VIABILITY METHOD—SHOOT TIPS

NLGRP CLONAL MS CITRUS SHOOT-TIP-MICROGRAFTING 1

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NLGRP_CLONAL_MS_CITRUS_SHOOT-TIP-MICROGRAFTING_1

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Introduction
In 2012, we published a method for cryopreserving shoot tips excised from citrus vegetative buds derived from greenhouse or screenhouse-grown stock plants and recovered using an in vitro micrografting technique. Using this method, warmed shoot tips are recovered by grafting onto in vitro seedling rootstocks. This eliminates the need for optimized tissue culture media for diverse citrus cultivars. The availability of this method has increased the likelihood of adoption of citrus shoot tip cryopreservation techniques in citrus collections worldwide.

Source of Plant Material
Cryogenic storage (vapor phase)

Plant Material Description
Shoot tips on foil strips inside of cryo vials

Warming
Remove foil strips from vials and warm foil strips in room temperature unloading solution until melted and shoot tips are floating in unloading solution; incubate for 20 minutes

Regrowth/Viability Conditions
Prepare the seedling rootstocks
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 and plate 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 until the PVS2 solution is melted and shoot tips are floating. 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, trim about 0.2-0.5 mm of plant tissue off of the basal end to create a fresh cut surface. It is important that the cut be very straight and the shoot tip is kept hydrated (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 (preferred). Culture micrografted plants at 25°C under 16 hour photoperiod provided by fluorescent lights (90 µmol m2 sec-1 or ~7100 lux).

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 (~4 weeks) the micrografted scion will elongate and form a shoot and no more trimming is required.

Regrowth/Viability Assessment

Evaluate for shoot regrowth from the scion after 8 weeks.

Comments

N/A

References

Volk, GM, Bonnart R, Krueger R, Lee R. 2012. Cryopreservation of Citrus shoot tips using micrografting for recovery. CryoLetters 33:418-426.

Volk GM, Bonnart R, Shepherd A, Krueger R, Lee R. 2015. Cryopreservation of Citrus for Long-term Conservation. Acta Hort. 1065:187-192.

Volk GM, Bonnart R, Shepherd A, Yin Z, Lee R, Polek ML, Krueger R. 2016. Citrus cryopreservation: Viability of diverse taxa and histological observations. Plant Cell Tissue and Organ Culture 128:327-334. DOI 10.1007/s11240-016-1112-4

Volk GM, Jenderek MM, Walters C, Bonnart R, Shepherd A, Skogerboe D, Hall BD, Moreland B, Krueger R, Polek ML. 2019. Implementation of Citrus shoot tip cryopreservation in the USDA-ARS National Plant Germplasm System. Acta Hort. 1234: 329-334.

Appendices

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

FM Stock Solution (Ferrous Sulfate EDTA) 200x: 500 mL
• Na2 EDTA = 1.865 g (add first, dissolve completely)
• FeSO4*7H2O = 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

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