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Response of Petunia Plants to SO_2 and of Detached Leaves, Leaf Discs, and Callus to Sodium Sulfite[1]

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Abstract. **Flowering plants of 5 cultivars of petunia (*Petunia hybrida* Vilm.), exposed to 2.8 ppm SO_2 (16 hours) under controlled environmental conditions, exhibited variation in SO_2 sensitivity based on the degree of water-soaked necrotic leaf lesions. The range of SO_2 absorption rates was ± 14% of the mean for the 5 cultivars; however, there was a 2-fold difference in sensitivity among the cultivars, indicating that variation in SO_2 absorption plays only a limited role in determining the genetic differences involved in the reaction of petunia to SO_2. Detached leaves from all cultivars were equally sensitive to a 10 mM Na_2SO_3 solution. The injury response of leaf discs to Na_2SO_3 solutions was monitored by chlorophyll extracts. Although the cultivars varied in sensitivity, the ranking differed from that observed for whole plants to SO_2. Callus cultures grown on Murashige and Skoog (MS) medium + naphthaleneacetic acid (NAA) at 2.0 mg/liter + benzylamino purine (BA) at 0.5 mg/liter were treated with Na_2SO_3. Their viability, when evaluated by triphenyl tetrazolium chloride (TTC), yielded a sensitivity ranking of the same cultivars which differed from that obtained with both leaf discs and whole plants. No differences in Na_2SO_3 sensitivity were observed for callus cultures of the cultivars cultured on MS + 1.0 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D). The dissimilar injury responses to $SO_3^=/HSO_3^-$ in different tests on explants of the same cultivars indicate that at each level of plant organization sensitivity to Na_2SO_3 is determined by different morphological and physiological factors from those which specify whole plant reaction to SO_2.**

The culture and regeneration of plant cells provide the plant breeder with potential methods for the improvement of crop species by selection for specific traits at the cellular level. If undifferentiated cells accurately reflect whole plant status, then selection of cells in culture would be more efficient than that based on plant populations in the greenhouse or field. Correlations of the responses of whole plants and their cells *in vitro* have been reported for a few host-pathogen systems (11, 13, 23). Similarly, cell selection experiments have successfully led to the recovery of specific variants *in vitro*, e.g., resistance to aluminum (19), amino acid analogs (5, 22, 24), and disease resistance (9), herbicide resistance and pyrimidine base analogs (14, 18). Unfortunately, plants have not been regenerated from most of the previously mentioned selected cell lines, so it is not known whether the selected traits would have been expressed by their whole plants.

Regenerated whole plants of *Zea mays* L. derived from cells resistant to *Helminthosporium maydis* Race T. pathotoxin were resistant to the disease, but had lost their cytoplasmic male-sterility (10). Diploidized regenerated tobacco plants of haploid *N.*

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tabacum cells resistant to the methionine analog methionine sulfoximine were not only resistant to the analog but were also resistant to *Pseudomonas tabaci* (5).

Of 7 herbicide (picloram) resistant *Nicotiana tabacum* cell lines selected, 1 could not be regenerated and 2 others were unstable in their expression of resistance. Plants regenerated from 4 of the lines expressed resistance as a monogenic dominant (6).

The objective of the research reported here was to determine the feasibility of an *in vitro* system for the selection of sulfur dioxide (SO_2) resistance in *Petunia hybrida*. *P. hybrida* was chosen because: 1) tissue culture techniques including callus induction and routine plant regeneration have been accomplished for both haploids and diploids (1,2); 2) SO_2 sensitive and non-sensitive cultivars have been identified and their physiology examined (7,8).

Materials and Methods

Plant material. Five petunia cultivars were used and their response on a whole plant basis to 2.5 ppm SO_2 according to Feder et al. (8) and seed source are shown in Table 1.

Plant culture. Seeds of the 5 cultivars were germinated on VSP (Bay Houston Towing Co., 55% sphagnum peat, 35% vermiculite, 10% perlite) planting medium under 16 hr of 80 $\mu E\ m^{-2}s^{-1}$ light (G.E. Cool White fluorescent tubes); after 2 weeks transplanted to AC 6/8 Cell Paks (G. J. Ball Co.) using the same planting medium. The plants were grown for 4 weeks under natural photoperiod (February to June, 1980) in a greenhouse supplemented with 680 $\mu E\ m^{-2}s^{-1}$ light (G.E. Cool White fluorescent tubes) for 10 hr daily at a minimum 25°C day-night temperature. The plants were fertilized at each watering with a 150 ppm N aqueous solution with the pH adjusted to 6.5 with phosphoric acid. Subsequently, the plants were transplanted into 300-ml plastic pots and grown at a minimum 25°C temperature under natural photoperiod conducive to flowering (March 15 to June, 1980) in the greenhouse, and watered with 0.5 Hoagland solution.

Plant fumigation. Plants, 12–16 weeks old with 2–3 flowers per main stem and lateral branches, were fumigated with SO_2 in the chamber system described by Bressan et al. (3). Fumigations were done at 2.8 ppm for 16 hr at 50% relative humidity under 60–90 watts m^{-2}. Plants from the same sowing date were fumigated on successive days to minimize plant age influence. Plants were evaluated for SO_2 sensitivity 72 hr after fumigation. Each leaf was detached at the petiole base with a razor blade and the percent necrotic leaf area was determined by visual estimation. Leaf area was measured by a Li-Cor Portable Area Meter (Model No. L1-3000). From these data total percent necrotic leaf area per plant was calculated.

Detached Leaf. Detached third and fourth fully expanded leaves from the main stem of 55–60 day old seedlings of the 5 cultivars were floated on either a control solution of 20 mM NaCl at pH 4–5.0 or 10 mM Na_2SO_3 at an initial pH of 5.0–5.4. After 24 hr in the light, leaves were rinsed and placed on moist filter paper in Petri dishes for 24 hr to allow symptom development. The injury symptoms were water soaking with loss of turgor and necrotic lesions. Each rating was a visual determination of the approximate percent of damaged leaf area. Four leaves per cultivar were floated simultaneously and their necrotic values were averaged. The treatments were applied 4 times, using fresh leaves each time, and the data were analyzed using analysis of variance for a randomized complete block design with blocks over time (15).

Leaf discs. Ten leaf discs (5.0 mm) from the 2nd through 5th fully expanded leaf of the main stem of the 5 cultivars were placed in a control solution of 20 mM NaCl at pH 4.0–4.5 or 2.5, 5.0, or 7.5 mM Na_2SO_3 at pH 5.0–5.5 and placed in diffuse light (40 lux). After 3 hr, leaf discs were rinsed twice with distilled water and returned to their respective Petri dishes containing 10 ml of deionized distilled water at 800 lux for 16 to 20 hr. The 10 leaf discs were combined, and chlorophyll was extracted with 80% ethanol. Chlorophyll in the 80% ethanol extracts was measured spectrophotometrically at 665 nm. The raw data were standardized by dividing the values of the treatments by the value of the controls for each cultivar. The treatments were applied at 3 different times to provide blocks, and the analysis of variance was computed for a randomized complete block design with 15 treatments: 5 cultivars and 3 $NaSO_3$ concentrations.

Callus cultures. Two culture media based on MS salts (20) and vitamins of Nitsch and Nitsch (21) containing 30 g/liter sucrose and 8 g/liter agar were used for callus initiation and growth. In addition, medium designated MSNB had 2.0 mg/liter NAA and 0.5 mg/liter BA, whereas medium MSD had 1.0 mg/liter 2,4-D. For callus initiation, seeds of the 5 cultivars were sterilized with 0.5% NaOCl for 1 to 2 min, rinsed 4 times with sterile distilled water and placed on MSNB and MSD in 100 × 15 mm Petri dishes and wrapped with Parafilm. The seeds were germinated under 20 $\mu E\ m^{-2}s^{-1}$ (G.E. Cool White fluorescent tubes). Stem and leaf explants from the seedlings were placed on MSNB and MSD where callus formation occurred in the dark at 28°C within 3 weeks and was routinely subcultured thereafter twice a month. Triphenyl tetrazolium chloride (TTC) at a concentration of 0.5% was used as a vital stain for callus exposed to the Na_2SO_3 solutions. The TTC solution was filter-sterilized (0.22 μm pore size) and 5 ml was added to each Petri dish 24 hr later. The efficacy of TTC stain in measuring callus viability was determined by correlating callus growth rate and TTC scoring of control to Na_2SO_3 treated callus. First, the relationship of fresh to dry weight of the

Table 1. Plant response of 5 petunia cultivars to fumigation with SO_2.

Cultivar	Source	Plant injury[z] (%)	No. plants fumigated	Leaf injury[y] (%)	Mean leaf area (cm^2)	Mean SO_2 absorbance (nl/cm^2) ± SD
Calypso	Pan American	26	5	35 a	601	687 ± 103
Cherry Blossom	Pan American	27	4	17 c	655	587 ± 129
Victory	Harris	32	5	19 c	692	663 ± 54
Warrior	Harris	42	6	17 c	811	544 ± 50
Lilac Time	Harris	20	6	27 b	850	540 ± 143

[z]Feder et al. (8) Mean value for 3 levels of SO_2 exposure.
[y]Mean separation by Duncan's multiple range test, 5% level.

callus tissue was determined (r = 0.981***, significant at 0.01%.). Subsequently, fresh weight was used directly to determine growth rate, and 2 sets of experiments on MSNB medium were conducted with 3 samples of callus of each cultivar. Two samples were stained with TTC and the 3rd was placed on fresh culture medium to determine the growth rate. Each callus sample was about 100 mg fresh weight when grown for 14 days. Growth rate was calculated as the ratio of the final to the initial weight.

Preliminary trials indicated that the sulfite solutions lost potency due to oxidation. Therefore, fresh solutions were prepared by filter sterilization. A 10 mM stock solution at pH 5.5 was prepared by dissolving 10 millimoles (1.25 g) of Na_2SO_3 in 500 ml of distilled water. The solution was simultaneously titrated and brought to volume by adding 500 ml of water containing 0.7 ml of concentrated HCl. Test solutions of 7.5 mM, 5.0 mM, and 2.5 mM Na_2SO_3 were made by dilution. At pH 5.5 the predominant ion is HSO_3^-.

The experimental design was a split-plot with Na_2SO_3 concentrations as the main plot and cultivars as sub-plots. Exposure of callus was carried out: 1) 2 samples of callus (about 100 mg) of each cultivar were placed on MSNB and MSD medium in 10.0 cm Petri dishes, 2) 5 days later each dish of callus was treated with 10 ml of either a control solution of 20 mM NaCl at pH 4.2 or with Na_2SO_3, 3) the solutions were removed after 3 hr and the callus rinsed with 10 ml of filter-sterilized deionized distilled water, 4) TTC staining and visual evaluation were performed as previously described. A scale from 0 to 10 on the basis of color development, 10 being the most viable was used. The rating values of the two callus pieces of each cultivar were averaged. The data were standardized by dividing treatment ratings by that of the controls multiplied by 10. There were 15 treatments: 3 Na_2SO_3 concentrations and 5 cultivars, and 6 blocks on MSNP medium and 7 blocks on MSD. Data were analyzed separately for each medium using analysis of variance for a split-plot randomized design.

Results

Plant fumigation. Plants fumigated with SO_2 exhibited typical water soaked areas followed by yellowing and browning within 72 hr. Irrespective of the extent of necrosis, the 5 cultivars exhibited injury in a similar pattern, not limited to interveinal areas. Immature leaves were primarily necrotic at the margins. The percent leaf necrosis varied among the 5 cultivars with 'Calypso' and 'Lilac Time' more sensitive than 'Cherry Blossom', 'Victory' and 'Warrior' which were non-sensitive and statistically comparable in their response (Table 1). The range in SO_2 absorbance was from 540 nl cm^{-2} for 'Lilac Time' to 687.2 nl cm^{-2} for 'Calypso'; considering the SD there were no differences in mean SO_2 absorbance.

Whole leaf. Injury induced by exposing detached whole leaves to 10 mM Na_2SO_3 solutions consisted of water soaking with loss of turgor, yellowing, and necrotic lesions. Variation among the 4 treatment applications was substantial (treatment variance/error variance was 25.2), but no differences were observed among the 5 cultivars tested (Table 2).

Leaf disc. The damage of leaf discs exposed to Na_2SO_3 solutions consisted of bleaching of the tissue to a tan color. Since the values given (Table 2) are chlorophyll absorbance, larger values represent less injury. Thus, 'Cherry Blossom' and 'Calypso' did not differ from each other, and were less sensitive than the other cultivars. Similarly, 'Calypso, 'Victory', and 'Warrior' were not different from each other and together with 'Cherry Blossom' were all less sensitive than 'Lilac Time'. The Na_2SO_3 effect on leaf discs was also concentration dependent as 2.5, 5.0 and 7.5 mM gave 54.2, 23.9 and 18.1 standardized absorbance values respectively (LSD 5% = 5.9; for all cultivars).

Callus cultures. Callus cultures of the 5 cultivars were readily established on MSNB and MSD media. Callus growth of some lines was faster on MSNB than on MSD and *vice-versa*, but was sufficient to subculture all cultures on the same day; thus providing uniformity in the conduct of experiments. There also were coloration and friability differences; most obvious was the constant brownish color of 'Lilac Time' on MSNB medium, but it still gave a purple coloration with the TTC test.

Correlation coefficients were calculated for growth rate with visual rating of TTC staining. Correlation using each raw datum value was highly significant (r = 0.638***). Correlation of raw data summed over blocks, over cultivars, and over cultivars + blocks was highly significant (r = 0.784**, 0.890**, and 0.994**, respectively). The correlation of each value standardized as percent of control was significant (r = 0.438* significant at 5% level). Therefore TTC staining was a quick, convenient and reliable means of operationally defining callus cell viability.

No interaction occurred between Na_2SO_3 concentration and cultivars. On MSNB, TTC values of 8.3, 4.8, and 1.9 were observed at 2.5, 5.0 and 7.5 mM Na_2SO_3 respectively (LSD 5% = 1.38); whereas on MSD they were 7.9, 3.3, and 0.5 for all cultivars (LSD = 1.3). The effect of the same Na_2SO_3 concentrations between the 2 media was not different by *t*-test.

Table 3 shows the comparison among cultivars within a medium. Since there was no interaction, cultivar values were averaged over blocks and Na_2SO_3 concentrations. Only 'Cherry Blossom' cultured on MSNB was less sensitive than the other cultivars, whereas no differences among cultivars occurred on MSD. Correlation coefficients were determined for the TTC ratings of the controls with the sum of the normalized ratings of the 3 treatments. When all blocks and all cultivars were analyzed individually the correlations were highly significant for MSNB (r =

Table 2. Response of detached leaves and leaf discs of 5 petunia cultivars to Na_2SO_3.

Cultivar	Detached leaves necrosis (%)	Leaf discs standardized absorbance (O.D. 665 nm)
Cherry Blossom	61.6	41.5
Calypso	51.6	34.3
Victory	63.1	32.2
Warrior	55.6	32.1
Lilac Time	65.6	20.2
LSD 5%	–	7.7

Table 3. Mean TTC viability rating combined for callus of 5 petunia cultivars on MSNB or MSD media exposed for 3 hr to 2.5, 5.0, or 7.5 mM Na_2SO_3.

Cultivar	Mean standardized viability MSNB	Mean standardized viability MSD
Cherry Blossom	7.29	3.55
Lilac Time	4.93	3.60
Victory	4.67	3.41
Calypso	4.66	5.38
Warrior	3.57	3.60
LSD 5%	1.40	–

0.560**) and MSD (r = 0.440**) analyzed separately, and highly significant if MSNB and MSD were analyzed together (r = 0.467***). Correlation coefficients were determined for means over blocks of controls with means of sums of normalized ratings for each cultivar. The correlation was significant on MSNB (r = 0.906*) where there was a concurrent significant difference among the means of the cultivars (Table 3). However, on MSD the correlation was not significant (r = 0.265) and there was a concurrent lack of differences among the cultivar means.

Graphs were plotted for the responses of leaf discs, callus on MSNP medium, and callus on MSD medium to the various concentrations of Na_2SO_3. The dose responses to Na_2SO_3 of material of these 2 levels of biological organization were similar (Fig. 1).

Discussion

The 5 cultivars employed in this study had previously been ranked on a whole plant basis with respect to SO_2 sensitivity (8; Table 1). In our SO_2 fumigations, 4 of the same cultivars exhibited comparable SO_2 responses, but we found 'Victory', previously classified as sensitive, to be resistant. Even though the sensitivity rankings were comparable, the range in percent leaf damage was about 10-fold lower in the previous study by Feder et al. (8) than we observed. The discrepancy in cultivar sensitivity may in part be due to the exposure conditions in that Feder et al. (8) used greenhouse exposure chambers, but herein they were conducted in a plexiglass chamber located in the laboratory.

Elkiey and Ormrod (7) found that SO_2 caused only slight increases in leaf diffusion resistance at both 50% and 90% relative humidity indicating a limited role for stomate activity in determining the SO_2 reaction in petunia. Our findings further substantiate this observation since on a whole plant basis, both sensitive and non-sensitive cultivars exhibited similar SO_2 absorbance rates. The SO_2 fumigated plants did not vary appreciably in leaf area which could have influenced SO_2 absorbance. Thus, either genetically determined internal morphological or physiological differences are operative, after presumed equal concentration

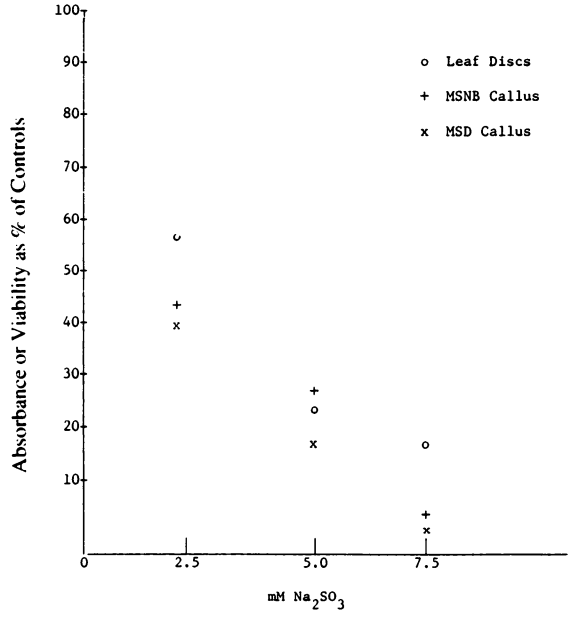

Fig. 1. The effect of Na_2SO_3 concentration on the mean standardized absorbance of chlorophyll extracts of petunia leaf discs and on the mean normalized TTC viability ratings of petunia callus cultures. Exposures were for 3 hr.

entry of SO_2 into leaf tissue, to control the SO_2 sensitivity response of petunia. Petunia thus differs markedly from most members of the Cucurbitaceae where differences in SO_2 absorption rate appear to be the primary determinant of cultivar resistance (3). Leaf discs of selected Curcubitaceae genera when floated in $KHSO_3$ solutions exhibited a sensitivity similar to that of the whole plants to SO_2, except for cucumber, which lead Bressan et al. (4) to conclude that after the stomate, other barriers such as biochemically determined cell membrane differences may control sensitivity. Their results on cucumber and those presented herein indicate that the genetic basis of SO_2 resistance is generally whole plant related and not always reflected by leaf, organ, or cultured, cells alone.

Detached leaves of petunia treated in Na_2SO_3 solution failed to exhibit the sensitivity differences among the cultivars anticipated from the ranking of whole plants. Environmental factors mainly influenced the Na_2SO_3 response as evidenced by the large block variance compared to the cultivar and error variances. However, the fact that the injury response of detached leaves, water soaking with loss of turgidity, chlorosis and necrosis, paralleled that of SO_2 treated plants provided an important link between the 2 methods of evaluation. There are 3 factors that may have obscured delineation of the cultivars by the detached leaf method: 1) the environmental variance, 2) the visual rating system in which equal emphasis was given to water soaked damage, chlorosis and necrosis; 3) the resistance mechanism(s) which became inoperative upon leaf detachment.

Experiments with leaf discs were more definitive than those with detached whole leaves since the results were objectively quantified by chlorophyll determination and found reproducible. A significant aspect was that the dose response of leaf discs was similar to that of the callus cultures (Fig. 1). The concentrations of 2.5 mM, 5.0 mM, and 7.5 mM Na_2SO_3 correspond to 45, 91, and 136 ppm of SO_2 moles dissolved in water. According to Malhotra and Hocking (17), at low concentrations of SO_2 gas there is about a thousand-fold higher concentration in the water phase than in the gas phase at equilibrium. Therefore, we assumed that the experimental solutions used to treat leaf discs and callus cultures would be similar to SO_2 gas mixtures of concentrations of about 0.045, 0.091, and 0.136 ppm. These concentrations are an order of magnitude lower than that given whole plants.

Even though the experiments with leaf discs were reliable, the responses did not correlate with those of the whole plants. Namely, 'Cherry Blossom' had relatively low sensitivity on a whole plant basis but did not differ as leaf discs from 'Calypso' which was sensitive on a whole plant basis. 'Warrior' had relatively low sensitivity as a whole plant but was sensitive as leaf discs. Therefore, excising leaf discs and exposing them to an aqueous Na_2SO_3 solution was a sufficient perturbation of the whole plant system to alter the pattern of relative sensitivity. For whole plants, the primary mode of entry of SO_2 gas into the leaf is through the stomata as evidenced by the fact that most injury occurs when they are open (12, 16). As mentioned previously, stomate activity plays only a limited role in determining whole plant petunia sensitivity. Such limited activity must also be the case with discs in the Na_2SO_3 solutions, based on the progression of chlorosis, which appeared initially at the cut edge and diffused toward the center.

The treatment of callus cultures with Na_2SO_3 injured the cells. This damage was not due to pH or to excessive ionic strength as demonstrated by experiments in which the pH and NaCl required about 10 times the ionic strength before injury symptoms developed. The symptom from high salts prior to TTC staining was browning of the callus instead of bleaching as caused by Na_2SO_3.

Aside from 'Cherry Blossom' on MSNB, there were no differences among the cultivars in callus response. As whole plants, 'Cherry Blossom', 'Victory', and 'Warrior' were damaged less than 'Calypso' and 'Lilac Time'. Therefore, no correlation was observed between the response of cells to Na_2SO_3 and that of whole plants to SO_2. There was a significant correlation between the viability of the callus cultures, as measured by TTC staining of the controls, and their response to Na_2SO_3 treatment. Cultures of lower viability were damaged disproportionately more than the more viable ones. This same correlation existed when the cells were subjected to NaCl stress. Therefore, the variations and differences among blocks and cultivars including 'Cherry Blossom' on MSNB were not only correlated with, but may also have been due to variation in viability (vigor) of the cultures. This does not necessarily imply that the resistance of 'Cherry Blossom' on MSNB was not genetic in nature. The difference in viability (vigor) *in vitro* could have a genetic as well as an environmental component. However, this possible genetic component was apparently not the same one involved in determining the physiochemical structures directly affected by SO_2; for if it were, then 'Cherry Blossom' should have been resistant on MSD as well as on MSNB.

We conclude that tissue culture techniques are useful to the study of the physiology of SO_2. However, no consistent relationship in SO_2 resistance between explants and whole plants was found in *Petunia hybrida*. Thus, although the whole plant presents a complicated SO_2 physiology mechanism, it is a critical factor to consider when extrapolating from explant to organism.

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