Rootstocks used for historical citrus cultivated in pots. I. In vitro propagation

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Abstract

Several experiments were carried out to develop protocols for *in vitro* propagation of citrus rootstocks using shoot-tip explants from seedlings. At the beginning Murashige & Skoog (MS) medium was supplemented with different concentrations of Kinetin (6- Furfurylaminopurine), later nutrient medium consisting of Driver and Kinyuki salt formula (DKW) and Kinetin was used ammended with BAP (6-Benzylaminopurine). These tests were performed with explants of Sour Orange (*Citrus aurantium* L.), Alemow (*Citrus macrophylla* Wester), Volkameriana (*Citrus volkameriana* Ten. et. Pasq.) and Poncirus (*Poncirus trifoliata* (L.) Raf). In other experiments, explants of Citrumelo (*Citroncirus citrumelo, C. paradisi* x *P. trifoliata* Raf.) and Citrange (*Citrange troyer,* hybrid between *C. sinensis* Osbeck and *P. trifoliata* Raf.) were cultured on MS mineral elements and then were tested on DKW medium with different concentrations of BAP, GA3 (Gibberellic acid) and IBA (3- Indolbutyric acid). The highest number of axillary shoots per explant was obtained on DKW medium supplemented with only BAP at concentration of 0.8 mg/l (Poncirus), 1.0 mg/l (Citrange), 0.6 mg/l (Citrumelo). At the end of three subcultures shoot multiplication rate was 1:8.94 Poncirus, 1:6.44 Citromelo and 1:5.83 Citrange. Different concentrations of IBA (3- Indolbutyric acid) and NAA (1- Naphtylacetic acid) were tested for *in vitro* root induction obtaining different responses, scarce rooting for Poncirus (68% of shoots developed roots on medium with NAA) and good results for Citromelo and Citrange (100% of shoots developed roots on peat/perlite substrate wetted with MS formula solution and added with IBA).

Keywords: *Citrus aurantium, C. macrophylla, C. volkameriana, Poncirus trifoliata,* Troyer citrange, micropropagation.

INTRODUCTION

Most species of Citrus trees (Rutaceae family) belong to the genus Citrus. There are plants from the Far East and specifically southern China, northern Burma and Annan, but they have been present for centuries in European gardens, especially in Tuscany, where the Medici (XVI century) began the cultivation then making them the emblem of their noble lineage. Since then "*lush collars of Seville oranges and cedar formed pleasant scenes of green against the walls and buildings, while the vases and bowls of oranges, lemons, limes, bitter oranges and bergamot alternated seasonally, with great expenditure of human labor, between the closed of orangeries*

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and the open loggias and gardens "(Citrus, fruit and grapes in the Florence of the Medici painter, Bartolomeo Bimbi, monograph 1982).

In the second half of the 15th century Citrus plants became an indispensable element for the design of the gardens in Tuscany, examples of what are still the parks of Villa Castello and Boboli Gardens in Florence, where, even today we can admire the Medici 's ancient collections. *Citrus aurantium*, commonly known as sour orange or Melangolo, has played a key role in the production of these plants having been the first rootstock for citrus trees in pots, used for this aim from the Renaissance to today, as this species is widely used with the same purpose in today's nurseries (Tintori et al., 2000).

In fact, sour orange as a rootstock ensures immunity to infection and rot gummosis, evoked by *Phytophthora*, and has good grafting affinity for all species. Usually citrus rootstocks are propagated by seed or cutting, then grafted with the cultivar. After a description of the various rootstocks currently used (Table 1), aim of our work was to investigate their micropropagation to develop easier and efficient protocols to obtain clonal material for commercial production.

MATERIALS AND METHODS

Shoot-tip culture establishment. Five-year seedlings of Sour orange, Alemow, Trifoliate orange and Volkamer lemon, grown in a glasshouse at 27/15 ± 2° C day/night temperature, were used as a source of explants. Actively growing shoot tips, 10-15 mm, were collected in April, soaked in flowing water for 15 min and dipped in 40% ethanol for a few seconds, then surface disinfected with 20% sodium hypochlorite solution (7% available chlorine; 0,4% active chlorine in the final solution) supplemented with a few drops of Tween 20 and stirred gently for 25 min. The basal portions of shoots injured by the disinfectant were removed.

Successively other two surface disinfection protocols were tested: 1) the shoot tips were dipped in 80% ethanol and surface disinfected with 20% sodium hypochlorite solution for 25 min.; 2) the disinfection was performed by 80% ethanol and then 25% sodium hypochlorite solution for 25 min. Three rinses with sterile distilled water were then carried out at the end of each protocol. Shoot tips, isolated under aseptic conditions, were inoculated into culture tubes (25 × 150 mm) containing 20 ml of MS nutrient medium. Visual inspection of the medium at the base of the explant provided evidence of some contaminants (bacteria and fungi).

Citrumelo and Troyer citrange *in vitro* cultures were already available in our lab (three months old), but the multiplication medium still needed to be improved.

Culture media. Shoot establishment was initially tested on the basic nutrients consisting of MS medium (Murashige and Skoog, 1962), adjusted to pH 5.8 with KOH and solidified with 6 g/l agar-agar (Fluka) and sterilized by autoclaving for 20 min at 120°C, 1 atm. Cultures into glass jars of 500 ml volume, each holding 100 ml of culture medium and containing 10 explants, were used for each treatment and maintained at 23±1°C under 16/8 h day/night by cool white fluorescent light at 40-50 μmol m^{-2} s^{-1}. In later experiments, the nutrient medium consisted of DKW medium (Driver and Kinyuki, 1984) and was solidified with a combination of agar-agar (Fluka) and carrageenan (Frimulsion Cesalpina) at a concentration of 5 g/l and 2.5 g/l, respectively.

Shoot proliferation. In the first experiment, freshly prepared explants of Sour orange, Alemow, Volkamer lemon and Trifoliate orange were cultured on two different media:

- M1 - MS mineral elements with the following additions (mg/l): Myo-inositol 100, Thiamine HCL 1.0, Glycine 2.0, Nicotinic acid 0.5, Pyridoxine 0.5, Kinetin (6-Furfurylaminopurine) 0.4, Sucrose 30 g/l. The pH was adjusted to 5.3 and the medium solidified with agar (Fluka) 6.0 g/l.
- M2 – the same medium (MS) but with half concentration of the mineral elements and Kinetin was further increased up to the concentration of 0.8 mg/l.

In the second experiment, explants of Citrumelo and Troyer citrange were established on M1 medium containing 0.2 mg/l BAP, 0.2 mg/l GA3 (Gibberellic acid 3) and 0.06 mg/l IBA (3-Indolylbutyric acid) instead of Kinetin.

Successively (experiment 3), explants of these two rootstocks and of Trifoliate orange were cultured on DKW medium without GA_3 and IBA, while BAP was further increased to a concentration of 1.0 mg/l for the Troyer citrange and 0.6 mg/l for Citrumelo and Trifoliate orange, respectively. The pH was adjusted to 5,8 and the media solidified with agar and carrageenan in a concentration of 5.0 g/l and 2.5 g/l, respectively. Multiplication rate was determined by recurrent subcultures of 3 cycles of 15 days each for Citrumelo and Troyer citrange, and of 10 days each for Trifoliate orange.

Rooting. Actively growing shoots of Citrumelo and Troyer Citrange were cultured in DKW medium (macro and microelements and the following substances, mg/l): Thiamine HCL 1.0, Glycine 2.0, Nicotinic acid 0.5, Pyridoxine 0.5, Myo-inositol 100, Sucrose 30 g/l. The pH was adjusted to 5,5 and the media were solidified with 5.0 g/l agar and 2.5 g/l carrageenan. In the first experiment, different auxin treatments were compared:

1) IBA (3- Indolbutyric acid) at a concentration of 0.6 mg/l. After 30 days microshoots were transferred on the same medium without auxin. After 15 days the number of rooted shoots was detected.
2) NAA (1 - Naphtylacetic acid) at a concentration of 1.0 mg/l and after seven days in the dark, microshoots were transferred in the light on the same medium without auxin. After 15 days the number of rooted shoots was determined.

In the second experiment, microshoots were cultured on a substrate, consisting of a mixture of TKS1 peat and perlite in a 1:1 ratio, placed in 500 ml-volume jars (200 ml per jar) and sterilized at 120 °C for 20 minutes to form a mechanical support for the implantation of microcuttings. Then the mixture was wetted aseptically with a liquid substrate (30 ml of medium) containing MS macro and microelements and IBA 0.8 mg/l, before implantation of the microshoots. The experiment was conducted in the light and after 34 days, the number of rooted shoots and the number of roots per shoot were detected.

- In the third experiment, microcuttings were placed on DKW substrate for root induction containing 2.0 mg/l of NAA and kept for 3 days in the dark, and then transferred to the same substrate of the 2nd experiment (peat + perlite), but wetted with liquid medium without auxin. After 34 days the number of rooted microshoots and the number of roots per shoot were detected.

In the fourth experiment, Trifoliate orange microcuttings were tested for rooting on DKW medium with the following treatments:

1) IBA at a concentration of 0.3 mg/l. After 24 days microcuttings were transferred to auxin-free medium. After 15 days the number of rooted microshoots were recorded.
2) IBA increased to 0.6 mg/l. The number of rooted shoots were detected after 25 days.
3) NAA at a concentration of 0.3 mg/l. The number of rooted shoots was detected after 25 days.

Statistical analysis

All experiments were repeated twice and one-way analysis of variance and comparisons between means were made according to Tukey's multiple range test at $P \leq 0.05$ by using the SigmaStat 11 software. For all experiments of proliferation, each explant (6 per pot) was considered a replicate. Three pots were established for each rootstock.

For rooting experiments, 30 replicates (10 microshoots/pot) of Citrumelo and Troyer Citrange and 18 replicates (6 microshoots/pot) of Trifoliate orange were tested in each treatment.

Results and Discussion

Surface disinfection

The protocol 1, performed on shoots of Trifoliate orange and Alemow treated with 40% ethanol and 20% hypochlorite, gave poor results with only 5% of the explants being successfully disinfected in both rootstocks (Table 2). The disinfection of Volkamer lemon and Sour orange, using 20 % hypochlorite and ethanol 80% (protocol 2), also showed unsatisfactory results (Table 2). The best results were obtained for all four rootstocks, with 80% ethanol in the pretreatment followed an immersion in a 25% hypochlorite solution (protocol 3), obtaining 95% of explants disinfected of Sour and Trifoliate orange, while lower rates were performed in Alemow (55%) and Volkamer lemon (75%). The non-contaminated explants resulted viable and growing after 15 days of visual inspection of medium.

After sterilization, the two media (M1 and M2), as used by Russo et al. (2008) for *Citrus clementina*, added with 0.4 mg/l Kinetin in a similar way of El-Morsy and Millet (1996) on *Citrus aurantium* and Al-Khayri and Al-Bahrany (2001) on *Citrus aurantifolia*, showed bad results in terms of multiplication. The replacement of the M&S formula with that of DKW had a positive effect on the development of microshoots, as observed by Perez-Tornero et al. (2008) on *Citrus limon*.

Proliferation phase

In the first experiment, only the Trifoliate orange microshoots found the M1 substrate suitable for the establishment and proliferation, reaching a proliferation rate of 1:5.9 after three cycles of cultivation of 15 days each one (Table 3). Microcuttings were extremely miniaturized and light green. Sour orange, Volkamer lemon and Alemow did not give satisfactory results in the same medium, highlighting several issues such as hyperhydricity, shoot curling, abnormal callus production, so to determine the abandonment of the *in vitro* culture. Similar results (data not shown) were obtained with the M2 medium and the increase of Kinetin concentration (0.8 mg/l in both media) caused only more symptoms of hyperhydricity.

In the second experiment, regarding Citrumelo and Troyer Citrange, already established in vitro, and Trifoliate orange, the replacement of the MS salt formula with that of DKW had a very positive effect on the appearance and proliferation of the microshoots that looked well miniaturized and without hyperhydricity, also showing a proliferation rate of 1:5.8, 1:6.4 and 1:8.9 in Troyer citrange, Citrumelo and Trifoliate orange, respectively (Table 4).

For the three rootstocks, subcultures should not exceed 20 days in order not to fall into the starvation and yellowing of the tissues.

Rooting phase

Initially, microcuttings of all rootstocks were inserted onto a DKW medium with 0.6 mg/l of IBA. After 24 days, only 5.5% of the Trifoliate orange microcuttings were rooted (Table 5). Their transfer on an auxin-free medium increased the rooting percentage (16,6% after 10 days). When IBA concentration was doubled, the rhizogenesis response reached 24,2%, while NAA auxin use gave the best result (33,3%).

In the first rooting test, Citrumelo microshoots placed on a medium containing 0.6 mg/l IBA, reaching only 6.6% of rooting after 30 days. Their transfer onto a free-auxin substrate determined an increase in the rooting percentage (Table 5) and produced plantlets with one or two vital roots and small amounts of callus at the base (Figure 1). This increase led to formulate a hypothesis based on a biological effect of auxin, which after a positive effect on the rhizogenesis induction, could exert an inhibitory action on the development of the roots, similarly as Galzy (1972) observed on grapevine.

In the second test the replacement of IBA with another auxin (NAA 1 mg/l) in combination with 7 days of darkness caused yellowing and starvation of the rootstocks microcuttings. Timely transfer to auxin-free medium allowed the microshoots to regain the green color and a good rooting response equal to 53.3% (Table 5). The results of more trials show that dark exposure did not have any rhizogenic effect on microcuttings.

According to Pierik and Steegmans (1975), also the low oxygenation of the agarized media could cause limited root formation *in vitro*, therefore, medium aeration at the proximal end of the of the microcuttings could favor rooting. In fact, in the third test, the best results were obtained using a more porous substrate, such as a mixture of peat/perlite sterilized, in substitution of the medium agar, wetted with a solution prepared with half-strength DKW mineral salts solution and containing 0.8 mg/l IBA.

On this medium Citrumelo microcuttings reached 100% of rooting and an average of root number equal to 1.2. Similarly, Troyer citrange showed a rooting capacity with a noticeable percentage of 30% and with one root for each microcutting (Table 6 and Figure 2).

In the fourth trial, the short auxin treatment was effective without causing the negative problems of more prolonged exposure. In fact, after 30 days Citrumelo microshoots still showed 100% of rooting, while Troyer citrange showed a significant increase in the rooting

percentage reaching 70% of microcuttings rooted (Table 7). No significant increase was observed in the number of roots per plantlet.

All the tests conducted on Poncirus (Table 8) showed a modest rhizogenic capacity for this rootstock. Montoliu et al. (2010) obtained on Carrizo citrange similar results (30.2% of rooted microshoots after 40 days of culture) with a NAA treatment at the concentration of 1 mg/l (5.4 μM). However, their experimentation, which also included combinations of two auxins (IBA and NAA), led to the conclusion that the highest percentage of rooting (68%) was obtained with 10.8 μM of NAA (about 2 mg/l) and addition of GA3 (0.3 μM).

Conclusions

The potential to produce new citrus rootstocks by either conventional means or in combination with modern technologies is substantial and demonstrated (Castle, 2010). Therefore, it is interesting to consider creating new rootstocks in the future with modern tools to identify genetic elements related to specific traits and to transfer them from one source to another, and quickly confirm that the desired event has occurred, allow creative concepts for rootstock improvement. As the knowledge base increases, perhaps new rootstocks designed in response to concerns could more readily be produced, and the aim of this research was that to contribute in this direction and to support modern molecular studies and breeding. As a conclusive result, some aspects of citrus micropropagation have been clarified (optimized medium formula and plant growth regulators), others need to be further investigated and investigated (influence of substrate aeration or auxin action time for rooting).

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Tables

Table 1. Short descriptions of characteristics, advantages and disadvantages of rootstocks tested in this research.

Scientific name	Common name	Characteristics and notes
***Citrus aurantium* L.**	Sour orange, Common sour orange, or Standard sour orange, also known as Bitter orange	It can stand several degrees of frost for short periods. In proper conditions the tree can attain exceptional longevity. Sour orange is one of the oldest and most widely used rootstocks for other citrus plants. It is suitable for almost all the most common commercially grown citrus types. It would probably continue to be the preferred rootstock worldwide if it were not susceptible to the Tristeza virus. It is somewhat tolerant to salinity, alkalinity and less than optimal drainage, and is relatively tolerant to cold, cotton foot rot and *Phytophthora* (Castle, 2010). Grapefruit and orange yields on sour orange are moderate, with average fruit size but good quality. Its advantages**:** *Phytophthora* tolerant, waterlogging tolerant, cold tolerant, blight tolerant, alkaline tolerant, salt tolerant, high sugar content, medium fruit size, trees of medium vigour. Disadvantages: nematode susceptible, Tristeza (TCV) susceptible.
***Poncirus trifoliata* (L.) Raf.**	Trifoliate orange	It is included in the Citrus because the trifoliate orange and some of its hybrids provide valuable rootstock varieties for other citrus types and because the fruits of some of its hybrids approach edibility. The plant is a highly distinctive deciduous shrub or small tree with very large stout spines and small compound leaves with winged petioles and three leaflets. Seedlings are important in most citrus-growing areas as rootstocks for various Citrus and related species (Castle et al., 2004). As an outdoor ornamental, this plant is commonly grown in the warm temperate regions of China, Japan, western Europe, and the eastern United States and is sometimes used as a hedge, for which it is very effective. It has long been the most important rootstock in Japan, primarily for the Satsuma mandarins, and is increasingly being employed in Australia, California, and Argentina. Although remarkably different from other citrus in nearly all respects, Citrus trifoliata hybridises freely with other Citrus species. Beginning in Florida in 1897 and continuing for several decades, many crosses were made between the trifoliate orange and other citrus species and some with other genera. From this work came a series of bigeneric hybrids - the citranges, citrumelos, citrandarins, citremons, citradias, and citrumquats - a few of which are of horticultural importance or promise. Advantages: Phytophthora resistant,

		nematode resistant, tristeza immune, cold tolerant, tolerates waterlogging, good fruit quality, compact tree. Disadvantages: dislikes high pH soil, dislikes highly acid soil, sensitive to salinity, sensitive to calcareous soils, drought sensitive, exocortis susceptible, slow growing in nursery.
***Citrus volkameriana* V.Ten. & Pasq.**	Volkamer lemon	This variety has been known for three centuries. It was first thought of as a cross of lemon and sour orange. More recently it has been described as a variant of mandarin lime. Slightly smaller than lemon trees, it flowers and bears fruit profusely. This and the attractively dense foliage make it an excellent ornamental tree. The Volkamer lemon is used as rootstock for other citrus types because of its resistance to many diseases (Castle, 2010). Advantages: It is tolerant to flooding, drought tolerant, tristeza (CTV) tolerant, exocortis (CEV) tolerant, high yield, high vigour, salt tolerant, alkalinity tolerant. Disadvantages: phytophthora susceptible, low cold tolerance, low blight tolerance, nematode susceptible, low sugar/solids, large tree size
Citrus paradisii* Mafe *x* *Poncirus trifoliata	Citrumelo	It is a cross of grapefruit and trifoliate orange. Citrumelo is sensitive to high chloride levels in soil and irrigation water but is more salt tolerant than other trifoliate hybrids such as Carrizo and Troyer citranges. It is sensitive to high pH soils and is unsuitable for highly calcareous soils (Castle, 1987). Soils with a clay content greater than 25-30% may restrict root growth. It is unsuitable for heavy clay soils and is also sensitive to over-watering. However, in trials in South Australia, it appears more tolerant to waterlogging than Troyer and Carrizo citranges. Citrumelo has moderate drought tolerance and is highly cold tolerant. It has some resistance to *Phytophthora* root and collar rots but is less resistant than *Poncirus trifoliata*. Tolerant to citrus nematode (*Tylenchulus semipenetrous*). Trees have good tolerance to citrus tristeza virus (TCV). Cultivars grafted on Citrumelo are susceptible to exocortis (scalybutt). Budwood for propagation should be obtained from the Auscitrus propagation scheme to ensure freedom from citrus exocortis viroid (CEV). Citrumelo is a superior rootstock for grapefruit producing high yields of large, excellent quality fruit with high juice content. It tends to overgrow most orange scion cultivars. It is incompatible with Eureka lemon and is not recommended for Imperial mandarin due to cincturing and overgrowth at the bud union. It is incompatible with Meyer lemon. Citrumelo has been used as a rootstock for Murcott tangor in Queensland.

		Advantages: Phytophthora resistant, drought tolerant, nematode resistant, tristeza tolerant, cold tolerant, highly polyembryonic. Disadvantages: dislikes high pH soil, dislikes clay soil, sensitive to salinity and waterlogging, sensitive to calcareous soils, overgrows orange scions.
Citrus sinensis* x *Poncirus trifoliata	Troyer citrange	Originated as a hybrid of the Washington navel orange crossed with trifoliate orange pollen that was made at Riverside, California in 1909. After twenty-five years from the first field trial it had become the rootstock most employed and is much in demand elsewhere. The tree is vigorous, upright spreading, and medium-large with rather slender, thorny branchlets, foliage moderately dense, evergreen to semi-evergreen. Leaves dark green, medium in size, and mainly trifoliate, occasionally unifoliate. Productive and hardy. Tolerant to lime-induced chlorosis and the tristeza virus (Castle, 1987). Advantages: Phytophthora tolerant, nematode tolerant, Tristeza tolerant, cold tolerant, highly polyembryonic, moderately vigorous. Disadvantages: dislikes high pH soil, dislikes clay soil, sensitive to salinity and waterlogging, sensitive to calcareous soils, overgrows mandarin scions.
***Citrus macrophylla* Wester**	Alemow	It is a hybrid of Celebes papeda (*Citrus celebica*) with a species of the subgenus Citrus, probably a pomelo (*Citrus maxima*). It has been tried as a rootstock for lemons in California. It is sometimes cultivated in Cebu, Philippine Islands. The fruits are very large, 8.5 to 10 cm in diameter, subglobose to oblong, narrowed at the base, with a rough, transversely corrugated, but rather thin skin. Very sensitive to cold and sore dry but has good affinity with all cultivars of citrus (Castle et al., 2004). Alemow is recommended as a dwarfing rootstock for old-line 'Temple.' (Levy et al., 1993). Advantages: resistance to phytophthora (foot rot), flood tolerant, drought tolerant, blight tolerant, CEV tolerant, high yield, large fruit size, high vigour, alkalinity tolerant, good salt tolerance. Disadvantages: low cold tolerance, low blight tolerance, tristeza susceptible, nematode susceptible.

Table 2. Effect of surface disinfection protocols (% of non-contaminated explants) on explants of citrus rootstocks (Volkamer lemon, Sour orange, Trifoliate orange and Alemow). Different letters indicate statistically significant difference within the treatments, for P≤0.05 as determined by Tukey's test, n = 18.

Treatment	**Volkameriana**		**Sour orange**	
ethanol 80% + Na-hypochlorite 20%	22,5 a		5 b	
	Poncirus		**Alemow**	
ethanol 40% + Na-hypochlorite 20%	5 a		5 a	
	Volkameriana	**Sour orange**	**Poncirus**	**Alemow**
ethanol 80% + Na-hypochlorite 25%	75 b	95 a	95 a	55 c

Table 3. Number of microshoots produced on average from each microcutting of four rootstocks (Alemow, Sour orange, Trifoliate orange and Volkamer lemon) after three cycles of *in vitro* cultivation on M1 and M2 media (three pots). Different letters indicate significant statistically difference among the rootstocks, for P≤0.05 as determined by Tukey's test, n = 18.

Rootstock	**M1**	**M2**
Alemow	1.6 ±0.18 c	1.4 ± 0.15 c
Sour orange	1.9 ±0.25 bc	1.7 ± 0.21 c
Trifoliate orange	5.9 ± 0.22 a	5.5 ± 0.23 a
Volkamer lemon	2.1 ± 0.20 b	2.0 ± 0.16 b

Table 4. Number of microshoots produced on average from each microcutting of three rootstocks (Citrumelo, Troyer citrange and Trifoliate orange), after three cycles of *in vitro* cultivation on DKW medium (three pots). Different letters indicate significant statistically difference among the rootstocks, for P≤0.05 as determined by Tukey's test, n = 18.

Rootstock	**DKW**
Citrumelo	6.4 ± 0.15 b
Troyer citrange	5.8 ± 0.18 c
Trifoliate orange	8.9 ± 0.14 a

Table 5. Rooting percentage of Citrumelo, Troyer citrange, Trifoliate orange microcuttings cultivated on DKW medium with different auxin concentration (IBA or NAA, the latter only in dark conditions). The "zero" treatments mean that microcuttings were transferred to an auxin-free substrate after 10 days. Different letters indicate significant statistically difference among the treatments, for P≤0.05 as determined by Tukey's test, n = 18.

Auxin type and concentration	**IBA (0.6 mg/l)**	**IBA (0 mg/l)**	**NAA (1.0 mg/l)**	**NAA (0 mg/l)**
Citrumelo	6.6 c	23.3 b	10.5 c	53.3 a
Troyer citrange	0.0 c	6.6 b	8.3 b	36.5 a
Trifoliate orange	16.5 d	24.2 c	33.3 b	68.4 a

Table 6. Rooting percentage and number of roots for microcutting of Citrumelo and Troyer citrange rootstocks cultivated *in vitro* on a peat/perlite substrate (30 days), wetted with liquid medium containing 0.8 mg/l IBA. Different letters indicate significant statistically difference among the rootstocks, for P≤0.05 as determined by Tukey's test, n = 18.

Rootstock	Rooting (%)	Mean root number
Citrumelo	100 a	1.2 a
Troyer citrange	30 b	1.0 a

Table 7. Rooting percentage and number of roots for microcutting of Citrumelo and Troyer citrange rootstocks cultivated in vitro on DKW medium, containing 2.0 mg/l of NAA, and then transferred on a peat/perlite substrate (30 days), wetted with liquid medium auxin-free. Different letters indicate significant statistically difference among the rootstocks, for P≤0.05 as determined by Tukey's test, n = 18.

Rootstock	Rooting (%)	Mean root number
Citrumelo	100 a	1.3 a
Troyer citrange	70 b	1.1 a

Table 8. Rooting percentage and number of roots for microcutting of Trifoliate orange rootstock cultivated in vitro on DKW medium, containing different auxin (IBA or NAA) and concentration of IBA (0.3 and 0.6 mg/l). Different letters indicate significant statistically difference among the rootstocks, for P≤0.05 as determined by Tukey's test, n = 18.

Treatment	Rooting (%)	Mean root number
IBA 0.3 mg/l	16.6 c	1.3 a
IBA 0.6 mg/l	22,2 b	1.2 a
NAA 2.0 mg/l	68,3 a	1.5 a

Figures

Figure 1 - Citrumelo microshoots rooted after in vitro cultivation on a DKW medium containing 0.6 mg/l IBA and transferred onto a free-auxin substrate. This protocol produced plantlets with one or two vital roots and poor callus.

Figure 2 - Citrumelo (left) and Troyer citrange (right) microshoots cultivated *in vitro* on a peat/perlite substrate (30 days), wet with liquid medium containing 0.8 mg/l IBA.