Available online: www.notulaebotanicae.ro

Print ISSN 0255-965X; Electronic 1842-4309

Not Bot Horti Agrobo, 2017, 45(2):383-391. DOI:10.15835/nbha45210900

Original Article

In vitro Propagation and Assessment of Genetic Relationships of Citrus Rootstocks Using ISSR Molecular Markers

Constantinos SALIS[1], Ioannis E. PAPADAKIS[1], Spyridon KINTZIOS[2], Marianna HAGIDIMITRIOU[2]*

[1]*Agricultural University of Athens, Department of Crop Science, Iera Odos 75, 118 55 Athens, Greece; saliskonstantinos@yahoo.com; papadakis@aua.gr*

[2]*Agricultural University of Athens, Department of Biotechnology, Iera Odos 75, 118 55 Athens, Greece; skin@aua.gr; marianna@aua.gr (*corresponding author)*

Abstract

The behavior of six citrus rootstocks, *Volkameriana*, *Citrumelo* 'Swingle', *Citrange* 'Carrizo', *Poncirus trifoliata* 'Serra', *Poncirus trifoliata* 'Rubidoux' and *Poncirus trifoliata* 'Flying Dragon', in *in vitro* propagation was studied and compared for shoot proliferation and rooting. In addition, the genetic relationships among the rootstocks studied and other Citrus species, using the Inter-Simple Sequence Repeats (ISSR) molecular markers, were investigated. Nodal explants of three months old shoots were used in Murashige and Skoog medium supplemented with N6-benzyladenine (BA) for shoot proliferation and with naphthaleneacetic acid (NAA) for rooting. The rootstock *Volkameriana* showed a statistically significant higher number of shoots (1.81), shoot length (15.14 mm) and number of leaves per explant (5.81), while all three *Poncirus trifoliata* rootstocks showed the lowest numbers. The number of roots and root length per explant were evaluated at the end of the rooting phase. The rootstock 'Swingle' showed a higher number of roots per explant (4.2) followed by 'Flying Dragon' (3.93) and 'Carrizo' (3.23) rootstocks. The rootstocks 'Swingle' (140.8 mm), *Volkameriana* (148 mm) and 'Flying Dragon' (131.12 mm) had significantly higher root length per explant compared to 'Carrizo' (31 mm) and 'Rubidoux' (34.5 mm). The ISSR molecular marker technique used in the present study grouped successfully the different species, varieties and rootstocks studied, revealing their genetic variability. The genetic variability observed among the rootstocks ranged between 0.29 (*Poncirus trifoliata* 'Serra' and *Citrumelo* 'Swingle') and 0.60 (*Volkameriana* and *Citrumelo* 'Swingle'). The response of the rootstocks studied in *in vitro* propagation however is not related to their genetic affinity.

Keywords: citrus, *in vitro*, ISSR, micropropagation, molecular markers, PCR, rootstocks

Introduction

The genus *Citrus* belongs to the Rutaceae family, sub-family Aurantioideae, tribe Citrae and sub-tribe Citrinae (Reuther *et al*., 1967). Currently, there are two main classification systems of the *Citrus* genus, the W. T. Swingle system and the T. Tanaka system. According to Swingle (1943), the citrus genus is classified in two sub-genera the *Citrus* or *Eucitrus* sub-genus and the *Papeda* sub-genus. As centre of origin for the *Citrus* genus is considered the South-East Asia, and mostly the area between China and India (Gmitter and Hu, 1990; Soost and Roose, 1996).

The genus *Citrus* is cultivated in more than 100 countries, mostly in tropical and sub-tropical areas, and it represents one of the most important commercial fruit crops in terms of economic value and human nutrition. According to FAO (2015) in the year 2013/14 121,273.2 thousand tons of citrus were produced globally, of which 15,022.9 thousand tons were used as exportable products. China ranked first in citrus products, reaching 29,567 thousand tons (2013/14), while countries of the Mediterranean region were the largest exporters with 12,282 thousand tons for the same year. The rootstock plays an important role in a productive orchard, since it affects the characteristics of the scion, such as growth, plant and fruit shape, fruit colour and weight, content of phytochemicals in fruits and juices, post-harvest storability (Ritenour *et al*., 2004; Rouphael *et al*., 2010; Orazem *et al*.,

Received: 15 May 2017. Received in revised form: 20 Aug 2017. Accepted: 24 Aug 2017. Published online: 15 Sep 2017.

2011; Turhan *et al*., 2011; Castle *et al*., 2016; Papadakis, 2016). In addition, rootstock helps the rootstock-scion combination to adapt to different abiotic factors, such as drought, flooding, salinity, mineral deficiency and toxicity, metal toxicity, heat, cold, soil temperature and oxygen, pH etc. (Papadakis *et al*., 2004; Colla *et al*., 2010; Hartmann *et al*., 2013; Savvas *et al*., 2010; Ghrab *et al*., 2014; Castle *et al*., 2016) and different biotic factors such as fungal and bacterial pathogens, virus, diseases, insects or nematodes (Mudge *et al*., 2009; Shokrollah *et al*., 2009; Roistacher *et al*., 2010; Louws *et al*., 2010; Castle *et al*., 2016). The choice of rootstock depends on its durability to enemies and diseases. Also, rootstocks affect the development of the graft, therefore the time of entering in production (Vasilakakis and Therios, 2006). Reduction of tree size without affecting production or plant health is a desirable characteristic (Soost and Roose, 1996). Citrus are considered sensitive to salinity, though some species show same tolerance to salt concentration (Storey and Walker, 1999; Ben-Hayyim and Moore, 2007). Rootstock's benefits except salinity resistance, are higher-yield and better growth, higher photosynthesis, water content and elevated concentrations of antioxidants and abscisic acid and lower contents of sodium or chloride, compared to ungrafted plants (Colla *et al*., 2010; Penella *et al*., 2016).

The propagation of *Citrus* rootstocks is commonly based on nucellar seeds (Barlass and Skene, 1982). This way of multiplication, though, has its limitation regarding the nucellar polyembryony, levels of heterozygosity, serious pathogen infections etc. Plant tissue culture approach solves the above problems by ensuring mass availability at plant material, maintaining clonal uniformity under given environmental conditions and preserving their selected traits (Hartmann *et al*., 2004). Furthermore, *in vitro* propagation systems can guarantee the production of pathogen-free material.

The morphogenic responses of *Citrus* cultured *in vitro* are depending on the genotype, explant type and culture medium (Carimi and De Pasquale, 2003; Perez-Tornero *et al*., 2010). In *Citrus*, the formation of adventitious organogenesis of shoots and buds has been observed (Barlass and Skene, 1982; Gmitter *et al*., 1992). This procedure is controlled by hormones, as the presence of BA cytokinin is decisive for the existence of organogenesis, but the ideal concentration depends on the genotype of the plant (Barlass and Skene, 1982).

The identification of species and varieties, as well as their genetic relationships, is possible with the aid of various markers like morphological, biochemical, cytogenetic, but the most efficient are the molecular (DNA) markers, which are based on the differences in their DNA sequence (Bretting and Widrlechner, 1995). In *Citrus*, markers that use PCR (Polymerase Chain Reaction) technology, have successfully been used, such as ISSR (Scarano *et al*., 2002), RAPD (Asadi and Isshiki, 2003), RFLP (Fang *et al*., 1997) and SCAR (Nicolosi *et al*., 2000).

The present study aimed to investigate the response to *in vitro* propagation (proliferation and rooting) of six *Citrus* rootstocks: 1. *Volkameriana*, used for its tolerance to tristeza virus and soil calcium, and give to the grafted variety early entering in production, 2. *Citrumelo* 'Swingle', used for its resistance to *Phytophthora* spp., nematodes, and tolerance to low temperatures (Gmitter *et al*., 2009; Vasilakakis and Therios, 2006), 3. *Citrange* 'Carrizo', used for its tolerance to tristeza virus and *Phytophthora* spp, and giving to the grafted variety high production and large size fruits with good quality, and three rootstocks coming from *Poncirus trifoliate*, used for their resistance to tristeza virus (Mestre *et al*., 1997), their resistance to low temperatures and their resistance to nematodes and *Phytophthora* spp, 4. *Poncirus trifoliata* 'Serra', 5. *Poncirus trifoliata* 'Rubidoux' and 6. *Poncirus trifoliata* 'Flying Dragon', with the later giving dwarf tree characteristics. In addition, the genetic relationships of the rootstocks studied, in combination with other *Citrus* species, have been investigated using the Inter-Simple Sequence Repeats (ISSR) molecular marker technique in order to reveal possible associations of the genotypes to *in vitro* response.

Materials and Methods

Plant material

Shoots of 3-year-old mother plants of three widely used rootstocks *Volkameriana*, *Citrumelo* 'Swingle', *Citrange* 'Carrizo' and three rootstocks used at a lesser extent, *Poncirus trifoliata* 'Serra', *Poncirus trifoliata* 'Rubidoux' and *Poncirus trifoliata* 'Flying Dragon', cultivated at the farms of 'Hellenic Plants' nursery (Xylokastro, Greece, 38.0773° N, 22.6327° E) were collected and used as plant material for the present study. Fifteen more samples from different *Citrus* species and varieties were also obtained from the same nursery. The biological material (Table 1) was originally acquired from CRSFA (Italy), the Pomology Institute of Poros (Greece) and the Agricultural University of Athens orchard.

Micropropagation

The collected shoots were defoliated and cut into node explants with a single node each of 1 to 3 cm in length. The following protocol for decontamination was used: the explants were washed out with a solution of commercial bleach 20% for 7 min, followed by two 5 min washes with sterilized water, a solution of ethanol 70% for 1 min and finally, three washings with sterilized water.

The basal medium used was MS medium (Murashige and Skoog, 1962), supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar. For the proliferation phase the MS medium was supplemented with five different concentrations of N6-Benzyladenine (BA) (0, 0.5, 1, 2 and 4 ppm). For rooting stage, explants were transferred under aseptic conditions to MS medium supplemented with 30 g L^{-1} sucrose, 6 g L^{-1} agar and five different concentrations of naphthaleneacetic acid (NAA) (0, 0.5, 1, 2 and 4 ppm). After the addition of growth regulators and adjustment of pH medium to 5.7-5.8, 10 ml of medium was dispensed into 150 × 20 mm culture tubes. The culture tubes with medium were sterilized in autoclave at 121 °C for 21 min. Cultures were grown at 24±1 °C, with a 16 h photoperiod. Proliferation responses were evaluated after 60 days and rooting responses after 70 days in culture.

DNA markers

DNA from young healthy leaves from the rootstocks studied and from different *Citrus* species and varieties was extracted using the PowerPlant kit. Concentration and purity of the DNA was measured at 260 and 280 nm with a spectrophotometer (Unicam Helios γ). Five ISSR primers (UBC 807, UBC 810, UBC 812, UBC 817 and UBC818) were used for the study. DNA quality was also checked with electrograph of Agarose gel 1% (w/v). The final concentrations for the PCR reaction were: 35ng DNA, 2.0mM $MgCl_2$, 200mM dNTPs, 1.0 Unit Taq and 0.5 mM primer (Tripolitsiotis *et al*., 2013).

DATA analysis

Micropropagation data were analysed using analysis of variance (ANOVA) and the 'PASW Statistics 18' statistical package (SPSS Inc., Chicago, USA). For rooting phase, means of control (0 ppm NAA) and NAA-treated (2 ppm NAA) explants of each genotype were compared using Student's *t-test*, at a significance level of $p \leq 0.05$. Genotype effects on proliferation and rooting stages, regardless of auxin (NAA) or cytokinin (BA) levels, respectively, were evaluated using the Duncan's multiple range test ($p \leq 0.05$). The same test was applied for the comparison of the effects of various BA concentrations in different proliferation traits of each genotype. The genetic similarities, obtained from the use of molecular markers, were calculated using the Jaccard algorithm and the dendrogram was constructed using the UPGMA (unweighted pair group method with arithmetic means).

Results and Discussion

Proliferation phase

Cytokinins and auxins are the most important plant growth regulators for shoot proliferation and rooting, respectively, in *Citrus* rootstocks explants (El-Morsy and Millet, 1996; Harada and Murai, 1996; Ghorbel *et al*., 1998; Murkute *et al*., 2008). In many species, BA has been found to be more effective than other cytokinins (like Kinetin) in inducing shoot development (Pattnaik *et al*., 1996; Yadav *et al*., 1990). BA has been the most commonly used plant growth regulator for proliferation of *Citrus* shoots (Carimi and De Pasquale, 2003). In the present study, the presence of BA increased the proliferation percentage in all rootstocks studied, except *Volkameriana*, which presented 100% shoot induction also in the control explants (0 ppm) (Table 2). Highest proliferation percentage for most of the rootstocks was observed at a concentration of 2 ppm BA. Tallón *et al*. (2012), reported also in *Citrus* rootstocks 'Alemow' and 'Cleopatra' the highest shoot proliferation using 2 ppm of BA. Rootstocks C*itrange* 'Carrizo' and *Volkameriana* also presented 100% shoot induction in 1 ppm of BA (Table 2). Many studies (Marques *et al*., 2011; Sharma *et al*., 2009; Pena *et al*., 1995; Rani *et al*., 2004) reported in various *Citrus* species highest proliferation percentage in that 1 ppm of BA. Also, it was observed that by increasing the BA concentration from 2 to 4 ppm, proliferation percentage was decreased in most of the rootstocks studied. This observation comes in agreement with several researchers (El-Morsy and Millet, 1996; Normah *et al*., 1997; Al Bahrany, 2002) stating that higher concentrations of BA reduced shoot induction.

The presence of BA significantly increased the number of shoots per explant in *Volkameriana*, *Citrange* 'Carrizo' and *Poncirus trifoliata* 'Serra'. Maximum number of shoots per explant was observed in *Volkameriana* at 1 ppm of BA (Table 2). Different researchers (Begum *et al*., 2008; Marques *et al*., 2011) observed in *Citrus aurantium* (sour orange) that 1 ppm of BA is the optimum concentration for maximum number of shoots per explant. *Citrange* 'Carrizo'

Table 1. Rootstocks and varieties used in the present study, place of origin, and species

No	Samples	Place of origin	Species/origin
2	'Rubidoux'	CRSFA (Italy)	*P. trifoliata*
3	*Siamelo*	CRSFA	*C. reticulata× C. sinensis × C. paradisi*
4	'Flying Dragon'	CRSFA	*P. trifoliata*
5	'Serra'	CRSFA	*P. trifoliata*
7	*C. aurantium*	CRSFA	*C. aurantium*
20	*Alemow*	CRSFA	*C.limon*
13	'Citrumelo 4475'	Pomology Institute of Poros (Greece)	*P. trifoliata × C. paradisi*
15	C. l. 'Eyreka'	CRSFA	*C. limon*
17	C. l. 'Meyer'	CRSFA	*C.limon*
21	C. s. 'Navellate'	CRSFA	*C. sinensis*
19	C. s. 'Tarocco Rosso'	CRSFA	*C. sinensis*
9	C. p. 'Marsh Seedless'	CRSFA	*C. paradisi*
22	*P. trifoliata*		*P. trifoliata*
23	'Swingle'	CRSFA	*P. trifoliata × C. paradisi*
27	'Citrumelo 1452'	CRSFA	*P. trifoliata × C. paradisi*
24	*Volkameriana*	CRSFA	*C. medicax C. limon*
6	*Cleopatra*	CRSFA	*C. reticulata*
28	'Troyer'	CRSFA	*P. trifoliata × C. sinensis*
29	'Carrizo'	Pomology Institute of Poros	*P. trifoliata × C. sinensis*

Table 2. Shoot proliferation percentage, number of shoots per explant, total length of shoots per explant and number of leaves per explant as affected by genotype and BA concentration (0, 0.5, 1, 2 or 4 ppm) in culture medium

Genotypes	Cytokinin (BA, ppm)	Shoot proliferation percentage (%)	Number of shoots per explant	Total shoot length per explant (mm)	Number of leaves per explant
'Carrizo'	Control (0)	47.20	0.72 a	6.29 a	2.21 a
	0,5	73.45	1.05 b	8.67 abc	3.70 bc
	1	100.00	1.18 b	9.79 bc	4.91 d
	2	100.00	1.20 b	10.40 c	4.44 cd
	4	100.00	1.09 b	7.45 ab	3.50 b
Volkameriana	Control (0)	100.00	1.30 a	12.00 a	3.85 a
	0,5	100.00	1.89 b	19.52 b	6.10 bc
	1	89.39	2.11 b	18.83 b	7.22 c
	2	100.00	2.05 b	14.61 ab	7.16 c
	4	81.67	1.75 b	10.31 a	4.75 ab
'Swingle'	Control (0)	21.12	1.00 a	8.33 ab	2.00 a
	0,5	92.03	1.00 a	5.66 a	3.08 a
	1	65.00	1.14 a	8.28 ab	4.14 a
	2	100.00	1.00 a	10.83 ab	2.16 a
	4	100.00	1.12 a	13.25 b	3.37 a
'Flying Dragon'	Control (0)	60.74	0.60 a	4.10 a	2.60 a
	0,5	100.00	1.00 a	6.40 a	5.10 ab
	1	100.00	1.07 a	6.14 a	6.00 b
	2	60.40	0.60 a	2.60 a	3.60 ab
	4	40.67	0.63 a	3.27 a	3.00 a
'Serra'	Control (0)	12.50	0.11 a	0.88 a	0.41 a
	0,5	43.70	0.43 ab	2.62 ab	1.81 ab
	1	64.20	0.64 bc	4.21 bc	2.78 bc
	2	81.20	0.93 c	6.00 c	3.75 c
	4	66.60	0.66 bc	2.80 ab	2.86 bc
'Rubidoux'	Control (0)	6.34	1.00 a	15.00 b	1.00 a
	0,5	46.30	1.16 a	6.33 a	4.50 bc
	1	63.00	1.00 a	5.45 a	5.36 c
	2	60.75	0.90 a	8.90 a	3.20 bc
	4	52.63	0.75 a	4.81 a	2.31 ab

Different letters in the same column and within each genotype indicate significant differences at P<0.05 (Duncan's multiple range test)

and *Poncirus trifoliata* 'Serra' showed highest number of shoots per explant at 2ppm of BA (Table 2). Perez-Tornero *et al*. (2010) in *Citrus limon* varieties 'Fino 49', 'Fino 77' and 'Messina', and Tallón *et al*. (2012) in *Cleopatra* rootstock, also stated that maximum number of shoots per explant were obtained at 2 ppm of BA. On the other hand, Kitto and Young (1981), noted that in 'Carrizo' highest number of shoots per explant was obtained using higher cytokinin concentrations, like 5 ppm of BA. Shoot length was not significantly affected by the presence of BA in all rootstocks studied, except *Volkameriana*, in which significant differences were shown among the different concentrations of BA, and 'Rubidoux', in which the presence of BA significantly decreased shoot length. Tallón *et al.* (2012), observed that the presence of BA did not affect significantly the shoot length in sour orange, but significantly affected the shoot length in *Alemow* and *Cleopatra* rootstocks. Perez-Tornero *et al.* (2010), in various lemon varieties, and Savita Singh *et al.* (2011) in *Citrus jambhiri* 'Lush', noted that BA presence significantly increased shoot length. In the present study, total shoot length per explant was significantly increased in *Volkameriana*, 'Carrizo' and 'Serra', in the presence of BA, but did not significantly affect the total shoot length per explant of *Citrumelo* 'Swingle' and *Poncirus trifoliata* 'Flying Dragon' (Table 2).

Rooting phase

The kind and the concentration of the auxin used in the culture media, play a key role in *in vitro* rooting and is affected by the plant species and the variety (George, 1996). The presence of auxin, including NAA or IBA, in the culture media is generally necessary to promote rooting in *Citrus in vitro* cultures (Carimi and De Pasquale, 2003). The most effective auxins for rooting are NAA, IBA and IAA (Bhojwani and Razdan, 1996). Rooting in some varieties is favored by a medium containing more than one hormone, like *Citrus reticulata* 'Blanco' and *Citrus limon* Burm. f. (Singh *et al*., 1994), while in others NAA alone induces rooting, like *Poncirus trifoliata* L. (Starrantino and Russo, 1980). In the present study, the presence of NAA in the culture media increased the rooting percentage in all rootstocks studied. In most of the rootstocks, maximum rooting percentage was obtained in the presence of 2 ppm NAA (Table 3). This finding is in agreement with different

researchers (Edriss and Burger, 1984; Tallón *et al*., 2012; Gill *et al*., 1995), stating that, in various *Citrus* species, 2 ppm of NAA was the optimum concentration for maximum rooting percentage. In addition, other studies have shown that maximum rooting, in various *Citrus* species, can be obtained in lower NAA concentrations, such as 1 ppm (Kitto and Young, 1981; Bordon *et al*., 2000; Al Bahrany, 2002; Rathore *et al*., 2007) or 0.5 ppm of NAA (Savita Singh *et al*., 2011). Rootstock *Poncirus trifoliata* 'Rubidoux' in control (0 ppm) obtained 0% rooting in explants (Table 3). Similarly, Al Bahrany (2002) observed that *Citrus aurantifolia* had 0% rooting in the absence of NAA.

The presence of auxin significantly increased the number of roots per explant in all rootstocks studied, except *Citrange* 'Carrizo' and *Volkameriana*. Rootstocks *Poncirus trifoliata* 'Flying Dragon', *Citrumelo* 'Swingle' and *Poncirus trifoliata* 'Rubidoux' presented the highest number of roots per explant using 2 ppm of NAA (Table 3), *Volkameriana* with 1 ppm of NAA and *Citrange* 'Carrizo' with 4 ppm of NAA (data not shown). Al Bahrany (2002) observed that the number of roots per explant in *Citrus aurantifolia* 'Swing' is increased with the simultaneous increase of NAA in the media, and 2 ppm of NAA was the optimum concentration for maximum number of roots per explant. Tallón *et al.* (2012), also observed in Sour Orange that maximum roots per explant were obtained with 2 ppm of NAA. Furthermore, in *Cleopatra (*Tallón *et al*., 2012), *Citrus macrophylla* (Ghorbel *et al*., 1998; Bordon *et al*., 2000) and *Citrus reticulata* 'Blanco' (Gill *et al*., 1995) 1 ppm of NAA was the optimum concentration of auxin for maximum number of roots per explants.

Total root length per explant was not significantly affected by the presence of NAA, in all rootstocks studied, except *Poncirus trifoliata* 'Rubidoux'.

Genotype effect on proliferation and rooting phases

One of the most important factors affecting the proliferation and rooting process of the explants is genotype. Genotype influences shoot formation and rooting (Cezar *et al*., 2015). Gomes *et al.* (2010) stated that the genotype of the donor plants is also a factor interfering with the multiplication. Also, Scaltsoyiannes *et al.* (1998), observed that in *Juglans regia,* genotype plays a crucial role in micropropagation.

Comparing the rootstocks studied, *Volkameriana* presented statistically significant higher number of shoots per explants (Fig. 1) as well as statistically significant higher total shoot length per explants (Fig. 2), followed by *Citrange* 'Carrizo' and *Citrumelo* 'Swingle'. This is in agreement with Carimi and De Pasquale (2003) who noted that the number of shoots per explants is different depending on genotype studied. The *Poncirus trifoliata* 'Serra' rootstock presented statistically significant lower number of shoots per explant and, along with *Poncirus trifoliata* 'Flying Dragon' and *Poncirus trifoliata* 'Rubidoux', statistically significant lower total shoot length per explant. Statistically significant higher root number per explant was obtained by *Poncirus trifoliata* 'Flying Dragon' and *Citrumelo* 'Swingle', followed by *Citrange* 'Carrizo' (Fig. 3). *Volkameriana* presented statistically significant lower number of roots per explant, along with *Poncirus trifoliata* 'Rubidoux'. Regarding the total root length per explants, statistically significant higher length was obtained from *Volkameriana, Poncirus trifoliata* 'Flying Dragon' and *Citrumelo* 'Swingle' rootstocks, while lower length was observed in *Citrange* 'Carrizo' and *Poncirus trifoliata* 'Rubidoux' (Fig. 4).

Genetic variability of the rootstocks studied

The use of molecular techniques has made taxonomic classification of *Citrus* species possible and more accurate. Knowledge of genetic variability and relationships among different genotypes is an important factor for the efficient exploitation of varieties' potential (Russell *et al*., 1997). Molecular markers differ in variability rates of detection and their efficiency depends on the species used (Lonn *et al*., 1995). The ISSR molecular markers technique used in the present study separated successfully 21 different samples belonging to different species, varieties and the rootstocks studied. Fang *et al.* (1997), used 46 ISSR molecular markers on various *Citrus* species, but only 11 of them were found to be polymorphic. Pasquale *et al.* (2006), used 11 ISSR and 6 RAPD molecular markers in order to test the genetic variability of five sour orange clones.

Table 3. Rooting percentage of the regenerated shoots, number of roots per explants, total root length per explants, drying rate and fresh weight of roots per length as affected by genotype and the concentration of NAA (0 or 2 ppm) in the culture medium

Genotypes	Auxin (a-NAA, ppm)	Rooting percentage (%)	Number of roots per explant	Total root length per explant (mm)	Drying rate (%)	Fresh weight of root per length (mg/mm)
'Carrizo'	Control (0)	31.20	2.60	156.40	00.00	0.012
	2	72.13	3.62 ns	142.25 ns	12.00 ns	0.023 *
Volkameriana	Control (0)	23.70	1.25	33.00	75.00	0.025
	2	73.65	1.64 ns	30.00 ns	57.00 ns	0.021 ns
'Flying Dragon'	Control (0)	64.24	1.50	84.16	00.00	0.015
	2	100.00	5.40 *	159.30 ns	30.00 ns	0.019 ns
'Swingle'	Control (0)	50.80	2.00	129.00	00.00	0.012
	2	100.00	5.66 **	148.00 ns	57.00 ns	0.018 ns
'Rubidoux'	Control (0)	00.00	0.00	0.00	00.00	0.00
	2	22.45	1.50 *	69.00 **	100.00 ***	0.015 *

Asterisks in parenthesis indicate significant differences between control and 2 ppm BA-treated plants of the same genotype, at P<0.05 (*), P<0.010 (**) or P<0.001 (***); n.s. indicates non-significant differences (P>0.05) (Student's t-test)

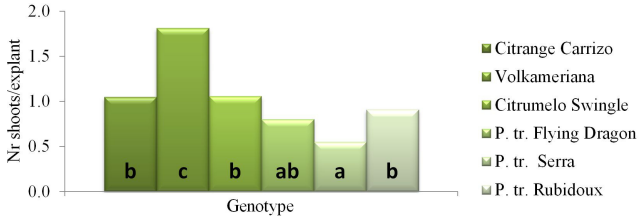

Fig. 1. Number of shoots per explant, of the genotypes tested in culture medium MS supplemented with different levels of BA. Different letters indicate significant differences among genotypes at P<0.05 (Duncan's multiple range test)

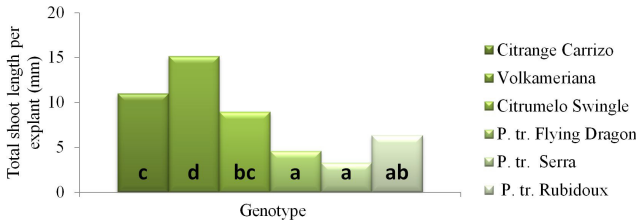

Fig. 2. Total shoot length per explant of the genotypes tested in culture medium MS supplemented with different levels of BA. Different letters indicate significant differences among genotypes at P<0.05 (Duncan's multiple range test)

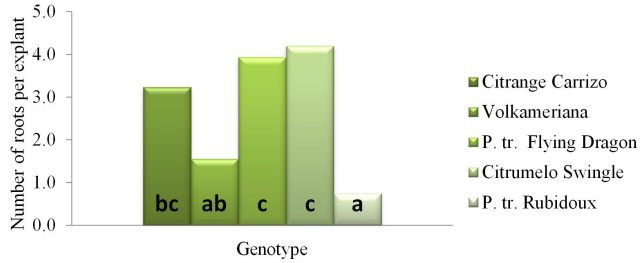

Fig. 3. Number or roots per explant, of the genotypes tested, in culture medium MS supplemented with different levels of NAA. Different letters indicate significant differences among genotypes at P<0.05 (Duncan's multiple range test)

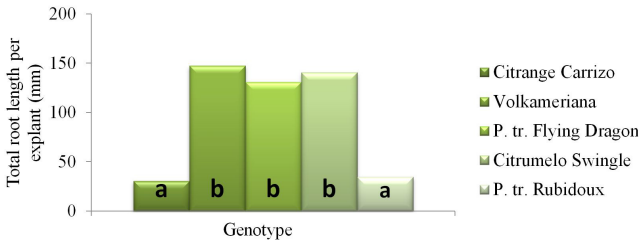

Fig. 4. Total length of roots per explant, of the genotypes tested, in culture medium supplemented with different levels of NAA. Different letters indicate significant differences among genotypes at P<0.05 (Duncan's multiple range test)

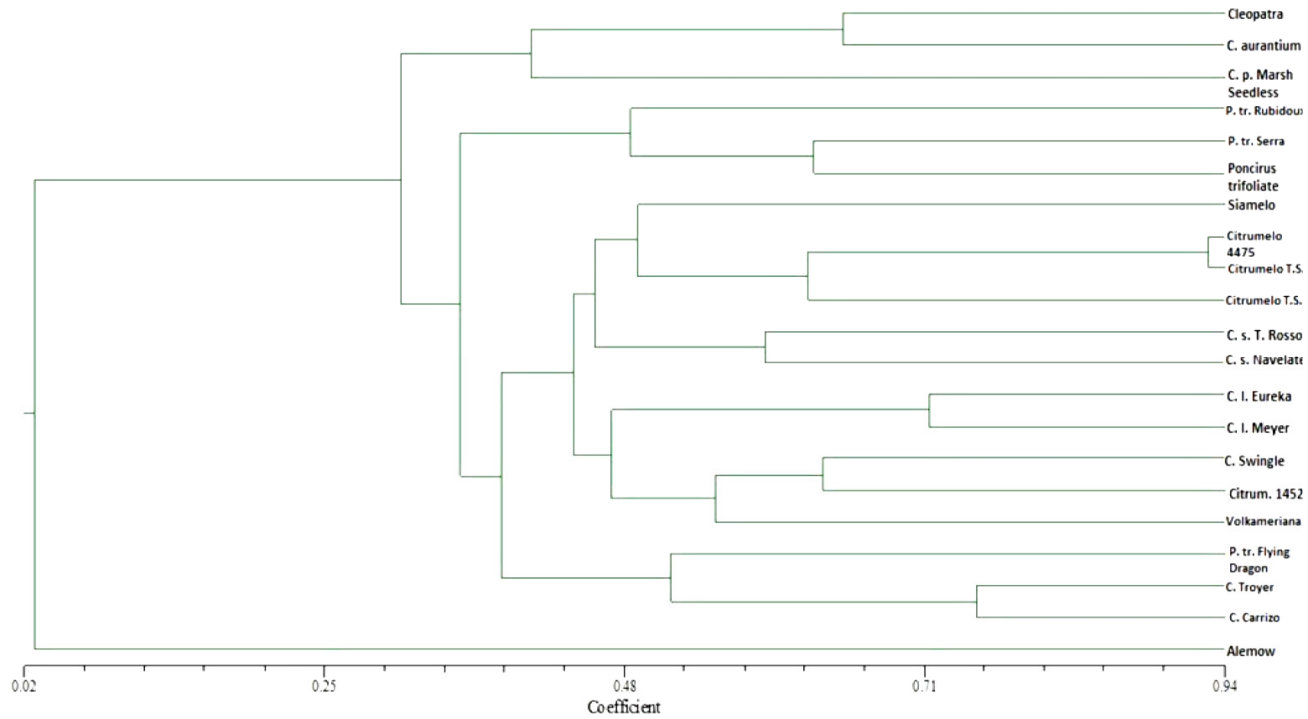

Fig. 5. UPGMA dendrogram for *Citrus* rootstocks and varieties based on five ISSR markers

According to the cluster (Fig. 5), among the rootstocks studied for their *in vitro* responses, higher genetic similarity was presented between *Volkameriana* and *Citrumelo* 'Swingle' (0.60), followed by *Citrumelo* 'Swingle' and *Citrange* 'Carrizo' (0.59). Amar *et al.* (2011), also observed a high genetic similarity (0.82) between *Citrumelo* and *Citrange*. Likewise, Romdhane *et al.* (2016), placed *Citrange* 'Carrizo' and *Citrumelo* 'Swingle' in a same genetic group. On the contrary, *Poncirus trifoliata* 'Serra' and *Citrumelo* 'Swingle' showed low genetic affinity, followed by *Volkameriana* and *Poncirus trifoliata* 'Serra' (0.32).

Poncirus trifoliata, *Poncirus trifoliata* 'Rubidoux' and *Poncirus trifoliata* 'Serra' were grouped together, with higher genetic similarity observed between *Poncirus trifoliata* and *Poncirus trifoliata* 'Serra' (0.63). The genotypes *Citrange* 'Carrizo' and *Citrange* 'Troyer' showed high genetic

similarity (0.75) and were grouped together with *Poncirus trifoliata* 'Flying Dragon' in the dendrogram (Fig. 5). Several studies (Uzun *et al*., 2009; Tripolitsiotis *et al*., 2013) using ISSR, RAPD and SRAP markers, revealed that *Citrange* 'Carrizo' and *Citrange* 'Troyer' are very similar genetically. *Citrumelo* '4475' presented high genetic similarity (0.93) with one sample of *Citrumelo* '4475' which came from micropropagation and lower genetic similarity with the second sample of *Citrumelo* '4475' which came also from micropropagation (Fig. 5). It is possible that the first sample which came from micropropagation was taken from the same donor plant with the sample of *Citrumelo* '4475', while the second sample might have come from a different donor plant. *Citrumelo* 'Swingle' and *Citrumelo* '1452' were grouped together in another branch of the cluster. In addition, Romdhane *et al. (*2016), placed 'Swingle' in a group with 'Troyer' and Amar *et al.* (2011), observed high genetic similarity between *Citrumelo* and *Citrange* 'Citrange'. *Volkameriana* was grouped together with C*itrus limon* 'Eureka' and C*itrus limon* 'Meyer' and this was in agreement with the findings of Uzun *et al.* (2009). Similarly, Tripolitsiotis *et al.* (2013) observed that *Volkameriana* presents genetic similarities with various lemon varieties and several researchers (Golein *et al*., 2012; Hamza, 2013) place *Volkameriana* in the same genetic group with different varieties of Lime.

Conclusions

Genotype affected significantly the proliferation percentage, the number of shoots and the total shoot length per explant. The results of the *in vitro* proliferation stage indicated that, among the six rootstocks studied, *Volkameriana* had the higher number of new shoots, shoot length and number of leaves per explant, while all three *Poncirus trifoliata* rootstocks ('Serra', 'Rubidoux' and 'Flying Dragon') showed the lowest numbers. Genotype affected also significantly the rooting percentage, number of roots and total root length per explant. Among the rootstocks studied, *Citrumelo* 'Swingle', presented significantly higher number of roots and total root length per explant. The presence of BA increased the proliferation percentage in all the rootstock studied, except *Volkameriana*. Highest proliferation percentage was observed at 2 ppm of BA for most of the rootstocks studied. BA presence significantly increased the number of shoots and the total shoot length per explant in *Citrange* 'Carrizo', *Volkameriana* and *Poncirus trifoliata* 'Serra' rootstocks. The presence of NAA increased the rooting percentage in all the rootstocks studied. In most rootstocks, maximum rooting was obtained at 2 ppm of NAA. The presence of NAA increased the number of roots per explant in the rootstocks studied, except *Citrange* 'Carrizo'. On the other hand, NAA presence did not affect significantly the total root length per explant in any of the rootstocks studied, except *Citrange* 'Carrizo'. The study of the genetic relationships showed that the rootstocks studied presented a broad genetic base with great genetic variability but response to *in vitro* propagation is not related to their genetic affinity.

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