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Role of gamma radiation in changing phytotoxic effect of elevated level of ozone in *Trifolium alexandrinum* L. (Clover)

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ABSTRACT

The present study was conducted on clover (*Trifolium alexandrinum* L. cv Wardan), to investigate the effect of ambient and elevated (ambient +10 ppb O_3) ozone (O_3) on plants grown in open top chambers (OTCs) germinated from gamma (γ) irradiated seeds. Dry seeds were subjected to irradiation with 0, 5, 10 and 20 krad doses of γ rays from ^{60}Co source. Dose dependent differential responses were observed on growth and biomass, photosynthetic pigments, metabolites, antioxidative defense system of plant. Growth parameters and biomass of plants were severely affected under elevated O_3 with increasing radiation doses, except, 5 krad which showed a reverse trend of response. Photosynthetic pigments and total soluble proteins were also reduced with higher dose of γ radiation and elevated O_3. Reactive oxygen species formation and membrane damage increased significantly to different extents. Plants grown from seeds irradiated with low dose (5 krad) of γ irradiation depicted more induction of antioxidants (enzymatic and non–enzymatic) than higher doses suggesting their high ameliorative capability against elevated O_3. Principal component analysis has also confirmed that plants grown from 5 krad γ irradiated seeds performed better against O_3 depicting reduction in negative effect against elevated O_3. The experimental findings evidently showed that 5 krad γ radiations altered the O_3 induced stress and thus minimized the loss in biomass of the test plant.

Keywords: *Elevated ozone, phytotoxic effect, reactive oxygen species, Trifolium alexandrinum L., gamma radiation*

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1. Introduction

Air quality in India is progressively being degraded as a consequence of rapid development in industrial and transport sectors during the last three decades (Agrawal et al., 2005). As a result, the level of tropospheric ozone (O_3) has been increased to concentrations that can adversely affect the plants and other living organisms (Tripathi and Agrawal, 2012). Phytotoxicity of O_3 is due to its oxidative capacity through induction of reactive oxygen species (ROS) in plant cells, such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), superoxide radicals ($^{\cdot}O_2^-$) and singlet oxygen ($^1O_2$) (Pell et al., 1997). Ozone diffuses through stomatal pores at the leaf surface, dissolves and decomposes rapidly to produce toxic ROS, which are capable of initiating oxidative events led to visual symptoms of O_3 injury, retardation of growth and severe loss of yields in crop plants (Sarkar et al., 2010).

Concentrations of O_3 continuously increased under current emissions trends due to increased emission of O_3 precursors and projected to increase about 20–25% by 2050 and 40–60% by 2100 (Meehl et al., 2007). Critical levels for crops and semi–natural vegetation assumes that concentrations of O_3 exceeding a threshold of 40 ppb during daylight hours are harmful for vegetation defined as AOT40, for agricultural crops, a 3 month AOT40 of 3 ppm h during the growing seasons (Mills et al., 2007). Detrimental effect of O_3 was also identified in grassland communities including sensitive species such as clover due to O_3 pollution (Mills et al., 2011). Monitoring of O_3 concentrations in Asia indicates that monthly mean O_3 concentrations now commonly reach 50 ppb during important agricultural growing seasons which is high enough to cause deleterious effects on plants (Emberson et al., 2009). Plants have acquired capacity to stimulate various antioxidative enzymatic and non–enzymatic defense mechanisms which remove ROS to alleviate cellular damage caused by O_3 (Calatayud et al., 2003). There are several chemicals that have been used to protect plants from O_3 induced stress under controlled ambient conditions (Manning, 2000). The use of chemicals that cause stomatal closure such as phenyl mercuric acetate and monoethyl esters of decenylsuccinic acid can protect plants from entry of O_3 into leaves. An approach which is commonly followed to assess the O_3 injury in plants is the use of ethylenediurea (N–[2–(2–oxo–1–imidazolidinyl) ethyl]–N phenylurea) was first reported by Carnahan et al. (1978). Gamma radiation was used in plants as it interacts effectively with atoms and molecules in cells, particularly water, to produce free radicals which affect differentially physiological and biochemical processes of the plants (Kovacs and Keresztes, 2002). Low dose of γ irradiation prior to seed sowing may stimulate awakening of the young embryo, led to increase cell division, growth, enzyme activation and yield of the plants (Moussa, 2011).

The present study is a first attempt in order to investigate the level of amelioration by applying γ radiation against elevated O_3 on plants. Therefore, the objective of the present study was to investigate whether pre–treatment of seeds of *Trifolium alexandrinum* L. (clover) with different doses of γ radiation has any ameliorative effect on its germination and growth at ambient and elevated levels of O_3 under natural field conditions.

2. Material and Methods

2.1. Experimental site

The experiment was carried out at the Botanical Garden of Banaras Hindu University at a suburban area of Varanasi, situated in the eastern Gangetic plains of Indian subcontinent at 25°14′ N latitude, 82°03′ E longitude, and 76.19 m above sea level.

2.2. Experimental design

Experiment was designed as split plot in which O_3 exposure is main plot and gamma (γ) radiation treatments as sub plot. Dry and healthy seeds of clover cv Wardan was irradiated with 0, 5, 10, 20 and 25 krad dose of gamma rays (^{60}Co) at Floriculture Section, National Botanical Research Institute, Lucknow. Plants germinated from γ irradiated seeds were exposed with two levels of O_3 [non filtered ambient air (AO) and non–filtered ambient air +10 ppb elevated O_3 (EO)]. Therefore, plants grown at ambient level of O_3 were designated as ($AO\gamma_0$, $AO\gamma_5$, $AO\gamma_{10}$, and $AO\gamma_{20}$) with corresponding elevated O_3 exposed plants as ($EO\gamma_0$, $EO\gamma_5$, $EO\gamma_{10}$ and $EO\gamma_{20}$).

2.3. Experimental setup

The experiment was performed in open top chambers (OTCs) installed at experimental site under natural field conditions by following the design of Bell and Ashmore (1986). Each OTCs are of 1.5 m diameter and 1.8 m height, consisting of an aluminum frame work covered by 0.25 mm thick polyethylene cover. At the base of the chamber polyethylene cover was double layered with holes perforated at specific distances throughout to ensure uniform gas distribution inside the OTC. Each OTC was connected to a heavy duty air blower via conducting duct. Flow rate of the blower was adjusted so as to allow three times air changes per minute. There were three replicate chambers for each treatment. Plants were exposed with elevated O_3 in the respective OTCs with the help of O_3 generator (Model Systrocom, India) attached to the respective blowers, for proper mixing of O_3 with the air entering inside the chamber with daily O_3 fumigation 6 h day^{-1} (09:00–15:00 h) of local time.

2.4. Raising the plants

Gamma irradiated seeds of *Trifolium alexandrinum* L. cv Wardan were sown in three rows inside the OTCs. Seeds were obtained from Indian Grassland and Fodder Research Institute, Indian Council of Agricultural Research, Jhansi, India. Plants were thinned after one week of germination and maintain a uniform distance of 15 cm, manual weeding was done time to time over the entire course of experiment and plants were irrigated regularly to maintain uniform soil moisture. Recommended doses of fertilizer (N, P, K 40:30:40 kg ha^{-1} as urea, single superphosphate, and muriate of potash, respectively) were added during the preparation of the field.

2.5. Ozone monitoring

Ozone concentrations were monitored by using non–dispersive UV absorption photometric O_3 analyzer (Model APOA 370, HORIBA Ltd., Japan) for 9 h day^{-1} from 09:00 to 18:00 h during the study period. Ambient O_3 monitoring was done continuously with O_3 analyzer at the experimental site and elevated O_3 monitoring was done at regular interval of time from air sample drawn through a 15 m long inert Teflon tube (0.35 cm diameter) placed randomly above the canopy of plants. Exposure index for O_3, i.e. AOT40 (accumulated O_3 over a threshold concentration of 40 ppb during daylight hours) was calculated by using the following formula (Mills et al., 2007):

$$\text{AOT } 40 = \sum_{i=1}^{n} [CO_3 - 40]\, i \qquad (1)$$

For CO_3>40 ppb; (AOT40 ppb h), where, CO_3 is the hourly O_3 concentration in parts per billion (ppb), i is the index, n is the number of hours with CO_3>40 ppb over the 3–month growing period that has been set as the evaluation period for respective crops.

2.6. Plant sampling and analysis

Growth parameters and biomass. Plants were randomly selected and samplings were done at 40, 70 and 100 days after germination (DAG). For growth and biomass determinations, monoliths of 10×10×20 cm^3 containing intact roots were carefully dug out at random from each OTC. Growth parameters recorded were: plant height, number of leaves plant^{-1} and leaf area. Leaf area was measured using portable leaf area meter (Model LI–3000, LI–COR, Inc., USA). For biomass determination, plants parts were oven dried (80 °C) till constant weight achieved then weighed separately and added to get total biomass of each plant expressed as g plant^{-1}.

Lipid peroxidation and reactive oxygen species. Malondialdehyde (MDA) content, a product of lipid peroxidation (LPO) was estimated by thiobarbituric acid (TBA) reaction and reactive oxygen species i.e., hydrogen peroxide content (H_2O_2 content), superoxide radical production rate ($^{\cdot}O_2^-$ production rate) and solute leakage were determined by methods already described by Mishra et al. (2013).

Photosynthetic pigments. Random samples of plant's leaves were taken in triplicate from each OTC at 40, 70 and 100 DAG. Total chlorophyll and carotenoid contents were extracted from leaf samples by using 10 mL of 80% acetone, optical densities were measured at 480, 510, 645, and 663 nm and estimated by using the formulae given by Maclachlan and Zalik (1963) and Duxbury and Yentsch (1956), respectively.

Metabolites and antioxidants. Ascorbic acid content, total protein content, total phenol and activities of antioxidative enzymes i.e., ascorbate peroxidase activity (APX), peroxidase activity (POD) expressed as purpurogallin formation, superoxide dismutase activity (SOD) measured as 50% reduction of nitroblue tetrazolium and glutathione reductase activity (GR) were assessed according to methods described in Tripathi et al. (2011).

Statistical analyses. The significance of differences between treatments was calculated by Student's *t*–test at different sampling intervals. To analyze individual and interactive effects of gamma (γ) radiation, O_3 treatment and age of plant on the assessed parameters, three–way analysis of variance (ANOVA) was conducted. Pearson's correlation test was also done to explore the correlation among changes in various observed parameters at 70 DAG. For the multivariate analysis of the assessed parameters, principal component analysis (PCA) was performed. The entire data sets were subjected to PCA based on the correlation matrix with the rotation method of Varimax with Kaiser normalization. The entire statistical tests were performed by using SPSS software (SPSS Inc., version 16.0).

3. Results

3.1. Ozone monitoring

Eight hourly monitoring of O_3 was conducted during the experimental period reveals that average ambient O_3 concentration was 47.4 ppb ranging from 27.3 to 63.3 ppb. While average elevated O_3 concentration during experimental period was 55.6 ppb ranging from 35.9 to 69.8 ppb (Figure 1). The AOT40 value was calculated as 3 896.8 ppb h under ambient O_3 level, while the AOT40 under elevated O_3 concentration was 5 302.9 ppb h.