Superoxide Dismutase a Possible Protective Enzyme against Sulphur Dioxide Injury in Cajanus cajan and Amaranthus paniculatus Leaf Discs Under Light and Dark Conditions

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Abstract:
The effect of varying concentrations of aqueous SO\(_2\) (0, 10, 20, 30, 40, 50, 100 and 250 ppm) on pigeonpea (Cajanus cajan (L.) Millsp. cv. PDM1), a C\(_3\) plant and amaranth (Amaranthus paniculatus L. a local cultivar), a C\(_4\) plant were selected for the present study on antioxidative metabolism. Ascorbic acid content and ascorbic acid oxidase activity decreased due to the continuous incubation of both the plant leaf discs in aqueous SO\(_2\). The decline was more in amaranth. The polyphenol oxidase activity increased in pigeonpea whereas it decreased in amaranth. The oxyradical detoxifying systems such as sulphite oxidation and activities of superoxide dismutase, catalase and peroxidase, exhibited differences between pigeonpea and amaranth. Pigeonpea showed continuous increase of both superoxide dismutase and peroxidase activity to SO\(_2\). In amaranth superoxide dismutase increased whereas the peroxidase activity decreased continuously to SO\(_2\). Catalase activity decreased in response to SO\(_2\) in both the plant species studied.

Keywords: Amaranth, aqueous SO\(_2\), catalase, peroxidase, pigeonpea, polyphenol oxidase activity, superoxide dismutase.

INTRODUCTION

Sulfur dioxide (SO\(_2\)) is one of the most common and harmful air pollutants. The physiological processes underlying the phytoxicity of SO\(_2\) are rather unclear. Sulphite ions formed due to the hydration of SO\(_2\) in cell sap are very reactive and the reaction with various cellular compounds is believed to be the major cause of SO\(_2\) phytoxicity (De Kok, 1990). On the other hand, it has been proposed that the toxicity of SO\(_2\) is due to the formation of superoxide anions and hydrogen peroxide (H\(_2\)O\(_2\)) generated during the oxidation of sulphite to sulphate (Nageswara Rao and Alscher 1991; Rao, 1992). In this view, plants tolerate SO\(_2\) by scavenging the toxic oxygen free radicals through a sequence of events involving metabolites (Rao and Dubey, 1993) like some of the enzymes, such as superoxide dismutase, catalase and peroxidase to reduce lipid peroxidation.

Ascorbic acid is considered to play an interesting role in combating SO\(_2\) injury of plants up to certain concentration thresholds of SO\(_2\). Ascorbic acid defends SO\(_2\) toxicity by scavenging phenoxy radicals, sulphite radicals, and superoxide radicals, that are formed by the reaction of sulphite radicals with oxygen. Ascorbic acid is also involved in controlling the rate of oxidation of sulphite to sulphate in the apoplast in order to prevent uncontrolled radical reactions (Takahama et al., 1992). Rao (1981) has demonstrated a positive correlation between increased ascorbic acid content and tolerance of plants to SO\(_2\). Shimazaki et al., (1980), showed that ascorbic acid being a scavenger of superoxide radicals may inhibit SO\(_2^-\) induced chlorophyll degradation. Relatively high concentrations of SO\(_2\) reduce ascorbic acid content of leaves (Nandi et al., 1984; Varshney and Varshney (1984). Ascorbic acid starts declining even before the appearance of foliar injury symptoms (Keller and Schwager, 1977). It was reported that a decrease in ascorbic acid content results in a reduction of yield of crop plants under SO\(_2\) exposure (Nandi et al., 1981). It was noted that SO\(_2\) inhibits the ascorbic acid oxidase activity in the leaves (Haisman, 1974; Nandi et al., 1984).

Sulphur dioxide enhances the production of additional free oxyradicals in plants. Free radicals such as superoxide radical, hydroxyl radical and sulphite radical were generated in plants as a result of oxidation of SO\(_2\) in leaves (Asada, 1980; Peiser et al., 1982). Free radical production may lead to inactivation of proteins (Fridovich, 1986), peroxidation of lipid membranes (Peiser and Yang, 1977, 1978) and bleaching of chlorophyll and carotenoids (Shimazaki et al., 1980). In order to delete the deleterious effect of free radicals, plants develop defensive mechanisms which include both enzymatic and non-enzymatic (Lee and Bennet, 1982; Rao, 1992; Allen, 1995). Evidence for the direct involvement of oxygen radicals in SO\(_2\) toxicity is well established (Bowler et al., 1992). The sequence of free radical generation during the aerobic oxidation of SO\(_2\) is represented in the following equations (Yang, 1970; Hayan et al., 1972):

**Initiation**

\[
\begin{align*}
\text{SO}_3^{2-} + \text{O}_2 & \rightarrow \text{SO}_4^{2-} + \text{O}_2^- \\
\text{H}_2\text{SO}_3 & \rightarrow \text{HSO}_3^- + \text{H}^+ \\
\text{SO}_3^- + \text{H}_2\text{O} & \rightarrow \text{H}_3\text{O}^+ + \text{SO}_4^{2-} \\
\text{SO}_4^{2-} + \text{H}_2\text{O} & \rightarrow \text{HSO}_3^- + \text{H}_3\text{O}^+ \\
\text{HSO}_3^- & \rightarrow \text{H}_2\text{O} + \text{SO}_2 + \text{H}^+ \\
\end{align*}
\]
Preparation of aqueous sulphur dioxide

Sulphur dioxide was prepared in the laboratory by reacting sodium metabisulphite with concentrated H₂SO₄. The generation of gas was collected into distilled water. Aqueous sulphur dioxide was generated by the reaction:

SO₂⁻ + O₂ → SO₃⁻
SO₃⁻ + SO₂⁻ → SO₄⁻ + SO₄²⁻
SO₄⁻ + SO₃⁻ → SO₄²⁻ + SO₅⁻
SO₄⁻ + OH⁻ → SO₄²⁻ + OH⁺

Termination

Superoxide dismutase (SOD) acts as a superoxide radical scavenger under oxidative stress. Plant chloroplasts contain high quantities of superoxide dismutase which catalyses the dismutation of two superoxide radicals to produce hydrogen peroxide. Sulphur dioxide induced superoxide dismutase activity was noted in Populus euramericana (Tanaka and Sugahara, 1980), and in pea (Jager et al., 1985). Sulphur dioxide tolerant plants exhibited a significant increase in superoxide dismutase activity than the sensitive plants (Agarwal et al., 1987; Nageswar Rao and Alsch, 1991; Rao and Dubey, 1993). The H₂O₂ formed in response to SO₂ is disposed off by catalase and peroxidase to water and oxygen.

Catalase is an enzyme present in nearly all plant cells predominantly in peroxisomes, which catalyses the following reaction: The activity of catalase in response to SO₂ is varied. Continuous decline in catalase activity was noticed in Oryza sativa (Nandi et al., 1984). However in maize, SO₂ toxicity led to an initial rise followed by a decline in the activity of catalase (Matters and Scandalios, 1987). Nandi et al. (1984) reported that the tetrameric catalase molecules of SO₂ exposed plants possibly degrade into monomeric catalase sub-units leading to the loss of its activity.

Peroxidases occur in different forms in plants and need a substrate (R) to convert hydrogen peroxide to water and oxygen. Sulphur dioxide enhances the peroxidase activity in several plants (Horsmann and Wellburn, 1975; Murray, 1984). However the increased rates of peroxidase activity varies from species to species (Murray, 1985b; Varshney and Varshney, 1985). The combined action of superoxide dismutase, catalase, and peroxidase efficiently eliminates OH⁻ and H₂O₂ and act as defensive mechanisms against SO₂ in plants. The degree of activity of these enzymes being dependent on the tolerance capacity of the plants (Bor-Hung, 1994). The sensitivity of plant species therefore depends upon the activities of these enzymes. Hence, it was intended to study the activities of these enzymes in pigeonpea and amaranth in response to aqueous SO₂ exposure.

MATERIALS AND METHODS

Plant material

Seeds of pigeonpea (Cajanus cajan (L.) Millsp. cv. PDM1), a C₃ plant, is an important pulse crop and amaranth (Amaranthus paniculatus L. a local cultivar), a C₄ plant, is popular green leafy vegetable consumed all over India were selected for present study.

Preparation of aqueous sulphur dioxide

Sulphur dioxide was prepared in the laboratory by reacting sodium metabisulphite with concentrated H₂SO₄ and the generated gas was collected into distilled water. Aqueous SO₂ concentration was determined titrimetrically according to the method of Vogel (1961). Fresh stock solution of 1000 ppm concentration was prepared and from it the various concentrations of SO₂ were prepared by diluting with distilled water. The pH was adjusted to 6.9 by adding dilute NaOH. It was reported that 1 ppm SO₂ in air gives 1000 ppm in aqueous solution (Puckett et al., 1973; Saunders and Wood, 1973; Malhotra, 1977).

Effect of incubation of leaf discs under light and dark conditions

Seeds were washed with distilled water and surface sterilized with 0.01 M mercuric chloride and were raised in earthen pots filled with soil containing farm yard manure and soil in the ratio of 1:3. The plants were watered on alternate days. The plants were grown under a natural photoperiod of approximately 12 h and average day temperatures of 31 ± 2 °C and 21 ± 1 °C at night at Andhra university experimental farm. Fully expanded third leaves from top of 1-month old pigeonpea and amaranth plants grown separately in earthenware pots for this purpose were harvested from 20 plants at 8:00 a.m. Discs of 1.0 cm diameter were cut from the leaves and floated with abaxial surface downwards in petri dishes containing 0, 10, 20, 30, 40, 50, 100 and 250 ppm aqueous SO₂. The petri dishes were covered with glass lids and sealed with silicon grease. Some sets of leaf discs were exposed to light of 195 µ mol m⁻² s⁻¹ and other sets of leaf discs were wrapped in aluminum foil to obtain dark conditions. All the leaf discs were exposed to a temperature of 30 ± 2 °C. The leaf discs were allowed to incubate 24 h in light and dark conditions. The leaf discs exposed to zero SO₂ concentration were termed as controls. The leaf disc samples were collected at 6, 12, 18 and 24 h of incubation, washed twice with distilled water to remove traces of aqueous SO₂ and used for analysis.

Ascorbic acid content

Ascorbic acid content was estimated according to the method of Roe (1964) which is based on the reduction of the dye, 2,6-dichlorophenol indophenol (DCIP) by ascorbic acid (AA) from its pink color in the acid medium to the colorless leuco form. One gram of sample leaf discs were macerated thoroughly and rapidly with 10 ml of 5% (w/v) metaphosphoric acid using a mortar and pestle. The maceration was completed within 1 or 2 min. and the homogenate was made up to 20 ml with 5% metaphosphoric acid. It was filtered through filter paper. Duplicate samples of 10 ml aliquots were titrated with DCIP reagent until a pink end point which persists for 15 sec was obtained. The quantity was calculated using the formula:

\[ \text{D} = \frac{1}{L \times S \times \frac{W}{A}} = \text{mg of AA per g material} \]

where

\[ L = \text{ml of DCIP reagent used in the titration.} \]
\[ S = \text{mg of AA reacting with 1 ml of reagent.} \]
\[ D = \text{volume of the extract in ml} \]
\[ A = \text{volume of the aliquot titrated in ml} \]
\[ W = \text{weight of the sample in g} \]

Ascorbic acid oxidase (EC 1.10.3.3)

Ascorbic acid oxidase activity was assayed according to the method of Povolotskaya and Sedenka (1956) as modified by Gopalachari (1963). Two hundred ng
sample leaf discs were grounded in a pre-cooled glass mortar, using cold phosphate-citrate buffer (pH 5.0). The homogenate was filtered through glass wool and made up to 25 ml with the same buffer solution. Four ml of the enzyme extract was added to each of the three 50 ml conical flasks followed by 2.5 ml of phosphate-citrate buffer, pH 5.0. In the first two flasks, 2.5 ml of 0.2% ascorbic acid was added and they were kept for 30 min with occasional shaking. At the end of 30 min, 1 ml of 10% (w/v) TCA was added to stop the enzyme activity. In the third flask which served as blank, 4 ml of the extract and 1 ml of 10% TCA was added immediately followed by 2.5 ml buffer and 2.5 ml ascorbic acid.

One ml of the solution from each flask was taken into 50 ml conical flask, diluted with 5 ml of distilled water and was titrated against 0.001 N 2,6-DCIP reagent till a permanent pink colour was obtained. From the differences in the titre values, ascorbic acid oxidase activity was expressed as mg of ascorbic acid oxidised in 30 min per gram weight of the tissue. The calculation of the activity of enzyme was simplified using the formula

\[ A = \frac{V}{W} \times 0.088 \times 10 \times \text{x \ldots \ldots \ldots} = \text{-----} \]

Where \( A \) = ascorbic acid oxidase activity (expressed as mg ascorbic acid oxidised in 30 min per gram weight).

\( V \) = difference in titre values between control and treatment in 1 ml.

\( W \) = fresh weight in grams of plant material.

The factor 0.088 represents 1 ml of 0.001 N 2,6-DCIP is equal to 0.088 mg of ascorbic acid.

**Polyphenol oxidase**

Polyphenol oxidase activity was determined according to the method of Kar and Mishra (1976). The procedure similar to peroxidase was followed for obtaining the enzyme extract of polyphenol oxidase. Five ml of the assay mixture for the polyphenol oxidase activity comprised: 125 \( \mu \) moles of phosphate buffer, pH 6.8, 50 \( \mu \) moles of pyrogallol, and 1 ml of the 20 times diluted enzyme extract. This was incubated for 5 min at 25°C. The reaction was stopped by adding 0.5 ml of 5% \( \text{H}_2\text{SO}_4 \) (v/v). The amount of purpurogallin formed was determined by taking the absorbance at 420 nm on 150-20 UV-VIS spectrophotometer (Hitachi, Japan). The enzyme activity was expressed in absorbance unit. The 0.1 difference was taken as one unit of enzyme activity.

**Superoxide dismutase (EC 1.15.1.1)**

One g of plant material was homogenized in 5 ml of 50 mM phosphate buffer (pH 7.0) containing 1% polyvinyl pyrrolidone. The homogenate was filtered and centrifuged at 15,000 x g for 10 min in refrigerated centrifuge. The supernatant obtained was used as enzyme extract. All steps in the preparation of enzyme extract were carried out at 0-4°C. An aliquot of 0.1 ml of the enzyme extract was used for the determination of protein content by using the method of Lowry et al. (1951). The activity of superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium, adopting the method of Beauchamp and Fridovich (1971). The reaction mixture 3 ml contained 40 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 \( \mu \)M nitroblue tetrazolium, 2 \( \mu \)M riboflavin, 0.1 mM EDTA and 0.1 ml of enzyme extract. Riboflavin was added last and the test tubes were shaken and placed 30 cm below light source consisting of two 15 W fluorescent lamps. The reduction was started by switching on the light. The reaction was allowed to take place for 90 min and was stopped by switching off the light. Then the tubes were covered with a black cloth. The absorbance of the solution was measured at 560 nm on Systronics 112 spectrophotometer. The reaction mixture which was not exposed to the light did not develop colour and served as control. \( \log A_{560} \) was plotted as a function of the volume of enzyme extract used in the reaction mixture. From the resultant graph, the volume of the enzyme extract corresponding to 50% inhibition of the reaction was read and considered as one enzyme unit.

**Catalase (EC 1.11.1.6)**

Catalase activity was estimated by the permanganate method of Povolotskaya and Sedenka (1956) as followed by Gopalachari (1963) with slight modification. Two hundred mg of leaf discs were weighed and was ground in a precooled glass mortar with few ml of cold phosphate buffer pH (7.0). The extract was filtered through glass wool and made up to 25 ml with the same buffer solution. Two ml of the enzyme extract was taken in a conical flask and 1 ml of 0.045 M hydrogen peroxide was added. Exactly after 5 min, one ml of 12% \( \text{H}_2\text{SO}_4 \) was added to stop the enzyme activity and titrated with 0.05 N potassium permanganate. The end point was denoted by the pink colour of the solution which lasts for 30 sec. A blank was run simultaneously as above substituting 2 ml of the enzyme extract with 2 ml phosphate buffer. The difference in the titre values gave the activity of catalase and was expressed as mg \( \text{H}_2\text{O}_2 \) destroyed per 5 min per g plant tissue by using the following equation:

\[ \text{mg } \text{H}_2\text{O}_2 \text{ destroyed in } 5 \text{ min by } 1 \text{ g leaf discs} = \frac{V}{W} \times \frac{25}{0.85} \]

where

\[ V = \text{difference between blank and sample titre values} \]

\[ W = \text{fresh weight in grams} \]

\[ 2 = \text{extract taken in ml} \]

The factor 0.85 represents 1 ml of 0.05 N \( \text{KMnO}_4 \) equal to 0.85 mg of \( \text{H}_2\text{O}_2 \).

**Peroxidase (EC 1.11.1.7)**

Peroxidase activity was estimated by the method followed by Kar and Mishra (1976). Two hundred mg of each sample was homogenized with 10 ml of 0.1 M phosphate buffer, pH 6.8 and centrifuged at 17,000 x g in a refrigerated centrifuge. The supernatant was taken as the enzyme extract. The assay mixture for the peroxidase comprised 125 \( \mu \) moles of phosphate buffer (pH 6.8), 50 \( \mu \) moles of pyrogallol, 50 \( \mu \) moles of \( \text{H}_2\text{O}_2 \) and 1 ml of enzyme extract. This was incubated for 5 min at 25°C after which time the reaction was stopped by adding 0.5 ml of 5% \( \text{H}_2\text{SO}_4 \). The amount of purpurogallin formed was determined by taking the absorbance at 420 nm in Schimadzu (UV-240) spectrophotometer. A similarly treated blank by substituting distilled water for the enzyme extract was used for zero setting. The enzyme activity was expressed as absorbance units (0.1 difference in absorbance value was taken as one unit of enzyme activity) per mg protein. The protein content of the enzyme extract was determined by the method of Lowry et al. (1951).
RESULTS

Ascorbic acid

The control leaf discs have always exhibited higher values of ascorbic acid content than the SO$_2$ treated leaves. Ascorbic acid content was conspicuously reduced in response to SO$_2$ even at the early stages of incubation in both the pigeonpea and amaranth. The difference between control and SO$_2$ treated leaves was greatest at the end of the experimental period. Further the degree of decline of ascorbic acid content was more conspicuous under dark. The SO$_2$ effect appeared more conspicuous on the amaranth than on the pigeonpea leaf discs. The ascorbic acid content in control leaves of pigeonpea under light exposure was 0.386 mg at 6 h and 0.365 mg at 24 h respectively. The corresponding values at 250 ppm SO$_2$ treatment was 0.162 and 0.050 mg respectively. On the other hand for amaranth, it was 0.853 mg at 6 h and 0.659 mg at 24 h for the controls and the corresponding values for 250 ppm SO$_2$ treatment was 0.132 and 0.030 mg at 6 and 24 h respectively. The same trend of ascorbic acid content was registered under dark exposure also in both pigeonpea and amaranth (Fig. 1 a,b,c,d).

Ascorbic acid oxidase

The ascorbic acid oxidase activity decreased continuously in the control leaf discs of both the plant species from 6 to 24 h of incubation under light and dark conditions. Ascorbic acid oxidase activity was observed initially more in the lower SO$_2$ concentration and at the initial stages followed by a continuous decline in the higher concentrations and at later stages in both the plant species. Higher SO$_2$ concentrations reduced the ascorbic acid oxidase activity. Further the reduction in the activity of ascorbic acid oxidase was more under dark than under light exposure. The reduction of ascorbic acid oxidase activity was observed more in amaranth than in pigeonpea leaf discs (Fig. 2 a,d,c,d).

Polyphenol oxidase activity

The polyphenol oxidase activity of control leaf discs of both the plant species showed a continuous increase from 6 to 24 h of incubation. Pigeonpea and amaranth differed in their response to aqueous SO$_2$. Pigeonpea exhibited a continuous increase of polyphenol oxidase activity from 6 to 24 h period of SO$_2$ incubation both under light and dark conditions. The values of SO$_2$ treated pigeonpea leaf discs were always greater than their respective control leaf discs. Maximum polyphenol oxidase activity was observed at 250 ppm SO$_2$. On the other hand amaranth leaf discs initially exhibited greater polyphenol oxidase activity at the 10 ppm SO$_2$ followed by a decline with increasing SO$_2$ concentration and

Figure-1: The effect of aqueous SO$_2$ on ascorbic acid content of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.), a and b - Pigeonpea; c and d – Aamaranth, --- under light; — under dark ○- 0 ppm; □-10 ppm; ×-20 ppm; ∆-30 ppm; ○-40 ppm; ●-50 ppm; ■-100 ppm; ▲-250 ppm

Figure-2: The effect of aqueous SO$_2$ on the ascorbic acid oxidase activity in pigeonpea and amaranth leaf discs (Vertical lines represent S.E.), a and b - Pigeonpea; c and d – Aamaranth, ---- under light; --- under dark ○- 0 ppm; □-10 ppm; ×-20 ppm; ∆-30 ppm; ○-40 ppm; ●-50 ppm; ■-100 ppm; ▲-250 ppm

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duration of exposure. The lowest values of polyphenol oxidase activity were registered at 250 ppm treated leaf discs, both under light and dark conditions (Fig. 3 a,b,c,d).

Superoxide dismutase

The results presented in the figure 4a,b,c,d indicate that the leaf discs of both pigeonpea and amaranth exposed to aqueous SO$_2$ exhibited an increase in the superoxide dismutase activity compared to their respective controls both in the light and dark. The controls always exhibited lower values than SO$_2$ treatments throughout the investigation. Superoxide dismutase activity increased with increasing SO$_2$ concentrations and duration of incubation. Among the treatments the catalase activity reached a minimum in 250 ppm SO$_2$ treatment at 24 h in both pigeonpea and amaranth. In pigeonpea the reduction of catalase activity at this concentration at 24 h was 13.42 fold in the light and 14.51 fold in the dark conditions over their respective controls. Amaranth showed 9.84 fold reduction in light and 37.67 fold reduction in dark over their respective controls. When light and dark treatments were compared the decline becomes more conspicuous in the dark. Further in between pigeonpea and amaranth (Fig. 4a,b,c,d).

Catalase

The catalase activity declined continuously from 6 to 24 h of incubation both in the control and SO$_2$ treated leaf discs of pigeonpea and amaranth and reaches its lowest activity at 24 h. However, the control leaf discs always exhibited higher values than SO$_2$ treated leaf discs at all the stages of study. The rate of activity of catalase declined more rapidly with increasing aqueous SO$_2$ concentration and time of incubation in both the plant species. Among the treatments the catalase activity reached a minimum in 250 ppm SO$_2$ treatment at 24 h in both pigeonpea and amaranth. In pigeonpea the reduction of catalase activity at this concentration at 24 h was 13.42 fold in the light and 14.51 fold in the dark conditions over their respective controls. Amaranth showed 9.84 fold reduction in light and 37.67 fold reduction in dark over their respective controls. When light and dark treatments were compared the decline becomes more conspicuous in the dark. Further in between pigeonpea and amaranth (Fig. 4a,b,c,d).

Figure-3: The effect of aqueous SO$_2$ on the polyphenol oxidase activity in pigeonpea and amaranth leaf discs (Vertical lines represent S.E.), a and b - Pigeonpea; c and d - Amananth, --- under light; — under dark ○ - 0 ppm; □ - 10 ppm; × - 20 ppm; Δ - 30 ppm; ○ - 40 ppm; ● - 50 ppm; ■ - 100 ppm; ▲ - 250 ppm

Figure-4: The effect of aqueous SO$_2$ on the activity of superoxide dismutase (SOD) in pigeonpea and amaranth leaf discs (Vertical lines represent S.E.), a and b - Pigeonpea; c and d - Amananth, --- under light; — under dark ○ - 0 ppm; □ - 10 ppm; × - 20 ppm; Δ - 30 ppm; ○ - 40 ppm; ● - 50 ppm; ■ - 100 ppm; ▲ - 250 ppm
amaranth, catalase activity was more affected in amaranth in response to SO\textsubscript{2} (Fig. 5 a,b,c,d). In amaranth the effect of SO\textsubscript{2} on peroxidase activity was not conspicuous. Lower concentration of 10 ppm increased the peroxidase activity up to 18 h followed by a decline. However, with increasing concentrations of SO\textsubscript{2} the enzyme activity decreased, reaching a minimum at 250 ppm under light conditions. A similar trend was also noticed under dark exposure (Fig. 6 c,d).

**DISCUSSION**

Ascorbic acid scavenges the free radicals and protects the plants from SO\textsubscript{2} injury (Keller and Schwager, 1977; Grill et al., 1979; Takahama et al., 1992) Ascorbic acid acts as an antioxidant. Ascorbic acid also plays an important role in the reduction of SO\textsubscript{2} to less reactive compounds in plants. The presence of ascorbic acid also stimulate CO\textsubscript{2} fixation in isolated chloroplasts (Isherwood and Mapson, 1962; Champigny and Gibbs, 1969). Reduction of ascorbic acid content in responses to SO\textsubscript{2} was observed in several plants (Varshney and Varshney, 1984; Murray, 1985a). Aqueous SO\textsubscript{2} reduced the ascorbic acid content in the leaf discs of incubated leaf discs were 8.83, 6.89, 6.58 and 5.67 folds respectively (Fig. 6 a,b).
pigeonpea and amaranth. The degree of its reduction was dependent on SO$_2$ concentration and duration of exposure (Fig. 1 a,b,c,d). Maximum reduction of ascorbic acid content was noted in 250 ppm SO$_2$ treatment. Further, the reduction was more conspicuous in dark (Fig. 1 b,d). The highly reactive oxy radicals promote the oxidation of ascorbic acid to dehydroascorbic acid, leading to the reduction of ascorbic acid content in the leaf discs of pigeonpea and amaranth (Fridovich and Handler, 1961; Varshney and Varshney, 1984). The decrease in ascorbic acid content was relatively more in amaranth than in pigeonpea.

The activity of ascorbic acid oxidase increased in the initial stages of lower SO$_2$ concentrations followed by a decline with increasing concentration and duration of SO$_2$ exposure in both the plant species (Fig. 2 a,b,c,d). The decrease was more in dark than in light (Fig. 2 b,d). These findings suggest that ascorbic acid oxidase alone is not the sole agent for the degradation of ascorbic acid in the leaf discs of pigeonpea and amaranth under SO$_2$ exposure. The possible role of free radicals are also involved in the degradation of ascorbic acid (Varshney and Varshney, 1984).

The polyphenol oxidase activity varied between pigeonpea and amaranth in response to SO$_2$ exposure. A continuous increase in polyphenol oxidase activity with increasing SO$_2$ concentration and duration of exposure was registered in pigeonpea. Several folds increase was noted in 250 ppm SO$_2$ treatment in both light and dark conditions (Fig.3 a,b). However in amaranth an initial rise of polyphenol oxidase activity at 10 ppm SO$_2$ exposure was observed followed by its continuous decline with increasing concentration of SO$_2$ revealing the sensitivity of polyphenol oxidase in amaranth (Fig. 3 c,d). Elevated polyphenol oxidase activity to SO$_2$ has been reported in horse chestnut (Aesculus hippocastanum). Polyphenol oxidase activity may also be considered as an index of pollutant stress including SO$_2$, NO$_2$ and hydrocarbons (Godzik, 1967). Therefore it was suggested that the assay of polyphenol oxidase may be used as a diagnostic test for the selection of SO$_2$-resistant genotypes (Wellburn et al., 1976). The increased activity of polyphenol oxidase in pigeonpea indicates its relative tolerance to SO$_2$ than amaranth.

The photooxidation of SO$_2$ was associated with the generation of superoxide radicals (O$_2^-$) (Asada and Kiso, 1973; Peiser and Yang, 1985). The dismutation of superoxide radicals lead to the production of H$_2$O$_2$ and O$_2$.$^2$. Interactions among the H$_2$O$_2$, O$_2$.$^2$ and chloroplast components produce the hydroxyl radicals (OH) and singlet oxygen (O$_3$), (Weiser and Benson, 1966; Allen, 1975; Asada et al., 1977; Tanaka et al., 1978; Takashashi et al., 1980). The H$_2$O$_2$ scavenging enzymes in chloroplasts such as ascorbate peroxidase, glutathione reductase, and catalase were sensitive to SO$_2$. The inactivation of these enzymes led to the accumulation of H$_2$O$_2$ in chloroplasts (Tanaka et al., 1982a). Tanaka et al., (1982b) reported that accumulation of H$_2$O$_2$ in response to SO$_2$ in spinach leaves suppressed photosynthesis by inhibiting the calvin cycle enzymes.

The superoxide dismutase was active in both the leaf discs of pigeonpea and amaranth (Fig. 4 a,b,c,d) which is considered to involve in the elimination of O$_2^-$ radicals formed during the chain reactions of SO$_2$ oxidation. Interestingly the levels of peroxidase in response to SO$_2$ increased in pigeonpea and it declined in amaranth (Fig. 6 a,b,c,d). On the other hand the catalase activity declined continuously in both the plant species. H$_2$O$_2$ formed due to superoxide dismutase activity is to be disposed off by peroxidase and catalase, otherwise the accumulation of H$_2$O$_2$ may affect leaf metabolism (Fridovich, 1978, 1986). The decreased activity of peroxidase and catalase in response to SO$_2$ may not completely scavenge H$_2$O$_2$ formed and therefore may affect the photosynthetic rate of both the plant species (Kaiser, 1976), though the impact may differ between the two plant species (Winner and Mooney, 1980b). It was also demonstrated that at high CO$_2$ concentrations, the C$_3$ plants were less sensitive to SO$_2$ than the C$_4$ plants (Carlson and Bazzaz, 1985).

Plants develop certain defensive mechanisms to eliminate the deleterious affects of free radicals (O$_2^-$, OH and SO$_2^-$) that were generated during SO$_2$ transformations in plants (Asada and Kiso, 1973; Peiser and Yang, 1977; Tanaka et al., 1982a; Bowler et al., 1992). The activity of superoxide dismutase, eliminate superoxide radicals and accumulate hydrogen peroxide (Kaiser, 1976; Fridovich, 1975, 1978; Tanaka and Sugahara, 1980; Halwit, 1987; Tanaka et al., 1982b). The scavenging of hydrogen peroxide is indispensable in SO$_2$ exposed plants to lessen its toxicity. Catalase and peroxidase acts as protectants against hydrogen peroxide damage in plants (Nakano and Asada, 1980; Bowler et al., 1992). An increase in the superoxide dismutase activity was registered in both the pigeonpea and amaranth leaf discs with increasing concentration and duration of SO$_2$ exposure (Fig. 4 a,b,c,d). Higher levels of superoxide dismutase was observed in 250 ppm SO$_2$ treatments in both the plant species at 24 h exposure. The superoxide dismutase activity was more in dark than in light in both the plant species (Fig. 4 b,d). Further, the enzyme activity was more in amaranth than in pigeonpea with respect to controls leading to higher accumulation of hydrogen peroxide in amaranth.

It was suggested that SO$_2$-induced increase of superoxide dismutase was associated itself with SO$_2$ tolerance in plants (Tanaka and Sugahara, 1980; Andrea et al., 1994; Miszalaski and Niewiadomska, 1994). However, this may not be true always (Nageswar and Alsch Scheer, 1991; Bor-Hung Sheu, 1994). Interestingly the increased superoxide dismutase activity in amaranth does not correlate with SO$_2$ tolerance, perhaps may be due to its more sensitivity to other photosynthetic parameters (Saraswathi and Madhava Rao, 1995). In recent studies transgenic tobacco plants were also used to assess the correlation between tolerance and superoxide dismutase activity in plants. Though the transgenic tobacco plants showed 30 to 50 fold increase of superoxide dismutase activity, they however, were not resistant to superoxide toxicity as expected (Pitcher et al., 1991).

The changes in the levels of catalase activity in response to SO$_2$ were presented in the figure 5 a,b,c,d. The catalase activity declined continuously with increasing concentration and duration of aqueous SO$_2$ exposure in both the plant species. The decline was more under dark than under light condition in both the plant species. Further the relative degree of decline in catalase activity was more in amaranth (Fig. 5 c,d). The decrease in catalase activity in both the plant species suggest the possible accumulation of hydrogen peroxide in the SO$_2$ treatments over their respective controls which led to foliar injury.
Peroxidase activity increased with increasing concentration and duration of SO\textsubscript{2} exposure in pigeonpea both under light and dark conditions (Fig. 6 a,b). Several fold increase in the activity of peroxidase was noted at 250 ppm SO\textsubscript{2} of 24 h exposure. However, in amaranth an initial rise of peroxidase activity at lower SO\textsubscript{2} concentration was followed by a continuous decline with increasing SO\textsubscript{2} concentration (Fig. 6 c,d). Effect of SO\textsubscript{2} on peroxidase activity varies from species to species (Murray, 1984; Nandi et al., 1984; Farooq et al., 1985; Varshney and Varshney, 1985; Pfanz et al., 1990; Takahama et al., 1992; Nast et al., 1993; Andrea et al., 1994; Bor-Hung Sheu, 1994). Therefore, it may be assumed that peroxidase is closely associated with SO\textsubscript{2} defensive system of pigeonpea rather than of amaranth. To mitigate and repair oxidative damage, plants have evolved an efficient antioxidant system that includes enzymes such as superoxide dismutase, catalase, and peroxidase that function to scavenge ROS (Sharma and Dubey, 2007).

CONCLUSIONS

The ascorbic acid content of both pigeonpea and amaranth decline in response to aqueous SO\textsubscript{2}. The decline was more in dark and the decline was more expressed in amaranth. The ascorbic acid oxidase activity increased initially for a short period in lower SO\textsubscript{2} concentrations. A continuous decline was observed at higher aqueous SO\textsubscript{2} concentrations. However the activity of polyphenol oxidase differed in pigeonpea and amaranth in response to SO\textsubscript{2} exposure. Pigeonpea exhibited many fold increase of polyphenol oxidase in response to SO\textsubscript{2}. On the other hand, the activity of polyphenol oxidase decreased continuously in amaranth indicating its sensitiveness to SO\textsubscript{2}. The activity of polyphenol oxidase was recorded more in dark. The activities of oxynitrogen scavenging enzymes such as superoxide dismutase, catalase and peroxidase differed between pigeonpea and amaranth. The activity of superoxide dismutase increased with increasing SO\textsubscript{2} concentration in both the plant species studied, though it was recorded slightly more in amaranth. On the other hand, catalase activity decreased with increasing SO\textsubscript{2} concentration and duration of exposure in both the plant species. However, the decline was more in amaranth. The activity of peroxidase increased rapidly with increasing SO\textsubscript{2} concentration in pigeonpea, whereas in amaranth its activity decreased throughout the investigation period.

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