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Critical Temperature: A Quantitative Method of Assessing Cold Tolerance

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Abstract

Critical temperature (T_C), defined as the highest temperature at which freezing injury to plant tissues can be detected, provides a biologically meaningful and statistically defined assessment of the relative cold tolerance of plant tissues. A method is described for calculating critical temperatures in laboratory freezing studies that use electrical conductivity as a viability assay, using analysis of variance as a means of partitioning variance and estimating error. Evidence presented indicates that critical temperatures are strongly correlated with field assessments of winter injury, sufficiently precise to detect subtle differences in cold tolerance, and highly repeatable from year to year. It is suggested that the critical temperature method of assessing cold tolerance can be extended to a diversity of plant species and studies.

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Introduction

Development of procedures to differentiate between cold hardy and tender plant material is a prerequisite to conducting research on cold tolerance in plants. Either survival or a subjective rating of visible winter injury in the field is commonly used for this purpose. However, such approaches are limited by the inconsistent severity of test winters and confounding effects of field injury related to other causes. Laboratory freezing studies overcome these limitations by providing cold tolerance information for any plant or plant tissue at any time of year. One difficulty associated with laboratory studies is in determining the degree of injury to plant tissue after freezing. Several tissue viability procedures have been devised for use in cold hardiness studies, and their effectiveness and reliability have been discussed by several investigators (Parker 1963; van den Driessche 1969; Stergios and Howell 1973; Blazich and others 1974; Timmis 1976).

Selection of viability tests to accompany laboratory freezing studies depends on many factors, including species, plant tissue, time, and the overall objectives of the research. There is probably no one best method for all species or conditions. Regardless of which procedure is adopted, it is desirable to relate laboratory results to actual field assessments of winter injury when possible. Generally, a viability test that is objective, relatively quick, and allows the use of small quantities of plant tissue is preferred. Also, it is desirable that a single meaningful expression of relative cold tolerance, such as a killing temperature, LT_{50}, or some other definable index of hardiness, can be derived from viability results for purposes of comparison and quantitative analysis.

Electrical conductivity is an effective procedure for evaluating tissue injury following freezing tests. This method, as first described by Dexter and others (1932), is based on the principle that live cells quickly lose the ability to regulate their contents when cell membranes or transport systems, or both, are damaged (Palta and Li 1978). As a result, electrolytes diffuse into solution, causing an increase in conductivity of the solution. Thus, the greater the injury to plant tissue due to low temperature, the higher the conductivity of the extract (Levitt 1980).

In early studies, comparisons of conductivity of leachates from frozen and unfrozen samples were used to establish the presence and extent of freezing injury. These comparisons were useful, but not exact, because total electrolytes varied for different samples. Stuart (1939) and Wilner (1960) improved on the procedure by expressing the amount of cell electrolytes released after freezing as a percentage of the total electrolytes released after heat-killing. This measure of injury has been useful for comparing relative cold tolerance of tissue exposed to various temperatures, but does not provide a single definable expression of cold hardiness. Flint and others (1967) converted the percentage release of electrolytes to an index of injury (I_t) scale in which an unfrozen control sample was given a value of zero and a heat-killed sample a value of 100. After determining I_t for samples exposed to a series of test temperatures, a temperature corresponding to any selected I_t could be found and used as an expression of hardiness to compare samples. Although this procedure provides relative cold tolerance information for any specific I_t, interpretation of results can be difficult because the I_t selected for comparison is necessarily arbitrary with respect to all samples, and the relative cold tolerance of samples can vary with the specific I_t selected. Zhang and Willison (1987) measured electrolyte leakage from frost-injured samples after 1 hour and 18 hours in deionized water and found that the temperature corresponding to half the "differential percent leakage" (DPL) was similar to LT_{50} estimates derived from fluorescein diacetate staining. However, this procedure has not been tested with woody plants, would require establishing a consistent relationship between DPL's and fluorescein diacetate staining throughout the period of cold tolerance development, and requires additional conductivity measurements.

In making cold tolerance comparisons, it is desirable to identify a definable and biologically meaningful point of comparison among all samples. Killing temperature would be a useful criterion, but it is difficult to determine when a particular sample of plant tissue is dead. Also, tissue death under laboratory conditions may not be the most meaningful representation of freezing injury under field conditions. In red spruce (*Picea rubens* Sarg.), for example, freezing injury to needles on current-year shoots often occurs, but rarely results in mortality of those shoots or associated buds (DeHayes and others, in press). An equally definable, and perhaps more meaningful, criterion is the temperature corresponding to the point of earliest detectable freezing injury. This "critical temperature" can be calculated easily using the percentage release of electrolytes from control samples and samples frozen at various test temperatures. The objective of this paper is to describe procedures for the calculation of critical temperatures, where critical temperature (T_c) is defined as the highest temperature at which statistically significant freezing injury to plant tissues can be detected.

Methods

The procedure we describe for calculating critical temperatures presupposes that cold tolerance comparisons are being made among several treatments (cultivars, seedlots, plant tissues, or fertilizers, for example) in established experiments that follow a randomized complete block design. However, the procedure can be adapted to accommodate other experimental designs. In addition, it is assumed that (1) tissue samples representative of each treatment are exposed to a series of decreasing test temperatures, (2) each treatment is replicated at each test temperature, and (3) electrical conductivity is used as a measure of tissue viability. Test temperatures should be chosen so that samples from all treatments will not be injured at the highest test temperature, but will sustain at least some injury at the lowest test temperature. Several samples from each treatment should be maintained as controls, since control samples form the basis from which critical temperatures are computed. Because there is potential for noninjurious subfreezing temperatures to influence electrolyte leakage, through changes in membrane permeability or active transport systems (Palta and others 1977; Zhang and Willison 1987), differences in electrical conductivity between unfrozen and slightly frozen samples may be mistakenly perceived as evidence of tissue injury. As a result, although unfrozen (approximately 1°C) controls are commonly used, we recommend using nondamaging subfreezing temperatures (approximately −5°C) as controls for studies with northern woody plants.

Steps for calculation of critical temperatures are as follows:

1. After freezing and thawing, determine tissue viability of each sample (including controls) using electrical conductivity. Autoclave the samples and remeasure conductivity. Assure that conductivity of the sample leachate has equilibrated before conductivity measurements are made (usually 20 to 24 hours for freeze-injured conifer needles and 15 to 20 hours for autoclaved tissue).

2. Calculate relative conductivity (C_r) of each sample as $C_r = (C_f/C_t) \times 100$, where C_f = conductivity of the leachate after freezing (or control), and C_t = total conductivity after autoclaving.

3. Perform an analysis of variance (ANOVA) on relative conductivities following the model outlined in Table 1. It may be necessary to transform data to equalize variances. The ANOVA is used to partition variance, rather than for tests of significance, so that an accurate, unbiased estimate of experimental error can be generated and used to provide a measure of variation around relative conductivity observations. This model is selected because it reflects the superimposition of the laboratory freezing study over the established experiment and appropriately incorporates sources of error associated with both facets of the cold tolerance investigation. Alternative models (such as a split-plot ANOVA) may also be appropriate, but will require additional computations (such as pooling of multiple error terms) to generate an accurate estimate of variation around relative conductivity observations.

4. From the error mean square generated by ANOVA (Table 1), compute a critical value ($P \leq 0.01$ is suggested) to be used in contrasting the relative conductivity of control and frozen samples. Although a least significant difference (LSD) can serve as a critical value, Dunnett's procedure is designed specifically for restricted comparisons of controls and treatments (Steel and Torrie 1960) and therefore provides a more appropriate critical value.

5. Add this critical value to the mean relative conductivity of control samples for each replicate of each treatment to determine the lowest relative conductivity that differs significantly from controls. If C_r's of controls differ substantially among treatments, it may be necessary to adjust controls to a common number.

6. Compute the critical temperature (T_c) corresponding to this relative conductivity by interpolating between the two test temperatures within which range the calculated conductivity value lies. Interpolation assumes a linear relationship between temperature and relative conductivity between these two test temperatures.

7. Determine T_c for each replicate of each treatment and evaluate treatment differences in critical temperature with statistical methods appropriate for the design of the established experiment.

Thus, critical temperature is the highest temperature at which freezing injury to each treatment can be detected, or the temperature corresponding to the earliest statistically significant increase in relative conductivity due to freezing injury (Fig. 1). Critical temperature, however, is not intended to be indicative of the temperature associated with tissue death, but rather the temperature at which initial freezing injury occurs.

Table 1.—Model for an analysis of variance to partition variation in relative conductivity (C_r) and provide an estimate of error used in calculating critical temperatures (T_c) from laboratory freezing studies of plant material in established experiments following randomized complete block designs.

Source of Variation	Degrees of Freedom[a]	Comments
Total	rpt–1	
Replication	r–1	Laboratory replications; however, field blocks should be consistent with laboratory replications so that error is minimized.
Test Temperature (T.T.)	t–1	Differences in C_r among test temperatures computed across treatments and replications.
Treatment (Trt.)	p–1	Differences in C_r among treatments computed across test temperatures and replications (that is, mean treatment conductivity). Provides some, not all, information on treatment differences in cold tolerance; may be confounded with nonfreezing-related treatment differences.
T.T. x Trt.	(t–1)(p–1)	Differential C_r response of treatments to decreasing test temperatures. Provides additional information on treatment differences in cold tolerance (see "Treatment" comments above).
Error	(r–1)(tp–1)	Variation around C_r observations; includes sources of error from laboratory-imposed and pre-existing components of the study and replication interactions. Mean square used to generate the critical value for calculating T_c.

[a]Where r, t, and p = numbers of replications, test temperatures, and treatments, respectively.

Discussion

Effectiveness of critical temperatures

We have used electrical conductivity and critical temperatures to compare the cold tolerance of needles from 30 populations of ponderosa pine (*Pinus ponderosa* Laws.) on 17 dates, and twigs from 24 populations of green ash (*Fraxinus pennsylvanica* Marsh.) on 12 dates. The material was derived from rangewide provenance tests of ponderosa pine in southern Michigan (Wright and others 1969) and green ash in central Pennsylvania (Steiner 1983). For each species, T_c's varied considerably among populations and were strongly associated with climatic conditions at the geographic origin of each population. Field assessments of needle or twig winter injury (recorded as percentage of visible tissue injury) made at the end of winter for each population were highly correlated with winter T_c for both ponderosa pine ($r = 0.88$, 28 d.f., $P<0.01$) and green ash ($r = 0.81$, 21 d.f., $P<0.01$). The strong correspondence between critical temperatures and field injury demonstrates the effectiveness of critical temperatures in providing an accurate estimate of relative cold tolerance.

Equally important, however, critical temperatures were sufficiently precise to detect subtle cold tolerance differences among populations that appeared equally hardy (zero percent injury) in field tests. Such information is valuable because it permits ecological interpretations or predictions, such as the maximum level of cold tolerance that a species can attain or the northern limit at which a species, population, or cultivar can be planted.

Cold tolerance assessments and seasonal cold acclimation-deacclimation curves derived from critical temperatures also have been highly repeatable from month to month and year to year. For instance, repeated fall and winter critical temperatures for ponderosa pine populations were strongly correlated ($r = 0.79$ and 0.89, respectively, 28 d.f., $P<0.01$) over a 2-year period. Critical temperatures also have been effective in discerning cold tolerance differences between fertilizer treatments (DeHayes and others 1989), foliage sample storage conditions,[1] and different age needles in red spruce and balsam fir (*Abies balsamea* (L.) Mill.) (DeHayes and others, in press), and between different tissues in pitch pine (*Pinus rigida* Mill.) (Berrang and Steiner 1986). The effectiveness of critical temperatures in providing interpretable, accurate, and repeatable cold tolerance information on several species and multiple populations and tissues of single species lends credibility to this quantitative method.

[1] DeHayes, D.H.; Waite, C.E.; Ingle M.A. Storage temperature and duration influence cold tolerance of red spruce foliage. Forest Science. In press.

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ERRATA SHEET--GTR-NE-134

Page 3.--Footnote 1 should read:

[1]DeHayes, D. H.; Waite, C. E.; Ingle, M. A. **Storage temperature and duration influence cold tolerance of red spruce foliage.** Submitted to Forest Science.

Page 6.--The fifth entry under References should read:

DeHayes, D. H.; Waite, C. E.; Ingle, M. A.; Williams, N. W. [In press]. **Winter injury susceptibility and cold tolerance of current and year-old needles of red spruce trees from several provenances.** Forest Science.