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PRUNUS SHOOT TIP CRYOPRESERVATION PROTOCOL

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Introduction into tissue culture and micropropagation

In vitro cultures are established from field-grown trees. Young flushes of growth are harvested after budbreak in the springtime. The leaves are removed from the young branch and the branch is cut into single-node sections for surface sterilization. Nodal sections are surface sterilized with 70% isopropanol for 3 minutes and then rinsed three times with tap water. The nodal sections are then treated with 10% bleach (0.825% sodium hypochlorite) for 10 minutes and transferred into a laminar flow hood. They are then rinsed three times with sterile, distilled water. Each nodal section is placed into a test tube containing Prunus *S*hoot Maintenance Medium (MS salts, 30 g L^{-1} sucrose, 0.25 mg L^{-1} BAP, 0.5 mg L^{-1} kinetin, 7 g L^{-1} agar at pH 5.7). Non-contaminated cultures are multiplied until there is enough plant material to harvest shoot tips for cryopreservation. In vitro cultures are grown with a 16 h light/8 h dark photoperiod with 85 µmol m^{-2} s^{-1} light provided by fluorescent bulbs.

Shoot tip excision and cryopreservation

Two millimeter shoot tips are excised from the apical buds derived from 4- to 6- week old in vitro-grown plants. The shoot tips are plated onto *Prunus* Preculture Medium (1/2 strength MS, 0.3 M sucrose, 0.4 M proline, 0.1 mM salicylic acid, 1 mM glutathione (reduced form), 8 g L^{-1} agar, pH 5.7) for three days at 25°C in the dark.

Shoot tips are removed from the preculture medium and placed in Loading Solution (2 M glycerol + 0.4 M sucrose + 1/2 strength MS salts) for 20 min at 22°C. The loading solution is removed and replaced with Plant Vitrification Solution 2 (PVS2; Sakai et al. 1990) at 0°C for 75 to 90 minutes. The shoot tips are then placed onto a thin layer of PVS2 on foil strips and plunged into liquid nitrogen (LN) and transferred to labeled cryovials.

Long term storage

Cryovials are placed onto cryocanes, which are then placed into labeled sleeves in cans. They will be maintained in the liquid or vapor phase of liquid nitrogen for long-term storage. A total of 170 shoot tips are processed per accession, with 10 shoot tips placed on each foil, and one foil placed into each cryovial. Fifteen cryovials (of 10 shoot tips each) are cryopreserved in the base collection, and one cryovial is warmed for a recovery event. The remaining 2 cryovials are available for viability assessments.

Warming and viability assessment

Shoot tips are warmed by removing the foil strips from the cryovials and then placing the foil strips into room temperature Unloading Solution (1/2 strength MS + 1.2 M sucrose at pH 5.7) for 20 min. Shoot tips

are then plated onto Prunus Recovery Medium #1 (1/2 strength MS macro elements (-NH_4), MS microelements and vitamins, 30 g L^{-1} sucrose, 0.1 mg L^{-1} BAP, 0.1 mg L^{-1} kinetin, 0.1 mg L^{-1} GA_3, 8 g L^{-1} agar, pH 5.7). After two weeks of culture in the dark on Prunus Recovery Medium #1, shoot tips are transferred to Prunus Recovery medium #2 (1/2 MS macro elements (+NH_4) + MS micro elements and vitamins, 30 g L^{-1} sucrose, 0.25 mg L^{-1} BAP, 0.25 mg L^{-1} kinetin, 0.1 mg L^{-1} GA_3, 8 g L^{-1} agar, pH 5.7). Shoot tips are then grown at 50 µmol m^{-2} s^{-1} provided by fluorescent lights. Shoot tips are grown for 8 weeks and transferred to fresh medium as needed (*P. persica* was transferred biweekly). Shoot tip survival and regrowth data are collected after 8 weeks.

References

Sakai A, Kobayashi S, Oiyama I. 1990. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. Var. Brasiliensis Tanaka) by vitrification. Plant Cell Rep. 9(1):30-33. doi: 10.1007/BF00232

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NLGRP Prunus Cryopreservation Media/Solution Formulations

In vitro culture media

Prunus Shoot Maintenance Medium (1L)

- Murashige & Skoog Basal Medium with Vitamins (Phytotechnology Labs M519) = 4.43 g
- Sucrose= 30 g
- BAP (0.1 mg/mL stock)= 2.5 mL
- Kinetin (0.1 mg/mL stock)= 5 mL
- Bring to volume
- Agar= 7 g
- pH= 5.7 final

Prunus Preculture Medium (250 mL)

- Murashige & Skoog Basal Medium with Vitamins (Phytotechnology Labs M519) = 0.55 g
- Sucrose (0.3 M)= 25.7 g
- Proline (0.4 M)= 11.51 g
- Glutathione, reduced form (Phytotechnology Labs G3399) (1 mM)= 19.22 mg
- Salicylic acid (0.1 mM) (1mg/mL stock)= 3.45 mL
- Bring to volume
- Agar= 2 g
- pH= 5.7 final

Prunus Recovery Medium #1 (-NH_4) (500 mL)

- Murashige & Skoog Modified Basal Salt Mixture (Phytotechnology Labs M571) (-NH4)= 0.67 g
- Murashige & Skoog Micronutrient Salt Base (Phytotechnology Labs M554)= 0.025g
- MS vitamins (1000x stock)= 0.5 mL
- Sucrose= 15 g
- BAP (0.1 mg/mL stock)= 0.5 mL
- Kinetin (0.1 mg/mL stock)= 0.5 mL
- GA3 (0.1 mg/mL stock = 0.5 mL
- Bring to volume
- Agar= 4 g
- pH= 5.7 final

Prunus Recovery Medium #2 (+NH_4) (500 mL)

- Murashige & Skoog Macronutrient Salt Base (Phytotechnology Labs M502)= 1.1 g
- Murashige & Skoog Micronutrient Salt Base (Phytotechnology Labs M554)= 0.05 g
- MS vitamins (1000X)= 0.5 mL
- Sucrose= 15 g
- BAP (0.1 mg/mL stock)= 1.25 mL
- Kinetin (0.1 mg/mL stock)= 1.25 mL
- GA3 (0.1 mg/mL stock)= 0.5 mL
- Bring to volume
- Agar= 4g
- pH= 5.7 final

MS Vitamin Stock (1000x): 30 mL

- Murashige & Skoog Vitamin Powder (1000x) (Phytotechnology Labs M533) = 3.09 g
- Bring to volume
- Dispense into 1 mL aliquots and freeze

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Cryopreservation Solutions

Liquid Preculture Medium, 0.3M Sucrose + ½ MS: 1 L
- Sucrose = 102.69 g
- Murashige & Skoog Basal Medium with Vitamins (Phytotechnology Labs M519) = 2.22 g
- Bring to volume
- pH = 5.8
- Dispense 25mL per 150 X 25 mm glass culture tubes

Loading Solution, 2M glycerol + 0.4M Sucrose + ½ MS: 1 L
- Glycerol = 184.2 g
- Sucrose = 136.9 g
- Murashige & Skoog Basal Medium with Vitamins (Phytotechnology Labs M519) = 2.22 g
- Bring to volume
- pH = 5.8
- Dispense 25 mL per 150 X 25 mm glass culture tubes

Plant Vitrification Solution 2 (PVS2): 250 mL
- Glycerol (30% w/v) = 75 g ***weigh this first in flask***
- Ethylene glycol (15% w/v) = 33.8 mL
- DMSO (dimethyl sulfoxide) (15% w/v) = 34.1 mL
- Sucrose (0.4 M) = 34.25 g
- Murashige & Skoog Basal Medium with Vitamins (Phytotechnology Labs M519) = 0.55 g
- Bring to volume
- pH = 5.8
- Filter sterilize using 0.45 micron syringe filter or Stericup filter units
- Dispense into sterile glass or plastic tubes, seal and refrigerate

Unloading Solution, 1.2 M Sucrose + ½ MS: 1 L
- Sucrose = 410.76 g
- Murashige & Skoog Basal Medium with Vitamins (Phytotechnology Labs M519) = 2.22 g
- Bring to volume
- pH = 5.8 final
- Dispense 25 mL per 150 X 25 mm glass culture tubes

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NLGRP PVS2 Cryoprotectant Preparation, 250 mL

1) Weigh out **75 g of glycerol** in a 250 mL Erlenmeyer flask
2) Place a stir bar into flask and put flask onto a stir plate
3) While stirring, add **33.8 mL of ethylene glycol** using a graduated pipette
4) Add **34.1 mL of DMSO** using a graduated pipette
5) Add **34.25 g of sucrose**
6) Add distilled water to bring the volume in flask to ~225 mL
7) Add **0.55 g of MS Salts + Vitamins powder** (M519, Phytotechnology Labs or use MS stock solutions)
8) **Bring to final volume of 250 mL** using a graduated cylinder
9) **Adjust pH up or down** to 5.8 using 0.5 M potassium hydroxide or hydrochloric acid solutions, respectively
10) **Filter sterilize** using .45µm Stericup filter unit or syringe filter
11) Transfer into sterile plastic or glass containers, seal and refrigerate

PVS2 Cryoprotectant composition for 250 mL

- Glycerol (30% w/v) = 75 g ***weigh this first in flask***
- Ethylene glycol (15% w/v) = 33.8mL
- DMSO (dimethyl sulfoxide) (15% w/v) = 34.1mL
- Sucrose (0.4 M) = 34.25g
- Phytotechnology Labs M519 (MS salts + vitamins) = 0.55g
- pH=5.8