Evaluation of impact of tropospheric ozone on Gladiolus cultivars using Ethylenediurea

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Abstract: Tropospheric ozone (O_3) is one of the most damaging air pollutants negatively affecting trees and crops in terms of growth and yield. But there is sparse information on O_3 effects on ornamental plants. In the present study we used an antiozonant ethylenediurea (EDU) to examine the impact of ambient O_3 on ornamental cultivars of *Gladiolus* named Vedio and Tigerflame. The cultivars were planted under natural field condition and treated with foliar EDU spray (200 and 400 ppm) at 7 days interval. The plant sampling was done at vegetative and flowering stage. During the study period, average O_3 concentration was 60 ppb. The results showed that both EDU treated cultivars showed enhanced levels of chlorophyll, GSH, GR and CAT, especially at flowering phase. Lipid peroxidation level was significantly reduced in EDU treated plants. There were no changes in stomatal conductance and photosynthesis. Vase life of the florets was increased in comparison to non-EDU treated plants. This study showed that high ambient O_3 is responsible for the damage of this economically important ornamental crop and that more Gladiolus varieties need to be tested against high O_3 stress.

Key words: Antioxidant, EDU, Gladiolus, Ozone, Vase life

1. Introduction

Ambient ozone (O_3) is considered as one of the most widespread pollutant which is highly toxic to both vegetation and human health. O_3 is a secondary air pollutant whose concentration has been increasing steadily as a result of excessive fossil fuel consumption, use of nitrogen fertilizers along with increase in vehicular emissions, urbanization and population growth (Meehl et al., 2007). It has been estimated that between 2015 and 2050 there will be an increase of 20-25% in worldwide O_3 level and by 2100 the increase will be between 40-60% (Meehl et al., 2007). O_3 is a potent oxidant, which induces phytotoxicity and said to be one of the major factor for worldwide reduction in agriculture productivity thus posing a serious threat to food security and agro- economy of agriculture based countries such as India (Cho et al., 2011; Oksanen et al., 2013; Feng et al., 2015). It has been reported that the ambient O_3 levels are increasing especially in northern part of India (Oksanen et al., 2013). This has serious implications for Indo-Gangetic Plains region which lay in Northern India, the primary agriculture site of the country thus threatening the livelihood of Indian population.

Ozone enters into plant system through stomatal pores. Once inside the leaves, O_3 quickly reacts with various compounds to yield reactive oxygen species (ROS) like hydrogen peroxide, superoxide radical, hydroxyl radical etc. that oxidize plant tissues and results in impaired photosynthesis, protein and chlorophyll degradation and early senescence (Cho et al., 2011;Ainsworth et al., 2012). The physiological and metabolic alteration caused by O_3 induces oxidative stress leading to unwanted modifications in crop quality thus causing yield reduction. In order to scavenge the excess ROS production plants have well developed antioxidant defense system. The detoxification process is a complex network involving different types of metabolites, antioxidant and antioxidative enzymes like CAT, SOD, GR and reduced glutathione (GSH) (Cho et al., 2011).

Several techniques have been used to study the deleterious effects of ambient O_3 on various crop and trees species; though all the techniques have their own advantages and disadvantages (Manning and Krupa, 1992). The most important method used to assess impact of O_3 on plants is OTCs though it has several disadvantages like high maintenance and chamber effects. To overcome the disadvantages of chamber effects and to assess the responses under natural field condition, use of antiozonant EDU has been established as the most successful and convenient method (reviewed in Manning et al., 2011; Agathokleous et al., 2015). Ethylene diurea (EDU) has been extensively used as a chemical protectant against O_3 injury in various crops, trees and horticultural crops (Singh et al., 2015). Though the mode of mechanism of EDU is not very clear, several experiments have shown that EDU induces changes in the activities of antioxidants and antioxidative enzymes. Elevation in levels of antioxidative enzymes (SOD, GR, CAT, APX) by EDU were observed in different crops like potato, wheat, mustard (as reviewed in Singh et al.,2015) and rice (Pandey et al., 2015). This chemical in recent time has gained popularity as an alternative option to open top chambers in assessment of O_3 sensitivity of various crops and their cultivar specific responses in natural field conditions. EDU has been proven effective in screening out ozone tolerant and sensitive cultivars under ambient O_3 conditions especially in areas where electricity is limited (Pandey et al., 2014; 2015).

Gladiolus (*Gladiolus grandiflora*) belonging to family Iridaceae is a very popular ornamental plant and is also known as queen of bulbous flowers (Bhattacharjee and De, 2005) due to its magnificent inflorescence with florets of dazzling colours. Hence, it occupies a prime position in the floriculture industry of India with an estimated production of 106 crore cut flowers. Floral senescence is the major problem regarding the post harvest management of gladiolus cut flowers. Oxidative stress is one of the processes that regulate the senescence in gladiolus which is independent of ethylene pathway (Woltering and van Doorn, 1988). Although gladiolus is fine indicator of fluoride pollution and is also susceptible to salt and water stress (Hashish et al., 2015), no studies have been carried out in relation to O_3 stress. As O_3 induced ROS accelerates senescence, it is hypothesized that O_3 stress may play a role in accelerating petal senescence thus affecting its vase life and lowering the economic value. Globally, information regarding impact of O_3 and EDU on yield of various food crops such as wheat, maize, rice, mustard and soybean are available, though information on ornamental crops and on flower production is very limited. At present in Indian scenario there are no studies to assess the impact of O_3 induced toxicity on flower quality in any ornamental crops.

The present study is designed to explore the responses and sensitivity of two cultivars of *Gladiolus grandiflora* (Vedio and Tigerflame) towards O_3 on the basis of biochemical and post harvest quality (vase life) parameters. EDU application was done in order to ameliorate the negative impacts of O_3 in natural field condition in a highly urbanised area of Lucknow, Uttar Pradesh, India. Our hypothesis was that (i) ambient

O_3 concentrations are sufficiently high negatively affect gladiolus growth and vase life (ii) enhancement of antioxidative defense system by EDU in gladiolus could be an important factor in combating O_3 phytotoxicity. This work may be helpful in further screening of various cultivars of gladiolus and other economically important ornamental crops for tolerance against O_3 phytotoxicity in sites experiencing high O_3 concentrations.

2. Materials and methods

2.1.Experimental plot preparation and crop cultivation

The experimental plot was at the National Botanical Research Institute (CSIR-NBRI) (26° 55′ N, 80° 59′ E), Lucknow, India, situated 132 m above sea level. The plot of 20 m^2 sizewas divided into 6 subplots with size of 2*1.5 m^2. Before planting of corms (bulbs) the total plot was treated with 70 g m^{-2} of DAP (Diammonium phosphate) and potash murate along with 200 g m^{-2}neem cake and 10 g m^{-2}forate to prevent infection of the bulbs from soil microbes. The bulbs were treated with 0.2% bavistin for 15-20 minutes to prevent from fungal infection (Chandel and Raj, 2010). The distance between the rows was maintained at 30 cm while distance of 20-25cm was maintained between the bulbs planted.

Two commercially available cultivars of *Gladiolus grandiflora*, named Vedio and Tigerflame were selected for the present work. The cultivars varied in their flower colour with Vedio having light purple coloured flower while Tigerflame had orange-red colour flower. The corms of both the cultivars were obtained from Floriculture department of CSIR -NBRI and these cultivars are widely grown in IGP region.

2.2.EDU treatment

EDU was kindly provided by Prof. W.J. Manning, University of Massachusetts, USA. Treatments of EDU were divided into 2 different concentrations i.e. 200 ppm and 400 ppm and were applied in the form of foliar spray on 17 days old plants. Treatments were carried out at an interval of 7 days and non EDU plants were sprayed with water. The abbreviations used were: VC-Vedio control, VT1- 200 ppm EDU, VT2-400 ppm EDU; TFC-Tigerflame control, TFT1-200 ppm EDU, TFT2-400 ppm EDU.

2.3.Ambient ozone monitoring

The monitoring of ambient O_3 was done by automatic ozone analyzer (2B Tech , model-106L) for 8 h day^{-1} (from 9.30 to 17.50) at the experimental plot throughout the experimental period.

2.4.Total chlorophyll

Total chlorophyll activity was estimated according to Arnon (1949) with 80% acetone.

2.5.Lipid Peroxidation

Malondialdehyde (MDA) content, a product of lipid peroxidation was estimated by thiobarbituric acid (TBA) reaction according to Heath and Packer (1968).

2.6.Antioxidant and antioxidative enzymes estimation

Biochemical analyses in leaf samples were carried out at two different stages; vegetative phase at 54 days after sowing (DAS) and flowering phase at 96 DAS. The leaves were collected from three randomly selected plants from each treatment and were immediately frozen in the liquid nitrogen and stored at -80°C till further analyses.

The activities of antioxidant glutathione; reduced (GSH), oxidized (GSSG), and total (GSH+GSSG) were assayed by an enzyme recycling assay described by Griffith (1980). The principle of the assay is based on oxidation of glutathione by DTNB to TNB with the enzyme glutathione reducatse (GR) in the presence of NADPH.

Superoxide dismutase (SOD) activity was assayed according to Beyer andFridovich (1987) using NBT method. The reaction mixture contained 50 mM phosphate buffer, pH 7.8, 9.9 mM L-methionine, 57 µM NBT, 0.025% (w/v) Triton X-100, 0.0044% (w/v) riboflavin and absorbance was noted at 560 nm.

Catalase (CAT) activity was assayed in reaction mixture containing 50 mM phosphate buffer (pH 7.0) according to Rao et al. (1996). The reaction was initiated by 10% H_2O_2. The reduction of absorbance was recorded at 240 nm by spectrophotometer.

Ascobate peroxidase (APX) activity was assayed according to Chen & Asada, (1989) with reaction mixture containing 50 mM phosphate buffer (pH 7.0) with 2 mM ascorbic acid. Decrease in absorbance of H_2O_2 that was consumed at 290 nm was recorded by spectrophotometer.

Activity of glutathione reductase (GR) was estimated according to Smith et al. (1988) by recording the increase in absorbance due to reduction of DTNB (5, 5'-dithiobis-(2-nitro-benzoic acid)) to TNB (5'-thio-2-nitrobenzoic acid) at 412 nm.

Protein estimation was carried out according to the method of Bradford (1976).

2.7.Post harvest parameters

Gladiolus spikes were harvested at 2-3 florets break stage and immediately brought to the lab for analysis of vase life. Flower spikes were re-cut (under water) and were placed in glass jars under controlled conditions (25±2 ^{O}C, 70±5% RH and 14 h day light).

2.8.Statistical Analysis

The experiment was conducted in randomised manner with three replicates of each treatment of Gladiolus cultivars Vedio and Tigerflame. Statistical analysis of the data was done following the methods of analysis of variance (One Way ANOVA) by SPSS 16.0 Software version by Duncan t- test and significance of data is analysed at $p \leq 0.05$.

3. Results and Discussion

3.1.Ambient O_3 concentrations and climatic conditions

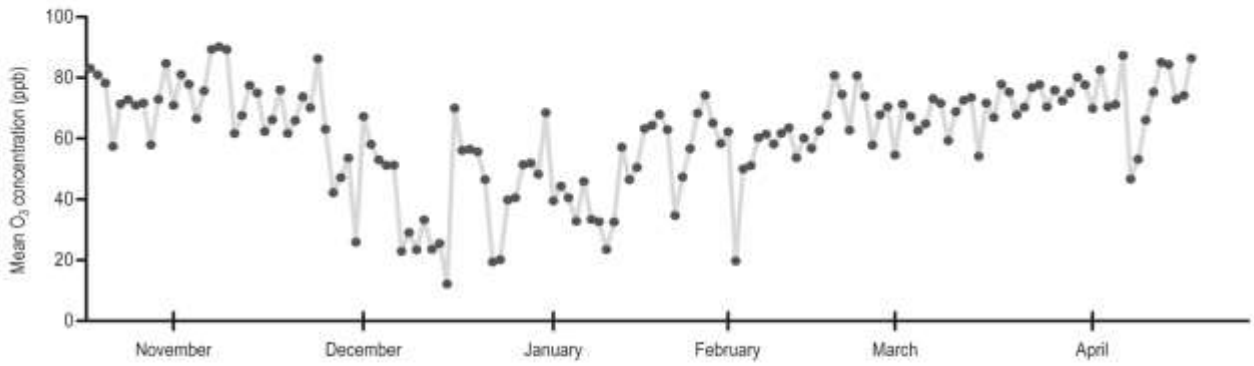

Figure 1:Mean ozone (O_3) concentration (ppb, 8 h daily average) during the growing season of Gladiolus (November–April).

Monthly averages of O_3 during experimental period (November - April) were 74.4, 50.3, 44.2, 56.9, 67 and 73 ppb, respectively (Fig.1). O_3 concentrations were low during months of December and January as there were frequent precipitation and occurrence of fog which dissolved the precursors responsible for O_3 formation. High levels of O_3 were found in November, March and April reaching up to 80 ppb because of favourable climatic conditions. The reason behind prevailing high O_3 in this region is increase in vehicular and anthropogenic emissions which along with favourable meteorological conditions like high temperature, sunlight and low humidity contribute to formation of O_3 (Singh et al., 2010a). There have been many reports of high ambient O_3 concentrations from cities like Delhi, Varanasi, Chandigarh, Ahmadabad, Agra, Bhubaneswar, Cochin and Pune (reviewed in Oksanen et al., 2013).

3.2.Effect of EDU on total chlorophyll

High O_3 induced phytotoxicity leads to membrane degradation of chloroplasts which hampers the photosynthetic apparatus. EDU ameliorates the negative effects of O_3 by maintaining membrane integrity thusleading to high chlorophyll content. In the present study both EDU treated cultivars showed significant increase in chlorophyll content in VT2 at vegetative phase and in TF2 at flowering phase (Figs. 2 c,d). O_3 is known to be very toxic to chlorophyll pigments (Cho et al., 2011). Increase in level of chlorophyll content is one of the prominent plant responses to EDU (as reviewed in Manning 2011). In the present study maintenance of high chlorophyll content in EDU treated plants signifies the protective mechanism of EDU. Induction of higher antioxidative enzymes and non-enzymatic activities by EDU leads to reduction in membrane peroxidation of chloroplasts which is said to be the major reason in retention of high chlorophyll content (Rai et al., 2015).

3.3.Reduction in lipid peroxidation by EDU

Lipid peroxidation is the marker for ROS induced membrane damage which is magnified during O_3 induced phytotoxicity (Singh et al., 2010a; Cho et al., 2011). Protection imparted by EDU results in low level of lipid peroxidation. In the present study EDU treated plants showed decreased MDA content by 42% and 58% in VT1 and VT2 and 18% and 50% in TF1 and TF2, respectively at vegetative stage in comparison to non EDU treated plants (Fig. 2a). Similar results have been reported by Singh et al., (2009) in wheat and Pandey et al. (2014) in mustard. At flowering stage reduction in MDA content in TF1 and TF2 was 48% and 55%, respectively which was more than vegetative phase (Fig. 2b). Reduction in lipid peroxidation in EDU treated plants in the present study signifies the protective nature of EDU and also demonstrates the sensitivity of gladiolus cultivars towards O_3. Pre-treatment of plants with EDU conferred protection to plants against O_3-induced losses of glycerolipids and chlorophyll (Whitaker et al, 1990). Low level of MDA content in all EDU treated plants can be correlated with increase in antioxidative defense system by EDU which we will see in the following section.

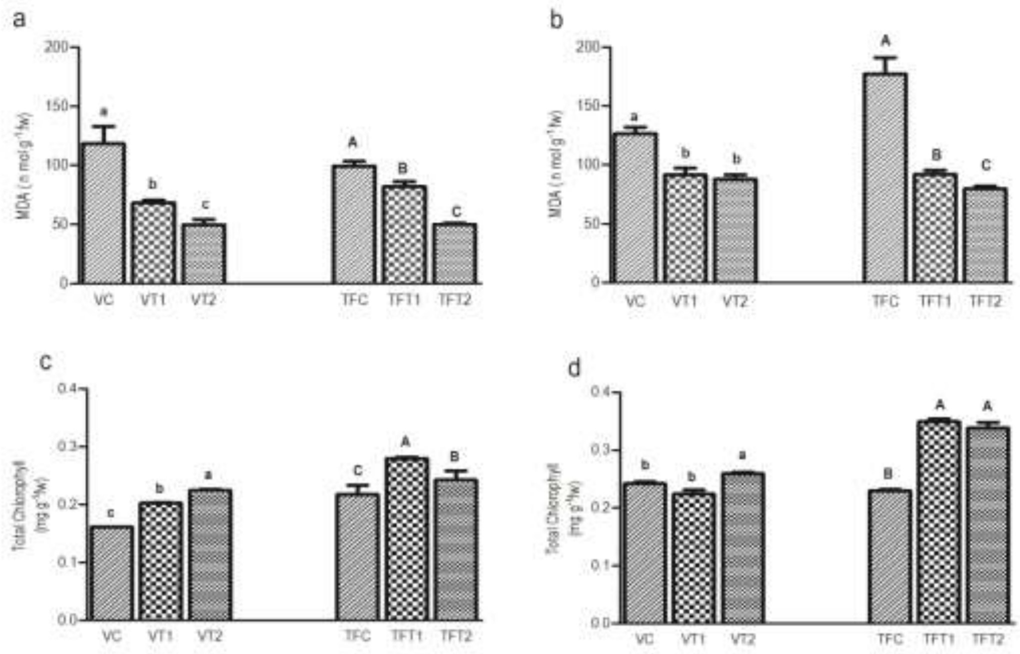

Figure 2: Effect of EDU treatments (200 ppm and 400 ppm) (mean ±standard error) (n = 3) on MDA content (a,b) and Total chlorophyll (c,d) in two Gladiolus cultivars (Vedio and Tigerflame). Letters (a,b,c) and (A,B,C) denote significance level in Vedio and Tiger flame, respectively according to One way ANOVA post hoc Duncan's test ($p \leq 0.05$).

3.4. EDU alleviates O_3 induced phytotoxic effect by enhancing antioxidant activities

Antioxidants and antioxidative enzymes are the core component in plant's defence against abiotc/biotic induced oxidative stress. As reviewed in Manning (2011), it has been suggested that EDU acts after its entry into the plants through stomata and enhances the activities of antioxidative defence system; the exact mechanism, though, is still not known. In the present study, we observed positive effect of EDU in maintaining antioxidative defense activities in both the cultivars. SOD which is first line of defence against reactive oxygen species, showed significantly higher activities in both the cultivars at both the stages (Figs. 3 A, E). At vegetative stage, SOD activity was increased by 36% and 29 % in VT2 and TF2, respectively. The SOD activity was further enhanced at flowering stage by 59% and 34% in VT2 and TF2, respectively

(Fig. 3 E). Increased activity of SOD is known to provide tolerance against high O_3 exposure (Singh et al., 2014; Rai et al., 2015). Similar observations were reported in various crops like rice (Pandey et al., 2015) and potato (Hassan, 2006). Significantly high CAT activities in both varieties of gladiolus were observed at both the stages. As such the baseline CAT activity was more at flowering stage in both the varieties. Maximum increase was recorded in Vedio at vegetative stage being, 95.5% and 70% in VT1 and VT2, respectively than non-EDU treated Gladiolus (Figs. 2 B). At flowering stage increments of 29% and 47.5% in VT1 and VT2, respectively were observed while in TFT1 and TFT2 the activity increased by 25% and 53%, respectively **(**Fig. 3 F). CAT plays an important role in H_2O_2 detoxification (Singh et al., 2009; Singh et al., 2014). Increased activity of CAT in EDU treated plants as compared to non EDU treated plants in the present study clearly depicts the variation in cultivar responses towards O_3. Although both EDU concentrations were effective, gradual increase in CAT activity with increased concentration of EDU (400 ppm) especially at flowering stage can be correlated with increasing ambient O_3 concentrations. Pandey et al. (2015), working with 18 rice varieties, reported that SOD and CAT were the most responsive parameters across all varieties in response to EDU.

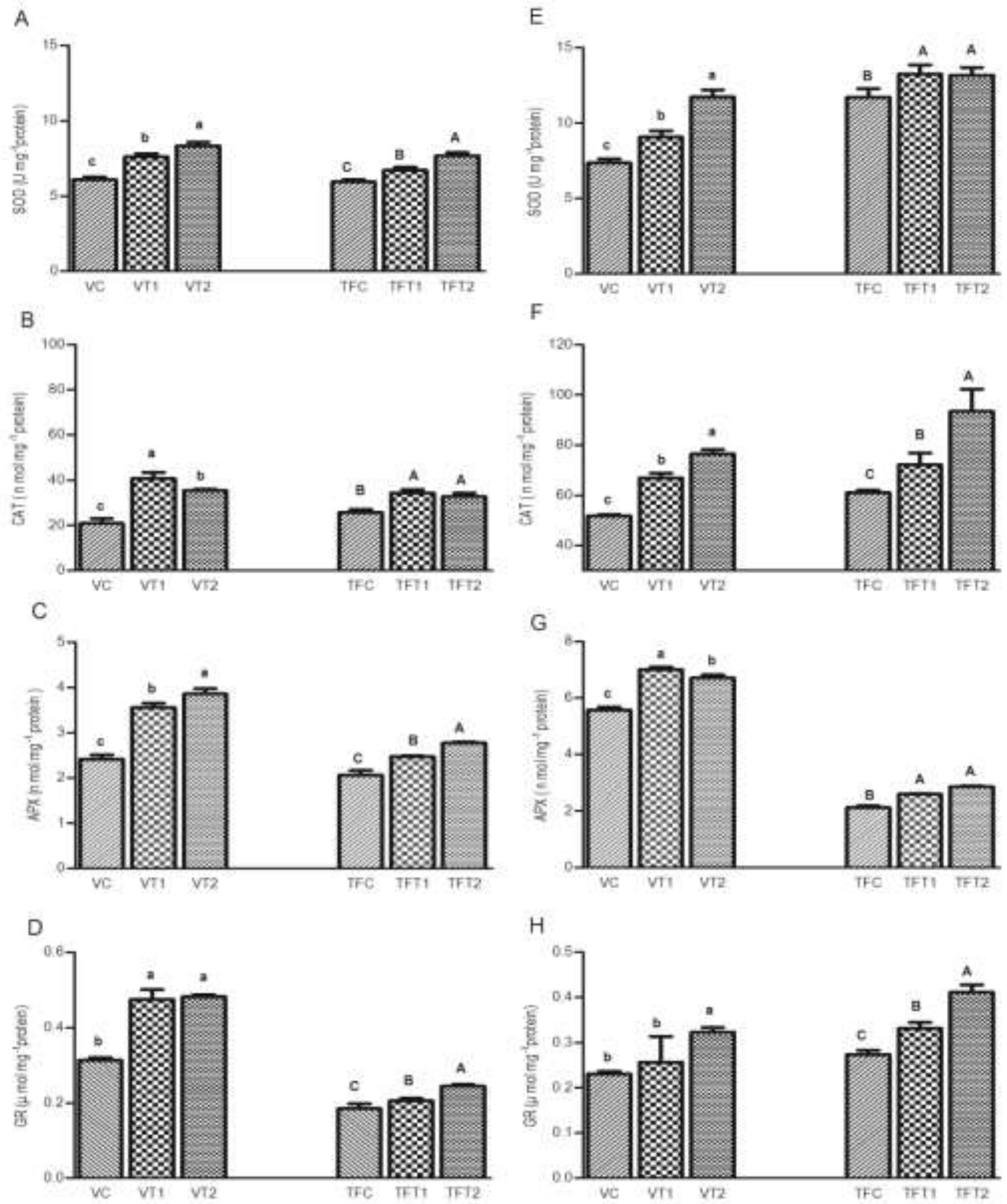

Figure 3: Effects of EDU treatment (200 and 400 ppm) (mean±standard error) (n=3) on SOD (A, E), CAT (B, F), APX (C, G) and GR (D, H) activities in two Gladiolus cultivars at the vegetative (A–D) and flowering phase (E–H). Letters (a,b,c) and (A,B,C) denote significance level in Vedio and Tiger flame, respectively according to One way ANOVA post hoc Duncan's test ($p \leq 0.05$).

APX and GR are the prominent enzymes of ascorbate-glutathione cycle. In the present study higher activity of APX in all EDU-treated cultivars in comparison to non EDU treated ones at both the stages were found. The strongest response was recorded in Vedio at vegetative stage with 47% and 59 % increase in VT1 and VT2, respectively over their control followed by Tigerflame having 20% and 34% increase in TF1 and TF2, respectively (Fig. 3 C). Low APX activity in non EDU treated gladiolus cultivars and high APX activity in EDU treated gladiolus cultivars denotes elevation in the enzyme activity by EDU which leads to proper utilization and regeneration of ascorbic acid and detoxification of H_2O_2 thus providing protection against O_3 induced toxicity (Rai et al., 2015). Significant increase in APX activity in both the gladiolus cultivars at different EDU concentrations suggests this enzymes' greater sensitivity to O_3 (Singh et al., 2009). Similarly increased GR activity was observed in EDU treated plants of Vedio and Tigerflame. Increased activity of GR plays a significant role in maintaining GSH/GSSG ratio (Pandey et al., 2014). At vegetative stage, an increase of 51% and 53% was observed in Vedio while in Tigerflame increment was only 11% and 31% as compared to non EDU treated plants (Fig. 3D). At flowering stage there was 21% and 47% increase in GR activity in Tigerflame (Fig. 3H). High GR activity in both developmental stages with 400 ppm of EDU indicate that higher concentration of EDU was much more effective than 200ppm EDU in providing protection against O_3 induced phytotoxicity. Increased EDU induced GR activity was also seen in crops like maize (Singh et al. 2014) and potato (Hassan, 2006).

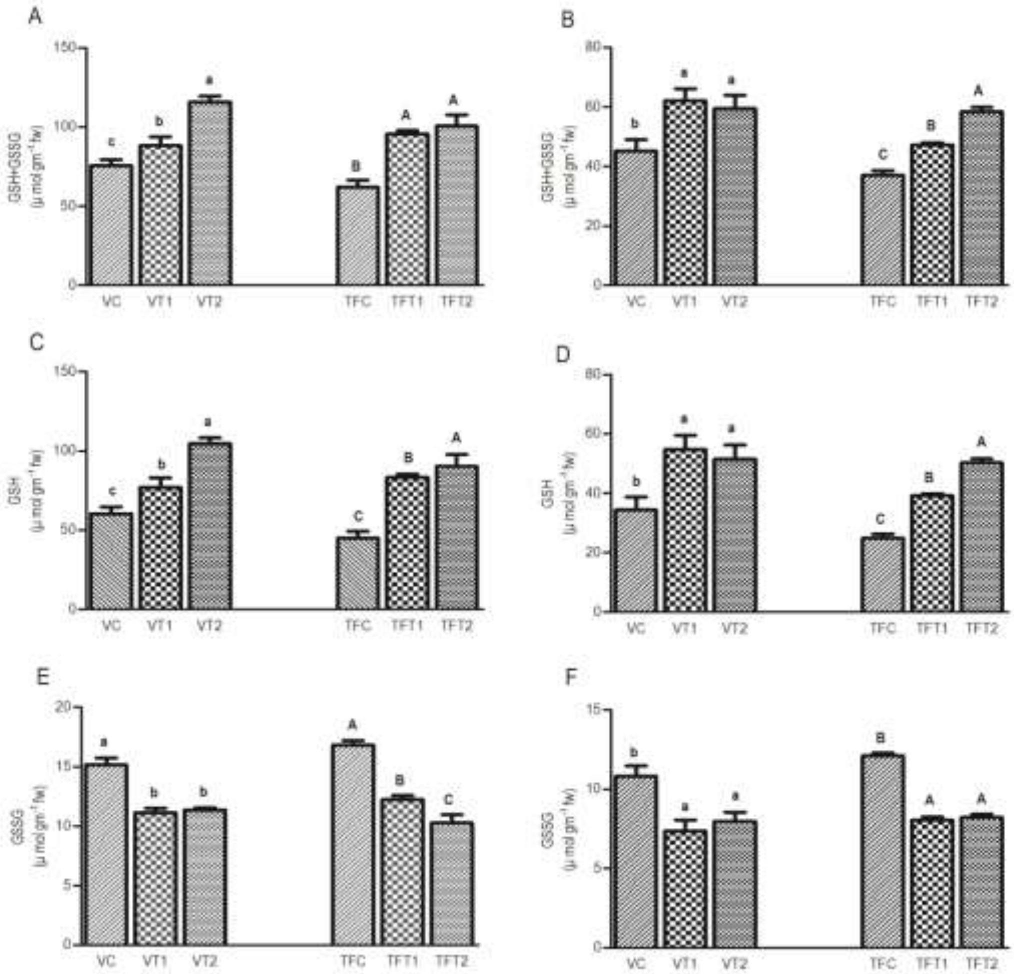

Figure 4:- Effects of EDU treatment (200 and 400 ppm) (mean±standard error) (n=3) on total GSH (A,B), GSH (C,D) and GSSG (E,F) content in two Gladiolus cultivars (Vedio and Tigerflame)at the vegetative (A-E) and flowering phase (B-F). Letters (a,b,c) and (A,B,C) denote significance level in Vedio and Tigerflame, respectively according to One way ANOVA post hoc Duncan's test ($p \leq 0.05$).

EDU induced changes in glutathione concentrations (GSH, GSSG, and total glutathione) are shown in Fig 4. There were significant increments in total glutathione (GSH+GSSG) level in the EDU treated plants compared to non EDU treated plants in both the varieties (Figs. 4 A, B).The content of reduced glutathione (GSH) increased significantly in both the varieties at vegetative phase with strongest increases in Tigerflame being 84% and 98% in TFT1 and TFT2, respectively than Vedio (27% and 73% increase in VT1 and VT2) (Figs. 4 C, D). Concomitantly the levels of oxidized glutathione (GSSG) were decreased consistently in both varieties at both sampling stages, maximum being in Tigerflame by 27.2% and 38.8% in TF1 and TF2, respectively (Figs. 4 E,F) . EDU maintains the level of reduced glutathione (GSH) to oxidised glutathione (GSSG) thus counteracting the inhibitory effect of O_3 (Hassan, 2006). In the present study also, plants treated with two different concentrations of EDU showed significant rise in GSH level along with decrease in GSSG with T2 (400 ppm of EDU) being the most effective at both vegetative and flowering stages. Thus our study shows that EDU maintains the GSH/GSSG ratios hence protecting cellular environment from oxidation.

The results of the present work clearly showed that both the varieties were sensitive towards O_3 and application of EDU protected the varieties against phytotoxic effects by enhancing activities of antioxidant defence system. This helped in keeping toxic free radicals in control thus maintaining the membrane stability (Singh et al., 2009) and important cellular processes.

3.5. Protection conferred by EDU increases post harvest quality

Table 1: Number of bloomed flowers in EDU and non EDU treated Gladiolus cultivars Vedio and Tigerflame

	Vedio			Tigerflame		
No. of Days	**Control**	**200 ppm**	**400ppm**	**Control**	**200 ppm**	**400 ppm**
1st day	5.67±1.53	5.01±2.8	4.01±2.9	5.5±0.7	4±2.08	6.3±0.6
3rd day	7.00±1.00	7.33±0.58	6.25±1	5.5±0.7	8±0.58	
5th day	3.33±1.15	5.33±0.58	3.5±1	4±0.01	6±0.58	6.3±0.6
7th day	2.00±1	3.33±0.58	2.00±1	1.5±0.7	3±0.58	2.3±0.6
8th day	-	1±0.01	1±0.01	-	1±0.58	1±0.01

Accelerated premature senescence is a major negative impact of O_3 induced phytotoxicity affecting yield (Oksanen et al., 2013; Feng et al., 2015). This phenomenon is counteracted by antiozonant EDU thus maintaining or enhancing yield (Singh et al., 2009; Feng et al., 2010; Pandey et al., 2014; 2015). In the present study application of EDU increased numbers of mature spikes in Tiger flame while there were no significant changes in cultivar Vedio (Fig.5). Number of more matured spikes in EDU treated Tiger flame as compared to non EDU treated plants show sensitivity of the gladiolus cultivar. Application of EDU extended the vase life of both the cultivars by nearly 24 hours **(**Figs. 6 A, B). To check the longevity of petals, the number of bloomed flowers on 1st ,3rd,5th,7th and 8th day were noted (Table 1). Delayed petal senescence was observed on 3rd and 5th day in all EDU treated plants in comparison to non EDU ones. Increase in shelf life was also observed in EDU treated plants where it went till 8th day in comparison to control. Increase in vase life by 24 hours in EDU treated plants can be attributed to increased antioxidative defense system by EDU which prompted scavenging of ROS thus lowering the level of lipid peroxidation maintaining a stabilized membrane system leading to enhanced vaselife (Manning et al., 2011; Singh et al., 2015) . Gimeno et al. (2004) reported reduction in the number of inflorescences, floral buds and affect flower biomass under elevated O_3. Although no study with EDU on ornamental crops has been reported, the mechanism of EDU protection on improvement of post harvest quality can be correlated with ROS scavenging chemicals like thiamine and salicylic acid which are known to delay petal senescence in salt and water stressed gladiolus cultivars (Hatamzadeh et al., 2012;Hashish et al., 2015). Thus our results clearly show that prevailing O_3 levels are having deleterious impact on this important ornamental crop. Although the complete mechanism of EDU protection is still unknown, effective scavenging of ROS by higher level of antioxidative enzymes and antioxidant glutathione in EDU treated gladiolus plants, reduction in lipid peroxidation and high chlorophyll content can be attributed for enhancing vaselife in gladiolus.

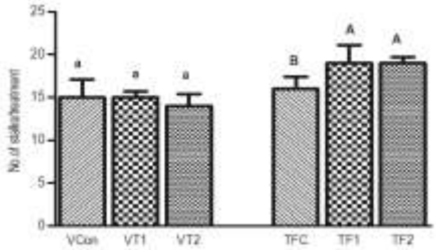

Figure 5: Effect of EDU on spike production on two gladiolus cultivars (Vedio and Tigerflame)

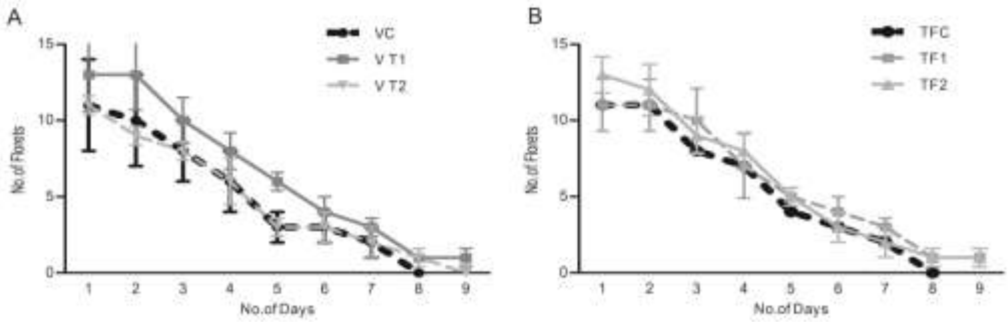

Figure 6:- Effect of EDU on post harvest quality (vase life) on two gladiolus cultivars (Vedio and Tigerflame).

4. Conclusion

The present study showed that ambient O_3 concentrations in Lucknow are very high and sufficient enough to have adverse impact on different biochemical attributes of two gladiolus cultivars. EDU protected the plants against negative impacts of high ambient O_3. EDU treated plants showed higher levels of antioxidative enzymes (SOD, CAT, APX, and GR) and antioxidant (GSH) which helped gladiolus plants to maintain membrane integrity and higher levels of chlorophyll. Based on our results, Tigerflame was more sensitive to O_3 as it was more responsive to EDU. As floriculture is becoming an important and lucrative industry, more studies are needed on the effects of O_3 on ornamental plants.

Acknowledgement: - Authors are thankful to the Director, CSIR-NBRI for support. This work was funded by Academy of Finland (project no. 138161).

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