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RESPONSES OF PETUNIA CULTIVARS TO SULFUR DIOXIDE OR SODIUM SULFITE AT DIFFERENT LEVELS OF BIOLOGICAL ORGANIZATION

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ABSTRACT

RESPONSES OF PETUNIA CULTIVARS TO SULFUR DIOXIDE OR SODIUM SULFITE AT DIFFERENT LEVELS OF BIOLOGICAL ORGANIZATION

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In order to evaluate a possible mutation-selection system for SO_2 resistance in Petunia hybrida, the responses of five cultivars of P. hybrida to treatments of SO_2 or Na_2SO_3 solutions at various levels of plant organization were studied. Whole plants were fumigated with SO_2 at 5 ppm for 16 hours and rated according to the amount of necrotic and chlorotic lesions on the leaves. Leaf discs were treated with Na_2SO_3 solutions at the concentrations of 2.5, 5.0, and 7.5 mM and evaluated by measuring the absorbances at 665 nm of ethanol extracts of chlorophyll. Callus cultures were treated the same as leaf discs but were evaluated by a triphenyl tetrazolium chloride (TTC) staining procedure to determine viability.

At each level of organization studied there were significant differences among the responses of the various cultivars. The trend of the responses of whole plants was similar to that reported in the literature. However, the rank of the cultivars according to their responses as leaf discs was not the same as that of the whole plants. Likewise, the rank of the cultivars according to the responses of

callus cultures grown on one medium was different from either the rank of whole plants or of leaf discs. There were no significant differences among the responses of callus cultured on a second medium. A significant correlation existed between the response of the callus cultures and the viability determined by the TTC staining procedure.

Because of the lack of correlation among the responses of whole plants, leaf discs, and callus cultures, it was concluded that responses of P. hybrida at these various levels of organization to SO_2 or its ions in solution were not primarily determined by the physiochemical systems directly affected by SO_2. Alternatively, the responses were possibly determined by other factors that were unique for each level of organization. Therefore, at this time, the most effective method of selection of desirable phenotypes for resistance to SO_2 in P. hybrida is to screen whole plants by fumigation with SO_2.

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INTRODUCTION

The ability to culture and regenerate plant cells and protoplasts *in vitro* give the plant breeder a potential method to improve crop species by mutation induction and selection of specific traits at the cell level. Since mutation rates are as low as 10^{-6} to 10^{-7} (10,11,38, 49,64,65), large populations must be screened to find desirable mutants; therefore, selection of cells in culture is more manageable than selection of whole plants in greenhouses or fields. However, four biological criteria must be met in order for this method to be effective for the plant breeder. Firstly, mutation of cells in culture must be possible or natural genetic variants must exist. Secondly, the desired characteristic must be expressed in culture in such a way that variants with the desired characteristic can be selected. Thirdly, whole plants must be capable of being regenerated from the selected variant cells. And fourthly, the regenerated plants must express the desired trait that was selected in culture.

Experiments have been conducted which demonstrate the feasibility of mutation and selection of specific mutants *in vitro*. Mutants resistant to various amino acid analogs (11,17,49,64,65,66), pyrimidine base analogs (33,37,42), and streptomycin (5,38) have been successfully selected. Also, some "leaky" auxotrophic mutants have been isolated (10). However, whole plants could not be regenerated from most of the

selected cell lines; therefore, it is not known whether those selected traits would have been expressed by whole plants.

The criterion that the desired trait be expressed by cells *in vitro* and by whole plants is paramount. Research studies have been done which indicate that certain whole plant traits can be expressed by cells in culture. For example, the single dominant gene conferring resistance to *Phytophthora infestans* race 0 in *Nicotiana tabacum* was expressed by cells in callus culture under very defined and stringent conditions (24). Correlation of responses between whole plant and cells *in vitro* has been reported for other host-pathogen systems (20,22,28,62). Recently, the unique physiological characteristic of low photo-respiration of C_4 plants was expressed by cells *in vitro* and was, therefore, not dependent on the unique Kranz anatomy of C_4 plants (31). The resistance and susceptibility of four lines of *N. tabacum* to ozone injury were also expressed when cells grown *in vitro* were exposed to ozone (T. Rice and P. Carlson, personal communication).

There are relatively few examples where traits selected at the cellular level *in vitro* were expressed by their regenerated plants. Cells of *Zea mays* L. from Texas male-sterile cytoplasm lines were selected for resistance to *Helminthosporium maydis* Race T pathotoxin (C. Green, personal communication). Regenerated whole plants were resistant to the disease, but the plants had lost their male-sterile cytoplasmic characteristic. Haploid *N. tabacum* cells that were resistant to the methionine analog, methionine sulfoximide were selected *in vitro* (11). Diploidized regenerated plants were not only resistant

to the analog but were also resistant to Pseudomonas tabaci. The toxin of P. tabaci is also an analog of methionine. Haploid N. tabacum cells resistant to streptomycin have also been selected in vitro (38). Diploidized regenerated plants were not tested directly for resistance, but leaf discs of resistant and control plants were placed on a medium containing streptomycin. Discs of the control plant produced some white callus, while discs of the mutant produced large amounts of green callus. Reciprocal crosses of the mutant plant with control plants showed that the resistance was maternally inherited.

The project reported herein was undertaken to study the feasibility of an in vitro cultural system for developing sulfur dioxide, SO_2, resistance in Petunia hybrida. P. hybrida was chosen as the experimental plant species since tissue culture techniques have been previously reported. Callus induction and subsequent plant regeneration has been accomplished for both diploid and haploid types (3,4,18,54). Protoplast isolation and whole plant regeneration was reported for diploid (18) and haploid Petunia (4). Most recently, protoplasts of P. hybrida and P. parodii were fused to produce somatic hybrids (51). Furthermore, much of the cytology, genetics, breeding, and taxonomy of Petunia has been established (7,8,16,17,21,29,40,44,45,50,52,58).

The problem of SO_2 resistance was selected since Petunia hybrida cultivars exhibit susceptibility to ambient air pollution, of which SO_2 is the main component world-wide (6). Also, numerous cultivars have been evaluated with respect to SO_2 susceptibility on a whole plant basis (15). Likewise, some of the detrimental modes of action of SO_2

on plant cell physiology have been examined. Reported effects include direct interference with photosynthetic CO_2 fixation and energy metabolism *via* inhibition by SO_3^{-2}, inhibition of various enzymes by redox products of SO_3^{-2} and cellular metabolites, and interference with membrane function and ultrastructure (37). Despite the many possible modes of action for SO_2, single gene mutations for SO_2 resistance might be detected because 1) certain processes could be more sensitive to SO_2 than others; and, a mutation of that system to resistance would increase overall resistance, and 2) genetic differences for SO_2 resistance have been reported on a whole plant basis (15). There are, however, potential limitations in this type of research. Correlations between whole plant and *in vitro* cell responses, with respect to SO_2 damage, may not exist since the stomata may play an important role on a whole plant basis. Obviously, stomata play no role in unorganized cells cultured *in vitro*. Likewise, since cultures are grown in the dark, photosynthetic fixation of CO_2 will not be a factor in conferring SO_2 susceptibility *in vitro*.

LITERATURE REVIEW

Physiology of SO_2 Injury

Probably no plant species is immune to SO_2 injury, but there is great variability amongst species relative to the concentrations necessary to produce phytotoxicity. For example, the eastern white pine (*Pinus strobus*) showed damage after exposure to 100 parts per billion (ppb) SO_2 for eight hours (14). In contrast, duckweed (*Lemna minor* L.) was not affected by SO_2 at levels as high as 300 to 600 ppb (9). In fact, the variable response to SO_2 damage even extends to the cultivar level. Certain petunia (*Petunia hybrida*) cultivars sustained minimal damage (about 1%) while others suffered extensive damage (19%) when exposed to the identical treatment of 2.5 parts per million (ppm) SO_2 for 1 hour (15).

Symptoms of SO_2 phytotoxicity range from reduced growth to cellular and whole plant death. With broadleaf species the chronic symptoms are interveinal chlorosis. Acute injury first appears as water-soaking and loss of turgor followed later by dead areas, bleached ivory to tan in color. If other pigments are present which were not concurrently destroyed by the SO_2, then the necrotic areas may appear red or black. Monocots usually develop necrotic streaks along the midveins. If the leaf blade has a bend, often the necrosis begins at the bend. Injury to species with needles begins at the tips of the needles and extends downward. Mild exposure often results in chlorosis and premature drop

of older needles. A high dose results in water-soaking which later changes from green to reddish brown. A succession of exposures results in distinct reddish brown banding of the needles (30).

Since SO_2 enters through the stomata, the leaf is most susceptible when the stomata are wide open. This generally occurs at high light intensities from 10 AM to 2 PM together with high relative humidity, adequate plant moisture and moderate temperatures. Therefore, plants are most sensitive to SO_2 in late spring and early summer. Middle-aged leaves are most sensitive and older leaves are most resistant to injury probably because of variation in the number, size, and activity of the stomata (26).

Due to the high solubility of SO_2 in water, H_2SO_3 is first produced in the cell before reacting with other chemical components (37). The pKa's of H_2SO_3 are 1.8 and 7.2 (32); therefore, the predominant ions in the cell sap are sulfite (SO_3^{-2}) and hydrogen sulfite (HSO_3^-).

Most plants are able to withstand and actually utilize a certain level of SO_2 in the atmosphere. Alfalfa (Medicago sativa) exposed to chronic, non-phytotoxic concentrations of SO_2 converted the sulfite ions predominantly to sulfate ions (61). Some sulfur was actually incorporated into organic compounds. Furthermore, if sulfate in the soil was low, the fumigated alfalfa had more organic sulfur than the non-fumigated plants. Mitochondria of etiolated oat seedlings (Avena sativa) had an enzyme, sulfite oxidase, capable of converting sulfite to sulfate (60). Similar utilization of SO_2 in plant growth was shown to occur with duckweed, Lemna minor L. (9). Sulfur from SO_2 was converted

to sulfate and then incorporated into amino acids and sulfoquinovose of sulfolipids. Duckweed had an efficient sulfite oxidizing system, and tolerated a relatively high concentration of SO_2 in the atmosphere.

Trees can also incorporate SO_2 in normal metabolism (55). White birch (Betula papyrifera Marsh), a species sensitive to SO_2, and pin oak (Quercus palustris Muenchh.), a species resistant to SO_2, were planted at two locations in Ohio. One location was low in SO_2 (less than 4 ppb) and the other was high in SO_2 (27 ppb) but below injury levels. The sensitive species, birch, grew better in the high SO_2 environment, while the resistant species, pin oak, grew worse in the high SO_2 environment. The author thinks that perhaps the SO_2 environment acted as a fertilizer for the birch, but actually was deleterious to the pin oak growth since the SO_2 stimulated stomatal closure.

If SO_2 in excess of plant requirements enters, no matter how slowly, chronic injury marked by chlorosis will occur (37). This chlorosis is due to excess sulfate--oxidized sulfite--producing an excess ion effect (30). If the excess SO_2 enters faster than the cell can convert it to sulfate, then the damage is the direct toxic effect of sulfite. Mesophyll cells are primarily affected, and the chloroplasts become plasmolyzed or bleached.

Various effects of SO_2 damage to chlorophyll, chloroplasts, and photosynthetic capabilities have been reported. Rao (53) showed that a 24-hour exposure to 5 ppm SO_2 degraded chlorophyll a of lichen. Chlorophyll a of lichen was affected more than chlorophyll b (52). Chloroplasts of Vicia faba underwent a reversible swelling when exposed

to 250 ppb SO_2 for one hour (63). Sulfur dioxide, even at levels indicating no visible signs of injury, reduced the amount of chlorophyll of cotyledons and primary needles and the dry weight of seedlings of Pinus resinosa (13). Chloroplast structure as well as photosynthetic activity, as measured by the Hill reaction, was impaired by SO_2 (36). Chloroplast integrity correlated well with the photosynthetic activity of Pinus contorta Dougl. needles. Chloroplasts from the basal portion of the needle were affected the least and showed the most photosynthetic activity; the opposite was true for chloroplasts from the tip of the needle.

Aside from affecting chloroplast integrity and chlorophyll content, SO_2 also disrupted enzymes involved in photosynthesis. Sulfite ions affected the activity of ribulose-1,5-diphosphate carboxylase, which fixes CO_2 during photosynthesis, of isolated spinach (Spinacia oleracea) chloroplasts (67). SO_3^{-2} was a non-competitive inhibitor with the substrate ribulose diphosphate with K_i of 14 mM SO_3^{-2}. Sulfite was a competitive inhibitor with the substrate hydrogen carbonate ion with a K_i of 3 mM SO_3^{-2}. Finally, sulfite acted as a non-competitive inhibitor with the cofactor Mg^{+2} with a K_i of 9.5 mM SO_3^{-2}. Therefore, the first substrate to be affected by sulfite in the system was dissolved CO_2.

Sulfur dioxide or SO_3^{-2} reacts with other cellular constituents. Bailey et al. (1) showed that .3 M SO_3^{-2} was capable of breaking sulfhydryl groups of proteins. Even though high concentrations of cellular sulfite is unlikely, such reactions at lower concentrations are possible,

perhaps to a lesser, but still damaging extent. More recently, sulfite was established to react mostly with interchain disulfide bonds rather than with intra-chain disulfide bonds (12).

Inhibition of ATP formation in plant mitochondria by SO_3^{-2} was demonstrated (2). Bean (Phaseolus vulgaris) cotyledon and maize (Zea mays) coleoptile mitochondria exposed to 3 mM SO_3^{-2} produced ATP at 77 and 70% of the controls, respectively. At 10 mM SO_3^{-2}, production of ATP was reduced to 56 and 44%. Even though bean was more sensitive to SO_2 in vivo than maize, the responses of the mitochondria in vitro were not significantly different from each other. Therefore, this inhibition did not account for all of the plant sensitivity to SO_2 air pollution.

Meudt and Werner (41) showed that sulfite affects indole-3-acetic acid (IAA) oxidation. Sodium metabisulfite at a concentration of .05 mM stimulated the initial reactions of IAA oxidation but inhibited the subsequent formation of the other intermediates. They hypothesized that the sulfite ions were scavenging the free radicals necessary for the further reactions. They also found that the higher concentration of 1 mM HSO_3^- inhibited even the initial reactions of IAA oxidation.

Using polyuridine (poly U), Shapiro (56) showed that 1 M sodium bisulfite produced sulfonated residues on the uracil moeity. When such residues were present, poly U would not base-pair with poly A. Likewise, the ability of polyribouracil to code for phenylalanine incorporation into protein was increasingly reduced as the number of sulfonated residues was increased. At 2.6% of saturation, there was

only 54% incorporation of phenylalanine in comparison to the control; and at 10.5% of saturation, there was only 8% incorporation. Shapiro suggested that a slight degree of modification of mRNA (perhaps a single uracil being sulfonated) could block translation.

Along the same lines, treatment with 1 to 10 mM bisulfite degraded the double stranded DNA of the phage T7 (22). Manganese ion was necessary, presumably to catalyze the autoxidation of SO_3^{-2} with water to form the radicals SO_2^- and $H_2O_2^-$. These radicals were responsible for the DNA breakdown. Detectable damage occurred after only 5 minutes of exposure to SO_3^{-2}.

In addition to causing damage by direct chemical reaction with vital cellular components, sulfite was able to react with cellular constituents to form other inhibitor substances. Alpha-hydroxy-2-pyridinemethanesulphonate (αHPMS), glyoxal bisulfite, as well as sodium bisulfite, were shown to inhibit CO_2 fixation in the light by Atriplex spongiosa, a C_4 plant. However, there was no light-CO_2-fixation inhibition on the C_3 plant Atriplex hastata. These components also inhibited the enzymes glycolate oxidase and phosphoenolpyruvate carboxylase of both species (48).

Murray and Bradbeer (43) found that αHPMS inhibited CO_2 fixation by spinach chloroplasts by 50% at a concentration of 7 mM. They also found that phosphoenolpyruvate carboxylase was competitively inhibited at a concentration of 1 mM αHPMS. Hydroxysulfonates also inhibited NADH:malate dehydrogenase in the mitochondria and peroxisomes.

In summary, SO_2 when in solution as bisulfite (HSO_3^-) or sulfite (SO_3^{-2}) interfered in a number of ways in cellular systems. It reacted with membranes as evidenced by swelling and plasmolysis of chloroplasts; it acted as a competitive inhibitor of a number of enzymes such as ribulose-1,5-diphosphate carboxylase and those concerned with ATP production in the mitochondria; it denatured protein by breaking sulfhydral bonds; it disrupted protein synthesis by reacting with uracil residues of mRNA's; it disrupted genetic information by degrading DNA; and it formed a number of secondary compounds that disrupted normal cellular functions. At this time, however, investigations are unable to definitely establish which specific reaction(s) is(are) the most important in determining SO_2 damage of plants.

Mutation and Selection of Plant Cells in Tissue Culture

A breeding system using mutation and selection *in vitro* must have several characteristics if it is to be effective in crop improvement. 1) Mutants must exist, either by occurring spontaneously or by induction with a mutagenic agent, 2) the desired characteristic must be expressed at the cellular level so that the desired cell types can be selected, 3) the mutant cells must remain totipotent so that whole plants can be regenerated from them, and 4) the characteristic must be expressed in the regenerated whole plant. The following literature review reveals that certain characteristics at the whole plant level are expressed at the cellular level, that mutation and selection of specific variant cell types *in vitro* are possible, and that in some systems, characteristics

mutated and selected at the cellular level are expressed in plants regenerated from the selected cells.

Most of the work that demonstrates expression of whole plant characteristics at the cellular level have been host-pathogen interaction systems. Helgeson et al. (25) used callus culture of two similar lines of Nicotiana tabacum L. in their research on the pathogen Phytophthora infestans par. As whole plants, one was resistant and the other susceptible to race 0 of the pathogen, and both were susceptible to race 1. The resistance was conferred by a single dominant gene. Under proper combinations of hormones and temperature treatment, the resistance and susceptibility of the two lines to race 0 was expressed in culture. Both lines in culture were susceptible to race 1. On a medium with another hormonal composition, both lines were susceptible to race 0; however, freshly excised pith also showed no differences in resistance to race 0. Likewise, at temperatures above 27°C the resistant line became susceptible. However, seedlings of the resistant line also became susceptible at temperatures above 32°C. Because of the specificities of the lines to the races of the pathogen and because of the similar temperature responses, the differences in susceptibility were apparently due to the genetic expression of resistance in callus culture. Furthermore, plants regenerated from the two cell lines retained their race specific resistance-susceptibility. Further experiments were done with essentially genetically identical lines except for resistance of one line to race 0 (24). F_1, F_2 and F_3 generations and testcrosses of homozygous recessive (susceptible) lines with the F_1

were tested as whole plants and as callus. There was (near) perfect correlation between the response of each of the whole plants and its respective callus to both race 0 and race 1. This further substantiated the claim that the dominant gene for resistance to race 0 was expressed at the cellular level.

An earlier and less comprehensive study was done by Ingram and Robertson (28) on the interaction of Phytophthora infestans and tissue cultures of Solanum tuberosum. Resistance-susceptibility of two cultures was expressed by large cell aggregates in culture, and, to a lesser extent, by cells in suspension culture. Also, living cells were necessary to inhibit stimulation of the pathogen; that is, dead cells of the resistant cultivar stimulated P. infestans growth. Further research using two races of the pathogen revealed that the resistance-susceptibility in culture to both races correlated with the whole plant responses (27).

A similar study was done by Warren and Routley (62) on Phytophthora infestans race 0 and Lycopersicon esculentum. Again, resistance-susceptibility was expressed in tissue culture.

Resistance of nonsterile cytoplasm Zea mays and the susceptible Texas male-sterile cytoplasm Zea mays to Helminthosporium maydis race T pathotoxin was expressed in callus culture (20). Furthermore, variants were selected within the susceptible Texas male-sterile cytoplasm maize cultures that were resistant to the toxin. Analysis of mitochondrial activity from both normal cytoplasm cells and from the selected variant cells showed that the mitochondria were not affected by the toxin, but

mitochondria from the Texas male-sterile cytoplasm cells were affected by the toxin. No plants could be regenerated from these variants, due to their age in culture. However, in a later experiment (C. Green, personal communication), plants were regenerated from selected variants; they were resistant to the toxin, but no longer had male-sterile cytoplasm.

A most interesting correlation between whole plant response and cellular response was reported by Kennedy (31) on the photorespiration of Portulaca oleracea, a C_4 plant, and Streptanthus tortuosus, a C_3 plant. It was formerly believed that the reduced level of photorespiration of C_4 plants was related to their unique "Kranz anatomy" of the leaves. However, C_4 suspension cultures had about a 1:1 light:dark photorespiration rate, like the whole plant, and the C_3 cultures had about a 3:1 light:dark photorespiration rate, like the whole plant. Furthermore, photorespiration of P. oleracea callus was little affected by O_2 concentration, while photorespiration of S. tortuosus callus increased with increased O_2 concentration. Again, these responses are typical of C_4 and C_3 whole plant responses. The dark respiration of plants and callus of both species were the same at all O_2 concentrations. He concluded that the C_4 reduced photorespiration rate was a cellular based phenomenon and was not due to the "Kranz anatomy".

Four cultivars of Nicotiana tabacum were assayed for ozone damage in vitro. As whole plants, two were resistant to damage by ozone and two were susceptible. Growth and cell viability of the resistant cultivars in culture were reduced less by ozone than the susceptible ones (T. Rice and P. Carlson, personal communication).

The previously mentioned experiments, except those of Gegenbach and Green, utilized existing differential responses of whole plants to demonstrate similar responses in tissue culture. There have been a number of instances where cells in vitro have been mutated and selected for certain characteristics, but in many instances whole plants could not be regenerated from them. In some cases, mutagens such as ethyl methanesulphonate (EMS) (10,11) or N-methyl-N-nitro-N-nitrosoguanidine (NTG) have been used to induce mutations (23,33,47); in other instances (5,19,38,39,49,64,65,66) naturally occurring or spontaneous mutations have been selected. The predominant selection technique involved growing callus or suspension cultures on media that contained toxic chemicals. Only those mutants that were resistant to the toxic chemicals continued to grow. Thus, those resistant mutants were selected. Cells resistant to amino acid analogs (11,19,49,64,65,66), base analogs (33, 39,47), pathotoxins (20), and streptomycin (5,38) have been selected by using this technique.

Another scheme was used by Carlson (10) to isolate auxotrophic mutants. Single haploid cells of Nicotiana tabacum were mutated by a treatment of 0.25% EMS for 1 hr. Survival of the treated cells was 47 to 68%. The cells were grown in the dark on a minimal medium containing 5-bromodeoxyuridine (BUdR) for 36 hours. The growing (autotrophic) cells incorporated BUdR. Then the cells were plated-out on a supplemental medium and exposed to light, thus killing the autotrophs. Presumptive auxotrophs grew on the supplemented medium in the light, since they had not incorporated BUdR while on the minimal medium.

From an original cell population of about 1.75×10^6, 119 calli were selected. Only six were found to be auxotrophs requiring either nucleic acids, vitamins, or amino acids. Although the auxotrophs were haploids, they grew slowly on unsupplemented media and have been called "leaky" auxotrophs. The explanation put forth for these incomplete auxotrophs was that N. tabacum is an allopolyploid, and hence, the haploid cells may still have multiple genes for some (many) traits.

There are three reported instances where mutated cells have been selected for certain traits, whole plants have been regenerated from these selected cells, and the selected traits have been expressed by the whole plant. The work of Green (personal communication) has already been mentioned. Carlson (11) mutated cells of haploid N. tabacum by treatment with EMS. He then selected mutants resistant to the methionine analog, methionine sulfoximide (MSO). Three selected cell lines were diploidized, and whole plants were regenerated. Plants were found to be resistant to MSO and to the pathogen Pseudomonas tabaci. The toxin of P. tabaci is also an analog of methionine. The plants of two lines had increased levels of free methionine. The resistance was due to a single semi-dominant gene, as crosses with susceptible lines gave a 1:2:1 F_2 ratio. The third mutant was thought due to possibly recessive genes at two loci with additive effects, as the F_1 progeny gave about a 9:3:3:1 F_2 segregating ratio.

The third instance of regenerated plants expressing a trait selected for in vitro was reported by Maliga, Sz.-Breznovits, and Márton (38). They selected haploid N. tabacum cells in callus culture

that were resistant to streptomycin. Plants were regenerated from spontaneously diploidized cells. Leaves of the resistant mutant and a susceptible line were placed on a medium with streptomycin. The leaf discs from the resistant plant produced green callus; whereas, leaf discs from the susceptible plant produced very little white callus. (It may be debated as to whether or not this demonstrated that the intact plant is resistant to streptomycin.) They performed crosses between resistant and susceptible lines and found that the resistance was only inherited when the resistant plant was the female parent. This suggested that resistance to streptomycin was determined by cytoplasmic gene(s).

The above reviews reveal that cells can be mutated and selected *in vitro*, and desired traits can be expressed by whole plants regenerated from the selected cells. The purpose of this project was to study the feasibility of an *in vitro* cultural system for developing SO_2 resistance in *Petunia hybrida*. This was accomplished by studying the response to SO_2 (or its ions in solution) of whole plants, leaf discs, and *in vitro* callus cultures to determine if the resistance and susceptibility reactions were similar at the different levels of plant organization. Of particular interest was whether or not the responses of callus cultures correlated with whole plant responses because mutation and selection could be carried out on callus cultures.

MATERIALS AND METHODS

Plant Material

Seven cultivars of *Petunia hybrida* were used in these experiments. The Michigan State University accession numbers, cultivar names, flower types (G = grandiflora, M = multiflora), SO_2 responses according to Feder *et al.* (15), and the sources of seeds are given in Table 1. Cultivars 990, 993, 997, and 1000 were used in preliminary experiments for establishing procedures for tissue culturing. Cultivars 1003, 1006, 1012, 1015, and 1016 were used for whole plant, leaf, leaf disc, and tissue culture experiments.

Whole Plant Experiments

Eight month old plants, with flowers removed, were exposed to a steady stream of air with 5 ppm SO_2 for 16 hours under artificial light of 1.5 mwatts/cm^2 (ca. 1000 ft-c). Exposures were conducted by Dr. R. Bressan in the exposure chamber of Dr. P. Filner of the Michigan State University Plant Biology Laboratories. One plant each of 1003, 1006, 1012, 1015, and 1016 were repotted from a 4" clay pot to a 4" plastic pot, and the tops of the pots were wrapped in "Parafilm" in order to prevent absorption of SO_2 by the soil. Injury consisted of wilting, chlorosis, and necrosis. Twenty-four hours after the end of the exposure, the plants were rated as follows: 1) the number of leaves with no injury, 0-25% injury, 25-50% injury, and greater than 50%

Table 1. *Petunia hybrida* cultivars used to study the response of various levels of biological organization to treatments of SO_2 gas or Na_2SO_3 solutions.

Accession Number	Cultivar Names	Flower Type[1]	SO_2 Response[2]	Source
990	Victory	M	Susceptible	Harris Seed Co.
993	Warrior	G	Susceptible	Harris Seed Co.
997	Blue Danube	Double G	Resistant	Pan Am Seed Co.
1000	Comanche	M	Not tested	Dr. J. B. Power
1003	Calypso	G	Resistant	Pan Am Seed Co.
1006	Cherry Blossom	G	Resistant	Pan Am Seed Co.
1012	Victory	M	Susceptible	Harris Seed Co.
1015	Warrior	G	Susceptible	Harris Seed Co.
1016	Lilac Time	G	Resistant	Harris Seed Co.

[1]M = multiflora, G = grandiflora.

[2]As determined by Feder *et al.* (15).

injury were counted, 2) these numbers were normalized on the basis of 100 leaves per plant, and 3) an R value was calculated using the formula

$$R = (1a + 2b + 3c)/3$$

where

a = normalized number of leaves with 0-25% injury
b = normalized number of leaves with 25-50% injury
c = normalized number of leaves with 50-100% injury.

Two exposures were conducted and the data were analyzed using analysis of variance (AOV) for a randomized complete block design.

Whole Leaf Experiments

The third or fourth fully expanded leaves of 1003, 1006, 1012, 1015, and 1016 were floated in petri dishes containing 50 cc of either a control solution of 20 mM NaCl at pH 4-5 or 10 mM Na_2SO_3 at an initial pH of 5.0-5.4. After 24 hours, the leaves were rinsed and placed on moist filter paper in petri dishes for another 24 hours to allow symptoms to develop. Symptoms were water-soaking with loss of turgor and yellow or necrotic lesions. Each rating was the visual determination of the approximate percent of the leaf area that was injured. Four leaves were floated together and their values were averaged. Four treatments were conducted and AOV was calculated for a randomized complete block design.

Leaf Discs

Leaf discs of the 2nd through 5th fully expanded leaves of 1003, 1006, 1012, 1015, and 1016 were made using a 5 mm cork borer. All the

leaf discs of each cultivar were put in a beaker of water and stirred in order to randomize them. Ten discs of each cultivar were placed in a petri dish containing 30 cc of either a control solution of 20 mM NaCl at pH 4.0-4.5 or a treatment solution of either 2.5, 5.0, or 7.5 mM Na_2SO_3 at pH 5.0-5.5. After 3 hours the solutions were removed. The leaf discs were rinsed with distilled water and returned to their respective petri dishes with 10 cc of distilled water. The dishes were placed under 1000 ft-c for an additional 16 to 20 hours. Chlorophyll was extracted by placing the 10 leaf discs in a test tube containing 80% ethanol. The tubes were placed in a boiling water bath and the ethanol was boiled until the volume was reduced to a few cc's. A 2nd and 3rd extraction were carried out using 5 cc each time. The three extracts were combined and brought to a volume of 10 cc by adding 80% ethanol. The absorbance at 665 nm was determined using a Beckman Model 25 Spectrophotometer. The raw data was normalized by dividing the values of the treatments by the value of the control for each cultivar. Three treatments were conducted, and the AOV was computed for a randomized complete block design with 15 treatments, 5 cultivars and 3 Na_2SO_3 concentrations.

Lower Leaf Epidermal Strips

Epidermal strips were peeled from the under side of fully expanded young leaves. Three stains were used in an attempt to determine viability of freshly prepared strips: 1) triphenyl tetrazolium chloride (TTC) at a concentration of 0.5%--viable cells become purple within 24

hours, 2) phenosafranin at a concentration of 0.1%--nonviable cells are stained red after a few minutes, and 3) neutral red at a concentration of 0.01%--viable cells take up the stain and are red after 20 minutes. Neutral red was also used to stain epidermal strips treated concurrently with leaf discs.

Tissue Culture

Media. Culture media contained the salts of Murashige and Skoog (42), the vitamins of Nitsch and Nitsch (46) for anther culture of N. tabacum, 30 g/liter sucrose and 8 g/liter agar. Medium designated MSNP had 2 ppm naphthalene acetic acid (NAA) and 0.5 ppm N^6 benzyl-aminopurine (6-BAP) as hormones added to the previously stated salts and vitamins. The medium designated PM had 1 ppm 2,4-dichlorophenoxy-acetic acid (2,4-D) as the sole hormonal component.

Callus culture initiation. 1) Stem sections of 990, 993, 997, and 1000 were washed in 95% ethanol for about 5 minutes, rinsed with water, and surface disinfested in 0.5% NaOCl with a few drops of Tween 20 added. After 10 minutes the tissue was rinsed twice with sterile water. With the damaged ends removed, the stem sections were placed on medium PM. Callus that was produced was excised and transferred to fresh medium. 2) Seed of 1003, 1006, 1012, 1015, and 1016 were surface disinfested with 0.5% NaOCl for 1 to 2 minutes, rinsed with sterile water and placed on media MSNP and PM. Seeds were germinated under 300 ft-c. Seeds germinated as normal seedlings or directly produced callus. Only seedling stems and leaves were cut-up and returned to the callus-inducing media since the origin of callus emerging directly from the

seed could have been from maternal tissue. Once callus was induced in the dark, it was subcultured about twice per month. All cultures were maintained at prevailing room temperature. This method of callus initiation was used in order to avoid contamination problems encountered when using explants of greenhouse grown plants.

Viability staining. Triphenyl tetrazolium chloride (TTC) at a concentration of 0.5% was used as a vital stain. The solution was filter-sterilized using a Millipore filter of 0.22 micron pore size; 3 to 5 cc were added to each 100x15 mm petri dish. In the preliminary experiments the stain was added immediately after the treatment solutions were removed. Viability was scored between one and 24 hours later. For the later experiments, TTC was added 24 hours after removal of the treatment solutions. Scoring was done 24 hours later. In order to determine the effectiveness of staining in measuring viability, a correlation was determined between the growth rate and TTC scoring of control and treated tissue. First, a correlation between fresh weight and dry weight of the callus tissue was determined and found to be very highly significant ($r = 0.981^{***}$) so that fresh weight was used. Then two blocks of experiments on PM medium were run with three samples of tissue of each cultivar instead of two (see section on cultivar by Na_2SO_3 factorial experiments). Two samples were stained with TTC, and the third sample was used to determine growth rate. The samples were about 100 mg fresh weight and were grown for 13 days. Growth rate was the ratio of the final to the initial weight.

Preliminary experiments. A number of preliminary experiments were performed on callus to determine proper experimental conditions for treatment with Na_2SO_3. Firstly, the effects of ionic strength and pH on callus cultures were determined by adding 5 cc of a solution of either 100 mM, 50 mM, 12.5 mM, 6.25 mM or 3.125 mM NaCl at a pH of either 5.2-5.7 or 2.9-3.0. The cultivars used were 990 and 997. Treatment was for 3 hours, and the calli were stained with 3 cc of TTC and scored the next day. Secondly, duration of exposure to Na_2SO_3 solutions was determined by treating calli of 990, 993, and 997 with either distilled water or 1000 ppm (ca. 8 mM) Na_2SO_3 for times of 1, 6, 24, or 49 hours. TTC was added immediately after removal of the treatment solutions, and the calli were scored 24 hours later. Thirdly, the potency of Na_2SO_3 solutions was evaluated zero, 1, 4, 24, and 48 hours after preparation. Five cc of either distilled water or 1500 ppm Na_2SO_3 solutions were added at appropriate times to calli of 990, 993, and 997. Treatment was for two hours. Fourthly, the effect of pH on Na_2SO_3 damage was determined by adding 3 to 5 cc of solutions of 500 ppm Na_2SO_3 dissolved in liquid PM medium at a pH of 7.0, 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, and 2.0 to cultures of 990, 993, and 997. Titration of the Na_2SO_3 solution to pH 2.0 caused evolution of some SO_2 gas. Treatment was for 24 hours, 2.5 cc of TTC were added, and calli were scored the following day.

Cultivar by Na_2SO_3 concentration factorial experiments. From the results of the preliminary experiments the following experimental procedure was used to treat callus cultures of 1003, 1006, 1012, 1015,

and 1016. Due to oxidation of sulfite to sulfate and subsequent loss of potency and the escape of SO_2 from water solutions of Na_2SO_3, especially at high temperatures, it was necessary to prepare fresh solutions by filter sterilization. The solutions were prepared as follows: a 10 mM stock solution at pH 5.5 was prepared by dissolving 10 millimoles (1.26 g) of Na_2SO_3 in 500 cc of distilled water; and the solution was simultaneously titrated and brought up to volume by adding 500 cc of water to which 0.7 cc of concentrated HCl was added. Solutions of 7.5 mM, 5.0 mM, and 2.5 mM Na_2SO_3 were made by dilution with distilled water. At pH 5.5 the predominant form of ion was HSO_3^-.

The experimental design was a split-plot with Na_2SO_3 concentrations as the main plot treatment split by cultivars. The treatments of the calli were carried out as follows: 1) two samples of calli (about 100 mg) of each of the five cultivars were placed together (in random positions by cultivar) on petri dishes of either PM or MSNP medium, 2) three to seven days later each dish of calli was treated with 10 cc of either a control solution of 20 mM NaCl at pH of about 4.2 or a treatment solution of 2.5 mM, 5.0 mM or 7.5 mM Na_2SO_3, 3) solutions were removed 3 hours later using sterile, disposable pipettes, 4) 10 cc of filter-sterilized water were used to rinse the cultures, 5) 24 hours after initiation of the experiment, the dishes were treated with 5 cc of filter-sterilized 0.5% solution of TTC, and 6) dishes were assigned random numbers and visually rated from 0 to 10 on the basis of color development, 10 being the most viable, 24 hours after application of the TTC. The rating values of the two pieces of each cultivar were

averaged. The data were normalized and analyzed as ratings of treatments divided by controls multiplied by 10 for convenience. This normalized rating was actually percent of control divided by 10. Since large amounts of callus and much manipulation of materials were necessary to run an experiment, they were usually carried out two blocks at a time. Blocking was by callus source and by day of experiment. There were 6 blocks on MSNP medium and 7 on PM medium. Data were analyzed using AOV for a split-plot design.

NaCl stress experiment. Calli of 1003, 1006, 1012, 1015, and 1016 were treated with 0, 3, 6, or 9% NaCl solutions for 24 hours. The TTC staining solution was added after removal of the NaCl solutions and rinsing with sterile water. Three replications were run, and the AOV was performed for split-plot design with NaCl concentrations as the main plot split by cultivars. Data were normalized as percent control divided by 10.

NaCl, Na_2SO_3 double stress experiments. A number of double stress experiments were carried out on 1006 callus cultures to determine the effect of environmentally induced variability of viability on SO_2 response. In the first attempt, callus was subjected to a stress of 5% NaCl solution with a control of sterile water. After 24 hours, the solution or water was removed, the callus was rinsed, and subjected to either a second stress of 5 mM Na_2SO_3 or a control of 20 mM NaCl. Treatment time was for 24 hours. The TTC stain was added after another 24 hours. In the second experiment, the first stress was either 2, 3, 4, or 5% NaCl and the second stress was 3.5 mM Na_2SO_3. In the third

experiment, the first stress was 2 or 4% NaCl and the second stress was 5 mM Na_2SO_3. And in the fourth experiment, the first stress was 2 or 4% NaCl and the second stress was 2.5 or 5.0 mM Na_2SO_3. Appropriate AOV's were performed when possible, and correlation coefficients were determined for ratings of controls (NaCl treatment) vs. normalized ratings of treatments (Na_2SO_3 treatment).

RESULTS

Whole Plant Experiments

Exposing whole plants to 5 ppm SO_2 for 16 hours resulted in damage consisting of wilting, chlorosis, and necrosis. Cultivars 1003, 1006, and 1016 were most resistant, while 1012 was less resistant, and 1015 was susceptible (Table 2). This ranking was similar to that of Feder et al. (15) where 1003, 1006, and 1016 were resistant and 1012 and 1015 were susceptible. The AOV is given in the Appendix as are the AOV's of the results of the other experiments.

Whole Leaf Experiments

Injury caused by exposing whole leaves to 10 mM Na_2SO_3 solutions consisted of water-soaking with loss of turgor, yellowing, and necrotic lesions (Table 3). Variation among the four blocks was substantial (block variance ÷ error variance was 25.2), but no differences were observed among the cultivars tested.

Leaf Disc Experiments

The damage to leaf discs exposed to Na_2SO_3 solutions consisted of bleaching of the tissue to a tan color (Tables 4 and 5). Since the values given are absorbances by chlorophyll, the larger the value is, the less damage occurred. There were significant differences among the cultivars and among the Na_2SO_3 concentrations, but there was no interaction between these two variables. The effect of Na_2SO_3 concentration

Table 2. Mean R values of undetached petunia leaves after 16 hours exposure to 5 ppm SO_2 gas.

Cultivar	1006	1003	1016	1012	1015
Mean R value	19.2	21.6	23.3	28.2	57.7

($L.S.D._{.05}$ = 13.27)

Table 3. Mean percent damage to whole detached leaves after 24 hours exposure to 10 mM Na_2SO_3 of initial pH of 5.0 to 5.5.

Cultivar	1003	1015	1006	1012	1016
Mean percent damage	51.6	55.6	61.6	63.1	65.6

($L.S.D._{.05}$ = 25.5)

Table 4. Normalized absorbances of chlorophyll extracts of leaf discs of 5 petunia cultivars averaged over Na_2SO_3 concentrations after 3 hours exposure to 2.5, 5.0 or 7.5 mM Na_2SO_3 at initial pH of 5.0 to 5.5.

Cultivar	1006	1003	1012	1015	1016
Mean normalized absorbance (665 nm)	41.47	34.32	32.18	32.10	20.20

($L.S.D._{.05}$ = 7.66)

Table 5. The effect of Na_2SO_3 concentration on mean normalized absorbances of chlorophyll extracts of petunia leaf discs, all cultivars, after 3 hours exposure to solutions at initial pH of 5.0 to 5.5.

Na_2SO_3 concentration	2.5 mM	5.0 mM	7.5 mM
Mean normalized absorbance (665 nm)	54.21	23.88	18.07

($L.S.D._{.05}$ = 5.93)

was straightforward with treatments of 5.0 and 7.5 mM causing significantly greater damage to the leaf discs than the treatment of 2.5 mM. However, the differences among the cultivars as leaf discs were not the same as observed with whole plants. Cultivar 1006 was still rated as resistant, but 1003, 1012, and 1015 were intermediate. The line 1016, which was resistant as a whole plant, was very susceptible to injury when tested in the leaf disc assay.

Notably, plants of cultivar 1016 appeared to be a lighter green than the others. This was confirmed by a significantly lower chlorophyll content in the leaf discs of the controls. Consequently, there was a significant correlation between the chlorophyll content of the controls and the sum of the normalized chlorophyll contents of the treatments. However, there were no significant differences among the mean chlorophyll contents of the controls of the other cultivars. The correlation was not significant if 1016 was excluded. Therefore, the significant differences for Na_2SO_3 damage between 1006 and 1012 and 1006 and 1015 could not be contributed to differences in the initial chlorophyll content of the leaf discs.

Epidermal Strips

No extensive experiments were carried out using epidermal strips because a suitable staining procedure to demonstrate viability could not be devised. Freshly prepared epidermal strips did not stain with TTC even after 24 hours of exposure. Freshly prepared epidermal strips took up the phenosafranin stain indicating that the cells in these tissues were no longer viable. Strips exposed to Neutral Red stain revealed

viable and non-viable regions. However, when strips were exposed to control and 7.5 mM Na_2SO_3 treatment solutions (exposed with leaf discs), no differences between control and Na_2SO_3 treated strips could be determined. Therefore, all staining procedures were deemed unreliable.

Tissue Culture Experiments

General growth of callus. Unorganized callus cultures of 1003, 1006, 1012, 1015, and 1016 were established on both MSNP and PM media. The callus growth of some lines was better on MSNP than on PM and vice-versa (see Table 6). However, growth was sufficient to allow subculturing of all cultures on the same day, so that callus of all the cultivars for a given experiment were identical with respect to age and the number of subcultures. Aside from callus growth rate differences among the cultivars, there were coloration and friability differences. Most obvious was the constant brownish color of the callus of line 1016 on MSNP medium.

Table 6. Callus growth rates of 5 petunia cultivars on two media as determined by the ratio of the fresh weight after 12 days to the initial fresh weight.

Cultivar	1003	1006	1012	1015	1016
Growth rate on MSNP	3.30	4.50	1.26	2.06	2.80
Growth rate on PM	1.46	4.30	2.77	2.41	1.55

Correlation of growth and visual rating of TTC staining. 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**). Correlations of raw data summed over blocks, over cultivars, and over cultivars and blocks were highly significant (r = 0.784**, 0.890**, and 0.994**, respectively). The correlation of each value normalized as percent of control was significant (r = 0.438*). Therefore, TTC staining was a quick, convenient and reliable means of operationally defining viability.

Effects of ionic strength and pH. The effects of treatment of calli with solutions of ionic strength to 100 mM and pH to 3.0 are shown in Table 7. There were no effects of ionic strength and pH in the range that the callus cultures were subjected to in the later experiments with Na_2SO_3, where ionic strength was less than 20 mM and the pH did not drop below 4.0.

Table 7. Effect of ionic strength and pH on TTC viability ratings of petunia callus cultures after 3 hours exposure.

NaCl conc. (mM)	3.125		6.25		12.5		25		50		100	
Cultivar	990	997	990	997	990	997	990	997	990	997	990	997
pH 5.5	5	9	4	9	4	9	4	8	4	9	3	7
pH 3.0	4	8	4	10	3	5	4	8	4	9	4	8

Duration of exposure to Na_2SO_3. The results of exposure of callus cultures to 1000 ppm Na_2SO_3 for various durations are shown in Table 8. Much of the damage occurred after an hour of exposure; hence, duration of exposure for subsequent experiments was 3 hours to allow for a more thorough treatment. A longer exposure was not chosen because the potency of Na_2SO_3 solutions decreased rapidly (see the next section).

Table 8. Mean TTC viability ratings of petunia callus cultures after 1, 6, 24 and 49 hour exposure to 1000 ppm of Na_2SO_3 at initial pH of 5.5.

	Cultures	1 hr.	6 hrs.	24 hrs.	49 hrs.
Control	990	5.5	8.5	8.0	9.0
	993	8.5	8.5	8.5	9.5
	997	8.5	9.0	9.0	*
Treatment	990	5.0	4.5	4.5	0.0
	993	1.5	1.5	0.0	0.0
	997	0.5	0.0	0.0	0.0

*Contaminated

Potency of Na_2SO_3 solutions. Callus cultures were exposed to either a water control or 1500 ppm Na_2SO_3 treatment 0, 1, 4, 24, and 48 hours after preparation of the solutions. Damage to callus decreased with time after preparation; therefore, the solutions had to be freshly prepared before use (Table 9).

Table 9. Mean TTC viability ratings of petunia callus after 2 hours exposure to 1500 ppm Na_2SO_3 starting 1, 4, 24, and 48 hours after preparation of the solution.

Time after preparation:		0 hr.	1 hr.	4 hrs.	24 hrs.	48 hrs.
Control	990	5.5	7.5	9.5	6.0	--
	993	7.0	9.5	9.5	10.0	--
	997	7.0	10.0	10.0	10.0	--
Treatment	990	1.5	1.5	6.0	5.5	8.0
	993	0.0	0.0	0.0	4.0	9.0
	997	0.5	0.0	0.0	4.5	9.0

Effect of pH on Na_2SO_3 damage. Callus cultures were exposed to 500 ppm Na_2SO_3 solutions at different pH's (Table 10). The effect of pH was dramatic (Figure 1, the values are the means over cultivars). Since Na_2SO_3 solutions become more acidic due to oxidation of HSO_3^- to HSO_4^- and the ionization of HSO_4^- to H^+ and SO_4^{-2}, solutions of Na_2SO_3 must be prepared at a high initial pH so that the solutions do not approach the critical pH of about 3.5 during the course of an experiment. The increase in damage closely follows the increase in the theoretical concentration of H_2SO_3 molecules in solution (pk_a=1.8). This, perhaps, indicates more uptake of the uncharged H_2SO_3 molecules in comparison to the charged HSO_3^- ions.

Table 10. The effect of pH on mean TTC viability ratings of petunia callus after 24 hours exposure to 500 ppm Na_2SO_3.

pH:		7.0	6.5	6.0	5.5	5.0	4.5	4.0	3.5	3.0	2.5	2.0
Cultivar	990	8.0	7.0	7.0	8.0	7.0	9.0	7.0	7.0	8.0	5.0	3.0
	993	10.0	10.0	8.0	10.0	8.0	8.0	9.0	10.0	8.0	4.0	0.0
	997	10.0	9.0	8.0	8.0	5.0	9.0	8.0	7.0	4.0	1.0	0.0

Cultivar by Na_2SO_3 concentration factorial experiments. The effects of various Na_2SO_3 concentrations on callus cultures of various cultivars are shown in Table 11. Since the AOV's showed no interaction between Na_2SO_3 concentrations and cultivars, the means are averages over blocks and cultivars. The higher the mean, the less damage occurred. The effects of each concentration within a given medium were significantly different from each other (by L.S.D.), but the effect of

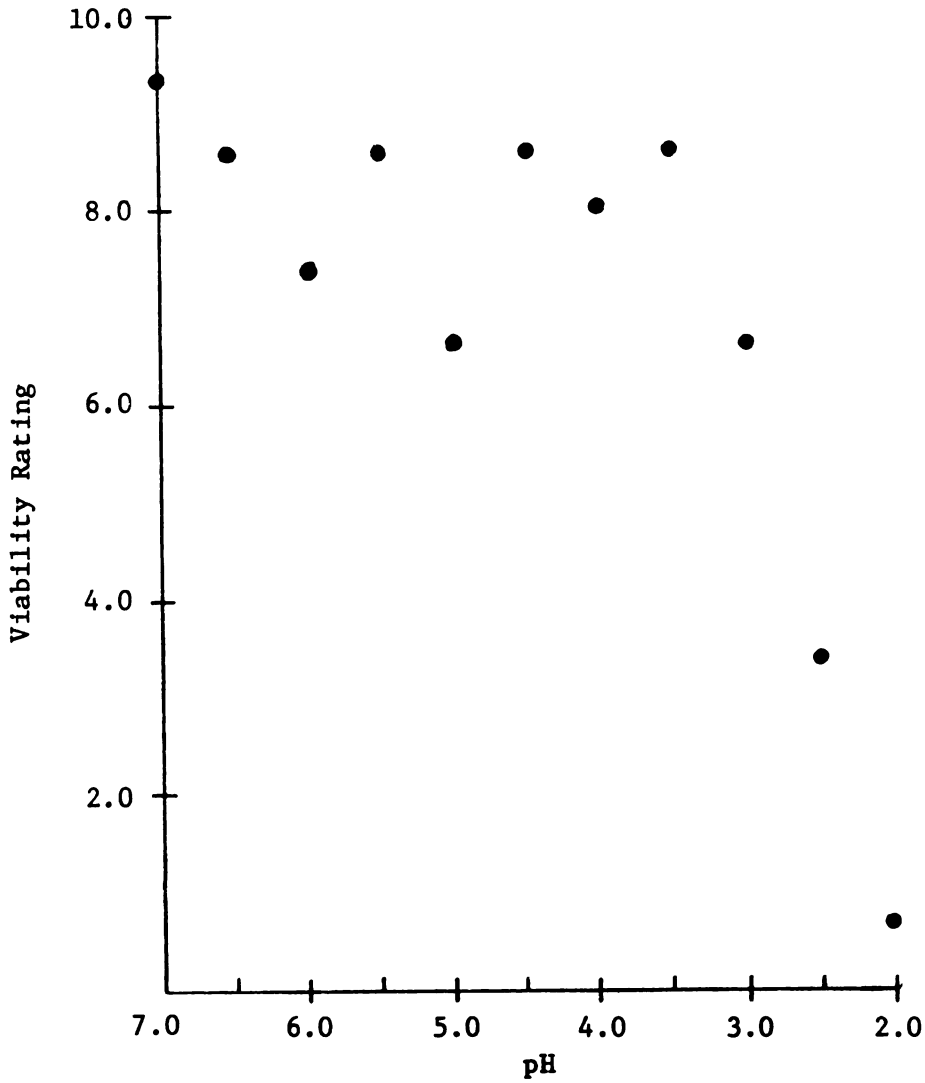

Figure 1. The effect of pH on mean TTC viability ratings of petunia callus after 24 hr exposure to 500 ppm Na_2SO_3.

the same concentration between the two media were not significantly different from each other (by the t-test).

Table 11. The effect of Na_2SO_3 concentrations on mean TTC viability ratings of petunia callus, all cultivars, after 3 hours exposure to solutions at initial pH of 5.0 to 5.5.

Na_2SO_3 concentration	2.5 mM	5.0 mM	7.5 mM
Mean normalized viability MSNP[1]	8.34	4.84	1.92
Mean normalized viability PM[2]	7.92	3.32	0.5

[1] ($L.S.D._{.05}$ = 1.28)

[2] ($L.S.D._{.05}$ = 1.26)

Table 12 shows the comparison among the cultivars within a medium. Again, since there was no interaction, cultivar values are averages over blocks and Na_2SO_3 concentrations. Duncan's multiple range test (34) was used for mean separation. Only 1006 cultured on MSNP was significantly different from the others while there were no significant differences among the cultivars on PM. The analyses of pairs of blocks that were run together are shown in Table 13. The results are not consistent with each other. This was probably due to some environmentally induced variation of critical physiology as reflected by variation of the viability of the controls.

Correlation coefficients were determined for the ratings of the controls with the sum of the normalized rating of the three treatments. If all blocks and all cultivars were analyzed individually, then the correlations were highly significant for MSNP (r = 0.560**) and PM

Table 12. Mean TTC viability ratings of the callus of 5 petunia cultivars after 3 hours exposure to 2.5, 5.0 or 7.5 mM Na_2SO_3 at initial pH of 5.0 to 5.5. AOV showed no significant[2] differences among the cultivars on PM medium.

Cultivar	1006	1016	1012	1003	1015
Mean normalized viability MSNP[1]	7.29	4.93	4.67	4.66	3.57
Cultivar	1003	1016	1015	1006	1012
Mean normalized viability PM[2]	5.38	3.60	3.60	3.55	3.41

[1] ($L.S.D._{.05}$ = 1.40)

[2] ($L.S.D._{.05}$ = 1.62)

Table 13. Mean TTC viability ratings of the callus of 5 petunia cultivars after 3 hours exposure to 2.5, 5.0, and 7.5 mM Na_2SO_3. Analysis by pairs of blocks treated simultaneously.

Cultivars	1006	1003	1015	1012	1016
Means, MSNP, blocks III & IV	7.05	6.20	3.92	3.75	3.30
Cultivars	1006	1016	1003	1012	1015
Means, MSNP blocks V & VI	5.17	5.13	2.72	2.25	1.00
Cultivars	1003	1015	1006	1016	1012
Means, PM, blocks II & III	6.76	4.15	3.92	2.08	0.53
Cultivars	1003	1015	1006	1016	1012
Means, PM, blocks IV & V	5.75	3.71	3.03	2.80	2.77
Cultivars	1016	1012	1006	1003	1015
Means, PM, blocks VI & VII	5.62	5.02	4.83	4.43	3.32

(r = 0.440**) analyzed separately, and very highly significant if MSNP and PM 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 MSNP (r = 0.906*) where there was a concurrent significant difference among the means of the cultivars (Table 12). However, the correlation was not significant on PM (r = 0.265 ns) where there was not a concurrent significant difference among the means of the cultivars. Correlation coefficients between controls and corresponding sums of the normalized ratings within cultivars were also determined (Table 14). Generally, these were not significant.

Table 14. Correlation coefficient within cultivars of mean TTC viability ratings of controls with the sum of the normalized TTC viability ratings of the Na_2SO_3 treatments.

Cultivars	1003	1006	1012	1015	1016
MSNP (df = 4)	0.819**	0.317	0.312	0.638	0.604
PM (df = 5)	0.950**	0.195	0.539	0.103	0.390
MSNP & PM (df = 11)	0.738**	0.310	0.438	0.454	0.437

Results of NaCl experiments. The AOV of experiments of stressing callus with various concentrations of NaCl showed no significant differences among the cultivars, although the effect of the stress was significant. Also, the appearance of the NaCl-stressed callus was different from Na_2SO_3-damaged callus, the former being brown, and the latter being bleached. Correlation coefficients were determined for individual

controls with the sum of the normalized ratings (r = 0.438 ns) and for means over blocks (r = 0.878*). The value for the means over blocks was significant at the 5% level. Also, the Spearman rank coefficient (59) for the means over blocks was 1.00; i.e., perfect correlation.

Results of double stress experiments. Results of the first double stress experiment are given in Table 15. Each value is an average of the ratings of four pieces of callus. Although the NaCl treated callus was damaged disproportionately more by Na_2SO_3 than the callus not treated with NaCl, this difference was not significant. This was because the treatment with Na_2SO_3 on the callus not treated with NaCl was not significantly different from zero. Also, it was felt that the NaCl stress was too damaging. Consequently, the second experiment was conducted. The correlation coefficient between the rating of the controls and the normalized rating of the treatment was 0.439 and was not significant. However, AOV showed that the treatment of 3.5 mM Na_2SO_3 was also not significant. Therefore, a third experiment was conducted. Not only was there bacterial contamination, but the concentration of 5 mM Na_2SO_3 was too high since most of the ratings were close to 0% viability. The fourth experiment yielded some useful information. The raw data are shown in Table 16. The bacterial contamination of the 4% NaCl, 2.5 mM Na_2SO_3 complicated any analysis. However, it is obvious from the raw data that the stressed callus was not damaged by Na_2SO_3 disproportionately more than the unstressed callus. The correlation coefficient using OmM Na_2SO_3 as controls and the sum of the normalized ratings of 2.5 mM and 5.0 mM for the treatments of the 0% and 2% NaCl

Table 15. Mean TTC viability ratings of callus of cultivar 1006 after 24 hours exposure to 5% NaCl and/or 2 hours exposure to 5 mM Na_2SO_3.

NaCl	Na_2SO_3	Block I	Block II	Block III
-	-	8.00	2.00	9.75
-	+	0.50	0.00	1.25
+	-	1.50	1.00	1.50
+	+	0.00	0.00	0.00

Table 16. TTC viability ratings of callus of cultivar 1006 after 21 hours exposure to NaCl solutions and 3 hours exposure to Na_2SO_3 solutions.

	Rep.	I	II	III	IV	V
0% NaCl	0.0 mM Na_2SO_3	9	9	9	9	9
	2.5 mM Na_2SO_3	9	9	9	8	8
	5.0 mM Na_2SO_3	7	6	6	7	7
2% NaCl	0.0 mM Na_2SO_3	7	8	7	7	8
	2.5 mM Na_2SO_3	9	9	8	9	9
	5.0 mM Na_2SO_3	2	3	3	4	5
4% NaCl	0.0 mM Na_2SO_3	3	4	4	3	3
	2.5 mM Na_2SO_3	Contaminated				
	5.0 mM Na_2SO_3	3	4	5	3	7

groups was 0.363, which was not significant. The correlation coefficient between OmM and 5.0 mM normalized to the OmM value of the 0, 2, and 4% NaCl groups was 0.632 which was significant. The 4% NaCl stressed callus being damaged disproportionately less than the others. From the results of these four experiments it is obvious that although the double stress experiment was logical, it was difficult to obtain consistent results. Also, it is apparent that variation in the susceptibility to damage by Na_2SO_3 was not strictly related to environmentally induced variation within a given cultivar.

Dose Response of Leaf Discs and Callus Cultures to Na_2SO_3

Leaf discs and callus on MSNP and PM treated with Na_2SO_3 were evaluated by dose response curves. The response of material of two levels of biological organization were in close agreement with one another (Figure 2).

Summary of Experiments

The ranking for SO_2 or Na_2SO_3 injury of the various cultivars at the different levels of biological organization are given in Table 17. The ranking was different for each level of organization.

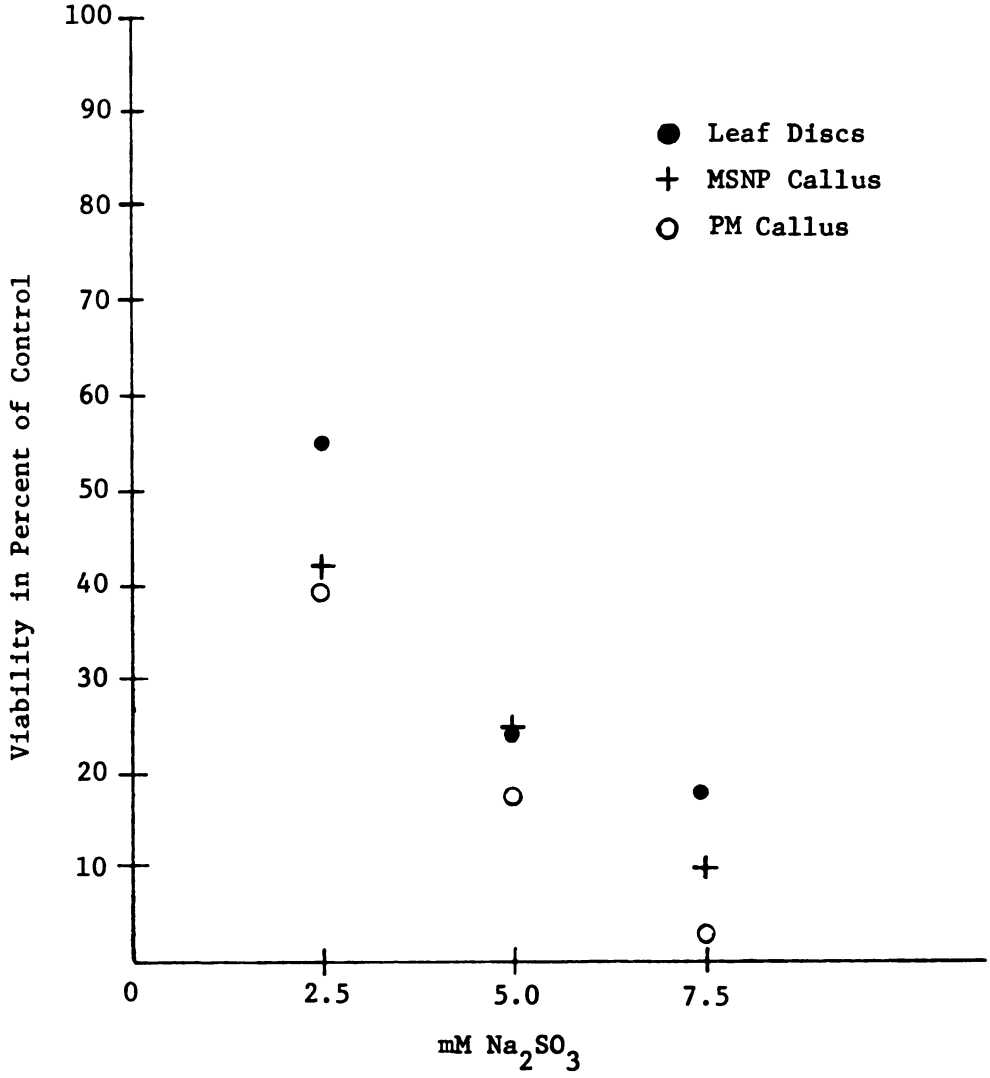

Figure 2. The effect of Na_2SO_3 concentration on the mean normalized absorbance of chlorophyll extracts of petunia leaf discs and on the mean normalized TTC viability ratings of petunia callus cultures. Exposures were for 3 hr.

Table 17. Rank of 5 petunia cultivars to SO_2 or Na_2SO_3 susceptibility at different levels of biological organization.

	Least susceptible			Most susceptible	
Whole Plant	1006	1003	1016	1012	1015
Leaf Disc	1006	1003	1012	1015	1016
Callus culture MSNP	1006	1016	1012	1003	1015
Callus culture PM	1003	1016	1015	1006	1012

DISCUSSION

Although the results of fumigating whole plants with SO_2 were from only two experiments, the overall trend of cultivar susceptibility was the same as reported by Feder et al. (15). Since variances were not reported by Feder et al. (15), significant differences in the ratings of the cultivars could not be determined. However, there seems to be one point of discrepancy: plants were treated by Feder et al. (15) with 2.5, 5.0 or 10.0 ppm SO_2 for one hour; whereas, in this report, plants were treated with 5.0 ppm for 16 hours in order to obtain a similar degree of injury. The difference in exposure times may have been necessary to compensate for the difference in stomatal opening associated with a difference in light intensity. Plants fumigated by Feder et al. (15) were in chambers in the greenhouse with a minimum illumination of 2.7 mwatts/cm^2; whereas, in this experiment, plants were under artificial lighting of 1.5 mwatts/cm^2. Relative humidity, temperature, CO_2 concentration, etc., could also have affected stomatal opening which in turn could have affected SO_2 injury.

Experiments using whole leaves were not useful in determining variation for SO_2 response due to cultivar differences. Environmental factors dominated the response as evidenced by the large block variance in relation to the cultivar and error variances. However, the experiments were useful in providing the necessary link between treatment

with SO_2 gas and treatment with Na_2SO_3 solutions. Although leaves in control solutions exhibited water-soaking, they did not lose turgidity as did the Na_2SO_3 treated leaves. Plants fumigated with SO_2 showed similar symptoms of water-soaking and wilting. Unlike the controls, the treated leaves also exhibited chlorotic and necrotic lesions as did fumigated plants. The use of Na_2SO_3 solutions is further justified by the results obtained by various authors (1,2,12,22,41,48,56,60,67) who also used Na_2SO_3 or $K_2S_2O_5$ solutions instead of SO_2 gas.

Experiments with leaf discs were more reliable than with whole leaves because 1) the effects were reproducible and consistent, and 2) the results were easier to quantify. Whole leaves were rated visually and equal weight was given to water-soaked damage, chlorosis, and necrosis; whereas, leaf discs were evaluated on an objective basis by spectrophotometric analysis of chlorophyll content. Another significant aspect of the leaf experiments was that the dose response of the discs was similar to that of the callus cultures (see Figure 2). Furthermore, these dose responses were of physiological significance as well. The concentrations of 2.5 mM, 5.0 mM, and 7.5 mM Na_2SO_3 correspond to 160, 320, and 480 ppm SO_2 dissolved in water. According to Malhotra and Hocking (37), 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, the experimental conditions for leaf discs and callus cultures were equivalent to exposures of SO_2 gas at concentrations of about 0.16, 0.32, and 0.45 ppm. These concentrations are an order of magnitude lower than that given to whole plants by Feder et al. (15) and herein.

Although the experiments with the leaf discs were physiologically significant, the results did not correlate with the whole plant response. Namely, 1016 was resistant as whole plants but was susceptible as leaf discs. Moreover, 1015 was susceptible as whole plants but was intermediate as leaf discs. Therefore, the making of leaf discs and exposing them to an aqueous Na_2SO_3 solution was sufficient pertubation of the whole plant-SO_2 gas system to allow different genetically controlled morphological and physiological factors to confer resistance or susceptibility. By-passing of the stomata is a logical hypothesis for the difference of response of whole plants in comparison to leaf discs because the primary mode of entry of SO_2 gas into the leaf is through the stomata as evidenced by the fact that most injury to a given plant occurs when the stomata are open (26,35). Likewise, the fact that significant damage occurs to leaf discs at a concentration that is an order of magnitude lower suggests that the stomata are no longer the primary point of entry. Instead the solutions seem to enter through the cut edges of the disc and diffuse towards the center of the disc as some discs remained green in the center while the edges were bleached.

The treatment of callus cultures with Na_2SO_3 damaged the cells. This damage was not due to pH or to excessive ionic strength as demonstrated by preliminary experiments of varying pH and NaCl concentrations. The NaCl stress experiments required approximately 10 times the ionic strength before symptoms developed, and the symptom before staining was browning of the cells instead of bleaching of the cells as caused by Na_2SO_3 treatment.

Aside from 1006 cultured on MSNP, there were no consistent significant differences among the cultivars when placed in culture. As whole plants, the 1003, 1006, and 1016 cultures were damaged less than 1012 and 1015. Therefore, no correlation was observed between *in vitro* cultured cells and their whole plant response to SO_2 (Na_2SO_3). There was a significant correlation between the viability of the cell cultures, as measured by TTC staining of the controls, and their response to Na_2SO_3 treatment. Less viable cultures were damaged disproportionately more than the more viable cultures. This same correlation existed when the cells were subjected to NaCl stress. Therefore, the variations and differences among blocks and cultures including 1006 on MSNP was 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 1006 on MSNP was not genetic in nature. The difference in viability (vigor) *in vitro* could have had a genetic as well as an environmental component. However, this possible genetic component was not the same one involved in determining the physiochemical structures directly affected by SO_2; for if it were, then 1006 should have been resistant on PM as well as on MSNP.

The idea that genetic factors are partially responsible for the resistance of 1006 on MSNP and not totally due to environmental factors is substantiated by three other observations. There was basically no correlation between viability and response within a given cultivar. If environmental factors were totally responsible for this correlation among the cultivars, then the correlation should have existed within

the cultivars. Likewise, there was no correlation between viability and response in the double stress experiment on 1006 when the viability was purposely altered. Again, if the correlation was due to environmental effects then correlation should have existed in these experiments. Helgeson *et al.* (25) found that although a dominant gene was expressed in culture on one medium, that gene was not expressed on another medium. Hence, there was a genetic-environment interaction. Therefore, it seems that the differences in response to SO_2 damage in culture was not simply due to environmentally induced differences in viability. Genetic factors affecting SO_2 response, perhaps by affecting viability *in vitro*, were involved as well, for there was a definite genetic-environmental interaction: response of the cultivars on medium PM was different from the response on medium MSNP.

Experiments using plant material at three different levels of organizations--whole plants, organs (leaf discs), and cells *in vitro*--have given different results when treated with SO_2 (Na_2SO_3). As whole plants, 1015 was significantly more susceptible to SO_2 injury than the rest. As leaf discs, 1016 was significantly more susceptible to Na_2SO_3 injury than the rest and 1006 was significantly more resistant than 1012, 1015, and 1016, but not 1003. Finally, as cells in culture, 1006 was significantly more resistant than the rest on medium MSNP, but not on medium PM. All of these differences could have had a genetic basis for SO_2 response at each level of organization. By artificially removing the constraints and complexities going from whole plants to cells in culture, different genetic systems affecting the response to SO_2

(Na_2SO_3) treatment may have been unmasked; but apparently the genetic systems of the different cultivars determining the proteins and/or structures that are physiochemically affected by SO_2 or its ions were not exclusively compared. Such a comparison would involve removing one more constraint; that is, testing and comparing proteins and/or structures in a cell-free system. Unfortunately, the most physiologically important system(s) directly involved with SO_2 injury are not known, although many systems have been implicated and, therefore, such comparisons would be of questionable significance at this time. Ballantyne (2) found that SO_2 injury was not related to mitochondrial response. Although Phaseolus vulgaris was more susceptible to SO_2 injury than Zea mays as whole plants, the responses of isolated mitochondria in vitro were not significantly different from each other. Likewise, a selection system involving cell-free proteins or structures would be impractical for a mutation-selection breeding scheme because plants can not be regenerated from selected, resistant, proteins or structures as from cells in culture.

Filner and Bressan (personal communication) have shown with cucurbits (whole plants) that resistance and susceptibility to SO_2 injury among cultivars correlated with SO_2 uptake by the plants. Furthermore, leaf discs of the various cultivars floated in $K_2S_2O_5$ solutions showed no significant differences in injury. They concluded that genetic differences in cuburbits as whole plants was stomate related and not cellularly related. Their results and the results presented herein lead to the same conclusion: the genetic basis of resistance

of whole plants to SO_2 damage is whole plant related and not leaf, organ, or cellularly related.

A speculative model may be presented in order to summarize the responses and the significance of the differences of the responses at different levels of organization. As whole plants stomata may be primarily responsible for resistance or susceptibility. Their number, size, response to SO_2 by closing, or a combination of these may be important. As leaf-discs differences in active transport of HSO_3^- or physiochemical structure of the intercellular matrix may lead to differential uptake. Likewise, a hypersensitive reaction to physical injury by cells at the cut surface could form a partial barrier to HSO_3^- uptake by alteration of the intercellular matrix to a less permeable form or by the release of cellular components that oxidize sulfite to sulfate. And general physiological vigor may be necessary in order for HSO_3^- to be actively excluded by cells in culture. The importance of this model is not its accuracy but the idea that resistance and susceptibility could be controlled by different genetic systems at different levels of organization and that none of these genes could have any direct influence in determining the proteins and structures that are injured by exposure to SO_2 (Na_2SO_3).

In conclusion, this project has demonstrated two ideas of importance in studying the genetics and physiology of traits of complex systems and the use of such studies in cell culture techniques for plant improvement. In general, tissue culture is a useful system in studying the physiological genetics of certain traits. By removing the

complexities and interactions of organized tissues, organs, or whole plants, the traits of the unconstrained cells can be studied. Likewise, the physiological genetics of a trait may be different at each level of organization. In particular, by removing these constraints, it has been shown that at different levels of organization, different genetic systems may be important in determining responses of Petunia hybrida to treatment with SO_2 (Na_2SO_3). Consequently, the genetic basis for SO_2 resistance apparently does not involve the genes that determine the proteins and/or structures that are damaged by SO_2 or its ions in solution; but rather resistance involves genes that control indirect effects such as SO_2 uptake by stomata. Therefore, at this time the most effective method of selection of desirable phenotypes for resistance to SO_2 in Petunia hybrida is to screen whole plants by fumigation with SO_2.

APPENDIX

RECOMMENDATIONS

To further understand the physiology and genetics of SO_2 resistance, the following approaches are recommended:

1) Investigate the possible correlation of the genetics of stomate parameters and SO_2 damage by studying inbred parents, F_1, F_2, etc., generations of Petunia hybrida cultivars 1006 and 1015, as the response of these two cultivars to SO_2 treatment as whole plants were at extremes.

2) Study whole plant responses as influenced by manipulating stomatal function.

3) Use tissue culture of divergent species and/or genera in order to obtain a wide genetic base of SO_2 resistance to see if there are genetic differences in proteins and structures of the cell affected by SO_2.

4) Compare cell-free protein and/or structures implicated in SO_2 damage of these divergent species and/or genera to possibly determine which systems are most significant at the subcellular level.

APPENDIX

TABLES OF AOV's

Table A1. AOV: Whole Plants.

Source	df	SS	MS	F	$F_{.05}$
Total	9	2292			
Blocks	1	198			
Cv's	4	2002	501	21.78	6.39
Error	4	92	23		

Table A2. AOV: Whole Leaves.

Source	df	SS	MS	F	$F_{.05}$
Total	19	24554			
Blocks	3	20733	(6911)	(25.22)	
Cv's	4	532	133	0.49	3.26
Error	12	3289	274		

Table A3. AOV: Leaf Discs.

Source	df	SS	MS	F	$F_{.05}$
Total	44	16330			
Blocks	2	504			
Treatments	14	14065			
Cv's	4	2109	527.25	8.38	2.71
Na_2SO_3	2	11295	5647.50	89.80	3.34
Cv x Na_2SO_3	8	661	82.63	1.31	2.29
Error	28	1761	62.89		

Table A4. AOV: MSNP Medium.

Source	df	SS	MS	F	$F_{.05}$
Mp[1] total	17	935.9			
Blocks	5	263.8			
Na_2SO_3	2	622.5	311.25	62.75	4.10
Error a	10	49.6	4.96		
Sp[2] total	89	1347.5			
Mp total	17	935.9			
Cv's	4	134.3	33.58	7.61	2.52
Cv x Na_2SO_3	8	12.9	1.61	0.37	2.10
Error b	60	264.4	4.41		

[1] Mp = Mainplot

[2] Sp = Subplot

Table A5. AOV: PM Medium.

Source	df	SS	MS	F	$F_{.05}$
Mp total	20	1148			
Blocks	6	98			
Na_2SO_3	2	980	490.0	84.0	3.88
Error a	12	70	5.83		
Sp total	104	1765			
Mp total	20	1148			
Cv's	4	57	14.25	2.05	2.50[1]
Cv x Na_2SO_3	8	59	7.38	1.05	2.07[1]
Error b	72	501	6.96		

[1]These F values are for 70 degrees of freedom for the error variance.

Table A6. AOV: NaCl Stress.

Source	df	SS	MS	F	$F_{.05}$
Mp total	8	371			
NaCl	2	290	145	10.75	5.14
Error a	6	81	13.5		
Sp total	44	623			
Mp total	8	371			
Cv's	4	44	11	2.53	2.78
Cv x NaCl	8	104	13	3.00	2.36
Error b	24	104	4.33		

Table A7. AOV: Second Double Stress.

Source	df	SS	MS	F	$F_{.05}$
Mp total	9	1396			
Blocks	1	58			
NaCl	4	1237	309.25	12.24	5.19
Linear	1	1232	1232	48.79	7.71
Residual	3	5	1.66	ns	
Error a	4	101	25.25		
Sp total	19	1529			
Mp total	9	1396			
Na_2SO_3	1	7	7	.301	
NaCl x Na_2SO_3	4	10	2.5	.107	
Error b	5	116	23.2		

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