Effect of Propyl Isothiocyanate on Antioxidant Enzymes of Garden Cress Seedlings Under in vitro Condition

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ABSTRACT

In this study effects of different levels of propyl isothiocyanate on some physiological parameters and antioxidant enzyme activities 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 soluble proteins were significantly decreased, while reduced carbohydrates, proline and antioxidant enzymes activities increased in seedlings which have been under oxidative stress. One of the major tissue damage following exposure to stress in plants is caused by the oxidative stress. Thus propyl isothiocyanate appears to cause oxidative stress and activation of the plant’s defenses parameters.

INTRODUCTION

Inside the cell hydrolysis of glucosinolate by myrosinase enzyme (thioglucoside glucohydrolase) produces a spectrum of products which the most important of them is isothiocyanate. 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 members of Brassicaceae family. Glucosinolates are organic anions include D- thioglucose and sulfonatedoxime that form an important and unique group of secondary metabolites in seeds, roots, and the leaves of plants. When glucosinolates are adjacent myrosinase enzyme (after mechanical injury or ulcer), the enzymes cause the hydrolysis of glucosinolate compounds in the presence of water. Hydrolysis products include aglycone, glucose, and sulfate. The aglycone part is unstable and for the formation of isothiocyanate, thiocyanates, nitrils, ocsasolydintions, epinitrils are rearranged based on reaction glucosinolates. Isothiocyanate have the functional group of N=C=S. Since isothiocyanates react with amino group and sulphydryl peptideid, it is probable that they influence the function of peptides [34]. Most of the natural isothiocyanate in plants are derived from chemical changes caused by glucosinolate. Natural isothiocyanate like allyl isothiocyanates are used as flavors, aroma and fungicides [34]. These compounds have a wide range of environmental activities including anti-oxidant, anti-bacteria, anti- fungal, anti-nematode, and anti-insect activities [34]. Isothiocyanates are highly reactive, so they cause oxidative reactions in plant and produce active oxygen [34]. In fact, these compounds have a double role; in high densities they have poisonous effects on cells [12]. 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 hydrolysis 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 reductase, S-methyl transferase, N-acetyl transferase). Usually stresses disturb the cellular electron transfer in different compartments and result in producing reactive oxygen species (ROS). One of the important tissue injuries that are created by placing plants under stress is the increase of different kinds of reactive oxygen and the creation of oxidative stress. Most of the metabolic processes produce reactive oxygen species. Plant cells and their organelles like chloroplast, mitochondria and peroxisome use anti-oxidant defense

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system to protect themselves against poisonous oxygen [7]. It seems that the ability of high plants in scavenging radicals of poisonous oxygen is a significant factor to tolerate environmental stresses. Performed experiments under in vitro condition show that most of the enzymes and secondary compounds protect the plants against oxidative injuries. Study of 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 to environmental stresses [7]. Despite the existence of few reports on isothiocyanate, physiological mechanism of plant against these compounds is not completely clear [12,14]. So, in this research the effect of propyl isothiocyanate on physiological responses and the activities of catalase, ascorbate peroxidase and guaiacol peroxidase in cress seedlings were studied. Isothiocyanate are among the compounds of Brassicaceae 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 [28]. Plants utilize several mechanisms to respond the stresses and regarding the fact that plant mechanism is not clear in response to isothiocyanate, this research is performed to study the effective role of isothiocyanate compounds in defense responses through induction of antioxidant system of plant and finding the most appropriate density of these compounds.

MATERIALS AND METHODS

The mature and sterilized seeds of cress (Lepidium sativum L.) were grown on MS medium [15]. All cultures then were kept in the culture room with a 16/8- h light/dark photoperiod at 25 ± 2 ºC for 20 days. Aqueous emulsion of propyl isothiocyanate at concentrations: 0, 0.01, 0.1, and 1 mM were prepared and aseptically (cabinet laminar) using a sterile syringe was treated on seedlings in containers. Plants treated with isothiocyanate then cultured for 3 days at the culture room. After 3 days, seedlings were frozen in liquid nitrogen and freeze at - 80 ºC and were used to measure physiological and biochemical parameters.

Measurement of chlorophyll and carotenoids:

Measurement of chlorophyll and carotenoids was determined according to [15]. 0.05 g of frozen leaf was homogenized in 10 ml acetone 80%. This solution contains chlorophyll a, b and carotenoids. The absorbance of each sample was read at 646.8, 663.20 and 470 nm. The amount of chlorophylls and carotenoids was expressed as mg g-1 FW.

Measurement of anthocyanin:

Measurement of total anthocyanin was determined according to modified [33]. method using acidified ethanol (Ethanol: HCl 99: 1 v/v). 0.05 g of frozen leaf was homogenized in 2.5 ml acidified ethanol and then kept at 25°C for 24 h in the dark. The extract was centrifuged at 4000 g for 10 min at room temperature. The absorbance of each supernatant was read at 550 nm. The extinction coefficient 33,000 (mol-1 cm-1) was used to calculate the amount of total anthocyanin and it was expressed as μ mol g-1 FW.

Measurement of proline:

Proline content was estimated using ninhydrin reaction [4]. A small portion (0.5 g) of leaves or roots was homogenized with 10 ml of 3% (w/v) sulphosalicylic acid, and passed through Whatman filter paper no. 2. Then ninhydrin reagent (2 ml) (Sigma) and glacial acetic acid (2 ml) were added to 2 ml of the filtered extract. The mixture was incubated at 100°C for 1 h, and the reaction was terminated by placing it on ice. The reaction mixture was extracted with 4 ml toluene, and absorption of chromophore was measured at 520 nm, against toluene as blank, using spectrophotometer (Shimadzu UV-160, Japan). Proline content was calculated using L-proline (Sigma) as a standard curve.

Measuring the amount of reducing carbohydrates:

Reducing carbohydrates content was measured by adapting Somogyi-Nelson’s method [30]. Approximately 0.05 g of fresh leaves and roots were extracted with 10 ml distilled water. The mixture was boiled in a boiling water bath, cooled and filtered. Then 2 ml of the extract was mixed with 2 ml of alkaline copper tartarate and the reaction mixture was heated for 20 min (Alkaline copper tartarate was prepared by dissolving 4 g anhydrous sodium carbonate, 0.75 g tartaric acid and 0.45 g hydrated cupric sulphate in 80 ml of distilled water and finally made up to 100 ml). Two ml of phosphomolibdate solution was added and the intensity of blue color was measured at 600 nm using spectrophotometer. D-glucose was used as standard. The reducing sugar content was expressed as mg/g FW.

Enzyme extraction and assay:

For enzyme extraction 0.1 g of shoot was homogenized using a mortar and pestle with 1 ml of 100 mM sodium phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 1% polyvinylpyrrolidone (PVP). The whole
extraction procedure was carried out on ice. The homogenates were then centrifuged for 30 min at 14000 rpm at 4°C and supernatants were used for protein and enzyme activity measurement.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to the method of Nakano and Asada [21]. The reaction buffer for APX activity contained 50 mM sodium phosphate buffer (pH 7), 0.5 mM ascorbic acid, 0.1 mM EDTA, 1.25 mM H2O2 and 0.05 ml enzyme extract in a final volume of 1 ml. Ascorbate oxidation was measured at 290 nm for 1 min with extinction coefficient of 2.8 mM-1 cm-1.

Catalase (CAT, EC 1.11.1.6) activity assay was also carried out according to the method of Aebi (1984) [1]. The decrease in H2O2 was measured at 240 nm and activity was calculated as H2O2 μM consumed per minute (extinction coefficient 39.4 mM-1 cm-1).

Guaiacol peroxidase enzyme activity was determined according to Lin and kao [16]. The reaction buffer containing 50 mM phosphate buffer (pH =7), Guaiacol 9 mM and 19 mM hydrogen peroxide. Absorption at wavelength of 470 nm was measured for 1 min with extinction coefficient of 6.26 mM-1 cm-1.

Determination of protein content:
The amount of total soluble protein of shoot was measured according to [5]. Absorption intensity of extractions was determined in wave length 595 nm and the results were reported according to mg/g FW.

Statistical Analysis:
All experiments were carried out in three replications and mean values ± standard deviation were presented. Data were subjected to ANOVA using the statistical package SPSS and the mean differences were compared by Duncan test at p < 0.05.

Results:

Chlorophyll a, b, total chlorophyll and carotenoids:
The amount of chlorophyll a, b and total chlorophyll and carotenoids significantly reduced under propyl isothiocyanate stress. Concentrations of 1 and 0.1 and 0.01 mM propyl isothiocyanate decreased chlorophyll a, respectively 72, 55 and 51% compared with control (Figure a1). Propyl isothiocyanate at 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. b1). The amount of total chlorophyll at concentration of 1, 0.1 and 0.01 mM propyl isothiocyanate than control seedlings respectively 62, 51 and 40% decreased (Figure c 1). Propyl isothiocyanate treatment caused significant reduction in the amount of carotenoids (Fig. d 1).

Anthocyanin content:
Anthocyanin content of leaf increased under propylisothiocyanate stress compared with control. Propylisothiocyanate at concentrations of 1 and 0.1 mM has increased anthocyanin content of leaf 54.1 and 24.1 compared to control. Concentration of 0.01 mM showed no significant differences.

The amount of reducing carbohydrates:
Propyliothiocyanate 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 control in the shoot. Sugars in the roots at concentrations of 1, 0.1 and 0.01 mM propylisothiocyanate increased significantly respectively 78.1, 77.1 and 46.1 percent compared to controls. The highest amount of sugar in 1 mM and the lowest amount of sugar at 0.01 mM propylisothiocyanate was observed.

Proline content of root and shoot:
Proline of roots increased compared to control at 1 mM propyliothiocyanate. Also, Proline of shoot increased compared to control. Significant differences between treatments in root proline at 0.1 and 0.01 mM was observed. Propyliothiocyanate at concentrations of 1 and 0.1 mM increased leaf proline respectively 6.1, 3.1 percent as compared to control (Figure 4).

Antioxidant enzymes activities in shoots:
The activity of all enzymes increased under propylisothiocyanate stress. Significant increase in catalase activity at concentrations 0.1 and 0.01 mM isothiocyanate compared to controls was observed. The lowest catalase activity at 1 mM and the highest catalase activity at 0.01 mM was obtained (Figure a 5). Ascorbat peroxidase activity at concentration of 0.01, 0.1, and 1 mM propyl isothiocyanate, respectively, 6/5, 4/2 and 8/1 fold against the controls increased (Figure b 5). At 0.01, 0.1 and 1 mM propylisothiocyanate, activity of guiacolperoxidase in leaves, respectively 2.6 , 4 and 3-fold increased compared with the control seedlings. At a concentration of 0.01 mM, maximum enzyme activity was observed (Fig. c 5).
Fig. 1: Effect of Propyl isothiocyanate on the amount of chlorophyll a (a), chlorophyll b (b), total chlorophyll (c) and carotenoids of Lepidium sativum (d). Values represent the mean of three replicates and dissimilar letters are significantly different according to Duncan’s test. (P ≤ 0.05).

Fig. 2: Effect of Propyl isothiocyanate on the amount of anthocyanin in Lepidium sativum. Values represent the mean of three replicates and dissimilar letters are significantly different according to Duncan’s test. (P ≤ 0.05).
Fig. 3: Effect of Propyl isothiocyanate on the amount of reducing carbohydrates in *Lepidium sativum*. Values represent the mean of three replicates and dissimilar letters are significantly different according to Duncan's test. (P ≤ 0.05).

Fig. 4: Effect of propyl isothiocyanate on root (a) and shoot proline (b) of *Lepidium sativum*. Values represent the mean of three replicates and dissimilar letters are significantly different according to Duncan's test. (P ≤ 0.05).
Protein content of shoot:

The amount of protein of shoot in all treated seedlings by propyl isothiocyanate than the control group decreased. At concentrations of 1, 0.1 and 0.01 mM, reduced protein levels, respectively 40, 20 and 10% compared to control.

![Graph showing protein content](image)

**Fig. 6**: Effect of propyl isothiocyanate on total protein content of leaf of *Lepidium sativum*. Values represent the mean of three replicates and dissimilar letters are significantly different according to Duncan’s test. (P ≤ 0.05).

Discussion:

The findings of this research indicate that propyl isothiocyanate stress induces physiological 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 stresses may stop metabolic processes by preventing enzyme activity. The reduction of chlorophyll in plants under stress is probably either because of controlling chlorophyll synthesis enzymes activity or the increasing of chlorophyll pigment disintegration [24]. Propyl isothiocyanate treatment in concentrations of 1, 0.1, 0.01 mM significantly reduced total chlorophyll of the cress seedlings compared to control [12]. Also, it was reported that propyl isothiocyanate treatment causes chlorophyll reduction (about 50%) in five-week-old seedlings of arabidopsis shoot [12]. Furthermore, in the present study propyl isothiocyanate has decreased the amount of carotenoids in leaf rather than the control seedlings. The free radicals produced during stress cause disintegration of photosynthesis and non-photosynthesis pigment as a result of which the pigments are decreased [24]. Carotenoids cause the protection of photosynthesis system against extra photons and oxidative stress such as reaction to chlorophyll to prevent the formation of active radicals of oxygen. In fact, carotenoids as a protective system against induced oxidative stress 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 [26]. So it is possible that the reduction of carotenoid amount under propyl isothiocyanate treatment is caused by the role of these pigments in protection of leaf chlorophyll against oxidative stress caused by isothiocyanats.

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 polyphenolic compounds and are among the most important secondary components of plants. These components are derivatives of phenyl propanoid. Anthocyanin as a group of soluble flavonoids is synthesized in the pathway of flavonoid biosynthesis at the end point [17]. By creating oxidative stress in plant, antioxidant genes expression [31] and induction of phenyl propanoid pathway especially Flavonoid biosynthesis are increased [11]. In this study, a significant difference in amount of anthocyanin is not observed at 0.01 propyl isothiocynats and the control, but at 1 and 0.1 mM propyl isothiocyanate, the amount of anthocyanin increased by 58 and 25 percent respectively. The same result is reported about the increase of flavonoids during environmental stress [20]. In this research, it seems that propyl isothiocyanate treatment causes removing of free radicals of oxygen and also plant adaptation to stress condition by increasing anthocyanin and flavonoids. In a research, [6] it was reported that antioxidant enzyme activities and anthocyanin content in blueberries are powerfully in contact with each other and are increased in cold weather[30]. Also Soleycka et al. (1999) studied effect of low temperature stress on anthocyanin content 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 stress of isothiocynats showed a significant increase compared to the control plants. To retain ion balance and osmotic regulation in vacuoles and cytoplasm, the plants accumulate low molecular weight compounds such as proline, glycine, betaine and sugars like glucose and fructose that are collectively called asmolite [20]. Furthermore, it seems that soluble sugars play an important role in relation with reactive oxygen species. Sugars are also needed for performing antioxidative processes like pentose phosphate pathway [3,8] and carotenoid biosynthesis. The reduction of saved starch and the increase of sugars are reported under stress situations [22]. Analysis of gene transcription has verified that sugar signaling is associated with the oxidative stress control. Different stresses like salinity,
drought, low temperature and heavy metal toxicity that directly or indirectly cause the accumulation of reactive oxygen species lead to the accumulation of soluble sugars that act as an adaptive mechanism to stress condition [23]. In the present study, it seems that the increase of carbohydrate is because of dealing with oxygen radicals generated under isothiocyanate stress. Proline plays an important role in stress tolerance in plant like anti-oxidant activity of proline. Proline can remove singlet oxygen and it also can play a role in protecting proteins against denaturation [2]. The results gained from measuring antioxidant enzyme activity under isothiocyanate stress show that propyl isothiocynats causes an increase in the activity of catalase enzyme and more tolerance to stress. The highest amount of catalase activity was observed at 0.01 mM of propyl isothiocynats. The balance between producing reactive oxygen species and anti-oxidant enzyme activity determines the way that oxidative signals occur [19]. Induction of antioxidant enzyme activity is a general adaptation of a plant against oxidative stresses [10]. 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 stress through hydrogen peroxide detoxification [18]. In high concentrations of propyl isothiocynats, catalase enzyme may break by proteases induced by oxidative stress that is reported in old pea leaves [25]. Decrease in antioxidant enzyme activities in high levels of stress, can be caused through enzyme molecular breakage by free radicals of oxygen [25]. Based on the results of this research, propyl isothiocynats has also increased the activity of ascorbate peroxidase enzyme compared to the control seedlings in shoots. The highest activity of this enzyme is observed at 0.01 mM of propyl isothiocynats. The increase of ascorbate peroxidase activity by abiotic stresses can be a sign of the beginning of antioxidant defense. Ascorbate peroxidase enzyme has participated in ascorbate-glutathione cycle and so has caused the removal of hydrogen peroxide. One study showed that allyl isothiocynats treatment at first causes the increase and then the decrease of enzyme activity of ascorbate peroxidase in blueberries [33]. Enzyme activity of gayacule peroxidase has increased under different concentrations of isothiocynats. Gayacule peroxidase enzyme catalyzes hydrogen peroxide- dependent oxidation of the substrate. This enzyme also stimulates lignin biosynthesis and creates physical barrier against the stresses and protects tissues against the free active radicals [9]. In the present research, the decrease in the activities of these enzymes in high concentrations of propyl isothiocynats may occur through inhibition of the enzyme or inactivation or down regulation of them so that the high intensity stress has opposite effect on the activity of the enzyme. The other results of this research include reduction of the amount of soluble proteins in plants under isothiocynats stress. One of the reasons of protein decrease in this level of stress might be increased production of free radicals of oxygen. These radicals inhibit protein synthesis or lead to protein denaturation [27]. It can also be due to reduced photosynthesis, and reduced materials required for protein synthesis under stress conditions [13].

Conclusion:
The results of this study showed that oxidative stress induced by moderate doses of propyl isothiocynates, induced physiological responses and antioxidant defense of cress seedlings under in vitro condition. It can be important in plant adapt and cope against stress condition. Probably, propyl isothiocynats in cress seedlings mediates the responses related to defense system of anti-oxidant.

REFERENCES


