

COLD ACCLIMATION AS A METHOD TO IMPROVE SURVIVAL
OF CRYOPRESERVED RUBUS MERISTEMS

Barbara M. Reed

Department of Horticulture, Oregon State University and
National Clonal Germplasm Repository
33447 Peoria Road
Corvallis, Oregon 97333 USA

SUMMARY

Tissue culture plantlets of Rubus spectabilis Pursh., R. idaeus L. 'Heritage' and Rubus spp. 'Logan Thornless' and 'Merton Thornless' were cold acclimated (25°/-1°C day/night temperatures) for one week preceding excision of apical meristems for cryopreservation. Meristems were slowly frozen to -40°C followed by immersion in liquid nitrogen for 1 hour. Survival in non-acclimated plants ranged from 18-41% with 51-67% survival in acclimated meristems. Acclimated R. spectabilis and 'Merton Thornless' retained their high survival rates following 30, 60 and 180 days of storage in liquid nitrogen.

KEY WORDS

Tissue culture, liquid nitrogen, cold hardening, Rubus, cryopreservation.

INTRODUCTION

Cold acclimation of plants and plant tissues has been studied extensively (10,13). Wide variation exists in the requirements for cold adaption such that some plants require only cold temperatures to initiate

acclimation while others must be subjected to both photoperiodic and temperature modification (16). Studies of cold acclimation of tissue cultures have been attempted with both positive and negative results (4). Callus cultures of Chrysanthemum morifolium cultivars were acclimated to survive to -16°C (2) and acclimated Populus callus tissues have also survived freezing (14). Acclimated Dianthus meristem cultures frozen to -196°C produced a survival rate double that of non-acclimated meristems (15); however, Cornus stolenifera tissue cultures failed to acclimate (6). In vitro shoot cultures of apple and saskatoon (Amelanchier alnifolia) acclimated at 2°C for 10 weeks survived exposures $4-8^{\circ}\text{C}$ colder than non-acclimated cultures (3). Shoot tip cultures of 'Jonathan' apples survived freezing to liquid nitrogen temperatures following cold acclimation of 6 weeks at 4°C in the dark. All recovery took the form of callus tissue (9). Cold conditioning of pea and strawberry in vitro cultures prior to cryopreservation did not improve their survival rates (7, 8).

The purpose of this study was to investigate cold acclimation as a method of increasing survival rates of cryopreserved Rubus species and cultivars.

MATERIALS AND METHODS

Apical meristems were obtained from tissue-cultured plantlets of Rubus spectabilis Pursh, R. idaeus L. 'Heritage' and Rubus spp. 'Logan Thornless' and 'Merton Thornless'. Stock cultures of the four accessions were maintained on Murashige and Skoog (MS) medium (11) containing adenine sulfate ($43\ \mu\text{M}$), N-(phenylmethyl)-1H-purine-6-amine (BA) ($10\ \mu\text{M}$), 1H-indole-3-butanoic acid (IBA) ($2\ \mu\text{M}$), 3% sucrose and 0.6% agar (pH 5.7). Growth room conditions were maintained at 25°C with a 16/8 hr (light/dark) photoperiod.

Cold acclimation conditions as adapted from Angelo (1) were 8/16 hr (light/dark) photoperiod with 25°C days and -1°C nights for 1 week. The cryoprotectant used was PGD, a combination of 10% polyethylene glycol, 10% glucose and 10% dimethylsulfoxide (Me_2SO) in water (5). PGD was filter sterilized ($0.45\ \mu\text{m}$) before use. Four or five replicates of ten meristems ($0.5-1.0\ \text{mm}$) of each were used per experiment. Excised meristems from cold acclimated or non-acclimated plants were grown for 48 hr on MS medium with the addition of 5% Me_2SO . Acclimated meristems were returned to acclimating conditions while non-acclimated apices were maintained under growth room conditions. Shoot tips were transferred to 0.25 ml chilled liquid medium in

1.2 ml cryotubes following incubation on the Me₂SO supplemented medium. Treatment consisted of the addition of chilled PGD (-1°C) drop by drop up to 1.2 ml over a 30 minute period. The meristems were held in the PGD solution for 1/2 hr at -1°C before freezing. Samples were frozen to -40°C in 1 ml PGD at a rate of .8°/min in a programmable controlled-temperature freezing chamber. Freezing rates were monitored in both a sample vial and the chamber. Following controlled freezing to -40°C, samples were immersed in liquid N₂ at -196°C, held there for one hour, then thawed quickly by warming in a 40°C water bath for 60 sec. followed by a 23°C water bath. After thawing, samples were diluted with liquid medium, drained on sterile filter paper and transferred to solid MS medium. Regrowth was under previously described growth room conditions. Control meristems were subjected to all treatments (including cryoprotection) but not frozen. Meristems frozen for longer than one hour were transferred to an LD40 liquid nitrogen refrigerator and stored in the liquid phase.

RESULTS AND DISCUSSION

Meristems of non-acclimated plants frozen for one hour produced callus or organized meristem growth in 18 to 41% of plants tested (Table 1). This is similar to our earlier study which produced growth rates of 14 to 40% (12). In contrast, acclimated plant recovery rates were 51 to 67%. This increase is similar to that noted in Dianthus (15) where cold acclimation doubled the survival rates and raised the differentiation rate from 2% to near 60%. In this study cold acclimation greatly increased survival of three of the four Rubus accessions frozen in liquid N₂. 'Jonathan' apple meristem cultures frozen in liquid nitrogen produced increased survival following acclimation. However, only callus was produced in both acclimated and non-acclimated meristems (9).

In this study both callus and normal growth were observed on control and experimental treatments of acclimated and non-acclimated plants. Unfrozen control meristems maintained high survival rates.

Storage of meristems for extended periods is also of interest. Survival rates of small numbers of meristems of two Rubus stored for various lengths of time are listed in Table 2. Samples were frozen at the same time then removed from the storage container at the designated intervals. Although variability is present in these small samples, regrowth is shown to be possible after 6 months of storage in liquid nitrogen. Recovery of

Table Growth of cold acclimated and non-acclimated Rubus meristems frozen for one hour at -196°C following pretreatment.

| <u>Rubus Accession</u> | Percent Viability ^z | | | |
|------------------------|----------------------------------|--------------------------------------|-------------------------------------|------------------------------------|
| | Acclimated | | Non-acclimated | |
| | Control | Frozen | Control | Frozen |
| <u>R. spectabilis</u> | 100 ^x 5) ^y | 66±17 ^x (14) ^y | 88±4 ^x (18) ^y | 88±7 ^x (0) ^y |
| Heritage | 100 20) | 67±10 (20) | 95±5 (6) | 28±5 17) |
| Logan Thornless | 100 20) | 67±9 (47) | 73±13 33) | 18±9 (25) |
| Merton Thornless | 96±4 29) | 51±8 13) | 85±5 37) | 41±5 (23) |

x mean ± S.E.M.

y Percent callus

Viability noted as percentage of meristems showing regrowth 30 days after thawing (n=50)

Table 2. Regrowth of s. ng acclimated Rubu :ems following storage in liquid nitrogen for to 180 days

| <u>Rubus Accession</u> | Percent Viability ^c | | | |
|------------------------|--------------------------------|---------|---------|----------|
| | Day | 30 days | 60 days | 180 days |
| <u>R. spectabilis</u> | 00 (20 ^y | 60 20) | 100 (0) | 100 (0) |
| Merton Thornless | 60 40) | 40 0) | 40 (40 | 20 (0) |

y Percent callus

Viability noted as percentage of meristems showing regrowth 30 days after thawing (n=5)

plants with normal apical growth is high for R. spectabilis and variable for 'Merton Thornless'.

Cold acclimation of in vitro cultures as a method to improve recovery from cryopreservation appears to be promising for those plants with the potential for hardening. Cryopreservation successes with Dianthus (15), apple (9) and Rubus (12) indicate that with proper acclimation techniques many plants may be candidates for storage in liquid nitrogen. Close attention should be paid to the duration of acclimation as well as photoperiod to allow for maximum hardening (16). As with cold acclimation in whole plants, the species and cultivar differences within each genus and species will need to be taken into account.

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REFERENCES

- E. Angelo, Minn. Agr. Exp. Sta. Tech. Bul. 135:7-19 (1939).
2. L.J. Bannier and P.S. Steponkus, J. Amer. Soc. Hort. Sci. 101(4):409-412 (1976).
3. K.L. Caswell, N.J. Tyler and C. Stushnoff, HortScience 21(5):1207-1209 (1986).
4. T.H.H. Chen and L.V. Gusta in: Cell culture and somatic cell genetics of plants, Vol. 3, Academic Press, NY, pp 527-535 (1986).
5. B.J. Finkle and J.M. Ulrich, Plant Physiol. 63:598-604 (1979).
6. L.H. Fuchigami and C.J. Weiser, Plant Physiol. 46:845-846 (1970).
7. K.K. Kartha, N.L. Leung and O.L. Gamborg, Plant Science Letters 15:7-15 (1979).
8. K.K. Kartha, N.L. Leung and K. Pahl, J. Amer. Soc. Hort. Sci. 105(4):481-484 (1980).
9. C.C. Kuo and R.D. Lineberger, HortScience 20(4):764-767 (1985).
10. J. Levitt, Responses of plants to environmental stresses. Academic Press, NY (1972).
- T. Murashige and F. Skoog. Physiol. Plant. 15:473-497 (1962).

12. B.M. Reed and H.B. Lagerstedt, HortScience 22(2):302-303 (1987).
13. A. Sakai and W. Larcher, Frost survival of plants, responses and adaptation to freezing stress. Springer - Verlag, Berlin (1987).
14. A. Sakai and Y. Sugawara, Plant and Cell Physiol. 14:1201-1204 (1973).
15. M. Seibert and P.J. Wetherbee, Plant Physiol. 59: 1043-1046 (1977).
16. C.J. Weiser, HortScience 5:403-409 (1970).