

Effect of elevated ozone and varying levels of soil nitrogen in two wheat (*Triticum aestivum* L.) cultivars: Growth, gas-exchange, antioxidant status, grain yield and quality

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ABSTRACT

Tropospheric ozone (O_3) is a phytotoxic air pollutant causing a substantial damage to plants and agriculture worldwide. Plant productivity is affected by several environmental factors, which interact with each other. Studies related to interactions involving O_3 and different levels of nitrogen (N) are still rare and elusive. In the present study we grew two wheat cultivars (HD2967 and Sonalika) in open top chambers (OTC) under ambient (AO) and elevated O_3 (EO) (ambient + 20 ppb O_3) and provided two levels of N fertilization; (a) recommended nitrogen (RN), (b) 1.5 times the recommended N (HN). Growth (root/shoot ratio, leaf number and leaf area), biomass, gas-exchange (stomatal conductance (g_s), photosynthesis (A), transpiration (E), chlorophyll fluorescence (F_v/F_m), physiological (chlorophyll and carotenoids), biochemical [antioxidant activity, lipid peroxidation (MDA)] parameters and leaf N content were measured at the vegetative and reproductive phases. Yield attributes (spike weight plant⁻¹, grain weight plant⁻¹, grain numbers plant⁻¹, husk weight plant⁻¹, straw weight plant⁻¹, 1000 grain weight, harvest index) and seed N content were analyzed at the final harvest stage.

Grain yield plant⁻¹ was decreased in Sonalika under EO irrespective of different levels of N fertilization. Seed N content decreased by 3.9% and 5.6% in HD2967 and Sonalika, respectively, under EO at RN treatment. Antioxidant defense played an important role in protecting the plants against O_3 stress which was enhanced under HN treatment. Response of antioxidants varied between the cultivar, growth phase (at the vegetative or reproductive phase) and the N levels (RN or HN). Cultivar HD2967 was characterized by higher biomass, g_s and stronger antioxidant protection system, while, Sonalika showed early senescence response (decreased leaf number plant⁻¹, g_s) and greater resources allocation towards eco-physiological parameters (increased A and F_v/F_m) at the vegetative phase, resulting in the significant decrease in the yield attributes. Further study warrants the need to screen a large number of cultivars in relation to their response to various levels of N fertilization to minimize the yield losses under highly O_3 polluted areas.

1. Introduction

Tropospheric ozone (O_3) is the most important air pollutant adversely affecting crop production mainly due to its high phytotoxicity and prevalence over agriculturally important regions of the world (Agathokleous et al., 2015; Agathokleous, 2017; Ainsworth, 2016; Emberson and Büker, 2008; Tai et al., 2014). Although increasing trends of surface level O_3 have levelled off or been slightly negative in USA and Europe (Klingberg et al., 2014). However trends of O_3 are highly variable at geographic level, for example, in South Asia the O_3

concentrations are predicted to increase by 40 – 60% compared with the current O_3 level of ~40 ppb by 2100 (Fowler et al., 2008; Meehl et al., 2007; Ohara et al., 2007; Osborne et al., 2016; Sun et al., 2014; Brauer et al., 2015) reported that O_3 concentration has increased by 20% and 13% in India and China, respectively from 1990 to 2013. Developing countries such as India and China are strongly affected due to rising O_3 pollution owing to their fast economic growth rate, industrial developments and weak legislation, responsible for increased levels of O_3 precursors leading to formation of O_3 (Agathokleous et al., 2016; Cooper et al., 2014; Fowler et al., 2008; Oksanen et al., 2013; Pandey

Abbreviation: A, Photosynthesis; AO, Ambient Ozone; AOT40, Accumulated O_3 exposure over threshold of 40 ppb; APX, Ascorbate peroxidase; ASA, Ascorbic acid; CAT, Catalase; DAG, Days after germination; E, Transpiration; EO, Elevated ozone; F_v/F_m , maximal photochemical efficiency of PSII; GR, Glutathione reductase; g_s , Stomatal conductance; HI, Harvest Index; HN, High nitrogen dose; MDA, Malondialdehyde; N, Nitrogen content; O_3 , Tropospheric ozone; OTC, Open Top Chambers; POD, Peroxidase; RN, Recommended nitrogen dose; ROS, Reactive oxygen species; RuBP, Ribulose 1,5-bisphosphate; SOD, Superoxide dismutase; Spkltg, Spike length plant⁻¹; Spkwt, Spike weight plant⁻¹; Strawwt, Straw weight

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et al., 2015; Pleijel et al., 2018; Wilkinson et al., 2012).

O₃ enters the leaves through stomata after which it rapidly transforms to generate reactive oxygen species (ROS) which damages the internal plant tissues and interferes with various physiological and biological functions such as decreased photosynthesis, stomatal conductance and dry matter production leading to production losses (Tai and Martin, 2017).

Plant growth and crop production affected by the soil N availability in the form of inorganic N fertilizers. Approximately, 85 million to 90 million metric tons (MMT) of nitrogenous fertilizer is added to soil per year global and is further predicted to increase to 240 MMT by the year 2050 (Good et al., 2004). Increasing demand for the food supply indicates that the demands for N fertilizers and agricultural land are also likely to grow substantially (Foley et al., 2011; Mueller et al., 2012). Yendrek et al. (2013) reported that sufficient N application may partially ameliorate the negative effects of O₃ on leaf area, above ground biomass and root biomass although effects were not significant in their study. Similarly, Singh et al. (2009) reported positive effects of 1.5 times recommended NPK fertilizer application on mustard as compared to the plants grown at the recommended dose of NPK under ambient-O₃ conditions in terms of better growth and biomass.

Wheat is an important crop for the global food supply supporting nearly two-third of the world's population with an annual production of more than 650 MMT (Fowler et al., 2008; Wu et al., 2016). Wheat has been classified as an O₃-sensitive crop with the critical level (AOT40) for 5% yield reduction at 3.3 ppm h (Mills et al., 2007). India is the second largest wheat producing country, averaging about 12% global production, contributing substantially to the food security worldwide (Burney and Ramanathan, 2014).

The present study aims to elucidate (1) the effect of O₃ on the grain yield of two important wheat cultivars under ambient and elevated levels of O₃ (2) to understand the mechanism(s) by which additional N fertilization can affect the response of the plants to high-O₃ conditions (3) to evaluate the effects of EO and N and their interactions on the growth yield and grain quality.

2. Material and method

2.1. Experiment site and plant material

The study was conducted in the Botanical Garden, Department of Botany, Institute of Science, Banaras Hindu University, Varanasi situated in the Eastern-Gangetic plains of the Indian subcontinent (25°14'N and 82°03'E at 76.19 m above sea level) during the months of November 2016–March 2017. Two cultivars of wheat HD2967 and Sonalika were selected for the study both are high yielding and disease resistant and are widely grown in the Indo-Gangetic region which is considered to be one of the most fertile and agriculturally important region of India.

2.2. Plant cultivation and experimental design

Seeds were obtained from the Department Of Genetics and Plant Breeding, Institute of Agriculture Science, Banaras Hindu University, Varanasi, India. Seeds were hand sown within the spacing of 20 cm inside each open-top chamber (OTC). N was applied at two different levels, at recommended N (RN) of NPK fertilization (120:60:60 kg ha⁻¹ as urea, single superphosphate, and muriate of potash, respectively) and the 1.5 times the recommended dose of N (HN) (180:60:60 kg ha⁻¹). N was applied in three splits 50% (with full doses of P and K) during the field preparation and remaining in two equal doses of 25% each at 30 and 60 days after germination (DAG), respectively. Weeding was done manually four times during the entire experimental period. Chambers were irrigated time to time to maintain the uniform moisture regime in the soil.

The experiment was conducted in the cylindrical OTC having 1.5 m

diameter and 1.8 m in height. The chambers receiving ambient-O₃ (AO) served as a control while with the elevated-O₃ (EO) (ambient + 20 ppb) served as the treatments. O₃ fumigation started on 15 November 2016. The plants were exposed under EO (10:00–14:00 h, which coincides with the maximum O₃ concentration in the ambient condition) for 4 h day⁻¹, 7 days week⁻¹, until the seed maturation. O₃ fumigation was done using O₃ generator (Faraday; A2G, Coimbatore, India). Our experiment was a complete randomized block having three chambers (n = 3) for each treatment. The average AO in the present study was 52.4 ppb and EO dose was selected based on the prediction by Solomon (2007) SRES A2 scenario which predicted that ambient O₃ levels will increase by 20–40% by 2050.

2.3. Ozone monitoring and AOT 40 calculations

O₃ monitoring at the experimental plot was carried out with a (Model APOA 370, HORIBA Ltd., Kyoto, Japan) analyzers from 9.00 to 17.00 throughout the growth period h. AOT 40 (accumulated exposure over a threshold of 40 ppb) was also calculated as the exposure index for the O₃ concentration, as described by De Leeuw and Van Zantvoort (1997).

2.4. Plant sampling

Plant samplings were done at 40 DAG (vegetative phase) and 80 DAG (reproductive phase) and the yield parameters estimated at the final harvest phase (122 DAG and 112 DAG for HD2967 and Sonalika, respectively). The plants were sampled from the center of the plot to avoid the edge effects. Five plants were randomly selected for the growth and morphological parameters. Plant sampling for growth (root and shoot length, root and shoot biomass, leaf number and leaf area plant⁻¹) were done at the vegetative and the reproductive phase. For the root biomass measurement, a monolith of 10 cm × 10 cm × 20 cm were dug and roots were separated under running tap water to remove the soil particles. Plants parts were kept in the oven at 80 °C, until the final weight reached to a constant value.

2.5. Biochemical analysis

Leaf samples were collected for various antioxidant activity analysis at vegetative and the reproductive phase. Three to four fully mature leaves were collected from three randomly selected plants for each treatment and each chamber. Leaves samples were frozen in the liquid nitrogen and kept at –80 °C until further analysis.

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content by thiobarbituric (TBA) acid method (Heath and Packer, 1968). Superoxide dismutase (SOD) activity was measured as 50% reduction of nitrobluetetrazolium (NBT) according to Beyer and Fridovich (1987). Catalase activity (CAT) was assayed by following the decrease in the absorbance at 240 nm as H₂O₂ was consumed (Rao et al., 1996). Peroxidase (POD) expressed as μm purpurogallin formed was estimated by the method of Britton and Mehley (1955). The activity of ascorbate peroxidase (APX) was measured by following the rate of H₂O₂ dependent oxidation of ascorbic acid (Chen and Asada, 1989). Glutathione reductase (GR) activity was assayed by following the increase in absorbance at 412 nm when 5, 5'-dithiobis-(2-nitro-benzoic acid) (DTNB) was reduced by glutathione to form TNB (Smith et al., 1988). Ascorbic acid content (ASA) was determined by using 2,6-dichlorophenol-indophenol dye reduction method (Keller and Schwager, 1977).

2.6. Gas-exchange, F_v/F_m and chlorophyll measurements

Stomatal conductance (g_s), net photosynthesis (A), transpiration (E), and maximal photochemical efficiency of PS II (F_v/F_m) were measured on the second youngest fully mature leaves from three randomly selected plants of both the wheat cultivars in each treatment. All the

measurements were performed using CIRAS-3 (PP Systems, Amesbury, MA). The CO_2 concentration ($400 \mu\text{mol mol}^{-1}$), photosynthetic photon flux density was $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$, leaf temperature (25°C) and relative humidity (50–60%) was maintained using an automatic control device on the CIRAS-3. F_v/F_m measurements were carried out by using CIRAS-3 after the leaves were dark-adapted for 30 min.

Chlorophyll contents were estimated using the method of Arnon (1949). Carotenoid content was calculated according to Parsons et al. (1984) from the absorbance values at 480 and 510 nm using UV–VIS spectrophotometer (Model 2203, Systronics, India)

2.7. Yield attributes and grain quality

Grain yield was calculated at the final harvest phase. Fifteen plants were sampled from each treatment and different yield attributes such as spike length (spklt), spike weight plant^{-1} (spkwt), grain weight plant^{-1} , number of grains plant^{-1} , straw weight plant^{-1} (strawwt), 1000 grain weight and harvest index (HI) (ratio of grain weight and the above ground biomass at maturity) were assessed at the harvest stage. Estimation of total N content in the leaves was performed at the vegetative and the reproductive phase and in the grains at the final harvest by using Gerhardt Digestion and Distillation system-2000 (Model KB8S, Germany).

2.8. Statistical analyses

Data of growth, morphology, biomass, gas-exchange and yield attributes were subjected to three way analysis of variance (ANOVA) to examine the individual and combined effect of cultivar, O_3 (ambient, elevated) and N treatment (RN and HN). The data were rank-transformed resulting in non-parametric ANOVA as described by Zar (1974). The significantly different mean for different parameters between AO and EO were tested using the Student's *t*-test. All the statistical tests were performed using SPSS software (SPSS Inc., version 17.0). The test was carried out separately for all the developmental stages (vegetative, reproductive and harvest).

3. Results

The average O_3 concentration was 52.4 (AO) and 59.6 ppb (EO) (day time average based on hourly values between 09.00 and 17.00 h) and it ranged from 44.9 ppb to 57.5 and 55.4–64.6 under AO and EO conditions, respectively (Fig. 1). AOT40 was 11.9 ppm h and 18.3 ppm h under AO and EO chambers, respectively at the end of the experimental period (Fig. 1).

Minimum temperature ranged from 6.8 to 27.6°C and maximum temperature ranged 15.4– 41.3°C , relative humidity ranged from 15% to 95% during the experimental period. High average O_3 concentrations were recorded both in the months of February (54.8 ppb) and in March (55.4 ppb) at AO. Maximum average temperature (33.4°C) and sunshine hours (8.7 h) were recorded in the month of March 2017.

3.1. Leaf N content and yield attributes

Leaf N content increased by 74% in HD2967 and decreased by 14% in Sonalika at the vegetative phase under EO at HN treatment as compared to AO and HN treated plants (Supplementary fig. 1). Seed N content decreased by 3.9% and 5.6% in HD2967 and Sonalika, respectively under EO at RN treatment as compared to AO at RN treatment (Supplementary fig. 1). While no significant change was observed in both the cultivars under EO at HN. Grain weight has been considered to be an important quality trait in wheat under EO conditions (Pleijel and Uddling, 2012). Sonalika showed significant reduction in grain weight plant^{-1} under EO at both RN/HN treatments indicating its sensitivity against O_3 -induced damages.

In HD2967, grain weight plant^{-1} was decreased by 10.2%

(insignificant) similarly; other yield attributes did not show significant changes under EO at RN/HN treatments, except there was an increase in the husk weight plant^{-1} under EO at RN treatment. Contrary to this, Sonalika showed significant reduction in grain weight plant^{-1} by 18% and 19% under EO at RN and HN treatments, respectively. Spike length was reduced by 8.7% under EO at RN whereas spike weight plant^{-1} has increased under EO at RN/HN treatment only in Sonalika. Husk weight plant^{-1} , straw weight plant^{-1} and 1000 grain weight decreased under EO at HN treatment as compared to plants grown under AO at HN conditions in Sonalika (Fig. 2).

A significant correlation was observed between grain yield plant^{-1} and leaf N content at both the vegetative ($R^2 = 40.4\%$) and the reproductive phase ($R^2 = 57.1\%$) (Supplementary fig. 2). Multivariate ANOVA result displayed significant interactions of $\text{O}_3 \times \text{N}$ and $\text{O}_3 \times \text{Cv}$ on husk weight plant^{-1} and 1000 grain weight plant^{-1} and $\text{N} \times \text{Cv}$ on spike weight plant^{-1} , straw weight plant^{-1} and 1000 grain weight (Table 1).

3.2. Growth and morphology

Biomass was reduced under EO and RN treatment, whereas, it increased at HN treatment in both the test cultivars at both the sampling phases, the only exception for increased biomass was in Sonalika at the harvest phase. Significant interactive effects of $\text{O}_3 \times \text{N} \times \text{Cv}$ were observed at the vegetative and reproductive phases (Supplementary fig. 3). Root/shoot ratio was increased (at the vegetative) and decreased (at the reproductive), in HD2967 at EO and RN treatment, while, no significant change was observed in root/shoot ratio in Sonalika, except, an increase under EO at RN treatment at the reproductive phase (Supplementary fig. 4).

In HD2967, leaf number plant^{-1} decreased by 39% at the vegetative phase and showed no significant difference at the reproductive phase while, decreased by 41% (at vegetative) and 14% (at reproductive) in Sonalika. Leaf area plant^{-1} was decreased by 59% in HD 2967 at the vegetative phase and at the flowering phase (insignificant) under EO at both RN/HN treatments (Supplementary fig. 5). Sonalika showed no significant change in leaf area plant^{-1} at the vegetative phase whereas reduced significantly by 35% at the flowering phase under EO at both RN/HN treatments. Significant effects of individual factors of O_3 , N, Cv and their interactions with growth parameters have been shown in the supplementary Table 1.

3.3. Photosynthetic pigments and gas-exchange parameters

Chlorophyll decreased in HD2967 at the reproductive phase, and at the vegetative phase in Sonalika under EO at both RN/HN treatments. Both the cultivars showed decrease in the carotenoid content at reproductive phase under EO at levels of N treatments (Fig. 3H). Physiological parameters A and g_s reduced under EO conditions, irrespective of RN and HN treatments. HD2967 had higher intrinsic g_s at the vegetative phase as compared to Sonalika. Sonalika showed a significant decline in g_s by 40% under EO at RN at the vegetative phase and A and E decreased under EO at both RN and HN treatment (Fig. 3C, D and F) with exception of A which has increased by 16% in Sonalika (at the vegetative phase) under EO at HN treatment. E rate significantly declined by 33% and 42% for both the cultivars HD2967 and Sonalika, respectively, under EO and at HN treatment as compared to the plants under AO and HN treatment at the reproductive phase. F_v/F_m decreased in both the cultivars under EO at both RN and HN treatments except in Sonalika (at vegetative phase) it increased under EO and HN treatment. There was an interaction between $\text{O}_3 \times \text{N} \times \text{Cv}$ for most of the gas-exchange parameters such as g_s and E at both the growth phases (Table 2).

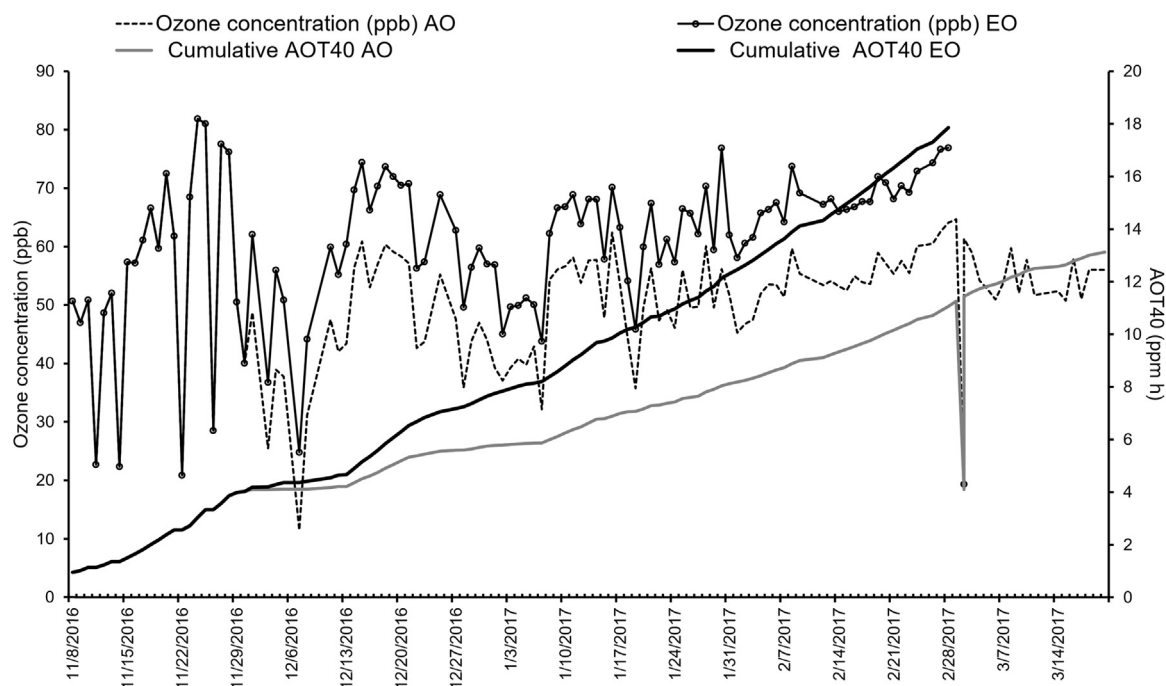


Fig. 1. Variation in 8 h (9:00–17:00) average ozone concentration and AOT40 values in ambient ozone (AO) and elevated ozone (EO) during the experimental period (8 November 2016–20 March 2017).

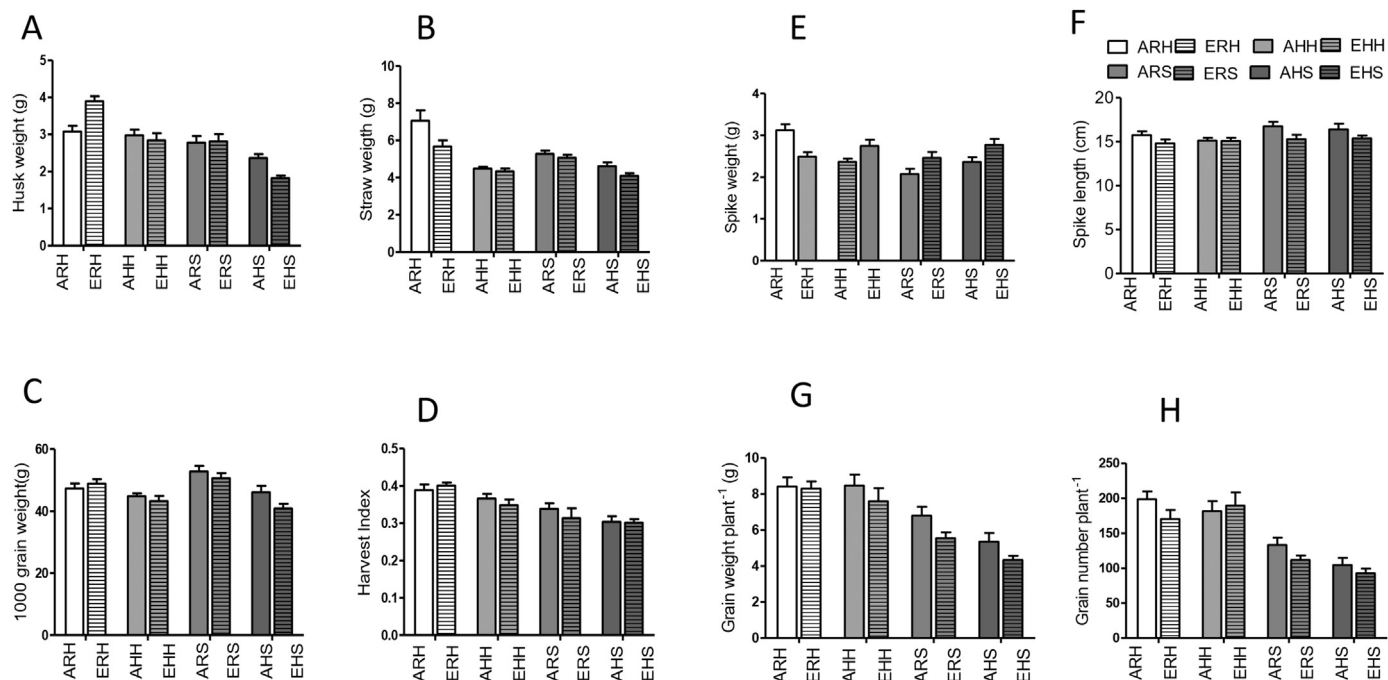


Fig. 2. Yield parameters of two wheat cultivars (HD2967 and Sonalika) shown as the mean and standard error (n = 5) for each treatment under ambient (AO) and elevated ozone (EO) at two different levels nitrogen recommended (RN) and 1.5 times recommended nitrogen (HN) (A) Husk weight; (B) straw weight plant⁻¹; (C) 1000 grain weight; (D) harvest index; (E) spike weight plant⁻¹; (F) spike length; (G) grain weight plant⁻¹; (H) grain number plant⁻¹. ARH, Ambient RN HD2967; ERH, Elevated RN HD2967; AHH, Ambient HN HD2967; EHH, Elevated HN HD2967; ARS, Ambient RN Sonalika; ERS, Elevated RN Sonalika; AHS, Ambient HN Sonalika; EHS, Elevated HN Sonalika.

3.4. MDA content and antioxidant defense

MDA content increased in HD2967 at both the vegetative and reproductive phase (insignificant) whereas increased significantly in Sonalika, under EO at HN treatment (Fig. 4A). SOD increased in HD2967 at the vegetative phase with other enzymatic antioxidants (CAT, APX, GR, POD) and ASA content showing a significant increase

indicating the severity of stress faced by the plants at the vegetative phase. Between the two tested cultivars, HD2967 showed a wider range of antioxidative protection as evident with the increased SOD, CAT, APX (vegetative) (Fig. 4B, C and D), APX (reproductive) (Fig. 4J) and POD activity (vegetative and reproductive) (Fig. 4E and K). Sonalika showed an increase of CAT activity at both the growth phases (Fig. 4C and I), while APX and GR activity increased only at the reproductive

Table 1

F ratios and levels of significance of multivariate ANOVA test for different parameters of HD2967 and Sonalika. Significant results of three-way ANOVA are marked with asterisks (* $P < 0.05$ and ** $P < 0.01$) for ozone (O_3), nitrogen (N), cultivar (Cv), and their interactions ($O_3 \times N$, $O_3 \times Cv$, $N \times Cv$, $O_3 \times N \times Cv$).

Parameters	O_3	N	Cv	$O_3 \times N$	$O_3 \times Cv$	$N \times Cv$	$O_3 \times N \times Cv$
Spike length plant ⁻¹	9558.9**	323.6	5442.6*	2915.5	295.4	190.2	213.6
Spike weight plant ⁻¹	10,857.2**	8866.5**	5928.5*	1998.0	172.4	8028.2**	652.9
Grain weight plant ⁻¹	4367.1*	7926.3**	53,352.0**	197.0	840.1	2132.8	462.9
Grain numbers plant ⁻¹	2078.7	4077.0*	60,739.6**	725.1	59.1	1935.1	118.2
Husk weight plant ⁻¹	0.4	24,945.5**	35,721.4**	7232.4**	2922.7*	1245.8	7.6
Straw weight plant ⁻¹	1155.7	3392.4**	44,749.5**	1283.1	1255.7	46,968.3**	219.5
1000 grains wt	3457.2**	23,731.1**	79,197.9**	1361.7*	1295.7*	2775.7**	125.0
Harvest index	722.8	7913.7**	36,476.2**	483.6	1274.7	377.8	2347.5

phase (Fig. 4J and L). Interestingly, both the test cultivars showed increased MDA content which reflects the oxidative damage experienced by plants due to high- O_3 conditions. Significant effects of individual factors of O_3 , N, Cv and their interactions have been shown in the Table 3.

4. Discussion

Between the two tested cultivars Sonalika proved to be more sensitive to elevated O_3 as compared to HD2967. Although, both the cultivars differed in response to HN under EO i.e., Sonalika (sensitive cultivar) invested more of its resources in improving growth eco-physiological processes such as, A and F_v/F_m , whereas HD2967 (tolerant cultivars) made investment of its resources not only in improving stimulated growth but also stimulated its antioxidant defense system

efficiently thus able to maintain the grain yield in better way as compared to the Sonalika under high- O_3 environment. Sonalika did not spend much of its resources in the antioxidant defense system except at the reproductive phase. Increase in the husk weight plant⁻¹ in HD2967 indicates the strategy adopted by tolerant cultivar by translocation of more photosynthates towards the husk in order to provide protection to the reproductive organs under EO and RN, similar structure related strategies have been reported for wheat (Gupta et al., 2017), mustard (Pandey et al., 2014). Higher N dose application may contribute to better resource allocation towards the vegetative growth as compared to reproductive structures. Our study indicates higher resource allocation pattern in Sonalika i.e. in spite the biomass was increased (at the reproductive phase) most of the yield attributes (husk weight plant⁻¹, straw weight plant⁻¹ and 1000 grain weight) decreased under EO at RN/HN with the only exception of spike weight plant⁻¹ in Sonalika at

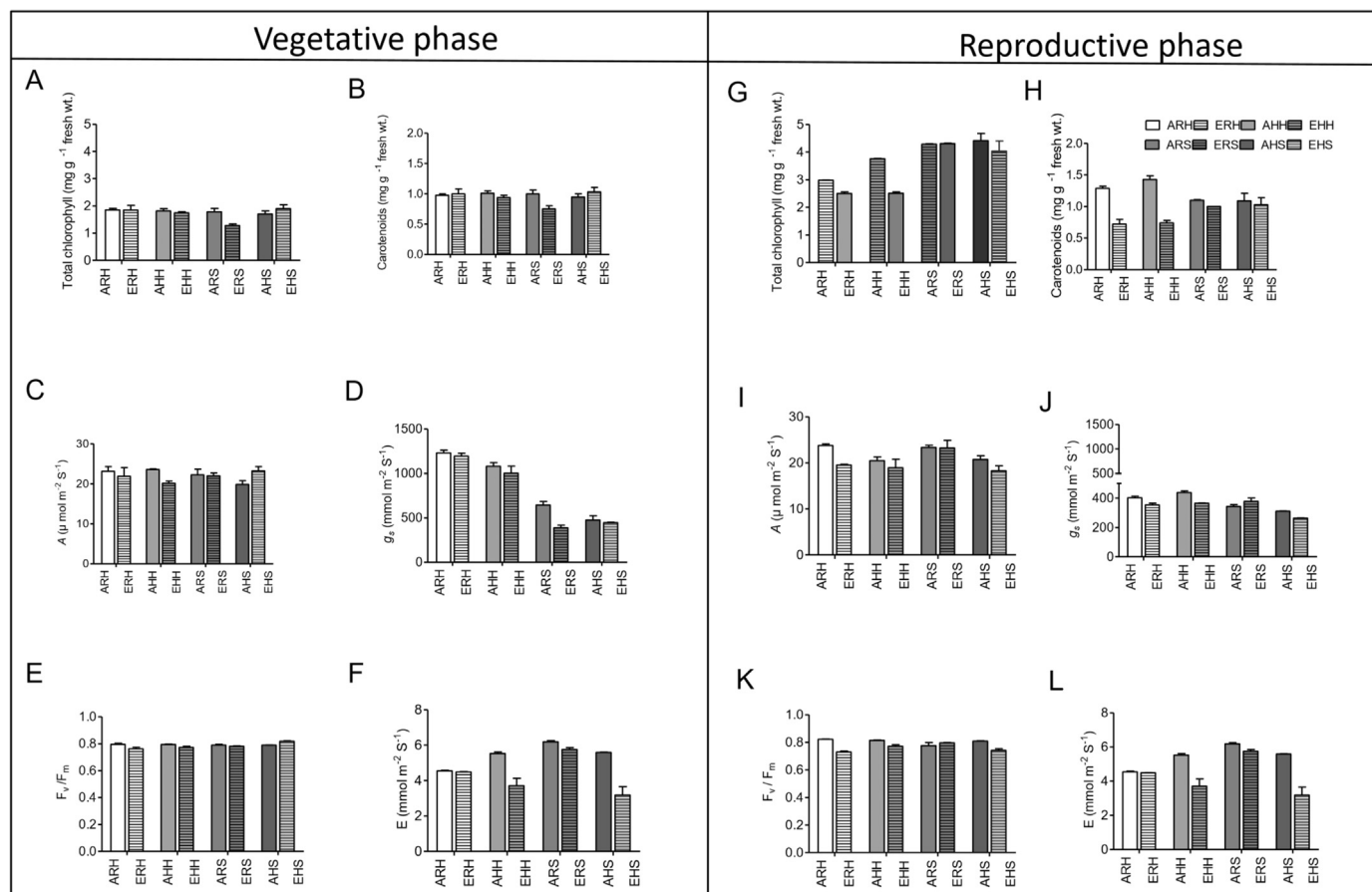


Fig. 3. Effect on chlorophyll content, carotenoid, stomatal conductance (g_s), net photosynthesis (A), maximal photochemical efficiency of PSII (F_v/F_m) and transpiration measured on the youngest fully mature leaves, at vegetative (40 DAG) and flowering phase (80 DAG), from three randomly selected plants in each treatment on two wheat cultivars (HD2967 and Sonalika) shown as the mean and standard error ($n = 3$) for each treatment under ambient (AO) and elevated ozone (EO) at two different nitrogen levels recommended (RN) and 1.5 times recommended nitrogen (HN).

Table 2

F ratios and levels of significance of multivariate ANOVA test for Chlorophyll, carotenoid, g_s , A , F_v/F_m and E at vegetative (V) and flowering (F) phase of growth in HD2967 and Sonalika. Significant results of three-way ANOVA are marked with asterisks (* $P < 0.05$ and ** $P < 0.01$) for ozone (O_3), nitrogen (N), cultivar (Cv), and their interactions ($O_3 \times N$, $O_3 \times Cv$, $N \times Cv$, $O_3 \times N \times Cv$).

Parameters	O_3	N	Cv	$O_3 \times N$	$O_3 \times Cv$	$N \times Cv$	$O_3 \times N \times Cv$
Total Chlorophyll (V)	0.022398	3.048556	0.420576	2.820709	0.033458	3.285251	8.662875 **
Total Chlorophyll (F)	15.2521 **	0.538375	17.15686 **	3.321569	5.490756 *	4.437535	0.471148
Carotenoids (V)	0.087802	3.521824	0.027099	1.822156	0.087802	2.195043	9.375271 **
Carotenoids (F)	100.5461 **	0.908518	0.071228	0.117744	34.02801 **	3.211067	0.76897
g_s (V)	21.24801 **	0.487787	168.7549 **	1.354965	1.354965	0.782628	33.87411 **
g_s (F)	9.283887 **	51.86701 **	169.4885 **	27.69821 **	99.43734 *	22.17391 **	135.3453 **
A (V)	0.747478	0.430178	0.747478	0.794327	4.736398	0.056883	2.787267
A (F)	2.445276	26.83117 **	1.375468	6.963305 *	12.37921 **	1.614264	0.009552
F_v/F_m (V)	17.41515 **	5.558458 *	16.37877 **	2.404813	3.720951	7.904617 **	3.624307
F_v/F_m (F)	12.88814 **	0.474709	6.260629 *	16.79101 **	89.57789 **	0.579907	0.064434
E (V)	6.740196 *	16.09848 **	188.6832 **	3.745544	5.349822	0.055704	11.87389 **
E (F)	0.408194	44.49057 **	42.46854 **	11.20804 **	50.83881 **	56.06892 **	68.13842 **

HN treatment. Black et al. (2000) reported that plants can adopt some structure related strategies under EO.

It is noteworthy that Singh et al. (2009, 2012) suggested the protective role of higher dose of NPK fertilizer (1.5 times the recommended dose) against ambient O_3 in terms of improved growth, biomass and yield in mustard (*Brassica campestris* L.) as compared to the plants grown in filtered air chambers (almost no O_3). Detailed information on the interactive effect of elevated- O_3 and varying levels of N fertilization

are not sufficiently related to functional traits that are involved in pollutant uptake and behavior of antioxidant defense mechanism in particular.

4.1. Prevalence of high O_3 concentration during the reproductive phase

Maximum O_3 concentrations were recorded during the reproductive phase especially during the months of February to March and this

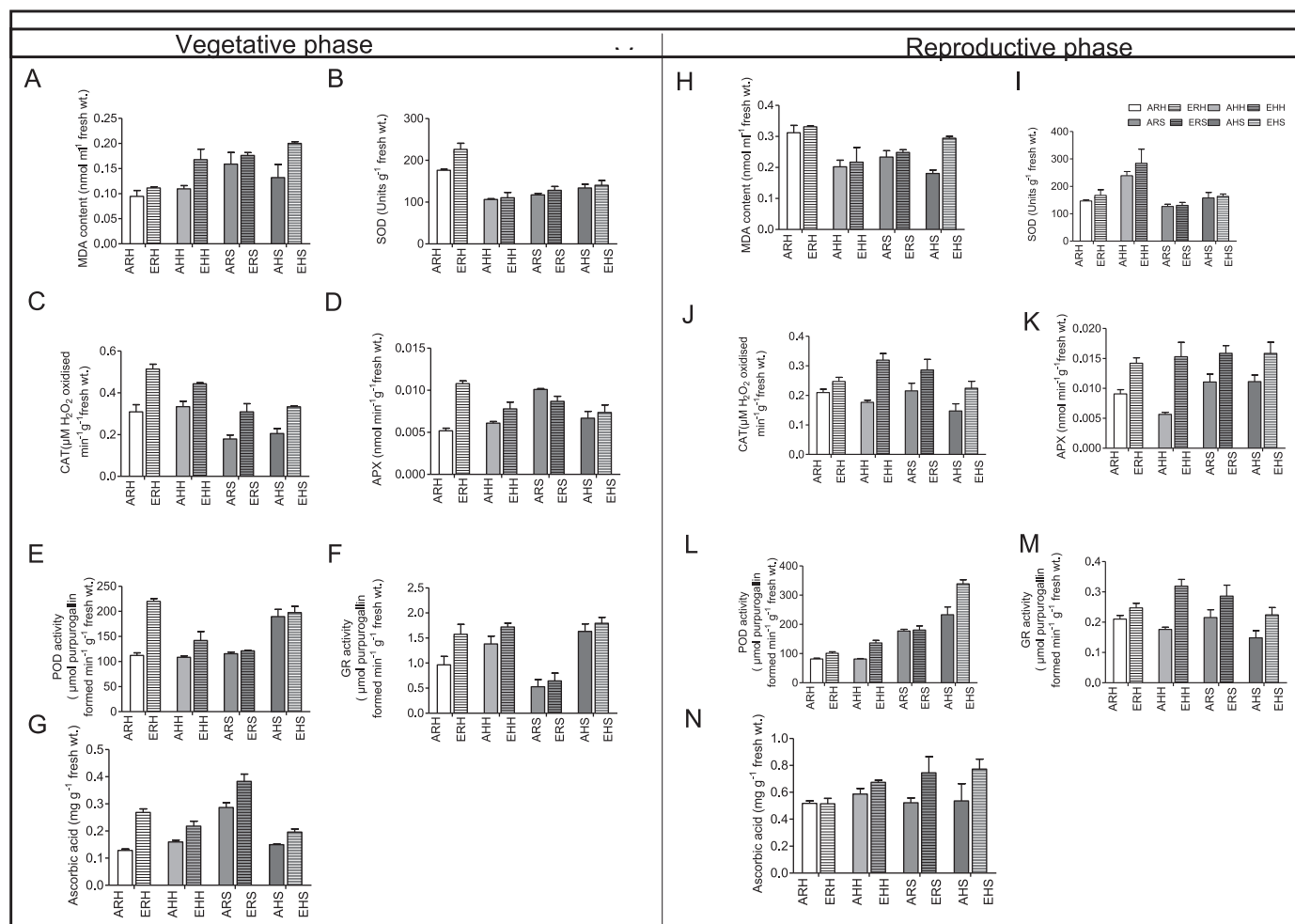


Fig. 4. Effect on MDA, SOD, CAT, APX, POD, GR and ascorbic acid at vegetative (A-G) and flowering phase (H-N), on two wheat cultivars (HD2967 and Sonalika) shown as the mean and standard error (n = 3) for each treatment under ambient (AO) and elevated ozone (EO) at two levels of nitrogen; recommended (RN) and 1.5 times recommended nitrogen (HN).

Table 3

F ratios and levels of significance of multivariate ANOVA test for different parameters antioxidant parameters of two wheat cultivars HD2967 and Sonalika. Significant results of three-way ANOVA are marked with asterisks (*P < 0.05 and **P < 0.01) for ozone (O₃), nitrogen (N), cultivar (Cv), and their interactions (O₃ × N, O₃ × Cv, N × Cv, O₃ × N × Cv).

Parameters	O ₃		N		Cv		O ₃ x N		O ₃ x Cv		N x Cv		O ₃ x N x Cv	
ASA (V)	20.79039	**	22.63755	**	0.982533		24.56332	**	3.423581		14.18777	*	17.88646	*
ASA (F)	21.83288	**	14.36388	**	5.703504	*	0.326146		9.703504	*	5.458221	*	0.973046	
SOD(V)	2.259366		18.4438	**	0.737752		0.414986		0.114		41.49856	*	0.04611	
SOD (F)	0.524322		22.15259	**	16.58986	*	0.03277	*	0.008193		0.991295		0.204813	
CAT (V)	43.21538	**	0.034615		44.86154	*	0.65		0.015385		0.465385		0.865385	
CAT (F)	35.95506	**	4.197253	*	1.68789		4.197253		0.249688		4.197253	*	2.399501	
APX (V)	92.48	**	2.88		38.72	*	5.12	*	9.102222	*	2.275556		78.54222	**
APX (F)	9.941748	**	0.349515		5.592233	*	15.53398	**	3.883495		0.15534		15.53398	**
GR (V)	16.94312	**	53.75801	**	9.530507	*	0.810755		9.530507	*	7.296794	*	0.148914	
GR (F)	23.2068	**	3.541076	*	73.63173	**	1.637394		5.09915	*	30.19263	**	2.997167	
POD (V)	89.94595	**	5.621622	*	4.790021	*	5.621622	*	38.45322	**	67.35967	**	1.629938	
POD (F)	23.04762	**	17.19048	*	246.8571	*	8.047619	*	9.333333	*	8.047619	**	0.047619	
MDA (V)	21.75063	**	0.060251		1.822594		0.810042		0.483682		0.001674		5.062762	*
MDA (F)	7.588011	*	12.59074	**	4.030518		1.833243		3.147684		13.60436	**	1.055041	

coincides well with the grain filling stage the most sensitive stage of crop to O₃-induced damages. AOT40 values exceeded the critical O₃ levels for O₃-sensitive crops (Critical AOT40 values of 3.3 ppm h for day light hours over a 3 months growing season for the wheat (Mills et al., 2007). Similar average O₃ concentrations of 55.6 ppb have been reported India (Pandey et al., 2015; Rai and Agrawal, 2014; Singh et al., 2014). Recently, Lal et al. (2017) reported that the maximum wheat production loss of about 9 million ton annually is caused due to the high concentration of ambient-O₃ in an agriculturally important region of Northern India.

4.2. Yield attributes and grain quality

Most of the yield attributes decreased in Sonalika, such as, grain weight plant⁻¹ and spike length plant⁻¹ under EO and RN treatment. In addition, Sonalika did not show any improvement in yield attributes even at HN indicating that the additional N was not sufficient to completely protect the plants against O₃-mediated damages at high-O₃ conditions (especially at reproductive phase). Insignificant change in the yield attributes in HD2967 indicates, that the cultivar is less sensitive to O₃-induced damage and under HN the extra N applied helps in the up-regulating the antioxidant defense system. Pleijel and Uddling (2012) considered grain weight as an important quality trait in wheat plants under elevated O₃ condition. Grain weight plant⁻¹ showed a significant correlation with the leaf N content at both the vegetative (R² = 40.4%) and at the reproductive phase (R² = 57.1%). Seed N decreased in both the cultivars indicating the reduction in acquisition of nutrient (N) as a result of weakened plant vitality under high-O₃ environment. Pleijel (2012) reported that increased grain protein content under high-O₃ may be due to dilution/concentration of nitrogen with an increase/decrease in biomass yield a so called growth dilution effect. Earlier studies suggest that O₃-sensitivity can be linked to various physiological (g_s) and biochemical (antioxidant activity) changes, still some additional functional traits such as leaf longevity and leaf N content may also play important role in determining O₃-sensitivity in plants as also observed in the present study (Li et al., 2016).

4.3. Growth, biomass and morphology

Leaf area plant⁻¹ was decreased under EO conditions irrespective of N levels (RN and HN) in both the cultivars HD2967 (vegetative), Sonalika (reproductive phase). Previous literatures suggested the ameliorative effects of additional N fertilization on the total leaf area. Additional N may increase leaf area plant⁻¹ by prioritizing resources (carbon and nutrients) towards the leaf (Ibrahim et al., 1998; Cooke et al., 2005; Li et al., 2012; Carriero et al., 2016). Leaf number did not

change for both the test cultivars under EO and at HN indicating that additional N treatment was able to maintain the photosynthate for repair and hence maintained the leaf formation, the only exception was in Sonalika at the reproductive phase which may be due to O₃-induced premature leaf senescence causing reduction in leaf number. There is evidence in the scientific literature that EO affects important metabolic processes leading to the reduction in carbon assimilation (Fares et al., 2013), growth (Hoshika et al., 2013), leaf area (Wittig et al., 2009), stomatal conductance, transpiration and dry matter production (Andersen (2003); Biswas et al. (2008); Zhu et al. (2011) in O₃-sensitive species/cultivars.

Yendrek et al. (2013) reported that leaves number did not change if the N fertilizer is sufficient. While, Pandey and Agrawal (1994) reported production of more leaves with smaller leaf area in polluted environment. O₃-mediated leaf senescence may be linked to metabolic changes that mimic the endogenous response that occur during the normal progression of senescence (Fiscus et al., 2005; Pell et al., 1995; Noodén, 1988). The negative effect of EO at HN treatment on leaf area plant⁻¹ indicates the severity of high-O₃ conditions (reproductive phase) in Sonalika.

Biomass increased under EO at HN as compared to plants grown at AO and at RN in both the test cultivars at both phases (vegetative and the reproductive). Interestingly, HD2967 maintained higher biomass throughout the experiment indicating that more of the photosynthetic assimilates was allocated in increasing the biomass under EO and at HN treatment. HD2967 cultivar exhibited better resource partitioning under high-O₃ as compared to Sonalika. HD2967 showed increased root/shoot ratio in response to EO condition at RN treatment (vegetative phase), while, at reproductive (high-O₃ conditions) plants shifted more of its resources towards the shoot as evident by lower root/shoot ratio in order to repair and maintain growth against O₃ induced damage under EO conditions. At HN treatment, root/shoot ratio did not show changes (at vegetative and reproductive phase) suggesting the protective role of additional N against high-O₃. In Sonalika, root/shoot ratio remained constant under EO and at RN treatment (vegetative phase) and at HN treatment (reproductive phase), except slight increase under RN treatment (reproductive). HD2967 was characterized by higher biomass, decreased (at reproductive phase) root/shoot ratio, higher g_s and increased activities of various antioxidants. Pääkkönen and Holopainen (1995) reported that additional N favors increased leaf area and confers more resistance against O₃ stress in terms of increased biomass production, as also indicated in the present study in HD2967. High-O₃ condition favors the increased demand for the carbon in the shoot for the repair and the maintenance as compared to the root system (Zheng et al., 2002; Singh et al., 2018) similar to findings shown in the present study for cultivar HD2967.

4.4. Photosynthetic pigment, gas-exchange

Chlorophyll content and *A* are highly correlated parameters (Fleischer, 1935). In this study chlorophyll and carotenoid contents and *A* declined in both the cultivars irrespective of N levels RN or HN (at reproductive phase). Decrease in the chlorophyll content in Sonalika (reproductive phase) under EO and at RN treatment indicates sensitivity of the Sonalika cultivar against prevailing high-O₃ conditions. N is an essential element in the chlorophyll biosynthesis being a cofactor for the glutamyl-tRNA reductase enzyme needed for the chlorophyll production from 5-aminolaevulinic acid (Tanaka and Tanaka, 2007). Emerson (1929) also reported a strong correlation between the chlorophyll content and *A*. Leaf N content increased in HD2967 at the vegetative phase, while decreased in Sonalika at the vegetative phase. In previous studies high-N availability has been associated with the increased *g_s* rate as observed in this study for HD2967 (Yamori et al., 2011; Urairi et al., 2016). Overall, HD2967 maintained higher intrinsic *g_s* under EO at the vegetative phase as compared to Sonalika. Though these difference for *g_s* between the cultivars were insignificant at the reproductive phase. Sonalika showed decrease in the *g_s* under EO and at RN treatment as compared AO at RN. Although *g_s* improved under EO conditions at HN treatment. Increased O₃-sensitivity has been associated with the increased *g_s* in wheat cultivars resulting to higher stomatal flux (Biswas et al., 2008; Pleijel et al., 2006). Possible role of N induced decrease in the *g_s* has been documented in several species (Sun et al., 2016). Application of HN to the plants provided partial protection against high-O₃ conditions at the reproductive phase.

Increase in the *A* at HN treatment was observed in Sonalika at the vegetative phase which can be attributed to the increase in the Ribulose 1,5-bisphosphate (RuBP) carboxylase activity (Maheshwari et al., 1993) even under EO at HN treatment. Positive effects of HN treatment on the process related to photochemistry resulting in enhanced *A* has been reported in previous studies (Fusaro et al., 2017). The mechanism associated with the increased *A* at HN treatment further reflects the possibility of investment of the available N in protein required to enhance *A*. Therefore decline in the N content of leaf in Sonalika at the vegetative phase could have been caused by higher resource allocation to increase the *A*. In addition, increased *F_v/F_m* ratio in Sonalika at the vegetative phase also supported the view that N was partially allocated to light harvesting complex. It increases the capacity to manage the energy flow through the photosystem even at low *g_s*, indicating that the HN treatment could be useful in ameliorating the detrimental effects of O₃ and photosystem functionality (Palmroth et al., 2014).

4.4.1. Biochemical parameters

Most of the antioxidant activity response related to oxidative stress varied between the cultivars, growth phase and N levels (RN or HN) in response to EO. MDA content is an indicator to oxidative stress and increased in the reproductive phase (Sonalika) indicates the sensitivity against O₃-mediated damage. In addition, increase in the MDA content at the vegetative phase of HD2967 indicates increased stress levels since an early stage of growth. Most of the antioxidant activity was up-regulated under EO and for both the cultivars in terms of increased activity though the array of enzymes varied between the cultivars. Additional N (HN) under EO treatment favored the enhanced antioxidant activity in both the cultivars.

HD2967 showed increase in the activity of ROS scavengers such as, SOD, CAT and APX which play an important role in scavenging superoxide anion (O₂^{•−}) and hydrogen peroxide (H₂O₂) and were higher under EO and HN treatment as compared to AO and RN at the vegetative phase. Increased ASA content in HD2967 (vegetative phase) indicates its potential role by directly scavenging the O₃ in the apoplast and also as a reductant for the activity of APX to convert H₂O₂ into non toxic form (Burkey et al., 2006). Conversely, in spite of similar levels of ROS formed in HD2967 (Table 3), SOD, APX and POD activities did not show significant changes in Sonalika at vegetative phase but played

important role in ROS scavenging at the reproductive phase indicating the possible involvement of nitric oxide (NO) under EO with HN treatment. This view is supported by the fact that there was no significant difference in the MDA content between two tested cultivars. At HN treatment plants may have higher NO production which is involved in triggering the antioxidant response or can also directly scavenge the ROS produced and concomitantly increased the *A* in Sonalika (at vegetative phase) (Das et al., 2015; Neill et al., 2003; Vainonen and Kangasjarvi, 2015).

The differences in the response of the two tested cultivars indicate differential strategies adopted by cultivars under EO condition and at different levels of N fertilization depending on their developmental phase (at the vegetative and reproductive phase) i.e., cultivar HD2967 was able to sense the stress at an early stage with up-regulation of its antioxidant defense in response to ROS produced at the vegetative phase. Black et al. (2000) reported that plants can adopt some structure related strategies under EO. On the contrary, Sonalika invested its resources to maintain the key eco-physiological parameters such as *A*, *F_v/F_m* and biomass though, at latter growth phase (reproductive) up-regulated the antioxidant defense which is considered to be the most sensitive phase for reproductive events and grain setting. During the grain filling phase, plant suffered from extremely high-O₃ concentrations, thereby indicating allocation of sources more for internal cell regulation (antioxidant defense) at the cost of reduction in grain yield plant^{−1} and other yield attributes. Growth, *A* and yield attributes were affected under high-O₃ environment and N availability in the soil. In a recent meta-analysis, Yendrek et al. (2013) reported that N fertilization may partially ameliorate the O₃-mediated adverse effects in tobacco plants.

5. Conclusions

The present study demonstrated the differential response of the two cultivars of wheat provided with different N dose under high-O₃ conditions. Interaction of O₃ and N varies with cultivars, developmental stage and prevailing O₃ concentrations. The results pointed out that application of higher dose of N was not able to provide full protection to the plants against O₃ toxicity. However, cultivar HD2967 showed higher biomass, efficient resource partitioning, enhanced antioxidant defense system with less reduction in economic yield against O₃ at higher N dose. It can be concluded that based on the above facts, cultivar HD2967 with higher antioxidative capacity against O₃ irrespective of N fertilization is suitable for cultivation in areas experiencing higher O₃ concentration.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ecoenv.2018.04.014>.

References

- Agathokleous, E., 2017. Perspectives for elucidating the ethylenediurea (EDU) mode of action for protection against O₃ phytotoxicity. *Ecotoxicol. Environ. Saf.* 142, 530–537.
- Agathokleous, E., Saitanis, C.J., Koike, T., 2015. Tropospheric O₃, the nightmare of wild plants: a review study. *J. Agric. Meteorol.* 71 (2), 142–152.

- Agathokleous, E., Paoletti, E., Saitanis, C.J., Manning, W.J., Sugai, T., Koike, T., 2016. Impacts of ethylenediurea (EDU) soil drench and foliar spray in *Salix sachalinensis* protection against O₃-induced injury. *Sci. Total Environ.* 573, 1053–1062.
- Ainsworth, E.A., 2016. Understanding and improving global crop response to ozone pollution. *Plant J.* 90 (5), 886–897.
- Andersen, C.P., 2003. Source-sink balance and carbon allocation below ground in plants exposed to ozone. *New Phytol.* 157 (2), 213–228.
- Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 24, 1–15.
- Beyer, W.F., Fridovich, I., 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal. Biochem.* 161 (2), 559–566.
- Biswas, D.K., Xu, H., Li, Y.G., Sun, J.Z., Wang, X.Z., Han, X.G., Jiang, G.M., 2008. Genotypic differences in leaf biochemical, physiological and growth responses to ozone in 20 winter wheat cultivars released over the past 60 years. *Glob. Change Biol.* 14 (1), 46–59.
- Black, V.J., Black, C.R., Roberts, J.A., Stewart, C.A., 2000. Tansley Review No. 115 Impact of ozone on the reproductive development of plants. *New Phytol.* 147 (3), 421–447.
- Brauer, M., Freedman, G., Frostad, J., Van Donkelaar, A., Martin, R.V., Dentener, F., Dingenen, R.V., Estep, K., Amini, H., Apté, J.S., Balakrishnan, K., 2015. Ambient air pollution exposure estimation for the global burden of disease 2013. *Environ. Sci. Technol.* 50 (1), 79–88.
- Britton, C., Mehley, A.C., 1955. Assay of catalase and peroxidase. *Methods Enzymol.* 2, 764–775.
- Burkey, K.O., Neufeld, H.S., Souza, L., Chappelka, A.H., Davison, A.W., 2006. Seasonal profiles of leaf ascorbic acid content and redox state in ozone-sensitive wildflowers. *Environ. Pollut.* 143 (3), 427–434.
- Burney, J., Ramanathan, V., 2014. Recent climate and air pollution impacts on Indian agriculture. *Proc. Natl. Acad. Sci.* 111 (46), 16319–16324.
- Carriero, G., Brunetti, C., Fares, S., Hayes, F., Hoshika, Y., Mills, G., Tattini, M., Paoletti, E., 2016. BVOC responses to realistic nitrogen fertilization and ozone exposure in silver birch. *Environ. Pollut.* 213, 988–995.
- Chen, G.X., Asada, K., 1989. Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. *Plant Cell Physiol.* 30 (7), 987–998.
- Cooke, J.E., Martin, T.A., Davis, J.M., 2005. Short-term physiological and developmental responses to nitrogen availability in hybrid poplar. *New Phytol.* 167 (1), 41–52.
- Cooper, O.R., Parrish, D.D., Ziemke, J., Balashov, N.V., Cupeiro, M., Galbally, I.E., Gilge, S., Horowitz, L., Jensen, N.R., Lamarque, J.F., Naik, V., 2014. Global distribution and trends of tropospheric ozone: an observation-based review. *Elem. Sci. Anthropocene* 2 (pp000029).
- Das, P., Nutan, K.K., Singla-Pareek, S.L., Pareek, A., 2015. Oxidative environment and redox homeostasis in plants: dissecting out significant contribution of major cellular organelles. *Front. Environ. Sci.* 2 (70), 1–11.
- De Leeuw, F.A.A.M., Van Zantvoort, E.D.G., 1997. Mapping of exceedances of ozone critical levels for crops and forest trees in the Netherlands: preliminary results. *Environ. Pollut.* 96 (1), 89–98.
- Emberson, L., Bükler, P., 2008. Ozone: a threat to food security in South Asia. Policy brief. Stockholm Environment Institute, Univ. of York, UK. (www.sei.se).
- Emerson, R., 1929. The relation between maximum rate of photosynthesis and concentration of chlorophyll. *J. General. Physiol.* 12 (5), 609–622.
- Fares, S., Vargas, R., Detto, M., Goldstein, A.H., Karlik, J., Paoletti, E., Vitale, M., 2013. Tropospheric ozone reduces carbon assimilation in trees: estimates from analysis of continuous flux measurements. *Glob. Change Biol.* 19 (8), 2427–2443.
- Fiscus, E.L., Booker, F.L., Burkey, K.O., 2005. Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. *Plant, Cell & Environment* 28, pp. 997–1011.
- Fleischer, W.E., 1935. The relation between chlorophyll content and rate of photosynthesis. *J. General. Physiol.* 18 (4), 573–597.
- Foley, J.A., Ramankutty, N., Brauman, K.A., Cassidy, E.S., Gerber, J.S., Johnston, M., Mueller, N.D., O'Connell, C., Ray, D.K., West, P.C., Balzer, C., 2011. Solutions for a cultivated planet. *Nature* 478 (7369), 337–342.
- Fowler, D., Amann, M., Anderson, F., Ashmore, M., Cox, P., Depledge, M., Derwent, D., Grennfelt, P., Hewitt, N., Hov, O., Jenkin, M., 2008. Ground-level ozone in the 21st century: future trends, impacts and policy implications. *R. Soc. Sci. Policy Report* 15 (08), 1–132. (www.royalsociety.org).
- Fusaro, L., Palma, A., Salvatori, E., Basile, A., Maresca, V., Karam, E.A., Manes, F., 2017. Functional indicators of response mechanisms to nitrogen deposition, ozone, and their interaction in two Mediterranean tree species. *PLoS One* 12 (10) (p.e0185836).
- Good, A.G., Shrawat, A.K., Muench, D.G., 2004. Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? *Trends Plant Sci.* 9 (12), 597–605.
- Gupta, S.K., Sharma, M., Majumder, B., Maurya, V.K., Lohani, M., Deeba, F., Pandey, V., 2017. Impact of Ethylene diurea (EDU) on growth, yield and proteome of two winter wheat varieties under high ambient ozone phytotoxicity. *Chemosphere*.
- Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts: i. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* 125 (1), 189–198.
- Hoshika, Y., Pecori, F., Conese, I., Bardelli, T., Marchi, E., Manning, W.J., Badea, O., Paoletti, E., 2013. Effects of a three-year exposure to ambient ozone on biomass allocation in poplar using ethylenediurea. *Environ. Pollut.* 180, 299–303.
- Ibrahim, L., Proe, M.F., Cameron, A.D., 1998. Interactive effects of nitrogen and water availabilities on gas exchange and whole-plant carbon allocation in poplar. *Tree Physiol.* 18 (7), 481–487.
- Keller, T., Schwager, H., 1977. Air pollution and ascorbic acid. *For. Pathol.* 7 (6), 338–350.
- Klingberg, J., Engardt, M., Karlsson, P.E., Langner, J., Pleijel, H., 2014. Declining ozone exposure of European vegetation under climate change and reduced precursor emissions. *Biogeosciences* 11 (19), 5269–5283.
- Lal, S., Venkataramani, S., Naja, M., Kuniyal, J.C., Mandal, T.K., Bhuyan, P.K., Kumari, K.M., Tripathi, S.N., Sarkar, U., Das, T., Swamy, Y.V., 2017. Loss of crop yields in India due to surface ozone: an estimation based on a network of observations. *Environ. Sci. Pollut. Res.* 24 (26), 20972–20981.
- Li, H., Li, M., Luo, J., Cao, X., Qu, L., Gai, Y., Jiang, X., Liu, T., Bai, H., Janz, D., Polle, A., 2012. N-fertilization has different effects on the growth, carbon and nitrogen physiology, and wood properties of slow-and fast-growing *Populus* species. *J. Exp. Bot.* 63 (17), 6173–6185.
- Li, P., Calatayud, V., Gao, F., Uddling, J., Feng, Z., 2016. Differences in ozone sensitivity among woody species are related to leaf morphology and antioxidant levels. *Tree Physiol.* 36 (9), 1105–1116.
- Maheshwari, M., Nair, T.V.R., Abrol, Y.P., 1993. Senescence and nitrogen remobilization. *Proc. Indian Natl. Sci. Acad.* B59 (3 and 4), 245–256.
- Meehl, G.A., Arblaster, J.M., Tebaldi, C., 2007. Contributions of natural and anthropogenic forcing to changes in temperature extremes over the United States. *Geophys. Res. Lett.* 34 (19).
- Mills, G., Buse, A., Gimeno, B., Bermejo, V., Holland, M., Emberson, L., Pleijel, H., 2007. A synthesis of AOT40-based response functions and critical levels of ozone for agricultural and horticultural crops. *Atmos. Environ.* 41 (12), 2630–2643.
- Mueller, N.D., Gerber, J.S., Johnston, M., Ray, D.K., Ramankutty, N., Foley, J.A., 2012. Closing yield gaps through nutrient and water management. *Nature* 490 (7419), 254–257.
- Neill, S.J., Desikan, R., Hancock, J.T., 2003. Nitric oxide signalling in plants. *New Phytol.* 159 (1), 11–35.
- Noodén, L.D., 1988. The phenomena of senescence and aging. In: Noodén, L.D., Leopold, A.C. (Eds.), 'Senescence and aging in plants', pp. 1–50.
- Ohara, T., Akimoto, H., Kurokawa, J., Horii, N., Yamaji, K., Yan, X., Hayasaka, T., 2007. An Asian emission inventory of anthropogenic emission sources for the period 1980–2020. *Atmos. Chem. Phys.* 7 (16), 4419–4444.
- Oksanen, E., Pandey, V., Pandey, A.K., Keski-Saari, S., Kontunen-Soppela, S., Sharma, C., 2013. Impacts of increasing ozone on Indian plants. *Environ. Pollut.* 177, 189–200.
- Osborne, S.A., Mills, G., Hayes, F., Ainsworth, E.A., Bükler, P., Emberson, L., 2016. Has the sensitivity of soybean cultivars to ozone pollution increased with time? An analysis of published dose-response data. *Glob. Change Biol.* 22 (9), 3097–3111.
- Pääkkönen, E., Holopainen, T., 1995. Influence of nitrogen supply on the response of clones of birch (*Betula pendula* Roth.) to ozone. *New Phytol.* 129 (4), 595–603.
- Palmroth, S., Bach, L.H., Nordin, A., Palmqvist, K., 2014. Nitrogen-addition effects on leaf traits and photosynthetic carbon gain of boreal forest understory shrubs. *Oecologia* 175, 457–470.
- Pandey, A.K., Majumder, B., Keski-Saari, S., Kontunen-Soppela, S., Pandey, V., Oksanen, E., 2014. Differences in responses of two mustard cultivars to ethylenediurea (EDU) at high ambient ozone concentrations in India. *Agric., Ecosyst. Environ.* 196, 158–166.
- Pandey, A.K., Majumder, B., Keski-Saari, S., Kontunen-Soppela, S., Mishra, A., Sahu, N., Pandey, V., Oksanen, E., 2015. Searching for common responsive parameters for ozone tolerance in 18 rice cultivars in India: results from ethylenediurea studies. *Sci. Total Environ.* 532, 230–238.
- Pandey, J., Agrawal, M., 1994. Evaluation of air pollution phytotoxicity in a seasonally dry tropical urban environment using three woody perennials. *New Phytol.* 126 (1), 53–61.
- Parsons, T.R., Maita, Y., Lalli, C.M., 1984. Determination of chlorophylls and total carotenoids: spectrophotometric method. Parsons, TR, Y. Maita and CM Lalli. A manual of chemical and biological methods for seawater analysis. Pergamon Press, Oxford, pp. 101–112.
- Pell, E.J., Sinn, J.P., Johansen, C.V., 1995. Nitrogen supply as a limiting factor determining the sensitivity of *Populus tremuloides* Michx. to ozone stress. *New Phytol.* 130, 437–446.
- Pleijel, H., 2012. Effects of ozone on zinc and cadmium accumulation in wheat—dose-response functions and relationship with protein, grain yield, and harvest index. *Ecol. Evol.* 2 (12), 3186–3194.
- Pleijel, H., Uddling, J., 2012. Yield vs. Quality trade-offs for wheat in response to carbon dioxide and ozone. *Glob. Change Biol.* 18 (2), 596–605.
- Pleijel, H., Eriksen, A.B., Danielsson, H., Bondesson, N., Sellden, G., 2006. Differential ozone sensitivity in an old and a modern Swedish wheat cultivar—grain yield and quality, leaf chlorophyll and stomatal conductance. *Environ. Exp. Bot.* 56 (1), 63–71.
- Pleijel, H., Broberg, M.C., Uddling, J., Mills, G., 2018. Current surface ozone concentrations significantly decrease wheat growth, yield and quality. *Sci. Total Environ.* 613, 687–692.
- Rai, R., Agrawal, M., 2014. Assessment of competitive ability of two Indian wheat cultivars under ambient O₃ at different developmental stages. *Environ. Sci. Pollut. Res.* 21 (2), 1039–1053.
- Rao, M.V., Paliyath, G., Ormrod, D.P., 1996. Ultraviolet-B and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. *Plant Physiol.* 110 (1), 125–136.
- Singh, A.A., Agrawal, S.B., Shahi, J.P., Agrawal, M., 2014. Assessment of growth and yield losses in two *Zea mays* L. cultivars (quality protein maize and nonquality protein maize) under projected levels of ozone. *Environ. Sci. Pollut. Res.* 21 (4), 2628–2641.
- Singh, P., Agrawal, M., Agrawal, S.B., 2009. Evaluation of physiological, growth and yield responses of a tropical oil crop (*Brassica campestris* L. var. Kranti) under ambient ozone pollution at varying NPK levels. *Environ. Pollut.* 157 (3), 871–880.
- Singh, P., Singh, S., Agrawal, S.B., Agrawal, M., 2012. Assessment of the interactive effects of ambient O₃ and NPK levels on two tropical mustard varieties (*Brassica campestris* L.) using open-top chambers. *Environ. Monit. Assess.* 184 (10), 5863–5874.
- Singh, S., Singh, P., Agrawal, S.B., Agrawal, M., 2018. Use of Ethylenediurea (EDU) in

- identifying indicator cultivars of Indian clover against ambient ozone. *Ecotoxicol. Environ. Saf.* 147, 1046–1055.
- Smith, I.K., Vierheller, T.L., Thorne, C.A., 1988. Assay of glutathione reductase in crude tissue homogenates using 5, 5'-dithiobis (2-nitrobenzoic acid). *Anal. Biochem.* 175 (2), 408–413.
- Solomon, S. (Ed.), 2007. *Climate change 2007-the physical science basis: Working group I contribution to the fourth assessment report of the IPCC 4* Cambridge university press.
- Sun, J., Feng, Z., Ort, D.R., 2014. Impacts of rising tropospheric ozone on photosynthesis and metabolite levels on field grown soybean. *Plant Sci.* 226, 147–161.
- Sun, J., Ye, M., Peng, S., Li, Y., 2016. Nitrogen can improve the rapid response of photosynthesis to changing irradiance in rice (*Oryza sativa* L.) plants. *Sci. Rep.* 6, 31305.
- Tai, A.P., Martin, M.V., 2017. Impacts of ozone air pollution and temperature extremes on crop yields: spatial variability, adaptation and implications for future food security. *Atmos. Environ.* 169, 11–21.
- Tai, A.P., Martin, M.V., Heald, C.L., 2014. Threat to future global food security from climate change and ozone air pollution. *Nat. Clim. Change* 4 (9), 817–821.
- Tanaka, R., Tanaka, A., 2007. Tetrapyrrole biosynthesis in higher plants. *Annu. Rev. Plant Biol.* 58, 321–346.
- Urairi, C., Tanaka, Y., Hirooka, Y., Homma, K., Xu, Z., Shiraiwa, T., 2016. Response of the leaf photosynthetic rate to available nitrogen in erect panicle-type rice (*Oryza sativa* L.) cultivar, Shennong265. *Plant Prod. Sci.* 19 (3), 420–426.
- Vainonen, J.P., Kangasjarvi, J., 2015. Plant signalling in acute ozone exposure. *Plant Cell Environ.* 38 (2), 240–252.
- Wilkinson, S., Mills, G., Illidge, R., Davies, W.J., 2012. How is ozone pollution reducing our food supply? *J. Exp. Bot.* 63 (2), 527–536.
- Wittig, V.E., Ainsworth, E.A., Naidu, S.L., Karnosky, D.F., Long, S.P., 2009. Quantifying the impact of current and future tropospheric ozone on tree biomass, growth, physiology and biochemistry: a quantitative meta-analysis. *Glob. Change Biol.* 15 (2), 396–424.
- Wu, H., Li, Q., Lu, C., Zhang, L., Zhu, J., Dijkstra, F.A., Yu, Q., 2016. Elevated ozone effects on soil nitrogen cycling differ among wheat cultivars. *Appl. Soil Ecol.* 108, 187–194.
- Yamori, W., Nagai, T., Makino, A., 2011. The rate-limiting step for CO₂ assimilation at different temperatures is influenced by the leaf nitrogen content in several C3 crop species. *Plant, Cell Environ.* 34 (5), 764–777.
- Yendrek, C.R., Leisner, C.P., Ainsworth, E.A., 2013. Chronic ozone exacerbates the reduction in photosynthesis and acceleration of senescence caused by limited N availability in *Nicotianasyvestris*. *Glob. Change Biol.* 19 (10), 3155–3166.
- Zar, J.H., 1974. *Biostatistical Analysis*. Prentice-Hall, Englewood Cliffs, N.J (620 pp).
- Zheng, Y., Shimizu, H., Barnes, J.D., 2002. Limitations to CO₂ assimilation in ozone-exposed leaves of *Plantago major*. *New Phytol.* 155 (1), 67–78.
- Zhu, X., Feng, Z., Sun, T., Liu, X., Tang, H., Zhu, J., Guo, W., Kobayashi, K., 2011. Effects of elevated ozone concentration on yield of four Chinese cultivars of winter wheat under fully open-air field conditions. *Glob. Change Biol.* 17 (8) (2697–270).