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Freeze Preservation of Apical Meristems of *Rubus* in Liquid Nitrogen

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Abstract. Shoot tip explants from tissue culture plantlets of five *Rubus* accessions (*Rubus leucodermis* Torr. & Gray, *R. spectabilis* Pursh, *R. idaeus* L. 'Heritage', *Rubus* spp. 'Logan Thornless' and 'Merton Thornless') were frozen slowly (0.8°C/min) to -40° and then rapidly to -196° in the presence of cryoprotectants. Following rapid thawing, regrowth as organized apical growth or as callus occurred on agar media. A combination of polyethylene glycol (PEG), glucose, and dimethylsulfoxide (PGD) was the most successful cryoprotectant.

The need to preserve plant germplasm for future generations is well-recognized (4). The National Clonal Germplasm Repository in Corvallis, Ore. is charged with the collection and maintenance of potentially useful genetic material of several fruit and nut crops, including *Rubus* species and cultivars. Presently, more than 700 different *Rubus* accessions are maintained at the repository. A large

amount of time and energy is required to maintain these plants, and the possibility of loss due to accident or disease exists. The development of methods to store germplasm collections in liquid N₂ could provide savings in labor and space and assure a reliable backup to present methods of storage. Currently, reports indicate that apical meristems

of a dozen species from ten genera have been frozen successfully using liquid N₂ (2, 3, 6-9, 11-15).

Initial studies with cryoprotectants showed the effects of single substances or of two protectant chemicals combined (16). Currently, many studies are using prefreezing treatments with mixtures of several cryoprotectants (5). Growth of meristems on agar media and in cryoprotectant solutions have improved survival of frozen cells and meristems. The addition of 5% dimethylsulfoxide (Me₂SO) to agar media and subsequent growth for 48 hr prior to freezing was shown to enhance survival of pea and strawberry meristems (9, 10).

Both established and novel cryopreservation methods need to be applied in order to develop cryopreservation systems for plants that have not been frozen successfully in liquid N₂. This study describes the methods used to freeze meristems successfully of several species and cultivars from the genus *Rubus*.

Apical meristems were obtained from tissue-cultured plantlets of *Rubus leucodermis* Torr. & Gray, *R. spectabilis* Pursh, *Rubus idaeus* L. 'Heritage', and *Rubus* spp. 'Logan Thornless' and 'Merton Thornless'. Stock

Table 1. Viability of control cultured shoot tips grown on 5% Me₂SO agar for 48 hr and incubated in various cryoprotectant solutions at room temperature for 1 hr.

<i>Rubus</i> accession	Viability (%) ²			
	Me ₂ SO (5%)	Me ₂ SO (10%)	PGD	PTD

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Table 2. Viability of cultured shoot tips frozen to -196°C following 48 hr of growth on 5% Me_2SO agar and incubation in various cryoprotectant solutions for 1 hr at room temperature. Samples were frozen to -40° , plunged into liquid N_2 for 1 hr, then thawed in 40° water.

Rubus accession	Viability (%) ^z			
	Me_2SO (5%)	Me_2SO (10%)	PGD	PTD
			40 ± 1	19 ± 1
			63 ± 12	16 ± 1
			14 ± 4	12 ± 1
			25	9 ± 1
			25 ± 12	19 ± 3

^zViability noted as percentage of shoot tips showing regrowth 1 month after thawing (two cryotubes with five shoot tips each per treatment, three trials; n = 30).

^yMean ± SE.

Table 3. Normal regrowth of surviving shoot tips following freezing to -196°C for 1 hr.

Rubus accession	Percent normal growth			
	Me_2SO (5%)	Me_2SO (10%)	PGD	PTD
	50 (4) ^z	100 (1)	75 (12)	66 (6)
	--- (0)	33 (3)	79 (19)	40 (5)
	100 (1)	50 (2)	50 (4)	75 (4)
	--- (0)	--- (0)	75 (8)	100 (3)
	50 (2)	100 (1)	62 (8)	100 (6)

^zTotal number of stem tips showing growth as normal meristems 1 month after thawing in parentheses.

cultures of the five accessions were maintained on Anderson's medium (1) containing adenine sulfate (43 μM), *N*-(phenylmethyl)-1*H*-purin-6-amine (BA) (10 μM), 1*H*-indole-3-butanoic acid (IBA) (2 μM), 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.

Cryoprotectant chemicals used were 5% Me_2SO in water; 10% Me_2SO in water; 10% PEG, 10% glucose, and 10% Me_2SO (PGD); and 10% PEG, 10% trehalose, and 10% Me_2SO (PTD). All solutions were filter-sterilized (0.45 μM) before use.

Thirty meristems were used per treatment for each accession. Excised meristems (0.6 to 1.0 mm) were grown for 48 hr on Anderson's medium with the addition of 5% Me_2SO . Shoot tips were transferred to 0.25 ml 5% Me_2SO in 1.8-ml cryotubes following incubation on the Me_2SO -supplemented media. Treatment in 1.8-ml cryotubes (five meristems/tube, two tubes/replication, 3 replications) consisted of the addition of the desired chilled (1°C) cryoprotectants to the 5% Me_2SO solution, drop-by-drop, over a 30-min period up to 1.8 ml. The meristems were held in the cryoprotectant solutions for 1 hr at 23° before freezing.

Samples were frozen to -40°C in 0.5 ml of the desired cryoprotectant at a rate of 0.8 $^{\circ}$ /min in a programmable controlled-temperature freezing chamber. Freezing rates were monitored by thermocouples in both a sample vial and the chamber. Following controlled freezing to -40° , samples were immersed in liquid N_2 at -196° , held there for 1 hr, then thawed quickly by warming in a 40° water bath for 90 sec followed by a room temperature water bath. After thawing, samples were drained on sterile filter paper and transferred to petri dishes containing Anderson's medium with 1 μM BA. Regrowth was under growth room conditions. Controls were meristems subjected to all treatments (including cryoprotectants) but not frozen.

The cryoprotectants used were chosen to represent those commonly used in cryopres-

ervation (5% and 10% Me_2SO), to apply one which had been used only with suspension cultured cells (PGD), and to test a new combination of promising chemicals (PTD).

The unfrozen control meristems exhibited varying survival rates (Table 1). Control meristems that survived produced normal growth. Of the frozen meristems, the 5% Me_2SO treatment resulted in surviving tissue in three clones (Table 2). Of these survivors, most grew as organized meristems, the rest as callus. Ten percent Me_2SO resulted in an equally low survival rate. The PGD treatment, formerly used for suspension-cultured cells, provided the best protection as evidenced by survival rates of both control and frozen cells. All five accessions exhibited their highest survival rates when frozen with PGD. Normal meristem regrowth was the highest with this cryoprotectant (Table 3). PTD, a new, untested cryoprotectant, also produced moderate survival rates for all five clones and high rates of normal meristem development. Since clonal reproducibility is important in cryogenic storage, the high percentage of normal meristem regrowth produced by meristems frozen in either PGD or PTD is important. *Rubus leucodermis* exhibited the greatest overall survival, followed by *R. spectabilis*. The other three *Rubus* accessions survived some of the treatments with moderate rates of success.

Dormant buds of *Rubus* have been frozen successfully in liquid N_2 (11), but until now no successful attempts have been made to preserve apical meristems in this manner. This screening trial of five accessions and four cryoprotectants confirms that *Rubus* apical meristems can be frozen successfully at -196°C and regrown. Combination cryoprotectants (i.e., PGD and PTD) appear to be the most promising for further studies, as they produced the highest percentage of normal meristem regrowth in these tests. Although variation in survival rates occurred, all of the accessions were afforded some degree of protection by these two chemical mixtures.

The use of cryopreservation for the storage of *Rubus* meristems shows definite promise. Improved methods to increase survival rates would enable *Rubus* to be stored in liquid N_2 . Long-term storage using modifications of these methods is now an option to be investigated.

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