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Stomatal and Photosynthetic Responses of *Cichorium intybus* Leaves to Sulfur Dioxide Treatment at Different Stages of Plant Development

Bhupinder Dhir, Mahmooduzzafar, Tariq Omar Siddiqi, and Muhammad Iqbal*
Department of Botany, Hamdard University, Hamdard Nagar, New Delhi 110 062, India

Fifty-day-old *Cichorium intybus* Linn. plants were exposed to 1 ppm sulfur dioxide gas, 2 h per day for 7 consecutive days. Their leaves as well as those from the control plants were sampled at pre-flowering, flowering, and post-flowering stages to study their morphological, physiological, and biochemical responses to SO_2 stress. The number, dimensions, area, and biomass of leaves were less in the treated plants. Length and width of stomatal apertures on both epidermises were greater for leaves exposed to SO_2. The Stomata were longer on the adaxial epidermis, but shorter on the abaxial epidermis, except at the pre-flowering stage. Stomatal widths varied widely. Compared with the controls, the abaxial epidermis on treated leaves showed consistently lower stomatal densities as well as stomatal indices. This was also true for the adaxial epidermis during the post-flowering stage. The photosynthetic rate and stomatal conductance were reduced in the SO_2-exposed plants, but intercellular CO_2 concentrations increased at the pre-flowering stage and, subsequently, declined. Chlorophyll *a*, carotenoid, and total chlorophyll contents increased at the pre-flowering stage, and then decreased. The level of chlorophyll *b* was reduced throughout plant development compared with the untreated controls.

Keywords: Biomass, chlorophyll, *Cichorium intybus*, photosynthesis, stomatal behavior

Sulfur dioxide, a major air pollutant, affects plant growth by interfering with physiological processes, and by influencing pigment concentrations, bioaccumulations, and cell structures (Darrall, 1989). Photosynthesis, respiration, stomatal activity, transpiration, and the transport process also are adversely affected. Various physiological functions may be altered when changes occur in the permeability of the plasma membrane (Treshow, 1984), enzymatic activities (Sandman and Gonzales, 1989), or metabolic functions (Nandi et al., 1986).

Upon entering the leaf through the stoma, SO_2 is rapidly dissolved in the aqueous phases of the leaf tissues, resulting in the formation of sulfites (SO_3^{2-}) and bisulfites (HSO_3^-). These and other ionic species influence cellular pH by generating protons. The number of stomata and the size of their apertures determine the extent of uptake. SO_2 may induce stomata to either open or close, depending on plant species, SO_2 concentration, duration of exposure, and environmental conditions at the time of exposure.

Cichorium intybus Linn., a member of the Asteraceae, is an erect, rough, and relatively glandular, perennial herb that can reach 1 m in height. It is a medically important species whose extracts are effective in curing jaundice, liver enlargement, gout, and rheumatic complaints (Kirtikar and Basu, 1991). In this study, we examined how its leaves respond to SO_2 stress during different stages of plant development.

*Corresponding author; fax +91-11-608-8874
e-mail root@hamduni.ren.nic.in

MATERIALS AND METHODS

Seeds of *C. intybus* Linn. were sown 20 cm apart in rows separated by 30 cm, in different beds during October. Plants were adequately watered and manured. When the plants were 50 days old, some were treated with 1 ppm SO_2 in a locally fabricated fumigation chamber. The gas was released through a tube from a cylinder, and was controlled by a regulator. Plants were treated for 2 h/day (7.30 a.m. – 9.30 a.m.) for 7 d. Fully expanded, mature leaves from both the control and the fumigated plots (10 plants each) were collected 80 days after fumigation (DAF) (pre-flowering stage), 100 DAF (flowering stage), and 120 DAF (post-flowering stage).

The area, length, and width of the leaves were measured with an LI-3000A Portable Leaf Area Meter (LICOR Inc. Lincoln, NE, USA). To estimate the biom-

ass, leaves were oven-dried at 80°C for 48 h and weighed on a digital balance. Fully expanded leaves from the controls and the treated plants were fixed in FAA (10 mL formaldehyde, 10 mL acetic acid, and 90 mL of 50% ethyl alcohol), and transferred to 70% alcohol after three days. Epidermal peels of the leaves, obtained by boiling the cut pieces in nitric acid (Ghouse and Yunus, 1972), were washed with tap water, then processed in an ethanol series for dehydration, stained with safranin, and mounted on Canada balsam. Dimensions of the stomatal apparatus and apertures were measured with an ocular micrometer scale in a compound light microscope. Stomatal density (SD) per mm^2 was determined and stomatal index (SI) was calculated by the formula of Salisbury (1927).

Photosynthetic rate, stomatal conductance, and intercellular CO_2 level were measured in-situ by clamping a leaf in the chamber of an LI-6200 Portable Photosynthesis System (LICOR). Transient (<1 min) exchange rates of H_2O vapor and CO_2 were then determined in this closed system. Chlorophyll amounts were measured by using DMSO (dimethyl sulfoxide), per the method of Hiscox and Israelstam (1979). The chlorophyll and carotenoid contents were then estimated by the formulae of Duxbury and Yentsch (1956), and MacLachlan and Zalik (1963), respectively. Absorbance was recorded at 480, 510, 645, and 663 nm on a DU 640B Spectrophotometer (Beckman, Franklin, TN, USA).

Field data were obtained over a three-year period. They were then analyzed with a Student's 't' test.

RESULTS

Leaf Dimensions

During each developmental stage, the SO_2-exposed plants had considerably fewer leaves than did the controls (Table 1). The length and width of treated leaves were significantly reduced only at the pre-flowering stage. Single- as well as total-leaf areas were always smaller in the treated plants, the latter parameter being significantly less. The maximum difference (82%) occurred at the pre-flowering stage. Leaf biomass was also reduced significantly at each growth

Table 1. Foliar characteristics of control and SO_2-treated plants of *C. intybus* at three developmental stages. Means ± SD are based on 100 readings for individual leaf traits and 10 readings for the total leaf estimates.

Parameter	Control	Treated	% Variation
Leaves per plant			
Pre-flowering	146.00 ± 29.81	76.33 ± 15.52	−47.72**
Flowering	292.00 ± 16.67	184.00 ± 43.36	−36.98**
Post-flowering	436.00 ± 80.36	328.33 ± 71.16	−24.69**
Leaf length (cm)			
Pre-flowering	20.76 ± 6.33	7.61 ± 5.17	−63.34**
Flowering	20.51 ± 5.82	19.53 ± 3.12	-4.77^{NS}
Post-flowering	29.03 ± 1.19	28.65 ± 2.03	-1.31^{NS}
Leaf width (cm)			
Pre-flowering	10.06 ± 0.52	9.23 ± 0.28	−8.25**
Flowering	10.46 ± 1.34	10.73 ± 0.61	$+2.58^{NS}$
Post-flowering	10.76 ± 0.09	10.90 ± 0.43	$+1.30^{NS}$
Single leaf area (cm^2)			
Pre-flowering	14.79 ± 2.50	8.44 ± 1.94	−42.93**
Flowering	27.75 ± 5.60	26.50 ± 2.82	−4.50*
Post-flowering	28.41 ± 4.60	26.79 ± 2.69	-5.70^{NS}
Total leaf area (cm^2)			
Pre-flowering	3976.86 ± 413.16	721.52 ± 108.01	−81.85**
Flowering	4441.36 ± 304.45	2256.43 ± 113.69	−49.19**
Post-flowering	8046.40 ± 270.05	4902.27 ± 185.31	−39.01**
Total leaf biomass (g)			
Pre-flowering	29.88 ± 3.32	6.73 ± 1.64	−77.47**
Flowering	31.08 ± 6.85	12.22 ± 1.60	−60.68**
Post-flowering	34.14 ± 6.28	26.50 ± 2.60	−22.37**

**, Significant at 1% level; *, Significant at 5% level; NS, Non-significant.

stage. Variations between the treated and control plants, for any of these parameters, generally were greater at the pre-flowering stage (Table 1).

Stomatal Features

Stomatal apertures on either epidermis of the treated plants were greater than for the controls (Table 2). Variations in aperture width were also similar (Table 2, Fig. 1). The difference between treated and control leaves for stomatal length on the adaxial epidermis was consistently significant; on the abaxial epidermis, stomata were considerably shorter during the late stages of treated-plant development. Stomatal widths on both epidermises of the treated leaves varied inconsistently, showing a relative gain in the pre-flowering stage, insignificant change during flowering, followed by a post-flowering decline.

SD on the adaxial epidermis of treated plants decreased significantly during the post-flowering stage, but was consistently low on the abaxial epidermis. Trends for SI followed those of SD on both surfaces, with the highest variation occurring at the post-flowering stage (Table 2).

Photosynthetic Capacity

The photosynthetic rate and stomatal conductance decreased over time for treated and untreated plants. Both parameters were relatively low under SO_2 stress, compared with higher readings for the controls (Fig. 2). The difference in conductance was more prominent during the late developmental stages. Intercellular CO_2 concentrations, which increased slightly over time, did not vary significantly between treated plants and controls (Table 3).

Pigments

Chlorophyll and carotenoid contents were highest during flowering and lowest in the post-flowering stage. Variations between the control and treated plants were negligible at the pre-flowering stage, but contents in the treated plants decreased significantly in subse-

Table 2. Stomatal responses of *C. intybus* plants to SO_2 treatment during different stages of plant growth. Means ± SD are based on 100 individual readings.

Parameter	Control	Treated	% Variation	Control	Treated	% Variation
	Adaxial epidermis			Abaxial epidermis		
Stomatal aperture length (μm)						
Pre-flowering	20.41 ± 2.79	22.88 ± 2.61	+12.10**	18.75 ± 2.51	22.87 ± 3.27	+21.97**
Flowering	24.23 ± 2.15	25.25 ± 3.01	+4.20*	22.63 ± 2.81	23.77 ± 2.67	+5.03**
Post-flowering	25.30 ± 2.45	27.65 ± 5.26	+9.28**	27.60 ± 4.86	29.50 ± 3.13	+7.40**
Stomatal aperture width (μm)						
Pre-flowering	15.00 ± 3.08	15.34 ± 1.70	$+2.26^{NS}$	14.93 ± 2.00	16.18 ± 1.61	+8.37*
Flowering	14.00 ± 2.37	15.27 ± 2.12	+9.09**	13.50 ± 3.13	13.60 ± 2.83	$+0.74^{NS}$
Post-flowering	8.75 ± 1.42	12.00 ± 2.17	+37.14**	10.25 ± 2.94	12.95 ± 1.60	+26.34**
Stomatal length (μm)						
Pre-flowering	25.08 ± 3.14	26.11 ± 2.94	+4.10*	26.83 ± 2.44	27.12 ± 1.51	$+1.08^{NS}$
Flowering	26.75 ± 1.67	27.75 ± 2.46	+3.73*	29.75 ± 1.75	27.12 ± 3.08	−8.84**
Post-flowering	28.53 ± 1.80	29.90 ± 3.11	+4.80*	31.50 ± 4.28	29.83 ± 2.45	−5.30**
Stomatal width (μm)						
Pre-flowering	19.00 ± 3.16	28.63 ± 2.91	+50.68**	27.50 ± 3.70	31.20 ± 3.33	+13.45**
Flowering	18.37 ± 2.52	19.75 ± 4.69	$+7.51^{NS}$	22.34 ± 6.97	21.70 ± 4.64	-2.86^{NS}
Post-flowering	17.75 ± 4.27	15.88 ± 1.74	−10.53**	20.00 ± 5.34	15.10 ± 1.89	−24.72**
Stomatal density (mm^{-2})						
Pre-flowering	7.37 ± 1.02	8.13 ± 1.82	+10.21*	13.96 ± 2.32	11.45 ± 2.72	−17.97**
Flowering	6.81 ± 1.24	7.26 ± 2.28	$+6.60^{NS}$	13.65 ± 2.32	10.17 ± 1.83	−25.49**
Post-flowering	6.00 ± 2.81	3.86 ± 1.43	−35.66**	9.06 ± 2.70	7.45 ± 1.07	−17.77**
Stomatal index (%)						
Pre-flowering	27.63 ± 5.30	29.48 ± 4.71	+6.69*	39.63 ± 0.99	32.39 ± 4.08	−18.26**
Flowering	24.48 ± 0.99	25.16 ± 1.01	$+2.77^{NS}$	36.00 ± 1.01	28.16 ± 4.71	−21.77**
Post-flowering	18.18 ± 4.71	14.28 ± 2.02	−21.45**	32.72 ± 4.71	20.83 ± 0.70	−36.33**

**, Significant at 1% level; *, Significant at 5% level; NS, Non-significant.

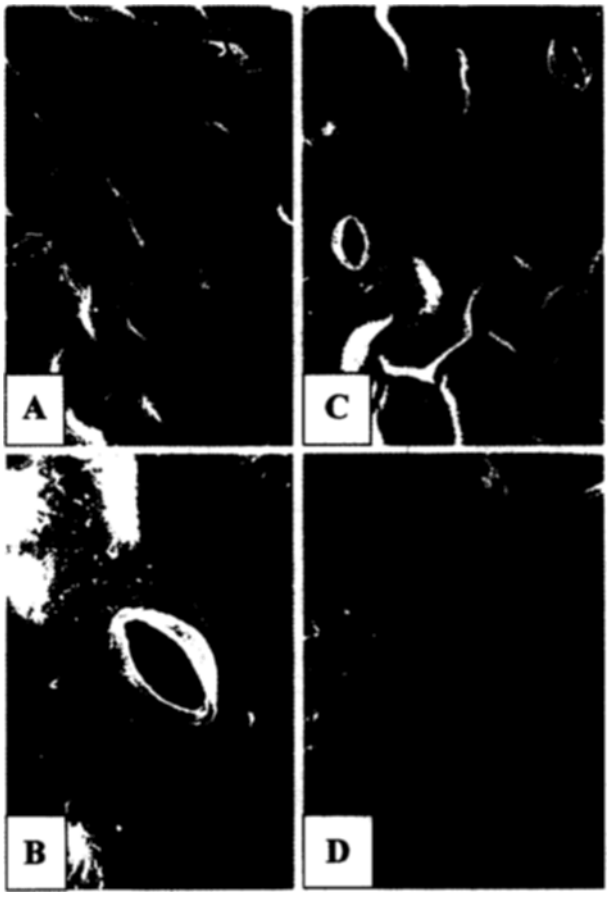

Figure 1. Electron micrographs of adaxial epidermis of *C. intybus* leaves showing wide-open stomata in SO_2-exposed samples (c and d) compared with control (a and b). a and c at 650X; b and d at 1800X.

quent stages -- the maximum difference occurring post flowering (Table 3).

DISCUSSION

The extent of leaf damage from pollutants is normally related to the amount of stomatal intake. Significantly fewer and smaller leaves evoke a direct effect on leaf growth. Reductions in leaf size may occur as a natural adaptation because the smaller the leaf, the less the absorption of noxious gases, as Gridhar (1981) noted in *Impatiens balsamina* growing near a thermal power plant. The decline in leaf biomass is apparently correlated with reductions in leaf area.

Treatment with SO_2 may disturb the stomatal aperture and the shape of the stomatal complex, thereby inducing changes in foliar responses related to con-

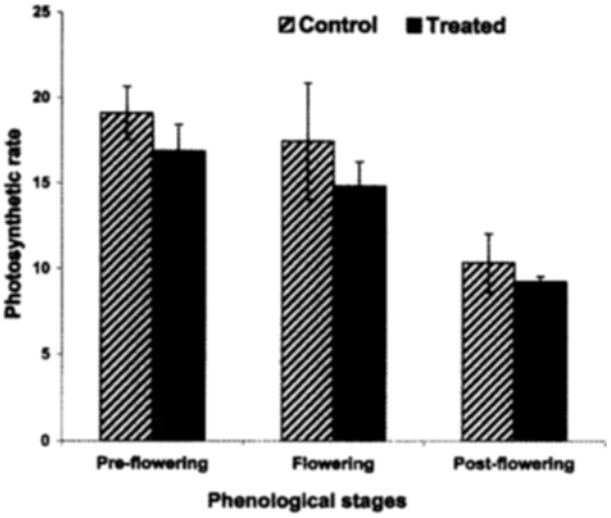

Figure 2. The net photosynthetic rate [expressed in µmol (CO_2) m^{-2} s^{-1}] in the leaves of control and SO_2-exposed *C. intybus* plants at different phenological stages. Observations are based on 10 independent readings for each sample. Differences were statistically significant at each stage.

ductance and CO_2 fixation. The influence of SO_2 on stomatal opening appears to be related to reduced turgor in the subsidiary cells. This loss of turgor is induced by changes in membrane permeability, which may account for the increase in the stomatal aperture (Mansfield and Pearson, 1996). In the present study, the reduced surface area of the *Cichorium* leaves meant fewer stomata could be accommodated, thereby reducing the volume of noxious pollutants entering the leaves. Sulfites, formed by the conversion of SO_2 in leaves, may inhibit the activities of PEP carboxylase (Ziegler, 1973) and NADP-malate dehydrogenase (Ziegler, 1974), both of which are involved in the formation of malate. These sulfites may also reduce the malate content (Kondo et al., 1984), which could be associated with regulation of the stomatal opening.

Photosynthesis may decline in response to damage in the photosynthetic apparatus, reduced pigment contents, and competition between SO_2 and CO_2 for foliar conductance or for active sites on carboxylases (Levitt, 1980). High concentrations of SO_2 also inhibit some Calvin cycle enzymes (Ziegler, 1973), thereby suppressing photosynthesis. Acidification of the stroma and accumulation of H_2O_2, as induced by SO_2, could also inactivate several enzymes necessary for CO_2 fixation (Alscher, 1984). Stomatal conductance in several woody angiosperms increases after exposure to low levels of SO_2, but can decrease in response to high doses (Steubig and Fangmeier, 1987; Cao, 1989).

Table 3. Physiological responses and pigment concentration of leaves of control and SO_2-treated *C. intybus* plants at different stages of plant growth. Means ± SD are based on 10 independent readings.

Parameter	Control	Treated	% Variation
Stomatal conductance (μmole $m^{-2}s^{-1}$)			
Pre-flowering	0.99 ± 0.19	0.89 ± 0.15	−10.00^{NS}
Flowering	0.84 ± 0.20	0.59 ± 0.02	−29.76**
Post-flowering	0.56 ± 0.06	0.42 ± 0.13	−25.00**
Intercellular CO_2 (ppm)			
Pre-flowering	350.23 ± 18.04	362.86 ± 37.72	+3.60^{NS}
Flowering	372.86 ± 15.70	367.50 ± 4.56	−1.43^{NS}
Post-flowering	379.62 ± 5.30	374.62 ± 26.16	−1.31^{NS}
Chlorophyll *a* (mg g^{-1} fr. wt.)			
Pre-flowering	0.38 ± 0.008	0.39 ± 0.01	+2.63^{NS}
Flowering	0.47 ± 0.006	0.43 ± 0.01	−8.51**
Post-flowering	0.28 ± 0.008	0.23 ± 0.01	−17.85**
Chlorophyll *b* (mg g^{-1} fr. wt.)			
Pre-flowering	0.31 ± 0.0012	0.31 ± 0.005	0.00^{NS}
Flowering	0.42 ± 0.0014	0.36 ± 0.004	−14.28**
Post-flowering	0.25 ± 0.0012	0.21 ± 0.008	−16.00**
Carotenoids (mg g^{-1} fr. wt.)			
Pre-flowering	0.25 ± 0.0012	0.26 ± 0.001	+4.00*
Flowering	0.31 ± 0.0024	0.29 ± 0.001	−6.45**
Post-flowering	0.19 ± 0.0012	0.16 ± 0.002	−15.78**
Total chlorophyll (mg g^{-1} fr. wt.)			
Pre-flowering	0.67 ± 0.000	0.68 ± 0.000	+1.49^{NS}
Flowering	0.86 ± 0.000	0.75 ± 0.000	−12.79**
Post-flowering	0.51 ± 0.002	0.43 ± 0.002	−15.68**

**, Significant at 1% level; *, Significant at 5% level; NS, Non-significant.

Inhibition of photosynthesis may lead CO_2 to accumulate in the substomatal cavity, sometimes prompting stomatal closure (Atkinson and Winner, 1987; Veljovic-Jovanovic et al., 1993). In the present study, however, the decreased SD may have been the reason for the low photosynthetic rate in the treated plants.

SO_2 stress causes photosynthetic pigment contents to decline in a variety of angiosperm species (Iqbal et al., 2000). Superoxide radicals may form when sulfites react with chlorophyll under illumination (Shimazaki et al., 1980; Williams and Banerjee, 1995). In SO_2-affected tissue, intracellular reactions at pH 2.2 to 3.5 may degrade chlorophyll molecules into photosynthetically inactive phaeophytin molecules (Rao and LeBlanc, 1966). In contrast, at pH > 3.5, SO_2 can affect the thylakoid membrane of the chloroplast (Peiser and Yang, 1978).

In general, the leaf morphology in our *C. intybus* plants was most affected by sulfur dioxide treatment in the pre-flowering stage, whereas pigment contents were maximally reduced at the post-flowering stage. These dissimilar responses may be indicative of a differential plant potential to resist pollution stress at various developmental stages.

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