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Effects of arbuscular mycorrhizal fungi on wheat growth, physiology, nutrition and cadmium uptake under increasing cadmium stress

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Key words: Arbuscular mycorrhizal fungi, Wheat, Cadmium toxicity, nutrient contents, Antioxidant enzymes.

Abstract

A pot culture experiment was carried out to study the alterations in growth, biochemical activities and cadmium (Cd) uptake by wheat (*Triticum aestivum*) inoculated with or without arbuscular mycorrhizal (AM) fungi in sterilized soil with addition of different Cd levels (0, 100, 300, 600 mg.kg^{-1}). In Mycorrhizal (M) plants, root colonization rates were significantly lower with the addition of high Cd concentration (600 mg.kg^{-1}). AM inoculation increased shoot and root biomass at 100 mg kg^{-1} Cd addition but cause a reduction at 300 and 600mg.kg^{-1}. Shoot and root Cd concentrations in mycorrhizal (M) plants were lower at all levels (0, 100, 300 and 600 mg.kg^{-1}) and Cd accumulation and uptake efficiency were lower in M plants. AM inoculation improved shoot and root P nutrition at all Cd levels. In addition, mycorrhization also cause to improved shoot nutrients uptake (N, P, K, Ca, Mg, Na), chlorophyll, carotene, protein and sugar contents as compared to NM plants. Cd toxicity induced proline accumulation and significant reduction of antioxidant enzyme activities (SOD, POD, CAT, APX) were observed in NM plants however proline contents were lower in M except the higher Cd concentration (600 mg.kg^{-1}). The results support the view that AMF can improve the capability of reactive oxygen species (ROS) and reduce Cd concentration in plants to protect wheat (*Triticum aestivum* L.) from Cd stress. Hence, AM fungi in combination with wheat is suitable for reduction of Cd toxicity and also shows a potential role in phytostabilization of soil moderately polluted with Cd.

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Introduction

Heavy metal contamination is a major environmental problem in the world (Davis, 2003). Cadmium (Cd) is a non-essential element and highly toxic to humans, animals and plants. It disrupts the metabolic processes of both plants and animals and is considered as one of the most phytotoxic heavy metal pollutants (Aravind and Prasad, 2003). Cd enters into the environment through both natural and anthropogenic sources. The natural sources include weathering of rocks, forest fires and volcanic eruptions. The anthropogenic sources that cause to increase the natural limit of Cd towards toxic include industrial waste release, use of fertilizers in agriculture that has a huge quantity of Cd which directly transferred to food chain and poses hazards for both human and animal health (Schutzendubel *et al*., 2002). In most of the plant species, Cd is readily translocated towards roots and accumulated in leaves (Lopez-Millan *et al*., 2009).

High levels of Cd cause to generate toxic free radicals i.e reactive oxygen species (ROS) produce oxidative stress in plants (Dixit *et al*., 2001). These toxic free radicals (ROS) react with biomolecules like lipids, proteins, pigments, nucleic acid cause lipid peroxidation, harm to cell membranes and inhibit enzymatic activity resulting in disruption of cell functioning. On the other hand, plants has been developed an antioxidant defense mechanism which stimulate the functioning of antioxidative enzymes and fight against these toxic radicals to protect plants from stess (Li*et al*., 2009). The antioxidative defense mechanism consist of both enzymatic and non-enzymatic antioxidants. Antioxidative enzymes include superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR). Non-enzymatic antioxidants include lipid soluble membrane associated antioxidants (e.g, a-tocopherol and b-carotene) and water soluble reductants (e.g., ascorbic acid and glutathione).

The role of arbuscular mycorrhizal fungi (AMF) for land remediation has been commonly studied (Smith and Read, 1997). Several studies reported the mutual symbiosis between AM fungi (AMF) and roots of terrestrial plants by increasing plant biomass and uptake of immobile nutrients such as P, Zn Cu (Sheng *et al*., 2009) and reducing metal toxicity to plants by decreasing root to shoot heavy metal (HM) translocation and shoot HM concentrations (Smith and Read, 1997). AMF secreted several compounds that cause to precipitate the metals in polyphosphate granules present in soil, adsorping metals to fungal cell walls and chelation of Cd inside the fungus (Gaur and Adholeya, 2004). However, the effect of AMF on metal uptake in plants is still in controversy. Previous reports suggested uptake of metals by AMF with decreased or increased translocation to shoots or sometimes even no uptake effects were studied (Gao *et al*., 2010).

Wheat is one of the oldest and most widely cultivated crops. According to FAO reports, in 2007 world production of wheat was 607 million tons making it the third most produced cereal after maize and rice. Cd is considered as one of the most toxic heavy metal causing serious problems in crops (Prasad, 1995). The toxic effects of Cd have been widely studied in different plant species is known to reduce or inhibit plant growth and development (Shahabivand *et al*., 2012). AMF can exert positive effects on terrestrial plants under Cd contamination (Shahabivand *et al*., 2012; Medina *et al*., 2010a).

However, the association between AMF colonization and accumulation of toxic elements in crops is an area of considerable interest relating to both production of safe food and bioremediation strategies (Smith and Read, 2008). There is scarce information about the effects of AMF on physiological and biochemical changes of cereal crops especially wheat under Cd stress. Therefore, this study was carried out to examine the effects of AMF (combination of Glomus species) on physiology, biochemical contents and shoot and root Cd accumulation in *Triticum aestivum* under different Cd contamination levels.

Materials and methods

Plant materials

Wheat seeds (*T. aestivum*) were obtained from the Department of Crop Science, National Agriculture Research Centre, Islamabad. Seeds were surface sterilized (10 min, 3% Chlorox) and gently washed by deionized water five times and germinated on sterile wet filter paper (Xin Hua No.101, China) in Petri dishes at 28°C for 48 hours. These were selected for uniformity before sowing. Five pre-germinated seeds were sown per pot and the plants were allowed to grow for 8 weeks.

Preparation of soil

The experimental soil and sand were collected from the top layer (0-20cm) in the vicinity of Quaid-i-Azam University, Islamabad. The soil and sand were air-dried and sieved with a 2-mm diameter sieve for analysis. The soil and sand were air-dried and sieved with a 2-mm diameter sieve for analysis. Soil was chemically characterized with a pH (6.7), T. Phosphorus (4.3 $mgkg^{-1}$), T. Potassium (19.5 $mgkg^{-1}$), Calcium (34.45 $mgkg^{-1}$), Magnesium (42.50 $mgkg^{-1}$), Extractable nitrate nitrogen (1.04 $mgkg^{-1}$), Extractable potassium (1.45 $mgkg^{-1}$), Extractable phosphorus (1.53 $mgkg^{-1}$), Zinc (1.50 $mgkg^{-1}$), Nickel (1.33 $mgkg^{-1}$), Copper (30.3 $mgkg^{-1}$), Cadmium (1.60 $mgkg^{-1}$), Iron (28.51 $mgkg^{-1}$), Lead (1.6 $mgkg^{-1}$), Chromium (4.25 $mgkg^{-1}$) and Manganese (10.4 $mgkg^{-1}$) respectively. The soil and sand were autoclaved-sterilized (121°C, 2 h) in order to eliminate native AM fungal propagules and other microorganisms. The soil was manually mixed with sand in ratio of 1:3 (v/v). The mixture of soil and sand were used as growth medium of plants. $CdCl_2$ was added to the growth medium as Cd stress at the concentrations of 100, 300 and 600 mg/kg respectively.

Fungal inoculum

The AMF used was the mixture of different Glomus species with dry soil substrates obtained from the AMF collection maintained by the company (Agrauxine) in France. Spores and dried sand-soil mixture (growth medium) were used in mycorrhizal inoculated treatments. Each pot received 50 g of AM fungal inoculum containing about 2500 spores per pot. AMF inoculation was performed during the transplantation process and was not provided in non-mycorrhizal treatments.

Pot experiment and growth conditions

A pot culture experiment was carried out under growth chamber conditions consisted of a completely randomized design with six replicates. Each pot (10cm diameter and 12cm height) contained 2kg growth medium plus 50g inoculum to mycorrhizal treatments, while the same amounts of growth medium were added to non-mycorrhizal treatments. The treatments were either inoculation or non-inoculation of the AM fungi and the addition of four Cd concentrations to the soil (0, 100, 300 and 600mg/kg). The experimental pots were placed in the growth chamber under conditions of 14 h of light, 10 h darkness, 28/20°C day/night temperature, relative humidity of 50-65% day. Water lost was replaced daily by top watering with deionized water at 24th intervals during growth period to maintain the moisture of the soil at about 60% until the end of the experiment. Each pot was irrigated with long Ashton's nutrient solution (20ml) every week. Six pots per treatment were used and plants were harvested after 60 d for chemical analysis.Roots and shoots of the harvested wheat samples were rinsed with tap water to remove soil particles and then carefully washed with deionized water.

Plant measurements and analysis

AM Root colonization

Root mycorrhizal colonization was estimated after clearing and staining (Koske and Gemma, 1989) using the grid-line intersect method (Giovannetti and Mosse, 1980). The stained roots were then mounted on glass slides (5 pieces of root per slide) for examination with an eyepiece cross-hair. Colonization percentage of mycorrhiza was estimated for each sample by examination of one hundred 1cm long pieces of roots.

Plant biomass

At harvest, roots and shoots were separated. Sub-

samples of fresh roots were taken to assess mycorrhizal colonization. Fresh weights of total roots and sub-samples were measured. Leaves and remaining roots were rinsed with tap water and then with deionized water. Tissues were weighed after oven drying at 60°C for 72 h and then ground to < 0.25 mm in a stainless mill. The percentage of water content in remaining roots and total root fresh weight were used to estimate total root dry weight.

Plant growth

The growth performance including stem diameter, shoot and root height, breadth and area were recorded. Height and diameter were measured by precision straight edge (Sword fish, China) and vernier caliper (ECV15°C, China).

Macro and Micro-Nutrients Analysis

After dry weight determination, the oven dried tissue samples (shoots and roots) were ground and digested in HNO_3 (70%) and H_2O_2 using the microwave digestion system (CEM-MDS 2000). The digest was filtered using What man No. 42 filter paper and made up to 50 ml by using deionized water. The metal contents (Na, K, Ca, Mg, Co, Cr, Cu, Fe, Ni, Pb, Mn, Cd, Zn) in plant tissues (shoot and roots) were determined by using atomic absorption spectrophotometer (Varian FAAS-240). Total Phosphorus (P) in plant digest was determined by ammonium-vanadomolyb date method (Ryan *et al*., 2001). Total N was determined by Kjeldahl method (Van Schouwenberg and Walinge, 1973).

Biochemical Analysis

Chlorophyll content in the fresh leaves (50mg) of the plant was measured in 10cm^3 dimethyl sulfoxide (DMSO) by using the method of Hiscox and Israelstam (1979). Carotenoid content was determined by the method of Duxbury and Yentsch (1956). Proline content of leaves was estimated by using the method of Bates *et al.* (1973). Sugar content of flag leaves was estimated by following the method of Dubois *et al.* (1956). Protein content in the leaves (50mg) of the plants was measured using Bovine Serum Albumen (BSA) as a standard Lowry *et al.* (1951).

Antioxidant enzymes

For enzyme analysis, fresh samples of leaves (300 mg each) were ground in a chilled mortar and extracted with 3 ml of 100 mm potassium phosphate buffer (pH 7.5). The homogenate was centrifuged at 12,000 rpm for 15 min. The supernatant was used for the estimation of antioxidant enzyme activities. Superoxide dismutase (SOD) activity was assessed spectrophotometrically at 560 nm based on the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) as described by method of Beauchamp and Fridovich (1971). One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50%. The activity of POD was measured by following the method of Gorin and Heidema (1976). Catalase (CAT) activity was determined by the method of Goel *et al.* (2003). The activity of Ascorbate peroxidase (APX) was measured by estimating the rate of ascorbate oxidation. The change in absorbance was monitored at 290 nm Nakano and Asada (1981).

Quality Control Measurements

The chemicals used were analytical grade and obtained from Sigma, Aldrich and Merck. All the analyses were performed in triplicates under standard optimizing conditions. Analytical data quality of metals in soil and plant samples was ensured through repeated analysis (n=6) of roots and shoot samples. The blank reagent and standard reference soil (NIST, 2709 San Joaquin) and plant materials (NIST, 1547 Peach leave) of National Institute of science and Technology were included in each sample batch to verify the accuracy and precision of the digestion procedure. Recoveries of metals from the plant tissues were found to be 99%. The blanks were run after five samples.

Statistical analysis

Data on physiological parameters, biochemical contents, antioxidant enzymes and root colonization were analyzed with two way analysis of variance (ANOVA) technique using statistix (version 8.1) software. For significant F value, Tukey test was used for mean comparison at 5% level.

Results

Plant biomass and Mycorrhizal colonization

Fig 1a and b shows the effects of cadmium toxicity on M and NM wheat shoot and root biomass. Wheat seedlings were greatly influenced by AMF inoculation with significantly higher shoots and root biomass production. The biomass of M seedlings were higher in both root and shoot than those of NM seedlingsat each concentration of Cd. In NM seedlings, the reduced biomass was observed with the increase of Cd concentration. The same trend was observed in both shoot and root parts of plants. In 100 mg.kg^{-1} soil, a significant increase ($P<0.05$) was noted in shoot and root biomass of M inoculated plants with Glomus species. While the decrease in the trend was observed at 300 and 900 mg.kg^{-1} concentration in both inoculated and non-inoculated plant shoot and root.

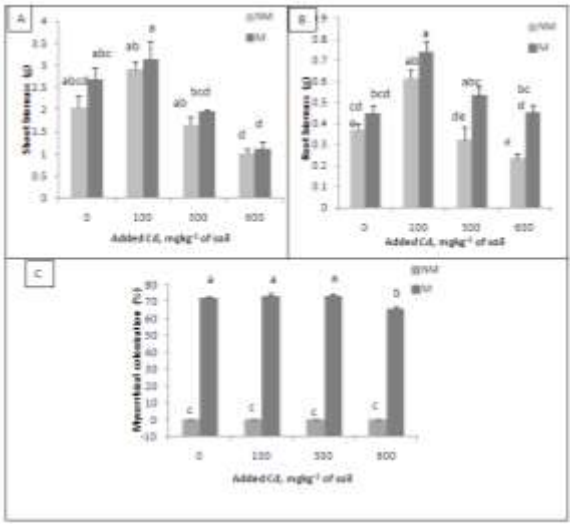

Fig. 1. Effect of increasing cadmium concentration on mycorrhizal and non mycorrhizal wheat plants: (a) Shoot biomass, (b) Root biomass, (c) Root colonization. The data shown are the means and standard error. The different letters above the bars indicates significant difference by Tukey test ($P < 0.05$).

Fig. 1c. shows the percentage of AM fungi colonization with roots of wheat (*Triticum aestivum*) plants. The result indicated that AMF colonization was not detected in non-inoculated treatments while all the inoculated treatments (M) showed high colonization rates with abundantly formed arbuscules and hyphal structures. From the results, it is obvious that the symbiotic relationship between *Triticum aestivum* and AMF can be well established under Cdstress conditions. The highest colonization 72.7% and 72.9% appeared at the 100 and 300mg/kg Cd concentration, while the lowest colonization 65.28 % appeared at 600 mg/kg Cd concentration. Mycorrhizal colonization of inoculated roots ranged between 60 and 70%.Cd addition negatively influenced M root colonization and it is diminished linearly with the increase of metal concentration in soil.

Effect on plant growth

Table 1 shows the effect of increasing Cd concentration on growth of M and NM wheat plants. M wheat plants exhibited higher growth than NM wheat plants. Results showed that Cd concentration (300 ad 600 mgkg^{-1}) cause a reduction in plant growth in both M and NM plants. At 100 mg kg^{-1}Cd, a significant improvement mainly in the M plants was observed. Generally, M plants exhibited significantly higher shoot and root length, breadth and area production than NM plants at 0, 100mgkg^{-1}Cd. Meanwhile, non-inoculated plants exhibited slower growth with increasing Cd concentrations even though the soil used in the experiments received P fertilization. The significant positive changes and better root and shoot growth were observed in M plants at Cd concentrations (0,100, 300mgkg^{-1}) as compared NM plants. The reduction in plant root and shoot growth was observed at highest Cd concentration (600mgkg^{-1}) in both M and NM plants.

Plant Phosphorus (P) uptake

Fig. 2. shows the effect of mycorrhizal inoculation on plant Pnutrition with increasing Cd concentrations. The improved P nutrition was observed in all M plants as compared to NM plants. In the experiment, the shoot of M plants maintained higher P level than NM plants at0, 100 mg kg^{-1} Cd in soil but not at 300 and 600 mg kg^{-1}. At this level, plant growth also was very limited. The decreasing trend was observed as the concentration of Cd increased in NM plants. The slight increase in P content was observed at 100

mg.kg^{-1} Cd concentration in both M and NM plants as compared to control plants.

Table 1. Effects of increasing cadmium concentration on growth of mycorrhizal and non-mycorrhizal wheat.

Experiment		Shoot			Root		
Cd, mg kg^{-1}		Length (cm)	Breadth (cm)	Area (cm 2)	Length (cm)	Breadth (cm)	Area (cm2)
0	M	19.17±0.5620^{a}	0.185±0.0328^{a}	5.75±0.3648^{a}	17.415±0.7509^{a}	0.19±0.0417^{a}	5.445±0.4271^{a}
	NM	14.445±0.5035^{bc}	0.135±0.0286^{bc}	3.18±0.1915^{cd}	11.655±0.7047^{cd}	0.115±0.0235^{cd}	2.2±0.1804^{cd}
100	M	19.925±0.8236^{a}	0.165±0.0126^{ab}	5.89±0.4785^{a}	15.525±0.6937^{ab}	0.17±0.0410^{ab}	5.06±0.3548^{a}
	NM	15.435±1.2115^{b}	0.1125±0.0393^{c}	2.975±0.4017^{cd}	11.97±0.3399^{cd}	0.105±0.0359^{d}	2.05±0.1748^{d}
300	M	16.605±0.8872^{ab}	0.1575±0.0116^{ab}	4.765±0.2709^{ab}	14.04±0.5478^{bc}	0.16±0.0308^{ab}	3.495±0.2780^{b}
	NM	13.814±0.7114^{bc}	0.1075±0.0373^{c}	2.735±0.2712^{cd}	10.571±0.5410^{d}	0.097±0.0101^{d}	1.93±0.2316^{d}
600	M	13.918±0.8906^{bc}	0.1625±0.0425^{ab}	4.03±0.3756^{bc}	13.23±0.5620^{bc}	0.145±0.0426^{bc}	3.225±0.2205^{bc}
	NM	11.735±0.6884^{c}	0.0995±0.0430^{c}	2.54±0.2898^{d}	9.65±0.6237^{d}	0.089±0.0428^{d}	1.75±0.1421^{d}

The data shown are the means and standard error. Value within each column marked with different letters means difference significant at P <0.05.

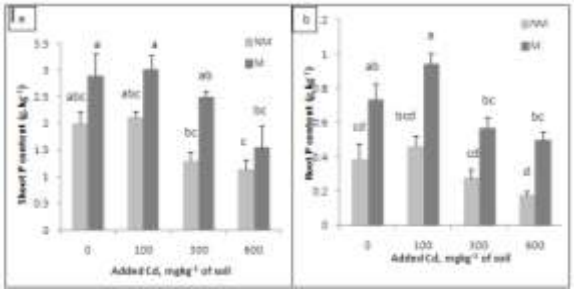

Fig. 2. Phosphorus concentration in: (a) shoots and (b) roots of mycorrhizal (M) and non-mycorrhizal (NM) wheat plants in response to Cd addition to soil. Means (n = 3) with the different letters are significantly different ($P< 0.05$) by the Tukey test.

Cd uptake in wheat plants

Fig 3 shows the linear correlation in plant tissues between Cd concentration in soil and plant uptake, increased cd uptake with increasing soil Cd concentration. NM plants accumulated more concentration of Cd in shoots and roots at all Cd treatments (100, 300, 600 mgkg^{-1}) than M plants. The trend of Cd concentrations in both M and NM plants were statistically different at all Cd concentrations (100, 300, 600 mgkg^{-1}) except control in which no Cd concentration was applied.

In control treatments when no Cd was applied, shoot and root Cd uptake were similar but as Cd application rate increased, shoot Cd uptake increased much less in M plants than root Cd uptake. The uptake of Cd decreased in M shoot as compared with NM plants. However, the increased concentration of Cd was observed in both shoot and root of NM plants as compared to M plants as the application of Cd increased from 100-600 mgkg^{-1} in soil. The more accumulation observed at the highest Cd concentration of 600mgkg^{-1}.In the experiment, the Cd was retained mainly in the roots. In M plants, Cd translocation to shoot was lower at low Cd concentration (100 mgkg^{-1}) than at moderate and high Cd concentrations (300 and 600mg kg^{-1}), in which the roots accumulated around 90% of the absorbed metal. As the Cd concentration increased in soil, the more Cd accumulated in root as compared to shoot part of plant. In M plants, the lower Cd uptake was observed in shoot part of plant as compared to NM plants in all Cd concentrations.

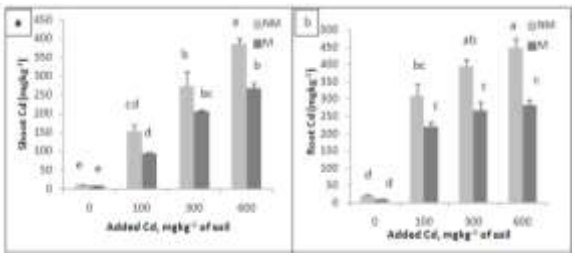

Fig. 3. Cd concentrations in (a) shoot; and (b) root of mycorrhizal (M) and non-mycorrhizal (NM) wheat growing in soil with increasing Cd concentrations, respectively. The different letters above the bars indicate significant difference between treatments. Bars represent standard error; M: black dots and NM: light grey dots.

Plant nutrient contents

Table 2a and 2b shows the effect of increasing Cd concentration on plant macro and micronutrient contents (K, N, Ca, Mg, Na, Fe, Mn, Ni and Cu). M inoculation showed significant positive changes as compared to NM plants. In general, increase in concentrations of nutrients (N, K, Ca, Mg, Na, Fe, Cu) were observed in all M treatments except Mn, Zn and Ni, in which the trend was decreased in mycorrhizal treatments as the concentration of Cd was increased. The statistical significance was obtained for all the nutrients in M and NM plants at 0, 100, 300 and 900 mg kg–^{1}Cd. The highest Cd addition (600 mg.kg^{-1}) had a harmful effect on the concentration of the analyzed nutrients as there was a significant decrease in their contents as the concentration of Cd increased in both inoculated and non-inoculated plants.

Table 2a. Macronutrients **(**K, N, Ca, Mg, Na) concentrations in shoots and roots of mycorrhizal (M) and non-mycorrhizal (NM) wheat grown in soils with increasing Cd concentrations.

Experiment (Cd, mgkg^{-1})		K	N	Ca	Mg	Na
Shoot		**g.kg^{-1}**				
0	NM	21.467±1.0476 abc	0.7533±0.0606 bcd	25.73±1.0016 ab	11.343±0.9531 abc	11.363±0.9915 bc
	M	28.513±1.0729 ab	1.0967±0.0726 ab	30.673±1.5510 a	12.41±0.3407 a	15.253±0 .3182 ab
100	NM	29.187±2.0502 a	0.8367±0.0669 bc	19.253±0.8027 c	11.77±0.2386 ab	8.5267± 0.6055 c
	M	31.93 ± 3.6344 a	1.48±0.1976 a	24.623±0.791 b	12.19±0.3951 a	17.66± 0.4844 a
300	NM	22.31±1.7439 abc	0.4633±0.0524 cd	14.117±0.9353 d	10.813±0.4901 abc	7.73± 1.2104 c
	M	23.437±3.5656 abc	1.3833±0.1049 a	17.703±1.2410 cd	11.243±0.648 abc	15.557±1.1823 ab
600	NM	15.267±1.5884 c	0.3367±0.0318 d	12.903±0.3170 d	9.1133±0.2356 c	6.4667±0.6393 c
	M	17.693±1.2127 bc	0.8533±0.0722 bc	15.323±0.094 cd	9.6667±0.2051 bc	9.2467±0.3527 c
Roots		**g.kg^{-1}**				
0	NM	9.91±0.3470 ab	0.2533±0.0371 cd	10.25±0.2022 c	6.0467±0.1040 ab	6.8267±0.1506 bc
	M	10.253±0.2341 ab	0.4633±0.0376 ab	11.037±0.4092 bc	7.08±0.0971 a	8.5433±0.6380 ab
100	NM	10.817±1.0822 ab	0.37±0.0265 abc	12.78±0.2307 ab	6.9667±0.0857 a	6.2467±0.4464 cd
	M	11.027±0.7451 a	0.4767±0.0318 a	13.327±0.4138 a	7.21±0.5243 a	9.7833±0.2226 a
300	NM	7.9767±0.3982 ab	0.22±0.0265 d	10.45±0.4636 c	6.1833±0.3860 ab	5.72± 0.3470 cd
	M	8.8133±0.1534 ab	0.3267±0.0291 bcd	12.08±0.1418 abc	6.3933±0.2431 ab	9.3867±0.3702 a
600	NM	7.64±1.2529 b	0.19±0.0115 d	10.917±0.8887 bc	5.2733±0.0865 b	4.7767±0.2074 d
	M	8.0733±0.0788 ab	0.2367±0.0291 cd	11.473±0.4179 abc	5.45±0.4455 b	6.33± 0.2627 cd

Data are presented as mean values ± SD (n = 3) and have been analyzed by two way analysis of variance. Means followed by the same letter within columns are not significantlydifferent by Tukey's test at the 5% level.

Table 2b. Micronutrients (Cu, Mn, Zn, Fe and Ni)concentrations in shoots and roots of mycorrhizal (M) and non-mycorrhizal (NM) wheat grown in soils with increasing Cd concentrations.

Experiment (Cd, mgkg^{-1})		Cu	Mn	Zn	Fe	Ni
Shoot		**(mg.kg^{-1})**				
0	NM	15.87±1.0340 d	72.447±2.2754 bc	33.137±2.5304 a	40.623±3.5274 cd	6.4867±0.2042 bc
	M	20.133±0.7405 bcd	56.47±2.3111 cd	20.83± 1.3501 b	75.963±2.7729 a	5.2133±0.5346 C
100	NM	17.807±0.8475 cd	90.487±4.3295 ab	28.363±4.3834 ab	33.663±2.6062 d	9.8467±0.4606 a
	M	18.253±0.5745 cd	61.953±4.9271 cd	24.4±2.2697 ab	48.743±1.8065 bc	6.94±0.2479 bc
300	NM	25.56±1.5408 ab	69.47±5.9127 cd	26.27±1.6493 ab	36.857±0.8311 cd	7.0033± 0.8521 abc
	M	23.77±1.8930 abc	52.537±1.8987 d	27.45±0.5820 ab	49.34±1.9038 b	8.2667±0.9654 ab
600	NM	28.39±0.9443 a	95.103±4.2755 a	18.307±0.8769 b	31.37±1.7157 d	6.34±0.5877 bc
	M	24.56±2.6743 abc	102.11±3.6651 a	19.26±0.6096 b	46.58±3.2725 bc	6.0533±0.3638 bc

Experiment (Cd, mgkg^{-1})		Cu	Mn	Zn	Fe	Ni
Root		(mg.kg^{-1})				
0	NM	22.253±1.3575 d	54.85±6.0658 bcd	17.51±1.0879 cde	19.253±0.8141 e	10.21±0.1908 cde
	M	28.77± 0.8016 cd	47.45±2.6511 d	28.107±1.8072 bc	28.5±1.4814 cd	7.4767±0.5496 e
100	NM	30.39± 2.9042 bcd	60.173± 4.0114 bcd	15.967±0.4042 de	32.34±2.0216 bcd	18.143±0.8141 a
	M	36.16± 1.1747 abc	49.593±2.1896 cd	31.347±3.8966 ab	42.133±1.7833 a	12.103±0.7591 bcd
300	NM	42.54± 2.2739 a	65.72±3.7120 abc	14.76±0.1747 e	29.457±0.4180 bcd	13.943±0.8141 bc
	M	40.937±4.0135 ab	53.757±3.0010 cd	37.183±1.3720 b	38.367±2.4940 ab	10.787±0.2354 bcde
600	NM	45.5± 2.8501 a	71.067±2.2059 ab	26.847±1.1106 bcd	27.76±2.3023 de	14.45±1.1877 ab
	M	39.873±1.9515 abc	78.703±2.0030 a	42.03±4.2672 a	36.98±2.4185 abc	10.03±1.1780 de

Data are presented as mean values ± SD (n = 3) and have been analyzed by two way analysis of variance. Means followed by the same letter within columns are not significantlydifferent by Tukey's test at the 5% level.

The result of the experiment clearly indicated that mycorrhization positively influenced the nutrient contents of wheat enhancing concentrations of K, N, Ca, Mg, Na, Fe, Cu in shoot but decrease in concentration of Mn, Zn and Ni was observed. However, the increase in root contents of K, N, Ca, Mg, Na, Fe, Cu, Zn, Fe was observed in M plants while decrease in Mn and Ni was recorded. In NM plants, the increase of soil Cd concentrations caused reductions in K, N, Ca, Na, Mg contents in shoot while increase in Cu, Mn, Ni and Fe contents was observed. However, the increase in Cu, Mn, Fe, Ni nutrient contents were observed inroots of NM plants with increasing Cd concentration. While decrease in N, Ca, Na, K and Na concentrations in roots of NM wheat plants.

Plant biochemical Analyses

Fig 4 a, b and c shows the effect of mycorrhiza on relative chlorophyll and carotene contents with increasing Cd concentrations. The concentration of chlorophyll (4a and b) and carotene contents (4c) in M plants were significantly higher than those of NM plants at each Cd concentration (100, 300, 600 mgkg^{-1}). The highest chlorophyll a and b contents were observed at Cd concentration of 100 mgkg^{-1} in both M and NM plants. The lowest chlorophyll and carotene contents were observed at Cd concentration (300 and 900 mgkg^{-1}) in both inoculated and inoculated treatments.

Fig 4d shows the proline content in wheat plants that are increased linearly as Cd concentrations increased in soil. The significant differences were found due to AMF inoculation in plants. M plants showed lower proline levels in control and 100 mgkg^{-1} Cd concentration. The proline contents was increased at 300 and 600 mgkg^{-1} Cd concentration in NM plants. Total proline contents in plants were drastically increased with the increase of Cd in soil except at lower Cd concentration of 100 mgkg^{-1}.

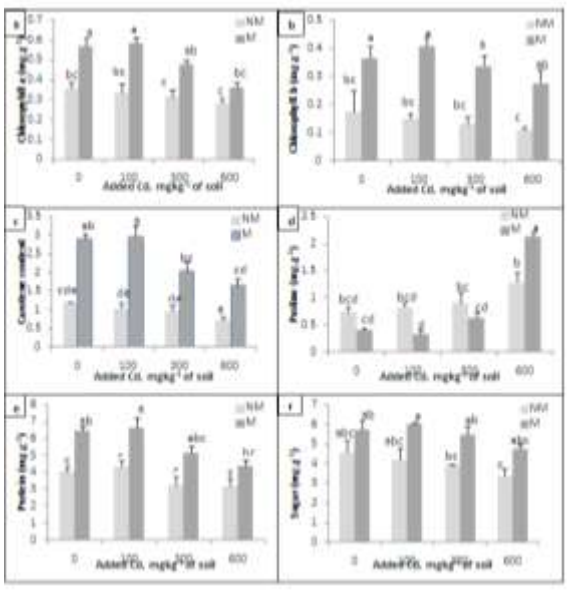

Fig. 4. Biochemical contents (a and b) Chlorophyll a, b content, (c) Total carotene content, (d) Proline contents, (e) Protein contents, and (f) Sugar, in leaves of mycorrhizal (M) and non-mycorrhizal(NM) wheat in response to increasing Cd concentrations in soil (R^2: coefficient of determination; $P< 0.05$ significant by the Tukey test (5%) for M and NM

means for each Cd concentration; M: black dots and black lines and NM: light grey dots and lines.

Fig 4e shows the effect of increasing Cd level on sugar content of wheat in both M and NM treatments. M plants showed significantly higher sugar contents than NM plants at each Cd concentration (100, 300, 600 mgkg^{-1}). In NM plants, total sugar contents were linearly decreased with the increase of Cd concentration (0, 100, 300 and 600 mgkg^{-1}) but comparatively increase in contents were observed in M plants.

Antioxidant enzyme activities

Fig 5 shows the antioxidant (SOD, CAT, APX, POD) activities in M and NM plants at different Cd concentrations (0, 100, 300, 600 mgkg^{-1}). Fig 5a shows the trend was decreasing as the Cd addition to soil increased in both M and NM plants. In NM plants, SOD activity decreased as the Cd addition to soil increased, except at Cd concentration of 100mgkg^{-1}. In M plants, an increase in activity was observed at all Cd concentration as compared to NM plants. In NM leaves, the SOD activity was low as compared to M plants and a little increase in concentration was observed as more Cd concentration applied.

Fig 5b shows the effect of Cd on POD contents in M and NM plants. The statistically significant increase in leaf POD activity was observed in wheat plants after treatment with different Cd concentration. On the contrary, in mycorrhizal treatment Zn exposure led to significant decrease at the highest Cd concentration of 600mgkg$^{-1.}$

Fig 5c shows the effect of Cd on CAT contents in M and NM plants. Leaf CAT activity significantly decreased in response to increasing Cd concentrations in NM and M plants. The decrease in activity was observed at the highest concentration of Cd in M plants. In NM plants, the highest activity of CAT was found at 100 and 300 mgkg-1. Fig 5d shows the plant treated with Cd showed significant increase in leaf APX activity. The trend of APX was same asSOD and POD activity. The highest APX activity was observed at 100 mg.kg^{-1} in M and NM plants.

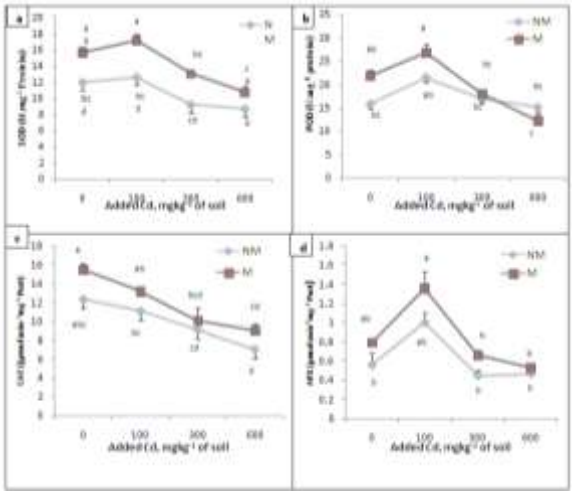

Fig. 5. Antioxidant enzymes activity (a) SOD activity, (b) POD content, (c) CAT activity, (d) APX activity, in leaves of mycorrhizal (M) and non-mycorrhizal (NM) wheat plants in response to cadmium addition to soil. Means (n = 3) with the different letters are significantly different ($p < 0.05$) by the Tukey test. M: black color bars and NM: light grey. Bars represent standard error.

Discussion

The study showed that AMF formed mycorrhizal symbiosis in wheat plants under Cd stress. Some physiological and biochemical parameters of wheat plants such as biomass, nutrients, chlorophyll, carotene, soluble sugar content, soluble protein content and antioxidant enzyme activity could be improved by some AMF species under Cd toxicity. In this study, Cd stress had a strong effect on AMF development and colonization decreased with the increase of Cd concentrations. Mycorrhizal colonization decreased as the concentration of Cd increased in soil. Pawlowska and Charvat (2004) reported that spores of Glomus species differed markedly in their sensitivities to Cd, Pb, and Zn exposures. G. mosseae was more sensitive at different levels of Cd in the soil.

In contrary, Shahabivand *et al.* (2012) reported that Cd toxicity does not affect the root colonization by arbuscular mycorrhizal fungi. These results

demonstrate that different levels of compatibility between host plants and AMF isolates may occur under conditions of Cd toxicity (Zhang *et al.* 2007). The toxic effects of Cd have widely been studied in different plant species and Cd is known to reduce or inhibit plant growth. In the present work, with increasing Cd in the soil, shoot length and shoot and root dry weights were decreased in NM plants. The same results were reported by Lopez-Millan *et al.* (2009) in tomato and in barley by Wu *et al.* (2008). However, AMF association cause to increase the shoot, root length and area. These positive effect was associated to the mycorrhiza contribution in uptake of host mineral nutrient especially immobile soil nutrients and the resistance of Cd uptake (Hu *et al.*, 2013).

Arbuscular mycorrhizal fungi are proposed for improving yield (Covacevich *et al.*, 2007), because it is known that mycorrhizal roots acquire P more efficiently than non-mycorrhizal roots especially at low soil fertility levels. High available P may be detrimental to mycorrhizal colonization and may limit their benefits in agrosystems (Hu *et al.*, 2013).

Mycorrhizas enhance the metal tolerance of host plants in soils containing high concentrations of toxic metals (Lux and Cumming, 2001). In the present study, Cd concentrations in the shoots and roots of wheat plants were always decreased by mycorrhizal inoculations to different degrees independent of the soil Cd concentrations. A possible mechanism of this effect is the ability of AMF to bind heavy metals by fungal hyphae outside and inside the roots.

Numerous experimental studies have indicated that in heavy metal contaminated soils, mycorrhizal plants usually had higher root metal concentrations but lower shoot metal concentrations compared with nonmycorrhizal plants (Chen *et al.*, 2005). Inoculation of plant species including soybeans, maize and lettuce with AM fungi decreases Zn and Cd concentrations in plant leaves at high soil metal concentrations and increases metal concentrations of plant leaves at low soil metal concentrations (Guo *et al.*, 1996). Cd concentration in roots was more than that of soil Cd, indicating that the Cd absorption mechanism for roots is an active process in wheat. It is suggested that the mechanisms of Cd absorption in roots and xylem loading are related to an energy-dependent active process (Ueno *et al.*, 2011).

In contrary, Fernandez *et al.*, (2009) reported shoots and roots of mycorrhized plants accumulated more Cd than non mycorrhized ones. This higher metal accumulation was observed in mycorrhized exposed to high Zn. This might be due to the reason that different AMF ecotypes can exhibit different degrees of metal tolerance. It can be attributed to fact that isolates from habitats contaminated with heavy metals are generally more metal tolerant than isolates from non-contaminated soils.

The result of the study indicated that AMF inoculation enhanced the chlorophyll formation in wheat plants. The alterations in chlorophyll content can be due to the result of nutrient deficiencies and the reaction of plants to the environments in which they survive. This chlorophyll deficiency might be due to Cd induced oxidative stress (Gallego *et al.*, 1996). Wu and Xia (2006) reported the chlorophyll content of non mycorrhizal wheat plants is reduced under Cd stress which may affect the synthesis of chlorophyll enzyme, thereby, reducing the photosynthesis of the plants and reduce the growth of plants. The soluble protein and sugar content in wheat plants increased with Cd addition especially in mycorrhizal plants at a high Cd addition level (100 mg. kg^{-1}). This might be due to the fact that wheat plants especially mycorrhizal plants could increase the ability to withstand adversity by delaying protein degradation and maintaining normal metabolism of proteins. In this study, the enzyme activity results showed that AM symbiosis significantly influenced these enzymes to different degrees to respond to the invasion from the environment, which might be the result of a complex interaction among the AMF, plants and Cd stress.

Conclusion

It is concluded from the results of the present study that the symbiotic relationship between the wheat plants and AMF was well established under Cd stress. AM colonization increased the biomass, growth of plants and availability of essential nutrients to wheat plants. The SOD, CAT and POD responses were associated with AM colonization and the induced activities of the enzymes by AMF contribute to the enhanced plant growth and Cd tolerance of mycorrhizal associated plants under Cd stress. However, it is not clear how AM symbiosis affects the antioxidant enzyme activities and induces the resistant Cd associated material metabolism under Cd stress. Thus, we consider that some of the antioxidant enzymes in AMF are induced by Cd stress which is important for revegetation in soils contaminated with heavy metals.

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