Pak. J. Bot., 49(1): 47-55, 2017.

EFFECTS OF AMBIENT OZONE ON REACTIVE OXYGEN SPECIES AND ANTIOXIDANT METABOLITES IN LEAVES OF PEA (*PISUM SATIVUM* L.) PLANTS

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Abstract

The differential response of two pea plants (*Pisum sativum* L. cultivars Little Marvel and Victory) to ambient O_3 grown under open top chambers (OTCs) was analyzed and compared. Reactive oxygen species (ROS) generation, antioxidant metabolites such as ascorbate/glutathione as well as a series of enzymes for scavenging ROS were analyzed, all aiming to reveal the differential behavior of two closely related plants when exposed to ambient O_3.

Antioxidant levels and activities of related enzymes in response to ambient were noticeably different among Little Marvel and Victory plants. However, the response was cultivar-specific. There was higher accumulation of ROS and relatively lower induction of antioxidants and more inhibition in photosynthetic rates in Victory than Little Marvel. There was a good correlation between tolerance to O_3 and high endogenous levels of antioxidant metabolites such as ascorbate (As), glutathione reductase (GR), superoxide dismutase (SOD), reduced (GSH) and oxidized glutathione (GSSG) in pea plants. These portrays a higher sensitivity of Victory to ambient O_3.

To the best of our knowledge, this is one of the very few studies attempted to describe the changes in contents of antioxidants and activities of related enzymes in leaves of two closely related cultivars to further ourunderstanding on the defense mechanism and strategies under ambient O_3. The results highlighted the possible roles of antioxidants in O_3 detoxification through activation an adaptive survival mechanism allowing the plant to complete its life cycle even under oxidative stressful conditions.

Key words: Ambient ozone. Oxidative stress. Defense system. Pea (*Pisum sativum* L. cultivars Little Marvel and Victory) plants.

Introduction

Tropospheric ozone (O_3) is one of the most significant phytotoxic pollutant causing alterations in physiological and biochemical processes worldwide (Ranieri *et al*., 1999; Hassan, 2006; Ismail *et al*., 2014; Mishra & Agrwal, 2014, Pellegrini *et al*., 2015), changes in crop quality and reductions in growth and yield (Dizengremel *et al*., 2008; 2009; 2012; Hatata *et al*., 2013; Taia *et al*., 2013; Sarkar *et al*., 2015; Yi *et al*., 2016).

Ismail *et al*. (2014) reported that ground level O_3 at a rural site in northern Egypt was about 77.81 nl l^{-1} with AOT40 of 28.141 ppm.h. These concentrations inhibited net photosynthetic rates, altered stomatal response and induced visible injury symptoms in leaves of three cultivars of pea (*Pisum sativum* L) plants.

The mechanism of plant response to O_3 is not fully understood (e.g. Severino *et al*., 2007; Pellegrini *et al*., 2015; Basahi *et al*., 2016).

However, the reductions in growth and yield are directly proportional to the decline in photosynthetic efficiency which in turn is associated with leaf injury (Nouchi, 1993; Wahid *et al.*, 2011; 2012; Ashmore *et al.*, 2005; Ismail *et al*., 2014; Ghude *et al.*, 2014).

The interplay between antioxidant defense system and reactive oxygen species (ROS) is a key metabolic pathway for plant growth, development and acclimatization to environmental stressors (Shao *et al*., 2008, del-Rio, 2015). Induction of different ROS species, such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide anion (O_2^-) and hydroxyl radical ($\cdot OH$), is stimulated upon decomposition of O_3 inside cells (Ranieri *et al*., 1999; Hassan, 2006). These ROS can cause chlorosis and chlorophyll degradation, lipid peroxidation and protein denaturation (Wu & Tiedemann 2002; Hassan & Twefik, 2006, Pellegrini *et al*., 2015). Plants have evolved protective scavenging systems in response to these ROS. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (GPX), as well as the enzymes of the ascorbate-glutathione cycle (Halliwell-Asada cycle): ascorbate peroxidase (APX), glutathione reductase (GR), monodehydro-ascorbate reductase (MDHAR) and dehydroascorbate reductase (DHA) provide endogenous defense against the accumulation of harmful concentrations of ROS (Wu &Tiedemann, 2002; Mishra & Agrawal, 2014).

Superoxide dismutase (SOD) is ubiquitous metaloenzyme that catalyses the dismutation of H_2O_2 and O_2 (Rubio *et al*., 2002; Shao *et al*., 2008). The superoxide radical is a potential precursor of the highly oxidizing $\cdot OH$ and therefore SOD is a crucial defense of the cell (Shao *et al*., 2008; Singh *et al*., 2014).

There are specific scavengers for these ROS generated by living cells to protect them from harmful effects of ROS (Pasqualini *et al*., 2001; Rubio *et al*., 2002). O_2^-,H_2O_2, $^1O_2$ are scavenged by SOD, hydrogen-donor peroxidases (ascorbate "APX" and guaiacol "GPX") and carotenoids, respectively (*e.g.* Hassan *et al*., 2013). Nevertheless, beside the above mentioned ROS – scavenging enzymes there is also other important part of the antioxidant defense array which is metabolites. The

most copious ones are ascorbate (AS), dehydroascorbate (DHA), reduced and oxidized glutathione (GSH and GSSG, respectively) (Marabottini *et al*., 2001; Mishra & Agrawal, 2014).

AS plays a defensive role both as direct scavenger ad by involvement in the xanthophylls cycle (Basahi *et al*., 2014, del-Rio, 2015). Ascorbate/glutathione cycle (enzymatic system includes SOD, AP, DHA and GR) is another powerful system in chloroplasts against ROS where GR recycles GSSG to GSH to maintain reducing power (Ivanov, 2003; Rai & Agrawal, 2008). Moreover, DHA and GSH could act as dual metabolites; react directly with O_3 by preventing its penetration into plasma membrane and help in degradation of O_2^- andH_2O_2 (Feng *et al*., 2010; Bortolin, 2014).

Contents of antioxidants and their related enzyme activities are known to be increased in response to oxidative-stress promoting factors which modify plant responsiveness to global climate change, including an increase in ground levels of O_3 (Shao *et al*., 2008; Burkart, 2013; Sarkar *et al*., 2015).

Pea plants (*Pisum sativum* L) is a common leguminous vegetable in the Middle East, it is very sensitive to O_3. Ismail *et al*. (2014) studied the photosynthetic response of three cultivars of pea (Little Marvel, Perfection and Victory). They found that Cultivar Victory is more susceptible to O_3 injury, in terms of declines in net photosynthetic rates, photosynthetic efficiency, and chlorophyll contents, than cultivar Little Marvel, while cultivar perfection showed intermediate sensitivity. Therefore, due to their differential sensitivity, our investigation was designed to evaluate the response of two O_3 differentially sensitive cultivars of Pea (*Pisum sativum* L. cultivars Little Marvel, and Victory) under natural conditions using open top chambers (OTCs).

Although there are many studies on O_3-induced ROS, the mechanisms underlying differential sensitivity of closely related species or even cultivars are poorly understood and fragmentary.

The present study was aimed to insight and further our knowledge on the changes in levels of antioxidant defense system in leaves of pea (*Pisum sativum* L. cultivars Victory and Little Marvel) in order to understand the mechanisms by which ambient O_3 differentially affects these plants.

Materials and Methods

Experimental design and plant rising

Seeds of two cultivars pea plants (*Pisum sativum* L. cultivars Little Marvel and Victory) were washed with distilled water and hand sown into a loamy clay soil with a pH 7.7 under six portable open-top chambers (OTCs). There were six rows in each chamber, the between-row distance was 30 cm, and each cultivar was planted in two rows in each OTC. Two weeks after sowing, when the first true leaf expanded, the seedlings were thinned to one per lot. There were 20 plants per row, thus there were 40 plant/cultivar/ chamber. The plants were irrigated manually with tap water once a week for the whole growing season (88 days).

No pesticides or fertilizers were applied.

OTCs were distributed in a split plot design; three chambers received charcoal-filtered air (FA) and the others received ambient non-filtered air (AA). Air filtration experiments with OTCs were carried out in between 15 November 213 and 10 February 2014 (09:00–17:00 h Egyptian local time). Air was distributed into the chambers by means of two perforated semicircular annuli attached to the wall of the chamber at heights of 0.50 and 1.00 m above ground level. Ventilation was continuous at a rate of 1800 mh^{-1} (2.5 air changes a minute).

Details of experimental facilities and plant culture were discussed elsewhere (Ismail *et al*., 2014).

Ambient air monitoring: AQM60 was used to monitor O_3, SO_2 and NO_x. It has an internal zero air scrubber for regular calibration and span self-adjustment. Air was sampled in OTCs with the help of Teflon tube placed above canopy of the plants. AOT40 (accumulated ozone over a threshold concentration of 40 ppb) value was calculated according to Ismail *et al.* (2014). Metrological parameters were recorded by Automatic Weather Sensors (AWS, GRWS 100, USA).

Sampling of plants for antioxidants bioassay: Fourteen days after sowing (DAS), when the first true leaf expanded, plants were harvested destructively at 5-d intervals for determination of enzymatic and non-enzymatic antioxidants to cover different stages of development. Four plants of both cultivars were selected randomly from each OTC (FA and AA) at a time, hence making a set of twelve replication for each treatment (n = 12). The youngest fully expanded trifoliate leaves from top of the plant canopy were selected for analyses.

Antioxidant enzymes assays: Leaves were cut from each treatment and immersed in liquid nitrogen and kept in a deep freezer at - 80^{o}C until the analyses were performed (Lee *et al*., 1997; Hassan, 2006; Hassan *et al*., 2013). Samples were weighed and ground at about ^{o}C in 25 m Tris–HCl buffer containing 3 mM $MgCl_2$ (Sigma Aldrich, USA). After centrifugation at 20 000 rpm for 15 min (Hettich, EBA 21, ZENTRIFUGEN, Germany), the supernatants were used for the enzyme bioassays and the results were expressed on protein basis (Bradford, 1976; Pasqualini *et al*., 2001; Sarkar *et al*., 2015).

All assays were performed using a final volume of 1 mL, with at least duplicate assays undertaken on each sample. Moreover, the assays were end-point determinations (Hassan, 2006).

Glutathione was analysed with a Schimadzu R.F. 1201 high performance liquid chromatography (HPLC), and peaks were detected by a fluorescence detector using an excitation wavelength of 340 nm and an emission wavelength of 420 nm. Total glutathione (GS) and oxidized glutathione (GSSG) were quantified by comparing peak areas with known standards. Reduced glutathione (GSH) was calculated by subtracting GSSG from GS (Lee *et al*., 1997; Hassan 2006).

Ascorbate (AS) and dehydroascorbate (DHA) were determined according to Kampfenkel *et al.* (1995) and the modified method of Ribas *et al.* (2010) This analysis is based on the reduction from $Fe^{3+}$to Fe^{2+} of the ascorbic acid (AS), followed by the spectrophotometric determination of

ion Fe^{2+} complexed with 2,2-dipiridil. Total AS was determined in a reaction mixture of 1.0 ml containing 50 μl apoplastic fluid, 100 μl phosphate buffer 0.2 m (pH 7.4), 50 μl DDT 10mm. After shaking and incubation for 20 min at 42ºC in water bath, 50 μl of 0.5% NEM were added, with a following incubation for 1 min at room temperature. AS levels were determined in the same way with exception for the increasing of DDT and NEM which were substituted by water. Coloring was obtained by adding of 250 μl 10% trichloroacetic acid (TCA), 200 μl of 42% phosphoric acid, 200 μl of dipiridil dissolved in 70% ethanol and 100 μl of 3% phosphoric acid. After shaking, a mixture was incubated for 40 min at 42ºC and the absorbance values of the final coloured solutions were recorded at 525 nm. Concentrations of DHA were calculated from the difference of total AS and AS. Corrections were made for color development in the absence of sample. AS and DHA (both from Sigma) were dissolved in 6% (w/v) TCA and used for calibration (Foyer *et al*., 1989; Ribas 2010).

SOD (EC 1.15.1.1) activity was monitored according to Lee *et al*. (1997). The extraction mixture contained 13 mM L-methionine, 63 µM nitro blue tetrazolium and 2 µM riboflavin (pH 7.8). The ability of the extract to inhibit the photochemical reduction of nitro blue tetrazolium was determined at 560 nm (Schimadzu UV-1201 spectrophotometer). The amount of the extract resulting in 50% inhibition of nitro blue tetrazolium reaction is defined as one unit of SOD activity (Hassan, 2006).

GPX (EC, 1.11.1.7) activity was determined by adding 50 mM phosphate buffer (pH 6.1), 1% H_2O_2 and 1% guaiacol to the extract, and the absorbance was determined at 470 nm (Mishra & Agrawal, 2014).

APX (EC, 1.11.1.11) activity was determined according to Maehly & Chance (1954). The reaction mixture contained 50 mM potassium phosphate, 0.5 mM ascorbate, 0.1 mM ethylenedimethyl tartaric acid (EDTA) and 0.1 mM H_2O_2, and the absorbance was determined at 290 nm.

Glutathione reductase (GR; EC, 1.6.4.2) activity was determined (Lee *et al*., 1997). The enzyme activity was monitored by measuring a decrease in absorbance at 334 nm resulting from oxidation of reduced nicotine amide dinucleotide (NADH) (6.2 mM^{-1}). The assay mixture contained 0.1 M Tris–HCl (pH 8.0), 1 mM EDTA, 0.1 mM NADH and 1 mM oxidised glutathione (GSSG) and the leaf extract. Protein oncentrations of leaf extracts were determined as described earlier (Bradford, 1976).

It is worth to mention that levels of antioxidants were determined in leaves without any visible injury symptoms (Bender *et al*., 1994; Hassan, 2006). The leaves without foliar injury symptoms were selected to avoid any losses in contents of antioxidants in the chlorotic or necrotic areas.

Measurements of hydrogen peroxide: The H_20_2 assay followed the method of Wu & Tiedemann (2002) and modified method of Hassan (2006). Fifteen leaf discs (10-mm diameter) were submerged in 750 µL reagent mixture containing 0.05% guaiacol and horseradish Peroxidase (350 µL L^{-1}, 250 U mL^{-1}) in 25 mM sodium phosphate buffer (pH 7.0) and incubated for 2 h at 20ºC in the dark. Then, a volume of 250 µL was transferred into 96-well microtitreplates and the absorbance was immediately measured at 450 nm in a plate reader photometer (SLT, Spectra, Dixons Ltd, Pure Chemicals for Laboratories, Switzerland).Commercial H_2O_2, which was used for standard curves, was calibrated by titration with $KMnO_4$.

Data analysis: data were subjected to one-way analysis of variance (ANOVA), with O_3 as a factor, followed by a LSD test and p values ≤ 0.05 were considered significant (using the STATGRAPHICS statistical package, Package 3, UK), based on chamber replicates.

Moreover, the individual and interactive effects of age (A), cultivar (Cv), and treatment (T) on different biochemical traits were analysed using multivariate analysis of variance (ANOVA). The significance of difference between treatments was calculated using "paired sample test.

Results

Metrological parameters are recorded in Table 1. Air temperatures, light intensity, precipitation, relative humidity were 22.5ºC, 1345 µmol $m^{-2}s^{-1}$, 128 mm $year^{-1}$ and 60%, respectively.

Reduced glutathione (GSH) showed no significant response ($p>0.05$) in leaves of Little Marvel plants, except for 25 and 30 DAS, where it was found to decease by 25% (Fig. 1). However, GSH was reduced by about 26% in leaves of Victory plants over the entire period of experiment. The effect started 35 days after treatment (DAT). The maximum impairment was at 60 DAT, where ambient O_3 caused 40% reduction in its content. Contents of oxidized (GSSG) and total glutathione were increased by 90% and 39% in Cv. Little Marvel, while they were decreased by 14% and 37%, respectively in cv. Victory in response to ambient O_3 (Fig. 1). Contents of total glutathione followed the same pattern.

L-ascorbate (AS) and total ascorbate were decreased in Little marvel and Victory plants by 21 and 41%, and by 15 and 39 %, respectively (Fig. 2). DHA responded differently to ambient O_3, while it was decreased by about 33% in Little Marvel plants, it was not affected ($p>0.05$) in Victory plants (Fig. 2).

Results of multivariate ANOVA revealed that variations recorded in GS and AS were significant due to A, Cv, T and their interactions except A × T (Table 2).

On the other hand, Activities of glutathione reductase (GR), ascorbate peroxidase (APX), superoxide dismutase (SOD) and guaiacol peroxidase (GPX) were increased by 31, 61, 13 and 57%, respectively, in leaves of Little marvel plants exposed to ambient O_3 when compared to those grown in filtered air (Figs. 3-6). Nevertheless, activities of SOD, GR, GPX showed insignificant response to ambient O_3 in Victory plants (Figs. 3, 5, 6), while APX was found to increase by 13% at 40 DAT (Fig. 4).

Moreover, there were significant interaction between age (A), cultivar (Cv) and treatment (T) singly and in combination on peroxidases "APX and GPX" and GR except A x Cv in GR ($p<0.05$) (Table 1). On the other hand, SOD showed significant difference with A, Cv x T and A x Cv x T ($p<05$) (Table 2).

The rate of H_2O_2 induction was significantly increased at all the ages of sampling in both cultivars grown in ambient air at all sampling dates as compared to plants grown in FA. H_2O_2 significantly increased by 13% and 39% in Little Marvel and Victory plants at 70 DAT, respectively (Fig. 7). Results of multivariate ANOVA revealed that variations recorded and were significant due to A, Cv, T and their interactions except A x Cv (Table 2).

Table 1. Metrological parameters recorded during the course of the experiment.

Air temperatures (ºC)	light intensity (µmol $m^{-2}s^{-1}$)	Precipitation (mm $year^{-1}$)	relative humidity (%)
22.5	1345	128	60

Table 2. Levels of significance of multivariate ANOVA test for different parameters of Little Marvel and Victory.

Parameter	GSH	GSSG	TG	AA	DHA	TA	SOD	GPX	GR	APX	H_2O_2
A	*	**	**	**	n.s.	n.s.	*	*	*	*	*
Cv	**	***	***	***	*	**	n.s.	***	**	***	*
T	***	***	***	**	*	***	n.s.	**	***	**	**
A X Cv	*	*	*	*	n.s.	n.s.	n.s.	*	n.s	*	n.s.
A X T	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	*	*	*
Cv X T	***	**	**	*	**	n.s.	*	**	***	***	**
A X Cv X T	***	***	*	**	*	**	*	*	**	**	*

A = Age; Cv = Cultivar; T = Treatment; TG = Total glutathion; TA = Total ascorbate. *, ** and *** are significant at $p \leq 0.05$, $0.01 < p < 0.05$ and $p \leq 0.01$, respectively

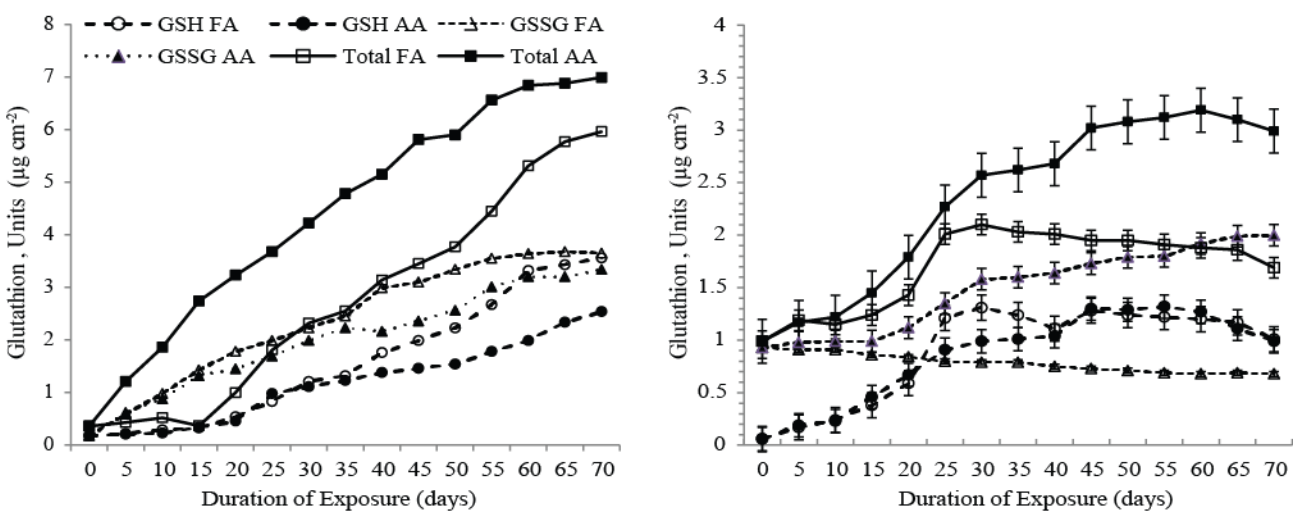

Fig. 1. Changes in glutathione contents in leaves of Little Marvel and Victory Plants grown at either filtered air (FA) or ambient air (AA). GSH = reduced glutathione, GSSG = oxidized glutathione. (n = 12 ± 1 S.E. of the means).

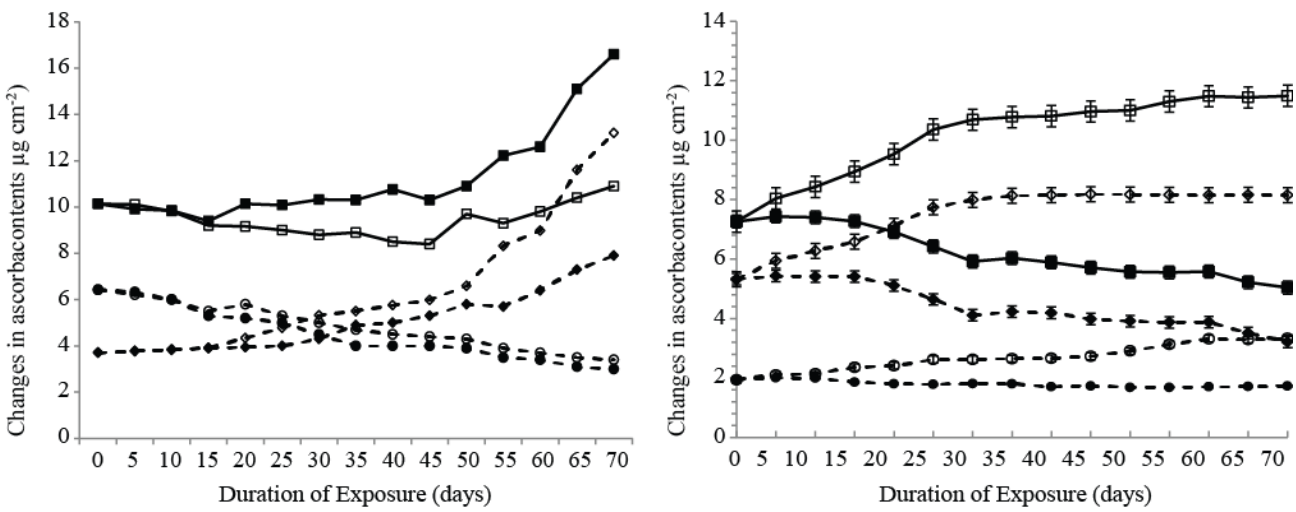

Fig. 2. Changes in ascorbate contents in leaves of Little Marvel and Victory plants. AS = L-ascorbate, DHA = dehydroascorbate reductase. (Legends as Fig. 1).

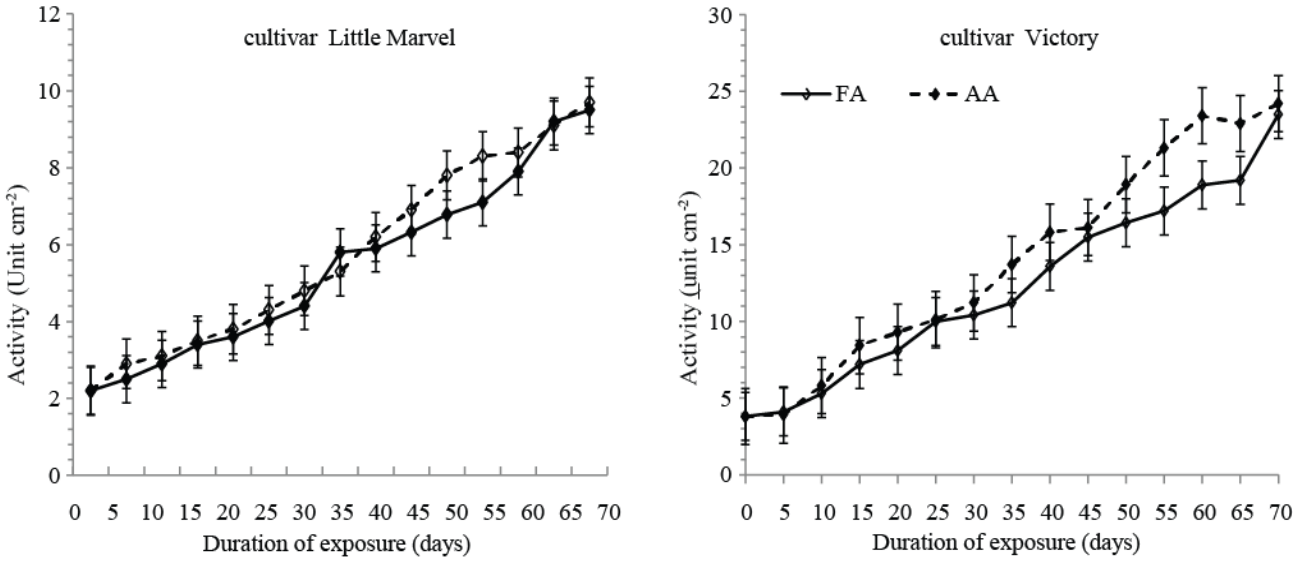

Fig. 3. Changes in the activities of superoxide dismutase (SOD) in leaves of Victory (a) and Little Marvel (b) plants. Legends as Fig. 1.

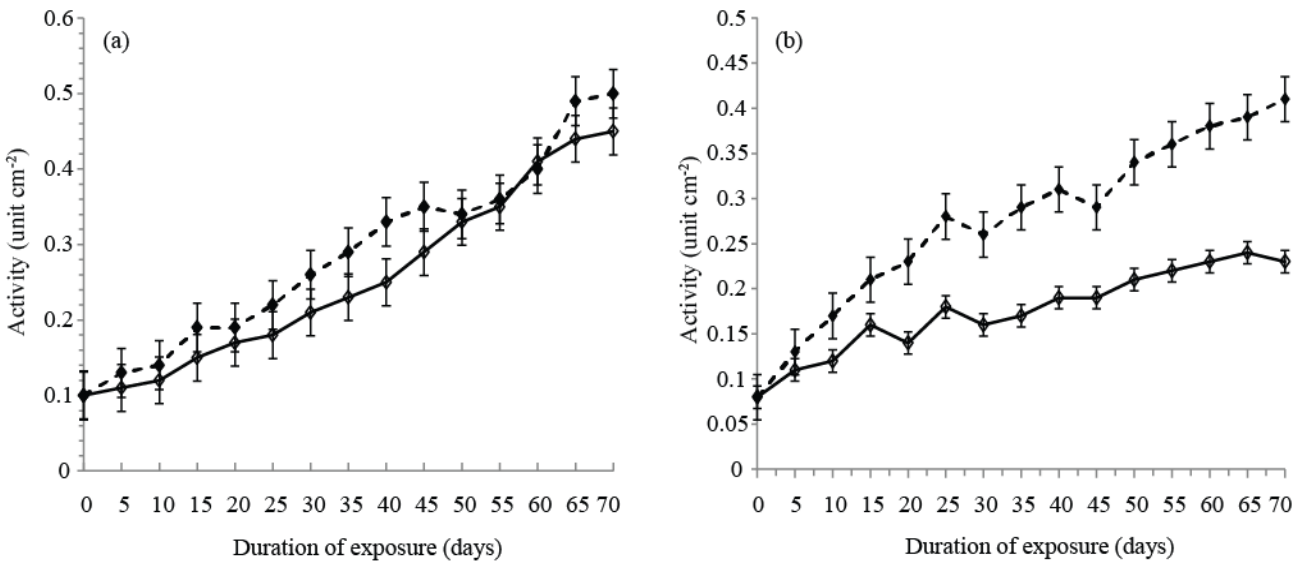

Fig. 4. Changes in the activities of guaiacol peroxidase (GPX) in leaves of Victory (a) and Little Marvel (b) plants. Legends as Fig. 1

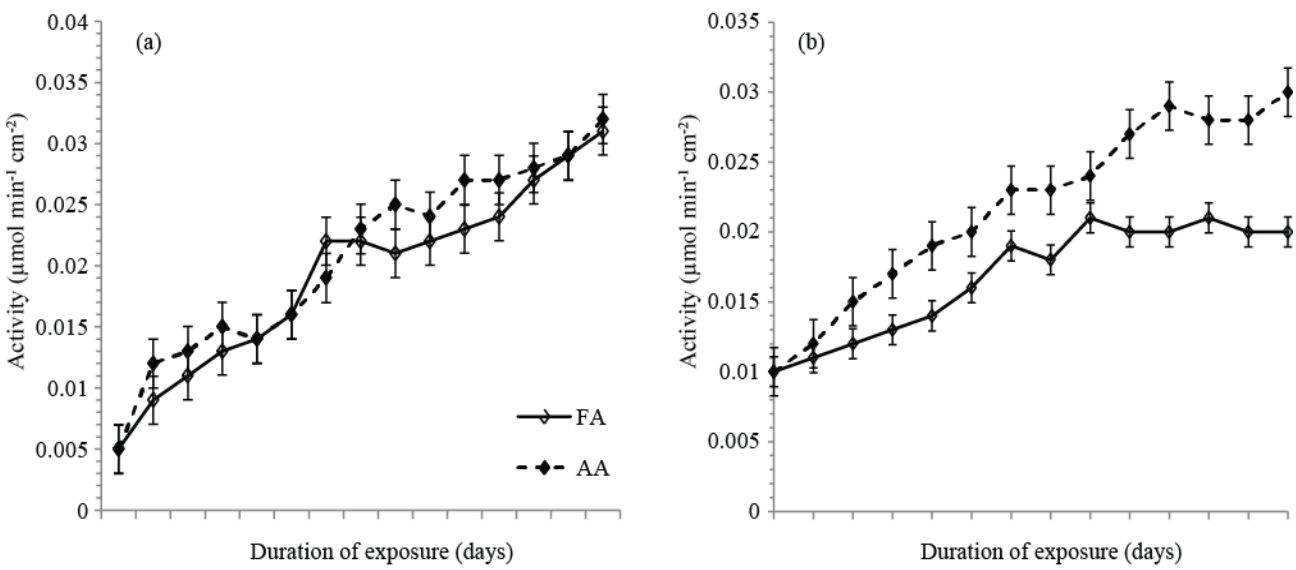

Fig. 5. Changes in the activities of glutathione peroxidase (GR) in leaves of Victory (a) and Little Marvel (b) plants. Legends as Fig. 1.

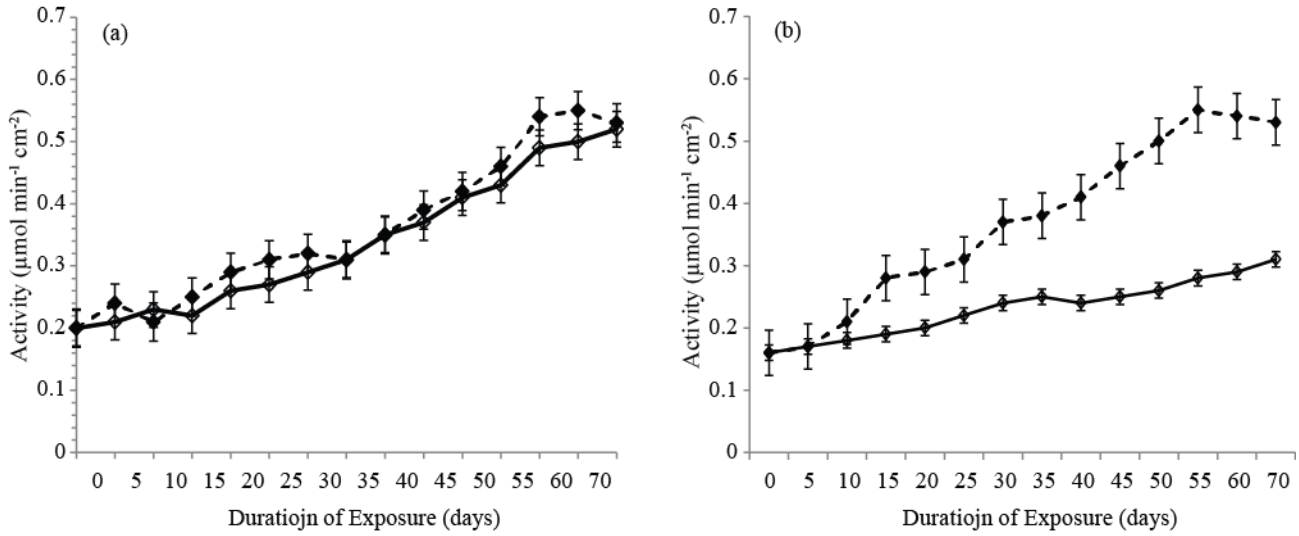

Fig. 6. Changes in the activities of ascorbate peroxidase (APX) in leaves of Victory (a) and Little Marvel (b) plants. Legends as Fig. 1.

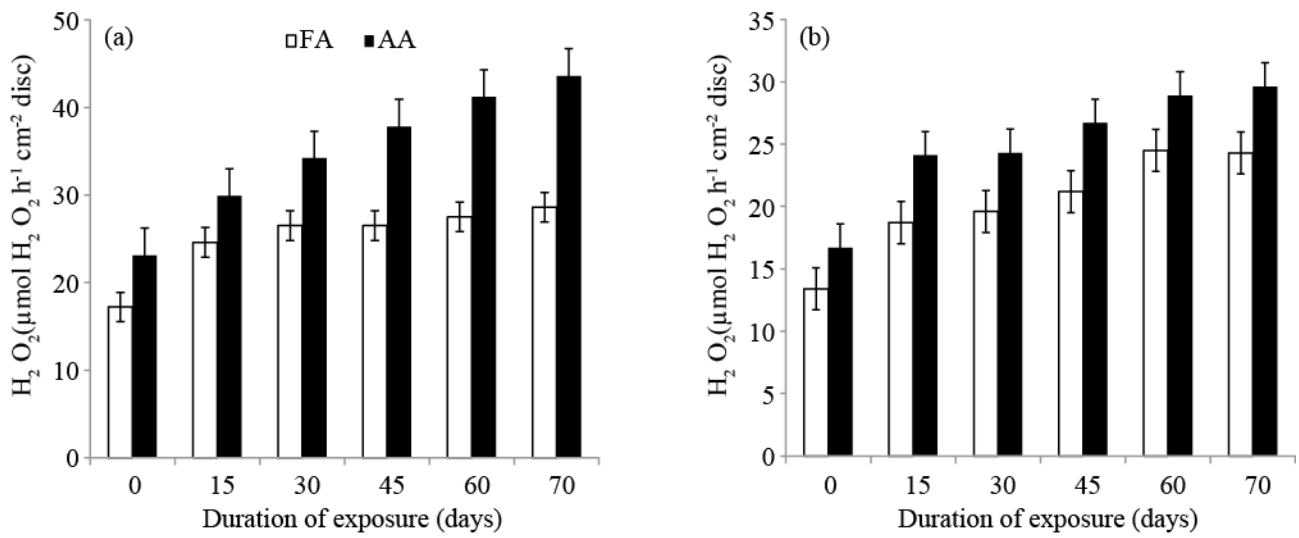

Fig. 7. Effects of exposure to ambient air on hydrogen peroxide (H_2O_2) content in Little Marvel and Victory plants. Columns represent means of six replicates ± 1 SE.

Discussion

Responses of antioxidant levels and activities of related enzymes in response to ambient O_3 in the present study were noticeably different among pea plants (*P. sativum* L. cultivars Little Marvel and Victory).

It is strongly believed that oxidative stress-induced changes in activities of antioxidant enzymes could be due to formation of new isoforms or may be due to alteration in the existing isoforms (Mishra and Agrawal, 2014). This warrants further investigation.

AS has a strong affinity to be oxidized to DHA (e.g. Bender *et al*., 1994); this explains its marked reduction in its level in plants exposed to ambient O_3 in the present study. Moreover, the percentage reduction in cv. Little Marvel was higher than that in cv. Victory. This is in agreement with the results of Sakaki *et al*. (1983), Lee *et al*. (1984), and Nouchi (1993) on rice, soybean and, spinach, respectively. However, activity of DHA was increased after exposure to ambient O_3 in cv. Little Marvel, which would suggest a rapid metabolic oxidation of As into DHA as a protective mechanism against phytotoxic effects of O_3 (Shao *et al*., 2008, Pellegrini *et al*., 2015). This was not the case in Victory plants, where AS was decreased while activity of DHA was not affected by ambient O_3 ($p>0.05$). This suggests that the enzyme did not act as a protective system as it did in the case of Little Marvel plants.

O_3 can induce a significant increase in peroxidase activities, "APX and GPX" (Pasqualini *et al*., 2001; Hassan, 2006; Mishra & Agrawal, 2014; Sarkar *et al*., 2015).An increase in GPX in Little Marvel Plants in the present study indicates production of H_2O_2 in response to ambient O_3 and there was a strong correlation between both parameters ($R^2 = 0.134$, data not shown). This is in agreement with the results of Bender *et al*. (1994) on wheat, Nouchi (1993) and Sarkar *et al*. (2015) on rice, Pasqualini *et al*. (2001) on tobacco; and Mishra & Agrawal (2014) and mung beans. O_3-induced H_2O_2 could reduce the rate of enzymes degradation and/or activates their biosynthesis (Bortolin *et al*., 2014), leading to activation and induction of specific H_2O_2 scavenging

enzyme system and hence a higher resistance of Little Marvel to ambient O_3.

Production of APX and GPX in little marvel plants exposed to ambient O_3 indicates the adaptation to O_3 and increasing ability of plants to scavenge toxic oxygen species as a protective mechanism against oxidative stress (Pellegrini *et al*., 2015). Moreover, increasing activities of these enzymes is an indicator of oxidative stress and production of H_2O_2.

Both GR and APX are integral parts of the ascorbate glutathione cycle and efficient H_2O_2 scavengers; their induction is a strategic management of O_3 induced oxidative stress (Calatayud *et al*., 2002; 2004; Cho *et al*., 2008; 2011). Their activities were increased in Little Marvel plants, while cultivar Victory showed insignificant response in the present study. This depicts and further our assumption that Little Marvel plants are more tolerant to O_3 than Victory ones. Moreover, they are strongly correlated to AS as they help in its regeneration and utilization (Cho *et al*., 2008). Our results shows that, the antioxidant activities of GR and APX are strongly correlated to endogenous levels of the antioxidant metabolites ascorbic acid and glutathione content and this is in agreement with Pasqualini *et al.* (2001) and Mishra & Agrawal (2014).

SOD plays a key protective role against phytotoxic effects of O_3 (Sarkar *et al*., 2015). It scavenges O_2^- and catalyzes its dismutation to H_2O_2 and O_2 (Sing *et al*., 2014). There is inconsistency and discrepancy in the literature regarding its activity with both increases and decreases have been reported. Its activity in rice leaves was increased after exposure to 90 nll^{-1} O_3, while it was decreased after exposure to 50 nll^{-1} O_3 (Nouchi 1990). These variations were related to duration of exposure (Nouchi, 1990). Recently, Singh *et al*. (2014) found higher SOD activity in O_3-treated maize leaves compared to plants grown under filtered air conditions. Moreover, Mishra & Agrawal (2014) found a significant increase in SOD activity in a resistant tropical mung bean (*Vigna radiata* L.) cultivar, while the sensitive one showed no significant response. This is in agreement with our results, as Victory plants showed no response while Little Marvel ones showed an increase in SOD content in plants exposed to ambient O_3 at 55 and 60 DAT. Nevertheless, it remains to determine whether the resistance or susceptibility of plants to ambient O_3 is correlated to SOD activities. Contrasting ozone sensitivities in pea cultivars could be a scribed to the role that the constituents of the ascorbate–glutathione cycle. Lower induction of SOD is responsible for inhibition in production of superoxide radicals necessary for detoxification of H_2O_2 and vice versa, and this is the case in our study (Sarkar *et al*., 2015).

Recently, Gill *et al*. (2015) stated that SOD catalyzes conversion of $O_2^{\bullet-}$ to O_2 and H_2O_2 to protect plants against potential consequences caused by these superoxide radicles. Hence, it represents the first line of defense against abiotic stress-accrued enhanced ROS and its reaction product.

Our results point out that increased activities of antioxidant enzymes most likely caused ozone resistance. This protective mechanism of against harmful oxidative stress, described both on levels of ROS and the corresponding scavenging enzymes, is reported in pea plants for the first time. Plant hormones could play roles in the response of plants, this warrants a further investigation in the future.

In conclusions there is an array of enzymatic antioxidants in plant cells to counteract the oxidative stress induced by O_3 and its-induced ROS. This array is responsible for differential sensitivity of pea cultivars (Little Marvel and Victory) subjected to chronic O_3 stress inside open-top chambers. Moreover, the degree of inducibility of the system clearly discriminates between ozone-sensitive cv. Victory and ozone tolerant cv. Little Marvel since the system is more readily triggered in the tolerant cultivar. Moreover, the results of the present study would add up in understanding differential sensitivity of cultivars against O_3 and help in developing O_3-resistant cultivars by using conventional breeding techniques to increase the yield to feed the rapidly growing populations in developing countries.

Acknowledgment

Authors would like to thank Professor Roy Harrison of Birmingham University, UK for revising this manuscript authors are indebted to anonymous reviewers for their valuable comments.

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(Received for publication15 January 2016)