Effects of Arbuscular Mycorrhizal Fungi on Growth, Nutrient Status and Biochemical Activities of *Triticum aestivum* L. and *Medicago sativa* L. with increasing Zinc and Cadmium Concentrations

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In

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BY

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2013

APPROVAL CERTIFICATE

This is to certify that the dissertation entitled **"Effects of Arbuscular Mycorrhizal Fungi on Growth, Nutrient Status and Biochemical Activities of *Triticum aestivum* L. and *Medicago sativa* L. with increasing Zinc and Cadmium Concentrations''** submitted by **Sadia Kanwal** is accepted in its present form by the Department of Environmental Sciences, Quaid-i-Azam University Islamabad, Pakistan, as satisfying the dissertation requirement for the degree of M.Phil in Environmental Biology and Ecotoxicology

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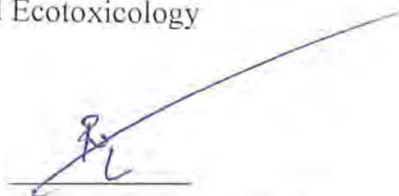

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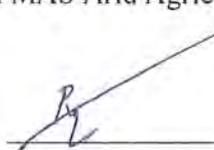

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DECLARATION

The whole of the experimental work including laboratory and field described in this thesis was carried out by me in the Laboratory of Environmental Sciences, Faculty of Biological Science, Quaid-i-Azam University, Islamabad. This thesis is conducted under Research Project that is funded by Higher Education Commission. The findings and conclusion are of my investigation with discussion of my research supervisor Dr. Riffat Naseem Malik. No part of this work has been presented for any other degree.

Sadia Kanwal

This thesis is dedicated to my loving

Parents

for their guidance, endless support

and encouragement

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LIST OF Abbreviations

S.no	Abbreviations	Words
1	AMF	Arbuscular mycorrhizal fungi
2	ANOVA	Analysis of variance
3	APX	Ascorbate peroxidase
4	BSA	Bovine Serum Albumen
5	Ca	Calcium
6	Cr	Chromium
7	CAT	Catalase
8	Cd	Cadmium
9	Co	Cobalt
10	Cu	Copper
11	DMSO	Dimethylsulfoxide
12	DW	Dry weight
13	ERH	Extraradical hyphae
14	FAAS	Flame atomic absorption spectrophotometer
15	Fe	Ferrous
16	FW	Fresh weight
17	GDP	Gross domestic product
18	GR	Glutathione reductase
19	IRH	Intraradical hyphae
20	K	Potassium
21	Mg	Magnesium
22	Mn	Manganese
23	Na	Sodium
24	NBT	Nitro blue tetra zolium salt
25	Ni	Nickel
26	NIST	National institute of science and technology
27	P	Phosphorus
28	Pb	Lead
29	POD	Peroxidase
30	ROS	Reactive oxygen species
31	SOD	Superoxide dismutase
32	VAM	Vesicular arbuscular mycorrhiza
33	Zn	Zinc

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3.81	Analysis of Variance Table for Zn ($mg.kg^{-1}$)

Abstract

The effects of mycorrhizal symbiosis on metal accumulation and plants tolerance are not commonly studied in cereal crops or medicinal plants under metal stress. The objective of this study was to assess the impact of mycorrhiza on wheat (*Triticum aestivum* L.) and alfalfa (*Medicago sativa* L.) plants with the increase of Zinc (Zn) and cadmium (Cd) toxicity. The experiment was conducted under controlled laboratory conditions in a completely randomized design. Zn and Cd uptake, some biochemical and physiological parameters were studied in eight weeks old wheat and alfalfa plants in response to inoculation or not with arbuscular mycorrhizal fungi (AMF) and with the increase of Zn (0, 100, 300, 900 $mgkg^{-1}$) and Cd concentrations (0, 100, 300, 600 $mgkg^{-1}$) in soil. The results showed that mycorrhizal (M) plants generally 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 accumulation of Zn and Cd in shoot parts of plants. Plants 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) nutrition at all Zn and Cd concentrations except the highest Zn (900 $mgkg^{-1}$) and Cd (600 $mgkg^{-1}$) concentration which cause to decrease the biomass leading to significant alterations in some biochemical contents such as proline, antioxidant enzymes in leaves and also in nutrients (N, P, K, Cu, Ni, Fe, Mn). Zn and Cd toxicity cause to increase the accumulation of proline in shoots and significant changes in antioxidant enzyme activities were also observed however proline contents were lower in M inoculated plants. Results confirmed that AMF protected wheat and alfalfa plants against Zn and Cd toxicity. This develops a positive association between fungus and plants that cause to improve plants performance. Hence, Glomus species was able to form an efficient symbiosis with wheat and alfalfa plants in moderately contaminated Zn and Cd soils (300 $mg.kg^{-1}$) and play an important role in food quality and safety.

Chapter 1

INTRODUCTION

Introduction

1.1 Soil heavy metal contamination

Soil comprises of mainly the rock, minerals and organic matter. The introduction of harmful substances or products into the environment is defined as pollution. Contamination is the presence of contagious material that renders a substance poisoned or harmful. Environmental pollution with inorganic contaminants is mainly due to excessive concentrations of heavy metals built up in the air, water and soil (Alloway, 2005). This reduces the ability to sustain life in that particular environmental matrix. Environmental pollution due to heavy metals is a global as well as regional and local issue. The industrial development is the main cause of biosphere pollution which has been increasing with the increase of development in industrial sector (Nriagu, 1989).

The natural sources are also cause of metals toxicity which includes dust, soil, volcano gas and forest fires etc (Fargasova, 1999). Nowadays, the most prominent is metal toxicity of the soil and mainly originates from increasing industrial and urban activities. The toxicity of metals depends on the concentration in which they are present in the soil (Smith and Read, 1997). The contamination of ecosystem is due to the transfer of harmful substances into non-polluted sites (Gaur and Adholeya, 2004). Previous studies indicated that the contamination of environment due to heavy metals had a bad effects on growth and development of plants and mineral nutrient status that eventually effects the plants structure (Mc Grath et al., 2001). Several researches indicated the main reasons for reduced plant growth are mainly the detrimental effect of metals toxicity on plant nutrient contents (Israr et al., 2006).

1.2. Zinc and Cadmium as an environmental contaminant

Among heavy metals, some selected metals are required as an important nutrient for normal plant growth, development and play structural roles in proteins (Aravind and Prasad, 2003). Zinc (Zn) is among those important nutrients that are required by plants in minute amounts. It also plays a role in membranes protection of biological organisms from oxidative damage, rupture of plasma membrane and changes

in permeability of membranes (Bettger and O'Dell, 1981). However, the excessive amount of Zn is also the main cause of toxicity in plants. The toxicity symptoms of Zinc in plants are nutrients deficiencies, inhibition of growth, yellowing of leaves and reduction of photosynthesis process (Todeschini et al., 2011; Cambrolle et al., 2012). Zn excessive concentrations cause toxicity in soil that harm directly to microbes present in soil which lead to reduction of crop productivity and are dangerous for food chain or food web (Hassan and Aarts, 2011).

Cadmium (Cd) is a non essential element and toxic to all biological organisms. Cd toxicity is detrimental for the morphological and physiological functions of organisms including humans, plants and animals. It is considered as one of the most phytotoxic heavy metal pollutants (Das et al., 1997). The main causes of Cd toxicity includes both natural and anthropogenic sources through which Cd enters into the environment. The natural ways through which Cd levels increases in the environment are rocks weathering, forest fires and volcanic eruptions. The anthropogenic sources that 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 entered into the food chain and poses hazards for both humans and animals health (Schützendübel et al., 2002). Many of the studies revealed that Cd exposure cause various abnormalities in plants like leaf chlorosis, biomass reduction and carbon integration (Krupa, and T. Baszynski, 1995) produce oxidative stress (Schutzendubel et al., 2002) cause to close stomata and decrease plant water uptake, damage root tips, growth and elongation (Milone et al., 2003; Liu et al., 2003; Daud et al., 2009), decrease nutrient uptake, inhibit photosynthesis and stunted plant growth (Das et al., 1997; Sgherri et al., 2002). Roots are mostly affected by heavy metals than shoots because of more roots metals accumulation (Yadav, 2010; Rascio and Navari-Izzo, 2011).

Metals toxicity cause to disturb various metabolic processes in plants. The harmful effects of metals toxicity on plants is recognized by effects such as inhibition of plant growth, less productivity and biomass, damage the structure of plants tissues or by alterations in various physiological and metabolic processes (Taylor, 1984).The level of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radicals are also increased with increase of metals toxicity (Israr et

al., 2006). The more ROS production in shoot and root of plants can stimulate oxidative stress. This stress cause to harm the structure of biomolecules in plant tissues such as lipid, protein and nucleic acid (Shalata and Tal, 1998). The antioxidant defense system comprises of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione reductase (GR) and ascorbate peroxidase (APX). These antioxidant system are the natural defense mechanism that provide protection to plants by removing ROS and H_2O_2 in cells. The mechanism of ROS elimination cause to lessen the oxidative damage in plants tissues that is the response of plants to environmental stress (Mittler, 2002).

1.3. Phytoremediation

Nowadays, there are a lot of expensive practices and techniques using for cleaning the metal polluted areas. The use of costly measures for the removal of heavy metals from contaminated sites are the main cause of prohibition of these techniques usage in many areas. Without some remediation techniques, the polluted soil become exposed to humans that cause many harmful effects. In addition to various physical ways used for cleaning of heavy metals contaminated areas, phytoremediation is a beneficial process or technique that is the use of different plants to eliminate pollutants and toxic metals. This is very useful technique which is still been explored. The use of plants and soil in combination to remove or lessen the toxicity of harmful metals or contaminants from the environment is defined as phytoremediation (Salt et al., 1998). Plants are used to cover the soil surface in order to avoid erosion, reduce water seepage and serve as a barrier to stop direct contact with the soil, to remove the contamination via extraction of the metal from the soil (Berti and Cunningham, 2000).

1.4. Role of Mycorrhiza in phytoremediation

The useful function of mycorrhiza in reduction of phytotoxicity has been generally known. Several studies revealed that plants in association with AMF have evolved different useful mechanisms. These mechanisms are the main cause to improve nutritional value, reduce metal uptake by immobilization of metals in roots and intracellular chelation by formation of metal complex in vacuoles (Andrade et al.,

2008). Studies reported that at seedling stage, there are a lot of plants dependent on mycorrhizal interactions (Andrade et al., 2009b). The toxic effects of metals varies in all part of plants by immobilization in inner or outer hyphal cell wall parts or by combination of metals with compounds secreted by fungus such as glomalin or by partitioning of metals inside fungus cells (Joner et al., 2000; Redon et al., 2009; Kaldorf et al., 1999; Vodnika et al., 2008). AMF reduce the uptake of metals in plants by decreasing the metal concentration in soil so they act as metal sinks (Joner et al., 2000). In this way, AMF form a more suitable environment for plants found in soils with high metal toxicity (Göhre and Paszkowski, 2006).

Mycorrhizal fungi are proven to be helpful in agriculture because they have the ability to improve nutrients uptake that help to improve the growth of plants, their structure and development. Many reports indicated that AMF play an important role in limiting nutrients uptake especially phosphorus (P) availability to plants (Chen et al., 2005, 2010). Moreover, AMF increase the plants nutrition by various modifications in root systems such as by enhancing root length and growth especially branches of roots. This extensive root system are the main reason of absorbing nutrient contents from large surface area of soil (Padilla and Encina, 2005).

1.5. Fungus-root interaction

Literature shows that in all ecosystems, mycorrhizal fungi play a beneficial role in the increased uptake of nutrients from the soil (Bonfante and Perotto, 1995). The term "mycorrhiza" means 'fungus root' and originates from the Greek word "Mykes" mean 'fungus' and "Rhizo" mean 'root' (Friberg, 2001). The term was first used by a German Plant Pathologist named Frank in 1855 to explain the symbiotic association between plant roots and fungi. This beneficial association is indicated by the exchange of nutrients between plants roots and fungi where carbon in the form of sugars flows towards the fungus and nutrients move towards the plant (Sylvia et al., 1983). Mycorrhizal fungi enhance the flow of inorganic nutrients mainly phosphorus to the plants through extensive network of hyphae (Van der Heijden et al., 1998). The other plant fungus interactions are different from mycorrhizal fungi because of their capacity to provide pathway for nutrient exchange which occurs within cells of the plant

(Brundrett, 2004). More than 80% of plant species are associated with mycorrhizal fungi including vascular, non-vascular plants and some important crops such as carrots, maize, leek, coffee, cocoa, soybeans, citrus fruits, tomatoes and pepper (Muchovej, 2004; Bonfante and Perotto, 1995).

1.6. Arbuscular mycorrhizal fungi

AM fungi have three major parts: i) the root that provides carbon in the form of sugars to the fungus, ii) fungal structures present within cortical cells of plant roots that provide interaction between fungus and the plant cytoplasm and, iii) the extraradical hyphae that assist in nutrients and water uptake (Smith and Read, 1997). The AMF association cause to increase the phosphorus (P) contents of plants. Previous studies showed that about 80% of mycorrhizal associated plants obtained P content from the fungus (Marschner and Dell, 1994). Nutrients like N, K, Mg, Cu and Zn present in soil in very minimum amount and in soluble form are also provided by AM (Marschner and Dell, 1994; Smith and Read, 1997; Clark and Zeto, 2000). Reports indicated that AM fungi were named as vesicular arbuscular mycorrhizal fungi (VAM) but some studies reported that not all genera of AM produce vesicles so the name arbuscular mycorrhizal fungi was adopted (Friberg, 2001).

Vesicles are the fungal structures that are basically the carbon storage compartments of fungi and are lipid rich contents. AM fungi are mostly found in three genera of Glomeromycota: *Glomus, Acaulospora and Enthrophospora* (Isaac, 1992). However, the formation of a particular genera depends on environmental conditions such as high or low P levels because this high or low P levels affects the development of vesicles (Smith and Read, 1997). The other main structures of AM fungi involved in the roots colonisation are intraradical hyphae (IRH), extraradical hyphae (ERH) and extraradical auxiliary cells (EAC). IRH are the pathways for the fungi through which it can extends its hyphae to short distances of the root cortical cells forming colonisation units such as arbuscules and vesicles (Morton and Benny, 1990). ERH are absorptive hyphae that colonise the rhizosphere for nutrients. This is also known as infective hyphae that moves towards and along root surfaces forming new entry points and also said as the reproductive hyphae that form fertile spores after roots colonisation

(Nagahashi and Douds, 1997). Extraradical auxiliary vesicles act as lipid storage structures and are involved in nuclei partitioning and nutrients (phosphorus/carbon) uptake.

1.7. Symbiotic benefits of AM fungi

The major benefits of AM fungi is the increase of growth and productivity in AM associated plants (Friberg, 2001; Cuenca et al., 1998; Thomson, 1991). The main benefits of AM fungi to host includes increased nutrient uptake, increased tolerance to root diseases, drought resistance, tolerance to toxic heavy metals and improved soil aggregation and structure. Mycorrhizal plants should be introduced in contaminated soils to regenerate contaminated sites by limitizing the increase uptake of metals due to alteration of the root systems (Khan et al., 2009).

1.7.1 Effect of AMF on plant growth

The association of plants with mycorrhiza cause to increase the nutritional contents of plants especially P uptake (Smith and Read, 1997). This is the main reason of increased growth in plants that enhance the capacity of plants from harmful effects of heavy metals due to dilution effect of biomass (Jarrell and Beverly, 1981; Meharg and Cairney, 2000). The increase of nutrients uptake cause to improve the growth of plants. P is considered as a limiting nutrient and present in deficient amount in soil solution but mycorrhizal associated plants taken up P in the form of phosphates (Li et al., 1991; Jakobsen et al., 2002). AMF association cause to increase the uptake of phosphates by decrease the uptake of heavy metals in plants. The soil structure also improve in AMF associated plants because of glomalin that is excreted by external hyphae of fungi (Rillig and Steinberg, 2002). These benefits help to maintain biodiversity of plants and also play a role in stability of ecosystem (Van der Heijden et al., 1998; Koide and Dickie, 2002). Fungal hyphae extends into the soil that cause to increase the surface area of soil for more nutrients uptake and hence limiting nutrient i.e P uptake is also increased (Smith and Read, 1997).

1.7.2 Role of AMF in nutrients uptake

Macro and micronutrients are necessary for plant growth in different quantities. Micronutrients are required in reasonable amounts and result in toxicity disorders when present in high levels or deficiencies when present in very low levels (Ashman and Puri, 2002). Micronutrients have been reported to affect the crops production such as rice, wheat and legumes (Johnson et al., 2000; Dhillon et al., 1983). Heavy metals, at very low concentrations, are essential for plant growth e.g. lead and nickel (Ashman and Puri, 2002). Furthermore, it has been reported that AM fungi also have the ability to sequester these nutrients and reduce uptake to the plant roots when nutrients are in high concentrations (Turnau et al., 2003).

Phosphorus is the second essential nutrient after nitrogen (N), required for plant growth and development is found in many soils in organic and complex inorganic forms (phytic acid). Plants usually cannot readily take P in an organic or complex inorganic forms due to its low solubility and mobility (Schachtman et al., 1998). Inorganic phosphates present in soils in very low amounts that take plants in a very limited amounts. Thus, AM fungi increase nutrient uptake through the network of extraradical hyphae into the surrounding soil and hydrolysing any unavailable sources of P with the help of secreted enzymes such as phosphatase (Carlile et al., 2001; Koide and Kabir, 2000; Amaranthus, 1994; Schachtman et al., 1998). The enzyme phosphatase produced by AM fungal extraradical hyphae hydrolyses and releases P from organic P complexes and facilitates the absorption of P and other nutrients thereby creating a depletion zone around the roots (Li et al., 1991). These depletion zones limits the rate of P uptake by non-mycorrhizal plants but gives mycorrhizal plants a greater advantage because of the ability of AM fungal ERH to extend this nutrient depletion zone to enhance absorption (Liu et al., 2000).

1.7.3 Role of AMF in toxicity reduction

The toxicity of metals mostly depends on the metal quantity in which they are present in the soil (Smith and Read, 1997). It is also reported that metal toxicity decrease in plants associated with AMF (Marques et al., 2007; Andrade et al., 2008). These metals arise from a variety of sources in the form of acid rain, dust containing

these metals, wash waters from polluted soils and from various industrial or agricultural activities (Gaur and Adholeya, 2004). However, it is reported by literature that the toxicity of different heavy metals varies for different plants and it is mostly dependent on their actual concentration, oxidation state in the soil, soil pH, organic matter content, cation exchange capacity and redox potential (Entry et al., 2002). Additionally, AM fungi alleviate stunted growth of plants that is due to presence of toxic metals by binding to these metals in the root zone with the help of extraradical mycelium and altering the plant cells ability to capture the metals. The potential use of AM fungi was reported in detoxification of environment polluted with heavy metals and in phytoremediation (Khan et al., 2009).

1.8. Contamination status in agriculture sector: Pakistan

Pakistan is the seventh most populous country in the world and agriculture is the backbone of country's economy. Being a developing country, over 70 percent of population living in the rural areas are dependent on agriculture for their needs (Government of Pakistan, 2005). Since 1947, the rate of agriculture contribution in country's gross domestic product has been decreased from 54 percent to 24.6 percent. This decrease percentage of GDP in agriculture sector in the following years is because of lower food quality that indicates the disability to implement the policies by the government authorities. Agriculture sector is an important phenomenon and exerts both favorable and un-favorable consequences on environment (Ambreen, 1993).

The main cause of poor food quality is the usage of pesticides and fertilizers in excessive amounts. Because of expensive fertilizers, the use of sewage sludge in agriculture sector is very common phenomenon nowadays. This sewage sludge is untreated and used as a fertilizers in various crops and vegetables in agriculture sector. The use of metal polluted sites for the agriculture purposes are the main drawback in Pakistan. These polluted areas are the basic reason of poor quality of crops and vegetables and cause to increase the risk of food insecurity and a lot of health problems in humans. The remediation measures are adopted for the removal of contaminants from these polluted sites. Nowadays, one of the cost effective method is phytoremediation. This technique of removing the contaminants is considered as a very

cost effective and environment friendly system. The use of sewage sludge and irrigation of crops with contaminated water as a source of nutrients are one of the main cause of increasing the rate of contaminants in agriculture. The application of fertilizers and pesticides is also one of the main cause of increasing toxicity in soil and plants (Qadir et al., 1997).

The direct use of untreated wastewater is common in most cities which are due to the lack of alternative water sources and about 26% of the domestic vegetables are cultivated with wastewater in Pakistan (Ensink-Jeroen et al., 2004). In many cities of Pakistan such as in Quetta, wastewater is also directly used for irrigation to raise the net output of vegetables which are considered as a major source of organic manure and plant nutrients (Ahmad et al., 1992). The application of toxic effluents for irrigation purpose may also result in raising the level of macro (Na, K, Mg, Ca) and micro-essential elements (Fe' Mn, Zn) as well as trace and toxic elements (Pb, Ni, Cd, Cr and Co) which may adversely affect the metabolic pathways of humans (Shah and Riazullah, 2003). It is also reported in many studies that some of the toxic heavy metals present in sludge may enter in the food chain through plants or animals that cause to pollute the surface and ground water and thus cause health hazards (Hue, 1994; Cui et al., 2005; Bi et al., 2006).

1.9. Wheat (*Triticum aestivum*): a staple food

Wheat is a staple food and an important food grain with high biomass potential that contain high level of vitamins, minerals and cellulose fibers. The food security of a country shows access to food for all and its timely availability. The food security of Pakistan is fragile due to application of various fertilizers and pesticides in agriculture area. The production declined to 18-19 million tons in the following years and the country soon became net importer instead of exporter (Abbas et al., 2006). The main causes of wheat low productivity are non availability of certified seed, irrational use of fertilizers, weed infestation, untimely sowing, scarcity and un-timely application of irrigation water, drought prone varieties and genetic instability of new cultivars, inadequate adoption of technology to achieve genetic potential and continuous use of wheat cotton rotation.

Wheat is one of the most important crops grown and consumed in Pakistan. It plays major role in fulfilling the human needs. Wheat is the main source of carbohydrates, proteins and certain inorganic micronutrients that are important for normal growth and development of humans. Wheat plant absorbs macro and micronutrients from soil and eventually accumulated in the grains. When accumulation is under the permissible limits then it is safe for food and major crops (Das, 1990). But when accumulation exceeds the permissible limits, it exerts toxic hazards and may produce variety of diseases in humans, plants and animals. This also adversely effect the environment and also to export system (Irshad et al., 1997). The presence of toxic metals cause serious diseases in humans even at low concentration (Avena, 1979).

1.10. Alfalfa *(Medicago sativa)*: a medicinal plant

Alfalfa *(Medicago sativa)* is a medicinal plant and comprising in the family of pea plant i.e. Fabaceae. It is a perennial legume from three to twelve years, which mostly depend upon climate and variety. The growth of alfalfa plant is more than 1 meter (3 ft) and length of root is about more than 4.5 meters (Singh et al., 2010). This plant is widely used for medicinal purposes and also for grazing all over the years. It has been reported that *Medicago sativa* (alfalfa) accumulate heavy metals more than the permissible levels in plant tissues (Rechcigl, 1988). Several studies indicated that AM inoculation with Glomus species enhance the growth and biomass of *Medicago species* and decreased Cd concentrations in plant shoots (Zaefarian et al., 2011). The main benefit of AMF associated plants are the increased uptake of limiting nutrients that are available to plants in a very limited amounts like P (Barea et al., 2011).

In addition, alfalfa plant is a leguminous specie used for medicinal purposes. A very limited research has been carried out on the behavior and response of these plant species (Wheat and Alfalfa) against metals toxicity (Junior et al., 2006). There are also limited studies related to interaction of metal stress and mycorrhiza in association with these plants. Reports showed many of the researches done in relation to association of other plants species with metals and mycorrhiza.

1.11. Hypothesis and Objectives

The hypothesis of the present study is that "Mycorrhizal (M) plants (wheat and alfalfa) would grow in a better way than non-mycorrhizal (NM) plants with increasing Zn and Cd concentrations, enhance tolerance, increase nutrient status and eventually plant growth".

The objectives of the study are to:

- Assess the metals (Zn, Cd) tolerance of the arbuscular mycorrhizal fungi in wheat and alfalfa plants.
- Investigate the influence of arbuscular mycorrhiza on plant growth at high Cd and Zn concentrations.
- Analyze phosphorus and other nutrients uptake in mycorrhizal and non mycorrhizal plants at high Cd and Zn concentrations.
- Evaluate biochemical and other antioxidant enzymatic activities (SOD, POD, CAT, APX) in mycorrhizal and non mycorrhizal plants under stress conditions (high Cd and Zn concentrations).

Chapter 2

MATERIALS AND METHODS

Materials and Methods

2.1. Soil collection and preparation

Soil samples were collected from the top layer (0-20cm) in the vicinity of Quaid-i-Azam University, Islamabad. The soil was initially air-dried and then sieved with a 2-mm diameter sieve. Then soil was further processed for physico-chemical 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, 1h) to remove local AM fungal spores and other microbes. The soil and sand were mixed in ratio of 3:1. The soil samples were analyzed in replicates .

2.1.1. Physicochemical analysis of soil

The procedures used for the physicochemical analysis of the soil are as follows.

- **Determination of pH and EC:** Soil suspension was prepared with deionized water in a ratio of (1:2), and was stirred at regular intervals for 30 minutes on shaker (Tandon et al., 2005). The pH and EC of the suspension was recorded on pH and EC meter (SM-802) .
- **Soil digestion procedure:** Soil (0.5gm) was digested in a combination of nitric-perchloric acid (1:2). The contents of solution were heated to the boiling temperature of 180°C for 15-20 minutes. Samples were digested until dense white fumes of acid appear. At this stage, the suspended matter was like white sand particles. The process was completed within 40 minutes. The mixture was cooled and 50 mL volume was made (Olsen and Sommers, 1982).

- **Nutrients Analysis:** The digested soil filtrate was used for the analysis of different macronutrients and micronutrients.

- **Total phosphorus:** Total phosphorus (P) was determined by ammonium heptamolybdate method (Ryan et al., 2001). Sample digest (5 mL) and ammonium vanadomolybdate reagent (10 mL) were mixed. A standard curve was prepared by putting 5mL of each standard (2-10 ppm). The reagent blank sample was also prepared with 10 mL vanadomolybdate solution. The blank was processed in the same way as the samples. The absorbance of samples, blank and standards were read at 410 nm wavelength after 10 minutes.

- **Extractable Phosphorus:** Extractable phosphorus was determined by mehlic extraction method (Mehlic, 1984). Soil samples (6 g) and Mehlic extracting reagent (50 mL) were mixed. Extracting reagent was prepared by dissolving ammonium nitrate (80 g), ammonium fluoride EDTA stock (16 mL), acetic acid (46 mL), conc. nitric acid (3.28 mL) into 3 L pure water. The final volume was brought to 4 L with deionized water. The extract was filtered, mixed with deionized water and mixed reagent in a ratio of (1:19:5). Mixed reagent was prepared by addition of 140 mL stock reagent and 0.74 g ascorbic acid. For the preparation of stock reagent, 12 g ammonium molybdate, 0.2908 g antimony potassium tartrate and 148 mL conc. sulphuric acid were added to 2 L volume of deionized water. All the samples were read after 30 minutes at 880nm wavelength using spectrophotometer .

- **Nitrate Nitrogen:** Nitrate-N in soil samples were measured by using spectrophotometer (Model: Hach, DR 5000). 10g air dried soil (2-mm) was added into 50 mL copper sulfate solution (0.02 N). The solution was shaken and filtrate was collected. 0.1% chromotropic acid (1 mL) and concentrated sulfuric acid (6 mL) were added into 3 mL filtrate. Standards and blank was made and proceeded as for the samples. All standards, blanks and samples were read at 430 nm wavelength after 45 minutes (Sims and Jackson, 1971) .

- **Determination of Metals in digest:** In the soil digest, the metals including Na, K, Ca, Mg, Cr, Fe, Cu, Ni, Co, Mn, Pb, Zn and Cd were analyzed using atomic absorption spectrophotometer (Varian FAAS-240) in the Department of

Environmental science, Quaid-i-Azam university, Islamabad. The analyses were performed in triplicates under standard optimizing conditions. The blank samples and standard reference soil (NIST, 2709 San Joaquin) of National Institute of Science and Technology were run with samples for the accuracy and precision. The analyses were within the confidence limit of 95% .

2.2. Biological material

- **Mycorrhizal inoculum:** The inoculum of different Glomus species was used with dry soil substrates obtained from the company (Agrauxine) in France. Spores of Glomus species were used in mycorrhizal inoculated treatments. Each Pot (15 cm diameter and 14 cm height) used in the experiment contained 2 kg soil and sand mixture plus 30 g of AMF inoculum to M treatments, while the same quantity of soil and sand combination were added to NM treatments. Each pot contained approximately 1500 spores at the time of sowing. AMF inoculation was performed during the transplantation process and was not provided in non-mycorrhizal treatments
- **Plants:** Seeds of wheat (*Triticum aestivum* L.) and alfalfa (*Medicago sativa*) were obtained from Department of Crop Science, National Agriculture Research Centre, Islamabad.

2.3. Experimental design and Procedure

- **Place of Experiment:** Three different experiments, one with Zn in association with wheat (Experiment 1), second Cd in association with wheat (Experiment 2) another Zn and Cd in association with *Medicago sativa* (Experiment 3) were conducted under greenhouse conditions in Growth chamber in Laboratary of Environmental Science, Quaid-i-Azam university, Islamabad. The treatments were AMF inoculated or non-inoculated in soil with the addition of four concentrations of Zn (0, 100, 300 and 900 mg kg^{-1}); and Cd (0, 100, 300 and 600 mg kg^{-1}). There were total 8 treatments with 6 replicates. Each pot (15 cm diameter and 14 cm height) contained 2 kg growth medium plus 30 g of AM fungal inoculum to

mycorrhizal treatments. Plants were placed during growth process in the growth chamber (12 h light per day at temperature of 15-30°C). During the experiment, the deionized water (10 mL) and Long Ashton's nutrient solution (20 mL) were used every week to maintain the moisture level (60%) in soil. The plants were harvested after 60 days of sowing. During the plant growth period, the temperature at day time ranged from 20°C to 30°C and at night time ranged from 15°C to 20°C (Plate 1.1) .

- **Petri Plates Preparation:** After autoclaving, the wrapped petri-plates were dried in the oven at specified temperature (90°C). After drying, petri plates were un-wrapped inside the sterilized conditions of the laminar flow for the preparation of plates for germination of seeds.

- **Sterilization of Collected Seeds:** Seeds were surface sterilized (10 min, 3% Chlorox), washed with deionized water and dried with filter papers (Xin Hua No.101, China). Seeds were placed at 28°C for 48 hours in petri dishes for germination. Five germinated seeds were sown in each pot and the plants were grown in growth chamber for 8 weeks (Plate 1.2) .

2.3.1. Experiment 1

In this experiment, Zn treatments provided to plants of wheat (*Triticum aestivum*). Four Zn addition levels (0, 100, 300 and 900 $mgkg^{-1}$) were applied as $ZnCl_2$ solution.

- **Treatments involved in Experiment 1:** The treatments involved in experiment are as follows.

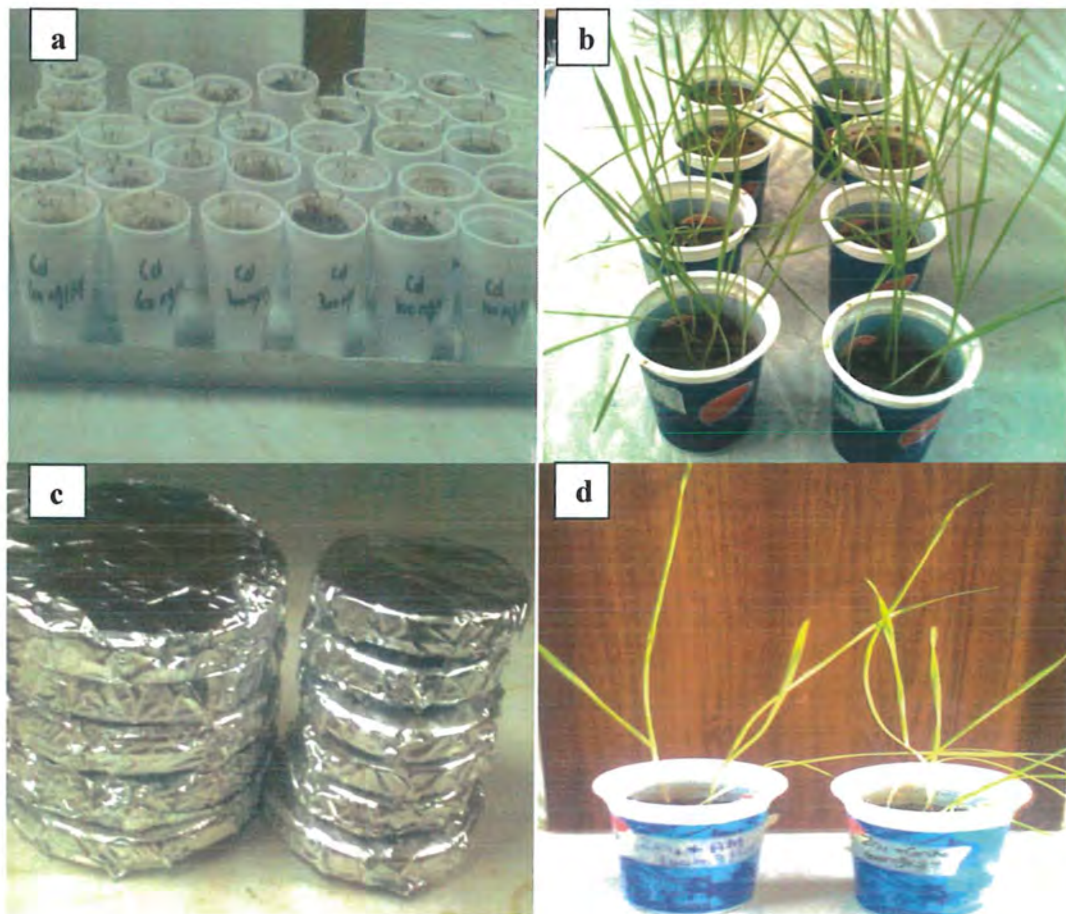

Plate 1.1: Figure showing place of Experiment: a) Alfalfa (*Medicago sativa*). b) Wheat (*Triticum aestivum*) c) Petri plates preparation for seed germination d) Wheat plant after 6 weeks of growth.

- **Treatment 1 and 2 (0mgkg^{-1} Zn):** In these treatments, 2.0 kg soil was placed directly in the pot. Five germinated seeds of wheat (*Triticum aestivum*) were sown in each pot of AMF inoculated and non-inoculated treatments with four replicates. There was no Zn concentration provided in these two treatments.
- **Treatment 3 and 4 (100mgkg^{-1} Zn):** In these treatments, 2.0 kg soil was mixed with 100mgkg^{-1} of Zinc after 2 weeks of transplantation of seedlings in pots. Five germinated seeds of wheat (*Triticum aestivum*) were sown in each pot of AMF inoculated and non-inoculated treatments with four replicates.
- **Treatment 5 and 6 (300mgkg^{-1} Zn):** In both of these treatments, 2.0 kg soil was mixed with 300mgkg^{-1} of Zinc after 2 weeks of transplantation of seedlings in pots. Five germinated seeds of wheat *(Triticum aestivum)* were sown in each pot of AMF inoculated and non-inoculated treatments with four replicates.
- **Treatment 7 and 8 (900mgkg^{-1}Zn):** In these treatments, 2.0 kg soil was treated with 900mgkg^{-1} of Zinc after 2 weeks of transplantation of seedlings in pots. Five germinated seeds of wheat *(Triticum aestivum)* were sown in each pot of all treatments.

2.3.2. Experiment 2

In Experiment 2, the Cd treatments provided to plants of wheat (*Triticum aestivum*). Four Cd addition levels (0, 100, 300 and 600 mgkg^{-1}) were applied as $CdCl_2$ solution mixed with the soil in all treatments. All the solutions were made in deionized water.

- **Treatments in Experiment 2:** The treatments involved in this experiment are as follows.

- **Treatment 1 and 2 (0mgkg^{-1} Cd):** In these treatments, 2.0 kg soil was placed directly in the pot. Five germinated seeds of wheat (*Triticum aestivum*) were sown in each pot of AMF inoculated and non-inoculated treatments with four replicates. There was no Cd concentration provided in these two treatments.
- **Treatment 3 and 4 (100mgkg^{-1}Cd):** In these treatments, 2.0 kg soil was treated with 100mgkg^{-1} of Cd after 2 weeks of transplantation of seedlings. Five germinated seeds of wheat (*Triticum aestivum*) were sown in each pot of AMF inoculated and non-inoculated treatments with four replicates.

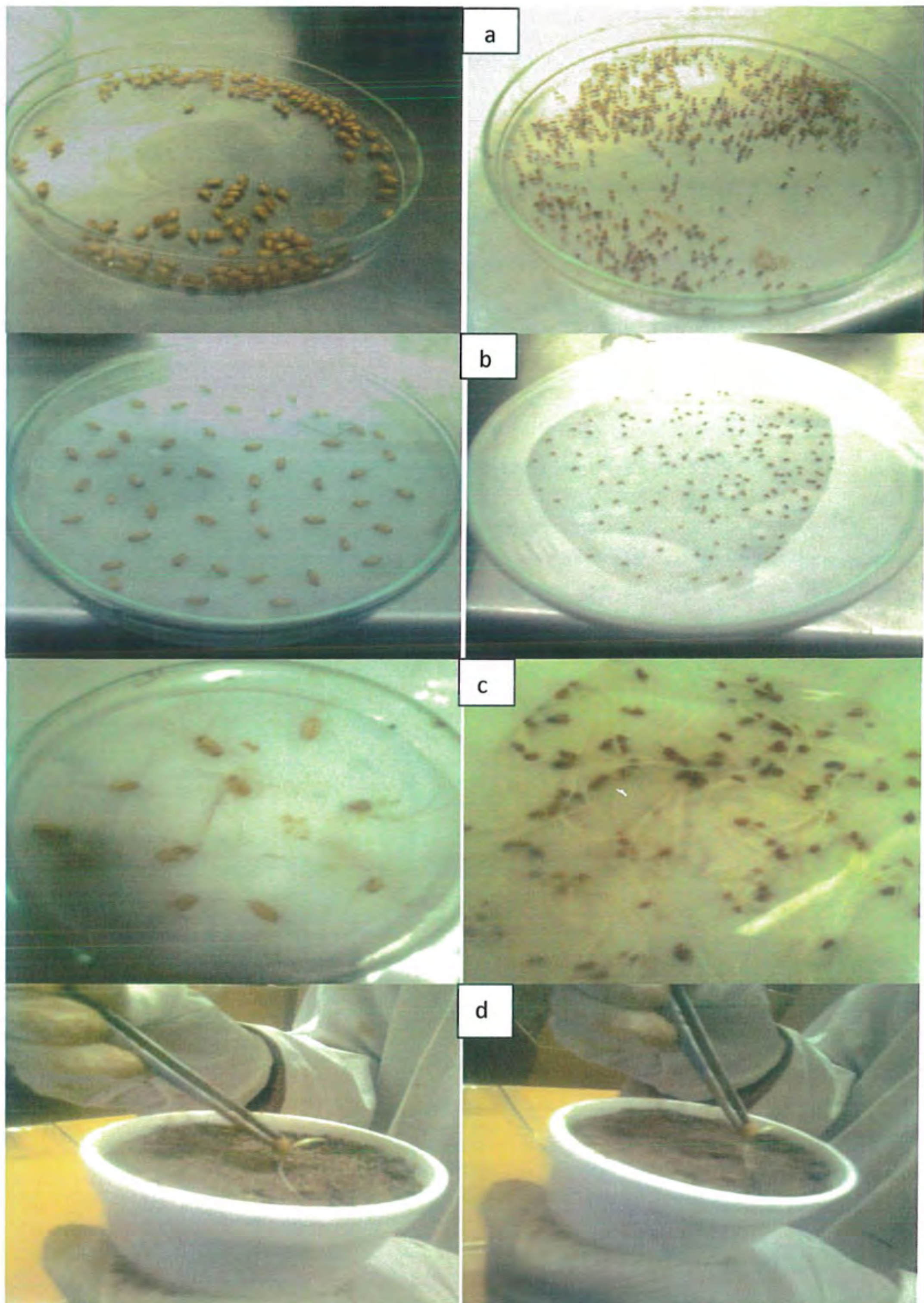

Plate 1.2: Figures shows experimental steps from plants (Wheat and Alfalfa) seed germination to sowing of i.e. a) Sterilization and washing of seeds with Chlorox 3(%) and deionized water, b) Seeds germination on moist filter paper, c) Seeds radicals after 48hrs germination, d) Seeds sowing in pots

- **Treatment 5 and 6 (300mgkg^{-1} Cd):** In both of these treatments, 2.0 kg soil was treated with 300mgkg^{-1} Cd after 2 weeks of transplantation of seedlings.
  Five germinated seeds of wheat *(Triticum aestivum)* were sown in each pot of AMF inoculated and non-inoculated treatments with four replicates.
- **Treatment 7 and 8 (900mgkg^{-1}Cd):** In these treatments, 2.0 kg soil was treated with 900mgkg^{-1} of Cadmium after 2 weeks of growth of seedlings. Five germinated seeds of wheat *(Triticum aestivum)* were sown in each pot of AMF inoculated and non-inoculated treatments with four replicates.

2.3.3. Experiment 3

In Experiment 3, Zn and Cd treatments separately provided to alfalfa (*Medicago sativa*) plants. Four Zn (0, 100, 300 and 900 mgkg^{-1}) and Cd (0, 100, 300 and 600 mgkg^{-1}) addition levels were applied as analytical grade $ZnCl_2$ and $CdCl_2$ solution mixed thoroughly with the soil inoculated or noninoculated with AM fungal inoculum. The solutions were prepared in deionized water and mixed with the soil manually.

- **Treatments involved in Experiment 3:** The treatments involved in experiment are as follows.

- **Treatment 1 and 2 (Control):** In these treatments, 2.0 kg soil was placed directly in the pot. Five germinated seeds of alfalfa (*Medicago sativa*) were sown in each pot of AMF inoculated and non-inoculated treatments with four replicates. There was no Zn and Cd concentration provided in these two treatments.
- **Treatment 3 and 4 (100mgkg^{-1} Zn):** In these treatments, 2.0 kg soil was treated with 100mgkg^{-1} of Zn and Cd separately after 2 weeks of transplantation of seedlings in pots. Five germinated seeds of alfalfa (*Medicago sativa*) were sown in each pot of AMF inoculated and non-inoculated treatments with four replicates.
- **Treatment 5 and 6 (300mgkg^{-1} Zn):** In both of these treatments, 2.0 kg soil was treated with 300mgkg^{-1} of Zn and Cd separately after 2 weeks of transplantation of seedlings in pots. Five germinated seeds of alfalfa (*Medicago sativa*) were sown in each pot of AMF inoculated and non-inoculated treatments with four replicates.
- **Treatment 7 and 8 (900mgkg^{-1}Zn):** In these treatments, 2.0 kg soil was treated with 900mgkg^{-1} of Zn and Cd separately after 2 weeks of transplantation of

seedlings in pots. Five germinated seeds of alfalfa (*Medicago sativa*) were sown in each pot of AMF inoculated and non-inoculated treatments with four replicates.

2.4. Harvesting of plants

After growth period of 8 weeks, shoots and roots of plants were harvested and preserved separately. Samples of roots were washed and cleaned with deionized water to remove soil particles. For measurement of AM colonization rate, fresh roots samples were also collected. Samples were collected and preserved at 4°C for analysis of antioxidant enzymes (Plate 1.3).

2.5. Plants Analysis

Plants were further analysed for physiological measurements and chemical analysis.

2.5.1. Determination of Plant Growth and colonization

- **Root and shoot growth:** The roots and shoots were washed gently with tap water then oven dried at 70°C. Samples were weighed after cooling in desiccators. The growth parameters measured including shoot and root length, breadth and area .

- **Plant biomass:** Plant biomass was analysed by determining plant dry weight (DW) and fresh weight (FW) just after plant harvest, 56 days from seed germination. Plants tissues i.e shoot and root were washed initially with deionized water to clean the samples from soil. Shoot and roots were weighed separately. The fresh weight of samples were measured. The samples were then oven dried at 60°C for 72 hours and ground to < 0.25 mm in a stainless mill. The dry weights were also measured. The total shoot and root biomass were measured by analyzing the difference of water content in fresh and dry shoot and root samples .

- **Evaluation of Mycorrhizal colonization:** Root samples were collected and preserved separately for the assessment of mycorrhizal colonization. Roots were cleaned and stored in a vial by mixing with solution of formaline acetic acid alcohol (FAA). The colonization percentage of fungus with plants roots were assessed by following the method of Phillips and Hayman (1970) and Liu and Chen (2005).

Root samples were first mixed with 2.5% KOH for 30 min. Samples were washed with deionized water three times after mixing with KOH. Samples were then dipped in HCL (2%) for 5 min and then washed with H_2O_2 for 10 min. After bleaching with hydrogen peroxide, root samples were washed with deionized water and then stained with trypan blue (0.05%). After this procedure, fungi stained blue and ready for assessment of percentage root colonization. Microscopic examination was done by placing the stained root segments on a glass microscopic slide (1 mm-1.2 mm thick). Root segments were covered with cover slip and pressed with finger for 1 min. The stained roots on microscope were observed under 100× magnifications. The AMF colonization was not observed in the non-inoculated plants (Plate 1.4). The colonization percentage was estimated for each treatment by measurement of about forty 1-cm long pieces of roots, and expressed as the following formula .

$$\text{Colonization } \% = \frac{\text{Number of colonized roots parts}}{\text{Total number of examined root samples}} \times 100\%$$

2.5.2. Chemical Analysis of Plants

Plant samples (roots and shoots) of wheat and alfalfa were further analyzed for physiological activity, biochemical contents, nutrient analysis and antioxidant enzymes.

2.5.2.1. Determination of Biochemical contents

Chlorophyll and Carotenoid content: The Chlorophyll content was determined by using the method of Hiscox and Israelstam (1979). Leaf material (0.05g) was firstly extracted by using 10 mL dimethylsulfoxide (DMSO). The sample was heated at 65°C for 4 hours. The absorbance of sample extracts for chlorophyll a and b was measured at 665 and 645 nm. Chlorophyll contents were calculated by following the Eq 1, 2 (Arnon, 1949)". The carotene content was determined by following the method of Lichtenthaler and Wellburn (1983) and calculation was done by using Eq 3.

$$Chl.a\,(mg/g) = 1.07\,(OD663) - 0.09\,(OD645)\ldots\ldots Eq\ 1$$

$$Chl.b\,(mg/g) = 1.77\,(OD645) - 0.280\,(OD663)\ldots\ldots Eq\ 2$$

$$Carotenoid\ content = AOD \times 4\ldots\ldots Eq\ 3$$

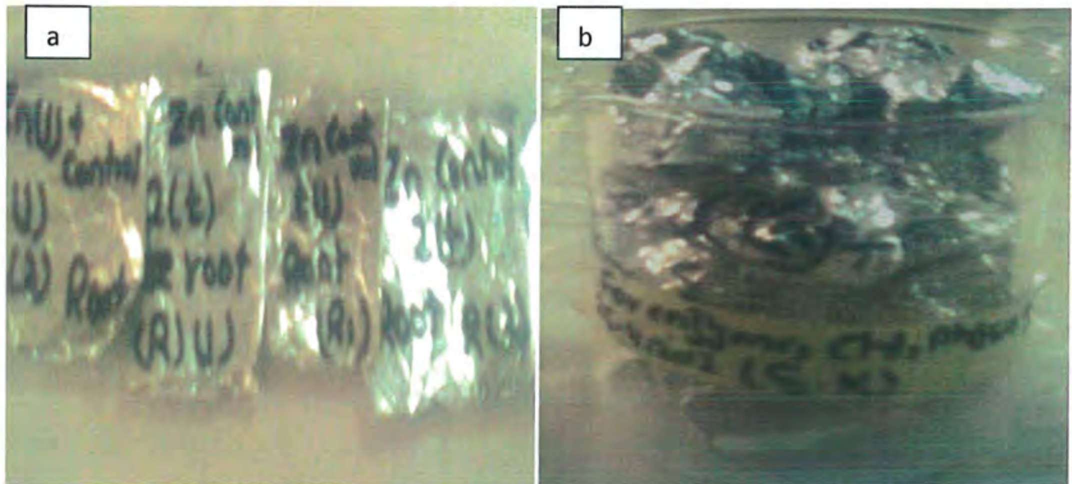

Plate 1. 3: Figure showing preservation of plants samples (shoots and roots) for: a) Analysis of heavy metals. b) Analysis of antioxidant enzymes

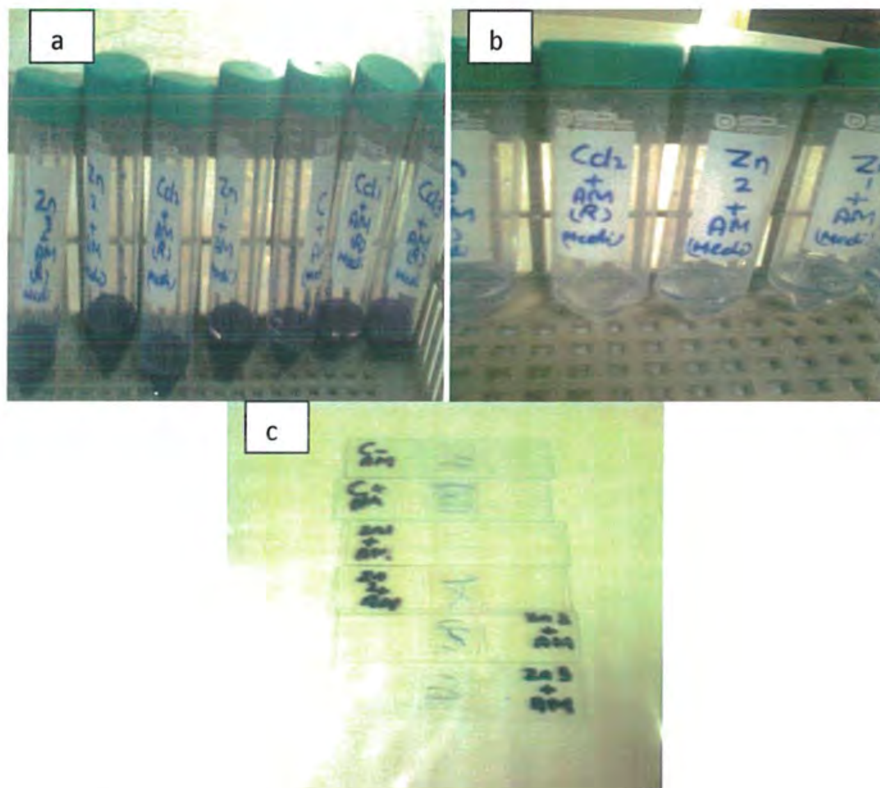

Plate 1.4: Figures showing procedure of evaluating Root-Mycorrhizal colonization: a) Staining with trypan blue (0.05%). b) clarifying with HCl (2%). c) Preparation of slides for microscopic view.

- **Proline Content:** Proline content of leaves was assessed by using the procedure of Bates et al. (1973). Plant material (0.1g) was mixed with 4 mL sulfosalicylic acid (3.0%) in mortar. The solution was kept overnight at specific temperature of 5°C. The mixture was centrifuged at 3000 rpm for 5 min at room temperature. The supernatant was then extracted and transfer to new tube. Then mixed with acedic ninhydrin reagent (4 mL). The mixture was shaken and heated in water bath for 1 h. After this procedure, the suspension in the tubes were placed to cool and the mixture was extracted with toluene (4 mL). The absorbance of the supernatant was read at 520 nm. The Proline content was expressed as $mg.g^{-1}$. The proline content of the unknown sample was determined with reference to the standard curve .

- **Sugar Content:** Fresh leaf material was analysed for sugar content by following the procedure of Dubois et al. (1956). Plant material (0.5g) was mixed in a mortar with deionized water (10 mL). The mixture was centrifuged for 5 min at 3000 rpm. After centrifugation, supernatant was extracted and transfer to new tubes. Phenol (1 mL) was added to 0.1mL of supernatant. Samples were then placed in room temperature for 1 hr. After incubation, concentrated H_2SO_4 (5 mL) was added to the samples. The samples were read at 420 nm wavelength and measured sugar content was expressed as $mg.g^{-1}$. The sugar content of unknown sample was assessed with reference to standard curve made of glucose

- **Protein Content:** Protein content of fresh leaves was assessed by using the procedure of Lowry et al. (1951). Leaves (0.1 g) were homogenized with 1 mL of sodium phosphate buffer (pH 7.5). The suspension was centrifuged at 3000 rpm for 10 min. After centrifugation, the supernatant (0.1 mL) was taken and deionized water was added to make the total volume of 1 mL. After this process, Reagent A (1 mL) was added in the 1 mL of sample solution. Reagent A was made by addition of 1 g Na_2CO_3, 0.2 g NaOH (0.1 N), 0.5 g Na-K tartrate, 0.25g $CuSO_4$ in 100 mL of deionized water. The suspension was shaken for 10 minutes. After that reagent D (0.1 mL) was added. Reagent D was made by mixing of folin phenol reagent with deionized water in the ratio of 1:1. The contents of protein in the samples was read at 650 nm after 30 min. Protein contents was expressed as $mg.g^{-1}$. The protein

content in unknown samples were analyzed with reference to standard curve made by using standard BSA (Bovine Serum Albumen).

2.5.2.2. Determination of Antioxidant enzymes Assays

Antioxidant enzymes were determined by homogenizing 0.5g of fresh leaves in 5ml of 50mM phosphate buffer. The suspension was centrifuged at 13000 rpm for 20 min at 4°C. The supernatant was extracted and used to analyze SOD, POD, CAT and APX activities.

- **Superoxide dismutase:** The activity of superoxide dismutase (SOD) was analyzed by using the procedure of Beauchamp and Fridovich (1971). The enzyme extract (0.1 mL) was mixed with 3 mL of reaction mixture. The reaction mixture was prepared by dissolving methionine (13mM), EDTA (0.1mM), riboflavin (0.002mM) and (NBT) nitro blue tetra zolium salt (0.075 mM) in 3 mL of 0.05 M sodium phosphate buffer (pH 7.8). The suspension was placed in light chamber to start the reaction. The temperature was maintained at 30°C for 1 hour in light chamber. Similar sample solutions were placed in dark chamber. The samples were read at 560 nm using spectrophotometer. SOD activity was presented as U/mg protein .

- **Peroxidase activity:** The POD content was assessed by using the procedure of Gorin and Heidema (1976). Enzyme extract (0.1 mL) was dissolved in reaction mixture (1.35 mL). Reaction mixture was prepared by mixing of 100 mM MES buffer (pH 5.5), 0.05% H_2O_2 and 0.1% phenylenediamine. The samples were read at 485 nm for 3 min via the spectrophotometer. The POD content was expressed as U /mg protein.

- **Catalase activity:** The Catalase (CAT) activity was examined by the using the procedure of Goel et al. (2003). Reaction mixture (3 mL) was added in enzyme extract (0.1 mL). The reaction mixture was prepared by dissolving 50mM phosphate buffer (pH 7) and H_2O_2 (15 mM). The CAT activity was determined at

240 nm wavelength by using spectrophotometer. The CAT activity was expressed as U/mg protein.

- **Ascorbate Peroxidase activity:** The activity of APX was analyzed by following the method of Nakano and Asada (1981). The enzyme extract (50–100 µL) was mixed with reaction mixture. The reaction solution contained 50 mM phosphate buffer (pH 7.0), EDTA (0.1 mM), Ascorbic acid (0.5 mM), H_2O_2 (0.1 mM). The APX activity was read at 290 nm and expressed as U/mg protein.

2.5.2.3. Plant mineral nutrient analysis

- **Plant digestion procedure:** Plant tissues (roots and shoots) were digested in nitric-perchloric acid (2:1). Plant material (1 g) was placed in acid reagent (10 ml). The mixture was heated in digestion chamber and temperature was gradually increased up to ~ 230 °C. The contents were heated till the formation of brown NO_2 fumes ceases and dense white fumes of $HClO_4$ appeared in the flask. The solution was further heated until the volume of 3–5 mL was left. The process was completed until the solution was colorless. Volume (50 mL) was made up with deionized water. The solution was used for the determination of various macro and micronutrients (Ryan et al., 2001).

➢ **Total Phosphorus in digest:** Total Phosphorus (P) in plant digest was determined by wet digestion procedure. Plant material was digested and 10ml of the digest filtrate was taken. 10 mL ammonium-vanadomolybdate reagent was added in the filtrate. Standards and blank reagent were also prepared and processed as for the samples. The curve was made for standards and the P concentration of unknown samples were analyzed with reference to curve. The blank, standards, and samples were read at 410-nm wavelength after 30 minutes on spectrophotometer (Ryan et al., 2001).

- **Total Nitrogen:** Total N content was analyzed by using the Kjeldahl method (Van Schouwenberg and Walinge, 1973). Two steps involved in the analysis of total nitrogen in plant samples.
  - **Digestion:** Plant sample (1 g) was dried at 60°C in an oven (overnight) and cooled in a desiccator. About 0.25 g dry plant material was taken and sulfuric-salicylic (20ml) mixture, sodium thiosulfate (2g), catalyst mixture (4g) and 3-4 pumice boiling granules were added and proceeded with the digestion until the mixture clears. The tubes were removed from the block-digester and allowed them to cool for about 20 minutes. The contents were agitated thoroughly and digested for 2 hours. After the digestion procedure, the contents were cooled at room temperature and finally made the volume of 250 mL. At least one reagent blank (no plant) was added with one batch of samples.
  - **Distillation:** Distillation and titration apparatus was warmed for 10 minutes. Before distillation, 10 N sodium hydroxide (7ml) solution was added in 25 mL aliquot respectively and then distillation was started. About 35 mL distillate was diluted and distillation flask was removed and connect an empty 100mL distillation flask to the distillation unit. Water was drained from the condenser jacket and apparatus was steamed out for 90 seconds before connecting the next sample. There was two standards and two blanks (reagent blanks) proceeded with each batch of distillation.

  - **Determination of Metals in digest:** In the soil acid extracts (Olsen and Sommers, 1997), the concentrations of heavy metals including Na, K, Ca, Mg, Cr, Co, Cu, Ni, Fe, Zn, Pb, Mn and Cd were determined using atomic absorption spectrophotometer (Varian FAAS-240) in the Department of Environmental science, Quaid-i-Azam University, Islamabad. The plant samples were analyzed in replicates.

2.6. Quality Assurance and Control

All the samples were analyzed in triplicates under standard optimizing conditions. The standard reference materials for soil (NIST, 2709 San Joaquin), plants

(NIST, 1547 Peach leave) and blank reagent were run with each batch of samples. These reference materials were processed to confirm the accuracy and precision of physico-chemical analysis. All the analyses were within the confidence limit of 95%.

2.7. Statistical Analysis

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

Chapter 3

RESULTS

Results

3.1 Part 1: Wheat and AMF association under Zinc stress

3.1.1 Mycorrhizal colonization

Fig 1.1 shows the percentage colonization of AM fungi with roots of wheat (*Triticum aestivum*) plants. The result indicated that AMF colonization was not observed in non-mycorrhizal plants. The higher colonization were observed in mycorrhizal plants because more arbuscules and hyphal structures were observed. Only minor differences in mycorrhizal frequency were found in inoculated plants. From the results, it is obvious that the symbiotic relationship between *Triticum aestivum* and AMF can be well developed at high zinc concentrations. At the Zn concentration (100 mg/kg), highest rate of colonization that is about 72.16 % was detected. While at the Zn level (900 mg/kg), lower rate of colonization that is 67.56 % was observed. The percentage of colonization in M inoculated plants at all three Zn concentration lies between 60 and 70%.

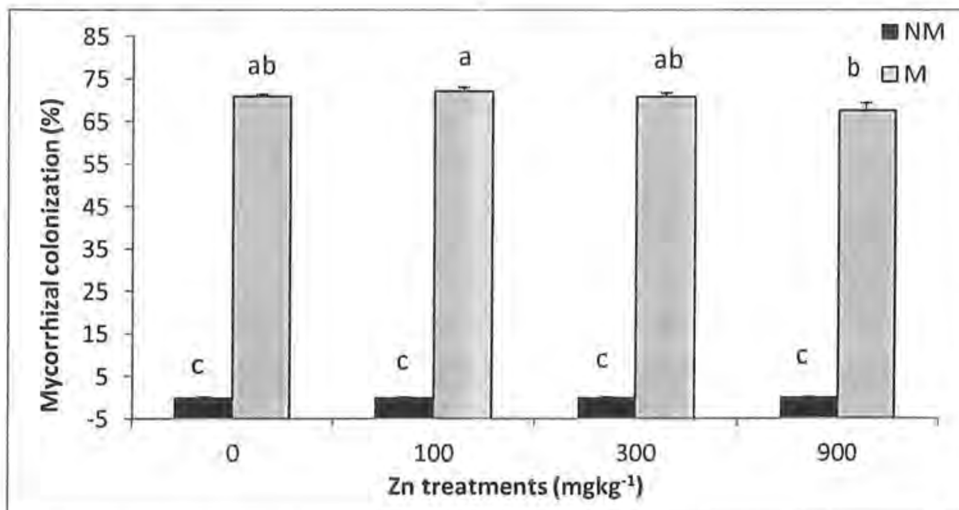

Fig 1.1 Arbuscular mycorrhizal fungi colonization with roots of wheat (*Triticum aestivum*) plants. Data are presented as means ± SE of the mean. Bars represent standard error. The different letters above the bars shows significant difference between treatments according to Tukey test *($P<0.05$)*.

3.1.2 Plant growth

Fig. 1.2 shows the effect of increasing zinc concentrations on growth of plants in M and NM wheat seedlings. The interaction of wheat plants and AM fungi species had significant effect on the plant growth and biomass in soil with the addition of soil zinc concentrations. Fig. 1.2 (a and b) shows that higher shoot and root biomass was observed in mycorrhizal associated wheat plants. At all Zn concentrations (100, 300, 900), the root and shoot biomass in M plants were greater than those of NM plants. As the Zn levels increased from 100 to 900 $mgkg^{-1}$, the biomass of plant tissues was decreased. The same trend was observed in both shoot and root parts of plants. In 100 and 300 $mgkg^{-1}$ soil, a significant increase ($P < 0.05$) was observed in shoot and root biomass of mycorrhizal inoculated plants with Glomus species. While the decrease in the trend was observed at 900 $mgkg^{-1}$ Zn concentration in both shoot and root parts of plants.

Fig. 1.2 (c-h) shows that plant growth was increased in AMF associated wheat plants as compared to NM plants at all Zn concentrations. Results showed that Zn concentration (900 $mgkg^{-1}$) cause a severe decrease of plant growth. At 100 and 300 mg $kg-^{1}$ Zn, a clear difference was observed in M associated plants. The results indicated that more shoot and root length, breadth and area was observed in M associated plants than NM plants at 0, 100 and 300 $mgkg^{-1}$ Zn. However, NM plants had slower and stunted growth at higher Zn toxicity. Therefore, all the results showed that wheat associated with mycorrhiza had improved growth as increased Zn concentrations (0, 100, 300 $mgkg^{-1}$) in soil except for the maximum Zn concentration (900 $mgkg^{-1}$) in which significant reduction in biomass was observed in all treatments.

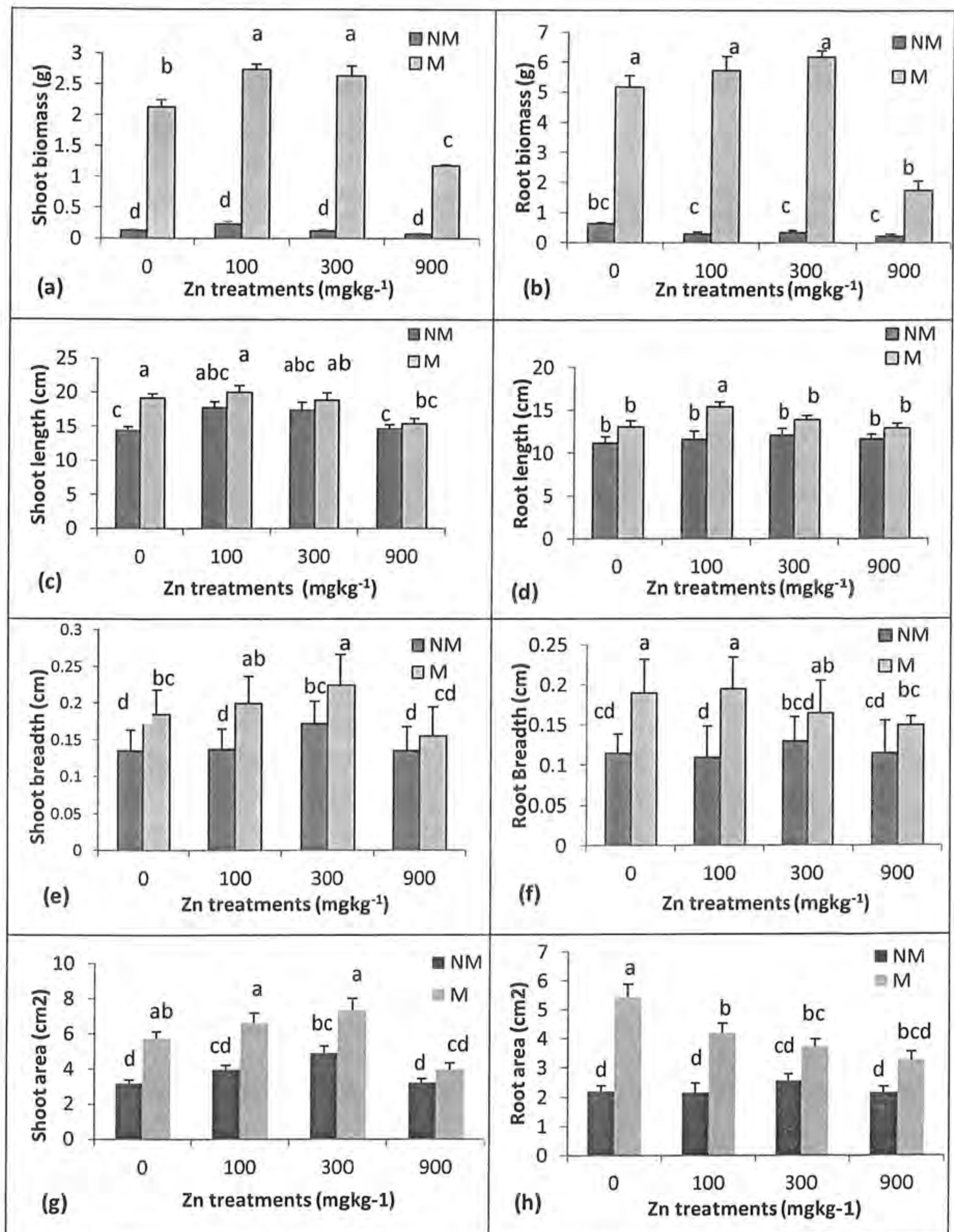

Fig 1.2. Effect of increasing zinc concentration on mycorrhizal and non mycorrhizal wheat seedlings: (a. b) Plant shoot and root biomass, (c.d) Plant shoot and root length, (e. f) shoot and root breadth, (g. h) shoot and root area.The data shown are the means and standard error. The different letters above the bars indicates significant difference by Tukey test ($P < 0.05$).

3.1.3. Plant nutrient status

The nutrient contents in wheat plants was increased by mycorrhizal inoculation especially P content (Table 1.1). Fig. 1.3 shows that at Zn concentration (100 and 300 $mg.kg^{-1}$), M plants had higher P contents in both shoot and root except at the highest Zn concentration (900 $mg.kg^{-1}$). Plant growth was also very stunted at the higher Zn concentration in both M and NM plants. Table 1.1 shows nutrient contents in both mycorrhizal (M) and non-mycorrhizal (NM) wheat plants with increasing soil Zn concentrations.

In general, increase in concentrations of nutrients (N, P, K, Ca, Mg, Na, Fe, Cu) were observed in M treatments (100 and 300 $mgkg^{-1}$) except Mn and Ni, in which the trend was decreasing in mycorrhizal treatments as the concentration of Zn was increased. Non significant results were obtained in Ca and Mg at all Zn concentrations. Zn addition (900 $mgkg^{-1}$) had a harmful effect on nutrient contents of plants. The decrease concentrations of all nutrients were observed at highest soil Zn concentration in both M and NM plants. The experiment showed that mycorrhiza had a positive effects on nutrient contents of wheat plants that cause to enhance the concentrations of P, K, N, Ca, Mg, Na, Cu and Ni. In M plants, the concentration of Mn and Fe was decreased in shoot part of plant. However, the increase of N, Cu, Mn and Ni were observed in roots part of plant. In NM plants, the reduction of nutrients like K, P, N, Mn, Ni and Fe were recorded in the shoots with increasing Zn concentrations. While, the Cu and Na was increased with increase of Zn concentration in shoots of NM plants. The increase of nutrient contents in N, Ca, Na, K and Ni were observed in roots of the wheat plants with soil increasing Zn level.

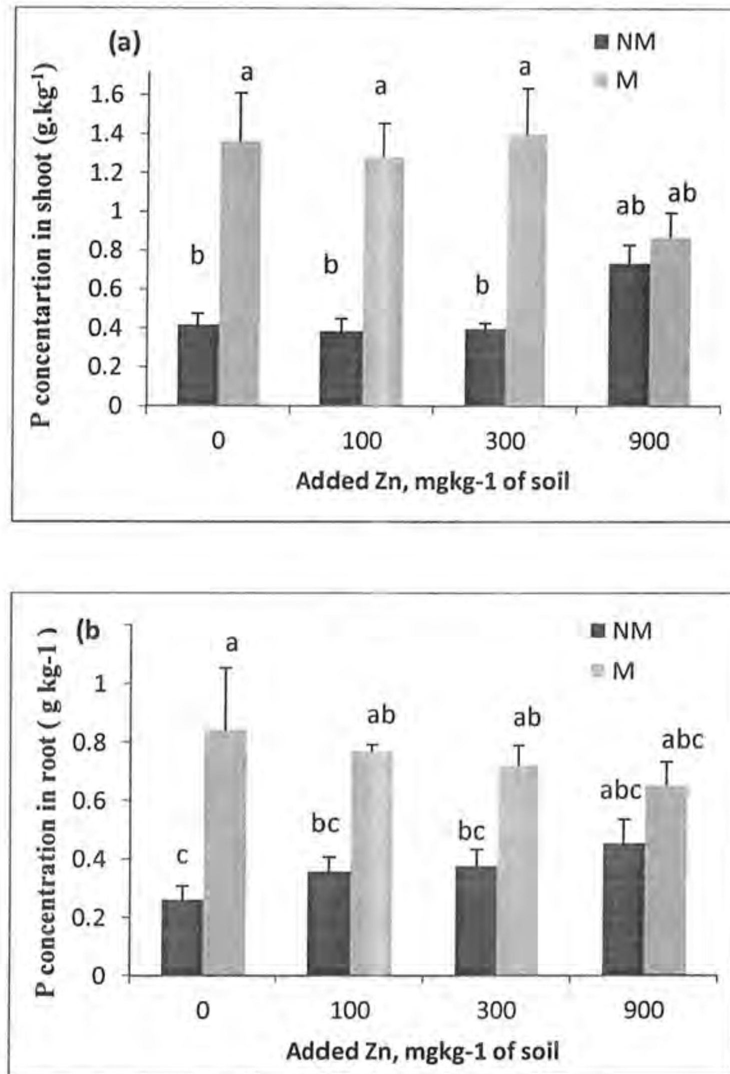

Fig 1.3. Phosphorus concentration in: (a) shoots and (b) roots of mycorrhizal (M) and non-mycorrhizal (NM) wheat plants in response to Zn addition to soil. Means (n = 3) with the different letters are significantly different (*P< 0.05*) by the Tukey test.

Table 1.1 Concentration of nutrients measured in shoots and roots of mycorrhizal (M) and non-mycorrhizal (NM) wheat seedlings grown in soils

Experiment (Zn, $mgkg^{-1}$)		K	P	N	Ca	Mg	Na	Cu	Mn	Fe	Ni
Leaves+stems		g.kg -1						mgkg -1			
0	NM	24.927 ± 2.6509 ab	0.4200 ± 0.0551 b	0.2567 ± 0.0233 cd	16.563±1.8148 a	10.620±0.6526 ab	18.523 ± 0.6479 bc	9.7200 ± 0.7514 c	167.77 ± 15.061 ab	55.880 ± 2.8630 bc	11.900 ± 1.4619 abc
	M	30.437 ± 5.1117 a	1.3633 ± 0.2469 a	0.5167 ± 0.0273 bc	12.820±0.7240 a	8.4767±0.7656 ab	22.507 ± 1.0287 ab	16.253 ± 0.6567 a	132.76 ± 7.3253 bc	94.800 ± 2.3908 a	7.7500 ± 1.0707 c
100	NM	19.073 ± 2.3507 ab	0.3867 ± 0.0606 b	0.2800 ± 0.0208 cd	14.657±1.8712 a	11.800 ±1.0601 a	18.387 ± 1.3594 bc	11.950 ± 0.9110 bc	171.11 ± 19.766 ab	45.080 ± 5.5245 c	15.800 ± 0.6799 a
	M	26.520 ± 2.6951 ab	1.2867 ± 0.1695 a	0.8033 ± 0.1257 ab	14.943±1.0199 a	10.653 ±0.8045 ab	23.133 ± 1.4546 ab	18.250 ± 0.4819 a	113.62 ± 10.174 c	64.533 ± 4.3521 b	9.3967 ± 0.5584 bc
300	NM	21.657 ± 1.2451 ab	0.4000 ± 0.0265 b	0.2400 ± 0.0379 cd	14.310±0.5024 a	9.2900 ±0.4789 ab	15.613 ± 1.6034 cd	12.127 ± 0.9167 bc	135.67 ± 7.8387 bc	46.023 ± 4.2411 bc	9.7033 ± 0.2510 bc
	M	27.997 ± 1.4801 ab	1.4033 ± 0.2338 a	0.8467 ± 0.0762 a	15.167±0.7503 a	9.2800 ±0.8253 ab	25.547 ± 2.1620 a	18.960 ± 0.9349 a	91.437 ± 6.5882 c	53.063 ± 4.0966 bc	8.6000 ± 0.3126 bc
900	NM	16.073 ± 1.4694 b	0.7367 ± 0.0906 ab	0.2167 ± 0.0328 d	11.280±1.0660 a	7.7767 ±1.1893 b	11.510 ± 0.5541 d	15.547 ± 0.4620 ab	170.40 ± 8.7727 ab	39.260 ± 3.1892 c	14.607 ± 1.2584 a
	M	17.697 ± 0.5622 b	0.8700 ± 0.1270 ab	0.6267 ± 0.0491 ab	12.363±1.3879 a	8.7300±0.3912 ab	14.140 ± 0.9361 cd	17.360 ± 1.1837 a	192.92 ± 1.8018 a	50.547 ± 4.0839 bc	12.763 ± 0.7354 ab
						Roots					
0	NM	10.807 ± 0.8940 c	0.2600 ± 0.0473 c	0.1643 ± 0.0160 bc	2.8967±0.3325 bc	4.4767 ± 0.6196 ab	7.4067 ± 0.5881 b	10.947 ± 0.7717 cd	52.057 ± 1.1984 cd	30.273 ± 2.8794 b	5.3300 ± 0.5508 b
	M	16.270 ± 1.7304 abc	0.8433 ± 0.2106 a	0.2800 ± 0.0265 ab	4.4233±0.4943 ab	5.9467 ± 0.3645 a	9.8267 ± 0.1393 a	16.780 ± 0.8600 ab	49.810 ± 4.0093 d	37.590 ± 4.0771 ab	4.6867 ± 0.2730 b
100	NM	13.467 ± 0.5957 bc	0.3567 ± 0.0491 bc	0.1700 ± 0.0140 bc	3.0967±0.2601 bc	4.8267 ± 0.4157 ab	7.7100 ± 0.3499 b	8.8067 ± 1.1851 d	76.147 ± 4.2048 ab	49.987 ± 3.2701 a	5.9833 ± 0.3262 ab
	M	11.293 ± 0.5877 c	0.7700 ± 0.0208 ab	0.3200 ± 0.0346 a	4.7500±0.4200 a	6.1833 ± 0.4366 a	9.9167 ± 0.1690 a	12.920 ± 0.8905 bcd	56.410 ± 3.1158 cd	50.997 ± 4.2143 a	4.7100 ± 0.3635 b
300	NM	18.547 ± 0.7647 ab	0.3767 ± 0.0546 bc	0.1167 ± 0.0203 c	2.5233±0.2981 c	4.9533 ± 0.0410 ab	7.3267 ± 0.3018 bc	8.1567 ± 0.8442 d	80.147 ± 2.6444 ab	42.000 ± 4.8182 ab	6.1733 ± 0.1048 ab
	M	16.177 ± 0.5700 abc	0.7200 ± 0.0681 ab	0.3433 ± 0.0291 a	4.3567±0.3524 ab	5.2933 ± 0.2256 ab	8.1733 ± 0.2700 b	14.067 ± 1.7152 bc	67.233 ± 2.6548 bc	46.510 ± 2.3832 ab	5.1467 ± 0.2896 b
900	NM	17.737 ± 1.2603 ab	0.4533 ± 0.0809 abc	0.0700 ± 0.0153 c	2.6633±0.2226 c	3.3233 ± 0.4997 b	4.7333 ± 0.3467 d	12.347 ± 0.4798 bcd	86.947 ± 5.2867 a	35.877 ±2.9880 ab	7.0833 ± 0.7714 ab
	M	19.533 ± 1.7776 a	0.6533 ± 0.0788 abc	0.2600 ± 0.0289 ab	2.9900±0.1779 bc	3.4800 ± 0.3365 b	5.7500 ± 0.1762 cd	20.917 ± 0.7835 a	90.123 ± 3.6968 a	41.640 ± 4.6277 ab	8.3667 ± 0.8604 a

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

3.1.4. Zinc uptake and distribution in Plants

Fig 1.4 (a and b) shows that Zn concentration was increased in plants tissues (shoot and root) with increase of Zn concentration in soil. More accumulation of Zn was recorded in NM inoculated plants as compared to M inoculated plants at all Zn treatments (100, 300, 900 $mgkg^{-1}$). The trend of Zn was same in both M and NM associated plants except highest soil Zn concentration (900 $mgkg^{-1}$) in which more Zn concentrations were recorded in both shoot and root parts of plants. In control plants, the same Zn uptake was observed in both shoot and root part of plants. It was also observed from the results that shoot Zn uptake was more as compared to root part of plants in M associated plants as compared to NM plants. The concentration of Zn increased in both M and NM plants at all Zn concentrations of 100-900 $mgkg^{-1}$. The more Zn accumulation was recorded at the Zn concentration of 900 $mgkg^{-1}$.

Fig 1.4 c shows the distribution of Zn concentration in shoot and root part of wheat plants. The result shows that Zn was more accumulated in root part as compared to shoot part of plants. At high Zn concentrations (300 and 900 $mgkg^{-1}$), the more uptake was observed in both shoot and root part of plants as compared to lower Zn concentration (100 $mgkg^{-1}$). The distribution pattern also shows that different plant organs has different pattern of accumulation. At all Zn concentrations, more concentration of Zn was recorded in NM plants. The root accumulates more Zn as compared to shoot.

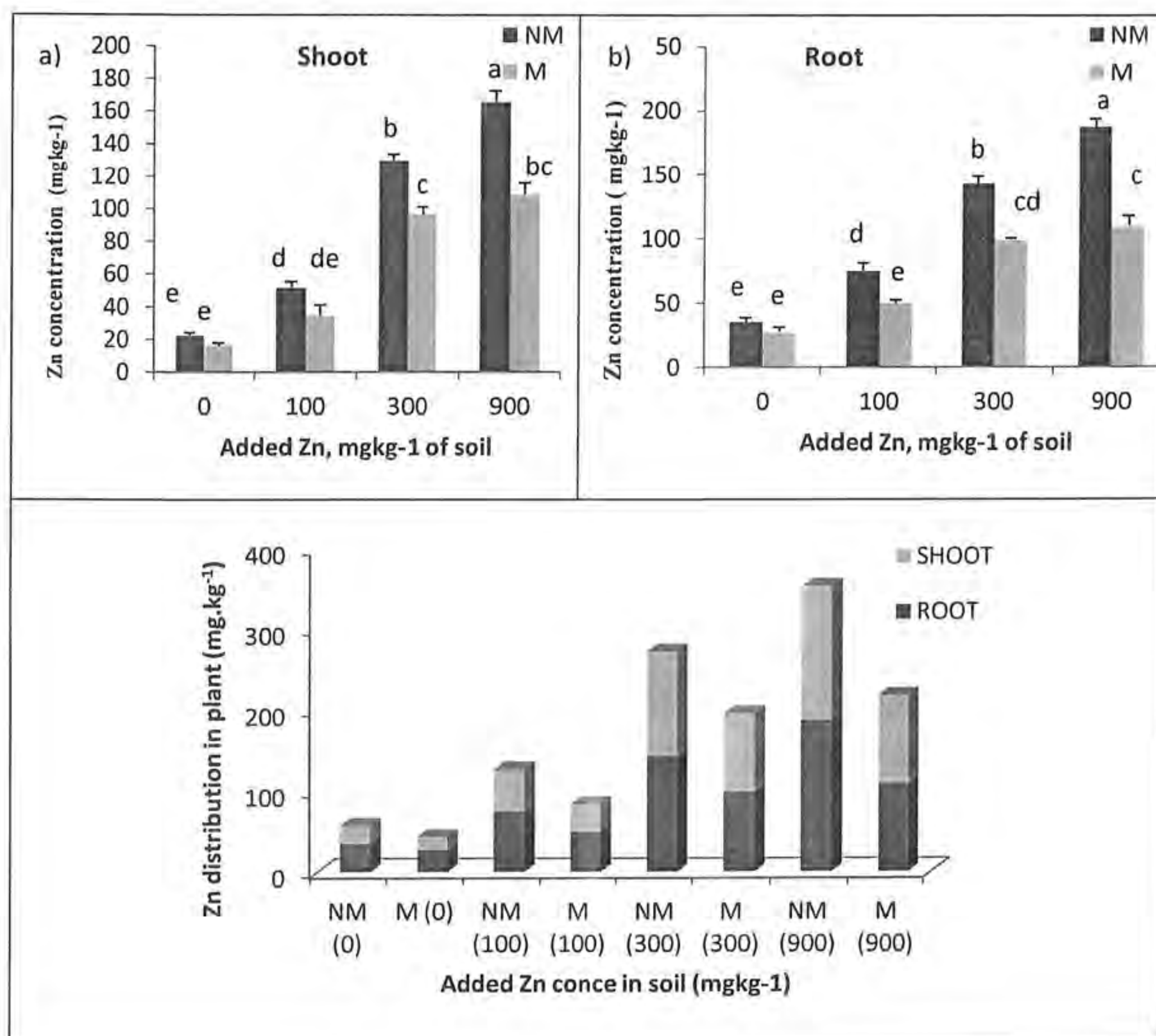

Fig 1.4. Zn concentrations in (a) shoot (b) root, and (c) Zinc distribution; of mycorrhizal (M) and non-mycorrhizal (NM) wheat seedlings growing in soil with increasing Zn concentrations, respectively. The different letters above the bars indicate significant difference between treatments. Bars represent standard error; NM: black bars and M: light grey bars.

3.1.5. Plant biochemical Analyses

Fig 1.5 (a, b and c) shows the relative chlorophyll and carotene contents in M and NM associated plants. The chlorophyll (a, b) and carotene contents (c) in M plants were more than of NM plants at all Zn concentration (100, 300, 900 $mgkg^{-1}$). The highest contents of chlorophyll a and b were observed at Zn concentration of 100 and 300 $mg.kg^{-1}$, while the lowest contents were observed at highest Zn concentration (900 $mgkg^{-1}$) in both inoculated and inoculated treatments. Fig 1.5d shows that as the Zn concentration increased in soil, the level of proline in wheat plants was also increased. M inoculated plants showed lower proline contents at all Zn concentrations than NM inoculated plants. The proline contents were more at highest Zn concentration (900 $mgkg^{-1}$) in both M and NM plants. Fig 1.5e shows the sugar contents with increasing Zn concentrations in mycorrhizal and non mycorrhizal plants. The sugar content was higher in M associated plants as compared to NM associated plants at all Zn concentrations (100, 300, 900 $mgkg^{-1}$). In NM plants, the sugar contents was decreased as the concentration of Zn increased in soil. At higher Zn concentration (900 $mgkg^{-1}$), the lower sugar content was observed in both M and NM plants.

3.1.6. Antioxidant enzyme activities

Fig 1.6 shows the activities of selected antioxidant enzymes i.e SOD, CAT, APX and POD in both M and NM plants at the different Zn concentrations (0, 100, 300, 900 $mgkg^{-1}$). Fig 1.6a shows that in Zn treatments (100 and 300 $mgkg^{-1}$), increased SOD activity was observed in NM plants. But at higher Zn concentration (900 $mgkg^{-1}$), the reduced activity of Zn was recorded. The increase SOD activity was observed at 100 and 300 mg $kg-^{1}$. However, its content was decreased at higher Zn concentration (900 mg $kg-^{1}$). In NM leaves, the SOD activity was low as compared to M plants and a little increase in concentration was observed as more zinc concentration applied. The highest activity of SOD in M plants was observed at 300 $mgkg^{-1}$. Fig 1.6b shows the activity of POD at increasing Zn concentrations in M and NM associated plants. The POD content was increased as the concentration of Zn increased in soil. The results showed that at highest Zn concentration (900 $mgkg^{-1}$), POD activity was decreased in both M and NM inoculated plants. Fig 1.6c shows the leaf CAT activity at all Zn

concentrations (0, 100, 300, 900 mg.kg^{-1}). The trend was same as in POD content. Fig 1.6d shows the trend of APX in M and NM inoculated plants at different Zn concentrations. Increased APX activity was recorded as the concentration of Zn increased in the soil. The highest APX activity was observed at 300 mg.kg^{-1} in M plants.

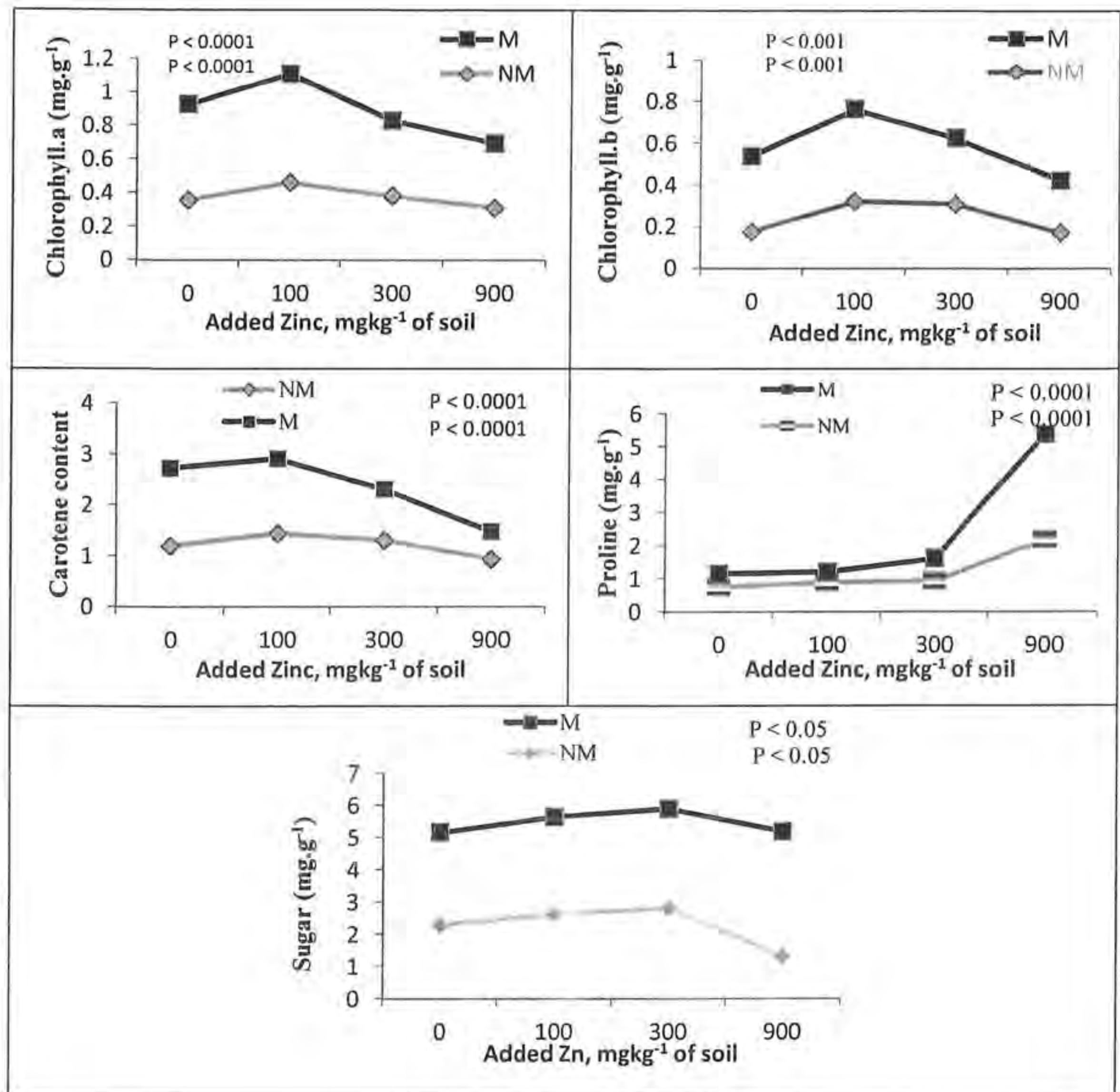

Fig 1.5. Biochemical contents (a and b) Chlorophyll a, b content, (c) Total carotene content, (d) Proline contents, and (e) sugar contents in leaves of mycorrhizal (M) and nonmycorrhizal(NM) *Triticum aestivum* in response to increasing Zn concentrations in soil (R^2: coefficient of determination; $P< 0.05$ significant by the Tukey test (5%) for M and NM means for each Zn concentration; M: black dots and black lines and NM: light grey dots and lines).

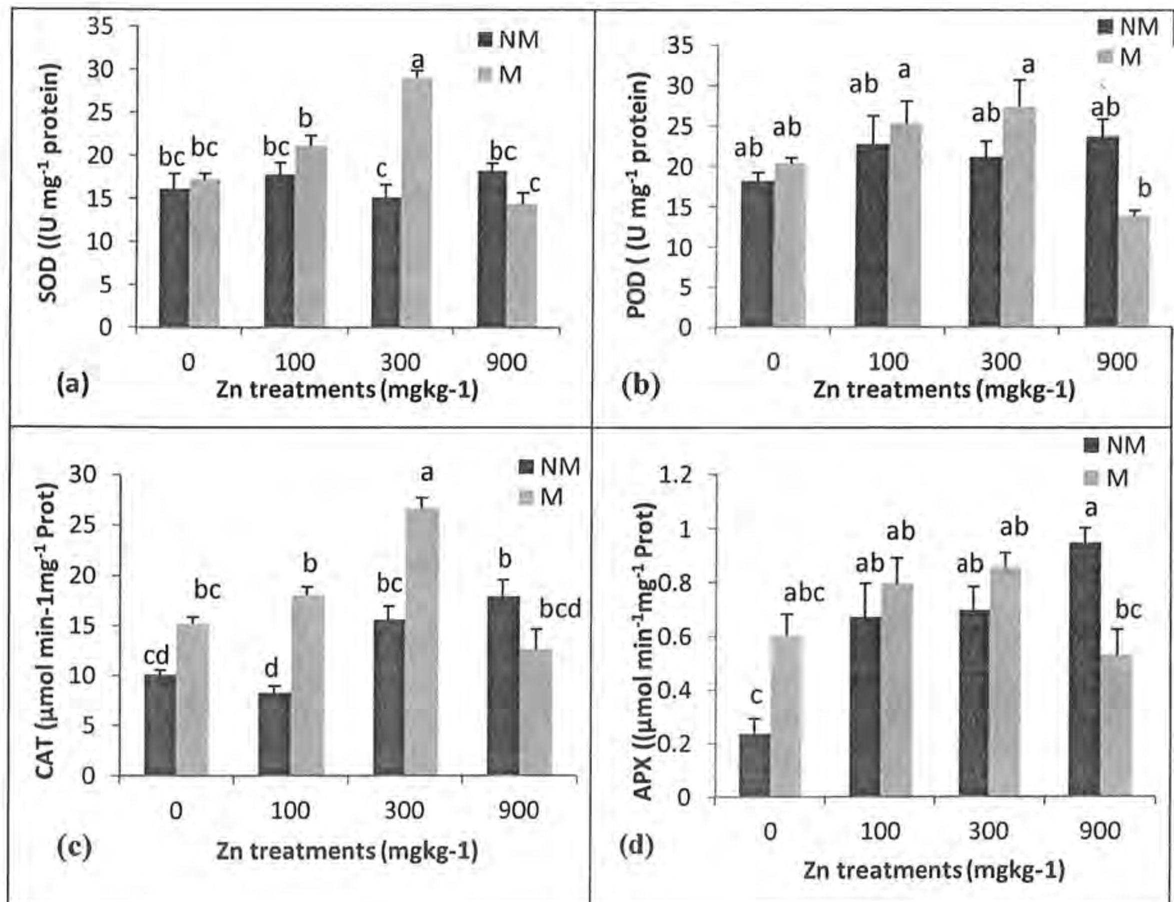

Fig 1.6. Antioxidant enzymes activity (a) SOD activity, (b) POD content, (c) CAT activity, (d) APX activity, in leaves of mycorrhizal (M) and nonmycorrhizal (NM) wheat plants in response to Zn addition to soil. Means (n = 3) with the different letters are significantly different ($p < 0.05$) by the Tukey test. NM: black color bars and M: light grey. Bars represent standard error.

3.2. Part 2: Wheat and AMF association under Cadmium stress

3.2.1. Plant biomass and Mycorrhizal colonization

Fig 2.1 (a and b) shows the effect of cadmium toxicity on M and NM wheat shoot and root biomass. The results indicated that shoot and root biomass was increased in AMF inoculated plants. The biomass of M seedlings were higher in both root and shoot than those of NM seedlings at each concentration of Cd. In NM seedlings, the reduced biomass was observed as the Cd concentration increased in soil. The same trend was observed in both shoot and root parts of plants. In 100 $mgkg^{-1}$ 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 $mgkg^{-1}$ concentration in both M and NM plants.

Fig 2.1c shows the percentage of AM fungi colonization with roots of wheat (*Triticum aestivum*) plants. The result indicated that AMF colonization was not observed in non-mycorrhizal treatments while in all the mycorrhizal treatments (M), more percentage of colonization was observed. The rate of colonization was examined in the form AMF structures i.e arbuscules and hyphal structures. From the results, it is obvious that the symbiotic relationship between *Triticum aestivum* and AMF can be developed under Cd toxic environment. The more percentage of about 72.7% and 72.9 % were recorded at the lower (100 $mgkg^{-1}$) and moderate(300 $mgkg^{-1}$) Cd levels. However, the lower rate of colonization 65.28 % appeared at 600 mg/kg Cd concentration. The percentage colonization in M associated plants at all Cd concentrations ranged between 60 and 70%. Cd addition negatively influenced M root colonization and it is decreased as the concentration of Cd increased in soil.

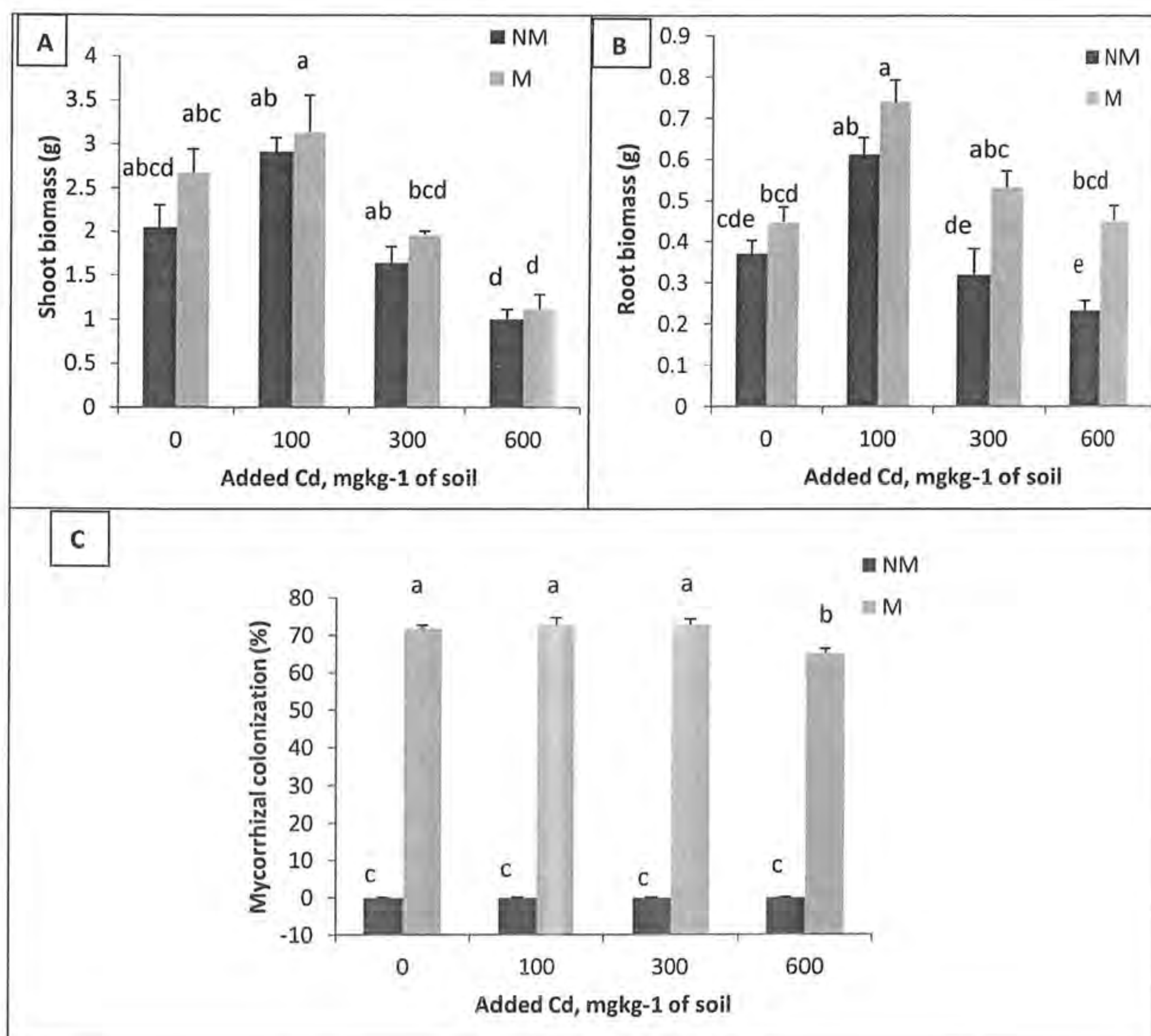

Fig 2.1. Effect of increasing cadmium concentrations on mycorrhizal and non mycorrhizal wheat plants: (a) Shoot biomass, (b) Root biomass, (c) Root colonization. The data shown are the means and standard error. The different letters above the bars indicates significant difference by Tukey test (*$P < 0.05$*).

3.2.2. Plant growth

Table 2.1 shows growth response of wheat plant on M and NM plants in Cd stress conditions. M wheat plants exhibited higher growth than NM wheat plants. Results showed that Cd concentration (300 ad 600 $mgkg^{-1}$) cause decrease in growth of plants in all treatments whether inoculation or not with mycorrhiza. Positive effects on plant growth was recorded at lower Cd concentration of 100 mg kg^{-1}. Plant length, breadth and area was increased in M inoculated plants at 100 mg kg^{-1} concentration of Cd. Meanwhile, non-inoculated plants exhibited slower growth with increasing Cd concentrations.

3.2.3. Cd uptake and distribution in Plants

Fig 2.2 (a and b) shows the linear correlation in plant tissues between Cd concentration in soil and plant uptake, increased cd uptake with increasing soil Cd concentration. The more concentration of Cd was recorded in NM associated plants tissues at all Cd treatments (100, 300, 600 $mgkg^{-1}$) than M plants. In control treatments when no Cd was applied, shoot and root Cd uptake were similar. The results also indicated that shoot Cd uptake increased much less in M plants than root Cd uptake with increase of Cd toxicity in plants. The Cd uptake was decreased in M shoot as compared with NM plants. However, the increased concentration of Cd was observed in all parts of NM plants in comparison to M plants as the application of Cd increased from 100-600 $mg.kg^{-1}$ in soil. The more accumulation observed at the highest Cd concentration of 600 $mg.kg^{-1}$. For analysis of Cd distribution in the wheat, plants were separated into shoot and root (Fig. 2.2c). In the experiment, more Cd accumulation was observed in roots of wheat plants. In M plants, Cd translocation to shoot was lower at low Cd concentration (100 $mg.kg^{-1}$). While lower uptake was recorded at Cd levels (300 and 600 mg kg^{-1}). It was also observed that Cd accumulation varies in different plant tissues with different concentration levels. In soil containing 300 and 600 $mg.kg^{-1}$ Cd, Cd range was higher in shoot and roots of NM plants as compared to M plants respectively. 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.

Table 2.1 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 $P < 0.05$.

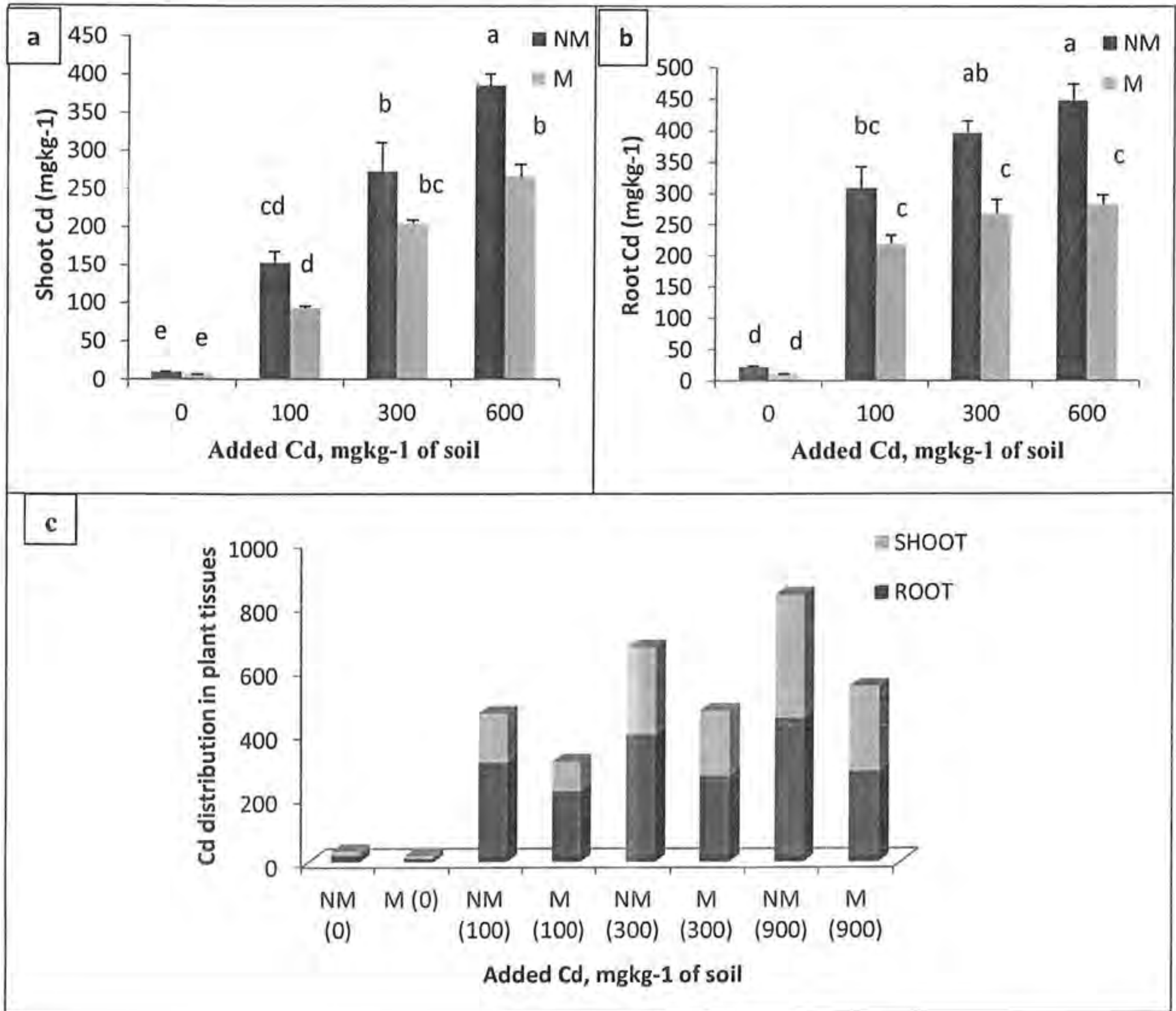

Fig 2.2. Cd concentrations in (a) shoot (b) root, and (c) cadmium distribution in plant tissues; of mycorrhizal (M) and non-mycorrhizal (NM) wheat growing in soil with increasing Cd concentrations, respectively. The different letters above the bars indicate significant difference between treatments. Bars represent standard error; NM: black bars and M: light grey bars.

3.2.4. Plant Phosphorus (P) uptake and mineral nutrition

Fig 2.3 (a and b) shows the effect of mycorrhizal inoculation on plant P nutrition with increasing Cd concentrations. In M plants, the more P was recorded in plant tissues than NM associated plants. The decreasing trend was observed as the concentration of Cd increased in NM plants. The slight increase in P content was observed at 100 $mgkg^{-1}$ Cd concentration in both M and NM plants as compared to control plants.

Table 2.2 shows the effect of increasing Cd concentration on plant mineral nutrition (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 all Cd addition levels. The highest Cd addition (600 $mgkg^{-1}$) had a detrimental effects on nutrient contents of plants in M and NM inoculated plant tissues. The result of the experiment clearly indicated that mycorrhization positively influenced the mineral nutrition 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 nutrients K, N, Ca, Mg, Na, Fe, Cu, Zn, Fe was recorded in M inoculated plant roots while reduced concentrations of Mn and Ni was observed. 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 recorded in NM root part of plants with increasing Cd concentration. While decrease in N, Ca, Na, K and Na concentrations in roots of NM wheat plants.

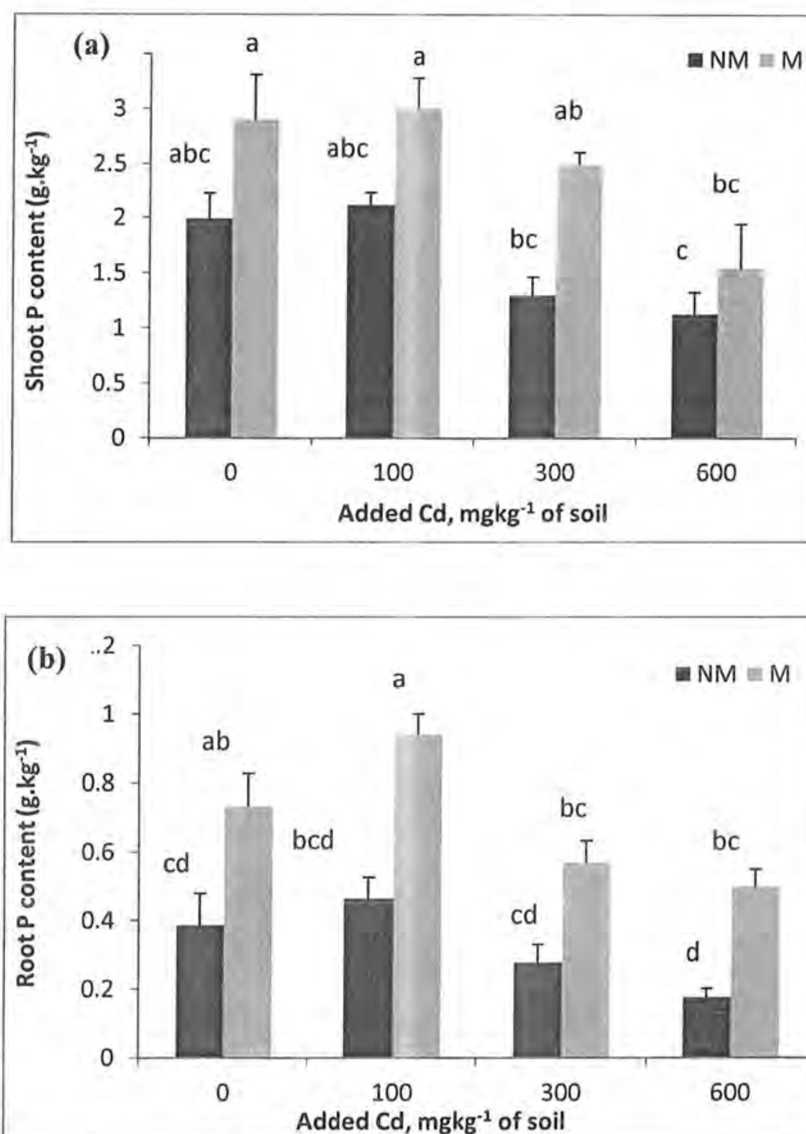

Fig 2.3. Phosphorus concentration in: (a) shoots and (b) roots of mycorrhizal (M) and non-mycorrhizal (NM) wheat plants in response to Cd addition to soil. Means (n = 3) with the different letters are significantly different (*P< 0.05*) by the Tukey test.

Table 2.2. K, N, Ca, Mg, Na, Fe, Mn, Ni and Cu 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	Cu	Mn	Zn	Fe	Ni
Shoot		g.kg^{-1}					mgkg^{-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	15.87±1.0340 d	72.447±2.2754 bc	33.137±2.5304 a	40.623±3.5274 bcd	6.4867±0.2042 bc
	M	28.513±1.0729 ab	1.0967±0.0726 ab	30.673±1.5510 a	12.41±0.3407 a	15.253± .3182 ab	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	29.187±2.0502 a	0.8367±0.0669 bc	19.253±0.8027 c	11.77±0.2386 ab	8.5267± .6055 c	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	31.93 ±3.6344 a	1.48±0.1976 a	24.623±0.7912 b	12.19±0.3951 a	17.66± 0.4844 a	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	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	25.56±1.5408 ab	69.47±5.9127 cd	26.27±1.6493 ab	36.857±0.8311 cd	7.0033± .8521 abc
	M	23.437±3.5656 abc	1.3833±0.1049 a	17.703±1.2410 cd	11.243±0.6481 abc	15.557± 1.1823 ab	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	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	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	17.693±1.2127 bc	0.8533±0.0722 bc	15.323±0.0941 cd	9.6667±0.2051 bc	9.2467± 0.3527 c	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
Roots											
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	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	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	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	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	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	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	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	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	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	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	40.937± 4.0135 ab	53.757±3.0010 cd	37.183±1.3720 ab	38.367±2.4940 ab	10.787±0.2354 bcde
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	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	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	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 ± SE (n = 3) and have been analyzed by two way analysis of variance (ANOVA). Means followed by the same letter within columns are not significantlydifferent by Tukey's test at the 5% level.

3.2.5. Plant biochemical Analyses

Fig 2.4 (a, b and c) shows the effect of mycorrhiza on relative chlorophyll and carotene contents with increasing Cd concentrations. The concentration of chlorophyll (2.4 a and b) and carotene contents (2.4c) in M plants were greater at all concentrations of Cd (100, 300, 600 mgkg^{-1}). The highest chlorophyll a and b contents were observed at Cd concentration of 100 mgkg^{-1} in all treatments. The lowest chlorophyll and carotene contents were observed at Cd concentration (300 and 900 mgkg^{-1}) in both inoculated and inoculated treatments. Fig 2.4d shows the proline content in wheat plants with increased Cd concentrations in soil. M inoculated plants showed lower proline levels in control and 100 mgkg^{-1} Cd concentration. The proline content was increased at 300 and 600 mgkg^{-1} Cd concentration in NM plants. As the concentration of Cd increased in soil, proline contents were also enhanced except at lower Cd concentration of 100 mg.kg^{-1}. Fig 2.4e 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.

3.2.6. Antioxidant enzyme activities

Fig 2.5 shows the antioxidant (SOD, CAT, APX, POD) activities in M and NM plants at different Cd concentrations (0, 100, 300, 600 mgkg^{-1}). Fig 2.5a shows that 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. In M plants, an increase in activity was observed at all Cd concentrations 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 2.5b shows the effect of Cd on POD contents in M and NM plants. The POD activity was increased in plants at different Cd concentrations. On the contrary, in mycorrhizal treatment Zn exposure led to significant decrease at highest Cd addition level (600 mg.kg^{-1}). Fig 2.5c indicates effect of Cd on CAT contents in M and NM plants. Reduced CAT activity was recorded with increased Cd addition levels. In mycorrhizal and non mycorrhizal associated plants, the CAT activity was more at 100 and 300 mg.kg^{-1}.

Fig 2.5d shows the APX activity in wheat plants with increasing Cd concentrations in all treatments. The results showed that APX activity was increased with the increase of Cd concentrations. The highest APX activity was observed at 100 mg.kg^{-1} in M and NM plants.

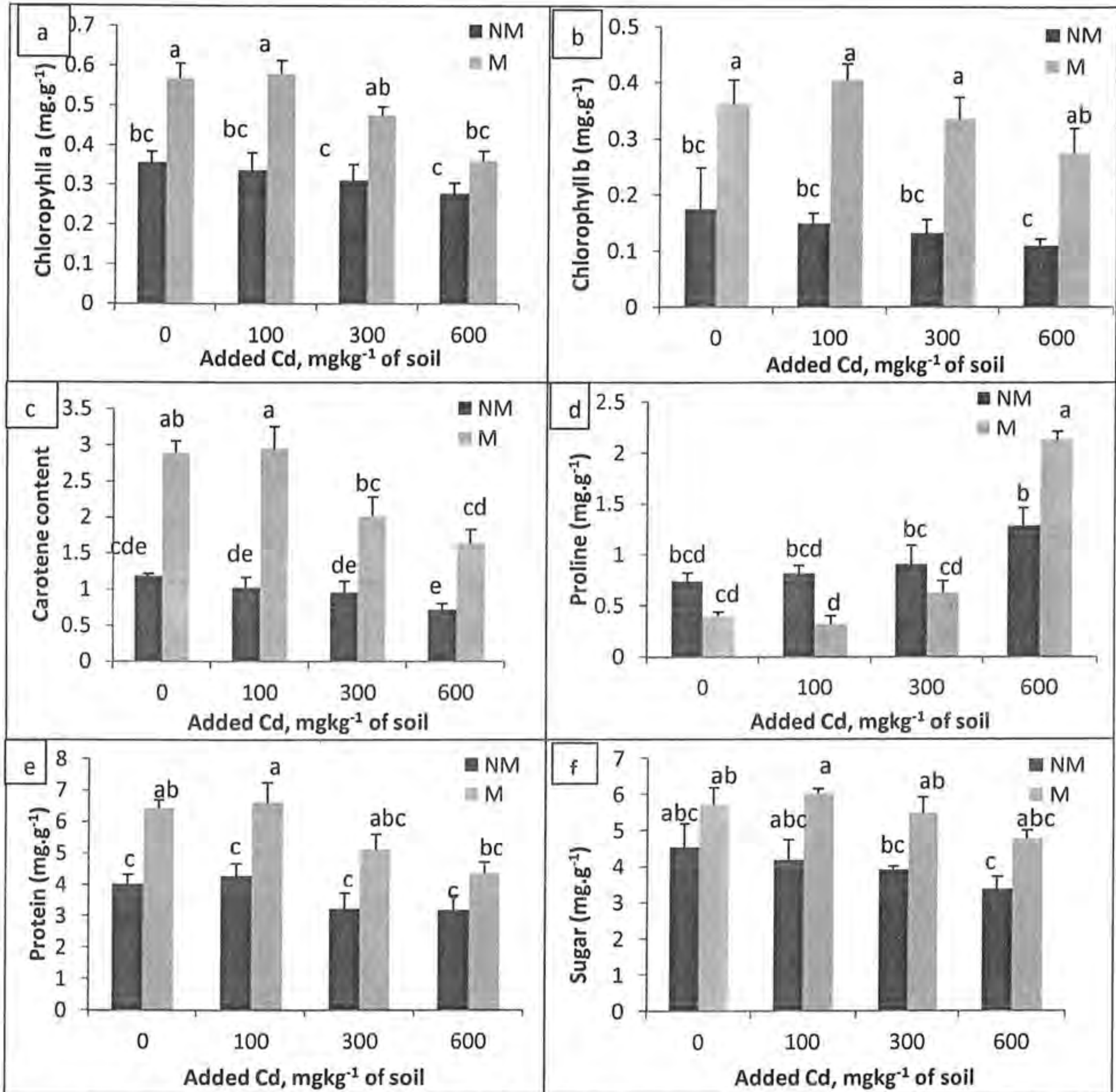

Fig 2.4. Biochemical contents (a and b) Chlorophyll a, b content, (c) Total carotene content, (d) Proline contents, (e) Protein contents, and (f) Sugar, in leaves of mycorrhizal (M) and nonmycorrhizal(NM) wheat in response to increasing Cd concentrations in soil (R^2: coefficient of determination; $P< 0.05$ 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.

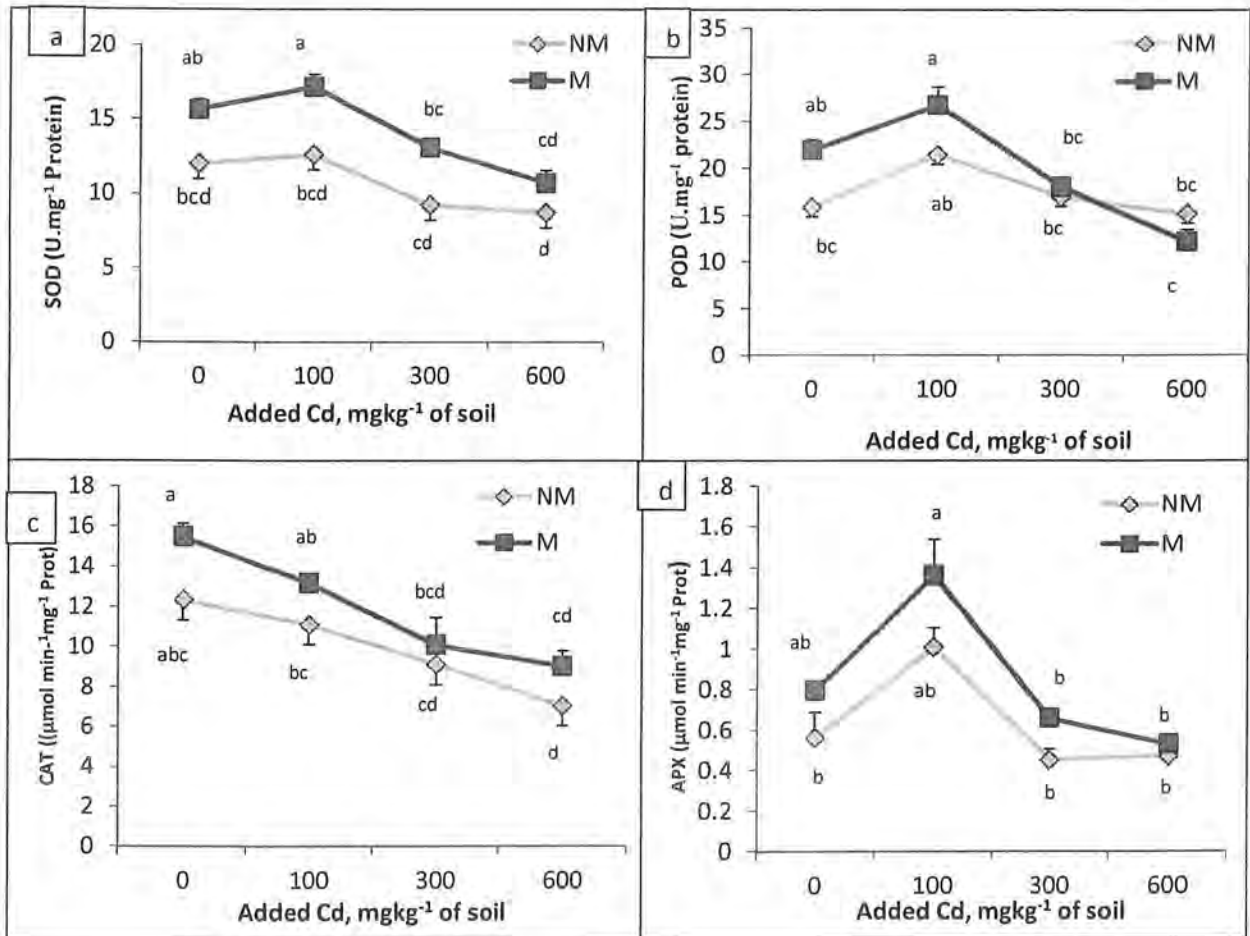

Fig 2.5. Antioxidant enzymes activity (a) SOD activity, (b) POD content, (c) CAT activity, (d) APX activity, in leaves of mycorrhizal (M) and nonmycorrhizal (NM) wheat plants in response to cadmium addition to soil. Means (n = 3) with the different letters are significantly different ($p < 0.05$) by the Tukey test. M: black color lines and NM: light grey lines. Bars represent standard error.

3.3. Part 3: Alfalfa and AMF association under Zinc and Cadmium stress

3.3.1. Mycorrhizal colonization of roots

Fig 3.1 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 detected in non-mycorrhizal associated plants, while more colonization percentage was recorded in mycorrhizal inoculated plants. The colonization was detected in roots with formation of arbuscules and hyphal structures. Fig 3.1a shows the highest colonization of 70% was found at 100 $mgkg^{-1}$ Zn concentration. The trend was decreasing as the concentration of Zn increased at 300 $mgkg^{-1}$ and 900 $mgkg^{-1}$. Fig 3.1b shows the root colonization of alfalfa plants with increasing Cd concentrations. 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 $mgkg^{-1}$. 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.

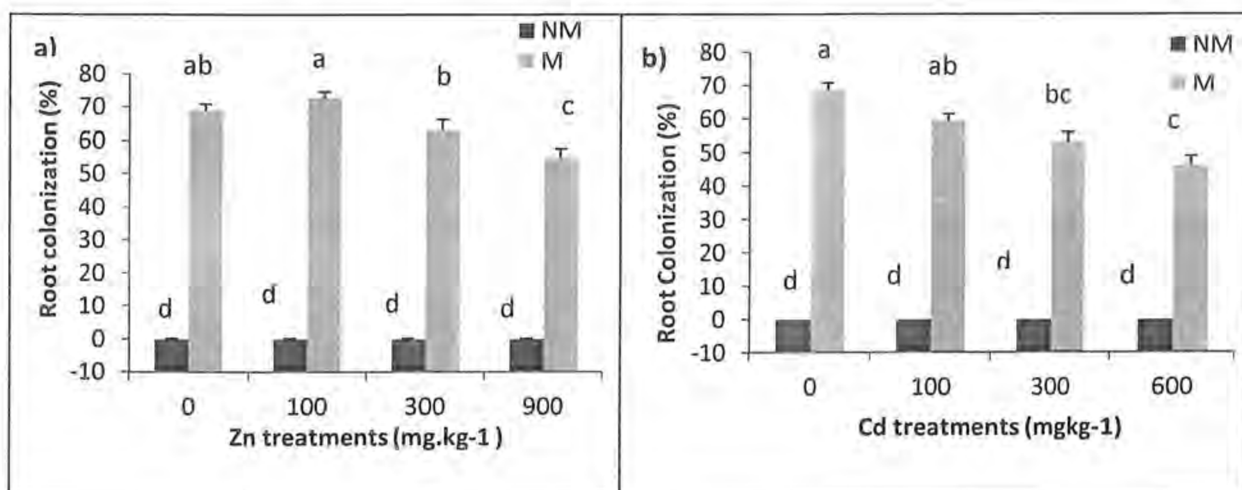

Fig 3.1. Percentage of root length colonized (% RLC) of mycorrhizal (M) and non-mycorrhizal (NM) *Medicago sativa* plants in soils with increasing Zn and Cd concentrations. M and NM means with different letters are significantly different by the Tukey test (5%).

3.3.2. Plant Growth and Biomass

Fig 3.2a shows the growth response of alfalfa plants with Zn increasing concentrations in M and NM inoculated treatments.The results showed that plant growth and biomass was reduced 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 biomass of plant tissues was increased in M treated plants in all the Zn concentrations (100, 300 and 900 $mgkg^{-1}$). While reduction in biomass was recorded in NM treated plants as the toxicity of Zn enhanced in soil. The highest trend was recorded at 100 $mgkg^{-1}$ Zn concentration in M and NM plants, while lowest biomass was recorded at 300 and 900 $mg.kg^{-1}$ Zn concentrations. 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 at Zn concentration (100 $mgkg^{-1}$). However, M inoculated plants had significant positive effects on plants growth and biomass at all Zn treatments as compared to NM plants.

Fig 3.2b shows the growth response of alfalfa plants with increasing cadmium concentrations in both M and NM plants. The plant growth and biomass was reduced in non-inoculated (NM) treatments under increasing Cd 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 Cd treatments (100, 300, 600 $mgkg^{-1}$), While reduction in biomass was observed in NM inoculated plants as the Cd concentration increased in soil. The highest trend was recorded in control plants in M and NM plants, while lowest biomass was recorded at all Cd addition levels. 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 all treatments. While the reduction in trend was recorded at all cd addition levels (100, 300 and 600 $mgkg^{-1}$) in both M and NM plants. However, M inoculated plants had significant positive effects on plants growth and biomass at all Cd treatments as compared to NM plants.

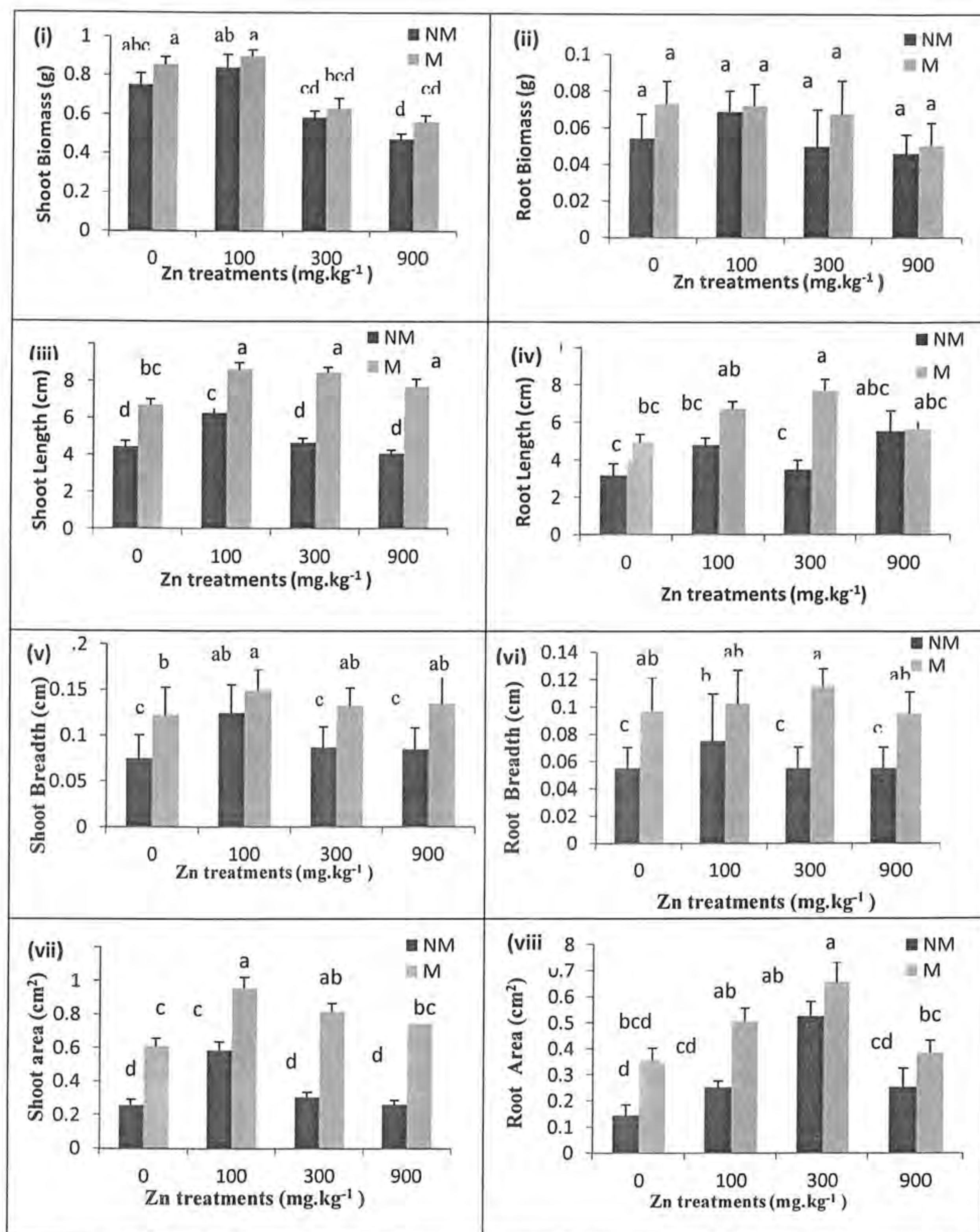

Fig 3.2a. Effects of increasing zinc concentrations on growth and biomass of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants: (i, ii) Shoot and root biomass, (iii, iv) Shoot and root length, (v, vi) Shoot and root breadth, (vii, viii) Shoot and root area. M and NM means with different letters are significantly different by the Tukey test (5%).

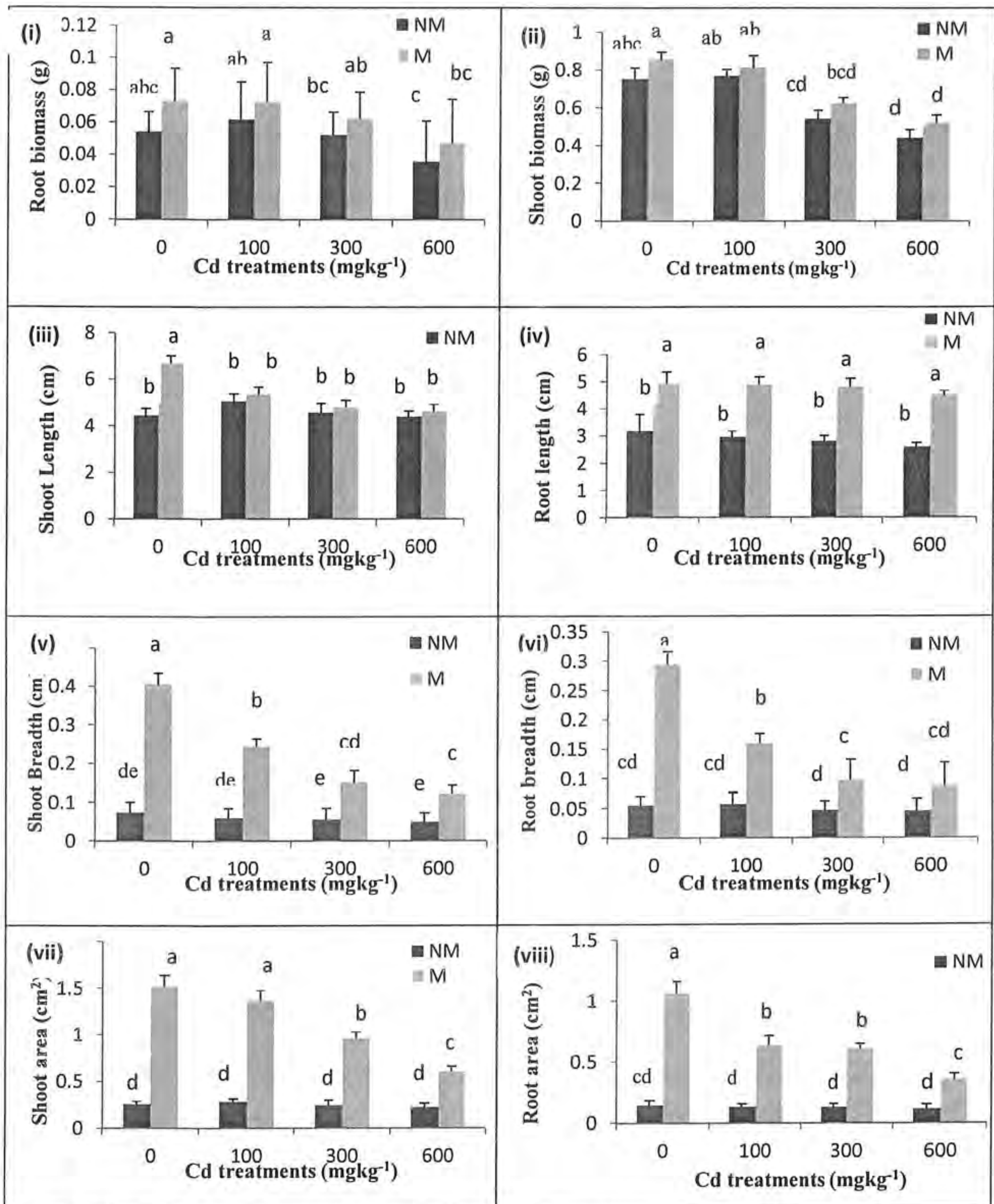

Fig 3.2b. Effects of increasing cadmium concentrations on growth and biomass of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants: (i, ii) Shoot and root biomass, (iii, iv) Shoot and root length, (v, vi) Shoot and root breadth, (vii, viii) Shoot and root area. M and NM means with different letters are significantly different by the Tukey test (5%).

3.3.3. Plant Mineral Nutrition

Table 3.1 shows nutrients status in alfalfa plants after treatments with different Zn addition levels. In general, the increased nutrient contents (N, K, Ca, Mg, Na, Fe, Cu) were observed in M treatments (100 and 300 $mgkg^{-1}$) except in Mn and Ni, in which the trend was decreasing in mycorrhizal treatments as the concentration of Zn was increased. In NM plants, the significant results were recorded for Ca, K, Na, P, N, Mn in M and NM treated plants. However, the non-significant results were recorded for Cu and Mg at all Zn concentrations. The Zn concentration (900 $mgkg^{-1}$) had a harmful effects on nutrients status because reduced nutrient contents were observed at this concentration in both M and NM associated 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. It was also depicted from the results that the increase of Zn concentrations in soil cause decrease of N, K, P, Ni, Fe and Mn contents shoot part of plants. The Cu and Na was increased with increase of Zn addition levels. 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 3.2 shows nutrients status of alfalfa plants with enhancing Cd addition levels in M and NM associated plants. 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 nutrients K, P, Na, N, Ca, Mg, Fe showed significant results in shoot tissues but not significant results were obtained in Mn and Ni at all Zn concentrations. The highest Zn concentration (900 $mgkg^{-1}$) had harmful effects on uptake of nutrients in M and NM treated plants. The experiment indicated that mycorrhizal inoculation significantly affects the mineral nutrition of alfalfa plants. In NM inoculated plants, Zn increasing concentrations cause reduction in N, K, Na, Ca, Mg, Fe, Mn, Ni and Zn contents in the shoots part of plants. The increase of Zn cause to increase the Cu contents in shoot of NM treated plants. The decrease of nutrient contents were observed in roots part of alfalfa plants with increasing soil Cd concentration.

Table 3.1. Concentration of nutrients measured in shoots and roots of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants grown in soils with increasing Zn concentrations

Experiment (Zn, mgkg^{-1})		N (g.kg^{-1})	K (g.kg^{-1})	Ca (g.kg^{-1})	Mg (g.kg^{-1})	Na (g.kg^{-1})	Mn (mg.kg^{-1})	Fe (mg.kg^{-1})	Ni (mg.kg-1)	Cu (mg.kg^{-1})
Leaves +stem										
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	179.35± 18.715 a	73.030 ±4.3985 abc	12.813±1.6108 ab	8.040 ±0.7744 a
	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	133.87±6.5347 ab	92.547± 2.5647 a	8.740 ± 0.8173 b	8.953± 0.8108 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	164.25±17.651 ab	61.837± 3.1418 bc	14.090 ± .6005 ab	10.147 ± 0.8714 a
	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	113.14±16.125 ab	70.483± 4.2766 abc	9.103 ± 1.1201 ab	11.453 ± 0.5167 a
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	109.79±16.967 ab	56.30 ± 8.0698 bc	14.983±1.9024 ab	10.473 ± 2.0368 a
	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	95.06± 7.9648 b	83.567± 7.4396 ab	9.4 ± 0.5541 ab	15.577 ± 4.0707 a
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	118.22±10.116 ab	48.640± 8.1033 c	15.363±1.2835 a	10.873 ± 0.7902 a
	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	125.48±16.293 ab	52.807± 5.6784 c	10.857±0.7449 ab	11.763 ± 1.0327 a
Roots										
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	81.890 ± 8.0254 ab	38.263± 10.026 a	8.77 ± 0.8260 ab	9.377 ± 0.4824 ab
	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	57.25 ± 9.4086 ab	45.68 ± 5.8473 a	5.813 ± 0.9385 b	11.177 ± 0.8312 ab
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	63.573 ± 12.077 ab	54.93 ± 4.9003 a	8.48 ± 0.5522 ab	9.413 ± 0.5820 ab
	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	46.923 ± 3.1745 b	65.51 ± 4.4623 a	6.373 ± 0.5500 ab	13.82 ± 0.9789 a
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	96.073 ± 4.3756 a	48.93 ± 4.2158 a	10.067±0.7166 a	10.85 ± 1.5069 ab
	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	45.513 ± 7.0815 b	52.62 ± 8.9798 a	5.78 ± 0.9777 b	13.143 ± 1.4814 a
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	82.947 ± 13.586 ab	46.227± 10.878 a	9.88 ± 0.7850 ab	7.54 ± 0.6116 b
	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	92.56 ± 9.3940 a	47.403± 5.5555 a	7.36±0.7900 ab	9.397 ± 0.5174 ab

Data are presented as mean ± 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 3.2. Concentration of nutrients measured in shoots and roots of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants grown in soils with increasing Cd concentrations.

Experiment (Cd, mgkg-1)		N (g.kg-1)	K (g.kg-1)	Ca (g.kg-1)	Mg (g.g-1)	Na (g.kg-1)	Mn (mg.kg-1)	Fe (mg.kg-1)	Ni (mg.kg-1)	Cu (mg.kg-1)	Zn (mg.kg^{-1})
Leaves +stem											
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	71.733± 6.0350 a	46.543±4.0441 ab	6.29±0.9585 a	9.507±0.6093 a	26.3±3.1701 a
	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	54.213± 4.3947 a	50.853±4.3645 a	5.7467±0.7115 a	5.027±0.5155 b	17.813±2.3157 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	57.060±4.7509 a	31.807±5.9041 b	4.4133±0.5190 a	11.45±0.7044 a	15.88±1.8618 b
	M	1.26±0.2629 a	13.82 ± 0.9771 abc	20.033± 2.8451 ab	13.523± 0.6393 bc	12.150±2.0924 a	49.323± 2.2645 a	46.747±1.7822 ab	4.8467±0.7568 a	9.983±0.1235 a	13.593±0.8810 b
300	NM	0.6567± 0.0649 ab	7.627±1.2209 d	13.913±0.7760 b	9.36± 0.4496 c	5.410±0.3635 bc	55.14±5.5078 a	42.257±3.7790 ab	3.9233±0.2200 a	13.447± 1.2788 a	14.257±0.3852 b
	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	52.033± 9.3103 a	50.86±3.2926 a	3.6933±0.1642 a	11.587±1.4921 a	12.62±1.5305 b
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	66.657±11.871 a	32.843±2.0503 ab	6.0367±0.7799 a	13.817±1.2432 a	13.857±0.9727 b
	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	73.693± 8.9930 a	40.813±3.0488 ab	4.5633±0.3148 a	10.143±1.4313 a	14.477±0.6064 b
Roots											
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	57.557± 3.3463 ab	26.523±2.0653 ab	7.433±0.9616 ab	19.033±3.1755 ab	13.923±0.9034 abc
	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	49.573± 2.1903 bc	29.61±3.2810 a	6.88±0.8600 ab	14.847±1.1866 b	16.7±0.5730 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	64.85± 3.9822 a	17.953±1.7402 cde	10.483±1.0721 a	16.697±1.6717 b	14.887±1.9581 abc
	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	43.747± 4.2026 bc	21.517±2.1262 bcd	7.517± 0.5279 ab	18.597±0.6569 ab	11.070±0.7778 bc
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	50.353± 3.1850 abc	16.817±0.8762 cde	5.463±0.4869 b	21.290±1.4043 ab	10.643±1.0712 bc
	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	38.180±1.9500 c	23.437±2.6793 abc	4.783±0.8417 b	24.89±0.9469 a	16.027±1.6120 ab
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	58.177± 2.4011 ab	14.51±0.6416 e	9.74±1.0761 a	14.557±1.2846 b	9.577±0.4736 c
	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	47.737± 2.6273 bc	16.287±1.2858 de	7.857±0.8027 ab	13.677±0.3877 b	13.117±0.6697 abc

Data are presented as mean ± 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.

3.3.4. Plant Phosphorus (P) uptake

Figure 3.3 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. Fig. 3.3a shows that alfalfa plants had higher shoot and root P at lowest Zn concentration (100 $mgkg^{-1}$), while the P content was decreased at the Zn concentration of 300 and 900 mgkg-1 in both M and NM plants. At these levels, the reduced growth of plants was recorded. The trend of P uptake was decreased as the Zn concentration was increased. Figure 3.3b 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 $mgkg^{-1}$. The enhanced P level was recorded in plant tissues where no Cd concentration was applied.

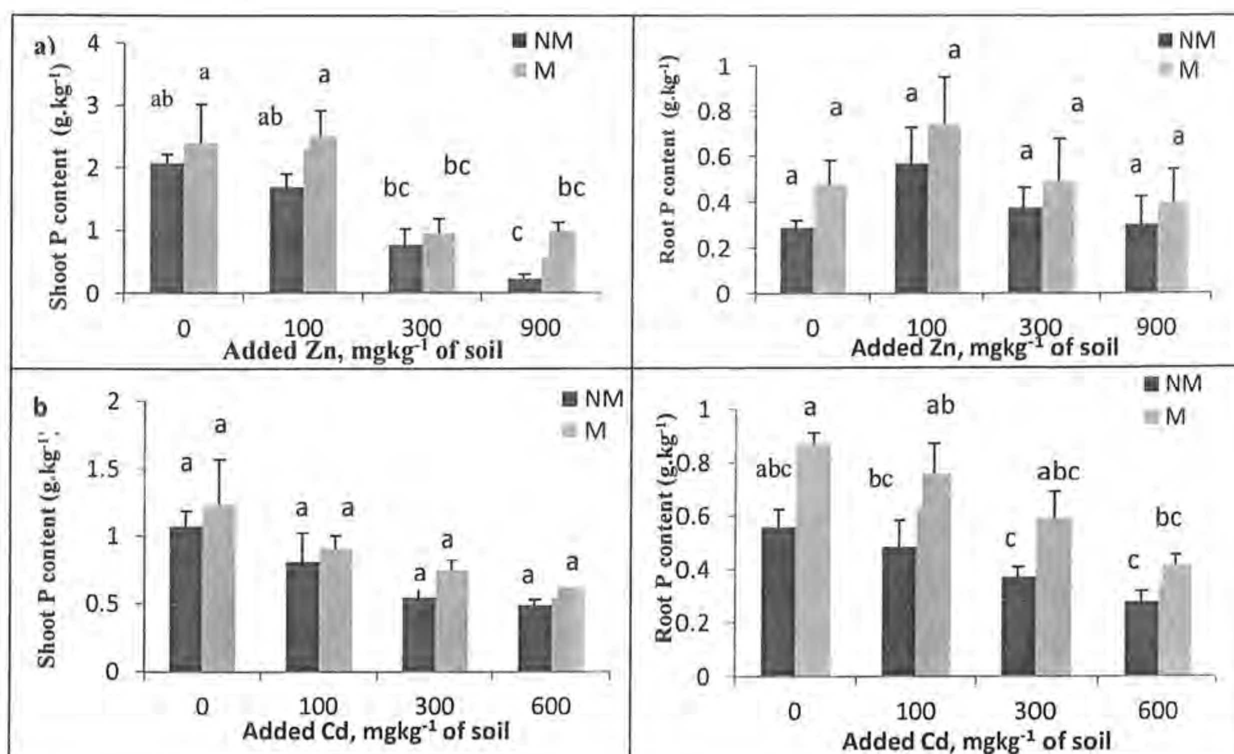

Fig 3.3. Phosphorus contents in: (a) shoots and (b) roots of mycorrhizal (M) and non-mycorrhizal (NM) in alfalfa plants in response to Zn and Cd addition to soil. Means (n=3) with the different letter are significantly different (*P< 0.05*) by the Tukey test.

3.3.5. Zn and Cd translocation and distribution in alfalfa plants

Fig 3.4 shows the uptake of Zn and Cd in alfalfa plant with increasing Zn and Cd concentrations. The Zn and Cd concentration was increased in shoot and root parts of plants as the metal addition levels increased in soil. However, the results indicated that NM plants accumulated more concentration of metals in plant at all Zn (100, 300, 900 $mgkg^{-1}$) and Cd (100, 300, 600 $mgkg^{-1}$) treatments than M inoculated plants. In control treatments when no Zn and Cd was applied, shoot and root Zn and Cd uptake were similar. The results indicated that shoot uptake of Zn and Cd was less in comparison to root metals uptake in mycorrhizal associated plants. However, Zn and Cd uptake was more in shoot of NM plants. As the application of Zn and Cd increased, the concentration increased in shoot and root of NM plants as compared to M plants.

Fig. 3.5 shows the distribution of Cd and Zn in alfalfa plant tissues in both M and NM treatments with increase of Cd and Zn concentrations in soil. Zn and Cd distribution were observed in shoot and root of plants. For analysis of Zn and Cd distribution in alfalfa, . In the experiment, the Zn and Cd accumulated mainly in the roots. Fig 3.5a shows that Zn translocation to shoot was lower at low Zn concentrations (100 mg kg^{-1}) than moderate and high Zn concentrations (300 and 900 $mg.kg^{-1}$), in which the roots accumulated more Zn than shoot part of plants. Fig 3.5b shows Cd distribution in shoot and root parts of plants. The results indicated that in M inoculated plants, more Cd accumulated in root as compared to shoot part of plants. In NM plants, the more Cd contents observed in shoot part of plants. As the concentration of Cd increased from 100-600 $mg.kg^{-1}$, the more accumulation was observed in shoot and root part of plant but lower uptake was recorded in M inoculated plants as compared to NM inoculated plants.

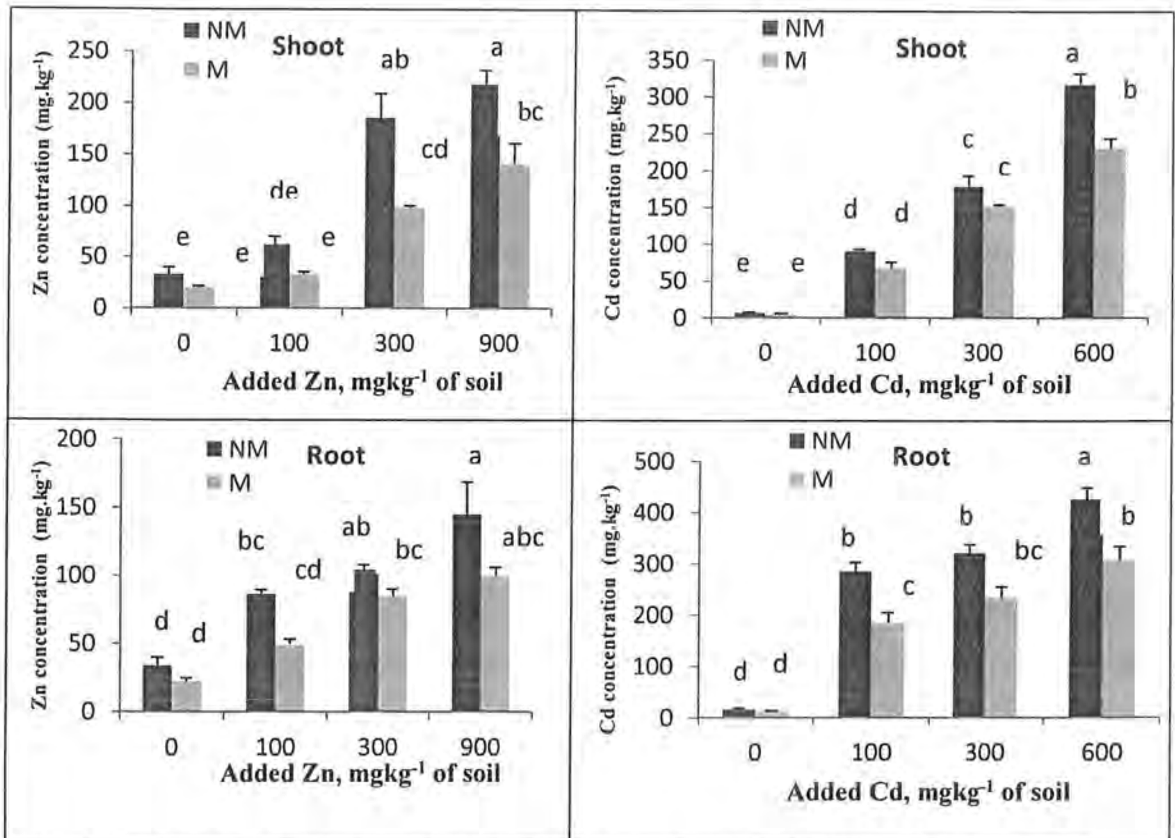

Fig 3.4. Cd and Zn concentrations in shoots and roots of mycorrhizal (M) and non-mycorrhizal (NM) *Medicago sativa* plants growing in soil with increasing Cd or Zn concentrations, respectively. Means (n = 3) with the different letters are significantly different (*P< 0.05*) by the Tukey test. (NM: black bars ; M: light grey bars).

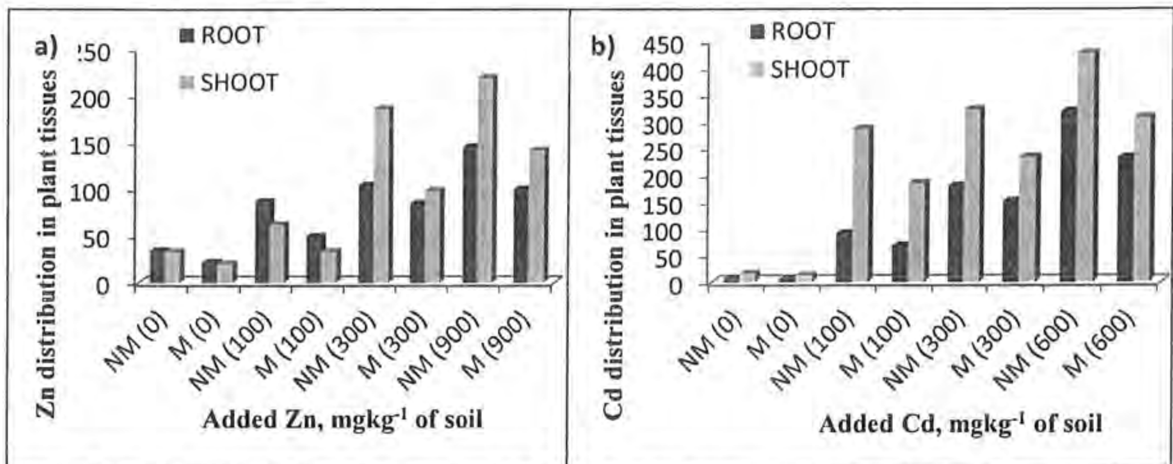

Fig. 3.5. Cd and Zn distribution in (a) shoot and (b) root of mycorrhizal (M) and non-mycorrhizal (NM) *Medicago sativa* plants in soil with increasing Cd and Zn concentrations in soil, respectively.

3.3.6. Effects of metals (Zn and Cd) on biochemical analysis

Fig 3.6 (a and b) shows the biochemical indicators in M and NM inoculated plants with increasing Zn and Cd concentrations. In general, relative chlorophyll and carotene contents was significantly higher in M inoculated plants as compared to NM inoculated plants at each Zn and Cd concentration. Fig 3.6a 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 lowest Zn addition level (100 $mgkg^{-1}$) in both inoculated treatments. The lowest sugar level was observed at highest Zn concentration (900 $mg.kg^{-1}$). The sugar content was decreased linearly as the Zn concentration increased in soil in M and NM associated plants. The sugar content recorded was higher in M inoculated plants than NM plants at all Zn additions (100, 300, 900 $mgkg^{-1}$). Results showed that increased proline content was recorded as the concentration of Zn level increased in soil from 100 to 900 $mg.kg^{-1}$ of soil. In NM plants, higher proline levels was recorded as compared to M inoculated plants.

Fig 3.6b shows the effects of increasing Cd concentrations on biochemical contents of M and NM inoculated plants. The reduced chlorophyll a, b and carotene contents were observed at all Cd induced concentrations. 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 $mgkg^{-1}$ Cd concentration. In M plants, the higher chlorophyll and carotene contents were observed as compared to NM plants at all Cd concentration (100, 300, 600 $mgkg^{-1}$). The sugar content was decreased linearly as the concentration of Cd increased in soil. However, the more sugar contents was observed at all Cd concentration (100, 300, 600 $mgkg^{-1}$) in mycorrhizal treated plants. But the lowest contents were observed in non-mycorrhizal plants. The proline content was increased as the Cd concentrations increased in soil. At Cd concentration (100 $mgkg^{-1}$), the lower proline content was recorded in both M and NM treated plants. The higher proline content was observed at 900 $mgkg^{-1}$ of Cd concentration.

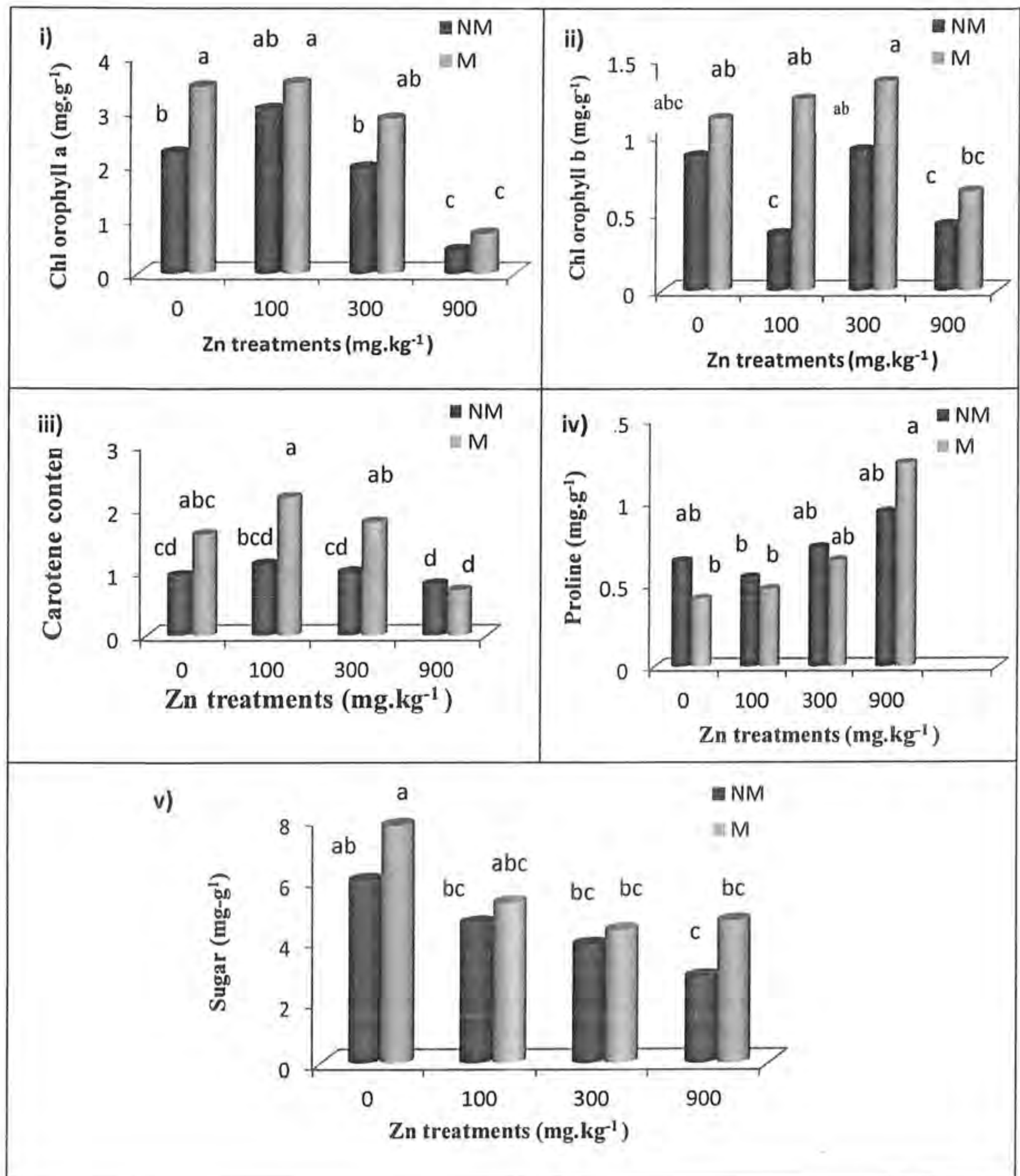

Fig 3.6a. Biochemical contents (i and ii) Chlorophyll a, b content, (iii) Total carotene content, (iv) Proline contents, and (v) sugar contents in leaves of mycorrhizal (M) and nonmycorrhizal(NM) *Medicago sativa* in response to increasing Zn concentrations in soil. Means (n = 3) with the different letters are significantly different (*P< 0.05*) by the Tukey test. NM: black bars and M: light grey bars).

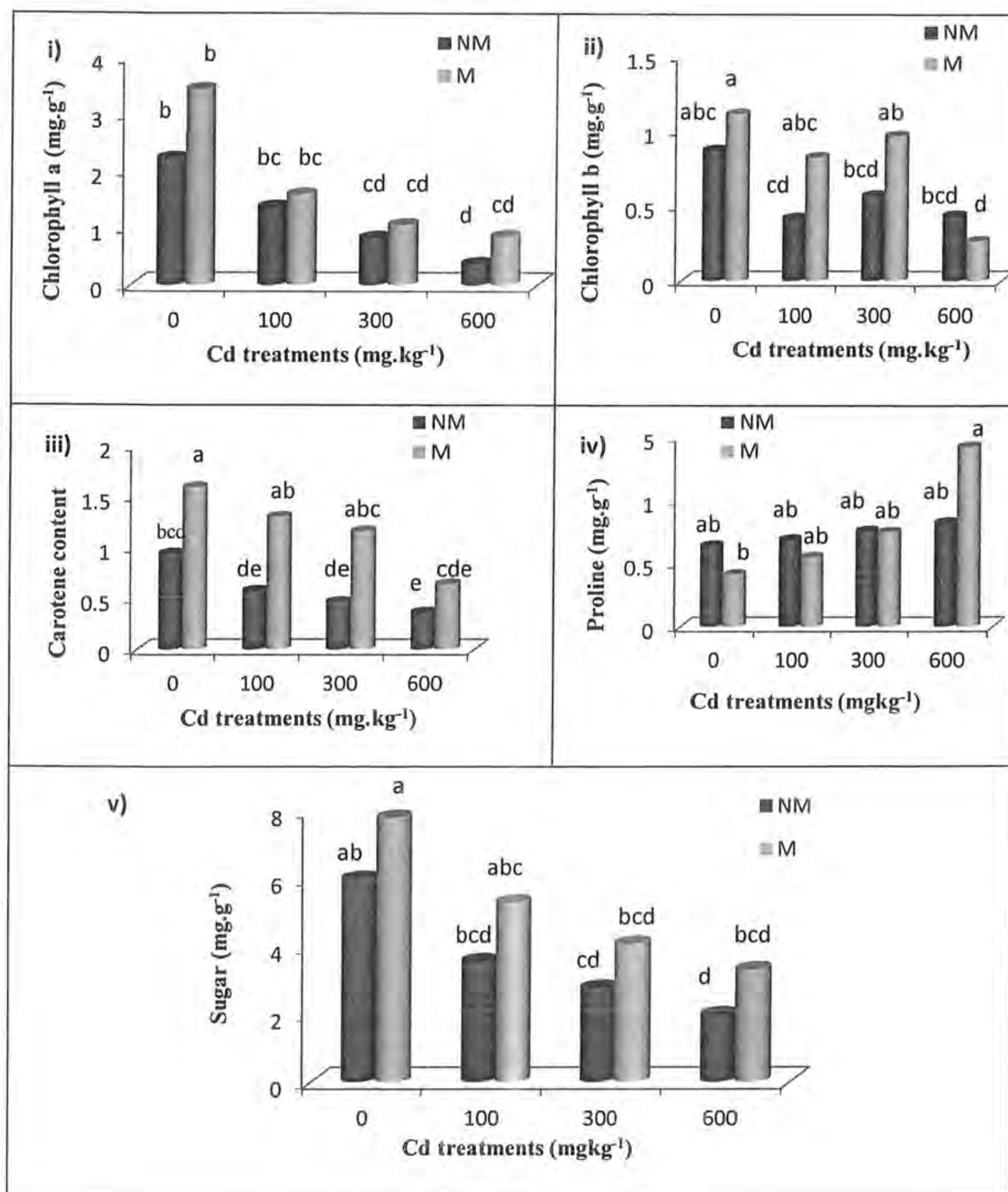

Fig 3.6b. Biochemical contents (i and ii) Chlorophyll a, b content, (iii) Total carotene content, (iv) Proline contents, and (v) sugar contents in leaves of mycorrhizal (M) and nonmycorrhizal(NM) *Medicago sativa* in response to increasing Cd concentrations in soil. Means (n = 3) with the different letters are significantly different (*P< 0.05*) by the Tukey test. NM: black bars and M: light grey bars).

3.3.7. Effects of metals (Zn and Cd) on antioxidant enzyme activities

Fig 3.7 shows the response of antioxidant enzymes in alfalfa plants with Zn and Cd additions levels in both M and NM treatments. Fig 3.7a shows the trend of SOD, CAT, APX and POD activities in M and NM plants with increasing Zn concentration (0, 100, 300, 900 $mg.kg^{-1}$). In NM plants, SOD activity increased with increasing Zn addition to soil, except at Zn concentration of 900 $mgkg^{-1}$. In M plants, the same trend was recorded but the activity of enzyme was better than metal stressed soil in NM treatments. The enhanced SOD activity was recorded at 100 and 300 $mg.kg^{-1}$ Zn concentrations in M treated plants. The reduced content was observed at highest Zn concentration (900 $mg\ kg^{-1}$).

The POD activity was increased with the increase of Zn concentration in alfalfa plants. The highest POD activity was observed at Zn addition level (100 $mgkg^{-1}$) in both M and NM treatments. The reduced POD activity was recorded at 300 and 900 $mgkg^{-1}$ Zn concentration. The CAT activity induced in the similar manner as the SOD activity. The increased CAT activity was observed as the level of Zn increased except at the highest Zn concentration (900 $mgkg^{-1}$) in M and NM inoculated plants. The results showed that the activity of APX was reduced as the Zn concentration increased in soil except at 100 $mgkg^{-1}$ in which the increased activity was recorded. The highest APX activity was observed at 100 mgkg-1 in M and NM inoculated plants.

Fig 3.7b shows the enzymatic response (SOD, CAT, APX, POD) of alfalfa plants in M and NM treatments with enhancing concentrations of Cd (0, 100, 300, 600 $mg.kg^{-1}$). In NM plants, the reduced SOD activity was recorded as more Cd stress induced in soil. 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 $mgkg^{-1}$ Zn concentration. The highest POD activity was observed at Zn addition level (300 $mgkg^{-1}$) in all inoculated treatments. The increased POD content was recorded in M treated plants as compared to NM inoculated plants at all Zn concentrations. As the concentration of Zn increased in soil, the activity of CAT was reduced in both M and NM inoculated plants. The results

indicated that APX activity was reduced as the Cd concentration increased in soil. The Cd concentrations (0, 100, 300, 600 $mgkg^{-1}$) caused to decrease the APX activity in both M and NM inoculated plants.

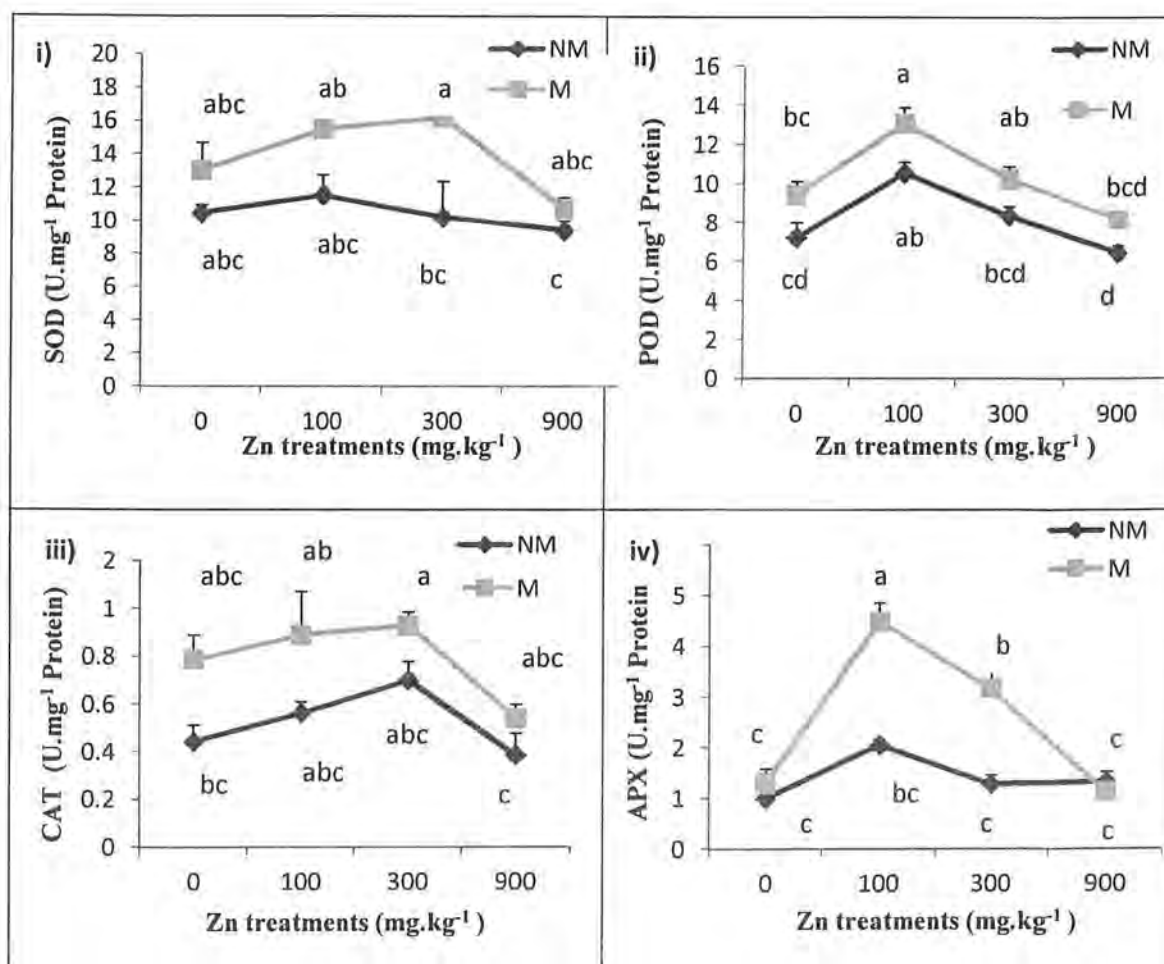

Fig 3.7a. Antioxidant enzymes activity (i) SOD activity, (ii) POD activity, (iii) CAT activity, (iv) APX activity, in leaves of mycorrhizal (M) and nonmycorrhizal (NM) alfalfa plants in response to Zn addition to soil. Means (n = 3) with the different letters are significantly different ($p < 0.05$) by the Tukey test. NM: black color lines and M: light grey lines. Bars represent standard error.

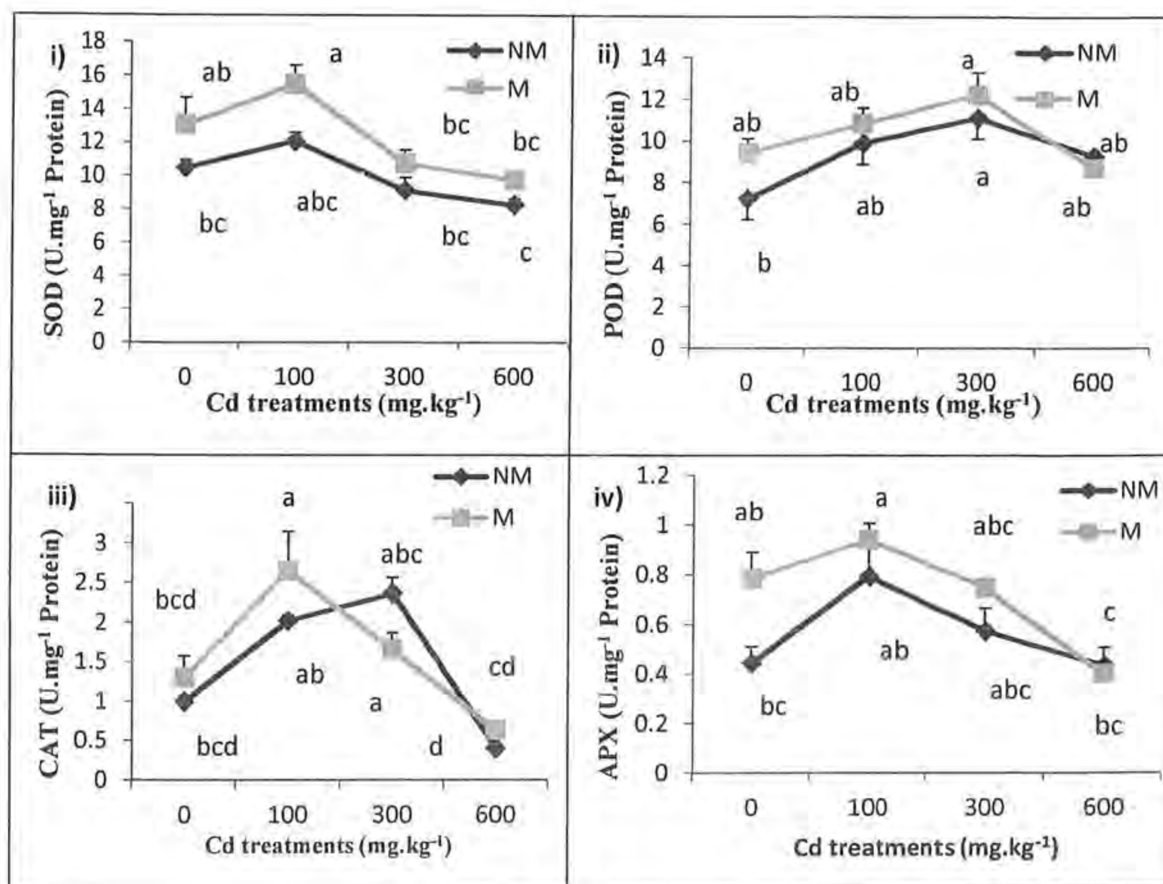

Fig 3.7b. Antioxidant enzymes activity (i) SOD activity, (ii) POD activity, (iii) CAT activity, (iv) APX activity, in leaves of mycorrhizal (M) and nonmycorrhizal (NM) alfalfa plants in response to Cd addition to soil. Means (n = 3) with the different letters are significantly different ($p < 0.05$) by the Tukey test. NM: black color lines and M: light grey lines. Bars represent standard error.

Chapter 4

DISCUSSION

Discussion

4.1. Part 1: Wheat and AMF association under Zinc stress

4.1.1. Effect of AMF on wheat growth, biomass and colonization under Zn toxicity

It has been reported that AM fungi can reduce Zn toxicity in plant species in association with AMF and have better growth and biomass of plants in comparison to non-inoculated plants (Arrigada, 2005). The results indicated that better wheat growth was observed in AMF-inoculated plants. While at Zn concentrations > 300mg/kg may inhibit root and shoot growth indicating Zn phytotoxicity. Similarly, some other reports indicated the same results (Andrade et al., 2010; Karagiannidis and Nikolaou, 2000; Chao and Wang, 1990). Gratao et al. (2005) reported that Zn is an essential nutrient that is required by plants in a very small quantities. However, the higher Zn concentrations (above 300 $mg.kg^{-1}$ Zn) is toxic for the growth and development of plants. Higher concentration of Zn may cause to disturb various physiological and morphological functions in plants. Zn toxicity also cause to produce oxidative stress in plants which directly affects the metabolic pathways.

The present study showed the fact that plant growth and biomass was increased at low Zn concentrations in soil and had no toxic effects on plants growth, development and metabolic processes. This may be due the deficiency of Zn nutrient in control soil. It also shows the fact that plants require Zn in limited amounts. The plant growth and biomass was not inhibited with lower and moderate Zn concentrations (100 $mg.kg^{-1}$ and 300 $mg.kg^{-1}$) in soil. This is due to the higher pH value of soil which is about 7.8. The more concentrations of Zn may have been immobilized at high pH and lower translocate towards the upper part of plants. This cause the lower Zn uptake towards the plant tissues even present at higher concentrations in soil.

The study showed that Zn toxicity had a deleterious effects on percentage of plant root colonization by fungi. The root colonization was decreased as the concentration of Zn increased in soil. The same result was reported in previous studies that mycorrhizal colonization decreased with increase of Zn toxicity in soil (Gildon and Tinker, 1983; Marques et al., 2006). The results of previous studies indicated microbes and plants in soil are also affected by the toxicity of heavy metals. Some studies also showed that mycorrhizal colonization was not affected from the increase of metals concentrations in soil. On the

contrary, other authors revealed that higher colonization was recorded in Zn toxic soils (Hildebrandt et al., 1999 and Diaz et al., 1996; Whitfield et al., 2004 ; del Val et al., 1999; da Silva et al., 2005; Wang et al., 2005a ; Chen et al., 2003; Li et al., 2011). This is due to the inoculation and usage of AMF from different environments. Because AMF isolated from heavy metals contaminated soils have more ability to persist in toxic soils. They have more accumulation capacity of metals than mycorrhizal spores isolated form less contaminated soils (Leung et al., 2006).

4.1.2 Effect of AMF inoculation on plant Zn uptake

The present study indicated that when Zn present in lower amounts in soil, the translocation of Zn towards mycorrhizal associated plants was increased. However, as the Zn concentrations increased in soil, the mycorrhiza provides protection to plants against Zn toxicity and more Zn was accumulated in roots. This shows the protective effects of mycorrhiza on plants against toxicity of metals. The same result was reported by Wong et al. (2007). The Zn concentration in plants tissues was increased as the level of Zn increased in soil whether the plants are inoculated or not with mycorrhiza. However, it is suggested that more Zn was accumulated in root of mycorrhizal plants and subsequently the uptake towards shoot was reduced. The lower growth and biomass was observed in non mycorrhizal plants because the more Zn was more accumulated in shoot and root parts of plants. This indicates the fact that fungi accumulated more Zn in the mycelium of mycorrhizal associated plants. (Colpaert et al., 1992). Some studies reported that AMF can be used for phytoremediation purposes due to binding of excessive quantities of metals in the mycelium and lower metals uptake towards edible part of host plants (Andrade et al., 2008; Marques et al., 2006, Toler et al., 2005 and Chen et al., 2003). Chen et al. (2001) reported in their study that Zn contents in the mycelium of fungi is 10 times more than shoot and roots parts of plants. Kaldorf et al. (1999) reported that more Zn was detected in the fungal structures such as vesicles present in root cortex. All of the evidences in the previous reports indicated that chelation of metals with fungal structures and immobilization of metals within mycelium are among the main factors that causes the lower translocation of metals in plants tissues. So these are the main factors that are involved in reduction of heavy metals toxicity within plants by using arbuscular mycorrhizal fungi. It is also studied that AM fungi cause to enhance the metals

uptake in plants when they are present in deficient amounts. There is still a need of exploring the mechanisms that are involved in increased metals uptake of plants. The effects of mycorrhiza on translocation of metals in plants is dependent upon the plant type, AMF species and particularly the type and concentration of metals in a soil.

It is reported that moderate Zn contamination was prevented by AM fungus in host plants. This is due to immobilization of maximum quantity of Zn in the fungus mycelium. This mechanism is the main reason that cause to decrease the metal concentrations in soil and eventually phytotoxicity is also prevented. Soil pH is also the main factor that prevented the metals uptake by solubilization in soil. This alteration in soil pH is the important factor through which phytotoxicity is reduced. Some researchers reported the activity of some compounds in reduction of metals toxicity like glomalin secreted by fungus (Joner et al., 2000) and role of extrametrical mycelium is also suggested (Gonzalez- Chavez et al., 2002). These two factors play a role in reducing the metals content in soil and plants. Mycorrhiza has various detoxification processes such as chelation of metals via extraradical mycelium and secretion of fungal compounds such as glomalin (Christie et al., 2004; Janouskova et al., 2007).

4.1.3. Effects of AMF on plant P uptake and nutrient status under Zinc toxicity

The results of the present study indicated that the mycorrhizal inoculation increased the nutrient contents at all Zn concentrations in wheat plants. While the nutrients uptake was inhibited in the non-mycorrhizal plants at all Zn addition levels. The P uptake and enhanced growth rate was observed in AMF-associated plants and it is one of the most useful effects on plants grown in metal toxic conditions. This increase of P content and other nutrients such as N, Mg, Ca and K in M associated plants cause to enhance the growth and productivity. The same results were reported by many authors (Chen et al., 2003, 2004; Feng et al., 2003; Christie et al., 2004; Wang et al., 2005a). The reason of enhanced plant growth and nutrient contents in M associated plants are due to increased absorption area due to more volume of soil explored by extra-radicular hyphae that is the main cause to increase the nutrient contents and directly enhance the growth of plants (Rhodes and Gerdemann, 1975).

The study indicated that mycorrhizae enhance the P concentration in shoot part of plants at moderate (300 $mgkg^{-1}$) and higher Zn concentration (900 $mgkg^{-1}$). The enhanced P

content in AMF interactive plants is the beneficial effects documented in a variety of plants exposed to metal stress. De Vos et al. (1993) reported that nutrient contents in plants are mostly deficient due to presence of toxic concentrations of heavy metals. This cause to decrease the nutrient uptake in plants that directly affects the growth and structure of roots and cell membrane. Another report showed that heavy metals cause damage in physiological and metabolic pathways of plants by inducing alterations and deficiencies in uptake of nutrients (Rossini Oliva et al., 2009). The mycelium of fungus helps to absorb the limiting nutrients especially P from the soil. The P absorbs by the extrametrical mycelium comprising of native and applied soil P. The better uptake of P nutrient in AMF associated plants is accredited to its active uptake from soil to plants. The arbuscules are the main sites of transfer of nutrients towards the plants (Smith and Read, 1997; Turnau and Przybylowicz, 2003).

In the present study, mycorrhizae improved the proficiency of N acquisition under Zn stress. There is generally a short provision of essential nutrients in combination with excessive metals at heavy metal polluted areas (Chen et al., 2005a). The increase of N acquisition in mycorrhizal inoculation under metal Cu, Cd, Ni, Pb and Zn exposure that aid host plants against metal stress (Blaudez et al., 2000a). It might be due to the presence of arbuscular mycorrhiza through which a host plant can acquire more nutrients and plant resistance to metal toxicity can be improved.

Several authors reported that AMF inoculation enhanced the uptake of nutrients in plants including P, N, K, Ca, Mg, Na that directly exert a positive effects on growth and development of plants (Clark and Zeto, 2000; Schweiger and Jakobsen, 2000). It is thought mostly that plants having interactions with AMF are unable to grow without mycorrhiza (Harrier, 2003). Plant mineral nutrition can be disturbed by heavy metals inducing deficiencies in essential nutrients uptake and affecting root growth and cell membrane structure (De Vos et al., 1993). Joner et al. (2000) revealed that Glomus species had a high metal binding capacity with toxic metals and prevent their uptake towards plants.

In the present study, the reduced K, Mg and Cu contents were observed in shoot of NM plants with increasing Zn concentrations. The same results were reported by Hall (2002) and Bonnet et al. (2000). This might be due the rupture of membranes at high metal concentrations that cause leakage of different nutrients.

4.1.4. Effects of AMF on biochemical activities under Zinc toxicity

The present study showed that soluble proline contents in M and NM inoculated plants increased with the increase of Zn toxicity in soil. Several reports indicated that rate of amino acids increased in plant tissues under metal toxicity (Herrera-Rodriguez et al., 2007). The increase of amino acids showing a protective action against metal stress (Sharma and Dietz, 2006). Similar results related to present studies was reported by several authors. The proline content was increased in different plant species as the metal toxicity increased in soil (Sharma and Dietz, 2006; Alia et al., 1995). The proline also cause to reduce the oxidative stress and generation of free radicals (Alia et al., 1995). Limited studies are reported on the response of proline contents under metal toxic conditions. The increased proline contents also the indicator of toxic concentration of metals. Moreover, lower proline and amino acid contents were observed in M than NM plants.

Several studies showed the reduction of root and shoot growth and decrease in total chlorophyll at high metal concentration (Khan et al., 2009; Paivoke and Simola LK, 2001). The increase of metals concentration cause toxicity in all plants resulted in chlorosis, necrosis, inhibition of growth and finally death (Mallick et al., 2011). Present study showed that biochemical contents were increased in M plants i.e chlorophyll and carotene contents as compared to NM with increasing Zn concentration. The decrease of chlorophyll content due to deficiency of metals is rare. The metals like Mg and Fe are essential for the metabolism of chlorophyll (Rosen et al., 1977). While the reduced P level is among one of the main factors that cause to reduce the chlorophyll content (Jiang et al., 2007). It is also observed that oxidative stress cause harm to chloroplasts because Zn toxicity cause to increase the production of reactive oxygen species (ROS) (Tewari et al., 2008).

The present study reported that antioxidant enzymes i.e SOD, APX, CAT and POD was enhanced in M associated plants with increasing Zn concentrations. Foyer and Noctor (2005) reported that antioxidants have a vital role in inducing defense mechanism in plants. Schutzendubel and Polle (2002) indicated the same results that alterations in activities of antioxidant enzymes are limited in M treated plants with the increase of metal toxicity. This might be due to the protective effect of SOD against reactive oxygen species (ROS) that prevents the cell from oxidative damage. It is suggested that AMF association form beneficial

mycorrhizal association in Zn stressed soils that cause to increase plant growth, nodulation and nutritional value.

Andrade et al. (2008) reported that mycorrhizal roots showed lower POD activity under metal stress than non-mycorrhizal associated plants. It is also reported that M inoculation generate less oxidative stress that is the function of fungal symbiont proteins (Ouziad et al., 2005 and Hildebrandt et al., 2007). As Zn is essential micronutrient and cofactor of SOD that cause to enhance the antioxidant enzymatic activity in associated plants which is beneficial to control the free radicals (Cakmak, 2000; Wang et al., 2007; Gao et al., 2010). Hacisalihoglu et al. (2003) observed that SOD activity was less in plants grown over Zn deficient soil. Furthermore, research on response of other antioxidant enzymes should be carried out in future studies to better understand the mechanism against metal stress.

4.2. Part 2: Wheat and AMF association under cadmium stress

4.2.1. Effect of AMF on wheat growth, biomass and colonization under Cd toxicity

The present study indicated positive effects of mycorrhizal association on wheat plants with the increase of cadmium toxicity. The growth, biomass and colonization percentage was enhanced in mycorrhizal associated plants with the increase of Cd concentrations. This shows that mycorrhiza is proven to be useful under heavy metals toxicity. Gaur and Adholeya (2004) reported the same results that mycorrhiza exert significant positive results and increase nutrient uptake and biochemical activities. However, the Cd toxicity also cause to decrease the plant mycorrhizal association like in the present study higher concentration of Cd (600 $mg.kg^{-1}$) decrease the metabolic and physiological processes. The colonization of mycorrhiza reduced with the increase of heavy metals toxicity in soil (Gildon and Tinker, 1983; Weissenhorn and Leyval, 1993; Hildebrandt et al., 1999). Pawlowska and Charvat (2004) reported that response of Glomus species varies with different toxic metals concentrations. However, Chen et al. (2004) and Shahabivand et al. (2012) revealed that Cd stress does not affect the colonization percentage of plant roots by AM fungi. This might be due to association linkage between plants type, metal concentrations and AMF species (Zhang et al., 2006).

In the present work, the shoot and root length, breadth and area was reduced with increase of Cd toxicity in wheat plants without mycorrhizal association. Similar results were reported in tomato plants (Lopez-Millan et al., 2005; Cao et al., 2003) and in barley (Wu and Zhang, 2002). AM colonization enhanced the plant growth indicating the fact that AMF has positive effects on plant physiological functions. These positive effects was linked to mycorrhizal interaction that play a role in uptake of mineral nutrients especially limited soil nutrients (Marschner and Dell, 1994; Gajewska et al., 2006; Hu et al., 2009; Xu et al., 2008).

4.2.2. Effects of AMF on plant P uptake and nutrients under Cd toxicity

The results of the present study indicated that arbuscular mycorrhizal fungi are helpful in enhancing the yield and productivity of host plants. The same result was reported by Hu et al. (2009). This is due to more P content acquired by mycorrhizal associated plants than nonmycorrhizal roots. The high contents of P is also harmful for agrosystems and have a negative effects on plants physiological and metabolic processes. The stress alleviation of heavy metals in host plants might be related to enhanced P uptake (Cumming et al., 1986). AM fungi had higher shoot and root biomass and P content compared to non-AM plants (Bai et al., 2008). The improved nutrient contents especially P content is another way through which AM fungi has positive effects on preventing heavy metals toxicity and prevention of mineral nutrition of plants (Chen et al., 2003, 2004; Christie et al., 2004; Wang et al., 2005a). Smith et al. (2003) reported that AM fungi can give the main supply of P content towards plants and unaffect the growth and development of plants.

4.2.3. Effect of AMF on plant Cd uptake

The mycorrhizal inoculation increased the metal tolerance of plants and create a resistance against metal toxicity in different plant species (Cumming et al., 1986). However, the process involved in reducing the metal uptake of plants is still unknown and also differ as according to association of fungal species and metal types (Kelly et al., 2005). The study reported that Cd translocation in plants decreased by inoculation of mycorrhiza in soil. While, the enhanced uptake of Cd was observed in plants that are not inoculated with mycorrhizal spores. Hua et al. (2009) reported fungi play a role in decrease uptake of toxic metals by binding the metals to the outside and inside of hyphae. The plant enhanced growth

and nutrient uptake was mostly associated with decreased metal stress. The metal reduction is associated with the biomass dilution effect (Meharg and Cairney, 2000; Janouskova et al., 2007).

Several studies indicated that mycorrhizal plants had lower metal content in shoot part of plants as compared to root part (Dehn and Schuepp, 1989; Loth and Hofner, 1995; Chen et al., 2005a, b). Studies showed that heavy metals were lower in AM fungi colonized roots than the non-colonized plants (Gildon and Tinker, 1983; Joner and Leyval, 2000; Li and Christie, 2001). The Cd concentration in root was more than that of shoot part of plant because Cd absorption mechanism for roots is an active process in wheat. It is reported that the process of Cd accumulation in roots are linked with energy-dependent active process (Ueno et al., 2008; Mori et al., 2009).

However, the opposite results were reported by Fernandez et al. (2005). The shoots and roots of mycorrhized plants had more Cd concentration than non mycorrhizal plants. This might be due to the reason that different AMF ecotypeshas different degrees of metal accumulation. It is also due to the reason that isolates from sites polluted with heavy metals are generally more metal tolerant and can be more effective in reducing metal uptake from roots to shoots than fungal spores from non-polluted sites (Weissenhorn et al., 1993; Hildebrandt et al., 1999).

4.2.4. Effects of AMF on biochemical activities under increasing Cd toxicity

The result of the study showed AMF association increased the biochemical contents in wheat plants under Cd toxicity. The same results were reported by authors (Tang et al., 2009). 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. The chlorophyll content of non mycorrhizal associated wheat plants is decreased with increasing Cd toxicity. Heavy metal stress cause to inhibit the formation of chlorophyll and inhibit the process of photosynthesis and growth in plants (Wu and Xia, 2006).

The present study showed that soluble protein and sugar content in wheat plants increased with Cd addition levels. However, Cd toxicity (600 $mg.kg^{-1}$) reduced the soluble sugar and proteins availability in plants. The same results were reported by Wu and Xia

(2006). This might be due the reason that toxic heavy metals increase the degradation of proteins and normal metabolism of amino acids.

At normal conditions, plants have well developed antioxidant defense system that decrease the production of ROS and maintain the balance. Heavy metals cause to increase the formation of ROS and indicate specific oxidative stress responses (Cuypers et al., 2009). In the present study, AMF association increase the activity of antioxidant enzymes and decrease the production of reactive oxygen species (ROS). The results are different in several reports that is due to the difference of fungus and plant species (Mittova et al., 2004).

4.3. Part 3: Alfalfa and AMF association under Zinc and cadmium stress

4.3.1. Effect of AMF on growth, biomass and colonization of alfalfa plants 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. Several studies reported that AMF protects the host plants from toxic effects of metals (Or1owska et al., 2011; Weissenhorn et al., 1993). 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 deleterious effects on colonization of plants associated with AM fungi as compared to plants with no fungal interaction. Similar results were indicated by different authors (Dueck et al., 1986; Bethlenfalvay and Franson, 1989; Malekzadeh et al., 2010; Shetty et al., 1994; Gupta et al., 2000). The reduced growth in plants grown under high levels of Zn and Cd was due to interference of these metals with P uptake by plants.

Vivas et al. (2003a) also indicated the use of vesicular arbuscular mycorrhizal (VAM) fungi at polluted areas enhanced biomass of plants with increasing concentrations of Zn and Cd in the soil. Several studies showed that root colonization in AMF associated plants were not reduced in heavy metals contaminated soils (Cardoso, 1985; Schenck and Hinson, 1973). In M associated plants, increased plant growth and biomass was observed that might be due to higher P concentrations and other nutrients uptake such as N, S, Mg and K. The increased nutrient uptake in plants is due to the higher volume of soil colonized by AMF via extraradicular hyphae that cause to enhance the plant growth (Rhodes and Gerdemann, 1975).

Reports also showed the useful effects of mycorrhiza on plant grown in metals stressed soils (Barua et al., 2010; Jankong and Visoottiviseth, 2008; Ultra et al., 2007; Xia et al., 2007; Yu et al., 2005). In some studies, the presence of toxic concentrations of heavy metals does not decrease the percentage of roots colonized by AM fungi (Smith et al., 2010).

4.3.2. Effect of AMF on Zn and Cd uptake in Alfalfa plants

The study indicated that AMF associated plants decrease the uptake of Zn and Cd in alfalfa plants that also cause to stabilize the soil. This might be due to the better nutrients contents and growth of plants in AMF interactive plants that cause to lower the toxic effects of heavy metals (Chen et al., 2007). The mycorrhizal associated plants decrease the uptake of heavy metals in plants by changing the pH of soil that cause to dissolution of metals. This solubilization of metals due to change of pH of soil is a defense mechanism that prevent the plants from heavy metals toxicity (Wang et al., 2007a).

Moreover, the secretion of compounds like glomalin or exudates from extra-matrical hyphae cause to reduce the metal contents in plants by chelation and immobilization of metals (Joner et al., 2000; Wang et al., 2007; Yan-De et al., 2007). Del Val et al. (1999) reported in their study that mycorrhizal spores isolated from heavy metals contaminated soils are more able to tolerate the metals and effective in plants HM uptake than those collected from non-contaminated environments. AMF inoculation is useful strategy that cause to enhance the growth and nutrient of plants in soil contaminated with Zn and Cd. Mycelium of fungus had more metal absorption capacity.

However it is also reported in previous studies that heavy metals toxicity also cause to negatively effect the sensitivity of AMF association with plants. This directly affects the colonization rate of plants and functioning of arbuscules and vesicles are also decreased. Some previous reports indicated that Cd, Pb and Zn were accumulated in roots that shows the filtering mechanisms of mycorrhizal plants (Leyval et al., 1997; Orlowska et al., 2011; Weissenhorn et al., 1995).

4.3.3. Effects of AMF on plant P uptake and mineral nutrition under Zn and Cd toxicity

The study indicated that AMF inoculated plants had 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 (Yan-De et al., 2007). Studies showed that legumes need more P contents for nitrogen fixation and nodulation development. So the normal growth and development also depends on mycorrhizal fungi (Rossini Oliva et al., 2009). The extra metrical mycelium explore the huge volume of soil that is the main cause to enhance the P content in soil and plants. This is the main mechanism of obtaining P rom the soil by mycorrhizal fungi (Gonzalez-Chavez et al., 2002).

The reduction of toxic effects of heavy metals is due to the sequestering of metals in plant associated hyphae so the toxic effects of metals on plant metabolic process might reduce. In NM plants, the reduced concentrations of P was observed that is due to association of toxic metals with nutrient uptake of alfalfa plants (Rufkyikri et al., 2000; Vogel Mikus et al., 2006). These results indicated the beneficial effects of AM fungi in the protection of plants and alleviation of toxic effects of heavy metals. However, more researches are still required to study the response of AM fungi in different plant with increasing metals stress. The effects of Zn stress on plant physiological conditions depends on deficient amount of Fe in plants (Chavan and Banerjee, 1980). In the present study, the increased level of Zn also cause to increase the level of Mn uptake in plants. Increase of Mn uptake cause yellowing of the leaves and resulting in chlorosis.

4.3.4. Effects of AMF on biochemical activities under Zn and Cd toxicity

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 $mgkg^{-1}$) and Cd (600 $mgkg^{-1}$). Farshian et al. (2007) reported the reduced chlorophyll content as the concentration of Zn increased in soil. This is due to that increase Zn stress cause reduction of Fe uptake in plant tissues which is needed for chlorophyll synthesis and

therefore causes chlorosis in plants. Rufkyikri et al., (2000) reported same results that AM plants possesss greater amount of chlorophyll in comparison with non-AM plants. An inhibition of Zn translocation to shoot was also reported in mycorrhizal maize seedlings (Khan et al., 2009).

The present study reported the increased protein and sugar contents in mycorrhizal inoculated plants. Farshian et al., (2007) found the same results that total protein content enhanced in M inoculated treatments but reduced contents was observed in the absence of fungi. This also indicated the mechanisms related to physiological interactions between AM fungus and plants. Rauser (1999) and clements (2001) reported the role of stress proteins such as phytochelatins and metallotheoneines in reduction of heavy metals in plants (Ott et al., 2002; Burleigh et al., 2003; Tong et al., 2004). 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. The same trend was reported by authors (Farshian et al., 2007; Wu and Xia, 2006). This may be due to decrease chlorophyll content and abnormal structure of chloroplast which leads to low photosynthetic efficiency.

The antioxidant enzymes system is a defense system in plants that also cause alterations with increasing heavy metal concentrations (Dazy et al., 2008; Sobrino-Plata et al., 2009). In the present study, SOD and POD activities increased with increasing metals concentrations showing the plant protective system against oxidative stress. Peroxisomes is an organelle in which CAT is found (Distefano et al., 1999). In metals toxic conditions, the ROS production was enhanced (Cuypers et al., 2009). In the present study, AMF association affects the enzymatic activity in different ways as according to environment reponses. The results are not completelt similar to the previous studies. The difference in results are due to variations in fungal and plant species (Mittova et al., 2004).

Concluding Remarks

It is concluded from the result of the present study that mycorrhizal association with wheat and alfalfa 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 plants. AMF protect the wheat and alfalfa plants against metal toxicity and also beneficial for nutrient uptake. The results are consistent with the suggestions (Chen et al., 2002; Li and Christie, 2001; Zhu et al., 2001) that 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 wheat and alfalfa especially when it grows in soils with high concentration 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. The maintenance and preservation of mycorrhizal symbiosis in orchards and in crop plants in the field may contribute to a more sustainable agro-ecosystems. 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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APPENDICES

Appendices

Part 1: Wheat and AMF association under Zinc stress

Appendix 1.1. Analysis of Variance Table for Root area (cm^2)

Source	DF	SS	MS	F	P
rep	19	24.533	1.291		
Inoculati	1	142.507	142.507	88.63	0.0000
trt	3	25.356	8.452	5.26	0.0019
Inoculati*trt	3	30.626	10.209	6.35	0.0005
Error	133	213.856	1.608		
Total	159	436.878			
Grand Mean 3.2136		CV 39.46			

Appendix 1.2. Analysis of Variance Table for Root Breadth (cm)

Source	DF	SS	MS	F	P
rep	19	0.02463	0.00130		
Inoculati	1	0.13225	0.13225	90.49	0.0000
trt	3	0.01075	0.00358	2.45	0.0662
Inoculati*trt	3	0.02075	0.00692	4.73	0.0036
Error	133	0.19437	0.00146		
Total	159	0.38275			
Grand Mean 0.1462		CV 26.14			

Appendix 1.3. Analysis of Variance Table for Root Length (cm)

Source	DF	SS	MS	F	P
rep	19	208.23	10.960		
Inoculati	1	283.16	283.157	33.28	0.0000
trt	3	142.67	47.555	5.59	0.0012
Inoculati*trt	3	130.13	43.377	5.10	0.0023
Error	133	1131.45	8.507		
Total	159	1895.64			
Grand Mean 13.002		CV 22.43			

Appendix 1.4. Analysis of Variance Table for SA (cm^2)

Source	DF	SS	MS	F	P
rep	19	90.703	4.774		
Inoculati	1	177.725	177.725	63.31	0.0000
trt	3	141.673	47.224	16.82	0.0000
Inoculati*trt	3	24.931	8.310	2.96	0.0346
Error	133	373.351	2.807		
Total	159	808.383			
Grand Mean 4.8691		CV 34.41			

Appendix 1.5. Analysis of Variance Table for SB (cm)

Source	DF	SS	MS	F	P
rep	19	0.00869	0.00046		
Inoculati	1	0.08556	0.08556	68.32	0.0000
trt	3	0.06156	0.02052	16.39	0.0000
Inoculati*trt	3	0.01006	0.00335	2.68	0.0496
Error	133	0.16656	0.00125		

Total	159	0.33244			
Grand Mean 0.1681	CV 21.05				

Appendix 1.6. Analysis of Variance Table for Shoot Length (cm)

Source	DF	SS	MS	F	P
rep	19	441.58	23.241		
Inoculati	1	208.62	208.621	17.81	0.0000
trt	3	342.57	114.191	9.75	0.0000
Inoculati*trt	3	88.92	29.641	2.53	0.0599
Error	133	1557.77	11.713		
Total	159	2639.46			
Grand Mean 17.241	CV 19.85				

Appendix 1.7. Analysis of Variance Table for Carotene

Source	DF	SS	MS	F	P
rep	2	0.0920	0.04601		
Inoculati	1	5.3922	5.39222	192.69	0.0000
trt	3	3.4924	1.16414	41.60	0.0000
Inoculati*trt	3	2.0040	0.66800	23.87	0.0000
Error	14	0.3918	0.02798		
Total	23	11.3725			
Grand Mean 1.6890	CV 9.90				

Appendix 1.8 Analysis of Variance Table for Chlorophyll a ($mg.g^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.00391	0.00196		
Inoculati	1	0.20962	0.20962	44.15	0.0000
trt	3	0.31649	0.10550	22.22	0.0000
Inoculati*trt	3	0.10027	0.03342	7.04	0.0041
Error	14	0.06647	0.00475		
Total	23	0.69677			
Grand Mean 0.4703	CV 14.65				

Appendix 1.9. Analysis of Variance Table for Chlorophyll b ($mg.g^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.00065	0.00032		
Inoculati	1	0.06139	0.06139	16.19	0.0013
trt	3	0.09385	0.03128	8.25	0.0021
Inoculati*trt	3	0.02432	0.00811	2.14	0.1412
Error	14	0.05308	0.00379		
Total	23	0.23329			
Grand Mean 0.2934	CV 20.99				

Appendix 1.10. Analysis of Variance Table for Proline ($mg.g^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.4943	0.24713		
Inoculati	1	0.0040	0.00400	0.02	0.8843
trt	3	18.7425	6.24748	34.32	0.0000
Inoculati*trt	3	2.2607	0.75357	4.14	0.0270
Error	14	2.5484	0.18203		
Total	23	24.0498			
Grand Mean 1.1693	CV 36.49				

Appendix 1.11. Analysis of Variance Table for Root biomass (g)

Source	DF	SS	MS	F	P
rep	2	0.310	0.155		
Inoculati	1	112.970	112.970	601.70	0.0000
trt	3	19.962	6.654	35.44	0.0000
Inoculati*trt	3	17.215	5.738	30.56	0.0000
Error	14	2.629	0.188		
Total	23	153.085			
Grand Mean 2.5438		CV 17.03			

Appendix 1.12. Analysis of Variance Table for Root length colonization (%)

Source	DF	SS	MS	F	P
rep	2	4.1	2.1		
Inoculati	1	29694.0	29694.0	18850.0	0.0000
trt	3	17.3	5.8	3.66	0.0390
Inoculati*trt	3	17.3	5.8	3.66	0.0390
Error	14	22.1	1.6		
Total	23	29754.8			
Grand Mean 35.175		CV 3.57			

Appendix 1.13. Analysis of Variance Table for Shoot biomass (g)

Source	DF	SS	MS	F	P
rep	2	0.0803	0.0401		
Inoculati	1	24.7863	24.7863	1732.23	0.0000
trt	3	2.6488	0.8829	61.70	0.0000
Inoculati*trt	3	1.9621	0.6540	45.71	0.0000
Error	14	0.2003	0.0143		
Total	23	29.6779			
Grand Mean 1.1588		CV 10.32			

Appendix 1.14. Analysis of Variance Table for sugar (g)

Source	DF	SS	MS	F	P
rep	2	0.3459	0.17293		
Inoculati	1	5.4913	5.49127	7.99	0.0135
trt	3	0.5686	0.18954	0.28	0.8419
Inoculati*trt	3	5.1352	1.71172	2.49	0.1028
Error	14	9.6221	0.68729		
Total	23	21.1630			
Grand Mean 2.7342		CV 30.32			

Appendix 1.15. Analysis of Variance Table for APX

Source	DF	SS	MS	F	P
rep	2	0.00228	0.00114		
Inoculati	1	0.19984	0.19984	8.58	0.0110
trt	3	0.38748	0.12916	5.54	0.0101
Inoculati*trt	3	0.44248	0.14749	6.33	0.0062
Error	14	0.32619	0.02330		
Total	23	1.35826			
Grand Mean 0.6687		CV 22.82			

Appendix 1.16. Analysis of Variance Table for CAT

Source	DF	SS	MS	F	P
rep	2	7.950	3.975		
Inoculati	1	167.270	167.270	43.07	0.0000
trt	3	257.680	85.893	22.11	0.0000
Inoculati*trt	3	256.703	85.568	22.03	0.0000
Error	14	54.375	3.884		
Total	23	743.978			
Grand Mean 15.643		CV 12.60			

Appendix 1.17. Analysis of Variance Table for POD

Source	DF	SS	MS	F	P
rep	2	18.311	9.1557		
Inoculati	1	0.082	0.0817	0.01	0.9428
trt	3	94.723	31.5743	2.06	0.1512
Inoculati*trt	3	286.622	95.5405	6.24	0.0065
Error	14	214.187	15.2991		
Total	23	613.925			
Grand Mean 21.655		CV 18.06			

Appendix 1.18. Analysis of Variance Table for SOD

Source	DF	SS	MS	F	P
rep	2	13.335	6.6677		
Inoculati	1	7.843	7.8433	2.00	0.1787
trt	3	180.748	60.2493	15.40	0.0001
Inoculati*trt	3	272.880	90.9601	23.25	0.0000
Error	14	54.774	3.9124		
Total	23	529.581			
Grand Mean 18.708		CV 10.57			

Shoot nutrients

Appendix 1.19. Analysis of Variance Table for Ca ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
Rep	2	33.362	16.6810		
Inoculati	1	0.863	0.8626	0.30	0.5916
trt	3	38.448	12.8160	4.48	0.0210
Inoculati*trt	3	23.141	7.7136	2.70	0.0860
Error	14	40.060	2.8615		
Total	23	135.874			
Grand Mean 14.013		CV 12.07			

Appendix 1.20. Analysis of Variance Table for Cu ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
Rep	2	10.753	5.377		
Inoculati	1	173.021	173.021	112.30	0.0000
trt	3	38.815	12.938	8.40	0.0019
Inoculati*trt	3	25.514	8.505	5.52	0.0103
Error	14	21.569	1.541		

Total	23	269.673			
Grand Mean 15.021		CV 8.26			

Appendix 1.21. Analysis of Variance Table for K ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
Rep	2	44.422	22.211		
Inoculati	1	164.117	164.117	8.56	0.0111
trt	3	376.082	125.361	6.54	0.0055
Inoculati*trt	3	28.848	9.616	0.50	0.6874
Error	14	268.550	19.182		
Total	23	882.020			
Grand Mean 23.047		CV 19.00			

Appendix 1.22. Analysis of Variance Table for Mg ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
Rep	2	4.6822	2.34112		
Inoculati	1	2.0651	2.06507	1.08	0.3173
trt	3	27.3574	9.11914	4.75	0.0174
Inoculati*trt	3	8.1614	2.72048	1.42	0.2795
Error	14	26.8828	1.92020		
Total	23	69.1489			
Grand Mean 9.5783		CV 14.47			

Appendix 1.23. Analysis of Variance Table for Mn ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
Rep	2	77.8	38.88		
Inoculati	1	4891.2	4891.19	12.05	0.0037
trt	3	14113.5	4704.51	11.59	0.0004
Inoculati*trt	3	5599.4	1866.46	4.60	0.0193
Error	14	5680.8	405.77		
Total	23	30362.6			
Grand Mean 146.96		CV 13.71			

Appendix 1.24. Analysis of Variance Table for Na ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
Rep	2	18.762	9.381		
Inoculati	1	170.027	170.027	36.93	0.0000
trt	3	273.505	91.168	19.80	0.0000
Inoculati*trt	3	45.951	15.317	3.33	0.0507
Error	14	64.460	4.604		
Total	23	572.705			
Grand Mean 18.670		CV 11.49			

Appendix 1.25. Analysis of Variance Table for Nitrogen ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
Rep	2	0.01886	0.00943		
Inoculati	1	1.21500	1.21500	112.84	0.0000
trt	3	0.11850	0.03950	3.67	0.0386
Inoculati*trt	3	0.10143	0.03381	3.14	0.0590
Error	14	0.15074	0.01077		
Total	23	1.60453			
Grand Mean 0.4733		CV 21.92			

Appendix 1.26 Analysis of Variance Table for P ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
Rep	2	0.23136	0.11568		
Inoculati	1	3.33015	3.33015	56.47	0.0000
trt	3	0.03890	0.01297	0.22	0.8810
Inoculati*trt	3	0.75635	0.25212	4.28	0.0244
Error	14	0.82557	0.05897		
Total	23	5.18233			
Grand Mean 0.8583		CV 28.29			

Appendix 1.27 Analysis of Variance Table for Zn ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
Rep	2	67.1	33.6		
Inoculati	1	4929.8	4929.8	63.45	0.0000
trt	3	56342.7	18780.9	241.73	0.0000
Inoculati*trt	3	2205.5	735.2	9.46	0.0011
Error	14	1087.7	77.7		
Total	23	64632.8			
Grand Mean 78.250		CV 11.26			

Appendix 1.28 Analysis of Variance Table for Fe ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
Rep	2	164.45	82.22		
Inoculati	1	2206.08	2206.08	52.75	0.0000
trt	3	3241.18	1080.39	25.83	0.0000
Inoculati*trt	3	899.14	299.71	7.17	0.0038
Error	14	585.50	41.82		
Total	23	7096.35			
Grand Mean 56.148		CV 11.52			

Appendix 1.29 Analysis of Variance Table for Ni ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
Rep	2	0.047	0.0233		
Inoculati	1	68.344	68.3438	25.17	0.0002
trt	3	84.984	28.3279	10.43	0.0007
Inoculati*trt	3	25.917	8.6390	3.18	0.0570
Error	14	38.008	2.7149		
Total	23	217.299			
Grand Mean 11.315		CV 14.56			

Root Nutrients

Appendix 1.30 Analysis of Variance Table for Ca ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.4328	0.2164		
Inoculati	1	10.6933	10.6933	30.39	0.0001
trt	3	3.9370	1.3123	3.73	0.0368
Inoculati*trt	3	2.1047	0.7016	1.99	0.1613
Error	14	4.9266	0.3519		
Total	23	22.0944			
Grand Mean 3.4625		CV 17.13			

Appendix 1.31 Analysis of Variance Table for Cu $(mg.kg^{-1})$

Source	DF	SS	MS	F	P
rep	2	6.159	3.080		
Inoculati	1	223.748	223.748	74.53	0.0000
trt	3	132.062	44.021	14.66	0.0001
Inoculati*trt	3	15.232	5.077	1.69	0.2145
Error	14	42.029	3.002		
Total	23	419.231			
Grand Mean 13.117		CV 13.21			

Appendix 1.32 Analysis of Variance Table for K $(g.kg^{-1})$

Source	DF	SS	MS	F	P
rep	2	14.317	7.1584		
Inoculati	1	2.768	2.7676	0.83	0.3772
trt	3	161.249	53.7495	16.15	0.0001
Inoculati*trt	3	62.357	20.7856	6.25	0.0065
Error	14	46.593	3.3280		
Total	23	287.282			
Grand Mean 15.479		CV 11.79			

Appendix 1.33. Analysis of Variance Table for Mg $(g.kg^{-1})$

Source	DF	SS	MS	F	P
rep	2	0.5858	0.29290		
Inoculati	1	4.1417	4.14170	8.07	0.0131
trt	3	16.3556	5.45188	10.62	0.0007
Inoculati*trt	3	2.0707	0.69023	1.34	0.3001
Error	14	7.1879	0.51342		
Total	23	30.3417			
Grand Mean 4.8104		CV 14.90			

Appendix 1.34 Analysis of Variance Table for Mn $(mg.kg^{-1})$

Source	DF	SS	MS	F	P
rep	2	53.74	26.87		
Inoculati	1	377.31	377.31	9.61	0.0078
trt	3	4406.83	1468.94	37.42	0.0000
Inoculati*trt	3	479.83	159.94	4.07	0.0283
Error	14	549.59	39.26		
Total	23	5867.30			
Grand Mean 69.859		CV 8.97			

Appendix 1.35. Analysis of Variance Table for Na $(g.kg^{-1})$

Source	DF	SS	MS	F	P
rep	2	0.7476	0.3738		
Inoculati	1	15.7950	15.7950	52.14	0.0000
trt	3	48.5394	16.1798	53.41	0.0000
Inoculati*trt	3	2.9193	0.9731	3.21	0.0557
Error	14	4.2414	0.3030		
Total	23	72.2428			
Grand Mean 7.6054		CV 7.24			

Appendix 1.36. Analysis of Variance Table for Nitrogen (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	0.00212	0.00106		
Inoculati	1	0.17459	0.17459	97.23	0.0000
trt	3	0.02205	0.00735	4.09	0.0279
Inoculati*trt	3	0.01044	0.00348	1.94	0.1698
Error	14	0.02514	0.00180		
Total	23	0.23434			
Grand Mean 0.2155		CV 19.66			

Appendix 1.37. Analysis of Variance Table for P (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	0.05561	0.02780		
Inoculati	1	0.88935	0.88935	34.29	0.0000
trt	3	0.00075	0.00025	0.01	0.9986
Inoculati*trt	3	0.11415	0.03805	1.47	0.2662
Error	14	0.36313	0.02594		
Total	23	1.42298			
Grand Mean 0.5542		CV 29.06			

Appendix 1.38. Analysis of Variance Table for Zn (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	86.3	43.2		
Inoculati	1	8928.6	8928.6	107.96	0.0000
trt	3	51361.9	17120.6	207.02	0.0000
Inoculati*trt	3	3998.2	1332.7	16.12	0.0001
Error	14	1157.8	82.7		
Total	23	65532.9			
Grand Mean 91.045		CV 9.99			

Appendix 1.39. Analysis of Variance Table for Fe (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	20.07	10.034		
Inoculati	1	129.73	129.735	2.77	0.1182
trt	3	916.32	305.441	6.52	0.0055
Inoculati*trt	3	32.43	10.810	0.23	0.8734
Error	14	655.56	46.826		
Total	23	1754.12			
Grand Mean 41.859		CV 16.35			

Appendix 1.40. Analysis of Variance Table for Ni (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	0.8906	0.44529		
Inoculati	1	1.0334	1.03335	1.27	0.2787
trt	3	26.9074	8.96914	11.03	0.0006
Inoculati*trt	3	6.0710	2.02367	2.49	0.1031
Error	14	11.3884	0.81346		
Total	23	46.2908			
Grand Mean 5.9350		CV 15.20			

Part 2: Wheat and AMF association under cadmium stress

Appendix 2.1. Analysis of Variance Table for Root area (cm^2)

Source	DF	SS	MS	F	P
rep	19	36.175	1.904		
trt	3	49.987	16.662	12.27	0.0000
Inoculati	1	217.300	217.300	160.04	0.0000
trt*Inoculati	3	25.909	8.636	6.36	0.0005
Error	133	180.585	1.358		
Total	159	509.955			
Grand Mean 3.1446		CV 37.05			

Appendix 2.2. Analysis of Variance Table for Root Breadth (cm)

Source	DF	SS	MS	F	P
rep	19	0.02425	0.00128		
trt	3	0.02695	0.00898	5.93	0.0008
Inoculati	1	0.16770	0.16770	110.72	0.0000
trt*Inoculati	3	0.00185	0.00062	0.41	0.7485
Error	133	0.20145	0.00151		
Total	159	0.42220			
Grand Mean 0.1339		CV 29.07			

Appendix 2.3. Analysis of Variance Table for Root Length (cm)

Source	DF	SS	MS	F	P
rep	19	176.47	9.288		
trt	3	233.24	77.748	10.93	0.0000
Inoculati	1	669.49	669.492	94.09	0.0000
trt*Inoculati	3	37.20	12.401	1.74	0.1613
Error	133	946.40	7.116		
Total	159	2062.80			
Grand Mean 13.007		CV 20.51			

Appendix 2.4. Analysis of Variance Table for SA (cm^2)

Source	DF	SS	MS	F	P
rep	19	47.308	2.490		
trt	3	39.080	13.027	5.67	0.0011
Inoculati	1	202.781	202.781	88.23	0.0000
trt*Inoculati	3	11.591	3.864	1.68	0.1742
Error	133	305.693	2.298		
Total	159	606.454			
Grand Mean 3.9857		CV 38.04			

Appendix 2.5. Analysis of Variance Table for SB (cm)

Source	DF	SS	MS	F	P
rep	19	0.03964	0.00209		
trt	3	0.02150	0.00717	4.08	0.0082
Inoculati	1	0.11610	0.11610	66.14	0.0000
trt*Inoculati	3	0.00115	0.00038	0.22	0.8833
Error	133	0.23346	0.00176		
Total	159	0.41185			
Grand Mean 0.1406		CV 29.81			

Appendix 2.6. Analysis of Variance Table for Shoot Length (cm)

Source	DF	SS	MS	F	P
rep	19	193.25	10.171		
trt	3	545.03	181.675	13.34	0.0000
Inoculati	1	503.35	503.355	36.96	0.0000
trt*Inoculati	3	47.08	15.694	1.15	0.3306
Error	133	1811.51	13.620		
Total	159	3100.22			
Grand Mean 15.631		CV 23.61			

Appendix 2.7. Analysis of Variance Table for Carotene

Source	DF	SS	MS	F	P
rep	2	0.3319	0.1660		
trt	3	3.0869	1.0290	11.17	0.0005
Inoculati	1	11.8399	11.8399	128.58	0.0000
trt*Inoculati	3	1.0558	0.3519	3.82	0.0343
Error	14	1.2892	0.0921		
Total	23	17.6038			
Grand Mean 1.6786		CV 18.08			

Appendix 2.8. Analysis of Variance Table for Chlorophyll a ($mg.g^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.00196	0.00098		
trt	3	0.08203	0.02734	7.95	0.0024
Inoculati	1	0.18505	0.18505	53.83	0.0000
trt*Inoculati	3	0.02169	0.00723	2.10	0.1458
Error	14	0.04813	0.00344		
Total	23	0.33886			
Grand Mean 0.4089		CV 14.34			

Appendix 2.9. Analysis of Variance Table for Chlorophyll b ($mg.g^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.00484	0.00242		
trt	3	0.02618	0.00873	3.33	0.0507
Inoculati	1	0.24629	0.24629	93.88	0.0000
trt*Inoculati	3	0.00647	0.00216	0.82	0.5032
Error	14	0.03673	0.00262		
Total	23	0.32051			
Grand Mean 0.2434		CV 21.05			

Appendix 2.10. Analysis of Variance Table for Proline ($mg.g^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.02273	0.01137		
trt	3	5.29788	1.76596	42.52	0.0000
Inoculati	1	0.02797	0.02797	0.67	0.4256
trt*Inoculati	3	1.70615	0.56872	13.69	0.0002
Error	14	0.58150	0.04154		
Total	23	7.63622			
Grand Mean 0.9076		CV 22.46			

Appendix 2.11. Analysis of Variance Table for Protein (mg.g^{-1})

Source	DF	SS	MS	F	P
rep	2	0.8731	0.4365		
trt	3	11.6187	3.8729	7.03	0.0041
Inoculati	1	22.7371	22.7371	41.30	0.0000
trt*Inoculati	3	1.3925	0.4642	0.84	0.4928
Error	14	7.7083	0.5506		
Total	23	44.3296			
Grand Mean 4.6650		CV 15.91			

Appendix 2.12. Analysis of Variance Table for sugar (g)

Source	DF	SS	MS	F	P
rep	2	0.6536	0.3268		
trt	3	4.3668	1.4556	2.93	0.0703
Inoculati	1	13.2908	13.2908	26.76	0.0001
trt*Inoculati	3	0.3634	0.1211	0.24	0.8643
Error	14	6.9523	0.4966		
Total	23	25.6270			
Grand Mean 4.7542		CV 14.82			

Appendix 2.13. Analysis of Variance Table for Root biomass (g)

Source	DF	SS	MS	F	P
rep	2	0.00491	0.00245		
trt	3	0.38810	0.12937	22.10	0.0000
Inoculati	1	0.15042	0.15042	25.69	0.0002
trt*Inoculati	3	0.02115	0.00705	1.20	0.3444
Error	14	0.08196	0.00585		
Total	23	0.64653			
Grand Mean 0.4633		CV 16.51			

Appendix 2.14. Analysis of Variance Table for Root length colonization (%)

Source	DF	SS	MS	F	P
rep	2	0.3	0.2		
trt	3	59.5	19.8	5.01	0.0145
Inoculati	1	29988.1	29988.1	7567.79	0.0000
trt*Inoculati	3	59.5	19.8	5.01	0.0145
Error	14	55.5	4.0		
Total	23	30162.9			
Grand Mean 35.348		CV 5.63			

Appendix 2.15. Analysis of Variance Table for Shoot biomass (g)

Source	DF	SS	MS	F	P
rep	2	0.7386	0.36932		
trt	3	12.6176	4.20588	33.50	0.0000
Inoculati	1	0.6144	0.61440	4.89	0.0441
trt*Inoculati	3	0.2203	0.07343	0.58	0.6348
Error	14	1.7578	0.12555		
Total	23	15.9487			
Grand Mean 2.0633		CV 17.17			

Shoot nutrients

Appendix 2.16. Analysis of Variance Table for Ca (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	8.687	4.344		
trt	3	734.392	244.797	82.26	0.0000
Inoculati	1	99.878	99.878	33.56	0.0000
trt*Inoculati	3	8.113	2.704	0.91	0.4617
Error	14	41.663	2.976		
Total	23	892.733			
Grand Mean 20.041		CV 8.61			

Appendix 2.17. Analysis of Variance Table for Cu (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	8.126	4.063		
trt	3	352.223	117.408	17.89	0.0000
Inoculati	1	0.311	0.311	0.05	0.8309
trt*Inoculati	3	54.062	18.021	2.75	0.0823
Error	14	91.874	6.562		
Total	23	506.595			
Grand Mean 21.793		CV 11.75			

Appendix 2.18. Analysis of Variance Table for Fe (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	12.06	6.03		
trt	3	1382.57	460.86	23.30	0.0000
Inoculati	1	2288.13	2288.13	115.70	0.0000
trt*Inoculati	3	507.11	169.04	8.55	0.0018
Error	14	276.87	19.78		
Total	23	4466.75			
Grand Mean 45.392		CV 9.80			

Appendix 2.19. Analysis of Variance Table for K (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	3.447	1.723		
trt	3	609.060	203.020	12.20	0.0003
Inoculati	1	66.767	66.767	4.01	0.0649
trt*Inoculati	3	29.743	9.914	0.60	0.6282
Error	14	233.008	16.643		
Total	23	942.024			
Grand Mean 23.725		CV 17.20			

Appendix 2.20. Analysis of Variance Table for Mg (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	1.1975	0.59876		
trt	3	25.8176	8.60588	11.18	0.0005
Inoculati	1	2.2878	2.28784	2.97	0.1067
trt*Inoculati	3	0.4200	0.14002	0.18	0.9069
Error	14	10.7746	0.76961		
Total	23	40.4977			
Grand Mean 11.069		CV 7.93			

Appendix 2.21. Analysis of Variance Table for Mn (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	132.14	66.07		
trt	3	5194.65	1731.55	39.69	0.0000
Inoculati	1	1111.26	1111.26	25.47	0.0002
trt*Inoculati	3	996.60	332.20	7.61	0.0029
Error	14	610.76	43.63		
Total	23	8045.41			
Grand Mean 75.072		CV 8.80			

Appendix 2.22. Analysis of Variance Table for Nitrogen (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	0.01066	0.00533		
trt	3	0.96558	0.32186	10.63	0.0007
Inoculati	1	2.20220	2.20220	72.71	0.0000
trt*Inoculati	3	0.26545	0.08848	2.92	0.0709
Error	14	0.42401	0.03029		
Total	23	3.86790			
Grand Mean 0.9004		CV 19.33			

Appendix 2.23. Analysis of Variance Table for Na (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	6.719	3.360		
trt	3	114.605	38.202	11.41	0.0005
Inoculati	1	209.391	209.391	62.57	0.0000
trt*Inoculati	3	41.911	13.970	4.17	0.0263
Error	14	46.854	3.347		
Total	23	419.481			
Grand Mean 11.475		CV 15.94			

Appendix 2.24. Analysis of Variance Table for P (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	0.4047	0.20236		
trt	3	5.7132	1.90439	9.43	0.0012
Inoculati	1	4.3776	4.37760	21.69	0.0004
trt*Inoculati	3	0.4808	0.16027	0.79	0.5173
Error	14	2.8259	0.20185		
Total	23	13.8023			
Grand Mean 2.0612		CV 21.80			

Appendix 2.25. Analysis of Variance Table for Zn (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	7.789	3.8943		
trt	3	286.220	95.4066	6.27	0.0064
Inoculati	1	74.942	74.9420	4.93	0.0435
trt*Inoculati	3	179.253	59.7510	3.93	0.0316
Error	14	212.957	15.2112		
Total	23	761.161			
Grand Mean 24.752		CV 15.76			

Appendix 2.26. Analysis of Variance Table for Cd (mg.kg^{-1} \)

Source	DF	SS	MS	F	P
rep	2	1546	773		
trt	3	343520	114507	139.25	0.0000
Inoculati	1	23900	23900	29.06	0.0001
trt*Inoculati	3	10277	3426	4.17	0.0264
Error	14	11512	822		
Total	23	390756			
Grand Mean 174.28		CV 16.45			

Appendix 2.27. Analysis of Variance Table for Ni (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	2.1479	1.07396		
trt	3	25.8662	8.62208	8.46	0.0019
Inoculati	1	3.8480	3.84800	3.77	0.0724
trt*Inoculati	3	13.7744	4.59147	4.50	0.0207
Error	14	14.2739	1.01956		
Total	23	59.9105			
Grand Mean 7.0188		CV 14.39			

Appendix 2.28. Analysis of Variance Table for APX

Source	DF	SS	MS	F	P
rep	2	0.13236	0.06618		
trt	3	1.75473	0.58491	14.22	0.0002
Inoculati	1	0.27307	0.27307	6.64	0.0220
trt*Inoculati	3	0.06533	0.02178	0.53	0.6695
Error	14	0.57604	0.04115		
Total	23	2.80153			
Grand Mean 0.7283		CV 27.85			

Appendix 2.29. Analysis of Variance Table for CAT

Source	DF	SS	MS	F	P
rep	2	3.328	1.6641		
trt	3	123.013	41.0042	23.75	0.0000
Inoculati	1	24.970	24.9696	14.46	0.0019
trt*Inoculati	3	3.475	1.1584	0.67	0.5839
Error	14	24.171	1.7265		
Total	23	178.957			
Grand Mean 10.910		CV 12.04			

Appendix 2.30. Analysis of Variance Table for POD

Source	DF	SS	MS	F	P
rep	2	10.102	5.051		
trt	3	334.104	111.368	18.19	0.0000
Inoculati	1	34.153	34.153	5.58	0.0332
trt*Inoculati	3	78.073	26.024	4.25	0.0248
Error	14	85.707	6.122		
Total	23	542.139			
Grand Mean 18.539		CV 13.35			

Appendix 2.31. Analysis of Variance Table for SOD

Source	DF	SS	MS	F	P
rep	2	6.447	3.2236		
trt	3	101.662	33.8875	19.48	0.0000
Inoculati	1	74.730	74.7301	42.96	0.0000
trt*Inoculati	3	5.183	1.7276	0.99	0.4246
Error	14	24.354	1.7396		
Total	23	212.377			
Grand Mean 12.376		CV 10.66			

Root nutrients

Appendix 2.32. Analysis of Variance Table for Ca (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	0.2734	0.13670		
trt	3	19.7330	6.57767	9.67	0.0010
Inoculati	1	4.6464	4.64640	6.83	0.0204
trt*Inoculati	3	1.1803	0.39343	0.58	0.6386
Error	14	9.5205	0.68003		
Total	23	35.3536			
Grand Mean 11.539		CV 7.15			

Appendix 2.33. Analysis of Variance Table for Cu (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	59.60	29.799		
trt	3	1169.49	389.831	25.57	0.0000
Inoculati	1	9.59	9.589	0.63	0.4410
trt*Inoculati	3	155.40	51.799	3.40	0.0479
Error	14	213.46	15.247		
Total	23	1607.53			
Grand Mean 35.803		CV 10.91			

Appendix 2.34. Analysis of Variance Table for Fe (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	26.38	13.191		
trt	3	582.67	194.222	19.47	0.0000
Inoculati	1	518.10	518.103	51.95	0.0000
trt*Inoculati	3	0.61	0.202	0.02	0.9959
Error	14	139.63	9.974		
Total	23	1267.39			
Grand Mean 31.849		CV 9.92			

Appendix 2.35. Analysis of Variance Table for K (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	1.1997	0.5999		
trt	3	36.8537	12.2846	8.28	0.0020
Inoculati	1	1.2467	1.2467	0.84	0.3747
trt*Inoculati	3	0.3279	0.1093	0.07	0.9731
Error	14	20.7587	1.4828		
Total	23	60.3868			
Grand Mean 9.3137		CV 13.07			

Appendix 2.36. Analysis of Variance Table for Mg ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.5198	0.25990		
trt	3	9.4130	3.13767	11.62	0.0004
Inoculati	1	1.0375	1.03750	3.84	0.0701
trt*Inoculati	3	0.7659	0.25532	0.95	0.4450
Error	14	3.7791	0.26994		
Total	23	15.5154			
Grand Mean 6.3254		CV 8.21			

Appendix 2.37. Analysis of Variance Table for Mg ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	3.39	1.697		
trt	3	1956.16	652.053	15.88	0.0001
Inoculati	1	186.60	186.595	4.54	0.0512
trt*Inoculati	3	365.61	121.870	2.97	0.0681
Error	14	574.83	41.059		
Total	23	3086.59			
Grand Mean 60.164		CV 10.65			

Appendix 2.38. Analysis of Variance Table for Nitrogen ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.00041	0.00020		
trt	3	0.15401	0.05134	17.23	0.0001
Inoculati	1	0.08284	0.08284	27.79	0.0001
trt*Inoculati	3	0.02071	0.00690	2.32	0.1201
Error	14	0.04172	0.00298		
Total	23	0.29970			
Grand Mean 0.3171		CV 17.22			

Appendix 2.39. Analysis of Variance Table for Sodium ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	2.1815	1.0907		
trt	3	22.4148	7.4716	25.47	0.0000
Inoculati	1	41.1340	41.1340	140.23	0.0000
trt*Inoculati	3	5.8343	1.9448	6.63	0.0052
Error	14	4.1065	0.2933		
Total	23	75.6711			
Grand Mean 7.2017		CV 7.52			

Appendix 2.40. Analysis of Variance Table for Phosphorus ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.00743	0.00372		
trt	3	0.46418	0.15473	11.15	0.0005
Inoculati	1	0.77760	0.77760	56.05	0.0000
trt*Inoculati	3	0.03093	0.01031	0.74	0.5439
Error	14	0.19423	0.01387		
Total	23	1.47438			
Grand Mean 0.5058		CV 23.29			

Appendix 2.41. Analysis of Variance Table for Zinc ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	10.73	5.36		
trt	3	508.91	169.64	10.06	0.0009
Inoculati	1	1516.07	1516.07	89.87	0.0000
trt*Inoculati	3	107.19	35.73	2.12	0.1438
Error	14	236.18	16.87		
Total	23	2379.07			
Grand Mean 26.719		CV 15.37			

Appendix 2.42. Analysis of Variance Table for Cadmium ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	554	277		
trt	3	447707	149236	117.63	0.0000
Inoculati	1	59369	59369	46.79	0.0000
trt*Inoculati	3	19725	6575	5.18	0.0129
Error	14	17762	1269		
Total	23	545117			
Grand Mean 244.50		CV 14.57			

Appendix 2.43. Analysis of Variance Table for Nickel ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	3.438	1.719		
trt	3	118.973	39.658	20.47	0.0000
Inoculati	1	100.246	100.246	51.74	0.0000
trt*Inoculati	3	9.935	3.312	1.71	0.2108
Error	14	27.125	1.938		
Total	23	259.716			
Grand Mean 12.143		CV 11.46			

Part 3a: Alfalfa and AMF association under Zinc stress

Appendix 3.1. Analysis of Variance Table for Root area (cm^2)

Source	DF	SS	MS	F	P
Rep	19	1.1983	0.06307		
TRT	3	2.5720	0.85732	16.00	0.0000
Inoculati	1	1.3079	1.30791	24.42	0.0000
TRT*Inoculati	3	0.1122	0.03740	0.70	0.5547
Error	133	7.1245	0.05357		
Total	159	12.3149			
Grand Mean 0.3851		CV 60.10			

Appendix 3.2. Analysis of Variance Table for Root Length (cm)

Source	DF	SS	MS	F	P
Rep	19	163.42	8.601		
TRT	3	77.19	25.730	3.93	0.0100
Inoculati	1	162.61	162.611	24.85	0.0000
TRT*Inoculati	3	85.59	28.530	4.36	0.0058

Error	133	870.27	6.543		
Total	159	1359.08			
Grand Mean 5.2694		CV 48.54			

Appendix 3.3. Analysis of Variance Table for Root Breadth (cm)

Source	DF	SS	MS	F	P
Rep	19	0.02125	0.00112		
TRT	3	0.00538	0.00179	2.28	0.0823
Inoculati	1	0.07225	0.07225	91.95	0.0000
TRT*Inoculati	3	0.00538	0.00179	2.28	0.0823
Error	133	0.10450	0.00079		
Total	159	0.20875			
Grand Mean 0.0813		CV 34.50			

Appendix 3.4. Analysis of Variance Table for SA (cm^2)

Source	DF	SS	MS	F	P
Rep	19	1.0145	0.05339		
TRT	3	2.4870	0.82900	21.62	0.0000
Inoculati	1	7.4075	7.40753	193.17	0.0000
TRT*Inoculati	3	0.2069	0.06897	1.80	0.1505
Error	133	5.1001	0.03835		
Total	159	16.2161			
Grand Mean 0.5691		CV 34.41			

Appendix 3.5. Analysis of Variance Table for Shoot length (cm)

Source	DF	SS	MS	F	P
Rep	19	45.785	2.410		
TRT	3	83.977	27.992	16.29	0.0000
Inoculati	1	358.202	358.202	208.51	0.0000
TRT*Inoculati	3	19.663	6.554	3.82	0.0116
Error	133	228.481	1.718		
Total	159	736.108			
Grand Mean 6.3788		CV 20.55			

Appendix 3.6. Analysis of Variance Table for Shoot Breadth (cm)

Source	DF	SS	MS	F	P
Rep	19	0.01992	0.00105		
TRT	3	0.03267	0.01089	13.90	0.0000
Inoculati	1	0.07014	0.07014	89.52	0.0000
TRT*Inoculati	3	0.00392	0.00131	1.67	0.1768
Error	133	0.10420	0.00078		
Total	159	0.23086			
Grand Mean 0.1141		CV 24.54			

Appendix 3.7. Analysis of Variance Table for APX

Source	DF	SS	MS	F	P
rep	2	0.13550	0.06775		
trt	3	0.41307	0.13769	6.75	0.0048
Inoculati	1	0.41160	0.41160	20.17	0.0005
trt*Inoculati	3	0.03479	0.01160	0.57	0.6450
Error	14	0.28567	0.02040		
Total	23	1.28063			
Grand Mean 0.6540		CV 21.84			

Appendix 3.8. Analysis of Variance Table for CAT

Source	DF	SS	MS	F	P
rep	2	0.5002	0.25008		
trt	3	17.8878	5.96258	35.63	0.0000
Inoculati	1	7.3214	7.32136	43.74	0.0000
trt*Inoculati	3	7.0632	2.35441	14.07	0.0002
Error	14	2.3431	0.16737		
Total	23	35.1156			
Grand Mean 1.9627		CV 20.84			

Appendix 3.9. Analysis of Variance Table for Carotene

Source	DF	SS	MS	F	P
rep	2	0.21123	0.10562		
trt	3	2.50803	0.83601	15.22	0.0001
Inoculati	1	2.14802	2.14802	39.12	0.0000
trt*Inoculati	3	1.09228	0.36409	6.63	0.0052
Error	14	0.76877	0.05491		
Total	23	6.72833			
Grand Mean 1.2717		CV 18.43			

Appendix 3.10. Analysis of Variance Table for Chlorophyll a ($mg.g^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.2916	0.14578		
trt	3	24.9909	8.33031	54.97	0.0000
Inoculati	1	3.1653	3.16527	20.89	0.0004
trt*Inoculati	3	0.7731	0.25769	1.70	0.2126
Error	14	2.1215	0.15153		
Total	23	31.3423			
Grand Mean 2.2785		CV 17.08			

Appendix 3.11. Analysis of Variance Table for Chlorophyll b ($mg.g^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.12891	0.06445		
trt	3	1.19867	0.39956	7.56	0.0030
Inoculati	1	1.18182	1.18182	22.36	0.0003
trt*Inoculati	3	0.41267	0.13756	2.60	0.0932
Error	14	0.73993	0.05285		
Total	23	3.66200			
Grand Mean 0.8689		CV 26.46			

Appendix 3.12. Analysis of Variance Table for POD

Source	DF	SS	MS	F	P
rep	2	2.307	1.1534		
trt	3	67.021	22.3403	20.60	0.0000
Inoculati	1	26.083	26.0834	24.05	0.0002
trt*Inoculati	3	0.597	0.1991	0.18	0.9058
Error	14	15.185	1.0846		
Total	23	111.193			
Grand Mean 9.1800		CV 11.34			

Appendix 3.13. Analysis of Variance Table for SOD

Source	DF	SS	MS	F	P
rep	2	29.148	14.5738		
trt	3	44.716	14.9053	5.04	0.0142
Inoculati	1	71.208	71.2081	24.06	0.0002
trt*Inoculati	3	17.815	5.9383	2.01	0.1594
Error	14	41.429	2.9592		
Total	23	204.316			
Grand Mean 12.137		CV 14.17			

Appendix 3.14. Analysis of Variance Table for root length colonization (%)

Source	DF	SS	MS	F	P
rep	2	11.4	5.7		
trt	3	272.4	90.8	10.88	0.0006
Inoculati	1	25266.9	25266.9	3028.88	0.0000
trt*Inoculati	3	272.4	90.8	10.88	0.0006
Error	14	116.8	8.3		
Total	23	25939.8			
Grand Mean 32.447		CV 8.90			

Appendix 3.15. Analysis of Variance Table for root biomass (g)

Source	DF	SS	MS	F	P
rep	2	0.00038	1.915E-04		
trt	3	0.00157	5.225E-04	4.72	0.0177
Inoculati	1	0.00074	7.370E-04	6.66	0.0218
trt*Inoculati	3	0.00032	1.058E-04	0.96	0.4403
Error	14	0.00155	1.106E-04		
Total	23	0.00455			
Grand Mean 0.0605		CV 17.40			

Appendix 3.16. Analysis of Variance Table for shoot biomass (g)

Source	DF	SS	MS	F	P
rep	2	0.01386	0.00693		
trt	3	0.49138	0.16379	29.12	0.0000
Inoculati	1	0.03227	0.03227	5.74	0.0312
trt*Inoculati	3	0.00353	0.00118	0.21	0.8882
Error	14	0.07874	0.00562		
Total	23	0.61978			
Grand Mean 0.7008		CV 10.70			

Appendix 3.17. Analysis of Variance Table for Proline ($mg.g^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.00602	0.00301		
Inoculati	1	0.00244	0.00244	0.04	0.8364
trt	3	1.31469	0.43823	7.95	0.0024
Inoculati*trt	3	0.22577	0.07526	1.37	0.2940
Error	14	0.77178	0.05513		
Total	23	2.32070			
Grand Mean 0.7038		CV 33.36			

Appendix 3.18. Analysis of Variance Table for Sugar

Source	DF	SS	MS	F	P
rep	2	5.0033	2.5017		
Inoculati	1	8.5562	8.5562	7.54	0.0158
trt	3	35.3697	11.7899	10.38	0.0007
Inoculati*trt	3	2.3552	0.7851	0.69	0.5723
Error	14	15.8971	1.1355		
Total	23	67.1816			
Grand Mean 4.9254		CV 21.63			

Shoot nutrients

Appendix 3.19. Analysis of Variance Table for Calcium ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	12.370	6.1851		
Inoculati	1	91.650	91.6504	16.20	0.0013
trt	3	271.970	90.6566	16.03	0.0001
Inoculati*trt	3	3.618	1.2061	0.21	0.8856
Error	14	79.200	5.6571		
Total	23	458.809			
Grand Mean 15.799		CV 15.05			

Appendix 3.20. Analysis of Variance Table for Cu ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	15.858	7.9291		
Inoculati	1	25.297	25.2971	2.68	0.1241
trt	3	62.857	20.9525	2.22	0.1314
Inoculati*trt	3	18.769	6.2565	0.66	0.5890
Error	14	132.303	9.4502		
Total	23	255.085			
Grand Mean 10.910		CV 28.18			

Appendix 3.21. Analysis of Variance Table for K ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	26.574	13.287		
Inoculati	1	66.967	66.967	5.37	0.0362
trt	3	666.217	222.072	17.80	0.0000
Inoculati*trt	3	2.626	0.875	0.07	0.9749
Error	14	174.620	12.473		
Total	23	937.004			
Grand Mean 19.962		CV 17.69			

Appendix 3.22. Analysis of Variance Table for Mg ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	9.359	4.6797		
Inoculati	1	18.975	18.9748	3.51	0.0820
trt	3	82.465	27.4885	5.09	0.0137
Inoculati*trt	3	6.228	2.0760	0.38	0.7661
Error	14	75.672	5.4051		
Total	23	192.699			
Grand Mean 14.717		CV 15.80			

Appendix 3.23. Analysis of Variance Table for Mn ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	1453.8	726.88		
Inoculati	1	4059.6	4059.64	6.57	0.0225
trt	3	9663.5	3221.15	5.21	0.0126
Inoculati*trt	3	3365.1	1121.71	1.82	0.1905
Error	14	8648.2	617.73		
Total	23	27190.2			
Grand Mean 129.90		CV 19.13			

Appendix 3.24. Analysis of Variance Table for Nitrogen ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.01336	0.00668		
Inoculati	1	0.07150	0.07150	10.91	0.0052
trt	3	0.06535	0.02178	3.32	0.0509
Inoculati*trt	3	0.00171	0.00057	0.09	0.9660
Error	14	0.09178	0.00656		
Total	23	0.24370			
Grand Mean 0.2629		CV 30.79			

Appendix 3.25. Analysis of Variance Table for Sodium ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	65.03	32.516		
Inoculati	1	369.23	369.233	11.62	0.0042
trt	3	305.01	101.669	3.20	0.0563
Inoculati*trt	3	83.96	27.988	0.88	0.4747
Error	14	444.91	31.779		
Total	23	1268.15			
Grand Mean 19.620		CV 28.73			

Appendix 3.26. Analysis of Variance Table for P ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.8775	0.43875		
Inoculati	1	1.6120	1.61202	6.65	0.0219
trt	3	12.6462	4.21541	17.39	0.0001
Inoculati*trt	3	0.4465	0.14883	0.61	0.6171
Error	14	3.3931	0.24236		
Total	23	18.9753			
Grand Mean 1.4567		CV 33.80			

Appendix 3.27. Analysis of Variance Table for Zn ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	1300	649.9		
Inoculati	1	15924	15924.3	35.43	0.0000
trt	3	97420	32473.5	72.26	0.0000
Inoculati*trt	3	5862	1954.0	4.35	0.0231
Error	14	6292	449.4		
Total	23	126798			
Grand Mean 99.200		CV 21.37			

Appendix 3.28. Analysis of Variance Table for Fe ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	326.38	163.19		
Inoculati	1	1331.76	1331.76	14.25	0.0020
trt	3	3137.25	1045.75	11.19	0.0005
Inoculati*trt	3	492.71	164.24	1.76	0.2014
Error	14	1308.39	93.46		
Total	23	6596.49			
Grand Mean 67.402		CV 14.34			

Appendix 3.29. Analysis of Variance Table for Ni ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	12.456	6.228		
Inoculati	1	137.521	137.521	28.83	0.0001
trt	3	17.410	5.803	1.22	0.3403
Inoculati*trt	3	1.893	0.631	0.13	0.9392
Error	14	66.788	4.771		
Total	23	236.068			
Grand Mean 11.919		CV 18.33			

Root Nutrients

Appendix 3.30. Analysis of Variance Table for Ca ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	1.0744	0.53720		
Inoculati	1	7.1177	7.11770	3.51	0.0821
trt	3	26.2443	8.74812	4.31	0.0238
Inoculati*trt	3	2.5183	0.83943	0.41	0.7459
Error	14	28.4147	2.02962		
Total	23	65.3694			
Grand Mean 9.2279		CV 15.44			

Appendix 3.31. Analysis of Variance Table for Cu ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	1.879	0.9396		
Inoculati	1	40.223	40.2227	13.38	0.0026
trt	3	45.794	15.2648	5.08	0.0138
Inoculati*trt	3	6.825	2.2751	0.76	0.5367
Error	14	42.087	3.0062		
Total	23	136.808			
Grand Mean 10.590		CV 16.37			

Appendix 3.32. Analysis of Variance Table for K ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.251	0.1255		
Inoculati	1	34.464	34.4641	12.46	0.0033
trt	3	161.160	53.7200	19.42	0.0000
Inoculati*trt	3	7.772	2.5908	0.94	0.4491
Error	14	38.721	2.7658		
Total	23	242.368			
Grand Mean 12.777		CV 13.02			

Appendix 3.33. Analysis of Variance Table for Mg (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	0.247	0.1237		
Inoculati	1	9.538	9.5382	2.09	0.1707
trt	3	135.849	45.2830	9.90	0.0009
Inoculati*trt	3	10.793	3.5978	0.79	0.5211
Error	14	64.026	4.5733		
Total	23	220.454			
Grand Mean 8.1754		CV 26.16			

Appendix 3.34. Analysis of Variance Table for Mn (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	1014.4	507.21		
Inoculati	1	2536.1	2536.08	12.28	0.0035
trt	3	3184.7	1061.55	5.14	0.0133
Inoculati*trt	3	2763.5	921.18	4.46	0.0213
Error	14	2891.4	206.53		
Total	23	12390.1			
Grand Mean 70.841		CV 20.29			

Appendix 3.35. Analysis of Variance Table for Nitrogen (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	0.00998	0.00499		
Inoculati	1	0.04682	0.04682	20.26	0.0005
trt	3	0.05830	0.01943	8.41	0.0019
Inoculati*trt	3	0.00435	0.00145	0.63	0.6092
Error	14	0.03236	0.00231		
Total	23	0.15180			
Grand Mean 0.2000		CV 24.04			

Appendix 3.36. Analysis of Variance Table for Na (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	11.681	5.8404		
Inoculati	1	19.278	19.2783	10.76	0.0055
trt	3	151.198	50.3993	28.14	0.0000
Inoculati*trt	3	16.786	5.5953	3.12	0.0599
Error	14	25.077	1.7912		
Total	23	224.020			
Grand Mean 10.375		CV 12.90			

Appendix 3.37. Analysis of Variance Table for P (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	0.50011	0.25005		
Inoculati	1	0.12760	0.12760	4.08	0.0629
trt	3	0.34591	0.11530	3.69	0.0381
Inoculati*trt	3	0.00915	0.00305	0.10	0.9601
Error	14	0.43783	0.03127		
Total	23	1.42060			
Grand Mean 0.4554		CV 38.83			

Appendix 3.38. Analysis of Variance Table for Zn ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	21.6	10.79		
Inoculati	1	4842.2	4842.20	15.74	0.0014
trt	3	28877.5	9625.83	31.30	0.0000
Inoculati*trt	3	1056.3	352.10	1.14	0.3653
Error	14	4306.0	307.57		
Total	23	39103.6			
Grand Mean 78.293		CV 22.40			

Appendix 3.39. Analysis of Variance Table for Fe ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	486.46	243.231		
Inoculati	1	196.02	196.025	1.33	0.2690
trt	3	1077.81	359.271	2.43	0.1086
Inoculati*trt	3	76.89	25.630	0.17	0.9127
Error	14	2070.99	147.928		
Total	23	3908.18			
Grand Mean 49.945		CV 24.35			

Appendix 3.40. Analysis of Variance Table for Na ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.6904	0.3452		
Inoculati	1	52.8363	52.8363	25.87	0.0002
trt	3	6.5066	2.1689	1.06	0.3965
Inoculati*trt	3	4.0224	1.3408	0.66	0.5921
Error	14	28.5900	2.0421		
Total	23	92.6458			
Grand Mean 7.8154		CV 18.28			

Part 3b: Alfalfa and AMF association under Cadmium stress

Appendix 3.41. Analysis of Variance Table for Root area (cm^2)

Source	DF	SS	MS	F	P
rep	19	1.2336	0.0649		
inoculati	1	11.3890	11.3890	209.70	0.0000
trt	3	2.8063	0.9354	17.22	0.0000
inoculati*trt	3	2.3947	0.7982	14.70	0.0000
Error	133	7.2232	0.0543		
Total	159	25.0469			
Grand Mean 0.3983		CV 58.52			

Appendix 3.42. Analysis of Variance Table for Root length (cm)

Source	DF	SS	MS	F	P
rep	19	68.557	3.608		
inoculati	1	143.831	143.831	79.22	0.0000
trt	3	5.844	1.948	1.07	0.3629

inoculati*trt	3	0.302	0.101	0.06	0.9827
Error	133	241.464	1.816		
Total	159	459.998			
Grand Mean 3.8469		CV 35.03			

Appendix 3.43 Analysis of Variance Table for Root breadth (cm)

Source	DF	SS	MS	F	P
rep	19	0.02831	0.00149		
inoculati	1	0.47306	0.47306	205.53	0.0000
trt	3	0.29869	0.09956	43.26	0.0000
inoculati*trt	3	0.25114	0.08371	36.37	0.0000
Error	133	0.30613	0.00230		
Total	159	1.35734			
Grand Mean 0.1056		CV 45.42			

Appendix 3.44. Analysis of Variance Table for Shoot area (cm^2)

Source	DF	SS	MS	F	P
rep	19	2.0079	0.1057		
inoculati	1	29.7965	29.7965	334.89	0.0000
trt	3	5.6093	1.8698	21.01	0.0000
inoculati*trt	3	4.6477	1.5492	17.41	0.0000
Error	133	11.8336	0.0890		
Total	159	53.8949			
Grand Mean 0.6872		CV 43.41			

Appendix 3.45. Analysis of Variance Table for Shoot Length (cm)

Source	DF	SS	MS	F	P
rep	19	49.718	2.6167		
inoculati	1	22.171	22.1712	12.69	0.0005
trt	3	28.604	9.5347	5.46	0.0014
inoculati*trt	3	30.253	10.0842	5.77	0.0010
Error	133	232.347	1.7470		
Total	159	363.093			
Grand Mean 5.0053		CV 26.41			

Appendix 3.46. Analysis of Variance Table for Shoot breadth (cm)

Source	DF	SS	MS	F	P
rep	19	0.08114	0.00427		
inoculati	1	1.16111	1.16111	254.94	0.0000
trt	3	0.56553	0.18851	41.39	0.0000
inoculati*trt	3	0.41422	0.13807	30.32	0.0000
Error	133	0.60573	0.00455		
Total	159	2.82772			
Grand Mean 0.1457		CV 46.32			

Appendix 3.47. Analysis of Variance Table for APX

Source	DF	SS	MS	F	P
rep	2	0.02127	0.01063		
Inoculati	1	0.14690	0.14690	7.59	0.0155
trt	3	0.61213	0.20404	10.54	0.0007
Inoculati*trt	3	0.10615	0.03538	1.83	0.1883
Error	14	0.27093	0.01935		

Total	23	1.15738
Grand Mean 0.6393		CV 21.76

Appendix 3.48. Analysis of Variance Table for CAT

Source	DF	SS	MS	F	P
rep	2	0.5602	0.28012		
Inoculati	1	0.0762	0.07624	0.65	0.4338
trt	3	12.2856	4.09522	34.88	0.0000
Inoculati*trt	3	1.5016	0.50052	4.26	0.0246
Error	14	1.6435	0.11740		
Total	23	16.0672			
Grand Mean 1.5020		CV 22.81			

Appendix 3.49. Analysis of Variance Table for Carotene

Source	DF	SS	MS	F	P
rep	2	0.27917	0.13959		
Inoculati	1	2.07682	2.07682	91.71	0.0000
trt	3	1.80297	0.60099	26.54	0.0000
Inoculati*trt	3	0.19462	0.06487	2.86	0.0744
Error	14	0.31702	0.02264		
Total	23	4.67060			
Grand Mean 0.8850		CV 17.00			

Appendix 3.50. Analysis of Variance Table for Chlorophyll a ($mg.g^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.2136	0.10680		
Inoculati	1	1.7180	1.71797	14.39	0.0020
trt	3	17.2407	5.74690	48.13	0.0000
Inoculati*trt	3	1.0035	0.33451	2.80	0.0785
Error	14	1.6717	0.11941		
Total	23	21.8475			
Grand Mean 1.4733		CV 23.45			

Appendix 3.51. Analysis of Variance Table for Chlorophyll b ($mg.g^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.07000	0.03500		
Inoculati	1	0.29002	0.29002	8.08	0.0130
trt	3	1.30932	0.43644	12.16	0.0003
Inoculati*trt	3	0.32953	0.10984	3.06	0.0631
Error	14	0.50251	0.03589		
Total	23	2.50137			
Grand Mean 0.6851		CV 27.65			

Appendix 3.52. Analysis of Variance Table for POD

Source	DF	SS	MS	F	P
rep	2	5.0033	2.5017		
Inoculati	1	5.0234	5.0234	3.04	0.1030
trt	3	40.0817	13.3606	8.09	0.0023
Inoculati*trt	3	6.0684	2.0228	1.23	0.3374
Error	14	23.1129	1.6509		
Total	23	79.2896			
Grand Mean 9.8308		CV 13.07			

Appendix 3.53. Analysis of Variance Table for SOD

Source	DF	SS	MS	F	P
rep	2	2.833	1.4164		
Inoculati	1	30.578	30.5778	13.56	0.0025
trt	3	80.023	26.6743	11.83	0.0004
Inoculati*trt	3	3.529	1.1762	0.52	0.6744
Error	14	31.577	2.2555		
Total	23	148.539			
Grand Mean 11.127		CV 13.50			

Appendix 3.54. Analysis of Variance Table for root length colonization (%)

Source	DF	SS	MS	F	P
rep	2	29.8	14.9		
Inoculati	1	19463.8	19463.8	1848.64	0.0000
trt	3	412.6	137.5	13.06	0.0002
Inoculati*trt	3	412.6	137.5	13.06	0.0002
Error	14	147.4	10.5		
Total	23	20466.3			
Grand Mean 28.478		CV 11.39			

Appendix 3.55. Analysis of Variance Table for root biomass (g)

Source	DF	SS	MS	F	P
rep	2	0.00008	0.00004		
Inoculati	1	0.00100	0.00100	20.01	0.0005
trt	3	0.00234	0.00078	15.62	0.0001
Inoculati*trt	3	0.00007	0.00002	0.50	0.6894
Error	14	0.00070	0.00005		
Total	23	0.00420			
Grand Mean 0.0576		CV 12.27			

Appendix 3.56. Analysis of Variance Table for shoot biomass (g)

Source	DF	SS	MS	F	P
rep	2	0.01472	0.00736		
Inoculati	1	0.03604	0.03604	6.54	0.0228
trt	3	0.45025	0.15008	27.24	0.0000
Inoculati*trt	3	0.00281	0.00094	0.17	0.9148
Error	14	0.07714	0.00551		
Total	23	0.58096			
Grand Mean 0.6663		CV 11.14			

Appendix 3.57. Analysis of Variance Table for Proline (mg.g^{-1})

Source	DF	SS	MS	F	P
rep	2	0.10373	0.05187		
Inoculati	1	0.01904	0.01904	0.18	0.6776
trt	3	1.23721	0.41240	3.90	0.0322
Inoculati*trt	3	0.63097	0.21032	1.99	0.1617
Error	14	1.47887	0.10563		
Total	23	3.46983			
Grand Mean 0.7580		CV 42.88			

Appendix 3.58. Analysis of Variance Table for Sugar (mg.g^{-1})

Source	DF	SS	MS	F	P
rep	2	5.4139	2.7070		
Inoculati	1	14.1681	14.1681	14.11	0.0021
trt	3	61.0953	20.3651	20.29	0.0000
Inoculati*trt	3	0.3121	0.1040	0.10	0.9566
Error	14	14.0534	1.0038		
Total	23	95.0428			
Grand Mean 4.3550		CV 23.01			

Appendix 3.59. Analysis of Variance Table for Calcium (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	155.76	77.882		
inoculati	1	4.97	4.969	0.39	0.5410
trt	3	1023.90	341.300	26.97	0.0000
inoculati*trt	3	55.01	18.335	1.45	0.2709
Error	14	177.16	12.655		
Total	23	1416.80			
Grand Mean 18.147		CV 19.60			

Appendix 3.60. Analysis of Variance Table for Cu (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	18.651	9.3254		
inoculati	1	49.421	49.4214	20.98	0.0004
trt	3	100.207	33.4023	14.18	0.0002
inoculati*trt	3	9.340	3.1134	1.32	0.3068
Error	14	32.975	2.3553		
Total	23	210.594			
Grand Mean 10.620		CV 14.45			

Appendix 3.61. Analysis of Variance Table for K(g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	24.785	12.392		
inoculati	1	19.207	19.207	4.20	0.0596
trt	3	427.580	142.527	31.17	0.0000
inoculati*trt	3	4.024	1.341	0.29	0.8295
Error	14	64.008	4.572		
Total	23	539.604			
Grand Mean 10.605		CV 20.16			

Appendix 3.62. Analysis of Variance Table for Mg (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	22.676	11.338		
inoculati	1	13.756	13.756	2.63	0.1271
trt	3	609.264	203.088	38.84	0.0000
inoculati*trt	3	5.109	1.703	0.33	0.8068
Error	14	73.207	5.229		
Total	23	724.013			
Grand Mean 12.695		CV 18.01			

Appendix 3.63. Analysis of Variance Table for Mn (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	75.97	37.986		
inoculati	1	170.56	170.560	0.97	0.3410
trt	3	1199.13	399.708	2.28	0.1244
inoculati*trt	3	468.40	156.133	0.89	0.4706
Error	14	2457.43	175.531		
Total	23	4371.49			
Grand Mean 59.982		CV 22.09			

Appendix 3.64. Analysis of Variance Table for Nitrogen (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	0.13051	0.06525		
inoculati	1	0.71070	0.71070	12.16	0.0036
trt	3	0.35091	0.11697	2.00	0.1602
inoculati*trt	3	0.31775	0.10592	1.81	0.1913
Error	14	0.81843	0.05846		
Total	23	2.32830			
Grand Mean 0.7696		CV 31.42			

Appendix 3.65. Analysis of Variance Table for Sodium (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	3.673	1.8364		
inoculati	1	20.646	20.6462	6.85	0.0203
trt	3	118.202	39.4005	13.07	0.0002
inoculati*trt	3	11.452	3.8173	1.27	0.3240
Error	14	42.209	3.0149		
Total	23	196.182			
Grand Mean 7.3408		CV 23.65			

Appendix 3.66. Analysis of Variance Table for P (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	0.08181	0.04090		
inoculati	1	0.14260	0.14260	1.94	0.1857
trt	3	1.25568	0.41856	5.69	0.0092
inoculati*trt	3	0.00941	0.00314	0.04	0.9878
Error	14	1.03059	0.07361		
Total	23	2.52010			
Grand Mean 0.8096		CV 33.51			

Appendix 3.67. Analysis of Variance Table for Cd(mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	312	155.8		
inoculati	1	7225	7224.9	29.90	0.0001
trt	3	240666	80222.1	331.99	0.0000
inoculati*trt	3	5884	1961.5	8.12	0.0022
Error	14	3383	241.6		
Total	23	257470			
Grand Mean 131.68		CV 11.80			

Appendix 3.67. Analysis of Variance Table for Fe (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	70.61	35.303		
inoculati	1	481.24	481.242	11.20	0.0048
trt	3	581.90	193.967	4.51	0.0205
inoculati*trt	3	87.74	29.245	0.68	0.5785
Error	14	601.75	42.982		
Total	23	1823.23			
Grand Mean 42.840		CV 15.30			

Appendix 3.68. Analysis of Variance Table for Ni (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	4.9971	2.49855		
inoculati	1	1.2331	1.23307	1.30	0.2737
trt	3	16.0150	5.33834	5.62	0.0096
inoculati*trt	3	2.8268	0.94228	0.99	0.4251
Error	14	13.3004	0.95003		
Total	23	38.3724			
Grand Mean 4.9392		CV 19.73			

Appendix 3.69. Analysis of Variance Table for Zn (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	11.967	5.9835		
inoculati	1	52.127	52.1265	5.67	0.0320
trt	3	288.977	96.3256	10.47	0.0007
inoculati*trt	3	68.347	22.7822	2.48	0.1041
Error	14	128.789	9.1992		
Total	23	550.206			
Grand Mean 16.100		CV 18.84			

Root nutrients

Appendix 3.70. Analysis of Variance Table for Ca (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	1.6362	0.8181		
inoculati	1	5.5008	5.5008	4.92	0.0436
trt	3	49.3817	16.4606	14.71	0.0001
inoculati*trt	3	5.5257	1.8419	1.65	0.2238
Error	14	15.6608	1.1186		
Total	23	77.7054			
Grand Mean 5.6263		CV 18.80			

Appendix 3.71. Analysis of Variance Table for Cu (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	14.474	7.2371		
inoculati	1	0.070	0.0704	0.01	0.9230
trt	3	253.357	84.4523	11.61	0.0004
inoculati*trt	3	52.238	17.4128	2.39	0.1121
Error	14	101.849	7.2750		
Total	23	421.989			
Grand Mean 17.948		CV 15.03			

Appendix 3.72. Analysis of Variance Table for K (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	1.1163	0.5582		
inoculati	1	3.2413	3.2413	1.79	0.2018
trt	3	37.3508	12.4503	6.89	0.0044
inoculati*trt	3	0.5642	0.1881	0.10	0.9563
Error	14	25.2988	1.8071		
Total	23	67.5715			
Grand Mean 5.5225		CV 24.34			

Appendix 3.73. Analysis of Variance Table for Mg (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	1.8549	0.9275		
inoculati	1	16.2691	16.2691	6.25	0.0254
trt	3	14.3794	4.7931	1.84	0.1858
inoculati*trt	3	3.0941	1.0314	0.40	0.7576
Error	14	36.4219	2.6016		
Total	23	72.0195			
Grand Mean 10.128		CV 15.93			

Appendix 3.74. Analysis of Variance Table for Mn (mg.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	78.44	39.22		
inoculati	1	1002.33	1002.33	37.09	0.0000
trt	3	397.98	132.66	4.91	0.0155
inoculati*trt	3	147.07	49.02	1.81	0.1909
Error	14	378.35	27.02		
Total	23	2004.16			
Grand Mean 51.272		CV 10.14			

Appendix 3.75. Analysis of Variance Table for N (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	0.05906	0.02953		
inoculati	1	0.09882	0.09882	6.21	0.0259
trt	3	0.70453	0.23484	14.76	0.0001
inoculati*trt	3	0.04458	0.01486	0.93	0.4503
Error	14	0.22274	0.01591		
Total	23	1.12973			
Grand Mean 0.4683		CV 26.93			

Appendix 3.76. Analysis of Variance Table for Na (g.kg^{-1})

Source	DF	SS	MS	F	P
rep	2	1.9932	0.99658		
inoculati	1	2.0126	2.01260	0.92	0.3546
trt	3	13.3448	4.44828	2.03	0.1565
inoculati*trt	3	3.4339	1.14463	0.52	0.6745
Error	14	30.7367	2.19548		
Total	23	51.5212			
Grand Mean 5.4354		CV 27.26			

Appendix 3.77. Analysis of Variance Table for P ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	0.04158	0.02079		
inoculati	1	0.33135	0.33135	22.34	0.0003
trt	3	0.46962	0.15654	10.55	0.0007
inoculati*trt	3	0.02642	0.00881	0.59	0.6295
Error	14	0.20769	0.01484		
Total	23	1.07665			
Grand Mean 0.5425		CV 22.45			

Appendix 3.78. Analysis of Variance Table for Cd ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	1285	643		
inoculati	1	36987	36987	35.40	0.0000
trt	3	402305	134102	128.35	0.0000
inoculati*trt	3	11847	3949	3.78	0.0354
Error	14	14628	1045		
Total	23	467052			
Grand Mean 223.68		CV 14.45			

Appendix 3.79. Analysis of Variance Table for Fe ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	120.146	60.073		
inoculati	1	84.901	84.901	15.76	0.0014
trt	3	501.396	167.132	31.03	0.0000
inoculati*trt	3	18.908	6.303	1.17	0.3562
Error	14	75.397	5.386		
Total	23	800.748			
Grand Mean 20.832		CV 11.14			

Appendix 3.80. Analysis of Variance Table for Ni ($g.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	5.045	2.5224		
inoculati	1	13.878	13.8776	6.48	0.0233
trt	3	58.203	19.4011	9.05	0.0014
inoculati*trt	3	5.797	1.9324	0.90	0.4649
Error	14	29.998	2.1427		
Total	23	112.921			
Grand Mean 7.5196		CV 19.47			

Appendix 3.81. Analysis of Variance Table for Zn ($mg.kg^{-1}$)

Source	DF	SS	MS	F	P
rep	2	8.285	4.1427		
inoculati	1	23.305	23.3051	6.31	0.0249
trt	3	47.724	15.9079	4.31	0.0239
inoculati*trt	3	72.378	24.1260	6.53	0.0055
Error	14	51.728	3.6949		
Total	23	203.420			
Grand Mean 13.243		CV 14.51			

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