

# A BIOCHEMICAL STUDY ON EFFECT OF CALCIUM HYDROXIDE AS NEUTRALIZING TOXIC EFFECT OF SO<sub>2</sub> ON SELECTED PLANTS BELONGING TO FAMILY *BRASSICACEAE*

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## ABSTRACT

The effect of SO<sub>2</sub> on biochemical in (*Brassica juncea* [L.] Czern. and Coss. cv. Pusa Bold; *Raphanus sativus* L.cv. Mino Early Long White). In exposed seedlings SO<sub>2</sub> concentration ranging (653, 1306, 2612 & 3918 µg m<sup>-3</sup> SO<sub>2</sub>). Resulted accumulation of metabolites especially ascorbic acid is the adaptive mechanisms that operate in plants, when exposed to SO<sub>2</sub> stress. Appreciable decrease due to SO<sub>2</sub> was observed in ascorbic acid content of *Raphanus sativus* plants. Carbohydrate level in plant is also influenced by SO<sub>2</sub> exposures, the possible reason for decreased sugar content in plants under SO<sub>2</sub> stress. All four concentrations of SO<sub>2</sub> caused reduction in carbohydrate in both crops except at 653 µg m<sup>-3</sup> of SO<sub>2</sub> where a rise in carbohydrate level was seen. A perusal of literature suggests that SO<sub>2</sub> had a marked impact on protein content of leaves and seeds. They reported that protein degradation under SO<sub>2</sub> stress was either due to decreased photosynthesis or due to inhibition of protein synthesis. Appreciable decrease due to 653 µg m<sup>-3</sup> and 3918 µg m<sup>-3</sup> of SO<sub>2</sub> was observed in the protein content of leaves as well as seeds. A marked decline found in the nitrogen & phosphorus content of SO<sub>2</sub> fumigated plants. Minerals like nitrogen and phosphorus in leaves of *Brassica juncea* and *Raphanus sativus* were recorded to be substantially reduced following the long-term exposures of 653, 1306, 2612 and 3918 µg m<sup>-3</sup> of SO<sub>2</sub>. Out of the two crops, SO<sub>2</sub> caused more decline in leaf nitrogen content of *Raphanus sativus* than that of *Brassica juncea*. The experimental crops, on exposure to SO<sub>2</sub> had shown decline in their phosphorus content. The reductions in carbohydrate, protein and mineral content were directly related to decline in chlorophyll content of treated seedlings. Results indicate that calcium hydroxide treatments afforded protective effects against SO<sub>2</sub> as evidenced by enhancement in plant length, biomass accumulation, leaf area and yield of both crops. When SO<sub>2</sub> exposed plants were periodically sprayed with calcium hydroxide their growth performance was improved. The effect of SO<sub>2</sub> on biochemical breakdown and rate of photosynthesis was due mostly to the specific direct action of SO<sub>2</sub> and was not a function of increased acidity.

## INTRODUCTION

SO<sub>2</sub> is one of the major air pollutants in industrialized areas that can damage vegetation, and it is one of the most prevalent phytotoxic air pollutants and causes substantial damages to green plant. An accompanying effect of SO<sub>2</sub> toxicity is the appearance of reactive oxygen species by transferring the electron to oxygen via the photosynthetic electron transport chain, radicals can be generated (Eltner, 1982). This is favoured by inhibition of key enzymes of the Calvin cycle, which has been observed after SO<sub>2</sub> fumigation (Jager *et al.*, 1986). The resulting superoxide reacts with either spontaneously or is enzymatically catalyzed to H<sub>2</sub>O<sub>2</sub>. The enzyme needed

for this reaction is superoxide dismutase, which plays an important role in defense against SO<sub>2</sub> toxicity (Tanaka and Sugahara, 1980). The present study was initiated to determine if low concentration of SO<sub>2</sub> have any effect on the process of photosynthesis without producing visual symptoms. The effect of SO<sub>2</sub> was studied on the following aspects of plant biochemical: (a) Effect on changes in antioxidant level, (b) Effect on alterations in carbohydrate, protein and metabolism, (c) Effect on Changes in mineral content

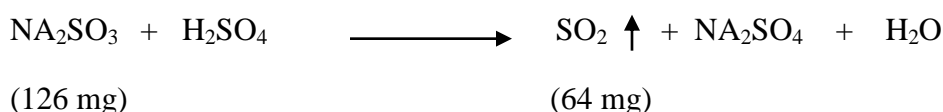
## MATERIAL AND METHOD

### *Growth Conditions*

Plants from the family Brassicaceae (*Brassica juncea* (L.) Czern.and Coss.cv.Pusa Bold; *Raphanus sativus* L.cv.Mino Early Long White) seeds were washed with sterile distilled water and then treated with 0.1% mercuric chloride for 5 minutes and finally washed with sterile distilled water for 15 minutes. Surface sterilized seeds were allowed to imbibe water for 6 hrs.and thereafter sown on petriplates lined with cotton over which Whatman no.40 filter paper was placed. Seeds were placed on filter paper. For each variety, five sets each having 150 seeds were maintained. Then seeds were sown in polythene bags/earthen ware containing garden soil. The soil was sandy loam and homogenously mixed with farmyard manure. All experiment will carried out in a fumigation chamber (1m\*1m\*1m) made of iron rods. Each chamber was portable and covered with transparent polythene sheets. Then Plants were exposed to SO<sub>2</sub> from 5<sup>th</sup> day onwards. Plants of each species were divided into five sets. Out of five four sets were exposed to four different concentration of SO<sub>2</sub> (653, 1306, 2612, 3918 µg m<sup>-3</sup>) while the fifth one was used as control.

### *SO<sub>2</sub> Treatment*

SO<sub>2</sub> was prepared by allowing a reaction of dilute sulphuric acid (10% H<sub>2</sub>SO<sub>4</sub>) and sodium sulphite (NA<sub>2</sub>SO<sub>3</sub>) under controlled condition of temperature and humidity. Complete reaction of 1M NA<sub>2</sub>SO<sub>3</sub> with 10% H<sub>2</sub>SO<sub>4</sub> produces 1M SO<sub>2</sub> or 126 mg of NA<sub>2</sub>SO<sub>3</sub> yields 64 mg SO<sub>2</sub>. The chemical reaction for SO<sub>2</sub> preparation is as follows-



Hence, 1.968 of NA<sub>2</sub>SO<sub>3</sub> is required to produce 1 mg (1000 µg m<sup>-3</sup>) of SO<sub>2</sub>. Therefore, on the basis of this equation 1.285, 2.571, 5.142, 7.713 mg of NA<sub>2</sub>SO<sub>3</sub> were used to obtain 653, 1306, 2612, 3918 µg m<sup>-3</sup> of SO<sub>2</sub>, respectively, inside the exposure system. The plants were given the treatment of SO<sub>2</sub> on alternate day for 2 hrs.

### *Calcium hydroxide treatment*

Calcium hydroxide (0.5% aqueous solution) was used for amelioration studies. Results of germination studies showed that lower concentration of SO<sub>2</sub> (653 and 1306 µg m<sup>-3</sup>) did not cause any appreciable reduction. On the contrary, higher concentration (2612 and 3918 µg m<sup>-3</sup>) of SO<sub>2</sub> proved to be highly toxic. Plants treated with 2612 and 3918 µg m<sup>-3</sup> of SO<sub>2</sub> were selected for amelioration studies and calcium hydroxide was used as an ameliorating agent.

For ameliorating study set treated with 0.5% Ca(OH)<sub>2</sub>, 0.5% Ca(OH)<sub>2</sub>+2612 µg m<sup>-3</sup> SO<sub>2</sub>, 2612 µg m<sup>-3</sup> SO<sub>2</sub>, 0.5% Ca(OH)<sub>2</sub>+3918 µg m<sup>-3</sup> SO<sub>2</sub> and 3918 µg m<sup>-3</sup> SO<sub>2</sub> were designated as C,C+T<sub>1</sub>, T<sub>1</sub>,C+ T<sub>2</sub> and T<sub>2</sub> sets, respectively. The calcium hydroxide solution was sprayed on SO<sub>2</sub> (2612 µg m<sup>-3</sup> and 3918 µg m<sup>-3</sup> SO<sub>2</sub>) treated plants once in a week with the help of a sprayer.

The study on both plants was carried out till maturity (90 d in *Brassica juncea* and 120 d in *Raphanus sativus*). The observations for various attributes were recorded in 15 d, 30 d, 45 d, 60 d, 75 d, 90 d old plants of *Brassica juncea* and *Raphanus sativus* respectively.

### ***Growth Analysis***

Plants grown on polythene bags/pots were taken out and roots were rinsed several times in deionized water to remove unwanted nutrients from root surface. Excess moisture was removed using absorbent towels.

### ***Phenological analysis***

#### ***Carbohydrate Extraction & Determination***

Total carbohydrate amount was estimated by anthrone colorimetric method (Yemm and Willis, 1954). For carbohydrate estimation, plant parts (root/leaf/seed) were placed in oven for 24 hrs at 80°C. Dried sample (50 mg) was crushed in 2.5 ml of 2.5 N HCl and then kept on boiling water bath for 3 hours. After 3 hours sodium carbonate was added to it till effervescence ceases. Final volume was made 25 ml with distilled water. Again it was centrifuged and 4 ml of anthrone reagent (200mg anthrone in 100 ml HCl) was added to 5 ml of sample and placed in water bath for 8 min. It was cooled and optical density was recorded at 630 nm. Calibration curve of glucose was used for estimation of carbohydrate in mg g<sup>-1</sup> dry weight.

#### ***Protein Extraction & Determination***

Protein estimation in leaves/root/seed was carried out according to method developed by Lowry *et al.* (1951). Fresh tissue (50 mg) was homogenised in 5 ml chilled tris maleate buffer and centrifuged at 2000 rpm for ten minutes. To 1 ml of supernatant 1 ml of TCA (20%) was added and kept in refrigerator for overnight. Next day, reaction mixture was again centrifuged and pellet was dissolved in 0.1 N NaOH (3 ml). After two hours, 1 ml of above solution was mixed with 5 ml alkaline copper tartarate solution. Ten min. later, 1 ml of folin ciocalteu reagent (double diluted) was added and solution was kept in dark for 30 min. Then optical density (OD) of sample was recorded at 660 nm and amount of protein (mg g<sup>-1</sup> fresh weight) was determined with the help of standard curve of bovine serum albumin (BSA).

#### ***Nitrogen Extraction & Determination***

Nitrogen content in various plant parts (roots/leave/seed) was determined according to method developed by Snell and Snell (1954). Dried plant sample (50 mg) was digested in 2 ml of H<sub>2</sub>O<sub>2</sub> (30%) and 5 ml of conc.

solution and placed on hot plate for 30 min. Again, 3 ml of  $H_2O_2$  was added and kept on hot plate for another 60 min or till the digest become clear. After cooling, 1 ml of digest was taken out in a test tube and 3 ml of Nessler's reagent and 1 ml of distilled water was added to this. Optical density of coloured solution was recorded with the help of spectrophotometer at 425 nm. Total amount of nitrogen was calculated by preparing a calibration curve of ammonium sulphate and expressed as  $mg\ N_2\ g^{-1}$  dry weight.

### ***Phosphorus Extraction & Determination***

Phosphorus content was estimated according to Olsen's (1954) method. 50 mg dry material (root/shoot/leaves) was homogenized in 10 ml of  $NaHCO_3$  (4.2 g/l) and pinch of activated charcoal was added to this solution. It was kept on a shaker for 30 min. and then centrifuged at (2000 rpm) for 10 min. In a test tube 5 ml of filtrate and 5 ml molybdate reagent (15 g ammonium molybdate + 30 ml distilled water + 348 ml conc. HCl + add distilled water to make 1 liter) was added and swirled. After that, 1 ml of working  $SnCl_2$  solution (prepared by mixing 1 ml stock  $SnCl_2$  solution (40%) and 60 ml distilled water) was added to reaction mixture. Final volume was made upto 25 ml. Optical density of each sample was recorded at 660 nm. Calibration curve prepared by potassium hydrogen phosphate ( $KH_2PO_4$ ) was used to calculate the amount of phosphorus in  $mg\ g^{-1}$  dry weight.

### ***Ascorbic acid Extraction & Determination***

Ascorbic acid was determined with help of titration method (Sadasivam and Manickam, 1992). The quantitative estimation of ascorbic acid was based on the fact that ascorbic acid reduces the blue coloured dye 2, 6 dichlorophenol indophenol (DCPIP) to a colourless form. Following reagents were prepared for ascorbic acid estimation–

1. 4% oxalic acid (4 g oxalic acid was dissolved in 100 ml distilled water)
2. DCPIP solution (53 mg DCPIP and 42 mg sodium bicarbonate was dissolved in 200 ml distilled water)
3. Stock solution of ascorbic acid (100 mg ascorbic acid was dissolved in 100 ml of 4% oxalic acid in a standard flask)
4. Working solution of ascorbic acid (It was prepared by diluting 10 ml of stock solution to 100 ml with the help of distilled water)

5 ml of working solution was taken out in 100 ml conical flask. 10 ml of oxalic acid (4%) was added to it and titrated against the dichlorophenol indophenol. Appearance of pink colour, which persisted for few minutes, was considered as end point. Volume of dye consumed ( $V_1$ ) was considered as equivalent to amount of ascorbic acid. Fresh tissue (50 mg leaf/root) was homogenised in small volume of oxalic acid (5ml) and then filtered. Final volume was made upto 100 ml with oxalic acid. 5 ml of this solution was taken out and 10 ml of oxalic acid was added to it and titrated against dichlorophenol indophenol. The volume ( $V_2$ ) of dye consumed was noticed. Amount of ascorbic was calculated with the help of following formula–

$$\text{Ascorbic acid} = \frac{0.5}{V_1} \times \frac{V_2}{5} \times \frac{100}{\text{wt. of sample}}$$

(mg g<sup>-1</sup> fresh weight)

**RESULT & DISSCUSION**

The seeds of some cultivars (*Brassica juncea*,) exposed to 653 µg m<sup>-3</sup> SO<sub>2</sub> show better germination and seedling growth than control ones. The reductions in carbohydrate, protein and mineral content were directly related to decline in chlorophyll content of treated seedlings. On the contrary ascorbic acid contents of treated seedlings increased than those of control ones. All four concentrations of SO<sub>2</sub> caused reduction in carbohydrate in both crops except at 653 µg m<sup>-3</sup> of SO<sub>2</sub> where a rise in carbohydrate level was seen. SO<sub>2</sub> affects photosynthesis process adversely either by interfering with the electron flow in electron transport chain of chloroplast system (Puckett *et al.*, 1974) or by causing swelling of thylakoid in chloroplast (Wellburn *et al.*, 1972). Protein

**Table 1a Estimation of some biochemical components in leaves of *Raphanus sativus* fumigated with different concentrations of sulphur dioxide at 15 d and 30 d plant age.**

Attribute	Plant age (d)													
	15 d							30 d						
	Concentration of Sulphur dioxide (µg m <sup>-3</sup> )							Concentration of Sulphur dioxide (µg m <sup>-3</sup> )						
						CD							CD	
	0	653	1306	2612	3918	5%	1%	0	653	1306	2612	3918	5%	1%
Ascorbic acid (mg g <sup>-1</sup> f. wt.)	0.514	0.546	0.526	0.527	0.518	0.135	0.144	0.754	0.749	0.654	0.598	0.624	0.135	0.292
Air pollution tolerance index (APTI)	9.224	9.357	8.798	8.745	8.776			9.307	9.110	8.943	8.790	8.6321		

**Table 1b Estimation of some biochemical components in leaves of *Raphanus sativus* fumigated with different concentrations of sulphur dioxide at 15 d and 30 d plant age.**

Attribute	Plant age (d)						
	30 d						
	Concentration of Sulphur dioxide ( $\mu\text{g m}^{-3}$ )					CD	
	0	653	1306	2612	3918	5%	1%
Carbohydrate ( $\text{mg g}^{-1}$ d. wt.)	31.069 $\pm 0.232$	29.208 $\pm 0.233$	26.582 ** $\pm 1.182$	24.390* * $\pm 0.356$	23.443* * $\pm 0.704$	2.532	2.713
Protein ( $\text{mg g}^{-1}$ f. wt.)	25.120 $\pm 1.318$	19.843* * $\pm 2.224$	16.676 ** $\pm 0.967$	14.776* * $\pm 1.318$	9.287** $\pm 0.967$	5.253	5.627
Nitrogen ( $\text{mg g}^{-1}$ d. wt.)	2.602 $\pm 0.035$	2.547 $\pm 0.027$	2.210* * $\pm 0.016$	1.414** $\pm 0.034$	1.278** $\pm 0.020$	0.066	0.071
Phosphorus ( $\text{mg g}^{-1}$ d. wt.)	0.533 $\pm 0.006$	0.512 $\pm 0.013$	0.509 $\pm 0.009$	0.405** $\pm 0.013$	0.479** $\pm 0.020$	0.033	0.035

Table 2a Estimation of some biochemical components in leaves of *Raphanus sativus* treated with  $\text{SO}_2$  and calcium hydroxide at 15 d plant age.

Attribute	Plant age (d)							
	15 d							
	Treatment						CD	
	Control	C	C+T <sub>1</sub>	C+T <sub>2</sub>	T <sub>1</sub>	T <sub>2</sub>	5%	1%
Ascorbic acid ( $\text{mg g}^{-1}$ f. wt.)	0.514 $\pm 0.034$	0.531 $\pm 0.019$	0.539 $\pm 0.016$	0.515 $\pm 0.004$	0.527 $\pm 0.029$	0.518 $\pm 0.044$	0.068	0.095
Air pollution tolerance index (APTI)	9.224	8.923	8.830	8.753	8.745	8.776	□	□

C : 0.5%  $\text{Ca(OH)}_2$ , C + T<sub>1</sub> : 0.5%  $\text{Ca(OH)}_2$  + 2612  $\mu\text{g m}^{-3}$   $\text{SO}_2$ , C + T<sub>2</sub> : 0.5%  $\text{Ca(OH)}_2$  + 3918  $\mu\text{g m}^{-3}$   $\text{SO}_2$ , T<sub>1</sub> : 2612  $\mu\text{g m}^{-3}$   $\text{SO}_2$ , T<sub>2</sub> : 3918  $\mu\text{g m}^{-3}$   $\text{SO}_2$

Table 2b Estimation of some biochemical components in leaves of *Raphanus sativus* treated with  $\text{SO}_2$  and calcium hydroxide at 15 d plant age.

Attribute	Plant age (d) 15 days							
	Treatment						CD	
	Control	C	C+T <sub>1</sub>	C+T <sub>2</sub>	T <sub>1</sub>	T <sub>2</sub>	5%	1%
<b>Carbohydrate</b> (mg g <sup>-1</sup> d. wt.)	19.355 ± 0.441	18.230 ± 0.585	17.920 ± 0.116	17.454* * ± 0.232	16.795* * ± 0.242	15.980* * ± 0.373	1.187	1.676
<b>Protein</b> (mg g <sup>-1</sup> f. wt.)	7.810 ± 0.967	6.499 ± 0.633	7.654 ± 0.967	6.021 ± 0.365	6.101 ± 1.00	4.432** ± 0.633	2.491	3.517
<b>Nitrogen</b> (mg g <sup>-1</sup> d. wt.)	0.840 ± 0.016	0.843 ± 0.024	0.817* * ± 0.012	0.777** ± 0.019	0.705** ± 0.020	0.668** ± 0.011	0.060	0.085
<b>Phosphorous</b> (mg g <sup>-1</sup> d. wt.)	0.227 ± 0.024	0.248 ± 0.013	0.245 ± 0.008	0.243 ± 0.011	0.240 ± 0.019	0.232 ± 0.100	0.042	0.059

forms the structural and functional basis of the cell. Appreciable decrease due to 653 µg m<sup>-3</sup> and 3918 µg m<sup>-3</sup> of SO<sub>2</sub> was observed in the protein content of leaves as well as seeds. Inhibition of nitrogenous parameters such as protein and total nitrogen content upon exposure of high doses of SO<sub>2</sub> may be due to interaction at different sites to inhibit a large number of enzymes having functional sulfhydryl group resulting in disruption of protein synthesis pathways (Saxena and Saxena, 1999). Minerals like nitrogen and phosphorus in leaves of both test crops were recorded to be substantially reduced following exposures of 653, 1306, 2612 and 3918 µg m<sup>-3</sup> of SO<sub>2</sub>. Nitrogen and phosphorus actively participate in various physiological activities and any imbalance in the optima of these biochemical components ultimately perturbs the growth and yield. Out of the two crops, SO<sub>2</sub> caused more decline in leaf nitrogen content of *Raphanus sativus* than that of *Brassica juncea*. Phosphorus plays a vital role as energy carrier for important reaction of plant life like the oxidation of sugars with the aid of several enzymes and in the initial reaction in photosynthesis & SO<sub>2</sub> had shown decline in their phosphorus content. Phosphorus deficiency could be one of the reasons for inhibition of photosynthesis. In *Brassica juncea*, ascorbic acid accumulation was increasing with increase in concentration of SO<sub>2</sub>. On the contrary, appreciable decrease due to SO<sub>2</sub> was observed in ascorbic acid content of *Raphanus sativus* plants. Reduction in level of ascorbic acid in pollutant exposed plants has been ascribed to enzyme toxicity and sulphonation of -SH groups (Mapson, 1958). One of the significant findings of this study is that calcium hydroxide works as an antidote against SO<sub>2</sub> toxicity in test crops. A strong protective effect of calcium hydroxide against ill-effects of SO<sub>2</sub> was reported by many workers (Rao *et al.*, 1985; Kirk-Othmer, 1991; Budavari 1989). The growth improvement due to calcium hydroxide was ascribed to its acid neutralizing capacity. Calcium hydroxide ionizes in water to form calcium (Ca<sup>+2</sup>) and hydroxyl (OH<sup>-</sup>) ions, forming a strong base or alkali which effectively neutralizes the acidity of soil solution (NOSB, 2002).

### Changes in antioxidant level

Noctor and Foyer (1998) investigated that ascorbic acid is one of the most extensively studied antioxidants and has been detected in majority of cell types, organelles and apoplast. Mapson (1958) and Materna (1972) observed that amount of ascorbic acid was decreased by SO<sub>2</sub> exposure. They also revealed the fact that species having higher level of ascorbic acid are more tolerant to SO<sub>2</sub> stress. Singh and Rao (1983) also found a direct relation between tolerance to pollution and amount of ascorbic acid. They derived a new measure of tolerance APTI (air pollution tolerance index) which represents tolerance level of plants to air pollution. They found that APTI values were dependent on ascorbic acid content of leaves.

### **Alterations in carbohydrate and protein metabolism**

Carbohydrate level in plant is also influenced by SO<sub>2</sub> exposures. According to Nandi (1984) and Pande and Mansfield (1985), the possible reason for decreased sugar content in plants under SO<sub>2</sub> stress might be related to the increased respiration that deplete most of the food reserves.

A perusal of literature suggests that SO<sub>2</sub> had a marked impact on protein content of leaves and seeds. Earlier studies in this regard were made by Godzik and Linskens (1974), Sij and Swanson (1974), Constantinidou and Kozlowski (1979), Davies *et al.* (1981) and Singh *et al.* (1985). They reported that protein degradation under SO<sub>2</sub> stress was either due to decreased photosynthesis or due to inhibition of protein synthesis.

### **Changes in mineral content**

Mishra (1980) reported a marked decline in the nitrogen content of SO<sub>2</sub> fumigated *Arachis hypogaea* plants. Such reduction in nitrogen and phosphorus content of SO<sub>2</sub> treated plants was also recorded by Agrawal *et al.* (1985). Peuke and Tischner (1994) exposed spruce seedlings to various doses of SO<sub>2</sub> and results of their study showed decrease in nitrate-nitrogen content of SO<sub>2</sub> fumigated seedlings.

### **CONCLUSION**

*Brassica juncea* was most resistant cultivar to SO<sub>2</sub> while *Raphanus sativus* proved to be most sensitive cultivar. Accumulation of metabolites especially ascorbic acid and proline are the adaptive mechanisms that operate in plants, when exposed to SO<sub>2</sub> stress. Accumulation of ascorbic acid in plants exposed to SO<sub>2</sub>, can be employed as sensitive biochemical indicator to SO<sub>2</sub> stress. Study reports that initial concentration of SO<sub>2</sub> (653 µg m<sup>-3</sup>) caused slight stimulation in growth of *Brassica juncea*. This suggests that plants of *Brassica juncea* have the capacity to assimilate higher levels of sulphur as compared to other crops simply because of its higher requirement of the element. Thus *Brassica juncea* can tolerate or grow better up to a certain critical level of SO<sub>2</sub>, the level of which is higher than other crops. The present investigation led us to believe that calcium hydroxide acts as an antidote against SO<sub>2</sub> stress in two test crops i.e. *Brassica juncea* and *Raphanus sativus*.

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