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Co-inoculation of *Glomus* and *Pseudomonas* enhances the cadmium stress tolerance in maize plants (*Zea mays*)

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Abstract
Cadmium tolerant Arbuscular Mycorrhizal (AM) fungi *Glomus* sp. (AM_1) and bacteria *Pseudomonas* sp. (PS_1) were isolated from the soil polluted with high concentration of cadmium in Coimbatore district of Tamil Nadu. These isolates along with two standard cultures *G. mosseae* (AM s) and *P. putida* (PS s) in sole and combinations were used for green house experiment in Cd spiked soil at varying levels of Cd (0, 75 and 125 ppm). Observations were recorded in the maize plants on 30 and 60 DAS. The combined inoculation of AM fungi with *Pseudomonas* sp. was found to be effective in enhancing the growth of the plant and tolerance of incremental levels of Cd compared with sole inoculation and uninoculated control. *G. mosseae* (AM s) with *Pseudomonas* sp. (PS 1) inoculated plants registered highest total chlorophyll content (28.9 percent increase over sole inoculation of *G. mosseae* (AM s)). Total phenol content (3.1 µg of catechol produced/g) and soluble protein content in root (5.79 mg/g) was evidenced higher in *Glomus* sp. (AM 1) with *Pseudomonas* sp. (PS 1) inoculated plants at 125 ppm of Cd on 30 DAS.

Keywords: Cadmium, *Glomus*, *pseudomonas*, chlorophyll, soluble protein

Introduction
Heavy metals are one of the most important pollutants in the environment and their toxicity related issues are increasing significantly and causing ecological, evolutionary, nutritional and environmental stresses. Environmental pollution by metals became extensive as mining and industrial activities increased in the late 19thand early 20th century (Pinto *et al.*, 2004) [19]. Among these metals, Cadmium is a non-essential element that negatively affects plant growth and development. It is recognized as an extremely significant pollutant due to its high toxicity and large solubility in water (Pinto *et al.*, 2004) [19]. Cadmium can make alterations in nutrients uptake by plants since, it has a negative or detrimental effects on the availability of soil nutrient or through a reduction in the population of soil microbes (Moreno *et al.*, 1999) [18]. It was reported that, plant physiological parameters *viz.*, stomatal opening and closing, transpiration, respiration and photosynthesis have been adversely affected by cadmium in nutrient solutions, but the uptake of metal into plants are more easily done from nutrient solutions than from the soil (Sanita di Toppi and Gabrielli, 1999) [23]. The visible symptoms are chlorosis, leaf rolls and stunting of growth shown in cadmium toxicity affected plants. Cadmium produces alterations in the functionality of membranes by inducing lipid peroxidation (Fodor *et al.*, 1995) [7] and disturbances in chloroplast metabolism by inhibiting chlorophyll biosynthesis and reducing the activity of enzymes involved in CO_2 fixation (De Filippis and Ziegler, 1993) [4]. The heavy metals *viz.*, cadmium is associated with oxidative stress induced plant damage and changes in the plant metabolism *viz.*, uptake of nutrient, pigment production, protein and chlorophyll synthesis or activity of isozymes) and enzyme concentration to stress metabolism (Monteiro *et al.*, 2009) [17].
The process of using microorganisms or their enzymes and products to return the natural environment altered by contaminants to its original condition is called as “Bioremediation”. Soil microorganisms are known to play a key role in mobilization and immobilization of metal cations, thereby changing their availability to plants.

High metal concentrations in soil were toxic to bacteria and fungi. Metal tolerance in soil microorganisms has been studied for using them in bioremediation of metal contamination. Various microbial species such as *Pseudomonas*, *Klebsiella*, *Proteus* and *Staphylococcus* have been shown to be relatively efficient in the bioaccumulation of different heavy metals from polluted effluents (Hussein *et al.*, 2001) [9]. It was reported that *Pseudomonas putida* can accumulate Cd in the medium in the form of poly β hydroxylbutyrate granules and also reduces the ethylene production by ACC deaminase activity. Mycorrhizas are among the extracellular strategies to avoid metal toxiciy (Jentshcke and Godbold, 2000) [11]. However, only few studies have presented direct evidence of the alleviation of metal toxicity by micorrhizal fungus (Schutzendubel and Polle, 2002) [25].

Arbuscular mycorrhizal fungi provide a direct physical link between soil and plant roots increasing soil nutrient exploitation and transfer of minerals and colonizing plant growing on heavy metal contaminated habitats and can able to take up the heavy metals and other metals which were immobilized in hyphae or mycelium. Therefore AM fungi combined with *Pseudomonas* would be an efficient tool in strengthening the plants in withstanding cadmium contaminated soils.

Materials and Methods

A pot culture experiment was conducted in the green house of the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore with the standard isolates *G. mosseae* and *P. putida*. Pots (30 x 28 cm size) were filled with 5 Kg of double sterilized soil which was the mixture of red soil, sand and farmyard manure in the ratio of 2:1:1. Soil was mixed with the recommended dose of fertilizers, 300:20:200 of N: P: K mg/kg of soil. Quarter of N, full P and K was applied as basal and remaining N was applied at 45th days after sowing. Cadmium was applied in the form of cadmium chloride ($CdCl_2$) at the rate of 75 and 125 mg/kg. Pure inoculum of *G. mosseae* (AM s) and *Glomus* sp. (AM_1) were applied @ 50 g/pot (containing 8 - 10 spores/g of inoculum) as a thin layer, 5 cm below the seeds prior to sowing. Two isolates of *Pseudomonas viz*., PS_1 and *Pseudomonas putida* (PS s) 48 h old broth cultures having the population load of 10^9 cells/ml at the rate 50 ml broth/pot was inoculated prior to sowing. Maize seeds Var. CO 1 obtained from the Department of Millets, Tamil Nadu Agricultural University, Coimbatore was used for the study. The seeds were surface sterilized and sown at 3 cm depth, and maintained three plants per pot.

Treatment Details

Two factor factorial CRBD was followed for pot culture experiment. Factor 1 was Cd concentrations (C1 – 0ppm, C2 -75 ppm and C3- 125 ppm) and factor 2 was sole and combinations of microorganisms with 9 treatments and 3 replications. The details are as follows.

Plant samples were collected randomly on 30 and 60 DAS for estimating total chlorophyll (Sadasivam and Manickam, 1992) [22] in leaves, total phenols (Malick and Singh, 1980) [16] and soluble protein (Lowry *et al.*, 1951) [14] in both root and shoot. The data was statistically analyzed and presented in table.

T_1 Absolute control
T_2 *Pseudomonas* sp.,
T_3 *Pseudomonas putida*
T_4 *Glomus* sp.,
T_5 *Glomus mosseae*
T_6 T_2+T_4
T_7 T_3+T_4
T_8 T_2+T_5
T_9 T_3+T_5

Results and Discussion

Total chlorophyll

The total chlorophyll content of maize plants was estimated on 30 and 60 DAS and presented in Table 1. The total chlorophyll content was increased with increasing concentrations of Cd in all the treatments. The high content of chlorophyll was recorded in T_9 (6.8 mg/g) (*G. mosseae* with *P. putida*) and control T_1 recorded (2.8 mg/g) at 125 ppm of cadmium on 30 DAS. But T_6 (*Glomus* sp. with *Pseudomonas* sp.) and T_9 (*G. mosseae P. putida*) were on par with each other on 30 DAS. Overall increase in chlorophyll content was noticed on 60 DAS than 30 DAS. The treatment T_8 (*G. mosseae* with *Pseudomonas* sp.) had recorded 7.49 mg/g of chlorophyll over control (3.89 mg/g) at 125 ppm of Cd on 60 DAS. It was showed 28.9 percent increase in chlorophyll content over T_5 *G. mosseae* sole inoculation at 125 ppm on 60 DAS. The same trend was reported by Rai *et al*., 2005 and Cheng *et al*., 2002 [2], that the increased Cd concentration gradually decreased the concentration of photosynthetic pigment chlorophyll a, b and total chlorophyll. Cd have a direct effect on the structure, composition and functioning of photosystem II domains in the thylakoid membrane of the plants (Becerril *et al.*, 1988) [1]. Since AM fungi enhanced the P nutrition availability to plants in Cd stress condition had compensated the photosynthetic efficiency of plants. Heavy metal contamination in soil was associated with iron deficiency (Wallace *et al.*, 1992) [29], low iron content results in chlorosis, since it inhibits both chloroplast development and chlorophyll biosynthesis (Imsande, 1998) [10]. On contrary to this finding, total chlorophyll was progressively increased with higher levels of Cd were observed in AM fungi and *Pseudomonas* sp. treated plants. It was agreed with the results of Tripathi *et al*. (2005) [28], who reported that the reduction in chlorophyll content at 110 µg/ml of $CdCl_2$ was reduced by the inoculation of *P. putida* strain KNP9. However the microbial iron siderophore complex produced by *Pseudomonas* could take up by plants as an iron source, thus the effect of iron deficiency was alleviated in Cd contaminated soils.

Table 1: Effect of combined inoculation of AM fungi and *Pseudomonas* sp. on total chlorophyll of maize crop on 30 and 60 DAS at different Cd levels

Treatments/Concentration of Cd (ppm)	Total chlorophyll (mg/g)					
	30 DAS			60 DAS		
	C_1 (0 ppm)	C_2 (75 ppm)	C_3 (125 ppm)	C_1 (0 ppm)	C_2 (75 ppm)	C_3 (125 ppm)
T_1	1.85	3.11	2.80	3.35	3.52	3.89
T_2	3.53	3.90	4.20	4.12	4.97	5.28
T_3	2.37	3.20	4.17	3.16	4.25	4.92
T_4	4.69	5.10	5.90	4.63	4.86	5.36
T_5	3.62	4.20	5.40	4.82	5.32	5.81
T_6	5.20	5.90	6.20	5.26	5.73	6.13
T_7	4.31	5.12	6.08	5.58	6.21	6.42
T_8	4.92	5.72	6.12	6.83	7.15	7.49
T_9	4.13	6.22	6.80	6.13	6.42	6.91
	S.Ed	CD (0.05)		S.Ed	CD (0.05)	
T	0.113	0.227		0.129	0.259	
C	0.065	0.131		0.074	0.149	
T x C	0.196	0.393		0.223	0.448	

Total phenolics

Total phenolics were measured in both shoot and root of maize plants on 30 and 60 DAS. The results were presented in Table 2. The total phenolics content was increased with increasing levels of Cd on both 30 and 60 DAS irrespective to the inoculated and uninoculated plants. Total phenol content was significantly increased in T_6 (*Glomus* sp. with *Pseudomonas* sp.) in both shoot and root. T_6 had recorded the highest content of total phenolics in root (3.1 µg of catechol produced/g) and slightly decreased in shoot (1.97 µg of catechol produced/g) at 125 ppm on 30 DAS. It was observed that the isolate *Glomus* sp. had shown 116.4 and 146 percent increase in total phenolics content when combined with *Pseudomonas* sp. in shoot and root respectively at 125 ppm on 30 DAS over its sole inoculation. In general total phenolics was increased on 60 DAS in all the treatments and T_6 *Glomus* sp. with *Pseudomonas* sp.) has recorded the highest content (4.63 µg of catechol produced/g of root) followed by T_8 (*G. mosseae* with *Pseudomonas* sp.) (4.12 µg of catechol produced/g of root) over control (1.86 µg of catechol produced/g of root) on 60 DAS. The combined inoculation significantly increased the total phenolics than the sole treatments. Dai *et al.* (2006) [3] reported that there was no significant difference in total phenolic content over control and Cd treated plants at initial stage. Increased time of exposure increased the total phenolic content over control plants. Increase in phenolic content in response to exposure to heavy metal Cd has been noted in several plants such as *Arabidopsis thaliana* (Lummerzheim *et al.*, 1995) [15], birch (Loponen *et al.*, 1998) [13], *Phyllanthus tenellus* (Santiago *et al.*, 2000) [24], *Nymphaeae* (Lavid *et al.*, 2001) [12], and Scots Pine (Schutzendubel *et al.* 2001) [26]. Our results also supported these findings that the total phenolics was increased on 60DAS 4.63 µg of catechol produced/g of fresh root tissue than 3.1 µg of catechol produced per g of fresh root tissue on 30 DAS. The main mechanism for cadmium accumulation was based on the binding of cadmium by polymerized phenolics in *Nymphaeae* (Lavid *et al.*, 2001) [12]. Phenolics contribute, together with ascorbate to H_2O_2 destruction in the phenol coupled ascorbate peroxidase (APX) reaction (Polle *et al.*, 1997) [20], and thus protect plants from oxidative stress. The increased production of total phenolics in *Glomus* and *Pseudomonas* interactions was observed and indicated the effective removal of reactive oxygen species with other antioxidant enzymes.

Table 2: Effect of combined inoculation of AM fungi and *Pseudomonas* sp. on total phenolics of maize crop on 30 and 60 DAS at different Cd levels

Treatments/ Concentration of Cd (ppm)	Total phenolics (µg of catechol produced/g)											
	Shoot						Root					
	30 DAS			60 DAS			30 DAS			60 DAS		
	C_1 (0 ppm)	C_2 (75 ppm)	C_3 (125 ppm)	C_1 (0 ppm)	C_2 (75 ppm)	C_3 (125 ppm)	C_1 (0 ppm)	C_2 (75 ppm)	C_3 (125 ppm)	C_1 (0 ppm)	C_2 (75 ppm)	C_3 (125 ppm)
T_1	0.03	0.10	0.14	0.08	0.61	1.13	0.05	0.09	0.16	0.13	0.97	1.86
T_2	0.08	0.14	0.43	0.26	1.63	1.85	0.27	0.23	0.56	0.45	1.90	2.14
T_3	0.07	0.13	0.52	0.13	0.87	1.65	0.16	0.20	0.92	0.28	1.14	1.98
T_4	0.09	0.23	0.91	0.92	1.56	2.11	0.35	0.39	1.26	1.26	2.00	2.49
T_5	0.12	0.47	1.13	0.84	1.61	1.82	0.57	0.64	1.65	1.12	1.84	2.10
T_6	0.62	0.94	1.97	1.93	2.54	4.09	1.54	1.63	3.1	2.16	3.17	4.63
T_7	0.73	1.12	1.85	1.32	2.41	3.27	1.28	1.32	2.63	1.64	2.73	3.91
T_8	1.25	1.26	2.68	1.19	3.19	3.74	1.86	1.73	2.97	1.94	3.66	4.12
T_9	0.39	1.09	1.75	1.87	2.37	3.07	0.95	1.21	2.13	2.10	2.81	3.27
	S.Ed	CD (0.05)		S.Ed	CD (0.05)		S.Ed	CD (0.05)		S.Ed	CD (0.05)	
T	0.024	0.048		0.048	0.097		0.033	0.066		0.057	0.114	
C	0.013	0.027		0.028	0.056		0.019	0.038		0.032	0.066	
T x C	0.041	0.083		0.084	0.168		0.057	0.116		0.098	0.198	

Soluble protein

Soluble protein was measured in both shoot and root of maize plants on 30 and 60 DAS. The results were presented in Table 3. It was observed that the soluble protein content was increased with increasing levels of Cd on both 30 and 60 DAS. The treatment T_6 (*Glomus* sp. with *Pseudomonas* sp.) had recorded the highest content of soluble protein in root (5.79 mg/g), and it was decreased in shoot (4.27 mg/g) at 125 ppm on 30 DAS. The percentage increased over control was 236.6 and 281.2 in root and shoot respectively.

The overall protein content was significantly increased on 60 DAS in all the treatments. The same treatment had recorded the highest content in root (6.37 mg/g) followed by T_8 (*G. mosseae* with *Pseudomonas* sp.) (5.94 mg/g) over control (1.17 mg/g) at 125 ppm on 60 DAS. The combined inoculation was increased the soluble protein than the sole treatments. Abiotic stress reduced the total soluble protein content in non-mycorrhizal plants than mycorrhizal plants. The total soluble protein content was increased in highest Cd concentration due to the mycorrhizal colonization. Ewais (1997) [6] showed that soluble protein content was increased in roots than shoots. The decrease in protein content in shoot may be due to the metabolic disorder leading to the inhibition of protein synthesis (Delhaize *et al.*, 1989) [5]. The decrease in protein content under heavy metal stress was due to decreased chlorophyll content and hence decreased the photosynthesis. Hou *et al.*, (2007) [8] reported that the protein content and photosynthesis was strongly inhibited by heavy metals in *Lemma minor*. Cd could induce DNA damage such as single and double strand breaks, modified bases leads to reduction in protein synthesis. The increase in soluble protein content was influenced by mycorrhizal association by way of increasing the total chlorophyll content and *Pseudomonas* by reducing the iron deficiency there by increasing the photosynthesis under Cd stress condition. (Sinha and Mukherjee 2008) [27].

Table 3: Effect of combined inoculation of AM fungi and *Pseudomonas* sp. on soluble protein of maize crop on 30 and 60 DAS at different Cd levels

Treatments/ Concentration of Cd (ppm)	Soluble protein (mg/g)											
	Shoot						Root					
	30 DAS			60 DAS			30 DAS			60 DAS		
	C_1 (0 ppm)	C_2 (75 ppm)	C_3 (125 ppm)	C_1 (0 ppm)	C_2 (75 ppm)	C_3 (125 ppm)	C_1 (0 ppm)	C_2 (75 ppm)	C_3 (125 ppm)	C_1 (0 ppm)	C_2 (75 ppm)	C_3 (125 ppm)
T_1	0.09	0.62	1.12	0.13	0.67	1.08	0.23	0.84	1.72	0.12	0.94	1.17
T_2	0.16	1.83	1.63	0.83	1.39	2.63	1.28	2.15	3.14	1.16	2.38	3.71
T_3	0.74	1.16	2.10	0.47	1.51	2.19	0.87	1.62	2.80	0.92	1.82	3.28
T_4	0.96	1.28	2.52	1.26	2.49	3.16	1.45	2.91	3.08	1.80	3.56	4.24
T_5	1.27	2.53	3.28	0.98	2.16	1.98	1.60	3.15	4.19	1.40	2.73	4.00
T_6	2.38	3.17	4.27	1.95	3.27	5.82	3.57	4.14	5.79	2.31	4.98	6.37
T_7	2.13	2.64	3.94	1.74	2.10	4.70	2.75	3.10	4.24	2.26	2.81	4.90
T_8	2.64	3.61	4.62	1.53	2.25	5.12	3.12	4.37	5.10	2.10	3.17	5.94
T_9	1.93	2.71	3.90	1.40	2.67	4.09	2.57	3.21	4.73	1.96	3.25	5.01
	S.Ed	CD (0.05)		S.Ed	CD (0.05)		S.Ed	CD (0.05)		S.Ed	CD (0.05)	
T	0.059	0.118		0.061	0.122		0.075	0.151		0.078	0.156	
C	0.034	0.068		0.035	0.070		0.043	0.087		0.045	0.090	
T x C	0.102	0.205		0.106	0.212		0.130	0.262		0.135	0.271	

Summary and Conclusion

Based on the results discussed above cadmium toxicity in plants as well as in soil was reduced by the inoculation of AM fungi with *Pseudomonas* sp. Standard cultures *G. mosseae* with *P. putida* have recorded higher total chlorophyll content at higher levels of Cd on 30 DAS. But at 60 DAS T_8 *G. mosseae* with *Pseudomonas* sp. recorded 28.9 percent increase in total chlorophyll content over sole inoculation of *G. mosseae* (T_5) at 125 ppm of Cd. Total phenolics and soluble protein content was increased with increasing levels of Cd in all the treatments and all the stages of observation. Among the treatments T_6 had recorded highest phenol content in root at 125 ppm on 30 DAS and 60 DAS. The same treatment had recorded the highest content of soluble protein in root and it was decreased in shoot at 125 ppm on 30 DAS. The phytochemical changes in the plant facilitate the accumulation of Cd in the roots and reduced the translocation of Cd to shoots. It can be concluded that consortium of microorganisms can be utilized for the bioremediation of Cd contaminated soil in a cost-effective manner.

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