GENETIC STUDIES OF WINTER HARDINESS IN *PISUM SATIVUM* L.

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Courtney Joy Holdt

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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

SUPERVISORY COMMITTEE:

Dr. Kevin McPhee
Chair

Dr. Edward Deckard

Dr. Thomas DeSutter

Dr. Francois Marias

Dr. Phil McClean

Approved:

April 17, 2017
Date

Dr. Harlene Hatterman-Valenti
Department Chair

ABSTRACT

Pea (*Pisum sativum* L.) is valued for its high protein content and symbiotic nitrogen fixation. The use of pea as a green manure and cover crop in rotations has been increasing. Pea is grown as a winter crop but current varieties are not able to survive the harsh winter of areas like North Dakota. This study has developed a protocol to select for winter hardiness in a greenhouse setting using a freeze chamber. Selections were made after acclimating for 4 weeks and freezing to -8°C. The protocol was used on a subset of two recombinant inbred line populations, Pril-1 ('Shawnee'/ 'Melrose') and Pril-2 ('Medora'/ 'Melrose'). The results of the greenhouse study correlated to field survival of both populations. The implementation of this protocol detected three QTL associated with winter hardiness, corresponding to previously discovered QTL. The use of this protocol will decrease the time needed for selection of winter hardy varieties.

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CHAPTER 1. JUSTIFICATION AND OBJECTIVES

Justification

Pea (*Pisum sativum* L.) is a member of the Leguminoseae family and broadly classified as a pulse crop. Pea was domesticated in the Middle East and was often grown in rotation with small grains (Zohary and Hopf, 1973). Legumes are dicotyledonous crop species characterized by their ability to fix atmospheric nitrogen in symbiotic association with Rhizobium bacteria. Classification as a pulse refers to a crop that is harvested for its dry seed held within a pod and that can be used for human and animal consumption. Legume crops are an excellent source of protein, can be beneficial in crop rotations, and can serve as a green manure intended to return fixed nitrogen and other nutrients to the soil (Araújo et al., 2014). These benefits have resulted in the maintenance of legume crops in cereal-based crop rotations. Production of dry pea in the United States increased by almost 44% between 2012 and 2013 (FAOSTAT, 2015). In the same year, North Dakota experienced an increase in dry pea production of approximately 25%, raising the area planted in 2013 to nearly 120,000 hectares (USDA, 2015). The Sustainable Agriculture Research and Education (SARE) program 2013-2014 reported that 55% of U.S. farmers in the U.S. use cover crops in crop rotations that included legumes. Pea production in North Dakota is exclusively spring-sown, due to harsh winter conditions; however, the potential for fall-sown pea and the agronomic benefits it offers has generated interest in including fall-sown pea crops in rotations. Austrian winter pea is grown in Oregon and Washington as a fall planted crop (USDA, 2015). The winter pea crop is used as a cover crop in rotation with cereal crops, primarily wheat (*Triticum*).

Current market classes for pea include green and yellow dry pea, marrowfat pea, and garden pea (McPhee, 2003). Marrowfat pea is the smallest market class and is used as a snack

item. The garden pea includes edible peas often consumed in the pod or shelled as immature seed. Dry peas are often split in processing and used for human and animal consumption. Superior visual quality is required for the human consumption market. Dry pea has 18-30% protein, 35-50% starch, and 4-7% fiber making them a good source of protein and nutrition. Recently, there has also been interest in utilizing pea as a protein supplement. This requires fractionation of the pea seed into many different components (Pietraski and Janz, 2010). This growing market is increasing the potential uses of pea and pea products such as pea flour for a gluten free option.

Fall planting offers producers the opportunity to sow pea in drier soil conditions in September, compared to the cool, wet conditions often experienced in the spring, allowing for better stand establishment. When sown directly into standing stubble, fall plantings can reduce soil compaction and damage to soil structure by reducing the amount of field work done by heavy machinery in one season (McPhee, 2003). The addition of pea to a crop rotation can provide added nitrogen, reduce disease cycles and aid in pest and weed management. By increasing some soil organisms, a pea crop added to a rotation can not only break disease cycles but also reduce the occurrence of diseases such as root rots (Krupinsky et al., 2002). While an increase in pea yield as a result of fall planting has not been proven, increases in wheat yield following a pea crop have been observed (Chen et al., 2012). When comparing wheat production in a wheat-wheat versus a pea-wheat rotation, the benefits of pea were large enough to affect the wheat yield. A study looking at nitrogen mineralization found crops following pea had greater nitrogen mineralization than those following canola or wheat (Beckie et al., 1997). After harvest, higher levels of nitrogen were also found in the plots with pea than with flax plots. The ability of

pea, and other legume crops, to fix nitrogen allows for greater nitrogen availability for the subsequent crop in the rotations (Grant et al., 2002).

Current winter pea varieties are only able to withstand winters in the northwestern part of the United States where conditions are relatively mild, with air temperatures averaging around -3°C to -1°C. North Dakota experiences significantly colder temperatures, with temperatures averaging between -17°C and -12°C. Development of winter pea varieties better suited to colder winter temperatures could positively impact the production of field pea in the Great Plains and Midwest states. Acclimation is vital for plant survival in cold conditions. One of the most important factors for winter survival is a strong plasma membrane (McKersie and Leshem, 1994). The plasma membrane is important for providing protection to cells and maintaining the viability of the crop. Cold tolerance gained through acclimation is due to many different factors. Some of the key changes include protein structure, lipid concentration, and enzyme activities (Uemura and Kawamura, 2014).

Breeding for winter pea varieties has been slow due to the need for consistent winter conditions to make adequate selections. With winter temperatures and snowfalls varying from year to year it is difficult to select for true winter hardy lines. The high level of uncertainty associated with the weather has spurred research into alternate screening procedures.

In order to produce improved winter pea varieties, a better understanding of the genes controlling winter hardiness in pea is needed. Six winter freeze damage (WFD) QTL were identified in a study by Lejeune-Hénaut et al. (2008) that mapped near the *Hr* gene, associated with delayed flowering. These QTL were also associated with *Vrn1*, a gene important for winter hardiness in barley and wheat. Gilmour et al. (2000) studied a group of binding factors, *CBF3*, linked to cold tolerance and noticed an increase in sugars, such as glucose and raffinose, led to

increased cold tolerance. Quantitative trait loci analysis completed by Dumont et al. (2009) found QTL controlling raffinose levels were associated with two of the freeze tolerance QTL from the work of Lejeune-Hénaut et al. (2008).

Knowledge of QTL can be used to conduct marker assisted selection (MAS) which can shorten the time it takes to produce a desired variety (Sleper and Poehlman, 2005). Marker assisted selection is able to shorten selection time by determining which lines have the desired traits without the need for as many winter field trials. Past studies have shown that conducting winter trials can be challenging and the results are not always consistent (Auld et al., 1983; Swensen and Murray, 1983; Liesenfeld et al., 1986). To date, only one study by Dumont et al. (2009) has reported results from a controlled setting that agrees with those from a field trial for pea. A controlled setting protocol was developed with the understanding that a 4-week acclimation period followed by freezing within a range of -7°C to -9°C allows for adequate detection of differences between lines (Swensen and Muray, 1983; Liesenfeld et al. 1986).

Objectives

The main objective of this study was to increase the efficiency of selecting winter hardy pea genotypes that are able to withstand the harsh winters of North Dakota and the Midwest. To achieve this objective, a protocol for testing winter hardiness in controlled settings must be developed. With the use of this protocol, a better understanding of winter hardiness in pea will be gained through QTL analysis.

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CHAPTER 2. LITERATURE REVIEW

Origin of field pea

Pea (*Pisum sativum* L.) was first domesticated in the Near East during the Neolithic era (7000 to 6000 B.C.) (Zohary and Hopf, 1973). Smýkal et al. (2011) expands this origin to include the Mediterranean. Zohary and Hopf (1973) report evidence of smooth seed coats found on pea in the sixth and seventh millennium. These findings suggest that pea was one of the first crops to be domesticated in the Middle East. Early domestication makes it difficult to pinpoint an area of origin because much of the Mediterranean and Middle East has seen much change (Smykal et al., 2011). Domestication of pea is believed to have taken place relatively close to the same time as the domestication of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) in the Neolithic Age (Zohary and Hopf, 1973). Records of planting peas with wheat and barley provide evidence that pea crops were considered well suited to growing in rotation with small grains. The production of pea quickly spread throughout the Middle East and into Europe.

Pisum is composed of three groups: *P. fulvum, P. abyssinicum,* and a complex group comprised of *P. elatius, P. sativum, P. humile* and *P. arvense* (Vershinin et al., 2003; Zohary and Hopf, 1973; Marx, 1977). Jing et al. (2010) used retrotransposons to develop a theory for the domestication of *Pisum* centered around *P. elatius* and *P. fulvum* as the wild species. While much uncertainty revolves around the domestication of current *P. sativum*, research now seems to be in agreement with a theory proposed by Jing et al. (2010) where selections of *P. elatius* were first made by farmers in the Fertile Crescent. From there, domestication spread across Southern Euroasia into the Indian subcontinent and Himalayan region. This first diversification event gave rise to Afghan ecotypes. The modern cultivated pea is believed to be the product of a second diversification event of a *P. elatius* sub-group, *P. sativum*. Weeden (2007) also suggests

that selections of *P. elatius* were carried through trade routes with large amounts of domestication occurring around the same time as the Kingdom of Egypt fell. The domestication of *P. abyssinicum* is known with much more certainty. A hybrid seed, formed from a cross between *P. elatius* and *P. fulvum*, is believed to have been transported to the region around modern day Ethiopia where it developed into a new species, *P. abyssinicum* (Jing et al., 2010). Genetic relationships suggest the following countries of origin for the four major species: *P. fulvum*, Israel and Syria; *P. abyssinicum*, Ethiopia; *P. elatius*, countries in the Eastern Mediterranean; *P. sativum*, Afghanistan, Nepal and South Central Asia.

Pea was brought to North America by Christopher Columbus where it quickly spread to much of the continent (Wade, 1931). The first report of a distinction between garden pea (*P. sativum* L.) and forage pea (*P. arvense* L. also referred to as *Pisum sativum*) was by Ruellius in 1563. While the spread of *Pisum* to Europe is not entirely clear, analysis of amplified fragment length polymorphisms (AFLPs) showed a clear distinction between pea samples of European origin and those of Asian origin (Dyachenko et al., 2014). The peas brought by Columbus would be of a European origin and most likely a variation of *P. sativum*, which originated from the Middle East and surrounding areas.

Pea is grown in semi-arid climates around the world. In 2010, Canada and the United States were the top producers of dry pea, followed by India, the Russian Federation, and France (Saskatchewan Ministry of Agriculture, 2015). In the United States, pea production for processing, mostly canning and freezing purposes, is located in Wisconsin, Minnesota, and Washington (Muehlbauer and McPhee, 1997). Production of dry pea, which can be used for both animal and human consumption, is greatest in Montana and North Dakota (USDA, 2015). Dry pea in North Dakota has shown higher yields compared to Montana. In 2015, 438,275 ha of dry

pea were harvested in the United States with 151,757 of those ha produced in North Dakota (USDA, 2015).

Uses and market classes

Pea is utilized as animal feed, green manure, and for human consumption. The major market classes for pea include fresh crop types such as canning, freezing, and edible pod (snap and snow pea) as well as mature dry seed types including marrowfat, smooth green, smooth yellow and Austrian winter peas (Muelhbauer and McPhee, 1997). Smooth yellow or green dry peas are sold whole or as a split product. There is a growing industry aimed at utilizing peas in new forms. Peas can be broken down and separated into starch, protein and fiber that can be used to replace ingredients such as flour or act as a supplement (Pietrasik and Janz, 2010). Pea flour is a common way of integrating pea, and is an extra source of protein, in pasta or snack foods. Fractionated pea products have shown to have a lower glycemic index and are gluten free.

Marrowfat peas have a larger green seed with a distinctly dimpled appearance. Marrowfat pea is used to produce snack items such as wasabi peas. Edible pea pod varieties, also known as snap peas, as well as freezer and canning peas, use green cotyledon varieties while freezer peas have a darker hue than canning peas. The final market class, 'Austrian' winter pea (AWP), is most commonly used as animal feed or green manure but, when necessary, can be used as split yellow pea. The AWP varieties are derived from *P. sativum ssp. arvense* L. 'Austrian' winter pea is currently only produced in Idaho, Montana and Oregon (USDA, 2015). A total of 8,498 hectares of AWP were harvested in 2015. A steady increase in AWP production has been seen since 2013 when only 5,665 hectares were harvested. Canada has been the leading worldwide producer for dry pea for the last 15 years (2000-2014) (FAO, 2016).

Growth habits

Described as an annual climbing herb, a pea plant is comprised of 20-25 nodes on average, with petioles, leaflets and stipules at each node (Muehlbauer and McPhee, 1997; Elzebroek and Wind, 2008). The cotyledons of field pea remain below the soil surface, characteristic of hypogeal emergence (Muehlbauer and McPhee, 1997). This trait allows for regrowth if injury to the above ground plant occurs. While eight distinct leaf phenotypes can be observed in pea, the normal leaf and semi-leafless varieties are the most common. Semi-leafless varieties are often favored due to reduced lodging and less foliar disease associated with the plants ability to stay more upright. Reduced lodging has also been correlated with an increase in yield (Kielpinski and Blixt, 1982). Pea has both determinate and indeterminate growth; however, nearly all pea varieties in production are indeterminate which allows for production of pods at successive nodes throughout the growing season (Muehlbauer and McPhee, 1997).

Pea, like wheat and many other crops, has both spring- and fall-sown varieties. Common traits associated with winter hardiness across different species include: prostrate growth, branching and reduced height (Lejeune-Hénaut et al., 2008). Differences between spring and winter-types are observed in internode length, branching and leaf shape (Markarian and Andersen, 1966). Low growing plants with branching often represent winter-type pea plants. A study by Markarain and Andersen (1966) found that rosette formation, increased vegetative growth at the top of the plant, is necessary for a plant to be winter hardy. The formation of this rosette in the fall correlated with the survival of certain lines in the spring. This finding was further confirmed by Lejeune-Hénaut et al. (2008) when studying the role of the *Hr* gene in winter hardy lines. The study of winter hardiness in *Arabidopsis* showed that lines with increased protein expression related to increased winter hardiness grew shorter to the ground, sprawling

rather than standing straight, flowered later than control plants, and had shorter petioles (Gilmour et al., 2000). Lines with these phenotypic characteristics were found to perform better in winter hardiness studies. In pea, a delay in growth or transition to the reproductive stage was observed with fall-sown crops experiencing flowering earlier than the spring-sown crop (Lejeune-Hénaut et al., 2008; Chen et al., 2006). Only producing vegetative growth in the fall allows for better winter survival and gives the crop an advantage over other crops in the spring.

Fall-sown pea fits well into crop rotations and allows for a longer growing period in the spring resulting in the crop flowering during the cooler period of the season (Chen et al., 2006). Gan et al. (2002) noted that pea planted earlier in the spring consistently had higher yield than later spring planting dates. Drought stress is often experienced later in the spring season thus giving an advantage to earlier planted pea crops that produce more vegetative growth before the stress occurs. High temperatures during the summer months can lead to poor pod formation and seed set (McPhee, 2003). Fall plantings allow for establishment and vegetative growth to occur in warmer and drier soil conditions than those often found in spring.

Agronomic benefits

Legume crops offer the opportunity to reduce fertilizer applications while maintaining yields due to symbiotically fixed nitrogen additions to the plant (Araújo et al., 2015). Along with human and animal consumption, pea can be used as a green manure which benefits the following crop. A survey by Sustainable Agriculture Research and Education (SARE) conducted in 2013-2014 found 55% of U.S. farmers use cover crops, including a legume. The addition of pea in crop rotations and as a cover crop can improve the cropping system in many ways. The ability of pea to form symbiotic relationships with bacteria that fix nitrogen makes it a valuable component of rotations with non-nitrogen fixing crops (Grant et al., 2002). A pea crop is able to add nitrogen

to promote yield and reduce nutrient depletion of the soil. In a study comparing pea stubble to wheat and canola stubble, there was 42% greater nitrogen mineralization on pea stubble (Beckie et al., 1997). While continuous cropping is generally a negative practice, continuous cropping with a pulse crop in the rotation may provide more nitrogen via mineralization and have long term benefits (Grant et al., 2002). When pulse crops are included in rotations an increase in nitrogen found in the crop planted following a pea crop, as well as a reduced need for nitrogen fertilizer, has been observed. Winter wheat yields increased with the inclusion of field pea in the crop rotation (Chen et al., 2012).

The inclusion of pea in crop rotations provides benefits in addition to increasing available nitrogen. Crop rotations are important for managing the spread of pests and disease as well as moisture and nutrient levels in the soil (Krupinsky et al., 2002). Observations have also indicated the inclusion of pea in a rotation can enrich the population of beneficial organisms found in the soil and reduce the risk of diseases on cereal crops. The ability to sow pea in the fall reduces the potential risk for soil damage that can occur when wet fields are worked too early in the spring. Spring pea, along with other legume cover crops, can increase the wet aggregate stability of a field, allowing for protection of soil structure from damage in a wet spring (Blanco-Canqui et al., 2013). The same study also found fields with spring pea as a cover crop had reduced runoff and significantly lowered rates of sediment loss. By maintaining soil structure, the cover crop can also reduce the amount of soil lost due to wind and water erosion. Inclusion of pea in crop rotations allows for field work to be more evenly distributed across the fall and spring planting seasons. A fall planted pea crop may also experience an increase in biomass since pod filling will occur during the cooler months of the summer and drought will be less of an issue for more mature plants (Chen et al., 2006).

Winter hardiness

Winter hardiness, as defined by McKersie and Leshem (1994) in "Stress and Stress Coping in Cultivated Plants", is a composite of stress tolerances including tolerances of freezing, ice-encasement, flooding, heaving, desiccation, and snow molds. The winter hardiness trait desired in most winter crops, is not as clearly understood as flower color or plant height. Winter hardiness is a quantitative trait due to the large variation in levels of hardiness produced among varieties within a species (Parodi et al., 1983). Plants able to withstand freezing conditions will generally utilize protective mechanisms to maintain viability in cold temperatures and only experience lasting injury due to the freezing process itself (Uemura and Kawamura, 2014). A plant can survive frost damage, but if intercellular damage is experienced, survival is limited. Cold events cause changes in protein structure, lipid concentration, and enzyme activity that leads to multiple changes in the cell.

The ability of plants to withstand freezing conditions is directly affected by cold acclimation. An acclimation period involves a slow transition to low temperatures. Extracellular freezing forms ice crystals between cells when the temperature is gradually decreased (Guy, 1990). Without acclimation or when the temperature drops suddenly, intracellular freezing occurs and ice crystals form within cells causing them to rupture. Equilibrium freezing gradually decreases the liquid level between cells while non-equilibrium freezing causes a sudden drop in liquid level, creating large, destructive ice crystals (reviewed by Olien, 1967). Equilibrium freezing is less detrimental than non-equilibrium. A strong plasma membrane is required to protect cell integrity from ice damage (McKersie and Leshem, 1994). The plasma membrane is maintained by a combination of lipids and proteins. Many of the protective protein and lipid

concentrations observed in freeze tolerant plants are produced during the acclimation period (Uemura and Kawamura, 2014).

Studies have found an increase of abscisic acid (ABA) in plants tolerant to freezing conditions (McKersie and Leshem, 1994; Levitt, 1980). Higher levels of ABA have not only been linked to increased winter hardiness in pea but are also hypothesized to play a role in the induction of cold-regulated (COR) proteins (Welbaum et al., 1997). Transcription factors play a key role in gene expression. There are an estimated 45 genes involved in a group of transcripts known as C-repeat binding factor (CBF) transcripts that have been linked to cold tolerance (Fowler and Thomashow, 2002). A total of 306 cold induced genes were identified in a study of *Arabidopsis* by Fowler and Thomashow (2002). Sixty of these genes were not affected by CBF transcripts and 41 of the remaining genes were found to be up- or down-regulated in all CBF-expressing lines. The genes influenced by the CBF genes have a wide range of functions, some of which are: transcription, signaling, cell defense and cellular biogenesis. A group of COR genes have been linked to an increased accumulation of winter hardiness (Gilmour et al., 1998; Artus et al., 1996). A study by Gilmour et al. (1998), which investigated the function of COR genes suggests that the chain reaction, leading to increased winter hardiness, begins with the accumulation of the CBF transcripts. In this study, high levels of both COR and CBF gene products correlated with an increase in winter hardiness. The COR genes were not found to be linked to each other in the genome at all despite the CBF genes being in sequential order on chromosome 4 of *Arabidobsis*. In a study where CBF1 genes were over expressed, COR gene expression was identified without low temperature treatment (Jaglo-Ottosen et al., 1998). This study confirms that CBF genes are responsible for the expression of COR genes, and they are not necessarily triggered only by cold. While COR gene expression is controlled by CBF transcripts,

Fowler and Thomashow (2002) found 60 genes linked to cold tolerance that were not expressed in plants with induced CBF expression. This indicates that there are multiple pathways leading to increased cold tolerance. One example of this was observed when Gilmour et al. (2000) investigated the change in sugars when plants were acclimated to cold temperatures. An accumulation of glucose, fructose, sucrose and raffinose were measured when expression of CBF3, a gene belonging to the CBF transcripts family, was induced in plants. An increase in two enzymes related to sucrose production, sucrose- phosphate- synthase (SPS) and sucrose synthase (SuSy), were also measured but levels were the same between the CBF3 and control plants that were cold acclimated. These results suggest that another pathway must be increasing the sugar levels since these two enzymes do not react to changing CBF3 levels.

Proline levels are linked to many stresses in plants such as drought, salinity and cold tolerance (Nanjo et al., 1999). Specifically, in plants where the degradation of proline was prohibited, an increase in tolerance to cold stress was seen. Proline is found to protect the integrity of cell membranes and even reduce the formation of ice crystals within cells during freezing (Rudolph and Crowe, 1985). Higher proline levels were correlated with normal ATPase levels and less damage of the cell membranes was seen. Low levels of proline can cause increased cell damage due to high levels of ATPase present in the cell. Specifically, in *Arabidopsis*, *CBF3* genes are linked to the accumulation of sugars but also proline (Gilmour et al., 2000). The expression of the proline biosynthetic enzyme Δ-pyrroline-5-carboxylate synthase (P5CS) was linked to higher levels of proline in cold conditions. A significant increase in P5CS levels was observed in plants with CBF3 overexpressed again, suggesting a link between the CBF transcripts and cold tolerance. Abscisic acid is not only linked to increased cold tolerance but has also been shown to increase proline levels in cold treated maize cells (Chen and Li,

2002). This increase in proline is due to lower levels of lipid peroxidation, which can lower the integrity of the cell and cause the loss of important substrates. Greater cold tolerance was seen in transgenic *Arabidopsis* lines, anti-ProDH, where proline degradation was stopped via anti-sense cDNA than in the wild-type. The study by Chen and Li (2002) is another example of the positive relationship between proline and cold tolerance. This yet again shows that cold tolerance requires some combination of many factors, be it an increase in CBF transcripts, ABA levels, proline, or all three.

Vernalization also plays a part in the winter hardiness of certain crops. Amasino (2004) defined vernalization as the process in which flowering is promoted by long periods of cold exposure. Vernalization genes in winter wheat (*VRN1, VRN2, and VRN3)* are responsible for suppressing flowering genes until the vernalization period is complete (Amasino, 2004). The *FRI* and *FLC* genes, which are key to pathways controlling flowering, play a role in initiating a winter annual habit as well. In winter hardy varieties, delayed flowering is a key characteristic for survival of the crop. Vernalization is required to initiate the transition from the vegetative to reproductive stage and increases low temperature tolerance (Fowler et al., 2001). A transition from vegetative to reproductive growth too early would decrease the energy storage and, therefore, the ability of the plant to survive the winter months to complete maturity in the spring. The transition from the vegetative phase to the reproductive phase must be delayed to provide more cold tolerance during the winter months. A greater tolerance to low temperatures has been associated with later transitioning from vegetative to reproductive growth, which is beneficial in fall plantings. While current winter pea varieties do not require a true vernalization to flower in spring, the *Hr* gene has been linked to linkage group III in pea (Murfet, 1973). Current varieties will flower if planted in the spring but this transition will occur much later than spring varieties.

The *Hr* gene is responsible for prolonging the vegetative phase of winter pea, when the *Sn* gene is also present. The prolonging of the vegetative phase, along with the inhibition of flowering due to the *Sn* gene leads to winter varieties not flowering until longer days in spring (Murfet, 1971; Murfet, 1973).

Enhancing breeding efforts

To improve current crop varieties, a breeder must observe phenotypic differences, make selections, and evaluate new progeny. This selection process can take many generations when only using phenotypic data. Marker assisted selection (MAS) increases efficiency and decreases time requirements of the selection process in breeding programs (Lande and Thompson, 1990). Marker assisted selection can reduce time and labor by selecting for desired traits earlier in the breeding process. Three key components required for MAS are: a genetic linkage map, molecular markers for identifying QTL related to the trait, and the development of PCR markers related to the locus of interest (Sleper and Poehlman, 2005). In theory, when a certain gene and marker combination is identified, a population can be screened for that gene and only lines with the desired marker allele will be advanced. Marker assisted selection has proven useful in situations for traits that are difficult to manage or are environmentally specific. Genetically, they have proven useful for backcross programs for maintaining a recessive allele, and for pyramiding of traits, such as for disease resistance (reviewed by Xu and Crouch, 2008). When selections are based on phenotypic data only, there is the potential to lose a desired allele due to the masking of heterozygotes. This loss can be minimized, in some situations, by using markers to select for multiple traits at one time with MAS (Xu and Crouch, 2008). Over the years, many types of molecular markers have been developed. Restriction fragment length polymorphisms (RFLP) were some of the first markers used to develop molecular maps (Sleper and Peohlman, 2005).

The use of RFLPs and amplified fragment length polymorphisms (AFLPs) were discontinued due to the use of radioactive material required to visualize polymorphisms. These markers were replaced by random amplified polymorphic DNA (RAPDs). With the development of simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers, which are both codominant and more accurate, the use of RAPDs has decreased. Single nucleotide polymorphisms can increase the accuracy and efficiency of MAS since they are often closely linked to the desired loci (Xu and Crouch, 2008). Past markers, such as RFLPs or RAPDs, were typically near but not at the desired loci allowing for the trait to potentially be lost due to recombination.

Quantitative traits are very challenging to select and improve since they are controlled by more than one gene throughout the genome. Agronomic traits such as yield and winter hardiness are just a few of the important traits that are classified as quantitative. Although MAS has been successful when selecting for a quantitative trait, such as disease resistance, it may fall short with regards to many other quantitative traits. Since these traits are controlled by multiple loci within the genome, MAS is only able to identify loci involved with the variation being observed that has previously been linked to a marker (Meuwissen et al., 2001; Schmidt et al., 2016). Genomic selection (GS) more accurately identifies quantitative trait loci because it accounts for variation that takes place across multiple chromosomes (Meuwissen et al., 2001). In barley, GS was proven successful, with the potential to be more efficient, in selecting for malting quality, an important quantitative trait (Schmidt et al., 2016). Studies have shown that GS can provide advantages over phenotypic selection, both in accuracy and efficiency, when applied to breeding programs (Michel et al., 2016; Meuwissen et al., 2001; Schmidt et al., 2016). A study in winter wheat found GS to identify 15% more lines correctly compared to phenotypic selection when

looking at grain yield (Michel et al., 2016). The accuracy of these predictions was also 36% higher than predictions made using only phenotypic selections.

Injury quantification

Injury caused by biotic and abiotic stresses can reduce the fitness of a crop leading to yield loss, or in the case of a cover crop, poor stand and ground cover. One major area of study which focuses on the measurement and comparison of such injury is plant pathology. Pathology studies focus on disease development, progression and location. When evaluating lines for disease resistance it is important to take all these components into consideration. Disease progression can be quantified using an area under disease progress curve (AUDPC) (Jeger and Vilijanen-Rollinson, 2001). An area under the curve can be beneficial since it takes into account both the injury level detected as well as the rate at which the injury occurred (Hernandez et al., 1993). To account for both parameters, the linear regression and the mean of the trait being measured are used to calculate one AUDPC value. Some variation of an AUDPC equation is often desired because it allows for the rate of disease progression to be considered in one value given to each sample (Shaner and Finney, 1977). A study by Haynes and Weingartner (2004) looked at potato blight resistance with AUDPC values calculated with different amounts of observation data and found that the results agreed with the AUDPC calculated at the end of the experiment. This suggests the number of observations necessary to make distinctions among lines can be reduced. Three requirements need to be considered when using AUDPC; 1) the injury or effect should be measured as a rate of infection; 2) the samples should all be exposed for the same amount of time; and 3) if environment interacts with the disease, results may be skewed (Jeger and Vilijanen-Rollinson, 2001). The same principles that are applied to disease progression can also be applied to injury from abiotic stresses.

Pea genetics

The pea genome is composed of seven chromosomes and is roughly 4.4 Gb in size. There is currently no complete genome sequence for pea, however; many consensus maps consistently place genes on the seven linkage groups (Ellis et al., 1992; Gilpin et al., 1997; Laucou et al., 1998; Loridon et al., 2005; Aubert et al., 2006; Duarte et al., 2014; Tayeh et al., 2015). Some of the first consensus maps were formed using RAPD, RFLP and AFLP markers. As science has advanced, SNP markers have increased maker density on the latest consensus maps. These consensus maps are used to map areas of interest such as vine length, seed color, and photoperiod response. The latest consensus map by Tayeh et al. (2015) was 794.9 cM in length and consisted of 15,079 SNP markers.

Synteny between pea and various legume crops has been identified. The largest amounts of synteny with pea are found with lentil (*Lens culinaris*), *M. truncatula* and chickpea (*Cicer arientinum* L.) (Table 1) (Aubert et al., 2006; Sindhu et al., 2014; Leonforte et al., 2013; Duarte et al., 2014). Some syntenic blocks have also been seen when comparing to *L. japonicas* and pigeon pea (*Cajanus cajan* L.).

Table 1. Synteny relationships among linkage groups in pea, *M. truncatula*, lentil and chickpea (Findings combined from: Aubert et al., 2006; Sindhu et al., 2014; Leonforte et al., 2013).

pea	*M. truncatula*	lentil	chickpea
I	5	5	2,8
II	1	1,5	4
III	3,2	3	7
IV	8	7	7
V	7	6	2
VI	2,6	2	1,2,8
VII	4,8	4	6

While macrosynteny between *Arabidopsis* and legume species is not common, some synteny has been observed for genes related to flowering between *Arabidopsis* and *Medicago*

(Hecht et al., 2005). Homologs of *VRN1*, responsible for vernalization in *Arabidopsis*, were found in pea. Sequences for all but two of the autonomous gene pathways in *Arabidopsis* were found in pea, suggesting a level of synteny. Hecht et al. (2005) found the position of *M. truncatula* genes related to flowering to be similar to *GIGAS* and *Hr*, two flowering genes in pea. These genes are located on chromosome V and chromosome III of pea, respectively. Despite the absence of two important flowering genes, *FRI* and *FLC*, sequences showing similar responses have been located in *M. truncatula* and pea through synteny relationships. Since the pea genome is not sequenced, these synteny relationships can be used to learn more about the pea sequence and areas of interest. For instance, the quantitative trait loci (QTL) for frost tolerance in *M. truncatula* is similar to the frost tolerance QTL identified in pea by Lejeune-Hénaut et al. (2008) (Tayeh et al., 2013). Since *M. truncatula* has a sequenced genome, this relationship could help better understand the genetics of winter hardiness in pea. The pea genome is significantly bigger than some of these species it is found to have synteny with. This is due in part to the high number of transposable elements and genetic repeats which also make sequencing the pea genome challenging (Kaló et al., 2004).

To fully understand how winter hardiness in pea is controlled, sequencing the whole genome of pea will be beneficial. Currently, there is an International Consortium of Pea Genome Sequencing group working on achieving this goal.

Current winter hardiness research

Winter hardiness is historically tested through field trials but there is interest in developing a protocol for testing in controlled settings due to the time and effort required for field trials (Auld et al., 1983; Swensen and Murray, 1983; Liesenfeld et al., 1986). This concept is supported from the results that confidently identified winter hardiness genes in plants through

field trials (Lejeune-Hénaut et al., 2008). While some studies found similar results between field and controlled setting trials, others were inconclusive. From the results of a controlled freeze experiment, Swensen and Murray (1983) were able to suggest that studies with freezing temperatures between -6 and -9°C could detect differences in winter hardiness between pea genotypes. These parameters were also suggested by the work of Fiebelkorn (2013) who found -8°C and a three-week acclimation period to give the most differential survival among lines. A study conducted by Dumont et al. (2009) is one of the first to produce reliable results from both field experiments and experiments carried out in controlled settings. The development of an accurate protocol for testing winter hardiness in a controlled setting needs to be reevaluated since many of these studies were conducted more than twenty years ago.

In grains the expression of photoperiod responsive genes were found to be linked to cold tolerance (Lejeune-Hénaut et al., 2008). Still, there is uncertainty as to whether this connection can be made in peas and other legumes as well. A connection between frost tolerance and photoperiod response was noted by Lejeune-Hénaut et al. (2008). Previous studies have found an acclimation period of four weeks at 4°C and a freezing episode ranging from -7 to -9°C to be able to make distinctions between spring- and winter-type genotypes (Swensen and Murray, 1983; Liesenfeld et al. 1986). Dumont et al. (2009) detected differences in winter hardiness with only an 11-day acclimation period and a 5-day freezing period among a population carrying the *Hr* allele determined by Lejeune-Hénaut et al. (2008) to be linked with freezing tolerance. Winter hardiness was measured by evaluating the amount of dead tissue at the end of the recovery period, and the levels of electrolyte leakage and RuBisCo. In *M. truncatula* lower levels of electrolyte leakage and a higher chlorophyll content index were associated with greater freezing tolerance (Avia et al., 2013).

Liesenfeld et al. (1986) concluded that as few as three additive genes could be responsible for winter hardiness differences among genotypes. These genes are associated with traits such as flowering and rosette formation in pea. Lejeune-Hénaut et al. (2008) confirmed the *Hr* locus in pea to be responsible for the initiation of flowering due to photoperiod ques. Another finding from this study was that the *Hr* locus was linked with winter frost damage (WFD) on linkage group (LG) 3. Lejeune-Hénaut et al. (2008) confirmed a link between *Vrn1* genes and increased winter hardiness in barley and wheat. QTL mapping has been used to observe the relationship between plant response to winter hardiness and response to developmental genes (Lejeune-Hénaut et al., 2008). Six QTL have been linked to winter hardiness in pea from the study conducted by Lejeune-Hénaut et al. (2008). The three QLT most consistently associated with winter hardiness, across locations in the study, were: WFD 3.1, WFD 5.1 and WFD 6.1, found on LG3, LG5 and LG6, respectively, of the linkage map created. Mt-FTQTL6, a QTL linked to freeze tolerance in *M. truncatula,* is syntenic to QTL WFD 6.1 (Tayeh et al., 2013). Additional QTL were found on LG1, LG4 and LG6 of *M. truncatula* (Avia et al., 2013). A study in lentil, another crop syntenic with pea, found three QTL related to winter survival on LG4, LG3 and LG6 (Kahraman et al., 2004). Further investigation by Kahraman et al. (2004) into the synteny between 11 different legume species and the markers associated with Mt-FTQTL6 found eight markers to be common among all species. Dumont et al. (2009) confirmed the connection between the QTL found on LG5 and LG6 and detected the QTL on LG6 in both controlled environment and field experiments. This study also showed an increase in RuBisCo and raffinose levels with a positive impact on the winter hardiness level in pea. The QTL for RuBisCO and raffinose, as well as electrolyte leakage, were found to associate with QTL for frost damage on LG5 and LG6. A QTL related to branching was also found on LG5 which is relevant due to the

increase in branching associated with winter hardy lines. This study confirms that other components, not just the *Hr* allele and photoperiod response, are involved in cold acclimation in pea.

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CHAPTER 3. DETECTION OF WINTER HARDINESS IN PEA (*PISUM SATIVUM* L.)

Introduction

Pea (*Pisum sativum* L.) is a legume crop most commonly used for human and animal consumption. The nitrogen fixation that results from the symbiotic relationship with rhizobium has made pea a positive addition to crop rotations and cover crop mixtures that improves soil health, interrupts disease and pest cycles, and reduces the amount of nitrogen fertilizer needed (Araújo et al., 2015; Grant et al., 2002; Krupinsky et al., 2002). Given that pea is a cool-season crop, the planting of pea as a winter cover crop has been an area of growing interest.

In the northwestern part of the United States, specifically in Washington and Montana, there has been success in growing pea varieties that can survive through the winter months as a cover crop. Increases in wheat yield following a planting of pea has been documented but further studies are needed (Chen et al., 2012). The problem for winter pea is predominately in states such as North Dakota and Minnesota where the winter months experience far colder temperatures and snow remains for longer. Low temperatures around -12°C can occur in an average North Dakota winter. Currently, there are no pea varieties that are able to withstand these conditions.

The development of new winter varieties can take longer than the development of spring varieties due to the selection process. In order to make selections on a winter variety, temperatures must be low enough to cause variation among lines but not so cold to kill them. Winter conditions, both temperature and snowfall, vary greatly from year to year and make it difficult to obtain multiple years of usable data for winter selections among breeding lines. The selection time needed to identify winter varieties would be decreased if selections could be made in a controlled environment, such as a greenhouse and freeze chamber. Currently, selection

studies (Swensen and Murray, 1983; Dumont et al., 2009) have not consistently found a freezing protocol that also correlates with field performance. Along with the use of a controlled environment, marker assisted selection (MAS) and genomic selection (GS) could also shorten the selection process (Lande and Thompson, 1990; Schmidt et al., 2016). Both of these techniques utilize the knowledge of markers near associated loci to aid in selections. With a quantitative trait, such as winter hardiness, quantitative trait loci (QTL) are identified because there is not one specific gene linked to its inheritance.

A protocol was developed using known varieties to confirm its function. The protocol was then used to collect phenotypic data from two populations. Quantitative trait analysis was conducted with this phenotypic data, as well as data from the field study to identify genetic regions related to winter hardiness.

Materials and methods

Greenhouse study

Establishment of methodology- Experiment 1

Genetic material. Sixty-two germplasm lines were tested for tolerance to freezing conditions using an ESPEC BTU-433 (Hudsonville, MI) benchtop freeze chamber. This set of germplasm was composed of 44 breeding lines and 18 known varieties (Table 2). Eight of the known varieties were ‘Austrian’ winter pea (AWP) types, known to typically have greater winter hardiness.

Table 2. Winter pea genotypes screened in experiment 1 for freezing tolerance.

Variety	Market class	Vine type*	Leaf type	Cotyledon color
Apache	CSC	short	semi-leafless	yellow
April	AWP	short	semi-leafless	yellow
Aravis	CSC	short	normal	yellow
Assas	AWP	long	normal	yellow
Cheyenne	CSC	short	semi-leafless	yellow
Dove	CSC	short	semi-leafless	green
EFB333	AWP	long	normal	yellow
Fenn	AWP	long	normal	yellow
Grana	CSC	short	normal	yellow
Granger	AWP	long	semi-leafless	yellow
Lynx	CSC	short	semi-leafless	green
Melrose	AWP	long	normal	yellow
Natura	CSC	short	semi-leafless	yellow
Picard	AWP	long	normal	yellow
Romack	AWP	long	normal	yellow
Specter	CSC	long	semi-leafless	yellow
Whistler	CSC	short	semi-leafless	yellow
Windham	CSC	short	semi-leafless	yellow
PS0017018	CSC	long	normal	yellow
PS0230F063	CSC	short	semi-leafless	green
PS0230F092	CSC	short	semi-leafless	green
PS0230F210	CSC	long	normal	yellow

Table 2. Winter pea genotypes screened in experiment 1 for freezing tolerance (continued).

Variety	Market class	Vine type*	Leaf type	Cotyledon color
PS03100635	CSC	long	normal	yellow
PS03100848	CSC	long	normal	yellow
PS03101120	CSC	long	normal	yellow
PS03101160	CSC	short	semi-leafless	green
PS03101170	CSC	short	semi-leafless	green
PS03101205	CSC	short	semi-leafless	green
PS03101247	CSC	short	semi-leafless	green
PS03101269	CSC	long	semi-leafless	green
PS05300069	CSC	short	normal	red
PS05300075	CSC	short	normal	red
PS05300077	CSC	short	normal	red
PS05300078	CSC	short	normal	red
PS05300083	CSC	short	normal	red
PS05300108	CSC	short	semi-leafless	yellow
PS05300126	CSC	long	semi-leafless	yellow
PS05300205	CSC	short	semi-leafless	green
PS05300213	CSC	long	semi-leafless	green
PS05300225	CSC	short	normal	green
PS05300228	CSC	short	normal	green
PS05300234	CSC	short	semi-leafless	green
PS05300239	CSC	short	semi-leafless	green
PS06300003	CSC	long	semi-leafless	green

Table 2. Winter pea genotypes screened in experiment 1 for freezing tolerance (continued).

Variety	Market class	Vine type*	Leaf type	Cotyledon color
PS06300007	CSC	long	semi-leafless	green
PS06300008	CSC	long	semi-leafless	green
PS06300016	CSC	short	semi-leafless	green
PS06300017	CSC	short	semi-leafless	green
PS06300022	CSC	short	semi-leafless	green
PS06300024	CSC	short	semi-leafless	green
PS06300028	CSC	short	semi-leafless	green
PS06300048	CSC	long	semi-leafless	green
PS06300050	CSC	long	semi-leafless	green
PS06300057	CSC	short	semi-leafless	yellow
PS06300060	CSC	short	semi-leafless	yellow
PS06300061	CSC	long	semi-leafless	yellow
PS06300064	CSC	long	semi-leafless	yellow
PS06300075	CSC	long	semi-leafless	yellow
PS06300108	CSC	long	normal	yellow
PS06300119	CSC	short	semi-leafless	yellow
PS06300142	CSC	long	semi-leafless	yellow
PS06300190	CSC	long	semi-leafless	yellow

* refers to internode length; CSC, clear seeded coat; AWP, Austrian Winter Pea

Experimental design. The genotypes were divided into six sets for each experimental run due to space constraints in the freeze chamber. Each set was arranged in a randomized complete block design (RCBD) with 12 genotypes, two of which were checks, and 6 replicates for a total

of 72 plants per freeze cycle. ‘Melrose’, a variety known to be winter hardy, and ‘Whistler’, a spring type variety, were used as checks in each set. Three experimental runs were completed. The first experimental run only contained 50 genotypes while the other two runs contained all 62 genotypes (Table A1). Twelve genotypes were added after running the first run to increase the diversity of the experiment. Seeds were scarified and sown at a depth of 7mm in 5mm x 5mm pots filled with Pro-Mix Flex (Premier Horticulture Inc. Quakertown, PA), and fertilized with Osmocote Plus 15-9-12, 3-4 month formula (Everris Inc.). The pots were watered daily. Greenhouse conditions were maintained at 25/20°C day/night temperature, and 14/10 hr day/night photoperiod during the growth period.

Two-week old seedlings were acclimated at 4°C for 4 weeks under T8 32 watt Tri Phosphor 6500K fluorescent lamps (Hatch Lighting, Tampa, FL) with a 12-hour photoperiod and watered as needed. After the acclimation period, the trays were loaded into the freeze chamber equipped with a Watlow Series F4S/D programmer and a YOKOGAWA FX1000 Paperless Recorder (Tokyo, Japan). The recorder was used to collect temperature readings, from 20 probes arranged throughout the chamber, during the freeze cycle. Replicates 1-3 of each set were placed on the bottom shelf of the freezer and 4-6 placed on the top for consistency. The freezing cycle began at 4°C with a reduction in temperature to -8°C at a rate of 1°C /hour. The minimum temperature, -8°C, was held for one hour, and then increased 1°C /hour to 4°C. Once the freeze chamber cycle was complete, the trays were moved back to the acclimation chamber for 72 hr. After this brief acclimation period, the plants were moved to the greenhouse where they were scored approximately every 72 hr, with the first score being taken on the day they were moved to the greenhouse. A total of seven scores were collected for each set and the plants were scored on

a 1 to 9 scale (adapted from Fiebelkorn, 2013) (Table 3). Scores were given based on the percentage of living tissue present (example of scoring found in Figure A8).

Table 3. Descriptions of visual scores for pea plants subjected to freezing stress.

Score	Visual ID
1	Plant is completely green with or without regrowth
2	Plant has minimal freezing damage
3	Plant has at least 75% living tissue
4	Plant has between 50-75% living tissue
5	Plant has 50% of tissue living
6	Plant has between 25-50% living tissue
7	Plant has 25% living tissue
8	Plant is almost dead but still has some living tissue
9	Plant is completely dead

The injury scores were used to calculate the area under the injury curve (AUIC) for each pot using the following equation:

$$AUIC = \sum_{i=1}^{n} \left(\frac{y_i + y_{i+1}}{2}\right) (t_{i+1} - t_i) \quad (1)$$

where "i" represents the scoring interval, "y" represents the injury score given and "t" is the number of days after freezing. The AUIC values were used to represent the level of winter hardiness for each genotype. Smaller AUIC values indicate greater predicted winter hardiness for that line.

Statistical analysis. Hartley's test for homogeneity was used to determine if the three experimental runs could be combined. Runs were only combined for analysis if the F-max value, calculated as the ratio between the largest mean square and the smallest mean square, was less than 10. Analysis of variance was completed as an RCBD using the general linear model (GLM)

procedure in with SAS® software version 9.3 (SAS Institute, Cary, NC) to evaluate the differences in AUIC values. Error mean squares were used to evaluate expected mean squares to determine the proper F-test for each factor. The assumption was made that sets had no effect within runs due to being planted only two days apart.

Phenotyping of recombinant inbred lines

Genetic material. Two recombinant inbred line (RIL) populations were created in 2005 at Washington State University, Pullman, WA (Table 4). 'Melrose', the common parent among the two populations, is reported to survive the winter months and flower in early June when planted in the fall in northern Idaho, where it was developed (Auld et al., 1978). 'Shawnee', one of the susceptible parents, is a large seeded, yellow cotyledon variety, which does not produce vine branching (Muehlbauer, 2002). The third parent, 'Medora', is a spring variety with a green cotyledon and semi-leafless morphology.

Table 4. Identifying traits of the parents used to create Pril-1 and Pril-2.

	Melrose	Shawnee	Medora
Seasonal type	winter	spring	spring
Cotyledon color	yellow	yellow	green
Branching	present	absent	absent
Leaf type	normal	normal	semi-leafless
Vine length	long	long	short
Powdery mildew resistance	absent	present	present
Neoplasm	present	absent	.

"." unknown

Freeze chamber study for Pril-1 and Pril-2. The RIL population, denoted as Pril-1 ('Shawnee' / 'Melrose'), was tested using the freeze chamber protocol denoted as FCS-1 (Freeze Chamber Study-1), to gain phenotypic data for quantitative trait loci (QTL) analysis. Due to space constraints, 160 lines selected according to seed availability from Pril-1 were divided into 10 sets. Each set contained the parents of the population as controls. The sets were organized as

an RCBD with 4 replicates of 16 genotypes in each set. Each set was tested according to the detailed protocol established and described in the establishment of methodology section. The study was repeated three times. A second study, FCS-2, using the second RIL population ('Medora'/'Melrose'), denoted as Pril-2, was completed on 32 lines (16 susceptible and 16 winter-hardy) using the same protocol used for Pril-1. The susceptible and winter-hardy lines were selected based on flowering date and branching data collected from the field. The lines were sorted by flowering date with the twenty-five earliest and twenty-five latest flowering lines being grouped. The early flowering lines were hypothesized as susceptible and further narrowed down by selecting lines with fewer branches. The winter hardy lines were hypothesized to be late flowering lines with greater degree of branching.

Statistical Analysis. Analysis of variance was conducted as previously described for Experiment 1 using PROCGLM (SAS 9.3®), for FCS-1 and FCS-2 to determine differences among lines. LSmeans were calculated using SAS 9.3® and were used for QTL analysis. Hartley's test for homogeneity was used to determine if experimental runs could be combined for analysis. Correlation analysis was performed among experimental runs for both populations using SAS 9.3®.

Field study

Experimental design

Field studies to evaluate the winter hardiness of Pril-1 and Pril-2 were planted on 24 September 2015 and 15 September 2016, near Prosper, North Dakota. Field trials for both Pril-1 and Pril-2 were set up as an RCBD with two replicates. The experiments were planted as two row plots, 3m in length. Both populations were sown into standing wheat stubble at a targeted stand density of 12 plants/m^2. The standing stubble was approximately 10-15 and 20-25 cm in

height in 2015 and 2016, respectively. In 2015, weed control was carried out with a pre-emergence application of Assure II (Quizalofop P-ethly) and Tomahawk (glyphosate) followed by spring application of Assure II. No weed control measure was used in 2016.

Data collection

Stand counts for each plot were taken in the fourth week of October for both years of the study and were collected on the second meter of the first row in each plot. Stand counts were repeated on 18 May 2016 and were not taken for the second year of the study (2016-2017). The 2015 experiment was also scored for injury on 15 December after a significant frost event had occurred using the same scoring as the freeze chamber experiment (Table 3). No winter injury scores were collected for 2016 due to no injury prior to snow cover. Percentage of survival for each RIL was calculated by comparing the fall and spring stand counts. Data were collected for both populations during the summer of 2016 in the field for the following phenotypic traits: branching, first flower, powdery mildew resistance, and prostrate growth. These traits were used to anchor the linkage maps.

Statistical Analysis

Analysis of variance for percent survival was conducted using PROCGLM (SAS 9.3®). Correlation analysis was performed between AUIC values, from FCS-1 and FCS-2, and field survival ratings for Pril-1 and Pril-2 from 2015-2016 using SAS 9.3®.

Linkage map generation and QTL analysis

Single nucleotide polymorphisms (SNPs) markers were generated using standard genotype-by-sequencing (GBS) methodology (Elshire et al., 2011) in the laboratory of Dr. Xuehui Li, NDSU Dept. Plant Sciences. Leaf tissue was collected from 266 and 232 individuals for Pril-1 and Pril-2, respectively. Library preparations were completed using a modified version

of the methods described by Poland et al. (2012). Isolation of DNA was completed using a glass filter plate extraction procedure with AcroPrep™ Advance 96-well filter plates (Pall Life Sciences). After DNA samples were normalized they were digested with restriction enzyme *Ape*KI and barcodes were ligated. Individual samples were pooled to form libraries, and the libraries were PCR-amplified. Libraries were sequenced by the Texas A&M University sequencing center using an Illumina HiSeq2000 instrument. SNPs were identified from sequence data using TASSEL3 and UNEAK pipeline software. Single nucleotide polymorphisms discovered in the UNEAK pipeline were filtered for coverage in the population and for severe segregation distortion.

Genetic linkage maps were developed for both Pril-1 and Pril-2 using SNP marker genotypes. Linkage groups (LG) in Pril-1 were anchored using the genes for powdery mildew (*er-1*), flower color (*a*) and neoplasm (*np*). Linkage groups for Pril-2 were anchored using leaf type (*af*), flower color, powdery mildew (*er-1)* and vine length (*le*). The raw SNP data, for both Pril-1 and Pril-2, were filtered to remove SNP loci that were missing from more than 50% of the lines. The 6116 raw SNP loci for Pril-1 were further filtered to identify polymorphism in reference to the parents of the population. 2558 monomorphic loci were excluded. The remaining 3558 SNP loci were further filtered for segregation distortion. Finally, loci with a heterozygous call for either of the parents were also excluded to avoid errors that could occur from manually assigning alleles to these SNP calls. The final number of SNPs used for mapping Pril-1 was 1507. The 13,268 raw SNP loci for Pril-2 were filtered the same as described for Pril-1. Given the large number of SNP loci remaining for Pril-2, additional SNP loci were excluded if calls were missing for at least 40% of the individuals leaving 3245 loci suitable for mapping. Maps were created using JoinMap4.0 (Van Ooijen, 2011). The extra exclusion of SNP loci was

necessary since JoinMap cannot handle more than 4000 loci. Loci were excluded from mapping if they were missing greater than 50% of calls for both populations. Given the large number of SNPs in Pril-2, SNP loci with significant segregation distortion ($p<0.1$) were excluded. In Pril-1 SNP loci with segregation distortion at $p<0.05$ were excluded. SNPs were assigned to seven linkage groups at a minimum LOD value of 5. The Haldane mapping function was used to order SNP loci for both populations. To gain more information for the produced linkage maps, pea genetics map positions were compared to physical map positions of related-species. BLASTN (NCBI; https://www.ncbi.nlm.nih.gov) was used to align 64 base pair tag sequences containing the SNP marker sequences from this study to *M. truncatula* and chickpea sequences. Microsoft Excel was used to visualize linkage map positions with physical positions of the best hit on the two related genomes.

Quantitative trait loci analysis was performed using QGene v4.0 (Joehanes and Nelson, 2008) and QTL IciMapping v4.1 (Meng et al., 2015). A 0.5cM scan interval was used in QGene for both populations. Percent survival from the field study and the AUIC values from FCS-1 and FCS-2 were used for the analysis. Composite interval mapping (CIM), with cofactors selected by the software, and a 1000 permutation test was run to identify significant QTL at $\alpha = 0.05$. Composite interval mapping was also run in IciMapping with a 1 cM scan interval and 1000 permutation test.

Results

Greenhouse study

Establishment of methodology- Experiment 1

The first experimental run contained 50 different genotypes. ‘Igloo’ was excluded from all analyses since it was not included in subsequent runs. Only 49 lines from run 1 that were common across all runs were analyzed. AUIC values for the three experimental runs ranged from

62 to 147 (Table A1). Each set within a run was analyzed separately as an RCBD (not shown). Hartley's test for homogeneity showed that sets within runs could be analyzed together (Table A5). The runs were then analyzed separately across all sets again as an RCBD (Table A2-A4). Analysis of each run individually showed effect of genotype on AUIC values to be statistically significant with no significant impact from replicates. The F-max values from Hartley's test for homogeneity allowed for all runs to be analyzed together (Table A6). The combined analysis showed genotype and run to be significant while genotype x run and replicate within run were not significant (Table 5).

Table 5. ANOVA for AUIC across all runs of experiment 1.

Source	DF	Mean square	F value	Pr>F
run	2	23423.79	40.87	<0.0001***
genotype	61	3407.01	8.77	<0.0001***
genotype x run	109	388.31	1.12	0.2105
rep(run)	15	573.12	1.65	0.0567

ns, not significant; ***, p<0.0001

Phenotyping of recombinant inbred lines

The current protocol was able to detect a wide range of differences in winter hardiness based on AUIC values. A high level of consistency in the protocol was seen in both FCS-1 and FCS-2. When the top 25% of lines with the lowest AUIC values were compared, 10 and 4 lines were found to be common across runs for FCS-1 and FCS-2, respectively (Table 6-7). These results show that 25% and 50% of the top quartile of FCS-1 and FCS-2, respectively, were consistent across three experimental runs. The AUIC values ranged from 77-150 and 73-150 in FCS-1 and FCS-2, respectively (Table A7-A8). An AUIC value of 150 indicates a line died in less than six days after freezing. Hartley's test for homogeneity on FCS-1 sets and FCS-2 sets (Table A15 and A17) allowed for each run to be analyzed separately (Table A9-A14). The genotypic effect was significant in all three runs of FCS-1 and FCS-2 with no effect from

replicates. A combined analysis was justified for FCS-1 and FCS-2 by Hartley's test for homogeneity (Table A16 and A18). The combined analysis of FCS-1 showed that run, genotype, and the genotype x run interaction were significant (Table 8). Only replicates within run had no significant effect on AUIC values in FCS-1. All sources of variation for FCS-2 were significant in the combined analysis (Table 9). Run and genotype x run interaction were significant at the $p<0.05$ level indicating their effect was not as significant as genotype and replicate within run ($p<0.0001$).

Table 6. Common lines among the top 25% of each experimental run of FCS-1.

Line	Run 1	Run 2	Run 3
Pril-1-085	84.00	116.25	123.75
Pril-1-225	84.75	114.00	111.75
Pril-1-223	92.25	122.25	104.63
Pril-1-025	94.87	112.50	127.13
Melrose	108.04	117.34	124.74
Pril-1-200	108.73	113.25	110.25
Pril-1-035	110.62	114.75	127.50
Pril-1-063	112.12	100.50	126.38
Pril-1-030	114.75	117.75	126.00
Pril-1-056	115.12	88.13	128.25

Table 7. Common lines among the top 25% of each experimental run of FCS-2.

Line	Run 1	Run 2	Run 3
Melrose	100.13	84.19	73.88
Pril-2-063	93.75	94.88	96.38
Pril-2-077	107.25	98.25	85.88
Pril-2-247	94.88	85.13	81.75

Table 8. ANOVA for AUIC combined across all runs of FCS-1.

Source	DF	Mean square	F value	Pr>F
run	2	12115.62	33.01	$<0.0001^{***}$
genotype	161	1157.47	3.42	$<0.0001^{***}$
genotype x run	317	338.24	1.45	$<0.001^{***}$
rep (run)	9	367.07	1.57	0.1175

ns, not significant; *** $p<0.0001$

Table 9. ANOVA for AUIC combined across all runs of FCS-2.

Source	DF	Mean square	F value	Pr>F
run	2	6076.30	5.21	0.0314*
genotype	33	4079.77	11.26	<0.0001***
genotype x run	66	362.30	1.37	0.0405*
rep (run)	9	1166.38	4.42	<0.0001***

ns, not significant; *, p<0.05; ***, p<0.0001

Correlation coefficients between runs ranged from 0.37-0.47 and 0.70-0.83 in FCS-1 and FCS-2, respectively, and were significant at p<0.0001 (Tables 10 and 12). Correlation between AUIC, the number of branches and first flower date for both FCS-1 and FCS-2 detected no significant correlation between AUIC and either branching or flowering date (Tables 11 and 13). The number of branches and flowering date did have a significant correlation (p<0.01) in both experiments.

Table 10. Correlation coefficients with pairwise two-sided p-values for AUIC values across all runs of FCS-1.

	Run 1	Run 2	Run 3
Run 1	1.00	0.40 <0.0001***	0.48 <0.0001***
Run 2	0.40 <0.0001***	1.00	0.37 <0.0001***
Run 3	0.48 <0.0001***	0.37 <0.0001***	1.00

***, p<0.0001

Table 11. Correlation coefficients with pairwise two-sided p-values for AUIC values, presence of branching and flowering date combined across all runs of FCS-1.

	AUIC	Br†	FlwrDt‡
AUIC	1.00	-0.04 0.59^{ns}	0.03 0.71^{ns}
Br	-0.04 0.59^{ns}	1.00	0.24 0.0030**
FlwrDt	0.03 0.71^{ns}	0.24 0.0030**	1.00

ns, not significant; **, p<0.01
† branching
‡ first flower date

Table 12. Correlation coefficients with pairwise two-sided p-values for AUIC values across all runs of FCS-2.

	Run 1	Run 2	Run 3
Run 1	1.00	0.83 <0.0001***	0.70 <0.0001***
Run 2	0.83 <0.0001***	1.00	0.78 <0.0001***
Run 3	0.70 <0.0001***	0.78 <0.0001***	1.00

***, p<0.0001

Table 13. Correlation coefficients with pairwise two-sided p-values for AUIC values, presence of branching and flowering date combined across all runs of FCS-2.

	AUIC	Br†	FlwrDt‡
AUIC	1.00	0.19 0.29^{ns}	-0.26 0.13^{ns}
Br	0.19 0.29^{ns}	1.00	0.52 0.0018**
FlwrDt	-0.26 0.13^{ns}	0.52 0.0018**	1.00

ns, not significant; **, p<0.01
† branching
‡ first flower date

Survival scores at 6 days after freezing were normally distributed and provided the greatest discernment between the greatest and least tolerant genotypes in FCS-1 (Figure A1-A3). This same result was observed in Run 1 and Run 2 of FCS-2 (Figure A4-A6). Similar results were reported in a study in *M. truncatula* where the highest amount of variability in freezing tolerance was seen two weeks after freezing (Avia et al., 2013).

Field study

Due to limited seed availability, only 256 lines from Pril-1 and 164 lines from Pril-2 were analyzed in the first year of the study (2015-2016). Four and two lines in Pril-1 and Pril-2, respectively, did not have adequate stand establishment for evaluation. The second year of the study (2016-2017) contained 278 and 258 RILs from Pril-1 and Pril-2, respectively. The winter conditions were mild for 2015-2016. The lowest temperature from November through February was -25°C with high temperatures reaching 2°C at the end of February (NDAWN, 2016). While the winter of 2016-2017 did experience the same low of -25°C between November and February, there also was an increase in temperatures causing most of the snow to dissipate in mid-February. A high of 6°C was recorded mid-February. This thaw was then followed by freezing temperatures with lows of -17°C. Percent survival was calculated for each plot using the fall and spring stand counts (Tables A19-A20). The susceptible parents of both populations, 'Shawnee' and 'Medora', had zero survival in the first year of the study (2015-2016). Spring stand counts for the second year of the study (2016-2017) were not collected due to complete winter kill within the plots. 'Melrose', the resistant parent in both populations, survived in all but one out of 14 plots, 10 in the Pril-1 study and 4 in the Pril-2 study (2015-2016). Some plots received a percent survival greater than 100. This is due to late germination after the fall stand counts were taken. Analysis of variance detected significant variation due to genotype for Pril-1 and Pril-2

(p<0.01 and p<0.05, respectively) and variation due to replications was not significant (Tables 14-15). In the first year of the field study, 7% of the Pril-1 lines had greater than or equal to 75% survival and less than 1% of Pril-2 lines had greater than or equal to 75% survival. Across Pril-1 and Pril-2, 50% and 64% of the entries, respectively, had no survival (Figure 1-2). Injury scores for 2015-2016 ranged from 1-7 based on the 1-9 injury scale. The injury scores were not always reflected in the overall survival of lines (Tables A19-A20). Since only one replicate of lines was scored in 2015-2016 these data were not analyzed statistically.

Table 14. ANOVA for field survival of Pril-1 genotypes (2015-2016).

Source	DF	MS	F value	Pr>F
Genotype	251	1945.31	1.52	0.0008**
Rep	1	0.70	0	0.9815ns

ns, not significant; **, p<0.01;

Table 15. ANOVA for field survival of Pril-2 genotypes (2015-2016).

Source	DF	MS	F value	Pr>F
Genotype	161	499.59	1.33	0.0384*
Rep	1	554.37	1.47	0.2264ns

ns, not significant; *, p<0.05;

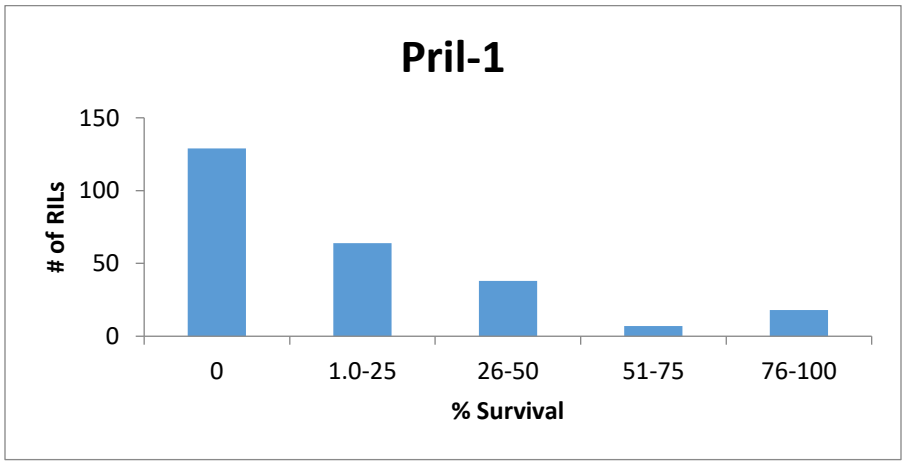

Figure 1. Frequency analysis for survival of Pril-1 in 2015-2016 Prosper, ND.

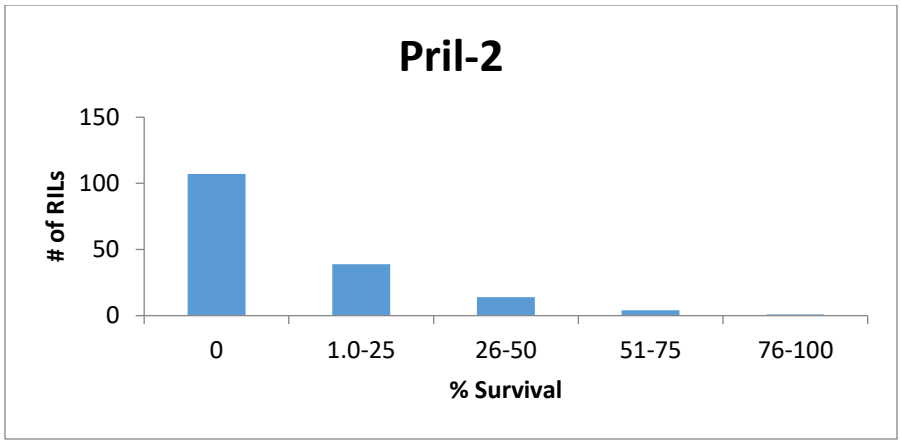

Figure 2. Frequency analysis for survival of Pril-2 in 2015-2016 Prosper, ND.

Twenty-two lines from Pril-1 were common in the top 25% of both the field (2015-2016) and the greenhouse (FCS-1) study (Table 16). Only two lines, including Melrose, the winter hardy check, performed in the top 25% in the field and greenhouse experiments for Pril-2 (Table 17). Correlation between AUIC values from the combined analysis of the greenhouse studies and the average survival of each line calculated from stand counts in the field study showed a significant negative correlation ($p<0.001$) in both Pril-1 and Pril-2 (Tables 18-19).

Table 16. Lines in common within the top 25% in the field study (2015-2016) and FCS-1 for Pril-1.

Line	Field survival (%)	AUIC value
Pril-1-021	100.00	116.25
Pril-1-024	133.33†	107.00
Pril-1-025	100.04	111.50
Pril-1-041	50.00	119.00
Pril-1-046	66.67	119.88
Pril-1-056	77.74	110.50
Pril-1-063	28.57	113.00
Pril-1-077	37.50	121.50
Pril-1-085	43.33	108.00
Pril-1-090	26.67	118.75
Pril-1-093	43.75	121.00
Pril-1-123	187.50	113.63
Pril-1-125	91.67	119.38
Pril-1-125	91.67	119.38
Pril-1-126	87.50	118.88
Pril-1-128	47.50	114.38
Pril-1-140	50.00	122.25
Pril-1-143	35.71	122.13
Pril-1-144	47.62	114.75
Pril-1-209	49.96	113.00
Pril-1-249	33.33	120.88
Melrose	96.67	116.71

† survival greater than 100% was due to late germination

Table 17. Lines in common within the top 25% in the field study (2015-2016) and FCS-2 for Pril-2.

Line	Field survival (%)	AUIC value
Pril-2-025	25.00	106.13
Melrose	42.32	86.06

Table 18. Correlation coefficients with pairwise two-sided p-values for AUIC values and field survival rates (2015-2016) combined across all runs of FCS-1.

	AUIC value	Field survival
AUIC value	1.00	-0.38 <0.0001***
Field survival	-0.38 <0.0001***	1.00

***,p<0.0001

Table 19. Correlation coefficients with pairwise two-sided p-values for AUIC values and field survival rates (2015-2016) combined across all runs of FCS-2.

	AUIC value	Field survival
AUIC value	1	-0.64 0.0018**
Field survival	-0.64 0.0018**	1

**, p<.001

Linkage map generation and QTL analysis

The linkage map produced for Pril-1 was constructed using GBS data from 266 individuals and 1507 SNP loci. This linkage map consisted of seven linkage groups with a total map size of 1368 cM. The map for Pril-2 was constructed using 232 individuals and 3245 SNP loci. Seven linkage groups were established for Pril-2 with a final map size of 1419 cM. Anchor traits were used to determine which linkage groups in the populations correspond to the pea consensus linkage groups according to Loridon et al. (2005). Linkage groups representing pea consensus LGs are indicated by roman numerals from here forward. Four linkage groups in each population were anchored to the consensus linkage groups (Tables 20-21). Linkage group 7 of Pril-1 and LG6 of Pril-2 were anchored to LGI of the consensus map based on the gene for leaf type, *af*. Linkage group 4 of both Pril-1 and Pril-2 were anchored to LGII of the consensus map based on the presence of *a* for flower color. Linkage group 1 of Pril-1 and LG5 of Pril-2 were

anchored to LGIII of the consensus map by the *le* gene. Linkage group 5 of Pril-1 and LG7 of Pril-2 were anchored to LGVI of the consensus map by the gene for powdery mildew resistance, *er-1*.

Marker density for the Pril-1 linkage map ranged from 0.79-1.28 markers per cM. The marker density was higher for the Pril-2 linkage map with a range of 2.02-2.55 markers per cM. The BLAST analysis found 206 hits and 469 hits with *M. truncatula* for Pril-1 and Pril-2, respectively, at an E-value threshold of 10^{-10}. The BLAST analysis against chickpea only found 165 and 348 hits for Pril-1 and Pril-2, respectively. The resulting BLAST hits were used to align the LGs of Pril-1 and Pril-2 to LGs of *M. truncatula* and chickpea (Tables 22-23). All LGs from Pril-1 and Pril-2 that were anchored to pea consensus LGs had the same corresponding M. *truncatula* and chickpea LG assignments with the exception of one. Linkage group 6 of Pril-2, anchored to pea consensus LGI found hits aligned to LG7 of chickpea which was not seen for LG7, pea consensus LGI, of Pril-1.

Table 20. Predicted consensus linkage group, the anchor trait, number of SNP loci, length and average marker density for each linkage group of the Pril-1 linkage map.

Linkage group	Predicted *Pisum* linkage group	Anchor trait	Number of SNP loci	Length (cM)	Average marker density
1	III	*le*	324	257	1.26
2			229	216	1.06
3			266	208	1.28
4	II	*a*	235	191	1.23
5	VI	*er-1*	172	184	0.93
6			167	167	1.00
7	I	*af*	114	145	0.79
		Map Total	**1507**	**1368**	

Table 21. Predicted consensus linkage group, the anchor trait, number of SNP loci, length and average marker density for each linkage group of the Pril-2 linkage map.

Linkage group	Predicted *Pisum* linkage group	Anchor trait	Number of SNP loci	Length (cM)	Average marker density
1			502	210	2.39
2			413	200	2.07
3			541	220	2.46
4	II	*a*	518	203	2.55
5	III	*le*	495	227	2.18
6	I	*af*	399	172	2.32
7	VI	*er-1*	377	187	2.02
		Map Total	**3245**	**1419**	

Table 22. Linkage groups from Pril-1 and how they align with the *Pisum* consensus map based on anchor traits, and *M. truncatula* and chickpea based on BLAST results.

Pril-1	Pisum	*M. truncatula*	Chickpea
1	III	3	5,1†
2		4,8	7
3		4,8	6
4	II	1	4
5	VI	2	1
6	V	7	6
7	I	5	2

† when more than one LG is listed, the first linkage group had the large amount of synteny with the Pril-1 LG

Table 23. Linkage groups from Pril-2 and how they align with the *Pisum* consensus map based on anchor traits, and *M. truncatula* and chickpea based on BLAST results.

Pril-2	*Pisum*	*M. truncatula*	Chickpea
1		4,8†	7
2	V	7	3
3		4,8	6
4	II	1	4
5	III	3	5,1
6	I	5	2,7
7	VI	2,6	1

† when more than one LG is listed, the first linkage group had the large amount of synteny with the Pril-2 LG

Quantitative trait analysis was conducted using Qgene and QTL IciMapping. Analysis with QGene for QTL related to winter hardiness, based on AUIC values from FCS-1, produced two significant QTL peaks above the $\alpha = 0.05$ cutoff of LOD = 4.7 determined by permutation tests for Pril-1 (Table 24). A third QTL on LGII was identified at an LOD of 4.2. The QTL on LGIII had a confidence interval (CI) from 44-47cM and explained 13.9% of the variation. The QTL found on LG6 had a CI from 38-40cM and explained 16% of the observed variation. The final QTL on LGII had a CI from 121-127cM and explained 12% of the variation. All three QTL related to winter hardiness detected for Pril-1 by Qgene were confirmed with QTL IciMapping (Table 24, Figure 3). The significant LOD values as well as percent variation were similar for all three QTL. Regression analysis showed the combined effect of the QTL identified accounted for 24% of variation in AUIC. Analysis of QTL related to field survival of Pril-1 using Qgene found significant QTL on LG III, CI from 53-55cm, and LG 6, CI from 60-63cM (Table 25). These QTL were confirmed by QTL IciMapping which also found a significant peak on LG5.

The QTL analysis of FCS-2 by QGene and QTL IciMapping was inconclusive. Analysis of AUIC values from FCS-2 by QGene showed a significant QTL on LGIII but it was found on the opposite end of the linkage group in regards to the QTL found in Pril-1. The QTL peak was located at 201cM and a CI from 200-202 cM on LGIII (data not shown). The analysis of field survival for Pril-2 was inconclusive.

Table 24. Chromosome location, flanking markers, LOD and R^2 values for winter hardiness (AUIC) QTL identified by CIM analysis using Qgene and QTL IciMapping for FCS-1.

Program	Chromosome	Markers flanking QTL peak	Position (cM)	LOD	R^2
QGene	1 (III)	TP174523, TP129363	45.5	5.1	0.14
	4 (II)	TP17649,TP125600	123	4.2	0.12
	6	TP126940,TP145328	39	5.8	0.16
QTL IciMapping	1 (III)	TP174523, TP152133	45	5.0	0.11
	4 (II)	*A*,TP125600	125	3.8	0.08
	6	TP126940,TP176037	39	5.936	0.13

Table 25. Chromosome location, flanking markers, LOD and R^2 values for field survival QTL located by CIM analysis using Qgene and QTL Icimapping for Pril-1.

Program	Chromosome	Markers flanking QTL peak	Postion (cM)	LOD	R^2
Qgene	1 (III)	TP27549, TP168060	54	7.7	0.14
	6	TP111778, TP129236	62	14.0	0.24
QTL IciMapping	1(III)	TP3868, TP55407	75	6.7	0.09
	3	TP169441, TP146422	111	4.0	0.05
	5(VI)	TP293331, TP76360	94	4.1	0.05
	6	TP156265, TP91337	64	14.5	0.22

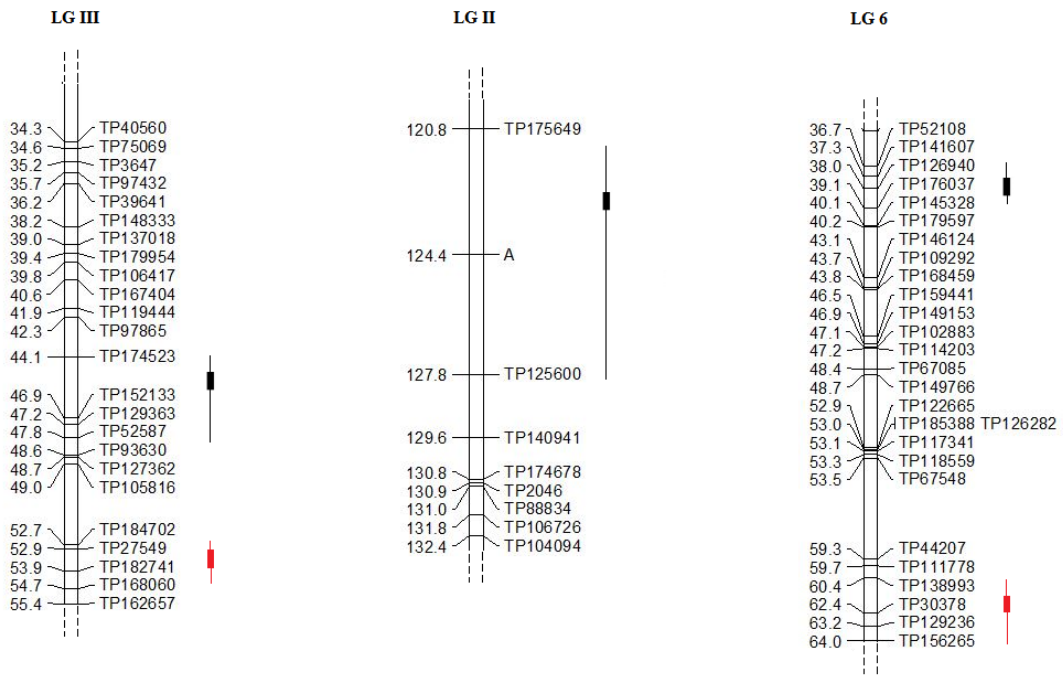

Figure 3. QTL for winter hardiness (black) and field survival (red) identified for Pril-1 with Qgene and supported by QTL IciMapping on LG1, LG4 and LG6.

Discussion

The results from the greenhouse protocol were analyzed as an RCBD by excluding set as a source of error. The assumption that sets were equal within runs was made due to the fact that the sets were planted only two days apart. The freeze chamber produced consistent temperatures supporting the assumption that the sets were treated the same throughout the experiment and therefore did not need to be analyzed as a source of error. Experiment 1, which was conducted to establish a reliable protocol, confirmed that the protocol used can in fact detect responses to freezing that are consistent and statistically significant across a diverse set of genotypes when runs were analyzed separately. It is unclear why there was variation due to runs when the experiment was analyzed across runs. This aligns with other studies that used a 4-week acclimation period and a minimum temperature around -8°C (Swensen and Murray, 1983;

Liesenfeld et. 1986). The ANOVA and correlation results confirm the protocol detected differences in response to freezing among lines. Four weeks of acclimation was chosen despite Fiebelkorn (2013) finding a slight decrease in survival after four weeks of acclimation compared to three weeks. For further confirmation of the results found here, this protocol could also be run with a 3-week acclimation period. Since the protocol requires two weeks for growth, four weeks for acclimation, and three weeks for recovery, the runs were tested across multiple months which produced slight variations in the greenhouse room temperatures. This could have caused the injury of some runs to progress more quickly. The amount of water the plants received after freezing was critical and excess water caused some plants to die quicker due to damaged roots. Due to experimental constraints, it is possible that the lines died quicker than if they were frozen as a large plant, as would occur in the field.

Temperatures in the freezing chamber and within the canopy of the samples were consistent during FCS-1 and FCS-2 (Figure A7, only FCS-1 shown). Slight variation was observed when comparing the temperature measurements taken by probes in individual pots. This variation could be due to moisture levels of each pot and the small size of the pots. The combined analysis for FCS-1 detected significant differences due to not only genotype but also run, and the genotype x run interaction. The combined analysis of FCS-2 showed significance across all sources of variation. Ideally, the effect of run, replicate within run and genotype x run would not be significant. Variation among runs could be due to the three runs of FCS-1 being conducted over approximately a three month period during the summer. Slight variation in greenhouse conditions, specifically ambient temperature, could have affected the recovery period and rate of death. Despite these other sources of variation being significant, the fact that genotypes were significantly different shows that the protocol is able to detect differences among

lines. Variation among runs for FCS-2 may have been less, compared to FCS-1, since the experiment was run during the winter months and greenhouse conditions were more constant. Significant replicate within run for FCS-2 is most likely due to the selection of lines for both extremes. Overall, it is important to note that there was no variation due to replicates when experimental runs were analyzed separately but genotype was always significant indicating the ability of the protocol to detect genotypic differences in winter hardiness consistently. Further modifications to the protocol should be made to reduce the variation due to runs in the combined analysis to enhance the repeatability of the protocol.

Significant correlation coefficients ($p<0.0001$) between all runs for FCS-1 and FCS-2 confirms the repeatability of the protocol despite variation due to runs being significant. Due to the correlation found by Lejeune-Hénaut et al. (2008) between a winter freezing damage (WFD) QTL and the *Hr* gene for flowering, a negative correlation between AUIC values, representing winter hardiness in this study, and first flower date would have been expected but this correlation was not significant in either FCS-1 or FCS-2. The assumption is that lines with a later flowering date should produce lower AUIC values, indicating greater winter hardiness. It is important to point out the significant correlation ($p<0.01$) found between branching and first flower date in both experiments. While both traits did not correlate with AUIC as expected, this finding confirms the assumption that there is a link between branching and a later flowering date. Phenotypic traits such as increased branching, prostrate growth and later flowering date have been linked to increased winter hardiness in pea (Lejeune-Hénaut et al., 2008). A correlation with these traits would have provided further support for the hypothesis that the AUIC value is representative of winter hardiness. Correlation coefficients between AUIC from the greenhouse study and field survival were significant ($p<0.0001$, $p<0.01$) for FCS-1 and FCS-2, respectively.

While AUIC was not correlated to branching or flower date, this negative correlation to field survival suggests that the AUIC values obtained with this greenhouse protocol are related to actual winter survival in the field. This correlation suggests that accurate selections for winter hardiness could be made in the greenhouse.

Distribution of freezing survival scores narrowed at each time point and scores at day six followed a normal distribution and represented the greatest distinction between the greatest and least freezing tolerant lines in FCS-1. Scores at day nine maintained much of the variation among lines, but scores beyond day nine progressively narrowed the differences between the extremes. This suggests that significant differences could be detected in the earlier stages of recovery, decreasing the recovery and scoring period needed for adequate selections. This finding concurs with previous studies that found the best detection date was two weeks after freezing (Avia et al., 2013; Dumont et al., 2009). This same distribution was not as prevalent in FCS-2 most likely due to the selection process of the lines and the lower number of lines evaluated. A study of potato blight resistance supports reducing the number of times scores are collected (Haynes and Weingartner, 2004). The AUDPC values calculated for blight resistance using a smaller number of injury scores was comparable to the values received at the end of the trial. These results could be due to the largest amount of injury occurring shortly after the treatment.

Due to poor planting conditions for the first year of the field study (2015-2016) only 252 and 162 lines of Pril-1 and Pril-2, respectively, had adequate stand establishment for evaluation in at least one replicate. Unfortunately, ‘Shawnee’, the susceptible parent for Pril-1 could not be evaluated in the first year due to poor establishment. The field conditions at planting for the second year of the study (2016-2017) were ideal and there were no lines that lost both replicates to poor stand establishment. It is interesting to note that the injury scores given in the first year of

the field study (2015-2016) did not correlate with the survival seen (Appendix 10 and 11). From the comparison of the top lines in both the field study and the greenhouse study (Table 16), five lines from Pril-1 were found to have better AUIC values then 'Melrose', the winter hardy check, and survival greater than 75%. From the results of this study Pril-1-021, Pril-1-024, Pril-1-025, Pril-1-056 and Pril-1-123 would be chosen to have the greatest winter hardiness in the population and tested further. Due to the large thaw experienced in February 2017, the stand counts from the second year of the study most likely would not have correlated with the first year (2015-2016).

A larger number of SNP loci were identified through GBS for Pril-2, resulting in more markers being available to construct a linkage map compared to Pril-1. A total of 652 SNP loci were common among the two populations. This allowed for comparison of LGs between the maps and to anchor the maps to the consensus pea chromosomes where anchor traits were present in one but not both populations. Both maps had a high marker density, higher than the 0.17 marker/cM density seen in Loridon et al. (2005), which allowed good resolution to identify markers linked to each QTL.

Analysis using QGene and QTL IciMapping confirmed three QTL affecting winter hardiness in Pril-1 based on AUIC values. The QTL located on LGIII appears to be in close proximity to *Hr* (Murfet, 1973; Lejeune-Hénaut et al., 2008). This assumption was based on the knowledge that *Hr* is closely linked to *m*, the gene for seed mottling and at the opposite end of the LG than the npl gene. The gene for pod neoplasm (*np*), was used to anchor LGIII in the Pril-1 linkage group map and was placed at 223 cM on LGIII with the QTL peak found at 45cM. The QTL found on LGIII is in agreement with a WFD QTL found by Lejeune-Hénaut et al. (2008). The QTL analysis using percent field survival found QTL on LGIII and LGVI, which agree with the analysis of AUIC values, but also located QTL on LG3 and LG6 of the Pril-1 linkage map.

The QTL found on LGII, LG3, LG5 and LG6 of Pril-1 cannot be confirmed to align with any QTL identified by Lejeune-Hénaut et al. (2008). This is because LG3 and LG6 of Pril-1 were not anchored to the consensus map and therefore could not be confirmed to match QTL previously found on linkage groups I and V by Lejeune-Hénaut et al. (2008). The knowledge of synteny between pea, *M. truncatula* and chickpea could help close this gap. From the BLAST results we can hypothesize: LGIV is represented by LG2 and LG1 of Pril-1 and Pril-2, respectively; LGV is represented by LG6 and LG2 in Pril-1 and Pril-2, respectively; LGVII is represented byLG3 of both populations. With this knowledge the QTL found on LG6 of Pril-1 could coincide with the QTL found on LGV by Lejeune-Hénaut et al. (2008), providing more confirmation in this protocol's ability to detect winter hardiness among lines. The QTL for winter hardiness found on LGVI could be related to the winter tolerance found in *M. truncatula* since that LG aligns with the *M. truncatula* LG where a QTL for winter tolerance was detected by Avia et al. (2013). This synteny relationship could help further explain winter hardiness in pea. Due to the low amount of phenotypic data collected for FCS-2, the population was not adequate for a conclusive QTL analysis of Pril-2 relating to AUIC values and field survival in these studies. The QTL located on LGIII in Pril-2, while inconclusive, was located near the *Le* gene which correlates with the WFD 3.2 found by Lejeune-Hénaut et al. (2008). Despite QTL analysis for Pril-2 being inconclusive, the proximity of this QTL to the WFD 3.2 previously recorded supports the validity of the protocol developed.

No correlation was found between AUIC value with either flowering date or branching (Tables 11-13) suggesting that these traits were not sufficient for selecting winter hardy lines. The selection of extremes from Pril-2 for FCS-2 was not an accurate representation of winter hardy and winter susceptible lines. Both the choice of traits for selection and the number of lines

selected for the study affected the ability to complete QTL analysis. The fact that QTL analysis of AUIC values or field survival, which had 162 data points, for Pril-2 found no significant peaks leads to the hypothesis that the population may not be segregating for traits associated with winter hardiness.

To gain a more complete picture of what genetic factors are important for winter hardiness, phenotyping and QTL analysis should be completed on full Pril-1 and Pril-2 populations. The measurement of chlorophyll content and electrolyte leakage in plant tissues was studied as an indicator of winter hardiness (Avia et al., 2013). It would be beneficial to measure these two traits with the greenhouse protocol for further QTL analysis. It would also be beneficial to compare the phenotype data to percent field survival from at least two more years of field trials.

The protocol developed here is able to detect differences in winter hardiness consistently, in a controlled environment, and as an accurate representation of winter field response. The minimization of variation due to run would be necessary in order to use this protocol for selections in a breeding program. The freeze response detected aligned with previous QTL knowledge related to winter hardiness in pea. Completion of the pea genome sequencing project will provide additional insight and markers to complement those identified by Lejeune-Hénaut et al., (2008) to aid in the selection of varieties with greater winter hardiness.

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CHAPTER 4. CONCLUSION

An increased level of winter hardiness in pea varieties would be beneficial to crop rotations and cover crops being used in areas such as North Dakota. Along with simply being another crop in a crop rotation, which can decrease weed and pest pressure, a pea crop can also provide nitrogen and in some cases, increase yield of the following crop (Chen et al., 2006; Beckie et al., 1997; Grant et al., 2002). The use of cover crops is of interest to the agronomic world as an addition to crop rotations as well as in no till environments where wind and water erosion can be detrimental.

Winter hardiness is related to an acclimation period that crops must go through. During this time, changes in protein, sugar, and lipid levels help to stabilize cellular structures against freezing damage (McKersie and Leshem, 1994; Uemura and Kawamura, 2014). Another key component for maintaining the viability of a winter crop is a vernalization period. Vernalization is a requirement of certain day length and temperature before a crop will enter the reproductive phase (Amasino, 2004). This is important to ensure that the crop does not flower before over wintering, as this can reduce the energy source the crop will need for regrowth and not allow the crop to acclimate to the cold conditions. While pea does not have a true vernalization period, winter pea varieties do become dormant before entering the reproductive phase in the fall. This allows them to reach maturity earlier in the spring, which can benefit yield (Chen et al., 2006).

Field testing to select for winter hardiness is challenging in this and other studies due to unpredictable winter conditions. To make accurate selections, a breeder needs to evaluate a breeding line over multiple years and environments. Unpredictable winter conditions generate inconsistent data from year to year. An extreme winter will not allow for selections to be made if the whole study is killed, or mild conditions may not kill non-winter hardy genotypes. Studies

have been conducted in controlled environments to test winter hardiness in pea (Auld et al., 1983; Swensen and Murray, 1983; Liesenfeld et al., 1986). Unfortunately, the results did not prove consistent or did not correlate to what was seen in the field.

The development of a protocol for detection in a controlled setting could decrease the time necessary to select and produce superior winter hardy lines. The use of molecular markers, along with a dense molecular map, can help decrease this selection time. With a knowledge of QTL related to winter hardiness, markers can be used to help select for lines with the desired traits through marker assisted selection (MAS) or genomic selection (GS) (Meuwissen et al., 2001; Schmidt et al., 2016). Access to the pea genome sequence will generate the ultimate high resolution molecular marker map for genetic populations. The maps produced for Pril-1 and Pril-2 have large numbers of quality SNP markers, but the ability to understand the physical position of the markers and their associated QTL will be advanced with the genome sequence. The use of QTL analysis to identify specific areas of a genome that can be screened will further decrease the time needed to develop improved lines. The QTL identified in this study correlates to previous work by Lejeune-Hénaut et al. (2008). While no new QTL were confidently detected, these findings do confirm that the AUIC values collected from the screening protocol and used for QTL analysis do correlate with winter hardiness. Further studies should be conducted to confirm the results found here and confirm the QTL identified here.

The protocol developed in this study proved to detect differences in response to freezing conditions in a controlled environment. Not only were differences detected but the protocol was repeatable across experimental runs and populations. Twenty-two lines, including the winter hardy check, were in the top quartile of FCS-1 across all three experimental runs (Table 8). These lines from Pril-1 can be selected as winter hardy. Significant correlation ($p<0.001$)

between percent survival in the field and AUIC values obtained with the greenhouse protocol confirm that the protocol can detect a level of true winter hardiness among lines for both Pril-1 and Pril-2. While slight improvements could be made to increase efficiency, the protocol could replace some of the field trials currently needed to select for winter hardy lines. The QTL analysis confirmed that the winter hardiness measured with this protocol is related to other winter hardy studies in pea by presenting similar QTL as were found by Lejeune-Hénaut et al. (2008).

There are still more trials to be run but the work done in this study has confirmed a greenhouse protocol able to detect differences in winter hardiness among lines. The variation due to runs needs to be reduced to prove a higher level of consistency can be obtained with this protocol. The inability to perform QTL analysis on Pril-2 showed that there is a size requirement for studies using this protocol to identify QTL in a population. Greenhouse selections for winter hardiness could be made on a population of around 160 lines in approximately 90 days. An initial selection in the greenhouse, made using the protocol developed here, would reduce the number of lines entered into costly and risky field studies, which is a benefit to a variety development program.

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APPENDIX

Table A1. Average AUIC values for each genotype from experiment 1 for all three experimental runs.

	Run 1	Run 2	Run 3
Apache	113.25	119.55	140.50
April	101.25	116.75	133.50
Aravis	120.75	117.57	142.95
Assas	107.00	99.75	112.25
Cheyenne	136.75	119.25	145.84
Dove	99.00	125.92	125.72
EFB333	91.50	90.50	97.00
Fenn	83.75	86.25	98.00
Grana	93.00	95.25	92.75
Granger	73.25	89.50	103.25
Lynx	103.17	122.00	132.21
Melrose	72.82	84.32	97.03
Natura	84.33	118.50	137.50
Picard	80.50	83.86	100.25
Romack	89.25	82.41	98.00
Specter	81.88	117.75	130.00
Whistler	115.53	116.29	133.14
Windham	95.42	117.05	126.85
PS0017018	69.75	82.00	94.15
PS0230F063	105.38	113.96	118.50
PS0230F092	108.75	109.12	117.50
PS0230F210	77.14	97.58	105.25
PS03100635	79.75	77.62	108.50
PS03100848	92.75	86.25	102.50
PS03101120	67.84	88.25	92.73
PS03101160	.	119.85	126.25
PS03101170	124.24	99.16	114.00
PS03101205	.	110.50	128.64
PS03101247	.	133.00	134.75
PS03101269	80.75	102.75	108.25
PS05300069	121.50	108.50	135.77
PS05300075	111.00	107.50	138.00
PS05300077	100.50	106.75	126.65
PS05300078	112.00	107.50	128.90
PS05300083	.	122.08	119.28
PS05300108	.	88.25	113.89
PS05300126	116.50	94.69	118.51
PS05300205	.	110.00	130.09
PS05300213	.	121.23	128.38

Table A1. Average AUIC values for each genotype from experiment 1 for all three experimental runs (continued).

	Run 1	Run 2	Run 3
PS05300225	109.00	114.75	129.31
PS05300228	131.00	128.63	127.81
PS05300234	131.50	120.68	128.50
PS05300239	127.92	123.08	136.60
PS06300003	89.53	106.50	119.75
PS06300007	.	109.11	127.06
PS06300008	.	117.77	118.26
PS06300016	122.25	104.43	122.00
PS06300017	114.00	115.60	129.14
PS06300022	113.75	119.76	132.50
PS06300024	120.75	112.75	120.14
PS06300028	127.75	120.42	138.35
PS06300048	.	114.09	109.76
PS06300050	104.50	116.91	101.24
PS06300057	.	100.25	131.54
PS06300060	.	121.80	129.57
PS06300061	.	84.22	111.46
PS06300064	81.89	110.13	117.10
PS06300075	97.50	111.50	135.00
PS06300108	100.00	118.32	126.62
PS06300119	113.41	124.00	134.50
PS06300142	118.85	122.23	134.71
PS06300190	123.00	102.77	126.95

‘.’ Missing data;

Table A2. ANOVA for AUIC for run 1 of experiment 1.

Source	DF	Mean square	F value	Pr>F
Genotype	48	2030.18	3.42	$<0.0001^{***}$
Rep	5	849.09	1.43	0.2145^{ns}

ns, not significant; ***, p<0.0001

Table A3. ANOVA for AUIC for run 2 of experiment 1.

Source	DF	Mean square	F value	Pr>F
Genotype	61	1257.48	4.78	<0.0001***
Rep	5	513.23	1.95	0.0859ns

ns, not significant; ***, p<0.0001

Table A4. ANOVA for AUIC for run 3 of experiment 1.

Source	DF	Mean square	F value	Pr>F
Genotype	61	1310.88	5.52	<0.0001***
Rep	5	357.05	1.5	0.1883ns

ns, not significant; ***, p<0.0001

Table A5. Error mean squares and F-max calculated according to Hartley's Test for Homogeneity for each set from all experimental runs from experiment 1.

Run 1		Run 2		Run 3	
Set	ErrorMS	Set	ErrorMS	Set	ErrorMS
1	597.20	1	613.49	1	190.12
2	344.31	2	197.67	2	132.82
3	556.76	3	271.53	3	181.27
4	482.24	4	189.20	4	350.72
5	492.24	5	149.76	5	211.21
		6	78.14	6	404.40
F-max	1.73	F-max	7.63	F-max	2.23

Table A6. Error mean squares calculated from ANOVA for set interactions for all experimental runs of experiment 1.

Run	ErrorMS
1	594.18
2	263.06
3	237.38
F-max	2.50

Table A7. Average AUIC values for all lines of FCS-1.

Line	Run 1	Run 2	Run 3	Combined
Pril-1-019	134.60	127.65	144.75	135.66
Pril-1-021	93.38	117.38	138.00	116.25
Pril-1-023	137.25	119.65	139.36	132.09
Pril-1-024	77.63	122.63	120.75	107.00
Pril-1-025	94.88	112.50	127.13	111.50
Pril-1-026	132.16	131.25	146.25	136.55
Pril-1-027	135.00	132.75	138.00	135.25
Pril-1-028	131.25	137.25	127.13	131.88
Pril-1-030	114.75	117.75	126.00	119.50
Pril-1-031	127.13	124.50	143.25	131.63
Pril-1-035	110.63	114.75	127.50	117.63
Pril-1-036	113.25	145.50	148.50	135.75
Pril-1-038	96.75	126.75	129.00	117.50
Pril-1-039	121.52	120.75	140.25	127.51
Pril-1-041	119.25	115.50	122.25	119.00
Pril-1-044	99.52	140.33	144.75	128.20
Pril-1-046	117.75	100.13	141.75	119.88
Pril-1-047	118.50	122.81	134.25	125.19
Pril-1-051	113.25	121.50	133.50	122.75
Pril-1-052	144.62	122.25	142.50	136.46
Pril-1-053	124.50	119.25	143.25	129.00
Pril-1-054	151.13	140.62	150.08	147.28
Pril-1-055	136.50	123.00	138.75	132.75
Pril-1-056	115.13	88.13	128.25	110.50
Pril-1-058	145.60	103.66	131.97	127.08
Pril-1-059	143.25	123.75	140.25	135.75
Pril-1-060	130.81	134.90	149.15	138.29
Pril-1-061	145.62	150.275	134.35	143.42

Table A7. Average AUIC values for all lines of FCS-1 (continued).

Line	Run 1	Run 2	Run 3	Combined
Pril-1-062	123.00	120.75	138.75	127.50
Pril-1-063	112.13	100.50	126.38	113.00
Pril-1-064	151.19	138.13	138.88	142.73
Pril-1-065	141.00	125.25	133.50	133.25
Pril-1-066	140.95	124.20	141.83	135.66
Pril-1-067	105.75	135.00	121.50	120.75
Pril-1-069	133.50	128.25	139.50	133.75
Pril-1-070	144.75	138.42	146.25	143.14
Pril-1-071	102.00	116.25	135.75	118.00
Pril-1-073	117.75	128.25	134.25	126.75
Pril-1-075	117.00	138.75	138.00	131.25
Pril-1-077	104.63	122.63	137.25	121.50
Pril-1-079	122.25	132.43	129.00	127.89
Pril-1-081	102.75	123.75	127.50	118.00
Pril-1-083	120.75	125.25	132.00	126.00
Pril-1-085	84.00	116.25	123.75	108.00
Pril-1-087	131.25	138.75	129.00	133.00
Pril-1-089	129.00	129.00	138.00	132.00
Pril-1-090	107.25	107.25	141.75	118.75
Pril-1-091	132.00	135.00	148.50	138.50
Pril-1-092	120.00	117.75	132.00	123.25
Pril-1-093	125.63	120.75	116.63	121.00
Pril-1-094	144.00	125.25	138.00	135.75
Pril-1-095	135.38	124.50	136.50	132.13
Pril-1-096	146.25	121.87	139.89	136.00
Pril-1-098	147.75	142.50	140.25	143.50
Pril-1-100	132.75	124.50	146.37	134.54
Pril-1-101	143.25	123.00	138.00	134.75
Pril-1-102	123.00	128.63	136.50	129.38
Pril-1-103	134.25	125.25	140.25	133.25
Pril-1-104	136.50	137.25	143.25	139.00
Pril-1-105	128.25	111.75	133.50	124.50
Pril-1-106	129.75	116.25	126.75	124.25
Pril-1-107	138.00	123.75	143.25	135.00
Pril-1-108	136.50	116.25	147.75	133.50
Pril-1-109	133.50	113.25	125.25	124.00
Pril-1-110	139.50	122.41	143.25	135.05
Pril-1-111	138.00	137.25	142.50	139.25

Table A7. Average AUIC values for all lines of FCS-1 (continued).

Line	Run 1	Run 2	Run 3	Combined
Pril-1-112	127.50	131.25	128.25	129.00
Pril-1-113	121.13	129.75	136.50	129.13
Pril-1-114	132.90	138.75	147.00	139.55
Pril-1-115	141.00	126.75	126.00	131.25
Pril-1-116	125.63	117.75	128.25	123.88
Pril-1-117	135.75	135.00	143.25	138.00
Pril-1-118	137.25	131.25	134.25	134.25
Pril-1-120	121.50	134.25	139.50	131.75
Pril-1-121	124.50	127.50	136.50	129.50
Pril-1-122	153.30	142.09	140.25	145.21
Pril-1-123	116.63	115.50	108.75	113.63
Pril-1-124	147.23	133.50	146.24	142.32
Pril-1-125	117.75	114.38	126.00	119.38
Pril-1-126	112.88	128.25	115.50	118.88
Pril-1-128	101.63	112.50	129.00	114.38
Pril-1-132	119.25	135.00	145.50	133.25
Pril-1-139	120.75	123.00	136.50	126.75
Pril-1-140	116.25	130.50	120.00	122.25
Pril-1-142	112.88	130.50	135.00	126.13
Pril-1-143	99.38	130.50	136.50	122.13
Pril-1-144	97.50	111.75	135.00	114.75
Pril-1-145	129.75	132.75	144.75	135.75
Pril-1-146	137.62	148.55	144.53	143.56
Pril-1-147	120.75	132.75	138.00	130.50
Pril-1-148	143.25	138.75	144.00	142.00
Pril-1-149	117.00	131.25	130.50	126.25
Pril-1-150	107.63	131.25	136.50	125.13
Pril-1-151	123.75	123.00	134.25	127.00
Pril-1-152	-	134.42	140.03	-
Pril-1-154	127.50	135.21	145.50	136.07
Pril-1-155	-	148.71	-	-
Pril-1-156	142.50	135.00	145.50	141.00
Pril-1-158	147.78	146.25	148.20	147.41
Pril-1-159	128.25	126.00	133.50	129.25
Pril-1-160	127.50	122.25	146.25	132.00
Pril-1-161	135.98	145.11	120.04	133.71
Pril-1-164	145.50	137.73	135.44	139.56
Pril-1-165	138.78	132.00	144.75	138.51

Table A7. Average AUIC values for all lines of FCS-1 (continued).

Line	Run 1	Run 2	Run 3	Combined
Pril-1-166	145.50	142.50	143.25	143.75
Pril-1-167	119.18	144.43	135.75	133.12
Pril-1-168	148.50	136.50	145.50	143.50
Pril-1-169	126.75	129.00	97.50	117.75
Pril-1-170	127.50	139.83	132.00	133.11
Pril-1-171	147.00	133.50	132.75	137.75
Pril-1-172	135.00	123.75	128.25	129.00
Pril-1-176	140.25	144.75	137.25	140.75
Pril-1-177	131.25	143.25	139.50	138.00
Pril-1-180	112.50	141.75	140.25	131.50
Pril-1-181	121.88	140.25	133.50	131.88
Pril-1-182	132.00	147.00	143.25	140.75
Pril-1-183	123.00	140.25	136.50	133.25
Pril-1-184	133.50	138.00	139.50	137.00
Pril-1-185	130.50	144.00	148.50	141.00
Pril-1-186	131.25	142.50	149.25	141.00
Pril-1-187	147.00	150.00	144.75	147.25
Pril-1-188	144.29	147.73	147.48	146.50
Pril-1-189	141.00	142.50	143.25	142.25
Pril-1-190	128.25	141.75	144.00	138.00
Pril-1-191	133.50	141.00	140.25	138.25
Pril-1-192	138.00	147.75	145.50	143.75
Pril-1-193	127.50	147.75	141.00	138.75
Pril-1-195	150.91	149.91	150.96	150.59
Pril-1-197	111.00	127.50	125.25	121.25
Pril-1-198	132.75	144.75	119.40	132.30
Pril-1-200	108.73	113.25	110.25	110.74
Pril-1-201	121.50	126.00	123.75	123.75
Pril-1-203	123.75	111.59	117.38	117.57
Pril-1-204	101.25	120.38	129.75	117.13
Pril-1-205	137.25	133.50	146.25	139.00
Pril-1-206	118.50	129.75	122.25	123.50
Pril-1-207	111.00	112.13	130.50	117.88
Pril-1-209	100.50	124.88	113.63	113.00
Pril-1-216	141.38	135.00	141.00	139.13
Pril-1-217	133.50	145.50	137.25	138.75
Pril-1-219	138.75	129.00	129.75	132.50
Pril-1-220	125.25	141.75	137.25	134.75

Table A7. Average AUIC values for all lines of FCS-1 (continued).

Line	Run 1	Run 2	Run 3	Combined
Pril-1-221	127.40	139.59	148.50	138.50
Pril-1-223	92.25	122.25	104.63	106.38
Pril-1-224	136.26	142.50	146.25	141.67
Pril-1-225	84.75	114.00	111.75	103.50
Pril-1-226	99.16	144.034	139.39	127.53
Pril-1-227	111.75	125.25	137.25	124.75
Pril-1-229	101.25	140.25	123.36	121.62
Pril-1-232	135.75	142.50	140.92	139.72
Pril-1-233	115.88	129.75	113.25	119.625
Pril-1-235	.	.	146.02	.
Pril-1-236	145.62	136.50	118.85	133.65
Pril-1-237	138.75	129.76	138.00	135.50
Pril-1-238	121.50	133.50	129.00	128.00
Pril-1-242	131.25	138.75	123.33	131.11
Pril-1-246	107.63	140.12	121.50	123.08
Pril-1-248	114.00	139.50	133.50	129.00
Pril-1-249	103.88	121.50	137.25	120.88
Pril-1-256	118.50	130.50	125.63	124.88
Melrose	108.04	117.34	124.74	116.71
Shawnee	138.43	135.93	141.38	138.58

“.”, missing data

Table A8. Average AUIC values for all lines in FCS-2.

Line	Run 1	Run 2	Run 3	Combined
Pril-2-013	146.25	150.00	149.25	148.50
Pril-2-014	134.25	139.50	136.50	136.75
Pril-2-017	124.50	124.50	88.50	112.50
Pril-2-020	134.25	139.50	133.50	135.75
Pril-2-025	121.50	120.75	76.13	106.13
Pril-2-032	125.25	105.75	101.25	110.75
Pril-2-040	125.25	139.50	125.25	130.00
Pril-2-053	124.13	127.50	109.50	120.38
Pril-2-058	130.13	130.13	95.25	118.50
Pril-2-063	93.75	94.88	96.38	95.00
Pril-2-067	133.50	136.50	124.50	131.50
Pril-2-076	124.57	138.70	148.54	137.27
Pril-2-077	107.25	98.25	85.88	97.13

Table A8. Average AUIC values for all lines in FCS-2 (continued).

Line	Run 1	Run 2	Run 3	Combined
Pril-2-102	141.75	146.25	111.00	133.00
Pril-2-130	113.63	112.50	114.00	113.38
Pril-2-133	147.00	130.50	132.00	136.5
Pril-2-134	142.50	135.00	144.75	140.75
Pril-2-139	133.50	128.63	109.50	123.88
Pril-2-159	122.63	138.75	129.75	130.38
Pril-2-161	146.25	143.48	138.00	142.58
Pril-2-168	132.75	111.38	112.50	118.88
Pril-2-169	140.25	129.75	114.75	128.25
Pril-2-176	146.25	147.00	144.00	145.75
Pril-2-183	140.25	131.25	147.00	139.50
Pril-2-184	126.00	108.75	88.50	107.75
Pril-2-186	145.50	133.88	111.00	130.13
Pril-2-217	132.38	115.88	103.88	117.38
Pril-2-218	135.38	142.50	132.38	136.75
Pril-2-228	135.75	117.00	130.50	127.75
Pril-2-233	126.75	117.00	106.13	116.63
Pril-2-234	150.00	143.25	141.00	144.75
Pril-2-247	94.88	85.13	81.75	87.25
Medora	145.88	147.00	143.63	145.50
Melrose	100.13	84.19	73.88	86.06

Table A9. ANOVA including set interactions for AUIC for run 1 of FCS-1.

Source	DF	Mean square	F value	Pr >F
Genotype	158	951.07	3.27	$<0.0001^{***}$
Rep	3	447.07	1.54	0.204^{ns}

ns, not significant; ***, p<0.0001

Table A10. ANOVA including set interactions for AUIC for run 2 of FCS-1.

Source	DF	Mean square	F value	Pr >F
Genotype	160	514.68	2.52	$<0.0001^{***}$
Rep	3	173.68	0.85	0.4672^{ns}

ns, not significant; ***, p<0.0001

Table A11. ANOVA including set interactions for AUIC for run 3 of FCS-1.

Source	DF	Mean square	F value	Pr >F
Genotype	160	402.09	1.97	<0.0001***
Rep	3	480.45	2.35	0.0713ns

ns, not significant; ***, p<0.0001

Table A12. ANOVA with set interactions for AUIC for run 1 of FCS-2.

Source	DF	Mean square	F value	Pr >F
Genotype	33	1003.25	5.02	<0.0001***
Rep	3	1012.94	5.07	0.0026ns

ns, not significant; **, p<0.01; ***, p<0.0001

Table A13. ANOVA with set interactions for AUIC for run 2 of FCS-2.

Source	DF	Mean square	F value	Pr >F
Genotype	33	1527.16	6.02	<0.0001***
Rep	3	146.75	0.58	0.6307ns

ns, not significant; ***, p<0.0001

Table A14. ANOVA with set interactions for AUIC for run 3 of FCS-2.

Source	DF	Mean square	F value	Pr >F
Genotype	33	2279.57	6.73	<0.0001***
Rep	3	2339.45	6.91	0.0003**

ns, not significant; **, p<0.01; ***, p<0.0001

Table A15. Error mean squares and F-max calculated according to Hartley's Test for Homogeneity for each set from all experimental runs of FCS-1.

Run 1		Run 2		Run 3	
Set	ErrorMS	Set	ErrorMS	Set	ErrorMS
1	391.62	1	308.45	1	225.69
2	229.31	2	151.83	2	192.41
3	190.22	3	177.12	3	157.19
4	209.61	4	241.46	4	168.17
5	275.74	5	159.37	5	156.25
6	278.00	6	131.85	6	165.85
7	173.60	7	179.48	7	304.14
8	219.99	8	62.43	8	100.74
9	540.37	9	383.07	9	326.71
10	318.15	10	170.18	10	214.47
F-max	3.112	F-max	6.14	F-max	3.24

Table A16. Error mean squares calculated from ANOVA for set interactions for all experimental runs in FCS-1.

Run	ErrorMS
1	290.88
2	204.42
3	204.14
F-max	1.42

Table A17. Error mean squares and F-max calculated according to Hartley's Test for Homogeneity for each set from all experimental runs of FCS-2.

Run 1		Run 2		Run 3	
Set	ErrorMS	Set	ErrorMS	Set	ErrorMS
1	269.43	1	306.97	1	384.35
2	133.91	2	219.08	2	293.56
F-max	2.012	F-max	1.40	F-max	1.31

Table A18. Error mean squares calculated from ANOVA for set interactions for all experimental runs in FCS-2.

Run	ErrorMS
1	199.90
2	253.86
3	338.75
F-max	1.70

Table A19. Injury score for replicate one and average percent survival of Pril-1 in the field study (2015-2016).

Line	Injury score	% survival
Pril-1-001	5	5.00
Pril-1-003	3	37.50
Pril-1-004	2	111.67
Pril-1-005	4	0.00
Pril-1-010	2	76.67
Pril-1-012	3	12.50
Pril-1-014	3	0.00
Pril-1-015	7	4.17
Pril-1-016	3	16.67
Pril-1-017	5	7.14
Pril-1-018	3	0.00
Pril-1-019	3	40.00
Pril-1-020	4	30.00
Pril-1-021	3	100.00
Pril-1-022	4	15.00
Pril-1-023	3	0.00
Pril-1-024	3	133.33
Pril-1-025	4	100.00
Pril-1-026	4	16.67
Pril-1-027	2	0.00
Pril-1-028	6	0.00
Pril-1-029	4	16.67
Pril-1-030	5	16.67
Pril-1-031	5	25.00
Pril-1-032	5	37.50
Pril-1-033	0	11.11
Pril-1-034	5	0.00
Pril-1-035	2	25.00
Pril-1-036	7	0.00
Pril-1-037	6	50.00
Pril-1-038	5	0.00
Pril-1-039	0	0.00
Pril-1-040	5	0.00
Pril-1-041	2	50.00
Pril-1-042	6	75.00
Pril-1-043	5	16.67
Pril-1-044	4	0.00
Pril-1-045	6	0.00

Table A19. Injury score for replicate one and average percent survival of Pril-1 in the field study (2015-2016) (continued).

Line	Injury score	% survival
Pril-1-046	2	66.67
Pril-1-047	5	50.00
Pril-1-048	5	0.00
Pril-1-049	5	50.00
Pril-1-050	4	25.00
Pril-1-051	5	0.00
Pril-1-052	6	0.00
Pril-1-053	.	0.00
Pril-1-054	.	0.00
Pril-1-055	.	0.00
Pril-1-056	.	77.78
Pril-1-057	6	0.00
Pril-1-059	3	0.00
Pril-1-060	3	0.00
Pril-1-061	5	0.00
Pril-1-062	3	75.00
Pril-1-063	3	28.57
Pril-1-064	4	0.00
Pril-1-065	7	0.00
Pril-1-066	2	0.00
Pril-1-067	4	0.00
Pril-1-068	6	0.00
Pril-1-069	7	0.00
Pril-1-070	6	0.00
Pril-1-071	6	0.00
Pril-1-072	6	5.00
Pril-1-073	7	0.00
Pril-1-074	4	16.67
Pril-1-075	4	0.00
Pril-1-077	6	37.50
Pril-1-078	6	16.67
Pril-1-079	3	0.00
Pril-1-080	5	11.54
Pril-1-081	5	0.00
Pril-1-082	4	0.00
Pril-1-083	6	0.00
Pril-1-085	4	43.33
Pril-1-087	5	0.00

Table A19. Injury score for replicate one and average percent survival of Pril-1 in the field study (2015-2016) (continued).

Line	Injury score	% survival
Pril-1-088	3	0.00
Pril-1-089	6	50.00
Pril-1-090	4	26.67
Pril-1-091	4	0.00
Pril-1-092	4	12.50
Pril-1-093	3	43.75
Pril-1-094	7	33.33
Pril-1-095	5	0.00
Pril-1-096	2	83.33
Pril-1-097	5	18.75
Pril-1-098	5	8.33
Pril-1-099	3	45.71
Pril-1-100	4	0.00
Pril-1-101	4	37.14
Pril-1-102	3	0.00
Pril-1-103	6	0.00
Pril-1-104	7	0.00
Pril-1-105	6	40.00
Pril-1-106	3	50.00
Pril-1-107	4	4.17
Pril-1-108	4	58.33
Pril-1-109	3	25.00
Pril-1-110	3	32.50
Pril-1-111	5	20.00
Pril-1-112	4	0.00
Pril-1-113	5	8.33
Pril-1-114	4	0.00
Pril-1-115	3	37.50
Pril-1-116	3	25.00
Pril-1-117	5	0.00
Pril-1-118	4	0.00
Pril-1-120	4	0.00
Pril-1-121	3	7.14
Pril-1-122	3	0.00
Pril-1-123	3	187.50
Pril-1-124	3	4.17
Pril-1-125	2	91.67
Pril-1-126	3	87.50

Table A19. Injury score for replicate one and average percent survival of Pril-1 in the field study (2015-2016) (continued).

Line	Injury score	% survival
Pril-1-127	4	0.00
Pril-1-128	3	47.50
Pril-1-129	6	0.00
Pril-1-130	5	4.17
Pril-1-131	4	100.00
Pril-1-132	4	25.00
Pril-1-133	6	25.00
Pril-1-134	3	0.00
Pril-1-135	7	0.00
Pril-1-136	5	0.00
Pril-1-137	4	0.00
Pril-1-139	4	83.33
Pril-1-140	6	50.00
Pril-1-142	3	33.33
Pril-1-143	2	35.71
Pril-1-144	3	47.62
Pril-1-145	4	0.00
Pril-1-146	2	25.00
Pril-1-147	5	16.67
Pril-1-148	4	0.00
Pril-1-149	2	50.00
Pril-1-150	4	0.00
Pril-1-151	4	0.00
Pril-1-152	1	75.00
Pril-1-153	4	0.00
Pril-1-154	3	0.00
Pril-1-155	0	0.00
Pril-1-156	3	0.00
Pril-1-158	2	0.00
Pril-1-159	4	12.50
Pril-1-160	5	0.00
Pril-1-161	2	66.67
Pril-1-162	4	0.00
Pril-1-163	4	0.00
Pril-1-164	6	8.33
Pril-1-165	2	31.25
Pril-1-166	6	0.00
Pril-1-167	5	0.00

Table A19. Injury score for replicate one and average percent survival of Pril-1 in the field study (2015-2016) (continued).

Line	Injury score	% survival
Pril-1-168	5	0.00
Pril-1-169	2	0.00
Pril-1-170	2	25.00
Pril-1-171	6	0.00
Pril-1-172	4	0.00
Pril-1-174	6	0.00
Pril-1-176	4	175.00
Pril-1-177	5	25.00
Pril-1-178	5	0.00
Pril-1-179	3	29.17
Pril-1-180	2	66.67
Pril-1-181	5	0.00
Pril-1-182	1	0.00
Pril-1-183	2	25.00
Pril-1-184	1	0.00
Pril-1-185	5	25.00
Pril-1-186	2	0.00
Pril-1-187	4	0.00
Pril-1-188	2	0.00
Pril-1-189	3	0.00
Pril-1-190	2	14.29
Pril-1-191	5	0.00
Pril-1-192	2	0.00
Pril-1-193	6	0.00
Pril-1-194	3	0.00
Pril-1-195	2	29.17
Pril-1-196	3	0.00
Pril-1-197	3	18.75
Pril-1-198	2	0.00
Pril-1-199	5	0.00
Pril-1-200	2	0.00
Pril-1-201	3	80.00
Pril-1-202	4	0.00
Pril-1-203	6	0.00
Pril-1-204	2	25.00
Pril-1-205	3	0.00
Pril-1-206	2	25.00
Pril-1-207	2	0.00
Pril-1-208	2	83.33

Table A19. Injury score for replicate one and average percent survival of Pril-1 in the field study (2015-2016) (continued).

Line	Injury score	% survival
Pril-1-209	3	50.00
Pril-1-210	5	8.33
Pril-1-212	4	0.00
Pril-1-215	2	100.00
Pril-1-216	2	25.00
Pril-1-218	4	0.00
Pril-1-219	3	0.00
Pril-1-220	2	25.00
Pril-1-221	2	0.00
Pril-1-223	4	15.00
Pril-1-224	4	0.00
Pril-1-225	3	0.00
Pril-1-226	3	0.00
Pril-1-227	3	0.00
Pril-1-228	2	0.00
Pril-1-229	2	0.00
Pril-1-230	5	25.00
Pril-1-231	4	0.00
Pril-1-232	4	12.50
Pril-1-233	4	25.00
Pril-1-234	0	0.00
Pril-1-235	0	0.00
Pril-1-236	4	0.00
Pril-1-237	5	0.00
Pril-1-238	3	0.00
Pril-1-242	6	16.67
Pril-1-243	3	0.00
Pril-1-245	4	16.67
Pril-1-246	2	0.00
Pril-1-247	2	5.00
Pril-1-248	3	33.33
Pril-1-249	3	33.33
Pril-1-251	5	25.00
Pril-1-252	6	50.00
Pril-1-253	4	12.50
Pril-1-254	5	25.00
Pril-1-255	4	6.25
Pril-1-256	4	29.17

Table A19. Injury score for replicate one and average percent survival of Pril-1 in the field study (2015-2016) (continued).

Line	Injury score	% survival
Pril-1-257	5	0.00
Pril-1-258	4	0.00
Pril-1-259	4	5.56
Pril-1-260	4	0.00
Pril-1-261	7	0.00
Pril-1-262	4	0.00
Pril-1-263	6	20.00
Pril-1-264	4	0.00
Pril-1-265	6	0.00
Pril-1-267	3	31.25
Pril-1-268	6	14.55
Pril-1-269	3	25.00
Pril-1-270	6	0.00
Pril-1-272	6	0.00
Pril-1-273	2	42.86
Pril-1-274	6	0.00
Pril-1-275	7	0.00
Pril-1-276	5	10.00
Pril-1-277	4	5.56
Pril-1-279	4	150.00
Pril-1-280	6	0.00
Pril-1-281	5	0.00
Pril-1-283	6	0.00
Pril-1-284	2	33.33
Pril-1-285	5	14.29
Melrose	2	96.67
Shawnee	0	0.00

“.”, data missing

Table A20. Injury score for replicate one and average percent survival of Pril-2 in the field study (2015-2016).

Line	Injury score	% survival
Pril-2-003	4	0.00
Pril-2-004	4	0.00
Pril-2-005	5	0.00
Pril-2-008	4	0.00
Pril-2-009	3	100.00
Pril-2-012	4	0.00

Table A20. Injury score for replicate one and average percent survival of Pril-2 in the field study (2015-2016) (continued).

Line	Injury score	% survival
Pril-2-013	6	0.00
Pril-2-016	4	16.67
Pril-2-017	5	5.00
Pril-2-018	3	10.00
Pril-2-019	3	25.00
Pril-2-020	6	0.00
Pril-2-021	4	0.00
Pril-2-022	6	0.00
Pril-2-023	2	52.08
Pril-2-024	3	50.00
Pril-2-025	3	25.00
Pril-2-026	4	0.00
Pril-2-027	3	8.33
Pril-2-028	6	0.00
Pril-2-029	4	0.00
Pril-2-030	2	11.81
Pril-2-031	4	0.00
Pril-2-032	6	0.00
Pril-2-033	5	0.00
Pril-2-034	3	25.00
Pril-2-037	3	8.33
Pril-2-038	6	11.69
Pril-2-039	5	0.00
Pril-2-040	4	0.00
Pril-2-042	6	0.00
Pril-2-043	3	0.00
Pril-2-044	4	0.00
Pril-2-046	6	25.00
Pril-2-048	3	0.00
Pril-2-049	4	0.00
Pril-2-051	3	22.92
Pril-2-052	6	7.14
Pril-2-053	2	0.00
Pril-2-054	2	25.00
Pril-2-055	2	16.67
Pril-2-056	5	12.50
Pril-2-057	2	0.00
Pril-2-061	2	41.67

Table A20. Injury score for replicate one and average percent survival of Pril-2 in the field study (2015-2016) (continued).

Line	Injury score	% survival
Pril-2-069	3	0.00
Pril-2-070	5	0.00
Pril-2-072	2	0.00
Pril-2-076	3	0.00
Pril-2-077	5	0.00
Pril-2-078	4	0.00
Pril-2-079	6	0.00
Pril-2-081	6	0.00
Pril-2-083	5	0.00
Pril-2-085	4	0.00
Pril-2-086	7	0.00
Pril-2-087	5	42.50
Pril-2-088	3	0.00
Pril-2-089	4	30.00
Pril-2-090	5	0.00
Pril-2-091	2	55.56
Pril-2-092	4	0.00
Pril-2-094	2	17.50
Pril-2-095	5	12.50
Pril-2-097	6	0.00
Pril-2-101	4	0.00
Pril-2-106	3	8.33
Pril-2-108	5	7.14
Pril-2-111	7	50.00
Pril-2-112	5	0.00
Pril-2-113	5	0.00
Pril-2-114	4	0.00
Pril-2-115	2	0.00
Pril-2-116	3	0.00
Pril-2-117	3	0.00
Pril-2-118	6	5.56
Pril-2-119	6	33.33
Pril-2-120	4	33.33
Pril-2-124	6	50.00
Pril-2-125	1	0.00
Pril-2-126	5	0.00
Pril-2-127	3	0.00
Pril-2-128	6	0.00

Table A20. Injury score for replicate one and average percent survival of Pril-2 in the field study (2015-2016) (continued).

Line	Injury score	% survival
Pril-2-129	6	0.00
Pril-2-132	3	12.50
Pril-2-134	5	0.00
Pril-2-137	2	35.71
Pril-2-138	5	0.00
Pril-2-139	7	0.00
Pril-2-144	5	0.00
Pril-2-145	6	5.00
Pril-2-149	5	0.00
Pril-2-150	5	12.50
Pril-2-151	4	0.00
Pril-2-155	5	0.00
Pril-2-158	4	0.00
Pril-2-160	1	25.00
Pril-2-162	2	0.00
Pril-2-163	3	33.33
Pril-2-165	3	0.00
Pril-2-166	7	8.33
Pril-2-167	5	0.00
Pril-2-168	6	5.00
Pril-2-170	3	0.00
Pril-2-171	6	0.00
Pril-2-173	3	0.00
Pril-2-174	5	0.00
Pril-2-176	6	0.00
Pril-2-177	6	25.00
Pril-2-178	4	16.67
Pril-2-181	3	0.00
Pril-2-183	6	0.00
Pril-2-185	4	0.00
Pril-2-186	3	0.00
Pril-2-188	6	12.50
Pril-2-190	5	0.00
Pril-2-192	6	0.00
Pril-2-194	2	66.67
Pril-2-195	5	0.00
Pril-2-196	4	0.00
Pril-2-197	4	0.00

Table A20. Injury score for replicate one and average percent survival of Pril-2 in the field study (2015-2016) (continued).

Line	Injury score	% survival
Pril-2-199	6	25.00
Pril-2-200	2	0.00
Pril-2-203	3	0.00
Pril-2-204	4	0.00
Pril-2-205	5	0.00
Pril-2-207	4	0.00
Pril-2-208	7	0.00
Pril-2-210	7	20.00
Pril-2-213	7	10.00
Pril-2-214	4	36.25
Pril-2-215	5	0.00
Pril-2-216	5	0.00
Pril-2-217	5	10.00
Pril-2-219	5	6.25
Pril-2-220	4	0.00
Pril-2-221	5	0.00
Pril-2-222	3	26.25
Pril-2-223	3	16.25
Pril-2-225	2	41.25
Pril-2-226	7	0.00
Pril-2-227	6	0.00
Pril-2-228	6	12.50
Pril-2-229	2	0.00
Pril-2-230	2	67.86
Pril-2-233	6	0.00
Pril-2-234	4	0.00
Pril-2-235	5	0.00
Pril-2-238	4	0.00
Pril-2-239	3	20.00
Pril-2-240	5	0.00
Pril-2-241	3	0.00
Pril-2-242	6	0.00
Pril-2-246	3	0.00
Pril-2-249	4	15.00
Pril-2-251	3	0.00
Pril-2-252	4	0.00
Pril-2-253	3	0.00
Pril-2-254	4	0.00

Table A20. Injury score for replicate one and average percent survival of Pril-2 in the field study (2015-2016) (continued).

Line	Injury score	% survival
Pril-2-256	5	0.00
Pril-2-258	4	0.00
Pril-2-260	4	0.00
Pril-2-263	7	0.00
Melrose	2	42.78
Medora	6	0.00

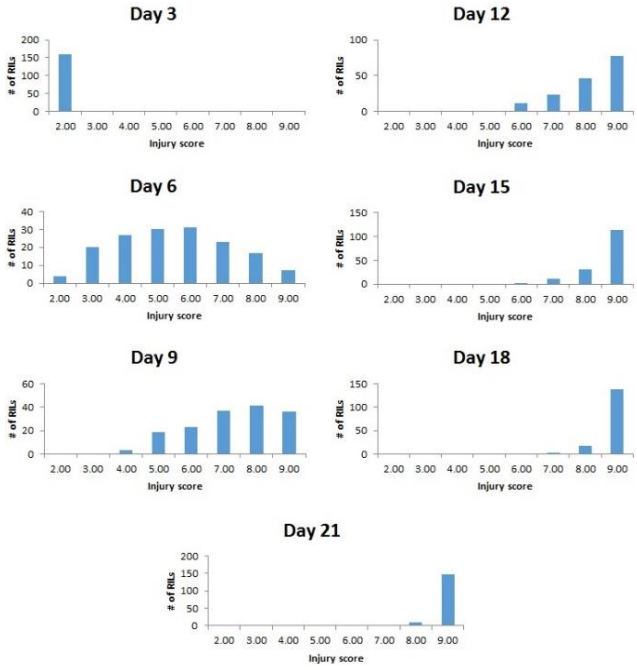

Figure A1. Histograms of scores given for FCS-1 run 1 recorded on day 3, 6, 9, 12, 15, 18 and 21.

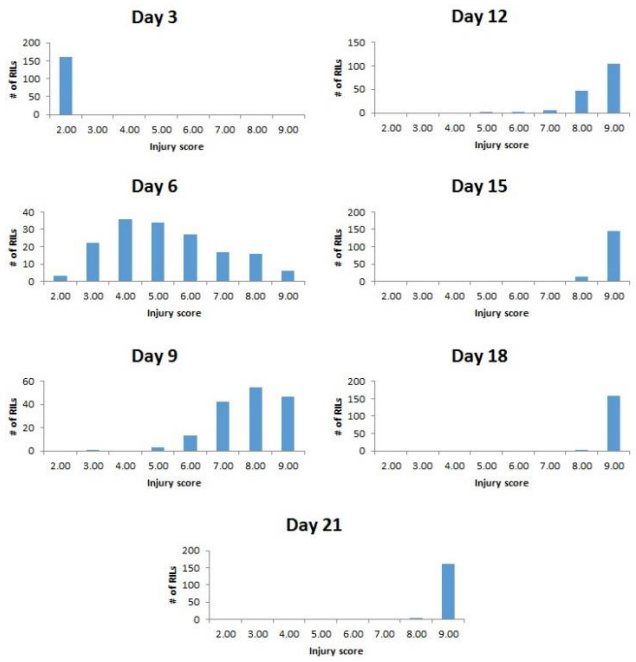

Figure A2. Histograms of scores given for FCS-1 run 2 recorded on day 3, 6, 9, 12, 15, 18 and 21.

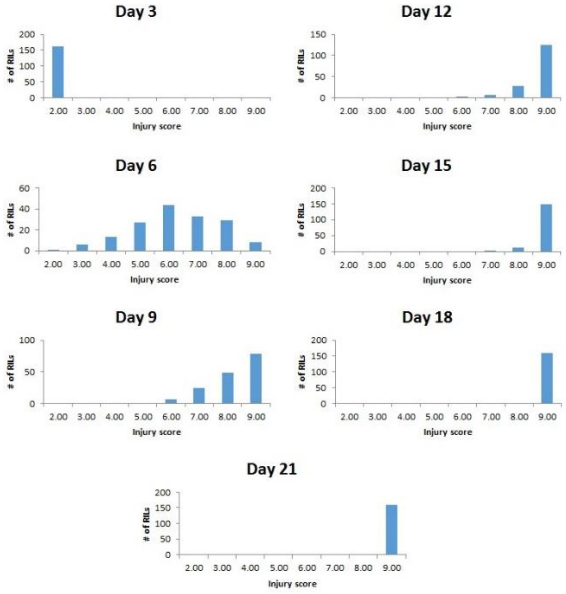

Figure A3. Histograms of scores given for FCS-1 run 3 recorded on day 3, 6, 9, 12, 15, 18 and 21.

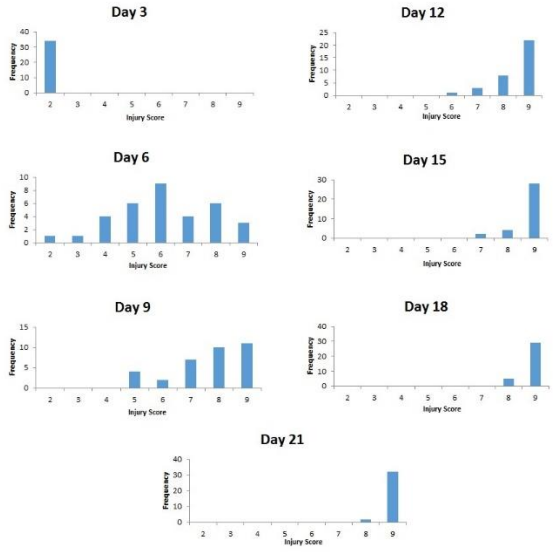

Figure A4. Histograms of scores given for FCS-2 run 1 recorded on day 3, 6, 9, 12, 15, 18 and 21.

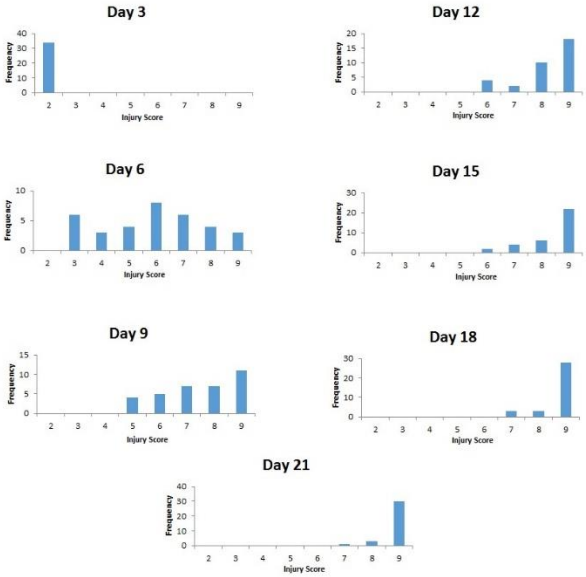

Figure A5. Histograms of scores given for FCS-2 run 2 recorded on day 3, 6, 9, 12, 15, 18 and 21.

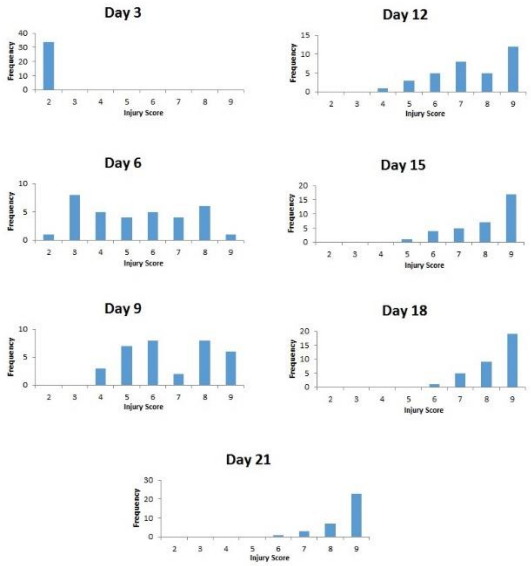

Figure A6. Histograms of scores given for FCS-2 run 3 recorded on day 3, 6, 9, 12, 15, 18 and 21.

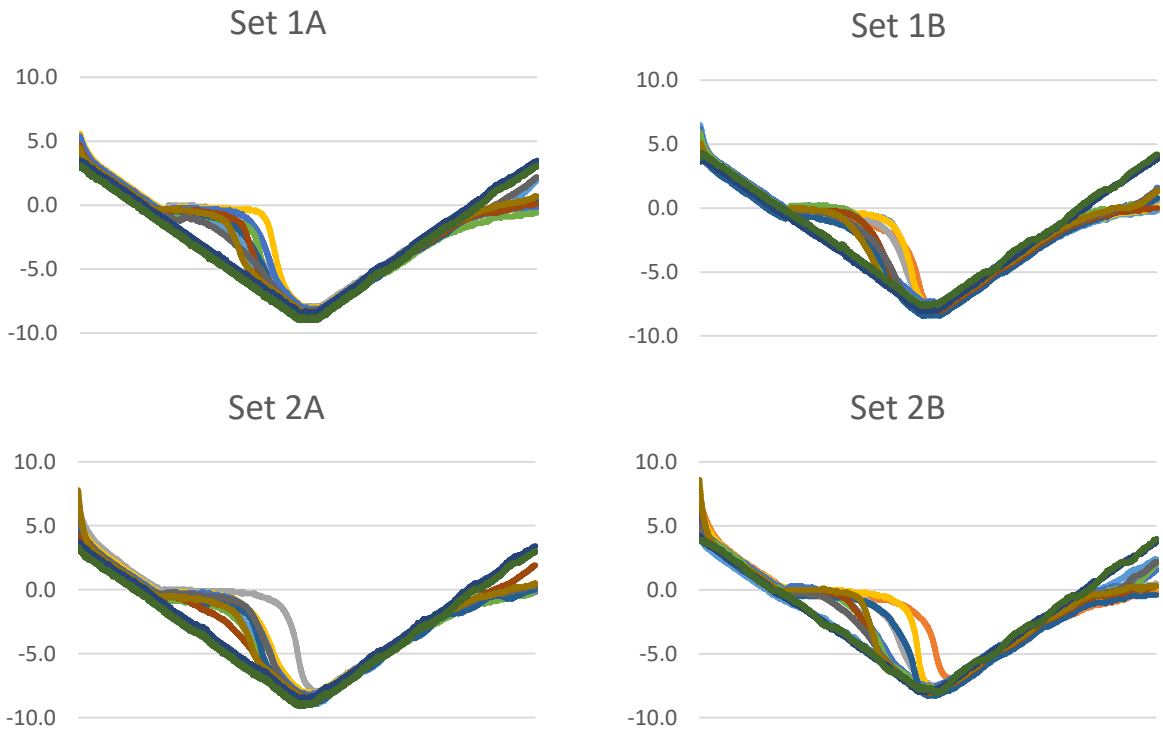

Figure A7. Twelve temperature measurements taken for tray 1 and tray 2, 'A' and 'B' respectively, in each set of run 1 while in the freeze chamber for FCS-1. Channel 11 and 12 corresponded to the temperature within the canopy and aerial temperature, respectively.

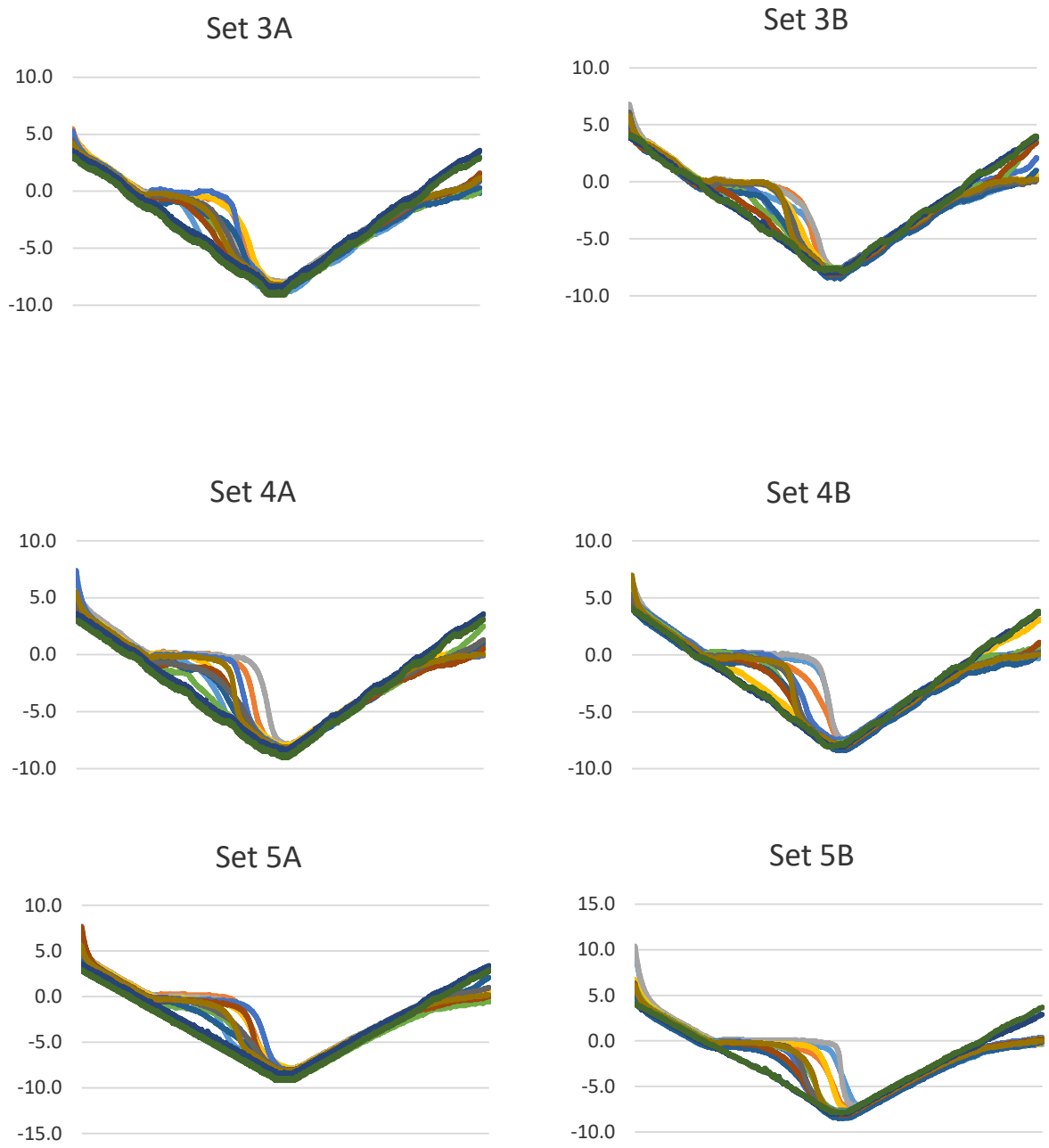

Figure A7. Twelve temperature measurements taken for tray 1 and tray 2, 'A' and 'B' respectively, in each set of run 1 while in the freeze chamber for FCS-1 (continued). Channel 11 and 12 corresponded to the temperature within the canopy and aerial temperature, respectively.

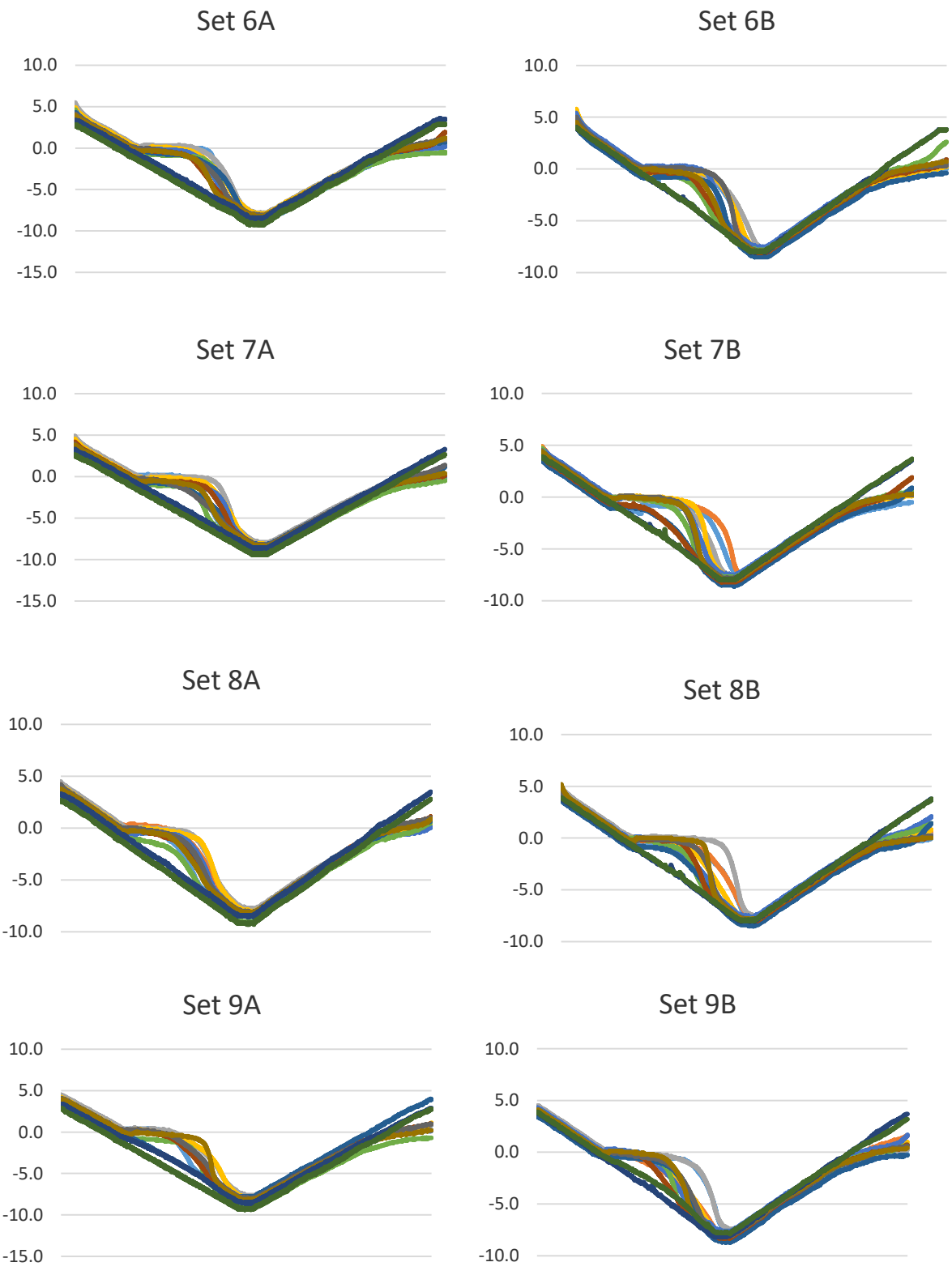

Figure A7. Twelve temperature measurements taken for tray 1 and tray 2, 'A' and 'B' respectively, in each set of run 1 while in the freeze chamber for FCS-1 (continued). Channel 11 and 12 corresponded to the temperature within the canopy and aerial temperature, respectively.

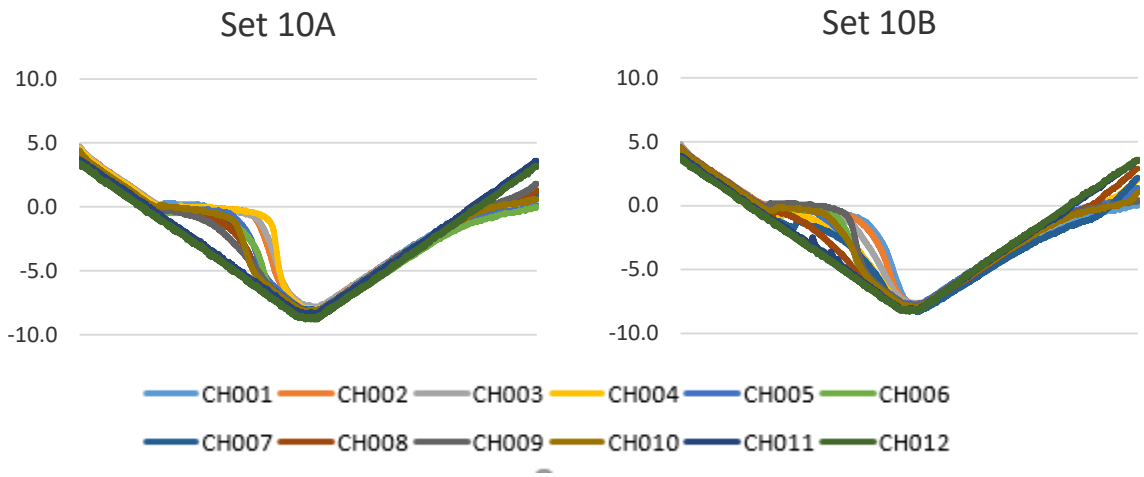

Figure A7. Twelve temperature measurements taken for tray 1 and tray 2, 'A' and 'B' respectively, in each set of run 1 while in the freeze chamber for FCS-1 (continued). Channel 11 and 12 corresponded to the temperature within the canopy and aerial temperature, respectively.

Figure A8. Example of injury scores given to plants based on the amount of living tissue.