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High Variation in Resource Allocation Strategies among 11 Indian Wheat (*Triticum aestivum*) Cultivars Growing in High Ozone Environment

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Received: 26 December 2018; Accepted: 23 January 2019; Published: 28 January 2019

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Abstract: Eleven local cultivars of wheat (*Triticum aestivum*) were chosen to study the effect of ambient ozone (O_3) concentration in the Indo-Gangetic Plains (IGP) of India at two high-ozone experimental sites by using 300 ppm of Ethylenediurea (EDU) as a chemical protectant against O_3. The O_3 level was more than double the critical threshold reported for wheat grain production (AOT40 8.66 ppm h). EDU-grown plants had higher grain yield, biomass, stomatal conductance and photosynthesis, less lipid peroxidation, changes in superoxide dismutase and catalase activities, changes in content of oxidized and reduced glutathione compared to non-EDU plants, thus indicating the severity of O_3 induced productivity loss. Based on the yield at two different growing sites, the cultivars could be addressed in four response groups: (a) generally well-adapted cultivars (above-average yield); (b) poorly-adapted (below-average yield); (c) adapted to low-yield environment (below-average yield); and (d) sensitive cultivars (adapted to high-yield environment). EDU responses were dependent on the cultivar, the developmental phase (vegetative, flowering and harvest) and the experimental site.

Keywords: cultivars; EDU (ethylenediurea); grain yield; India; ozone; wheat

1. Introduction

Tropospheric ozone (O_3) is a phytotoxic pollutant causing substantial damage to agricultural production and food security [1–7]. O_3-induced loss in plant productivity has been estimated to range between 14 and 26 billion US$ on the global scale [8]. Modelling studies suggest a further increase in O_3 concentrations, especially in the East and South Asian regions due to the increased O_3 precursor emissions that result from high population density, rapid industrial growth and favorable climatic conditions [9–11]. Tropospheric O_3 concentration has increased in India and China by 20% and 13%, respectively, from the year 1999 to 2013 [12]. Recent studies have shown very high O_3 concentrations in India, particularly in the Indo-Gangetic Plains (IGP) region, which is one of the most fertile agricultural land areas facing high pollution and population loads [13–15]. By 2030, the population of India is projected to increase further by 300 million people (United Nations Population Division [16,17]. Therefore, due to limited arable land, food security in India is under threat. The selection of O_3-tolerant crops or cultivars can be an important strategy for food security in the area suffering from high-O_3 concentrations [3].

O_3 can adversely affect crop productivity either directly causing the oxidative damage as a result of the production of the reactive oxygen species (ROS) or indirectly as a greenhouse gas [3]. O_3 enters the leaves mainly through stomatal pores. After entry, O_3 is rapidly dissolved in the apoplast and it generates ROS that finally causes an imbalance in the redox status of the cells. This eventually leads to cellular damage or programmed cell death [18–20]. O_3 modifies the plant metabolism by adversely affecting the photosynthetic carbon assimilation, stomatal regulation and plant growth leading to reduced crop yield [21,22].

India is a major producer of wheat (*Triticum aestivum*) accounting 12% of the total wheat production in the world [23]. Wheat is sensitive to O_3-induced damage [17,24]. The critical O_3 level for 5% yield reduction, a three-month Accumulated Ozone exposure over the Threshold of 40 ppb (AOT40) for three months is 3 ppm h, for wheat [25]. Recently, Ghude et al. [24] estimated the production loss of 3.5 ± (0.8) million tons of wheat in India. Despite the obvious sensitivity of wheat to O_3, large scale screening of Indian wheat cultivars in the field conditions has been scarcely done due to technical limitations [26,27]. So far, responses of 14 wheat cultivars cultivated in India have been tested in open top chambers for their O_3 sensitivity to elevated O_3 of 30 ppb with respect to ambient O_3, resulting in lower gas exchange rates, biomass, and yield [27].

A chemical protectant, EDU [ethylenediurea; N-(2-2-oxo-1-imidazolidinyl) ethyl]-N′- phenyl urea N-(2-2-oxo-1-imidazolidinyl) ethyl]-N′-phenyl urea) was first introduced by Carnahan et al. [28]. Thereafter, several reports have shown that the use of EDU can specifically prevent the O_3 injury as well as decrease the growth and yield losses in the ambient field conditions [13,15,29–31]. EDU application is a useful and cost-effective method for the large-scale screening of plant materials for ozone-tolerance/sensitivity, particularly in remote areas, lacking electricity and infrastructures for O_3 exposure or its removal [32].

In this study, we compared the productivity and performance of 11 Indian wheat cultivars throughout the growing season in the ambient field conditions at two high-ozone experimental sites, NBRI (urban) and Banthra (semi-urban), in the IGP region of India. EDU application was used as a research tool to protect the plants against high-ozone stress at both experimental sites. The plants with and without the application of EDU were measured for growth, gas-exchange antioxidants and yield attributes in two developmental phases and two sites. The aim was (1) to classify the cultivars into four adaptation groups, according to their yield (grain weight), and thereafter (2) to indicate the key parameters linked to adaptation strategies for each adaptation group.

2. Materials and Methods

2.1. Experimental Sites and Plant Material

A field study was carried out from 6 December 2011 to 29 March 2012 at two experimental sites (1) the Botanical garden of the CSIR-National Botanical Research Institute, NBRI (26°55′ N, 80°59′ E) in Lucknow and (2) at the Banthra (26°45′ N, 80°53′ E) approximately 25 km from the Lucknow city, India (Figure 1). The NBRI site represents an urban site with soil type of sandy loam (sand 50%, silt 33%, clay 17%; pH 8.4 and electrical conductivity 231.1 μS cm^{-1}), and Banthra a semi-urban site with silt clay loam (sand 14.5%, silt 53.5%, clay 32%; pH 8.4 and electrical conductivity 219 μS cm^{-1}) soil. Eleven locally important wheat (*Triticum aestivum* L.) cultivars were chosen for the present study (Table S1).

Figure 1. The location of experimental sites in Lucknow, state of Uttar Pradesh, India.

2.2. Experimental Design and EDU Application

The field size was 400 m^2 at the both experimental sites. The field was divided into six plots; three for ambient O_3-grown (non-EDU treated) and three for EDU-treated plants, where non-EDU treated were sprayed with water. Each plot had 11 subplots of 1.5 × 1.5 m in dimension, one for each of the 11 cultivars. The distance between the subplots was 0.5 m. Seeds were sown in each subplot in rows with 25 cm spacing. The recommended dose of NPK fertilisation (120:60:60 kg ha^{-1}) was applied during the field preparation. At first, nitrogen was applied as basal dose which included a full dose of potassium and phosphorus, the second and third doses of nitrogen were applied after 30 and 60 days of sowing (DAS) as top dressing.

EDU was applied at 300 ppm concentration as a foliar spray to individual plants until its entire foliage was visibly saturated. EDU treatment was started after 15 DAS and continued at an interval of 15 days until the final harvest phase. Application of EDU was done on a cloud free day to avoid risk of washing away. The choice of 300 ppm EDU concentration was based on the earlier experiments by Feng et al. [33] suggesting the concentrations at 200–400 ppm range as the most effective in ameliorating effects of high-O_3 concentration in field conditions. Paoletti et al. [34] also demonstrated that 300 ppm of EDU concentration was effective to halt the O_3-induced ROS formation in *Phaseolus vulgaris*. EDU was obtained from Prof. W.J. Manning, University of Massachusetts, Amherst, USA.

2.3. Ozone Monitoring

Ambient O_3 monitoring was carried out with a 2B Tech Ozone Monitor (106-L) for 8 h day^{-1} (9.00 to 17.00) regularly at the NBRI site, and on weekly basis at the Banthra site using the same device (Figure 2). For the NBRI site, the AOT40 (accumulated exposure over a threshold of 40 ppb) exposure index for the O_3 concentration was calculated as described by De Leeuw and Zantvoort [35].

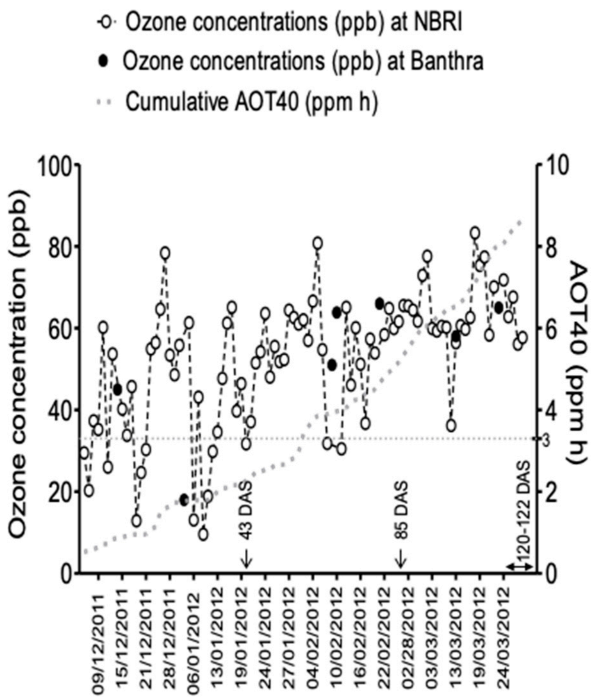

Figure 2. Variation in 8 h (9:00 to 17:00) average ozone concentration at NBRI (open circles) and Banthra (closed circles) sites. Accumulated Ozone exposure over the Threshold of 40 ppb (AOT40) accumulation during the experimental period (6 December 2011 to 29 March 2012) is indicated by grey dashed line. Horizontal line indicates the AOT40 threshold for wheat (3 ppm h). Arrows denote the sampling dates for analyses at vegetative phase (43 days of sowing (DAS)), flowering phase (85 DAS) and harvest phase (120–122 DAS).

2.4. Biomass and Yield Attributes

Harvest index (HI, the ratio of grain yield and the above ground biomass at maturity), 1000 grain weight, grain weight plant^{-1}, and inflorescence weight plant^{-1}, were measured from three randomly selected plants for each cultivar in both treatments (n = 3) at 120–121 DAS at NBRI and 122 DAS at the Banthra site. Above ground biomass was measured for three plants for each cultivar in both treatments at the harvest phase (n = 3).

2.5. Physiological and Biochemical Measurements

The second youngest fully mature leaves were measured for photosynthetic rate (A), stomatal conductance (g_s), and the maximum quantum yield of primary PSII photochemistry (F_v/F_m, the ratio of variable fluorescence to maximum chlorophyll fluorescence) with Li-COR 6400, gas exchange portable photosynthesis system (Li-COR, Lincoln, Nebraska, USA) with a fluorescence chamber (LFC6400-40; Li-COR). Three randomly selected plants of each wheat cultivar in each treatment were measured (n = 3) at the vegetative phase (42–45 DAS) and at the flowering phase at (84–87 DAS) at both experimental sites. The CO_2 level inside the leaf cuvette was maintained as 400 μmol mol^{-1}, photosynthetic photon flux density was 1200 μmol mol^{-1}, and leaf temperature was 25 °C.

Leaf samples were collected for the biochemical analyses twice: at the vegetative phase at 43 DAS and at the flowering phase at 85 DAS. The measurements were performed on three randomly selected plants within each cultivar for each treatment (n = 3). Leaf samples were frozen in liquid nitrogen and stored at −80 °C until further analyses. Total chlorophyll content was calculated using equation given in Arnon [36]. The total carotenoid content was calculated from the absorbance values at 480 and 510 nm according to Parsons et al. [37].

Lipid peroxidation in the leaf tissue was determined as 2-thiobarbituric acid (TAB) reactive metabolite, mainly malondialdehyde, Heath and Packer [38]. The Bradford [39] method was used to measure the protein concentration using bovine serum albumin (BSA sigma) as the concentration standard. Superoxide dismutase activity (SOD) was measured using the photochemical NBT method, Beyer and Fridovich [40]. Catalase (CAT) activity was measured by following the reduction in the absorbance at 240 nm as H_2O_2 was consumed Rao et al. [41]. Reduced glutathione (GSH) and oxidized glutathione (GSSG) content were measured by enzyme recycling assay as illustrated by Griffith [42].

2.6. Statistical Analyses

To test the effects of EDU treatment, cultivar and their interaction, two-way ANOVA was performed with SPSS software (SPSS Inc., version 21.0), separately for the vegetative, flowering and the harvest phase and two experimental sites.

To test the differences in the grain weight plant^{-1} for all the 11 tested cultivars, a linear regression was conducted between the mean grain weight plant^{-1} of all the cultivars at each experimental site and EDU treatment (as a numerical measure of the overall quality of the environment) and the individual grain weight of each of the 11 cultivars in the experimental site and treatment combinations. This technique was originally used by Finlay and Wilkinson [43], in order to test the performance of barley in different environments and time scale.

The cultivars were classified in four groups (a–d) based on whether the mean grain weight plant^{-1} of each cultivar was above or below the mean grain weight plant^{-1} of all cultivars (site-mean) in the two environments (NBRI and Banthra). The site mean had a regression coefficient of 1 and the cultivars with clearly higher or lower regression coefficient were considered sensitive or insensitive to environmental change, respectively. The groups were named as: (a) "Well-adapted cultivars" that had an above average grain weight plant^{-1} in all environments. (b) "Poorly adapted cultivars" that had a below average grain weight plant^{-1} in all environments (c) "Cultivars adapted to high yield environments" whose grain weight plant^{-1} was higher than the mean in high-yield environments, but lower than the mean in low-yield environments: They had a regression coefficient clearly higher than 1 indicating strong environmental response in yield. (d) "Cultivars adapted to low yield environments" whose grain weight plant^{-1} was higher than the mean in low yield environments, but lower than mean in high-yield environments. Their regression coefficient was less than 1. Details of the technique used have been illustrated in Pandey et al. [32]. The Spearman correlation of grain yield plant^{-1} with the other measured parameters was tested. The analysis was performed separately for pooled data with all the cultivars and for each cultivar response group.

3. Results

Detailed O_3 data was collected at the NBRI site throughout the study. Less frequent measurements from the Banthra site followed the same pattern. Daily mean O_3 concentrations were above 40 ppb during most of the growing season for wheat, especially during the flowering phase (in February and March), although high daily O_3 concentrations were observed throughout the experiment (Figure 2). The average O_3 concentrations (day time average based on hourly values between 09:00 and 17:00 h) of 45, 45, 57 and 65 ppb were recorded for December, January, February and March, respectively. The average ambient O_3 concentration was 52.8 ppb and ranged between 9.6 and 83.3 ppb during the growth period of wheat. Accumulated Ozone exposure over the Threshold of 40 ppb (AOT40 exposure) was 8.66 ppm h at NBRI site (Figure 2).

3.1. Yield and Biomass in Response to EDU Treatment

The yield attributes (HI, grain weight plant^{-1} and weight of inflorescence) were generally higher in EDU-treated than in non-EDU treated plants, particularly at NBRI site (Figure 3, Figure S1). However, large variation between cultivars in the response to EDU for all yield parameters was evident by the significant Cv $\times$ EDU treatment interaction in ANOVA (Table 1 and Figure S1).

Biomass was significantly higher with EDU treatment than in non-EDU treated plants for all the cultivars at the NBRI site (Figure 3A). Since both the grain yield and the biomass were higher in response to EDU, HI was slightly lower in response to EDU treatment at NBRI site (Figure 3A). At Banthra, biomass decreased, and grain yield remained the same, and thus HI improved with EDU treatment, which is indicated by the median in the box-plot suggesting that more than 50% of the cultivars showed improved HI (Figure 3B).

The 11 cultivars represented different response groups in a regression analysis of grain weight plant^{-1}: (a) three cultivars (Kundan, WR544 and PBW550) were generally well-adapted with above-average yield (Figure 4A), (b) three cultivars (PBW373, PBW154, HUW234) were poorly-adapted with below-average yield (Figure 4A); (c) two cultivars (PBW343, LOK1) were adapted to low-yield environments (Figure 4B); and (d) three cultivars (PBW502, WH711 and DBW17) were adapted to high-yield environments (Figure 4B). The cultivars adapted to high-yield environments (WH711 and DBW17) had the poorest grain yield of the whole experiment at the NBRI site at ambient O_3 conditions.

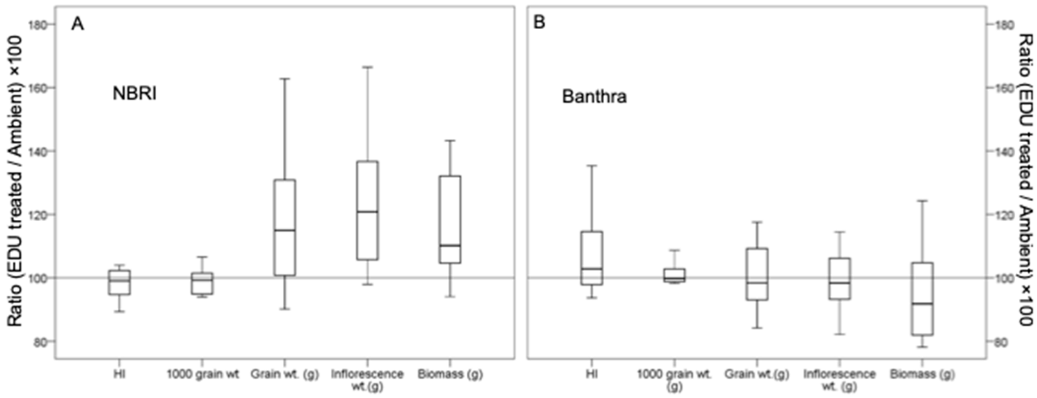

Figure 3. The effect of Ethylenediurea (EDU) treatment on various yield attributes across all the cultivars {(based on relative values, e.g., harvest index with non-EDU/harvest index with EDU treatment) × 100)}. The median of each parameter is shown as the horizontal bar in each box, and the upper and the lower sides of a box represent the first and the third quartile values of the distribution respectively. Harvest index (HI), 1000 grain weight plant^{-1} (1000 grain wt.), grain weight plant^{-1} (grain wt.), inflorescence weight plant^{-1} (wt. of inflorescence) and biomass.

Table 1. F ratios and levels of significance of multivariate ANOVA test for different parameters of all the 11 tested cultivars. Significant results of two-way ANOVA are marked with asterisks (* $p < 0.05$ and ** $p < 0.01$) for Cultivar, Treatment (EDU) and Cultivar × treatment (EDU) at experimental sites; NBRI and Banthra at vegetative, flowering and harvest phase. Superoxide dismutase activity (SOD), catalase activity (CAT), reduced glutathione (GSH), oxidised glutathione (GSSG), malondialdehyde content (MDA), total chlorophyll (Tchl), carotenoid (Caro), photosynthesis (A), stomatal conductance (gs), ratio of variable to maximal chlorophyll fluorescence (Fv/Fm), harvest index (HI), 1000 grain weight plant^{-1} (1000_grain wt), inflorescence weight plant^{-1} (Inflorescence wt) and biomass.

	NBRI						**Banthra**					
	Vegetative			**Flowering**			**Vegetative**			**Flowering**		
	Cultivar	**Treatment (EDU)**	**Cultivar × treatment (EDU)**	**Cultivar**	**Treatment (EDU)**	**Cultivar × treatment (EDU)**	**Cultivar**	**Treatment (EDU)**	**Cultivar × treatment (EDU)**	**Cultivar**	**Treatment (EDU)**	**Cultivar × treatment (EDU)**
SOD	23.36 **	175.10 **	25.63 **	1.86	0.03	1.81 **	48.16 **	956.17 **	28.25 **	45.78 **	0.04	35.04 **
CAT	27.26 **	46.27 **	25.94 **	17.56 **	24.40 **	14.79 **	67.83 **	0.34	17.77 **	169.41 **	307.79 **	108.07 **
GSH	4.71 **	1.82	6.67 **	14.86 **	16.82 **	19.63 **	10.78 **	699.47 **	12.57 **	11.36 **	15.86 **	18.38 **
GSSG	10.80 **	25.17 **	19.12 **	7.62 **	76.36 **	14.48 **	26.24 **	82.41 **	7.02 **	36.15 **	24.56 **	21.41 **
MDA	12.97 **	3.56	8.45 **	46.62 **	12.06 **	14.02 **	17.69 **	238.44 **	26.32 **	18.05 **	82.85 **	5.71 **
T Chl	41.92 **	58.73 **	26.84 **	10.60 **	8.53 **	15.20 **	1614.38 **	2919.22 **	2101.01 **	327.17 **	3314.84 **	128.99 **
Caro	25.94 **	27.69 **	15.56 **	24.52 **	2.47	12.26 **	1549.28 **	1622.39 **	2027.76 **	514.43 **	1993.56 **	158.96 **
A	2.08 *	1.15	4.63 **	17.43 **	18.21 **	23.80 **	11.19 **	33.61 **	11.16 **	25.52 **	2.38	9.98 **
gs	3.22 **	0.37	1.65	13.89 **	3.03	11.31	6.33 **	31.85 **	5.93 **	39.34 **	7.13 **	3.30 **
FvFm	1.79	0.08	1.09	2.97 **	2.98	1.69	0.65	4.10 *	1.51	0.96	0.23	0.93

Harvest parameters				**Harvest parameters**			
HI	16.04 **	6.03 **	2.74 **	HI	67.73 **	22.33 **	15.31 **
1000_grain wt	401.47 **	55.75 **	51.52 **	1000_grain wt	331.58 **	62.61 **	31.57 **
Inflorescence wt	12.58 **	96.61 **	6.80 **	Inflorescence wt	12.56 **	0.66	3.44 **
Grain_wt	10.01 **	34.61 **	3.93 **	Grain_wt	10.78 **	0.00	3.39 **
Biomass	14.65 **	81.30 **	8.24 **	Biomass	12.95 **	20.95 **	12.12 **

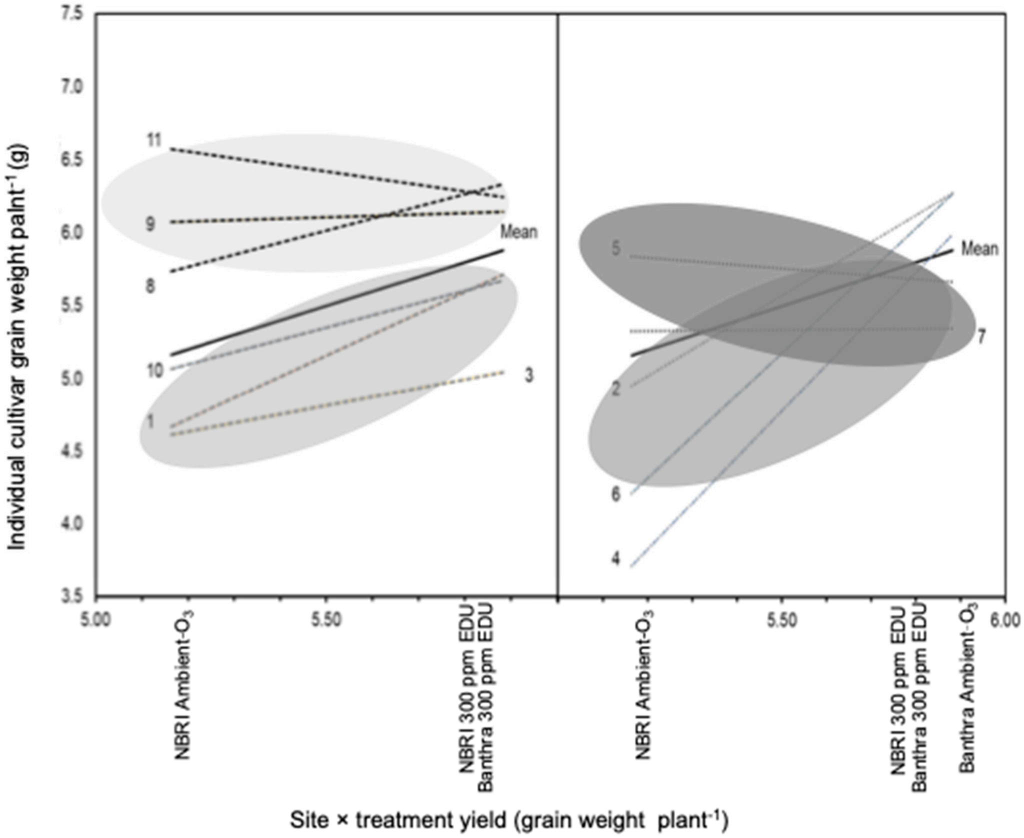

Figure 4. Regressions between the mean grain weight plant^{-1} of all the cultivars at each site and treatment (x-axis) and the individual grain weight plant^{-1} (y-axis) of each of the 11 wheat cultivars. (**A**) Grouping of well adapted (8, 9 and 11) cultivars and poorly adapted (1, 3 and 10) cultivars. (**B**) Grouping of cultivars adapted to low-yield conditions (5 and 7) and cultivars adapted to high-yield (2, 6 and 4) conditions. (1) PBW-373, (2) PBW-502, (3) PBW-154, (4) WH711, (5) PBW-343, (6) DBW-17, (7) LOK-1, (8) KUNDAN, (9) WR-544, (10) HUW-234, (11) PBW-550.

3.2. Gas Exchange and Pigments

Gas exchange was affected by cultivar, EDU, developmental stage and study site. Although impact of EDU on A and g_s was variable among the cultivars as shown by the significant Cv × EDU treatment interactions (Table 1 and Figure S2) EDU-treated plants tended to have higher A and g_s than non-EDU ones (significantly only in Banthra at vegetative phase) (Supplementary Figures S3 and S5).

Cultivars differed from each other also in the contents of pigments (chlorophyll, carotenoids) throughout the experiment at both experimental sites (Figure S4). Contents of chlorophyll and carotenoids differed among the cultivars and treatments in a similar way. Significantly lower contents of chlorophyll and carotenoids were detected in EDU-treated plants than non-EDU ones in the flowering phase at both experimental sites (Figure S5). EDU-treated plants had higher chlorophyll and carotenoid content than the non-EDU treated plants at the vegetative phase at NBRI (Figure S5), whereas they had similar or even lower contents of pigments at the flowering phase at both experimental sites (Figure S5).

3.3. Biochemical Measurements

EDU treatment had a significant effect on all measured biochemical parameters (MDA, CAT, GSH, GSSG, SOD), but the responses varied among the cultivars, developmental phases and experimental sites throughout the experiment (Table 1 and Figures S3, S6 and S7). Lipid peroxidation (MDA content) tended to be lower in EDU-treated plants than non-EDU ones at both experimental sites (except for

Banthra at the vegetative phase) (Figure S7). EDU-treated plants had higher SOD activity and GSH content than non-EDU treated ones in Banthra at vegetative stage, while EDU-treated plants had lower SOD activity than non-EDU treated ones (NBRI, vegetative phase), GSSG content (NBRI, flowering phase; Banthra, vegetative phase) than non-EDU ones (Figure S7).

3.4. Correlations of Measured Parameters and Grain Yield

The strongest positive correlations to grain yield across all response groups were found for inflorescence weight^{-1} and biomass, except for the cultivars adapted to low-yield environments (Table 2). HI showed a positive correlation with grain yield in the cultivars adapted to high-yield environments. The strongest negative correlations to grain yield were found for CAT activity (except for the cultivars adapted to high-yield conditions) and GSSG (except for the cultivars adapted to low-yield conditions) at the flowering phase. The grain yield of the well-adapted cultivars showed a positive correlation with A at the flowering phase (Table 2). The grain yield of the poorly-adapted cultivars showed positive correlation with A and gs at the vegetative phase, followed by strong negative correlations with the CAT, GSSG and MDA at the flowering stage (Table 2). The grain yield of the cultivars adapted to low-yield conditions showed negative correlations with chlorophyll content at the vegetative phase and CAT at the flowering phase (Table 2). In the cultivars adapted to high-yield environments, strong negative correlation was found with SOD at the vegetative phase, accompanied by positive correlations with CAT, GSH and GSSG. At the flowering phase, positive correlation with SOD and contents of chlorophyll and carotenoids were accompanied with negative correlations with CAT, GSSG and gas exchange parameters.

Table 2. Correlation of the different parameters with the grain yield plant^{-1} for the groups assigned from the Finlay method. Significant correlations are in bold. Positive correlations (light grey) and negative (grey) are presented in the table. Superoxide dismutase activity (SOD), catalase activity (CAT), reduced glutathione (GSH), oxidised glutathione (GSSG), malondialdehyde content (MDA), chlorophyll (chl), carotenoid (Caro), photosynthesis (A), stomatal conductance (g_s), ratio of variable to maximal chlorophyll fluorescence (F_v/F_m), harvest index (HI), 1000 grain weight plant^{-1} (1000_grain wt), inflorescence weight plant (Inflorescence wt) and biomass.

Parameters	Cultivars 8,9,11 (Well-adapted)	Cultivars 1,3,10 (Poorly Adapted)	Cultivars 5,7 (Low-yield Condition)	Cultivars 2,4,6 (High-yield Condition)	All Cultivars
Vegetative					
SOD	−0.151	0.011	0.166	−0.614 **	−0.313 *
CAT	0.005	0.294	0.244	0.156	0.311 *
GSH	0.125	0.060	−0.240	−0.149	0.033
GSSG	0.277	−0.542 **	0.192	0.490 **	0.325 *
MDA	−0.246	0.163	0.087	−0.007	−0.024
Chl	−0.263	0.230	−0.472 *	−0.079	−0.087
Car	−0.320	0.294	−0.401	−0.221	−0.12
A	−0.285	0.441 **	−0.204	0.132	0.144
g_s	−0.281	0.344 *	−0.218	0.245	0.26
F_v/F_m	0.049	−0.055	0.116	0.062	0.06
Flowering					
SOD	−0.033	0.155	−0.364	0.590 **	0.137
CAT	0.222	−0.472 **	−0.408 *	−0.629 **	−0.373 *
GSH	0.266	0.067	0.236	−0.203	0.191
GSSG	−0.132	−0.379 *	0.401	−0.543 **	−0.322 *
MDA	0.010	−0.618 **	−0.222	−0.178	−0.251
Chl	0.202	−0.010	0.132	0.384 *	0.236
Car	−0.117	0.116	0.053	0.410 *	0.198
A	0.383 *	0.147	−0.078	−0.533 **	−0.141
gs	0.189	−0.093	−0.140	−0.653 **	−0.257
FvFm	0.284	0.012	−0.125	−0.098	0.107
Final harvest					

Table 2. *Cont.*

Parameters	Cultivars 8,9,11 (Well-adapted)	Cultivars 1,3,10 (Poorly Adapted)	Cultivars 5,7 (Low-yield Condition)	Cultivars 2,4,6 (High-yield Condition)	All Cultivars
HI	0.209	0.064	0.327	0.659 **	0.426 **
1000_grain wt	−0.082	−0.425 **	−0.097	0.243	−0.246
Inflorescence wt	0.614 **	0.599 **	−0.033	0.695 **	0.737 **
Biomass	0.472 **	0.618 **	0.138	0.694 **	0.776 **

4. Discussion

In this study EDU application was used as ozone-protectant to indicate the severity of the O_3-induced damage in wheat production in an agriculturally important region suffering from high pollution in a highly populated area of India. The O_3 concentration increased gradually during the growing period of wheat from December to March, particularly at the grain filling phase (February to March), which has been considered to be the most sensitive stage to O_3 damage, especially for wheat [44]. The critical three-month O_3 level for wheat (3 ppm h) [25] was not reached at the vegetative phase, but it was attained before the flowering phase resulting in O_3 exposure that was double than the estimated damage threshold by the harvest time. Accordingly, our results indicate the strong impact of O_3 in the flowering and harvest stage. AOT40 values and the average O_3 concentrations were in line with the other studies performed in this region of India reviewed by Oksanen et al. [13] and Ainsworth [3], e.g., with mustard (*Brassica campestris*) [45] and clover (*Trifolium alexandrium* L.) [27].

4.1. Biomass, Allocation Strategies and Grain Yield

Our experiment showed clear differences in antioxidant and gas exchange parameters among the cultivars, adaptation groups and the two developmental phases. These results can be linked to O_3-tolerance and O_3-defence strategies, because plants treated with EDU application can be regarded to represent clean-air controls. O_3 tolerance of the plants can be linked to two important strategies, the regulation of stomatal conductance and the potential to detoxify the ROS generated in the course O_3 degradation [14,46–48]. Previous studies have also indicated that O_3-sensitive cultivars tend to allocate more of their resources to defense actions in response to O_3 limiting biomass [27,45,49,50].

In the present study, biomass accumulation showed a positive significant correlation with the grain yield. The associations between the grain yield and other parameters in this study indicated that the grain yield of the well-adapted cultivars was not associated with the biochemical parameters, but rather the higher the yield was correlating with high photosynthesis (*A*) at the flowering stage (Table 2). Poorly-adapted cultivars showed positive correlations with gas exchange rates during the vegetative stage, which may indicate high O_3 uptake, accompanied by weak antioxidative defense through GSSG and CAT. Cultivars adapted to low-yield conditions were limited by chlorophyll content and poor defense by CAT. Cultivars adapted to high-yield conditions (including EDU protection) are relying on high antioxidative defense through CAT, GSH, GSSG during the vegetative stage, with negative correlations (trade-off) with SOD. At the flowering stage, antioxidant status was reversed and accompanied by low gas exchange rates but high contents of chlorophyll and carotenoids. Thus, our study indicated that defense strategies are complex and may vary during the development. Clearly, low grain yield in our experiment was associated with low CAT activity but high GSSG content at the flowering phase for most of the cultivar groups. GSSG content has been shown to accumulate in response to O_3, as well as GSH content and total glutathione [51]. Higher total glutathione content has been associated with high tolerance to O_3 in poplar trees [51]. Singh et al. [49] have exposed 14 wheat cultivars to elevated (ambient +30 ppb) O_3 and classified them in three different classes: sensitive, intermediately sensitive, and tolerant cultivars based on the cumulative stress response matrix using growth, physiological and yield. Two cultivars included also in our study, i.e., the well-adapted Kundan and the high-yield environment adapted PBW502, were classified by Singh et al. [49] as O_3-tolerant and intermediately sensitive, respectively, which was in accordance with our classification despite the different grouping method. Reduced biomass due to O_3 stress may also be attributed to several other physiological and biochemical events in the developmental phase of the plants, for example decline in Rubisco activity [50]. Pleijel and Uddling [52] reported that O_3 can significantly reduce the proportion of above-ground biomass converted to grains, on the contrary, in the present study, biomass accumulation showed a positive significant correlation with the grain yield.

The higher biomass and yield with EDU treatment compared to ambient field conditions reflect the positive effects of EDU in those parameters, which are often negatively affected by O_3 [53,54]. In a meta-analysis by Feng et al. [33] the increase of the above-ground biomass by 6.7% was reported with EDU treatment. Similar biomass enhancements with EDU treatment under high O_3 have been reported in wheat (*Triticum aestivum* L.) [55], mustard (*Brassica campestris* L.) [45], rice (*Oryza sativa* L.) [32] and pea (*Phaseolus vulgaris* L.) [56]. In addition to positive impact of EDU, in the present study indicated that the resource allocation strategies in response with EDU differed among the wheat cultivars and between experimental sites. At NBRI, the wheat plants showed more efficient resource allocation towards grains in response to EDU treatment which was accompanied by improved biomass and slight decrease in HI. However, at Banthra, the biomass was lower with EDU-treated plants than non-EDU ones and HI was slightly improved (due to decrease in above-ground biomass) and grain yield was not higher with EDU-treatment than in non-EDU treated plants.

4.2. EDU as a Tool to Reveal Ozone Impact

In the present study, EDU responses were not only limited to growth, gas-exchange or the biochemical parameters, but also showed that the prevailing O_3 concentration had an adverse effect on yield attributes, reflected as reduced grain yield at the harvest phase. EDU-mediated increase in the antioxidant defense (SOD, CAT, APX and GR), growth parameters, biomass, and yield attributes have been reported in previous studies under high O_3 conditions. The activation of the antioxidative defense and EDU responses, however, are related to severity of the oxidative stress [11,13,29,30,32,45,50,55,57–60].

The positive impact of EDU on gas exchange and photosynthesis related parameters is not well-established, as evidenced also in our experiment showing the high variation among the cultivars. Feng et al. [33], Hassan et al. [61] and Manning et al. [57] have reported that EDU did not show any clear effect on the g_s and A. On the other hand, a positive impact of EDU on g_s in rice [59] and wheat [26] and on A, g_s, light reaction and F_v/F_m in pea (*Phaseolus vulgaris* L.) [56] have been reported, especially on O_3 sensitive cultivars, which is in accordance with our results at vegetative phase at Banthra. The higher chlorophyll content in non-EDU grown plants than in EDU-treated plants during the flowering phase (NBRI and Banthra) may indicate O_3-induced compensatory responses in the newly formed leaves, as all the measurements were conducted on the youngest fully mature leaves. Such compensatory responses appearing as increased shoot weight plant^{-1} [32,62] leaf greenness, and photosynthetic adjustment [32,63] have been reported in response to high O_3. The similarity of responses between chlorophyll and carotenoid content across the cultivars was expected because of the similarity in the regulation of their biosynthesis [64].

Our experiment demonstrates the applicability of EDU as a surface treatment in large-scale screening for O_3-tolerance in wheat cultivars in different environments. A recent study by Ashrafuzzaman et al. [31] also suggest that EDU did not interfere with the gene-regulations and did not affect the tolerance of the plants to other abiotic stresses, such as iron toxicity, zinc deficiency and salinity stresses, under O_3-stress conditions in rice, which further strengthen the potential use of EDU in field conditions. Several other studies with rice [32,55,59] and wheat [50,55] have also reported the usefulness of EDU in the field conditions in identifying O_3-tolerant cultivars. In the present study, the EDU-responses varied not only among the cultivars, but also due to growth phase and experimental site, as reported also in pea [65], mustard [45], rice [32,59] and wheat [50]. Although the exact mechanism for the mode of action of EDU still unclear, it has been demonstrated that the nitrogen present in EDU has no role in fertilization, growth regulation, or grain yield under O_3-free conditions [2,59].

EDU is currently not commercially available and can thus be applied for research purposes only. Earlier studies with EDU suggest the range between 100 and 300 ppm (100 to 300 mg L^{-1}) to be the most effective concentration in ameliorating negative effects against O_3 without having any toxic effects of its own [2]. The concentration of 300 mg EDU L^{-1} was also recommended as the upper limit for toxicity in a toxicological bioassay in *Lemna minor* [66]. Manning et al. [30] reported that EDU did not show any constitutive effects on the crops in O_3-free control conditions.

5. Conclusions

Our experiment with EDU application at two different high-ozone environments indicated high variation in the resource allocation and the defense strategies in the Indian wheat cultivars. The well-adapted cultivars in our study, i.e., Kundan, WR544 and PBW550 showed a high yield regardless of the site in the IGP area of India. In these well-adapted cultivars, the grain yield was related to high net assimilation (*A*) at the flowering stage of the development and high biomass accumulation at the end of the experiment. On the other hand, all other response groups showed high stomatal conductance and net assimilation at vegetative phase and low antioxidant defense (CAT activity, glutathione content) at vegetative and flowering phases. The cultivars that were able to maintain high antioxidative defense and net assimilation capacity ended up with higher yield indicating higher ozone tolerance. It is clear that a wide screening of wheat cultivars is necessary to improve food-security for crops in areas experiencing high O_3 concentrations. Based on our results, high throughput screening will reveal high differences among cultivars and help to find the key parameters to be studied.

Supplementary Materials: The following are available online at http://www.mdpi.com/2225-1154/7/2/23/s1.

Author Contributions: E.O. conceived the project. A.K.P. and B.M. performed the experimental part. V.P. supervised the experiments. A.K.P., S.K.-S. (Sarita Keski-Saari), S.K.-S. (Sari Kontunen-Soppela), E.O., V.P. performed writing reviewing and editing.

Funding: This study was funded by the Academy of Finland, project no 138161. AKP also acknowledges FinCEAL Plus Research Visit Grant project No. 51770 for the financial support to travel to Finland.

Acknowledgments: The authors express a sincere thanks to W.J. Manning, Department of Plant, Soil and Insect Science, University of Massachusetts, Amherest, USA, for providing EDU for this study. AP and BM thank the Academy of Finland and for the financial support. Jenna Lihavainen is acknowledged for long insightful discussions.

Conflicts of Interest: The authors declare no conflict of interest.

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