Responses of two cultivars of *Trifolium repens* L. to ethylene diurea in relation to ambient ozone

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Received 08 July 2009; revised 07 September 2009; accepted 30 October 2009

Abstract

Three ethylene diurea (EDU) concentrations (0, 150 and 300 mg/L) were used to evaluate the negative impact of ozone (O$_3$) on two cultivars of *Trifolium repens* L. cv. Vardan and Bundel grown under natural field conditions in a suburban area of Varanasi, India. Mean O$_3$ concentrations varied from 30.3 to 46.6 µg/L during the experimental period. Higher photosynthetic pigments and ascorbic acid concentrations were noticed in both EDU-treated cultivars over non-EDU-treated ones, but a reverse trend was found for lipid peroxidation. Growth parameters and biomass also showed increments under EDU treatment of both cultivars. The ratio of variable fluorescence to maximum fluorescence increased significantly in Vardan but not in Bundel upon EDU treatment. Results revealed that EDU concentration of 300 mg/L was more effective to combat the oxidative stress as well as protecting plants from O$_3$ injury symptoms. The test cultivar Vardan is relatively sensitive to O$_3$, thereby can be used as a bioindicator of O$_3$ pollution in areas having higher O$_3$ concentrations. Results also indicated that Bundel has more efficient antioxidant defense system than Vardan and hence was more tolerant to O$_3$ stress.

Key words: *Trifolium repens*; ozone; oxidative stress; ascorbic acid; photosynthetic pigments

DOI: 10.1016/S1001-0742(09)60223-0

Introduction

Injudicious planning in urban development and industrial sector without due regard to sustainable development is leading to serious environmental hazards. These changes are posing a risk to the well being of plants, animals and human health.

Ozone (O$_3$) is a widespread secondary photochemical air pollutant, which occurs naturally at ground level in low concentrations and is considered the most significant phytotoxic air pollutant world wide. Ozone levels often exceed limits set to protect humans and vegetation (EEA, 2007). The current level of O$_3$ exceeds the tolerance threshold of many plants, thus impairing plant growth, reducing crop yield and altering the composition of the plant community (Davison and Barnes, 1998; Singh et al., 2009). Background concentration of O$_3$ in the troposphere has doubled in the past decade and there are evidences of an increase in annual mean values ranging from 0.1 to 1 µg/L per year (Coyle et al., 2003). Tropospheric O$_3$ is formed in presence of sunlight through the chemical interaction of reactive volatile organic compounds and oxides of nitrogen (NO$_x$) that results from high-temperature combustion of fossil fuels. More importantly, O$_3$ can be transported long distances from urban to rural areas through wind movement (Chameides et al., 1994; Rai and Agrawal, 2008).

Most of the work on long-term variations in tropospheric O$_3$ is based on photochemical models. Quantitative estimates of ground level O$_3$ are limited over the Indian subcontinent. In Varanasi City, annual average O$_3$ concentrations varied from 8 to 25 µg/L during 1989–1990 (Pandey and Agrawal, 1992). High concentrations of O$_3$ varying between 9.4 and 128.3 µg/L have been reported for the same period at an urban site in Delhi (Varshney and Aggarwal, 1992). A detailed monitoring program conducted at a suburban area of Varanasi during 2004–2006 showed average daytime O$_3$ concentrations of 56, 42 and 32 µg/L during summer, winter and rainy seasons, respectively (Rai et al., 2007; Tiwari and Agrawal, 2009). Upon comparison of data of 2004–2006 from earlier monitoring records (1989–1990) for the same area, it was observed that average O$_3$ concentrations increased by 32%, 41% and 36%, respectively during summer, winter and rainy seasons. Maximal O$_3$ concentration during summer can be explained by favourable meteorological factors such as high solar radiation, longer day light period, high temperature, stagnant wind patterns and low humidity.

Ethylene diurea (EDU) has been used extensively to detect plant injury caused by ambient O$_3$ in a bioindicator program in Europe (Manes et al., 1990; Sanders et al., 1992). EDU is potentially used as a research tool for...
O$_3$ injury survey work and plant response assessment in remote areas particularly in developing regions where electricity and funding are limited (Bytnerowicz et al., 1993; Hassan et al., 1995; Blum et al., 1998; Tiwari et al., 2005). EDU protects plants from premature senescence, pigment degradation and helps in maintenance of higher nutrient levels to allow successful growth and reproduction (Toivonen et al., 1982; Brunsch-Warti et al., 1995a, 1995b; Eckardt and Pell, 1996; Blum and Didyk, 2007). EDU is mainly applied as a soil drench or foliar spray to crops and vegetables and sometimes also as a stem injection in tree species. However, it is known that EDU remains in apoplastic region of plants for 10 days, without entering into cells suggesting that EDU itself acts against O$_3$ injury (Gatta et al., 1997). In order to be effective, EDU must be applied at regular intervals (Manning, 2000).

Species of clover (Trifolium repens) are considered relatively sensitive to O$_3$ (Fagnano et al., 2004). Subterranean clover has been used to investigate crop responses to O$_3$ at many sites across Europe (Sanders et al., 1992) within the framework of the United Nations/Economic Commission for Europe International Co-operative Program on the effects of air pollution and other stresses on crops and non-woody plants (UN/ECE ICP Vegetation; formerly ICP Crops). This program was aimed to verify the long term critical level of O$_3$ for growth and yield reduction and the short term critical level for protection of crops against visible injury (Benton et al., 1995).

*Trifolium repens* L. (clover) is a major fodder crop grown throughout India. Field experiment was conducted on two cultivars of clover (Vardan and Bundel) considered a good bioindicator of O$_3$ pollution to determine the effectiveness of EDU against O$_3$ stress under truly ambient field conditions in a suburban area of Varanasi City, Uttar Pradesh, India. Dose response studies with EDU and clover plants may be helpful in screening various clover cultivars for sensitivity to O$_3$ injury and biomass loss in areas experiencing elevated concentrations of O$_3$.

### 1 Experimental procedures

#### 1.1 Experimental site

The experiment was conducted at the Botanical garden, Banaras Hindu University, Varanasi (25°18’N latitude and 83°1’E longitude and 76.19 m above the sea level in the eastern Gangetic plains of Northern India) during the growing season of clover (December 2008 to February 2009). The mean monthly minimum and maximum temperatures varied from 10.5 to 11.5°C and from 24.1 to 27.2°C. Total rainfall was 0.6 mm in January. Mean maximal monthly relative humidity ranged from 84.5% to 94.5%. Sunshine hours ranged from 4.2 to 9.4 hr (Table 1). Soil of the study site was sandy loam with soil pH varying from 7.0 to 7.2, and organic carbon concentration ranged from 0.6% to 0.7%.

#### 1.2 Plant materials and growth conditions

Two cultivars of *T. repens* (Vardan and Bundel) obtained from the “Indian Grassland and Fodder Research Institute, Jhansi” (India) were grown in 18 plots of 1 m × 1 m (9 plots for each cultivar) on December 6, 2008. The plot was prepared by ploughing to a 20-cm depth. Each plot had 30 plants with a distance of 15 cm between plants. Recommended doses of fertilizer (NPK 40:30:40 kg/ha as urea, single superphosphate, and muriate of potash, respectively) were added during the preparation of the field. Plots were irrigated to maintain uniform moisture approximately near to field capacity.

#### 1.3 EDU application

Plants were treated with three EDU concentrations (0, 150 and 300 mg/L). Six plots of each cultivar were treated with 150 and 300 mg/L EDU while the other three received the same amount of deionized water. EDU solution was freshly prepared each time, using deionized water, and applied as a soil drench (100 mL/plant) 10 days after germination (DAG) between 9:00 and 10:00 at intervals of 10 day up to 70 DAG.

#### 1.4 Ambient ozone monitoring

Twelve hourly O$_3$ monitoring was done using an automatic O$_3$ analyzer (Horiba, APOA-370, Japan) from 8:00 to 20:00 daily until plants were mature. Air samples were collected with a Teflon tube (0.35 cm diameter) placed above the canopy of the plants.

#### 1.5 Plant sampling and analysis

##### 1.5.1 Growth parameters

For biomass and growth determinations, three plants were sampled from each plot at 20, 40 and 60 DAG by carefully digging monoliths 10 cm length × 10 cm width × 20 cm depth. These were thoroughly washed under running tap water to remove soil particles adhering to the roots. Plant samples were analyzed for root and shoot length, number of leaves and leaf area. Leaf area was measured with a portable leaf area meter (Model LI-3000, LI-COR, Inc., USA). For biomass determination, plants were oven dried (80°C) to constant weights.

##### 1.5.2 Physiological and biochemical parameters

Chlorophyll fluorescence was determined at 40 DAG between 10:00 and 11:00 using portable plant efficiency analyzer (Model, MK29414, Hansatech Instrument Ltd.,

<table>
<thead>
<tr>
<th>Month-year</th>
<th>Total rainfall (mm)</th>
<th>Temperature (°C)</th>
<th>Relative humidity</th>
<th>Sunshine time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Max</td>
<td>Min</td>
<td>(%) (Min) (%)</td>
</tr>
<tr>
<td>December 2008</td>
<td>0</td>
<td>24.3</td>
<td>11.5</td>
<td>84.8</td>
</tr>
<tr>
<td>January 2009</td>
<td>0.6</td>
<td>24.1</td>
<td>10.7</td>
<td>85.4</td>
</tr>
<tr>
<td>February 2009</td>
<td>0</td>
<td>27.2</td>
<td>10.5</td>
<td>94.8</td>
</tr>
</tbody>
</table>
Leaf clips for dark adaptation were placed on the adaxial side of leaves 30 min before measurement at an excitation irradiance of 2000 μmol/(m²·sec). Minimum fluorescence ($F_0$), maximum fluorescence ($F_m$), variable fluorescence ($F_v$) and ratio of variable and maximum fluorescence ($F_v/F_m$) were calculated.

Biochemical analyses were conducted in leaves of both cultivars sampled in triplicates from plants at 40 and 60 DAG. Malondialdehyde (MDA) concentration, a product of lipid peroxidation was estimated by thiobarbituric acid (TBA) reaction following the protocol of Heath and Packer (1968). Photosynthetic pigments were extracted from leaf samples in 10 mL of 80% acetone. After centrifugation, the optical densities of the supernatant were measured at 480, 510, 645 and 663 nm wavelength and the amount of total chlorophyll and carotenoids were calculated using the formulae given by Maclachlan and Zalik (1963) and Duxbury and Yentsch (1956), respectively. Ascorbic acid in leaf samples were extracted in oxalic acid and Na-EDTA and the concentrations were estimated by using 2,6-dichlorophenol indophenol (DCPIP) dye reduction method of Keller and Schwager (1977).

1.6 Statistical analysis

The data of growth, biomass and biochemical parameters were analyzed by three-way ANOVA test for examining the individual and interactive effects of age, cultivar and treatment. Duncan’s multiple range tests were performed as post hoc for various measures subjected to one-way ANOVA test. Statistical tests were performed using SPSS software (SPSS Inc., version 10.0).

2 Results and discussion

The $O_3$ monitoring data recorded during 3 months of experiment are summarized in Fig. 1. Ozone concentration showed monthly variations with maximum mean concentration in February (46.6 μg/L). Singh et al. (2009) reported higher mean $O_3$ concentration ranging from 34.2 to 54.2 μg/L between December and March 2006–2007. Rai et al. (2007) reported a higher mean concentration of $O_3$ (48 μg/L) at a suburban area of Varanasi during 2005. Tiwari et al. (2005) reported that $O_3$ concentrations frequently exceeded 40 and 50 μg/L in February and March during 2002–2003 at a suburban area of Varanasi. High $O_3$ concentration in February may be due to high light intensity and warm temperature conducive for $O_3$ formation.

Clover leaves showed brown to reddish discoloration and water soaked areas in expanding trifoliates of non-EDU-treated plants at 40 DAG. In the later stages of development, tip burning was also observed at 60 DAG whereas no visible injury was observed in EDU-treated plants. EDU treatment has been shown to reduce visible $O_3$ injury symptoms on potato leaves in an area experiencing mean $O_3$ concentration of 50 μg/L (Hassan, 2006). Szantoi et al. (2009) found a significant linear decrease of 13.9%, 13.2%, 11.1%, and 9.2% in percent leaves injured after 0, 200, 400 and 600 mg/L EDU treatments of Rudbeckia laciniata exposed at 12 hr mean $O_3$ concentration of 73 μg/L in open top chambers.

The extent of lipid peroxidation (LPO) measured as MDA concentration in leaves was lower in both EDU-treated cultivars than in non-EDU-treated ones at both the samplings (Fig. 2). Lipid peroxidation varied significantly due to all the factors except age × treatment (Table 2). Ozone after entering inside the leaves forms reactive oxygen species (ROS) (Mehlhorn et al., 1990), which alter membrane permeability and allow cell contents to leak into extracellular spaces leading to ionic imbalance within the cells (Dominy and Heath, 1985). The greater induction of MDA concentration in non-EDU-treated plants of Vardan clearly depicts its greater sensitivity compared to Bundel. Whitaker et al. (1990) reported reduction in ROS

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### Table 2: Results of three way ANOVA test showing levels of significance for various growth and biochemical parameters of *Trifolium repens* L.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Age</th>
<th>Cultivar</th>
<th>Treatment</th>
<th>Age × Cultivar</th>
<th>Age × Treatment</th>
<th>Cultivar × Treatment</th>
<th>Age × Treatment × Cultivar</th>
</tr>
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<tbody>
<tr>
<td>Root length</td>
<td>***</td>
<td>***</td>
<td>NS</td>
<td>***</td>
<td>*</td>
<td>**</td>
<td>*</td>
</tr>
<tr>
<td>Shoot length</td>
<td>***</td>
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<td>***</td>
<td>***</td>
<td>*</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Number of leaves</td>
<td>***</td>
<td>NS</td>
<td>***</td>
<td>NS</td>
<td>***</td>
<td>***</td>
<td>***</td>
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<tr>
<td>Leaf area</td>
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<td>***</td>
<td>***</td>
<td>***</td>
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<td>***</td>
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<tr>
<td>Root biomass</td>
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<td>***</td>
<td>***</td>
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<td>***</td>
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<td>Shoot biomass</td>
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<td>***</td>
<td>***</td>
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<tr>
<td>Leaf biomass</td>
<td>***</td>
<td>***</td>
<td>***</td>
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<td>***</td>
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<tr>
<td>Total biomass</td>
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<td>***</td>
<td>***</td>
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<td>***</td>
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<tr>
<td>Total chlorophyll</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
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<td>***</td>
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<tr>
<td>Carotenoids</td>
<td>***</td>
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<tr>
<td>Ascorbic acid</td>
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<tr>
<td>Lipid peroxidation</td>
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<td>***</td>
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<td>***</td>
</tr>
</tbody>
</table>
| *P < 0.05; **P < 0.01; ***P < 0.001; NS: not significant.*
production in 500 mg/L EDU-treated snap bean grown at 400 µg/L O₃ in open top chambers leading to prevention of peroxidation and loss of membrane glycerolipids and steryl composition. A significant reduction of 17.7% in MDA concentration of 400 mg/L EDU-treated mung bean over non-EDU ones at mean O₃ concentrations of 52.9 to 64.5 µg/L was reported (Singh et al., 2010).

Three way ANOVA tests showed significant variations in total chlorophyll and carotenoid concentrations due to all the factors and their interactions (Table 2). Total chlorophyll concentration was significantly higher in both (150 and 300 mg/L) EDU-treated plants of Vardan at both the ages, whereas in Bundel at 60 DAG (Fig. 2). EDU has been shown to act like cytokinin to prevent chlorophyll degradation (Lee and Chen, 1982). Increase of 28.2% in carotenoids content was observed in 300 mg/L EDU-treated Vardan whereas a decrease of 14.2% in 300 mg/L EDU-treated Bundel was observed at 60 DAG. Blum and Didyk (2007) also reported that the concentrations of chlorophyll a and b in leaves of T. subterraneum cv. Geraldton treated with EDU were higher compared to control when mean O₃ concentration exceeded 30 µg/L. In another study conducted in Saudi Arabia, Al-Qurainy (2008) reported consistently higher chlorophyll a, b and total on application of 250 mg/L EDU. Higher levels of chlorophyll and carotenoids under EDU treatment were also observed in Solanum tuberosum (Eckardt and Pell, 1996), Vigna radiata (Agrawal et al., 2005; Singh et al., 2010), Triticum aestivum (Agrawal et al., 2004; Singh et al., 2009) and Beta vulgaris (Tiwari and Agrawal, 2009). Ozone induced ROS production is implicated for damage to chloroplast membranes and associated molecules (Sakaki et al., 1983). By maintaining a higher carotenoid concentration in leaves under EDU treatments, chlorophyll damage due to O₃ was prevented (Lichtenthaler, 1987).

Membrane integrity and its peroxidation are correlated with the levels of antioxidants (Calatayud et al., 2002). Ascorbic acid concentration remained high in both cultivars treated with EDU as compared to non-EDU-treated ones at both the ages of observations (Fig. 2), but a decline was observed with age. Highly significant variations in ascorbic acid concentration due to individual factors and their interactions (P < 0.001) were noticed (Table 2). An increase of 13.8% in ascorbic acid was recorded in EDU-treated plants over non-EDU-treated ones at a mean O₃ concentration of 34 µg/L (Agrawal et al., 2005). Ascorbic acid is the most abundant antioxidant in leaf cells and has the potential to react directly with O₃ detoxification (Runeckles and Chevone, 1992). Increases (Singh et al., 2010) as well as no change (Gillespie et al., 1998) in ascorbic acid content of EDU-treated compared to non-EDU-treated plants were reported.
Table 3  Variations in chlorophyll fluorescence (mV) parameters of *Trifolium repens* L. at different EDU treatments at 40 DAG (mean ± 1 SE)

<table>
<thead>
<tr>
<th>EDU treatment</th>
<th>$F_o$</th>
<th>$F_v$</th>
<th>$F_m$</th>
<th>$F_v/F_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vardan</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mg/L</td>
<td>501.00 ± 2.88 a</td>
<td>1567.33 ± 41.95 a</td>
<td>2068.00 ± 39.71 a</td>
<td>0.757 ± 0.0036 b</td>
</tr>
<tr>
<td>150 mg/L</td>
<td>453.00 ± 10.21 b</td>
<td>1713.33 ± 54.17 a</td>
<td>2166.00 ± 28.35 a</td>
<td>0.797 ± 0.0017 a</td>
</tr>
<tr>
<td>300 mg/L</td>
<td>419.00 ± 3.78 b</td>
<td>1658.66 ± 41.59 a</td>
<td>2077.66 ± 44.39 a</td>
<td>0.798 ± 0.00318 a</td>
</tr>
<tr>
<td><strong>Bundel</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mg/L</td>
<td>435.00 ± 11.13 a</td>
<td>1805.33 ± 32.297 a</td>
<td>2240.33 ± 35.89 a</td>
<td>0.805 ± 0.00448 a</td>
</tr>
<tr>
<td>150 mg/L</td>
<td>453.33 ± 15.03 a</td>
<td>1782.33 ± 84.902 a</td>
<td>2233.33 ± 192.50 a</td>
<td>0.797 ± 0.00203 a</td>
</tr>
<tr>
<td>300 mg/L</td>
<td>453.33 ± 11.35 a</td>
<td>1779.00 ± 93.29 a</td>
<td>2232.33 ± 102.764 a</td>
<td>0.796 ± 0.00569 a</td>
</tr>
</tbody>
</table>

Within each group, values not followed by same letters are significantly different at $P < 0.05$.

Behavioral difference in the fast kinetics of chlorophyll a fluorescence in response to EDU was observed during the present study particularly in Vardan, where a significant increase of about 5.4% in $F_v/F_m$ ratio was observed (Table 3). $F_v/F_m$ ratio indicates the photochemical efficiency of PSII and decrease in this parameter in non-EDU-treated plants clearly depicts $O_3$-induced photoinhibition (Krause, 1988). Higher $F_v/F_m$ ratio of EDU-treated plants of Vardan is maintained due to higher level of chlorophyll concentration compared to EDU-treated Bundel. Significant increments in $F_v/F_m$ ratio of EDU-treated palak (*B. vulgaris*) plants at a site experiencing 52 to 73 µg/L $O_3$ (Tiwari and Agrawal, 2009) and three wheat cultivars grown at a site having 34.2 to 54.2 µg/L $O_3$ (Singh et al., 2009) were reported. In this study, a significant increase in $F_v/F_m$ ratio of Vardan suggests a greater sensitivity of this cultivar to $O_3$ than Bundel as EDU is known to protect plants more at higher $O_3$ levels.

The overall effects of EDU treatments on lowering of lipid peroxidation and consequent increase in antioxidant ascorbic acid led to protection of photosynthetic pigments and electron transport chemistry. This efficiency of EDU treatment is also reflected in positive modifications in growth and biomass accumulation. Root and shoot lengths increased in EDU-treated plants compared to non-EDU-treated ones in both the cultivars of clover (Fig. 3).

![Fig. 3 Root and shoot lengths, number of leaves and leaf area of two cultivars of *Trifolium repens* L. at different EDU treatments at 20, 40 and 60 DAG (mean ± 1 SE). Bars with different letters in each group show significant difference at $P < 0.05$.](image-url)
Responses of two cultivars of Trifolium repens L. to ethylene diurea in relation to ambient ozone

Fig. 4 Root, shoot, leaf and total biomass of two cultivars of Trifolium repens L. at different EDU treatments at 40 and 60 DAG (mean ± 1 SE). Bars with different letters in each group show significant difference at $P < 0.05$.

Statistical analysis also showed significant variations in root length due to individual factors except treatment and their interactions (Table 2). Increase in shoot length upon EDU treatment has been reported in mungbean (Agrawal et al., 2005) and palak (Tiwari and Agrawal, 2009). Disturbances in various metabolic activities, impairment of photosynthetic capacity and early senescence due to high levels of $O_3$ lead to reductions in plant growth (Agrawal et al., 2005; Hassan, 2006; Singh et al., 2009, 2010). Numbers of leaves were higher by 32.8% and 23.3% in 300 mg/L EDU-treated Vardan and Bundel at 60 DAG. Agrawal et al. (2004) reported 13.6%, 21.0%, and 10.6% increases in number of leaves upon 500 mg/L EDU treatment of three wheat cultivars. In contrast, there are several studies showing that the number of leaves did not vary significantly due to EDU treatment to $T. subterraneum$ (Tonneijck and Van Dijk, 1997), $V. radiata$ (Agrawal et al., 2003) and $T. aestivum$ (Tiwari et al., 2005). Leaf area was significantly higher in 300 mg/L EDU-treated Vardan at 40 DAG and in Bundel at both the ages compared to their respective controls (Fig. 3). Increments in leaf area were also found in soybean (Wahid et al., 2001) and mungbean (Singh et al., 2010) under EDU treatment.

Biomass accumulation increased with increasing EDU application rates at both the ages in Vardan, but decreased at 150 mg/L EDU at 40 DAG in Bundel compared to non-EDU-treated ones (Fig. 4). Significant variations were found in root, shoot and leaf biomass due to all the individual factors (Table 2). Total biomass reduced by 42.2% and 46.2% in Vardan and 23.2% and 21% in Bundel at 40 DAG and 60 DAG, respectively in non-EDU-treated plants compared to 300 mg/L EDU-treated ones. Significant increments in total biomass of 400 mg/L EDU-treated mungbean (Singh et al., 2010) and in five cultivars of wheat (Singh and Agrawal, 2009) in a suburban area having high mean $O_3$ concentrations were reported. In our experiment, a significant increase in root biomass was found for both the cultivars at both ages and EDU
treatments. Shoot and leaf biomass also increased significantly for both the cultivars at 60 DAG. Higher biomass in EDU-treated plants may be attributed to maintenance of high levels of photosynthetic pigments and antioxidant ascorbic acid leading to reduction in lipid peroxidation in the cells, which ultimately maintains the membrane integrity. Significant increases in root, shoot and total biomass of turnip and radish (Hassan et al., 1995) and mungbean (Agrawal et al., 2005) after EDU treatment of 500 mg/L were reported in an area having high O₃ concentrations. However, Szantoi et al. (2009) reported significant reductions of 10.5%, 32.9% and 24% in root biomass and 11.9%, 29.6% and 22% in total biomass at 200, 400 and 600 mg/L EDU-treated R. laciniata plants, respectively, compared to non-EDU-treated ones.

3 Conclusions

*Trifolium repens* is a well known bioindicator of O₃ pollution. *Trifolium subterraneum* has a threshold limit of 30 μg/L for visible injury symptoms. The present study clearly showed that O₃ concentration exceeded the threshold limit in a three-month experimental period and significantly increased the oxidative stress in both cultivars leading to significant losses of photosynthetic pigments and photosynthetic efficiency which ultimately led to reduction in plant growth and biomass accumulation. EDU treatment protected both the cultivars from visible injuries and helped to retain their higher antioxidant potential, which reduced the damage of cellular membrane as depicted by lower lipid peroxidation. EDU concentration of 300 mg/L showed a better protection for various parameters including biomass as compared to 150 mg/L EDU. Cultivar Bundel showed a higher tolerance to O₃ than Vardan, which showed higher biomass accumulations as well as photosynthetic efficiency under EDU treatments compared to non-EDU-treated ones. However, further research is required on different plants, particularly at molecular levels to understand the exact role of EDU in ameliorating the oxidative stress induced by O₃.

Acknowledgments

The authors wish to express their sincere thanks to authorities of Banaras Hindu University and Ministry of Environment and Forests, Govt. of India for financial support, Head, Department of Botany, Banaras Hindu University for laboratory facilities and to Prof. W. J. Manning, Department of Plant, Soil and Insect Sciences, University of Massachusetts, Amherst, USA for providing EDU as gift.

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