

## Evaluation of laboratory tests for determining the lethal temperature of *Vitis labruscana* BAILEY Concord roots exposed to subzero temperatures<sup>1)</sup>

by

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### Die Auswertung von Labortests zur Bestimmung der letalen Gefriertemperatur für die Wurzeln von *Vitis labruscana* BAILEY cv. Concord

**Zusammenfassung:** Die Wurzeln 1jähriger dormanter Concordreben wurden in einer Gefriertruhe, deren Temperatur sich stündlich um  $-2^{\circ}\text{C}$  erniedrigte, abgestuften Frosttemperaturen ausgesetzt. Eine erste Temperaturserie fiel in Intervallen von  $-5^{\circ}\text{C}$  von  $0^{\circ}\text{C}$  bis auf  $-30^{\circ}\text{C}$  ab, ein zweites Temperaturprogramm mit Temperaturstufen von  $-2^{\circ}\text{C}$  erstreckte sich zwischen  $0$  und  $-20^{\circ}\text{C}$ . An Rebwurzeln von 1—3 mm und von 4—6 mm Durchmesser wurden die elektrische Leitfähigkeit (EC), die Reduktion von Triphenyltetrazoliumchlorid (TTC) und die Gewebebräunung (TB) nach Kälteeinwirkung geprüft. Ferner wurden Wachstums- und Überlebenstests durchgeführt. Aufgrund dieser Prüfverfahren wurde für die Concordwurzeln eine Letaltemperatur von ungefähr  $-5^{\circ}\text{C}$  ermittelt. Pflanzen, deren Wurzeln  $-10^{\circ}\text{C}$  ausgesetzt waren, wuchsen zwar zunächst, starben aber später ab. Die durch EC- und TTC-Tests ermittelten Letaltemperaturen der Wurzeln waren den Ergebnissen der Überlebenstests vergleichbar. Obwohl der TB-Test nur qualitative — histologische — Aussagen erlaubt, kann er doch in Verbindung mit den anderen Tests aufschlußreiche Ergebnisse liefern.

**Key words:** cold, resistance, frost damage, analysis, variety of vine.

### Introduction

Subzero temperatures of  $-15^{\circ}\text{C}$  or colder occur in the Yakima Valley, Washington on an average of every 3 years. Two recent winters which have caused severe damage to grapevines occurred in 1978—79 and 1983—84. The result of such cold spells because of lack of snow cover is deep soil freezing. It is generally believed that the primary cause of vine mortality is root damage (1).

Relatively little information is available on the cold resistance of grape root tissues as compared to above-ground parts. CARRICK (3) using 10 cm long root segments 2 mm in diameter tested the cold resistance of 6 grape cultivars grown in New York in a chamber surrounded by a mixture of ice and common salt to drop the temperature to  $-18^{\circ}\text{C}$ . Tissue temperature was not measured. On the basis of the number of roots injured by visual observation he classified the cultivars studied as resistant and not resistant to cold.

Studies on cold hardiness with other plants have demonstrated that the root system is considerably less cold hardy than stem tissues of the same plant under field conditions (5, 8, 18, 21, 22). STUART (17) froze artificially 1- to 2-year-old roots of apple seedlings from 31 parent varieties, but the hardiness of the progenies could not be cor-

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related with hardiness of the parents. Roots farther from the stem and younger are less hardy than those closer to the stem and older (10, 13). Reduced moisture content of the roots appears to be related to hardiness (23). Severity of root injury depends upon fall hardening conditions, nutritional status of the plant, rates of freezing and thawing, minimum temperatures, duration of minimum temperatures, age and depth of rooting (4, 6, 7, 8, 9, 10, 11, 19, 20).

The generally accepted procedure to estimate cold hardiness under controlled conditions is to freeze plant tissues to desired temperatures and then thaw and determine the degree of survival of low temperature injury. The tests to assess cold hardiness following artificial or natural freezes include electrical conductivity methods (22, 23), tetrazolium chloride reduction tests (12, 16), visual discoloration or physical integrity of tissues (16), growth and recovery tests, xylem pressure potential and chlorophyll fluorescence methods (2).

Experiments were started at the Irrigated Agriculture Research and Extension Center, Prosser, Washington to determine the effect of subzero temperatures on young Concord vines. The objectives were to: i) determine the freezing temperatures at which Concord roots would be injured or killed; ii) test the reliability of electrolytic and colorimetric tests developed for other crops for determining freezing injury to grape tissues; and iii) study the anatomical features associated with partially and severely freeze-damaged and killed root and stem tissues.

### Materials and methods

Three hundred 1-year-old dormant Concord vines grown in a nursery were dug in January of 1980 and 1981 and stored until the experiments on freezing began. The lowest air temperature these plants were exposed to outdoors was  $-4^{\circ}\text{C}$  in 1980 and  $-3^{\circ}\text{C}$  in 1981. The plants were sorted for uniformity in bundles of 25 and stored for 2 weeks in the cold room at  $1^{\circ}\text{C}$  in moist peat moss. Peat moss was removed from roots, and tops pruned to 3 buds. Rootings were placed in a plastic bag with stem portion protruding. Only the roots were exposed to subzero temperatures. The bag was tied with a string just below the lowest bud. Temperature was measured by copper-constantin thermocouples inserted in roots of 1–3 mm and 4–6 mm diameter and in the stem portion just below the bud and coupled to a recorder. The plants were used for the following tests:

#### Survival test

A set of 10 dormant plants at each temperature were placed in a freezer at  $0^{\circ}\text{C}$ , which was designed to drop the temperature of the roots at  $-2^{\circ}\text{C}/\text{h}$ . In two separate experiments temperatures of  $-5$  to  $-30^{\circ}\text{C}$  (6 temperatures) with  $-5^{\circ}\text{C}$  decrements and temperatures of  $-2$  to  $-20^{\circ}\text{C}$  with  $-2^{\circ}\text{C}$  decrements (10 temperatures) were used. The plants were left in the freezer for 5 min after the desired temperature was attained. Out of the 10 plants exposed to each temperature 5 plants/temperature (5 single vine replicates) were used for the survival test in each experiment. After exposure to the respective temperature and thawing at room temperature ( $25^{\circ}\text{C}$ ), they were planted in the greenhouse to test survival. This was done in 1980 and also in 1981. Bud break and shoot growth were taken as an indication of survival. After 2 months, the test was terminated. The shoot length was measured, roots were exposed and examined for the presence of fibrous roots. The other 5 plants were saved for electrical conductivity (EC), triphenyl tetrazolium chloride test (TTC) and tissue browning tests (TB).

### Electrical conductivity test

After thawing, roots were separated into small (1—3 mm in diameter) and large (4—6 mm in diameter) roots. A 10 g sample of small and large roots from each temperature was cut to 5 cm segments in length and soaked in 200 ml of distilled water in an Erlenmeyer flask for 4 h at 25 °C. Electrical conductivity of the diffusate was measured in micro mhos ( $\mu\text{mhos}$ ) on a conductivity bridge (Model No. RC 16B1 — Industrial Instruments, NJ). The electrical conductivity of water was subtracted from the readings of the diffusate.

### Triphenyl tetrazolium chloride test

a) Extraction of formazan. — A 100 g sample of root segments from each size group previously exposed to subzero temperatures was weighed. After transferring to a test tube, 3 ml of 0.6 % TTC in 0.05 M  $\text{Na}_2\text{HPO}_4$ - $\text{KH}_2\text{PO}_4$  buffer, pH 7.4 and 0.05 % (v/v) Ortho X 77 wetting agent was added and incubated at 30 °C for 15 h. TTC was drained and tissue rinsed with distilled water. The red color due to production of formazan was extracted repeatedly with 10 ml of ethanol until all the color was extracted. The color was read on Bausch and Lomb Spectronic 21 Spectrophotometer at 530 nm. About 90 % of the formazan was recovered from the root tissues by repeated extractions.

b) Visual observations of roots from TTC test. — Root samples 5 cm in length of 1—3 and 4—6 mm in diameter, previously exposed to subzero temperatures were placed in a petri dish (2 pieces of root of each diameter to a dish), submerged in the TTC solution and incubated at 30 °C for 15 h. There were 5 replications. At the end of the incubation period, the roots were examined for red color and graded on a scale of 1—5, 1 having no color and 5 having intense red color.

### Tissue browning test

The root segments of 1—3 and 4—6 mm diameter previously exposed to the respective temperatures and thawed were used for the tissue browning test. Thin longitudinal and cross sections of roots were made with a razor blade and examined microscopically and macroscopically for tissue browning. Cross sections of stem just above the lowest bud were also made and examined for browning. The browning was rated on a scale of 1—5, 1 showing no browning and 5 showing intense browning.

### Sectioning of grape buds to detect injury

Two buds from each of the 5 vines used for survival, EC and TTC tests were sectioned with a razor blade and examined microscopically for injury indicated by browning.

## Results and discussion

All control plants survived. The shoots of the control plants were significantly longer compared to plants exposed to  $-5$  and  $-10$  °C (Table, upper part). Numerous fibrous roots were found on the control plants, their number diminishing as the temperature was lowered to  $-10$  °C. No fibrous roots or shoots were observed on plants exposed to  $-15$  °C and lower, indicating death of the roots. In the temperature regime  $-2$  to  $-20$  °C, plants exposed to  $-8$  °C and lower died (Table, lower part).

Electrical conductivity of root diffusate increased as the temperature was lowered from 0 to  $-30$  °C (Fig. 1). There was a gradual increase in electrical conductivity of dif-

Survival of 1-year-old Concord plants and their roots after exposure to subzero temperatures  
 Überlebensrate 1jähriger Concordreben und ihrer Wurzeln nach Einwirkung verschiedener Gefrier-temperaturen

Temp. °C	% survived	Shoot length (cm)	Roots %		Remarks
			Old lignified	New non- lignified	
Control	100	41 a <sup>1)</sup>	100	100	Numerous fibrous roots
- 5	80	21 b	80	65	Few fibrous roots
- 10	20	3 c	20	15	Plant died subsequently
- 15	0	0 d	0	0	Dead — no fibrous roots
- 20	0	0 d	0	0	Dead — no fibrous roots
- 25	0	0 d	0	0	Dead — no fibrous roots
- 30	0	0 d	0	0	Dead — no fibrous roots
Control	100	45 a	100	100	Numerous fibrous roots
- 2	100	40 a	100	100	Few fibrous roots
- 4	80	20 b	80	80	Few fibrous roots
- 6	20	20 b	80	40	Very few fibrous roots
- 8	0	0 c	20	20	Plant died subsequently
- 10	0	0 c	0	0	Dead — no fibrous roots
- 12	0	0 c	0	0	Dead — no fibrous roots
- 14	0	0 c	0	0	Dead — no fibrous roots
- 16	0	0 c	0	0	Dead — no fibrous roots
- 18	0	0 c	0	0	Dead — no fibrous roots
- 20	0	0 c	0	0	Dead — no fibrous roots

<sup>1)</sup> Means in the columns separated by Duncan's Multiple Range Test, 5 % level.  
 Data represents the mean of 1980 and 1981.

fusate up to  $-8^{\circ}\text{C}$  for roots of 1—3 mm diameter and up to  $-6^{\circ}\text{C}$  for the 4—6 mm diameter roots (Fig. 2). At lower temperatures, there was a sudden and dramatic increase in electrical conductivity. It appears that as the temperature is lowered there is an increase in cell permeability up to  $-6$  and  $-8^{\circ}\text{C}$  for roots of 1—3 and 4—6 mm diameter. This may indicate injury to root cells. Below these temperatures, injury may have become severe, resulting in the death of the cells. PALTA *et al.* (11) working on the freezing injury of onion (*Allium cepa*) bulbs, showed increased electrical conductivity of the diffusate after severe freezing ( $-11^{\circ}\text{C}$ ).

They stated that the semipermeable properties of the cell are uninjured but that ion and sugar transport mechanisms are damaged by freezing. They concluded that the final injury following freezing and thawing cannot be evaluated from the conductivity of the diffusate immediately after thawing. Since grape root tissues are lignified, the same conclusion may or may not apply here. Based on the survival test (Table),  $-5^{\circ}\text{C}$  is the lowest temperature which the grape roots survived. The roots exposed to  $-10^{\circ}\text{C}$  grew but subsequently died possibly because of the severe injury. There was a large increase in electrical conductivity from  $-5$  to  $-10^{\circ}\text{C}$  indicating injury at  $-10^{\circ}\text{C}$  (Figs. 1 and 2). Root injury may not be fully explained by the EC alone, however, this method is simple and is useful as an indicator of root injury.

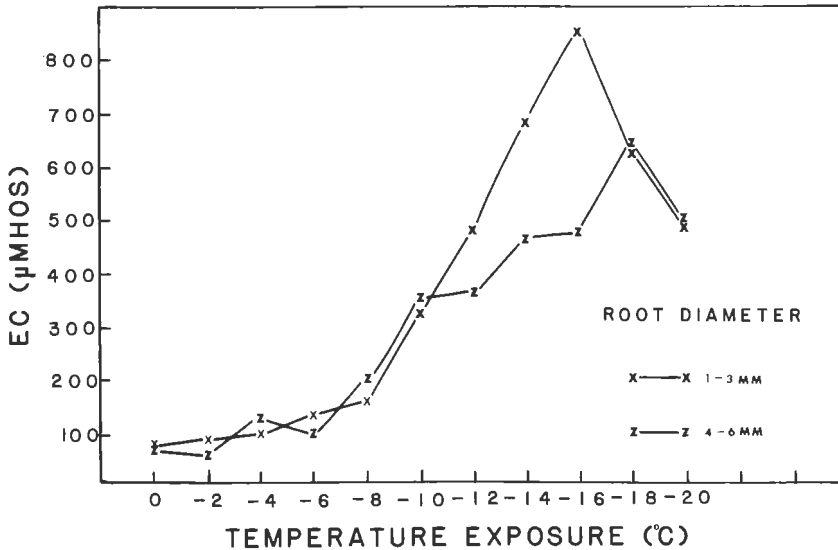
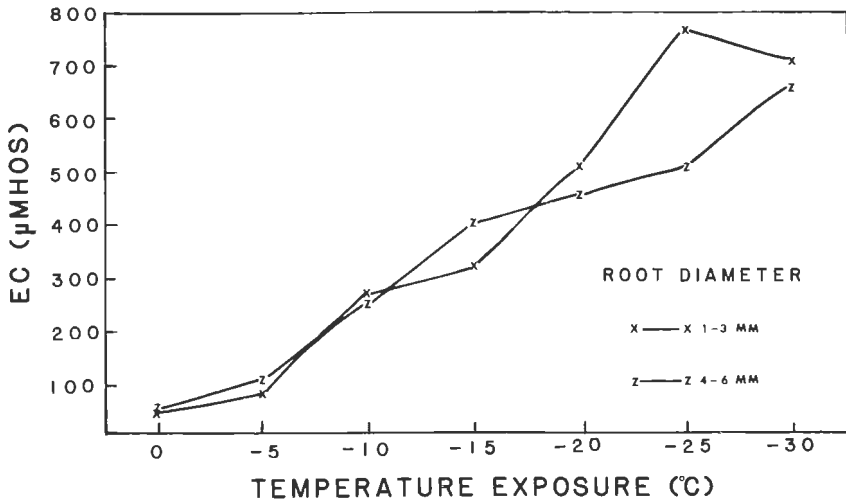


Fig. 1 (top): Electrical conductivity of diffusate from small and medium-sized Concord roots after exposure to various temperatures (down to  $-30^{\circ}\text{C}$ ). After the desired temperatures were reached, the roots were left at that temperature for 5 min. The regression equations with  $\text{EC} = y$  and temperature =  $x$  are: 1-3 mm root diameter,  $y = -25.36x + 3.82$ ,  $v = -0.97$ ; 4-6 mm root diameter,  $y = -19.67x + 5.78$ ,  $v = -0.97$ .

Fig. 2 (bottom): Electrical conductivity of diffusate from small and medium-sized Concord roots after exposure to various temperatures (down to  $-20^{\circ}\text{C}$ ). After the desired temperatures were reached, the roots were left at that temperature for 5 min. The regression equations with  $\text{EC} = y$  and temperature =  $x$  are: 1-3 mm root diameter,  $y = -31.26x + 24.04$ ,  $v = -0.74$ ; 4-6 mm root diameter,  $y = -31.78x + 17.77$ ,  $v = -0.96$ .

The amount of formazan produced by root tissue samples exposed to subzero temperatures decreased as the temperature was lowered from unfrozen control to  $-30^{\circ}\text{C}$  in  $-5^{\circ}\text{C}$  decrements and down to  $-20^{\circ}\text{C}$  in  $-2^{\circ}\text{C}$  decrements (Figs 3 and 4). There was a sudden drop in absorbance from  $-5$  to  $-10^{\circ}\text{C}$  with the decrease continuing at lower temperatures. Similarly, a sudden drop in absorbance was noticed when the temperature was lowered below  $-6^{\circ}\text{C}$  for the 1–3 mm roots. It is not clear why there was increase in absorbance from 0 to  $-6^{\circ}\text{C}$ . This indicates that near  $-5^{\circ}\text{C}$  a sudden change occurs in the root tissues. Root samples placed in petri dishes and submerged in TTC solution exhibited red color throughout the vascular system at  $-5^{\circ}\text{C}$ , and at  $-10^{\circ}\text{C}$  a faint pink color was noted. The intensity of the color was higher in phloem than in the xylem. Below  $-10^{\circ}\text{C}$ , no color developed in root segments indicating death of the tissues.

The question arises as to what temperature is lethal for Concord roots. By examining the data from the survival test (Table), electrical conductivity test (Figs. 1 and 2) and triphenyl tetrazolium chloride test (Figs 3 and 4) we find that near  $-5^{\circ}\text{C}$  electrical conductivity increased and absorbance decreased at a rapid rate. The survival test also showed that plants exposed to  $-10^{\circ}\text{C}$  did not survive but those exposed to  $-5^{\circ}\text{C}$  did. Based on these tests, the conclusion can be drawn that the lethal temperature of Concord roots in this test is  $-5^{\circ}\text{C}$ .

One serious drawback of the TTC test (12) is that even if the triphenyl tetrazolium chloride is reduced to formazan derivative, the plant may subsequently die. STEPONKUS and LAMPHEAR (15) tried to overcome this disadvantage by working out a formula. The minimum amount of TTC reduction by tissue sample required to insure survival at a later date is 50 % and the killing point of the tissue was defined as the temperature where 50 % reduction value is reached. This formula was derived by working with the stem segments of *Hedera helix*. Since grape roots are different from the stem tissues of *Hedera*, in our experiment, the survival test was made the basis for determining the corresponding values for formazan and electrical conductivity which will indicate lethal temperatures for roots.

Sectioning of grape buds to detect injury revealed that buds were not injured when the root temperature was dropped. Microscopic examination of the sectioned root tissues to detect browning revealed that uninjured tissues in the control remained bright and clear while the injured ( $-10^{\circ}\text{C}$ ) were discolored and brown but did not disintegrate. Browning was noticed in cambium, immature xylem cells close to the cambium and in the wood rays and cortex. The extent of browning increased as the temperature was lowered. Browning was considerably less at  $-5^{\circ}\text{C}$  than at  $-10^{\circ}\text{C}$  and lower temperatures. Examination of the discolored tissues following freezing and thawing is tedious and time consuming. Though not quantitative, the browning test reveals injury to tissues which cannot be seen by other methods (14). The order of hardness of tissues could not be determined by this study.

From the results obtained under conditions prevailing in this study, based on the survival test, EC and TTC, and rating of tissue browning in cross sections, it appears that the lethal temperature of Concord grape roots is near  $-5^{\circ}\text{C}$ . CARRICK (3) reported

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Abb. 1 (oben): Elektrische Leitfähigkeit des Diffusates kleiner und mittelgroßer Concordwurzeln nach Einwirkung verschiedener Gefriertemperaturen (bis  $-30^{\circ}\text{C}$ ). Nach Erreichen der jeweiligen Temperatur verblieben die Wurzeln noch 5 min in diesem Temperaturbereich.

Abb. 2 (unten): Elektrische Leitfähigkeit des Diffusates kleiner und mittelgroßer Concordwurzeln nach Einwirkung verschiedener Gefriertemperaturen (bis  $-20^{\circ}\text{C}$ ). Nach Erreichen der jeweiligen Temperatur verblieben die Wurzeln noch 5 min in diesem Temperaturbereich.

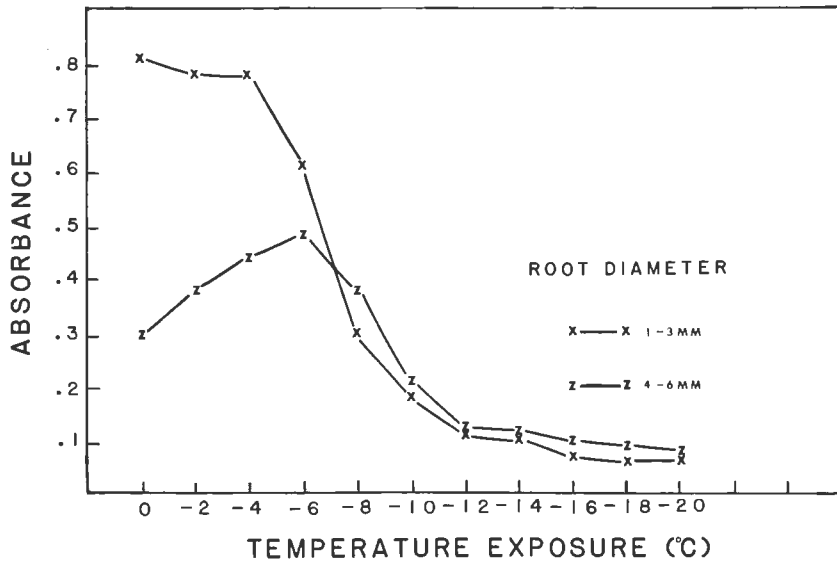
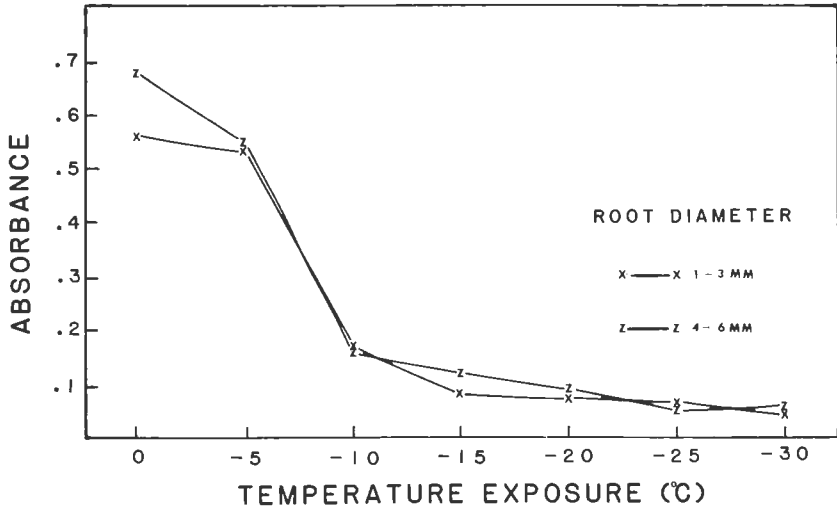


Fig. 3 (top): Absorbance of formazan at 530 nm from small and medium-sized Concord roots after exposure to various temperatures (down to  $-30^{\circ}\text{C}$ ). After the desired temperatures were reached, the roots were left at that temperature for 5 min. The regression equations with absorbance =  $y$  and temperature =  $x$  are: 1-3 mm root diameter,  $y = 0.0009x^2 + 0.0463x + 0.6065$ ,  $r = 0.87$ ; 4-6 mm root diameter,  $y = 0.0009x^2 + 0.0470x + 0.7056$ ,  $r = 0.88$ .

Fig. 4 (bottom): Absorbance of formazan at 530 nm from small and medium-sized Concord roots after exposure to various temperatures (down to  $-20^{\circ}\text{C}$ ). After the desired temperatures were reached, the roots were left at that temperature for 5 min. The regression equations with absorbance =  $y$  and temperature =  $x$  are: 1-3 mm root diameter,  $y = 0.0022x^2 + 0.0892x + 0.9361$ ; 4-6 mm root diameter,  $y = 0.0006x^2 + 0.0075x + 0.4037$ .

that roots of Concord survived  $-10^{\circ}\text{C}$  under New York conditions. It is not known if tissue temperature reached  $-10^{\circ}\text{C}$  in CARRICK's experiment because it was not measured.

Both EC and TCC reduction tests were suitable for evaluating grape root hardiness although the findings of PALTA *et al.* (11) regarding EC throw some doubt on the reliability of this method for all tissues. The method needs confirmation. Even though tissue browning and survival tests are slow, tedious and qualitative, they are still reliable. Similar conclusions were drawn by STERGIOS and HOWELL (16) while working with stem tissues of certain small fruits.

These studies have provided valuable information on the suitability of laboratory tests for determining the lethal temperature of Concord roots. It is known that there are wide differences in the cold hardiness of different *V. vinifera* varieties (1). In the Pacific Northwest where subfreezing temperatures occur at periodic intervals, there is need to do similar research with cold tender *V. vinifera* varieties and rootstocks.

### Summary

The roots of 1-year-old dormant Concord plants were subjected to subzero temperatures in a cold box programmed to lower the temperature at  $-2^{\circ}\text{C}/\text{h}$ . Temperatures down to  $-30^{\circ}\text{C}$  with  $-5^{\circ}\text{C}$  decrements and  $-20^{\circ}\text{C}$  with  $-2^{\circ}\text{C}$  decrements were used. Electrical conductivity (EC), triphenyl tetrazolium chloride reduction (TTC), and tissue browning (TB) tests were conducted on roots of 1–3 and 4–6 mm in diameter. Growth and survival tests were also conducted. Based on these tests, the lethal temperature of Concord roots was near  $-5^{\circ}\text{C}$ . The plants exposed to  $-10^{\circ}\text{C}$  grew but subsequently died. Lethal temperature of roots indicated by EC and TTC was comparable to that obtained from the survival test. TB tests, though qualitative, were useful in evaluating root injury to tissues and can be used in conjunction with other tests.

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Abb. 3 (oben): Absorption des aus kleinen und mittelgroßen Concordwurzeln extrahierten Formazans bei 530 nm nach Einwirkung verschiedener Gefriertemperaturen (bis  $-30^{\circ}\text{C}$ ). Nach Erreichen der jeweiligen Temperatur verblieben die Wurzeln noch 5 min in diesem Temperaturbereich.

Abb. 4 (unten): Absorption des aus kleinen und mittelgroßen Concordwurzeln extrahierten Formazans bei 530 nm nach Einwirkung verschiedener Gefriertemperaturen (bis  $-20^{\circ}\text{C}$ ). Nach Erreichen der jeweiligen Temperatur verblieben die Wurzeln noch 5 min in diesem Temperaturbereich.



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