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# Freezing Tolerance of Tissue Cultured *Rubus* Plants[1]

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## Abstract

Tissue cultured 'Perron Red,' 'September Red,' and 'Canby' red raspberry, 'Shawnee' blackberry, and 'Royalty' purple raspberry plants 7 to 12 cm in height were subjected to artificial freezing tests. Apical meristems and roots of 'Perron Red' survived -8°C when whole plants were frozen at 1°C/hr; however, after seven days under mist in a greenhouse, plants subjected to -2°, -6°, or -8°C had less root regrowth than unfrozen controls. Shoot dry weight of plants subjected to temperatures below -2°C was reduced and root dry weight of plants exposed to -8°C was adversely affected when measured after 26 days in the greenhouse. When only shoots of whole plants were frozen, the lowest survival temperature (LST) for apical meristems of 'Canby' was -8°C, the LSTs of 'Perron Red', 'September Red,' and 'Shawnee' were all -12°C, and 'Royalty' remained uninjured at -14°C. Despite meristem survival, severe freezing injury to mature leaves of all cultivars occurred by -10°C. In another experiment, mefluidide (N-[2,4-dimethyl-5-[[(trifluoro-methyl)-sulfonyl]amino]-phenyl]acetamide) was applied to plants at 5 mg·l$^{-1}$ to determine if the growth regulator provided protection against low temperature injury. The LST of mefluidide-treated plants did not differ from the controls.

## Introduction

Tissue cultured brambles are often recommended for planting because they are uniform, easy to handle, and free of certain diseases that may be present in material propagated from field-grown plants. While tissue cultured plants are generally pathogen free, they may be more susceptible to environmental stresses before they have acclimated to field conditions when compared to conventionally propagated, dormant plant material (1).

Donnelly and Vidaver (2) and Donnelly *et al.* (4) reported that aseptic culture influenced both the anatomical and physiological characteristics of young red raspberry plants. Tissue cultured plantlets had smaller, thinner leaves with less compact palisade cells than those of field-grown plants. Additionally, these plantlets had few filiform trichomes and adaxial, abaxial, and peripheral stomata on leaves. In contrast, leaves of field-grown plants had numerous trichomes and only a few stomata. Peripheral stomata were absent. Thus, transplant shock and lowered water stress tolerance of tissue cultured material at planting were attributed to the altered leaf anatomy. In another study (3), $CO_2$ uptake by leaves of *in vitro* raspberry plants was half that of *ex vitro* plants. Differences in photosynthetic capacity were associated with restricted gas exchange in the young tissue cultured plants.

In addition to water stress resistance and photosynthetic activity, the tolerance of tissue cultured plants to subfreezing temperatures may be a critical factor in survival since spring frosts can occur after field planting. The objectives of this study were to determine the freezing tolerance of selected tissue cultured *Rubus* cultivars and to determine if low temperature injury could be reduced with an application of mefluidide.

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## Materials and Methods

*Plant materials.* Commercially micropropagated 'Perron Red' raspberry plants (approximately 10 cm tall) were received on 3 May 1988 and stored at 2°C until testing. All experiments on these plants were initiated within the following 12 days. On 20 May 1988, micropropagated 'Shawnee' blackberry, 'Royalty' purple raspberry, and 'September Red' and 'Canby' raspberry plants that were approximately 7, 8, 10, and 12 cm tall, respectively, were obtained from a commercial nursery. These plants were stored at 2°C under fluorescent lights (20 $\mu mol \cdot s^{-1} \cdot m^{-2}$) to prevent leaf senescence until experiments were performed. Freezing tests on these plants were initiated within the following 16 days.

*Freezing tolerance of whole 'Perron Red' plants.* On 5 May 1988, twenty-four 'Perron Red' plants were wrapped in moist paper towels and placed in 25 x 200 mm test tubes. The tubes were then inserted into a rack in a temperature-controlled ethylene glycol bath (Neslab RTE 220, Newington, NH) at -1°C. The top of the rack was constructed of a one cm thick sheet of styrofoam between layers of plexiglass. Each plant was positioned in a test tube so that roots and shoots were below the surface of the coolant. A 36 gauge copper-constantan thermocouple was placed in close proximity to the apical meristem of selected plants to monitor tissue temperature. Thermocouple output was recorded on a Honeywell Electronik 112 multipoint recorder. Plants were held at -1°C for one hr before they were seeded with ice. Seeding was accomplished by touching the moist paper towel surrounding each plant with a thin wire which had been dipped in liquid nitrogen. After seeding, a foam plug was placed in the top of each tube and samples were held at -1°C for an additional hour before cooling was initiated at a rate of 1°C/hr. Four plants were removed at 2° intervals from -2°, -4°, -6°, and -8°C, placed in thermos bottles precooled to 2°C, and thawed for 24 hr in a cold room at 2°C. Unfrozen control plants stored at 2°C were also included in the experiment. After thawing, plants were planted in 28 x 54 cm flats containing vermiculite and placed on a mist propagation bench in a greenhouse at 25°(day)/20°C (night). After seven days under mist (12 sec mist every 6 min), the number of uninjured leaves per plant and the length of new growth of five roots per plant were recorded. Subsequently, the plants were transplanted into Pro-Mix BX (Premier Brands, Inc., New Rochelle, NY) in 12.7-cm-diameter pots, fertilized with Osmocote (14N-6.2P-11.6K, 5 g/pot), and allowed to grow in the greenhouse for 26 days. Shoots and roots were then rinsed free of media and oven-dried 24 hr at 80°C to determine dry weight. Root growth and shoot and root dry weight data were subjected to an analysis of variance and means were separated by a least significant difference (LSD) test. Data from the unfrozen control were deleted and orthogonal contrasts were performed to test the effect of temperature on root growth and dry weight.

*Freezing tolerance of shoots.* On 17 May 1988, shoots of individual 'Perron Red' plants were wrapped in a moist paper towel and a foam plug was placed at the root-shoot junction of each plant. Plants were then inserted into test tubes with the shoots in a downward position and placed in the temperature bath at -1°C so that the roots remained above the level of the coolant. Plants were held at -1°C for one hr before they were seeded with ice. Ice seeding was accomplished by inserting a thin wire, dipped in liquid nitrogen, through a small glass tube extending through the foam plug and

touching it against the moist paper towel surrounding the shoot. A moist piece of paper towel was placed over the roots to prevent desiccation during the experiment. Thermocouples were attached near the apical meristem and to the roots to monitor tissue temperature. Samples were then cooled as described above. Four test tubes were removed from the bath at 2° intervals from -6° to -14°C. Samples were then thawed at 2°C, planted in trays of vermiculite, and placed under mist for seven days. Apical meristems of plants were then examined for oxidative browning under a dissecting microscope to determine the lowest survival temperature (LST).

Similarly, on 24 May 1988, shoots of 'September Red' and 'Canby' raspberry, 'Shawnee' blackberry, and 'Royalty' purple raspberry were frozen to -8°, -10°, -12°, and -14°C. Three replications of each cultivar were used. After exposure at each temperature, plants were removed from the bath, thawed at 2°C, and placed under mist for seven days. Apical meristems were examined for oxidative browning to determine the LST.

*Freezing tolerance of excised apical meristems.* On 5 June 1988, a three cm long section of tissue including the apical meristem was excised from tissue cultured 'September Red' and 'Royalty' plants. Tissue was wrapped in a moist paper towel and placed in test tubes. Foam plugs were then inserted in the tubes to prevent desiccation. The freezing test was performed as described above and three samples per cultivar were removed at 2° intervals from -8° to -14°C. After thawing at 2°C for 24 hr, samples were incubated at 25°C and 100% RH for three days before the apical meristems were examined for browning.

*Freezing tolerance of mefluidide-treated 'Shawnee' plants.* Immediately after plants were received the roots were rinsed free of media and the plants were placed in trays of vermiculite under mist in the greenhouse for three days. Analytical grade mefluidide was dissolved in 5 ml of acetone and then diluted with 1 ml of Tween-20 surfactant and water to a final concentration of 5 $mg \cdot l^{-1}$ mefluidide. An atomizer was used to spray plants to runoff (≈5 ml spray solution/plant). Treated plants and untreated controls were left in vermiculite trays in the greenhouse at 25° (day)/20°C (night) for three days. Shoots of plants were subjected to freezing temperatures as previously described and 3 replications of each treatment were removed at each test temperature (-8°, -10°, -12°, and -14°C). After thawing at 2°C, plants were placed under mist in the greenhouse for 7 days and then evaluated for injury to determine the LST.

## Results and Discussion

*Freezing tolerance of whole 'Perron Red' plants.* After seven days under mist, all plants exposed to subfreezing temperatures had five to seven uninjured leaves (data not shown). Leaves exhibiting injury were always mature, while the youngest 2 to 3 leaves (including the meristem) did not exhibit injury symptoms After plants were removed from the mist and allowed to grow for 26 days in the greenhouse, those exposed to -2°, -6°, and -8°C had less new root growth than unfrozen controls when five roots per plant were measured (Table 1). However, only plants subjected to -8°C had less root dry weight than the unfrozen controls. Root dry weight exhibited a linear response to temperature. Shoot dry weight was lower in plants exposed to all test temperatures below -2°C.

*Freezing tolerance of shoots.* Almost all mature leaves were necrotic and minimal root regrowth was observed

**Table 1. Root growth and root and shoot dry weight of 'Perron Red' raspberry after whole plants were exposed to subfreezing temperatures[z]**

| Tissue temperature (°C) | New root[y] growth (mm) | Root dry wt.[x] (mg) | Shoot dry wt. (mg) |
|---|---|---|---|
| Unfrozen control | 21 | 405 | 1338 |
| -2 | 11 | 323 | 1013 |
| -4 | 14 | 280 | 850 |
| -6 | 10 | 305 | 813 |
| -8 | 3 | 163 | 583 |
| LSD (5%) | 9 | 139 | 458 |
| Significant effects[w] | | | |
| $Temperature_L$ | NS | * | NS |
| $Temperature_Q$ | NS | NS | NS |
| $Temperature_C$ | NS | NS | NS |

[z]Means of the temperature treatments and the unfrozen control were separated by LSD.
[y]Means represent the length of new growth of five roots per plant. Measurements were recorded after plants were placed in the greenhouse under mist for seven days.
[x]Measurements were obtained after plants were placed in the greenhouse for 26 days.
[w]Linear (L), quadratic (Q), and cubic (C) orthogonal contrasts were determined by regression. NS, not significant or *, significant at the 5% level.2

on 'Perron Red' plants subjected to temperatures below -8°C. Apical meristems, however, remained uninjured at -10°C and one of four survived -12°C. Mature leaves of 'Canby' exposed to -6°C were necrotic and minimal new root growth occurred after seven days under mist. The LST for apical meristems of 'Canby' was -8°C. Necrosis of mature leaves and minimal root regrowth was observed at all temperatures below -8°C on 'September Red,' 'Shawnee,' and 'Royalty.' The LSTs of apical meristems of 'September Red' and 'Shawnee' were both -12°C. 'Royalty' meristems remained uninjured at -14°C (the lowest test temperature). The LSTs for treatment replicates of 'Canby,' 'September Red,' and 'Shawnee' were identical (i.e., no treatment variance was present), therefore, a statistical analysis of the data was not performed.

Since the root temperature of all plants remained above 2°C throughout the freezing experiment, the lack of new root growth appears to be associated with low temperature injury to mature leaves. Donnelly and Vidaver (4) demonstrated that leaves produced after plantlets were transferred from the tissue culture medium were photosynthetically active. Although photosynthetic rates in the bramble plants were not measured, the lack of root regrowth observed in this study may have been due to a freeze induced reduction of photosynthesis in the mature leaves and/or a depletion of carbohydrate stored in the roots.

*Freezing tolerance of excised apical meristems.* The LST for excised meristems of 'September Red' was -12°C, while two of the three excised meristems of 'Royalty' remained uninjured at -14°C. Results from this experiment were similar to those in which the intact shoots were frozen. Apparently, the longer exposure of these meristems at 2°C (16 vs 4 days for experiments on shoots) did not result in increased tolerance to low temperature. Since freezing tests were not performed on plants prior to exposure at 2°C, the rate of acclimation (if it occurred) is not known. Nevertheless, the lack of acclimation of *Rubus* meristems after 16 days appears to be similar to that of

spinach leaves exposed to low temperature. Fennell and Li (5) measured a rapid increase in freezing tolerance of spinach leaves during the first three days at 5°/2°C day/night temperature, but the rate of acclimation decreased dramatically thereafter.

*Freezing tolerance of mefluidide-treated 'Shawnee' plants.* The LST for meristems of untreated 'Shawnee' control plants was -8°C (a dehardening of 4°C when compared to the 'Shawnee' plants tested above and presumably due to the three day growth period in the greenhouse). A similar result was observed in mefluidide-treated plants except that one plant remained uninjured at -10°C. Zhang and Li (8) reported that mefluidide at concentrations of 2 or 5 ppm increased the freezing resistance of wheat (*Triticum aestivum* L.) seedlings by 3° to 4°C. Cucumber (*Cucumis sativus* L.) and corn (*Zea mays*) have also been protected from chilling injury (i.e., injury at temperatures from 0° to 15°C) by mefluidide treatment (6, 7). The reason for the lack of response in the tissue cultured blackberry plants is not known but may have been the result of a low mefluidide dosage, lack of absorption, and/or physiological differences in blackberry plant tissues. For example, Tseng *et al.* (7) postulated that mefluidide treatment alters plasma membrane characteristics of corn and, thereby, protects this sensitive cellular structure from chilling injury. Similar protection may not be possible in woody species such as blackberry.

The apical meristems of all tissue cultured brambles studied here were relatively tolerant to freezing temperatures and survived exposure to at least -8°C. However, injury to mature leaves and reductions in root growth occurred at warmer temperatures. In 'Perron Red' raspberry, apical meristems survived to -10°C, but shoot dry weight was adversely affected at all temperatures below -2°C and root dry weight decreased linearly with temperature. Similarly, mature leaves of 'Canby' and 'September Red' raspberry, and 'Shawnee' blackberry, and 'Royalty' purple raspberry were injured at higher temperatures than their apical meristems. Reductions in new root growth appeared to be associated with freezing injury to mature leaves. In conclusion, while apical meristems of tissue cultured brambles may survive below -8°C, subsequent growth may be impaired due to injury at higher temperatures in other plant parts.

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