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Chilling Effects on Antioxidant Systems of Maize (*Zea mays* L.)

BY

D. Mark Hodges

Thesis submitted to
the School of Graduate Studies and Research
in partial fulfilment of the requirements for the Ph.D.
degree in Biology

University of Ottawa
Ottawa, Ontario
1995

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ABSTRACT

When chilled, plants produce greater amounts of toxic oxygen compounds than they do under non-stressed conditions. These toxic oxygen compounds have the potential to cause severe damage to plants. Plants have evolved antioxidant systems which can scavenge these toxic oxygen compounds and thus allow for the avoidance of their detrimental effects. The purpose of this thesis was to characterize antioxidant capacities of differentially chilling sensitive lines of maize (*Zea mays* L.) in order to test the hypothesis that the most chilling sensitive lines would have less antioxidant capacity and, hence, less ability to scavenge damaging toxic oxygen compounds, than the more tolerant lines.

Three objectives were set to test this hypothesis. The first objective was to select out relatively chilling sensitive and tolerant inbred maize based on their physiological responses to chilling. This first objective was successfully met by subjecting the inbred lines to laboratory chilling tests at the germination stage and early growth stages. Field trials which assessed physiological parameters at both the emergence and early growth phases were then carried out which confirmed laboratory results.

The second objective of this thesis was to test if the selected chilling sensitive inbred maize lines had less antioxidant capacities than the tolerant. To this end, activities of the antioxidant enzymes catalase (CAT; EC

1.11.1.6), ascorbate peroxidase (ASPX; EC 1.11.1.11), superoxide dismutase (SOD; EC 1.15.1.1), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), and glutathione reductase (GR; EC 1.6.4.2), along with concentrations of the antioxidant compounds ascorbate, glutathione, ß-carotene, and α-tocopherol and levels of the general metabolic indicators of chilling stress (carbohydrates, chlorophyll, and soluble proteins) were assessed. These parameters were assessed at three developmental stages (first, third and fifth leaf stages) and under control (25°C) and both short- and long-term chilling (11°C) regimes. This second objective was successfully met.

Significantly lower percent of control activities of the antioxidant enzymes ASPX, MDHAR, and CAT, were observed in the most chilling sensitive inbred line as compared to the tolerant at the first leaf stage of development. The percent of control concentration of the antioxidant compound ß-carotene was significantly lower in the chilling sensitive relative to the chilling tolerant inbred maize lines under both short- and long-term chilling treatments for the first leaf stage. Lower levels of ß-carotene may, in conjunction with reduced activities of ASPX, CAT, and MDHAR, have led to increased production of damaging toxic oxygen compounds in the chilling sensitive relative to the tolerant lines, limiting chilling tolerance at the earliest stages of inbred maize development. There were no significant differences between chilling sensitive and tolerant plants in respect to the

percent of control concentrations of total ascorbate and glutathione under short-term chilling for all leaf stages, but as the long-term chilling treatment progressed and the plants aged, percent of control concentrations of these compounds increased until they ultimately became significantly higher in the chilling sensitive relative to the tolerant lines. There were no significant differences between percent of control activities of any of the antioxidant enzymes between the chilling sensitive and tolerant lines at the fifth leaf stage of development under long-term chilling. As the ability of the chilling sensitive line to detoxify toxic oxygen compounds increased as the plants aged from the first to the fifth leaf developmental stage, the susceptibility to chilling of the sensitive maize line potentially decreased.

Inbreds are prone to inbreeding depression due to their homozygosity and are frequently less vigorous than hybrids. This raised the question of whether relatively chilling sensitive maize would exhibit similar antioxidant capacities than those more tolerant for both inbreds and hybrids. If so, would the same antioxidant enzymes and/or compounds be limiting in both inbreds and hybrids? Thus the third objective of this thesis was designed to determine if there were similar results for antioxidant capacities and metabolic indicator concentrations for the chilled hybrids as there were for the inbred maize lines. Thus, a complete diallel cross between the above selected inbreds was made, and the resulting

hybrids then classified as relatively chilling sensitive or tolerant depending upon how they performed in the laboratory germination and early growth stage screening tests with confirmation in the field. The hybrids were grown until the third leaf stage under short-term chilling (11°C) and assessed for activities of the antioxidant enzymes and concentrations of antioxidant compounds and metabolic stress indicators.

This third objective was successfully met. The chilling sensitive maize hybrids were found to have lower, although not significantly so, percent of control activities of CAT, MDHAR, and ASPX than the chilling tolerant hybrids, paralleling the results of the parental inbreds. As well as with the inbreds, percent of control concentrations of total ascorbate and glutathione were not significantly different between the chilling sensitive and tolerant hybrids. Although hybrid maize was observed to be better able to induce and cycle ascorbate, results suggest that similar antioxidant mechanisms exist between both inbred and hybrid maize. The combining abilities for the physiological growth parameters (germination and early growth), antioxidant enzyme activities and concentrations of antioxidant compounds and carbohydrates demonstrated that screening of hybrid maize for chilling sensitivity should be done with the hybrids themselves, as it is not possible to accurately predict chilling sensitivity of hybrids based on the chilling sensitivity of their parental inbreds.

Résumé

Lorsqu' exposées à de basses températures, les plantes produisent beaucoup plus de composés oxygénés toxiques que lorsqu'elles croissent dans des conditions non-stressantes. Ces composés oxygénés toxiques peuvent potentiellement causer des dommages sévères aux plantes. Les plantes ont donc développé, au cours de leur évolution, des systèmes antioxydants qui servent à récupérer ces composés oxygénés toxiques. Les plantes évitent donc ainsi les effets néfastes de ces composés. Le but de cette thèse est de caractériser les capacités antioxydantes de lignés endogames de maïs (Zea mays L.) ayant différentes sensibilités au froid afin de vérifier l'hypothèse que les lignées de maïs plus sensibles au froid auront une capacité antioxydante plus faible. En d'autre mots, les plantes les plus sensibles au froid auront une plus faible capacité de récupérer ces composés oxygénés toxiques.

Trois objectifs ont été établis afin de vérifier cette hypothèse. Le premier était de sélectionner des lignées endogames de maïs sensibles au froid, ainsi que des lignées endogames tolérantes, en se basont sur leurs réponses physiologiques au froid. Nous avons réalisé ce premier objectif en exposant les lignées de maïs à des tests de basse température en laboratoire durant le stade de germination ainsi que durant le stade de croissance juvénile. Par la suite, nous avons mesuré des paramètres physiologiques sur le terrain lors de l'émergence de la plante ainsi que pendant la croissance primaire. Ces derniers tests ont confirmé les résultats obtenus en laboratoire.

Le deuxieme objectif de cette thèse était de vérifier si les lignées endogames de maïs sensibles au froid ont des capacités antioxydantes moindre que celles tolérantes au

froid. Afin de mettre ceci à l'épreuve, nous avons mesuré l'activité de plusieurs enzymes antioxydants, soient la catalase (CAT; EC 1.11.1.6), l'ascorbate peroxidase (ASPX; EC1.11.1.11), la superoxide dismutase (SOD; EC 1.15.1.1), la monodéhydroascorbate réductase (MDAR; EC 1.6.5.4), et la glutathione réductase (GR; EC 1.6.4.2), ainsi que les concentrations de composés antioxydants (l'ascorbate, le glutathione, le ß-carotène et l'α-tocophérol) et les niveaux d'indicateurs généraux de stress au froid (hydrates de carbones, chlorophylle et protéines solubles). Ces paramètres ont été étudiés à trois différents stades de développement (première, troisième et cinquième feuille) chez des plantes témoins (25°C) ainsi que chez des plantes soumises à une basse température (11° C) pour une brève ou longue période.

Lors du premier stade de développement de la feuille, l'activité des enzymes ASPX, MDHAT et CAT s'est avérée significativement moins élévée ches les plantes sensibles au froid comparées aux plantes tolérantes. D'ailleurs, la concentration de l'antioxydant ß-carotène était significativement plus faible chez les lignées sensibles exposées à une basse température, tient sur une courte qu'une longue période. Des niveaux faibles de ß-carotène peuvent, en conjonction avec les activités réduites des enzymes ASPX, CAT et MDHAR, semblent responables de l'augmentation de la quantité de composés oxygénés toxiques ches les lignées sensible, limitant ainsi la tolérance au froid de ces plantes au stade primaire du développement. De courtes periodes d'exposition au froid ne provoquent cependant aucune différence significative entre les lingées sensibles et tolérantes en ce qui a trait aux concentrations totales d'ascorbate et de glutathione, peu importe le stade de développement. Cependant, les concentrations de

ces composés augmentent progressivement chez les lingées sensibles exposées au froid pour une longue période. En contraste, l'activité des enzymes antioxydants au stade 5 feuilles n'est pas affectée par une longue période d'exposition au froid. La capacité de la lignée sensible au froid à éliminer les composés oxygénés toxiques augmente avec le développement de la plante et sa susceptabilité au froid diminue.

Les lignées endogames sont souvent moins vigoureuses que les hybrides à cause de leur homozygotie. Donc, il se peut que les lignées endogames de maïs relativement sensibles au froid montrent des capacités antioxydantes moindres que les lignées endogames et les hybrides plus tolérantes. Si cela est le cas, il s'agit de savoir si les enzymes et/ou composés antioxydants sont les facteurs limitants. Dans cet optique, le troisième objectif de cette thèse a été élaboré afin de déterminer s'il y a des capacités antioxydantes et des concentrations d'indicateurs métaboliques similaires chez des hybrides exposés au froid que chez des lignées endogames de maïs. Donc, nous avons réalisé un croisement diallélique complet entre les lignées endogames sélectionnées. Les hybrides résultants ont été classés comme étant relativement sensibles au froid ou tolérants, selon la façon que les plantes réagissaient aux tests réalisés durant les stades de germination et de croissance primaire. Les hybrides ont été exposés pour une courte durée à une température de 11°C. Ensuite, l'activité des enzymes antioxydants, les concentrations des composés antioxydants et les indicateurs de stress métabolique ont été mesurés.

Nous avons observé chez les hybrides de maïs sensibles au froid que l'activité des enzymes CAT, MDHAR et ASPX est légèrment plus faible, mais de façon non

significative, que chez les hybrides tolérants au froid, un résultat semblable à celui obtenu avec les lignées endogames parentales. Tout comme chez les lignées endogames, les concentrations totales d'ascorbate et de glutathione chez les hybrides sensibles et tolérants au froid n'étaient pas significativement différentes. Bien que nous ayons observé que les hybrides de maïs sont mieux capables d'induire et de recycler l'ascorbate, nos résultats indiquent que des mécanismes antioxydants similaires existent chez les lignées de maïs hybrides et chez les lignées endogames. Étant donné les effets combinés des paramètres physiologiques de croissance (germination et croissance primaire), des activités des enzymes antioxydants et des concentrations des composés antioxydants ainsi que des hydrates de carbones, nous constatons que la procédure de sélection des hybrides de maïs pour la tolérance au froid devrait être effectuée avec les hybrides eux-mêmes. Il semble en effet que l'on ne peut prédire avec précision la tolérance au froid des hybrides en se basent sur la tolérance au froid de leurs parents de lignée endogame.

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LIST OF ABBREVIATIONS

AsA	-	reduced ascorbate
ASPX	-	ascorbate peroxidase
BSA	-	bovine serum albumin
CAT	-	catalase
CHU	-	corn heat units
DAsA	-	dehydroascorbate
DTT	-	dithiothretitol
DHAR	-	dehydroascorbate reductase
DMSO	-	dimethyl sulphoxide
EDTA	-	ethylene-diaminetetra-acetic acid
HCL	-	hydrochloric acid
GCA	-	general combining ability
GR	-	glutathione reductase
GSH	-	reduced glutathione
GSSG	-	oxidized glutathione
MDHAR	-	monodehydroascorbate reductase
NADH	-	nicotinamide adenine dinucleotide
NADPH	-	nicotinamide adenine dinucleotide phosphate
PEP	-	phosphenolpyruvate
PVPP	-	polyvinylpyrrolidone
SCA	-	specific combining ability
SOD	-	superoxide dismutase
TCA	-	trichloroacetic acid

CHAPTER ONE

INTRODUCTION

1.1 An Oxygenated Environment

Oxygen is an essential component in the life of all aerobic organisms. However, life with oxygen carries with it a potential danger. Although molecular oxygen (O_2) itself is not toxic, it can produce by-products which are highly reactive and which can pose a potential for severe cellular damage or lethality. These toxic oxygen compounds play significant roles in the peroxidation of essential phospholipids (Senaratna et al., 1987; Matsuo et al., 1990; Zheng and Yang, 1991) and in both nucleic acid (Elstner, 1982; Imlay and Lin, 1988; Monk et al., 1989) and protein (Lesser and Shick, 1989; Casano and Trippi, 1992) denaturation. For plants, typical symptoms of the presence of these toxic oxygen compounds are inhibition of chloroplast development (Poskuta et al., 1974; Salin, 1988), early leaf and cotyledon senescence (Dhindsa et al., 1981; Li and Mei, 1989), chloroplast membrane damage (Knox and Dodge, 1985; Salin, 1988), damage to cellular lipids and DNA (Prinsze et al., 1990), and the inactivation of many types of enzymes resulting from the alteration of proteins (Halliwell, 1981; Kalt-Torres and Huber, 1987) and subsequent attacks by proteases (Landry and Pell, 1993).

Plants produce toxic oxygen compounds in greater

quantities when exposed to environmental stresses such as drought, high light intensities, high ambient O_2 pressures, ozone or sulfur dioxide, some pathogens (Bowler et al., 1992; Pastori and Trippi, 1992), relatively high concentrations of NaCl (Gossett et al., 1994), herbicides, metals (Foyer et al., 1994a), and chilling temperatures (Sonoike and Terashima, 1994; Terashima et al., 1994). The differential capacity of plants exposed to chilling stress to scavenge these toxic oxygen compounds forms the basis of this thesis.

1.2 Toxic Oxygen Compounds

Toxic oxygen compounds arise from O_2 as products of its reduction or its excitation to the singlet state (Cornic and Briantais, 1991; Foyer et al., 1994a). A compete reduction of oxygen requires four electrons with water being the end product (Salin, 1988; Scandalios, 1993). Conversely, the oxidation of water would result in the liberation of four electrons (Salin, 1988). When the reduction of oxygen proceeds in univalent steps, reactive intermediates are produced (Halliwell and Gutteridge, 1985). Among these are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH) (Halliwell and Gutteridge, 1985) (Fig. 1). Excitation of O_2 can lead to the production of singlet oxygen ($^1O_2$) (Larson, 1988; Foyer et al., 1994*a*):

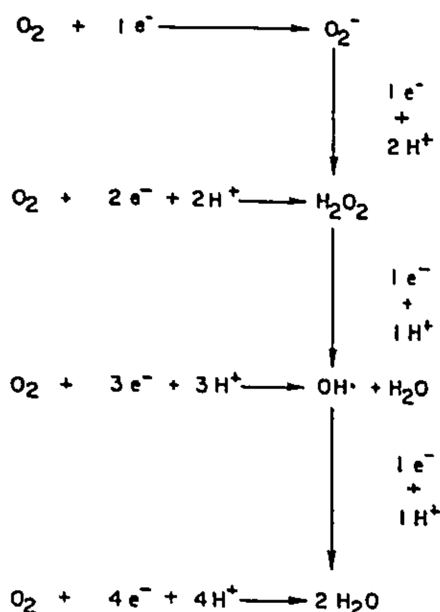


Figure 1. Pathways in oxygen reduction: formation of active oxygen intermediates (from Salin, 1988).

1.2.1 Superoxide

As a result of O_2's spin restriction, it has a much greater tendency to react with radical species and unpaired electrons than with substrates that donate pairs of electrons (Salin, 1988). Therefore the simplest route of O_2 reduction is via the univalent pathway, leading to the production of the superoxide anion (O_2^-) (Salin, 1988). Molecules of O_2^- can also be produced from the univalent oxidation of H_2O_2 (Salin, 1988). The generation of O_2^- has been observed in mitochondria, chloroplasts, glyoxysomes, microsomes, peroxisomes, and nuclei of many types of living organisms (del Rio et al., 1989).

These O_2^- molecules can arise as catalytic byproducts from normal aerobic metabolism involving electron transport chains (Foyer et al., 1994*a*; Foyer et al., 1994*b*) and enzymes such as nitropropane dioxygenase, aldehyde oxidase, galactose oxidase, and xanthine oxidase (Halliwell, 1981; Palm et al., 1991; Ushimaru et al., 1992; Scandalios, 1993). This toxic oxygen compound may also arise from non-enzymatic processes involving the autooxidation of such substrates as ferredoxins, hydroquinones, thiols, and reduced hemoproteins (Fridovich, 1976).

Superoxide itself is not as highly toxic as are some of the other toxic oxygen compounds (*e.g.* $\cdot OH$, $^1O_2$), although within biological membranes it can act as a powerful nucleophile and base (Alscher and Amthor, 1988). More importantly, however, O_2^- can react non-enzymatically with H_2O_2 in the Haber-Weiss reaction to form the hydroxyl radical (Cakmak et al., 1993), a highly destructive compound; this reaction can only occur in the presence of certain transition metal ions or metal chelates (Alscher and Amthor, 1988; Mishra et al., 1993; Gossett et al., 1994). Superoxide also appears to convert the metal ion Mn^{+2} into a more reactive species (Halliwell, 1981). As well, O_2^- is recognized as being associated with the elicitation of plant stress responses (Foyer et al., 1994*b*).

The dismutative elimination of O_2^- can occur spontaneously or under the catalytic influence of the enzyme superoxide

dismutase, which catalyses a reaction leading to the production of H_2O_2 and O_2 (Cakmak and Horst, 1991; Irigoyen et al., 1992; Hérouart et al., 1994). Compounds such as ascorbate (Walker and McKersie, 1993; Gossett et al., 1994) and α-tocopherol (Wise and Naylor, 1987; Fryer, 1992) also remove O_2^- directly in a non-catalysed reaction.

1.2.2 Hydrogen Peroxide

Hydrogen peroxide itself is the most stable of the toxic oxygen compounds since its two outer orbitals are completely filled (Salin, 1988). As with O_2^-, H_2O_2 can act as both a mild reductant and an oxidant, although it is a much stronger nucleophilic oxidizing agent (Salin, 1988). Also like O_2^-, H_2O_2 is relatively unreactive; it does not react efficiently with organic substrates, but does tend to form complexes with transition metals (Salin, 1988). However, a major mode of toxicity of H_2O_2 itself that does exist occurs through the oxidation of SH-groups (Salin, 1988). As well, H_2O_2 can inhibit key enzymes of the Calvin Cycle (Scandalios, 1993; Elstner and Osswald, 1994) and can interact with O_2^- in the Haber-Weiss reaction to form the highly unspecific and toxic ·OH radical (Salin, 1988). As H_2O_2 can readily diffuse across membranes (Alscher and Amthor, 1988; Bowler et al., 1992), indirectly the presence of this compound can be detrimental. In general, however, H_2O_2 itself is relatively unreactive and

any observed toxic effects seem to occur only at non-physiological concentrations, although in the presence of metal catalysts, the toxicity is greatly enhanced (Fridovich, 1976). Some pathways require H_2O_2 as an oxidizer for coupling of such aromatic compounds in plant cell walls as cinnamic acids esterified to polysaccharides, cross-linking Tyr residues in structural proteins, and the monomeric precursors of lignin (Liu et al., 1995). The presence of H_2O_2, which can be mediated by salicylic acid (Sánchez-Casa and Klessig, 1994), has been postulated to be a secondary messenger in its own right (Foyer et al., 1994*b*), and has been demonstrated to increase concentrations of free cytosolic calcium, a ubiquitous secondary messenger for cellular responses (Price et al., 1994).

Molecules of H_2O_2 have been observed to be produced in chloroplasts (Cakmak and Marscher, 1992), peroxisomes (Badger, 1985), and mitochondria (Halliwell, 1981; Prasad et al., 1994*a*). Production of H_2O_2 can also occur through ß-oxidation of fatty acids and peroxisomal photorespiration reactions involving glyoxylate oxidation (Scandalios, 1993). Catalase is the enzyme directly responsible for the removal of H_2O_2 with the primary reaction product of H_2O_2 elimination being H_2O (Volk and Feierabend, 1989; Schöner and Krause, 1990). Ascorbate can also directly remove H_2O_2 (Walker and McKersie, 1993) in a reaction requiring the enzyme ascorbate peroxidase (Nakano and Asada, 1987). The compound glutathione and the

enzymes guaiacol peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase are all indirectly involved in H_2O_2 removal (Del Longo et al., 1993; Gossett et al., 1994).

1.2.3 Hydroxyl Radical

A free radical, so called because it contains an unpaired electron, always begets another radical when it reacts with a non-radical (Salin, 1988). A chain reaction will be produced, and will only come to an end when stable products are formed: hydroxylated products and hydroperoxides in the case of oxygen dependent reactions and fragmentation or dismutation products when no oxygen is involved (Saran et al., 1988).

The hydroxyl radical (·OH) is the product of the univalent reduction of H_2O_2 and is one of the strongest oxidizing agents known (Salin, 1988; Scandalios, 1993). This reduction is known as the Haber-Weiss reaction, and involves a reaction between O_2^- and H_2O_2 which is catalysed by certain transition metal ions or metal chelates (Alscher and Amthor, 1988; Mishra et al., 1993; Gossett et al., 1994). Copper and iron ions present in the plant systems can serve this function (Alscher and Amthor, 1988).

$$O_2^- + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$$

The hydroxyl radical is an extremely unspecific, highly reactive species which will react with the first available

substrate (Scandalios, 1993). These substrates include enzymes, small metabolites, nucleic acids, and membranes, making the potential for lethality of the ·OH radical quite high (Salin, 1988). In particular, ·OH is very reactive towards proteins and may cause modification of almost all amino acid residues, covalent cross-linking, and fragmentation, resulting in loss of function and an increased susceptibility to degradation by proteolytic enzymes (Prinsze et al., 1990).

Molecules of ·OH are very reactive and will not undergo many collisions before reacting. Thus specific scavengers for ·OH are not very feasible; the best strategy is to prevent it's production, to minimize the damage caused and/or repair any damage done (Rabinowitch and Fridovich, 1983). Ascorbate (Walker and McKersie, 1993), glutathione (Foyer et al., 1994a; Gossett et al., 1994), and α-tocopherol (Wise and Naylor, 1987; Fryer, 1992) are compounds that can react directly with ·OH in order to neutralize it.

1.2.4 Singlet Oxygen

When electronically excited species of oxygen are formed due to one of the outer shell electrons being elevated to a higher orbital and the spin is inverted, the resulting antiparallel spin is referred to as the singlet state (Salin, 1988). There are two excited states of singlet oxygen, the

second being extremely short-lived and rapidly inactivated by collisional quenching to form the first singlet state, which has a lifetime lasting long enough to allow for chemical reactions with other compounds to occur (Knox and Dodge, 1985).

Molecules of $^1O_2$ can be produced from various sources, such as by-products of lipoxygenase activity (Foyer et al., 1994a). However, the conditions which most favour singlet oxygen production are found within the actively photosynthesizing chloroplast (Knox and Dodge, 1985). The major mechanism of formation is by energy transfer from photoexcited compounds such as chlorophyll (Scandalios, 1993; Foyer et al., 1994a). Photo-excited chlorophyll is in the singlet state, which is used to transfer energy or electrons. However, it may undergo radiative decay (fluorescence) or may convert to the triplet chlorophyll state which can then interact with ground-state O_2 to form $^1O_2$ (Larson, 1988; Foyer et al., 1994a).

Singlet oxygen can take part in addition reactions to enes and dienes to form hydroperoxides and endoperoxides, which propagate free radicals in chain-type reactions (Larson, 1987). They also result in peroxidized lipids which lead to weakened and altered membranes (Larson, 1987). Specific amino acids, such as histidine, methionine, and tryptophan, are susceptible to oxidation by singlet oxygen, possibly resulting in protein disruption and enzyme deactivation (Knox and Dodge,

1985).

The primary means of quenching singlet oxygen within the chloroplast are the carotenoid pigments (Knox and Dodge, 1985; Dodet, 1991; De La Rivas, 1993). In terms of quantity, lutein and ß-carotene are the most important, and to a lesser extent, violoxanthin and α-carotene (Knox and Dodge, 1985). Compounds such as ascorbate (Walker and McKersie, 1993), glutathione (Gossett et al., 1994) and α-tocopherol (Wise and Naylor, 1987; Fryer, 1992) can also react directly with and quench $^1O_2$.

1.3 Chilling Effects on Plants

Temperature is an important environmental factor which determines both latitudinal and longitudinal distributions of plants (Öquist, 1983). Those plants seriously injured or killed by temperatures above the freezing point of the tissue are considered chilling-sensitive, while those able to continue growth near 0°C are deemed chilling resistant (Graham and Patterson, 1982).

1.3.1 Visible Symptoms of Chilling Injury

Many important crops of tropical or subtropical origin, such as corn (*Zea mays* L.), are sensitive to low temperatures (Koscielnak, 1993; Koroleva et al., 1994). The physiological

dysfunctions which may occur upon exposure of chilling-sensitive species to low temperatures lead to a variety of visible symptoms, the extent of which is a function of temperature extremes (Lyons et al., 1979). Chilling can affect the germination capacity of seeds of such sensitive species as cotton, corn, and tomatoes (Lyons et al., 1979; Eagles and Brooking, 1981). Once the plant has become established, chilling can result in water loss and wilting (Wilson, 1976; Koscielniak, 1993); the closure of leaf stomates, directly or indirectly, at chilling temperatures, along with increased water viscosity and reduced root permeability (Miedema, 1983), impairs the ability to transport water (Lyons et al., 1979; Stamp, 1984). Chilling can also result in lower intercellular CO_2 concentrations since the stomates have a role in both the diffusive transfer of water vapour and CO_2 (Öquist, 1983). Reduced accumulation of dry mass in roots and shoots also occurs under chilling (Stamp, 1984). Thus percents germination and viability, average time taken to germinate, dry mass, and percent water content are all good physiological indicators of chilling stress.

Conditioning or hardening chilling sensitive tissues by exposing them to temperatures just slightly above the chilling range for a period of time before placing them at the injurious temperature threshold can often prepare them to withstand low temperatures for a somewhat longer period of time before exhibiting visible symptoms of chilling injury

(Lyons et al., 1979).

1.3.2 Chilling Stress at the Molecular Level

At the molecular level, apparent loss of cell turgor, vacuolization, reduction in the volume of both the cytoplasm and the vacuole or protein bodies, apparent deposition of new material in the cell walls, general disorganization of organelles, and a general loss of cytoplasmic structure have all been reported (Iker et al., 1976). Possible mechanisms of chilling injury include lipid membrane phase-separation (Parkin et al., 1989; Hariyadi and Parkin, 1993), weakened hydrophobic bonding resulting in effects on protein-protein and protein-lipid interactions (Patterson and Graham, 1987; Parkin et al., 1989), and effects on secondary messengers of chilling stress (Price et al., 1994; Sánchez-Casa and Klessig, 1994).

An interesting feature of the chilling phenonemon is its reversibility following short-term exposure to a chilling treatment; the physiological dysfunctions resulting from the molecular changes induced at low temperatures can be reversed (or repaired) if the tissue is returned to non-chilling temperatures before the occurrence of actual injury (Lyons et al., 1979. This is especially so with meristematic tissues in both roots and shoots, but the functional photosynthetic system is susceptible to irreversible damage induced by even

short-term chilling (Stamp, 1984).

1.3.2.1 Chilling Effects on Membranes

The loss of electrolytes as a result of chilling can be correlated to the physical effects of chilling temperatures on changes in membrane permeability and/or membrane integrity (Lyons et al., 1979). Low temperatures may cause discrete membrane lipid domains, normally in a fluid phase, to change to a gel phase, thus resulting in phase separations of the lipids (Patterson and Graham, 1987; Parkin, 1987; Hariyadi and Parkin, 1993). A higher permeability of the membranes under chilling stress may injure the cell because of an efflux or accumulation of ions and metabolites (Stamp, 1984). It has been suggested that the physical state of the lipids affects the properties of the membrane-bound enzymes, so that their activation energies increase when the temperature falls below the phase shift temperature of lipids (Raison, 1973).

1.3.2.2 Chilling Effects on Hydrophobic Interactions

Protein-protein and protein-lipid interactions may become disturbed by chilling temperatures which decrease the strength of hydrophobic bonding (Patterson and Graham, 1987; Parkin, 1989; Hariyadi and Parkin, 1993). Weakening of these bonds leads to protein subunit dissociation and/or polypeptide

unfolding (Parkin et al., 1989). Subjection to cold also causes the dissociation of the microtubules and actin cytoskeletons (Patterson and Graham, 1987).

1.3.2.3 Chilling Effects on Secondary Messengers

Plants use secondary messengers such as calcium (Minorsky, 1985; Parkin et al., 1989; Monroy et al., 1993; Price et al., 1994) and salicylic acid (Sánchez-Casa and Klessig, 1994) to perceive and/or compensate for chilling stress. Loss of compartmentation due to phase separation of lipid membranes, and chilling effects on such metabolic components as the calcium ATPase pump, may lead to inability of these secondary messengers to protect against chilling injury (Parkin et al., 1989; Price et al., 1994). Loss of calcium compartmentation also leads to impaired control of ATP production and may trigger senescent processes as well, further exacerbating chilling-induced injury (Parkin et al., 1989).

1.3.3 Chilling Effects on Enzymatic Activity

Many of the effects of temperature on the activity of enzymes can be demonstrated as changes in the maximum reaction rate (V max) and the Michaelis-Menten constant (Km), both of

which vary with temperature (Graham and Patterson, 1982; Patterson and Graham, 1987) and thus environmental kinetic energy (Hochachka and Somero, 1984). Temperature not only affects the enzyme-substrate affinity due to lower kinetic energies and altered enzyme and/or substrate shapes due to weakened hydrophobic bonding, but also affects the strength of hydrophobic binding of activators and inhibitors which are vital components in the allosteric control of many enzymes (Graham and Patterson, 1982; Guy, 1990). Some enzymes are so affected by cold that they are inactivated; these typically have complex structures and dissociate into subunits upon cooling, probably because the hydrophobic interactions which stabilize the multimeric aggregates are weakened by the decrease in temperature (Patterson and Graham, 1987). Often enzymes, such as ATPases and peroxidases, have isozymic variants which are better suited for lower temperatures (Guy, 1990). Enzymatic function is also dependent upon cellular pH which maintains the protonation levels of the imidazo groups essential for proton exchange during catalysis (Patterson and Graham, 1987). As a decrease in temperature often leads to an increased [H^+], and thus lowered pH, due to weakened hydrophobic bonds and altered metabolism, the cytoplasmic pH must be returned to a higher value by H+ consuming-reactions (Patterson and Graham, 1987).

1.3.4 Chilling Effects on Metabolites

Plants experience many other metabolic disturbances as a result of exposure to chilling temperatures and often assessment of these allow for good markers of chilling stress. Soluble proteins have been observed to increase due to prolonged low temperatures (Graham and Patterson, 1982; Guy, 1990). Sugars, both reducing and non-reducing also increase under prolonged chilling (Levitt, 1980; Fry et al., 1993; Hurry et al., 1994). Starch generally accumulates upon short-term chilling exposure (Mitchell and Madore, 1992), but often concentrations decrease over long-term periods of chilling (Paul et al., 1992; Castonguay et al., 1995). Total chlorophyll content is generally lower in cold sensitive plants subjected to cool temperatures when compared with those exposed to a warmer regime (Stamp, 1987; Brüggemann and Linger, 1994; Humbeck et al., 1994).

1.3.5 Chilling Effects on Photosynthesis and Toxic Oxygen Compound Production

In terms of the photosynthetic process, enzymatic steps of the electron transport chain in the chloroplast thylakoids (Brüggemann and Dauborn, 1993), the coupling to photophosphorylation, the enzymes in the carbon reduction cycle in the stroma (Humbeck et al., 1994; Byrd et al., 1995), and the transport mechanisms of the photosynthetic products

from the chloroplasts (Long, 1983; Öquist, 1983) are all affected by temperature.

It has been demonstrated that chilling of sensitive plants in light is much more damaging to the photosynthetic apparatus than chilling in darkness (Wise and Naylor, 1987; Krause, 1988; Mishra et al., 1993). This occurs because chilling under light leads to an increase in the amount of toxic oxygen compounds present in plant systems (MacRae and Ferguson, 1985; Hodgson and Raison, 1991; Tsang et al., 1991; Havaux and Davaud, 1994; Sonoike and Terashima, 1994; Terashima et al., 1994). The reason that chilling under even moderate light intensities is more damaging to plants than chilling in darkness is due to the process of chilling-induced photoinhibition.

Under non-chilling, high-light conditions, acceptor-side photoinhibition occurs when over-reduction of the primary plastoquinone acceptor of PSII (Q_A) by strong illumination inhibits the normal electron transfer through Q_A, which in turn leads to the recombination of the primary charge pair and hence to triplet formation (Sonoike and Terashima, 1994). The triplet reacts with oxygen to form $^1O_2$ which causes the destruction of P-680, the reaction-centre chlorophyll of PSII (Sonoike and Terashima, 1994). Degradation of the D1 protein, one of the reaction-centre subunits, is also induced (Sonoike and Terashima, 1994). For donor-side photoinhibition, the activity of the donor side of PSII is insufficient to reduce

P-680, and $^1O_2$ accumulates on the donor side (Sonoike and Terashima, 1994). However, exposure of the plant to chilling conditions can induce photoinhibition of photosynthesis at much lower light intensities than would occur under optimal temperatures (Wise and Naylor, 1987; Schöner and Krause, 1990). This chilling-induced photoinhibition, at least initially, occurs at PSI, not PSII (Havaux and Davaud, 1994; Sonoike and Terashima, 1994; Terashima et al., 1994). Under chilling conditions, CO_2 concentrations may be low due to stomatal closures (Öquist, 1983), and energy-consuming carbon metabolism (Cakmak and Marschner, 1992; Elstner and Osswald, 1994) and repair processes in the chloroplast are restricted (Öquist, 1983). This results in an over-energization of the photosystem reaction-centres resulting from an inadequate supply of the natural electron acceptor $NADP^+$. Molecular oxygen may act as both an electron acceptor for the PSI electron transport chain at the iron-sulphur centres and for reduced ferredoxin, producing superoxide (Long, 1983) which then destroys the iron-sulphur centres of PSI, leading to a cascade production of toxic oxygen compounds (Sonoike and Terashima, 1994). Alternatively, the destruction of the iron-sulphur centres may occur first, leading to the recombination of an early electron acceptor and P-700 and resulting in the formation of triplet chlorophyll and hence $^1O_2$, which then may inactivate P-700 itself (Sonoike and Terashima, 1994).

1.3.6 Chilling and C3 and C4 Plants

C4 plants, such as maize, have a higher optimum temperature for photosynthesis than do C3 plants (Berry and Björkman, 1980; Long, 1983). In both systems, the carbon reduction cycle of the C3 pathway is important, but the C4 plants also have systems such as PEP-carboxylase for concentrating CO_2 so that a high partial pressure can be maintained at the carboxylating site, even under conditions unfavourable to CO_2 uptake (Stamp, 1984; Hatch, 1992). The C4 species are considered as an adaption for maintaining a high rate of photosynthesis in warm and dry habitats. Most C4 plants are thermophilic (*i.e.* more or less chilling-sensitive) because, in general, the mechanisms used for developing heat tolerance in photosynthetic enzymes and membrane function, in both C3 and C4 plants, are completely different from those for developing cold tolerance of soluble enzymes and membranes (Öquist, 1983).

C4 species are more readily injured when exposed to chilling temperatures than are C3's (Long, 1983; Hatch, 1992). A decrease in the capacity to assimilate CO_2 when chilled is greater in C4s than C3's, and has been found to be directly proportional to the light received during the chilling period (Long, 1983; Hatch, 1992). Several reasons for the reduction in the capacity to assimilate CO_2 in C4's have been investigated. Pyruvate orthophosphate dikinase (EC 2.7.9.1) is an enzyme which catalyzes the synthesis of PEP in the

photosynthetic cycle. The purified enzyme from maize has been demonstrated to reversibly dissociate from a tetramer to less active, or possible inactive, dimeric or monomeric forms at 11°C and below (Long, 1983). The photosynthetic PEP-carboxylase of C4 plants is cold sensitive as well and could account, at least partially, for a depressed photosynthetic rate in C4 plants exposed to cold (Graham and Patterson, 1982) although Long (1983) states that there is no evidence of rate limitation under chilling for this key enzyme. The rate of transfer of assimilated carbon from the C4 di-carboxylates, malate and aspartate, into C3 cycle intermediates, have been demonstrated to decrease under chilling (Long, 1983). A lack of NADPH for conversion of oxaloacetate to malate may also occur if the photosynthetic electron transport processes were blocked by cold (Graham and Patterson, 1982). Moreover, it has been suggested that this reversible reduction in capacity to assimilate CO_2 coupled with a lack of photorespiration, an alternative pathway for the utilization of light-generated reducing power, may make C4 species more prone to chilling-dependent photoinhibition (Long, 1983).

C4 species, due to their greater potential for injury upon exposure to chilling, are thus ideally suited for research dealing with chilling effects. Maize is the one of most significant crops grown in temperate climates (Long, 1983). Adaptation of this subtropical C4 plant to cool climates has only been achieved through its ability to grow

and reproduce in the short period of each year when mean temperatures exceed 10°C (Stamp, 1987; Zemetra and Cuany, 1991).

1.4 Toxic Oxygen Compound Scavenging Systems

During the course of evolution, plants have developed complex antioxidant system(s) to protect themselves from toxic oxygen compounds. Essentially, the antioxidant system can be classified into one of three classes: (1) the antioxidant enzymes, (2) the water soluble antioxidant compounds, and (3) the lipid soluble, membrane-associated antioxidant compounds. The antioxidant enzymes are composed of superoxide dismutase (SOD), ascorbate peroxidase (ASPX), catalase (CAT), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Mishra et al., 1993; Foyer et al., 1994a; Gossett et al., 1994). Ascorbate and glutathione are examples of water soluble antioxidants, and the lipid soluble antioxidants include β-carotene and α-tocopherol (Chauhan et al., 1992; Walker and McKersie, 1993). The photosynthetic electron transport system is the major potential source of toxic oxygen compounds in plants (Asada, 1994; Foyer et al., 1994a). Although these antioxidant enzymes and compounds can be found in various organelles, those located in the chloroplasts thus hold special significance. Various of these antioxidant enzymes

and compounds form a toxic oxygen compound scavenging system known as the glutathione-ascorbate cycle (Volk and Feierabend, 1989; Walker and Mckersie, 1993; Edwards et al., 1994) (Fig. 2).

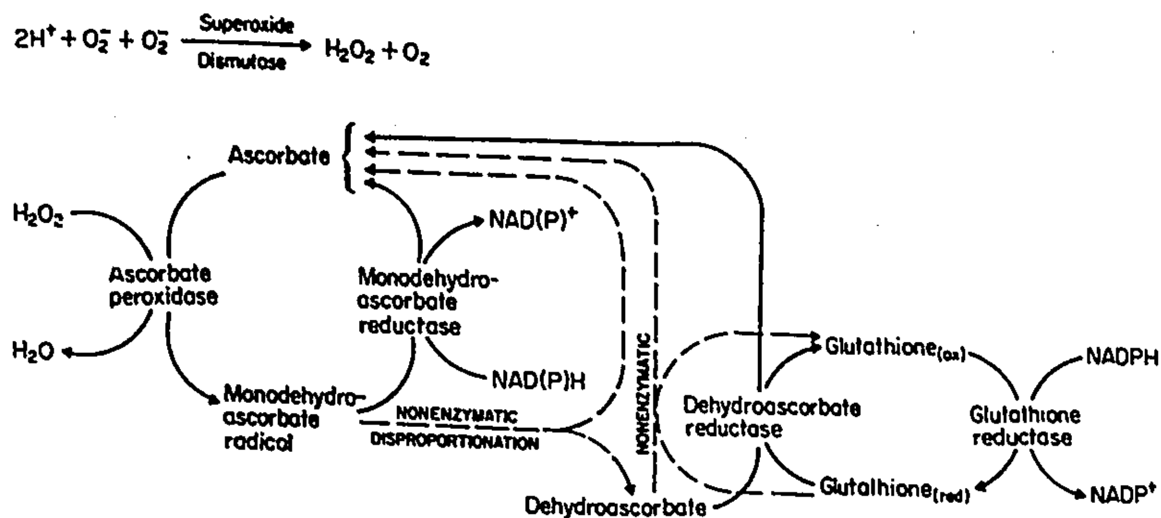
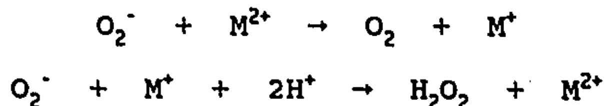


Figure 2. The O_2^- and H_2O_2 scavenging system of the chloroplast. Solid lines indicate enzyme catalyzed reactions and dashed lines indicate non-enzyme catalyzed reactions (from Jahnke et al., 1991)

1.4.1 Superoxide Dismutases

Superoxide dismutases (SOD; EC 1.15.1.1) are a group of metallo-enzymes which accelerate the conversion of two O_2^-

molecules to H_2O_2 (Fig. 2) (Olmos et al., 1994). This mechanism involves the alternate reduction and oxidation of the metal associated with the enzyme (Salin, 1988):



$$O_2^- + M^{2+} \rightarrow O_2 + M^+$$
$$O_2^- + M^+ + 2H^+ \rightarrow H_2O_2 + M^{2+}$$

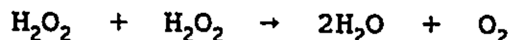
Three forms of the SOD enzyme have been characterized and have been named for the metals found in the reaction centres: iron SOD (FeSOD), manganese SOD (MnSOD), and copper-zinc SOD (Cu/ZnSOD) (de Jesus et al., 1989; Sen Gupta et al., 1993a; Scandalios; 1993). The amino acid sequences and 3-D protein structures of the FeSOD and MnSOD enzymes have been shown to be quite similar, indicating a close evolutionary relationship, whereas those of Cu/ZnSOD are different, suggesting very little, if any, evolutionary relationship to the other two (Parker et al., 1986; Bowler et al., 1992). In maize the multiple nuclear genes *Sod1, Sod2, Sod3, Sod4,* and *Sod5* encode the differentially compartmentalized SOD isozymes: SOD-1 (chloroplastic), SOD-3 (mitochondrial), and SOD-2, SOD-4, and SOD-5 (cytosolic) (Acevedo and Scandalios, 1991).

It appears that, in regards to plant tissue, at least two of these forms of SOD are present, and in some cases all three (Salin, 1988). It has been demonstrated that two distinct enzymes with SOD activity are present in chloroplasts: Cu/ZnSOD in the stroma and a MnSOD bound to the thylakoid

membrane (Sen Gupta et al., 1993*a*). MnSODs are also found in the mitochondria (Alscher and Amthor, 1988; Bowler et al., 1992) and have been reported to be present in glyoxysomes (Salin, 1988). Cu/Zn SODSs are also found in the cytosol (Scandalios, 1993; Sen Gupta et al., 1993*b*). FeSODs have been found only in a few families of plants, such as the Ginkogoaceae and Cruciferae, and are associated with the chloroplasts (Salin, 1988).

1.4.2 Catalases

Catalases (CAT; EC 1.11.1.6) are responsible for the removal of H_2O_2 in biological systems (Sánchez-Casa and Klessig, 1994). CATs are heme-containing tetrameric enzymes which catalyse the reaction (Jablonski and Anderson, 1982; Williamson and Scandalios, 1994):



$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$$

CATs are generally found only in the glyoxysomes and peroxisomes (Halliwell, 1981; Volk and Feierabend, 1989; Scandalios, 1993) and in the mitochondria (Chandlee et al., 1983; Prasad et al., 1994*a*) of plant cells and therefore have no direct role in removing H_2O_2 from the chloroplasts (Salin, 1988). In maize, three CAT isozymes exist, CAT-1 in the scutellum of germinating embryos and mature pollen, milky

endosperm, and scutella of embryos during kernel development, CAT-2 in the peroxisomes and glyoxysomes, and CAT-3 in the mitochondria (Avecedo and Scandalios, 1991; Williamson and Scandalios, 1994). CATs are not robust enzymes as they are susceptible to photoinactivation and degradation (Volk and Feierabend, 1989; Hertwig et al., 1992). They are also limited in their effectiveness, not only because of their general localisation in the glyoxysomes and peroxisomes, but because they have a relatively low affinity for H_2O_2 (Foyer et al., 1994a). However, as H_2O_2 can readily diffuse through membranes (Bowler et al., 1992), and since H_2O_2 can be produced in the peroxisomes by the oxidation of glycolate (Alscher and Amthor, 1988), the localisation of CAT does not become detrimental to plant survival. As well, since H_2O_2 can interact with O_2^- in the Haber-Weiss reaction to form the highly unspecific and toxic ·OH radical (Salin, 1988), any means of removal of this toxic oxygen compound would be important for plant survival.

1.4.3 Ascorbate Peroxidase

Peroxidases, such as ascorbate peroxidase (ASPX; EC 1.11.1.11) which uses ascorbate as a substrate, are important for removal of H_2O_2 from plant systems (Fig. 2) (Nakano and Asada, 1987; Cakmak and Marschner, 1991; Jahnke et al., 1991; Del Longo et al., 1993). ASPX differs from other peroxidases

which have high turnover for guaiacol in that they do not catalyze the oxidation of guaiacol (Nakano and Asada, 1981). The guaiacol-dependent peroxidases can only catalyze the oxidation of ascorbate at 1-3% that of their oxidation of guaiacol (Nakano and Asada, 1981). ASPX is located mainly in chloroplasts (Salin, 1988; Sankhla et al., 1992; Sen Gupta et al., 1993; Gossett et al., 1994) and in the cytosol (Foyer et al., 1994*b*) where it peroxidizes ascorbate to monodehydroascorbate in the presence of H_2O_2 (Fig. 2) (Jahnke et a., 1991; Foyer et al., 1994*a*).

1.4.4 Monodehydroascorbate Reductase

Hossain et al. (1984) demonstrated that the primary reaction product of the chloroplast glutathione-ascorbate cycle was the monodehydroascorbate radical (Fig. 2). The monodehydroascorbate radical can also be produced from such non-enzymic reactions as oxidation of ascorbate by $O_2^{\cdot}$ and ·OH (Asada and Takahashi, 1987; Mishra et al., 1993; Gossett et al., 1994). Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), using NADPH as a donor, was suggested to account for the regeneration of ascorbate from the monodehydroascorbate radicals produced from the ascorbate peroxidase enzyme activity (Fig. 2) (Hossain et al., 1984). The monodehydroascorbate radical is reduced by NADPH to ascorbate in a reaction catalyzed by monodehydroascorbate reductase, and

then disproportionates spontaneously to dehydroascorbate and ascorbate (Hossain et al., 1984; Schöner and Krause, 1990; Ushimaru et al., 1992; Mishra et al., 1993). As well, monodehydroascorbate radicals can be reduced by acting as a terminal electron acceptor of the photosynthetic electron transport system (Foyer et al., 1994*a*).

MDHAR has been reported in the cytosol, mitochondria, microsomes, and chloroplasts of plant cells (Alscher and Amthor, 1988)

1.4.5 Glutathione Reductase

Glutathione reductase (GR; EC 1.6.4.2) was first proposed to be involved in the ascorbate-glutathione cycle to reduce oxidized glutathione (GSSG) to the reduced form (GSH) by Halliwell and Foyer (1978). This reaction occurs in the presence of NADPH (Fig. 2) (Salin, 1988; Jahnke et al., 1991; Edwards et al., 1994). Not only is the presence of GSH important for the ascorbate-glutathione cycle, but it also serves as an oxidant scavenger (Schmidt and Kunert, 1986; Mahan and Burke, 1987). Although the majority of glutathione reductase found within plant cells is located in the chloroplast, smaller amounts are present in the mitochondria and cytosol (Alscher and Amthor, 1988; Edwards et al., 1994). Glutathione reductase isolated from corn mesophyll chloroplasts were found to be in the form of a heterotetramer and is different from the pea GR enzymes in both structure and

weight (Mahan and Burke, 1987).

1.4.6 Ascorbic Acid

Water soluble ascorbate eliminates H_2O_2 in a pivotal reaction catalyzed by ascorbate peroxidase (Fig. 2) (Jahnke et al., 1991; Foyer et al., 1994*b*; Gossett et al., 1994). This compound has also been demonstrated to react directly with and detoxify O_2^-, $\cdot OH$, and $^1O_2$ (Halliwell, 1981; Larson, 1988; Walker and McKersie, 1993) and can act synergistically with and regenerate α-tocopherol (Packer et al., 1979; Finckh and Kunert, 1985), another antioxidant compound. The regeneration of reduced ascorbate can be made possible either by the monodehydroascorbate radical acting as a photosynthetic electron acceptor (Foyer et al., 1994*a*) or reduction by the enzymes MDHAR and dehydroascorbate reductase (DHAR; EC 1.8.5.1) and another antioxidant compound, glutathione (Jahnke et al., 1991; Foyer et al., 1994*b*) (Fig. 2). Ascorbate exists in relatively high concentrations in many cellular environments, such as the stroma of the chloroplasts and the cytosol (Alscher and Amthor, 1988; Larson, 1988). Foyer et al. (1983) demonstrated that only 30% of the total available ascorbate in spinach (*Spinacia oleracea* L.) was present in the chloroplast. However, in addition to the recycling of ascorbate within the chloroplast, the chloroplast can take up ascorbate from the cytosol by virtue of a translocator located

on the chloroplast envelope (Foyer et al., 1994*a*).

1.4.7 Glutathione

The simple tripeptide water-soluble thiol compound glutathione ultimately serves to re-reduce ascorbate in the glutathione-ascorbate cycle (Fig. 2) although it can also react directly with and quench $^1O_2$ and $\cdot OH$ species (Larson ,1988; Foyer et al., 1994*b*). Glutathione may also directly remove H_2O_2 through the catalytic action of non-specific peroxidases (Salin, 1988; Foyer et al., 1991). Furthermore, it has a major function in preventing inactivation of enzymes due to oxidation of thiol groups; reduced glutathione (GSH) is more easily available than are enzyme thiol groups and thus will be preferentially oxidized (Halliwell, 1981; Foyer and Halliwell, 1986). GSH can also reactivate some enzymes that have been inhibited by previous exposure to oxygen, presumably by reducing their oxidized -SH groups (Halliwell, 1981) and plays major roles in sulphur transport and in regulating gene expression in response to environmental stress (Edwards et al., 1994). The regeneration of reduced glutathione can be made possible by GR in a NADPH-requiring reaction (Fig. 2) (Foyer and Halliwell, 1976; Jahnke et al., 1991; Foyer et al., 1994*b*). This compound is present in high concentrations both in chloroplasts and in the cytosol (Alscher and Amthor, 1988). Gillham and Dodge (1986) have demonstrated in pea (*Psium*

sativum L.) that 90% of the total cellular glutathione can exist outside the chloroplasts.

1.4.8 ß-carotene

The lipid-soluble carotenoids have a recognized role in acting as the photoreceptive "antenna pigments" for photosynthesis where they absorb certain wavelengths of light that are not absorbed by the chlorophylls. However, ß-carotene is also considered to be a powerful quencher of singlet oxygen (Halliwell and Gutteridge, 1985; Larson, 1988) and can quench excess chlorophyll excitation energy not readily passed on through the photosystem (Halliwell, 1981; Knox and Dodge, 1985; De La Rivas, 1993) and can thereby reduce potential photoinhibition. The rate constant for ß-carotene's quenching of singlet oxygen exceeds that for the reaction of singlet oxygen with most unsaturated fatty acids by 4 to 5 orders of magnitude, thus allowing a relatively low concentration of ß-carotene to effectively protect chloroplastic membrane lipids from reactions of singlet oxygen leading to peroxidation (Larson, 1988).

1.4.9 α-tocopherol

Chloroplasts contain large amounts of lipid-soluble α-tocopherol, a scavenger whose lipophilic nature enables it to

deal effectively with the toxic oxygen compounds O_2^-, •OH, and $^1O_2$ (Wise and Naylor, 1987; Fryer, 1992; Gossett et al., 1994) and to act as a lipid oxidation chain-breaker and a lipid peroxidation trap (Fryer, 1992; Walker and McKersie, 1993). Although present in smaller concentrations than ascorbic acid, it is considerably more lipophilic and has been found to be the more potent antioxidant, particularly with respect to lipid peroxidation (Packer et al., 1979). A single molecule of α-tocopherol can undergo approximately 100 singlet oxygen quenching events before it is oxidized to α-tocopherolquinone (Wise and Naylor, 1987).

It had been proposed that ascorbate acts as an antioxidant-synergist with α-tocopherol and that both compounds could act together as a powerful antioxidant system in cells (Leung et al., 1981). In this system, α-tocopherol acts as the primary antioxidant, while ascorbate oxidation regenerates α-tocopherol (Gossett et al., 1994); peroxidation seems to be determined by both the amount and the ratio of these two antioxidant compounds present in the plant (Finckh and Kunert, 1985).

1.5 Effect of Chilling on Antioxidant Systems

Chilling under light leads to an increase in the amount of toxic oxygen compounds present in plant systems (Havaux and Davaud, 1994; Sonoike and Terashima, 1994; Terashima et al.,

1994). This is due to chilling-induced photoinhibition and low-temperature inhibition of other metabolic pathways and repair processes, including a potential initial decrease in scavenger system capacities (Schöner and Krause, 1990).

In maize, relatively short-term stresses leading to production of toxic oxygen compounds result in decreases in activity of SOD (Del Longo et al., 1992) and both increases (Pastori and Trippi, 1992) and decreases (Jahnke et al., 1991) in activities of ASPX and GR. However, it has been observed in other plant species that prolonged oxidative stress can generally induce or enhance activities of SOD (Schöner and Krause, 1990; Bowler et al., 1992; Foyer et al., 1994a), ASPX (Melhorn et al., 1987; Cakmak and Marschner, 1992; Mishra et al., 1993), and GR (Schmidt and Kunert, 1986; Pastori and Trippi, 1992; Foyer et al., 1994a, Gossett et al., 1994) and concentrations of ascorbate, glutathione (Smith, 1985; Schmidt and Kunert, 1986; May and Leaver, 1993; Foyer et al., 1994a) and carotenoids (Schöner and Krause, 1990; Mishra et al., 1993; Walker and McKersie, 1993). Long-term oxidative stress in terms of activities of SOD, GR, or ASPX or concentrations of ascorbate, carotenoids, or α-tocopherol have not been studied in maize. However, glutathione concentrations have been observed to increase in maize in response to prolonged oxidative stress (Smith, 1985).

It is postulated that there is an alteration in antioxidant system biosynthesis brought about by the presence

of toxic oxygen compounds (Schöner and Krause, 1990; Perl-Treves and Galun, 1991; Foyer et al., 1994*b*). The production of toxic oxygen compounds in plants is perhaps a general alarm signal which serves to alert metabolism and gene expression for possible modifications (Schöner and Krause, 1990; Foyer et al., 1994*b*). The general increase in activities of SOD, ASPX, and GR may not, however, be solely due to increases in gene expression or steady state mRNA levels (Tsang et al., 1991; Foyer et al., 1994*b*), but also to subtle changes in the intracellular distribution and differential sensitivity to photooxidation between different isozymic forms with different substrate affinities (Edwards et al., 1994; Foyer et al., 1994*a*).

Activity of MDHAR has been observed to increase under both short-term (Jahnke et al., 1991; Mishra et al., 1993) and long-term (Schöner and Krause, 1990) chilling stress, or not to change at all (Walker and McKersie, 1993), relative to control plants in response to chilling stress. In maize, short-term chilling exposure led to increased MDHAR activity, but as the chilling treatment was sustained, the increase in MDHAR activity was not as pronounced as in the short-term, although the activity was still higher than that of controls (Jahnke et al., 1991). As MDHAR activity is known to be regulated by its metabolites (Hossain et al., 1984), changes in its activity may be correlated with similar changes in production of its substrate, the mondehydroascorbate radical

(Fig. 2) (Mishra et al., 1993). The production of this monodehydroascorbate radical is dependent upon the rate of scavenging of H_2O_2 by ascorbate and ascorbate peroxidase (Fig. 2) (Gossett et al., 1994).

CAT has been observed to decrease in response to low temperatures in various crop plants (MacRae and Ferguson, 1985; Volk and Feierabend, 1989; Schöner and Krause, 1990). In darkness, up-regulation of CAT is an important means of maintaining low H_2O_2 levels (Prasad et al., 1994*b*). Feierabend et al. (1992) found no changes in CAT levels of chill-shocked, dark-grown maize. Under illumination, however, photoinactivation of catalase occurs which is mediated through light absorption by both the enzyme bound heme group and chloroplast pigments (Volk and Feierabend, 1989; Schöner and Krause, 1991). Similar results in maize have been observed under both short-term (Feierabend et al. 1992) and long-term (Prasad et al. 1994*b*) chilling stress. Concomitant resynthesis of CAT can compensate for the loss of CAT and can maintain constant levels of this enzyme under light (Hertwig et al., 1992; Mishra et al., 1993). However, low temperature inhibition of CAT biosynthesis may well slow down the mechanisms which, under normal control conditions, would compensate for the loss of active enzyme.

Levels of α-tocopherol have generally been shown to decrease upon chilling (Wise and Naylor, 1987; Walker and McKersie, 1993; Gosset et al., 1994). As α-tocopherol

compounds are one of the first antioxidants affects by oxidative stress, they may be the first line of defence for protecting photosynthetic pigments (Wise and Naylor, 1987).

As chilling under light leads to an increase in the amount of damaging toxic oxygen compounds present in plant systems (Schöner and Krause, 1990; Hodgson and Raison, 1991; Tsang et al., 1991; Havaux and Davaud, 1994; Sonoike and Terashima, 1994; Terashima et al., 1994), the question whether plants having higher antioxidant capacities can withstand chilling to a higher degree than plants with lower antioxidant capacities has been asked. However, the vast majority of past research attempting to determine if different responses of antioxidant systems existed between relatively chilling sensitive and tolerant plants have compared two or more different species (MacRae and Ferguson, 1985; Wise and Naylor, 1987; Hodgson and Raison, 1991; Jahnke et al., 1991; Walker and McKersie, 1993). When comparing responses in different species, it must be taken into account that the genetic and metabolic mechanisms behind one species's response may be quite different from another's. The advantage of working with different lines or cultivars of one species exhibiting differential responses is that the mechanisms involved in both relative sensitivity and resistance can be studied in a similar genotype, thus reducing the complexity of genetic differences and variability. Recent work comparing

antioxidant capacities between differentially drought sensitive maize lines (Del Longo et al., 1993) and differentially salt sensitive cotton (*Gossypium hirsutum* L.) cultivars (Gossett et al., 1994) has been reported.

The following work assesses antioxidant capacities and levels of general metabolic indicators of chilling stress between differentially chilling sensitive lines of maize. It was proposed that relatively chilling sensitive maize would have less antioxidant enzyme and/or compound capacities than relatively chilling tolerant maize. Less capacity to scavenge toxic oxygen compounds in the chilling sensitive line compared to the tolerant lines could lead to an accumulation of toxic oxygen byproducts and hence to greater damage while under chilling. This work is unique in that this is the first time antioxidant capacity comparisons have been made between relatively chilling sensitive and tolerant lines within a single species of higher plants. The study of differentially chilling sensitive lines within the same species eliminates some of the possible genetic differences and variability which occurred in past work of this nature. Furthermore, for the first time, both the antioxidant capacities and the metabolic indicators of chilling stress were assessed in four inbred lines and the twelve hybrids resulting from a full diallel cross of the parental inbreds. The purpose of this was two-fold. Firstly, it was to evaluate the combining abilities of the antioxidant and indicator parameters between inbreds and

hybrids and to determine if hybrid performance relative to these parameters could be predicted from inbred performance. Secondly, this was performed to determine if any of these antioxidant enzymes and compounds and/or metabolic indicators could be useful in a novel screening technique for chilling sensitivity for both inbred and hybrid lines of maize.

1.6 Thesis Objectives

In order to determine if relatively chilling sensitive maize would have less antioxidant enzyme and/or compound capacities than that of relatively chilling tolerant maize, lines of maize exhibiting differential sensitivity to chilling had to be identified. Selection of relatively chilling sensitive and tolerant maize based on their physiological responses to chilling, the first objective of this thesis, was done by subjecting inbred lines to two newly developed laboratory model chilling tests, one at the germination stage and the other at the early growth (fourth-leaf) stage. Field trials were then carried out to confirm laboratory model results.

The second objective of this thesis was to test if the selected chilling sensitive inbred maize lines had less antioxidant capacities than the tolerant. To this end, activities of the antioxidant enzymes CAT, ASPX, SOD, MDHAR, and GR, along with concentrations of the antioxidant compounds

ascorbate, glutathione, ß-carotene, and α-tocopherol and levels of the general metabolic indicators of chilling stress sugars, starch, chlorophyll, and soluble proteins were assessed. Part of the second objective was to determine if antioxidant enzyme and compound capacities and the concentrations of the indicators would change with plant age and with length of exposure to chilling. Thus, these parameters were assessed at three developmental stages (first, third and fifth leaf stages) and under both short- and long-term chilling temperature regimes.

As inbreds are prone to inbreeding depression due to their homozygosity, they are frequently less vigorous than hybrids. This raises the question of whether relatively chilling sensitive lines would have potentially less antioxidant capacities than more tolerant lines for both inbreds and hybrids. If so, would the same antioxidant enzymes and/or compounds be limiting in both inbreds and hybrids? To answer this question, the third objective of this thesis was designed to determine if there were similar results for antioxidant capacities and metabolic indicator concentrations for the chilled hybrids as there were for the inbred maize lines. As well, the feasibility of a screening test for chilling sensitivity in both inbred and hybrid maize lines could be determined. Furthermore, evaluations of the general and specific combining abilities of these parameters between hybrids and their parental inbreds could be performed in order

to determine if hybrid chilling sensitivity relative to physiological developmental characteristics (germination and early growth), antioxidant enzyme activities and antioxidant compound and metabolic indicator concentrations could be predicted from those of the inbreds. Thus, a complete diallel cross between the above selected inbreds was made, and the resulting hybrids then classified as relatively chilling sensitive or tolerant depending upon their performance in the laboratory germination and early growth stage screening tests as per the inbreds above. Field trials were performed to confirm laboratory results. The hybrids were grown until the third leaf stage under short-term chilling, and assessed for antioxidant enzyme activities and concentrations of antioxidant compounds and metabolic indicators.

In this thesis, the lower antioxidant capacity of the chilling sensitive inbred and hybrid relative to the tolerant maize lines is reported. As well, it is demonstrated that a screening test for chilling sensitivity of both inbred and hybrid maize lines using activities of CAT, ASPX, and MDHAR and concentrations of carbohydrates would be feasible. It has also been shown that hybrid levels of antioxidants and metabolic indicators, as well as physiological germination and early growth parameters, cannot be predicted from those of their parental inbreds. Furthermore, this thesis had led to the development of two new laboratory screening tests for identification of differentially chilling sensitive maize at

both the germination and early growth stages.

This thesis is presented in the form of six papers, either of published or submitted status. Each chapter, from **Chapter 2 - Chapter 7**, represents the reproduction of one paper. In order to shorten the length of this work, however, it should be noted that if methodology was repeated, a reference to a "Materials and Methods" section of a previous chapter is made. The references have also been removed from the end of each paper from **Chapter 2 - Chapter 7** and have been combined into one general **References** section at the end of this thesis which also contains the references from the **Introduction** and the concluding **Chapter 8**. **Chapter 8** provides an overview of the results and a synthesis of the "Discussion" sections of each chapter with the view to future work.

CHAPTER TWO

A CHILLING RESISTANCE TEST FOR INBRED MAIZE LINES

The following chapter is a reproduction of a paper published in the Canadian Journal of Plant Science (1994, 74:687-691) by D.M. Hodges, R.I. Hamilton, and C. Charest. The principal author (D.M. Hodges) accumulated and interpreted all data. The second author (Dr. R.I. Hamilton) produced and contributed seed for the inbred maize lines used in these experiments.

The following chapter partially details the fulfilment of the first objective of this thesis: identifying inbred lines of maize exhibiting differential sensitivity to chilling. Relatively chilling sensitive and tolerant maize lines were to be selected based on their physiological responses to chilling at the germination stage. A laboratory germination test based on exposure of maize seeds to a temperature regime which mimicked natural field conditions was developed to determine chilling resistance. The percent germination, percent viability, and average time taken to germinate were the parameters measured. The results of all three parameters corresponded in indicating which line was chilling susceptible, and the percents germination and viability in which lines were chilling tolerant. Field trials were sown and percent of emergence and average time to emergence

recorded in order to confirm the results of our laboratory model. Our laboratory model was successful in that the lines demonstrated to be most chilling susceptible by the laboratory germination test and those lines selected to be most chilling tolerant by the laboratory percent germination and viability assays corresponded to those classified as such by the field percent emergence.

Results from this germination test were combined with those from an early growth (fourth leaf stage) phase test (Chapter Three) in order to confirm that the maize lines exhibiting differential sensitivity to chilling at germination exhibited similar properties at a later stage of development.

2.1 ABSTRACT

A laboratory germination test based on exposure of seeds to a temperature regime derived from natural conditions was developed to determine chilling resistance in maize (Zea mays L.). Seven inbred lines were exposed to a temperature regime approximating Ottawa, Ontario's (Lat. 45° 24' N, Long. 75° 43' W) spring climate. Seeds were subjected to a range of maximum (16hr) and minimum (8hr) temperatures corresponding to the dates spanning April 15 (10.3/0.4°C) to May 30 (21.0/9.1°C) in controlled temperature germinators. A control germination test used a constant 25°C. The percent germination, percent viability, and average time taken to germinate were measured. The results of all three parameters corresponded in indicating which line was chilling susceptible, and the percents germination and viability in which lines were chilling tolerant. Field trials were sown in Ottawa in the early spring of 1992 and 1993 and percent of emergence and average time to emergence recorded. The line demonstrated to be most chilling susceptible by the laboratory germination test and those lines selected to be most chilling tolerant by the laboratory percent germination and viability assays corresponded to those classified as such by the field percent emergence.

Key Words: Maize - Germination Test - Chilling Tolerance & Sensitivity

2.2 INTRODUCTION

The sensitivity of a crop species to chilling frequently restricts the environments to where it can be cultivated. Maize (Zea mays L.) grown in temperate regions is often subjected to chilling conditions before and after emergence which can lead to disruption of development (Cutworth and Shaykewich 1990; Hope et al. 1992). The introduction of maize lines with high potentials for emergence under chilling conditions in temperate regions would aid in stand establishment and in potentially stabilizing and optimizing grain yields.

Most previous methods of determining tolerance or susceptibility of maize to chilling have relied on controlled germination under cool, constant temperatures (Barla-Szabo and Dolinka 1988; Tekrony and Egli 1991). It has been important to select a proper temperature for use in these procedures; too high a temperature will not adequately test the chilling response, and too low a temperature will stop all development together. This does not reflect the temperature nor the soil conditions that the seeds experience in the field. There have been few reports of chilling responses of inbred lines (Mock and McNeill 1979) since most studies have used hybrids (Sowinski and Maleszewski 1990; Hope et al. 1992). Cold tolerance appears to be genetically controlled but inherited with maternal effects related to germination and early seedling growth (Maryam and Jones 1983; Aidun et al. 1991).

Inbreds have a genetic integrity which will be maintained over generations and which can contribute a superior agronomic performance to hybrids (Hallauer et al. 1988), an important factor of concern to breeders and seedsmen.

The objective of this study was to evaluate a laboratory chilling sensitivity test designed to approximate spring field conditions using a group of elite inbreds.

2.3 MATERIALS AND METHODS

2.3.1 Plant Material

The inbred lines of corn (Zea mays L.) CO251 (dent), CO266 (flint), CO304 (flint/dent), CO305 (dent), CO306 (dent), CO308 (dent), and F2 (flint) were previously observed to differ in their ability to germinate and grow under chilling conditions (data unpublished), and thus were chosen for this study. The elite CO and F2 inbreds were produced in the 1990 and 1992 Agriculture Canada nursery, Ottawa, Canada. All lines were produced in the same nursery in the same year. The CO inbreds were developed by Agriculture Canada and the F2 inbred originated from l'Institut National en Recherche Agronomique (INRA), France.

2.3.2 Germination Conditions

After pretreatment with the fungicide Thiram, 5 replicates of 20 seeds per line were placed, embryo up, in covered plastic petri dishes (100 x 15 mm) lined with Whatman #1 filter paper. Germination was initiated by adding 5 ml distilled water to each dish. Further additions necessary to keep the filter paper moist were made once every two days until the end of the experiment. The dishes were placed into dark controlled germinators (Conviron Model I18) at a constant humidity (98 % plus) and under a controlled temperature regime. This regime approximates Ottawa's (Lat. 45° 24' N, Long. 75° 43' W) spring climate using values corresponding to the average air temperatures recorded over a 30-year period (Edey et al., 1968), starting from April 15 (10.3°C max / 0.4°C min) to May 30 (21.0°C max : 9.1°C min) (16 hr max:8 hr min) (Table 2.1). Temperature settings were altered every 5 days during the 45 day cycle. The thermal environment was evaluated by calculating the corn heat units (CHU) (Table 2.1) (Brown 1969). The seeds were checked once a day, and seedlings which had obtained a coleoptile length of at least 1 cm were removed and scored as germinated. Germination was calculated for each line as the percent of seeds which achieved this coleoptile length. The average time to germinate was defined as the average length of time necessary for the seed to achieve a 1 cm coleoptile. At the end of the temperature cycle, seeds that had failed to germinate were

tested for viability using tetrazolium chloride (Bennett and Loomis, 1948). Viability was calculated for each line as the percent of seeds which had either germinated or whose embryo and/or scutellum stained pink. Germination trials were repeated in the same germinator at three different times for a total of 300 seeds per line.

Another set of 3 repeats in time of 5 replicates of 20 seeds per line were used as controls; these were prepared in the same manner and subjected to a constant temperature of 25°C.

2.3.3 Field Test

Eight replicates of 25 seeds/line pretreated with Thiram were sown in the field at Ottawa on April 22, 1992 and April 26, 1993 in a medium textured soil (Allendale sandy loam), consisting of 8" of sand overlying clay. In addition, a light sandy loam (Uplands sandy loam) and a heavy clay (Rideau clay) textured soils were seeded on April 27, 1993. Seeds were hand planted at a depth of 5 cm in rows 2.5 m long and 0.4 m apart in a randomized complete block design. A data logger was programmed to record soil temperatures at a 5 cm depth in five random positions of the experiment. Five day averages of the field soil temperatures were then calculated (Table 2.1). However, in the 1993 medium textured soil site, a malfunction due to a lightning strike on the data logger required that an

approximation of the field temperatures be made by averaging temperature data from the heavy clay and the light sandy soil sites. Fields were checked daily, and any seedling whose coleoptile protruded from the soil was scored as emerged. Emergence and average time to emergence were defined as the percent of seed which had emerged from the soil and the average time it took to emerge, respectively. Observations were made until 25 days from date of seeding (May 17 in 1992 and May 22 in 1993). At this point, at least one of the lines in all the plots had reached the fourth-leaf stage of development. Corn heat units (CHU) were calculated from field sites using the maximum and minimum 5 day average shade temperatures recorded 1 m above-ground (Table 2.1).

2.3.4 Statistical Analysis

Analyses of variance and LSD tests using a Statistical Analysis System (SAS) package (Version 6.06.01) running PROC ANOVA, PROC GLM, and LSMEANS tests were conducted. Results of these analyses demonstrated high repeatability between replicates within lines for both the laboratory and the field tests. Correlations coefficients (n=7) were calculated between the laboratory and the field parameters averaged over the two years.

2.4 RESULTS

2.4.1 Laboratory Experiment

In the 25°C control regime, all lines germinated at 90% and above (Table 2.2). CO308 (96.6%) was least viable (Table 2.2), and the lines CO266 (4.3 days) and CO251 (4.5) took the shortest times to germinate, and F2 (5.8) the longest (Table 2.3).

The variable temperature chilling regime indicated that CO251 (65.4%) and F2 (71.7%) were the lines having the lowest percentage of seeds to germinate, while CO304 (100%) and CO305 (99.7%) had the highest (Table 2.2). These percent germination results corresponded ($r=0.799$, $P=0.032$) to the percent viabilities of the lines which indicated that CO251 (78.7%) was least viable at the low temperatures (Table 2.2). Under the chilling regime, CO266 (19.8 days) took the shortest and F2 (24.4) the longest average times to germinate (Table 2.3).

Since significant differences in the three recorded parameters were found between lines exposed to the control regime (Tables 2.2, 2.3), the ratio of chilling to control values provided a tool for determining relative chilling resistance. Lines with lower ratios for percents germination and viability and higher ratios for average time taken to germinate could then be classified as more chilling sensitive. For germination, the chill:control ratio of CO251 (0.69) was

the lowest, followed by F2 (0.77), (0.89), CO306 (0.89), CO308 (0.91), CO304 (1.00), CO305 (1.00), then CO266 (1.02) (Table 2.2). These germination ratios were correlated with the viability ratios (r=0.805, P=0.023), of which CO251 (0.80) was the lowest (Table 2.2). For the average time to germinate, the ratios showed F2 (4.21) to be the lowest, followed by CO305 (4.32), CO306 (4.35), CO308 (4.39) CO266 (4.60), CO304 (4.69), then CO251 (4.78) (Table 2.3).

2.4.2 Field Temperatures

The maximum and minimum temperatures measured at the 5 cm soil depth in the 1992 and 1993 spring field trials differed from those calculated by averaging 30 years of past air data (Table 2.1). Both maximum and minimum temperatures were lower in the heavy clay soil than in the light sandy soil in the initially warm part of the 1993 field trials (Table 2.1). However, during the subsequent cooler period, the heavy soil remained warmer than the light textured soil (Table 2.1). The accumulated corn heat units (ΣCHU) demonstrated that the shade temperatures above the light soil were higher than those above the medium followed by the heavy textured soils (Table 2.1).

2.4.3 Field Experiment

For the 1992 spring field trial, which was sown in the

medium textured soil, the percent emergence of the seven lines ranged from 69% (CO251) to 87% (CO304) (Table 2.2). The percent emergence of CO251 was not significantly different from those of CO266 or CO306 (Table 2.2). The group with the highest percent emergence included CO308, CO305, and CO304 (Table 2.2). In this medium soil type in 1993, CO266, CO251, F2, CO306, and CO308 had the lowest percents emergence (69-71%), and CO304 (80.5%) the highest (Table 2.2). The percents emergence of the lines sown in the light textured soil in 1993 were not significantly different from each other, and ranged from 52.5% (CO251) to 60.3% (CO304) (Table 2.2). On the heavy textured soil in 1993, CO251, CO266, F2, and CO308 were rated as the lowest (40-48%), and CO306 (60%) and CO304 (67%) the highest (Table 2.2). These percent emergence results were correlated (r=0.766, P=0.037) with those of the laboratory percent germination test.

The average times to emerge in the medium textured soil in the 1992 field trial exhibited a narrow range from 17.7 (CO251) to 18.9 days (F2) (Table 2.3). This range was somewhat wider in the 1993 medium soil field trial. The lines taking the shortest and longest time to emerge were again CO251 (11.7 days) and F2 (14.5) (Table 2.3). In the light textured soil, CO266, CO251, CO305, CO304, and CO306 required the shortest time to emerge (10.9-11.7 days), while F2 (14.5) and CO308 (12.7) took the longest (Table 2.3). In the 1993 heavy soil trial, CO306, CO251, CO308, CO305, and CO304

required the least time to emerge (15.1-16.1 days), and F2 (17.5) took the longest (Table 2.3).

2.5 DISCUSSION

The objective of this study was to investigate whether a laboratory chilling test approximating natural, spring field temperatures could be verified in the field. The most chilling susceptible line identified by this laboratory variable temperature chilling test and, furthermore, those lines selected to be most chilling tolerant by the percents germination and viability, corresponded to those classified as such by the field percent emergence.

Chilling:control ratios for each line in the laboratory test were calculated since these ratios take into account inherent genetic differences among lines which became apparent under control conditions. Thus, actual effects of chilling on the individual lines can be assessed. These ratios for germination, viability, and the average time taken to germinate selected CO251 as being most affected by chilling. This line was also selected by the percents germination and viability as most sensitive to chilling by the low temperature test alone. Thus, low percents of germination and viability, as well as low percents of emergence by extrapolation, are good indicators of chilling sensitivity. The percent emergence results from the field trials implied that CO251,

CO266, and F2 were chilling susceptible, and F2 was selected for as such according to average time to emergence. Similarly, F2 took the longest average time to germinate under the low temperature test alone. The three laboratory germination and the field percent emergence tests thus corresponded in the selection of CO251 as most chilling susceptible.

The percents germination and viability tests at chilling temperatures, and when expressed as chilling:control ratios, indicated that CO266, CO304, and CO305 were the most chilling tolerant. This suggests that percents germination and viability, as well as percent emergence, are good indicators of chilling tolerance as well as sensitivity. CO304, CO305, and CO306 were also selected as tolerant by field percentage emergence. Average time to germination in the laboratory chilling test and the corresponding chilling:control ratios indicated that F2 and CO266, respectively, were more chilling tolerant. The average time to emergence, however, implied that CO251 and CO306 were more tolerant. Thus, there exists a discrepancy in the relative rankings of the inbreds in their tolerance to chilling when measured either as percents germination, viability, or emergence, or when measured as average times to germinate or emerge. As percent emergence, or percent germination , is a significant criterion of a selection program under chilling conditions, it is clear that the average time to germinate or to emerge is not a

distinguishing parameter for this set of genotypes. Minor differences in the relative ranking of the lines from the four field tests also demonstrated that the interactive effect of soil temperature and texture, which was also indicated by the ΣCHUs, was a factor in the emergence of the inbreds.

There is evidence that a high percentage of germination is associated with rapid emergence under both field and controlled environments (Mock and McNeill 1979; Eagles and Brooking 1981; Maryam and Jones 1983). Past methods of predicting the emergence potential of maize lines under field conditions with laboratory screening techniques have seldom been successful when only one parameter was measured (Barla-Szabo and Dolinka 1988). This is a result of emergence relying on the simultaneous effects of several environmental factors such as temperature and moisture availability. The majority of laboratory screening procedures for prediction of field emergence potentials have been developed by exposure of the seeds to a constant, cool temperature, often around 11°C (Barla-Szabo and Dolinka 1988; Hope et al. 1992). The concept of the present test regime is not only to negate the problem of an appropriate single screening temperature, but to alter the exposure temperature to approximate that of a specific local environment. This would simulate, to an extent, the temperature environment to which the seeds would be exposed under field conditions. An accurate laboratory test to simulate crop field emergence potential, such as the one

proposed here, would be of economic benefit for plant breeders and producers.

2.6 ACKNOWLEDGEMENTS

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Table 2.1. Five day averages of maximum and minimum temperatures (°C) and accumulated corn heat units (ΣCHU) from either the laboratory germination test or 1992 medium (sand and clay) and 1993 light (sandy loam), medium, and heavy (clay) textured soil field trials

Date	Laboratory test[z]			Field trials[y]											
				1992			1993								
				Medium soil			Light soil			Medium soil			Heavy soil		
	Max.	Min.	ΣCHU	Max.	Min.	ΣCHU	Max.	Min.	ΣCHU	Max.	Min.	ΣCHU	Max.	Min.	ΣCHU
Apr 15	10.3	0.4	3.2												
Apr 20	11.7	2.4	16.4												
Apr 25	13.4	2.5	42.8	17.2	5.9	56.3	19.7	10.1	85.9	17.0	9.5	70.9	14.3	8.9	52.2
Apr 30	15.9	4.3	84.2	12.2	5.2	78.9	26.6	11.6	198.5	20.5	11.2	165.5	14.3	10.0	113.2
May 5	18.1	5.1	129.1	22.8	10.0	175.0	21.5	10.3	291.6	18.4	10.3	245.4	15.3	10.4	178.3
May 10	16.2	3.8	173.7	24.2	12.3	285.4	20.0	9.0	374.2	18.4	9.1	322.7	17.2	9.3	249.6
May 15	17.9	6.3	235.3	26.5	14.1	383.1	18.7	11.8	467.5	20.2	12.6	422.7	21.2	13.5	359.5
May 20	19.4	6.4	303.4												
May 25	20.6	8.3	386.6												
May 30	21.0	9.1	473.2												

[z]Laboratory temperatures and ΣCHU were calculated from 30 year averages.
[y]Field temperatures were recorded at a 5 cm depth and the ΣCHU were calculated from shade temperatures measured 1 m above-ground.

Table 2.2 Control (25°C), chilling (variable temperature), and the chill : control ratio of both percent viability and germination from the laboratory germination test and the percent emergence from the 1992 and 1993 field trials on light (sandy loam), medium (sand and clay), and heavy (clay) textured soils

	% Viability			% Germination			% Emergence			
							1992	1993		
Line	Control	Chill	Chill/ Control	Control	Chill	Chill/ Control	Medium soil	Light soil	Medium soil	Heavy soil
CO251	98.4^{ab}(2)^{z}	78.7^{a}(1)	0.80(1)	94.8^{ab}(4)	65.4^{a}(1)	0.69(1)	69.0^{a}(1)	52.5^{a}(1)	69.5^{a}(2)	40.0^{a}(1)
CO266	100.0^{b}(5)	99.7^{b}(6)	1.00(5)	90.0^{a}(1)	91.6^{c}(5)	1.02(6)	70.5^{ab}(2)	54.0^{a}(3)	69.0^{a}(1)	43.0^{a}(2)
CO304	100.0^{b}(5)	99.7^{b}(6)	1.00(5)	100.0^{b}(6)	100.0^{d}(7)	1.00(5)	87.0^{c}(7)	60.3^{a}(7)	80.5^{b}(6)	67.0^{c}(7)
CO305	100.0^{b}(5)	96.0^{b}(4)	0.96(2)	100.0^{b}(6)	99.7^{d}(6)	1.00(5)	84.0^{c}(6)	55.1^{a}(5)	75.0^{ab}(5)	58.5^{bc}(5)
CO306	99.7^{b}(4)	96.7^{b}(5)	0.97(3)	99.4^{b}(5)	88.5^{bc}(4)	0.89(3)	77.5^{abc}(3)	56.0^{a}(6)	71.0^{a}(4)	60.0^{c}(6)
CO308	96.6^{a}(1)	94.7^{b}(2)	0.98(4)	92.7^{a}(2)	84.4^{b}(3)	0.91(4)	81.5^{c}(5)	55.0^{a}(4)	71.0^{a}(4)	48.0^{ab}(4)
F2	99.0^{b}(3)	95.0^{b}(3)	0.96(2)	93.1^{a}(3)	71.7^{a}(2)	0.77(2)	79.5^{bc}(4)	53.0^{a}(2)	70.0^{a}(3)	47.0^{ab}(3)

^{z}Numbers in parentheses next to values indicates relative ranking within columns.

$^{a-d}$Indicates differences among the values within tests. Values with the same letter are not significantly different at the 5% level according to Fisher's LSD.

Table 2.3. Control (25°C), chilling (variable temperature), and the chill : control ratio of average time to germinate from the laboratory germination test and the average time to emerge from the 1992 or 1993 field trials in light (sandy loam), medium (sand and clay), and heavy (clay) textured soils

	Average time to germinate (days)			Average time to emerge (days)			
				1992	1993		
Line	Control	Chill	Chill/ Control	Medium soil	Light soil	Medium soil	Heavy soil
CO251	4.5^{ab}(2)[z]	21.5^{b}(3)	4.78(7)	17.7^{a}(1)	11.1^{a}(2)	11.7^{a}(1)	15.6^{a}(2)
CO266	4.3^{a}(1)	19.8^{a}(1)	4.60(5)	18.1^{a}(5)	10.9^{a}(1)	12.9^{b}(5)	16.4^{ab}(5)
CO304	4.8^{b}(4)	22.5^{c}(6)	4.69(6)	18.0^{a}(4)	11.4^{a}(4)	13.0^{b}(6)	16.1^{a}(4)
CO305	4.7^{bc}(3)	20.3^{b}(2)	4.32(2)	18.1^{a}(5)	11.3^{a}(3)	12.7^{b}(3)	15.9^{a}(3)
CO306	5.1^{c}(5)	22.2^{c}(4)	4.35(3)	17.9^{a}(3)	11.7^{ab}(5)	12.4^{b}(2)	15.1^{a}(1)
CO308	5.1^{c}(5)	22.4^{c}(5)	4.39(4)	17.8^{a}(2)	12.7^{bc}(6)	12.8^{b}(4)	15.9^{a}(3)
F2	5.8^{d}(6)	24.4^{d}(7)	4.21(1)	18.9^{b}(6)	13.0^{c}(7)	14.5^{c}(7)	17.5^{b}(6)

[z]Numbers in parentheses next to values indicates relative ranking within columns.

[a-d]Indicates differences among the values within tests. Values with the same letter are not significantly different at the 5% level according to Fisher's LSD.

CHAPTER THREE

A CHILLING RESPONSE TEST FOR EARLY GROWTH PHASE MAIZE

The following chapter is a reproduction of a paper published in the Agronomy Journal (*in press*) by D.M. Hodges, R.I. Hamilton, and C. Charest. The principal author (D.M. Hodges) accumulated and interpreted all data. The second author (Dr. R.I. Hamilton) produced and contributed seed for the inbred maize lines used in these experiments.

In order to complete the first objective of this thesis, identifying inbred lines of maize exhibiting differential sensitivity to chilling, the identification of relatively chilling sensitive and tolerant maize lines based on their physiological responses to chilling at the fourth leaf early growth stage was performed. The data from the early growth phase test was then compared to that of the germination stage (**Chapter Two**) to see if lines could be selected for differential sensitivity to chilling based on similar performance at both developmental stages. A new model laboratory screening technique for the determination of chilling responses in maize at the early growth phase was developed. Total, shoot, and root dry mass, and the shoot and root water content were the responses measured. Field trials were sown in Ottawa and the shoot dry mass recorded at the

fourth-leaf stage. The chill:control ratios of the shoot dry mass of seven of the eight lines from the laboratory were correlated with the averaged field shoot dry mass (r=0.780, P=0.032). The laboratory model for evaluating chilling differential chilling sensitivity was supported in that the lines indicated by shoot dry mass to be the most chilling resistant or the most sensitive in the laboratory responded similarly in the field.

Results from this early growth selection process, and from the germination screening test were, in general, not analogous in the ranking of chilling sensitivity of the eight inbred lines originally screened. However, these results did allow for the selection of one chilling sensitive (CO251) and three chilling tolerant (CO255, CO304, and CO308) maize inbreds based on similar rankings performed from physiological growth parameters assessed from both the germination and early growth phase developmental stages.

3.1 ABSTRACT

Results from a new laboratory screening technique for the determination of chilling responses in maize (Zea mays L.) at the early growth phase were compared to those obtained from the field. This laboratory technique consisted of growing plants hydroponically in styrofoam rafts floated on Hoagland's solution at a constant low temperature. Seedlings of eight inbred maize lines were pinned into the rafts and placed in controlled environment chambers at 11°C and 98% relative humidity for 28 d until the fourth-leaf stage. A control test used a constant 25°C for 7 d, at which point all lines were at the fourth-leaf growth stage. Total, shoot, and root dry mass, and the shoot and root water content were the responses measured. Comparisons between the chilling and the chilling:-control ratios showed that they were good indicators of chilling tolerance. Field trials of the same eight inbreds were sown in Ottawa (Lat. 45° 24'N, Long. 75° 43' W) in the early spring of 1992 and 1993 and the shoot dry mass recorded at the fourth-leaf stage. The chill:control ratios of the shoot dry mass of seven of the eight lines from the laboratory were correlated with the averaged field shoot dry mass (r=0.780, P=0.032). Thus, this laboratory screening procedure was successful in that the lines indicated by shoot dry mass to be the most chilling resistant or the most sensitive in the laboratory responded similarly in the field.

3.2 INTRODUCTION

Maize is one of the most economically important plants grown in North America and is often subjected to cool conditions after sowing which can disrupt development (Cutworth and Shaykewich, 1990; Hope et al., 1992).

Maize lines that respond well under chilling conditions in temperate regions would aid in optimizing yields. Predicting field performance at chilling temperatures based on laboratory screening tests would be useful, not only for predicting emergence potential (Hodges et al., 1994), but also for predicting early growth potential. In maize, low temperature reduces shoot (Miedema, 1982) and root (Pahlavanian and Silk, 1988) growth rates and water content (Miedema, 1983). Dry masses and water contents of shoot and roots might therefore be potentially good indicators of chilling tolerance.

Few previous methods of determining the degree of tolerance of maize lines to chilling have used inbred lines (Mock and McNeill, 1979); most studies have involved hybrids (Markowski, 1988; Hope et al., 1992). Inbred lines tend to be more susceptible to cold than hybrids (Janowiak and Markowski, 1987). Furthermore, the screening of inbreds, which can retain the traits and combining abilities for the subsequent generation and study of valuable hybrids, is an efficient way for breeders to select for cold tolerance.

Most methods of determining chilling responses have

measured germination and emergence responses (Menkir and Larter, 1985; Tekrony and Egli, 1991; Zemetra and Cuany, 1991; Hodges et al., 1994), rather than early growth. Maize seedlings are damaged more by constant rather than fluctuating low temperatures (Stamp, 1987). Many of the laboratory tests which determine differential chilling sensitivity of early growth phase inbred and hybrid lines of maize, as well as other species, place them in a potting medium and subject them to constant temperatures (AOSA, 1983; Janowiak and Markowski, 1987; Eagles, 1988; Markowski, 1988). Few of these tests have compared laboratory results with those obtained from the field. Moreover, it is difficult to separate roots from potting medium to measure dry mass and water content.

The objective of this study was to evaluate if an early growth phase laboratory screening technique recently developed and in current use by the Corn Breeding Program at the Plant Research Centre, Agriculture Canada, would reflect field chilling response conditions.

3.3 MATERIALS AND METHODS

3.3.1 Plant Material

The following inbred lines of maize were used: CO251 (dent), CO255 (flint), CO266 (flint), CO304 (flint/dent), CO305 (dent), CO306 (dent), CO308 (dent), and F2 (flint). The

lines were chosen on the basis of previous observations of their differences in ability to germinate and grow under chilling conditions (Hodges et al., 1994). The seeds for the 1992 and 1993 laboratory and field trials were produced at the Agriculture Canada, Ottawa, nurseries in 1990 and 1992, respectively. All lines tested were produced in the same nursery in the same year. The elite CO inbreds were developed by Agriculture Canada, and F2 originated from l'Institut National en Recherche Agronomique, France.

3.3.2 Laboratory Test

Two replicates each of 36 seeds per line were germinated on paper towels moistened with distilled water in dark germinators (Conviron Model I8, Conviron, Winnipeg, Manitoba, Canada) at 25°C and a constant relative humidity (98%+) for 5 d at which time coleoptile lengths ranged from 1-cm and 3.5-cm, depending upon genotype. Rectangular styrofoam rafts (50 cm length by 24 cm width by 2 cm depth) were prepared by drilling five rows of 10 holes (1 cm diam.) (Figure 3). Maize seedlings with a coleoptile length representative for that genotype were pinned into the bottom of the raft, with the coleoptile orientated vertically in the hole (Figure 3). The raft was placed in a tray of Hoagland's solution in a growth chamber (Conviron Model PGW-36, Conviron, Winnipeg, Manitoba, Canada) (16L:8D) at 11°C, 98%+ relative humidity. The plants

were grown for 28 d, by which time all lines were at the fourth-leaf stage of development. Of the 36 plants per raft, the inside 24 plants were harvested, the seed removed, the root and shoot separated and fresh weights obtained, and then oven-dried at 85°C to determine dry mass and percent moisture. The thermal environment in the germinator and growth chambers were characterized using corn heat units using the calculation: daily corn heat unit = $[3.33(T_{max}-10°C)-0.084(T_{max}-10°C)^2 + 1.8(T_{min}-4.4°C)]/2$ (Brown, 1969). This test was repeated twice in time in the same growth chamber for a total of 96 seedlings per line.

Another set of two replicates of 36 seeds per line was used as controls. They were treated in a similar manner except for being exposed to a 25°C growth chamber temperature for 7 d until the fourth-leaf stage instead of 28 d at 11°C. The genotype-specific range in coleoptile length before pinning was not considered critical since the pre-pinning germination procedure at 25°C for 5 d was performed for both control and chilling treatments. Thus any line-specific differences in coleoptile length at the pre-pinning stage would be nullified. This test was also repeated twice in time in the same growth chamber for a total of 96 seedlings per line.

3.3.3 Field Test

Eight replicates of 25 seeds per line pretreated with Thiram (tetramethylthiuram disufide) were field sown in Ottawa (Lat. 45° 24'N, Long. 75° 43'W) on a medium textured soil consisting of 20 cm of sand overlying clay (Allendale sandy loam [ASL m.p.d.]) on 27 Apr. 1992 and in this location as well as on a light sandy loam (Uplands sandy loam [Usl]) and a heavy clay (Rideau clay [Rc]) textured soils on 2 May 1993. Seeds were hand-planted at a depth of 5 cm in rows 2.5 m long and 0.4 m apart in a randomized complete block design. A data logger recorded soil temperatures at a 5-cm depth in five random sections of the field; 5-d averages of the soil temperatures were then calculated. However, for the 1993 field trial in the medium textured soil site, a malfunction in the data logger led to a loss of information. When the first of the eight lines reached the fourth-leaf stage (24 d for both years), all lines were harvested, the shoots removed, and the dry weight determined as above. As the root systems were difficult to remove from the soil, the dry mass of the root was not determined in the field experiments.

Corn heat units were also calculated for the site of the experiment using the maximum and minimum 5-d average shade temperatures recorded 1 m above-ground.

3.3.4 Statistical Analysis

Analyses of variance and least significant difference tests for the laboratory and field experiments were performed using PROC ANOVA and PROC GLM (SAS, 1989). The laboratory treatments were analyzed as a completely randomized design (line x temperature) and the field trials as a randomized complete block design (line x field site). Correlation coefficients (n=8) were calculated among the laboratory responses and between the shoot dry weights of the laboratory and the averaged shoot dry mass for the four field sites using MSTAT (1988). This average was performed after demonstration that there was no significant line x field site interaction.

3.4 RESULTS AND DISCUSSION

Results of the analyses of variance demonstrated high repeatability between the replicates within the lines for both the laboratory and the field experiments. Significant laboratory line x temperature effects were observed (data not shown). No significant line x field site interactions were demonstrated (data not shown).

The total and shoot dry mass chill:control ratios from the growth chamber studies were correlated across lines ($r=0.979$, $P<0.001$). Ranking of the lines indicated that F2 was the most chilling resistant, followed by CO255, CO308, CO306 and CO304, CO251, CO266, then CO305 (Table 3.1). The

root dry mass ratio suggested the ranking of the lines, from most chilling resistant to susceptible, to be F2 and CO255, CO306, CO308, CO251, CO304, and CO305 and CO266 (Table 3.1). The chill:control ratios, calculated from the chilling and control parameters obtained in the laboratory, are indicative of the lines' absolute responses to chilling. They correct for, as much as possible, the inherent genetic differences that each line would exhibit under the optimal (control) temperature of 25°C (Tables 3.1, 3.3). This ratio removes extraneous factors, such as genotypical variations in leaf length and width from the actual responses of the lines' exposure to chilling temperatures. The fact that some of the values are above unity does not indicate that growth is better under long-term chilling than at control temperatures. It is extremely difficult for the morphological stage of development to coincide exactly for all the lines between the 11°C chilling and 25°C control regimes at harvest. Slight developmental stage differences between the laboratory control (25°C) and chilling (11°C) treatments and the field trials are inevitable but can be disregarded since all the maize lines were harvested at the same time for each test and therefore simple, relative comparisons are being performed. Thus, these ratios produce a relative ranking of the eight lines based on their chilling tolerance. The relative ranking of chilling sensitivity derived from the chill:control dry mass ratios for total, shoot, and root dry mass responses approximated those

expressed by their respective absolute chilling values (Table 3.1). This implies that total, shoot, and root dry mass are good potential indicators of chilling sensitivity.

The thermal environments for the two laboratory regimes, as determined by the total cumulative corn heat units, were: 552.4 for the 5 d 25°C germination followed by 28 d 11°C chilling period, and 408.9 for the 7 d 25°C control regime (Table 3.2).

The chill:control ratios for the percent water content of both the laboratory grown shoot and roots indicated that CO266 was the most chilling resistant and both F2 and CO255 were the most sensitive lines (Table 3.3). Ranking of the ratios was similar to the absolute chilling values for both the shoot ($r=0.968$, $P<0.001$) and root ($r=0.965$, $P<0.001$) (Table 3.3). One manifestation of chilling sensitivity is internal water stress (Wilson, 1976). This stress can be partly due to increased water viscosity at lower temperatures (Stamp, 1984), phase shifts in lipid membranes leading to reduced root permeability (Miedema, 1983), and slow closure of stomata (Rhodes, 1987). Exposure to chilling of maize has been observed to result in an increase in stomatal apertures in a chilling susceptible variety (Mustardy et al., 1982) and has been observed to decrease the permeability of roots to water (Miedema, 1983).

Results of the shoot dry mass from the four field trials were significantly correlated with each other (data not

shown). The shoot dry mass consistently indicated that CO255 was the most chilling resistant line (Table 3.4). The 1992 medium and 1993 light soils showed no significant differences in indicating that CO266, F2, CO251, and CO304 were the most chilling sensitive (Table 3.4). In the 1993 medium soil, there was no significant difference in all the lines except CO255. The lines F2 and CO251 were the most susceptible to chilling in the heavy soil field trial (Table 3.4). The corn heat unit values recorded in the field trials were similar to those from the laboratory growth chambers (Table 3.2). The differences in the accumulated corn heat units between the soil types in the 1993 field tests can be attributed to such characteristics as soil compaction and texture. The light, coarse, sandy loam soil responds rapidly to ambient temperatures due to the relatively large air spaces between the soil particles (Brady, 1984). The fine-particled heavy clay soil is less porous, resulting in a comparably slower response to the surrounding thermal environment (Olson and Sander, 1988). The mixed sand over clay medium textured soil exhibits temperature response characteristics of both its compositional attributes (Brady, 1984). Unseasonably warm temperatures in the early part of the 1993 season resulted in a comparably larger accumulation of corn heat units in the light as to the heavy textured soils. Cooler temperatures near the end of the 1993 trials, however, led to a relative decrease in accumulated corn heat units in the light soil for

this period as compared to the heavy.

Dry mass results from the laboratory and field tests thus corresponded in the selection of the most chilling resistant lines, with F2 being a notable exception. In laboratory and field dry mass experiments, CO255 was the line most tolerant to chilling stress. These laboratory and field trials also corresponded in indicating CO266 was one of the most chilling sensitive lines. Conversely, CO266 was indicated by the percent water content to be the most chilling resistant and F2 and CO255 the most sensitive. CO305, suggested to be one of the more chilling susceptible by the laboratory dry mass parameters, was only medially sensitive by the percent water content and the field trials. The shoot dry mass from the field trials implied that F2 was one of the most sensitive to chilling, whereas it was indicated by the laboratory dry mass results to be one of the more resistant. The chill:control ratios of the shoot dry weights from the growth chamber test were not correlated with those from the four averaged field sites ($r=0.065$, $P=0.879$). When a scatter diagram was produced, F2 was observed to be an outlier (Figure 4). With F2 excluded, the growth chamber and field tests became significantly correlated ($r=0.780$, $P=0.032$). These differing results suggests that F2 responds differently than the other lines to one of these test growth conditions. Laboratory percent germination of the same genotypes was significantly correlated with field percentage emergence in selecting F2 as

fairly chilling susceptible (Hodges et al., 1994). This may imply that, through an unknown mechanism, the constant chilling at 11°C in the laboratory tests until the fourth-leaf stage somehow has a positive, perhaps acclimatory, effect on the growth and development of F2 as compared to the other lines. Furthermore, the ranking of the lines from chilling resistant to sensitive derived from the percent water content was essentially the opposite of that indicated by the laboratory and field dry mass responses. These results suggest that percent water content was not a distinguishing parameter for this set of genotypes.

This laboratory technique is an efficient means of rapidly examining large numbers of different lines in the same chamber, which can be useful for rapid screening of germplasm. The roots can be easily isolated from the shoots and examined. Methods such as the AOSA cold test method (AOSA, 1983), which rely on growing plants under chilling conditions in soil of known moisture content, make it difficult to fully remove and clean the root and rarely take into account the nature of the soil, a physically and biologically variable material. In our screening technique concerns of soil adhesion and incomplete removal of plant material from soil can be negated, and thus dry masses of shoots and roots, as well as percent water contents, are responses that can be quite easily measured. Plants can also be easily removed from the styrofoam rafts and transplanted into fields or greenhouses.

Most laboratory screening techniques used for predicting field performance of maize lines have subjected seeds to a constant, low temperature, and measured a variable related to the seeds' ability to germinate, such as percent emergence and/or percent germination (Barla-Szabo and Dolinka, 1988; Tekrony and Egli, 1991; Hope et al., 1992). Few attempts to develop a laboratory screening method for chilling responses in maize have studied correlation with responses in the field at the early growth phase. Young maize seedlings at this stage of development may be exposed to relatively low, frequently damaging, temperatures. Using our technique not only results in an accurate prediction of low temperature field performance of maize lines, but facilitates separation of plant parts for studies of both shoot and root fresh and dry masses and percent water contents.

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Table 3.1. Total, shoot, root dry mass (grams) and the chill:control ratio for control (25°C) and chilling (11°C) regimes from the laboratory growth chamber test.

Line	Laboratory dry mass								
	Total			Shoot			Root		
	Control	Chill	Chill/Control	Control	Chill	Chill/Control	Control	Chill	Chill/Control
	—— g ——			—— g ——			—— g ——		
C0251	0.157^{bc}	0.117^{c}	0.75	0.125^{ab}	0.099^{b}	0.79	0.032^{bc}	0.018^{cd}	0.77
C0255	0.150^{bc}	0.196^{b}	1.32	0.107^{bc}	0.144^{b}	1.29	0.040^{ab}	0.051^{a}	1.23
C0266	0.085^{d}	0.061^{d}	0.71	0.065^{c}	0.048^{c}	0.78	0.022^{c}	0.012^{d}	0.55
C0304	0.146^{c}	0.127^{c}	0.88	0.109^{bc}	0.103^{b}	0.93	0.036^{ab}	0.026^{bc}	0.71
C0305	0.189^{ab}	0.130^{c}	0.69	0.153^{ab}	0.105^{b}	0.70	0.037^{ab}	0.030^{bc}	0.68
C0306	0.173^{bc}	0.165^{bc}	0.95	0.142^{ab}	0.131^{b}	0.89	0.032^{bc}	0.034^{bc}	1.09
C0308	0.215^{a}	0.236^{a}	1.09	0.166^{a}	0.183^{a}	1.02	0.051^{a}	0.049^{a}	0.96
F2	0.107^{d}	0.163^{bc}	1.50	0.079^{c}	0.125^{b}	1.60	0.030^{bc}	0.036^{ab}	1.23

$^{a-d}$Indicates differences among the values within tests. Values within the same column with the same letter are not significantly different at the 5% level according to Fisher's LSD.

Table 3.2. Temperatures (°C) and total accumulated corn heat units (ΣCHU) recorded in the laboratory chilling and control tests and during the 1992 and 1993 field trials in either light (sandy loam), medium (sand and clay), or heavy (clay) textured soils.

	Test[†]	days	Temperature Max.	Min.	ΣCHU
		– no. –	°C		units
A.	Laboratory				
	(1) Chilling	5	25.0	25.0	170.3
		28	11.0	11.0	382.1
	(2) Control	12	25.0	25.0	408.9
B.	Field[‡]				
	(1) 1992 Medium soil	5	17.2	5.9	56.3
		5	12.2	5.2	78.9
		5	22.8	10.0	174.9
		5	24.2	12.3	285.4
		5	26.5	14.1	383.1
	(2) 1993 Light soil	5	19.7	10.3	85.9
		5	26.6	11.6	198.5
		5	21.5	10.3	291.6
		5	19.9	9.0	374.2
		5	18.7	11.8	463.5
	(3) 1993 Heavy soil	5	14.3	8.9	52.2
		5	14.3	10.0	113.2
		5	15.3	10.4	178.3
		5	17.2	9.3	249.6
		5	21.2	13.5	359.5

[†]Data on 1993 medium soil was lost due to data logger malfunction.

[‡]Field temperatures were recorded at a 5-cm depth and averaged over 5 d. The ΣCHU were calculated from 5-d averages of shade temperatures measured 1 m above-ground using the formula daily CHU = $[3.33(T_{max}-10°C)-0.084(T_{max}-10°C)^2 + 1.8(T_{min}-4.4°C)]/2$ (Brown, 1969).

Table 3.3. Shoot and root percent water content and the chill:control ratio for the control (25°C) and chilling (11°C) from the laboratory growth chamber test.

	Shoot			Root		
Line	Control	Chill	Chill/ Control	Control	Chill	Chill/ Control
	%			%		
CO251	89.07[b]	85.92[d]	0.96	94.31[ab]	89.09[c]	0.94
CO255	88.91[b]	83.35[fg]	0.94	93.74[bc]	86.32[f]	0.92
CO266	90.17[a]	89.52[a]	0.99	94.64[a]	92.73[a]	0.98
CO304	90.27[a]	88.49[b]	0.98	94.10[abc]	90.93[b]	0.97
CO305	90.40[a]	86.74[c]	0.96	93.91[bc]	89.20[c]	0.95
CO306	89.33[b]	83.82[f]	0.94	93.47[c]	88.04[d]	0.94
CO308	90.34[a]	84.97[e]	0.94	93.65[c]	87.53[de]	0.93
F2	90.50[a]	83.05[g]	0.92	94.70[a]	87.38[e]	0.92

[a-d]Indicates differences among the values within tests. Values within the same column with the same letter are not significantly different at the 5% level according to Fisher's LSD.

Table 3.4. Shoot dry mass (grams) from the 1992 and 1993 field trials on light (sandy loam), medium (sand and clay), and heavy (clay) textured soils.

	Field shoot dry mass			
	1992	1993		
Line	Medium soil	Light soil	Medium soil	Heavy soil
	g			
C0251	0.143^{bc}	0.070^{bc}	0.068^{b}	0.062^{cd}
C0255	0.243^{a}	0.111^{a}	0.111^{a}	0.101^{a}
C0266	0.128^{c}	0.075^{bc}	0.070^{b}	0.065^{bc}
C0304	0.132^{c}	0.081^{c}	0.073^{b}	0.081^{b}
C0305	0.181^{b}	0.086^{b}	0.081^{b}	0.080^{b}
C0306	0.182^{b}	0.088^{b}	0.079^{b}	0.082^{b}
C0308	0.164^{b}	0.084^{b}	0.081^{b}	0.081^{b}
F2	0.129^{c}	0.065^{c}	0.067^{b}	0.049^{d}

[a-d]Indicates differences among the values within tests. Values within the same columns with the same letter are not significantly different at the 5% level according to Fisher's LSD.

3.6 FIGURE LEGENDS

FIGURE 3 ILLUSTRATION OF PINNING PROCEDURE FOR LABORATORY EARLY GROWTH PHASE MAIZE

Styrofoam block (50 cm x 24 cm x 2.5 cm) containing 1 cm diameter holes drilled 5 cm apart (1). Maize seeds are pinned in bottom of holes with coleoptile and root orientated up and down, respectively (2). Styrofoam block is then floated in tray of Hoagland's solution (3). Maize plant at fourth-leaf stage (4). Removal of pin allows for easy extraction of plant from block.

FIGURE 4 SCATTER DIAGRAM OF FIELD SHOOT DRY MASS VERSUS LABORATORY CHILL:CONTROL RATIOS OF SHOOT DRY MASS FOR INBRED MAIZE

Scatter diagram of the chill:control ratios of the shoot dry masses of the growth chamber controlled environment and the shoot dry masses (grams) of the four averaged field sites. The regression line excludes F2.

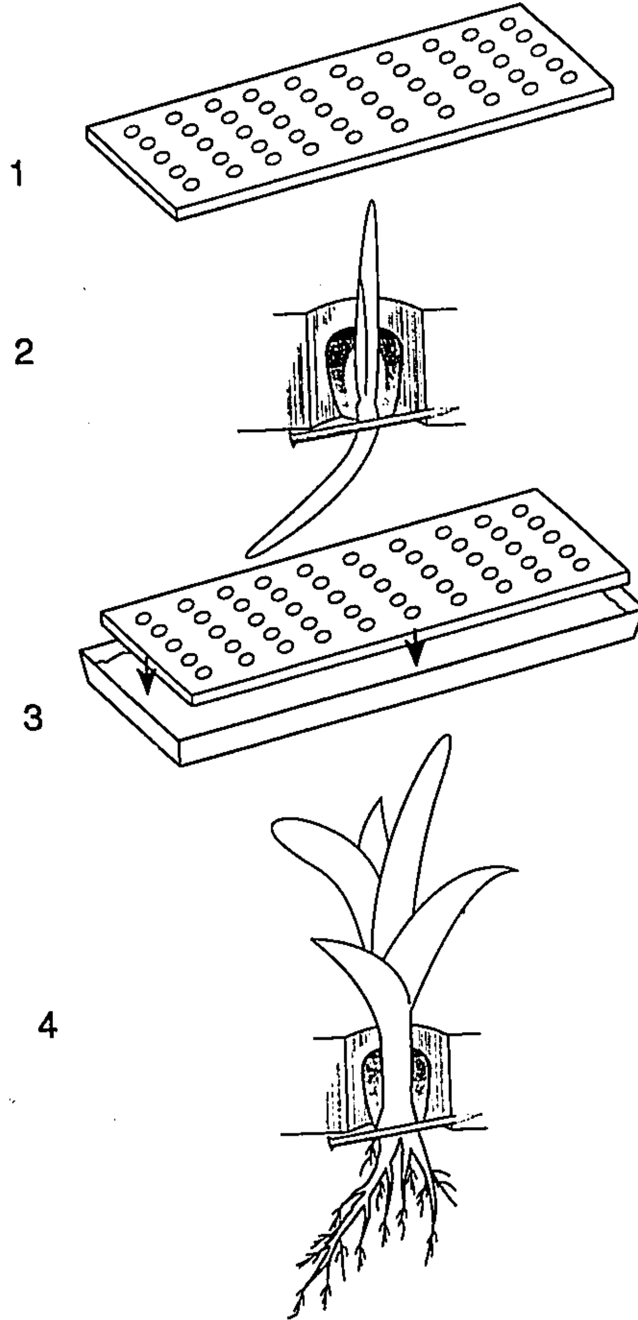


Figure 3

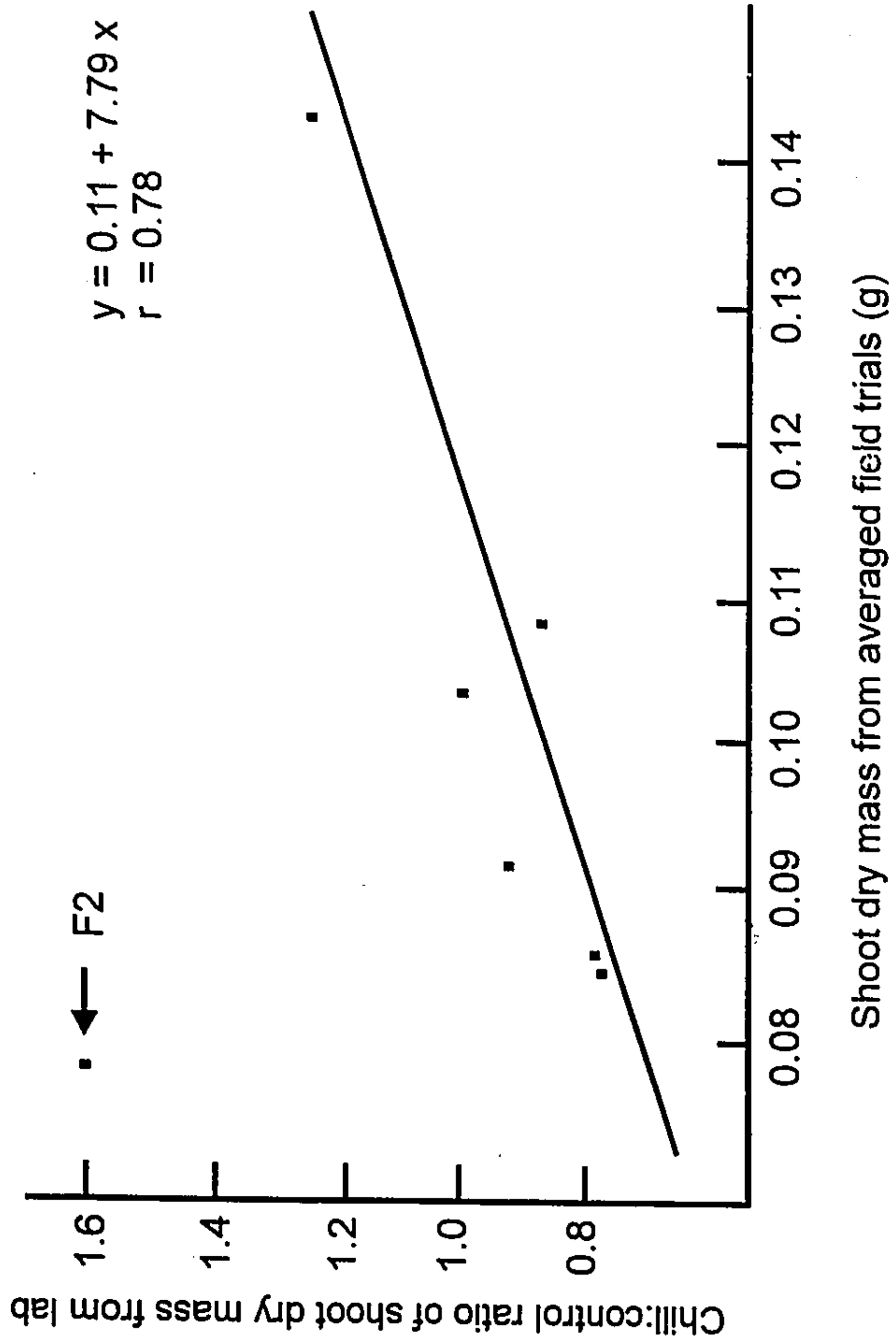


Figure 4

CHAPTER FOUR

ANTIOXIDANT ENZYME RESPONSES TO CHILLING STRESS IN DIFFERENTIALLY SENSITIVE INBRED MAIZE LINES

The following chapter is a reproduction of a paper submitted by D.M. Hodges, C.J. Andrews, D.A. Johnson, and R.I. Hamilton. The principal author (D.M. Hodges) accumulated and interpreted all data. The fourth author (Dr. R.I. Hamilton) produced and contributed seed for the inbred maize lines used in these experiments.

With the selection of relatively chilling sensitive (CO251) and tolerant (CO255, CO304 and CO308) inbred lines of maize by the germination (**Chapter Two**) and early growth (**Chapter Three**) screening tests, the second thesis objective of testing if the chilling sensitive CO251 had less antioxidant capacities, and experienced more chilling stress as measured by the metabolic indicators of chilling stress, than the tolerant lines when assessed at three developmental stages under both short-term and long-term chilling regimes could be met. **Chapter Four** describes the differences between the differentially chilling sensitive inbred lines in terms of activities of the antioxidant enzymes CAT, SOD, ASPX, GR, and MDHAR and the concentrations of the metabolic indicators total, reducing, and non-reducing sugars and starch.

Reduced percent of control activities of CAT, ASPX, and

MDHAR were demonstrated in the chilling sensitive CO251 as compared to the tolerant lines. However, enzyme results also suggested that the most chilling sensitive maize line becomes less so as the plants age from the first to the fifth leaf stages. These data support the model that the chilling sensitive line had less antioxidant enzymatic capacities than did the tolerant ones and that this may contribute to limited chilling tolerance, at least at the early stages of development, in maize. Changes in levels of sugar and starch supported the germination (**Chapter Two**) and early growth (**Chapter Three**) physiological parameters in selecting for differential chilling sensitivity, indicating a more rapid disruption of the carbohydrate utilization in comparison to photosynthetic rates in the chilling sensitive line under short-term chilling shocks and suggesting a greater degree of acclimation in the tolerant lines over longer periods of chilling.

The concentrations of the antioxidant compounds and the metabolic indicator chlorophyll of these inbred maize lines grown under the same conditions are presented in **Chapter Five** of this thesis.

4.1 ABSTRACT

Antioxidant enzyme activities were determined at three developmental stages (first, third and fifth leaf) of four inbred lines of maize (*Zea mays* L.) exhibiting differential sensitivity to chilling. Plants were exposed to a photoperiod of 16:8 L:D for one of three treatments: (a) control (25°C) for 2, 5, and 10 days, (b) control treatment plus an exposure to a short-term chilling shock of 11°C 1 d prior to harvesting, and (c) long-term (11°C constant) chilling exposure for 8, 19, and 29 days. Catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (ASPX; EC 1.11.1.11), superoxide dismutase (SOD; EC 1.15.1.1), glutathione reductase (GR; EC 1.6.4.2), and monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activities were assessed. Reducing and non-reducing sugars and starch were determined as general metabolic indicators of stress. Percent of control activities of ASPX and MDHAR were most reduced in the chilling sensitive relative to the tolerant lines for the first leaf stage under the short-term chilling treatment. Percent of control activities of CAT and GR became highest in this line compared to the chilling tolerant lines as the plant aged. Percent of control concentrations of sugars were highest in this sensitive line after 1-d chilling for the first and third leaf stages, and starch percent of control concentrations highest in this line for all three stages. Under long-term chilling exposure, percent of control activities of ASPX and MDHAR at the first

leaf stage and CAT at the first and third leaf stages were most depressed in the chilling sensitive line. There were no significant differences between percent of control activities of any of the antioxidant enzymes between chilling sensitive and tolerant lines at the fifth leaf stage. Sugar and starch percent of control concentrations were lowest in the chilling sensitive line under the prolonged chilling treatment. Antioxidant enzyme results suggest that the most chilling sensitive maize line became less sensitive to chilling-induced oxidative stress as the plants aged from the first to the fifth leaf stages. Reduced percent of control activities of CAT, ASPX, and MDHAR may contribute to limiting chilling tolerance at the early stages of development in maize. Changes in levels of sugar and starch indicate a more rapid disruption of the carbohydrate utilization in comparison to photosynthetic rates in the chilling sensitive line under short-term chilling shocks and suggests a greater degree of acclimation in the tolerant lines over longer periods of chilling.

Key words - Antioxidant enzymes, differential chilling sensitivity, maize, soluble carbohydrates, *Zea mays*

4.2 INTRODUCTION

Electrons from metabolic pathways can often react with O_2

during normal aerobic metabolism to produce toxic oxygen compounds such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH) (Halliwell and Gutteridge 1985). For plants, the presence of these O_2 byproducts is potentially lethal as they have been observed to play a significant role in peroxidation of essential phospholipids (Matsuo et al. 1990; Zheng and Yang 1991), and in protein (Prinsze et al. 1990; Casano and Trippi 1992) and nucleic acid degradation (Imlay and Lin 1988).

Plants, as well as other organisms, have evolved antioxidant systems in order to protect against these toxic species of oxygen. Superoxide dismutases (SOD; EC 1.15.1.1) are a group of enzymes which accelerate the conversion of O_2^- to H_2O_2 (Salin 1988). Both catalases (CAT; EC 1.11.1.6) and various forms of peroxidases are responsible for the removal of H_2O_2 from biological systems (Gossett et al. 1994). CAT is located mainly in the glyoxysomes, peroxisomes, cytosol, and mitochondria (CAT-3, maize) (Prasad et al. 1994*a*) but not in the chloroplasts. Thus, it is the peroxidases which are responsible for the removal of H_2O_2 from chloroplasts by way of the glutathione-ascorbate cycle. In this cycle, ascorbate is peroxidized to the monodehydroascorbate radical by ascorbate peroxidase (ASPX; EC 1.11.1.11) (Hossain et al. 1984). This radical can then either be reduced back to ascorbate by NADPH-requiring monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), or it can non-enzymatically disproportionate to

dehydroascorbate, which is then reduced back to ascorbate by glutathione by the action of dehydroascorbate reductase (DHAR; EC 1.8.5.1) (Jahnke et al. 1991). Subsequent glutathione reduction occurs by glutathione reductase (GR; EC 1.6.4.2) in another NADPH-requiring reaction (Halliwell and Foyer 1978). Ultimately, this cycle results in H_2O_2 being removed by photosynthetically generated NADPH (Salin 1988).

Chilling conditions may lead to an increase in the amounts of toxic oxygen compounds present in plant systems (Wise and Naylor 1987; Hodgson and Raison 1991; Tsang et al. 1991). Chilling of sensitive plants, such as maize (*Zea mays* L.), in light is more damaging to the photosynthetic apparatus than chilling in darkness (Krause 1988; Somersalo and Krause 1989). This is due to a process known as chilling-induced photoinhibition. This process is characterized by an overenergization of the photosystem reaction centres generally resulting from an inadequate supply of the natural electron acceptor $NADP^+$. The $NADP^+$ pool can be limited by a reduction in the CO_2 fixation rate (Cakmak and Marschner 1992; Elstner and Osswald 1994), restricted carbon metabolism being a symptom of low temperature stress (Schöner and Krause 1990). Molecular oxygen may then become reduced instead of $NADP^+$ at the Fe-S centres or by ferredoxin, producing O_2^- (Long 1983).

The duration of chilling stress can have important effects on chilling sensitive plants. Physiological dysfunctions resulting from molecular changes induced at low

temperature can be reversed if the tissue is returned to non-chilling temperatures before the dysfunction becomes persistent (Lyons et al., 1979). Prolonged chilling can lead to metabolic damage which may, however, be alleviated to an extent due to hardening or conditioning (Stamp, 1984). Comparisons between short- and long-term chilling treatments on antioxidant responses have not appeared in the literature. Similarly, very little work has been reported on chilling sensitivity in relation to the age of plants subjected to chilling stress.

Short-term (< 3 d) chilling conditions often result in the accumulation of sugars and starch in leaves (Mitchell and Madore 1992). Longer term chilling treatments in species such as tomato (*Lycopersicon esculentum* L.) (Brüggemann et al. 1992), alfalfa (*Medicago sativa* L.) (Castonguay et al. 1995), sunflower (*Helianthus annuus* L.), and rape (*Brassica napus* L.) (Paul et al. 1992), however, have resulted in starch formation being impaired. The accumulation of solutes such as proteins (Guy 1990) and sugars (Rhodes 1987; Olien and Clark 1993) has been linked to acclimation effects.

Several studies comparing different species have reported that chilling resistant species or strains have a greater antioxidant capacity than other species which are sensitive (Jahnke et al. 1991; Walker and McKersie 1993; Gossett et al. 1994). The following work assessed the differences in antioxidant enzyme capacity of inbred maize lines exhibiting

differential chilling sensitivity. To our knowledge, this is the first time antioxidant capacity comparisons have been made between chilling sensitive and resistant lines within a single species of higher plants. This would thus reduce the complexity of genetic differences which result from comparisons between species. Soluble carbohydrate levels were determined as general metabolic indicators of chilling stress. In order to study the effects of chilling duration on growth stage in young maize seedlings, plants were harvested at the first, third, and fifth leaf stages under both short- and long-term chilling treatments. Reduced capacities of CAT, ASPX, and MDHAR to detoxify toxic oxygen compounds may limit the ability to tolerate low temperature stress in the chilling sensitive relative to the tolerant lines. Changes in levels of sugar and starch indicate a more rapid disruption of the carbohydrate assimilate utilization in comparison to photosynthetic rates in the chilling sensitive line under short-term chilling shocks and suggests a greater degree of acclimation in the tolerant lines over longer periods of chilling.

Abbreviations:

ASPX, ascorbate peroxidase; CAT, catalase; GR, glutathione reductase; MDHAR, monodehydroascorbate reductase; SOD, superoxide dismutase.

4.3 MATERIALS AND METHODS

4.3.1 Plant material

The four inbred lines of maize (*Zea mays* L.) selected for study exhibited differential sensitivity to chilling based upon germination and emergence (Hodges et al. 1994) and early growth dry mass parameters (Hodges et al. 1995) (Table 4.1) in both the laboratory and the field. One line, CO251, was shown through all tests to be the most chilling sensitive of eight original inbred lines tested. The other three lines, CO255, CO304, and CO308, depending upon the growth parameter assayed, were demonstrated to be either the most, or one of the more, chilling tolerant tested. These elite inbreds were produced in the 1993 Agriculture Canada nursery, Ottawa, Canada.

Material was germinated for 5 days in the dark at 25°C, until the coleoptile was approximately 2-cm long, and then pinned into rectangular styrofoam rafts and floated on trays containing Hoaglands solution (Hodges et al. 1995) in a growth chamber (Conviron E-15). The photocycle was 16:8 L:D with a photon flux rate of 450-500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The tissue was harvested at the first, third and fifth leaf stages under three experimental growth conditions: 25°C constant (control), 11°C 1-d (short-term chilling shock), and 11°C constant (long-term chilling exposure). For the 25°C control experiments, the material reached these stages in 2, 5, and 10 days from pinning. For the 11°C 1-d treatment, the material was

harvested on the same schedule as the controls, except for the exposure to 11°C 1-d prior to harvesting. Leaves were harvested from the 11°C long-term chilling treatment at 8, 19, and 29 days from pinning for the first, third, and fifth leaf stages, respectively. The pooling of leaves from approximately 20, 12, and 10 plants for the first, third, and fifth leaf plants, respectively, was necessary for 1.0 gram of fresh tissue. For the third and fifth leaf stages the two most recently expanded leaves were harvested in a 50:50 ratio. The main midrib was removed from all leaf tissue.

4.3.2 Enzyme assays

Leaves (1.5 g fresh material) were extracted by grinding approximately 30 sec in a mortar and pestle with 0.5 g of inert sand, 0.25 g of polyvinylpolypyrrolidone, and 20 mL of chilled 50 m*M* phosphate buffer (pH 7.8). The extraction buffer for the ASPX assay contained 0.2 *m*M ascorbate. Extracts were then centrifuged (Dupont Instruments Sorvall RC-5B) at 12000 g for 20 min at 2°C. For CAT, ASPX, and GR assays, the extracts were desalted by passage through a Sephadex G-25 column pre-equilibrated with 50 m*M* phosphate buffer (pH 7.8).

All enzymes were assessed spectrophotometrically on a Milton Roy Spectronic 1001 Plus equipped with a Haake F3 digital water bath for temperature control. The three cuvette assay temperatures (25°C, 15°C, and 10°C) had been previously

calibrated with a YSI 44TE tele-thermometer.

CAT (EC 1.11.1.6) activity was assayed in a reaction mixture containing 0.85 mL of 50 m*M* phosphate buffer (pH 7.8), 0.5 mL of 30 m*M* H_2O_2, and 0.15 mL of the extract in a method following Aebi (1983). Activity was determined by following the decomposition of H_2O_2 at 240 nm.

ASPX (EC 1.11.1.11) activity was determined in a method modified from Gossett et al. (1994). The reaction mixture contained 0.5 mL of 100 m*M* phosphate buffer (pH 7.8), 0.1 mL of 1.0 m*M* EDTA, 0.1 mL of 1.5 m*M* ascorbate, 0.1 mL of 1.0 m*M* H_2O_2, and 0.2 mL of extract. Activity was determined by following the ascorbate-dependent decomposition of H_2O_2 at 265 nm.

SOD (EC 1.15.1.1) activity was assayed by following the reduction of cytochrome *c* at 550 nm in a procedure modified from McCord and Fridovich (1969). The reaction mixture contained 0.95 mL of solution A consisting of 25 mL of 50 m*M* phosphate buffer (pH 7.8), 0.5 m*M* xanthine in 0.001 *N* NaOH, 0.08 m*M* cytochrome *c*, and 0.2 mL of solution B consisiting of 0.06 mL of 0.2 U/mL xanthine oxidase (units as defined by Sigma Chemical Co.) in 5.0 mL of 0.1 m*M* EDTA disodium salt alone or with the extract. Activity was determined by monitoring at 550 nm the inhibition of the reduction rate of cytochrome *c* between mixtures with and without the enzyme extract. Unlike the classical procedure whereby the amount of enzyme required to produce 50 % inhibition of the reduction of

cytochrome *c* is determined, this method simply subtracts the absorbance of the reaction containing the enzyme from that which does not. Although similar data were generated in comparisons between these two methods, this method proved to be more replicable (data not shown).

GR (EC 1.6.4.2) activity was determined following Goldberg and Spooner (1983). The reaction mixture contained 0.8 mL of 100 m*M* phosphate buffer (pH 7.8), 0.1 mL of 100 m*M* oxidized glutathione, 0.1 mL of 15 m*M* EDTA disodium salt, 0.02 mL of 10 m*M* NADPH in 1% (w/v) $NaHCO_3$, and 0.3mL of extract. Activity was determined by following the oxidation of NADPH at 340 nm.

MDHAR (EC 1.6.5.4) was assayed according to the method following Hossain et al. (1984). Each reaction mixture contained 0.5 mL of 50 m*M* tris-HCL buffer (pH 7.8), 0.05 mL of 0.125 % Triton-X, 0.1 mL of 0.2 m*M* NADH, 0.1 mL of 2.5 m*M* ascorbate, 0.05 mL of 0.5 mg 12.5 U/mL ascorbate oxidase (units as defined by Sigma Chemical Co.) in 10.0 mL H_2O, and 0.2 mL of extract. The reaction was followed by measuring the decrease in absorbance at 340 nm due to NADH oxidation.

4.3.3 Protein determinations

Protein concentration was determined in extracts used in analysis of the enzymes spectrophotometrically at 595 nm using the Bio-Rad Protein Assay Dye Reagent Concentrate (catalogue

number 500-0006) in a method based on Bradford (1976). Bovine gamma-globulin (0.25-1.4 mg/mL) was used as a standard reference.

4.3.4 Sugar assay

Reducing and non-reducing sugars were extracted by grinding 0.5 g of frozen tissue with 0.25 g of inert sand and 5 mL of deionized water in a mortar and pestle. The extract was transferred with a further 20 mL deionized water into an Erlenmeyer flask and then boiled for 20 min. After cooling, the extracts were filtered (Whatman #4) and the final volume adjusted to 25 mL. Reducing sugars were determined following Nelson (1944). Total sugars were determined with invertase (ß-D-fructofuransoside fructohydrolase EC 3.2.1.36) (10 units$\cdot$ml^{-1}) in a method following Westhafer et al. (1982). Non-reducing sugars were determined by subtracting total sugars from reducing sugars. Glucose (0.045-0.18 mg/mL) was used as the standard reference.

4.3.5 Starch assay

Starch was extracted by grinding 0.75 - 1.0 g frozen tissue with 2.5 mL of 8 *M* HCl and 0.2 g of inert sand in a mortar and pestle. The extract was transferred with 20 mL DMSO into an Erlenmeyer flask and then incubated at 60°C in a controlled

water bath (Thelco 83) for 1 h. After rapid cooling, the addition of 2.5 mL of 8 *M* NaOH was followed by diluting to 50 mL with 0.112 *M* citrate buffer (pH 4.0). For each test, 0.1 mL of the extract was used. Starch concentration was determined spectrophotometrically at 340 nm using a coupled enzyme system (Boehringer-Mannheim test combination, catalogue number 207 748). In this system, starch becomes hydrolysed to D-glucose by amyloglucosidase (EC 3.2.1.3). The D-glucose formed is then converted by hexokinase (EC 2.7.1.1) to glucose-6-phosphate. Glucose-6-phosphate then becomes oxidized by $NADP^+$ and glucose -6-phosphate dehydrogenase (EC 1.1.1.49) to form gluconate-6-phosphate. The resulting formation of NADPH is monitored at 340 nm.

4.3.6 Statistical Analysis

All enzyme and metabolite assays were based on at least two readings from four independent replicates. The effects of inbred line, temperature regime, and growth stage on enzymatic activities and carbohydrate concentrations were analyzed by a three factor completely randomized ANOVA. Enzyme and carbohydrate data from control treatments are given as means ± SE. Data from both short-term and long-term chilling treatments are expressed as percent of control and are shown as means ± SE. Significance of enzymatic Q_{10}'s and the percent germination (Hodges et al., 1994) and total dry mass (Hodges

et al., 1995) data (Table 4.1) were calculated using Duncan's Multiple Range Test ($P \leq 0.05$).

4.4 RESULTS

4.4.1 Chilling effects on enzymatic activities

Profiles of the enzymatic activities for control (25°C) material at various developmental stages for the four inbred maize lines are presented in Table 4.2. Basal control activities of CAT, ASPX, and MDHAR were generally highest in all three leaf stages in the chilling sensitive CO251 maize line when assayed at 25°C (Table 4.2). Unlike the other antioxidant enzymes, control levels of ASPX and MDHAR decreased or plateaued at a lower level as the plants aged. No other distinguishing enzymatic characteristics could be determined between the chilling sensitive and chilling tolerant lines at control temperatures. Standard errors of four independent samples at control temperatures are presented in Table 4.2. Similar values from plants at chilling temperatures were proportionately the same or less (data not shown).

All enzyme activities after short- or long-term chilling were expressed as percentage of the control values shown in Table 4.2. Thus, after chilling for 1 d, percents of control of activities of ASPX and MDHAR were significantly lower in

the chilling sensitive CO251 line relative to the tolerant lines (Table 4.3, Figures 5-7). There were no substantial differences in SOD between the chilling sensitive and tolerant lines (Table 4.3, Figures 5-7). There was an increase in CAT and GR percent of control activities in the chilling sensitive line as the plants aged, ultimately expressing activities significantly greater than those of the chilling tolerant lines (Table 4.3, Figures 5-7). There were no significant differences between the chilling sensitive and tolerant lines in terms of percent of control activities of SOD, ASPX, and MDHAR at the fifth leaf stage (Figure 7).

After chilling for 8 d, percent of control activities of MDHAR in the chilling sensitive CO251 line were significantly lower than those of the chilling tolerant lines (Table 4.3, Figure 5). This difference between the lines was lost as chilling exposure increased (Figures 5-7). Percent of control activity of this enzyme was generally higher for the long-term as compared to the short-term chilling treatments. Percent of control activity of ASPX was significantly more reduced in the chilling sensitive line relative to the tolerant lines for the first leaf stage under long-term chilling. Percent of control activity of CAT was significantly lower in this sensitive line for the first and third leaf developmental stages (Table 4.3, Figures 5,6). There was no distinguishable difference in percent of control activities of SOD between chilling sensitive and tolerant lines under long-term chilling

(Table 4.3, Figures 5-7). Percent of control activities of GR for the chilling sensitive line were no different from those of the chilling tolerant lines at the first leaf stage, but increased to the highest, although not significantly, by the fifth leaf stage (Figures 5-7). Overall values of SOD were observed to decrease during chilling as the plants aged while those of GR were constant and well in excess of control values.

There were no statistically significant differences in the Q_{10}'s (25°C/15°C) between the differentially chilling sensitive lines (data not shown).

4.4.2 Chilling effects on carbohydrates

During growth at control temperatures, total sugars increased from the first to the fifth leaf stage in the chilling sensitive line, but decreased in the tolerant lines (Table 4.4). Levels of non-reducing sugars were low at the first leaf stage in all lines, but increased through the growth stages. At control temperatures, non-reducing sugars generally remained substantially lower than reducing sugars. Similar results were obtained with absolute values of reducing and non-reducing sugars under both short- and long-term chilling treatments (data not shown). After 1 d chilling, total sugars at all growth stages increased markedly over the controls in the chilling susceptible line, whereas the

increase was substantially less in the tolerant lines (Figure 8). These increases resulted from changes in both reducing and non-reducing components, but far significantly more non-reducing sugars were accumulated relative to the controls in the chilling susceptible CO251 (Table 4.3, Figure 8). After 8 days of chilling (the first leaf stage), reducing and non-reducing sugars were again highest relative to the controls in the chilling sensitive line CO251 (Table 4.3, Figure 8). However, with continued chilling this trend was reversed, and by the fifth leaf stage, total sugars were significantly highest for the chilling tolerant lines.

Starch concentrations increased approximately 15-fold from first to fifth leaf stage when grown under control conditions, but were generally lower in the chilling sensitive CO251 line (Table 4.5). After 1 d chilling, starch content significantly increased relative to the controls at all growth stages and more so in the chilling sensitive CO251 (Tables 4.3,4.5). Similar to the sugars, particularly the non-reducing sugars, this trend was reversed during long-term chilling exposure whereby, although starch contents decreased relative to controls at all growth stages, the chilling tolerant lines maintained the significantly highest amounts (Tables 4.3,4.5).

4.5 DISCUSSION

The toxic oxygen compound H_2O_2 can inhibit key enzymes of the Calvin cycle (Badger 1985, Elstner and Osswald 1994) and can interact with O_2^- in the Haber-Weiss reaction to form the highly unspecific and reactive •OH radical (Salin 1988). As H_2O_2 can readily diffuse across membranes (Bowler et al. 1992), higher levels of antioxidant activity would allow for a greater degree of scavenging and thus lessen its detrimental effects.

During long-term chilling exposure up to the third leaf stage, percent of control activity of CAT, which detoxifies H_2O_2, exhibits less relative activity in the line more susceptible to chilling injury. When chill-shocked for 1 d, however, percent of control activity of CAT in the chilling sensitive line, although no different from the tolerant lines, increases as the plant ages until it is the highest when assayed at the fifth leaf stage. It has been shown that H_2O_2 levels are elevated in chilled maize seedlings and that up-regulation of CAT is an important means of maintaining low H_2O_2 levels in dark-grown chilling acclimated tissue (Prasad et al. 1994*a*). Long-term acclimation has been observed to lead to a decrease in percent of control activity of CAT in spinach (*Spinacia oleracea* L.) (Schöner and Krause 1990) and various other plant species (MacRae and Ferguson 1985). Feierabend et al. (1992) found no changes in CAT levels of chill-shocked, dark-grown maize. Only after exposure to light was catalase

activity rapidly decreased, presumably due to photoinactivation. This has been observed in both short-term (Feirabend et al. 1992; Mishra et al. 1993) and long-term (Schöner and Krause 1990) chilling periods. Our results for the short-term chilling treatment indicated that CAT activity generally did not fall below that of the non-chilled control. It is possible that, over long-term exposure to chilling, the most chilling sensitive line CO251 would be less able than the more tolerant lines to replace photoinactivated CAT. When exposed to the short-term chilling treatments, CAT activity may not yet be fully affected by photoinactivation.

The lower percent of control activity of MDHAR in the chilling sensitive line for the first leaf stage under both short-term and long-term chilling exposure suggests that the activity of this enzyme is rapidly decreased due to cold at the earlier growth stages, but then through either resynthesis or production of more tolerant isozymes, eventually it recovers as the plant ages. In comparing a relative chilling sensitive *Zea mays* L. cultivar with the more tolerant tillers of *Zea diploperennis* L., Jahnke et al. (1991) found a transient higher activity, compared to control, in the 1-5 days chill stressed *Z. mays* cultivar. However, Walker and McKersie (1993) found no increase in the activity of MDHAR of chilling sensitive *Lycopersicon esculentum* L. and the more tolerant *L. hirstum* L., or differences in activities between the two species, when exposed to cold for 3-d. Thus there is

conflicting evidence on the activity of this enzyme.

Activity of ASPX has been observed to increase when chilled in leaves of such plants as spinach (Schöner and Krause 1990) and wheat (*Triticum aestivum* L.) (Mishra et al. (1994). The lower percent of control activity of the H_2O_2-detoxifying ASPX in the chilling sensitive maize line for both short- and long-term chilling treatments at the first leaf stage implies that, as with MDHAR, the activity of this enzyme is more inhibited in this line than in the tolerant for the earlier growth stages.

It is noteworthy that whether under short- or long-term chilling, either CAT and/or MDHAR and ASPX, all playing an important role in H_2O_2 removal, have percent of control activities which are more depressed in the chilling sensitive line at the first leaf stage. This strongly suggests that the sensitivity of CO251 to chilling-induced oxidative stress is greater at this stage. Reduced ascorbate cycling due to depressed ASPX and MDHAR activities would not only lead to reduced H_2O_2 detoxification, but also to reduced scavenging of O_2^-, $\cdot OH$, and $^1O_2$. As the plant aged under the long-term chilling treatment, increasing percent of control activities of CAT, ASPX, GR, and MDHAR in the chilling sensitive line relative to the tolerant plants may compensate for the initially lower H_2O_2-detoxifying and ascorbate-regenerating capacity of this line at the earlier stages of development. These H_2O_2 molecules can be otherwise removed through direct

contact with CAT, with ascorbate in a reaction catalyzed by ASPX, and through reoxidation by GR of glutathione (Foyer et al. 1994*b*). Glutathione can then be reduced by either the direct removal of H_2O_2 in reactions catalyzed by non-specific peroxidases (Salin 1988; Foyer et al. 1991) or by non-enzymatically removing other potentially damaging active oxygen species such as O_2^- and ·OH (Larson 1988). MDHAR serves to regenerate ascorbate (Hossain et al. 1984). Reduced ascorbate can not only remove H_2O_2 in a reaction catalyzed by ASPX, but can scavenge $^1O_2$, O_2^-, and ·OH by direct contact (Walker and McKersie 1993). Oxidative stress has been shown to induce or enhance levels of SOD, GR, and ASPX (Foyer et al. 1994*b*), suggesting that chilling sensitive line has accumulated greater levels of toxic oxygen compounds due to its initially lower activities of H_2O_2-scavenging and ascorbate-regenerating enzymes.

Growth stage is apparently an important factor in maize chilling sensitivity. Certain antioxidant enzyme capacities are lowest at the first leaf stage in the most chilling sensitive line in comparison to the tolerant lines for both the short-term and long-term chilling treatments. Both short-term and long-term chilling results suggest that as the chilling sensitive plants age from the first to the fifth leaf stage, they are more able to maintain or rapidly synthesize these enzymes in response to chilling-induced oxidative stress. Differences, however, do exist between results from

the short-term and long-term chilling treatments. Under short-term chilling, percent of control activities of ASPX and MDHAR are lowest in the first leaf stage of the chilling sensitive line relative to the tolerant. Under long-term chilling, percent of control activities of CAT, ASPX, and MDHAR are lowest in this line at this stage. At the fifth leaf stage, short-term chilling results indicate that percent of control activities of CAT and GR are significantly higher in the most chilling sensitive line, whereas under long-term chilling no significant differences exist for the enzymes between lines. This would suggest that long-term chilling is more detrimental to maize than short-term chilling, regardless of the developmental stage.

Soluble sugars generally accumulate in plants such as winter rye (*Secale cereale* L.) (Koster and Lynch 1992), alfalfa (*Medicago sativa* L.) (Castonguay et al. 1995) and wheat (Perras and Sarhan 1984) under chilling conditions leading to acclimation. Chilling of 1 to 8 days in maize led to accumulations of sugars in all lines with the greatest increase in the chilling sensitive CO251. However, further chilling led to an accumulation of total sugars in the chilling tolerant lines only. Although starch levels increased in response to short-term chilling, they were found to fall below control levels upon long term chilling from 8 - 29 days. Although Farrar (1988) states that starch commonly accumulates at low temperatures, this is not the case with

maize chilled for these periods. Our results are consistent with the relatively long-term results of Castonguay et al. (1995) with alfalfa, Brüggemann et al. (1992) with tomato (*L. esculentum* L.) and sunflower (*Helianthus annuus* L.) and Paul et al. (1992) with rape (*Brassica napus* L.). However, our results indicating that starch accumulated as a response to short-term treatments agree with those of Mitchell and Madore (1992) with muskmelon (*Cucumis melo* L.) .

The chilling sensitive line CO251 demonstrated the highest and lowest accumulation of sugars and starch in the short- and long-term chilling treatments, respectively, relative to the tolerant lines. Carbohydrates commonly increase in source leaves under chilling conditions mainly as a result of the rate of assimilate utilization being depressed more than the rate of photosynthesis (Farrar 1988). Then CO251, being more chilling sensitive than the other lines, would experience a greater decrease in its rate of assimilate utilization in the short term, resulting in greater accumulation of sugars and starch. In the long term, however, the more chilling tolerant lines presumably become acclimated to a greater degree, though this may not be apparent from the antioxidant results. Since one of the manifestations of acclimation is the increase in solutes such as sugars (Perras and Sarhan 1984; Koster and Lynch 1992), this would account for the greater accumulation in the chilling tolerant maize lines. Pollock and Lloyd (1987) demonstrated that the enzymes

involved in starch formation were much more sensitive to low temperatures than those involved in sucrose synthesis. This may explain starch levels being much more reduced in relation to controls than were total sugars under the long-term chilling treatments.

Carbohydrate levels, as general metabolic indicators of chilling stress, correspond with previously reported growth parameters such as percent germination and percent emergence (Hodges et al. 1994) and early growth (fifth leaf stage) dry mass (Hodges et al. 1995) in demonstrating the ranking in terms of differential chilling sensitivity of these lines.

In conclusion, reduced capacities of CAT, ASPX, and MDHAR in the chilling sensitive line to detoxify toxic oxygen compounds may limit its ability to cope with low temperature stress at early stages of development. As the maize seedlings developed, the increasing percent of control activities of CAT, ASPX, GR, and MDHAR in the most chilling sensitive line relative to the tolerant lines suggest that this line becomes less sensitive to chilling-induced oxidative stress as the plants age. Antioxidant compounds may also play a critical role in conferring chilling tolerance. Preliminary results for relative percent of control concentrations of glutathione and ascorbate show that, although initially there were no significant differences between the chilling sensitive and tolerant lines under short-term chilling, they increase to be significantly highest in the chilling sensitive line as the

chilling exposure lengthens (manuscript submitted). Accompanying changes in levels of sugar and starch indicate a more rapid disruption of the carbohydrate assimilate utilization in comparison to photosynthetic rates in the chilling sensitive line under short-term chilling shocks and suggests a greater degree of acclimation in the tolerant lines over longer periods of chilling. These enzyme and metabolic indicator data point to the differing abilities of these lines in responding to, and tolerating, chilling stress.

4.6 ACKNOWLEDGEMENTS

This work was funded in part by a grant from Natural Sciences and Engineering Council of Canada to D.A.J.

Table 4.1. Control and chill:control ratios of the percentages germination and of the fourth-leaf stage total dry masses of the four inbred maize lines. Germination and dry mass data are from Hodges et al. (1994) and Hodges et al. (1995), respectively. For controls, means followed by the same letter are not significantly different at the 0.05 level according to Fisher's LSD.

	Percentage germination		Total dry mass	
Line	Control	Chill:control	Control	Chill:control
CO251	94.8[ab]	0.69	0.157[b]	0.75
CO255	95.2[ab]	0.99	0.150[b]	1.32
CO304	100.0[b]	1.00	0.146[b]	0.88
CO308	92.7[a]	0.91	0.215[a]	1.09

Table 4.2. CAT (μmol H_2O_2 decomposed/min/mg prot.), ASPX (mmol H_2O_2 decomposed/min/mg prot.), SOD (μmol cytochrome *c* conserved/min/mg prot.), GR (μmol NADPH oxidized/min/mg prot.), and MDHAR (μmol NADH oxidized/min/mg prot.) activities assayed at 25°C in three leaf stages of the chilling sensitive CO251 and chilling resistant CO255, CO304, and CO308 inbred lines of maize grown under 25°C control treatment.

	1st leaf stage	3rd leaf stage	5th leaf stage
CAT			
CO251	216.5± 32.4	217.2± 12.3	138.9± 11.6
CO255	120.9± 9.3	166.9± 5.9	44.8± 11.2
CO304	83.5± 13.2	206.1± 27.2	108.0± 12.1
CO308	86.7± 13.2	172.8± 19.7	83.2± 10.8
ASPX			
CO251	3.2± 0.1	1.9± 0.3	2.6± 0.1
CO255	2.3± 0.1	1.5± 0.2	2.6± 0.0
CO304	1.6± 0.0	1.3± 0.3	1.2± 0.1
CO308	1.8± 0.1	1.6± 0.1	1.4± 0.1
SOD			
CO251	182.0± 9.6	288.7± 46.3	91.9± 8.6
CO255	140.4± 20.8	285.4± 20.1	73.9± 7.1
CO304	144.8± 16.4	296.5± 24.1	70.3± 5.1
CO308	166.8± 22.4	245.3± 14.0	83.3± 6.8
GR			
CO251	42.8± 1.4	28.3± 2.5	13.8± 0.5
CO255	44.8± 1.5	38.7± 1.8	10.4± 1.5
CO304	31.2± 3.5	35.0± 7.6	8.2± 0.7
CO308	59.6± 3.0	59.1± 10.9	13.1± 0.8
MDHAR			
CO251	75.1± 4.6	19.1± 3.6	19.1± 1.1
CO255	53.9± 2.1	12.1± 1.0	11.9± 0.6
CO304	49.6± 6.0	12.5± 0.4	6.9± 0.9
CO308	39.5± 1.1	19.1± 2.9	17.3± 2.9

Table 4.3 Levels of significance for a three factor ANOVA for activities of CAT, ASPX, SOD, GR, and MDHAR assayed at 25°C and concentrations of total sugars (TS), reducing sugars (RS), non-reducing sugars (NRS), and starch of the four inbred maize lines; *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, N.S. not significant.

Source	CAT	ASPX	SOD	GR	MDHAR	TS	RS	NRS	starch
Line	***	***	***	***	***	***	***	***	***
Temperature Regime (TR)	***	***	***	***	***	***	***	***	***
Growth Stage (GS)	***	***	***	***	***	***	***	***	***
Line x TR	***	***	*	N.S.	***	***	***	***	***
Line x GS	N.S.	***	N.S.	*	***	***	***	***	**
TR x GS	N.S.	***	***	***	***	**	***	**	***
Line x TR x GS	***	***	N.S.	*	***	***	***	***	**

Table 4.4. Reducing, non-reducing, and total soluble sugars (mg/g/fr.wt.) extracted from the three leaf stages of the chilling sensitive CO251 and chilling tolerant CO255, CO304, and CO308 inbred lines of maize grown under the 25°C control treatment.

Line	1st leaf stage			3rd leaf stage			5th leaf stage		
	Reducing sugars	Non-reducing sugars	Total sugars	Reducing sugars	Non-reducing sugars	Total sugars	Reducing sugars	Non-reducing sugars	Total sugars
CO251	1.17 ± 0.14	0.06 ± 0.01	1.23 ± 0.14	1.04 ± 0.20	0.13 ± 0.06	1.17 ± 0.19	1.35 ± 0.16	0.77 ± 0.11	2.12 ± 0.17
CO255	1.75 ± 0.04	0.26 ± 0.19	1.75 ± 0.08	0.38 ± 0.03	0.78 ± 0.11	1.16 ± 0.10	0.83 ± 0.04	0.29 ± 0.07	1.16 ± 0.08
CO304	1.69 ± 0.23	0.36 ± 0.15	2.05 ± 0.15	1.25 ± 0.30	0.22 ± 0.02	1.18 ± 0.35	1.08 ± 0.36	0.36 ± 0.05	0.98 ± 0.19
CO308	1.70 ± 0.16	0.09 ± 0.03	1.79 ± 0.13	0.92 ± 0.08	0.21 ± 0.03	1.12 ± 0.07	1.07 ± 0.04	0.42 ± 0.12	1.18 ± 0.09

Table 4.5. Starch concentrations (mg/g/fr.wt.) of the three leaf stages of the chilling sensitive CO251 and chilling resistant CO255, CO304, and CO308 inbred lines of maize grown under 25°C (control), 11°C short-term chilling, and 11°C long-term chilling treatments. Figures in parentheses indicates the changes in starch concentration expressed as percent of control.

Line	Leaf stage	Control (25°C)	11°C short-term	11°C long-term
CO251	1	0.66 ± 0.26	2.72 ± 0.09 (415.7)	0.04 ± 0.01 (5.6)
	3	6.47 ± 0.97	30.64 ± 2.51 (473.6)	0.64 ± 0.18 (9.9)
	5	15.56 ± 6.50	34.45 ± 1.99 (221.4)	0.39 ± 0.14 (2.5)
CO255	1	2.25 ± 0.28	3.84 ± 1.37 (170.4)	0.89 ± 0.09 (39.4)
	3	7.60 ± 3.02	16.46 ± 3.13 (216.6)	1.16 ± 0.38 (15.2)
	5	20.83 ± 2.43	37.05 ± 4.63 (177.9)	6.74 ± 1.71 (32.3)
CO304	1	2.91 ± 0.30	4.80 ± 1.06 (164.9)	1.46 ± 0.79 (50.0)
	3	5.80 ± 1.00	19.89 ± 4.27 (343.1)	1.87 ± 0.59 (32.3)
	5	26.83 ± 0.94	33.25 ± 2.28 (123.9)	1.97 ± 0.62 (7.3)
CO308	1	2.11 ± 0.09	6.14 ± 1.91 (290.6)	0.26 ± 0.07 (12.2)
	3	7.83 ± 0.46	16.31 ± 3.02 (208.4)	6.23 ± 1.41 (79.6)
	5	30.06 ± 2.99	30.29 ± 3.05 (100.8)	2.23 ± 0.86 (7.4)

4.7 FIGURE LEGENDS

FIGURE 5 PERCENT OF CONTROL (CHILL:CONTROL) OF ANTIOXIDANT ENZYME ACTIVITIES FOR THE FIRST LEAF STAGE OF INBRED MAIZE

Percents of control of CAT, ASPX, SOD, GR, and MDHAR of the first leaf developmental stage in the chilling sensitive CO251 and chilling tolerant CO255, CO304, and CO308 inbred maize lines for 11°C short-term (a) and 11°C long-term chilling (b) periods. Vertical bars represent the mean ± SE of four replications.

FIGURE 6 PERCENT OF CONTROL (CHILL:CONTROL) OF ANTIOXIDANT ENZYME ACTIVITIES FOR THE THIRD LEAF STAGE OF INBRED MAIZE

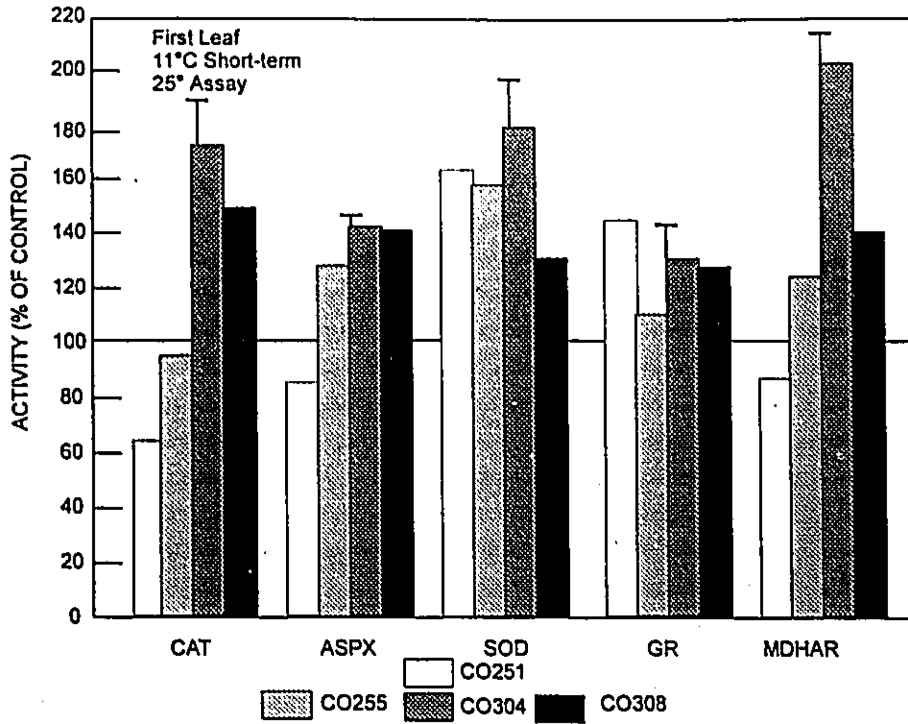
Percents of control of CAT, ASPX, SOD, GR, and MDHAR of the third leaf developmental stage in the chilling sensitive CO251 and chilling tolerant CO255, CO304, and CO308 inbred maize lines for 11°C short-term (a) and 11°C long-term chilling (b) periods. Vertical bars represent the mean ± SE of four replications.

FIGURE 7 PERCENT OF CONTROL (CHILL:CONTROL) OF ANTIOXIDANT ENZYME ACTIVITIES FOR THE FIFTH LEAF STAGE OF INBRED MAIZE

Percents of control of CAT, ASPX, SOD, GR, and MDHAR of the fifth leaf developmental stage in the chilling sensitive CO251 and chilling tolerant CO255, CO304, and CO308 inbred maize lines for 11°C short-term (a) and 11°C long-term chilling (b) periods. Vertical bars represent the mean ± SE of four replications.

FIGURE 8 PERCENT OF CONTROL (CHILL:CONTROL) OF TOTAL, REDUCING, AND NON-REDUCING SUGAR CONCENTRATIONS FOR INBRED MAIZE

Percents of control of reducing (RS), non-reducing (NRS), and total (TS) soluble sugars of the first, third, and fifth leaf developmental stages in the chilling sensitive CO251 and chilling tolerant CO255, CO304, and CO308 inbred maize lines for (a) 11°C short-term and (b) 11°C long-term chilling periods. Vertical bars represent the mean ± SE of four replications.



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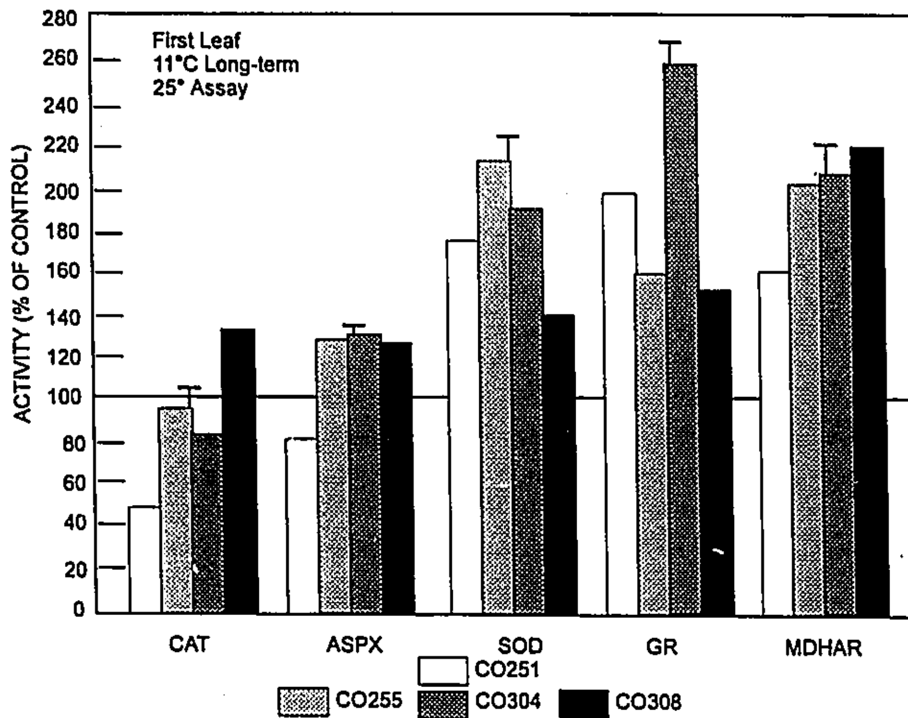

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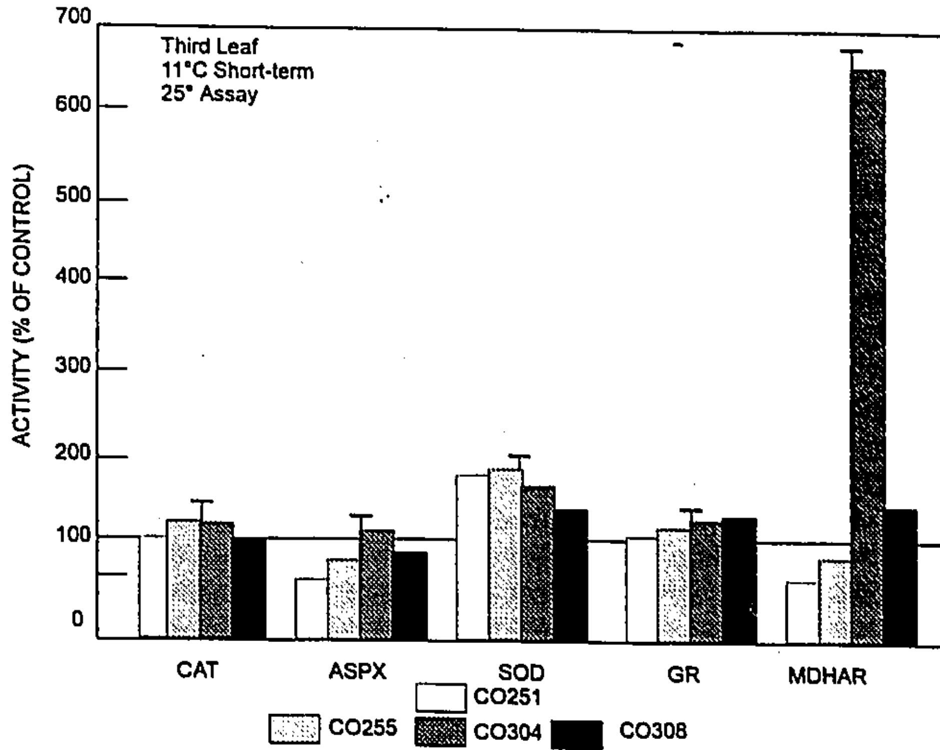
Figure 5



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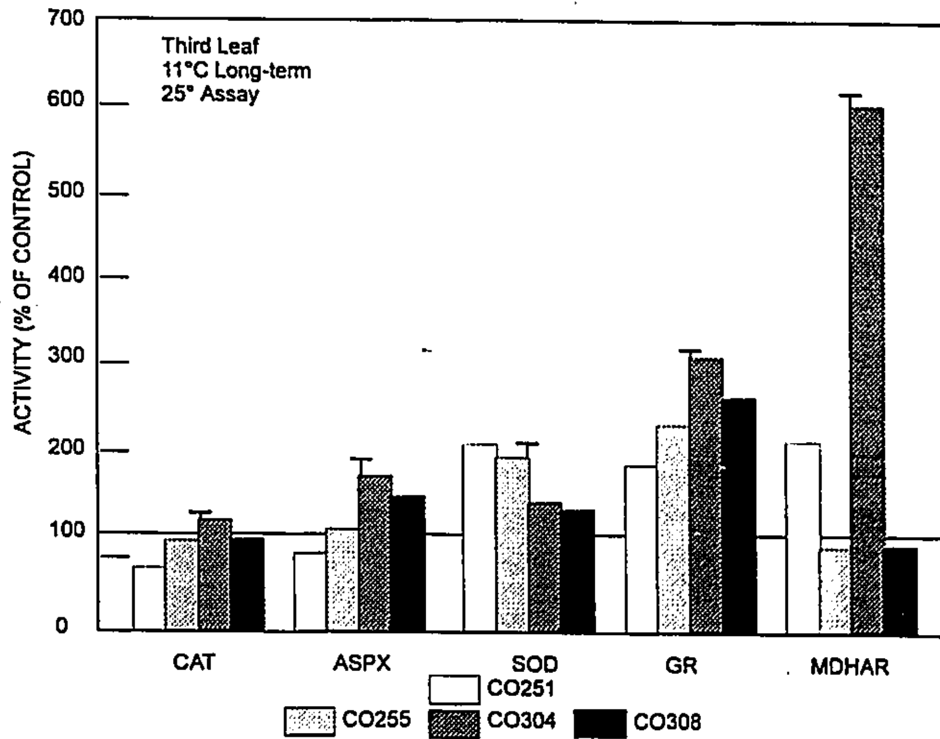

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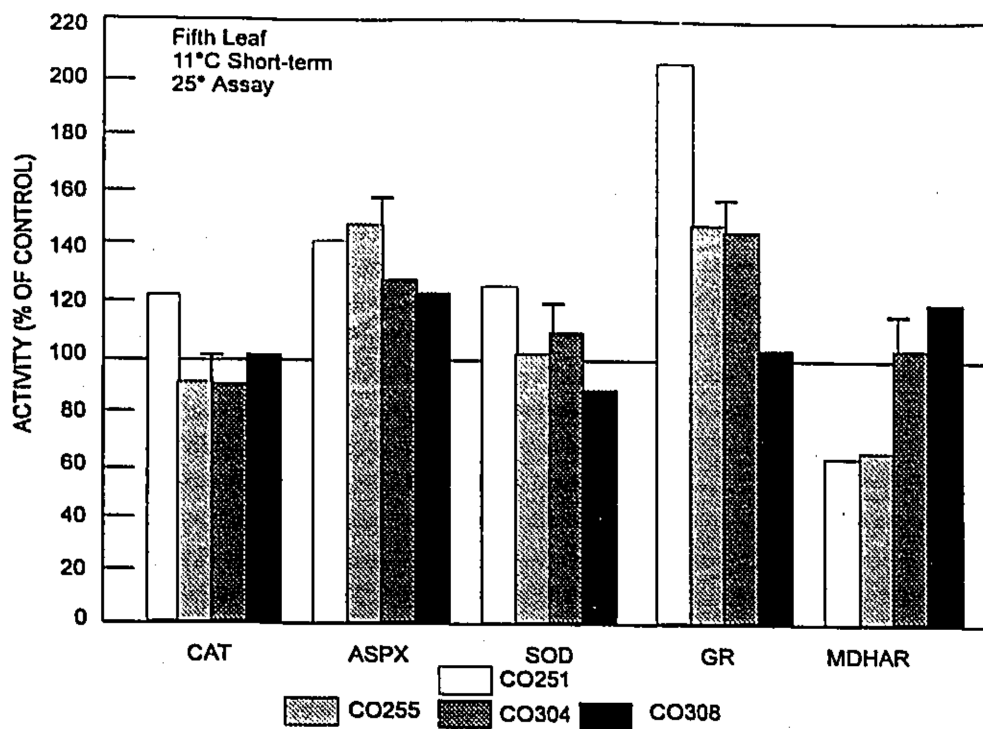
Figure 6



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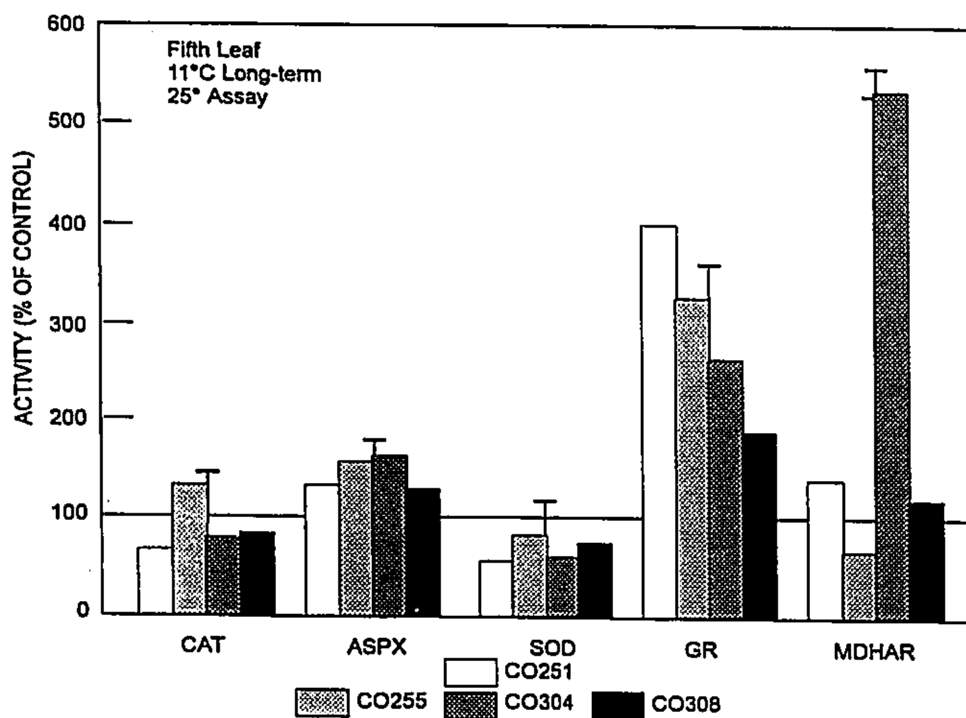

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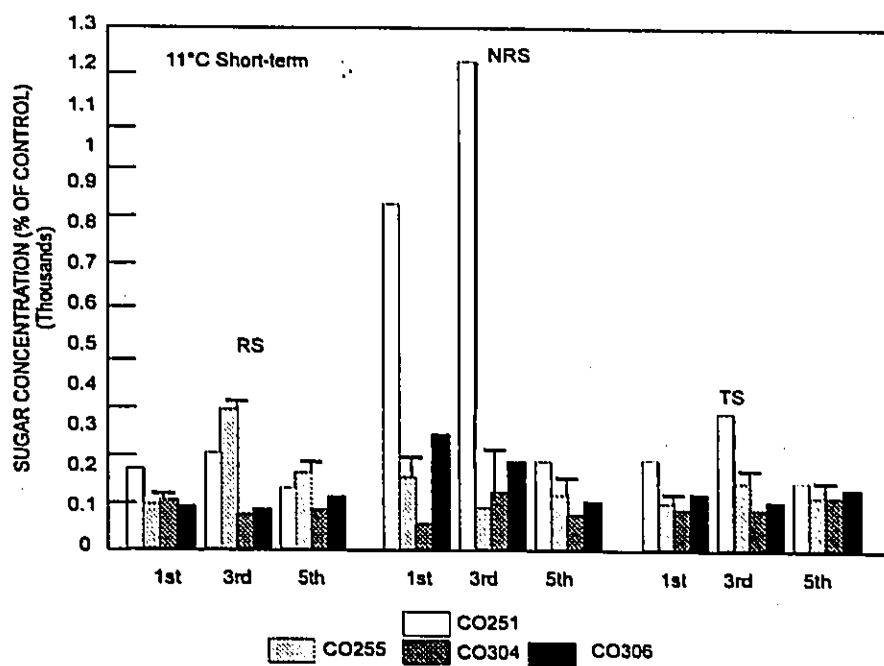
Figure 7



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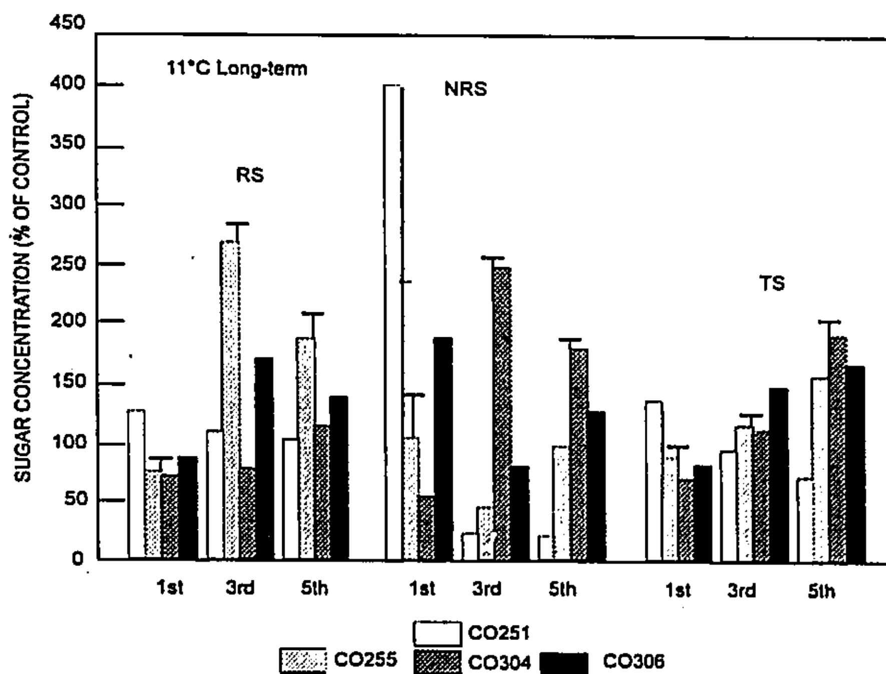

(b)

Figure 8

CHAPTER FIVE

ANTIOXIDANT COMPOUND RESPONSES TO CHILLING STRESS IN DIFFERENTIALLY SENSITIVE INBRED MAIZE LINES

The following chapter is a reproduction of a paper submitted by D.M. Hodges, C.J. Andrews, D.A. Johnson, and R.I. Hamilton. The principal author (D.M. Hodges) accumulated and interpreted all data. The fourth author (Dr. R.I. Hamilton) produced and contributed seed for the inbred maize lines used in these experiments.

In order to complete the second objective of this thesis, *i.e.* to assess the antioxidant capacities and metabolic indicators of chilling stress of differentially sensitive maize inbreds, the concentrations of the antioxidant compounds ascorbate, glutathione, ß-carotene, and α-tocopherol and of the metabolic indicator chlorophyll were measured at the three developmental stages under both short-term and long-term chilling treatments.

It was found that percent of control levels of ß-carotene were lowest in the chilling sensitive relative to the tolerant lines at the first leaf stage under both short- and long-term chilling treatments. Percent of control DAsA/AsA ratios were lowest in the chilling sensitive relative to the tolerant

lines for all three stages under both short- and long-term chilling treatments. Percent of control concentrations of ß-carotene and total ascorbate and glutathione in this chilling sensitive line increased as the plant aged under long-term chilling exposure until they were significantly higher or no different relative to the tolerant lines. However, lower percent of control ratios of DAsA/AsA in the chilling sensitive lines relative to the tolerant lines at all stages under both short- and long-term chilling treatments suggest that this sensitive line did not a effectively recycle ascorbate, regardless of the developmental stage. These results, along with those of the antioxidant enzyme activities described in **Chapter Four** of this thesis, support the model that the chilling sensitive inbred maize line CO251 had less antioxidant scavenging capacities than did the tolerant lines at the earlier stages of development. Initially reduced toxic oxygen compound scavenging capacity would reduce chilling tolerance as the damage due to these compounds would be greater than for the lines with relatively higher antioxidant capacity. However, as the ability of the chilling sensitive line to detoxify toxic oxygen compounds increased as the plants aged from the first to fifth leaf developmental stages, as measured by antioxidant enzyme (**Chapter Four**) and compound capacities, the susceptibility to chilling of the sensitive maize line potentially decreased correspondingly. Chlorophyll content, initially significantly lower in the first leaf stage

in the chilling sensitive line for both short- and long-term chilling treatments, increased compared to the tolerant lines as the plants aged. The relative concentrations of this metabolite indicated that CO251 experienced an initially greater susceptibility to chilling at the earlier developmental stages, and that this line became less sensitive as it aged.

5.1 ABSTRACT

Antioxidant compound levels were determined at three developmental stages (first, third, and fifth leaf) of four inbred lines of maize (*Zea mays* L.) exhibiting differential sensitivity to chilling in order to determine if chilling sensitive maize had lower concentrations of these compounds than the tolerant lines. Plants were exposed to a photoperiod of 16:8 L:D for one of three treatments: (a) control (25°C) for 2, 5, and 10 days, (b) control treatment plus an exposure to a short-term chilling treatment of 11°C 1 d prior to harvesting, and (c) long-term (11°C constant) chilling exposure for 8, 19, and 29 days. Total ascorbate, DAsA/AsA, total glutathione, GSH/GSSG, and contents of ß-carotene and α-tocopherol were assessed. Chlorophyll contents were determined as indicators of chilling stress. Lower percent of control concentrations of ß-carotene in the chilling sensitive relative to the tolerant lines for the first leaf stage under both short- and long-term chilling treatments may have led to an increase in the toxic oxygen compound levels in the sensitive line. Percent of control concentrations of ß-carotene and total ascorbate and glutathione in the chilling sensitive line increased as the chilling treatment progressed and the plant aged until they ultimately became either significantly higher or no different relative to the tolerant lines. However, lower percent of control ratios of DAsA/AsA in the chilling sensitive line relative to the tolerant lines

at all stages under both short- and long-term chilling treatments suggest that this sensitive line did not as effectively recycle ascorbate, regardless of developmental stage. Results, in combination with those of antioxidant enzymes from our previous work, suggest that this sensitive line becomes less sensitive to chilling-induced oxidative stress as it ages.

5.2 INTRODUCTION

Toxic oxygen compounds may be produced as byproducts from metabolic pathways even under optimal growth conditions. In plants, those metabolic pathways driven by or associated with the light reactions of photosynthesis are particularly prone to generate such toxic oxygen compounds as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH) (Halliwell and Gutteridge, 1985). These compounds are generally produced through photoinhibitory processes of the photosynthetic electron transport chain (Foyer et al., 1994a). The presence of these toxic oxygen byproducts is potentially lethal as they have been observed to play a significant role in the peroxidation of phosopholipids (Matsuo et al., 1990; Zheng and Yang, 1991), the denaturation of proteins (Casano and Trippi, 1992; Bowler et al., 1992), and the degradation of nucleic acids (Bowler et al., 1992).

Chilling temperatures may lead to an increase in the

amounts of toxic oxygen compounds present in chilling-sensitive plants such as maize (*Zea mays* L.), mainly due to chilling-induced photoinhibition (Hodgson and Raison, 1991; Sonoike and Terashima, 1994; Terashima et al., 1994, Havaux and Davaud, 1994). In this process, an over-energization of the photosystem reaction centres results from an inadequate supply of the natural electron acceptor $NADP^+$, the pool of which can be lowered by a reduction in the CO_2 fixation rate (Öquist and Huner, 1993; Sonoike and Terashima, 1994). As the rate of carbon fixation is reduced as a result of low temperature stress (Schöner and Krause, 1990, Foyer et al., 1994a), molecular oxygen may act as an electron acceptor in place of $NADP^+$, producing O_2^- (Robinson, 1988).

Plants have evolved antioxidant systems with which to protect themselves against toxic oxygen compounds. Antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and ascorbate peroxidase (ASPX, EC 1.11.1.11) can eliminate these oxygen compounds (Scandalios, 1993; Hodges et al., 1995 manuscript submitted). In addition to these enzymes, antioxidant compounds such as ascorbate, glutathione, ß-carotene, and α-tocopherol play important roles in the removal of these toxic oxygen byproducts.

Ascorbate is an extremely important antioxidant in plants. It can react directly with and detoxify O_2^-, •OH, and singlet oxygen ($^1O_2$) (Walker and McKersie, 1993), and can

remove H_2O_2 through the processes of the glutathione-ascorbate cycle (Gossett et al., 1994). Reduced ascorbate can be regenerated by the enzymes monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and dehydroascorbate reductase (DHAR, EC 1.8.5.1) and another antioxidant thiol compound, glutathione (Jahnke et al., 1991; Foyer et al., 1994a, Gossett et al., 1994). Glutathione can also be oxidized by reacting directly with $^1O_2$ and $\cdot OH$ species (Foyer et al., 1994b). The resulting oxidized glutathione (GSSG) is then converted back to the reduced form by glutathione reductase (GR, EC 1.6.4.2) (Foyer and Halliwell, 1976).

The membrane-associated antioxidant α-tocopherol can scavenge $O_2^{\cdot}$, $\cdot OH$, and $^1O_2$ (Wise and Naylor, 1987; Fryer, 1992). Ascorbate can reduce tocopheryl radicals produced through the oxidation of α-tocopherol and thus regenerate α-tocopherol (Packer et al., 1979). Another lipid soluble antioxidant compound is ß-carotene, which may quench singlet oxygen and quench the excess chlorophyll excitation energy not readily passed on through the photosystem (Knox and Dodge, 1985).

The duration of chilling stress can have important effects on chilling sensitive plants. Physiological dysfunctions resulting from molecular changes induced at low temperature can be reversed if the tissue is returned to non-chilling temperatures before the dysfunction becomes persistent (Lyons et al., 1979). Prolonged chilling stress can lead to metabolic damage which may, however, be alleviated

to an extent due to hardening or conditioning (Stamp, 1984). Comparisons between short- and long-term chilling treatments on antioxidant compound responses have not appeared in the literature. Similarly, very little work has been reported on chilling sensitivity in relation to the age of plants subjected to chilling stress.

A previous study comparing differentially chilling sensitive inbred lines of maize subjected to short- and long-term chilling temperatures demonstrated that a chilling sensitive line had less antioxidant enzyme capacities than did the tolerant lines (Hodges et al., manuscript submitted). The following work assesses differences in the antioxidant compound concentrations between these inbred maize lines in order to determine if this chilling sensitive line has less antioxidant compound capacities as well. To our knowledge, this is the first time antioxidant capacity comparisons have been made between chilling sensitive and tolerant plants within a single species of higher plants. This reduces the complexity of genetic differences which result from comparisons between different species. To further the study of the effects of chilling durations on growth stage in young maize seedlings, plants were harvested at the first, third, and fifth leaf stages under both short-term and long-term chilling treatments. Results, in combination with those of antioxidant enzymes from our previous work, suggest that this sensitive line becomes less sensitive to chilling-induced

oxidative stress as it ages.

5.3 MATERIALS AND METHODS

5.3.1 Plant material

The four elite inbred maize (*Zea mays* L.) lines selected for this study exhibited differential sensitivity to chilling based upon germination and emergence (Hodges et al., 1994) and early growth dry mass (Hodges et al., 1995) parameters in both the laboratory and the field. The line CO251 was shown to be the most chilling sensitive of eight original inbred lines tested, and CO255, CO304, and CO308 were demonstrated to be either the most, or one of the more, chilling tolerant depending upon the growth parameter assayed (Hodges et al., 1994; Hodges et al., 1995). These elite inbreds were produced in the 1994 Agriculture Canada nursery, Ottawa, Canada.

Material was germinated and grown as in section 4.3.1 of this thesis. Leaves were harvested from the 11°C long-term chilling treatment at 8, 19, and 29 d from pinning for the first, third, and fifth leaf stages, respectively. For the third and fifth leaf stages, the two most recently expanded leaves were harvested in a 1:1 ratio. The main midrib was removed from all leaf tissue. The pooling of leaves from approximately 20, 12, and 10 plants for the first, third, and fifth leaf stages, respectively, was necessary for 1.0 gram of

fresh tissue.

5.3.2 Ascorbate determination

Reduced ascorbate (AsA), dehydroascorbate (DAsA), and total ascorbate (AsA + DAsA) were determined in a method modified from Gossett et al. (1994). Over ice, 1 g frozen tissue was ground up with inert sand and 10 mL of 5% *m*-phosphoric acid with a mortar and pestle. The homogenate was centrifuged at 22 000 x *g* for 15 min. Total ascorbate was determined in a reaction mixture consisting of 200 μL of supernatant, 500 μL of 150 m*M* KH_2PO_4 buffer (pH 7.4) containing 5 m*M* EDTA, and 100 μL of 10 m*M* dithiothretitol (DTT) to reduce DAsA to AsA. After 10 min at room temperature, 100 μL of 0.5% (w/v) *N*-ethylmaleimide was added to remove excess DTT. AsA was assayed in a similar manner except that 200 μL of deionized H_2O was substituted for DTT and *N*-ethylmaleimide. Colour was developed in both series of reaction mixtures with the addition of 400 μL of 10% TCA, 400 μL of 44% *o*-phosphoric acid, 400 μL of α,α'-dipyridyl in 70% ethanol, and 200 μL of 30g L^{-1} $FeCL_3$. The reaction mixtures were incubated at 40°C for 1 hr and assayed spectrophotometrically (Milton Roy Spectronic 1001 plus) at 525 nm. Ascorbate standards of between 0.01 and 0.5 m*M* ml^{-1} ascorbate in *m*-phosphoric acid were analyzed in the same manner as extracts. For each sample, DAsA was estimated from the difference of total

ascorbate and AsA.

5.3.3 Glutathione determination

Oxidized glutathione (GSSG), reduced glutathione (GSH), and total glutathione (GSSG + GSH) were determined in a method adapted from Gossett et al. (1994). Over ice, 1 g frozen tissue was ground up with inert sand and 5 mL of ice-cold 6% *m*-phosphoric acid (pH 2.8) containing 1 m*M* EDTA in a mortar and pestle. The homogenate was centrifuged at 22 000 x *g* for 15 min, and the supernatant removed and then filtered through a 0.45 μm ultrafilter. Two solutions were then prepared. Solution A consisted of 8 mL of 110 m*M* $Na_2PO_4 \cdot 7H_2O$, 8 mL of 40 m*M* $NaH_2PO_4 \cdot H_2O$, 18 mL of 5 m*M* EDTA, 38 mL of 0.3 m*M* 5,5'-dithiobis-(2-nitrobenzoic acid), and 10 mL of 0.4 ml L^{-1} BSA. Solution B consisted of 1 mL of 1 m*M* EDTA, 5 mL of 50 m*M* imidazole, 5 mL of 0.2 ml L^{-1} BSA, and 5 mL of 1.5 units glutathione reductase. Total glutathione was measured in a reaction mixture consisting of 400 μL of solution A, 320 μL of solution B, 400 μL of a 1:50 dilution of the extract in 5% Na_2HPO_4 (pH 7.5), and 80 μL of 0.15 m*M* NADPH. The reaction rate was measured spectrophotometrically by following the change in absorbance at 412 nm for 10 min. For GSSG, 1 ml of the 1:50 extract dilution was initially incubated with 40 μL of 2-vinylpyridine at 25°C for 1 hr. A standard curve was developed by preparing solutions of 0.002 - 0.0001 g ml^{-1} GSH

in 6% of *m*-phosphoric acid (pH 2.8) containing 1 mM EDTA, diluting 1:2000 with the 5% Na_2PO_4, and analyzing in the same manner as the extracts. GSH was estimated as the difference between total glutathione and GSSG.

5.3.4 Beta-carotene determination

Beta-carotene was extracted from plant tissue in a method modified from Lichtenthaler (1987). Under dim light and over ice, 1 g material was ground up with 5 ml acetone, and inert sand in a mortar and pestle. The mortar was washed with 5% ascorbic acid in deionized H_2O and the wash mixed with the acetone. A subsequent 2.5 ml hexane addition, followed by vortexing and centrifugation (1000 X *g*) was performed, and the top layer removed. This hexane extraction was repeated twice. The hexane extract was then dried down under nitrogen in darkness and the residue taken up in 0.5 ml chloroform. Extracts were stored under nitrogen at -75°C until assayed.

Beta-carotene concentration was determined by HPLC in a method modified from Miller and Yang (1985) using a LKB 2150 binary pump, LKB 2151 variable wavelength monitor, and a LKB 2152 controller. The column was a CSC-sil 80A/ODS2 (4.6 mm X 25 cm, 5-μm) fitted with a Hamilton C18 pre-column. The column was eluted isocratically with a solvent system consisting of methanol-acetonitrile-chloroform (25:60:15) at a flow rate of 1.5 ml min^{-1}. Standards were prepared using a

stock solution of 0.125 mg ml^{-1} ß-carotene in chloroform. Extraction efficiencies averaged 92% with dosed samples. The retention time of ß-carotene was 15.5 min.

5.3.5 Alpha-tocopherol determination

Alpha-tocopherol was extracted from 1.0 g plant material in a similar manner as was ß-carotene except that the dried down hexane residue was redissolved in 0.5 ml 1:1 pyridine:bis(trimethylsilyl)trifluoroacetamide (BFSTA) (v/v) containing 0.034 mg α-tocopherol acetate as an internal standard, and the extracts stored under argon at -75°C.

Alpha-tocopherol concentration was determined by gas chromatography (GC) in a method modified from Walker and McKersie (1993). A J&W Scientific DB-17 fused silica column attached to a Varian 3400 Gas Chromatograph was standardized with 1:1 pyridine:BFSTA. Standards were prepared using 0.067 mg ml^{-1} α-tocopherol in 1:1 pyridine:BFSTA. The retention times of α-tocopherol and α-tocopherol acetate were 19.7 and 23.3 min, respectively.

5.3.6 Chlorophyll determination

Chlorophyll was determined in 1 g fresh plant tissue as described by Arnon (1949).

5.3.7 Statistical Analysis

Antioxidant compound and chlorophyll assays were based on at least two readings from four independent samples. The effects of inbred line, temperature regime, and growth stage on antioxidant compound and chlorophyll concentrations were analyzed by a three-factor completely randomized ANOVA (Table 5.1). Data from control treatments are given as means ± SE. Data from both short-term and long-term chilling treatments are shown as means ± the averaged SE.

5.4 RESULTS

Total ascorbate and the dehydroascorbate/ascorbate (DAsA-/AsA) ratios for the maize inbred lines grown at 25°C are presented in Table 5.2 and total glutathione and the reduced/oxidized glutathione (GSH/GSSG) ratios in Table 5.3. Concentrations of both total ascorbate and glutathione of the chilling sensitive maize inbred line were not significantly different from the tolerant lines throughout all three developmental stages except for glutathione in the third leaf stage, where it was significantly higher (Tables 5.1,5.3). The DAsA/AsA ratios were highest and the GSH/GSSG ratios lowest in this line (Tables 5.2-5.3). Control concentrations of ß-carotene were significantly higher in this chilling sensitive line for the first leaf developmental stage, but were not significantly different for the third or fifth leaf

stages (Tables 5.1, 5.4). Both control concentrations of α-tocopherol and chlorophyll were not significantly different highest in the sensitive line compared to the tolerant lines for all three leaf developmental stages (Tables 5.1,5.4).

All antioxidant compound and chlorophyll concentrations after short- or long-term chilling were expressed as percents of their respective control values. Thus, after chilling for 1 d, percent of control values for concentrations of total ascorbate and total glutathione of the chilling sensitive line were not significantly different from those of the tolerant inbred maize lines for the three developmental stages (Tables 5.1-5.3). The percent of control DAsA/AsA and GSH/GSSG ratios were generally lower in the chilling sensitive line as compared to the tolerant lines for all leaf stages (Tables 5.2-5.3). Percent of control levels of ß-carotene after the 1 d short-term chilling treatment were significantly lower in the chilling sensitive as compared to the tolerant lines for the first leaf stage, but were not significantly different for the third or fifth leaf stages (Table 5.1, Figures 9-11). Percent of control levels of α-tocopherol for the chilling sensitive line were not significantly different from the tolerant lines for all three growth stages (Table 5.1, Figure 9-11). Percent of control concentrations of chlorophyll for the short-term chilling were not significantly different between the chilling sensitive and tolerant lines for all three leaf stages (Table 5.1, Figures 9-11). There were no

significant differences between chilling sensitive and tolerant lines for the chlorophyll a:chlorophyll b ratios (data not shown).

Under long-term chilling (11°C), percent of control of concentrations of total ascorbate in the chilling sensitive maize line were not significantly different from the tolerant lines after 8 days (first leaf stage) and 19 days (third leaf stage) chilling, but were significantly higher after 29 days (fifth leaf stage) chilling (Tables 5.1-5.2). Percents of control of the DAsA/AsA ratios were always lowest in this sensitive line for all three developmental stages (Table 5.2). Similar to total ascorbate, percentages of control of total glutathione in the chilling sensitive line were no different from those of the chilling tolerant lines for the first leaf stage, but increased to be significantly highest for the third and fifth leaf developmental stages (Tables 5.1,5.3). There were no discernable differences between the lines in respect to the GSH/GSSG ratios (Table 5.3). Percent of control of ß-carotene concentrations were significantly lowest in the chilling sensitive compared to those of the tolerant lines for the first leaf stage, but were not significantly different for the third or fifth leaf stage under long-term chilling (Table 5.1, Figures 9-11). Percent of control levels of α-tocopherol of the chilling sensitive line were not significantly different from those of the tolerant lines for all three leaf stages (Table 5.1, Figures 9-11). Percent of control

concentrations of chlorophyll were significantly lower in the chilling sensitive relative to the tolerant lines for the first leaf stage of development under long-term chilling, but at the third and fifth leaf stages, there were no significant differences between the sensitive and tolerant lines (Table 5.1, Figures 9-11). As with the short-term chilled maize, there were no differences between chilling sensitive and tolerant lines for the chlorophyll a:chlorophyll b ratios (data not shown).

5.5 DISCUSSION

The lower percent of control ß-carotene content found in the chilling-sensitive relative to the chilling tolerant maize lines at the first leaf developmental stage under both short- and long-term chilling treatments could be a basis for greater toxic oxygen compound accumulation in this sensitive line. Beta-carotene has been demonstrated to be able to quench $^1O_2$ and excited triplet states of chlorophyll (De La Rivas et al., 1993). As production of both $^1O_2$ and triplet states of chlorophyll occurs during photoinhibition (Long et al., 1994; Sonoike and Terashima, 1994), less ß-carotene content as observed here would lead to less available protection from this detrimental process which is capable of producing even more toxic oxygen compounds, particularly under chilling (Terashima et al., 1994). As decreases in carotenoid contents

have been observed under both short-term chilling in the relatively chilling-sensitive species tomato (*Lycopersicum* spp. L.) (Walker and McKersie, 1993) and chilling-tolerant wheat (*Triticum asetivum* L.) (Mishra et al., 1993) and long-term chilling in the chilling-tolerant species spinach (*Spinacia oleracea* L.) (Schöner and Krause, 1990), ß-carotene content would appear to be an important contributor to chilling tolerance.

As the maize lines aged from the first to the fifth leaf stages under the long-term chilling treatment, there was an increase in the percent of control concentrations of total ascorbate and glutathione in the chilling sensitive line until the levels for both were significantly higher than those of the chilling tolerant lines. As oxidative stress induces or enhances ascorbate and glutathione levels (Smith, 1985; May and Leaver, 1993; Foyer et al., 1994a), this supports the concept that the chilling sensitive line CO251 had initially accumulated greater levels of toxic oxygen compounds relative to the tolerant lines. As well, significantly lower percent of control activities in the chilling sensitive line of ascorbate peroxidase and monodehydroascorbate reductase in the first leaf stage from both short- and long-term chilling treatments and catalase for the first and third leaf stages under long-term chilling, would have also contributed to greater oxidative stress in this line (Hodges et al., manuscript submitted).

In all lines, when short- and long-term chill stressed, percent of control levels of total glutathione were increased over the controls to a greater degree than total ascorbate. This is similar to results of air-stressed rice (*Oryza sativa* L.) (Ushimaru et al., 1992) and low-temperature exposed rye (*Secale cereale* L.) (Volk and Feierabend, 1989) and wheat (Badiani et al., 1993).

The DAsA/AsA chill:control ratio was lowest in the chilling sensitive line CO251, and most especially so during the long-term chilling exposure. Since there was more reduced than oxidized ascorbate in the chilling sensitive line, this suggests that this sensitive line did not as effectively cycle ascorbate as did the tolerant lines. This in turn indicates that there would have been less potential scavenging of H_2O_2 and other toxic oxygen compounds by ascorbate in this sensitive line. Less percent of control activities of ascorbate peroxidase and monodehydroascorbate reductase relative to the tolerant lines for the first leaf stage for both short- and long-term chilling treatments may have accounted for some of the lower ascorbate-related scavenging potential CO251 (Hodges et al., manuscript submitted). A larger glutathione pool in this chilling sensitive line may have also contributed to greater concentrations of AsA relative to DAsA, as the glutathione pool has been demonstrated to be involved in recycling of the ascorbate pool (Foyer et al., 1991).

The GSH/GSSG ratios of the chilling sensitive line for the short- and long-term chilling terms were not discernably different from those of the tolerant lines. As there were higher concentrations of total glutathione in the sensitive line, the maintenance of these similar ratios was consistent with the finding that glutathione reductase activity was higher in this line after prolonged chilling exposure (Hodges et al., manuscript submitted).

Percent of control levels of α-tocopherol in the chilling sensitive relative to the tolerant lines were not significantly different for any of the leaf developmental stages from short- or long-term chilling treatments. Wise and Naylor (1987), in experiments with chill-stressed cucumber (*Cucumis sativus* L.) and pea (*Pisum sativum* L.), demonstrated that α-tocopherol concentration appeared to be implicated in long-term protection of photosynthetic pigments. As percent of control levels of α-tocopherol of the chilling sensitive line were never significantly different from those of the tolerant lines, our findings lend support to this theory. Apparently, at least in maize, α-tocopherol is not one of the factors which limit chilling tolerance.

Percent of control concentrations of chlorophyll, as a general indicator of chilling damage (McWilliam and Naylor, 1967; Koscielniak, 1993; Brüggemann and Linger, 1994; Humbeck et al., 1994), were demonstrated to increase in the chilling sensitive line from the significantly lowest at the first leaf

stage under the long-term chilling treatment to significantly no different than the chilling tolerant lines as the plants aged. These observed dramatic changes in chlorophyll content could indicate that a combination of antioxidant compounds and/or enzymes resulted in a greater reduction of damaging effects of toxic oxygen compounds upon the chilling sensitive line's photosynthetic systems as the plant aged. The increasing percent of control values in the chilling sensitive relative to the tolerant inbred maize lines of levels of ß-carotene, as well as percent of control activities of catalase, ascorbate peroxidase, monodehydroascorbate reductase, and glutathione reductase (Hodges et al., manuscript submitted), for the short-term chilling treatment as the plants aged from the first to the fifth leaf developmental stage suggests that sensitivity of this line to chilling-induced oxidative stress declined as it aged. These results were parallelled by the long-term chilling treatment, where percent of control levels of total ascorbate, glutathione, ß-carotene, and chlorophyll, as well as percent of control activities of these same enzymes (Hodges et al., manuscript submitted), in the chilling sensitive line increased to become either significantly higher of no different from the tolerant maize lines as the plants aged.

Growth stage is apparently an important factor in maize chilling sensitivity. Both short-term and long-term chilling results suggest that as the chilling sensitive plants aged

from the first to the fifth leaf stage, they were more able to maintain or synthesize these compounds in response to chilling-induced oxidative stress. There are similarities between results of the short- and long-term chilling treatments as well. Percent of control concentrations of ß-carotene were lowest for the first leaf stage and percent of control levels of DAsA/AsA are lowest for all stages in the chilling sensitive line for both chilling treatments. Differences, however, do exist between results from the short-term and long-term chilling treatments. Under short-term chilling, percent of control concentrations of total ascorbate and glutathione of the chilling sensitive line were not significantly different from the tolerant lines for all three growth stages. Under long-term chilling, percent of control concentrations of total ascorbate and glutathione of the chilling sensitive line increased to be significantly higher than in the tolerant lines. This would confirm, as with the antioxidant enzyme results (Hodges et al., manuscript submitted), that long-term chilling is more detrimental to maize than short-term chilling.

In conclusion, the percent of control levels of the antioxidant compound ß-carotene were lowest in the chilling sensitive relative to the chilling tolerant inbred maize lines under both short- and long-term chilling treatments for the first leaf stage. Initially lower percent of control levels of ß-carotene may, in conjunction with reduced activities of

certain antioxidant enzymes (Hodges et al., manuscript submitted), have led to increased production of toxic oxygen compounds in the chilling sensitive relative to the tolerant lines at the earlier stages of development. Percent of control concentrations of ß-carotene and total ascorbate and glutathione increased in the chilling sensitive line, as did the activities of particular antioxidant enzymes (Hodges et al., manuscript submitted), as the chilling treatment progressed and the plants aged until they ultimately became significantly higher or no different relative to the tolerant lines. However, lower percent of control ratios of DAsA/Asa in the chilling sensitive line relative to the tolerant lines at all stages under both short-term and long-term chilling treatments suggests that this sensitive line did not as effectively recycle ascorbate, regardless of developmental stage. As the ability of the chilling sensitive line to detoxify toxic oxygen compounds increased as the plants aged from the first to fifth leaf developmental stages, the susceptibility to chilling of the sensitive maize line is suggested to decrease correspondingly.

5.6 ACKNOWLEDGEMENTS

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Table 5.1 Levels of significance for a three factor ANOVA for concentrations of total ascorbate (TOT AsA), reduced ascorbate (AsA), dehydroascorbate (DAsA), total glutathione (TOT GLUT), reduced glutathione (GSH), oxidized glutathione (GSSG), β-carotene (β-carot), α-tocopherol (α-tocoph), and chlorophyll (CHL); *** P≤0.001, ** P≤0.01, * P≤0.05, N.S. not significant.

Source	ASA	ASA	DAsA	GLUT	GSH	GSSG	β-carot	α-tocopherol	CHL
Line	***	***	***	***	***	***	***	***	*
Temperature Regime (TR)	***	***	***	***	***	***	***	***	***
Growth Stage (GS)	*	***	***	***	***	***	***	***	***
Line x TR	**	***	***	***	***	***	***	***	***
Line x GS	**	**	**	***	***	N.S.	***	***	**
TR x GS	***	***	***	***	***	***	***	**	***
Line x TR x GS	***	***	**	***	***	N.S.	**	N.S.	***

Table 5.2. Total concentration of ascorbate (mmol/g fr.wt.) and the dehydroascorbate/ascorbate (DAsA:AsA) ratio for control (25°C) and the chill:control ratios for the short-term and long-term chilling (11°C) treatments for the chilling-sensitive CO251 and chilling-tolerant CO255, CO304, and CO308 inbred maize lines at the first-, third-, and fifth-leaf stages of development. The results represent the mean ± SE of four replications.

	Control		Chill:Control			
			Short-term		Long-term	
Line	Total	DAsA/ AsA	Total	DAsA/ AsA	Total	DAsA/ AsA
(A) First Leaf Stage						
CO251	0.77±0.05	1.76	1.23±0.09	0.49	1.09±0.03	0.42
CO255	0.60±0.05	1.18	1.34±0.13	0.39	1.14±0.08	0.60
CO304	0.68±0.03	1.39	1.29±0.06	0.39	0.83±0.04	0.78
CO308	0.54±0.04	1.61	1.09±0.10	0.61	1.29±0.09	0.66
(B) Third Leaf Stage						
CO251	1.38±0.12	2.42	0.36±0.04	0.04	0.70±0.07	0.40
CO255	0.94±0.08	1.38	0.43±0.04	0.21	0.91±0.06	3.21
CO304	0.61±0.04	1.54	0.57±0.03	0.10	1.07±0.05	0.91
CO308	0.73±0.02	1.76	0.44±0.01	0.32	0.66±0.02	0.57
(C) Fifth Leaf Stage						
CO251	0.99±0.04	0.14	0.42±0.04	7.85	1.34±0.12	1.27
CO255	0.90±0.04	0.09	0.51±0.03	7.89	0.89±0.10	5.00
CO304	0.88±0.03	0.07	0.47±0.04	17.59	0.84±0.06	4.90
CO308	0.91±0.01	0.03	0.43±0.02	39.76	0.82±0.03	6.35

Table 5.3. Total concentration of glutathione (μmol/g fr.wt.) and the reduced/oxidized glutathione (GSH:GSSG) ratio for control (25°C) and the chill:control ratios for the short-term and long-term chilling (11°C) treatments for the chilling-sensitive CO251 and chilling-tolerant CO255, CO304, and CO308 inbred maize lines at the first-, third-, and fifth-leaf stages of development. The results represent the mean ± SE of four replications.

	Control		Chill:Control			
			Short-term		Long-term	
Line	Total	GSH/ GSSG	Total	GSH/ GSSG	Total	GSH/ GSSG
(A) First Leaf Stage						
CO251	448.0±76.4	0.18	2.21±0.27	0.12	2.84±0.36	0.92
CO255	285.5±70.3	0.43	2.96±0.51	0.09	1.78±0.33	0.70
CO304	185.4±47.4	0.61	3.06±0.48	0.09	5.47±1.35	0.18
CO308	107.9± 6.0	0.62	2.02±0.17	0.25	2.47±0.18	0.38
(B) Third Leaf Stage						
CO251	229.6±49.1	0.26	1.10±0.26	0.68	5.54±0.69	0.73
CO255	163.9±31.9	0.34	1.34±0.25	0.76	2.71±0.57	1.40
CO304	67.4±19.7	0.55	2.36±0.64	0.62	3.16±0.70	0.69
CO308	49.6± 9.9	0.62	3.93±1.14	1.32	3.25±0.67	2.41
(C) Fifth Leaf Stage						
CO251	278.3±75.2	0.13	0.69±0.15	1.01	2.05±0.35	1.62
CO255	148.5±42.8	0.22	0.95±0.21	1.30	1.37±0.25	0.96
CO304	106.8±31.3	0.15	1.57±0.37	1.41	1.15±0.27	1.99
CO308	46.1± 7.6	0.96	2.72±0.52	0.17	1.87±0.38	0.46

Table 5.4. Concentrations of ß-carotene (μg/g fr.wt.), α-tocopherol (μg/g fr.wt.), and chlorophyll (mg/g fr.wt.) for the control treatments for the chilling-sensitive CO251 and chilling-tolerant CO255, CO304, and CO308 inbred maize lines at the first-, third-, and fifth-leaf stages of development. The results represent the mean ± SE of four replications.

Line	ß-carot	α-tocoph	Chl
(A) First Leaf Stage			
CO251	29.50±2.70	141.1±14.2	0.48±0.03
CO255	14.22±2.10	84.7±10.3	0.31±0.04
CO304	12.55±0.83	135.9± 5.4	0.31±0.07
CO308	19.44±2.36	105.9±12.7	0.32±0.10
(B) Third Leaf Stage			
CO251	26.45±4.09	222.9±54.9	0.27±0.02
CO255	22.90±5.90	312.2±22.2	0.29±0.04
CO304	13.79±1.74	328.2±40.0	0.73±0.06
CO308	12.42±3.16	242.8±31.3	0.63±0.10
(C) Fifth Leaf Stage			
CO251	14.20±1.27	421.6±19.9	0.46±0.14
CO255	19.89±3.43	419.1±22.1	0.43±0.12
CO304	15.85±1.21	524.1±30.8	0.78±0.09
CO308	5.85±1.42	276.7±25.0	0.68±0.09

5.7 FIGURE HEADINGS

FIGURE 9 CHILL:CONTROL RATIOS OF THE LEVELS OF ß-CAROTENE, α-TOCOPHEROL, AND CHLOROPHYLL AT THE FIRST LEAF STAGE OF INBRED MAIZE

Chill:control ratios of concentrations of ß-carotene (ß-carot), α-tocopherol (α-tocoph), and chlorophyll (Chl) of chilling-sensitive CO251 and chilling-tolerant CO255, CO304, and CO308 inbred maize lines at the first-leaf developmental stage grown under both short-term (1 d) (11°C) and long-term (8 d) chilling (11°) treatments. Error bars represent mean ± SE of four independent replicates for each line and compound.

FIGURE 10 CHILL:CONTROL RATIOS OF THE LEVELS OF ß-CAROTENE, α-TOCOPHEROL, AND CHLOROPHYLL AT THE THIRD LEAF STAGE OF INBRED MAIZE

Chill:control ratios of concentrations of ß-carotene (ß-carot), α-tocopherol (α-tocoph), and chlorophyll (Chl) of chilling-sensitive CO251 and chilling-tolerant CO255, CO304, and CO308 inbred maize lines at the third-leaf developmental stage grown under both short-term (1 d) (11°C) and long-term (19 d) chilling (11°) treatments. Error bars represent mean ± SE of four independent replicates for each line and compound.

FIGURE 11 CHILL:CONTROL RATIOS OF THE LEVELS OF ß-CAROTENE, α-TOCOPHEROL, AND CHLOROPHYLL AT THE FIFTH LEAF STAGE OF INBRED MAIZE

Chill:control ratios of concentrations of ß-carotene (ß-carot), α-tocopherol (α-tocoph), and chlorophyll (Chl) of chilling-sensitive CO251 and chilling-tolerant CO255, CO304, and CO308 inbred maize lines at the fifth-leaf developmental stage grown under both short-term (1 d) (11°C) and long-term (29 d) chilling (11°) treatments. Error bars represent mean ± SE of four independent replicates for each line and compound.

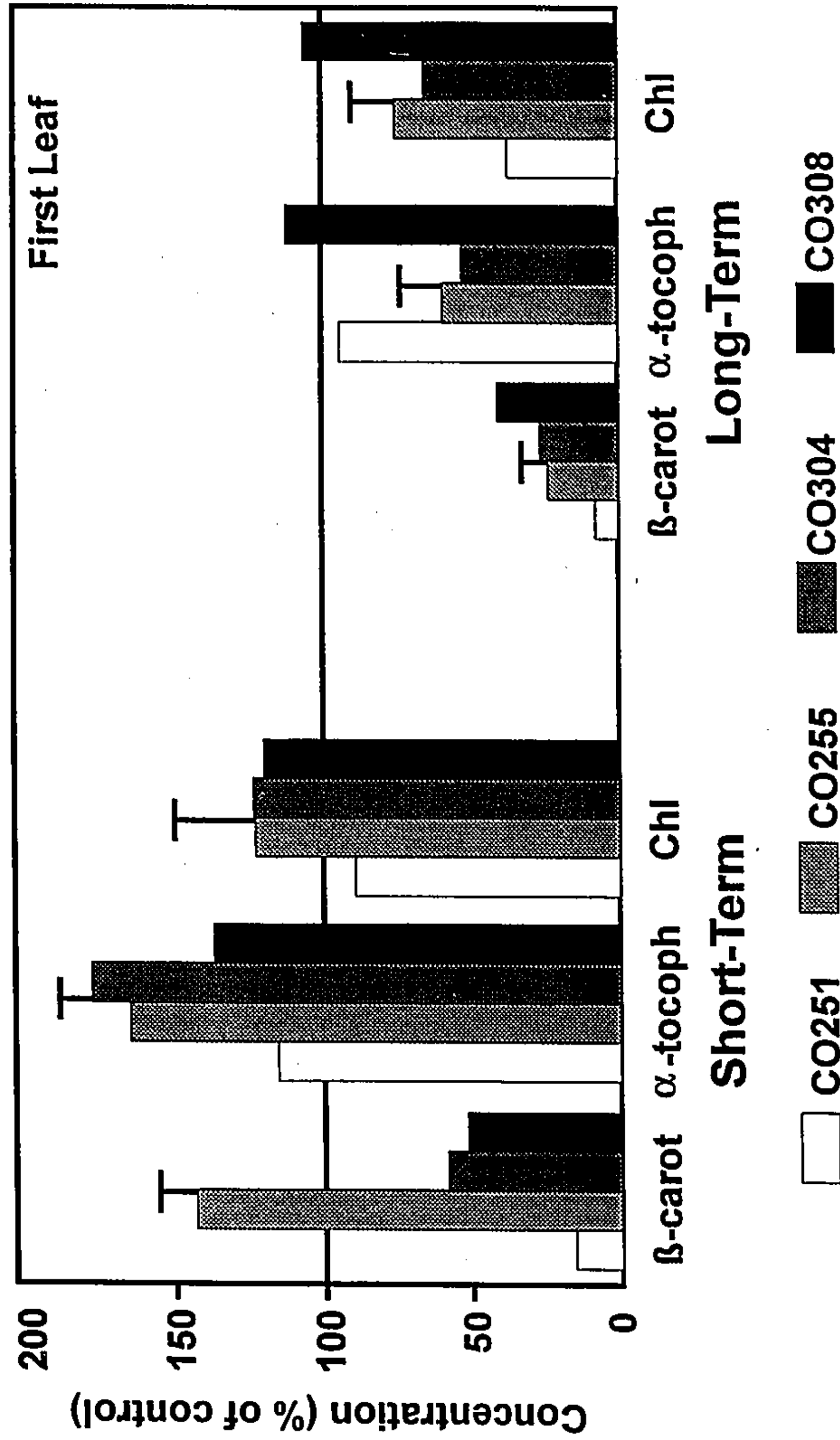

Figure 9

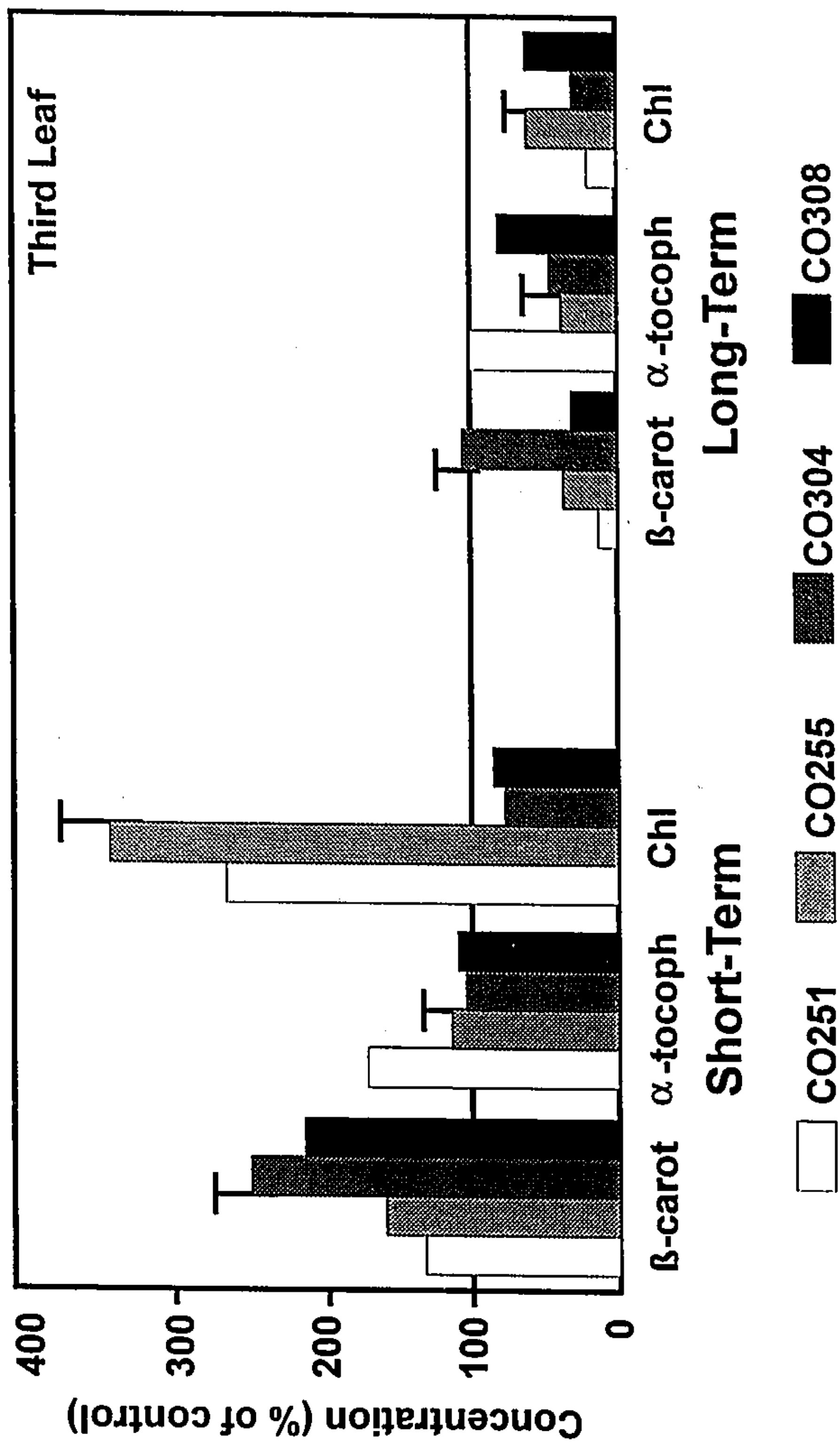

Figure 10

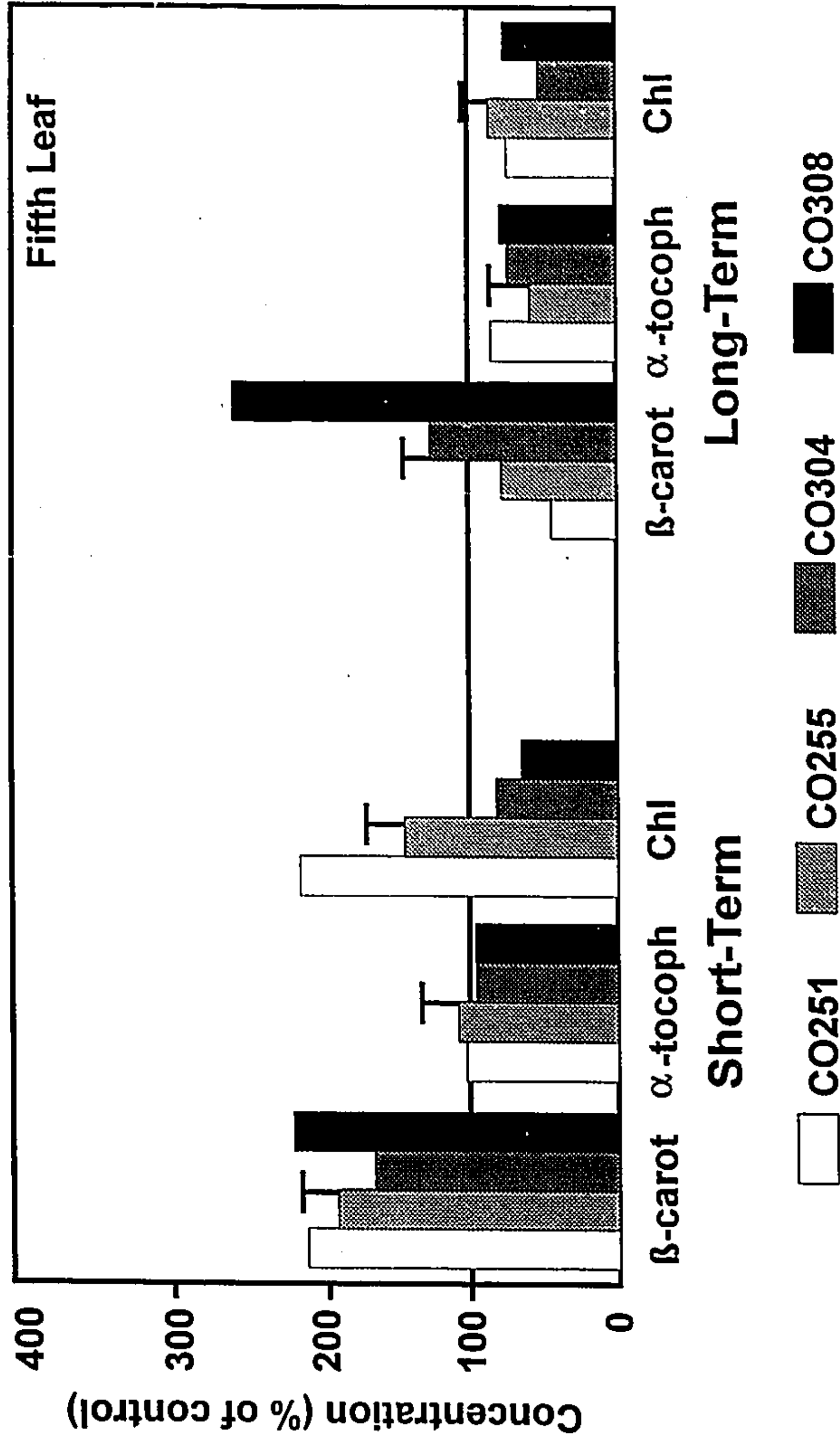

Figure 11

CHAPTER SIX

SENSITIVITY OF HYBRID MAIZE TO CHILLING AND THEIR COMBINING ABILITIES AT TWO DEVELOPMENTAL STAGES

The following chapter is a reproduction of a paper submitted by D.M. Hodges, C.J. Andrews, D.A. Johnson, and R.I. Hamilton. The principal author (D.M. Hodges) accumulated and interpreted all data. The fourth author (Dr. R.I. Hamilton) produced and contributed seed for the hybrid maize lines used in these experiments.

The third objective of this thesis was to determine if there were similar results for antioxidant capacities and metabolic indicator concentrations for the chilled hybrids as there were for the inbred maize lines of the second objective. To this end, a complete diallel cross between the above selected inbreds was made. However, to fulfil this third objective, the hybrids from this cross had to be classified as relatively chilling sensitive or tolerant depending upon how they performed in the laboratory germination and early growth stage screening tests as described for the inbreds in **Chapter Two** and **Chapter Three**. Field trials were performed to confirm laboratory results.

Results demonstrated that, unlike the inbreds, it was only possible to accurately evaluate differential chilling

sensitivity of hybrids at the early growth stage, suggesting that the germination/emergence and early growth stages are under the control of different genetic factors. Thus, to evaluate chilling tolerance of maize, it is important to examine plants at both the germination/emergence and early growth stages. Maternal effects were observed, but were much more pronounced at the fourth-leaf stage than at the germination/emergence stage. It was also demonstrated that, based on the physiological growth parameters assessed both in the laboratory and in the field, it is not possible to accurately predict hybrid maize cold tolerance from knowledge of the inbreds' responses.

As the antioxidant enzyme and compound capacities and the concentrations of the metabolic indicators of chilling stress were to be assessed at the third-leaf developmental stage of the hybrids, results from the early growth (fourth-leaf) growth test were of more significance. Thus, the lines with CO308 as the maternal parent (CO308xCO251, CO308xCO255, and CO308xCO304) were identified as being more chilling sensitive than the other maize hybrids. Results for antioxidant enzyme activities and concentrations of antioxidant compounds and metabolic indicators of chilling stress are described in **Chapter Seven** of this thesis.

6.1 ABSTRACT

Twelve maize (*Zea mays* L.) hybrids from a complete diallel of four inbreds differing in their sensitivity to chilling were subjected to two laboratory screening tests at the germination and early growth stages in order to discern differences in chilling sensitivity between the two developmental stages. Hybrids were subjected to either a laboratory germination test which exposed them to a variable chilling regime approximating Ottawa, Ontario's (Lat. 45° 24'N, Long. 75° 43'W) natural spring climate or to an early growth phase test where the hybrids were grown at 11°C until the fourth-leaf stage. Field trials were sown in Ottawa in May of 1993 and 1994 and corresponding parameters to those evaluated in the laboratory were assessed. Estimates of the general combining abilities (GCA) and specific combining abilities (SCA) were performed. Results demonstrated that it is only possible to accurately evaluate chilling tolerance of maize by examining plants at both the germination/emergence and early growth stages as these two stages may be under the control of different genetic factors. Maternal effects were much more pronounced at the fourth-leaf stage than at the germination/emergence stage. Based on the physiological growth parameters assessed both in the laboratory and in the field, it is not possible to accurately predict hybrid maize cold tolerance from knowledge of the inbreds' responses.

Abbreviations: CHU, corn heat units; GCA, general combining ability; SCA, specific combining ability.

6.2 INTRODUCTION

Maize (*Zea mays* L.) is an important economic crop, but as it is of sub-tropical origin, chilling temperatures are a major environmental constraint which limit its range of adaption (Cutworth and Shaykewich, 1990; Koscielniak, 1993). The introduction of maize with high potentials of emergence and growth in cold soils has long been an important objective of maize breeders in temperate regions with short growing seasons (Eagles and Brooking, 1981).

Damage to plant tissues subjected to chilling stress can occur through many metabolic processes. Low temperatures may cause the membrane lipids, normally in a fluid phase, to transform to a gel phase, thus resulting in phase separation of the lipids (Parkin et al, 1989). Chilling can also enhance photoinhibition, leading to production of toxic oxygen compounds (Sonoike and Terashima, 1994). As well, chilling can result in disturbed protein-protein and protein-lipid interactions due to decreased relative strength of hydrophobic bonding (Parkin et al., 1989).

Chilling sensitive plants, such as maize, can exhibit several observable characteristics of chilling stress such as reduced germination/emergence and increased time taken to

germinate/emerge (Eagles and Brooking, 1981; Hodges et al., 1994), loss of turgor (Wilson, 1976; Koscielniak, 1993; Hodges et al., 1995), and reduced dry mass accumulation (Stamp, 1984).

The majority of past efforts to select chilling tolerant maize have relied mainly on results from either the laboratory (Mock and McNeill, 1979; Janowiak and Markowski, 1987; Markowski, 1988) or the field (Mosely et al., 1984) but seldom both. The few methods which have examined both laboratory and field parameters only studied either germination/emergence (McConnell and Gardner, 1979; Hodges et al., 1994) or early growth (Stamp et al., 1986; Hodges et al., 1995). Those that have directly examined both germination/ emergence and early growth parameters in maize, however, have only done so under laboratory conditions (Janowiak and Markowski, 1987). In this laboratory work, no correlation was observed between the cold tolerance of seedlings and the seed germinability of maize inbred and hybrids at chilling temperatures (Janowiak and Markowski, 1987).

Two laboratory screening methods were recently developed for inbred maize lines which determined the potential of both germination/emergence (Hodges et al., 1994) and early growth phase (fourth-leaf) development (Hodges et al., 1995) under chilling temperatures. Furthermore, these laboratory results from both developmental stages were successfully correlated with those from the field (Hodges et al., 1994; Hodges et al.,

1995). Rankings of chilling sensitivity of the inbreds subjected to these screening methods were, to some extent, different between the germination/emergence and early growth stages. However, inbreds because of the homozygosity, often suffer from inbreeding depression and thus hybrids, due to heterosis, are generally more vigourous by comparison (Hallauer et al., 1988). No studies on chilling sensitivity at both the germination/emergence and early growth phase stages of both laboratory and field grown maize hybrids have as of yet been undertaken. Moreover, a complete diallel cross of maize lines has not been analyzed to examine the performance of both germination/emergence and early growth phase laboratory and field parameters.

The objectives of this research were two-fold. Relative chilling sensitivity of twelve maize hybrids, resulting from a complete diallel cross of four differentially sensitive inbred lines, were to be evaluated in both the laboratory and the field with the recently developed germination and early growth phase screening tests (Hodges et al., 1994; Hodges et al., 1995) in order to discern differences in chilling sensitivity between developmental stages. As well, estimates of general combining abilities (GCA), specific combining abilities (SCA), and reciprocal effects were assessed to examine the performance in regards to germination/emergence and early growth parameters of both the maize inbreds in hybrid combinations (GCA) and of the resulting hybrids based on their

parental performance (SCA).

6.3 MATERIALS AND METHODS

6.3.1 Plant Material

The twelve maize hybrids (*Zea mays* L.) studied were produced from a complete diallel cross of four parental inbreds previously selected for differential chilling sensitivity (Hodges et al., 1994; Hodges et al., 1995). One of these four inbreds, CO251, was classified as chilling sensitive, and the other three, CO255, CO304, and CO308 were demonstrated to be relatively more chilling tolerant (Hodges et al., 1994; Hodges et al., 1995). These parental inbreds were classified as such according to two recently developed screening tests for chilling sensitivity at the germination (Hodges et al., 1994) and early growth phase (Hodges et al., 1995) stages. Diallel crosses of these inbreds to produce the hybrid seed used in the following tests were performed in the 1992 and 1993 Agriculture and Agri-Food Canada nursery, Ottawa, Canada.

6.3.2 Germination/Emergence Test

The laboratory germination and field emergence tests were

performed as in sections 2.3.2 and 2.3.3 of this thesis, respectively. The eight replicates of 25 seeds/line were sown in the field at Ottawa on May 5, 1993 and May 4, 1994. Observations were made until approximately 25 days from the date of seeding (May 30, 1993 and 1994).

6.3.3 Early Growth Phase Test

The laboratory early growth phase test was conducted as described in section 3.3.2 of this thesis. Laboratory results were confirmed in the field as described in section 3.3.3 of this thesis. The eight replicates of 25/seeds line were sown in the field at Ottawa on May 5, 1993 and May 4, 1994. Observations were made until approximately 25 days from the date of seeding (May 30, 1993 and 1994).

6.3.4 Statistical Analysis

Analyses of variance and least significant difference tests for the laboratory and field experiments were performed using PROC ANOVA and PROC GLM (SAS, 1989). The laboratory treatments were analyzed as a completely randomized design (hybrid x temperature) and the field trials as a randomized complete block design (hybrid x field site). Correlation coefficients (n=12) were calculated among the laboratory and field responses using MSTAT (1988). The field results for

percentage emergence, average time taken to emerge, and shoot dry weight were averaged over the fields and years after demonstration that there was no significant hybrid x field site interaction. Diallel analysis was based on Griffing's (1956) model I, method 1 for a non-random (fixed) population and all p^2 combinations, where p is the number of parents. The components of variance ratio $2\sigma^2_g/(2\sigma^2_g+\sigma^2_s)$ was computed for the chilled material to determine the relative importance of additive and non-additive gene effects (Baker, 1978). Analysis was conducted on all laboratory and field parameters.

6.4 RESULTS

6.4.1 Field Temperatures

The maximum and minimum temperatures measured at the 5-cm soil depth in the 1993 and 1994 spring field trials were similar to those calculated by averaging 30 yr of past air data (Table 6.1). The values of the accumulated corn heat units (ΣCHU) after 25 days in the field for the 1993 medium (422.7) and the 1994 light (379.5), medium (333.5), and heavy (390.5) soils were similar to those values obtained in both the laboratory germination (473.2) and early growth phase (control:408.9; chilling:382.1) tests.

6.4.2 Laboratory Germination/ Field Emergence

Under the laboratory 25°C control regime, the hybrid maize hybrid CO308xCO304 (97.7 %) was significantly least viable (Table 6.2). All hybrids germinated at 96.6 % or better, and the hybrids with CO308 as the maternal parent took a significantly longer average time to germinate (Table 6.3).

When the hybrids were germinated under the laboratory variable chilling temperature regime, CO308xCO304 had the significantly lowest percentage viability (81.5 %) and germination (82.7 %) (Table 6.3). The hybrids CO255xCO251 and CO255xCO308 had the highest percentages viability and germination. Percentage viability and germination under chilling temperatures were significantly correlated ($r=0.943$, $P \le 0.001$). Under this laboratory chilling regime, CO304xCO308 took the longest (35.26 d), and CO255xCO308 (28.24 d) the shortest average time to germinate (Table 6.3).

As there were significant differences between hybrids in the control results for each parameter assessed (Tables 6.2, 6.3), the ratio of chilling to control values proved essential for determining relative sensitivity to chilling (Hodges et al., 1994; Hodges et al., 1995). Hybrids with lower ratios for percentages viability and germination, and higher ratios for average times taken to germinate could then be characterized as chilling sensitive (Hodges et al., 1994). The chill values obtained for percentages viability and germination from the hybrids when chilled were significantly

correlated with those of their respective chill:control ratios (data not shown). For viability and germination, CO308xCO304 had the lowest chill:control ratios with values of 0.85 and 0.82, respectively (Table 6.2). Values for the other hybrids ranged from 0.94 to 1.01 for both parameters. The chill:control ratios of viability and germination were significantly correlated ($r=0.912$, $P\leq0.001$). For the average time taken to germinate, the ratios clearly exhibited maternal effects with the hybrids with either CO251 or CO308 as maternal parents having the lowest values (Table 6.3).

As there were no significant hybrid x field site interactions, the percentage emergence and average time taken to emerge values for the respective hybrids were averaged over the fields and years. For percentage emergence, CO308xCO251 (78.3 %) had the lowest value, and CO255xCO304 (90.3 %) the highest (Table 6.2). Maternal effects were again apparent for the average time taken to emerge, in which hybrids with CO255 as the maternal parent took significantly less time to emerge, followed by the hybrids with CO251, than CO304 and CO308 as maternal parents (Table 6.3).

6.4.3 Laboratory and Field Early Growth Phase

Maternal effects between hybrids were much more apparent in results from the early growth phase than from the germination/emergence tests, particularly in the chill:control

ratios.

Total and shoot dry masses of the laboratory control-grown hybrids were highest when CO308 was the maternal parent (Table 6.5). Values for the hybrids with CO308 as the maternal parent ranged from 0.190 to 0.239 g for the total dry mass and 0.157 to 0.197 g for the shoot dry mass. The dry mass of the roots was highest in both hybrids with CO255 (0.035 to 0.049 g) and CO308 (0.032 to 0.043 g) as maternal parents. In terms of maternal effects, it was difficult to distinguish which hybrid group had the lowest total, shoot, and root dry masses. Similarly, there were no readily discernable differences between maternal groups with respect to shoot and root percentage water content (Table 6.5).

Upon chilling in the laboratory, hybrids with CO251 as the maternal parent generally had the lowest total (0.071 to 0.093 g) and shoot (0.051 to 0.061 g) dry masses, and both hybrids with CO251 and CO304 as the maternal parents the lowest root dry mass (Table 6.4). Total dry mass was significantly correlated to both shoot ($r=0.996$, $P\leq0.001$) and root ($r=0.930$, $P\leq0.001$) dry masses. Those hybrids with CO308 as the maternal parent had the lowest percentage water content in the shoot (64.61 to 67.96 %) (Table 6.5). There was no discernable difference between the maternal groups for the percentage root water content.

Again, the chill:control ratios were essential to differentiate between chilling sensitive and tolerant hybrids.

Those hybrids with the lowest ratios for total, shoot, and root dry masses and percentage water content are classified as the most chilling sensitive (Hodges et al., 1995). Values obtained for total, shoot, and root and the percentage water content from the chilled hybrids were significantly correlated with their respective chill:control ratios (data not shown). Total dry mass chill:control ratios were significantly correlated with those of shoot ($r=0.994$, $P \leq 0.001$) and root ($r=0.896$ $P \leq 0.001$) dry masses in demonstrating that hybrids with CO308 as the maternal parent were the lowest with values between 0.34 to 0.44, 0.30 to 0.36, and 0.43 to 0.63 for total, shoot, and root dry masses, respectively (Table 6.4). The chill:control ratio for the percentage water content of the shoot again demonstrated maternal effects, with hybrids with CO308 as the maternal parent having the lowest values (0.70 to 0.75) (Table 6.5). There were no readily discernable differences between maternal hybrid groups in respect to percentage water content of the root.

As there were significant hybrid x field interactions, the field shoot dry mass values for the respective hybrids were averaged over fields and years. Generally, the hybrids with CO304 as the maternal parent had the lowest shoot dry mass (0.071 to 0.087 g) and the hybrids with CO255 as the maternal parent the greatest (0.091 to 0.126 g) (Table 6.6). As a results of the high values from the laboratory total and shoot dry masses, these did not correlate with the shoot dry

mass of the averaged field. However, when the three hybrids with CO304 as the maternal parent were removed from the equation, the field shoot dry masses of the other nine hybrids became significantly correlated with those of total dry mass for both the chilled ($r=0.902$, $P=0.001$) and the chill:control ratio ($r=0.810$, $P=0.006$) values, and the shoot dry mass for both the chilled ($r=0.934$, $P\leq0.001$) and the chill:control ratio ($r=0.803$, $P=0.007$) values.

6.4.4 Diallel Analysis

The analyses of variance of the general combining ability effects (GCA), specific combining ability effects (SCA), and reciprocal effects are shown in Table 6.7. Data for the inbreds for the germination/emergence and early growth phase tests from both the laboratory and field are from previous work (Hodges et al., 1994; Hodges et al., 1995). The components of variance ratio were 0.16, 0.33, and 0.92 for the chilled laboratory percentages germination and viability, and average time taken to germinate, respectively. For the early growth phase, the components of variance were 0.40, 0.14, and 0.85 for the chilled total , shoot, and root dry masses, respectively, and 0.22 and 0.19 for the percent water content of the shoot and root, respectively. For the field tests, the components of variance were 0.05, 0.33, and 0.32 for the percentage emergence, average time taken to emerge, and shoot

dry mass, respectively.

There were significant differences in the mean squares of GCA effects for all laboratory germination and early growth parameters and with field percentage emergence, but not for both field average time taken to emerge and shoot dry mass (Table 6.7). Significant differences existed in the mean squares of SCA effects for all laboratory and field parameters with the exception of laboratory control percentage viability and shoot dry mass, laboratory chill root dry mass and field average time taken to emerge. Significant reciprocal effects existed for all laboratory chilled values, all laboratory control values except for percentage viability, and for the field shoot dry mass.

Estimates of GCA, SCA, and reciprocal effects are given in Table 6.8. Negative effects are associated with chilling sensitivity and positive effects are associated with chilling tolerance.

From GCA estimates, it is apparent that the original chilling sensitive inbred CO251 and chilling tolerant inbred CO304 had worse than expected performance in hybrid combinations when chilled for percentages viability and germination (Table 6.8). The two other chilling tolerant original inbreds CO255 and CO308 had better than expected performance for these two parameters. Only the parental inbred CO304 had better than expected performance in regards to the average time taken to germinate when chilled; the

others performed worse than expected. The inbreds CO251 and CO304 performed better than expected for percentage water content of both shoot and root, while CO255 and CO308 had considerably poorer performance. The original chilling sensitive inbred CO251 performed worse than expected for total, shoot, and root dry masses while the other chilling tolerant hybrids performed either as expected or better. This CO251 inbred also performed worse than expected in hybrid combinations for the field parameters percentage emergence, average time taken to emerge, and shoot dry mass. With the exception of CO308 and CO255, which performed worse than expected for percentage emergence and average time taken to emerge, respectively, the tolerant parental inbreds performed better than expected.

The SCA estimates indicated that, in general, the hybrids with CO308 as the maternal parent had worse performance when chilled than expected based on the parental performance of percentages viability, germination and emergence, total and shoot dry masses, and average time taken to emerge (Table 6.8). The hybrids with CO251 as the maternal parent, however, performed better than expected, and better than any other maternal group, for percentages viability, germination, and emergence. There were few other distinguishing differences observed between the four maternal groups.

Reciprocal effects served to demonstrate maternal effects of the original four inbred maize hybrids in producing the

twelve hybrids through the complete diallel cross.

6.5 DISCUSSION

An objective of this work was to evaluate both in the laboratory and in the field the relative chilling sensitivity of twelve hybrid maize hybrids by using recently developed screening methods at both the germination/emergence and early growth phase (fourth-leaf) developmental stages. Differences between hybrids in regards to chilling sensitivity were greater at the early growth phase than at the germination/emergence stage. Maternal effects were observed in the germination/emergence stage but were more pronounced in the early growth phase.

In the germination/emergence stage, the chill:control ratios for the respective laboratory parameters of the hybrids were fairly closely grouped. Viability and germination chill:control ratios selected CO308xCO304 as the most chilling sensitive hybrid and the maternal group of CO255 among the most tolerant. The field percentage emergence and average time taken to emerge, the latter demonstrating pronounced maternal effects, agreed with the chill:control ratio of viability and germination in indicating the hybrids with CO255 as the maternal parent as being the most tolerant. However, the laboratory average time taken to germinate, another parameter demonstrating maternal effects, selected the

hybrids with CO308 as the maternal parent as being the most tolerant and the hybrids with CO251 as the maternal parent as the most sensitive of the hybrids. Thus, as with the inbred results (Hodges et al., 1994), the average times taken to germinate and emerge did not agree in selecting for relative chilling sensitivity. As percentage emergence, or percentage germination, is a significant criterion for selection programs under chilling conditions (McConnel and Gardner, 1979), it is clear that the average time taken to emerge or to germinate is not a distinguishing parameter for selection processes for at least this set of genotypes, inbred or hybrid. However, as there are only small differences between values, and as maternal effects are not as pronounced for percentages viability, germination, and emergence as they were for the average times taken to germinate or emerge, it is more difficult than with the inbreds (Hodges et al., 1994) or with hybrids of the early growth phase to assess differential chilling sensitivity among hybrids at the germination/emergence stage. As strong maternal effects have been observed for both percentage germination (Eagles and Hardacre, 1979) and time to germination (Maryam and Jones, 1983) in other maize lines, the fact that these hybrids don't express them as clearly for percentages viability, germination and emergence as they did for average times taken to germinate and emerge suggests that the physiological parameters may be under the control of different genetic factors. As well, the

differences between the values of percentages viability, germination, and emergence were smaller for the hybrids than for the inbreds at this stage could be a manifestation of inbreeding depression. Similar conclusions were reached by Janowiak and Markowski (1987).

The range between values was greater for the fourth-leaf early growth stage parameters of the maize hybrids, although still not as great as they were for their parental inbreds (Hodges et al., 1995). As well, maternal effects were much more pronounced at this later stage. Chill:control ratios of laboratory total, shoot, and root dry masses and percent water content of the shoot agreed in selecting the hybrids with CO308 as the maternal parent as the most chilling sensitive and those with CO304 as the maternal parent as the most chilling tolerant of the hybrids. The shoot dry mass from the field implied that the hybrids with CO304 as the maternal parent was one of the most chilling sensitive, whereas it was indicated by the laboratory dry mass and percent water content of the shoot to be the most resistant. When the data from the hybrids with CO304 as the maternal parent was removed, however, the laboratory total and shoot dry mass results correlated with those of the field. Substantially lower temperatures in the field than in the laboratory may have affected the CO304 maternal group more than any other. Past work with maize has shown laboratory dry masses exposed to chilling conditions to be well correlated with field dry mass

grown under cool conditions (Stamp et al., 1986, Hodges et al., 1995).

Although the range between values of the germination/emergence parameters were sufficiently large for clearly evaluating differential chilling sensitivity in the inbreds (Hodges et al., 1994), this was not so for the hybrids at this stage. Thus it was only possible to accurately evaluate differential chilling sensitivity of hybrids at the early growth stage, unlike the situation with the inbreds where evaluation could be performed at both the germination/emergence and early growth stage phases. However, both inbred (Hodges et al., 1994; Hodges et al., 1995) and hybrid results suggest that maize responses to chilling changed as the plants aged, as some lines which were initially chilling sensitive or tolerant at the germination/emergence stage altered their sensitivity to chilling at the early growth stage. For example, the hybrids with CO255 as the maternal parent was selected as being most chilling tolerant at the percentage/emergence stage but was not so at the early growth phase stage. Chilling sensitivity of both inbred and hybrid maize lines at the two developmental stages may be under the control of different genetic factors. A similar conclusion was reached for both inbred and hybrid maize lines by Janowiak and Markowski (1987) although Mock and McNeill (1978) found that field percentage emergence and seedling dry mass were significantly correlated in 34 maize inbreds.

The observation of maternal effects in the hybrids, more pronounced at the fourth-leaf early growth stage, has appeared in many studies of cold tolerance in maize. The genetic nature of chilling sensitivity in maize has previously been observed to be complexly inherited because of significant maternal or cytoplasmic effects associated with inheritance of germination or early seedling growth potential (Eagles and Hardacre, 1979; Maryam and Jones, 1983). However, Aidun et al. (1991) found no maternal effects among six early growth phase reciprocal maize hybrids involving a common parent and, contrary to our results, concluded that the inheritance of cold tolerance may be restricted to the germination/emergence phase of maize development.

General combining abilities (GCA) indicate the average performance of a parent in hybrid combination. Specific combining abilities (SCA) indicate which hybrid combinations do better or worse than expected based upon the GCA of the lines involved (Griffing, 1956). Estimates of GCA effects indicated that, when chilled, CO251, the most chilling sensitive of the parental inbreds (Hodges et al., 1994; Hodges et al., 1995), had the poorest combining abilities compared to the other relatively chilling tolerant inbreds CO255, CO304, and CO308 for (Hodges et al., 1994; Hodges et al., 1995) for laboratory percentages viability and germination, and total, shoot, and root dry masses and field percentage emergence and shoot dry mass. However, SCA effects demonstrated that this

chilling sensitive inbred generally had better combining abilities as a maternal parent when chilled than CO308 for laboratory percentages viability and germination, percent water content of the shoot, total and shoot dry mass, and all field parameters. Estimates of SCA effects for hybrids with CO308 as the maternal parent, the most chilling sensitive hybrids at the early growth stage, demonstrated that these hybrids generally performed worse than any others for total and shoot dry masses and percentage water content of the shoot. The relative importance of GCA and SCA in determining progeny performance was assessed by calculating the ratio of the components of variance as outlined by Baker (1978). These ratios were generally not close to unity for any of the parameters measured. These results, and those of the reciprocals, suggest that it is not possible to accurately predict chilling sensitivity of the hybrids based solely upon the performance of GCA effects of its parents or the parental inbred reaction to chilling stress. This disagrees with the results of Maryam and Jones (1983), who found that the performance of hybrids, based on days to germination, greatly depended upon the parental inbred lines. This disagreement may be in part due to discrepancies between time to germination/emergence and percentage germination/emergence found in an earlier report (Hodges et al. 1994). The results of this work do agree with McConell and Gardner (1979) and Aidun et al. (1991), who, after measuring physiological growth

parameters, also determined that inbred maize cold tolerance cannot be used to accurately predict hybrid cold tolerance.

In conclusion, it is only possible to accurately evaluate chilling tolerance of inbred or hybrid maize by examining plants at both the germination/emergence and early growth stages as these two stages may be under the control of different genetic factors. Maternal effects were much more pronounced at the fourth-leaf early growth phase stage than at the germination/emergence stage. Finally, this work has shown that, based on these physiological growth parameters, it is not possible to accurately predict hybrid maize cold tolerance from that of inbreds.

6.6 ACKNOWLEDGEMENTS

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Table 6.1. Five day averages of maximum and minimum temperatures (°C) and accumulated corn heat units (ΣCHU) from both the laboratory germination and early growth phase tests and the 1993 medium (sand and clay) and 1994 light (sandy loam), medium, and heavy (clay) textured soil field trials.

Date	Laboratory test			Field trials[y]											
				1993			1994								
				Medium soil			Light soil			Medium soil			Heavy soil		
	Max.	Min.	ΣCHU	Max.	Min.	ΣCHU	Max.	Min.	ΣCHU	Max.	Min.	ΣCHU	Max.	Min.	ΣCHU
(A) Germination test[z]															
Apr 15	10.3	0.4	3.2												
Apr 20	11.7	2.4	16.4												
Apr 25	13.4	2.5	42.8												
Apr 30	15.9	4.3	84.2												
May 5	18.1	5.1	129.1												
May 10	16.2	3.8	173.7	17.0	9.5	70.9	22.9	5.7	78.5	18.8	8.7	76.5	21.3	8.6	86.5
May 15	17.9	6.3	235.3	20.5	11.2	165.5	17.4	5.4	133.0	14.0	4.4	104.5	15.3	6.6	134.5
May 20	19.4	6.4	303.4	18.4	10.3	245.4	18.0	5.6	191.5	16.3	6.1	156.5	16.9	6.6	192.0
May 25	20.6	8.3	386.6	18.4	9.1	322.7	25.0	8.6	288.0	24.6	9.7	257.5	24.5	9.5	291.5
May 30	21.0	9.1	473.2	20.2	12.6	422.7	23.1	8.5	379.5	18.4	9.0	333.5	23.4	10.0	390.5
(B) Early growth phase test															
(I) Control															
12 days	25.0	25.0	408.9												
(II) Chilling															
5 days	25.0	25.0	170.3												
28 days	11.0	11.0	382.1												

[z]Temperatures and ΣCHU were calculated for the laboratory germination test from 30 year averages.
[y]Field temperatures were recorded at a 5 cm depth. The ΣCHU were calculated from shade temperatures measured 1 m aboveground using the formula daily CHU = $[3.33(T_{max}-10°C)-0.084(T_{max}-10°C)^2 + 1.8(T_{min}-4.4°C)]/2$ (Brown, 1969).

Table 6.2. Control (25°C), chilling (variable temperature), and the chill : control ratio of both percent viability and germination from the laboratory germination test and the percent emergence from averaged 1993 and 1994 field trials.

Hybrid	% Viability			% Germination			% Emergence
	Control	Chill	Chill/ Control	Control	Chill	Chill/ Control	
CO251xCO255	100.0^{b}	97.0^{c}	0.97	99.7^{b}	94.8^{bcd}	0.95	83.7^{abc}
CO251xCO304	99.7^{b}	96.3^{bc}	0.97	100.0^{b}	94.2^{bc}	0.94	82.0^{ab}
CO251xCO308	100.0^{b}	97.0^{c}	0.97	99.7^{b}	98.2^{cd}	0.98	81.1^{ab}
CO255xCO251	100.0^{b}	99.7^{c}	1.00	100.0^{b}	99.7^{d}	1.00	89.3^{c}
CO255xCO304	99.7^{b}	99.3^{c}	1.00	99.0^{b}	96.0^{bcd}	0.97	90.3^{c}
CO255xCO308	100.0^{b}	99.7^{c}	1.00	100.0^{b}	99.0^{cd}	0.99	84.0[illegible]
CO304xCO251	99.7^{b}	93.3^{b}	0.94	96.6^{a}	91.2^{b}	0.94	78.5^{a}
CO304xCO255	99.7^{b}	98.3^{c}	0.99	99.3^{b}	93.8^{bc}	0.94	78.7^{ab}
CO304xCO308	100.0^{b}	99.0^{c}	0.99	99.0^{b}	95.2^{bcd}	0.96	84.3^{abc}
CO308xCO251	99.7^{b}	98.3^{c}	0.99	96.7^{a}	97.6^{cd}	1.01	78.3^{a}
CO308xCO255	99.3^{b}	98.7^{c}	0.99	98.6^{b}	97.3^{cd}	0.99	84.3^{a}
CO308xCO304	97.7^{a}	82.7^{a}	0.85	99.0^{b}	81.5^{a}	0.82	84.5^{bc}

$^{a-d}$Indicates differences among the values within tests. Values with the same letter are not significantly different at the 5% level according to Fisher's LSD.

Table 6.3. Control (25°C), chilling (variable temperature), and the chill : control ratio of average time to germinate from the laboratory germination test and the average time to emerge from the averaged 1993 and 1994 field trials.

Hybrid	Average time to germinate (days)			Average time to emerge (days)
	Control	Chill	Chill/ Control	
CO251xCO255	3.07[a]	30.91[b]	10.07	15.90[c]
CO251xCO304	3.20[ab]	34.61[de]	10.81	15.54[bc]
CO251xCO308	3.33[b]	31.24[b]	9.38	16.22[cd]
CO255xCO251	3.20[ab]	29.25[a]	9.14	14.93[a]
CO255xCO304	3.43[b]	34.07[d]	9.93	14.55[a]
CO255xCO308	3.13[ab]	28.24[a]	9.02	14.99[ab]
CO304xCO251	3.69[c]	32.89[c]	8.91	16.34[cde]
CO304xCO255	3.76[cd]	31.43[b]	8.36	16.62[de]
CO304xCO308	3.71[cd]	35.26[e]	9.50	17.25[f]
CO308xCO251	4.09[e]	31.58[b]	7.72	16.81[e]
CO308xCO255	3.92[de]	28.48[a]	7.27	16.23[cd]
CO308xCO304	4.34[f]	34.07[d]	7.85	16.96[ef]

[a-f]Indicates differences among the values within tests. Values with the same letter are not significantly different at the 5% level according to Fisher's LSD.

Table 6.4. Total, shoot, root dry mass (grams) and the chill:control ratio for control (25°C) and chilling (11°C) regimes from the laboratory early growth phase test.

	Laboratory dry mass								
	Total			Shoot			Root		
Hybrid	Control	Chill	Chill/ Control	Control	Chill	Chill/ Control	Control	Chill	Chill/ Control
	g			g			g		
CO251xCO255	0.127^{de}	0.058^{d}	0.46	0.098^{cd}	0.043^{d}	0.44	0.029^{g}	0.015^{i}	0.52
CO251xCO304	0.113^{e}	0.060^{d}	0.53	0.092^{cd}	0.047^{d}	0.51	0.021^{j}	0.013^{k}	0.62
CO251xCO308	0.136^{de}	0.075^{d}	0.55	0.105^{cd}	0.056^{cd}	0.53	0.032^{f}	0.019^{h}	0.56
CO255xCO251	0.129^{de}	0.087^{cd}	0.67	0.094^{cd}	0.061^{cd}	0.65	0.035^{e}	0.026^{d}	0.74
CO255xCO304	0.165^{cd}	0.122^{bc}	0.74	0.129^{bcd}	0.098^{bc}	0.76	0.037^{d}	0.024^{e}	0.65
CO255xCO308	0.162^{cd}	0.141^{b}	0.87	0.112^{cd}	0.113^{b}	1.01	0.049^{a}	0.028^{c}	0.57
CO304xCO251	0.117^{e}	0.077^{d}	0.66	0.098^{cd}	0.062^{cd}	0.63	0.019^{k}	0.015^{j}	0.79
CO304xCO255	0.114^{e}	0.196^{a}	1.72	0.088^{d}	0.165^{a}	1.88	0.025^{i}	0.031^{b}	1.24
CO304xCO308	0.163^{cd}	0.219^{a}	1.34	0.135^{bc}	0.179^{a}	1.33	0.028^{h}	0.040^{a}	1.43
CO308xCO251	0.239^{a}	0.082^{cd}	0.34	0.197^{a}	0.059^{cd}	0.30	0.042^{c}	0.018^{i}	0.43
CO308xCO255	0.210^{ab}	0.093^{cd}	0.44	0.167^{ab}	0.061^{cd}	0.36	0.043^{b}	0.022^{f}	0.51
CO308xCO304	0.190^{bc}	0.071^{d}	0.37	0.157^{ab}	0.051^{d}	0.32	0.032^{f}	0.020^{g}	0.63

$^{a-k}$Indicates differences among the values within tests. Values within the same column with the same letter are not significantly different at the 5% level according to Fisher's LSD.

Table 6.5. Shoot and root percent water content and the chill:control ratios of the control (25°C) and chilling (11°C) from the laboratory growth phase test.

Hybrid	Shoot			Root		
	Control	Chill	Chill/ Control	Control	Chill	Chill/ Control
	%	%		%	%	
CO251xCO255	92.73^{a}	80.61^{bc}	0.87	95.44^{ab}	92.19^{bc}	0.97
CO251xCO255	90.94^{a}	80.05^{bc}	0.88	95.35^{ab}	92.06^{bc}	0.97
CO251xCO308	90.51^{a}	78.79^{c}	0.87	94.03^{b}	90.41^{de}	0.96
CO255xCO251	90.57^{a}	82.66^{b}	0.91	94.27^{b}	92.05^{bc}	0.98
CO255xCO304	91.08^{a}	68.54^{d}	0.75	95.06^{ab}	91.60^{bcd}	0.96
CO255xCO308	91.69^{a}	79.91^{bc}	0.87	94.57^{ab}	88.50^{f}	0.94
CO304xCO251	91.40^{a}	81.91^{bc}	0.90	95.71^{a}	89.93^{ef}	0.94
CO304xCO255	90.47^{a}	80.63^{bc}	0.89	94.92^{ab}	90.79^{cde}	0.96
CO304xCO308	91.89^{a}	87.33^{a}	0.95	94.95^{ab}	90.87^{cde}	0.96
CO308xCO251	91.57^{a}	69.13^{d}	0.75	94.81^{ab}	92.58^{b}	0.98
CO308xCO255	92.05^{a}	64.61^{e}	0.70	95.21^{ab}	91.87^{bc}	0.96
CO308xCO304	91.55^{a}	67.96^{d}	0.74	95.04^{ab}	94.21^{a}	0.99

$^{a-f}$Indicates differences among the values within tests. Values within the same column with the same letter are not significantly different at the 5% level according to Fisher's LSD.

Table 6.6 Shoot dry mass (grams) from the averaged 1993 and 1994 field trials.

Hybrid	Field shoot dry mass
	g
CO251xCO255	0.073[c]
CO251xCO304	0.080[c]
CO251xCO308	0.087[bc]
CO255xCO251	0.096[bc]
CO255xCO304	0.139[a]
CO255xCO308	0.126[ab]
CO304xCO251	0.071[c]
CO304xCO255	0.071[c]
CO304xCO308	0.087[bc]
CO308xCO251	0.082[c]
CO308xCO255	0.086[c]
CO308xCO304	0.089[bc]

[a-c]Indicates differences among the values within tests. Values within the same columns with the same letter are not significantly different at the 5% level according to Fisher's LSD.

Table 6.7. Analysis of variance of a complete diallel cross of laboratory control (25°C) and chilling results for % germination, % viability, average time to germinate, total, root, and shoot dry masses, and % water shoot and root, and field results for % emergence average time to emerge, and shoot dry mass.

		Mean Square	
Source	df	Control	Chill
(II) Germination/emergence			
Laboratory % germination			
GCA	3	6.39*	152.21***
SCA	6	6.13***	110.44***
Reciprocal	6	1.89	19.50***
Error	30	1.49	2.77
Laboratory % Viability			
GCA	3	3.05***	52.94***
SCA	6	0.84	35.17***
Reciprocal	6	1.61**	17.07***
Error	30	0.46	3.57
Laboratory average time to germinate			
GCA	3	0.76***	17.41***
SCA	6	0.93***	1.87***
Reciprocal	6	0.17***	1.20***
Error	30	0.01	0.27
Field % emergence			
GCA	3	130.70***	
SCA	6	144.00***	
Reciprocal	6	16.58	
Error	45	16.72	
Field average time to emergence			
GCA	3	1.56	
SCA	6	3.06	
Reciprocal	6	0.42	
Error	45	1.59	

Table 6.7. Analysis of variance for the diallel cross (cont'd).

Source	df	Mean Square Control	Mean Square Chill
(II) Early growth phase			
Laboratory total dry mass			
GCA	3	0.005***	0.006**
SCA	6	0.001*	0.003*
Reciprocal	6	0.001***	0.001*
Error	45	0.000	0.001
Laboratory shoot dry mass			
GCA	3	0.003***	0.003***
SCA	6	0.000	0.002***
Reciprocal	6	0.001***	0.002***
Error	45	0.000	0.000
Laboratory root dry mass			
GCA	3	0.002***	0.000***
SCA	6	0.000*	0.000
Reciprocal	6	0.000*	0.000*
Error	45	0.000	0.000
Laboratory % water shoot			
GCA	3	0.84***	23.96***
SCA	6	1.48***	31.40***
Reciprocal	6	0.18**	71.55***
Error	45	0.02	1.75
Laboratory % water root			
GCA	3	0.26***	3.34***
SCA	6	0.64***	5.97***
Reciprocal	6	0.21***	2.82***
Error	45	0.03	0.33
Field shoot dry mass			
GCA	3	0.01	
SCA	6	0.10***	
Reciprocal	6	0.03***	
Error	45	0.01	

*,**,*** indicate significance at the 0.05, 0.01, and 0.001 level, respectively.

Table 6.8. Estimates of general and specific combining abilities and reciprocal effects of a complete diallel cross of laboratory control (25°C) and chilling results for % germination, % viability, average time to germinate, total, root, and shoot dry masses, and % water shoot and root, and field results for % emergence average time to emerge, and shoot dry mass.

	Laboratory tests									
	% Germination		% Viability		Avg. time germ.		% Water shoot		% Water root	
Hybrid	Control	Chill	Control	Chill	Control	Chill	Control	Chill	Control	Chill
CO251	-0.51	-4.50*	-0.23	-3.44	-0.21	-0.12	-0.32*	+1.57	+0.07	+0.26
CO251xCO255	+0.96	+4.13**	+0.65	+2.63	-0.49***	+0.53	+0.57***	+2.11	+0.17	+1.93***
CO251xCO304	+1.45	+7.34***	+0.22	+2.82	-0.31***	-0.77	+0.47***	-0.99	+0.54***	-0.68
CO251xCO308	+0.44	+7.20	-0.77	+4.61*	-0.21*	+0.24	+0.11	-4.87***	-0.15	+0.84
CO255	+1.23	+4.52*	+0.69	+2.68	-0.07	-2.17***	-0.22	-1.08	-0.09	-0.74
CO255xCO251	+0.17	+2.43	+0.00	+2.20	+0.07	-0.83	-0.33*	+1.02	-0.58***	-0.07
CO255xCO304	+0.54	+0.31	+0.32	-0.99	-0.31***	+0.24	+0.09	-4.75***	+0.16	+0.52
CO255xCO308	-0.17	-2.32	-0.35	-0.35	-0.49***	-0.77	+0.85***	-3.83***	+0.47**	+0.42
CO304	-0.78	-2.87	-0.73	-0.14	+0.07	+2.84***	+0.15	+1.37	+0.22	+0.75
CO304xCO251	-1.68	-1.85	-1.52*	-1.50	+0.26**	-0.90	+0.23	+0.93	+0.18	-1.06*
CO304xCO255	+1.67	-0.59	+0.17	+1.18	+0.16	-1.32**	-0.19	+6.04***	-0.07	-0.40
CO304xCO308	+1.50	-4.71***	+0.55	-3.86*	-0.17	+0.53	+0.33*	-0.97	+0.34*	+1.40**
CO308	+0.08	+2.85	+0.27	+0.90	+0.21	-0.54	+0.38*	-1.89	-0.20	-0.27
CO308xCO251	-1.50	-1.02	-1.50	+0.67	+0.40***	+0.16	+0.53***	-4.73***	+0.39*	+1.08*
CO308xCO255	-0.68	-0.57	-0.50	-0.17	+0.42***	+0.12	+0.17	-7.65***	+0.32*	+1.78***
CO308xCO304	-0.17	-6.85***	-0.17***	-6.50***	+0.30	-0.60	-0.17	-9.76***	+0.11	+1.68***

*,**,*** Significantly different from 0 at the 0.05, 0.01, and 0.001 probability levels, respectively

Table 6.8. Estimates of general and specific combining abilities and reciprocal effects (cont'd).

	Laboratory tests						Field trials		
	Total dry mass		Shoot dry mass		Root dry mass.				
Hybrid	Control	Chill	Control	Chill	Control	Chill	% Emergence to emerg.	Avg. time dry mass	Shoot
CO251	-0.02*	-0.04	-0.01	-0.03*	-0.00	-0.01*	-4.17	-0.28	-0.05
CO251xCO255	+0.00	-0.03	-0.01	-0.02*	-0.02**	-0.01**	+6.01	+0.06	+0.11
CO251xCO304	-0.01	-0.02	-0.01	-0.02*	+0.00	+0.00	+3.35	+0.51	+0.06
CO251xCO308	+0.02*	-0.03	+0.01	-0.02*	+0.00	+0.00	+6.19	+0.97	+0.08
CO255	+0.00	+0.01	-0.01	+0.01	+0.00	+0.01*	+5.01	-0.47	+0.01
CO255xCO251	+0.00	+0.02	+0.00	+0.01	+0.00	+0.01**	+0.83	-0.49	+0.11
CO255xCO304	+0.00	+0.02	+0.01	+0.01	+0.00	+0.00	+1.54	+0.68	+0.16
CO255xCO308	+0.00	-0.04	+0.00	-0.03**	+0.00	-0.01**	+1.49	+0.26	+0.05
CO304	-0.01	+0.00	-0.01	+0.01	+0.00	+0.00	+1.34	+0.32	+0.04
CO304xCO251	+0.00	+0.01	+0.00	+0.01	+0.00	+0.00	-3.60	-0.35	-0.07
CO304xCO255	+0.03***	+0.04	-0.02**	+0.03**	+0.00	+0.00	-4.39	-0.06	+0.02
CO304xCO308	+0.00	+0.01	+0.01	+0.00	+0.00	+0.00	+5.74	+0.95	+0.20**
CO308	+0.04***	+0.02	+0.03*	+0.02*	+0.01*	+0.01*	-2.17	+0.43	+0.00
CO308xCO251	+0.05***	+0.00	+0.05***	+0.00	+0.01**	+0.01**	-3.18	-0.43	+0.11
CO308xCO255	+0.03***	-0.03	+0.03***	-0.03**	+0.00	+0.01**	-1.59	-0.08	-0.07
CO308xCO304	+0.01	-0.08*	+0.01	-0.06***	+0.00	-0.01**	-2.06	-0.85	+0.24***

*,**,*** Significantly different from 0 at the 0.05, 0.01, and 0.001 probability levels, respectively

CHAPTER SEVEN

ANTIOXIDANT ENZYME AND COMPOUND RESPONSES TO CHILLING STRESS AND THEIR COMBINING ABILITIES IN DIFFERENTIALLY SENSITIVE HYBRID MAIZE LINES

The following chapter is a reproduction of a paper submitted by D.M. Hodges, C.J. Andrews, D.A. Johnson, and R.I. Hamilton. The principal author (D.M. Hodges) accumulated and interpreted all data. The fourth author (Dr. R.I. Hamilton) produced and contributed seed for the hybrid maize lines used in these experiments.

With the identification of the chilling sensitive and hybrid maize lines (**Chapter Six**), the third objective of this thesis, to determine if there were similar results for antioxidant capacities and metabolic indicator concentrations for the chilled hybrids as there were for the inbred maize lines of the second objective could be met. As well, the feasibility of a screening test for chilling sensitivity in both inbred and hybrid maize lines could be determined. Furthermore, evaluations of the general and specific combining abilities of these parameters between hybrids and their parental inbreds could be performed in order to determine if hybrid chilling sensitivity relative to antioxidant enzyme and metabolic indicators could be predicted from those of the inbreds. Results demonstrated that when the hybrids were

chilled, activities of CAT, MDHAR, and ASPX were lowest and concentrations of carbohydrates highest in the most chilling sensitive hybrids as they were with the parental inbreds of **Chapter Four**. Differences between lines in antioxidant capacities to detoxify toxic oxygen compounds may be important in differential chilling sensitivity. The higher concentration of carbohydrates in the chilling sensitive hybrid lines after chilling indicated that their rate of assimilate utilization was reduced by the chilling treatment more so than those of the chilling tolerant lines. Activities of ASPX, CAT, and MDHAR and concentrations of carbohydrates were negatively correlated. Percent of control activities of CAT, MDHAR, and ASPX, and concentrations of carbohydrates would thus make excellent indicators of chilling sensitivity in a screening technique for chilling sensitivity of both inbred and hybrid lines of maize. However, results of the GCA and SCA estimates demonstrated that it is not possible to accurately predict chilling sensitivity of hybrids based on the chilling sensitivity of their parental inbreds.

7.1 ABSTRACT

Activities of antioxidant enzymes and concentrations of antioxidant compounds and carbohydrates were assessed in twelve leaves of differentially sensitive maize (*Zea mays* L.) hybrids, resulting from a complete diallel cross of four original inbreds, in order to determine if these antioxidant and metabolic parameters would be useful in a proposed screening technique for chilling sensitivity in maize. Leaves of the maize hybrids were harvested at the third leaf stage under either constant (control) 25°C or under 11°C for 1 d prior to harvesting. The photocycle was 16:8 L:D with a photon flux rate of 450-500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Catalase (CAT; EC 1.11.1.6), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), and ascorbate peroxidase (ASPX; EC 1.11.1.11) activities and concentrations of total ascorbate and glutathione and the ratios of DAsA/AsA and GSH/GSSG were assessed. Levels of reducing and non-reducing sugars and starch were determined as general metabolic indicators of chilling stress. Estimates of the general combining abilities (GCA) and specific combining abilities (SCA) were performed. When the plants were chilled, percent of control activities of CAT, MDHAR, and ASPX were lowest and concentrations of carbohydrates highest in the most chilling sensitive hybrids. Differences between hybrids in antioxidant capacities to detoxify toxic oxygen compounds may be important in differential chilling sensitivity. The higher percent of control concentration of carbohydrates in the

chilling sensitive hybrids after chilling indicated that their rate of assimilate utilization was reduced by the chilling treatment more so than those of the chilling tolerant hybrids. Percent of control activities of ASPX, CAT, and MDHAR and concentrations of carbohydrates were negatively correlated. Activities of CAT, MDHAR, and ASPX, and concentrations of carbohydrates would thus make excellent indicators of chilling stress in a screening technique for chilling sensitivity of maize. However, results of the GCA and SCA estimates demonstrated that it is not possible to accurately predict chilling sensitivity of hybrids based on the chilling sensitivity of their parental inbreds.

Abbreviations: AsA, reduced ascorbate; ASPX, ascorbate peroxidase; CAT, catalase; DAsA, dehydroascorbate; GCA, general combining ability; GSH, reduced glutathione; GSSG oxidized glutathione; MDHAR, monodehydroascorbate reductase; SCA, specific combining ability.

7.2 INTRODUCTION

Exposure to sub-optimal chilling temperatures may lead to an increased proliferation of toxic oxygen compounds present in chilling sensitive plants such as maize (*Zea mays* L.). These toxic oxygen byproducts, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH), may

be produced under low temperatures due to chilling-induced photoinhibition (Hodgson and Raison, 1991; Sonoike and Terashima, 1994; Terashima et al., 1994; Havaux and Davaud, 1994). In this process, over-energization of the photosystem reaction centres can result from restricted carbon metabolism, a symptom of low temperature stress (Schöner and Krause, 1990). This would lead to an inadequate supply of the natural photosystem electron acceptor $NADP^+$ (Cakmak and Marschner, 1992; Öquist and Huner, 1993). Molecular oxygen may then become reduced instead of $NADP^+$ at the iron-sulphur centres or by ferredoxin, producing O_2^- (Long, 1983; Hodgson and Raison, 1991). Alternatively, oxygen may be reduced directly by PS I, producing O_2^-which then destroys the iron-sulphur centres of PS I (Sonoike and Terashima, 1994).

Plants have evolved antioxidant systems to protect themselves against these toxic oxygen byproducts. Antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), located in the chloroplasts, mitochondria, and cytosol, remove O_2^- (Salin, 1988). Others such as the mainly chloroplast-localized ascorbate peroxidase (ASPX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), and glutathione reductase (GR; EC 1.6.4.2) (Scandalios, 1993), and catalase (CAT; EC 1.11.1.6), principally located in the peroxisomes and glyoxysomes (Prasad et al., 1994), can function in removing H_2O_2 (Foyer et al., 1994a). Often interacting with these antioxidant enzymes are antioxidant

compounds such as ascorbate and glutathione, both intrinsically involved in the glutathione-ascorbate cycle. In this cycle, ascorbate (AsA) is peroxidized to the monodehydroascorbate radical by ascorbate peroxidase (Hossain et al., 1984). This radical can then either be reduced back to ascorbate by monodehydroascorbate reductase, or it can non-enzymatically disproportionate to dehydroascorbate (DAsA), which is then reduced back to ascorbate by glutathione (Jahnke et al., 1991). The resulting oxidized glutathione (GSSG) can be subsequently reduced (GSH) by glutathione reductase (Halliwell and Foyer, 1978). Ascorbate can also react directly with and detoxify O_2^-, ·OH, and singlet oxygen ($^1O_2$) (Walker and McKersie, 1993). Glutathione can also be oxidized by reacting directly with $^1O_2$ and ·OH species (Foyer et al., 1994b).

Past studies comparing different species have demonstrated that chilling sensitive species have a lower antioxidant capacity than do tolerant species (Jahnke et al., 1991; Walker and McKersie, 1993). Previous work comparing antioxidant enzyme activities and compound concentrations in differentially chilling sensitive inbred maize lines demonstrated that, at the first leaf stage under short-term (1 d) chilling at 11°C, percent of control activities of MDHAR and ASPX (Hodges et al., manuscript submitted a) were lowest in the most chilling sensitive line. At the third leaf stage, percent of control activities of CAT, MDHAR, and ASPX as well

as concentrations of total ascorbate and glutathione and the DAsA/AsA ratio (Hodges et al., manuscript submitted b), were lowest, although not significantly so, in the most chilling sensitive inbred line. These results suggested that the chilling sensitive line had less capacity to detoxify toxic oxygen compounds than the tolerant lines. Concentrations of sugars and starch, measured as metabolic indicators of chilling stress, were significantly highest in this short-term chilled sensitive line (Hodges et al., manuscript submitted a). Short-term chilling of chilling-sensitive species, such as maize or tomato (*Lycopersicum* spp.), often results in the accumulation of carbohydrates such as sugars and starch in leaves (Mitchell and Madore, 1992). Carbohydrate levels from maize inbreds indicated that the chilling sensitive line experienced a greater depression in the rate of assimilate utilization relative to the rate of photosynthesis than did the tolerant lines (Hodges et al., manuscript submitted a).

As demonstrated by phenotypic parameters, cold tolerance of maize inbreds was not an accurate predictor of cold tolerance of maize hybrids (Aidun et al., 1991). Inheritance of cold tolerance has significant maternal effects associated with germination and early seedling growth (Maryam and Jones, 1983). Twelve hybrids exhibited maternal effects when selected for chilling sensitivity (Hodges et al., manuscript submitted c). The purpose of the current study was to determine if activities of the antioxidant enzymes CAT, MDHAR,

and ASPX, concentrations of the antioxidant compounds ascorbate and glutathione, as well as the DAsA/AsA and GSH/GSSG ratios, and concentrations of carbohydrates as metabolic chilling indicators, would give similar results in these twelve differentially chilling sensitive hybrids as they did in the parental inbreds of previous work (Hodges et al., manuscript submitted a; Hodges et al., manuscript submitted b). The usefulness of these parameters as screening tools for chilling sensitivity was also examined.

7.3 MATERIALS AND METHODS

7.3.1 Plant material

The twelve maize (*Zea mays* L.) hybrids studied exhibited differential sensitivity to chilling based upon germination and emergence (Hodges et al. 1994) and fourth-leaf early growth dry mass and percentage water tests (Hodges et al. 1995) in both the laboratory and the field (Hodges et al., manuscript submitted c). A complete 4 x 4 diallel cross was made with the maize inbreds CO251, CO255, CO304, and CO308 in 1994 at the Central Experimental Farm, Ottawa, ON, Canada. These inbreds were chosen on the basis of our previous work which indicated that they differ in chilling sensitivity (Hodges et al. 1994; Hodges et al. 1995) for chilling sensitivity. One of the inbred lines, CO251, was shown

throughout all tests to be the most chilling sensitive (Hodges et al. 1994; Hodges et al. 1995). The other three inbred lines, CO255, CO304, and CO308, depending upon the growth parameter assayed, were demonstrated to be either the most, or one of the more, chilling tolerant tested (Hodges et al. 1994; Hodges et al. 1995). The fourth-leaf early growth test demonstrated that there were maternal effects and that CO308xCO251, CO308xCO255, and CO308xCO304 were the relatively most chilling sensitive (Hodges et al., manuscript submitted c).

Material was germinated for 5 d in the dark at 25°C, until the coleoptile was approximately 2-cm long, and then pinned into rectangular styrofoam rafts and floated on trays containing Hoaglands solution (Hodges et al. 1995) in a growth chamber (Conviron E-15). The photocycle was 16:8 L:D with a photon flux rate of 450-500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The tissue was harvested at the third leaf stage under either constant (control) 25°C or under 11°C for 1 d prior to harvesting (chilling). For the 25°C control experiments, the material reached the third leaf stage 5 d after pinning. The two most recently expanded leaves were harvested. The main midrib was removed from all leaf tissue.

7.3.2 Enzyme assays

Leaves were extracted and activities of CAT, ASPX, and

MDHAR were assessed as in section 4.3.2 of this thesis.

7.3.3 Metabolite Assays

Concentrations of reduced ascorbate (AsA), dehydroascorbate (DAsA), and total ascorbate (AsA + DAsA) were determined as described in section 5.3.2 of this thesis.

Concentrations of oxidized glutathione (GSSG), reduced glutathione (GSH), and total glutathione (GSSG + GSH) were determined as described in section 5.3.3 of this thesis.

Concentrations of reducing and non-reducing sugars were determined as described in section 4.3.4 of this thesis.

Starch levels were determined as in section 4.3.5 of this thesis.

Protein concentrations were determined as described in section 4.3.3 of this thesis.

Chlorophyll content was determined as described in section 5.3.6 of this thesis.

7.3.4 Statistical Analysis

All enzyme, compound, and metabolite assays were based on at least two readings from four independent replicates. Data from control treatments are given as means ± SE. Data from the chilling treatment are expressed as the percent of control and are shown as means ± the averaged SE.

Correlation coefficients (n = 12) were calculated between respective chill:control ratios from all parameters assessed using MSTAT (1988).

Diallel analysis was based on Griffing's (1956) model I, method 1 for a non-random (fixed) population and all p^2 combinations, where p is the number of parents. Analysis was conducted on all antioxidant enzyme and compound and metabolite data. The components of variance ratios $2\sigma^2_g/(2\sigma^2_g + \sigma^2_s)$ were computed to determine the relative importance of additive and non-additive gene effects (Baker, 1978).

7.4 RESULTS

7.4.1 Chilling Effects on Antioxidant Enzyme Activities:

Profiles of the enzymatic activities for control-grown (25°C) material for the twelve maize hybrids are presented in Table 7.1. Similar values at chilling temperatures were proportionately the same or less (data not shown). Basal control activities of MDHAR and ASPX were generally highest in the chilling sensitive hybrids with CO308 as a female parent relative to the tolerant hybrids when assayed at both 25°C and 10°C (Table 7.1).

All antioxidant enzyme activities and concentrations of antioxidant compounds and metabolites in chilled seedlings were expressed as percent of control values. When chilled for

1 d, percent of control values of CAT generally fell below 100% when assayed at both 25°C and 10°C, while MDHAR values increased above 100% at both assay temperatures (Figure 12). Percent of control values of ASPX were above 100% for hybrids with either CO251 or CO255 as female parents, but fell below 100% for the hybrids with CO304 or CO308 as maternal parents at both assay temperatures.

Percent of control values of MDHAR and ASPX were lowest in the chilling sensitive hybrids with CO308 as the maternal parent relative to the tolerant hybrids when assayed at 25°C (Figure 12). Although CAT percent of control activity was lower for these chilling sensitive hybrids, they were demonstrated to be lowest when assayed at 10°C. Percent of control activities of MDHAR and ASPX were among the lower when assayed at 10°C. The percent of control activities of CAT assayed at 25°C were significantly correlated with those of MDHAR ($r=0.776$, $P=0.003$) and ASPX ($r=0.737$, $P=0.005$) and the percent of control activity of MDHAR was significantly correlated with that of ASPX ($r=0.738$, $P=0.005$). The percent of control activities of MDHAR assayed at 10°C were significantly correlated with those of ASPX ($r=0.856$, $P \le 0.001$).

The percents of control of enzymatic activities assayed at 25°C and 10°C were significantly correlated (CAT: $r=0.856$, $P \le 0.001$; MDHAR: $r=0.94$, $P \le 0.001$; ASPX: $r=0.981$, $P \le 0.001$) (Figure 12).

7.4.2 Chilling Effects on Antioxidant Compound Concentrations

Control grown chilling sensitive hybrids with CO308 as the maternal parent generally had lower total ascorbate and glutathione concentrations than did the chilling tolerant hybrids (Table 7.2). As well, DAsA/AsA and GSH/GSSG ratios were generally the highest in these chilling sensitive hybrids.

When chilled for 1 d, percent of control values for total ascorbate and glutathione, as well as DAsA/AsA, dramatically increased above 100% (Figure 13). Percent of control values for GSH/GSSG generally fell below 100%.

Although there were no discernable differences in percent of control of total ascorbate and glutathione and GSH/GSSG ratios between the chilling sensitive and tolerant hybrids. when chilled, DAsA/AsA ratios were generally highest in the chilling sensitive hybrids (Figure 13). The percent of control DAsA/AsA ratios were significantly negatively correlated with all percent of control activities of CAT, MDHAR and ASPX assayed at both 25°C and 10°C (data not shown). The percent of control of total glutathione was significantly correlated with those of ASPX activities assayed at both 25°C ($r=0.761$, $P=0.003$) and 10°C ($r=0.790$, $P=0.002$).

7.4.3 Chilling Effects on Metabolite Concentrations

In control plants there were no discernable differences concentrations of the sugars, starch, or proteins between the chilling sensitive and tolerant hybrids (Table 7.3).

When chilled for 1 d, concentrations of total, reducing and non-reducing sugars, and starch generally increased (Table 7.3). Percent of control values revealed that total sugars, reducing and non-reducing sugars, and starch levels were highest in the chilling sensitive hybrids with CO308 as the maternal parent as compared to the tolerant hybrids. Far more non-reducing sugars were accumulated in all hybrids relative to reducing sugars. The percent of control of total sugars was significantly negatively correlated with activities of CAT, MDHAR, and ASPX assayed at both 25°C and 10°C (data not shown). The percent of control concentrations of starch were significantly negatively correlated with activities of MDHAR and ASPX assayed at both temperatures (data not shown) and significantly positively correlated with percentage of control concentrations of total ascorbate ($r=0.852$, $P\leq0.001$) and total sugars ($r=0.686$, $P=0.013$).

Protein levels, upon chilling, declined the most in the chilling sensitive hybrids with CO308 as the maternal parent relative to the tolerant hybrids (Table 7.3). Percent of control concentrations of proteins were significantly correlated with all parameters assessed except for concentrations of total ascorbate and glutathione, and

reducing sugars, and the GSH/GSSG ratio (data not shown)

There were no discernable differences in the percent of control of chlorophyll content between the chilling sensitive and tolerant hybrids when they were chilled (data not shown).

7.4.4 Diallel Analysis

The analyses of variance of the general combining ability effects (GCA), specific combining ability effects (SCA), and reciprocal effects of the antioxidant enzyme activities and antioxidant compound and metabolite concentrations are shown in Table 7.4. Data for inbred antioxidant enzyme activities and antioxidant compound and metabolite concentrations are from previous work (Hodges et al., manuscript submitted a, Hodges et al. (manuscript submitted b).

For control-grown material, there were significant differences in the mean squares of GCA effects of activities of CAT (25°C assay), MDHAR (10°C assay), ASPX (25°C assay), and concentrations of total ascorbate and glutathione, reducing and non-reducing sugars, and starch (Table 7.4). Significant differences existed for the mean squares of SCA effects of all parameters tested except MDHAR (25°C assay) and ASPX (10°C assay) activities. There were significant reciprocal effects for CAT (10°C assay) and MDHAR (10°C assay) activities and in all antioxidant compound and metabolite concentrations except for proteins.

For chilled material, there were significant differences in mean squares for GCA effects for all parameters assessed except for CAT (10°C assay) and MDHAR (25°C assay) activities and protein concentration (Table 7.4). The components of variance ratios were 0.47, 0.38, and 0.24 for CAT, MDHAR and ASPX activities, respectively, assayed at 25°C and 0.11, 0.34, and 0.34 for CAT, MDHAR, and ASPX activities, respectively, assayed at 10°C. For the antioxidant compound concentrations, these ratios were 0.25, 0.07, and 0.25 for total ascorbate, AsA, and DAsA, respectively, and 0.39, 0.25, and 0.21 for total glutathione, GSH, GSSG, respectively. For the concentrations of the metabolic indicators of chilling stress, the components of variance ratios were 0.34, 0.67, and 0.03 for total, reducing, and non-reducing sugars, respectively, and 0.10 and 0.15 for starch and chlorophyll, respectively. Significant differences for SCA effects were found for the mean squares of all parameters except protein concentrations. Significant reciprocal effects were found for all parameters assessed except for CAT (25°C assay) activities and protein concentrations.

Estimates of GCA, SCA, and reciprocal effects are given in Table 7.5. Negative effects are associated with chilling sensitivity and positive effects are associated with chilling tolerance. From GCA estimates, it is apparent that the original chilling-sensitive inbred CO251 had better than expected performance in hybrid combinations when chilled for

activities of CAT and ASPX, and total ascorbate and glutathione, and total, reducing and non-reducing sugar concentrations (Table 7.5). The original chilling tolerant inbreds CO304 and CO308 have worse than expected performance in hybrid combinations than CO251 when chilled for activities of CAT and ASPX, and concentrations of total ascorbate and glutathione, and total and reducing sugars. The CO255 inbred had worse than expected performance than CO251 when chilled for activities of CAT and concentrations of total ascorbate and glutathione, total, reducing and non-reducing sugars, and starch.

The SCA estimates suggested that hybrids with CO308 as the maternal parent had worse performance when chilled than expected based on the parental performance for activities of CAT and ASPX, and concentrations of total ascorbate and glutathione, total and reducing sugars, and proteins (Table 7.5). The hybrids with CO251, CO255, and CO304 as maternal parents had better than expected based on the parental performance when chilled than the hybrids with CO308 as the female parent for activities of CAT and ASPX, and concentrations of total ascorbate.

Reciprocal effects demonstrated the maternal effects of the original four parental inbred maize lines in producing the twelve hybrids through the complete diallel cross.

7.5 DISCUSSION

A decrease in the activity of CAT relative to controls, under both short- (Feierabend et al., 1992; Mishra et al., 1993) and long-term chilling treatments (Schöner and Krause, 1990), has been suggested to occur through photoinactivation of this enzyme when it is exposed to both light and cold (Volk and Feierabend, 1989; Feierabend et al., 1992). The percent of control activity of CAT in the maize hybrids also decreased relative to the controls upon short-term (1 d) chilling (11°C), with the chilling sensitive hybrids with CO308 as the maternal parent experiencing the greatest decrease relative to the more tolerant hybrids. Although the most chilling-sensitive parental inbred line (CO251) also had low percent of control CAT activity relative to the tolerant inbreds, there was not a general decrease below 100% of the controls when short-term chilled (Hodges et al., manuscript submitted a). Presumably, the CAT enzymes of the inbreds are less immediately photoinactivated.

Activity of ASPX has been observed to increase during short-term chilling in wheat (*Triticum aestivum* L.) (Mishra et al., 1993) and in both a chilling sensitive cultivar of maize and the more tolerant *Zea diploperennis* L. (Jahnke et al., 1991). As with the parental inbreds from a previous work (Hodges et al., manuscript submitted a), percent of control activities of ASPX at the third leaf stage were both above and below 100%, but were lowest in the chilling sensitive relative

to the tolerant hybrids. Production and turnover of this enzyme may be more inhibited in these chilling sensitive maize lines than in the tolerant.

Percents of control for the activity of MDHAR have been demonstrated to increase under short-term chilling in such plants as tomato (*Lycopersicum* spp.) (Walker and McKersie, 1993). Similar results were observed with these maize hybrids in the current work and in general with the parental inbreds from previous research (Hodges et al., manuscript submitted a).

Thus, when the maize hybrids are exposed to the short-term chilling treatment, percent of control activities of CAT, MDHAR, and ASPX were lowest in the chilling sensitive hybrids with CO308 as the maternal parent relative to the tolerant hybrids. As CAT is primarily responsible for detoxifying H_2O_2, as are ASPX and MDHAR which also serve to regulate reduced ascorbate and thus indirectly function to detoxify other toxic oxygen compounds, lower activities of these three antioxidant enzymes may limit the abilities of the chilling sensitive hybrids to cope with low temperature stress relative to the tolerant hybrids. Activities of CAT and ASPX have been demonstrated to be higher in salt tolerant cultivars of cotton (*Gossypium hirsutum* L.) than salt sensitive (Gossett et al., 1994). Lower activities of CAT, ASPX and MDHAR, levels of which were significantly correlated with one another, were also found in the chilling sensitive inbred maize line

relative to the tolerant (Hodges et al., manuscript submitted a). Hydrogen peroxide can readily diffuse across membranes (Bowler et al., 1992) and can inhibit key enzymes of the Calvin cycle (Badger, 1985; Elstner and Osswald, 1994), as well as interact with O_2^{-} in the Haber-Weiss reactions to form the highly unspecific and reactive ·OH radical (Salin, 1988). Thus, detoxification of H_2O_2 is extremely important in controlling levels of toxic oxygen compounds present within plant tissues. Reduced ascorbate cycling due to depressed ASPX and MDHAR activities would not only lead to reduced H_2O_2 detoxification, but also to reduced scavenging of O_2^{-}, ·OH, and $^1O_2$. Differences in capacities between lines to detoxify these toxic oxygen compounds may be important in the exhibition of differential chilling sensitivity.

As with the inbreds (Hodges et al., manuscript submitted b), percent of control concentrations of total ascorbate and glutathione were not found to be different after short-term chilling at the third leaf stage between the chilling sensitive and tolerant hybrids. This differs from short-term chilling results of two varieties of tomato differing in their chilling sensitivity (Walker and McKersie, 1993), where the concentrations of these two antioxidant compounds were less in the chilling sensitive plants. The DAsA/AsA ratios were highest in the chilling sensitive hybrids, which may be partially due to reduced percent of control activity of MDHAR. Similar results for this DAsA/AsA ratio have been observed in

salt tolerant and sensitive cotton (Gossett et al., 1994) and in chill-stressed tomato (Walker and McKersie, 1993). However, the opposite was seen in the chilling sensitive inbred maize line, where the DAsA/AsA ratio was lowest (Hodges et al., manuscript submitted b). This, together with the fact that the hybrids induced approximately ten fold more total ascorbate when short-term chilled than did the inbreds, suggests that the maize hybrids have a greater capacity to induce and cycle ascorbate than do their parental maize inbreds.

When chilled, the chilling sensitive hybrids demonstrated the highest percent of control accumulation of sugars and starch levels, both of which were significantly correlated with one another. Similar results were observed with the chilling sensitive maize inbred (Hodges et al., manuscript submitted a). Carbohydrates commonly increase in source leaves under chilling conditions mainly due to the rate of assimilate utilization being depressed more than the photosynthetic rate (Farrar, 1988). As the chilling sensitive lines experience a greater decrease in their rate of assimilate utilization in the short-term, as evidenced by the lower soluble protein levels than the tolerant maize, this would result in greater carbohydrate accumulation.

Estimates of GCA and SCA effects demonstrated that CO251, the most chilling sensitive of the parental inbreds (Hodges et al., 1994; Hodges et al., 1995), which also had the lowest

percent of control activities of CAT, MDHAR, and ASPX and highest concentrations of carbohydrates (Hodges et al., manuscript submitted a), had a much better combining ability than expected as a maternal hybrid parent in terms of these parameters than did CO308, one of the most tolerant of the parental inbreds. The relative importance of GCA and SCA in determining progeny performance was assessed by calculating the ratio of the components of variance as outlined by Baker (1976). These ratios were not close to unity for any of the parameters assessed. These results, as well as those of the reciprocals, suggest that the performance of the hybrids cannot be predicted based solely upon the GCA effects of its parents or the parental inbred response to chilling. This disagrees with the results of Maryam and Jones (1983), also in maize, who found that the performance of hybrids, based on days to germination, greatly depended upon the parental inbred lines. This disagreement may in part be due to the discrepancies between time to germination /emergence and percent germination/emergence which occur in evaluating differential chilling sensitivity (Hodges et al., 1994; Hodges et al., manuscript submitted c). Significantly, however, these antioxidant capacity and carbohydrate results of the present work do agree with Aidun et al. (1991), who, after measuring physiological growth parameters such as shoot length and leaf colour, determined that inbred maize cold tolerance cannot be used to accurately predict hybrid cold tolerance.

The chilling sensitive maize hybrids were found to have lower percent of control activities of CAT, MDHAR, and ASPX, and higher concentrations of carbohydrates than the chilling tolerant hybrids. These results paralleled those of the parental inbreds (Hodges et al., manuscript submitted a). Similarly as with the inbred lines, there were no discernable differences between the chilling sensitive and tolerant hybrids in terms of concentrations of ascorbate and glutathione. Lower activities of CAT, MDHAR, and ASPX in the chilling sensitive inbred and hybrid maize would lead to less capacity to scavenge toxic oxygen compounds than in the tolerant maize, allowing for a greater accumulation of these toxic oxygen byproducts, and hence, greater damage. Differences in capacities between lines to detoxify these toxic oxygen compounds may be important in the exhibition of differential chilling sensitivity. The higher concentration of carbohydrates in the chilling sensitive inbreds and hybrid maize after chilling indicates that their rate of assimilate utilization is reduced by the chilling treatment more so than those of the chilling tolerant maize. The percent of control activities of CAT, MDHAR, and ASPX were negatively correlated with the concentration of carbohydrates. These biochemical parameters would thus make excellent indicators of chilling stress in a screening technique for chilling sensitivity for both inbred and hybrid maize, and potentially other plant species as well. The results of this research also

demonstrated that screening of hybrid maize for chilling sensitivity should be done with the hybrids themselves, as it is not possible to accurately predict chilling sensitivity of hybrids based on the chilling sensitivity of their parental inbreds.

7.6 ACKNOWLEDGEMENTS

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Table 7.1. Control (25°C) activities of catalase (CAT) (μmol H_2O_2 decomposed/min/mg protein), monodehydroascorbate reductase (MDHAR) (μmol NADH oxidized/min/mg protein) and ascorbate peroxidase (ASPX) (mmol H_2O_2 decomposed/min/mg protein) assayed at 25°C and 10°C at the third-leaf stage in twelve hybrids lines of maize.

Hybrid	CAT 25°C	CAT 10°C	MDHAR 25°C	MDHAR 10°C	ASPX 25°C	ASPX 10°C
CO251xCO255	223.2±19.2	194.4±19.8	37.6± 9.5	11.9± 2.3	1.59±0.1	1.53±0.1
CO251xCO304	242.8± 5.9	188.3±11.4	51.7±11.7	19.1± 3.0	1.43±0.1	1.06±0.1
CO251xCO308	258.5±17.5	192.7±24.4	63.7± 3.3	21.7± 2.0	1.42±0.1	1.21±0.1
CO255xCO251	208.6±15.8	228.5±16.5	40.8± 4.3	14.7± 0.5	1.52±0.2	1.31±0.1
CO255xCO304	210.0±17.7	177.4±33.2	39.1±14.1	11.2± 2.1	1.32±0.2	1.04±0.1
CO255xCO308	178.1±32.7	209.0±23.1	43.3±16.3	15.6± 4.6	1.42±0.1	1.32±0.1
CO304xCO251	277.3±15.8	285.0±21.4	30.0± 2.7	12.2± 0.6	1.47±0.1	1.49±0.1
CO304xCO255	242.6±17.9	225.9±13.8	38.9± 5.5	15.0± 1.0	1.59±0.1	1.48±0.1
CO304xCO308	243.2±11.4	233.6± 3.7	39.7± 2.8	17.6± 1.5	1.36±0.1	1.13±0.1
CO308xCO251	223.3±10.8	209.4± 9.8	53.9± 2.8	23.4± 0.7	1.60±0.1	1.43±0.0
CO308xCO255	215.5± 9.0	201.7±11.4	61.5± 7.1	27.6± 3.8	1.68±0.1	1.48±0.1
CO308xCO308	220.6± 2.9	205.4±20.6	81.7±11.1	38.7± 4.0	1.67±0.1	1.19±0.1

Table 7.2. Total concentrations of ascorbate (mmol/g fr. wt.) and glutathione (μmol/g fr. wt.) and the DAsA/AsA and GSH/GSSG ratios at the third- leaf stage of the twelve hybrid maize lines grown as controls (25°C).

Hybrid	Total Ascorbate	DAsA/ AsA	Total Glutathione	GSH/ GSSG
CO251xCO255	0.72±0.08	2.57	503.1± 41.2	0.62
CO251xCO304	0.59±0.03	2.82	412.9± 34.2	0.66
CO251xCO308	0.68±0.14	2.79	395.2± 84.3	1.52
CO255xCO251	0.50±0.05	4.81	456.8± 42.0	0.31
CO255xCO304	0.52±0.01	5.29	561.1± 16.8	2.11
CO255xCO308	0.45±0.00	4.82	319.9± 16.4	0.52
CO304xCO251	0.77±0.02	11.85	976.9±132.0	1.71
CO304xCO255	0.87±0.03	9.05	1094.2± 36.7	0.70
CO304xCO308	0.77±0.05	8.53	830.8± 57.7	0.56
CO308xCO251	0.66±0.05	7.45	363.3± 26.3	1.76
CO308xCO255	0.47±0.01	12.73	387.1± 75.4	1.32
CO308xCO304	0.50±0.09	21.83	387.1± 41.4	1.77

Table 7.3. Concentrations of total, reducing (red.), and non-reducing (non-red.) sugars (mg/g fr. wt.), starch (mg/g fr. wt.) and proteins (mg/g fr. wt.) of the third leaf-stage of the twelve maize hybrids grown under control (25°C) conditions and their respective chill:control ratios when grown under 11°C for 1 d.

Hybrid	Total Sugars	Chill: Control	Red. Sugars	Chill: Control	Non-Red. Sugars	Chill: Control	Starch	Chill Control	Prot.	Chill: Control
CO251xCO255	1.47±0.11	1.26	1.36±0.07	0.89	0.18±0.07	5.55	15.74±2.12	0.37	18.04±2.04	101.2
CO251xCO304	1.12±0.07	1.61	0.82±0.12	1.52	0.30±0.09	1.85	8.36±1.82	1.09	18.00±0.82	98.7
CO251xCo308	1.11±0.14	1.81	0.79±0.11	1.39	0.32±0.09	2.86	10.62±2.22	1.49	18.71±1.87	90.2
CO255xCO251	1.28±0.03	1.23	1.18±0.02	0.83	0.08±0.04	7.24	12.90±2.45	1.13	17.33±1.46	108.9
CO255xCO304	1.25±0.08	1.16	1.06±0.11	0.88	0.25±0.05	2.06	22.44±2.69	1.01	16.20±0.91	114.0
CO255xCO308	1.72±0.13	0.94	1.05±0.13	0.88	0.67±0.25	1.05	23.82±3.86	0.78	17.33±2.54	104.6
CO304xCO251	1.95±0.05	1.70	1.54±0.09	1.08	0.41±0.13	4.01	25.02±4.37	1.48	15.13±1.26	99.3
CO304xCO255	1.67±0.05	1.44	1.22±0.08	1.07	0.46±0.05	2.45	32.17±4.19	1.27	17.53±1.97	96.0
CO304xCO308	1.84±0.11	1.38	1.45±0.09	0.92	0.39±0.06	3.05	31.26±5.32	1.05	16.72±0.45	95.0
CO308xCO251	1.27±0.10	2.56	0.92±0.03	2.00	0.34±0.08	4.08	22.97±1.19	1.65	18.23±1.02	78.8
CO308xCO255	1.18±0.11	2.23	1.02±0.11	1.44	0.16±0.05	7.71	22.33±3.21	1.68	18.12±0.86	83.4
CO308xCO304	1.08±0.03	2.04	0.96±0.01	1.34	0.12±0.02	7.60	17.46±2.08	2.90	17.70±0.43	79.6

Table 7.4. Analysis of variance for control (25°C) and chilling (11°C 1 d) activities of catalase (CAT), monodehydroascorbate reductase (MDHAR), ascorbate peroxidase (ASPX) assayed at 25°C and 10°C, and concentrations of total ascorbate, total glutathione, total sugars, reducing sugars, non-reducing sugars, starch, and proteins of a diallel cross.

Source	df	Mean Square	
		Control	Chill
CAT (25°C Assay)			
GCA	3	2382.1***	2072.2***
SCA	6	6191.3***	691.6*
Reciprocal	6	417.4	409.9
Error	45	8191.4	179.9
CAT (10°C Assay)			
GCA	3	627.5	649.6
SCA	6	6684.3***	893.5*
Reciprocal	6	1166.9*	1249.7***
Error	45	404.1	258.4
MDHAR (25°C Assay)			
GCA	3	322.8	192.0
SCA	6	321.9	1329.4***
Reciprocal	6	215.6	727.4***
Error	45	304.7	76.6
MDHAR (10°C Assay)			
GCA	3	63.3***	48.5*
SCA	6	25.5***	65.4***
Reciprocal	6	73.4***	76.4***
Error	45	5.9	14.0
ASPX (25°C Assay)			
GCA	3	354977.4***	35212.2***
SCA	6	366602.1***	182566.4***
Reciprocal	6	15601.8	269772.6***
Error	45	13074.3	28241.8
ASPX (10°C Assay)			
GCA	3	942552.6	473101.8***
SCA	6	826934.9	192630.2***
Reciprocal	6	1504486.4	168596.9***
Error	45	932478.1	12986.1

Table 7.4. Analysis of variance for the diallel cross (cont'd).

Source	df	Mean Square	
		Control	Chill
Total Ascorbate			
GCA	3	0.055***	0.067***
SCA	6	0.065***	1.298***
Reciprocal	6	0.023***	0.045***
Error	45	0.003	0.007
Total Glutathione			
GCA	3	54748.9***	428763.2***
SCA	6	126532.3***	1160185.8***
Reciprocal	6	67200.7***	592631.2***
Error	45	3040.0	39666.9
Total Sugars			
GCA	3	0.017	1.329***
SCA	6	0.045*	0.535***
Reciprocal	6	0.150***	0.561***
Error	45	0.020	0.028
Reducing Sugars			
GCA	3	0.127*	0.445***
SCA	6	0.109*	0.144***
Reciprocal	6	0.111*	0.089***
Error	45	0.034	0.013
Non-Reducing Sugars			
GCA	3	0.057***	0.511***
SCA	6	0.045***	0.570***
Reciprocal	6	0.033**	0.968***
Error	45	0.009	0.014
Starch			
GCA	3	39.8***	115.4**
SCA	6	136.1***	7976.6***
Reciprocal	6	60.4***	195.9***
Error	45	7.3	22.36
Proteins			
GCA	3	0.820	89.449
SCA	6	13.376***	91.774
Reciprocal	6	1.028	2.514
Error	45	1.872	48.729

*,**,*** indicate significance at the 0.05, 0.01, and 0.001 level, respectively.

Table 7.5. Estimates of general and specific combining abilities and reciprocal effects of control (25°C) and chilling (11°C 1 d) activities of catalase (CAT), monodehydroascorbate reductase (MDHAR), ascorbate peroxidase (ASPX) assayed at 25°C and 10°C and concentrations of total ascorbate, total glutathione, total sugars, reducing sugars, non-reducing sugars, starch, and proteins of a diallel cross.

	CAT				MDHAR				Total Proteins	
Line	Control 25°C	Chill 25°C	Control 10°C	Chill 10°C	Control 25°C	Chill 25°C	Control 10°C	Chill 10°C	Control	Chill
CO251	+23.92	+21.21	+12.52	+8.43	+4.72	-6.89	-0.55	-1.65	-0.37	-1.09
CO251xCO255	+6.09	+26.91*	+19.57	+6.45	-4.87	+26.03***	-1.01	+5.60	-0.91	+1.01
CO251xCO304	+35.71	+2.50	+39.78*	+17.44	+14.54	-7.06	-2.91	+0.35	-1.75	-6.24*
CO251xCO308	+22.94	-1.26	+11.24	-2.42	+7.80	+13.65	+1.31	+1.85	+0.36	+0.86
CO255	-17.25	-3.54	-5.13	-11.06	-8.39	-0.02	-3.44	-0.31	+0.38	-0.41
CO255xCO251	-7.29	+19.74	+17.06	-12.95	+1.64	+4.40	+1.56	+2.78	-0.35	-3.03
CO255xCO304	+43.13	-4.18	+22.43	+7.49	-5.43	+2.37	-2.57	-0.19	-2.22*	-5.69
CO255xCO308	+15.03	+11.24	+33.22	+9.09	+14.50	+19.36**	+3.23	+6.05	-1.13	+1.19
CO304	-2.63	+0.06	-0.15	+6.15	+5.12	+4.04	+0.64	-1.61	+0.10	+4.69
CO304xCO251	+17.25	+9.51	+48.36*	+14.55	+20.41	-22.74**	-3.41	-8.14*	-1.43	-1.37
CO304xCO255	-2.63	+0.06	+24.29	+19.04	-0.08	-27.29***	+1.94	-8.46*	+0.68	-0.80
CO304xCO308	+35.49	+7.49	+42.36*	+19.36	-0.73	+9.74	+3.37	-3.66	-1.35	-5.52
CO308	-4.04	-17.74	-7.23	-3.51	-1.45	+2.87	+3.35	+3.57	-0.12	-3.19
CO308xCO251	-12.56	-14.14	+8.34	-20.05	-4.90	-15.29*	+0.85	-4.95	-0.22	-1.25
CO308xCO255	+18.71	-14.11	-3.61	-33.33*	+9.06	-25.64***	+6.03**	-7.66*	+0.39	-1.51
CO308xCO304	-11.30	-9.39	-14.23	-38.33*	+11.01	+3.10	+12.86***	-0.58	+0.50	-3.19

*, **, *** Significantly different from 0 at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 7.5. Estimates of general and specific combining abilities and reciprocal effects (cont'd).

Line	ASPX				Total Ascorbate		Total Glutathione	
	Control	Chill	Control	Chill	Control	Chill	Control	Chill
CO251	+180.20	+123.69	-55.96	+85.65	+0.12*	+0.07	-4.19	+195.86
CO251xCO255	-474.31***	+192.49	-681.09	+85.02	-0.19***	+0.47***	+27.90	+263.94
CO251xCO304	-175.33	-62.42	-375.70	-31.33	-0.10	+0.47***	+149.36**	+383.49*
CO251xCO308	-194.28*	-18.78	+53.89	+9.42	-0.07	+0.33***	+34.49	+610.63***
CO255	+179.99	+217.70	+447.03	+302.99*	-0.01	+0.02	+6.50	+95.79
CO255xCO251	-35.05	+102.55	-105.64	-0.28	-0.11*	-0.13	-23.18	-658.81***
CO255xCO304	-170.68	-170.79	+994.14	-199.05*	+0.06	+0.50***	+271.32***	+921.26***
CO255xCO308	-157.95	-236.79	-369.78	-319.87**	-0.15**	+0.31***	-0.46	-93.70
CO304	-221.15	-148.49	-3.15	-231.47	-0.04	+0.04	+100.00	+42.23
CO304xCO251	+22.76	-443.31**	+216.94	-386.18***	+0.09	-0.21**	+282.04***	-227.10
CO304xCO255	+137.65	-291.08*	+2106.03*	-299.46**	+0.17***	+0.23**	+266.41***	-596.88***
CO304xCO308	+94.42	-303.16	-158.16	-247.78*	+0.05	+0.37***	+161.49***	-259.95
CO308	-139.05	-192.90	-387.92	-157.16	-0.07	-0.13	-102.32	-333.89
CO308xCO251	+89.88	-442.13**	+110.55	-263.70*	-0.01	-0.06	-14.44	-940.15***
CO308xCO255	+129.59	-567.50***	+80.84	-444.10***	+0.01	+0.01	+33.53	-218.83
CO308xCO304	+35.23	-5.16	+33.78	-14.81	-0.13*	-0.13	-221.84***	-65.27

*, **, *** Significantly different from 0 at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 7.5. Estimates of general and specific combining abilities and reciprocal effects (cont'd).

Line	Total Sugars		Reducing Sugars		Non-Reducing Sugars		Starch	
	Control	Chill	Control	Chill	Control	Chill	Control	Chill
CO251	-0.02	+0.57**	-0.01	+0.35*	-0.06	+0.15	-3.17	-2.73
CO251xCO255	+0.05	-0.65***	+0.26	-0.36***	-0.22*	-0.51***	+0.66	-8.66*
CO251xCO304	+0.15	+0.05	-0.09	-0.11	+0.17*	-0.03	+1.32	-3.39
CO251xCO308	-0.05	-0.49***	-0.13	-0.12	+0.09	+0.45***	+1.95	+1.37
CO255	+0.01	-0.11	-0.07	-0.13	+0.12	-0.22*	+0.09	-3.78
CO255xCO251	-0.12	-0.05	-0.08	-0.01	-0.02	-0.03	-1.42	+4.36
CO255xCO304	+0.05	-0.24	-0.22	+0.04	-0.01	+0.04	+8.68***	+6.48
CO255xCO308	+0.12	-0.01	+0.10	+0.03	-0.01	+0.40***	+4.97*	+3.58
CO304	+0.05	-0.07	+0.18	-0.12	-0.06	-0.21*	+1.80	+3.71
CO304xCO251	+0.41***	+0.75***	+0.36*	+0.21*	+0.05	+0.55***	+8.33***	+13.91**
CO304xCO255	+0.21	+0.61***	+0.37*	+0.18	+0.10	+0.30***	+4.86*	+9.11*
CO304xCO308	+0.09	+0.19	+0.02	+0.20	+0.01	+0.42***	+4.54*	+9.78*
CO308	-0.04	-0.39*	+0.10	-0.10	-0.00	+0.28*	+1.28	+2.79
CO308xCO251	+0.02	-0.28	-0.08	+0.38***	+0.01	+1.16***	+6.18*	+11.05*
CO308xCO255	-0.27*	-0.13	-0.02	+0.22*	-0.26**	+0.96***	-0.75	+9.55*
CO308xCO304	-0.38**	-0.81***	-0.24	-0.02	-0.14	+0.49***	-6.90**	+8.90*

*, **, *** Significantly different from 0 at the 0.05, 0.01, and 0.001 probability levels, respectively.

7.7 FIGURE HEADINGS

FIGURE 12 PERCENT OF CONTROL (CHILL:CONTROL) OF ANTIOXIDANT ENZYME ACTIVITIES ASSAYED AT 25°C AND 10°C OF HYBRID MAIZE

(TOP) Percentage of control ratios of the activities of catalase (CAT), monodehydroascorbate reductase (MDHAR), and ascorbate reductase (ASPX) assayed at 25°C of the twelve hybrid maize lines. Those with CO308 as the maternal parent are chilling sensitive. Numbers in the top-right hand corner are the standard errors averaged over all lines for each enzyme. Bars represent the mean of four independent samples for each line and enzyme.

(Bottom) Percentage of control ratios of the activities of catalase (CAT), monodehydroascorbate reductase (MDHAR), and ascorbate reductase (ASPX) assayed at 10°C of the twelve hybrid maize lines. Those with CO308 as the maternal parent are chilling sensitive. Numbers in the top-right hand corner are the standard errors averaged over all lines for each enzyme. Bars represent the mean of four independent samples for each line and enzyme.

FIGURE 13 PERCENT OF CONTROL (CHILL:CONTROL) OF ANTIOXIDANT COMPOUND CONCENTRATIONS OF HYBRID MAIZE

Percentage of control ratios of the concentrations of total ascorbate and glutathione and the dehydroascorbate/reduced ascorbate (DAsA/AsA) and reduced glutathione/oxidized glutathione (GSH/GSSG) ratios of the twelve hybrid maize lines. Those with CO308 as the maternal parent are chilling sensitive. Numbers in the top-right hand corner are the standard errors averaged over all lines for each parameter. Bars represent the mean of four independent samples for each line and parameter.

Figure 12

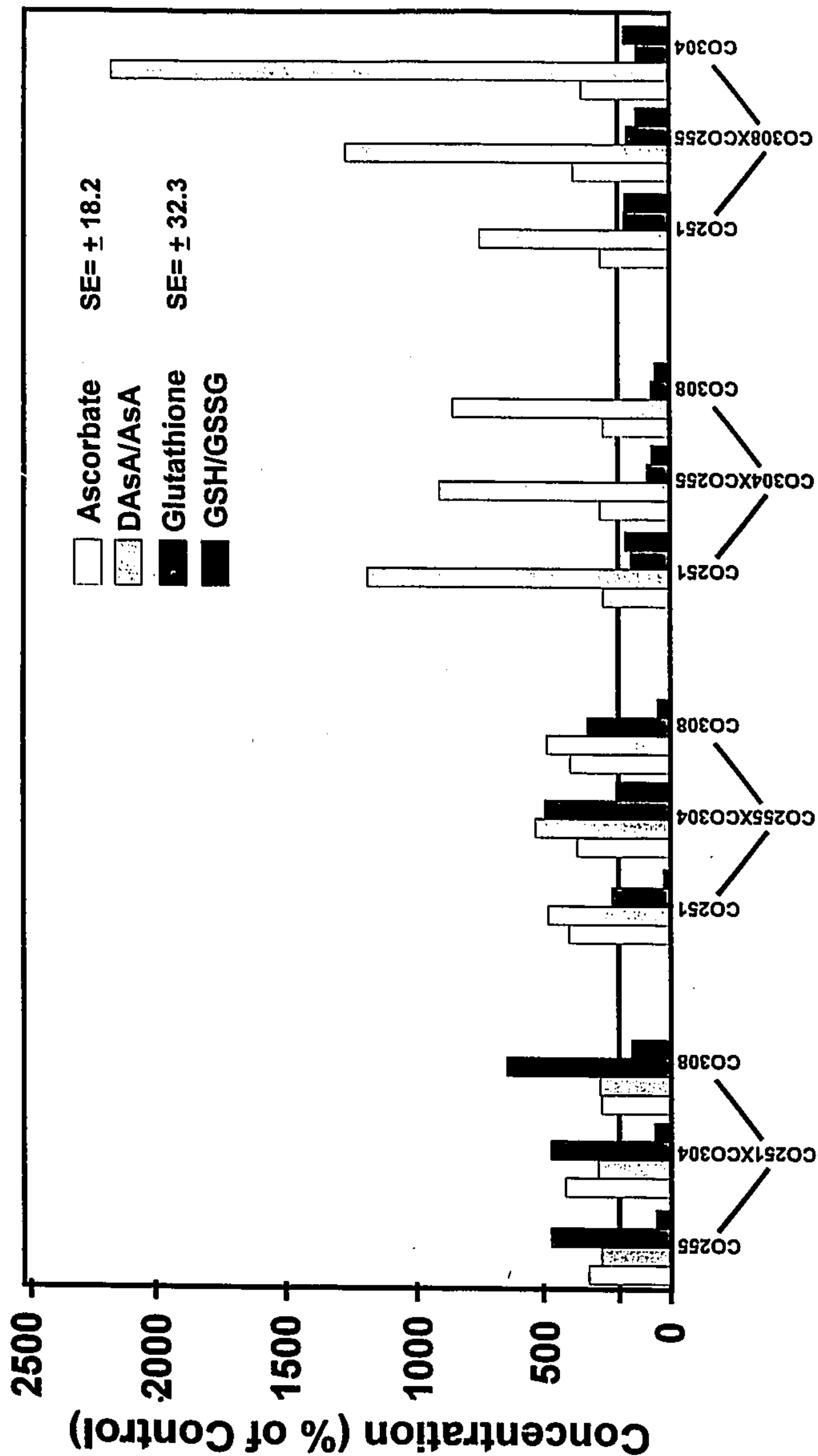

Figure 13

CHAPTER EIGHT

8.1 Conclusions

The purpose of this thesis was to characterize antioxidant capacities of differentially chilling sensitive lines of maize (*Zea mays* L.). The hypothesis was that the most chilling sensitive maize lines would have less antioxidant enzyme and/or compound capacities than that of relatively chilling tolerant maize. In this model, chilling sensitive maize lines would have less ability than the tolerant lines to scavenge toxic oxygen compounds and would consequently experience a greater accumulation of these toxic oxygen byproducts and, hence, greater damage. To this end, three objectives were set. The first objective was to select differentially chilling sensitive maize inbred lines. The second objective was to assess the antioxidant capacities of these inbred lines, and the third objective was to determine if hybrid maize lines would exhibit similar results for antioxidant capacities as for the inbreds. The feasibility of a screening test for chilling sensitivity of both inbred and hybrid maize lines in relation to antioxidant capacities could then be determined. As well, since the hybrid lines were a result of a complete diallel cross between the inbreds, evaluations of the general and specific combining abilities for the assessed parameters could be estimated and the predictive value of the chilling tolerance of hybrids based on

these parameters from those of the inbreds could be determined.

This first objective was successfully met. Differential chilling sensitivity of inbred maize lines was characterized with newly developed laboratory germination (percentages germination and viability, and average time taken to germinate) (Hodges et al., 1994) and early growth (total, shoot, and root dry masses, and percent water content of shoot and root) (Hodges et al., 1995) tests, results of which were confirmed in the field. The inbred CO251 was selected as being most chilling sensitive, and the lines CO255, CO304, and CO308, as more tolerant. As part of the third objective, the hybrids were also similarly classified as relatively chilling sensitive or tolerant. The lines with CO308 as the maternal parent (CO308xCO251, CO308xCO255, and CO308xCO304) were classified as most chilling sensitive of the hybrids.

Once these inbred and hybrid lines were characterized as relatively chilling sensitive or tolerant, the second and third objectives of this research, the assessment of antioxidant capacities between differentially sensitive inbred and hybrid maize lines, were also successfully met.

For the inbreds, activities of the antioxidant enzymes CAT, ASPX, SOD, MDHAR, and GR, along with concentrations of the antioxidant compounds ascorbate, glutathione, ß-carotene, and α-tocopherol and levels of the general metabolic indicators of chilling stress (sugars, starch, chlorophyll,

and soluble proteins) were assessed. As well, to determine if antioxidant enzyme and compound capacities and concentrations of the indicators would change with plant age and with length of exposure to chilling, these parameters were assessed at three developmental stages (first, third and fifth leaf stages) and under both short- and long-term chilling temperature regimes. Although the leaves harvested for the third and fifth leaf stages were the two most recently expanded, and thus cannot be considered a true indicator of the chronological age of the plant, the tissue was harvested in a standardized fashion between maize lines. Consequently, such a harvesting procedure allows for relative comparisons to be performed between lines, regardless of differences between developmental age of the leaf and chronological age of the entire plant. For the hybrids, activities of the antioxidant enzymes CAT, ASPX, and MDHAR, concentrations of the antioxidant compounds ascorbate and glutathione, and concentrations of sugars, starch, proteins, and chlorophyll, the metabolic indicators of chilling stress, were assessed at the third leaf stage under the short-term chilling treatment.

For both the inbreds and hybrid maize lines, the carbohydrate levels, as general metabolic indicators of chilling stress, corresponded with the initial laboratory germination and early growth parameters in ranking the lines in terms of differential chilling sensitivity.

Reduced activities of the antioxidant enzymes ASPX,

MDHAR, and CAT were observed in the most chilling sensitive inbreds and hybrids as compared to the tolerant lines, particularly at the first leaf stage of both short-term and long-term chilling treatments. These enzymes play a significant role in H_2O_2 removal. As H_2O_2 can inhibit key enzymes of the Calvin cycle (Elstner and Osswald, 1994) and can interact with O_2^- to form the highly toxic ·OH radical (Salin, 1988), less ability to scavenge this compound may lead to more chilling-related damage in the chilling sensitive line. Reduced ascorbate regeneration due to depressed MDHAR activity would not only lead to reduced H_2O_2 detoxification, but to reduced scavenging of O_2^-, $^1O_2$, and ·OH (Walker and McKersie, 1993).

In the inbred lines, levels of the antioxidant compound ß-carotene were significantly lower in the chilling sensitive relative to the chilling tolerant lines under both short- and long-term chilling treatments for the first leaf stage. Lower levels of ß-carotene may, in conjunction with reduced activities of ASPX, and CAT and/or MDHAR, have led to increased production of toxic oxygen compounds in the chilling sensitive relative to the tolerant lines, limiting chilling tolerance at the earliest stages of inbred maize development.

As the inbred maize plants aged under long-term chilling treatment, the increase in percent of control activities of CAT, ASPX, MDHAR, and GR in the chilling sensitive line as compared to the tolerant lines may follow from the initially

lower percent of control activities of CAT, ASPX, and MDHAR in this sensitive. As well, lower concentrations of ß-carotene under long-term chilling at the first leaf stage of development would also lead to less potential for toxic oxygen compound scavenging in the chilling sensitive line compared to the tolerant lines. Increased percent of control activities of CAT, ASPX, MDHAR, and GR may represent a compensation for the initially lower H_2O_2-detoxifying and ascorbate-regenerating capacity of this line at the earlier stages of development. These H_2O_2 molecules can be otherwise removed through direct contact with CAT, with ascorbate in a reaction catalysed by ASPX, and through re-oxidation glutathione by GR (Foyer et al., 1994a). Glutathione can then be reduced by either the direct removal of H_2O_2 in reactions catalysed by non-specific peroxidases (Salin, 1988; Foyer et al., 1991) or by non-enzymatically removing other potentially damaging active oxygen species such as O_2^- and ·OH (Larson, 1988). As the long-term chilling period progressed and the plants aged, it was also demonstrated in the inbred lines that the concentrations of total ascorbate and glutathione in the chilling sensitive line increased until they ultimately became significantly higher relative to the tolerant lines.

Oxidative stress has been shown to induce or enhance levels of SOD, GR, and ASPX, and concentrations of ascorbate and glutathione (Foyer et al., 1994a), suggesting that the chilling sensitive line has accumulated greater levels of

toxic oxygen compounds under long-term chilling due to its initially lower CAT, MDHAR, and ASPX activities and concentrations of ß-carotene. As the antioxidant compounds, unlike the antioxidant enzymes, only increased in the chilling-sensitive line compared to the tolerant lines under long-term chilling, it is suggested that the antioxidant enzymes respond more rapidly to oxidative stress than do antioxidant compounds in maize. Thus, as the ability of the chilling sensitive line to detoxify toxic oxygen compounds increased as the inbred maize plants aged from the first to fifth leaf developmental stages, the susceptibility to chilling of the sensitive maize line potentially decreased correspondingly, *i.e.* the most chilling sensitive line became less so. This model is supported by measurements of the chlorophyll content, which is significantly lowest in the chilling sensitive line compared to the tolerant lines at the first leaf stage under both short- and long-term chilling but not significantly different at the fifth leaf stage. As chlorophyll content is a good indicator of chilling damage (Koscielniak, 1993; Brüggemann and Linger, 1994), increased contents in the chilling sensitive line as it aged would suggest that a combination of antioxidant enzymes and compounds resulted in a greater reduction of damaging effects of the toxic oxygen compounds upon its photosynthetic apparatus.

Our hypothesis was supported in that the most chilling

sensitive inbred and hybrid maize lines were demonstrated to have less antioxidant capacities than the tolerant lines, particularly at the earlier stages of development. Less capacity of the chilling sensitive lines to scavenge toxic oxygen compounds at these earlier stages would have led to more accumulation of these toxic oxygen byproducts relative to the tolerant lines, and thus the chilling sensitive lines would have experienced greater damage from these toxic oxygen compounds. Differences in capacities between lines to detoxify these toxic oxygen compounds may be important in the exhibition of differential chilling sensitivity between lines of the same species. However, other mechanisms which impart differential sensitivity to chilling must be taken into account as well. These include such inherent differences between relatively chilling sensitive and tolerant plants as effectiveness of protective measures against chilling-induced photoinhibition (e.*g*. the xanthophyll cycle (Koroleva et al., 1994)), differences in membrane composition (Parkin et al., 1989) and both secondary messenger compartmentalization and effectiveness (Parkin et al., 1989), and in different general metabolic pathways and capacities. These differences, as well as antioxidant capacities, all probably play some role in conferring differential chilling sensitivity within and between species. The relative importance of the role that toxic oxygen compound damage, and thus antioxidant capacities, plays in chilling injury compared to other chilling-induced

manifestations, such as phase transitions in membranes or weakened hydrophobic bonding, is not known.

Different antioxidant capacities have been observed between chilling sensitive and tolerant species of maize (*Zea mays* L. and *Z. diploperennis* L.) (Jahnke et al., 1991) and tomato (*Lycopersicum esculetnum* L. and *L. hirsutum* L.) (Walker et al., 1993). Different antioxidant capacities have also been observed between salt sensitive and tolerant lines of cotton (*Gossypium hirsutum* L.) and drought sensitive and tolerant maize (Pastori and Trippi, 1992; Del Longo et al., 1993). In all cases, the most sensitive plants had, to some extent, less antioxidant capacities than did the tolerant, although they generally differed in which enzyme(s) and/or compound(s) was/were lower. These results, along with those of the differentially chilling sensitive maize inbreds and hybrids from this thesis, would tend to suggest that antioxidant capacities play an important role in the exhibition of differential sensitivity to stress. This stress can not only take the form of chilling, but of drought and salt and presumably other environmental stresses which could also lead to toxic oxygen compound accumulation. Thus, the differential sensitivity of maize and other plants to various stresses which allow for the accumulation of toxic oxygen compounds is strongly suggested to be at least partially due to differences in antioxidant capacities.

The actual measurement of toxic oxygen compound

accumulation would be beneficial in supporting the biological significance of the chilling sensitive maize having less antioxidant capacities. The standard procedure of measuring levels of toxic oxygen compounds invovles the indirect measurement of lipid peroxidation. This involves determining the concentrations of malondialdehyde (MDA), which is a product of lipid peroxidation (Mishra et al., 1993). However, this procedure only indirectly measures levels of $^1O_2$ and $\cdot OH$, the compounds which peroxidize lipids. Furthermore, other free radicals can peroxidize lipids and can consequently affect results. Unless one can assume that a constant ratio exists between levels of O_2^-, H_2O_2, $\cdot OH$, and $^1O_2$, this procedure will only provide approximations of levels of $\cdot OH$, and $^1O_2$; levels of O_2^- and H_2O_2 can not be assessed. However, other methods do exist for the determination of levels of these toxic oxygen compounds. Often these methods, such as ESR spin-trapping of O_2^- (Hodgson and Raison, 1991) are labour-intensive and technically difficult, and can frequently be dramatically influenced by such variables as moisture content (McKersie, 1991). However, future assessment of levels of the toxic oxygen compounds within chilling sensitive and tolerant maize would provide a valuable contribution to the evidence presented in this thesis that the chilling sensitive plants, having initially lower antioxidant capacities, had consequently accumulated greater amounts of these damaging toxic oxygen compounds than the tolerant maize, particulary at

the earlier stages of development.

The reason(s) why some inbred and hybrid maize lines have less antioxidant capacities than others is unclear. It may be that metabolic pathways in either the synthesis of antioxidants or the perception of oxidative stress may be more sensitive to chilling in these lines. It may also be that some of the antioxidant enzymes of the chilling sensitive lines have less active isozymes, or ones which dissociate more readily, under chilling than do more tolerant maize. Research into this area needs to be done.

The results of this thesis indicate that more than one pathway for eliminating toxic oxygen compounds contribute to conferring chilling tolerance and that these depend, to some extent, upon the stage of development. The lower percent of control activities of MDHAR, CAT, and ASPX and concentrations of ß-carotene in the chilling sensitive maize lines at the early stages of development for both short- and long-term chilling treatments suggests that levels of these antioxidants may be limiting to chilling tolerance. However, different maize lines exhibiting tolerance to chilling demonstrated different favourable configurations of these antioxidants, as well as of the metabolic indicators of chilling stress, and any ranking of relative tolerance to chilling based upon these antioxidant and metabolic parameters did not always agree with the original ranking of differential chilling sensitivity identified by the physiological growth parameters. For

example, CO255 was characterized by the early growth phase test to be the most chilling tolerant of the chosen inbreds yet did not always have the highest antioxidant capacities compared to the other lines at the earlier stages of development. It is possible that once capacities of particular antioxidants are above a certain critical threshold, maize, depending upon it's genetic background, may cope with oxidative stress through different antioxidant configurations. Testing of this hypothesis in maize and other plant species will require further experimentation.

Part of the third objective was to determine if a screening test for chilling sensitivity could be developed based upon the antioxidant capacities and concentrations of the general metabolic indicators of chilling stress of the differentially chilling sensitive hybrid maize. This part of the third objective was successfully met. In the hybrid maize, activities of the antioxidant enzymes CAT, MDHAR, and ASPX were negatively correlated with the concentration of carbohydrates. In effect, lower activities of these enzymes are correlated to a metabolic parameter which reflects relative chilling sensitivity. These biochemical parameters would thus make excellent indicators of chilling stress in a screening technique for chilling sensitivity for both inbred and hybrid maize, and potentially other plant species as well.

Another part of the third objective was to determine, using general and specific combining ability estimates, if the

hybrid chilling sensitivity could be predicted from that of their parental inbreds. This part of the third objective was successfully met. The combining abilities for the physiological development parameters (germination and early growth stages), antioxidant enzyme activities and concentrations of carbohydrates also demonstrated that screening of hybrid maize for chilling sensitivity should be done with the hybrids themselves, as it is not possible to accurately predict chilling sensitivity of hybrids based on the chilling sensitivity of their parental inbreds. This disagrees with the results of Maryam and Jones (1983), also in chilled maize, who found that the performance of hybrids, based on days to germination, greatly depended upon the parental inbred lines. This disagreement may in part be due to the discrepancies between time to germination /emergence and percent germination/emergence which occur in evaluating differential chilling sensitivity (Hodges et al., 1994). However, the findings of this thesis do agree with McConell and Gardner (1979) and Aidun et al. (1991), who, after measuring physiological growth parameters, also determined that inbred maize cold tolerance cannot be used to accurately predict hybrid cold tolerance.

The genetic nature of chilling sensitivity in maize has previously been observed to be complexly inherited because of significant maternal or cytoplasmic effects associated with inheritance of germination or early seedling growth potential

(Eagles and Hardacre, 1979; Maryam and Jones, 1983). However, as the genes for CAT, ASPX, and MDHAR are nuclear encoded (Mishra et al., 1993; Mittler et al., 1994; Suzuki et al., 1994), the maternal effects exhibited by these enzymes are unlikely to emanate from maternal transmission of cytoplasmic plastids. More work must be performed in regards to maternal inheritance in maize.

In summary, this thesis demonstrated lower antioxidant capacities of the chilling sensitive inbred and hybrids relative to the tolerant maize, although, at least in the inbreds, these capacities increase as the plants age. This suggests that at the earlier stages of development, the chilling sensitive maize have less capacities to scavenge toxic oxygen compounds than the tolerant maize, and thus experience greater damage. The chilling sensitive maize became less so, however, as they aged to the fifth leaf developmental stage, as their antioxidant capacities and thus their toxic oxygen compound scavenging potential, increased with age. As well, it has been demonstrated that a screening test for chilling sensitivity of both inbred and hybrid maize using percent of control activities of CAT, ASPX, and MDHAR and concentrations of carbohydrates would be feasible. It has also been shown that hybrid levels of antioxidants and metabolic indicators, as well as physiological germination and early growth parameters, cannot be predicted from those of their parental inbreds. Furthermore, this thesis had led to

the development of two new laboratory screening tests for identification of differentially chilling sensitive maize at both the germination and early growth stages.

8.2 Future Research

This thesis by no means completes this story. Although this thesis has answered questions pertaining to antioxidant capacities in differentially chilling sensitive maize, during the execution of this thesis more questions and ideas for further avenues of exploration with respect to this area have arisen.

Examples of future work relating to the results of this thesis which should be considered are the measurements of the toxic oxygen compounds themselves present in differentially chilling sensitive maize. Although direct measurements of $O_2^{\cdot-}$, H_2O_2, $\cdot OH$ and $^1O_2$ would be ideal, these compounds, except for H_2O_2, are difficult to measure and the results presently lack specificity and/or sensitivity. However, indirect measurements of lipid peroxidation, such as ethane and malondialehyde production, can be employed. Levels of these toxic oxygen compounds could be used to support the notion that reduced antioxidant capacities in the most chilling sensitive maize compared to the tolerant results in greater toxic oxygen compound accumulation in the sensitive maize and, hence, to greater chilling damage. As well, activities of

DHAR and other peroxidases could also be assessed in order to further characterize the relative differences in antioxidant capacities between differentially stress-sensitive maize.

Using such methods as gel electrophoresis, western blotting, and specific activity stains, the chilling-induced induction of isozymic variants for each of the enzymes could be assessed and their activities, as well as their induction kinetics, could be described. Moreover, these isozymic variants could be isolated and their enzymatic kinetics (*e.g.* Vmax, Km) characterized. Further to this, organellar purification experiments could be undertaken to determine if certain organelles change their concentrations of antioxidant enzymes or compounds to a greater degree than do others. For example, this approach could test if ASPX is induced in the chloroplast under chilling stress to a greater degree than it is induced in the cytosol, or vice versa. As well, the ASPX, CAT, and MDHAR from the differentially chilling sensitive maize could be isolated to determine if there are any differences between enzymes from chilling sensitive or tolerant maize which could account for differences in activities.

Future work could also employ genetic manipulation of maize germplasm so that the expression of the appropriate isozymes of antioxidant enzymes such as ASPX, CAT and MDHAR in relatively chilling sensitive maize is increased. Although studies of transgenic plants with increased levels of some antioxidant enzymes such as SOD have met with mixed success in

terms of stress tolerance (Tepperman and Dunsmuir, 1990; Sen Gupta et al., 1993*a*; Sen Gupta et al., 1993*b*), the increased expression of more than one antioxidant enzyme or compound may lead to a more enhanced tolerance of photooxidative stress. Many interacting factors are involved in the overall defence against oxidative stress, and it may not always be possible to enhance stress tolerance through manipulation of single components alone as they may not be the single factor that is limiting the ability to withstand stress. Activities of ASPX, MDHAR, and CAT were demonstrated to be lowest in the most chilling sensitive inbred and hybrid maize at the earlier stages of development, thus genetic manipulations in view of increasing their complements could possibly lead to increased levels of chilling tolerance among maize. Whether or not genetic manipulations of these antioxidant complements in other plant species would increase their chilling tolerance would depend upon how similar their results for the antioxidant capacities would be with maize. If similar, the selective breeding or genetic manipulation of both maize and other plant species could eventually lead to increased chilling tolerance, a significant achievement for economically important crops in temperate countries such as Canada. Increased chilling tolerance would allow for sowing in more northerly areas and would lower existing mortality rates of crops sown into cool, spring soils, both of which would lead to greater overall yields.

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