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Changes in Lipid Metabolism by Thin Layer Chromatography in Leaf Discs of *Cajanus Cajan* and *Amaranthus Paniculatus* under Aqueous Sulphur Dioxide

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----------ABSTRACT----------
The impact of elevated aqueous SO_2 (0, 10, 20, 30, 40, 50, 100 and 250 ppm) on lipid metabolism by thin layer chromatography and lipid peroxidation of pigeonpea (Cajanus cajan (L.) Millsp. cv. PDM1), a C_3 plant and amaranth (Amaranthus paniculatus L. a local cultivar), a C_4 plant leaf discs under light and dark conditions has been studied. The total lipids, phospholipids, individual phospholipids and glycolipids decreased both in pigeonpea and amaranth in response to aqueous SO_2. The reduction of lipid metabolism was expressed more in dark than in light exposed leaf discs of both the plant species. In between pigeonpea and amaranth the lipid peroxidation was observed more in the later and was always greater under dark than under light conditions.
Keywords: *Amaranthus paniculatus, aqueous SO_2, Cajanus cajan, light and dark, lipid metabolism, lipid peroxidation.*

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I. INTRODUCTION

Air pollution can directly affect plants via leaves or indirectly via soil acidification. When exposed to airborne pollutants, most plants experienced physiological changes before exhibiting visible damage to leaves (Liu and Ding, 2008). Sulfur dioxide occupies leading position as an air pollutant due to its potential hazard for vegetation as well as due to its wide distribution over the world has been reported to induce visible injury to leaves and leads to reduction in photosynthetic pigments inhibition of metabolic processes and suppression of growth and yield of plants of natural and agricultural ecosystems (Agrawal and Agrawal, 1991). This type of leaf injury is characterized by reduced rate of physiological processes, such as photosynthesis, respiration, lipid and protein synthesis etc. Injury takes place at biochemical level in the form of inactivation of enzymes, disorganization of cellular membranes and alteration of organic and inorganic ingredients of plants. In the cells, SO_2 dissolves to give bisulfite and sulfite ion, sulfite is toxic and at low concentrations it is metabolized by chloroplasts to sulfate, which is non toxic and at sufficiently low concentrations, bisulfite and sulfite are effectively detoxified by plants and SO_2 air pollution then provides a sulfur source for the plant (Zeiger, 2006).

Lipids are the important constituents of biological membranes store energy and perform several cellular functions (James and Nicholas, 1966; Kates and Marshall, 1975; Mudd and Garcia, 1975). The principal plant lipids of most plant membranes are phospholipids, glycolipids and sterols. Leaves in general have a lipid content of about 10% of the dry weight, whereas leaf chloroplasts and unicellular algae have much higher lipid content. The major phospholipids are phosphatidyl cholin, phosphatidyl ethanolamine, phosphatidyl glycerol and phosphatidyl insitol. The two most abundant glycolipids are monogalactosyl diglycerides and digalactosyl diglycerides. Sulphur dioxide causes a marked reduction in the lipid content of the leaves. Since chloroplasts are presumed as the major targets of SO_2 action, it is interesting to know its effects on lipid components of chloroplast membrane (Shaw *et al*., 1976). Exposure of *Pinus contorta* and *Pinus banksiana* to aqueous SO_2 showed a marked inhibition in the *de novo* synthesis of phospholipids, glycolipids and neutral lipids. The intensity of SO_2 inhibition was dependent on its concentration and duration of exposure. The effect was however transitory in nature, as the inhibition was partially or completely reversed upon removal of plants from the SO_2 exposure (Khan and Malhotra, 1977; Malhotra and Khan, 1979). However, reduction in linolenic acid content and an increase in palmatic acid content in both the leaves suggested that SO_2 inhibited both the elongation and desaturation processes. The decline in the linolenic acid content may be due to SO_2-stimulated free radical peroxidation reactions.

This is indicated by the accumulation of malondialdehyde in SO_2 treated leaves (Khan and Malhotra, 1977). Lizada and Yang (1981) also attributed the peroxidation of linolenic acid and linolenic acid to the free radicals which were generated from the oxidation of sulphite to sulphate.

Peiser *et al*. (1982) studied lipid peroxidation in spinach chloroplasts, taking ethane as a measure of lipid peroxidation. Ethane formation required sulphite and light and its formation occurred with concomitant oxidation of sulphite to sulphate. In the dark, both ethane formation and sulphite oxidation were inhibited. These two activities were also effectively inhibited by free radical scavengers like ascorbate or superoxide dismutase.

Malondialdehyde formation can also be used as a measure of lipid peroxidation. Shimazaki *et al*. (1980) observed increased malondialdehyde content in SO_2 fumigated spinach leaves. Malondialdehyde formation was inhibited when the free radical scavengers like ascorbate, glutathioine and superoxide dismutase were introduced into the leaf homogenate. This support the view that membrane damage is caused mainly by lipid peroxidation (Peiser *et al*., 1982). Recent work of Jager *et al*. (1985) showed that the concentration of malondialdehyde, which is a product of lipid peroxidation was significantly higher in SO_2 polluted plants (Heath and Packer, 1968; Dindsha *et al*., 1981). Present investigation is aimed for the analysis of lipid metabolism by thin layer chromatography and lipid peroxidation of various levels of sulphur dioxide pollution on two crop plants pigeonpea and amaranth leaf discs under light and dark conditions.

II. MATERIALS AND METHODS

Plant material

Seeds of pigeonpea (*Cajanus cajan* (L.) Millsp. cv. PDM1), a C_3 plant is an important pulse crop of India and amaranth *(Amaranthus paniculatus* L. a local cultivar), a C_4 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 H_2SO_4 and the generated gas was collected into distilled water. Aqueous SO_2 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 SO_2 were prepared by diluting with distilled water. The pH was adjusted to 6.9 by adding dilute NaOH. It was reported that 1 ppm SO_2 in air gives 1000 ppm in aqueous solution (Puckett *et al.,* 1973; Saunders and Wood, 1973; Malhotra, 1977).

Effect of aqueous SO_2 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 ^oC and 21 ± 1 ^oC 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 SO_2. The petri dishes were covered with glass lids and sealed with silican 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 ^oC. The leaf discs were allowed to incubate 24 h in light and dark conditions. The leaf discs exposed to zero SO_2 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 SO_2 and used for analysis.

Total lipids

Extraction and estimation: Total lipid extraction was carried out according to the method of Bligh and Dyer (1959). One g treated and control leaf discs were washed and macerated with 14.4 ml boiling mixture of chloroform : methanol : water (1:2:0.6 V/V/V). The contents were filtered using muslin cloth. The filtrates were transferred into stoppered test tubes and the residues were reextracted with 8.0 ml of hot methanol and again transferred to stoppered tubes. To these tubes, 12 ml each of chloroform was added. After keeping the samples over night at 0°C in deep freeze, 11.2 ml of water was added to each tube, shaken well and centrifuged for phase separation. The final ratio of chloroform: methanol: water in each tube was 2:2:1.8 (V/V/V). The aqueous phases were removed with suction. The lipid phases were washed thrice with methanol: water (2:1.8 V/V) mixture. The contents were centrifuged in a refrigerated centrifuge at 3000 x g for 10 min in order to remove the water soluble compounds. The lipid extracts were evaporated to dryness.

The dried lipid residues were taken into chloroform and transferred to pre-weighed bottle and evaporated to dryness under nitrogen. The bottles were kept in a vaccum dessicator over potassium hydroxide under reduced pressure for several hours and weighed again. The difference between the weights was taken as the weight of the total lipid present in each sample. The total lipid of each sample was dissolved in chloroform, made upto 5 ml and was preserved at 0°C in tightly stoppered standard flask until further analysis. The total lipid content was expressed as mg lipid/g fresh weight.

Total phospholipids: The total phospholipid content was determined as the phosphorus content of the phospholipids procedure outlined by Bartlett (1959) and those of total glycolipids were determined by the phenol-sulphuric acid method. The lipid samples were taken into test tubes marked with 10 ml. The organic solvent was removed by passing compressed air into the test tubes. One ml of 60% perchloric acid was added to each tube and digested at 170-180°C on a heating mantle. Digestion was continued until the samples were clear. After digestion, to each tube 4.5 ml of 0.44% ammonium molybdate reagent was added, followed by 0.2 ml of 1-amino-2-naphthol-4-sulphonic acid reagent. The contents of the tubes were shaken well and heated over a boiling water bath for 10 min. After cooling, the volume was adjusted to 10.0 ml with glass distilled water. The colour intensity of each sample was measured at 660 nm using Schimadzu (UV 240) Spectrophotometer. Aliquots of KH_2PO_4 solution containing 2 g phosphorus/ml were used as standards. Blanks were run simultaneously. For computing the total phospholipid content, the lipid phosphorus was multiplied by factor 2.5. The results were expressed as mg phosphorus/g fresh weight.

Individual phospholipids

Preparation of TLC plates: TLC plates were prepared by spreading slurry of silica gel – G (50g in 100 ml of water) of 0.5 cm thickness with the help of an applicator. The plates were allowed to dry at room temperature and before starting the experiment, the gels on the plates were activated by heating at 110°C for one hour.

Application of the sample: The lipid samples in chloroform were applied to the TLC plates with the help of a micropipette.

Chromatography chambers: The Chromatography chambers are lined with filter paper and were saturated with the solvent at least one hour before use. The plates were developed in solvent system until the solvent front was about 2 cm from the top edge.

Solvent system: The solvent system contained chloroform, methanol, acetic acid and water in the ratio of 100:20:10:3 respectively.

Detection of spots: The plates after running were air dried at room temperature for 20 min. The phospholipid species were detected with iodine vapour. The individual lipid species thus separated, were marked with a fine dissecting needle. Most of the iodine was allowed to evaporate before removal of spots, small amount of iodine if any did not interfere with the quantitative analysis of phosphorus.

Conformation of phospholipids using phosphate reagent was carried out by the method of Skipski and Barclay (1969).

Removal of spots: The individual phospholipids resolved on the TLC plates were removed with a razor blade carefully. A drop of water was placed on each of the spots to facilitate easy removal. The silica gel scrapings containing the phospholipid species were transferred to a centrifuge tube with the help of a spatula. To ensure complete transfer of material, the remaining periphery of the spot was scrapped off from the plate was placed vertically and tapped to allow scrapings to fall on to glazed paper. This powder was pooled with previously removed material. This procedure minimized losses of phospholipids. The area in which there were no lipid spots was used as blank.

Quantitative determination of individual phospholipids: The quantitative analysis of phospholipids were determined according to Bartlett (1959) and those of glycolipids were determined by phenol-H_2SO_4 method of Dubois *et al*. (1956) and Hayashi *et al*., 1992.

III. RESULTS

Total lipids

The total lipid content of the leaf discs of pigeonpea and amaranth decreased with increasing aqueous SO_2 concentration and incubation time. The pigeonpea showed a reduction of 2.66 fold in light and 3.11 fold in dark with respect to their controls at 250 ppm SO_2 concentration at the end of 24 h incubation period. The corresponding values of amaranth were a reduction of 3.06 fold under light and 4.25 fold under dark with respect to their controls. The loss of total lipid content was always more in amaranth (Fig.1 A,B,C,D).

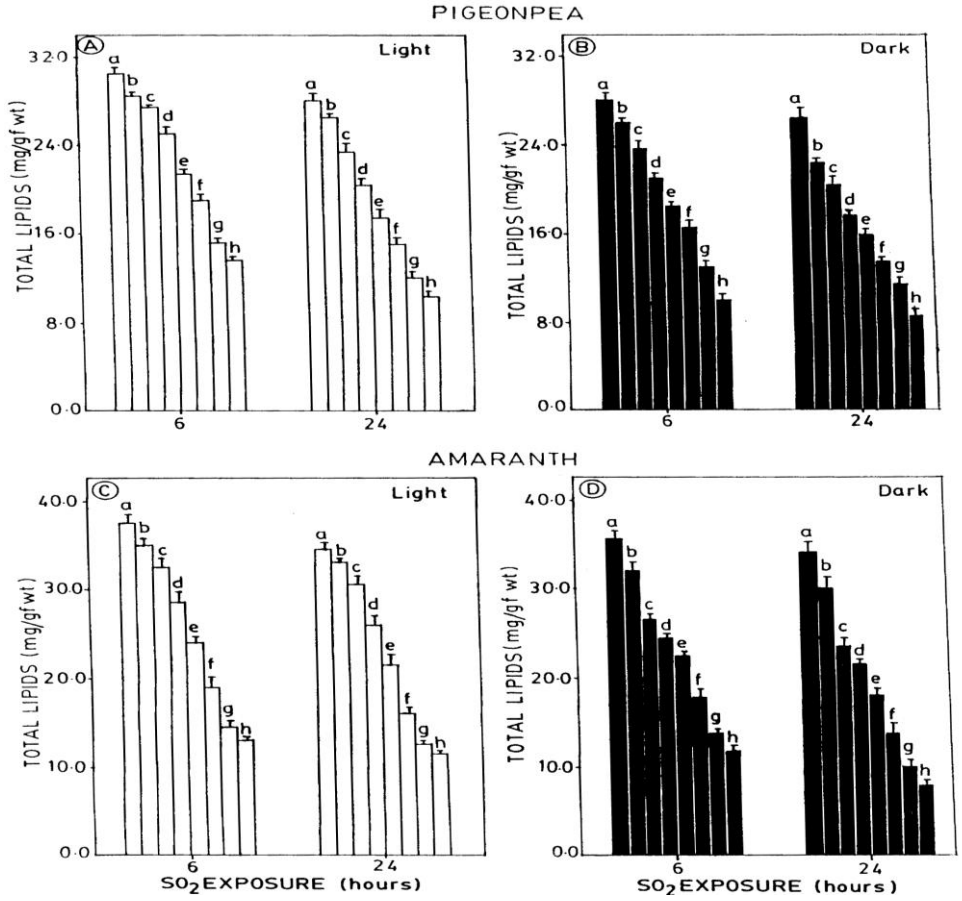

Fig.1:The effect of aqueous SO_2 on total lipid content of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.).
A and B - Pigeonpea; C and D - Amaranth; □ - under light; ■ - under dark
a - 0 ppm; b -10 ppm; c - 20 ppm; d - 30 ppm; e - 40 ppm; f - 50 ppm; g - 100 ppm; h - 250 ppm

Total phospholipids

The leaf discs that were subjected to different concentrations of SO_2 incubation resulted in a gradual decline of phospholipids with increasing SO_2 concentration and incubation period, in both the plant species. The phospholipid content showed a maximum reduction at 250 ppm SO_2 of 24 h incubation. A reduction of 2.24 fold in light and a reduction of 3.71 fold in dark over the respective controls were recorded in pigeonpea at 250 ppm SO_2 of 24 h incubation. In amaranth a reduction of 3.43 fold under light and a reduction of 3.65 fold under dark exposure over the respective controls were recorded at 250 ppm SO_2 of 24 h incubation period. In between pigeonpea and amaranth the loss of phospholipids were more in amaranth in response to SO_2 exposure (Fig. 2 A,B,C,D).

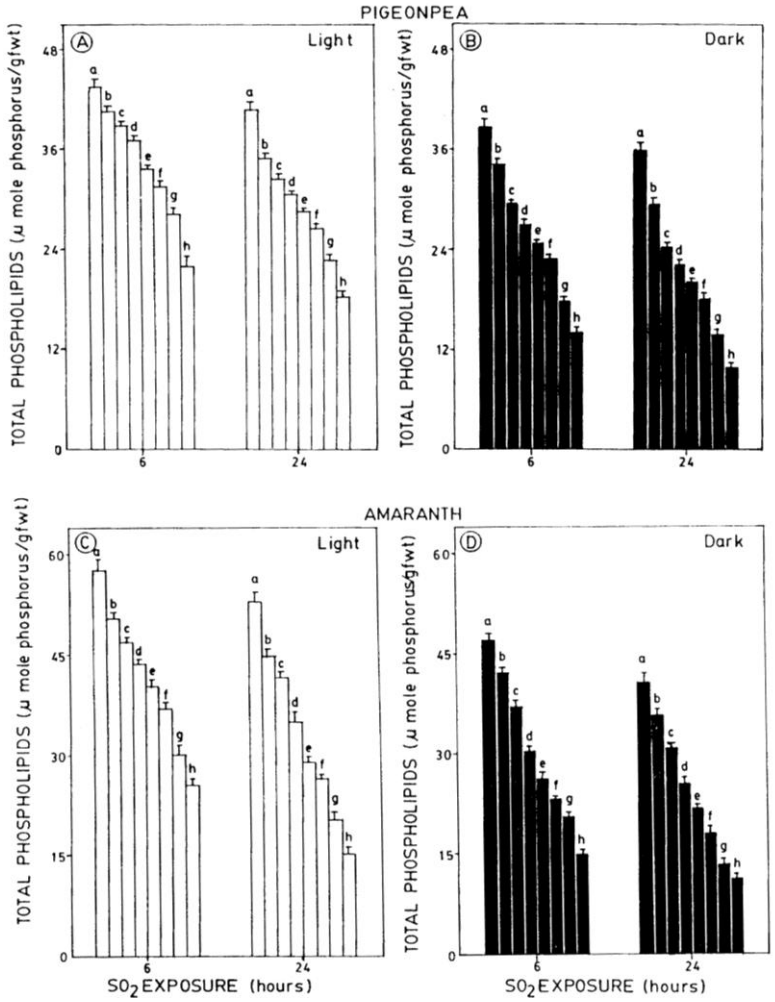

Fig.2: The effect of aqueous SO_2 on total phospholipids of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.).
A and B - Pigeonpea; C and D - Amaranth; □ - under light; ■ - under dark
a - 0 ppm; b -10 ppm; c - 20 ppm; d - 30 ppm; e - 40 ppm; f - 50 ppm; g - 100 ppm; h - 250 ppm

Individual phospholipids

The studies on the individual phospholipids were confined to 0, 30, 100 and 250 ppm aqueous SO_2 exposed leaf discs of 6 h incubation period. Figure 3 a,b,c,d represents the changes in the levels of phospholipids and glycolipids in the control and SO_2 treated leaf discs of both pigeonpea and amaranth. The SO_2 incubation of leaf discs caused a gradual decline in the phospholipid and glycolipid contents with increasing SO_2 concentration. The reduction of phospholipid and glycolipid contents were more under dark than under light exposure.

Analysis of the phospholipids and glycolipids by thin layer chromatography indicated that pigeonpea and amaranth differed in their response to SO_2. Of the phospholipids the phosphatidyl ethanol affected more in pigeonpea, where as phosphatidyl glycerol affected more in amaranth. Among the glycolipids, digalactosyl diglyceride affected in both the plant species. The per cent reduction of phospholipids and glycolipids over the controls of 250 ppm SO_2 treated leaf discs of pigeonpea were 36.85% for phosphatidyl glycerol, 42.22% for phosphatidyl cholin, 46.46% for phosphatidyl ethanolamine, 38.24% for monogalactosyl diglyceride and 46.39% for digalactosyl diglyceride (Figs. 3 A,B,C,D; 4 A,B,C,D and 5 A,B,C,D).

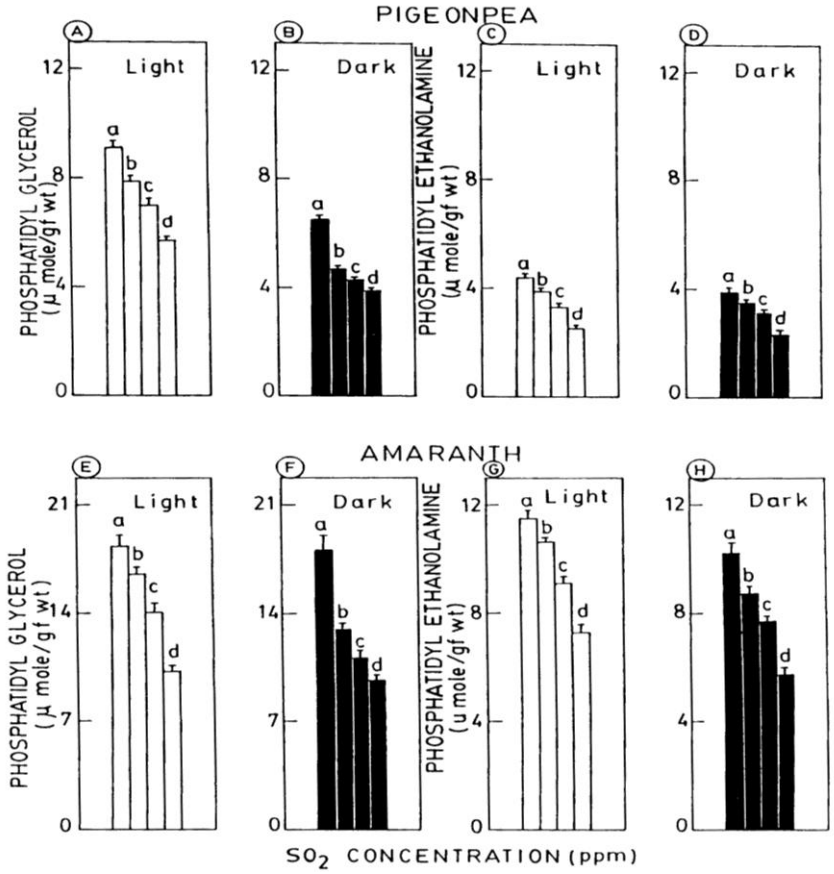

Fig.3: The effect of aqueous SO_2 on the phosphatidyl glycerol and phosphatidyl ethanolamine contents of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.).
A, B, C and D - Pigeonpea; E, F, G and H - Amaranth; □ - under light; ■ - under dark
a - 0 ppm; b - 30 ppm; c - 100 ppm; d - 250 ppm

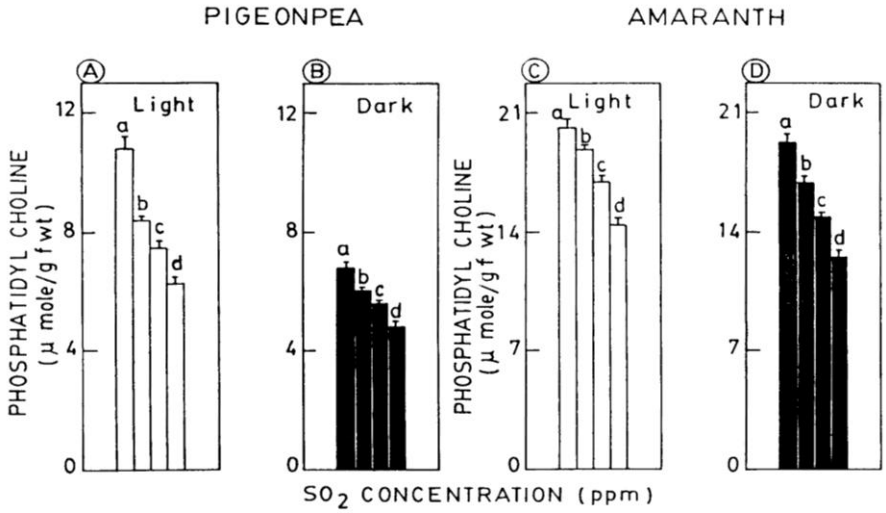

Fig 4: The effect of aqueous SO_2 on the phosphatidyl choline content of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.).
A and B - Pigeonpea; C and D - Amaranth; □ - under light; ■ - under dark
a - 0 ppm; b - 30 ppm; c - 100 ppm; d - 250 ppm

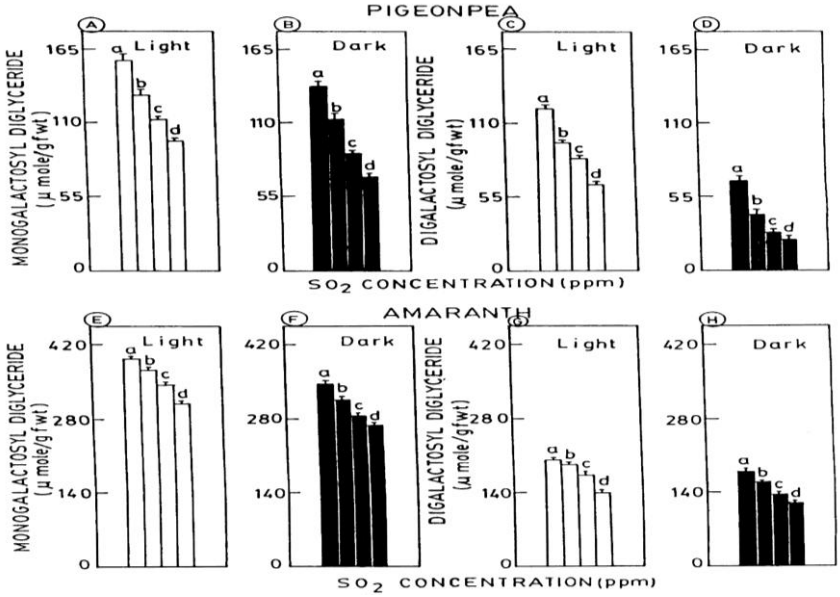

Fig.5: The effect of aqueous SO_2 on monogalactosyl diglyceride and digalactosyl diglyceride contents of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.).
A, B, C and D - Pigeonpea; E, F, G and H - Amaranth; □ - under light; ■ - under dark
a - 0 ppm; b - 30 ppm; c - 100 ppm; d - 250 ppm

The corresponding values of reduction for amaranth leaf discs at 250 ppm SO_2 concentrations were 44.34% for phosphatidyl glycerol, 28.58% for phosphatidyl cholin, 37.50% for phosphatidyl ethanolamine, 21.43% for monogalactosyl diglyceride and 31.10% for digalactosyl diglyceride (Figs. 3 E,F,G,H; 4 C,D, and 5 E,F,G,H).

Lipid Peroxidation

The changes in lipid peroxidation, as a measure of malondialdehyde formation, was not conspicuous in the control leaf discs of both pigeonpea and amaranth. Increasing SO_2 concentration and duration of SO_2 exposure however, increased the malondialdehyde content of both the plant species.

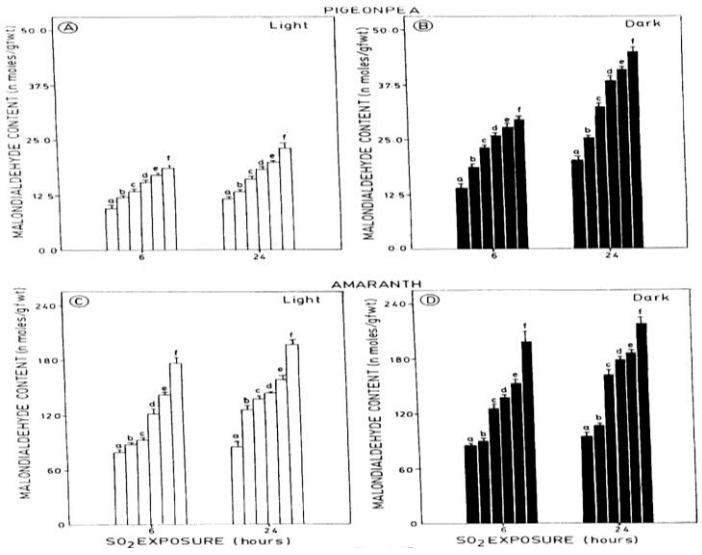

Fig.6: The effect of aqueous SO_2 on the lipid peroxidation of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.).
A and B - Pigeonpea; C and D - Amaranth; □ - under light; ■ - under dark
a - 0 ppm; b -10 ppm; c - 20 ppm; d - 30 ppm; e - 40 ppm; f - 50 ppm; g - 100 ppm; h - 250 ppm

Maximum malondialdehyde content was noticed in the leaf discs of pigeonpea and amaranth at 250 ppm SO_2. Lipid peroxidation was always greater under dark than under light conditions. In between pigeonpea and amaranth, lipid peroxidation was several fold more active in amaranth (Fig. 6 A,B,C,D).

IV. DISCUSSION

Lipids and phospholipids play an important role in the structure and function of plant cells. Leaf chloroplasts contain more lipid content and the major lipids include glycolipids which account for about 40% of total lipids (Kates, 1970). Glycolipids have shown to be involved in the structural integrity of thylakoids which are associated with electron transport system of photosynthesis (Shaw *et al*., 1976, Barber, 1987).

The total lipid content of leaf discs of pigeonpea and amaranth reduced with increasing SO_2 concentration and duration of exposure. However, the decline in lipid content was more in amaranth than in pigeonpea. Further, the reduction was more in dark than in light in both the plant species (Fig. 1 a,b,c,d). The total phospholipid content was also reduced considerably in response to SO_2 exposure in the leaf discs of both pigeonpea and amaranth. The reduction was more in amaranth. This reduction was expressed more in dark than in light (Fig. 2 a,b,c,d). Aqueous SO_2 reduced the lipid and phospholipid contents in pine needles of *Ulmus americana* and affected biomembrane integrity (Malhotra and Khan, 1979, Constantinidou and Kozlowski, 1979b). It is presumed that the reduction of lipid and phospholipid contents in response to SO_2 exposure may be a consequence of their reduced synthesis or increased lipase activity or lipid peroxidation either individually or in their different combinations (Mudd *et al*., 1971; Malhotra and Khan, 1979; Constantinidou and Kozlowski, 1979b; Nouchi and Toyama, 1988; Covello *et al.*, 1989).

Phospholipids and glycolipids constitute a major part of chloroplast lipids of leaves and play an important role in the functioning of photosynthetic systems of plants. The composition and distribution of these lipids differ in C_3 and C_4 plant chloroplasts (Munshi *et al*., 1991). Aqueous SO_2 affects individual phospholipids namely phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl glycerol and glycolipids, namely, monogalactosyl diglyceride and digalactosyl diglyceride in both the plant species. The data in the figures 3 to 5 shows the changes in the levels of different phospholipids and glycolipids of both pigeonpea and amaranth in response to SO_2 exposure. The magnitude of their decline was dependent on the concentration of SO_2. Phosphatidyl ethanolamine was effected more in pigeonpea. On the other hand, phosphatidyl glycerol was more effected in amaranth. Among the glycolipids, digalactosyl diglyceride was reduced much in both the plant species in response to SO_2. The reduction of all individual phospholipids and glycolipids was more in dark exposed leaf discs of both the plant species. Exposure of pine needles to aqueous SO_2 showed a marked inhibition in the *de novo* synthesis of phospholipids and glycolipids (Malhotra and Khan, 1979). One of the reasons for this reduction of lipid content may be attributed to the lipid peroxidation of chloroplast membranes (Shaw *et al*., 1976). The differential response of pigenonpea and amaranth in relation to their lipid and phospholipid contents may be attributed to the differences in the lipid composition of C_3 and C_4 plants (Munshi *et al*., 1991).

Exposure of leaf discs of pigeonpea and amaranth to aqueous SO_2 increased lipid peroxidation. Malondialdehyde increased two folds in amaranth than in pigeonpea (Fig. 6 a,b,c,d). In addition the lipid peroxidation was more pronounced in dark exposed leaf discs (Fig. 6 b,d). The formation of malondialdehyde is considered as a measure of lipid peroxidation (Heath and Packer, 1968; Dhindsa *et al*., 1981). Rao and Duby (1993) reported different levels of lipid peroxidation in soybean cultivars in response to SO_2. It may be therefore assumed that plant species susceptible to SO_2 contain more content of malondialdehyde than tolerant species (Jager *et al*., 1985). The involvement of free radicals $O_2^{\cdot-}$, $OH^{\cdot}$ and $^1O_2$ in initiating lipid peroxidation is well known (Fridovich, 1975, 1978; Dhindsa *et al*., 1981). The oxyradical formation increases under oxidative stress (Bowler *et al*., 1992). Sulphur dioxide also contribute to the oxyradical production in leaves (Peiser and Yang, 1977, 1979; Shimazaki *et al*., 1980; Tanaka and Sugahara, 1980; Lizada and Yang, 1981; Peiser *et al*., 1982, Jagar *et al*., 1985; Covello *et al*., 1989;Sandmann and Gonzales, 1989).

V. CONCLUSIONS

The malondialdehyde content, a sensitive marker of lipid peroxidation increased with increasing SO_2 concentration and duration of exposure in both pigeonpea and amaranth leaf discs. However, the increase was more in amaranth and it was more so in dark. The total lipids, phospholipids and individual phospholipids and glycolipids declined with increasing SO_2 concentration and duration of exposure. Interestingly digalactosyl diglyceride affected more in both the plant species.

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