Journal of Plant Physiology and Breeding

2014, 4(1): 23-33 ISSN: 2008-5168

UV Radiation Induced Changes of Phenolic Compounds and Antioxidant Enzymes in Okra (*Hibiscus esculents* L.) Seedlings

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Received: August 19, 2012 Accepted: June 15, 2014
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Abstract
The influences of different ultraviolet (UV-A, UV-B and UV-C) radiation treatments on phenolic compounds (total phenol, anthocyanin and flavonoid), lipid peroxidation content and behavior of the some enzymes such as polyphenoloxidase (PPO), phenylalanin ammonia–lyase (PAL), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), catalase (CAT) and glutathione reductase (GR) were studied in okra (*Hibiscus esculents* L.) seedlings. results showed that UV-B and UV-C treatments increased UV absorbing compounds and lipid peroxidation contents as compared with the control and UV-A treatment plants. UV-A treatment did not significantly affect the activity of antioxidant enzymes (APX, GPX, CAT, GR), but in plants treated with UV-B and UV-C, APX, GPX, CAT and GR activity were significantly increased when compared with the control plants. Also activity level of PPO and PAL in the shoots of okra plants increased significantly in response to UV-B and UV-C treatments. These results suggest that the increased phenolic compounds and antioxidant system activity in the UV-B and UV-C treatments may be considered as biomarkers of intensity of UV radiation stress. However, more research is necessary to elucidate the precise role that the antioxidant system plays under ultraviolet radiation stress.

Keywords: Antioxidant Enzymes; Okra seedling; Oxidative Stress; Phenolic compounds; Ultraviolet radiation

Abbreviations: APX- Ascorbate peroxidase, CAT- Catalase, GR- Glutathione Reductase, GSH- Reduced glutathione, GSSG- Glutathione disulfide, H_2O_2- Hydrogen peroxide, MDA– Malondialdehyde, PPO- Polyphenoloxidase, PAL- Phenylalanins ammonia–lyase, GPX- Guaiacol peroxidase, ROS- Reactive Oxygen Species, TBA- Thiobarbituric acid, TCA- Trichloroacetic acid, NBT- Nitroblue etrazolium

Introduction

The growing ozone depletion could lead to an increase of solar UV-B radiation reaching the Earth's surface (Costa *et al.* 2002). The increase of ultraviolet (UV-B) radiation can have many direct or indirect effects on plants including DNA and membrane injuries, photosynthetic or hormone systems disorders (Caldwell *et al.* 1989; Rozema *et al.* 1997; Jansen *et al.* 1998) As a result, UV radiation causes a multitude of physiological and biochemical changes in plants, such as changes in morphology, phenology and biomass accumulation (Caldwell *et al.* 1995). Furthermore, UV radiation induces increased amounts of reactive oxygen species (ROSs), UV-absorbing pigments and flavonolglycosides (Ren *et al*. 2007). ROSs induces oxidative damage to various cellular constituents, such as lipids, proteins and eventually causing the death of plant cells (Del Rio *et al*. 2003). To overcome this, cells are equipped with enzymatic and non-enzymatic mechanisms to eliminate or reduce the UV damaging effects (Khatun *et al*. 2008). ROS_s are efficiently eliminated by antioxidant enzymes such as catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) and non-enzymatic antioxidants such as ascorbic acid (AA), tocopherols, carotenoids and reduced glutathione (GSH) (Zhang *et al.* 2005; Janknegt *et al*. 2008). Previous experiments on different plant seedlings

have shown that enhanced UV radiation increases activity of the antioxidant enzymes and concentration of secondary metabolites (Ravindran *et al.* 2008). Some plants are more tolerant to UV-B than other because they produce a variety of secondary metabolites that effectively absorb UV radiation and prevent it from penetrating into the leaf mosophyll cells. (Indrajith and Ravindran 2009). Ruhland *et al.* (2005) reported that increased concentrations of these phenolics in response to solar radiation may not only act as a sunscreen against potentially damaging UV, but may also partially ameliorate damage caused by increased ROS. Previous results have also shown that phenolic second metabolites such as flavonoids and cinnamic acid derivatives are accumulated in epidermal cells by UV irradiation (Mori *et al.* 2005). Short-term studies of the biological effects of ultraviolet at the seedling stage can be useful for investigations of physiological mechanisms and of differences in resistance to UV radiation among cultivars (Kumagai *et al.* 2001). Okra (*Hibiscus esculents* L.) which belongs to the Malvaceae family, is cultivated as a food source in many tropical countries and is best known as a summer vegetable in the southern region of Iran, It is also a popular home garden vegetable and a good source of many nutrients including vitamins B and C, fiber, calcium and folic acid (Hegazi and Hamideldin 2010).

The aim of this work was to study the effect of different UV radiation on changes in some antioxidant enzymes (PPO, PAL, APX, GPX, CAT, GR) activity and phenolic compounds (total phenol, anthocyanin, flavonoid) in okra (*Hibiscus esculents* L.) seedlings.

Material and Methods

Growing conditions and treatments

This study was conducted at the Urmia University. Pre-soaked seeds of okra (Clemson Spineless cultivar that was obtained from Bakker Brothers Co., NOORD- Scharwoude, Holland) were germinated in the incubator at 25 ºC for 4 days. These seeds were then transferred to plastic pots filled with sand and were irrigated alternately with half- strength Hogland solution and distilled water. Seedling were grown in the greenhouse with diurnal regime of 16 h light at 27-29 ºC and 8 h dark at 18-20 ºC, The plants were grown in 60 pots, When the primary leaves fully developed, they divided into 4 sets of 15 pots. One set served as the control and other sets received different UV radiations. UV-A radiation was applied with two insecticide lamps (F20/BL-Hitachi, Japan), UV-B radiation was applied with two (15 w) UV-B lamps (LF-215 m. 312 nm) and UV-C radiation was applied by a germicidal lamp (TUV/G30T8, Philips, Holland) for 8 days. The biologically effective level of UV-A, UV-B and UV-C radiation were 11 kJ $m^{-2}d^{-1}$, 8.6 kJ $m^{-2}d^{-1}$ and 4.5 kJ $m^{-2}d^{-1}$, respectively. The treatments were arranged as completely randomized design with three replications.

Determination of UV absorbing pigments

Anthocyanin content

Fresh leaf tissue was homogenized in 10 ml of acidified methanol (1:99, HCl: methanol). The homogenate was centrifuged at 6000 g for 10 min. Extract was incubated at room temperature in dark

for 24 h .Then the absorbance was read at 550 nm (Fulcki and Francis 1968).

Total flavonoid content

Flavonoid content was assayed by the method of Chang *et al.* (2002). Fresh leaf tissue was homogenized in 1 ml deionized water. This solution (0.5 ml) was mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The samples were incubated at room temperature for 40 min. Then the reaction mixture absorbance was measured at 415 nm.

Total phenol content

Fresh leaf tissue was homogenized in 10 ml of 80% methanol. The homogenate was centrifuged at 10000 g for 15 min (Horii *et al.* 2007). To 1 ml of extract, 9 ml of deionized water and 1 ml folin ciocalteu reagent were added. After 5 min, 10 ml of 7% Na_2Co_3 was added to the mixture; the solution was diluted to 25 ml volume and mixed. The samples were incubated at room temperature for 90 min; the reaction mixture absorbance was measured at 750 nm (Marinova *et al.* 2005).

Polyphenoloxidase (EC 1.10.3.1, PPO) activity

PPO activity was measured using a modification of the procedure by Siriphanich and Kader (1985). The fresh weight of leaves (5 g) were homogenized in 45 ml of 0.1 M phosphate buffer (pH 6.5) with a chilled mortar and pestle. The homogenate was filtered through 4 layers gauze and centrifuged at 10000g for 20 min at 4 ^oC. The reaction mixture contained 1.95 ml phosphate buffer (pH 6.5), 1 ml 20 mM caffeic acid, 50 µl enzyme extract in the final volume of 3.02 ml. PPO activity was determined in the homogenates by measuring the increase in absorption at 420 nm.

Phenylalanins ammonia–lyase (EC 4.3.1.5, PAL) activity

PAL activity was measured using a modification of the procedure of Ke and Saltveit (1986). The fresh weight of leaves (5g) were homogenized in an ice cold mortar using 50 Mm borate buffer (pH 8.5) containing 5 mM 2-Mercaptoethanol and 2% PVP. After that, the homogenate was filtered through 4 layers gauze and centrifuged at 10000g for 20 min at 4 ^oC. PAL activity in supernatant was assayed by adding 0.55 ml of 100 Mm L-phenylalanine to 5 ml of the crude extract. After heating at 40 ^oC for 60 min the absorbance was measured at 290 nm.

CAT, APX, GPX and GR activity

Enzyme extraction was prepared for assaying CAT, APX, GR and GPX using a modified method of Kang and Saltveit (2002). For this purpose, 0.5 g of fresh tissues were placed in an ice cold mortar using 50 Mm Tris-HCl buffer (pH 7.5) containing 3 mM $MgCl_2$, 1 mM EDTA and 1% PVP. After that, the homogenate was centrifuged at 5000g for 20 min. For APX extraction, a buffer containing 0.2 mM AA was also used. The supernatant was stored at the -80 ^oC and used for the assay of enzymes activities. Ascorbate peroxidase (EC 1.11.1.11, APX) activity was measured in a 2.8 ml reaction volume containing 0.1 mM EDTA, 0.5 mM AA, 0.2 ml 1% H_20_2 and 0.1 ml enzyme extract. The decomposition of H2O2 was followed by measuring the decrease in absorbance at 290 nm. (ε: 2.8 mM^{-1} cm^{-1}) (Asada 1992). Guaiacol

peroxidase (EC 1.11.1.7, GPX) activity was measured using a modification of the procedure of Upadhyaya *et al.* (1985). The reaction mixture contained 2.5 ml phosphate buffer (pH 7), 1 ml 1% guaiacol, 1 ml 1% H_2O_2 and 0.3 ml enzyme extract in the final volume of 4.8 ml. GPX activity was determined in the homogenates by measuring the increase in absorption at 470 nm (extinction coefficient of 26.6 mM/cm). Catalase (EC 1.11.1.6, CAT) activity was determined in a 2.9 ml reaction volume containing 2.5 ml phosphate buffer (pH 7.4), 0.1 ml H_2O_2 and 0.3 ml enzyme extract. CAT activity was determined in the homogenates by measuring decrease in absorption at 240 nm (extinction coefficient of 4.7 mM/cm) (Aebi 1984). Glutathione reductase (EC 1.6.4.2 GR) activity was measured using a modification of the procedure of Foyer and Halliwel (1976). The reaction mixture consisted of 50 Mm phosphate buffer (PH 7) with 2.5 Mm $MgCl_2$, 0.5Mm GssG, 0.2 mM NADPH and 0.3ml enzyme extraction in the final volume of 2.8 ml. GR activity was determined in the homogenates by measuring the decrease in absorbance at 340 nm due to NADPH oxidation (ε: 6.2 mM^{-1} cm^{-1}).

Malondialdehyde content

MDA content was estimated using the procedure described by Heath and Packer (1968). An amount of 0.2 g fresh weight was homogenized in 5 ml of 1% (w/v) trichloroacetic acid (TCA) and then centrifuged at 8000 g for 10 min at 4 ºC. To 1 ml of the aliquot of supernatant, 1 ml of 20% TCA containing 0.5% (w/v) thiobarbituric acid (TBA) was added. The solution was heated for 30 min at 95 ºC. The reaction was stopped by placing the reaction tubes in an ice bath for 10 min. The samples were re-centrifuged at 8000 g for 5 min. The absorbance of supernatant was recorded at 532 nm and 600 nm.

Statistical analysis

The data was statistically analyzed using one way analysis of variance followed by Duncan's multiple range test (DMRT). All statistical analyses were performed with the SPSS 19 Program. Standard error of means was also calculated for presentation of the Figures.

Results

The effects of different Ultra violet radiation treatments on the content of several UV absorbing compounds in okra leaves were shown in Table 1. We showed that Ultra violet radiation treatments increased UV absorbing compounds in compared with the control, UV-A treatment not significantly affect on UV absorbing compounds content, but, plants grown under UV-B and UV-C treatments showed a higher concentration of UV absorbing compounds than control and UV-A treated seedling.

Table 1 shows that the exposure to ultraviolet radiation had a significant effect on PAL and PPO activity in seedling. PAL and PPO activity were significantly increased, in UV-B and UV-C treated in Compared with the control. UV-A treatment not significantly affects on PAL and PPO activity when compared to control. Increased MDA content was observed in UV treated seedling (Figure 1). The highest accumulation of MDA was noticed in UV-B and UV-C treated seedling when compared to control seedlings. but in UV-A treated plants there was no significant increase in comparison with the controls. In this

study, increased antioxidant enzymes activity such as CAT, APX, GR and GPX were observed in UV treated . CAT activity in the shoot and roots of okra plant were shown in (Figure 2). CAT activity was a significant increase in the shoot and roots of UV treatments compared to the control , and the highest value was observed at UV-B and UV-C treatments groups. Changes in APX activity of shoots and roots of okra plants at different ultraviolet treatment are shown in (Figure 3). In the shoot and root of plants treated with UV-C and in shoot of plants treated with UV-B, APX activity was significantly increased when compared to the control plants. But, no significant changes in APX activity were observed in the root of plants treated with UV-B and in shoot and root of plants treated with UV-A when compared to the control plants. The effects of different Ultra violet radiation treatments on the GPX activity in okra shoot and root were investigated and the results are shown in (Figure 4).The GPX activity in the shoot of okra showed the highest activity at different ultraviolet treatments comparing with control, The GPX activity of roots also showed a similar trend. UV-B and UV-C treatment had a significant effect on GPX activity in comparison with the controls. Figure 5 showed that the exposure to ultra violet radiation increased the GR activity in shoot and root when compared whit control plants. In the plants treated with UV-B and UV-C, GR activity was significantly increased when compared to the control plants (Figure 5). But in the plants treated with UV-A, GR activity was not significantly increased when compared to the control plants (Figure 5).

Table 1. The effects of different ultraviolet radiation treatments on UV-absorbing pigments content, PAL and PPO activity in okra plants

	Control	UV-A	UV-B	UV-C
PAL (µg cinamate hg^{-1} Fw^{-1})	4.16 ±0.07+ c	4.4 ± 0.24 bc	4.92 ± 0.06 b	6.27 ± 0.14 a
PPO (µmol $min^{-1}g^{-1}$ Fw^{-1})	0.809 ±0.09 b	1.6 ± 0.07 b	2.32 ± 0.08 a	2.49 ± 0.173 a
Total phenol (µmol g^{-1} Fw	32.49 ±1.70 b	35.29 ±1.76 b	48.89 ± 1.96 a	55.96 ± 3.69 a
Total Flavonoid (µg g^{-1} Fw)	0.288 ±0.01 c	0.330 ±0.011 c	0.403 ± 0.005 b	0.469 ± 0.019 a
Antocyanin (µmol g^{-1} Fw)	0.170 ±0.009 c	0.173±0.013 c	0.257 ± 0.0128	0.304 ± 0.006 a

Mean±SE; n = 3), P≤ 0.05.

Discussion

Ultraviolet radiation enhancement disturbs plant metabolism and cause oxidative injury by increasing the production of reactive oxygen species (ROS) (Indrajith and Ravindran 2009). ROSs lead to the oxidation of biomolecules (i.e. lipids, proteins and nucleic acids) or even cell death (Xin *et al.* 2009). Plants have evolved various protective mechanisms to eliminate or reduce ROSs. MDA that is used as an indicator reflecting the degree of cell injury resulting from ROS, is a quantity that could represent the degree of membrane lipid peroxidation (Tang *et al.* 2010). Van Hasselt (1974) reported that MDA is the peroxidation product of polyunsaturated fatty acid, in particular linolenic acid. The increase in

the MDA content observed in this experiment was also reported in other published results about peanut seedlings (Tang *et al.* 2010), cassia seedlings (Agarwal 2007) and sunflower cotyledons (Costa *et al.* 2002). In our study MDA content significantly correlated with the UV-B and UV-C treatment (Figure 1), which may be another evidence for the occurrence of oxidative stress in the okra plant. UV exposure significantly increased total phenol, total flavonoied and anthocyanin content. Similar results about the increase in UV absorbing compounds have been observed in durum wheat (Balouchi *et al.* 2009), cucumber (María Luisa Tapia 2010) and *Phaseolus trilobus* (Ravindran *et al*. 2008). It is thought that UV-absorbing compounds accumulate primarily in the epidermal layers or leaves and stems, and provide a means of protection against UV-B damage and subsequent cell death by protecting DNA from dimerization and breakage (Strack 1997). According to Flint and Caldwell (1983) UV-B-induced accumulation of flavonoids in the epidermis has a substantial impact on screening UV radiation from underlying tissues. Flavonoids may also have a key role as free radical scavengers in the response mechanisms to excess light stress (Tattini *et al*. 2000). Anthocyanins are suggested to protect chloroplasts from excess irradiance due to their ability to absorb light between 400 and 600 nm (Gould *et al*. 2000). Total phenolics and flavonoids have shown high correlation with antioxidant activity (Yoo *et al.* 2008). Results of this experiment about the increase in the synthesis of flavonoids and anthocyanins are in concordance with the previously published works for *Phaseolus trilobus* seedlings (Ravindran *et al*. 2008). As a conclusion it can be argued that increase in flavonoids, anthocyanins and other phenolic compounds prevents the penetration of UV radiation to sensitive tissues and reduce oxidative damage in okra plants.

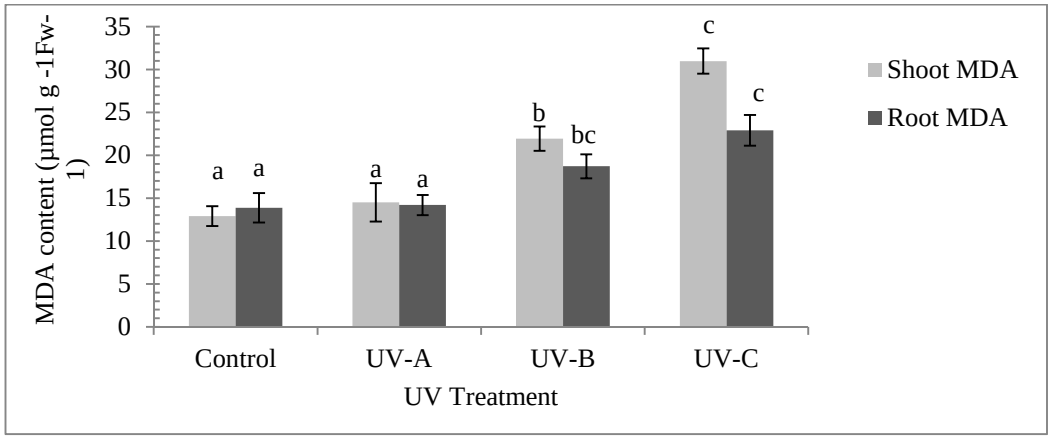

Figure 1. Effects of different ultraviolet radiation treatments on MDA content as compared with the control plants (Mean ± SE, n= 3), P ≤ 0.05

A marked increase of the PAL and PPO activity in the shoots of okra plant in this study could represent an adaptive response against UV radiation. The increase in PAL and PPO activities indicated that okra plants had the capacity to adapt to UV treatment by increasing its phenolic compounds and removing ROSs. PAL is a basic cellular enzyme associated with a host of functions and biosynthesis of diverse phenylpropanoids and thus constitutively expressed in young seedlings (Hahlbrock and Scheel 1989). Increase in PAL activity of *Phyllanthus amarus* L. treated with UV was observed by Indrajith and Ravindran (2009). Liu *et al.* (1995) observed that UV-B irradiance in barley primary leaves prolonged the activity of PAL, resulting in an enhanced accumulation of UV absorbing compounds. We also observed an increase of PPO activity in the shoots of okra. Other researchers have also reported the enhancement of PPO activity in *Crotalaria juncea* L. (Balakrishnan *et al.* 2005) and *Phyllanthus amarus* (Indrajith and Ravindran 2009). Polyphenols have the capacity to prevent DNA oxidative damage and to reduce lipid peroxidation by neutralizing and detoxifying the radicals produced in the process of exposure to oxidative stress (Xin *et al.* 2009).

The present results show that the okra plant responds to UV radiation with changes in the levels of antioxidant enzymes. APX and CAT are two major scavengers of hydrogen peroxide (H_2O_2) and breakdown of H_2O_2 to H_2O and O_2 (Zhang *et al.* 2008). APX and CAT activity in okra increased in response to UV-B and UV-C exposure (Figures 2 and 3). The maximum level of increase in the APX activity was larger than that of CAT. This is because that APX is present throughout the cell and has a higher substrate affinity in the presence of ASA as a reductant agent, whereas CAT is present only in the peroxisome and has a low substrate affinity (Zhang *et al.* 2008). Activation of antioxidant enzymes by UV has earlier been observed in several plant species with respect to CAT and APX (Wang *et al.* 2009; Tang *et al.* 2010).

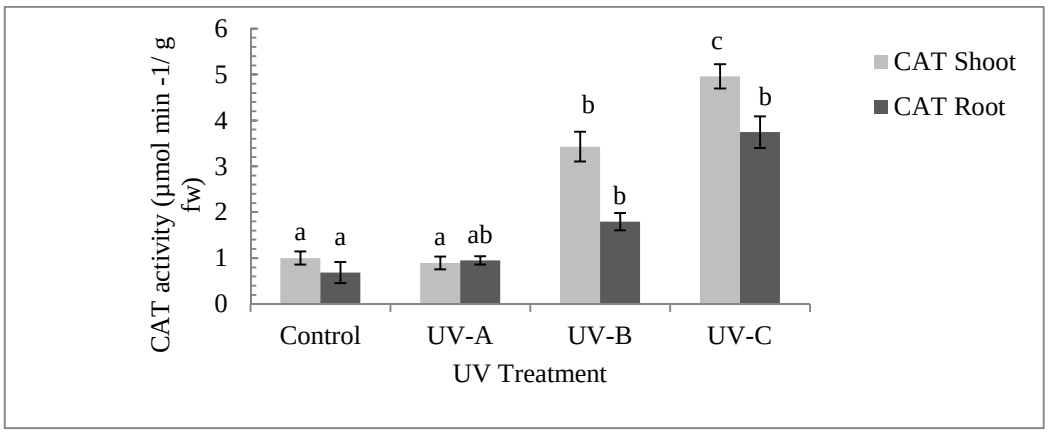

Figure 2. Effects of different ultraviolet radiation treatments on CAT activity as compared with the control plants (Mean ± SE, n = 3), P≤ 0.05.

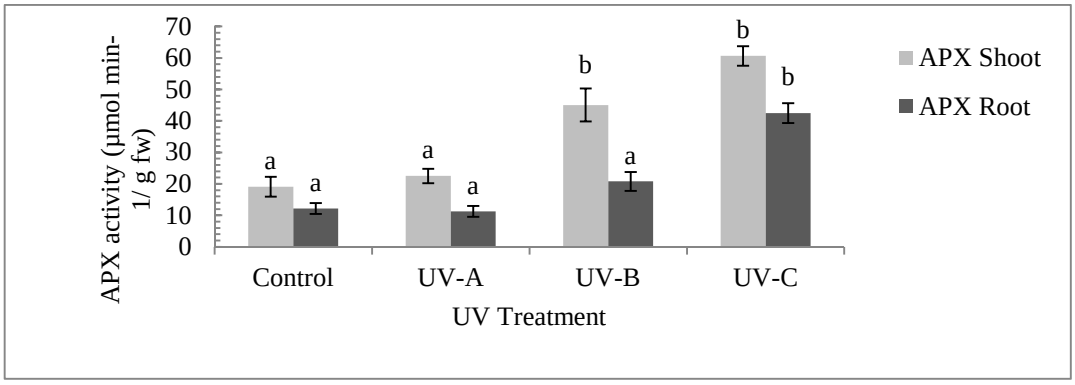

Figure 3. Effects of different ultraviolet radiation treatments on APX activity as compared with the control plants (Mean ± SE, n= 3), P≤0.05.

Guaiacol peroxidase (GPX) activity increased by UV-B and UV-C in the shoots and roots of okra plants (Figure 4) which can be considered as an evidence for the role of GPX in detoxification of ROSs that were induced under UV treatment. Furthermore, increase in POX activity might be the result of increase in de novo protein synthesis or the activation of enzymes already present in plant cells to diminish ROS deleterious effects (Van Assche and Clijsters (1990). GPX is an important member of peroxidases, and used as a substrate for the measurement of peroxidase (POX) activity (Hiraga *et al.* 2001). POXs are widely distributed in plants tissues involved in growth, development and senescence processes of plants. The activity of POXs prevents H_2O_2 damage to cell membranes, proteins and DNA (Di Giulio *et al.* 1989). GR is the key enzyme of the ascorbat- glutation cycle for the removal of H_2O_2 in different cellular compartments (Jiménez *et al.* 1997). The conversion of glutathione disulfide (GSSG) to glutathione (GSH) which is catalyzed by GR enzyme was correlated with the change in GSH/GSSG mole ratios that plays an important role in the cellular redox status and in signal transduction of several transcription and metabolic processes (Hatata and Adel Abdel-Aal 2008). Costa *et al.* (2002) also reported the increase in GPX and GR activity for sunflower Cotyledons. Our results showed that UV-B and UV-C treatment increase the ROSs production in okra (*Hibiscus esculents* L.) seedlings.

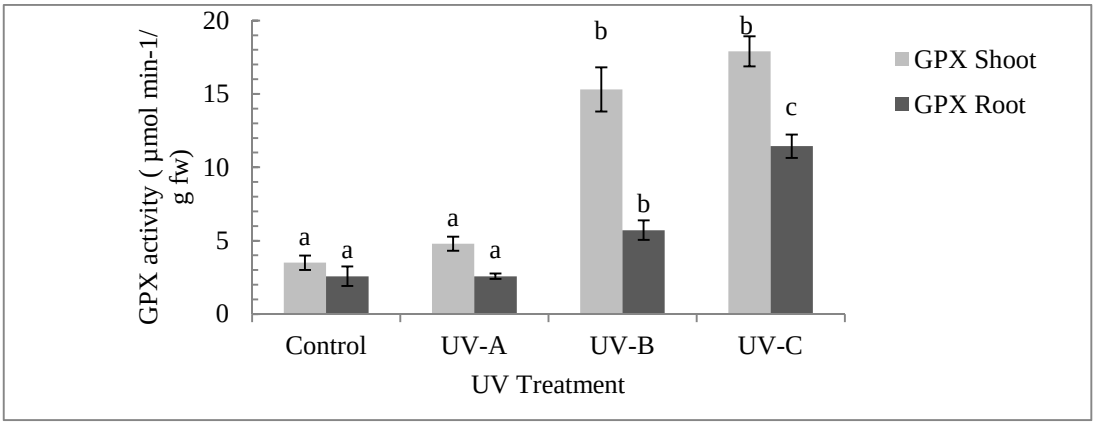

Figure 4. Effects of different ultraviolet radiation treatments on GPX activity as compared with the control plants (Mean ± SE, n= 3), P≤ 0.05.

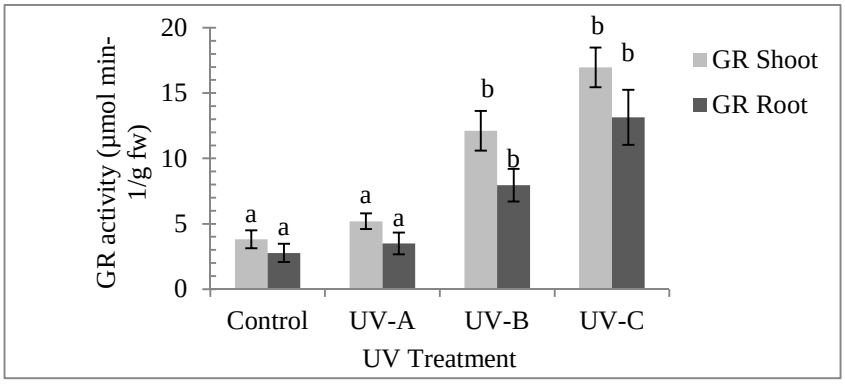

Figure 5. Effects of different ultraviolet radiation treatments on GR activity as compared with the control plants (Mean ± SE, n= 3), P≤ 0.05.

In conclusion, the increase in lipid peroxidation and UV absorbing compounds and also changes in antioxidant enzymes activity in okra seedlings had a clear relationship with UV treatments. The significant increase in antioxidant enzymes and UV absorbing compounds might protect the plants against major deleterious effects of UV radiation. Furthermore, the coordinated increase in the activities of PAL and PPO under UV radiation, should play an important role in the antioxidant defense of the okra leaves.

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