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Copper Toxicity Influence on Antioxidant Enzymes Activity in Tomato Plants and Role of Arbuscular Mycorrhizal Fungus *Glomus etunicatum* in the Tolerance of Toxicity

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Abstract: Soil microorganisms have been shown to possess several mechanisms capable of altering metal bioavailability for uptake into roots. In addition, root mycorrhizal associations have been shown to affect the rate of metal uptake. There is evidence that exposure of plants to excess concentrations of heavy metals such as Cu results in oxidative injury. In this study, effect of arbuscular mycorrhizal fungus *Glomus etunicatum* on tolerance of Cu toxicity in tomato plants was studied. In order to prepare seedling medium, we used washed and sterilized sand and agricultural soil. Tomato seeds were surface sterilized and planted in two pots. One filled just with sterilized sand (for non-mycorrhizal treatments) and the other filled with sterilized sand mixed with *G. intraradices* mycorrhizal inoculum. We were certain about complete colonization after 4 weeks, so we transferred three seedlings to each main pot. Plants grew in growth chamber for nine weeks. During growth period plants received modified Hoagland's solution (with half P content) with Cu concentration of 0, 1.5, 3.5, 5.5, 7.5 mM $CuSO_4$ in triplicates. Antioxidant enzymes activity, Ascorbate Peroxidase (APX) and Guaiacol Peroxidase (GPX) and Root Length Colonization (RLC) percentage in mycorrhizal and non-mycorrhizal plants were measured. APX activity in mycorrhizal shoots increased but there was no significant correspondent increase in roots of these plants. GPX activity in mycorrhizal roots increased but there was no significant correspondent increase in shoots of these plants. Activity of this enzyme in roots and shoots of mycorrhizal plantshigher than non-mycorrhizal plants. Estimation of root length colonizatinon by gridline intersect method, increase in Cu concentration, colonization percentage decreased significantly. The data show the possible role of mycorrhiza in plant protection against Cu toxicity.

Key words: Tomato, copper, mycorrhiza, *Glomus etunicatum*, colonization percentage

INTRODUCTION

Copper (an essential micronutrient) is frequently related to soil pollution and is potentially toxic to living organisms. Copper (Cu) is necessary for plant growth in low concentrations, a structural part of enzymes and is uptaken as divalent cation (Cu^{2+}) or Cu chelate. Cu, however, is often present in high concentrations that are toxic enough to biota.

When higher plants are subjected to environmental stresses, such as water stress and salinity, a variety of Reactive Oxygen Species (ROS) such as superoxide anion radical (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and singlet oxygen ($1O_2$) are induced (Elstner, 1982; Jung, 2004). As a consequence, higher plants induce efficient antioxidant systems to protect them against oxidative injury (Asada, 1999). The antioxidant systems consist of antioxidant enzymes including superoxide dismustase (SOD), Guaiacol Peroxidase (G-POD), catalase (CAT), Glutathione Reductase (GR), Ascorbate Peroxidase (APX) and non-enzymatic antioxidants including ascorbate (ASC) and glutathione (GSH), which are designed to minimize the concentrations of O_2d_ and H_2O_2.

Arbuscular Mycorrhiza (AM) is one of the most widespread Mycorrhizal associations between soil microorganisms and higher plants (Malekzadeh *et al*., 2007a). Ninety percent of the earth's land plant species form symbiotic associations with Arbuscular Mycorrhizal Fungi (AMF) (Gadkar *et al*., 2001). Providinga direct physical link between soil and plant roots, the Arbuscular Mycorrhiza (Am) fungi are important rhizospheric microorganisms. They can increase plant uptake of nutrients especially relatively immobile elements such as P, Zn and Cu (Ryan and Angus, 2003) and consequently, they increase root and shoot biomass and improve plant growth. It has been indicated that Am fungi can colonize plant roots in metal contaminated soil (Vogel-Mikus *et al*., 2005), while their effects on metal uptake by plants are conflicting. In slightly metal contaminated soil, most

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studies show that Am fungi increased shoot uptake of metals (Weissenhorn *et al*., 1995), while in severely contaminated soil, Am fungi could reduce shoot metal concentration and protect plants against harmful effects of metals (Li and Christie, 2001; Malcova *et al*., 2003).

As a result of this symbiotic association between AM fungi and host plants, P content also has an effect on physiological parameters in plants (Johnson, 1984; Paradi *et al*., 2003). One of the physiological parameters is the increase in photosynthesis.

AMF-colonized plants are generally more resistant to stresses caused by drought, salt, heavy metals or attack by pathogens. These positive effects of the fungi on the growth of plants often result from an improved nutrient supply and can partly be due to complex and not easily resolved interactions between the symbiotic partners. In contrast, AMF-colonized roots of plants from soils severely polluted by high concentrations of heavy metals show lower amounts of heavy metals than non-colonized plants (Schuepp *et al*., 1987; El-Kherbawy *et al*., 1989; Weissenhorn *et al*., 1995; Weinstein *et al*., 1995; Kaldorf *et al*., 1999; but see also opposite data by (Gildon and Tinker, 1983; Killham and Firestone. 1983), (Rufyikiri *et al*., 2000; Ottet *et al*., 2002; Rabie, 2005; Vogel-Mikus *et al*., 2005).

The present study was undertaken to thoroughly evaluate reactive oxygen metabolism, including ROS, antioxidant enzymes and non-enzymatic antioxidants, in leaves and roots of tomato plant inoculated with *G. etunicatum* under Cu toxicity conditions.

However, information about activities of antioxidant enzymes in AM and non-AM plants is scarce.

MATERIALS AND METHODS

Seeds of tomato (*Lycopersycium esculentum* Mill) were germinated in a moist mix of soil and sand in polystyrene trays. There 28 day old seedlings, uniform in size, were transplanted into 30 plastic pots filled with sterilized sand. Half of the pots received the AMF *Glomus etunicatum* and Trappe by placing 30 g (moist weight) of inoculums is soil below the tomato seedling prior to planting. The AMF inoculum (consist of soil and root fragments and spores) (This inoculum was prepared in trap culture of *G. etunicatum* in the greenhouse) was placed directly adjacent to each seedling root to facilitate fungal colonization of plant roots. Control treatments received no AMF inoculum.

The plants were grown in a greenhouse in Urmia university under natural photoperiods (28/20°C day/night 6000/10000 lux light in tensity) for 13 weeks during which only distilled water was applied. In addition, twice a week, each pot was supplied with 100 mL of a nutrient solution containing: five Cu concentration (0, 1.5, 3.5, 5.5, 7.5 Mm $CuSO_4$) added to Hogland nutrient solution (with half P concentration).

The symbiotic fungal partner, *Glomus etunicatum*, was produced in a soil:and (1/1, v/v) mixture using maize as the host plant. Inoculum of *Glomus etunicatum* (30 g), consisting of spores, external mycelium and AMF colonized roots, was laid around the seed. The same amount of sterilized inoculum was laid into the control pots. The roots were cleared and stained by using the methods by Philips and Hayman (1970). The percentage of mycorrhizal colonization (F %) was estimated by the grid line intersect method (Giovanetti and Mosse, 1980). At the end of the experiment, plants were harvested 10 weeks after seed sowing. Plant shoots was separated, dried (70°C 48 h).

For extraction of antioxidative enzymes, roots were homogenized with 0.1 M sodium phosphate buffer (pH 6.8) in a chilled pestle and mortar. The homogenate was centrifuged at 12,000 g for 20 min and the resulting supernatant was used for determination of enzyme activity. The whole extraction procedure was carried out at 4°C. The decrease in the concentration of ascorbate was recorded at 290 nm. The enzyme activity was calculated from the initial rate of the reaction using the extinction coefficient of ascorbate (2.8 mM_1 cm_1 at 290 nm).

The whole extraction procedure was carried out at 4°C. APOD and GR were assayed as described previously (Chang and Kao, 1998).

RESULTS

Figure 1 shows the effect of Cu treatment on the activity of APX in the shoots of mycorrhizal and non-mycorrhizal tomato plants. APX activity in mycorrhizal shoots increased significantly ($p<0.05$) by increase in Cu concentration, but there was no significant correspondent increase in roots of these plants (Fig. 2). Activity of this enzyme in non-mycorrhizal plant increased significantly ($p<0.05$), but Activity of this enzyme in roots and shoots of mycorrhizal plants higher than non-mycorrhizal plants.

Figure 3 shows, GPX activity in mycorrhizal roots increased significantly ($p<0.05$) by increase in Cu concentration, but there was no significant correspondent increase in roots of these plants (Fig. 4). Activity of this enzyme in non-mycorrhizal plant increased significantly ($p<0.05$), but Activity of this enzyme in roots and shoots of mycorrhizal plants higher than non-mycorrhizal plants.

Estimation of root length colonizatinon by gridline intersect method, increase in Cu concentration, colonization percentage decreased significantly. The data show the possible role of mycorrhiza in plant protection against Cu toxicity.

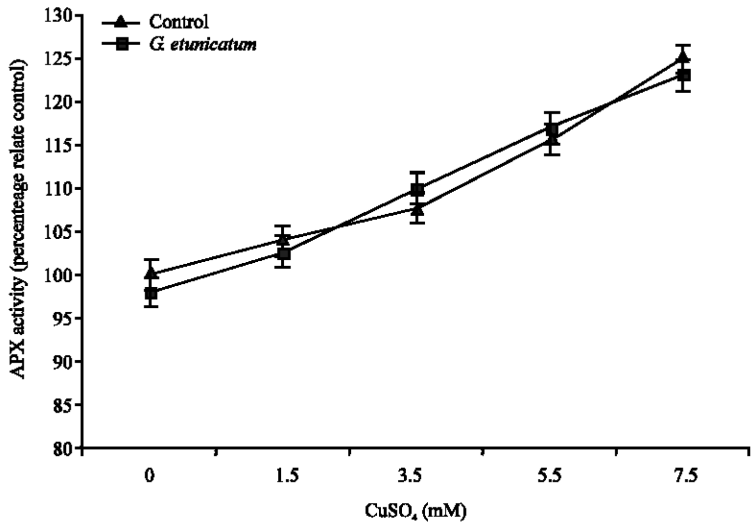

Fig. 1: The effects of Cu treatment on the activity of APX in the shoots of mycorrhizal and non-mycorrhizal tomato plants treated with $CuSO_4$

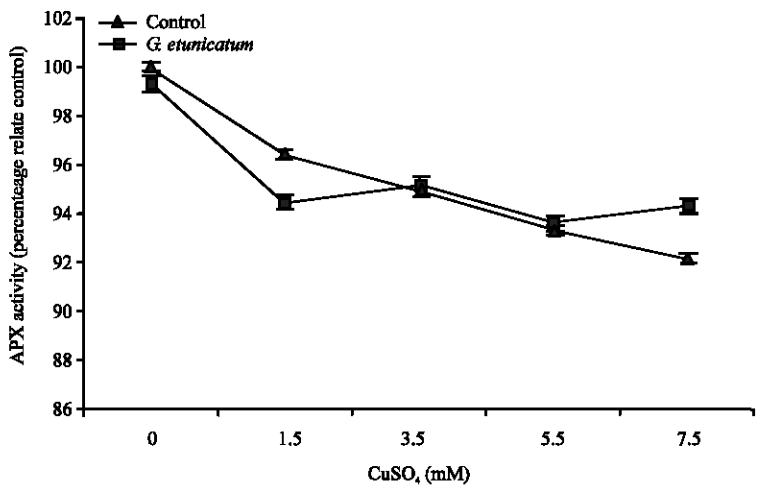

Fig. 2: The effects of Cu treatment on the activity of APX in the roots of mycorrhizal and non-mycorrhizal tomato plants treated with $CuSO_4$

Figure 5 showed that the development of Am symbiosis varied between Cu concentration. Root colonization of tomato plants was significantly reduced and negatively correlated by the presence of heavy metals in nutrient solution, it was decreased from 57% in control to 34% in the 7.5 mM concentration.

The data show the possible role of mycorrhiza in plant protection against Cu toxicity.

DISCUSSION

The protective mechanisms adapted by plants to scavenge free radicals and peroxides include several antioxidative enzymes such as SOD, APX, GR, CAT and GPX. The antioxidative enzymes are important components in preventing the oxidative stress in plants as is based on the fact that the activity of one or more of these enzymes is generally increased in plants when exposed to stressful conditions (Malekzadeh *et al*., 2007b).

Figure 1-4 indicates antioxidant enzyme (APX and GPX) activities of Am tomato plants showed significant higher values in polluted soil than in non-polluted one. Based on these results, the mechanisms related to physiological interactions between the Am fungus and tomato plants involve increased protein synthesis as

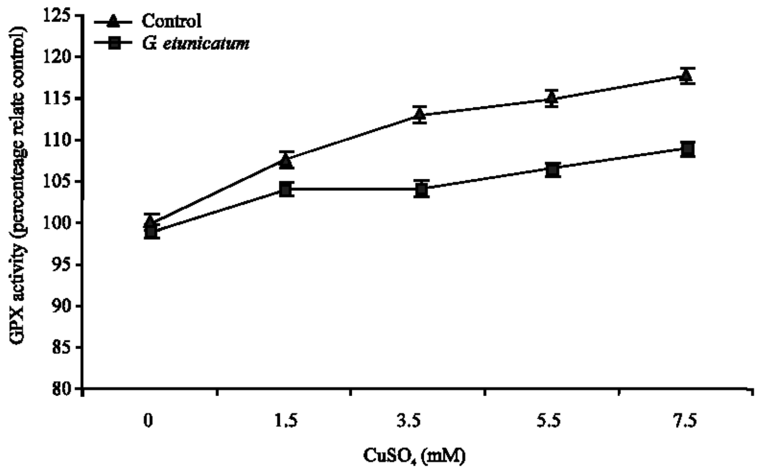

Fig. 3: The effects of Cu treatment on the activity of GPX in the shoots of mycorrhizal and non-mycorrhizal tomato plants treated with $CuSO_4$

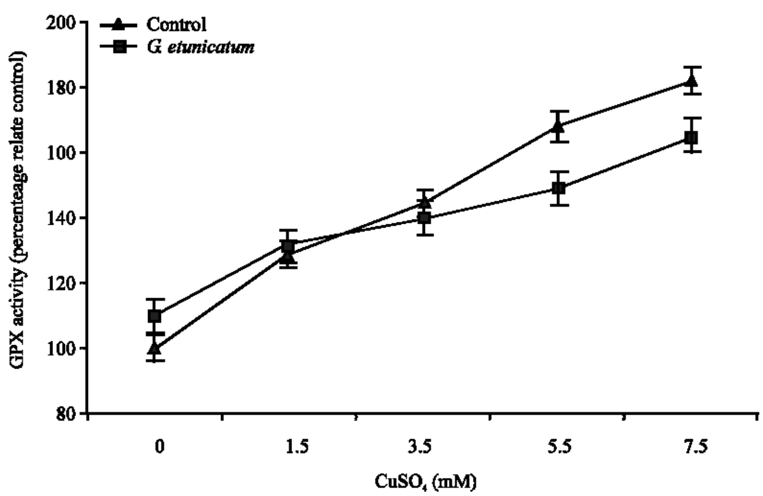

Fig. 4: The effects of Cu treatment on the activity of GPX in the roots of mycorrhizal and non-mycorrhizal tomato plants treated with $CuSO_4$

well as induction of antioxidant enzymes to avoid heavy metal-mediated oxidative stress. If so, the author attributed the reduced heavy metal toxicity effects in Am tomato plants to antioxidative protection through detoxification of heavy metals, chelation through metal-binding proteins (peptides) and dilution through increased plant growth induced by Am fungi. This finding supports results from some dissipated studies reporting that the role of antioxidative enzyme system as well as improved growth as possible mechanisms for plant protection against high accumulated toxic heavy metals in the shoots (Ott *et al*., 2002; Burleigh *et al*., 2003; Tong *et al*., 2004).

This finding supports results from numerous studies reporting that Am fungi often protect plants against high accumulation of toxic elements in the shoots, as it was reported for Al (Rufyikiri *et al*., 2000), Cd (Yu *et al*., 2004), Zn (Burleigh *et al*., 2003), Pb (Malcova *et al*., 2003), U (Rufyikiri *et al*., 2004), Cu (Gonzalez-Chavez *et al*., 2002) and As (Fitz and Wenzel, 2002).

Estimation of root length colonizatinon by gridline intersect method, resulted in 50% decrease between controls (0 mM) and 7.5 mM and showed that by increase in Cu concentration, colonization percentage decreased significantly.

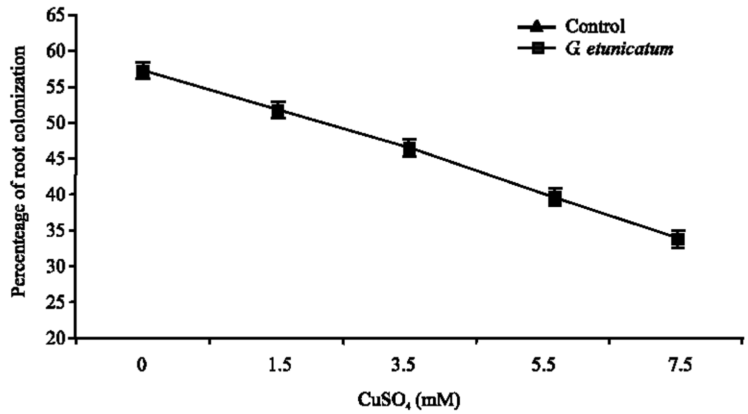

Fig. 5: The effects of Cu treatment on the percentage of root colonization with *Glomus etunicatum* in tomato plants

This finding is in line with results of Leyval *et al.* (1995), Weinstein *et al.* (1995), Hildebrandt *et al.* (1999) and Vogel-Mikus *et al.* (2005) who reported that sensitivity of Am symbionts to heavy metal contaminated soil expressed as a reduction in spore germination, hyphal growth or root colonization, had been proved previously in number of studies. Another interesting result in Fig. 5 was that the presence of Am fungi inoculations would increase the metal tolerance index of tomato plants compared with non-Am plants that grew in heavy metal polluted soil. This result emphasizes that Am fungi could be potentially effective in protecting plants exposed to high levels of heavy metal. The Am fungi ability to alleviate heavy metal stress of plants grown in heavy metal contaminated soil was previously proved by Rufyikiri *et al.* (2000), Hildebrandt *et al.* (2002) and Burleigh *et al.* (2003).

These results overwhelmingly indicate that the behavior of Am fungi in the protection of plants from heavy metal toxicity might differ according to host plant and metal concentration which can not generalized. Therefore additional researches are needed to explore the behavior of Am fungi in various plant species and families for plant protection in heavy metal polluted soil. Nevertheless, the beneficial effects of the Am fungus, observed in this study, arouse an interest in considering the role of Am fungi in plant-based strategies of remediation of highly heavy metal contaminated soils.

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