

## Responses to ABA and Cold Acclimation Are Genotype Dependent for Cryopreserved Blackberry and Raspberry Meristems<sup>1</sup>

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The meristems of cold-acclimated cultivars and species of *Rubus* (blackberry and raspberry) can be successfully cryopreserved through optimization of cryoprotectants, cooling rates, and cold acclimation treatments. Genotypes that do not cold acclimate are difficult to cryopreserve and new methods are needed for this group of plants. The effects of abscisic acid (ABA) (50  $\mu$ M) and 7-day cold acclimation (CA) treatments on the survival of cryopreserved apical meristems was determined for five *Rubus* genotypes (Blackberries: *Rubus cissoides* Cunn. and *Rubus* hybrid cvs. Hillemeier and Silvan, and Raspberries: *R. parvifolius* L., and *R. parviflorus* Nutt.). ABA alone did not significantly improve the survival of any of the genotypes tested. The combination of ABA and CA significantly improved the survival of *R. cissoides*, but neither was effective alone. Cold acclimation with or without ABA significantly improved the survival of meristems of 'Hillemeier,' 'Silvan,' and *R. parvifolius* L. Survival of *R. parviflorus* Nutt. meristems was not significantly affected by any of the treatments. In contrast to cytokinin effects in other culture systems, the cytokinin benzyladenine did not significantly affect survival of 'Hillemeier' and showed no interaction with ABA or CA. Murashige and Skoog-based recovery medium produced higher survival rates than did Anderson's medium for the nine genotypes tested. Overall, genotype played a large role in the survival of cryopreserved *Rubus* meristems. In this system ABA does not substitute for CA, but in some genotypes may interact with CA to increase survival through a synergistic effect. © 1993 Academic Press, Inc.

Meristems of many *Rubus* genotypes can be cryopreserved using optimum cryoprotectants, freezing rates, and cold acclimation treatments (11). Improved cryopreservation methods are needed for many tropical plants and those that do not respond to cold acclimation. Cold acclimation can be induced by abscisic acid (ABA) in cell cultures of many plant species (2, 8, 10). Maximum hardiness is often obtained within 7 days of ABA treatment and is indicated by increases in starch grains, lipid bodies, sugar content, and dry weight similar to that produced by cold acclimation (16).

Cytokinins sometimes counteract or alter the normal action of ABA (3, 7). For exam-

ple, freezing tolerance induced by ABA can be reduced or totally inhibited by the presence of kinetin in the growth medium, but this effect varies with genotype (10). Present methods of *Rubus* cryopreservation use a growth medium containing the cytokinin benzyladenine (BA) and have moderate survival rates (11). The interactive effect of cytokinins, cold acclimation, and ABA in the *Rubus* cryopreservation system is unknown.

Survival and the rate of shoot development from meristems following cryopreservation are dependent on the culture medium used in the recovery period (19). Variations among genotypes indicate that a single recovery medium is not always suitable for all members of a genus (18, 19). The medium on which a plant is grown also influences its vigor in culture and may affect the tolerance of meristems to cryopreservation (12).

This study compared ABA and cold ac-

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climation pretreatments for improving the survival of cryopreserved *Rubus* meristems from five genotypes; examined the interaction between ABA treatment and the cytokinin BA in the growth medium; and compared the effect of two commonly used recovery media on meristem survival rates for nine genotypes.

#### MATERIALS AND METHODS

**Media.** Micropropagated shoots of *Rubus* accessions (species and cultivars) were multiplied in Magenta GA7 boxes (Magenta Corp., Chicago, IL) on pH 5.7 base medium: Murashige and Skoog (MS) (9) salts and vitamins with 3% sucrose, 1 mg N<sup>6</sup>-BA, 0.05 mg indole-3-butyric acid, 0.01 mg GA<sub>3</sub> (Sigma, St. Louis, MO) and 3.5 g BiTek agar (Difco, Detroit, MI), and 1.45 g Gelrite (Kelco, San Diego, CA) per liter. After autoclaving, ABA ( $\pm$ cis/trans isomer, Sigma) (50  $\mu$ M) in dimethyl sulfoxide (DMSO, Sigma) was added to the medium, and an equivalent amount of DMSO was added to the non-ABA treatments (15). The final concentration of DMSO in the medium was 0.1%. For recovery, the same MS formulation or Anderson's medium (1) with the same growth regulator concentrations and 3 g agar and 1.25 g Gelrite were used.

**Pretreatment.** *In vitro* plants were cold-acclimated (CA) for 1 week in a growth chamber with 22°C 8 h days (20  $\mu$ E M<sup>-2</sup> s<sup>-1</sup>) and -1°C 16 h nights (11). Regular growth room conditions were maintained at 25°C with a 16/8 h (light/dark) photoperiod (30  $\mu$ E M<sup>-2</sup> s<sup>-1</sup>). Apical meristems were dissected from *in vitro* plants pretreated for 1 week with the appropriate medium and environmental condition. Dissected meristems (0.8 mm) of each treatment were grown for 48 h on basal medium with 3.5 g agar and 1.75 g Gelrite per liter and with 5% DMSO under the same growth regulator and environmental conditions as the parent plants. ABA concentrations used were determined in preliminary experiments.

**Cryopreservation.** Following pretreat-

ment, meristems were transferred to 0.25 ml liquid medium in 1.2-ml plastic cryotubes on ice. The cryoprotectant PGD (4), a mixture of 10% each of polyethylene glycol (MW 8000, Sigma, St. Louis MO), glucose, and DMSO in water, was added dropwise up to 1.2 ml over 30 min. A 30-min equilibration period on ice was followed by removal of excess cryoprotectant down to 1 ml. Samples were frozen to -35 at 0.8°C/min in a programmable controlled-temperature cooling chamber (Cryomed, New Baltimore, MI) and then immersed in liquid nitrogen (LN) for 1 h. Vials were thawed for one min. in 45°C water, then cooled in 23°C water until the ice was completely melted. The cryoprotectant was drained from the vials and replaced with liquid MS medium. Meristems were drained on sterile filter paper and plated on recovery medium for regrowth. Ten or twenty treated meristems and five controls (not frozen) were tested for each replicate of each accession with three or four replicates per experiment.

**Recovery.** Meristems were plated on 2 ml medium in sterile 24-well plates (Costar, Cambridge, MA) for regrowth under standard growth room conditions. Total survival (shoots and callus) was evaluated at 1 month for the formation of shoots or callus. Shoot survival included only those meristems exhibiting organized shoot growth 1 month after thawing. The basal MS medium was used in all except one recovery experiment in which both MS and Anderson's medium were used.

**Histology.** For histological study, meristems were fixed in pH 6.8 phosphate-buffered (0.02 M) 3% glutaraldehyde for 1-3 days at 4°C, dehydrated with an acetone:water series, infiltrated and embedded in Spurr's low-viscosity epon mixture (Polysciences, Warrington, PA). Sections 10-15  $\mu$ m thick were stained with toluidine blue or methylene blue.

**Data analysis.** Data were analyzed with MSTAT software (Michigan State Univer-

sity, East Lansing, MI) using factorial analysis and mean separation with Duncan's multiple range test.

## RESULTS AND DISCUSSION

### *Effects of Cold Acclimation and ABA*

Cold acclimation significantly improved survival of most *Rubus* genotypes either with or without ABA; however, a 7-day  $5 \times 10^{-5}$  M ABA pretreatment produced no significant increases in total or meristem survival for plants grown under warm conditions (Table 1). Factorial analysis showed that the interaction of pretreatment and genotype was significant ( $P \leq 0.01$ ) for total survival percentages. The effect of pretreatment was also significant ( $P \leq 0.01$ ) for the percentage shoot survival. The addition of 50  $\mu$ M ABA failed to produce significant increases in total survival for any of the warm-grown or for three of five cold-acclimated plants. 'Hillemeier' and *R. cissoïdes* showed significantly greater total and shoot survival with CA/+ABA than

with any other treatment. This appears to be a synergistic effect for *R. cissoïdes* since it was unaffected by CA alone or by ABA under warm conditions but total survival and shoot survival tripled following pretreatment with the combination of ABA and CA. CA with or without ABA increased survival over warm-grown plants in four genotypes and for CA alone with *R. parviflorus* (Table 1).

The response of *Rubus* meristems differs markedly from the effect of ABA on cell cultures. The induction of cold acclimation by ABA treatment has been observed for cell cultures of winter wheat (ABA  $7.5 \times 10^{-5}$  M) (2), birdsfoot trefoil (ABA  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  M) (8), and alfalfa (ABA  $5 \times 10^{-5}$  M) (10). Bromegrass cells treated with ABA at warm or cold temperatures developed more freezing resistance than cells cultured at 3°C without ABA (14). Ishikawa *et al.* (6) found 25 to 30°C to be the optimum temperature range for  $7.5 \times 10^{-5}$  M ABA-induced acclimation of *Bromus inermis* suspension cultures. In the present study the survival of *Rubus* plantlets grown at 25°C

TABLE 1  
Percentage Survival after Cryopreservation of *Rubus* Meristems Grown with 0.44  $\mu$ M Benzyladenine (BA) and Pretreatments of 50  $\mu$ M Abscisic Acid (ABA) and/or 1 Week Cold Acclimation (CA), 4 Weeks After Thawing

Genotype	Percentage survival following thawing*			
	Warm/- ABA	Warm/+ ABA	CA/- ABA	CA/+ ABA
Hillemeier	16.9 $\pm$ 1.1 e (16.9 $\pm$ 1.1) z	27.5 $\pm$ 4.3 e (15.0 $\pm$ 2.9) z	51.0 $\pm$ 9.1 cd (21.3 $\pm$ 1.3) yz	77.5 $\pm$ 7.2 ab (52.5 $\pm$ 1.4) wx
Silvan	26.1 $\pm$ 9.3 e (10.5 $\pm$ 6.1) z	14.7 $\pm$ 6.0 e (7.5 $\pm$ 4.3) z	74.5 $\pm$ 6.1 ab (26.0 $\pm$ 8.1) yz	47.5 $\pm$ 10.1 d (12.5 $\pm$ 4.3) z
<i>R. parvifolius</i>	16.9 $\pm$ 4.0 e (16.9 $\pm$ 4.0) z	15.0 $\pm$ 1.0 e (6.7 $\pm$ 3.9) z	84.2 $\pm$ 6.3 a (36.7 $\pm$ 13.5) xy	65.2 $\pm$ 0.9 bc (46.1 $\pm$ 0.4) x
<i>R. parviflorus</i>	20.8 $\pm$ 6.2 e (15.8 $\pm$ 9.1) z	10.0 $\pm$ 2.9 e (10.0 $\pm$ 2.9) z	45.9 $\pm$ 5.2 d (45.9 $\pm$ 5.2) x	16.1 $\pm$ 3.5 e (16.1 $\pm$ 3.5) z
<i>R. cissoïdes</i>	24.3 $\pm$ 6.2 e (19.5 $\pm$ 6.1) yz	24.4 $\pm$ 0.3 e (21.9 $\pm$ 1.1) yz	28.29 $\pm$ 1.9 e (23.1 $\pm$ 1.1) yz	76.7 $\pm$ 4.8 ab (66.2 $\pm$ 10.9) w

Note. Survival includes both shoot and callus growth. In parentheses, meristem survival includes only shoots which are green and growing 6 weeks after thawing ( $N = 4$  experiments of 10 meristems each). Survival of unfrozen controls was 100%. Mean separation by Duncan's multiple range test ( $P \leq 0.05$ ). Means followed by the same letter are not significantly different. Means of total survival (abcde) and shoot survival (wxyz) are considered separately.

\* Mean  $\pm$  standard error.

was not improved by 7-day ABA pretreatment before cryopreservation at the same concentration. This lack of acclimation may be due to inhibition of transport to apical buds in stressed plants (5) or inherent differences in response to ABA by diverse genera (10). Further studies of this response are necessary to determine if this is truly a CA/ABA synergism as well as the mode of action.

#### Interactions Between BA and ABA

Results for blackberry cv. Hillemeier showed that BA does not inhibit the survival of cryopreserved meristems. Only the pretreatment temperature was significant in the survival of 'Hillemeier' meristems following cryopreservation in LN. BA did not significantly change the survival percentage with or without either cold acclimation or ABA, and interactions were not apparent in either case. Total survival (shoots and callus) following CA (mean 71%) was significantly greater than that for warm-grown meristems (mean 14.8%) in all cases. Shoot survival was also significantly greater for CA plants of all pretreatments, but the effects of BA and ABA were not significant for meristems within a temperature treatment (Table 2). In contrast to these results, freezing tolerance of alfalfa cell cultures requires the deletion of kinetin from the growth medium and the use of both low-temperature and ABA treatments (10).

#### Recovery Medium Effects

Recovery medium experiments showed significant effects for medium type ( $P \leq 0.001$ ) and genotype ( $P \leq 0.01$ ), but interactions were not significant. Overall, meristems of the nine *Rubus* genotypes had significantly higher survival rates (mean 42.2%) when grown on MS medium than on Anderson's medium (mean 19.3%) ( $P \leq 0.001$ ). Genotype effects varied considerably from blackberry cv. Hillemeier with good survival on both media to *R. hirsutus* with low survival rates on both (Table 3).

TABLE 2

The Influence of Cold Acclimation, Benzyladenine (BA 0.44  $\mu M$ ) and Abscisic Acid (ABA 50  $\mu M$ ) Pretreatments on the Percentage Shoot Survival of *Rubus* Hybrid Cv. Hillemeier Meristems Frozen in Liquid Nitrogen, Thawed, and Grown for 4 Weeks

BA/ABA treatment	Percentage survival following thawing*	
	Grown at 25°C	Cold acclimated
- BA/- ABA	5.0 $\pm$ 3.5 b	42.0 $\pm$ 12.1 a
- BA/+ ABA	13.8 $\pm$ 4.3 b	52.0 $\pm$ 11.3 a
+ BA/- ABA	7.2 $\pm$ 4.1 b	40.5 $\pm$ 11.6 a
+ BA/+ ABA	7.3 $\pm$ 3.0 b	43.8 $\pm$ 13.5 a

Note. Survival reported as meristems with shoot growth at 4 weeks after thawing ( $N = 4$  experiments of 20 meristems each). Survival of unfrozen controls was 100%. Mean separation by Duncan's multiple range test ( $P \leq 0.05$ ). Means followed by the same letter are not significantly different.

\* Mean  $\pm$  standard error.

Differences in survival were significant for five of the nine individual genotypes ( $P \leq 0.05$ ) and in all cases survival was higher on MS medium than on Anderson's medium.

TABLE 3

Survival following Cryopreservation of Meristems of *Rubus* Species and Cultivars Pretreated with 1-Week Cold Acclimation and Grown for 4 Weeks on Two Types of Culture Medium

Genotype	Percentage survival*	
	Anderson	MS
Hillemeier		56.2 $\pm$ 9.8 a
Kotata		62.1 $\pm$ 3.1 a
Mandarin		33.3 $\pm$ 15.6 a
ORUS 1362		33.3 $\pm$ 25.4 a
<i>R. grabowskii</i> Weihe ex. Gunther et al.	30.0 $\pm$ 17.3 a	39.7 $\pm$ 23.3 a
<i>R. hirsutus</i> Thunb.	3.3 $\pm$ 1.7 a	10.7 $\pm$ 3.2 a
<i>R. leucodermis</i> Doug. ex Tor. & Gray	1.7 $\pm$ 1.7 b	28.3 $\pm$ 6.0 a
<i>R. ulmifolius</i> f. <i>bellidiflorus</i> Schott	11.8 $\pm$ 4.4 b	51.1 $\pm$ 6.7 a
Silvan	24.7 $\pm$ 14.4 b	65.4 $\pm$ 4.8 a
Mean of Medium Types	19.3 $\pm$ 3.7 b	42.2 $\pm$ 5.0 a

Note. Survival as meristems at 4 weeks after thawing ( $N = 3$  experiments of 20 meristems each). Survival of unfrozen controls was 100%. Means in rows followed by the same letter are not significantly different [Duncan's multiple range test ( $P \leq 0.05$ )].

\* Mean  $\pm$  standard error.

*Rubus* is a diverse genus and the response of species and cultivars to standard media formulations varies considerably (13). This is consistent for the genotypes tested in this study. Most grow better on MS medium although some will also grow moderately well on Anderson's medium, which contains lower nitrogen and micronutrient concentrations than MS and includes the cytokinin adenine sulfate as a regular component. We have not tested the effect of adenine sulfate in pretreatment medium on survival of cryopreserved meristems. Different survival rates among genotypes have in some cases been shown to be due to the recovery medium (17–19). The medium on which a plant is grown influences its vigor in culture and a suboptimum medium may contribute to decreased survival following cryopreservation (12).

#### Histology

Histological examination of frozen and control meristems showed normal meristematic growth on those that formed shoots. Any callus formed by recovering meristems remained as callus and did not regenerate shoots, thus preserving the clonal integrity of the sample (data not shown). Callus production in cryopreserved *Rubus* may result from wounding during excision or from freeze-induced injury. In other studies, the amount of callus production appears to be genetically controlled but may also be due to growth regulators present in the medium (18). Genetic control seems likely with *Rubus* since the amount of callus production was high in all treatments for some genotypes and low for others. Improved recovery of shoots from cryopreserved meristems through media improvements has been proposed for several genera (18, 19).

#### CONCLUSIONS

The effect of genotype played a large role in the survival of *Rubus* meristems following cryopreservation. Recovery media tests showed differential genotype response;

however, meristems grown on MS-based medium had higher survival in general than those on Anderson's medium. In contrast to cytokinin effects in other culture systems, BA did not significantly affect survival and produced no interaction with ABA or CA. Cold acclimation significantly improved survival of four genotypes and survival of *R. cissoides* was tripled by combined ABA and CA pretreatments. It appears that in this system, ABA does not substitute for CA pretreatments but in some genotypes it may interact with CA to increase survival through a synergistic effect.

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