Comparing Callus Growth with Discoloration and Electrical Conductivity as Measures of Stem Injury after Freezing Woody Plants

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ADDITIONAL INDEX WORDS. cold hardiness, winter hardiness, controlled freezing, crabapples, *Malus*, *Sorbus*, freezing injury, tetrazolium chloride

ABSTRACT. Most of 36 crabapple and 19 other woody plant taxa demonstrated the ability, when dormant, to grow a continuous row of callus along the cambial region on split-stem pieces within 5 to 7 days of incubation at 25 °C. The ability to grow callus after freezing tests was compared with discoloration and electrical conductivity for determining laboratory freeze injury to selected taxa. Hardiness levels were determined using the procedures of callus growth, discoloration, and electrical conductivity after freezing stem pieces of Jack crabapple [*Malus baccata* (L.) Borkh. 'Jacki'], pink bud Sargent crabapple [*M. sargentii* Rehd. 'Rosea'], Mary Potter crabapple [*Malus* sp. 'Mary Potter'], and snowberry mountainash [*Sorbus discolor* (Maxim.) Maxim.]. Sampling dates for laboratory freezing tests were chosen to represent midwinter cold hardiness and partial hardiness of either late fall or early spring. There was a high correlation between discoloration and callus ratings for most plants; however, the two methods usually did not identify the same critical temperature (T_{50}) for injury. The critical temperatures identified by callus growth was often 3 to 6 °C lower than for discoloration. For many taxa, callus growth was easier to see than discoloration of cambium and phloem, providing a less subjective evaluation of injury. TTC (2,3,5-triphenyl tetrazolium chloride) treatment was sometimes useful to identify callus growth that died after forming. The critical temperature (T_c), the highest temperature at which relative electrical conductivity differed significantly from the control temperature, was higher in most cases, indicating less cold hardiness than the T_{50} for callus and discoloration. The callus procedure may have value for evaluating injury to the cambial zone from freezing and other plant stresses because it determines the ability of the plant to continue growth.

Laboratory freezing studies are useful to identify cold-hardiness levels of woody plants. Many methods have been used to quantify injury to woody stem pieces following laboratory freezing (Calkins and Swanson, 1990). Rating discoloration or browning of injured cambium, phloem and xylem of stem tissue has become a common procedure for evaluating injury (Dirr et al., 1993; Fuchigami et al., 1971; Holt and Pellett, 1981; Lapins, 1961; McNamara and Pellett, 1996; Stergios and Howell, 1973; Väinölä et al., 1997). Frozen tissues, after thawing, are incubated at warm temperatures for several days to two weeks then dissected and evaluated for discoloration. The cambial zone must survive to ensure plant survival; however, we have observed that discoloration of this region is often difficult to detect when assessing injury.

Electrical conductivity is another common method for evaluating tissue injury following freezing tests. The reliability of the procedure has varied depending on taxa, age, and tissue type (Calkins and Swanson, 1990). After thawing, electrolytes diffuse into solution causing an increase in conductivity. Conductance readings are taken after freezing and again after heat-killing the samples. Relative conductivity (C_r) is the percentage of total electrolytes released after heat killing (Stuart, 1939). This method measures electrolyte release from all injured cells, not only the cells critical to plant survival, and usually shows a range of increasing injury at decreasing temperatures rather than an identified killing point (Ketchie et al., 1972). The discoloration method usually identifies a distinct temperature below which tissues are discolored even though cells in the tissues critical for survival may

not all die at the same temperature. DeHayes and Williams (1989), using the electrical conductivity method, described a critical temperature (T_c) as the highest temperature at which statistically significant freezing injury to plant tissues could be detected. The highest freezing temperature resulting in a significantly higher C_r than the control temperature was considered to be the critical temperature resulting in freezing injury. This procedure was used to detect critical temperatures for needles of different red spruce, *Picea rubens*, provenances (DeHayes et al., 1990). The same procedure identified lethal temperatures to azalea stems which were similar to those causing discoloration (Väinölä et al., 1997).

The ability to grow callus on wounded stem pieces may be a reliable method to determine whether critical tissues are viable after freezing, but to our knowledge, little research has been reported. Stergios and Howell (1973) considered stems alive after freezing if stem pieces callused on the ends or budbreak occurred. Callus is produced on wounded, live stems by cells in the cambial zone, a region including the cambium, and xylem and phloem mother cells on either side of the cambium that continue to divide (Fahn, 1982). "Callus may be formed by division of parenchyma cells of the phloem, cortex or vascular rays, but it is formed mostly in the cambium" according to Fahn. Stems must survive freezing before they can produce callus from wounding of the cambial region, therefore survival would indicate little or no injury to the cambial zone. Discoloration of visible stem phloem and xylem does not necessarily represent injury to cambial zone tissues which are critical for survival and may be a less accurate measure of injury affecting survival than callus growth. The cambial zone is only a small portion of the visible stem tissue therefore, it is difficult to evaluate its discoloration without being biased by discoloration of secondary phloem and xylem.

Two experiments were conducted to answer the following questions: 1) Can dormant woody plants reliably develop observable callus on wounded stem pieces? and 2) How do the freezing

Received for publication 22 Dec. 1997. Accepted for publication 8 Apr. 1998. We appreciate the assistance of Maureen O'Connell for statistical analysis and Jeff Tricou for computer graph preparation. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

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temperatures that reduce callus growth of stem pieces compare with those causing discoloration and the critical temperature determined by the electrical conductivity method?

Materials and Methods

EXPERIMENT 1. Split-stem samples of selected crabapples and other deciduous trees were incubated to determine their potential for vigorous callus growth. Twigs of the most recent growth were collected from 19 different taxa growing at the University of Vermont Horticultural Research Center in South Burlington on 16 Nov. 1994, 13 Mar. 1995, and 28 Oct. 1996 (Table 1). Samples were also collected from 36 crabapple taxa on 7 Dec. 1994 and 28 Oct. 1996 at the same location (Table 2). Ten 1-cm stem pieces of each taxa were split longitudinally or stripped of bark on one side and wrapped in moist paper towels and incubated at 25 °C for various periods from 5 to 12 d, then evaluated for callus growth.

Callus growth was rated 1 to 10, where 0 = no callus and 10 = a continuous line of callus along the cambial zone on both sides of the longitudinally cut stem piece. For example, a rating of 9 indicated that 90% of the linear cambial zone grew callus. Means of 10 samples were calculated on each date (Tables 1 and 2).

Callus growth of some taxa is small and hard to see. Tetrazolium chloride (TTC) treatment was used after evaluating callus growth to demonstrate which taxa callus could reduce TTC to the red color of formazan that helped to visualize callus. Callused pieces were placed in TTC solution, 1 g·L⁻¹ with four drops of Tween 20 (polyoxyethylene sorbitan monolaurate), for 6 h. Longer and shorter exposure to TTC was less effective for most taxa. Red colored callus was rated 0 to 10, where 0 = no callus and 10 = continuous row of red callus along the cambial region.

EXPERIMENT 2. Stem samples of the most recent growth were collected on several dates from 25-year-old trees of Mary Potter, pink bud Sargent, and Jack crabapples and snowberry mountainash

growing at the University of Vermont Horticultural Research Center. Sampling dates were chosen to represent midwinter cold hardiness and partial hardiness of either late autumn or early spring. The terminal and basal two nodes were removed and the remaining twigs were cut into 1-cm internodal sections. Two lots of 10 stem pieces of each taxa were placed singly in test tubes in an alcohol bath at 0 °C for 30 min then exposed to one of a series of six freezing test temperatures. The test temperatures were chosen to encompass the expected killing temperature for the date. Temperature was lowered at 5 °C/h with tubes removed at 3 °C intervals. Tubes with stem pieces were placed at 5 °C for 8 to 10 h to thaw after removal from the bath.

After thawing, the 10 pieces to be observed for callus growth were cut longitudinally. These and the uncut pieces to be rated for discoloration were wrapped tightly in moist paper toweling, placed in polyethylene bags and incubated at 25 °C for 6 or 9 d. There was little or no fungal growth after incubation except on some stem pieces frozen at test temperatures much colder than the T₅₀.

For electrical conductivity measurements, 10 mL of distilled water was added to tubes containing stem samples. There were 10 stem pieces (one piece per tube) for each temperature. The tubes were placed in a 5 °C refrigerator for 48 h, then into a 20 °C bath for 1 h before measuring conductivity. Samples were then autoclaved for 20 min, cooled in the bath and remeasured for conductivity. The relative conductivity (C_r) of each sample was calculated as $C_r = (C_f/C_t) \times 100$ where C_f = conductivity of leachate after freezing (or control), and C_t = total conductivity after autoclaving. The critical temperature (T_c) causing injury was determined by ANOVA and the Dunnett's Test ($P \leq 0.01$) as the highest temperature at which relative conductivity differed significantly from the control temperature (SAS Institute, Cary, N.C.).

Stem samples in this study were not ice-nucleated because adding ice or water before freezing to the samples for electrical conductivity evaluation would make effusate volume control

Table 1. Callus growth² and callus plus tetrazolium chloride (TTC)³ rating means of longitudinally cut stem pieces after incubation at 25 °C for various periods.

Taxon	Period (d)					
	5	12	7	10	7	TTC
	16 Nov. 1994		28 Oct. 1996		13 Mar. 1995	
<i>Acer ginnala</i> Maxim.	---	---	3.9	0.4	---	---
<i>Acer truncatum</i> Maxim.	4.2	0.8	3.9	0.4	2.5	0
<i>Cercis canadensis</i> L.	---	---	8.2	0	---	---
<i>Corylus colurna</i> Bunge	3.8	1.8	8.1	0	1.0	0
<i>Crataegus crusgalli</i> L.	9.7	10.0	10.0	10.0	10.0	8.9
<i>Crataegus laevigata</i> (Poir.) DC.	9.3	0	---	---	9.2	10.0
<i>Crataegus phaenopyrum</i> (L. F.) Med.	8.7	---	9.8	9.6	9.6	9.2
<i>Maackia amurensis</i> Rupr. & Maxim.	10.0	10.0	9.6	9.2	10.0	2.5
<i>Magnolia kobus</i> DC.	7.6	10.0	8.5	8.0	9.5	0
<i>Magnolia stellata</i> (Zucc.) Maxim. 'Royal Star'	3.7	9.7	9.4	9.8	8.4	0
<i>Magnolia xloebneri</i> Kache 'Merrill'	6.0	8.6	10.0	9.8	9.9	0.3
<i>Sorbus alnifolia</i> (Sieb. & Zucc.) C. Koch	10.0	10.0	10.0	10.0	10.0	7.9
<i>Sorbus discolor</i> (Maxim.) Maxim.	10.0	10.0	10.0	10.0	10.0	10.0
<i>Sorbus esserteauiana</i> Koehne	10.0	10.0	10.0	10.0	10.0	9.6
<i>Sorbus folgeri</i> (Schneid.) Rehd.	10.0	---	10.0	10.0	10.0	9.8
<i>Sorbus japonicum</i> (Decne.) Hedl.	10.0	10.0	10.0	10.0	9.3	3.8
<i>Syringa pekinensis</i> Rupr.	8.9	9.5	---	---	10.0	1.0
<i>Ulmus wilsoniana</i> Schneid. Prospector	---	---	9.5	8.8	---	---
<i>Viburnum sieboldii</i> Miq.	5.3	7.6	9.9	9.0	6.6	0

²Mean of 10 samples rated 1 to 10, where 1 = no callus and 10 = continuous row of callus in cambial zone.

³Ratings of red callus after a 6-h treatment with 2,3,5-triphenyl tetrazolium chloride (TTC), where 1 = no red callus and 10 = continuous row of red-colored callus in cambial zone.

difficult. Ice nucleation by introducing stem samples to test tubes with partially thawed 1 mL of frozen water was compared, in a separate study, to no nucleation on crabapple and mountainash stems (data not included). While the nucleated samples had a one to three degree lower LT_{50} in some cases, the relationship between callus growth and discoloration was similar to this study.

The methods for visually quantifying injury were: rating callus growth on split stems and rating separate pieces for discoloration of cambium or phloem on split stems. The callus growth was rated 1 to 10 as in Expt. 1. Stem samples were cut longitudinally 6 d after freezing and discoloration of phloem and cambial zone was rated 1 to 3 where 1 = obvious discoloration, 2 = uncertain and, 3 = no discoloration.

At temperatures near the point of injury, callus appeared to grow on some samples and then a portion of it died. This may have occurred when surrounding tissues were killed by previous freezing temperatures. The same TTC procedure used in Expt. 1 was used to identify callus growing from cells surviving freezing injury which died later apparently because of injury to surrounding

tissues. Red colored callus was rated 0 to 10, where 0 = no callus and 10 = continuous row of red callus along the cambial region. If callus developed along 90% of the cambial region, but only 60% of the total cambial length colored, the TTC rating was 6.

Spearman rank-order correlation coefficients were determined for ratings of callus growth at 6 and 9 d, callus plus TTC at 9 d, and discoloration ratings of the three crabapple cultivars and snow-berry mountainash (SAS Institute). The temperature (T_{50}) at which 50% of the stem samples showed injury was calculated using the Probit Procedure (SAS Institute). For callus and callus plus TTC evaluations, stems were considered injured when ratings were <10 . For discoloration, stems were considered injured when ratings were <3 . The proportion injured at each temperature equaled the number of observations injured/number of observations. The proportions were analyzed using maximum-likelihood estimates of regression parameters with a logistic function for the regression model. The probit analysis gives the estimated temperature (T_{50}) for $p = 0.5$, as well as lower and upper 95% confidence limits. This estimate could not be made when test temperatures were either too

Table 2. Callus growth ratings^z of split stem pieces of *Malus* taxa after incubation at 25 °C for various periods.

Taxon	Period (d)			
	5	10	7	10
	7 Dec. 1994		28 Oct. 1996	
Adirondack	---	---	10.0	10.0
Almey	10.0	10.0	10.0	10.0
Beauty	9.2	8.9	10.0	10.
Bob White	10.0	2.5	9.4	9.6
Candied Apple	10.0	9.5	---	---
Dolgo	9.6	9.8	7.8	9.2
Donald Wyman	9.0	9.0	9.4	9.8
Dorothea	10.0	8.6	8.9	9.0
Eric	8.6	6.8	8.8	9.0
Evelyn	10.0	10.0	9.0	9.0
Flame	9.5	7.2	10.0	10.0
Golden Harvest	10.0	10.0	---	---
Golden Hornet	10.0	10.0	9.9	10.0
Helen	6.6	8.8	8.3	6.8
Henrietta Crosby	10.0	10.0	8.5	9.8
Hopa	9.6	8.7	9.9	9.3
Indian Summer	9.3	8.3	10.0	8.6
Irene	9.2	10.0	7.9	8.4
Jay Darling	10.0	8.3	9.7	9.0
Klehms	10.0	10.0	7.5	7.7
Marshal Oyama	9.2	10.0	9.6	10.0
Mary Potter	6.8	9.6	10.0	10.0
Prairie Maid	---	---	10.0	9.6
Radiant	9.2	9.3	8.1	7.8
Red Baron	9.8	10.0	10.0	9.6
Red Silver	10.0	10.0	9.6	10.0
Royalty	9.1	7.8	7.0	1.8
Snowbank	10.0	9.3	8.1	7.9
Strathmore	10.0	10.0	9.7	9.7
Van Eseltine	6.3	7.5	9.6	10.0
<i>Malus xatrosanguinea</i> (Späth) Schneid.	9.1	9.8	9.1	9.4
<i>M. baccata</i> (L.) Borkh. Gracilis	7.3	0.2	8.2	1.5
<i>M. baccata</i> Jacki	10.0	5.5	10.0	10.0
<i>Malus xpurpurea</i> (Barbier) Rehd. Lemoinei	10.0	10.0	9.1	9.0
<i>M. sargentii</i> Rosea	9.8	9.9	9.9	10.0
<i>Malus xzumi</i> (Matsum.) Rehd. Calocarpa	10.0	6.6	9.6	9.6

^zMeans of 10 samples rated 1 to 10, where 1 = no callus and 10 = continuous row of callus in cambial zone.

Fig. 1. Callus growth from cambial zone on split stem of *Malus* 'Mary Potter' after incubation at 25 °C for 7 d.

low, resulting in injury at all temperatures, or not low enough to cause injury.

Results

EXPERIMENT 1. Callus growth occurred along the cambial zone of longitudinally cut stem pieces (Fig. 1). Most taxa had substantial callus growth on at least one date and some showed variable callus growth among dates. *Crataegus*, *Sorbus*, and *Malus*, members of Rosaceae, showed vigorous callus growth after 5 and, or 7 d (Tables 1 and 2). *Cercis*, *Corylus*, *Magnolia*, *Syringa*, *Ulmus* and *Viburnum* had callus ratings ≥ 8 on at least one date. *Acer ginnala* and *Acer truncatum* were reluctant callus formers receiving low ratings.

The length of time for callus growth varied among species.

Magnolia and *Viburnum* increased callus ratings from 5 to 12 d (Table 1). *Acer*, *Corylus*, *Cercis*, and *Crataegus laevigata* ratings decreased over time as a result of death of some callus cells.

Most *Malus* taxa had mean ratings ≥ 9 after 5 or 7 d. Several taxa had increased ratings by day 10 while others had decreasing ratings (Table 2). Of 36 *Malus* taxa, only two had mean callus ratings < 9 in all incubation trials. Variations in callus ratings appeared to result from differences in vigor of stems. Stems of *Malus* with smaller diameter and shorter length generally produced less vigorous callus.

A period between 5 and 10 d incubation at 25 °C appears to be the shortest time for stems to produce a vigorous, continuous row of callus along the cambium. Five days was generally necessary for most uniform callus growth of the taxa studied although the vigor of growth depended on apparent vigor of stem pieces. Some stem pieces grew callus faster than others of the same taxa. In separate experiments (data not presented), callus growth was faster at 25 than 20 °C.

EXPERIMENT 2. There was a significant correlation ($p \leq 0.05$) between the discoloration and callus ratings of injury after six or nine d incubation for three crabapple taxa on most test dates (Table 3). The correlation between discoloration and callus + TTC ratings were significant for crabapples on six of seven dates. For snowberry mountainash, coefficients were significant ($p \leq 0.05$) between discoloration and callus on two of the three dates after 6 d and on one date after 9 d incubation. Significant correlation coefficients indicate a relationship between the data from injury ratings, however they do not determine the critical temperature for injury.

The T_{50} s for callus ratings of Jack, Mary Potter, and pink bud Sargent crabapple and snowberry mountainash were lower (more hardy) than for discoloration on most test dates (Fig. 2 A and B). After exposure to low temperatures, Mary Potter (−38 °C, 3 Jan. 1996), pink bud Sargent crabapple (−41 °C, 12 Jan. 1996) and Jack crabapple (−44 °C, 12 Dec. 1995) had mean callus ratings of 9.4, 8.9, and 10, respectively, indicating little or no injury, and discoloration ratings of 1.1, 1.0, and 1.5 indicating severe injury (data not shown). Confidence intervals for T_{50} s were usually 3 to 6 °C and, in some cases the T_{50} for callus or discoloration may have been within the confidence intervals for the other measure. However, these measures do not indicate which is the better indicator of freezing injury.

A portion of the callus growth on some samples exposed to near critical temperatures died after forming. The living callus was

Table 3. Spearman correlation coefficients ($p > f$) between stem discoloration ratings² and callus ratings 6 or 9 d after laboratory freezing and tetrazolium chloride (TTC) treatment.

Taxa	Date	Callus		Callus + TTC
		6 d	9 d	9 d
<i>Malus baccata</i> Jacki	12 Dec. 95	---	0.66 (0.16)	0.85 (0.03)
	29 Mar. 96	0.97 (<0.01)	0.97 (<0.01)	0.85 (0.03)
<i>Malus</i> Mary Potter	15 Nov. 95	0.65 (0.17)	0.87 (0.02)	0.83 (0.04)
	3 Jan. 96	0.97 (<0.01)	0.98 (<0.01)	0.98 (<0.01)
<i>Malus sargentii</i> Rosea	12 Mar. 96	0.85 (0.03)	0.98 (<0.01)	0.98 (<0.01)
	12 Jan. 96	0.88 (0.02)	0.84 (0.04)	0.84 (0.04)
	16 Mar. 96	0.88 (0.02)	0.84 (0.03)	0.78 (0.07)
<i>Sorbus discolor</i>	4 Dec. 95	0.89 (0.02)	0.77 (0.07)	0.93 (0.01)
	15 Jan. 96	0.95 (<0.01)	0.90 (0.02)	0.34 (0.51)
	27 Mar. 96	0.40 (0.43)	0.66 (0.15)	0.95 (<0.01)

²Discoloration of stems was rated 1 to 3, where 1 = brown (discolored), 2 = uncertain, and 3 = no discoloration. Callus was rated 1 to 10, where 0 = no callus and 10 = continuous row of callus along cambial zone. Callused stems treated with TTC were rated 1 to 10, where 1 = no red callus and 10 = continuous row of red-colored callus in cambial zone.

demonstrated by red color from TTC treatment. T_{50} s for TTC-treated callus were often higher (less hardy), but within the 5% confidence intervals of the callus ratings (Fig. 2). Rating means for callus treated by TTC were usually lower than for callus alone (data not shown). Red-colored callus from the TTC procedure was easier to see and, therefore, to rate than callus alone for most crabapples, *Sorbus* and *Crataegus* species (Table 1, Fig. 2). The TTC procedure was not effective for demonstrating live callus for *Maackia*, *Magnolia*, *Syringa pекinensis*, or *Viburnum sieboldii* because red color failed to develop. However, other taxa readily developed red colored callus with the TTC procedure. Ratings of red callus were low where callus had died and for taxa that needed longer than 6 h for red color to develop.

The critical temperature, T_c , calculated from the electrical conductivity method was generally higher in most cases than the T_{50} for visual measures of injury (Fig. 1). The T_c was comparable to the T_{50} for discoloration of *Malus baccata* 'Jacki' on 12 Dec. 1995 and lower than *Malus* 'Mary Potter' discoloration on 14 Nov. 1995, but those were the only exceptions.

Discussion

Each injury evaluation method measures a different phenomenon. It is important to evaluate injury to the tissue most critical to continued plant development rather than to all living cells in the organ of interest if the intent is to determine what temperatures the plant will tolerate. The tissues that survive will determine whether growth can continue.

The highest freezing temperature resulting in a significantly higher relative conductivity than the control did not identify a critical temperature causing injury. The T_c calculated from the electrical conductivity method for *Malus* stems was generally higher than the T_{50} for discoloration or callus methods. The increased conductivity at the higher freezing temperatures may partially result from reversible injury to cells. Palta et al. (1977a, 1977b) observed that onion cells frozen at -4°C for 12 d released 85% of electrolytes when thawed, but were still alive as revealed by protoplasmic streaming and ability to plasmolyze. They concluded that the effusate after freezing to nonkilling temperatures should not be used to evaluate injury and predict survival, since the tissue may recover.

Zatylny et al. (1996) identified a lethal temperature, LT_{40} (temperature giving an index of 40% electrolyte conductance), which corresponded with temperatures causing discoloration of raspberry canes. Their procedure has merit, however, the electrical conductivity method does not differentiate between tissues critical for plant survival (Calkins and Swanson, 1990). Our results showed a range of increasing conductivity of effusate at decreasing temperatures without a distinct injury point, an observation consistent with Ketchie et al. (1972).

Discoloration of stem tissue has become one of the most accepted methods for determining injury after laboratory freezing of woody plants. Most researchers using discoloration of stem pieces after freezing tests report observations of phloem, cambium and xylem. Our experience with 1-year-old stems of deciduous plants, especially crabapples, is that phloem and cambium discoloration are difficult to discern with the naked eye or low magnification. Secondary xylem tissue is much larger and easier to observe than the cambial zone which is most critical for continued cell division and plant survival. We suspect that most reported discoloration observations of first year stems were influenced more by color of the secondary xylem than by phloem or the cambial zone.

Most taxa tested in our studies developed callus on wounded stem pieces, providing a technique that may be useful for evaluating freeze damage and other causes of stress injury to cambium. Since callus growth originates mostly from cells in the cambial zone (Fahn, 1982), it may be a better indicator of a plant's ability to continue growth than discoloration and other indicators of plant injury. Although discoloration has become a standard in hardness evaluations, several reports observe that regrowth is a better or

Fig. 2. Comparison of stem cold hardness of (A) *Malus* taxa and (B) *Sorbus discolor* using three visual methods for evaluating freezing injury [discoloration of phloem, cambium, or xylem; callus growth of split stem pieces; red color of callus treated with tetrazolium chloride (TTC)] and T_c (electrical conductivity). T_c is the highest temperature at which relative conductivity differed from the control. Vertical bars on T_{50} s represent confidence intervals ($p \leq 0.05$). Confidence intervals couldn't be calculated for T_{50} s showing no bars because insufficient injury was observed at the lowest test temperatures.

equal indicator of injury than discoloration (Holubowicz et al., 1982; Lapins, 1961; Pellett and Carter, 1981). Callus development on split stem pieces is a form of regrowth. Most taxa in this research developed vigorous, easy-to-rate, callus. Callus growth, as a measure of freeze injury, was easier to see and less subjective to rate than discoloration of phloem and cambium.

Some callus appeared to die soon after forming. Freezing damage to some, but not all, cells in the cambial zone may have stimulated callus growth by remaining cells. Injury to some cells may stimulate callus growth from surviving cells just as wounding stimulates callus growth of adjacent living cells. These isolated callus cell groups may not survive when surrounding tissues are killed. We observed, in some cases of near-critical-freezing temperatures, that callus developed then died perhaps because surrounding injured cambial zone cells could not support them. This dead callus was easier to identify when it didn't turn red from TTC treatment. The TTC procedure requires an extra step in the injury evaluation process, but may help determine whether callus is alive and, for some taxa, make callus more visible.

This study did not demonstrate whether the callus procedure is a more or less accurate measure of the cold hardiness than discoloration. However, callus growth from the cambial zone of many taxa was much easier to see than discoloration of the same tissues, thereby increasing accuracy of reporting injury. Discoloration reflects both the amount of injury and the amount of subsequent tissue oxidation whereas callus growth is a direct indicator of cell survival, and the capacity of surviving cells to divide.

We used a 1 to 3 rating scale for discoloration and many reports use only two, injured and noninjured, but callus growth was rated on a 1 to 10 scale. Since it has a larger scale, callus ratings provide a more accurate indication of the extent of injury than the discoloration method which rates samples either dead or alive or uses a more limited scale.

The callus method for determining the regrowth ability of frozen plant stems has potential for those taxa that readily grow callus. The procedure may also be useful for determining regrowth ability from other plant stresses such as drought, high temperature or air pollution.

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