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Effect of SO_2 - NO_2 fumigation on wooden tree seedlings in open top chamber system

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Abstract. The present study has been performed on one year old tree saplings of *Azadirachta indica* (L.), *Cassia siamea* (L.), *Dalbergia sissoo* (Roxb.), *Eucalyptus rostrata* (L.), *Mangifera indica* (L.) and *Schyzygium cumini* (L.) in order to assess the effect of exposure of SO_2-NO_2, alone and combination of two gases. Tree saplings have been exposed to an average of 495 μg m^{-3} SO_2 and 105 μg m^{-3} NO_2 for 40 d at the rate of 4 h d^{-1} during 10:00 am to 01:00 pm in OTC. Total chlorophyll, specific leaf area (SLA), nitrate reductase (NR) activity, foliar protein, free proline content and free amino acids (AAs) of foliage have been the plant parameters, taken into consideration to evaluate the effect of gaseous exposure. Exposure of two gases has caused reduction in total chlorophyll content ($P < 0.05$, 0.01). Physiological and biochemical process has been seemed to be altered noticeable due to the combined effect of SO_2+NO_2 followed by SO_2 alone ($P < 0.05$, 0.01). NO_2 mediated stress has produced, stimulatory and inhibitory responses in tree saplings. Results reveal that tree saplings have been attempted to absorb the NO_2 through N assimilation pathway. *E. rostrata*, *C. siamea* have been emerged as moderate tolerant to SO_2 mediated stress followed by *A. indica*. Response pattern of *S. cumini*, *M. indica* and *D. sissoo* set them as good indicators of SO_2 - NO_2 exposure. Effects of two gases on tree saplings have been found to be synergistic.

Keywords: Open Top Chamber (OTC); SO_2; NO_2; stimulatory-inhibitory response; tree saplings

1. Introduction

Atmospheric gases like SO_2, NO_2 and O_3 are considered damaging pollutants and their phytotoxicity are well-known (Ozolincius *et al*. 2005, Paoletti *et al*. 2010). These polluting gases enter in leaves through stomata following the same diffusion pathway of the CO_2 (Zeiger 2006). Sulphur dioxide (SO_2) is considered as widespread air pollutant, and its environmental effects include acidification of soils, lakes and rivers and damage to plants and crops. Nitrogen-containing air pollutants (NO, NO_2 and NH_3) can affect vegetation indirectly, via chemical reactions in the atmosphere, or directly after being deposited on vegetation, soil or water (WHO 2000).

Phytotoxicity of SO_2 on growth suppression and yield reduction has been well documented (Katiyar 2000, Katiyar and Dubey 2001). In addition to SO_2, NO_2 is also considered an important phytotoxic agent (Muzika *et al*. 2004). Lower concentration NOx species (NO_2, NO) can induce a

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variety of physiological alteration and reduce the growth rate without producing identifiable substantial damaging features (Katiyar and Dubey 2000, 2001, Bach *et al*. 2004, Singh *et al*. 2003, 2005, Rajput and Agrawal 2005). It is also assumed that the combined effect of $SO_2 + NO_2$ can cause more harmful effects than the sum of their individual effects (Bach *et al*. 2004, Muzika *et al*. 2004).

Open top chambers (OTCs) are very applicable in the study of plant-pollutants exposure analysis especially for wooden trees at early stages. Using OTCs, plants can be exposed to individual pollutant as well as analyzed under natural environmental conditions. Moreover, the sapling age is a critical phase in the life cycle of all seed plants and it becomes more crucial when planted in stressful environment (Wright and Westoby 1999).

Thus, in this study it is hypothesized that saplings age of wooden trees may be crucial for exposure of SO_2, NO_2 gaseous. Quantification of altered physiological and biochemical characteristics in tree saplings, due to the exposure of SO_2 NO_2 gases could be used as indicator species of pollutant gases in tropical environment.

2. Materials and methods

2.1 Tree saplings

The selection of tree species was done according the types of wooden trees found in and around the Ujjain city (23.182778°N 75.777222°E). Seeds of six tree species *Azadirachta indica* (L.), *Cassia siamea* (L.), *Dalbergia sissoo* (Roxb.), *Eucalyptus rostrata* (L.), *Mangifera indica* (L.) and *Schyzygium cumini* (L.) were grown in earthen pots containing black cotton soil and kept in greenhouse. Average day/night temperature inside the greenhouse was 24.1°C (± 1.28) / 16.3°C (± 1.44) at the same time as day/night relative humidity inside the green house was 55% (± 2.1) and 66% (± 3.9), respectively. This study was carried out during 1997-1998.

2.2 Exposure chamber

One-year-old tree saplings were transferred into exposure chambers and were preconditioned for 72 h. In this study exposure chambers were essentially thick plastic enclosures, one cubic meter sized, a cubical frame of welding iron rods. As name indicates the top of the chamber was left open. Air, inside the chamber was supplied through perforated tube for uniform distribution and supply of gases to the plants kept inside. The air temperatures within the chamber were 30°C (± 4.39) / 21.8°C (± 1.2) day/night respectively, whereas day and night relative humidity (Rh) were 56% (± 4.9) and 69% (± 2.9) respectively.

2.3 Design of experiment

Experiment was divided into four sets with triplicate based on the supply air inside the open top chamber (OTC) -

Set A: No supply of outside air
Set B: Outside air supply with sulphur dioxide gas (SO_2)
Set C: Outside air supply with nitrogen dioxide gas (NO_2)
Set D: Outside air supply with mixture of $SO_2 + NO_2$ gas.

2.4 Generation of sulphur dioxide and nitrogen dioxide

Sulphur dioxide was generated by bubbling air of known speed through an impinger containing 1% aqueous sodium metabisulphite (Hashimato and Tanaka 1980). Nitrogen dioxide was generated by bubbling SO_2 gas through dilute HNO_3 at a fixed rate.

2.5 Measurement of gases

The generated and available gaseous concentration at chamber inlet was measured with regular interval using Toxic Gas Monitor 555 (CEA Inst. USA). The plants were exposed to 495 (± 3.09) $\mu g\ m^{-3}$ SO_2 and 105 (± 2.91) $\mu g\ m^{-3}$ NO_2 and same concentration in combination for 40 d at the rate of 4 h d^{-1} during 10:00 am to 01:00 pm. A cumulative dose of SO_2 and NO_2, given to the tree saplings seedlings was 79200 and 16800 units for SO_2 and NO_2, respectively.

2.6 Plant analysis

After 40 d exposure of two gases foliage of tree saplings were sampled and part of fresh leaves samples was analyzed for total chlorophyll content (Arnon 1949), nitrate reductase (NR) activity (Srivastava and Mathur 1980), free proline (Bates *et al*. 1973) and foliar protein content (Lowry *et al*. 1951). Another part of fresh leaves has been used for measuring leaf area and dry weight, after keeping the material in oven at 80°C for 24 h. Data were used to calculate specific leaf area (SLA) (Rao and Dubey 1988). Finally dry leaves of each species have been used for analysis of free amino acid (AAs) of leaves following the method of Jayaraman (1981).

2.7 Statistical Analysis

Whole experiment has been divided in four set (each in triplicate) including control set kept with no supply of pollutant gases. Magnitude of changes in metabolic pool of plants has been quantified calculating the percentage difference between exposed and unexposed saplings. Moreover, paired *t*' test has been applied in order to evaluate the significant differences between quantitative values of various parameters of unexposed and exposed wooden tree saplings. The assumptions of ANOVA have been met to facilitate the analysis of interactions between gaseous exposure and plant response using two-way analysis of variance.

3. Results

Total chlorophyll content has always been higher in unexposed tree saplings as shown in Fig. 1. Analysis of total chlorophyll content reveals that 40 d exposure of 495 $\mu g\ m^{-3}$ SO_2 and 105 $\mu g\ m^{-3}$ NO_2 have brought about reduction in photosynthetic pigment content. Reduction in total chlorophyll content has been found on higher range in *A. indica* (13.93%), *E. rostrata* (12.29%), *M. indica* (10.96%) and *S. cumini* (10.35%) as a result of exposure of SO_2. Decline in total chlorophyll content owing to combined exposure of SO_2 and NO_2 has been varied from 9 to 20% in all tree saplings, being better in *S. cumini* (19.56%) and *C. siamea* (19.31%). Decrease in total chlorophyll content has been noticed only 2 to 6% in all tree sapling due to exposure of NO_2. Paired *t*' test has revealed that SO_2 singly as well as accompanying to NO_2 has been able to produce significant reduction in total chlorophyll content in all tree saplings ($p < 0.05$). At the same time, statistically significant decrease in total chlorophyll content has been noticed in *S. cumini* and *A. indica* ($p < 0.05$) due to exposure of NO_2 (Table 1).

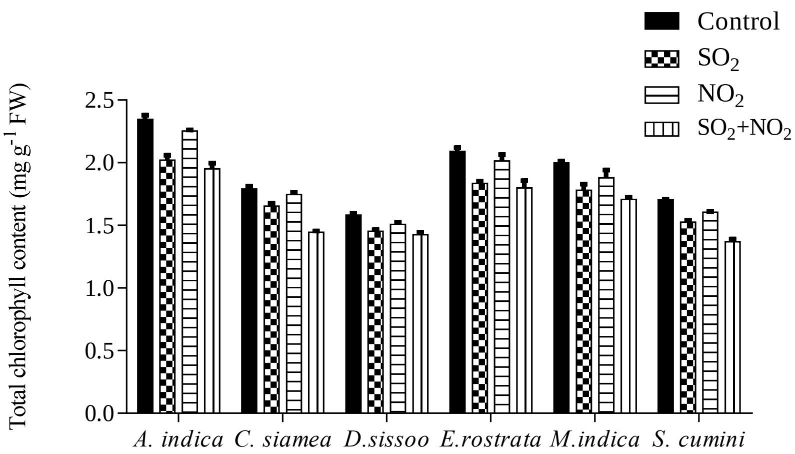

Fig. 1 Total chlorophyll content (mg g^{-1} fresh wt.) in tree saplings due to exposure of SO_2, NO_2 (Vertical bars & lines are means & standard error respectively)

Table 1 Maximum to minimum of effect of SO_2-NO_2 exposure in tree saplings

Pollutants	Total chlorophyll content	Specific leaf area	NR activity	Free amino acids	Foliar protein Content	Proline content
SO_2	*A. indica** *E. rostrata** *M. indica** *S. cumini** *D. sissoo** *C. siamea**	*D. sissoo*^{ns} *M. indica** *A. indica** *E. rostrata** *C. siamea** *S. cumini*^{ns}	*S. cumini** *M. indica*^{ns} *D. sissoo** *A. indica*^{ns} *C. siamea*^{ns} *E. rostrata**	*M. indica** *S. cumini** *A. indica** *D. sissoo** *E. rostrata** *C. siamea**	*D. sissoo** *M. indica** *A indica** *S. cumini*^{ns} *C. siamea** *E. rostrata*^{ns}	*D. sissoo** *M. indica** *A. indica** *C. siamea** *E. rostrata*^{ns} *S. cumini**
NO_2	*M. indica*^{ns} *S. cumini** *D. sissoo*^{ns} *A. indica** *E. rostrata*^{ns} *C. siamea*^{ns}	*S. cumini** *D. sissoo*^{ns} *E.rostrata*^{ns} *M. indica** *A. indica** *C. siamea*^{ns}	*M.indica*^{ns} *S. cumini** *D. sissoo** *A. indica*^{ns} *E. rostrata** *C. siamea*^{ns}	*A. indica** *C. siamea** *S. cumini** *E. rostrata** *D. sissoo** *M. indica**	*D. sissoo** *C. siamea*^{ns} *S. cumini*^{ns} *A. indica*^{ns} *M .indica*^{ns} *E. rostrata**	*M. indica*^{ns} *D. sissoo*^{ns} *C. siamea** *A. indica*^{ns} *S. cumini*^{ns} *E. rostrata*^{ns}
$SO_2 + NO_2$	*S .cumini** *C. siamea** *A. indica** *M. indica** *E. rostrata** *D. sissoo**	*E. rostrata** *C. siamea** *A. indica** *S. cumini** *D. sissoo*^{ns} *M. indica**	*S. cumini** *D. sissoo** *A. indica*^{ns} *M. indica*^{ns} *C. siamea*^{ns} *E. rostrata**	*A. indica** *C. siamea** *S. cumini** *D. sissoo** *M. indica** *E. rostrata**	*M. indica** *S. cumini** *D. sissoo*^{ns} *C. siamea** *A. indica*^{ns} *E. rostrata*^{ns}	*M. indica** *C. siamea** *D. sissoo** *E. rostrata*^{ns} *A. indica** *S. cumini**

*, ns = Results of paired *t*' test applied between exposed and unexposed tree saplings.
* = Significant paired *t* test ($p < 0.05$),
^{ns} = Not significant

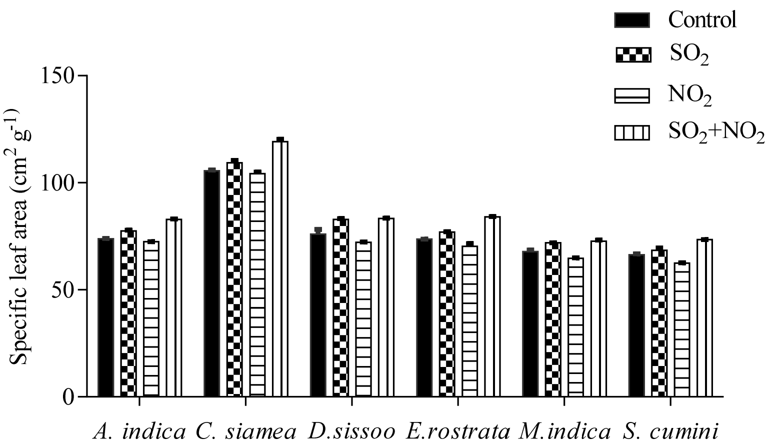

Fig. 2 Specific Leaf Area (SLA) (cm^2 g^{-1}) in tree saplings due to exposure of SO_2, NO_2 (Vertical bars & lines are means & standard error respectively)

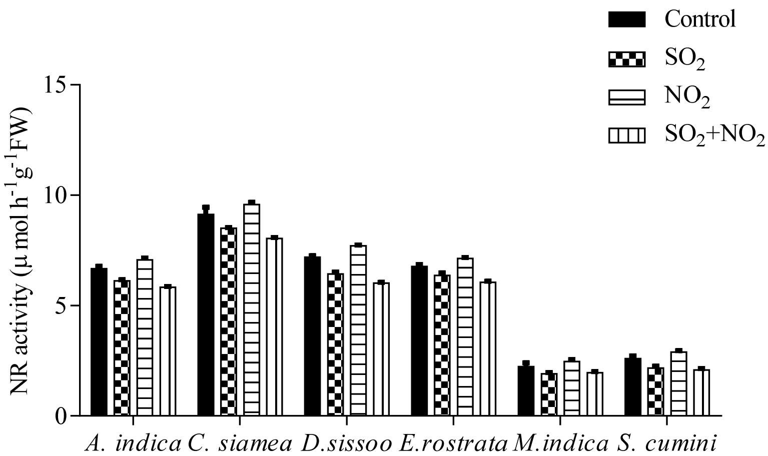

Fig. 3 Nitrate reductase (NR) activity (µmol $h^{-1}g^{-1}$ fresh wt.) in tree saplings due to exposure of SO_2, NO_2 (Vertical bars & lines are means & standard error respectively)

Enhanced SLA is mark of unpleasant consequences of pollutant or any stress conditions. In this experiment, increased or decreased SLA has been strongly dependent upon the nature of gas. Exposure of NO_2 has brought about decrease in SLA by 2 to 6% over their respective controls. In contrast, SO_2 alone or in combination with nitrogen dioxide has shown an increase of 4 to 14% respectively in exposed tree saplings as given in Fig. 2. As result of exposure of SO_2 singly and accompanying NO_2, a significant differentiation in SLA has been observed in *A. indica*, *C. siamea*, *E. rostrata* and *M. indica*, *S. cumini* ($p < 0.05$). Similar results have been found in *C. siamea*, *M. indica* and *S. cumini* as a consequence of exposure of NO_2.

Nitrate reductase activity has been increased in all tree saplings in response to exposure of NO_2 from 5 to 14% in tree saplings, with significantly increased in *A. indica*, *D. sissoo,* and *E. rostrata* ($p < 0.05$). Fig. 3 has explained that NR activity has been dropped from 10 to 19% in all tree species as one of the effect of combined exposure of SO_2+NO_2, considerable diminution in *A.*

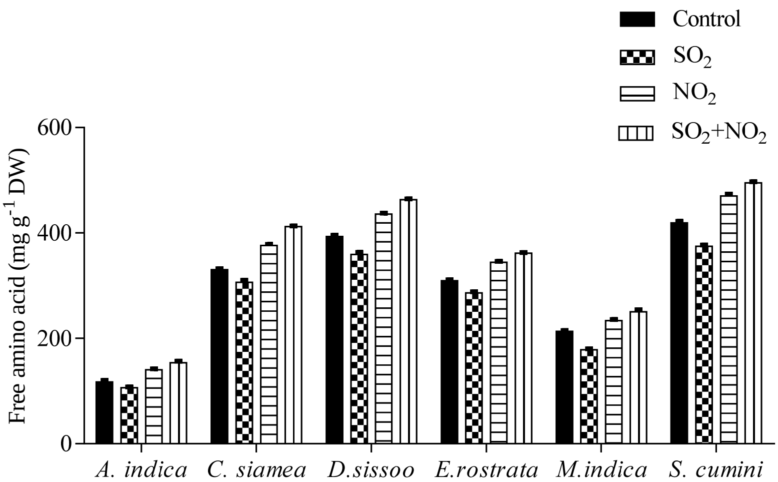

Fig. 4 Free amino acid (AA) content (μg g^{-1} dry wt.) in tree saplings due to exposure of SO_2, NO_2 (Vertical bars & lines are means & standard error respectively)

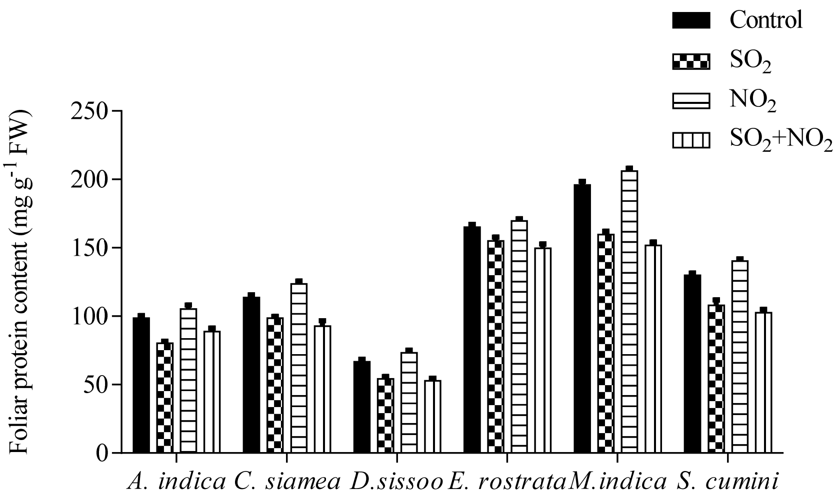

Fig. 5 Foliar protein content (mg g^{-1} fresh wt.) in tree saplings due to exposure of SO_2, NO_2 (Vertical bars & lines are means & standard error respectively)

indica and *E. rostrata* ($p < 0.05$). The significant reduction in NR activity has been distinguished in D. *sissoo*, *E. rostrata* and *S. cumini* ($p < 0.05$) as a result of SO_2 exposure. It is important to note that NR activity has been reduced from 5 to 16% in the saplings corresponding to their controls due to exposure of SO_2.

A distinct shift has been observed in the content of free amino acids in all combination of gaseous exposure. Amino acids contents have been decreased from 7-16% over their respective controls due to exposure of SO_2. On the other hand, exposure of combination of $SO_2 + NO_2$ seems to be synergistic, as it has enhanced the free amino acid contents from 16 to 31% in all tree saplings corresponding to their controls. Exposure of NO_2 has significantly increased the content of amino acid from 9 to 19% in all saplings as pointed out in Fig. 4.

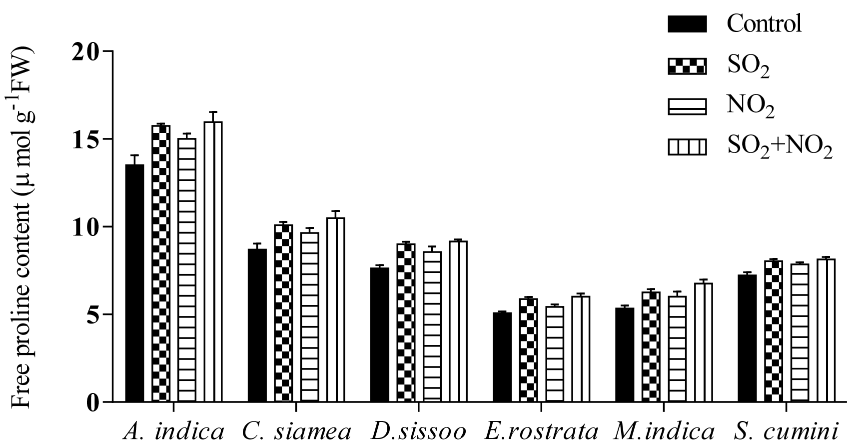

Fig. 6 Free proline content (μmol g^{-1} fresh wt.) in tree saplings due to exposure of SO_2, NO_2 (Vertical bars & lines are means & standard error respectively)

Increase in content of free amino acid seems to alter the amount of protein content, as a consequence of combined exposure of SO_2 and NO_2. Foliar protein content has been declined from 9 to 22% in all tree saplings in response to combined exposure of $SO_2 + NO_2$. Whereas exposure of SO_2 alone has been capable to cause 6 to 18% decrease in content of foliar protein in all tree saplings corresponding to their controls. A favorable effect has been seen in the foliar protein content of tree saplings due to exposure of NO_2, since amount of foliar protein content has been increased from 2 to 9% as compared to their controls as revealed within the Fig. 5.

Free proline has always been increased in exposed tree saplings over their respective controls. It has been increased by 13 to 26% in all tree saplings attributable to combined exposure of $SO_2 + NO_2$. Sulphur dioxide exposure has shown the increase of 11 to 18% in proline content in tree saplings. At the same time, exposure of NO_2 has increased the level of proline from 7 to 12% in all tree saplings (Fig. 6). Paired *t*' test illustrate that statistically significant increase in amount of free proline has been observed in *C. siamea* ($p < 0.05$).

Changes in chlorophyll content, SLA, NR activity, free AAs, foliar protein content indicated the adverse effects of exposure of two gases. As shown by the analysis of variance, exposure of SO_2, NO_2 have significantly stimulated the quantity of all plant parameters analyzed in this study. The effects of SO_2 and NO_2 have been found to be the cause of phytotoxicity in tree saplings, characteristically to exposure of SO_2 singly and in combination of NO_2. Table 1 illustrates the maximum to minimum effect of gaseous exposure in various parameters of tree saplings.

4. Discussion

Sulphur dioxide (SO_2) is known as a strongly damaging air pollutant. After conversion into sulphite and bisulfate ions in aqueous solution, it becomes a strong nucleophilic agent that attacks numerous compounds in the cell. At low concentrations plants effectively detoxified the bisulfite and sulfite, consequently, SO_2 turned into accessible to plants as sulfur source (Zeiger 2006). Sulphite oxidase (*SO*) is an enzyme essential for detoxifying excessive amounts of sulphite in the cell which is important for the survival of the plant (Lang *et al*. 2007).

Similarly, NO_2 dissolves in cells and gives rise to nitrite ions (NO_2^-) (toxic at high concentrations) and nitrate ions (NO_3^-) that enter into nitrogen metabolism, if they had been absorbed through the roots (Zeiger 2006). On the other hand, plants have the ability to assimilate the NO_2 gas through nitrate reductase and nitrite reductase to ammonia (WHO 2000). It has been confirmed that one third of the total nitrogen derived from the NO_2, taken up through the leaves stomata, does not convert in inorganic or organic nitrogen (Takahashi *et al*. 2007).

Magnitude of toxicity of SO_2 and NO_2 gases might be dependent according to the the amount of *SO* enzyme, nitrogen assimilation pathway as well as antioxidants of plants. Green plants defend themselves against different stresses, such as O_3, SO_2, NOx using their antioxidant system (Tiwari and Agrawal 2011). Ascorbic acid, peroxidase, superoxide dismutase, catalase are the enzymes that play vital role in scavenging free radicals in plants system (Tripathi and Gautam 2007). However, continuous high level of exposure of pollutant gases may reduce the activities of the antioxidant systems in plants without visible injury (Chauhan and Joshi 2010) and may lead to loss in photosynthetic pigments (Pandey 2005) resulting in large changes in photosynthesisc rates and productivity (Ali *et al*. 2008, Calder *et al*. 2010).

Reduction in chlorophyll content due to air pollutant gaseous exposure have also been reported by Katiyar and Dubey (2000), Pandey (2005), Rajput and Agrawal (2005), Saquib *et al*. (2010), Singh *et al*. (2005). Combined exposure of SO_2 and NO_2 has reduced the growth and development of tree (Muzika *et al*. 2004). At low concentration, NO_2 has been found to produced the favorable effect on plant growth as well as phytotoxicity has been turned out at exposure of higher concentration of NO_2 (Ma *et al*. 2007, WHO 2000).

Gaseous exposure has led to closure of stomata and thereby decrease in biochemical fixation of CO_2 by photosynthesis (Ali *et al*. 2008, Calder *et al*. 2010) and caused the decline in dry matter production and yielding too (Ali *et al*. 2008). Decrease in dry weight of leaves of all tree saplings due to the exposure of SO_2 alone as well as combined with NO_2 has correlated with the effect on specific leaf area (SLA). Decrease in leaf area has been reported in plants exposed to air pollutant gases (Pandey 2005). The SLA represents the plant growth and its physiological conditions. Increasing the amino acid production and protein synthesis has improved added dry matter production in NO_2 treated leaves (Sabratnam and Gupta 1988).

The toxic effects of the SO_2 exposure includes decrease in the nitrate reductase (NR) activity, as observed in all tree saplings. This toxic effect was reported by Kumawat *et al*. (2003). Exposure of SO_2 has caused the reduction in pool of free amino acids and the content of foliar protein in plants (Kumawat *et al*. 2003, Anjali *et al*. 2012). Decrease in the total protein content has also been evident in leaves of soybean after fumigation of $SO_2 + NO_2$ (Hamid and Jawed 2009).

Despite the phytotoxic nature of NO_2, plants have the ability to take up atmospheric NO_2 and incorporate it into different nitrogen pools within the plant (Vallano and Sparks 2007). Direct foliar uptake is a direct addition of N to plant metabolism and could potentially more readily influence the plant growth compared to soil deposited *N* (Sparks 2009).

Nitrate reductase activity often controls the overall assimilation rate of nitrate. There are two distinct pools for nitrate in plant tissues i.e., storage and metabolic pools, only nitrate of the metabolic pool functions as a substrate for NR activity and contributes to organic nitrogen. In the present investigation, increase in NR activity may be due to the more availability of nitrate in the metabolic pool of the plants due to exposure of NO_2 as reported by Tripathi and Gautam (2007).

Proline has been found to be accumulated within the leaves when the plants were subjected to environmental stresses like SO_2 and NO_2 (Katiyar and Dubey 2000, 2001, Kumawat *et al*. 2003). Similar pattern of results has also been reported in this study. Accumulation of proline in plants under stress is a result of the reciprocal regulation of two pathways: increased expression of proline synthetic enzymes and repressed activity of proline degradation (Peng *et al*. 1996). Proline may play a role in minimizing the damage caused by dehydration (Mohammadkhani and Heidari 2008).

It is apparent that *M. indica*, *S. cumini*, *D. sissoo* have been emerged as good indicator of SO_2 exposure. Simultaneously, *E. rostrata* has been exhibited better tolerance to simulated SO_2 treatment as compare to *C. siamea* and *A. indica*. The response of tree saplings has been prominent and severe due to combined exposure of $SO_2 + NO_2$. The species *E. rostrata*, *C. siamea* and *A. indica* showed the maximum potential tolerance to the combined exposure of SO_2 and NO_2.

When tree saplings were treated with NO_2, no clear gradation could be distinguished for chlorophyll content, SLA and proline. On the other hand, increase in contents of N-enriched parameters like NRA (4-14%), foliar protein (2-9%) and AAs (9-19%) were apparent in all tree saplings as consequence of NO_2 exposure. Thus, it can be concluded that fraction of NO_2 might have been utilized by tree saplings, as a sole nitrogen source at very early stage of development. Nitrate reductase activity was maximum in *M. indica* and *S. cumini* but further utilization of NO_2 through *N* assimilation pathway was not apparent in *M. indica* and *S. cumini*.

Analysis of various plant parameters illustrate the three possible mechanisms of response of tree saplings to exposure of SO_2, NO_2 – Nitrogen assimilation pathway of tree saplings may be rely upon excess accumulation of inorganic form of S/N. SO_2 conferred loss in AAs might be one of the possible reason of higher accumulation of inorganic *S* (sulphate) and no conversion in organic *S* (AAs) and lead to a decrease in protein content.

The magnitude of quantitative changes of different plant parameters of tree sapling caused by SO_2-NO_2 exposure has been seemed to be low. This may be attributable to the gases diffusion to the surrounding environment during the exposure through the open top chambers. Stomata might be closed during peak day hours (1200-1300 h) and restrict the entry of gases inside leaves. Other factors like metabolic activities of plant system, leaf age, plant age, stomatal conductance, active detoxifying system have governed the response of the trees to air pollution (Katiyar and Dubey 2001, 2002, Schmidt *et al*. 1990).

Even though under stress condition, many plants show a shift in biomass allocation in order to increase carbon gain or to enhance uptake of water and nutrients (Mooney and Winner 1988). Young leaves and plants have the ability to reduce the negative consequence of gases, it appears that has been governed.

5. Conclusions

In conclusion, tree saplings are more sensitive towards combined exposure of SO_2 and NO_2. We found that *M. indica*, *S. cumini*, and *D. sissoo* have been emerged as indicator tree species. No clear gradation in tree sapling could be made at this point of investigation owing to NO_2 exposure. At sapling stage *E. rostrata*, *C. siamea* and *A. indica* have been provided evidence to be moderate tolerant tree species. Effect SO_2-NO_2 seems to be synergistic rather than additive.

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