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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.

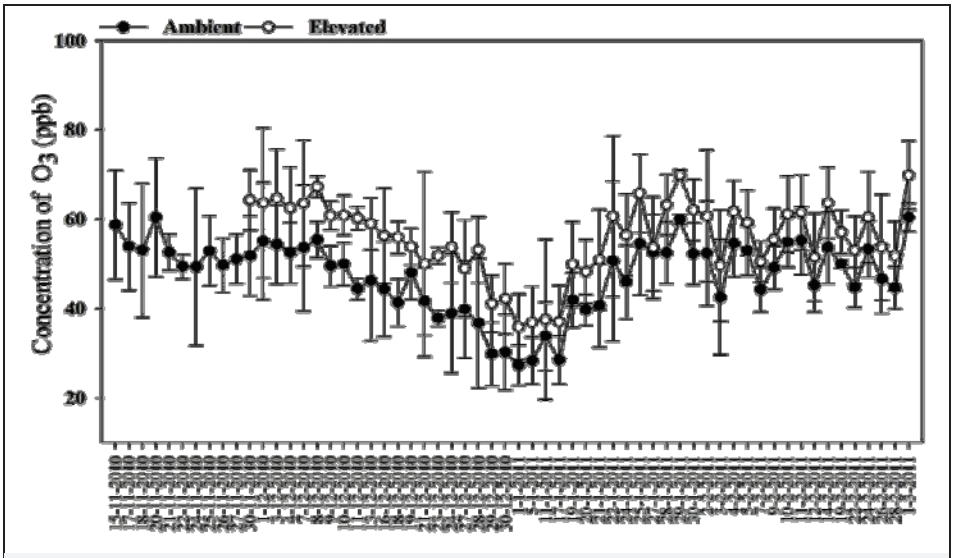

Figure 1. *Monitoring of ambient and elevated levels of O_3 concentration (ppb) during experimental period.*

3.2 Seed germination rate (%)

Seed germination test after γ irradiation of seeds revealed significant reduction in the germination with increasing γ dose in test plant. Maximum germination of 90% was observed in non–treated seeds (0 krad) followed by 5 krad (82%), 10 krad (73%), 20 krad (55%), and minimum germination of 45% were observed in the seeds irradiated with 25 krad of γ radiation therefore, LD_{50} was obtained at 25 krad dose of γ irradiated seeds (Figure 2).

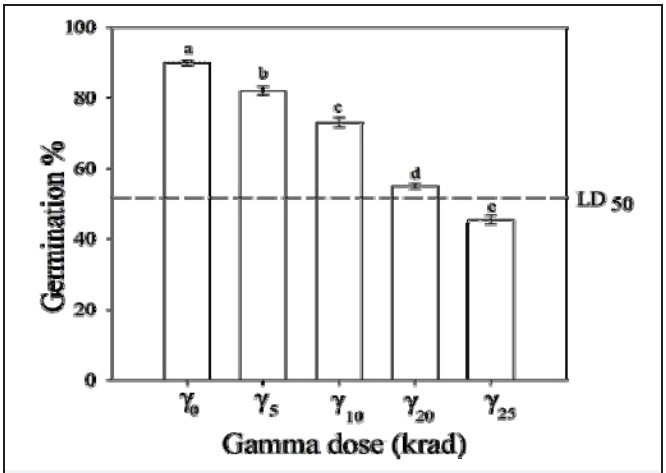

Figure 2. *Variation in germination % and LD_{50} in seeds of Clover irradiated with γ rays (0, 5, 10, 20 and 25 krad), different letters on bars indicate significant differences among different γ doses irradiated seeds at p<0.05 according to Duncan's test.*

3.3. Growth parameters and total biomass

Variation in plant height was observed in plants germinated from seeds pre–treated with γ irradiation. Maximum reduction of 36.4% was observed in $EO\gamma_{20}$ at 100 DAG as compared to other treatments. However, plant height increased significantly in $EO\gamma_5$ treatment by 11.3 and 12.1% at 40 and 100 DAG, respectively (Table 1; see the Supporting Material, SM, Figure S1). Reduction in number of leaves $plant^{-1}$ observed with increasing dose of γ radiations except at 5 krad where an increment was recorded. Leaf area also followed the same trend with all the treatments and, maximum reduction of 13.8% (70 DAG) was observed in $EO\gamma_{20}$ while minimum reduction of 12.1% (100 DAG) was observed in $EO\gamma_0$. Simultaneously, significant increment by 11.5% observed in $EO\gamma_5$ at 40 DAG than plants germinated with seeds exposed with same dose of γ radiation under ambient conditions of O_3 (Table 1). Total biomass was negatively affected by elevated O_3 exposure in 33% in $EO\gamma_0$, 24.1% in $EO\gamma_{10}$ and 36.4% in $EO\gamma_{20}$ except in $EO\gamma_5$ where a significant increment of 12.2% was recorded at 100 DAG (Table 1).

Three way ANOVA showed significant variations in plant height and total biomass due to age (A), gamma radiation (γ) and O_3 treatment (T) and their interactions (A×γ, A×T, γ×T and A×γ×T). Number of leaves varied significantly with A, γ, T and γ×T while leaf area varied significantly with individual factor of A, γ, T and their interactions A×γ, G×T, and A×γ×T (see the SM, Table S1).

3.4. Lipid peroxidation and reactive oxygen species

A significant increment in malondialdehyde concentrations was observed in all the treatments with maximum increment of (23.7%) in $EO\gamma_{20}$ followed by $EO\gamma_0$ (20.4%), $EO\gamma_{10}$ (18.6%) and minimum in $EO\gamma_5$ 15.9% at 40 DAG (Figure 3).

Significant increment in formation of reactive oxygen species ($^{\cdot}O_2^-$ and H_2O_2) was observed in all the treatments. Maximum increase in H_2O_2 content was found in $EO\gamma_{20}$ (43.2%) followed by $EO\gamma_{10}$, $EO\gamma_0$, and minimum was observed in $EO\gamma_5$ (19.4%) at 40 DAG (Figure 3). Similar trend of increments was followed by $^{\cdot}O_2^-$ production rate and solute leakage in all the treatments. Maximum increase in $^{\cdot}O_2^-$ production rate and solute leakage were found in $EO\gamma_{20}$ (26.6 and 33.1%, respectively), at 40 DAG, while, minimum increments were noticed at $EO\gamma_5$ (15.7 and 9.3%, respectively) at 70 DAG (Figure 3). Differential foliar injury symptoms in all treatments as small pale yellow and brown flecks appeared on upper surface that turned into bifacial necrosis which was more severe in plants germinated from higher dose of γ irradiated seeds (see the SM, Figure S1).

Three–way ANOVA revealed that LPO varied significantly with all individual factors (A, γ and T) and their interactions. H_2O_2 content varied only with individual factors while, $^{\cdot}O_2^-$ production rate showed significant variation of A and γ and solute leakage showed significant variations due to A, γ, A×γ and A×T (see the SM, Table S1).

Table 1. *Variation in plant height, number of leaves, leaf area and total biomass of clover plants pre–treated with different doses of γ radiation (0, 5, 10 and 20 krad) under ambient (AO) and elevated (EO) levels of O_3. Values are Mean±SD*

Parameter/Treatment	40 DAG	70 DAG	100 DAG
	Plant height (cm)		
$AO\gamma_0$	12.66±0.57	24.11±0.98	45.11±2.90
$EO\gamma_0$	8.53±0.39 [a]	16.97±0.74 [c]	30.27±2.06 [a]
$AO\gamma_5$	12.93±0.58	25.93±0.99	48.02±2.95
$EO\gamma_5$	14.40±0.57 [a]	28.85±0.98 [c]	53.85±2.92 [b]
$AO\gamma_{10}$	12.24±0.43	21.64±0.82	38.95±2.21
$EO\gamma_{10}$	8.81±0.53 [a]	16.29±0.94 [a]	29.53±2.22 [a]
$AO\gamma_{20}$	12.23±0.58	20.47±0.92	38.18±2.33
$EO\gamma_{20}$	8.74±0.46 [c]	13.54±0.95 [a]	24.28±2.60 [a]
	Number of leaves plant^{-1}		
$AO\gamma_0$	96.67±5.67	72.05±6.62	123.67±8.60
$EO\gamma_0$	82.67±5.67 [b]	62.64±4.94 [d]	108.67±9.67 [c]
$AO\gamma_5$	98.80±5.29	74.22±6.38	121.47±8.44
$EO\gamma_5$	107.04±6.28 [a]	82.78±7.75 [c]	134.71±8.49 [d]
$AO\gamma_{10}$	91.89±6.91	62.62±5.34	120.23±8.81
$EO\gamma_{10}$	80.33±4.56 [a]	55.32±3.56 [c]	106.33±8.54 [c]
$AO\gamma_{20}$	82.22±4.67	52.75±5.44	110.56±9.34
$EO\gamma_{20}$	73.22±6.56 [a]	45.45±3.56 [c]	99.22±9.90 [d]
	Leaf area (cm^2)		
$AO\gamma_0$	50.02±2.44	85.45±2.43	119.78±7.51
$EO\gamma_0$	45.18±2.38 [c]	77.27±1.60 [a]	108.90±8.38 [a]
$AO\gamma_5$	50.95±2.23	89.76±2.67	115.68±8.59
$EO\gamma_5$	55.78±2.21 [b]	97.59±3.96 [a]	127.51±7.49 [d]
$AO\gamma_{10}$	52.03±2.28	85.12±2.01	122.17±8.25
$EO\gamma_{10}$	44.86±3.19 [b]	75.95±3.99 [a]	108.22±7.41 [c]
$AO\gamma_{20}$	50.59±2.15	76.94±3.48	117.61±7.85
$EO\gamma_{20}$	43.42±2.10 [c]	69.84±3.70 [d]	108.44±8.73 [d]
	Total Biomass (g plant^{-1})		
$AO\gamma_0$	12.66±1.11	24.11±1.98	45.11±1.48
$EO\gamma_0$	8.53±1.14 [a]	16.97±1.75 [a]	30.27±1.56 [c]
$AO\gamma_5$	12.93±1.14	25.93±1.98	48.02±1.65
$EO\gamma_5$	14.02±1.13 [a]	28.85±1.92 [a]	53.85±1.72 [c]
$AO\gamma_{10}$	12.24±1.15	21.64±1.52	38.95±1.62
$EO\gamma_{10}$	8.81±1.13 [a]	16.29±1.55 [a]	29.53±1.83 [c]
$AO\gamma_{20}$	12.23±1.28	20.47±1.12	38.18±1.63
$EO\gamma_{20}$	7.74±0.24 [d]	13.54±1.15 [c]	24.28±1.70 [b]

[a] *p<0.001,*
[b] *p<0.01,*
[c] *p<0.5,*
[d] *not significant*

3.5. Photosynthetic pigments

Total chlorophyll and carotenoids in plants showed varying degree of reductions with all the treatments. The extent of reduction in total chlorophyll was maximum in $EO\gamma_{20}$ (32.7%) followed by $EO\gamma_{10}$ (26.8%), $EO\gamma_0$ (23.0%) and minimum in $EO\gamma_5$ (11.0%) at 40 DAG (Figure 4). Elevated O_3 exposure exhibited reduction in carotenoid content and magnitude varied with γ irradiation doses with maximum reduction in $EO\gamma_{20}$ (17.9%) and minimum in $EO\gamma_5$ (6.8%) at 100 DAG (Figure 4). Three–way ANOVA revealed significant variations in total chlorophyll due to A, γ, T, A×γ and γ×T and carotenoids due to all the individual factors and their interactions except due to A×γ×T (see the SM, Table S1).

3.6. Metabolites

Variation in increment of total phenol was observed in all treatments at all the ages with maximum increment was detected at $EO\gamma_{20}$ (17.1% at 40 DAG) and lowest in $EO\gamma_5$ (6.3% at 70 DAG) (Figure 5). Maximum significant reduction of protein by 23.0% at 100 DAG was found in treatment $EO\gamma_0$ and minimum reduction of 15.1% was observed in $EO\gamma_5$ at 70 DAG (Figure 5). Ascorbic acid content also increased significantly at $EO\gamma_5$ (23.6%) while minimum increment of 5.3% was observed in $EO\gamma_{20}$ at 70 DAG (Figure 5).

Three–way ANOVA depicted that total phenol varied significantly with A, γ, T and due to their interactions except A×γ×T. Protein varied significantly A, γ, T and γ×T, while ascorbic acid varied significantly only with individual factors A, γ and T (see the SM, Table S1).

3.7. Antioxidative enzymes activities

Antioxidative enzymes activities showed significant variations with different doses of γ radiation. APX activity increased maximally in $EO\gamma_5$ (12.0% at 70 DAG) and least in $EO\gamma_{20}$ (5.5%) at

100 DAG. Plants germinated from 5 krad irradiated seeds showed maximum increase of POD activity while showed lesser increments with increase in γ dose. Activity of SOD showed maximum increment of 16.9% at 70 DAG was observed in $EO\gamma_5$ and minimum increment of 6.3% was observed in $EO\gamma_{10}$ at 70 DAG. In treatment $EO\gamma_5$, GR activity was highest, moreover, other treatments showed less stimulation in the activity of this enzyme (Figure 6).

Three–way ANOVA revealed that APX activity varied significantly with A, γ, T, A×T and γ×T. POD activity varied significantly with A, γ, T, A×γ, A×T. SOD activity varied significantly with A, γ, T and γ×T. However, GR activity varied significantly with A, γ, T and A×γ. (see the SM, Table S1).

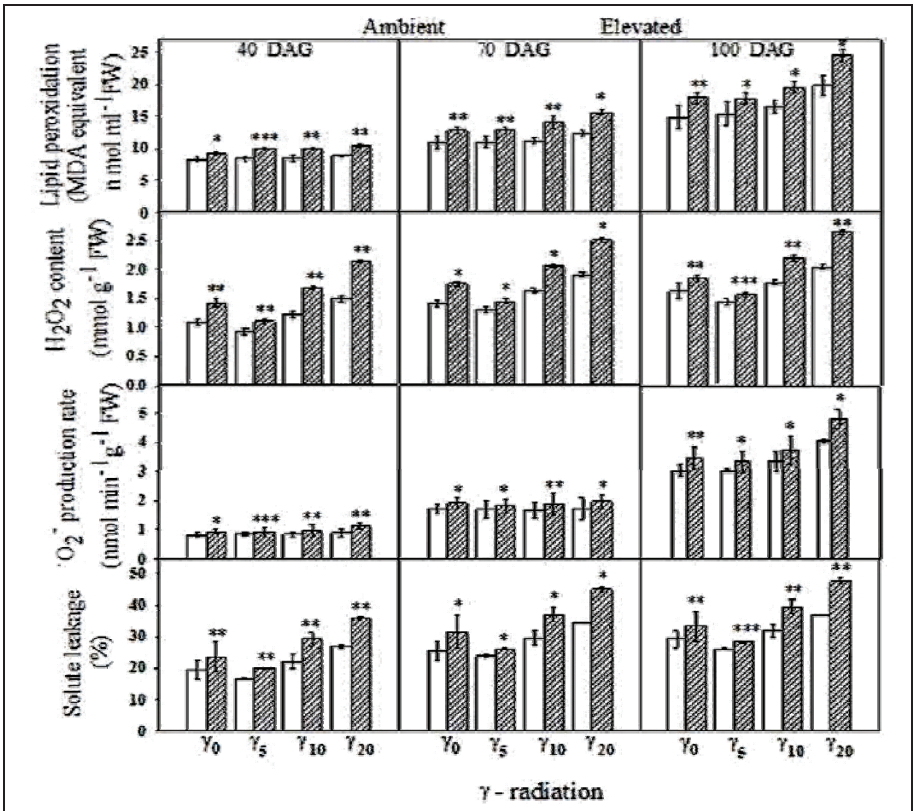

Figure 3. *Effect of ambient and elevated O_3 on LPO, H_2O_2 content, $^{\cdot}O_2^-$ production rate and solute leakage of clover plants pre–treated with different doses of γ radiation (Mean±SD). Levels of significance are *p<0.5, **p<0.01, ***p<0.001, NS=not significant.*

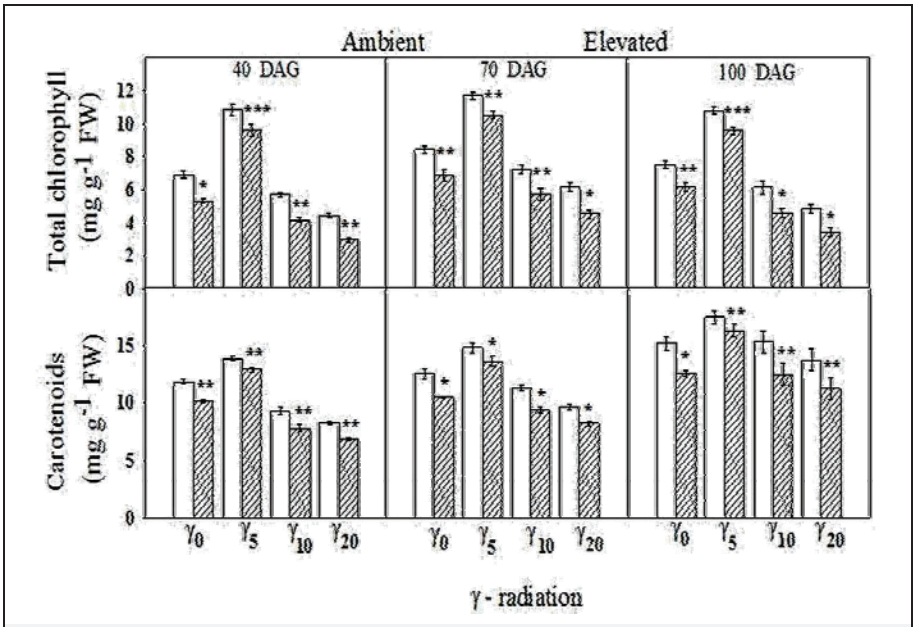

Figure 4. *Effect of ambient and elevated O_3 on total chlorophyll and carotenoids of clover plants pre–treated with different doses of γ radiation (Mean±SD). Levels of significance are *p<0.5, **p<0.01, ***p<0.001, NS=not significant.*

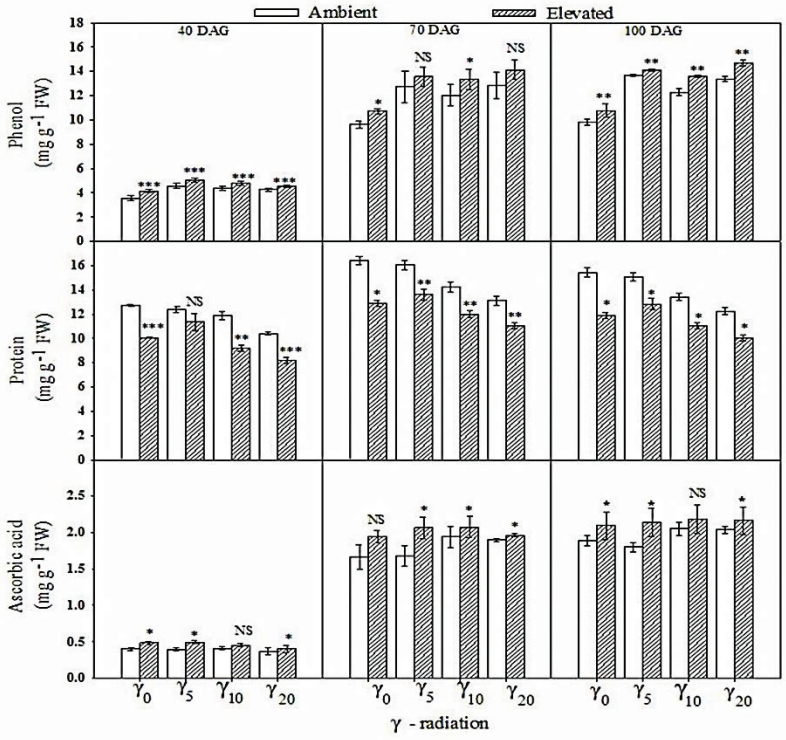

Figure 5. *Effect of ambient and elevated O_3 on phenol, protein and ascorbic acid of clover plants pre–treated with different doses of γ radiation (Mean±SD). Levels of significance are *p<0.5, **p<0.01, ***p<0.001, NS=not significant.*

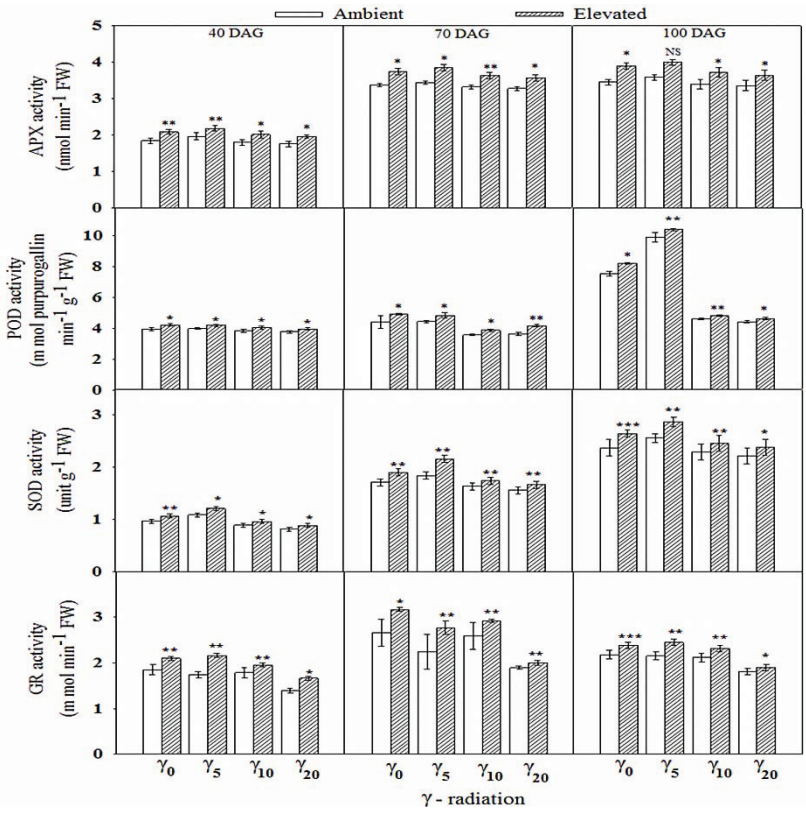

Figure 6. *Effect of ambient and elevated O_3 on APX, POD SOD and GR activities of clover plants pre–treated with different doses of γ radiation (Mean±SD). Levels of significance are *p<0.5, **p<0.01, ***p<0.001, NS=not significant.*

Various assessed parameters of plants germinated from different γ doses (0, 5, 10 and 20 krad) exposed with O_3 were subjected to PCA (Figure 7). Morphological and biochemical trait variance were extracted into two components by four PCA approaches for different γ doses. In all PCA analysis, two components accounted respective grouping of the assessed parameters. The result of PCA of plants germinated from 0 krad γ irradiated seeds against ambient and elevated levels of O_3 showed that plant height, number of leaves, leaf area, total biomass, total chlorophyll and carotenoids had their respective loading values more than 0.5 grouped for the first component, while LPO, antioxidative enzymes, metabolites, and ROS had values more than 0.5 grouped for the second component. Similarly PCA results of 10 krad and 20 krad follows the similar grouping of variables as that of 0 krad. While, plants germinated from 5 krad dose of γ irradiation showed variation in distribution of variables then three γ doses. Therefore, it is clear from PCA result that 0, 10 and 20 krad showed reductions in morphological parameters, total biomass, photosynthetic pigments and total protein which are located towards the positive end of the component 1 axis while, increase in the metabolites, ROS, LPO and antioxidative enzymes are located towards the positive end of component 2 axis. However, PCA result of 5 krad showed grouping of morphological and biochemical parameters in component 1 as these parameters performed better against O_3 depicting that 5 krad reduced the negative effect elevated O_3 while, protein and photosynthetic pigments were least affected and grouped in negative end of the component 2 (Figure 7).

4. Discussion

Variations were observed in O_3 concentrations during the experimental period as prevailing tropical climatic conditions of Varanasi favor O_3 formation (Tripathi et al., 2011). Differential responses were observed in plants grown from γ irradiated seeds exposed with ambient and elevated levels of O_3.The stimulating effects of γ radiation on seed germination may be designated to the activation of RNA or protein synthesis, occurred during the early stage of seeds germination after irradiation (Abdel–Hady et al., 2008). High dose of γ irradiation may increase the frequency of chromosomal damage which stimulates reduction in seed germination percentage led to the reduction in plant growth and development (Borzouei et al., 2010). Gamma irradiation showed significant negative correlation with plant height, number of leaves and leaf area (see the SM, Table S2). Growth performance of the plants deteriorated because of high radiation dose may block the cellular DNA, hence slow down the plant growth (Mokobia and Anomohanran, 2005). Although, plants germinated from seeds pre–treated with low dose of γ radiation (5 krad) grew better under elevated O_3 might be due to more investment of photo-assimilates to the vegetative parts, its utilization for repair, and activation of antioxidative defense systems in response to O_3. Gamma radiation predominantly effects the formation of ROS caused due to water radiolysis in the plant cell. ROS may induce photolytic degradation by oxidation, and damaging membrane structures by peroxidation led to increase permeability and, eventually, causes cell death. Positive correlation was observed in response of plants germinated from different γ doses and exposure with elevated level of O_3 led to further increase in the level of ROS and membrane damage (see the SM, Table S2).

Photosynthetic pigments were found to be highly sensitive to radiation as γ radiation may modify the plastid structures like thylakoids and altered photosynthetic pigment content (Kovacs and Keresztes, 2002). Ling et al. (2008) also obtained lower chlorophyll content from γ irradiated plantlets as compared to non–irradiated plantlets of sweet orange (*Citrus sinensis*).

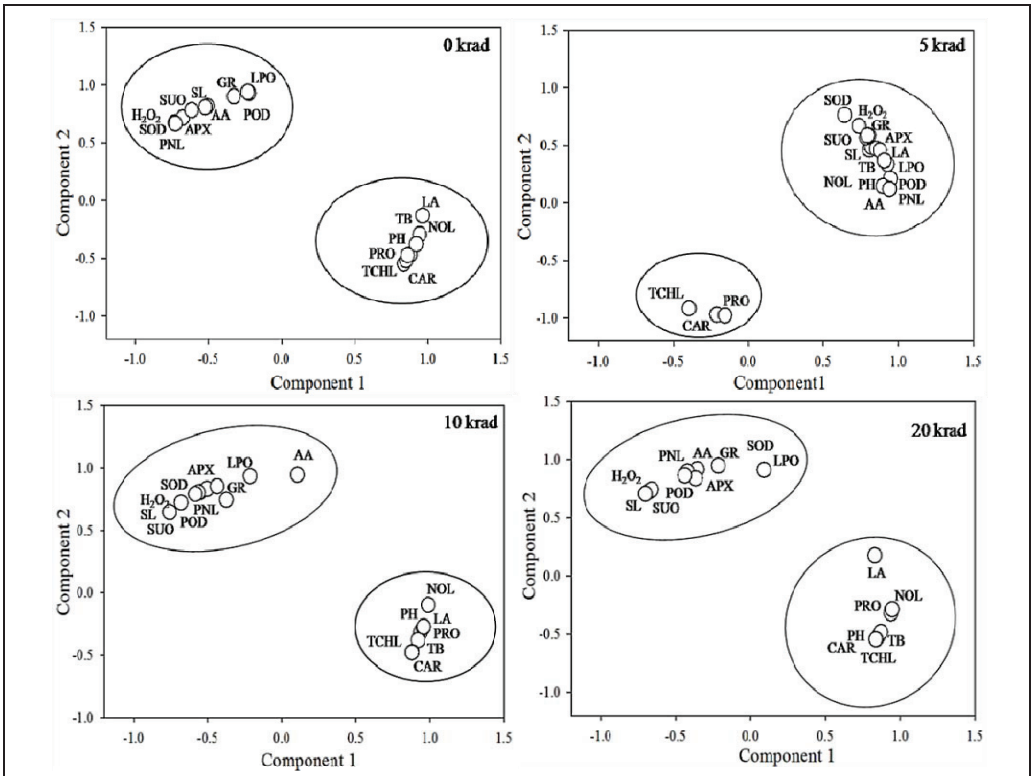

Figure 7. *Loading plots for all the observed parameters assessed in clover plants germinated from different doses of γ irradiated seeds (0, 5, 10 and 20 krad) exposed with ambient and elevated levels of O_3 on principal components. Each symbol represents the parameters taken into account i.e., PH– plant height, NOL– number of leaves, LA– leaf area, TB– total biomass, TCHL– total chlorophyll, CAR– carotenoids, LPO– lipid peroxidation, H_2O_2– H_2O_2– content, SUO–O_2^- production rate, SL– solute leakage, AA– ascorbic acid, PRO– protein content, PNL– phenol content, APX– APX activity, POD– POD activity, SOD– SOD activity and GR– GR activity.*

Observed increase in total phenolic contents was beneficial for antioxidant properties and showed positive correlation with γ irradiation and O_3 treatment (see the SM, Table S2). Total phenols correlated with plant resistance against many stresses and its increment of total phenols in γ irradiated plants has also been reported by Lee et al. (2009). Reductions in protein content with increment in dose of γ radiation and showed significant negative correlation between γ irradiation and protein content (see the SM, Table S2). Protein contents were reduced in plants exposed with elevated O_3. Total protein content showed differences depending on the dose of γ radiation which significantly influencing ROS production subsequently causes modification on structure by breakage of covalent bond of peptide chain, fragmentation and aggregation of proteins (El–Beltagi et al., 2011).

Among the non–enzymatic antioxidants, ascorbic acid is the most abundant antioxidant in plants serves as an integral weapon in defense against ROS. Increment in ascorbic acid was less with higher dose of γ irradiation might be because of its utilization by APX as electron donor in the neutralization of H_2O_2 in cytosol and in different cellular compartments, therefore, negative correlation was observed between ascorbic acid and γ radiation (see the SM, Table S2). Similarly, higher ascorbic acid content was also reported in two cultivars of wheat under elevated O_3 (Mishra et al., 2013) and plants germinated from γ irradiated seeds (Mohammed et al., 2012).

Antioxidant enzymes such as APX, POD, SOD and GR functions as effective quenchers for ROS operates with the sequential and simultaneous actions and their level may determine differential level of sensitivity among plants. Activities of enzymes involved in ROS scavenging were also altered by γ irradiation as the activities of peroxidase, catalase and superoxide dismutase in radish (*Raphanus sativus*) leaves were enhanced by γ irradiation dose of 10 Gy (Lee et al., 2003). Low dose γ radiation led to an efficient induction of antioxidative enzymes involved in ROS scavenging (Zaka et al., 2002) as these radicals do not kill the cells, but rather produce genetic abnormalities and immediately triggers anti-oxidative defense systems by modulating the activities. Therefore, positive correlation was observed between antioxidative enzymes and γ irradiation treatment (see the SM, Table S2).

The present study clearly showed that negative impact of O_3 on total biomass of the test plant with increased concentration of O_3 excluding the plants germinated from 5 krad γ irradiation. Significant negative correlation was observed between total biomass and γ irradiation (see the SM, Table S1). Further, γ irradiated seeds of the low dose of 5 krad may be helpful to ameliorate the O_3 induced stress and minimized loss of biomass grown under elevated O_3.

Overview of the differential response of plants germinated from different γ doses under ambient and elevated levels of O_3 extracted from PCA. Different γ doses induced noticeable changes in assessed parameters with varied magnitude. Plants germinated from 5 krad γ irradiation favorably affected various morphological and biochemical characteristics of plants leading to significant increment in the biomass as compared to other γ doses. Five krad treatments was affected favorably led to significant increment in biomass as compared to other γ doses. Therefore, 5 krad dose of γ radiation might be helpful in protecting plants against O_3 stress as it induce higher activities of antioxidative enzymes and metabolites required for repair. Thus, cumulative effect of biochemical parameters resulted in the enhancement of growth and biomass against O_3 in plants germinated from lower dose of γ radiation.

5. Conclusions

Present study showed that elevated level of O_3 (ambient +10 ppb) is high enough to cause negative effects on growth, biomass and biochemical characteristics of plants. Results clearly revealed that varying response was observed in plants germinated with different γ radiation doses. Findings also imply that plants grown with low dose of γ irradiation (5 krad) treated seeds showed better response against ambient and elevated levels of O_3 by counteract the oxidative stress as compared to higher doses of γ radiation. Study further points out that low dose of γ irradiation may be helpful in ameliorating the O_3 induced damage and thus minimizing the losses in yield.

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Supporting Material Available

F–values and significance level for various growth parameters, total biomass, LPO, reactive oxygen species, photosynthetic pigments, metabolites and antioxidative enzymes of clover plants as obtained by three way ANOVA test (Table S1), Correlation coefficient (*r*) between various measured parameters under different γ radiation and O_3treatment at 70 DAG (Table S2), Variation in plant height and foliar injury symptoms in different treatments ($EO\gamma_0$, $EO\gamma_5$, $EO\gamma_{10}$ and $EO\gamma_{20}$) of *Trifolium alexandrinum* L. (Figure S1). This information is available free of charge via the Internet at http://www.atmospolres.com

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