CHAPTER 9

Cold Tolerance

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Wheat is grown across a wide range of environments and is considered to have the broadest adaptation of all cereal crop species (Briggle and Curtis, 1987). This broad adaptation is due, to a large extent, to wheat's cold tolerance, i.e. the ability to withstand temperatures much lower than 1-4ºC, considered the minimum temperature for growth (Figure 1).

In a general sense, cold tolerance in wheat should refer to performance at temperatures lower than the optimum for growth (about 20ºC), and there are definitely differences in the growth rate of cultivars at low temperatures and, consequently, in their adaptation to cool climate. However, the term "cold tolerance" is most frequently used to describe a plant's response to freezing temperatures, which have more dramatic effects on the crop. Most often, freezing temperatures affect autumn-sown wheat during winter. Freezing tolerance refers to the broader term of "winter hardiness," an attribute of autumn-sown cereals that is responsible for differences in "winter survival" or "overwintering."

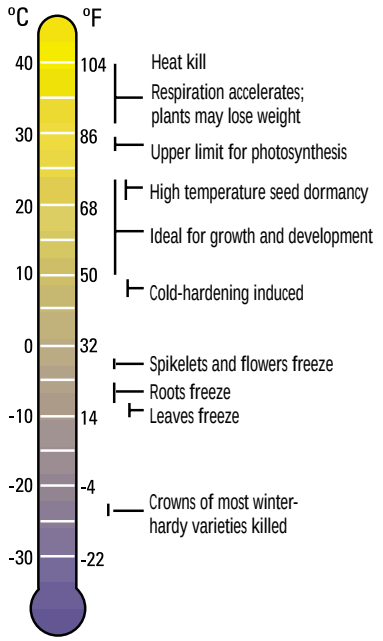

Figure 1. General range of favorable and unfavorable (stress) temperatures for wheat.

Winter survival is defined by Blum (1988) as "the final integrated plant response to a multitude of stresses involved during and after freezing stress, including both external-physical and biotic stresses." Even if plants are not winter-killed, they can be affected by freezing temperatures that may damage the leaf, causing reduction in leaf area, delayed growth, and plant debilitation. Considerable variation for winter hardiness exists among cultivars, which justifies dedicating extensive efforts to this breeding objective (Pictures 1 and 2).

Less often, freezing temperatures can occur during late frosts in spring, causing leaf or spike injury. Unhardened leaves can tolerate -4 to -8ºC (Gusta and Chen, 1987), but the reproductive tissue of the developing ear is considerably less resistant to freezing and may be injured at -1.8ºC (Single and Marcellos, 1974). Differences in what is generally called "frost tolerance" are less pronounced, although waxy or hairy lemma, palea, and awns are thought to delay formation of ice in the tissue. Due to the limited genetic variation for frost tolerance, breeding efforts have been directed mostly to escaping frost by selecting for later flowering.

Picture 1. Differential winter damage in head rows.

Picture 2. Differential winter damage in plots.

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It should be noted that low, non-freezing temperatures (below 10ºC) at the critical stage of meiosis can also have dramatic effects on wheat by causing male-sterility and, consequently, low yields. Genetic differences in the response to this stress are known to exist (Qian et al., 1986, Saulescu et al., 1997) but, because of its relatively rare occurrence, little effort is directed towards breeding for tolerance other than selecting for an appropriate flowering time that allows plants to escape the stress.

Stress Factors Involved in Winterkill

The reasons for winterkill in wheat, as well as the extent of the damage, vary greatly from region to region and from year to year. The main factors causing winterkill (alone or in combination) are related to low temperature *per se* (such as extreme air or soil temperatures, below the critical temperature of a particular wheat cultivar):

- inadequate hardening, due to late emergence in autumn or a sudden drop in temperature;
- long periods of cold-induced desiccation (Gusta et al., 1997a);
- prolonged periods of low sub-zero temperatures; in particular, mid-winter temperatures below -15ºC result in the rapid loss of winter hardiness (Gusta et al., 1997b);
- alternate freezing and thawing, which causes increased injury from ice crystal growth with each freeze (Olien, 1969).

Another factor responsible for winterkill is ice encasement, a major cause of plant death in areas of high rainfall and fluctuating temperatures during winter (Andrews et al., 1974). Ice has high thermal conductivity and can aggravate the effect of low temperatures. It also has low gas permeability and may, in extreme cases, smother or suffocate plants by depriving them of oxygen (Poltarev et al., 1992).

Finally, low temperatures or snow can cause indirect damage through:

- frost heaving due to the formation of ice in the soil. The ice pushes the plants upward, breaking and exposing the roots;
- snow mold, caused by fungi in areas with long-lasting snow cover. The most damaging fungus affecting winter survival is pink snow mold (*Microdochium nivale* (Fries) Samuel and Hallet), previously known as *Fusarium nivale* (Fr) Ces. (Hömmö, 1994). Although *Microdochium nivale* cannot survive freezing, it is tolerant to low temperatures and severely damages plants in the 0-5ºC temperature range. Other, less important fungi causing snow mold are *Typhula* spp., the pathogen for speckled snow mold or typhula blight, and *Sclerotinia borealis*, which causes sclerotinia snow mold.

The relative importance of stress factors causing winterkill can vary greatly among regions. In the Ukraine, an analysis of data from the last 100 years showed that winterkill was caused by low temperatures in 35% of cases, by alternate freezing and thawing in 26% of cases, and by ice encasement in 22% of years when significant winter damage occurred (Poltarev et al., 1992). Wisniewski et al. (1997) stated that the critical factors that affect winter survival in Poland are low temperature, freeze-induced desiccation, and infection by pathogenic fungi.

Gusta et al. (1997a) reported that the main factors responsible for winterkill in the Great Plains of North America are long periods of cold-induced desiccation, poor acclimation conditions in autumn, and unpredictable timing and duration of extremely cold temperatures, whereas the primary cause of winterkill in western Canada is freeze-induced desiccation. For eastern North America, Olien (1967) found that winterkill is most likely to occur during low temperature stress following a midwinter thaw, when the crown tissues have high moisture content. Correct evaluation of the frequency with which these or other factors can affect winter survival in the target area is essential for making a better choice of parents and testing procedures in a breeding program; this can also improve resource allocation efficiency.

Wheat plants can cope with each of the above mentioned winter stress factors through different genetic and physiological mechanisms. For example, a plant's freezing tolerance and snow mold resistance are based on different genetic mechanisms (Hömmö, 1994). However, the basic process behind most events leading to winterkill is freezing, or formation of ice in plant tissues (Figure 2). Freezing damage is in general not a consequence of low temperature *per se*, but rather the result of cellular dehydration brought about by extra-cellular ice crystallization. Cellular membranes have been recognized as the primary sites of freezing injury (Hincha and Schmitt, 1994).

Freezing tolerance is defined as the ability of plants to survive ice formation in extracellular tissues without significant damage to membranes or other cell components.[3] It is the result of physiological, chemical, and physical

[3] For the sake of clarification, it should be noted that intracellular ice formation is always lethal. The chemical potential in the intracellular solution must be equal to the chemical potential of the external solution or the ice. This equilibrium is attained through removal of intracellular water. To avoid cellular dehydration under freezing stress, the osmotic potential of intracelluar solutions is increased as the osmotic potential of extracellular solution decreases. For a detailed discussion of the physiological processes during freezing stress, see Blum (1988).

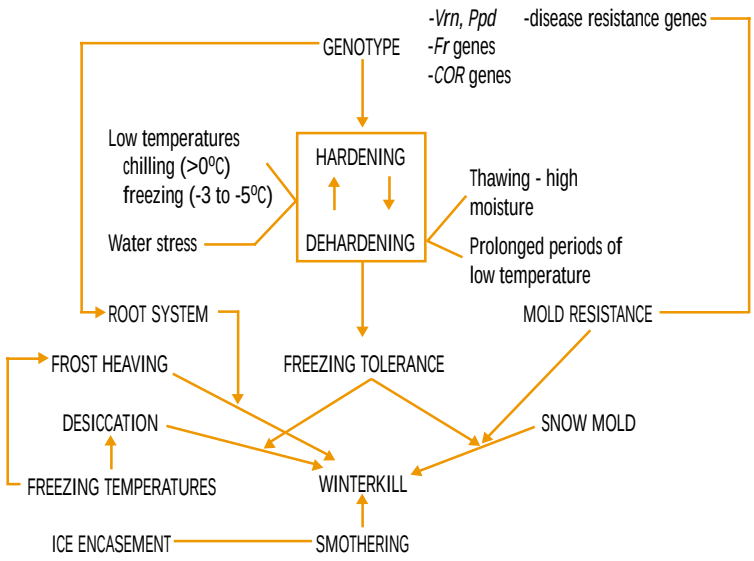

Figure 2. Diagram of the processes involved in winterkill and winter hardiness.

reactions, and of changes in plant cell structure that take place at appropriate developmental stages, under suitable environmental conditions. This process is called hardening or acclimation.

Acclimation proceeds in two stages, depending on the sequential action of chilling (>0ºC) and freezing (-3 to -5ºC) temperatures. A decrease of water potential in tissues, due to decreased osmotic potential (because of sugar accumulation in vacuoles), is the most important feature in the first stage of plant acclimation. It is correlated with a significant increase in the abscisic acid (ABA) level and results in modification of protein synthesis. There are great differences among cereals regarding the above 0ºC temperature at which acclimation is initiated. Winter rye starts at much warmer temperatures, which makes its acclimation period longer than that of winter wheat. Spring wheat and spring barley do not initiate acclimation at temperatures above 2ºC (Gusta et al., 1997a). Reversible modifications of membrane properties, which result in further decrease of water potential in parenchymatic tissue, seem to play a main role in the frost-dependent stage of acclimation (Kacperska, 1994).

Low temperatures act as the primary inducing factor, but stresses other than low temperature (water stress, wind, etc.) can also induce a certain level of freezing tolerance. Low temperature itself or secondary factors (ABA, sucrose fatty acids, and water status), produced in response to the primary signal, can result in conformational changes in either membrane and/or proteins and/or an ABA hormone receptor, and this, in turn, can result in the regulation of genes involved in cold acclimation (Gusta et al., 1997a). Several genes have been identified in various plants as low-temperature-inducible (lti) or cold regulated (COR). These genes may have a cryoprotective effect on cellular membranes (Thomashow, 1993).

The fact that frost-resistant cultivars harden faster and deharden more slowly than frost-susceptible genotypes suggests that acclimation may have different threshold induction temperatures in cultivars differing in cold tolerance. In barley, a higher degree of frost resistance is associated with a higher threshold induction temperature for the accumulation of COR proteins (Rizza et al., 1994).

Freezing tolerance is not a static condition, for it changes with time, temperature, soil and plant moisture, nutrition, and physiological age and status. It depends largely on the cold acclimation or hardening processes. Indeed, differences in freezing tolerance of unhardened plants of different cultivars are negligible, while considerable differences can be detected after full hardening. The hardening process can be stopped, reversed, and restarted. Generally, under natural conditions, the dynamics of freezing tolerance are characterized by three stages (Prasil et al. 1994):

- a hardening period, in autumn, when cold tolerance is acquired;
- a period of tolerance maintenance, when the critical or lethal temperature varies, depending on temperature fluctuations in winter;
- a dehardening period, generally at the end of winter, when plants lose their cold tolerance.

Each stage is influenced not only by genotypical (vernalization requirement and photoperiodic response) or developmental (age of plants) factors, but also by environmental ones. For example, if water content is altered by flooding or desiccation, the cold hardiness of winter cereals changes dramatically (Metcalfe et al., 1970). Similarly, prolonged periods of low sub-zero temperatures decrease the freezing tolerance of winter wheat seedlings significantly. The most cold tolerant, fully hardened winter wheats can tolerate -15ºC for only around six days, and survive at -18ºC for only 24 h and at -23ºC for only 12 h (Gusta et al., 1982). After being exposed to low temperatures for a longer period, the seedlings' acquired freezing tolerance is greatly reduced, and they are killed at much higher temperatures than in early winter.

Cultivars with similar freezing tolerance in early winter vary greatly in their

ability to cope with long periods of sub-freezing temperatures. The duration and intensity of sub-zero temperature is therefore a main factor determining the loss of freezing tolerance and consequent winterkill in winter wheat. (Gusta et al., 1997a). Freezing tolerance is also known to be influenced by plant nutrition (Freyman and Kaldy, 1979), herbicides (Freyman and Hamman, 1979), viral infection (Paliwal and Andrews, 1979), and seed-borne diseases such as common bunt (*Tilletia foetida* and *T. caries)* (Veisz, 1997). This is why, to detect genotypic differences in freezing tolerance, it is important to keep all environmental factors as uniform as possible.

Traits Associated with Freezing Tolerance in Wheat

Freezing tolerance is the result of complex physiological mechanisms involving many cell and plant traits. Numerous studies have shown that the genetic control of cold tolerance is complex and can be regarded as polygenic. As many as 15 out of 21 chromosomes in wheat have been found to influence tolerance to low temperatures (Stushnoff et al., 1984). Nevertheless, major genes such as *Fr1*, closely linked but separable from the *Vrn1* gene on chromosome 5A, and *Fr2*, linked but easily separated from *Vrn3* on chromosome 5D, were shown to have a large effect on low temperature response (Snape et al., 1997).

The association between freezing tolerance and vernalization requirements can therefore be partially explained by the linkage between major genes controlling freezing tolerance and two genes that control growth habit. In addition, the vernalization genes have been identified as key developmental factors responsible for the duration of expression of low-temperature-induced structural genes (Fowler et al., 1996b). Recent data show that the regulatory influence exerted by the vernalization genes over low-temperature-induced structural gene expression occurs at the transcriptional level (Fowler et al., 1996a).

Winter wheats initiate hardening at higher temperatures than spring wheats, and the latter harden only to a very limited extent. Similarly, in spring, completely vernalized winter wheats only reharden to the level of spring cereals. Obviously, a high level of hardening can only be achieved in "dormant," not rapidly developing, plants; therefore, a strong association exists between the degree of vernalization and the degree of freezing tolerance that can be achieved in winter cereal seedlings (Roberts, 1990a). On the other hand, several northern European wheat cultivars with very long vernalization requirements only have moderate freezing tolerance (Gusta et al., 1997a). Braun (1997) found a highly significant correlation (r=0.67-0.77) between growth habit and freezing tolerance, which suggests that only 45-60% of the variation for cold hardiness can be attributed to the differences in vernalization requirements.

Freezing tolerance was found to be associated with prostrate growth type. A gene controlling prostrate growth was found to be closely linked with *Fr1* and *Vrn1* on chromosome 5A (Roberts, 1990b). Genetic linkage is probably not the only explanation for the observed association, since a prostrate plant is also less exposed to low temperatures and desiccation, and better protected by snow.

It is worth mentioning that prostrate growth type, found in dormant juvenile plants in autumn, can also occur in cultivars with low vernalization requirements but high photoperiod response. In wheat such cultivars are usually only moderately winter hardy, but in barley some of the most cold tolerant cultivars are known to be day-length sensitive, with a low vernalization requirement.

Both high vernalization requirement and day-length response, as well as other mechanisms that cause "winter dormancy" and delay development to the generative stage (differentiation of the meristem), are generally associated with lateness. In an experiment with winter oat, populations selected for higher levels of winter hardiness were also later maturing and often taller than desired (Marshall, 1976). A similar association has also been observed in wheat. This association can create difficulties in breeding early and short winter hardy cultivars. However, no effect of height alone on winter hardiness was found in a comparison of winter wheat height isolines derived from the cultivar Yogo (Allen et al., 1986). This suggests that the association between winter hardiness and plant height may depend on the height genes involved and on genetic background.

Cell length, as measured in stomatal guard cells, was also found to be associated with cold hardiness, as was leaf length and height of hardened plants (Limin and Fowler, 1994; Roberts, 1990b). Although the association is not very strong, these traits have the advantage of being easily determined on individual plants.

Hardening induces significant changes in many of the plant's biochemical and physiological characters. Phenotypic differences in these characters are often associated with freezing tolerance. For example, tissue water content (Limin and Fowler, 1994), accumulation of simple sugars or polysaccharides (Olien et al., 1986), free proline accumulation in leaves and shoots (Dörffling et al., 1990), and accumulation of specific cold-regulated proteins (Houde et al.,

1992) in hardened plants were found to be correlated with freezing tolerance.

Genetic linkage is the most likely explanation for the association between certain gliadin blocks, as identified by electrophoresis, and freezing tolerance (Sasek et al., 1984). Such associations can be useful only in specific crosses, involving specific parents. Despite the large number of correlations with other traits, none is high enough to justify replacing direct freezing tests.

Breeding Approaches

Handling a complex trait such as winter hardiness in a breeding program is a difficult task, due to the large number of genes involved and the numerous interactions with the environment. But the main difficulty in breeding cold tolerant wheat is that high freezing tolerance is generally associated with lower yields and later maturity.

Many traits that are associated with freezing tolerance, such as delayed spring growth or small cells, can have negative effects on yield, especially in rainfed environments where rapid growth in early spring and earliness are important to avoid late drought and high temperatures. Besides, every additional breeding objective will slow down genetic progress for all other traits of interest. Therefore, the breeding objective should not be to maximize winter hardiness, but to develop cultivars with the minimum winter hardiness necessary for a given target area. As Fowler et al. (1981) pointed out, in general the most successful winter wheat cultivars have only marginally greater winter hardiness than the minimum required for the area in which they are grown.

Definition of the minimum hardiness required for a given region is not a simple job. It should be based on assessment of the winterkill risk, based both on weather data and information about cultivar performance in the area. A careful analysis of historical weather data, including minimum temperatures and time of their occurrence, is useful, but not sufficient. The same low temperature can have rather different effects on wheat plants, depending on prior temperature regimes and other factors, which determine the level of hardening achieved. Some of the crop models, such as CERES, have a sub-routine that simulates hardening and winterkill, and these could be used for a more correct estimation of the winterkill risk.

A simpler, but very useful approach, is to use winterkill data of cultivars with varying levels of winter hardiness that have been cultivated in the target area for a long time and for which long-term records for overwintering are available. In general, it is not difficult to identify a cultivar grown for many years in a region with only occasional and not very serious winterkill. A frequency of 1 out of 10 years with some winterkill can generally be considered acceptable. But the accepted risk can be higher or lower, depending on economical, social, and other factors. If such a cultivar is identified, it is the best definition of the minimum level of hardiness required for the area and should be used as a check in all cold hardiness tests. Additional checks should be used to provide a range of hardiness.

When wheat cultivars with higher levels of freezing tolerance are selected to lower the risk of winter damage, it should be remembered that the resulting yield penalty in years without freezing stress may be far greater than the advantage of better winter survival in years with severe winters.

Obviously, the breeding strategy for developing winter hardiness will depend on the ratio between the hardiness level in the gene pool used and the minimum necessary for the target area. If most parents used in crosses have a winter hardiness equal to or higher than the accepted minimum, maintaining this level is a relatively easy task that can be accomplished through the application of mild selection pressure against the rare, less hardy segregants. On the other hand, if a large number of parents are not winter hardy enough, as is the case in programs that use spring x winter crosses, higher selection pressure is advisable, beginning with the early generations, to increase chances of recovering an acceptable level of hardiness. Early generation selection against spring growth habit, as suggested by Braun (1997), can be very efficient.

Breeding for winter hardiness is much more difficult in areas marginal for winter wheat cropping where the minimum required hardiness is at or above the maximum available cold hardiness potential. As Grafius (1981) pointed out, there “has been a notable absence of improvement in the maximum cold hardiness potential of cereals in this century”, and this “inability of plant breeders to increase maximum cold tolerance levels suggests that all of the available cold tolerance genes had been previously concentrated in hardy land races within winter cereal species.”[4] Recovering this maximum level of hardiness in higher yielding genotypes is only possible by applying very high selection pressure in large segregating populations.

[4] Cereals differ greatly in their ability to survive low temperatures. The most cold tolerant rye cultivars are killed at around –34ºC, wheat cultivars at around –23ºC, and barley at around –18ºC.

Transgressive segregation for freezing tolerance has only been recorded in crosses among medium or less hardy parents, but not among the most hardy parents. There are hopes that interspecific hybridization can bring in new genes from species with higher freezing tolerance (such as rye), but to date no such transfer has been successful for common wheat.

Durum wheat generally has much lower winter hardiness than bread wheat, so breeding for freezing tolerance is more difficult. However, for areas where winter durums are superior to spring durums, breeding for winter hardiness has to be a high priority. The best winter hardiness is found in cultivars derived from interspecific crosses with bread wheat, and such crosses, as well as transgressive segregation in intraspecific crosses, will probably allow further progress in this respect.

Methods and Techniques

Field testing

Whenever winter conditions differentiate genotypes for winter survival, evaluation of winter hardiness in the field is desirable. Field evaluation allows large-scale, inexpensive characterization of breeding materials against the full range of factors affecting winter survival, whereas controlled freeze tests measure only low temperature tolerance. For this reason, most breeding programs, regardless of available resources, favor field testing to measure winter survival (Fowler et al., 1993), despite the disadvantages described below.

Levitt (1972) defined a "test winter," or "differential winter," as a winter severe enough to kill the most tender plants and damage those of intermediate hardiness to various degrees. Unfortunately, from a plant breeder's point of view, winters with "good" differentiation among genotypes for their winter hardiness are infrequent, even in areas that require a high level of cold tolerance.

It should be stressed that winterkill is often not only the result of low temperature stress, but also of the interaction of a range of factors, which most likely will not all occur in a given year or location. Therefore, multilocational testing can give better information on winter hardiness, especially if locations are selected to provide higher probability of winterkill.

Years with milder winters than a "test winter" may sometimes exert some selection pressure for winter hardiness, based on leaf damage and color (Picture 3). Although the correlation with actual winter hardiness is not very high, scores based on leaf damage are helpful for discarding the less cold tolerant lines, provided notes are taken when symptoms are most visible (a very short period, only 2-3 days) in spring, before active growth begins.

An additional problem of field testing for winter hardiness is the great variability within a field due to non-uniform snow cover, soil preparation, planting depth, soil and plant moisture, etc. To cope with this problem, the following are highly recommended:

- Plant one or two check cultivars of known winter hardiness every few rows.
- Take notes on replicated nurseries, preferably in small plots. Marshall et al. (1981) consider short (0.5-1.5 m long), replicated, single-row plots as the most efficient and reliable method of selecting under field conditions.
- Make every effort to improve uniformity in the field (especially soil preparation). It has even been suggested that the top soil be completely replaced with a homogeneous mixture, carefully leveled over a coarse base to provide uniform drainage.
- Use special data-handling procedures that allow controlling and reducing environmental errors.

Fowler and Gusta (1979) developed a field survival index (FSI) based on the relative winter hardiness of winter wheat cultivars tested in more than 60 trials over a five-year period. The FSI uses:

Picture 3. Leaf damage and discoloring after a mild winter.

- only data from plots with partial winterkill;
- differences in percent winterkill among entries in a block, rather than actual percent winterkill in each plot;
- a moving average.

Although calculations and efforts involved in determining the FSI may seem tedious, the index provides a very robust measure for comparing the winter hardiness of cultivars. Other approaches such as a "nearest neighbor analysis" may also be useful.

Enhancing winter stress in the field

The probability of differential winterkilling in a natural winter environment can be increased by using simple procedures:

- planting wheat on ridges, 20-30 cm high, from which snow is usually blown out, leaving plants more exposed to low temperatures and desiccation (Nam et al., 1982);
- planting wheat in wooden or cement boxes placed above the ground, preferably in an open field, to allow lower temperatures to be reached at the crown level, producing higher winterkilling than in field planted wheat;
- leaving plots without snow cover by temporarily covering them during snowfalls, or by gently removing newly fallen snow from the plots.

As in normal field testing, use of several checks of known winter hardiness, repeated every few plots or rows, is highly recommended.

Artificial testing

The irregular occurrence of natural conditions that satisfactorily differentiate genotypes has led many plant breeders to develop artificial techniques for assessing the freezing resistance of their materials. Already in 1956, Dexter concluded that the results of such tests were generally well correlated with field assessments of winter hardiness, and recent methodological refinements have improved the correlation. On the other hand, Fowler et al. (1981, 1993) concluded that, although controlled environments should allow more rigid control of freezing conditions, comparative studies suggested that field trials usually provide more repeatable results and have lower experimental errors. The decision on which method to apply should largely depend on how frequently field testing results in "good" differentiation and on the equipment available for screening under artificial conditions. Wherever possible, both methods should be applied.

Most methods used in wheat breeding programs are direct, i.e., they are based on exposing plants or seedlings to controlled freezing in artificial climate facilities, such as freezing cabinets, growth chambers, etc. However, there are indirect methods in which plants are not exposed to freezing; instead, their freezing tolerance is estimated based on biochemical changes induced by hardening or on the presence of molecular markers associated with genes involved in controlling winter hardiness (Table 1).

Direct freezing tests

Many methods have been suggested and are used in winter wheat breeding programs around the world for artificial testing of freezing tolerance. They differ in the way plants are sown and prepared for testing, in the hardening and freezing procedures, and in the way freezing damage is assessed.

In many cases, sowing is done in boxes, flats, or pots, which makes it easy to handle, regardless of weather conditions, but is relatively laborious. The other choice is to pick up plants from breeding plots in the field. This approach is more economical, but picking plants from the field is dependent on weather conditions and can be hampered by snow cover or frozen soil.

Table 1. Approaches used for testing freezing tolerance in wheat.

	Hardening	Exposure to freezing		Assessment
Direct	- Natural in the field - In growth chambers - Combined	- Field (regular or special locations) - Field enhanced (ridges, boxes above ground, snow removal) - Freezing cabinets - Immersed in a refrigerated solution	- Plants in boxes - Plants from field, transplanted (in boxes, rootrainers, moist sand) - Crowns (in polyethylene bags, tubes, sand) - Seedlings	- Plant survival - Leaf damage - Root regrowth - Cell membrane damage (electrolyte leakage) - Tissue viability - Tissue electrical conductivity - Fluorescence - Enzyme activity
Indirect	- Natural in the field - In growth chambers - Combined	No		- Tissue water content - Free proline - COR proteins - Tissue electrical resistance of: - Seedlings - Crowns
	No	No		- Molecular markers

Hardening is most easily done under natural conditions, either by placing the boxes or pots outdoors or by picking up hardened plants from the field (Picture 4). The main disadvantage of this approach is the lack of control over the hardening level. Results from such freezing tests are not reproducible, and the test temperature must be adjusted for each test, according to the hardening level, to properly differentiate genotypes.

Hardening in growth chambers under controlled temperature and light regimes can achieve a controlled level of hardening, but is expensive and requires space in growth chambers for about 30 days. A workable compromise is to use natural field conditions for the first stage of hardening and then transfer the boxes or transplanted plants to growth chambers with a controlled environment for the second stage of hardening, which may take only 24-130 h. It is important that plants be collected before they are covered by snow.

Freezing response shows significant cultivar x hardening-duration interactions (Jenkins and Roffey, 1974). Therefore, ideally, the frost resistance of a genotype should be assessed over a range of hardening and freezing regimes; however, this is not practicable when testing large numbers of early-generation selections. Several alternative hardening regimes can be selected, depending on the breeding requirements:

- natural hardening, which better reflects the situation in farmers' fields. Many years of testing are needed to characterize freezing tolerance of a genotype using this method;
- an "average hardening" regime, representative of most years in the area. Various hardening regimes are used by different testing programs (see Table 2);
- striving for maximum level of hardening, corresponding to "potential freezing tolerance" or "static freezing resistance."[5]

Picture 4. Hardening of plants sown in wooden boxes, using a vegetation house.

There are several options for exposing wheat plants to low temperatures. Often boxes or pots in which wheat has been planted are placed directly in freezing chambers. This has the advantage of not disturbing the plants before stress exposure, but requires larger freezing cabinets and a longer exposure period, due to thermal inertia of the large amount of soil.

Most important for plant survival, the crown meristem must be able to produce new roots and tillers, so some methods expose just the crowns to freezing temperatures. Crowns are prepared by trimming the upper part of the plant to 2-3 cm above the crown and the roots to 0.5-1 cm (Figure 3). Crowns are then put into plastic bags, vials, tubes, moist sand, etc. (Fowler et al. 1981, Gusta et al. 1978). To avoid the work involved in trimming, other methods expose plants that have been transplanted from the field into small boxes, trays, or some type of supporting device (e.g., roottrainers) with a small amount of moist sand or soil (Poltarev, 1990; O'Connor et al., 1993; Ryabchun et al., 1995). Using young seedlings has the advantage of reducing test duration and the amount of soil needed, but, as differential survival of seedlings is more difficult to obtain, evaluation is usually based on leaf damage (Figure 4). Larsson (1986) found a very good correlation between seedling leaf damage and field winter hardiness.

Most methods use freezing cabinets with controlled air temperature. However, to achieve better temperature control, Jenkins and Roffey (1974) used a refrigerated bath with ethylene glycol, in which pots with plants were immersed. Most authors recommend a

[5] Ryabchun et al. (1995) recommend adding 36 h at -5ºC, 56 h at -7ºC, 24 h at -9ºC, and 14 h at -10ºC of artificial hardening to the level achieved through natural hardening in the field by 14-25 November under conditions in Kharkov, Ukraine.

Table 2. Main characteristics of methods used for assessing freezing tolerance in cereals.

Author	Planting	Hardening	Exposure	Freezing	Recovery	Assessment
Jenkins and Roffey (1974)	In paper pots, 1.9 cm diameter and 6.4 cm deep	In growth chambers at 8/5 ºC for around 30 days	Pots placed in glass tubes, immersed in a refrigerated bath with 40% ethylene glycol	Solution chilled 2ºC/h to -4.5 At -4.5 ºC for 7 1/2 h; Decreased 2ºC/h to -9 At -9 ºC for 11 h	Solution heated to +1 ºC in 7 h	Electrical resistance of leaves, by clipping 2 platinum electrodes 2 cm apart through lamina of first leaf
Fowler et al. (1981)	Plants from the field	In the field	Crowns above (trimmed 3 cm and 0.5 cm below the crown) placed in aluminum dishes with moist sand	Equilibrated 12 h at -3 ºC Decreased 2ºC/h to 5 test temp separated by 2 ºC intervals Dishes removed when test temp. is reached	At 0 ºC for 15 h Planted in soil-perlite-peat At 15 ºC, 3 weeks	Plant survival
Larsson (1986)	In plastic boxes with mixture of peat and sand Grown 2 weeks in glasshouse	In growth chambers at +1ºC, 20-30 days	Seedlings in plastic boxes	Decreased 1ºC/h to 5 test temp. separated by 1.3ºC intervals		Foliar damage on primary leaves
Poltarev (1990)	Plants from the field Transferred in boxes or trays	In the field	Plants in boxes or trays	Decreased 2-3 ºC/h to 2 test temp. at 2 ºC interval	Increased 2-3 ºC/h 15-16 days at 20-22 ºC or 3 days at 24-26 ºC	Plant survival
O'Connor et al. (1993)	Plants from the field transplanted to folding rootrainers.	In the field	Plants in rootrainers	Decreased 2ºC/h to 8 test temp. separated by 2 ºC intervals	Thawing at 4 ºC for 15-20 h Recovery at 17 ºC for 3 weeks	Plant survival
Ryabchun et al. (1995)	Plants from the field Transferred in wooden boxes or special trays	In the field + artificial 36h at -5ºC 56h at -7ºC 24h at -9ºC 14h at -10ºC	Plants in boxes or trays	Decreased 1ºC/h Exposed for 24 h at -16, -18, -20 and -22 ºC	Increased 2-3 ºC/h to -2ºC, then 1ºC/h Crowns planted in soil at +20ºC for 15-16 days	Plant survival
Fedoulov (1997)	In wooden boxes	In natural field conditions + artificial 24h at -5ºC	Plants in wooden boxes	Decreased 1ºC/h Exposed for 24 h at -17 to -20 ºC	Increased 1ºC/h 24 h at +5 ºC 21 days in greenhouse	Plant survival
Tischner et al. (1997)	In wooden boxes (38x26x11 cm)	Artificial 7 days at +3 to -3ºC 4 days at -4ºC	Plants in wooden boxes	24 h at -15ºC	In phytotron	Plant survival
Dencic et al. (1997)	In pots 20 cm deep	In the field + 24h at 0ºC	Plants in pots	24h at -15ºC 96h at -17 ºC	120h at +5 to +7ºC	Plant survival + leaf damage

gradual decrease in temperature (by 1-3ºC/h), but direct exposure to the test temperature can also be used (Dencic, 1997; Tischner et al., 1997).

Difficulty in reproducing cold acclimation conditions severely limits the resolution of controlled-freeze tests that employ a single minimum (test) temperature. Therefore, it is best to use of a series of test temperatures, usually separated by 2 ºC intervals, to determine the LT_{50}, i.e., the lowest test temperature at which 50% or more of the plants of a wheat genotype survive freezing (Fowler and Limin, 1997).

As stated above, cold tolerance of winter cereals is reduced by prolonged exposure to sub-lethal temperatures and, consequently, both minimum temperature and exposure time are important variables in controlled-freeze test procedures. For economic reasons, most methods prefer shorter exposures to lower temperatures, but longer exposures might be advantageous if thermal inertia is large or if freezing cabinets have limitations in reaching lower temperatures. Thomas et al. (1988) recommended prolonged

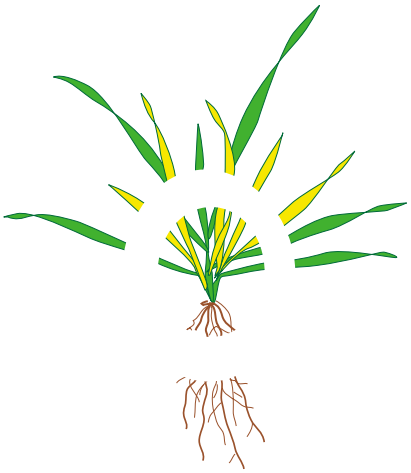

Figure 3. Crown prepared for artificial freezing test by trimming the upper part of the plant to 2-3 cm above the crown and the roots to 0.5-1.0 cm.

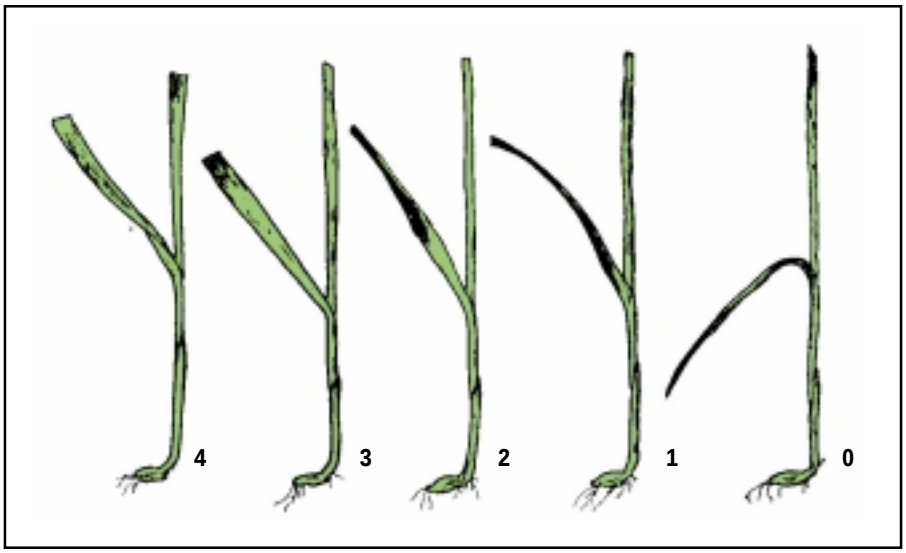

Figure 4. Grading freezing injuries in seedlings.
Source: Larsson (1986).

freezing of dark-hardened seedlings for rating and selecting winter wheats for winter survival.

There are small variations among methods for the recovery procedures. Most authors recommend a gradual increase in temperature until thawing, followed by a 2-3 week recovery period at 15-22 ºC. To reduce this long period when greenhouse facilities are used, Poltarev (1990) recommends transferring the plants at higher temperatures (24-26 ºC) where survival can be observed after only 3 days, provided great care is taken to avoid desiccation.

After the recovery period, freezing damage is usually assessed either by plant survival counts or by visually scoring leaf damage (Pictures 5 and 6). However, such indices are to some extent subjective, have high experimental errors, and involve a considerable delay between freezing and survival assessment. Many techniques have been proposed to evaluate the effect of freezing on plants by measuring other traits, such as:

- electrical conductivity of solutions resulting from exosmosis after freezing. Electrical conductivity, which depends on electrolyte content, is measured using a solution analyzer after freezing (initial EC) and again the next day, after killing tissue by immersing test tubes in a 80 ºC water bath for 1 h (final EC). Percent electrolyte leakage at each temperature is defined as: EL (%) = (initial EC / final EC) x 100. Lethal temperatures are determined by fitting data with a sigmoidal response curve and using the inflection point of the sigmoidal response curve to predict the lethal temperature (Fry et al., 1993);
- electrical resistance of plant tissue (Jenkins and Roffey, 1974);
- chlorophyll fluorescence analysis is rapid, sensitive, non-destructive to the plant, relatively cheap, and able to detect injury before visible symptoms occur. Onset of chilling injury is accompanied by a decrease in chlorophyll fluorescence *in vivo*; if the chilling treatment is prolonged, chlorophyll fluorescence eventually declines to zero (Wilson and Greaves, 1990). Unfortunately, the need for costly equipment limits the use of this type of analysis;
- tissue viability, estimated by using chemicals (such as tetrazolium or acid fuxine) that change color in the presence of oxidation reactions. This approach is very time consuming and labor intensive (Poltarev, 1990);
- enzyme activity (Bolduc et al., 1978).

Picture 6. Differential recovery of crowns submitted to artificial freezing.

Picture 5. Differential damage in plants grown in boxes, after artificial freezing.

These methods for assessing freezing damage have lower coefficients of variation and produce results without delay after freezing, but require special equipment and more laboratory work. This probably explains why most breeding programs still rely on determining plant survival.

Table 2 summarizes the main characteristics of methods used for directly evaluating freezing tolerance in wheat. This could serve as inspiration for adapting a method fitted to available equipment and conditions.

Cultivars can be compared not only for their hardening potential or maximum freezing tolerance, but also for the stability of their freezing tolerance and the ability to reharden (Prasil et al., 1994), which, in some areas, may be equally important. Three approaches have been used for evaluating these traits:

1. repeating freezing tests several times during the winter, based on the assumption that plants are naturally subjected to variable conditions leading to dehardening and rehardening;
2. exposing plants to controlled thawing and rehardening before the freezing test. For example, at the Odessa Institute, hardened plants are subjected to thawing at 10-12ºC for 120 h with continuous light, rehardened at -2 to -4ºC for 24 h and then frozen at -12ºC for 24 h (Litvinenko and Musich, 1997);
3. estimating the stability of the hardening condition based on the time needed to fulfill the vernalization requirements. To estimate the time needed to complete vernalization, Poltarev et al. (1992) recommended planting the genotypes in several boxes in the field and then transferring them to a greenhouse at about 20ºC and continuous light, at 47, 55, 62, 68, 74, and 82 days after planting. Growing point development is evaluated after one month in the greenhouse, and the number of plants that show spikelet differentiation is recorded.

The authors have established a close correlation between the length of time to complete vernalization and the stability of freezing tolerance. Even if this correlation is not general (see Gusta et al., 1997a), it can be useful in selecting for stability of freezing tolerance, especially when segregants with low and very low vernalization requirements are common in a breeding program.

Indirect freezing tests

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

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

Proline is thought to play a protective role in plants subjected to several stresses, including frost. Considerable amounts of free proline accumulate in leaves and shoots during cold hardening, and proline accumulation is positively correlated to genotype-specific frost tolerance (Dörffling et al., 1990). Measuring proline content after hardening can therefore provide information about potential freezing tolerance of cultivars.

There is a close correlation between the degree of freezing tolerance and the accumulation of a specific cold-regulated (COR) wheat protein (WCS120). The corresponding antibody discriminates between frost-resistant and frost-susceptible wheat cultivars (Houde et al., 1992). Therefore this protein can be used as a molecular marker to select for freezing tolerance.

Tissue electrical resistance of eight-day-old seedlings was found to be correlated with freezing tolerance. Used as a selection tool, it is very convenient in breeding schemes that employ artificial climate for accelerating generations (Musich, 1987; Litvinenko and Musich, 1997).

Restricted fragment length polymorphisms (RFLPs) and other molecular markers may also be used to detect the presence of alleles having positive effects on winter hardiness.

Although very attractive, indirect methods generally describe only some of the mechanisms involved in the control of genotypic differences in freezing tolerance, and therefore can probably exploit only part of the genetic potential available in a breeding program. Besides, they are generally more expensive and therefore restricted to stronger research programs.

Conclusions

Little progress has been made in breeding for increased tolerance to low temperature stress since the introduction of the winter wheat variety Minhardi at the beginning of this century (Grafius, 1981). However, this statement refers to the absolute minimum temperature wheat plants can survive. Most of the winter wheat growing areas in the world do not require wheat varieties with such a high level of winter hardiness. Consequently, the main breeding objective in many winter wheat breeding programs is not to lose the winter hardiness level present in commercial cultivars, rather than to increase it.

This target is often reached through routine field screening. The costs related to screening in controlled environments or using other indirect methods are probably one of the reasons why the measurement of winter survival in the field is still the standard procedure for most winter wheat breeding programs. With the identification of genes that

control frost resistance and the development of markers, it is likely that some of the problems related to field testing and/or controlled environment screening will be overcome. However, field testing will remain for some time to come the final measure of the winter hardiness of a wheat cultivar.

To increase the efficiency of breeding for winter hardiness in wheat, we recommend:

- identifying priorities among stress factors involved in winterkill across the target area. Evaluate long-term data on temperature fluctuation, snow cover, diseases, etc.;
- estimating the minimum freezing tolerance needed in the target area to reduce the risk of significant winter damage to an acceptable level;
- establishing a set of check cultivars, preferably with a long growing history in the region, representing the maximum and minimum winterkill risk assumed;
- taking every opportunity to select in the field to reduce the frequency of winter-tender lines;
- adapting an artificial freezing procedure suitable to the available facilities and potential. Standardize planting, hardening, freezing, recovery, and assessment procedures to increase reproducibility; and
- creating a database on winter hardiness of potential parents and advanced lines. Avoid crosses where none of the parents has the desired level of hardiness.

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