The assessment of non-pathogenic related effects of the seed treatment Stamina on germinating maize under cold stress

by

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ABBREVIATIONS

ADP	Adenosine Di-Phosphate
AOSA	Association of Official Seed Analysts
ATP	Adenosine Tri-Phosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
FAD	fully oxidized Flavin Adenine Dinucleotide
FADH$_2$	reduced Flavin Adenine Dinucleotide
GMO	Genetically Modified Organism
GS	growth stage
H_2O_2	Hydrogen Peroxide
IL	ion leakage
KCN	Potassium Cyanide
kPa	Kilopascal
LT$_{50}$	Lethal Temperature at which 50% injury occurs
MRGR	Mean Relative Growth Rate
NADH	Nicotinamide adenine dinucleotide
NBT	Nitro Blue Tetrazolium
PCR	Polymerase Chain Reaction
PPB	Potassium Phosphate Buffer
Pi	Inorganic Phosphate
Q	Quinone, can be in multiple forms
Rpm	repetitions per minute
ROS	Reactive Oxygen Species
RR2	RounduUp Ready 2
SAS	Statistical Analysis Software
SOD	Superoxide Dismutase; can be in forms of Cn/Mn/Zn, depending on metial ion cofactor
Stamina	Seed treatment that contains active ingredient pyraclostrobin and binders
TCA	Tri-Carboxylic Acid
YG3	Yieldgaard Triple Stack
YSI	Yellow Springs Instruments

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ABSTRACT

Maize is an important crop grown in the Midwest. Low temperatures in this region can cause irreversible damage to maize, limiting stand establishment and ultimately affecting yield. Fungal contamination also contributes to poor stand establishment, affecting yield. A new seed treatment fungicide for maize (Stamina) has shown positive effects on maize plants under cold stress, not related to protection against fungi. The goal of this project was to determine whether the seed treatment Stamina protects maize seedlings from cold stress or freezing injury by enhancing superoxide dismutase (SOD) activity in maize seedlings. In this study, three seed treatments (Stamina and a binding agent, binding agent only, and no treatment) were used for each of three seed lots. Seeds were planted and grown at 10°C for seven days, followed by four days at 25°C. At the end of the four days at 25°C, seedlings were moved back to 10°C for ten days to provide additional stress. Seedling height, dry weight, and SOD were measured at the end of the 25°C treatment (Point A), as well as at the end of the second stress (Point B). Freeze injury was measured at Point B at three temperatures: -1.0°C, -1.5°C, and -2.0°C. Stamina treated seedlings were taller and contained greater SOD activity at Point A, compared to seedlings treated with binders or no treatment. Stamina treated seedlings were taller and heavier than seedlings treated with binders or no treatment at Point B, but no differences in SOD activity were found. The effects of temperature, seed lot, and treatment were variable over all levels in the freezing assay. Variability in injury was most evident at -1.5°C. These results indicate Stamina provides protection against oxidative stress at Point A, evidenced by increased SOD activity. This leads to greater performance in seedling height. Mean relative growth rate measurements indicate Stamina is providing some level of protection against the chilling stress at Point B, as Stamina treated seedlings are both taller and heavier, even though SOD activity is not enhanced. It is difficult to assess the effectiveness of Stamina on freeze injury due to inconsistencies in freeze injury on seedlings treated with binders. Seed lot differences were important across all aspects of the study, having an effect on seedling height, dry weight, SOD activity and freeze injury.

CHAPTER ONE - LITERATURE REVIEW

Low Temperature Stress

Crop tolerance to low temperatures is a valuable agronomic trait. Crops adapted to temperate climates, such as small grains, are resistant to chilling temperatures ranging from 0-10°C. Crops of tropical origin, such as maize, can be irreversibly damaged by these temperatures, with performance affected by extended periods of low temperature. Current agronomic trends call for earlier spring planting to maximize yields, increasing the likelihood that germinating maize seedlings will spend at least some portion of early development under suboptimal temperatures (Lauer et al, 1999). Low temperatures induce oxidative stress (Prasad et al, 1994a) in the form of reactive oxygen species (ROS). ROS, such as superoxide anions (O_2^-) and hydrogen peroxide ($H_2O_2^-$), are generated by the partial reduction of molecular oxygen (O_2). Since ROS can damage proteins, lipids, and DNA, production and elimination of them must be managed by the plant. Research continues in the area of low temperature stress and its implications on ROS production and its effects on metabolism.

Respiration

Respiration is the controlled oxidation of reduced carbon to produce carbon dioxide and water (Siedow and Umbach, 1995). Although many reduced carbon compounds are oxidized, such as fatty acids, glycerol, or amino acids, the most common substrates are carbohydrates. The complete oxidation of sucrose releases a large amount of free energy, which is coupled to the conversion of ADP and P_i to ATP. Respiration can be divided into three phases: glycolysis, which is anaerobic, the TCA cycle and mitochondrial electron transport, both of which are aerobic. The effect of low temperature stress on respiration depends on a number of factors, such as severity, duration of stress, adaptation of species, and specific tissues, to name a few. Respiration will decline during low temperature stress, increase during re-warming, and decline to pre-chilled levels after recovery (Lyons, 1973). Each phase of respiration can be affected by low temperature stress in various ways, leading to an overall decline in respiration during the stress period. Phosphofructokinase (Graham and Patterson, 1982) and glyceraldehyde-3-phosphate dehydrogenase (Guy, 1990), two enzymes used in glycolysis, are inactivated by low temperature stress. If these two enzymes are inactivated, conversion to pyruvate is reduced, leading to less metabolite for the TCA cycle. Isocitrate dehydrogenase, an enzyme present in the TCA cycle, is inactivated by low temperature stress (Duke et al, 1977). One of the products of the TCA cycle, $FADH_2$, is thus lowered by low temperature stress, as less metabolite is available for reduction. These inactivations and reductions illustrate how respiration is differentially impacted by low temperatures.

Mitochondrial Electron Transport

Products of glycolysis and the TCA cycle, NADH and $FADH_2$, are used during oxidative phosphorylation during mitochondrial electron transport (Siedow and Umbach, 1995), by donating their electrons to the electron transport chain. The electron transport chain contains structures which are effectively the same set of electron carriers found in the mitochondria of other organisms. The individual proteins are found in the inner mitochondrial membrane, with the transfer of electrons leading to a simultaneous transport of protons across the inner mitochondrial membrane at complexes I, III, and IV (Siedow and Umbach, 1995, Moller, 2001). The flow of electrons between compounds creates an electrochemical gradient across the inner membranes of the mitochondria. The energy in this gradient pumps protons across the mitochondrial matrix, which are used to produce ATP, while yielding water and consuming oxygen in the process. The electron transport chains of plants contain an alternative oxidase (Vanlerberghe et al, 1997), the function of which has been the focus of much research.

Complex I

Complex I, also known as NADH dehydrogenase or NADH-Q oxidoreductase, oxidizes NADH generated from the TCA cycle. This enzyme couples the transfer of two electrons from NADH to ubiquinone with the translocation of four protons through the membrane (Vedel et al, 1999, Moller 2001). In the presence of KCN, ROS have been produced, indicating that Complex I, which is the beginning of the pathway for electron flow, could possibly contribute to ROS production (Rich and Bonner 1978, Purvis et al, 1995). In addition, Complex I is a major source of superoxide production by molecular oxygen undergoing a partial reduction (Braidot et al, 1999).

Complex II

Complex II, also known as succinate dehydrogenase or succinate-Q oxidoreductase, oxidizes succinate to fumarate, with FAD as the hydrogen acceptor. $FADH_2$ produced by the oxidation of succinate does not dissociate from the enzyme; instead, two electrons are transferred to iron-sulfur clusters of the enzyme, which passes the electrons to ubiquinone (Yankovskaya et al, 2003). Succinate dehydrogenase is the physical link between the TCA cycle and mitochondrial electron transport. There is no indication of direct ROS production at succinate dehydrogenase, but it has been reported that succinate dehydrogenase is inactivated by low temperature stress (Lyons and Raison, 1970) due to membrane injury. This can possibly contribute to free radical production by restricting electron flow.

Q-Cycle/Pool

The Q cycle is made up of coenzyme Q, also known as ubiquinone. Quinones can exist in multiple oxidation states: a fully oxidized state (Q), a semiquinone form (QH•), a semiquinone radical ion ($Q\bullet^-$), and ubiquinol (QH_2). The function of the Q-cycle is ultimately to funnel electrons from a two-electron carrier of fully reduced coenzyme Q, to a one electron carrier, cytochrome c (Verkhovsky et al, 1999, Shultz et al, 2001). In the first half of the cycle, two electrons of fully reduced QH_2 molecule are transferred; one to cytochrome c (as catalyzed by Complex III) and the other to a bound coenzyme Q molecule in a second binding site, forming the semiquinone radical. In the second half of the cycle, a second fully reduced QH_2 molecule gives up its electrons; one to cytochrome c, and the other to reduce the semiquinone radical ion to QH_2, which results in an uptake of two protons from the matrix. Both the cytochrome and alternative oxidases obtain their electrons from the fully reduced form of coenzyme Q; hence, the Q-cycle is the branching point for the pathways. The level of reduction of the Q-pool determines the ultimate pathway for electron transfer (Dry et al, 1989, Moore et al, 1991).

Complex III

Complex III, also known as Q-cytochrome c oxidoreductase or the bc_1 complex, catalyzes the transfer of electrons from fully reduced ubiquinone to cytochrome c, and is coupled to transmembrane proton translocation (Hatefi, 1985, Trumpower et al, 1994). Complex III contains two types of cytochromes, b and c_1. Each cytochrome contains a heme prosthetic group, which alternates the oxidation state of its iron atom during electron transport. Complex III is a major site of ROS production (Raha and Robinson, 2000, Moller, 2001). In addition, it has also been reported that ROS production is completely inhibited by

KCN, indicating that Complex III may have been the only source of ROS before KCN addition (Puntarulo et al, 1988, Purvis et al, 1995).

Complex IV and V (ATP Synthase)

Complex IV, or cytochrome c oxidase, is the terminal enzyme of the respiratory chain and catalyzes the transfer of electrons to molecular oxygen. This reaction is coupled to the pumping of protons across the mitochondrial inner membrane from the matrix to the cytoplasmic side (Capaldi, 1990). The reaction is thermodynamically favorable, with the free energy captured in the form of a proton gradient for subsequent use in ATP synthesis. Cytochrome c oxidase is inhibited by cyanide (Hackett, 1960).

Complex V, or ATP Synthase, uses a proton-motive force to drive the synthesis of ATP from ADP and inorganic phosphate (Boyer, 1997). ATP Synthase is not part of electron transfer per se, as electrons do not flow through the structure, but it maintains a role in oxidative phosphorylation by using the proton gradient to drive the synthesis of ATP. Thus, when certain complexes are inhibited, the result is fewer protons transferred across the inner membrane, and less ATP is produced.

Alternative Oxidase

The mitochondria of plants possess an alternative respiratory pathway that branches from the cytochrome pathway at the ubiquinone pool and contains a single terminal oxidase (Moore and Siedow, 1991, Vanlerberghe and McIntosh, 1997). This pathway is non-phosphorylating, bypasses two of the three energy coupling sites of the cytochrome pathway, and reduces oxygen to water, similar to that of the cytochrome oxidase (Moore et al, 1978). It has been proposed that alternative oxidase may serve to limit mitochondrial ROS production (Purvis, 1997).

Reactive Oxygen Species

Low temperatures reduce the activity of cytochrome oxidase by decreasing cytochrome oxidase protein levels, and increase the activity of the alternative oxidase by increasing alternative oxidase protein levels (Leopold and Musgrave, 1979, Van de Venter, 1985, Prasad et al, 1994b). When electron flow through the cytochrome pathway is inhibited with antimycin or KCN, blocking transfer at Complex III, the alternative pathway is enhanced (Vanlerberghe and McIntosh, 1992). As mentioned previously, the blocking of the cytochrome pathway leads to increased ROS production because the ubiquinone pool becomes over-reduced. It is reasonable to assume that the alternative oxidase functions to regulate the ubiquinone reduction level, in an attempt to modulate ROS production when the cytochrome pathway is inhibited. This was initially proposed by Purvis and Shewfelt (1993) and supported (Millenaar et al, 1998) by studies on roots with cytochrome inhibitors. While many functions have been proposed, it appears that the alternative oxidase diminishes ROS production (Purvis, 1997, Popov et al, 1997, Maxwell et al, 1999).

Antioxidants and Superoxide Dismutase

Superoxide can be converted into O_2 and H_2O_2 by superoxide dismutase (SOD). There are three distinct types of SOD, all based on the metal ions they have at their active sites: Cu/ZnSOD, generally found in the cytosol, MnSOD, found in the mitochondria, or FeSOD, usually found in prokaryotes and in some plants (Duke and Salin, 1985). H_2O_2 can react with another superoxide anion and form the hydroxyl radical via the Haber-Weiss reaction. The hydroxyl radical is the most reactive of ROS, causing lipid peroxidation, denaturation of proteins, and DNA mutation. Therefore, a scavenging system similar to SOD, catalase in this case, must be in place to break down H_2O_2 into water and oxygen gas.

Since increased production of ROS has been associated with injury symptoms of various environmental stresses, such as chilling (Wise and Naylor, 1987), a means to manage these ROS is needed. Increased tolerance to environmental stresses that induce ROS production is correlated with an increased capacity to detoxify ROS (Kendall and McKersie, 1989, Malan et al, 1990). In addition, transgenic plants over-expressing SOD exhibit enhanced tolerance of oxidative stress (McKersie et al, 1993).

Freezing Tolerance and Ion Leakage

Plants exposed to low, non-zero temperatures are reported to have an increased tolerance to freezing temperatures (Steponkus, 1984, Hughes & Dunn, 1996, Thomashow, 1999). Studies indicate the membrane systems of the cell are the primary site of freezing injury in plants, with the damage resulting primarily from the severe dehydration associated with freezing (Steponkus et al, 1993, Thomashow, 1999). As temperatures decrease below 0°C, ice formation is initiated in the intercellular space, due to the fact the extracellular fluid has a higher freezing point than intracellular fluid. The formation of extracellular ice results in a drop in water potential outside the cell, due to the fact the chemical potential of ice is less than that of liquid water at a given temperature. It was demonstrated that freeze-induced dehydration can cause multiple forms of membrane lesions, such as expansion-induced lysis, lamellar-to-hexagonal II phase transitions, and fracture jump lesions (Steponkus et al, 1993, Thomashow, 1999).

Studies with maize leaves have shown enhanced electrolyte leakage at low temperature stress (Creencia, 1971), with Raison (1973) having shown a relationship between membrane function/temperature and membrane lipid fluidity on the other, suggesting that chilling injury derives from effects on membrane lipids. Therefore, a key function of cold acclimation is to stabilize membranes against freezing injury. Numerous physiological changes occur during cold acclimation: increase in cold-stress proteins (Arora and Wisniewski, 1994; Arora et al, 1997), the accumulation of cryoprotectants such as proline (Wanner and Junttila, 1999), and increases in the unsaturated-to-saturated fatty acid ratio of phospholipids in the plasma membrane (Palta et al, 1993), which is the best-documented mechanism involved in membrane stabilization (Thomashow, 1999). Ion leakage resulting from decreased membrane integrity of freeze-damaged tissues can be measured to determine the freezing tolerance of several species (Arora and Palta, 1991, Nunes and Smith, 2003, Thapa et al, 2008). The temperature at which 50% injury occurs is termed LT_{50} (Burr et al, 1990), and LT_{50} is used as a measure of relative freezing tolerance (Palta et al, 1978, Sakai et al, 1986, Arora and Palta, 1991, Arora et al, 1992, Webb et al, 1994, Welling et al, 2002, Nunes and Smith, 2003).

As mentioned previously, chilling stress has been associated with an increased production of ROS. However, other stresses, such as freezing (Kendall and McKersie, 1989), also result in increased production of ROS. Generally speaking, tolerance to chilling and/or freezing correlates with an increased capacity to detoxify ROS (Kendall and McKersie, 1989). Since an over-expression of SOD has been shown to enhance tolerance to oxidative stress, it stands to reason that an over-expression would also enhance freezing tolerance and protect against freezing injury. In fact, McKersie et al, (1993) examined a transgenic alfalfa plant that contained more SOD activity in leaves, greater tolerance of the herbicide acifluorfen (which is used to generate oxidative stress), and greater regrowth after freezing stress. The tolerance of these stresses was altered by the presence of the Mn-SOD transgene, indicating ROS are involved in the injury response due to freezing.

Pyraclostrobin and Strobilurin Fungicides

The strobilurins are an important class of agricultural fungicides, whose discovery was inspired by a group of natural β-methoxyacrylates, such as strobilurin A and oudemansin A. The fungicidal activity of the strobilurins stem from their ability to inhibit mitochondrial respiration by binding at the Q_o site of cytochrome b, which is part of Complex III. When the inhibitor binds, it blocks electron transfer, which disrupts the energy cycle within the fungus by halting ATP production. These fungicides are extremely important, as no inhibitors with this specific binding interaction had been previously identified (Bartlett et al, 2002). An important feature of these types of fungicides is that they possess an extremely broad spectrum of activity resulting in protection against Ascomycetes, Deuteromycetes, Basidiomycetes, and Oomycetes (Ammerman et al, 1992, Heaney and Knight, 1994, Bartlett et al, 2001). A majority of the strobilurins, most notably pyraclostrobin, has protectant, curative, translaminar and locosystemic properties, leading to a broad application window (Ammermann et al, 2000).

Apart from their fungicidal effects, strobilurins can cause long-term changes in the metabolism and growth of the treated plants resulting in higher biomass and yield (Jabs et al, 2002). Additionally, kresoxim-methyl has been shown to delay senescence in wheat (Grossman and Retzlaff, 1997). Treatment with pyraclostrobin increased SOD activity in barley leaves at GS 55, and led to a strong reduction of superoxide production (Jabs et al, 2002), while azoxystrobin exhibited an increase in similar antioxidant enzymes (Wu and von Tiedemann, 2001). In addition to ROS production, pyraclostrobin treatment resulted in a strong reduction of ethylene production.

Disease control by strobilurins remains excellent for the most part. However, as with most fungicides, resistance can become a problem. Strobilurins are known as Q_oI compounds, since they bind to the Q_o site, as mentioned previously. For most of the pathogens in which Q_oI resistance has been reported, it appears the mechanism for resistance is a point mutation at G143A, whereby the amino acid alanine is substituted with glycine (Barlett et al, 2002). This has been detected in at least seven species of plant pathogenic fungi. Measures have been taken in an attempt to keep resistance to a minimum, such as limiting the number of applications of all Q_oI cross-resistance groups, alternation with effective compounds from different resistance groups, and the possible mixture with fungicide partners, where appropriate. In addition, PCR methodologies are used to track resistance genes and point mutations (Bartlett et al, 2002), in an attempt to advance the understanding of Q_oI resistance. The impact of resistance to Q_oI compounds has been limited so far (Gisi et al, 2000).

Project Goals

Damage caused by low temperature stress has been a problem in maize in the corn belt of the Midwest. Low temperatures can be a limiting factor in stand establishment, which can lead to reduced yields. Additionally, infection by fungal pathogens early in development result in poor stand establishment, also contributing to lower yields. Protection against both of these stresses would without a doubt improve the early development of a maize seedling. Stamina, a new strobilurin (pyraclostrobin) fungicide, has shown positive effects on early maize development that are not related to pathogenic fungi (BASF, personal communication, June 2010). Therefore, it was the goal of this project to determine whether the seed treatment

Stamina protects maize seedlings from cold stress or freezing injury by enhancing SOD activity in maize seedlings.

CHAPTER TWO - MATERIALS AND METHODS

Plant Materials

One seed lot each of three different hybrids commonly used in agriculture and having the same GMO traits, namely round-up ready two (RR2) and yieldgard triple stack (YG3), were chosen for this study. The seed lots chosen contained reasonably low fungal pathogens, as determined by seed health tests (Barnett and Hunter, 1987, McGee, 1988, Alexopoulos and Mim, 1996). Standard germination and cold tests were used to assess the quality of the seed lots. Germination can be defined as the emergence and development from the seed embryo of those essential structures indicative of the ability to produce a normal plant under favorable conditions (AOSA Seed Vigor Testing Handbook, 2002). The cold test gives an estimate of emergence under less than ideal conditions, by providing high soil moistures and low temperatures to simulate early spring field conditions (AOSA Seed Vigor Testing Handbook, 2002). Three seed treatments were used for each of the three seed lots: the active ingredient pyraclostrobin (Stamina) and binders (Color Red and CF Clear), binders only (blank), and no treatment (control).

Fifty seeds of each seed lot by treatment combination were germinated by the standard germination protocol (AOSA Rules For Testing Seeds, 2005), with minor modifications. Seeds were placed in a germination chamber at 25°C ± 2°C with germination counts made 7 days after set up. Seedlings were classified as normal, abnormal, and dead, according to the AOSA Rules For Testing Seeds (2005). One hundred seeds of each seed lot by treatment combination were used in the cold test (AOSA Vigor Testing Handbook, 2002), using seven days for both the cold and optimum temperatures. Again seedlings were classified according to the AOSA.

Growth Conditions for Low Temperature Stress Treatments

Seeds of each seed lot by treatment combination were planted in a similar fashion to the cold test mentioned previously, with minor modifications. Seeds were placed at 10°C for 7 days after which they were allowed to grow at 25°C environment for 4 days. At the end of the 4 days at 25°C, the seeds were moved back to the 10°C environment for another 10 days to provide additional stress to the developing seedlings. Since this system had been evaluated earlier (BASF, personal communication, June 2010) and was obviously stressful, we used it for our stress conditions. The temperature, time, and duration treatments are graphically represented in Figure 1.

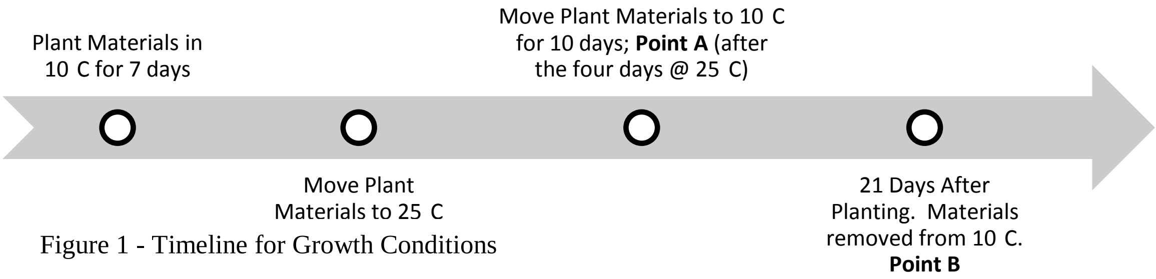


Figure 1 - Timeline for Growth Conditions

Seedling Measurements

Seedling height was calculated on emerged seedlings as the distance from the surface of the soil to the extended leaf tip. Measurements were taken at both Points A & B (Figure 1). Seedlings that had unique levels of damage or were not otherwise consistent with the bulk of seed lot were not used for measurements. These included seedlings that contained significant leaf damage, lesions, necrotic spots, etc, that were non-symptomatic with fungal contamination. Dry weight was also measured on seedlings harvested at both Points A & B. Measurements were taken by cutting the seedlings at the soil surface level and placing them in pre-weighed, labeled, 120 ml Qorpak® jars. Jars were placed in an oven, with the lids off, at 60°C for three days, a modification of the protocol used by Khodary (2004). The jars were then weighed again. The dry weight was calculated as the total weight of the jar and dried seedling minus the initial weight of the jar.

Freeze Injury Tests

Freezing injury tests were performed on seedling leaves harvested at Point B and placed in a controlled freeze-thaw regime. The effects of the freezing treatments were assessed by measuring electrolyte leakage as described by Lim et al, (1998), with minor modifications. Leaves were excised above the mesocotyl node of the seedling. The leaf collar was included to keep the leaves intact, and the samples were placed in 25 x 200 mm test tubes containing 200 µL de-ionized water. The test tubes containing leaves were cooled in a temperature-controlled glycol bath (Isotemp 3028; Fisher Scientific, Pittsburgh, PA).

Ion leakage was assessed at three different temperatures: -1.0°C, -1.5°C, and -2.0°C. In preliminary studies, the LT_{50} of treated seedlings was around -1.5°C (Appendix C). Previous work on rhododendron (Lim et al, 1998) has shown ice nucleation is initiated at -1.0°C by adding small ice crystals to the samples. This is done to initiate freezing in the sample, and to avoid deep super-cooling (Thomashow, 1999). Preliminary studies were run on seed lot three initially, as an abundance of seed lot three was on hand, to determine if there was a noticeable difference in LT_{50} between Stamina treated seedlings and untreated controls. After preliminary trials did not show much (if any) difference in the LT_{50} of Stamina treated seedlings and untreated seedlings in seed lot three, the untreated controls from each seed lot were run to determine if LT_{50} would differ among seed lots. The LT_{50} was similar in all seed lots (Appendix C). Even though the LT_{50} was similar across seed lots and treatments, there was variability in freeze injury at different temperatures. Little variation occurs in freeze injury in any seed lot at temperatures cooler than -2.0°C (Appendix C). The same can be said for temperatures warmer than -1.0°C. The greatest variation in freeze injury in preliminary studies was usually at -1.5°C. With little variation in freeze injury occurring at -1.0°C, it would be difficult to detect differences in freeze injury among seed treatments. Likewise, with little variation occurring in freeze injury at temperatures equal to or colder than -2.0°C, differences in freeze injury due to treatment would also be difficult to detect. If there are differences in freeze injury due to Stamina, it is likely they will occur at -1.5°C, due to greater variability of freeze injury. It would not be possible to determine LT_{50} of each treatment by seed lot combination in the final experiment, as space in the glycol bath was limited. Because of this, three temperatures were chosen for the study; -1.0°C, because ice nucleation needs to occur (Lim, 1998), -1.5°C, in an effort to detect differences in freeze injury due to treatments, and -2.0°C. Including the two temperatures where little variation occurs (-1.0° and -2.0°C) also provides a comparison to preliminary studies, to assure that the variation followed a similar pattern.

The seedling leaves were kept at 0°C in the glycol bath for 40 minutes, then the temperature was lowered to -1°C for one hour. After one hour, ice nucleation was initiated by dropping small ice crystals into the tubes. After an additional hour at -1°C, the samples were cooled at a rate of ½ degree per hour until -2.0°C was reached. Frozen samples for each treatment were removed from the glycol bath and both frozen samples and control samples were thawed on ice overnight. Samples were removed from ice and thawed at 4°C for 1 h, and then allowed to thaw for an additional hour at room temperature. Twenty milliliters of de-ionized water was added to all samples, followed by roughly 10 minutes of vacuum infiltration (three times for 3 ½ min each at ~ 100 kPa) and shaking for 1 h at 250 rpm on a gyratory shaker. An initial ion leakage measure was taken with a YSI, model 3100 conductivity meter. Samples were then autoclaved at 121°C for 20 min and allowed to slowly reach room temperature over the next 2-3 h, after which the total ion leakage was measured. Initial leakage was expressed as a percentage of the total ion leakage value, and percentage of leakage for each temperature was converted to a % injury by the following equation:

$$\% \text{ Injury} = \frac{-}{-} \text{ X } 100$$

where % IL(t) and % IL(c) are measurements of percentage of ion leakage from the respective treatment temperature and the unfrozen control, respectively.

Superoxide Dismutase Enzyme Assay

SOD was assayed on the basis of its ability to inhibit the photochemical reduction of nitro blue-tetrazolium (Beauchamp, Fridovich, 1971) and was assessed at both Points A & B. The method of Stewart and Bewley (1980) was used, with the following modifications. A 150 mg sample of leaf tissue was excised, frozen in liquid nitrogen, and ground into a fine powder. The resultant powder was not allowed to thaw before it was dissolved in 1 mL of pre-chilled potassium phosphate buffer, pH 7.8, and kept on ice until centrifugation at 12,000 g for 20 min at 4°C. The reaction mixture for assaying SOD activity was constructed from the resulting supernatant. Specifically, the 3-mL reaction mixture used contained final concentrations of 25 mM potassium phosphate buffer, 13 mM methionine, 75 µM nitro blue-tetrazolium, 10 µM EDTA, and 50 µL of SOD enzyme extract. Riboflavin, at a final concentration of 2 µM, was added and the reaction mixture was vortexed and placed below a light bank consisting of two 15-watt fluorescent tubes. The light was switched on and the reaction allowed to run for ~ 30 min, after which the light was switched off and the tubes were covered with a black garbage bag. Absorbance by the reaction mixture was read at 560 nm. A non-irradiated reaction mixture served as the blank to read as an absorbance of zero. One unit of SOD activity was defined as the amount of enzyme needed to reach 50% inhibition of the reaction not containing the enzyme (control). Protein content of the enzyme extract was determined according to Lowry (1951).

Statistical Analysis

All experiments were arranged as a randomized complete block with time as the blocking factor. The experiments consisted of four replications and seed lots and treatments were considered fixed factors. Seedling heights were taken on thirty individual seedlings and averaged for each replication. Seedling dry weight was comprised of five individual measurements averaged for each replication and freezing injury was comprised of four individual measurements per temperature, averaged for each replication. Finally, three individual measurements were assayed from one 150 mg sample for SOD activity, and the results averaged for each replication. Analyses of variance were conducted using the GLM procedure of SAS. Differences between means for germination, cold test, and seedling height/dry weight were evaluated by Fisher’s least significant difference (LSD) test. Differences between means for freezing tolerance and SOD were separated using a PDIFF statement in GLM, and comparing means of interest. In all cases differences of $P \leq 0.05$ were considered significant.

CHAPTER THREE - RESULTS

Germination

Stamina increased the percentage of normal seedlings in the standard germination test. The increase was associated with a decrease in the percentage of abnormal seedlings (Table 1). Stamina had no effect on dead seeds. The standard germination results of the three seed lots were not different (Table 2).

Table 1 - Effect of Treatment on Normal and Abnormal Germination, and dead seeds.

Treatment	% Normal [A]	% Abnormal	% Dead
Stamina	98.50 a	0.83 c	0.67 e
Binders	96.67 b	2.50 d	0.83 e
No Treatment	96.00 b	3.00 d	1.00 e

[A] *Values represent the mean of twelve replicates averaged over three seed lots*

[B] *Means within the same column followed by the same letter are not different at $P \leq 0.05$*

Table 2 - Effect of Seed Lot on Normal and Abnormal Germination, and dead seeds.

Seed Lot	% Normal [A]	% Abnormal	% Dead
One	96.83 a	2.00 b	1.17 c
Two	97.33 a	2.17 b	0.50 c
Three	97.00 a	2.17 b	0.83 c

[A] *Values represent the mean of twelve replicates averaged over three treatments*

[B] *Means within the same column followed by the same letter are not different at $P \leq 0.05$*

Cold Test

Stamina increased the percentage of normal seedlings in the cold test. Since the seeds were covered with a sand:soil mixture, un-emerged seedlings were not classified as abnormal or dead. Thus, only the percentage of normal seedlings is reported. Treatment and seed lot affected cold test results (Table 3).

Table 3 - Percent Germination in Cold Test by treatment and seed lot.

Treatment	% Norm [A]	Seed Lot	% Norm
Stamina	92.33 a	One	90.67 b
Binders	90.00 b	Two	93.25 a
No Treatment	89.50 b	Three	87.92 c

[A] *Values represent the mean of twelve replicates averaged over three seed lots or treatments*

[B] *Means within the same column followed by the same letter are not different at $P \leq 0.05$*

Seedling Height

Stamina treated seedlings were taller than seedlings treated with binders or no treatment at both sampling Points A and B (Table 4). Seed lot two was the tallest at both Points A and B. Seed lot three was taller than seed lot one at Point A. Seed lots one and three were the same height at Point B (Table 5). In addition, there was a treatment by seed lot interaction (Figure 2).

Table 4 - Effect of Treatment on seedling height at Point A (after 25°C stage) and Point B (after second stress).

Treatment	**Height (cm)** [A]	
	Point A [B]	Point B
Stamina	11.18 a	11.99 a
Binders	10.85 b	11.83 b
No Treatment	10.82 b	11.85 b

[A] *Values represent the mean of twelve replicates per time period, averaged over all three seed lots*

[B] *Means within the same column followed by the same letter are not different at $P \leq 0.05$*

Table 5 - Effect of Seed Lot on seedling height.

Seed Lot	**Height (cm)** [A]	
	Point A [B]	Point B
One	10.43 c	11.35 b
Two	11.58 a	12.97 a
Three	10.84 b	11.36 b

[A] *Values represent the mean of twelve replicates per time period, averaged over all three treatments*

[B] *Means within the same column followed by the same letter are not different at $P \leq 0.05$*

Seedling Dry Weight

Stamina increased dry weight relative to the other seed treatments at Point B but not at Point A (Table 6). Seed lot affected dry weight at both Points A & B (Table 7).

Table 6 - Effect of Treatment on seedling weight at Point A (after 25°C stage) and Point B (after second stress).

Treatment	**Dry Weight (mg)** [A]	
	Point A [B]	Point B
Stamina	31.46 a	37.12 a
Binders	31.43 a	33.64 b
No Treatment	31.09 a	32.63 b

[A] *Values represent the mean of twelve replicates per time period, averaged over all three seed lots*

[B] *Means within the same column followed by the same letter are not different at $P \leq 0.05$*

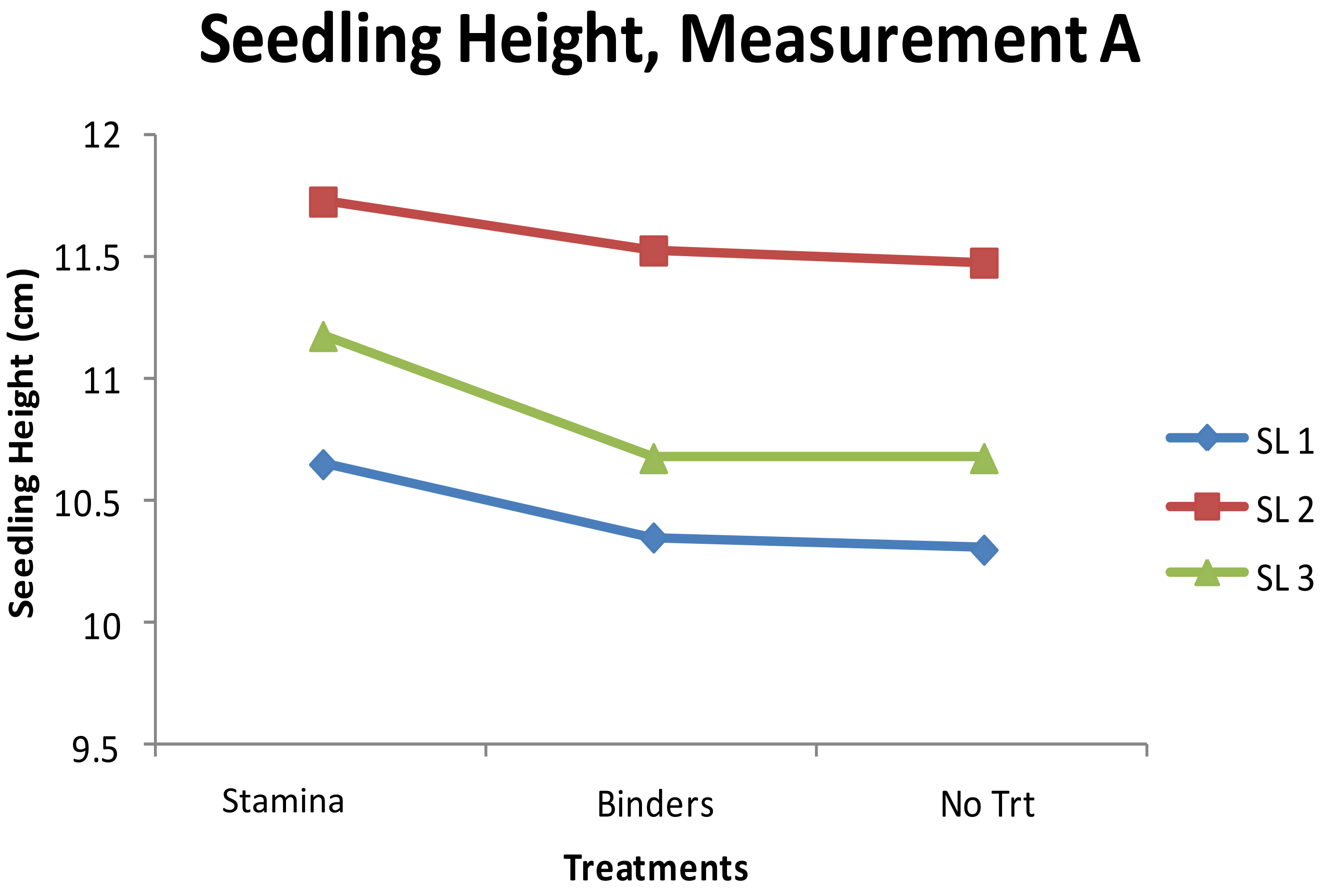


Figure 2. Seed lot (SL) by treatment interaction for seedling height, at Point A.

Table 7 - Effect of Seed Lot on seedling weight at Point A (after 25°C stage) and Point B (after second stress).

Seed Lot	**Dry Weight (mg)** [A]	
	Point A [B]	Point B
One	26.90 c	26.66 c
Two	37.21 a	44.70 a
Three	29.90 b	32.03 b

[A] Values represent the mean of twelve replicates per time period, averaged over all three treatments

[B] Means within the same column followed by the same letter are not different at $P \leq 0.05$

Growth Rate Assessments

Stamina treated seedlings had a lower mean relative growth rate (MRGR), or the growth rate relative to its previous growth measurement, for seedling height from Point A to Point B. However, Stamina increased the MRGR for seedling dry weight from Point A to Point B (Table 8). Seed lot affected MRGR for both height and weight.

Table 8 - Relative Growth Rate of treated seedlings between Point A and Point B for height and weight.

Treatment	**MRGR Height (day^{-1})**	**MRGR Weight (day^{-1})**
Stamina	0.00687 b	0.01509 a
Binders	0.00859 a	0.00539 b
No Treatment	0.00903 a	0.00359 b
Seed Lot		
One	0.00847 b	-0.00089 b
Two	0.01137 a	0.01823 a
Three	0.00469 c	0.00673 b

MRGR = Mean relative growth rate. Calculated by [ln(B) - ln(A)]/[t(B) - t(A)]. Ln(A) or Ln(B) are equal to the natural logarithm of the value for seedling height or dry weight at either Point A or B. t(A) or t(B) refers to the values in days after planting

Means within the same column followed by the same letter are not different at $P \leq 0.05$

Freezing Injury

Stamina did not affect the injury at -2.0°C in any of the three seed lots. The effect of Stamina in decreasing injury was variable at -1.5°C; Stamina decreased injury in comparison to the binders treatment in seedlings from seed lot one, a decrease in injury compared to no treatment in seedlings from seed lot two, and no decrease in injury in seedlings from seed lot three. Stamina decreased the injury at -1.0°C when comparing to the binders treatment in seed lot one. In all other seed lots, Stamina did not decrease the injury at -1.0°C (Table 9). However, the seed lot x treatment x temperature interaction was significant. The treatment by temperature interaction can be seen over all levels of seed lot (Appendix A1, Figure 4-6). The lot x treatment x temperature interaction was sliced by lot x temperature, to show treatment effect (Appendix A2). When focusing on freeze injury at only -1.5°C, a treatment by temperature interaction was significant (Figure 3). The binders treatment was inconsistent in its effects on freeze injury when focusing on only -1.5°C (Table 10). Main effects at -1.5°C (Table 11) are significant for seed lot and treatment.

SOD Enzyme Activity

Stamina increased the SOD activity of seedlings at Point A, but did not increase the SOD activity of seedlings at Point B. The SOD activity of seedlings treated with binders only and no treatment increased their SOD activity from Point A to Point B, but Stamina treated seedlings showed no such increase (Table 12). Seed lot affected SOD activity at both Points A & B (Table 13).

Table 9 - Percent Injury of seedlings, specific to seed lot, treatment, and temperature effects.

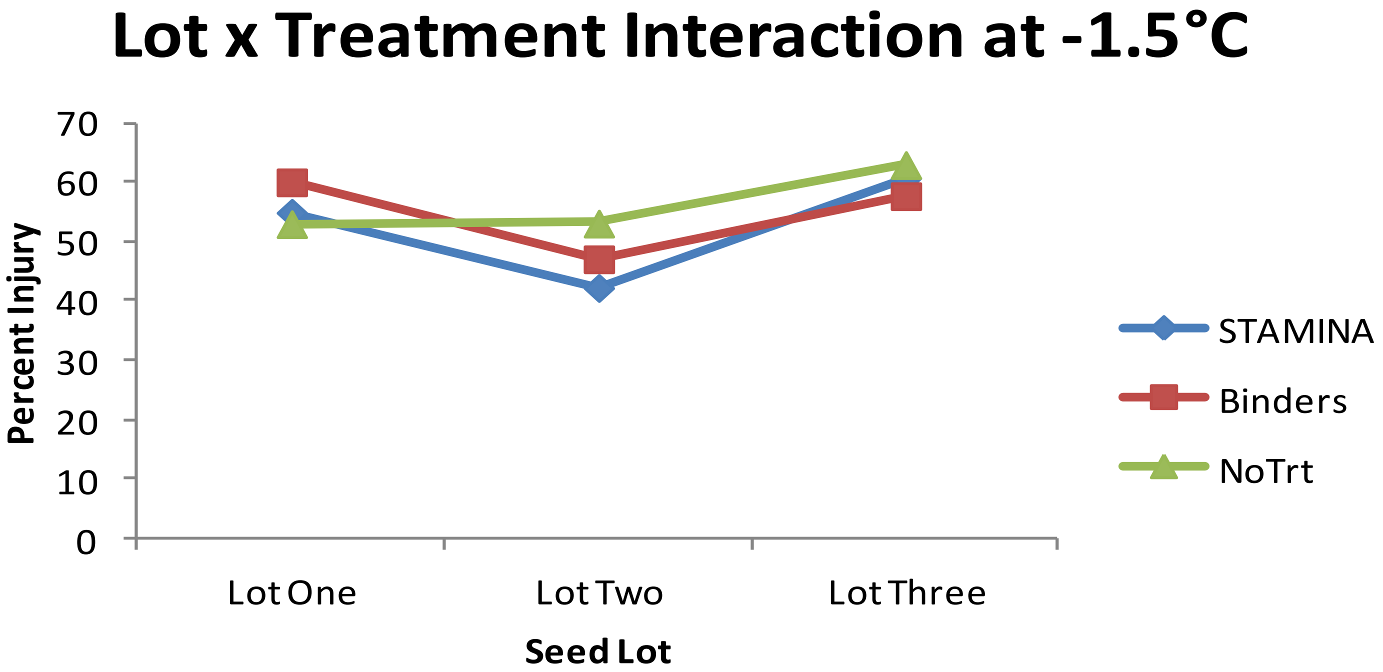
		Temperature (°C) [A]		
Seed Lot	**Treatment**	**-1.0**	**-1.5**	**-2.0**
		Percent Injury		
One	Stamina	35.25 a[B]	54.93 c	63.47 d
	Binders	42.64 b	60.06 d	62.58 d
	No Treatment	38.38 ab	53.01 c	59.92 d
Two	Stamina	37.88 a[B]	42.25 ab	56.98 d
	Binders	33.74 a	47.09 bc	56.60 d
	No Treatment	34.67 a	53.10 d	58.29 d
Three	Stamina	42.84 a[B]	60.76 bc	67.36 d
	Binders	47.00 a	57.73 b	66.51 d
	No Treatment	44.05 a	62.98 cd	67.27 d

[A] *Values represent the percent injury averaged over four replicates per lot-treatment-temperature combination*

Table 10 - Percent Injury of seedlings at -1.5°C, specific to seed lot and treatment effects.

Seed Lot	**Treatment**	**Percent Injury**
One	Stamina	54.93 ab
	Binders	60.06 b
	No Treatment	53.01 a
Two	Stamina	42.25 a
	Binders	47.09 ab
	No Treatment	53.10 b
Three	Stamina	60.76 a
	Binders	57.73 a
	No Treatment	62.98 a

[A] *Values represent the percent injury averaged over four replicates per lot-treatment-temperature combination*



Figures 3. Seed lot by treatment interaction in freeze injury at -1.5°C

Table 11 - Freeze Injury at -1.5°C for seed lot and treatment.

Treatment	**Freeze Injury**	**Seed Lot**	**Freeze Injury**
Stamina	52.65 a	One	56.00 b
Binders	54.96 ab	Two	47.48 a
No Treatment	56.36 b	Three	60.49 c

Values followed by different letters are considered significant at the P $\leq$ 0.05 level for each individual test

Table 12 - Effect of Treatment on SOD activity at Point A (after 25°C stage) and Point B (after second stress).

Treatment	**SOD (U/mg protein)** [A]	
	Point A [B]	Point B [B]
Stamina	8.91 a	8.91 a
Binders	8.04 b	9.31 a
No Treatment	8.04 b	9.05 a

[A] *Values represent the mean of twelve replicates per time period, averaged over all three seed lots*

[B] *Time periods are defined in Materials and Methods, Figure 1*

Table 13 - Effect of Seed Lot on SOD activity at Point A (after 25°C stage) and Point B (after second stress).

Seed Lot	**SOD (U/mg protein)** [A]	
	Point A [B]	Point B [B]
One	9.31 a	10.12 a
Two	7.73 b	8.43 b
Three	7.95 b	8.72 b

[A] *Values represent the mean of twelve replicates per time period, averaged over all three treatments*

[B] *Time periods are defined in Materials and Methods, Figure 1*

CHAPTER FOUR - DISCUSSION

The standard germination and cold tests were conducted as quality assurance tests on the seed lots. Standard germination test results for seed lots indicate that the potential of each seed lot under optimal conditions was the same. Stamina, however, did increase the germination performance under the optimal conditions of the standard germination test. This increase, though not large, was associated with a reduction in the percentage of abnormal seedlings; this is possibly due to fungal infection being controlled by Stamina. While the potential performance of the seed lots under favorable conditions was the same, performance under the stresses in the cold test differed. Seed lot two had the highest cold test performance (93.25%) and seed lot three the lowest (87.92%). It could be assumed, based on the results of the cold test, that seed lot two had the highest vigor among the seed lots. It is probable, given the performance of seed lots in standard germination (Table 2) and cold germination (Table 3), that these seed lots might be found in commercial channels (Dr. Allen Knapp, Iowa State University, Agronomy, Personal Communication, November 2012).

Differences in seedling height can be attributed to genetics, as many studies have shown genetic variability for germination of maize at low temperatures (Pinnell 1949, Pollmer 1969, Grogan 1970, Pesev 1970, Gubbels 1974). Stamina positively influenced seedling height across all lots at Point A; however there was a seed lot by treatment interaction (Figure 2). The interaction is apparent in seed lot three, where there is a greater difference in height between Stamina and binder treated seedlings than the other two seed lots. A CONTRAST statement in SAS was used to determine that seedlings treated with Stamina are taller than seedlings treated with binders only or no treatment in each individual seed lot (Appendix B, P-values in parameter comparisons). In addition, seedlings treated with binders from each individual seed lot are the same as untreated seedlings within the same lot. Given this fact, it is not unreasonable to focus on the positive main effects of Stamina on seedling height, as long as the importance of seed lot and how it affects results is kept in mind.

Seedling dry weight measurements were not affected by seed treatment at Point A (Table 6) but were affected by seed lot (Table 7). It is unclear why seedling height was affected by treatment and seed lot yet seedling dry weight was not. Given the growth conditions for the test (Figure 1), it is possible that these seedlings experienced primarily elongation growth early in seedling development. Germination and seedling growth are dependent upon genetics, environment, and seedling vigor, among other factors.

After the first measurement, seedlings were placed back at 10°C and the second biological measurement was made at Point B (Figure 1). Seedlings treated with Stamina were taller and heavier than seedlings treated with either binders only or no treatment. Given the fact that seedlings were of varying heights and weights, MRGR values were calculated according to Fisher (1921) in an effort to compare growth from different experimental treatments relative to their starting point, in this case, Point A. MRGR was calculated by taking the difference in the natural logarithms for seedling height or weight from Points B and A, divided by the period of growth (10 days). Natural logarithms of the growth measurements were used as growth follows a logarithmic pattern. Taking the logarithms of the growth measurements allowed for easier comparisons of growth among seedlings. Seedling growth is affected by genetics and their interaction with the environment. This can be seen by the differences in seedling height and dry weight MRGR for seed lots (Table 8). It is unclear how Stamina aids in increasing the MRGR for seedling dry weight in treated seedlings. In addition, the overall seed quality differences of each seed lot, as well as the level of fungal contamination, likely factor into increases in MRGR.

Stamina treated seedlings had higher SOD activity than seedlings from the other treatments at Point A (Table 10). These results are consistent with previous observations that increased SOD activity can be correlated with a higher tolerance to oxidative stress (Kendall and McKersie, 1989, Malan et al, 1990, Bowler et al, 1991, McKersie et al, 1993, Pitcher and Zilinkskas, 1996) resulting in increased performance.   The SOD activity varied by seed lot as well, with seed lot expressing increased SOD activity relative to other seed lots. Interestingly, even though the first seed lot contained greater SOD activity than seed lots two and three after Point A, the seedlings from seed lot one were not the tallest nor were they the heaviest. This could be attributed to the fact that high SOD activity can be indicative of the amount of stress experienced by the seedlings. SOD is induced (Bowler et al, 1992) by increased production of ROS, so it would not be uncommon for a significant amount of variation in activity to occur. Differences in seed quality could cause seedlings to experience varying levels of stress, causing varying levels of ROS production. This could differentially induce SOD activity. It would not be uncommon to see variability among seed lots from the same variety or different varieties. For instance, given that seed lot two had high seedling vigor, it is likely there is less ROS activity. This would lead to lower SOD activity, as ROS would not be present to induce antioxidant activity (Bailly, 2004). Seed deterioration due to increased ROS activity could also lead to higher levels of SOD activity, which could be possible in seed lot one.

The SOD activity of Stamina treated seedlings was the same as the SOD activity of seedlings treated with binders only or no treatment at Point B (Table 10). In fact, seedlings treated with binders only or no treatment both increased in SOD activity (15% and 12%, respectively) compared to measurements at Point A, while Stamina treated seedlings did not. The fact that SOD activity was not increased in Stamina treated seedlings could indicate a certain level of protection. Since SOD activity is inducible due in part to the amount or duration of stress, it is possible Stamina treated seedlings were not as stressed at Point B due to some level of protection; this could be due to the treatment or some genetic differences. However, given that all three seed lots increased their SOD activity (9%, 9%, and 10%, respectively) at Point B, it is likely oxidative stress was present, and stressful enough to induce SOD activity. Taking this into consideration, it is likely Stamina was the cause for increased protection against oxidative stress in treated seedlings, by managing ROS.

It is interesting to note that Stamina treated seedling had higher SOD activity at Point A but not Point B. One possible explanation for this is the nature of test. SOD was assayed as a snapshot in time at two different points, under differing conditions (Figure 1). At Point A, the seedlings had been exposed to an initial stress and then allowed to grow under ideal conditions for four days, while at Point B, SOD was assayed from seedlings under stress. SOD activity is unknown at other times throughout the test. It is possible Stamina elevates SOD activity for a longer amount of time after exposure to stress.

There was no consistent effect of Stamina on freezing injury (Figure 1). This inconsistency is seen in Figures 4-6 (Appendix A1) and is a function of how the study was conducted. Two temperatures, -1.0°C and -2.0°C, were included to assure the variation observed was similar to preliminary studies. Given that preliminary studies exhibit little variation in freeze injury at -1.0 and -2.0°C, it makes sense to see why the three way interaction is largely seen at -1.5°C; there is more variability in freeze injury at this temperature. Thus, it is not surprising the interaction is present at the temperature where the most variation occurred.

The results of the freeze injury may also be related to overall seed quality. If focus is placed on only -1.5°C, an interaction is observed. The seed lot with the highest vigor (two) had the lowest percent injury at -1.5°C. Seed deterioration occurs at the cellular level and affects the integrity and functional capacity of the membranes. It is not unreasonable to assume seed lots had varying amount of deterioration, which could lead to variation in freezing injury. In addition, lots were not selected on basis of whether they were cold resistant or sensitive. Large differences in percent injury at -1.5°C among seed lots (Table 13) suggest seed lot plays a vital role in the severity of injury at freezing temperatures. It should also be noted that the response of the binders treatment was inconsistent in the freezing injury assay (Tables 9 and 10). Further research will be needed to determine the possible cause of this inconsistency.

Previous studies have shown an increased production of ROS associated with freezing (Kendall and McKersie, 1989), and that tolerance to freezing is correlated to an increased capacity to detoxify ROS (Kendall and McKersie, 1989, Malan et al, 1990, Van Camp et al, 1996). As mentioned previously, elevated SOD activity may indicate elevated levels of ROS. Given the fact that SOD activity of Stamina treated seedlings was not higher than seedlings treated with binders or no treatment at Point B (Table 10), when freezing injury was assayed, it is likely the protection received was not great enough to stabilize the membranes against freeze injury.

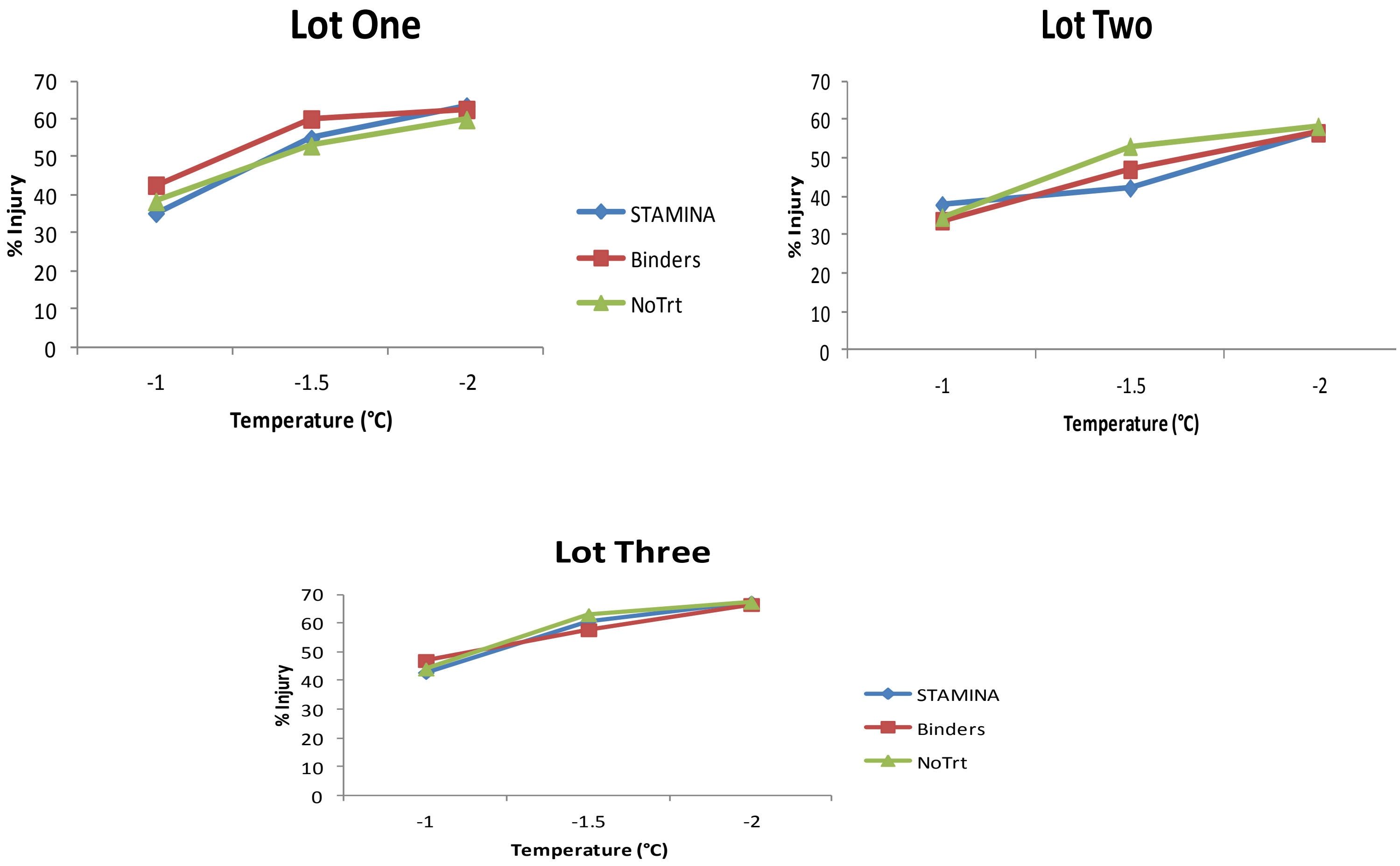
CONCLUSIONS

Stamina is a strobilurin fungicide seed treatment used on maize. Its primary objective is to protect against fungal pathogens. The purpose of this study was to investigate the physiological effects Stamina had on SOD activity and freeze injury, unrelated to fungal contamination. Even when fungal contamination is reasonably low, as in these seed lots, Stamina affects seedling performance at chilling temperatures in the laboratory early during the germination period. This can be seen in a small elevation in SOD activity, which has previously been shown to provide protection against oxidative damage (Kendall and McKersie, 1989, Malan et al, 1990, Bowler et al, 1991, McKersie et al, 1993, Pitcher and Zilinkskas, 1996). While these differences are not large, the effect is evidenced by enhanced seedling performance, as seen by increased seedling height of Stamina treated seedlings compared to seedlings treated with binders or no treatment.

While Stamina treated seedlings did not contain elevated SOD activity after the second stress, it is reasonable to assume the treatment did provide some amount of protection to the seedlings, as MRGR are higher for dry weight, leading to heavier seedlings. Seed lot differences were important across all aspects of the study, having an effect on seedling height, dry weight, SOD activity, and freeze injury. The effect of the binders treatment was inconsistent in the freeze injury assay. This makes it difficult to assess the effectiveness of Stamina in this scenario. In addition, it is possible the assay used is not as valuable in maize as it might be in other plants.

Further research needs to be done to expand upon laboratory results to determine how these results translate to field performance.

APPENDIX A: SEED LOT BY TEMPERATURE BY TREATMENT INTERACTION FOR FREEZING INJURY



Figures 4-6. Figure 4 (upper left) is the treatment by temperature interaction over seed lot 1. Figure 5 (upper right) is the treatment by temperature interaction over seed lot 2. Figure 6 (lower middle) is the treatment by temperature interaction over seed lot 3.

Seed Lot/treatment/temperature interaction sliced by seed lot/temperature interaction to show effect of treatment.

Seed Lot	**Temperature (°C)**	**P - Value**
One	-1.0	0.0159*
	-1.5	0.0180*
	-2.0	0.3100
Two	-1.0	0.2054
	-1.5	0.0005*
	-2.0	0.7548
Three	-1.0	0.2146
	-1.5	0.1033
	-2.0	0.9242

[*] *Shows significance at the $p \leq 0.05$ level.*

APPENDIX B: SEED HEALTH INFORMATION FOR SEED LOTS

Seed Lot and Treatment	**Fusarium**	**Penicillium**	**Aspergillus**	**Rhizopus**	**Other**
Seed Lot #1 Stamina	0	0	0	0	0
Seed Lot #1 Stamina	0	0	1	1	0
Seed Lot #1 Stamina	0	0	1	0	0
Seed Lot #1 Stamina	2	2	0	0	0
Seed Lot #1 Binders	0	0	4	8	0
Seed Lot #1 Binders	0	0	4	0	0
Seed Lot #1 Binders	3	3	2	3	1
Seed Lot #1 Binders	3	3	2	0	0
Seed Lot #1 No Treatment	6	6	9	0	0
Seed Lot #1 No Treatment	4	4	3	0	1
Seed Lot #1 No Treatment	6	6	12	5	0
Seed Lot #1 No Treatment	9	9	9	0	2
Seed Lot #3 Stamina	0	0	0	0	0
Seed Lot #3 Stamina	1	0	0	0	0
Seed Lot #3 Stamina	1	0	0	0	0
Seed Lot #3 Stamina	0	0	0	0	0
Seed Lot #3 Binders	0	2	2	2	0
Seed Lot #3 Binders	3	5	6	0	0
Seed Lot #3 Binders	2	1	0	2	0
Seed Lot #3 Binders	1	1	2	1	0
Seed Lot #3 No Treatment	0	5	8	0	1
Seed Lot #3 No Treatment	0	5	0	0	0
Seed Lot #3 No Treatment	5	7	2	0	0
Seed Lot #3 No Treatment	7	5	6	0	1
**Seed Lot #2	1	8	2	0	3

Results based on seed health tests performed at Iowa State University Seed Science Center. Numbers indicate # of seeds infected out of 100. **Seed Lot #2 based on seed health tests performed by BASF. Numbers indicate # of seeds infected out of 120

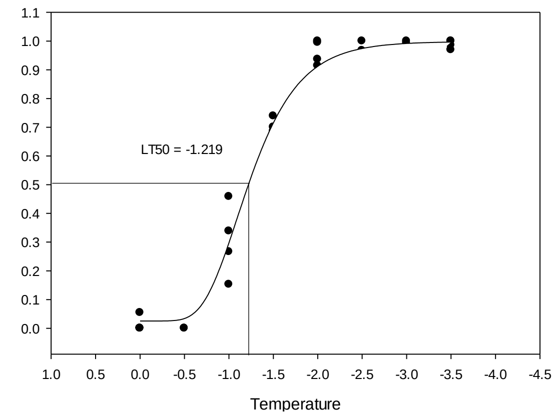
APPENDIX C: LINEAR CONTRAST COMPARISONS OF SEEDLING HEIGHT AT POINT A MEASUREMENT

```
Seedling Height Analysis
After 4 Day Grow-Out
Linear Contrasts

The GLM Procedure

Dependent Variable: height   cm

                                       Sum of
Source                      DF        Squares     Mean Square    F Value    Pr > F

Model                       23    10.25222222      0.44574879      68.77    <.0001

Error                       12     0.07777778      0.00648148

Corrected Total             35    10.33000000


R-Square     Coeff Var      Root MSE    height Mean

0.992471      0.735230      0.080508       10.95000


Source                      DF    Type III SS     Mean Square    F Value    Pr > F

blk                          3     0.49444444      0.16481481      25.43    <.0001
sl                           2     8.03166667      4.01583333     619.59    <.0001
blk*sl                       6     0.59055556      0.09842593      15.19    <.0001
trt                          2     0.98666667      0.49333333      76.11    <.0001
blk*trt                      6     0.04222222      0.00703704       1.09    0.4234
sl*trt                       4     0.10666667      0.02666667       4.11    0.0251


                                                           Standard
Parameter                                Estimate             Error    t Value    Pr > |t|

AI vs Binders at Seed Lot 1            0.30000000        0.05692750       5.27      0.0002
AI vs Control at Seed Lot 1            0.35000000        0.05692750       6.15      <.0001
Binders vs Control at Seed Lot 1       0.05000000        0.05692750       0.88      0.3970
AI vs Binders at Seed Lot 2            0.20000000        0.05692750       3.51      0.0043
AI vs Control at Seed Lot 2            0.25000000        0.05692750       4.39      0.0009
Binders vs Control at Seed Lot 2       0.05000000        0.05692750       0.88      0.3970
AI vs Binders at Seed Lot 3            0.50000000        0.05692750       8.78      <.0001
AI vs Control at Seed Lot 3            0.50000000        0.05692750       8.78      <.0001
Binders vs Control at Seed Lot 3       0.00000000        0.05692750       0.00      1.0000
```

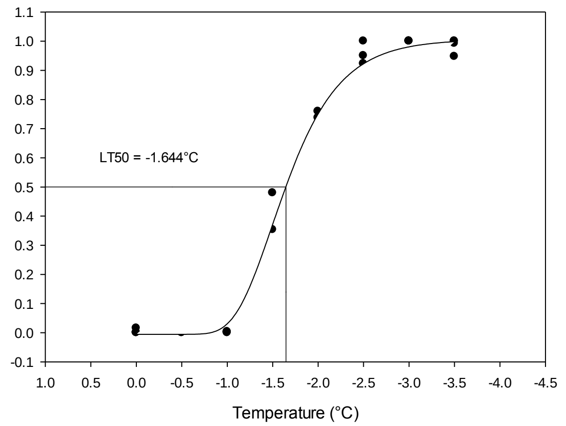
Results based on PROC GLM procedure in SAS.

APPENDIX D: COMPARISON OF LT50 AND FREEZE INJURY IN PRELIMINARY TRIALS

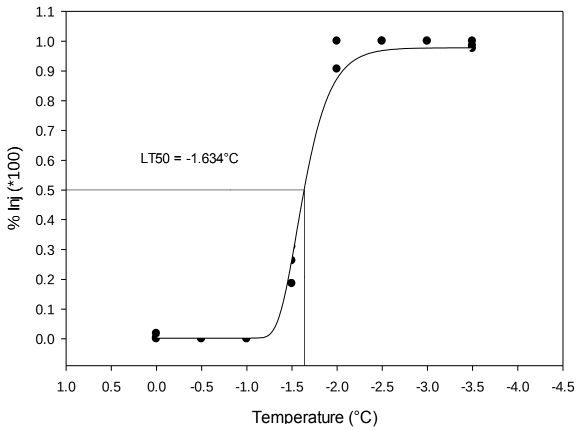
Seed Lot 3, STAMINAS 3/16/12



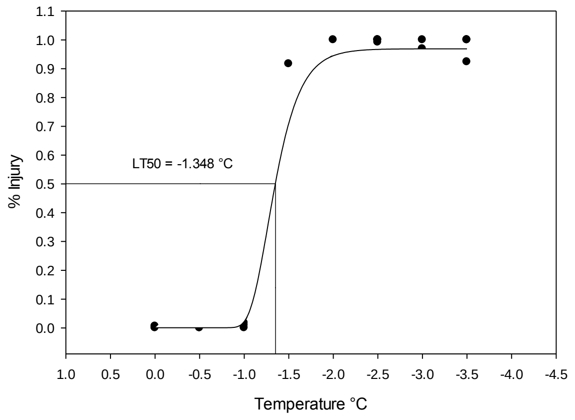
Seed Lot 3, STAMINAS 4/24/12



Seed Lot 3, Untreated, 3/16/12

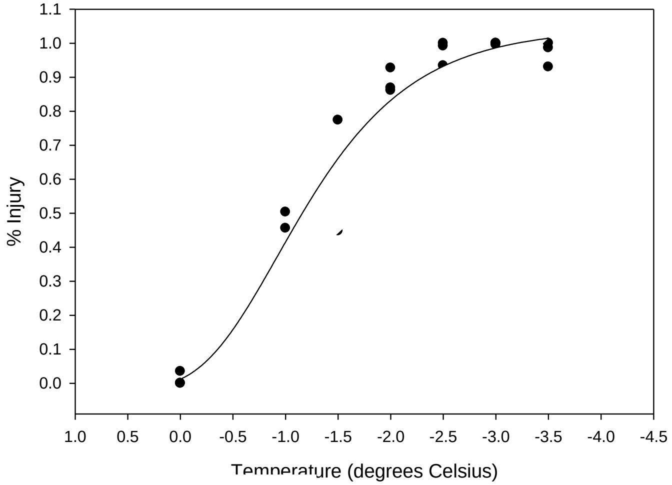


Seed Lot 3, Untreated 4/24/12

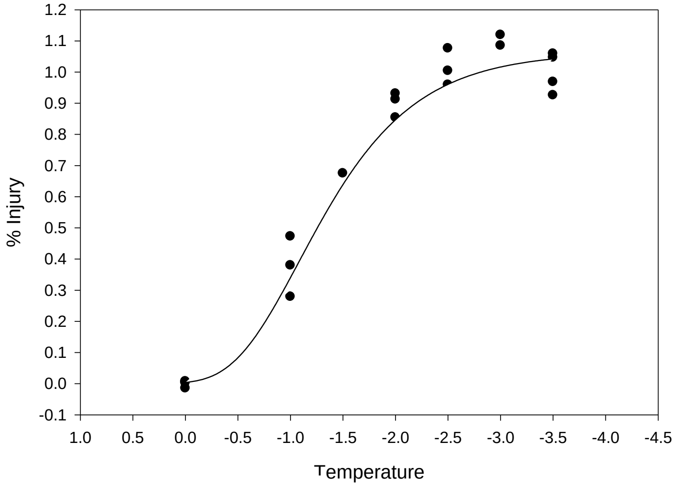


Raw data of freeze injury calculations in preliminary studies. Done according to Materials and Methods

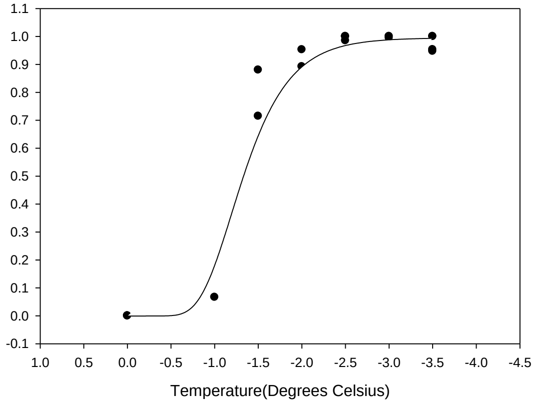
Seed Lot 1, Untreated 6/12/12



Seed Lot 2, Untreated 6/12/12



Seed Lot 3, Untreated 6/12/12



Raw data of freeze injury on untreated seed lots from preliminary trials.

APPENDIX E: STATISTICAL ANALYSIS OF SEEDLING HEIGHT AT POINTS A & B

```
options nocenter ls=89 ps=51 pageno=1 frmdlim='=';
title 'Seedling Height Analysis';
title2 'After 4 Day Grow-Out';
data sh4;
input blk lot trt height;
label height = 'cm';
cards;
1      1      1      10.8
1      1      2      10.6
1      1      3      10.5
1      2      1      12.0
1      2      2      11.8
1      2      3      11.6
1      3      1      11.3
1      3      2      10.7
1      3      3      10.7
2      1      1      10.6
2      1      2      10.2
2      1      3      10.1
2      2      1      11.7
2      2      2      11.6
2      2      3      11.6
2      3      1      10.9
2      3      2      10.4
2      3      3      10.3
3      1      1      10.7
3      1      2      10.4
3      1      3      10.4
3      2      1      11.8
3      2      2      11.5
3      2      3      11.5
3      3      1      11.3
3      3      2      10.8
3      3      3      10.7
4      1      1      10.5
4      1      2      10.2
4      1      3      10.2
```

```
4      2      1      11.4
4      2      2      11.2
4      2      3      11.2
4      3      1      11.2
4      3      2      10.8
4      3      3      11.0
;
run;
title3 'Complete Analysis - After 4 Day Grow-Out';
proc glm data=sh4;
class blk lot trt;
model height = blk lot blk*lot trt blk*trt lot*trt;
test h=lot e=blk*lot;
test h=trt e=blk*trt;
means trt / lsd;
means lot / lsd;
means lot*trt/lsd;
run;
title 'Seedling Height Analysis';
title2 '21 Days After Planting';
data sh21;
input blk lot trt height;
label height = 'cm';
cards;
1      1      1      11.6
1      1      2      11.4
1      1      3      11.3
1      2      1      13.0
1      2      2      12.8
1      2      3      12.6
1      3      1      11.5
1      3      2      11.5
1      3      3      11.6
2      1      1      11.4
2      1      2      11.2
2      1      3      11.3
2      2      1      13.2
2      2      2      13.0
2      2      3      13.2
```

```
2      3      1      11.4
2      3      2      11.3
2      3      3      11.3
3      1      1      11.5
3      1      2      11.2
3      1      3      11.3
3      2      1      13.0
3      2      2      12.9
3      2      3      12.9
3      3      1      11.4
3      3      2      11.3
3      3      3      11.3
4      1      1      11.4
4      1      2      11.3
4      1      3      11.3
4      2      1      13.1
4      2      2      12.9
4      2      3      13.0
4      3      1      11.4
4      3      2      11.2
4      3      3      11.1
;
run;
title3 'Complete Analysis - 21 Days After Planting';
proc glm data=sh21;
class blk lot trt;
model height = blk lot blk*lot trt blk*trt lot*trt;
test h=lot e=blk*lot;
test h=trt e=blk*trt;
means trt / lsd;
means lot / lsd;
means lot*trt/lsd;
run;
```

```
==========================================================================================

Seedling Height Analysis                             12:15 Tuesday, September 4, 2012   1
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure

    Class Level Information

Class         Levels    Values

blk                4    1 2 3 4

lot                3    1 2 3

trt                3    1 2 3


Number of Observations Read          36
Number of Observations Used          36

==========================================================================================

Seedling Height Analysis                             12:15 Tuesday, September 4, 2012   2
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure

Dependent Variable: height   cm

                                        Sum of
Source                      DF         Squares     Mean Square    F Value    Pr > F

Model                       23     10.25222222      0.44574879      68.77    <.0001

Error                       12      0.07777778      0.00648148

Corrected Total             35     10.33000000


R-Square     Coeff Var      Root MSE    height Mean

0.992471      0.735230      0.080508       10.95000
```

```
Source                      DF       Type I SS     Mean Square    F Value    Pr > F

blk                          3      0.49444444      0.16481481      25.43    <.0001
lot                          2      8.03166667      4.01583333     619.59    <.0001
blk*lot                      6      0.59055556      0.09842593      15.19    <.0001
trt                          2      0.98666667      0.49333333      76.11    <.0001
blk*trt                      6      0.04222222      0.00703704       1.09    0.4234
lot*trt                      4      0.10666667      0.02666667       4.11    0.0251


Source                      DF     Type III SS     Mean Square    F Value    Pr > F

blk                          3      0.49444444      0.16481481      25.43    <.0001
lot                          2      8.03166667      4.01583333     619.59    <.0001
blk*lot                      6      0.59055556      0.09842593      15.19    <.0001
trt                          2      0.98666667      0.49333333      76.11    <.0001
blk*trt                      6      0.04222222      0.00703704       1.09    0.4234
lot*trt                      4      0.10666667      0.02666667       4.11    0.0251


      Tests of Hypotheses Using the Type III MS for blk*lot as an Error Term

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

lot                          2      8.03166667      4.01583333      40.80    0.0003

==========================================================================================

Seedling Height Analysis                          12:15 Tuesday, September 4, 2012   3
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure

Dependent Variable: height   cm

      Tests of Hypotheses Using the Type III MS for blk*trt as an Error Term

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

trt                          2      0.98666667      0.49333333      70.11    <.0001

==========================================================================================

Seedling Height Analysis                          12:15 Tuesday, September 4, 2012   4
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure
```

```
t Tests (LSD) for height

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.


Alpha                             0.05
Error Degrees of Freedom            12
Error Mean Square             0.006481
Critical Value of t            2.17881
Least Significant Difference    0.0716


Means with the same letter are not significantly different.


       Mean          N    trt

A      11.18333     12    1

B      10.85000     12    2
B
B      10.81667     12    3

==========================================================================================

Seedling Height Analysis                             12:15 Tuesday, September 4, 2012   5
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure

t Tests (LSD) for height

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.


Alpha                             0.05
Error Degrees of Freedom            12
Error Mean Square             0.006481
Critical Value of t            2.17881
Least Significant Difference    0.0716
```

```
Means with the same letter are not significantly different.


       Mean          N    lot

A      11.57500     12    2

B      10.84167     12    3

C      10.43333     12    1

==========================================================================================

Seedling Height Analysis                            12:15 Tuesday, September 4, 2012   6
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure


Level of     Level of           ------------height-----------
lot          trt          N             Mean          Std Dev

1            1            4       10.6500000       0.12909944
1            2            4       10.3500000       0.19148542
1            3            4       10.3000000       0.18257419
2            1            4       11.7250000       0.25000000
2            2            4       11.5250000       0.25000000
2            3            4       11.4750000       0.18929694
3            1            4       11.1750000       0.18929694
3            2            4       10.6750000       0.18929694
3            3            4       10.6750000       0.28722813

==========================================================================================

Seedling Height Analysis                            12:15 Tuesday, September 4, 2012   7
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure

    Class Level Information

Class         Levels    Values

blk                4    1 2 3 4

lot                3    1 2 3
```

```
trt                3    1 2 3


Number of Observations Read          36
Number of Observations Used          36

=========================================================================================

Seedling Height Analysis                            12:15 Tuesday, September 4, 2012   8
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure

Dependent Variable: height   cm

                                        Sum of
Source                      DF         Squares     Mean Square    F Value    Pr > F

Model                       23     21.36583333      0.92894928     109.65    <.0001

Error                       12      0.10166667      0.00847222

Corrected Total             35     21.46750000


R-Square     Coeff Var      Root MSE    height Mean

0.995264      0.774027      0.092045       11.89167


Source                      DF       Type I SS     Mean Square    F Value    Pr > F

blk                          3      0.03416667      0.01138889       1.34    0.3064
lot                          2     20.80166667     10.40083333    1227.64    <.0001
blk*lot                      6      0.31166667      0.05194444       6.13    0.0039
trt                          2      0.18166667      0.09083333      10.72    0.0021
blk*trt                      6      0.02500000      0.00416667       0.49    0.8027
lot*trt                      4      0.01166667      0.00291667       0.34    0.8430


Source                      DF     Type III SS     Mean Square    F Value    Pr > F

blk                          3      0.03416667      0.01138889       1.34    0.3064
lot                          2     20.80166667     10.40083333    1227.64    <.0001
blk*lot                      6      0.31166667      0.05194444       6.13    0.0039
trt                          2      0.18166667      0.09083333      10.72    0.0021
blk*trt                      6      0.02500000      0.00416667       0.49    0.8027
```

```
lot*trt                      4      0.01166667      0.00291667       0.34    0.8430


      Tests of Hypotheses Using the Type III MS for blk*lot as an Error Term

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

lot                          2     20.80166667     10.40083333     200.23    <.0001

==========================================================================================

Seedling Height Analysis                             12:15 Tuesday, September 4, 2012   9
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure

Dependent Variable: height   cm

      Tests of Hypotheses Using the Type III MS for blk*trt as an Error Term

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

trt                          2      0.18166667      0.09083333      21.80    0.0018

==========================================================================================

Seedling Height Analysis                             12:15 Tuesday, September 4, 2012  10
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure

t Tests (LSD) for height

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.


Alpha                              0.05
Error Degrees of Freedom             12
Error Mean Square              0.008472
Critical Value of t             2.17881
Least Significant Difference     0.0819


Means with the same letter are not significantly different.
```

```
       Mean          N    trt

A      11.99167     12    1

B      11.85000     12    3
B
B      11.83333     12    2

=========================================================================================

Seedling Height Analysis                            12:15 Tuesday, September 4, 2012  11
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure

t Tests (LSD) for height

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.


Alpha                             0.05
Error Degrees of Freedom            12
Error Mean Square             0.008472
Critical Value of t            2.17881
Least Significant Difference    0.0819


Means with the same letter are not significantly different.


       Mean         N    lot

A      12.96667     12    2

B      11.35833     12    3
B
B      11.35000     12    1

=========================================================================================

Seedling Height Analysis                            12:15 Tuesday, September 4, 2012  12
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure
```

```
Level of     Level of             ------------height-----------
lot          trt          N              Mean          Std Dev

1            1            4        11.4750000       0.09574271
1            2            4        11.2750000       0.09574271
1            3            4        11.3000000       0.00000000
2            1            4        13.0750000       0.09574271
2            2            4        12.9000000       0.08164966
2            3            4        12.9250000       0.25000000
3            1            4        11.4250000       0.05000000
3            2            4        11.3250000       0.12583057
3            3            4        11.3250000       0.20615528
```

APPENDIX F: STATISTICAL ANALYSIS OF SEEDLING DRY WEIGHT AT POINTS A & B

```
options nocenter ls=89 ps=51 pageno=1 frmdlim='=';
title 'Seedling Dry Weight Analysis';
title2 'After 4 Day Grow-Out';
data sdw4;
input blk lot trt weight;
label weight = 'mg';
cards;
1     1     1     30.0
1     1     2     29.5
1     1     3     28.5
1     2     1     39.5
1     2     2     43.9
1     2     3     39.7
1     3     1     26.7
1     3     2     33.8
1     3     3     32.6
2     1     1     23.4
2     1     2     23.3
2     1     3     22.4
2     2     1     35.8
2     2     2     32.8
2     2     3     29.5
2     3     1     27.2
2     3     2     29.8
2     3     3     27.1
3     1     1     28.0
3     1     2     26.0
3     1     3     27.8
3     2     1     41.5
3     2     2     39.6
3     2     3     37.0
3     3     1     31.8
3     3     2     30.2
3     3     3     33.6
4     1     1     30.5
4     1     2     25.4
4     1     3     28.0
4     2     1     34.0
4     2     2     33.8
```

```
4      2      3      39.4
4      3      1      28.8
4      3      2      29.4
4      3      3      27.5
;
run;
title3 'Complete Analysis - After 4 Day Grow-Out';
proc glm data=sdw4;
class blk lot trt;
model weight = blk lot blk*lot trt blk*trt lot*trt;
test h=lot e=blk*lot;
test h=trt e=blk*trt;
means trt / lsd;
means lot / lsd;
means lot*trt/lsd;
run;
title 'Seedling Dry Weight Analysis';
title2 '21 Days After Planting';
data sdw21;
input blk lot trt weight;
label weight = 'mg';
cards;
1      1      1      31.4
1      1      2      25.9
1      1      3      31.1
1      2      1      44.4
1      2      2      49.7
1      2      3      37.9
1      3      1      35.7
1      3      2      30.8
1      3      3      30.3
2      1      1      25.3
2      1      2      23.9
2      1      3      21.6
2      2      1      50.0
2      2      2      37.7
2      2      3      36.1
2      3      1      31.1
2      3      2      28.7
```

```
2      3      3      26.0
3      1      1      28.1
3      1      2      25.8
3      1      3      26.4
3      2      1      52.8
3      2      2      41.3
3      2      3      45.6
3      3      1      38.0
3      3      2      31.8
3      3      3      33.1
4      1      1      26.1
4      1      2      28.1
4      1      3      26.2
4      2      1      45.0
4      2      2      48.3
4      2      3      47.6
4      3      1      37.5
4      3      2      31.7
4      3      3      29.6
;
run;
title3 'Complete Analysis - 21 Days After Planting';
proc glm data=sdw21;
class blk lot trt;
model weight = blk lot blk*lot trt blk*trt lot*trt;
test h=lot e=blk*lot;
test h=trt e=blk*trt;
means trt / lsd;
means lot / lsd;
means lot*trt/lsd;
run;
```

```
==========================================================================================

Seedling Dry Weight Analysis                        11:50 Tuesday, September 4, 2012   1
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure

    Class Level Information

Class         Levels    Values

blk                4    1 2 3 4

lot                3    1 2 3

trt                3    1 2 3


Number of Observations Read          36
Number of Observations Used          36

==========================================================================================

Seedling Dry Weight Analysis                        11:50 Tuesday, September 4, 2012   2
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure

Dependent Variable: weight   mg

                                        Sum of
Source                      DF         Squares     Mean Square    F Value    Pr > F

Model                       23      963.327778       41.883816       8.92    0.0002

Error                       12       56.324444        4.693704

Corrected Total             35     1019.652222


R-Square     Coeff Var      Root MSE    weight Mean

0.944761      6.915574      2.166496       31.32778


Source                      DF       Type I SS     Mean Square    F Value    Pr > F
```

```
blk                         3     182.7344444      60.9114815      12.98    0.0004
lot                         2     675.5605556     337.7802778      71.96    <.0001
blk*lot                     6      40.3038889       6.7173148       1.43    0.2807
trt                         2       1.0072222       0.5036111       0.11    0.8991
blk*trt                     6      42.9372222       7.1562037       1.52    0.2512
lot*trt                     4      20.7844444       5.1961111       1.11    0.3976


Source                     DF     Type III SS     Mean Square    F Value    Pr > F

blk                         3     182.7344444      60.9114815      12.98    0.0004
lot                         2     675.5605556     337.7802778      71.96    <.0001
blk*lot                     6      40.3038889       6.7173148       1.43    0.2807
trt                         2       1.0072222       0.5036111       0.11    0.8991
blk*trt                     6      42.9372222       7.1562037       1.52    0.2512
lot*trt                     4      20.7844444       5.1961111       1.11    0.3976


      Tests of Hypotheses Using the Type III MS for blk*lot as an Error Term

Source                     DF     Type III SS     Mean Square    F Value    Pr > F

lot                         2     675.5605556     337.7802778      50.29    0.0002

==========================================================================================

Seedling Dry Weight Analysis                        11:50 Tuesday, September 4, 2012   3
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure

Dependent Variable: weight   mg

      Tests of Hypotheses Using the Type III MS for blk*trt as an Error Term

Source                     DF     Type III SS     Mean Square    F Value    Pr > F

trt                         2      1.00722222      0.50361111       0.07    0.9328

==========================================================================================

Seedling Dry Weight Analysis                        11:50 Tuesday, September 4, 2012   4
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure
```

```
t Tests (LSD) for weight

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.


Alpha                             0.05
Error Degrees of Freedom            12
Error Mean Square             4.693704
Critical Value of t            2.17881
Least Significant Difference    1.9271


Means with the same letter are not significantly different.


         Mean      N    trt

A       31.4583     12    2
A
A       31.4333     12    1
A
A       31.0917     12    3

==========================================================================================

Seedling Dry Weight Analysis                         11:50 Tuesday, September 4, 2012   5
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure

t Tests (LSD) for weight

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.


Alpha                             0.05
Error Degrees of Freedom            12
Error Mean Square             4.693704
Critical Value of t            2.17881
Least Significant Difference    1.9271


Means with the same letter are not significantly different.


          Mean      N    lot
```

```
A       37.2083     12    2

B       29.8750     12    3

C       26.9000     12    1

=========================================================================================

Seedling Dry Weight Analysis                         11:50 Tuesday, September 4, 2012   6
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure


Level of     Level of           ------------weight-----------
lot          trt          N             Mean          Std Dev

1            1            4       27.9750000       3.23560916
1            2            4       26.0500000       2.57487864
1            3            4       26.6750000       2.86516434
2            1            4       37.7000000       3.41467422
2            2            4       37.5250000       5.20088134
2            3            4       36.4000000       4.75604878
3            1            4       28.6250000       2.29836899
3            2            4       30.8000000       2.02649122
3            3            4       30.2000000       3.37737571

=========================================================================================

Seedling Dry Weight Analysis                         11:50 Tuesday, September 4, 2012   7
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure

    Class Level Information

Class         Levels    Values

blk                4    1 2 3 4

lot                3    1 2 3

trt                3    1 2 3


Number of Observations Read          36
```

```
Number of Observations Used          36

==========================================================================================

Seedling Dry Weight Analysis                        11:50 Tuesday, September 4, 2012   8
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure

Dependent Variable: weight   mg

                                        Sum of
Source                      DF         Squares     Mean Square    F Value    Pr > F

Model                       23     2449.970000      106.520435       8.30    0.0002

Error                       12      154.055556       12.837963

Corrected Total             35     2604.025556


R-Square     Coeff Var      Root MSE    weight Mean

0.940839      10.39726      3.583010       34.46111


Source                      DF       Type I SS     Mean Square    F Value    Pr > F

blk                          3      132.925556       44.308519       3.45    0.0515
lot                          2     2059.833889     1029.916944      80.22    <.0001
blk*lot                      6       34.959444        5.826574       0.45    0.8291
trt                          2      133.137222       66.568611       5.19    0.0238
blk*trt                      6       58.149444        9.691574       0.75    0.6179
lot*trt                      4       30.964444        7.741111       0.60    0.6679


Source                      DF     Type III SS     Mean Square    F Value    Pr > F

blk                          3      132.925556       44.308519       3.45    0.0515
lot                          2     2059.833889     1029.916944      80.22    <.0001
blk*lot                      6       34.959444        5.826574       0.45    0.8291
trt                          2      133.137222       66.568611       5.19    0.0238
blk*trt                      6       58.149444        9.691574       0.75    0.6179
lot*trt                      4       30.964444        7.741111       0.60    0.6679

      Tests of Hypotheses Using the Type III MS for blk*lot as an Error Term
```

```
Source                      DF     Type III SS     Mean Square    F Value    Pr > F

lot                          2     2059.833889     1029.916944     176.76    <.0001

==========================================================================================

Seedling Dry Weight Analysis                        11:50 Tuesday, September 4, 2012   9
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure

Dependent Variable: weight   mg

      Tests of Hypotheses Using the Type III MS for blk*trt as an Error Term

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

trt                          2     133.1372222      66.5686111       6.87    0.0281

==========================================================================================

Seedling Dry Weight Analysis                        11:50 Tuesday, September 4, 2012  10
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure

t Tests (LSD) for weight

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.


Alpha                              0.05
Error Degrees of Freedom             12
Error Mean Square              12.83796
Critical Value of t             2.17881
Least Significant Difference     3.1871


Means with the same letter are not significantly different.


          Mean      N    trt

A       37.117     12    1

B       33.642     12    2
```

```
B
B        32.625     12    3

==========================================================================================

Seedling Dry Weight Analysis                        11:50 Tuesday, September 4, 2012  11
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure

t Tests (LSD) for weight

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.


Alpha                            0.05
Error Degrees of Freedom           12
Error Mean Square            12.83796
Critical Value of t           2.17881
Least Significant Difference   3.1871


Means with the same letter are not significantly different.


          Mean      N    lot

A        44.700     12    2

B        32.025     12    3

C        26.658     12    1

==========================================================================================

Seedling Dry Weight Analysis                        11:50 Tuesday, September 4, 2012  12
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure


Level of     Level of           ------------weight-----------
lot          trt          N            Mean          Std Dev

1            1            4      27.7250000       2.71830217
1            2            4      25.9250000       1.71731379
```

```
   1            3            4       26.3250000       3.87932554
   2            1            4       48.0500000       4.04103947
   2            2            4       44.2500000       5.70701323
   2            3            4       41.8000000       5.65036872
   3            1            4       35.5750000       3.14258386
   3            2            4       30.7500000       1.43874946
   3            3            4       29.7500000       2.92175746
```

APPENDIX G: STATISTICAL ANALYSIS OF SOD ENZYME ACTIVITY AT POINTS AND B

```
options nocenter ls=89 ps=51 pageno=1 frmdlim='=';
title 'SOD Analysis';
title2 'After 4 Day Grow-Out';
data sod4;
input blk lot trt SOD;
label SOD = 'U/mg protein';
cards;
1      1      1      12.62
1      1      2      9.31
1      1      3      8.99
1      2      1      7.70
1      2      2      7.15
1      2      3      7.69
1      3      1      8.46
1      3      2      7.19
1      3      3      8.18
2      1      1      9.42
2      1      2      8.60
2      1      3      8.90
2      2      1      7.80
2      2      2      8.12
2      2      3      8.42
2      3      1      9.33
2      3      2      8.22
2      3      3      6.80
3      1      1      8.98
3      1      2      8.28
3      1      3      8.33
3      2      1      7.14
3      2      2      6.84
3      2      3      .
3      3      1      8.70
3      3      2      7.73
3      3      3      7.44
4      1      1      10.69
4      1      2      8.26
4      1      3      9.28
4      2      1      7.39
4      2      2      9.27
```

```
4       2       3       7.90
4       3       1       8.70
4       3       2       7.47
4       3       3       .
;
run;
title3 'Complete Analysis - After 4 Day Grow-Out';
proc glm data=sod4;
class blk lot trt;
model SOD = blk lot blk*lot trt blk*trt lot*trt;
test h=lot e=blk*lot;
test h=trt e=blk*trt;
lsmeans trt / pdiff stderr;
lsmeans lot / pdiff stderr;
lsmeans lot*trt / pdiff stderr;
run;
title 'SOD Analysis';
title2 '21 Days After Planting';
data sod21;
input blk lot trt SOD;
label SOD = 'U/mg protein';
cards;
1       1       1       9.56
1       1       2       9.68
1       1       3       9.35
1       2       1       8.91
1       2       2       9.86
1       2       3       9.97
1       3       1       8.95
1       3       2       9.39
1       3       3       9.97
2       1       1       7.61
2       1       2       12.21
2       1       3       9.88
2       2       1       10.61
2       2       2       7.54
2       2       3       5.99
2       3       1       7.81
2       3       2       9.16
```

```
2      3      3      7.73
3      1      1      10.72
3      1      2      11.72
3      1      3      10.82
3      2      1      9.57
3      2      2      7.20
3      2      3      8.78
3      3      1      6.96
3      3      2      9.21
3      3      3      8.88
4      1      1      10.25
4      1      2      9.41
4      1      3      10.24
4      2      1      8.02
4      2      2      7.17
4      2      3      .
4      3      1      7.95
4      3      2      9.12
4      3      3      .
;
run;
title3 'Complete Analysis - 21 Days After Planting';
proc glm data=sod21;
class blk lot trt;
model SOD = blk lot blk*lot trt blk*trt lot*trt;
test h=lot e=blk*lot;
test h=trt e=blk*trt;
lsmeans trt / pdiff stderr;
lsmeans lot / pdiff stderr;
lsmeans lot*trt / pdiff stderr;
run;
```

```
==========================================================================================

SOD Analysis                                        13:54 Tuesday, September 11, 2012   1
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure

    Class Level Information

Class         Levels    Values

blk                4    1 2 3 4

lot                3    1 2 3

trt                3    1 2 3


Number of Observations Read          36
Number of Observations Used          34

==========================================================================================

SOD Analysis                                        13:54 Tuesday, September 11, 2012   2
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure

Dependent Variable: SOD   U/mg protein

                                       Sum of
Source                      DF        Squares     Mean Square    F Value    Pr > F

Model                       23     36.69476985      1.59542478       2.48    0.0690

Error                       10      6.44378310      0.64437831

Corrected Total             33     43.13855294


R-Square     Coeff Var      Root MSE      SOD Mean

0.850626      9.566379      0.802732      8.391176


Source                      DF       Type I SS     Mean Square    F Value    Pr > F
```

```
blk                          3      2.46910850      0.82303617       1.28    0.3346
lot                          2     16.29616087      8.14808044      12.64    0.0018
blk*lot                      6      4.60770024      0.76795004       1.19    0.3836
trt                          2      6.04516417      3.02258208       4.69    0.0366
blk*trt                      6      1.45811361      0.24301894       0.38    0.8775
lot*trt                      4      5.81852246      1.45463062       2.26    0.1351


Source                      DF     Type III SS     Mean Square    F Value    Pr > F

blk                          3      2.16045422      0.72015141       1.12    0.3875
lot                          2     16.22982388      8.11491194      12.59    0.0019
blk*lot                      6      4.75944214      0.79324036       1.23    0.3671
trt                          2      5.75982780      2.87991390       4.47    0.0410
blk*trt                      6      1.48007663      0.24667944       0.38    0.8739
lot*trt                      4      5.81852246      1.45463062       2.26    0.1351


      Tests of Hypotheses Using the Type III MS for blk*lot as an Error Term

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

lot                          2     16.22982388      8.11491194      10.23    0.0117

=========================================================================================

SOD Analysis                                        13:54 Tuesday, September 11, 2012   3
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure

Dependent Variable: SOD   U/mg protein

      Tests of Hypotheses Using the Type III MS for blk*trt as an Error Term

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

trt                          2      5.75982780      2.87991390      11.67    0.0085

=========================================================================================

SOD Analysis                                        13:54 Tuesday, September 11, 2012   4
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure
Least Squares Means
```

```
                           Standard                     LSMEAN
trt      SOD LSMEAN           Error    Pr > |t|      Number

1        8.91083333      0.23172870      <.0001           1
2        8.03666667      0.23172870      <.0001           2
3        8.03922619      0.27696878      <.0001           3


      Least Squares Means for effect trt
     Pr > |t| for H0: LSMean(i)=LSMean(j)

           Dependent Variable: SOD

i/j             1             2             3

   1                     0.0236        0.0365
   2       0.0236                      0.9945
   3       0.0365        0.9945



==========================================================================================

SOD Analysis                                        13:54 Tuesday, September 11, 2012   5
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure
Least Squares Means

                           Standard                     LSMEAN
lot      SOD LSMEAN           Error    Pr > |t|      Number

1        9.30500000      0.23172870      <.0001           1
2        7.73082143      0.25981974      <.0001           2
3        7.95090476      0.25981974      <.0001           3


      Least Squares Means for effect lot
     Pr > |t| for H0: LSMean(i)=LSMean(j)

           Dependent Variable: SOD

i/j             1             2             3

   1                     0.0011        0.0030
   2       0.0011                      0.5689
   3       0.0030        0.5689
```

```
=========================================================================================

SOD Analysis                                        13:54 Tuesday, September 11, 2012   6
After 4 Day Grow-Out
Complete Analysis - After 4 Day Grow-Out

The GLM Procedure
Least Squares Means

                                  Standard                  LSMEAN
lot    trt      SOD LSMEAN           Error    Pr > |t|      Number

1      1        10.4275000       0.4013659      <.0001           1
1      2         8.6125000       0.4013659      <.0001           2
1      3         8.8750000       0.4013659      <.0001           3
2      1         7.5075000       0.4013659      <.0001           4
2      2         7.8450000       0.4013659      <.0001           5
2      3         7.8399643       0.5341980      <.0001           6
3      1         8.7975000       0.4013659      <.0001           7
3      2         7.6525000       0.4013659      <.0001           8
3      3         7.4027143       0.5341980      <.0001           9


                       Least Squares Means for effect lot*trt
                        Pr > |t| for H0: LSMean(i)=LSMean(j)

                               Dependent Variable: SOD

i/j         1        2        3        4        5        6        7        8        9

   1            0.0095   0.0210   0.0004   0.0011   0.0031   0.0166   0.0006   0.0011
   2   0.0095            0.6537   0.0802   0.2061   0.2745   0.7512   0.1217   0.1003
   3   0.0210   0.6537            0.0367   0.0997   0.1524   0.8941   0.0567   0.0521
   4   0.0004   0.0802   0.0367            0.5653   0.6295   0.0464   0.8035   0.8785
   5   0.0011   0.2061   0.0997   0.5653            0.9941   0.1243   0.7415   0.5230
   6   0.0031   0.2745   0.1524   0.6295   0.9941            0.1824   0.7848   0.5886
   7   0.0166   0.7512   0.8941   0.0464   0.1243   0.1824            0.0713   0.0634
   8   0.0006   0.1217   0.0567   0.8035   0.7415   0.7848   0.0713            0.7163
   9   0.0011   0.1003   0.0521   0.8785   0.5230   0.5886   0.0634   0.7163


=========================================================================================

SOD Analysis                                        13:54 Tuesday, September 11, 2012   7
21 Days After Planting
```

```
Complete Analysis - 21 Days After Planting

The GLM Procedure

    Class Level Information

Class         Levels    Values

blk                4    1 2 3 4

lot                3    1 2 3

trt                3    1 2 3


Number of Observations Read          36
Number of Observations Used          34

=========================================================================================

SOD Analysis                                   13:54 Tuesday, September 11, 2012   8
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure

Dependent Variable: SOD   U/mg protein

                                        Sum of
Source                      DF         Squares     Mean Square    F Value    Pr > F

Model                       23     46.78995703      2.03434596       1.24    0.3757

Error                       10     16.41981944      1.64198194

Corrected Total             33     63.20977647


R-Square     Coeff Var      Root MSE      SOD Mean

0.740233      14.04499      1.281398      9.123529


Source                      DF       Type I SS     Mean Square    F Value    Pr > F

blk                          3      3.55539869      1.18513290       0.72    0.5616
lot                          2     19.17694276      9.58847138       5.84    0.0209
blk*lot                      6      8.30733502      1.38455584       0.84    0.5646
trt                          2      1.10130583      0.55065292       0.34    0.7228
```

```
blk*trt                    6      4.69047194      0.78174532       0.48    0.8118
lot*trt                    4      9.95850278      2.48962569       1.52    0.2699


Source                    DF     Type III SS     Mean Square    F Value    Pr > F

blk                        3      3.55035660      1.18345220       0.72    0.5621
lot                        2     16.85503611      8.42751806       5.13    0.0293
blk*lot                    6      8.64255833      1.44042639       0.88    0.5441
trt                        2      0.95597417      0.47798708       0.29    0.7536
blk*trt                    6      4.66052917      0.77675486       0.47    0.8139
lot*trt                    4      9.95850278      2.48962569       1.52    0.2699


      Tests of Hypotheses Using the Type III MS for blk*lot as an Error Term

Source                    DF     Type III SS     Mean Square    F Value    Pr > F

lot                        2     16.85503611      8.42751806       5.85    0.0389

=========================================================================================

SOD Analysis                                 13:54 Tuesday, September 11, 2012   9
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure

Dependent Variable: SOD   U/mg protein

      Tests of Hypotheses Using the Type III MS for blk*trt as an Error Term

Source                    DF     Type III SS     Mean Square    F Value    Pr > F

trt                        2      0.95597417      0.47798708       0.62    0.5714

=========================================================================================

SOD Analysis                                 13:54 Tuesday, September 11, 2012  10
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure
Least Squares Means

                           Standard                    LSMEAN
trt      SOD LSMEAN           Error    Pr > |t|      Number

1        8.91000000      0.36990787      <.0001           1
```

```
2        9.30583333      0.36990787      <.0001           2
3        9.05041667      0.52312872      <.0001           3


      Least Squares Means for effect trt
     Pr > |t| for H0: LSMean(i)=LSMean(j)

           Dependent Variable: SOD

i/j              1             2             3

   1                      0.4667        0.8309
   2        0.4667                      0.6985
   3        0.8309        0.6985


NOTE: To ensure overall protection level, only probabilities associated with pre-planned
      comparisons should be used.

==========================================================================================

SOD Analysis                                   13:54 Tuesday, September 11, 2012  11
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure
Least Squares Means

                          Standard                  LSMEAN
lot      SOD LSMEAN          Error    Pr > |t|      Number

1        10.1208333      0.3699079      <.0001           1
2         8.4297222      0.4271328      <.0001           2
3         8.7156944      0.4271328      <.0001           3


      Least Squares Means for effect lot
     Pr > |t| for H0: LSMean(i)=LSMean(j)

           Dependent Variable: SOD

i/j              1             2             3

   1                      0.0135        0.0322
   2        0.0135                      0.6238
   3        0.0322        0.6238


NOTE: To ensure overall protection level, only probabilities associated with pre-planned
```

```
      comparisons should be used.

==========================================================================================

SOD Analysis                                        13:54 Tuesday, September 11, 2012  12
21 Days After Planting
Complete Analysis - 21 Days After Planting

The GLM Procedure
Least Squares Means

                                  Standard                  LSMEAN
lot    trt      SOD LSMEAN           Error    Pr > |t|      Number

1      1         9.5350000       0.6406992      <.0001           1
1      2        10.7550000       0.6406992      <.0001           2
1      3        10.0725000       0.6406992      <.0001           3
2      1         9.2775000       0.6406992      <.0001           4
2      2         7.9425000       0.6406992      <.0001           5
2      3         8.0691667       0.9060855      <.0001           6
3      1         7.9175000       0.6406992      <.0001           7
3      2         9.2200000       0.6406992      <.0001           8
3      3         9.0095833       0.9060855      <.0001           9


                       Least Squares Means for effect lot*trt
                        Pr > |t| for H0: LSMean(i)=LSMean(j)

                               Dependent Variable: SOD

i/j         1        2        3        4        5        6        7        8        9

   1            0.2079   0.5662   0.7821   0.1093   0.2160   0.1045   0.7353   0.6460
   2   0.2079            0.4687   0.1340   0.0112   0.0360   0.0107   0.1211   0.1468
   3   0.5662   0.4687            0.4009   0.0406   0.1012   0.0387   0.3689   0.3607
   4   0.7821   0.1340   0.4009            0.1714   0.3018   0.1643   0.9507   0.8141
   5   0.1093   0.0112   0.0406   0.1714            0.9114   0.9785   0.1889   0.3589
   6   0.2160   0.0360   0.1012   0.3018   0.9114            0.8940   0.3241   0.4166
   7   0.1045   0.0107   0.0387   0.1643   0.9785   0.8940            0.1811   0.3483
   8   0.7353   0.1211   0.3689   0.9507   0.1889   0.3241   0.1811            0.8534
   9   0.6460   0.1468   0.3607   0.8141   0.3589   0.4166   0.3483   0.8534
```

APPENDIX H: STATISTICAL ANALYSIS OF FREEZE INJURY OF ALL THREE TEMPERATURES AT POINT B

```
options nocenter ls=89 ps=51 pageno=1 frmdlim='=';
title 'Freezing Tolerance Analysis';
data A;
input blk lot trt temp injury;
label injury = '% Injury';
cards;
1      1      1      1      28.51
1      1      1      2      46.70
1      1      1      3      61.02
1      1      2      1      39.27
1      1      2      2      55.00
1      1      2      3      60.75
1      1      3      1      34.48
1      1      3      2      45.62
1      1      3      3      53.87
1      2      1      1      30.03
1      2      1      2      42.95
1      2      1      3      51.26
1      2      2      1      30.69
1      2      2      2      43.20
1      2      2      3      51.30
1      2      3      1      28.73
1      2      3      2      48.39
1      2      3      3      54.29
1      3      1      1      41.88
1      3      1      2      59.57
1      3      1      3      66.82
1      3      2      1      42.96
1      3      2      2      49.09
1      3      2      3      60.20
1      3      3      1      41.37
1      3      3      2      56.98
1      3      3      3      65.69
2      1      1      1      40.09
2      1      1      2      55.41
2      1      1      3      61.02
2      1      2      1      47.32
2      1      2      2      58.38
```

2	1	2	3	64.15
2	1	3	1	36.24
2	1	3	2	61.38
2	1	3	3	60.36
2	2	1	1	39.02
2	2	1	2	41.96
2	2	1	3	56.70
2	2	2	1	37.00
2	2	2	2	48.67
2	2	2	3	53.36
2	2	3	1	35.63
2	2	3	2	54.44
2	2	3	3	60.27
2	3	1	1	55.58
2	3	1	2	61.27
2	3	1	3	65.81
2	3	2	1	51.75
2	3	2	2	56.22
2	3	2	3	63.32
2	3	3	1	41.86
2	3	3	2	65.11
2	3	3	3	64.56
3	1	1	1	42.45
3	1	1	2	65.36
3	1	1	3	70.95
3	1	2	1	48.11
3	1	2	2	63.95
3	1	2	3	63.88
3	1	3	1	47.65
3	1	3	2	51.96
3	1	3	3	66.61
3	2	1	1	49.53
3	2	1	2	44.39
3	2	1	3	61.68
3	2	2	1	41.15
3	2	2	2	52.71
3	2	2	3	61.22
3	2	3	1	43.13
3	2	3	2	60.69

```
3     2     3     3     65.45
3     3     1     1     37.99
3     3     1     2     54.40
3     3     1     3     71.14
3     3     2     1     58.04
3     3     2     2     59.04
3     3     2     3     72.62
3     3     3     1     51.70
3     3     3     2     63.62
3     3     3     3     69.99
4     1     1     1     29.93
4     1     1     2     52.26
4     1     1     3     60.90
4     1     2     1     35.84
4     1     2     2     62.91
4     1     2     3     61.52
4     1     3     1     35.06
4     1     3     2     53.08
4     1     3     3     58.82
4     2     1     1     32.92
4     2     1     2     39.70
4     2     1     3     58.28
4     2     2     1     26.12
4     2     2     2     43.77
4     2     2     3     60.50
4     2     3     1     31.18
4     2     3     2     48.86
4     2     3     3     53.16
4     3     1     1     35.91
4     3     1     2     67.79
4     3     1     3     65.67
4     3     2     1     35.23
4     3     2     2     66.55
4     3     2     3     69.89
4     3     3     1     41.25
4     3     3     2     66.20
4     3     3     3     68.85
;
run;
```

```
title2 'RCBD Complete Analysis';
proc glm data=a;
class blk lot trt temp;
model injury = blk lot blk*lot trt blk*trt lot*trt blk*lot*trt temp blk*temp lot*temp trt*temp
blk*lot*temp blk*trt*temp lot*trt*temp;
test h=lot*trt e=blk*lot*trt;
test h=trt*temp e=blk*trt*temp;
test h=lot e=blk*lot;
test h=trt e=blk*trt;
test h=temp e=blk*temp;
lsmeans trt*temp / pdiff stderr;
lsmeans lot*trt*temp / pdiff stderr;
lsmeans lot*trt*temp / slice=lot*temp;
run;
```

```
=========================================================================================

Freezing Tolerance Analysis                         13:30 Tuesday, September 11, 2012   1
RCBD Complete Analysis

The GLM Procedure

    Class Level Information

Class         Levels    Values

blk                4    1 2 3 4

lot                3    1 2 3

trt                3    1 2 3

temp               3    1 2 3


Number of Observations Read         108
Number of Observations Used         108

=========================================================================================

Freezing Tolerance Analysis                         13:30 Tuesday, September 11, 2012   2
RCBD Complete Analysis

The GLM Procedure

Dependent Variable: injury   % Injury

                                        Sum of
Source                      DF         Squares     Mean Square    F Value    Pr > F

Model                       83     14785.96610       178.14417      16.01    <.0001

Error                       24       267.12841        11.13035

Corrected Total            107     15053.09451


R-Square     Coeff Var      Root MSE    injury Mean

0.982254      6.400918      3.336218       52.12093


Source                      DF       Type I SS     Mean Square    F Value    Pr > F
```

```
blk                          3     1258.546981      419.515660      37.69    <.0001
lot                          2     2044.456580     1022.228290      91.84    <.0001
blk*lot                      6      142.012880       23.668813       2.13    0.0874
trt                          2       37.431424       18.715712       1.68    0.2073
blk*trt                      6       23.885324        3.980887       0.36    0.8983
lot*trt                      4      189.957170       47.489293       4.27    0.0095
blk*lot*trt                 12      256.717748       21.393146       1.92    0.0837
temp                         2     9465.563785     4732.781893     425.21    <.0001
blk*temp                     6      415.668741       69.278123       6.22    0.0005
lot*temp                     4       95.970793       23.992698       2.16    0.1049
trt*temp                     4       94.038331       23.509583       2.11    0.1105
blk*lot*temp                12      219.463881       18.288657       1.64    0.1451
blk*trt*temp                12      245.840387       20.486699       1.84    0.0983
lot*trt*temp                 8      296.412074       37.051509       3.33    0.0105


Source                      DF     Type III SS     Mean Square    F Value    Pr > F

blk                          3     1258.546981      419.515660      37.69    <.0001
lot                          2     2044.456580     1022.228290      91.84    <.0001
blk*lot                      6      142.012880       23.668813       2.13    0.0874
trt                          2       37.431424       18.715712       1.68    0.2073
blk*trt                      6       23.885324        3.980887       0.36    0.8983
lot*trt                      4      189.957170       47.489293       4.27    0.0095
blk*lot*trt                 12      256.717748       21.393146       1.92    0.0837
temp                         2     9465.563785     4732.781893     425.21    <.0001
blk*temp                     6      415.668741       69.278123       6.22    0.0005

=========================================================================================

Freezing Tolerance Analysis                         13:30 Tuesday, September 11, 2012   3
RCBD Complete Analysis

The GLM Procedure

Dependent Variable: injury   % Injury

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

lot*temp                     4       95.970793       23.992698       2.16    0.1049
trt*temp                     4       94.038331       23.509583       2.11    0.1105
blk*lot*temp                12      219.463881       18.288657       1.64    0.1451
blk*trt*temp                12      245.840387       20.486699       1.84    0.0983
lot*trt*temp                 8      296.412074       37.051509       3.33    0.0105

    Tests of Hypotheses Using the Type III MS for blk*lot*trt as an Error Term
```

```
Source                      DF     Type III SS     Mean Square    F Value    Pr > F

lot*trt                      4     189.9571704      47.4892926       2.22    0.1280

    Tests of Hypotheses Using the Type III MS for blk*trt*temp as an Error Term

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

trt*temp                     4     94.03833148     23.50958287       1.15    0.3810

      Tests of Hypotheses Using the Type III MS for blk*lot as an Error Term

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

lot                          2     2044.456580     1022.228290      43.19    0.0003

      Tests of Hypotheses Using the Type III MS for blk*trt as an Error Term

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

trt                          2     37.43142407     18.71571204       4.70    0.0591

      Tests of Hypotheses Using the Type III MS for blk*temp as an Error Term

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

temp                         2     9465.563785     4732.781893      68.32    <.0001
=========================================================================================

Freezing Tolerance Analysis                         13:30 Tuesday, September 11, 2012   4
RCBD Complete Analysis

The GLM Procedure
Least Squares Means

                     injury        Standard                   LSMEAN
trt    temp          LSMEAN           Error    Pr > |t|      Number

1      1         38.6533333       0.9630832      <.0001           1
1      2         52.6466667       0.9630832      <.0001           2
1      3         62.6041667       0.9630832      <.0001           3
2      1         41.1233333       0.9630832      <.0001           4
2      2         54.9575000       0.9630832      <.0001           5
2      3         61.8925000       0.9630832      <.0001           6
```

```
3      1         39.0233333       0.9630832      <.0001           7
3      2         56.3608333       0.9630832      <.0001           8
3      3         61.8266667       0.9630832      <.0001           9


                       Least Squares Means for effect trt*temp
                        Pr > |t| for H0: LSMean(i)=LSMean(j)

                             Dependent Variable: injury

i/j         1        2        3        4        5        6        7        8        9

   1            <.0001   <.0001   0.0823   <.0001   <.0001   0.7882   <.0001   <.0001
   2   <.0001            <.0001   <.0001   0.1027   <.0001   <.0001   0.0118   <.0001
   3   <.0001   <.0001            <.0001   <.0001   0.6061   <.0001   0.0001   0.5734
   4   0.0823   <.0001   <.0001            <.0001   <.0001   0.1362   <.0001   <.0001
   5   <.0001   0.1027   <.0001   <.0001            <.0001   <.0001   0.3131   <.0001
   6   <.0001   <.0001   0.6061   <.0001   <.0001            <.0001   0.0005   0.9618
   7   0.7882   <.0001   <.0001   0.1362   <.0001   <.0001            <.0001   <.0001
   8   <.0001   0.0118   0.0001   <.0001   0.3131   0.0005   <.0001            0.0005
   9   <.0001   <.0001   0.5734   <.0001   <.0001   0.9618   <.0001   0.0005


NOTE: To ensure overall protection level, only probabilities associated with pre-planned
      comparisons should be used.

=========================================================================================

Freezing Tolerance Analysis                         13:30 Tuesday, September 11, 2012   5
RCBD Complete Analysis

The GLM Procedure
Least Squares Means

                            injury        Standard                    LSMEAN
lot    trt    temp          LSMEAN           Error    Pr > |t|      Number

1      1      1         35.2450000       1.6681090      <.0001           1
1      1      2         54.9325000       1.6681090      <.0001           2
1      1      3         63.4725000       1.6681090      <.0001           3
1      2      1         42.6350000       1.6681090      <.0001           4
1      2      2         60.0600000       1.6681090      <.0001           5
1      2      3         62.5750000       1.6681090      <.0001           6
1      3      1         38.3575000       1.6681090      <.0001           7
1      3      2         53.0100000       1.6681090      <.0001           8
1      3      3         59.9150000       1.6681090      <.0001           9
2      1      1         37.8750000       1.6681090      <.0001          10
2      1      2         42.2500000       1.6681090      <.0001          11
2      1      3         56.9800000       1.6681090      <.0001          12
```

```
2      2      1         33.7400000       1.6681090      <.0001          13
2      2      2         47.0875000       1.6681090      <.0001          14
2      2      3         56.5950000       1.6681090      <.0001          15
2      3      1         34.6675000       1.6681090      <.0001          16
2      3      2         53.0950000       1.6681090      <.0001          17
2      3      3         58.2925000       1.6681090      <.0001          18
3      1      1         42.8400000       1.6681090      <.0001          19
3      1      2         60.7575000       1.6681090      <.0001          20
3      1      3         67.3600000       1.6681090      <.0001          21
3      2      1         46.9950000       1.6681090      <.0001          22
3      2      2         57.7250000       1.6681090      <.0001          23
3      2      3         66.5075000       1.6681090      <.0001          24
3      3      1         44.0450000       1.6681090      <.0001          25
3      3      2         62.9775000       1.6681090      <.0001          26
3      3      3         67.2725000       1.6681090      <.0001          27



=========================================================================================

Freezing Tolerance Analysis                        13:30 Tuesday, September 11, 2012   6
RCBD Complete Analysis

The GLM Procedure
Least Squares Means

                     Least Squares Means for effect lot*trt*temp
                        Pr > |t| for H0: LSMean(i)=LSMean(j)

                             Dependent Variable: injury

i/j         1        2        3        4        5        6        7        8        9

   1          <.0001   <.0001   0.0045   <.0001   <.0001   0.1995   <.0001   <.0001
   2   <.0001            0.0014   <.0001   0.0398   0.0035   <.0001   0.4231   0.0453
   3   <.0001   0.0014            <.0001   0.1610   0.7070   <.0001   0.0002   0.1446
   4   0.0045   <.0001   <.0001            <.0001   <.0001   0.0823   0.0002   <.0001
   5   <.0001   0.0398   0.1610   <.0001            0.2970   <.0001   0.0064   0.9515
   6   <.0001   0.0035   0.7070   <.0001   0.2970            <.0001   0.0005   0.2707
   7   0.1995   <.0001   <.0001   0.0823   <.0001   <.0001            <.0001   <.0001
   8   <.0001   0.4231   0.0002   0.0002   0.0064   0.0005   <.0001            0.0074
   9   <.0001   0.0453   0.1446   <.0001   0.9515   0.2707   <.0001   0.0074
  10   0.2760   <.0001   <.0001   0.0549   <.0001   <.0001   0.8397   <.0001   <.0001
  11   0.0067   <.0001   <.0001   0.8717   <.0001   <.0001   0.1120   0.0001   <.0001
  12   <.0001   0.3940   0.0111   <.0001   0.2041   0.0261   <.0001   0.1054   0.2255
  13   0.5295   <.0001   <.0001   0.0009   <.0001   <.0001   0.0620   <.0001   <.0001
  14   <.0001   0.0028   <.0001   0.0713   <.0001   <.0001   0.0011   0.0192   <.0001
  15   <.0001   0.4878   0.0076   <.0001   0.1549   0.0182   <.0001   0.1417   0.1721
  16   0.8087   <.0001   <.0001   0.0025   <.0001   <.0001   0.1309   <.0001   <.0001
```

```
  17   <.0001   0.4436   0.0002   0.0002   0.0069   0.0005   <.0001   0.9716   0.0080
  18   <.0001   0.1672   0.0380   <.0001   0.4610   0.0820   <.0001   0.0347   0.4982
  19   0.0037   <.0001   <.0001   0.9315   <.0001   <.0001   0.0695   0.0002   <.0001
  20   <.0001   0.0210   0.2611   <.0001   0.7700   0.4486   <.0001   0.0031   0.7241
  21   <.0001   <.0001   0.1124   <.0001   0.0050   0.0538   <.0001   <.0001   0.0043
  22   <.0001   0.0026   <.0001   0.0769   <.0001   <.0001   0.0012   0.0176   <.0001
  23   <.0001   0.2481   0.0226   <.0001   0.3321   0.0508   <.0001   0.0571   0.3625
  24   <.0001   <.0001   0.2105   <.0001   0.0116   0.1085   <.0001   <.0001   0.0101
  25   0.0010   0.0001   <.0001   0.5556   <.0001   <.0001   0.0239   0.0009   <.0001
  26   <.0001   0.0023   0.8356   <.0001   0.2282   0.8660   <.0001   0.0003   0.2066
  27   <.0001   <.0001   0.1203   <.0001   0.0054   0.0580   <.0001   <.0001   0.0047

                     Least Squares Means for effect lot*trt*temp
                        Pr > |t| for H0: LSMean(i)=LSMean(j)

                             Dependent Variable: injury

i/j        10       11       12       13       14       15       16       17       18

   1   0.2760   0.0067   <.0001   0.5295   <.0001   <.0001   0.8087   <.0001   <.0001
   2   <.0001   <.0001   0.3940   <.0001   0.0028   0.4878   <.0001   0.4436   0.1672
   3   <.0001   <.0001   0.0111   <.0001   <.0001   0.0076   <.0001   0.0002   0.0380

=========================================================================================

Freezing Tolerance Analysis                         13:30 Tuesday, September 11, 2012   7
RCBD Complete Analysis

The GLM Procedure
Least Squares Means

                     Least Squares Means for effect lot*trt*temp
                        Pr > |t| for H0: LSMean(i)=LSMean(j)

                             Dependent Variable: injury

i/j        10       11       12       13       14       15       16       17       18

   4   0.0549   0.8717   <.0001   0.0009   0.0713   <.0001   0.0025   0.0002   <.0001
   5   <.0001   <.0001   0.2041   <.0001   <.0001   0.1549   <.0001   0.0069   0.4610
   6   <.0001   <.0001   0.0261   <.0001   <.0001   0.0182   <.0001   0.0005   0.0820
   7   0.8397   0.1120   <.0001   0.0620   0.0011   <.0001   0.1309   <.0001   <.0001
   8   <.0001   0.0001   0.1054   <.0001   0.0192   0.1417   <.0001   0.9716   0.0347
   9   <.0001   <.0001   0.2255   <.0001   <.0001   0.1721   <.0001   0.0080   0.4982
  10            0.0760   <.0001   0.0924   0.0007   <.0001   0.1866   <.0001   <.0001
  11   0.0760            <.0001   0.0014   0.0514   <.0001   0.0037   0.0001   <.0001
  12   <.0001   <.0001            <.0001   0.0003   0.8717   <.0001   0.1126   0.5831
  13   0.0924   0.0014   <.0001            <.0001   <.0001   0.6977   <.0001   <.0001
  14   0.0007   0.0514   0.0003   <.0001            0.0005   <.0001   0.0177   <.0001
```

```
  15   <.0001   <.0001   0.8717   <.0001   0.0005            <.0001   0.1509   0.4787
  16   0.1866   0.0037   <.0001   0.6977   <.0001   <.0001            <.0001   <.0001
  17   <.0001   0.0001   0.1126   <.0001   0.0177   0.1509   <.0001            0.0374
  18   <.0001   <.0001   0.5831   <.0001   <.0001   0.4787   <.0001   0.0374
  19   0.0460   0.8046   <.0001   0.0008   0.0844   <.0001   0.0020   0.0002   <.0001
  20   <.0001   <.0001   0.1224   <.0001   <.0001   0.0904   <.0001   0.0034   0.3065
  21   <.0001   <.0001   0.0002   <.0001   <.0001   0.0001   <.0001   <.0001   0.0008
  22   0.0007   0.0556   0.0003   <.0001   0.9690   0.0004   <.0001   0.0162   <.0001
  23   <.0001   <.0001   0.7549   <.0001   0.0001   0.6363   <.0001   0.0614   0.8119
  24   <.0001   <.0001   0.0005   <.0001   <.0001   0.0003   <.0001   <.0001   0.0019
  25   0.0152   0.4541   <.0001   0.0002   0.2094   <.0001   0.0006   0.0008   <.0001
  26   <.0001   <.0001   0.0179   <.0001   <.0001   0.0123   <.0001   0.0003   0.0586
  27   <.0001   <.0001   0.0002   <.0001   <.0001   0.0001   <.0001   <.0001   0.0009

                     Least Squares Means for effect lot*trt*temp
                        Pr > |t| for H0: LSMean(i)=LSMean(j)

                                Dependent Variable: injury

i/j        19       20       21       22       23       24       25       26       27

   1   0.0037   <.0001   <.0001   <.0001   <.0001   <.0001   0.0010   <.0001   <.0001
   2   <.0001   0.0210   <.0001   0.0026   0.2481   <.0001   0.0001   0.0023   <.0001
   3   <.0001   0.2611   0.1124   <.0001   0.0226   0.2105   <.0001   0.8356   0.1203
   4   0.9315   <.0001   <.0001   0.0769   <.0001   <.0001   0.5556   <.0001   <.0001
   5   <.0001   0.7700   0.0050   <.0001   0.3321   0.0116   <.0001   0.2282   0.0054
   6   <.0001   0.4486   0.0538   <.0001   0.0508   0.1085   <.0001   0.8660   0.0580

==========================================================================================

Freezing Tolerance Analysis                        13:30 Tuesday, September 11, 2012   8
RCBD Complete Analysis

The GLM Procedure
Least Squares Means

                     Least Squares Means for effect lot*trt*temp
                        Pr > |t| for H0: LSMean(i)=LSMean(j)

                                Dependent Variable: injury

i/j        19       20       21       22       23       24       25       26       27

   7   0.0695   <.0001   <.0001   0.0012   <.0001   <.0001   0.0239   <.0001   <.0001
   8   0.0002   0.0031   <.0001   0.0176   0.0571   <.0001   0.0009   0.0003   <.0001
   9   <.0001   0.7241   0.0043   <.0001   0.3625   0.0101   <.0001   0.2066   0.0047
  10   0.0460   <.0001   <.0001   0.0007   <.0001   <.0001   0.0152   <.0001   <.0001
  11   0.8046   <.0001   <.0001   0.0556   <.0001   <.0001   0.4541   <.0001   <.0001
  12   <.0001   0.1224   0.0002   0.0003   0.7549   0.0005   <.0001   0.0179   0.0002
```

```
  13   0.0008   <.0001   <.0001   <.0001   <.0001   <.0001   0.0002   <.0001   <.0001
  14   0.0844   <.0001   <.0001   0.9690   0.0001   <.0001   0.2094   <.0001   <.0001
  15   <.0001   0.0904   0.0001   0.0004   0.6363   0.0003   <.0001   0.0123   0.0001
  16   0.0020   <.0001   <.0001   <.0001   <.0001   <.0001   0.0006   <.0001   <.0001
  17   0.0002   0.0034   <.0001   0.0162   0.0614   <.0001   0.0008   0.0003   <.0001
  18   <.0001   0.3065   0.0008   <.0001   0.8119   0.0019   <.0001   0.0586   0.0009
  19            <.0001   <.0001   0.0909   <.0001   <.0001   0.6142   <.0001   <.0001
  20   <.0001            0.0100   <.0001   0.2109   0.0226   <.0001   0.3561   0.0109
  21   <.0001   0.0100            <.0001   0.0004   0.7210   <.0001   0.0755   0.9707
  22   0.0909   <.0001   <.0001            0.0001   <.0001   0.2232   <.0001   <.0001
  23   <.0001   0.2109   0.0004   0.0001            0.0011   <.0001   0.0356   0.0005
  24   <.0001   0.0226   0.7210   <.0001   0.0011            <.0001   0.1476   0.7485
  25   0.6142   <.0001   <.0001   0.2232   <.0001   <.0001            <.0001   <.0001
  26   <.0001   0.3561   0.0755   <.0001   0.0356   0.1476   <.0001            0.0812
  27   <.0001   0.0109   0.9707   <.0001   0.0005   0.7485   <.0001   0.0812


NOTE: To ensure overall protection level, only probabilities associated with pre-planned
      comparisons should be used.

=========================================================================================

Freezing Tolerance Analysis                         13:30 Tuesday, September 11, 2012   9
RCBD Complete Analysis

The GLM Procedure
Least Squares Means

                            injury
lot    trt    temp          LSMEAN

1      1      1         35.2450000
1      1      2         54.9325000
1      1      3         63.4725000
1      2      1         42.6350000
1      2      2         60.0600000
1      2      3         62.5750000
1      3      1         38.3575000
1      3      2         53.0100000
1      3      3         59.9150000
2      1      1         37.8750000
2      1      2         42.2500000
2      1      3         56.9800000
2      2      1         33.7400000
2      2      2         47.0875000
2      2      3         56.5950000
2      3      1         34.6675000
2      3      2         53.0950000
2      3      3         58.2925000
```

```
3      1      1         42.8400000
3      1      2         60.7575000
3      1      3         67.3600000
3      2      1         46.9950000
3      2      2         57.7250000
3      2      3         66.5075000
3      3      1         44.0450000
3      3      2         62.9775000
3      3      3         67.2725000

=========================================================================================

Freezing Tolerance Analysis                         13:30 Tuesday, September 11, 2012  10
RCBD Complete Analysis

The GLM Procedure
Least Squares Means

            lot*trt*temp Effect Sliced by lot*temp for injury

                               Sum of
lot    temp        DF         Squares     Mean Square    F Value    Pr > F

1      1            2      110.129017       55.064508       4.95    0.0159
1      2            2      106.253017       53.126508       4.77    0.0180
1      3            2       27.382550       13.691275       1.23    0.3100
2      1            2       37.662050       18.831025       1.69    0.2054
2      2            2      236.140650      118.070325      10.61    0.0005
2      3            2        6.336517        3.168258       0.28    0.7548
3      1            2       36.558067       18.279033       1.64    0.2146
3      2            2       55.617617       27.808808       2.50    0.1033
3      3            2        1.759517        0.879758       0.08    0.9242
```

APPENDIX I: STATISTICAL ANALYSIS OF FREEZE INJURY ON ONLY -1.5°C AT POINT B

```
options nocenter ls=89 ps=51 pageno=1 frmdlim='=';
title 'Freezing Tolerance Analysis';
title2 '-1.5 C Only';
data A;
input blk lot trt injury;
label injury = '% Injury';
cards;
1     1     1     46.70
1     1     2     55.00
1     1     3     45.62
1     2     1     42.95
1     2     2     43.20
1     2     3     48.39
1     3     1     59.57
1     3     2     49.09
1     3     3     56.98
2     1     1     55.41
2     1     2     58.38
2     1     3     61.38
2     2     1     41.96
2     2     2     48.67
2     2     3     54.44
2     3     1     61.27
2     3     2     56.22
2     3     3     65.11
3     1     1     65.36
3     1     2     63.95
3     1     3     51.96
3     2     1     44.39
3     2     2     52.71
3     2     3     60.69
3     3     1     54.40
3     3     2     59.04
3     3     3     63.62
4     1     1     52.26
4     1     2     62.91
4     1     3     53.08
4     2     1     39.70
4     2     2     43.77
```

```
4      2      3      48.86
4      3      1      67.79
4      3      2      66.55
4      3      3      66.20
;
run;
title3 'RCBD Complete Analysis';
proc glm data=a;
class blk lot trt;
model injury = blk lot blk*lot trt blk*trt lot*trt;
test h=lot e=blk*lot;
test h=trt e=blk*trt;
means trt / lsd;
means lot / lsd;
lsmeans lot*trt / pdiff stderr;
run;
```

```
==========================================================================================

Freezing Tolerance Analysis                          15:12 Thursday, November 1, 2012   1
-1.5 C Only
RCBD Complete Analysis

The GLM Procedure

    Class Level Information

Class         Levels    Values

blk                4    1 2 3 4

lot                3    1 2 3

trt                3    1 2 3


Number of Observations Read          36
Number of Observations Used          36

==========================================================================================

Freezing Tolerance Analysis                          15:12 Thursday, November 1, 2012   2
-1.5 C Only
RCBD Complete Analysis

The GLM Procedure

Dependent Variable: injury   % Injury

                                         Sum of
Source                      DF          Squares     Mean Square    F Value    Pr > F

Model                       23      2084.009044       90.609089       5.60    0.0017

Error                       12       194.126056       16.177171

Corrected Total             35      2278.135100


R-Square     Coeff Var      Root MSE    injury Mean

0.914787      7.359044      4.022085       54.65500


Source                      DF        Type I SS     Mean Square    F Value    Pr > F
```

```
blk                          3      306.962256      102.320752       6.33    0.0081
lot                          2     1048.033317      524.016658      32.39    <.0001
blk*lot                      6      258.826528       43.137755       2.67    0.0699
trt                          2       84.417317       42.208658       2.61    0.1146
blk*trt                      6       72.175661       12.029277       0.74    0.6256
lot*trt                      4      313.593967       78.398492       4.85    0.0147


Source                      DF     Type III SS     Mean Square    F Value    Pr > F

blk                          3      306.962256      102.320752       6.33    0.0081
lot                          2     1048.033317      524.016658      32.39    <.0001
blk*lot                      6      258.826528       43.137755       2.67    0.0699
trt                          2       84.417317       42.208658       2.61    0.1146
blk*trt                      6       72.175661       12.029277       0.74    0.6256
lot*trt                      4      313.593967       78.398492       4.85    0.0147


      Tests of Hypotheses Using the Type III MS for blk*lot as an Error Term

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

lot                          2     1048.033317      524.016658      12.15    0.0078

==========================================================================================

Freezing Tolerance Analysis                         15:12 Thursday, November 1, 2012   3
-1.5 C Only
RCBD Complete Analysis

The GLM Procedure

Dependent Variable: injury   % Injury

      Tests of Hypotheses Using the Type III MS for blk*trt as an Error Term

Source                      DF     Type III SS     Mean Square    F Value    Pr > F

trt                          2     84.41731667     42.20865833       3.51    0.0979

==========================================================================================

Freezing Tolerance Analysis                         15:12 Thursday, November 1, 2012   4
-1.5 C Only
RCBD Complete Analysis

The GLM Procedure
```

```
t Tests (LSD) for injury

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.


Alpha                             0.05
Error Degrees of Freedom            12
Error Mean Square             16.17717
Critical Value of t            2.17881
Least Significant Difference    3.5776


Means with the same letter are not significantly different.



               Mean      N    trt

     A       56.361     12    3
     A
B    A       54.958     12    2
B
B            52.647     12    1

=========================================================================================

Freezing Tolerance Analysis                          15:12 Thursday, November 1, 2012   5
-1.5 C Only
RCBD Complete Analysis

The GLM Procedure

t Tests (LSD) for injury

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.


Alpha                             0.05
Error Degrees of Freedom            12
Error Mean Square             16.17717
Critical Value of t            2.17881
Least Significant Difference    3.5776


Means with the same letter are not significantly different.
```

```
          Mean      N    lot

A        60.487     12    3

B        56.001     12    1

C        47.478     12    2

=========================================================================================

Freezing Tolerance Analysis                          15:12 Thursday, November 1, 2012   6
-1.5 C Only
RCBD Complete Analysis

The GLM Procedure
Least Squares Means

                    injury        Standard                LSMEAN
lot    trt          LSMEAN           Error    Pr > |t|      Number

1      1        54.9325000       2.0110427      <.0001           1
1      2        60.0600000       2.0110427      <.0001           2
1      3        53.0100000       2.0110427      <.0001           3
2      1        42.2500000       2.0110427      <.0001           4
2      2        47.0875000       2.0110427      <.0001           5
2      3        53.0950000       2.0110427      <.0001           6
3      1        60.7575000       2.0110427      <.0001           7
3      2        57.7250000       2.0110427      <.0001           8
3      3        62.9775000       2.0110427      <.0001           9


                       Least Squares Means for effect lot*trt
                        Pr > |t| for H0: LSMean(i)=LSMean(j)

                             Dependent Variable: injury

i/j         1        2        3        4        5        6        7        8        9

   1            0.0966   0.5119   0.0008   0.0173   0.5304   0.0631   0.3455   0.0152
   2   0.0966            0.0290   <.0001   0.0007   0.0307   0.8104   0.4276   0.3252
   3   0.5119   0.0290            0.0026   0.0594   0.9766   0.0185   0.1232   0.0043
   4   0.0008   <.0001   0.0026            0.1147   0.0025   <.0001   0.0001   <.0001
   5   0.0173   0.0007   0.0594   0.1147            0.0563   0.0004   0.0028   0.0001
   6   0.5304   0.0307   0.9766   0.0025   0.0563            0.0195   0.1295   0.0046
   7   0.0631   0.8104   0.0185   <.0001   0.0004   0.0195            0.3073   0.4502
   8   0.3455   0.4276   0.1232   0.0001   0.0028   0.1295   0.3073            0.0896
   9   0.0152   0.3252   0.0043   <.0001   0.0001   0.0046   0.4502   0.0896
```

APPENDIX J: STATISTICAL ANALYSIS OF SEEDLING GERMINATION

```
options nocenter ls=89 ps=51 pageno=1 frmdlim='=';
title 'Standard Germination Analysis';
data A;
input blk lot trt germ;
label germ = '% Normal Germination';
cards;
1      1      1      100
1      1      2      100
1      1      3      96
1      2      1      100
1      2      2      96
1      2      3      94
1      3      1      98
1      3      2      98
1      3      3      96
2      1      1      98
2      1      2      94
2      1      3      96
2      2      1      100
2      2      2      96
2      2      3      98
2      3      1      96
2      3      2      98
2      3      3      96
3      1      1      98
3      1      2      96
3      1      3      96
3      2      1      100
3      2      2      98
3      2      3      96
3      3      1      98
3      3      2      98
3      3      3      96
4      1      1      98
4      1      2      94
4      1      3      96
4      2      1      98
4      2      2      96
```

```
4       2       3      96
4       3       1      98
4       3       2      96
4       3       3      96
;
run;
title2 'RCBD Complete Analysis';
proc anova data=a;
class blk lot trt;
model germ = blk lot blk*lot trt blk*trt lot*trt;
test h=lot e=blk*lot;
test h=trt e=blk*trt;
means trt / lsd;
means lot / lsd;
run;
```

```
=========================================================================================

Standard Germination Analysis                           11:12 Sunday, August 5, 2012   1
RCBD Complete Analysis

The ANOVA Procedure

    Class Level Information

Class         Levels    Values

blk                4    1 2 3 4

lot                3    1 2 3

trt                3    1 2 3


Number of Observations Read          36
Number of Observations Used          36

=========================================================================================

Standard Germination Analysis                           11:12 Sunday, August 5, 2012   2
RCBD Complete Analysis

The ANOVA Procedure

Dependent Variable: germ   % Normal Germination
```

```
                                     Sum of
Source                      DF          Squares     Mean Square    F Value    Pr > F

Model                       23      87.00000000      3.78260870       3.52    0.0137

Error                       12      12.88888889      1.07407407

Corrected Total             35      99.88888889


R-Square     Coeff Lot      Root MSE     germ Mean

0.870968      1.067817      1.036375      97.05556


Source                      DF        Anova SS     Mean Square    F Value    Pr > F

blk                          3      6.55555556      2.18518519       2.03    0.1628
lot                          2      1.55555556      0.77777778       0.72    0.5048
blk*lot                      6     14.44444444      2.40740741       2.24    0.1103
trt                          2     40.22222222     20.11111111      18.72    0.0002
blk*trt                      6     13.11111111      2.18518519       2.03    0.1390
lot*trt                      4     11.11111111      2.77777778       2.59    0.0906


        Tests of Hypotheses Using the Anova MS for blk*lot as an Error Term

Source                      DF        Anova SS     Mean Square    F Value    Pr > F

lot                          2      1.55555556      0.77777778       0.32    0.7358


        Tests of Hypotheses Using the Anova MS for blk*trt as an Error Term

Source                      DF        Anova SS     Mean Square    F Value    Pr > F

trt                          2     40.22222222     20.11111111       9.20    0.0149

Standard Germination Analysis                           11:12 Sunday, August 5, 2012   3
RCBD Complete Analysis

The ANOVA Procedure

t Tests (LSD) for germ

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.
```

```
Alpha                           0.05
Error Degrees of Freedom          12
Error Mean Square           1.074074
Critical Value of t          2.17881
Least Significant Difference   0.9219


Means with the same letter are not significantly different.


           Mean      N    trt

A       98.5000     12    1

B       96.6667     12    2

B       96.0000     12    3

Standard Germination Analysis                            11:12 Sunday, August 5, 2012   4
RCBD Complete Analysis

The ANOVA Procedure

t Tests (LSD) for germ

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.


Alpha                           0.05
Error Degrees of Freedom          12
Error Mean Square           1.074074
Critical Value of t          2.17881
Least Significant Difference   0.9219


Means with the same letter are not significantly different.


           Mean      N    lot

A       97.3333     12    2

A       97.0000     12    3

A       96.8333     12    1
```

APPENDIX K: STATISTICAL ANALYSIS OF SEEDLING COLD TEST RESULTS

```
options nocenter ls=89 ps=51 pageno=1 frmdlim='=';
title 'Standard Germination Analysis';
data A;
input blk lot trt germ;
label germ = '% Vigor';
cards;
1     1     1     96
1     1     2     94
1     1     3     93
1     2     1     96
1     2     2     95
1     2     3     96
1     3     1     91
1     3     2     92
1     3     3     91
2     1     1     92
2     1     2     90
2     1     3     89
2     2     1     95
2     2     2     94
2     2     3     92
2     3     1     88
2     3     2     85
2     3     3     87
3     1     1     92
3     1     2     89
3     1     3     87
3     2     1     95
3     2     2     90
3     2     3     90
3     3     1     88
3     3     2     86
3     3     3     86
4     1     1     91
4     1     2     88
4     1     3     87
4     2     1     94
4     2     2     92
```

```
4      2      3      90
4      3      1      90
4      3      2      85
4      3      3      86
;
run;
title2 'RCBD Complete Analysis';
proc anova data=a;
class blk lot trt;
model germ = blk lot blk*lot trt blk*trt lot*trt;
test h=lot e=blk*lot;
test h=trt e=blk*trt;
means trt / lsd;
means lot / lsd;
run;
```

```
==========================================================================================

Standard Germination Analysis                           11:20 Sunday, August 5, 2012   1
RCBD Complete Analysis

The ANOVA Procedure

    Class Level Information

Class         Levels    Values

blk                4    1 2 3 4

lot                3    1 2 3

trt                3    1 2 3


Number of Observations Read          36
Number of Observations Used          36

==========================================================================================

Standard Germination Analysis                           11:20 Sunday, August 5, 2012   2
RCBD Complete Analysis

The ANOVA Procedure

Dependent Variable: germ   % Vigor

                                        Sum of
Source                      DF         Squares     Mean Square    F Value    Pr > F

Model                       23     377.0555556      16.3937198      17.11    <.0001

Error                       12      11.5000000       0.9583333

Corrected Total             35     388.5555556


R-Square     Coeff Lot      Root MSE     germ Mean

0.970403      1.080381      0.978945      90.61111


Source                      DF        Anova SS     Mean Square    F Value    Pr > F

blk                          3     126.3333333      42.1111111      43.94    <.0001
lot                          2     170.7222222      85.3611111      89.07    <.0001
```

```
blk*lot                     6       8.8333333       1.4722222       1.54    0.2478
trt                         2      54.8888889      27.4444444      28.64    <.0001
blk*trt                     6      11.3333333       1.8888889       1.97    0.1494
lot*trt                     4       4.9444444       1.2361111       1.29    0.3280


        Tests of Hypotheses Using the Anova MS for blk*lot as an Error Term

Source                     DF        Anova SS     Mean Square    F Value    Pr > F

lot                         2     170.7222222      85.3611111      57.98    0.0001


        Tests of Hypotheses Using the Anova MS for blk*trt as an Error Term

Source                     DF        Anova SS     Mean Square    F Value    Pr > F

trt                         2     54.88888889     27.44444444      14.53    0.0050

=========================================================================================

Standard Germination Analysis                              11:20 Sunday, August 5, 2012   3
RCBD Complete Analysis

The ANOVA Procedure

t Tests (LSD) for germ

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.


Alpha                            0.05
Error Degrees of Freedom           12
Error Mean Square            0.958333
Critical Value of t           2.17881
Least Significant Difference   0.8708


Means with the same letter are not significantly different.


          Mean      N    trt

A       92.3333     12    1

B       90.0000     12    2

B       89.5000     12    3
```

```
=========================================================================================

Standard Germination Analysis                           11:20 Sunday, August 5, 2012   4
RCBD Complete Analysis

The ANOVA Procedure

t Tests (LSD) for germ

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise
error rate.


Alpha                              0.05
Error Degrees of Freedom             12
Error Mean Square              0.958333
Critical Value of t             2.17881
Least Significant Difference     0.8708


Means with the same letter are not significantly different.


         Mean      N    lot

A       93.2500     12    2

B       90.6667     12    1

C       87.9167     12    3
```

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