NAPPO
North American Plant Protection Organization
Organización Norteamericana de Protección a las Plantas
MEXICO - USA - CANADA

NAPPO TREATMENT PROTOCOLS

TP No. 02
Shoot-Tip Micrografting (STG)

The Secretariat of the North American Plant Protection Organization
1431 Merivale Road, 3rd Floor, Room 140
Ottawa, Ontario, Canada K2B 0B9
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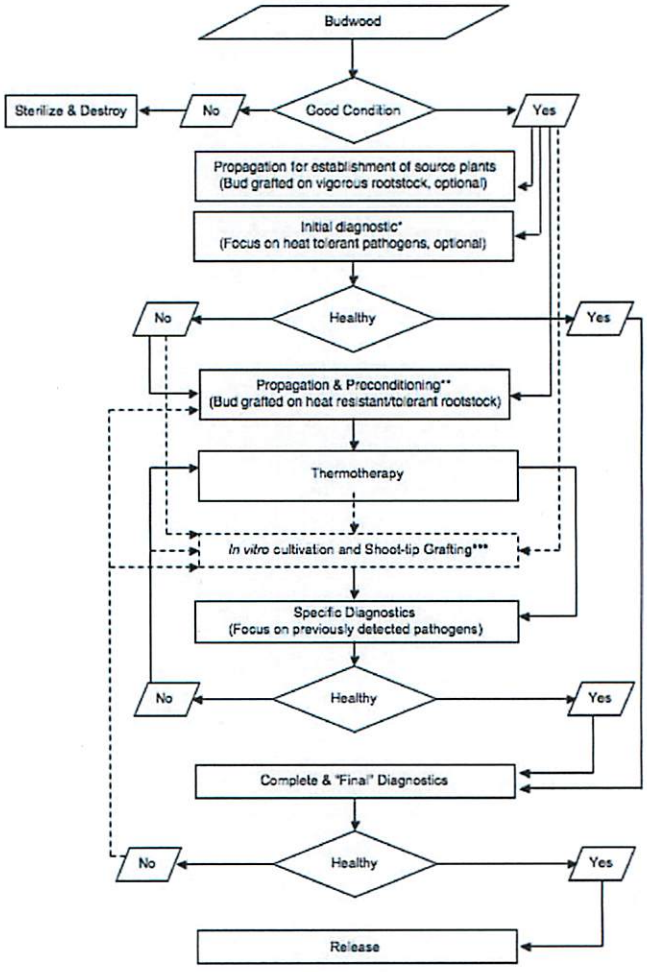
Active ingredient	NA
Treatment type	Biological
Target pest	Viruses, viroids, spiroplasmas, and other pathogens transmitted by grafting.
Target regulated articles	*Citrus* spp.
Treatment schedule	Micrografting can be done during any season when apices are available. A typical schedule or program would be: Day 1: Prepare medium to grow rootstock. Day 2: Prepare materials to plant seeds; remove seed tegument. Day 3: Plant seeds. Day 6: Defoliate the trees (source of apices). Day 15: Prepare medium for micrografting. Day 16 – 18: Collect apices; micrograft. Day 24, 31, 38: Check micrografting. Note: For further details see section 3.
Other relevant information	Micrografting treatment should be used as part of the measures applied in a certification program for propagative material free of pathogens. The following chart represents the normal flow of the procedures used for thermotherapy and shoot-tip micrografting treatments: 

	*An initial diagnosis is recommended. This will help to compare the phytosanitary condition of the material before and after the treatment for those pathogens that were detected (not all pathogens are detected with the initial diagnosis). Nevertheless, the propagated material** can be directly subjected to shoot-tip micrografting and/or thermotherapy. ***Shoot-tip micrografting and/or thermotherapy can be complementary treatments. The plant material can be either subjected to both treatments or to one or the other. This protocol refers specifically to micrografting.
References	Brown L. G., L. L. Breman. 1996. Introduction of Citrus Germplasm into Florida. Plant Pathology Circular No. 379. Fla. Dept. Agric. & Consumer Services. Citrus Clonal Protection Program (CCPP). Shoot-tip micrografting. Riverside, California. Frison E., M. Taher. 1991. Technical Guidelines for the Safe Movement of Citrus Germplasm. FAO/IBPGR. Navarro L. 1979. Microinjerto de ápices caulinares *in vitro* para la obtención de plantas de agrios libres de virus. Instituto Nacional de Investigaciones Agrarias. Centro de Levante, Moncada (Valencia). Bol. Serv. Plagas, 5: 127 – 148. Navarro L. 1981. Citrus shoot-tip grafting *in vitro* (STG) and its applications: a review. Proc Int Soc Citriculture pp 452-456. Navarro L., C.N. Roistacher, and T. Murashige. 1975. Improvement of shoot-tip grafting *in vitro* for virus-free citrus. J. Amer. Soc. Hort. Sci. 100:471-479. Navarro L. and J. Juarez. 1977. Elimination of citrus pathogens in propagative budwood. II. *In vitro* propagation. Proc. Int. Soc Citriculture 3:973-987. Roistacher C.N., L. Navarro, and T. Murashige. 1976. Recovery of citrus selections free of several viruses, exocortis viroid, and *Spiroplasma citri* by shoot-tip grafting *in vitro*. Proc 7th Conf Intern Org Citrus Virol. IOCV, Riverside. Pp 186-193. RSPM 16. 2013. *Integrated Measures for the Movement of Citrus Propagative Material.* Ottawa, NAPPO. Wisler G. C., L. G. Brown, and C. L. Schoulties. 1996. A Manual for Introduction of Citrus Germplasm into Florida. Fla. Dept. Agric. & Consumer Services.

Feasibility and applicability

Procedure for carrying out the phytosanitary treatment

The following is a summary from Brown and Breman (1996); CCPP (ND); Frison and Taher (1991); Navarro (1979, 1981); Navarro et al. (1975); Roistacher et al. (1976); Wisler et al. (1996).

For the shoot-tip micrografting technique, it is necessary to:

1. Obtain rootstock seed and germinate seedlings *in vitro*
2. Obtain young shoots
3. Micrograft
4. Re-grafting on conventionally grown seedlings or
5. Plant the micrografted plant directly into soil

1. Obtaining the rootstock

- Make sure the seeds used for rootstock production are not infected by any seed-borne pathogen.
- When seeds (Troyer or Carrizo) have received fungicide treatment, it is necessary to eliminate the fungicide with a wash.
- Soak the seed for 24 hours in distilled water (change the water every 8 hours).
- Place the seeds in distilled water at 52 °C for 10 minutes. Once the time has elapsed, place the seeds in water at room temperature.
- Peel (remove) both inner and outer integuments of seeds. Store peeled seed on moistened filter paper in a clean Petri dish in a refrigerator. Seeds can be peeled up to 24 hours before planting.
- When ready to plant the seeds, prepare the seed germination medium and place 25 ml into sterile 25 X 150 mm culture tubes.
- Wrap groups of 10 peeled (shelled) seeds in a gauze. Disinfect the seeds in a laminar flow hood, immersing them in 10% sodium hypochlorite (commercial formulation) plus 1% Tween 20 for 10 minutes. Wash three times with sterile distilled water and, using aseptic techniques, plant two seeds in each sterile culture tubes containing the seed germination medium. Cover with kaputs.
- Keep the tubes at 27 °C in the dark, until the seedlings reach grafting size, approximately in 2-3 weeks.

2. Obtaining the young shoots

Shoots can be obtained directly from the field or from plants grown in greenhouses by defoliating the plant. For plants grown in greenhouses, all leaves are removed from the tree and all young, soft growth is cut off. Usually 10 – 15 days are needed to obtain an apex with an appropriate size for micrografting (1-2 cm). Growth depends on time of year and variety. The tree can be moved to a warmer or cooler room if tips are growing too slowly or too quickly.

Axillary buds cultivated *in vitro* are another alternative.

- Budwood approximately 10 cm long with 4-6 buds are preferable. Budwood with only one bud is an alternative.
- Wash with ethanol 95% and rinse with water to eliminate dust.
- In a laminar flow hood, disinfect them by immersion in 10% sodium hypochlorite and 1% Tween 20 for 10 minutes, wash three times with sterile distilled water. Make a fresh angled

cut on one end of a budstick and place the end in a sterile culture tube containing approximately 25 ml of medium to acquire tips for STG. Alternatively, the culture tubes may be filled with approximately 1/3 volume of horticultural sand supplemented with 25 ml of 4.3 g/L MS salts (pH 5.7) and the budsticks planted into the sand.

- Keep the tubes containing the budwood at a temperature between 25 – 30 °C, and illuminated at 1000 lux/16 hours.

Most of the buds will sprout between 7 – 14 days. It is important to use shoots measuring less than 5 cm long to avoid apices that are in abscission stage.

3. Micrografting

The following steps should all be done using aseptic technique under a laminar flow hood. All instruments should be sterilized with 10 % sodium hypochlorite (commercial formulation).

Rootstock

- Remove the rootstock from the culture medium with tweezers and place in a Petri dish under a dissecting scope.
- Remove the tip of the rootstock, leaving 1 – 2 cm, and cut the root to 4-6 cm. Remove the cotyledons and axillary buds.
- Holding the seedling firmly, use a scalpel to make a cut in a triangular shape (1 mm) on the bark of the rootstock without touching the pith. Make the triangle below the upper cut. Alternatively, an inverted T cut may be used.
- Place the seedling aside in the Petri dish away from the light.

Shoots

- Use fresh, 1-2 cm long apices in good condition.
- In a laminar flow hood, disinfect the shoots by immersion in 5% sodium hypochlorite (commercial formulation) and 1% Tween 20 for 10 minutes; wash three times with sterile distilled water.
- Pick up a shoot-tip with inverse tweezers and place under the dissecting scope.
- Holding the shoot-tip as close as possible to the tip area, use a scalpel to remove the primordial leaves, leaving the meristem and 2 – 3 leaf primodia.
- Dip the razor sliver or scalpel into 10 % sodium hypochlorite (commercial formulation), then water, and slice the tip off. Work near the end of the blade and replace it when the tips do not separate easily (usually 5 – 10 cuts).

Micrografting

- With the tip still on the blade, place the apex on the triangular cut or T cut. The base of the apex should overlap with the base of the triangle. If the T cut is used, the tip should be flat on the surface of the cut and the edges of the cut are closed over the tip.
- Pick up the grafted seedling with forceps and place the root tip into the hole of the Heller support (using medium prepared as per 5.b.1. below). Lower the support into the culture tube so that it is level with the medium. Cap the tube and place it in the rack.
- Alternatively, pick up the grafted seedling with forceps and place grafted seedling into the tube with the graft recovery media (using medium prepared as per 5.b.2 below). Cap the tube and place it in the rack.
- After 4 – 5 grafts, dip tools in alcohol and place on a clean towel to dry.
- Keep the micrografts in the culture room or chamber at a temperature of 26 – 27 °C and illuminated at 1000 lux/16 hours, for 5-8 weeks or until the plants show at least two to three expanded leaves from the apex.
- Check periodically the micrografts and remove the lateral shoots produced by the rootstock aseptically with scissors.

4. Re-grafting
   - When the scion has pushed and grown in size to about 1 – 2 cm, plants can be re-grafted onto conventionally grown seedlings.
   - Use a vigorous disease free rootstock, such as rough lemon or *C. volckameriana*, and cut to about 30 cm using a sterilized clipper.
   - Using a sterilized grafting knife, make a T cut in the rough lemon's bark.
   - Remove the STG from the tube and cut off the roots and any adventitious shoots with a sterilized, sharp knife. Slice the STG behind the graft so that there is a flat area to place in contact with the rough lemon's cambial tissue.
   - Carefully slide the STG into the T cut and wrap with budding tape.
   - Place a plastic bag over the plant and keep it in the greenhouse in the shade.
   - After two weeks, loosen the plastic bag from the pot but leave the bag over the plant and leave the plant in the shade. Three to four days later entirely remove the bag from the plant but keep it in the shade for an additional week.
   - Move the plant out of the shade and sucker as needed.

When the scion is 2 to 3 months old, diagnostic tests should be performed on the material that has been micrografted to corroborate its phytosanitary condition. A number of methodologies can be used for this aim. In a plant certification program biological diagnosis is performed through indexing (i.e. grafting of the treated material in different plants used as indicators of disease) and/or through a laboratory diagnosis using serological and molecular techniques. Please consult RSPM 16: 2013 Annexes 1 and 2, on the accepted diagnostics tests for the different pathogens that infest citrus.

5. Media

5a. Medium for planting seeds

1. To a 4 L beaker, add 1900 ml distilled water.
2. Add 8.66 g Murashige and Skoog's salts and dissolve.
3. Check pH of solution and add 1N NaOH to bring pH to 5.7 ~ 0.1.
4. Transfer solution to 4 L Erlenmeyer flask and add distilled water to bring solution to 2000 ml.
5. Add 4 g agar replacement and shake flask gently.
6. Cap flask with foil and autoclave for 7 minutes.
7. Set up two racks of 40 culture tubes each.
8. When autoclaving is complete, remove flask and shake carefully until agar replacement is mixed in the solution.
9. Measure 25 ml water into one tube and use it with approximate 25 ml media. Make sure clamp is on the tube, fill funnel with media and dispense 25 ml into each tube.
10. Wash funnel and flask with hot water to remove the agar replacement.
11. Put plastic caps on tubes and autoclave for 15 minutes. Place a tray on top of the tubes to hold plastic caps on during autoclaving.
12. Remove sterile tubes to cabinet in dust-free room to cool.

5b.1. Medium for micro-grafting (liquid, using Heller support)

A. Supplies:
1. 4 L beaker
2. M-S salts; 8.66 g
3. White's vitamins; 20 ml
   - Nicotinic acid; 25 mg
   - Pyridoxine; 25 mg
   - Thiamine He1; 5 mg
   - Distilled water; 250 ml

With these supplies prepare the White's vitamins in a 250 ml volumetric flask and store in a capped and labelled bottle in the refrigerator.

4. Inosital; 200mg
5. Sucrose; 150 g
6. pH equipment:
   - 1N NaOH
   - 7.0 buffer
   - Squirt bottle to rinse electrode
   - Tissue wipes to dry electrode
   - Beaker
7. 80 (25 X 150 mm) culture tubes in two racks
8. Stand and funnel
9. Heller support supplies:
   - Whatman filter paper, 9 cm
   - Small test tube in holder
   - Wood skewer
10. 80 plastic caps

B. Procedure:
1. Add about 1800 ml distilled water to 4 L beaker.
2. Add 8.66 gm M-S salts, 20 ml Whites vitamins, 200 mg inosital, and 150 g sucrose.
3. Stir on magnetic stirrer until sucrose is dissolved.
4. Adjust pH to 5.7 + 0.1.
5. Add distilled water to bring solution to 2000 ml.
6. Set up two racks of 40 tubes each.
7. Measure 25 ml water into one tube and use this to approximate 25 ml media.
   Be sure clamp is on tubing, fill funnel with media and dispense 25-ml into each culture tube.

5b.2. Alternate medium for micro-grafting (gel, no Heller support used)

A. Supplies
1. 4-L beaker
2. M-S salts, 8.86 g
3. White's vitamins; 20 ml
   - Nicotinic acid; 25 mg
   - Pyridoxine; 25 mg
   - Thiamine He1; 5 mg
   - Distilled water; 250 ml
4. Sucrose, 150 g
5. Agar, plant micropropagation grade, 14 g
6. pH equipment:
   - 1N NaOH
   - 7.0 buffer
   - Squirt bottle to rinse electrode
   - Tissue wipes to dry electrode
   - Beaker
7. 80 (25 X 150 mm) culture tubes in two racks
8. Stand and funnel
9. 80 plastic caps

B. Procedure:

1. Add about 1800 ml distilled water to 4 L beaker.
2. Add 8.66 gm M-S salts, 20 ml Whites vitamins, 200 mg inosital, 150 g sucrose and 14 g agar, plant micropropagation grade.
3. Stir on magnetic stirrer until sucrose is dissolved.
4. Adjust pH to 5.7 + 0.1.
5. Add distilled water to bring solution to 2000 ml.
6. Set up two racks of 40 tubes each.
7. Heat solution on stir-plate to dissolve agar, watching closely to prevent over "cooking".
8. Measure 25 ml water into one tube and use this to approximate 25 ml media. Be sure clamp is on tubing, fill funnel with media and dispense 25-ml into each culture tube.

5c. Media to acquire tips for STG

A. Materials:

1 L distilled water
4.3 g M-S Salts
2 g agar replacement (or microbiological grade agar)
10 ml White's vitamins (25 mg Micotinic Acid, 25 mg Pyridoxine, 5 mg Thiamine HCl in 250 ml distilled water)

B. Procedure:

Add M-S Salts and White's vitamins into water and dissolve. Adjust pH to 5.7, transfer to a 2 L flask and add agar replacement. Autoclave for 7 minutes, remove and carefully shake in a circular motion to spread agar replacement in solution. Fill 25 x 150 ml culture tubes with approximately 25 ml media. Cover test tubes with plastic caps and autoclave for 20 minutes. Remove tubes and let cool.

Cost of typical treatment facility and operational running costs if appropriate

The micro-grafting technique requires laboratory equipment such as: scale, autoclave, dimmer, glasses, several substances (vitamins, salts, etc.), stereoscopic microscope, laminar flow hood, incubator, which represents an approximate investment of US $ 80,000.

Greenhouses are also necessary for propagating micrografted seedlings.

Commercial relevance, including affordability

Diseases caused by virus, viroids and other similar organisms cause significant economic losses in the worldwide citrus production. Some diseases cause plant death and others decrease production and fruit quality, causing loss of vigour and plant longevity. Viruses and other disease agents may be transmitted by bud grafting. Once grafting takes place, the disease can remain latent in the plant for many years. Buds obtained from a plant with latent diseases will produce sick plants. Countries with advanced citrus production have been successful applying certification programs that use disease free plants, especially those caused by virus, virus like organisms, and other graft transmissible pathogens.

These virus free plants are obtained from citrus plant varieties that have desirable agronomic features but that have been affected by one or more diseases caused by virus or similar organisms. For this purpose, techniques that allow obtaining virus free plants from sick individuals are used. Micrografting is a technique used for this purpose.

Extent to which other NPPOs have approved the treatment as a phytosanitary measure

Countries with a certification program for propagative material such as Spain, USA, etc., use micrografting in their clean plant programs.

Availability of expertise needed to apply the phytosanitary treatment
• Knowledge of the technique • Preparation of solutions • Knowledge or expertise on aseptic techniques and handling at a micro scale.
Versatility of the phytosanitary treatment
It can also be potentially applied to other perennial crops. It can eliminate a range of pathogens.
The degree to which the phytosanitary treatment complements other phytosanitary measures
Micrografting is another technique to eliminate pathogens in citrus propagative material. Both micrografting and thermotherapy techniques can complement each other. Micro-grafting can be done using apices from a tree that has undergone thermotherapy and conversely thermotherapy can be used with buds from a tree that has already undergone shoot-tip micrografting. Since both techniques have their strong and weak points, sometimes it is better to use both techniques in parallel. For example, some pathogens that are difficult to eliminate with thermotherapy (i.e., *Spiroplasma citri)* can be eliminated with shoot-tip micrografting, and others that are hard to eliminate with shoot-tip micrografting (i.e. citrus tatter leaf virus) can be eliminated with thermotherapy. If the propagative material is infected with more than one pathogen, both techniques may be required. Also, it is possible to use a type of thermotherapy at 32° C for a few weeks to pre-condition the material for shoot-tip micrografting. This pre-conditioning can also eliminate psorosis, a virus hard to eliminate with micrografting.
Summary of available information of weaknesses of the treatment or potential undesirable side-effects
The micrografting technique is based on the hypothesis that plant pathogens do not infect the meristem since the mother plant's vascular system has not been in contact with the meristem, which has not yet differentiated. Once the plant is obtained through this technique, it should be subject to pathogen testing (biological, immunochemical, serological, molecular testing, etc.) in order to determine with certainty that the plant is free from specific pathogens.
Applicability of treatment with respect to specific regulated article/pest combinations
Shoot-tip micrografting is known to be effective against several viruses including: *Citrus tristeza closterovirus*, citrus vein enation virus, citrus tatter leaf virus, *Citrus psorosis ophiovirus* and others; citrus viroids causal agents of diseases such as Exocortis and Cachexia; and other graft-transmissible pathogens such as *Spiroplasma citri.*
Technical viability
This is a commercial treatment and it is extensively applied in many countries.
Phytotoxicity and other effects on the quality of regulated articles, when appropriate
NA
Consideration of the risk of the target organism having or developing resistance to the treatment
NA

Review

NAPPO Treatment and Diagnostic Protocols are subject to periodic review and amendment. The next review date for this NAPPO protocol is 2020. A review of any NAPPO Protocol may be initiated at any time upon the request of a NAPPO member country.

Approval

This Protocol was approved by the North American Plant Protection Organization Executive Committee on October 19, 2009, revised on March 4, 2015 and is effective from this date.

Approved by:

Greg Wolff
Executive Committee Member
Canada

Osama El-Lissy
Executive Committee Member
United States

Javier Trujillo Arriaga
Executive Committee Member
Mexico