Characterization of inducible cold tolerance in photosynthetic maize seedlings and the behavior of selected xanthophyll compounds

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Characterization of inducible cold tolerance in photosynthetic maize seedlings and the behavior of selected xanthophyll compounds

by

Karen Elaine Grote

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Crop Production and Physiology

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2004
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Signatures have been redacted for privacy
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LITERATURE REVIEW

Importance

Cold temperature limits plant growth, production, and distribution. Maize is an economically important crop of subtropical origin. In the subtropical climates, cold temperatures are not prevalent through the majority of the year. Maize, which evolved under such conditions, is consequently sensitive to cold temperatures. As maize production has moved out of the equatorial climates, plants are more routinely exposed to cold temperatures during the growing season.

Planting under low temperature stress affects two components of the establishment process; germination (Hodges et al., 1997b) and seedling growth (Blacklow, 1972; Miedema, 1982). Improving germination and growth under cold temperatures would reduce the susceptibility of maize to damage during early seedling growth, reduce the risk of stand failure and enable earlier planting in the spring. Early planting results in earlier stand establishment and canopy closure, increased competition against early season weeds, elongation of the growing season, and avoidance of the hot, dry conditions that normally plague pollination and seed set (Mock and Pearce, 1975).

The development of cold tolerance is critical to take advantage of the benefits of early planting, which could cause yield improvement and increased yield stability across most production environments (Tollenaar and Wu, 1999). Cold tolerance can be considered both an innate and acquired trait. While some species/lines are intrinsically more capable of functioning and surviving cold temperature stress, others species/lines develop or induce
tolerance only by exposure to particular environmental cues. Cold acclimation is the process by which the cold or freezing tolerance of a plant is increased after exposure to a sub-optimal temperature (Pearce, 1999). The acclimation phenomenon is relatively common in freezing tolerant plants such as Arabidopsis (Wilhelm and Thomashow, 1993) and wheat (Ohno et al., 2001). Although acclimation has been documented in several cold sensitive species including cucumber (Erez et al., 2002), tomato (Vallejos, 1991), and maize, it has not been extensively characterized in cold sensitive species. Because of the differences between freezing and low or cold temperature damage, acclimation in cold sensitive species will be defined and measured differently than acclimation in freezing tolerant species.

Currently, cold sensitive plants rely predominately upon intrinsic, or constitutive, levels of resistance to cold temperature stress. Plants capable of acclimation can utilize both constitutive and inducible tolerance mechanisms. Characterization of acclimation in cold sensitive species could provide insight into the development of cold tolerance, and could potentially provide researchers with valuable information for the generation of new cold tolerant varieties.

**Types of Stress and Response**

Changes occur during acclimation which protect the plant from subsequent stress (Palva, 1994; Palva and Heino, 1998). Since acclimation requires exposure to temperatures below the growth optimum, there is a possibility that damage occurs during the sub-optimal temperature treatment and the development of tolerance. The acclimation process could follow two basic paths: acclimation could induce damaging stress which is repaired, or it could induce a shift in plant metabolism that increases stress tolerance without stress-

Stress can be broken into two categories: elastic and plastic. Elastic strains are freely reversible, do not require metabolic energy for repair (Levitt, 1980), and can be difficult to quantify. Plastic strains are either irreversible or require the expenditure of metabolic energy for repair. Stress tolerance is normally the terminology applied to plastic resistance, or the ability to prevent irreversible strains and injurious physical or chemical changes (Levitt, 1980). Plastic stress injury depends greatly on the temperature and duration of stress. Just as insufficient stress levels will not cause differentiation of susceptibility due to lack of stress, extreme stress will not effectively discriminate between tolerance levels as a result of exceeding survivability in all lines or varieties of a species regardless of variation in tolerance (Hardacre and Greer, 1989).

Plastic stress injury also depends upon the length of exposure in addition to the severity of stress. Stress duration directly impacts the ability of the plant to function at the low temperature and to recover from it (Creencia and Bramlage, 1971). If acclimation does cause some level of cold damage, the quantitative aspect or cumulative affect of plastic injuries are particularly important. As the length of the acclimation treatment increases, the accumulation of damage can also increase which can affect the advantageous— or possibly the deleterious— physiological change resulting from the acclimation treatment. Temperatures that are too severe will damage the plant while temperatures that are too close to optimal might not induce tolerance to lower temperatures if the improvements in tolerance are in response to the mild stress of the acclimation treatment. In freezing tolerant species, the length of acclimation have been shown to affect the efficacy of the treatment (Rife and
Zeinali, 2003). In cold sensitive species, acclimation duration could potentially influence the capacity to improve tolerance due to the quantitative aspect of the mild acclimation stress.

**Acclimation to Freezing Temperatures**

Acclimation to freezing temperatures are fairly well understood (Steponkus et al., 1998; Thomashow, 1999; Xin and Browse, 2000); consequently, its mechanisms can serve as a model for the development of cold acclimation theories and strategies in cold sensitive plants. Freezing temperatures can induce phase changes within cellular membrane, which cause them to lose osmotic responsiveness and become permeable to water (Xin and Browse, 2000) allowing water movement out of the cell. This can exacerbate damage both to the plasma and internal membranes.

Since cellular membranes are the primary site of injury under freezing conditions (Lyons et al., 1979), most of the processes of freezing acclimation aid in their protection (Pearce, 1999). Uemura and Steponkus (1994) found that the extent of acclimation in spring oat and winter rye was dependent upon unsaturated lipid content of the membranes which was found to increase during acclimation and prevent tight packing of membrane lipids and promote fluidity and proper permeability. The chloroplast targeted, acclimation induced (Wilhelm and Thomashow, 1993), cold regulated (COR) proteins stabilize membranes and prevent phase transitions and membrane lesions (Steponkus et al., 1998).
Cold tolerance in Maize

It is necessary to understand the changes in plant physiology under cold temperature stress and the associated damage in order to characterize acclimation and the development of tests for the phenomena associated with it. Maize seedlings are susceptible to cold temperature-induced damage during germination, and the heterotrophic (Eagles, 1982) and autotrophic (Hardacre and Eagles, 1980) developmental stages. The physiological processes of a seedling change dramatically during the early phase of growth, particularly during the transition from heterotrophic growth to autotrophic growth. Autotrophic and heterotrophic seedlings differ in ability to endure low temperatures (Hardacre and Eagles, 1980) such that varieties tolerant to cold temperatures at one developmental stage are not necessarily tolerant at the other. This disparity in tolerance due to developmental stage necessitates testing for cold tolerance at both the autotrophic and heterotrophic stages to accurately evaluate the cold tolerance of any particular line of maize (Hodges et al., 1997b).

There is a wide range of tolerance or susceptibility levels to cold temperature stress in maize, which can allow for selection and improved varieties of maize (Eagles, 1979). The development of cold tolerant maize lines includes testing both inbred and hybrid stock. The inheritance of germination and seedling growth in cold temperatures is highly complex and can be obscured by maternal effects which influence the traits (Maryam and Jones, 1983). Although Aidun et al. (1991) concluded that inbred cold tolerance could not be accurately used to predict hybrid cold tolerance, their research indicated that the cold tolerant inbred lines did tend to impart cold tolerance to hybrids in spite of no common patterns of inheritance among the selected inbreds. Others have found that inbred performance in growth chambers held at cold temperatures can be used to predict hybrid germination and
early season growth in cold field conditions (Revilla et al., 2000) and that there are
differences among inbreds for the ability to pass cold tolerance to hybrids (Hodges et al.,
1997). Testing of inbred lines for selection and breeding of cold tolerant hybrid lines can
effectively predict hybrid performance at low temperatures, thereby increasing the
information that can be included in developing a cross and potentially reducing the number
of crosses that are required for the development of a cold tolerant line.

Both the heterotrophic and autotrophic growth stages in maize contain variation for
cold tolerance (Brandolini et al., 2000; Hodges et al., 1994; Janowiak and Markowski, 1987).
The assignment of a tolerance value to a particular line is complex because relative tolerance
levels among lines will depend upon the developmental age of the seedling, the means of
quantifying tolerance, and the conditions under which tolerance is determined since the
temperature (Hardacre and Turnbull, 1986), duration, and timing of the stress treatment
(Taylor and Rowley, 1971) will influence the severity and the response to stress. Regardless
of the many variations in testing strategies, lines can be grouped into relative tolerance
groups. Even within a certain tolerance group, not all lines respond to cold temperatures
equally. Cold stress affects many plant components and processes, which suggests that there
are many mechanisms for preventing cold-induced damage. Prevention and/or repair of the
physiological changes that occur under stress conditions are key functions in cold tolerance.

**Physiological Changes under Cold Temperatures**

Cold soil and air temperatures slow germination and reduce the number of seeds that
germinate and develop into normal seedlings (Furter and van de Venter, 1990; Blacklow,
While cold tolerant lines may germinate equally under optimal and stressful conditions, germination of susceptible varieties can drop to 40% or lower as a result of cold temperatures (Brandolini et al., 2000), or can be prevented completely (Janowiak and Markowski, 1987). Seeds germinating at cold temperatures have higher seed water content at the time of radicle and shoot initiation (Blacklow, 1972), and low (6%) moisture kernels are more susceptible to cold-induced damage than 16% moisture kernels (Cal and Obendorf, 1972). Decreased membrane fluidity are also related to reductions in germination at low temperatures (De Santis et al., 1999). Taken together, these findings suggest that a portion of the reduction in germination at low temperatures is related to the uptake of water.

After germination, the seedling is dependent upon heterotrophic growth until approximately the second leaf stage or the development of photosynthetic competence (Cooper and Macdonald, 1970). Cold temperatures substantially reduce heterotrophic growth rate (Brandolini et al., 2000), or in extreme cases, cause it to cease completely (Blacklow, 1972). Reductions in heterotrophic growth can have major impacts on stand establishment, the length of the heterotrophic growth phase, and the eventual attainment of photosynthetic competence for sustained, season long growth. Although heterotrophic growth rates, measured in grams biomass accumulated per day, are less than autotrophic growth rates (Brandolini et al., 2000), they can be relatively large on a relative growth rate basis due to the small size of the seedlings. Growing tissues are more sensitive to stress than older, non-growing tissues (Bewley and Larsen, 1982), with the mesocotyl being particularly sensitive to cold temperatures during the early stages of development (Stewart et al., 1990). As seedling age increases, the developing leaves can also sustain significant damage (Prasad and Stewart, 1998), which can reduce future photosynthetic potential.
Reductions in seedling growth are related to the effect of cold stress on metabolic activity, particularly its effect on respiration and the production of energy for growth (Prasad et al., 1994a). The slowing of respiration can have many negative effects on seed germination and seedling development and, in some cases, can kill young plants. Cold temperatures decrease electron transport and ATPase activity in mitochondria (Prasad et al., 1994a), which can lead to lowered energy production and reduced growth at low temperatures. Activity of the alternative oxidase pathway, which also reduces energy production by dissipating energy as heat, tends to increase at cold temperatures (Stewart et al., 1990). Chilling-induced stress can damage the mitochondrial membrane, further reducing respiration and preventing normal function following the removal of the stress (Stewart et al., 1990; Levitt, 1980). In pre-emergent seedlings, the ability to recover following cold stress is partially due to the resumption of mitochondrial function (Prasad et al., 1994a) in conjunction with the severity of the cold stress. Taken together, these facts suggest that tolerance to low temperatures during the heterotrophic stage of development is related to mitochondrial function and the production of energy to support non-photosynthetic tissue and growth.

In contrast, autotrophic cold tolerance appears to be related to the ability to maintain photosynthetic growth under cold temperatures (Hardacre and Greer, 1989). Cold tolerance has been linked to increased CO₂ assimilation rates and higher chlorophyll concentrations under cold temperatures, which suggests that the cold tolerant lines were able to continue photosynthesis at relatively high rates under low temperatures (Hardacre and Greer, 1989). Cold temperatures will reduce photosynthetic rates in most varieties of maize regardless of relative tolerance level (Taylor and Rowley, 1971). Reduced photosynthetic rate decreases
autotrophic growth rates, increases the time needed to reach maturity, and reduces the rate of
leaf appearance (Hardacre and Turnbull, 1986). Delays in the development of leaf area can
reduce light interception, which further decreases growth potential, and can reduce possible
yields by decreasing total carbon assimilation over the course of the season. On the
biochemical level, cold temperatures reduce the activity of Calvin-Benson cycle enzymes
(Kingston-Smith et al., 1997) which are partially responsible for reductions in growth and the
inability to efficiently utilize the energy captured by the photosynthetic apparatus (Hardacre
and Greer, 1989; Leipner et al., 1999).

A portion of the reduction in photosynthetic rate is fostered by cold-induced changes
in chloroplast structure and composition (Nie et al., 1995). Low temperatures inhibit
chlorophyll synthesis and reduce accumulation of thylakoid proteins (Robertson et al., 1993).
Since chlorophyll content is related to the ability to harvest light energy, reductions in its
quantity will impact the level of energy harvested for use in carbon fixation. Thylakoid
stacking is reduced under cold temperatures (Taylor and Craig, 1971), which can influence
the distribution of membrane localized proteins, including the photosystems. Spatial
separation of the photosystems and other thylakoid proteins can potentially affect the
efficiency of conversion of light energy to chemical energy, which can disrupt the normal
function of all energy requiring processes such as carbon reduction.

Membranes have many important roles within the cell and are very susceptible to
damage under cold temperatures. Membranes are the site of electron excitation in
photosynthesis and act as selectively permeable barriers for the generation of electrical and
chemical gradients in the mitochondrial and chloroplast for energy production. Membrane
lipids also act as a semipermeable barrier between the symplast and apoplastic space. The
permeability of membranes, including those of the chloroplast and mitochondria, increases under cold stress (Janowiak and Markowski, 1987). The increased permeability of chloroplast and mitochondria membranes could reduce the gradients necessary for energy production, and consequently decrease available energy for growth and repair. Cold temperatures can induce mild water stress conditions within cells (Wolfe, 1991). The increased permeability of the plasma membrane and the movement of cellular water to move into the apoplastic space could cause such conditions.

Janowiak and Markowski (1987) found a high correlation between the level of electrolyte leakage, as measured by electroconductivity, through the membranes and the survival of seedlings following stress. Theoretically, membranes that are damaged will be more permeable and will release more intracellular metabolites into the water, which will result in higher electroconductivity. The correlation between this indirect measurement of membrane damage and seedling survival was high. Thus, the extent of membrane leakage, which can be indicative of damage, appears to be closely related to the ability to survive cold stress.

In addition to causing damage directly to the membranes, cold temperatures can also affect the function and localization of membrane proteins, such as the protein components of the photosystems, and enzymatic reactions, such as the production of ATP by ATPase (Jian et al., 1999). In the thylakoid membranes, protein D1 is a very temperature sensitive component of PSII and this sensitivity has been associated with low photosynthetic competence under cold temperatures. Cold temperatures affect D1 metabolism and synthesis in addition to the stability/activity of the protein. Temperatures below 20°C reduce D1 biosynthesis and alter its metabolism (Bredenkamp and Baker, 1994) such that its abundance
is greatly reduced at cold temperatures (Robertson et al., 1993). Under excess light conditions, such as those found in conjunction with cold temperature stress, irreversible structural changes occur in D1 that expose a cleavage site of the protein, which are followed by protein degradation (Zer and Ohad, 1995). The loss of D1 protein prevents normal energy transfer through the photosystems and, consequently, significantly reduces the capacity to photosynthesize at low temperatures. D1 replacement following alleviation of stress is a major factor in the recovery of photosynthetic competence (Fryer et al., 1995).

Most thylakoid proteins are reduced under cold temperatures and are unevenly distributed between the mesophyll and bundle sheath tissues, which contributes to the low photosynthetic activity at low temperatures. Under cold stress, 20-30% of leaf tissue cells completely lack several thylakoid proteins (Robertson et al., 1993). The inability to become stabilized in the membrane is partially responsible for the absence of several key proteins (Nie and Baker, 1991). The uneven distribution of thylakoid proteins (Robertson et al., 1993) in leaf tissue could ultimately contribute to the low overall photosynthetic capacity developed at low temperatures. Upon alleviation of cold stress, the synthesis of chlorophyll and other thylakoid proteins rapidly increase, and eventually the chloroplast structure and protein composition of the thylakoids can be indistinguishable from plants grown at 25°C. Despite apparent return to normal levels of chloroplast proteins and pigments, plants do not regain a proportional level of photosynthetic competence (Nie et al., 1995). Heterogeneity of recovery in the chloroplasts of the mesophyll cells along with possible instability of the accumulated proteins/pigments in the thylakoid membranes (Nie and Baker, 1991) could ultimately contribute to the inability to regain photosynthetic competence. The stabilization
of proteins within membranes appears to be an important component of cold tolerance (Bergantino et al., 1995).

Light in conjunction with low temperatures can cause irreversible damage to the photosynthetic capacity of leaves (Taylor and Rowley, 1971). Slowed enzymatic action and reduced photosynthate export reduce carbon assimilation, but the rate of light energy capture by chlorophyll molecules remains relatively constant in spite of the reductions in energy usage under cold temperature stress. As a result, the ratio of energy capture and photosynthetic electron transport to carbon assimilation increases (Fryer et al., 1998). The inability of CO$_2$ reduction to keep pace with that of light energy capture increases the likelihood that excitation energy or electrons will be donated to molecules other than CO$_2$. Carbon assimilation decreases while the probability of production of reactive oxygen intermediates (ROIs) increases. ROIs damage most biological molecules including lipids, proteins, and nucleic acids and come in many forms, including superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH$^-$$^\text{)}$. Superoxide and hydroxyl radicals are particularly damaging to biological systems, but they are rapidly converted to hydrogen peroxide, which is not as damaging and is more easily managed by the plant antioxidant system. Despite its relative manageability, hydrogen peroxide levels can increase to damaging levels (Prasad et al., 1994b). Kingston-Smith et al. (1999) found that 14°C grown plants had hydrogen peroxide levels that were 300% of 20°C grown plants (Kingston-Smith et al., 1999). These plants grew slower and had more cold-induced damage, which potentially was the result of the high hydrogen peroxide levels. Hydrogen peroxide is highly concentrated in the mesophyll cells of maize leaves (Doulis et al., 1997; Pastori et al., 2000),
which may be related to the greater damage that occurs in that cell type (Robertson et al., 1993) in comparison to the bundle sheath cells.

The combinations of physiological changes which occur at cold temperatures initiate the formation of ROIs; consequently, oxidative stress is a large component of cold-induced damage. Cold temperature tolerance and susceptibility in maize have been linked to antioxidant capacity (Pastori et al., 2000) and the uneven distribution of antioxidants and antioxidant-regenerating enzymes between the mesophyll and bundle sheath cells (Doulis et al., 1997). While overexpression of superoxide dismutase resulted in higher antioxidant capacity in maize leaves and reduced electrolyte leakage, it did not improve photosynthetic efficiency or growth at low temperatures (Van Breusegem et al., 1999). Although this data appears to counter the role of antioxidants in cold stress tolerance, antioxidant content has been shown to be related to differences in cold tolerance between lines (Hodges et al., 1997a; Massacci et al., 1995), treatments (Leipner et al., 1997; Prasad, 1997), and tissue types (Doulis et al., 1997; Pastori et al., 2000).

Although ROIs are damaging when allowed to accumulate, moderate levels appear to be involved in stress-responsive signaling and regulation of gene expression (Desikan, Mackerness et al. 2001). Prasad et al. (1994) found that low levels of accumulated hydrogen peroxide early in the stress treatment signaled the production of antioxidant enzymes which then could prevent the accumulation of damaging levels of ROIs. The signaling abilities of ROIs were substantiated by the induction of cold tolerance via application of exogenous hydrogen peroxide, and the subsequent changes in gene expression. ROIs have also been implicated in stress responsive gene expression in several other species including rice (Lee et al., 2001) and soybean (Delledonne et al., 2001). An oxidative stress responsive promoter
has been identified in tobacco (Garretton et al., 2002), which indicates that ROIs can directly affect gene expression. The current body of knowledge regarding the role of ROIs in stress strongly supports the dual roles of signaling molecule and damaging compound. Low levels of ROIs, such as those which would be produced under mild stress conditions, appear to closely be related to signaling and induction of tolerance mechanisms.

The hormone abscisic acid (ABA) also increases under stress conditions and appears to be involved in signaling stress tolerance. ABA accumulates rapidly under cold stress conditions (Janowiak et al., 2002). Its accumulation is involved in both signaling and the direct protection of cellular components. ABA is capable of inducing the expression of mlip15, a maize bZIP transcription factor also regulated by cold temperatures (Kusano et al., 1995), and SCOF-1, a soybean transcription factor whose constitutive activity induced cold-regulated gene expression in transgenic Arabidopsis (Kim et al., 2001). ABA treatment has also been shown to increase the activity and abundance of ROI scavenging enzymes (Zhu and Scandalios, 1994). Research by Chen and Li (Chen and Li, 2002) suggests that ABA has a direct role in the protection of membranes as well. ABA-treated cells had less lipid peroxidation and were able to retain more intracellular proline than controls. The production and presence of ABA appear to be an important element in the development of cold tolerance due to its role in signal transduction and direct protection against cold related damage.

**Acclimation in Maize**

The means of selection for improved cold tolerance in maize have typically assessed plant performance when maintained at a constant stress temperature, or when placed directly into the stress temperature from optimal conditions. These means of selection do not provide
the environmental conditions necessary for the induction of cold tolerance; consequently, this has resulted in the selection for improved constitutive tolerance to cold stress. Maize is capable of inducing tolerance to cold temperatures if provided particular environmental conditions during a period of acclimation. Ideally, acclimation would improve plant growth and function both during the stress and recovery relative to plants that not treated with an acclimation period. Acclimation treatment could also induce the slowing of metabolic processes during cold stress, which could prevent the accumulation of damage to cellular systems. Lower levels of damage at the cold temperatures would not require as much time or energy for repair; consequently, the plant that had suffered significant reductions in function during the stress could recover to higher levels at a faster rate. Very little is known of the mechanisms of acclimation in cold sensitive species, thus, further characterization of the phenomena could significantly enhance the knowledge of cold tolerance in maize and could contribute to improvements in overall stress tolerance. The development of a generalized procedure for characterizing relative acclimation capabilities is necessary both for assessing the effects of acclimation, and for rating the capacity of individual lines for acclimation.

Acclimation has been documented in a range of genotypes under a range of environmental conditions. Acclimation was observed in both heterotrophic seedlings (Anderson et al., 1995; Anderson et al., 1994; Prasad and Stewart, 1998; Prasad et al., 1994a; Prasad et al., 1994b) and photosynthetic plants (Leipner et al., 1997; Verheul et al., 1995). Current literature includes a wide range of acclimation (Anderson et al., 1994; Kingston-Smith et al., 1999; Verheul et al., 1995) and stress treatments (Kingston-Smith et al., 1999; Leipner et al., 1997; Verheul et al., 1995) where differences are found in temperature, duration, and timing of application.
Determination of the acclimation event can be assayed by several measurements such as plant survival (Anderson et al., 1994) and photosynthetic parameters including chlorophyll fluorescence, quantum yield of photosynthesis, and chlorophyll content (Verheul et al., 1995). The type of measurement will be influenced by the developmental stage of the plant and the unique test conditions, and will also have an effect on the assessment of acclimation capability. Obviously, photosynthetic rate should not be used to determine acclimation in a pre-emergent seedling, but the choice between seedling growth and tissue death is less unambiguous. These assessments measure very different types of injury, and each appears to be better qualified under particular circumstances. Significant damage could occur before tissue death and is not accounted for when only measuring plant survival. In photosynthetic plants, the most logical choices for determination of acclimation contain some means of assessing photosynthetic capacity of the plant since a large part of cold-induced damage is centered on the chloroplasts, light harvesting, and carbon assimilation. Within the range of options available to gauge photosynthetic capacity, there are many subtle differences which can have a large effect on the experimental outcome. When the developmental stage of the seedling, the environmental circumstances of the experiment, and the means of assessment are considered jointly, it is apparent that the current body of literature regarding acclimation in maize is fairly broad-reaching in that a wide range of processes within the plant could be responsible for an acclimation induced improvements in performance.

In non-photosynthetic seedlings, acclimation is related to protecting mitochondrial function during cold stress. Pre-emergent seedlings, which had been acclimated for 3 days at 14°C, maintained electron flow through cytochrome oxidase and had lower activity of the alternative oxidase than in non-acclimated stressed seedlings after exposure to 4°C stress and
a period of recovery at 22°C (Prasad et al., 1994a). The acclimated seedlings also accumulated more dry weight during this time (Anderson et al., 1994). Higher levels of energy flow through the cytochrome oxidase would result in higher energy production than flow through the alternative oxidase, which leads to the possibility that the maintenance of efficient energy production could be involved in new growth and repair to cellular components at ambient conditions. Seedling survival was also significantly influenced by the acclimation treatments. Following the ten day recovery period at 22°C, 68% of the acclimated seedlings were still alive - as determined by seedling necrosis, mesocotyl constriction, or lack of obvious growth - whereas only 22% of the non-acclimated plants survived (Anderson et al., 1994). The increase in survival could be due to protection from damage during the cold stress in addition to the elevated available energy for repair.

In autotrophic plants, a period of acclimation prior to dropping to stress temperatures can improve photosynthesis during stress and increase the level of recovery. There are many means of assessing photosynthetic rate and efficiency and although some of these parameters are not directly related to plant growth, they are indirectly related to plant performance and function. Changes in carbon dioxide uptake and oxygen evolution directly imply that acclimation affects photosynthesis at low temperatures. Verhuel et al. (1995) found that acclimated plants had higher yields of oxygen evolution at stress temperatures than non-acclimated plants. Acclimation also reduced chlorophyll degradation (Leipner et al., 1997), which would affect light capture during stress. The preservation of chlorophyll and the light harvesting complexes would not only affect the ability to photosynthesize at low temperatures, but also the speed at which the plants are able to recover from the stress due to
the lack of need for new synthesis of chlorophyll. In sorghum, acclimation improved carbon
dioxide uptake only slightly during stress but significantly upon the removal of stress and
following a period of recovery (Taylor and Rowley, 1971). Reduced levels of chlorophyll in
the acclimated seedlings corresponded with higher post-stress photosynthetic rates, which
suggest that decreased photosynthesis may have prevented damage that would inhibit
recovery following stress.

Function and efficiency of the photosystems can be assessed indirectly by changes in
chlorophyll fluorescence. The ratio of oxidized to reduced Q$_{a}$, measured as the
photochemical quenching of fluorescence, in leaves acclimated at 15°C remained high under
cold temperature stress, indicating re-oxidation of Q$_{a}$ was not a limiting factor in
photosynthetic efficiency (Haldimann et al., 1996). These acclimated plants were also less
susceptible to photoinhibitory conditions. Light-independent damage to the reaction centers
is lower in acclimated plants (Verheul et al. 1995), as determined by the maximum rate of
induced rise in chlorophyll fluorescence. Greater quenching of the dark level of fluorescence
(Haldimann et al., 1996) and the rapid development and higher level of non-photochemical
quenching (Leipner et al. 1997) indicated that acclimation had a significant effect on excess
energy dissipation mechanisms in the plant. Overall, these findings suggest that a period of
exposure to sub-optimal temperatures, the acclimation treatment, improves photosynthetic
function possibly both during and after cold stress, and that the photosystems and were better
equipped to dissipate the excess energy resulting from light intensities that exceeded the
capacity for use at low temperatures.
ENERGY DISSIPATION AND XANTHOPHYLL CYCLE

Energy dissipation and the prevention of ROI generation appear to be particularly important in the development of cold tolerance. Non-photochemical quenching (NPQ) is the primary means of dissipating excessive light energy as heat. Chlorophyll generally does not release excitation energy as heat; consequently, for energy dissipation to occur other pigments either accept excitation energy from chlorophyll or directly absorb the light energy, preventing it from interacting with chlorophyll, and release it as heat (Demmig-Adams et al., 1996). Carotenoid compounds are particularly well suited for protection under high light conditions due to the high number of conjugated double bonds found within these molecules (See Figure 1). This conformation allows particular carotenoids to accept excitation energy from chlorophyll and interact with ROIs if necessary (Krinsky, 1979). Some of the carotenoids are better suited for this activity than others. It has been shown that the xanthophyll cycle intermediate zeaxanthin and the related compound lutein are directly involved in the dissipation of excess light energy and photoprotection (Niyogi et al., 1997), while others, such as violaxanthin and neoxanthin, are not directly involved in energy dissipation.
The xanthophyll cycle is comprised of violaxanthin, antheraxanthin, and zeaxanthin. These compounds are derivatives of \( \beta \)-carotene and undergo reversible interconversions via epoxidases and de-epoxidases in the thylakoid membranes (Yamamoto, 1979) depending upon the energy status of the chloroplast and the environmental conditions. Under high light conditions, the pH of the lumen decreases which is necessary for the activity of violaxanthin de-epoxidase and the formation of zeaxanthin through the intermediate antheraxanthin. The
conversion antheraxanthin to zeaxanthin is much faster than the conversion of violaxanthin to antheraxanthin (Hartel et al., 1996); consequently, any accumulation of antheraxanthin is transient due to its rapid conversion to zeaxanthin. Very little zeaxanthin or antheraxanthin is found under non-stress conditions (Haldimann et al., 1995; Leipner et al., 2000).

Zeaxanthin rapidly accumulates under high light conditions such as those experienced by cold stressed plants and is predominately responsible for energy dissipation (Demmig et al., 1987; Demmig-Adams and Adams, 1996). Lutein is formed from α-carotene and is structurally and functionally similar to zeaxanthin, but it is not directly involved in the xanthophyll cycle. Lutein is also involved in energy dissipation, but probably not to the extent of zeaxanthin due to its presence under non-stress conditions and the negative effects it would have on photochemistry under non-stress conditions.

Zeaxanthin and lutein, which differs from zeaxanthin only in the conformation around a single bond, are able to accept energy from activated chlorophyll molecules. Singlet chlorophyll is the first excited species and is short-lived. Singlet chlorophyll can form triplet chlorophyll, which has a much longer life span and is able to interact with other compounds such as oxygen to form ROIs (Krinsky, 1979). Once the carotenoid molecule is activated by singlet or triplet chlorophyll, the energy is not normally transferred back and consequently released in a non-photochemical process as heat. By accepting excitation energy from chlorophyll, and thus preventing the formation of ROIs, the xanthophylls and carotenoids provide protection from light induced damage under cold temperature stress. Carotenoids may also react directly with activated oxygen to prevent damage (Krinsky, 1979). Free zeaxanthin in the thylakoid membranes has also been shown to prevent lipid peroxidation (Niyogi et al., 1997).
Growth at low temperatures tends to increase the overall quantity of xanthophylls in relationship to chlorophyll (Haldimann et al., 1995) and leaf area (Demmig et al., 1987). The acclimation treatment, which is potentially a mild stress due to the sub-optimal temperature conditions, can influence the quantity and the relative content of xanthophylls and carotenoids. Plants which had been grown at 15°C contained more zeaxanthin at the beginning of the stress treatment, were capable of accumulating more zeaxanthin over the course of stress, and did not degrade lutein and zeaxanthin as rapidly as the non-acclimated plants (Leipner et al., 1997). These acclimated plants had higher zeaxanthin (de-epoxidated) to violaxanthin (epoxidated) ratio (Leipner et al., 1997), which corresponded with elevated NPQ. A high zeaxanthin to violaxanthin ratio can indicate photoprotection of the photosynthetic apparatus (Demmig-Adams et al., 1996). Plants developed under sub-optimal temperatures were generally less susceptible to cold temperatures than plants developed at optimal temperatures due to the increase in xanthophyll content of the leaves (Haldimann et al., 1996).

Since a large component of cold stress is managing the light energy which exceeds photosynthetic capacity, the development of tolerance will involve mechanisms for energy dissipation. Carotenoid compounds, particularly those of the xanthophyll cycle, are the generally thought to be responsible for accomplishing this task. The relationship between these carotenoids and acclimation to cold temperatures in cold sensitive species has only slightly been explored, but their role in the development of cold tolerance in photosynthetic plants and their changes in composition at low temperature suggest that these compounds could be very responsive to an acclimation treatment. Thus, these compounds are potentially
very important in acclimation to low temperatures in photosynthetic plants, and their role in acclimation-induced tolerance should be further characterized.

**SUMMARY**

Differences in cold tolerance between lines or varieties can be attributed to a set of physiological characteristics that increase the ability of the plant to perform at low temperatures while also maintaining the integrity of plant composition such that recovery to normal function is possible upon alleviation of the stress. In the process of acclimation at sub-optimal temperatures, changes occur such that these physiological characteristics are induced or elevated which then increases stress tolerance in the acclimated plants relative to plants which do not undergo an acclimation period.

Oxidative stress is a major component of cold damage in both heterotrophic and autotrophic seedlings. Even low to moderate light intensities can exceed photosynthetic capacity at low temperatures, which can lead to the production of ROIs. Prevention and dissipation of these molecules are critical to stress tolerance. Xanthophylls function both in the dissipation of excess energy via non-photochemical quenching and the quenching generated radicals. These compounds are responsive to growth at low temperatures and have been implicated in the maize stress tolerance and the acclimational phenomena. The research presented in this thesis explores the development of cold tolerance by acclimation and the potential role of the xanthophyll compounds.
ABSTRACT

Cold temperatures reduce growth in autotrophic maize plants primarily by reducing photosynthetic activity. Three maize lines of putatively different cold tolerance levels – B73, Co255, and A619 – were assessed for the ability to acclimate to cold temperatures. Seedlings were grown at 25°C until the 4th leaf stage, at which time the temperature was dropped to 15°C for 3 or 7 days of acclimation treatment, which was then followed with 7 days of 10°C, or directly to 10°C for the non-acclimated treatment. Apparent photosynthetic rate and chlorophyll fluorescence were measured on the first, third, and seventh days of stress and after a period of recovery at 25°C. Results indicate that a period of acclimation did improve plant performance both during and after the stress treatment. On the first day of stress, photosynthetic rate and efficiency of acclimated plants were slightly depressed in comparison to the non-acclimated, but by the third day photosynthetic rates of acclimated A619 and Co255 were higher the non-acclimated plants. All acclimated plants were able to return to Fv/Fm levels that were equal to the initial unstressed values while the recoveries of non-acclimated plants did not. The maize lines used in this experiment showed improvements in photosynthetic rate and efficiency as a result of the acclimation treatments and that there is variation for this response which could be used for selection and cold tolerance improvement.

INTRODUCTION

As maize production has migrated farther from its tropical origins, plants are routinely exposed to cold temperatures, which can have a large effect on maize growth and development over the course of the growing season. An understanding of plant response to cold temperatures and the development of cold tolerant varieties is important for increasing yield stability across the environments in which maize is grown. The mechanisms of cold tolerance are highly complex and integrate many plant biological systems. According to Levitt (1980) the ability of plants to withstand low temperatures can take on two forms:
avoidance and tolerance. Although plants are unable to physically avoid cold temperatures, growth and metabolism can slow, thus avoiding growth in unsuitable conditions, preventing the accumulation of damage, and allowing plant recovery with the alleviation of the stress (Creencia and Bramlage, 1971). Such a phenomena could be similar to dormancy in overwintering species. Alternatively, some plants can continue to function and grow at low temperatures while maintaining the ability to recover from the cold stress. Both mechanisms provide the plant with the ability to endure low temperatures.

The environment affects plant physiology and composition (De Santis et al., 1999). Plants that develop at low temperatures have lower chlorophyll content (Kingston-Smith et al., 1999), decreased starch accumulation (Hodges et al., 1997c), higher antioxidant quantities (Leipner et al., 1997), reduced thylakoid protein concentration (Nie and Baker, 1991), and lower photosynthetic activity (Taylor and Rowley, 1971) than plants that are grown under more optimal conditions. Exposure to and growth at sub-optimal temperatures prior to highly stressful temperatures could induce changes in the plant that enhance growth at the lower temperatures (Anderson et al., 1994; Kingston-Smith et al., 1999). Cold acclimation is the development of improved cold tolerance as the result of exposure to sub-optimal temperatures. Acclimation to cold, non-freezing temperatures in cold sensitive species has not been documented as extensively as cold acclimation in freezing tolerant species, but non-photochemical quenching (Leipner et al. 1997), antioxidant status (Haldimann et al., 1996; Leipner et al., 1997), and plant survival (Anderson et al., 1994) have been shown to increase in acclimated maize seedlings.
We report here that a period of acclimation can improve photosynthetic rates and chlorophyll fluorescence under particular environmental conditions and that variation for acclimation exists. The mechanisms and extent of tolerance in these lines appear to be differentially affected by the acclimation, or lack of acclimation, treatment. These results indicate the acclimation could be used as a screen for tolerance as well as a tool to identify the physiological characteristics that enhance the ability to withstand and recover from cold temperature stress.

MATERIALS AND METHODS

Three maize inbreds – A619, B73, and Co255 – were assayed for an acclimation response. A619 is of low to moderate cold tolerance (Pietrini et al., 1999). B73 (Mock and McNeill, 1979) and Co255 (Hodges et al., 1995) are tolerant.

All plants were grown to the 4th leaf stage, under near-optimal conditions in growth chambers, prior to applying the acclimation and stress treatments. The initial conditions consisted of light at 350 µmole photons m⁻² s⁻¹ (Deleens and Brulfert, 1983) provided by a mixture of fluorescent and halogen bulbs. Air and leaf temperature were maintained 25°C (Haldimann et al., 1996) for optimal seedling growth. Air flow into the chamber was 20 cubic feet per minute. Four seeds were planted in each of 5 3-liter pots for each inbred. Pots were filled with Sunshine © brand potting mix, and the seeds planted approximately 2.5cm deep. Following planting, the pots were watered with tap water as needed until day 7, at which time the plants were watered 3 times weekly with Miracle-Gro Excel (approximately
30 mg pre-mixed concentrate/gallon water). In addition, the plant fertility regimen was supplemented with calcium nitrate (950mg/L), magnesium sulfate (490mg/L), and ferric citrate (300mg/L) once weekly. At the first leaf stage the seedlings were thinned from 4 plants to 3, and then thinned from 3 plants to 2 at the second leaf stage, and thinned for the final time at the third leaf stage to one seedling per pot.

The growth chamber temperature was dropped to either 15°C for the acclimation treatments or to 10°C for the non-acclimated treatment (the control treatment) immediately following the 4th leaf measurements. The acclimation treatments were maintained at 15°C (Leipner et al., 1997) for either 3 or 7 days. The plants were then stressed for 7 days at 10° and recovered for 4 days at 25°C. The non-acclimated control treatment was 7 days at 10°C followed by a four days of recovery at 25°C. The treatments were replicated 3 times in a Latin square design using chambers as columns and replications as rows.

Apparent photosynthesis and chlorophyll fluorescence were measured on 5 plants of each inbred in each treatment with a LI-COR 6200 Portable Photosynthesis System and a Walz PAM-2000 Fluorometer, respectively. Measurements were taken at the 4th leaf stage, first day of stress, third day of stress, seventh day of stress, and at recovery approximately to the 5th leaf stage.

Data was analyzed with PROC MIXED from SAS. The 4th leaf photosynthesis and chlorophyll fluorescence measurements were used as a covariate in models to account for
any pre-treatment differences among plants. Photosynthetic rate and Fv/Fm data were highly skewed due to outlier points which were large in magnitude but rare in occurrence. Thus, the analysis was completed using the natural logarithm transformed data to reduce the effect of outlying data points. Means estimates on the log scale were backtransformed to the original units in tables for ease of interpretation. Analysis on the percentage change in photosynthetic rate and Fv/Fm over time also required natural logarithm transformation and is presented as the back-transformed means.

RESULTS

Acclimation treatment effects on cold tolerance of the inbreds were assessed by comparing apparent photosynthetic rate and efficiency of plants placed directly into low temperature stress conditions at 10°C for 7 days to those which were acclimated for either 3 or 7 days at 15°C before being placed in the same low temperature stress. Photosynthetic rates (µmoles m⁻² s⁻¹) and maximum quantum efficiency of Photosystem II as measured by chlorophyll fluorescence (Fv/Fm) dropped significantly during 7 days of 10°C stress in both the acclimated and non-acclimated plants.

Photosynthetic Rate

At the 4th leaf stage, B73 had higher photosynthetic rates than either A619 (p<0.0001) or Co255 (p<0.0001) in all treatments. There were no significant differences between the acclimated and non-acclimated plants at the 4th leaf stage (Table 1).
Day One of 10°C Stress

Photosynthesis was significantly lower after one day of cold stress than at the unstressed 4th leaf stage (p<0.0001 for all inbred*treatment combinations). The photosynthetic rates of non-acclimated B73, Co255, and A619 were lower than the initial 4th leaf measurements and were significantly different for all comparisons. In both acclimation treatments, B73 and Co255 had approximately equal percentage reductions (Table 2). Inbred A619 had the lowest photosynthetic rates in all treatments (Table 1). B73 had the highest photosynthetic rates with the exception of the 3-day acclimated plants that were equal to Co255 (Table 1).

Acclimation did not significantly change the reduction in photosynthetic rate between the unstressed 4th leaf and the one-day stress values. Non-acclimated plants tended to have higher photosynthetic rates after 24 hours of 10°C stress, but were not statistically different than acclimated plants. Across all treatments, photosynthetic rates ranged from 2.53 μmoles CO₂ m⁻² s⁻¹ in acclimated A619 to 7.10 μmoles CO₂ m⁻² s⁻¹ in B73 in the non-acclimated treatment.

Third Day of 10°C Stress

The photosynthetic rates of non-acclimated plants continued to decline from 24 h stress to the third day (p<0.0001 for all inbreds) of stress. The photosynthetic rate of the acclimated inbreds dropped less rapidly or slightly increased relative to the non-acclimated controls in this same time period (Table 2). On the third day of stress, photosynthetic rates of non-acclimated A619 plants were 62% lower than on the first day of stress. In comparison, the 3-
day acclimated A619 showed an 8% increase and the 7-day acclimated plants dropped only
32% from the day one value. Whereas the Co255 non-acclimated control plants dropped
65% from the first day of stress values, the 3-day acclimated and the 7-day acclimated plants
dropped only 31% and 21%, respectively (Table 2). The decrease in the photosynthetic rate
of non-acclimated B73, although insignificant, tended to be slightly greater than in the
acclimated plants, but the photosynthetic rates were still higher than the photosynthetic rates
of either 3 or 7-day acclimated plants (Table 1).

As a result of the smaller decline between the first and third days of stress in the acclimated
plants, Co255 and A619 maintained higher photosynthetic rates on the third day of stress
than the non-acclimated plants (Table 1). The non-acclimated photosynthetic rates of A619
and Co255 were 1.35 and 1.82 µmoles CO₂ m⁻² s⁻¹, respectively. The 3-day acclimated
plants were photosynthesizing at 2.75 µmoles CO₂ m⁻² s⁻¹ in A619 (p=0.1288) and 4.10
µmoles CO₂ m⁻² s⁻¹ in Co255 (p=0.0956). The plants acclimated for 7-day also tended to
have higher photosynthetic levels than the non-acclimated plants in both inbreds.

Between the third and seventh days of stress at 10°C, the decrease in photosynthetic rate was
more severe in non-acclimated plants than the 3-day acclimated plants (Table 2). The
percentage reduction in non-acclimated B73 was over three times as great as the acclimated
plants (p=0.0577). In the 3-day acclimated A619 plants, the percent reduction was
approximately half of the non-acclimated plants (p=0.0512). Co255 also experienced a
larger percent reduction in the non-acclimated than the 3-day acclimated plants while the
change in the 7-day acclimated Co255 was slightly higher than the non-acclimated plants.
Seventh Day of 10°C Stress

On the seventh day of 10°C stress, the photosynthetic rates of B73 were still higher than both A619 and Co255 in all treatments in spite of the large reductions in photosynthetic rate. In the non-acclimated plants, B73 had significantly higher photosynthetic rates than Co255, which was significantly higher than A619 (Table 1). Although B73 maintained the highest photosynthetic rates of all inbreds, it suffered a severe reduction in photosynthetic rate relative to the third day of stress. Inbreds A619 and Co255, which were both photosynthesizing at relatively low levels on the third day of stress, do not appear to decrease as much as B73 between the third and seventh day of stress. On the third day of stress, non-acclimated B73 photosynthetic rates (4.44 µmoles CO₂ m⁻² s⁻¹) were 2.62 µmoles CO₂ m⁻² s⁻¹ higher than Co255 (1.82 µmoles CO₂ m⁻² s⁻¹) and 3.09 µmoles CO₂ m⁻² s⁻¹ higher than A619 (1.35 CO₂ µmoles m⁻² s⁻¹). On the seventh day of stress, the apparent photosynthesis of all inbreds had decreased, but the decrease in B73 plants was more than A619 and Co255 which resulted in B73 being only 0.99 µmoles CO₂ m⁻² s⁻¹ higher than Co255 and 1.42 µmoles CO₂ m⁻² s⁻¹ higher than A619 (Table 1). Although the inbreds were still all significantly different, the magnitude of the difference between them was reduced substantially.

In both acclimation treatments, the photosynthetic rates of B73 and Co255 were equal and significantly higher than A619 on the seventh day of stress. The trend for improved photosynthetic rates in acclimated plants identified at the third day of stress was also evident on the seventh day of stress. The 3-day acclimated A619 and Co255 plants maintained
photosynthetic rates that were approximately 4 (p=0.1167) and 3.5 times higher than the non-acclimated plants (p=0.1358), respectively (Table 1). Inbred B73, which had not responded favorably to acclimation at the first or third day of stress, appeared to have slightly higher photosynthetic rates in the 3-day acclimated plants on the seventh of the 10°C stress.

After the acclimation and/or stress treatments, the plants were returned to 25°C for 4 days (approximately to the 5th leaf stage) to assess the ability of the inbreds to recover from the different stress treatments. The plants did not recover from any of the acclimation and/or stress treatments to the initial unstressed photosynthetic rates (p<0.0001 for all comparisons). However, the acclimated plants tended to recover to a photosynthetic rate closer to the pre-stress level. In acclimated Co255, photosynthetic rates recovered to approximately 70% of the pre-stress rate. The non-acclimated plants recovered to only 48% of the pre-stress level (Table 2). The difference in percent recovery observed in Co255 was statistically significant for both acclimation treatments (p=0.0373 and p=0.0465 for the 3 and 7 day treatments, respectively).

Chlorophyll Fluorescence

Chlorophyll fluorescence (Fv/Fm) was significantly lower on the first day of the 10°C stress than the unstressed 4th leaf measurement (p<0.0001 for all inbred*treatment combinations). Fv/Fm dropped from 0.75 to 0.8 for all inbreds to between 0.38 and 0.54 for all inbreds in all treatments on the first day of the stress treatment. There were no differences in Fv/Fm among the inbreds in any of the treatments or due to acclimation on the first day of stress.
By the third day of exposure to 10°C, the non-acclimated inbreds were all significantly different. Non-acclimated B73 had significantly higher Fv/Fm values than A619 (p=0.0011), which was significantly higher than Co255 (p<0.0001) (Table 3). Chlorophyll fluorescence of non-acclimated Co255 decreased 74% from the first to the third day of stress. This was a larger reduction than either A619 (57%, p<0.0001) or B73 (46%, p<0.0001) (Table 4). In the acclimated plants, A619 had lower Fv/Fm than B73 (p=0.0052 for the 3-day and p=0.0061 for the 7-day acclimation treatment). Co255, which had significantly lower Fv/Fm than A619 in the non-acclimated plants, had slightly higher values than A619 in the acclimation treatments, and was not significantly lower than B73 (Table 3).

Both the 3 and 7-day acclimated plants had significantly smaller reductions in Fv/Fm than the non-acclimated plants from the first to the third day of 10°C stress. In all inbreds, the non-acclimated plants dropped from the highest Fv/Fm on the first day of stress to the lowest on the third. Averaged across inbreds, the 3-day acclimated plants were able to maintain Fv/Fm levels that were approximately 80% of the first day of stress levels. The non-acclimated plants suffered a severe reduction in Fv/Fm from the first to the third day, declining to only 60% of the day one value (Table 4).

Both the 3-day (p=0.026) and 7-day (p=0.012) acclimation treatments improved Fv/Fm values in Co255 on the third day of the cold stress. While Fv/Fm of non-acclimated Co255 was lower than either A619 (p=0.0003) or B73 (p<0.0001), it was equal to or better than the other inbreds in the acclimation treatments. The improvements in cold tolerance as the result
of acclimation were still evident at the seventh day of the stress treatment (p=0.0353 and p=0.0194 for the 3 and 7 day treatments, respectively).

Following four days at 25°C, non-acclimated A619, B73, and Co255 had Fv/Fm values of 0.62, 0.66, and 0.62, which were significantly lower than the unstressed 4th leaf values of 0.79 (p=0.012), 0.80 (p=0.05), and 0.75 (p=0.05), respectively. Fv/Fm values in acclimated plants were equal to the 4th leaf stage values for all inbreds (p>0.2 for A619, p>0.4 for B73 and p>0.5 for Co255).

DISCUSSION

Quality of Photosynthesis and Fv/Fm Measurements

Comparisons between photosynthetic rates and Fv/Fm of the non-acclimated control and the 3 or 7-day acclimated plants were the basis for assessing acclimation ability. While other experiments have utilized non-photochemical quenching (Leipner et al., 1997; Leipner et al., 2000), enzyme activation (Kingston-Smith et al., 1999), or the antioxidant content of acclimated plants (Leipner et al., 1997) as a means of determining acclimation to cold temperatures, photosynthetic rate and efficiency are a higher scale of measurement which reflects the integration of many physiological processes in plants. These measurements were sufficient to differentiate between inbreds within treatments as well as between the acclimated and non-acclimated plants.
Photosynthetic rates dropped from approximately 12-15 \( \mu \text{mol} \text{ CO}_2 \text{ m}^{-2} \text{ s}^{-1} \) at 25°C to less than 10\( \mu \text{mol} \text{ CO}_2 \text{ m}^{-2} \text{ s}^{-1} \) on the first day of stress and to less than 5\( \mu \text{mol} \text{ CO}_2 \text{ m}^{-2} \text{ s}^{-1} \) by the seventh day. The plants did not recover to the pre-stress levels following four days at 25°C in any of the treatments. The inability to recover to pre-stress photosynthesis levels was also found by Nie et al. (1995) and Taylor et al. (1971). The lack of recovery could either be a consequence of an irreversible strain on the photosynthetic system, or an insufficient length of time for repair of the system. The inability to recover photosynthesis to pre-stress levels could be due to altered chloroplast structure (Nie et al., 1995), temperature-sensitivity of photosystem protein synthesis (Bredenkamp and Baker, 1994), reduced enzyme action (Kingston-Smith et al., 1997), or accumulation of photosynthetic end products (Sun et al., 2002) due to reduced growth. Additionally, the photosynthetic capacity of the 4th leaf could decrease with age and the development of new, younger leaves (Usuda, 1984).

At the 4th leaf stage, Fv/Fm of all inbreds was between 0.75 and 0.80, which is in the typical range for healthy, unstressed plants (Krause and Weis, 1991). During the subsequent stress, Fv/Fm dropped as low as 0.08 in Co255 and 0.13 in B73 and A619. Unlike apparent photosynthesis, photosynthetic efficiency did recover to levels that were equal to the pre-stress values in the acclimated plants. The ability to recover Fv/Fm indicates that photoinhibition is not the sole cause of reduced photosynthesis at the recovery stage. The plants were able to re-oxidize QA- and maintain electron flow through the photosystems, which would require an alternate sink other than CO\(_2\) assimilation (Fryer et al., 1998) since there apparent photosynthesis did not recover to pre-stress levels.
Performance of Inbred Lines of Known Tolerance in the Non-Acclimated Control

The selected inbreds, which were of putatively known tolerance levels, responded in unexpected ways to the acclimation and stress treatments. In the non-acclimated control treatment, Co255 photosynthetic rate was more similar to that of A619, the cold susceptible line, than B73, a tolerant line, and Fv/Fm was lower than that of A619. Inbred Co255 is putatively cold tolerant (Hodges et al., 1995), but under the conditions of this experiment would be assigned 'least tolerant' if assessment were based upon Fv/Fm performance in the non-acclimated treatment. Many of the tolerance assessments of inbred Co255 had been conducted in the heterotrophic stage of development (Hodges et al., 1997b) or did not directly assess plant photosynthetic rates or Fv/Fm (Hodges et al., 1995).

Research by Hodges et al. (1995) indicated that Co255 is cold tolerant and that the temperature for optimal growth of the inbred may be closer to 11°C than 25°C. Although the length of time needed to reach the 4th leaf stage was longer, Co255 accumulated more dry matter when grown at the constant 11°C. Conversely, in the research presented here, the photosynthetic rate and Fv/Fm of Co255 at the 4th leaf stage was not lower than either A619 or B73. Although this is not proof that the plants had adapted to the warmer environments, it does indicate the relative performance of Co255 may not have been reduced at warm temperatures relative to the other inbreds.

The environment in which a plant develops has been shown to affect development and composition. For example, plants grown under constant low temperature have significantly more unsaturated fatty acids than plants grown under warm conditions (De Santis et al.,
which tends to increase membrane fluidity under cold temperatures and decrease the likelihood of injury (De Santis et al., 1999; Moon et al., 1995). Hypothetically, a plant such as Co255 which is putatively well suited for growth at low temperatures, can adjust its metabolism such that growth is optimized at warm/optimal temperatures. This could result in many of the physiological processes that were partially responsible for its optimal growth at low temperatures being absent in plants which developed at 25°C.

Although Co255 had very low photosynthetic rates under the stress conditions, the plants recovered to values that were equal to B73, and were significantly higher than A619. Chlorophyll fluorescence also recovered to a level at which it is equal to the two other inbreds as well as the unstressed 4th leaf stage measurement. In spite of the severe reductions in PSN rate and Fv/Fm during the 10°C stress, Co255 maintained the capacity for recovery under the conditions of this experiment. The ability to recover from cold stress is an important component of cold tolerance can be separate from the ability to tolerate and grow at cold temperatures. Such phenomena would support the theory that some plants are capable of ‘shutting down’ to avoid growth and the accumulation of damage at low temperatures, which either is not related to recovery or contributes to recovery due to reduced accumulation of repair-requiring damage. The low photosynthetic rates and Fv/Fm during the 10°C stress could be the result of a low temperature “avoidance” – that is avoidance of metabolic activity – mechanism. The inhibition of photosynthesis and the dissipation of energy through mechanisms other than photochemistry possibly reduced the amount of cumulative damage occurring over the seven days of cold stress, which could be related to the greater extent of recovery observed in the Co255 plants. The amount of
recovery in non-acclimated Co255 was far greater than B73. After four days of 25°C, at which time the photosynthetic rates of B73 and Co255 were equal, the non-acclimated B73 had recovered to a value 2.9 times greater than the photosynthetic rate on the seventh day of cold stress while Co255 recovered to a level 4.7 times the seventh day rate. The severe down regulation of photosynthetic rate and efficiency could be a key to the differential recovery between the inbreds.

**Effect of Acclimation**

On the first day of the 10°C stress, the acclimated plants typically had lower photosynthetic rates and Fv/Fm values than non-acclimated plants. Although the selected acclimation temperature, 15°C, was not as severe as the 10°C stress, it is significantly lower than the optimum growth temperature for maize and is a stress (Greaves, 1996). The duration of cold treatment has been shown to be proportional to the effect of cold stress on CO₂ assimilation (Long et al., 1983). The cumulative effect of the relatively mild stress over the course of 3 or 7 days of acclimation could be responsible for the lower values in the acclimated plants on the first day of stress. On the first day of stress, the non-acclimated plants had only been exposed to 24 hours of 10°C stress, which did not have the physiological consequences of the cumulative effect of the mild stress of the acclimation treatment.

The moderate stress of the acclimation treatment positively affected cold tolerance in A619 and Co255 as duration of the 10°C stress increased. The acclimated plants were able to maintain values closer to the day one levels while the non-acclimated plants dropped significantly more, which resulted in values less than that of the acclimated plants. In
addition to the overall trend for improved photosynthesis in acclimated plants, both 3 and 7-
day acclimated Co255 showed an improvement in Fv/Fm on both the third and seventh days of stress, which suggest that plant photosynthetic efficiency and capacity (Fracheboud et al., 1999) were higher in the acclimated plants than the non-acclimated plants.

Although the p-values listed for the comparisons between acclimated and non-acclimated plants are higher than the typically reported 0.05 level, the observed differences appear to have biological significance. Differences between treatments are similar or greater than the differences between inbreds within treatments that can be labeled significant at the 0.05 level, but have fewer degrees of freedom for significance testing than for comparisons between inbreds within a treatment.

The improvements in A619 and Co255 photosynthetic rates were likely due to changes generated during the acclimation treatment which improved tolerance to cold temperature stress. The lack of acclimation benefits on the first day of stress could be due to cumulative stress of the acclimation treatment. Alternatively, the low apparent photosynthetic rates on the first day of stress could be due to the down regulation of photosynthesis as a response to acclimation. This slowing of photosynthesis early in the stress could prevent the formation of damaging compounds. For example, a rapid slowing of photosynthesis following the application of stress was observed in salt-tolerant rice while salt-intolerant rice did not respond quickly and died within 24 hours (Kawasaki et al., 2001). The ability to reduce damage early in the stress could prevent the accumulation of damage to cellular components and could be responsible for the improvements in cold tolerance observed later in the stress
treatment. Without the signal or adjustment from the acclimation treatment, the non-acclimated plants maintained high photosynthetic rates which could have promoted the formation of reactive intermediates. The synthesis and accumulation of such damaging compounds at the onset of stress could be responsible for the significant decline in photosynthesis and Fv/Fm from day one levels in the non-acclimated plants.

**Acclimation as a Screening Method**

The acclimation and non-acclimation treatments differ in their ability to differentiate inbreds both early and late in the stress treatment. For acclimation to be an effective screen for assaying cold tolerance, it should optimally differentiate lines based upon their relative tolerance levels.

Based upon the inbred ranking in the treatments and over the course of stress, it is apparent that the means by which tolerance is assessed, the timing of assessment, and the experimental conditions considerably influence the evaluation of the lines. On the seventh day non-acclimated A619 had significantly higher Fv/Fm than Co255 while the 7-day acclimated Co255 was significantly higher than A619. Under the conditions of this experiment and on the seventh day of stress, the non-acclimated treatment indicates that A619 is more tolerant than Co255 while the reverse – that Co255 is more tolerant than A619 – is supported by the performance of the 7-day acclimated plants. The non-acclimated treatment, which was originally best able to separate the cold tolerance of the inbreds based Fv/Fm, gradually became less efficient over the course of the stress period until there were no differences between inbreds in Fv/Fm at the recovery stage. These results could partially be due to
accumulation of damage over the 7 days of the stress treatment which exceeded the capacity for repair/function in any of these selected lines.

In summary, we have shown that maize seedlings are capable of acclimating to cold temperatures given specific environmental conditions, and that there is genetic variation for the trait. The improvements observed in the acclimated plants are the result of physiological or biochemical changes that occur during the acclimation treatment that alter plant physiology to tolerate cold temperatures. The behavior of the inbreds in the non-acclimated treatment did not support the previously established tolerance estimations. While B73 consistently had the highest photosynthetic rates and chlorophyll fluorescence values, putatively tolerant Co255 had severely reduced performance under stress. In contrast, the acclimation treatment was most beneficial to Co255, which may be related to its ability to adapt, or in this case acclimate, to low temperatures. Further characterization and optimization of the acclimation phenomena in maize is required prior to utilizing this trait as a means of selection for improved inbreds and hybrids. Due to the highly unexpected behavior of the selected lines, the means by which tolerance levels are currently classified should be reevaluated and/or modified.
Table 1 Backtransformed photosynthetic rate means of non-acclimated control and the 3 and 7 day acclimation treatment plants during cold stress and recovery. P-values are listed for differences between the non-acclimated and the acclimation treatment. Within a treatment*time combination, values that are preceded by different letters are significantly different at the $p=0.05$ level.

<table>
<thead>
<tr>
<th>Time</th>
<th>Inbred</th>
<th>Non-acclimated</th>
<th>3-day Acclimation</th>
<th>7-day Acclimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4th leaf</td>
<td>A619</td>
<td>a 12.74</td>
<td>a 13.67 ns</td>
<td>a 12.38 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>b 14.7</td>
<td>b 15.38 ns</td>
<td>b 14.2 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>a 13.52</td>
<td>a 13.37 ns</td>
<td>a 12.06 ns</td>
</tr>
<tr>
<td>Day 1 stress</td>
<td>A619</td>
<td>a 3.49</td>
<td>a 2.53 ns</td>
<td>a 2.89 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>b 7.10</td>
<td>b 6.36 ns</td>
<td>b 5.70 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>c 5.21</td>
<td>b 5.87 ns</td>
<td>c 4.31 ns</td>
</tr>
<tr>
<td>Day 3 stress</td>
<td>A619</td>
<td>a 1.35</td>
<td>a 2.75 (p=0.1288)</td>
<td>a 2.01 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>b 4.44</td>
<td>b 4.48 ns</td>
<td>b 4.48 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>c 1.82</td>
<td>b 4.10 (p=0.0956)</td>
<td>c 3.42 ns</td>
</tr>
<tr>
<td>Day 7 stress</td>
<td>A619</td>
<td>a 0.46</td>
<td>a 1.84 (p=0.1167)</td>
<td>a 0.83 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>b 1.88</td>
<td>b 3.74 ns</td>
<td>b 2.12 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>c 0.89</td>
<td>b 3.13 (p=0.1358)</td>
<td>b 1.88 ns</td>
</tr>
<tr>
<td>Recovery</td>
<td>A619</td>
<td>a 3.60</td>
<td>a 5.64 ns</td>
<td>a 3.10 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>b 5.58</td>
<td>b 8.85 ns</td>
<td>b 7.85 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>b 5.58</td>
<td>b 9.12 ns</td>
<td>b 8.00 ns</td>
</tr>
</tbody>
</table>
Table 2 Percentage changes in photosynthetic rate as exposure to cold stress increases. P-values are listed for comparisons between the acclimated and non-acclimated controls. Within a treatment*time combination, values that are preceded by different letters are significantly different at the p=0.05 level.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inbred</th>
<th>Non-acclimated</th>
<th>3-day acclimation</th>
<th>7-day acclimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>First day stress relative to 4th leaf</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A619</td>
<td>a -0.72</td>
<td>a -0.81 ns</td>
<td>a -0.77 ns</td>
<td></td>
</tr>
<tr>
<td>B73</td>
<td>b -0.5</td>
<td>b -0.57 ns</td>
<td>b -0.59 ns</td>
<td></td>
</tr>
<tr>
<td>Co255</td>
<td>c -0.62</td>
<td>b -0.56 ns</td>
<td>b -0.65 ns</td>
<td></td>
</tr>
<tr>
<td>Third day stress relative to first leaf</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A619</td>
<td>a -0.62</td>
<td>a 0.08 (p=0.0006)</td>
<td>a -0.32 (p=0.0251)</td>
<td></td>
</tr>
<tr>
<td>B73</td>
<td>b -0.36</td>
<td>b -0.27 ns</td>
<td>a -0.19 ns</td>
<td></td>
</tr>
<tr>
<td>Co255</td>
<td>a -0.65</td>
<td>b -0.31 (p=0.011)</td>
<td>a -0.21 (p=0.0035)</td>
<td></td>
</tr>
<tr>
<td>Seventh day stress relative to third leaf</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A619</td>
<td>a -0.65</td>
<td>a -0.31 (p=0.0512)</td>
<td>a -0.59 ns</td>
<td></td>
</tr>
<tr>
<td>B73</td>
<td>ab -0.56</td>
<td>a -0.16 (p=0.0577)</td>
<td>a -0.54 ns</td>
<td></td>
</tr>
<tr>
<td>Co255</td>
<td>b -0.52</td>
<td>a -0.22 ns</td>
<td>a -0.71 ns</td>
<td></td>
</tr>
<tr>
<td>Four days recovery relative to 4th leaf</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A619</td>
<td>a -0.58</td>
<td>a -0.55 ns</td>
<td>a -0.66 ns</td>
<td></td>
</tr>
<tr>
<td>B73</td>
<td>a -0.52</td>
<td>b -0.4 ns</td>
<td>b -0.39 ns</td>
<td></td>
</tr>
<tr>
<td>Co255</td>
<td>a -0.52</td>
<td>c -0.29 (p=0.0373)</td>
<td>b -0.31(p=0.0465)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3 Comparison of chlorophyll fluorescence between non-acclimated control and the 3 and 7 day acclimation treatment plants. P-values are listed for differences between the non-acclimated and the acclimation treatment. Within a treatment*time combination, values that are preceded by different letters are significantly different at the $p=0.05$ level

<table>
<thead>
<tr>
<th>Time</th>
<th>Inbred</th>
<th>non-acclimated</th>
<th>3-day Acclimation</th>
<th>7-day Acclimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4th leaf</td>
<td>A619</td>
<td>a 0.79</td>
<td>a 0.79 ns</td>
<td>a 0.78 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>a 0.8</td>
<td>a 0.79 ns</td>
<td>a 0.8 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>a 0.75</td>
<td>a 0.77 ns</td>
<td>a 0.74 ns</td>
</tr>
<tr>
<td>day 1 stress</td>
<td>A619</td>
<td>a 0.46</td>
<td>a 0.39 ns</td>
<td>a 0.41 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>a 0.54</td>
<td>a 0.45 ns</td>
<td>a 0.47 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>a 0.46</td>
<td>a 0.38 ns</td>
<td>a 0.42 ns</td>
</tr>
<tr>
<td>day 3 stress</td>
<td>A619</td>
<td>a 0.20</td>
<td>a 0.27 ns</td>
<td>a 0.30 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>b 0.29</td>
<td>b 0.37 ns</td>
<td>b 0.41 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>c 0.12</td>
<td>ab 0.31 (p=0.026)</td>
<td>ab 0.36 (p=0.012)</td>
</tr>
<tr>
<td>day 7 stress</td>
<td>A619</td>
<td>a 0.13</td>
<td>a 0.20 ns</td>
<td>a 0.20 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>a 0.15</td>
<td>b 0.28 ns</td>
<td>b 0.26 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>b 0.08</td>
<td>ab 0.22 (p=0.0353)</td>
<td>b 0.26 (p=0.0194)</td>
</tr>
<tr>
<td>Recovery</td>
<td>A619</td>
<td>a 0.62</td>
<td>a 0.74 ns</td>
<td>a 0.65 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>a 0.66</td>
<td>a 0.78 ns</td>
<td>b 0.69 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>a 0.62</td>
<td>a 0.76 ns</td>
<td>b 0.69 ns</td>
</tr>
</tbody>
</table>
Table 4 Change in chlorophyll fluorescence as exposure to cold stress. P-values are listed for comparisons between the acclimation treatments and non-acclimated controls. Within a treatment*time combination, values that are preceded by different letters are significantly different at the \( p=0.05 \) level.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inbred</th>
<th>Non-acclimated</th>
<th>3-day acclimation</th>
<th>7-day acclimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>First day stress relative to 4(^{th}) leaf</td>
<td>A619</td>
<td>a -0.41</td>
<td>a -0.54 ns</td>
<td>a -0.47 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>a -0.32</td>
<td>b -0.41 ns</td>
<td>a -0.40 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>a -0.4</td>
<td>a -0.50 ns</td>
<td>a -0.46 ns</td>
</tr>
<tr>
<td>Third day stress relative to first</td>
<td>A619</td>
<td>a -0.57</td>
<td>a -0.30 ((p=0.009))</td>
<td>a -0.26 ((p=0.0043))</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>b -0.46</td>
<td>a -0.19 ((p=0.0211))</td>
<td>a -0.10 ((p=0.0059))</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>c -0.74</td>
<td>a -0.21 ((p&lt;0.0001))</td>
<td>a -0.12 ((p&lt;0.0001))</td>
</tr>
<tr>
<td>Seventh day stress relative to third</td>
<td>A619</td>
<td>a -0.32</td>
<td>a -0.17 ns</td>
<td>a -0.40 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>a -0.46</td>
<td>a -0.19 ((p=0.0687))</td>
<td>a -0.41 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>a -0.37</td>
<td>a -0.22 ns</td>
<td>a -0.33 ns</td>
</tr>
<tr>
<td>Four days recovery relative to 4(^{th}) leaf</td>
<td>A619</td>
<td>a -0.19</td>
<td>a -0.06 ns</td>
<td>a -0.16 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>b -0.16</td>
<td>a b -0.03 ns</td>
<td>ab -0.13 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>c -0.17</td>
<td>b -0.01 ns</td>
<td>b -0.07 ns</td>
</tr>
</tbody>
</table>
PART 2. XANTHOPHYLL CONTENT IN ACCLIMATED AND NON-ACCLIMATED MAIZE SEEDLINGS AND THEIR POTENTIAL ROLES IN THE DEVELOPMENT OF COLD TOLERANCE

ABSTRACT

Under cold temperatures, even moderate levels of light can exceed photosynthetic capacity and increase the chance of photo-oxidative damage. Xanthophylls protect plants from high light stress by dissipating excess energy and preventing damage to the photosynthetic apparatus. This study was conducted to determine the effect of a 15°C acclimation treatment on the formation and accumulation of selected xanthophyll compounds under 10°C stress conditions. Maize seedlings were grown to the 4th leaf stage, acclimated and/or placed into the 10°C stress for 1, 3 or 7 days, and then returned to 25°C for a period of recovery. Lutein, zeaxanthin, violaxanthin, and neoxanthin levels from plant leaves were assessed by HPLC analysis. Xanthophyll compounds were highly responsive to the acclimation treatments. In general, the acclimated plants contained less neoxanthin and more zeaxanthin than the non-acclimated plants. Violaxanthin content was unaffected by acclimation. Improvements in plant tolerance to stress due to the acclimation treatment did not align directly with changes in the content of any individual xanthophyll compound. The results of this experiment can not conclusively determine the role of the selected carotenoids in the development of stress tolerance, but do indicate that the compounds are involved in plant response to cold temperature stress.

INTRODUCTION

Most environmental stresses are intensified under high light conditions. Even under relatively low light intensities, absorbed light energy can exceed the capacity of the plant to use the energy under stress conditions (Fryer et al., 1998) which can result in photoinhibition of photosynthesis (Massacci et al., 1995). Xanthophylls are oxygenated carotenoids and have many functions within plants, including protection of the plant from a variety of stresses including drought, heat stress, and cold temperatures. These compounds are involved in the dissipation of excess light energy and the prevention and removal of reactive molecules. Under high light conditions, the zeaxanthin is formed by the de-epoxidation of violaxanthin...
in the xanthophyll cycle (Yamamoto, 1979). Zeaxanthin, along with the structurally similar compound lutein, are primarily responsible for the development of non-photochemical quenching of excess energy and heat dissipation (Niyogi et al., 1997).

The xanthophyll composition in maize leaves changes in response to growth at sub-optimal temperatures (Haldimann et al., 1996; Leipner et al., 1997) and exposure to low temperature stress (Demmig et al., 1987). Growth at sub-optimal acclimation temperatures can provide plants the appropriate environmental conditions for modification of the photosynthetic apparatus and increased tolerance to low temperatures under more severe temperature stress (Grote et al., ant. 2005; Kingston-Smith et al., 1999; Leipner et al., 1997; Verheul et al., 1995). The goal of this study was to assess the role of xanthophyll compounds in the development of cold tolerance in three inbreds of established tolerance levels and to determine the effect of acclimation on their accumulation.

MATERIALS AND METHODS

Xanthophylls were quantified in acclimated and non-acclimated plants of three maize inbreds of differing tolerance levels to low temperature stress. A619 is of low to moderate cold tolerance (Pietrini et al., 1999). B73 (Mock and McNeill, 1979) and Co255 (Hodges et al., 1995) are tolerant.

Plants were grown according to Grote et al. (exp 2005). Samples were taken at the 4th leaf stage, first day of stress, third day of stress, seventh day of stress, and following four days of 25°C as a recovery period. Two plants per inbred per treatment were sampled at each time
point by cutting a 3 cm segment from the middle of the 4th leaf of each plant and immediately freezing them in liquid nitrogen. Samples were lyophilized using a Labconco Freeze Dry System Freezone 4.5 and weighed.

Lyophilized leaf segments were ground in liquid nitrogen and extracted with 5 mL ice cold 80% (v/v) acetone. Samples were shaken, on ice, for 15 min and centrifuged at 4°C for 12 min at 5,000 g. A 1.0 mL aliquot was placed in amber HPLC vials and bubbled with nitrogen gas for immediate analysis by HPLC. The HPLC protocols were based upon Leipner et al. (2000) with the following modifications. Solvent A was comprised of acetonitrile:methanol:0.1M Tris HCl:ethyl acetate (80:10:10:1, by volume). The chromatographic system was Waters 510 HPLC with a Zorbax ODS C18 non-endcapped column (250mm long, 4.6mm i.d.; 5µm particle size).

Detection was at 445 nm using a Waters 486 Tunable Absorbance Detector. Peaks were identified according to their retention times in comparison to standards. Lutein and zeaxanthin standards were obtained from Roche Vitamins Ltd. Neoxanthin and violaxanthin standards were generated by thin layer chromatography and quantified spectrophotometrically (Hager and Meyer-Bertenrath, 1966)

Data was analyzed with PROC MIXED from SAS. Distributions of the xanthophylls data were highly skewed and treatment variances were not homogenous. As a result, a natural logarithm transformation of the data was analyzed to account for the outlying data points.
Means estimates on the log scale were backtransformed to the original units for ease of interpretation.

RESULTS

4th Leaf Stage

As expected, only trace quantities of zeaxanthin were found at the unstressed 4th leaf stage and there were no differences among inbreds (data not shown). Inbreds A619 and B73 had more violaxanthin per gram dry weight than Co255 (p=0.0098 and p=0.0389, respectively). On an area basis, B73 contained more lutein than A619 (p=0.047) and Co255 (p=0.0066). There were no differences in neoxanthin content among inbreds.

Day One 10°C Stress

Zeaxanthin content increased significantly after one day of exposure to 10°C stress. On a dry weight basis, A619 had significantly more zeaxanthin than B73 (p<0.0001) and Co255 (p<0.0001). A619 and Co255 had approximately 2.7 and 2.4 times as much zeaxanthin as violaxanthin while the ratio of zeaxanthin to violaxanthin in B73 was 0.92. Acclimation increased zeaxanthin content per unit area in A619 (Table 1).

Violaxanthin levels were significantly lower on the first day of stress compared to the unstressed 4th leaf stage, but there were no differences in violaxanthin content between acclimated and non-acclimated plants. Inbred B73 had the highest violaxanthin levels on the first day of stress. A619, which had approximately the same violaxanthin content as B73 at the unstressed 4th leaf stage, had significantly less violaxanthin on the first day of stress.
Following 24 hours at 10°C, B73 contained more lutein than A619 (p=0.0013 and p=0.0482), which had more than Co255 (p=0.0006 and p=0.0151) both per unit area and per gram dry weight, respectively. Non-acclimated B73 had significantly more lutein per gram dry weight than the 7-day acclimated plants (p=0.0259). This trend was observed in most non-acclimated plants, but the differences were not significant (Table 2).

Non-acclimated B73 had more neoxanthin per gram dry weight after one day of stress than either the 3-day (p=0.0413) or the 7-day (p<0.0001) acclimated plants. In the 7-day acclimated plants, A619 had lower neoxanthin levels than the non-acclimated A619 (p=0.0635) (Table 2). There were no differences in neoxanthin levels between acclimated and non-acclimated Co255 on the first day of stress.

**Day Three of 10°C Stress**

The acclimation treatments continued to reduce neoxanthin levels on the third day of stress compared the non-acclimated treatment. Non-acclimated A619 and B73 had significantly more neoxanthin per gram dry weight than 7-day acclimated A619 and B73 (p=0.0012 and p=0.0001, respectively) and the 3-day acclimated B73 (p=0.05111). This was also observed on the first day of stress. Neoxanthin levels per gram in Co255, not affected by acclimation on the first day of stress, were now significantly higher in the non-acclimated plants than the 3-day acclimated plants (p=0.0466). On an area basis, only 3-day acclimated Co255 had significantly less neoxanthin than the non-acclimated Co255.
The lutein content of acclimated plants continued to be lower than non-acclimated plants. Averaged across inbreds, the lutein content of non-acclimated plants contained 1.959 µmole/g while the 3 and 7-day acclimated plants had 1.812 µmole/g (p=0.0056) and 1.541 µmole/g (p<0.0001), respectively. Seven days of acclimation reduced lutein content in all inbreds, but the 3-day acclimation treatment lowered lutein levels only in Co255 (p=0.0555) (Table 2). Co255 was the only inbred in which acclimation reduced lutein content on an area basis (p=0.0694) (Table 1).

Violaxanthin levels were not affected by acclimation treatment, but were affected by inbreds. B73 had significantly more violaxanthin than either A619 or Co255 on both a dry weight and an area basis (Tables A and B). The large amount of violaxanthin in addition to the low zeaxanthin detected in B73 resulted in a lower ratio of zeaxanthin to violaxanthin than either A619 (p=0.0003) or Co255 (p=0.0008). Both A619 and Co255 had approximately three times as much zeaxanthin as violaxanthin while B73 maintained an approximately 1:1 ratio.

A619 had significantly more zeaxanthin than either B73 (p<0.0001 per gram dry weight and 0.0632 per m²) or Co255 (p<0.0001 per gram dry weight and p=0.0003 per m²). On an area basis, B73 had significantly more zeaxanthin than Co255 (p=0.049), but the two inbreds were statistically equal when compared per gram dry weight. The acclimation treatment did not affect zeaxanthin content on an area or dry weight basis on the third day of stress.
Day Seven of the 10°C Stress

Whereas neoxanthin levels were higher in the non-acclimated plants on the first and third days of the stress period, on the seventh day of stress the 3-day acclimated A619 (p=0.0205) and Co255 (p=0.0345) contained significantly more neoxanthin per m² than the non-acclimated plants. The 7-day acclimated B73 and Co255 had significantly less neoxanthin per gram than the non-acclimated plants (p=0.0055 and p=0.0438, respectively). This followed the trend that had been established on the first and third days of stress.

In both the acclimated and non-acclimated plants, B73 contained significantly more violaxanthin than either A619 or Co255 and subsequently had a significantly lower Z/V than A619 (p=0.0052). In the 7-day acclimated plants, Co255 contained significantly more violaxanthin per gram than A619 while the 3-day acclimated A619 had significantly higher levels than Co255.

Lutein concentrations per gram in non-acclimated B73 and Co255 were significantly greater than the 7-day acclimated plants (p=0.03 and p=0.0221, respectively), which was also found on the third day of stress. Averaged across inbreds, the 3-day acclimation treatment plants had 61.65 µmole/m² lutein while the non-acclimated plants had only 51.64 µmole/m² (p=0.0423).

On the seventh day of stress, the acclimation treatments did not affect the zeaxanthin content of any inbred based on either dry weight or leaf area. This had also been observed on the third day of stress. A619 had higher zeaxanthin concentrations than B73 in all treatments...
(p<0.0001) and Co255 only in the 7-day acclimation treatment (p=0.009). A619 and Co255 had equal levels of zeaxanthin in the 3-day acclimated and non-acclimated plants.

After seven days of 10°C stress, plants were returned to 25°C for four days for a recovery period. The acclimation treatments did not significantly alter the quantity of any of the measured xanthophylls following the 4 days of growth at 25°C. Zeaxanthin and violaxanthin content following the recovery period were equal to the pre-stress levels in all inbreds and treatments. Lutein contents following the recovery period were equal to the original unstressed 4th leaf stage levels.

**DISCUSSION**

Analyses of Leaf Area and Leaf Weight

Xanthophylls were quantified on both a leaf area and weight basis. Both analyses indicated that xanthophyll content responded to the acclimation treatments and that the xanthophyll content varied among inbreds. General trends were similar in both xanthophyll analyses based on dry weight and leaf area, but there were several points of disparity. The selected inbreds had significantly different leaf mass to area ratios with B73 having the highest at all time points (data not shown). Thicker leaves will tend to have more weight per given area. The distribution of chloroplasts and photosystems (Anderson and Andersson, 1988; Taylor and Craig, 1971) will vary in the leaf under different environmental conditions which could potentially differ among inbreds, and will influence the outcome of analysis on dry weight and area. When analyzed on an area basis, it is assumed that the chloroplasts and photosystems are predominately clustered near the leaf epidermis and that leaf thickness does
not affect xanthophyll quantity in the given area. Conversely, analysis per gram dry weight does not assume that leaf thickness is irrelevant. Both analyses provide information that is relevant to the investigation of the role of the xanthophylls in low temperature stress tolerance.

Xanthophyll Content in Inbreds of Putatively Known Tolerance

At the unstressed 4th leaf stage, B73 had more total xanthophylls per m² than A619 and Co255 but was approximately equal to A619 on a dry weight basis. The difference in total carotenoid composition is primarily due to the larger quantity of violaxanthin and lutein in B73 leaves. The high violaxanthin content of B73 provides a larger pool of violaxanthin which can rapidly be converted to zeaxanthin under stress conditions. The availability of free, unbound violaxanthin in the membrane limits the rate of conversion to zeaxanthin by violaxanthin de-epoxidase (Yamamoto and Bassi, 1996); thus the quantity of violaxanthin in the leaves could influence the development of stress tolerance. However, the conversion of violaxanthin to zeaxanthin is faster and less energy dependent than synthesis of zeaxanthin from β-carotene, and would decrease the amount of time required for the plant to respond to the stress.

A greater amount of zeaxanthin was expected to be formed under stress in B73 due to the initial high violaxanthin content, but this was not found. The least cold tolerant inbred, A619, had a higher zeaxanthin content and Z/V than either B73 or Co255 during the cold stress. Additionally, the total carotenoid content of Co255, which is putatively moderate to highly tolerant to cold temperatures, was lower than that of A619, which is considered more
cold susceptible. Although these findings tend to refute the role of the xanthophylls and particularly zeaxanthin in stress tolerance, it does not disprove the photoprotective role of the compounds. The combination of stress conditions and the selected inbreds should be considered because the 10°C temperature treatment may not provide an equal stress level for all plants (Levitt, 1980). In drought-stressed wheat plants, zeaxanthin content increased more in drought-susceptible cultivars than drought-tolerant cultivars (Loggini et al., 1999). This was attributed to the continuance of electron transport in the tolerant cultivar which effectively prevented the accrual of excess energy and the consequent formation of zeaxanthin. If cold tolerant B73 was capable of maintaining energy balance under cold temperatures stress and was not experiencing the same stress ‘load’ as A619, the formation of zeaxanthin from violaxanthin may not be favored as greatly and consequently less violaxanthin was converted.

Neoxanthin

Acclimated plants tended to have lower neoxanthin levels than the non-acclimated plants throughout the stress period. Neoxanthin is formed from the violaxanthin and does not participate in the xanthophyll cycle or energy dissipation. Its formation effectively removes violaxanthin from the xanthophyll cycle and consequently prevents rapid conversion to zeaxanthin and the development of non-photochemical quenching. As a general trend, acclimated plants maintain higher photosynthetic rates and Fv/Fm values than the non-acclimated plants (Grote et al., ant. 2005). The lower cold tolerance in the non-acclimated plants may partially be due to the formation of neoxanthin and its inability to participate in the xanthophyll cycle and energy dissipation.
Elevated neoxanthin levels in the 3-day acclimated Co255 and A619 plants coincide with improved photosynthetic rates and higher Fv/Fm, which counters the hypothesis that its formation and removal from the xanthophyll cycle reduces cold tolerance. Increases in synthesis of zeaxanthin from β-carotene could act to balance the removal of potential photoprotectants, or neoxanthin could be a component of another tolerance pathway.

Neoxanthin, along with all members of the xanthophyll cycle, is an intermediate in the production of abscisic acid (ABA) (Li and Walton, 1990), which has been shown to be involved in the stress response. ABA typically increases under stress conditions and is thought to be involved in the development of cold tolerance (Anderson et al., 1994; Chen and Li, 2002; Li et al., 1998; Xin and Li, 1993). The enzyme that cleaves neoxanthin to xanthoxin is a main point of control for the production of ABA (Qin and Zeevaart, 1999) so the changes observed in neoxanthin content of the acclimated and non-acclimated plants could be indicative of ABA production. Li and Walton (1990) found a relationship between the increase in ABA content of dark-grown, water-stressed leaves and the reduction in the xanthophylls neoxanthin and violaxanthin. The correlation between xanthophyll content and ABA may not be as strong under light conditions due to a higher total level of xanthophylls and the new synthesis of xanthophylls to replace the depleted cycle. The reductions in neoxanthin could be indicative of increased ABA production, which is involved in the development of tolerance in the acclimated plants (Anderson et al., 1994).
Lutein

Lutein is structurally similar to zeaxanthin and is also involved in energy dissipation (Niyogi et al., 1997). The acclimation treatments tended to decrease lutein content, which would hypothetically decrease the ability of the plant to withstand the high light stress accompanying cold temperature. Both the 3 and 7-day acclimated Co255 plants contained significantly less lutein on the third day of stress than the non-acclimated plants. At the same time, the photosynthetic efficiency was higher in both the 3 and 7-day acclimated plants, and the 3-day acclimated plants maintained higher photosynthetic rates (Grote et al., ant. 2005).

If the reductions in lutein were the result of increased preference for the formation of β-carotene and subsequently zeaxanthin from γ-carotene rather than α-carotene and lutein (Cunningham et al., 1996), the reduction in lutein would be a response of the shift in metabolism which favors the production of the xanthophyll cycle intermediates and zeaxanthin.

On an area basis, the most tolerant inbred as defined by photosynthetic rates and efficiency, B73 (Grote et al., ant. 2005), contained the most lutein at the 4th leaf stage and on each day of the cold stress but was equal to A619 when analyzed on a dry weight basis. High lutein content in the leaves may be a component of the physiological basis for cold tolerance in B73, but its abundance in cold susceptible A619 indicates that it is only one component of a complex system regulating plant response to low temperature stress.
Violaxanthin and Zeaxanthin

Acclimation treatments did not affect violaxanthin content in subsequent stress temperatures, but did influence the quantity of zeaxanthin per unit area and the ratio of zeaxanthin to violaxanthin. On the first day of the 10°C stress, acclimated A619 had significantly more zeaxanthin than the non-acclimated plants, but no concurrent significant reduction in violaxanthin. Zeaxanthin is formed either from the de-epoxidation of violaxanthin in the thylakoid membranes or by new synthesis from β-carotene (Yamamoto, 1979). The increased production of zeaxanthin in the acclimated plants was not due to the increased conversion from violaxanthin, so it appears that the increase in zeaxanthin content was due to new biosynthesis.

A619 and B73 had approximately equal levels of violaxanthin at the 4th leaf stage, but on the first day of stress the violaxanthin content of A619 was significantly less than B73. The reduction in violaxanthin in A619 relative to B73 could be due to increased conversion to zeaxanthin, as indicated by the higher Z/V in A619, reduced synthesis, or increased removal from the xanthophyll cycle via formation of neoxanthin. On the seventh day of stress, the violaxanthin content of A619 was higher than Co255 in the 3-day acclimation treatment while the opposite was found in the 7-day acclimation treatment. The reversal in accumulation of violaxanthin potentially affected the ability of the plants to recover from the cold stress. Photosynthesis in the 3-day acclimated A619 plants recovered to a much higher level than in the 7-day acclimated plants and to a level much closer to the photosynthetic rates of Co255 (Grote et al., ant. 2005). The larger quantity of violaxanthin in the 3-day acclimated A619 could be due to reduced conversion to zeaxanthin, which could be due to
less excess light, better energy balance, and consequently less severe stress load. If the plant
was under less stress on the seventh day of stress, its ability to recover is better than that of a
plant which is under a severe stress.

Haldimann et al. (1995) showed that plants acclimated at 15°C had higher levels of
zeaxanthin than plants grown at 25°C. The mild stress during the 15°C acclimation treatment
might have been sufficient to increase the production of zeaxanthin in all inbreds, but it was
only still apparent in A619 after 24 hours, possibly due to increased production of zeaxanthin
in B73 and Co255 in the non-acclimated plants. By the third day of stress, the acclimated
A619 plants did not contain significantly more zeaxanthin than the non-acclimated plants.
This was primarily due to an increase in the zeaxanthin content of the non-acclimated plants
rather than reductions in the acclimated plants. The 3-day acclimation treatment did not
significantly improve A619 photosynthetic rate or efficiency on the first day of stress, but did
tend to increase photosynthetic rate as the length of the stress period increased (Grote et al.,
ant. 2005). The accumulation of zeaxanthin during the acclimation treatment in A619 may
have prevented damage to the photosynthetic apparatus by preventing the formation of
reactive oxygen species or other radicals that are formed when light energy exceeds the
capacity for use by the plant. Consequently, the accumulation of zeaxanthin reduced the
cumulative effect of the cold stress as evident by the improved photosynthetic performance
on the third day of stress.

The acclimation treatments did not affect the accumulation of zeaxanthin at any time over the
course of the seven day stress period. Since acclimation increased Co255 Fv/Fm and
photosynthesis on the third and seventh days of stress (Grote et al., ant. 2005) without any significant accumulation of zeaxanthin, other compounds or processes within the plant appear to be responsible for the acclimation-induced improvements in Co255 on the third day of stress.

In summary, xanthophylls are involved in stress tolerance and are responsive to the acclimation treatments, but the improvements in photosynthetic rate and efficiency do not appear to align with the changes in any particular compound. Overall, cold tolerant B73 had the greatest xanthophyll quantity, but it accumulated less zeaxanthin and maintained a lower Z/V ratio than A619 and Co255 when exposed to 10°C. The cold susceptible inbred, A619, also had fairly large quantities of xanthophylls and accumulated larger amounts of zeaxanthin, which appeared to be generated both from violaxanthin conversion and new biosynthesis. According to current theory, higher zeaxanthin content should increase excess energy dissipation and protect the plant from photoinhibition of photosynthesis. Under the experimental conditions of this research, we found that the most tolerant line, based upon photosynthetic rate and Fv/Fm, had the lowest foliar zeaxanthin concentrations. We hypothesize that B73 was more capable of maintaining electron flow through the photosystems and continuing carbon fixation at 10°C than A619, which resulted in a relatively lower stress level in B73. We have shown that increased zeaxanthin does not necessarily result in increased tolerance as determined by photosynthetic rate and efficiency. Rather, the zeaxanthin content appears to be closely related to the effect that the particular stress has on the function of a specific line; the high zeaxanthin content of A619 could actually be related to its lack of tolerance as determined by these measurements of plant
performance (Fryer et al., 1995). The changes observed in neoxanthin could be indicative of ABA production. The flux of carbon through the xanthophyll cycle to ABA production is a potential consequence of acclimation and should be further studied. The role of ABA in the development stress tolerance could be integral to an acclimation phenomenon in cold sensitive species.
Table 1  Backtransformed means of selected carotenoids on an area basis in response to acclimation treatment. The p-values listed are for comparisons between the acclimated plants and the non-acclimated controls. Values that are preceded by different letter with a time*treatment combination are significantly different at the p=0.05

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>µmoles neoxanthin/m²</th>
<th>µmoles violaxanthin/m²</th>
<th>µmoles lutein/m²</th>
<th>µmoles zeaxanthin/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>day one stress</td>
<td>Inbred</td>
<td>non-acclimated 3-day acclimation</td>
<td>non-acclimated 7-day acclimation</td>
<td>non-acclimated 3-day acclimation</td>
<td>non-acclimated 7-day acclimation</td>
</tr>
<tr>
<td>A619</td>
<td>ab 44.7</td>
<td>a 44.26 ns</td>
<td>a 38.86 ns</td>
<td>ab 14.88</td>
<td>a 10.49 ns</td>
</tr>
<tr>
<td>B73</td>
<td>a 53.52</td>
<td>a 47.47 ns</td>
<td>a 42.10 (p=0.0029)</td>
<td>a 23.57</td>
<td>b 25.53 ns</td>
</tr>
<tr>
<td>Co255</td>
<td>b 38.47</td>
<td>a 40.04 ns</td>
<td>b 32.79 ns</td>
<td>b 10.07</td>
<td>a 8.00 ns</td>
</tr>
<tr>
<td>day 3 stress</td>
<td>Inbred</td>
<td>non-acclimated 3-day acclimation</td>
<td>non-acclimated 7-day acclimation</td>
<td>non-acclimated 3-day acclimation</td>
<td>non-acclimated 7-day acclimation</td>
</tr>
<tr>
<td>A619</td>
<td>a 47.47</td>
<td>a 44.70 ns</td>
<td>a 38.47 ns</td>
<td>a 11.70</td>
<td>a 10.70 ns</td>
</tr>
<tr>
<td>B73</td>
<td>a 53.52</td>
<td>a 54.60 ns</td>
<td>a 41.26 ns</td>
<td>a 16.28</td>
<td>b 34.47 ns</td>
</tr>
<tr>
<td>Co255</td>
<td>a 46.53</td>
<td>b 32.14 (p=0.0030)</td>
<td>a 38.47 ns</td>
<td>a 9.21</td>
<td>a 8.50 ns</td>
</tr>
<tr>
<td>day 7 stress</td>
<td>Inbred</td>
<td>non-acclimated 3-day acclimation</td>
<td>non-acclimated 7-day acclimation</td>
<td>non-acclimated 3-day acclimation</td>
<td>non-acclimated 7-day acclimation</td>
</tr>
<tr>
<td>A619</td>
<td>a 34.12</td>
<td>a 46.53 (p=0.0025)</td>
<td>a 40.04 ns</td>
<td>a 7.92</td>
<td>a 21.12 ns</td>
</tr>
<tr>
<td>B73</td>
<td>b 49.90</td>
<td>a 53.52 ns</td>
<td>a 41.68 ns</td>
<td>b 25.28</td>
<td>b 30.6 ns</td>
</tr>
<tr>
<td>Co255</td>
<td>a 35.52</td>
<td>a 46.06 (p=0.0345)</td>
<td>a 34.81 ns</td>
<td>a 6.55</td>
<td>c 11.13 ns</td>
</tr>
</tbody>
</table>


Table 2. Backtransformed means of selected carotenoid content per gram dry weight in response to acclimation treatment. Table values that are preceded by different letters are significantly different at the p=0.05 level.

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>non-acclimated 3-day acclimation</th>
<th>7-day acclimation</th>
<th>Treatment</th>
<th>non-acclimated 3-day acclimation</th>
<th>7-day acclimation</th>
<th>Treatment</th>
<th>non-acclimated 3-day acclimation</th>
<th>7-day acclimation</th>
<th>Treatment</th>
<th>non-acclimated 3-day acclimation</th>
<th>7-day acclimation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmoles violaxanthin/gram dry weight</td>
<td></td>
<td></td>
<td>pmoles violaxanthin/gram dry weight</td>
<td></td>
<td></td>
<td>pmoles lutein/gram dry weight</td>
<td></td>
<td></td>
<td>pmoles zeaxanthin/gram dry weight</td>
<td></td>
</tr>
<tr>
<td>day one stress</td>
<td>A619</td>
<td>a 1.79</td>
<td>a 1.88 ns</td>
<td>a 1.40 (p=0.0065)</td>
<td>0.59</td>
<td>0.44 ns</td>
<td>0.39 ns</td>
<td>a 1.99</td>
<td>a 2.02 ns</td>
<td>a 1.77 ns</td>
<td>a 0.77</td>
<td>a 1.39 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>a 1.72</td>
<td>b 1.35 (p=0.0143)</td>
<td>b 0.92 (p=0.0001)</td>
<td>0.76</td>
<td>0.71 ns</td>
<td>0.67 ns</td>
<td>a 2.12</td>
<td>b 1.73 ns</td>
<td>b 1.36 (p=0.00326)</td>
<td>a 0.38</td>
<td>b 0.63 ns</td>
</tr>
<tr>
<td></td>
<td>Co255</td>
<td>a 1.46</td>
<td>b 1.62 ns</td>
<td>a 1.23 ns</td>
<td>0.37</td>
<td>0.28 ns</td>
<td>0.21 ns</td>
<td>a 1.68</td>
<td>c 1.40 ns</td>
<td>b 1.36 ns</td>
<td>a 0.37</td>
<td>b 0.66 ns</td>
</tr>
<tr>
<td>day 3 stress</td>
<td>A619</td>
<td>a 2.06</td>
<td>a 1.80 ns</td>
<td>a 1.45 (p=0.0012)</td>
<td>a b 0.54</td>
<td>a 0.44 ns</td>
<td>a 0.38 ns</td>
<td>a 2.56</td>
<td>a 2.14 ns</td>
<td>a 1.84 (p=0.0136)</td>
<td>a 1.36</td>
<td>a 1.39 ns</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>a b 1.92</td>
<td>a b 1.50 (p=0.0011)</td>
<td>a b 0.7 (0.0001)</td>
<td>a 0.65</td>
<td>b 0.94 ns</td>
<td>b 0.86 ns</td>
<td>a b 2.41</td>
<td>a 1.93 ns</td>
<td>a b 1.67 (p=0.0095)</td>
<td>b 0.84</td>
<td>b 0.72 ns</td>
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<td></td>
<td>Co255</td>
<td>a 1.67</td>
<td>b 1.28 (p=0.0666)</td>
<td>a b 1.37 ns</td>
<td>b 0.33</td>
<td>c 0.25 ns</td>
<td>a 0.45 ns</td>
<td>a 1.88</td>
<td>a 1.43 (p=0.0535)</td>
<td>a 1.42 (p=0.0145)</td>
<td>b 0.81</td>
<td>b 0.75 ns</td>
</tr>
<tr>
<td>day 7 stress</td>
<td>A619</td>
<td>a 1.60</td>
<td>a 1.85 ns</td>
<td>a 1.57 ns</td>
<td>a 0.38</td>
<td>a 0.83 ns</td>
<td>a 0.34 ns</td>
<td>a 2.05</td>
<td>a 2.36 ns</td>
<td>a 2.05 ns</td>
<td>a 1.21</td>
<td>a 1.33 ns</td>
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<tr>
<td></td>
<td>B73</td>
<td>a 1.69</td>
<td>a 1.61 ns</td>
<td>a 1.12 (p=0.0055)</td>
<td>b 0.86</td>
<td>a 1.19 ns</td>
<td>b 0.97 ns</td>
<td>a 2.27</td>
<td>a 2.34 ns</td>
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<td>b 0.79 ns</td>
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<tr>
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<td>b 1.25 (p=0.0438)</td>
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<td>a 0.44 ns</td>
<td>b 0.65 ns</td>
<td>a 1.87</td>
<td>a 2.05 ns</td>
<td>b 1.46 (p=0.0211)</td>
<td>b 0.87</td>
<td>a 1.09 ns</td>
<td>b 0.90 ns</td>
</tr>
</tbody>
</table>
REFERENCES


Grote, K et al. anticipated 2005. Induction of cold tolerance by sub-optimal temperature treatment in maize seedlings.


Acknowledgements

I would like to thank my major professor, Dr. Allen Knapp, for helping me over the last 2+ years. Even if we didn’t agree, he would always listen to my opinion. I can honestly say that this thesis is the result of my work and my thought.

My husband deserves a lot of credit for dealing with me while I was working on my master’s project. It was pretty stressful for both of us.

I also want to thank my friends in the department for listening to me when things weren’t working like they should have been.