CITRUS CRYOPRESERVATION AND MICROGRAFTING PROTOCOL

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Citrus Cryopreservation

Obtain citrus budwood from greenhouse- or screenhouse-grown plants. Cuttings should come from vegetative budwood at the triangular growth stage that is about 3-5mm in diameter. Collect material 2-6 weeks after growth flushes have occurred; the material should not be actively growing and should be somewhat "hard."

Remove leaves and petioles. If needed, budwood may be stored up to 4 weeks at 4°C in sealed plastic bags. However, it is best to use within 2 to 3 days especially if material is tender or is small in diameter.

Cut budwood into nodal sections. Surface disinfect sections in 70% isopropanol for 2 minutes. Drain the isopropanol and rinse three times with tap water, then add 10% bleach with Tween 20 (1 drop per 100 mL) and shake for 10 minutes. In a laminar flow hood, drain the bleach water and rinse nodal sections 3 times with sterile distilled water.

Excise axillary shoot tips from non-elongated buds. The final size should be about 1-2 mm. Make sure to leave some plant tissue as a base for the shoot tip because this will need to be trimmed in order to make a clean cut before micrografting. Put excised shoot tips into Liquid Preculture Medium in a small Petri dish (35 x 10mm). Leave shoot tips overnight in the Liquid Preculture Medium at 25°C in darkness.

After the overnight preculture treatment, remove the preculture medium with a pipette and immerse shoot tips into a Loading Solution (2M glycerol + 0.4M sucrose + ½x MS) for 20 minutes at 22°C. Use a pipette to remove the Loading Solution from the shoot tips.

Add the Plant Vitrification Solution 2 for 30 to 60 minutes at 0°C, this step can be done on a tub of crushed ice. Use 30 minutes in PVS2 for smaller shoot tips (1mm) and 60 minutes for larger shoot tips (2mm).

While shoot tips are being treated with PVS2, prepare foil strips with PVS2 droplets. Place autoclaved foil strips (about 2-3 cm in length and 0.5 cm wide) on a sterile Petri dish on the crushed ice. With a pipette, make 5 PVS2 droplets either individually or as one long channel on a foil strip. Begin moving shoot tips to the droplets/channel (2-5 shoot tips per droplet, 10 shoot tips per strip) shortly before the 30 or 60 minutes is up.

At 30 or 60 minutes, begin to plunge foil strips into liquid nitrogen. Transfer foil strips into cryovials, then place cryovials into cryocanes, and finally cryocanes into cryoboxes for long-term storage in liquid nitrogen vapor.

Citrus micrografting for recovery after cryopreservation

Prepare the rootstock seedlings

The seedling rootstocks must be prepared about 2-4 weeks before processing shoot tips (newer seeds will germinate more quickly than older seeds). Peel the white seed coat (leaving the brown "skin" underneath the seed coat intact) from *Poncirus trifoliata* x *Citrus sinensis* "Carrizo" seeds and surface sterilize in 70% isopropanol for 2 minutes and rinse three times with tap water. Then add 20% bleach with Tween 20 (1 drop per 100 mL) for 20 minutes, transfer to the laminar flow hood then rinse three times with sterile distilled water.

Transfer peeled and surface sterilized seeds to Citrus Seed Germination Medium in 25 x 150 mm test tubes with 25 mL of medium per tube (1-2 seeds per tube). Maintain the seedlings in darkness at 25°C until germination and growth. Seedlings with a height of about 3-7 cm are ready to use for micrografting; this may take about 3-6 weeks. There may be more than one seedling per seed due to the occurrence of nucellar embryos. The nucellar embryos can also be used for micrografting.

Warm the cryopreserved shoot tips

To warm shoot tips, pour room temperature Unloading Solution into a medium size Petri dish (60 x 15mm). Using forceps, quickly pull the foil strips from the liquid nitrogen and immerse in the Unloading Solution. Leave the shoot tips in this solution for 20 minutes.

Plate the shoot tips onto the Citrus Shoot Tip Recovery Medium and leave shoot tips on the medium overnight at 25°C in darkness.

Micrograft the shoot tips into the rootstocks

To prepare seedlings for micrografting, trim the seedling shoot to about 1 cm above the cotyledonary node and the root to 3-4 cm.

To prepare the shoot tip, basally trim about 0.2 mm of plant tissue off to create a fresh cut surface. It is important that the cut is very straight and to not let the shoot tip dry out (dip shoot tip into Shoot Tip Dip/Rinse Solution if needed).

Make a 2 mm deep incision to bisect the cut surface of the rootstock and then make a perpendicular cut to the edge of the seedling. This cut can be difficult to make with a normal scalpel blade; a microblade with a blade holder will make this step much easier. It should appear as if a ledge or a shelf was made at the top of the seedling. If working slowly, place the prepared rootstock into Shoot Tip Dip/Rinse Solution to prevent it from drying out. Prepared rootstocks can be stored in this solution temporarily while micrografting.

Gently place the shoot tip on the edge of the shelf cut that was made into the rootstock and carefully firm the shoot tip into place making sure there is good contact between the shoot tip and rootstock cut

edges. If necessary, add a small amount of Shoot Tip Dip/Rinse Solution with fine forceps to the area around the graft (there should only be a thin film of liquid present and not a pool).

Then carefully insert the grafted seedling into a 25 x 150mm test tube containing 25 mL of Citrus Micrograft Recovery Medium. Seal tubes with parafilm or plastic sealing film. Culture micrografted plants at 25°C under 16 hour photoperiod provided by fluorescent lights (100 µmol m^2 sec^{-1}).

Shoot tip regrowth and trimming

After 2 weeks, the tissues will have joined together and micrografts should be examined weekly for growth. The rootstock may begin to grow adventitious shoots which will inhibit the growth of the scion, so these adventitious shoots need to be trimmed off until the scion becomes well established. The easiest way to do this is to remove the grafted seedling from the tube and then carefully trim off the adventitious shoots without disturbing the scion. Re-insert the grafted seedling into the medium.

Eventually the micrografted scion will elongate and form a shoot.

References

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NLGRP Citrus Cryopreservation & Micrografting Media/Solution Formulations

In vitro culture media

Citrus Seed Germination Medium: 2 L

- Murashige & Skoog Basal Salt Mixture (Phytotechnology Labs M524) = 4.33 g
- Sucrose = 50 g
- Myo-inositol (powder) = 0.1 g = 100 mg
- FM Stock (MS ferric EDTA) (200x stock) = 10 mL
- Bring to volume with distilled water
- Agar = 14 g
- pH = 5.8 final
- Dispense 25 mL per 150 X 25 mm glass culture tubes
- Autoclave

Citrus Shoot Tip Recovery Medium: 500 mL

- Lloyd & McCown Woody Plant Basal Mixture (Phytotechnology Labs L154) = 1.15 g
- MS Vitamins (1000x stock) = 0.5 ml
- Sucrose = 25 g
- Bring to volume
- Agar = 3.5 g
- pH = 5.8 final
- Autoclave
- Dispense into 60 X 15mm Petri plates (12 mL/plate)

Citrus Micrograft Recovery Medium: 1 L

- Murashige & Skoog Basal Salt Mixture (Phytotechnology Labs M524) = 4.33 g
- White's vitamin stock (100x stock) = 10 mL
- Sucrose = 75 g
- Bring to volume
- Agar = 7 g
- pH = 5.8
- Dispense 25 mL per 150 X 25mm glass culture tubes w/clear caps
- Autoclave

Stock Solution Recipes for Medium Preparation

FM Stock Solution (MS ferric EDTA) 200x: 500 mL

- Na_2 EDTA = 1.865 g (add first, dissolve completely)
- $FeSO_4*7H_2O$ = 1.39 g
- Adjust volume to 450 ml
- Boil and allow to cool
- Bring to volume
- Refrigerate at 3-5C in darkness

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

White's Vitamin Stock (100x): 250 mL

- Nicotinic acid = 25 mg = 0.025 g
- Pyridoxine HCl = 25 mg = 0.025 g
- Thiamine HCl = 5 mg = 0.005 g
- Myo-inositol = 2500 mg = 2.5 g
- Bring to volume
- Dispense into small vials (10 mL/vial) and freeze

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

Shoot Tip Dip/Rinse Solution, 3% sucrose + MS solution: 1 L

- Sucrose = 30 g
- Murashige & Skoog Basal Medium with Vitamins (Phytotechnology Labs M519) = 4.43 g
- Bring to volume
- pH = 5.8
- Dispense 25 mL per 150 X 25 mm glass culture tubes

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