



# Effect of Shortage Mineral Elements (Nitrogen, Phosphorus, Potassium, Magnesium and Calcium) on Antioxidant Enzymes Activity (Peroxidase, Glutathione Reductase, Ascorbate Peroxidase and Catalase) in Fenugreek (*Trigonella foenum graecum*)

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## ABSTRACT

When needing nutrient elements hold suitable degree for plants, they performed their physiological and biochemical processes properly and so there will be no need to use defensive mechanism. In doing so, its growth and function level increases. In this study, effect of shortage nutrient elements (Nitrogen, Phosphorus, Potassium, Magnesium and Calcium) on antioxidant enzymes activity such as Peroxidase, Glutathione Reductase, Ascorbate Peroxidase and Catalase were studied. The observations indicate that Ascorbate Peroxidase, Peroxidase and Catalase activity in root and shoot was reduced in all the shortage level of mineral elements than blank level. But Peroxidase activity in phosphorous shortage was increased than blank level. In plants with Magnesium shortage, Glutathione Reductase activity in root was decreased than blank. While in plants with Potassium, Calcium and Nitrogen, Glutathione Reductase activity was increased. In relation with Glutathione Reductase measurement in plants shoot with Nitrogen, Phosphorous and Magnesium shortage showed no significant changes, while in Calcium and Potassium shortage was decreased than blank level.

### Keywords:

Fenugreek (*Trigonella foenum graecum* L.), Mineral Elements, Antioxidant Enzymes

## INTRODUCTION

Fenugreek with scientific name (*Trigonella foenum graecum* L.) from Fabaceae family is grass plant, annual plant, 10 to 50 cm length, with alternate and trifoliate compound leaves. It has single flowers with yellow color, violet or inclines to white with length of 0/8 to 1/8 cm. It has legume fruit that is amphitropous (recurve) with 3 to 11 cm of length and 5 to 20 number of angle seeds with 4 to 6 mm length and 2 to 3 mm width. Its color changes from yellow to brown (Moatar and Ardakani, 1999). This plant have perfumed tasty and is medicinal. Leaf extraction, root and stem have a variety of medical effect that is suitable for diabetes, cholesterol, and children bone tuberculosis and dyspepsia treatment. Also Root, shoot and seeds of Fenugreekis are very rich in respect to secondary metabolic content and they have different Steroid Saponins such as Diosgenin or Mogenine, Ticogenine, Neoticogenine. Important amongst the plant alkaloid is Trigoneline in that its concentration in Fenugreek seeds is 36%. Most important among the Flavonoid compounds in this plant is Ornithine, Viticsine and Quercetin. In addition fix oil and protein compound are found in plant seed (Zahedi, 2005), such as oxidative damages that create at oxygen radicals effect; we can refer oxidative damage to lipids, proteins and DNA and production of reactive oxygen species caused to lipids peroxidation (Chen and Asada, 2000) and protein degradation (Jiang and Huang, 2001). But in front of such damages, plants are equipped to antioxidant defence like Super Oxide Dismutase (SOD) and Catalase (CAT) into Reactive Oxygen Species (ROS) (Agarwal et al., 2005). ROS are considered to comparison compatible and tolerance to different biotic and Abiotic stress (Dat et al., 2009). Activity of enzymes such as Catalase (CAT), Peroxidase (POD) and Super Oxide Dismutase (SOD) are caused of damaging activity of ROS product in cells and ROS production in plant cells cause increase of enzymes activity such as Catalase, Peroxidase and Super Oxide Dismutase (Dat et al., 2009). Peroxidase participates in many of the growth and development stages, and defense mechanisms in plants. Gayacol Peroxidase is available in different parts of plant cells such as cytosol, membranes, nucleus and mitochondria and connecting to cell walls. Hallyl and Exton (1972) carried out an experiment on cytochemical evaluation of Peroxidase in Pea (*Pisum sativum* L.) root and appointed enzyme activity in root cap, epiderm, endoderm, pheloem and xylem. Also, Catalase changes of peroxide hydrogen ( $H_2O_2$ ) to water and oxygen. This enzyme are found in all aerobic eukaryotic and is very important in deletion of peroxide hydrogen in peroxysome by oxidase and fatty acids production by beta oxidation in Glyoxalate cycle (photorespiration) and Purine catabolism. Catalase has been separated as one of the first enzymes in the country. Molecular weights of all form of enzyme are more than 220000 KD. Several form of catalase have

been described in many plants. Tree form of this enzyme has cloned of corn (Redinbaugh et al., 1998; Sacandalias, 1990). Tree cloned of this enzyme in corn named CAT-1, CAT-2 and CAT-3 that has been described on separate and different chromosomes is regulated independently (Sacandalias, 1990). GSSG is reduced by NADPH with Glutation Reductase. Ivanov et al. (2002) described oxygen reduction in plastoquenon source in tilacoid membrane. The importance of this finding in relation to Mohler reaction at Invitro condition is that there is need for more experiments. Antioxidant systems are fundamental for rebuff of AOS toxicity. Ascorbate is the most solute antioxidant in chloroplast. It has been proved that high content of antioxidants in *Qnercuse ilexare* serves as a strategy for defeating high light stress and low warm degree. Smirnoff (2002) in his work, carried out a significant study on the identification of several role of ascorbic acid in photosynthesis and light protection. Ascorbate biosynthesis is control by light. Biosynthetic pathway for ascorbate in plants has a particular trend. Oxidation of 1, 4-L-Galactanolacton with one of L-Galactose dehydrogenase is depended on NAP settlement in the internal membrane of mitochondria. High level of ascorbate is obligatory in high photo oxidative stress. It is clear that high-level antioxidants are responsible for degradation kinds of ROS in plants in its growing state found in high light environment in comparison with low light regimes. Also plants in high light environmental conditions have xanthophyll cycle independent to energy. Ascorbate Peroxidase use two molecule of ascorbic acid thereby creating  $H_2O_2$  to water along with two molecule MDHA (MDA) (radical with short long term) that have not connection with MDHA. MDHA reduced by MDHA Reductase that GSH is substrate.

## MATERIALS AND METHODS

### Plant Materials

Plant seeds of Fenugreek (*Trigonella foenum graecum*) were prepared in Iran Jungle and Pasture Research Institute. The study was done under controlled condition and in Botany Laboratory located in Islamic Azad University of Tonekabone Branch since spring of 2011. Also seeds were same size. For antiseptic of seeds, sodium hypochlorite (5%) was put in this solution for 10 min and then washed in distilled water (Falahati, 2006). Seeds cultivated in petri dish with medium size (number of 8) were placed into germinator with temperature of 25 °C (centigrade) until after germination. After 4 days growing, seedlings were cultivated in pots with 30 cm in length and diameter. Without delay pots were placed in growth room with temperature 22-24 °C (dark period) and 25-27 °C (light period) with light period of 16 h. The first week were used for planting in pots with 40 mm distilled water. Next was the supply of solutes after first

week irrigation have been done by 40 ml Hogl and then solution. The number of pots taking 5 treatments and one evidence in every pot is 4 repetitions (Rajesh, 2004). Plantlets were transplanted in aerated solution culture having nutrient composition: 2.0 mM  $\text{KNO}_3$ , 2.0 mM  $\text{Ca}(\text{NO}_3)_2$ , 1.0 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.67 mM  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 0.05 mM NaCl, 0.05 mM Fe-EDTA, 5.0\_M  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.5\_M  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.5\_M,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 16.5\_M  $\text{H}_3\text{BO}_3$ , 0.1\_M  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.05M  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.05\_M  $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$ . The pH of the nutrient solution was adjusted at  $6.7 \pm 0.2$  before supply. On fourth day after transplantation, pots were grouped into seven lots having three pots each. Whereas, plants in lot 1 continued to receive complete nutrient solution (Control), lots 2–7 were treated differentially by withdrawing the supply of salts of specific macronutrient elements. The withdrawn salts were replaced by equivalent quantities of alternate salts in order to maintain the osmoticum of the nutrient solution and to ensure adequate supply of other elements. In case of N deficiency,  $\text{KNO}_3$  and  $\text{Ca}(\text{NO}_3)_2$  were replaced by equivalent amounts of  $\text{K}_2\text{SO}_4$  and  $\text{CaCl}_2$ ; in case of K and Ca deficiencies respectively,  $\text{KNO}_3$  and  $\text{Ca}(\text{NO}_3)_2$  were replaced by equivalent amounts of  $\text{NaNO}_3$ ; In case of P deficiency,  $\text{Na}_2\text{HPO}_4$  was replaced by equivalent amounts of NaCl and for developing Mg,  $\text{MgSO}_4$  was replaced by equivalent amounts of  $\text{Na}_2\text{SO}_4$  and  $\text{MgCl}_2$ , respectively, and in K deficiency, Fe-EDTA was replaced by Fe-EDTA. The volume of nutrient solution was made-up daily by de-ionized water. The nutrient solution was refreshed every alternate day. The glasshouse conditions during the experiment were: maximum PPFD (12:00 h) 1170–1350  $\mu\text{mol m}^{-2} \text{ s}^{-1}$ , daily maximum and minimum temperatures 34.3–40.5 and 24.4–30.6  $^\circ\text{C}$ , respectively, and RH (9:00 h) 60–70%. The average photoperiod was  $12.3 \pm 0.2$  h.

#### Determination of Peroxide Content

Root and shoot tissues (0.005 g) were homogenized by mortar-pestle and homogenizer respectively in 0.02ml of 100mM potassium phosphate buffer (pH 6.8). Homogenate was filtered through 2 layers of cheese cloth and then centrifuged at 1800 g for 20 min at 4 $^\circ\text{C}$ . Modified method of Bernt and Bergmeyer (1974) was used to determine the  $\text{H}_2\text{O}_2$  content of the supernatant. Peroxidase enzyme was used and the reaction is started in an aliquot of 0.005 ml of supernatant and 2.5 ml of peroxidase reagent (0.87 mM potassium phosphate, pH 7.0, 0.005 % (W/V) o-dianizidine, 0.4 mg Peroxidase/ml) at 30 $^\circ\text{C}$ . After 10 minutes of incubation was stopped by adding ml of 1N Perchloric acid and centrifuged with sigma 3K 30 centrifuges at 5000 g for minutes. Absorbance was read at 436 nm.  $\text{H}_2\text{O}_2$  content was determined using the extinction coefficient 39.4  $\text{Mm}^{-1} \text{ cm}^{-1}$ .

#### Determination of APX Activity

Wang spectrometric assay (Wang et al., 1991) was used to determine the APX activity. Tissue of 0.05 g was weighed and powdered with liquid nitrogen in a mortar. Powder was homogenized with 1 ml of suspension solution containing 0.006 mM Tris-HCL (pH 7.2), 2% PVP, 0.3 mM EDTA and 0.035 mM ascorbate. The suspension was centrifuged at 12000 g for 20 minutes at 4  $^\circ\text{C}$  and suspension was used for the enzyme assay. Enzyme extract containing 100 mg protein, determined by Bradford Method (1976), was added into assay medium containing 0.01mM potassium phosphate buffer (pH 6.6), 0.0044 mM ascorbate, 0.085 mM  $\text{H}_2\text{O}_2$ . The reaction was started by the addition of peroxide. The decrease in the ascorbate concentration was recorded at 290 nm with Shimadzu double-beam spectrophotometer for 3 minutes; the enzyme activity was calculated from the initial rate of the enzyme. Standard curve of range 0-0.5 mM Ascorbate was used (Extinction coefficient of ascorbate = 2.8  $\text{mM}^{-1} \text{ cm}^{-1}$  at 290 nm). Corrections were made for the non-enzymatic reduction of the Peroxide with Ascorbate.

#### Determination of CAT Activity

Catalase activity was determined according to the method of Chonce et al.(1995), 0.005 g tissue was ground with liquid nitrogen by using cold mortar and pestle and then suspended with suspension solution containing 0.006 mM Tris-HCL (pH 7.8). After filtering through 2 layers of cheese cloth, the suspensions were centrifuged at 12000 g for 20minutes at 4  $^\circ\text{C}$ . Supernatant was taken for the enzyme assay. Enzyme extract containing 100 mg protein, determined by Bradford Method (1976), was added into assay medium containing 0.01 mM potassium phosphate buffer (pH 7.0), 0.002 mM  $\text{H}_2\text{O}_2$ . The enzyme extract. The decrease in absorbance was recorded at 240 nm with Shimadzu double-beam spectrophotometer for 2 minutes. The enzyme activity was calculated from the initial rate of the enzyme (Extinction coefficient of  $\text{H}_2\text{O}_2=40 \text{ mM}^{-1} \text{ cm}^{-1}$  at 240 nm).

#### Determination of GR Activity

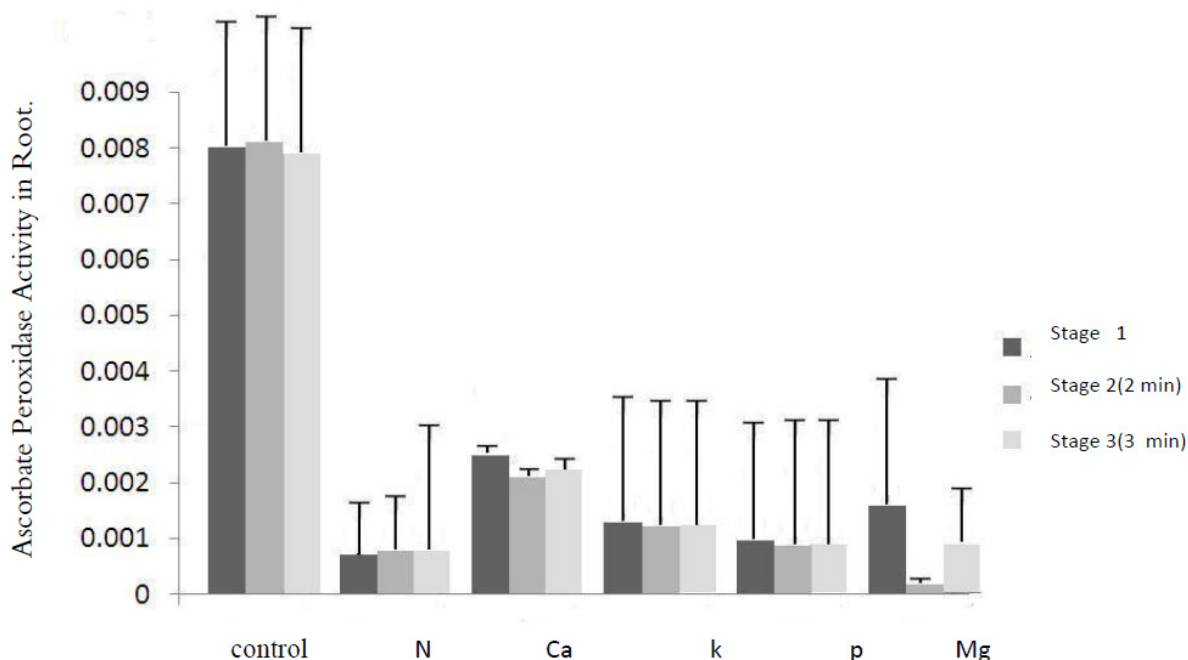
Glutathion Reductase activity was determined according to the method of Sgherri et al. (1994) that 0.01 g (Shoot/Root) tissue was ground with liquid nitrogen and the powder was suspended in 1.5 ml of suspension solution containing 100 mM potassium phosphate buffer (pH 7.0), 0.01 mM  $\text{Na}_2$  EDTA and 2% insoluble PVP. The GSSG dependent oxidation of NADPH was monitored by the decrease in Absorbance at 340 nm at 30  $^\circ\text{C}$ . Enzyme extract containing 100 mg protein determined by Bradford Method (1976), was added into assay solution containing 0.04 mM potassium phosphate buffer (pH 7.5), 0.2 mM GSSG, 0.002 mM NADPH, in a final volume of 1 ml. The reaction was started by the addition NADPH. The enzyme activity was calculated for the background absorbance at 340 nm, without NADPH. The enzyme activity was calculated from the initial rate

of enzyme after subtracting the non-enzymatic oxidation. Extinction coefficient of NADPH = 6.2 mM. Experiments in completely random framework, with 3 replications were performed. Data analysis, variance and comparison of means were done by Danken experiment and SPSS software; for drawing of diagrams, Excel software was used.

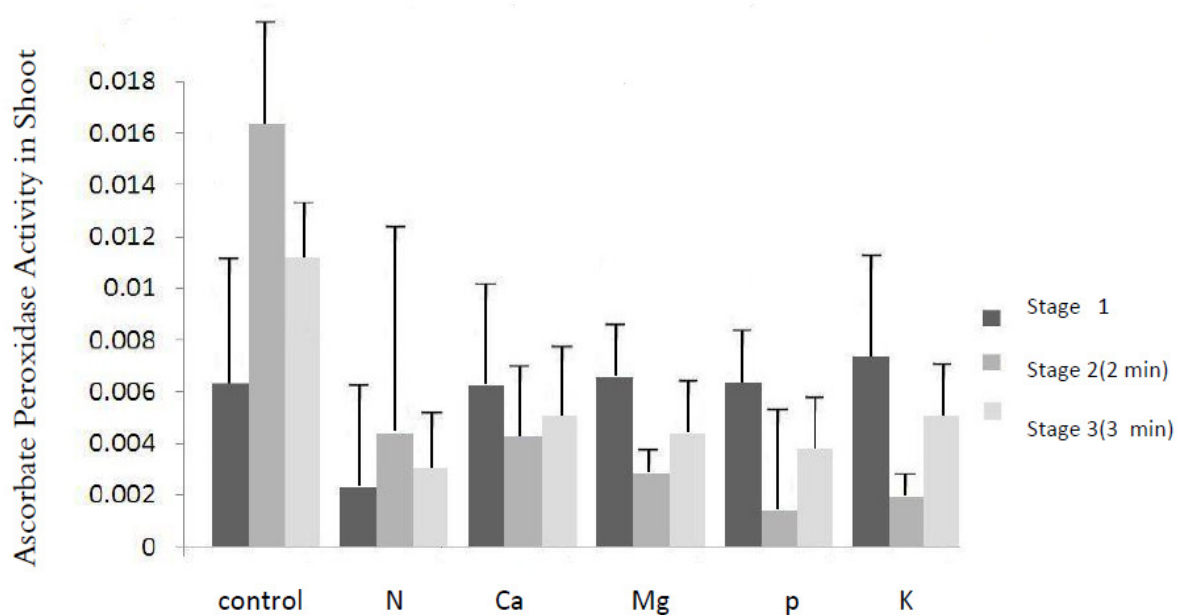
## RESULTS

Done on plants and shortage effect of mineral elements on antioxidant enzymes activity showed an increase or a decrease of some antioxidant enzymes activation. In this study, also, results showed much decrease in antioxidant enzymes activity. In Ascorbat Peroxidase enzyme activity in root, there was meaningful decrease than blank level in all shortages of mineral elements, while in two and tree stages that Ascorbate Peroxidase measurement was performed in 3 minute after absorption, results showed that Ascorbate Peroxidase activity had meaningful decrease in all shortages than blank level (Figure 1). Also, Ascorbate Peroxidase measurement in shoot, in 3 stages of measurement was decreased than blank level in all of mineral elements

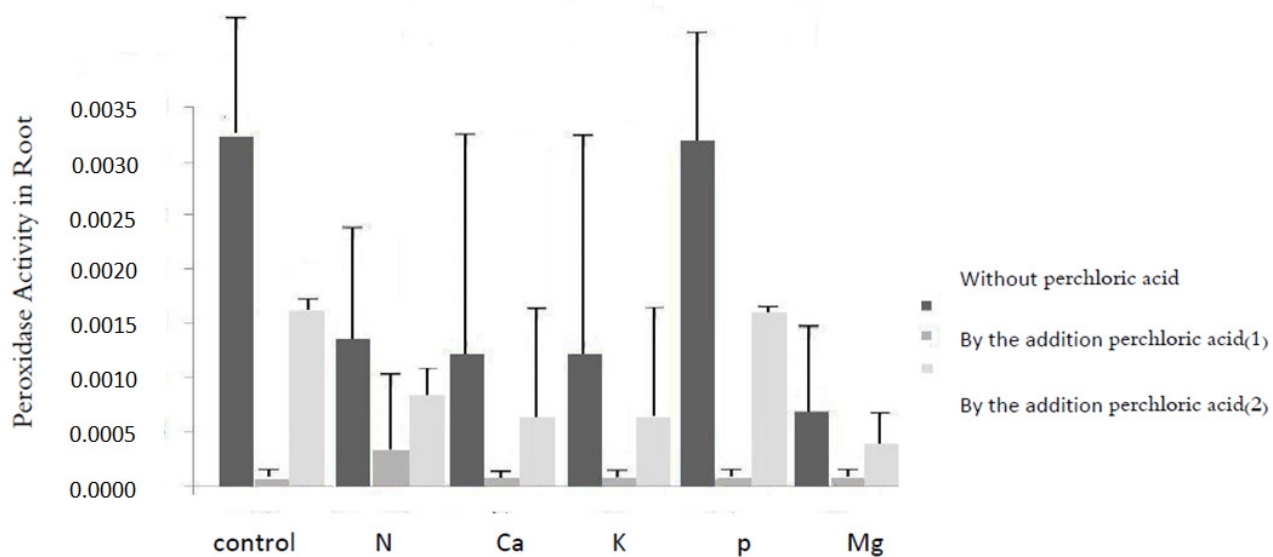
shortage (Figure 2). The Peroxidase enzymes activation in root, in the 3 stages of measurement, was observed to have showed meaningful decreased activity than the blank level in the all levels of shortage except in phosphorous (Figure 3). Peroxidase enzyme activity in shoot, also, showed meaningful decrease in all levels of shortage than blank in the 3 stages of measurement (Figure 4). Catalase activity measurement in root, in the 3 stages of measurement showed meaningful decrease in all levels of shortage than the control level, except in phosphorous shortage (Figure 5). Also, Catalase activity in shoot, in the 3 stages of measurement showed meaningful decrease in all plants with levels of shortage (Figure 6). Glutation Reductase activity measurement in root of plants with levels of magnesium shortage showed significant decrease than the blank level, while in plants with phosphorous, calcium, nitrogen and potassium shortages were observed to have showed meaningful increase than the blank level (Figure 7). Also, Glutation Reductase measurement in shoot with nitrogen, phosphorous and magnesium shortages showed no meaningful difference than the control level, while in plants with calcium and potassium shortage showed meaningful decrease (Figure 8).



**Figure 1: Effect of Mineral Macro Element Shortage on Ascorbate Peroxidase Activity in Root**



**Figure 2: Effect of Mineral Macro Element Shortage on Ascorbate Peroxidase Activity in Shoot**



**Figure 3: Effect of Mineral Macro Element Shortage on Peroxidase Activity in Root**

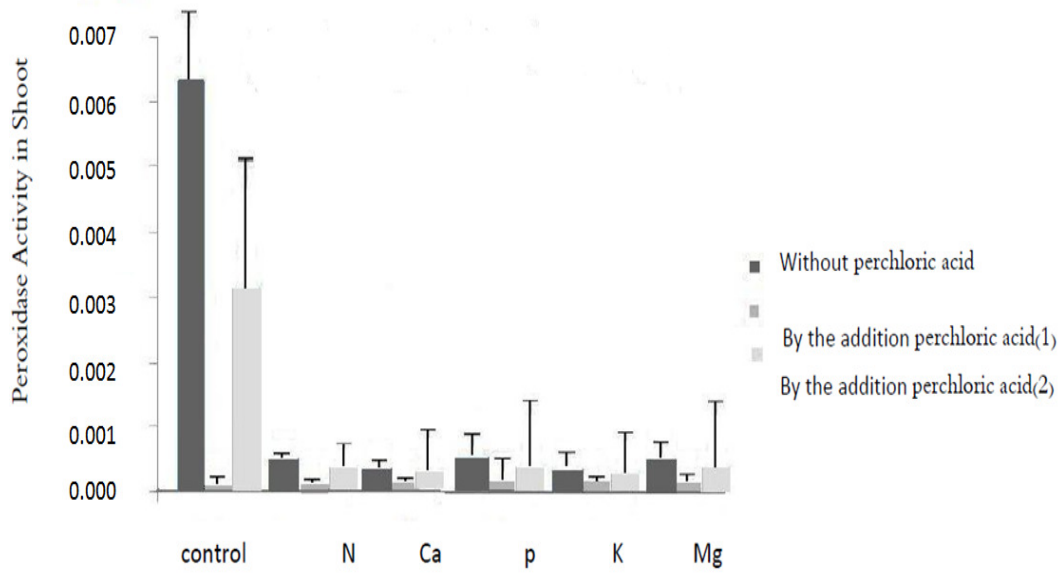


Figure 4: Effect of Mineral Macro Element Shortage on Peroxidase Activity in Shoot

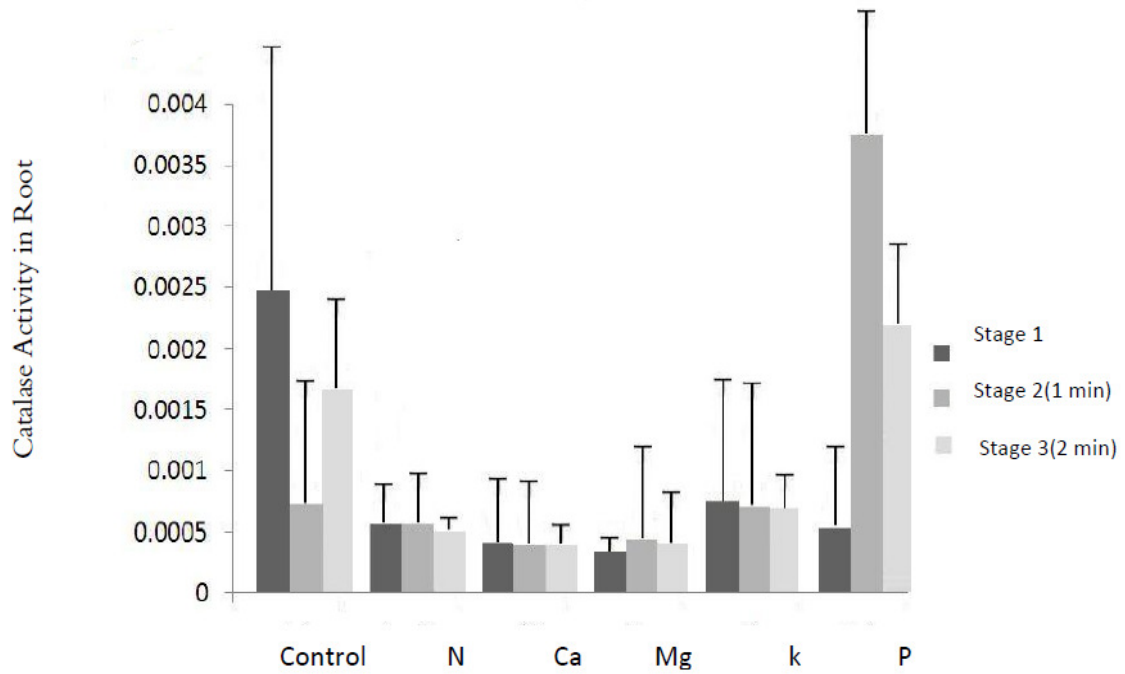


Figure 5: Effect of Mineral Macro Element Shortage on Catalase Activity in Root

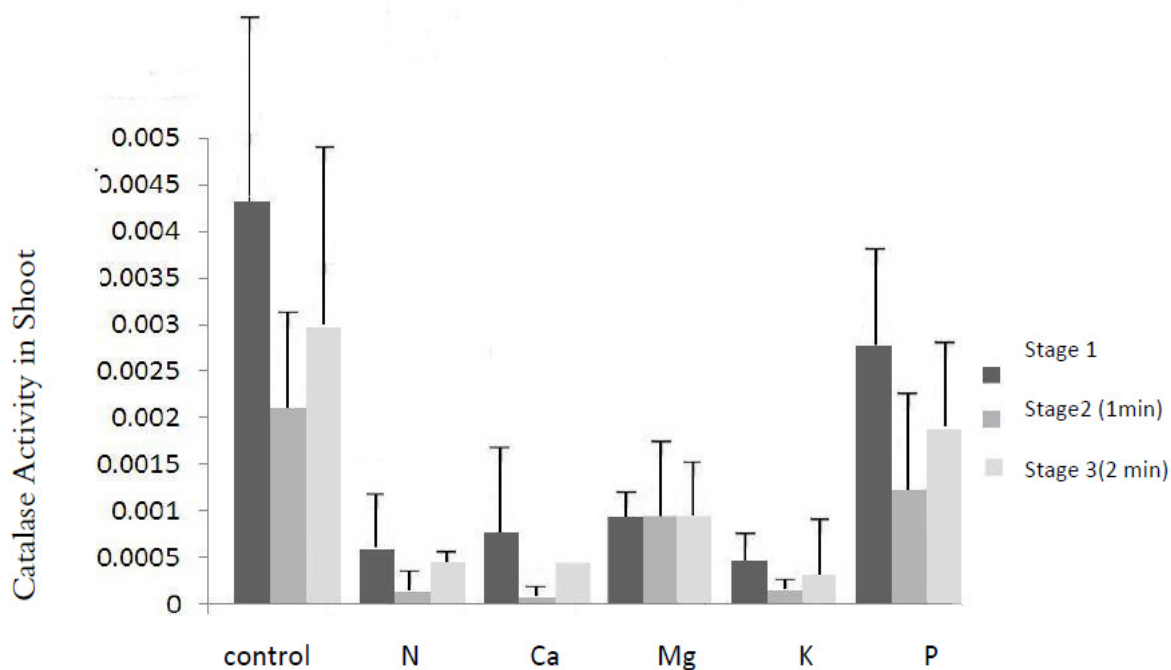


Figure 6: Effect of Mineral Macro Element Shortage on Catalase Activity in Shoot

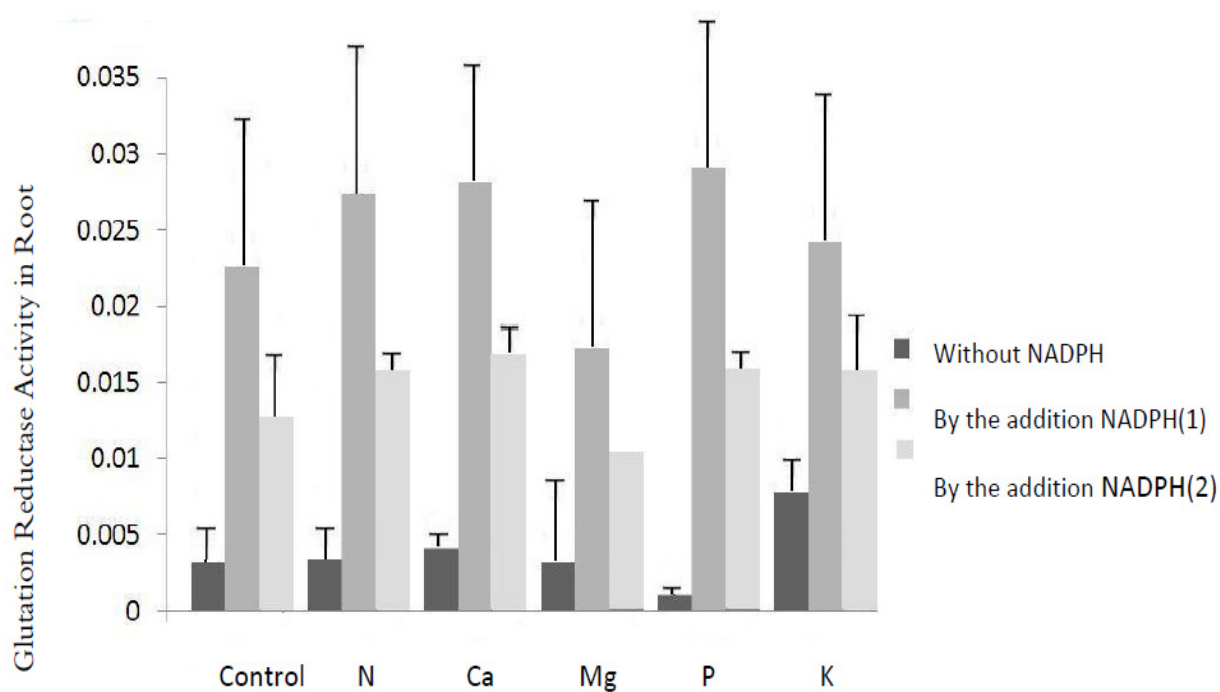
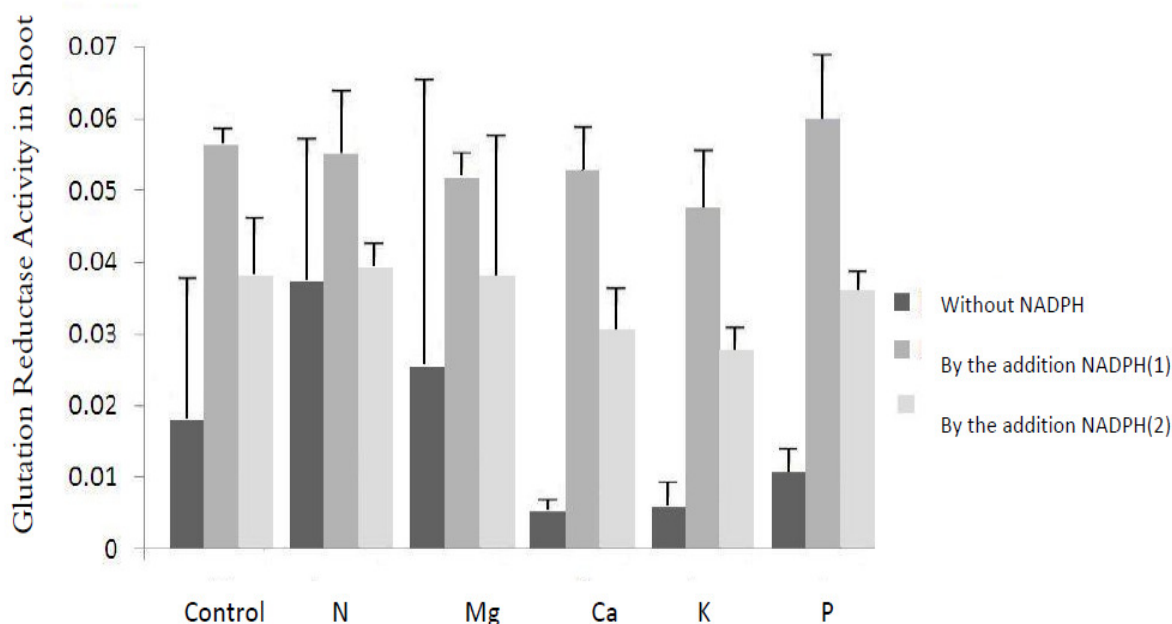


Figure 7: Effect of Mineral Macro Element Shortage on Glutathione Reductase Activity in Root



**Figure 8: Effect of Mineral Macro Element Shortage on Glutathione Reductase Activity in Shoot**

## DISCUSSION

When needing nutrient elements put in suitable content for plants, they performed their physiological and biochemical process properly and so there is no need of defensive mechanisms. In doing so, it adds to their growth and function level (Mohammad and Mozahreh, 2003). Recent studies showed that 60% of agricultural soils as element shortage of mineral element and nutrient element has growth limitation (Cakmak, 2003). Unlike potassium, it takes no part in tissues structures but performed important roles in physiological processes such as photosynthesis, transporting of nutrient compounds to sources, turgor pressure, leakage of stomata, osmotic adjustment, enzymically activation, cell development and membrane polarization (Salardini and Mojtahedi, 1989; Mengel and Kirkby, 2000; Marschner, 1995). Potassium plays a special role under environmental stresses condition for plant survival. In shortage of potassium levels condition, plants sensitive increase to environmental stresses (Cakmak, 2003). In stresses condition, ROS production in plants stimulates severely (Cakmak and Marschner, 2005). In stress time, usually enzymes activity such as Super Oxide Dismutase (SOD), Ascorbate Peroxidase and Glutathione Reductase, stimulate (Mishra et al., 1995). As in this study, we were also face with a significant decrease and increase of enzymes. The studies on Sunflower variety in drought stress effect showed that antioxidant enzymes activity in drought stress condition was increased, but there are no positive relation between production stability and enzymes activation in Sunflower variety (Habibi et al., 2004). According to Gang et al. (2005) experiments, about silicon effects on wheat in drought

stress conditions was studied, they found that in drought stress condition, silicon application caused increment in antioxidant enzymes activity (SOD, CAT and GR). Generally, the important factors that determine the increase of Super Oxide Desmutase (SOD) in plants consist of using herbicides like Paraquat, increase of SO<sub>2</sub> in atmosphere, drought stress condition and high concentration of zinc and magnesium (Ananeiva et al., 2002). Kafi and Mahdavi (1998) reported that increment of Catalase activity has vital role in decrement of Peroxidase effects in plants such as Wheat, Barley, Soybean and Pea in environmental stresses conditions. Nutrient elements consumption is one of the ways in increment of plants function according to soil and water test (Kikha and Fanaie, 2005). Aman et al. (2000) showed that drought stress was caused by increased antioxidant enzymes activity. In live organisms detoxification of H<sub>2</sub>O<sub>2</sub> (Peroxide Hydrogen) are performed by Catalase and Glutathione Peroxidase, and Catalase is used only in Peroxisome and Gelioxysome; in plant cells that are of metal ions as cofactors. Peroxidase and Catalase in several physiological cycles consist of response to environmental stresses that increase mediator production such as H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup> and OH<sup>-</sup> (Paranhos et al., 1999). Catalase and Ascorbate Peroxidase are considered the most important H<sub>2</sub>O<sub>2</sub> or peroxide hydrogen gathering. Decrease of Catalase and Ascorbate Peroxidase activity in Alvand and Zarine variety caused increment of Peroxide hydrogen (H<sub>2</sub>O<sub>2</sub>) concentration, in spite of Haber-Vayz reaction performed which caused a decrease of enzymes activity in Calvin cycle, such as Ribulose Mono Phosphate Kinase and Bi Phosphatase (Asada, 2000). The decrease of these enzymes in Calvin cycle can cause

decrease of NADPH and NADP production and increase of ROS in chloroplast that degradation of biomolecules like lipids. Also enzymes activity such as Fe-SOD and Cu/Zn-SOD are sensitive to high concentration of Peroxide Hydrogen ( $H_2O_2$ ) (Mitler, 2002). Pierce (1986) reported that repress of plant growth and decrement of biomass production to shortage levels different from nutrient elements may contribute to decrease of photosynthesis because in plants with magnesium shortage caused of chloroplast pigment decrement,  $CO_2$  photosynthetic stability enzyme and photosynthetic pathways decrement, and effective on Rubisco Carboxylase enzyme dependent to light phase. Ruffy et al. (1988) reported that shortage of potassium level which caused attaching of  $CO_2$  are delay by decrement opening stomata that cause decrease of plant growth. Marschner (1995) demonstrated that the more repress supply are found in plants growth supplying Ca, S and N shortage level, that probably is fix and plants caused to Ca, S and N shortage level. Increase of POD activity has been observed under S and P shortage levels (Chunhua et al., 1998). In the work of Paranhos et al. (1999), increase of Ascorbate Peroxidase in *Digitalis thapsi* plants with Calcium shortage was showed. While Juszcuk et al. (2001) reported that APX activity increase in crops family with Phosphorous shortage. Also Rajesh (2004) performance work in relation to macro element shortage on Maize plants (*Zea mays* L.) and increment of Ascorbate Peroxidase activity was showed in plants with Potassium, Magnesium and Nitrogen shortage. In this study, Ascorbate Peroxidase and Peroxidase activity decreases in root, and shoot plants and in all the levels of shortages observed. In relation to Catalase activity, in this study Catalase activity in all the levels of shortages was decreasing, except in Phosphorous shortage. Also, in relation with Glutation Reductase activity in all the levels of shortages showed no meaningful difference than the control level.

## ACKNOWLEDGMENTS

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