Screening for low temperature stress tolerance in maize: Protocol development and testing

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Screening for low temperature stress tolerance in maize:
Protocol development and testing

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Signatures have been redacted for privacy
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ABSTRACT

Maize (Zea mays L.), is considered by many to be one of the most meaningful crops grown in the USA and in many countries around the world. Despite of its sub-tropical origins, maize endures many unfavorable conditions effecting growth and development. With a wide genetic background, many desirable cold tolerant traits in maize have been identified, but there are still many more to be discovered. The method of identifying these various mechanisms and traits of cold tolerance is the purpose of my study.

Three maize inbred (A619, B73, CO255) lines that all have illustrated various genetic differences were used to determine: electrical conductivity, percent potassium leakage as a result of membrane permeability and dry weight accumulation.

Seedlings were grown at 22/24°C until the 4th leaf stage at which time they were transferred to the experimental treatment regime of 8/6°C for 48 hours and 9 hours. After stress treatments regime plants were returned to the control temperature of 22/24°C to recovery until the 5th leaf stage.

There were significant differences found among all growth parameters tested. Inbred line B73 was found to have the highest percentage of electrical conductivity when tested at 48 hours of 8/6°C. B73 was also recorded to have
the highest amount of potassium leached as a result of treatment regime. When comparing the amount of dry weight accumulation CO255 was the largest of the three inbred lines tested. But when dry weight was compared before and after treatment B73 had the largest amount of growth after recovery from the treatment regime. This suggest that though CO255 was the largest of all the inbred lines tested, B73 had a greater amount of dry weight accumulation after or during recovery.

The evaluation of these inbred lines did not allow a definite evaluation of the whole genetic background of the experimental lines tested, but chilling did induce the seedlings to respond in such a way that desirable characteristics could be identified.
INTRODUCTION

Unfavorable weather conditions, particularly when soils are wet and the environment is cool, can limit the ability of maize seeds to germinate rapidly and produce vigorous seedling. The ability to survive cold temperatures and continue to grow in unfavorable weather conditions provides a species with a competitive advantage by lengthening the effective growth season and/or positioning the plant to capitalize on favorable weather periods during the growing season (Greaves, 1996).

The range of temperatures within which a plant can grow has been defined as "one of the most important ecological factors governing the natural distribution of species and the yield potential of crops" (Greaves, 1996). Resistance or sensitivity to stress can depend on the species, the genotype, and the developmental age of the plant (Bray et al., 2000).

In unfavorable conditions, cold tolerant genotypes may be sown earlier than usual, thus allowing the achievement of several agronomic advantages, such as early development of canopy, a decrease in soil evaporation because of shading, and anthesis before the dry and hot days of mid-summer (Pendleton, 1965).

It is the purpose of this study to analyze specific laboratory techniques that are easily reproducible and can be employed as a screening tool for the investigation of the various mechanisms of cold tolerance. The development of such a screening tool is the focus of this research.
LITERATURE REVIEW

Cold Tolerance

Cold tolerance is a complex quantitative trait that is expressed following exposure of plants to temperatures that approach freezing (Fowler et al. 1993; Fowler et al. 1996) and is associated with several physiological and biochemical alterations in the plant. Results of numerous research studies with field crops can be summarized to help us understand the genetics of cold tolerance, the range of genetic variability for gene pools within species and the potential sources of new exploitable genetic variability (Grafius, 1981, Stushnoff et al. 1984). The results of these studies can demonstrate the complex nature of the mechanism the control cold tolerance.

Cold tolerance is dependent upon an integrated system of structural, regulatory and developmental genes. The evaluation of two populations of Iowa Stiff Stalk Synthetic by Mock and Eberhart (1972) have evaluated estimates of genotypic variations and heritabilities, and suggested that this germplasm could be improved for cold tolerance.

Pinnell (1949), Helgason (1953) and Grogan (1970) all have reported that the genetic nature of cold tolerance in maize is complex because of significant maternal or cytoplasmic effects associated with inheritance of germination and early growth. Grogan (1970) also concluded that heterosis influenced germination and growth at cool temperatures and also concluded that “an additive multiple-factor genetic system conditioned cold tolerance in maize.” Pesev (1970) considered inheritance of
cold tolerance to be rather complex and concluded that the better stand establishment of single crosses over inbreds was attributable to complementary gene action.

Neal (1949) and Haskell et al. (1949) early in the investigation of cold tolerant material reported in their findings, the highly complex and difficult background in the understanding of the genes which control cold tolerance and reported a significant difference between maize lines and cultivars with respect to their capability to germinate to low temperature.

Challenges in Conventional Breeding and Screening Protocols

Visual observations in the field have been the conventional screening process for chilling sensitivity in many breeding programs, which can vary largely based on the experience and knowledge of the individual making the observations. A technique is needed that will give rapid, reliable, quantitative assessment of the injuries caused to plants by chilling and the critical temperatures at which those injuries occur. Heritability is another variable used in selection of an inbred, which can be reduced under conditions of stress and vary from year to year (Greaves, 1996). Thus, an adequate screening protocol is needed to isolate the potential of cold tolerance materials and at the same time mimic unfavorable field conditions.

Effects of Oxidative stress on plants

Cold temperatures in common with many other stresses may cause oxidative stress, which (Prasad, 1997) considers to be a secondary factor. A number of the
factors contributing to protection against oxidative stress are expressed at higher levels or with increased activity in cold stressed plants.

Numerous defense mechanisms, both enzymatic and non-enzymatic, are present in the cells for protection from oxidative injury. Growth at low temperatures provokes oxidative stress in plants.

Oxygen free radicals can mediate chilling injury by acting as agents that can cause injury to membranes and photosystems (Prasad, 1994a; 1994b). According to Lyons and Raison (1970), oxidative stress must be considered as a secondary response to a primary lesion in a redox enzyme. Shewfelt & Erickson (1991) proposed that lipid peroxidation would also alter the physical properties of membrane lipids, and thereby inhibit the function of membrane-bound proteins contributing to the development of visual symptoms of chilling injury.

Oxidative stress is a major damaging factor in plants exposed to the combination of high light intensities and other stresses which limit photosynthesis such as drought and low temperature (Jahnke et al. 1991; Massacci et al. 1995).

Membrane fluidity is significant in the identification of chilling sensitivity and membranes that are susceptibility to lipid peroxidation (De Santis et al., 1999). Equally important is the capacity for antioxidant defense available to protect membranes and other components from oxidative damage. Whether this is the case or not, the antioxidant defenses appear to provide crucial protection against oxidative damage in plants grown at low temperatures.

**Mitochondrial Effects**
Mitochondria are critical organelles in the metabolic production of energy in the cell. The competence and the stability of mitochondria are very important for seedlings to survive low-temperature stress, especially during early seedling growth. Therefore, a better understanding of molecular and biochemical mechanisms of chilling responses could provide clues for “genetic manipulation” of chilling-sensitive species for achieving better growth and higher crop yields (Purvis and Shewfelt, 1993). An immediate response of chilling-sensitive tissues to low temperature is a depression of mitochondrial respiration (Lyons and Raison, 1970). Their results also suggested that a phase change occurs in mitochondrial membrane components during low-temperature stress (Lyons and Raison, 1970). Chilling injury impairs mitochondrial function by depressing the respiratory activity, the electron transport and ATPase activity (Jahnke et al., 1991). Changes in these processes seem to be, but not completely, under the control of translation. An oxidative stress maybe partially responsible for the irreversible damage to the mitochondrial membrane (Doulis et al., 1997).

Tolerance to low temperatures is a complex and multi-faceted trait requiring adaptation of many cellular processes. In maize, mitochondrial functions are impaired in chilled seedlings (De Santis et al., 1999) while chilling induced photoinhibition of photosynthesis is a major factor limiting yield (Prioul, 1996; Fryer et al., 1998). Both studies implicate that chilling-induced oxidative stress can be the cause of lost function. This suggests that antioxidant defense is central to survival of sub-optimal temperatures (Prasad, 1997; Hodges et al., 1997). In support of this view, antioxidant enzymes activities have been used to screen maize populations for
chilling resistance (Hodges et al., 1997). It has also been reported that, mesophyll and bundle sheath cells of maize leaves clearly have very different capacities of antioxidant defense at optimal temperatures (Doulis et al., 1997; Burgener et al., 1998) and this difference is even more pronounced at low growth temperatures.

Low Temperature affect on plant membranes

The primary and immediate response to low temperature in chilling-sensitive species is a "reversible change in the physical state of the membranes, above the critical temperature of transition the membranes posses the fluidity essential for normal physiological processes, whereas below that temperature the fluidity is restricted" (Pearce, 1999). The severity of the symptoms is "a function of the temperature extreme, duration of exposure, the morphological and physiological condition of the plant material at exposure, and the time between exposure and appearance of the symptom or dysfunction" (Pearce, 1999).

The biochemical events that follow the loss of membrane fluidity include interference with function of those enzymes embedded in or closely associated with a membrane, reduced energy supply, loss of compartmentation, ion leakage and similar events that disrupt normal metabolism and lead to imbalances and loss of function (Lyons, 1973). Many authors have reported that the primary sites of the chilling effect appear to be plant membranes (Yoshida, 1991; Brauer et. al. 1991). The evidence of membrane damage caused by chilling has been found by different methods including measurements of electrolyte leakage, electron spin resonance and fluorescence depolarization, changes in lipid composition, freeze-fracture electron microscopy, X-ray diffraction (Lyons, 1973; Du Pont, 1989; Yoshida, 1994).
Temperature is likely to also have a direct effect on the fluidity, viscosity, or phase-state of membrane lipids (Lyons, 1973; Dupont, 1989). Since the permeability of cell membranes is related to the phospholipid content (Nasyroya et al. 1984) the possibility arises that chilling may bring about some change in the phospholipid composition of leaf membranes.

**Effects of Chilling Injury on plants**

Physiological injury to plants at low, non-freezing temperature is termed chilling injury (Lyons et al. 1979), and is characterized by such diverse symptoms as reduced seedling vigor and growth, reduced yield and quality, inhibited photosynthesis, abnormal fruit ripening, and increased disease susceptibility (Saltveit and Morris, 1990). The exact method by which low temperatures cause this alternation is unclear, but it has been suggested that “a conformational change in membranes or a change in the activation energy of enzymes is the cause” (Hugly et al., 1990). These recent studies of the molecular mechanism of chilling injury using genetically altered plants have confirmed that sensitivity to chilling is related to the lipid composition of membrane (Hugly et al., 1990). A great deal of existing data indicates that a cellular membrane is involved in the expression of chilling injury, if not in the actual transduction of chilling temperatures into a physiological signal (Lyons, 1973; Lyons et al. 1979; Ono and Murata, 1982; Patterson et al., 1984).

An injury caused by brief exposure to severe chilling temperature can result in substantial loss of leaf area and root systems (Dupont, 1989). Miedema et al. 1987, demonstrated that chilled seedlings in the dark for 3-9 days after emerging resulting
in different types of injury such as narrow chlorotic cross-bands, necrosis of young leaf tissue, necrosis of mature leaf tissue and whole plant tissue.

Responses to chilling include a decrease in water uptake presumably due to reductions in root conductance and leakage of endogenous solutes due to loss of membrane integrity (Bolger et al. 1992). Growth reduction seems to be caused by alternations in the balance existing between cell production and differentiation. This is followed by the induction of a differential response in the capacity to resume growth when warmer temperatures resume (Barlow and Adam, 1989).

**Photosynthesis**

Although exposure to low temperature can adversely affect many physiological processes, the effect on photosynthesis is considered to be a particularly important factor contributing to the poor early establishment of the crop (Baker et al. 1983). Exposure to low temperatures has been reported to cause “perturbations in specific enzyme activities, for which genotypic variation can exist” (Stamp, 1987). A restriction in carbon metabolism has been connected to an increased susceptibility to photosynthetic stress at low temperatures; which promotes an energization of the photosynthetic apparatus and therefore the formation of reactive oxygen species (Prasad, 1994a). Photoinhibition will typically occur when the rate of light (photon) captured significantly exceeds the ability of the chloroplast to transfer and convert this trapped light energy into chemical energy (Nie et al. 1992). The combination of continual light captured without efficient transfer and the activity of trapped energy into carbon assimilation can be
catastrophic because the trapped energy is lost to electron acceptors such as oxygen which becomes chemically activated forming free radicals (Alberda, 1969). These radicals rapidly cause cell damage resulting in a loss in cellular integrity.

Several studies have shown that during chilling (0 to 12°C), the photosynthetic apparatus can be damaged by light, a process termed chilling-dependent photoinhibition of photosynthesis (Powles, 1983). Maize has also been shown to suffer chilling dependent photoinhibition in controlled environments and in the field when grown in temperate climates (Alberda, 1969; Stamp, 1987).

**Electrical Conductivity**

Damage to plants caused by exposure to low temperatures is generally divided into two categories, "freezing injury" and the "chilling injury" caused to plants by exposure to cold in a temperature range (Stushnoff et al. 1984). Changes in conductivity have in the past been utilized as a measurement of various other types of injury e.g. reaction to toxins (Osterhout, 1922), to concentrated salt solutions, to electric shocks and to freezing (Wilner, 1961). The cell membranes are believed to contribute to the electrical resistance of plant tissue. Changes in the membrane as a result of cold temperatures can cause a rise in conductivity for two reasons: a decrease in resistance or the leakage of electrolytes into the intercellular spaces, where they are more available for ionic conduction (Saltveit, 2002). However, there is increasing evidence that electrolyte leakage denotes irreversible cell damage and occurs long after many other events in the cell membranes (Yoshida, 1991). Although still alive, the cells may have suffered an injurious increase of passive permeability.
The electrical conductivity test is based on the amount of naturally occurring electrolytes that diffuse out of the cells following the cold exposure (Dexter, 1956). Measuring solute leakage from plant tissue is a longstanding method for estimating membrane permeability in relation to environmental stresses, growth and development, and genotypic variation. Recognizing that electrolytes content of plant tissue can vary among samples, (Dexter et al. 1930) was one of the first to recommend expressing electrolyte leakage as an index percentage of total electrolytes present in the tissue due to these variation.

The basic assumption of ion leakage measured by electrical conductivity is that the greater the injury of the living tissue, the greater the efflux of ions from the cells (Tai et al., 1986). Effusate can be defined as a solution of substances removed from plant tissue by immersion in water. In recent literature, the assumption seems to have been made that the efflux is mainly from dead cells, and therefore one measures the chilling injury by determining the percentage of cells killed (Sukumaran et al.1991)

Chilling injury has also been reported to result in the leakage of ions and sugars from the cells (Palta et al. 1977). He also stated that the more severely injured the cell the less capable they become to reabsorb the solution from the intercellular spaces. Cell membranes are one of the first targets of many plant stresses and it is generally accepted that the maintenance of their integrity and stability. Vainola and Repo, (2000) has reported that electrolyte leakage measurements seems to be correlated with several physiological and biochemical parameters conditioning the plant responses to environmental conditions such as
spectral reflectance. Antioxidative enzyme synthesis, stomatal resistance, osmotic potential, and leaf rolling index (Huang and Liu, 2000) are also biochemical parameters that have been associated with electrolyte leakage. Electrolyte leakage has also been recommended for the identification of stress resistant cultivars in several crop species (Leopold et al. 1982; Stevanovic et al. 1997).

According to Prasil and Zamecnik (1998), electrolyte leakage from plant tissue in deionized water can be considered as a function of time. An excelled rate of leakage can occur from the intercellular free space regions followed by slower releases across the plasma membrane and then tonoplast (Bajji et al. 2002). As time passes stressing conditions can lead to stabilization in the amount of leakage during subsequent rehydration which can suggest the hardening of the stressed tissue (Leopold et al. 1981).

Studies, which analyzed this aspect electrolyte leakage in other species, concluded that an important part of this electrical conductivity may be attributed to potassium and its unidentified counteranions (Palliotti et al. 1996; Palta et al. 1977; Scherbakova et al. 1983). Bajji et al. 2002, have reported that potassium is undoubtedly the major inorganic ion recorded in the effusate tested.

Ultrastructural membrane changes that permit electrolyte leakage and ion imbalance in the cell, have been show to result form chilling injury Lyons (1973). Many authors have also reported that the main cause of injuries in severe chilling may be phase transitions in the membranes causing their greater permeability or total dysfunction (Lyons, 1973; Bagnall and Wolfe, 1982; Wilson and McMurdo, 1981).
We know from cited reports that, a stressful environment can result in numerous physiological symptoms which can result in membrane damage. Among the symptoms of membrane damage are a lessening of the Hill reaction of chloroplast (Boyer, 1976); a lowering of the efficiency of photosynthesis (Boyer, 1976); a lowering of the respiratory rates of mitochondria (Koeppe et al. 1973); an increase in leakage of solutes from leaf tissue (Gupta, 1977). Dexter et al. 1930 was one of the first scientist that observed a component of chilling injury; an increased leakage of solutes following stress.

**Potassium**

Potassium plays a key role in a vast array of physiological processes vital to plant growth from protein synthesis to maintenance of plant/water balance. Presently the knowledge of biochemistry and physiology mechanisms that control stress tolerance in plants suggest that the membranes are important cellular targets common to different stresses (Levitt, 1980). Palta (1977) and Pukacki (1986) have proposed that the major cation that leaks out of the cell is know to be potassium (K), thus enhancing the efflux of ions and result from a higher efflux than the influx rate resulting in damage. Potassium was selected in my study as a representative ion because it is one of the major inorganic ions present in the cell membrane of the plant. Palta (1977) has provided independent verification of a strong relationship between electrical conductivity and potassium in aqueous solutions containing effusate from plant tissue. This will in turn lead to concentration gradients, which are recognized as a major component of the electrochemical driving force from even movements across the concentration gradient.
Potassium is also known to play an important role as an osmotic substance and it may also influence the physical status of the cell membrane (Briskin, 1986). Potassium is a part of the cytoplasmic solution and helps to lower the osmotic potential; resulting in lower water loss from the leaves and better water uptake by the plants roots (Marshlener, 1995). Potassium predominately exists as a free or absorptive bound cation, and can therefore be displaced very easily on the cellular level (Epstein et al. 1969).

High mobility of potassium in plants may explain the major functional characteristics of potassium as the main cation involved in the neutralization of charges and as the most important inorganic osmotic active substance (Clarkson and Hanson 1980). Effusate (leakage) of onion bulb scales potassium ions plus counterions, accounted for almost all the increase in electrical conductivity (Palta, 1977). He has also proposed a possible explanation for the increase in ion leakage: passive permeability of cell to potassium is increased by freezing and low temperatures, it is also possible that counterions or freezing and low temperatures inactivates the active uptake mechanism of potassium and possible other counterions. It is been suggested Stadelmann (1974) that the reason for this damage is probably by the replacement of calcium in the plasma membrane by potassium; thus making the cell membrane bound structure weaker.

Premachandra (1989) observed that sugars and potassium were the primary osmotic contributors and that potassium contributed more than sugar to osmotic concentration under low moisture and temperature levels. And while the primary osmoticum differs somewhat among crops, potassium and sugar are the main
contributors to osmotic adjustment in many species of plants (Jones, 1980). The increase in total ion leakage, K+ leakage specifically, was reported to be a sensitive indicator of chilling injury in grapefruit callus tissue (Forney et al. 1990) and in whole oranges, limes and grapefruit (Pantastico et al. 1968).

Concentration gradients are also recognized as a major component of the electrochemical driving force for ion movement. It is also well-known that both leaf water content and K+ content, which together determine internal concentration, vary with age stress history and other factors (Wyn, 1979). Whereas ion uptake in plants receives considerable attention, the release of solute from the plant into the environment is less investigated. Considerable amounts of solutes are lost after cell damage and cell death. Although the mechanism of potassium and its absorption in the plant cell is not fully understood; potassium neutralizes various anions and other compounds within the plant, helping to stabilize pH (Kochian, 1989). Epstein et al. 1963, has proposed that potassium present in the cell determines how many of the enzymes can be activated and the rates at which these chemical reactions can proceed. Active uptake of potassium across a cell membrane has been found to result from either a direct or indirect coupling of H+/K+ exchange (H+efflux/K+ influx) Briskin (1986). Serrano (1985) suggested that the plasma membrane of higher plants contain an electrogenic proton pumping ATPase, which uses the chemical energy of ATP to drive the extrusion of protons into the external medium. This will turn leads to a product of a pH gradient and also an electrical potential, which is more positive outside than inside.
It was also reported by Arora (1991) that these gradients constitute a proton motivating force, which is believed to drive "carrier-mediated" active influx of various ions across the plasma membranes. In addition, evidence is accumulating in favor of plasma membrane ATPase as a primary sire of alteration following several stresses, chilling, ozone and salinity (Gronwald et al. 1990).

Measurements of loss of electrolytes have been used in many studies assessments of tissue damage in studies of freezing (Sukumaran and Weiser, 1972) and chilling (Wright and Simon, 1973) and possibly reflect the level of membrane integrity. Lewis et al. (1964) have reported that they have found a significant correlation between electrolyte leakage and chilling injury. Thus eluding to the theory that leakage and exudation are useful in revealing genetic difference among lines of chilling tolerant and intolerant plants.

MATERIALS AND METHODS

Genotypes

Three maize [Zea mays L.] inbred lines were used in this experiment: chilling-tolerant North American dent C0255, originating from Ottawa research station (Hodges et al. 1997, Aidun et al. 1991), Canada; chilling moderate from the U.S. Corn Belt from Iowa Stiff Stalk Synthetic dent B73 (Mock et al. 1979, Brandolini et al. 2000, Messmer et al. 1992); and chilling sensitive cornbelt dent originating from University of Minnesota A619 (USA) (Hardacre et al. 1980, Eagles et al. 1981, Pietrini et al. 1999). These three inbred lines were selected because of their ability to survive in cool temperatures below 12°C (Hodges et al.1997, Mock et al. 1979, Eagles et al. 1981). The U.S. Corn Belt dent inbred lines used for this study are
typical of the lines used in the production of many commercial hybrids in the U.S, New Zealand and many other temperate regions of the world.

Seeds of these three inbred lines were produced in 2001 at Iowa State University research farm. At maturity ears were picked, dried at 25 to 30°C with low humidity and hand shelled. Until time of study in November 2002, the seed lots were stored in a cold room set at 4.4°C, 40% relative humidity.

**Cultural**

All seeds were germinated in 9 x 10-inch pots filled with "Redi-Earth" potting mix, which containing vermiculite, peat moss mix (Redi-Earth Hummert Cat. # 10-2030) and grown at a temperature of 22/24°C in a controlled environment growth chamber. Seedlings were grown at a density of eight plants per pot and were watered once every other day. At the 2½ leaf stage plants were transplanted into 4 x 4 -inch plastic pots, with the density of one plant per pot containing "Redi-Earth" potting mix. Leaf stages are defined as the number of leaves with visible ligules (Soldati et. al., 1999).

Seedlings remained in the 4 x 4 -inch plastic pots for the duration of the study. Each chamber contained 73 plants per inbred line tested, which totaled 220 seedlings per growth chamber. After plants were transferred to 4 x 4 -inch pots Scott's Miracle Grow Excel fertilizer was added into watering regime every other day.

Once seedlings had reached the 4th leaf stage, the growth chamber temperature settings were changed to one of the three day/night temperature regimes: 24/22°C (control), 10/8°C for 48 hours (treatment one), 10/8°C for 96 hours (treatment two). One of the three chambers was kept as a control and maintained
the control temperature of 22/24°C for the duration of the experiment. After the stress temperature regime was completed, chambers designated for treatment regimes were returned to control temperature of 22/24°C and the plants allowed to recover to the 5th leaf stage at which time growth measurements were taken and project was then terminated. Measurements were taken before the cold treatments were applied, immediately after the application of the cold treatments and at the 5th leaf stage.

Environmental

Three growth chambers were used to conduct this study, each chamber was programmed in such a way that the lights, temperature and relative humidity of all three were similar in operation. At the initiation of the study all three chambers were set at a control temperature of 24/22°C (day/night) with a relative humidity of approximately 70% and a light regime of 12 hours photoperiod (day/night), at 375 μmol photons m⁻² s⁻¹ photosynthetically active radiation (PAR). Light was provided by florescent tubes (Sylvania cool white, VHO) and incandescent bulbs (100 W, Sylvania). Light regimen was 12 hour photoperiod day/night, with 375 μmol photons m⁻² s⁻¹ PAR were used in the experiments. Photosynthetically active radiation (PAR) was measured with a quantum sensor (Li-185, LiCor, Lincoln, NE, USA). These settings were kept constant with the exception of temperature throughout the three replications of the research study.

Experimental

Electrical Conductivity Test
Six randomly selected plants per inbred line were removed from each of the three growth chamber. Twenty leaf disc per plant were punched (using a Swingline three-hole notebook puncher) from each of the six selected plants at each designated testing intervals. Leaf disc where punched and then placed into a plastic weigh boat were they were mixed by hand and randomly placed into six labeled 100ml glass Pyrex test tubes. Twenty ml of double deionized (DDI) water was added to each of the six test tubes. Test tubes were then transferred to a vacuum chamber for fifteen minutes (Manley et al. 1996). After vacuum infiltration, plastic tops were placed on test tubes and they were then transferred to a platform shaker (Innova Model 2300 large platform shaker) for two hours to initiate initial electrolyte leakage. After the shaking period, initial electrolyte was measured (Oakton electroconductivity meter model #510) at room temperature (Couto et al. 1998). Test tubes were then autoclaved at 240°C for twenty minutes to heat-kill plant tissue (Mackay, 1994). Test tubes were removed and let cool back to room temperature of 24°C. After cooling, the solute in the test tubes was measured for final electrolyte leakage. Leakage was expressed as the ratio (leakage of live tissues/leakage of heat-killed tissues) x 100.

**Atomic Absorption Spectrometry (AAS)**

The preparation of plant materials for atomic absorption spectrometry was identical to the preparation for the electrical conductivity testing. AAS measurements were made using an Perkin Elmer Aanalyst 300 spectrometer (Si et al. 1999; Hu et al. 1999; Hendershott et al. 1986). After removing the designated test tubes from the platform shaker for potassium measurements; three ml aliquots of solute from each test tube containing 20ml of solute was removed and measured for initial potassium
leakage by AAS (Hamze et al. 1984; Page, 1982; Sparks, 1996). Remaining solute was then transferred to autoclave for twenty minutes at 240°C to heat-kill plant tissue. After autoclaved test tubes were allowed to cool back to room temperature of 24°C and final potassium leakage was measured by AAS. Leakage is expressed as the ratio (potassium leakage as a result of treatment regime/potassium leakage after autoclaving) x 100.

**Fresh Weight Accumulation**

The fresh weight accumulation and biomass accumulation techniques are viewed as useful method in comparing plant growth at various developmental stages (Janowiak and Markowski, 1987; Hodges et al. 1997). Plants for fresh weight accumulation measurements where selected in identical fashion as the previous measurements have been described.

Each plant was washed and separated into roots and shoots and placed into small coin envelopes. Each plant part was place into individual envelopes and labeled. Envelopes were then weighed individual for fresh weight and placed into oven set at 70°C for 48 hours. After drying, envelopes were allowed to cool back to room temperature in a bench top dessicator after which time they were weighted for dry weight (Shipley, 2002).

**Experimental Design**

The study was conducted using a split-plot design. The temperature regimes were the whole-plot treatment factor and the genotypes and times of exposure were the split-plot factors. The whole-plot portion of the experiment was arranged as a Latin square with runs and chambers as blocking factors. All experiments were
repeated three times with similar results. Data were subjected to ANOVA and LSD values were calculated when significant differences (P= 0.05) were detected.

**Electrical Conductivity Results**

Electrical conductivity measurements, from both stress treatments of 8/6°C for 48 and 96 hours were found to be significant (Table 1). There were no significant differences in electrical conductivity when inbred lines were tested at the fourth leaf stage at the control temperature of 22/24°C. However, when stressed for 48h at 8/6°C the electrical conductivities of all three inbreds were significantly different with inbred CO255 having the lowest electrical conductivity and B73, the highest (P ≤ 0.05). Under prevailing theories regarding electrical conductivity, these results indicate that, at 48 hours of treatment, B73 suffered greatest amount of injury of the three inbred lines tested.

When the three selected inbred lines were treated at 8/6°C for 96 hours, the electrical conductivities of all three inbred lines were again, significantly different. However, this time, B73 and CO255 both had higher amounts of electrical conductivity while A619 had the lowest level.

**Potassium Leakage Results**

Small, but significant differences were found in the amount of potassium leached as a result of the treatment regimes. After 48 hours of treatment at 8/6°C, B73 lost the highest percent of potassium K⁺ of the three inbred lines tested (Table 2). A619 also showed elevated amounts of K⁺ leached from leaf tissue, but this measurement was not found to be statistically significant (P≤0.05), compared to CO255. One commonly identified mechanism that can influence the relative amount
of K in the plant is the interference of Na with K at the site of uptake in the roots (Epstien, 1972; Waisel, 1972). This interference could explain the lower potassium readings in plants that were treated with the treatment regime of 8/6 C° when compared to those at control temperatures of 22/24 C°.

Differences were also found in K+ measurements taken after recovery at the 5th leaf stage. All three inbred lines were found to be significant when measured at the control treatment 22/24 C° and at 48 hours of stress treatment. Though both treatments had recorded differences, the plants measured at 22/24 C° had the highest amount of potassium leakage from leaf tissue when compared.

No significant differences in percent potassium leakage were found after 96 hours of exposure to 8/6 C° (Table 2).

**Fresh Weight Accumulation Results**

Variations in inbred fresh weight accumulation were observed as a result of experimental treatments (Table 3). When the fresh weight of plants measured at control temperatures 22/24°C and after plants were treated with 48 hours of stress treatment at 8/6°C were compared a significant difference was found among plants that were subjected to temperature regime 8/6°C. There was no significant difference found among inbred lines treated for 96 hours at 8/6°C (Table 4), which maybe a function of chambers used for study and measurement of control taken at that given time. With further investigation it can be identified that plant that were subjected to 8/6°C had significantly less growth than those that were grown at 22/24°C. Among the plants treated with 48 hours of stress, inbred line CO255 had the largest amount of fresh weight accumulation when compared to the other inbred
lines tested (Table 3). A possible explanation can be that CO255 has been reported by Hodges et al. 1994 &1995 to be a cold tolerant inbred line, thus giving CO255 a growth advantage over B73 and A619 when subjected to experimental treatments. But as the plants continued to grow and were return to the temperature of 22/24°C until the 5th leaf stage; the difference among the plants grown for 48 hours at 8/6 °C and the plants that maintained a constant temperature of 22/24°C were found to be no longer significant.

After comparison of plants treated with temperature regime of 48 hours or 96 hours at 8/6°C and plants that were maintained at 22/24°C, a reduction of growth due to temperature was found (Table 5). After further investigation of 48 hours at temperature 8/6°C was shown to have the greatest affect on over plant growth when compared across treatments. After inbred lines were returned to 22/24°C after treatment regime and compared to plants that were grown at a constant 22/24°C for the duration of the study. A significant difference was also found amount inbred lines tested that had been subjected to a stress temperature regime (Table 5). When compared to plants grown at 22/24°C, plants that were treated with the temperature regime of 8/6°C for 96 hours showed the least amount of growth when tested at the 5th leaf stage (Table 4).

CONCLUSIONS

Several different responses to low temperature exposure were observed. In the electrical conductivity study, the temperature at which plant injury occurred, was expected to be indicated by a sharp increase in electrolyte leakage with declining temperature. This was a characteristic recorded in our current study. This increase
could be due to a membrane phase change as described by Lyons and Raison (1970), and/or a delay and disruption of membrane organization during low temperature as described by Bramlage et al. (1978) in soybean seeds. According to these theories, maize cultivars with the ability to adapt to lower temperature or have no distinct temperature for an increased leakage should be the varieties with the greater cold tolerance. As electrolyte leakage is greatly influenced by both plant and leaf age (Premachandra et al. 1987), all experimental measurements were taken at the same seedling stage.

Reported by Prasil and Zamecnik (1998), electrolyte leakage from plant tissue in deionised water is a function of time. And rapid leakage would occur from the intercellular free space regions followed by slower releases across the plasma membrane and then to the tonoplast. Bajji et al. 2002, has also reported that an initial high leakage level may influence the sensitivity of a plant to cold injury. Bajji, also concluded that a plant with good cold tolerance would be one that exhibits both low initial leakage and a low temperature tolerance for electrolyte loss. Damage to plants and membranes could also result through accumulation of toxic metabolites following differential effects on enzymatic reaction rates (Lyons, 1973).

Exposure of plants to 8/6 C° for 48 hours did cause significantly more injuries than those plants treated at 8/6 C° for 96 hour of treatment (Table 3 & 4). But not all cold related injuries and mechanisms of physiological injuries have been explained so far. In the opinion of many authors the main causes of injuries in severe chilling may be phase transition in membranes causing greater permeability or total dysfunction (Lyons, 1973; Bagnall and Wolfe, 1978; Wilson and McMurdo, 1981).
Many authors have claimed from previous studies that the greater the accumulation of dry matter, the extension growth and fresh weight under chilling conditions, the higher the cold resistance of genotypes (Cal et al. 1972; Stamp, 1981). And such characteristics as maternal effects can be more pronounced in response to adaptation to cold temperatures (Hodges et al. 1997). Some instances inbred lines that were initially chilling or tolerant may alter their sensitivity to chilling as they grow (Hodges et al. 1997).

Exposure of maize seedlings to lower temperatures had an unfavorable effect on fresh weight accumulation of the seedlings tested. The exposure of maize seedling to low temperature leads to a decrease in fresh weight accumulation of the whole plant (shoots and roots). The method of a decrease in fresh weight appears to have a direct effect on the plant's ability to avoid injury. This conclusion can be drawn after viewing the recorded observations at the 5th leaf stage. It was recorded that there was no significant difference in growth among plants that were treated with low temperature regime from those that were grown for the duration of the study at 22/24°C. The fact that plant measured at the 5th leaf stage show no significant signs of being different maybe any example of the plant ability to avoid injury and slow growth.

Verheul et al. 1995, reported that transferring maize plants to suboptimal temperatures strongly reduced absolute and relative growth rate compared to plants grown at or near optimal temperature (22/24C), and who also considered that the reduction of growth was less in the chilling tolerant lines than in the chilling sensitive lines. As leaves were exposed to lower temperatures (Stamp, 1981 and Baker et al.
1983) reported that this lowering in temperature resulted in a decreased in the Calvin cycle activity (e.g. slower regeneration of Rubisco). This decrease in the Calvin cycle can have disastrous effect on plant growth.

The method of measuring leaf dry matter content and fresh weight can reflect the trade-off in plant functions between a rapid production of biomass and an efficient conservation of nutrients (Poorter et al. 1999). It has also been proposed that methods of measuring such as specific leaf area and/or leaf dry mass be measured routinely in screening programs (Westoby, 1998; Weiher et al. 1999; Wilson et al. 1999). But because of the sensitivity of specific leaf area and dry leaf mass to water status and the time of day at which the leaves are sampled, it has been suggested by Reich et al. 1992, that the leaves should be young and fully expanded when measured. This was a practiced method in when taking measurements in this study.

Shipley, 2002, believes that it is important to know how the physiological and morphological differences contribute to relative growth rate and fresh weight variation. Thus it is therefore necessary to also measure net assimilation rates which measurements the plants daily net photosynthetic rate, which can be weighted by the rate of change in plant carbon content to study these differences (Shipley et al. 1999; Pooter, 1989).

Potassium is primarily present in the cytoplasm and only some K+ (<10%) is bound to the functional exchange groups on the cell walls (Brown et al. 1985). Increased concentration of K+ is associated with an attempt to maintain homeostasis though to increase osmotic activity (Kim et al. 1993).
The feature of potassium uptake being higher when plants are grown at 25 to 33°C range has been reported by (Lahav et al. 1982; Wheily et al. 1990). Thus, the increased percent leakage of K in the control environment 24/22°C could result from a higher amount of potassium in the control plants. There is a possible explanation to these results. We can assume that K uptake is influenced by several processes. Each of the processes has a different temperature response. Another explanation could be that K uptake is affected by certain inhibitors which are highly active at the 24°C range, but lose their activity whether at low temperature or at high ones.

The high amount of K measurements at 22/24 C in the leaves may indicate that K uptake is limited by processes other than those of the energy metabolism. The response of cytosolic K in each cell, maybe related either to difference in the K concentration gradients that the active K transporter can maintain. Or to differences in the variation properties of outward rectifying K channels in the plant cell within epidermal cells having a higher probability resulting in the loss of K through the cell (Walker et al. 1996). These changes in K are believed to cause a decrease in biochemical processes and a decline in growth (Leigh et al. 1984.)

It seems possible to believe that multiple responses to low temperature would explain the intricate genetic background for cold tolerance in maize. Several investigators (Pinnell, 1949; Pesev, 1970; Grogan, 1970; Cal et al. 1972) concluded that cold tolerance in maize was a complex quantitative trait.

The differences found in this study are consistent with the data of other authors indicating similar differences in traits associated with cold tolerance in Zea mays. Further work will be needed to determine the relationship of the responses
described in this study and their relative importance to the further understanding of cold tolerance and their relationship to seedling cold tolerance. To minimize the chance of elimination germplasm with good cold tolerance, screening methods will need to consider a wide variety of different plant responses to low temperature.

described in this study and their relative importance to the further understanding of cold tolerance and their relationship to seedling cold tolerance. To minimize the chance of elimination germplasm with good cold tolerance, screening methods will need to consider a wide variety of different plant responses to low temperature.
Table 1. Percent electrical conductivity means by variety and treatment averages. Values are means of 3 experimental replicates, six plants per inbred line tested. Means within the same column followed by the same letter are not significantly different at the 0.05 level of probability according to LSD test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Variety</th>
<th>4th leaf-stage</th>
<th>Time of Measurement</th>
<th>8/6°C for 48h</th>
<th>8/6°C for 96h</th>
<th>5th leaf-stage</th>
<th>Variety Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>24/22°C</td>
<td>A619</td>
<td>2.4a</td>
<td></td>
<td>2.42a</td>
<td>2.43a</td>
<td>2.52a</td>
<td>2.44</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>2.43a</td>
<td></td>
<td>2.48a</td>
<td>2.54a</td>
<td>2.47a</td>
<td>2.48</td>
</tr>
<tr>
<td></td>
<td>CO255</td>
<td>2.48a</td>
<td></td>
<td>2.51a</td>
<td>2.55a</td>
<td>2.52a</td>
<td>2.51</td>
</tr>
<tr>
<td>Averages</td>
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<td></td>
<td></td>
<td>2.43</td>
<td>2.47</td>
<td>2.50</td>
<td>2.50</td>
</tr>
</tbody>
</table>

| 8/6°C for 48hr | A619    | 2.56a          | 2.77a               | -             | 2.49a         | 2.6       |
|               | B73     | 2.49a          | 2.93b               | -             | 2.56a         | 2.66      |
|               | CO255   | 2.6a           | 2.65c               | -             | 2.56a         | 2.6       |
| Averages     |         |                |                     | 2.55          | 2.78          | 2.53       | 2.62           |

| 8/6°C for 96hr | A619    | 2.41a          |                     | 2.74a         | 2.43a         | 2.52       |
|               | B73     | 2.5a           |                     | 3.1b          | 2.45a         | 2.68       |
|               | CO255   | 2.51a          |                     | 2.9c          | 2.47a         | 2.62       |
| Averages     |         |                |                     | 2.47          | -             | 2.91       | 2.45           | 2.60 |

* Leakage is expressed as the ratio (leakage of live tissue/leakage of heat killed tissue) x 100
Table 2. Percent potassium means of leaf disc by variety and treatment averages. Values are means of 3 experimental replicates, six plants per inbred line tested. Means within the same column followed by the same letter are not significantly different at the 0.05 level of probability according to LSD test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Variety</th>
<th>4th leaf-stage</th>
<th>8/6C° 48h</th>
<th>8/6C° 96h</th>
<th>5th leaf-stage</th>
<th>Variety</th>
<th>Means</th>
</tr>
</thead>
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<td></td>
<td></td>
<td>24C</td>
<td>8/6C for 48hr</td>
<td>8/6C for 96hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A619</td>
<td>13.05a</td>
<td>12.3a</td>
<td>12.04a</td>
<td>13.8a</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>13.03a</td>
<td>11.47a</td>
<td>9.62b</td>
<td>9.62b</td>
<td>10.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO255</td>
<td>12.46a</td>
<td>11.9a</td>
<td>11.66a</td>
<td>11.51c</td>
<td>11.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.84</td>
<td>11.89</td>
<td>11.1</td>
<td>11.64</td>
<td>11.86</td>
<td></td>
</tr>
<tr>
<td>8/6C for 96hr</td>
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<td>12.68a</td>
<td>-</td>
<td>9.54a</td>
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<td></td>
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<td>13.02</td>
<td>-</td>
<td>10.37</td>
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<td>9.38a</td>
<td>9.61</td>
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<td></td>
<td>B73</td>
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<td>8.9a</td>
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<td>9.93</td>
<td></td>
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<td></td>
<td>CO255</td>
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<tr>
<td></td>
<td></td>
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<td>-</td>
<td>8.11</td>
<td>8.64</td>
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</tr>
</tbody>
</table>

* Leakage is expressed as the ratio (potassium leakage as a result of treatment regime/potassium leakage after autoclaving) x 100.
Table 2. Percent potassium means of leaf disc by variety and treatment averages. Values are means of 3 experimental replicates, six plants per inbred line tested. Means within the same column followed by the same letter are not significantly different at the 0.05 level of probability according to LSD test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Variety</th>
<th>4th leaf-stage</th>
<th>8/6C° 8/6C°</th>
<th>5th leaf-stage</th>
<th>Variety</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>24C</td>
<td>A619</td>
<td>13.05a</td>
<td>12.3a</td>
<td>12.04a</td>
<td>13.8a</td>
<td>12.79</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>13.03a</td>
<td>11.47a</td>
<td>9.62b</td>
<td>9.62b</td>
<td>10.93</td>
</tr>
<tr>
<td></td>
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<td>12.46a</td>
<td>11.9a</td>
<td>11.66a</td>
<td>11.51c</td>
<td>11.88</td>
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<td>11.89</td>
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<td>11.86</td>
</tr>
<tr>
<td>8/6C for 48hr</td>
<td>A619</td>
<td>11.2a</td>
<td>12.68a</td>
<td>-</td>
<td>9.54a</td>
<td>11.14</td>
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<tr>
<td></td>
<td>B73</td>
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<td></td>
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<td>11.04c</td>
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<td>-</td>
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<td>11.63</td>
</tr>
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<td>8/6C for 96hr</td>
<td>A619</td>
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<td>7.59a</td>
<td>8.02a</td>
<td>9.47</td>
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<tr>
<td></td>
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<td>12.26</td>
<td>-</td>
<td>8.11</td>
<td>8.64</td>
<td>9.67</td>
</tr>
</tbody>
</table>

* Leakage is expressed as the ratio (potassium leakage as a result of treatment regime/potassium leakage after autoclaving) x 100.
Table 4. Fresh weight of six plants by variety and treatment averages at 22/24C° and 96 hours of treatment at 8/6C°. Values are means of 3 experimental replicates. Means within the same column followed by the same letter are not significantly different at the 0.05 level of probability according to LSD test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Variety</th>
<th>4th leaf-stage</th>
<th>22/24C° at 96h</th>
<th>5th leaf- Variety stage</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>22/24C°</td>
<td>A619</td>
<td>1.64a</td>
<td>4.19a</td>
<td>5.38a</td>
<td>3.73</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>1.01b</td>
<td>3.92b</td>
<td>5.16a</td>
<td>3.36</td>
</tr>
<tr>
<td></td>
<td>CO255</td>
<td>2.15c</td>
<td>7.81c</td>
<td>10.1b</td>
<td>6.68</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variety</th>
<th>4th leaf-stage</th>
<th>8/6C° for 96h</th>
<th>5th leaf- Variety stage</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>8/6C° for 96hrs.</td>
<td>A619</td>
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<td>10.21a</td>
</tr>
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<tr>
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<td>CO255</td>
<td>1.64a</td>
<td>5.56c</td>
<td>9.93a</td>
</tr>
</tbody>
</table>

* [ (root1 + shoot1 at time of interest) − (root1 + shoot1 at 4th leaf stage) ] / (root1 + shoot1 at the 4th leaf stage)
Table 5. Overall temperature effect across inbred variety averages at 22/24°C (T1), 48 hours of treatment at 8/6°C (T2), 96 hours of treatment at 8/6°C (T3) and the measurements taken at the 5th leaf stage (T4). Values are means of 3 experimental replicates.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time of Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1</td>
</tr>
<tr>
<td>22/24°C</td>
<td>27.98a</td>
</tr>
<tr>
<td>8/6°C for 48 hrs</td>
<td>22.68b</td>
</tr>
<tr>
<td>8/6°C for 48 hrs</td>
<td>24.44b</td>
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REFERENCES


