Micropropagation of peach rootstocks and cultivars

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Abstract

Peach (*Prunus persica* (L.) Batsch) is one of the most popular stone fruits, commercially produced largely in Mediterranean and, to a lesser extent, in continental climatic conditions. Several breeding programs with different aims release annually large numbers of new cultivars. Micropropagation offers a suitable method to provide the growers of sufficient quantities of rootstocks, as well as of pathogen-free planting material of old and new cultivars.

An effective four-step micropropagation procedure for cultivar and rootstock production is described here, based on the use of modified MS and WPM media. The health status of the initial shoot tips is very important, also because the growth and proliferation rate of shoot cultures from virus-infected clones are generally very poor. Proliferation and elongation phases depend on the major macro-elements, as well as the content and ratio of plant growth regulators. It is important to grow the cultures at 22°C, as hyperhydricity may develop at higher temperatures. Although sucrose is the most common carbon source used during proliferation and rooting, for some peach cultivars and rootstocks the replacement of sucrose (10 g l^{-1}) with glucose (20 g l^{-1}) in the rooting medium improves the rooting and survival rates of plants in the acclimatization phase. The rooting rate of the rootstock 'Cadaman' is improved with the chelated form of iron FeEDDHA at 150 mg l^{-1}. Rooted plants are acclimatized in greenhouse under high humidity conditions.

Key Words 'Cadaman', 'GF-677', inter-specific hybrid rootstocks, 'PeDa', 'PeMa', *Prunus persica* (L.) Batsch, shoot-tip culture, stone fruits

1. Introduction

The peach (*Prunus persica* L. Batsch), originated from China, is produced commercially in 80 countries worldwide on about 1.6 million ha. In 2010 the peach and nectarine fruit harvest was 20.3 million tonnes, which constitutes 53% stone fruit traded worldwide ***(1)***. Peach and nectarine cultivation is mainly restricted to the Mediterranean climates that are characterized by warm to hot, dry summers and mild,

wet winters. Peach has a very long ripening period which starts from the middle of March and finishes early November (Northern Hemisphere), and from end-September until end-March (Southern Hemisphere). Currently, there are several peach breeding programs in the USA, Europe, Latin America, Australia, Asia and to a smaller extent in Africa and Australia, aimed to improve the characteristics of existing cultivars. Today, for instance, the plum pox virus (PPV) and the European stone fruit yellow phytoplasma (ESFY) cause the most serious losses to the peach growers; their control in infected orchards is not effective, however, possible prevention is by planting healthy peach trees. Commercial peach cultivation requires not only new cultivars, but also vegetative propagated rootstocks suited to the various production conditions.

Tissue culture techniques can provide new cultivars to meet the demand of market. The rapid multiplication of elite cultivars ***(2-5)***, production of pathogen-free plants ***(6-8)***, breeding of early ripening peach varieties ***(9)*** and gene transfer studies ***(10-12)*** are important objectives for micropropagation. The micropropagation system, independent of the growing season, is ideally suited to fulfil these requirements. The propagation of healthy material from gene collection and breeding work is fundamental especially when pathogen-free material is unavailable. Phytotherapy is a crucial step to carry out micropropagation ***(6-8)***. This will markedly improve the growth and propagation rate of clones infected by PPV and ESFY.

In case of peach, several reports are available, dealing with the development and optimization of individual steps of the micropropagation cycle, as well as of complete protocols for specific rootstocks and cultivars ***(2- 5, 13, 14)***. This manuscript describes a stepwise protocol for shoot induction, propagation, elongation, rooting and subsequent acclimatization, suitable for *in vitro* mass production to a wide range of rootstocks and cultivars with some minor modifications.

2. Materials

2.1. Decontamination of explants and transfer of tissue culture plants

1. Tap water.
2. Contact fungicide solution.
3. Commercial bleach solution, diluted to have 0.7% of active chloride (*see* **Note 1**).
4. Autoclaved tap water.

5. 70% ethanol (v/v).
6. Sterile 500 ml Erlenmeyer flasks / 500 ml glass bottles with wide neck (4 cm).
7. Sterilized filter paper.
8. Instruments for micropropagation: pruning shears, scalpels with blades, forceps.
9. Glass bead sterilizer.
10. Laminar flow benches.

2.2. Preparation of culture media

1. 1 M concentrated solution of KOH and HCl for pH adjusting.
2. Chemicals of the medium/fertilization components according to the modified MS ***(15)***, WPM ***(16)*** and Jacquiot ***(17)*** formulations shown in **Tables 1** to **4**, and dissolved in distilled water.
3. Glassware to measure and to mix the medium components.
4. Sterilized paper (size about 10 x 18 cm).
5. 200 ml screw cap culture vessels/magenta jars.
6. Semi-permeable plastic cling wrap to close the culture vessels.
7. Double distilled water for preparing stock solutions.
8. Laboratory facilities: balances, magnetic stirrer, pH meter, microwave, autoclave, fridge.

2.3. Growth room for in vitro culture

1. Growth room with shelves mounted with warm white fluorescent lights, like OSRAM L 58/30 or TUNGSRAM F 29.
2. Air conditioner.

2.4. Acclimatization of micropropagated plants to ex vitro conditions

1. Greenhouse with mist benches.
2. Tap water.
3. Plastic tray “TEKU JP 3050/72”, (PÖPPELMANN GmbH and Co. Pf 1160 D-49378 Lohne Germany). The tray size is 50 x 28 cm with 72 cells (4 x 3.7 x 4 cm).
4. Potting mixture consists of peat : perlite in a ratio 1 : 1.

3. Methods

The protocol described here follows a classic four-step micropropagation method **(Fig.1)**.

3.1. Preparation and sterilization of culture media

1. Prepare separate stock solutions for the media according to **Table 1**. Store the stock solutions in a laboratory fridge at 4°C. Stock solution can be substituted with master mix powders from commercial companies by following the instruction for preparation.
2. Use stock solutions of the correct volumes to prepare three different media, i.e., establishment/elongation, shoot proliferation and rooting medium.
   To calculate the required volume of the stock solution for one liter of medium:
3. Prepare a stock solution of microelements, as indicated in **Table 2**. Add 1 ml l^{-1} to each medium.
4. Prepare a stock solution of Jacquiot vitamins **(Table 3)**. Add 1 ml l^{-1} to the proliferation medium only.
5. Dissolve the correct amount of sucrose or glucose into the medium.
6. Adjust the pH according to the required medium, (**Table 1**), using 1M KOH or 1M HCl.
7. Heat the medium, add 0.6% (w:v) agar (e.g., Oxoid n°5); continue heating and stirring the medium until the agar is dissolved.
8. Pour 50 ml warm medium into 200 ml culture vessels or 15 ml into a test tube and close.
9. Autoclave the medium at 121°C for 20 minutes.
10. Store the medium-containing culture vessels/test tubes in shelves. If they are kept at room temperature, discard when older than one month.

3.2. Stock plants and shoot decontamination

1. Collect actively growing shoot tips, about 15 cm long from 3 to 5 year-old trees, growing in pots (4-liter pot is a good size) in the greenhouse (*see* **Note 2, 3, 4** and **5**).
2. Cut back all the leaves, leaving about 0.5 cm of petioles.
3. Cut the shoots into sections, containing 2 buds. Discard the very weak shoot tips about 3 cm and the terminal 2-cm shoot sections. Use buds in the middle part of the shoots for tissue culture.
4. For decontamination, treat about 20 shoot sections in a 500 ml flask with 70% ethanol for 1 min, followed by immersion in the bleach solution, prepared according to section 2.1 and add few drops of wetting agent "Tween 80" to increase the efficacy. Close the flask and keep on a magnetic stirrer for 30 min. **(***see* **Note 6** and **7)**.
5. Transfer the flask into the laminar flow hood and rinse the shoot sections 3 times with autoclaved distilled water. Dry them with sterile filter paper.
6. Remove 0.2 cm of the bottom parts of the 2-bud sections using sterile instruments and put 10 sections into one culture vessel.
7. Repeat the decontamination procedure the following day, except for the ethanol treatment.
8. Cut 0.2 cm piece from the bottom ends of the 2-bud sections and place the 2-bud sections individually into the test tubes, then transfer them into the culture room.

3.3. Culture and maintenance of explants

1. Use the medium prepared according to section 3.1.
2. For culture establishment, place 2-bud sections into test tubes (one per tube to avoid losses due to contamination), containing the medium for culture establishment. About 100 shoot explants per genotype are necessary for successful establishment.
3. Grow the explants in a culture room at 22°C, under a 16h light/8h dark photoperiod, with a light intensity of 40–45 μmol m^{-2} s^{-1}, provided by daylight fluorescent lamps (*see* **Note 8**).
4. Eliminate contaminated explants daily.

5. During the first two months, transplant cultures to fresh medium every 2 weeks.
6. Isolate shoots, developed from buds, when they are about 1 cm long, and transfer them to the propagation medium ***(13)***, 5 to 7 shoots per culture vessel (*see* **Note 9**).
7. Grow the cultures on the proliferation medium (*see* **Note 10** and **11**) for about three weeks. Three to five new shoots start to develop during this period. Transfer the shoot clusters to the elongation medium with a fresh cut at the base, without isolating the new shoots to allow small buds and shoots to grow to about 1.5 cm long shoots which are cut from the dominant shoot. Multiplication and elongation phases alternate until the required quantity of rootable shoots are produced. On average, the culture period on the proliferation medium is 3 weeks; while on the elongation medium is 10-14 days (*see* **Note 12**).
8. Different types of shoots develop on the elongation medium: 1) shoots shorter than 0.8 cm should be kept together and transferred to the proliferation medium for further multiplication, 2) shoots from 0.8 to 1.5 cm are suitable for further propagation, 3) the elongated shoots (about 1.5 cm) can be moved to the root induction medium (*see* **Note 13**) or back to the proliferation medium if the quantity of cultures is still not sufficient.

3.4. Shoot rooting

1. Transfer elongated shoots into vessels containing gelled root induction medium, ten shoots per vessel (*see* **Note 14** and **15**).
2. Keep the vessels in the culture room, under the climatic conditions described in section 3.3.3.
3. Generally, rooting starts in about 10 days and is completed in 21 days.

3.5. Acclimatization of micropropagated plants to ex vitro conditions

1. Remove rooted shoots with 3 to 5 roots (about to 0.5 cm long) from the medium. Clean root surface from the medium residuals to avoid contamination. Transfer rooted shoots into planting trays described in part 2.4.3 filled with a substrate (as described in part 2.4.4) for micropropagated

plantlets, wetted until run off, and place in the greenhouse *(see* **Note 16** and **17**).

2. Spray or drench the plantlets with a fungicide (Proplant^{TM} at the manufacturer's recommendation) in order to protect them from fungal infection.
3. Cover the plantlets with a plastic sheet to protect from direct sunshine and to keep the humidity around the plantlets close to saturation (*see* **Note 18**).
4. Shade the greenhouse in order to maintain temperature lower than 30°C, as well as to protect the plantlets from direct sunshine.
5. Water the plantlets carefully.
6. After the first 7 to 10 days, depending on weather conditions open the plastic sheet gradually to accustom plantlets to the greenhouse humidity. During this time, it is important to check periodically the plants for fungal infection and, when necessary, to treat them with an appropriate fungicide, i.e. captan or benomyl.
7. After 6 to 8 weeks, transplant plantlets into individual containers (6 x 6 x 9 cm) filled with a mixture of sand (40%), peat (43%), alginate (15%) and organic matters (FLORASCA Hungaria) and placed in open field, under irrigated and shaded conditions.
8. Fertilize the container plants 1-2 times a week with a solution (E. SZŰCS, Institute for Fruitgrowing and Ornamentals), (**Table 4** and *see* **Note 19**).
9. Under continental climatic conditions, micropropagated rootstocks, acclimatized in spring, can be grafted at the end of summer/early autumn.

4. Notes

1. Commercial bleach (NaClO) is generally at a concentration of 3.5% (v/v) of active chloride.
2. Two to three weeks before isolation and establishment of explants, transfer actively growing potted plants to the greenhouse, with an interior temperature 24±4°C. Apply a preventative contact fungicide, e.g., captan or benomyl.

3. If potted trees are not available, cut shoots from trees in spring (April in the Northern Hemisphere, November in Southern Hemisphere) or when secondary growth occurs in July (Northern Hemisphere) under continental climatic conditions. New shoots from the middle part of the tree are suitable for establishment of tissue culture.
4. Cultivar identification and health control of the stock plants is very important especially in case of mass production ***(6- 8)***.
5. Sometimes trees are maintained only in the field. In case of field originated explants the establishment step is time consuming, careful and repeated disinfection treatments may be required. It is always very important to select healthy mother plants especially if field derived.
6. In case of heavily fungal infected explants the bleach solution can be substituted with the more effective, but also more dangerous for cell viability, use 0.5% mercuric chloride solution for 1 minute. Mercuric chloride is highly toxic; hence use a dust filter mask, safety gloves and glasses. After the use, collect this solution separately and discard under certified controlled conditions (refer to the specific instructions of your Country).
7. If plants are infected with viral pathogens, such as PPV or ESFY, the disinfection treatment will not eliminate the pathogens, and phytotherapy is required.
8. Several peach cultures are highly sensitive to the temperature of the culture room; 'Cadaman' rootstock and 'Cresthaven' and 'Suncrest' cultivars. At temperatures higher than 22°C, shoot hyperhydricity can occur. Hyperhydrated cultures are swollen, the tissues are shiny and unfit for further propagation, and should be discarded.
9. The original explant often appears as a dominant shoot, surrounded by smaller shoots originated by axillary and basal buds. These *de-novo* formed shoots, because of their small size (from 0.2 to 0.8 cm) are still not suitable for isolation and subculturing.
10. The plant growth regulator ratio and content of the medium should be determined every time for each specific rootstocks or cultivars. The medium in **Table 1** was developed for micropropagation of the rootstock 'GF-677', the most widely used in peach orchards. For the multiplication of the

rootstock 'Cadaman', for instance, 0.05 mg l^{-1} 1-naphthalene acetic acid (NAA) is required, while 6-benzylaminopurine (BAP) and adenine-sulphate concentrations should be reduced to 0.3 mg l^{-1}, L-glutamine to 30 mg l^{-1} and the gibberellic acid (GA_3) has to be omitted.

11. The peach cultivars propagated in our laboratory ('Babygold 6', 'Biscoe', 'Creshaven', 'Fantasia', 'Frederica', 'Redhaven' and 'Suncrest') require a BAP concentration of 1 mg l^{-1} and indole-3-butyric acid (IBA) at 0.05 mg l^{-1}.
12. The 'PeDa', a Hungarian *Prunus persica* x *P. davidiana* hybrid rootstock, requires 950 mg l^{-1} K_2SO_4 instead of 1900 mg l^{-1} KNO_3 in the elongation medium.
13. Cold storage (at 4°C) of shoots on elongation medium for 1 to 3 months before rooting can improve the rooting rate
14. For the rooting medium, higher rooting percentages are achieved when FeEDTA is replaced with FeEDDHA ***(18)***.
15. The rootstock 'Cadaman' requires increased potassium (1400 mg l^{-1}) and FeEDDHA (150 mg l^{-1}), as well as reduced nitrogen (600 mg l^{-1}) concentration in the rooting medium for achieving a proper root development ***(2)***.
16. Spring time (from middle February till end of May) or September (Northern Hemisphere) is the preferable period for acclimatization under continental climatic conditions.
17. The water used for irrigation should be at greenhouse temperature.
18. The plastic sheet must not touch the plants.
19. Dilute the stock solution in **Table 4** 100-fold with tap water before use. For calcium nutrition the normal tap water (used for dilution) is generally enough. Tap water contains about 100-150 mg l^{-1} Ca.

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Tables

Table 1. Culture medium composition developed for the micropropagation of 'GF-677'. The medium is based on Murashige and Skoog ***(15)*** and Woody Plant Medium ***(16)***. Note that formulations are expressed in mg l^{-1}, while the concentrations of stock solutions are expressed in mg/100 ml.

Culture medium components	Establishment Elongation (mg l^{-1})	Proliferation (mg l^{-1})	Rooting (mg l^{-1})	Stock solutions (mg/100 ml)
NH_4NO_3	1,650	200	1,650	10,000
$Ca(NO_3)_2 \times 4\ H_2O$	-	600	600	10,000
$CaCl_2 \times 2\ H_2O$	440	-	-	1,000
KNO_3	1,900	900	900	10,000
$MgSO_4 \times 7\ H_2O$	370	180	180	10,000
KH_2PO_4	170	140	140	10,000
FeEDDHA	-	-	120	400
FeNaEDTA	20	20	-	400
$CoCl_2 \times 6\ H_2O$	0.03	0.03	0.03	
$CuSO_4 \times 5\ H_2O$	0.03	0.03	0.03	
H_3BO_3	3.00	3.00	3.00	
KI	0.30	0.30	0.30	
$MnSO_4 \times 4\ H_2O$	11.00	11.00	11.00	
$Na_2MoO_4 \times 2\ H_2O$	0.30	0.30	0.30	
$ZnSO_4 \times 7\ H_2O$	4.00	4.00	4.00	
Myo-inositol	100.00	500.00	100.00	1,000
Nicotinic acid	0.50	1.00	-	50
Piridoxine HCl	0.50	-	-	50
Thiamine HCl	0.10	1.00	0,40	10
Glycine	2.00	-	-	100
Ca-pantothenate		0.50	-	
Biotine		0.10	-	
Riboflavine		0.10	-	
p-Amino-benzoic-acid		1.00	-	

Folic-acid		0.01	-	
IAA	1.00	-	-	10
NAA	-	0.005	-	10
IBA	-	-	0.50	10
BAP	0.06	0.50	-	10
GA_3	0.10	0.25	-	10
L-glutamine	-	50.00	50.00	1,000
Adenine sulphate	-	0.50	-	10
Glucose	30,000	-	20,000	
Sucrose	-	20,000	-	
Agar (Oxoid no.5)	6,000	6,000	6,000	
Distilled water (ml)	1,000	1,000	1,000	
Medium pH	5.2	5.5	5.8	

Table 2. Stock solution of microelements

Medium components	Concentration (mg/100 ml)
$ZnSO_4$.x $7H_2O$	400
H_3BO_3	300
$MnSO_4$ x $4H_2O$	1,100
$CuSO_4$ x $5H_2O$	3
Na_2MoO_4 x $2H_2O$	30
$CoCl_2$.x $6H_2O$	3
KI	30

Table 3. Stock solution of the Jacquiot vitamins ***(17)***

Medium components	Concentration (mg/100 ml)
Thiamine HCl	100
Ca-pantothenate	50
Biotine	10
Riboflavine	10
p-Amino-benzoic-acid	100
Nicotinic acid	100
Folic acid	1

Table 4. Stock solution for fertilization of container grown-plants in peat based growing medium or in rock wool

Components for nutrition solution	Concentration (g/10 l)
NH_4NO_3	480
$MgSO_4 \cdot 7H_2O$	430
KNO_3	264
Fe-EDTA / Fe-EDDHA, (9-10 % Fe)	20
$MnSO_4 \cdot H_2O$	13,44
$CuSO_4 \cdot H_2O$	0,48
$ZnSO_4 \cdot 7H_2O$	0,576
$Na_2B_4O_7$	3,6
$[(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0,07
H_3PO_4 (84 % P_2O_5)	90 ml

Figure Legends

Figure 1. The four-step method for micropropagating peach rootstocks and cultivars (Photo: P. Balla)

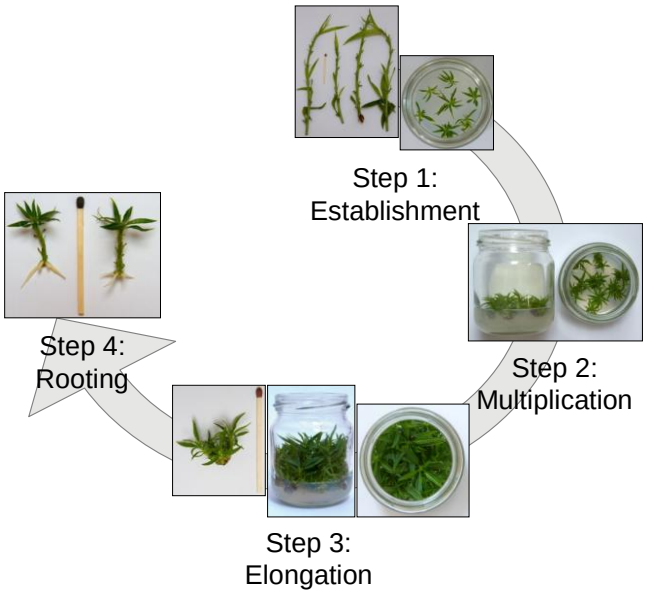

Fig. 1. The four-step method for micropropagating peach rootstocks and cultivars (Photo: P. Balla)