Estimating Cold Stress in 'Beautiful Arcade' Apple Roots using Electrical Impedance Analysis

Jean-Pierre Privé¹ and
M.I.N. Zhang²

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Summary. 2,3,5-triphenyl tetrazolium chloride (TTC) staining, electrical conductivity, and electrical impedance (Z) analyses were used to assess freezing injury of 'Beautiful Arcade' apple (*Malus × domestica* Borkh.) roots taken in late March from either the field or 3C-refrigerated storage (cold-stored). Lethal temperature (LT₅₀) levels using TTC or electrical conductivity occurred at colder temperatures than those found using Z. Techniques varied in their ability to detect changes in cell viability with increasing cold stress. Listed in order of decreasing responsiveness they are electrical impedance (Z), electrical conductivity, and TTC vital staining. With the most sensitive technique, Z, two parameters—extracellular and total tissue electrical

resistance—were about five and eight times lower (indicating more injury) for roots from the field than from cold storage. The smaller values obtained from the field roots were probably due to natural in-field freeze-thaw cycling before the controlled cold-stress tests in the laboratory. More importantly, the impedance technique provided more detailed information than TTC or electrical conductivity about how apple roots respond to cold stress and how Z may provide some insight into freeze-thaw history before injury assessment. Although this technique shows potential, future studies are required to render a complete physiological significance to the impedance parameters involved in calculating Z.

In breeding for more freeze-resistant plants, there is the challenge of evaluating many samples for their survival under different developmental stages and under various freezing stresses. Viability frequently is measured by testing regrowth or tissue browning. While these two methods produce the most reliable data on survival, they are both time-consuming and require large amounts of samples (Stergios and Howell, 1973). Other methods are simpler to use but are less reliable. These include triphenyl tetrazolium chloride (TTC) reduction (Towill and Mazur, 1975), electrical conductivity (DeHayes et al., 1990; Ketchie et al., 1972; Murray et al., 1989; Raese, 1983), and earlier studies using in situ electrical impedance measurements (Lougheed et al., 1983; Tattar and Blanchard, 197). A problem with these methods is that they have worked for some species but not for others (Stergios and Howell, 1973).

The principle of electrical impedance is much like Ohm's law. In a direct current (DC) circuit, $R = V/I$ (resistance = voltage/current), while in an alternating current (AC) circuit, Ohm's law is in the form $Z = v/i$ (Z = electrical impedance). In an AC field, the ionic fluid of biological tissue acts as a conductor and the membranes act as capacitors. There is no inductive component in biological tissue, therefore the impedance consists of resistance and capacitive reactance. Because of the passive electrical properties, tissue impedances are related to

cellular ionic content and membrane structures. Impedance therefore varies according to the nature of the tissue and its physiological state. Recently, impedance techniques in plant physiology have improved in both measurement and analysis (Repo et al., 1994; Stout et al., 1987; Zhang and Willison, 1991a, 1991b). As this newer technique is based on measurement and analysis over a wide range of frequencies, it is almost independent of measurement apparatus or the individual frequencies used for measurement; therefore it overcomes some previously identified disadvantages of the technique (Lougheed et al., 1983).

A successful application of impedance spectroscopy to plant physiology depends on several factors: 1) determination of tissue impedance by using an accurate measurement technology; 2) a proper model to represent plant tissue; and 3) a proper technique to analyze the measurement data in reference to the corresponding model(s). Presently, a four-terminal measurement provides the best results for (1) (Zhang, personal communication). With respect to (2), the goal of applying impedance spectroscopy to plants is to quantify physiological status in terms of model parameters. A model parameter should correspond to a cellular component or structure, so that each model parameter has a biological meaning. With tissues of uniform cell size, a lumped model is suitable, and each parameter can represent a biological meaning. Unfortunately, with highly differentiated tissues such as roots or shoots, the lumped model is unsatisfactory, and a distributed model provides greater suitability, but not without a cost. The cost is that it is difficult to render biological meaning to some of the parameters in the distributed model (Repo and Zhang, 1993). With (3), the complex nonlinear least squares analysis is the most powerful method capable of fitting complicated models and is recommended for users interested in extracting maximum information from their measurement data.

With the recent developments in measurement and data analysis, plant impedance analysis has emerged as a new tool for in situ measurement. The present study examines the power and limitation of this technique in monitoring 'Beautiful Arcade' apple roots for cold-stress injury.

¹Fruit crop physiologist, Agriculture and Agri-Food Canada, Bouctouche NB Canada E0A 1G0. To whom reprint requests should be addressed.

²Researcher, Department of Physiology and Cell Biology, University of Texas Medical School, Houston, Texas 77030.

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Materials and methods

Plant material. 'Beautiful Arcade' seedlings were started in the greenhouse in 1992 and then placed in refrigerated storage (3C) at the end of the season. In 1993, the most uniform seedlings were set in a nursery (field) while others were established in 3-liter (20-cm-diameter) pots containing a peatlite growing medium (Nova Mix; Annapolis Valley Peat Moss, Aylesford, N.S.). Potted and field plants were watered and fertilized as required. Once the leaves had fallen off in Fall 1993, the potted plants were placed in refrigerated storage (3C) while the field plants were left in the nursery. Before the controlled-freezing tests in the laboratory, the cold-stored plants were never exposed to subzero temperatures, whereas the field plants were subjected to natural in-field freeze-thaw cycling during the entire winter. Soil temperatures taken at 10 cm revealed that there was one freeze-thaw event in November, were two in December and was one before harvest in March. The soil remained frozen between 26 Dec. and 26 Mar. Field plants were dug from the nursery on 31 Mar. when soil temperatures were about 5C, potted plants were removed from cold storage, and roots from both environments were subjected to the freeze-thaw treatments.

Freeze-thaw treatment. Preliminary studies (unpublished data) found no differences in Z parameters for root segments taken either near the shank or at the extremity of the root. Consequently, root pieces were randomly chosen throughout the root system for this study. Roots (2 to 4 mm in diameter) were selected from field and cold-stored plants and cut into 3 to 4-cm-long segments. After a brief rinsing following cutting, six root segments were placed in each test tube and submerged in a microprocessor controlled cryobath (Haake F3-Kcryostat, Haake Mess-Technik GmbH, Germany), while the controls were placed in a refrigerated storage at 3C. In the freezing samples, ice nucleation was initiated at -3C and the frozen samples were then kept at -3C for 14 h (overnight). Subsequent freezing was programmed to have a cooling rate of 4.8 C/h and 1 h holding at the preselcted test temperatures of -6, -9, -12, -15, and -18C. Samples were taken out at each temperature.

LT₅₀ values for all methods were

designated to corresponding cold-stress temperatures when values dropped by about 50% of the control temperature.

One advantage of measuring tissue impedance as an indicator of physiological status is that the tissue is not destroyed while useful data are being obtained. This permitted root segments to be analyzed first for Z, then by the destructive TTC staining and electrolyte-leakage methods.

Electrical impedance and analysis. Since apple roots contain a diversity of cell sizes and types, a distributed model was used to represent the plant tissue. Electrical impedance spectra between 100 Hz and 800 kHz were measured by a computer-controlled HP 4284A Precision LCR Meter (Zhang and Willison, 1991a) using needle electrodes (Zhang and Willison, 1992b). The impedance spectra were analyzed by a complex nonlinear least squares (CNLS) program, BIA (written in Visual Basic and available upon request from MINZ for \$100). BIA is modified from MODEL3 in Zhang and Willison, 1991b, 1992a) based on Cole-Cole function (Eq. [1]) (see Repo and Zhang, 1993; Zhang et al., 1994).

$$Z = R_{\infty} + (R_0 - R_{\infty}) / [1 + (j\omega\tau)^{\Psi}] \quad [1]$$

where R_0 = extracellular resistance; R_{∞} = total tissue resistance; τ = time constant; and Ψ = distribution factor of the time constant.

Eq. [1] has exactly the same form as the formula developed by Cole and Cole (1941) for characterizing dielectrical behavior. The same form has been used in materials science (Sandifer and Buck, 1974) as well as in biological impedance analysis (Ackmann and Seitz, 1984; Greenham et al., 1982) In this model, Z is impedance; R_0 corresponds to extracellular resistance or the resistance at extremely low frequency; R_{∞} corresponds to total tissue resistance or the resistance at extremely high frequency as though all membranes were destroyed; τ is a generalized time constant of the tissue; Ψ is a distribution

factor of the time constant (Ackmann and Seitz 1984; Repo and Zhang 1993); j is a complex number operator $[(-1)^{1/2}]$, and ω angular frequency of the alternate current. τ and Ψ are parameters that characterize the whole system and they describe the distribution of cell sizes.

In Cole-Cole function parameter optimization, unit weighting statistical procedures were used for all impedance spectral analyses. In most analyses, the full range impedance data (100 Hz to 800 kHz) were used. With seriously injured tissues, a low-frequency section of the Cole-Cole plot sometimes showed obvious deviation from the main section. Here, we only analyzed the section that was stable among replicates.

Parameter normalizaation. Impedance parameters were normalized to values for 1 cm³ of tissue. In this report, all data are normalized and all resistances are expressed as ohm-cm⁻¹.

Tetrazolium staining. After each of the test temperatures was reached, six root segments were cut into halves longitudinally and placed in a 2 × 15-cm test tube and stained with 0.08% TTC according to the method of Chen and Gusta (1983). After 18 h incubation in the dark at 20C, root segments were rinsed with distilled water. Phloem tissues were examined visually for the percentage of tissue that had stained red, i.e., live phloem vs. tissue that had not stained, i.e., injured. These results, expressed

Fig. 1. Percent injury using TTC vital staining or electrical conductivity following freezing stress in 'Beautiful Arcade' apple roots from either cold storage or the field. Electrical conductivity was performed only on field roots.

Fig. 2. Cole-Cole function parameters in relation to freezing stress in 'Beautiful Arcade' roots taken from cold storage (3C) in late March. (A) τ ; (B) R_0 ; (C) R_∞ ; (D) Ψ .

as percent injury, also were tested using the extraction method and were found to be similar. Therefore, only visual ratings are reported.

Electrical conductivity. Only field-collected roots were used to assess the cold injury of the root segments using the electrolyte leakage method. After each of the test temperatures, three root segments were placed individually in 2×15 -cm test tubes with 15 ml of water and shaken at 100 rpm for 20 h. Afterwards, conductivity of the bathing solution was measured; samples were then heated

to 90°C for 20 min, reshaken for another 20 h, and conductivity was measured again. The results are expressed as percent injury determined by the amount of electrolyte leakage after each of the preselected temperatures.

Results

Tetrazolium staining. Vital staining with TTC and visual estimation of phloem viability after controlled freezing provided a rapid means of analyzing freeze-thaw injury in apple roots. This technique revealed no noticeable differences in cell viability for either cold-stored or field-collected roots down to -6°C (Fig. 1). Thereafter, the survival rates differed and the resulting LT_{50} for field-collected root segments was about -9°C, whereas it

was about -15°C for the cold-stored roots. At temperatures of -12°C and lower, no red stain was visible (100% injury) to the field-roots, whereas >40% of the cold-stored roots were still viable at -18°C.

Electrical conductivity. Measurements of electrical conductivity derived from cellular leakage due to cold stress also provided a rapid means for analyzing freeze-thaw injury. Although the conductivity method was more sensitive to temperature change than the TTC method, similar LT_{50} values were found for field-collected root segments, i.e., -9°C (Fig. 1). No data are available for cold-stored roots.

Impedance results. Graphs of extracellular resistance (R_0), total tissue resistance (R_∞), a generalized time constant of the tissue (τ), and a distribution factor of the time constant (Ψ) are shown for apple roots taken either from cold storage (Fig. 2) or dug from the field (Fig. 3).

Some interesting differences between the two treatments were found. Despite whether roots were taken from cold storage or the field, changes in R_0 and τ were evident as cold stress temperatures decreased (Figs. 2 and 3, A and B), while R_∞ and Ψ were much less influenced (Figs. 2 and 3, C and D). As cold stress increased, τ and R_0 decreased linearly for the cold-stored roots (Fig. 2 A and B) and exponentially for the field roots (Fig. 3 A and B). This resulted in an associated LT_{50} of -9°C for the cold-stored roots and -3 to -6°C for the field roots. This suggests that there was more evidence of injury in the field-roots than in the cold-stored roots. What is more important, a decrease in impedance using the Cole-Cole function for R_0 and τ was detected after a freeze-thaw of only -3°C. This suggests that Z is very sensitive to tissue changes caused by even minor cold stress. Besides the unfrozen samples, i.e., 3°C, R_∞ remained unchanged with increasing cold stress (Figs. 2C and 3C), while Ψ seemed only partially influenced at the lower temperatures (Figs. 2D and 3D). Another major difference between the cold-stored and field roots was the range of values, i.e., the scale for the R_0 and R_∞ parameters. Initial values at 3°C for R_0 (6350 ohm-cm⁻¹) and R_∞ (893 ohm-cm⁻¹) from cold-stored roots were five and eight times greater than for their corresponding values in field-exposed roots (1093 and 115 ohm-cm⁻¹,

respectively; (Figs. 2 and 3, B and C). By contrast, τ (0 to 12 μ s) and Ψ (0 to 0.8) had similar ranges in values for either cold-stored or field roots (Figs. 2 and 3, A and D).

Discussion

As found in many other tissues (Ackmann and Seitz, 1984; Greenham et al., 1982; Repo and Zhang, 1993), the Cole-Cole function (Eq. [1]) is a suitable model for describing electrical properties in apple roots. The power of the impedance technique in apple root-stock analysis lies in the interpretation of the parameters in Eq. [1] in physiological terms.

The two most valuable parameters, extracellular resistance (R_o) and a generalized time constant (τ), were the most responsive to freeze-thaw stress in cold-stored and field roots, decreasing linearly and exponentially, respectively, with increasing cold stress. In comparison, TTC vital staining and electrical conductivity were least and intermediate, respectively, in their response to decreasing temperatures. Concurrently, despite the lack of response between 4 and -6°C in both environments using TTC vital staining, Z (R_o and τ) and electrical conductivity provided additional information within these minimal stress levels. Similar phenomena were observed in Z in Scots pine (*Pinus sylvestris* L.) needles (Repo et al., 1994) and turnip (*Brassica rapa* L.) root tissues (Zhang et al., 1994). The decrease in R_o is mostly due to release of intracellular electrolytes to extracellular space (Zhang and Willison, 1992a, 1992b). A decrease in R_o will contribute to some decrease in τ , and a decrease in membrane capacitance from membrane damage will be another factor in causing the decrease in τ (Repo et al., 1994). At present, it is speculated that the release of electrolytes to extracellular space is due to membrane rupturing and resealing during freeze-thaw cycling. Nevertheless, the event of rupture-reseal does not necessarily involve membrane functional loss in nonlethal freeze-thaw stresses (Zhang et al., 1994).

The impedance technique may provide not only physiological information about the injury level of the roots but also about the freeze-thaw history before assessment. In the present study, the field roots were exposed to a total of four freeze-thaw

events, whereas the cold-stored roots never experienced subzero temperatures before the controlled freezing tests. Naturally, all techniques reported more injury to the field roots following treatment, but only the extracellular resistance, R_o , and the total tissue resistance, R_∞ , were able to detect differences in tissue integrity before the tests. The values for R_o and R_∞ in cold-stored roots were five and eight times larger than the corresponding values from the field roots. While it is not completely clear what factors produced this great difference in R_o and R_∞ between the two environments, we speculate that field freezing before our sampling for controlled stress in the laboratory is responsible for their low values in the field. Earlier studies showed that cyclic freezing in red on-

Fig. 3. Cole-Cole function parameters in relation to freezing stress in 'Beautiful Arcade' roots dug from the field in late March. (A) τ ; (B) R_o ; (C) R_∞ ; (D) Ψ .

ion (*Allium cepa* L.) scale tissues produced a cumulative effect in electrolyte leakage to extracellular space, and more freeze-thaw cycles produced more tissue injury (Zhang and Willison, 1990). The present tests also showed that more injury was produced in field than in the cold-stored roots. It is believed that the excessive injury of field roots compared to cold-stored roots is probably due to natural in-field freeze-thaw cycling before our controlled stress tests in the laboratory. Future studies will be aimed at examining the influence of freeze-thaw cycling in apple roots.

All methods analyzed suggest that the apple roots taken from the field in late March, i.e., after roots had been exposed to natural freeze-thaw cycles throughout the winter and early spring, suffered more injury than roots taken from cold storage. This is not surprising, since it is known that apple trees and other crops reacclimate with warming spring temperatures (Howell and Weiser, 1970; Ketchie, 1985). However, roots were not examined in those studies and may react differently than above-ground portions of the tree (Quamme, personal communication). Nonetheless, what is more important to realize is the limitation in analyzing root tissues collected from the field during winter. Results from the field roots will always be based on a second freezing of the thawed samples regardless of the technique. By contrast, the cold-stored roots never experienced any freezing before the preselected test temperatures. Therefore, any comparison between the two environments should be interpreted cautiously. Nevertheless, electrical impedance seems particularly useful for evaluating root tissues that are difficult to evaluate by recovery, tissue browning, or DTA techniques.

The experimental data gave us two lines of useful information: 1) the capability of the impedance technique in detecting a sample's historical events, in this case the in-field freezing and thawing during sampling before controlled freezing in the laboratory; and 2) the practical difficulty in the assessment of actual injury levels in field plants during the winter season. Measuring injury by laboratory technique will be affected by the freeze-thaw history a plant experiences in the field in two ways: a) the thawing related injury and b) the thawing-induced deacclimatization.

It is easy to control (b) by reducing the time required for thawing and cleaning up the soil from the root system, but it is impossible to control (a) such that the soil could be removed without complete thawing. Although the Z technique looks promising, it will need to be compared with a recovery method, such as regrowth, to validate its usefulness and power as a tool for testing cold-stress tolerance in plant tissues. Additional information also is required to render more physiological

significance to the parameters in this distributed Cole-Cole model. Nonetheless, its potential as a nondestructive technique that can detect subtle differences in tissue integrity would be an asset for breeding programs worldwide.

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