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Impact of Ethylene diurea (EDU) on growth, yield and proteome of two winter wheat varieties under high ambient ozone phytotoxicity

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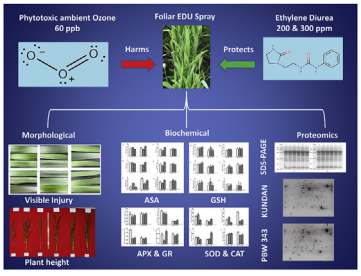
HIGHLIGHTS

- Ethylene Diurea (EDU) enhanced tolerance to high ambient phytotoxic ozone stress in wheat.
- EDU treated plants showed higher antioxidant capacity in the ascorbate-glutathione cycle.
- In yield spikelet number and 1000 grain weight increased significantly in Kundan cultivar.
- Protein related to growth and yields were expressed differentially for better performance.

GRAPHICAL ABSTRACT



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ABSTRACT

The present study evaluated the impact of high ambient O_3 on morphological, physiological and biochemical traits and leaf proteome in two high-yielding varieties of wheat using ethylene diurea (EDU) as foliar spray (200 and 300 ppm). Average ambient ozone concentration was 60 ppb which was more than sufficient to cause phytotoxic effects. EDU treatment resulted in less lipid peroxidation along with increased chlorophyll content, biomass and yield. EDU alleviated the negative effects of ozone by enhancing activities of antioxidants and antioxidative enzymes. Two dimensional electrophoresis (2DGE) analysis revealed massive changes in protein abundance in Kundan at vegetative stage (50% proteins were increased, 20% were decreased) and at flowering stage (25% increased, 18% decreased). In PBW 343 at both the developmental stages about 15% proteins were increased whereas 20% were decreased in abundance. Higher abundance of proteins related to carbon metabolism, defense and photorespiration conferred tolerance to EDU treated Kundan. In PBW343, EDU provided incomplete protection as evidenced by low abundance of many primary metabolism related proteins. Proteomic changes in response to EDU treatment in two varieties are discussed in relation to growth and yield.

1. Introduction

Indo-Gangetic Plains (IGP), which is the most productive and cultivated area of India, is facing high tropospheric ozone concentrations due to increasing anthropogenic and biogenic emissions of

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precursor compounds (Lal et al., 2012; reviewed in Oksanen et al., 2013; Agathokleous et al., 2015). Relative yield losses for North west- Indo-Gangetic plains (NW-IGP) region, based on the AOT40 (Average ozone threshold over 40 ppb) metrics, ranged from 30 to 42% for wheat, 22–26% for rice, 3–5% for maize to 47–58% for cotton (Sinha et al., 2015). Tropospheric O_3 is presently viewed as a well-known and growing problem that suppresses crop productivity on a large scale (Mauzerall and Wang, 2001; Fuhrer and Booker, 2003; Bytnerowicz et al., 2007; reviewed in Oksanen et al., 2013). It has been reported that every 1% decrease in crop yields would result in 0.36% decrease of India's GDP (Gadgil and Gadgil, 2006). Modelling-based studies to assess the extent and magnitude of ozone (O_3) risk to agriculture in Asia suggest that yield losses of 5–20% for important crops may be common in areas experiencing elevated O_3 concentrations (Emberson et al., 2009). Ozone is a powerful oxidant and the mechanism(s) leading to chronic O_3 damage are less well characterized, but morphological and physiological symptoms include: lower stomatal conductance, leaf chlorosis, accelerated senescence, and a general decrease in green leaf area and plant productivity, decreased photosynthetic activity due to decreased RuBisCO activity and chlorophyll content, (Morgan et al., 2003; Ashmore, 2005; Fuhrer, 2009; Pandey et al., 2014, 2015).

It is very complex to understand plant response to ozone, as plant have developed several different mechanisms to cope from oxidative stress resulting from chronic exposure of ozone (Overmyer et al., 2008). Moreover different development stages (vegetative and flowering) have responded differently (Black et al., 2007) e.g. short term acute effect is different from long term chronic impact.

An aromatic compound ethylenediurea (N-[2-(2-oxo-l-imidazolidinyl) ethyl] -N′-phenyl urea) commonly abbreviated as EDU is used as ozone protectant for plants and tree in scientific studies (Agathokleous et al., 2015). As EDU specifically protect plants against ambient O_3, it is very useful to ascertain O_3 effects on field grown plants (Manning et al., 2011). EDU has been shown to protect leaves from ozone injury (reviewed by Manning et al., 2011) and has been used in the field (e.g., Paoletti et al., 2007, 2009).

Currently, India is second largest producer of wheat in the world after China with about 12% share in total world wheat production (www.fao.org). Impacts of O_3 have been studied on wheat in various experiments (Agrawal et al., 2002; Sarkar and Agrawal, 2010; Sawada et al., 2012; review in Oksanen et al., 2013) but so far no study has been done to evaluate response of wheat leaf proteome to EDU under field condition.

Based on the previous works, we know that O_3-sensitive plant species are more responsive to EDU, e.g. wheat (Agrawal et al., 2003; Rajput and Agrawal, 2005; Rai et al., 2007), rice (Pandey et al., 2015; Ashrafuzzaman et al., 2017), mustard (Singh et al., 2011; Pandey et al., 2014), and linseed (Tripathi et al., 2011). Therefore we hypothesize that there will be massive protein level changes in O_3- sensitive wheat variety. To test this hypothesis, we studied impact of EDU on growth, physiology, yield and proteome of two wheat varieties differing in O_3 sensitivity. The objective of the present study was to gain better insight in to the EDU induced changes on leaf protein expression in wheat varieties and their possible biological significance.

2. Material and methods

2.1. Experimental site and study plots

The field experiments were conducted at CSIR-National Botanical Research Institute garden in Lucknow city, (26°55′ N latitude, 80°59′ E longitude and at an altitude of 113 m) Uttar Pradesh, India. Twenty four number plots of 9 m^2 were prepared. Two plots were used for ozone toxicity experiments and rest 22 were used for main experiments. Plots were randomized using random number generator for sowing of seeds and treatments with the statistical software tool (SPSS Inc., version 16). Eleven plots were selected for each variety in which three served as control and four for each treatment.

2.2. Plant material and its cultivation

2.2.1. Wheat (Triticum aestivum L.)

Wheat (*Triticum aestivum* L.) varieties Kundan and PBW-343 were selected for the experiments which are widely grown varieties of north-eastern plains of Indo-Gangetic plains. These are modern varieties released in 2001 and 1996, respectively. Kundan is recommended for rainfed condition with limited inputs; the variety is double dwarf and its height ranges from 80 to 100 cm and is the only cultivated dwarf variety that has distinct pubescent glumes and is highly resistant against rusts whereas PBW 343 (developed in Punjab Agricultural University (PAU), Ludhiana, India) is recommended for irrigated condition, has wide adaptability, high degree of resistance to rusts (brown & yellow) and tolerance to Kernel Bunt. The grains are amber, semi hard to hard, good straw strength resulting in high yield.

2.2.2. Seed sowing and fertilization doses

Seeds were manually sown in field at rate of 15*30 cm (i.e. distance between plants was 15 cm and between two row −30 cm) using recommended agronomic practices including fertilizer doses given as urea (120 kg ha $^{-1}$), superphosphate (60 kg ha^{-1}) and muriate of potash (40 kg ha^{-1}). One third dose of N and full doses of P and K were given as basal dressing and another two doses of N were given as a top dressing after 60 and 90 days of germination. Plants were thinned after one week of germination to maintain a uniform distance of 15 cm. Manual weeding was done from time to time and plants were irrigated regularly to maintain uniform soil moisture.

2.3. Doses response test for EDU

A preliminary experiment was conducted to decide effective EDU doses. Kundan and PBW 343 were sown in field and foliar spray of EDU was started after 15 days of germination in the concentration range of 100–500 ppm (2 EDU sprays at the interval of 7 days) while control plants were sprayed with distilled water. Total chlorophyll concentration was recorded using SPAD-502 (Konica-Minolta, Osaka, Japan). Plants were harvested for height and biomass after 30 days of germination. Both the varieties of wheat exhibited better biomass and height in dose response test at 200 and 300 ppm concentrations of EDU while remaining concentrations of EDU (100, 400 and 500 ppm) were not as much effective (Figs. S1 and S2). So we selected 200 and 300 ppm EDU treatment for our main experiment. We have used abbreviations: KA= Kundan control, K1=Kundan 200 ppm EDU, K2= Kundan = 300 ppm EDU; PA= PBW control, P1 = PBW 200 ppm EDU, P2 = 300 ppm EDU.

2.4. Ozone monitoring and AOT 40 calculations

Ambient ozone monitoring was carried out by 2B Tech Ozone Monitor (106-L) on an average 8 h (9:00 to 17:00) at the experimental site throughout the experiment. Accumulated Ozone Threshold 40 ppb (AOT 40) was calculated for exposure index for ozone, as described by De Leeuw and Van Zantvoort (1997).

2.5. Application of EDU

Ethylenediurea was kindly provided by Prof. W.J. Manning, University of Massachusetts, USA. Two EDU treatments (200, 300 ppm) and one control treatment (distilled water) were used. EDU treatments were initiated at 15 DAG (days after germination). The entire foliage was sprayed early in morning until it was visibly drenched and the treatment was repeated weekly throughout the experiment till final harvesting of plant.

2.6. Physiological parameters

2.6.1. Assessment of ozone visible injury

Plants were regularly surveyed over the growing seasons for any visible injury. Ozone visible injury occurred as dark stippling on the upper leaf surface.

2.6.2. Biomass sampling

Plants were sampled for biomass at three different stages; vegetative stage at 60 DAG, flowering stage at 100 DAG, and harvest stage at 143 DAG. Five different plants were selected randomly from the subplots. In order to obtain an intact root system, a monolith was carefully dug out and first kept in water, then washed with running tap water to remove the adhered soil. The roots and the shoots were separated and dried in a hot air oven at 65 °C until the weight reached a constant value.

2.6.3. Gas exchange parameters

Net photosynthesis (Pn), stomatal conductance (gs) and maximal photochemical efficiency of PSII (*Fv/Fm*) were measured on the youngest fully mature leaves, at 55 DAG and 93 DAG, from twelve randomly selected plants of both varieties in the each treatment. All measurements were performed using a Li-COR 6400 gas exchange portable photosynthesis system (Li-COR, Lincoln, Nebraska, USA) with a fluorescence chamber (LFC6400–40; Li-COR). The CO_2 levels inside the leaf cuvette were maintained at 400 μmol mol^{-1}, leaf temperature was 25 °C, photosynthetic photon flux density (PPFD) was 1200 μmol m^{-2} s^{-1} and relative humidity was 60–80%. *Fv/Fm* measurements were carried out after the leaves were dark-adapted for 30 min.

2.6.4. Total chlorophyll

Chlorophyll content was measured non-destructively using a SPAD-502 (Konica- Minolta, Osaka, Japan) device two times during the growth period, at the vegetative stage (55 DAG) and at flowering stage (93 DAG) on ten randomly selected plants from each treatment. SPAD values have a strong correlation to chlorophyll concentration but the relationship is non-linear (Uddling et al., 2007).

2.7. Biochemical analysis

Leaf samples for the antioxidative enzyme analysis were collected at two different developmental stages. Two to three fully mature leaves were taken from three randomly selected plants from each treatment immediately frozen in liquid-N_2 and were stored at −80 °C until further analyses. Protein content was estimated according to Bradford method (1976). The level of lipid peroxidation (as malondialdehyde (MDA) equivalents) in the leaf tissue was measured using the 2-thiobarbituric acid (TBA) method (Heath and Packer, 1968).

Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) were determined as per Beyer and Fridovich (1987), Rao et al. (1996), Chen and Asada (1989), and Smith et al. (1988), respectively. Ascorbate and glutathione contents were measured as per Hodges et al. (1996) and Griffith (1980), respectively.

2.8. Yield parameters

Shoot weight $plant^{-1}$, inflorescence weight $plant^{-1}$, spikelet no. $spike^{-1}$, spike length, grain no. $plant^{-1}$, grain weight $plant^{-1}$ and harvest Index (measurement of crop yield: the weight of grain as a ratio of the total biomass of the plant) were measured for twelve plants for each treatment after the final harvest.

2.9. Proteomic analysis

2.9.1. Protein extraction

Total leaf protein was extracted after Damerval et al. (1986). In brief, samples were crushed in liquid-N_2 with mortar and pestle and resulting powder was extracted with buffer containing 50 mM Tris-HCl (pH 8.0), 25 mM EDTA, 500 mM thiourea and 0.5% β-mercaptoethanol (β-ME). The resulting extract was mixed with 10% cold Trichloro acetic acid (TCA) and 0.07% β-ME, and left overnight at −20 °C. Next day, after centrifugation pellet was washed three times with cold acetone and 0.07% β-ME. The pellet was then vacuum dried, solubilized in 0.1 M Tris-HCl (pH 8.0), 50 mM EDTA and 2% β-ME. Proteins were extracted with 2.5 mL Tris-buffered phenol.10 mL, 0.1 M ammonium acetate in methanol was added to phenol phase and left overnight at −20 °C. Next day, after centrifugation pellet was dissolved in 0.1 M ammonium acetate in methanol and 1% β-ME and then washed twice with cold acetone and 1% β-ME. Dried pellet was re-suspended in a solubilization buffer consisting of 7 M urea, 2 M Thiourea, 2% CHAPS, 20 mM DTT, and 0.5% v/v immobilized pH gradients buffers. Protein was quantified by the Bradford assay with BSA as the standard.

2.9.2. Sample preparation for SDS-PAGE

Samples were prepared for SDS-PAGE, with 1X protein sample loaded in stacking gel with 2X gel loading buffer (50 mM Tris-HCL, 2% SDS, 10% [v/v] glycerol, 0.05 M DTT and 0.1% bromophenol blue). Fifty μg proteins were loaded on 5% stacking and separating gel of 12% polyacrylamide with an acrylamide: Bis ratio of 29:1. Electrophoresis was performed at a constant 70 V in Tris-Glycine tank buffer. Gels were stained with 0.5% brilliant blue G and distained with 10% glacial acetic acid in 50% methanol. Image analysis was performed using Image Quant TL 7.0 (GE Healthcare).

2.9.3. 2D Gel electrophoresis

Two-dimensional gel electrophoresis (2-DGE) was carried out as described in Lehesranta et al. (2005). Extracted protein samples (120 μg) were passively rehydrated overnight on immobilized pH gradients (IPG) strips (7 cm, pH 4–7) with 135 μl of rehydration buffer (7 M Urea, 2 M Thiourea, 2% CHAPS, 20 mM DTT, 0.5% v/v immobilized pH gradients buffers) at room temperature. Isoelectric focusing (IEF) was conducted on Ettan IPGphore-3 (GE Healthcare). The focusing was done as follows: 250 V for 1 h, 500 V for 1 h, 1500 V for 2 h, and 4000 V for 2 h and 6000 V for 2 h for a total of 21.2 kVh. The focused strips were equilibrated twice (1% w/v DTT and 2.5% w/v Iodoacetamide) for 10 min in 5 mL of equilibration solution (6 M urea, 30% w/v glycerol, 2% w/v sodium dodecyl sulfate (SDS), and 50 mM Tris-HCl buffer, pH 8.8). Electrophoresis was carried out on 12% polyacrylamide slab gels initially for 30 min at 70 V and then at 120 V in Tris-Glycine tank buffer. Gels were stained with 0.5% brilliant blue-G and destained with 10% glacial acetic acid in 50% methanol. Triplicate gels were analyzed using Image Master 2D Platinum 7.0 and relative volume (% volume) was taken as increased/decreased (1.5 fold or more) (Data S1 and Data S2). This

procedure permitted to normalize experimental variations due to protein loading and staining.

2.9.4. Protein identification

Protein identification was performed according to Koistinen et al. (2002). Briefly, excised gel particles were first destained and then dehydrated by washing three times with 25 mM ammonium bicarbonate (ABC) containing 50% acetonitrile. Resulting gel particles were dried in a speedvac and rehydrated in equal volumes of 0.1 mg mL^{-1} trypsin and 50 mM ABC. Gel particles were immersed in 25 mM ABC and samples were digested overnight at 37 °C. Peptides were extracted twice with 50% acetonitrile (ACN)/1% trifluoro acetic acid (TFA). The recovered peptides were concentrated to a final volume of 20 μl.

2.9.5. MS and MS/MS

MALDI-MS and MS/MS analyses were carried out by 4800 Proteomic Analyzer (Applied Biosystems) with TOF/TOF optics. Spotting mixture was prepared by mixing 0.5 μl of protein sample with 0.5 μl of matrix solution (5 mg mL^{-1} a-Cyano-4-hydroxycinnamic acid in 50% ACN containing 0.1% TFA). The mass spectrometer was externally calibrated with a standard mass mixture of angiotensin I, Glu-fibrino-peptide B, ACTH (1–17), and ACTH (18–39). For MS/MS calibration, the instrument was externally calibrated with fragment of Glufibrino-peptide B. The monoisotopic peptide masses obtained from MALDI-TOF/TOF were analyzed by the 4000 Series Explorer software version 3.5 (ABI). Protein identification was performed with the online Mascot software (http://www.matrixscience.com) with the generated mass signal against Swiss Prot, NCBInr and MSDB databases. Database searches parameters were: monoisotopic mass accuracy, <100 ppm; missed cleavages, 1; carbamidomethylation of cysteine as fixed modification and oxidation of methionine, N-terminal pyroglutamylation (peptide) and N-terminal acetylation (protein) as variable modifications.

2.10. Statistical analysis

The effects of EDU treatment, variety and their interaction on all the measured parameters were analyzed by two-way ANOVA and mixed model ANOVA. The vegetative, flowering and harvest stages were analyzed separately, since different blocks were sampled for the assays where the number of replicates was three. Significance of the difference of EDU treatment within variety was tested with one-way ANOVA followed by Duncan's post hoc test. The difference at $P < .05$ was considered to be significant. The data were log 10-transformed when needed to meet the ANOVA requirements. All the analyses were carried out with SPSS software (SPSS Inc., version16.0).

3. Results

3.1. Meteorological data, average ozone concentration and visible injury

The weather of Lucknow is characterized by dry tropical monsoon climate. Maximum and minimum temperatures varied from 20 to 39 °C and 9–27 °C, respectively during the experimental period (Nov, 2013 to Apr, 2014). Mean sunshine hours were least in December and maximum in April (Table S1).

Average ambient ozone concentration during the experiment was 60 ppb. It ranged from 15 to 100 ppb during experimental period. High ozone concentrations were recorded in November, December and March in comparison to January and February (Fig. 1). AOT 40 calculation showed maximum peaks in mid vegetative stage and flowering stage (Table 1).

Foliar injuries in terms of interveinal chlorosis and chlorotic stippling were first observed on adaxial surface of non-EDU treated leaves of Kundan (Fig. 2). Injury was identified as O_3-like because it was either absent or very less in EDU treated leaves and apparently more severe in older than in younger leaves.

3.2. Morphological, physiological and biochemical analysis

One way and mixed model Anova analysis showed significant increase in plant height, biomass and total chlorophyll (Table 2 and Table S2). Photosynthesis, fluorescence and chlorophyll content did not show any significant change due to EDU treatment in both the varieties (Fig. S3). Biomass was significantly affected due to all factors excepting Cv*stage. Significant increases were observed in shoot weight and total biomass in K1, at flowering stage, (Figs. S4E and S4F). Significant increase was also recorded in root length P1 (vegetative stage) and K1 (flowering stage) and in shoot length K1 (flowering stage) (Fig. S5). Actual contribution percentage (ACP) test was shown in Table S3. Total chlorophyll was also positively impacted by EDU (Fig. S6). The extent of lipid peroxidation (measured as MDA equivalents) was lower in both the EDU treated varieties than non-treated ones, especially in K1 and K2 at flowering stage (Fig. S7B). Ascorbate content showed variable response; at vegetative stage it showed increment only at K2 while at flowering stage, it was significantly increased in P2 and K1 (Fig. S8). All the antioxidative enzymes showed significant variation due to EDU treatment in both the varieties at both the stages (Figs. S8 and S9).

Table 1
Average ozone (ppb) and AOT 40 (ppb/hrs/day) during different developmental stages.

Developmental Stages	Germinating stages	Vegetative stages	Flowering stages	Harvesting stages
Avg Ozone (ppb)	58.90	41.84	63.13	75.13
AOT 40 (ppb/hrs/day)	18.86	1.80	23.09	35.09

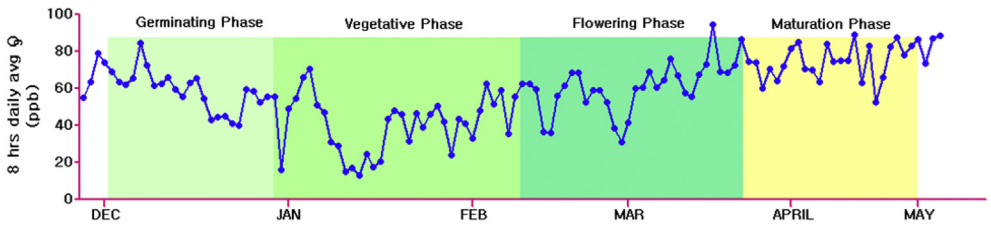

Fig. 1. Daily ozone concentrations (8 h daily average) during the study period (28th November 2013–29th April 2014). The different stages for wheat growth shown in coloured boxes, and dots denote the date of the month.

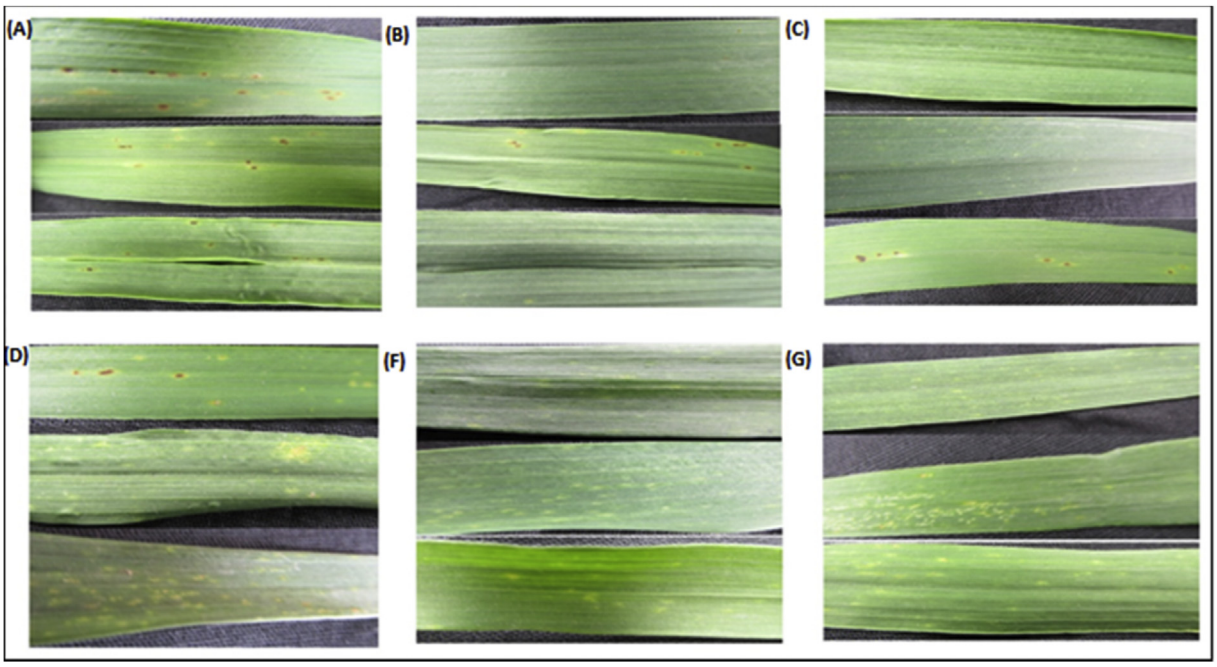

Fig. 2. Injury profile in leaves of wheat (*Triticum aestivum* L.) at 70 DAG. A,B,C represents KA, K1, K2 and D,E,F represents PA, P1 and P2 respectively.

Statistical analyses showed that variations in SOD, APX, CAT and GR were significant due to all the factors excepting in APX at stage*-treatment (Table S2) (Figs. S10 and S11).

3.3. Yield parameters

There were variety specific variations in yield parameters, while EDU treatment only impacted 1000 grain weight. Significant increase in 1000 grain weight was observed in K1 and K2 (Fig. S12G). One way ANOVA analysis showed increase in spike length in P1 and P2 at harvest stage (Fig. S12D). Mixed model Anova analysis revealed variety specific changes rather than treatment specific (Table S4).

3.4. Proteomic analysis

3.4.1. SDS-PAGE analysis

Image quant TL 0.7 analysis showed significant difference between treatments and control as well as between varieties. In PBW 343, 17 bands were analyzed, out of which 9 were more abundant and 6 were less abundant. While in Kundan 25 bands were analyzed out of which 12 were more abundant, 11 were less abundant. In PBW 343, out of 17 bands, 15 were identified while in Kundan out of 25, 16 bands were identified ($P < .05$) (Figs. S13A and B). In both the varieties, Rubisco large subunit (LSU) and small subunit (SSU) were increased mostly but some bands also showed decreased expression. Proteins expression pattern during SDS-PAGE analysis are shown in Tables S5 and S6.

3.4.2. 2D gel analysis and differential protein expression under EDU treatment

Three representative gels (Data S3) were analysed for each treatment within each variety (Figs. 3 and 4). Around 400 protein spots were reproducibly detected on brilliant blue G (commassie) stained gels within each treatment in both the varieties. A number of significantly differentially abundant proteins ($P < .05$) were observed. It is interesting to note that Kundan showed massive protein changes (more at vegetative stage) than PBW 343 at both EDU treatments. In Kundan (among 92 identified proteins) about 50% proteins were more in abundance and 20% less in abundance at vegetative stage while at flowering stage (among 92 identified proteins) the percent increase/decrease was 25%/18%, while in PBW 343 (99 proteins-vegetative and 87 proteins-flowering), only about 15% proteins were more in abundance and 20% proteins were less in abundance at both the stages (Table S7) (Fig. S14).

These identified proteins were categorized into different functional categories as shown in Fig. 5 (A, B, C and D) and listed on the basis of functions (Table 3, Tables S8 and S9). Proteins were also sorted according to varieties and developmental stages in Venn diagram (Fig. S15).

In photosynthesis category 7 proteins were identified and these proteins showed variable response, e.g., Rubisco LSU was both up and down regulated in response to EDU (Table 3). Moreover, Oxygen evolving enhancer (OEE) protein was more abundant in Kundan at vegetative stage while it was more abundant in PBW 343 at flowering stage. Cyt b6f protein was more in Kundan while less abundant in PBW. Out of 14 proteins related to carbon metabolism, Fructose bis phosphate aldolase (FBPase), hydrolase, Rubisco activase and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) were differentially expressed. In energy metabolism, ATP synthase beta subunit was less abundant in Kundan while more abundant in PBW. In antioxidant defense, germin like protein was more in Kundan and less in PBW. All the identified proteins in both treatments and developmental stages of both varieties have been shown in Table 3 and Tables S8 and S9.

4. Discussion

In this study, impacts of high ambient ozone concentrations on two important local Indian varieties of *Triticum aestivum* L. were studied using EDU treatments under field condition. EDU treatment resulted in a series of integrated morphological, biochemical and

Table 2
Effect of EDU treatment on different parameters related to growth, biomass, physiology, antioxidant, antioxidative enzymes and MDA content in wheat varieties. Values represent mean ± standard error (n = 3); different letters indicate significant differences between EDU treatments within each cultivar (P < .05) by one way Anova.

S. No	Parameters (unit)	Kundan Vegetative			Kundan Flowering			PBW 343 Vegetative			PBW 343 Flowering		
		CON	200 ppm	300 ppm	CON	200 ppm	300 ppm	CON	200 ppm	300 ppm	CON	200 ppm	300 ppm
1	Root length (cm)	13.6 ± 1.2^{A}	13.2 ± 1.3^{A}	13.20 ± 0.9^{A}	12.95 ± 0.96^{B}	15.24 ± 1.47^{AB}	14.82 ± 1.12^{A}	12.80 ± 0.8^{b}	15.20 ± 0.9^{a}	12.20 ± 0.7^{b}	18.63 ± 0.85^{b}	15.24 ± 1.47^{a}	$16.93 \pm 0.85a^{b}$
2	Shoot length (cm)	76.4 ± 1.5^{B}	82.8 ± 1.1^{A}	77.80 ± 1.1^{B}	93.73 ± 2.83^{A}	99.06 ± 2.54^{A}	104.56 ± 2.58^{A}	57.20 ± 1.0^{c}	63.40 ± 1.4^{b}	67.60 ± 1.0^{a}	90.59 ± 0.85^{a}	94.83 ± 0.85^{a}	93.01 ± 0.79^{a}
3	Root weight (g)	0.31 ± 0.02^{B}	0.43 ± 0.04^{A}	0.40 ± 0.01^{AB}	0.87 ± 0.05^{C}	1.31 ± 0.08^{B}	1.76 ± 0.08^{A}	0.27 ± 0.03^{b}	0.45 ± 0.01^{a}	0.25 ± 0.02^{b}	2.08 ± 0.20^{a}	2.34 ± 0.22^{a}	1.42 ± 0.12^{b}
4	Shoot Weight (g)	3.26 ± 0.30^{A}	4.37 ± 0.77^{A}	3.39 ± 0.08^{A}	17.31 ± 0.08^{B}	18.51 ± 0.64^{AB}	19.72 ± 0.85^{A}	1.25 ± 0.09^{c}	2.10 ± 0.04^{a}	1.69 ± 0.02^{b}	18.50 ± 2.14^{a}	17.13 ± 0.48^{a}	14.68 ± 0.19^{a}
5	Total Biomass (g)	3.57 ± 0.32^{A}	4.80 ± 0.81^{A}	3.79 ± 0.10^{A}	18.18 ± 0.12^{B}	19.82 ± 0.72^{AB}	21.48 ± 0.92^{A}	1.52 ± 0.12^{c}	2.55 ± 0.06^{a}	1.95 ± 0.05^{b}	20.58 ± 2.33^{a}	19.47 ± 0.70^{a}	16.10 ± 0.30^{a}
6	Photosynthesis (μmol mol^{-1})	21.57 ± 0.74^{A}	23.43 ± 0.79^{A}	22.73 ± 0.44^{A}	9.76 ± 1.08^{A}	10.88 ± 0.76^{A}	12.48 ± 0.69^{A}	21.30 ± 2.44^{a}	23.57 ± 0.43^{a}	24.30 ± 1.35^{a}	8.63 ± 1.44^{a}	11.89 ± 0.61^{a}	2.85 ± 1.28^{a}
7	Conductance ($m^{-1}s^{-1}$)	0.26 ± 0.01^{A}	0.28 ± 0.01^{A}	0.26 ± 0.01^{A}	0.13 ± 0.06^{A}	0.26 ± 0.03^{A}	0.28 ± 0.00^{A}	0.20 ± 0.01^{a}	0.25 ± 0.01^{a}	0.22 ± 0.03^{a}	0.32 ± 0.05^{a}	0.26 ± 0.02^{a}	0.13 ± 0.08^{a}
8	*Fv/Fm*	0.72 ± 0.01^{A}	0.76 ± 0.00^{A}	0.74 ± 0.01^{A}	0.72 ± 0.01^{A}	0.76 ± 0.00^{A}	0.75 ± 0.01^{A}	0.76 ± 0.01^{a}	0.76 ± 0.01^{a}	0.74 ± 0.01^{a}	0.72 ± 0.01^{a}	0.74 ± 0.01^{a}	0.74 ± 0.01^{a}
9	Total Chlorophyll (SPAD unit)	36.70 ± 1.4^{B}	42.15 ± 0.77^{A}	42.95 ± 1.12^{A}	30.14 ± 2.26^{B}	32.76 ± 2.71^{A}	31.83 ± 2.71^{A}	40.68 ± 2.58^{b}	45.06 ± 1.13^{a}	47.21 ± 0.69^{a}	31.95 ± 2.11^{b}	35.55 ± 2.41^{a}	34.37 ± 2.78^{a}
10	TASA (μmol g^{-1} FW)	13.69 ± 0.05^{B}	11.40 ± 0.01^{C}	16.31 ± 0.12^{A}	8.26 ± 0.03^{C}	9.52 ± 0.07^{A}	8.44 ± 0.04^{B}	12.94 ± 0.01^{b}	14.28 ± 0.01^{c}	11.55 ± 0.02^{a}	8.55 ± 0.04^{b}	6.96 ± 0.01^{c}	10.58 ± 0.02^{a}
11	ASA (μmol g^{-1} FW)	8.36 ± 0.02^{A}	8.55 ± 0.02^{B}	12.00 ± 0.03^{C}	5.17 ± 0.01^{B}	5.72 ± 0.03^{A}	4.95 ± 0.01^{C}	11.69 ± 0.02^{a}	11.31 ± 0.05^{b}	10.44 ± 0.04^{c}	5.48 ± 0.03^{b}	4.38 ± 0.02^{c}	6.74 ± 0.02^{a}
12	DHA (μmol g^{-1} FW)	5.32 ± 0.07^{A}	2.85 ± 0.03^{C}	4.30 ± 0.13^{B}	3.09 ± 0.03^{C}	3.79 ± 0.10^{A}	3.49 ± 0.02^{B}	1.24 ± 0.02b	2.9 ± 0.06^{a}	1.11 ± 0.04^{b}	3.06 ± 0.07^{b}	2.58 ± 0.03^{c}	3.84 ± 0.00^{a}
13	TGSH (μmol g^{-1} FW)	9.44 ± 1.23^{A}	14.19 ± 1.45^{A}	9.89 ± 1.45^{AB}	11.46 ± 0.57^{A}	13.25 ± 0.92^{A}	11.62 ± 0.44^{A}	11.38 ± 1.81^{a}	13.67 ± 0.81^{a}	15.27 ± 0.61^{a}	10.28 ± 0.25^{b}	12.20 ± 0.38^{a}	11.37 ± 0.39^{ab}
14	GSH (μmol g^{-1} FW)	6.38 ± 1.32^{A}	11.01 ± 1.80^{A}	6.30 ± 1.10^{A}	-1.15 ± 0.88^{A}	0.51 ± 1.24^{A}	-0.10 ± 1.03^{A}	7.16 ± 1.86^{a}	9.24 ± 1.06^{a}	10.22 ± 0.91^{a}	3.76 ± 0.34^{a}	2.75 ± 0.94^{a}	3.89 ± 0.22^{a}
15	GSSG (μmol g^{-1} FW)	3.05 ± 0.11^{A}	3.17 ± 0.41^{A}	3.59 ± 0.09^{A}	12.61 ± 0.50^{A}	12.74 ± 0.60^{A}	11.72 ± 0.65^{A}	4.22 ± 0.20^{a}	4.42 ± 0.34^{a}	5.05 ± 0.31^{a}	6.51 ± 0.50^{b}	9.44 ± 0.58^{a}	7.48 ± 0.55^{b}
16	Catalase (μmol mg^{-1} protein^{-1} min^{-1})	18.99 ± 0.77^{B}	15.84 ± 0.19^{C}	28.22 ± 0.72^{A}	31.75 ± 0.98^{A}	8.78 ± 0.34^{C}	11.33 ± 0.00^{B}	42.82 ± 0.72^{b}	51.37 ± 3.47^{a}	22.05 ± 1.02^{c}	11.93 ± 0.68^{b}	18.22 ± 0.88^{a}	5.00 ± 0.34^{c}
17	SOD (μmol mg^{-1} protein^{-1} min^{-1})	4.25 ± 0.07^{B}	4.98 ± 0.09^{B}	10.87 ± 0.43^{A}	6.19 ± 0.09^{A}	4.47 ± 0.07^{C}	4.91 ± 0.08^{B}	6.20 ± 0.44^{a}	6.21 ± 0.18^{a}	4.25 ± 0.08^{b}	4.72 ± 0.07^{b}	5.34 ± 0.10^{a}	5.46 ± 0.10^{a}
18	APX (μmol mg^{-1} protein^{-1} min^{-1})	1.71 ± 0.10^{A}	1.58 ± 0.10^{A}	1.72 ± 0.02^{A}	0.96 ± 0.04^{B}	1.05 ± 0.01^{B}	1.30 ± 0.04^{A}	1.63 ± 0.19^{a}	1.22 ± 0.18^{a}	1.42 ± 0.04^{a}	4.32 ± 0.13^{a}	3.65 ± 0.15^{b}	3.28 ± 0.26^{b}
19	GR (μmol mg^{-1} protein^{-1} min^{-1})	0.08 ± 0.01^{A}	0.06 ± 0.00^{B}	0.07 ± 0.00^{AB}	0.19 ± 0.01^{A}	0.15 ± 0.01^{B}	0.18 ± 0.00^{A}	0.18 ± 0.00^{a}	0.06 ± 0.00^{b}	0.07 ± 0.00^{b}	0.19 ± 0.01^{a}	0.06 ± 0.01^{b}	0.24 ± 0.01^{a}
20	MDA content (μmol g^{-1} FW)	7.98 ± 1.20^{A}	8.52 ± 0.51^{A}	7.75 ± 1.52^{A}	7.51 ± 0.19^{A}	8.99 ± 1.27^{AB}	6.82 ± 0.51^{B}	15.96 ± 1.02^{a}	13.07 ± 0.94^{b}	11.67 ± 0.91^{ab}	17.67 ± 0.91^{a}	14.26 ± 1.41^{a}	13.22 ± 2.34^{a}

Abbreviations: TASA, Total ascorbate; ASA, ascorbate; DHA, dehydroascorbate; TGSH, total glutathione; GSH, glutathione; GSSG, oxidised glutathione; SOD, superoxide dismutase; APX, ascorbate peroxidase; GR, glutathione reductase; MDA, Malondialdehyde; FW, Fresh weight.

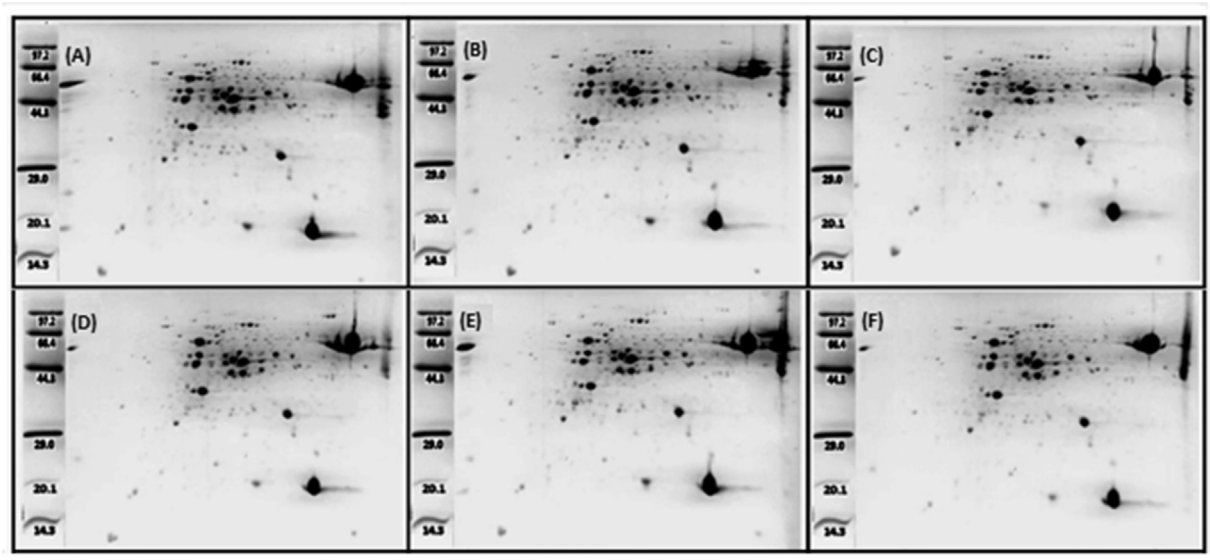

Fig. 3. Effect of EDU application on protein expression pattern of two *Triticum aestivum* varieties Kundan and PBW 343 leaves at vegetative stage. 12% gel, 120 μg loading, pH range 4–7, Brilliant blue G stained two-dimensional gel representing control (A, D), 200 ppm EDU dose (B, E) and 300 ppm EDU dose (C, F). for details, see materials and methods. (A, B, C = Kundan and D, E, F = PBW 343).

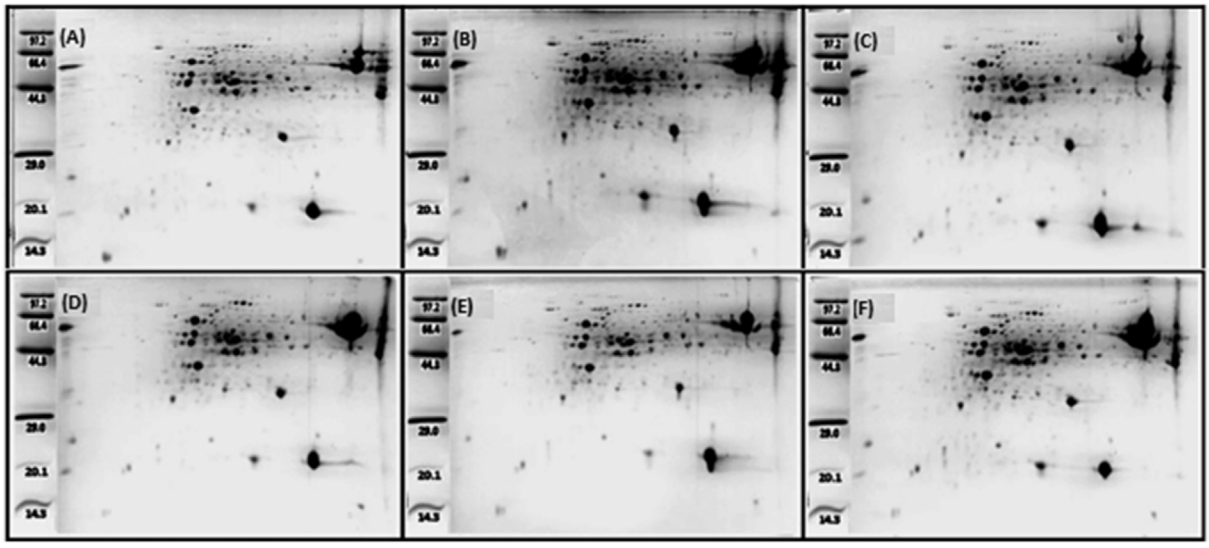

Fig. 4. Effect of EDU application on protein expression pattern of two *Triticum aestivum* varieties Kundan and PBW 343 leaves at flowering stage. 12% gel, 120 μg loading, pH range 4–7, Brilliant blue G stained two-dimensional gel representing control (A, D), 200 ppm EDU dose (B, E) and 300 ppm EDU dose (C, F), for details, see materials and methods. (A, B, C = Kundan and D, E, F = PBW 343).

proteomic changes in two wheat varieties. Our results showed that Kundan was more responsive to EDU application indicating that it is more sensitive to prevailing O_3 concentrations.

4.1. Meteorological conditions and ambient ozone

Maximum temperature recorded during experimental period was in March (29 °C) whereas minimum temperature was in January (13 °C). Mean hourly concentrations of ambient ozone (60 ppb) during the experimental period often exceeded the threshold value of 40 ppb at experimental site, causing injury to the wheat leaves. Maximum 78 ppb ozone was recorded in the harvest stage in the month of January. These concentrations are in line with several other measurements in India, as reviewed in Oksanen et al. (2013).

4.2. Visible injury and total chlorophyll

Ozone-induced symptoms were similar to those reported by Sanders and Benton (1998) and Tonneijck (1983) in Reid-grown beans. EDU protected or partially protected bean plants against ozone injury in laboratory studies (Kostka-Rick and Manning, 1993) or in exposure studies in open-top chambers and in field exposures to ambient ozone (Heagle, 1989). EDU treatments reduced the number of injured leaves and the degree of injury due to O_3 (Fig. 2) and increased total chlorophyll in both the varieties and stages (Fig. S6). Decrease in visible ozone injury and increase in total chlorophyll in EDU treatment is also reported by Bortier et al. (2001), Hassan, 2006), Paoletti et al. (2007), Basahi et al. (2016). These results confirm that EDU provided protection from visible leaf injury induced by O_3. EDU enhances anti-oxidant defense and

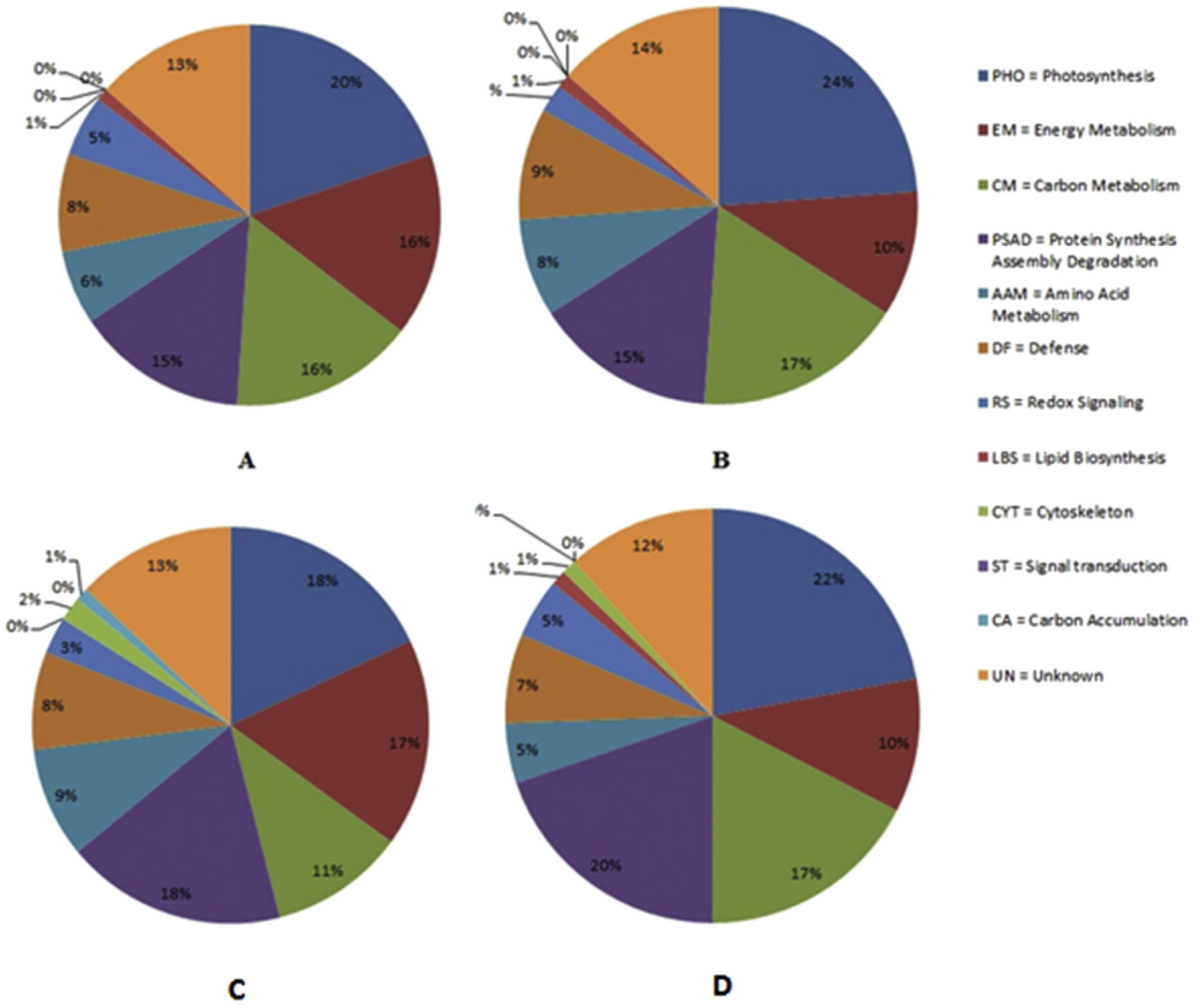

Fig. 5. Effect of EDU applications on proteins function of two stages of *Triticum aestivum* varieties of Kundan (A = Vegetative stage, B= Flowering stage) and PBW 343 (C= Vegetative stage, B= Flowering stage) in leaves.

reduces reactive oxygen species (ROS) in ozone-sensitive varieties, resulting in less damage to PS-I and PS-II under ozone stress (Guidi et al., 2010). Kundan was more responsive to EDU as it showed less visible injury in 200 ppm EDU treatment than PBW 343.

4.3. Morphological, physiological and biochemical parameters

Both the EDU dose positively impacted the growth and biomass of wheat in both the developmental stages (Table 2). Similar results are also reported by Pandey et al. (2014, 2015) in mustard and rice, respectively. Morphological and biochemical parameters have been used as markers of the sensitivity of plants to O_3 phytotoxicity (Singh et al., 2010; Sarkar and Agrawal, 2010). The varieties showed very different growth patterns (regarding biomass accumulation), particularly after the vegetative stage: PBW 343 and Kundan both varieties showed continuous increase in biomass in both the developmental stage except in shoot weight (Fig. S4). Previous studies have shown that ozone stress can lower carbon fixation, enhance foliar and root respiration, shift the partitioning of carbon into different chemical forms, and disrupt carbon and nutrient allocation patterns (Friend et al., 1992; Friend and Tomlinson, 1992; Kelly et al., 1993; Laurence et al., 1994; Baker et al., 1994; Samuelson and Kelly, 1996; Scagel and Andersen, 1997) (Fig. S3). There was less lipid peroxidation in both the wheat varieties showing effective protection by EDU against high ambient O_3, as plant membranes are one of the first target of O_3 injury. There were reduced MDA contents in PBW 343 at vegetative stage and in Kundan at flowering stage under EDU treatments indicating both varieties responded differently at different growth stages. Results show EDU responsiveness in Kundan, indicating that Kundan is sensitive to ozone and EDU enhanced its tolerance capability. Antioxidants like ascorbate and glutathione play important roles in combating oxidative stress (Noctor, 2006). In our study, antioxidative defense was elevated in response to EDU in both varieties and there were significant interaction between the variety and the EDU treatment.

There were increased activities of APX and GR enzymes together with high amounts of SOD and CAT in EDU treated wheat leaves which helped in the detoxification of ROS more efficiently. Similar EDU induced increases of ascorbate and APX activity have been reported in sensitive varieties of wheat (Singh et al., 2009), ash tree

Table 3
List of EDU- responsive leaf proteins in two wheat (*Triticum aestivum* L.) varieties i.e. kundan and PBW 343 at two different developmental stages analysed from two-dimensional gel electrophoresis (2-DGE) and identified by mass spectrometry (MS). Values (mean of three replicate gels within each treatment) represent fold changes with threshold of 1.5 fold increased or decreased.

S. No.	Protein names	Functions[a]	Kundan, Vegetative stage		Kundan, Flowering stage		PBW 343, Vegetative stage		PBW 343, Flowering stage	
			200 ppm	300 ppm	200 ppm	300 ppm	200 ppm	300 ppm	200 ppm	300 ppm
Photosynthesis related proteins										
1	Rubisco LSU	RubP carboxylase/oxygenase activity	1.62↑	1.60↑	−2.21↓	-	−1.85↓	−2.68↓	−2.09↓	1.85↑
2	Oxygen evolving enhancer protein	PS II Regulation	3.79↑	2.85↑	−1.75↓	−2.06↓	-	-	1.93↑	-
3	Cyt-b6f complex	Electron transport	-	1.88↑	3.03↑	-	−3.18↓	-	−4.33↓	−4.15↓
4	a/b- binding protein	Light harvesting	2.48↑	3.41↑	6.27↑	4.39↑	−6.17↓	-	−1.98↓	-
5	Ferredoxin dependant- NADP(H) reductase	Electron transport	2.08↑	1.85↑	−1.59↓	9.14↑	-	-	−6.87↓	-
6	Rubisco SSU	Monooxygenase activity	-	-	-	-	1.49↑	-	−2.09↓	−1.56↓
7	Photosystem I subunit VII	Oxidoreductase activity	2.05↑	2.75↑	-	1.77↑	-	-	−1.92↓	−2.02↓
Carbon metabolism proteins										
8	Rubisco activase (chloroplastic)	ATP binding	2.73↑	-	-	−1.50↓	-	-	-	-
9	Rubisco activase- α	ATP binding	-	-	-	-	4.43↑	1.90↑	1.50↑	-
10	Hydrolase	Epoxide hydrolase activity	−2.28↓	1.56↑	-	1.99↑	5.33↑	−2.25↓	3.65↑	3.15↑
11	Triosephosphate isomerase	Carbohydrate metabolism	1.93↑	2.17↑	-	−2.19↓	−1.75↓	-	−6.90↓	-
12	Phosphoglycolate phosphatase	Carbohydrate metabolism	2.94↑	3.07↑	−1.53↓	-	−5.17↓	-	−2.70↓	−1.76↓
13	Phosphoribulo kinase	ATP binding	−2.22↓	−2.00↓	-	-	−3.20↓	-	−1.57↓	-
14	Fructose bisphosphate aldolase	Glycolytic Process	2.16↑	2.52↑	1.89↑	−2.18↓	-	3.01↑	-	2.02↑
15	Enolase	Glycolytic Process	−1.81↓	-	1.76↑	2.78↑	2.07↑	-	−1.41↓	−1.54↓
16	Carbonic anhydrase	Carbon utilization	3.79↑	13.72↑	4.39↑	-	-	−16.96↓	−1.76↓	−9.79↓
17	Isocitrate dehydrogenase	TCA cycle	−4.42↓	-	−2.23↓	-	-	-	-	-
18	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase activity	-	−1.60↓	−1.77↓	-	-	-	-	-
19	Transeketolase	Transketolase activity	-	-	-	-	-	−1.73↓	−2.20↓	-
20	Ribose phosphate isomerase	Ribose metabolism	-	−2.44↓	-	-	−2.60↓	−1.55↓	−2.08↓	-
21	Malate dehydrogenase	TCA cycle	-	-	-	-	2.83↑	3.60↑	-	1.58↑
Energy metabolism related proteins										
22	Thioredoxin reductase	ROS removal	-	−1.57↓	-	-	−2.06↓	-	-	-
23	Thioredoxin dependant peroxidase	Peroxidase activity	-	-	-	-	-	−2.26↓	-	-
24	Peroxiredoxin	Peroxidase activity	-	-	-	-	-	−2.23↓	-	-
25	ATP synthase α- subunit	ATP synthesis	2.82↑	1.69↑	-	−1.65↓	-	−2.78↓	1.65↑	-
26	ATP synthase β- subunit	ATP synthesis	−4.45↓	-	−5.79↓	−2.55↓	−2.32↓	-	4.60↑	-
27	ATP synthase ε- subunit	ATP synthesis	-	-	-	-	-	−1.98↓	-	-
28	Rhodanese like domain	Senescence	-	-	-	-	-	3.27↑	−1.63↓	-
29	Presequence protease	Peptidase activity	−1.62↓	-	2.12↑	-	-	−1.57↓		
30	Chain A, crystal structure of PSBP	Assisting water splitting							−2.82↓	−2.32↓
31	Vacuolar proton ATPase	Proton pump	−2.22↓	-	-	−1.64↓	-	-	-	-
32	Aminomethyl transferase	Transaminase activity	−1.86↓	−2.11↓	-	-	-	-	-	-
Protein synthesis assembly and degradation										
33	Rubisco LSU binding protein (α and β)	Protein refolding	−1.58↓	−1.55↓	-	−1.69↓	2.19↓	-	1.51↑	5.68↑
34	Chaperone protein ClpC2	Protein metabolism	−15.64↓	-	−1.65↓	-	−2.32↓	−2.30↓	-	2.24↑
35	Trigger factor	Protein folding/transport	−1.80↓	-	-	1.99↑	-	-	-	-
36	30S Ribosomal protein	Protein synthesis	2.76↑	4.23↑	−1.90↓	−3.11↓	-	-	-	-
37	Cell division protease ftsh	Cell division	-	-	-	-	−1.64↓	-	−1.54↓	−2.71↓
38	20Kda chaperonine	Protein folding	-	-	-	-	−2.36↓	-	1.68↑	-
39	ATP dependant clp protease binding protein	Protein unfolding	-	2.38↑	−2.29↓	−2.38↓	2.46↑	3.01↑	1.62↑	-
40	70Kda heat shock protein	Stress response	−1.60↓	−1.76↓	−1.50↓	−1.80↓	-	-	-	-
41	Methionine synthase	Methionine synthesis	1.76↑	1.94↑	−4.67↓	-	-	-	-	-
42	Elongation Factor	Translation elongation	−2.17↓	-	-	-	-	1.50↑	-	-
43	Glycine rich RNA binding protein	RNA binding	-	-	-	-	−3.08↓	−3.34↓		
44	Triticain -α	Peptidase activity	-	−1.52↓	-	-	1.74↑	-	1.93↑	1.65↑
45	Eukaryotic initiation factor 4A	Translation initiation	-	-	−1.74↓	-	-	-	-	−1.71↓
Amino Acid metabolism										
46	Ketol acid reductoisomerase	NADP binding	3.07↑	3.52↑	-	−1.52↓	-	4.97↑	8.28↑	5.82↑
47	Plastid glutamine synthetase	Glutamine biosynthesis	-	−1.62↓	-	−2.91↓	−2.70↓	−7.87↓	-	-
48	Thioredoxin H-type 4	Cell redox homeostasis	2.04↑	2.13↑	−5.07↓	-	−1.78↑	−1.88↓	-	-
49	Thiamine thiozole synthase	Thiamine/thiozole synthesis	−1.58↓	-	-	-	−1.75↓	−9.11↓	-	-
50	Ubiqutin like protein	Ligase activity	-	-	-	-	−4.93↓	−1.91↓	-	-
Defence										
51	Germin like protein	Nutrient reservoir	1.98↑	2.41↑	-	5.48↑	-	-	−5.55↓	-
52	L-Ascorbate peroxidase	Defense response	-	2.42↑	1.50↑	-	-	-	-	-
53	Catalase	H_2O_2 catabolism	−1.61↓	-	−3.53↓	6.20↑	-	-	-	-
54	Superoxide dismutase	First line of defense	2.34↑	-	2.66↑	-	−3.73↓	-	-	-
Photorespiration										
55	Phophoglycerate phosphatase	Phosphatase activity	2.94↑	−1.53↓	3.07↑	-	-	-	-	-
56	Glycine decarboxylate	Glycine metabolism	1.80↑	3.47↑	-	-	−2.67↓	−2.89↓	-	-
57	Glycine dehydrogenase	Glycine degradation	−1.78↓	−1.52↓	-	−3.23↓	-	-	-	-
58	Serine glyoxylate amino transferase	Transamination	-	-	-	-	-	-	2.24↑	−1.57↓

(*continued on next page*)

Table 3 (*continued*)

S. No.	Protein names	Functions[a]	Kundan, Vegetative stage		Kundan, Flowering stage		PBW 343, Vegetative stage		PBW 343, Flowering stage	
			200 ppm	300 ppm	200 ppm	300 ppm	200 ppm	300 ppm	200 ppm	300 ppm
Cytoskeleton										
59	Actin depolymerising protein	Actin binding	-	-	-	-	−3.08↓	−2.28↓	2.32↑	2.67↑
60	Humon tumor like protein	-	-	-	-	-	−2.02↓	−1.78↓	1.78↑	2.52↑
Redox signaling										
61	Leucine amino peptidase	Peptidase activity	-	-	1.50↑	2.17↑	-	-	-	-
62	Nucleoside diphosphate	NDP kinase activity	3.50↑	1.73↑	-	-	−1.66↓	−1.86↓	-	-

Abbreviations: LSU, Large subunit; SSU, Small sub unit; (↑) Denotes, Increased expression of proteins; (↓) denotes, Decreased expression of proteins; (-) denotes, No change.
[a] Functions of the proteins as per "www.uniprot.com".

(Paoletti et al., 2008), mung bean (Singh et al., 2010), carrot (Tiwari and Agrawal, 2010) and spinach (Tiwari and Agrawal, 2009).

In the present study, increased levels of ASA in EDU treated wheat in both varieties provided protection against ozone stress as reported by Mittler and Zilinskas (1992), Smirnoff (2005), Athar et al. (2008). Metalloenzyme SOD is the first line of defense against the toxic effects of elevated ROS levels. The positive responses of antioxidant and antioxidative enzymes (Chalapathi Rao and Reddy, 2008; Reddy and Raghavendra, 2006) showed that EDU protected plants from harmful effects of ozone and enhanced the defense mechanism by increasing SOD and CAT more prominently in Kundan variety.

4.4. Yield parameters

The differences between the varieties in terms of overall yields were surprisingly small and were not correlated to the biomass in two earlier stages of development. It means that plants invested most of its resources for an active antioxidative defense system. Thus, our study demonstrated that biomass accumulation and allocation pattern is not strongly correlated with yield in two varieties of wheat under EDU treatment. This phenomenon has been reported by Pandey et al. (2014) in two varieties of mustard treated with EDU, where one variety showed stronger antioxidative defense throughout the experiment whiles other variety senesced early and allocated greater resources for yield. Authors further opined that there may be different structure-related strategies among the varieties to compensate for ozone-induced losses, as reviewed in Black et al. (2000). Only 1000 grain weight and spikelet length were significantly increased in Kundan with EDU treatment. Feng et al. (2010), in a meta-analysis, also reported varied yield responses in various crops due to EDU treatment.

4.5. Role of protein expression in EDU protection in two wheat varieties

EDU effects on photosynthetic machinery showed very contrasting results in 2 varieties of wheat (Table 3). Rubisco LSU (Protein no. 1) and SSU (Protein no. 6) showed differential expression at both stages in Kundan and PBW that's why we probably did not get EDU effect on photosynthesis. Rubisco LSU is the most abundant photosynthetic protein and is an important target of O_3 (Sarkar et al., 2015). Many previous studies showed that both the quantity and activity of Rubisco is decreased under O_3 stress. Torres et al. (2007) and Bohler et al. (2007) reported decreased abundance of OEE under higher O_3 exposure. EDU affected OEE protein (Protein no. 2) differentially at two stages in Kundan whereas PBW showed more abundance only at flowering stage. Cyt-b6f complex (Protein no. 3) and a/b binding proteins (Protein no. 4), which contribute to photo protection and PS-II integrity, were increased in Kundan and decreased in PBW under EDU treatment. Increase in Ferredoxin dependant- NADP(H) reductase (FNR) (Protein no. 5) expression might help better transport of electron in Kundan to facilitate photosynthesis. FNR functions as electron donors to various cellular proteins, such as glutamate synthase etc. (Lea-Smith et al., 2016). FNR protein was significantly less abundant in PBW at flowering stage; this indicates that photosynthetic machinery was not performing better under EDU treatment.

Carbonic anhydrase (CA) (Protein no. 16) is an essential part of inorganic carbon transport and its assimilation by the cell (DiMario et al., 2017) and both varieties responded very differently. High abundance of CA in Kundan might be taken as an adaptive response under EDU treatment. After anthesis, leaves play important role transporting assimilates to developing grains (Thomas, 2013). Triose phosphate isomerase (TPI) (Protein no. 11), Phosphoglycolate phosphatase (PGP) (Protein no. 12) and Phosphoribulo kinase (PRK) (Protein no. 13) proteins are essential part of Calvin cycle and glycolysis. Their abundance increases starch accumulation resulting in significant higher biomass during flowering and grain filling stages in Kundan (Fig. S4F). FBPase (Protein no. 14) and Enolase (Protein no. 15) are integral part of Calvin cycle and effectively control starch synthesis. Their more abundance in Kundan and PBW show positive effect of EDU in carbon metabolism under high ambient O_3. Isocitrate dehydrogenase (ICDH) (Protein no. 17) and GAPDH (Protein no. 18) were less abundant in Kundan. Chloroplastic Transketolase (TK) (Protein no. 19) assists pyruvate dehydrogenase in transfer of carbon from glycolysis to Krebs cycle (Kosová et al., 2011). Its decrease resulted in poor performance of PBW under EDU treatment. The less abundance of Ribose phosphate isomerase (RPI) (Protein no. 20) and cytosolic Malate dehydrogenase (MDH) (Protein no. 21) under EDU treatment obviously affected carbon metabolism in PBW.

Increased ATP synthase α (Protein no. 25) and decreased ATP synthase β subunits (Protein no. 26) reduce ATP production through photophosphorylation and thereby affect the Calvin cycle in photosynthesis (Agrawal et al., 2002). These results suggest that EDU may enhance glucose catabolism, which is necessary for faster senescence process, detoxification and repair of damages caused by ozone stress. It was impeded in flag leaves of Kundan, which showed a high sensitivity to ozone.

Trigger factor (TF) (Protein no. 35) is involved in protein folding and it also assists in enzymatic activity (Scholz et al., 1997) increases and N mobilization during grain formation which was reflected in 1000 grain weight in Kundan. Heat shock protein (HSP) (Protein no. 40) is known to act as molecular chaperones in protein quality control (Timperio et al., 2008) and was not responsive to EDU. 30S Ribosomal protein (RP) (Protein no. 36) helps in protein synthesis and its increased abundance would have helped in protein synthesis at vegetative stage in Kundan. Rubisco LSU binding protein (α and β) (Protein no. 33) had mixed abundance response to EDU treatment. Cell division proteases ftsh (Protein no. 37), 20Kda chaperonine (Protein no. 38), Triticain-α (Protein no. 44) are involved in protein synthesis and degradation. FtsH protease

activity has been related with the deprivation of oxidatively damaged D1 protein in vivo in higher plants (Bailey et al., 2002; Sakamoto et al., 2002). Decrease in protein folding related proteins might decrease synthesis of new proteins during flowering stage in Kundan, resulting in less grain numbers. Triticain-α is involved in maturation of developing seeds (Kiyosaki et al., 2009) and its increased abundance in EDU treatment at flowering stage would have helped in better seed development in Kundan. Proteins like glutamine synthetase (Protein no 47), have role in grain formation and their decreased abundance affected grain numbers in both the varieties. Ketol acid reductoisomerase (Protein no. 46), involved in branched chain amino acid synthesis, has been involved in abiotic stress tolerance (Hao et al., 2015). This enzyme was increased specifically in Kundan at vegetative and PBW at flowering stage conferring tolerance.

Superoxide dismutase (SOD) (Protein no. 54) acts as first line of defence in abiotic stress (reviewed in: Gill and Tuteja, 2010) and its activity was increased in K1at both the stages depicting better protection under EDU treatment. Germin like protein (Protein No. 51) and APX (Protein no. 52) are commonly induced by multiple stresses (Agrawal et al., 2002; Pandey et al., 2015). Their induction showed better adaptation of EDU treated plants in high ambient ozone. Decrease in catalase (Protein no. 53) in Kundan over PBW, are surprising as several authors have reported increase in response to EDU (Singh et al., 2009; Pandey et al., 2014).

Leucine amino peptidase (Protein no. 61) is involved in redox signaling by assisting protein catabolism. Increase in K2 at flowering stage may have maintained osmoregulation in Kundan during protein metabolism.

Photorespiration is involved in protection against not only O_3 but also other stresses through the dissipation of excess reducing energy, and as a source of ROS, which act as stress signals (Igamberdiev et al., 2004; del Río et al., 2006; Foyer et al., 2009; Sandalio et al., 2013; Voss et al., 2013). We found better induction of some proteins (Proteins nos 55, 56) related to photorespiration in Kundan than in PBW. Strong inhibition of photo respiratory enzymes, including glycolate oxidase and hydroxypyruvate reductase, has been reported in ozone treated soybean (Booker et al., 1997) and poplar (Bagard et al., 2008) leaves.

5. Conclusion

Our results clearly showed that prevailing O_3 concentrations around Lucknow unfavorably impacted both wheat varieties. EDU application protected plants against negative impacts of O_3. EDU treated plants had less lipid peroxidation, higher biomass and antioxidant activity which resulted in better yield in Kundan. Proteomics revealed that EDU modulated higher abundance of proteins related to carbon metabolism, defense and photorespiration which conferred tolerance to Kundan. Our results clearly show differential EDU protection against O_3 stress in two wheat varieties. In PBW, EDU provided incomplete protection as evidenced by many down regulated proteins of primary metabolism although this did not result in yield reduction. More studies are necessary to clarify how EDU impacts different metabolic pathways using mutants. This would further help elucidate mechanism of EDU action in crop plants.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2017.12.150.

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