Refinement of the Triphenyl Tetrazolium Chloride Method of Determining Cold Injury

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Summary. The method of evaluating cold injury in woody plants by the use of triphenyl tetrazolium chloride has been refined to eliminate bias associated with visual differentiation between varying degrees of tetrazolium reduction and to predict tissue survival at a later date. An advantage of the method described here is that a small amount of tissue (50-100 mg) is required; this, therefore, allows for hardness determinations at precise locations on the plant. The high correlation between cold injury and triphenyl tetrazolium chloride reduction may be due to cofactor and substrate limitations rather than inactivation of dehydrogenases.

A prerequisite to conducting research in the area of cold hardness is a reliable method to determine tissue viability. Ideally, the method should eliminate bias associated with visual observations, be based on a quantitative system that could be analyzed statistically, utilize small quantities of tissue, be relatively quick, and be capable of predicting the future performance of the plant.

Primarily the triphenyl tetrazolium chloride (TTC) test has been used in the past as a qualitative response, and if the red formazan derivative was produced, the tissue was considered to be viable (5, 7, 8, 10). However, only limited provisions have been made to distinguish between varying degrees of tetrazolium reduction; as a result, differences have been reported as dark pink, pale pink, streaked pink or pink (10). Another serious disadvantage of the TTC test (9) is that even if the sample of tissue reduces the TTC to the formazan derivative, the plant may die the next day. Therefore, any refinement of the TTC test must enable one to ascertain if tissue samples viable at the time of testing will be indicative of the future viability of the plant from which they were taken.

Materials and Methods

Investigations in refinement of the TTC test utilized both leaf discs and stem segments of Hedera helix L. var. Thorndale (English Ivy) plants grown in a greenhouse. Where designated, plants were artificially cold acclimated at 5° for 6 weeks under 600 ft-c of light provided by incandescent and cool white fluorescent lamps. Leaf and stem samples were frozen under controlled conditions with a decline in temperature of 3.3° per hour. The samples were then kept at the desired low temperature for 2 hours, and survival was determined after thawing for 12 hours at 5°.

The procedure for the refined TTC test is as follows: 1) Weigh previously frozen material to 100 ± 10 mg (fr wt) samples. Stem sections are cut to a maximum length of 1.0 cm and 0.7 cm leaf discs are removed from leaf samples. 2) Place tissue samples in 17 x 120 mm test tubes graduated to 10 ml and add 3.0 ml of 0.6% (w/v) TTC in 0.05 M Na2HPO4-KH2PO4 buffer (pH 7.4) + 0.05% (v/v) wetting agent (Ortho X-77) and infiltrate under vacuum. A 0.6% TTC solution was used to insure that TTC was in excess (fig 1). The wetting agent and infiltration under vacuum insured uniform uptake by the tissue. 3) Incubate at 30° for 15 hours. During the incubation period no attempts were made to control bacterial contamination. However, reduction attributable to contamination was less than 2% of the total reduction. This figure was calculated by rapidly freezing samples at -50° to insure death and then assaying tissue samples by the TTC test. The total amount of formazan produced was less than 2% of that produced by unfrozen controls. 4) Drain the TTC solution and rinse the tissue once with distilled water. Extract the samples with 7 ml of 95% (v/v) ethanol in a boiling water bath. The water-insoluble formazan was extracted with various solvents; ethyl acetate, 80% (v/v) ethanol and 95% (v/v) ethanol. The most effective solvent was 95% ethanol. A 5 minute extraction period is adequate for leaf samples, while woody

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tissue must first be sliced and then extracted for 10 minutes. Utilizing these procedures, 98% of the formazan was recovered in the initial extraction. The extracts are cooled and made up to a 10 ml volume with 95% ethanol. 5) Record the absorbance at 530 m\(\mu\). While the reduced TTC has a \(\lambda_{\text{max}}\) at about 490 m\(\mu\), there is interference from endogenous plant pigments at this wavelength (fig 2). Therefore, 530 m\(\mu\), a wavelength still in the region of high absorption by the reduced TTC but minimum absorption by tissue pigments, was chosen for assaying the formazan derivative.

**Results and Discussion**

To facilitate interpretation of the data and to compare results of different experiments, the amount of formazan produced (\(A_{230}\)) by tissue samples exposed to freezing temperatures was expressed as a percentage of the amount of formazan produced by unfrozen (5\(^\circ\)) control samples. Results of a typical freezing test are presented in figure 3. The amount of formazan produced rapidly declines after a temperature of \(-4^\circ\) is reached. The question arises as to what temperature is lethal. That is, tissue from cuttings exposed to \(-7^\circ\) is still able to reduce the TTC to the formazan derivative, but is this indicative of continued in vivo survival of the cuttings? To answer this question, the results of a number of such tests represented in figure 3 were compiled and the results are presented in figure 4. Individual plants of varying degrees of cold hardness were separated into 2 node-2 leaf cuttings and subjected to the temperatures indicated on the abscissa of figure 4. Following thawing, 1 leaf and the basal section of the stem were assayed for their ability to reduce TTC. The remaining portion of the cutting was placed in moist vermiculite at 21\(^\circ\), and survival of the stem and leaf portions was visually determined after 4 weeks. In figure 4, the percent reduction was plotted with respect to the freezing temperature. The percent reduction was represented with a dot if the corresponding leaf or stem segment was alive after the 4-week period, and a circle was used if it was dead. Cuttings, samples of which had a 50% reduction value or greater, were alive after the 4-week period. Conversely, samples which had a percent

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*Fig. 1. Influence of TTC concentration on amount (\(A_{230}\)) of formazan extracted with 95% (v/v) ethanol from ivy leaf discs and stem segments incubated for 15 hours at 30\(^\circ\).*

*Fig. 2. Absorption spectra of 1) reduced TTC (formazan obtained from Nutritional Biochemicals), 2) 95\% (v/v) ethanol extract of ivy leaf discs incubated in a solution of 0.6\% (w/v) TTC in 0.05 M NaH\(_2\)PO\(_4\)-K\(_2\)HPO\(_4\) buffer (pH 7.4) for 15 hours, 3) 95\% (v/v) ethanol extract of leaf discs incubated in a 0.05 M NaH\(_2\)PO\(_4\)-K\(_2\)HPO\(_4\) buffer (pH 7.4) for 15 hours.*

*Fig. 3. The amount (\(A_{230}\)) of TTC reduction by ivy leaf discs and stem segments frozen at various temperatures expressed as a percentage of the amount of TTC reduction by unfrozen control samples.*
reduction value of 50 or less were viable at the time of assay, but the cuttings died during the following 4 weeks. Thus, the minimum amount of TTC reduction by tissue samples required to insure survival of the cutting at a later date is 50%, and the killing point of the cutting is defined as the temperature where a 50% reduction value is reached. Based on this definition, the killing point of the tissue presented in figure 3 is extrapolated to be $-6.5^\circ$ for stem tissue and $-8.1^\circ$ for leaf tissue.

Thus, it is possible to compensate for the prime objection (9) to the TTC test that even if a tissue sample reduces the TTC to the formazan derivative, the cutting or plant may die the next day. In addition, the method provides results that are indicative of cutting or plant survival after 4 weeks in only 15 hours. The small quantity of tissue required allows for hardiness determinations at precise locations on individual plants. Statistical evaluation of these and succeeding experiments (approximately $5 \times 10^4$ observations) indicates a standard error of a mean of 1.5° was routinely achieved using this method.

The decrease in TTC reduction with cold injury may be due to cold inactivation of dehydrogenases, as dehydrogenase activity is difficult to demonstrate in previously frozen tissue (2,12). However, demonstration of specific dehydrogenases by tetrazolium in fresh tissue is difficult due to non-specific tetrazolium reduction. Freezing (to allow substrates to diffuse from the cells) and subsequent addition of specific substrates and cofactors makes possible the demonstration of specific dehydrogenases (3,4). Previously frozen sections required additions of substrates and cofactors for intense staining; sections stained weak without cofactors and not at all without substrates. Thus, the high correlation between cold injury and TTC reduction in a system where exogenous substrates and cofactors are not added (as in the refined TTC test), is not due to cold inactivation of dehydrogenases but is due to cofactor and substrate limitations. These limitations could be caused by inactivation of enzymes other than dehydrogenases required for the continued synthesis of substrates and cofactors. The irreversible inactivation of oxidative phosphorylation by freezing (1) represents such a key step leading to a depletion of substrates and cofactors and subsequent decrease in the amount of TTC reduction in a manner similar to the inhibition of TTC reduction by 2,6-dinitrophenol (11). Substrate and cofactor limitations may also arise due to diffusion or dilution of the substrates. Since freezing disrupts lipo-protein complexes and affects membrane permeability (6), intracellular localization and concentration of substrates at enzyme sites may be greatly diminished.
Literature Cited


