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RESEARCH ARTICLE

AQUEOUS SULPHUR DIOXIDE EFFECTS ON NUCLEIC ACID METABOLISM OF LEAF DISCS UNDER LIGHT AND DARK CONDITIONS OF *CAJANUS CAJAN* AND *AMARANTHUS PANICULATUS*

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ABSTRACT

The effect of elevated aqueous SO_2 (0, 10, 20, 30, 40, 50, 100 and 250 ppm) on total RNA and DNA contents, Ribonuclease (RNase) activity, incorporation of radioactive isotopes of ^{3}H-uridine and ^{14}C-thymidine of pigeonpea (*Cajanus cajan* (L.) Millsp. cv. PDM1) and amaranth (*Amaranthus paniculatus* L. a local cultivar) leaf discs under light and dark conditions has been studied. The synthesis of RNA and DNA were reduced in both the leaf discs incubated in aqueous SO_2. In between the pigeonpea and amaranth aqueous SO_2 stimulated gradually with increase RNase activity was more in amaranth under dark than light. The ^{3}H-uridine incorporation rate into RNA and ^{14}C-thymidine incorporation rate into DNA was recorded higher values in amaranth than in pigeonpea observed under light conditions.

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INTRODUCTION

The presence of unaccepted level of foreign gaseous and particulate matters in the atmosphere is referred to as air pollution (Odigure, 1999). Air pollution is generally caused by automobiles, aircraft, industrial plants, power generation systems, construction projects and solid wastes (Javed *et al*., 2009). Air pollutants may inter into plant tissues via stomata and elevate the level of reactive oxygen species (ROS) causing serious damage to the DNA, proteins and lipids (Sharma and Davis, 1997; Hippeli and Elstner, 1996). SO_2 causes a decline in the RNA content of bean (Beckerson and Hofstra, 1979). SO_2 is considered to interfere with ribonuclease activity and RNA content of leaves, inducing an early senescence in leaves (Craker and Starbuck, 1972). Sulphite also reacts through a free radical chain mechanism with certain DNA and RNA molecules (Inoue *et al.,* 1972; Kitamura and Hayatsu, 1974). Although the exact nature of such reactions is not known, evidences indicate that the free radicals generated during the autooxidation of bisulphite was responsible for the glycosidic bond cleavage of pyrimidine nucleosides (Hayatsu and Miller, 1972; Peiser and Yang, 1985). In spite of the importance of nucleic acids in protein synthesis and related phenomena, not much information is available on the impact of SO_2 on these important macromolecules of pigeonpea and amaranth.

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MATERIALS AND METHODS

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.

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.

Nucleic acids

DNA and total RNA

Extraction of nucleic acids were carried out according to the method of Schmidt and Thannhauser (1945) as modified by Smillie and Krotkov (1960). One g of leaf discs were homogenized with 15 ml of cold methanol and centrifuged. The residue was reextracted twice with 15 ml of cold methanol and centrifuged. The residue was then stirred for 2 minutes with 15 ml of 5% TCA (w/v) at 4 ^oC and centrifuged. Washing with 5% TCA was repeated second time. The resulting residue was further extracted with 15 ml of each of the following solvents (i) 95% ethanol (twice), (ii) ethanol-ether (2:1) and (iii) ether. For each washing the solvent was allowed to boil for 20 sec. The residue was carefully dried, powdered and used for further extraction of either DNA or RNA.

For the estimation of DNA, dried residue was extracted with 15 ml of 5 % perchloric acid at 90^oC for 15 min. The contents were allowed to cool down to room temperature and centrifuged. The precipitate was washed with 5% perchloric acid at 90 ^oC for 5 min cooled to room temperature and centrifuged. The combined supernatants were made up to a known volume with 5 % perchloric acid.

The deoxyribose content of the DNA extract was estimated by following the method of Burton (1968). Two ml of DNA extract was mixed with 2 volumes of diphenylamine reagent. A blank containing 5% perchloric acid but no DNA was similarly prepared. All the tubes were incubated for 17 h at 30^oC and the absorbance at 600 nm was measured in a schimadzu (UV-240) spectrophotometer. The standard curve was prepared using calf thymus DNA. To estimate RNA, the original dried residue after the removal of lipid phosphorus was hydrolysed for 16 h with 15 ml of 0.3 N KOH at 37 ^oC. The digest was cooled to 0^oC. Magnesium chloride was added to 10^{-3} M and acidified to pH 2.0 with perchloric acid. An equal volume of 95% ethanol was added, contents were allowed to stand for 20 min at 0 ^oC and centrifuged. The residue was washed with 1% perchloric acid. The supernatant and the washings were combined and adjusted to pH 8.0 with KOH and allowed to stand for 2 h at 0 ^oC and centrifuged. The precipitate was washed with cold water. The combined supernatants were allowed to run down through a column (5 cm x 0.5 cm) of Dowex-I (chloride form 200 mesh). The column was first washed with 0.01 M NaCl and the nucleotides that were retained on the column were eluted with HCl-NaCl solution (20 ml HCl +5.6 g NaCl in 240 ml of water). Twenty five ml of the elute was collected.

The ribose content of the elute was estimated following the method of Markham (1955) using orcinol reagent. 0.5 ml of RNA extract was mixed with 5.0 ml of orcinol reagent in a tube which was then stoppered and heated for 8 min on a water bath at 100 ^oC. It was then cooled and the absorbance was measured at 660 nm in Schimadzu (UV-240) spectrophotometer. A similarly treated blank was used for zero setting. The standard curve was prepared using yeast RNA.

Ribonuclease (E.C.2.7.7.6)

Ribonuclease (RNase) activity of the leaf discs were determined according to the methods based on McDonald (1955) and Tuve and Anfinsen (1960) as followed by Bagi and Farkas (1967). Five g of the material was homogenized in distilled water at 0-5 ^oC. The homogenate was filtered through four layers of cheese cloth and extract was centrifuged for 10 min at 20,000 X g in a centrifuge. The supernatant was used for the assay of RNase activity. The assay system contained the following components 0.5 ml of enzyme extract, 0.5 ml of 7.5 mg/ml yeast RNA solution and 0.5 ml of 0.1 M acetate buffer at pH 5.5 and was incubated at 37 ^oC in a water bath for 30 min. The reaction was stopped by adding 1.5 ml of McFadyes reagent, stirred and allowed to stand overnight at 4 ^oC. The mixture was centrifuged and diluted to 1:10 with distilled water and the absorbance at 260 nm was measured using Schimadzu (UV-240) Spectrophotometer against '0' time control. The activity was expressed as absorbance at 260 nm/min/mg protein. Protein content of the enzyme extract was estimated by the method of Lowry *et al.* (1951).

^{14}C- thymidine incorporation

The SO_2 treated leaf discs were washed and placed in 5 cm petri dishes containing 10 ml of ^{14}C-thymidine (specific activity 10 m Ci/mM, BARC, Bombay) at the concentration of 1 µ Ci/ml and then incubated at 30 ± 1^oC under darkness. After different periods of incubation for 12, 24, 36 and 48 h they

were collected, washed thrice with distilled water and used for analysis. The extraction of nucleic acids was carried out by the method of Ingle (1963). The leaf discs were homogenized in 5 % perchloric acid. The resultant homogenate was centrifuged for 20 min at 31,500 X g and the residue was washed thrice with 5% perchloric acid. The acid insoluble fractions were further washed thrice with the mixture of ethanol : ether : chloroform (2:2:1). The supernatant was discarded. The final pellet was incubated in 5% perchloric acid at 70 ^{o}C for 20 min and centrifuged at 31,500 X g for 5 min. The residue was washed once again. The nucleic acid content was determined by UV absorption using Schimadzu (UV-240) spectrophotometer. The amount of ^{14}C-thymidine incorporated into nucleic acid was determined by the measurement of ^{14}C activity on a "Automatic liquid Scintillation System" LSS 34 ECIL.

^{3}H-uridine incorporation

A similar procedure as carried out for ^{14}C-thymidine incorporation was followed except that the ^{14}C-thymidine was replaced by ^{3}H-uridine (specific activity 1 µ Ci/ml).

RESULTS

Total RNA

The RNA content in the control leaves of both pigeonpea and amaranth always maintained higher values than the SO_2 treated leaf discs. The SO_2 treatments altered the levels of RNA in both the plant species. The reduction of RNA content in response to SO_2 depends on the concentration and incubation period. The effect was more conspicuous in higher SO_2 concentrations in both the pigeonpea and amaranth. Among the SO_2 treated leaf discs, the 250 ppm treated ones exhibited maximum decline. Pigeonpea exhibited a decline of 2.34 fold under light and 2.78 folds under dark at 250 ppm SO_2 of 24 h duration. Amaranth exhibited a decline of 3.57 fold in light and 5.00 fold in dark exposed leaf discs treated with 250 ppm SO_2 of duration. The reduction in RNA content was more under dark in both the species. Amaranth exhibited more conspicuous declined than pigeonpea (Fig.- 1 a,b,c,d).

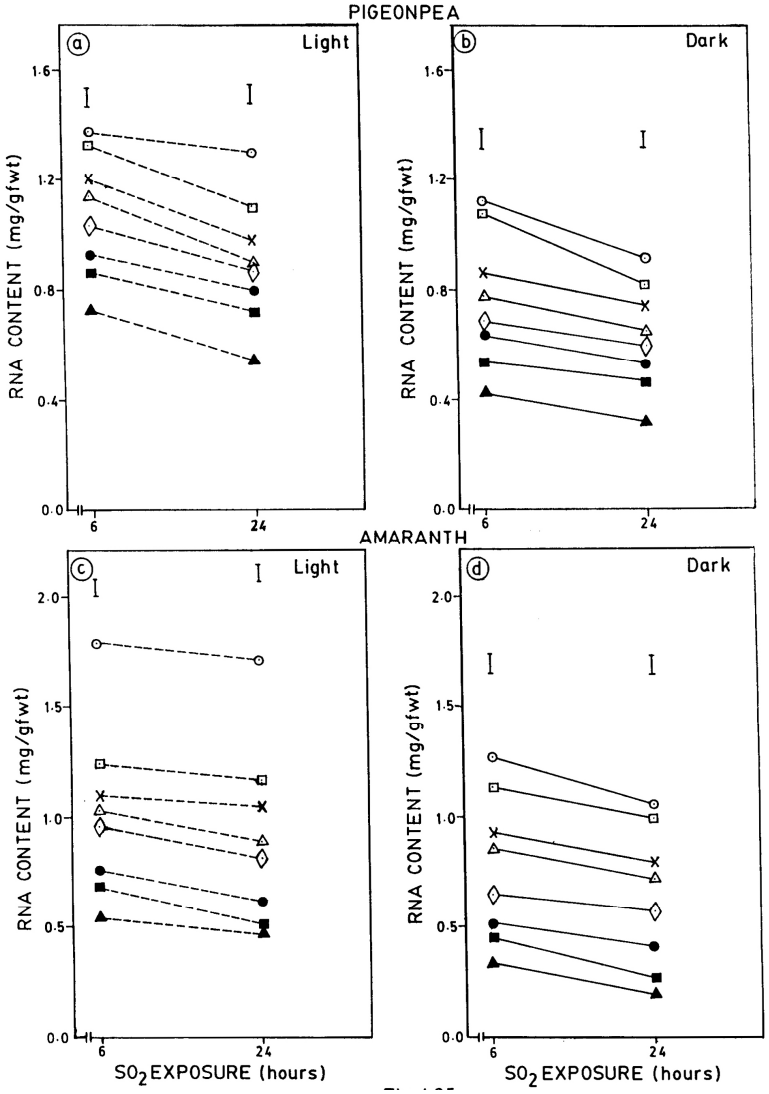

Figure 1. The effect of aqueous SO_2 on RNA content of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.), a and b - Pigeonpea; c and d – Amaranth, ---- under light; — under dark ○- 0 ppm; □-10 ppm; ×-20 ppm; -30 ppm; -40 ppm; ●-50 ppm; ■-100 ppm; ▲-250 ppm

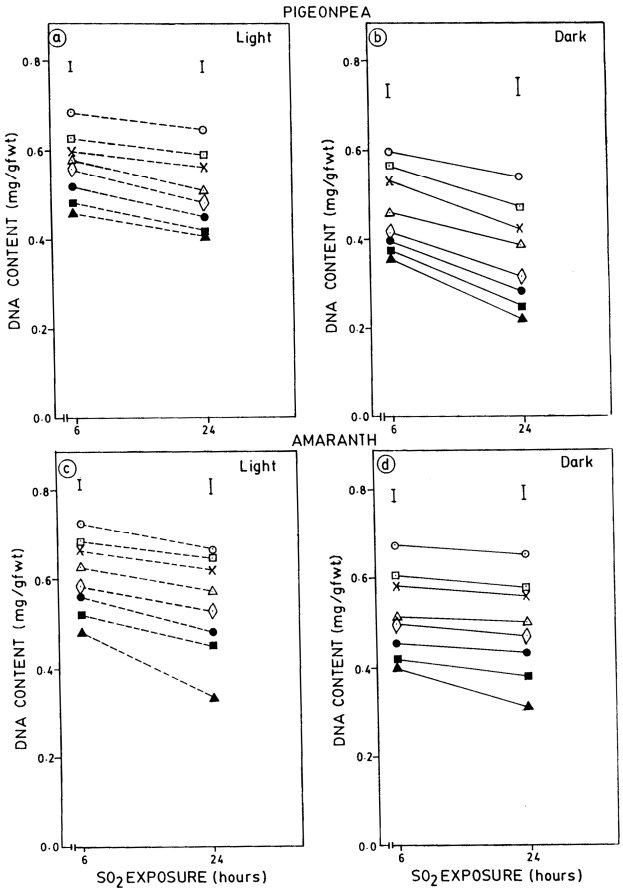

Figure 2. The effect of aqueous SO_2 on DNA content of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.), a and b - Pigeonpea; c and d – Amaranth, ---- under light; — under dark ○- 0 ppm; □-10 ppm; ×-20 ppm; -30 ppm; -40 ppm; ●-50 ppm; ■-100 ppm; ▲-250 ppm

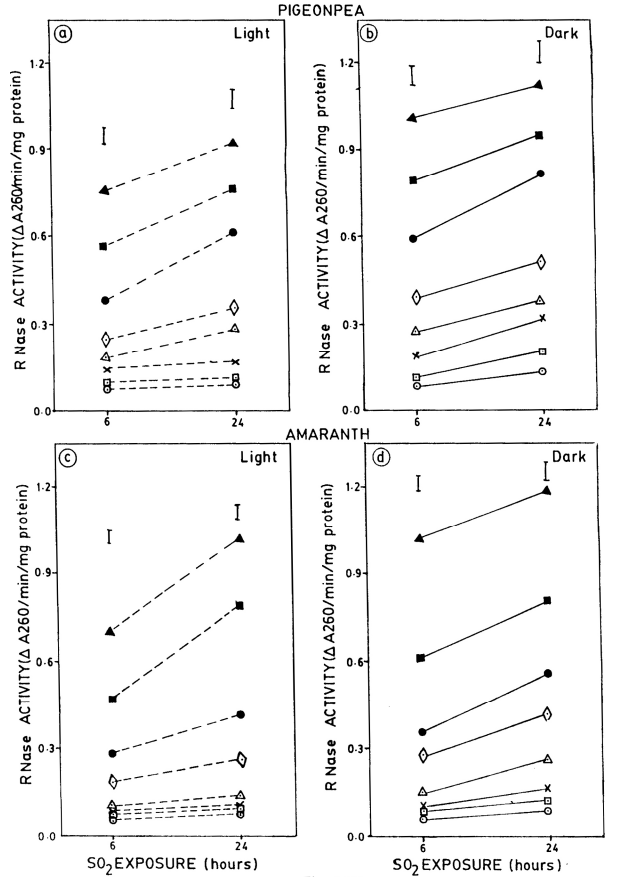

Figure 3. The effect of aqueous SO_2 on the RNase activity of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.), a and b - Pigeonpea; c and d – Amaranth, ---- under light; — under dark ○- 0 ppm; □-10 ppm; ×-20 ppm; -30 ppm; -40 ppm; ●-50 ppm; ■-100 ppm; ▲-250 ppm

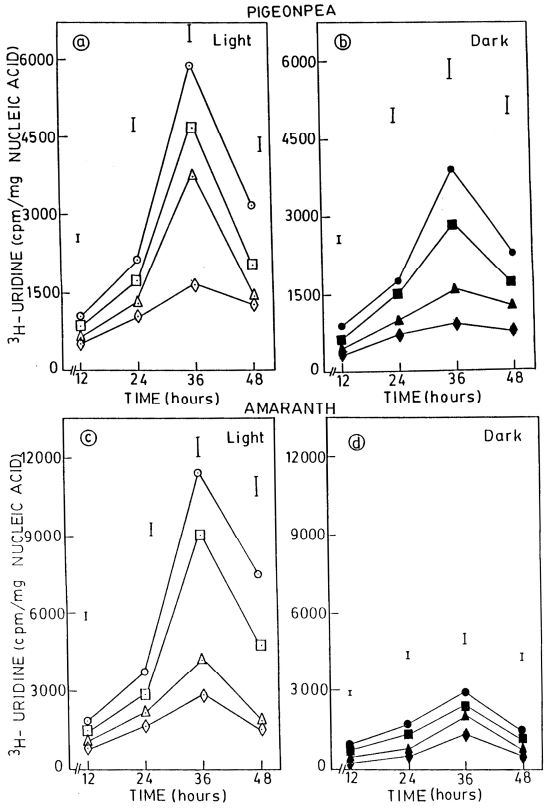

Figure 4. The effect of aqueous SO_2 on ^{3}H-uridine incorporation into nucleic acids of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.), a and b - Pigeonpea; c and d - Amaranth, under light : ○- 0 ppm; □-30 ppm; -100 ppm; -250 ppm; under dark : ●-0 ppm; ■-30 ppm; ▲-100 ppm♦-250 ppm

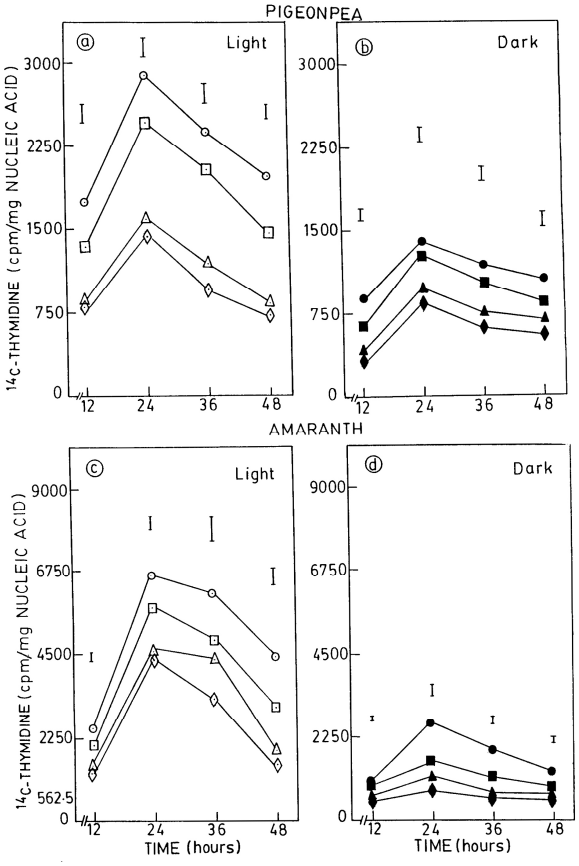

Figure 5. The effect of aqueous SO_2 on ^{14}C-thymidine incorporation into nucleic acids of the leaf discs of pigeonpea and amaranth (Vertical lines represent S.E.), a and b - Pigeonpea; c and d - Amaranth, under light : ○- 0 ppm; □-30 ppm; -100 ppm; -250 ppm; under dark : ●-0 ppm; ■-30 ppm; ▲-100 ppm♦-250 ppm

Total DNA

The control leaf discs of pigeonpea and amaranth exhibited greater content of DNA than SO_2 treated leaf discs. The decrease of DNA becomes more conspicuous with increasing SO_2 concentration and duration of incubation. Maximum decline was noticed at 250 ppm SO_2 concentration in both the plant species. Pigeonpea reached a decline of 1.56 fold under light and 2.46 fold under dark at 250 ppm SO_2 concentration of 24 h. The corresponding values of amaranth were 1.96 fold under light and 2.62 fold under dark. The loss of DNA content was more in dark in both the plant species (Fig.- 2a, b, c, d).

RNase activity

The RNase activity of leaf discs increased from 6 to 24 h of incubation. The SO_2 treated leaf discs always exhibited higher values when compared to the corresponding controls. Thus aqueous SO_2 stimulated the RNase activity gradually with increase in its concentration. Among the SO_2 treated leaf discs maximum activity was noticed at 250 ppm SO_2. At this concentration the rise in RNase activity in pigeonpea was 10.28 fold under light and 10.51 fold under dark at 24 h. The corresponding rise in amaranth was 12.64 fold under light and 13.42 fold under dark. In between pigeonpea and amaranth the activity recorded more in amaranth. Further the activity was conspicuous under dark (Fig. - 3 a,b,c,d).

^{3}H-uridine incorporation

The ^{3}H-uridine incorporation into RNA was carried out in the leaf discs incubated in aqueous SO_2 for 6 h only. The time course of incorporation of ^{3}H-uridine in different aqueous SO_2 treatments were carried out form 12 to 48 h under both light and dark conditions. The ^{3}H-uridine incorporation into RNA in all the SO_2 treatments including their respective controls increased up to 36 h followed by a reduction at 48 h of incorporation. More incorporation of ^{3}H-uridine was observed in the controls than in the SO_2 treatments of both pigeonpea and amaranth leaf discs. The rate of incorporation in treatments were decreased with increasing SO_2 concentration. The ^{3}H-uridine incorporation in both the plant species was higher under light than under dark. Further ^{3}H-uridine incorporation rate into RNA was higher in amaranth than in pigeonpea (Fig.- 4a,b,c,d).

^{14}C-thymidine incorporation

The ^{14}C-thymidine incorporation into DNA was also carried out in the leaf discs incubated in aqueous SO_2 for 6 h only. The time course of incorporation of ^{14}C-thymidine in different aqueous SO_2 treatments were carried out from 12 to 48 h under both light and dark conditions. The rate of incorporation of all the SO_2 treatments including the control showed an early increase followed by a decline. In the controls of both pigeonpea and amaranth, ^{14}C-thymidine incorporation increased upto 24 h followed by a decline. Though the SO_2 treated leaf discs of both pigeonpea and amaranth registered an initial increase in the incorporation of ^{14}C-thymidine, they always exhibited lower values than their respective controls. In both the plant species more incorporation was observed under light conditions. The rate of incorporation recorded higher values in amaranth than in pigeonpea (Fig.- 5 a,b,c,d).

DISCUSSION

Aqueous SO_2 reduced the levels of RNA in the leaf discs of both pigeonpea and amaranth. The decline was closely associated with the increasing concentration of SO_2 and duration of exposure. The degree of decline in RNA content was relatively more in dark than in light treatments. The decline in RNA content was noted more in amaranth than in pigeonpea (Fig.- 1 a,b,c,d). The decrease in RNA content was attributed to the non-availability of soluble precursors/or increased RNA hydrolysis (Craker and Starbuck, 1972), or breakdown of the pre-formed RNA molecules under increased SO_2 exposure. Evidences also indicate that free-radicals generated during sulphite oxidation were also responsible for the glycosidic bond cleavage of pyrimidine nucleosides of RNA molecules (Kitamura and Hayatsu, 1974).

The activity of ribonuclease increase during normal leaf senescence (Sacher *et al*., 1975). It was considered interesting to find out the effect of SO_2 on ribonuclease activity of the leaf discs of pigeonpea and amaranth. The studies revealed that the ribonuclease activity increased with increasing SO_2 concentration. The ribonuclease activity appeared relatively more in amaranth, and it was more pronounced in dark (Fig. - 3 a,b,c,d). The RNA content of the discs of both pigeonpea and amaranth under SO_2 exposure was closely associated with ribonulease activity.

The effect of aqueous SO_2 on the incorporation of ^{3}H-Uridine by the leaf discs of pigeonpea and amaranth exhibited varied responses. Aqueous SO_2 decreased the ability of ^{3}H-uridine incorporation in the leaf discs of both the plant species. The control leaf discs registered higher incorporation than SO_2 treated leaf discs in both the pigeonpea and amaranth. The greatest incorporation of ^{3}H-uridine was registered at 24 h stage in all the treatments. However, the control leaf discs incorporated more ^{3}H-uridine than SO_2 treated leaf discs. The lowest ^{3}H-uridine incorporation was registered in 250 ppm SO_2 treated leaf discs of both the plant species (Fig.- 4 a,b,c,d). The decrease in the synthesis of RNA may be due to the inactivation of RNA synthesizing enzymes or damage to RNA synthesizing organelles by the free radicals generated during the SO_2 metabolism of leaf discs. The decreased incorporation of ^{3}H-uridine however suggests that the damage to RNA synthesizing complex was not total. Exposure of leaf discs of pigeonpea and amaranth to SO_2 led to a considerable decline of DNA content. Increased SO_2 concentration and duration of exposure increased the degree of decline in both the plant species. Further the decline was more in dark than in light exposed leaf discs (Fig. - 2 a,b,c,d). Highest decline was registered at 250 ppm SO_2 in both pigeonpea and amaranth leaf discs. It is presumed that the free radicals may be involved in the cleavage of phosphodiester bonds of DNA, leading to decreased DNA content (Hayatsu and Miller, 1972; Inoue *et al*., 1972).

The incorporation of ^{14}C thymidine into DNA was more in control leaf discs of pigeonpea and amaranth than their

treatments. The degree of reduction depends on the SO_2 concentration. The greatest uptake of ^{14}C-thymidine of all the SO_2 treatments was registered at 36 h imbibitions (Fig. - 5 a,b,c,d). It can be presumed that the decline in DNA synthesis may be due to the free radicals produced during the chain reactions of SO_2 oxidation (Brandle and Erismann, 1973; Ziegler, 1975).

Conclusion

The total RNA and DNA contents declined in all the SO_2 treated leaf discs. The decline was gradual and becomes more sharp with increasing SO_2 concentration and duration of exposure. RNA content was more affected than DNA content in both the plant leaf discs studied in response to SO_2 exposure. RNase activity increased in response to aqueous SO_2 exposure in both the plant species. Incorporation of radioactive isotopes of ^{3}H-uridine and ^{14}C-thymidine exhibited a decline in the synthesis of RNA and DNA with increasing SO_2 concentration.

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