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Chemical and Natural Plant Extract in Ameliorating Negative Impact of Tropospheric Ozone on Wheat Crop: A Case Study in a Part of Semiarid North West India

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ABSTRACT

Current rise in concentration of tropospheric ozone (O_3) has deleterious effect on growth and yield of crops. Wheat is one of the most O_3 sensitive crops. Application of Chemical antioxidants may be beneficial to lessen the harmful effects of O_3 on crops. A two year study growing wheat (variety PBW550) was carried at the farms of Indian Agricultural Research Institute, New Delhi in 2010–11 and 2011–12 to evaluate the efficacy of some natural and environment friendly antioxidant chemicals in ameliorating the negative impacts of elevated ozone on growth and productivity of wheat. The treatments were i) charcoal filtered air (CF) ii) 1% ascorbic acid (AA), iii) 100 ppm quercetin (Q), iv) 10% marigold leaves extract (*Tagetes patula* var pusa arpita) (T) and v) elevated O_3 control (C). Additional 25–35 ppb of O_3 over the ambient levels was maintained in all open top chambers except the CF treatment. The seasonal daily average O_3 concentration in ambient air was 38 ppb in 2010–11 and 29 ppb in 2011–12 during the crop growth period. The exogenous application of antioxidants increased the levels of endogenous leaf ascorbic acid. The activity of antioxidant enzymes (superoxide dismutase, catalase and peroxidase) was lowered by 5 to 34% in antioxidants sprayed plants as compared to elevated O_3 control (C). The application of antioxidants increased the yield of wheat by 23–26% in AA, 13–15% in Q and 8–10% in T as compared to C. The micronutrient (Cu, Fe, Zn and Mn) content of wheat grains on application of exogenous ascorbic acid was higher. The application of antioxidant chemicals was effective in alleviating the negative impacts of elevated O_3 on enzyme activity and nutritional quality of wheat which increased the growth and yield of the crop.

Keywords: Tropospheric ozone; Wheat; Antioxidant; Ascorbic acid; Stress enzyme.

INTRODUCTION

Tropospheric ozone (O_3), a powerful oxidizing agent, is responsible for more damage to vegetation than any other air pollutant. Current O_3 concentration is considerably higher in the Northern Hemisphere than the Southern Hemisphere, with background monthly mean O_3 concentration in the Northern Hemisphere ranging from 35 to 50 ppb (Emmons *et al.*, 2010). Mean monthly O_3 concentrations often reaches 50 ppb during important crop growing periods in Asia (EANET, 2006). Future tropospheric O_3 concentrations are expected to vary with emissions of O_3 precursors and climate change scenarios. Global photochemical models project that under current emission scenarios; further increase in O_3 concentrations by 2030 is expected in several parts of Asia (Dentener *et al.*, 2006). Large areas of India show ozone values above the AOT40 threshold limit (3000 ppb h for 3 months). Simulated AOT40 values are found to be substantially higher throughout the year over the most fertile Indo-Gangetic plains than the other regions of India, which can have an adverse effect on plants and vegetation in this region (Deb Roy *et al.*, 2009).

Ozone is one of the most phytotoxic air pollutant affecting growth and yield of plants. There are reports of decrease in yield of cereals including wheat due to tropospheric O_3 in

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the South Asian subcontinent (Wahid, 2006; Singh and Agrawal, 2009). According to Van Dingenen *et al.* (2009) the global crop yield loss due to tropospheric O_3 was estimated to be worth $14–26 billion for the year 2000, about 40% of which may be occurring in China and India. India is the second largest wheat producing country (88.31 Mt) with a productivity of 3.06 Mt ha^{-1} (MOA, 2011–12). Ghude *et al.* (2014) reported the present-day ozone-induced damage to wheat and rice in India to be 3.5 ± 0.8 Mt and 2.1 ± 0.8 Mt respectively which was sufficient to feed roughly 35% of population below poverty line in India. In another recent study by Sinha *et al.* (2015) relative yield losses based on the AOT40 metrics ranged from 27–41% for wheat which amounted to a crop production losses of 20.8 Mt in fiscal year 2012–2013 and 10.3 Mt in fiscal year 2013–2014 for Punjab and Haryana jointly.

Ozone enters the leaf interior through stomata and interacts with the constituents of apoplast, inducing the formation of damaging reactive oxygen species (ROS) that can initiate multiple oxidation events in the cells (Apel and Hirt, 2004). Alteration in the dark and light reactions of photosynthesis, lipid peroxidation and changes in membrane permeability are some of the events widely documented in O_3 stressed plants (Calatayud and Barreno, 2004). In response, plants have evolved certain biochemical mechanisms, such as alteration in antioxidant enzyme activities and metabolite concentrations to avoid the negative influence of ROS on cells. An efficient antioxidant system comprises non-enzymatic antioxidants (ascorbate, salicylate, glutathione, tochopherols etc.) and enzymatic antioxidants like superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Foyer and Noctor, 2003).

Reduced growth and yield in plants exposed to O_3 may be due to reductions in stomatal conductance (gs), net photosynthetic CO_2 assimilation (A), and carboxylation efficiency (Morgan *et al.*, 2003). Long and Naidu (2002) attributed these reductions to a loss of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and decreased Rubisco activity. RuBisCO, is an enzyme involved in the first major step of carbon fixation, a process by which atmospheric carbon dioxide is converted by plants to energy-rich molecules such as glucose.

Enhanced production of antioxidants for augmented O_3 tolerance can be achieved through exogenous application of specific antioxidants (Ashraf and Foolad, 2007). It has been observed that exogenous application of antioxidants such as ascorbic acid in *Vigna radiata* (Yoshida *et al.*, 1994), quercetin, catechin and marigold leaves extract in clover plants counteracted O_3 induced growth inhibition (Blum and Didyk, 2007). At the present day O_3 levels also the antioxidants showed significant increments in yield in a preliminary field study in potato crop (unpublished). However there are not many studies on the application of synthetic and natural antioxidant chemicals on the plant defence mechanisms especially in cereal crops. As the ozone levels are increasing and there are different scenarios for the rate of increase in ozone levels in future. Some projections say that ozone is projected to increase at a rate of 10 ppb per decade from 2000 to 2020 (Austin and Butchart, 2003). Simulations for the period 2015 through 2050 project increases in ozone of 20 to 25% (Grewe *et al.*, 2001; Hauglustaine and Brasseur, 2001), and simulations through 2100 indicate that ozone may grow by 40 to 60% (Stevenson *et al.*, 2000, Hauglustaine *et al.*, 2005). Impacts are going to be most severe over India and China, in recent decades (Fishman *et al.*, 2010). Keeping these variable scenarios we tried to evaluate what would be the impact of antioxidant chemicals on wheat yields under elevated ozone. The present study was carried out to assess the efficacy of exogenously applied antioxidants on enzyme activity, yield and nutritional quality of wheat grown under elevated O_3.

METHODS

Study Area

Location, Climate and Soil Characteristics

A field experiment was conducted growing wheat (variety-PBW550) during *rabi* season (December–April) in 2010–11 and 2011–12 at the experimental farm of the Indian Agricultural Research Institute, New Delhi, India (Fig. 1). The site is located in the Indo-Gangetic alluvial tract at 28°40'N and 77°12'E, at an altitude of 228 m above mean sea level. The climate of the region is subtropical, semi-arid and January is the coldest month of the year with a minimum temperature ranging from 5 to 7°C. The mean maximum and minimum temperatures from December to April are 36.6 and 22.6°C, respectively. The mean annual normal rainfall is 650 mm, while July and August are the wettest months. The annual mean pan evaporation is about 850 mm.

The alluvial soil **(**Typic Ustochrept**)** of experimental site was having a sandy loam texture (46% sand, 33% silt and 21% clay) and had a bulk density of 1.42 g cm^{-3}, pH (1:2 soil:water) of 8.61, electrical conductivity of 0.158 dS m^{-1}, CEC of 7.3 C mol (p^+) kg^{-1}; and organic carbon, total N, Olsen P, and ammonium acetate extractable K contents of 0.317%, 156.8 kg ha^{-1}, 14.71 kg ha^{-1}, and 176.23 kg ha^{-1}, respectively. The experimental soil was under rice-wheat rotation for the past two years.

Treatments and Crop Management

The experiment was carried out growing wheat under five treatments in open top chambers (OTC) of 3 meter diameter and 2.5 meter height (Table 1). Five OTCs were selected separately for studying the effect of each treatment. All the treatments consisted of three replicates. In the CF treatment air was blown in OTC through charcoal filters to reduce O_3 levels in the ambient atmosphere by around 80–85%. In other treatments, additional 25 to 35 ppb of O_3 was blown with the help of ozone generators (Systocom, Varanasi, India). Generated ozone was blown into the open top chambers along with the ambient air with the help of blowers. The UV radiation from the UV lamps present in the ozone generators oxidized the oxygen present in ambient air to O_3. Ozone levels in ambient air and inside the OTCs were monitored by an automated continuous ozone analyzer (Model APOA-370, Horiba, Germany), using a cross flow modulated ultraviolet absorption method. PBW550, a new wheat variety, recommended for cultivation in north-

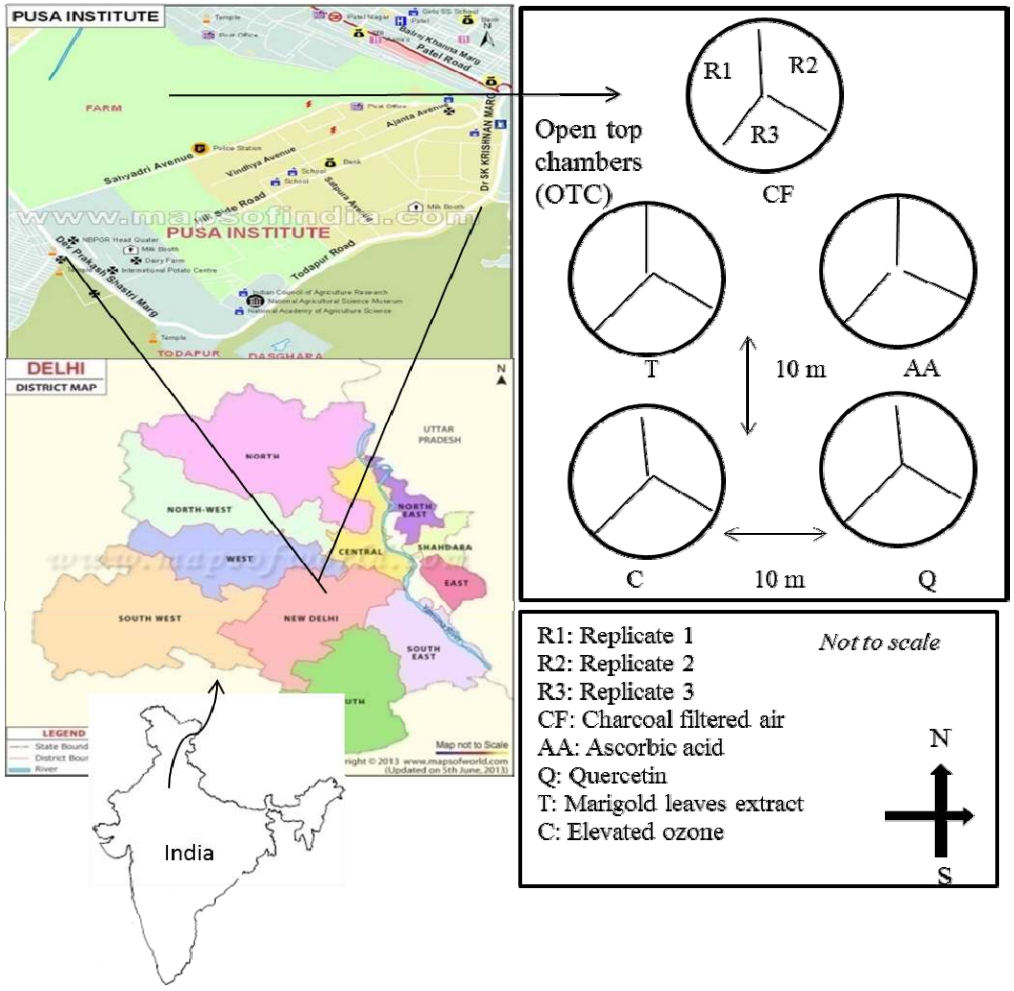

Fig. 1. Block diagram for experimental arrangement along with study area.

Table 1. Treatments.

Sl No.	Treatment	Concentration of antioxidants used	Mode of application	Ozone (O_3) concentration
1	Ascorbic acid (AA)	1% ascorbic acid water solution	Foliar spray	Ambient O_3 + 25–35 ppb O_3
2	Quercetin (Q)	100 ppm quercetin water acetone solution	Foliar spray	Ambient O_3 + 25–35 ppb O_3
3	Marigold leaves extract (T) (*Tagetes patula* L.)	10% marigold leaves water extract	Foliar spray	Ambient O_3 + 25–35 ppb O_3
4	Charcoal filtered air (CF)	No antioxidant	-----	< 10 ppb O_3
5	Elevated ozone Control (C)	No antioxidant	-----	Ambient O_3 + 25–35 ppb O_3

western plains of India was used in our study. The wheat plants were exogenously sprayed with 1% Ascorbic acid (AA), 100 ppm Quercetin (Q) and 10% Marigold leaves extract (*Tagetes patula* var. pusa arpita) (T). The solution of each antioxidant chemical was freshly prepared using distilled water except quercetin where along with distilled water acetone was also used. Foliar spray of antioxidants was carried out six times during the crop duration. It was started one week after crown root initiation stage (CRI) and sprayed between 11:00 and 12:00 h at 10 days interval upto 85 DAS. Initial foliar sprays were done at 10 mL plant^{-1} then gradually increased to 50 mL plant^{-1} and finally reached upto 100 mL plant^{-1}. Fresh leaves of french marigold (*Tagetes patula* var. pusa arpita) (growing time during mid-December to mid-February) was collected and macerated in porcelain mortar with warm distilled water (40–50°C). The extracts were filtered and applied to test plants as a leaf spray immediately after preparation (within 1–2 hours after leaves collection). The marigold extract concentration was 10% as calculated by fresh weight. The seeds were sown at a spacing of 20 × 20 cm (row × row). The plots were treated with recommended dose of NPK fertilizer (120:60:40) applied in the form of urea, diammonium phosphate and muriate of potash. In this study elevated O_3 was taken as a

control to compare the efficiency of the antioxidant chemicals in reducing the impacts of elevated O_3 to crop growth and yield.

Plant Sampling and Analysis

The different oxidative stress enzymes activity (Superoxide Dismutase, Catalase, Peroxidase) and rubisco activity was measured in the third leaf from above of the wheat plants. Leaf samples were collected and frozen immediately in liquid nitrogen (N_2) and stored at –80°C for further analysis. The stress enzymes activity was measured at two growth stages viz. flowering and milky stage at 70 and 100 DAS in 2010–11 and 74 and 102 DAS in 2011–12 respectively. While Rubisco enzymes activity was measured at flowering and milky stages of the crop in 2011–12. The sugar, starch and protein content was measured in biomass at the above mentioned growth stages. In the wheat grains sugar, starch and protein content was measured at the time of final harvesting. Single-leaf net photosynthetic rates and stomatal conductance were measured with portable photosynthesis systems (LI-6400-40 Portable Photosynthesis System). Final harvesting of the crop was carried out on 134 DAS and 131 DAS in the years 2010–11 and 2011–12 respectively. For yield and biomass determination, plant parts were oven dried (65°C) till constant weight was achieved. Yield parameters of average number of tillers, average number of spikes, average number of grains spike^{-1}, grain yield, total biomass and 1000 grain weight were recorded. The harvest index (economic yield/biological yield) of the crop under different treatments was recorded.

Measurement of Ascorbic Acid in Leaf

Ascorbic acid content in leaf was determined as described by Mukherjee and Choudhuri (1983). Leaf material (0.25 g of the third leaf) was extracted with 10 mL of 6% trichloroacetic acid. Four mL of the extract were mixed with 2 mL of 2% dinitrophenyl hydrazine (in acidic medium) followed by the addition of one drop of 10% thiourea (in 70% ethanol). The mixture was boiled for 15 min in a water bath and after cooling at room temperature, 5 mL of 80% (v/v) sulfuric acid (H_2SO_4) were added to the mixture at 0°C. The absorbance was recorded at 530 nm.

Extraction and Analysis of Antioxidant Enzymes, Determination of Rubisco Activity, Sugar, Starch and Soluble Protein in Leaves and Grains

Superoxide dismutase (SOD) activity was estimated by the method explained by Dhindsa *et al.* (1981). Catalase (CAT) was assayed according to Aebi (1984). Peroxidase (POX) assay was done according to the method by Castillo *et al.* (1984). The rubisco enzyme activity was estimated as per Fair *et al.* (1973). **S**ugar and starch estimation was carried out as per method adopted by Mc. Cready *et al.* (1950). Estimation of soluble protein was carried out by Bradford Dye Binding method (Bradford, 1976).

Macro and Micro Nutrients

Macro and micro nutrients (Cu, Fe, Zn, Mn, Ca, Mg, K, Na) content of the wheat grain was determined with the help of Atomic Absorption Spectrophotometer (AAS), (Jackson, 1973).

Statistical Analysis

The statistical analysis of the data was carried out using SAS 9.1 statistical software. The treatment means were compared at $p < 0.05$ level of probability using student t-test and working out LSD values.

RESULTS AND DISCUSSION

Ozone (O_3) Flux

Variations in O_3 concentration were observed during the crop growth period. The seasonal average ambient O_3 concentrations were 38 ppb in 2010–11 & 29 ppb in 2011–12 respectively (Fig. 2). Ozone concentrations in the elevated O_3 control (C) and in the antioxidant treatments (AA, Q and T) were 25–35 ppb O_3 above the ambient levels. Lower average seasonal ambient O_3 concentrations were observed during the second year of the study. Higher ambient O_3 concentration was observed during the flowering period i.e., February to March as compared to the vegetative phase i.e., December and January. During winter (December and January) reduction in the solar radiation, low temperatures and fog result in less photochemical production of O_3 (Sinha *et al.*, 2015). Hayes *et al.* (2011) found concentrations of O_3 exceeding a threshold of 40 ppb are harmful for vegetation and in this study during 2010–11 and 2011–12 the average elevated O_3 concentration was 69 ppb & 61 ppb respectively (Fig. 2). AOT 40 for 2010–11 and 2011–12 was 13.40 ppm hr and 12.61 ppm hr respectively in the elevated ozone treatments.

Ascorbic acid is the most abundant antioxidant in plants and act as first line of defense against O_3, participates to target ROS and prevent O_3 induced damage (Turcsanyi *et al.* 2000) was one of the antioxidants used in the study. Another compound used in the study was quercetin, which is a flavanoid. Flavonoids are the most common group of secondary metabolites of higher plants with polyphenolic structure. Flavonoids were found to scavenge directly the most of reactive oxygen radicals formed during O_3 oxidation of apoplast components, including superoxide anions, hydrogen peroxide, and hydroxyl radicals (Grace and Logan 2000). Another extract used in the study was marigold leaves extract which is known to contain high levels of secondary metabolite such as quercetagetin, quercetagetin-7-*O*-arabinosyl-galactoside, quercetagetin-3-*O*-arabinosyl-galactoside, quercetagetin-7-*O*-glucoside, patuletin, patuletin-7-*O*-glucoside and isorhamnetin in its extract (Faizi *et al.*, 2008) which have antioxidant properties.

The objective of our study was to test the efficacy of the protective effect of antioxidant chemicals (AA, Q and T) against O_3 damage. Exogenous application of antioxidant chemicals caused a significant increase in leaf ascorbic acid content (Figs. 3(a), 3(b), Figs. 4(a) and 4(b)). As shown in Table 1 the application of various antioxidant chemicals was done six times during the entire crop growth period of wheat upto 85 DAS starting from 27 DAS in both the years of study. The leaf ascorbic acid content was highest at 86

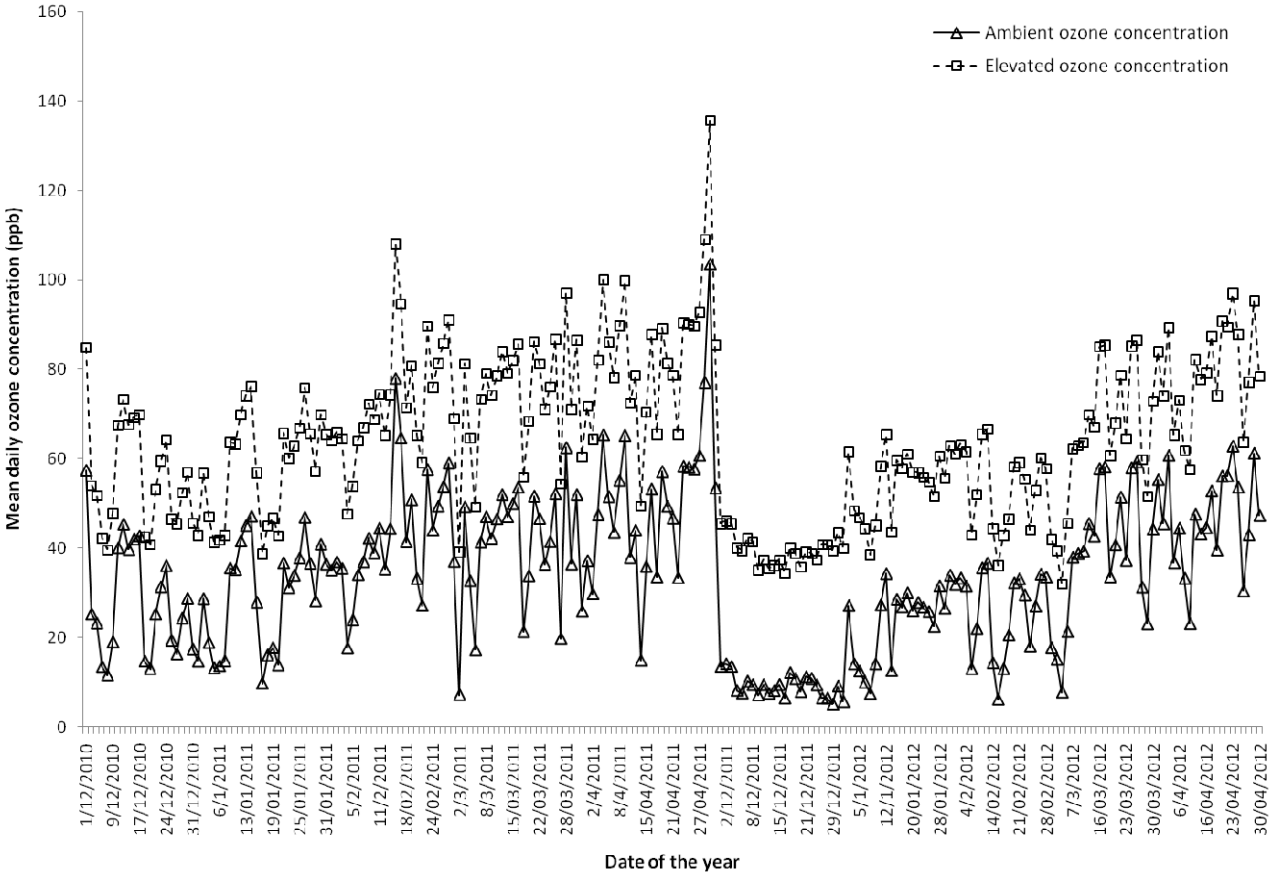

Fig. 2. Variations in daily mean O_3 concentration during experimental period (2010–12) at ambient and elevated levels of O_3 during wheat growing season (dates given in X-axis).

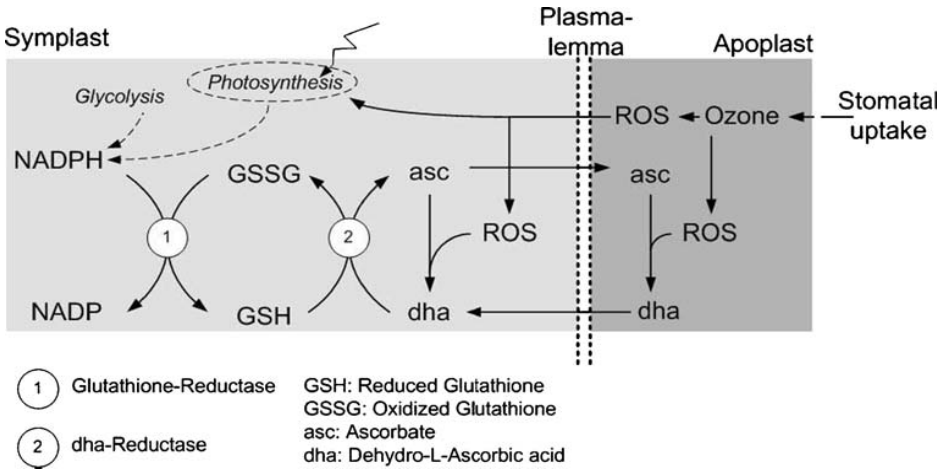

Fig. 3. Simplified scheme of antioxidative defence systems (Ascorbate-glutathione cycle) in cells based on the recycling of ascorbate (asc), which in the apoplast captures ROS produced by penetrating ozone (Fuhrer, 2009).

DAS (flowering stage) after application of AA treatment (6.1 mg g^{-1} fresh wt) followed by flavanoids Q and T. In the treatment CF, plants were at low O_3 and other pollutant levels; the leaf ascorbic acid content was lowest in this treatment. It is known that the concentration of ascorbic acid in the apoplast increases when plant is under stress and ROS are being generated. Chaudhary and Agrawal (2014) reported after foliar application of ascorbic acid under elevated ozone on mung bean higher level of leaf ascorbic acid content as compared to treatment with no ascorbic acid. Ascorbic acid in the apoplast minimizes O_3 injury by two types of reactions one by direct ozonolysis between ascorbic acid and O_3 and another by enzymatic deactivation of peroxides generated during O_3 decomposition (Fig. 3)

(Castillo and Greepin, 1988).

Lower average seasonal O_3 concentrations were observed during the second year of the study. In our study leaf ascorbic acid concentration were found to be lower during the second year as compared to the first year.

Antioxidant Enzymes Activity

Ozone enters the mespohyll cells through the stomata and is converted into ROS of superoxide anion (O^{2-}), hydroxyl radicals (OH^*) and H_2O_2 (Wohlgemuth *et al.*, 2002). Plants metabolise these ROS by invoking the antioxidant enzyme mechanism and this leads to an increase in the concentration of enzymes like catalase, peroxidase and super oxide dismutase. All the three antioxidant enzymes activity (SOD, POX and CAT) increased under elevated O_3 levels in the control treatment as shown in Figs. 4(a), 4(b), 5(a) and 5(b). On the contrary it decreased on application of antioxidants (AA, Q and T) at different growth stages.

From Figs. 4(a), 4(b), 5(a) and 5(b) at flowering stage the SOD and CAT activity was highest in elevated O_3 (C). At flowering stage the SOD activity was significantly lower in AA and Q but not in T treatment in the year 2010–11 (Figs. 4(a) and 4(b)). In 2011–12 at this stage the decrease in SOD was significant for AA but was not significant for Q and T treatments (Figs. 5(a) and 5(b)). The decrease in the SOD activity in AA, Q and T was 22, 13 and 6% in 2010–11 and 23, 12 and 7% in 2011–12 respectively as compared to elevated O_3 control (C). This showed that AA

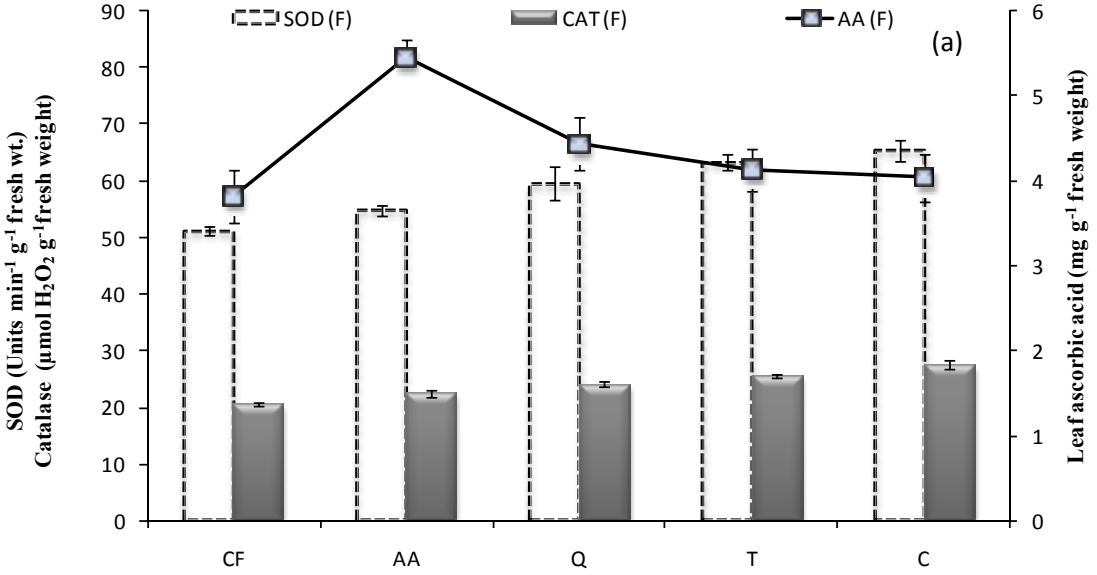

Fig. 4(a). Superoxide dismutases (SOD) (Units min^{-1} g^{-1} fresh weight), Catalase (µmol H_2O_2 g^{-1} fresh weight) enzyme activity and leaf ascorbic acid concentration (mg g^{-1} fresh weight) in different treatments at flowering stage of wheat (2010–11).

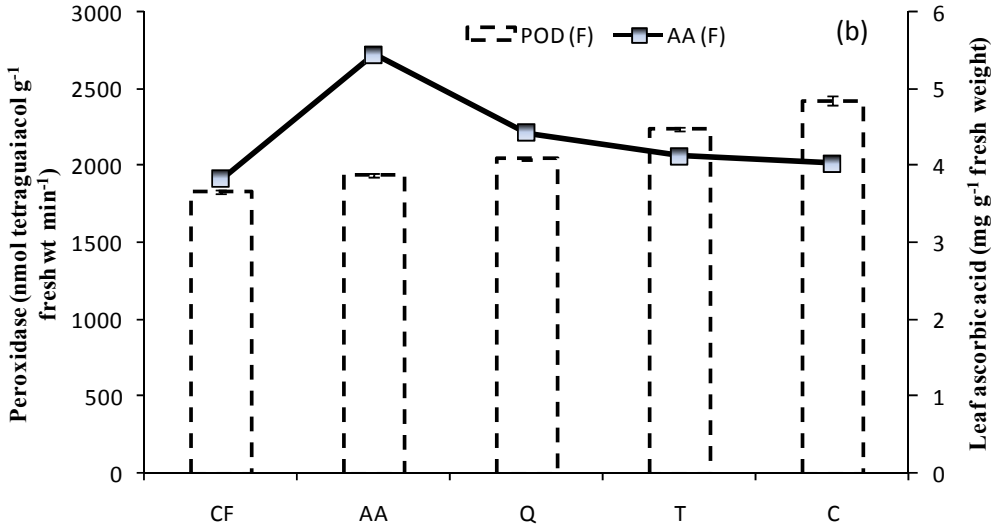

Fig. 4(b). Peroxidase (nmol tetraguaiacol g^{-1} fresh weight min^{-1}) enzyme activity and leaf ascorbic acid concentration (mg g^{-1} fresh weight) in different treatments at flowering stage of wheat (2010–11).

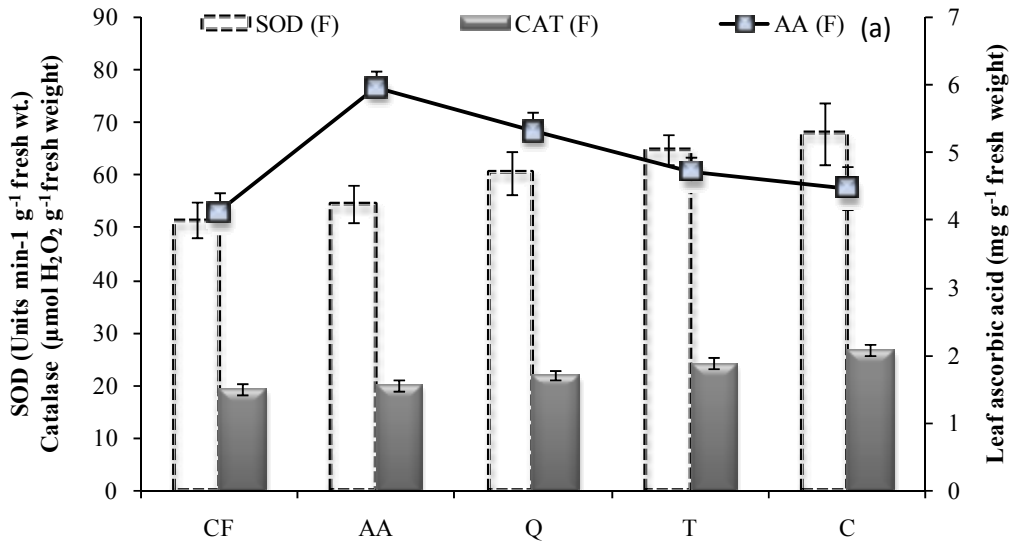

Fig. 5(a). Superoxide dismutases (SOD) (Units min^{-1} g^{-1} fresh weight), Catalase (µmol H_2O_2 g^{-1} fresh weight) enzyme activity and leaf ascorbic acid concentration (mg g^{-1} fresh weight) in different treatments at flowering stage of wheat (2011–12).

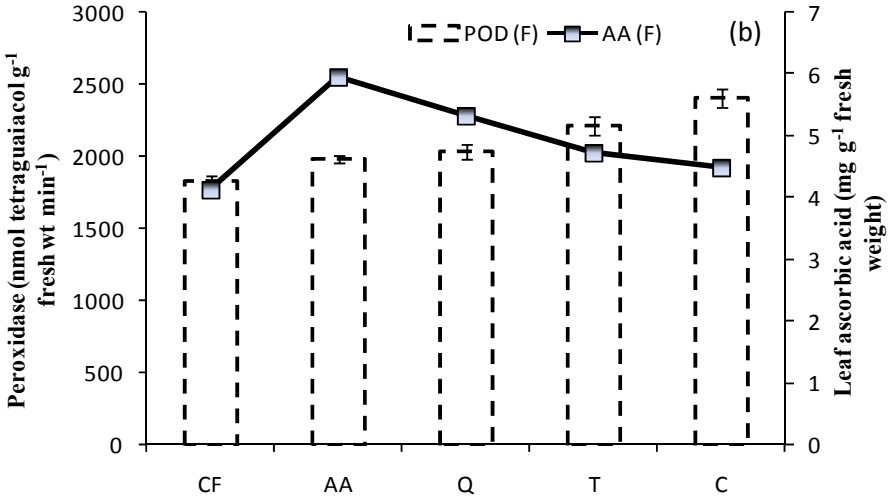

Fig. 5(b). Peroxidase (nmol tetraguaiacol g^{-1} fresh weight min^{-1}) enzyme activity and leaf ascorbic acid concentration (mg g^{-1} fresh weight) in different treatments at flowering stage of wheat (2011–12).

was most effective in reducing the O_3 stress on plants as compared to Q and T. The percentage decrease in CAT enzyme activity (Fig. 5(a)) in antioxidant treatments (AA, Q and T) at flowering was 22, 14 and 7% in 2010–11 and 25, 18 and 10%- in 2011–12 as compared to elevated O_3 control (C). An increase in POX activity was observed on exposure of plants to elevated O_3. The activity of this enzyme in the antioxidant treated plants decreased as compared to elevated O_3 (C) treatment during the crop growth period (Figs. 4(a), 4(b), 5(a) and 5(b)). The percentage decrease in POX activity at flowering stage as compared to control for the antioxidant treatments AA, Q and T was 25, 19 and 8% (2010–11) and 22, 19 and 10% (2011–12).

Superoxide dismutases are enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. Thus, they are an important antioxidant defence in nearly all cells exposed to O_3. Several studies (Mishra *et al.*, 2013) observed that O_3 exposure enhanced superoxide dismutase, peroxidases, glutathione reductase, and ascorbate peroxidase. The CAT enzymes catalyze the dismutation of two molecules of H_2O_2 to water and O_2 under stress condition. The higher leaf apoplatic ascorbic acid in the antioxidant treatments may be sufficient to scavenge the ROS; this may be the reason of lower superoxide dismutases, catalase and peroxidases like antioxidants enzymes activity in these treatments. The lower SOD activity in the antioxidant treatments as compared to C indicates the antioxidant chemicals were effective in reducing the O_3 stress on plants and thus generation of ROS.

Chaudhary and Agrawal (2014) observed higher H_2O_2 content in ascorbic acid treated mung bean leaves as compared to non treated plants which resulted in a

concomitant increase in catalase (CAT) and peroxidise (POX) enzyme activity. In the current study conducted on wheat the activity of CAT and POX in antioxidant treated plants (AA, Q, T) was lower as compared to non treated plants. This lower CAT and POX activity may be due to increased endogenous concentration of antioxidants like ascorbic acid which scavenged more superoxide radicals thereby reducing H_2O_2 formation.

Photosynthetic Rate (P_N), Stomatal Conductance (gs) and Rubisco Enzyme Activity

A lowering in P_N was observed in plants grown under elevated O_3 (C) at 36% in flowering stage as compared to the plants grown under sub-ambient levels of O_3 in the CF treatment (Fig. 6). P_N of the plants sprayed with antioxidants (AA, Q and T) was significantly higher at flowering stage and followed the same trend at milking stage also as compared to plants grown under elevated O_3 alone (C). The P_N in antioxidant treated plants (AA, Q and T) significantly increased by 23, 17 and 12% in 2010–11 and 22, 18 and 10% in 2011–12 respectively as compared to elevated O_3 (C) at flowering stage. The stomatal conductance (gs) ranged from 0.297 to 0.395 ($molm^{-2}$ s^{-1}) and 0.346 to 0.431 ($molm^{-2}$ s^{-1}) in all the antioxidant treated plants at flowering in 2010–11 and 2011–12 respectively (Fig. 7). In 2010–11 there was a significant increase in gs at flowering stage of plants treated with AA, Q and T (32, 19 and 10%) respectively as compared to C.

Rubisco enzyme is an important component of photosynthetic machinery. The rubisco enzyme activity was measured in the year 2011–12 at flowering and milking stages of the crop (Fig. 8). At flowering stage rubisco enzyme activity was significantly higher in all the antioxidant treated plants as compared to elevated O_3 alone (C). Rubisco activity

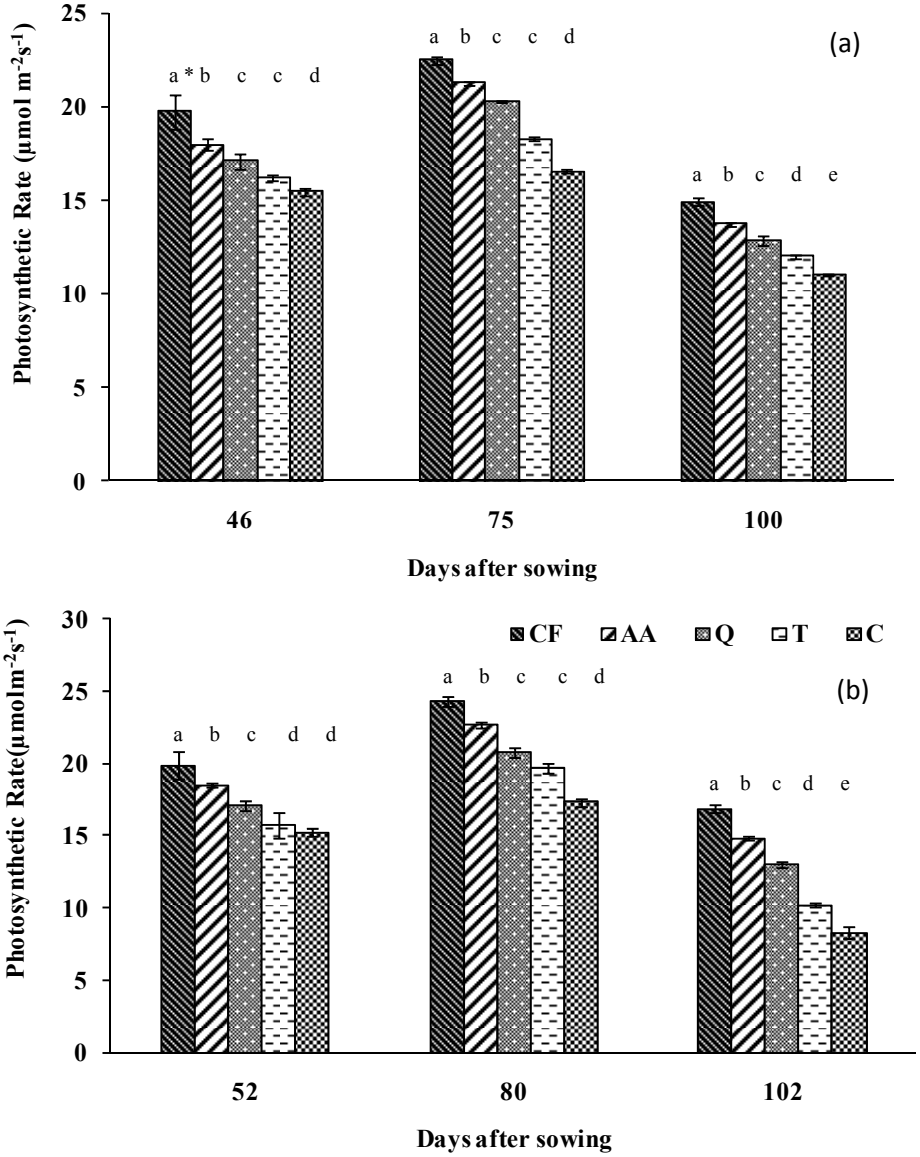

Fig. 6. Photosynthetic rates (P_N) (µmol m^{-2} s^{-1}) at tillering, flowering and milky stage (a) 2010–11, (b) 2011–12 (**Means with the same letter are not significantly different at $P < 0.05$*).

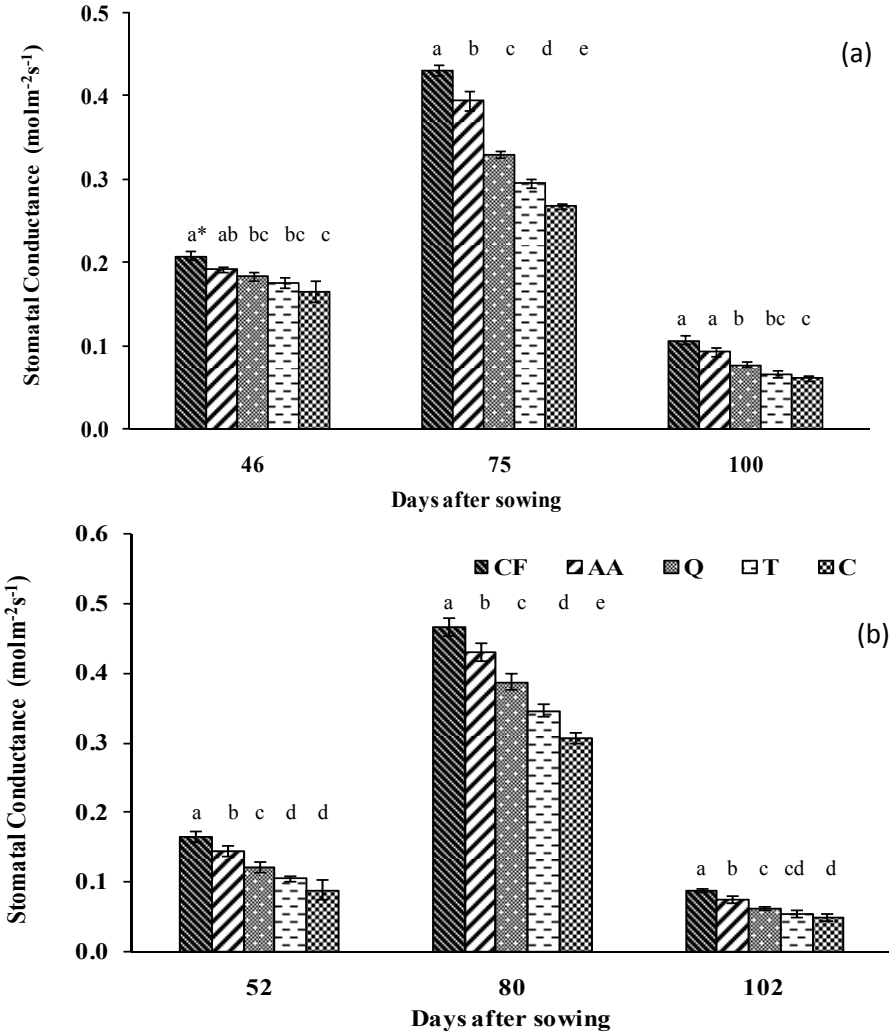

Fig. 7. Stomatal conductance (gs) at tillering, flowering and milking stage (a) 2010–11, (b) 2011–12 (* *Means with the same letter are not significantly different at P < 0.05*).

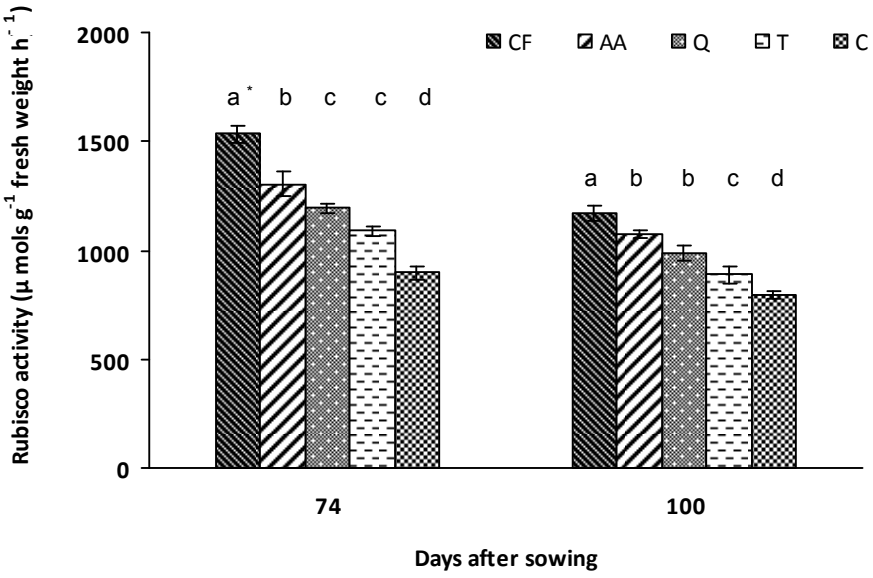

Fig. 8. Rubisco enzyme activity (µmols g^{-1} fresh weight h^{-1}) under different antioxidant treatments in wheat during 2011–12 (* *Means with the same letter are not significantly different at P < 0.05*).

at this stage increased by 31, 25 and 18% in the antioxidant treatments AA, Q and T respectively as compared to C. Here also the highest activity was found in AA followed by Q and T treatments.

Elevated O_3 is known to decrease soluble protein and Rubisco content in a number of plants, including wheat. Different studies (Cho *et al.*, 2008) indicate that O_3 damages the photosynthetic machinery leading to a progressive loss in the amount as well as activity of Rubisco. Mishra *et al.* (2013) reported a reduction in Rubisco activity in wheat under higher levels of O_3 which supports the findings of our study.

Yield of Wheat

Elevated O_3 concentration significantly lowered the yield of wheat (Tables 2(a) and 2(b)). There was a significant decline in average no. of tillers m^{-2}, average no. of spikes m^{-2}, average no. of grains $spike^{-1}$, total biomass, total grain weight and 1000 grain weight under elevated O_3 as compared to charcoal filtered air which had less than 10 ppb of O_3 during the crop growth period. The increase in average no. of tillers m^{-2} in 2010–11 was 14 and 7% in the antioxidant treatments AA and Q and 15% in CF as compared to elevated O_3 control. In 2011–12 the increase in average no. of tillers m^{-2} in antioxidant treatments was 24, 19 and 17% in AA, Q and T respectively and by 30% in CF as compared to C.

The average no. of spikes m^{-2} increased by 26, 20 and 10% in 2010–11 and 26, 23 and 21% in 2011–12 in AA, Q and T treatments respectively as compared to C. In the year 2010–11 there was no significant increase observed in average no. of grains $spike^{-1}$ in the antioxidants treated plants as compared to C but it significantly increased in case of CF. Wahid (2006) also observed a similar increased in grains $spike^{-1}$ under filtered air in wheat. This was due to higher translocation of carbohydrates to sink, leading to higher grain filling and grain weight in filtered air. Tomer *et al.* (2015) observed accelerated senescence which shortened the grain filling duration and there by reduced average no. of grains $spike^{-1}$ and ultimately to lower grain yield under elevated O_3. Total biomass increased by 8, 6 and 3% in AA, Q and T in 2010–11 and 12, 5 and 3% in 2011–12 respectively as compared to control (C) at the time of final harvesting. In CF treatment it increased by 12% in 2010–11 and 16% in 2011–12 respectively as compared to C.

The highest yield among antioxidant treated plants was in AA followed by Q and T treatments. The yield of wheat in AA, Q and, T treatments were 23%, 13% and 10% higher as compared to C in 2010–11 and 17, 13 and 6% higher in 2011–12 respectively. Apart from CF the AA treatment was most effective in providing protection followed by Q and T treatments. The increase in yield in antioxidant treatments might be due to increased P_N. This increased P_N could compensate for the inhibitory effects of O_3 on carbon assimilation. Feng *et al.* (2008) in a meta-analysis of O_3 impact on wheat found that seasonal O_3 concentrations averaging 42 ppb lowered yield by 18%, compared with CF air controls. In our study we also observed a decrease in yield of wheat by 31% and 26% as compared to CF treatment with seasonal average of 68 and 59 ppb O_3 in the elevated O_3 treatment in 2010–11 and 2011–12 respectively.

Harvest index is the ratio of Economic yield to the biological yield. HI was also higher in antioxidant treatments

Table 2(a). Yield and Harvest parameters under different antioxidant treatments in wheat (2010–11).

Treatments	Avg. No. of tillers (m^{-2})	Avg. No. of spikes (m^{-2})	Avg. No. of grains (per spike)	Total biomass (g m^{-2})	Total grain weight (g m^{-2})	1000 grain weight (g)	Harvest index
	Mean	Mean	Mean	Mean	Mean	Mean	Mean
CF	373^{a*}	363^{a}	43^{a}	1127^{a}	544^{a}	42^{a}	0.48^{a}
AA	369^{a}	351^{a}	39^{b}	1077^{b}	484^{b}	41^{ab}	0.44^{b}
Q	343^{b}	322^{b}	39^{b}	1044^{c}	430^{c}	40^{b}	0.41^{c}
T	333^{bc}	289^{c}	37^{b}	1013^{d}	415^{c}	38^{c}	0.41^{c}
C	319^{c}	259^{d}	37^{b}	987^{e}	374^{d}	38^{c}	0.37^{d}

CF- Charcoal filtered air, AA- Ascorbic acid, Q- Quercetin, T- Marigold leaves extract, C- Elevated ozone Control.
* *Means with the same letter are not significantly different at $P < 0.05$.*

Table 2(b). Yield and Harvest parameters under different antioxidant treatments in wheat (2011–12).

Treatments	Avg. No. of tillers (m^{-2})	Avg. No. of spikes (m^{-2})	Avg. No. of grains (per spike)	Total biomass (g m^{-2})	Total grain weight (g m^{-2})	1000 grain weight (g)	Harvest index
	Mean	Mean	Mean	Mean	Mean	Mean	Mean
CF	453^{a*}	436^{a}	49^{a}	1542^{a}	586^{a}	44^{a}	0.44^{a}
AA	416^{b}	397^{b}	45^{ab}	1462^{b}	522^{b}	42^{b}	0.44^{a}
Q	392^{c}	381^{c}	42^{b}	1351^{c}	496^{c}	41^{bc}	0.42^{b}
T	384^{d}	369^{d}	40^{bc}	1325^{d}	458^{d}	40^{cd}	0.39^{c}
C	318^{e}	293^{e}	35^{c}	1290^{e}	431^{e}	39^{d}	0.37^{d}

CF- Charcoal filtered air, AA- Ascorbic acid, Q- Quercetin, T- Marigold leaves extract, C- Elevated ozone Control.
* *Means with the same letter are not significantly different at $P < 0.05$.*

(AA, Q & T) as compared to C (Tables 2(a) and 2 (b)). There were 15, 8 and 7% increase in HI in 2010–11 and 16, 11 and 5% increase in 2011–12 in the antioxidant treatments (AA, Q & T) respectively as compared to C. HI indicates the partitioning of dry matter between grain and above ground biomass. Reduction in HI of plants under elevated O_3 as compared to CF and antioxidant treatments indicated that relatively less dry matter was partitioned into grain under O_3 stress. The presence of antioxidants increased the HI as they affected more (increased) the grain yield as compared to biomass yield in all the treatments, thus showing that there was a differential impact on partitioning of carbon in the antioxidant treatments. Higher HI obtained in the second year may be due to lower ambient O_3 levels as compared to the previous year.

Nutritional Quality

Sugar, Starch and Soluble Protein in Leaves and Grains

Elevated O_3 levels caused a decrease in the sugar, starch and soluble protein content of wheat leaf (Table 3). The increase was significant in all the antioxidant treatments as compared to C. At flowering stage leaf sugar increased by 14, 9 and 5% in 2010–11 and 18, 13 and 8 % in 2011–12 in AA, Q and T treatments respectively as compared to control (C). The leaf starch content was also higher at flowering by 31, 25 and 17% in 2010–11 and 28, 24 and by 18% in 2011–12 in AA, Q and T respectively as compared to control. In case of wheat grain (Fig. 9) the sugar content in 2010–11 study was significantly higher in the antioxidant treatments AA and Q but not in the treatment T as compared to control (C), however it was significantly higher in AA, Q and T in the second year (2011–12) of the study. The starch content in grains (Fig. 9) was significantly higher in all the antioxidant treatments as compared to control in both the years. The percentage increase in starch content was 15, 12 and 7% and 14, 8 and 4% in AA, Q and T treatments in the year 2010–11 and 2011-12 respectively as compared to control.

After application of second round of antioxidants to the plant the leaf protein content (Table 3) increased in AA and Q antioxidant treatments in both the years. The percentage increase in leaf protein content at this stage was 19, 15 and 7% in 2010–11 and 18, 13 and 7% in 2011–12 as compared to control. In both the years 2010–11 and 2011–12 increase in grain protein content in C was significant as compared to antioxidant treatments. The protein content was however more in antioxidant treatments than in CF.

In our study the sugar content of the antioxidant treated plants increased over elevated O_3 control but were lower than CF treatment. This may be due to reduction in O_3 induced stress due to increased concentration of endogenous antioxidants (leaf ascorbic acid) in apoplast after application of exogenous antioxidants. An earlier study done by Chaudhary and Agrawal (2014) found that ascorbic acid reduces O_3 induced stress by reduction in reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-) having high oxidation potential. This reduced ROS in plant lead to lower SOD, CAT and POX activity (as shown in previous section), higher rubisco enzyme activity and ultimately resulted in higher carbohydrate fixation. We observed a significant reduction in starch content in wheat leaf (Table 3) in C plants upon exposure to elevated O_3 alone as compared to CF treatment, which corroborates findings by other researchers (Braun *et al.*, 2004). On the contrary, in case of plants treated with antioxidants the starch content in leaves increased as compared to C. Ozone has been reported to affect the possibility of transport of assimilated products (Gelang *et al.*, 2000). The C-assimilation and transport was less affected in antioxidant treatments as compared to elevated O_3 alone treatment as O_3 induced stress was less in previous. In elevated O_3 treatment the leaf protein content was significantly lower than antioxidant treatments. Chernikova *et al.* (2000) observed a decrease in leaf protein content in soybean leaves on exposure of plants to elevated O_3 which showed similar line of finding in this study done on wheat.

The starch content of wheat grains in antioxidant treatments increased as compared to control (Fig. 9). Wahid (2006) reported reduced starch concentration in wheat grains on exposure to higher O_3 concentrations. The grain protein content was however more in AA, Q and T treatments as compared to CF. It has been seen by crop physiologists that the grain filling of starch and protein responds differently to stress. Ozone has been shown to shorten the duration of the leaves and impair the grain growth (Ojanperä *et al.*, 1992). The increased protein content in the wheat grain at higher O_3 concentrations can be viewed as an example of this general phenomenon. Several studies (e.g., Piikki *et al.*, 2008; Pleijel and Uddling, 2012) have shown that reduced grain yield of wheat is commonly associated with enhanced

Table 3. Leaf sugar and starch content (mg g^{-1} fresh weight) in wheat plant in different treatments.

Treatments	2010–11						2011–12					
	Leaf Sugar		Leaf Starch		Leaf Protein		Leaf Sugar		Leaf Starch		Leaf Protein	
	73 DAS	100 DAS	73 DAS	100 DAS	73 DAS	100 DAS	72 DAS	102 DAS	72 DAS	102 DAS	72 DAS	102 DAS
CF	75.42^{a}	47.24^{a}	42.50^{a}	37.30^{a}	143.34^{a}	56.27^{a}	82.74^{a}	54.33^{a}	46.34^{a}	37.34^{a}	151.03^{a}	58.59^{a}
AA	71.57^{b}	42.75^{ab}	39.37^{ab}	31.74^{b}	134.49^{ab}	50.29^{ab}	78.55^{ab}	50.33^{ab}	43.55^{ab}	35.42^{a}	142.71^{b}	53.93^{a}
Q	67.37^{c}	40.98^{ab}	35.95^{b}	28.91^{b}	128.99^{bc}	44.21^{bc}	73.52^{b}	45.63^{b}	41.07^{ab}	32.22^{b}	134.25^{c}	48.33^{b}
T	64.72^{c}	38.92^{b}	32.74^{b}	26.43^{b}	117.81^{cd}	39.32^{cd}	69.71^{b}	42.10^{b}	38.20^{b}	29.02^{c}	126.07^{c}	42.08^{c}
C	61.58^{d}	31.31^{c}	27.03^{c}	21.19^{c}	109.22^{d}	34.99^{d}	64.16^{c}	35.12^{c}	31.19^{c}	23.12^{d}	116.92^{d}	36.31^{d}

CF- Charcoal filtered air, AA- Ascorbic acid, Q- Quercetin, T- Marigold leaves extract, C- Elevated ozone Control.

[*] *Means with the same letter are not significantly different at $P < 0.05$.*

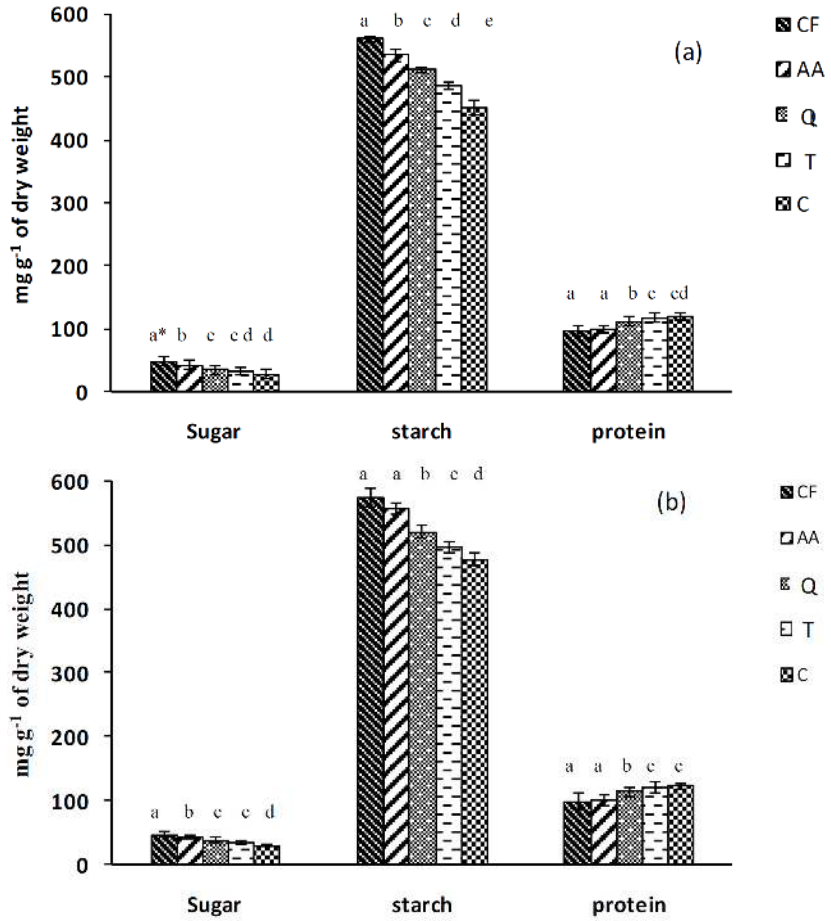

Fig. 9. Sugar, starch and protein content (mg g^{-1} of dry weight) of wheat grains in different treatments (* *Means with the same letter are not significantly different at P < 0.05*) at (a) 2010–11 and (b) 2011–12.

grain protein concentration. Although the protein content is negatively affected by O_3, the net yield of protein per unit area was positively influenced.

Micronutrients (Cu, Fe, Zn, Mn) and Macronutrients (Ca, Mg, K) in Grain

Less attention has been paid to the effects of O_3 on nutrient concentrations in herbaceous species. In our study an increase in the micronutrient content of wheat grain was observed on application of antioxidant chemicals to the wheat plant. The Copper (Cu) content in wheat was significantly higher in all the antioxidant treatments (AA, Q and T) than elevated O_3 control in 2010–11 (Table 4). The increase in Iron (Fe) in 2010–11 was 27, 21 and 14% in AA, Q and T respectively as compared to control. In 2011–12 the Fe was significantly higher in AA and Q by 21 and 17% but was not significantly higher in T as compared to C. Among antioxidant treatments AA had highest amount of Zn in both the years. There was no significant difference observed in Mn among the AA and Q treatments. The Mn content in wheat was higher in the second year as compared to the first year.

As contrast to micronutrients the macronutrient content showed the opposite trend. The Calcium (Ca) in grains increased in elevated O_3 control as compared to other treatments in both the years of study. No significant difference was observed in Q and T treatments. The increase in Ca under elevated O_3 was 26% as compared to CF air. Ca increased in antioxidant treatments T, Q and AA by 18, 13 and 7% as compared to CF in 2010–11. Magnesium (Mg) in wheat grains also increased in control as compared to CF. The Potassium (K) content in the grains increased with increase in O_3 concentration. No significant difference in K content was observed among AA and Q treatments.

Pleijel (2012) reported an increase in Zn content on exposure of plants to elevated O_3 which was in contrast to the results obtained in our study. In our study K content increased in grain in plants grown under elevated O_3 alone, Feng *et al.* (2008) reported enhanced concentrations of K in response to O_3 exposure.

CONCLUSIONS

The present study demonstrated that the exogenous application of antioxidant chemicals like ascorbic acid, quercetin and natural plant extract of marigold leaves caused a significant increase in leaf ascorbic acid contents which is the first line of defence in a plant system against O_3 induced ROS and damage caused by them. The concentration of ROS-detoxifying enzymes such as superoxide dismutase,

Table 4. Micronutrients and macronutrients content in wheat grain in different treatments.

Treatments	2010–11				2011–12				2010–11			2011–12		
	Cu	Fe	Zn	Mn	Cu	Fe	Zn	Mn	Ca	Mg	K	Ca	Mg	K
CF	6.62^{a*}	38.88^{a}	26.12^{a}	2.56^{a}	7.05^{a*}	40.22^{a}	28.12^{a}	3.07^{a}	79^{d}	632^{c}	2749^{d}	72^{c}	607^{d}	2696^{c}
AA	5.90^{b}	34.43^{b}	20.88^{b}	2.01^{b}	6.61^{a}	38.30^{a}	24.72^{b}	2.71^{b}	85^{cd}	652^{c}	2774^{c}	83^{bc}	620^{cd}	2725^{c}
Q	5.09^{c}	31.98^{b}	18.71^{c}	1.92^{b}	6.08^{b}	36.77^{a}	22.32^{c}	2.45^{bc}	90^{bc}	677^{bc}	2786^{c}	95^{ab}	636^{c}	2792^{b}
T	4.47^{d}	29.43^{b}	16.82^{c}	1.57^{c}	5.62^{c}	32.82^{ab}	19.90^{d}	2.13^{cd}	96^{b}	713^{ab}	2810^{b}	97^{ab}	675^{b}	2839^{ab}
C	3.92^{e}	25.26^{c}	14.85^{d}	1.45^{d}	5.22^{c}	30.40^{b}	17.50^{e}	1.87^{d}	107^{a}	745^{a}	2831^{a}	105^{a}	706^{a}	2863^{a}

CF- Charcoal filtered air, AA- Ascorbic acid, Q- Quercetin, T- Marigold leaves extract, C- Elevated ozone Control.
[*] *Means with the same letter are not significantly different at $P < 0.05$.*

peroxidase and catalase significantly reduced, alleviating the oxidative stress in wheat plants and increased yield and nutritional quality under elevated O_3 levels. There was an increase in micronutrient content under the application of antioxidant chemicals. Among the three antioxidant treatments the performance of ascorbic acid (AA) was found to be best in reducing oxidative stress followed by quercetin (Q) and marigold leaves extract (T). Although ascorbic acid and quercetin are chemical antioxidants but marigold leaves extract being naturally derived possesses negligible environmental impact. There is no economic use of marigold leaves and are available in plenty in South Asian region. These may be useful to the farmers as a natural antioxidant for reducing O_3 related yield losses, however more experiments are required for working out the cost benefit analysis.

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