Differences between wheat genotypes in damage from freezing temperatures
during reproductive growth

David P. Livingston III^{ab*}, Tan D. Tuong^a, Thomas G. Isleib^b, J. Paul Murphy^b

a. US Department of Agriculture – Agriculture Research Service, 840 Method Rd, Unit 3,
Raleigh, North Carolina, USA.

b. North Carolina State University, 840 Method Rd, Unit 3, Raleigh, North Carolina,
USA.

* Corresponding Author: Tel: 01 919 515 4324 *E-mail address:* dpl@ncsu.edu.

ABSTRACT

Cereal crops in the reproductive stage of growth are considerably more susceptible to injury from freezing temperatures than during their vegetative growth stage in the fall. While damage resulting from spring-freeze events has been documented, information on genotypic differences in tolerance to spring-freezes is scarce. Ninety wheat genotypes were subjected to a simulated spring-freeze at the mid-boot growth stage under controlled conditions. Spring-freeze tolerance was evaluated as the number of seeds per head at maturity after plants were frozen at -6°C. Plants that froze, as confirmed by infrared (IR) thermography, died shortly after thawing and consequently the heads did not mature. Only in plants that had no visible freezing (super-cooled) were heads able to reach maturity and produce seeds. In plants that super-cooled four genotypes had significantly higher seed counts after being exposed to freezing than three with the lowest. In addition, significant differences between genotypes were found in whole plant survival among those that had frozen. Genotypes with high whole-plant freezing survival were not necessarily the same as the super-cooled plants with the highest seed counts. Spring-freeze tolerance was not correlated with maturity suggesting that improvement in freezing tolerance could be selected for without affecting heading date. Spring-freeze tolerance was not correlated with freezing tolerance of genotypes of plants in a vegetative state, either under non-acclimated or cold-acclimated conditions indicating that vegetative freezing tolerance is not a good predictor of spring-freeze tolerance.

Key Words: Wheat; Spring Freeze; Heading; Infra Red Thermography; Supercooling; Barrier

1. Introduction

Fall-sown genotypes of cereal crops such as rye (*Secale cereale* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and oats (*Avena sativa* L.) are generally preferred by growers over their spring planted counterparts. A fall-planted crop usually has a higher yield and allows the opportunity to plant a second crop in areas where it can be harvested sufficiently early in the season. After the crop germinates in the fall, low, above-freezing temperatures induce cold-acclimation which makes fall-sown genotypes better able to withstand freezing temperatures during winter.

In addition to cold-acclimation, low temperatures also stimulate vernalization. This ensures that when temperature and day-length requirements are met in the spring (Zadoks growth stage 30; Zadoks et al., 1974), the plant will enter a reproductive phase and flower. Once the plant enters a reproductive phase, the mechanisms whereby cold-acclimation is induced are suspended (Limin and Fowler, 2006; Mafoozi et al., 2001) and the plant reverts to approximately the freezing tolerance of a non-acclimated plant. Because the plant has lost most of its freezing tolerance and can no longer cold-acclimate, an unexpected freeze can cause considerable damage to the plant, particularly during Zadoks growth stages 35 to 47 when the developing head is in the boot.

Information on the extent of damage due to spring-freezes is somewhat anecdotal and varies widely depending on weather conditions and stage of reproductive development. Losses from 30% to as high as 90% have been reported (Al-Issawi et al., 2012; Frederiks, et al., 2015; Fuller et al., 2007; Thakur et al., 2010). In contrast, yields in Kansas (Paulson and Heyne, 1983) and Oklahoma (Chatters and Schlehuber 1953), were reportedly higher in years when late spring freezes occurred. However, it is not clear how yields were impacted in specific areas of fields where freeze damage was originally observed.

With the exception of Reinhiemer et al. (2004) who made comparisons in field observations between barley plants at the same growth stage, most differences were observed between cultivars at different growth stages. Because early maturing cultivars consistently suffered more damage than those maturing later, differences between cultivars were considered to be due to differences in growth stage (Fredericks et al., 2015; Shroyer et al., 1995). This led to the conclusion that “little or no difference exists in susceptibility of wheat varieties at the same growth stage and therefore little opportunity to increase freezing resistance in improved varieties” (Shroyer et al., 1995).

1 However, Reinheimer et al. (2004) identified barley genotypes with low floret
2 sterility after a spring freeze and reported QTL associated with spring freeze tolerance.
3 Fuller et al. (2007) reported differences in freeze damage between two wheat cultivars
4 using electrical conductivity measurements. In addition, differences between wheat and
5 barley with regard to the tolerance of heads to freezing temperatures have been reported
6 (Livingston and Swinbank, 1947; Suneson, 1937; Waldron, 1932).

7 It is generally accepted that differences between genotypes in spring freeze
8 tolerance are a result of differences in maturity with early cultivars being more
9 susceptible than later ones (Fredricks et al., 2015). In fact, some researchers have
10 recommended planting later maturing cultivars as the best means to avoid damage caused
11 by spring freezes (Singles and Marcellos, 1981; Livingston and Swinbank, 1947).
12 However, to our knowledge a test under controlled conditions that would enable freezing
13 a selection of genotypes as they all reach the same growth stage has not been developed.

14 The purpose of this study was to devise a procedure to evaluate the ability of
15 winter wheat germplasm to withstand a spring freezing event while in the boot stage prior
16 to emergence from the flag leaf. In addition we wanted to determine if spring-freeze
17 tolerance could be predicted by measuring the freezing tolerance of genotypes in the
18 vegetative state as cold-acclimated plants and/or as non-acclimated seedlings.

2. Materials and Methods

2.1. Plant Materials

Ninety cultivars and germplasm lines submitted to the North Carolina Official Variety Test (NC-OVT) in 2013 (supplemental Table 1) were subjected to a spring-freeze simulation under controlled conditions. They were planted in Fafard #2 potting mix (Sungro Horticultural Distribution, Agawam, MA) in 2.5cm x 12 cm cone-tainers (Hummert, Int, Earth City, MO) suspended in racks containing 100 plants in a 10 by 10 grid (Fig. 1A). Seeds were germinated in a growth chamber at 13°C for 10 days. Under these conditions plants had emerged from the soil and had a single leaf. Racks containing plants were moved to a chamber at 3°C for 8 wk to induce vernalization and then transferred to a greenhouse at 20 to 25°C under 12-h supplemental light until mid boot stage (Z45 to Z47). Plants were watered daily and fertilized weekly with a dilute solution of Miracle Grow fertilizer (Scotts Co., Marysville, OH). Under these conditions each plant produced a single dominant tiller with a well-developed head. Occasional immature secondary and tertiary tillers were kept until after the plant was frozen. Secondary tillers produced after freezing were removed prior to harvesting the head of the primary tiller.

By growing plants individually in cone-tainers in staggered plantings (one to two weeks apart) it was possible to select individual plants of later maturing genotypes that were the same growth stage as earlier genotypes. All individual plants were therefore frozen at mid-boot, (Z45 to 47). This is the growth stage when most wheat cultivars in North Carolina are exposed to unexpected spring freezes. (Fig. 1C). This preliminary experiment was repeated three times; each experiment was considered a replication for a total of three replications.

The three hardiest, the three least hardy and four intermediate lines were selected from the preliminary test for a smaller, more detailed experiment, and to confirm results from the larger experiment. This experiment was conducted in three separate years with three replications in each year under the same conditions described above. Each replication contained 10 plants for a total of 90 plants of each genotype.

2.2. *Infrared Thermography*

In year 3 (2015), freezing of two representative cultivars was monitored by infrared thermography using a FLIR T620 video camera (FLIR Systems, Wilsonville, OR) with a 45° lens. The camera lens was inserted through a hole in the door of the freezer and monitored the freezing of multiple copies of AG South 2056 (hardy) and Merl (non hardy) (see Table 1) from 0°C to -6°C and until thawed. The camera was connected to a computer by USB cable and the freeze test was recorded using Research IR software (FLIR Systems, Wilsonville, OR) at 1 frame per second. Recordings are shown in grey-scale with white being hot and black cold. In Fig 3a, a color pallet was used to accentuate freeze-events; in this case, yellow was hot and purple was cold. A photograph (at a reduced opacity) of the unfrozen plants prior to freezing was overlaid onto the IR recordings using Adobe After Effects to allow better visual differentiation of tissues. Fig. 1 and 2 show the results of whole plant freezing while figures 3 and 4 is a close-up view of freezing primarily in the head region of plants that had frozen. Supplemental videos were time-lapsed to show the entire 8h freeze test in approximately 60 seconds.

2.3. *Spring-freeze simulation*

Several preliminary freeze tests of the NC-OVT resulted in the selection of a test temperature of -6°C because that temperature resulted in a nearly normal seed count (the same as in unfrozen controls) in the most hardy lines while seed counts of the least hardy lines were at or near zero. Both the large experiment (supplemental table #1) and smaller one (Table 1) were subjected to the same freezing procedure.

Whole plants in cone-tainers were placed in a freezer at 20°C with stems, leaves, and heads exposed to the air. A convective type cooling process was used to provide uniform cooling throughout the plant canopy and reduce gradients that would have confounded results (Reinheimer *et al.* 2004). Our purpose was to develop the simplest procedure that would allow screening multiple genotypes. The similarity of our results to those of Fuller *et al.* (2009) led us to conclude our procedure was a reasonable approximation of what would also be expected under radiative conditions.

The temperature inside the freezer was taken from 21°C to -6°C over a 9 hour period to simulate a sudden freeze. To measure the temperature within heads, copper-

constantan thermocouples were inserted into the center of the head on selected plants and temperatures were monitored on a computer.

When the freezer temperature reached 0°C, plants were misted with water and after freezing were visually inspected to ensure that all plants were covered with frost. The temperature was reduced to -6°C at 4.5C/h and held there for 2h. It was then raised back to 21°C in 5 hours.

Plants were left to mature in a greenhouse for 3 to 4 weeks before being evaluated. Plants were watered every other day until they began to turn yellow or brown. Many plants that were freeze-damaged grew new tillers during recovery but only the head from the single, primary tiller was assessed for freezing survival.

Spring-freeze survival was measured as the number of mature, plump seeds in the head of the primary tiller. The total number of seeds from 10 plants was averaged for each genotype for each rep for a total of 20 plants for each genotype per replication in the large experiment (supplemental Table #1) and 30 plants for each genotype in the smaller experiment (Table 1). Minor differences between genotypes in initial experiments (not shown) were found in the number of spikelets per plant but these differences did not affect the overall ranking when total seeds per plant were used. Unfrozen controls were grown in an identical manner and their seeds counted at maturity.

2.4. Winter-freeze simulation

To measure the freezing tolerance of plants in the vegetative state, two separate experiments were performed. One with non-acclimated (NA), 2 week-old seedlings at the first leaf stage and another with 8 week-old plants that had been cold-acclimated (CA). Plants were germinated and grown in cone-tainers as described above for the spring-freeze simulation, in growth chambers at 13°C and 300micro Moles of light for 12h per day. They were watered daily and fertilized twice weekly with Miracle Grow fertilizer.

Two week-old NA seedlings were frozen at -8°C by sprinkling ice shavings over plants and placing conetainers with the seedlings into modified upright freezers at -3°C. Soil in the cone-tainers took about 18h to completely freeze as determined by temperatures monitored using thermocouples placed in the soil. The temperature was then lowered 1C/h down to -8°C. After being held there for 2h it was raised to 3°C at

2C/h. After thawing, plants were allowed to recover for 3 weeks at 13°C and rated on a scale from 0=dead to 4=undamaged. Percent survival was calculated as the number of plants rated 1 or above, divided by the total number of plants.

CA plants were grown for 5 wks at 13°C and then acclimated for 3 wks at 3°C before being frozen like the NA plants. The test temperature in the case of the CA plants was -16°C. Both the NA and CA freeze tests were replicated three times using 10 individual plants in each replication.

2.5. *Statistical analysis*

Analysis of variance with standard F-tests and Tukey-Kramer means separation tests were done using SAS V.9.3 (SAS Institute, Cary, NC) to determine significance of percent survival and seed count.

A chi-square statistic was computed from a 10x2 contingency table to determine if whole plant survival (table 1) applied uniformly to all 10 lines tested. We compared a 40/40 (100% survival) survival rate with 39/40, 38/40, etc., to see how low the rate of survival had to be to be considered significantly different ($P<0.05$) from the 40/40 (100%) rate. This was followed by a comparison against the next highest rate and so on.

3. Results and Discussion

3.1. Infra-red thermography to detect freezing patterns

3.1.1. Freezing

Freezing always began in the soil when the chamber was between -0.5 and -3.0°C and always before any plant part froze (Supplemental Video #1). Despite leaves being at a lower temperature and covered with frozen water droplets, freezing almost always began at the base of the stem and proceeded upward into the leaves (Supplemental Video 1&2). Out of 36 plants that froze while being imaged with IR, only two froze from the top down. Hacker and Neuner (2007) reported that freezing began in stems and proceeded into leaves in several species of plants despite leaves being at a lower temperature. They suggest intrinsic ice nucleators within stems prompt stems to freeze before leaves (Hacker and Neuner, 2007). Wisniewski *et al.* (2014) discuss various compounds that could act as intrinsic nucleators including polysaccharides in *Prunus* woody-tissue, soluble polysaccharides in *Lobelia* and others.

We frequently observed a minor freezing event that was restricted to the lower part of the stem (crown) just after the soil froze (Supplemental Video #1). But, freezing did not proceed up the stem into the rest of the plant until the temperature was 2 or 3 degrees lower, if the plant froze at all. How some plants were able to resist ice growth from the crown into stems that are at the same or a lower temperature is unknown. But histological analysis of cereal crowns in a vegetative stage of growth show a considerable diversity of cell/tissue types (Livingston, *et al.*, 2013; Tannino and McKersie, 1985) with some winter cereals having a spherical layer of cells that apparently act as a barrier between live and dead tissue during recovery from freezing. Various parameters such as pore size and length of water conducting vessels as well as other structural barriers can have an effect on ice propagation (Aloni and Griffith, 1991; Hacker and Neuner, 2007; Wisniewski *et al.*, 2014; Zamecnik *et al.*, 1994). In addition, a significant difference between regions of the crown in carbohydrate content (Livingston *et al.*, 2009) and protein expression (Houde *et al.*, 1995) as well as antifreeze proteins (Wisniewski *et al.*, 2014) was reported during vegetative growth stages. More research will be needed with

1 wheat in the reproductive phase to determine precisely how ice propagation is promoted
2 or restricted in various tissues.

3 As the temperature was reduced to about -3 to -5°C, ice formation began in the
4 outermost, or oldest, leaf sheath at the bottom of the plant (Fig. 2B and Supplemental
5 videos) and then proceeded to the tip of that leaf. This sequence occurred despite the
6 presence of frost on all leaves and stems. When the outermost leaf had frozen nearly to
7 the leaf tip, the next younger leaf sheath on the stem began to freeze and proceeded along
8 the full length of that leaf (Supplemental Video #1). This pattern repeated itself until the
9 entire plant had frozen. Hacker and Neuner (2007) reported an “age-dependent freezing
10 pattern” and demonstrated that ice nucleation temperature was significantly lower in
11 immature leaves of *Eucalyptus pauciflora* than in older leaves and that freezing was
12 always slower in current year stems than in previous year stems of *Fagus sylvatica*. They
13 reported similar results with the evergreen *Taxus bacatta* (Hacker and Neuner, 2007).
14 Wheat is clearly an annual species and older leaves are only a few days more advanced
15 than younger leaves but, our results seem to agree with the observation that older leaves
16 freeze before younger ones. Usually, once the outermost leaf froze, the remaining leaves
17 quickly froze in sequence but we observed several instances when the oldest leaf froze
18 but the rest of the plant super-cooled (see Fig 2A, arrows for one example and
19 supplemental video #1).

20 When plants froze at -6°C it took from 25 to 30 seconds for freezing to progress
21 from the base of the plant to the tip of the topmost leaf. Ice propagation in individual
22 leaves was considerably faster at 2.0 to 2.5 cm per second. Single and Marcellos (1981)
23 mention propagation rates of up to 2 cm per sec in non acclimated wheat which, they
24 state, is equivalent to that in pure water.

25 Once this initial freeze of the entire plant occurred, a more prolonged freezing
26 began in all parts of the plant simultaneously (Supplemental Video #1). Hacker and
27 Neuner, (2007) described two phases of ice propagation with initial freezing in
28 *Eucalyptus pauciflora* leaves that tracked the vascular system and then a subsequent
29 prolonged freeze event that encompassed the mezophyl. Pierce and Fuller (2001)
30 describe a similar two-event freezing pattern in barley. Ashworth *et al.* (1995) suggest
31 that freezing begins in veins because they contain more water than other tissues.

32 About half way through the initial freezing phase a barrier appeared at the node
33 below the head (Fig. 3i, arrow). Single and Marcellos (1981) describe a nodal block that

1 was able to prevent the head from freezing down to -7°C . In our case the node did not
2 appear to prevent freezing into the head; but, the heat signature was very prominent up to
3 the base of the node with the node itself not freezing for at least several minutes. Before
4 this node was apparently breached, a second node above within the flag-leaf covering the
5 head began to freeze (Fig 3m-p, see arrow in p). This node was directly below the head
6 and in some freeze tests appeared to delay freezing into the head. Nodal blocks in several
7 plant species that slowed the spread of ice were described by Wisniewski et al (2014).
8 Fuller et al (2007) described a pause in freezing at a node in wheat but reported that in
9 some cases it prevented the head from freezing. We never observed this node to prevent
10 freezing of the head but it is possible that at a different temperature or growth stage this
11 node may act as a barrier to freezing as suggested (Fuller et al, 2007).

12 When whole plants froze, freezing in the head began slowly and appeared to
13 envelope the entire region of the head in compartments as if florets were freezing
14 separately (Fig 3u, arrow; supplemental video #2). This suggests barriers may exist
15 within the rachis of the head as described by Single and Marcellos (1981). However, in
16 our case none of the plants that froze showed any evidence of differential freezing within
17 the head.

18 19 3.1.2. Thawing

20
21 To confirm which plants had frozen, we continued IR imaging through the
22 thawing process. As the freezer temperature approached zero, plants which had frozen,
23 warmed at a slower rate than those which super-cooled due to the presence of ice
24 (particularly in heads). Consequently frozen plants remained darker (cooler) than the
25 background as the temperature was raised above 0.0°C (Fig 4a). In all cases where whole
26 plants froze the region containing heads was the last to thaw.

27 Even though plants all froze at different temperatures, all plants thawed at the
28 same temperature. This allowed a single image at 0.5°C to confirm which specific plants
29 froze. Or, more precisely, the image identified which plants still had ice in the head as
30 the background temperature was raised above zero.

31 Plants that had frozen (Fig. 4a, white arrows) quickly died and heads never
32 matured (Fig 4b, stems from plant which had frozen “f”). Those that super-cooled (Fig

4a, black arrows) produced a head that was partially or nearly completely fertile depending on the genotype (Fig. 4B and Table 1).

3.1.3. *Super-cooling*

Fuller et al. (2007) state that it is a common mistake to assume all plants exposed to freezing temperatures will freeze and that they will freeze at the same temperature. We agree because we found that even though the soil containing plants always froze between -0.5 and -3°C (Supplemental Video #1) not all plants subsequently froze even when temperatures were reduced to -6°C. We monitored freezing in 40 individual plants of Merl and 40 of AG2056 (see Table 1) in 8 separate experiments. Out of 80 plants, only 35 were confirmed by IR thermography to have frozen (18 Merl and 17 AG2056) even though all were misted with water and were observed to have frost on leaves and stems. A list of temperatures to which different plant species can super-cool was included by Single and Marcellos (1981). They state that non-acclimated wheat can be kept at -3 to -5°C for long periods of time without freezing. Fuller et al. (2007) observed water droplets freezing on a leaf that did not appear to trigger the leaf to freeze. Many times we observed unfrozen drops of water to freeze after leaves had frozen, as if the water drop was triggered to freeze by the leaf (not shown).

Super-cooling is a freeze avoidance mechanism that normally prevents freeze-damage in plants (Wisniewski et al., 2014). However, in this study we found seed set to be significantly reduced in florets that had super-cooled (Table 1). Fuller et al. (2009) reported that up to 90% of plants exposed to freezing temperatures (-6°C) super-cooled even when plants were misted and droplets of water had frozen on leaves and stems. They reported that seed-set in super-cooled plants were significantly lower than those in unfrozen controls (Fuller et al., 2009).

While it is possible that a minor, undetectable (at our resolution) freeze event occurred in a critical region of some florets, this seems unlikely considering the intense, visually obvious freeze event that spread throughout the head of plants that froze (Fig. 2, 3 and Supplemental Videos 1,2). While Al Issawi et al. (2012) observed partial freezing in heads that had been detached from plants, we never observed partial freezing of heads in intact plants. Nor did we observe freezing of stems up to node(s) below the head without freezing in the head as was described by Fuller et al. (2007) and Single and

1 Marcellos (1981). It may be possible that under other conditions we would observe
2 partial freezing but under conditions described here, without exception, plants which
3 completely froze died within 7 days and heads consequently never matured (Fig 4b).

4 Even in low above freezing temperatures (no ice present) florets in wheat
5 (Fredriks et al., 2015) can be permanently damaged. Thakur et al. (2010) provide a
6 detailed review of damage caused to reproductive tissues in various plant species,
7 particularly rice. These include “disruption of meiosis, tapetal hypertrophy, anther
8 protein degradation, pollen sterility and pollen tube disruption” (Thakur et al., 2010), any
9 of which could lead to partial or complete floret sterility. It seems reasonable to assume
10 that similar effects may be operative in wheat florets that were super-cooled. Research is
11 continuing to determine the specific damage to florets caused by super-cooling.

13 3.2. *Genotypic Differences*

15 Typical spring-freeze injury in wheat has been described as leaf chlorosis, white
16 awns, spikes trapped in boot, damage to lower stems and other effects on vegetative
17 structures (Shroyer et al., 1995). While we observed all these effects (not shown) in our
18 controlled freeze tests, floret sterility, resulting in low or no seed-set, was the most
19 convenient quantitative measure of damage. Hence, we evaluated freeze damage by
20 counting the number of seeds at maturity in the heads of plants that had been exposed to
21 freezing temperatures. Based on a sample of plants that were imaged by IR during
22 freezing we determined that plants with heads containing seeds were those that super-
23 cooled. Therefore, even though we describe our results as “freezing tolerance” it could
24 be argued that seed counts are actually a measure of freeze-avoidance.

26 3.2.1. *Seed Counts*

28 The freezing tolerance (Lt 50) of cold-acclimated (vegetative growth stage) wheat
29 under our controlled conditions was about -18°C (Table 1). However, as plants became
30 reproductive and entered the boot stage, a dramatic reduction in freezing tolerance
31 occurred with a decrease in seed count as well as whole plant survival after being
32 exposed to freezing temperatures at -6°C (Table 1). This was not unexpected since it is
33 well established that winter cereals loose much, if not all their acclimation after

1 transitioning from a vegetative to a reproductive growth phase (Limin and Fowler, 2006).
2 It is possible that at different reproductive growth stages, genotypes differ in their ability
3 to withstand spring freeze damage (Shroyer et al., 1995). Al Issawi et al., (2012)
4 evaluated wheat for spring freeze tolerance at a later growth stage (Z59 and Z65) and
5 reported reductions in fertility in plants exposed to freezing as we observed here.
6 However, they did not report differences in fertility between genotypes.

7 While there were significant differences in seed count between genotypes that
8 were exposed to freezing temperatures (Table 1) most of the 90 genotypes initially
9 evaluated had from 7 to 18 seeds per head (Supplemental Table 1) and were not
10 significantly different from each other. Of the 10 genotypes evaluated (Table 1), three
11 had approximately 20 seeds per plant; these three had significantly higher seed counts
12 than the five lowest which had around 5 seeds per plant.

13 In unfrozen plants, 50 seeds per head are supposedly typical of European wheat
14 (Al Issawi et al., 2012), but seed counts as low as 22 per head have been reported as
15 normal in winter wheat (Lyon and Kein, 2007). Under the conditions used in this study,
16 unfrozen control plants had an average of 24 seeds per plant, and ranged from 21 to 28
17 (Table 1). Because we did not detect significant differences ($p=0.05$) between unfrozen
18 genotypes we assumed 24 seeds per head to be an average control seed count for all
19 plants not exposed to freezing temperatures. This means that even the hardest genotype
20 (Progeny 870) had a 17% reduction in seed set when exposed to freezing temperatures.
21 The least hardy (AG South 2038) had an 83% reduction in seed set.

22 Resistance to spring freeze damage (reduced seed set) under controlled conditions
23 was apparently not related to heading date (Table 1) because mid and late maturing
24 genotypes evaluated under field conditions did not correlate to simulated spring-freeze
25 tolerance. Of the 90 genotypes evaluated in the NC-OVT only 10 were categorized as
26 “early” in field evaluations (Fig 5) and their seed counts ranged from a high of 20 to a
27 low of 8 seeds per head. Conversely seed counts in late maturing genotypes ranged from
28 25 to zero (Fig 5). This suggests that seed set after freezing is not closely linked to
29 maturity and while the heritability of spring freeze tolerance is currently unknown it
30 should be possible to transfer this trait into existing cultivars without affecting maturity.

31 32 3.2.2. *Whole plant survival*

1 While most plants did not freeze under our conditions, those that did freeze, as
2 evidenced by a visible exotherm (Fig 2), died within days after thawing and consequently
3 the head never matured (Fig 4). These plants were analyzed as either alive or dead using
4 a Chi Square.

5 The chi square calculated from a 10x2 contingency table was 29.296 with 9
6 degrees of freedom ($P=0.0006$), so we must conclude that the lines varied in their percent
7 survival from the mean of $35.5/40=88.8\%$. There were two statistical groupings
8 (designated “a” and “b” – Table 1) among the ten lines: those ranging from percent
9 survival of 92.5 to 87.5 (a) falling into both groups, *i.e.*, not differentiable from the
10 highest (100%) and lowest (72.5%) (b) survival rates (Table 1).

11 Fuller et al. (2007) reported differences between two wheat cultivars for
12 nucleation and freezing when plants were misted with distilled water prior to freezing but
13 found no differences in the presence of *Pseudomonas syringae* which is a well known
14 extrinsic nucleator (Wisniewski et al., 2014). In our case, all plants were misted with tap
15 water at 0°C and we do not know if extrinsic nucleation played a role in plants freezing.
16 Because the freezing resistant plants did not in fact freeze (that we could see) we propose
17 that this response to freezing temperatures is also a case of freeze avoidance.

18 Interestingly, plants that had high seed counts (super-cooled) were not necessarily
19 those with high whole-plant survival. For example Progeny 870 was the genotype with
20 the highest seed count (Table 1) and was also the genotype with the highest whole plant
21 survival. However, AG South 2056 also had a high seed count but had a significantly
22 lower whole plant survival. Conversely, AG South 2038 had the lowest seed count
23 (Table 1), but had 100% whole plant survival. This indicates that there are two distinct
24 mechanisms that determine two different responses to below freezing temperatures. One
25 response was related primarily to tissues within the floret that reflected a tolerance of
26 low, non-freezing temperatures (assayed by seed counts) and one with the ability to
27 prevent freezing in the plant. Additional research would be necessary before speculating
28 on the importance of intrinsic or extrinsic ice nucleators and/or anatomical or chemical
29 factors in differences between genotypes.

30 31 3.3 Relationship of winter-freeze to spring-freeze 32

Two genotypes with the highest spring-freeze tolerance also had high vegetative freezing tolerance (cold-acclimated); however overall, this was not the case (Table 1). Merl, and USG 3251 had excellent cold-acclimated freezing tolerance but poor spring-freeze tolerance. Conversely, P117 and AgriMAX1342 had a high level of spring-freeze tolerance (seed count) but low winter-freezing tolerance. We wondered if non-acclimated, 2-wk-old seedlings may have some inherent low level of tolerance that would be correlated to that in the reproductive phase but this was not the case, either in seed count or in whole plant survival (Table 1). For example Progeny 870 had high whole plant survival in the reproductive phase but low vegetative survival in NA, 2 week seedlings.

Unfortunately, this means that the freezing tolerance of seedlings or of cold acclimated plants is a poor predictor of spring-freeze tolerance. In fact, a correlation between winter and spring freeze tolerance of the 90 genotypes in the OVT indicated little if any relationship (Fig 6) with either cold acclimated plants or non acclimated, 2-week old seedlings. Fredricks et al. (2012) state that vegetative freezing resistance “does not necessarily confer superior resistance in reproductive stages”, which our results agree with.

4. Conclusion

Warm periods during winter are generally appreciated in temperate regions of the world. But if temperatures remain warm for too long, winter cereals can enter a reproductive phase and begin to head. This has been termed a “false spring” (Marino et al., 2011) and some researchers have concluded it is a phenomenon that is increasing in frequency due to climate change (Gu et al., 2008; Rigby and Porporato, 2008). If freezing conditions occur during this growth stage, complete failure of the crop is possible.

Under field conditions the extent of freeze-injury can vary depending on the position in the field, stage of growth, cultivar, and air temperature. Many times only a portion of the head is killed by freezing and in some cases new tillers developing after death of the main tiller will produce a crop, albeit a lesser one. Even slight freeze damage can affect the test weight and overall quality of the seed.

Using our procedure we were able to measure spring freeze tolerance (seed counts) under controlled conditions in up to 90 different genotypes, and to confirm the results in multiple experiments over time. Results reported here suggest that genetic

1 factors are involved in spring freeze tolerance and that aspects involved in freeze
2 avoidance can be exploited to improve existing cultivars.

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Figure Legends

Fig 1. Wheat plants for evaluation of genotypic response to spring-freeze simulation under controlled conditions.

A. Wheat plants under growing conditions. Each cone contains a single plant.

B. Alternating plants of AG2056 and Merl (Table 1) in freezer for infra-red video recording to confirm freezing of individual plants.

C. A closer view of the same set of plants in B showing heads inside flag leaf circled. See supplemental video 1 for a recording of the simulated spring-freeze of these plants.

Fig. 2. Still images from an infra-red recording of plants in Fig 1 being frozen. Heat given off by formation of ice is white in comparison to the background. Note the white color at the top of the cone-tainers indicating that the soil in every cone had frozen

A. Plants at -4.9°C with the oldest leaf near the bottom of the image freezing (2 arrows). The single arrow pointing upwards is the plant to which this leaf is attached. Despite the soil in the cone-tainer and the oldest leaf freezing, this plant never froze.

B. Plants at -5.9°C showing complete freezing of three plants. Arrows point to the position of the head within the flag leaf. See Supplemental Video #1 for a recording of the spring-freeze simulation.

Fig. 3. Close up images of two plants showing sequence of freezing from the base of the stem below the head up to the top of the leaf. The plant to the right never froze. Heat of freezing is represented by a yellow color. A is a visible image of the two plants with the flag leaf containing the head inside the dotted elongated oval.

The plant on the left underwent a rapid initial freeze (b-p). Arrow in b shows the point where the freezing front entered the video frame. After the initial freeze a secondary, slower freeze occurred but with more intense freezing (q-v) that included the head (elongated oval in v). Note the well defined freezing front in f-i suggesting freezing primarily in veins.

b-p are images taken one second apart. q to v are taken 1 minute apart. Arrow in I shows where a pause in freezing occurred at the lower node outside the flag leaf. Arrow in p shows a pause at the node just below the head inside the flag leaf. Arrow in u shows

what appears to be a floret inside the flag-leaf freezing separately. See Supplemental Video #2 for a recording of the complete freeze.

Fig 4. A. Closer view of plants from Fig 1 and 2 being thawed. Dark color indicates the presence of ice in a warmer background at 0.5°C. Dark arrows pointing downward indicate plants which super-cooled to -6°C and never froze. White arrows pointing upwards on darker heads indicate plants which froze. Note that even though plants which froze did so at different temperatures, all plants thawed at the same temperature, allowing this single image that confirmed which plants froze during the simulated spring freeze.

B. Heads of all 16 plants one month after freezing and thawing showing only the head of plants that had frozen (f) and those that supercooled (s). Note that only plants which super-cooled produced a head. In every case when the plant froze, the plant quickly died and consequently the head never matured. What is shown here is the remaining dried portion of the flag leaf that contains remnants of the head.

Fig 5. Seeds per plant compared to maturity of 90 wheat genotypes (see Supplemental Table) at the same growth stage that were exposed to freezing conditions (-6°C) in a simulated spring freeze event. The maturity of each genotype was evaluated separately under field conditions. Note that all 3 maturity levels have both high and low seed set, suggesting that response to freezing (with regard to seed set) is not closely linked to maturity. The 10 genotypes in Table 1 were selected from this population.

Fig 6. Relationship of seed count to survival during the vegetative growth stage in 90 genotypes (Supplemental Table). A. Cold-acclimated and B. Non-acclimated 2 week old seedlings. Note that no relationship was apparent under either cold or non-acclimated conditions.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

1 Table 1. Seed number per spike and whole plant survival in ten wheat genotypes exposed
2 to freezing temperatures while in mid-boot growth stage (Zadoks 45-47).

Genotype	Reproductive		Whole plant ³		Vegetative	
	Seed/Spike		Survival		% survival	
	Super-cooled ¹	not frozen ²	(%)	Maturity ⁴	NA ⁶	CA ⁷
Progeny 870	21 a ⁵	28	100a ⁵	late	8.3bc	84a
Progeny 185	20 a	26	100a	late	21abc	75ab
AG South 2056	19 ab	27	77.5b	late	21abc	58abc
Oakes	16 abc	23	90.0ab	med	0.0c	56abcd
AgriMaxx415	12 bcd	26	90.0ab	late	41ab	49abcd
USG 3251	11 cd	21	77.5 b	late	46a	73ab
DynaGro Yorktown	10 cd	25	92.5ab	med	28abc	19d
Merl	6.6 d	23	72.5b	late	54a	66abc
SY Harrison	6.6 d	21	87.5ab	late	38ab	38bcd
AG South 2038	4.4 d	25	100a	late	22abc	30cd
LSD (p=0.05)	7.9	NS	chi square 29.3		37	39

19 1. Mean of three replications in each of three years with 10 plants per rep (n==90).
20 These plants were exposed to freezing conditions but according to infra red thermography
21 they did not freeze at -6°C.

22 2. Unfrozen control. Mean of four replications with 10 plants per rep (n=40).

23 3. Chi square analysis, n=40 plants. These plants were exposed to freezing conditions
24 and according to infra-red thermography the ones which died had frozen.

25 4. Maturity measured separately under field conditions.

26 5. Numbers with the same letter within the column are not significantly different from
27 each other according to Tukeys HSD at p=0.05.

28 6. Non acclimated, two week old plants, frozen at -8°C.

29 7. Cold acclimated for three weeks, eight week-old plants, frozen at -18°C.