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In Vitro Adventitious Rooting of Carrizo Citrange Microshoots

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***Abstract*. The objective of this work was to develop an efficient in vitro rooting protocol for one of the most commercially used citrus rootstocks in Spain, Carrizo citrange (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf.). Single-node cuttings taken from greenhouse-grown plants were cultured in petri dishes containing basal Murashige and Skoog medium. Shoots from nodal stem segments were excised and cultured in a multiplication medium (basal medium supplemented with 1.8 μM 6-benzylaminopurine) to promote the development of axillary buds. Individual shoots (15 mm long) were treated with different hormones at several concentrations for root induction evaluations. The addition of activated charcoal (AC) to the culture medium was also explored. The addition of auxins to the culture medium enhanced rooting percentage. Optimal results were obtained when 1-naphthalene acetic acid (10.8 μM) and gibberellic acid (0.3 μM) were added to the culture medium. The addition of AC to the rooting medium resulted in negative effects on the percentage of rooted shoots but had a positive effect on number of roots per rooted shoot. Chemical names used: activated charcoal (AC); 6-benzylaminopurine (BA); 1-naphthalene acetic acid (NAA); gibberellic acid (GA_3); indole-3-butyric acid (IBA)**

The efficient de novo regeneration of plants from cell and tissue cultures is recognized as a prerequisite for the application of biotechnological approaches to crop improvement (Castellanos et al., 2008; Nehra et al., 2005).

During the last 30 years, it has become possible to regenerate plantlets from explants and callus in many types of plants. As a result, large-scale micropropagation protocols are available for a wide range of species and, at present, tissue-culture technology is widely used for the vegetative propagation of selected plants in horticulture and, to a lesser extent, in forestry (Debergh and Zimmerman, 1991; George et al., 2008).

Highly efficient and reproducible methods for in vitro regeneration through somatic embryogenesis or organogenesis are a prerequisite for clonal propagation of selected genotypes (Debergh and Zimmerman, 1991; George et al., 2008). Although plant regeneration through somatic embryogenesis systems has been reported in a number of species, the production of plants from axillary buds or shoots has proven to be the most generally applicable and reliable method of true-to-type in vitro propagation (George et al., 2008).

Micropropagation is used for the commercial-scale propagation of several woody species such as *Vitis*, *Prunus* rootstocks, *Malus*, and so on (George et al., 2008). However, in many others species, adventitious root formation is still a major problem. Root formation of woody species was found to be regulated by a great number of factors, including to a great extent auxins (Arena et al., 2005; Baksha et al., 2003; George et al., 2008). Some progress has been made in rooting of different species using different chemical or natural compounds in the rooting media (Fotso et al., 2004).

Research has indicated the activated charcoal (AC) alone or in combination with auxins can promote growth and development because of its capacity to adsorb inhibitory substances, decrease phenolic oxidation, buffer pH, or establish a dark environment simulating soil conditions (reviewed in Thomas, 2008).

There have been some reports on organogenesis from different types of explants in citrus genotypes as reviewed by Carimi (2001). The morphogenic responses of citrus cultured in vitro are influenced by the genotype, the explant type, and the culture medium. Several attempts to induce in vitro rhizogenesis have been described for different explants (nodal and internodal segments, hypocotyls, epicotyls, shoot tip, and internodal seedling stem sections) from several citrus species (*Citrus sinensis*, *Poncirus trifoliata*, *C. acida*, *C. aurantifolia*, and *C. limon*) through the addition of plant growth regulators in various combinations and concentrations (Al-Bahrany, 2002; Bordón et al., 2000; Chakravarty and Goswami, 1999; Hassanein and Azooz, 2003).

Because of the variable response of citrus species and cultivars to the in vitro environment, the objective of this study was to improve rooting efficiency of Carrizo citrange microshoots produced from cultures initiated from nodal explants. Carrizo citrange is a main citrus rootstock widely used in important citrus-producing areas such as Spain and California, where ≈90% of the new plantings are grafted onto citranges.

Materials and Methods

Plant material and culture media. Three-year-old greenhouse-grown plants of the citrus rootstock Carrizo citrange [*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf (CC)] were used as source of plant material. Stem pieces (15 cm long) were stripped of leaves, disinfected by immersion for 10 min in a 2% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Tween 20, followed by three rinses, 5 min each, in sterile water. Node stem segments (1 cm long) were cultured in petri dishes (10 cm diameter) with 25 mL of basal medium containing the inorganic salts of Murashige and Skoog (1962) supplemented with 0.55 mM i-inositol, 4.8 μM pyridoxine-HCl, 0.6 μM thiamine-HCl, 8 μM nicotinic acid, and 88.2 mM sucrose. The medium was solidified with 9 $g \cdot L^{-1}$ agar (Pronadisa, Madrid, Spain). The pH was adjusted to 5.7 ± 0.1 with 0.1 N NaOH before autoclaving.

Shoots recovered from nodal stem segments were excised from the explant and cultured in 150 × 20-mm tubes on a multiplication medium to promote the development of axillary buds. The multiplication medium consisted of basal medium supplemented with 1.8 μM 6-benzylaminopurine (BA). New shoots from axillary buds were excised when they reached 15 mm for use in subsequent experiments.

Throughout the experimental period, all cultures were maintained at 26 ± 2 °C with a 16-h photoperiod provided by cool-white fluorescent light (70 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Subcultures were performed every 2 weeks.

Effect of different plant growth regulators on in vitro rooting. Individual shoots (15 mm long) cultured into 150 × 20-mm tubes were subjected to different rooting treatments following procedures reported in the literature (Table 1). Rooting media consisted of basal medium supplemented with the following: 5.4 μM 1-naphthalene acetic acid (NAA) plus 0.3 μM gibberellic acid (GA_3) (R1); 8.1 μM NAA plus 9.8 μM 3-indolebutyric acid (IBA) (R2); 1.1 μM BA, 2.7 μM NAA plus 4.9 μM IBA (R3); 5.4 μM NAA (R4); and 2.7 μM NAA plus 9.8 μM IBA (R5) (Al-Bahrany, 2002; Bordón et al., 2000; Chakravarty and Goswami, 1999; Hassanein and Azooz, 2003). In all cases, the medium was solidified by the addition of 9 $g \cdot L^{-1}$ agar (Pronadisa). The pH was set at 5.7 ± 0.1 with 0.1 N NaOH before autoclaving.

According to preliminary rooting results, the following treatments were used for subsequent experiments: (R1.1) R1 plus 2.7 μM

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Table 1. Characteristics of the assayed rooting treatments.

Medium	BA (μM)	NAA (μM)	IBA (μM)	GA (μM)	PGR filter-sterilized	Dipping in IBA (50 μM)	AC (12.0 μM)	Reference
R1	0	5.4	0	0.3	No	No	No	Al-Bahrany, 2002; Bordón et al., 2000
R2	0	8.1	9.8	0	No	No	No	Al-Bahrany, 2002
R3	1.1	2.7	4.9	0	No	No	No	Hassanein and Azooz, 2003
R4	0	5.4	0	0	No	No	No	Chakravarty and Goswami, 1999
R5	0	2.7	9.8	0	No	No	No	Al-Bahrany, 2002
R1.1	0	8.1	0	0.3	No	No	No	NR
R1.2	0	10.8	0	0.3	No	No	No	NR
R5.1	0	2.7	9.8	0	Yes	No	No	NR
R5.2	0	2.7	9.8	0	No	Yes	No	NR
R5.3	0	2.7	9.8	0	No	No	Yes	NR
R5.4	0	2.7	9.8	0	No	Yes	Yes	NR

BA = 6-benzylaminopurine; NAA = 1-naphthalene acetic acid; IBA = indole-3-butyric acid; PGR = plant growth regulator; AC = activated charcoal; NR = the rooting treatment has not been reported in the literature.

NAA; (R1.2) R1 plus 5.4 μM NAA; (R5.1) R5 composition, but in this case, the plant growth regulators were filter-sterilized (instead of autoclaved) and added to the medium once cooled down to 60 °C after autoclaving; (R5.2) R5 plus dipping of basal part of explant in an IBA concentrate solution (50 μM) for 10 s before subculture onto R5 medium; (R5.3) R5 plus 0.25 $g \cdot L^{-1}$ AC; and (R5.4) R5.3 plus a basal dip of the explant in an IBA concentrate solution (50 μM) for 10 s before subculture.

Measurements after 10, 20, 30, and 40 d of culture in rooting media included percentage of rooted shoots and number of roots per explant.

Statistical analysis. For each set of experiments, 48 replicates were used per treatment, and the experiment was repeated twice. One-way analysis of variance and comparisons between means were made following the least significant difference test at $P \leq 0.05$ by using the STATGRAPHICS PLUS Version 5.1 (Statistical Graphics Corporation, Herndon, VA) software (Montoliu et al., 2009).

Results and Discussion

Regeneration of shoots from in vitro-cultured node explants. As was described for other species (Dewan et al., 1992; Pereira et al., 1995), shoot multiplication appeared to be highly affected by the concentration of BA. Although the micropropagation of CC was not the main objective of this work, it was observed that the omission of BA in the medium resulted in single shoots only, but addition of 1.8 μM BA stimulated shoot multiplication (data not shown).

Effect of different treatments on in vitro rooting. To improve in vitro rooting of CC shoots, different rooting media were used. The addition of BA together with NAA and IBA in the medium (R3) resulted in zero roots on CC shoots, even after 40 d of culture, in contrast to results obtained with *Citrus reticulata*, in which 85% of shoots rooted after 12 d culture (Hassanein and Azooz, 2003). Low rates of rooting were also observed in shoots cultured on medium containing exclusively NAA (R4) after 40 d on rooting medium.

The rooting process of different citrus genotypes in a culture media supplemented with 5.4 μM NAA and 0.3 μM GA_3 (R1) has been described. The percentage of rooted shoots was higher in R1 than the one observed in media containing other auxins in *Citrus aurantifolia* (Al-Bahrany, 2002) and was 28% for Troyer citrange (Bordón et al., 2000). In our study, as shown in Table 2, the highest rooting rates in CC shoots were observed in medium supplemented with 5.4 μM NAA and 0.3 μM GA_3 was added to the medium (R1) or when the medium contained NAA (2.7 μM) in addition to 9.8 μM IBA (R5). The pattern of rooting was different between shoots cultured in R1 or R5 medium throughout the experiment. In R1, the percentage of shoots that exhibited roots after 20 d of culture was 41.67% and this value remained almost constant throughout the rest of the experiment (45.45% at Day 40). In R5, only 23.21% of shoots had roots after 25 d in culture, which was significantly lower than the percentage observed in shoots cultured in R1 (42.00%). Rooting of shoots cultured in medium R5 progressively increased throughout the experimental period. At Day 30, the percentage of rooting in R5 was similar to that observed in R1 (39.87% in R5 versus 43.90% in R1) and at the end of the experiment (Day 40), shoots cultured in R5 exhibited the highest rooting rates (Table 2).

Number of roots per rooted shoot after different time periods is also shown in Table 2. In all cases, the number of roots per rooted shoot was similar after 25 d in culture regardless of the composition of the medium (with the exception of R3), ranging from 1.37 in R2 to 2.06 in R1 and R5. At Day 35, differences among treatments were evident with shoots in R5 showing the highest number of roots per shoot (5.08). At the end of the experiment (Day 40), the best results were obtained when R5 was used as culture medium (5.23) followed by R1 (4.14).

The presence of a higher number of adventitious roots on the shoots is highly recommended to ensure the success in the transfer of vitroplants to greenhouse conditions (George et al., 2008). It has been reported that in the genotypes *Citrus aurantifolia* (Al Bahrany, 2002) and *Citrus limon* (Pérez-Tornero et al., 2010), the number of roots formed per shoot increased in response to increasing IBA concentrations in combination with other auxin (NAA and indole-3-acetic acid, respectively) in the rooting medium. However, in CC, the best results were obtained when using 9.8 μM IBA plus 2.7 μM NAA in the rooting medium (R5) or 5.4 μM NAA in absence of IBA (R1) (Table 2).

In most plants, the addition of gibberellins in the culture media is detrimental for in vitro rooting (George et al., 2008); in others such as *Citrus lemon,* gibberellins had no effect (Pérez-Tornero et al., 2010). Our results pointed that, in the case of CC, the addition of GA_3 had a positive effect. Therefore, the percentage of rooting after 40 d of treatment increased from 30.20% (R4) to 45.45% in R1 (R4 plus 0.3 μM GA_3). Moreover, GA_3 had a positive effect on the number of roots per rooted shoot, with the differences statistically significant between R1 and R4 (4.14 and 2.13 roots/rooted shoot, respectively).

To improve in vitro rooting of CC shoots, the two media that provided better results (R1 and R5) were modified and new experiments performed. The percentages of rooted shoots in each medium throughout the experimental period are shown in Table 3. Shoots cultured in media derived from R1, in which NAA concentration was increased, resulted in higher rooting percentage than its precursor; R1.2 (R1 plus 5.4 μM NAA) provided the highest rate of rooting (68.18%). The percentage of rooted shoots in media derived from R5 was, in all cases, lower than that observed in R5. Neither of the following variations had a positive effect on rooting: filter sterilization of the plant growth regulators (instead of autoclaving, R5.1), dipping process of the explant in IBA solution (R5.2); addition of AC to the culture media (R5.3); or a combination of both dipping in IBA and AC (R5.4).

There is no an evident explanation for the lack of effectiveness of these treatments that have proved to be valuable in inducing rhizogenesis in other species (reviewed in Thomas, 2008). Data point to a specific behavior of this genotype important to take into consideration in future research.

Shoots cultured in media R5.1 and R5.2 exhibited the same pattern as those cultivated in R5 throughout the experimental period; the percentage of rooting was low for 25 d of treatment (Table 3). From Day 30, a higher increase in rooting was observed in shoots growing in R5, R5.1, and R5.2, achieving similar values of those obtained in shoots cultured in media containing NAA and GA_3 (R1 and R1.1).

When the number of roots per rooted shoot was considered (Table 3), significant differences were observed among treatments. Shoots cultured in media with high NAA plus GA_3 (R1.2) or low NAA plus IBA and AC (R5.3) gave the highest number of roots per rooted shoot (6.69 and 7.10, respectively). However, the pattern was different over time (Table 3).

Table 2. Effect of culture medium on percentage of rooted shoots and number of roots per rooted shoot in Carrizo citrange shoots cultured in vitro.[z]

		Days						
		10	15	20	25	30	35	40
Rooted shoots (%)	R1	12.50 ± 1.5 b[y]	30.11 ± 1.2 c	41.67 ± 1.2 d	42.00 ± 1.5 d	43.90 ± 1.2 d	44.07 ± 1.0 d	45.45 ± 0.3 d
	R2	7.01 ± 1.0 b	10.50 ± 1.6 b	12.00 ± 2.4 b	12.71 ± 1.3 b	13.03 ± 1.2 b	14.33 ± 1.3 b	14.04 ± 1.1 b
	R3	0.00 ± 0.0 a	0.00 ± 0.0 a	0.00 ± 0.0 a	0.00 ± 0.0 a	0.00 ± 0.0 a	0.00 ± 0.0 a	0.00 ± 0.0 a
	R4	9.07 ± 2.0 b	12.30 ± 1.4 b	22.00 ± 1.8 c	24.82 ± 1.1 c	27.32 ± 1.5 c	28.56 ± 1.2 c	30.20 ± 1.3 c
	R5	8.98 ± 1.3 b	11.01 ± 1.2 b	22.50 ± 1.7 c	23.21 ± 1.2 c	39.87 ± 1.2 d	44.32 ± 1.7 d	53.20 ± 1.8 e
Number of roots/rooted shoot	R1	1.02 ± 0.7 b	1.05 ± 0.4 b	1.98 ± 0.7 b	2.06 ± 0.3 b	2.07 ± 0.4 b	2.08 ± 0.2 b	4.14 ± 0.3 c
	R2	1.20 ± 0.4 b	1.20 ± 0.2 b	1.25 ± 0.4 b	1.37 ± 0.3 b	1.43 ± 0.2 b	1.56 ± 0.5 b	1.54 ± 0.3 b
	R3	0.00 ± 0.0 a	0.00 ± 0.0 a	0.00 ± 0.0 a	0.00 ± 0.0 a	0.00 ± 0.0 a	0.00 ± 0.0 a	0.00 ± 0.0 a
	R4	1.73 ± 0.1 b	1.73 ± 0.1 b	1.73 ± 0.1 b	1.79 ± 0.1 b	1.86 ± 0.1 b	1.99 ± 0.0 b	2.13 ± 0.1 b
	R5	0.94 ± 0.2 b	0.98 ± 0.4 b	1.06 ± 0.7 b	2.06 ± 0.3 b	3.07 ± 0.4 c	5.08 ± 0.2 c	5.23 ± 0.3 d

[z]Shoots were grown in R1, R2, R3, R4, or R5 medium. Each point corresponds to the average of 48 replicates ± SE.
[y]Data within each column, and for each parameter, followed by dissimilar letters differ significantly at $P \leq 0.05$.

Table 3. Effect of culture medium on percentage of rooted shoots and number of roots per rooted shoot in Carrizo citrange shoots cultured in vitro.[z]

		Days						
		10	15	20	25	30	35	40
Rooted shoots (%)	R1	12.50 ± 1.5 b[y]	30.11 ± 1.2 c	41.67 ± 1.2 c	42.00 ± 1.5 c	43.90 ± 1.2 c	44.07 ± 1.0 c	45.45 ± 0.3 c
	R1.1	11.48 ± 0.8 b	28.12 ± 1.3 c	45.45 ± 1.2 c	46.32 ± 0.9 c	48.00 ± 1.6 c	49.00 ± 1.6 c	55.32 ± 0.8 d
	R1.2	13.93 ± 1.6 b	35.02 ± 0.6 c	49.25 ± 1.2 c	57.98 ± 0.6 d	58.77 ± 1.0 d	59.60 ± 1.0 d	68.18 ± 0.9 e
	R5	8.98 ± 1.3 b	11.01 ± 1.2 b	22.50 ± 1.7 b	23.21 ± 1.2 b	39.87 ± 1.2 b	44.32 ± 1.7 c	53.20 ± 1.8 d
	R5.1	9.86 ± 0.5 b	12.96 ± 1.0 b	23.30 ± 1.0 b	25.84 ± 1.3 b	30.27 ± 0.9 b	34.22 ± 0.8 b	39.50 ± 0.7 b
	R5.2	11.95 ± 0.4 b	15.22 ± 1.6 b	20.50 ± 1.3 b	22.43 ± 0.9 b	32.98 ± 0.4 b	35.23 ± 0.4 b	38.90 ± 0.7 b
	R5.3	1.71 ± 0.7 a	3.21 ± 0.5 a	5.20 ± 1.1 a	7.91 ± 1.0 a	10.90 ± 1.6 a	15.11 ± 0.5 a	19.13 ± 0.4 a
	R5.4	1.00 ± 0.8 a	1.21 ± 0.9 a	2.10 ± 1.5 a	5.23 ± 1.0 a	6.22 ± 1.0 a	8.38 ± 0.9 a	10.76 ± 0.4 a
Number of roots/rooted shoot	R1	1.02 ± 0.7 a	1.05 ± 0.4 a	1.98 ± 0.7 b	2.06 ± 0.36 a	2.07 ± 0.4 a	2.08 ± 0.2 a	4.14 ± 0.3 a
	R1.1	1.50 ± 0.6 a	2.30 ± 0.4 b	2.60 ± 0.4 b	2.70 ± 0.43 a	3.00 ± 0.4 b	3.07 ± 0.3 a	4.11 ± 0.3 a
	R1.2	2.70 ± 0.2 b	2.50 ± 0.2 b	3.01 ± 0.5 c	4.30 ± 0.20 c	4.40 ± 0.2 c	5.67 ± 1.1 b	6.69 ± 1.4 c
	R5	0.94 ± 0.2 a	0.98 ± 0.4 a	1.06 ± 0.7 a	2.06 ± 0.36 a	3.07 ± 0.4 b	5.08 ± 0.2 b	5.23 ± 0.3 b
	R5.1	1.11 ± 0.7 a	1.56 ± 0.4 b	2.05 ± 0.3 b	3.32 ± 0.41 b	3.33 ± 0.4 b	3.11 ± 0.3 a	3.67 ± 0.3 a
	R5.2	0.50 ± 0.4 a	0.70 ± 0.2 a	1.00 ± 0.3 a	2.00 ± 0.32 a	2.10 ± 0.2 a	2.50 ± 1.3 a	3.10 ± 0.2 a
	R5.3	3.00 ± 0.3 b	3.00 ± 0.4 c	3.00 ± 0.5 c	3.60 ± 0.80 b	3.70 ± 0.4 c	3.70 ± 0.2 a	3.90 ± 1.0 a
	R5.4	0.70 ± 0.1 a	1.00 ± 0.2 a	3.00 ± 0.1 c	6.00 ± 0.13 d	7.00 ± 0.2 d	7.00 ± 0.1 c	7.10 ± 1.2 c

[z]Shoots were grown in R1, R1.1, R1.2, R5, R5.1, R5.2, R5.3, or R5.4 medium. Each point corresponds to the average of 48 replicates ± SE.
[y]Data within each column, and for each parameter, followed by dissimilar letters differ significantly at $P \leq 0.05$.

Different auxins at different concentrations provided diverse percentages of rooting when added to the culture medium. Best results, in terms of percentage of rooting, were obtained when 10.8 μM NAA and 0.3 μM GA_3 were added to the culture medium. AC had a negative effect on the percentage of rooted shoots when incorporated into the culture medium; however, it had a positive effect when the number of roots per rooted shoot was considered. Because of the highest percentages of rooting together with a high number of roots per rooted shoot were obtained when R1.2 was used as rooting media, we conclude that a combination of 10.8 μM NAA and 0.3 μM GA_3 in the culture medium is the best option for the development of in vitro adventitious roots in CC shoots.

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