DEVELOPMENT AND EVALUATION OF A GREENHOUSE TESTING PROCEDURE FOR COLD-HARDINESS IN HARD RED WINTER WHEAT

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Experiments were conducted to develop a greenhouse test for comparing the cold tolerance of winter wheat genotypes. Winter-survival is a complex trait and in field trials it has low heritability, consequently, selection will be facilitated by a dependable early generation test. Such a preliminary test of the ability of seedlings to survive subzero temperatures was evaluated and improved upon. The finalized version was then used to compare varieties in a duplicated greenhouse trial. The winter-survival of the same varieties have also been evaluated in field variety trials conducted independently by either this project or Ducks Unlimited. Finally, a selection experiment was done to further test the method. Only the first greenhouse trial correlated well with the Ducks Unlimited field trial data (more dependable data set). The selection trial showed no selection response. It appears that the test could be very useful following further modification to make it more robust.

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1. INTRODUCTION

Recently, North Dakota State University reinstated their winter wheat breeding program. Spring wheat is the number one cash crop in North Dakota and with improved cultural practices winter wheat can also be grown statewide. Hard red winter wheat accounts for 3-10% of the total wheat acreage in North Dakota (North Dakota Wheat Commission, 2014). The state of North Dakota is divided into four different areas of winterkill risk (USDA, 2012) and the level of cold-hardiness required of varieties that are planted in the coldest zones must equal that of Canadian winter wheat varieties.

The new varieties that need to be developed must have high levels of winter-hardiness. Differential winterkill is a prerequisite to effective selection among winter wheat genotypes in field survival trials (Fowler, 1979). Seasonal variation in winter conditions and cold exposure combined with the multi-faceted nature of winter survival makes this a lowly heritable trait. Early generation selection for cold hardiness (a component of winter survival) in breeding programs can therefore be a very useful breeding tool. Controlled-freeze tests on plants that have been cold acclimated in controlled environments have been used to measure low temperature tolerance. However, such tests have not been shown to be more advantageous than field evaluations (Thomas et al., 1988).

This project will aim to develop and evaluate a greenhouse testing procedure for cold hardiness of winter wheat that can also be applied to single plants. This will then be tested on segregating generations of specific cross combinations in an attempt to select plants with higher levels of cold hardiness. This project is different from previous studies on the winter-hardiness of winter wheat in that it will be exposing single seedlings to subzero temperatures at a very early stage of development.

2. LITERATURE REVIEW

Wheat (*Triticum aestivum* L.) is a grass species cultivated worldwide for its seed and is annually among the top three most produced crops in the world, along with maize and rice (FAOSTAT, 2013). Wheat is very important to the world's economy. Many foods come from the grain produced by wheat: breads, pastas, cakes, cookies, noodles, cereals, etc.

There are two different kinds of red winter wheat, i.e. hard red winter wheat and soft red winter wheat. Hard red winter wheat has hard, vitreous kernels and high-protein content and is used for bread, hard baked goods and as an adjunct in other flours to increase protein, for example in pastry flour for pie crusts. Soft red winter wheat is a soft endosperm and low-protein wheat that is used for cakes, pie crusts, biscuits, muffins, and self-rising flour (National Association of Wheat Growers, 2013).

Winter crop seedlings must survive numerous winter stresses. In order for them to survive the harsh winters, they must have an adequate level of winter-hardiness for the region in which they grow in. Winter-hardiness in a winter crop not only allows it to be grown during the winter, but also allows it to mature later and have a longer growing season. The injuries that a plant endures during the winter have been a subject of interest and importance to plant breeders and agriculturists for many years. Winter survival is defined by Blum (1988) as "The final integrated plant response to a multitude of stresses involved during and after freezing stress, including both external-physical and biotic stresses" (Sãulescu et al., 2001). Overwintering is made possible by two mechanisms, namely, the need for vernalization and the ability to withstand freezing temperatures (winter-hardiness); however, the relationship between the two processes is still not fully understood (Limin and Fowler, 2006).

Freezing is a major environmental stress, inflicting economic damage on crops and limiting the distribution of both wild and crop species. Therefore, understanding the effects of freezing and how it damages plants is of extensive practical importance. Improvements in imaging methods are providing a precise description of the initiation and spread of ice formation in plant tissue and of the nature of damage (Pearce, 2001). The development of winter-hardy genotypes is a primary tool for combating injury from freezing temperatures.

Winter-hardiness is the ability of a plant to withstand temperatures of 1 to -10°C for a long period of time. Winter-hardiness is different from frost resistance, which is the resistance to sub-zero temperatures and is usually associated with extreme exposure to cold conditions during early spring or during flowering. While winter-hardy wheat can survive subzero temperatures, the capacity to withstand subzero temperatures is not constitutive. A period of exposure to low, non-freezing temperatures are required in order to obtain freezing tolerance. This is why a plant's winter-hardiness has three stages: acclimation, mid-winter-hardiness, and de-acclimation. Plants cannot fully acclimate until the temperature drops well below the induction threshold, and the rate of acclimation is inversely proportional to temperature (Fowler, 2008; Ganeshan et al., 2008, 2009). The capacity to acquire freezing tolerance is closely associated with a requirement for vernalization, and maximum freezing tolerance is attained when plants are fully vernalized. Acclimation is a process triggered late in the fall by shorter days and a decrease in temperature. When the temperature gradually falls over an extended period of time, the plants have the ability to respond by initiating a series of physiological and biochemical changes in order to protect the plant from potential damage. This results in a more winter hardy crop. The second stage (mid-winter-hardiness) is defined as the lowest temperature in which a plant can survive without injury, after it has gone through the acclimation process, while reaching its maximum level of

winter-hardiness. The final stage is de-acclimation which refers to the decrease in winter-hardiness of plant tissue in response to the warming temperatures that occurs in late winter and early spring. The suitability of a cultivar for a particular climate depends not only on its maximum mid-winter-hardiness level, but also on the timing and rates of acclimation and de-acclimation in response to environmental cues (Fowler and Gusta, 1979).

Plants can be grouped into three different classes according to their low-temperature tolerance (Stushnoff et al. 1984). The first group includes frost tender plants that are sensitive to chilling injury and can be killed by short periods of exposure to temperatures just below freezing. They cannot tolerate ice in their tissues and readily exhibit frost injury symptoms that include a water soaked flaccid appearance with loss of turgor followed by rapid drying upon exposure to warm temperatures. Beans, corn, rice, and tomatoes are examples of plants in this category (Stushnoff et al. 1984). Low-temperature acclimation of plants in the second group allows them to tolerate the presence of extracellular ice in their tissues. Their frost resistance ranges from the broad-leafed summer annuals, which are killed at temperatures slightly below freezing, to perennial grasses that can survive exposure to -40^{o}C. As temperatures decrease the outward migration of intracellular water to the growing extracellular ice crystal causes dehydration stress that will eventually result in irreversible damage to the plasma membrane, which is the primary site of low-temperature injury. If ice nucleation does not occur at -3 to -5^{o}C, supercooling may result in intracellular freezing and death of individual cells (Stushnoff et al. 1984). The final group is made up of very cold hardy plants that are predominantly temperate woody species. Like the plants in the previous group, their lower limits of cold tolerance are dependent on the stage of acclimation, the rate and degree of temperature decline, and the genetic capability of tissues to accommodate extracellular freezing and the accompanying dehydration stress. Deep

supercooling allows certain tissues in plants from this group to survive low temperatures without the formation of extracellular ice. However, the most cold hardy species do not rely on supercooling and can withstand temperatures of -196 ºC (Stushnoff et al. 1984, Fowler et al. 2014).

The reasons for winterkill in wheat, as well as the extent of the damage, vary greatly from region to region and from year to year (Sãulescu et al., 2001). The main factors causing winterkill (alone or in combination) are related to low temperature per se (such as extreme air or soil temperatures below the critical temperature of a particular wheat cultivar); inadequate hardening, due to late emergence in autumn or a sudden drop in temperature; long periods of cold-induced desiccation (Gusta et al., 1997b); prolonged periods of low sub-zero temperatures; in particular, mid-winter temperatures below -15ºC result in the rapid loss of winter-hardiness (Gusta et al., 1997a); alternate freezing and thawing, which causes increased injury from ice crystal growth with each freeze (Olien, 1969).

Another factor responsible for winterkill is ice encasement, a major cause of plant death in areas of high rainfall and fluctuating temperatures during winter (Andrews et al., 1974). Ice has high thermal conductivity and can aggravate the effect of low temperatures. It also has low gas permeability and may, in extreme cases, smother or suffocate plants by depriving them of oxygen (Poltarev et al., 1992). Finally, low temperatures or snow can cause indirect damage through: frost heaving due to the formation of ice in the soil. The ice pushes the plants upward, breaking and exposing the roots; snow mold, caused by fungi in areas with long-lasting snow cover. The most damaging fungus affecting winter survival is pink snow mold (*Microdochium nivale* (Fries) Samuel and Hallet), previously known as *Fusarium nivale* (Fr) Ces. (Hömmö, 1994). Although *Microdochium nivale* cannot survive freezing, it is tolerant to low temperatures

and severely damages plants in the 0-5ºC temperature range. Other, less important fungi causing snow mold are *Typhula* spp., the pathogen for speckled snow mold or typhula blight, and *Sclerotinia borealis*, which causes sclerotinia snow mold (Hömmö, 1994).

Winter crops are planted from early to late fall depending on the region of the Northern Hemisphere where it is planted. They are planted in the fall so that the seeds can begin sprouting before freezing occurs. The apical meristem should be below the surface before the plant becomes dormant, to avoid being killed off during the winter. Once the soil temperature increases in the spring, the plant will break dormancy. The plant needs several weeks of cold temperatures before being able to flower; this is called vernalization (Fowler and Gusta, 1979).

Vernalization and photoperiod requirements have a major influence on plant development. Temperature and light are important abiotic stimuli that provide plants with diurnal and seasonal cues which enable them to adapt to environmental change. The autumn to winter decline in temperature and light that occurs in temperate regions act as cues enabling plants to anticipate the change in season and consequently prepare for the arrival of freezing temperatures by inducing or enhancing cold stress tolerance mechanisms (Winfield et al., 2010). Most cereal crops are daylight sensitive, which is when the day length affects apical development and the production of leaves and other developmental processes. Short-day conditions can be used to extend the vegetative phase which causes an increase in the number of leaves and delays the reproductive phase (Mahfoozi et al., 2001).

The plant goes through vernalization in order to prevent the transition to the reproductive phase in regions with cold winters. Once the vernalization requirement has been fulfilled the vegetative phase ceases. Winter crops gradually lose their ability to tolerate below-freezing

temperatures even when they are maintained at temperatures in the optimum range for low temperature acclimation (Mahfoozi et al., 2001).

Wheat has over 450 genes that are regulated by cold. Although these genes have been identified on the basis of their response to a cold stimulus, in many cases their specific function has not been discovered and their role in cold acclimation, if any, remains unknown. However, there are a good number of cold-regulated genes that have been assigned specific functions either as transcription factors that act up-stream in cold acclimation or as effector molecules that act to counter the potential damaging effects of cold stress (Winfield et al., 2010).

At least two genes control differences in winter wheat with respect to the length of the vernalization period (Mokanu and Fayt, 2008). The symbol *Vrd* (from the initial letters of the English words, vernalization requirement duration) was proposed to denote these genes (Stelmakh et al., 2005). The genes are called *Vrd1* and *Vrd2*. The *Vrd1* gene has been localized in chromosome 4A and the *Vrd2* gene in chromosome 5D. A possible third gene may be involved in the control of differences with respect to the duration of the vernalization period, localized on either chromosome 1A, 6A, or 4B (Fayt et al., 2007). The *Vrd1* gene induces heading in winter wheat plants following 20 to 35 days of vernalization, while the *Vrd2* gene induces heading following 40 to 45 days of vernalization. Genotypes, in which recessive alleles of both genes are present, form ears following 50 to 60 days of vernalization (Fayt, 2003). Winter wheat reaches its peak for low temperature tolerance between 42 and 49 days of vernalization. This is around the time that vernalization saturation occurs and marks the end of the vegetative phase. This can be measured by counting the final leaf number (FLN), which is used to determine intervals throughout the acclimation period to measure vernalization, the lower the FLN the longer the vernalization period (Fayt, 2003).

Once a winter wheat plant breaks dormancy in the spring, it can rejuvenate from undamaged crown tissue. If the crown tissue has been damaged at some point during winter the plant will die. Damage to the crown tissue is called winter-kill. This happens when the temperature of the soil fell below the minimum survival temperature. For that reason, the temperature at which the plant's crown is exposed is a critical factor that determines winter survival. The crown of the plant is normally located less than five centimeters below the soil surface. In order for the crown tissue to survive the freezing temperatures of the winter, it relies on warmth from the soil. In order to keep the heat in the soil, a protective layer of snow is required. As little as five centimeters of snow-cover greatly reduces the winter-hardiness required to survive the freezing temperatures; however, winter-kill can still occur (Fowler and Limin, 1997).

The lower limit of freezing tolerance of a plant population is measured as LT_{50}, the lethal temperature for 50% of the individuals. In single plants, LT_{50} is often determined as the loss of 50% of the electrolytes from plant tissues after freezing. As plants acclimate to low temperatures in autumn, they acquire freezing tolerance and the LT_{50} becomes progressively lower. In breeding programs, plants are often selected for increased freezing tolerance based on changes in LT_{50}; however, any progressive change that occurs during cold acclimation is correlated with the acquisition of freezing tolerance. Antifreeze proteins accumulate in the apoplast of winter cereals during cold acclimation as the plants acquire freezing tolerance, and directly influence the freezing process (Griffith et al., 2004).

Plant antifreeze proteins are unusual proteins: they have multiple, hydrophilic ice-binding domains that appear to function as inhibitors of ice recrystallization and ice nucleation (Griffith et al., 2004). Antifreeze activity was first reported in plants in 1992. Antifreeze activity is present

in overwintering plants only after they have been exposed to low temperatures and only in plants that tolerate the presence of ice in their tissues. Antifreeze activity has been observed in different parts of overwintering plants, including seeds, stems, crowns, branches, buds, petioles, leaf blades, flowers, and roots.

Low temperature affects water and nutrient uptake, membrane fluidity and protein and nucleic acid conformation, and dramatically influences cellular metabolism either directly through the reduction in the rate of biochemical reactions or indirectly through the large-scale reprogramming of gene expression (Winfield et al., 2010). A large number of genes with small effects and complex interactions determine the phenotypic expression of low temperature tolerance (Fowler and Limin, 1997). As a majority of these genes belong to the Late Embryogenesis–Abundant (LEA) family that commonly encode highly hydrophilic proteins, they are usually referred to as Cold-Responsive (COR)/LEA genes or simply COR genes. A positive correlation exists between the levels of COR gene expression and that of freezing tolerance (Winfield et al., 2010). It is a complex trait that involves freeze tolerance, desiccation, anoxia, ice-encasement, resistance to diseases, etc. Freeze tolerance has been considered as the primary limiting factor in most production regions (Galiba et al., 2008).

Winter-hardiness is a heritable and complex quantitative trait that is strongly influenced by environment (Fowler and Limin, 1997). Winter wheat that is developing winter-hardiness is able to adjust to the low, but above freezing, temperatures. This ability is accumulated over time and involves the fusion, or increased buildup, of particular subsets of proteins in plants. When plants freeze at subzero temperatures, they form ice in the intercellular spaces, xylem vessels, and tracheids. Freezing injury usually arises from cellular dehydration as intracellular water is lost to the growing extracellular ice. Injury can also occur when plants are frozen for prolonged

periods because ice undergoes a spontaneous process known as recrystallization, in which ice crystals coalesce to minimize their surface area. Although an insulating blanket of snow is protective against low temperatures, it can also cause problems by maintaining ambient temperatures near the melting point of plant tissues where ice recrystallizes quickly to form physically damaging masses of ice (Griffith et al., 2004).

With the major consequence of cold stress being dehydration and osmotic stress, several of the COR genes are dehydrins. Dehydrins are a distinct biochemical group of LEA proteins (known as LEA D-11 or LEA II) characterized by the presence of a lysine-rich amino acid motif, the K-segment. They are highly hydrophilic, soluble upon boiling and rich in glycine and polar amino acids. Their expression is induced by various environmental factors—heat, drought, salinity— that cause cellular dehydration. Extreme cold and frost can also lead to osmotic stress, and it has been shown that the induction and accumulation of dehydrins is an important part of the cold acclimation apparatus of cultivars of the winter cereals (Winfield et al., 2010).

Dehydrins have been linked to the *Wcs120* gene family of hexaploid wheat. When these proteins were detected in hexaploid wheat with an antibody produced against the protein encoded by the *Wcs120* gene, levels of all the recognized proteins were much higher in the more cold-tolerant lines (Limin et al., 1997). Therefore, the more Wcs120 proteins a plant processes, the greater the winter-hardiness.

The well-characterized wheat cold-specific (*WCS120*) gene family belongs to the Cor/Lea superfamily. The *WCS120* protein family members share homology with the Lea D11 dehydrins (Winfield et al., 2010). Members of the *WCS120* family of proteins are thought to play a significant role in frost tolerance because of their higher induction in winter-hardy compared to tender spring wheat plants. There is a close correlation between the degree of freezing tolerance

and the accumulation of *WCS120* wheat protein. The corresponding antibody discriminates between frost-resistant and frost susceptible wheat cultivars (Houde et al., 1992). Therefore this protein can be used as a molecular marker to select for freezing tolerance (Sãulescu et al., 2001).

Even though there are winter-hardiness indicators, controlled-freeze testing, and molecular markers, almost all of the winter wheat breeding programs still consider field testing as the final evaluation for winter-hardiness potential (Fowler and Limin, 1997). Some of the reasons why field testing is still the preferred method is because it is simple, inexpensive, does not require access to specialized facilities, and does not rely on cooperating programs with conflicting priorities. Some of the negatives about field testing are: complete winterkill or lack of it; it can only be done once per year, not every winter provides the same environment, and variations in amount of snow cover, soil moisture, soil fertility, disease, desiccation, soil heaving, smothering, and insect damage may affect results. These can make it difficult to identify small, but important differences among cultivars. Studies conducted under extreme winter conditions in western Canada showed that genotype by environment interaction for winter-hardiness is minute when compared to the error that is associated with individual measurements (Fowler et al., 1981).

Most screening methods used in wheat breeding programs are direct, i.e., they are based on exposing plants or seedlings to controlled freezing in artificial climate facilities, such as freezing cabinets, growth chambers, etc. However, there are indirect methods in which plants are not exposed to freezing; instead, their freezing tolerance is estimated based on biochemical changes induced by hardening or on the presence of molecular markers associated with genes involved in controlling winter-hardiness (Sãulescu et al., 2001).

Several alternative means of hardening can be employed, depending on the breeding requirements. Natural hardening, which better reflects the situation in farmers' fields is frequently used. Many years of testing are needed to characterize freezing tolerance of a genotype using this method. An "average hardening" regime, representative of most years in the area can be simulated. Various hardening regimes are used by different testing programs; including striving for the maximum level of hardening, corresponding to "potential freezing tolerance" or "static freezing resistance" (Sãulescu et al., 2001).

Skinner and Mackey (2009) performed a freezing survival test with plants that were grown in planting trays. Seeds from ten lines and five parent populations were planted in each planting tray. Plants were germinated and grown in a growth chamber under cool, white fluorescent lights with a 16 hour photoperiod until the seedlings reached the three-leaf stage. Relative humidity was not controlled. The plants were then transferred to 4°C with a 16 hours photoperiod for 35 days to induce cold acclimation prior to freezing survival tests. The plants were irrigated weekly with nutrient solution containing macro and micronutrients. Just prior to freezing, plants were counted, the trays were drenched with ice water and allowed to drain until drainage had essentially ceased, a layer of crushed ice was placed on the soil surface to nucleate ice formation, and freezing was carried out in a programmable freezer. The temperature of the plant growth medium in each tray was monitored using food piercing temperature probes and an internet-enabled temperature monitor was placed near the crown of the plants. The temperature was recorded every two minutes using a data capture script running on a remote computer. The temperature in the freezer was reduced from 4°C to the target temperature over an 8 hour period. Each population was exposed in separate trials to target temperatures of -10 and -12°C. The temperature was held at the target temperature for two hours then raised to 0°C over eight hours.

Each population was frozen to each target temperature twice at different times. Following freezing, the plants were held at 4°C for 24 hours, then moved to a greenhouse. Survival was scored as the proportion of plants that had regrown after five weeks.

Skinner and Mackey (2009) concluded that significant differences in abilities to survive freezing in saturated soil were found among wheat lines and their progeny populations. In part, these differences were due to differential abilities to tolerate changes in specific components of the freezing process. The genetic control of the ability to tolerate freezing in saturated soil appeared to be complex and included complementary gene action that favored increased freezing sensitivity. Nonetheless, it may be possible to improve the ability to tolerate freezing in saturated soil by genetically combining the abilities to survive various components of the freezing process.

Two microarray-based studies of wheat that were done by Winfield et al. (2010) elucidated the effect of low temperature on transcriptome reprogramming in two winter varieties (Harnesk and Solstice) and a spring variety (Paragon). In the 'cold-shock experiment', plants were rapidly transferred from 16 to 4° C and held for two days. Two days of exposure were chosen because it has been reported that many COR genes accumulate maximally within this period. In a second 'cold acclimation' experiment, designed to mimic a natural autumn to winter transition, plants were exposed to a gradual decline in temperature and light (quality and day length) over several weeks. Winfield et al.(2010) pointed out that a weakness of the majority of research to date is that it has been based on responses to rapid, dramatic changes in temperature that do not in any way represent conditions found in nature. In such studies, plants have been directly transferred from favorable conditions for active growth (c. 20°C) and placed at low, nonfreezing temperatures (usually 4 or 2°C). Their cold-shock experiment was of this kind and permitted them to make comparisons with the results from other such studies. The changes

observed under such conditions are unlikely to truly reflect those that occur when plants experience a gradual decline in light and temperature more typical of the change from autumn to winter (Winfield et al., 2010). In the cold-shock experiment, the authors saw high levels of induction of some of the early light-inducible proteins (ELIPs) that might have been interpreted as a cold response given no other information. However, when plants were exposed to a slow decline in temperature and light, little or no response was seen from these genes. Experimental design, therefore, is fundamentally important in being able to identify candidate cold-responsive genes. A great deal of attention has been paid to the events occurring when plants are exposed to a rapid fall in temperature. Much less attention has been directed towards the elucidation of the molecular mechanisms underlying responses to gradual changes in ambient temperature that might be more representative of the conditions experienced during a typical autumn—winter progression.

Using young seedlings has the advantage of reducing test duration and the amount of soil needed, but, as differential survival of seedlings is more difficult to obtain, evaluation is usually based on leaf damage (Sãulescu et al., 2001). Larsson (1986) found a very good correlation between seedling leaf damage and field winter-hardiness.

According to Sãulescu et al. (2001) most screening methods use freezing cabinets with controlled air temperature. However, to achieve better temperature control, Jenkins et al. (1974) used a refrigerated bath with ethylene glycol, in which pots with plants were immersed. Most authors recommend a gradual decrease in temperature (by 1-3ºC/hour), but direct exposure to the test temperature can also be used (Dencic et al., 1997; Tischner et al., 1997).

Difficulty in reproducing cold acclimation conditions severely limits the resolution of controlled-freeze tests that employ a single minimum (test) temperature (Sãulescu et al., 2001).

Therefore, it is best to use of a series of test temperatures, usually separated by 2ºC intervals, to determine the LT_{50} (Fowler et al., 1997).

Cold tolerance of winter cereals is reduced by prolonged exposure to sub-lethal temperatures and, consequently, both minimum temperature and exposure time are important variables in controlled-freeze test procedures. For economic reasons, most methods prefer shorter exposures to lower temperatures, but longer exposures might be advantageous if thermal inertia is large or if freezing cabinets have limitations in reaching lower temperatures (Sãulescu et al., 2001).

There are small variations among methods for the recovery procedures. Most authors recommend a gradual increase in temperature until thawing, followed by a 2-3 week recovery period at 15-22ºC (Sãulescu et al., 2001).

After the recovery period, freezing damage is usually assessed either by plant survival counts or by visually scoring leaf damage. However, such indices are to some extent subjective, have high experimental errors, and involve a considerable delay between freezing and survival assessment (Sãulescu et al., 2001).

These suggest that the main factor limiting selection for winter-hardiness is inefficient measurement of the differences for this trait. When selecting for winter-hardiness four questions must be considered (Fowler et al., 1981): 1) Do fully acclimated plants from genotypes with a range of winter-hardiness potential show significant differences for the character considered? 2) Are measurements of these characters sufficiently repeatable to allow for their use in prediction tests? 3) Are differences detected in these characters heritable? and, 4) What is the relationship of the differences in field survival? In addition to these four questions, the results of the test for

winter-hardiness should be able to distinguish among genotypes with a difference of less than 15% in Field Survival Index (FSI).

According to Limin and Fowler (1991) there appears to be three important factors interacting to determine cold hardiness in plants: (1) within and between various species there exist genes conferring different degrees of cold hardiness; (2) there appears to be a gene dosage effect; and (3) superimposed upon both the quantity and quality of cold-hardiness-conferring genes is the effect of cell size.

To date, no plant has been reported to have constitutive expression of antifreeze proteins; rather all studies have shown that transcripts and translation products of antifreeze protein genes accumulate during cold acclimation. The conditions used for cold acclimation mimic autumn when days become shorter and colder. Therefore, low temperature and day length are important environmental cues for antifreeze protein production (Griffith et al., 2004). However, Ganeshan et al. (2008) clearly showed that cold-responsive genes are differentially expressed between different tissues (crown and leaf) and pointed out that analyzing only the changes that occur in a single tissue will provide an incomplete picture of the events taking place in cold-treated plants. What is more, in winter cereals, it has been shown that whole-plant survival is dependent on the survival of specific tissues within the crown. The crown contains the meristematic regions from which all other tissues arise. The mature leaf tissue may die back after suffering cold damage, but the immature, meristematic tissue of the crown must survive to re-establish growth when permissive conditions return (Winfield et al., 2010).

Many scientists have tried to avoid problems related to direct freezing of plants (expensive freezing cabinets, high experimental error) by suggesting indirect methods that estimate the level of hardening instead of freezing damage (Sãulescu et al., 2001). Water content

in plants is reduced during hardening, especially in hardier genotypes. Water content after hardening was found to be correlated with winter survival (Fowler et al., 1981).

Winter wheat production in many areas is limited due to the freezing temperatures. Higher yields can be achieved by improving the winter-hardiness of winter wheat (Winfield et al., 2010). The inability of plant breeders to increase the maximum level of winter-hardiness in this century strongly suggests that all of the available winter-hardiness genes had previously been concentrated in hardy landraces of the winter cereal species (Fowler and Limin, 1997). Thus, even with all of the advances that were made in understanding the genetics behind winter crops, it has not been possible to produce super-hardy varieties. However, attempts to develop super-hardy varieties should continue, as they are the key to the expansion of winter crop production to the regions that require a level of winter-hardiness that exceeds that which was found in the land races that were selected by early farmers.

3. MATERIALS AND METHODS

This study was done to develop and evaluate an early-generation (seedling-based) screening test for cold-hardiness of wheat. The first experiments aimed to formulate and implement a test procedure. Over the course of the study this initial test procedure was further optimized as deemed necessary and following additional experimentation. In order to evaluate the ability of the test to identify cold-tolerant genotypes, two types of evaluation were done: Firstly, a set of genotypes was evaluated for cold-survival making use of the new test. Winter survival under field conditions of the same set of genotypes was then measured and the outcomes were compared. Secondly, two F_2 populations segregating for cold-hardiness were subjected to early-generation seedling selection over two generations. Upon completion of selection, the selected progenies were compared to unselected control populations.

3.1 Seedling cold tolerance test – its initial development

The first protocol was planned based on the results of O'Connor et al. (1993) and that of Mahfoozi et al. (2001).

Plastic planting trays containing six (6cm x 6cm x 8cm) cells were filled evenly with Sunshine LC1 professional growing mix and the peat mixture compacted leaving it 2 cm from the top of the cell. The peat mixture was saturated with water. The seeds were planted approximately 2 cm deep; covered with the peat mixture and lightly watered again to let the soil settle around the seed. The trays were left at room temperature for between one to four days, watered again and placed in a walk-in vernalization chamber with controlled temperature (4^{o}C), dimmed lighting, and air-flow. The trays remained in the vernalization chamber for a total of 56 days at 4°C. In some versions of the screening test the cold exposure was done after the first three weeks of vernalization; in other cases the cold exposure was postponed until after 56 days.

The cold test chamber is a programmable bench top chamber used to create low temperature extremes. Five minimum temperature programs, which were -11, -13, -15, -17, and -19°C were programmed into the chamber and used at different times during the study. Each program started with a twelve hour period (pre-freezing treatment) at -3°C. After this twelve hour soak, the temperature fell at a linear rate of 2°C per hour until the desired minimum temperature was reached. The time that it takes for the chamber to reach that minimum temperature ranged from four hours for -11°C to six hours for -15 °C and eight hours for -19°C. Once the minimum target temperature was reached, the samples remained at that temperature for twenty minutes. Next the program raised the temperature to 4°C over a one hour period. Once the cold test chamber reached 4°C, it remained there for twenty minutes. After the twenty minutes the chamber ended the program, and the samples were retrieved.

From the above it can be seen that the severity of a cold exposure treatment was not only determined by the minimum associated temperature but also by the duration of exposure to temperatures at and below -11°C.

Following the cold treatment the trays were either moved back to the vernalization chamber (three week vernalization) or a greenhouse (eight week vernalization). The greenhouse temperature was not controlled. The trays were fertilized with a 20-20-20 water soluble fertilizer. Over the next three weeks the surviving seedlings were evaluated and scored for viability and growth.

3.2. Preliminary evaluation of the test procedure

The purpose of this trial was to gain a first impression of how well the test might work; determine if the range of cold stress programs decided upon will differentiate winter-hardy and winter-sensitive genotypes; and finally, determine if the duration of seedling growth before the

onset of vernalization will affect the outcome of the test. This trial did not involve a full vernalization period – instead, the seedlings were only vernalized for three weeks which is believed to allow for full cold acclimation (Mahfoozi et al., 2001). The trial run for the cold test involved a split-pot (randomized block) experiment with two replications. Three minimum temperature programs (-11, -13, -15 °C) constituted the whole plot treatments. Each whole plot contained 12 subplots. The subplots were combinations of a variety X germination period. There were three varieties: Alsen (a spring wheat with very poor winter-hardiness), Overland (intermediate winter-hardiness) and Jerry (good winter-hardiness) and four germination periods (1, 2, 3, and 4 days of germination at room temperature prior to moving the trays to a vernalization chamber). Each subplot consisted of 16 cells and a single seed was planted in each cell. A pot marker was placed in each cell to identify the specific temperature X variety X replication combination.

The seedlings were evaluated three weeks after removal from the vernalization chamber. They were evaluated on an arbitrarily devised scale of 1-10 to evaluate the overall growth. With one being cold-sensitive and 10 being cold-tolerant.

3.3. A preliminary evaluation of twelve winter wheat varieties

Following the trial run, a broader range of winter wheat genotypes was evaluated in order to further refine the test conditions and to also determine whether differential profiles of a genotype's response to a range of cold treatments could be obtained. Twelve hard red winter wheat varieties that ranged from having poor winter-hardiness to excellent winter-hardiness were used. The varieties were Peregrine (excellent winter-hardiness), Norstar (excellent winter-hardiness), Jerry (good winter-hardiness), Radiant (good winter-hardiness), Overland (average winter-hardiness), Buteo (average winter-hardiness), Art (poor winter-hardiness), RCATL33

(unknown winter-hardiness), RCATTF203/2 (unknown winter-hardiness), Falcon (average winter-hardiness), Accipiter (excellent winter-hardiness), and Ideal (average winter-hardiness) (Dvorak, 2014).

A split-plot (randomized block) trial with three replications was used. The whole plot treatments consisted of five minimum temperature programs (-11, -13, -15, -17, -19°C). Each whole plot contained twelve subplots, which represented the twelve hard red winter wheat varieties. Each subplot consisted of eight cells, in each of which a single seed was planted, for a total of 96 cells in each whole plot. A pot marker was placed in each cell to identify the specific temperature X variety X replication combination.

The trays were left at room temperature for one day before being put in the vernalization chamber for 56 days. After the 56 day vernalization period the seedlings were put through one of the five minimum temperature programs. Once the cold treatment has been completed the seedlings were put in the vernalization chamber to thaw overnight. Next, the trays were moved to the greenhouse and fertilized with a 20-20-20 water soluble fertilizer. After three weeks the seedlings went through the evaluation procedure. This experiment was repeated in order to assess the repeatability of the experiment.

3.4. Evaluation of a first test modification: Timing of the low temperature exposure

When it appeared that the differences in survival of cold-sensitive and cold-tolerant genotypes were less consistent following vernalization for 56 days, it was decided to test whether results can be improved by doing the cold exposure treatment at an earlier stage (after three weeks). A split-plot (randomized block) trial with three replications was used. The whole plot treatments consisted of four minimum temperature programs (-11, -13, -15, -17°C). Each whole plot contained six subplots, which represented the six hard red winter wheat varieties (Jerry,

Decade (average winter-hardiness), Peregrine, Art, Hawken (poor winter-hardiness), and Glenn (spring wheat variety with very poor winter-hardiness) (Dvorak, 2014) within a replicate. Each subplot consisted of eight cells, in which a single seed was planted, for a total of 48 cells/seeds in each whole plot. A pot marker was placed in each cell to identify the specific temperature X variety X replication combination.

The trays were planted and left at room temperature for 24 hours, watered again and placed in a vernalization chamber where they remained for 21 days at 4°C. After 21 days, the trays were moved directly into the cold test chamber.

Following cold treatment the trays were returned to the vernalization chamber for 34 days, for a total of 56 days of vernalization and acclimation. The trays were then moved to the greenhouse and fertilized with a 20-20-20 water soluble fertilizer. After three weeks the seedlings went through the evaluation procedure.

3.5. Evaluation of a second test modification: Defining the size of the test seeds

The purpose of this experiment was to determine the extent to which variation in seed size of genotypes can distort differences in their cold tolerance, and whether it will be necessary to make an adjustment for this. Three varieties (Peregrine, Overland, and Art) were chosen based on their levels of winter-hardiness. A bulk seed sample of each was sieved for two minutes using Tyler sieves #7, #9, and #12 (openings 2.95 mm, 2.24 mm, and 1.65 mm, respectively). A split-plot (randomized block) trial with three replications was used. The whole plot treatments consisted of three minimum temperature programs (-11, -13, -15°C). Each whole plot contained nine subplots, which represented the three seed sizes of each of the three hard red winter wheat varieties within a replicate. Each subplot consisted of 10 cells, in which a single seed was planted, for a total of 90 cells/seeds in each whole plot. A pot marker was placed in each cell to

identify the specific temperature X variety-seed size X replication combination. This experiment followed the same procedure as the previous experiment (cold exposure done after three weeks; vernalization continued for a total of 56 days).

A germination test was done to determine the viability of the three seed sizes for each of the three hard red winter wheat varieties. Two petri dishes were used per seed size; 50 seeds were germinated per petri dish for a total of 100 seeds per seed size. The petri dishes were lined with filter paper and wetted. The seeds were placed directly onto the wet filter paper. Next the petri dishes were stored in re-sealable zipper storage bags at room temperature for 72 hours. After 72 and 120 hours the seeds that germinated were removed and counted.

3.6. The final revised testing procedure for cold-hardiness

Following the initial testing and modification of the screening protocol, the finalized procedure was as follows:

1. Fill plastic trays (6 cm X 6 cm X 8 cm pockets) with a peat mixture
2. Compact it leaving the mixture 2cm from the top of the cell
3. Plant the seeds approximately 2 cm deep
4. Cover the seed with the peat mixture and water lightly to let the peat settle around the seed
5. Saturate the peat with 20-20-20 water soluble fertilizer
6. Put the trays in a vernalization chamber for 21 days at 4°C
7. Remove trays from vernalization after 21 days and expose them to the appropriate minimum temperature program in a programmable cold chamber
8. Return the trays to 4°C to complete the remaining 35 days of a total of 56 days of vernalization

9. Move the trays to a greenhouse for three weeks with standard fertilization
10. Record the number of seedlings that have died for each entry (freeze-kill)
11. Evaluate the surviving seedlings by making one or more of the following measurements:
    a. Cut the seedlings close to the soil surface and record the total wet biomass (grams)
    b. Measure the height of the tallest tiller (cm)
    c. Count the number of seedlings that died as a result of freeze exposure

3.7. Validation of the cold-tolerance screening test – Comparison with field survival data

In order to make sure that the newly developed cold test does in fact differentiate wheat genotypes in terms of their cold tolerance, follow-up experiments were done. The first confirmation attempts involved measuring the field survival of the entries in the Hard Red Winter Wheat Variety Trials that were grown in North Dakota in 2013 and 2014, respectively. The average field survival of the varieties was then compared to the average performances of the same varieties in the seedling screening test.

2013 Hard Red Winter Wheat Variety Trial and Multiplication Plots: The entries that were involved are listed in Table 3.1. Field data were collected from the variety trials planted at Prosper, ND (Dr. Joel Ransom, NDSU Plant Sciences, Fargo, ND 58108) and from un-replicated seed multiplication strips planted at Casselton, ND (Dr. Francois Marais, NDSU Plant Sciences, Fargo, ND 58108).

2014 Hard Red Winter Wheat Variety Trial: The entries of this trial (Table 3.2) were evaluated at Prosper, ND (Dr. Joel Ransom) and Langdon, ND (Mr. John Lukach, Langdon Research Extension Center, 9280 107th Ave. N.E. Langdon, ND 58249).

The hard red winter wheat variety trial at Prosper, ND in 2013 and 2014 was a split-split plot design. The trial was planted with 17.78 cm rows and seven rows per plot. In 2013 there were six replications per variety and in 2014 there were three replications per variety. The seeds were planted at a density of 1.2 million seeds per acre.

The hard red winter wheat variety multiplication strips planted at Casselton, ND in 2013 were un-replicated plots that were 54.86 m with 17.78 cm rows, seven rows per plot and planted at 1.2 million seeds per acre. Each plot was split into six equal sized areas and measurements were made within each. The average count served as a single un-replicated observation for that variety.

The hard red winter wheat variety trial at Langdon, ND in 2014 was a split-split plot design. The trial was planted with 15.24 cm rows, seven rows per plot, and four replications per variety. The seeds were planted at a density of 1.2 million seeds per acre.

For the 2013 Hard Red Winter Wheat Variety Trial and un-replicated multiplication strips, stand counts were taken in November 2012 and in April 2013. A random stretch of 60.96 cm was measured out in a random row within each plot, and the seedlings within that distance were counted. For the 2014 Hard Red Winter Wheat Variety Trial, a stand assessment was made in early May 2014, in which a 1m x 1m square was used to demarcate a random area within each plot for counting the seedlings.

These measurements were subjected to analyses of variance and the results were used to compare the winterkill in the field trials with the data from seedling cold tests.

Table 3.1. Entries of the 2013 Hard Red Winter Wheat Variety Trial.

Variety	Winter-hardiness[1]	Source
Boomer	Good	North Dakota
Robidoux	Poor	Nebraska-Wyoming
Ideal	Intermediate	South Dakota
NI08708[a]	Poor	Nebraska
NE06545[a]	Intermediate	Nebraska
McGill	Intermediate	Nebraska
Moats	Good	Saskatchewan
Sunrise	Good	Saskatchewan
Lyman	Intermediate	South Dakota
Jerry	Excellent	North Dakota
AC Broadview	Good	Alberta
AC Emerson	Good	Canada
Flourish	Intermediate	Alberta
WB Grainfield	Intermediate	North Dakota
WB Matlock	Good	North Dakota
SY Wolf	Poor	North Dakota
Overland	Intermediate	Nebraska
Art	Poor	Kansas
Falcon	Intermediate	Saskatchewan
TX09D1037[b]	Poor	Texas
Peregrine[b]	Excellent	Saskatchewan
Decade[b]	Intermediate	North Dakota-Montana
Norstar[c]	Excellent	Alberta

[a]Included in the field trial, but not in the greenhouse trial due to seed un-availability at time of testing.

[b]Not included in the field trial, but used as parents in validation of the cold-tolerance screening test.

[c]Not included in the field trial, but included in the greenhouse trial for its excellent winter-hardiness.

[1]Winter-hardiness rating from Dvorak (2014).

Table 3.2. Entries of the 2014 Hard Red Winter Wheat Variety Trial.

Variety	Winter-hardiness[1]	Source
Robidoux	Poor	Nebraska-Wyoming
Ideal	Intermediate	South Dakota
McGill	Intermediate	Nebraska
Moats	Good	Saskatchewan
Lyman	Intermediate	South Dakota
Jerry	Excellent	North Dakota
AC Broadview	Good	Alberta
Flourish	Intermediate	Alberta
WB Grainfield	Intermediate	North Dakota
WB Matlock	Good	North Dakota
SY Wolf	Poor	North Dakota
Overland	Intermediate	Nebraska
Art	Poor	Kansas
Falcon	Intermediate	Saskatchewan

[1]Winter-hardiness rating from Dvorak (2014).

3.8. Validation of the cold-tolerance screening test – Selection of two early generation populations

The second validation attempt was in the form of a selection experiment (Fig. 3.1). Two F_2 populations were utilized. Cross 12K618 = TX09D1037 (poor winter-hardiness)/Decade (good winter-hardiness) was selected using the -13°C program. Cross 11K135 = Overland (intermediate winter-hardiness)/Peregrine (excellent winter-hardiness) (Dvorak, 2014) was selected using the -15°C program. In each population 500 seeds were evaluated and the best plants selected (10%) in each of two generations (Fig. 3.1). At the same time, in each population an unselected control group (500 plants) was maintained (a random 10% progressed to the next generation) over the same period. In the end the selected and unselected populations were compared to determine whether genetic progress was made.

The F_2: The top 10% surviving seedlings (50 plants) from the F_2 population and 10% of the unselected bulk (50 random plants) were retained to produce F_3 populations. Of each selected

plant, ten random F_3 seeds were used to establish the next generation. The F_3 seeds of the 50 random plants were bulked.

The F_3: Within the selected group, the best 10% of the F_3 were again selected. For the control set of F_3, 50 random plants were planted.

The F_4: An experiment was done to compare the selected and control populations. Two randomized complete block trials with six replications each were used. Two minimum temperature programs (-13°C for 12K618 and -15°C for 11K135) were employed. In each experiment, the seven populations (unselected F_2, unselected F_3, selected F_3, unselected F_4, selected F_4, and both parents) were included within a replicate. Each replicate X population combination consisted of sixteen cells, in each of which a single seed was planted, for a total of 96 cells (seeds) per population. The finalized cold-hardiness test procedure summarized in section 3.6 was used to evaluate cold-hardiness.

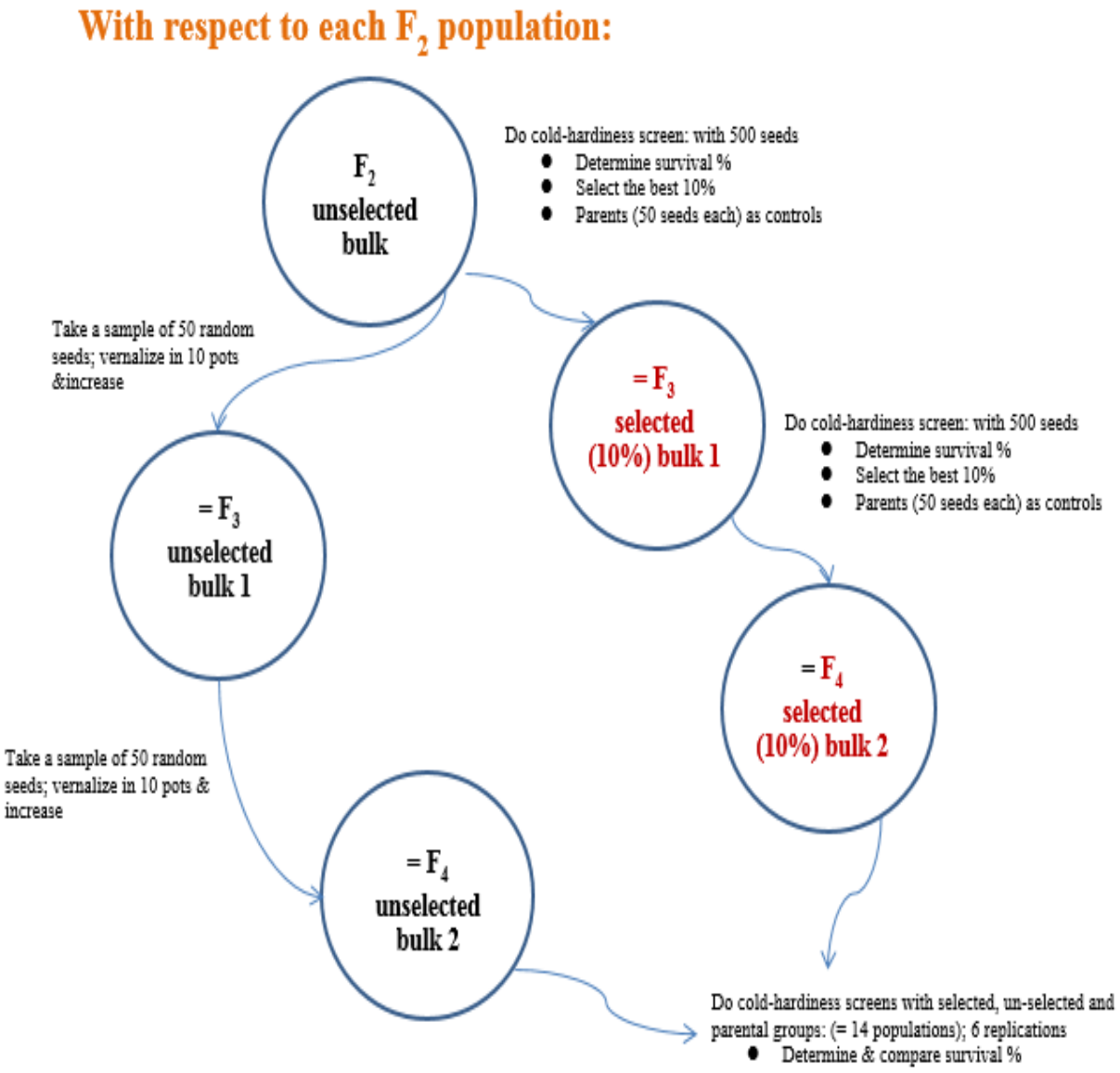

Figure 3.1. The scheme that was used to select for cold-tolerance in the F_2 and F_3 of two hybrid populations. The populations were 12K618 = TX09D1037/Decade (tested at -13ºC) and 11K135 = Overland/Peregrine (tested at -15ºC).

3.9. Statistical analyses

Statistical Analysis System (SAS) software provided by the SAS Institute Inc. (Cary, NC) through the North Dakota State University Plant Sciences Department license agreement was

used to perform the analyses of variance and to calculate correlations. Levels of significance were calculated at 1 and 5%.

4. RESULTS AND DISCUSSION

4.1. Initial development and preliminary evaluation of a seedling cold tolerance test

The first experiment aimed to (a) provide an initial indication of the effectiveness of the cold hardiness test and (b) to determine how long a germination period the seeds need before they can be put into the vernalization chamber. The varieties that were tested (at -11, -13 and -15^{o}C) were Alsen, Jerry, and Overland. The germination times that were used were 1-4 days. Following vernalization for 21 days the seedlings were removed from the chamber and subjected to the cold test. Since it was only a trial run, a full vernalization (56 days total) was not done. The treated seedlings were then allowed to grow for three weeks before they were evaluated using an arbitrarily devised scale of 1-10 to evaluate the overall growth; with one being cold-sensitive and 10 being cold-tolerant.

Since no seedlings survived in many of the variety X temperature treatment combinations, especially at the more severe temperatures, the data were not amenable to statistical analysis. At the lower temperatures, the means and variation about the means were small, whereas at the milder temperatures the sizes of the means and variances about the means were generally high. Thus, the error variances would have varied with the treatments that were applied and could not be averaged for mean comparison. Instead of doing statistical analyses, the means were simply derived for each treatment and the data summarized (Table 4.1).

Table 4.1. Mean seedling cold survival scores for three varieties evaluated at three freezing temperatures and four germination periods (2 replications, 16 plants per replicate).

Variety and germination period	Temperature		
	-11° C	-13° C	-15° C
Alsen 1 Day	2.1	1.0	1.0
Alsen 2 Days	2.9	1.0	1.0
Alsen 3 Days	1.0	2.1	1.0
Alsen 4 Days	1.0	1.0	1.0
Jerry 1 Day	7.8	3.1	6.8
Jerry 2 Days	8.1	7.4	7.1
Jerry 3 Days	8.3	7.9	5.5
Jerry 4 Days	8.9	7.2	6.3
Overland 1 Day	8.0	4.9	4.6
Overland 2 Days	8.4	4.6	3.9
Overland 3 Days	8.6	4.7	1.6
Overland 4 Days	6.3	6.1	3.3

The results of Table 4.1 show that the germination period didn't have a clear-cut effect on the cold hardiness of the plants and that a one day germination period will suffice. Apart from an outlying data point for Jerry (which can be ascribed to experimental error), Alsen, Jerry, and Overland reacted as expected when subjected to the cold testing procedure. Alsen performed the worst with 85% of the seedlings being killed at -11°C, 98% at -13°C, and 100% at -15°C. Jerry performed the best with 5% of seedlings killed at -11°C, 25% at -13°C, and 50% at -15°C. Overland was intermediate with 15% of the plants killed at -11°C, 35% killed at -13°C, and 60% killed at -15°C. These responses plus average ratings of the surviving plants in each treatment were in agreement with the known winter-hardiness of these varieties (Dvorak, 2014) and suggested that the preliminary test can be developed into an effective screening procedure.

During the evaluation of the seedlings using the arbitrary scale, it proved to be difficult to score intermediate plants that tillered more yet were shorter relative to plants that grew taller yet tillered less. As a consequence, it was decided that future evaluations may be more reliably done by simply measuring the length of the primary tiller of each surviving seedling, weighing the

freshly-cut (above ground) seedling biomass and counting the number of seedlings that get killed by the treatment (freeze-kill). The *primary shoot length* represents the average height of surviving seedlings after three weeks of greenhouse recovery and therefore does not take into account seedlings that had died and could therefore be a less consistent and informative measurement. *Seedling biomass* is the mass of the surviving plants at the end of three weeks (recovery) and therefore incorporates both freeze-survival and plant recovery. A potential disadvantage of this measurement is that physiological responses that are unrelated to cold survival per se (for example photoperiod response, tillering, etc) may distort the results. Greenhouse conditions vary with the time of year and are unlikely to relate well to seasonal field conditions. *Freeze-kill* is simply the number of plants of a variety that were killed in the cold stress test.

4.2. Preliminary evaluation of a larger set of winter wheat varieties

The primary purpose of this trial was to refine the test procedure and to determine how well it can differentiate among twelve winter wheat varieties that are known to differ in winter-hardiness. Furthermore, it was hoped that the results would suggest an optimal temperature program that could be used to routinely evaluate winter-hardiness in segregating populations. For this trial, seeds of twelve varieties were germinated for one day and then vernalized for the full period of 56 days when they were exposed to the cold treatment (-11, -13, -15, -17 and -19^{o}C). Following cold treatment, the seedlings were moved to a greenhouse for three weeks and evaluated by measuring the primary shoot lengths, seedling biomass and freeze-kill. Once again, lethality of many treatment combinations made it impossible to do standard analyses of variance. The mean measurements for the treatment combinations are given in Table 4.2.

Table 4.2. Mean measurements (over three replications; eight plants per replication) taken after 21 days on surviving seedlings of 12 winter wheat varieties/lines evaluated at five freezing temperatures.

Temp °C	Variety/ Line	Primary shoot (cm)	Seedling biomass (g)	Freeze-kill
-11	Peregrine	27.5	10.3	1.0
-11	Norstar	35.3	6.8	1.7
-11	Jerry	26.1	16.1	1.7
-11	Radiant	21.5	5.1	4.0
-11	Overland	29.6	5.8	1.3
-11	Buteo	21.9	6.2	3.7
-11	Art	23.6	4.1	2.0
-11	RCATTL33	35.4	19.3	1.0
-11	RACTTF203/2	30.8	10.8	0.3
-11	Falcon	23.2	15.1	1.4
-11	Accipiter	29.2	7.4	0.3
-11	Ideal	28.7	8.0	1.0

Temp °C	Variety/ Line	Primary shoot (cm)	Seedling biomass (g)	Freeze-kill
-13	Peregrine	28.7	6.9	1.4
-13	Norstar	24.2	4.8	3.3
-13	Jerry	29.6	5.7	0.3
-13	Radiant	15.7	1.4	6.0
-13	Overland	23.6	1.0	4.7
-13	Buteo	22.3	6.7	3.3
-13	Art	22.0	3.5	3.7
-13	RCATTL33	17.1	3.2	6.6
-13	RACTTF203/2	31.3	7.8	0.7
-13	Falcon	24.8	2.4	4.0
-13	Accipiter	23.5	3.4	2.0
-13	Ideal	24.3	2.3	5.3

Temp °C	Variety/ Line	Primary Shoot (cm)	Seedling biomass (g)	Freeze-kill
-15	Peregrine	20.7	1.3	4.0
-15	Norstar	24.9	1.7	4.3
-15	Jerry	24.5	2.8	2.3
-15	Radiant	10.7	1.6	7.3
-15	Overland	15.5	0.2	7.0
-15	Buteo	16.5	1.6	4.7
-15	Art	11.2	0.6	7.3
-15	RCATTL33	11.6	0.2	8.0
-15	RACTTF203/2	21.5	2.5	3.0
-15	Falcon	23.8	1.5	3.7

Temp °C	Variety/ Line	Primary shoot (cm)	Seedling biomass (g)	Freeze-kill
-17	Peregrine	10.2	2.4	7.0
-17	Norstar	6.9	0.1	6.7
-17	Jerry	12.9	0.2	7.3
-17	Radiant	2.8	0.0	7.3
-17	Overland	9.4	0.1	8.0
-17	Buteo	7.0	2.0	7.7
-17	Art	8.3	0.1	7.7
-17	RCATTL33	13.1	0.7	8.0
-17	RACTTF203/2	10.7	0.1	6.0
-17	Falcon	10.2	0.1	7.7

Table 4.2. Mean measurements (over three replications; eight plants per replication) taken after 21 days on surviving seedlings of 12 winter wheat varieties/lines evaluated at five freezing temperatures (continued).

Temp °C	Variety/ Line	Primary Shoot (cm)	Seedling biomass (g)	Freeze-kill
-15	Accipiter	22.3	2.1	3.0
-15	Ideal	12.7	2.3	3.0

Temp °C	Variety/ Line	Primary shoot (cm)	Seedling biomass (g)	Freeze-kill
-17	Accipiter	11.1	0.1	7.3
-17	Ideal	7.4	0.1	6.7

Temp °C	Variety/ Line	Primary shoot (cm)	Seedling biomass (g)	Freeze-kill
-19	Peregrine	8.8	0.1	8.0
-19	Norstar	9.2	0.1	8.0
-19	Jerry	14.7	0.2	6.3
-19	Radiant	0.3	0.0	7.7
-19	Overland	8.6	0.1	8.0
-19	Buteo	2.0	0.0	8.0
-19	Art	7.3	0.1	8.0
-19	RCATTL33	13.1	0.3	8.0
-19	RACTTF203/2	8.5	0.3	8.0
-19	Falcon	10.2	0.1	7.3
-19	Accipiter	9.2	0.1	8.0
-19	Ideal	7.0	0.1	8.0

In an attempt to consolidate the results from Table 4.2, into single values that would be easier to interpret, three simple arbitrary indices were derived (Table 4.3). The *primary shoot length index* was calculated as the weighted average height of surviving seedlings. Weights of 1-5 were assigned to the mean values obtained at -11 to -19^{o}C. The *seedling biomass index* was based on the weighted mean mass of surviving plants. Once again weights of 1-5 were assigned to the mean values obtained for -11 to -19^{o}C. Similarly, the *freeze-kill index* was obtained by applying weights of 1-5 to the proportions of freeze-killed plants observed at -11 to -19^{o}C. Index values were calculated with respect to each replication and measurement and used to perform analyses of variance which are summarized in Table 4.3.

Table 4.3. Average index values (over three replications; each containing eight plants) calculated for three winter-hardiness measurements. For comparison, the average normalized survival (% stand) calculated for varieties in the Ducks Unlimited Annual Variety Trials (Dvorak, 2014) is also provided. The number of trials used for each calculation is given in brackets.

Variety	Primary shoot length[1]	Variety	Seedling biomass[2]	Variety	Freeze-kill[3]	Variety	Normalized Survival (% Stand)[4]
Jerry	284.2	Peregrine	38.0	Jerry	8.8	Peregrine	85.7 (20)
RCATTF203/2	242.9	RCATTF203/2	34.9	RCATTF203/2	9.3	Accipiter	81.3 (23)
Falcon	235.6	Jerry	32.8	Accipiter	9.9	Radiant	78.8 (18)
Accipiter	233.7	Buteo	25.3	Peregrine	10.8	Jerry	78.2 (52)
Peregrine	231.7	Accipiter	20.4	Norstar	11.0	Buteo	76.6 (26)
Norstar	231.5	Ideal	20 .0	Falcon	11.0	Ideal	69.7 (8)
RCATL33	222.9	Norstar	18.3	Ideal	11.2	Falcon	68.5 (50)
Overland	203.0	RCATL33	16.8	Buteo	11.9	Overland	64.7 (24)
Ideal	199.7	Falcon	14.7	Art	12.8	Art	35.9 (19)
Art	165.0	Radiant	12.2	Overland	13.0	RCATTF203/2	n/a
Buteo	154.1	Art	12.0	Radiant	13.4	Norstar	n/a
Radiant	96.9	Overland	10.6	RCATL33	13.8	RCATL33	n/a
LSD[5]	83.2	LSD	18.5	LSD	2.5		

[1] Primary shoot length index value = primary shoot length at -11°C + 2(primary shoot length at -13°C) + 3(primary shoot length at -15°C) + 4(primary shoot length at -17°C) + 5(primary shoot length at -19°C).

[2] Seedling biomass index value = Biomass at -11°C + 2(biomass at -13°C) + 3(biomass at -15°C) + 4(biomass at -17°C) + 5(primary shoot length at -19°C).

[3] Freeze-kill index value = Proportion of plants killed at -11°C + 2(proportion of plants killed at -13°C) + 3(proportion of plants killed at -15°C) + 4(proportion of plants killed at -17°C) + 5(proportion of plants killed at -19°C).

[4] The mean winter survival stand percentage over a number of trials conducted in the Ducks Unlimited Annual Variety Trials via Steven Dvorak.

[5] LSD was calculated for a 5% mean comparison.

Table 4.4. Analysis of variance results based on replicated index values for three cold response variables measured in twelve varieties.

Dependent Variable: Primary shoot length					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	2	23170.1517	11585.0758	4.80	0.0186
Variety	11	80906.4967	7355.1361	3.05	0.0125
Error	22	53073.8417	2412.4473		
Corrected Total	35	157150.4900			

Dependent Variable: Seedling biomass					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	2	2419.6706	1209.8353	10.09	0.0008
Variety	11	2908.7289	264.4299	2.21	0.0550
Error	22	2636.9494	119.8613		
Corrected Total	35	7965.3489			

Dependent Variable: Freeze-kill					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	2	0.6950	0.3475	0.16	0.8534
Variety	11	83.6900	7.6082	3.50	0.0125
Error	22	47.8650	2.1757		
Corrected Total	35	132.2500			

Highly significant differences were seen among replications for the seedling height and biomass measurements; however, replications did not appear to have a significant effect on freeze-survival (Table 4.4). Thus, the differences among replications with respect to height and biomass probably stem from different greenhouse conditions during the recovery period. Significant genetic differences were seen among varieties/lines with respect to each measurement and the means are listed in Table 4.2. For comparison, survival (% stand) data based on field trials in North Dakota and kindly supplied by Dvorak (2014) were included where available.

From Table 4.2 and 4.3 it appears that the primary shoot length, biomass and freeze-kill responses were not always reflective of known cold-hardiness differences among the varieties and in particular, did not separate highly tolerant genotypes such as Radiant and Buteo (Dvorak, 2014) from intermediate (Overland and Ideal) and very cold-sensitive genotypes (such as Art). The above results would imply that unknown factors in addition to freeze tolerance could have affected the survival of seedlings. It is possible that a vernalization period of 56 days could deplete seedling nutrient reserves to such an extent that it will impact its viability and cold-resistance. In this regard initial seed vigor and seed size may then become very important. These aspects were therefore the focus of further experiments.

4.3. Does the duration of the vernalization/acclimation period affect cold survival?

The third experiment aimed to determine whether more consistent differences in cold-hardiness will emerge if the cold screening was done after three weeks of acclimation/ vernalization rather than 56 days. Following cold treatment after 21 days of vernalization, the seedlings were returned to the vernalization chamber for the remainder of the 56 day vernalization period. Five winter wheat and one spring wheat variety were subjected to four subzero temperature regimes. There were three replications and each temperature X variety X replication combination involved eight seedlings. Index values were calculated for each replication and entry and are listed in Table 4.5; however, an analysis of variance was not done in view of the small data set involved.

Table 4.5. Average index values (over three replications; each containing eight plants) calculated for three winter-hardiness measurements.

Variety	Primary shoot length index[1]	Variety	Seedling biomass index[2]	Variety	Freeze-kill index[3]
Peregrine	307.1	Peregrine	42.3	Peregrine	3.9
Jerry	294.2	Jerry	35.0	Jerry	4.2
Decade	147.7	Decade	13.5	Decade	8.2
Hawken	83.7	Hawken	4.5	Hawken	8.9
Art	28.4	Art	3.0	Art	9.5
Glenn	12.1	Glenn	1.3	Glenn	9.9

[1] Primary shoot length index value = primary shoot length at -11°C + 2(primary shoot length at -13°C) + 3(primary shoot length at -15°C) + 4(primary shoot length at -17°C).
[2] Seedling biomass index value = Biomass at -11°C + 2(biomass at -13°C) + 3(biomass at -15°C) + 4(biomass at -17°C).
[3] Freeze-kill index value = Proportion of plants killed at -11°C + 2(proportion of plants killed at -13°C) + 3(proportion of plants killed at -15°C) + 4(proportion of plants killed at -17°C).

The results of Tables 4.5 show that Peregrine, closely followed by Jerry, had the best cold survival. The freeze-kill index showed separation of highly tolerant genotypes from intermediate and very cold-sensitive genotypes. While the -17°C treatment was severe and survival was generally low, Peregrine, Jerry and Decade still had some survivors at this temperature. This would suggest that the -17°C treatment could be very useful for identifying only highly tolerant plants from segregating populations of select crosses. The high level of lethality even among very tolerant plants will, however, limit the numbers that can be selected. Decade followed by Hawken showed intermediate cold tolerance. Both varieties performed fairly well at -11 and -13°C yet their tolerance was sharply reduced at -15 and -17°C. Art is known to have poor winter-hardiness and could not survive temperatures below -11°C. As expected, the spring wheat Glenn was the worst performer. The assessment conformed very well to the field data of Dvorak (2014) who assigned normalized survival percentages as follows: Peregrine (85.7%), Jerry (78.2%), Decade (77.2%), Hawken (53.7%), and Art (35.9%).

The results showed that performing the cold screening test after 21 days of vernalization rather than 56 days, more accurately reflected the known differences in cold-hardiness among the varieties. It was then decided to change the test protocol accordingly and to determine whether seed size differences among varieties may also affect assessments of their cold-hardiness in this modified test procedure.

4.4. Evaluation of the effect of seed size on the test results

To see whether the size of the test seeds had an effect on the winter-hardiness of the seedlings evaluated after 21 days of vernalization, a bulk seed sample of each of the varieties Peregrine, Overland, and Art was sieved for two minutes using Tyler sieves #7, #9, and #12. Three minimum temperature programs were used (-11, -13, -15°C). Each variety X size X replication combination involved ten seeds. Germination tests were done beforehand with each size category to confirm its viability (Table 4.6). The mean measurements for the treatment combinations are given in Tables 4.7.

Table 4.6. The percentage of seeds that germinated in a five day period.

	Germination	
Variety X seed size	3 Days	5 Days
Art Small	97%	98%
Art Medium	98%	99%
Art Large	93%	98%
Peregrine Small	93%	98%
Peregrine Medium	96%	98%
Peregrine Large	97%	98%
Overland Small	97%	99%
Overland Medium	96%	98%
Overland Large	98%	99%

Table 4.7. Mean (thirty seedlings) evaluation measurements for three varieties and three seed sizes evaluated at four freezing temperatures following 21 days of vernalization.

Temp °C	Variety & seed size	Primary shoot (cm)	Seedling biomass (g)	Freeze-kill
-11	Art Small	16.7	1.2	8.7
-11	Art Medium	27.3	3.2	6.3
-11	Art Large	28.3	2.1	5.7
-11	Peregrine Small	28.2	4.9	3.7
-11	Peregrine Medium	28.3	3.9	3.7
-11	Peregrine Large	32.3	6.2	4.3
-11	Overland Small	29.4	4.6	4.7
-11	Overland Medium	33.1	6.4	6.0
-11	Overland Large	33.4	7.3	4.0

Temp °C	Variety & seed size	Primary shoot (cm)	Seedling biomass (g)	Freeze-kill
-13	Art Small	17.3	0.8	9.7
-13	Art Medium	16.6	0.9	9.7
-13	Art Large	0.0	0.0	10.0
-13	Peregrine Small	15.4	0.8	6.7
-13	Peregrine Medium	20.7	1.0	7.3
-13	Peregrine Large	17.2	0.5	6.3
-13	Overland Small	10.4	1.3	8.7
-13	Overland Medium	17.5	0.6	9.0
-13	Overland Large	11.2	0.5	7.7

Temp °C	Variety & seed size	Primary shoot (cm)	Seedling biomass (g)	Freeze-kill
-15	Art Small	0.0	0.0	10.0
-15	Art Medium	0.0	0.0	10.0
-15	Art Large	0.0	0.0	10.0
-15	Peregrine Small	10.1	0.1	9.0
-15	Peregrine Medium	8.7	0.1	8.7
-15	Peregrine Large	8.9	0.3	9.3
-15	Overland Small	9.8	0.7	9.7
-15	Overland Medium	0.0	0.0	10.0
-15	Overland Large	10.7	0.4	9.7

The germination tests showed that the seeds had 98-99% germination after five days and that this would not have been a cause of variation in the results that were produced. The results of Tables 4.7 furthermore showed that the size of the seed did not have an obvious and consistent effect on the cold survival of the seedlings that were evaluated in the 21 day test. Some of the larger seed sizes were sometimes out-performed by the smaller seed sizes for a specific cold treatment. Therefore, it appears unnecessary to allow for seed size differences in the 21 day test.

4.5. Validation of the 21-day cold-tolerance screening test – Comparison with field survival data

Based on the prior experiments, a final cold tolerance screening protocol was derived. This protocol is outlined in section 3.6 of the Materials and Methods. In an attempt to validate the cold-tolerance screening test and to compare the results with field survival data, the 2013 North Dakota Hard Red Winter Wheat Variety Trial entries were evaluated with the new testing procedure.

4.5.1. Results obtained following evaluation of variety trial entries with the finalized cold tolerance screening test

The same trial was done twice – the first trial (= *trial 1*) was completed in the spring of 2014 and the second trial (= *trial 2*) was completed in the late summer and fall of 2014. In each trial the same twenty varieties and lines were evaluated in three replications at four freezing temperatures (-11, -13, -15 and -17^{o}C). Each replication X treatment combination involved six test plants.

An index value was calculated for each variety X replicate combination and analyses of variance were done. The ANOVA results and mean values calculated for each measurement and entry are given in Tables 4.8 to 4.10 and Tables 4.11 to 4.13, respectively.

Table 4.8. Analysis of variance results with respect to trial 1 for the primary shoot length, biomass and freeze-kill indices.

Dependent Variable: Primary shoot length

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	2	170.9373	85.4687	0.05	0.9530
Variety	19	188720.8592	9932.6768	5.60	<.0001
Error	38	67432.7693	1774.5466		
Corrected Total	59	256324.5658			

Dependent Variable: Seedling biomass

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	2	32.2493	16.1247	0.90	0.4142
Variety	19	1159.6373	61.0335	3.41	0.0006
Error	38	679.2307	17.8745		
Corrected Total	59	1871.1173			

Dependent Variable: Freeze-kill

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	2	2.1003	1.0502	1.87	0.1687
Variety	19	62.4560	3.2872	5.84	<.0001
Error	38	21.3930	0.5630		
Corrected Total	59	85.9493			

Table 4.9. Analysis of variance results with respect to trial 2 for the primary shoot length, biomass and freeze-kill indices.

Dependent Variable: Primary shoot length					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	2	1819.2910	909.6455	0.31	0.7365
Variety	19	198252.7073	10434.3530	3.54	0.0004
Error	38	112091.2557	2949.7699		
Corrected Total	59	312163.2540			

Dependent Variable: Seedling biomass					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	2	566.6423	283.3212	3.85	0.0300
Variety	19	1723.8473	90.7288	1.23	0.2831
Error	38	2794.8577	73.5489		
Corrected Total	59	5085.3473			

Dependent Variable: Freeze-kill					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	2	6.8223	3.4112	4.03	0.0260
Variety	19	45.7573	2.4083	2.84	0.0030
Error	38	32.1977	0.8473		
Corrected Total	59	84.7773			

Table 4.10. Combined analysis of variance results with respect to trials 1 and 2 for the primary shoot length, biomass and freeze-kill indices.

Dependent Variable: Primary shoot length

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	5	29257.8884	5851.5777	2.22	0.0582
Variety	19	316467.2882	16656.1731	6.33	<.0001
Error	95	250030.3033	2631.8979		
Corrected Total	119	595755.4799			

Dependent Variable: Seedling biomass

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	5	2833.1987	566.6397	11.51	<.0001
Variety	19	1679.8783	88.4146	1.80	0.0342
Error	95	4677.6947	49.2389		
Corrected Total	119	9190.7717			

Dependent Variable: Freeze-kill

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	5	14.7307	2.9461	3.36	0.0077
Variety	19	78.5147	4.1324	4.71	<.0001
Error	95	83.2893	0.8767		
Corrected Total	119	176.5347			

The ANOVA for trial 1 showed highly significant differences among entries for primary shoot length, seedling biomass, and freeze-kill. Trial 2 only had significance among varieties for primary shoot length and freeze-kill. Seeding biomass in trial 2 was not significantly different among varieties. When the trials were combined the primary shoot length and freeze-kill measurements detected highly significant ($P<.0001$) differences among varieties while the genetic differences for biomass index were less pronounced at $P = 0.0342$.

Table 4.11. Average primary shoot length index values (over three replications; each containing six plants) for trials 1 and 2 plus the overall averages for the two trials combined.

Trial 1		Trial 2		Average	
Variety	Primary shoot length index[1]	Variety	Primary shoot length index	Variety	Primary shoot length index
Moats	236.7	Peregrine	309.2	Peregrine	237.8
Flourish	210.8	WB Matlock	207.5	Moats	203.0
AC Broadview	209.4	Norstar	196.7	Flourish	201.2
Norstar	168.9	Jerry	192.2	Norstar	182.8
Peregrine	166.2	Flourish	191.3	Jerry	177.5
Jerry	159.9	Boomer	179.1	AC Broadview	177.4
AC Emerson	118.0	AC Broadview	169.4	WB Matlock	154.5
Boomer	109.1	Moats	169.2	Boomer	151.3
WB Grainfield	108.4	Falcon	151.3	Falcon	125.5
WB Matlock	101.6	Overland	148.9	Overland	122.7
Falcon	99.7	Art	135.9	AC Emerson	114.1
Sunrise	99.0	Robidoux	117.8	Sunrise	103.9
Ideal	98.8	AC Emerson	110.0	Ideal	103.1
Overland	96.5	Sunrise	109.0	Lyman	96.9
Lyman	88.2	Ideal	107.6	WB Grainfield	94.5
TX09D1037	71.6	Lyman	105.6	Art	89.0
Decade	54.4	Decade	84.6	Robidoux	78.5
Art	42.2	WB Grainfield	80.6	TX09D1037	74.4
Robidoux	39.0	TX09D1037	77.3	Decade	69.5
SY Wolf	29.2	SY Wolf	60.6	SY Wolf	44.9
LSD[2]	69.7	LSD	89.8	LSD	58.8

[1] Primary shoot length index value = primary shoot length at -11°C + 2(primary shoot length at -13°C) + 3(primary shoot length at -15°C + 4(primary shoot length at -17°C).

[2] LSD was calculated for a 5% mean comparison.

Table 4.12. Average biomass index values (over three replications; each containing six plants) for trials 1 and 2 plus the overall averages for the two trials combined.

Trial 1		Trial 2		Average	
Variety	Seedling biomass index[1]	Variety	Seedling biomass index	Variety	Seedling biomass index
Moats	19.4	Overland	25.1	Boomer	19.4
Boomer	15.3	Peregrine	24.8	Peregrine	17.1
Norstar	12.3	Boomer	23.2	Overland	16.2
Flourish	10.9	Falcon	22.0	Jerry	15.1
Jerry	10.7	WB Matlock	21.0	Flourish	14.8
AC Broadview	10.4	Jerry	19.5	Norstar	14.6
Peregrine	9.5	Flourish	18.5	AC Broadview	14.1
AC Emerson	7.8	AC Broadview	17.8	Falcon	13.8
Ideal	7.4	Lyman	17.7	Moats	13.1
Lyman	7.4	Norstar	17.1	Lyman	12.5
Overland	7.2	Sunrise	16.7	WB Matlock	12.4
Sunrise	6.8	Art	14.7	Sunrise	11.7
Falcon	5.7	Decade	14.6	AC Emerson	10.1
TX09D1037	4.4	TX09D1037	13.2	Ideal	10.6
WB Grainfield	4.1	Ideal	12.7	Decade	8.8
WB Matlock	3.9	AC Emerson	12.4	TX09D1037	8.8
Robidoux	3.2	Robidoux	11.8	Art	8.4
Decade	2.9	WB Grainfield	11.7	WB Grainfield	8.3
Art	2.1	Moats	7.0	Robidoux	7.5
SY Wolf	1.9	SY Wolf	4.9	SY Wolf	3.4
LSD[2]	7.0	LSD	14.2	LSD	8.0

[1] Seedling biomass index value = Biomass at -11 º C + 2(biomass at -13ºC) + 3(biomass at -15ºC) + 4(biomass at -17ºC).

[2] LSD was calculated for a 5% mean comparison.

Table 4.13. Average freeze-kill index values (over three replications; each containing six plants) for trials 1 and 2 plus the overall averages for the two trials combined.

Trial 1		Trial 2		Average	
Variety	Freeze-kill index	Variety	Freeze-kill index	Variety	Freeze-kill index
Moats	6.0	WB Matlock	5.9	Peregrine	6.7
Jerry	6.6	Peregrine	6.2	AC Broadview	6.8
Flourish	6.7	AC Broadview	6.6	Flourish	6.8
Norstar	7.0	Boomer	6.7	Boomer	7.0
AC Broadview	7.1	Falcon	6.8	Moats	7.1
Peregrine	7.2	Flourish	6.9	Jerry	7.1
Boomer	7.4	Norstar	7.3	Norstar	7.2
Sunrise	7.9	Sunrise	7.5	WB Matlock	7.2
AC Emerson	8.0	Jerry	7.5	Falcon	7.6
WB Grainfield	8.0	Overland	7.6	Sunrise	7.7
Lyman	8.3	Art	7.7	Lyman	8.1
Falcon	8.4	Lyman	7.8	WB Grainfield	8.1
WB Matlock	8.4	Robidoux	7.9	AC Emerson	8. 1
Ideal	8.6	Moats	8.1	Overland	8.2
Overland	8.9	WB Grainfield	8.2	Ideal	8.4
TX09D1037	8.9	Ideal	8.3	Art	8.6
Robidoux	9.4	AC Emerson	8.3	Robidoux	8.7
Decade	9.4	Decade	8.9	TX09D1037	8.9
Art	9.5	TX09D1037	8.9	Decade	9.1
SY Wolf	9.6	SY Wolf	9.1	SY Wolf	9.3
LSD[2]	1.2	LSD	1.5	LSD	1.1

[1] Freeze-kill index value = Proportion of plants killed at -11ºC + 2(proportion of plants killed at -13ºC) + 3(proportion of plants killed at -15ºC) + 4(proportion of plants killed at -17ºC).

[2] LSD was calculated for a 5% mean comparison.

The data of the two trials did not consistently distinguish among the best and worst performers. Considering the top six entries for the three traits (Tables 4.11 to 4.13); Flourish, Peregrine, Norstar and Jerry were in the upper group for both trials with respect to the primary shoot length index. For the biomass index, Boomer and Jerry occurred among the top six for both trials. For the freeze-kill index Flourish, Broadview and Peregrine occurred among the top six in both trials. None of the varieties were placed in the top six for both trials and all three measurements. The long-term field data of Dvorak (2014) showed that Peregrine, Jerry, Broadview were among the most winter-hardy varieties; however, Flourish was among those with poor winter survival. Since Flourish is a recently released cultivar, fewer data were available for ranking it (Dvorak, 2014); however, it appears that the present measurements had failed to predict its field survival.

Regarding the group of six entries that rated worst for the two trials and the three indices (Tables 4.11 to 4.13) the following was found: Lyman, Decade and SY Wolf ranked in this group in both trials with respect to the primary shoot index. For the biomass index, Grainfield, SY Wolf and Robidoux occurred in this group for both trials. For the freeze-kill index TX09D1037, Decade and SY Wolf occurred among the worst six in both trials. Only SY Wolf was consistently rated among the bottom six by both trials and all measurements. Dvorak's (2014) data were in agreement with the placement of SY Wolf among the bottom six; however, ranked Art as the variety with the lowest survival. In the present data trial 1 consistently ranked Art in the lower group, however, trial 2 suggested that it had intermediate cold tolerance. Another inconsistency was with respect to Decade which often occurred among the bottom six; however, was ranked in the higher-intermediate group by the data of Dvorak (2014).

4.5.2. Winter-survival results obtained from the variety trials

Winter survival of the varieties evaluated with the laboratory screening test, was also estimated using the 2013 and 2014 hard red winter wheat variety trials. In 2013 all twenty varieties were evaluated at both Prosper and Casselton, ND. An analysis of variance was done and the results are presented in Table 4.14. The entry means are listed in Table 4.15. Fifteen of the varieties were also evaluated in the 2014 variety trial. Field survival measurements were taken at Prosper and Carrington and analyzed (Tables 4.17 and 4.16, respectively).

Table 4.14. An analysis of variance of field survival data obtained for 20 varieties included in the 2013 variety trial grown in Prosper ND.

Dependent Variable: Survival %					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	5	9952.8207	1990.5641	4.82	0.0006
Variety	18	19840.2616	1102.2368	2.67	0.0012
Error	90	37159.7901	412.8866		
Corrected Total	113	66952.8723			

Table 4.15. Average field survival results for the 2013 hard red winter wheat variety trials grown at Casselton, ND and Prosper, ND.

Variety	Field Survival % [1]		
	Casselton	Prosper	AVG
AC Emerson	69.8	85.2	77.5
NI08708	70.0	84.2	77.1
Boomer	77.3	73.8	75.6
Moats	62.4	85.3	73.9
Jerry	64.5	78.0	71.2
Flourish	70.6	69.6	70.1
Ideal	60.9	75.1	68.0
Falcon	53.0	80.8	67.0
WB Matlock	47.1	86.4	66.8
AC Broadview	56.5	72.9	64.7
Robidoux	62.1	66.2	64.1
SY Wolf	42.2	78.2	60.2
Sunrise	54.9	60.6	57.7
Lyman	60.7	51.7	56.2
NE06545	37.6	71.7	54.7
Overland	44.6	61.2	52.9
Art	29.7	73.0	51.3
WB Grainfield	34.1	64.8	49.5
McGill	43.0	55.2	49.1
AVG	54.8	72.3	
		LSD[2] 23.3	

[1] Stand counts were taken in November 2012 and in April 2013. A random stretch of 60.96 cm was measured out in a random row within each plot, and the seedlings within that distance were counted.

[2] LSD was calculated for a 5% mean comparison.

Table 4.16. An analysis of variance of field survival data obtained for 15 varieties included in the 2014 variety trials grown in Langdon, ND and Prosper ND.

Dependent Variable: Survival %					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	2	3124.0238	1562.0119	1.62	0.2067
Location	1	19413.4405	19413.4405	20.18	<.0001
Variety	13	21899.1548	1684.5504	1.75	0.0764
Location*Variety	13	16164.3929	1243.4148	1.29	0.2463
Error	54	51957.9762	962.1847		
Corrected Total	83	112558.9881			

Table 4.17. Average winter survival results for the 2014 hard red winter wheat variety trials planted at Langdon, ND and Prosper, ND.

Variety	Field Survival[1]		
	Prosper	Langdon	AVG
Art	161.0	173.0	167.0
AC Broadview	158.0	150.0	154 0
Lyman	172.0	121.0	146.50
Moats	166.0	119.0	142.5
SY Wolf	158.0	121.0	139.5
Flourish	163.0	111.0	137.0
Mcgill	128.0	140.0	134.0
Jerry	135.0	131.0	133.0
Falcon	149.0	109.0	129.0
WB Matlock	132.0	108.0	120.0
Overland	123.0	114.0	118.5
Robidoux	153.0	75.0	114.0
WB Grainfield	148.0	74.0	111.0
Ideal	124.0	97.0	110.5
AVG	148.0	117.0	LSD[2] 35.9

[1] A 1m x 1m square was used to demarcate a random area within each plot for counting the plants. The expected number of plants within the area was 297 (based on the seeding density).
[2] LSD was calculated for a 5% mean comparison.

Table 4.18. The average`e normalized survival (% stand) calculated for varieties in the Ducks Unlimited Annual Variety Trials and the number of trials used for each calculation (Dvorak, 2014).

Variety	Number of trials	Normalized Survival (% Stand)
Moats	2	102.7
Sunrise	2	101.3
Peregrine	20	85.7
AC Broadview	10	81.6
Jerry	52	78.2
Decade	15	77.2
Boomer	14	74.7
WB Grainfield	3	71.9
WB Matlock	14	70.1
Ideal	8	69.7
Falcon	50	68.5
Overland	24	64.7
Lyman	15	61.1
Flourish	3	56.8
SY Wolf	10	44.2
Robidoux	9	38.2
Art	19	35.9

In 2013 significant differences in the survival of the entries were seen yet in 2014 these differences were not significant at the 5% level (Tables 4.14 and 4.16). In 2013 the F-probability for significance of the varieties mean square was 0.0012 and in 2014 it was 0.0764 The F-probability was not significant for the location x varieties mean square, with a value of 0.2463 in 2014. There was no F- probability for the location x varieties mean square for the 2013 field variety trial, because only one replicated trial was used. Despite the fact that significant genetic differences were seen in 2013, neither of the two sets of means appeared to relate well to the ranking previously reported by Dvorak (2014) (Table 4.18); this could partly be due to limited data from only one trial site being available in 2013 and only two trial sites being available in 2014. For example, Art and SY Wolf that have poor winter-hardiness grouped with intermediate

(Lyman and Flourish), good winter-hardiness (Moats and WB Matlock), and excellent winter-hardiness (Jerry) varieties.

In order to better compare the mean values obtained with the different indices measured in the freeze-chamber tests and the survival in the variety trials, correlations were obtained and compared among the different traits.

4.5.3. Correlations involving laboratory and field tests of cold-hardiness

A correlation table was computed. All possible correlations were calculated among the mean indices of thirteen varieties that were common in the two laboratory evaluation trials, mean winter-survival in the 2013 and 2014 variety trials, and the normalized survival data provided by Dvorak (2014). The thirteen varieties were AC Broadview, Art, Falcon, Flourish, Ideal, Jerry, Lyman, Moats, Overland, Robidoux, SY Wolf, WB Grainfield, and WB Matlock. These correlations are shown in Table 4.19.

Table 4.19. Correlation matrix for the primary shoot length index (PSI), biomass index (BMI) and freeze-kill index (FKI) of the first (1), second (2) and combined (1&2) greenhouse trials; the field survival measurements of the 2013 and 2014 variety trials; and the long term normalized survival means from the Ducks Unlimited (DU) field trials provided by Dvorak (2014). **,* Indicate correlations that are significant at the 1% and 5% levels respectively, whereas ^{ns} indicate non-significance. These correlations were derived from the 13 varieties common to all the data sets.

	PSI (1)	BMI (1)	FKI (1)	PSI (2)	BMI (2)	FKI (2)	PSI (1&2)	BMI (1&2)	FKI (1&2)	Field 2013	Field 2014	Field DU
PSI (1)	1.00											
BMI (1)	0.90**	1.00										
FKI (1)	-0.96**	-0.89**	1.00									
PSI (2)	0.63*	0.49^{ns}	-0.59*	1.00								
BMI (2)	0.12^{ns}	-0.04^{ns}	-0.07^{ns}	0.59*	1.00							
FKI (2)	-0.37^{ns}	-0.10^{ns}	0.29^{ns}	-0.81**	-0.75**	1.00						
PSI (1&2)	0.94**	0.81**	-0.90**	0.86**	0.34^{ns}	-0.60*	1.00					
BMI (1&2)	0.67*	0.61*	-0.63*	0.78**	0.77**	-0.66*	0.79**	1.00				
FKI (1&2)	-0.88**	-0.70**	0.87**	-0.85**	-0.44^{ns}	0.72**	-0.96**	-0.79**	1.00			
Field 2013	0.07^{ns}	0.08^{ns}	0.02^{ns}	0.16^{ns}	-0.18^{ns}	-0.18^{ns}	0.12^{ns}	-0.09^{ns}	-0.08^{ns}	1.00		
Field 2014	0.18^{ns}	0.18^{ns}	-0.16^{ns}	0.15^{ns}	-0.09^{ns}	-0.10^{ns}	0.19^{ns}	0.05^{ns}	-0.17^{ns}	0.18^{ns}	1.00	
Field DU	0.79**	0.80**	-0.79**	0.42^{ns}	0.07^{ns}	-0.2^{ns}	0.71**	0.57*	-0.69**	0.13^{ns}	-0.09^{ns}	1.00

Based on the correlations above, when a correlation was done for the same index measured in the two trials the following became apparent: Only the primary shoot length indices had a significant positive correlation, with an R-squared value of 0.4. The seedling biomass and freeze-kill indices of the two trials were not correlated. The lack of correlation suggested that the repeatability of the two greenhouse trials were very low, in particular for the seedling biomass and freeze-kill indices. The ANOVA of Table 4.9 furthermore showed that trial 2 failed to detect significant genetic differences among entries for the biomass index due to a comparatively large entry X replication interaction. This worrying because the trial is largely performed under strictly controlled temperature conditions, both in the vernalization and cold freeze chamber and such poor correlations were unexpected. The different results of the two trials therefore relate to either the planting tray preparation or the greenhouse recovery period or both.

When the three indices were correlated among each other for the individual trials, the primary shoot length index and the seedling biomass index were positively correlated to each other as expected. As expected the freeze-kill index was negatively correlated with both primary shoot length and seedling biomass. The correlations for trial 2 were generally lower than for trial 1, yet were still significant. The correlation of different traits measured in different trials was only significant (5%) for the freeze-kill index of trial 1 and primary shoot length index of trial 2. This confirms the very dissimilar rating of the 13 varieties in the two trials.

The correlation between the 2013 (Prosper, ND) and the 2014 (Prosper, ND and Langdon, ND) variety trials were insignificant. This is not unexpected in view of the absence of genetic differences in the 2014 trials. In the 2014 data set the location effect was highly significant (Tables 4.16) while the location X variety interaction was non-significant. Varietal differences ($Pr > F = 0.0012$) were only observed for the 2013 data set. Thus, the error

component in both data sets was comparatively high and the mean values (particularly for the 2014 data set) were not very reliable indicators of genetic differences among genotypes. Also, the field data of the two years were not significantly correlated with either of the trial 1 or trial 2 greenhouse data, nor did it correlate with Dvorak's data. The 2013 and 2014 variety trials represented only one growing season and the plot to plot error variation was high. The Ducks Unlimited (DU) field trials provided by Dvorak (2014) on the other hand represents standardized means calculated over a large number of years and trials and can be expected to have better predictive value.

When the Ducks Unlimited field data were correlated with the indices from the greenhouse trials, only the trial 1 greenhouse data revealed strong and highly significant correlations. These correlations showed that the trial 1 greenhouse measurements could predict between 62-64% of the variation seen in the DU trials. This would imply that the ability to with-stand the low temperatures is a very significant component of winter-survival, yet is likely not the only determinant.

The trial 2 measurements were not correlated with Dvorak's data, whereas, the averages of trials 1 and 2 were significantly correlated with Dvorak's means. However, the magnitude of these latter correlations was reduced compared to the trial 1 correlations and confirm that the trial 2 data were unreliable.

In conclusion, the correlation data could mean that: (1) the greenhouse test developed and evaluated here does not provide a reliable and consistent measurement of cold tolerance; or more likely, (2) the test does measure a significant component of cold-survival, yet is very sensitive to fluctuation in the planting medium, the way the soil is compacted in the plant container, the seeding depth and water content of the tray at the time of the cold treatment (all three

measurements). In the case of main tiller length and biomass after three weeks of greenhouse growth, variable greenhouse conditions might also have affected the data.

4.6. Validation of the cold-tolerance screening test – Selection of two early generation populations

In a further attempt to validate the cold-tolerance screening test, two segregating populations were subjected to selection. The first population derived from cross 12K618 (TX09D1037/Decade) and was selected using the -13°C program. The second population (cross 11K135 = Overland/Peregrine) was selected using the -15°C program. The different selection temperatures were chosen based on the reported cold-hardiness of parents Decade (intermediate-good) and Peregrine (very good) (Dvorak, 2014). In each population 500 F_2 seeds were used to initiate selection while 50 random F_2 seeds were used to establish a control population. The best plants (10%) were selected in the F_2 and their F_3 was used for a second selection and thus selected F_3 and F_4 bulks were established. The two selected populations of each cross were then compared to the parents plus unselected F_2, F_3 and F_4 bulks. A total of 96 plants were compared per population. In order to accommodate the large number of plants in the freeze chamber, the test was performed using six replications each consisting of 16 plants per population.

4.6.1. The cross 12K618 results

An analysis of variance was done for cross 12K618 (Table 4.20) while the mean measurements for this cross are given in Table 4.21.

Table 4.20. Analysis of variance results for three variables measured in the parental, selected and control populations of cross 12K618 (TX09D1037/Decade).

Dependent Variable: Primary shoot length					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	5	819.5355	163.9071	5.46	0.0011
Population	6	253.359	42.2265	1.41	0.2442
Error	30	899.9095	29.997		
Corrected Total	41	1972.804			

Dependent Variable: Seedling biomass					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	5	932.1469	186.4294	5.03	0.0018
Population	6	622.8295	103.8049	2.80	0.0276
Error	30	1111.3448	37.0448		
Corrected Total	41	2666.3212			

Dependent Variable: Freeze-kill					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	5	151.8333	30.3667	4.56	0.0033
Population	6	34.3333	5.7222	0.86	0.5353
Error	30	199.6667	6.6556		
Corrected Total	41	385.8333			

Table 4.21. Measurement means (16 plants X six replicates = 96) for the selected and control populations of cross 12K618 (TX09D1037/Decade). Means followed by the same letter did not differ significantly at the 5% level of significance.

Temp ^{o}C	Population	Primary shoot length (cm)	Biomass (g)	Freeze-kill
-13	TX09D1037	34.0 B	7.9 BC	10.7 A
-13	Decade	27.6 B	4.6 C	11.8 A
-13	F_2	35.3 A	18.0 A	9.2 A
-13	F_3 Selected bulk	33.0 B	10.3 BC	11.2 A
-13	F_3 Unselected bulk	33.5 B	11.9 AB	11.2 A
-13	F_4 Selected bulk	32.3 B	11.7 ABC	11.8 A
-13	F_4 Unselected bulk	35.5 A	12.3 AB	10.0 A

For cross 12K618 (Table 4.2) highly significant replication effects were observed for all three measurements, however, significant variation among the seven populations occurred only in the case of seedling biomass. The seedling biomass mean values (Table 4.21) of the two parents TX09D1037 (7.9g) and Decade (4.6g) were not significantly different (5%). However, the F_2 population mean as well as the unselected F_3 and F_4 means were significantly higher than that of the parents, which might be indicative of transgressive segregation for initial growth in the greenhouse (photoperiod insensitivity) rather than cold survival per se. The unselected bulks did have a slightly higher seedling biomass than the selected bulks. Based on these results, the selection procedure did not improve the seeding biomass, when compared to the unselected bulks.

4.6.2. The cross 11K135 results

For cross 11K135, highly significant replication effects were also observed (Table 4.22). The only significant differences among the seven populations occurred in the case of freeze-kill. The freeze-kill averages are summarized in Table 4.23. Peregrine showed significantly lower mortality (13.5 of 16 seedlings) at -15 ^{o}C as compared to Overland (15.5 of 16 seedlings). However, none of the selected and unselected bulk populations performed significantly better than Overland, and with the exception of the unselected F_3 bulk, Peregrine survived significantly better than all of the segregating populations.

Table 4.22. Analysis of variance results for three variables measured in the parental, selected and control populations of cross 11K135 = Overland/Peregrine.

Dependent Variable: Primary shoot length					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	5	3114.7879	622.9576	4.10	0.0059
Population	6	1084.8348	182.8058	1.19	0.3390
Error	30	4563.6938	152.1231		
Corrected Total	41	8763.3164			

Dependent Variable: Seedling biomass					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	5	115.542	23.1084	6.84	0.0002
Population	6	16.7074	2.7846	0.82	0.5600
Error	30	101.3076	3.3769		
Corrected Total	41	233.557			

Dependent Variable: Freeze-kill					
Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Rep	5	27.6429	5.5286	7.36	0.0001
Population	6	18.619	3.1032	4.13	0.0038
Error	30	22.5238	0.7508		
Corrected Total	41	68.7857			

Table 4.23. Measurement means (16 plants X six replicates) for the selected and control populations of cross 11K135 (Overland/Peregrine). Means followed by the same letter did not differ significantly at the 5% level of significance.

Temp ^{o}C	Variety/Generation	Primary shoot length (cm)	Biomass (g)	Freeze-kill
-15	Overland	6.1 AB	1.4 A	15.5 A
-15	Peregrine	19.6 A	0.7 A	13.5 B
-15	F_2	11.7 AB	0.8 A	15.3 A
-15	F_3 Selected bulk	8.9 AB	1.0 A	15.0 A
-15	F_3 Unselected bulk	13.1 AB	2.1 A	14.5 AB
-15	F_4 Selected bulk	11.4 AB	0.3 A	15.2 A
-15	F_4 Unselected bulk	2.3 B	0.0 A	15.2 A

For cross 11K135 (Table 4.22) highly significant replication effects were observed for all three measurements, however, significant variation among the seven populations occurred only in the case of freeze-kill. The data clearly shows that the selection was meaningless. The procedure as executed did not accurately identify better progenies in the different selection stages and no progress was made in improving the mean performance of progenies.

5. SUMMARY

Freeze-kill is negatively correlated with primary shoot length and seedling biomass, while, the primary shoot length and seedling biomass are positively correlated. Since the three indices correlated well in both trials, it may not be necessary to measure all three. The biomass and freeze-kill indices are the easiest to measure; while primary shoot length does not appear to provide additional information and could be dropped.

The present version of the cold-tolerance test may not be robust enough. It provided good differentiation of the genotypes in the first exploratory trials and in trial 1, yet produced less consistent data afterwards and totally inconsistent data in the selection trial. This may either mean that it was not done as carefully (soil volume and compaction, plant depth) in the later trials as it was done initially. It may also, or in addition, mean that it will be necessary to control the greenhouse recovery phase better and perhaps employ a growth chamber with fixed light and temperature settings rather than the more fluctuating greenhouse conditions.

The better trait for selecting crosses in the early generations may be freeze survival as it can shorten the test procedure (can be evaluated after three weeks of vernalization and say ten days of recovery instead of 56 days vernalization followed by three weeks of greenhouse growth); selected seedlings can then be fully vernalized and transplanted to a greenhouse without further selection. It may be better to apply a harsh treatment that would kill 80-90% of the seedlings.

If appropriately and successfully modified, the procedure could be a useful tool for preliminary testing of the winter-hardiness of later generation inbred lines. The better trait for characterizing a small group of advanced breeding lines/varieties may be the biomass index: it can be used to test a smaller number of genotypes more precisely by involving larger numbers of

plants of the same genotype; also, it will not be necessary to complete the full vernalization period (evaluate after three weeks).

In order to make the screening procedure more robust variations in the planting medium, the way the soil is compacted in the plant container, the seeding depth and water content of the tray at the time of the cold treatment need to be evaluated. In addition the greenhouse recovery conditions need to be controlled more precisely, in particular light and temperature.

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