Determining the Extent of Frost Damage in Maize Seed Using the Tetrazolium Test

Mindy DeVries, Department of Agronomy, Iowa State University, Ames, IA

A. Susana Goggi, Department of Agronomy, Iowa State University, Ames, IA

Abstract

Frost damage in seed corn causes a significant decrease in seed quality. The tetrazolium (TZ) test has been used to identify types of physiological damage in several plant species, including frost damage in corn. Quantifying the amount of seed damaged by frost and the consequent loss of seed vigor are important for making management decisions. The current tetrazolium test procedures provide seed viability information but do not estimate seed vigor. In our project, the tetrazolium staining procedure was modified to allow separation of viable seeds into vigor categories. Vigor categories were defined based on Association of Official Seed Analysts (AOSA) recommendations, previous research, and current observations. The results from the tetrazolium test were compared with standard germination and saturated cold tests during the first 6 months of storage. The staining procedure aided in visual identification of frost damage. Although a strong correlation was found between seed viability as determined by the tetrazolium test, the standard germination test, and the saturated cold test, sorting seeds into vigor categories in the tetrazolium test did not improve the correlation between the tetrazolium test and the saturated cold test.

Introduction

Frost damage is costly to seed producers for two reasons. First, the seed decreases in value because of physiological damage and second, the extent of damage can only be assessed after seed harvest, drying, conditioning, and storage of damaged seed. The delay in assessing

seed deterioration is caused by the initially slow decline in seed vigor and viability. The tetrazolium test is traditionally used for determining viability, although its use for seed vigor determination is outlined in the Tetrazolium Testing Handbook [1] and Seed Vigor Testing Handbook [3]. The tetrazolium test is often considered the most accurate test for early determination of frost damage in seed corn. This test requires trained analysts and more labor than traditional germination tests, but it leads to faster results. Seed damage can be assessed in 24 hours using the tetrazolium test rather than waiting 7 to 8 days for the results of a germination test. Although the tetrazolium test is the first tool for identifying frost damage, germination tests should be used for evaluating subsequent damage in storage [5]. The results obtained with the tetrazolium test must correlate to either the standard germination test or a vigor test such as the cold test to be a valuable tool in seed testing.

The use of mechanical detection systems for evaluating tetrazolium -stained seeds has been explored [6, 12]; however, only the color ratio was measured. Scanning tools were used to calculate the ratio of red-stained tissue to white-unstained tissue. This value was termed the tetrazolium staining ratio and was used to determine the viability of the seed. However, the location of staining patterns is as critical as the staining intensity [4]. The precision of these mechanical evaluations is reduced because the location of stained and unstained tissue is not taken into account. Xie and Paulsen [12] increased the accuracy of evaluation by creating a larger algorithm. Their algorithm also accounts for the size of the unstained area based on the assumption that the larger the unstained area, the greater the likelihood that the seed is nonviable. However, they were unable to separate critical locations of unstained areas such as the radicle, plumule, and seminal roots from the entire embryo. This inability to identify staining patterns in critical regions limits the application of mechanical detection for diagnostic evaluations.

Therefore, skilled analysts are still necessary for interpreting the tetrazolium test for vigor determination.

The traditional staining procedure outlined in the Association of Official Seed Analysts (AOSA) Tetrazolium Testing Handbook [1] states that seeds should be imbibed overnight, bisected longitudinally through the embryo, placed in 0.1% tetrazolium chloride solution, and rinsed with water before evaluation. However, the traditional staining method is inadequate for accurately assessing frost damage. The embryos are stained dark, and staining patterns are difficult to differentiate. The vigor categories of seed stained by the traditional method, especially high and medium vigor, are too similar to separate. We feel that it is important to develop an improved staining method for the tetrazolium test and to clearly define vigor categories for this test specifically relating to frost damage. By improving the identification of frost damage in seed corn, seed producers and brokers have a better tool for making management decisions.

The objective of our research was to develop a consistent tetrazolium staining method and evaluation criteria for separating frosted seed into vigor categories. Additionally, we wanted to compare the numbers of seeds in these vigor categories to results from the standard germination and saturated cold tests.

Production of Frost-Damaged Seed Corn

Hybrids B73xIRF311 and Mo17xIRF311 were produced in isolation in 2003 and 2004 near Ames, IA. These two female lines were chosen to represent different genotypic tolerance to cold temperatures. B73 was developed by the federal-university corn breeding program at Iowa State University and is considered relatively tolerant to cold temperatures. Mo17 was developed by the federal corn breeding program at University of Missouri and is considered relatively

sensitive to cold temperatures. IRF311 pollinator is from the Iowa Research Foundation and was selected based on nick with B73 and Mo17. Samples were harvested at three moisture content ranges; 30-35%, 40–45%, and 50–55% moisture content fresh weight (fw) basis. Two field replications were used for this experiment. Ears were harvested with the husks and shank intact. Immediately following harvest, all ears were placed in a cold room at 50°F for a minimum of 4 hours to ensure uniform cooling rates. Control samples were then husked and dried, while remaining ears were frozen in a Conviron growth chamber (Controlled Environment Limited, Winnipeg, Manitoba, Canada). The frost cycle lasted 24 hours, beginning and ending at 50°F and including a minimum of 8 hours below 32°F and 2 hours at the minimum temperature 23°F. After the frost treatment, ears were husked and dried. Samples were dried on the ear in experimental sized forced air driers at a temperature below 90°F. All samples were shelled using an experimental sheller and stored in a cold room (50°F at 50% RH) until the time of testing.

Additional seed samples were harvested after the first natural frost event recorded on 2 October 2004. Data from these naturally frosted samples were included in the study as a comparison. However, seeds had reached physiological maturity (as determined by black layer formation) in the field before a natural frost; therefore, all naturally frosted samples were below 35% moisture fw at harvest. These samples were dried, shelled, and stored in the same manner as the artificially frosted seed samples.

Tetrazolium tests were conducted starting after all samples were shelled and were repeated every 6.5 weeks for 6 months. One hundred seeds were placed in moistened paper towels for 12 hours to hydrate. Seeds were bisected longitudinally through the embryonic axis. One-half of each seed was selected for evaluation. The selected half was placed with the cut surface on the moist paper towel until the entire sample had been cut. The seeds were then

soaked in water for 10 minutes. After this time had elapsed, the water was drained and replaced with 0.1% tetrazolium chloride solution (Sigma-Aldrich, St. Louis, MO). The seeds were allowed to stain for 1 hour and 10 minutes at room temperature. After staining, the tetrazolium solution was drained and seeds were rinsed. Samples were either evaluated immediately or returned to water and placed in a refrigerator for a maximum of 2 hours before evaluation.

Evaluation was done under a stereomicroscope (Fischer Scientific, Pittsburgh, PA). Seeds were placed into one of four categories based on the guidelines for the tetrazolium test as a vigor test, AOSA Seed Vigor Testing Handbook [3], and the criteria used by Moore and Smith [11]. Instead of using five viable categories as described by Moore and Goodsell [10], only three were used in keeping with the AOSA recommendations. Some additional observations were recorded during evaluation to assist with the identification of frost damage.

The standard germination (WG) test was conducted as described in the AOSA Rules for Testing Seeds [2] with the exception that only 100 seeds per seed lot were tested. Saturated cold (SC) test was conducted as described by Martin et al. [7] with the following changes. After 7 days at 10°C, the trays were moved to 25°C and alternating dark/light cycles of 4 hours each for 4 days. Only 100 seeds per seed lot were used. Evaluation of seedlings was completed according the AOSA Rules for Testing Seeds [2]. The standard germination test and the saturated cold test were done every 6.5 weeks for the first 6 months of storage.

Description of Vigor Categories

Previous work outlines methods for determining seed vigor using the tetrazolium test. Previous researchers have suggested alternative methods for evaluating seed damage, but the protocol and evaluation criteria were not clearly defined. Moore and Goodsell [10] found that their top three vigor categories (out of five) in the tetrazolium test were correlated to the cold

test. The difference between Moore and Goodsell's third and fourth vigor categories was that the embryos in the third category only showed beginning signs of deterioration at the cut surface. In the third category, the underlying tissues were stained normally and in the fourth category the degradation was more pronounced and the underlying tissues were deeply bruised and water soaked. We used similar criteria to designate between our medium and low vigor seed categories. If water soaking was present below the cut surface, the seed was categorized as low vigor. However, seeds that appeared watery or bruised in the plumule region were considered low vigor seeds independent of the underlying tissue.

The four categories are high vigor, medium vigor, low vigor, and nonviable. For seeds to be placed into the high vigor category (Figure 2), all critical embryo tissues must be intact and functional. Critical embryo tissues are shown in Figure 1. The predominant staining pattern is red spots on a white or slightly pink background. Slight damage may be found in the scutellum, but the remaining tissue is healthy. Staining patterns in damaged seeds can be classified into two distinct groups. In the first group, stained tissues have a higher red-to-white ratio. In the second group, stained tissues show patterns of concentric rings of white and red or purple indicating ice formation and injury. Moore [9] found that points of injury can be detected before changes in the standard germination test percentage and seed death because the tetrazolium test evaluates individual seed structures, which may vary in soundness and physiological integrity [8].

The seeds placed in the medium vigor category (Figure 3) have visible damage to the embryonic axis, but not severe enough to cause death and consequently lack of staining. The plumule tissue is intense red or has high red-to-white ratio, but rings or bruises are not visible. Bruising, evident by purple tissue and a watery appearance, may be observed in the radicle. However the meristem and majority of the plumule are still healthy. The mesocotyl region may

have an intense red color (Figure 3a), but it is not watery or purple. The seeds placed in the low vigor category (Figure 4) have extensive damage to the meristem and plumule tissues. Critical embryo structures are still alive as evident by staining. Staining color is a deep red to purple. Seeds included in this category are germinable, but may produce abnormal seedlings when planted in a vigor test. The embryo may be misshapen as seen in Figure 4b. Seeds in the high, medium, and low vigor categories are all germinable seeds. The final category consists of seeds that are ungerminable or nonviable. Ungerminable seeds are those that lack staining in one or more critical embryo structures. Either the plumule including the meristem (Figure 5a), or the radicle (Figure 5b), or more than one-third of the scutellum (Figure 5c) must be dead and unstained. Some seeds have completely unstained embryos where both the plumule and radicle tissue are dead. The characteristic staining pattern of frost-killed seeds is represented in Figure 6 where the embryonic axis is unstained, but the scutellum stained deep red with white areas.

Relationships among Tetrazolium, Standard Germination and Saturated Cold Tests

Tetrazolium test categories can be used to predict germination or vigor after several months of storage. Pearson correlation coefficients were calculated between one or more seed vigor categories from the tetrazolium test and the saturated cold test results at 3 and 6 months. The number of viable seeds in the tetrazolium test has a positive correlation ($r = 0.86$) to the percentage of normal seedlings in the saturated cold test after 6 months of storage (Figure 7). Although the nonfrosted control samples are clustered at the top of the graph where germination and vigor are high, the correlation coefficients of the nonfrosted ($r = 0.61$), naturally frosted ($r = 0.97$), and artificially frosted ($r = 0.93$) samples are all significant ($P < 0.05$). Samples exposed to a natural frost were more mature when the frost occurred; therefore, they do not exhibit as much damage as the artificially frosted samples that were frosted at higher moisture contents.

There were a few samples with low saturated cold test percentages (<40%). The positive correlation between the saturated cold test results after 3 and 6 months of storage and the tetrazolium test conducted immediately after the frost still exists when the data points with saturated cold test results <40% are removed from the analysis. More importantly, the correlation coefficients of the frost-damaged samples and nonfrosted samples remained similar to each other. The number of seeds in the high vigor category in the tetrazolium test at 0 months does not significantly correlate to the number of normal seedlings in the saturated cold test at any of the testing times (data not shown). Significant positive correlations ($P < 0.05$) were found between the standard germination and saturated cold test percentages compared with the total number of seeds in the high and medium vigor categories of the tetrazolium test (Table 2). However, the correlation coefficients improve when all viable categories (high, medium, and low vigor) are added together. The correlation coefficients and *P* values improve for both tests over all time periods (Table 2). This improved correlation indicates that seeds in the low vigor category are still able to produce normal seedlings in the saturated cold test after 6 months of storage in a cold room. Seeds in which only the plumule or radicle was dead and watery were recorded separately. Adding these seeds to the viable category did not improve the correlations, indicating that these "half-dead" seeds either produce abnormal seedlings or are unable to germinate.

Conclusions

We conclude that the new staining procedure facilitates the evaluation and interpretation of staining patterns in frost-damaged seeds. Classifying viable seeds into separate vigor classes can be beneficial for research purposes or as a diagnostic tool, but it is not necessary for obtaining good estimates of germination or vigor after 6 months of storage.

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Table 1 General description of the vigor categories based on the staining patterns of critical embryo tissues.

Category	Plumule Appearance	Radicle Appearance	Scutellum Appearance
High Vigor	Red or pink spots on a white background	Red or pink spots on a white background	Red spots on a pink or white background, may have regions of more intense red
Medium Vigor	Red or intense-red tissue	Red or intense-red tissue, may have purple tint or a watery appearance	Red or intense-red tissue, may have bruising or concentric rings
Low Vigor	Red to purple tissue with a watery appearance	Red or intense-red tissue, may have purple tint or a watery appearance	Red or intense-red tissue, may have bruising, concentric rings, or unstained areas less than 1/3 the scutellum
Ungerminable/ Unviable	Unstained, red, or purple tissue with a watery appearance	Unstained, red, or purple tissue with a watery appearance	Red or intense-red tissue, may have bruising, concentric rings, or unstained areas

Table 2 Pearson's Correlation Coefficients between the TZ test at 0 months of storage and the standard germination test and saturated cold test after 0, 3, and 6 months of storage.

	0 months		3 months		6 months	
	WG	SC	WG	SC	WG	SC
HML of TZ test at 0 months	0.68***	0.77***	0.77***	0.89***	0.77***	0.88***
HM of TZ test at 0 months	0.48 **	0.44*	0.57**	0.55**	0.49**	0.60**

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

Figure Captions

Figure 1 Critical embryo structures seen in a longitudinally bisected corn kernel: scutellum/cotyledon (a), coleoptile (b), plumule/shoot (c), meristem (d), mesocotyl region (e), seminal root (f), radicle/root (g), coleorhiza (h), and black layer (i).

Figure 2 A high vigor seed as indicated by the staining pattern of red spots on a white background. All critical seed structures are stained, and there is no damage present in the embryonic axis.

Figure 3 Medium vigor seeds with beginning signs of damage. Red-to-white ratio is much higher especially in the embryonic axis (a). Some seeds in this category will have a healthy scutellum (b), but the intense red staining of the plumule will classify them as medium vigor.

Figure 4 Low vigor seeds with extensive damage to the plumule. Plumule and radicle have a slightly watery or purple-tinted appearance (a). Extreme damage can be seen as deep purple-stained tissue (b). Areas of the scutellum are often bruised as seen in b near the base of the scutellum.

Figure 5 Ungerminable seeds as indicated by the absence of stained tissue in critical embryo structures. Plumule and meristem unstained (a). Radicle unstained (b). Unstained radicle and more than 1/3 of the scutellum unstained (c).

Figure 6 Typical frost-killed seed where the plumule, meristem, and center of the radicle are unstained, whereas all remaining tissues exhibit little damage.

Figure 7 Positive correlation ($r = 0.86$***) between TZ test results and the percentage of normal seedlings in the saturated cold test after 6 months of storage.

Figure 1

High Vigor Seed

Figure 2

Medium Vigor Seed

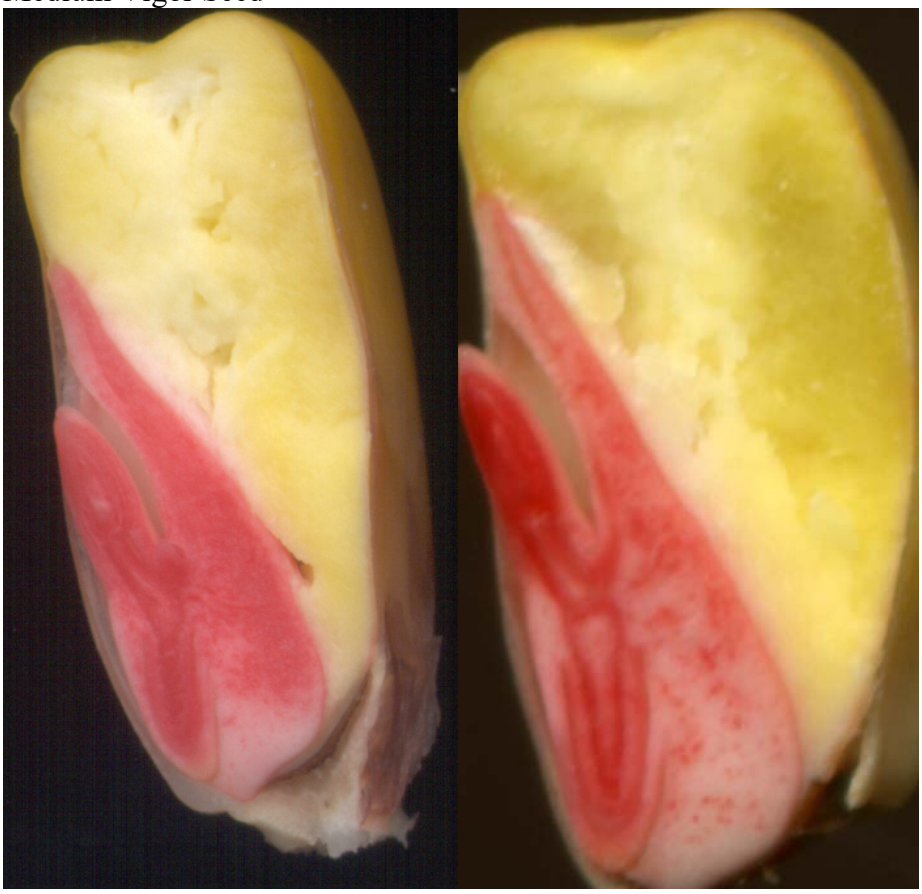


Figure 3

Low Vigor Seed

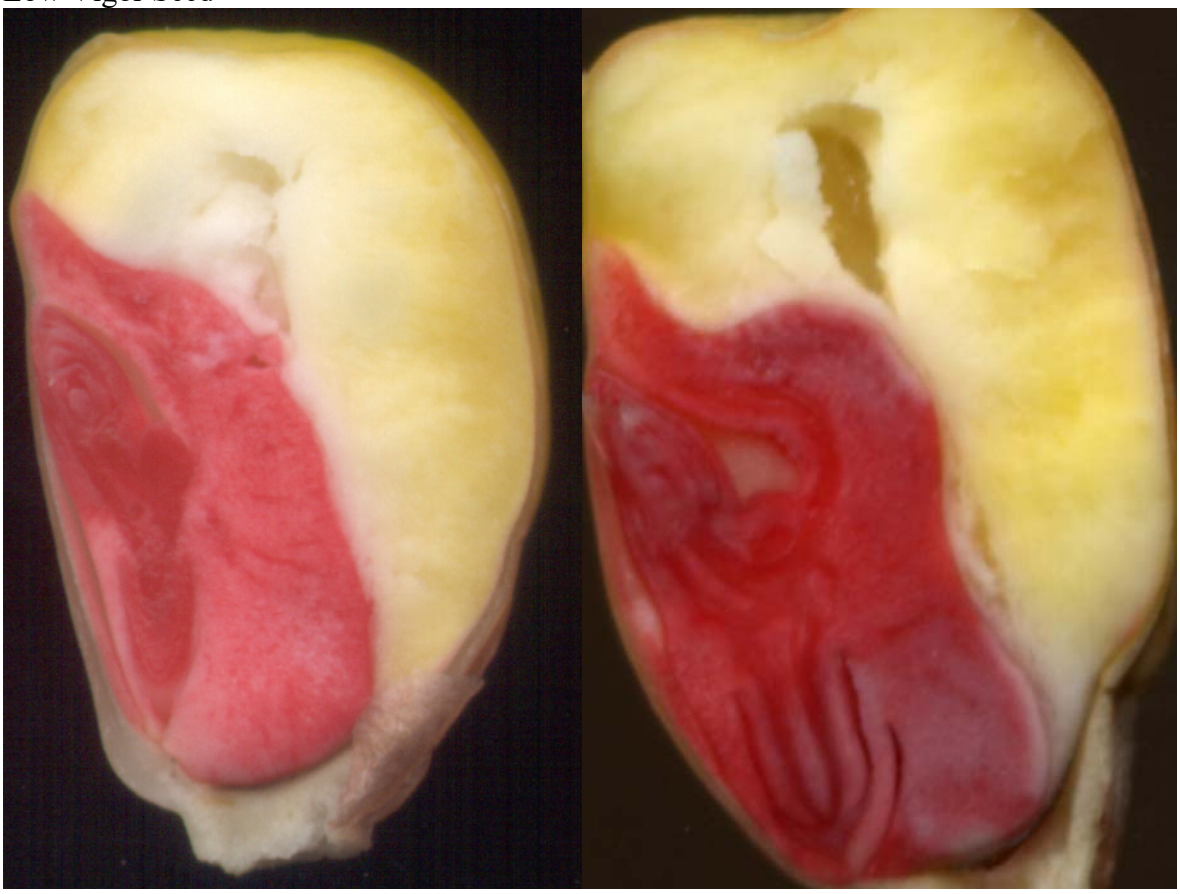


Figure 4

Ungerminable Seed (upper left) plumule and meristem dead (upper right) radicle and lower scutellum dead (bottom) more than one-half of the scutellum is dead

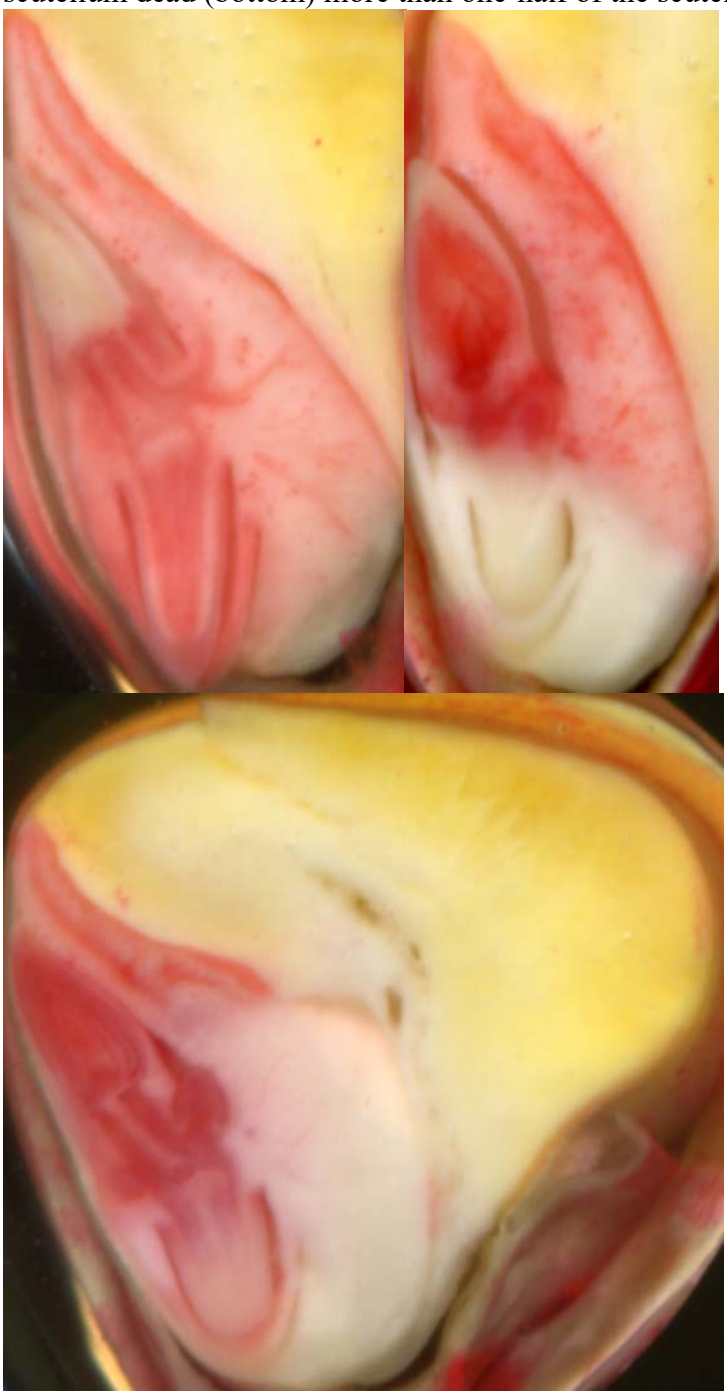
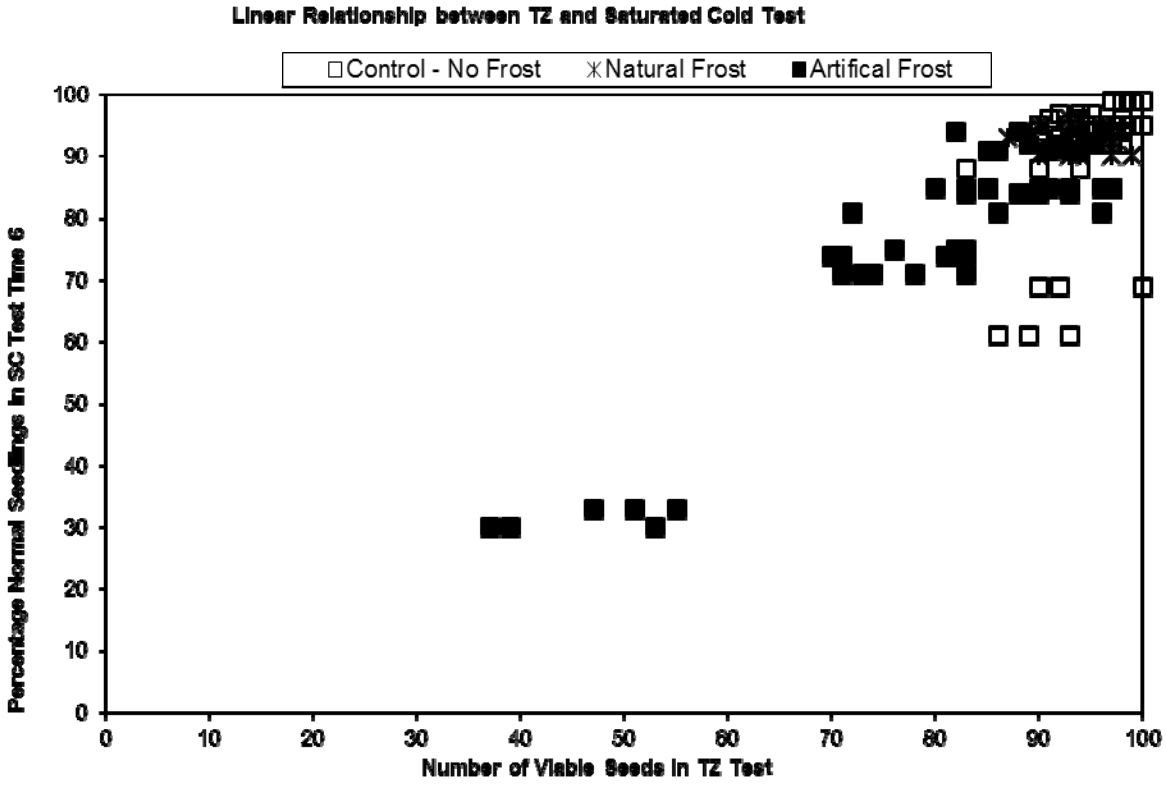


Figure 5
Typical Frost-killed Embryo

50 Figure 6



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