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Effects of Arbuscular Mycorrhizal Fungi on Metals Uptake, Physiological and Biochemical Response of *Medicago Sativa* L. with Increasing Zn and Cd Concentrations in Soil

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Abstract

The effect of mycorrhizal symbiosis on metal accumulation and plant tolerance are not commonly studied in medicinal plants under metal stress. The objective of this study was to assess the impact of mycorrhiza on alfalfa plants with the increase of Zn and Cd toxicity. The experiment was conducted under controlled laboratory conditions. Zinc (Zn) and cadmium (Cd) uptake, some biochemical and physiological parameters were studied in eight-week-old alfalfa plants in response to inoculation or not with arbuscular mycorrhizal fungi (AMF) and with the increase of Zn (0, 100, 300, 900 mg·kg^{-1}) and Cd concentrations (0, 100, 300, 600 mg·kg^{-1}) in soil. The results showed that mycorrhizal (M) plants exhibited tolerance to Zn and Cd up to 300 mg·kg^{-1} in comparison to non-mycorrhizal (NM) plants which exhibited a significant growth reduction at the same soil Zn and Cd level. M inoculation reduced the Zn and Cd accumulation in shoot and showed higher Zn and Cd contents in roots which showed a different Zn and Cd distribution in AMF associated or non-associated plants. Mycorrhizal plants increased phosphorus (P) contents at all Zn and Cd concentrations except the highest (600 and 900 mg·kg^{-1}) leading significant alterations in biochemical contents such as proline, antioxidant enzymes in leaves and also in nutrients (N, P, K, Cu, Ni, Fe, Mn). Zn and cadmium toxicity cause to increase the proline content in shoot of NM plants, however, proline contents are lower in M plants. Results confirmed that AMF protected alfalfa plants against Zn and Cd toxicity. Mycorrhizal colonization was able to form an efficient symbiosis with alfalfa plants in moderately contaminated Zn and Cd soils (300 mg·kg^{-1}) and play an important role in food quality and safety.

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Keywords

Zinc, Cadmium, Arbuscular Mycorrhizal Fungi, Alfalfa, Phosphorus, Antioxidant Enzymes

1. Introduction

Soil pollution by heavy metals due to anthropogenic activities is the most important problem nowadays because metals are noxious, persistent and non-biodegradable. They tend to accumulate readily in soils and organisms, mainly where effects of human activities are severe. Cadmium (Cd), a non-essential element, is considered as more toxic because it tends to accumulate more readily in the environment especially in biological organisms even at low concentrations in the environment, leading to harsh consequences [1]. There are many sources through which the level of heavy metals are increasing in the environment. The main sources are mining, refining, and electroplating activities.

Cd is rarely present alone in soil and is mostly linked to other heavy metals such as high levels of zinc (Zn). In polluted soils, Cd and Zn uptake in plants and soils are associated [2]. Zn as an essential element is a second major concern because of its toxicity. Zn toxicity may occur in soils polluted by human activities such as mining, smelting and application of sewage sludge in agricultural soils [3]. Many studies revealed the aspects which influenced the bioavailability of metals in plants. The most important factors which affects metal uptake in plants are: pH, redox potential, texture, organic matter, mineral composition, temperature and water regime [4].

In soil microorganisms, arbuscular mycorrhizal (AM) fungi are commonly studied because of their capacity to develop plant strength under toxic and inappropriate conditions [5]. Due to colonization with most of terrestrial plants, these symbiotic fungi enhance essential nutrients to plants such as low available P which cause to increase shoot biomass [6]. AM fungi increase acquisition of nutrients by increasing soil volume [7]. This strategy can be useful for host plants that are growing in unfavourable soil conditions like in nutrient deficient soils or in contaminated areas. AM fungi can also improve the plants growth and nutrient contents by decreasing the metals uptake in plants [8].

The non-essential elements such as Cd can also transfer by AM fungi towards plants and store them in roots [9]. However, AM fungi have differential effects on metal uptake and growth of host plant [10] as in some cases AM fungi enhance the uptake of nutrients and the growth of host plants [11]. AM fungi isolated from polluted areas are thought to have a better effect towards plants than isolates from non polluted soils [12]. The metal accumulation of AM fungi is not easy to consider because they cannot be grown without the plant. It has been reported that *Medicago sativa* (alfalfa) accumulate heavy metals concentrations more than the permissible levels in different plant tissues. This may be due to specialized chemical functional groups that could be responsible for metal accumulation. Improvements in plant mineral nutrition are mainly related to elemental uptake by extra-radical hyphae from the non-rhizosphere soil region and its transport to the root [13]. AMF inoculation has been shown to be advantageous for the development of sustainable agriculture in nutrient-deficient tropical soils showing AMF to be an undiscovered resource for sustainable management and soil conservation.

In the study, we used alfalfa as a test plant because it is one of the most popular species used for perennial grazing and is widely cultured on the global scale for medicinal purposes. *Medicago sativa* (alfalfa) is a flowering plant in the pea family Fabaceae. It is a perennial legume from three to twelve years, depending upon climate and variety [14]. The objective of this study was to investigate the responses and development of *Medicago sativa* plants in association or not with AMF with increasing Cd and Zn concentrations in soil. The uptake of nutrients and the distribution of Cd and Zn in different plant organs were evaluated. Additionally, total protein and proline contents in leaves were analysed as biochemical indicators of metal stress in alfalfa plants and discussed in relation to their mycorrhizal status. The hypothesis of the present study was that AMF associated-*Medicago sativa* plants would perform better under metal stress conditions than non-associated plants, improving tolerance, nutrition and consequently, plant growth.

2. Materials and Methods

2.1. Experimental Design and Soil Preparation

A pot culture experiment was installed under controlled laboratory conditions. The treatments were either in-

oculation or non-inoculation of the AM fungi and the addition of zinc (0, 100, 300 and 900 mg/kg) and cadmium concentrations (0, 100, 300, 600 mg/kg) to soil. The sample of soil and sand were collected from the top layer (0 - 20 cm) in the vicinity of Quaid-i-Azam University, Islamabad. The area has subtropical climate, with a mean temperature of 19˚C - 25˚C and an average rainfall of 31 mm. 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 $mg{\cdot}kg^{-1}$), T. Potassium (19.5 $mg{\cdot}kg^{-1}$), Calcium (34.45 $mg{\cdot}kg^{-1}$), Magnesium (42.50 $mg{\cdot}kg^{-1}$), Extractable nitrate nitrogen (1.04 $mg{\cdot}kg^{-1}$), Extractable potassium (1.45 $mg{\cdot}kg^{-1}$), Extractable phosphorus (1.53 $mg{\cdot}kg^{-1}$), Zinc (1.50 $mg{\cdot}kg^{-1}$), Nickel (1.33 $mg{\cdot}kg^{-1}$), Copper (30.3 $mg{\cdot}kg^{-1}$), Cadmium (1.60 $mg{\cdot}kg^{-1}$), Iron (28.51 $mg{\cdot}kg^{-1}$), Lead (1.6 $mg{\cdot}kg^{-1}$), Chromium (4.25 $mg{\cdot}kg^{-1}$) and Manganese (10.4 $mg{\cdot}kg^{-1}$) 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. $ZnCl_2$ and $CdCl_2$ were added to the growth medium as Zn and Cd stress respectively.

2.2. Inoculum of Glomus Species

The AMF used was the mixture of different Glomus sp 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 (10 cm diameter and 12 cm height) contained 2 kg growth medium plus 50 g of AM fungal inoculum to mycorrhizal treatments, while the same amounts of growth medium were added to non-mycorrhizal treatments. Each pot received approximately 2500 spores at the time of sowing. AMF inoculation was performed during the transplantation process and was not provided in non-mycorrhizal treatments.

2.3. Sterilization of Alfalfa Seeds

Seeds of alfalfa (*Medicago sativa* L.) were obtained from Department of Crop Science, National Agriculture Research Centre, Islamabad. Seeds were surface sterilized (10 min, 3% Chlorox) and gently washed by deionized water for several times at room temperature and then put them on the sterile moist filter papers (Xin Hua No. 101, China) in Petri dishes at 28˚C for 48 hours for germinating. 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. Seedlings were grown in the growth chamber with 12 h light per day at 25˚C - 35˚C. Water lost was replaced daily by top watering with deionized water and 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 (20 ml) every week. Six pots per treatment were used and seedlings were randomly harvested 60 days after sowing.

2.4. Evaluation of Mycorrhizal Colonization

Root mycorrhizal colonization was estimated after clearing and staining [15] using the grid-line intersect method [16]. 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.

2.5. 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 (ECV150C, China).

2.6. Plant Biomass

At harvest, roots and shoots were separated. Subsamples 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.

2.7. Analytical Determinations

2.7.1. Heavy Metals Quantification

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 Whatman 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-vanadomolybdate method [17]. Total N was determined by Kjeldahl method [18].

2.7.2. Biochemical Analysis

Chlorophyll content in the fresh leaves (50 mg) of the plant was measured in 10 cm^3 dimethylsulfoxide (DMSO) by using the method [19]. Carotenoid and sugar content was determined by the method [20]. Proline content of leaves was estimated by using the method [21]. Protein content in the leaves (50 mg) of the plants was measured using Bovine Serum Albumen (BSA) as a standard [22].

2.7.3. Assay of Enzyme Activity

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 [23]. 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 [24]. Catalase (CAT) activity was determined by the method [25]. The activity of Ascorbate peroxidase (APX) was measured by estimating the rate of ascorbate oxidation. The change in absorbance was monitored at 290 nm [26].

2.8. Quality Control Analysis

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.

2.9. Statistical Analysis

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.

3. Results

3.1. Mycorrhizal Colonization of Roots

Figure 1 shows the percentage of AMF colonization with roots of alfalfa (*Medicago sativa*) plants with increasing Zn and Cd concentrations. The results showed AMF colonization was not found in non-inoculated plants, while all the inoculated plants showed high colonization rates. The colonization was detected in roots with formation of arbuscules and hyphal structures. **Figure 1(A)** shows the highest colonization of 70% was found at 100 $mg \cdot kg^{-1}$ Zn concentration. The trend was decreasing as the concentration of Zn increased at 300 $mg \cdot kg^{-1}$ and 900 $mg \cdot kg^{-1}$. **Figure 1(B)** shows the root colonization of alfalfa plants with increasing Cd concentrations.

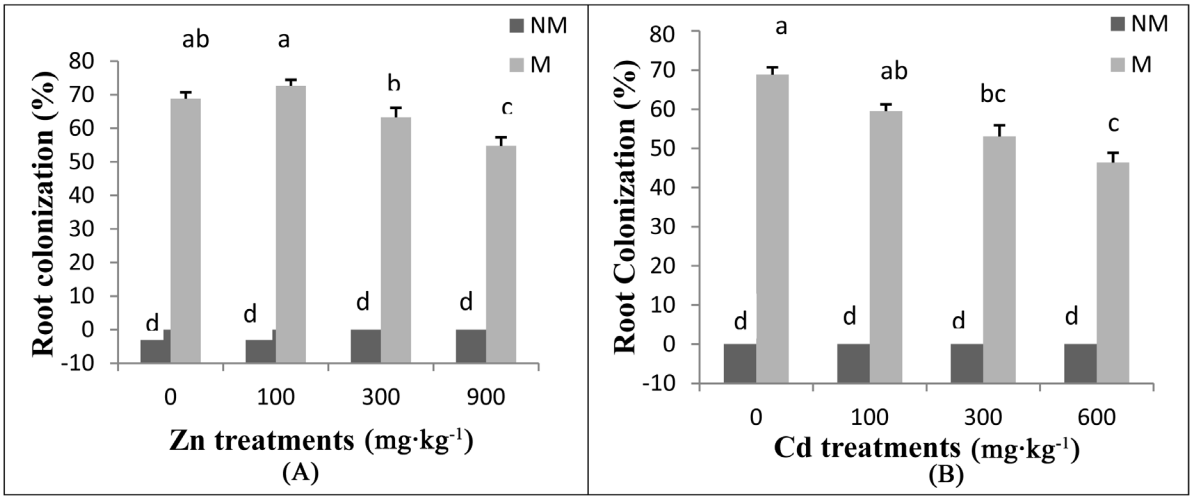


Figure 1. Colonization percentage of root length (% RLC) in mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants grown in soils with increasing Zn and Cd concentrations. M and NM means with different letters are significantly different by the Tukey test (5%).

The highest colonization was observed in control plants where no Cd concentration was applied. The trend observed was decreasing as the concentration of Cd increased from 100 to 300 mg·kg^{-1}. In general, the results showed that Zn and Cd addition negatively affects mycorrhizal root colonization and decreasing trend was observed with the increase of metal concentration in soil.

3.2. Plant Growth and Biomass

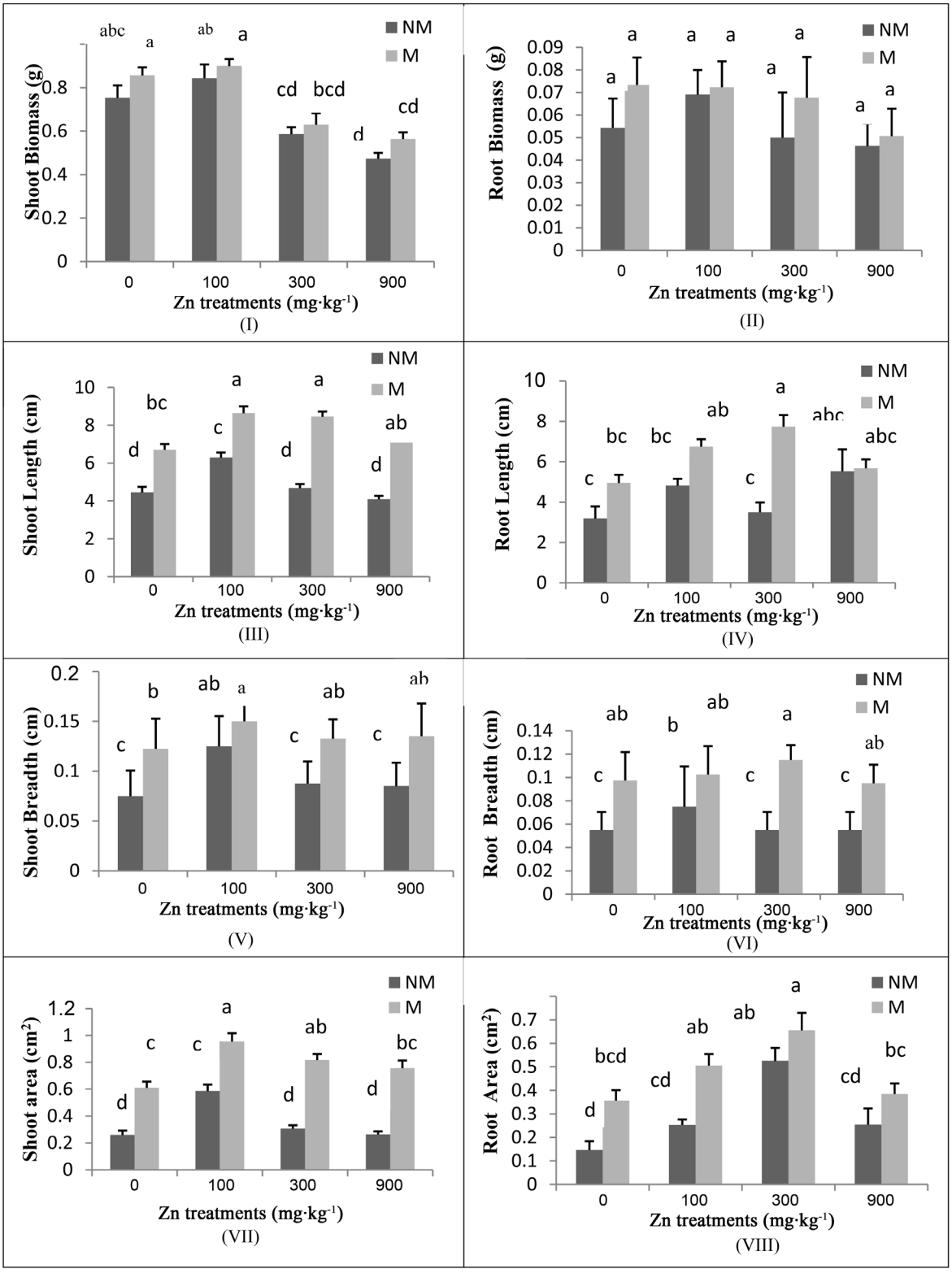
Figure 2(A) shows the effects of increasing zinc concentrations on growth and biomass of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants. The interaction of plants and AM fungi had very significant effects on growth and biomass of plants under increasing Zn stress. The reduced plant growth and biomass was observed in non-inoculated (NM) treatments under increasing Zn stress. However, the positive significant effects were observed on plants growth and biomass in mycorrhizal (M) inoculated treatments. The results showed that in M plants, shoot and root biomass was increased at all the Zn addition levels of 100, 300 and 900 mg·kg^{-1}, While reduction in biomass was observed in NM inoculated plants as the Zn concentration increased in soil. The highest trend was recorded at 100 mg·kg^{-1} Zn concentration in M and NM plants. While lowest biomass was recorded at 300 and 900 mg·kg^{-1} Zn concentration in both inoculated and non-inoculated treatments. The highest length, breadth and area of shoot and root tissues was observed at 100 mg·kg^{-1} Zn concentration in M and NM plants, while the reduction in trend was recorded at 300 and 900 mg·kg^{-1} of Zn concentration in both M and NM plants. However, M inoculated plants had significant positive effects on plants growth and biomass at all Zn treatments as compared to NM plants.

Figure 2(B) shows the effects of increasing cadmium concentrations on growth and biomass of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants. The interaction of plants and AM fungi had very significant effects on growth and biomass of plants under increasing Cd stress. The plant growth and biomass was reduced in non-inoculated (NM) treatments under increasing Cd stress. The highest trend was recorded in control plants in M and NM plants, while the lowest biomass was recorded at 100, 300 and 600 mg·kg^{-1} Cd concentration in both inoculated and non-inoculated treatments. The shoot and root growth was enhanced in M inoculated plants while decrease in growth was observed in NM inoculated plants. The highest length, breadth and area of shoot and root tissues was observed in control plants in both M and NM plants. While the reduction in trend was recorded at all cd addition levels (100, 300 and 600 mg·kg^{-1}) in both M and NM plants.

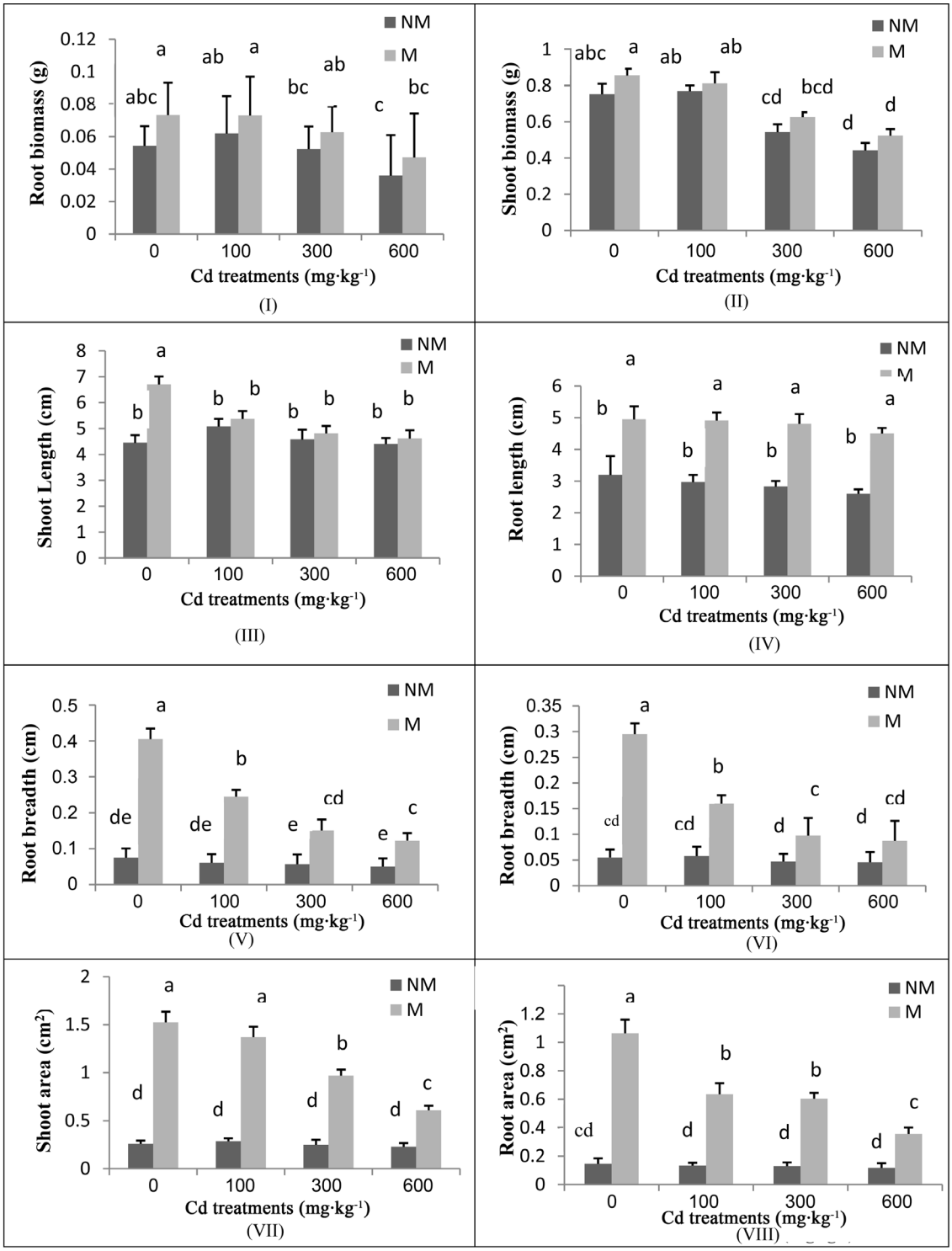
3.3. Plant Nutrient Contents

Table 1(A) and **Table 1(B)** show macro and micronutrients contents in shoots and roots of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants grown in soils with increasing Zn concentrations. In general, the increased

nutrient contents (N, K, Ca, Mg, Na, Fe, Cu) were observed in M treatments (100 and 300 mg·kg^{-1}) except in Mn and Ni, in which the trend was decreasing in mycorrhizal treatments as the concentration of Zn was increased.



(A)



(B)

Figure 2. (A) Effects of increasing zinc concentrations on growth and biomass of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants: (I, II) Shoot and root biomass, (III, IV) Shoot and root length, (V, VI) Shoot and root breadth, (VII, VIII) Shoot and root area. M and NM means with different letters are significantly different by the Tukey test (5%). (B) Effects of increasing cadmium concentrations on growth and biomass of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants: (I, II) Shoot and root biomass, (III, IV) Shoot and root length, (V, VI) Shoot and root breadth, (VII, VIII) Shoot and root area. M and NM means with different letters are significantly different by the Tukey test (5%).

Table 1. (A). Macronutrient contents measured in shoots and roots of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants grown in soils with increasing Zn concentrations. (B). Micronutrient contents measured in shoots and roots of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants grown in soils with increasing Zn concentrations.

(A)

Experiment (Zn, mg·kg^{-1})		N (g·kg^{-1})	K (g·kg^{-1})	Ca (g·kg^{-1})	Mg (g·kg^{-1})	Na (g·kg^{-1})
0	NM	0.26 ± 0.0458 ab	26.89 ± 4.2547 ab	17.747 ± 2.8582 abc	13.047 ± 1.5734 a	17.509 ± 5.1792 ab
	M	0.34 ± 0.0635 ab	29.783 ± 2.0910 a	22.997 ± 1.6896 a	15.747 ± 0.8079 a	29.135 ± 0.4642 a
100	NM	0.25 ± 0.0624 ab	17.863 ± 1.2801 bc	15.967 ± 1.2731 bcd	16.770 ± 2.2291 a	17.217 ± 1.3811 ab
	M	0.3667 ± 0.0318 a	22.187 ± 1.8409 abc	19.343 ± 0.5625 ab	17.337 ± 1.4834 a	23.307 ± 6.4599 ab
300	NM	0.2067 ± 0.0593 ab	16.07 ± 1.4586 c	11.217 ± 0.7254 cd	14.027 ± 1.0267 a	15.590 ± 1.8537 ab
	M	0.3267 ± 0.0353 ab	18.673 ± 0.7576 bc	14.747 ± 0.8079 bcd	16.897 ± 0.7442 a	26.696 ± 1.8572 ab
900	NM	0.116 ± 0.0296 b	12.343 ± 1.0002 c	10.45 ± 0.5021 d	11.467 ± 0.4378 a	12.477 ± 1.3603 b
	M	0.236 ± 0.0291 ab	15.887 ± 1.5258 c	13.927 ± 0.9324 bcd	12.443 ± 1.4404 a	15.033 ± 2.3617 ab
0	NM	0.18 ± 0.0346 abc	10.917 ± 0.7294 bcd	9.480 ± 0.4246 ab	10.583 ± 0.5605 ab	12.007 ± 0.9444 b
	M	0.2867 ± 0.0318 a	13.52 ± 1.3718 abc	11.45 ± 0.5700 a	11.443 ± 2.0367 a	16.673 ± 0.6343 a
100	NM	0.23 ± 0.0289 ab	14.157 ± 0.8920 abc	9.817 ± 1.1249 ab	9.043 ± 1.1767 ab	10.04 ± 0.8271 bc
	M	0.2767 ± 0.0233 a	18.193 ± 1.0045 a	10.027 ± 0.8233 ab	10.443 ± 1.1970 ab	11.217 ± 1.1799 bc
300	NM	0.13 ± 0.0173 bc	12.657 ± 1.0397 bcd	8.09 ± 0.5516 ab	5.443 ± 0.6451 ab	8.43 ± 0.6409 bc
	M	0.2467 ± 0.0410 ab	14.74 ± 0.8087 ab	9.433 ± 0.4902 ab	8.703 ± 0.9034 ab	9.11 ± 1.2677 bc
900	NM	0.0833 ± 0.0203 c	8.583 ± 0.6648 d	7.347 ± 0.9700 b	5.110 ± 1.2303 b	7.437 ± 0.5305 c
	M	0.1667 ± 0.0328 abc	9.447 ± 0.3163 cd	8.180 ± 0.9905 ab	4.633 ± 0.8287 b	8.083 ± 0.6759 c

(B)

Experiment (Zn, mg·kg^{-1})		Mn (mg·kg^{-1})	Fe (mg·kg^{-1})	Ni (mg·kg^{-1})	Cu (mg·kg^{-1})
			Shoot		
0	NM	179.35 ± 18.715 a	73.030 ± 4.3985 abc	12.813 ± 1.6108 ab	8.040 ± 0.7744 a
	M	133.87 ± 6.5347 ab	92.547 ± 2.5647 a	8.740 ± 0.8173 b	8.953 ± 0.8108 a
100	NM	164.25 ± 17.651 ab	61.837 ± 3.1418 bc	14.090 ± 0.6005 ab	10.147 ± 0.8714 a
	M	113.14 ± 16.125 ab	70.483 ± 4.2766 abc	9.103 ± 1.1201 ab	11.453 ± 0.5167 a
300	NM	109.79 ± 16.967 ab	56.30 ± 8.0698 bc	14.983 ± 1.9024 ab	10.473 ± 2.0368 a
	M	95.06 ± 7.9648 b	83.567 ± 7.4396 ab	9.4 ± 0.5541 ab	15.577 ± 4.0707 a
900	NM	118.22 ± 10.116 ab	48.640 ± 8.1033 c	15.363 ± 1.2835 a	10.873 ± 0.7902 a
	M	125.48 ± 16.293 ab	52.807 ± 5.6784 c	10.857 ± 0.7449 ab	11.763 ± 1.0327 a
			Roots		
0	NM	81.890 ± 8.0254 ab	38.263 ± 10.026 a	8.77 ± 0.8260 ab	9.377 ± 0.4824 ab
	M	57.25 ± 9.4086 ab	45.68 ± 5.8473 a	5.813 ± 0.9385 b	11.177 ± 0.8312 ab
100	NM	63.573 ± 12.077 ab	54.93 ± 4.9003 a	8.48 ± 0.5522 ab	9.413 ± 0.5820 ab
	M	46.923 ± 3.1745 b	65.51 ± 4.4623 a	6.373 ± 0.5500 ab	13.82 ± 0.9789 a
300	NM	96.073 ± 4.3756 a	48.93 ± 4.2158 a	10.067 ± 0.7166 a	10.85 ± 1.5069 ab
	M	45.513 ± 7.0815 b	52.62 ± 8.9798 a	5.78 ± 0.9777 b	13.143 ± 1.4814 a
900	NM	82.947 ± 13.586 ab	46.227 ± 10.878 a	9.88 ± 0.7850 ab	7.54 ± 0.6116 b
	M	92.56 ± 9.3940 a	47.403 ± 5.5555 a	7.36 ± 0.7900 ab	9.397 ± 0.5174 ab

Means (n = 3) with the different letters are significantly different (P <0.05) by the Tukey test.

The statistical significance was obtained for K, P, Na, N, Ca, Mn in M and NM plants but not significant results obtained in Cu and Mg at 0, 100, 300 and 900 mg·kg^{-1} Zn. The detrimental effect of highest Zn concentration (900 mg·kg^{-1}) was recorded on the concentration of the analyzed nutrients as there was a significant decrease in both inoculated (M) and non inoculated (NM) plants.

The result of the experiment indicated that mycorrhizal inoculation significantly affects the mineral nutrition of alfalfa plants. In M inoculated plants, the increase in K, N, Ca, Mg, Na, Cu, Ni was recorded in shoot part of plants but decrease in Mn and Fe contents was recorded. In M roots, the Fe, Ni, Cu contents was increased, while reduction of K, N, Ca, Mg contents was also observed. In NM inoculated plants, the increase of soil Zn concentrations caused reductions in K, P, N, Mn, Ni and Fe contents in the shoots except Cu and Na in which the increase in concentration was observed in shoots of NM plants. The increase of nutrient contents were observed in N, Ca, Na, K and Ni concentrations in roots of the alfalfa plants with increasing soil Zn concentration.

Table 2(A) and **Table 2(B)** show macro and micronutrients contents in shoots and roots of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants grown in soils with increasing Cd concentrations. In general, all nutrient contents were increased in M inoculated shoot part of plants except Mn, Ni, Cu, and Zn. However, the decrease in nutrient contents was observed in root part of plants except in Zn and Cu. The statistical significance was obtained for K, P, Na, N, Ca, Mg, Fe in shoot tissue but not significant results obtained in Mn and Ni at 0, 100, 300 and 900 mg·kg^{-1} Zn. The detrimental effect of highest Zn concentration (900 mg·kg^{-1}) was recorded on the concentration of the analyzed nutrients as there was a significant decrease in both inoculated (M) and non inoculated (NM) plants.

The result of the experiment indicated that mycorrhizal inoculation significantly affects the mineral nutrition of alfalfa plants. In NM inoculated plants, the increase of soil Zn concentrations caused decrease in K, N, Ca, Na, Mg, Fe, Mn, Ni and Zn contents in the shoots except Cu in which the increase in contents was observed in shoots of NM plants at all Zn concentrations of 100, 300, 900 mg·kg^{-1}. While, the decrease of nutrient contents were observed in roots part of alfalfa plants with increasing soil Cd concentrations.

3.4. Plant Phosphorus (P) Uptake

Figure 3 shows trend of plant phosphorus contents in M and NM inoculated plants under increasing Zn and Cd concentrations. The results showed that P content was increased in M inoculated root and shoot part of plants under Zn and Cd stress. However, the decreasing trend was recorded in NM root and shoot tissues of plants. In general, Plant P nutrition was improved by mycorrhizal inoculation. **Figure 3(A)** shows that M plants exhibitng significantly ($P < 0.001$) higher shoot and root P at Zn concentration of 100 mg·kg^{-1}, while the P content was decreased at the Zn concentration of 300 and 900 mg·kg^{-1} in both M and NM plants. In the experiment, the shoots of mycorrhizal plants maintained higher P level than shoots of non mycorrhizal plants in 0 and 100 mg·kg^{-1} Zn in soil, but not at 300 and 900 mg·kg^{-1}. At this level, plant growth also was very limited. The trend of P uptake was decreased as the Zn concentration was increased.

Figure 3(B) shows trend of plant P contents in M and NM inoculated plants under increasing Cd concentrations. The trend was decreasing as the concentration of Cd increased at 100, 300 and 600 mg·kg^{-1} in both M and NM plants. In control plants, the increased P contents were recorded in both shoot and root part of plants. However, the reduced P contents were recorded as the Cd concentration increased in both M and NM plants.

3.5. Zinc and Cd Uptake in Alfalfa Plants

Figure 4 shows the Zn and Cd concentration in plant tissues was linearly correlated to the soil concentration. The Zn and Cd concentration was increased in shoot and root parts of plants as the concentration of metals increased in soil. However, the results indicated that NM plants accumulated more concentration of Zn and Cd in the shoots and roots at all Zn (100, 300, 900 mg·kg^{-1}) and Cd (100, 300, 600 mg·kg^{-1}) treatments than M inoculated plants. In general, NM inoculated plants accumulated more Zn and Cd in both shoot and root parts of plants as compared to M inoculated plants. In control treatments when no Zn and Cd was applied, shoot and root Zn and Cd uptake were similar but as Zn and Cd application rate increased, shoot Zn and Cd uptake increased much less than root Zn and Cd uptake. The uptake of Zn and Cd decreased by M roots and shoots compared with NM plants. However, as the application of Zn and Cd increased, the concentration increased in shoot and root of NM plants as compared to M plants.

Table 2. (A) Macronutrient contents measured in shoots and roots of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants grown in soils with increasing Cd concentrations. (B) Micronutrient contents measured in shoots and roots of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants grown in soils with increasing Cd concentrations.

(A)

Experiment (Cd, mg·kg^{-1})		N (g·kg^{-1})	K (g·kg^{-1})	Ca (g·kg^{-1})	Mg (g·kg^{-1})	Na (g·kg^{-1})
0	NM	0.4867 ± 0.0233 b	15.967 ± 1.5458 ab	24.997 ± 2.8266 a	19.297 ± 2.5794 ab	7.523 ± 0.5344 abc
	M	1.03 ± 0.1127 ab	17.18 ± 2.6684 a	27.330 ± 3.7871 a	22.253 ± 2.4095 a	9.547 ± 0.5704 ab
100	NM	0.66 ± 0.1582 ab	10.62 ± 1.1116 bcd	25.623 ± 4.6407 a	11.957 ± 1.1261 c	8.303 ± 1.2822 abc
	M	1.26 ± 0.2629 a	13.82 ± 0.9771 abc	20.033 ± 2.8451 ab	13.52 ± 0.6393 bc	12.150 ± 2.0924 a
300	NM	0.6567 ± 0.0649 ab	7.627 ± 1.2209 d	13.913 ± 0.7760 b	9.36 ± 0.449 c	5.410 ± 0.3635 bc
	M	0.8133 ± 0.1870 ab	8.92 ± 0.8346 cd	12.453 ± 1.1903 b	10.447 ± 0.4101 c	6.993 ± 0.4668 bc
600	NM	0.5867 ± 0.0867 ab	4.627 ± 0.5434 d	9.873 ± 0.8239 b	7.137 ± 0.9732 c	4.417 ± 0.6567 c
	M	0.6633 ± 0.0639 ab	6.077 ± 0.7849 d	10.950 ± 0.9100 b	7.583 ± 0.7262 c	4.383 ± 0.4822 c
0	NM	0.53 ± 0.1021 abc	6.9533 ± 1.0552 ab	6.7067 ± 0.4461 ab	6.7067 ± 0.7216 ab	5.82 ± 0.7744 a
	M	0.8067 ± 0.0441 a	7.8467 ± 1.2444 a	9.2533 ± 0.8080 a	9.2533 ± 1.4912 a	6.5433 ± 1.3462 a
100	NM	0.5533 ± 0.1192 abc	5.2133 ± 0.6744 ab	5.33 ± 0.3500 b	5.33 ± 1.2052 b	5.62 ± 1.0283 a
	M	0.6367 ± 0.1161 ab	6.3667 ± 0.7002 ab	5.5467 ± 0.4086 b	5.5467 ± 0.4740 b	6.14 ± 0.8114 a
300	NM	0.3367 ± 0.0260 bc	4.5967 ± 0.4736 ab	4.8467 ± 0.9995 b	4.8467 ± 0.7882 b	4.6567 ± 0.5161 a
	M	0.4 ± 0.0458 bc	5.1133 ± 0.4388 ab	5.0267 ± 0.0845 b	5.0267 ± 0.5851 b	6.2567 ± 0.7213 a
600	NM	0.1967 ± 0.0498 c	3.8567 ± 0.4160 b	3.7067 ± 0.4677 b	3.7067 ± 0.7361 b	4.4867 ± 0.3886 a
	M	0.2867 ± 0.0406 bc	4.2333 ± 0.4532 ab	4.5933 ± 0.7188 b	4.5933 ± 0.6728 b	3.96 ± 0.6243 a

(B)

Experiment (Cd, mg·kg^{-1})		Mn (mg·kg^{-1})	Fe (mg·kg^{-1})	Ni (mg·kg^{-1})	Cu (mg·kg^{-1})
Shoot					
0	NM	71.733 ± 0.0350 a	46.543 ± 4.0441 ab	6.29 ± 0.9585 a	9.507 ± 0.6093 a
	M	54.213 ± 0.3947 a	50.853 ± 4.3645 a	5.7467 ± 0.7115 a	5.027 ± 0.5155 b
100	NM	57.060 ± 4.7509 a	31.807 ± 5.9041 b	4.4133 ± 0.5190 a	11.45 ± 0.7044 a
	M	49.323 ± 2.2645 a	46.747 ± 1.7822 ab	4.8467 ± 0.7568 a	9.983 ± 0.1235 a
300	NM	55.14 ± 5.5078 a	42.257 ± 3.7790 ab	3.9233 ± 0.2200 a	13.447 ± 1.2788 a
	M	52.033 ± 9.3103 a	50.86 ± 3.2926 a	3.6933 ± 0.1642 a	11.587 ± 1.4921 a
600	NM	66.657 ± 11.871 a	32.843 ± 2.0503 ab	6.0367 ± 0.7799 a	13.817 ± 1.2432 a
	M	73.693 ± 0.9930 a	40.813 ± 3.0488 ab	4.5633 ± 0.3148 a	10.143 ± 1.4313 a
Roots					
0	NM	57.557 ± 0.3463 ab	26.523 ± 2.0653 ab	7.433 ± 0.9616 ab	19.033 ± 3.1755 ab
	M	49.573 ± 2.1903 bc	29.61 ± 3.2810 a	6.88 ± 0.8600 ab	14.847 ± 1.1866 b
100	NM	64.85 ± 3.9822 a	17.953 ± 1.7402 cde	10.483 ± 1.0721 a	16.697 ± 1.6717 b
	M	43.747 ± 4.2026 bc	21.517 ± 2.1262 bcd	7.517 ± 0.5279 ab	18.597 ± 0.6569 ab
300	NM	50.353 ± 3.1850 abc	16.817 ± 0.8762 cde	5.463 ± 0.4869 b	21.290 ± 1.4043 ab
	M	38.180 ± 1.9500 c	23.437 ± 2.6793 abc	4.783 ± 0.8417 b	24.89 ± 0.9469 a
600	NM	58.177 ± 2.4011 ab	14.51 ± 0.6416 e	9.74 ± 1.0761 a	14.557 ± 1.2846 b
	M	47.737 ± 0.6273 bc	16.287 ± 1.2858 de	7.857 ± 0.8027 ab	13.677 ± 0.3877 b

Means (n = 3) with the different letters are significantly different (P <0.05) by the Tukey test.

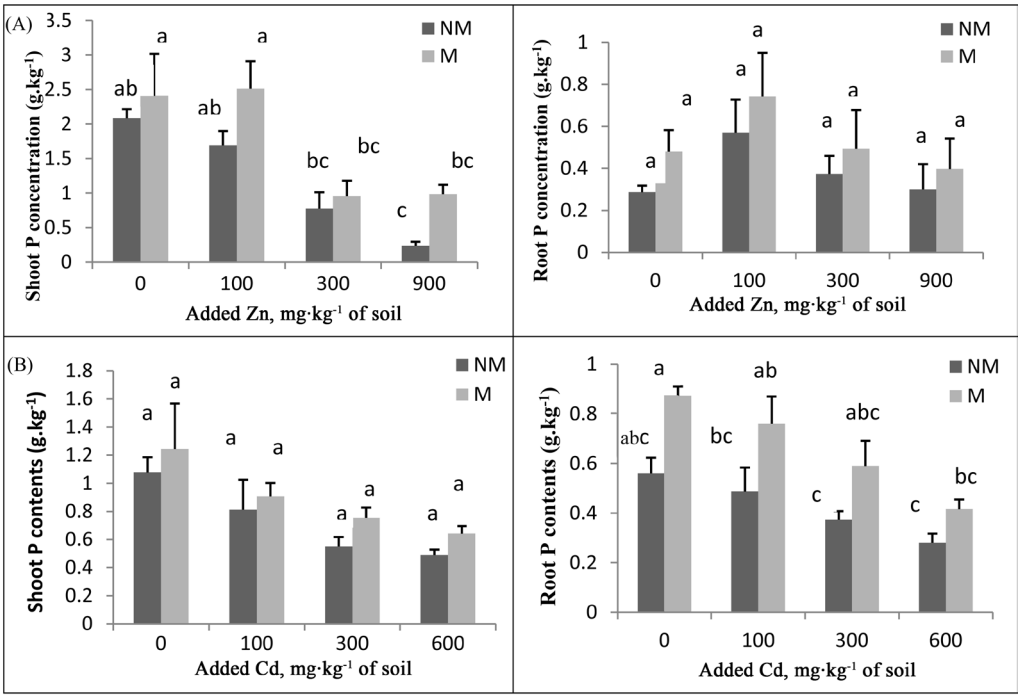


Figure 3. Phosphorus (P) contents in: (A) shoots and (B) roots of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants in response to Zn and Cd addition to soil. Means (n = 3) with the different letters are significantly different ($P < 0.05$) by the Tukey test (NM: black lines and M: light grey lines).

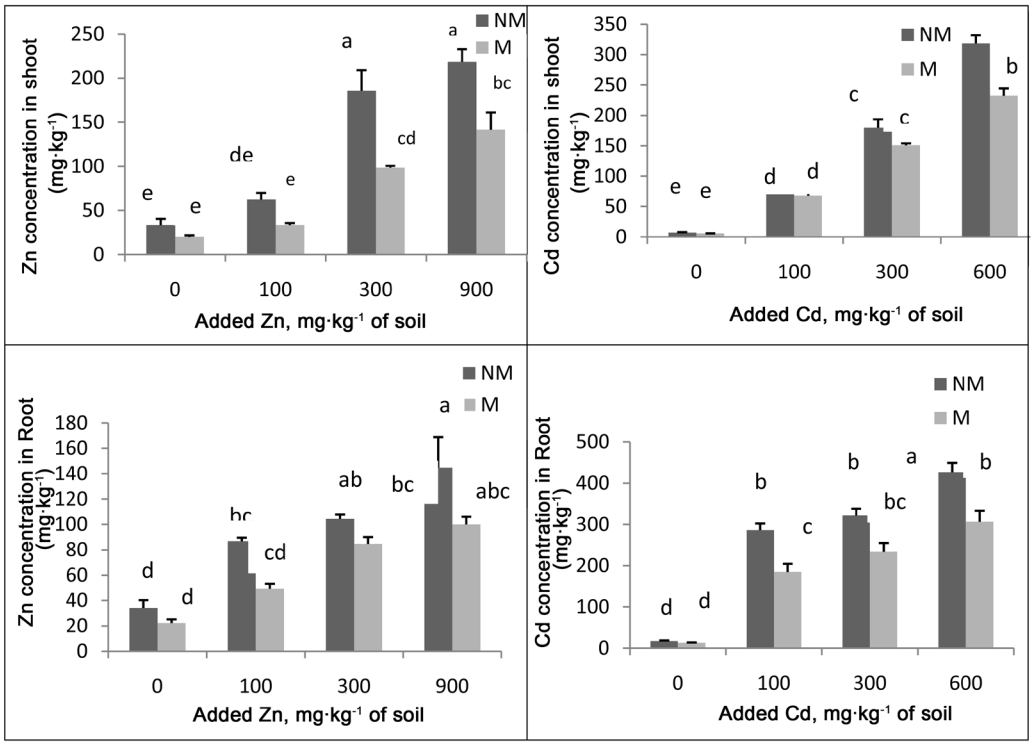
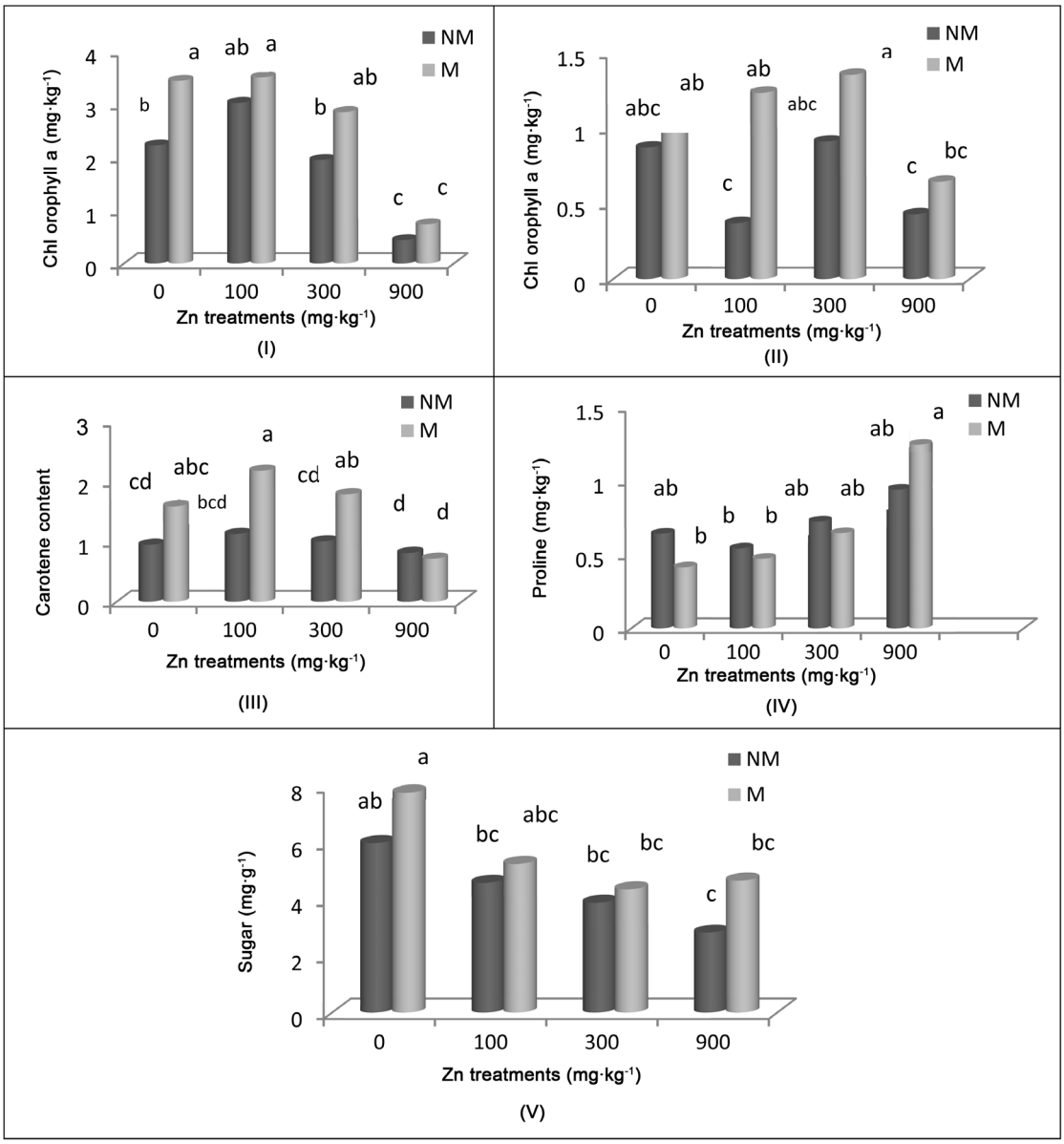


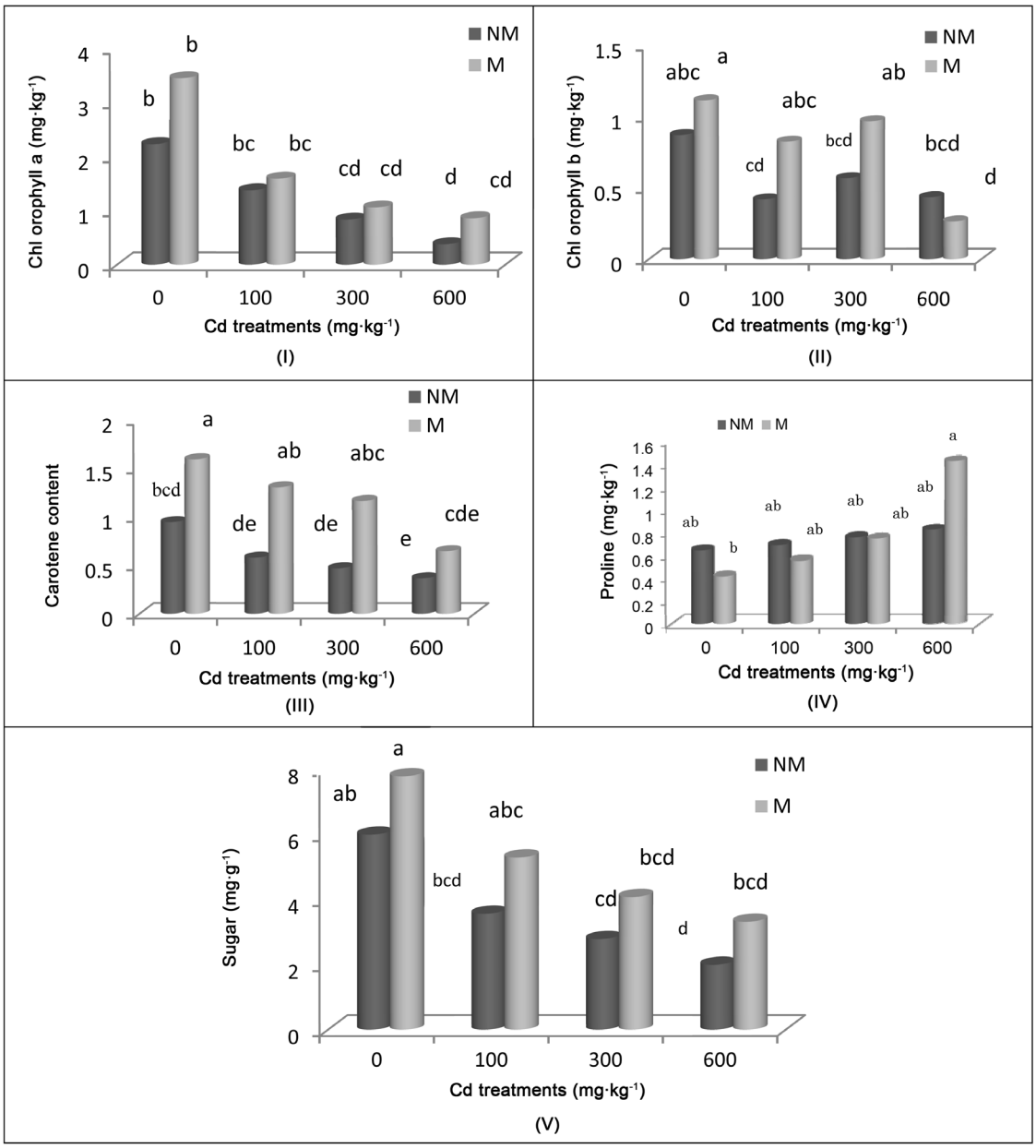
Figure 4. Cd and Zn concentrations in shoots and roots of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants growing in soil with increasing Cd or Znconcentrations, respectively. Means (n = 3) with the different letters are significantly different ($P < 0.05$) by the Tukey test (NM: black lines and M: light grey lines).

3.6. Effects of Metals Concentrations on Plants Biochemical Activities

Figure 5(A) and **Figure 5(B)** shows the biochemical indicators in M and NM inoculated plants with increasing Zn and Cd concentrations. In general, relative chlorophyll and carotene contents were significantly higher in M inoculated plants as compared to NM inoculated plants at each Zn and Cd concentration. **Figure 5(B)** shows the effects of Zn increasing concentrations on biochemical contents in M and NM inoculated plants. The highest contents of chlorophyll a and b contents were observed at Zn concentration of 100 mg·kg^{-1} in both inoculated and non-inoculated plants. The lowest contents were observed at highest Zn concentration of 900 mg·kg^{-1} in both inoculated and inoculated treatments. The sugar contents were decreased linearly as the Zn concentration increased in soil in both M and NM plants. The sugar content of M plants was significantly higher than those of NM plants at each Zn concentration (100, 300, 900 mg·kg^{-1}). The proline content in alfalfa plants increased as Zn concentrations increased in soil from 100 to 900 mg·kg^{-1} of soil. In NM plants, higher proline levels were recorded as compared to M inoculated plants. Total proline contents in plants were drastically increased with the increase in Zn concentration in the soil.



(A)



(B)

Figure 5. (a) Biochemical contents (I and II) Chlorophyll a, b content, (III) Total carotene content, (IV) Proline contents, and (V) sugar contents in leaves of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa in response to increasing Zn concentrations in soil. Means (n = 3) with the different letters are significantly different ($P < 0.05$) by the Tukey test. (NM: black lines and M: light grey lines). (b) Biochemical contents (I and II) Chlorophyll a, b content, (III) Total carotene content, (IV) Proline contents, and (V) sugar contents in leaves of mycorrhizal (M) and nonmycorrhizal (NM) *Medicago sativa* in response to increasing Cd concentrations in soil. Means (n = 3) with the different letters are significantly different ($P < 0.05$) by the Tukey test. NM: black lines and M: light grey lines).

Figure 5(B) shows the effects of increasing Cd concentrations on biochemical contents of M and NM inoculated plants. The results indicated that chlorophyll a, b and carotene contents were decreased as the concentration of Cd increased in soil. The higher chlorophyll and carotene contents were recorded in control plants in which no Cd was applied. The lower contents were recorded at 300 and 600 mg·kg^{-1} Cd concentration. In M plants, the higher chlorophyll and carotene contents were observed as compared to NM plants at all Cd concen-

tration (100, 300, 600 mg·kg^{-1}). The sugar content was decreased linearly as the concentration of Cd increased in soil. However, the more sugar contents was observed in M plants as compared to NM plants at all Cd concentration (100, 300, 600 mg·kg^{-1}). The proline contents were increased as the Cd concentrations increased in soil. The lower proline content was recorded at 100 mg·kg^{-1} of Cd concentration in both M and NM plants. The higher proline content was observed at 900 mg·kg^{-1} of Cd concentration.

3.7. Effects of Metals on Plants Antioxidant Enzyme Activities

Figure 6 shows the antioxidant enzymatic activity in leaves of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants in response to Zn and Cd addition to soil. **Figure 6(A)** shows the trend of SOD, CAT, APX and POD activities in M and NM plants with increasing Zn concentration (0, 100, 300, 900 mg·kg^{-1}). The trend was increasing in M and NM inoculated plants but their activity was induced in response to the increasing concentrations of Zn in the soil. In NM plants, SOD activity increased with increasing Zn addition to soil, except at Zn concentration of 900 mg·kg^{-1}. In M plants, an increase in SOD activity in response to 100 and 300 mg·kg^{-1} was observed, however the activity was reduced at 900 mg·kg^{-1} Zn concentrations.

Slight but statistically significant increase in leaf POD activity was observed in alfalfa plants after treatment with Zn concentration. The highest POD activity was observed at 100 mg·kg^{-1} Zn concentration in both M and NM plants. However, the decrease in POD activity was recorded at 300 and 900 mg·kg^{-1} Zn concentration. The CAT activity induced in the similar manner as the SOD activity. The activity was enhanced as the Zn concentration increased in the soil except at the highest Zn concentration (900 mg·kg^{-1}) in both M and NM plants. The APX activity was decreased as the Zn concentration increased in soil except at 100 mg·kg^{-1} in which the increased activity was recorded. In both M and NM plants, the trend of APX was same as the POD activity. The highest APX activity was observed at 100 mg·kg^{-1} in both M and NM plants.

Figure 6(B) shows the trend of SOD, CAT, APX and POD activities in M and NM plants with increasing Cd concentration (0, 100, 300, 600 mg·kg^{-1}). The trend was increasing in M and NM inoculated plants but their activity was induced in response to the increasing concentrations of Zn in the soil. In NM plants, SOD activity decreased with increasing Cd addition to soil, except at Cd concentration of 100 mg·kg^{-1}. In M plants, the increased SOD activity was observed at all Cd concentrations (0, 100, 300, 600 mg·kg^{-1}) as compared to NM inoculated plants. The reduced activity was recorded at 300 and 900 mg·kg^{-1} Zn concentration.

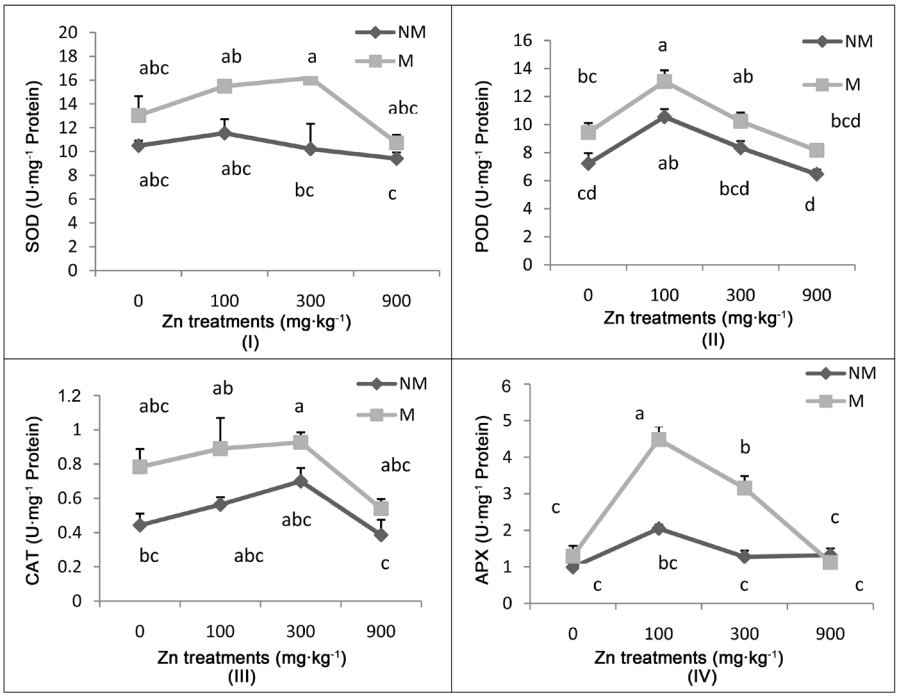
The leaf POD activity was increased in alfalfa plants as the concentration of Zn increased in soil. The lowest POD content was observed at 900 mg·kg^{-1} Zn concentration. The highest POD activity was observed at 300 mg·kg^{-1} Zn concentration in both M and NM plants. In M plants, the POD content was increased as compared to NM inoculated plants at all Zn concentration. The CAT activity was decreased as the Zn concentration increased in the soil except at the highest Zn concentration (100 mg·kg^{-1}) in both M and NM plants. The decreased APX activity was recorded as the Cd concentration increased in soil. The Cd concentrations (0, 100, 300, 600 mg·kg^{-1}) caused to decrease the APX activity in both M and NM inoculated plants.

4. Discussion

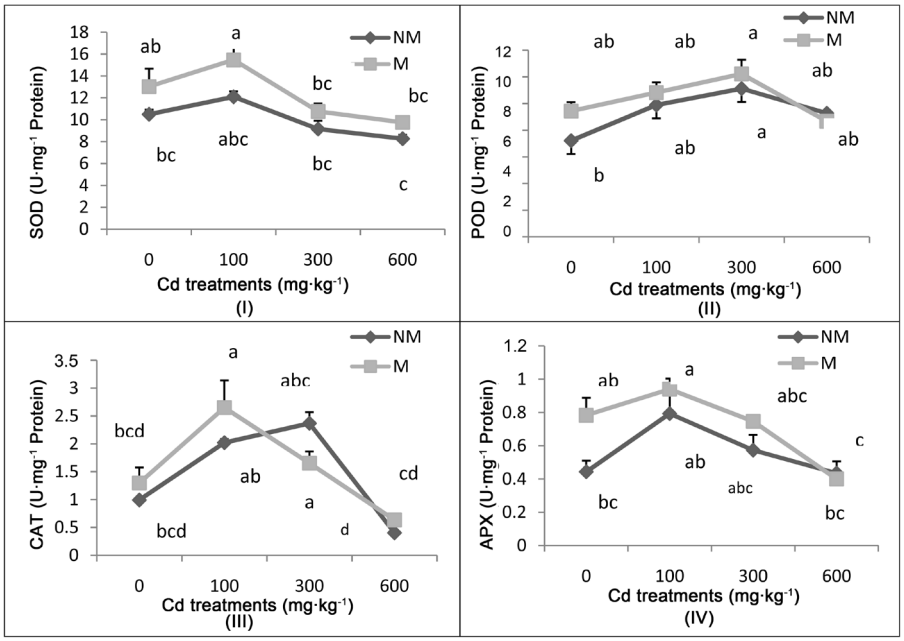
4.1. Effect of AMF Inoculation on Alfalfa Growth, Biomass and Colonization under Zn and Cd Toxicity

The results of the present study indicated increased alfalfa growth and biomass in the presence of AM fungi under Zn and Cd toxicity. However previous studies have shown that AMF has resistance to toxic metals found in the soil [27]. These results indicated that AM fungi were able to colonize plants roots under Zn and Cd polluted conditions and the useful effects of plant mycorrhizal interaction is primarily due to enhancement of P uptake by mycorrhizal fungus. The results also indicated that Cd and Zn toxicity had no negative effect on root colonization of plants with AM fungi interaction as compared to plants with no fungal interaction. The same results were reported by [28] [29]. The growth inhibition in plants grown under high levels of Zn and Cd was due to interference of these metals with P uptake by plants.

The benefits of the mycorrhizal symbiosis on plant growth and nutrition are well known and have been extensively studied for many plants. The application of Vesicular Arbuscular Mycorrhiza (VAM) fungi at contaminated sites increased plants biomass even at elevated levels of Zn and Cd in the soil [30]. It is also reported that root colonization of plants inoculated with non-indigenous AMF isolates to HM-contaminated soils was not



(A)



(B)

Figure 6. (A) Antioxidant enzymes activity (I) SOD activity, (II) POD activity, (III) CAT activity, (IV) APX activity, in leaves of mycorrhizal (M) and nonmycorrhizal (NM) alfalfa plants in response to Zn addition to soil. Means (n = 3) with the different letters are significantly different ($p < 0.05$) by the Tukey test. NM: black color lines and M: light grey lines. Bars represent standard error. (B) Antioxidant enzymes activity (I) SOD activity, (II) POD activity, (III) CAT activity, (IV) APX activity, in leaves of mycorrhizal (M) and nonmycorrhizal (NM) alfalfa 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. NM: black color lines and M: light grey lines. Bars represent standard error.

decreased by increasing the HMs additions to soil [31].

4.2. Effect of AMF on Zn and Cd Uptake in Alfalfa Plants

In the present study, the presence of AMF contributed more to the retention of Cd and Zn in alfalfa roots and also to soil stabilization. The reason of plant protection against Zn and Cd toxicity in plants inoculated with AMF may occur indirectly by enhancing plant nutrition and increasing plant growth therefore resulting in a diluting effect of Cd and Zn in plant tissues [32]. Also, mycorrhizal plants might actively diminish heavy metal uptake from soils by solubilising the metals via soil pH changes, which may be a defense strategy adopted by mycorrhizae to avoid or escape the negative impacts of high soil metal concentrations Furthermore, chelation/immobilization of metals by extraradical mycelium, glomalin, or exudates can sequester metals [33]. Therefore, inoculation of HM contaminated soils with AMF seems to be a strategy which can be suggested for enhancing plant growth in soil polluted with Zn and Cd. AM fungus sensitivity to excessive concentrations of heavy metals in the soil may have resulted in a negative effect on the functioning of the symbiosis. In addition to decrease rate of root colonisation, functioning of the symbiotic structures such as external mycelium and arbuscules are also decreased. Some previous reports suggested that Cd, Pb and Zn were strongly retained within roots of both mycorrhizal and non-mycorrhizal plants showing that plants have filtering mechanisms that cause to decrease the metal translocation to shoots [27].

4.3. Effects of AMF on Nutrients Uptake and Biochemical Contents under Zinc and Cd Toxicity

The present study reported that plants inoculation with AMF improved growth and shoot P, N, Fe, Mn and Zn uptake of plants in M inoculated plants polluted with Zn and Cd in comparison with only metals polluted soils. The beneficial effects of inoculation of plants and AM fungi on nutrients uptake may act as a protection mechanism that decreases Zn and Cd toxicity. The primary mechanism by which mycorrhizal fungi improve P uptake is through more extensive soil exploration rather than a unique capacity to mobilize sources of P not available to plants [34].

Mycorrhizal plants alleviate the severe effects of Zn and Cd by changing the translocation of metals and sequestering it in their hypha, so the toxic effects of Zn and Cd on photosynthesis and carbohydrate metabolism might decrease. The reduce amount of phosphorus observed in non-AM plants may be due to interference of toxic concentrations of Zinc and Cd with phosphorus uptake by alfalfa plants. The great amount of phosphorous in M plants emphasizes the enhancement of P uptake from the soil and its translocation to plants by the extra-radical mycelium of AM fungi [35]. These results indicated the beneficial effects of AM fungi in the protection of plants and alleviation of toxic effects of heavy metals. Therefore additional researches are needed to explore the behaviour of AM fungi in various plants species and familiar for plant protection under heavy metal stress.

The results of the study indicated that AMF associated alfalfa plants had better biochemical activities than non AMF plants under high Zn and Cd concentrations. However, the decreased chlorophyll and carotene content was observed at toxic concentrations of Zn (900 $mg \cdot kg^{-1}$) and Cd (600 $mg \cdot kg^{-1}$). [36] reported the same results that AM plants possesss greater amount of chlorophyll in comparison with non-AM plants. The increased protein and sugar contents were also reported in the present study. The process involves increase protein synthesis as well as induction of antioxidant enzymes to avoid heavy metal-mediated oxidative stress. In non mycorrhizal plants, reduction in total proteins content may be due to the toxic effects of Zn and Cd on cellular metabolism and protein synthesis.

5. Concluding Remarks

It is concluded from the result of the present study that mycorrhizal association with alfalfa plants has beneficial positive effects on growth, biochemical contents and antioxidant enzymatic activity. The plant grew faster, exhibited improved mineral nutrition and had higher yields than non-mycorrhizal seedlings. AMF protect the alfalfa plants against metal toxicity and also benefit for nutrient uptake. AM fungi immobilize heavy metals such as Zn and Cd in moderately polluted soils. The decrease Zn and Cd uptake in mycorrhizal plants could be associated with the decline of Zn and Cd availability resulting from the increase in soil pH caused by the AM fungi.

The obtained results indicate the importance of mycorrhization for alfalfa especially when it grows in soils with high levels of heavy metals. As some common agricultural practices and the increasing use of sewage sludge in agriculture may cause the accumulation of toxic metals in soils. Furthermore, experiments under field conditions should be performed to study the extent to which mycorrhizal fungi can alleviate Zn and Cd plant toxicity.

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