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CHANGES IN ABSCISIC ACID, STOMATAL CONDUCTANCE, AND ANTIOXIDANTS DURING LOW TEMPERATURE PRECONDITIONING AGAINST SO_2 INJURY IN CONTRASTING CULTIVARS OF COLEUS

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KRIZEK, D. T., TERRY, P. H., UPADHYAYA, A., CALDWELL, C. R. and MIRECKI, R. M. *Changes in abscisic acid, stomatal conductance, and antioxidants during low temperature preconditioning against SO_2 injury in contrasting cultivars of coleus.* BIOTRONICS **30**, 1–14, 2001. The influence of 5 days of low temperature preconditioning (13℃) on SO_2 sensitivity in relationship to abscisic acid (ABA) concentration, stomatal conductance, transpiration rate, and SO_2 injury was examined in two cultivars of coleus found previously to differ in stress tolerance: SO_2-sensitive 'Buckley Supreme' ('BS') and SO_2-insensitive 'Marty' ('M'). 'BS' plants grown at 20℃ for 5 days and then fumigated with SO_2 for 2 hr had 75% overall injury; those grown at 13℃ for 5 days had only 13% SO_2 injury. Corresponding 'M' plants given 20 and 13℃ pretreatment had 17 and 0% injury, respectively. This decrease in SO_2 sensitivity following preconditioning at 13℃ was associated with an increase in ABA concentration and a decrease in stomatal conductance and transpiration (Tn) rate, which presumably decreased the penetration of SO_2 into the leaf. Changes in the levels of several antioxidants and associated enzyme systems were also determined. Stress-insensitive 'M' contained 47% more ascorbate (AsA) and 17% greater ascorbate peroxidase (AP) activity than stress-sensitive 'BS.' Temperature preconditioning increased AP activity in 'BS' by 11% which was comparable to that found in 'M' at 20℃. Thus, the phytoprotection afforded

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against SO_2 by temperature preconditioning appears to involve both avoidance and detoxification mechanisms.

Key words: ABA; avoidance mechanisms; *Coleus blumei*; cross protection; detoxification mechanisms; SO_2 tolerance; stress tolerance; temperature hardening.

INTRODUCTION

Cultivar differences in coleus have been observed in response to a wide range of environmental stresses, including UV-B radiation, drought, sulfur dioxide (SO_2), chilling (*21, 24, 25, 27, 28*), and aluminum toxicity (*12*). Preliminary experiments conducted in our laboratories have indicated that hardening coleus and poinsettia plants for several days at 13℃, a temperature close to the critical phase shift of some cell membrane components, confers protection against subsequent exposure to chilling (*24*) and air pollutants (*27*).

The present study was conducted to examine the role of both avoidance and detoxification mechanisms involved in temperature preconditioning. The role of avoidance mechanisms was examined by determining whether the protection afforded by temperature pretreatment might involve changes in endogenous levels of abscisic acid (ABA), that in turn might alter stomatal behavior and transpiration rate, and if so, whether there were any differences in cultivar response to temperature pretreatment. Since ABA synthesis and/or accumulation is believed to be localized in the chloroplast (*17, 18*), measurements were also made of chlorophyll concentrations to determine possible correlations with ABA content. To investigate the role of detoxification mechanisms, measurements were made of the levels or activities of key antioxidants and associated enzymes to ascertain whether possible differences could account for the differences in stress tolerance between 'M' and 'BS.'

Oxidative damage is considered to be an early response of plant tissues sensitive to chilling (*16, 33*), air pollutants (*2*) and other environmental stresses (*8*). Such damage is caused by the generation of highly reactive free radical species and the oxidative breakdown of cell membranes through lipid peroxidation (*6, 35*). There are three primary classes of antioxidant defense systems: (a) the lipid soluble, membrane-associated antioxidants (e.g., α-tocopherol and β-carotene); (b) the water soluble reductants (e.g., ascorbate (AsA) and glutathione (GSH); and (c) enzymatic antioxidants [e.g., superoxide dismutase (SOD), catalase (CAT) and enzymes of the ascorbate/glutathione cycle] (*33*). In concert, they play an important role in scavenging free radicals generated during oxidative stress (*2, 8, 13*).

Chemical names used: ABA, abscisic acid; AP, ascorbate peroxidase (EC 1.11.1.11); AsA, ascorbate; CAT, catalase (EC 1.11.16); GR, Glutathione reductase (EC 1.6.4.2); GSH, reduced glutathione; GSSG, oxidized glutathione; PER, peroxidase (EC 1.11.1.7); SOD, superoxide dismutase (EC 1.15.1.1)

Ascorbate (AsA) is a key antioxidant that protects cell constituents against oxidative or photooxidative damage by scavenging hydrogen peroxide (H_2O_2) and hydroxyl radicals. This function is mediated by ascorbate peroxidase (AP), an enzyme that is specific to plants (*5*). In the chloroplast, the ascorbate enzyme system is important as a detoxification system and as a regulator of electron flow *in vivo* (*2*).

In addition to its role as a primary antioxidant, AsA is also an important secondary antioxidant, maintaining the α-tocopherol pool that scavenges radicals in the inner regions of membranes (*2*). Glutathione occurs widely in plant cells, and its reduced form (GSH) is another important antioxidant (*1, 2*). A high cellular content of GSH is maintained by glutathione reductase (GR), which catalyzes the reduction of GSSG to GSH. A high GSH/GSSG ratio appears to be required for detoxification of active oxygen species and for plant adaptation to environmental stress (*2, 34*).

MATERIALS AND METHODS

Plant material and cultural procedures

Plant material consisted of two cultivars of coleus [(*Coleus blumei* Benth.= *Solenostemon scutellarioidef* (L.) Codd] found previously to differ in sensitivity to a wide range of stresses, with 'Buckley Supreme' ('BS') being highly sensitive, and 'Marty' ('M') being relatively insensitive (*27, 28*). Cuttings were obtained from clonal stocks of the above cultivars and propagated in a charcoal-filtered greenhouse on a hot pad (29℃) under mist in a synthetic peat-vermiculite mix (Jiffy Mix, Jiffy Products of America, West Chicago, IL, USA*). After two weeks, cuttings were selected for uniformity, transplanted to 12.7 cm diam plastic pots containing peat-vermiculite mix, and kept under mist for 2–3 days to prevent transplanting shock. Plants were maintained in the greenhouse under a 16 hr photoperiod at 27±3℃ day temperature, 20±2℃ night temperature, ambient CO_2, and ambient relative humidity. Daylength was controlled by means of supplemental incandescent lamps which were kept on from 4:00 p.m. to 9:00 p.m. and from 5:00 a.m. to 8:00 a.m.

Temperature preconditioning

Ten days after transplanting, the cuttings were grown for 5 days in Revco (Asheville, NC) Model 511-38 growth chambers maintained at constant temperatures of either 13 or 20±0.5℃, 70±5% relative humidity, and ambient CO_2. Plants were grown under a 16 hr photoperiod at 320 μmol m^{-2} s^{-1} of photosynthetic photon flux (PPF) provided by 1500 mA cool white fluorescent lamps.

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Abscisic acid determination

ABA levels were determined on the third pair of leaves from the apex (the first pair was included if it was 2 cm or greater in length). One pair of leaves was collected from each of four plants. Approximately 5 to 10 g of leaves were harvested at approximately 1,430 hr, homogenized in 70% acetone, and extracted as described by Terry et al. (*30*).

Chlorophyll concentration

Chlorophyll measurements were made using six, 5 mm diam leaf discs or half leaf samples harvested from the fourth node from the apex. Three replicates were used per treatment. Tissue was extracted in 80% acetone, washed twice on successive days with 80% acetone, and brought to volume in graduated centrifuge tubes. The absorbance of the chlorophyll extract was measured at 645 and 663 nm, using a Gilford Model 330-N (Gilford Instruments Laboratories Inc., Oberlin, OH) Micro Sample Spectrophotometer. The concentrations of chlorophyll a, chlorophyll b, and total chlorophyll were determined as described by Arnon (*3*).

Plant water relations

After 5 days of temperature preconditioning, measurements of stomatal resistance and instantaneous transpiration rates were made on the fourth or fifth leaf from the top of the plant, on at least 4 replicates, using a LI-COR Model 1600 Steady State Porometer (Lincoln, NE). Stomatal conductances were obtained by taking the reciprocals of the stomatal resistances. Transpiration rates on a whole plant basis were obtained by enclosing each pot in a tightly secured polyethylene bag prior to placing the plants in the SO_2 fumigation chamber and then determining changes in fresh weight after 2 or 4 hr of fumigation and again 24 hr after the initial weighing. Transpiration rates were then calculated on a per hr basis.

SO_2 fumigation conditions

The SO_2 treated plants were fumigated in a Controlled Environments, Inc. (Conviron) Model PGW 36 growth chamber for 2 or 4 hr at 2 μmol mol^{-1} SO_2. The environmental conditions during fumigation were as follows: 25℃ temperature, 70% relative humidity, and 320 μmol m^{-2} s^{-1} of PPF provided by cool white fluorescent lamps. Plants were equilibrated in the chamber for 1–2 hr before SO_2 fumigation was begun. Sulfur dioxide concentration was monitored with a TECO Model 43 SO_2 analyzer (Thermo Electro Corp., Hopkinton, MA 01748). A TECO Model 143 calibrator containing a SO_2 permeation tube was used to calibrate the analyzer. The concentration of SO_2 was controlled with a Model MDCM-1 SO_2 Concentration Controller and Monitor (Tuttle Electronics, 3020 Bosseau Ave., Southold, NY 11971).

SO_2 injury ratings

Following fumigation, the plants were moved to a charcoal-filtered

greenhouse for evaluation of SO_2 injury. SO_2 injury was scored according to three criteria: a) percentage of leaves showing any damage; b) highest injury rating; and c) overall injury rating. Ratings were made 24 hr after fumigation. Damage was estimated on a scale of 0 (leaves without injury) to 100% (complete loss of leaf or 100% injury).

Enzyme extraction

Fresh leaf tissue was taken from three separate plants, fresh weights determined, and immediately homogenized with a Model PT0/35 polytron (Brinkmann Instruments, Inc., Westbury, NY) in 50 mM Tris–HCl (pH 7.0) containing 1 mM EDTA, 3 mM $MgCl_2$, and 1% polyvinylpyrrolidone (PVP) at 4℃. The supernatant recovered after centrifugation at 10,000 rpm for 20 min was used immediately for enzyme assays.

Enzyme assays

Activities of CAT and peroxidase (PER) were assayed by measuring the rate of disappearance of H_2O_2 using the method of Maehly and Chance (*23*). For CAT activity, the decrease in H_2O_2 was followed as a decline in absorbance at 240 nm ($\varepsilon = 39.4\ M^{-1}\ m^{-1}$). For guaiacol-dependent PER, the increase in absorbance at 470 nm was followed for 1 min ($\varepsilon = 2.67\ M^{-1}\ m^{-1}$). Reaction mixtures for both CAT and PER were the same as described by Upadhyaya et al. (*32*). SOD was assayed by measuring the ability to inhibit the photochemical reduction of nitroblue tetrazolium, following the procedure described by Upadhyaya et al. (*32*). The volume of enzyme extract corresponding to 50% inhibition of the reaction was considered as one enzyme unit (*7*).

AP was determined by measuring the decrease in absorbance at 290 nm as AsA was oxidized ($\varepsilon = 0.28\ M^{-1}\ m^{-1}$) (*4*). The reaction mixture contained 50 mM potassium phosphate (pH 7.5), 0.5 mM AsA, 1 mM EDTA, 0.1 mM H_2O_2, and enzyme extract. Correction was done for the low nonenzymatic oxidation of AsA by H_2O_2. In the experiments reported here, ascorbate was not added to the extraction buffer. However, addition of ascorbate to the extraction medium did not seem to have any significant effect on AP activity.

GR was measured by following the decrease in absorbance at 334 nm due to the oxidation of NADPH ($\varepsilon = 0.62\ M^{-1}\ m^{-1}$) (*19*). The reaction mixture contained 50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.1 mM NADPH, 1 mM GSSG, and enzyme extract. Substrate specificity was tested with 0.1 mM NADH instead of NADPH and was found to have only a negligible effect.

All enzyme activities were assayed at 25℃ and, except for SOD, they were calculated using their extinction coefficients.

Ascorbate analysis

AsA concentration was determined according to the procedure described by Senaratna et al. (*29*). Fresh leaf tissue was homogenized in 5% trichloroacetic acid (TCA) and centrifuged at 8,000 rpm for 20 min. The supernatant was collected in separate tubes and used for AsA determination. To 0.3 ml of

supernatant, 0.3 ml each of 40 μM 1,4-dithiothreitol (Cleland's reagent) in ethanol, 0.2 M sodium phosphate, and 1.2 M NaOH was added. The reaction mixtures were then incubated at room temperature for 10 min to allow for the conversion of dehydroascorbate to AsA. After this period, 0.3 ml of each of the following were added sequentially: 0.2 mM N-ethylmaleimide in ethanol, 20% TCA, 0.4 mM phosphoric acid in ethanol, 0.5% bathophenanthroline, and 18 μM ferric chloride in ethanol. The samples were then diluted to a final volume of 5 ml with ethanol and permitted to stand at room temperature for 1 hr. The absorbance was read at 534 nm and AsA concentrations were determined by comparison to a standard curve.

Measurement of glutathione

The concentrations of GSH and GSSG were determined by modifications of the method of Farris and Reed (*11*). Fresh leaf tissue was homogenized with a polytron in 15% aqueous perchloric acid containing 15 mM bathophenanthroline disulfide (BPDS). The homogenate was centrifuged at 11,000 g for 10 min with 250 μl of the supernatant used for derivatization. After addition of 10 μl of internal standard (0.2 mg ml^{-1} glutamy-glutamate (Glu-Glu) in 0.3% perchloric acid) the supernatant was carboxy-methylated and then derivatized with 2,4-dinitro-1-fluorobenzene.

Fifty (50) μl samples were injected into a Waters 600 series HPLC system equipped with a model 990 diode array detector and a 100 mm Machery Nagel nucleosil 120 (7 μ) amine cartridge column coupled to a 30 mm guard column with the same packing material. The dinitrophenol derivatives were separated using the same solvent system as Farris and Reed (*11*) except that the gradients were modified. After sample injection, the column was eluted with 80% solvent A (80% aqueous methanol)/20% solvent B (0.5 M sodium acetate in 64% aqueous methanol) for 5 min followed by a 15 min linear gradient to 1% solvent A and 99% solvent B. After holding for 10 min, the solvent proportions were then returned to the initial ratio over 1 min and the column washed for 4 min prior to the next sample injection. The absorbance at 365 nm was recorded and the peak areas integrated using the Water's chromatography system software. Identification and quantification of GSH and GSSG was performed using authentic standards derivatized as described above.

Statistical analysis

The statistical design was completely randomized. Differences referred to as significant were at $P \leq 0.05$ as determined by Analysis of Variance (Anova) using PC SAS version 6.04. Means of SO_2 injury ratings, transpiration rates, and stomatal conductance were subjected to analysis of variance using PC SAS version 6.04 and tested for significance at the 0.05 level of probability applying Duncan's multiple range test. Standard errors of the mean were determined for all data.

RESULTS

ABA and chlorophyll concentration

Temperature preconditioning had a significant effect on ABA concentration in both SO_2-sensitive 'BS' and SO_2-insensitive 'M' (Table 1). In both cases, leaves of plants grown for 5 days at 13℃ contained more ABA than those of plants grown at 20℃. However, the total chlorophyll concentration in 'M' was significantly greater at 20℃ than at 13℃, while the opposite was true in 'BS' (Table 1). Thus, there was no correlation between ABA concentration and chlorophyll concentration.

Water relations

Instantaneous measurements of stomatal conductance and transpiration rates of plants grown for 5 days at 13℃ were one third to one half as great as those treated at 20℃. This was true irrespective of the cv. (Table 2). At both temperatures, SO_2-sensitive 'BS' had significantly greater stomatal conductances and transpiration rates than did SO_2-insensitive 'M.' On a whole plant basis, similar reduction in transpiration rate was observed in low temperature preconditioned plants after 2 and 4 hr of fumigation (Table 3).

SO_2 injury

SO_2 injury was greatly reduced in 'BS' and completely prevented in 'M' by preconditioning plants for 5 days at 13℃ prior to fumigation. SO_2-sensitive 'BS' plants grown at 20℃ for 5 days and then fumigated for 2 hr had 75% overall SO_2 injury while those grown at 13℃ for 5 days had only 13% injury. Corresponding SO_2-insensitive 'M' plants had 17% and 0% injury. Increasing the duration of exposure to 4 hr increased SO_2 damage at 20℃ in 'BS' but had no effect at 13℃ in either cultivar (Table 3).

Table 1. Abscisic acid (ABA) and chlorophyll concentration in leaves of 'Buckley Supreme' and 'Marty' coleus grown in a growth chamber 5 days at 13 or 20℃.[z, y]

Coleus cultivar	Temperature Treatment ℃	ABA concentration (ng g^{-1} fr wt)	Chlorophyll concentration ($\mu g\ mg^{-1}$)
Buckley Supreme	13	21.70±2.30 a (12)	4.95±0.30 a (21)
	20	15.86±2.38 b (11)	4.43±0.17 b (21)
Marty	13	24.30±2.27 a (12)	4.49±0.15 b (18)
	20	11.11±2.26 b (12)	5.24±0.27 a (18)

[z]Plants grown in growth chamber under CWF lamps at 320 $\mu mol\ m^{-2}\ s^{-1}$ of PPF. Means ±SEM. Number of replicates shown in parentheses.

[y]Means for any variable not having a letter in common are significantly different at $p \leq 0.05$ applying LSM (ABA) or Duncan's multiple range test (Chl).

Table 2. Stomatal conductance and transpiration rate of 'Buckley Supreme' and 'Marty' coleus grown in a growth chamber 5 days at 13 or 20℃. Instantaneous measurements of stomatal conductance and transpiration rate made with a LI-COR steady state porometer.[z, y]

Coleus cultivar	Temperature treatment ℃	Stomatal conductance ($cm\ s^{-1}$)	Transpiration rate ($\mu g\ cm^{-2}\ s^{-1}$)
Buckley Supreme	13	0.33±0.02 c (27)	1.61±0.09 c (27)
	20	0.72±0.02 a (35)	3.48±0.12 a (35)
Marty	13	0.20±0.01 d (26)	1.10±0.07 d (26)
	20	0.59±0.06 b (28)	3.03±0.22 b (28)

[z]Plants grown in growth chamber under CWF lamps at 320 $\mu mol\ m^{-2}\ s^{-1}$ of PPF. Means ±SEM. Number of replicates shown in parentheses.
[y]Means for any variable not having a letter in common are significantly different at $p \leq 0.05$ applying Duncan's multiple range test.

Table 3. Effects of duration of SO_2 exposure (2 $\mu mol\ mol^{-1}$) and preconditioning temperature (13 or 20℃ for 5 days) on SO_2 injury and transpiration (Tn) rate of 'Buckley Supreme' and 'Marty' coleus. Transpiration rate determined on a whole plant basis after 2 and 4 hr of SO_2 fumigation and 24 hr later.[z]

Coleus cultivar	SO_2 hr	Temp ℃	SO_2 injury rating (%)			Tn rate ($g\ hr^{-1}$ $plant^{-1}$)		
			overall rating	highest rating	%lvs damaged	after SO_2 2 hr	after SO_2 4 hr	after SO_2 24 hr
Buckley Supreme	2	13	13.3 c	16.7 c	13.3 c	4.2 b	—	1.8 a
	2	20	75.0 b	75.0 b	81.7 b	7.4 a	—	2.0 a
	4	13	10.0 c	13.3 c	13.3 c	—	3.1 b	1.7 a
	4	20	90.0 a	90.0 a	95.0 a	—	5.3 a	1.7 a
Marty	2	13	0.0 d	0.0 d	0.0 d	1.8 c	—	1.1 b
	2	20	16.7 b	20.0 b	33.3 b	3.6 b	—	1.6 a
	4	13	0.0 d	0.0 d	0.0 d	—	1.8 c	1.1 b
	4	20	23.3 b	33.3 b	26.7 b	—	2.8 b	1.3 b

[z]Means for any variable having a different letter are significantly different at $p \leq 0.05$ applying Duncan's multiple range test.

Enzyme assays

Overall, coleus plants grown at 13℃ had 18% greater PER, 18% lower SOD, and 38% lower CAT activities relative to 20℃-grown controls (Table 4). Temperature preconditioning at 13℃ increased the enzyme activity of AP in 'BS' by 11% which was comparable to that found in 'M' at 20℃ (Table 5). However, preconditioning had no effect on GR activity in either cultivar (Table 5). Stress-insensitive 'M' had 17% more AP, 35% lower SOD activity, and 52% lower PER

Table 4. Catalase, peroxidase, and superoxide dismutase (SOD) activity in leaves of 'Buckley Supreme' and 'Marty' coleus after plants were grown 5 days at 13 or 20℃. Anova summary shown for overall effects and cv× temp interactions.[z, y]

Coleus cultivar	Temp ℃	Catalase μmol min^{-1} g fwt^{-1}	Peroxidase μmol min^{-1} g fwt^{-1}	SOD units min^{-1} g fwt^{-1}
Buckley Supreme	13	0.66±0.06 b	0.63±0.05 a	31.94±1.60 b
	20	1.26±0.08 a	0.60±0.04 a	38.05±2.25 a
Marty	13	0.84±0.05 b	0.36±0.02 b	20.14±1.04 d
	20	1.17±0.10 a	0.23±0.02 c	25.31±1.42 c
Source of variation		Anova summary (F values)		
cultivar (cv)		0.42^{NS}	158.60***	95.01***
temperature (temp)		47.43*	8.90**	20.09***
cv×temp		3.83^{NS}	3.47^{NS}	0.14^{NS}

[z]Means of 9 plants (from 3 expts) ±SEM. Means for any variable not having a letter in common are significantly different at $p \leq 0.05$ applying LSM.
[y]*, **, ***=significant at $p \leq 0.05$, 0.01, 0.001, respectively; [ns], not significant.

Table 5. Ascorbate peroxidase (AP) and glutathione reductase (GR) activity in leaves of 'Buckley Supreme' and 'Marty' coleus plants grown 5 days at 13 or 20℃. Anova summary shown for overall effects and cv×temp interactions.[x, y]

Coleus cultivar	Temp ℃	AP	GR
		μmol min^{-1} g fwt^{-1}	
Buckley Supreme	13	3.34±0.18 b	0.41±0.18 a
	20	3.02±0.13 b	0.40±0.30 a
Marty	13	4.10±0.15 a	0.46±0.02 a
	20	3.35±0.15 b	0.39±0.02 a
Source of variation		Anova summary (F values)	
cultivar (cv)		15.56***	0.58^{NS}
temperature (temp)		14.84***	2.59^{NS}
cv×temp		2.48^{NS}	1.08^{NS}

[z]Means of 9 plants (from 3 expts)±SEM. Means for any variable not having a letter in common are significantly different at $p \leq 0.05$ applying LSM.
[y]*, **, ***=significant at $P \leq 0.05$, 0.01, 0.001, respectively; [ns], not significant.

activity than stress-sensitive 'BS.' There were no cultivar differences in CAT or GR activity and no cultivar×temperature interactions (Tables 4 and 5).

Ascorbate analysis

There were significant cultivar differences in AsA concentration. Stress-insensitive 'M' contained 47% more AsA than did stress-sensitive 'BS.' There

Table 6. Ascorbate (AsA), reduced glutathione (GSH), oxidized glutathione (GSSG), and GSH/GSSG ratio in leaves of 'Buckley Supreme' and 'Marty' coleus grown 5 days at 13 or 20℃. Overall effects and cv× temp interactions shown in Anova summary.[z, y]

Coleus cultivar	Temp ℃	AsA	GSH	GSSG	GSH/GSSG
		mg g fwt^{-1}	nmol g fwt^{-1}	nmol g fwt^{-1}	
Buckley Supreme	13	3.2±0.2 b	92.0±7.6 c	6.0±0.6 b	18.1±4.1 bc
	20	3.5±0.2 b	124.4±12.2 ab	5.6±0.6 b	23.4±2.1 ab
Marty	13	5.0±0.3 a	132.2±11.7 a	4.6±0.4 b	28.8±2.6 a
	20	4.8±0.2 a	100.5±7.6 bc	8.5±0.7 a	13.5±1.8 c
Source of variation			Anova summary (F values)		
cv		101.18***	0.66^{NS}	1.56^{NS}	0.02^{NS}
temp		0.24^{NS}	0.00^{NS}	9.91*	3.25^{NS}
cv×temp		2.51^{NS}	10.24**	14.03**	13.61**

[z]Means±SEM. n=9 for AsA; 6 for GSH; and 3 for GSSG and GSH/GSSG ratio. Sources of variation: cv, cultivar, temp, temperature. Means for any variable not having a letter in common are significantly different at $P \leq 0.05$ applying LSM.
[y]*, **, ***=significant at $P \leq 0.05$, 0.01, 0.001, respectively; [ns], not significant.

were no differences, however, in AsA due to temperature preconditioning. There were no cultivar×temperature interactions (Table 6).

Glutathione

There was no overall cultivar or temperature effect for GSH, or GSH/GSSG ratio, and no overall cultivar effect for GSSG, based on an analysis of variance (Table 6). In general, temperature preconditioning at 13℃ decreased GSSG levels but there were cultivar×temperature interactions (Table 6). Consequently, low temperature preconditioning increased GSH in 'M,' but reduced it in 'BS.' GSSG was unaffected in 'BS' and reduced in 'M' to a level below that in 'BS.' Therefore, temperature pretreatment at 13℃ had opposite effects on the GSH/GSSG ratio in the two cultivars, doubling it (from 13.5 to 28.8) in stress-insensitive 'M' while decreasing it by 23% (from 23.4 to 18.1) in stress-sensitive 'BS.' At 20℃, the GSH/GSSG ratio in Marty was just over half that of 'BS,' while at 13℃, 'M' was 60% greater (Table 6).

DISCUSSION

Environmental stress has been shown to increase endogenous ABA levels of mesophyll tissue up to 40 fold. Less than 2% of the endogenous ABA in the mesophyll is required to induce stomatal closure (*17*). Thus, the increase in ABA which we obtained in coleus after 5 days of temperature preconditioning at 13℃ would be adequate to account for the decrease in stomatal conductance and transpiration rate measured in these plants. These changes in ABA

concentration and accompanying changes in stomatal conductance and transpiration rate (on both an instantaneous and whole plant basis), may provide a physiological basis for the amelioration of SO_2 injury found in coleus as well as in poinsettia (*20*) and may explain the cross protection afforded against SO_2 exposure by low temperature pretreatment.

Our results of temperature effects on ABA accumulation are consistent with those reported by Titov et al. (*31*) and Daie and Campbell (*10*) for tomato. Since data on intracellular localization of ABA in coleus are lacking, it is not possible to determine whether the poor relationship between ABA and chlorophyll levels in the two cultivars reflects a difference in the site of synthesis or degradation of these compounds or a difference in turnover rate (*9*).

Low temperature preconditioning has been found to be effective in reducing chilling injury in a number of chilling sensitive plants (*36*). These treatments have been found to result in various physiological changes. In cotton, these included increases in sugar and starch, and decreases in RNA, protein, and lipid -soluble phosphate. Low temperature preconditioning has been reported to reduce leakage of metabolites and prevent chilling injury in cotton seedlings subsequently held at 5℃ (*15*). According to Schöner and Krause (*26*), resistance of cold-hardened spinach to subsequent chilling mediated photoinhibition was correlated with increased SOD activity and increases in the levels of enzymes of the Halliwell-Asada pathway that scavenge H_2O_2 in the chloroplasts. Wang (*34, 35*) observed an increase in activities of SOD and GR during storage at 5℃ following low temperature preconditioning. Higher activities of both SOD and GR were measured in a SO_2-insensitive cultivar of *Pisum sativum* in comparison to a SO_2-sensitive cultivar (*22*).

In our experiments, SOD did not appear to play a role in temperature hardening since low temperature pretreatment reduced SOD activity. In fact stress-sensitive 'BS' had higher SOD activity than did stress-insensitive 'M.' Similar to the results of Wang (*34*), we found a decrease in CAT activity following growth of both coleus cultivars at 13℃ in comparison to 20℃.

Stress-insensitive 'M' had 47% higher AsA content than stress-sensitive 'BS.' However, temperature preconditioning did not have any effect on AsA content in either cultivar (Table 6). Overall, the enzyme activity of AP was also greater in 'M' than in 'BS;' temperature hardening at 13℃ increased AP activity in 'BS' to the level found in 'M' at 20℃. If antioxidants are involved in the cool -temperature induced protection against SO_2 injury and the inherent resistance of 'M', then the increase in AP activity may account in part for the increased stress tolerance observed in 'BS' following temperature hardening, although the data on associated enzyme activities were not consistent.

Higher PER activity has been reported in zucchini squash during storage at 5℃ (*35*). Other workers also found similar increases in PER activity following low temperature treatment (*37*). Peroxidases have several diverse biochemical functions in higher plants and are involved in the response of plants to stress (*14*). In our experiments, PER activity was almost twice as high in stress-sensitive 'BS' as compared to stress-insensitive 'M,' but was unaffected by low

temperature pretreatment.

A high GSH/GSSG ratio has been suggested to be important in conferring stress tolerance (*34*). However, in our study, temperature preconditioning at 13℃ had no effect on GSH or the GSH/GSSG ratio when data for the two cultivars were combined, as shown by the ANOVA (Table 6). Increased levels of GSSG would be indicative of peroxidative damage. However, in our study, we found no such correlation. Grown at 20℃, stress-sensitive 'BS' had a higher ratio than 'M' and yet was still more sensitive. After pretreatment at 13℃, 'BS' had a lower ratio than 'M' and was insensitive. The same pattern applied to GSH. Stress-insensitive cultivar 'M' had more GSH at 13℃ than at 20℃, (indicating that there was some peroxidation of GSH); 'BS' on the other hand had less GSH at 13℃ than at 20℃, which is just the opposite pattern to what one might expect. Likewise, GSSG was not correlated with stress sensitivity, since low temperature pretreatment did not significantly increase its level in 'BS' and since pretreatment reduced the GSSG level in 'M' to less than that of 'BS' at 20℃.

CONCLUSIONS

Our findings suggest that both avoidance and detoxification mechanisms are involved in conferring stress tolerance in coleus. The protective effect of low temperature pretreatment was mediated in large part by a build-up in ABA and a concomitant closure in stomatal aperture and reduction in transpiration rate, which in turn likely reduced the penetration of SO_2 into the leaf. Certain antioxidants, notably AsA and AP, also appeared to play a role in conferring stress tolerance in stress-tolerant 'M,' while other antioxidants, e.g., SOD, often implicated in stress tolerance, either showed no cultivar difference or were unaffected by temperature preconditioning. The increase in GSH/GSSG ratio found in 'M' during temperature hardening suggests that glutathione may also play a role in the phytoprotection against SO_2 afforded by low temperature preconditioning.

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