MATERIALS AND METHODS
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PLANT MATERIAL:

Saplings of the following four commonly occurring tree species were used for assessing their response both during SO₂ fumigation and the recovery regime:

- *Azadirachta indica* A.Juss.
- *Melia azadirach* Linn.
- *Syzygium jambolina* Lamk.
- *Morus alba* Linn.

These saplings were grown in earthen pots of 8" diameter, filled with garden soil and organic manure in 3:1 ratio. Six months old saplings of uniform height were selected for experiments. Eighteen potted saplings were taken for fumigation and another set of nine saplings were maintained as control.

Ethylene emission was compared between SO₂ exposed 12" long freshly cut twigs taken from adult, healthy trees growing at non-polluted site and SO₂ fumigated potted saplings of the same species. The following species were used for this study:

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- *Morus alba* Linn.
- *Syzygium jambolina* Lamk.

In addition, SO₂ induced ethylene emission was also measured in healthy twigs of important tree and shrub species commonly grown in the area. Twigs of the following species were taken:

- *Azadirachta indica* A.Juss.
- *Albizia lebbeck* Benth.
- *Alstonia scholaris* R.Br.
- *Bougainvillea spectabilis* Willd.,
- *Bauhinia variegata* Linn.
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Carissa carandas Linn.
Melia azadirach Linn.
Morus alba Linn.
Nerium indicum Mill.
Polyalthia longifolia Thw.
Syzygium jambolina Lamk.

**Fumigation Chamber:**

A dynamic, fumigation chamber of 1 m³ capacity made of glass was used in this study. It had an inlet at the base and an outlet at the top on the opposite side. A small electric fan of 10"x9" size was fixed inside the chamber to ensure uniform mixing of the fumigating gas.

**Sulfur dioxide generation:**

Sulfur dioxide was generated by bubbling air at a constant rate of 1.55 l/min. in an impinger containing 250 ml of the desired strength of sodium metabisulphite solution (Fig: 1). The sequence of reactions leading to SO₂ evolution are as follows:

\[
\begin{align*}
\text{NaHSO}_3 & \quad \rightarrow \quad \text{Na} + \text{HSO}_3 & (1) \\
\text{HSO}_3 + \text{H} & \quad \rightarrow \quad \text{H}_2\text{SO}_3 & (2) \\
\text{H}_2\text{SO}_3 & \quad \rightarrow \quad \text{H}_2\text{O} + \text{SO}_2 & (3)
\end{align*}
\]

Sulfur dioxide was introduced into the chamber through an inlet and its concentration inside the chamber was determined by scrubbing the gas from the exit port in an impinger containing 20 ml of sodium tetrachloromercurate (TCM) for 10 minutes. SO₂ forms a dichlorosulfito-mercurate complex with TCM. 5 ml
Fig: 1  Schematic representation of SO$_2$ generation
of pararosaniline and 2 ml formaldehyde (0.2% v/v) was added to the dichlorosulfito-mercurate complex which results in a pink colored solution. Optical density of this solution was measured spectrophotometrically at 548 nm. The SO$_2$ concentration has been expressed in ppm from the OD values using the formula described by West and Gaeke (1956)

\[
\text{ug SO}_2 \text{ m}^3 = \frac{(A - A_o) \times (10^3) \times B_s \times D}{V_r}
\]

where,

- $A =$ sample absorbance
- $A_o =$ reagent blank
- $10^3 =$ conversion of liters to cubic meters
- $V_r =$ the sample volume corrected to $25^\circ\text{C}$ and 760 mm Hg liters.
- $B_s =$ calibration factor, ug/unit of absorbance
- $D =$ Dilution factor

One ppm SO$_2$ = 2620 ug m$^3$ SO$_2$

EXPOSURE SCHEDULE:

**A:** Saplings of tree species grown in pots were fumigated with 0.5 ppm SO$_2$ for 4 hr everyday (from 0700 hr to 1100 hr), for six days. Thereafter plants were allowed to recover upto the eighteenth day.

**B:** The potted plants and twigs were fumigated together with 0.5 ppm SO$_2$ for 4 hr.

**C:** Healthy twigs of 11 species were exposed to a single cycle of 0.5 ppm SO$_2$ for 4 hr.
Diagrammatic representation of sampling schedule for plants subjected to exposure schedule (A).

*: Ethylene emission
Ascorbic acid

#: Leaf area, Leaf pH, Buffering capacity

Membrane Permeability Photosynthetic Pigments Proteins

Peroxidase Superoxide dismutase Glutathione reductase

Fig. 2
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D: Healthy twigs of 11 species were fumigated with 0.5 ppm SO₂ for 2 hr.

SAMPLING SCHEDULE:

A: Sampling of *Azadirachta indica* A. Juss., *Melia azadirach* Linn., *Morus alba* Linn., and *Syzygium amboinum* Lamk. were carried out to determine the response of these species to SO₂. Morphological, physiological and biochemical parameters were examined both during the fumigation period and the recovery regime (Fig: 2).

B: Leaves from the potted plants and twigs were detached from control as well as SO₂ exposed plants to measure their ethylene emission.

C: Ethylene emission measurements were carried out in control and SO₂ fumigated twigs.

MORPHOLOGICAL PARAMETERS:

Leaf area:

The leaf area was determined by drawing the outline of the leaf on 0.1 cm² grids and counting the number of squares covered by the leaf outline.
PHYSIOLOGICAL PARAMETERS:

Leaf pH:

One gram leaf sample was homogenized in 50 ml double distilled water. The homogenate was filtered through two layers of cheese cloth and pH of the filtrate was measured with the help of a calibrated Elico Digital pH Meter Model LI 120.

Buffering Capacity:

Buffering capacity of plants was measured according to the method of Pfanz and Heber, (1986). One gram leaf material was homogenized in 50 ml double distilled water. Homogenate was filtered and pH was measured with a calibrated pH meter. The homogenate was divided into two equal portions. In one, 0.1 N NaOH solution was added drop by drop till pH was 12 and the quantity of NaOH required was noted. To the second half, 0.1 N HCl was added drop wise till pH 2 was reached and the amount of HCl required was recorded.

The acid-alkali titration curves have been plotted using a single co-ordinate axis, with ml of alkali and acid added increasing to the right and left of the origin respectively.
Membrane permeability:

The membrane permeability was determined by estimating solute ion leakage from the cells using the method of Keller (1976). 10 small discs of 0.7 cm diameter were punched from the leaf lamina and blotted dry to remove water from the leaf surface. The leaf discs were placed in 50 ml deionized water for 4 hr. Electrical conductivity of the solution was measured by Spectra Digital Conductivity Meter which was calibrated against the standard 0.01 N KCl solution having specific electrical conductance of 141.3 µ mhos. The values obtained were multiplied by the cell constant.

BIOCHEMICAL PARAMETERS:

Photosynthetic Pigments:

Fresh leaves weighing 0.5 gm were homogenized in 20 ml of 80% acetone (Acetone : water v/v) in a pre-chilled mortar and pestle. The homogenate was filtered through two layers of cheese cloth. The filtrate was centrifuged at 3000 g for 15 minutes in Janetzki refrigerated centrifuge Model K - 24 at 4°C. The supernatant was decanted and the volume was made upto 25 ml with 80% acetone. Care was taken to shield the chlorophyll extract from bright light. The optical density was measured at 480, 510, 645, and 663 nm wavelength using JASCO spectrophotometer Model 7800 UV/VIS. The amount of chlorophyll a,
chlorophyll b and carotenoids was determined by using the formula described by Maclachlan and Zalik (1963).

\[
\text{Chlorophyll 'a' = } 12.3D_{663} - 0.86D_{645} \times V \\
\text{d x 1000 x W}
\]

\[
\text{Chlorophyll 'b' = } 19.3D_{645} - 3.6D_{663} \times V \\
\text{d x 1000 x W}
\]

\[
\text{Carotenoid = } 7.6D_{480} - 1.49D_{510} \times V \\
\text{d x 1000 x W}
\]

where

- \(D\) = optical density at 480, 510, 643, 663 nm respectively,
- \(V\) = volume of the chlorophyll extract in acetone (ml)
- \(d\) = length of light path (cm)
- \(W\) = fresh weight of leaves (g)

Ascorbic Acid:

The ascorbic acid content of leaf tissue was estimated by the method given by Roe (1954). Ascorbic acid standard solution was prepared by dissolving 100 mg of ascorbic acid in 500 ml of 0.5% oxalic acid solution. The solution is unstable, therefore the dye was standardized immediately. 2,6-dichloro-phenol-indophenol (DCPIP) dye was prepared by dissolving 50 mg sodium salt of DCPIP in 150 ml of double distilled water and then placed in an oven at 80°C for 5 min. 42 mg of NaHCO₃ was added to this solution and decanted into a 200 ml volumetric flask. After cooling and filtering, the volume was made up to 200 ml with double distilled water. The dye was stored in a dark bottle in a refrigerator.
where it remained stable for one week. For standardization, DCPIP dye was titrated against 5 ml of ascorbic acid solution until a pink end point, lasting for 15 seconds, was reached. As 5 ml of the standard ascorbic acid solution contains 1 mg of Vit C, the burette reading is the amount of dye required to oxidize 1 mg of ascorbic acid. The amount of ascorbic acid oxidized by 1 ml of the dye was then calculated.

0.5 gram fresh leaf tissue was homogenized in a pre-chilled mortar and pestle with 20 ml of 0.5 % oxalic acid solution. The homogenate was centrifuged at 1800 g in Janetzki refrigerated centrifuge Model K - 24 at 4° C for 15 min. 10 ml of supernatant was titrated with DCPIP dye till the pink colour persists for at least 15 seconds. The amount of ascorbic acid in the sample was calculated using the following formula:

\[
\text{mg AA in 1 gm sample} = \frac{(V \times T)}{W}
\]

where,
- \(V\) = Volume of dye in ml used for titration of extract
- \(T\) = AA equivalent of dye solution expressed as per ml of dye
- \(W\) = Weight of leaf material

**Ethylene Emmision:**

Whole leaves of the third node from the apex of plants were detached one hour after suspending fumigation for the measurement of ethylene emission. Leaf area was measured and individual leaves were placed in 68 ml capacity glass vials containing 2 ml distilled water. The glass vials were plugged with air-tight rubber stoppers which were fastened to the mouth of glass vials with the help of a tight sealing aluminium jacket to prevent gas leakage. The glass vials containing test leaves were incubated for 4 hr at room temperature in dark as dark incubation
enhances ethylene emission (Tingey, et al., 1976). Two ml of the gas sample was withdrawn from the vial headspace with a 2 ml hypodermic syringe. The gas sample was then injected into a Nucon Gas Chromatogram containing 80 Porapak R Column equipped with a flame ionization detector. Temperature was maintained at 80°C. The flow rate of the carrier gas (N₂) was maintained at 30 ml/min, hat of air was 2.5 l/min and of H₂ was kept at 30 ml/min.

A calibration curve was prepared from serial dilutions of standard ethylene gas (105 ppmv in Argon). The area of ethylene peak obtained on the gas chromatogram from the incubated leaf samples was noted and compared with the calibration curve. Ethylene emission has been expressed in ppm per cm² of leaf area, which can be converted to μmole/cm² by multiplying with 3.0357.

Protein:

Leaf protein was determined according to the method of Lowery et al. (1951). One gm of fresh leaf tissue was homogenized in 10 ml of 0.1 M phosphate buffer of pH 7.5 and centrifuged at 16000 rpm for 30 min in Janetzki refrigerated centrifuge Model K-24 at 4°C. The total volume of the supernatant was noted. Proteins in the supernatant were precipitated by adding 0.2 ml of 10% TCA solution to 2 ml of crude enzyme extract in a test tube. The precipitate was dissolved in 0.4 ml of 0.1 N NaOH. 0.2 ml of this was used for the protein test. To this 0.8 ml of water and 2.5 ml of protein reagent prepared by mixing 10 parts 2% Na₂CO₃ in 0.1 N NaOH, 0.1 part 2% Na-K tartarate and 0.1 part 1% CuSO₄ were added. After 15 min, 0.25 ml of Folin’s reagent (diluted 1:1 with distilled water) was added and the mixture was incubated in dark at room temperature for 30 min. OD was determined after 30 min. at 750 nm using JASCO spectrophotometer Model 7800 UV/VIS. Calibration curve for protein was made using 1% aqueous solution of bovine serum albumin according to
standard procedure and the amount of total soluble protein has been expressed as mg protein per gram leaf fresh weight.

Peroxidase (POD) (E.C.1.11.1.7):

Peroxidase was assayed according to the method of Bergmeyer (1971). 0.5 gram fresh leaf tissue was homogenized with 10.0 ml ice cold 0.1 M phosphate buffer (7.2) in a pre-cooled mortar and pestle. The homogenate was centrifuged at 16,000 g for 20 min in Janetzki refrigerated centrifuge K - 24 at 4° C. The clear supernatant was used for enzyme assay.

In a cuvette containing 1.95 ml phosphate buffer and 0.05 ml leaf extract, 0.25 ml guicol (1.0% v/v) solution was added. The assay mixture was shaken and OD at 436 nm was set zero with this. 0.25 ml of H₂O₂ solution was added to the reaction mixture. Change in OD was record at every 15 second interval for 3 minutes. Concurrently, protein estimation was carried out according to Lowery et al., 1951). The POD activity was expressed as IU per mg protein per gram fresh weight.

Superoxide Dismutase (SOD) (EC 1.15.1.1.):

Superoxide dismutase was assayed according to the method of Paoletti et al. (1986). Fresh leaf tissue weighing 0.5 gm was homogenized in a pre-chilled mortar and pestle with 5.0 ml chilled 100 mM Tea - Dea buffer (pH 7.4). The homogenate was centrifuged at 16,000 g for 25 min in Janetzki refrigerated centrifuge K - 24 at 4° C.

In a cuvette containing 1.6 ml Tea-Dea buffer, 0.05 ml EDTA/MnCl₂ (100 mM), 0.10 ml leaf extract, and 0.08 ml NADH (7.5 mM) was added. The assay
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mixture was shaken and OD was set zero at 340 nm with this solution. 0.2 ml mercaptoethanol was added to the assay mixture and the change in OD was noted after every 3 minutes for 15 min using JASCO Spectrophotometer Model 7800 UV/VIS. Concurrently, protein estimation was carried out according to Lowery et al., 1951). Activity of the enzyme has been expressed as IU per mg protein per gram fresh weight.

**Glutathione Reductase (GR) (EC 1.6.4.2):**

Three gram fresh leaf tissue was rapidly homogenized with a pre-cooled mortar and pestle in chilled 10 ml 0.66M STM buffer (pH 7.4) containing 0.3 M sucrose, 50 mM Tris-HCl, 1mM EDTA and 5 mM MgCl₂. The homogenate was filtered through cheese cloth and centrifuged in refrigerated centrifuge K - 24 at 4° C at 3,500 rpm for 15 min. The supernatant was discarded and the pellet was resuspended in buffer. The entire procedure was carried out under green light.

Chlorophyll content of the chloroplast suspension was determined as described earlier using the formula given by Maclachlan and Zalik (1963).

To determine the percentage intactness of the isolated chloroplasts, the oxygen evolution of the chloroplast suspension was measured with a Clark type oxygen electrode (Hansatech - CBID, UK) in aqueous media as described by Shimazaki and Sugahara (1979). Oxygen evolution was estimated by using 100 μl/l chlorophyll in 2 ml of 5 mM NH₄Cl, 4 mM K₃FeCN and 1 mM ethyl diamine. The mixture was illuminated for 2 minutes and the oxygen evolution was recorded on Omniscrrib chart recorder. Oxygen evolution has been expressed as μmol/mg chlorophyll/h.

Glutathione reductase is a stromal enzyme and its activity can be measured
only by lysing the chloroplast. For lysation of chloroplasts, 0.5 ml of the chloroplast suspension was taken in an eppendorf tube and centrifuged for 5 min at 5000 rpm at 4°C. The supernatant was discarded and the pellet was resuspended in 0.3 ml of 0.2 M potassium phosphate buffer by shaking gently to ensure lysing. It was again centrifuged at 5000 rpm for 5 min. The supernatant containing suspended stroma was used for GR assay.

Glutathione reductase was assayed according to the method of Foyer and Halliwell (1976). One ml assay mixture contained 50 ul NADPH, 50 ul GSSG, 100 ul chloroplast suspension and 800 ul 0.2 M potassium phosphate of pH 7.0. The decrease in OD at 340 mM was noted for 5 min. Activity has been expressed as nmol NADPH oxidised per min per mg protein.

Statistical analysis

Data of the different sets have been subjected to the following statistical treatments.

**Standard deviation** : The standard deviation was calculated according to the following formula (Snedecor & Cochran, 1967):

\[ S.D. = \sqrt{\frac{d^2}{n-1}} \]

where \( n \) = no. of samples,
\( d \) = deviation from the mean.
Coefficient of correlation: The measure of mutual relationship between two variables was calculated as

\[ r = \frac{\sum x_1 x_2}{\sqrt{\left( \sum x_1^2 \right) \left( \sum x_2^2 \right)}} \]

where \( r \) = correlation coefficient, \( x_1, x_2 \) are two different variables.