Effects of Copper Heavy Metal and Interaction With Nitric Oxide on Growth Parameters, Photosynthetic Pigment, Soluble Carbohydrate Content and Antioxidant Enzymes in *Portulaca oleracea* L.

Ferdous Fendereski, Mahlagha Ghorbanli* and Arian Sateei

Biology Department, Islamic Azad University, Gorgan Branch, Gorgan, Iran

Abstract

Copper is one of the heavy metal in plant that causes toxicity at high concentration via producing reactive oxygen species. Nitric oxide can protect cells from oxidative stress produce by reactive oxygen species. Effect of different concentrations of copper (1000, 1500 and 2000 µM) and interaction with nitric oxide (100 and 150 µM) were studied on growth parameters (shoot and root length) and some physiological factors (chlorophyll a, chlorophyll b, soluble carbohydrate), antioxidant enzymes (catalase, peroxidase and ascorbate peroxidase) in *Portulaca oleracea* L. For this purpose, an experiment was done in complete block random design with three replications under pot condition. Our result showed that nitric oxide treatment increased shoot and root length, shoot and root fresh weight as well as chlorophyll a and b content under copper stress. Therefore, in stressed plants treated with nitric oxide growth parameter improved and damage to pigments decreased. Copper stress did not affect soluble carbohydrate and increased antioxidant enzyme activity but nitric oxide increased soluble carbohydrate content and decreased antioxidant enzyme activity with exception of catalase and peroxidase. Pre-treatment with nitric oxide had protective role under copper treatment stress with interaction with ROS and photosynthetic pigments.

Keywords: copper toxicity; Nitric oxide; *Portulaca oleracea* L.

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Introduction

Metals with density higher than 5 g/cm^3 are categorized in heavy metal elements (Appenroth, 2010). 53 elements from 90 elements in nature are heavy metals. But all heavy metal are not important from biological point of view. In other hand, the problem cause by heavy metal is not related to high density of heavy metal. Copper in low concentration is an essential micro nutrient necessary for plant growth. This element require at least in six parts of plant cell including Cytosol, Endoplasmic reticulum, inner membrane of mitochondria, stroma of Chloroplasts, lumen of thylakoid and apoplast (Marschner, 1995). Copper is involved on many electron transfer reaction in

*Corresponding author
E-mail address: mahlagha.ghorbanli@yahoo.com
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photosynthesis and respiration process. Also, this element has a role in lipid and fatty acid metabolism. Copper is major compound of Chloroplast protein, plastocyanin. One of the major features of copper ion is its ability to attach small molecule such as oxygen as a ligand. Therefore, copper is a cofactor for many oxidase enzymes. One of the best known oxidase is mitochondrial cytochrome oxidase. Another enzyme is amine oxidase related to cell wall that catalyzes oxidation of Putrescine. Putrescine is involved on H_2O_2 production which is involved on lignification process, cross link with protein cell wall and program cell death (Moller and Mc Pherson,1998). Polyphenol oxidase is another enzyme that is found in thylakoid of some plant such as spinach but it is not found in Arabidopsis species (Kaiser et al., 1998 Schubert et al., 2002). This enzyme has a role in ROS defense system. Copper ion could be join ethylene via ethylene acceptor ETR1 is located on Endoplasmic reticulum and depends on copper (Rodríguez et al., 1999). Nitric oxide (NO) is known as biochemical messenger in plants (Hayat et al., 2010). Pretreatment with NO improved germination of wheat seeds and decrease oxidative stress against copper toxicity through increasing of SOD and CAT and reduction of lipooxygenase activity and MDA. NO involved on detoxification of H_2O_2 via modulation of antioxidant enzymes such as CAT, POD and AXP and keeping of cell redox couple and non-protein antioxidant including thiol, ascorbate (Tewari al., 2008).

Purslane (*Portulaca oleracea* L.) is an annual plant with C_4 photosynthesis pathway belongs to *Portulacaceae* family. It is tolerate against drought and salinity condition (Gorham, 1996; Khan et al., 2006). Purslane is a source of α-linolenic acid and β-carotene (Liu et al., 2000; Barbosa-Filho et al., 2008). It contains cumarin flavonoid and monoterpene glycoside, Dopamine, noradrenaline ferulic acid and adenosine (Sakai et al., 1996). Mucilage, pectin, protein, carbohydrate, non-saturated fatty acid, antioxidant, mineral nutrient (Iron, copper, manganese, potassium, calcium, phosphorus and selenium) were found in all parts of this plant. Antioxidant compound contain α-tocopherol, ascorbic acid and glutathione. Phytochemical experiment showed that this plant contain B1 and A vitamins, Quercetin, cinamic acid and cafeic acid flavonoids and also ω3 fatty acid (Miladi Gorji et al., 2007).

Materials and Methods

Seed of *Portulaca oleracea* L. were produced from Pakan bazr company, Isfahan, Iran. The experiment was conducted in Islamic Azad University of Gorgan, Iran. Seed were cultivated in pot based on completely random design with three replications under greenhouse condition.

Plants were treated with $CuSO_4$ solution (1000, 1500, 2000 µM) and Nitro sodium peroxide (100 µM) alone or $CuSO_4$ solution (1000, 1500, 2000 µM) and Nitro sodium oxide (150 µM) together for two weeks. Morphological parameters including shoot and root length and shoot and root dry weight. Some physiological parameters including chlorophyll a, chlorophyll b determined by Arnon method (1949), soluble carbohydrates were estimated by the method described by Irrigoyen et al. (1992).

Assay and protein extraction

Frozen leaves (0.5 g fresh weight) were homogenized in 5 ml Tris- Glycine buffer (pH 8.3). The homogenate was centrifuged at 12000 g for 10 min. All operations were performed at 4 ˚C. Protein contents were determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Measurement of catalase activity

Activity of catalase was measured in a reaction mixture consisting Tris-Glycine buffer (50 mM, pH 7.5), H_2O_2 (10 mM) and enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm (Pereira et al., 2002).

Measurement of peroxidase activity

Peroxidase activity was measured in a reaction mixture consisting acetate buffer (0.2 mM, pH 4.8), hydrogen peroxide (0.1 mM), benzidine (0.04 M) and enzyme extract. Enzyme activity was measured at 530 nm (Koroi, 1989).

Measurement of ascorbate peroxidase activity

Ascorbate peroxidase activity was measured according to the method of Nakano and Asada (1981). The reaction mixture consisted of enzymatic extract, L-1 sodium phosphate buffer (50 mM, pH 7), ascorbate (0.5 mM), hydrogen peroxide (0.1 mM), EDTA (0.1 mM) and enzyme extract. The reaction started after addition of the hydrogen peroxide, and the absorbance was measured by a spectrophotometer at 290 nm.

Experimental design and statistics:

The experiment was performed under random design. SPSS software was used. Interaction effect of copper stress and nitric oxide was performed with factorial design, LSD test was used to estimate significant difference of means, (*p < 0.05) was considered as a statistical significant difference.

Results

Shoot length

Shoot length decreased under copper stress (2000µM) showed significant reduction compared to control. But there was no significant difference between the control and other copper concentration. Under non-stress condition, there was significant difference between 100 µM NO and control. But 150 µM NO showed no significant difference with control. Under copper stress (1500 and 2000 µM) treatment with 150 µM caused significant increase in shoot length (P<0.05) (Fig. I).

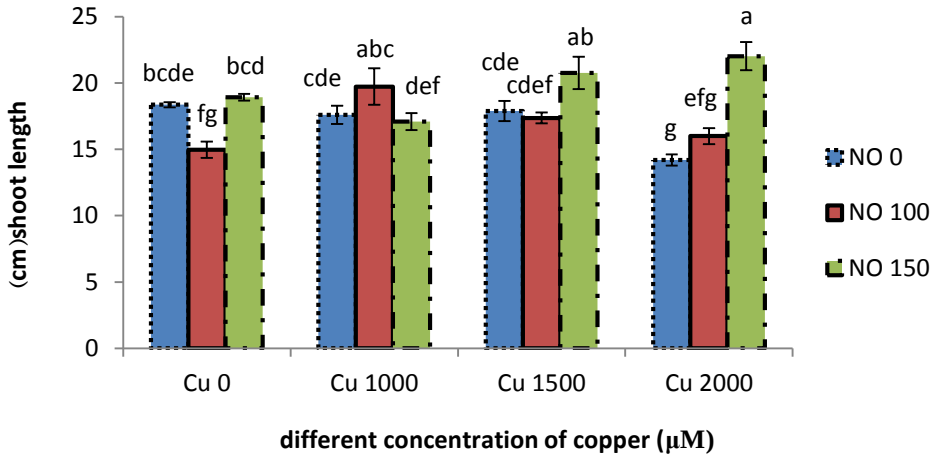

Fig. I. Effect of different concentration of copper (1000, 1500 and 2000 µM concentration) and nitric oxide (100 and 150 µM concentration) on shoot length (P<0.05)

Root length

Root length decreased under copper stress (2000µM) showed significant reduction compared to control. But there was no significant difference between the control and other copper concentration. Under non-stress condition, there was no significant difference between 100 µM NO and control. But 150 µM NO showed significant difference with control. Under copper stress (1500 and 2000 µM) treatment with 150 µM NO cause significant increase in root length Copper treatment (1500 µM) with 150 µM nitric oxide

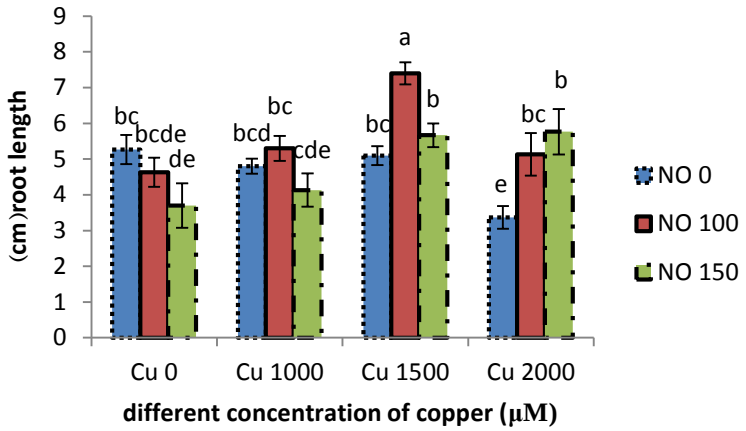

Fig. II. Effect of different concentration of copper (1000, 1500 and 2000 µM concentration) and nitric oxide (100 and 150 µM concentration) on root length (P<0.05)

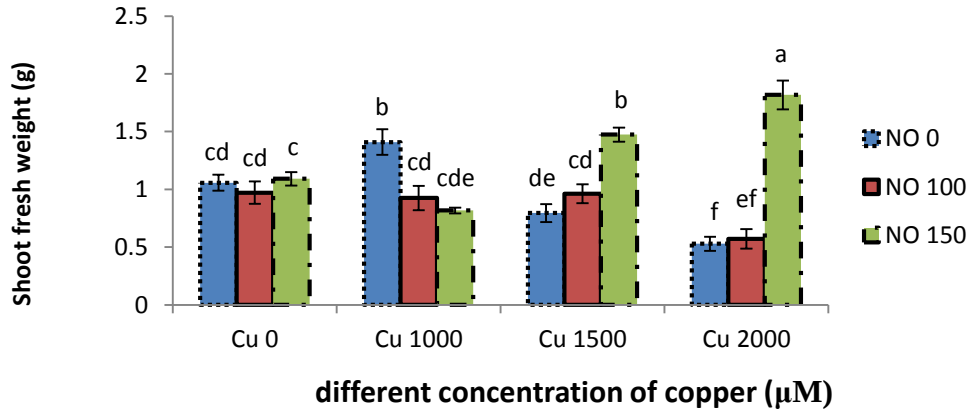

Fig. III. Effect of different concentration of copper (1000, 1500 and 2000 µM concentration) and nitric oxide (100 and 150 µM concentration) on shoot fresh weight (P<0.05)

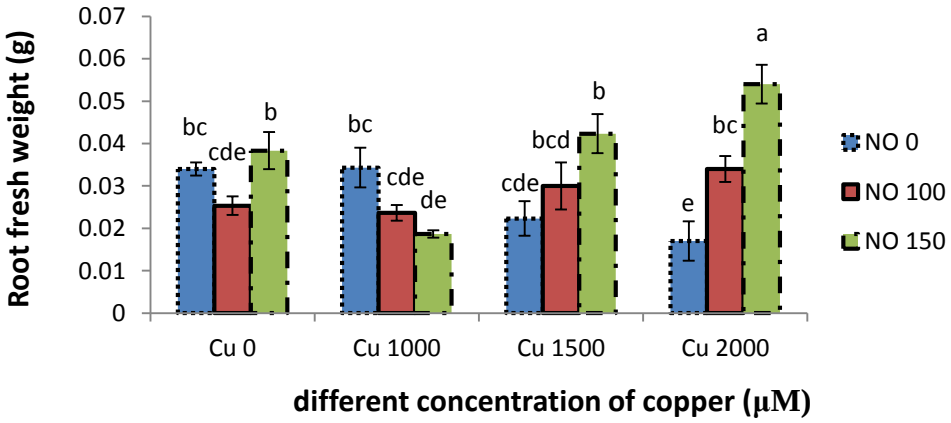

Fig.IV. Effect of different concentration of copper (1000, 1500 and 2000 µM concentration) and nitric oxide (100 and 150 µM concentration) on root fresh weight (P<0.05)

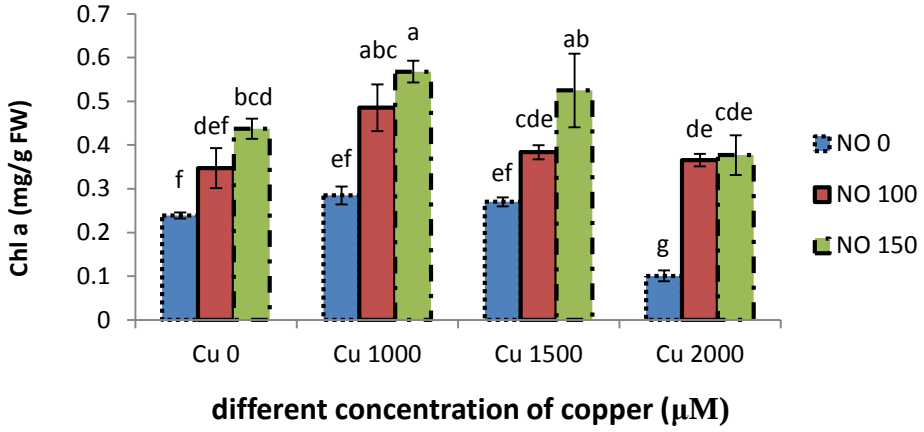

Fig.V.Effect of different concentration of copper (1000, 1500 and 2000 µM concentration) and nitric oxide (100 and 150 µM concentration) on chlorophyll a (P<0.05)

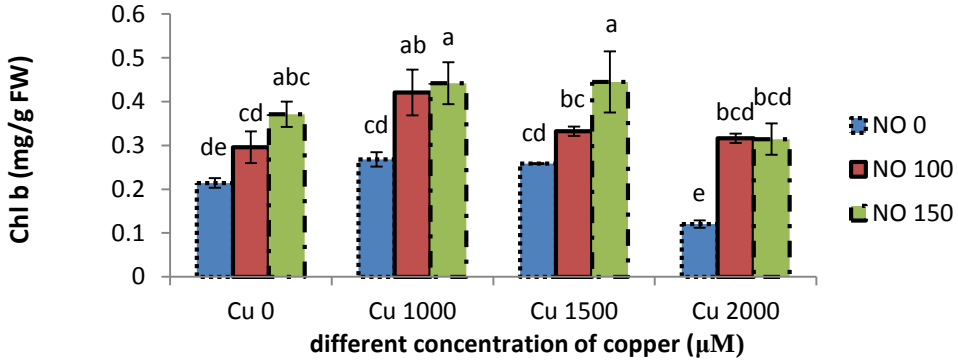

Fig.VI. Effect of different concentration of copper (1000, 1500 and 2000 µM concentration) and nitric oxide (100 and 150 µM concentration) on chlorophyll b (P<0.05)

showed significant increase in comparison to control plant (P<0.05) (Fig. II).

Shoot fresh weight

Shoot fresh weight under 1000 and 2000 µM concentration of copper showed significant increase and decrease, respectively. Under non-stress conditions, there was no significant difference between the NO treatment and control. Under 1000 µM of copper stress, both concentration of NO (100 and 150 mM) caused a significant reduction in Shoot fresh weight. Also, under copper stress of 1500 and 2000, 150 µM concentrations of NO caused a significant increase in shoot fresh weight compared to control (P<0.05) (Fig.III).

Root fresh weight

Under 2000 µM concentration of copper root fresh weight significantly decrease. Under non-stress conditions, there was no significant difference between other copper concentration and control. Under non- stress condition, there was no significant difference between NO treatments and control. Under 1000 µM and 1500 µM copper stress, 150 µM concentrations of NO

and under 2000 µM copper stress both concentration of NO (100 and 150 µM) caused a significant increase in root fresh weight compared to control (P<0.05) (Fig.IV).

Chlorophyll a

Under 2000 µM copper stress chlorophyll a content decrease significantly compare to control. But under non- stress condition and 15 µM concentration of NO chlorophyll a content increased. Under 1000 µM and 2000 µM copper stress and both NO concentration (100 and 150 µM) and Under 1500 µM copper stress and 150 µM concentration of NO chlorophyll a content increased compare to control plant (P<0.05) (Fig.V).

Chlorophyll b

Under 2000 µM copper stress chlorophyll b content decrease significantly compare to control. But under non- stress condition and both NO concentration (100 and 150 µM) chlorophyll b content increased significantly. Under 1000 µM and 2000 µM copper stress and both NO concentration (100 and 150 µM) and under 1500 µM copper stress and 150 µM concentration of NO chlorophyll b content increased compare to control plant. Also under 1000 and 1500 µM copper stress and both NO concentration (100 and 150 µM) chlorophyll b content increased compare to control plant (P<0.05) (Fig.VI).

Soluble carbohydrate of shoot

Under copper stress, soluble carbohydrate content of shoot show significant difference. Under non- stress condition, 150 µM concentration of NO soluble carbohydrate content increase significantly compare to control plant. Under 1000 µM concentration of copper and 100 µM concentration of NO as well as 1500 µM concentration of copper and 150 µM concentration of NO soluble carbohydrate content increased significantly (P<0.05) (Fig. VII).

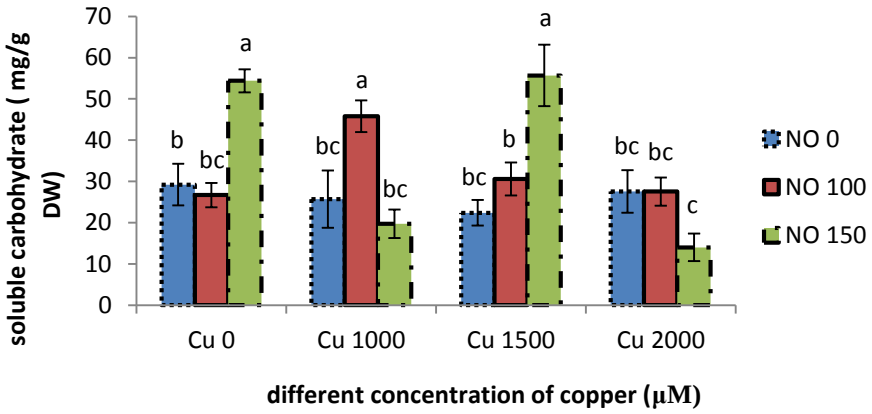

Fig.VII. Effect of different concentration of copper (1000, 1500 and 2000 µM concentration) and nitric oxide (100 and 150 µM concentration) on soluble carbohydrate (P<0.05)

Peroxidase enzyme activity

Peroxidase enzyme activity decrease significantly under copper stress condition. Under non- stress condition and both 100 and 150 µM concentration of NO Peroxidase enzyme activity decrease significantly compare to control plants. There was no significant difference among all copper stress treatments (P<0.05) (Fig. VIII).

Catalase enzyme activity

Catalase enzyme activity decreased significantly under copper stress condition compare to control plant. There was no significant difference in relation to Catalase enzyme activity under non- stress condition between 100 and 150 µM concentration of NO and control. Under 1000 µM of copper stress and 100 µM concentration of NO as well as under 1500 µM of copper stress and 150 µM concentration of NO, catalase enzyme

activity decrease significantly compare to control plants. There was no significant difference in relation to Catalase enzyme activity under 2000 µM of copper stress (P<0.05) (Fig. IX).

Ascorbate peroxidase enzyme activity

enzyme activity significantly decrease and increase, respectively. Under 1500 µM of copper stress and both concentration of NO ascorbate peroxidase enzyme activity decrease significantly compare to control plants. Also Catalase enzyme activity under 2000 µM of copper stress and 150

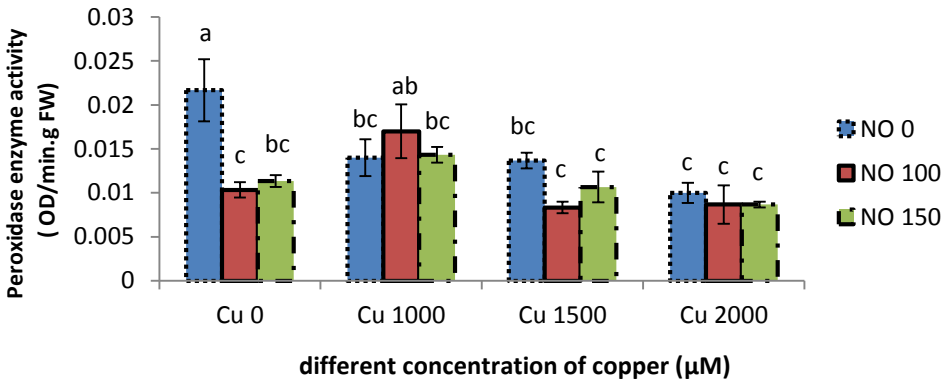

Fig. VIII. Effect of different concentration of copper (1000, 1500 and 2000 µM concentration) and nitric oxide (100 and 150 µM concentration) on Peroxidase enzyme activity (P<0.05)

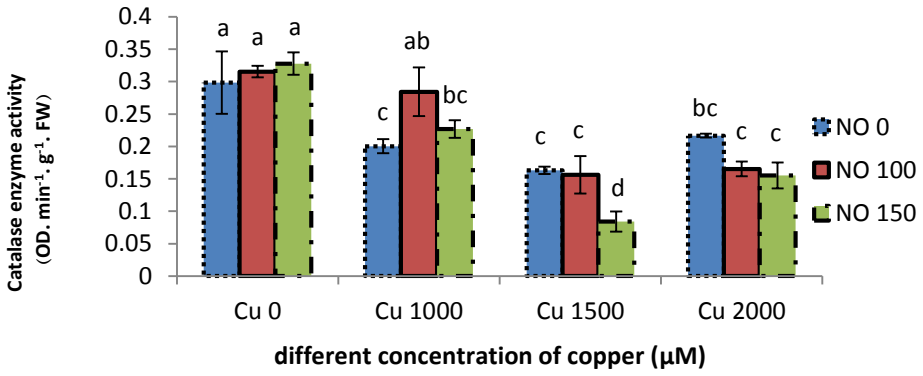

Fig.IX. Effect of different concentration of copper (1000, 1500 and 2000 µM concentration) and nitric oxide (100 and 150 µM concentration) on catalase enzyme activity (P<0.05)

Ascorbate peroxidase enzyme activity increase significantly under 1500 µM copper stress condition compare to control plant. Under non- stress condition ascorbate peroxidase enzyme activity increase significantly in both 100 and 150 µM concentration of NO. Under 1000 µM of copper stress and 100 µM and 150 µM concentration of NO, ascorbate peroxidase

µM concentration of NO decrease significantly (P<0.05) (Fig. X).

Discussion

According to our result, under copper stress condition, Shoot and root length as well as Shoot and root fresh weight decrease significantly compare to control. NO application cause to

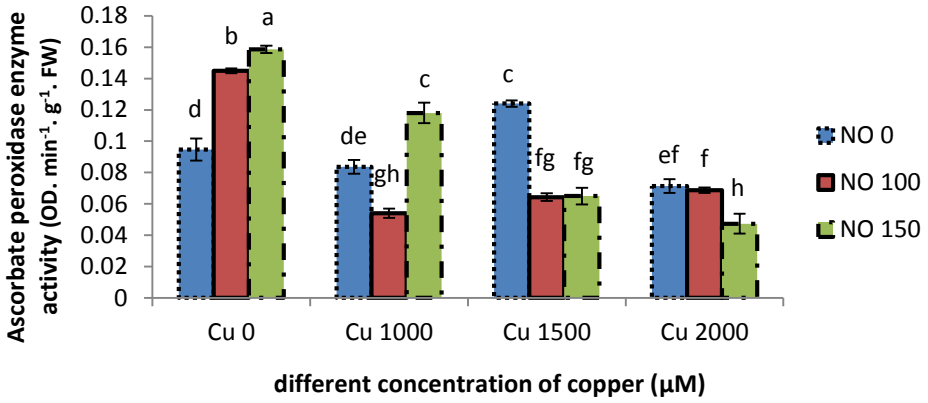

Fig. X. Effect of different concentration of copper (1000, 1500 and 2000 µM concentration) and nitric oxide (100 and 150 µM concentration) on ascorbate peroxidase enzyme activity (P<0.05)

increase Shoot and root length and also shoot and root fresh weight. It seems that No improves negative effect of copper stress. In *Arabidopsis thaliana*, high level of copper decrease growth of roots and shoots. This reduction was clear in root because absorption of copper was higher in roots (Lequeux et al., 2010). Copper via deleterious effect on physiological reaction cause abnormality in growth and development of plants (Yruela, 2005). Copper toxicity affect root morphology, cell proliferation and number of hairy roots (Sheldon and Menzies, 2005). Also, root tip became brown, root growth decreased and apical dominance demolished because of meristem cells death. Under this condition, number of secondary roots increase in order to absorption of water and mineral nutrition and plant survival (Raisi et al., 2010). Low level of copper had not significant effect on *Festuca arundinacea* and *Lolium perenne* root length. However high level of copper showed obvious signs of toxicity and after seven days root growth diminish and browning of root was observed. According to this research root and shoot growth decrease under high level of copper concentration and root growth was more sensitive than shoot growth against copper stress that are in agreement with our result (Sheldon and Menzies, 2004). SNP treatment in *Lepidium sativum* L. under copper stress increase fresh weight of root and shoot which are parallel with this research (Raisi et al., 2009). High level of copper decrease fresh and dry weight of root and shoot in wheat cultivars and *Chloris gayana* (El-Tayab et al., 2004; Sheldon and Menzies, 2004). Growth reduce under high level of copper because of Ethylene increase, cytokinin and polyamine decrease, inhibition of cell enlargement or effect of copper on metabolism and transporter of auxin (Aidid and Okamoto, 1993). Indeed, high level of copper affects indole acetic acid oxidase and lead to toxicity (Coombes et al., 1976). Low level of NO but not high level of NO increase fresh weight and leaf area. The possibility of a link between NO and roots grow with IAA exists; NO affect proton extrusion and calcium input via special channel and increase cell enlargement (Beligni and Lamattina, 2001). Treatment of leaf with high level of NO show stress signs (Leshem et al., 2000). High level of SNP had deleterious effect on root growth of *Cassia tora* and root growth under SNP treatment depend on time of exposure (Wang and Yang, 2005). In this research, NO stimulate root and shoot growth. It is proposed that auxin and NO activate same response in plants.

Effect of copper stress on photosynthetic pigments

Copper stress at 2000µM concentration decreased Chlorophyll a and Chlorophyll b content. Application of NO under stress condition increased Chlorophyll content. Also, 150 µM NO under non –stress condition increased Chlorophyll a and Chlorophyll b content. Reduction of Chlorophyll a content reported by other researchers (El-Tayab et al., 2010). Addition

of copper decreased Chlorophyll b and carotenoid in wheat cultivars (El-Tayab et al., 2010). Heavy metals stress cause oxidative stress and destroy chlorophyll content and reduces equilibrium of protein complexes of photosystem II system (Laspina et al., 2005). It seems significant reduction of photosynthetic pigment in copper-stressed plants due to their degradation in rye (Ali and al, 2003).

Prasad and his co-workers (2001) considered that Chlorophyll reduction by heavy metal is due to peroxidation of chloroplast membrane. After copper stress treatment, Chlorophyll reduction is related to enzymes involved in synthesis and degradation of Chlorophyll (Yruela, 2005). In Pea- nut Chlorophyll a and b content significantly reduce under copper stress treatment. This reduction was much greater than zinc stress samples that represent more toxic effect of copper on the leaves of peanut. Photosystem II in leaves of pea nut sensitive to high concentrations of copper (Gangrong and Qingsheng, 2009). SNP pre-treatment also were resulted in the high chlorophyll content in potatoes, lettuce and Arabidopsis (Beligni and Lamanttina, 2001).

Accordingly, the SNP prevent chlorosis between leaf veins and chlorophyll content in maize have increased in comparison with the control plants (Graziano et al., 2002). SNP improve also chlorophyll content reduction in age-related wheat seedlings (Turk et al., 2003); oxidative stress in tomato plants (Beligini and Lamattina, 1999) and salinity in corn (Zhang et al., 2006). SNP at a 100 mM concentrations and 100 nM concentration of NaCl increased the chlorophyll content of wheat seedlings grown in the dark and chlorophyll degradation caused by the herbicide paraquat and D. Kavat reduce (Beligni and Lamattina, 2001) which corresponded with the results of our findings. Protective effect of nitric oxide against oxidative stress induced by cadmium heavy metal were studied in sunflowers plant. These result Showed that the leaves of seedlings treated with 100 mM NO improve dry weight and chlorophyll degradation caused by exposure to heavy metals (Laspina et al., 2005).

Effect of copper stress and NO on soluble carbohydrate content

With increasing copper concentration no difference was observed between soluble carbohydrate content. Using NO at 150 µM concentration increase soluble carbohydrate. Soluble carbohydrate are important osmolyte which increase in response to drought stress. Increasing soluble carbohydrate cause to cell inflammation and inhibit cell plasmolysis. There are positive relationship between movement and adjustment of carbohydrate in order to acquire osmotic metabolite. Soluble carbohydrate decrease water lose by closing of stomata and protecting cell membrane. Also, carbohydrate induce abcisic acid that decrease water stress (Verlinden and Garica, 2004). Heavy metal change activity of water channels protein and close leaf stomata, therefore inhibit water movement in plant (Zhang and Tyerman, 1999).

Following cadmium accumulation in cells and reduction of water transfer, the amount of soluble carbohydrate in plant increased. This feature is a method for adaptation of plants for osmotic adjustment. In addition, soluble carbohydrate helps plants to increase their carbohydrate reserves to maintain the basal metabolism at an optimal level under stress condition (Verma et al., 2001). Contradictory results related to the effect of salinity on accumulation of soluble carbohydrate were existing in literature. Some studies have reported that under stress conditions, soluble carbohydrate increased (Pilon-Smits et al, 1995; Dubey and Singh, 1999; Kerepesi and Galiba, 2000) for example in *Cajanus cajan*, (Subbaro et al., 2000); sorghum (Gill et al., 2001); wheat (El-Tayeb and Ahmed, 2010); rice (Mostajeran and Rahimi-Eichi, 2009); soya (Ghorbanli and Niakan, 2005); potato (Masoudi-Sadaghiani et al., 2011); Arabidopsis (Rizhsky et al., 2004). Decreased content of soluble carbohydrate has been reported in other researches. Soluble carbohydrate content decreased in *Satureja hortensis* (Yazdanpanah et al., 2011); canola cultivars (Qasim et al., 2003) under salinity stress. Trehalose content was found at low amount under salinity stress in the nodules of alfalfa root (Fougere et al., 1991).

In sunflower were sensitive to salinity, the amount of soluble carbohydrate was less than resistant ones. In response to salinity in grapes reducing sugars increased and sucrose and starch content

decreased. In Barley, the amount of sucrose and reducing sugars increased. In soya sucrose and starch content decreased. In *Cenchrus penisetiformis*, the amount of sucrose, glucose and fructose decreased (Ashraf and Harris, 2009). Generally, soluble carbohydrate content under stress condition increased or unchanged (Pinheiro et al., 2001). All observable change under stress conditions are for Compatibility of plants adapt to various stresses associated synthesis activities and carbohydrate content (Gill et al., 2001). Hydroxyl group of alcoholic sugars and Hydroxyl group of water were kept Hydrophilic interactions with proteins and lipid membrane. In response to stress, leaf carbohydrate status changed and made a metabolic signal (Jang et al., 1997).

Effect of various copper stress and No on antioxidant enzyme activity

According to result, No was effective on antioxidant enzymes activity. Plant has special protective system including antioxidant enzymes and compounds in order to resist against oxidative damage of heavy metal ions (Ali et al., 2003). This system consist of antioxidant enzymes (Ascorbate peroxidase, superoxide dismutase, catalase, Glutathione reductase) and non-enzymatic compounds (ascorbic acid, Glutathione, carotenoid) (Groppa et al., 2007). Reaction of plants to heavy metal depends on plant species, metal concentration in soil and bioavailability of metals. The importance of antioxidant enzymes related to their ability of ROS scavenging and inhibition of oxidative damage (Khatun et al., 2008). Wong et al (2006) investigate antioxidant properties of 25 tropical plants. The lowest antioxidant activity was found in local celery and the highest antioxidant activity was observed in leaves of other plants like amaranth (Amaranthus), mint (Mentha), sweet potato. Resistance and acclimatization of plants against heavy metal are varied among plants (Mashhadi Akbar Boojar & Goodarzi, 2007). In potato under drought stress catalase activity increased (Masoudi-Sadaghiani et al., 2011). In plants antioxidant enzymes activity increased under oxidative stress of heavy metals and reaction of antioxidant enzymes depends on plant species. Antioxidant enzymes in plant extract had various performances and their activity related to experimental condition that influence on plant chemical compound with antioxidant activity (Wong et al., 2005). Indeed plant with high antioxidant enzyme activity had higher resistance against oxidative stress (Khatoun et al., 2008). Catalase activate under oxidative stress condition and can delete and digest H2O2 in plants (Khatoun et al., 2008). Therefore, peroxide hydrogen, the product of superoxide dismutase enzyme, is the substrate of peroxidase and catalase that deactivated by these enzymes (Garnczarska and Ratajczak, 2000). Various studies on antioxidant enzymes show that plant needs mix performances of superoxide dismutase, peroxidase and catalase antioxidant enzymes in order to protect plants against ROS species (Garnczarska and Ratajczak, 2000).. Seed of cucumber treated with 50 mM NaCl reduced the seed germination rate in short term and seed of germination but 50 µM exogenous SNP significantly increased the activity superoxide dismutase et catalase but no obvious effects of exogenous NO on peroxidase and ascorbate peroxidase (Fan et al , 2013). Cu treatment significantly inhibited the activity of POD and APX in both leaves and roots of tomato , and exogenous of NO supply greatly decreased the inhibition (Cui et al ;2010).

Abiotic stress such as heavy metal, drought and salinity directly and indirectly cause molecular damage in plants via production of reactive oxygen species. Nitric oxide at some concentration improves damages cause by abiotic stress. Purslane is resistance against copper stress up to 1500 µM. It seems NO inhibit ROS performance and decrease oxygen radicals damage. Growth parameters such as shoot and root length as well as shoot and root fresh weight decrease under copper stress and application of NO improves stress condition and increase Growth parameters. NO treatment under stress condition decrease damage of photosynthetic pigments. Antioxidant enzyme activity under copper stress decreased. NO treatment act as antioxidant and decrease antioxidant enzyme activity. NO increase soluble carbohydrate because of increase chlorophyll content and photosynthesis. According to our result NO as an antioxidant produce suitable condition for plant growth under copper stress condition.

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