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Evaluation of Different Methods to Assess Cold Hardiness of Peach Trees[1]

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Abstract. **The reliability, capability to predict survival, and convenience of 5 cold hardiness tests – triphenyl tetrazolium chloride reduction (TTC), electrolytic conductance (EC), ninhydrin-reactive compounds (NRC), trunk cambial browning (TCB), and bacterial canker development (BCD) – were evaluated using previous season's twigs of 'Redhaven' peach (*Prunus persica* (L.) Batsch) during the 1976-77 dormancy season. TTC, NRC, and EC were all significantly correlated with TCB as well as percent tree survival (PTS) in the field, and thus proved to be quantitative, reliable and capable of predicting survival at a later date. No statistical correlation was found between BCD and other hardiness tests.**

The generally accepted procedure to estimate cold hardiness under controlled conditions is to freeze plant tissue to desired temp, then thaw and determine degree of survival or injury. The various tests to assess cold hardiness, following artificial or natural freezes, that have been employed from time to time could be classified as: electrical methods (1, 5, 7, 9, 12, 21, 23, 24, 25); color or spectrophotometric determinations (11, 13, 14, 16, 17, 18, 22); visual discoloration or physical integrity of tissue (1, 3, 6, 12, 17, 18); growth and recovery tests (3, 5, 7, 9, 18, 21); and xylem pressure potential and chlorophyll fluorescence methods (2). A recently developed test (20) has also been used to evalute the effect of artificial freezing on the susceptibility of peach twigs to bacterial canker development (BCD) after inoculation with *Pseudomonas syringae* van Hall. Each of these methods has its own merits based on such crucial factors as rapidity, reliability, simplicity, convenience, and capability to predict survival. However, statistical correlations between most of these commonly used tests are lacking. There is a need, particularly in the U. S. Southeast, for rapid, reliable, and quantitative methods to evaluate cold hardiness in peach. The present research was designed to assess the aforesaid merits of and statistical correlations between trunk cambial browning (TCB), electrolytic conductance (EC), triphenyl tetrazolium chloride reduction (TTC), ninhydrin-reactive compounds (NRC), and bacterial canker development (BCD) methods to quantitatively estimate cold hardiness and predict % tree survival (PTS) of peach following artifical freezing of previous season's growth and natural freeze of whole trees in the orchard during 1976-77 season.

Materials and Methods

We selected 84 'Redhaven' peach trees grown on 7 seedling rootstocks replicated 4 times with 3 trees of each kind. For hardiness studies in the laboratory we used previous season's growth (twigs from 4 to 7 mm in diam), one 40-cm-long twig uniformly selected from each tree at biweekly intervals from mid-Nov. 1976 to late-Feb. 1977. The upper 20 cm of the twig including the tip was used for BCD, with the adjacent first internodal section being used for TTC. The next 3 consecutive internodal sections were utilized for EC and NRC tests as shown in Fig. 1.

Plant tissue was frozen in a Forma-Temp Jr Model 2154 M equipped with a circulating bath containing 70% anti-freeze (ethylene glycol base) in water. Individual sample groups from each tree, comprised of 1 section of twig with both nodes intact for TTC and 3 sections with only 1 (lower) node on each for EC and NRC, were placed in 15 ml glass vials, capped with rubber snap-caps and then securely screw-capped. These vials, held in test tube racks, were placed centrally in the bath and weighted to keep them from floating. The initial bath temp of 18°C was lowered at the rate of 2°/hr to freezing temp of −15° to −25° depending on temp outdoors for each sampling date. Samples were allowed to stay at freezing temp for at least 4 hr and then thawed for 2 to 3 hr before evaluation procedures (Fig. 1) were carried out.

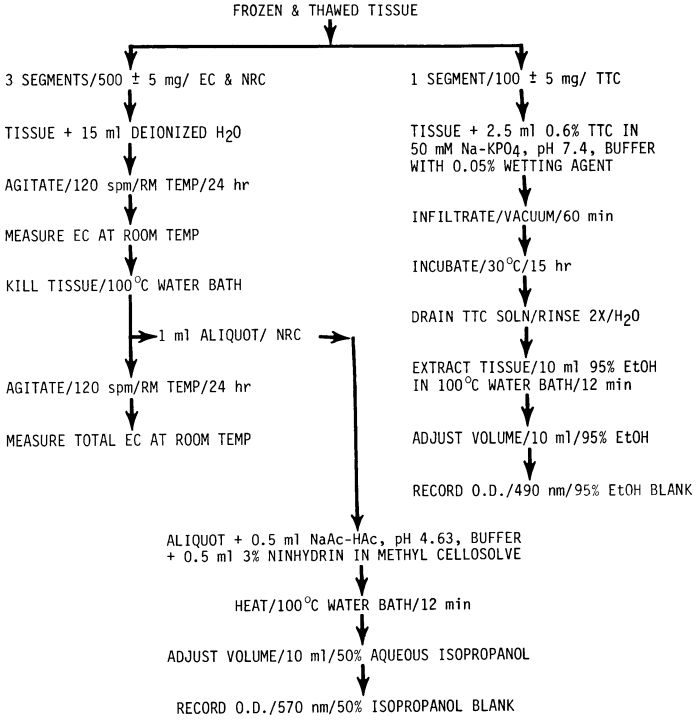

Fig. 1. Flow sheet for EC, TTC reduction, and NRC procedures.

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Trunk cambial browning. Two bark flaps were cut open at 30° angles to expose a small area of tissue in the cambial zone on the trunk of each tree at a point about 30 to 40 cm above the soil surface. We evaluated visually the severity of discoloration of cambial tissue using an arbitrary scale of 1 to 9, similar to that used by Lapins (9). The ratings we used were: 1 = tissue white, no browning (healthy tissue), 2 = very slight browning (nearly healthy tissue), 3 = slight browning (slight injury), 4 = slight to moderate browning (minor injury), 5 = moderate browning (pronounced injury), 6 = moderate to severe browning (very pronounced injury), 7 = severe browning (severe injury), 8 = severe to complete browning (very severe injury), and 9 = tissue completely brown, bark separated (dead or dying). Tree death was later confirmed upon complete tree collapse with leaves wilted and no apparent sign of growth recovery.

Triphenyl tetrazolium chloride reduction. We made minor modifications in the refined TTC procedure of Steponkus and Lanphear (17). Internodal segments, 5 to 10 mm long and weighing 100 ± 5 mg, were prepared from thawed samples. To each segment contained in a test tube, we added 2.5 ml of 0.6% 2,3,5-triphenyl tetrazolium chloride in 50 mM monobasic potassium phosphate –sodium hydroxide buffer (pH 7.4) plus 0.05% wetting agent (tergitol nonionic NPX, Union Carbide). Tissue samples were infiltrated *in vacuo* for 60 min and then were incubated at 30°C for 15 hr, followed by draining the TTC solution and rinsing the tissue twice with distilled water. Rinsed tissue was extracted with 10 ml of 95% EtOH in a 100° water bath for 10 to 12 min. After extraction, the final volume was adjusted to 10 ml with 95% EtOH and mixed thoroughly. Optical density (490 nm) of samples with a 95% EtOH blank was recorded using a Universal Spectrophotometer model 14, Coleman Instruments, Inc. We did not use unfrozen samples, since extreme variability made it impossible to separate rootstock effects.

Electrolytic conductance. For quantitative purposes, we slightly modified the conductivity method used by Ketchie et al.(7). Following freezing and thawing of tissue, 3 internodal segments 5 to 10 mm long and weighing 500 ± 5 mg from each tree were placed in screw-capped test tubes to which 15 ml deionized water was added. To facilitate leaching, the test tubes were agitated horizontally at 120 strokes per min at room temp for 24 hr. Electrolytic conductance of the leachate was measured with a Barnstead conductivity bridge model PM-70CB equipped with a dip-type, K = 1.0 cm^{-1}, conductivity cell, at room temp. Following this, the samples were killed by heating the test tubes in a 100°C water bath for 25 min. Killed samples were allowed to leach for an additional 24 hr while agitating before total electrolytic conductivity measurements were taken. The results were expressed as percent electrolytes = 100 × (conductivity of unboiled samples) / (conductivity of boiled samples).

Determination of ninhydrin-reactive compounds. For this technique we used the method described by Wiest et al. (22), with some necessary modifications. Since most of the unfrozen or frozen and unboiled samples did not produce measurable color, we used only frozen and boiled samples in this test. To 1 ml aliquots that were taken from killed samples (for EC test) before final leaching, we added 0.5 ml of 0.1 M sodium acetate-acetic acid buffer (pH 4.63) and 0.5 ml of 3% triketohydrindenehydrate (ninhydrin) in ethylene glycol monomethyl ether (methyl cellosolve). This mixture contained in test tubes was heated in 100°C water bath for 15 min. Immediately after removal from the bath, the volume in each test tube was adjusted to 10 ml with 50% isopropanol (by vol) in water and vigorously shaken. Optical density for Ruheman's purple in the samples compared to a 50% isopropanol blank was measured at 570 nm on the spectrophotometer used for the TTC test.

Bacterial canker development. Modifications of the methods reported by Vigouroux (19) and Klement et al. (8) were used as described by Weaver (20) to test the susceptibility of the excised peach twigs to BCD. To surface sterilize the twigs they were dipped in a 0.01% solution of sodium hypochlorite, rinsed in sterile water, dipped in 95% EtOH, and again rinsed in sterile water. The basal end of each twig was coated by inserting it to a depth of 2 cm in melted paraffin. Surface-sterilized twigs were then placed on water-saturated cotton in the bottom of glass tubes 25 cm long and 2.5 cm diam which had been sterilized in an autoclave for 60 min at 121°C and 15 psi. A 1-cm-long piece was cut off the apical end of each twig and the exposed cut end was immeditaely inoculated with a water suspension of *Pseudomonas syringae* (2.1×10^8 cells ml^{-1}). The tubes were sealed with aluminum foil and placed in an incubator maintained at 15°. After 7 days, the tubes were transferred to a freezing incubator set at –10° and left for 36 hr. The tubes were then returned to the 15° incubator, left for 10 days, and twigs were examined for bacterial canker injury. The length of the brown bark canker on each twig beginning at the inoculated end was measured and recorded.

Statistical analysis. Percent tree survival (PTS), TCB ratings, % electrolytes, and O.D. × 10^3 values for both TTC and NRC were all statistically analysed separately for analysis of variance for each sample date and means were separated by LSD. To test the relationship between different hardiness methods, we determined the correlation coefficient and tested for null hypothesis of zero correlation using t-values (15).

Results and Discussion

The data presented as EC, TTC, NRC, and BCD reported throughout the dormancy season of 1976-77 and comparative TCB ratings and PTS during the spring of 1977 are presented in Table 1. The correlations between different cold hardiness tests, except TTC and EC, were statistically significant at less than 5% level (Table 2). BCD did not show any correlation with other tests in the laboratory or field. Average length of bark cankers ranged from 4.7 to 6.0 mm but differences among rootstocks were not significant (Table 1).

The injury ratings in all tests were greater for less hardy tissues than for healthy tissues with greater cold hardiness. This trend prevailed throughout the dormancy season. Trees that produced higher values for EC, TTC, and NRC during

Table 1. Laboratory and field determinations of cold hardiness, tissue injury and survival of 'Redhaven' peach trees on seven seedling rootstocks.

Rootstocks[z]	Tree survival (%)	TCB[y] ratings	%EC[y]	TTC[y]	NRC[y]	BCD[y]
Lovell	100.00[x]	1.34[x]	27.9[w]	673[w]	108[w]	5.6[x]
Halford	100.00	1.83	27.5	704	117	4.9
NA8	100.00	1.92	27.2	697	116	4.7
152AI-2	97.92	2.21	29.3	695	118	5.4
HW 208	95.84	2.30	29.2	696	134	5.5
Siberian C	85.42	3.00	28.3	707	126	5.2
NRL 4	72.92	4.29	31.3	723	144	6.0
LSD 5%	4.93	0.90	2.6	24	25	2.3

[z]12 trees per rootstock.

[y]TCB = trunk cambial browning; % EC = percent electrolytes; TTC = triphenyl tetrazolium chloride (O.D. × 10^3, 490 nm); NRC = ninhydrin-reactive compounds (O.D. × 10^3, 570 nm); BCD = bacterial canker development (length, mm).

[x]Each value within columns represents the mean of 24 observations.

[w]Each value within columns represents the mean of 144 observations.

Table 2. Correlation coefficients between different methods of hardiness evaluation and the corresponding t-values with the levels of statistical significance.

Methods[z]	Correlation coefficient (r)	t-value[y]	Significance[x]
PTS vs. TCB	−0.9726	−9.42	***
PTS vs. NRC	−0.8332	−3.38	*
PTS vs. EC	−0.7955	−2.94	*
PTS vs. TTC	−0.7821	−2.81	*
TCB vs. NRC	0.8943	4.46	**
TCB vs. TTC	0.8636	3.83	*
TCB vs. EC	0.8262	3.28	*
NRC vs. TTC	0.7979	2.96	*
NRC vs. EC	0.8272	3.29	*
TTC vs. EC	0.5742	1.57	NS
BCD vs. Other tests	----	--	NS

[z]PTS = percent tree survival; TCB = trunk cambial browning; TTC = triphenyl tetrazolium chloride reduction; NRC = ninhydrin-reactive compounds; EC = electrolytic conductance; BCD = bacterial canker development.

[y]Calculated t-value = $r\sqrt{n-2}/\sqrt{1-r^2}$ at (n − 2) degrees of freedom.

[x]Significant at 5% (*); 1% (**); 0.1% (***); or not significant (NS).

dormancy also showed proportionally higher TCB ratings and trees that gave extremely high readings eventually died in the spring. Part or all of trees from which we obtained minimum values of 800 (O.D. × 10^3) in TTC, 250 (O.D. × 10^3) in NRC, or 40% EC died during or following leafing-out. The variability between different sampling dates increased substantially with the advance of the dormancy season. However, the overall trend in hardiness was not greatly affected by the variability, since we selected freezing temp to compensate for the variation in the prevailing outdoor temp. Injury which occurred in the field confirmed our laboratory findings.

Uninjured tissue from all sampling dates released less than 30% electrolytes. The relative shoot hardiness between sampling dates did not progress uniformly with the advance of the dormancy season as reported by Emmert and Howlett (5). However, Wilner (23) concluded that EC was not modified by seasonal variations or cultural practices suggesting its regulation by certain internal mechanisms probably inherent in their nature. The EC data in this study was significantly correlated with PTS, TCB, and NRC but not with TTC (Table 2). Percent electrolytes that diffused out of the tissue were found to be proportional to the severity of tissue injury (24). The extent of natural freeze damage in orchards can be determined earlier by this electrolytic procedure than by visual observations (7), and thus rough predictions can then be made by referring to % EC values.

Hardy tissues showed lower readings of TTC reduction compared to tender (non-hardy) tissue, which suggests that cold injury increased with increasing TTC values (Table 1). High correlation between cold injury and TTC may have been due to cofactor and substrate limitations rather than inactivation of dehydrogenases (17). This technique showed a significant negative correlation with PTS, and was directly correlated with TCB and NRC but not with EC. Stergios and Howell (18) did not find this method as reliable as EC for work on cherry and raspberry. However, in our study we found this test to be a simple, quick, reliable, and capable technique to predict peach tree survival at a later date.

Like the above methods, NRC was also directly correlated with cold injury; lower O.D. readings were obtained from hardy tissues and higher values indicated tissue tenderness. The NRC technique can be used as a quantitative method for estimating cold injury by freezing (13, 14). El-Mansy and Walker (4) reported high amino acid concn in peach in late summer and early fall; Lasheen and Chaplin (10) concluded that higher free amino acid concn in the tender 'J. H. Hale' peach were the result of reduced protein synthesis from the free amino acid pool. This would result in higher levels of amino acids and lower protein contents in tender cultivars and would produce higher NRC values than would hardy cultivars. The technique was significantly correlated with PTS (negatively), TCB, EC, and TTC. Wiest et al. (22) found the NRC method to be easily applicable for hardiness determinations with proven effectiveness since it uses small sample size. We found it reliable, convenient, and capable of predicting peach tree survival. It can successfully be used simultaneously with EC by utilizing the same leachate (Fig. 1).

In conclusion, we found the EC, NRC, and TTC techniques to be quick, convenient, reliable, and useful in predicting the cold hardiness of peach tissue in the field during early dormancy — before actual cold injury symptoms are apparent in the spring at the time of budbreak. All of these were significantly correlated with subsequent tree injury and death in the orchard. However, BCD was a time consuming technique which failed to separate rootstock differences and to correlate with other cold hardiness techniques.

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Effect of Peach Seedling Rootstocks and Orchard Sites on Cold Hardiness and Survival of Peach[1]

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Abstract. **Two-year-old 'Redhaven' peach (*Prunus persica* (L.) Batsch) trees on 7 different peach seedling rootstocks growing on short-life and on non-short-life sites were examined for cold hardiness of trunks using trunk cambial browning (TCB), and cold hardiness of twigs using tests for electrolytic conductivity (EC), triphenyl tetrazolium chloride (TTC), and ninhydrin-reactive compounds (NRC). It was found that Lovell, Halford, and NA 8 rootstocks invariably imparted more cold hardiness to 'Redhaven' budded onto them than other rootstocks tested, whereas maximum cold injury was sustained by trees on NRL 4 rootstock. Tree mortality was higher and cold injury was more severe on the short-life site than on the non-short-life site.**

Under growing conditions in the southeastern U.S., peach tree longevity plays a critical role in economic orchard operation. Average tree life has gradually declined in recent years, due mainly to the adverse effects of peach tree short life (PTSL), a term used to describe the sudden death of peach trees in late winter or early spring which from all appearances were healthy during the preceding growing season (2). The disease syndrome includes common symptoms of bacterial canker, blast, sour sap, die-back, sudden death, winter injury, freeze, or cold injury, etc. Typical tree death involves a sudden collapse of new growth in early spring due to trunk cambial browning and tissue disintegration. Cold injury to the trunk is initially associated with cambial browning and appears to occur during late winter dehardening of the trees (4, 5, 14). Trees on a site with a previous history of peach cultivation (short-life site) are generally much more susceptible to PTSL than those on a new site (non-short-life site) with no previous peach production (2, 12).

Rootstocks are known to influence scion physiology, including cold hardiness and dormancy (1, 2, 3, 6, 8, 9, 10, 11, 17). However, there are also reports indicating greater influence of scion cultivars on cold hardiness than that of rootstocks (16). The fact that the roots do not appear to have a rest period is of particular significance in this connection (5). In climates where severe root zone freezing may occur, absolute rootstock hardiness is critical, since once the root system is dead or severely injured, the scion will also surely die shortly after the trees leaf out in spring (8). Late-winter cold hardiness has been associated with peach tree longevity in the Southeast (2, 12). This study was designed to examine the effects of 7 peach seedling rootstocks and 2 orchard sites on the cold hardiness of 'Redhaven' peach trees.

Materials and Methods

Two-year-old 'Redhaven' peach trees on 7 different peach seedling rootstocks were tested. The rootstocks included 2 currently-recommended standards (Lovell and Halford), 2 Canadian selections (Siberian C and Harrow 208), a red-leafed Nemaguard selection (NRL 4), a North Carolina mountain natural selection (NA 8), and a North Carolina long-lived selection (152AI-2). The trees were grown on 2 sites with sandy clay loam soil in Peach County, Georgia. The sites were about 400 m apart and preplant fumigation with methyl bromide was used on the non-short-life site. The non-fumigated short-life site had been repeatedly planted with peach trees in recent years, many of which were confirmed PTSL victims.

The hardiness study was carried out on 40 cm twigs of the previous season's growth using tests for electrolytic conductance (EC), ninhydrin-reactive compounds (NRC), and triphenyl tetrazolium chloride reduction (TTC), and on the intact trees in the field using tree death and trunk cambial browning (TCB) observations during the dormant season of 1976-77. All tests and observations were conducted at biweekly intervals from Nov. 29, 1976 to Feb. 21, 1977. The first internodal section at

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