

Physiological responses and antioxidant enzyme activities in seedlings of *Lepidium sativum* L. under propyl isothiocyanate stress *In vitro*

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Abstract: Edible plant of the Imperial Order Brassicales, Brassicaceae family, genus *Lepidium*, *Sativum* species, scientific name (*Lepidium Sativum* L.). without Chorus annuals and a toothless man power to use it. We have many resources in the glucosinolate Brassicaceae family includes 350 genera and 3000 species alone is found to be. glucosinolate and builder of pre isothiocyanate are very stable, soluble in water, typically in the context of live and fresh isothiocyanate values than those from which they were derived, they appear. They There are many plant families Wallflower. More than 20 kinds of natural and synthetic isothiocyanate derivatives of them. glucosinolate hydrolysis products and as anti oxidants non can be used directly in comments. One of the major tissue damage following exposure to stress in plants is caused by the oxidative stress. In this study effects of different levels of propyl isothiocyanate on biochemical parameters and antioxidant enzyme activity such as ascorbate peroxidase, catalase and guaiacol peroxidase in seedlings of *Lepidium sativum* L. under *in vitro* was investigated. The seeds of *Lepidium sativum* (Garden Cress) were sterilized and cultured in MS medium. After 20 days, seedlings were treated with concentrations of 0.01, 0.1 and 1 mM propyl isothiocyanate (various levels of stress) under sterile condition. After 3 days, result showed chlorophyll a, chlorophyll b, total chlorophyll, carotenoids and protein were significantly decreased, while reduced sugar, proline and antioxidant enzymes were increased in seedlings which have been under oxidative stress. Thus propyl isothiocyanate appears to cause oxidative stress and activation of the plant's defenses parameters. The maximum and minimum respectively at concentrations of 1 mM and 0.01 mM propyl isothiocyanate observed.

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Introduction

Inside hydrolyze glucosinolate cell a spectrum of products are created by myrosinase enzyme (tioglucosiedglucohydrolyze) the most important of which is isothiocyanates. Recently, the biochemical and genetic studies performed on the *Arabidopsis* plant confirm the existence of Amino acid precursor in the glucosinolate biosynthesis pathway. glucosinolate exist in all of the cells (vacuoles) in different densities and in the shoots of all wallflowers and organic anions include D- tioglucose and sulfonatedoxime that form an important and unique group of secondary metabolites in seeds, roots, and the leaves of plants. When glucosinolates and myrosinase enzyme are adjacent (after mechanical injury or ulcer), the enzymes cause the hydrolyze of glucosinolate compounds in the presence of water. Hydrolyze products include aglycon, glucose, and sulfate. The aglycon part is unstable and for the formation of isothiocyanates, thiocyanates, nitrils, ocsasolydintions, epitionitrils are rearranged based on glucosynolats and reaction condition. Isothiocyanate have the functional group of N=C=S. since isothiocyanates react with amino group and sulfodryl

peptides, it is probable that they influence the function of peptides (Yan and Chen, 2007). Most of the natural isothiocyanates in plants are derived from chemical changes caused by glucosinolate. Natural isothiocyanates like alilisoithiocyanates are used as flavors, aroma and fungicides (Yan and Chen, 2007). These compounds have a wide range of environmental activities including anti-oxidant, anti-bacteria, anti- fungal, anti-nematode, and anti-insect activities (Yan and Chen, 2007). Isothiocyanates are highly reactive, so they cause oxidative reactions in plant and produce active oxygen (Yan and Chen, 2007). In fact, these compounds have a double role; in high densities they have poisonous effects on cells (Hara et al., 2010). But in lower densities indirectly cause cell defense. Vitamin c, vitamin E and carotenoids are direct anti-oxidants and they neutralize free radicals before they can damage the cells. glucosinolate and their hydrolyze products are considered as indirect anti-oxidants, because they don't directly neutralize free radicals, they act through regulation of the activities of xenobiotic metabolizing enzymes (phase 1 and phase 2 enzymes which launch delayed anti-oxidant activities). Phase 1

enzymes include cytochrome P450, and the enzymes of phase 2 are glutathione S-transferase, aldehyde dehydrogenase, S-methyltransferase, N-glucosyltransferase). Usually the tensions disturb the cellular electron transfer in different compartments and result in producing active oxygen species (ROS). One of the important tissue injuries that are created by placing plants under tension is the increase of different kinds of reactive oxygen and the creation of oxidative tension. Most of the metabolic processes produce active oxygen species. Herbal cells and their organelles like chloroplast, mitochondria and prokaryotes use anti-oxidant defense system to protect themselves against poisonous oxygen (Corpas et al., 2001). It seems that the ability of organic plants in sweeping radicals of poisonous oxygen is a significant factor for tolerating environmental tensions. Performed experiments in glass condition show that most of the enzymes and secondary compounds protect the plants against oxidative injuries. Reviewing anti-oxidant enzymes and determining the amount of their activities is one of the determining factors in reinforcing antioxidant system and as a result decrease or increase of plant resistance toward environmental tensions (Corpas et al., 2001).

Despite the existence of few reports on isothiocyanate, physiologic mechanism of plant against these compounds is not completely clear (Hara et al., 2010; Khokon et al., 2011). So, in this research the effect of propyl isothiocyanate on physiologic responses and the activities of catalase, ascorbate peroxidase and glutathione peroxidase anti-oxidant enzymes in cress seedlings is studied. Isothiocyanates are among the compounds of wallflower family and have a strong smell and taste. Garden cress is also an edible plant and is called scientifically (*Lepidium sativum* L.). a one-year-old plant that its glabrous and toothless leaves are consumed by humans (Sheel and Agarwal, 2010). Plants utilize several mechanisms to respond to the tensions and regarding the fact that plant mechanism is not clear in response to isothiocyanates, this research is performed to study the effective role of isothiocyanate compounds applied in defense responses through anti-oxidant system of plant and finding the most appropriate density of these compounds.

Materials and Methods

Seeds of cress (*Lepidium sativum* L.) were prepared from pure seed. Seeds with distilled water and then washed with 70% ethanol for 1 min and a solution of 20% sodium hypochlorite for 20 minutes were disinfected. No. 20 seed royal dishes containing culture medium containing MS media seeds were planted and cultivated for 20 days in a room with a

photoperiod of 16 h light and 8 hours dark, the temperature of 25 ± 2 °C humidity 98-95% were. Aqueous emulsion of propylisothiocyanate zero concentration, 0/01, 0/1, and 1 mM were prepared and aseptically (cabinet laminar) using a sterile syringe treated seedlings were planted into containers. Plants treated with isothiocyanate then cultured for 3 days at room conditions were maintained. After 3 days, seedlings were frozen in liquid nitrogen and freeze at -80 °C and were used to measure physiological and biochemical parameters.

Measurement of chlorophyll and carotenoids:

This measure is based on Lichtenthaler, 1987 took place. This method is based on a 0/05 g fresh weight of leaves and porcelain mortar containing 5 mL of acetone was 80% worn. Glass funnel with filter paper that was placed in a mortar content was smooth. Then, 10 mL of acetone were added. Volume of the solution was brought to 15 ml. This solution contains chlorophyll a, b and carotenoids. 3 ml of the sample extract was poured in absorption intensity at wavelengths of 646, 663, and 470 nm was read using a spectrophotometer. The results of measurements of photosynthetic pigments in terms of mg/g wet weight was calculated and presented.

Measurement of anthocyanin:

This measure is based on Wagner, 1979 took place. In the first, 0/05 g leaves with weight scales, then the 2/5 ml of acidified methanol (99 ml of methanol and 1 ml of hydrochloric acid) was worn. The total volume of the extract in a measuring cylinder 2/5 ml was reached. test tube and poured the juice for 24 hours in the dark and the temperature was 25 °C for 10 min and then centrifuged at 4,000 rpm speed, using atomic absorption spectrophotometer at a wavelength of 550 nm was read. To calculate concentration, molar extinction coefficient of 33,000 cm was considered and the results were calculated in terms of micromoles per gram wet weight.

Measurement of proline: content in leaves and roots of proline by Bate et al (1973) were measured. According to this method, 0/02 g of fresh leaf and root tissues were weighed and separated from each sample with 10 ml of 3 % in sulfosalicylic acid were abraded porcelain mortar. After centrifugation and addition of reagents ninhydrin and pure acetic acid supernatant, the samples were placed in a water bath for one hour. With the addition of toluene and separation of supernatant samples, the intensity of absorbance at a wavelength of 520 nm was read. Using the standard curve, the concentration of proline in terms of mg per g wet weight was calculated.

Measuring the amount of reducing sugars:

0.05 g of fresh leaves and roots were weighed and tissue samples were abraded with 10 ml of distilled water in a porcelain mortar. Reducing sugars in leaves and roots by Somogyi-Nelson (1952) were measured. Strong absorbance at 600 nm was read using a standard curve of soluble sugars concentration in mg per g wet weight was calculated.

Preparation of plant extracts for measuring enzyme:

The 10 g of shoots were weighed and placed into a mortar and 5 ml of phosphate-buffered saline containing polyvinyl pyrrolidone (PVP) was added. The pH of the buffer used in the 4/7, respectively. The tissue was pulverized in a mortar completely homogeneous solution was obtained, such that extract tissue samples were homogenized and stored at low temperature were performed on ice. Extract tissue samples at 10,000 rpm and 4 °C were centrifuged for 10 min. ascorbate peroxidase enzyme activity: APX enzyme activity according to the method of Nakano and Asada (1987) were measured. APX enzyme reaction mixture containing 625 ml phosphate buffer 0.2 mM EDTA, 175 ml of ascorbic acid, 50 ml and 50 ml of hydrogen peroxide was formed by bovine serum albumin. To measure the enzyme activity of APX, the reaction solution of 900 ml and 100 ml of the enzyme extract were mixed and reduced absorption at a wavelength of 290 nm curves were plotted for 60 seconds. One unit of enzyme activity is rapidly oxidized by APX $\mu\text{mol min}^{-1} \text{ } ^\circ\text{C}^{-1}$ was expressed as ascorbic acid at room temperature. This procedure was repeated three times and the average APX activity in each sample was calculated based on three times. The activity of ascorbate peroxidase using the molar extinction coefficient of 2.8 $\text{mM}^{-1}\text{cm}^{-1}$, respectively.

Measurement of catalase activity:

Catalase activity measured according to the method described by Aebi (1984) was performed. The reaction mixture containing 5 mM H₂O₂ concentration of 50 mM phosphate buffer with a pH of 7 was obtained. 150 μl and 1350 μl of enzyme extract was combined with the reaction mixture. Starting in H₂O₂ decomposition reaction began decreasing absorption at a wavelength of 240 nm over time was measured in a spectrophotometer. The activity of catalase using the molar extinction coefficient $\text{mM}^{-1}\text{cm}^{-1}$ 0.39/0, respectively.

Guaiacol peroxidase enzyme activity were measured: the size of the reaction mixture guaiacol peroxidase enzyme activity in accordance with the

method of Lin and Kao (1999) containing 50 mM phosphate buffer, pH =7 Guaiacol 9 mM NaCl and 19 mM hydrogen peroxide M is. Absorption at a wavelength of 470 nm was measured over 1 min size. Extinction coefficient, 6/26 mM cm^{-1} was considered. One unit of enzyme activity Guaiacol peroxidase as the enzyme in the formation of the min 1 mM Tetraguaiacol be defined.

Total protein concentration:

Protein was measured according to the method of Bradford (1976) was little changed. Extracts absorption wavelength nm 595 was read with a spectrophotometer UV-mini 1240 model Shimadzu. The amount of total soluble protein in terms of mg / g FW respectively.

Statistical Analysis

The experimental design used in a completely randomized design with three replicates per treatment was performed. Data analysis was performed using SPSS software. Duncan test was used to compare mean differences between treatments at $p \leq 0.05$ levels, respectively.

Results

Chlorophyll a, b, total chlorophyll and carotenoids: chlorophyll content of the seedlings in a, b and total carotenoids and stressed propylisothiocyanate significantly reduced. Concentrations of 1 and 0/1 and 0/01 mM methyl isothiocyanate decreased chlorophyll a, respectively 72, 55 and 51% compared with control (Figure a 1). propyl isothiocyanate at a concentration of 1 mM reduced chlorophyll b to 40% compared to controls, and the concentration of 0/1 and 0/01 respectively decreased 45 and 42 percent of chlorophyll b compared with controls (Fig. b 1). The amount of total chlorophyll concentration of 1 \cdot 0/1 and 0/01 mM propylisothiocyanate than control seedlings read 62, 51 and 40 % reduction (Figure c 1). propylisothiocyanate treatment resulted in a significant reduction in the amount of carotenoids. concentrations of 1, 0/1 and 0/01 mM isothiocyanate reduce carotenoid levels, respectively 82, 60 and 52% compared to controls was observed (Fig. d 1). Anthocyanin in leaf anthocyanin content was observed that the effect propylisothiocyanate control over grown seedlings. propylisothiocyanate at concentrations of 1 and 0/1 mM anthocyanin leaf in the order of 54/1 and 24/1 to comparable control has increased the anthocyanin concentration of 01/0 mM significantly different from controls did not (Fig. 2).

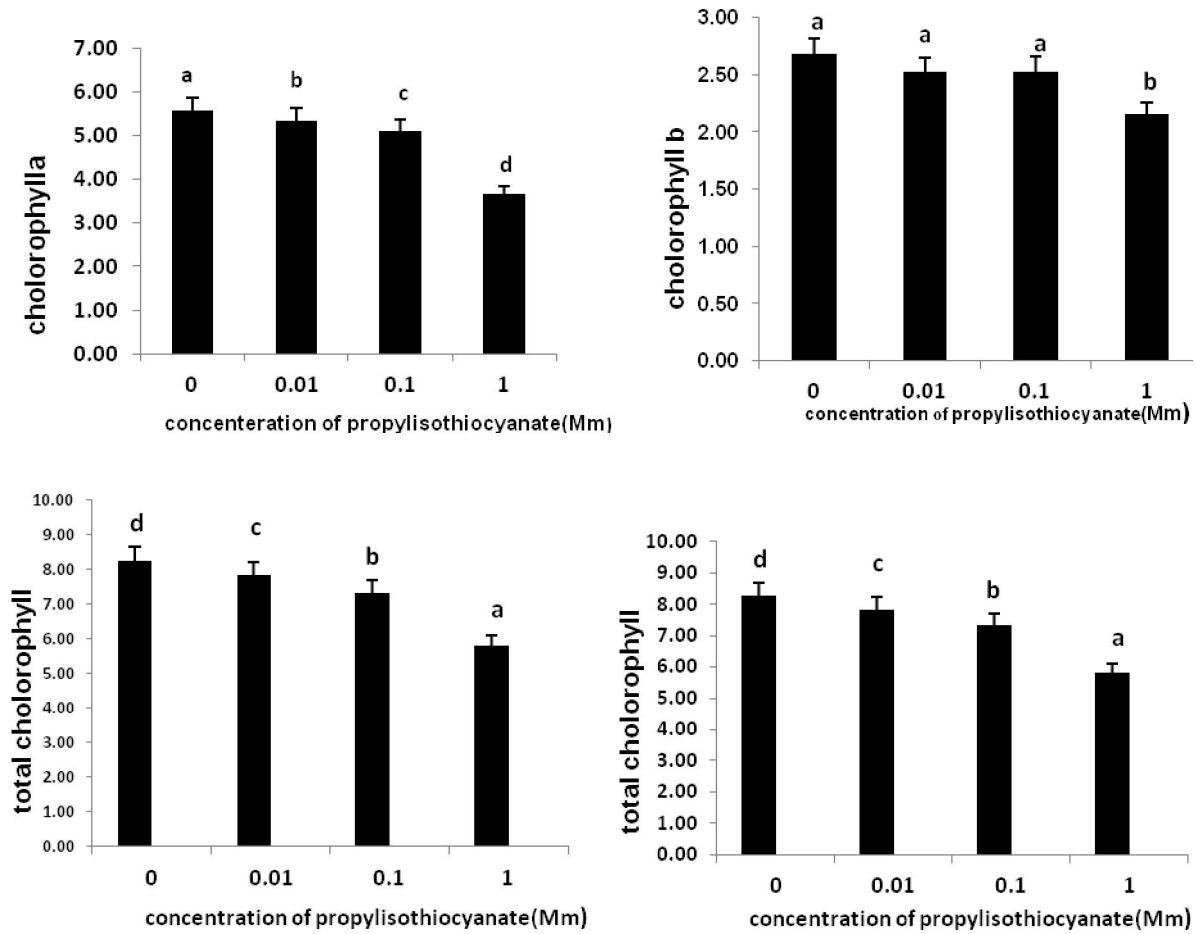


Figure 1. Propylisothiocyanate impact on the amount of chlorophyll a (a), chlorophyll b (b), total chlorophyll (c) and carotenoids (d). Values represent the mean of three replicates and dissimilar letters are significantly different according to Duncan's test. ($P \leq 0.05$).

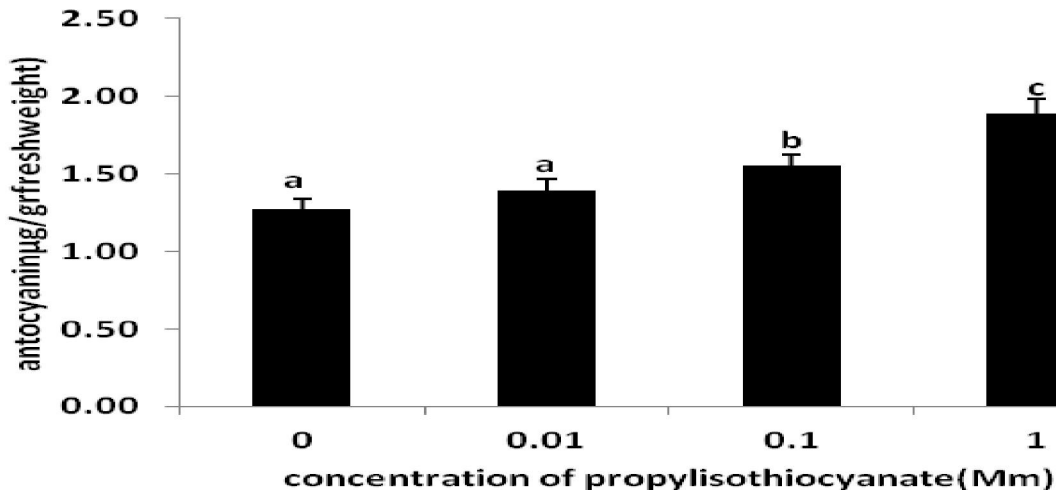


Fig 2. Propylisothiocyanate impact on anthocyanin in *Lepidium sativum*. Values represent the mean of three replicates and dissimilar letters are significantly different according to Duncan's test. ($P \leq 0.05$).

The amount of reducing sugars: sugars in the roots at concentrations of 1, 0/1 and 0/01 mM respectively propylisothiocyanate 78/1, 77/1 and 46/1 to have increased significantly compared to controls. propylisothiocyanate at concentrations of 1, 0/1 and

0/01 mM caused a significant increase in sugars respectively 4/4, 8/3 and 2/3 times compared with the shoot. The highest increase in the concentration of sugars in the lowest concentration of 1 mM and 0/01 mM was observed (Fig. 3).

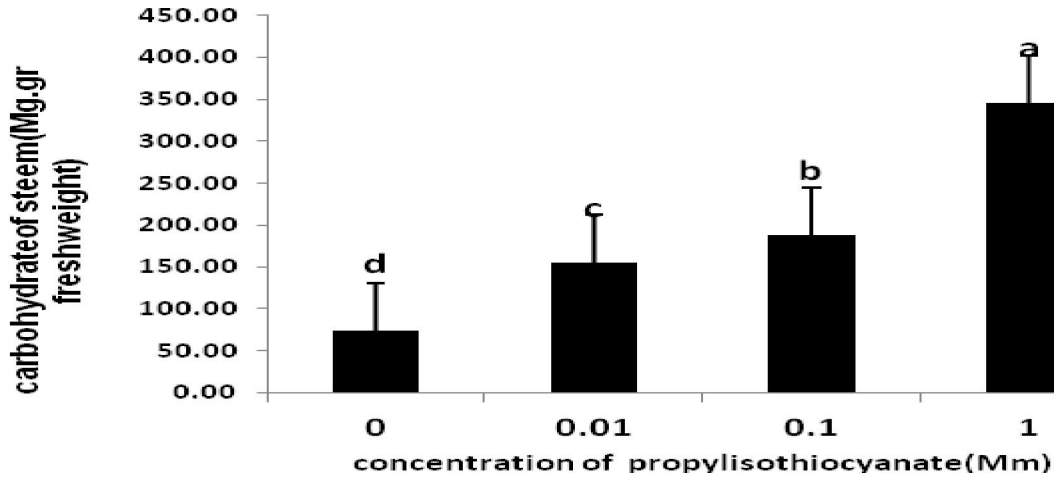


Figure 3. Proline root and shoot: root propylisothiocyanate concentration of 1 mM proline increased compared to control. Significant differences between treatments in root proline 0/1 and 0/01 mM and seedlings were observed. propylisothiocyanate concentrations of 1 and 0/01 mM increased leaf proline respectively 6/1, 3/1 as compared to control. Significant differences between treatments in leaf proline 0/01 mM and seedlings were observed (Figure 4).

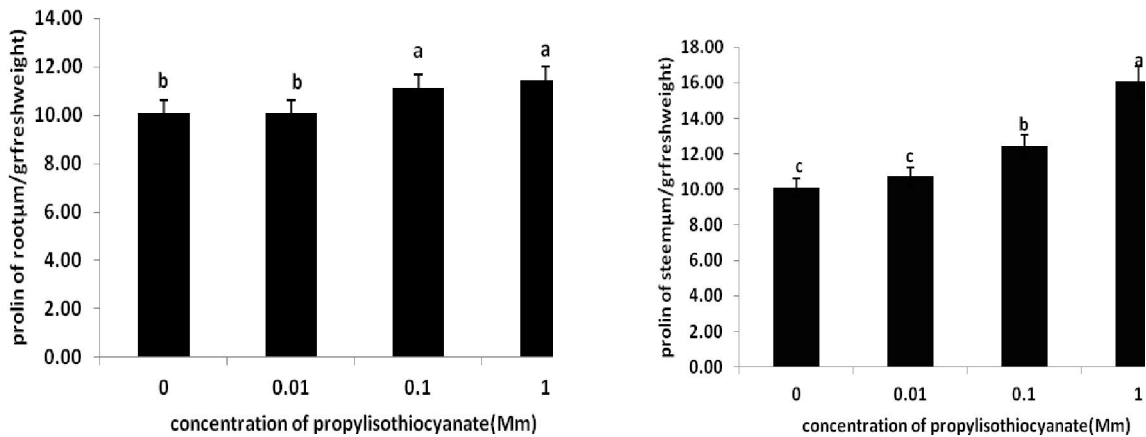


Figure 4. propylisothiocyanate effect on root proline (a) and shoots (b) of cress seedlings. Values represent the mean of three replicates and dissimilar letters are significantly different according to Duncan's test. ($P \leq 0.05$)

Antioxidant enzyme activities in shoots : The activity of each enzyme treated propylisothiocyanate increased. propylisothiocyanate in effect, a significant increase in enzyme activity at concentrations catalase 0/1 and 0/01 mM isothiocyanate compared to controls was observed. The lowest concentration of 1 mM and the concentration of 0/01 mM catalase activity was greatest. catalase enzyme activity at concentrations below 0/01 and 0/1 and 2 mM isothiocyanate the approximately 7 fold increase compared to control

(Figure a 5). Concentration of 0/01, 0/1, and 1 mM methyl isothiocyanate, activity level Skvrbatpraksydaz respectively 6/5, 4/2 and 8/1 against the seedling controls increased significantly (Figure b 5). Treatment concentrations of methyl isothiocyanate 0/01, 0/1 and 1 mM, enzyme activity in leaves guaiacoleperoxidase respectively 2/6, 4 and 3-fold increased compared with control seedlings. At a concentration of 0/01 mM Maximum enzyme activity was observed (Fig. c 5).

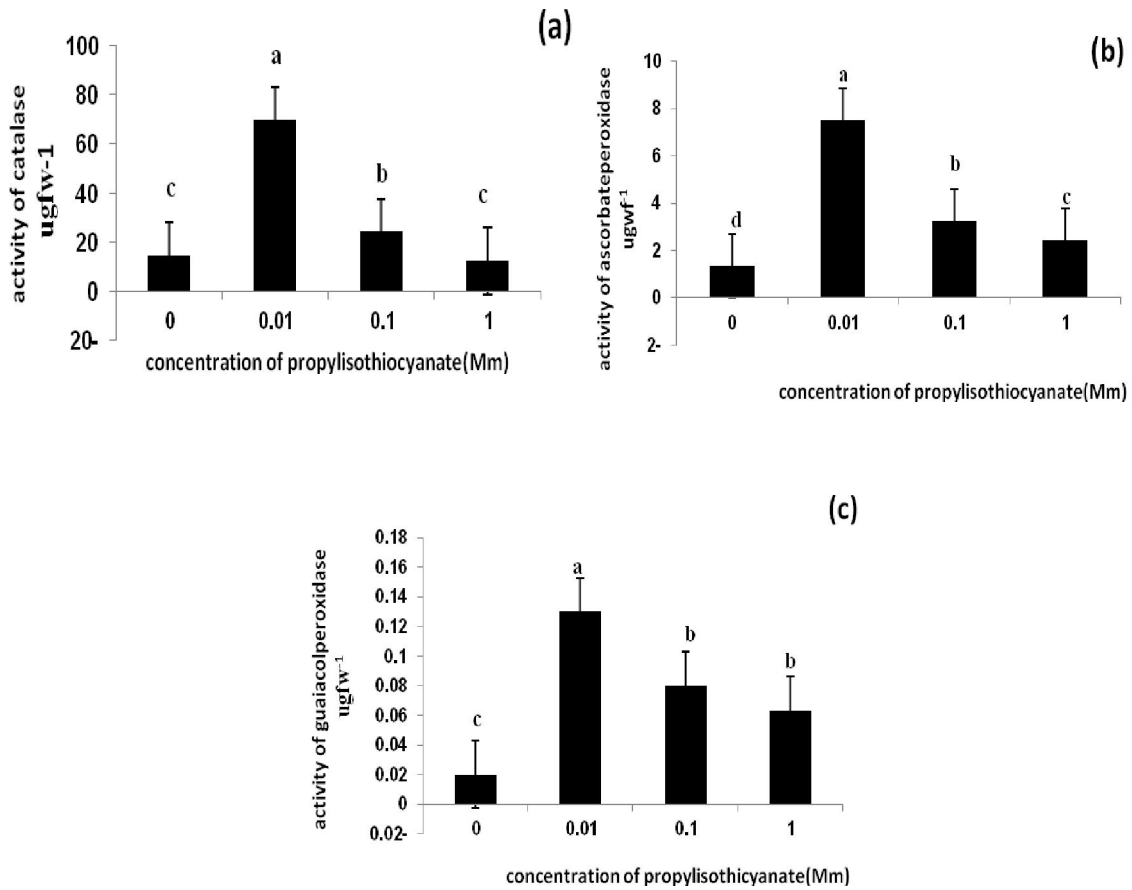


Figure 5. The amount of protein in shoot protein in seedling of propylisothiocyanate stressed than the control group decreased propylisothiocyanate at concentrations of 1, 0/1 and 0/01 mM, reduced protein levels, respectively 40, 20 and 10% compared to control.

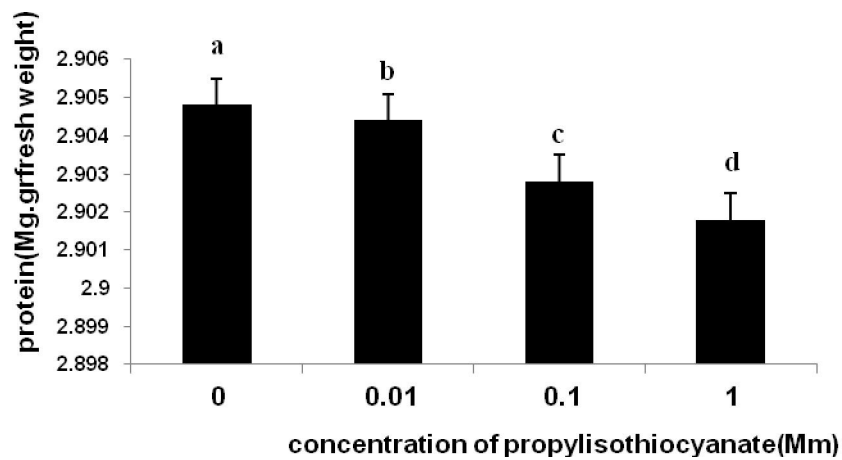


Figure 6. Propylisothiocyanate effect on total protein content in watercress leaf. Values represent the mean of three replicates and dissimilar letters are significantly different according to Duncan's test. ($P \leq 0.05$)

Discussion

The findings of this research indicate that propylisocyanine tension induces physiologic responses and activates antioxidant enzymes in cress seedlings. The amount of chlorophyll in plants is often estimated to evaluate the effects of environmental tensions. These tensions may stop metabolic processes by preventing enzyme activity. The reduction of chlorophyll in plants under tension is probably either because of controlling chlorophyll synthesis enzymes activity or the increasing of chlorophyll pigment disintegration (Sairamet *et al.*, 1998). Propyl isocyanine treatment in 1, 0/1, 0/01 mm density results in total amount of chlorophyll decrease related to the cress seedlings. Hara *et al.* (2010) also reported that propyl isocyanine treatment causes chlorophyll reduction (about 50%) in five-week-old seedlings of *Arabidopsis* shoot (Hara *et al.*, 2010). Furthermore, in the present study methyl isocyanine has decreased the amount of carotenoids in leaf rather than the control seedlings. The free radicals produced during tension cause photosynthesis and non-photosynthesis pigment disintegration as a result of which the pigments are decreased (Sairamet *et al.*, 1998). Carotenoids cause the protection of photosynthesis system against extra photons and oxidative tension such as reaction to chlorophyll to prevent the formation of active radicals of oxygen. In fact, carotenoids as a protective system against induced oxidative tension are disintegrated and destroyed. The photochemical suppression of induced chlorophylls by carotenoids results in the disruption of carotenoid structure and finally reduction in their amount (Sanita and Gabbrielli, 1999). So it is possible that the reduction of carotenoid amount under propyl isocyanine treatment is caused by the role of these pigments in protection of leaf chlorophyll against oxidative tension caused by isothiocyanates.

In this research, it is found that propyl isothiocyanate treatment has increased the amount of anthocyanin in cress seedling leaf compared to the control seedlings. Flavonoids are polyphonic compounds and are among the most important secondary components of plants. These components are derivatives of phenylpropanoid. Anthocyanin as a group of soluble Flavonoids is synthesized in the course of flavonoid biosynthesis at the end point (Mars *et al.*, 1995). By creating oxidative tension in plant, antioxidant genes expression (Vinyard *et al.*, 2005) and induction of phenyl propanoid course especially Flavonoid biosynthesis are increased (1995 Green and Fluhr.). In this study, a significant difference in amount of anthocyanin is not observed in methyl isothiocyanate and the control with the density of 0/01 mm, but in the density of 1 and

0/1 mm the amount of anthocyanin is increased by 58 and 25 percent respectively. The same result is reported about the increase of flavonoids during environmental tension (Murali and Teramura, 1986). In this research, it seems that propyl isothiocyanate treatment causes removing of free radicals of oxygen and also plant adaptation to tensioned condition by increasing anthocyanin and Flavonoids. In a research, Connor and *et al.* (2002) reported that antioxidant and anthocyanin enzyme activities in blueberries are powerfully in contact with each other and are increased in cold weather. Solecka and *et al.* (1999) reviewed the effect of cold tension (°C5-) on the amount of anthocyanin in cabbage. Their results showed the increase of anthocyanin compared to control seedlings. The amount of carbohydrate and proline in the seedling root and shoot under the tension of isothiocyanate showed a significant increase compared to the control plants. To retain ion balance and osmotic regulation in vacuoles and cytoplasm, the plants gather low molecular mass such as proline, glycine, betaine and sugars like glucose and fructose that are collectively called osmolyte (Parida *et al.*, 2002). Furthermore, it seems that soluble sugars play an important role in relation with reactive oxygen species. Sugars are also needed for performing anti-oxidative processes like pentose phosphate pathway (Barra *et al.*, 2003; Debnam *et al.*, 2004) and carotenoid biosynthesis. The reduction of saved starch and the increase of sugars are reported under tensioned situations (Parida *et al.*, 2002). Analysis of gene transcription has verified that sugar messaging is associated with the oxidative tension control. Different tensions like salinity, drought, low temperature and heavy metal toxicity that directly or indirectly cause the accumulation of reactive oxygen genes lead to the accumulation of soluble sugars that act as an adaptive mechanism to tension condition (Roitsch and Ehneß, 2000). In the present study, it seems that the increase of carbohydrate is because of dealing with oxygen radicals generated under isothiocyanate tension. Proline plays an important role in tolerating plant tension like anti-oxidative activity of proline. Proline can remove unique oxygen and the devastations originating from free radicals and it also can play a role in protecting proteins against denaturation (Alia and Mohanty, 1991). The results gained from measuring anti-oxidant enzyme activity under isothiocyanate tension show that methyl isothiocyanate causes an increase in the activity of catalase enzyme and as a result the toleration of tension becomes more. The highest amount of catalase activity was observed in the density of 0/01 mm of propyl isothiocyanate. The balance between producing reactive oxygen species and anti-oxidant enzyme activity determines the way that oxidative signals

occur (Moller *et al.*, 2007). Induction of anti-oxidant enzyme activity of a mechanism is the general adaptation of a plant against oxidative tensions (Foyer and Noctor, 2005). Catalase is the most important enzyme for removing oxygen peroxide and it happens by dividing it into water and oxygen. Induction of catalase activity results in overcoming oxidative tension through hydrogen peroxide detoxification (Mazhoudiet *al.*, 2002). In high densities of propyl isothiocyanate, catalase enzyme may break by proteases induced by oxidative tension that is reported in old pea leaves (Sandalio *et al.*, 2001). Decrease in anti-oxidant enzyme activities in high levels of tension, can be caused through enzyme molecular breakage by free radicals of oxygen (Sandalio *et al.*, 2001). Based on the results of this research, propyl isothiocyanate has also increased the activity of ascorbate peroxidase enzyme compared to the control seedlings in shoots. The highest activity of this enzyme is observed in 0/01 mm density of propyl isothiocyanate. The increase of ascorbate peroxidase activity by non-environmental reasons can be a sign of the beginning of anti-oxidant defense. Ascorbate peroxidase enzyme has participated in ascorbate-glutathione cycle and so has caused the removal of hydrogen peroxide. In a study, Wang and et al (2010) showed that allyl isothiocyanate treatment at first causes the increase and then the decrease of enzyme activity of ascorbate peroxidase in blueberries. Enzyme activity of guaiacol peroxidase has shown increase depending on the different densities of isothiocyanate. Guaiacol peroxidase enzyme catalyzes the oxidant related to the hydrogen peroxide. It also provokes lignin biosynthesis and creates physical barrier against the tensions and as a result the tissues which damage the cells are invigorated against the free active radicals (Fang and Kao, 2000). In the present research, the decrease in the activities of these enzymes in high densities of propyl isothiocyanate may be because of the controlling of these enzymes or deactivating of them in a way that in high levels of tension it has an opposite effect on the activities of defense enzymes and a decrease may be observed in the activities of anti-oxidant enzymes. The other results of this research include reduction of the amount of soluble proteins in plants under isothiocyanate tension. One of the reasons of protein decrease in this level of tension might be the increase in producing free radicals of oxygen because these compounds control protein synthesis (Sgherri and Navari-Izzo, 1995). We also can point out photosynthesis decrease and the reduction of required materials for protein synthesis under tension conditions (Havaux *et al.*, 1987).

Conclusion

The results acquired from this research indicate that oxidative tension originated by medium densities treatment of propyl isothiocyanate induces physiologic responses and activates antioxidant defense enzymes in cress seedlings in a glass and it can be important in plant adapt and cope against tension condition. Probably, propyl isothiocyanate in cress seedlings mediates the responses related to defense system of anti-oxidant.

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