid exudation pattern varies differently and significantly with different isolates of ectomycorrhizal fungi.

Jonnarth et al. (2000) found that Ni (17 μM) and Cd (0.44 μM) did not affect the exudation of low molecular weight organic acids in two experiments using *S. variegatus*, *P. involutus* or *R. roseolus* infected and non-mycorrhizal seedlings, but the possibility of organic acid production at higher metal concentrations cannot be excluded. In the present work also, positive correlation equation could be drawn between organic acid exudation and metal uptake in certain cases. Hence it can be said that,

organic acid exudation pattern varies differently with different metal uptake and no uniform correlation between organic acid exudation and metal uptake can be drawn when using natural substrate like fly ash.

On the basis of several regression equations, it can be said that metal uptake ability by ectomycorrhizal fungi is substrate specific and organic acid exudation by ectomycorrhizal fungi is strongly influenced by the metal uptake. Further, in case of natural substrate or natural habitat that contains wide array of different metals, organic acid exudation cannot be singly attributed to any particular metal *per se*. The finding supports the widespread role of low molecular weight organic acid as a function of tolerance, when exposed to metals in vitro. This was the first approach to evaluate statistically the effect of a natural substrate like fly ash on organic acid exudation by ectomycorrhizal fungi. This knowledge can be successfully employed for future field-based exploitation studies using suitable host.

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