

EXTENDED COLD ACCLIMATION AND RECOVERY MEDIUM ALTERATION IMPROVE REGROWTH OF *RUBUS* SHOOT TIPS FOLLOWING CRYOPRESERVATION

Yongjian Chang¹ and Barbara M. Reed²*

¹Department of Horticulture, Oregon State University, Corvallis, OR 97331, USA.

²USDA/ARS, National Clonal Germplasm Repository, 33447 Peoria Road, Corvallis, OR 97333, USA.

*To whom correspondence should be addressed; Email: reedbm@bcc.orst.edu

Summary

Extended cold acclimation (CA) of shoot cultures in alternating low temperatures [22°C with 8 h light ($10 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) / -1°C 16 h dark] improved the recovery of cryopreserved shoot tips. As the duration of CA prior to cryopreservation increased from 1 to 3 weeks, *Rubus parvifolius* L. shoot tip survival increased from 63% to 90% and shoot formation increased from 25% to 75%. Six to ten weeks of CA were required to achieve high survival and shoot formation in *R. caesius* L. and improve shoot survival from 8% to 70-80% and shoot formation from zero to 60-80%. Eliminating indole-3-butyric acid from the recovery medium decreased callus formation and increased direct shoot formation for both *R. parvifolius* and *R. caesius*. Histological studies showed that *R. parvifolius* shoot tips continued to grow and regenerated directly from the meristematic domes following liquid nitrogen exposure. The upper axillary buds often survived and regrew along with the apex. No shoots regenerated from callus produced on margins of leaf primordia and damaged meristematic domes.

Key words: cryopreservation, histology, recovery, shoot tip, raspberry, blackberry

Introduction

One problem often encountered in cryopreservation of shoot tips is the maintenance of organization within the meristem and its subsequent regrowth without inducing callus formation, since regeneration of shoots from callus increases the frequency of somaclonal variation (10). The genetic stability of regenerated plants can be ensured by developing appropriate protocols of cryopreservation and regrowth medium for each species and cultivar (16, 17). Although many *Rubus* (blackberry and raspberry) genotypes can be cryopreserved and have high survival following cryopreservation, the percentage of shoot formation of some genotypes is not high enough for germplasm storage and many genotypes produce too much callus (11, 13, 14). Long-term storage and maintenance of genetic stability in *Rubus* germplasm requires improved protocols.

Many plant species that grow in temperate areas have increased freezing tolerance following exposure to low, non-freezing and non-injurious temperatures, a process termed cold acclimation (CA). A one week cold acclimation pretreatment significantly improves survival and regrowth of some *Rubus* genotypes following liquid nitrogen (LN) exposure, but others, including *R. caesius* L. and *R. parvifolius* L., do not respond (12, 13). Extended CA treatments improved recovery of cryopreserved pear and silver birch meristems (2, 15). The effect of extended CA treatment on

recovery of *Rubus* shoot tips following LN exposure is not known.

The aim of this study was to improve shoot formation, reduce callus formation, and verify the direct regrowth of shoots from the meristems of cryopreserved *Rubus* shoot tips. We examined the effect of CA duration on the recovery of cryopreserved shoot tips and the effect of indole-3-butyric acid (IBA) in the recovery medium on shoot and callus formation. We also studied the histology of apices with and without LN exposure to observe the regrowth pattern of shoot tips and determine the origin of callus observed under some recovery conditions.

Materials and Methods

Plant Material

In vitro-grown plantlets of *R. parvifolius* L and *R. caesius* L were micropropagated in Magenta GA₇ boxes (Magenta Corp., Chicago, IL, USA) on NCGR-RUB medium, a modified MS medium (9) with doubled EDTA-Fe, 4.4 μM N⁶ - benzyladenine (BA), 0.49 μM IBA, 0.35% agar (Bitek agar, Difco, Detroit, MI, USA), 0.175% Gelrite (Kelco, San Diego, CA), and 3% sucrose (13). The pH was adjusted to 5.8 before autoclaving. The plantlets were subcultured every three weeks, and grown at 25°C with a 16 h light / 8 h dark photoperiod (40 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Both species are cold hardy and grow in north temperate climates.

Cold acclimation and pretreatments

Two weeks after the last subculture, plantlets were cold acclimated with alternating temperatures, a short photoperiod, and reduced irradiance [22°C with 8 h light (10 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) / -1°C 16 h dark] for 1 to 10 weeks. Controls were held under growth-room conditions without transfer. Dissected shoot tips (0.8-1.0 mm) from CA and control treatments were grown for 48 h on NCGR-RUB medium with 5% dimethyl sulfoxide (DMSO) under the same CA conditions as the parent plantlets.

LN exposure and recovery

Following the 48 h 5% DMSO pretreatment, shoot tips were transferred to 1.2 ml plastic cryo-vials containing 0.25 ml liquid NCGR-RUB medium at 0°C. The cryoprotectant PGD [a mixture of 10% each polyethylene glycol (MW 8000), glucose, and DMSO in liquid NCGR-RUB medium] was added dropwise up to 1.2 ml over 30 min (4). After 30 min equilibration at 0°C, the shoot tips were cooled to -35°C at 0.5°C/min in a programmable freezer (CRYO-MED, Forma Scientific, Mt. Clemens, Mich.) and then immersed in LN for 1 h. Vials were thawed in 45°C water for 1 min, then for 2 min in 23°C water. The cryoprotectant was removed and replaced with liquid NCGR-RUB medium. Shoot tips were recovered on NCGR-RUB medium without IBA in 24 cell plates, one shoot tip per cell (Costar, Cambridge, Mass.).

Effect of IBA on recovery

The effect of IBA on shoot tip recovery was examined by recovery of frozen shoot tips on NCGR-RUB medium with or without 0.49 μM IBA. Ten-week CA plantlets of *R. parvifolius* and *R. caesius* were used in this test. Shoot tips were cooled as described above, thawed and plated on medium with or without IBA in 24 cell plates for recovery under standard growth-room conditions.

Data collection

Shoot formation and/or survival data were taken four weeks after thawing. Shoot formation was the percentage of new shoots formed by cryopreserved shoot tips, and survival was the percentage of shoot tips producing either new shoots or callus. There were 20 shoot tips in a

vial for each treatment and five apices for unfrozen controls. The experiment was repeated 3 times.

Anatomical observations

Histological studies were conducted on *R. parvifolius* meristems recovered on NCGR-RUB medium without IBA. Ten shoot tips were fixed in FAA (50% alcohol: formalin: acetic acid =18:1:1) at 0, 3, 5, 7, 11, 15 and 21 days following thawing. After at least 48 h fixation, the samples were dehydrated with tert-butyl-alcohol (TBA) and water series, infiltrated with xylene and embedded in paraffin. The sections (10 µm thick) were cut with a rotary microtome (Spencer Lens CO., Buffalo, NY, USA) and stained with 1% safranin and 0.1% fast green (7).

Results and Discussion

The effect of cold acclimation on Rubus shoot tip survival and shoot formation

The two *Rubus* genotypes varied in the rate of response to CA (Fig. 1). One week of CA increased the survival of *R. parvifolius* following LN exposure from 23 to 63%, and shoot formation from 0 to 25%. *R. parvifolius* shoot formation continued to increase, and at 3 weeks CA reached 90% survival and 75% shoot formation. Shoot formation and survival remained high for the 10 weeks tested. For *R. caesius*, survival and growth gradually increased with CA duration. Shorter CA treatments (< 3 weeks) resulted in little shoot formation. Both shoot formation and survival of *R. caesius* reached 81.5% with 10 weeks CA. Optimal survival and regrowth was achieved after 2 weeks of cold acclimation for *R. parvifolius*, but after only 10 weeks for *R. caesius*. This observation is similar to that reported for other cold hardy genera. With pears length of CA is genotype dependent. Some genotypes require only one week CA, but others require more than 10 weeks (2). Silver birch shoots require more than 4 weeks CA to obtain greater than 40% recovery (15).

Successful cryopreservation protocols must be designed to minimize damage and maximize regrowth potential. Cold acclimation of *in vitro* plantlets takes advantage of the plant's natural defense against cold and increases cold hardiness of the tissues. There are many morphological, physiological and biochemistry changes in cells during plant cold acclimation. Many genes are involved in the overall cold acclimation process (3). Our studies showed that increased duration of CA greatly improved the regrowth of cryopreserved *R. parvifolius* and *R. caesius* meristems, which had low survival (36% and 23%) following 1-week CA in an earlier study (13). When the CA period was short, survival was low and most of the surviving shoot tips formed callus rather than shoots. This phenomenon indicated that the apices were seriously injured during the freezing/thawing process of cryopreservation, resulting in callus production during regrowth. Tissues and cells which have been cold acclimated for longer periods were more resistant to freezing stress and therefore were better able to recover following cryopreservation.

IBA effects on recovery

Callus proliferation rather than shoot growth was stimulated when IBA was included in the recovery medium (Fig. 2). *R. parvifolius* apices produced more callus on the IBA medium than did those of *R. caesius*. Callus production on medium without IBA dropped to zero or near zero for apices of both genotypes CA for 10-weeks. IBA, as a plant growth regulator promotes plantlet growth in culture and is very commonly added to *Rubus* multiplication medium (1, 12). However, IBA also stimulates callus formation from many kinds of tissues (6). After LN exposure, tissues of *Rubus* apices were partially damaged and that damage stimulated callus formation. In this case, if IBA was added to the recovery medium, additional callus was produced. The composition of the recovery medium is an important parameter to optimize the recovery of cryopreserved shoot tips. The cryopreserved shoot tips are considerably more fragile than the control shoot tips or a

micropropagated plantlet. The damage produced by freezing and dehydration of cells in the shoot tips induce callus production that is further encouraged by growth regulators in the recovery medium (6). Reducing IBA, a callus promoter, is therefore a logical choice for optimal recovery of cryopreserved *Rubus*.

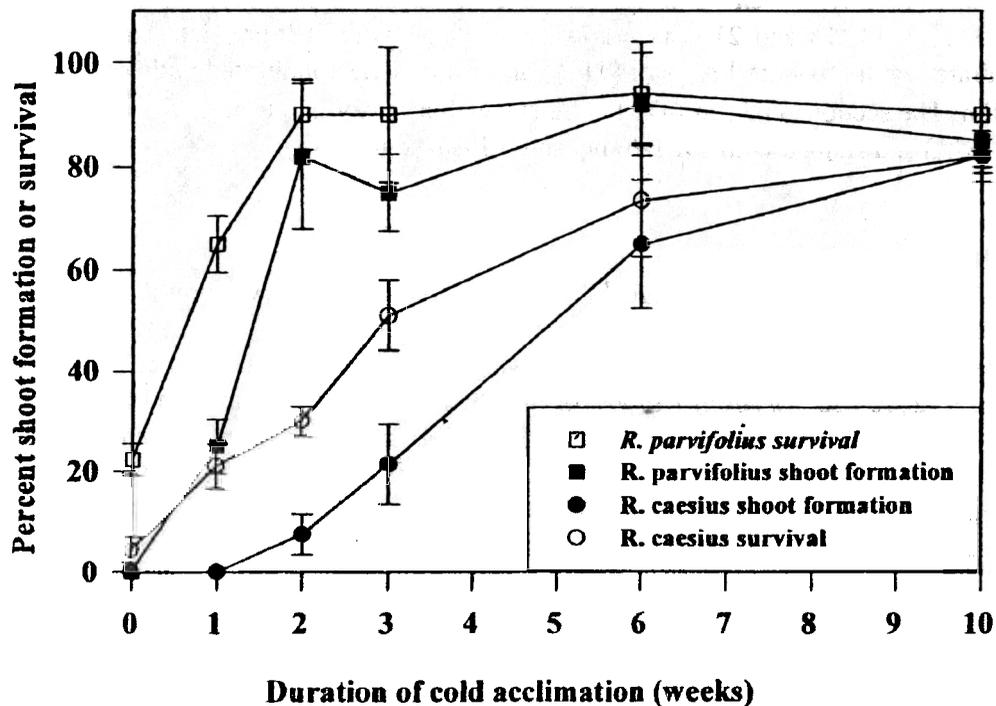


Fig. 1. The effect of 0 - 10 weeks cold acclimation on survival and shoot formation following LN exposure of *R. parvifolius* and *R. caesius* shoot tips recovered on NCGR-RUB medium without IBA.

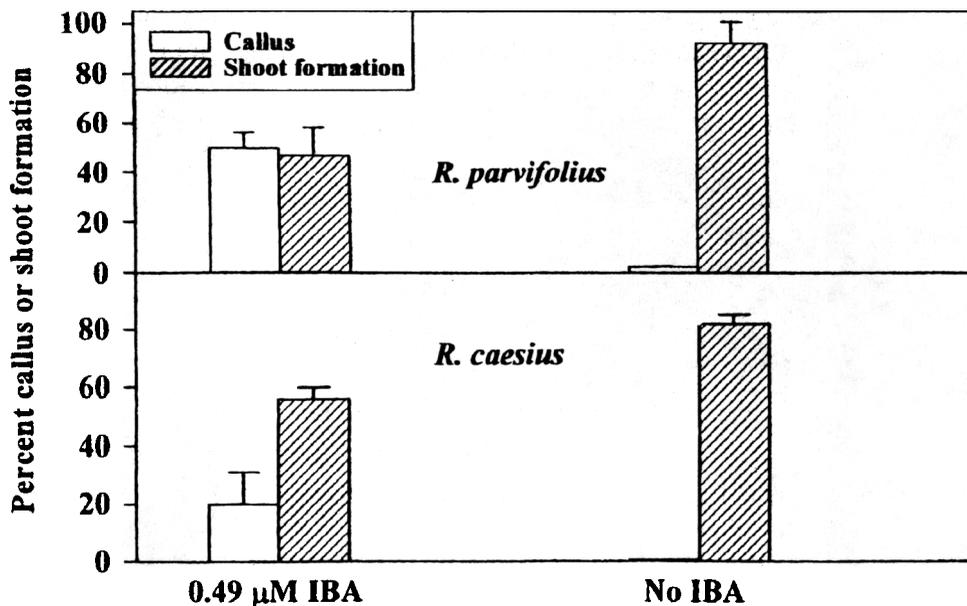


Fig. 2. The effect of IBA in NCGR-RUB recovery medium on callus and shoot formation of 10 weeks cold acclimated *R. parvifolius* and *R. caesius* shoot tips following LN exposure.

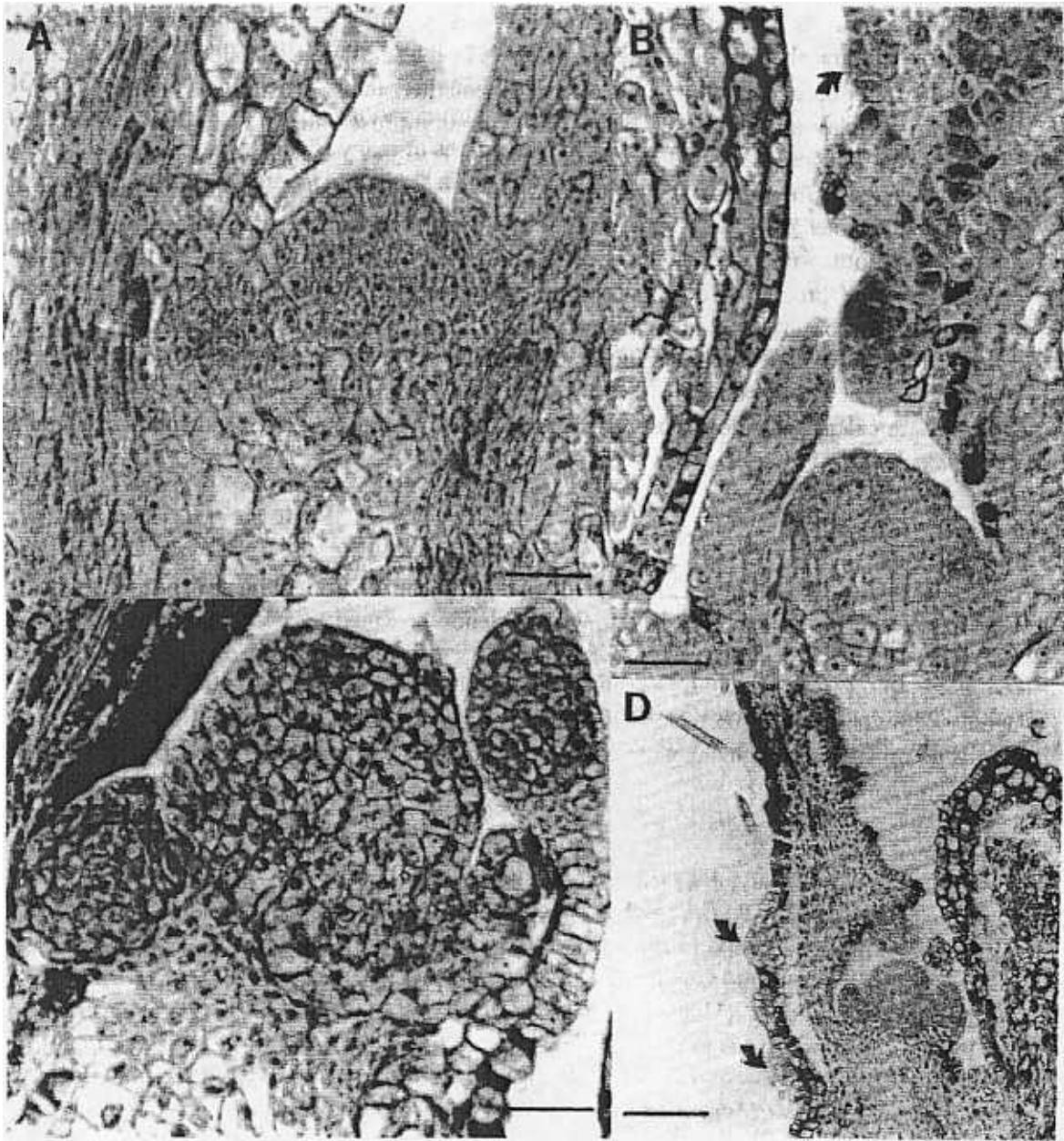


Figure 3. Longitudinal sections of cryopreserved meristems of *R. parvifolius* on NCGR-RUB medium without IBA. A). The non-frozen control meristem, bar = 40 μ M. B). The cryopreserved meristems continued to grow and developed directly from the meristematic dome (3-day reculture), some callus formed on leaf primordia (arrow), bar = 40 μ M. C). Axillary buds survived and grew along with the apical meristems (5-day reculture), bar = 40 μ M. D). Most of the callus (arrow) was produced from the margins of the leaf primordia (5-day reculture), bar = 160 μ M.

Histology of the meristems before and after cryopreservation

R. parvifolius shoot tips consisted of an apical dome and two or three leaf primordia (Fig. 3A). Following freezing/thawing, meristems continued to grow and develop directly when recovered on medium without IBA. The structure of frozen shoot tips was similar to that of the non-frozen controls (Fig. 3B). The axillary buds survived and grew along with the apical meristems (Fig. 3C). For some apices, growth was delayed for several days compared to the controls. Recovered shoot tips originated from the direct regrowth of either apical meristems or lateral meristematic domes. This

regrowth pathway is the most likely to ensure genetic stability of the plant material. Some callus formed on the apical meristems, lateral meristems or leaf primordia following damage (Fig. 3D). Callus and other tissues did not produce adventitious meristems. Histology is often used to evaluate the genetic stability of regenerated plants following cryopreservation (5, 8, 15). Haskins and Kartha (5) found that for pea meristems regrowth generally resumes at many sites and most of the surviving cells are located in the primordia leaf tissues. Other surviving cells are located laterally on the apical meristem. Ryyanen (15) found that callus forms from silver birch apices following cryopreservation, and some shoots are regenerated from this callus. Our observations demonstrated that this cryopreservation procedure and recovery medium produced *Rubus* shoots only from pre-existing meristems. Although some callus formed during recovery, no shoots regenerated from the callus. Thus, all new *Rubus* shoots originated from the pre-existing apical or lateral meristems. The observation that shoots develop directly from pre-existing apical and axillary meristems without intermediate callus formation provides evidence that cryopreservation maintains the genetic stability of *Rubus* plants.

Conclusions

Frozen-thawed *Rubus* shoot tips directly produced shoots from either apical meristems or axillary buds but not from callus. The histological evidence provided by this study indicated the safety of using meristems for *Rubus* germplasm storage. Shoot formation was greatly improved by increasing the duration of CA before freezing. Shoot formation was also increased and callus formation decreased by eliminating IBA from the shoot recovery medium. A protocol for cryopreservation of *Rubus* germplasm should include an extended CA period and recovery on medium without auxin.

References

1. Anderson, WC (1980) *Acta Horticulturae*, **112**, 13-20.
2. Chang, Y and Reed, BM (1997) *In Vitro Cellular & Developmental Biology*, **33**, 50A.
3. Chen, THH, Burke, MJ and Gusta, LV (1995) In "Biological Ice Nucleation and Its Application" Lee, Warren and Gusta (ed). APS Press, pp 115-135.
4. Finkle, BK and Ulrich, JM (1987) *Plant Physiology*, **63**, 598-604.
5. Haskins, RH and Kartha, KK (1980) *Canadian Journal of Botany*, **58**, 833-840.
6. Irvine, JE, Fitch, M and Moore, PH (1983) *Plant Cell Tissue and Organ Culture*, **2**, 141-149.
7. Johansen, DA (1940) *Plant Microtechnique*. 1st ed. McGraw-Hill, New York and London.
8. Mari, S, Engelmann, F, Chabrillange, N, Huet, C and Michaux - Ferrière, N (1995) *Cryo-Letters*, **16**, 289-298.
9. Murashige, T and Skoog, F (1962) *Physiologia Plantarum*, **15**, 473-497.
10. Razdan, MK and Cocking, EC (1997) In "Conservation of Plant Genetic Resources In Vitro" Razdan MK and Cocking, EC (ed). Oxford and IBH Publishing Co. Pvt. Ltd., India and M/S Science Publishers, Inc. USA, pp 3-26.
11. Reed, BM and Lagerstedt, HB (1987) *HortScience*, **22**, 302-303.
12. Reed, BM (1988) *Cryo-Letters*, **9**, 166-171.
13. Reed, BM (1993) *Cryobiology*, **30**, 179-184.
14. Reed, BM and Chang, Y (1997) In "Conservation of Plant Genetic Resources In Vitro" Razdan MK and Cocking, EC (ed). Oxford and IBH Publishing Co. Pvt. Ltd., India and M/S Science Publishers, Inc. USA, pp 67-105.
15. Ryyanen, L (1998) *Cryobiology*, **36**, 32-39.
16. Towill, LE (1988) *HortScience*, **23**, 839-941.
17. Withers, LA, Benson, EE and Martin, M (1991) *Cryo-Letters*, **9**, 114-119.