Chapter-6

Summary and conclusions

The overall results of the present study clearly showed that ozone (O₃) is a major air pollutant at the experimental site and drastically affecting the plants performance. Ozone is a potent air pollutant, important greenhouse gas and also recognized as a major component of global climate change. Presently, increasing trend of O₃ is well known to significantly impair the growth of wide variety of plants (Ainsworth et al. 2012), including forest trees, semi–natural vegetation and various crop plants. In the near future concentration of O₃ is expected to increase by more than 20% by the year 2050. Ozone levels in Asia are increasing with an alarming rate owing to rapid industrialization across the region. Particularly East, South–East and South Asia experience continued deterioration of their air quality mainly due to emissions of nitrogen oxides and other O₃ precursors because of rapid urbanization, industrialization and enormous increase in number of vehicles. Therefore, the current levels of O₃ are high enough to exceed the threshold of many plants and able to cause detrimental alterations in the exposed plants. The magnitude of plant response against O₃ varies significantly from crop to crop and from cultivar to cultivar (Mills et al. 2011; Singh et al. 2014a; Mishra and Agrawal, 2015).

Present study demonstrates the impact of ambient (present) and elevated (projected) levels of O₃ on various phenotypical, physiological, and biochemical characteristics of different clover and mung bean cultivars used for the experimental purpose. Favorable meteorological conditions such as high temperature, dry conditions and higher solar radiation attributed to higher concentrations of tropospheric O₃ in tropical areas. At the experimental site meteorological conditions favoured the O₃ formation and a strong correlation was observed between different meteorological parameters (temperature, sunshine hours and relative humidity) and O₃ concentration. Therefore, variation in concentration of O₃ was observed during mung bean and clover growth period at the experimental site. Thus, in view of deleterious effect of O₃ on plants, present experiment was performed to assess the impact of ambient and elevated levels of O₃ on clover and mung bean cultivars as follows:
1. Assessment of effect of ambient and elevated levels of O3 on clover and mung bean cultivars.

i. Experiment was designed to study the impact of O3 on clover and mung bean plants under field conditions. For this purpose, experiment was performed by using open top chambers (OTCs). During experimental period the mean ambient day time O3 concentration was recorded averagely above 40 ppb, which was high enough to causes negative impacts on plants. According to Meehl et al. (2007), O3 concentration is anticipated to rise by 20–25 % between 2015 and 2050 in the northern hemisphere, thus, in the present study, about, 20 % elevated O3 over ambient (10 ppb O3) was selected to expose the plants.

ii. The present study was carried out in the suburban area of Varanasi with the objective to analyze the effect of ambient and elevated levels of O3 on phenotypical, physiological and biochemical characteristics of different cultivars of clover and mung bean.

iii. Ozone monitoring was performed during day time at the experimental site for 9 h day\(^{-1}\) (0900–1800 h) throughout the growth period of the test plants. Hourly mean ambient O3 often exceeded 40 ppb during the experimental period; therefore, AOT40 values exceeded critical level and considered to be high enough to cause the phytotoxic effects. Consequently, O3 concentrations were recorded towards higher side in the summer months as compared to the winters referred to mung bean and clover growing periods, respectively. Varanasi experienced higher concentration of O3 during past few years reported from earlier studies conducted in rural and sub-urban regions of Varanasi (Rai et al. 2010, Sarkar and Agrawal, 2010b; Tripathi et al. 2011; Mishra et al. 2013a; Singh et al. 2014a; Rai et al. 2015). During present study ambient and elevated O3 concentration along with the AOT40 observed are shown in Table 1.
Table 1 Concentration of O\textsubscript{3} (ppb) and AOT40 (ppb h) during different experimental period at the study site.

<table>
<thead>
<tr>
<th>Year/months</th>
<th>Parameters</th>
<th>Ambient</th>
<th>Elevated</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010 (April-June)</td>
<td>Mean O\textsubscript{3}</td>
<td>64.1</td>
<td>70.9</td>
</tr>
<tr>
<td></td>
<td>AOT40</td>
<td>11491.9</td>
<td>12846.8</td>
</tr>
<tr>
<td>2010-2011 (November-March)</td>
<td>Mean O\textsubscript{3}</td>
<td>49.4</td>
<td>57.6</td>
</tr>
<tr>
<td></td>
<td>AOT40</td>
<td>7371</td>
<td>11727</td>
</tr>
<tr>
<td>2011 (April-June)</td>
<td>Mean O\textsubscript{3}</td>
<td>63.3</td>
<td>68.9</td>
</tr>
<tr>
<td></td>
<td>AOT40</td>
<td>11491.9</td>
<td>12846.8</td>
</tr>
<tr>
<td>2011-2012 (November-March)</td>
<td>Mean O\textsubscript{3}</td>
<td>52.7</td>
<td>58.3</td>
</tr>
<tr>
<td></td>
<td>AOT40</td>
<td>7871.5</td>
<td>11028.0</td>
</tr>
<tr>
<td>2012 (April-June)</td>
<td>Mean O\textsubscript{3}</td>
<td>65.4</td>
<td>71.1</td>
</tr>
<tr>
<td></td>
<td>AOT40</td>
<td>11617.1</td>
<td>14028.9</td>
</tr>
</tbody>
</table>

iv. Test plants were sampled at periodical interval to study the plant responses after exposure of elevated O\textsubscript{3}. Plant responses includes phenotypical characteristics (plant height, number of leaves, leaf area, foliar injury %, biomass and growth indices), physiological characteristics (photosynthetic rate, stomatal conductance and chlorophyll fluorescence) and biochemical characteristics (lipid peroxidation, solute leakage, free radical production rate, histochemical localization of ROS, ascorbic acid, protein, phenol, SOD, CAT, POD, APX, GR, PAL, photosynthetic pigments i.e. total chlorophyll and carotenoids and non-photosynthetic pigments i.e. flavonoids and anthocyanin) and reproductive parameters (number of flowers and pollen viability) also included. After the plant maturity, harvesting and yield characteristics (number and weight of seeds and pods) and seed quality characteristics (seed protein, total soluble sugar, starch and mineral nutrient content) were quantified. Cumulative sensitivity index (CSI) was calculated to evaluate the overall response against O\textsubscript{3}, designated as the sum of individual sensitivity responses of the assessed parameters which showed cultivar specific response.

v. Different growth parameters were negatively affected in clover and mung bean plants against O\textsubscript{3}. Comparison between different characteristics of growth parameters under ambient and elevated levels of O\textsubscript{3}, suggested differential response among test cultivar of both the test plants. Growth indices; RGR and NAR also followed the similar trend of reduction, thus the ascending order of hierarchy in clover cultivar was as follows: Wardan
vi. After plants exposed with elevated $O_3$, induction of free radical accelerated such as, $H_2O_2$ content and $'O_2^-$ production rate. Clover and mung bean plants showed increments in elevated $O_3$ exposed plants as compared to ambient ones. Cultivar specific variation depicted amongst the studied plants. Clover showed maximum induction of $H_2O_2$ content and $'O_2^-$ production rate in cultivar Wardan followed by Bundel, JHB-146, Mescavi, Saidi and Fahli. Mung bean cultivar showed the following ascending trend after exposure of $O_3$, $HUM-1 > HUM-2 > HUM-6 > HUM-26 > HUM-24 > HUM-23$. Histochemical localization of $H_2O_2$ and $'O_2^-$ also revealed high depositions in both the plants exposed with $O_3$. Therefore, histochemical localization technique proved to be a powerful tool in an early diagnosis of $O_3$ injury before the appearance of visible symptoms and can be an insight for screening the sensitivity or tolerance against $O_3$.

vii. Exposure of $O_3$ expressed higher membrane damage (LPO and solute leakage); magnitude of variation was assessed among the cultivars of both the test plants. In clover, cultivar Wardan showed maximum increment in LPO (29.3 %), while minimum in Fahli (14.1 %). Solute leakage was increased under elevated $O_3$ treatment with maximum significant increment in Bundel (28.3 %) and minimum in Fahli (8.0 %) at 30 DAG. Exposure of $O_3$ causes. Significant positive correlation was observed between LPO and solute leakage amongst all the cultivars of mung bean.

viii. Variations exist in appearance and magnitude of foliar injury amongst the test cultivars of both the test plants against elevated $O_3$. After fumigation of elevated $O_3$, test plants showed typical $O_3$ induced foliar injury symptoms as yellowing and chlorosis on adaxial surface of the leaves. According to the foliar injury % (FIP) among clover cultivars, Wardan showed highest FIP and indexed in category “severe” followed by Bundel and JHB-146 having moderate rate of injuries. Other cultivars; Fahli, Saidi and Mescavi were found to be comparatively less sensitive and indexed under categories “slight” and “very slight”. All the six cultivars of mung
bean showed visible foliar symptoms against O₃, ranging from 55.7 % as maximum in HUM-1 to 7.4 % as minimum in HUM-23. By scoring the extent of O₃ induced injury, Wardan was depicted “sensitive” amongst clover cultivars used in the study. Similarly, FIP of mung bean cultivars resulted HUM-1 to be most sensitive followed by HUM-2 and HUM-6.

ix. Elevated O₃ level significantly reduced the physiological parameters such as photosynthetic rate (Ps), stomatal conductance (gs) and chlorophyll fluorescence (Fv/Fm), which significantly reduced against O₃ in all the cultivars of both the test plants. Among all the cultivars of clover, reduction was observed not only in Ps but also in gs under elevated O₃ at all the ages of sampling. Mung bean also showed reductions in Ps, gs and Fv/Fm due to elevated O₃ were observed in all the cultivars and at all the ages of observations, being maximum for HUM-1 and minimum for HUM-23. Elevated O₃ exposure exhibited varying degree of chlorophyll loss in both the test plants. The magnitude of decline, however, varied between cultivars. Maximum significant reductions in total chlorophyll and carotenoids were observed in Wardan (57.3 and 39.2 %) and minimum in Fahli (16.1 and 9.4 %) of clover. Reduction in total chlorophyll was also observed in all the cultivars of mung bean against O₃, however, carotenoids increased significantly in all cultivars under elevated O₃. Minimum increment was observed in HUM-1 (23.0 %) and maximum in HUM-23 (47.9 %). Significant increment was also observed in anthocyanin and flavonoid contents in both the test plants against elevated O₃.

x. Both mung bean and clover exhibited variations in metabolites (ascorbic acid, phenol and protein content. Total phenol and ascorbic acid content showed the increments against O₃ and its order of hierarchy was as follows: Fahli > Mescavi > Saidi > JHB-146 > Bundel > Wardan. Mung bean cultivars also followed the increasing trend after exposure of O₃. However, reduction was observed in protein content of both the test plants. Plant tolerance against O₃ depends largely on the ability to detoxify O₃ and fluctuation in enzyme activities reflects the ability of plant performance against O₃. Elevated O₃ altered the activities of antioxidative enzymes (SOD, POD, APX, GR, CAT and PAL) with varied magnitude in various
culturists of both the test plants. Ozone being a potent inhibitor of reproductive structure reported to affect the viability of pollen and number of flowers. Against elevated $O_3$, plants showed delayed flowering and reduced pollen viability as compared to ambient ones and the response was cultivar specific. Therefore, loss of pollen viability under elevated $O_3$ treatment might affect the yield of both the plants.

xi. Ozone exposure disturbs the normal growth as evidenced by marked decrease in total biomass. Therefore, exposure of $O_3$ causes a marked reduction in total biomass in all the cultivars of clover. Reductions in total biomass were maximum for Wardan (37.5 %) followed by Bundel and JHB-146, Mescavi, Saidi and Fahli. Reduction in yield was also observed in all the mung bean cultivars under elevated $O_3$. Yield was reduced maximally in HUM-1 (15.4 %) followed by HUM-2, HUM-6, HUM-24, HUM-26 and minimum in HUM-23 (13.8 %).

xii. From the obtained results with reference to cultivar sensitive index (CSI), and foliar injury index, it is evident that all the test cultivars of clover showed differential level of sensitivity against $O_3$. Therefore, according to CSI classification of test cultivars, Wardan and Bundel ranked “sensitive” having the CSI $< -50$. Cultivar JHB-146 ranked “intermediate” CSI ($-16.1$) ranged between 10 and $-50$. While the rest of the cultivars (Fahli, Saidi and Mescavi) were found to be comparatively less sensitive as CSI ranged $>10$ and ranked “insensitive”.

xiii. Among mung bean cultivars HUM-1 and HUM-2 ranked “sensitive” with CSI $< -50$, while HUM-6 ranked “intermediate” and other three cultivars were found to be least sensitive as their CSI value ranged $> 10$ and ranked as “insensitive”.

xiv. Results of present study showed that cultivars of clover exhibited phenotypical, physiological, and/or alteration in its metabolism and suggested Wardan and Bundel to be sensitive. Sensitive cultivars can be successfully use as a tool to map $O_3$ effect as bioindicator under Indian conditions for areas experiencing higher concentration of $O_3$.

xv. Further, in view of the anticipated increasing concentrations of $O_3$ in near future, cultivars showed resistance against $O_3$ can be used for cultivation for getting maximum productivity.
2. To investigate the impact of elevated O\(_3\) on clover cultivars developed by gamma irradiated seeds.

According to the results of the objective to investigate whether pre–treatment of seeds of *Trifolium alexandrinum* L. (clover) with different doses of \(\gamma\)–radiation (0, 5, 10, 20 and 25 krad) has any ameliorative effect on its germination and growth under ambient and elevated levels of O\(_3\) under natural field conditions are summarized as follows:-:

i. Low dose of \(\gamma\)–radiation (5 krad) altered the O\(_3\) induced stress and thus minimized the loss in biomass of the test plant. Different cultivars of clover (*Trifolium alexandrinum* L.) Wardan, Bundel, JHB-146, Fahli, Saidi and Mescavi were grown in OTCs germinated from gamma (\(\gamma\)) irradiated seeds. Dry seeds were subjected to irradiation with 0, 5, 10 and 20 krad doses of \(\gamma\)–rays from \(^{60}\)Co source.

ii. Seed germination test after \(\gamma\)–irradiation of seeds revealed significant reduction in the germination with increasing \(\gamma\)–dose in test cultivars and \(LD_{50}\) was obtained at 25 krad dose of \(\gamma\)–irradiated seeds.

iii. Different growth parameters were negatively affected by O\(_3\) exposure and the response was varied with different doses of \(\gamma\)–radiation. Significant increment in formation of ROS (\('O_2^-\) and \(H_2O_2\)) was observed in all the treatments. Similar, increment were also observed in LPO and solute leakage in all the treatment with maximum increment in \(H_2O_2\) content was found in EO\(_{\gamma20}\) followed by EO\(_{\gamma10}\), EO\(_{\gamma0}\), and minimum were observed in EO\(_{\gamma5}\). The content of pigments showed varying degrees of reduction and the magnitude of reduction was affected by different \(\gamma\)–doses. Variation in metabolites such as total phenolics and ascorbic acid was observed among all the treatments in all test cultivars, therefore, increment was observed in all the cultivars with different treatment. Ozone evidently reduced the protein content in all the treatments in accordance with increase in dose of \(\gamma\)–radiation exceptionally, low \(\gamma\)–dose of 5 krad, depicted least reduction in all the cultivars. Antioxidative enzymes activities showed significant variations with different doses of \(\gamma\)–radiation. Cultivar specific variations were also observed with differential activities of antioxidative enzymes. Therefore, increased activity of antioxidative enzymes in all the treatment were arranged in the order as follows: EO\(_{\gamma5}\) > EO\(_{\gamma0}\) > EO\(_{\gamma10}\) > EO\(_{\gamma20}\).
while changes were in cultivars as follows: Fahli > Saidi > Mescavi > JHB-146 > Bundel > Wardan.

iv. Total biomass was negatively affected by elevated O₃ exposure in all the cultivars at all the treatments. Plants germinated from seeds irradiated with γ–radiation showed reduction except in EOγ₅ treatment where the increments in biomass were observed. A significant maximum reduction was observed in Wardan at EOγ₂₀ (36.7 %), while, minimum reduction was observed in Fahli (10.2 %) in EOγ₁₀ at 40 DAG. Treatment with EOγ₅ showed the increment in all the cultivars arranged as follow:-: Fahli > Saidi > Mescavi > JHB-146 > Bundel > Wardan. Therefore, results 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 O₃ by counteract the oxidative stress as compared to higher doses of γ–radiation. At 5 krad dose of γ–radiation, least response of measured parameters was observed among Fahli, Saidi, while Mescavi showed its greater resistance to O₃. Study further points out that lower dose of γ–irradiation may be helpful in ameliorating the O₃ induced damage and thus minimizing the losses in yield.

3. To assess the amelioration provided by foliar application of ascorbic acid on mung bean cultivars under ambient and elevated levels of O₃.

The present experiment clearly suggests that mean ambient O₃ concentration around Varanasi has unfavorably affected mung bean cultivars performance leading to significant loss in yield. Foliar spray of ascorbic acid (Asc) protected the plant against the negative impact of O₃ stress. The results showed cultivar specific variations in morphological and biochemical characteristics of mung bean due to foliar spray of ascorbic acid (Asc) under elevated O₃ summarized that exogenous application of Asc promotes plant's performance by providing protection against O₃ induced oxidative stress. It may also be used in screening of the mung bean cultivars against O₃ phytotoxicity. Experiment was performed on six mung bean cultivars (Vigna radiata L. HUM-1, HUM-2, HUM-6, HUM-23, HUM-24 and HUM-26) in OTCs under field conditions and Asc was applied as foliar spray prior to the exposure of ambient (AO+) and elevated (EO+) levels of O₃. Effect of exogenous application of Asc solution was examined on the growth, photosynthetic pigments, biochemical and yield
characteristics of mung bean cultivars against O$_3$. There were three replicate chambers for each treatment, i.e. ambient O$_3$ with ascorbic acid (AO+), elevated O$_3$ (ambient+10 ppb) with ascorbic acid (EO+) and ambient O$_3$ without ascorbic acid (AO) with same amount of distilled water, Ascorbic acid solution of 12.5 mM prepared freshly in double distilled water was applied as foliar spray (100 ml plant$^{-1}$). The foliar application of Asc definitely helped to stimulate plant response through enhancement of photosynthetic pigments, reducing membrane damage, higher activities of antioxidative enzymes and maintaining higher biomass and yield under elevated O$_3$. Plants showed better response with Asc under ambient and elevated levels of O$_3$ with cultivar specific variations in total biomass. Maximum increment of 33.7 and 28.2 % in HUM-1 was observed in AO+ and EO+, respectively, and least increment was observed in HUM-23 (18.7 and 15.6 %). There was the noticeable increment in the quality and quantity of seeds with the application of Asc. Cultivar HUM-1 was the most sensitive as it showed more ameliorative response against O$_3$ as compared to other test cultivars. The study certainly contributes to emphasize the possibility of utilization of Asc as phytomonitoring tool in screening the crop cultivars and also its usefulness in providing the protection against O$_3$ induced oxidative damage.

Therefore, the present study led to the following conclusions:

1. Monitoring of ambient concentration of O$_3$ depicted variations with higher concentration during warmer months of mung bean growth period.
2. Higher O$_3$ concentrations were recorded during the reproductive phase of both the test plants resulted marked reduction in yield.
3. During experimental period, AOT40 values exceeded the limit that has been shown to cause significant damage to plant.
4. Response of clover and mung bean cultivar under ambient and elevated levels of O$_3$, were evaluated with the following results:
   i. Exposure of O$_3$ causes significant negative effect on phenotypical, physiological, biochemical, biomass and yield characteristics of both the test plants.
   ii. Induction of reactive oxygen species accelerated after O$_3$ exposure led to membrane damage and visible leaf injury symptoms.
iii. Phenotypical manifestations such as appearance of specific foliar injury and growth reductions were observed in all the cultivars of both the test plants.

iv. Ozone triggers the destruction of pigments in plants and declining trend correlated with reduced photosynthetic rate of both the test plants under O\textsubscript{3} stress.

v. Differential sensitivities of cultivars against O\textsubscript{3} were assessed in correspondence with metabolite production and antioxidative potential.

vi. More deterioration in number and size of flowers and pollen viability observed in sensitive cultivars of both the test plants.

vii. Lower enhancements of antioxidants showed more susceptibility amongst Wardan and HUM-1 cultivars of clover and mung bean, respectively.

viii. Exposure of O\textsubscript{3} causes marked decrease in biomass and yield of various cultivars of both the test plants.

ix. According to CSI, susceptibility against O\textsubscript{3} among clover cultivars was arranged as: Wardan > Bundel > JHB-146 > Mescavi > Saidi > Fahli.

x. Similarly, mung bean cultivars were arranged as: HUM-1 > HUM-2 > HUM-6 > HUM-24 > HUM-26 > HUM-23.

5. Effect of \(\gamma\)-irradiation of seeds prior to sowing on various growth and biochemical characteristics of clover grown under ambient and elevated levels of O\textsubscript{3} are as follow:

i. Varying response was observed in seeds of different cultivars germinated after irradiation with different doses of \(\gamma\)-irradiation.

ii. Plants grown with low dose of \(\gamma\)-irradiated (5 krad) seeds depicted more induction of antioxidants than higher doses suggesting their higher ameliorative capacity against elevated O\textsubscript{3}.

iii. Irradiation of 5 krad dose of \(\gamma\)-radiation favorably affected various morphological and biochemical characteristics of plants and altered O\textsubscript{3} induced stress led to minimized the loss in biomass.
6. Role of foliar spray of ascorbic acid under ambient and elevated levels of ozone on various characteristics of mung bean can be summarized as follows:

   i. Foliar application of Asc helped to improve plant's performance against O$_3$ through enhancement of photosynthetic pigments, reducing membrane damage by scavenging reactive oxygen species (\(\cdot O_2^-\) and H$_2$O$_2$), higher activities of antioxidative enzymes resulted in maintaining higher biomass and yield.

   ii. Cultivar HUM-1 was most sensitive as it showed more ameliorative response against O$_3$ as compared to other test cultivars.

   iii. Foliar application of ascorbic acid proved its usefulness in assessing effects of O$_3$ and also in screening the sensitivity amongst mung bean cultivars.