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Ontogenetic changes in foliar features and psoralen content of *Psoralea corylifolia* Linn. exposed to SO_2 stress

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Abstract: *Field-grown Psoralea corylifolia plants were exposed to 0.5 ppm and 1.0 ppm concentrations of sulphur dioxide gas and sampled for observation at the pre-flowering, flowering and post-flowering stages of plant ontogeny. One ppm SO_2 concentration caused a significant decline in leaf number and leaf area per plant, total leaf dry weight, and the size and amount of midrib vasculature. The density and size of stomata decreased and many stomata were damaged. Interestingly, new epidermis developed over some of the damaged leaf stomata, thus showing a unique defence strategy against SO_2 stress through dedifferentiation of the epidermal cells. Decline in the concentrations of leaf chlorophylls and carotenoids in treated plants were up to 20% and 29% respectively. Stomatal conductance, intercellular CO_2 content and net photosynthetic rate lowered by over 52%, 20% and 35%, respectively, under the SO_2 stress. Concentration of psoralen, a basic linear furanocoumarin known for its use in the treatment of dermal diseases, was highest (5.32%) in seeds and lowest (0.28%) in roots. It was heavily reduced in SO_2 treated plants, the maximum decline occurring in seeds (86.70%) and leaves (56.27%). In the roots and shoots of the treated plants, it was low in pre-flowering stage, compared with the control, but showed a recovery during the post-flowering phase of plant growth.*

Key words: *Leaf vasculature, Photosynthesis, Psoralea corylifolia, Psoralen content, Sulphur dioxide*
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Introduction

Plant distribution, all over the globe, is dependent on the mode of interaction of plants with their surrounding environment, which in turn depends on the type of environment and the degree of sensitivity or resistance of plants to the environmental stress (Dwivedi and Tripathi, 2007; Tripathi and Gautam, 2007). Sulphur, an essential element for all living plants, is taken up by plants in the form of sulphate from the soil through roots. Additional sulphur, if required, can be obtained by plants from the atmosphere, mostly in the form of sulphur dioxide (SO_2), through leaf stomata (Khan *et al*., 2006). In the urban areas, atmospheric SO_2 level normally varies from 0.05 to 0.5 ppm but in the vicinity of SO_2 sources such as thermal power plants, it goes high and may exceed 2 ppm (Wali *et al*., 2007). High SO_2 concentrations are phytotoxic and disturb stomatal behavior, photosynthesis, transpiration, and formation of secondary metabolites (Agrawal, 2003; Wali *et al*., 2004). In SO_2-exposed plants, sulphur accumulation occurs mainly in the aerial parts through open stomata on leaves (Iqbal *et al*., 2005; Mandal, 2006). In the mesophyll, SO_2 readily dissolves in aqueous phases thereby forming sulphurous acid with dissociation products as sulphite, bisulphite and protons (Rennenberg and Polle, 1994; Rennenberg and Herschbach, 1996). The sulphite and bisulphite anions are phytotoxic.

Babchi (*Psoralea corylifolia* Linn.), a leguminous, bluish-purple or yellow flowered, annual herb (Fig.1), is used extensively in the Indian systems of medicine (Ayurveda and Unani) for curing dermal diseases such as leucoderma, leprosy and psoriasis. Seeds or fruits are the main plant part used for therapeutic purposes. The fruits are also known to be laxative, aphrodisiac, anthelmintic, anti-inflammatory and diuretic in febrile condition (Anonymous, 1989). Several secondary metabolites like furanocoumarins (psoralen and isopsoralen), flavonoids (bavachin, bavachinin, bavachalcone, psoralidin and their isomers) and a monoterpene phenol (bakuchinol) accumulate in seeds. Oral administration of acetone-extract of babchi seeds promotes bone calcification (Miura *et al*., 1996). Leaves are used in diarrhoea and roots are useful in the caries of teeth (Anonymous, 1989).

Now that much attention is being paid to the cultivation of medicinal plants and the yield of their active ingredients, it is desirable to know the impact of environmental stresses on the growth and yield of this taxa. The present study investigates the effects of different SO_2, concentrations representative of a polluted atmosphere, on growth parameters, photosynthetic efficiency and psoralen content of the babchi plant.

Materials and Methods

Plant raising, fumigation and sampling: Babchi seeds, obtained from the Indian Agricultural Research Institute, New Delhi, were sown 20 cm apart in rows separated by 30 cm from each other in different beds in the Hamdard University Campus. The soil of the site was light-textured sandy loam with a 7.2 pH and a 0.207 dS m^{-1} electrical conductivity. The levels of available nitrogen and sulphur in the soil were 51 ppm and 7.9 ppm, respectively.

The seedlings were allowed to grow undisturbed, with due watering and manuring, for 60 days after seed sowing. Sixty-day-old plants were fumigated, 2 hr in the morning, for four consecutive days in the month of July (at 34°C mean temperature and 64%

Fig. 1: A branch of *P. corylifolia* showing leaves and flowers

relative humidity). SO_2 (0.5 ppm and 1 ppm) was released separately from a gas cylinder, through a tube, into an especially designed fumigation chamber over the plots bearing the young babchi plants, and was controlled by a regulator. The plants growing on one of the plots were left un-fumigated and served as the control. Five plants from each of the control and the fumigated plots were collected at pre-flowering stage (20 days after fumigation), flowering stage (80 DAF) and post-flowering stage (140 DAF). Fully developed healthy leaves were selected for measuring the morphological, anatomical, functional and biochemical foliar characterstics. The experiment was repeated in field conditions for three consecutive years. The data obtained on different parameters in three successive years did not show any significant mutual variation. These were, therefore, pooled for obtaining the mean values presented in this communication.

Leaf morphology: The area and dimensions of detached leaves were measured using LI-3000A portable leaf area meter (LI-COR, Lincoln, USA) based on rectangular approximation method to provide 1 mm^2 resolution. The leaves were passed, one after the other, through the LI-3050A Transparent Belt Conveyer for their measurement. After each measurement, values were added to a secondary summing register so as to collect the accumulated leaf area data needed to estimate the whole plant leaf area. To find out the biomass of the leaf, the samples from treated and untreated plants were oven-dried separately at 80°C for 48 hr. The dry weight was determined on a digital balance.

Microtomy and microscopy: Fully expanded leaves collected from the control as well as treated plants were fixed in FAA (formaldehyde 10 ml, acetic acid 10 ml, 50% ethyl alcohol 90 ml) and transferred into 70% alcohol (V/V) after a week. To obtain the epidermal peels, pieces of about one cm^2 cut out from the region between midrib and leaf margin around the middle part of the leaf lamina were taken with 70% HNO_3 in a test tube and heated gently until the epidermal peels were separated from the mesophyll tissue (Ghouse and Yunus, 1972). The peels, washed with tap water, were stained with safranin and mounted in Canada balsam for microscopic study. Cross-sections (10 μm thick) of the leaf were obtained on a Reichert's sliding microtome with the help of potato pith; these were processed in the customary ethanol series for dehydration, stained with Heidenhain's haematoxylin and safranin and mounted in Canada balsam for study under microscope. Cell dimensions were measured with an ocular micrometer scale fitted in a compound microscope. Stomatal density on leaf epidermis was estimated by counting the number of stomata per microscopic field under 5x10 magnification. Photomicrographs were obtained with the help of an Olympus VENOX AH2 microscope with photographic attachment.

Chlorophyll content and photosynthetic efficiency: Following Hiscox and Israelstam (1979), the vials containing fresh leaves (0.1 g), washed with distilled water and dipped in 7 ml dimethyl sulphoxide (DMSO), were kept in oven at 65°C for 1.25 hr. One ml aliquot and 3 ml DMSO were taken in another tube and vortexed. Optical density was measured at wavelengths of 480, 510, 645 and 663 wavelength on a DU 640B Spectrophotometer (Beckman, USA). The chlorophyll and carotenoid contents were estimated by the formulae of Duxbury and Yentsch (1956) and MacLachlan and Zalik (1963), respectively.

Stomatal conductance (g_s), net photosynthetic rate (P_N) and intercellular CO_2 concentration (C_i) were measured *in situ*, between 10-11 AM on sunny days, by clamping fully expanded leaves in the leaf chamber (LI-6000-13, quarter liter) of a portable LI-6200 photosynthesis system (LICOR, Inc., Lincoln, USA) which measured the transient (<1min) exchange rates of H_2O vapour and CO_2 in a closed system, as described earlier (Wali *et al*., 2007)

Extraction and isolation of psoralen: One gram powder of dried leaves, stem, root and seeds each was extracted separately in chloroform. Following the method of Innocenti *et al*. (1977), the extracts were dissolved in chloroform, loaded on an analytical silica gel plate (Silica 60 G) and run in chloroform (the developing solvent) to isolate psoralen (Rf-0.40). The areas corresponding to psoralen were eluted separately for different plant parts with ethyl alcohol. The alcoholic solutions thus obtained were centrifuged, filtered and utilized for spectrophotometric determination. The UV-absorption spectra of the compounds were recorded on a Beckman DU 640B Spectrophotometer. An internal standard was used during psoralen extraction to control for losses. The band isolated from TLC was checked chromatographically for purity before doing spectroscopy.

The data obtained were analyzed statistically to determine the degree of authenticity of the results. Variations caused by 0.5

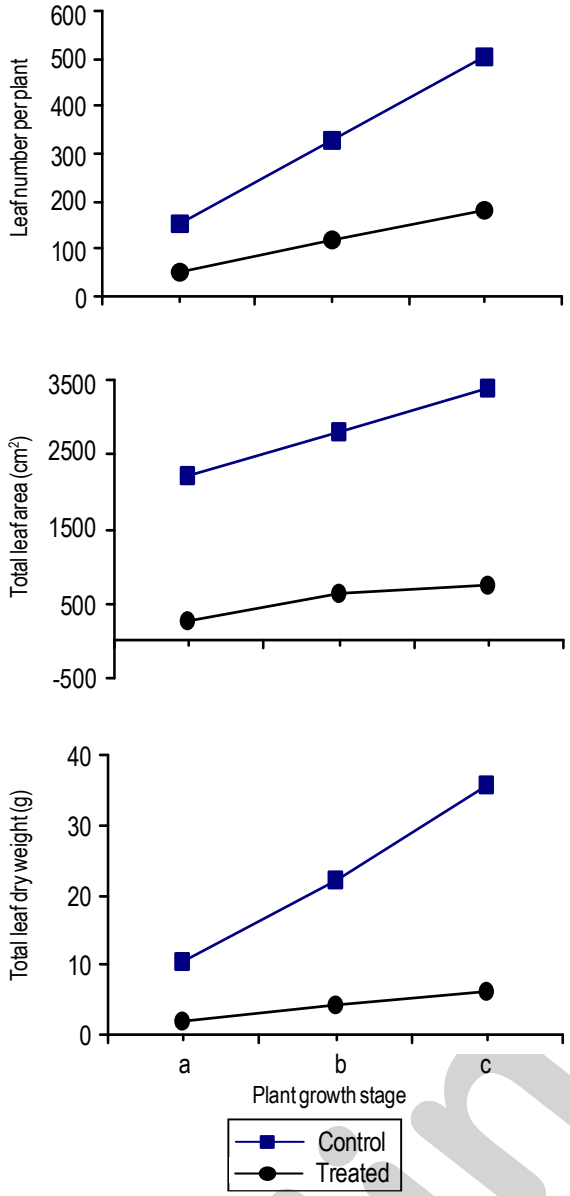

Fig. 2: Variation in foliar density, leaf area and leaf dry weight per plant in the control as well as SO_2 (1ppm)-treated *P. corylifolia* plants as observed at the (a) pre-flowering, (b) flowering and (c) post-flowering stages of plant growth. Values are the mean of five sets of readings obtained each of the 3 years of study

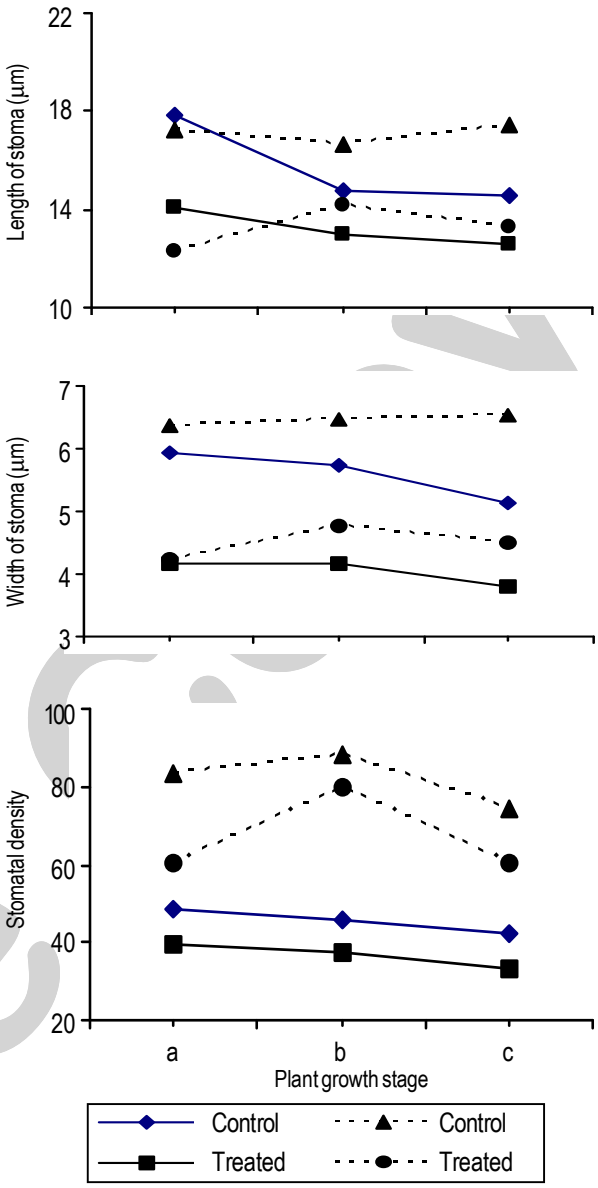

Fig. 3: Variation in some stomatal characterstics of both surfaces of leaf of the control and the SO_2 (1 ppm)-treated *P. corylifolia* plants as observed at the (a) pre-flowering, (b) flowering and (c) post-flowering stages of plant growth. Values are the mean of 25 x 3 independent readings. Continuous lines relate to the adaxial surface and interrupted lines to the abaxial surface

ppm SO_2 were largely insignificant (and hence not described in this report), whereas those due to 1.0 ppm SO_2 stress were significant and have formed the basis of the present discussion.

Results and Discussion

Foliar morphology and anatomy: In the present study, low SO_2 stress (up to 0.5 ppm) did not cause a considerable change in any of the parameters studied. This could be because the soil of the experimental site was basically S-deficient. A more or less similar effect of this concentration of SO_2 has been reported for *Calendula officinalis* (Wali *et al*., 2007). However, 1 ppm SO_2 fumigation made a difference. Compared with the control, number of leaves per plant was significantly less in each sampling of plants fumigated with 1 ppm SO_2. The reduction in leaf number was maximum (66%) at pre-flowering stage, and slightly less (64%) in later stages of plant growth (Fig. 2). The average leaf size and the total leaf area per plant showed a similar decline. Leaf width decreased more significantly in the early (pre-flowering and flowering) stages, whereas leaf length did so in the last two stages (data not given). The reduced leaf area, up to a maximum of 87% (in the pre-flowering stage), is indicative of

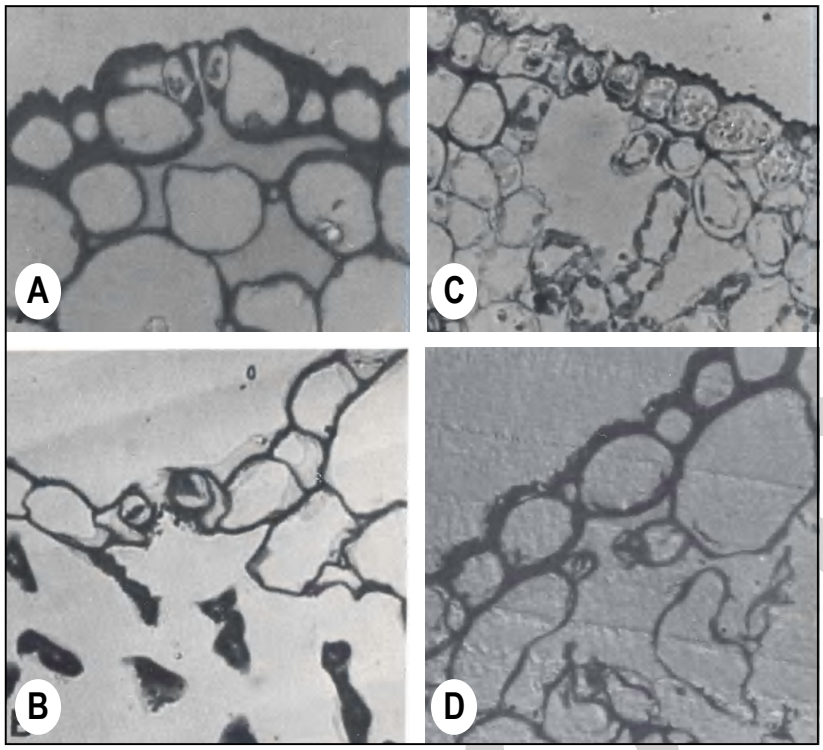

Fig. 4: Partial views of vertical sections of *P. corylifolia* leaves showing degeneration of stomata and the mesophyll tissue below stomata in the treated plants. 'A' shows healthy epidermal and mesophyll cells in the control, whereas 'B' and 'C' exhibit varied degree of damage to stomata and hypostomatal tissue due to heavy SO_2 stress. Formation of a new layer of epidermal cells above the degenerated stomata can be seen in 'D' (All at X 200)

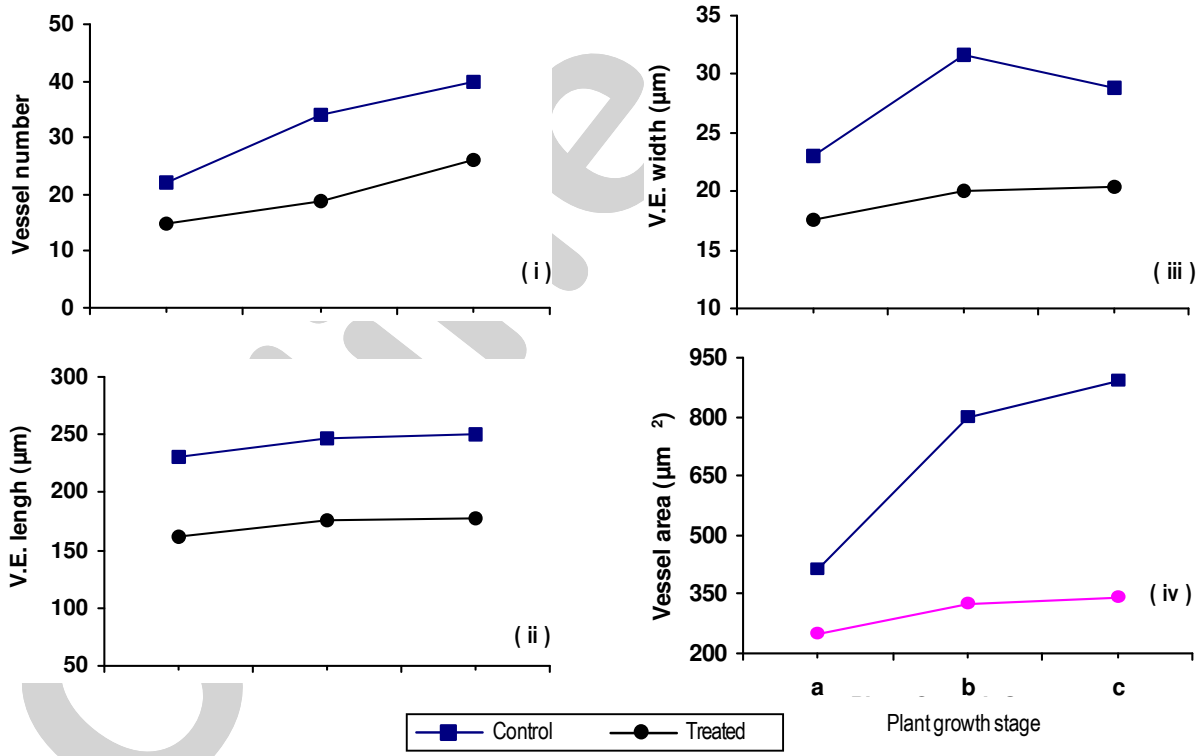

Fig. 5: Variation in the length and width of vessel elements and in the number and transectional area of vessels in the mid-rib xylem of leaves of the control and the SO_2 (1 ppm)-treated *P. corylifolia* plants as observed at the (a) pre-flowering, (b) flowering and (c) post-flowering stages. Values are the mean of 250 x 3 independent readings

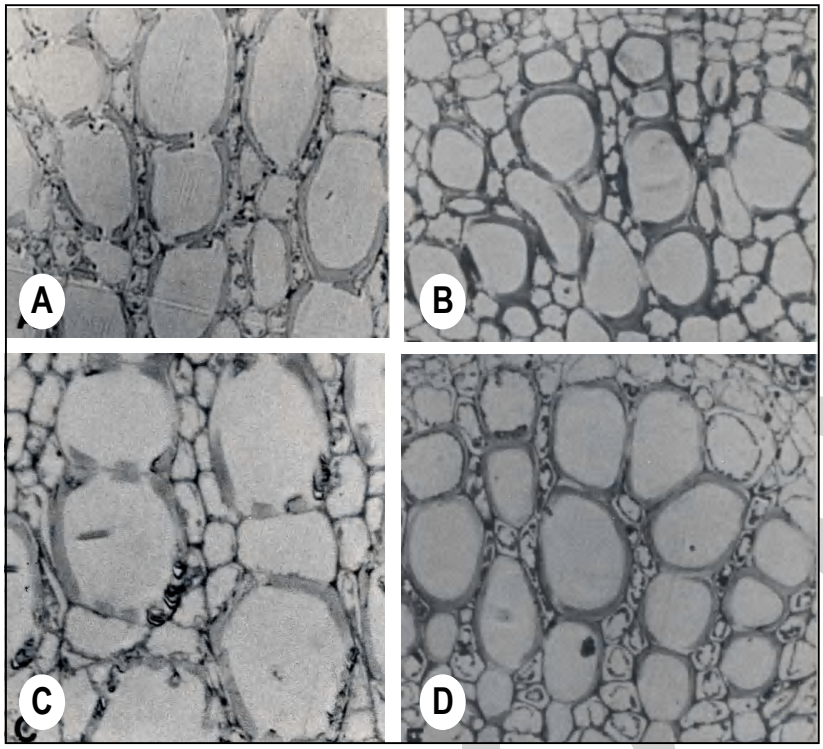

Fig. 6: Vertical sections of *P. corylifolia* leaves passing through the midrib. 'A' and 'C' show the xylem tissue of control plants in pre-flowering and post-flowering phases, respectively, whereas 'B' and 'D' represent the corresponding stages in SO_2 (1 ppm)-treated plants. Vessels are distinctly narrow in the treated samples; lignification is denser and the vessels to parenchyma ratio lower than in the control (All at X 200)

growth retardation or possibly a morphological adaptation to the stressful environment. In *Achyranthes aspera*, on the contrary, leaf size was found to increase considerably under the stress of smoke pollution (Dhir *et al.,* 1999). The total leaf dry weight in Babchi plants increased with plant age, but kept significantly low in the treated plants, as compared with the controls (Fig. 2). Variations were inconsistent and non-significant with 0.5 ppm SO_2. However, with 1 ppm concentration dry weight declined, showing a maximum decline (82%) in the post-flowering stage, which could be partly due to a bulk conversion of vegetative shoots into reproductive ones.

In the control plants, stomatal pore size and stomatal density on the adaxial leaf surface gradually decreased with the age of the plant. On the abaxial surface, pore dimensions remained nearly constant with growing age of the plant, but stomatal density increased till flowering and declined in the post-flowering stage. Both the parameters were significantly lower in the SO_2 (with 1 ppm)-treated plants than in the control, at each stage of plant growth (Fig. 3). The reduction in pore size was maximum in pre-flowering stage on both leaf surfaces (Fig. 3).

Low doses of SO_2 normally increase the stomatal aperture and hence the stomatal conductance, whereas high doses may close stomata (Majernik and Mansfield, 1970; Black and Black, 1979). In the present study, 0.5 ppm SO_2 did not cause any noticeable difference in aperture size but 1 ppm did. Suppression of stomatal opening and/or closure of stomata under high SO_2 concentrations or prolonged fumigations often involve accumulation of SO_2 in the sub-stomatal cavity (Cao, 1989; Dhir *et al*., 2001). The C_i may progressively increase while P_N declines by the increased SO_2 level, as observed in *Pelargonium zonale* (Veljovic-Jovanovic *et al*., 1993). Transpiration rate also declines, as reported for hydroponically grown pea plants and barley seedlings fumigated with SO_2 (Kaiser *et al*., 1993).

In the present study, the stomatal apparatus and the mesophyll cells were severely damaged and/or degenerated in the samples treated with 1 ppm SO_2, as shown in Fig. 4. The treatment caused a heavy damage to mesophyll; it was rather a shock to some of the cells below stomatal cavity as they were completely degenerated (Fig. 4B, C). Interestingly, however, in some developing leaves a layer of new epidermal cells was formed (Fig. 4D) over the damaged stomata during the subsequent phase of leaf growth. SO_2 may cause injury to all tissues of the leaf including the vascular tissues (Evans and Miller, 1975). High concentrations of SO_2 in the vicinity of smelters caused a greater damage to sub-stomatal mesophyll cells than to epidermal cells in some forest plants (Holopainen *et al*., 1992; Kukkola *et al*., 1994); an observation conforming to our present findings that despite having the epidermal cells intact some mesophyll cells were severely damaged in the pre-flowering stage of *P. corylifolia*. In later stages of plant growth, stomatal cells died and, curiously enough, new epidermal cells formed over the damaged stomata in some portions of the injured leaves. This was a unique protective measure adopted by the affected leaves, showing that some of the epidermal cells dedifferentiated to regain the meristematic potential and re-form

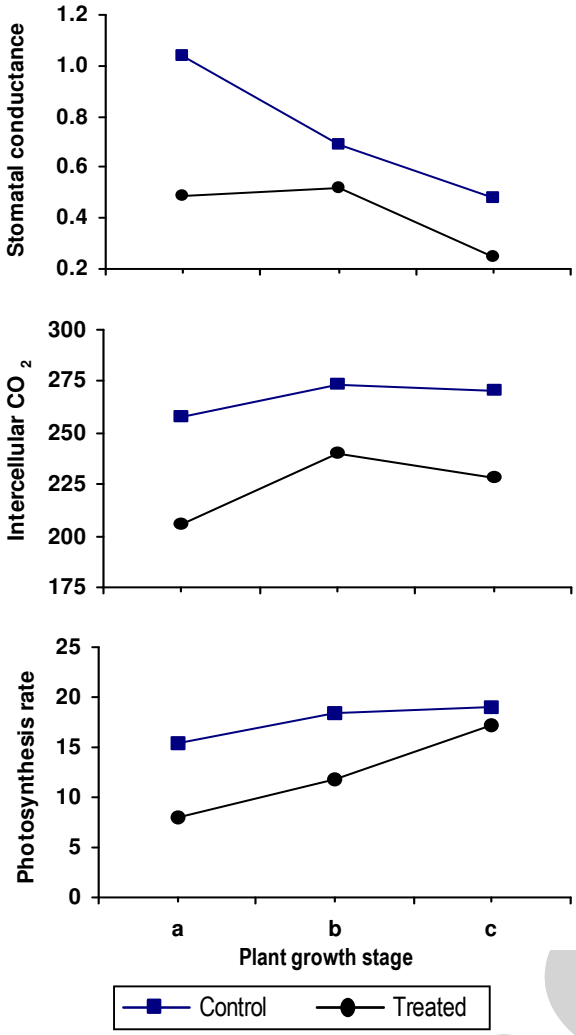

Fig. 7: Variation in stomatal conductance (mol m^{-2} s^{-1}), intercellular CO_2 (ppm) and the net photosynthetic rate (µmol m^{-2} s^{-1}) in the control and the SO_2 (1 ppm)-treated *P. corylifolia* plants as observrd at the (a) pre-flowering, (b) flowering and (c) post-flowering stages of plant growth. Values are the mean of 25 x 3 independent readings

a protective layer over the damaged tissue, a phenomenon hardly seen in earlier studies of foliar responses to environmental toxicity.

Figure 5 provides interesting data on leaf vasculature in relation to SO_2 stress. The number of vessels in midrib xylem, the length and width of vessel elements, and the cross-sectional area of midrib vessels were observed to increase with plant age. All these characters experienced a significant reduction in the SO_2 treated plants (Fig. 5). The degree of the vessel: parenchyma ratio declined in the treated material. These observations conform to the findings of Evans and Miller (1975). In the stem of *Calendula officinalis,* Wali *et al.* (2007) found a decrease in vessel diameter and density but an increase in the length of vessel elements under the influence of 1 ppm SO_2. Vessel-wall lignification was relatively denser in the treated samples than in the control (Fig. 6). This could be an adaptive defence strategy to provide strength to the reduced vasculature with fewer vessels.

Pigments and photosynthesis: Concentration of green pigments in the leaf continued to increase till the flowering, and declined afterwards in the control babchi plants (Table 1). Formation of chlorophyll was almost unaffected under the influence of 0.5 ppm SO_2 but markedly affected by 1 ppm treatment till the flowering stage. Concentration of chlorophyll *b* declined more severely than that of chlorophyll *a* during the pre- and post-flowering stages. The carotenoid content varied more or less like chlorophyll *a*, thus showing a highly significant sensitivity to SO_2 stress during the pre-flowering and flowering phases than in the later phase of plant life (Table 1).

Dorries (1932) proposed that SO_2 causes a local acidity in the chloroplasts, which splits Mg from chlorophyll converting it into pheophytin. This pheophytin, accumulated at the expense of chlorophyll, causes chlorosis and reduces photosynthesis. Chlorophyll content of leaves may be affected even by low concentrations of SO_2, if the release of the gas is constant (Gupta and Ghouse, 1987). Chlorophyll concentration in wheat plants grown in open top chamber declined as much as about 60% after exposure to 226 mg m^{-3} of SO_2 (Davieson *et al*., 1990). Carotenoids are sometimes more sensitive than chlorophyll, as was observed in *Oryza sativa* (Agrawal *et al*., 1982). In the present study, sensitivity of carotenoids to SO_2 far exceeded that of chlorophyll till the flowering stage, but a reverse situation marked the post-flowering phase. About one fourth of carotenoid content in the leaves of the treated plants had been lost till flowering, the degree of loss being considerably less in the post-flowering phase.

Figure 7 depicts physiological events related to carbon assimilation such as stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and rate of photosynthesis (P_N). The g_s decreased with the growing plant age. It was significantly low under SO_2 stress, the maximum decline occurring in the pre-flowering stage. The C_i, maximum in flowering stage, was significantly and consistently lower in SO_2 affected plants than in the control. The P_N increased with plant age and was lower in the treated plants than in the control throughout the plant ontogeny. The extent of decline varied from about 10-48%, with the maximum occurring at the pre-flowering stage and the minimum at the post-flowering stage.

SO_2 can affect the metabolic intermediates of photosynthesis and the photosynthetic electron transport. High SO_2 concentrations are known to inhibit the Calvin cycle enzymes (Tanaka *et al*., 1984). Recovery occurs through detoxification mainly due to chloroplast reactions (Veljovic-Jovanovic *et al*., 1993). P_N may decrease with increasing age of the needle/leaf and/or the rate of SO_2 deposition (Meng *et al*., 1995; Dhir *et al*., 2001). In our study, P_N was always lower in treated materials than in the control, the maximum difference figuring in early stages of plant development. In a recent study, however, the maximum decline in P_N has been reported in later stages of plant growth (Wali *et al*., 2007).

Table - 1: Pigment concentration (mg g^{-1} fresh weight) in the control and the SO_2 (1 ppm)-treated plants of *P. corylifolia* at different developmental stages. Values are the mean of 30 independent readings (5 plants x 2 replications x 3 years)

Parameters	Control mean ± SD	Treated mean ± SD	Percent decline
Chlorophyll *a*			
Pre-flowering	03.18 ± 00.17	02.88 ± 00.12	09.43 *
Flowering	03.92 ± 00.50	03.13 ± 00.14	20.15 **
Post-flowering	02.38 ± 00.16	02.19 ± 00.38	07.98 NS
Chlorophyll *b*			
Pre-flowering	01.84 ± 00.08	01.53 ± 00.10	16.85 **
Flowering	02.29 ± 00.18	01.87 ± 00.15	18.34 **
Post-flowering	01.41 ± 00.22	01.13 ± 00.18	19.85 **
Carotenoid			
Pre-flowering	02.50 ± 00.34	01.89 ± 00.06	24.40 **
Flowering	02.91 ± 00.34	02.07 ± 00.13	28.86 **
Post-flowering	01.33 ± 00.09	01.27 ± 00.16	04.51 NS

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

Table - 2: Psoralen concentration (%) in different parts of *P. corylifolia* plants grown in the natural and the SO_2 (1 ppm)-stressed atmospheric conditions. Values are the mean of 30 readings (5 plants x 2 replications x 3 years) per year

Organ / Stage	Control plants mean ± SD	Treated plants mean ± SD	Percent variation
Leaf			
Pre–flowering	0.85 ± 0.01	0.44 ± 0.01	48.35**
Flowering	0.98 ± 0.01	0.53 ± 0.05	46.03**
Post–flowering	0.72± 0.04	0.32 ± 0.01	56.27**
Stem			
Pre–flowering	0.46 ± 0.02	0.26 ± 0.03	43.44**
Flowering	0.54 ± 0.01	0.48 ± 0.01	11.11**
Post–flowering	0.28 ± 0.01	0.32 ± 0.03	08.53NS
Root			
Pre–flowering	0.27 ± 0.03	0.12 ± 0.01	35.63*
Flowering	0.34 ± 0.02	0.39 ± 0.01	16.96**
Post–flowering	0.29 ± 0.02	0.43 ± 0.02	48.43**
Seed	5.32 ± 0.62	0.71 ± 0.15	86.70**

* = Significant at 1%, ** = Significant at 5%, NS = Non-significant

Psoralen content: SO_2 interacts with plant metabolism, growth and yield, and may affect production of secondary metabolites. The chromatographic and spectrophotometric estimations revealed that seeds contain highest amount of psoralen, the leaves standing the second. SO_2 treatment caused a significant reduction (87%) in the psoralen content of seeds, whereas leaf psoralen content declined by 48%, 46% and 56% during pre-flowering, flowering and post-flowering stages respectively. The psoralen content in the stem was reduced significantly in the pre-flowering (43%) and flowering (11%) stages, but later showed a slight increase, compared with the control. In roots also, there was a significant reduction (36%) in the pre-flowering stage; albeit a significant increase was observed during the subsequent stages (Table 2).

Since the structural damage is often followed by the deposition of phenolic compounds or the lignification of cell walls around the damaged area, the production of secondary compounds in stressed plants may relate to the degree of cellular damage. The damage may induce biosynthesis or transport of carbon- or nitrogen-based secondary metabolites in the undamaged portions (Coleman and Jones, 1991).

Babchi plants have a symbiotic relationship with N-fixing bacteria. Bacterial nodules were abundantly present on the roots of control plants, while their number was markedly reduced in SO_2 treated plants. N deficiencies reduce the production of N-based alkaloids and increase the formation of C-based compounds (Gershenzon, 1984). Psoralen, being a C-based molecule, might accumulate heavily in the roots of the stressed plants in accordance with the above generalization. Further, the explanation for the increased psoralen content of roots of the treated plants during late stages of plant development, as observed in the present study, may lie in that the photosynthate produced at late stages of plant ontogeny is little consumed in the formation of new structures and may therefore

adopt the secondary metabolic pathway, with the ultimate product being transported to and stored finally in the roots.

Production of linear furanocoumarins including psoralen is influenced by changes in the environment and can be induced by environmental stresses including the acidic atmospheric condition (Dercks *et al*., 1990). Elevated CO_2 in the atmosphere may increase psoralen content in some species and decrease in others (Reitz *et al*., 1997). This shows that the carbon-nitrogen balance hypothesis, which predicts that increase in plant C-N ratio under elevated CO_2 condition ensures a greater allocation of the plant's carbohydrate resources to secondary metabolism resulting in a greater production of C-based secondary chemicals, does not seem to hold with reference to psoralen production.

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