Protein Aggregation, Radical Scavenging Capacity, and Stability of Hydrogen Peroxide Defense Systems in Heat-stressed Vinca and Sweet Pea Leaves

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ABSTRACT. Although heat stress injury is known to be associated with membrane dysfunctions, protein structural changes, and reactions of activated forms of oxygen, the underlying mechanisms involved are poorly understood. In this study, the relationships between thermotolerance and hydrogen peroxide (H_2O_2) defense systems, radical scavenging capacity [based on 1,1-diphenyl-2-picrylhydrazyl (DPPH) reduction], and protein aggregation were examined in vinca [*Catharanthus roseus* (L.) G. Don 'Little Bright Eye'], a heat tolerant plant, and sweet pea (*Lathyrus odoratus* L. 'Explorer Mix'), a heat susceptible plant. Vinca leaves were 5.5 °C more thermotolerant than sweet pea leaves based on electrolyte leakage analysis. Vinca leaf extracts were more resistant to protein aggregation at high temperatures than sweet pea leaf extracts, with precipitates forming at ≥ 40 °C in sweet pea and at ≥ 46 °C in vinca. Vinca leaves also had nearly three times greater DPPH radical scavenging capacity than sweet pea leaf extracts. Two enzymatic detoxifiers of H_2O_2 , catalase (CAT) and ascorbate peroxidase (APOX), demonstrated greater activities in vinca leaves than in sweet pea leaves. In addition, CAT and APOX were more thermostable in vinca, compared with sweet pea leaves. However, tissue H_2O_2 levels did not differ between controls and tissues injured or killed by heat stress in either species, suggesting that H_2O_2 did not play a direct role in acute heat stress injury in vinca or sweet pea leaves. Greater thermotolerance in vinca, compared with sweet pea, was associated with greater DPPH radical scavenging capacity, indicating that AOS other than H_2O_2 may be involved in acute heat stress injury.

Abiotic stresses, including temperature extremes, are among the primary causes of diminished plant quality, reduced crop yields, and loss of viability. Heat stress damages cellular structure and metabolic pathways, and contributes to secondary water stress (Levitt, 1980). Injury is evidenced by decreased leaf chlorophyll content (Liu and Huang, 2000), peroxidation of membrane lipids (Nanaiah and Anderson, 1992), and protein denaturation and aggregation (Salvucci et al., 2001). Acute heat stress injury probably involves changes in structure and function of membranes and proteins.

Temperature extremes can reduce enzymatic function and protein solubility through changes in intra- and intermolecular bonds of proteins. These noncovalent bonds can be broken at elevated temperatures, leading to denaturation, a complex process involving intermediate folding stages (Buchner et al., 1998). Heat shock proteins influence heat tolerance of plants by stabilizing folding intermediates (Jakob et al., 1995). If not protected, denatured proteins can aggregate irreversibly and form hydrophobic or electrostatic interactions with other unfolded proteins. Although scientists have recognized the differences between subtle conformational changes that may affect enzymatic activity and gross changes in solubility, it has been known for many decades that plant protoplasts coagulate at elevated temperatures (Levitt, 1980). Levitt (1980) cited reports from several researchers indicating that protein denaturation, cellular coagulation, and loss of viability were integrally related. Treatments that altered protein

stability had a corresponding effect on heat tolerance of bacteria and algae (Levitt, 1980).

In addition to protein stability, generation and reactions of activated oxygen species (AOS) are involved in plant responses to temperature stress. Activated oxygen species, including singlet oxygen, superoxide, hydrogen peroxide (H_2O_2), and the hydroxyl radical, can react with pigments, membranes, enzymes, and nucleic acids, thereby modifying their functions (Elstner, 1982). Autocatalytic peroxidation of membrane lipids triggered by AOS results in loss of membrane semipermeability (Basaga, 1989), one of the primary symptoms of stress injury (Foyer et al., 1997).

However, production of AOS in plants is not restricted to stressful conditions because formation occurs during fatty acid β -oxidation (del Rio et al., 1998) and photorespiration (Veljovic-Jovanovic, 1998). Activated oxygen species are also produced under normal conditions by membrane-associated oxidases (Desikan et al., 1996), and as byproducts from electron transport chains (Shewfelt and Purvis, 1995). Activated oxygen species can perform beneficial or deleterious functions in cells. For example, H_2O_2 benefits plants by polymerizing a lignin precursor during cell wall lignification (Gross, 1980), and can serve as a second messenger in signal transduction pathways leading to temperature stress acclimation (Foyer et al., 1997). Activated oxygen species can also contribute to senescence during normal developmental processes (Dhindsa et al., 1981). The type and concentration of AOS are key factors determining the beneficial or harmful functions of AOS. Hydroxyl radicals are so reactive that they normally cause random destruction at the point of formation. Less reactive molecules, such as H_2O_2 , can injure cells at high concentrations, or lead to acclimation at moderate levels (Foyer et al., 1994; Prasad et al., 1994).

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Several enzymatic and nonenzymatic antioxidant defense systems tightly control AOS concentrations to protect cells from damage (Noctor and Foyer, 1998). The primary enzymatic defenses include superoxide dismutase (SOD; EC = 1.15.1.1), catalase (CAT; EC = 1.11.1.6), peroxidase (POX; EC:1.11.1.7), ascorbate peroxidase (APOX; EC = 1.11.1.11), and glutathione reductase (GR; EC = 1.8.1.7). Nonenzymatic defenses include glutathione, α -tocopherol, ascorbate, β -carotene, hydroquinones, flavonoids, phenols, and phenolic acids (Elstner, 1982; Larson, 1988). Typically, antioxidants provide adequate protection against the deleterious effects of AOS (Alscher et al., 1997). However, increased AOS were observed in plants exposed to chilling (Wise and Naylor, 1987) and heat stress (Dat et al., 1998). Acclimation treatments can increase antioxidant activity, detoxifying heavy loads of AOS and providing protection against chilling injury (Prasad et al., 1999; Sala and Lafuente, 1999). Acclimated maize (*Zea mays* L.) seedlings had increased CAT activity and modestly increased H_2O_2 levels after chilling, but nonacclimated seedlings had large increases in H_2O_2 (Prasad et al., 1994). However, H_2O_2 content and activities of APOX and CAT were poorly correlated when comparing heat tolerant and susceptible wheat genotypes at different planting dates and stages of development (Sairam et al., 2000). Hydrogen peroxide levels were unchanged in heat-stressed pepper leaves (Anderson, 2002) and reduced in several plant species exposed to chilling temperatures (MacRae and Ferguson, 1985) despite reduced enzymatic defenses. The role of H_2O_2 in chilling injury appears to depend on plant species and environmental conditions before and during exposure. Hydrogen peroxide involvement in heat stress injury is not clear.

In addition to studies focusing on activities of enzymes involved in AOS detoxification, scavenging activity has been monitored using relatively stable free radicals. Kang and Saltveit (2002) reported increased 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity being associated with heat shock-induced chilling resistance in rice (*Oryza sativa* L.) seedlings. Antioxidant activity has also been determined in microorganisms (Abe et al., 1998) and plant leaf extracts (Masuda et al., 1999) using the DPPH radical.

Determining relationships between thermotolerance and AOS defenses, membrane integrity, and protein stability are essential in understanding the underlying basis for heat tolerance in plants. Therefore, the objectives of this study were to use a heat-tolerant bedding plant, vinca (*Catharanthus roseus* L.), and heat-susceptible sweet pea (*Lathyrus odoratus* L.) to 1) determine the relationship between leaf thermotolerance and the stability of CAT and APOX; 2) assess the effect of heat stress on endogenous H_2O_2 ; 3) evaluate if vinca and sweet pea leaves differed in DPPH radical scavenging capacity; and 4) test whether plant extracts differed in resistance to heat-induced aggregation and precipitate formation.

Materials and Methods

PLANT CULTURE. Vinca and sweet pea seeds were sown in 15-cm-diameter pots in a commercial potting mix (Universal mix, Strong-lite, Pine Bluff, Ark.) enriched with micronutrients. Amendments included dolomite (3.6 g·L⁻¹), superphosphate (0.7 g·L⁻¹), Micromax (The Scotts Co., Marysville, Ohio) (0.6 g·L⁻¹), and KNO₃ (0.6 g·L⁻¹). Plants were grown in controlled environment chambers (model PGW36; Conviron, Winnipeg, Man., Canada) at 24/20 °C (day/night) cycles, with 45% to 65% relative humidity. The chamber was programmed for 14-h photoperiods with

a photosynthetic photon flux density at canopy height of about 400 μ mol·m⁻²·s⁻¹. Plants were fertigated with 20N-8.6P-16.6K soluble fertilizer (J.R. Peters, Allentown, Pa.) at 0.7 g·L⁻¹ as needed, based on soil color and pot weight.

ELECTROLYTE LEAKAGE. Leaf thermotolerance was assessed by electrolyte leakage. Leaf disks (7 mm diam) from 8-week-old plants were prepared with a cork borer and placed in 25 × 150 mm test tubes with 2 mL distilled water. Only fully expanded, nonsenescent leaves were used. High temperature treatments were administered for 15 min by placing the test tubes into a circulating water bath at the desired temperature. Temperatures tested were 24 °C for the controls, 50 to 58 °C for vinca, and 44 to 52 °C for sweet pea, at 1 °C intervals. Three subsamples were assayed for each temperature exposure. After temperature treatments, 20 mL distilled water was added to the test tubes and samples were incubated on an orbital shaker for 22 h at 24 °C. Initial conductivity was measured using a conductivity meter (model 35; Yellow Springs Instrument Company, Yellow Springs, Ohio). Samples were then autoclaved for 20 min, followed by another 22 h incubation before final conductivity measurements were taken. Electrolyte leakage (EL) was computed as the ratio of initial to final conductivity × 100. The midpoint of the EL-temperature response curve (T_{mid}) was determined as described by Ingram (1985) using PROC NLIN (SAS Institute, Cary, N.C.).

THERMOSTABILITY OF CAT AND APOX. Leaf tissue (1 g), excluding the midrib, was cut into approximately 1-cm² pieces and placed in a 25 × 150 mm test tube with 2 mL distilled water for high temperature treatments. The test tubes were placed into a circulating water bath for 15 min for each high temperature exposure. Temperatures tested were 24 °C for the controls, 48 to 64 °C for vinca, and 42 to 58 °C for sweet pea, at 2 °C intervals. Heat-treated tissue was homogenized in a blender (Waring Products Div., Dynamics Corp., New Hartford, Conn.) along with 0.08 g polyvinylpyrrolidone (PVPP) and 25 mL potassium phosphate buffer (50 mM, pH 7.0), then filtered through Miracloth (Calbiochem-Novabiochem Corp., San Diego) premoistened with buffer. Filtrate was centrifuged at 16,000 g_n for 15 min and the supernatant was used as crude extract.

Catalase activity in the crude extract was determined spectrophotometrically by recording the decrease in absorbance at 240 nm for 10 s after a 10-s lag (Aebi, 1983). The 3 mL reaction mixture contained 1.5 mL of a 1:20 dilution of crude plant extract and 1.5 mL 30 mM H_2O_2 prepared in buffer, which initiated the reaction. Ascorbate peroxidase activity was determined spectrophotometrically by recording the decrease in absorbance at 290 nm for 50 s after a 10 s lag (Miyake et al., 1991). The 3 mL reaction mixture contained 1 mL of a 1:10 dilution of crude plant extract, 0.8 mL 50 mM phosphate buffer (pH 7.0) and 0.6 mL 1.0 mM ascorbate. The reaction was initiated by addition of 0.6 mL 0.5 mM H_2O_2 . All reagents were prepared in buffer. In both enzyme assays, crude plant extracts were analyzed in three subsamples.

Activity midpoint temperatures (T_{mid}) were calculated for APOX and CAT activity using PROC NLIN. The experiment was a split-plot arrangement in a randomized complete block design with five experimental dates (replications) constituting the blocks. Plant species were levels of the main unit factor, and EL, APOX, and CAT were levels of the split unit factor. Analysis of variance was performed using PROC MIXED (SAS Institute).

HYDROGEN PEROXIDE LEVELS IN CONTROL, INJURED, AND KILLED TISSUES. Leaf disks (7 mm diam) were prepared for measurement of EL and 0.5 g leaf tissue was cut into 1-cm strips for measurement of H_2O_2 . The leaf disks or strips were placed in 25 × 150 mm

test tubes with 2 mL distilled water and exposed to 24, 54, or 60 °C for vinca, and 24, 48, or 54 °C for sweet pea in a circulating water bath for 15 min.

Hydrogen peroxide content of the tissue was determined spectrophotometrically using a modification of the procedure described by Ngo and Lenhoff (1980). After the temperature treatment, 0.5 g leaf tissue was frozen in liquid nitrogen. Five milliliters chilled 5% trichloroacetic acid (TCA) was added and the tissue was homogenized using a mortar and pestle. The homogenate was filtered through Miracloth premoistened with TCA. The filtrate was vortexed with 0.1 g activated carbon and 0.04 g PVPP, then filtered through Miracloth again. The filtrate was centrifuged at 16,000 g_n for 15 min and the supernatant was used as crude plant extract. The 3-mL reaction mixture contained 1.92 mL 0.375 M sodium phosphate buffer (pH 6.5), 0.3 mL 3.3 mM 3-dimethylaminobenzoic acid (DMAB), 0.3 mL 0.07 mM 3-methyl-2-benzothiazolinonehydrazone (MBTH), and 0.18 mL buffer containing 10 ng type 1 horseradish peroxidase (0.25 units). All reagents were prepared in buffer, except MBTH, which was prepared in distilled water. The reaction was initiated by adding 0.3 mL crude plant extract containing about 1 mg protein. Samples were incubated at 24 °C for 24 h in the dark before measuring the absorbance at 590 nm. The experiment was conducted on three dates with three subsamples from each crude plant extract assayed on each date. The experiment was a split-plot arrangement with species as the main-plot factor and temperature treatment as the subplot factor. Analysis of variance was performed using PROC MIXED (SAS Institute).

DPPH RADICAL SCAVENGING. DPPH is a relatively stable radical that absorbs strongly at 517 nm in the oxidized form (Masuda et al., 1999). Solutions scavenging the radical reduce absorbance as the purple color is diminished, allowing spectrophotometric determination of radical scavenging in plant extracts. The procedure of Masuda et al. (1999), with minor modifications, was employed to measure DPPH radical scavenging activity. Leaf tissue (0.6 g) was ground in 60 mL methanol in a mortar containing washed sand. The mortar, pestle, and methanol were prechilled to 4 °C. The liquid was passed through Miracloth, then centrifuged at 16,000 g_n for 20 min. The supernatant was pipetted into test tubes and diluted with methanol to produce solutions from 1.25 to 10 mg fresh weight leaf tissue per mL solvent. Sixty microliters DPPH (5 mM in methanol) was added to bring the total volume to 3 mL, then tubes were vortexed. Duplicate samples were prepared without DPPH to subtract the background absorbance of the tissue solution. Absorbance at 517 nm was recorded using a spectrophotometer 30 min after adding DPPH. The tissue concentration reducing A_{517} by 50%, ED_{50} (Abe et al., 1998), was calculated by linear interpolation. The assay was conducted using three replications derived from different plants. Three subsamples per treatment combination were assayed for each replication. Analysis of variance was performed using PROC GLM (SAS Institute) for the response variable ED_{50} .

THERMOSTABILITY OF LEAF EXTRACTS. Leaf tissue (10.08 g) was homogenized in 175 mL MES (2-(N-morpholino)ethanesulfonic acid buffer (Sigma, St. Louis) (50 mM, pH 6.0) containing 1.08 g PVPP. Preliminary experiments with sweet pea and vinca leaf extracts prepared in distilled water yielded pH values of 6.0 for sweet pea and 6.1 for vinca extracts. Homogenates were filtered through Miracloth premoistened with buffer, then centrifuged at 16,000 g_n for 20 min at 24 °C. Supernatants were placed in test tubes with 4 mL per tube and three tubes per treatment combination, then exposed to test temperatures for 15 min. Sweet pea extracts

were exposed to 24 °C as a control, or 34 to 50 °C in 2 °C intervals. Test temperatures for vinca extracts were 24 °C (control), or 38 to 58 °C in 2 °C intervals. Three circulating baths at different temperatures were used to reduce the overall treatment duration. Test tubes removed from the high temperature treatments were placed in a water bath at ≈ 24 °C for 5 h before visual observations of precipitation and spectrophotometric determination of turbidity at 540 nm (Xu et al., 1998). Only the highest treatment temperatures for each species resulted in precipitation during or immediately after cooling. Turbid liquids either remained cloudy or formed macroscopic suspended aggregates that slowly settled to the bottom of the tubes over the course of the 5-h observation duration. It is likely that less turbid solutions that remained cloudy without forming precipitates in the bottom of the tubes would have formed precipitates eventually if the observation interval was extended beyond 5 h. Insoluble material that settled to the bottom of the tube was avoided when sampling liquid over precipitates for turbidity measurements. Protein contents of plant extracts were determined colorimetrically (Bradford, 1976) using ovalbumin (Sigma) as the standard. The randomized, complete block experiment was conducted on four dates (blocks) with three subsamples from each crude plant extract assayed on each date. Analysis of variance was performed using PROC GLM (SAS Institute) for the response variables protein concentration and precipitation temperature.

Results and Discussion

ELECTROLYTE LEAKAGE AND THERMOSTABILITY OF CAT AND APOX. Vinca leaves were 5.5 °C more thermotolerant than sweet pea leaves based on EL (Fig. 1A). Catalase activity was 6.0 °C more thermostable in vinca compared with sweet pea leaves (Fig. 1B), and APOX activity was 2.6 °C more thermostable in vinca than in sweet pea leaves (Fig. 1C). In addition to greater enzyme thermostability, control (24 °C treatment) activities of CAT and APOX were significantly greater in vinca leaves than in sweet pea. Control CAT activity was 65% greater and APOX activity was 161% higher in vinca controls than in sweet pea controls on a fresh weight basis. In vinca, T_{mid} values for APOX activity and EL were not significantly different (52.9 and 53.9 °C, respectively), but were lower than T_{mid} for CAT (56.0 °C). In sweet pea, T_{mid} values for APOX and CAT activities were not significantly different from each other (50.3 and 50.0 °C, respectively), but were higher than T_{mid} for EL (48.4 °C).

Although few studies have explored thermal stability of AOS defenses in plants, factors related to heat stability of bacterial pathogens has been a focus in food safety studies. Dallmier and Martin (1988) reported that CAT activity was reduced significantly when *Listeria monocytogenes* cell extracts were heated above 50 °C for 10 min. Temperatures causing a 50% reduction in activity ranged from 57.5 to 59.0 °C for the four strains of bacteria. Dallmier and Martin (1988) also reported genetic differences in baseline activity of unstressed cells. However, a relationship between antioxidant enzyme activity and cellular thermotolerance was not observed, which supports the current study.

The mechanism of injury in heat-stressed tissue may involve an increase in the rate of production of AOS, impairment of antioxidant defenses, or both (Bowler et al., 1992). Direct involvement of H_2O_2 in heat stress injury would involve increased levels of endogenous H_2O_2 , or increased sensitivity in heat-stressed plant tissue. If impaired enzymatic defenses contribute to injury, activities of antioxidant enzymes would decrease as the temperature increases,

resulting in elevated AOS levels before or coincident with injury. In the present study, T_{mid} values for the H_2O_2 -scavenging enzymes, CAT and APOX, were similar to or greater than T_{mid} values for EL in both plant species (Fig. 1). Temperature ranges in which

responses began differing from baseline levels were similar when comparing all three responses for a particular species. Ascorbate peroxidase activity in vinca leaves began decreasing between 48 and 52 °C, CAT activity started decreasing between 50 and 52 °C, and EL started to increase by 50 °C. Ascorbate peroxidase and CAT activities in sweet pea leaves began decreasing between 46 and 48 °C, and EL began increasing between 47 and 48 °C. Data were mostly consistent with coincident changes in membrane function, based on EL, and activities of H_2O_2 -scavenging enzymes as temperature increased.

Loss of enzyme activities at elevated temperatures may be due to conformational changes, production of inhibitors, diminished rates of enzyme synthesis and/or elevated enzyme degradation. Acute heat stress injury probably involves changes in protein structure that impair enzymatic function. Differences in enzyme stability between vinca and sweet pea could involve more thermostable isozymes of CAT and APOX in vinca. Alternatively, enzyme thermostability may have been moderated by protective mechanisms, such as molecular chaperones (Nagao et al., 1990). Depending on the type and amount, the protective machinery could have provided greater thermoprotection to the enzymes in vinca than sweet pea.

HYDROGEN PEROXIDE LEVELS IN CONTROL, INJURED, AND KILLED TISSUES. Enzyme thermal stability provided only indirect information on the role of H_2O_2 in heat stress injury to vinca and sweet pea leaves. Therefore, a more direct approach was taken by measuring endogenous H_2O_2 levels in control, injured, and killed tissues. Temperature selection for each species was based on EL, with the injurious temperature corresponding to T_{mid} and the killing temperature representing the lowest temperature that led to the EL maximum (Fig. 1A). These categories were arbitrarily assigned because we did not track tissue responses to determine the effects of these temperatures on tissue viability. In vinca, EL was 22% at 24 °C, increased to 64% at 54 °C, and reached 94% at the killing temperature of 62 °C (Table 1). Similarly, in sweet pea leaves, EL was 23% at 24 °C, increased to 59% at 48 °C, and reached 93% at 54 °C. No significant differences in the level of H_2O_2 were observed across the exposure temperatures or between the two species (Table 1). If H_2O_2 played a role in heat stress injury, an increase in the level of H_2O_2 or an increase in tissue sensitivity to H_2O_2 at elevated temperatures would have been expected. Although we did not attempt to determine whether tissue sensitivity to H_2O_2 changed with increased temperature, endogenous H_2O_2 levels did not increase following heat stress.

MacRae and Ferguson (1985) reported similar results in pea (*Pisum sativum* L.), mung bean (*Vigna radiata* (L.) Wilczek.), and cucumber (*Cucumis sativus* L.) exposed to chilling stress. After chilling, they observed a significant decrease in CAT activity, however, H_2O_2 content was unchanged or decreased depending on the species. Despite decreased APOX and CAT activities, H_2O_2

Fig. 1. Electrolyte leakage (A) and thermostability of catalase (CAT) (B) and ascorbate peroxidase (APOX) (C) in 8-week-old vinca and sweet pea. Leaf tissues were exposed to the indicated temperature for 15 min. Data represent means $\pm$ SE of 15 measurements (5 replications with 3 subsamples).

Table 1. Electrolyte leakage (EL) and hydrogen peroxide (H_2O_2) concentration in leaf tissue of 8-week-old vinca and sweet pea plants exposed to injurious or killing temperatures for 15 min. Means $\pm$ SE of nine measurements are reported.

Leaf tissue	Treatment	Temp (°C)	EL (%)	H_2O_2 ($\mu\text{mol g}^{-1}$ fresh wt)
Vinca	Control	24	22.0 $\pm$ 0.5 a ^z	2.7 $\pm$ 0.2 a
	Injured	54	64.1 $\pm$ 1.3 b	3.1 $\pm$ 0.4 a
	Killed	62	94.3 $\pm$ 0.5 c	3.3 $\pm$ 0.4 a
Sweet pea	Control	24	23.0 $\pm$ 0.6 a	2.7 $\pm$ 0.2 a
	Injured	48	58.7 $\pm$ 2.4 b	2.7 $\pm$ 0.2 a
	Killed	54	93.3 $\pm$ 0.6 c	2.2 $\pm$ 0.2 a

^zMeans within a column followed by the same letter are not significantly different at $P \leq 0.05$ using LSD.

Fig. 2. Radical scavenging capacity of leaf extracts was estimated spectrophotometrically by DPPH reduction. Absorbance at 517 nm by methanolic extracts of vinca and sweet pea leaves containing 5 mM DPPH was measured 30 min after addition of DPPH. Means $\pm$ SE of nine measurements are reported.

Fig. 3. Turbidity and precipitate formation in aqueous leaf extracts. Apparent absorbance at 540 nm by sweet pea and vinca leaf extracts after exposure to elevated temperatures for 15 min. Precipitates formed at ≥ 40 °C in sweet pea extracts and at ≥ 46 °C in vinca extracts. Means $\pm$ SE of nine measurements are reported.

decreased in rice (*Oryza sativa* L.) plants exposed to water stress (Boo and Jung, 1999). These studies suggest that impairment of enzyme defenses resulting in high levels of H_2O_2 may not be the mechanism of injury from chilling and water stress in these plants. However, another study found that H_2O_2 increased nearly 200% in rice shoot cultures chilled to 4 °C (Fadzillah et al., 1996). Although APOX activity was unchanged, elevated H_2O_2 content was associated with decreased CAT activity. Increased H_2O_2 in chilled cucumber seedlings was also accompanied by a decrease in CAT activity (Omran, 1980). Apparently, the effects of chilling on H_2O_2 are a function of plant species and environmental conditions before and during chilling. A similar situation may hold for heat stress since H_2O_2 increased in mustard seedlings (Dat et al., 1998), but not in pepper (Anderson, 2002), or the vinca and sweet pea plants in the present study.

DPPH REDUCTION. Vinca leaves had significantly greater DPPH radical scavenging capacity than sweet pea leaves (Fig. 2). Vinca extracts at 2.5 $mg \cdot mL^{-1}$ reduced absorbance by 87%, but sweet pea extracts only lowered absorbance 30% at this concentration. ED_{50} values, the concentrations reducing absorbance by 50% (Abe et al., 1998), were 1.5 $mg \cdot mL^{-1}$ for vinca and 4.1 $mg \cdot mL^{-1}$ for

sweet pea. Although the reactions involved, and their significance to stress injury are not known, the more thermotolerant plant had greater DPPH radical scavenging ability. Additional studies will be required to determine whether greater DPPH radical scavenging capacity translates to greater AOS poise in vivo.

THERMOSTABILITY OF LEAF EXTRACTS. We observed precipitates forming in extracts heated to elevated temperatures during experiments examining the thermostability of CAT and APOX. Subsequent experiments were performed to determine if species differences in bulk protein stability existed in vinca and sweet pea leaves. Vinca leaf extracts were significantly more resistant to heat-induced changes in solubility than sweet pea extracts. Precipitates formed at 46.7 ± 0.3 °C in vinca extracts and at 40.5 ± 0.3 °C in sweet pea (Fig. 3). As exposure temperatures increased from ambient (24 °C), turbidity increased to a maximum, then decreased as precipitates formed. Our observations of increasing turbidity followed by precipitation as temperatures increased were consistent with previous studies using egg white solids. Xu et al. (1998) described both soluble (turbid) and insoluble (coagulated) materials after heating. Vinca extracts were more thermostable than sweet pea extracts even though they contained significantly more protein (4.1 vs 3.0 $mg \cdot mL^{-1}$ extract). Control pea extracts were significantly more turbid than vinca controls in spite of a lower protein content, indicating that additional factors contributed to turbidity.

Turbidity development and precipitation in crude leaf extracts appears to be useful in examining bulk protein thermostability, similar to studies with individual proteins (Park et al., 2002), egg white solids (Xu et al., 1998), and protein-chaperone mixtures (Lee and Vierling, 1998). However, additional factors are involved in protoplasmic coagulation since $\approx 70\%$ of the proteins remained in solution following coagulation (data not presented).

In conclusion, differences in intrinsic thermostolerance between vinca and sweet pea leaves were correlated with DPPH radical scavenging activity and protein thermal stability. Although CAT and APOX activity were greater and more thermostable in vinca than sweet pea, differences in H_2O_2 were not observed following heat stress. Therefore, it is not likely that H_2O_2 plays a direct role in acute heat stress injury in these plants. However, the role of H_2O_2 and H_2O_2 defenses in acclimation responses has been established (Foyer et al., 1997; Kang and Saltveit, 2002). It would be instructive to further our understanding of plant thermostolerance by determining the nature of the involvement of reducing power and protein stability.

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