Understanding the Basics of Cold tolerance and its Basis in Agronomic Decisions for Winter Cereals on the Canadian Prairies.

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Summary
Cold tolerance is the most basic requirement for winter cereals on the prairies and considerable research efforts have been made throughout western Canada to improve cultivars and the production system. Upstream of this research is the underlying mechanism of cold tolerance including: how cell membranes deal with cold and freezing temperatures, the plants' genetic response to cold temperatures, and the adaptation of photosynthesis to low temperatures. Early, shallow planting of winter cereals optimizes the plant's ability to maintain photosynthesis and produce the building blocks for plant establishment and acclimation, ensuring the maximum cold tolerance potential of the cultivar is reached.

Introduction
Cold tolerance is the most basic requirement for winter cereals on the prairies. Simply put, if the seedlings established in the fall are not sufficiently cold tolerant they will not survive the winter. An extensive amount of work has been completed throughout western Canada to improve winter cereal cultivars, but the most important research in winter cereals has been on the production system. Improvement of the winter cereals production system has led to optimization of the growing environment for the plants, improving their acclimation conditions, ensuring the plants are sufficiently established and have the resources to survive the winter, and limiting their exposure to lethal freezing temperatures. This review of cold tolerance will consider the basics of how cold affects plant cells and the plant itself. Current testing methods and research on cold tolerance will be discussed and related to the way winter cereals are managed in the field.

Basics of cold tolerance in the plant cell
The majority of the freezing stress on a plant cell results from ice development in the region outside of the cell membrane known as the apoplast[1]. Formation of ice outside of the cell membrane reduces liquid water outside the cell creating a negative water balance or water potential. This negative water potential means that the cell must respond through moving water out of the cell to re-establish the proper water balance. The loss of free cellular water to ice leads to dehydration and in order to maintain turgor pressure, changes in the cell membrane are required[1]. There are several ways this can occur. In some cases, non-cold adapted cells contract to reduce cell surface area by forming endocytotic vesicles (absorption of outer membrane into the cell) (Figure 1). In cold adapted cells, exocytotic extrusions (Figure 1) are produced with no reduction of the cell surface size[2]. Exocytotic extrusions are finger-like sections of the membrane that protrude from the cell surface and give the cell a jagged-like appearance.

If thawing of the ice outside the cell occurs, the result is a change in water balance leading to an influx of water into the cell. Since the process of cell membrane expansion is slower than water balance adjustment, the cells that have undergone endocytotic vesicle formation can do very little to prevent swelling, which leads to the cell rupture and death (Figure 1)[2]. In non-cold adapted plants, this type of cell death occurs 30% and greater than 40% in oats and rye, respectively[3]. Survival of cold adapted plant cells is associated with the formation of exocytotic extrusions that are responsive to osmosis (no membrane apposition) (Figure 1). Since the membrane size remains the same, upon thawing it is able to expand without lysis (rupture of the cell membrane) occurring.

In cells that are exposed to severe dehydration through freezing, cellular membranes and membranes of cellular organelles (chloroplast and mitochondria) come in close proximity or apposition with each other leading to the membrane components interacting. The formation of membrane structures called inverted micellar intermediates, which lead to the development of interlamellar attachments and hexagonal II phase structures (Figure 1), compromise

the integrity of the membranes in the cell and cause the cell to be no longer responsive to osmosis (water flow back into the cell)[4]. If the freezing stress has caused extreme dehydration, the end result of these membrane interactions is cell death, even in cells that are cold acclimated.

The ability of the cell to become cold tolerant and perform adaptive changes in membrane structure does not occur rapidly. To reach maximum cold tolerance, cells must acclimate slowly under cool, above freezing temperatures over a number of weeks. Acclimation of the plant cell involves many diverse processes including cell membrane alterations, adjustment in cell metabolism to maintain photosynthetic capacity, increasing cell concentrations of solutes like sugars, and up-regulating proteins that are involved in a number cold adaptive cell processes[5]. All of these processes combine to increase and maintain the cold tolerance in the individual cells and the whole plant.

Cold acclimation of cell membranes has been shown to be associated with a change in membrane lipid composition. In response to cold temperatures, it has been shown that wheat and rye chloroplast membranes alter the ratios of structural lipids called glyceroglycolipids from monogalactosyldiglyceride to digalactosyldiglyceride. This change is associated with improved cold tolerance[6,7]. The change from monogalactosyldiglyceride to digalactosyldiglyceride results in the desaturation of the membrane, which limits the development of hexagonal phase II complexes through improved ability to bind to water molecules that reduces effects of dehydration[6]. As a result, lower freezing temperatures are required to remove the water that is bound to the membrane to induce these cell membrane compromising complexes (Figure 1).

Increased sugar content in cereals is associated with increased cold tolerance. A comparison of winter and spring wheat varieties found that during a four week acclimation period, the more cold tolerant variety accumulated the most sugars and the less cold tolerant spring wheat line accumulated lower amounts of sugars[8]. After one week of acclimation, all varieties showed large increases in mono- and disaccarides (glucose, fructose and sucrose). After two weeks, the levels of these sugars continued to increase in the cold tolerant winter wheat lines and levelled off in the tender spring wheat line. Furthermore, polysaccharide concentration (raffinose and fructans) continued to increase in the most cold tolerant winter wheat line. To add to this common trend, a gene expression study examining the effects of cold acclimation over a two week period found that a specific gene (galactinol synthase) related to polysaccharide development had sustained upregulation in the winter wheat variety compared to the spring wheat variety after two hours of acclimation[9]. Finally, Fowler et al. (1981) examined 36 wheat varieties and found that total sugars, as well as simple sugars (glucose and fructose) were all correlated to LT50 and field survival index (FSI). The strong correlation indicated the importance of sugars for winter survival.

Increased sugars in the cell are thought to have a number of roles in improving cold tolerance. First, the sugars can be used to maintain cell water potential by limiting the amount of water that leaves the cell due to ice formation in the apoplast. Sugars are also thought to associate with the membrane of the cell by replacing lost water and maintaining membrane fluidity[12]. Finally, sugars play a role as energy sources and building blocks for other important cold tolerance processes in the cell.

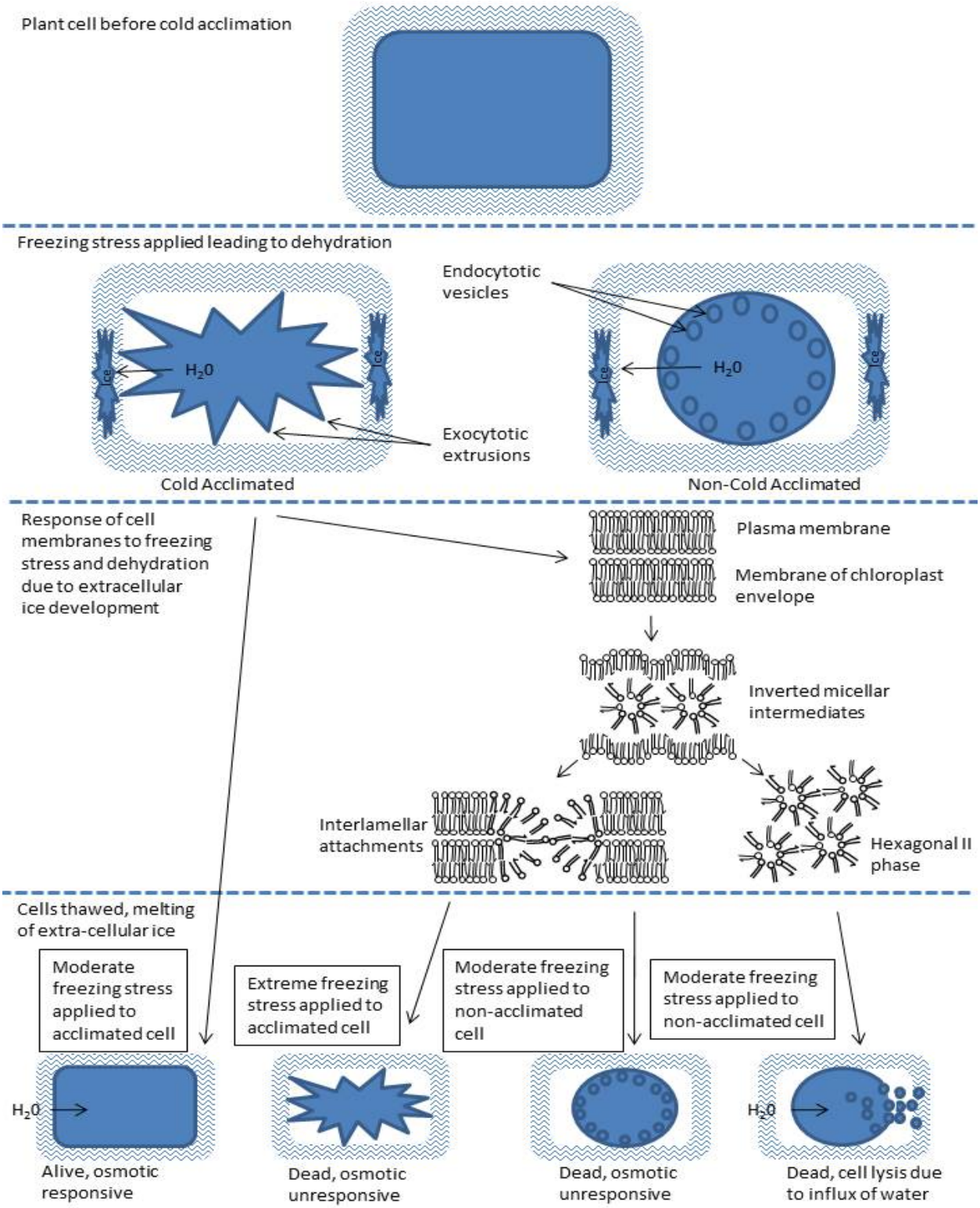

Figure 1. Effects of freezing temperatures on cold and non-cold acclimated cells of cereals (adapted from Ruelland et al. 2009 and Uemura et al. 2005).

In an attempt to understand the genetic basis of cold tolerance in cereals, a number of studies have used quantitative genetic and molecular mapping techniques to identify genetic regions or loci associated with cold tolerance. These loci have been named FROST RESISTANCE 1 and 2 (*FR-1* and *FR-2*). *FR-1* was initially discovered to be tightly linked[13] to VERNALIZATION (*vrn-1*), the requirement of the plant to be exposed to cool temperatures before reproductive growth occurs. Through plant physiology studies, it is thought that *FR-1* is a pleiotropic (one gene having an effect on multiple traits) effect of *vrn-1*[14], meaning that there may not be actual genes associated with cold tolerance at *FR-1*. Instead, it is thought that holding the plant in a vegetative phase through vernalization or any other means (e.g. photoperiod response) maintains cold tolerance[15,16,17]. Upon the fulfillment of vernalization requirement, a shift from vegetative to reproductive phase occurs, leading to a subsequent loss of cold tolerance. *FR-2*, likely the most important locus for cold tolerance, has been the focus of most of the cold tolerance research in cereals. It has been found to be associated with the regulation of a large portion (estimated 40%) of the cold tolerance in cereals[18,19,20].

Genetic mapping confirmed that *FR-2* is loosely linked to one of the winter habit genes (vernalization gene; *vrn-A1*) and DNA sequencing of *FR-2* in wheat and barley determined that this locus is composed of a cluster of C-repeat binding factor (*CBF*) genes[21,22]. *CBFs* are transcription factors that up regulate cold tolerance genes through binding to a conserved element in the promoters of cold tolerance or dehydration-responsive genes[23], as well as being associated with regulating genes responsible for phenotypic changes including the compact growth habit and thick leaves of a cold acclimated, cold tolerant plant[24]. It is not known which specific *CBF* genes are most important for cold tolerance or if it is an additive effect. Differences in cold acclimation induction temperatures among cereal species have been noted and are explained by *CBF* expression. In a study by Campoli et al. (2009), *CBF* gene expression patterns of rye, wheat and barley were examined at four different acclimation temperatures. The results showed that there were differences in cold acclimation induction temperatures across species. Rye showed signs of increased *CBF* expression at 18°C versus winter wheat and barley, which showed increased *CBF* expression at 10°C. This may explain some of the differences between species in terms of cold tolerance as rye starts to cold acclimate at much higher temperatures compared to wheat and barley[26]. Variation has also been found at this locus in barley for number of genes present, as well as the presence of a non-functional *CBF* pseudogene which may also explain some of the variability in cold tolerance among varieties[26].

Specific examples of genes from these gene families are *COR14b*, a protein that is found in the stroma of the chloroplast and is associated with protecting the photosystem from photodamage[27] and *WCS120* gene family[28], which belongs to the dehydrin group of proteins. The function of dehydrins is not known, but they are suspected to be involved in several processes important for cold tolerance including: scavenging for reactive oxygen species, protecting membrane lipids, and cryoprotecting enzymes[29]. Beyond these specific genes, cold acclimation is associated with complex changes in gene expression over the acclimation period. Monroy et al (2007) compared gene expression of one winter and one spring wheat variety over a two week cold-acclimation period and determined that 450 genes were differentially expressed (turned-on or turned off) between the two varieties. These genes include a number of regulatory genes like transcription factors, protein kinases, binding proteins, as well as genes involved in sugar metabolism, photosynthesis, stress response, cell wall proteins and also numerous genes with unknown function. A more recent study using the Affymetric Wheat Genome microarray, which can simultaneously survey 55,052 unique genes, determined that over 12,900 genes were affected during cold acclimation and vernalization[30].

For cold adaptive processes to occur, the plant cell must synthesize building blocks through harvesting light energy (photosynthesis) and utilizing the energy and fixed carbon (glucose/sucrose) in subsequent downstream biochemical reactions (glycolysis and citric acid cycle). Therefore, adjustment of photosynthesis to cold temperatures is perhaps the most crucial process for acclimation and is required for a plant to reach maximum cold tolerance[31,24]. When temperatures drop below the acclimation threshold, biochemical (enzymatic) processes slow significantly in comparison to the ability of the plants chlorophyll and antennae pigments to capture light. These slower biochemical processes cause an energy bottleneck, disrupting the balance of light energy captured compared to the light energy used by the plant (photostasis). The plant must deal with the excess of light energy or reactive oxygen species like hydrogen peroxide (H_2O_2) and superoxide (O_2-) will develop[5]. Production of these reactive oxygen species is a result of the

electron transport chain having no available electron acceptor (system is over-reduced) due to slower downstream biochemical processes. This results in significant damage to crucial chloroplast proteins that are required for photosynthesis and other processes. Since low temperatures limit the activity of enzymes that normally neutralize H_2O_2 and O_2-, the plant uses a short term strategy to adjust energy going into photosynthetic processes through releasing the light energy as heat. This method is known as non-photochemical quenching. The long term strategy of cold tolerant winter cereals, which has been shown to not occur in non-cold tolerant spring cereals, is the adjustment of the photosynthetic capacity (photochemical quenching) of the system. It has been shown that the approximate time for the photosynthetic system to adjust to cold acclimation temperatures is 3-4 weeks[32]. Adjustment of the photosynthetic system is associated with a specific change of the phenotypic growth habit of cold adapted, cold tolerant cereals (termed phenotypic plasticity) to a smaller compact form with thicker leaves. This growth habit, thought to be associated with *CBF* expression, allows for increased efficiency in the ability to absorb light energy (photons) and to carry out photosynthesis due to the thicker leaves having increased energy production per unit volume in comparison to a non-cold acclimated or non-cold tolerant cereals with greater leaf area[24]. This gain in efficiency also reduces the amount of non-metabolically productive non-photochemical quenching occurring. The decrease in vegetative growth in comparison to spring or non-acclimated cereals is thought to allow for the energy from photosynthesis to be used processes other than vegetative growth, like cold acclimation. Increased photosynthetic efficiency leads to an increase in available carbon metabolic products that are transported out of the leaf in the form of sucrose and fructans and stored in the vacuole of carbon sinks, mainly in the crown of the plant[33] where they are used as energy sources, building blocks for cold acclimation and cryoprotectants.

Methods for Testing Cold Tolerance in Cereals Indoors

A range of cold tolerance screening methodologies have been developed in cereals. The protocols all have similar main procedures and follow the general guideline laid out by Levitt (1980). Durations of cold acclimation vary, depending on the experiment, but are generally between two and six weeks. Before freeze testing commences, all methods have a short, sub-zero period for ice nucleation (-3 or -4 °C) that can vary in length from 12 hours to two days. Gradual freezing of the plants occurs at a rate of 2-3°C per hour. The time interval the plant material is maintained at sub-zero testing temperatures varies across protocols, ranging from removal once the material reaches the testing temperature to after many weeks of freezing[35,36].

All protocols thaw the plants slowly by placing them immediately at 0 °C or 4 °C[35,37] or slowly raising the temperature of the freezers 2 °C per hour and maintaining the freezer at 1 °C for as long as 15 hours (See Figure 2A and B for a typical set up). The plants are transplanted and placed in a greenhouse or growth room. Two to three weeks after freezing, plants (Figure 2C) are scored as alive or dead[35,37], or on a quantitative scale (e.g. 1-4) rating the extent of regrowth or damage[32,38,39]. Fowler and Carles (1979) carried the analysis of plant survival further using probit statistical analysis to calculate LT50 or lethal temperature at which 50% of the plants die to give a value to the degree of cold tolerance of the plants (Figure 2D).

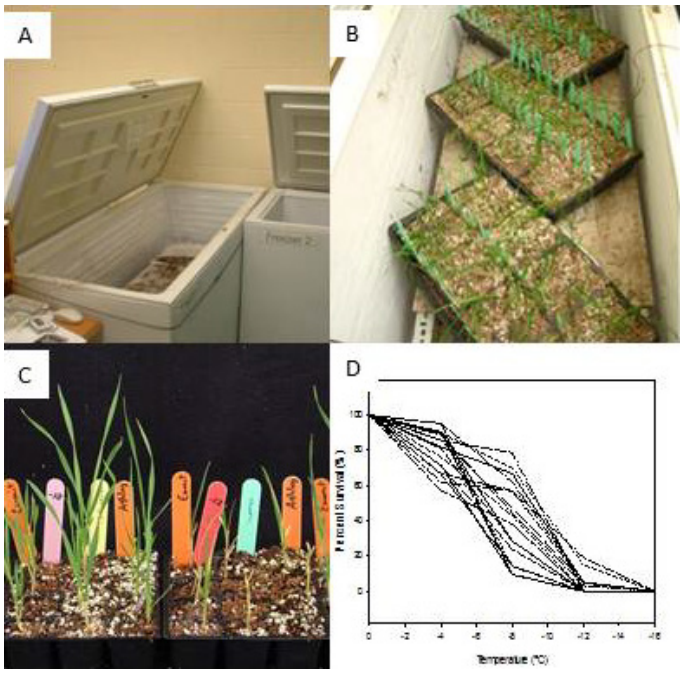

Figure 2. Examples of artificial freezing test equipment and set up (A and B), plants at evaluation stage two weeks after freezing (C) and, example data curves for cereal seedlings frozen over a series of freezing temperatures.

Methods for Testing Cold Tolerance in Cereals Outdoors - the Field Survival Index (FSI)

Due to the inconsistency and uneven winter kill across field seasons, locations and within-trial plots, the field survival index (FSI) was developed to provide an objective index of the winter hardiness of cereal varieties and agronomic treatments (stubble type, fertilizer rate, etc.) across a number of trials[40,41]. To calculate FSI, plots are first scored in the spring, after green-up, on a percent survival basis. Only plots showing a differential are used for the analysis (95% to 5% survival). Varieties with winter survival higher than 95% and lower than 5% are either not stressed enough to show a difference or were almost completely killed. Consequently, including this data in analyses tends to mask differences in winter survival and can often result in misleading conclusions. The computation of FSI is somewhat complex and requires the initial estimation of the relative difference in winter survival among varieties across locations. This relative value is then used in the estimation of the FSI value for each plot at each location to have 100% winter survival and the estimation of a FSI stress value for each plot which normalizes plot to plot variation in the data. Data resulting from the analysis for each variety grown at a location is then combined in an overall analysis across locations. This generates a cultivar ranking and a final FSI value for the specific cultivar that ultimately represents the minimum FSI value of a variety required to have no winterkill (100% survival) in a plot. A higher FSI value indicates that the variety has an increased level of winter hardiness. In some cases the percent differences between cultivars can be quite large. For example, the difference in FSI between Norstar, cold tolerant winter wheat (FSI=514) and Manitou non-cold tolerant spring wheat (FSI=160) is 354%. FSI is especially effective in lowering experimental error due to the use of the stress value calculations, as well as providing a true scale of winter survival among cereal varieties. Also, lines or varieties being tested do not need to be consistent across experiments to assign a FSI rating. As long as a control variety with a FSI value is present in the experiment, the relative FSI value can be calculated and compared to all other FSI values from previous experiments.

Application of Plant Physiology Knowledge to Agronomy Practices in the Field

Cultivars

The only winter cereals grown with any success in western Canada are fall rye, winter wheat and winter triticale (wheat-rye hybrid). Fall rye has impressive cold tolerance with LT50 values ranging between -26.5 to -33°C and FSI ratings ranging from 550-735 meaning that fall rye can be grown throughout the prairies. Winter wheat has less cold tolerance potential and ranges in LT50 values of -18 to -24 °C and FSI values of 420 to 514 are typical for western Canadian winter wheat varieties[41]. Extensive efforts have been made to increase cold tolerance of winter wheat varieties available to producers in Western Canada. However, almost no tangible gain has been made in cold tolerance since the release of Norstar, which has led to the conclusion that natural variation for cold tolerance in winter wheat has been exhausted[42]. Further efforts to examine cold tolerance and the possibility of transferring this trait into wheat includes crossing wheat with rye to establish the expression of cold tolerance of triticale[43], as well as crossing with wild relatives of wheat[44]. The most cold tolerant winter wheat variety developed in western Canada is Norstar (FSI=514) and a majority of the new winter wheat varieties have Norstar in their pedigree[45]. Current efforts in germplasm enhancement for improved cold tolerance have revolved around in-depth examination of the *FR-2* locus and examining genetic variation in cereal germplasm. This could lead to the discovery of new cold tolerance genes not currently employed in western Canadian germplasm[46] as well as the regulatory mechanism of these genes, which may allow selection for optimal epistatic (multiple genes) interactions[26].

Agronomy and Cold Tolerance

Date of Seeding and Seeding Depth

Considerable research on optimal planting dates has been conducted in western Canada[47,48,49,50]. All of these studies arrive at a similar conclusion; optimal planting date for winter survival and yield is generally 4 to 6 weeks before the onset of winter conditions[48,47](Figure 3). Seeding depth has also been examined with optimal seeding depth determined to be from 1 to 2.5 cm[51,52,48]. Since cold acclimation has been shown to be a light dependant process (requires photosynthesis) in winter cereals[27], meeting the optimum seeding date and depth facilitates fall plant establishment and ensures that the photosynthetic capacity is in place so the plant can produce a well-developed crown (a least three leaves) and be prepared to cold acclimate when soil temperatures begin to drop. Cold-acclimated cereal plants can continue to photosynthesize throughout the fall and winter, provided the temperatures remain warm enough and the leaves are intact[53]. Sugars (sucrose and fructans) produced over this period can be translocated to the crown to be used as energy, building blocks for acclimation processes, or as a solutes to maintain water balance in the crown cells. The effects of early plant establishment on sugar content has been determined under field and indoor environments where earlier seeded plants or plants under a longer acclimation period produced considerably higher amounts of fructans[54]. As a side note, higher levels of fructans in the plants acclimated over a longer period were also associated with improved tolerance to snow molds compared to plants acclimated for a shorter period of time, another factor in winter survival which is not discussed in this review (see Gaudet et al. 2001 for more details). It must also be noted that seeding too early can reduce winter survival and cold tolerance as it can lead to extension of the growing point in the crown towards the soil surface making it more exposed to cold temperatures or temperature fluctuations[55]. Exposure to plant diseases may also play a role in reduced winter survival of early planted winter cereals[56].

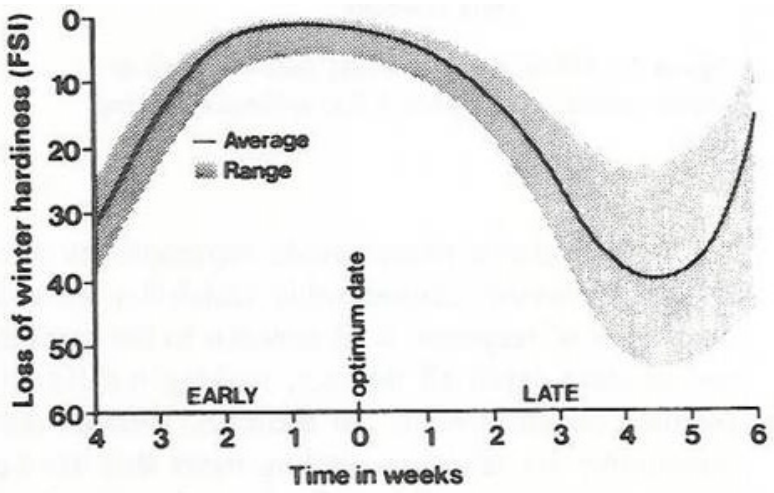

Figure 3. Effect of seeding date on winter survival based on the reduction of a cultivars field survival index (FSI) (From Fowler 1995).

Rotation

A major advance in winter cereal winter survival is the stubbling-in or planting of winter wheat into standing stubble in a no-till system. Planting into standing stubble does not have a direct effect on the cold tolerance of the plant per se. Instead, it modifies the plant environment as snow cover is deeper and more uniform in standing stubble versus fallow fields. Increased snow trapping is associated with warmer soil temperatures through the winter and a decrease in exposure of freezing temperatures that approach lethal LT50 values of the plant. Increased snow cover improves the chance of the plant to survive the winter, but prolonged and deep snow cover is associated with reduced winter survival and snow mold risks (Table 1). Sugar content is higher in plants seeded at an earlier seeding date[54] and therefore associated with cold acclimation and the ability to photosynthesize under cool temperatures. Resistance to deep snow cover and snow mold may also have to do with a reduced rate of sugar metabolism during the snow cover period, which is variety specific[57].

Table 1. Minimum cultivar field survival index values for an undamaged winter cereal stand based on depth of snow cover (From Fowler 1995).

Snow depth	FSI
Bare summerfallow	>650
5 cm snow cover	540
10 cm snow cover	430
>15 cm snow cover (snow mold risk)	<420

Fertilizer (Phosphorus/Nitrogen)

Fertilizer management plays a significant role in cold tolerance of winter cereals and soil testing is recommended before planting to optimize inputs and maximize cold tolerance and winter survival. Phosphorus appears to have the most direct effect on cold tolerance in winter cereals, although the mechanism of how it affects cold tolerance and winter survival is somewhat unclear. The literature indicates that phosphorus-limited treatments or mutant plants with lower levels of phosphorus in the leaves have an increase in cold tolerance. The reasoning for this is that phosphorus is a component of the building blocks (sugar-phosphate intermediates) of sucrose the end product of photosynthesis. If phosphorus is limited, production of sucrose is limited leading to an energy

bottleneck and a disruption of the energy balance in the cell. As a result the photosystem must adjust in the same way that it would under cold acclimation temperatures[58]. However, in the field, this relationship is generally not observed and it has been shown that too low or too high levels of phosphorus lead to a reduction in winter survival[59,60,61]. Therefore, optimum phosphorus levels are required. This disagreement with indoor cold tolerance experiments indicates that optimum phosphorus levels may be not directly associated with cold tolerance, but more likely linked to plant regrowth in the spring[61]. No significant effect of nitrogen levels have been consistently found in research completed in western Canada[41,61] and the majority of effects on winter survival are associated with method of nitrogen application; with seed placed nitrogen often causing ammonia phytotoxicity leading reduced stand establishment[62] and winter survival[63]. However, there have been numerous studies showing that excess nitrogen decreases cold tolerance[34,54,64]. One possibility may be that optimum nitrogen and phosphorus balances are required to maximize cold tolerance and winter survival which may be location and soil dependant[61,54,64].

Seed treatment

Seed treatments generally do not increase cold tolerance unless the seed has been infected previously with dwarf bunt, which is known to affect seedling cold tolerance[65,66]. Recently, it has been shown that seed treatments with a neonicotinoid insecticide improved cold tolerance in spring wheat[39]. Research completed in other plant species indicates that neonicotinoids elicit a salicylic acid response, which is associated with an increase in scavenging (neutralization) of reactive oxygen species[67]. As mentioned above, reactive oxygen species are responsible for damage of cellular membranes and photosynthetic reaction center complexes. Limiting this damage may allow the plant to cold acclimate more effectively. Further research is required and experiments to assess the effects of the neonicotinoid treatment on cold tolerance in winter wheat are currently being pursued.

Conclusion

Cold tolerance is the most basic requirement for winter cereals on the prairies. Basic research examining fundamental structures and processes (cell membranes, gene regulation and photosynthesis) confirms applied agronomic research. Shallow seeding 4-6 weeks before the onset of winter conditions enables quick plant establishment and maximizes cold tolerance through the plants ability to photosynthesize and produce the building blocks for cold acclimation. The plant cell uses these building blocks to alter membrane structure and transcribe of genes involved in preparing the plant for cold stress. Changes in plant cells that lead to maximum cold tolerance take 4-6 weeks or longer and coincide with the length in time from planting to the onset of winter conditions. Producers need to keep this in mind when planting winter cereals, as maximum cold tolerance and winter survival is directly related to a successful harvest in the summer.

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