HEAT STRESS, HYDROGEN PEROXIDE AND ANTIOXIDANT DEFENSES IN HORTICULTURAL CROPS

By

SONALI R. PADHYE

Bachelor of Science

College of Horticulture

Pune, India

1997

Submitted to the Faculty of the
Graduate College of
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2001

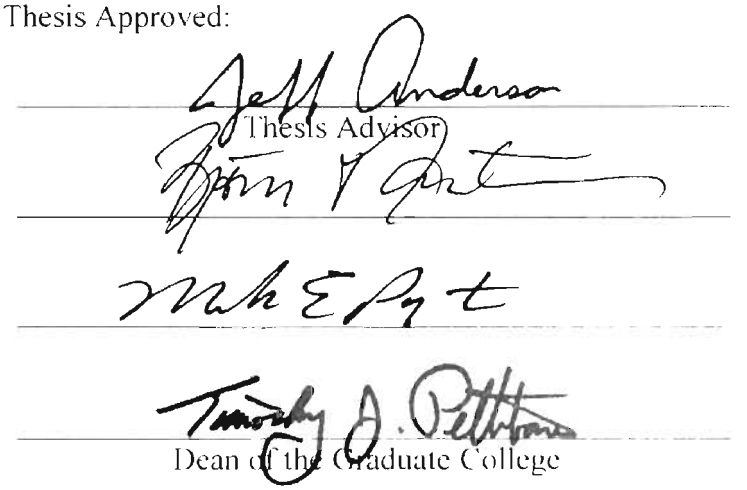
HEAT STRESS, HYDROGEN PEROXIDE AND

ANTIOXIDANT DEFENSES IN

HORTICULTURAL

CROPS

Thesis Approved:



Thesis Advisor

Dean of the Graduate College

ACKNOWLEDGEMENTS

I thank my advisor, Dr. Jeff Anderson for his guidance and valuable insight. His encouragement throughout my graduate program is sincerely appreciated. I thank Dr. Mark Payton for serving on my thesis committee and making the statistical analysis an enjoyable experience. I also appreciate his patient and friendly advice. I extend my thanks to Dr. Bjorn Martin for serving on my thesis committee as well as his valuable suggestions. I am grateful to Dr. Niels Maness and Dr. Shiping Deng for allowing me to use the spectrophotometer.

I wish to express my gratitude to Lynda Wells not only for her technical assistance, but also for being such a great friend. I could not have successfully accomplished this goal, if not for her pleasant company and strong support when it mattered the most.

I wish to express my sincere gratitude to Dr. Janet Cole for her valuable guidance and vision. I have thoroughly enjoyed serving as a teaching assistant for Dr. Lou Anella and Dr. Douglas Needham. I thank Tim Hooper for helping me with the plant care and for his friendship. I also extend my thanks to the faculty and administrative staff in the Department of Horticulture and Landscape Architecture for their help. The financial aid provided by Department of Horticulture and Landscape Architecture is sincerely appreciated.

I would like to take this opportunity to thank my colleagues Brian Jervis, Haregewoin Takele and Jennifer Barnes for their friendship, which I will cherish forever. I also am earnestly grateful to Haregewoin Takele, Lynda Wells and Veronica Tudor for accommodating me in their homes during the final phase of my thesis writing.

A very special thanks to Naroo for his love and support. Stillwater would never have been so beautiful without him.

I am indebted to all my family and friends for their love. Finally, I want to thank my parents for their unconditional love as well as moral and financial support. I wish to dedicate this work to my mother, who has always taken pride in even the smallest of my achievements.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Abiotic stresses are various environmental factors such as extreme temperatures, light intensity, water excess and deficit, salinity, various air pollutants, photodynamic herbicides and heavy metals. Exposure to abiotic stresses can have a profound effect on physiological processes, growth and development, productivity and sustainability of plants. Exposure to high temperature stress results in considerable pre-harvest and post-harvest crop losses. Hence, understanding the biological processes associated with heat stress is important. Comprehension of plant response to heat stress can aid in development of cultivars genetically engineered to sustain the future global warming (Paulsen, 1994). Although some aspects of heat stress, such as the heat shock response, are well characterized, the overall response of plants to heat stress is not yet well characterized in spite of its significance (Paulsen, 1994).

In order to comprehend the effect of heat stress, it is essential to understand injury and acclimation. The magnitude of high temperature sensed by a plant determines its outcome in injury or acclimation. Exposure to extreme heat stress results in injury, whereas exposure to moderate heat stress can result in acclimation, achieving thermotolerance to previously lethal temperatures.

Temperature Sensing by Plants

Temperature sensing by plants is in part a function of membrane fluidity, a physiological attribute vital for effective membrane functioning. At increasing

temperatures, the movement of lipids in the bilayer increases, whereas at decreasing temperatures, the lipid movement declines (Monson, 2001), resulting in the primary signal for temperature perception by plants. Membrane fluidity is influenced by several direct, as well as indirect factors. Besides the exposure temperature, membrane fluidity is directly affected by degree of unsaturation of fatty acids, ratio of cis and trans double bonds and presence of cholesterol (Leng, 2000). Membrane fluidity is indirectly affected by the configuration of membrane-associated proteins, changes in phosphorylation state of proteins, and influx of Ca^{2+} ions within the cell, through a feedback loop (Murata et al., 1997). The plant response to a given temperature depends not only on the exposure temperature, but also on the duration of exposure, since longer exposure to moderately high temperature can be as injurious as brief exposure to extremely high temperature (Georgieva, 1999).

Heat Shock Response

During rapid exposure to high temperatures, plants produce stress-adaptation proteins called heat shock proteins (HSPs) and this response is termed the heat shock response (HSR). HSR is highly conserved among most organisms, particularly in eukaryotes by similar gene-induction processes and presence of homologous major HSPs (Vierling, 1991). Nagao et al. (1990) described the well characterized HSR as follows. At higher temperatures, synthesis of normal proteins is temporarily decreased and heat shock messenger RNAs (HS mRNAs) are synthesized. Synthesis of most pre-existing mRNAs is temporarily terminated and mRNAs are preserved. Large amounts of HSPs are produced and accumulated. Prolonged heat stress results in decreased HS mRNA

synthesis, followed by decline in HSPs. Normal protein synthesis is resumed. Heat shock results in selective intracellular localization of HSPs in cytosol, chloroplast, ER and mitochondria (Waters et al., 1996), followed by delocalization at the time of recovery. HSR aids in acquiring tolerance to previously lethal high temperatures (Nagao et al., 1990). HSR protects plants from cellular injury due to heat shock and hence, is a critical protection mechanism.

Schoffl et al. (1998) explained the following model of heat shock factor (HSF) regulation. HSFs are constitutively produced as inactive monomers in plants. Exposure to heat shock results in oligomerization of HSFs and their activation to bind with heat shock elements (HSE). This promotes the transcription of HS mRNAs and production of HSPs.

Several functions are performed by HSPs during HSR. The contribution of HSPs as molecular chaperones is especially significant. HSPs act as armors to protect structures of other proteins during translocation. HSPs bind with proteins damaged due to heat stress and aid in correct refolding, thus preserving the protein structures (Vierling, 1991). Their contributions in repairing damaged proteins by annealing and removal of damaged structures by proteolysis are extremely significant in prevention of cellular damage and increasing thermotolerance. Several HSPs have been identified and characterized in different plant systems. However, specific functions of all of them have not yet been revealed. Also, the role of individual HSPs in attaining thermotolerance is not fully elucidated (Nguyen, 1994).

Injury

Based upon their ability to withstand high temperatures, plants can be classified as psychrophiles, mesophiles or thermophiles, stated in order from lowest to highest thermotolerance (Levitt, 1980). When plants are exposed to temperatures above their tolerance levels, injury occurs. Levitt (1980) classified heat-stress injury into primary and secondary types depending on direct or indirect involvement of heat stress in the mechanism of injury, respectively. Exposure to heat stress causes the transpiration rates to increase, resulting in secondary water stress, causing injury. Several mechanisms of primary heat-stress injury have been proposed.

Levitt (1980) described the classic mechanism of primary direct heat-stress injury as denaturation and aggregation of proteins. Exposure to higher temperatures for short duration causes temporary loss of three-dimensional structure of proteins, affecting their physical and chemical properties. This effect is denaturation of proteins and is usually reversible. However, prolonged heat stress results in permanent unfolding of proteins, called aggregation. Being irreversible, aggregation results in permanent loss of physical and chemical properties of proteins.

Levitt (1980) also explained the mechanism of primary indirect injury associated with longer-term exposure to moderately high temperatures. As temperatures increase, reaction rates increase due to the elevated kinetic energy. Since the temperature coefficients (Q_{10}) of reactions vary, imbalance can result when one reaction speeds up at a greater rate than another, paired reaction, thereby affecting the rate of production of reaction end products. Indirect injury occurs due to the imbalance resulting from

decreased production of essential metabolites and/or accumulation of metabolites to toxic levels, initiating "metabolic lesions" (Levitt, 1980).

In plants, photosynthesis is an energy-producing metabolism and respiration is an energy-consuming metabolism. At increasing temperatures both photosynthesis and respiration rates increase and exposure to higher temperature results in decline in the rates of photosynthesis and respiration. The temperature at which photosynthesis and respiration are at the same rates is termed the "temperature compensation point" (TCP). At temperatures higher than TCP, the rate of respiration is higher than the rate of photosynthesis and hence more energy is expended than being generated causing "starvation" or growth inhibition of plants (Levitt, 1980). At higher temperatures C-3 plants photorespire and as a result undergo net loss of energy which can also result in starvation or growth inhibition. Physiological aspects of photorespiration are covered elsewhere in this chapter.

High temperature can damage the plasma membrane and induce loss of membrane semi-permeability. This results in leakage of cellular electrolytes. The amount of electrolyte leakage (EL) is not only affected by exposure temperature, but is also affected by exposure duration (Ingram, 1985).

The high temperature-induced membrane injury is associated with peroxidation of polyunsaturated fatty acids in the plasma membrane (Liu and Huang, 2000). Basaga (1989) described the following mechanism of lipid peroxidation. Extraction of hydrogen atom from polyunsaturated fatty acids in cellular membranes forms another free radical which further reacts with O_2 and forms peroxy radicals. Peroxy radicals propagate the reaction by reacting with polyunsaturated fatty acids forming lipid peroxides by

extracting another hydrogen atom. This series of auto-oxidative reactions involved in lipid peroxidation has the potential to destroy the plasma membrane. Lipid peroxidation reactions initiated by extraction of a hydrogen atom summarized by Basaga (1989) are reviewed elsewhere in this chapter as autocatalytic reactions.

Enzymatic lipid peroxidation can be catalyzed by lipoxygenases (LOX) (Hildebrand, 1989; Siedow, 1991; Gardner, 1991). Although LOXs can utilize several substrates, linoleic and linolenic acids are the primary substrates utilized in plants (Hildebrand, 1989). Lipid peroxidation observed during exposure to biotic and abiotic stresses is mainly associated with oxidative damage caused by activated oxygen species (AOS) (Foyer et al., 1997).

Activated Oxygen Species

Exposure of plants to biotic and abiotic stresses can result in production of high levels of AOS. This phenomenon is termed "oxidative stress". AOS include singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$). Being free radicals, $^{\cdot}O_2^-$ and $^{\cdot}OH$ possess one or more unpaired electrons in the last atomic orbital, making them extremely reactive (Halliwell and Gutteridge, 1984; Mehlhorn and Wellburn, 1994). AOS can initiate autocatalytic reactions. Basaga (1989) and Mehlhorn and Wellburn (1994) summarized the autocatalytic reactions into "initiation", "propagation" and "termination" steps as described below. R is a membrane associated fatty acid and X is a radical initiating the autocatalytic reactions.

"Initiation" step: $RH + X \longrightarrow R + XH$

"Propagation" step: $R + O_2 \longrightarrow ROO$

$ROO + RH \longrightarrow ROOH + R$

$$2\ ROOH \longrightarrow ROO^{\cdot} + RO^{\cdot} + H_2O$$

$$RO^{\cdot} + ROOH \longrightarrow ROO^{\cdot} + ROH$$

"Termination" step: $2\ ROO^{\cdot} \longrightarrow O_2 +$ stable products

$$2\ R^{\cdot} \longrightarrow \text{stable products}$$

$$ROO^{\cdot} + R^{\cdot} \longrightarrow ROOR$$

$^1O_2$ exists in Σg and Δg forms, which are radical and non-radical forms, respectively. The radical form usually has two unpaired electrons and decays to the non-radical form before it can react with any biomolecules (Halliwell and Gutteridge, 1984; Asada and Takahashi, 1987; Cadenas, 1989; Foyer and Harbinson, 1994). Due to its ability to act as a nucleophile and extract electrons from biomolecules, Δg form can be deleterious (Cadenas, 1989).

$O_2^{\cdot}$ is a radical with negative charge and acts as an oxidizing agent as well as reducing agent. $^{\cdot}O_2^{-}$ can be formed as a result of a single electron acceptance by O_2. Addition of one more electron to $^{\cdot}O_2^{-}$ results in the formation of a peroxide ion (O_2^{2-}), which at physiological pH forms H_2O_2 (Halliwell and Gutteridge, 1984). H_2O_2 is formed when $O_2^{\cdot}$ undergoes dismutation reaction. $O_2^{\cdot}$ and H_2O_2 together participate in Haber-Weiss reaction or in the presence of metal ions, H_2O_2 participates in Fenton-type reactions, producing the most reactive AOS, OH. OH can react with any biomolecule available in its vicinity at diffusion rates, producing secondary radicals, thus, initiating autocatalytic reactions causing random destruction. Due to its tremendous reactivity, OH reacts with biomolecules available at its very site of production and this is the "site-specific effect" of OH (Asada and Takahashi, 1987). OH can initiate lipid peroxidation by extracting a hydrogen atom from a free fatty acid (Elstner, 1982).

The primary function of O_2 in plants is to act as an electron acceptor in respiratory electron transport. However, there are many reactions in which O_2 is converted into AOS

in order to protect the components of electron transport systems from over-excitation and over-reduction. During exposure to stress, energy requirements of plants decrease, posing danger to the components of photosynthetic electron transport system by the presence of excess electrons in photosystem II (Somersalo and Krause, 1990). In order to protect the components of photosynthetic electron transport system, excess electrons are transferred to O_2. As a consequence, AOS form and accumulate. Mehler reaction and photorespiration are two reactions associated with photosynthesis, which incorporate O_2 in AOS. During photosynthesis, at a very high O_2 concentration or very low CO_2 fixation rate, pseudocyclic electron flow occurs, during which electrons are transferred from H_2O to O_2, forming $O_2^{\cdot}$ (Ntuli, 1995). $O_2^{\cdot}$ undergoes dismutation reaction and H_2O_2 is formed. $O_2^{\cdot}$ can also decompose into H_2O_2 by reacting with cellular reductants such as ascorbate, glutathione, Mn^{2+} and reduced ferredoxin (Asada, 1994). As a result, the Mehler reaction generates a trans-thylakoid proton gradient. In photorespiration, the oxygenation reaction of ribulose bisphosphate carboxylase/oxygenase results in formation of 3-phosphoglyceric acid and glycolate. Glycolate enters peroxisomes where it is oxidized by glycolate oxidase forming intermediates such as H_2O_2 (Veljovic-Jovanovic, 1998). Photorespiration is favored by high levels of O_2, low levels of CO_2, high light intensities and high temperatures (Kimball, 2000). Photorespiration protects C-3 plants from photoinhibition by serving as an excess energy dissipation mechanism, thereby preventing overreduction of the components of photosynthetic electron transport (Krause, 1994). $^1O_2$ is formed by peroxisomal membrane polypeptides during normal conditions (Del Rio et al., 1998) and $^1O_2$ is also formed in chloroplasts as a result of reaction

between O_2 and triplet excited state chlorophyll (Asada and Takahashi, 1987; Bowler et al., 1994; Foyer and Harbinson, 1994).

AOS perform beneficial as well as harmful roles in a cell. In the absence of biotic or abiotic stress, H_2O_2 polymerizes a lignin precursor in the cell wall lignification process (Gross, 1980). In the event of exposure to biotic stress, AOS can perform several beneficial roles such as cross linking of cell wall to prevent pathogen entry, direct eradication of pathogens by acting as "antibiotics" and induction of hypersensitive response and systemic acquired resistance (Mehdy et al., 1996). If exposed to biotic or abiotic stress, AOS can induce signal transduction pathways, resulting in tolerance to previously lethal stress. Due to their extreme reactivity, AOS can react with several biomolecules, including pigments, membranes, nucleic acids and enzymes (Elstner, 1982) and modify them. The modification of biomolecules caused by AOS can be extremely harmful to the cell. AOS have been suggested to be one of the factors contributing to senescence (Dhindsa et al., 1981). Lipid peroxidation caused by AOS can lead to loss in membrane semi-permeability and is one of the primary mechanisms of stress-injury (Foyer et al., 1997).

The concentration of AOS is one of the key factors determining the beneficial or harmful roles to be performed by AOS, higher concentrations causing injury (Foyer et al., 1994) and moderate concentrations leading to acclimation. Moderate levels of AOS are believed to induce increased antioxidant activity, resulting in stress tolerance (Kerdnaimongkol et al., 1997). Moderate levels of AOS are also considered to act as secondary messengers to induce signal transduction pathways, leading to stress tolerance. Prasad et al. (1994) illustrated that in maize seedlings exposed to chilling temperatures in

the dark, severe oxidative stress resulted in injury, whereas moderate oxidative stress resulted in acclimation.

Antioxidant Defenses Against AOS

As described earlier, AOS generate in plants at very high rates even under normal environmental conditions. In order to protect the cells from the deleterious effects of AOS, it is vital to keep a tight control on AOS levels. This is achieved by a combination of several defense strategies against AOS, such as pre-emptive scavengers, compartmentalization of metal catalysts to stop formation of AOS (Asada and Takahashi, 1987; Basaga, 1990) and repairing the damage caused by AOS (Cadenas, 1989). Pre-emptive scavengers intercept AOS before they can react and cause cellular damage. Pre-emptive scavengers include several antioxidant defenses, which act in unison at both genetic and protein levels (Alscher et al., 1997), detoxifying AOS from various cellular locations, preventing cellular damage. "Any compound capable of detoxifying AOS without undergoing formation into destructive radical itself is an antioxidant" (Noctor and Foyer, 1998). Antioxidants are enzymatic and non-enzymatic. Non-enzymatic mechanisms include compounds like glutathione, alpha tocopherol, ascorbate, beta carotene, hydroquinones, flavonoids, phenols and phenolic acids (Elstner, 1982; Larson, 1988). Enzymatic mechanisms, defined as "enzymes which directly catalyze reactions intercepting AOS" (Noctor and Foyer, 1998) include superoxide dismutases (SOD; EC: 1.15.1.1), catalases (CAT; EC: 1.11.1.6), and peroxidases (POX; EC: 1.11.1.7).

Enzymatic Defenses Against AOS

SOD is a class of metal-containing enzymes and is the principle antioxidant defense against $^{\cdot}O_2^-$, breaking it down to H_2O_2, thus affecting the cellular concentrations of both $^{\cdot}O_2^-$ and H_2O_2 (Bowler et al., 1992).

$$2\ O_2^{\cdot-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad \text{(Bowler et al., 1992)}$$

SOD can contain Cu/Zn, Fe or Mn as a metal cofactor (Bowler, 1994; Camp et al., 1994) and is present in several cellular organelles. Cu/Zn SOD is found in chloroplasts, cytosol and mitochondria, Fe SOD is found in chloroplasts and Mn SOD is found in chloroplasts and peroxisomes (Del Rio et al., 1992). SOD is a very efficient catalyst and can detoxify O_2^- at diffusion rates (Bowler et al., 1994).

CAT is an important enzymatic defense against H_2O_2. Scandalios (1994) reviewed following properties of CAT. CAT is a class of tetrameric enzymes existing in multiple isoforms (CAT-1, CAT-2 and CAT-3, coded by genes *cat-1, cat-2* and *cat-3*) and is present in all aerobic organisms. CAT-1 and CAT-2 are associated with peroxisomes and glyoxysomes and CAT-3 is associated with mitochondria. CAT detoxifies photorespiratory H_2O_2 in peroxisomes and in glyoxysomes it detoxifies H_2O_2 produced during β-oxidation of fatty acids. CAT contains heme group as a cofactor. CAT has an extremely high rate of H_2O_2 detoxification. The efficiency of CAT in removal of H_2O_2 is limited due to the susceptibility of CAT to photoinactivation (Feierabend et al., 1992), its low specificity for H_2O_2 because of the required access of two H_2O_2 molecules at the CAT active site, and its limited cellular localization (Foyer and Harbinson, 1994). However, H_2O_2 can diffuse to distant cellular locations from its site of production and therefore, H_2O_2 produced at one site can be detoxified at the other. Hence, although

cellular locations of CAT are limited, the overall contribution of CAT in H_2O_2 detoxification can be significant.

The mode of action of CAT depends upon H_2O_2 concentration. Scandalios (1994) described the following mechanism of CAT action at low or high H_2O_2 concentrations:

In the presence of low levels of H_2O_2, CAT catalyzes the "peroxidatic" reaction, oxidizing available hydrogen donors.

$$RH_2 + H_2O_2 \xrightarrow{CAT} R + 2\,H_2O$$

Whereas, in the presence of high levels of H_2O_2, CAT catalyzes the "catalatic" reaction, with a very high rate of H_2O_2, detoxification.

$$2\,H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2$$

Scandalios (1994) further summarized the two-step mechanism involved in peroxidatic and catalatic reactions as:

"Step 1": CAT reacts with H_2O_2 forming a CAT-Fe-peroxide complex referred by Scandalios (1994) as "Compound-I".

$$CAT\text{-}Fe\text{-}OH + H_2O_2 \longrightarrow CAT\text{-}Fe\text{-}OOH + H_2O$$

Step 2: Depending upon the concentration of H_2O_2, peroxidatic or catalatic reactions occur.

"Peroxidatic reaction": In the presence of lower levels of H_2O_2, hydrogen donors reduce "Compound-I".

$$CAT\text{-}Fe\text{-}OOH + RH_2 \longrightarrow CAT\text{-}Fe\text{-}OH + H_2O + O_2$$

"Catalatic reaction": In the presence of higher levels of H_2O_2, "Compound-I" reacts with H_2O_2 to form H_2O and O_2.

$$CAT\text{-}Fe\text{-}OOH + H_2O_2 \longrightarrow CAT\text{-}Fe\text{-}OH + H_2O + O_2$$

The co-operative effect of SOD and CAT ensures detoxification of O_2^- and H_2O_2. This is a very important defense strategy, vital to avoid build up of O_2^- and H_2O_2, which can take part in the Haber-Weiss reaction forming more deleterious AOS, OH (Basaga, 1989).

$$O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + OH \qquad \text{(Basaga, 1989)}$$

Peroxidase (POX) is a class of non-specific enzymes that utilize diverse substrates like ascorbate, guaiacol, NADPH or cytochrome c for functioning (Asada, 1992). POX is a monomeric protein (Lagrimini et al., 1990). The H_2O_2-detoxifying POX can contain several prosthetic groups such as heme, flavin or seleno proteins and can detoxify H_2O_2 or other hydroperoxides (Asada, 1992).

$$AH_2 + H_2O_2\,(ROOH) \xrightarrow{POX} A + 2\,H_2O\,(ROH + H_2O) \qquad \text{(Asada, 1992)}$$

Asada (1992) explained the two-step mechanism employed by most POX containing the heme group as a cofactor in detoxifying H_2O_2 as follows:

$$2\,AH_2 + H_2O_2 \longrightarrow 2\,AH + 2\,H_2O$$
$$2\,AH + AH_2 \longrightarrow 2\,AH_2 + A$$

Ascorbate peroxidase (APOX; EC1.11.1.11) is the chief POX involved in H_2O_2 detoxification in plants and is substrate-specific for ascorbate (Asada and Takahashi, 1987). APOX contains the heme group as a cofactor (Creissen et al., 1994) and has a higher affinity for H_2O_2 than CAT. APOX is present in chloroplast and cytosol. The chloroplastic APOX can be further distinguished into stroma form and thylakoid membrane-bound form (Asada, 1994). APOX uses ascorbate as an electron donor for detoxification of H_2O_2, forming monodehydroascorbate (MDHA).

$$H_2O_2 + 2 \text{ Ascorbate} \xrightarrow{\text{APOX}} 2 \text{ Monodehydroascorbate} + 2\ H_2O$$

MDHA further gets converted into ascorbate and dehydroascorbate (DHA). DHA can be regenerated into ascorbate by a reaction catalyzed by DHA reductase, using glutathione (GSH) as a substrate. Product of this reaction, glutathione disulfide (GSSG) gets reduced back into GSH by a reaction catalyzed by GSH reductase, using the reducing power of NADPH. This cycle of reactions involved in elimination of H_2O_2 and regeneration of substrates is the ascorbate-glutathione cycle (Noctor and Foyer, 1998). Due to the diffusive nature of H_2O_2, both APOX and CAT can quench H_2O_2 escaped from the detoxifying action of the other (Foyer et al., 1994).

Hydrogen Peroxide

H_2O_2, being implicated in injury due to chilling stress, has been the focus of several stress studies. Increased production of H_2O_2 was observed in several plant systems, including transgenic tomato plants expressing an antisense catalase gene (ASTOMACAT1) exposed to chilling stress (Kerdnaimongkol and Woodson, 1999), cold-treated winter wheat (Okuda et al., 1991) and cucumber leaves exposed to chilling (Lee and Lee, 2000). In plants exposed to chilling stress, H_2O_2 can either induce oxidative damage, or can induce signal transduction, resulting in chilling tolerance (Prasad et al., 1994; Kerdanaimongkol et al., 1997). Levine et al. (1994) suggested that low levels of H_2O_2 can induce plant defense genes such as glutathione S-transferase and glutathione peroxidase and high levels of H_2O_2 can cause cell death in soybean cell cultures. Studies imply that the role of H_2O_2 in oxidative stress is concentration

dependent, high concentrations causing injury and low concentrations inducing the events leading to acclimation.

Under normal conditions plants produce H_2O_2 as a by-product of photorespiration and β-oxidation of fatty acids. H_2O_2 is formed in several cellular organelles, including peroxisomes, chloroplasts and mitochondria (Elstner, 1982). Dismutation reaction catalyzed by superoxide dismutase (SOD) generates H_2O_2 in chloroplasts (Bowler, 1992). Minor quantities of H_2O_2 can also be generated by reaction of $O_2^{\cdot}$ with some cellular reductants (Asada and Takahashi, 1987). In peroxisomes, action of oxidases such as glycolate oxidase, urate oxidase and acyl CoA oxidase generate H_2O_2 (Willekens et al., 1995).

H_2O_2 is not a radical and does not carry a charge. Due to the lack of charge, it can diffuse across the membranes and hence, the site of oxidative damage due to H_2O_2 can be distant from its site of production. Due to its diffusive nature, H_2O_2 can act as a secondary messenger (Foyer et al., 1997). H_2O_2 can participate in redox reactions and can react with thiol groups of proteins and enzymes, inactivating them. H_2O_2 can have deleterious effects at 10 mM (Bowler et al., 1992). However, it is a relatively stable molecule by itself. The primary mechanism of injury resulting from H_2O_2 is by participating either in the Haber-Weiss reaction with $O_2^{\cdot}$ or in Fenton-type reactions, in the presence of metal ions to form $^{\cdot}OH$ (Larson, 1988). Being the most reactive AOS with a very high redox potential, $^{\cdot}OH$ can cause considerable damage. Hence intercepting H_2O_2 and $O_2^{\cdot}$, the substrates for the formation of $^{\cdot}OH$, is vital to avoid injury.

Acclimation

Moderate heat stress results in acclimation, increasing the thermotolerance of plants to previously lethal temperatures. Although the degree of acclimation depends directly on the exposure temperature and duration, other factors like genotype (Ingram and Buchanan, 1984), previous exposure temperature and tissue age (Anderson et al., 1990) also have a significant effect on the acclimation potential of plants.

Exposure to abiotic stresses can induce increased levels of AOS. Moderate increase in the levels of AOS induces an increase in the antioxidant activity of one or several antioxidant enzymes (Prasad et al., 1994). Elevated antioxidant activity aids in quenching additional load of AOS and thus stress tolerance is acquired (Allen, 1995). However, tolerance thus provided is inadequate to quench very high loads of AOS.

In several studies increased levels of H_2O_2 have been observed (Okuda et al., 1991; Prasad et al., 1994; Kerdnaimongkol and Woodson, 1999; Lee and Lee, 2000). As previously stated, due to it diffusive nature, H_2O_2 has a potential to act as a secondary messenger to induce signal transduction pathways leading to acclimation (Levine et al., 1994). Attempts have been made to unmask the role of H_2O_2 in acclimation by inducing increase in its levels through inhibition of antioxidants. Dat et al. (1998) reported that salicylic acid, a CAT inhibitor induced heat acclimation in mustard seedlings. The thermotolerance thus achieved involved an increase in H_2O_2 levels. Similarly, acetylsalicylic acid, an artificial analog of salicylic acid induced thermotolerance in potato microplants (Lopez-Delgado et al., 1998). These findings suggest involvement of H_2O_2 in the induction of thermotolerance.

Signal Transduction

Signal transduction is a cascade of biochemical events, during which specific extracellular stimuli are converted into chemical signals, which trigger specific responses including changes in gene expression. Biotic and abiotic stresses act as extracellular stimuli, which are perceived by various receptors and converted into a chemical form. This step usually involves a secondary messenger, such as AOS (Bowler et al., 1992; Wu et al., 1995) to convey the chemical signal to a specific intercellular target molecule. Signal transduction results in induction of a cellular response, frequently due to changes in phosphorylation state of proteins leading to changes in gene expression (Seo et. al., 1997).

Contrary to well characterized animal signal transduction pathways, those in plant systems are not well understood. Although hypersensitive response and systemic acquired resistance resulting in resistance to pathogen attack have been studied, no single signaling pathway has yet been completely revealed. The available information about roles of specific biomolecules and precise events during signal transduction pathways is sketchy (Foyer et al., 1997).

AOS can trigger induction of signal transduction pathways in plants exposed to both biotic and abiotic stresses (Doke et al., 1994). Role of moderate increase in levels of H_2O_2 as a potential trigger to induce stress tolerance has been previously described. Price et al. (1994) reported that H_2O_2 caused an increase in the levels of cytosolic calcium ($[Ca^{2+}]_{cyt}$), indicating a possible role of Ca^{2+} in signal transduction. Knight et al. (1996) reported that both long-term as well as short-term treatments with H_2O_2 altered $[Ca^{2+}]_{cyt}$ in *Arabidopsis* and the response curve obtained was similar to the cold acclimation

response curve, suggesting a possibility of involvement of a common signal transduction pathway.

Calcium and Signal Transduction

$[Ca^{2+}]_{cyt}$ plays an important role in signal transduction. After the reception of signal, rapid influx of Ca^{2+} occurs into cytosol from both intra-cellular and extra-cellular Ca^{2+} stores. Knight et al. (1992) demonstrated that Ca^{2+} is released from the apoplast in cold shock, whereas it is released from intracellular stores such as vacuole in case of touch and wind. Thus, the type of stimulus received governed the source of Ca^{2+}. Intracellular Ca^{2+} influx occurs through voltage gated and IP_3 channels and extracellular Ca^{2+} influx primarily occurs through the voltage gated channels (Bush, 1993). However, influx may also occur through I_{CRAC}, channels involved in capacitative Ca^{2+} signaling or even through non-selective channels (Bush, 1993). The increased $[Ca^{2+}]_{cyt}$ activates Ca^{2+} binding target proteins, including calmodulin and Ca^{2+} dependant protein kinases (CDPKs). The Ca^{2+} signals are primarily deactivated by the action of ATPase pumps and also by H^+/Ca^{2+} antiport (Buchanan, 2000). ATPases, located in several cellular organelles, including plasma membrane, endoplasmic reticulum (ER) and tonoplast use ATP to delocalize Ca^{2+} into extracytosolic organelles (Buchanan, 2000). Thus, $[Ca^{2+}]_{cyt}$ is restored and Ca^{2+} signal is turned off. In order to achieve effective Ca^{2+} signaling, the difference maintained between $[Ca^{2+}]_{cyt}$ and extracytosolic Ca^{2+} concentration is substantial. $[Ca^{2+}]_{cyt}$ is maintained very low (100-200 nM) as opposed to the higher extracytosolic Ca^{2+} concentration (up to 1 mM) (Buchanan et al., 2000).

Several studies have indicated a role of Ca^{2+} in cold signaling (Monroy et al., 1993; Monroy and Dhindsa, 1995; Knight et al., 1996). Elevated levels of $[Ca^{2+}]_{cyt}$ have been reported in plants exposed to cold shock (Knight et al., 1996), chilling (Monroy and Dhindsa, 1995), touch (Braam, 1992), oxidative stress (Price et al., 1994), and heat shock (Biyaseheva et al., 1993; Gong et al., 1998). Few studies have been conducted to investigate the role of Ca^{2+} in heat acclimation. In tobacco seedlings, heat-shock resulted in increased $[Ca^{2+}]_{cyt}$ (Gong et al., 1998). The authors proposed that elevated $[Ca^{2+}]_{cyt}$ acted as a trigger to induce signal transduction pathways resulting in changes in the gene expression acquiring thermotolerance. In another study, Gong et al. (1997) examined the role of exogenous Ca^{2+} on thermotolerance in mustard seedlings and reported elevated Ca^{2+}, increased antioxidant activities and induced thermotolerance. In the same study, treatments with Ca^{2+} chelators and Ca^{2+} channel blockers lowered the antioxidant activities and increased injury, substantiating the role of Ca^{2+} in preventing injury due to heat stress. Exogenous Ca^{2+} decreased electrolyte leakage in heat-stressed roots of *Beta vulgaris* (Toprover and Glinka, 1976; Cooke et al., 1986). Exogenous Ca^{2+} treatments also enhanced the heat tolerance of tall fescue and Kentucky bluegrass (Jiang and Huang, 2001). However, external Ca^{2+} did not alleviate the high temperature induced growth retardation of wheat coleoptiles (Onwueme and Laude, 1972). The role of Ca^{2+} signaling in thermotolerance is not yet clear and awaits further investigation.

Summary

Heat stress has profound effects on growth and development of plants. To optimize the productivity of plants, comprehending injury and acclimation resulting from

high temperature is important. Exposure to biotic and abiotic stresses results in elevated levels of AOS. Although beneficial at moderate concentrations, AOS can have deleterious effects at higher concentrations due to their ability to react with biomolecules. H_2O_2 has been implicated in the mechanism of chilling injury when present at high concentrations and when present at moderate concentrations, H_2O_2 is believed to mediate cold acclimation. Due to its permeability across membranes, H_2O_2 may act as a secondary messenger in the signal transduction pathways. Enzymatic antioxidant defenses such as CAT and APOX play a significant role in keeping H_2O_2 concentrations under control. Modifications in the activities of antioxidant enzymes can alter cellular concentration of H_2O_2. The role of H_2O_2 in heat stress injury is not clear.

Objectives

1. To determine the relationship between the stability of hydrogen peroxide defense mechanisms and thermotolerance of vinca (*Catharanthus roseus*) and sweet pea (*Lathyrus odoratus*) leaf tissues.
2. To determine the effect of heat stress on endogenous H_2O_2 levels in vinca (*Catharanthus roseus*) and sweet pea (*Lathyrus odoratus*) leaf tissues.
3. To determine the effect of elevated H_2O_2 levels on thermotolerance of vinca (*Catharanthus roseus*) and sweet pea (*Lathyrus odoratus*) leaf tissues.

Literature Cited

Allen R.D. 1995. Dissection of oxidative stress tolerance using transgenic plants. Plant Physiol. 93: 196-205.

Alscher R.G., J.L. Donahue and C.L. Cramer. 1997. Reactive oxygen species and antioxidants: relationships in green cells. Physiol. Plant. 100: 224-233.

Anderson J.A., G. McCollum and W. Roberts. 1990. High temperature acclimation in pepper leaves. HortScience 25: 1272-1274.

Asada K. and M. Takahashi 1987. Production and scavenging of active oxygen in photosynthesis. In: Kyle D.J., Osmond C.B. and Arntzen C.J. (eds.). Photoinhibition. Elsevier, Amsterdam. pp. 227-287.

Asada K. 1992. Ascorbate peroxidase – a hydrogen peroxide-scavenging enzyme in plants. Physiol. Plant. 85: 235-241.

Asada K. 1994. Production and activation of active oxygen species in photosynthetic tissues. In: C.H. Foyer and P.M. Mullineaux (eds.). Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, Boca Raton, FL. pp. 77-104.

Basaga H.S. 1989. Biochemical aspects of free radicals. Biochem. Cell Biol. 68: 989-998.

Biyaseheva A.E., Y.G. Molotkovskii and L.K. Mamonov. 1993. Increase of free Ca^{2+} in the cytosol of plant protoplasts in response to heat stress as related to Ca^{2+} homeostasis. Russian Plant Physiol. 40: 540-544.

Bowler C.M., M.Van Montagu and D. Inze. 1992. Superoxide dismutase and stress tolerance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43: 83-116.

Bowler C., W. Van Camp, M. Van Montague and D. Inze. 1994. Superoxide dismutase in plants. Crit. Rev. Plant Sci. 13: 199-218.

Braam J. 1992. Regulated expression of the calmodulin-related TCH genes in cultured *Arabidopsis* cells: Induction by calcium and heat shock. Proc. Natl. Acad. Sci. (USA) 89: 3213-3216.

Buchanan B.B., W. Gruissem and R.L. Jones. 2000. Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Maryland. pp. 962-963.

Bush D.S. 1993. Regulation of cytosolic calcium in plants. Plant Physiol. 103: 7-13.

Cadenas E. 1989. Biochemistry of oxygen toxicity. Annu. Rev. Biochem. 58: 79-110.

Camp W.V., M. Van Montagu and D. Inze. 1994. Superoxide dismutases. In: C.H. Foyer and P.M. Mullineaux (eds.). Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, Boca Raton, FL. pp. 317-341.

Cooke A., A. Cookson and M.J. Earnshaw. 1986. The mechanism of action of calcium in the inhibition on high temperature induced leakage of betacyanin from beet root discs. New Phytol. 102: 491-497.

Creisson G.P., E.A. Edwards and P.M. Mullineaux. 1994. Glutathione reductase and ascorbate peroxidase. In: C.H. Foyer and P.M. Mullineaux (eds.). Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, Boca Raton, FL. pp. 343-364.

Dat J.F., H. Lopez-Delgado, C.H. Foyer and I.M. Scott. 1998. Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. Plant Physiol. 116: 1351-1357.

Del Rio L., L.M. Sandalio, J.M. Palma, P. Bueno and F.J. Corpas. 1992. Metabolism of oxygen radicals in peroxisomes and cellular implications. Free Radical Biol. Med. 13: 557-580.

Del Rio L.A., L.M. Sandalio, F.J. Corpas, E. Lopez-Huertas, J.M. Palma and G.M. Pastori. 1998. Activated oxygen mediated metabolic functions of leaf peroxisomes. Physiol. Plant. 104: 673-680.

Dhindsa R.S., P. Plumb-Dhindsa and T.A. Thorpe. 1981. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. 32: 93-101.

Doke N., Y. Miyura, M.S. Leandro and K. Kawakita. 1994. Involvement of superoxide in signal transduction: Responses to attack by pathogens, physical and chemical shocks, and UV irradiation. In: C.H. Foyer and P.M. Mullineaux (eds.). Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, Boca Raton, FL. pp. 177-197.

Elstner E.F. 1982. Oxygen activation and oxygen toxicity. Ann. Rev. Plant Physiol. 33: 73-96.

Feierabend J., C. Schaan and B. Hertwig. 1992. Photoinactivation of catalase occurs under both high- and low-temperature stress conditions and accompanies photoinhibition of photosystem II. Plant Physiol. 100: 1554-1561.

Foyer C.H and J. Harbinson. 1994. Oxygen metabolism and the regulation of photosynthetic electron transport. In: C.H. Foyer and P.M. Mullineaux (eds.). Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, Boca Raton, FL. pp. 1-42.

Foyer C.H., P. Descourvieres and K.J. Kunert. 1994. Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. Plant Cell Environ. 17: 507-523.

Foyer C.H., Lopez-Delgado H., J.H. Dat and I.M. Scott 1997. Hydrogen peroxide-and glutathione-associated mechanisms of acclimatory stress tolerance and signaling. Physiol. Plant. 100: 241-254.

Gardner H.W. 1991. Recent investigations into the lipoxygenase pathway of plants. Biochim. Biophys. Acta 1084: 221-239.

Georgieva K. 1999. Some mechanisms of damage and acclimation of the photosynthetic apparatus due to high temperature. Bulg. J. Plant Physiol. 25: 89-99.

Gong M., S. Chen. Y. Song and Z. Li. 1997. Effect of calcium and calmodulin on intrinsic heat tolerance in relation to antioxidant system in maize seedlings. Aust. J. Plant Physiol. 24: 371-379.

Gong M., A.H. van der Luit, M.R. Knight and A.J. Trewavas. 1998. Heat-shock-induced changes in intracellular Ca^{2+} level in tobacco seedlings in relation to thermotolerance. Plant Physiol. 116: 429-437.

Gross G. G. 1980. The biochemistry of lignification. Adv. Bot. Res. 8: 25-63.

Halliwell B. and J.M.C. Gutteridge. 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 219:1-14.

Hildebrand D.F. 1989. Lipoxygenases. Physiol. Plant. 76: 249-253.

Ingram D.L. and D.W. Buchanan. 1984. Lethal high temperatures for roots of three citrus root-stocks. J. Amer. Soc. Hort. Sci. 109: 189-193.

Ingram D.L. 1985. Modeling high temperature and exposure time interactions on *Pittosporum tobira* root cell membrane stability. J. Amer. Soc. Hort. Sci. 110: 470-473.

Jiang Y. and B. Huang. 2001. Effects of calcium on antioxidant activities and water relations associated with heat tolerance in two cool-season grasses. J. Exp. Bot. 52: 341-349.

Kerdnaimongkol K., A. Bhatia, R.J. Joly and W.R. Woodson. 1997. Oxidative stress and diurnal variation in chilling sensitivity of tomato seedlings. J. Amer. Soc. Hort. Sci. 122: 485-490.

Kerdnaimongkol K. and W.R. Woodson. 1999. Inhibition of catalase by antisense RNA increases susceptibility to oxidative stress and chilling injury in transgenic tomato plants. J. Amer. Soc. Hort. Sci. 124: 330-336.

Kimball J. 2000. http://www.ultranet.com/~jkimball/BiologyPages/C/C4plants.html

Knight M.R., S.M. Smith and A.J. Trewas. 1992.Wind-induced plant motion immediately increases cytosolic calcium. Proc. Natl. Acad. Sci. (USA) 89: 4967-4971.

Knight H., A.J. Trewavas and M.R. Knight. 1996. Cold calcium signalling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. Plant Cell 8: 489-503.

Krause G.H. 1994. The role of oxygen in photoinhibition of photosynthesis. In: C.H. Foyer and P.M. Mullineaux (eds.). Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, Boca Raton, FL. pp. 43-76.

Lagrimini L.M., S. Brandford and S. Rothstein. 1990. Peroxidase-induced wilting in transgenic tobacco plants. Plant Cell 2: 7-18.

Larson R.A. 1988. The antioxidants of higher plants. Phytochemistry 27: 969-978.

Lee D.H. and C.B. Lee. 2000. Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. Plant Sci. 159: 75-85.

Leng O.H. 2000. http://www.vjc.moe.edu.sg/dept/biology/webpage/cell%20membrane/THE%20CELL%20MEMBRANE%20lecture%202000/sld025.htm

Levine A., R. Tenhaken, R. Dixon and C. Lamb. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79: 583-593.

Levitt J. 1980. Responses of plants to environmental stresses. Academic Press. New York. Vol. 1. pp. 347-393.

Liu X. and B. Huang. 2000. Heat stress injury in relation to membrane lipid peroxidation in creeping bentgrass. Crop Sci. 40: 503-510.

Lopez-Delgado H., J.F. Dat, C.F. Foyer and I.M. Scott. 1998. Induction of thermotolerance in potato microplants by acetylsalicylic acid and H_2O_2. J. Exp. Bot. 49: 713-720.

Monroy A.F., F. Sarhan and R.S. Dhindsa. 1993. Cold-induced changes in freezing tolerance, protein phosphorylation and gene expression. Plant Physiol. 102: 1227-1235.

Monroy A.F. and R.S. Dhindsa. 1995. Low-temperature signal transduction: Induction of cold acclimation-specific genes of alfalfa by calcium at 25 °C. Plant Cell 7: 321-331.

Mehdy M.C., Y.K. Sharma, K. Sathasivan and N.W. Bays. 1996. The role of activated oxygen species in plant disease resistance. Physiol. Plant. 98: 365-374.

Mehlhorn H. and A.R. Wellburn. 1994. Man-induced causes of free radical damage to plants: O_3 and other gaseous pollutants. In: C.H. Foyer and P.M. Mullineaux (eds.). Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, Boca Raton, FL. pp. 155-174.

Monson R. 2001. http://spot.colorado.edu/~monsonr/2060lecture104.html

Murata N., and D.A. Los. 1997. Membrane fluidity and temperature perception. Plant Physiol. 115:875-879.

Nagao R.T., J.A. Kimpel and J.L. Key. 1990. Molecular and cellular biology of the heat-shock response. Adv. Genet. 28: 235-274.

Nguyen H.T. 1994. Genetic and molecular aspects of high temperature stress response. In: Physiology and Determination of Crop Yield. Boote K.J., Benett J.M., Sinclair T.R., Paulsen G.M. (eds.) ASA-CSSA-SSSA. pp 391-394.

Noctor G., and C.H. Foyer 1998. Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 249-279.

Ntuli M. 1995. http://www.und.ac.za/und/icd/citte/paper/net2/muzi.html

Okuda T., Y. Masuda, A. Yamanaka and S. Sagisaka. 1991. Abrupt increase in the levels of hydrogen peroxide in leaves of winter wheat is caused by cold treatment. Plant Physiol. 97: 1265-1267.

Onwueme I.C. and H.M. Laude. 1972. Heat induced growth retardation and attempts at its prevention in barley and wheat coleoptiles. J. Agric. Sci. 79: 331-333.

Paulsen G.M. 1994. High temperature responses of crop plants. In: Physiology and Determination of Crop Yield. Boote K.J., Benett J.M., Sinclair T.R., Paulsen G.M. (eds.) ASA-CSSA-SSSA. pp 365-389.

Prasad T.K., M.D. Anderson, B.A. Martin, and B.R. Stewart. 1994. Evidence of chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell 6: 65-74.

Price A.H., A. Taylor, S.J. Ripley, A. Griffiths, A.J. Trewavas and M.R. Knight. 1994. Oxidative signals in tobacco increase cytosolic calcium. Plant Cell 6: 1301-1310.

Scandalios J.G. 1994. Regulation and properties of plant catalases. In: C.H. Foyer and P.M. Mullineaux (eds.). Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, Boca Raton, FL. pp. 275-316.

Schoffl F., R. Prandl and A. Reindl. 1998. Regulation of the heat-shock response. Plant Physiol. 117: 1135-1141.

Seo S., H. Sano, and Y. Ohashi. 1997. Transgenic manipulation of signaling pathways of plant resistance to pathogen attack. Biotech. Annu. Rev. 3: 197-225.

Siedow J.N. 1991. Plant lipoxygenase: structure and function. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42: 145-188.

Somersalo S. and G.H. Krause. 1990. Reversible photoinhibition of unhardened and cold acclimated spinach leaves at chilling temperatures. Planta 180: 181-187.

Toprover Y. and Z. Glinka 1976. Calcium ions protect beet root cell membranes against thermally induced changes. Physiol. Plant. 37: 131-134.

Veljovic-Jovanovic S. 1998. Active oxygen species and photosynthesis: Mehler and ascorbate peroxidase reactions. Iugoslav. Physiol. Pharmacol. Acta 34: 503-522.

Vierling E. 1991. The roles of heat shock proteins in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42: 579-620.

Waters E., G. Lee and E. Vierling. 1996. Evolution, structure and function of the small heat shock proteins in plants. J. Exp. Bot. 47: 325-338.

Willekens H., D. Inze, M. Van Montagu, and W. Van Camp. 1995. Catalases in plants. Mol. Breed. 1: 207-228

Wingsle G. 1999. http://www.forestbiotech.com/project/p5.html

Wu G., B.J. Shortt, E.B. Lawrence, E.B. Levine, K.C. Fitzsimmons and D.M. Shah. 1995. Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell 7: 1357-1368.

CHAPTER II

ANTIOXIDANT ACTIVITY AND HYDROGEN PEROXIDE CONTENT IN HEAT STRESSED VINCA AND SWEET PEA

Introduction

Among the primary causes of reduction in crop yields are abiotic stresses, including extreme temperatures, water excess and deficit, salts, heavy metals, photodynamic herbicides and environmental pollutants. High temperature injury can result in considerable pre-harvest and post-harvest crop losses. High temperature injury may involve damage due to oxidative stress as speculated in heat-stressed creeping bentgrass (Liu and Huang, 2000). Oxidative stress is the overall impact of production of high levels of activated oxygen species (AOS). Besides high temperature, oxidative damage due to AOS was implicated in several other abiotic stresses such as chilling and freezing temperatures (Prasad et al., 1994; McKersie et al., 1997), heavy metal stress (Teisseire et al., 2000; Prasad et al., 1999; Lidon et al., 2000), severe water stress (Iturbe-Ormaetxe et al., 1998) and salinity stress (Dionisio-Sese and Tobita, 1998).

AOS include singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH). Production of AOS in plants is not restricted to stressful conditions. Under normal conditions, AOS are produced as a result of several mechanisms, including Mehler reaction and photorespiration. During Mehler reaction, transfer of electrons from H_2O to O_2 forms $O_2^{\cdot}$ and a trans-thylakoid proton gradient

(Ntuli, 1995). During photorespiration, the oxygenation reaction of ribulose bisphosphate carboxylase/oxygenase (Rubisco) forms glycolate, which enters peroxisomes and produces H_2O_2 by the enzyme glycolate oxidase (Veljovic-Jovanovic, 1998; Wingsle, 1999). H_2O_2 can also be formed by dismutation reaction of $O_2^{\cdot-}$ catalyzed by superoxide dismutase (SOD; EC: 1.15.1.1) (Bowler et al., 1992). $^1O_2$ can be generated by a reaction between O_2 and triplet excited state chlorophyll (Asada and Takahashi, 1987).

AOS can perform beneficial or deleterious functions in the cells under normal and stressful conditions. H_2O_2 polymerizes a lignin precursor in the cell wall lignification process under normal conditions (Gross, 1980). When exposed to abiotic stresses, AOS can perform beneficial functions such as induction of cell wall polymerization to prevent pathogen entry, action as "antibiotics" to directly eradicate pathogens and induction of hypersensitive response and systemic acquired resistance (Mehdy et al., 1996). When exposed to biotic or abiotic stress, AOS can induce signal transduction pathways, resulting in tolerance to previously lethal stress (Foyer et al., 1994). Harmful functions of AOS include reacting with several biomolecules including pigments, membranes, enzymes and nucleic acids (Elstner, 1982) and modifying their functions. AOS can contribute to senescence (Dhindsa et al., 1981). AOS can also cause autocatalytic peroxidation of membrane lipids, resulting in loss of membrane semipermeability (Basaga, 1989). Lipid peroxidation is one of the primary mechanisms of stress injury (Foyer et al., 1997). The concentration of AOS is one of the key factors determining the beneficial or harmful functions performed by AOS, higher concentrations causing injury (Foyer et al., 1994) and moderate concentrations leading to acclimation. Moderate levels of AOS are believed to induce increased antioxidant activity, resulting in stress tolerance

(Kerdnaimongkol et al., 1997). Prasad et al. (1994) illustrated that the consequence of severe oxidative stress was injury, whereas moderate oxidative stress led to acclimation in maize seedlings exposed to chilling temperatures in the dark.

In order to protect cells from the potential damage from AOS, several enzymatic and non-enzymatic antioxidant defense systems maintain AOS concentrations under tight control (Noctor and Foyer, 1998). The primary enzymatic defenses include SOD, catalase (CAT; EC: 1.11.1.6), and peroxidases (POX; EC:1.11.1.7). The non-enzymatic defenses are compounds like glutathione, alpha tocopherol, ascorbate, beta carotene, hydroquinones, flavonoids, phenols and phenolic acids, (Elstner, 1982; Larson, 1988). Under normal environmental conditions the antioxidants provide adequate protection against the deleterious effects of AOS (Alscher et al., 1997).

Changes in antioxidant activity have been the focus of several stress studies (Noctor and Foyer, 1998). Findings of some studies have shown a decrease in antioxidant activity as a result of stress (Fadzillah et al., 1996). In such instances it is postulated that decreases in antioxidant activity resulting in higher levels of AOS may be a mechanism of injury. However, in some studies antioxidant activity has been reported to increase as a result of stress (Prasad et al., 1999; Sala and Lafuente, 1999). In such instances, it is postulated that the increase in antioxidant activity occurs in order to detoxify the heavy loads of AOS and provide protection.

Among the AOS, H_2O_2 has been a primary focus of study due to its involvement in chilling-stress injury. Increased production of H_2O_2 was seen in several stress scenarios, including transgenic tomato plants expressing an antisense catalase gene (ASTOMACAT1) exposed to chilling stress (Kerdnaimongkol and Woodson, 1999),

cold-treated winter wheat (Okuda et al., 1991) and cucumber leaves exposed to chilling (Lee and Lee, 2000). H_2O_2 plays a twofold role in plants exposed to chilling stress, inducing not only oxidative damage, but also acting as a signal for induction of defenses, resulting in chilling tolerance (Prasad et al. 1994; Kerdanaimongkol et al. 1997). Levine et al. (1994) suggested that low levels of H_2O_2 could result in induction of plant defense genes such as glutathione S-transferase and glutathione peroxidase and high levels of H_2O_2 could induce cell death in soybean cell cultures. These studies suggest that the role of H_2O_2 in oxidative stress is concentration dependent, high concentrations resulting in injury and low concentrations mediating acclimation.

Plants produce H_2O_2 at optimal environmental conditions as a by-product of metabolic reactions like photorespiration, Mehler reaction and β-oxidation of fatty acids. H_2O_2 is relatively stable due to lower reactivity compared to other AOS. The lack of charge enables H_2O_2 to diffuse across membranes and due to this property, H_2O_2 has the potential to act as a secondary messenger (Foyer et al., 1997). H_2O_2 can exhibit deleterious effects such as enzyme activity inhibition at 10 mM (Bowler et al., 1992). The primary mechanism of injury due to H_2O_2 is acting as a substrate to form $^{\cdot}OH$ by participating either in the Haber-Weiss reaction with $O_2^{\cdot-}$ or in Fenton-type reactions, accepting electrons from metals (Larson, 1988). $^{\cdot}OH$ is the most reactive AOS with a very high redox potential and can cause considerable damage to the plant. Hence, intercepting H_2O_2 and $O_2^{\cdot-}$, the substrates for formation of $^{\cdot}OH$, is vital to avoid injury.

CAT and POX are the key enzyme classes responsible for H_2O_2 detoxification. Tetrameric, heme-containing CAT converts H_2O_2 to water and oxygen utilizing an energy efficient mechanism (Scandalios, 1994). The mode of action of catalase depends upon the

concentration of H_2O_2. At low concentrations of H_2O_2, catalase catalyzes the "peroxidatic" reaction, in which it oxidizes variety of hydrogen donors; whereas, at high concentrations of H_2O_2 it catalyzes the "catalatic" reaction which has a very high rate of detoxification (Scandalios, 1994). However, due to the required access of two H_2O_2 molecules at CAT active site for CAT to be functional, CAT has a very low affinity for H_2O_2 (Noctor and Foyer, 1998). CAT is present in peroxisomes and mitochondria (Scandalios et al., 1980). Plant POX are a class of monomeric glycoproteins containing several prosthetic groups that oxidize biomolecules utilizing wide range of substrates (Lagrimini, 1990). Ascorbate peroxidase (APOX; EC: 1.11.1.11) is a class of heme-containing POX (Creissen et al., 1994) that utilizes ascorbate as an electron donor for detoxification of H_2O_2 into water and monodehydroascorbate (Foyer et al., 1994). APOX detoxifies H_2O_2 in chloroplasts and cytosol (Creissen et al., 1994). Although APOX has a higher affinity for H_2O_2 than CAT, CAT activity in leaves is generally 100-1000 times higher than that of POX (Volk and Feierabend, 1989), making CAT the key enzyme responsible for detoxification of H_2O_2. On the other hand, chloroplasts generate large amounts of H_2O_2 and obtain protection against it from APOX. Thus, cellular activity of CAT as well as APOX is of paramount importance and diminution in CAT or APOX activity could result in increasing cellular H_2O_2 levels, causing injury.

Correlation between modification of antioxidant enzyme activities, changes in endogenous levels of H_2O_2 and injury due to various stresses have been the focus of several studies in order to determine the mechanism of injury. Increased levels of H_2O_2 and decreased antioxidant enzyme activities were correlated with injury due to chilling stress (Prasad, 1996). Mizuno et al. (1998) also reported an increase in both CAT and

APOX activities in potato tubers exposed to chilling stress. However, decreased CAT activity was seen in rice shoot cultures (Fadzillah et al., 1996), cucumber seedlings (Omran, 1980) and cucumber plants (Lee and Lee, 2000) exposed to chilling stress and mustard seedlings exposed to heat shock (Dat et. al., 1998). At injuring temperatures heat-stressed pepper leaves showed significant decrease in CAT activity (Anderson, unpublished data).

The role of H_2O_2 in injury due to heat stress is not clear. The objectives of this study were to 1) determine the relationship between the stability of hydrogen peroxide defense mechanisms and thermotolerance, 2) determine the effect of heat stress on endogenous H_2O_2 levels in plant tissues, 3) determine the effect of elevated H_2O_2 levels on thermotolerance. A heat-tolerant bedding species, vinca (*Catharanthus roseus*) and a heat-susceptible bedding species, sweet pea (*Lathyrus odoratus*), were used.

Materials and Methods

PLANT CULTURE AND AGE. Vinca (*Catharanthus roseus*) and sweet pea (*Lathyrus odoratus*) seeds were sown in 15-cm diameter pots in a commercial potting mix (Universal mix, Strong-lite, Pine Bluff, AR) enriched with micronutrients. Plants were grown in growth chambers programmed at 24/20°C (day/night) cycles, with 45-65% relative humidity. The chambers were programmed for 14 h photoperiod provided by fluorescent and incandescent bulbs. The light intensity at canopy height was ≈ 400 $\mu mol\,m^{-2}\,s^{-1}$. Plants were watered with soluble fertilizer (Peters, 20N-8.6P-16.6K) at $0.7 g\,L^{-1}$ as per requirement, judged by soil color and pot weight.

Since tissue age can affect thermotolerance (Anderson et al., 1990), preliminary experiments were conducted to determine suitable plant age for optimizing tissue response to heat stress. A significant relationship between tissue age and electrolyte leakage (data not presented) was not observed. Based on optimum physiological development stage, 8-week-old plants were selected for the experiments.

HIGH TEMPERATURE TREATMENTS. Seven-millimeter diameter leaf disks were punched out and immersed in 2 mL distilled water in 25 x 150 mm test tubes. High temperature treatments were given for 15 min by placing the test tubes into a circulating water bath set at a desired temperature. The temperature range used was 24° and 50-58°C for vinca and 24° and 44-52°C for sweet pea, at every 1°C interval. Three sub-samples were assayed per temperature exposure. After high temperature treatments, 20 mL 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 probe (Model 35, Yellow Springs Instrument Company, Yellow Springs, OH) and samples were autoclaved followed by another 22 h incubation. Final conductivity measurements were taken. Electrolyte leakage (EL) was computed as the ratio of initial to final conductivity X 100. EL was plotted against exposure temperatures and the temperature midpoint (T_{mid}) was computed as described by Ingram (1985).

THERMOSTABILITY OF CAT AND APOX. One gram leaf tissue, excluding the midrib, was cut into ≈ 1 cm^2 squares and immersed in 2 mL distilled water in a 25 x 150 mm test tube for high temperature treatments. The test tubes were placed into a circulating water bath for 15 min for each high temperature exposure. The temperature range used was 24°C and 48-64°C for vinca, and 24°C and 42-58°C for sweet pea, at

every 2°C interval. Heat-treated tissue was homogenized in a blender (Waring Products Div. Dynamics Corp. of America, New Hartford, CT) along with 0.075 g polyvinylpolypyrrolidone (PVPP) and 25 mL potassium phosphate buffer (50 mM, pH 7.0), then filtered through Miracloth (Calbiochem-Novabiochem Corp., San Diego, CA) pre-moistened with buffer. Filtrate was centrifuged at 16,000 g_n for 15 min and the supernatant was used as crude extract.

CAT activity was determined spectrophotometrically by the decrease in absorbance at 240 nm for 10 s after a 10 s lag (modification of the procedure described by Aebi, 1983). Three mL reaction mixture contained 1.5 mL 1:20 dilution of crude plant extract in buffer and 1.5 mL 30 mM hydrogen peroxide prepared in buffer, which initiated the reaction.

APOX activity was determined spectrophotometrically by the decrease in absorbance at 290 nm for 50 s after a 10 s lag (modification of the procedure described by Miyake et al., 1991). Three mL reaction mixture contained 1 mL 1:10 dilution of crude plant extract using buffer, 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 hydrogen peroxide. All reagents were prepared in buffer.

During both the enzyme assays, crude plant extract prepared using 1 g leaf tissue was analyzed spectrophotometrically in three sub-samples and the experiment was replicated five times. The experiment was a split-plot arrangement in a randomized complete block design. Experimental dates constituted the blocks, plant species were levels of the main unit factor, and EL, APOX and CAT were levels of the split unit factor. T_{mid} responses were calculated for APOX and CAT activity using PROC NLIN

(SAS Institute) in both vinca and sweet pea. The analysis of variance procedure was performed using PROC MIXED (SAS Institute).

EXPOSING LEAF TISSUE EXTRACTS TO HIGH TEMPERATURE TREATMENTS AND MEASUREMENT OF CATALASE ACTIVITY. One gram leaf tissue was homogenized along with 0.075 g PVPP and 25 mL potassium phosphate buffer (50 mM, pH 7.0) in a Waring blender. Leaf tissue extract was filtered through pre-moistened Miracloth and centrifuged at 16,000 g_n for 15 min. The leaf tissue extract was partially purified by sequentially passing it through G25, C2 and anion exchange columns conditioned with 1 mL water and 2 mL buffer. Sixteen x 100 mm test tubes containing 0.5 mL purified extract were placed in a circulating water bath for 15 min for each high temperature exposure.

Preliminary experiments indicated that CAT activity in vinca extracts started declining at lower temperatures compared to intact tissue. Therefore, the CAT response curve was optimized for vinca extracts by selecting a lower temperature range than for intact tissue. Conversely, in sweet pea extract the CAT activity decreased in a similar temperature range compared to intact tissue and therefore, extract was exposed to the same temperature range as intact tissue. The temperature range selected for both vinca and sweet pea was 24° and 42-58°C, at every 2°C interval. After the heat treatments, 9.5 mL phosphate buffer was added to the extracts and CAT activity was assayed as previously described. T_{mid}s were computed as previously described.

Three sub-samples were assayed per high temperature treatment. The experimental design was a completely randomized design and T_{mids} were the responses. The analysis of variance procedure was performed using PROC MIXED (SAS Institute).

MEASUREMENT OF H_2O_2 IN CONTROL, INJURED AND KILLED TISSUE. Seven-millimeter diameter leaf disks were punched for measurement of EL and 0.5 g leaf tissue was cut into strips excluding the midrib for measurement of H_2O_2. The leaf disks or strips were immersed in 2 mL distilled water in 25 x 150 mm test tubes 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 and were either assayed for EL or H_2O_2 content.

Hydrogen peroxide content of the tissue was determined spectrophotometrically (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 milliliter chilled 5% trichloroacetic acid (TCA) was added and the tissue was homogenized using a mortar and pestle. Homogenate was filtered through Miracloth pre-moistened with TCA. Filtrate was vortexed with 0.1 g activated carbon and 0.04 g PVPP and 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. Three mL reaction mixture contained 1.92 mL 0.375 M sodium phosphate buffer (pH 6.5), 0.3 mL of 3.3 μM 3-dimethylaminobenzoic acid (DMAB), 0.3 mL 0.07 μM 3-methyl-2-benzothiazolinonehydrazone (MBTH), and 10 ng type 1 horseradish peroxidase (0.25 units). All reagents were prepared in buffer, except MBTH, which was prepared in water. The reaction was initiated by adding 0.3 mL crude plant extract. The reaction was carried out at 24°C and samples were incubated for 24 h in the dark before measurement. H_2O_2 content was assayed by recording the absorbance at 590 nm. During each experiment a standard absorbance curve was generated by measuring a range of concentrations of H_2O_2. The curve thus obtained was used to calculate the H_2O_2 concentration in the tissue.

Three sub-samples were assayed from each crude plant extract. The experiment was a split-plot arrangement in a randomized complete block design. Experimental dates constituted the blocks. H_2O_2 concentration and EL were the responses. The analysis of variance procedure was performed using PROC MIXED (SAS Institute).

HEATING IN LIGHT AND DARK. Leaf disks were exposed to light and dark treatments using a circulating water bath lighted with incandescent bulbs. Light intensity was ≈ 650 $\mu mol \cdot m^{-2} \cdot sec^{-1}$. Seven-millimeter leaf disks were cut using a borer for the measurement of EL and 0.5 g leaf tissue was cut into ≈ 1 cm^2 squares for assaying endogenous H_2O_2. Erlenmeyer flasks containing 20 mL deionized distilled water and either three disks or 0.5 g tissue were placed in the lighted water bath. For heating in the dark, flasks were covered with aluminum foil. The water bath temperature was increased stepwise from 25°C to 53°C for vinca and from 25°C to 46.5°C for sweet pea, with a 15 min soak time after every 5°C increase. Samples were taken out after 25°C and the final temperature exposure. EL and H_2O_2 levels were assayed as previously described.

The experiment was a split-plot arrangement in a randomized complete block design. Experimental dates constituted the blocks. H_2O_2 concentration and EL were the responses. The analysis of variance procedure was performed using PROC MIXED (SAS Institute).

Results and Discussion

THERMOSTABILITY OF H_2O_2 DEFENSES. Antioxidant enzymes play a vital role in protecting cells from deleterious effects of AOS. There is a plethora of

literature available on modification of antioxidant enzyme activities during exposure to abiotic stresses (Noctor and Foyer, 1998). It is postulated that decreased antioxidant levels result in the production of excessive AOS, resulting in injury. 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). In the event of direct involvement of H_2O_2 in the mechanism of injury due to heat stress, one would expect to see impaired defenses and/or increased levels of endogenous H_2O_2 in heat stressed plant tissue. Also, if impairment of defenses results in injury, one would expect that as the temperature increases, activities of antioxidant enzymes would decrease, resulting in elevated AOS levels prior to, or coincident with, the occurrence of injury.

It was observed that at increasing temperatures, CAT and APOX activities decreased and then stabilized at a minimum, resulting in inverted sigmoidal curves (Fig. 1-4). The amount of electrolytes leaking from damaged membranes increased at increasing temperatures, then stabilized at a maximum, resulting in sigmoidal curves (Fig. 5,6). In this study, temperature midpoints (T_{mid}s) obtained by plotting EL against temperature as an index of thermotolerance were used. Ingram (1985) utilized T_{mid} obtained from a similar sigmoidal response curve from EL in *Pittosporum tobria* root cells to quantify the interaction between heat stress and membrane stability. T_{mid}s obtained by plotting enzyme activities against temperature were used as indexes of thermostability of enzyme activities. In vinca, T_{mid}s of sigmoidal curves for EL, APOX and CAT were 53.9°, 52.9°, and 56.0°C, respectively, and in sweet pea they were 48.4°, 50.5°, and 50.0°C, respectively. In vinca, T_{mid}s for APOX and EL were not significantly different, but they were significantly lower than the T_{mid} for CAT at $P \leq 0.05$ (Table 1). In

sweet pea, T_{mid}s for APOX and CAT were not significantly different from each other, but were significantly higher than the T_{mid} for EL. Also, T_{mid}s for EL, APOX and CAT in vinca were significantly higher than the corresponding values in sweet pea. The T_{mid}s indicate that CAT activity decreased in vinca at a significantly ($P \leq 0.05$) higher temperature than the temperature at which EL increased, and the temperature at which APOX activity decreased was not significantly different than that at which EL increased. In sweet pea both CAT and APOX activities decreased at a significantly higher temperature than that at which EL increased.

In this study, T_{mid}s have been used to quantify the injurious temperature. A T_{mid} is a mathematical midpoint of sigmoidal or inverted sigmoidal curve. T_{mid} value does not furnish information about another indicator of heat injury, the temperature at which EL begins increasing and enzyme activities begin decreasing. Since it is not known how much decline in the enzyme activity or increase in EL is physiologically significant, the discussion is solely based upon the numeric decrease or increase. APOX activity in vinca began decreasing between 48-50°C, and (Fig. 1) and CAT activity started decreasing between 50-52°C (Fig. 3) and EL started increasing between 52-53°C (Fig. 5). APOX and CAT activities in sweet pea began decreasing between 44-46°C (Fig. 2,4) and EL began increasing between 47-48°C (Fig. 6). Thus, in vinca and sweet pea the decline in the enzyme activities and increase in EL occurred in a similar temperature range.

Decline in the enzyme activities, followed by heat-stress injury would have made the involvement of defense impediment in heat-stress injury apparent. However, looking at both T_{mid}s and temperature ranges in which enzyme activities declined, the results indicate that impairment of defenses occurred either at a lower or similar temperature

than the increase in the EL in both vinca and sweet pea. Hence, the data did not present conclusive evidence of the involvement of enzyme defense impairment in the mechanism of injury in heat-stressed vinca and sweet pea leaf tissue.

Several reasons can be stated to explain the loss of enzyme activities at higher temperatures, including conformational change leading to decline in the activity, production of inhibitors, diminished rates of enzyme synthesis and/or elevated enzyme degradation. Also, the factors stated above may interact, finally determining the net enzyme activity. MacRae and Ferguson (1985) suggested that a change in enzyme configuration was a very strong possibility in CAT activity decline in chilling stress. Lazarov (1983) established that in the rat liver cells, for CAT to become active, the precursor apomonomer must pass through the peroxisomal membrane, where it acquires heme and aggregates to the active tetrameric form. Hence, damage to the peroxisomal membrane might affect CAT assembly, in turn affecting its activity. However, in spite of the destructive leaf extract preparation method used, measurable enzyme activity was seen in the experiments. Also, considering the time course of the enzyme assays, the primary focus was on the decrease in activity of existing enzyme. Therefore the factors related to enzyme production or degradation were important, but not the most significant. Exploring all the possibilities for the decrease in enzyme activities was beyond the scope of this study and needs further investigation. In this study, an increase in EL after heat treatments was observed, implying heat-induced damage to membranes that had a potential to influence the events associated with the modification in enzyme activities. Hence, the possibility that loss in enzyme activities might have been a consequence of the

events leading to loss of semipermeability of membranes of heat-stressed vinca and sweet pea leaf tissue was considered.

Vinca, a heat tolerant species was compared with a heat susceptible species, sweet pea. The higher T_{mid} for EL confirmed that vinca is more thermotolerant than sweet pea. CAT and APOX activities showed significantly higher thermostability in vinca than in sweet pea. One reason for the differences in enzyme stability could be the presence of more thermostable isozymes of CAT and APOX in vinca. High temperature could result in reversible denaturation or irreversible aggregation of proteins, and due to small differences in the amino acid sequences of isozymes, the effect of high temperature on their structure and/or conformation could be variable. If loss in CAT and APOX enzyme activity was a consequence of events related to loss of membrane semipermeability, it may explain why these enzymes were more thermostable in vinca than sweet pea. Protective mechanisms, such as molecular chaperones exist in plants to protect the protein structures from thermal denaturation or aggregation (Nagao et al., 1990), maintaining enzyme activities at higher temperatures. Depending upon the type and amount, the protective machinery could have provided higher thermoprotection to the enzymes in vinca than sweet pea.

In order to better understand the causes associated with decline in enzyme activities due to heat stress and the difference in thermostability of enzymes in heat tolerant and heat susceptible species, partially purified extracts of vinca and sweet pea leaf tissue were exposed to high temperature treatments and CAT activity was assayed. Although partial purification of tissue extract resulted in 48% decreased CAT activity in

vinca and 30% decreased CAT activity in sweet pea compared to the intact tissue controls. CAT activity was still measurable (data not presented).

T_{mid}s for CAT activity were 48.4° and 49.8°C in vinca and sweet pea extracts, respectively. Thus, compared to T_{mid} for intact vinca tissue (56 °C), T_{mid} for vinca extract decreased about 8.6°C. However, in sweet pea, T_{mid}s for intact tissue and extracts were similar. Due to procedural and time differences in conducting the two assays, the data from heat treatments of intact tissue and purified extracts were not pooled for a combined statistical analysis. Nonetheless, the decrease in T_{mid} in vinca purified extract as opposed to lack of change in T_{mid} in sweet pea purified extract was apparent. There was no significant difference between T_{mid}s for CAT activity in purified leaf tissue extracts of vinca and sweet pea.

Earlier discussion indicates the possibility of the events responsible for loss of membrane stability being a significant factor responsible for decrease in enzyme activities after exposure to high temperature treatments. Considerably lower T_{mid} for CAT activity in vinca extract compared to intact tissue was observed. Therefore, it seemed unlikely that loss in CAT activity was a secondary event resulting from heat-induced loss of membrane semipermeability.

T_{mid}s for vinca and sweet pea purified leaf tissue extract were not significantly different, questioning the likelihood of presence of more thermostable isozymes in vinca. A viable explanation for the similar T_{mid}s of vinca and sweet pea purified leaf tissue extracts as opposed to higher T_{mid} of intact vinca tissue than sweet pea was better thermoprotection due to the type or amount of existing protective mechanisms, such as molecular chaperones in intact vinca tissue. The observed loss in the former higher

thermostability of CAT activity in vinca could have been due to the effect of extract purification process on the existing protective mechanisms. As opposed to vinca, there seemed to be no alteration in thermostability of partially purified tissue extract of sweet pea, indicating probable differences in types of protective mechanisms.

The data were incapable of establishing the direct involvement of impairment of H_2O_2 defenses in heat stress injury. Hence, a more direct approach to determine the involvement of H_2O_2 in heat stress was taken by measuring the endogenous H_2O_2 in control, injured and killed tissue.

ENDOGENOUS H_2O_2 LEVELS IN CONTROL, INJURED AND KILLED TISSUE. In the event of direct involvement of H_2O_2 in heat stress injury, the endogenous levels of H_2O_2 would have been expected to increase with increasing temperatures. Therefore, endogenous levels of H_2O_2 in controls and tissue injured as well as killed by elevated temperature were measured. Temperature selection for each species was based upon EL, with the injurious temperature being the T_{mid} for EL and the killing temperature corresponding to the EL maximum (Table 2). In vinca, EL was 22% at 24 °C and increased to 64% at injurious temperature (54 °C) and further to 94% at killing temperature (62 °C). Similarly in sweet pea, EL was 23% at 24 °C and increased to 59% at injurious temperature (48 °C) and further to 93% at killing temperature (54 °C).

No significant differences in the levels of H_2O_2 at the given thermal regimes in vinca and sweet pea were observed (Table 2). Also, at the given thermal regimes there was no significant difference in H_2O_2 levels between the two species.

If H_2O_2 was a key player in heat stress injury, an increase in the level of H_2O_2 at elevated temperatures would have been expected. The observed lack of significant

difference in H_2O_2 levels at injurious temperatures may have been due to lack of production of additional H_2O_2 at higher temperatures or due to a corresponding increased degradation of H_2O_2, ultimately resulting in the same steady state H_2O_2 concentration. The lack of increase in observed H_2O_2 levels, in spite of increased production, could have been due to breakdown of H_2O_2 to $^{\cdot}OH$ by Haber-Weiss or Fenton-type reactions. However, in a similar study with rice (*Oryza sativa*) plants exposed to water stress, Boo and Jung (1999) reported a rapid decrease in H_2O_2 levels and no significant change in the concentration of catalytic iron, a recurrent substrate for the Haber-Weiss or Fenton-type reactions. Another possibility for static H_2O_2 level in spite of higher production could have been detoxification of H_2O_2 by antioxidants, which seems unlikely since in this study it was found that antioxidant activities decreased at injuring temperatures. Hence, it is more likely that the endogenous H_2O_2 levels did not alter as a result of heat stress.

MacRae and Ferguson (1985) reported results similar to these findings in pea (*Pisum sativum*), mung bean (*Vigna radiata*) and cucumber (*Cucumis sativus*) exposed to chilling stress. They observed a significant decrease in CAT activity, however, H_2O_2 content in these species did not increase significantly. Also, Boo and Jung (1999) reported that in rice plants exposed to water stress, APOX and CAT activities decreased but levels of $^{\cdot}O_2^-$ showed no change and levels of H_2O_2 decreased. The results from these studies suggest that impairment of enzyme defenses resulting in high levels of H_2O_2 may not be the mechanism of injury due to chilling and water stress. However, Fadzillah et al. (1996) reported that at 4°C, activity of CAT decreased and activity of APOX did not change in rice shoot cultures exposed to chilling stress. They also reported an almost fourfold increase in tissue levels of H_2O_2. They hypothesized that the loss in antioxidant

activity might have contributed to the increase in H_2O_2 levels and chilling injury. In another study with chilled cucumber seedlings, Omran (1980) reported a decrease in CAT activity with no change in POX activity and a subsequent increase in the levels of H_2O_2. It was postulated that the increase in levels of H_2O_2 might have resulted from reduced CAT activity. Similarly, Kuroda and Sagisaka (1998) studied changes in the levels of H_2O_2 in apple (*Malus domestica*) flower buds exposed to freezing injury and reported that high levels of H_2O_2 were produced following freezing. The authors proposed that elevated H_2O_2 impaired H_2O_2-scavenging mechanisms such as POX, further resulting in accumulation of H_2O_2, which played a role in the mechanism of injury by acting as a substrate in the formation of $^{\cdot}OH$.

In a study with wheat (*Triticum aestivum* L.) genotypes with varying susceptibility to high temperature stress, Sairam et al. (2000) found that tolerant genotypes had the highest APOX and CAT activity and the lowest H_2O_2 content at elevated temperature. They proposed that heat tolerance in wheat was associated with higher antioxidant levels responsible for detoxification of H_2O_2. Although in this study it was observed that the baseline APOX and CAT activities in vinca were 2.6 and 1.7 times higher than in sweet pea, respectively, no significant difference between the H_2O_2 level of a heat tolerant species, vinca, and a heat susceptible species, sweet pea was seen. Thus, no conclusive evidence of the association of antioxidants and H_2O_2 with the higher thermotolerance of vinca than sweet pea was observed in this study.

INCREASING ENDOGENOUS H_2O_2. Several scientists have used elevated endogenous H_2O_2 as a tool to investigate the role of H_2O_2 in abiotic stress acclimation and injury. Treatments with salicylic acid and its artificial analog, acetylsalicylic acid,

increased endogenous H_2O_2 levels and increased thermotolerance (Dat et al., 1998; Lopez-Delgado et al., 1998). Sairam and Srivastava (2000) incubated wheat leaf strips in 0.05-0.20 mM H_2O_2 solutions and observed a concentration-dependent decrease in membrane stability compared to controls, indicating elevated injury. In this study, no change in the thermostability of vinca was observed after floating leaf disks in 10 or 100 mM H_2O_2 solutions or after syringe-infiltrating vinca and sweet pea leaves with 10 or 100 mM H_2O_2 solutions (data not presented). Using transgenic plants deficient in catalase, Willekens et al. (1997) established that exposure to high light intensities could result in production of high levels of photorespiratory H_2O_2. The production of photorespiratory H_2O_2 in vinca and sweet pea and its effect on thermotolerance, as indicated by EL was examined by imposing heat treatments in light and dark. In the event of production of high levels of photorespiratory H_2O_2 in samples exposed to injurious temperatures in the light, greater injury would have been expected to occur compared to samples heated in the dark.

EL from vinca leaf disks held at 24°C was 13.8% and 15.4% in the presence and absence of light, respectively (Table 3). EL at injurious temperature (53°C) was 46.4% and 64.6% in the presence and absence of light, respectively. Similarly, EL from sweet pea leaf disks held at 24°C was 14.0% and 17.0% in the presence and absence of light, respectively (Table 3) and EL at injurious temperature (46.5°C) was 51.8% in the light and 49.2% in the dark. Injurious temperature resulted in significantly higher EL than control in both sweet pea and vinca (Table 3). In sweet pea, the presence or absence of light did not have a significant effect on EL at both control and injurious temperatures. In vinca, there was no significant difference in the EL due to the presence or absence of

light at 24°C. However, at injurious temperature the absence of light resulted in significantly higher EL than its presence. The mechanism of higher injury in dark is not known.

The presence or absence of light did not make a significant difference in the H_2O_2 levels in vinca or sweet pea. In vinca, at 24°C, endogenous H_2O_2 was 1.8 $\mu mol \cdot g^{-1}$ FW in the light and 1.9 $\mu mol \cdot g^{-1}$ FW in the dark (Table 3). At injurious temperature (53°C), H_2O_2 was 2.6 $\mu mol \cdot g^{-1}$ FW in both the presence, as well as absence of light. In pea, at 24 °C, H_2O_2 was 3.8 $\mu mol \cdot g^{-1}$ FW in the light and 3.9 $\mu mol \cdot g^{-1}$ FW in the dark. At injurious temperature (46.5°C), H_2O_2 was 3.8 $\mu mol \cdot g^{-1}$ FW in both the presence, as well as absence of light. In vinca, H_2O_2 in the injured tissue was significantly higher than in controls (Table 3). However, in sweet pea, there was no significant difference between H_2O_2 levels at the two thermal regimes. The findings suggest that the stepwise increase in temperature significantly elevated H_2O_2 levels in vinca, but not in sweet pea. Moreover, in both the species at injurious temperature there was no significant difference in endogenous H_2O_2 in the presence or absence of light, indicating lack of accumulation of photorespiratory H_2O_2. The lack of accumulation of photorespiratory H_2O_2 may have been due to a lack of production or rapid detoxification. However, it was previously seen that at injurious temperatures, CAT and APOX activities declined. The exact cause of lack of accumulation of photorespiratory H_2O_2 is not known.

The observed H_2O_2 levels in this experiment (Table 3), and the previous findings (Table 2) are not identical due to the variations in experimental protocols. While measuring the H_2O_2 levels in injured leaf tissue, vinca was exposed to 53° and sweet pea was exposed to 48°C for 15 min. The temperature selection was based upon EL as

previously stated. While inducing the production of photorespiratory H_2O_2, the increase in temperature was stepwise from 24° to 53°C in vinca and from 24° to 46.5°C in sweet pea, with a 15 min soak after every 5°C interval. Tissues were in the water bath the entire time during stepwise increase in temperature (vs. a single temperature exposure for 15 min). Preliminary experiments were conducted to select the temperature regimes where EL was closest to the target range (~ 50%). Consequently, the tissue was exposed to increasing temperatures for a much longer duration, which may explain higher H_2O_2 levels at injurious temperature (53°C in vinca and 46.5 °C in sweet pea) compared to controls (24°C). Another procedural change included discarding the bathing solution after temperature treatments to induce photorespiration. Whereas, while measuring the endogenous H_2O_2 in tissue exposed to different thermal regimes, 2 mL bathing solution was homogenized with the tissue during extraction. This could have resulted in different H_2O_2 levels in this experiment (Table 3) compared with the previous findings (Table 2). Another inconsistency observed was, while measuring H_2O_2 concentration at different thermal regimes, the data indicated that there was no significant difference between the H_2O_2 levels in the two species held at 24°C (Table 2). However, in the experiment where tissues were heated in light and dark, at control (24°C), sweet pea had significantly higher H_2O_2 levels than vinca. The cause of lower H_2O_2 in vinca and higher H_2O_2 in sweet pea while heating in light and dark was not identified.

Summary

The data indicate that CAT and APOX activities did not decline prior to increase in EL in vinca and sweet pea leaf tissue. Hence, there was no conclusive evidence that

impairment of enzymatic defenses, resulting in accumulation of injurious levels of H_2O_2 played a vital role in heat stress injury. In vinca both CAT and APOX were more thermostable than in sweet pea. The differences in thermostability of CAT and APOX in vinca and sweet pea could have been due to differences in type and amount of protein-protection mechanisms, such as molecular chaperones in the two species.

Exposing vinca and sweet pea purified leaf tissue extracts to high temperature treatments and measurement of CAT revealed that the thermostability of CAT in vinca and sweet pea did not differ. Also, the thermostability of vinca after exposing the purified extract to heat stress was lower than in intact tissue. The observed loss of CAT thermostability in vinca could have been an effect of partial purification process. Conversely, in sweet pea, although purifying the extract lowered CAT activity compared with intact tissue, it did not affect thermostability, suggesting probable differences in the types of protective mechanisms in the two species. The causes associated with decrease in CAT activity after exposure to heat stress need further exploration.

Measurement of H_2O_2 in control and tissue injured or killed due to heat stress showed no alterations in the H_2O_2 levels. Thus, the data did not corroborate the role of H_2O_2 in heat stress injury. The study of effect of production of photorespiratory H_2O_2 by exposing vinca and sweet pea tissue to injurious temperatures in the light showed no evidence of production of higher levels of H_2O_2 in the light than dark. Thus, the data neither substantiated the impairment of enzymatic defenses prior to heat stress injury, nor did it support the role of H_2O_2 in heat stress injury. The mechanism of heat stress injury is complex and may not be directly regulated by accumulation of higher levels of H_2O_2 in vinca and sweet pea.

Literature Cited

Aebi H.E. 1983. Catalase. In: H.V. Bergmeyer (ed.) Methods of enzymatic analysis. Verlag, Weinheim. pp. 273-286.

Alscher R.G., J.L. Donahue and C.L. Cramer 1997. Reactive oxygen species and antioxidants: Relationships in green cells. Physiol. Plant. 100: 224-233.

Anderson J.A., G. McCollum and W. Roberts. 1990. High temperature acclimation in pepper leaves. HortScience 25: 1272-1274.

Asada K. and M. Takahashi. 1987. Production and scavenging of active oxygen in photosynthesis. In: Kyle D.J., Osmond C.B. and Arntzen C.J. (eds.). Photoinhibition. Elsevier, Amsterdam. pp. 227-287.

Basaga H.S. 1989. Biochemical aspects of free radicals. Biochem. Cell Biol. 68: 989-998.

Boo Y.C. and J. Jung 1999. Water deficit-induced oxidative stress and antioxidant defenses in rice plants. J. Plant Physiol. 155: 255-261.

Bowler C.M., M. Van Montagu and D. Inze. 1992. Superoxide dismutase and stress tolerance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43: 83-116.

Creissen G.P., E.A. Edwards and P.M. Mullineaux. 1994. Glutathione reductase and ascorbate peroxidase. In: C.H. Foyer, P.M. Mullineaux (eds.) Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, Boca Raton, FL. pp. 343-364.

Dat J.F., H. Lopez-Delgado, C.H. Foyer and I.M. Scott. 1998. Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. Plant Physiol. 116: 1351-1357.

Dhindsa R.S., P. Plumb-Dhindsa and T.A. Thorpe. 1981. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. 32: 93-101.

Dionisio-Sese M.L. and S. Tobita. 1998. Antioxidant responses of rice seedlings to salinity tress. Plant Sci. 135: 1-9.

Elstner E.F. 1982. Oxygen aviation and oxygen toxicity. Ann. Rev. Plant Physiol. 33: 73-96.

Fadzillah N.M., V. Gill, R.P. Finch and R.H. Burdon. 1996. Chilling, oxidative stress and antioxidant responses in shoot cultures of rice. Planta 199: 552-556.

Foyer C.H., P. Descourvieres and K.J. Kumar. 1994. Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. Plant Cell Environ. 17: 507-523.

Foyer C.H., H. Lopez-Delgado, J.H. Dat, and I.M. Scott. 1997. Hydrogen peroxide-and glutathione-associated mechanisms of acclimatory stress tolerance and signaling. Physiol. Plant. 100: 241-254.

Gross G.G. 1980. The biochemistry of lignification. Adv. Bot. Res. 8: 25-63.

Ingram D.L. 1985. Modeling high temperature and exposure time interactions on *Pittosporum tobira* root cell membrane thermostability. J. Amer. Soc. Hort. Sci. 110: 470-473.

Iturbe-Ormaetxe I., P.R. Escuredo, C. Arrese-Igor and M. Becana. 1998. Oxidative damage in pea plants exposed to water deficit or paraquat. Plant Physiol. 116: 173-181.

Kerdnaimongkol K., A. Bhatia, R.J. Joly and W.R. Woodson. 1997. Oxidative stress and diurnal variation in chilling sensitivity of tomato seedlings. J. Amer. Soc. Hort. Sci. 122: 485-490.

Kerdnaimongkol K. and W.R. Woodson. 1999. Inhibition of catalase by antisense RNA increases susceptibility to oxidative stress and chilling injury in transgenic tomato plants. J. Amer. Soc. Hort. Sci. 124: 330-336.

Lagrimini L.M., S. Bradford and S. Rothstein. 1990. Peroxidase-induced wilting in transgenic tobacco plants. Plant Cell 2: 7-18.

Larson R.A. 1988. The antioxidants of higher plants. Phytochemistry. 27: 969-978.

Lazarov P.B. 1983. Biogenesis of peroxisomal content proteins: in vivo and in vitro studies. Methods Enzymol. 96: 721-728.

Lee D.H. and C.B. Lee. 2000. Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. Plant Sci. 159: 75-85.

Levine A., R. Tenhaken, R. Dixon and C. Lamb. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79: 583-593.

Lidon F.C. and M.G. Teixeira. 2000. Oxy radicals production and control in chloroplast of Mn-treated rice. Plant Sci. 152: 7-15.

Liu X. and B. Huang. 2000. Heat stress injury in relation to membrane lipid peroxidation in creeping bentgrass. Crop Sci. 40: 503-510.

McKersie B.D., J. Murnaghan and S.R. Bowley. 1997. Manipulating freezing tolerance in transgenic plants. Acta Physiol. Plant. 19: 485-495.

MacRae E.A. and I.B. Ferguson. 1985. Changes in catalase activity and hydrogen peroxide concentration in plants in response to low temperature. Physiol. Plant. 65: 51-56.

Mehdy M.C., Y.K. Sharma, K. Sathasivan and N.W. Bays. 1996. The role of activated oxygen species in plant disease resistance. Physiol. Plant. 98: 365-374.

Miyake C., F. Michihata and K. Asada. 1991. Scavenging of hydrogen peroxide in prokaryotic and eukaryotic algae: acquisition of ascorbate peroxidase during the evolution of cyanobacteria. Plant Cell Physiol. 32: 33-43.

Mizuno M., M. Kamei and H. Tsuchida 1998. Ascorbate peroxidase and catalase cooperate for protection against hydrogen peroxide generated in potato tubers during low-temperature storage. Biochem. Mol. Biol. Int. 44: 407-408.

Nagao R.T., J.A. Kimpel and J.L. Key. 1990. Molecular and cellular biology of the heat-shock response. Adv. Gen. 28: 235-274.

Ngo T.T. and H.M. Lenhoff. 1980. A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. Anal. Biochem. 105:389-397.

Noctor G. and C.H. Foyer. 1998. Ascorbate and glutathione: Keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 249-279.

Ntuli M. 1995. http://www.und.ac.za/und/icd/cittc/paper/net2/muzi.html

Okuda T., Y. Masuda, A. Yamanaka and S. Sagisaka. 1991. Abrupt increase in the levels of hydrogen peroxide in leaves of winter wheat is caused by cold treatment. Plant Physiol. 97: 1265-1267.

Omran R.G. 1980. Peroxide levels and activities of catalase, peroxidase and indoleacetic oxidase during and after chilling cucumber seedlings. Plant Physiol. 65: 407-408.

Prasad K.V.S.K., P. Paradha Saradhi and P. Sharmila. 1999. Concerted action of antioxidant enzymes and curtailed growth under zinc toxicity in *Brassica juncea*. Environ. Exp. Bot. 42:1-10.

Prasad T.K., M.D. Anderson, B.A. Martin, and B.R. Stewart. 1994. Evidence of chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell 6: 65-74.

Prasad T.K. 1996. Mechanisms of chilling-induced oxidative stress injury and tolerance in developing maize seedlings: changes in antioxidant system, oxidation of proteins and lipids, and protease activities. Plant J. 10: 1017-1026.

Sairam R.K., G.C. Srivastava and D.C. Saxena. 2000. Increased antioxidant activity under elevated temperatures: a mechanism of heat stress tolerance in wheat genotypes. Biol. Plant. 43: 245-251.

Sairam R.K. and G.C. Srivastava. 2000. Induction of oxidative stress and antioxidant activity by hydrogen peroxide treatment in tolerant and susceptible wheat genotypes. Biol. Plant. 43: 381-386

Sala J.M. and M.T. Lafuente. 1999. Catalase in heat-induced chilling tolerance of cold-stored hybrid Fortune mandarin fruits. J. Agric. Food Chem. 47: 2410-2414.

Scandalios J.G., W.F. Tong and D.G. Roupakias. 1980. Cat3, a third gene locus coding for a tissue-specific catalase in Maize: Genetics, intracellular location, and some biochemical properties. Mol. Gen. Genet. 179: 33-41.

Scandalios J.G. 1994. Regulation and properties of plant catalases. In: C.H. Foyer and P.M. Mullineaux (eds.). Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, Boca Raton, FL. pp. 275-316.

Teisseire H. and V. Guy. 2000. Copper-induced changes in antioxidant activities in fronds of duckweed (*Lemna minor*). Plant Sci. 153: 65-72.

Volk S. and J. Feierabend. 1989. Photoinactivation of catalase at low temperature and its relevance to photosynthetic and peroxide metabolism in leaves. Plant Cell Environ. 12: 702-712.

Veljovic-Jovanovic S. 1998. Active oxygen species and photosynthesis: Mehler and ascorbate peroxidase reactions. Iugoslav. Physiol. Pharmacol. Acta 34: 503-522.

Willekens H., S. Chamnongpol, M. Davey, M. Schraudner, C. Langebartels, M.V. Montagu, D. Inze and W.V. Camp. 1997. Catalase is a sink for H_2O_2 and is indispensable for stress defense in C_3 plants. EMBO J. 16: 4806-4816.

Wingsle G. 1999. http://www.forestbiotech.com/project/p5.html

Table 1. Temperature midpoints (T_{mids}) for ascorbate peroxidase (APOX) activity, catalase (CAT) activity and electrolyte leakage (EL) response curves in 8-week-old vinca and sweet pea leaf tissue exposed to a range of temperatures for 15 min. Means ± SE of five replications are reported.

	T_{mid} (°C)		
Species	APOX activity	CAT activity	EL
Vinca	52.9 ± 0.4 a[z]	56.0 ± 0.5 b	53.9 ± 0.4 a
Sweet pea	50.3 ± 0.8 x	50.0 ± 0.2 x	48.4 ± 0.1 y
P value for Species	0.0007	<0.0001	<0.0001

[z] Means within a row followed by the same letter are not significantly different at $P \leq 0.05$ using LSD.

Table 2. Electrolyte leakage (EL) and hydrogen peroxide (H_2O_2) concentration in 8-week-old vinca and sweet pea leaf tissue exposed to control, injurious and killing temperature for 15 min. Means ± SE of three replications are reported.

Species	Temperature (°C)		EL (%)	H_2O_2 ($\mu mol \cdot g^{-1}$ FW)
Vinca	Control	24	22.0 ± 0.5 a[z]	2.7 ± 0.2 a
	Injured	54	64.1 ± 1.3 b	3.1 ± 0.4 a
	Killed	62	94.3 ± 0.5 c	3.3 ± 0.4 a
Sweet pea	Control	24	23.0 ± 0.6 a	2.7 ± 0.2 a
	Injured	48	58.7 ± 2.4 b	2.7 ± 0.2 a
	Killed	54	93.3 ± 0.6 c	2.2 ± 0.2 a

[z] Means within a column followed by the same letter are not significantly different at $P \leq 0.05$ using LSD.

Table 3. Electrolyte leakage (EL) and hydrogen peroxide (H_2O_2) concentration in 8-week-old vinca and sweet pea leaf tissue exposed to control and injurious temperature in light and dark. Temperature increased stepwise with a 15 min soak after every 5 °C. Means ± SE of five replications are reported.

	Control		Injured	
Species	Light	Dark	Light	Dark
	EL (%)			
Vinca	13.8 ± 1.8 a[z]	15.4 ± 2.8 a	46.4 ± 4.2 b	64.6 ± 7.3 c
Sweet pea	14.0 ± 1.3 a	17.0 ± 1.8 a	51.8 ± 5.1 b	49.2 ± 5.9 b
P value for Species	0.97	0.79	0.38	0.02
	H_2O_2 (μmol g^{-1} FW)			
Vinca	1.8 ± 0.2 a[z]	1.9 ± 0.3 a	2.6 ± 0.3 b	2.6 ± 0.3 b
Sweet pea	3.8 ± 0.3 b	3.9 ± 0.3 b	3.8 ± 0.3 b	3.8 ± 0.3 b
P value for Species	0.02	0.02	0.12	0.10

[z] Means within a row followed by the same letter are not significantly different at $P \leq 0.05$ using LSD.

Table 4. Temperature midpoints (T_{mids}) for catalase (CAT) activity response curves in 8-week-old vinca and sweet pea partially purified leaf tissue extract exposed to a range of temperatures for 15 min. Means ± SE of three replications are reported.

Species	T_{mid} (°C)
Vinca	48.4 a[z]
Sweet pea	49.8 a

[z] Means within a column followed by the same letter are not significantly different at $P \leq 0.05$ using LSD.

Figure 1. Thermal stability of ascorbate peroxidase activity in vinca. Leaf tissues were exposed to the indicated temperature for 15 min. Enzyme activity was calculated as ascorbate consumed per gram fresh weight. Means ± SE of five replications are reported.

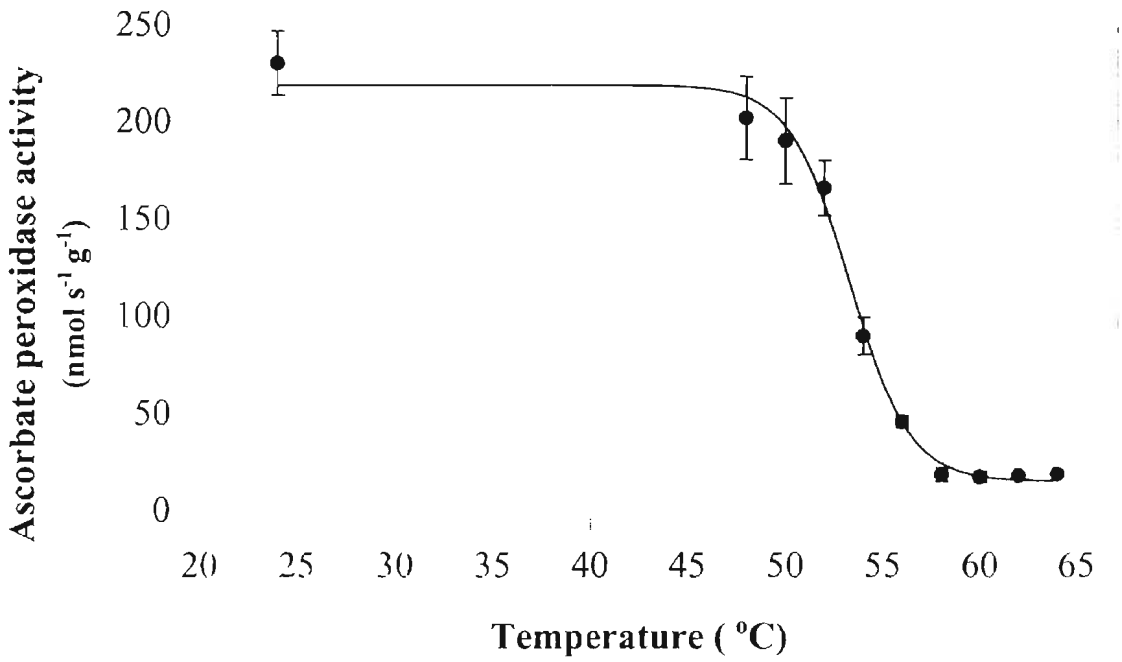


Figure 2. Thermal stability of ascorbate peroxidase activity in sweet pea. Leaf tissues were exposed to the indicated temperature for 15 min. Enzyme activity was calculated as ascorbate consumed per gram fresh weight. Means ± SE of five replications are reported.

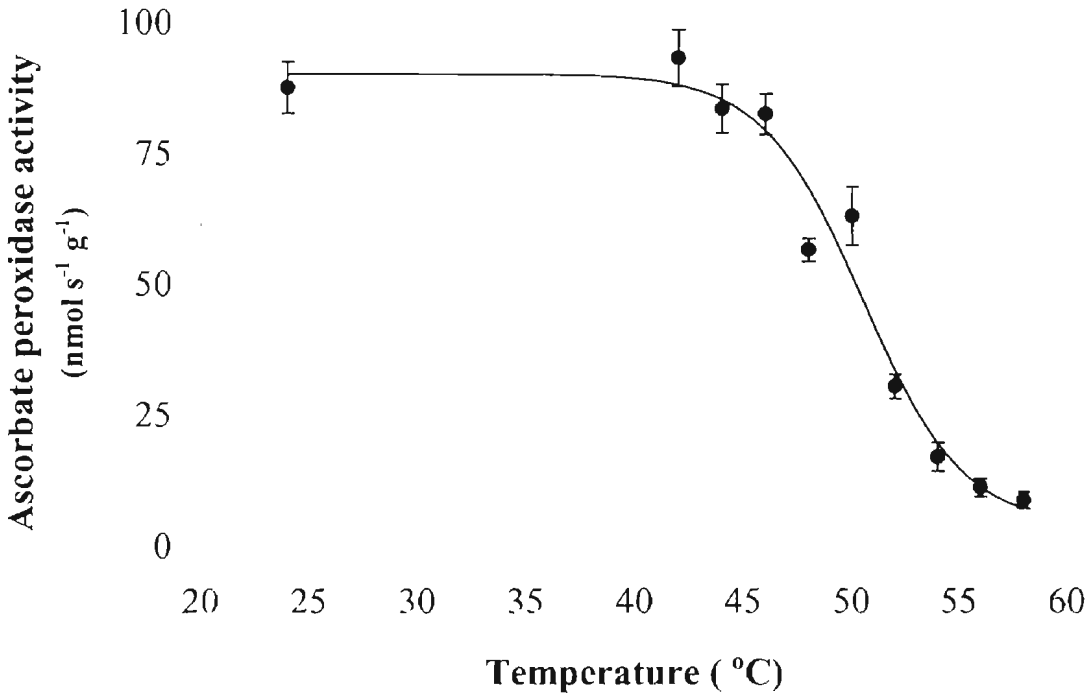


Figure 3. Thermal stability of catalase activity in vinca. Leaf tissues were exposed to the indicated temperature for 15 min. Enzyme activity was calculated as hydrogen peroxide consumed per gram fresh weight. Means ± SE of five replications are reported.

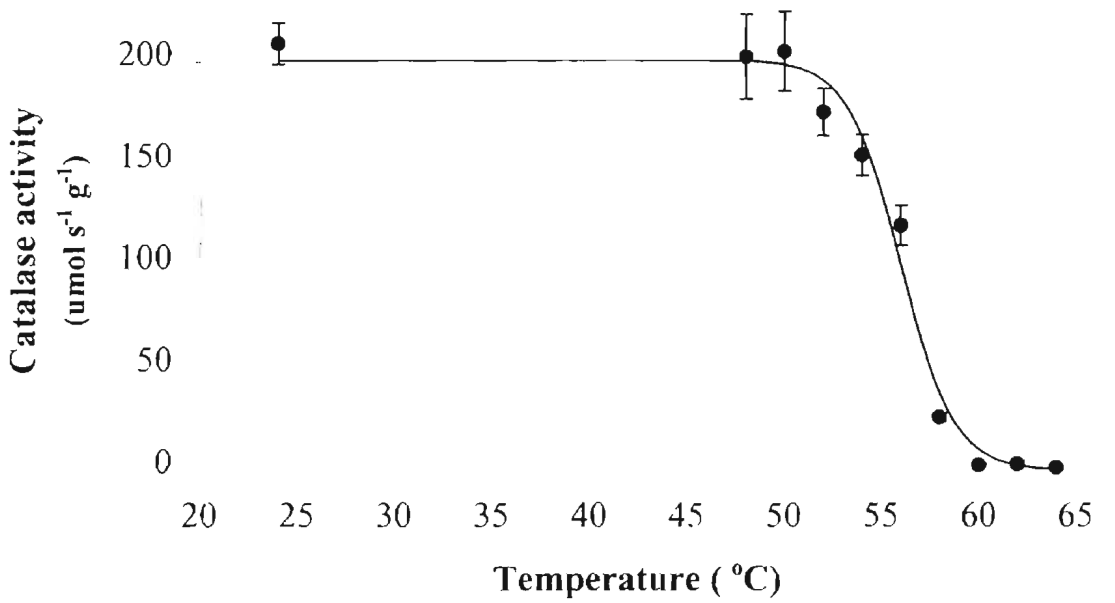


Figure 4. Thermal stability of catalase activity in sweet pea. Leaf tissues were exposed to the indicated temperature for 15 min. Enzyme activity was calculated as hydrogen peroxide consumed per gram fresh weight. Means ± SE of five replications are reported.

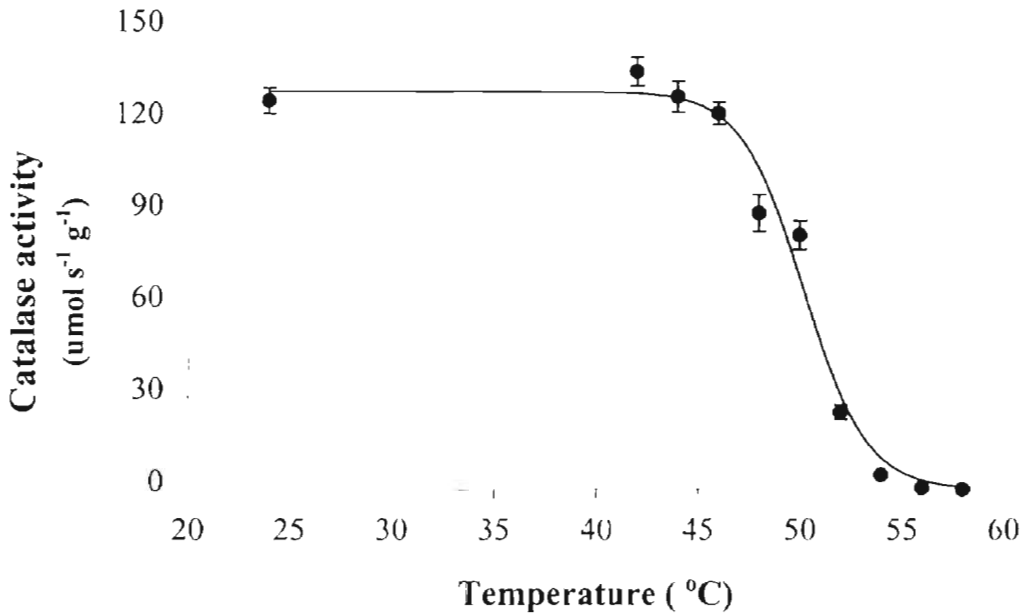


Figure 5. Electrolyte leakage from vinca leaf tissue exposed to a range of temperatures for 15 min each. Means ± SE of five replications are reported.

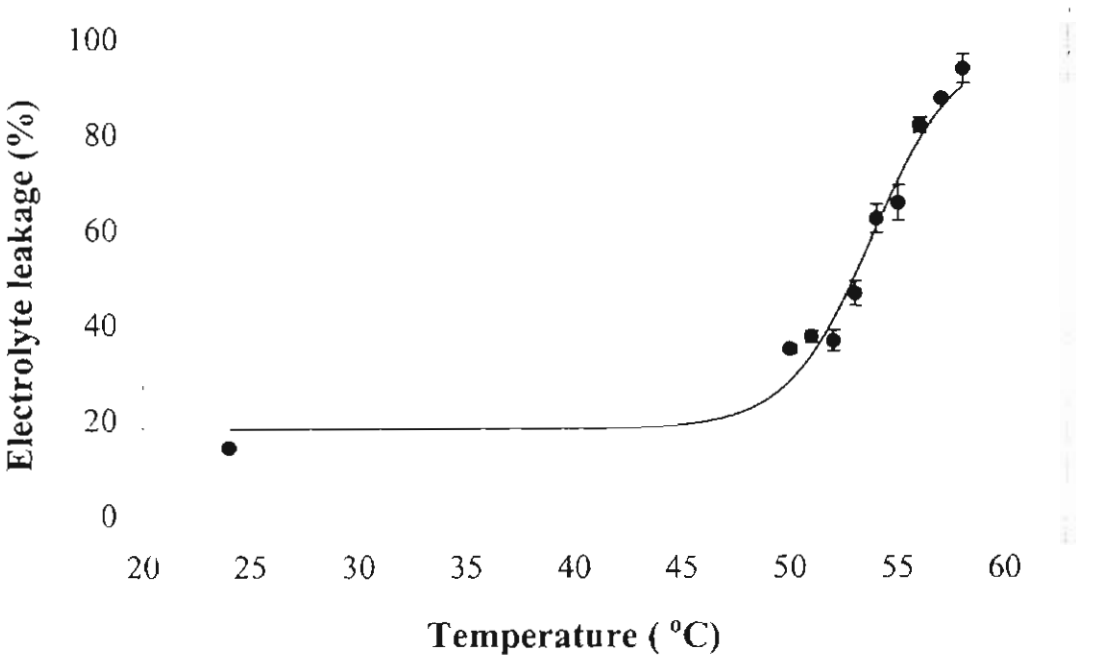


Figure 6. Electrolyte leakage from sweet pea leaf tissue exposed to a range of temperatures for 15 min each. Means ± SE of five replications are reported.

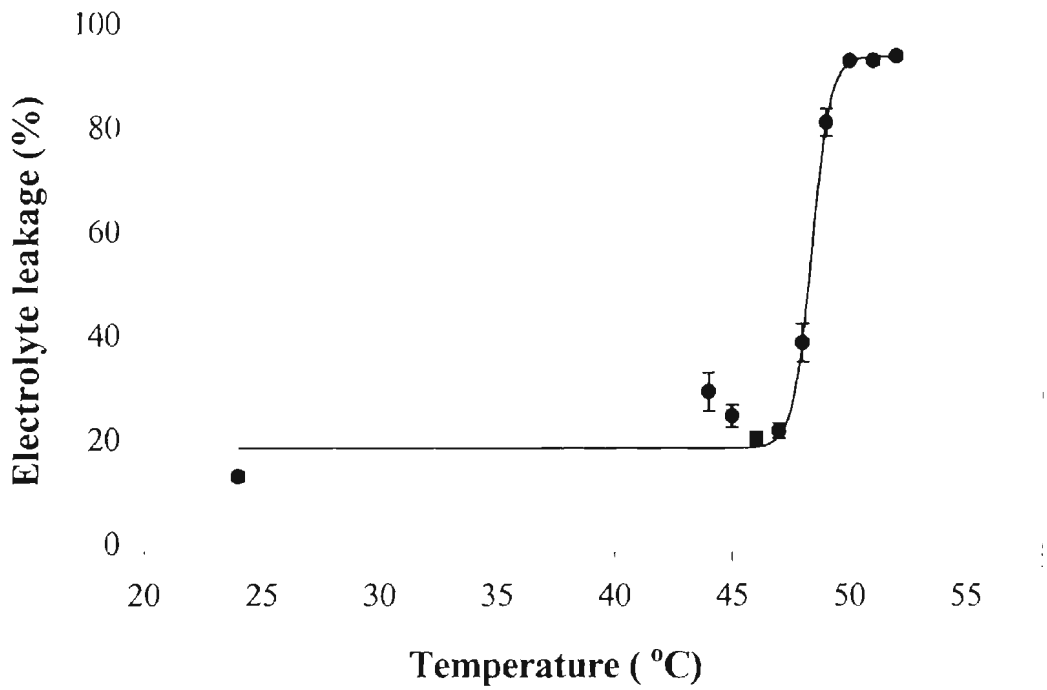
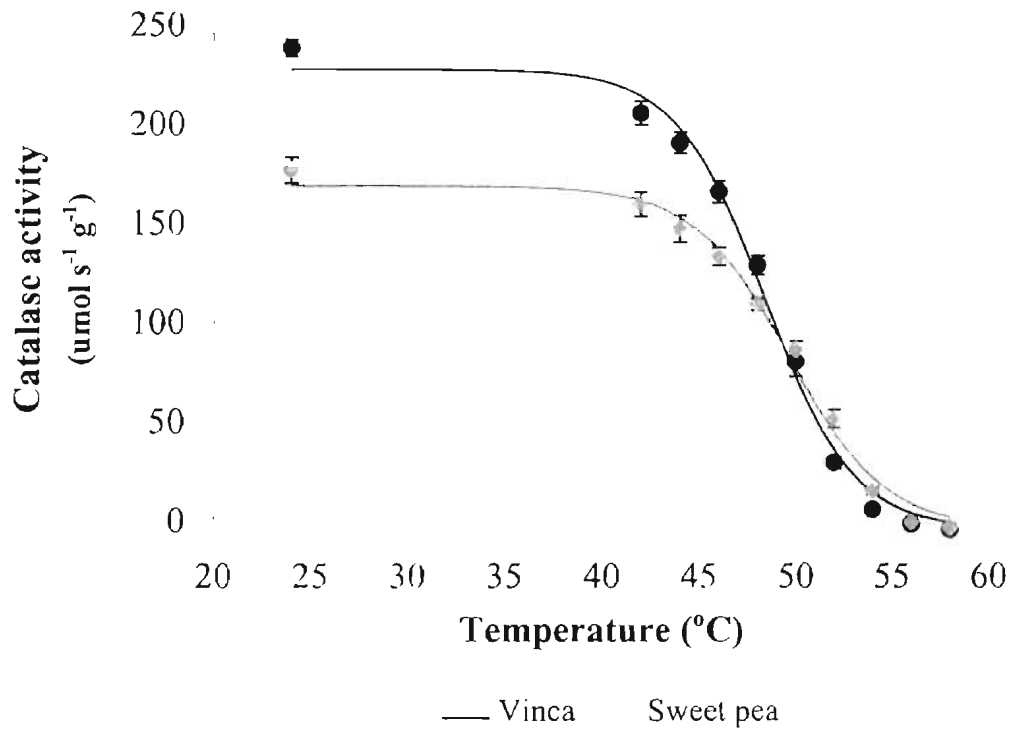


Figure 7. Thermal stability of catalase activity in vinca and sweet pea partially purified leaf tissue extracts exposed to the indicated temperature for 15 min. Enzyme activity was calculated as hydrogen peroxide consumed per gram fresh weight. Means ± SE of three replications are reported.



VITA

Sonali R. Padhye

Candidate for the Degree of

Master of Science

Thesis: HEAT STRESS, HYDROGEN PEROXIDE AND ANTIOXIDANT DEFENSES IN HORTICULTURAL CROPS

Major Field: Horticulture

Biographical:

Personal Data: Born in Thane, India, March, 12, 1976, the daughter of Ramesh and Mangala Padhye.

Education: Graduated from Parle College, Mumbai in July 1993; received Bachelor of Science degree in Horticulture from College of Horticulture, Pune in July 1997; Completed requirements for the Master of Science degree at Oklahoma State University in December, 2001.

Experience: Graduate Research Assistant, Department of Horticulture and Landscape Architecture, August 1999-August 2001, Oklahoma State University.

Professional Memberships: American Society for Horticultural Science, Pi Alpha Xi.