Plant Physiology and Biochemistry 154 (2020) 675–688

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Effects of ethylenediurea (EDU) on regulatory proteins in two maize (*Zea mays* L.) varieties under high tropospheric ozone phytotoxicity

Sunil K. Gupta[a,b,c,*], Marisha Sharma[a], Baisakhi Majumder[a], Vivek K. Maurya[a], Farah Deeba[a], Jiao-Lin Zhang[c], Vivek Pandey[a,b,**]

[a] Plant Ecology and Climate Change Science Div., CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow, 226 001, India
[b] Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh, 201 002, India
[c] CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, Yunnan, 666 303, China

ARTICLE INFO

Keywords:
Ozone phytotoxicity
EDU protection
Maize
Antioxidative defense
Protein expression
Mass spectrometry

ABSTRACT

Rising tropospheric ozone is a major threat to the crops in the present climate change scenario. To investigate the EDU induced changes in proteins, two varieties of maize, the SHM3031 and the PEHM5, (hereafter S and P respectively) were treated with three EDU applications (0 = control, 50 and 200 ppm) (hereafter 0 = A, 1 and 2 respectively) (SA, S1, S2, PA, P1, P2 cultivar X treatments). Data on the morpho-physiology, enzymatic activity, and protein expression (for the first time) were collected at the vegetative (V, 45 DAG) and flowering (F, 75 DAG) developmental stages. The tropospheric ozone was around 53 ppb enough to cause phytotoxic effects. Protective effects of EDU were recorded in morpho-physiologically and biochemically. SOD, CAT and APX together with GR performed better under EDU protection in SHM3031 variety than PEHM5. The protein expression patterns in SHM3031 at the vegetative stage (28% proteins were increased, 7% were decreased), and at the flowering stage (17% increased, 8% decreased) were found. In PEHM5, a 14% increase and an 18% decrease (vegetative stage) whereas a 16% increase and a 20% decrease (flowering stage) were recorded in protein expression. Some protein functional categories, for instance, photosynthesis, carbon metabolism, energy metabolism, and defense were influenced by EDU. Rubisco expression was increased in SHM3031 whereas differentially expressed in PEHM5. Germin like protein, APX, SOD, and harpin binding proteins have enhanced defense regulatory mechanisms under EDU treatment during prevailing high tropospheric O_3. The present study showed EDU protective roles in C4 plants as proven in C3.

1. Introduction

Ground-level ozone (O_3) is increasing at the rate of approximately 0.5–2% per year over the mid-latitudes of the Northern Hemisphere due to rapid industrialization and urbanization in the last three decades (IPCC, 2013; Simpson et al., 2014). Global tropospheric O_3 levels were around 50 ppb in the year 2000, already 25% above the AOT40 threshold proven for damage to sensitive plants (Bhatia et al., 2012). Due to its phytotoxicity, tropospheric O_3 has been recognized as one of the most hazardous and toxic air pollutants with a higher degree of negative impacts on global agriculture (Ashmore, 2005; Emberson et al., 2009; Singh et al., 2015). Various studies conducted on the Indian crops suggest their high vulnerability to ozone-induced damage, but unfortunately genetic variation among cultivars in response to O_3 has hardly been addressed (Oksanen et al., 2013; Peng et al., 2020). Global yield reduction's, due to ambient O_3, for maize, rice, wheat, and soybean have been estimated to be 6.1%, 4.4%, 7.1%, and 12.4% (mean of 2010–2012) annually, respectively (Mills et al., 2018). Economic losses for Europe based on ozone assessment studies on 23 crops, were estimated to be US$7.5 billion (Holland et al., 2006) and global crop production losses were estimated to have been 79–121 Mt worth US $11–18 billion (Avnery et al., 2011). Estimating the loss of crop

Abbreviations: ANOVA, Analysis of variance; AOT40, Accumulated ozone exposure above threshold of 40 ppb; APX, Ascorbate peroxidase; CAT, Catalase; DAG, Days after germination; GR, Glutathione reductase; GSH, Reduced glutathione; H_2O_2, Hydrogen peroxide; MDA, Malondialdehyde; NBT, Nitroblue tetrazolium; $O_2^{\cdot -}$, Superoxide radical; 2DGE, Two dimensional gel electrophoresis; SA, SHM3031control; S1, SHM3031 50 ppm EDU dose; S2, SHM3031 200 ppm EDU dose; PA, PEHM5 control; P1, PEHM5 50 ppm EDU dose; P2, PEHM5 200 ppm EDU dose

* Corresponding author. Plant Ecology and Climate Change Science Div., CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow, 226 001, India.
** Corresponding author. Plant Ecology and Climate Change Science Div., CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow, 226 001, India.
E-mail addresses: padavsunilkumar@yahoo.co.in (S.K. Gupta), v.pandey@nbri.res.in (V. Pandey).

https://doi.org/10.1016/j.plaphy.2020.05.037
Received 2 April 2020; Received in revised form 14 May 2020; Accepted 27 May 2020
Available online 18 June 2020
0981-9428/ © 2020 Elsevier Masson SAS. All rights reserved.

production from ground-level O_3 is valuable for understanding the potential benefits of reducing O_3 concentration and for projecting future food supply (Burney and Ramanathan, 2014).

Among the different effects of ozone on vegetation, visible injury in leaves is considered a valuable tool for the assessment of ozone impacts in the field and the detection of areas of high risk due to O_3 (Schaub et al., 2010). Ozone causes damage by entering the leaf intercellular air spaces via stomata, where it reacts with compounds in the exposed wet cell-wall surfaces, causing the production of damaging radicals and signaling that accelerates senescence (Long and Naidu, 2002; Fiscus et al., 2005). Photosynthetic efficiency and mesophyll conductance are also affected by ozone in the crops (Xu et al., 2019; Peng et al., 2020). This has led to the expectation that O_3 damage will be less in C4 plants (maize and sugarcane), given their intrinsically lower stomatal conductance, as well as for plants under drought stress, and in response to rising (CO_2) (McKee et al., 2000; Long and Naidu, 2002; Leitao et al., 2007; Yi et al., 2020). Different studies indicate that O_3 damages the photosynthetic machinery leading to a progressive loss in the amount as well as activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Agrawal et al., 2002; Cho et al., 2008). Light and dark reactions of chloroplast also get affected either directly or indirectly due to high ozone concentration (Fiscus et al., 2005).

Ethylenediurea (EDU) has been widely used as a research tool to reveal and evaluate the ozone-sensitivity in several crops and tree species (Paoletti et al., 2009; Feng et al., 2010; Manning et al., 2011; Oksanen et al., 2013). Protective capability of EDU was observed on reactive oxygen species (ROS) mechanism in wheat (Agrawal et al., 2005; Singh and Agrawal, 2009; Pandey et al., 2019), European Ash (Paoletti et al., 2008), mung bean (Singh et al., 2010a), carrot (Tiwari and Agrawal, 2010), maize (Singh et al., 2018) and in palak (Spinach) (Tiwari and Agrawal, 2009).

The present study comprises the evaluation of regulatory proteins together with morpho-physiological, biochemical, and yield in two maize varieties under EDU treatment. This is the first proteomic study under EDU treatment in the C4 crop. As we know, the morpho-physiological approach which reveals the changes in comparison with the given EDU treatments, whereas proteomic and biochemical response analyses the insight of the plant's metabolism under any prevailing conditions. Proteomic evaluation includes, identifying differential expression of proteins in response to EDU treatment in two maize varieties. The other study parameters include pigments estimation, lipid peroxidation (MDA equivalent content), antioxidants (Ascorbate and Glutathione), and the antioxidative enzymes: Ascorbate peroxidase (APX), Glutathione reductase (GR), catalase (CAT) and superoxide dismutase (SOD). Maize is considered as the third most important crop at the global context of which, two varieties SHM3031 (stress sensitive), and PEHM5 (stress tolerant) were selected for the present study.

2. Material and methods

2.1. Study site, climatic condition and plant material

The study was conducted at CSIR-National Botanical Research Institute garden in Lucknow, city of Uttar Pradesh, India. It is situated along the southern bank of river Gomati at 26055′ N latitude, 80059′ E longitude, and an altitude of 113 m in subtropical climates. Lucknow is characterized by a dry, tropical monsoon climate. Maximum average temperatures varied from 25 to 32 °C, and minimum average temperature varied from 14 to 27 °C, and a minimum of 60% and a maximum of 78% humidity was recorded during the study period. Meteorological parameters during the experimental period were provided in (Figs. S1A and S1B). The study site (total area of 225 m^2) contains 24 plots of 9 m^2. Plots were randomized using a statistical software tool (SPSS Inc., version 16) in this number were randomized and applied to the plots. In total 24 plots, half of the plots were taken for each variety. Plots were laid in such a way that the same treatment and variety couldn't get clash with each other. The soil is sandy loam (sand 50%, silt 33%, clay 17%) with a pH of 8.4 and electrical conductivity of 231.1 μs cm^{-1}. Two maize varieties SHM3031 (drought tolerant) and PEHM5 selected for the experiment were highly recommended and widely grown variety of north-eastern plain zone of India. SHM3031 is a late variety with a life cycle of 100–120 days, whereas PEHM5 is early with a life span of 90–100 days. 'Late' crop varieties require more cultivation days than the 'early' crop varieties, meaning early varieties can be harvested much quicker.

2.2. Crop management and sampling procedure

Caryopses of maize were sown in plots at a rate of 20 × 30 cm using recommended agronomic practices. Fertilizers were supplied in ample amounts: Plots were fertilized with NPK (120:60:40). One-third dose of N and full doses of P and K were given as basal dressing. Another two doses of N were given as a top dressing after 60 and 90 days after germination (DAG). There were about 8 plants in each row comprising 96 plants in each subplot, the edge of the subplot was skipped for sowing to reduce the edge effect. Plants sampling was performed at two different stages; the vegetative stage at 45 DAG and the flowering stage at 75 DAG for both varieties, while the harvest sampling was done at 90 DAG, at the end of the maturation stage. Five different plants were selected randomly from each subplot and treatment. 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 80 °C until the weight reached a constant value. Leaf samples for the enzyme and proteomic analysis were collected at vegetative and the flowering stage. Two to three fully mature leaves were collected from three randomly selected plants from each treatment. The leaves were immediately frozen in the liquid nitrogen and stored at −80 °C until further analyses.

2.3. EDU application

EDU was kindly provided by Prof. W.J. Manning, University of Massachusetts, USA. In the present study, EDU was applied as a foliar spray, in the mixing ratios 50 and 500 ppm control plants (0 ppm) were treated with distilled water. An EDU application started at 15 DAG and was repeated every 7 days.

A preliminary dose-response test was performed before the main experiments. Total chlorophyll concentration was recorded using SPAD-502 (Konica- Minolta, Osaka, Japan) once during the experiment. Plants were harvested for their height measurements and biomass after 30 days of sowing and 15 days of EDU treatments. For biomass, plant material was kept in the oven at 80 °C for one week. In both the parameters five replicates of plant materials were taken. Both the varieties of maize exhibited better biomass, height, and total chlorophyll at 50 and 200 ppm concentrations of EDU while remaining concentrations of EDU (100, 300, and 400 ppm) were not as effective (Tables S1 and S2 and Fig. S16). So, we have selected 50 and 200 ppm EDU for our main experiment.

2.4. Average ozone and AOT40 measurements

Ozone concentrations were recorded at plant height by an annually calibrated O_3 monitor (2B Tech Ozone Monitor (106-L)) on an average of 8 h (9:00 to 17:00). Ozone exposure indices were calculated during the growing season (July to December) as AOT40, i.e. the accumulated exposure above a threshold concentration of 40 ppb during daylight hours; as described by De Leeuw and Van Zantvoort (1997).

2.5. Physiological parameters

Gas exchange parameters include, net photosynthesis (Pn) and

stomatal conductance (gs) beside this maximal photochemical efficiency of PSII (Fv/Fm) were also measured on the youngest fully mature leaves, at 55 DAG and 93 DAG, from twelve randomly selected plants of both varieties in each treatment. Minimum 3 plants per subplot were recorded for each treatment. Physiological recordings were performed using a gas exchange portable photosynthesis measuring unit (Li-COR 6400, Lincoln, Nebraska, USA) with a fluorescence chamber (LFC6400–40; Li-COR). The CO_2 levels inside the leaf cuvette were maintained at 400 mmol mol^{-1}, photosynthetic photon flux density (PPFD) was 1200 μmol $m^{-2}s^{-1}$, leaf temperature was 25 °C, and relative humidity was 60–80%. Fv/Fm measurements were performed after the leaves were dark-adapted for 30 min (Basahi et al., 2016).

2.6. Pigments, MDA, antioxidant contents and antioxidant enzymes assay

The pigments estimation was performed following Arnon (1949) and Lichtenthaler (1987) method. Fresh leaves (0.05 g) were thoroughly homogenized in chilled 80% acetone in a mortar and pestle and the homogenates were centrifuged at 10,000 g for 10 min at 4 °C. The supernatants were collected, and the absorbance of the pigment extracts was measured at 663, 646, 510 and 480 nm using a UV–visible spectrophotometer (Spectra Max Plus; Molecular Devices, USA). The Chl a, Chl b, total chlorophyll, Chl a + b, Chl a/b ratios, and carotenoids were calculated (mg g^{-1} FW) (Fig. S6). Protein estimation was carried out according to the method of Bradford (1976) with Bovine serum albumin (BSA, Sigma) as standard, and absorbance was measured at 595 nm (μg ml^{-1} $Protein^{-1}$).

The level of lipid peroxidation in the leaf tissue was measured as the content of malondialdehyde (MDA) equivalents using the 2-thiobarbituric acid (TBA) method (Heath and Packer, 1968). Reduced ascorbate (ASA), dehydroascorbate (DHA), and total ascorbate were determined by following the method of Gillespie and Ainsworth (2007). Total glutathione, oxidized glutathione (GSSG) and reduced glutathione (GSH) were measured according to Griffith (1980).

Superoxide dismutase (SOD) activity was assayed using the photochemical NBT method Bayer and Fridovich (1987) based on SOD's ability to inhibit the reduction of nitro blue tetrazolium (NBT) to form formazan by superoxide. Catalase (CAT) activity was assayed by measuring the decrease in absorbance at 240 nm due to the utilization of H_2O_2 (Rao et al., 1996). The rate of hydrogen peroxide-dependent oxidation of ascorbic acid was estimated to study ascorbate peroxidase (APX) activity (Chen and Asada, 1989). The Glutathione reductase (GR) activity was assayed by following the increase in absorbance at 412 nm when 5,50-dithiobis-(2-nitro-benzoic acid) (DTNB) was reduced by glutathione to form TNB (Smith et al., 1988).

2.7. Harvest and yield attributes

In yield parameters shoot weight $plant^{-1}$, inflorescence weight $plant^{-1}$, grain weight $plant^{-1}$, 1000 grain weight, grain no. $plant^{-1}$, spike length $plant^{-1}$, and harvest index (measurement of crop yield: the weight of grain as a ratio of the total biomass of the plant) have been taken as parameters for yield analysis. Five replicates (n = 5) of plants were taken from each variety x EDU treatment.

2.8. Proteomics extraction and 2DGE

Leaf protein extraction was carried out according to Sharma et al. (2018). In brief, leaf tissue was grounded into a fine powder followed by extraction with 50 mM Tris-HCl, pH 8.0, 25 mM EDTA, 500 mM thiourea, and 0.5% β-mercaptoethanol. The extracted protein was kept overnight (at −20 °C) with the solution of 10% cold TCA and 0.07% β-mercaptoethanol. Overnight precipitated pellet was washed three times with cold acetone and 0.07% BME. Vacuum dried extract was then, mixed within 0.1 M Tris-HCl, pH 8.0, 50 mM EDTA, and 2% BME. Tris-buffered phenol (2.5 ml) was used to extract the proteins. After centrifugation, a protein was solubilized in the lower phenol phase and was collected carefully with the Pasteur pipette. Now protein was precipitated overnight with 0.1 M ammonium acetate in methanol at −20 °C. Now pellet was solubilized in 0.1 M ammonium acetate in methanol and 1% BME. After centrifugation, the precipitate was dried and re-suspended with (7 M urea, 2 M Thiourea, 2% CHAPS, 20 mM DTT, and 0.75% v/v immobilized pH gradients buffers). 2DGE was performed as described in Lehesranta et al. (2005). In brief, protein sample (120 μg) were rehydrated overnight on immobilized pH gradients (IPG) strips (7 cm, p^H 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 performed at 20 °C with an Ettan IPGphore-3. The voltage setting was as follows: 250 and 500V for 1h each, 1500 and 4000V for 2h each, 6000V for 2 h for a total of 21.2 kVh. After focusing strips were equilibrated two times (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) differing with 1% w/v DTT and by 2.5% w/v iodoacetamide, respectively. Electrophoresis was performed at a constant 70V and 120V. Staining was done using 0.5% brilliant blue G and destained with 10% glacial acetic acid in 50% methanol and images were acquired with a document scanner. The data were analysed using the Image Master 2D Platinum 7.0 software. Relative volume (% volume) was used to quantify and compare the spots. The criteria for defining the protein expression patterns were % volume increased/decreased at least 1.5-fold; More/Less abundant protein spot.

Protein digestions were performed according to Koistinen et al. (2002). Briefly, gel particles were treated with the 25 mM ABC (Ammonium bicarbonate) containing 50% acetonitrile (ACN) for dehydration. Vacuum dried destained particles rehydrated with 0.1 mg ml^{-1} trypsin and left overnight at 37 °C for digestion. Peptides were extracted with 1% TFA in 50% ACN and concentrated to a final volume of 20 μl. A 4800 proteomics analyzer (Applied Biosystems) with TOF/TOF optics was used for all MALDI-MS and MS/MS applications. Samples were prepared by mixing 0.5 ml of sample with 0.5 ml of matrix solution (5 mg mL^{-1} a-Cyano-4-hydroxycinnamic acid in 50% ACN containing 0.1% TFA) and spotted on stainless steel 384 well target plate. External calibration of the mass spectrometer was performed with a mixture of angiotensin I, Glu-fibrinopeptide B, ACTH (1–17), and ACTH (18–39). The instrument was externally also calibrated with a fragment of glu-fibrinopeptide B, for MS/MS. Based on mass signals, protein identification was performed online (http://www.matrixscience.com) to search proteins against Swiss Prot, NCBInr, and MSDB databases. The following 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 for protein database online.

2.9. Data analysis

Before statistical analysis, the data were first checked for fulfilling the normality assumption, using the Levene's test, and if necessary, were transformed. Two-way ANOVA was used to calculate the interaction between EDU treatment and variety. Morphological, physiological, and biochemical parameters were analysed using the actual contribution test (ACP). Since different plots were sampled for the assays where the N = 3 all the developmental stages were analysed separately. Duncan's post hoc test was performed to analyses the interaction of EDU treatment and varieties (One-way ANOVA). $P < 0.05$ was significant for all the analysed dataset. All the analyses were carried out with SPSS software (SPSS Inc., version16.0). Principal component analysis (PCA) was performed for on biochemical and physiological parameters to check variations among treatments, stages and varieties by the “Past 3” software. To test the separation between the

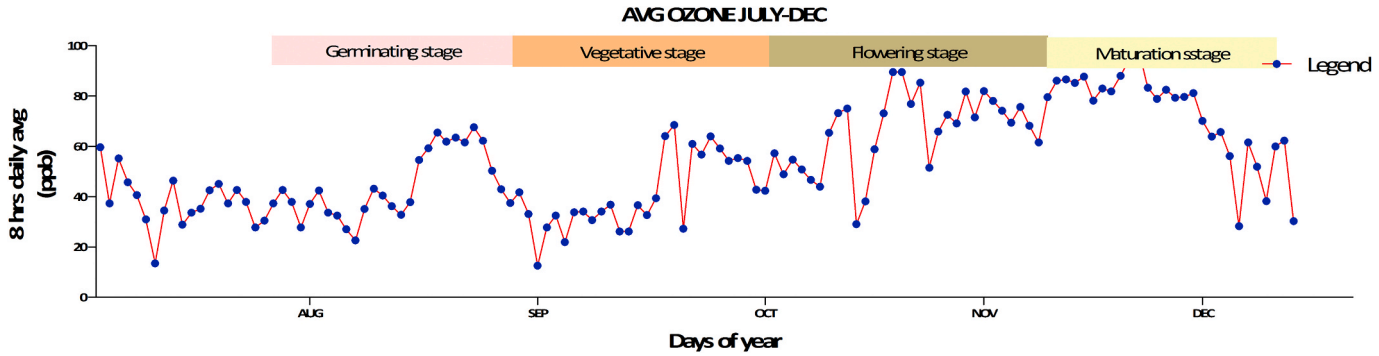

Fig. 1. Daily ozone concentrations (8 h average) during the study period. The different stages for maize growth shown in coloured boxes, and dots denote the date of the month. Germinating stages (0–25 DAG), vegetative stages (26–45 DAG), flowering stages (46–75 DAG), maturation stage (76–90 DAG).

two treatment classes, both variable importance in projection (VIP) (> 1) and the p(corr) (> 0.4) values was used as the cut-off for selecting the parameters responsible for group separation in the model (Wheelock and Wheelock, 2013).

3. Results

3.1. Average ozone and AOT40

The average ambient ozone during the study period was 53.21 ppb. It had been recorded that precipitation and cloudy weather were responsible for comparatively low ambient ozone during the study period rather than dry seasons. Precipitation leads to washout of the precursor responsible for ozone formation. The ozone levels since October were higher than until September because of higher precipitation rate in later months (Fig. 1). High ambient O_3 concentrations (hourly average) (> 40 ppb) prevailed particularly during the flowering and maturation stage. Maximum AOT40 concentrations were also recorded during mid-vegetative, flowering and maturation stages (Table 1).

3.2. Morpho-physiological and biochemical response

The ACP analysis showed that root length was significantly increased in both the varieties (SHM3031 and PEHM5) at both the developmental stages in EDU treatment (Table 2 & Fig. S2). It was more in SHM3031 as about 60–100% increase was recorded at the vegetative stage with both the EDU doses. Increase in root weight was significant in both SHM3031 and PEHM5; S1 (91%), S2 (68%), P1 (69%) and P2 (148%) at vegetative stage, while in the flowering stage significant increases were recorded at 60%, 31%, 172%, and 67% in S1, S2, P1, and P2, respectively (Table 2, Table S3 and Fig. S3). Responses in pigment content showed a significant increase in EDU treated plants in almost all the pigments type i.e. chlorophyll *a, b*, total, a + b, a/b ratio, and carotenoid content. Responses SHM3031 at the vegetative stage performed better for pigments content under 50 ppm of EDU dose, however, at the flowering stage, SHM3031 was also able to maintain good pigment quantity for both the EDU concentration (Table 2 & Fig. S6). No significant changes in the net photosynthetic rate (Pn) and stomatal conductance (gs) were observed in both the varieties and EDU treatment (Table 2 & Fig. S4). In both the varieties, 50 ppm EDU concentration showed less MDA equivalent content in the vegetative stage. At the flowering stage, 200 ppm EDU showed less MDA equivalent content in both the varieties as there was high ozone concentration during this stage (Table 2 & Fig. S7).

Total ASA content was more in SHM3031 at 50 ppm EDU treatment at the vegetative stage whereas it was more in 200 ppm EDU dose at the flowering stage. In, PEHM5 total ascorbate significantly increased only in 200 ppm EDU concentration at the flowering stage (Table 2 & Fig. S8). Reduced GSH showed a significant increase only in SHM3031 at the vegetative stage (Table 2 & Fig. S9). Higher oxidized glutathione (GSSG) levels were observed in SHM3031 and PEHM5 at the flowering stage. In the present study SOD activity was more at the flowering stage with 200 ppm EDU as compared to the vegetative stage in PEHM5. SHM3031 showed more SOD activity at the vegetative stage with both EDU treatments. Although Catalase activity was more at 50 ppm EDU treatment in SHM3031 was more responsive than 200 ppm, whereas 200 ppm EDU treatment was more responsive in PEHM5 (Table 2 & Fig. S10). APX activity was significantly increased in SHM3031 at both the developmental stages whereas PEHM5 showed increased activity only in 200 EDU treatments at the flowering stage. GR activity showed a significant increase at both the stages in SHM3031 whereas in PEHM5 only in 50 ppm of EDU treatment at vegetative stage (Table 2 & Fig. S11).

Table 1
Average ozone concentration (ppb 8 h average) and AOT 40 (ppm.h) during the entire study period. Germinating stages (0–25 DAG), Vegetative stages (26–45 DAG), Flowering stages (46–75 DAG), Maturation Stage (76–90 DAG).

Developmental Stages	Germinating stages	Vegetative stages	Flowering stages	Maturation Stage
Avg Ozone (ppb)	41.07	53.16	68.97	71.38
AOT 40 (ppm.h)	0.30	3.79	8.34	9.03

3.3. Comparisons between varieties for different parameters

The PCA plot showed differences among the varieties as the first principal component (34.43%). Variations amongs sensitive and tolrent variety were observed as PC 1 shown positive loading for GR V, CARO F, COND F, and GSSG F in SHM3031. Although as expected MDA content has negative loading in both developmental stages in PC1. Under prevailing high ozone concentration variation due to EDU treatment shown as the second principal component (PC2), explaining 23.72% of the total variation in the dataset (Fig. S12A). A clear separation was found between ambient and EDU treatment and between varieties.

Increased glutathione and pigment content were observed in the vegetative and flowering stages, respectively. Increase in SOD, CAT activities together with ASA, GSH content, while decreased GSSG content at the flowering stages (Fig. S12B), proved EDU enhanced enzymatic pool to cope prevailing high tropospheric ozone during study period.

3.4. Final harvest and yield attributes

One-way ANOVA showed significant increases in shoot weight plant^{-1}, inflorescence weight plant^{-1}, grain no. plant^{-1}, grain weight plant^{-1}, and 1000 grain weight. In S1 and P1, 29% and 23% increase in

Table 2
Effect of EDU on different parameters related to growth, biomass, physiology, antioxidant, antioxidative enzymes and MDA content. Values represent mean ± standard error (n = 3); different letters indicate significant differences between EDU treatments: 0 ppm (control), 50 ppm and 200 ppm within each cultivar ($P < 0.05$) by one way anova. (Abbreviations: TASA, total ascorbate; ASA, ascorbate; DHA, dehydroascorbate; TGSH, total glutathione; GSH, glutathione; GSSG, oxidized glutathione; SOD, superoxide dismutase; APX, ascorbate peroxidase; GR, glutathione reductase; MDA, Malondialdehyde; FW, Fresh weight.

S.No	Parameters (unit)	SHM3031 Vegetative			SHM3031 Flowering			PEHM5 Vegetative			PEHM5 Flowering		
	Treatments	CON	50 ppm	200 ppm	CON	50 ppm	200 ppm	CON	50 ppm	200 ppm	CON	50 ppm	200 ppm
1	Root length (cm)	11.0 ± 1.0^{B}	22.6 ± 2.3^{A}	18.0 ± 1.7^{A}	21.6 ± 0.8^{B}	28.6 ± 1.8^{A}	29.3 ± 2.2^{A}	12.0 ± 0.5^{b}	13.3 ± 0.8^{a}	16.3 ± 2.3^{a}	22.0 ± 2.1^{b}	31.0 ± 2.0^{a}	24.3 ± 0.9^{b}
2	Shoot length (cm)	112.0 ± 2.3^{B}	134.6 ± 4.1^{A}	131.0 ± 1.7^{A}	166.7 ± 3.7^{B}	195.0 ± 5.8^{A}	194.0 ± 4.0^{A}	121.0 ± 2.9^{b}	141.6 ± 3.5^{a}	134.3 ± 3.4^{a}	170.3 ± 4.1^{b}	202.0 ± 3.4^{a}	161.7 ± 5.5^{b}
3	Plant height (cm)	123.3 ± 2.6^{B}	157.3 ± 5.9^{A}	149.0 ± 1.7^{A}	188.3 ± 4.4^{B}	223.6 ± 4.5^{A}	223.3 ± 5.8^{A}	133.0 ± 3.2^{b}	154.67 ± 2.6^{a}	150.6 ± 6.1^{a}	192.3 ± 5.3^{b}	233.0 ± 5.1^{a}	186.0 ± 5.0^{b}
4	Root weight (g)	1.4 ± 0.02^{B}	2.6 ± 0.04^{A}	2.3 ± 0.2^{A}	4.9 ± 0.3^{C}	7.9 ± 0.09^{A}	6.5 ± 0.3^{B}	1.3 ± 0.2^{c}	2.2 ± 0.2^{b}	3.3 ± 0.3^{a}	3.3 ± 0.5^{c}	8.9 ± 0.2^{a}	5.5 ± 0.6^{b}
5	Shoot Weight (g)	20.2 ± 1.7^{A}	22.0 ± 0.3^{A}	23.8 ± 3.5^{A}	29.8 ± 3.0^{B}	47.0 ± 5.2^{A}	38.4 ± 2.8^{AB}	16.3 ± 2.9^{a}	27.3 ± 4.2^{a}	25.8 ± 1.8^{a}	28.4 ± 4.1^{b}	45.2 ± 4.5^{a}	31.9 ± 2.2^{b}
6	Total Biomass (g)	21.5 ± 1.7^{A}	24.6 ± 0.3^{A}	26.1 ± 3.3^{A}	34.7 ± 3.0^{B}	54.9 ± 5.3^{A}	44.9 ± 3.1^{AB}	17.6 ± 3.0^{b}	29.5 ± 4.4^{a}	29.1 ± 1.7^{a}	31.6 ± 4.6^{b}	54.1 ± 4.6^{a}	37.4 ± 2.5^{b}
7	Photosynthesis (μmol mol^{-1})	41.8 ± 1.5^{A}	48.0 ± 0.8^{A}	31.6 ± 2.5^{A}	11.4 ± 0.4^{A}	15.2 ± 0.1^{B}	13.0 ± 1.0^{A}	40.6 ± 5.7^{a}	42.2 ± 0.3^{a}	39.5 ± 6.0^{a}	17.2 ± 0.5^{a}	21.9 ± 0.3^{b}	20.8 ± 0.3^{a}
8	Conductance (m^{-1}s^{-1})	0.01 ± 0.0^{A}	0.02 ± 0.01^{A}	0.01 ± 0.0^{A}	0.01 ± 0.0^{A}	0.02 ± 0.0^{B}	0.01 ± 0.0^{A}	0.02 ± 0.0^{a}	0.02 ± 0.0^{b}	0.04 ± 0.02^{a}	0.01 ± 0.0^{a}	0.03 ± 0.0^{b}	0.04 ± 0.0^{a}
9	*Fv/Fm*	0.75 ± 0.0^{A}	0.76 ± 0.01^{A}	0.75 ± 0.01^{A}	0.72 ± 0.0^{A}	0.74 ± 0.0^{A}	0.75 ± 0.02^{A}	0.74 ± 0.01^{a}	0.75 ± 0.0^{a}	0.75 ± 0.01^{a}	0.72 ± 0.0^{a}	0.75 ± 0.0^{a}	0.75 ± 0.0^{a}
10	Chlorophyll *a* (mg g^{-1} FW)	17.7 ± 0.1^{B}	24.3 ± 0.4^{A}	18.6 ± 0.4^{B}	4.44 ± 0.1^{C}	9.02 ± 0.2^{B}	11.7 ± 0.3^{A}	15.6 ± 0.4^{c}	22.8 ± 0.2^{a}	21.7 ± 0.2^{b}	4.60 ± 0.0^{c}	7.73 ± 0.0^{b}	8.32 ± 0.0^{a}
11	Chlorophyll *b* (mg g^{-1} FW)	14.1 ± 0.1^{C}	17.9 ± 0.1^{A}	14.7 ± 0.2^{B}	1.83 ± 0.0^{C}	2.92 ± 0.0^{B}	3.18 ± 0.0^{A}	12.0 ± 0.3^{c}	17.83 ± 0.1^{a}	16.8 ± 0.2^{b}	1.65 ± 0.0^{c}	4.81 ± 0.3^{a}	2.30 ± 0.0^{b}
12	Total Chlorophyll (mg g^{-1} FW)	25.7 ± 0.1^{C}	33.7 ± 0.3^{A}	26.8 ± 0.4^{B}	4.73 ± 0.1^{C}	8.82 ± 0.2^{B}	10.8 ± 0.2^{A}	22.2 ± 0.6^{c}	32.6 ± 0.1^{a}	30.9 ± 0.3^{b}	4.65 ± 0.0^{c}	9.84 ± 0.4^{a}	7.73 ± 0.0^{b}
13	Chlorophyll *a*+b (mg g^{-1} FW)	31.2 ± 0.1^{B}	42.2 ± 0.5^{A}	33.3 ± 0.5^{B}	6.28 ± 0.2^{C}	11.9 ± 0.2^{B}	14.9 ± 0.3^{A}	27.6 ± 0.7^{c}	40.6 ± 0.2^{a}	38.6 ± 0.3^{b}	6.25 ± 0.0^{c}	12.5 ± 0.4^{a}	10.6 ± 0.0^{b}
14	Chlorophyll *a*/b (mg g^{-1} FW)	1.25 ± 0.0^{B}	1.35 ± 0.0^{A}	1.26 ± 0.0^{B}	2.42 ± 0.0^{C}	3.08 ± 0.0^{B}	3.68 ± 0.1^{A}	1.29 ± 0.0^{c}	1.27 ± 0.0^{a}	1.29 ± 0.0^{b}	2.78 ± 0.1^{b}	1.62 ± 0.1^{c}	3.61 ± 0.0^{a}
15	Carotenoids (mg g^{-1} FW)	8.03 ± 0.0^{B}	10.2 ± 0.1^{A}	8.02 ± 0.0^{B}	1.81 ± 0.1^{C}	3.62 ± 0.2^{B}	4.26 ± 0.0^{A}	6.99 ± 0.2^{c}	9.49 ± 0.0^{a}	8.44 ± 0.1^{b}	0.99 ± 0.0^{c}	1.80 ± 0.1^{b}	2.44 ± 0.0^{a}
16	MDA content (μmol g^{-1} FW)	64.6 ± 1.1^{A}	25.5 ± 0.7^{C}	40.1 ± 0.2^{B}	16.8 ± 0.1^{A}	16.2 ± 0.4^{A}	14.6 ± 0.1^{B}	34.5 ± 0.9^{a}	23.9 ± 1.2^{b}	31.8 ± 1.8^{a}	24.2 ± 0.2^{a}	23.5 ± 0.3^{a}	18.9 ± 0.3^{b}
17	TASA (μmol g^{-1} FW)	9.79 ± 0.3^{B}	10.6 ± 0.1^{C}	8.88 ± 0.1^{A}	7.92 ± 0.0^{B}	6.30 ± 0.0^{C}	10.4 ± 0.1^{A}	8.54 ± 0.0^{a}	8.15 ± 0.1^{b}	8.43 ± 0.1^{ab}	7.46 ± 0.0^{b}	5.83 ± 0.0^{c}	7.87 ± 0.0^{a}
18	ASA (μmol g^{-1} FW)	2.86 ± 0.0^{B}	5.92 ± 0.1^{A}	2.99 ± 0.0^{B}	3.45 ± 0.1^{B}	2.78 ± 0.1^{C}	5.27 ± 0.0^{A}	2.98 ± 0.0^{c}	3.63 ± 0.0^{b}	5.52 ± 0.1^{a}	3.43 ± 0.0^{b}	2.23 ± 0.1^{c}	3.68 ± 0.0^{a}
19	DHA (μmol g^{-1} FW)	6.93 ± 0.4^{A}	4.72 ± 0.1^{C}	5.88 ± 0.1^{B}	4.47 ± 0.1^{B}	3.52 ± 0.1^{C}	5.14 ± 0.1^{A}	5.55 ± 0.1^{a}	4.52 ± 0.1^{b}	2.91 ± 0.1^{c}	4.02 ± 0.0^{b}	3.59 ± 0.0^{c}	4.19 ± 0.0^{a}
20	TGSH (μmol g^{-1} FW)	8.07 ± 0.3^{B}	14.0 ± 0.3^{A}	14.8 ± 0.8^{A}	11.5 ± 0.6^{A}	12.9 ± 0.5^{A}	11.4 ± 0.5^{A}	8.52 ± 1.3^{a}	10.8 ± 0.2^{a}	9.51 ± 0.4^{a}	12.2 ± 0.4^{b}	13.8 ± 0.5^{ab}	14.9 ± 0.8^{b}
21	GSH (μmol g^{-1} FW)	4.41 ± 0.4^{B}	10.2 ± 0.2^{A}	11.0 ± 0.8^{A}	10.3 ± 0.6^{A}	9.41 ± 0.5^{A}	9.30 ± 0.4^{A}	6.02 ± 1.4^{a}	7.59 ± 0.2^{a}	4.73 ± 1.0^{a}	10.8 ± 0.4^{a}	11.3 ± 0.4^{a}	12.3 ± 0.8^{a}
22	GSSG (μmol g^{-1} FW)	3.66 ± 0.1^{A}	3.83 ± 0.2^{A}	3.78 ± 0.1^{A}	1.17 ± 0.0^{C}	3.49 ± 0.1^{B}	2.10 ± 0.1^{A}	2.49 ± 0.1^{b}	3.30 ± 0.0^{b}	4.78 ± 0.5^{a}	1.44 ± 0.0^{c}	2.43 ± 0.1^{b}	2.71 ± 0.0^{a}
23	SOD (μmol mg^{-1} protein^{-1} min^{-1})	13.7 ± 0.4^{B}	16.7 ± 0.4^{A}	16.6 ± 0.5^{A}	20.2 ± 1.0^{A}	23.1 ± 0.9^{A}	17.2 ± 0.7^{B}	16.5 ± 0.4^{b}	17,2 ± 0.4^{b}	21.4 ± 0.6^{a}	22.8 ± 1.2^{b}	23.7 ± 1.0^{b}	37.7 ± 2.2^{a}
24	CAT (μmol mg^{-1} protein^{-1} min^{-1})	5.92 ± 0.5^{C}	17.8 ± 0.9^{A}	15.4 ± 1.7^{B}	16.1 ± 0.6^{C}	31.1 ± 0.3^{A}	21.0 ± 0.9^{B}	4.65 ± 0.5^{c}	6.98 ± 0.8^{a}	15.1 ± 0.8^{b}	19.2 ± 0.8^{c}	26.8 ± 1.9^{b}	37.0 ± 1.8^{a}
25	APX (μmol mg^{-1} protein^{-1} min^{-1})	3.35 ± 0.1^{B}	4.58 ± 0.2^{A}	4.60 ± 0.3^{A}	1.64 ± 0.0^{B}	2.04 ± 0.2^{A}	2.14 ± 0.0^{A}	2.84 ± 0.2^{a}	2.25 ± 0.0^{b}	3.16 ± 0.1^{a}	1.65 ± 0.2^{b}	1.48 ± 0.0^{c}	2.19 ± 0.0^{a}
26	GR (μmol mg^{-1} protein^{-1} min^{-1})	0.06 ± 0.0^{B}	0.14 ± 0.0^{A}	0.12 ± 0.0^{A}	0.25 ± 0.0^{B}	0.34 ± 0.0^{A}	0.36 ± 0.0^{A}	0.08 ± 0.0^{b}	0.30 ± 0.0^{a}	0.11 ± 0.0^{b}	0.24 ± 0.0^{a}	0.24 ± 0.0^{a}	0.24 ± 0.0^{a}

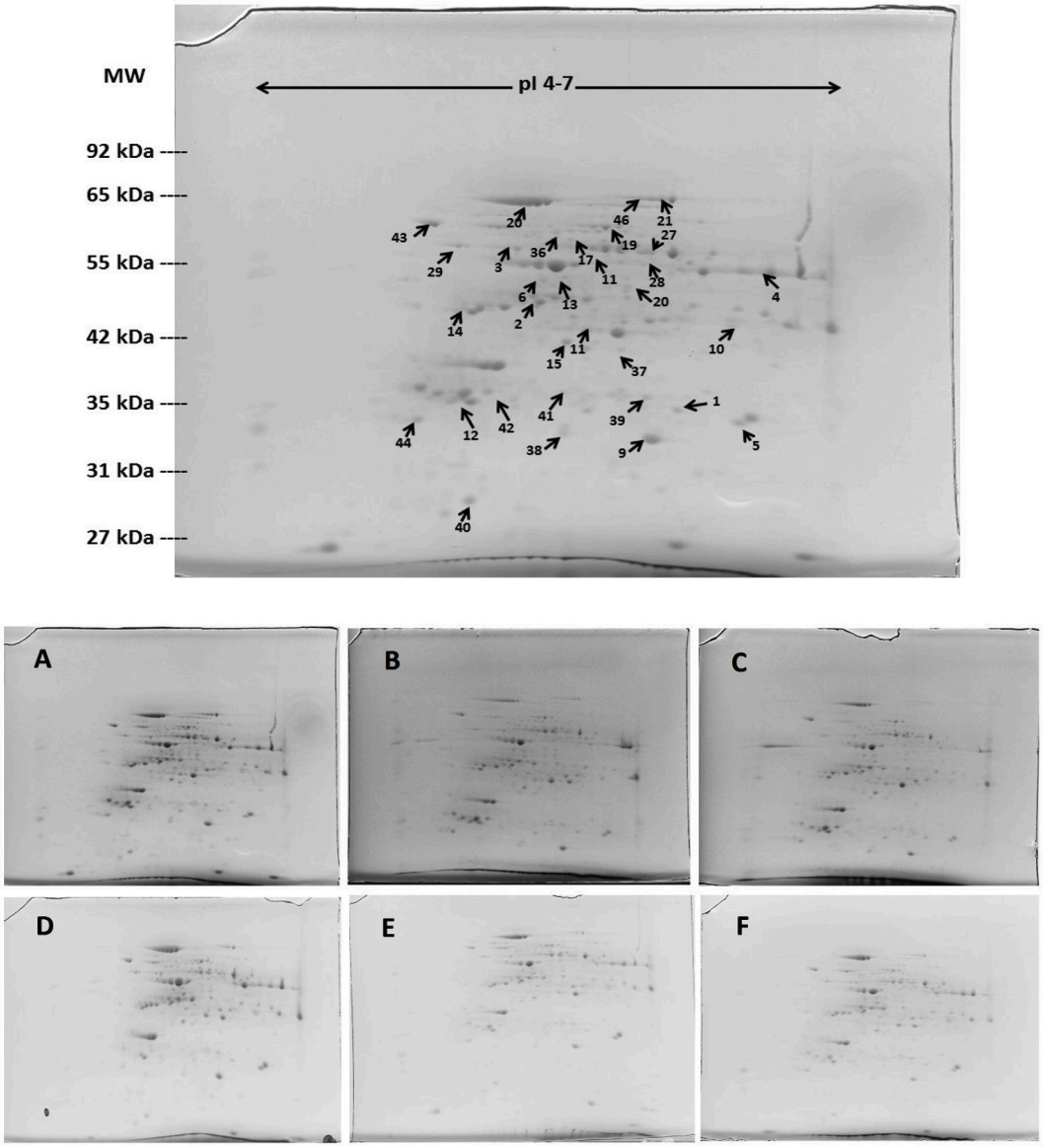

Fig. 2. Effect of EDU application on protein expression pattern of two maize varieties viz. SHM3031, and PEHM5 leaves at the vegetative stage. (12% gel, 120 μg loading, pH range 4–7, Brilliant blue G stained two-dimensional gels, arrows in main gel represents proteins identified, control (A, D), 50 ppm EDU dose (B, E) and 200 ppm EDU dose (C, F). (A, B, C = SHM3031 and D, E, F = PEHM5).

shoot weight were found, respectively. Inflorescence weight plant^{-1} showed 38% (S1) and 59% (P2) increase in EDU treatment. SHM3031 showed a significant increase in grain no. plant-1 (45%) whereas it was insignificant in PEHM5. Grain weight plant-1 was significantly increased in both the S1 and P2 by 30%. (Fig. S5).

3.5. Proteomics analysis

Leaf proteomics revealed increased or decreased expressions of proteins under two EDU doses in both varieties during two developmental stages (Figs. 2 and 3 & Fig. S13). Protein expression was more at the vegetative stage in both varieties, predominantly in SHM3031, than at the flowering stage (Table 3 and Supplementary Data S1). The number of identified proteins was 110 and 82 in SHM3031 at the vegetative and flowering stage, respectively. In PEHM5, 84 proteins were identified at the vegetative stage and 66 at the flowering stage. Identified proteins were functionally characterized into seven categories and mostly belonged to the maize. species (Figs. S14A, B, C & D). Photosynthesis and carbon metabolism were the two major dominating functional categories. Forty-four proteins of SHM3031 and 29 of PEHM5 were involved in carbon metabolism. The number of photosynthesis-related proteins was 28 each in SHM3031 and PEHM5. Many proteins related to protein synthesis, assembly, and degradation (15 in SHM3031 and 17 in PEHM5) were also identified. Defense category had 12 and 9 proteins in SHM3031 and PEHM5, respectively. There were 19 energy metabolism proteins in SHM3031 and 9 in PEHM5. Two proteins of SHM3031 and 1 protein from PEHM5 were categorized under lipid biosynthesis. Cytoskeleton category had one protein for SHM3031, while one protein from PEHM5 for cell transports (Table 3).

The effect of EDU on the proteins in varieties and developmental stages have been analysed using a Venn diagram (Fig. S15) showed 17 proteins common in both the varieties and developmental stages, whereas 21 were unique to SHM3031 and 17 proteins unique to PEHM5. Heat map analysis showed mostly more abundant (increased in expression) proteins in PEHM5 varieties at vegetative stage whereas SHM3031 variety shows less abundant (decreased in expression) of proteins during the vegetative stage (Fig. 5). Although at the flowering stage both the varieties have shown almost similar responses in terms of

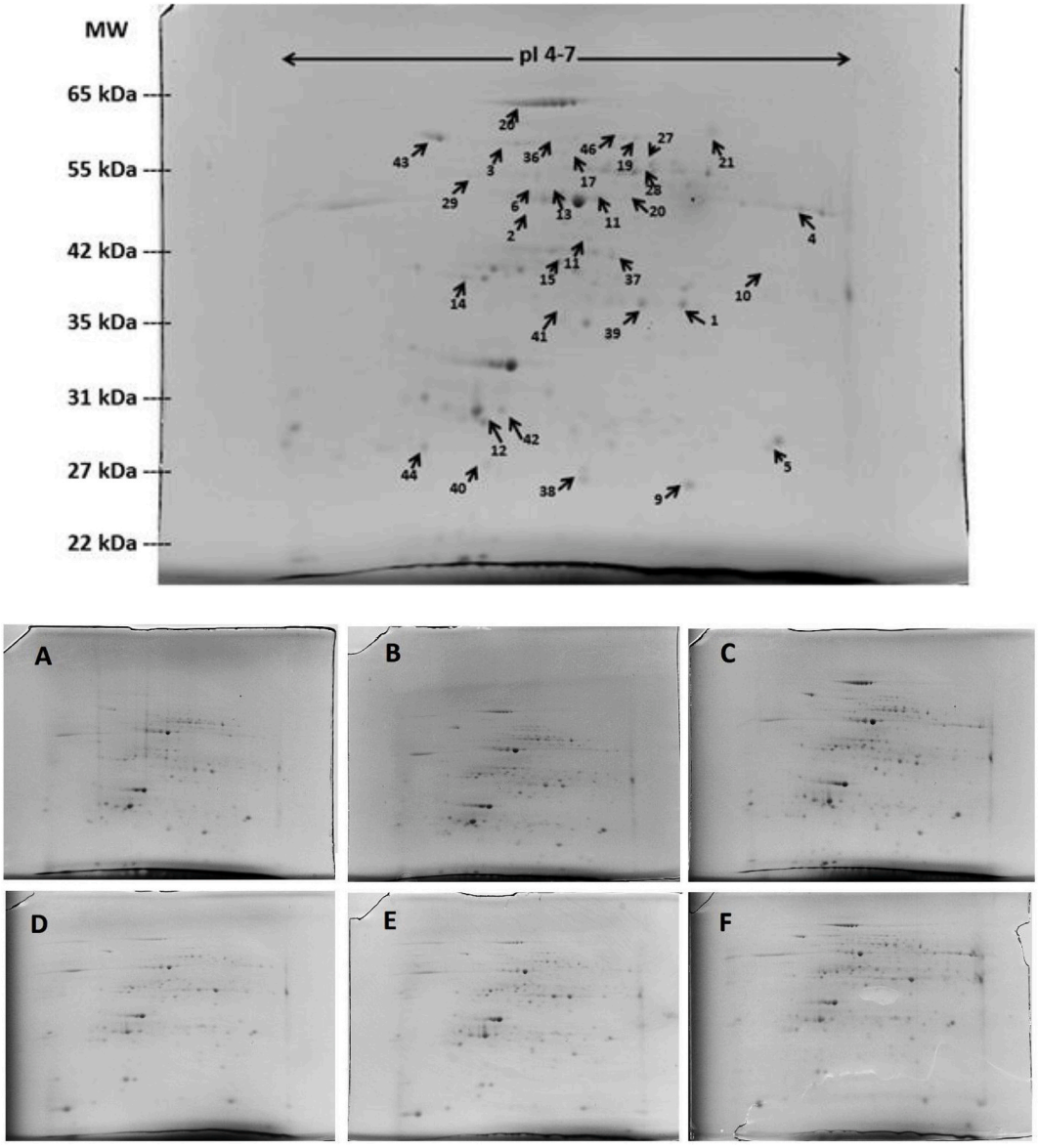

Fig. 3. Effect of EDU application on protein expression pattern of two maize varieties viz. SHM3031, and PEHM5 leaves at the flowering stage. (12% gel, 120 μg loading, pH range 4–7, Brilliant blue G stained two-dimensional gels, arrows in main gel represents proteins identified, control (A, D), 50 ppm EDU dose (B, E) and 200 ppm EDU dose (C, F). (A, B, C = SHM3031 and D, E, F = PEHM5).

protein expression. Proteins related to C4- photosynthesis were greatly influenced by the EDU treatment. Some of them like NADP- malic enzyme increased at vegetative and flowering stages whereas malate dehydrogenase showed differential expression for SHM3031 and increased expression for PEHM5 with 200 ppm of EDU treatment. These proteins were exclusive to maize; there change in expression shows EDU also has effects on the maize. Some of the defense related-proteins such as Germin like protein, Harpin binding protein, and 2- cys peroxiredoxin have shown mixed expression under EDU treatment. Expression patterns and details about their functions under different functional categories were shown in Table 3 and Supplementary Data S2. A schematic representation of the C4- Cycle, Calvin cycle, and photophosphorylation had been shown to represent EDU mediated changes in protein expression (Fig. 4).

4. Discussion

We demonstrated harmful effects of tropospheric ozone on maize using EDU as anti-ozonant supplement in the middle IGP region, of India. The higher concentration of ozone in troposphere resulted from high temperature, longer sunshine hours, and less relative humidity. In our study, the maximum O_3 concentration (71.38 ppb) was found during October and November, and minimum during peak rainfall months (July, August, and September). The lower O_3 concentration during rainy season was appeared due to washout of O_3 developing precursors (Feng et al., 2010). The annual average ozone concentration was 53 ppb, which was significantly above of threshold value (40 ppb) to cause injuries in both plants and animals (Oksanen et al., 2013). Deb Roy et al. (2009) also reported high ambient ozone (AOT 40) in the IGP region, India.

EDU has been widely used since long time in ambient fields to diagnose the effect of prevailing tropospheric ozone (Manning et al., 2011; Agathokleous et al., 2015). We selected two EDU concentration based on dose response test (Tables S1 and S2 and Fig. S16) conducted prior to main experiment. In dose response test 50 and 200 ppm EDU concentration showed optimum response for morphology, biomass and total chlorophyll. Singh et al. (2018) also used 200 ppm of EDU dose for two maize varieties at Varanasi area near to Lucknow during ozone

Table 3
List of EDU- responsive leaf proteins in two maize varieties viz. SHM3031 and PEHM5 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]	SHM3031, Vegetative stage	SHM3031, Flowering stage		PEHM5, Vegetative stage		PEHM5, Flowering stage	
			50 ppm	200 ppm	50 ppm	200 ppm	50 ppm	200 ppm	50 ppm	200 ppm
Photosynthesis related proteins										
1	Cytochrom b6f complex FeS	Electron transport	−2.87↓	–	−2.46↓	–	–	–	–	–
2	Chlorophyll a/b binding protein	Light harvesting	−1.77↓	–	−3.37↓	−2.08↓	2.38↑	−4.50↓	–	−3.72↓
3	Rubisco Activase	Rubisco activation	3.37↑	2.91↑	2.54↑	–	–	1.56↑	–	1.76↑
4	Rubisco LSU	Carboxylase/oxygenase activity	2.28↑	2.61↑	–	–	−1.80↓	1.76↑	−1.54↓	–
5	Oxygen evolving enhancer protein	PS II Regulation	−1.71↓	−1.60↓	–	–	3.68↑	5.11↑	−3.03↓	−1.79↓
6	Ferredoxin NADP- reductase	Electron transport	4.20↑	1.50↑	–	–	−2.17↓	–	–	–
7	Ferredoxin	Electron transport	–	−5.34↓	–	–	–	1.64↑	−4.98↓	–
8	Thylakoid lumenal 18 kDa protein	PS- II repair	–	–	−1.50↓	−2.12↓	1.54↑	–	–	–
Carbon metabolism proteins										
9	NADP- Malic enzyme	TCA cycle	–	4.33↑	1.67↑	2.91↑	1.66↑	–	–	1.97↑
10	Malate dehydrogenase	TCA cycle	1.73↑	−4.34↓	1.74↑	−1.82↓	–	2.52↑	–	1.65↑
11	Fructose-bisphosphate aldolase	Glycolytic Process	−2.25↓	–	1.62↑	−2.30↓	2.23↑	–	−1.64↓	–
12	Adenylate kinase	Kinase activity	–	1.50↑	−1.84↓	–	–	–	–	–
13	Glyceraldehyde-3-phosphate dehydrogenase1	Glycolytic Process	−1.56↓	−1.98↓	3.26↑	3.22↑	7.69↑	–	−5.16↓	–
14	Sedoheptulose bisphosphatase	Riboneogenesis	−1.62↓	–	1.56↑	–	–	–	–	7.60↑
15	Phospho glycerate kinase	Glycolytic process	2.21↑	−1.51↓	1.79↑	1.54↑	–	2.22↑	2.03↑	2.27↑
16	Glucose-1-phosphate adenylyl transferase	Starch biosyntheis	–	1.65↑	–	1.61↑	–	–	–	–
17	Enolase	Glycolytic Process	−2.41↓	–	−3.29↓	–	2.30↑	3.77↑	–	–
18	beta-D-glucosidase precursor	Glucose hydrolysis	−2.47↓	−1.70↓	–	2.19↑	2.59↑	–	–	–
19	Transketolase	Transketolase activity	−1.68↓	–	−1.54↓	1.96↑	2.71↑	3.20↑	–	3.03↑
20	Pyruvate orthophosphate	Pyruvate metabolism	–	−3.70↓	–	1.95↑	–	−1.52↓	–	–
21	Phosphoenolpyruvate carboxykinase	Pyruvate metabolism	1.88↑	–	1.55↑	–	1.85↑	–	–	–
22	beta-D-glucosidase precursor	Glycolytic Process	–	–	–	2.64↑	–	–	–	–
23	Carbonic anhydrase	Carbon utilization	–	–	–	1.57↑	–	–	–	–
24	GDP-mannose 3,5-epimerase	ascorbate biosynthesis	–	–	–	−1.86↓	2.28↑	–	–	2.71↑
25	Pyruvate, phosphate dikinase	Pyruvate metabolism	–	3.67↑	–	–	–	–	–	–
Energy metabolism related proteins										
26	ATP synthase subunit α	ATP synthesis	−2.21↓	−6.17↓	−1.64↓	−1.96↓	3.58↑	1.54↑	–	1.80↑
27	ATP synthase subunit β	ATP synthesis	−2.19↓	−2.54↓	2.13↑	−1.55↓	1.78↑	6.53↑	2.22↑	2.63↑
28	ATP synthase subunit γ	ATP synthesis	−1.52↓	–	–	−1.78↓	1.62↑	–	–	1.79↓-
Protein synthesis assembly and degradation										
29	Chloroplast protein synthase	Protein Synthesis	23.13↑	42.18↑	–	1.77↑	–	–	−3.49↓	18.1↓-
30	Elongation factor	Translation elongation	−1.56↓	−1.81↓	−1.58↓	–	3.27↑	1.77↑	–	5.85↓-
31	Translation initiation factor	Translation initiation	–	2.56↑	−1.79↓	−1.54↓	–	–	–	–
32	Chaperonin	Protein folding	−1.50↓	–	−1.55↓	−2.07↓	–	−1.63↓	–	1.61↑
33	Peptidyl-prolylcis-trans isomerase	FK506 binding	−2.49↓	–	–	–	–	–	–	−2.57↓
34	Peptidase beta subunit	Protein Maturation	–	1.57↑	–	–	–	–	–	–
35	Ftsh	Chapernone activity	–	4.53↑	–	–	–	–	3.43↑	–
36	RNA polymerase II	Translation	−4.98↓	2.00↑	–	–	2.33↑	6.06↑	–	2.56↑
Defense										
37	Germin-like protein	Nutrient reservoir	−1.73↓	–	−1.67↓	–	−3.43↓	−3.28↓	1.76↑	−2.46↓
38	Cytosolic Ascorbate Peroxidase	Defense response	1.55↑	–	1.72↑	8.76↑	−2.08↓	–	–	–
39	fruit protein PKIWI502	Oxidoreducatse activity	−3.21↓	−1.55↓	–	1.97↑	–	−1.61↓	−3.46↓	–
40	Superoxide dismutase	ROS removal	−1.88↓	–	–	–	–	–	–	–
41	Thioredoxin M-type	ROS removal	–	–	1.63↑	–	–	–	–	–
42	Harpin binding protein	Defense response	1.52↑	–	−1.57↓	–	4.51↑	–	–	–
43	heat shock protein	Response to heat	–	−3.14↓	−2.79↓	−2.82↓	4.86↑	3.53↑	–	–
44	2-cys peroxiredoxin	Response to stress	−1.69↓	2.61↑	−2.84↓	–	4.13↑	–	–	–
Cytoskeleton										
45	Actin family protein	Actin binding	2.70↑	3.60↑	–	1.57↑	–	–	–	–
Lipid biosynthesis										
46	Lipoxygenase	Lipid oxidation	−8.40↓	–	−3.23↓	–	5.91↑	5.25↑	–	–
Cell transport										
47	hypothetical protein ZEAMMB73_561858	–	–	–	–	–	–	2.67↑	−1.63↓	–

[a] Functions of the proteins as per "www.uniprot.com".; Abbreviations: LSU, large subunit; SSU, small sub unit; (↑) Denotes, increased expression of proteins; (↓) denotes, decreased expression of proteins; (-) denotes, no change.

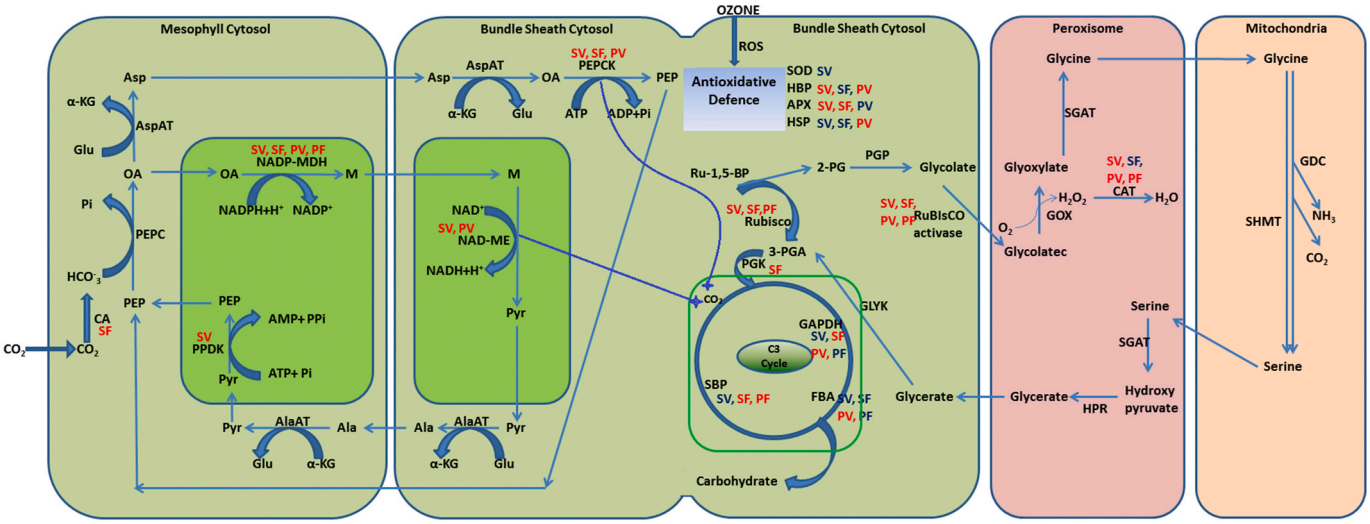

Fig. 4. Schematic representation showing effect of EDU treatment on C4-cyle, Calvin-cycle and photophosphorylation related proteins in two maize varieties. Red colour represents positive regulation and blue represents negative regulation of proteins. (SV- SHM3031 vegetative stage; SF- SHM3031 flowering stage; PV- PEHM5 vegetative stage; PF- PEHM5 flowering stage). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Abbreviations for proteins: FBA, fructose-bisphosphate aldolase; GADPH, glyceraldehyde-3-phosphate dehydrogenase; PEPC, phospho enol pyruvate carboxylase; NADP-MDH, NADP-malate dehydrogenase; PGK, phospho glycerate kinase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SBP, sedoheptulose-1,7-bisphosphatase; PPDK, pyruvate phosphate dikinase; PEPCK, phospho enol pyruvate corboxy kinase; CA, carbonic anhydrase; CAT, catalase; SOD, superoxide dismutase; HBP, harpin binding protein; HSP, heat shock protein; HPR, hydroxyl pyruvate reductase; SGAT, serine glyoxylate amino transferase; GDC, Glycine decarboxylase; GOX, Glycine oxidase.

Abbreviations for metabolites: F-6-P, fructose-6-phosphate; 3-PGA, 3-phosphoglycerate; Ru-1, 5-BP, ribulose-1, 5-bisphosphate; PEP, phospho enol pyruvate; OA, oxaloacetate; M, malate; Pyr, pyruvate.

assessment study. The EDU approach assumes that the chemical alleviates ozone effects on crops, while having no constitutive effects on plants (Ashrafuzzaman et al., 2017).

4.1. Morpho-physiological and biochemical response

EDU mediated significant increment were recorded in root length, shoot length, and plant height, in both the varieties (SHM3031, and PEHM5) and developmental stages (Vegetative and flowering) (Figs. S2 and S3). These results depict both the EDU dose 50 and 200 ppm have positive effects on maize morphology in ours study. A similar report had been published in previous works for *Vigna sps.* (Agrawal et al., 2005; Singh et al., 2010a). Root weight and shoot weight showed significant increase in both varieties states better EDU protection against prevailing high tropospheric ozone. Now it is well understood that EDU positively influences biomass as shown by Singh et al. (2018) in two sensitive varieties of maize and Astorino et al. (1995) in *Phaseolus vulgaris*. EDU mediated increase in biomass was observed at the flowering stage in S1 and S2 as compared to vegetative stage. This might because, at the earlier stage, relatively low tropospheric ozone leads to less EDU protection (Fig. 1). Several other crops such as spinach (Tiwari and Agrawal, 2009), carrot (Tiwari and Agrawal, 2010) mung bean (Singh et al., 2010a) and mustard (Pandey et al., 2014) also reported an increase in biomass with EDU treatment. In our study gas exchange parameters were not significantly affected under both the EDU treatment, except *Fv/Fm* (Fig. S4) in both the varieties. Effect of EDU on photosynthetic rate, stomatal conductance was also negatively reported in some previous studies on C3 crops (Pandey et al., 2014, 2015; Gupta et al., 2018). So, our results show EDU also do not effects gas exchange parameters in maize. Some of the previous studies show a net decrease in photosynthesis, stomatal conductance with increasing ozone concentrations (Samuelson and Kelly, 1996; Scagle and Andersen, 1997; Xu et al., 2019).

Since the decline in chlorophyll content is known to reflect the activation of leaf senescence (Munne-Bosch and Alegre, 2004), our results showed that 53 ppb of tropospheric ozone was able to induce the activation of senescence-related processes in control. Decrease in pigments content due to ozone stress was also observed in maize (Leitao et al., 2007; Li et al., 2017), as a result of oxidative stress in the chloroplast (Pellegrini et al., 2015). Chl a, b and total chlorophyll were reduced significantly in control than both the EDU treatment in the vegetative stage, whereas similar trend was observed for Chl a + b and total carotenoids, which was similar to those found by Singh et al. (2018). At the flowering stage, overall pigment content was decreased due to plant at the verge of senescence. Depletion in photosynthetic pigments implies a lowered capacity for light-harvesting, but in our study, it seems, EDU prolonged the senescence. It indicates control plants faced early senescence due to depletion of photosynthetic pigments than EDU treated. On the other hand, the Chl a/b ratio was increased by EDU in S1 and S2 whereas in PEHM5 it was only in the P2 at the flowering stage. No change in Chl a/b ratio was also occurred by ozone in Aleppo pine needles, indicating that Chl a, and b could also be equally depressed (Le Thiec and Manninen, 2003). The degradation of chloroplast absorbing pigments might be an adaptive response to limit the production of active oxygen species (AOS), mainly driven in chloroplasts by excess energy absorption in the photosynthetic apparatus, as suggested by Nyachiro et al. (2001) and Herbinger et al. (2002) as for drought-stressed plants. Increased total chlorophyll under EDU treatment was also reported in several crops viz. maize (Singh et al., 2018), mustard (Pandey et al., 2014), wheat (Gupta et al., 2018; Pandey et al., 2019) at the lucknow and nearby area.

The decline in ROS upon EDU treatment led to decreased peroxidation of membrane resulting in less foliar MDA equivalent content in S1 and S2. It shows SHM3031 is more EDU responsive to lipid peroxidation as compared to PEHM5 at the vegetative stage. Singh et al. (2018) also reported less lipid peroxidation in the case of sensitive varieties of maize with 200 EDU dose. An increase in ASA content in S1 at the vegetative and S2 at flowering stage likely enhanced antioxidant defense mechanism in SHM3031 as compared to PEHM5 under EDU treatment. At the flowering stage ozone concentrations was high, so

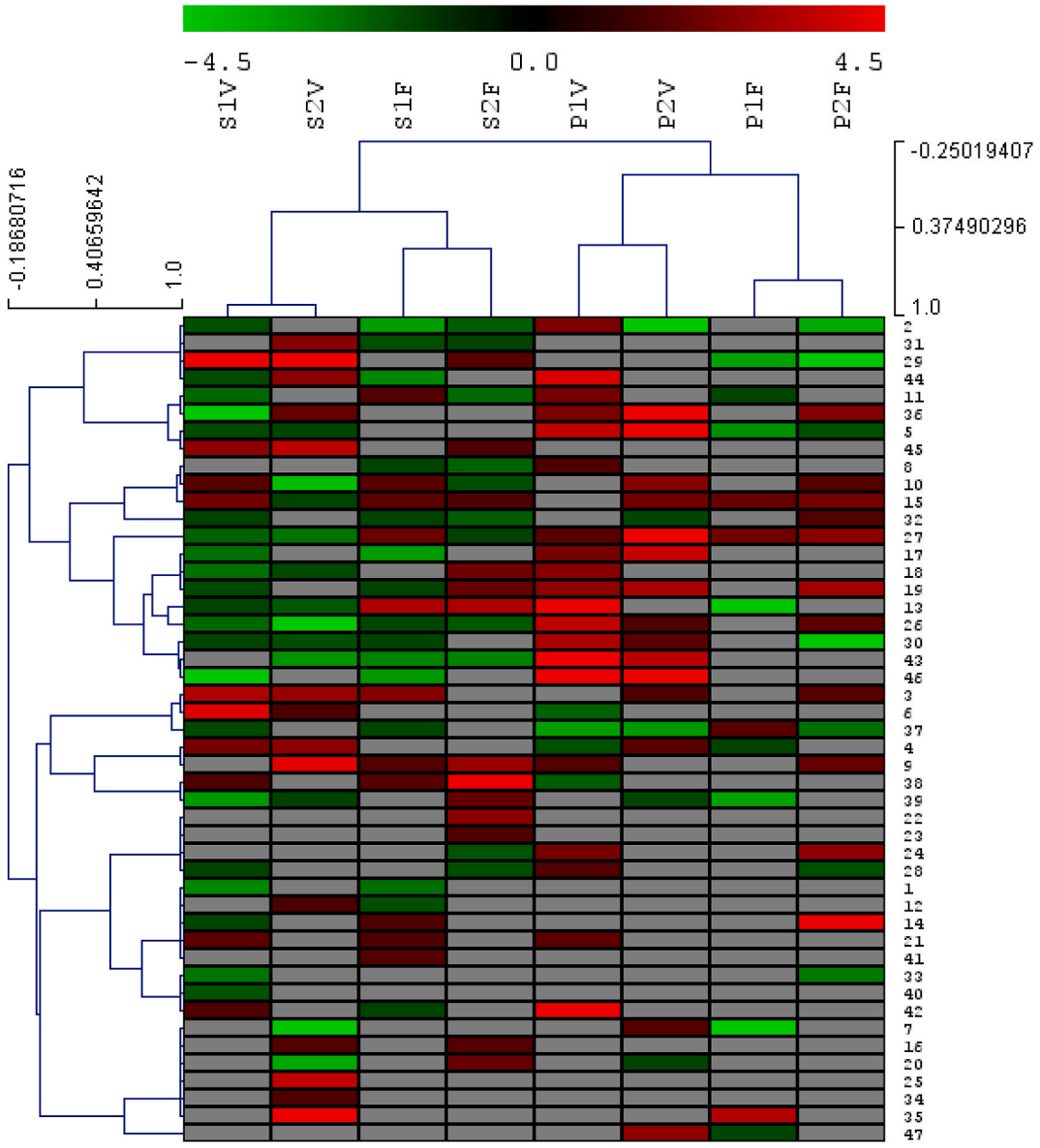

Fig. 5. Effect of EDU applications on proteins expression shown here by heat map at both the developmental stages in two maize varieties, SHM3031 and PEHM5 in leaves respectively. (S1V, SHM3031 50 ppm vegetative stage; P1V, PEHM5 50 ppm vegetative stages, numbers right side of heat map are protein numbers from Table 3) (Colour saturation: Red, increase expression of proteins; Green, decrease expression of proteins; Grey, no change in protein expression). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

greater protection of EDU was seen in SHM3031 at the flowering stage. An increase in ascorbic acid content under EDU treatment was also reported in maize (Singh et al., 2018), mustard (Pandey et al., 2014), and wheat (Gupta et al., 2018; Pandey et al., 2019). Higher GSSG content in both the varieties at the flowering stage depicts less EDU protection at both the doses. Variable responses for TGSH and reduced GSH between growth stage and varieties was also reported by Ranieri and Soldatini (1995) in *Phaseolus vulgaris*. These results are also well supported by Pandey et al. (2015) who found that EDU lowered the oxidative burden on rice plants by maintaining higher levels of GSH content compared to control (ambient O_3 treatment). A higher level of GSH content was also found in S1 and S2 at the vegetative stage depicting better EDU protection in SHM 3031 as compared to PEHM5.

EDU enhances the activity of SOD and CAT have previously been reported in snap bean (*Phaseolus vulgaris* L.) by Lee and Chen (1982) and Brunschon-Harti et al. (1995). SOD is first line of defense during ozone stress (Singh et al., 2010a). SOD and CAT activities in S1, S2, and P2 at both the stages were increased and maintained for better protection under high tropospheric ozone. It is well known that high ozone lowers the activity of SOD and CAT as reported in snap bean (Pitcher et al., 1992) and *Vigna radiata* L. (Singh et al., 2010c). SHM3031 performed better in both (APX and GR) the enzymatic activity in both the EDU treatment indicates it's more sensitivity to EDU and ozone. Increased GR activity was also recorded by Singh at al. (2018) in two maize varieties. It seems, EDU enhanced APX and GR activity in SHM3031 to maintain the enzymatic pool during high ozone stress. Increased APX activity in SHM3031 at the flowering stage also showed high use of ascorbate content during prevailing high ozone under EDU treatment (Gupta et al., 2018). It also proves APX maintained ascorbate content by scavenging ROS produced during high tropospheric O_3 levels in SHM3031. A similar increase in APX and GR activity was reported by Pandey et al. (2014) in two variety mustard. There were increased activities of ascorbate-glutathione (APX and GR) cycle enzymes together with high contents of ascorbate. Increased APX and GR activities with SOD and CAT surly helped in detoxification of ROS under EDU treatments.

4.2. Harvest and yield attributes

EDU has a protective role on the yield related parameters, it has already been established for different crop plants under high tropospheric and elevated levels of ozone (Rai et al., 2015; Gupta et al., 2018; Pandey et al., 2019; Yi et al., 2020). In the present study, a significant increase in inflorescence weight plant^{-1}, grain weight plant^{-1}, 1000 grain weight, grain no. plant^{-1} were recorded mostly with 50 ppm of EDU dose, depicting EDU protection in maize (Table 2 & Fig. S5). It was mainly because EDU helped to increase the size and number of grains after its application. It shows accumulation of biomass was comparatively low as grain numbers and grain weight increased during the maturation stage led to increased yield under both the doses of EDU treatment in SHM3031 (Fig. S5). Increase in 1000 grain weight was also found under EDU treatment two varieties of wheat (Gupta et al., 2018). Wang et al. (2007) conducted a similar study in China and showed increase in yield parameters in wheat and rice under EDU treatment. Grain weight and oil content of two mustard varieties viz. Kranti and "Peela sona" were reported to be increased by the EDU application (Pandey et al., 2014). A similar report was also shown by two maize cv. Buland and Prakash with 200 and 400 EDU dose (Singh et al., 2018). It shows SHM3031 (sensitive variety) have better EDU protection rather than a PEHM5 (tolrent variety) under high tropospheric ozone.

4.3. Proteomic response

It has been shown that Rubisco large subunit (Protein no. 4) (Table 3) can be cleaved by ROS (Luo et al., 2002). As higher abundance of Rubisco LSU was found at the vegetative stage in both the varieties under EDU treatment. Higher Rubisco abundance depicts lesser ROS production under EDU treatment (Gupta et al., 2018). In contrast, decreased expression of Rubisco (LSU and SSU) under high ozone exposure was reported in maize (Leitao et al., 2007). This shows ozone negatively affects photosynthetic machinery. We assume that EDU increases Rubisco activity, but it was not enough to reflect at the physiological level (Fig. S4), as reported in the case of wheat (Gupta et al., 2018). Increased Rubisco activase (Protein no. 3) showed a positive response with EDU treatments. Rubisco activase is involved in PS-II and directly participate in photosynthesis, their up-regulation showed a positive sign in EDU protection. Rubisco activase is required for the activation of Rubisco to perform its catalytic activity during carbon assimilation (Portis et al., 1986). Oxygen-evolving proteins (OEE) (Protein no. 5) play a crucial role in photosynthesis by controlling the O_2 evolution from water splitting complex and maintaining the stability of photosystem II (PSII) (Callahan et al., 1986; Sugihara et al., 2000). Decreased expression of OEE in S1, S2 (vegetative stage), P1, and P2 (flowering stage) showed less effect of EDU. While, in P1 and P2 (vegetative stage) increase was observed showing requirement for repairing protein damage caused by dissociation and for retaining the formation of oxygen. Torres et al. (2007) and Bohler et al. (2007) reported a decrease in OEE protein in their experiment under higher O_3 exposure.

Carbon metabolism proteins were differentially expressed in both maize varieties in response to both the EDU treatments. Glycolysis is a metabolic pathway that oxidizes glucose to generate ATP, reductant, and pyruvate (Plaxton, 1996). GAPDH (Protein no. 13) enzyme is involved in glycolysis and converts D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphates and maintains cellular ATP balance. GAPDH increased activity in S1 and S2 at flowering stage showed optimum balance of cellular ATP under high tropospheric ozone. Decrease in its expression under ozone fumigation experiments were already recorded in rice (Agrawal et al., 2002), and in wheat (Sarkar et al., 2010). Other proteins involved in carbon metabolism like fructose-bisphosphate aldolase (chloroplastic) (Protein no. 11), Malate dehydrogenase (Cytosolic) (Protein no. 10), phosphoglycerate kinase (Protein no. 15) and enolase (Protein no. 17) are the part of the Calvin cycle and glycolysis and their increased expression showed that they helped in starch accumulation resulting in higher biomass during flowering and maturation stage under EDU treatments in SHM3031. PGK (Protein no. 15) has catalytic activity, an increase in its activity in both the varieties showed better EDU protection during high tropospheric ozone (Ahmad Khan et al., 2013). Several research groups reported that PGK enhances dehydration-tolerance in osmotic-stressed banana meristem (Carpentier et al., 2007), and is induced in a short-period salt-stressed rice leaf lamina (Parker et al., 2006). Although PGK is related to the generation of energy, the reported results suggest that PGK may be implicated with O_3-tolerance in maize. A differential expression of FBPase (Protein no. 11) during the flowering stage in SHM3031 suggests that there was less EDU protection and more O_3 stress (Torres et al., 2007). FBPase is integral part of the Calvin cycle and regulate starch synthesis. Its increased expression in S2 at the flowering stage showed positive response of EDU on carbon metabolism in SHM3031. TK (Protein no. 19) has an important role in pentose phosphate pathways shunt and its increased activity showed a positive role in starch accumulation in SHM3031 and PEHM5 as both the varieties had better yield in EDU treatment. Carbonic anhydrase (CA) (Protein no. 23) is an essential part of inorganic carbon pool and its assimilation by the cell (Fukuzawa et al., 1992) and was increased in S2 at flowering stage. EDU mediated increased in CA suggest better assimilation of inorganic carbon leads to better biomass in SHM3031.

PEPCK (Protein no. 21) is an important enzyme which activates PEPC via phosphorylation. C4 plants must maintain a malate gradient to provide a carbon flow from the mesophyll, to the cells of the bundle sheath (Leegood, 1985). In our study increase in PEPCK expression in S1 at both stages and P1 at the vegetative stage provided better maintenance of malate gradient under EDU treatment in both varieties. This provided higher carbon assimilation leading to more biomass accumulation in SHM3031. Increased NADP-ME (Protein no. 9) activity leads to increased decarboxylation rate and higher malate consumption. The higher decarboxylation rate in the bundle sheath cells may facilitated by EDU positively influence the total photosynthesis rate (Calvin-Cycle) and ultimately more CO_2 fixation in SHM3031. Pyruvate phosphate dikinase (PPDK) (Protein no. 25) enzyme has been regarded as a putative rate-limiting factor for C4 photosynthesis (Beyel and Bruggemann, 2005; Dias and Brüggemann, 2007; Alfonso and Brüggemann, 2012). Its increases expression in S2 at the vegetative stage showed better C4 photosynthesis in SHM3031 under EDU treatment. Efficient mobilization of metabolites like pyruvate from old to young leaves for plant survival were facilitated through PPDK in the case of *Arabidopsis* (Taylor et al., 2007). NADP malate dehydrogenase (Protein no. 10) was increased in S1 and P2 at both the stage while decreased in S2 at both the stage. This enzyme along with PPDK and PEPCK are utilized in mesophyll for efficient fixation of CO_2 into malate via the carboxylation stage of the C4 pathway. Differential expression in case of SHM3031 of NADP malate dehydrogenase may have provided controlled fixation of CO_2 into malate under EDU treatment.

ATP synthase groups of proteins involved in the electron transport chain in chloroplast to generate energy. Enhanced activity of ATP synthase α and β subunit were found in elevated ozone conditions (Ahmad Khan et al., 2013). ATP synthase alpha (Protein no. 26), beta (Protein no. 27), and gamma (Protein no. 28) were decreased in SHM3031 and have increased expression in PEHM5 at both the stages. We predict, the energy requirement in PEHM5 to cope higher ozone stress was high because of less EDU protection. Whereas in SHM3031 less requirement of energy metabolism showed less production of ROS with 50 and 200 ppm of EDU dose. As SHM3031 is EDU responsive, these proteins were mostly down regulated, explaining the reduction in oxidative stress. This results again proved SHM3031 more EDU responsive than PEHM5 in present study. Different subunits of ATP synthase e.g. gamma was decreased in expression under ozone fumigation experiments (Agrawal et al., 2002; Sarkar et al., 2010).

High tropospheric O_3 can repress protein synthesis (Gupta et al.,

2018) and it was expected that EDU would protect this process. A highly significant increase in Chloroplast protein synthase (Protein no. 29) was found only in S1 and S2 where it increased by 23 and 42 times, respectively while it decreased in P1 and P2. Increased in Chloroplast protein synthase with both the EDU doses indicates N_2 mobilization during grain formation which was reflected in yield in SHM3031.

Increased, Ftsh (Protein no. 35) protease in S2 at the vegetative stage as it is integral part of thylakoid membrane (Olson, 1998). These results suggest EDU helped to provided ATP binding and cysteine t-RNA ligation during protein synthesis in SHM3031 at 200 ppm dose. Elongation Factor- Tu (Protein no. 30) promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes. An increase in its activity in P1 and P2 at the vegetative stage helped in protein biosynthesis in PEHM5. Its decreased abundance in case of SHM3031 showed less EDU impact for this protein. Chaperonin (Protein no. 32) and HSP 70 kDa (Protein no.43) are involved in protein folding and their decreased expression was quite interesting for us although both were increased in P2 at the flowering stage. It stabilizes the native structure of a protein by acting as a chaperone (Torres et al., 2007). An increase in its activity would have provided greater stability to proteins in adverse conditions for PEHM5 at the flowering stage. Lipoxygenase (Protein no. 46) is involved in fatty acid oxidation (Porta and Rocha-Sosa, 2002). Its decreased expression in S1 at both stages showed less oxidation of fatty acid under EDU treatment.

EDU protects plants by enhancing antioxidative defense systems as reviewed by Oksanen et al. (2013). APX (Protein no. 38) expression was increased in SHM3031 at both the developmental stage. It is an important enzyme in combating oxidative stress in plants (Agrawal et al., 2002; Sarkar et al., 2010; Pandey et al., 2014, 2015). Increase in APX activity in SHM3031 depicts better EDU protection of ROS born stress. Thioredoxin (Protein no. 41) was also increased in S1 at flowering stage. Thioredoxin role is to remove ROS and redox regulation of chloroplast enzymes during oxidative stress. Its increased expression in SHM3031 showed better EDU protection under high tropospheric ozone. Decrease in SOD (Protein no. 40), Harpin binding protein (Protein no. 42), and HSP (Protein no. 43) in SHM3031were quite interesting as several authors have reported increase in response to EDU (Singh et al., 2009; Pandey et al., 2014). Peroxiredoxin (Protein no. 44) protein helps in combating oxidative stress. It showed differential expression depicting less EDU protection in SHM3031. Moreover, this protein is translocated from intracellular to the cell walls by heat, H_2O_2 treatment as well as pathogen infection (Vallelian-Bindschedler et al., 1998).

Decrease in germin-like protein (Protein no. 37) under EDU treatment may cause by its translocation to the cell wall by O_3 stress. Its translocation to cell wall may inhibited O_3 entry inside the cell in SHM3031. Actin family protein (Protein no. 45) was increased in SHM3031 at both the developmental stages showed EDU provided better strength of the cellular skeleton under high tropospheric ozone.

This is the first systematic study of the extensive changes in maize protein profiling after EDU treatment, an antiozonant, providing basic information of the protein's expression (Table 3) under high tropospheric ozone. It has been recently argued that, if it is necessary to determine the relatively long-term or accumulated effects of pollutants on plants, it may be more suitable to determine regulatory protein expression. Proteins such as chloroplast protein synthesis, FNR, HSP, germin like protein, and APX have shown their regulatory role in coping higher tropospheric ozone and enhanced their activities under EDU treatment.

4.4. Comparing EDU response in two maize varieties

In SHM3031 increased antioxidative enzymes in response to tropospheric ozone, the antioxidative ability of this variety was enough to protect ROS prompted damages under both the EDU treatment. EDU prompted signaling (changes) might repaired proteins involved in light reactions in SHM3031. These proteins may have damaged during enhanced ROS formation in SHM3031 but protected by EDU. Light reaction, through PS-II and PS-I, produced enough ATP and reducing power under EDU treatment, which was needed for faster translocation of proteins involved in senescence of leaves in high tropospheric ozone. A consequent increase in Calvin cycle related enzymes led to the optimum fixation of metabolic CO_2 after C4 fixation in SHM3031. Consequently, yield induction in SHM3031 were due to inhibition of metabolites translocation and increased production of carbohydrate. In, SHM3031 defense-related proteins, for instance, Harpin binding proteins, germin like protein, APX, and SOD could minimize ozone prompt phytotoxicity and resulted in increased yield under EDU treatment. Although catalase activity was also increased in PEHM5 at both the developmental stages, but only one enzyme activities was not enough, so not reflected in yield. Proteins related to the light reaction and the Calvin cycle were decreased in PEHM5 under EDU treatment. Moreover, PEHM5 could utilizes the glycolytic enzymes such as PGK and enolase for translocation of N_2 metabolites from leaves to grain. Therefore, the grain yield of PEHM5 was not affected under EDU treatment.

5. Conclusion

In Summary, under conditions with high tropospheric ozone as those prevailing in Lucknow area. EDU conferred an important protection to sensitive maize variety, increasing morpho-physiological performance, maintaining an enhanced antioxidant capacity, and finally leading to higher biomass and/or grain yields. The magnitude of variations in biochemical parameters varied with stages and varieties. SHM3031 showed more induction in non-enzymatic antioxidants with respect to PEHM5 at both the developmental stages. Proteins involved in the C4- Cycle were better regulated by EDU, led to optimum fixation of atmospheric and metabolic CO_2 in maize during ozone stress. Proteins involved in defense mechanism also provided better protection through their optimum expression under EDU treatment. The key factor inducing the varieties differences in EDU responses is that one is sensitive and the other is tolerant. Our results proved, these two EDU doses 50 and 200 ppm were satisfactory in the IGP region to asses changes to prevailing high tropospheric ozone during rainy season. Besides better understanding the mechanisms of EDU effects on this C4 plant, these results are relevant for assessing the O_3 risk to this crop, especially in Asia, and eventually, for including O_3 influence in crop productivity models. The ozone problem in India has been partly covered by other urgent air quality issues such as the high levels of particulate matter, but current levels represent a serious threat both for human health and for food security. Measures to reduce ozone pollution in India are urgently needed.

Author's contribution

VP, and SKG designed the experiment. SKG, MS, and VKM did yield and physiological work. BM did the enzymatic work. SKG, MS , VKM, and FD did proteomic work. SKG, and VP analyzed the data and wrote the paper. SKG, ZJL, and VP revised the manuscript. All the authors approved the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Authors are grateful to Director, CSIR-NBRI for providing necessary facilities. SKG and MS are grateful to CSIR and UGC, respectively for

senior research fellowship. Funding for this work was provided by Council of Scientific & Industrial Research (CSIR), New Delhi, India [Project no. PSC112].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.05.037.

References

Agathokleous, E., Koike, T., Watanabe, M., Hoshika, Y., Saitanis, C.J., 2015. Ethylenediurea (EDU), an effective phytoproctectant against O_3 deleterious effects and a valuable research tool. J. Agric. Meteorol. 71, 185–195.

Agrawal, G.K., Rakwal, R., Yonekura, M., Kubo, A., Saji, H., 2002. Proteome analysis of differentially displayed proteins as a tool for investigating ozone stress in rice (*Oryza sativa* L.) seedlings. Proteomics 2 (8), 947–959.

Agrawal, S.B., Singh, A., Rathore, D., 2005. Role of ethylenediurea (EDU) in assessing impact of ozone on *Vigna radiate* L. plants in a suburban area of Allahabad (India). Chemosphere 61, 218–228.

Ahmad Khan, N., Komatsu, S., Sawada, H., Nouri, M.Z., Kohno, Y., 2013. Analysis of proteins associated with ozone stress response in soybean cultivars. Protein Pept. Lett. 20 (10), 1144–1152.

Alfonso, S.U., Brüggemann, W., 2012. Photosynthetic responses of a C3 and three C4 species of the genus *Panicum* (s.l.) with different metabolic subtypes to drought stress. Photosynth. Res. 112 (3), 175–191.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24 (1), 1.

Ashmore, M.R., 2005. Assessing the future global impacts of ozone on vegetation. Plant Cell Environ. 28, 949–964.

Ashrafuzzaman, M., Lubna, F.A., Holtkamp, F., Manning, W.J., Kraska, T., Frei, M., 2017. Diagnosing ozone stress and differential tolerance in rice (*Oryza sativa* L.) with ethylenediurea (EDU). Environ. Pollut. 230, 339–350.

Astorino, G., Margani, I., Tripodo, P., Manes, F., 1995. The response of *Phaseolus vulgaris* L. cv. Lit. to different dosages of the anti-ozonant ethylenediurea (EDU) in relation to chronic treatment with ozone. Plant Sci. 111 (2), 237–248.

Avnery, S., Mauzerall, D.L., Liu, J., Horowitz, L.W., 2011. Global crop yield reductions due to surface ozone exposure: 1. Year 2000 crop production losses and economic damage. Atmos. Environ. 45, 2284–2296.

Basahi, J.M., Ismail, I., Haiba, N.S., Hassan, I.A., Lorenzini, G., 2016. Assessing ambient ozone injury in olive (*Olea europaea* L.) plants by using the antioxidant ethylenediurea (EDU) in Saudi Arabia. Environ. Monit. Assess. 188 (6).

Bayer Jr., W.F., Fridovich, I., 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal. Biochem. 161, 559–566.

Beyel, V., Bruggemann, W., 2005. Differential inhibition of photosynthesis during preflowering drought stress in *Sorghum bicolor* genotypes with different senescence traits. Physiol. Plantarum 124 (2), 249–259.

Bhatia, A., Tomer, R., Kumar, V., Singh, S.D., Pathak, H., 2012. Impact of Tropospheric Ozone on Crop Growth and Productivity–A Review.

Bohler, S., Bagard, M., Oufir, M., Planchon, S., Hoffmann, L., Jolivet, Y., Hausman, J.F., Dizengremel, P., Renaut, J., 2007. A DIGE analysis of developing poplar leaves subjected to ozone reveals major changes in carbon metabolism. Proteomics 7 (10), 1584–1599.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brunschon-Harti, S., Fangmeier, A., Jager, H., 1995. Effects of ethylenediurea and ozone on the antioxidative system in beans (*Phaseolus vulgaris* L.). Environ. Pollut. 90, 95–103.

Burney, J., Ramanathan, V., 2014. Recent climate and air pollution impacts on Indian agriculture. Proc. Natl. Acad. Sci. U.S.A. 111 (46), 16319–16324.

Callahan, F.E., Becker, D.W., Cheniae, G.M., 1986. Studies on the photoactivation of the water-oxidizing enzyme II.Characterization of weak light photoinhibition of PSII and its light-induced recovery. Plant Physiol. 82 (1), 261–269.

Carpentier, S.C., Witters, E., Laukens, K., Van Onckelen, H., Swennen, R., Panis, B., 2007. Banana (*Musa* spp.) as a model to study the meristem proteome: acclimation to osmotic stress. Proteomics 7 (1), 92–105.

Chen, G., 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, 987–998.

Cho, K., Shibato, J., Agrawal, G.K., Jung, Y.H., Kubo, A., Jwa, N.S., Tamogami, S., Satoh, K., Kikuchi, S., Higashi, T., Kimura, S., Saji, H., Tanaka, Y., Iwahashi, H., Masuo, Y., Rakwal, R., 2008. Integrated transcriptomic, proteomics, and metabolomics analyses to survey ozone response in the leaves of rice seedling. J. Proteome Res. 7, 2980–2998.

De Leeuw, F., Van Zantvoort, E., 1997. Mapping of exceedances of ozone critical levels for crops and forest trees in The Netherlands: preliminary results. Environ. Pollut. 96, 89–98.

Deb Roy, S., Beig, G., Ghude, S.D., 2009. Exposure-plant response of ambient ozone over the tropical Indian region. Atmos. Chem. Phys. 9, 5253–5260.

Dias, M.C., Brüggemann, W., 2007. Differential inhibition of photosynthesis under drought stress in *Flaveria* species with different degrees of development of the C4 syndrome. Photosynthetica 45 (1), 75–84.

Emberson, L.D., Buker, P., Ashmore, M.R., Mills, G., Jackson, L.S., Agrawal, M., Atikuzzaman, M.D., Cinderby, S., Engardt, M., Jamir, C., Kobayashi, K., Oanh, N.T.K., Quadir, Q.F., Wahid, A., 2009. A comparison of North American and Asian exposure – response data for ozone effects on crop yields. Atmos. Environ. 43, 1945–1953.

Feng, Z., Wang, S., Szantoi, Z., Chen, S., Wang, X., 2010. Protection of plants from ambient ozone by applications of ethylenediurea (EDU): a meta-analytic review. Environ. Pollut. 158, 3236–3242.

Fiscus, E.L., Booker, F.L., Burkey, K.O., 2005. Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. Plant Cell Environ. 28 (8), 997–1011.

Fukuzawa, H., Suzuki, E., Komukai, Y., Miyachi, S., 1992. A gene homologous to chloroplast carbonic anhydrase (icfA) is essential to photosynthetic carbon dioxide fixation by *Synechococcus* PCC7942. Proc. Natl. Acad. Sci. Unit. States Am. 89 (10), 4437–4441.

Gillespie, K.M., Ainsworth, E.A., 2007. Measurement of reduced, oxidized and total ascorbate content in plants. Nat. Protoc. 2 (4), 871–874.

Griffith, O.W., 1980. Determination of glutathione and glutathione disulphide using glutathione reductase and 2-vinyl pyridine. Anal. Biochem. 106, 207–212.

Gupta, S.K., Sharma, M., Majumder, B., Maurya, V.K., Lohani, M., Deeba, F., Pandey, V., 2018. Impact of Ethylene diurea (EDU) on growth, yield and proteome of two winter wheat varieties under high ambient ozone phytotoxicity. Chemosphere 196, 161–173.

Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198.

Herbinger, K., Tausz, M., Wonisch, A., Soja, G., Sorger, A., Grill, D., 2002. Complex interactive effects of drought and ozone stress on the antioxidant defense systems of two wheat cultivars. Plant Physiol. Biochem. 40, 691–696.

Holland, M., Kinghorn, S., Emberson, L., Cinderby, S., Ashmore, M., Mills, G., Harmens, H., 2006. Development of a framework for probabilistic assessment of the economic losses caused by ozone damage to crops in Europe. Part of the UNECE International Cooperative Programme on Vegetation. Contract Report EPG 1/3/205. CEH Project No: C02309, NEW.

Intergovernmental Panel on Climate Change (IPCC), 2013. Fifth assessment report. http://www.ipcc.ch/report/ar5/index.shtml.

Koistinen, K.M., Hassinen, V.H., Gynther, P.A.M., Lehesranta, S.J., Keinänen, S.I., et al., 2002. Birch PR-10c is induced by factors causing oxidative stress but appears not to confer tolerance to these agents. New Phytol. 155, 381–391.

Le Thiec, D., Manninen, S., 2003. Ozone and water deficit reduced growth of Aleppo pine seedlings. Plant Physiol. Biochem. 41, 55–63.

Lee, E.H., Chen, C.M., 1982. Studies on the mechanisms of ozone tolerance: cytokininlike activity of N-[2-(2-oxo-1-imidazolidinyl) ethyl]-N'-phenylurea, a compound protecting against ozone injury. Physiol. Plantarum 56 (4), 486–491.

Leegood, R.C., 1985. The intercellular compartmentation of metabolites in leaves of *Zea mays* L. Planta 164 (2), 163–171.

Lehesranta, S.J., Davies, H.V., Shepherd, L.V.T., Nunan, N., McNicol, J.W., Auriola, S., Koistinen, K.M., Suomalainen, S., Kokko, H.I., Kärenlampi, S.O., 2005. Comparison of tuber proteomes of potato varieties, landraces, and genetically modified lines. Plant Physiol. 138, 1690–1699.

Leitao, Louis, Delacôte, Emilien, Dizengremel, Pierre, Le Thiec, Did ier, Biolley, Jean-Philippe, 2007. Assessment of the impact of increasing concentrations of ozone on photosynthetic components of MAIZE (*Zea mays* L.), a C4 plant. Environ. Pollut. 146 (1 March 2007), 5–8.

Li, P., Feng, Z.Z., Catalayud, V., Yuan, X.Y., Xu, Y.S., Paoletti, E., 2017. A meta-analysis on growth, physiological, and biochemical responses of woody species to ground-level ozone highlights the role of plant functional types. Plant Cell Environ. 40, 2369–2380.

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350–382.

Long, S.P., Naidu, S.L., 2002. Effects of oxidants at the biochemical, cell and physiological levels, with particular reference to ozone. In: Bell, J.N.B., Treshow, M. (Eds.), Air Pollution and Plant Life, second ed. Wiley, West Sussex, UK), pp. 69–88.

Luo, S., Ishida, H., Makino, A., Mae, T., 2002. Fe^{2+}-catalyzed site-specific cleavage of the large subunit of ribulose 1, 5-bisphosphate carboxylase close to the active site. J. Biol. Chem. 277 (14), 12382–12387.

Manning, W.J., Paoletti, E., Sandermann Jr., H., Ernst, D., 2011. Ethylenediurea (EDU): a research tool for assessment and verification of the effects of ground level ozone on plants under natural conditions. Environ. Pollut. 159, 3283–3293.

McKee, I.F., Mulholland, B.J., Craigon, J., Black, C.R., Long, S.P., 2000. Elevated concentrations of atmospheric CO_2 protect against and compensate for O_3 damage to photosynthetic tissues of field-grown wheat. New Phytol. 146 (3), 427–435.

Mills, G., Sharps, K., Simpson, D., Pleijel, H., Frei, M., Burkey, K., Emberson, L., Uddling, J., Broberg, M., Feng, Z.Z., Kobayashi, K., Agrawal, M., 2018. Closing the global ozone yield gap: quantification and cobenefits for multistress tolerance. Global Change Biol. 24, 4869–4893.

Munne-Bosch, S., Alegre, L., 2004. Die and let live: leaf senescence contributes to plant survival under drought stress. Funct. Plant Biol. 31, 203–216.

Nyachiro, J.M., Briggs, K.G., Hoddinott, J., Johnson-Flanagan, A.M., 2001. Chlorophyll content, chlorophyll fluorescence and water deficit in spring wheat. Cereal Res. Commun. 29 (1/2), 135–142.

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.

Olson, J.M., 1998. Chlorophyll organization and function in green photosynthetic bacteria. Photochem. Photobiol. 67 (1), 61–75.

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, A.K., Majumder, B., Keski-Saari, S., Kontunen-Soppela, S., Pandey, V., Oksanen, E., 2019. High variation in resource allocation strategies among 11 Indian wheat (*Triticum aestivum*) cultivars growing in high ozone environment. Climate 7 (2), 23.
Paoletti, E., Contran, N., Manning, W.J., Castagna, A., Ranieri, A., Tagliaferro, F., 2008. Protection of ash (*Fraxinus excelsior*) trees from ozone injury by ethylenediurea (EDU): roles of biochemical changes and decreased stomatal conductance in enhancement of growth. Environ. Pollut. 155, 464–472.
Paoletti, E., Contran, N., Manning, W.J., Ferrara, A.M., 2009. Use of antiozonant ethylenediurea (EDU) in Italy: verification of the effects of ambient ozone on crop plants and trees and investigation of EDU's mode of action. Environ. Pollut. 157, 1453–1460.
Parker, R., Flowers, T.J., Moore, A.L., Harpham, N.V., 2006. An accurate and reproducible method for proteome profiling of the effects of salt stress in the rice leaf lamina. J. Exp. Bot. 57 (5), 1109–1118.
Pellegrini, E., Francini, A., Lorenzini, G., Nali, C., 2015. Ecophysiological and antioxidant traits of Salvia officinalis under ozone stress. Environ. Sci. Pollut. Res. 22, 13083–13093.
Peng, J., Shang, B., Xu, Y., Feng, Z., Calatayud, V., 2020. Effects of ozone on maize (*Zea mays* L.) photosynthetic physiology, biomass and yield components based on exposure-and flux-response relationships. Environ. Pollut. 256, 1134–1166.
Pitcher, L.H., Brennan, E., Zilinskas, B.A., 1992. The antiozonant ethylenediurea does not act via superoxide dismutase induction in bean. Plant Physiol. 99 (4), 1388–1392.
Plaxton, W.C., 1996. The organization and regulation of plant glycolysis. Annu. Rev. Plant Biol. 47 (1), 185–214.
Porta, H., Rocha-Sosa, M., 2002. Plant lipoxygenases. Physiological and molecular features. Plant Physiol. 130 (1), 15–21.
Portis, A.R., Salvucci, M.E., Ogren, W.L., 1986. Activation of ribulosebisphosphate carboxylase/oxygenase at physiological CO_2 and ribulosebisphosphate concentrations by Rubisco activase. Plant Physiol. 82 (4), 967–971.
Rai, R., Agrawal, M., Choudhary, K.K., Agrawal, S.B., Emberson, L., Büker, P., 2015. Application of ethylene diurea (EDU) in assessing the response of a tropical soybean cultivar to ambient O_3: nitrogen metabolism, antioxidants, reproductive development and yield. Ecotoxicol. Environ. Saf. 112, 29–38.
Ranieri, A., Soldatini, G., 1995. Detoxificant systems in bean plants grown in polluted air: effects of the antioxidant EDU. Agric. Mediterr. 125, 375–386.
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, 125–136.
Samuelson, L.J., Kelly, J.M., 1996. Carbon partitioning and allocation in northern red oak seedlings and mature trees in response to ozone. Tree Physiol. 16, 853–858.
Sarkar, A., Rakwal, R., Bhushan Agrawal, S., Shibato, J., Ogawa, Y., Yoshida, Y., Kumar Agrawal, G., Agrawal, M., 2010. Investigating the impact of elevated levels of ozone on tropical wheat using integrated phenotypical, physiological, biochemical, and proteomics approaches. J. Proteome Res. 9 (9), 4565–4584.
Scagle, C.F., Andersen, C.P., 1997. Seasonal changes in root and soil respiration of ozone-exposed ponderosa pine (*Pinus ponderosa*) grown in different substrates. New Phytol. 136, 627–643.
Schaub, M., Calatayud, V., Ferreti, M., Brunialti, G., L€ovblad, G., Krause, G., Sanz, M.J., 2010. Assessment of Ozone Injury. Manual on Methods and Criteria for Harmonized Sampling, Assessment, Monitoring and Analysis of the Effects of Air Pollution on Forests. 1e22. UNECE ICP Forests Programme Co-ordinating Centre, Hamburg. Available at: http://icp-forests.net/.
Sharma, M., Gupta, S.K., Majumder, B., Maurya, V.K., Deeba, F., Alam, A., Pandey, V., 2018. Proteomics unravel the regulating role of salicylic acid in soybean under yield limiting drought stress. Plant Physiol. Biochem. 130, 529–541.
Simpson, D., Arneth, A., Mills, G., Solberg, S., Uddling, J., 2014. Ozone e the persistent menace: interactions with the N cycle and climate change. Curr. Opin. Environ. Sustain. 9, 9–19.
Singh, S., Agrawal, S., 2009. Use of ethylene diurea (EDU) in assessing the impact of ozone on growth and productivity of five cultivars of Indian wheat (*Triticum aestivum* L.). Environ. Monit. Assess. 159, 125–141.
Singh, S., Agrawal, S., Singh, P., Agrawal, M., 2010a. Screening three cultivars of *Vigna mungo* L. against ozone by application of ethylenediurea (EDU). Ecotoxicol. Environ. Saf. 73, 1765–1775.
Singh, S., Agrawal, S.B., Agrawal, M., 2009. Differential protection of ethylenediurea (EDU) against ambient ozone for five cultivars of tropical wheat. Environ. Pollut. 157, 2359–2367. https://doi.org/10.1016/j.envpol.2009.03.029.
Singh, S., Agrawal, M., Agrawal, S.B., Emberson, L., Buker, P., 2010c. Use of ethylenediurea for assessing the impact of ozone on mungbean plants at a rural site in a dry tropical region of India. Int. J. Environ. Waste Manag. 5, 125–139.
Singh, A.A., Singh, S., Agrawal, M., Agrawal, S.B., 2015. Assessment of ethylene diurea-induced protection in plants against ozone phytotoxicity. Reviews of Environmental Contamination and Toxicology ume 233. Springer International Publishing, pp. 129–184.
Singh, A.A., Chaurasia, M., Gupta, V., Agrawal, M., Agrawal, S.B., 2018. Responses of *Zea mays* L. cultivars 'Buland'and 'Prakash'to an antiozonant ethylene diurea grown under ambient and elevated levels of ozone. Acta Physiol. Plant. 40 (5), 1–15.
Smith, I.K., Vierheller, T.L., Thorne, C.A., 1988. Assay of glutathione reductase in crude tissue homogenates using 5,50-dithiobis(2-nitrobenzoic acid). Anal. Biochem. 175, 408–413.
Sugihara, K., Hanagata, N., Dubinsky, Z., Baba, S., Karube, I., 2000. Molecular characterization of cDNA encoding oxygen evolving enhancer protein 1 increased by salt treatment in the mangrove *Bruguiera gymnorrhiza* L. Plant Cell Physiol. 41 (11), 1279–1285.
Taylor, L., Nunes-Nesi, A., Parsley, K., et al., 2007. Abscisic acid and abiotic stress signaling. Plant Signal. Behav. 2 (3), 135–138.
Tiwari, S., Agrawal, M., 2009. Protection of palak (*Beta vulgaris* L. var All green) plants from ozone injury by ethylenediurea (EDU): roles of biochemical and physiological variations in alleviating the adverse impacts. Chemosphere 75, 1492–1499.
Tiwari, S., Agrawal, M., 2010. Effectiveness of different EDU concentrations in ameliorating ozone stress in carrot plants. Ecotoxicol. Environ. Saf. 73, 1018–1027.
Torres, N.L., Cho, K., Shibato, J., Hirano, M., Kubo, A., Masuo, Y., Iwahashi, H., Jwa, N.S., Agrawal, G.K., Rakwal, R., 2007. Gel-based proteomics reveals potential novel protein markers of ozone stress in leaves of cultivated bean and maize species of Panama. Electrophoresis 28 (23), 4369–4381.
Vallelian-Bindschedler, L., Mösinger, E., Métraux, J.P., Schweizer, P., 1998. Structure, expression and localization of a germin-like protein in barley (*Hordeum vulgare* L.) that is insolubilized in stressed leaves. Plant mol. Boil. 37 (2), 297–308.
Wang, X.K., Zheng, Q.W., Yao, F.F., Chen, Z., Feng, Z.Z., Manning, W.J., 2007. Assessing the impact of ambient ozone on growth and yield of a rice (*Oryza sativa* L.) and a wheat (*Triticum aestivum* L.) cultivar grown in the Yangtze Delta, China, using three rates of application of ethylenediurea (EDU). Environ. Pollut. 148, 390–395.
Wheelock, Å.M., Wheelock, C.E., 2013. Trials and tribulations of 'omics data analysis: assessing quality of SIMCA-based multivariate models using examples from pulmonary medicine. Mol. Biosyst. 9 (11), 2589–2596.
Xu, Y.S., Feng, Z.Z., Shang, B., Dai, L.L., Uddling, J., Tarvainen, L., 2019. Mesophyll conductance limitation of photosynthesis in poplar under elevated ozone. Sci. Total Environ. 657, 136–145.
Yi, Y., Cai, Z., Yi, L., Fang, S., Zhang, Y., Yang, D., Wang, Y., Liang, M., Wang, M., 2020. Direct observations ofatmospheric transport and stratosphere-troposphere exchange from high-precision carbon dioxide and carbon monoxide profile measurements. Adv. Atmos. Sci. 37, 608–616. https://doi.org/10.1007/s00376-020-9227-2.