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Full Length Research Paper

Effects of UV-B radiation and butylated hydroxyanisole(BHA) on the response of antioxidant defense systems in winter wheat (*Triticum aestivum L.*Yildirim) seedlings

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Effects of UV and butylated hydroxanisol (BHA) on the activities of antioxidant enzymes were studied on the leaves of winter wheat cultivars (*Triticum aestivum L.* Yildirim). Fifteen days old wheat seedlings were treated with UV radiation (240 nm, 3 day) before they are treated with BHA (20°C). Supplementary UV-B radiation and UV-B+BHA significantly decreased chlorophyll and total phenol contents. The activities of enzyme extracts; polyphenol oxidase, catalase, paraoxonase and peroxidase were determined in the leaves under normal, UV-B (<315 nm) and UV-B + BHA conditions for 4 days. The antioxidant enzymes affected and showed enhanced activities in peroxidase, paraoxonase and polyphenoloxidase (except catalase) in UV-B and then BHA irradiated seedling. UV-B and then BHA-treated winter wheat seedling tries to counteract high level of reactive oxygen species that are produced under UV-B stress through the increased activities of antioxidant enzyme. It brings to mind that BHA tries to counteract high concentrations of oxygen species produced under UV-B radiation stress through increase in UV absorbing compound and antioxidant enzymes. In this study, the changes in quantities of polypeptide in wheat was investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). While the accumulation and pattern of the polypeptides unchanged in UV-treated leaves, they increased in UV+BHA-treated leaves compared to the control. It is concluded that BHA can be involved in UV radition tolerance by regulating antioxidant enzyme activities.

Key words: Oxidative damage, butylated hydroxanisol (BHA), peroxidase, catalase, polyphenol oxidase, paraoxonase, UV, winter wheat.

INTRODUCTION

Due to thinness of the ozone layer, it absorbs less UV-B in sunlight. Due to thinness of the ozone layer, less UV-B radiation of the sun is adsorbed by ozone. The world's surface is exposed to UV-B radiation which is harmful for the plant growth, development and physiology. Excessive UV-B negatively affects the growth process and development of almost all of the green plants (Frohnmeyer and Staiger, 2003; Brian, 2011). A number of studies have been carried out to study whether this increased UV-B radiation has a significant impact on plants (Dėdelienė and Juknys, 2010). Plants' exposure to UV radiation stress cause production of deleterious free radicals/reactive oxygen species (ROS) such as singlet oxygen, superoxide radical, hydrogen peroxide, hydroxyl ion and free hydroxyl radical ($^1O_2$, $\cdot O^{2-}$, H_2O_2, OH^- and $\cdot OH$) which are produced in mitochondria, endoplasmic reticulum, micro-bodies, plasma membranes and chloroplasts (Escoubas et al., 1995; Olga et al., 2003). Reactive oxygen species presence and induce antioxidant

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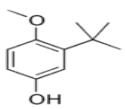
system to protect plants (Foyer and Noctor, 2005; Foyer and Graham, 2009; Parra-Lobata et al., 2009). Antioxidants can act by scavenging reactive oxygen species, by inhibiting their formation, by binding transition metal ions and preveting formation of OH /O_2^-, H_2O_2 (Zancan et al., 2008; Hideg et al., 2002; Mackerness et al., 1998; Foyer and Graham, 2009).

Antioxidants occur naturally in plants during stress. The antioxidant system includes enzymes such as peroxidase (POX, EC 1.11.1.7), catalase (CAT, 1.11.1.6), paraoxonase (PON, aryldialkylphosphatase, EC 3.1.8.1) etc...; besides vitamins, phytochemicals, minerals and food additives (Azzedine et al., 2011). SOD accelerates O^{2-} to H_2O_2 dismutation and is localized in cytosol, chloroplast, mitochondria and peroxisomes. POX, an iron heme protein, catalyses the reduction of H_2O_2 with a concurrent oxidation of a substrate, mostly located in cell wall and involved in oxidation of phenol compounds in terms of lignin synthesis. CAT also catalyses the reduction of H_2O_2 to water and molecular oxygen and is localized in mitochondria and peroxisomes, and absent in chloroplast (Kuk et al., 2003). Paraoxonase 1 (PON1) is a member of the multigene family of paraoxonases (PONs), which is observed in various tissues and cells (Primo-Parmo et al., 1996; La Du et al., 1999) including plants (Demir et al., 2011). There is also evidence in some studies that human serum paraoxonase (PON1)'s antioxidant function begins at the level of lipoprotein protection against oxidative modification by reactive oxygen species (ROS). The enzyme also reduces lipid hydroperoxides to hydroxides and presents a peroxidase-like activity, as PON1 seem to degrade hydrogen peroxide (H_2O_2), one of the ROS that is produced under oxidative stress (Aviram et al., 1998). Previous studies have shown that UV-B radiation and O_3 reduced biomass, yield, photosynthetic rate, chlorophyll, carotenoid and ascorbic acid contents and catalase activity, whereas increased total phenol content and peroxidase activity in wheat plants (*Triticum aestivum* L.) (Ambasht and Agrawal, 2003).

Butylated hydroxyanisole (BHA, 2-tert-Butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole) is a food additive and an aromatic organic compound. BHA has been widely used as antioxidants in the food industry including beverages, ice creams, candies, baked goods, instant mashed potatoes, edile fats and oils, breakfast cereals, dry yeast and sausages (Williams et al., 1999; Young, 1997; Verhagen et al., 1991). The structure of Butylated hydroxyanisole (BHA) is as follows:



It is very important to determine and evaluate the effects of UV-B on wheat which is used to produce bread for nutrition. In this study, a type of winter wheat (*T.aestivum L.* Yildirim) which is used for producing first- class bread was used. It can outlast drought and cold. It is thought that this winter wheat variety can also resist to UV-B effects which is another reason why it is selected as the wheat type.

In addition, it is thought that BHA, which is an antioxidant substance, has a protective role against UV stress on wheat leaves in the antioxidant system of plants. In this study, effects of UV- B radiation and BHA were investigated on the amount of activities of some antioxidant enzymes such as paraoxonase (PON 1), catalase (CAT), peroxidase (POX), polyphenol oxidase (PPO), protein and the phenolic compound in winter wheat (*T. aestivum L.* Yildirim).

MATERIALS AND METHODS

The growth of plants

In this study, *T. aestivum* L. Yildirim was used as a type of winter wheat. Before sowing, the plant seeds' surface were sterilized for 10 min. with 10:1 water/bleach (commercial NaOCl) solution and then washed five times with distilled water. The plants were grown hydroponically in a growth chamber under controlled environmental conditions for 15 days (day/night temperature of 22/20°C, relative humidity of 75%, and a 16-h photoperiod with photosynthetically active radiation at photon flux density of 400 µmol m^{-2} s^{-1}).

Plants were divided into three groups: (1) control, (2) plants that exposed to UV-B, (3) plants exposed to UV- B radiation and then exposed to BHA (10 mM). Three replicates were used in each experiment. Firstly, two groups of plants were exposed to UV-B (312 nm) radiation with a density of 5.8 W/m^2 and measured with a UV sensor model of Leybold Didactic, Germany, during 4 days. UV-B lamp was purchased from Philips. After the plant was exposed to UV-B, BHA solution (10 mM) was sprayed on a group of wheat leaves in plastic pots during 2 days. Distilled water was used to spray control plants. Then, 10 g wheat leaves were harvested from plants exposed to UV-B radiation, UV-B + BHA and harvested from control leaves of plants, respectively. All harvested leaves were used to determine the antioxidant enzymes, amount of protein and phenolic content.

Sampling and laboratory analysis

Harvested leaves (10 g) were carefully cut with a sharp bistoury into 2 cm lengths, and rinsed 6 times in distilled water to remove BHA from the surfaces of plants. Winter wheat (*T. aestivum L.* Yildirim) sample of 10 g fresh weight (FW) was frozen immediately in liquid nitrogen and stored at -80°C until assay. The enzyme extract for POX, PPO, CAT and PON was prepared by grinding frozen tissue with 50 ml extraction buffer [0.1 M potassium phosphate buffer, pH 7.5 containing 0.5 mM ethylene diamine tetra acetic acid (EDTA)]. The extract was centrifuged for 20 min. at 15,000 × *g* and the supernatant was used for enzymatic assay (Nadaroglu and Demir, 2009).

Determination of catalase activity

CAT (mM H_2O_2 reduced min^{-1} g^{-1} FW) assay was based on the absorbance at 240 nm on UV spectrophotometer, a decrease in absorbance was recorded awhile as described by Aebi (1984). The complete reaction mixture contained 1.5 ml of 100 mM potassium phosphate buffer (pH 7.0), 0.5 ml of 75 mM H_2O_2, 0.2 ml of enzyme

extract and 0.8 ml of distilled water.

Determination of peroxidase (POX) activity

POX activity (µmol tetraguaiacol min^{-1} g^{-1} FW) analysis is based on the increase in optical density due to the oxidation of guaiacol to tetraguaiacol (Castillo et al., 1984). The complete reaction mixture contained 1.0 ml of 100 mM potassium phosphate buffer (pH 6.1), 0.5 ml of 96 mM guaiacol, 0.5 ml of 12 mM H_2O_2, 0.1 ml of enzyme extract and 0.4 ml of distilled water. The enzyme activity was measured at 470 nm for 1 min. and calculated using the extinction coefficient (ε) of tetraguaiacol (26.6 mM/cm).

Determination of paraoxonase activity

Paraoxonase activity was determined at 25°C with paraoxon (diethyl *p*-nitrophenyl phosphate) (1 mM) in 50 mM *Tris*/HCl (pH 8.0) containing 1 mM $CaCl_2$. The enzyme analyze was based on the estimation of *p*-nitrophenol at 412 nm. Molar extinction coefficient of *p*-nitrophenol (ε = 18,290 $M^{-1}cm^{-1}$ at pH 10.5) was used to calculate enzyme activity (Gan et al., 1991; Renault et al., 2006; Demir et al., 2011). One enzyme unit was defined as the amount of enzyme that catalyzes the hydrolysis of 1 µmol of substrate at 25°C. Analyses were carried out using a spectrophotometer (PG Instrument T80, USA) (Kuo and La Du, 1995; Stafforini et al., 1990; Reiner and Radic, 1985).

Determination of polyphenol oxidase

PPO activity was determined by measuring the increase in absorbance at 420 nm with a spectrophotometer (UV-Beckman). 50 µl of crude extract was added to a 3 ml substrate mixture containing 0.20 M sodium phosphate buffer (pH: 6.5), 25 mM catechol. Enzyme activity was calculated from the linear portion of the curve. One unit of PPO activity was defined as the amount of enzyme that can cause an increase in absorbance of 0.001/min (Flurkey, 1986).

Determination of total phenolic content

Total phenolic content was determined (Wu et al., 2007) with a concomitant formation of a blue complex by reduction of Folin-Ciocalteu's reagentby phenolic compounds. In this study, 0.50 ml of the extract was mixed with 3.0 ml of distilled water and 0.25 ml of Folin-Ciocalteau reagent. Immediately, 0.75 ml of saturated sodium carbonated and 0.95 ml of distilled water were added. Then, the mixture was incubated for 30 min at 37°C; the absorbance was read at 765 nm using an UV-Vis spectrophotometer (T80 PG Instrument, USA). Total phenolic concentration of samples was calculated on the basis of a standard curve obtained by using gallic acid; and then calculations were expressed as milligrams of gallic acid equivalents (GAE) per gram of sample.

Determination of chlorophyll content

The chlorophyll content was analyzed according to the method described by Wintermans and De Mots (1965). Chlorophyll was extracted by 80% ethanol and measured spectrophotometrically at 654 nm.

Protein concentration

Total soluble protein concentration was determined spectrophotometrically by Bradford's method (Bradford, 1976), using bovine serum albumin (BSA) as the standard.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed control,control+BHA, UV and UV+BHA treated proteins. It was carried out in 3 and 10% acrylamide concentrations for the stacking and running gels, respectively, each of them containing 0.1 % SDS (Laemmli, 1970). The sample (20 µg) was applied to the electrophoresis medium. Brome tymol blue was used as tracking dye. Gels were stained in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol, 10% acetic acid and 40% distilled water for 1.5 h. It was destained by washing with 50% methanol, 10% acetic acid and 40% distilled water several times (Laemmli, 1970). The electrophoretic pattern was photographed (Figure 3).

Statistical analysis

All results were analysed by analysis of variance, and means were compared by Duncan's multiple range test.

RESULTS AND DICUSSION

It was thought that loss of plant species would start with the ozone layer depletion which accordingly starts reduction of global food stocks. Negative effects of UV lights on wheat *(T. aestivum L.* Yildirim), the basic raw material for the bread which is the most important basic food of the communities, will cause a global problem. UV-B enhancement disturbs plant metabolism and causes oxidative injury by enhancing the production of reactive oxygen species. Metabolism of reactive oxygen species depends on low molecular anti-oxidant systems as well as enzymes such as paraoxonase, peroxidase, polyhenoloxidase and catalase. Therefore, in this study, it was determined that antioxidant enzymes activities changed in winter leaves on which firstly UV-B radiation and then BHA which is known as an antioxidative food additive is applied.

Activities of PPO, CAT, PON and POX in winter wheat were decreased very little in BHA treatment. However, activity of PON was significantly increased under UV-B radiation and BHA treatments as 196.83 EU/g leaf and 249.32 EU/g leaf, respectively; with respect to the control value 52.49 EU/g leaf. POX activity was significantly increased at 5.8 W/m^2 UV-B treatments (10.53 and 2.15% over control values). Activity of PPO was increased at both UV-B radiation and BHA treatments as 15 and 27.2 EU/g leaf, respectively, with respect to the control value 10 EU/g leaf. CAT activity significantly was significantly decreased at 5.8 W/m^2 UV-B and then BHA treatments (66.7 and 46.7% over controls, respectively) (Figures 1 and 2).

The results suggest that excess UV-B radiation could promote and stimulate the generation of ROS leading to increase in the activities of antioxidant enzymes as a defense system induced antioxidant defenses protecting plant against major fatal effects of ROS (Mittler et al.,

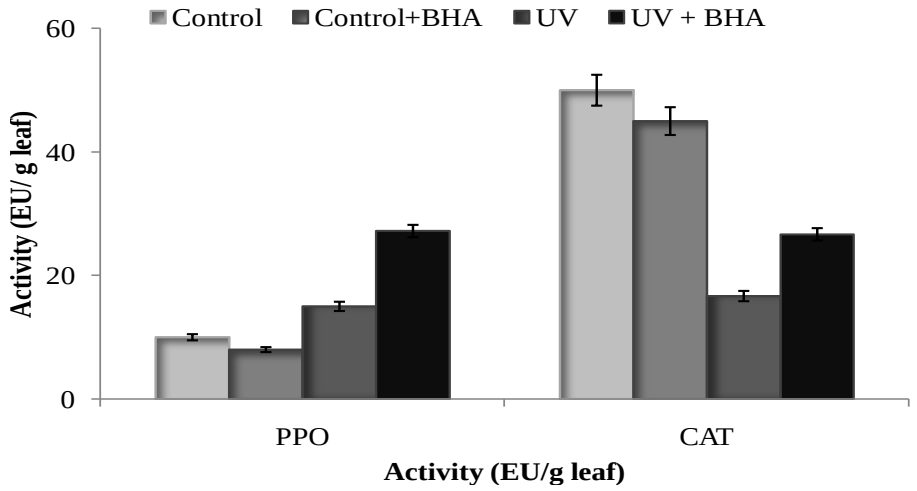


Figure 1. Effect of BHA, UV and UV + BHA on PPO and CAT antioksidant enzymes activity. Means with different letters are significantly different at $p<0.05$ based on Duncan's multiple range test

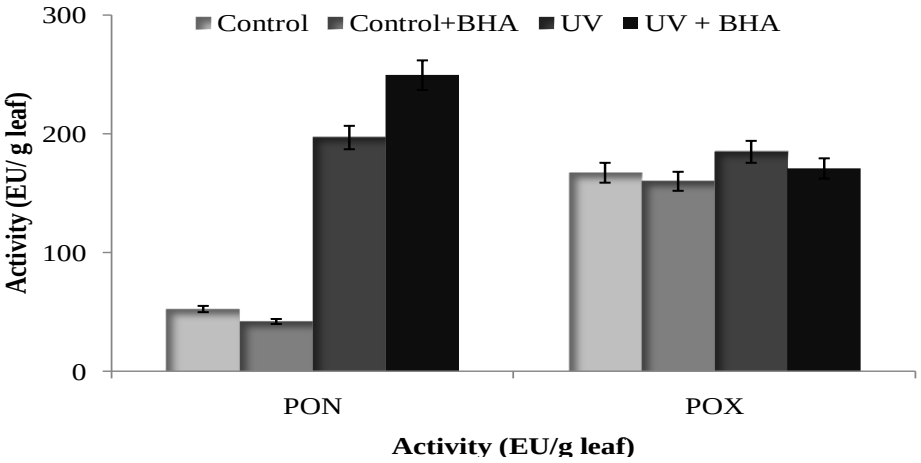


Figure 2. Effect of BHA,UV and UV + BHA on PON and POX antioksidant enzymes activity. Means with different letters are significantly different at $p<0.05$ based on Duncan's multiple range test.

2004; Fariduddin et al., 2009). The damage caused by these radicals which is indicated by the decrease in chlorophyll and total phenol contents was limited. This showed that water soluble non-enzymatic antioxidants and enzymatic antioxidant defense system represented by PON, POX, PPO and CAT are changeable in the winter wheat (*T. aestivum L.* Yildirim). The UV-B enhancement of CAT and POX activities which are both responsible for detoxification of H_2O_2 generated by PON, are probably equally important in the process. BHA has not increased antioxidant enzyme activities (PON, POX and PPO).

Chlorophyll content was greatly reduced in both treatments with UV-B and then BHA in winter wheat leaves (Table 1). It was shown that it was a possible damage in photosynthetic capacity of chloroplasts (Malanga et al., 1997; Kakani et al., 2004; Santos et al., 2004; Strid, 1993; Mackerness et al., 1998). In a field experiment with *Vigna radiate*, (Pat et al., 1999) and *Phyllanthus amarus* (Indrajith and Ravindran, 2009) an initial increase and subsequent decrease in chlorophyll content was observed, which is also reflected in this present study. Total phenol content also decreased for treated UV-B and then BHA as 22.64 and 22.13% respectively, with respect to control (Table 1).

Amount of total soluble protein in 1 g wheat was determined by using Bradford method and standard graphic of serum albumin. In 1 g wheat, the amount of protein for control, treated with UV-B radiation and treated with BHA was determined as 94.04 ± 1.78 µg of

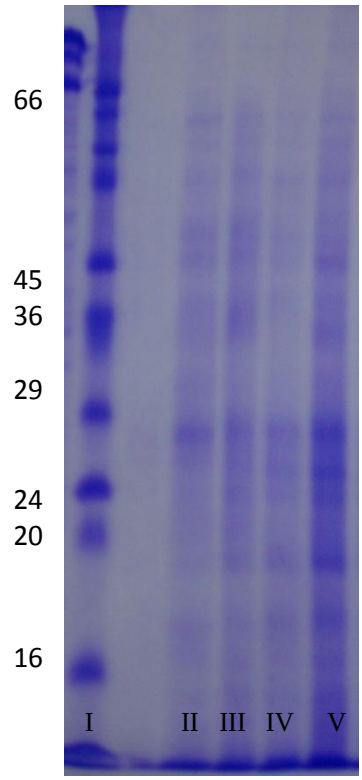


Figure 3. The electrophoretic pattern of antioxitative polypeptidesfrom winter wheat cultivars (*Triticum aestivum* L. Yildirim) after UV and BHA treatment (I: Standart; II: BHA; III:Control; IV: UV; V: UV+BHA).

Table 1. Effect of BHA, UV-B and UV-B+BHA treatments on the contents of chlorophyll, total phenol, total protein in winter wheat leaves.

Parameter	Total phenolic content (µg/ g leaf)		Chlorophyll content (µg g^{-1})		Total protein content (µg protein/ g leaf)	
	Mean ±SD	P	Mean ±SD	p	Mean ±SD	p
Control	2.39 ± 0.031	-	242.3 ± 2.16	-	94.04 ± 1.78	-
BHA	2.30 ± 0.02	p<0.05	235.8± 0.72	p<0.05	93.2 ± 1.05	p<0.05
UV	1.85 ± 0.02	p<0.05	226.4 ± 2.03	p<0.05	138.15 ± 1.20	p<0.05
UV + BHA	1.86 ± 0.016	p<0.05	206.5 ± 33.79	p<0.05	269.34 ± 0.91	p<0.05

protein, 138.15 ± 1.20 µg protein and 269.34 ± 0.91 µg protein, respectively. The results showed that the amount of protein increased in both treatments firstly UV-B and then BHA in wheat leaves. This causes an increase in the synthesis of the plant antioxidative enzymes as shown in previous studies (Agarwal, 2007). Amount of total phenolic substance, protein and chlorophyll contents were not changed significantly in winter wheat leaves only in BHA-treatment plants (Table 1).

While POX, PPO and PON enzyme activities were increasing, CAT activity inhibited after application of UV-B radiation on leaves (Figures 1 and 2). It is known that antioxidant enzymes in plants play an important role in response to the stress in plants. Previous studies have determined that UV-B enhanced oxidative stress and the generation of ROS (Mishra et al., 2011) and antioxidant defense system induced in *Arabidopsis thaliana* (Gao and Zhang, 2008), in *Oryza sativa* (Dai et al., 1997) and *Zea mays* seedlings (Carletti et al., 2003). Similar to our results, the increased levels of ROS trigger the activity of several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) (Mishra et al., 2011).

BHA, known as a food additive and antioxidant, enhanced antioxidative enzyme activities and accumulation of polypeptides in wheat after UV treatment. When

polypeptides control, UV and UV+BHA were compared, accumulation of polypeptides was increased by BHA at wheat leaves. Amount of polypeptides (especially, 20, 24, and 29 kDa) slightly decreased with UV-B stress and later highly increased with BHA (Figure 3). According to the results, it could be thought that BHA influences the mechanism of the formation of antioxidant enzymes.

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