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Influence of Aqueous SO_2 on Changes in the Leaf Discs of Free Amino Acid Composition and Free Organic Acid Metabolism under Light and Dark Conditions of *Cajanus Cajan* and *Amaranthus Paniculatus*

B. Sujatha
Professor, Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, India
B. Priyadarshini
Post Doctoral Fellow, Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, India
K. M. Priyanka
Research Scholar, Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, India
M. V. V. P. Kumar
Research Scholar, Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, India
Ch. Umamahesh
Research Scholar, Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, India
J. Saraswathi
Retd. Lecturer, Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, India

Abstract:
The influence of elevated aqueous SO_2 (0, 10, 20, 30, 40, 50, 100 and 250 ppm) on free amino acid composition, proline content and free organic acid content 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 studies of free amino acid composition were carried out at 0, 30 and 100 ppm aqueous SO_2 treated leaf discs in both pigeonpea and amaranth. Though small quantitative variations appeared among individual amino acids, the total quantity of amino acids increased in both the plant species with increasing SO_2 concentration and duration of exposure. Cysteine was absent in the light incubated amaranth but it appeared in the dark incubated SO_2 treatments. In pigeonpea, though initially absent cysteine appeared in the higher concentrations of SO_2. Methionine content was more in amaranth than in pigeonpea. Enhanced levels of proline content were registered at all SO_2 concentrations both in light and dark exposed leaf discs in pigeonpea than amaranth. The accumulation of free organic acid content was more conspicuous in amaranth than in pigeonpea under dark conditions rather than under light conditions in response SO_2.

Keywords: *Amaranth, amino acid composition, aqueous SO_2, leaf discs, organic acid content, pigeonpea.*

1. Introduction

Air pollution has become an extremely serious problem for the modern industrialized world. Air pollution may be defined as any atmospheric condition in which certain substances are present in such concentrations that may produce undesirable effects on man and ecosystem. These substances include gases (sulphur dioxide, nitrogen oxides, carbon monoxides, hydrocarbons, etc.), particulate matters (smoke, dust, fumes, aerosols, etc.), radioactive materials and many others. Air pollution may or will have harmful effects on living things and materials. It may interfere with biochemical and physiological processes of plants to an extent, which ultimately leads to yield losses (Heck *et al*., 1988).

In plants, sulfate is taken up from soil by high-affinity transporters. Sulfate is largely transported to shoots where it can be activated by ATP via ATP sulfurylase in the leaves. The product is reduced by 5'-adenylylsulfate (APS) reductase to sulfite which can be reduced to H_2S by sulfite reductase (Rausch and Wachter, 2005). Sulphur dioxide can also be produced endogenously from sulfur containing amino acids (Stipanuk *et al*., 2006). The endogenous production of SO_2 also suggests that it has a physiological role in plants.

Amino acids are the primary products of inorganic nitrogen assimilation. The effect of SO_2 on amino acid metabolism is complex and needs greater attention. Increasing concentration of SO_2 enhanced the concentration of many of the amino acids even before any visible foliar injury symptom appears (Godzik and Linskens, 1974; Malhotra and Sarkar, 1979; Buwalda *et al.,* 1990). Sulphur dioxide exposure particularly at high concentrations increases the pools of alanine, asparagine, glutamine, γ-amino-butyric acid, citrulline, valine, glycine, isoleucine, leucine, threonine, lysine, tyrosine, arginine, histidine, cysteine and methionine. The amino acids such

as glutamic and aspartic acids including glutamine exhibited certain varied responses depending upon the plant species studied, physiological status and phase of growth and the concentration and duration of SO_2 exposure (Godzik and Linskens, 1974; Malhotra and Sarkar, 1979; Buwalda *et al.,* 1988, 1990; Schlee, 1992).

Proline has enhanced slightly by low concentrations and short duration treatment of sulphite. However, it decreased significantly with increasing concentration and duration of exposure in *Trebouxia* species (Ewald and Schlee, 1983). The fall in proline content may be presumed due to the inhibition of proline synthesis and/or its oxidation by sulphite. Several enzymes such as glutamate dehydrogenase involved in amino acid metabolism have also been shown to be affected by SO_2 (Pahlich, 1971; Pahlich *et al.,* 1972).

Sulphur dioxide affects a number of enzymes and intermediates involved in organic acid metabolism. Organic acids act as intermediates of a number of metabolic end products and help to maintain cellular pH. Any change in their metabolism would therefore have an influence on plant growth and yield. Sulphur dioxide enhances free organic acids in plants resulting in increased pH of the cytosol causing damage to tissues. This may due to impaired activity of enzymes which are unable to utilize the organic acids in their metabolism under SO_2 impact. Keeping this in view, in the present investigation, it was intended to compare the sensitivity of pigeonpea, a C_3 plant and amaranth, a C_4 plant to aqueous SO_2.

2. Materials and Methods

2.1. Plant Material

Seeds of pigeonpea (*Cajanus cajan* (L.) Millsp. cv. PDM1), a C_3 plant is an important pulse crop 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.

2.2. 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).

2.3. 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°C and 21 ± 1°C at night at Andhra university experimental farm. Fully expanded third leaves from top of 1-month old pigeon pea 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.

2.4. Amino Acid Analysis

Changes in free amino acid composition were studied in the control, 30 and 100 pm aqueous SO_2 treatments of 6 and 24 h exposed leaf discs using LKB Automatic Amino Acid Analyser.

2.4.1. Extraction of Amino Acids

For amino acid analysis 200 mgs of plant material was homogenized in 80% alcohol. The alcohol was evaporated in vacuo and the residue was dissolved in citrate buffer, pH 2.2 and was made up to a known volume.

2.4.2. Running of Amino Acid Analyser

The amino acids were loaded and analysed on a cation exchange resin with buffer of carefully defined salt concentrations and pH as described in the 'Hand Book and Applications for LKB Biochrom Automatic Amino Acid Analyser' which was used for this analysis. Sodium salts used for buffer preparation is passed through a teflon coil placed in a boiling water bath. Before entering the coil, the column effluent is mixed with acetate buffer containing the reduced ninhydrin. This compound reacts with amino acids forming a dye complex. The absorption is determined in a flow photometer and registered on the chart of a recorder. The quantification and identification of the different amino acids were carried out using standard amino acids mixture consisting of aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine and ammonia. The LKB standard concentration is 2.5 µ mole/ml except for cysteine which is 1.25 µ mole/ml.

2.4.3. Peak Evaluation

Quantitative evaluation of the amino acids on a good chromatogram was performed by calculating the area under the peaks manually as described in the 'Hand Book and Applications for LKB Biochrom Automatic Amino Acid Analyser'

2.5. Proline

Proline content was estimated by the acid ninhydrin method of Troll and Lindsley (1955) as modified byTully *et al*. (1979).The extraction of proline was carried by grinding 200 mg of plant material in 5 ml of water and heating at 100°C for 30 min in sealed tubes. The cooled content was centrifuged and the supernatant was made up to a known volume.

For the estimation of proline, 5 ml of the extract was taken; 5 ml of glacial acetic acid and 5 ml of ninhydrin reagent were added and heated in a water bath for 1 h in test tubes with plastic screw caps. The solutions were cooled to room temperature, and the colour was extracted with 5 ml of toluene by shaking them vigorously for 5 min in a separating funnel. The phases were allowed to separate. The toluene phase was transferred to a cuvette and the absorbance determined at 515 nm on Milton Roy spectronic 1201 UV spectrophotometer. Standard curve was prepared by using known quantities of proline.

2.6. Free Organic Acids

Total free organic acids of control and treated leaf discs at different stages were determined according to the method of Ting and Dugger (1968). One gram of the sample leaf discs was taken, chopped into fine slices and boiled for 30 min with glass distilled water free of carbon dioxide. The homogenate was centrifuged at 5000 xg for 15 min and the supernatant was made up to a known volume. Ten ml of aliquots of the supernatant were titrated against 0.01 N NaOH using phenolphthalein as indicator and the results were expressed as milliequivalents of acid per 100 g fresh weight.

3. Results

3.1. Free Amino Acid Composition

The studies of free amino acid composition were carried out at 0, 30 and 100 ppm aqueous SO_2 treated leaf discs in both pigeonpea and amaranth. The free amino acid composition of pigeonpea and amaranth were presented in Tables 1; 2; 3 and 4. The variation in free amino acid composition in control leaves of pigeonpea and amaranth was not significant in response to incubation from 6 to 24 h time. However, SO_2 exposure greatly affected the free amino acid composition of pigeonpea and amaranth. The concentration of proline, cysteine, methionine and ammonium increased considerably in response to SO_2 compared to their respective controls. Though considerable variations were found in relation to the quantity of individual amino acid contents among the treatment, the total quantity of amino acids were increased with increasing SO_2 concentration and duration of exposure, in both the plant species.

	Duration of exposure time					
Amino acid	**6 h light**			**24 h light**		
	0 ppm	**30ppm**	**100ppm**	**0 ppm**	**30ppm**	**100ppm**
Aspartic acid	6.587	19.500	20.160	6.653	5.976	4.678
Threonine	---	---	---	2.191	7.711	6.452
Serine	2.400	2.811	7.129	2.425	3.961	6.205
Glutamic acid	0.293	2.734	3.181	0.487	5.076	10.152
Proline	0.175	2.797	5.390	0.240	4.043	11.952
Glycine	0.764	1.939	2.069	1.163	2.293	2.316
Alanine	1.415	4.790	12.204	1.950	5.444	16.845
Cysteine	0.925	2.744	4.400	1.094	4.214	6.912
Valine	1.768	4.119	5.445	1.852	4.166	6.960
Methionine	---	0.847	0.879	----	0.243	0.254
Isoleucine	0.218	0.233	0.286	0.435	3.223	4.667
Leucine	1.140	3.157	4.054	1.369	3.489	7.770
Tyrosine	---	2.079	3.108	1.881	2.170	4.196
Phenylalanine	---	1.191	1.656	1.518	2.254	3.037
Histidine	0.750	3.337	10.800	1.650	5.550	11.775
Lysine	0.461	0.600	1.514	0.699	3.046	3.277
Arginine	1.019	1.510	6.344	1.133	1.813	8.416
Total amino acids	17.196	54.388	88.619	26.740	64.672	115.864
Ammonia	0.023	1.693	2.617	1.505	2.321	2.878
Total amino acids with ammonia	17.939	56.081	91.236	28.245	66.993	118.742

Table 1: The effect of aqueous SO_2 on free amino acid composition of pigeonpea (µ mole amino acid/g f wt) under light.

	Duration of exposure time					
Amino acid	**6 h light**			**24 h light**		
	0ppm	**30ppm**	**100ppm**	**0ppm**	**30ppm**	**100ppm**
Aspartic acid	7.413	11.576	17.322	6.725	9.780	11.380
Threonine	5.868	8.134	8.605	6.452	7.954	9.557
Serine	1.651	5.546	6.469	2.546	8.912	10.167
Glutamic acid	2.499	5.537	6.618	3.281	21.342	27.342
Proline	----	----	1.434	0.598	1.304	1.845
Glycine	0.106	0.846	1.175	0.916	2.152	2.351
Alanine	3.527	12.008	16.112	5.771	13.719	18.182
Cysteine	----	-----	6.456	----	------	7.776
Valine	0.710	3.296	5.039	5.117	6.818	22.727
Methionine	0.142	1.271	2.333	0.165	0.769	2.260
Isoleucine	0.622	2.800	3.501	1.983	5.446	7.156
Leucine	0.709	3.040	5.574	2.868	9.940	20.101
Tyrosine	0.724	4.295	4.640	4.196	4.775	11.189
Phenylalanine	0.515	1.656	2.531	2.944	5.889	7.542
Histidine	1.939	2.553	5.250	2.700	3.000	16.800
Lysine	0.923	1.615	4.523	1.338	2.538	4.800
Arginine	1.359	1.378	13.595	2.869	5.265	14.048
Total amino acids	28.707	65.550	111.177	50.469	110.603	195.223
Ammonia	0.155	10.298	18.161	12.689	18.348	20.349
Total amino acids with ammonia	28.862	75.844	129.338	63.158	128.951	215.572

Table 2: The effect of aqueous SO_2 on free amino acid composition of pigeonpea (µ mole amino acid/g f wt) under dark.

	Duration of exposure time					
Amino acid	**6 h light**			**24 h light**		
	0ppm	**30ppm**	**100ppm**	**0ppm**	**30ppm**	**100ppm**
Aspartic acid	6.358	7.069	15.978	6.409	7.105	16.488
Threonine	---	-----	------	0.730	1.155	1.461
Serine	0.494	1.081	15.821	2.245	7.595	17.328
Glutamic acid	2.344	3.984	7.310	5.625	6.012	8.638
Proline	----	----	2.516	0.319	1.567	1.030
Glycine	0.940	1.763	7.088	2.938	4.467	22.453
Alanine	2.395	7.317	14.982	1.635	2.177	7.055
Cysteine	----	----	----	----	----	----
Valine	0.378	2.841	7.794	4.024	4.735	13.636
Methionine	----	2.359	5.929	---	2.542	6.779
Isoleucine	0.933	5.835	10.103	1.517	6.064	11.881
Leucine	0.316	2.894	5.667	3.047	3.160	11.972
Tyrosine	1.446	2.122	3.482	1.641	2.238	3.521
Phenylalanine	1.380	2.208	4.988	1.564	3.129	6.349
Histidine	2.100	3.408	3.570	2.895	5.550	6.300
Lysine	3.366	3.554	4.027	2.686	2.769	1.999
Arginine	2.184	3.021	3.654	2.832	5.287	7.703
Total amino acids	24.634	49.456	112.909	37.440	65.552	144.593
Ammonia	10.086	12.059	18.538	12.200	28.110	28.405
Total amino acids with ammonia	34.720	61.515	131.447	49.640	93.662	172.998

Table 3: The effect of aqueous SO_2 on free amino acid composition of amaranth (µ mole amino acid/g f wt) under light.

Cysteine and methionine showed marked differences in pigeonpea and amaranth in response to SO_2. Cysteine was absent in the light incubated amaranth but it appeared in the dark incubated SO_2 treatments. In pigeonpea, though initially absent cysteine appeared in the higher concentrations of SO_2. Methionine content was more in amaranth than in pigeonpea.

	Duration of exposure time					
Amino acid	**6 h light**			**24 h light**		
	0ppm	**30ppm**	**100ppm**	**0ppm**	**30ppm**	**100ppm**
Aspartic acid	7.010	14.670	16.620	9.397	15.106	27.552
Threonine	---	---	---	2.434	3.052	3.571
Serine	5.453	7.757	20.202	5.881	9.240	20.474
Glutamic acid	0.195	0.817	6.875	0.390	18.834	31.050
Proline	1.674	2.604	6.956	1.087	2.913	0.750
Glycine	1.293	3.233	7.406	2.040	4.973	7.658
Alanine	8.052	8.747	16.047	8.939	10.181	16.452
Cysteine	0.147	0.340	0.528	0.189	0.417	0.556
Valine	1.894	5.113	8.238	3.281	6.061	9.105
Methionine	0.413	0.423	0.544	1.179	1.525	1.806
Isoleucine	0.933	1.244	4.668	2.684	3.485	5.200
Leucine	2.406	3.378	6.929	2.912	3.733	7.130
Tyrosine	0.868	1.736	15.414	1.757	3.376	15.614
Phenylalanine	0.782	1.380	2.521	2.006	2.738	2.876
Histidine	4.650	4.806	11.025	2.024	2.839	4.514
Lysine	1.245	1.892	2.769	2.538	2.832	4.015
Arginine	1.736	2.356	5.841	1.613	4.695	6.486
Total amino acids	38.751	60.496	132.583	50.351	96.000	164.809
Ammonia	10.788	11.713	12.234	11.185	13.574	15.259
Total amino acids with ammonia	49.539	72.209	114.817	61.536	109.574	180.068

Table 4: The effect of aqueous SO_2 on free amino acid composition of amaranth (µ mole amino acid/g f wt) under dark.

3.2. Proline

The proline content was increased from 6 to 24 h incubation in all the leaf discs studied. However, the values were always lower in control compared to SO_2 treatments. Proline content increased in all the SO_2 treatments under light conditions. On the other hand, under dark conditions, lower SO_2 concentrations exhibited the greater values and higher SO_2 concentrations the lower value in amaranth. Pigeonpea recorded higher values of proline than amaranth at 250 ppm SO_2 treatment (Fig.1 a, b, c, d).

3.3. Free Organic Acid Content

Aqueous SO_2treatment enhanced the accumulation of free organic acid content in all the SO_2treated leaf discs in both the pigeonpea and amaranth. The accumulation of free organic acid content gradually increased with increasing SO_2concentration and incubation period and it was more pronounced in 250 ppm SO_2treatment in both the pigeonpea and amaranth. The accumulation of free organic acid content was more conspicuous under dark conditions rather than under light conditions. Moreover, maximum accumulation of free organic acid content with values of 75.00 meq in pigeonpea and 80.50 meq in amaranth were registered at 250 ppm SO_2 treatment of 24 h dark incubation. The accumulation of free organic acid content was conspicuous in amaranth than in pigeonpea in response SO_2 (Fig. 2 a, b, c, d).

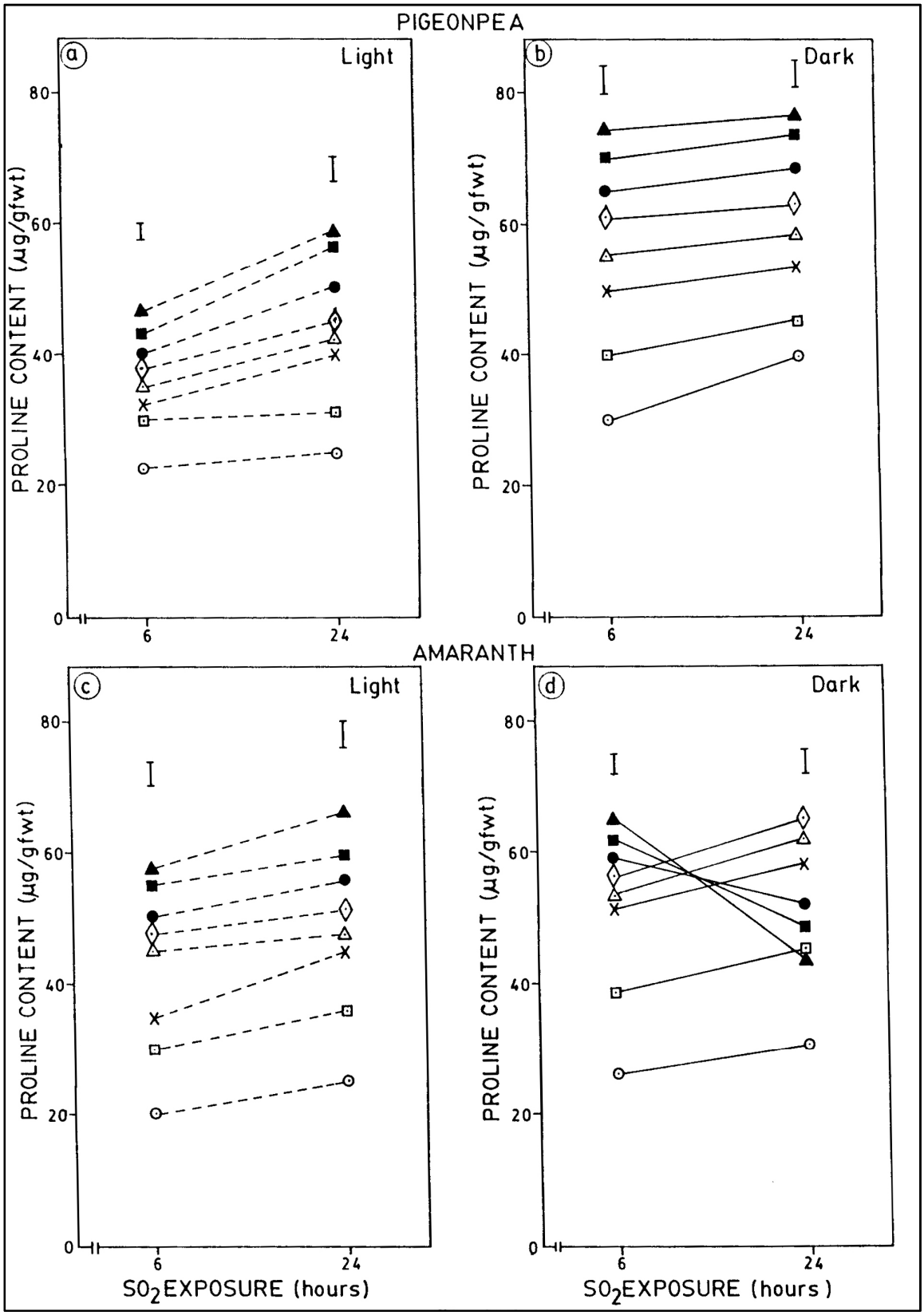

Figure-1: The effect of aqueous SO_2 on proline content of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.), a and b - Pigeonpea; c and d – Amaranth, ---- under light; — under dark
○-0 ppm; □-10 ppm; ×-20 ppm; Δ-30 ppm; ◊-40 ppm; ●-50 ppm; ■-100 ppm; ▲-250 ppm

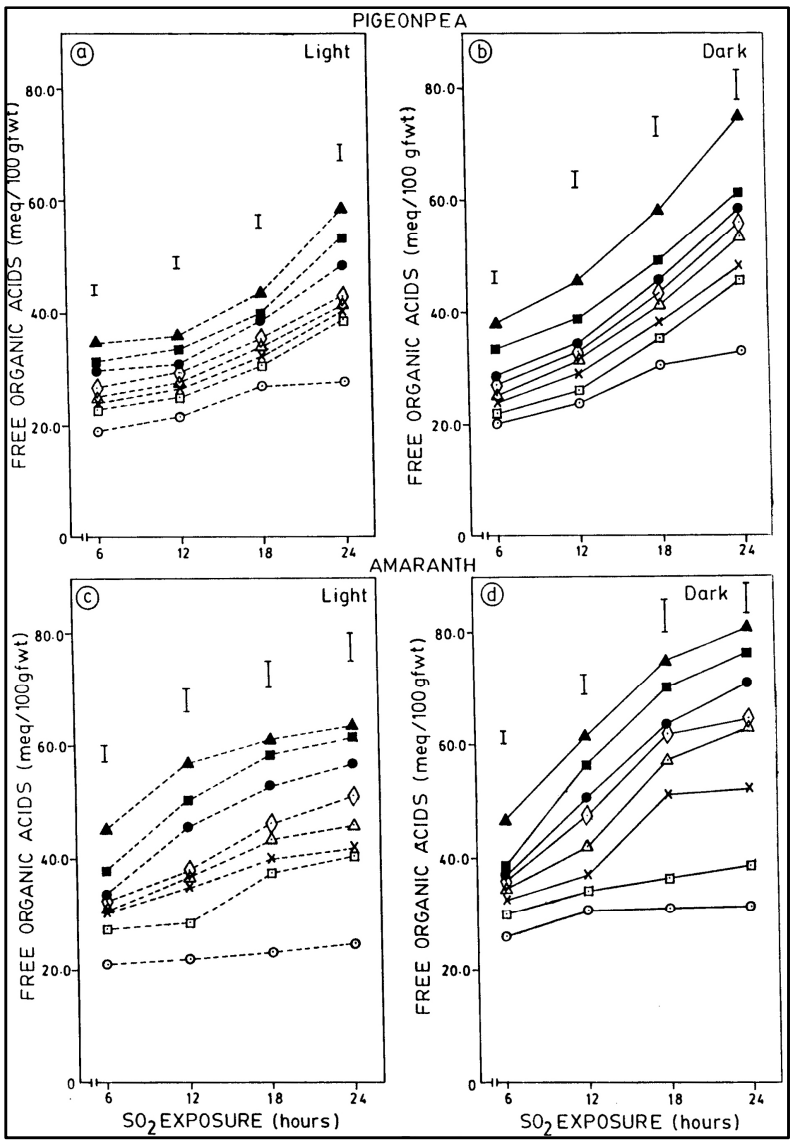

Figure 2: The effect of aqueous SO_2 on the free organic acid content of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.), a and b - Pigeonpea; c and d – Amaranth, ---- under light; — under dark
○-0 ppm; □-10 ppm; ×-20 ppm; Δ-30 ppm; ◊-40 ppm; ●-50 ppm; ■-100 ppm; ▲-250 ppm

4. Discussion

The changes in the free amino acid composition in response to SO_2 exposure of both the pigeonpea and amaranth were presented in tables 1, 2, 3 and 4. Though small quantitative variations appeared among individual amino acids, the total quantity of amino acids increased in both the plant species with increasing SO_2 concentration and duration of exposure. The levels of cysteine and methionine, the sulphur containing amino acids showed some differences in between pigeonpea and amaranth. In amaranth, cysteine was present in the dark exposed leaf discs but in pigeonpea it is absent at lower concentrations but present at higher concentrations of dark exposed leaves. However, occurrence of high levels of cysteine in the presence of excess sulphur both under light and dark conditions was registered in spinach leaf discs (Dekok *et al*., 1988). Cysteine at high concentrations is toxic to plants (Rennenberg, 1984). Proline content in response to SO_2 increased at lower concentrations and declined at higher concentrations in both the plant species. A decline in proline content in response to SO_2 was registered in pine needles (Malhotra and Sarkar, 1979). Interestingly, methionine, one of the sulphur containing amino acid was more in amaranth than in pigeonpea (Table 2, 4). Another interesting feature was that ammonia (NH_3) content increased with increasing SO_2 concentration in both the plant species (Godzik and Linskens, 1974; Van Dijk *et al*., 1986).

Free proline is known to accumulate in leaves of higher plants in response to a variety of environmental stresses including SO_2 (Stewart and Larher, 1980; Dashek and Erickson, 1981; Karolewski, 1984, 1989). Enhanced levels of proline were registered at all SO_2 concentrations both in light and dark exposed leaf discs in both the plant species (Fig. 1 a, b, c, d). However, prolonged exposure of leaf discs to SO_2 led to slight decline in proline content under dark conditions at higher SO_2 concentrations in amaranth (Fig. 1 c,d). The decline in proline content at higher concentrations of SO_2 was attributed to the inhibition of its synthesis or to an increased rate of

its oxidation (Ewald and Schlee, 1983). The accumulation of proline may be considered as an indication of the development of a defense mechanism against the toxic action of SO_2 (Jager and Pahlich, 1972; Godzik and Linskens, 1974; Malhotra and Sarkar, 1979; Karolewski, 1989).

Free organic acids play an important role in cell metabolism, mainly through respiratory metabolism by providing the carbon skeleton for the synthesis of amino acids and other cellular constituents (Towers and Martimer, 1954). The organic acids also act as intermediates of a number of metabolic end products and help to maintain cellular pH. Any change in their metabolism would therefore effect the physiology of the foliage. The free organic acids increased in the leaf discs of pigeonpea and amaranth in response to increasing concentrations of aqueous SO_2 and its duration of exposure (Fig.2 a, b, c, d). The accumulation of free organic acids was more in amaranth (Fig. 2 c,d). Dark incubation further enhanced the levels of free organic acids in the leaf disc of both the plant species (Fig. 2 b,d). The accumulation of free organic acids in response to SO_2 exposure interferes with different biochemical activities of the leaf cells (Sarkar and Malhotra, 1979; Malhotra and Khan, 1984).

5. Conclusions

An analysis of free amino acid composition was exhibited an increase in the levels of several individual amino acids in response to SO_2. Interestingly, sulphur containing amino acids and ammonia (NH_3) increased considerably under SO_2 exposure probably due to the damage of biomembrane intactness by SO_2. The increase in proline content was more in pigeonpea than in amaranth. Free organic acids increased in both pigeonpea and amaranth leaf discs in response to SO_2 as a consequence of altered leaf disc metabolism.

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