Effect of aqueous sulphur dioxide on the biochemical and antioxidant properties of *Malva sylvestris*

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SUMMARY

Leaf discs of *Malva sylvestris* were treated with different concentrations of aqueous sulphur dioxide (10-1000 ppm) under illumination for 4 hours to study changes in various biochemical and antioxidative properties of the plant. A concentration dependent decrease in the chlorophyll, pheophytin and carotenoid content was observed. Similar results were obtained for proteins. The amino acids, however, increased in response to increasing sulphur dioxide concentration. The exposed plants showed a concentration dependent decrease in starch as well as free sugars. A decrease in the level of total phenolics was also observed with increasing sulphur dioxide concentrations. A concentration dependent increase in the activity of superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, glutathione-*S*-transferase and glutathione peroxidase was observed. The lipid peroxidation increased significantly in response to increasing sulphur dioxide concentration.

Key words : Aqueous sulphur dioxide, Chlorophyll, proteins, Sugars, *Malva sylvestris*, Enzymes, Pheophytion.

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Growth and development of plants is affected by various environmental factors including the pollutants. Due to the recent industrial revolution and developments in the field of science and technology, a huge quantity of wastes in the form of industrial and agricultural effluents, sewage and domestic wastes, residues of pesticides, herbicides, insecticides, fertilizers, detergents and heavy metals, various toxic gases, flash etc. are regularly thrown into rivers, ponds, air, open places ultimately causing environmental pollution. These environmental problems have become so severe that various flora and fauna are in danger. Among the gaseous pollutants, sulphur dioxide is considered to be the most wide spread phototoxic air pollutant altering plant growth and metabolism.

Sulphur dioxide toxicity on vegetation has been well reviewed in terms of foliar injury and physiological and biochemical alterations (Khan and Khan,1993; Javeed *et al*., 1998; Masood *et al*., 2001, Agarwal and Deepak, 2003; Amin *et al*., 2007; Dar *et al*., 2008). Sulphur dioxide penetrates the leaves principally through the stomata and then diffuses through the cell membrane into the cytoplasm. In the cytoplasm, it is hydrated to form sulphurous acid which is converted to bisulfite and sulfite (Cotton and Wilkinson, 1980). These anions are highly phytotoxic. They are detoxified by oxidation to sulfate and then incorporated into the normal sulphur metabolic system (Huber *et al*., 1987; Takahama *et al*., 1992). In excess of a certain level, sulphur dioxide exerts its damaging effects. The damage produced by sulphur dioxide to plants includes membrane damage, chlorophyll destruction, interference with the activity of enzymes, plasmolysis, genetic material destruction and retarded growth and development (Ventaketashwar, 1992; Lee *et al*., 1997; Anuradha *et al*., 1999; Amin *et al*., 2007; Dar *et al*., 2008). However, the sequence leading to such disability is poorly understood. The present study has been used to determine changes in various biochemical and antioxidative properties which lead to tissue injury.

MATERIALS AND METHODS

Generation of aqueous sulphur dioxide:

Sulphur dioxide was generated by reducing hot concentrated sulphuric acid with copper turnings and estimated according to West and Gaeke (1956).

$$Cu + 2H_2SO_4 \rightarrow CuSO_4 + 2\,H_2O + SO_2$$

Exposure of leaf discs to aqueous sulphur dioxide:

Malva Sylvestris was purchased from local market and discs of 1 cm diameter each were cut from healthy leaves using a stainless steel cork borer. Leaf discs were treated with 10, 100 and 1000 ppm of aqueous sulphur dioxide for four hours in Petri dishes (15 x 20

mm) under illumination which was provided by a 100 W electric bulb. Treatment conditions were kept similar for each section.

Preparation of homogenate:

The experimental (aqueous sulphur dioxide treated) and control leaf discs were separated, washed, patted dry, weighed and homogenized in chilled water using pre chilled pestle and mortar. A 10 % (w/v) homogenate was prepared. The homogenate was filtered through eight layered muslin cloth. The filtered extract was centrifuged at 500g for 10 minutes at 4°C. The supernatant was subsequently used for various estimations.

Estimation of pigments:

Chlorophyll, pheophytin and carotenoids were extracted in 80 % acetone from fresh tissue. 2 ml of 10 % w/v whole homogenate was mixed with 8 ml acetone in a volumetric flask, shaken well and kept for 1-2 hrs at 4°C. The mixture was centrifuged at 5000 rpm at 4°C for 5 minutes. The extract was used for the estimation of pigments.

Estimation of proteins:

Pellet left after the extraction of pigments with acetone was dried and dissolved in 0.1 N NaOH and the volume made up to 5 ml with distilled water. The mixture was centrifuged at 5,000 rpm for 5 min. and supernatant was used for the estimation of protein by the method of Lowry *et al*. (1951) using BSA as standard.

Estimation of amino acid, phenolics, free sugars and starch:

After homogenization, 2 ml of whole extract was mixed with 8 ml of absolute alcohol, shaken well and centrifuged. The pellet left after centrifugation was re-extracted with hot alcohol. Pooled extract was used for the estimation of amino acids, phenolics (Farkas and Kiralay (1962) and total free sugars (Montgomery, 1957). The pellet left after alcohol extraction was used for starch estimation (Agarwal *et al*., 1982).

Assay of enzymes:

Super oxide dismutase (EC 1.15.1.1):

SOD was assayed by using the photochemical NBT method. The standard assay mixture contained, in a total volume of 3ml, 50mM phosphate buffer pH 7.8, 9.9mM L-Methionine, 57µM NBT, 0.025% (w/v) Triton X-100, and 0.00445 (w/v) riboflavin. The photo reduction of NBT (formation of purple formazon) was measured at 560 nm and an inhibition curve was made against different volumes of extract.

One unit of SOD was defined as that being present in the volume of extract that caused inhibition of photo reduction of NBT by 50%.

Catalase(EC 1.11.1.6):

Catalase activity was assessed by following the decomposition of H_2O_2 at 240 nm according to Cakman and Marschner (1992). The standard assay mixture contained, in a total volume of 3ml, 100mM phosphate buffer pH7.0, 6mM H_2O_2 and enzyme extract equivalent to 100 µg of protein. The reaction rates were corrected for the non enzymatic decomposition of H_2O_2 by running enzyme and substrate blanks simultaneously. The enzyme blank contained all the reagents except enzyme and substrate blank contained everything except substrate. Decomposition of H_2O_2 was measured as decrease in absorbance at 240 nm at every 1min. interval over a period of 6 min.

One unit of CAT activity was defined as the decomposition of 1 µmol H_2O_2/mg protein per min at pH 7.0 and 25°C.

Ascorbate peroxidase (EC 1.11.1.11):

Ascorbate peroxidase was assayed according to Chen and Asada (1989). The enzyme activity was determined by monitoring the oxidation of ascorbic acid at 290 nm.

The standard assay mixture contained, in total volume of 3 ml, 50mM phosphate buffer, pH 7.0, 0.6mM ascorbic acid and enzyme extract equivalent to 100 µg of protein. The reaction was initiated by the addition of 10µl of 10% (v/v) H_2O_2 and the oxidation of ascorbic acid was estimated by following the decrease in absorbance at 290nm at every 3min.interval over a period of 15 min. The non enzymatic reaction rates were corrected by running enzyme and substrate blanks simultaneously. The enzyme blank contained all the reagents except enzyme and substrate blank contained everything except substrate.

Glutamate reductase (EC 1.6.4.2)

Glutamate reductase activity was determined from the rate of NADPH oxidation as measured by the decrease in absorbance at 340 nm following the procedure of Foyer and Halliwell (1976). The standard assay mixture contained, in total volume of 1 ml, 0.1 M Tris buffer (pH 7.8), 2 mM EDTA, 50 µM NADPH, 0.5 mM GSSG, and the enzyme extract equivalent to 100 µg of protein. The reaction was initiated by the addition of NADPH and was carried out at 25°C. The initial velocity of the reaction

was determined, and activity was expressed as nmol of NADPH oxidized min^{-1} mg^{-1} protein. The non enzymatic reaction rates were corrected by running enzyme and substrate blanks simultaneously. The enzyme blank contained all the reagents except enzyme and substrate blank contained everything except substrate.

Glutathione-S-transferase (EC 2.5.1.18):

The activity of glutathione-S-transferase was determined by the method given by Li *et al*. (1995). The reaction mixture in a volume of 3 ml contained 50 mM phosphate buffer, pH 7.5,1 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and eluate equivalent to 100 µg of protein. The reaction was initiated by the addition of 1 mM GSH and formation of *S*-(2, 4-dinitrophenyl) glutathione (DNP-GS) was monitored as an increase in absorbance at 334 nm.

Glutatione peroxidase (EC 1.15.1.1):

GPOX activity was assayed by the method given by Edwards (1996). The enzyme activity was determined with cumene hydroperoxide as substrate using a glutathione reductase coupled assay to monitor the oxidation of GSH. The standard assay mixture contained in a final volume of 3 ml, 0.1 M phosphate buffer, pH 7.0, containing 0.2% (w/v) Triton X-100, 0.24 U GR, 1 mM GSH, 0.15 mM NADPH, and 1 mM cumene hydroperoxide. After addition of enzyme eluate equivalent to 100µg of protein, cuvettes were incubated at 30 °C for 10 min and NADPH was added to measure the basal rate of GSH oxidation by monitoring the absorbance at 340 nm for 3 min. The reaction was initiated by addition of cumene hydroperoxide and GPOX activity was expressed as change in absorbance at 340 nm.

One unit of GSH dependant peroxidase activity was equivalent to the oxidation of 1nmol of NADPH/mg of protein/min.at pH 7.

Lipid peroxidation assay:

Lipid peroxidation was assayed by usual method. The reaction mixture in a total volume of 1.0ml contained 0.5ml of white ghost (4mg protein/ml), PMS or microsomes (3mg protein/ml), 0.1 ml of ferric nitrate (20mM) and 0.1 ml of ascorbic acid (100mM). These models were pre incubated with various concentrations of plant extracts, wherever needed. The reaction mixture was incubated at 37°C in a shaking water bath for one hour. The reaction was stopped by the addition of 25% trichloro acetic acid (TCA). Tubes were centrifuged at 5000 rpm for 5 minutes. To the supernatant, 1ml or 1.67% thiobarbutaric acid (TBA) was added to each tube. All the tubes were then placed in a boiling water bath for 20 minutes. The tubes were then immediately cooled. Amount of TBARS formed in each sample was assessed by measuring the absorbance at 535nm against a reagent blank.

RESULTS AND DISCUSSION

Effect of aqueous sulphur dioxide on photosynthetic pigments:

Exposure of leaf discs to 10 ppm aqueous sulphur dioxide did not show any significant biochemical change.

Fig.1 represents the effect of aqueous sulphur dioxide on photosynthetic pigments. A significant decrease of 20

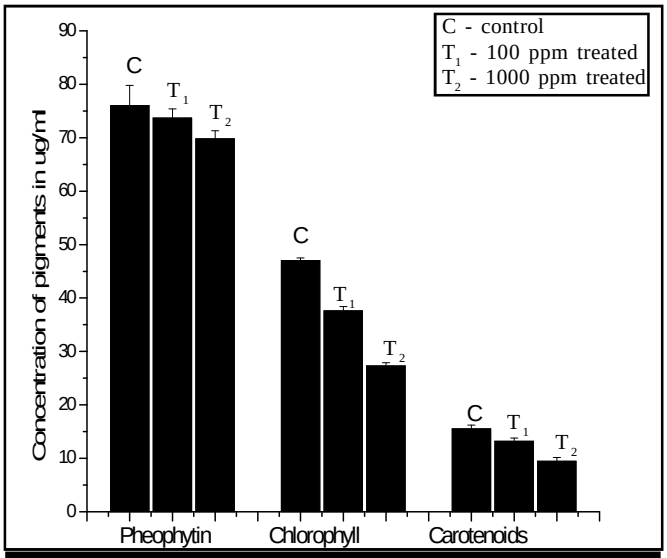

Fig. 1: Effect of aqueous sulphur dioxide on the pigments of *Malva sylvestris* leaf discs
Data represent the means ± SD of five separate experiments, P<0.05

and 42% was found in the chlorophyll content of *Malva sylvestris* exposed to 100 and 1000 ppm of aqueous sulphur dioxide. Phaeophytins decreased by 3% at 100 ppm and 8% at1000 ppm aqueous sulphur dioxide. The carotenoids decreased by 15 % and 37 % when exposed to 100 and 1000 ppm of aqueous sulphur dioxide.

Effect of aqueous sulphur dioxide on proteins and amino acids:

Fig. 2 houses the results of effects of aqueous sulphur dioxide on proteins and amino acids. *Malva sylvestris* showed a decrease of 20% in protein content at 100 ppm and at 1000 ppm, 46% reduction was observed. However, the amino acids slightly increased after exposure to aqueous sulphur dioxide. The increase was 2% at 100 ppm and 5% at 1000 ppm sulphur dioxide.

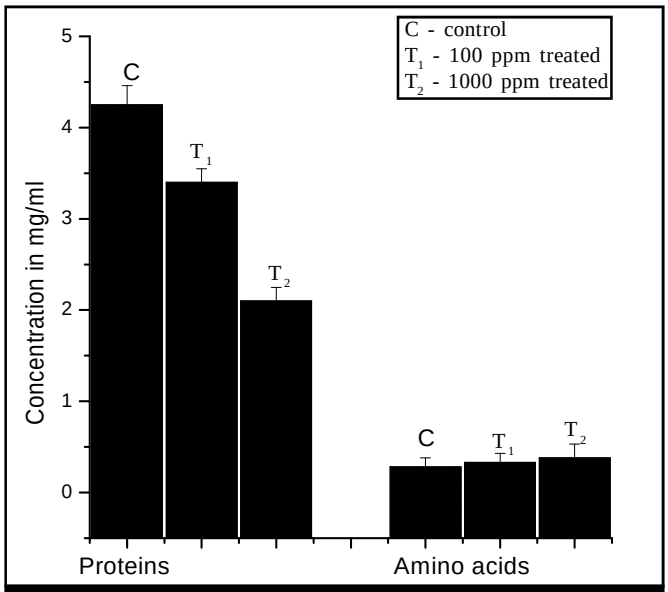

Fig. 2 : Effect of aqueous sulphur dioxide on proteins and amino acid content of leaf discs of *Malva sylvestris* Data represent the means ± SD of five separate experiments, P<0.05

Effect of aqueous sulphur dioxide on free sugars and starch:

The free sugars were found to decrease after exposure to aqueous sulphur dioxide. The decrease was 15 and 47% at 100 and 1000 ppm. The starch content showed a decrease of 10% at 100 ppm whereas at 1000 ppm a marked decrease of 30% was observed (Fig. 3).

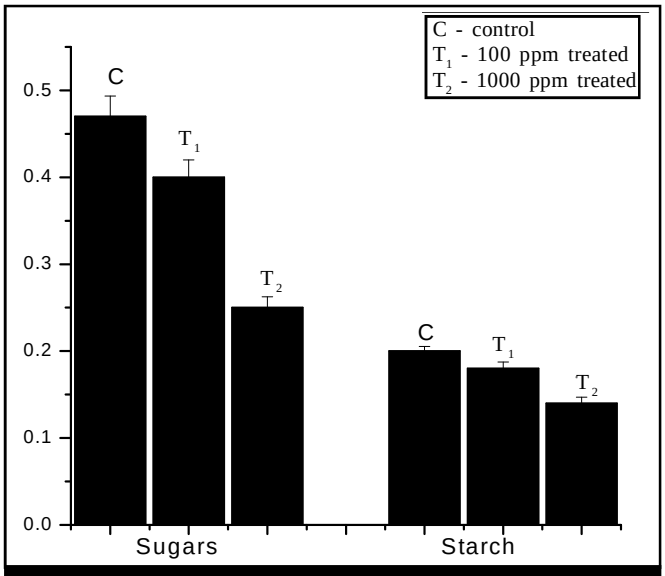

Fig. 3 : Effect of aqueous sulphur dioxide on sugars and starch content of leaf discs of *Malva sylvestris* Data represent the means ±SD of five separate experiments, *P*<0.05

Effect of aqueous sulphur dioxide on total phenolics:

Fig. 4 houses the results of the effect of aqueous sulphur dioxide on total phenolics. The results indicate that *Malva sylvestris* exhibited a decrease of 12% in total phenolics at 100 ppm and a marked decrease of 37 % at 1000 ppm sulphur dioxide.

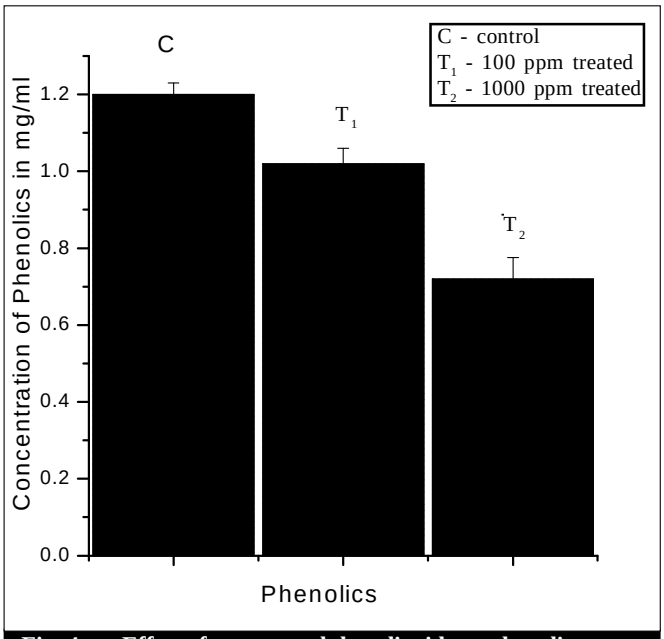

Fig. 4 : Effect of aqueous sulphur dioxide on phenolic content of leaf discs of *Malva sylvestris* Data represent the means±SD of five separate experiments, *P*<0.05

Effect of aqueous sulphur dioxide on activities of antioxidant enzyme:

Leaf discs of *Malva sylvestris* treated with different concentrations of aqueous sulphur dioxide showed different responses with respect to their antioxidant enzyme activity. SOD activity increased in the leaf discs of *Malva sylvestris* in response to increasing concentrations of aqueous sulphur dioxide. The increase was 13 % at 100ppm and 37% at 1000 ppm (Fig. 5). The catalase activity was 24% higher than control when exposed to 100ppm sulphur dioxide while as at 1000 ppm the activity increased by 47%. Ascorbate peroxidase activity increased by 25% at 100 ppm and at 1000pppm 70% increase was observed. Glutathione reductase activity also increased on exposure to aqueous sulphur dioxide. An increase of 28% and 80% was observed at 100 and 1000ppm aqueous sulphur dioxide. The activity of GST increased by only 10% at 100ppm while as the increase was 30% at 100ppm aqueous sulphur dioxide. Glutathione peroxidase also responded to sulphur dioxide exposure by increasing its activity. The increase was 15% at 100ppm and 45% at 1000pppm (Fig. 5).

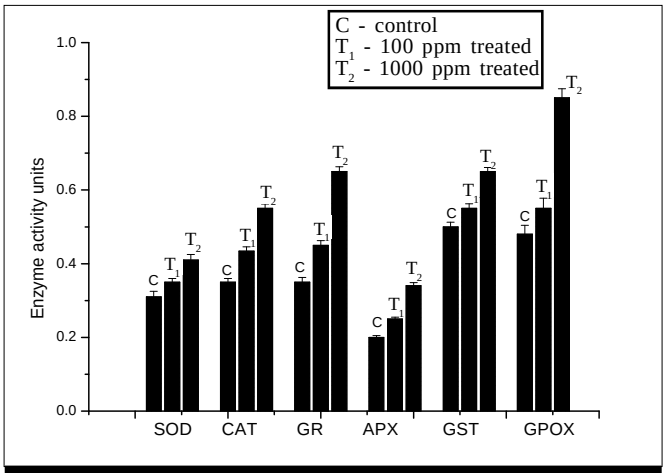

Fig. 5 : Antioxidant enzyme activities in discs of control and treated *Malva sylvestris*
Data represent the means±SD of five separate experiments, *P*<0.05

Effect of aqueous sulphur dioxide on post mitochondrial supernatant membrane lipid peroxidation:

In this study, PMS of sheep liver was used as a model and peroxidation was induced by Fe^{3+}/ ascorbic acid. The untreated extract of *Malva sylvestris* decreased the value of MDA formation from 8 to 3.5 n moles while as 100 ppm treated extract decreased the value from 8 n moles to 4.5 n moles and 1000 ppm treated decreased the value to 8 n moles to 6 n moles. The increased accumulation of lipid peroxides is indicative of enhanced production of toxic oxygen species (Fig. 6).

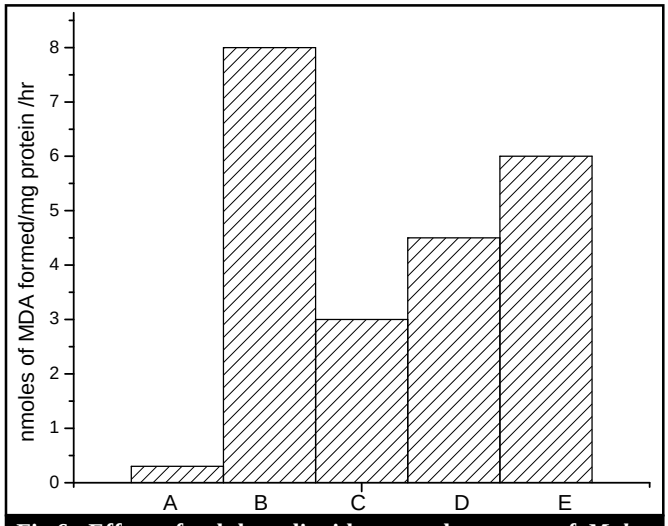

Fig 6: Effect of sulphur dioxide treated extracts of *Malva sylvestris* on post mitochondrial supernatant membrane lipid peroxidation in presence of various oxidants. A represents PMS alone (control), B represents PMS + Ferric nitrate (20mM) and Ascorbic acid (500mM), C represents PMS, Ferric nitrate (20mM), Ascorbic acid (500mM) and control plant extract, D represents PMS + Ferric nitrate/ Ascorbic acid and 100 ppm treated plant extract , E represents PMS + Ferric nitrate / Ascorbic acid and 1000 ppm treated plant extract.
Each value represents mean ± S.D of five experiments

Sulphur dioxide injury in plants depend mainly on entry through stomata and its diffusion into the mesophyll cells where it reacts with plant sap to form sulfite, bisulfite and other ionic species depending upon the surrounding pH (Pfanz *et al*., 1987). Both sulfite and bisulfite ions have been shown to be deleterious to plants (Winner *et al*., 1985) but the plants can overcome these phytotoxic effects by readily converting bisulfite and sulfite into sulfate ions which have been shown to be about 40 times less toxic (Huber *et al*., 1987). When the concentration of aqueous sulphur dioxide is more or the exposure time is long the plant is not able to take care of the entire aqueous sulphur dioxide load. The result is morphological as well as physiological injuries to the plant. Chlorophyll pigments are known to be damaged by sulphur dioxide (Masood *et al*., 1999; Amin *et al*., 2007; Dar *et al*., 2008). The present study showed a marked decrease in the chlorophyll content of *Malva sylvestris* upon exposure to sulphur dioxide. Sulphur dioxide can influence chlorophyll by various mechanisms including increased acidity, bleaching and conversion to phaeophytin by splitting Mg^{2+} and forming complexes with proteins (Rao and Le-Blanc, 1966). A simultaneous decrease in the pheophytin content was also observed, so phaeophytinization could not be responsible for the decrease in chlorophyll content. This has also been observed by Shimazaki *et al*., 1980.

Malva sylvestris showed a decrease in protein content in relation to sulphur dioxide. Similar results have been reported for a number of plants (Agarwal and Deepak, 2003; Amin *et al*., 2007; Dar, 2008). Higher levels of sulphur dioxide may break enzymes and proteins with disulfide bonds into thiosulphonates and thiols. Changes in amino acid content, and inactivation of enzyme in the sulphur dioxide treated plant leaf discs were held responsible for the decrease in protein synthesis (Prasad and Rao, 1982). A slight increase in the pool of amino acids was observed after sulphur dioxide exposure. The increase might be either due to the increased synthesis of amino acids or due to the disruption of proteins and various enzymes as a metabolic stress due to sulphur dioxide pollution.

Phenolics play an important role in the defense of plants against infections and their contents may vary during injury. A decrease in the level of total phenolics in *Malva sylvestris* indicated that this plant is susceptible to the attack of sulphur dioxide. Practically all higher plant phenolics are formed from shikimate; via shikimic acid pathway

(Kainulainen *et al*., 1995).The amount of shikimic acid has been observed to diminish by sulphur dioxide fumigation (Katoh *et al*., 1989; Katzel and Moller, 1993).

Many studies have been performed on the enhancement of plant tolerance to oxidative stress by modifying the plant antioxidant defense system (Allen, 1995; Vitoria *et al*., 2001). It has been shown that peroxidase, catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) are the major constituents of the plant antioxidant enzyme system, which operate by scavenging ROS (Prasad, 1997; Yun *et al*., 2000). In addition, it is believed that the changes in antioxidant enzymes induced by oxidative stress might be due to synthesis of new isozymes or enhancement of activities of existing antioxidant enzymes for metabolism of ROS. In *Malva sylvestris*, there has been observed a marked increase in the activity of catalase, peroxidase and GR which might be an important line of defense against sulphur dioxide. Several types of evidences suggest that free radical formation is one mechanism of sulphur dioxide phytotoxicity. Foliar application of free-radical scavengers, such as á-tocopherol, diphenylamine, or propylgallate, reduced foliar injury produced by sulphur dioxide (Chang *et al*., 1981). An increase in GR activity and new isozyme induction due to SO2 and ozone exposure were also reported in several plants including wheat seedlings (Rao *et al*., 1995). Moreover, there were differential responses of SOD in two pea cultivars during exposure to SO2. The cultivar progress showed an increased activity of SOD, whereas SOD activity decreased in the cultivar nugget in response to SO2 (Madamanchi *et al*., 1994). In the present study, the activity of SOD remained higher than the control at both concentrations. GSH content has been found to increase in leaves of the insensitive pea cv during exposure to SO_2, whereas in the sensitive *cv*, no increase was observed until the postexposure period, when photosynthesis was recovering (Alscher *et al*., 1987).

The results presented suggest that sulphur dioxide induced increase in the levels of antioxidative enzymes may represent a secondary defensive mechanism against oxidative stress that are not as direct as the primary defensive responses such as phytochelatins and vacuolar compartmentalization (Sanitá de Toppi and Gabbrielli, 1999). Antoxidative mechanisms seem to operate in additive way to cope effectively with any stress. The significant increase in the levels of SOD, CAT, APX, and GR even at 100 ppm exposure of aqueous sulphur dioxide may be indicative of a rapid signalling response.

Malva sylvestris was again found sensitive to sulphur dioxide pollution with a very to highly significant increase in lipid peroxidation. The membrane components, fatty acids can be damaged by sulfite. Lizada and Yang (1981) examined the peroxidation of linolenic and linoleic acids in the presence of bisulfite. Peroxidation and sulfite oxidation were inhibited by low concentration of the free radical scavenger's butylated hydroxytoluene, α-tocopherol and hydraquinone. Thus free radical oxidation of sulfite was responsible for the per oxidation of linoleic and linolenic acids.

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