High-efficiency Propagation of Mature 'Washington Navel' Orange and Juvenile 'Carrizo' Citrange Using Axillary Shoot Proliferation

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SUMMARY. In vitro axillary shoot proliferation can be used to increase availability of citrus (*Citrus*) types in high demand, while limiting somaclonal variation. However, established protocols could be improved to increase efficiency. Therefore, this study investigated some factors [plant growth regulators (PGRs), basal media, and successive subculturing] which affect the in vitro axillary shoot proliferation of mature 'Washington Navel' orange (*Citrus sinensis*) and juvenile 'Carrizo' citrange (*C. sinensis* × *Poncirus trifoliata*). In 'Washington Navel' orange, maximum axillary shoot induction (66.9% explants producing axillary shoots with a mean of 2.45 shoots per explant) was obtained in Driver and Kuniyuki walnut (DKW) medium supplemented with 0.1 mg·L⁻¹ 6-benzylaminopurine (BA), 0.05 mg·L⁻¹ naphthalene acetic acid (NAA) along with 1 mg·L⁻¹ 6-furfurylaminopurine [kinetin (kin)], whereas in 'Carrizo' citrange, axillary shoot production was greatest (82.6% and 87.5% of explants producing axillary shoots with a mean of 4.3 and 4.1 shoots per explant) at 1.0 or 2.0 mg·L⁻¹ BA in DKW medium. The initial nodal propagules (with basal tissue remaining from removed shoots) were repeatedly subcultured for six times every 4 weeks onto DKW medium with the same levels of PGRs used for initial culturing. Woody plant medium (WPM), Murashige and Skoog medium (MS), and DKW were also compared for rooting at quarter to full strength for salt components, all amended with 2.0 mg·L⁻¹ indolebutyric acid (IBA) and 0.5 mg·L⁻¹ NAA. MS at full strength provided the highest rooting in 'Carrizo' citrange (93%) and longest root length (58 mm), whereas half-strength MS provided the highest rooting in 'Washington Navel' orange (60% to 61%) and the longest roots (26 mm). Addition of 1 μM spermidine to the rooting medium enhanced root length only for 'Washington Navel' orange on full-strength MS, but accelerated rooting for both cultivars on all media. The plantlets were successfully transferred to greenhouse conditions, exhibiting normal development, with high uniformity, and no evidence of somaclonal variation.

Citrus trees are usually propagated by budding scions to greenhouse-grown nucellar rootstock seedlings. This requires

mature rootstock trees to produce seeds, and sufficient scion budwood from mature trees, to avoid delays in fruit production from juvenility. When a highly desirable rootstock or scion is introduced, the material available for propagation is often limited. Axillary shoot proliferation through tissue culture is an alternative propagation method that increases availability of material in high demand. This approach also facilitates use of

otherwise superior rootstocks that produce few nucellar embryos, and can provide a source of in vitro adult-phase plant material for genetic transformation, pathology studies, and in vitro conservation (Marutani-Hert et al., 2011; Pérez-Tornero et al., 2010).

The in vitro propagation of citrus has been explored by many workers using different sources of explants (Al Khayri and Al-Bahrany, 2001; De Oliveira et al., 2010; Dutt and Grosser, 2010; Marutani-Hert et al., 2011; Sharma et al., 2009). Axillary bud proliferation from nodal cultures is considered most suitable for commercial multiplication, minimizing genetic changes (Sharma et al., 2007) and providing fewer off-type shoots compared with adventitious organogenesis using internodes or callus (Debergh and Maene, 1989). In vitro axillary shoot production can be conducted in two ways. One approach is "linear nonproliferative culture," which simply accelerates development of buds grown on a single shoot per plant. This is used by many commercial tissue culture propagators with low or no exogenously applied growth regulators (M. Bordas, personal communication). Although there are no published data to compel conclusions, this is considered the most conservative approach and likely to generate the lowest level of somaclonal variation. However, the rate of propagule increase is much less than with the second method in which axillary bud-break is induced to produce numerous shoots per explant.

Despite extensive studies, the in vitro propagation of citrus is limited by poor growth and variable rooting of explants. In vitro propagation generally involves four stages: Stage 1 (initiation/establishment of aseptic cultures), Stage 2 (multiplication of propagules), Stage 3 (rooting of in vitro formed shoots), and Stage 4 (ex vitro acclimatization). Each stage is influenced by a number of variables,

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Units

To convert U.S. to SI, multiply by	U.S. unit	SI unit	To convert SI to U.S., multiply by
29.5735	fl oz	mL	0.0338
0.3048	ft	m	3.2808
2.54	inch(es)	cm	0.3937
25.4	inch(es)	mm	0.0394
1	ppm	mg·L ⁻¹	1
(°F - 32) ÷ 1.8	°F	°C	(°C × 1.8) + 32

with medium composition during multiplication greatly affecting the rate of propagule production (Carimi and De Pasquale, 2003). Many studies have focused on the influence of PGRs. MS medium (Murashige and Skoog, 1962) has been used for most studies on in vitro propagation in citrus (Marutani-Hert et al., 2011; Mukhtar et al., 2005; Rathore et al., 2007), but different basal salt media formulations may influence axillary bud/shoot proliferation.

For this report, we determined in vitro conditions to efficiently produce established ex vitro shoots starting with axillary shoot proliferation from juvenile 'Carrizo' citrange and mature 'Washington Navel' orange nodal segments. 'Washington Navel' orange is one of the most important fresh-fruit oranges in the United States (Boriss, 2006), and its mature tissue is generally considered recalcitrant to in vitro regeneration and transformation. Relevant existing literature reports an efficient transformation procedure using adventitious juvenile 'Washington Navel' orange tissue, but efficiency was reduced using older juvenile epicotyl sections (Bond and Roose, 1998). In another study, only 0.76 regenerated shoots per explant were produced using *Agrobacterium tumefaciens*-mediated genetic transformation of mature axillary buds from adult 'Newhall Navel' orange (He et al., 2011). It should be noted that the focus of these studies was not maximizing proliferation.

'Carrizo' citrange is an important citrus rootstock which generally confers the production of high-quality fruit in different citrus scion cultivars (Montoliu et al., 2010), and is quite amenable to in vitro propagation. However, limited research has been carried out on propagation using axillary citrus buds (Kanwar et al., 2013; Montoliu et al., 2010), and procedures from these studies are not suitable for large-scale multiplication.

A series of stepwise experiments was conducted in this study to maximize the establishment of new plants from axillary shoot proliferation. For each genotype, we first determined baseline auxin and cytokinin compositions in MS medium for maximum axillary shoot development. Next, we used these PGR levels to compare axillary shoot proliferation in

three nutrient salt formulations: WPM (Lloyd and McCown, 1980), MS, and DKW medium (Driver and Kuniyuki, 1984). For 'Washington Navel' orange only, we assessed the effects of gibberellic acid (GA) levels when added at two different times in culture development using the superior salt/auxin/cytokinin composition. We then evaluated axillary shoot proliferation through six subcultures with the superior salt/auxin/cytokinin/GA compositions, and finally assessed medium salt formulation/concentration and presence of spermidine on both rooting and ex vitro establishment after proliferation with the superior medium/PGR regimes. Collectively, these experiments provide methods to efficiently multiply 'Washington Navel' orange and 'Carrizo' citrange through axillary shoot proliferation.

Material and methods

Plant material and explant preparation

Nodal explants were used from greenhouse-grown 2-year-old seedling plants of 'Carrizo' citrange and 8-year-old 'Washington Navel' orange trees grafted on 'Swingle' citrumelo (*Citrus paradisi* × *P. trifoliata*), that had been purchased from a commercial nursery. Juvenile tissue was used for 'Carrizo' rootstock propagation as seedlings are routinely used for citrus rootstocks. Mature tissue is essential for scion propagation as juvenile scions typically have delays of many years before fruit is produced. Nodal source plants were drastically pruned to facilitate in vitro culture, stimulating basal bud sprouting to provide uniform vigorous growth. Elongated lateral shoots, at least 1 ft long, were stripped of their leaves and thorns, cut into small stem pieces (four to five nodes per shoot), then surface sterilized for 2 min in 70% (v/v) ethanol (Pharmco-AAPER, Brookfield, CT), followed by immersion in a 5% (v/v) commercial solution of sodium hypochlorite (Clorox, Oakland, CA) containing 0.1% (v/v) Tween 20 (Sigma-Aldrich, St. Louis, MO) for 20 min, and were rinsed with three changes of sterile double-distilled water. After disinfection, the nodal segments were cut (0.4–0.6 inch in length) and placed vertically in MS medium supplemented with 3%

sucrose (w/v) (Sigma-Aldrich) and 0.8% (w/v) agar (Sigma-Aldrich), with the medium pH adjusted to 5.7 ± 0.1 before autoclaving, and after autoclaving filter-sterilized PGRs were added as indicated below. Unless otherwise noted, all cultures were incubated under 16/8-h (day/night) photoperiod at 27 ± 2 °C.

In vitro shoot establishment and initial propagation

For all experiments, the explants were subcultured after 15 d and data were recorded after 45 d. Presence of shoots on each explant, number of shoots per explant, and length of shoots (millimeters) were recorded and statistically compared, following a completely randomized design. Six nodal segments were used per vessel and five vessels were used for each treatment per experiment. Each experiment was repeated twice.

COMPARISON OF PGR COMPOSITION. MS medium was used with a factorial combination of four BA (PhytoTechnology Laboratory, Shawnee Mission, KS) concentrations (0, 1, 2, and 3 mg·L⁻¹) and four NAA (PhytoTechnology Laboratory) concentrations (0, 0.25, 0.5, and 1 mg·L⁻¹) in 'Carrizo' citrange; and three BA concentrations (0, 0.1, and 0.5 mg·L⁻¹), along with three NAA concentrations (0, 0.05, 0.5 mg·L⁻¹), and three Kin (PhytoTechnology Laboratory) concentrations (0, 1, and 2 mg·L⁻¹) in 'Washington Navel' orange. Medium was poured (30-mL aliquots) in 100 × 20-mm plastic petri dishes (Corning, Corning, NY).

COMPARISON OF MEDIUM SALT COMPOSITION. To test the influence of the culture media during the multiplication stage, three basal media [MS, DKW, and WPM (all from PhytoTechnology Laboratory)] were evaluated. Unless otherwise stated, all media were supplemented with growth regulator composition of 1.0 mg·L⁻¹ BA for 'Carrizo' citrange, whereas 0.1 mg·L⁻¹ BA, 0.05 mg·L⁻¹ NAA, and 1 mg·L⁻¹ Kin were used for 'Washington Navel' orange. The media were poured (40-mL aliquots) in tissue culture vessels (Magenta™ GA-7; Sigma-Aldrich), instead of petri dishes to provide improved air flow.

GA₃ EFFECTS ON SHOOT ELONGATION. Because of the short

length of 'Washington Navel' axillary shoots, another experiment was performed to compare shoot elongation using GA₃ at 0, 0.5, and 1.0 mg·L⁻¹ (Sigma-Aldrich) added to the DKW shoot-induction medium (SIM; 0.1 mg·L⁻¹ BA, 0.05 mg·L⁻¹ NAA, and 1 mg·L⁻¹ Kin) either from initial explant culture (day 0) or in medium used for subculturing after bud induction (10–15 d). 'Carrizo' shoots were sufficiently long without GA₃, making this additional component unnecessary.

SUBCULTURING INITIAL EXPLANTS FOR FURTHER PROLIFERATION. After identifying the most effective media formulation for proliferation, subculturing for further proliferation was studied on the same medium for each genotype. DKW medium with 0.1 mg·L⁻¹ BA, 0.05 mg·L⁻¹ NAA, 1 mg·L⁻¹ Kin, and 0.5 mg·L⁻¹ GA₃ was used for 'Washington Navel' orange, whereas DKW medium with 1 mg·L⁻¹ BA was used for 'Carrizo' citrange. The shoots regenerated from the initial culturing were removed, and then the initial explants were subcultured every 4 weeks for 24 weeks. The transferred explants were the initial nodal explants trimmed to remove callus but leaving in place the basal stem tissue remaining from excised shoots. Multiplication parameters were calculated as the number of new shoots derived from one initial nodal explant at the end of each subculture.

Rooting induction and establishment of plantlets in the greenhouse

Shoots from the most effective shoot-multiplication medium (DKW medium with 0.1 mg·L⁻¹ BA, 0.05 mg·L⁻¹ NAA, 1 mg·L⁻¹ Kin, and 0.5 mg·L⁻¹ GA₃ for 'Washington Navel' orange; DKW medium with 1 mg·L⁻¹ BA for 'Carrizo' citrange) were used for this study following a completely randomized design. Three experimental units per treatment, each with twelve elongated shoots (1–1.5 cm long) were excised and transferred onto rooting media. All rooting media were supplemented with 3% sucrose, 0.25% Gelrite (Sigma-Aldrich), and then poured as 20-mL aliquots into 25 × 95-mm glass culture tubes (PhytoTechnology Laboratory), each of which received one shoot. The explants were incubated under white fluorescent light 16/8-h (day/night) photoperiod at 27 ± 2 °C for 4 weeks.

At this time, rooted microshoots were transplanted to soil cones, and were placed in an incubator at 27 °C, 30% relative humidity, and 16/8-h (day/night) photoperiod for 2 weeks. After 15 d, the plants were transferred to the greenhouse. Frequency of rooting and root length for ex vitro established plants were recorded after 45 d.

EFFECT OF BASAL SALTS ON ROOTING. The rooting media comparisons were MS, DKW, or WPM, each at different salt strength (full strength, half strength, and quarter strength), and all amended with 2.0 mg·L⁻¹ IBA (Sigma-Aldrich), and 0.5 mg·L⁻¹ NAA.

EFFECT OF SPERMIDINE ON ROOTING. Rooting on full- and half-strength MS salts amended with 2.0 mg·L⁻¹ IBA (Sigma-Aldrich) and 0.5 mg·L⁻¹ NAA was compared with and without 1 μM spermidine (Sigma-Aldrich).

Data analysis

All data were evaluated using the ASSISTAT/Statistical program package (Silva and Azevedo, 2009) compared through analysis of variance and Tukey's multiple comparison test at 5% probability for all the variables studied. For all proliferation studies, the individual experimental units were single culture vessels containing multiple explants. For rooting studies, in which test tubes contained single shoots, the experimental units were groups of 12 tubes.

Results and discussion

COMPARISON OF PGR COMPOSITION ON AXILLARY SHOOT PROLIFERATION. Axillary budbreak for both 'Carrizo' citrange and 'Washington Navel' orange was observed within 1 week of culture initiation. Shoot elongation was observed within 4 weeks, and data were collected 45 d after culture initiation. In 'Carrizo' citrange, both shoot production and shoot length were significantly ($P \leq 0.001$) affected by BA, NAA, and interaction between BA and NAA (Table 1). BA provided greatest shoot production at concentrations of 1.0 or 2.0 mg·L⁻¹ when used as the sole growth PGR. However, the percentage of explants with axillary shoots decreased drastically at 3.0 mg·L⁻¹ BA. The addition of NAA to BA did not improve the percentage of explants producing

shoots in 'Carrizo' citrange. The optimum growth regulator concentration for 'Carrizo' citrange was 1 or 2 mg·L⁻¹ BA, resulting in 82.6% and 87.5% of explants with axillary shoots with a mean of 4.3 and 4.1 shoots per explant, respectively, but the greatest shoot length (24.3 mm) was in medium amended with 1 mg·L⁻¹ BA. Therefore, in our study, media supplemented with 1 mg·L⁻¹ BA was used for further in vitro propagation of 'Carrizo' citrange nodal explants.

Preliminary studies in our laboratory (data not shown) demonstrated short shoots, defoliation, leaf chlorosis, and hyperhydricity in axillary shoots from 'Washington Navel' orange cultured on MS medium with BA ≥ 1 mg·L⁻¹. Therefore, further experiments were conducted with BA at 0.1 and 0.5 mg·L⁻¹ combined with Kin and NAA (Table 2). The percentage of explants producing axillary shoots, number of shoots per explant, and shoot length were affected by a three-way interaction between BA, NAA, and Kin concentrations (Table 2). In media supplemented with Kin and NAA, or Kin and BA, or Kin, BA, and NAA, shoots developed healthy branches and leaves. With BA or NAA alone, or a combination of BA and NAA alone, the percentage of responsive explants was reduced with poor shoot elongation. A maximum response of 66.9% of explants producing shoots with a mean of 2.45 shoots per initial explant was recorded in 0.1 mg·L⁻¹ BA, 1 mg·L⁻¹ Kin, and 0.05 mg·L⁻¹ NAA with a mean shoot length of 12.8 mm.

Cytokinin alone or in association with auxin has been frequently used in propagation of different citrus cultivars (Al-Bahrany, 2002; Al-Khayri and Al-Bahrany, 2001; Paudyal and Haq, 2000; Usman et al., 2005), but optimal PGR concentration and combination varies by citrus cultivar.

COMPARISON OF MEDIUM SALT FORMULATION ON AXILLARY SHOOT PROLIFERATION. No significant difference was observed between DKW and MS medium on percentage of explants producing shoots for either cultivar (Table 3). However, DKW media proved to be superior to MS medium for enhancing shoot length, 38.9 mm in 'Carrizo' citrange and 14.8 mm in 'Washington Navel' orange, compared with 22.3 mm mean shoot length in 'Carrizo' citrange and 10.7 mm in 'Washington Navel' orange cultured in

Table 1. Axillary shoot production of juvenile ‘Carrizo’ citrange nodal segments as influenced by 6-benzylaminopurine (BA) and naphthalene acetic acid (NAA) concentrations in Murashige and Skoog medium. Data are from two independent experiments and were recorded after 45 d of in vitro culture.

Treatments		Frequency of explants with shoots (%)	Mean shoots per initial explant (no.)	Length of shoots (mm) ^z
BA (mg·L ⁻¹) ^z	NAA (mg·L ⁻¹)			
—	—	16.6 fg ^y	2.4 c-e ^y	11.7 b-f ^y
1.0	—	82.6 a	4.3 a	24.3 a
—	0.25	13.3 gh	1.4 de	7.8 c-f
—	0.5	0 h	0 f	0 h
—	1.0	0 h	0 f	0 h
1.0	0.25	31.7 de	2.6 b-e	15.7 b
1.0	0.5	28.5 ef	2.5 b-e	7.2 d-g
1.0	1.0	9.3 gh	1.7 de	5.3 gh
2.0	—	87.5 a	4.1 a	15.1 b-d
2.0	0.25	54.8 bc	2.7 b-d	11.9 b-e
2.0	0.5	58.7 b	3.1 b	10.5 b-f
2.0	1.0	39.9 de	2.2 de	10.9 b-ef
3.0	—	41.3 c-e	2.7 b-d	13.0 b-d
3.0	0.25	37.8 de	3.0 bc	7.7 c-f
3.0	0.5	42.3 cd	2.3 c-e	5.8 d-g
3.0	1.0	33.3 de	2.8 b-d	7.3 d-g

Source		P value		
Effect on shoot propagation	df	Frequency of explants with shoots	Mean shoots per initial explant	Length of shoots
BA	3	<0.001 ^{***}	0.0834 ^{ns}	<0.001 ^{**}
NAA	3	<0.001 ^{**}	0.0671 ^{ns}	<0.001 ^{**}
BA × NAA	9	<0.001 ^{**}	0.0985 ^{ns}	<0.001 ^{**}

^z1 mg·L⁻¹ = 1 ppm, 1 mm = 0.0394 inch.

^yValues followed by a common letter in a column are not significantly different according to Tukey’s multiple range test ($P \leq 0.05$).

^x, ^{ns}, ^{**}Nonsignificant at $P > 0.05$, or significant at $P \leq 0.001$, respectively.

MS medium. WPM medium resulted in a low percentage of explants producing shoots and low number of shoots per initial explant compared with DKW. In ‘Carrizo’ citrange, DKW medium also improved the number of shoots per initial explant compared with MS or WPM. These results generally agree with other studies in different citrus cultivars (Pérez-Tornero et al., 2010; Tallón et al., 2012).

The shoots that developed in DKW medium were also morphologically different from those produced on MS and WPM. DKW medium produced fairly uniform, healthy green shoots (Fig. 1A), whereas shoots from WPM medium were excessively short and thin, with severe chlorosis, very small and narrow leaves. This may be associated with the low content of ammonium [NH₄⁺ (5 mM)] and nitrate [NO₃⁻ (9.7 mM)] in WPM medium (Bell et al., 2009).

The excellent shoot health attained during bud/shoot proliferation on DKW medium probably contributed to the improved shoot elongation observed on this medium.

DKW medium is enriched in calcium (Ca²⁺) compared with MS and WPM medium, was the only medium tested that contained nickel (Ni⁺), and the concentration of zinc (Zn²⁺) was 2× greater than in the other media. Both zinc and nickel are important nutrients for absorption of iron by plants (Tallón et al., 2012). George (1996) reported that calcium plays a major role in various physiological processes like cytokinin signal transduction; therefore, increased internal calcium may contribute to more cell division.

Tallón et al. (2012) showed a positive effect of high-salt (MS and DKW) media in tissue culture proliferation of alemow (*Citrus macrophylla*), sour orange (*Citrus aurantium*), and ‘Cleopatra’ mandarin (*Citrus reticulata*) citrus rootstocks. Their study indicated that nutrient-rich (high-salt) media improved proliferation for all citrus rootstock axillary shoot cultures, and explants growing in WPM produced a lower number of shoots per explants, shorter shoots, and lower productivity overall. On the other hand, Pérez-Tornero et al. (2010) reported that the basal medium did

not affect the micropropagation of lemon (*Citrus limon*), but explants on DKW medium were greener.

In our experiments, DKW medium was markedly superior for shoot proliferation since it provided a relatively high percentage of explants producing shoots, significantly increased ($P < 0.001$) shoot length, and resulted in minimal necrosis (data not shown) compared with the other media.

GA₃ EFFECTS ON SHOOT ELONGATION. In ‘Washington Navel’ orange, explants were cultured in DKW medium amended with 0.1 mg·L⁻¹ BA, 1 mg·L⁻¹ Kin, and 0.05 mg·L⁻¹ NAA (SIM) with and without the addition of 0.5 mg·L⁻¹ GA₃ either from initial explant culture (day 0) or in medium used for subculturing after bud induction (10–15 d). Addition of GA₃ produced more elongated shoots better suited for rooting or micrografting without affecting shoot formation frequency (Table 4). GA₃ initially included in SIM (day 0) or used in SIM for a transfer when buds first began to grow (day 10–15), resulted in longer shoots (20.6 mm up to 35.9 mm)

Table 2. Axillary shoot production of mature 'Washington Navel' orange nodal segments as influenced by 6-benzylaminopurine (BA), naphthalene acetic acid (NAA), and 6-furfurylaminopurine [kinetin (Kin)] in Murashige and Skoog medium. Data are from two independent experiments and were recorded after 45 d in vitro culture.

Treatments			Frequency of explants with shoots (%)	Mean shoots per initial explant (no.)	Length of shoots (mm) ^z
BA (mg·L ⁻¹) ^z	NAA (mg·L ⁻¹)	Kin (mg·L ⁻¹)			
—	—	—	7.8 j-l ^y	1.02 cd ^y	6.2 c-i ^y
—	—	1.0	38.9 d-f	1.12 cd	9.7 a-e
0.1	—	—	17.9 h-k	1.26 c	4.7 f-i
—	0.05	—	4.2 kl	0.84 d	4.1 g-i
—	0.5	—	2.6 l	1.02 cd	3.5 hi
—	0.05	1.0	59.3 a-c	1.53 bc	8.3 b-g
—	0.5	1.0	43.3 d-f	1.82 b	10.2 a-d
0.1	0.05	—	18.2 h-j	1.07 cd	5.7 e-i
0.1	0.5	—	14.2 i-l	1.01 cd	6.8 c-i
0.1	—	1.0	62.9 ab	1.94 b	7.3 c-h
0.1	0.05	1.0	66.9 a	2.45 a	12.8 a
0.1	0.5	1.0	32.9 e-g	1.40 bc	7.2 c-h
—	—	2.0	40.2 d-f	1.54 bc	10.7 a-d
—	0.05	2.0	43.8 de	1.20 c	11.5 ab
—	0.5	2.0	38.7 d-f	1.43 bc	7.5 b-h
0.1	—	2.0	45.9 c-e	1.55 bc	11.7 ab
0.1	0.05	2.0	51.0 b-d	2.14 a	8.8 a-f
0.1	0.5	2.0	22.6 g-i	1.15 cd	6.4 c-i
0.5	—	—	9.5 i-l	1.03 cd	3.2 hi
0.5	—	1.0	19.4 g-j	1.03 cd	5.9 d-i
0.5	0.05	—	11.7 i-l	1.4 bc	4.3 g-i
0.5	0.5	—	8.7 i-l	1.04 cd	2.7 i
0.5	0.05	1.0	39.4 d-f	1.61 bc	5.7 e-i
0.5	0.5	1.0	32.2 e-g	2.23 a	6.3 c-i
0.5	—	2.0	11.3 i-l	1.46 bc	4.1 g-i
0.5	0.05	2.0	29.7 f-h	1.25 c	3.8 hi
0.5	0.5	2.0	9.7 i-l	1.04 cd	4.2 g-i

Source

P value

Effect on bud/shoot propagation	df	Frequency of explants with shoots	Mean shoots per initial explant	Length of shoots
BA	2	<0.001***	<0.001**	0.0194*
NAA	2	<0.001**	<0.001**	<0.001**
Kin	2	<0.001**	<0.001**	<0.001**
BA × NAA	4	<0.001**	0.0756 ^{NS}	0.0636 ^{NS}
BA × Kin	4	<0.001**	<0.001**	0.0338*
NAA × Kin	4	<0.001**	<0.001**	0.0015**
BA × NAA × Kin	8	<0.001**	<0.001**	<0.001**

^z1 mg·L⁻¹ = 1 ppm, 1 mm = 0.0394 inch.

^yValues followed by a common letter in a column are not significantly different according to Tukey's multiple range test ($P \leq 0.05$).

^x, ^{NS}, *, **Nonsignificant at $P > 0.05$, or significant at $P \leq 0.05$ or 0.001, respectively.

compared with SIM devoid of GA₃ (12.4 mm). This result supports the findings reported for several citrus cultivars (Paudyal and Haq, 2000; Pérez-Tornero et al., 2010; Tallón et al., 2012), that the addition of GA₃ during shoot proliferation was beneficial for all cultivars studied.

SUBCULTURING INITIAL EXPLANTS AFTER SHOOT REMOVAL FOR FURTHER PROLIFERATION. To maximize production of usable shoots from tissue with limited availability, it is desirable to remove and use shoots from the initial explants

followed by subculturing the explants for additional shoot generation. To assess this, shoots regenerated from the nodal explants were removed and the initial explant (with basal tissue remaining from removed shoots) was repeatedly subcultured. New shoots suitable for rooting or micrografting were produced at greater than 1 shoot/explant in each round of subculturing. A significant decrease in multiplication rate was observed after the second subculture in 'Carrizo' citrange and remained constant afterward (Table 5), whereas in 'Washington

Navel' orange, multiplication rate declined after the first subculture and remained constant from second to fourth subculture, after which further decline in shoot formation was observed (Table 5). Several strategies were attempted to overcome this reduction (data not shown). According to Rathore et al. (2007), shoot multiplication of mature lemon from nodal explants was increased in subcultures by reducing initial BA, ammonium nitrate (NH₄NO₃), and potassium nitrate (KNO₃), and supplementing ammonium sulfate [(NH₄)₂SO₄] on

Table 3. Percentage of explants producing axillary shoots, number of shoots produced per explant, and length of shoots for ‘Carrizo’ citrange and ‘Washington Navel’ orange cultured in vitro with different salt formulation media: Driver and Kuniyuki walnut medium (DKW), Murashige and Skoog medium (MS), and woody plant medium (WPM). ‘Washington Navel’ orange media contained 0.1 mg·L⁻¹ 6-benzylaminopurine (BA), 0.05 mg·L⁻¹ naphthalene acetic acid, 1 mg·L⁻¹ 6-furfurylaminopurine (kinetin); whereas media for ‘Carrizo’ citrange contained 1 mg·L⁻¹ BA. Data are from two independent experiments and were recorded after 45 d in vitro culture.^z

Cultivar	Media	Frequency of explants with shoots (%)	Mean shoots per initial explant (no.)	Length of shoots (mm) ^z
Carrizo	DKW	87.3 a ^y	4.2 a ^y	38.9 a ^y
	MS	84.3 ab	3.2 bc	22.3 b
	WPM	79.9 b	2.9 c	18.9 bc
Washington Navel	DKW	61.8 A	2.3 A	14.8 A
	MS	57.9 A	2.4 A	10.7 BC
	WPM	48.3 B	1.5 B	8.2 C

^z1 mg·L⁻¹ = 1 ppm, 1 mm = 0.0394 inch.

^yValues followed by the same uppercase letters in the same column, or followed by the same lowercase letters in the same column are not significantly different according to Tukey’s multiple range test ($P \leq 0.05$).

Fig. 1. In vitro propagation of juvenile ‘Carrizo’ citrange and mature ‘Washington Navel’ orange. (A) In vitro axillary shoot production in nodal explants of ‘Carrizo’ citrange (top row is typical vessel and next typical shoot) and ‘Washington Navel’ orange (bottom row is typical shoot and next above is typical vessel) cultured on three different salt media formulations: Driver and Kuniyuki walnut medium (DKW), Murashige and Skoog medium (MS), and woody plant medium (WPM). (B) Rooted plantlets of ‘Washington Navel’ orange as affected by media salt formulation. (C) Propagated ‘Washington Navel’ orange plant (left) and propagated ‘Carrizo’ citrange plant (right) acclimated in greenhouse conditions.

the MS medium. However, in our work, no significant increase in multiplication rate was noted when these modifications of MS were used for the second and subsequent passage (data not shown), and never equaled subculture proliferation using DKW media. In ‘Carrizo’ citrange, there was no significant change in shoot length during subculturing (Table 5), whereas shoot length increased for ‘Washington Navel’ orange.

ROOTING INDUCTION AND ESTABLISHMENT OF IN VITRO AXILLARY SHOOTS IN SOIL. For rooting induction, healthy axillary shoots from the initial culture (on SIM optimized for each cultivar) were transferred into a range of media salt compositions, based on previous work in citrus (Al Bahrany, 2002); 2.0 mg·L⁻¹ IBA and 0.5 mg·L⁻¹ NAA were used in all rooting media.

Root formation and root growth in ‘Carrizo’ citrange and ‘Washington

Navel’ orange were significantly affected by basal salt formulation and the ionic strength of the medium (Table 6). Roots induced on all strengths of MS were of good quality with greater length compared with those from WPM and DKW media. Full-strength MS medium showed the greatest percentage of explants with root induction (92.7%) and greatest mean root length (58.2 mm) in ‘Carrizo’ citrange, whereas half-strength MS provided the greatest length of primary roots (26.3 mm) in ‘Washington Navel’ orange (Fig. 1B), with both full- and half-strength MS providing the greatest rooting percentage. Marked reduction in length of primary roots was observed in full-strength and half-strength DKW media, which may be associated with large callus formation on these media (data not shown). In this case, quarter-strength DKW medium exhibited a slight improvement in rooting but was still reduced compared with MS medium. WPM also displayed poor root formation and growth in both genotypes. One explanation for these results may be deleterious effects of lower nitrogen (N) concentration in WPM compared with MS (14.5 mm in WPM vs. 60 mm in MS). Root formation and growth are energy-demanding processes, in which sufficient inorganic components (salts) and carbohydrates are required (George, 1996).

Supplementation of MS full-strength rooting-induction medium with 1 μM spermidine significantly increased root length in ‘Washington Navel’ orange (Table 6; Fig. 1B), without altering percentage of shoots rooting, but had no significant effect

Table 4. Effect of gibberellic acid (GA₃) on shoot elongation for in vitro ‘Washington Navel’ orange axillary shoot propagation. GA₃ was added to the shoot-induction medium [Driver and Kuniyuki walnut medium supplemented with 0.1 mg·L⁻¹, 6-benzylaminopurine, 1 mg·L⁻¹ 6-furfurylaminopurine (kinetin), and 0.05 mg·L⁻¹ naphthalene acetic acid] either at the time of explant preparation (day 0) or after buds began to grow (10–15 d); control (shoot-induction medium devoid of GA₃). Data are from two independent experiments and were recorded after 45 d in vitro culture.

Treatments	GA ₃ (mg·L ⁻¹) ^z	Frequency of cultures with shoots (%)	Mean shoots per initial explant (no.)	Length of shoots (mm) ^z
Control	0	68.9 a ^y	3.9 a ^y	12.4 c ^y
Day 0	0.5	59.7 a	3.5 a	27.8 b
	1.0	61.2 a	3.4 a	21.2 b
Day 10	0.5	70.5 a	3.7 a	35.9 a
	1.0	65.2 a	3.8 a	20.6 b

^z1 mg·L⁻¹ = 1 ppm, 1 mm = 0.0394 inch.

^yValues followed by a common letter in a column are not significantly different according to Tukey’s multiple range test ($P \leq 0.05$).

Table 5. Effect of successive subcultures on shoot multiplication parameters for ‘Carrizo’ citrange and ‘Washington Navel’ orange on Driver and Kuniyuki walnut medium. ‘Washington Navel’ orange shoot-multiplication medium contained 6-benzylaminopurine (BA), 1 mg·L⁻¹ 6-furfurylaminopurine (kinetin), 0.05 mg·L⁻¹ naphthalene acetic acid, and 0.5 mg·L⁻¹ gibberellic acid; whereas for ‘Carrizo’ citrange, DKW contained 1 mg·L⁻¹ BA. Data are from two independent experiments and were recorded after 30 d in vitro for each subculture.^z

Cultivar	Subculture	Mean shoots per initial explant (no.)	Length of shoots (mm) ^z
Carrizo	1	2.25 a ^y	41.3 a ^y
	2	2.34 a	40.8 a
	3	1.83 b	39.7 a
	4	1.78 b	41.5 a
	5	1.63 b	40.2 a
	6	1.59 b	42.9 a
Washington Navel	1	2.04 A	13.8 CD
	2	1.65 B	15.5 C
	3	1.58 B	16.1 BC
	4	1.72 B	18.4 B
	5	1.05 C	21.7 A
	6	1.17 C	20.2 A

^z1 mg·L⁻¹ = 1 ppm, 1 mm = 0.0394 inch.

^yValues followed by the same uppercase letters in the same column, or followed by the same lowercase letters in the same column indicates no significant differences according to Tukey’s multiple range test ($P \leq 0.05$).

on rooting rates or root size in ‘Carrizo’ citrange. However, for both cultivars, rooting occurred more rapidly in culture media containing spermidine than in the corresponding media without spermidine (data not shown). Rooting initiation began at 10–15 d in media containing spermidine, whereas media without spermidine showed rooting initiation only after 21 d. It has been proposed that endogenous polyamines are involved in root induction (Mendes et al., 2011), and several studies have documented the benefit of spermidine during rhizogenesis (Tang and Newton, 2005; Verma and Mishra, 2005). Mendes et al. (2011) reported that supplementation of rooting-induction medium with 1–100 μ M spermidine significantly enhanced root formation and growth in sweet orange (*C. sinensis*). Amri and Shahsavari (2010) showed that exogenous spermidine treatment improved growth of root, shoot, and

total plant biomass in acid lime (*Citrus aurantifolia*).

Our results showed a marked enhancement in rooting induction of ‘Carrizo’ citrange axillary shoots compared with previous reports by Montoliu et al. (2010) and Kanwar et al. (2013), and root lengths were also greater than previously reported in other studies with citrus rootstocks (Tallón et al., 2012) and sweet orange (Mendes et al., 2011). The rooted shoots developed normally, and plants were transferred to the soil (Fig. 1C) with a survival rate of 100% in ‘Carrizo’ citrange and 82.5% in ‘Washington Navel’ orange.

Conclusion

In summary, the present study provides an efficient protocol for the propagation of ‘Carrizo’ citrange and ‘Washington Navel’ orange from nodal stem explants using axillary shoot proliferation, with similar protocols

probably suitable for closely related cultivars. DKW medium supplemented with 0.1 mg·L⁻¹ BA, 0.05 mg·L⁻¹ NAA, 1 mg·L⁻¹ Kin, and 0.5 mg·L⁻¹ GA₃, provided the best shoot proliferation response in ‘Washington Navel’ orange, whereas in ‘Carrizo’ citrange, best success was achieved on DKW medium with 1.0 mg·L⁻¹ BA. Although multiplication slowed after the first or second subcultures, proliferation continued with repeated subcultures and no further decline was observed through the six subcultures tested in ‘Carrizo’ citrange and the fourth subculture for ‘Washington Navel’ orange. For rooting in vitro, best results in ‘Carrizo’ were obtained with shoots cultured on full-strength MS medium supplemented with 2.0 mg·L⁻¹ IBA and 0.5 mg·L⁻¹ NAA, whereas for ‘Washington Navel’ orange the best results were obtained with half-strength MS amended with 2.0 mg·L⁻¹

Table 6. In vitro root formation and growth in ‘Carrizo’ citrange and ‘Washington Navel’ orange as affected by the ionic strength of three different media salt formulations [Driver and Kuniyuki walnut medium (DKW), Murashige and Skoog medium (MS), and woody plant medium (WPM)] all amended with 2.0 mg·L⁻¹ indolebutyric acid (IBA) and 0.5 mg·L⁻¹ naphthalene acetic acid (NAA). MS amended with 2.5 mg·L⁻¹ IBA and 0.5 mg·L⁻¹ NAA was also tested with and without spermidine (Spd) at 1 μM. Shoots were taken from ‘Carrizo’ citrange shoot proliferation DKW medium containing 1 mg·L⁻¹ 6-benzylaminopurine (BA) and ‘Washington Navel’ orange shoot proliferation DKW medium contains 0.05 mg·L⁻¹ NAA, 1 mg·L⁻¹ 6-furfurylaminopurine (kinetin), and 0.1 mg·L⁻¹ BA.^z

Media	Strength	‘Carrizo’		‘Washington Navel’	
		Plants rooting (%)	Mean length of roots formed (mm) ^y	Plants rooting (%)	Mean length of roots formed (mm)
DKW	Full strength	62.7 c ^y	10.1 d ^y	35.8 c ^y	8.5 d ^y
	Half strength	61.3 c	11.7 d	37.2 c	9.2 cd
	Quarter strength	77.2 b	19.8 d	40.0 c	11.2 c
MS	Full strength	92.7 a	58.2 a	60.8 a	15.7 c
	Half strength	78.3 b	49.6 bc	59.4 ab	26.3 ab
	Quarter strength	75.2 bc	42.3 c	50.9 b	21.6 b
WPM	Full strength	56.4 cd	12.5 d	17.5 e	11.9 c
	Half strength	42.3 e	14.3 d	22.5 de	12.6 c
	Quarter strength	47.8 cd	16.6 d	19.5 e	13.7 c
MS	Full strength + Spd	91.6 a	61.2 a	63.7 a	30.5 a
	Half strength + Spd	80.3 b	52.7 ab	68.7 a	33.9 a

^z1 mg·L⁻¹ = 1 ppm, 1 mm = 0.0394 inch.

^yData are from two independent experiments and were recorded after 45 d in vitro culture. Values followed by a common letter in a column are not significantly different according to Tukey’s multiple range test ($P \leq 0.05$).

IBA, 0.5 mg·L⁻¹ NAA, and 1 μM spermidine.

The micropropagation strategy that we report appears suitable to increase material available for propagation, with a total of 15.5 rooted ‘Carrizo’ citrange shoots per initial explant and 11.7 micrograftable ‘Washington Navel’ orange shoots (or 10.0 rooted cuttings) from each initial node used as an explant (calculated from data presented in this paper using the best procedure at each step for each cultivar). Since the material used for micropropagation is smaller than the material suited for conventional budding or grafting, this provides a complimentary tool for enhancing supply of citrus cultivars where demand exceeds supply. This method also provides material year-round suitable for subsequent transformation and regeneration experiments.

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