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Effect of various factors on shoot regeneration from citrus epicotyl explants

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Abstract

The effect of various treatments on shoot organogenesis from seedling epicotyl explants from various scion and rootstock polyembryonic citrus types was determined. Treatments included water source, gelling agent, explant insertion, seed size, light intensity, malachite green, nonionic surfactants, and sodium sulphate. Tap water, with the highest levels of SO_4^{2-}, Ca^{2+}, K^+, Mg^{2+}, and Na^+, resulted in the most shoots compared to the other 5 sources, suggesting a mineral nutrient effect. Carrageenan produced fewer shoots than agar and gellan gum. Explants inserted into the medium produced more shoots than those cultured on the surface, presumably because of better exposure to water and nutrients. Seed size, light intensity, malachite green, and sodium sulphate had no effect on the number of shoots regenerated. Triton X-100 at 0.1 % resulted in significantly fewer shoots; otherwise, nonionic surfactants had no effect.

Key words: Water, nonionic surfactants, gelling agents, malachite green, sodium sulphate, *Citrus sinensis* L. Osbeck. x *Poncirus trifoliata* L. Raf., *C. sunki* Hort. ex Tanaka. x *Poncirus trifoliata* L. Raf., *C. paradisi* Macf., *C. sinensis* L. Osbeck

Introduction

Citrus species are a major fruit crop worldwide and are consumed fresh as fruits, and processed, generally as juice. A citrus tree is typically grown on a rootstock. Some advantages of using a rootstock include tolerance to local biotic and abiotic conditions, control of tree size, earlier flowering and fruiting, and enhanced fruit quality. One of the earliest references to the use of rootstocks in citrus is the use of lemon as a rootstock to grow citrons in the Palestine area (Mudge *et al.*, 2009). Because of the significant interaction between the scion and the rootstock, modern plant breeding programs include the development of both scion and rootstock types. Thus, plant breeding methods must be useful for both citrus scion and rootstock variety development.

In vitro methods are used in plant breeding to achieve objectives that are impossible or difficult to achieve via conventional methods. For example, adventitious shoot formation or the *de novo* formation of meristems is an *in vitro* method often used in citrus breeding to produce transgenic plants (Moore *et al.*, 1992; Pena *et al.*, 1995; Gutiérrez-E *et al.*, 1997; Peña *et al.*, 1997; Cervera *et al.*, 1998; Cervera *et al.*, 1998; Luth and Moore, 1999; Costa *et al.*, 2002; Yu *et al.*, 2002; Almeida *et al.*, 2003; Almeida *et al.*, 2003; Li *et al.*, 2003; Khawale *et al.*, 2006; Cervera *et al.*, 2008; Dutt and Grosser, 2009; He *et al.*, 2011; Marutani-Hert *et al.*, 2012; Orbović *et al.*, 2012).The efficiency of shoot formation varies widely across citrus species and types, and is an important factor that affects the recovery efficiency of producing transgenic plants. Understanding the conditions that affect adventitious shoot formation is required to increase shoot regeneration efficiency across a broad range of genotypes and, consequently reduce the cost and broaden the applications that utilize adventitious shoot formation.

We report the results from a series of experiments that tested various treatments reported in the plant tissue culture literature to improve adventitious shoot formation.

Materials and methods

Plant material, explant source, and culture conditions: Seed of Carrizo citrange (*Citrus sinensis* 'Washington' L. Osbeck. x *Poncirus trifoliata* L. Raf.), Duncan grapefruit (*C. paradise* Macf.), Hamlin sweet orange (*C. sinensis* L. Osbeck) grapefruit (*C. paradise* Macf.), and US-812 (*Citrus sunki* Hort. ex Tanaka. x *P. trifoliata* L. Raf.) (Bowman and Rouse, 2006) were surface disinfested as follows: after removal of the seed coat, the seeds were soaked for 30 min in 50 mL of a 30 % bleach (5.25 % w/v sodium hypochlorite) solution with 3 drops of Tween 20. Seeds were then rinsed 3 times with sterile water, allowed to soak for 18 h in water, placed on the surface of MT basal medium (Murashige and Tucker, 1969) solidified with 8 % (w/v) Ultrapure Type A bacteriological agar (USB Corporation, Cleveland, OH, USA) in Magenta GA-7-3 vessels (Magenta Corporation, Chicago, IL, USA), and then incubated in the dark at 27 °C for 3-4 week. One-cm-long explants were excised from the epicotyl of the etiolated seedlings. Shoot regeneration experiments were conducted in growth cabinets at 27 °C over 6-2 week in the dark followed by 4 wk in the light. Light was provided by cool-white fluorescent lamps (30-55 µmol m^{-2} s^{-1}) with a 16-h photoperiod.

Responses measured and replication used: Shoot regeneration was measured by counting the number of shoots ≥ 2 mm, the minimum size for micrografting, on each epicotyl explant. For all experiments each treatment was measured from four 100 x 115 mm culture dishes, with each dish containing five epicotyl explants derived from a single seedling (1 seedling per dish). A replicate was a second set of four dishes with five explants per dish.

Effect of water source: Six water sources were tested using Carrizo. The experiment was as a single-factor, water source, design with six levels that included 1) tap from the lab, 2) drinking (Walmart, Bentonville, AR, USA), 3) distilled (Walmart,

Bentonville, AR, USA), 4) laboratory glass distilled, 5) Milli Q, and 6) reverse osmosis. The data was analyzed by one-way ANOVA followed by the Tukey's multiple comparison test. The mineral nutrient profile of each water source was determined by ion chromatography and included anions ($B(OH)_4^-$, Cl^-, F^-, NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}) and cations (Li^+, Na^+, $NH4^+$, K^+, Mg^{2+}, and Ca^{2+}) as described (U.S. Environmental Protection Agency, 1997).

Effect of gelling agent (agar, carrageenan, gellan gum): Three gelling agents were tested using Hamlin and US-812. The experiment was a single-factor design with gelling agent set at three levels that included 1) Ultrapure, Type A, bacteriological agar (Affymetrix, Santa Clara, CA, USA), 2) Gelcarin GP 812 carrageenan (PhytoTechnology Laboratories, Shawnee Mission, KS, USA), and 3) Culturegel™ Type I gellan gum (PhytoTechnology Laboratories, Shawnee Mission, KS, USA). The data was analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Effect of explant insertion: Explant insertion into the culture medium was tested. Explants were positioned horizontally "in" or "on" the medium. An explant "in" the medium was pushed into the medium so that the top of the explant was even with the surface of the medium. An explant "on" the medium was laid on the surface of the medium without insertion. The experiment was a 2-factor design of insertion (in, on) and cultivar (Hamlin, Carrizo). The data was analyzed by 2-way ANOVA.

Effect of seed size: Seed size was tested using Carrizo. First, a frequency distribution histogram was generated from the weights of five hundred Carrizo seed. Second, the experiment was a single-factor design with bin set at four levels that included 0-0.1, 0.1-0.2, 0.2-0.3, and 0.3-0.4 g. The data was analyzed by one-way ANOVA.

Effect of light intensity: Following the 2-week dark incubation period, light was tested at three levels (20, 39, and 89 $\mu E\ m^{-2}\ s^{-1}$) using Hamlin. The data was analyzed by one-way ANOVA.

Effect of malachite green: The aniline dye malachite green (Sigma-Aldrich, St. Louis, MO, USA) was tested at 4 concentrations (0, 0.001, 0.01 and 0.1 mM) using Hamlin. The data was analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test that compared each non-zero concentration to 0 mM.

Effect of nonionic surfactants: Three nonionic surfactants were tested using Hamlin. The experiment was a single-factor that included a 0 % surfactant control and eleven surfactant-concentration combinations as follows: Control (0 % surfactant), Pluronic F-68 (0.01, 0.1, and 1 %), Triton X-100 (0.0001, 0.001, 0.01, and 0.1 %), and Tween 20 (0.0001, 0.001, 0.01, and 0.1 %).

The data was analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test that compared each of the eleven surfactant-concentration treatments to the 0 % surfactant control.

Effect of sodium sulphate: Sodium sulphate, Na_2SO_4, was tested at 4 concentrations (0, 0.1, 1, and 2 mM) using Hamlin. The data was analyzed by one-way ANOVA.

Results and discussion

Effect of water source: The source of water used to make plant tissue culture medium is probably the most basic component of any medium. Thus, determining the effects of various available water sources is an important initial quality control step.

Six water sources were used and their ionic composition determined (Table 1). Sources that had levels of ions >= 1 mM included tap water (SO_4^{2-}, Ca^{2+}, K^+, Mg^{2+}, and Na^+) and Walmart drinking water (Na^+ and Cl^-). The remaining four sources had levels that were either "not detectable" or < 1 mM. A one-way ANOVA was conducted to compare the effect of the water samples on the number of shoots/explant of a size suitable for convenient shoot tip grafting (Table 2). The effect of water source on shoots produced was significant ($P<0.0001$). The mean separation analysis by Tukey's test revealed that tap water produced significantly more shoots compared to each of the other five sources, which did not significantly differ from each other (Fig. 1).

Tap water produced 40 % more shoots/explant than the average of the other water sources. This result suggested that mineral nutrition, specifically higher levels of SO_4^{2-}, Ca^{2+}, K^+, Mg^{2+}, and/or Na^+ were responsible for the greater number of shoots produced in media made with tap water. Because a complete analysis of the water for all ions and compounds was not done, an experiment that varied amount of the ions must be conducted to confirm their effect. Though tap water was the "best" source, its use is problematic due to repeatability issues. If tap water is used, an ionic composition analysis should be provided. Reconstructing tap water would require the use of software such as ARS-Media (http: //www.ars.usda.gov/services/software/download.htm?softwareid=148#downloadForm) that uses the linear programming algorithm previously reported (Niedz and Evens, 2006).

Table 1. The mineral nutrient profile of 6 water sources as determined by ion chromatography

Water Sources	Anions (mg L^{-1})							Cations (mg L^{-1})					
	$B(OH)_4^-$	Cl^-	F^-	NO_2^-	NO_3^-	PO_4^{3-}	SO_4^{2-}	Ca^{2+}	K^+	L^+	Mg^{2+}	Na^+	NH_4^+
Tap	n.d.	n.d.	< 1	< 1	< 1	< 1	48	23	3	< 1	6	50	1
Distilled (Walmart)	n.d.	< 1	n.d.	n.d.	n.d.	n.d.	n.d.	< 1	< 1	n.d.	< 1	< 1	< 1
Distilled (lab glass)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< 1	< 1	< 1	< 1	< 1	< 1
Drinking (Walmart)	n.d.	8	n.d.	n.d.	< 1	n.d.	n.d.	< 1	< 1	n.d.	< 1	9	n.d.
Milli-Q	n.d.	< 1	n.d.	n.d.	n.d.	n.d.	n.d.	< 1	< 1	n.d.	< 1	< 1	< 1
Reverse Osmosis	n.d.	< 1	n.d.	n.d.	n.d.	n.d.	n.d.	< 1	< 1	n.d.	< 1	< 1	< 1

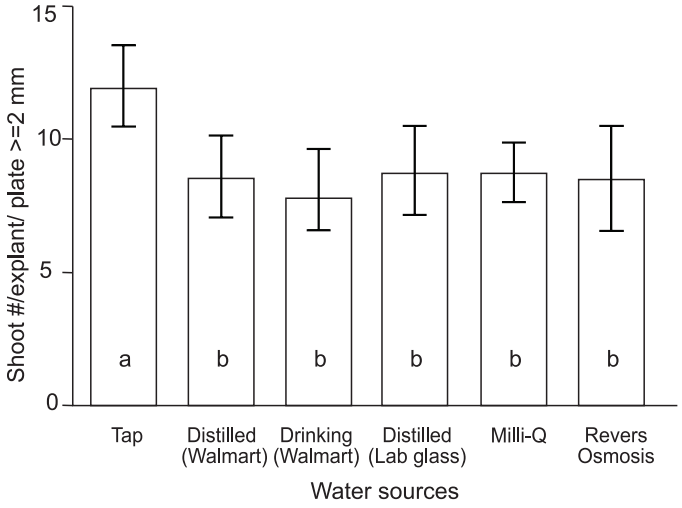

Fig. 1. The effect of water source on the number of shoots produced per explant from Carrizo explants that were >= 2 mm. Mean separation by Tukey's multiple comparison test where bars with different letters were significantly different. Bars expressed as mean ± standard deviation.

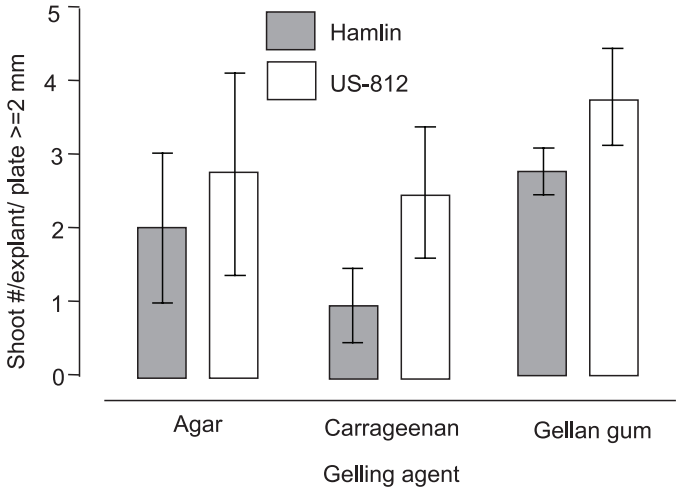

Fig. 2. The effect of 3 gelling agents and 2 citrus types on the number of shoots produced per explant that were >= 2 mm. Bars expressed as mean ± standard deviation.

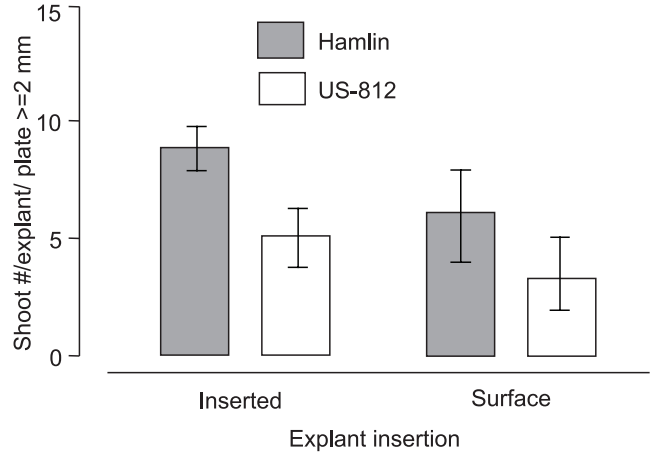

Fig. 3. The effect of explant insertion and variety on the number of shoots per explant >= 2 mm that were regenerated from Hamlin and Carrizo epicotyl explants. Bars expressed as mean ± standard deviation.

Effect of gelling agent (agar, carrageenan, gellan gum): The substrate used to support *in vitro* explants is another basic component of a plant tissue culture system. Determining the effects of various substrates is another important initial quality-control step. Three biopolymers, two algal and one bacterial, were selected and included agar, a "linear polysaccharide made up of alternating β(1, 3)- and α(1, 4)-linked galactose residues"; carrageenan, a "linear, sulphated polysaccharide based on a repeating disaccharide sequence of β-D-galactopyranose residues linked glycosidically through various positions" and, gellan gum, a "linear, anionic heteropolysaccharide based on a tetrasaccharide repeat unit 1, 3-β-D-glucose, 1, 4-β-D-glucouronic acid, 1, 4-β-D-glucose, and 1, 4-α-L-rhamnose (Stephen *et al.*, 2006).

A two-way ANOVA was conducted to determine the effects of 3 gelling agents and 2 citrus types on the number of shoots/explant (Table 3). The effects of gelling agent and variety were significant (P=0.0153 and P=0.0111, respectively), but the effect of the interaction between gelling agent and variety was not significant (P=0.7025). The mean separation analysis by Tukey's multiple comparison test on each set of gelling agents within each variety revealed only the carrageenan *vs*. gellan gum contrast of Hamlin sweet orange was significant at alpha 0.05 (Table 4); Hamlin shoot regeneration was reduced on carrageenan (Fig. 2). The significant variety effect on shoot regeneration was due to a greater number of shoots/explant produced by US-812 (Fig. 2). The high shoot organogenic capacity of US-812 relative to Hamlin may be because it is a *P. trifoliate* hybrid. Though we are not aware of studies that directly compare the shoot organogenic capacity of *P. trifoliata* to other citrus types, *P. trifoliata* hybrids typically have high shoot organogenic capacity when compared across studies (Burger and Hackett, 1986; Duran-Vila *et al.*, 1989; Sim *et al.*, 1989; Maggon and Deo Singh, 1995; Pérez-Molphe-Balch and Ochoa-Alejo, 1997; García-Luis *et al.*, 1999; Van Le *et al.*, 1999; Bordón *et al.*, 2000; Moreira-Dias *et al.*, 2000; Moreira-Dias *et al.*, 2001; Almeida *et al.*, 2002; Costa *et al.*, 2004; Da Silva *et al.*, 2005; Ali and Mirza, 2006; García-Luis *et al.*, 2006; Molina *et al.*, 2007; da Silva *et al.*, 2010; Marques *et al.*, 2011; Niedz and Evens, 2011). Because there are a large number of available gelling agents, each with complex colloidal effects, predicting their effects is difficult. Empirical testing may be the current best method to determine which ones work well for a particular species and application.

Effect of explant insertion: A two-way ANOVA was conducted to determine the effects of explant insertion, explants positioned on or in the medium, and citrus type on the number of shoots/explant that were produced (Table 5). The effects of explant insertion and variety were significant (P=0.0026 and P<0.0001, respectively), but the effect of the interaction of explant insertion x variety was not significant (P=0.4178). Explants inserted into the medium produced more shoots, and Carrizo produced more shoots/explant than Hamlin (Fig. 3). Insertion of explants into the medium resulted in the complete exposure of the cut ends to the culture medium and, consequently, better exposure of the cells to water, nutrients, and growth factors. The high shoot organogenic capacity of Carrizo may be due to it being a *P. trifoliata* hybrid like US-812.

Effect of seed size: Five hundred Carrizo seed were individually weighed. Seed weight ranged from 8.2 mg to 601 mg. A frequency distribution was generated using 4 bins that were each 100 mg wide (Fig. 4). Eleven seed exceeded the maximum size of the 300-400 mg bin and were not used; the single largest seed was 601 mg.

A one-way ANOVA was conducted to compare the effect of seed size bins on the number of shoots/explant that were produced (Fig. 5). The effect of seed size bins on shoots produced was not significant (P=0.6116); thus, sorting seeds by size is not required to improve shoot regeneration. Seed size can affect germination and growth (Keddy and Constabel, 1986; Zammit and Zedler, 1990; Bretagnolle *et al.*, 1995; Eriksson, 1999; Soltani *et al.*, 2002; Khurana and Singh, 2004), but there are few studies on the effect of seed size on *in vitro* responses. A study of the effect of large vs small seeds in barley on embryogenic callus induction and subsequent shoot regeneration in barley, observed that large seeds produced significantly more callus and shoots than small seed (Özgen *et al.*, 2007).

Table 2. ANOVA of the effect of six sources of water on the numbers of shoots >= 2 mm that were regenerated from Carrizo epicotyl explants

Factor	SS	df	MS	F	*P*-value
Water source[a]	120	5	24	$9_{(5, 66)}$	< 0.0001
Error	177	66	2.7		
Total	297	71			

[a] – Six sources that included 1) tapwater, 2) drinking (Walmart), 3) distilled (Walmart), 4) distilled (laboratory glass), 5) Milli Q, and 6) reverse osmosis.

Table 3. ANOVA of the effect of gelling agent on the number of shoots per explant >= 2 mm that were regenerated from Hamlin and US-812 epicotyl explants

Factor	SS	df	MS	F	*P*-value
Gelling agent[a]	8.78	2	4.39	$5.40_{(2, 17)}$	0.0153
Variety[b]	6.60	1	6.60	$8.12_{(1, 17)}$	0.0111
Gelling agent x Variety	0.59	2	0.29	$0.36_{(2, 17)}$	0.7025
Error	13.83	17	0.81		
Total	29.80	23			

[a] – Three gelling agents included 1) agar, 2) carrageenan, and 3) gellan gum.

[b] – Two citrus varieties included 1) Hamlin sweet orange, and 2) US-812 citrange.

Table 4. Tukey's multiple comparison test of gelling agents within Hamlin sweet orange and US-812 citrange

Comparisons	Mean difference	Significance
Hamlin		
agar vs. carrageenan	1.05	ns
agar vs. gellan gum	-0.75	ns
carrageenan vs. gellan gum	-1.80	*
US-812		
agar vs. carrageenan	0.300	ns
agar vs. gellan gum	-0.98	ns
carrageenan vs. gellan gum	-1.28	ns

Table 5. ANOVA of the effect of explant insertion and variety on the number of shoots per explant>= 2 mm that were regenerated from Hamlin and Carrizo epicotyl explants

Factor	df	MS	F	*P*-value
Explant insertion[a]	1	33.84	$11.82_{(1, 20)}$	0.0026
Variety[b]	1	67.20	$23.49_{(1, 20)}$	<0.0001
Explant insertion x Variety	1	1.96	$0.68_{(1, 20)}$	0.4178
Error	20	2.86		
Total	23			

[a] – Two horizontal insertion depths included 1) no insertion where the explant was laid on the surface of the medium, and 2) completely inserted where the top of the explant was even with the surface of the medium.

[b] – Two citrus varieties included 1) Hamlin sweet orange, and 2) Carrizo citrange.

Effect of light intensity: Light intensity can sometimes affect shoot regeneration as reported for a diverse array of plant species such as evergreen azalea (Hsia and Korban, 1998), muskmelon (Niedz *et al*., 1989), sugarcane (Sengar *et al*., 2011), apple (Magyar-Tábori *et al*., 2010), and cotton (Gupta *et al*., 2000). To the best of our knowledge, the effect of light intensity on shoot regeneration from citrus tissue explants has not been reported. However, a dark incubation period prior to incubation in the light was required for shoot regeneration from adult internode explants of sweet orange, grapefruit, and a citrange (Marutani-Hert *et al*., 2012). Following the dark incubation period, light was varied from 20 to 89 µE. A one-way ANOVA was conducted to compare the effect of light intensity on the number of shoots/explant that were produced from Hamlin epicotyl explants. The effect of light intensity was non significant (P=0.1831) (Fig. 6); thus, the range of 20 to 89 µE produced equivalent numbers of shoots/explant.

The effect of malachite green: The aniline dye malachite green was reported to promote shoot regeneration in raspberry leaf explants when used at less than 20 mg L-1(0.0548 mM) and

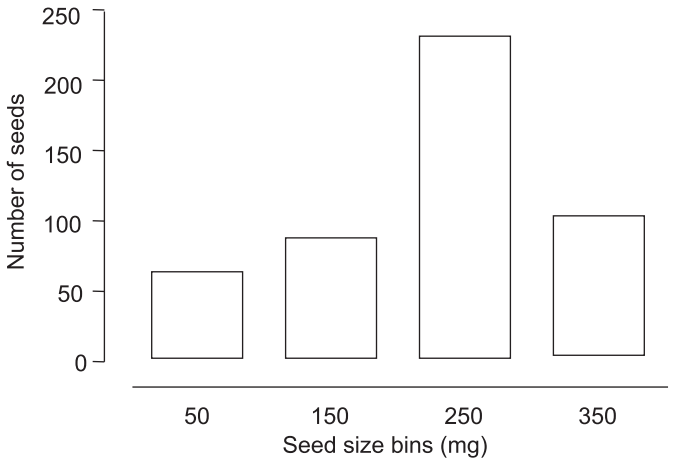

Fig. 4. Frequency distribution of five hundred Carrizo seed weights using 4 bins that were each 100 mg wide. Eleven seed exceeded the maximum size of the 300-400 mg bin and were not included in the experiment.

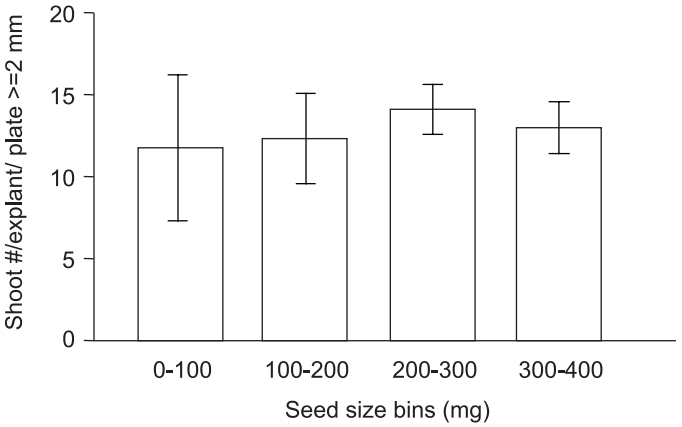

Fig. 5. The effect of seed size bins on the number of shoots/explant from Carrizo epicotyl explants that were produced. Bars expressed as mean ± standard deviation.

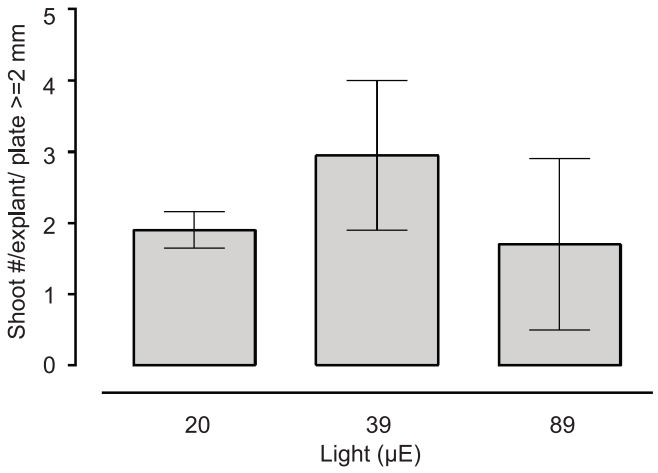

Fig. 6. The effect of light intensity on the number of shoots/explant from Hamlin epicotyl explants that were produced. Bars expressed as mean ± standard deviation.

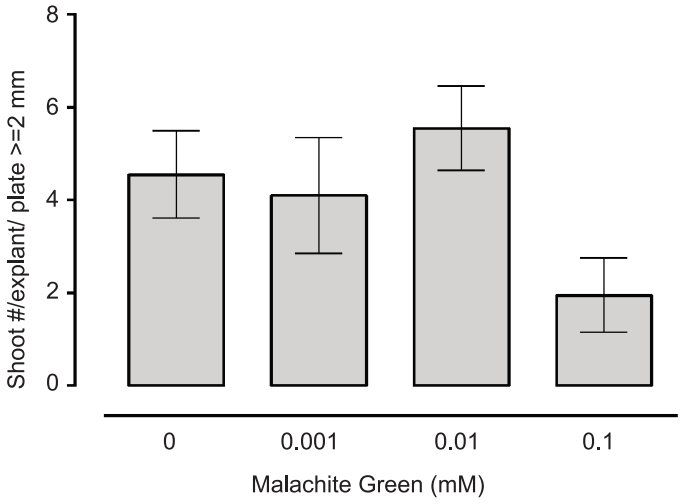

Fig. 7. The effect of the aniline dye malachite green on the number of shoots/explant from Hamlin epicotyl explants that were produced. Bars expressed as mean ± standard deviation.

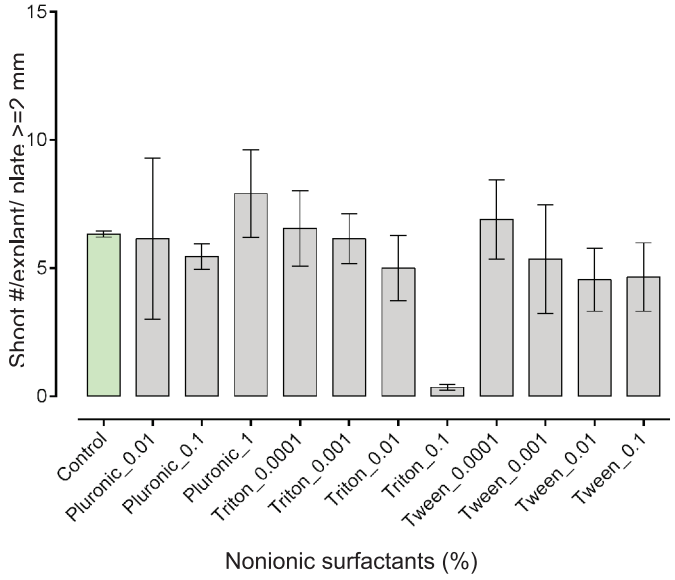

Fig. 8. The effect of 11 nonionic surfactants/concentration combinations on the number of shoots/explant from Hamlin epicotyl explants that were produced. Bars expressed as mean ± standard deviation.

for the propagation of blackberries (Herman, 1995); this is the only report we could find on the use of malachite green in plant tissue culture. Malachite green is used extensively as a biocide in aquaculture to control protozoan and fungal infections (Srivastava *et al*., 2004). To examine its effects in citrus a one-way ANOVA was conducted to compare the effect of malachite green at four concentrations (0, 0.001, 0.01, and 0.1 mM) using Hamlin epicotyl explants (Table 6). The effect of malachite green was significant (P=0.0018). A mean separation analysis by Dunnett's multiple comparisons test compared each concentration to the 0 mM level and revealed that significantly fewer shoots were produced from the 0.1 mM treatment compared to 0 mM (Fig. 7). These results are partially consistent with the findings for raspberry. No enhancement of shoot regeneration was observed, but the reduction in shoot regeneration occurred at 0.1 mM or 36.4 mg L-1, greater than the 20 mg L-1threshold reported for raspberry.

Effect of nonionic surfactants: Nonionic surfactants can enhance shoot regeneration (Khatun *et al*., 1993; Khatun *et al*.,

Table 6. ANOVA of the effect of Malachite Green on the numbers of shoots/explant >= 2 mm that were regenerated from Hamlin epicotyl explants

Factor	SS	Df	MS	F	*P*-value
Malachite Green[a]	27.7	3	9.3	$9.4_{(3, 12)}$	0.0018
Error	11.8	12	0.98		
Total	39.5	71			

[a] – Malachite Green tested at 4 levels – 0, 0.001, 0.01, and 0.1 mM.

Table 7. ANOVA of the effect of eleven non-ionic surfactant-concentration combinations on the numbers of shoots >= 2 mm that were regenerated from Carrizo epicotyl explants

Factor	SS	df	MS	F	*P*-value
Nonionicsurfactants[a]	154	11	14	$5.85_{(11, 35)}$	< 0.0001
Error	84	35	2.4		
Total	238	46			

[a] – Three non-ionic surfactants and a control that included 1) Pluronic F-68 (0.01, 0.1, 1%), 2) Triton X-100 (0.0001, 0.001, 0.01, 0.1%), 3) Tween 20 (0.0001, 0.001, 0.01, 0.1), and 4) control (no non-ionic surfactant).

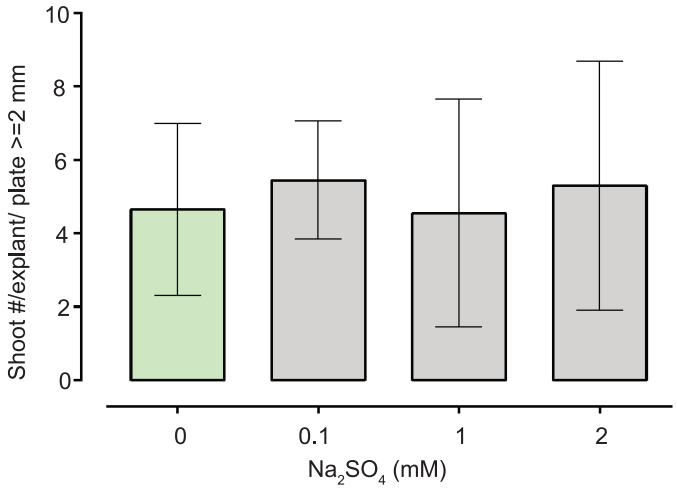

Fig. 9. The effect of sodium sulfate (Na_2SO_4) on the number of shoots/explant from Hamlin epicotyl explants that were produced. Bars expressed as mean ± standard deviation.

1993; Davey *et al*., 2003), including citrus (Cancino *et al*., 2001; Curtis and Mirkov, 2011). Though the mechanism is unknown, but may relate to the hydrophilic-hydrophobic balance (HLB) value that determines how easily the detergent can interact with the membrane's lipid component (Helenius and Simons, 1975; Curtis and Mirkov, 2011). The HLB numbers ranged from a high of 29 Pluronic F-68 to a low of 13.5 for Triton X-100; the HLB number of Tween 20 is 16.7. A one-way ANOVA was conducted to compare the effect of the nonionic surfactants/ concentration combinations on the number of shoots/explant that were produced from Hamlin epicotyl explants (Table 7). The effect of nonionic surfactant on shoots produced was significant (P<0.0001). The mean separation analysis by Dunnett's multiple comparisons test compared each surfactant/concentration to the zero surfactant control. The analysis revealed that Triton X-100 0.1 % treatment produced significantly fewer shoots (Fig. 8). The variance between our results and those previously reported for citrus suggests the existence of system specific effects. Thus, preliminary empirical testing is recommended.

The effect of sodium sulphate (Na_2SO_4): Sodium sulphate increased the number of shoots/nodal explant in the Indian medicinal plant *Vitex negundo* (Chandramu, 2003). A one-way ANOVA was conducted to compare the effect of Na_2SO_4 at four concentrations (0, 0.1, 1, and 2 mM) on the number of shoots/ explant that were produced from Hamlin epicotyl explants (Table 8). The effect of Na_2SO_4 was not significant (P=0.84) and revealed that supplementing MS medium with Na_2SO_4 up to 2 mM did not affect the number of shoots produced (Fig. 9). However, the effect on *Vitex negundo* may have been due to an interaction with sucrose. Though the authors did not analyze their data for interaction effects, they mentioned the effect and the data they presented in Fig. 2 does suggest a strong interaction between Na_2SO_4 and sucrose, where the increase in shoot number was only observed in the region of 0.29 mM Na_2SO_4 and 5-6 % sucrose. Because single factor experiments cannot detect interactions, our experiment would have not detected a Na_2SO_4 x sucrose interaction effect.

Table 8. ANOVA of the effect of sodium sulfate (Na_2SO_4) on the numbers of shoots/explant >= 2 mm that were regenerated from Hamlin epicotyl explants

Factor	SS	Df	MS	F	P-value
Na_2SO_4 [a]	2.5	3	0.8	$0.29_{(3, 12)}$	0.84
Error	34.6	12	2.9		
Total	37.1	15			

[a] – Na_2SO_4 tested at 4 levels – 0, 0.1, 1, and 2 mM.

The study indicate that water source, gelling agent, explant insertion influence shoot organogenesis from seedling epicotyl explants. Variable results under different factors suggest need for optimization of media and culture conditions and the results can be used for conducting further studies.

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References

Ali, S. and B. Mirza, 2006. Micropropagation of rough lemon (*Citrus jambhiri* Lush.): Effect of explant type and hormone concentration. *Acta Bot. Croat.,* 65: 137-146.

Almeida, W.A.B., F.A.A. Mourão Filho, L.E. Pino, R.L. Boscariol, A.P.M. Rodriguez and B.M.J. Mendes, 2003. Genetic transformation and plant recovery from mature tissues of *Citrus sinensis* L. Osbeck. *Plant Sci.,* 164: 203-211.

Almeida, W.A.B., F.A.A. Mourão Filho, B.M.J. Mendes, A. Pavan and A.P.M. Rodriguez, 2003. *Agrobacterium*-mediated transformation of *Citrus sinensis* and *Citrus limonia* epicotyl segments. *Scientia Agricola,* 60: 23-29.

Almeida, W.A.B., F.A.A. Mourão Filho, B.M.J. Mendes and A.P.M. Rodriguez, 2002. *In vitro* organogenesis optimization and plantlet regeneration in *Citrus sinensis* and *C. limonia*. *Scientia Agricola,* 59: 35-40.

Bordón, Y., J.L. Guardiola and A. García-Luis, 2000. Genotype affects the morphogenic response *in vitro* of epicotyl segments of citrus rootstocks. *Ann. Bot.,* 86: 159-166.

Bowman, K.D. and R.E. Rouse, 2006. US-812 citrus rootstock. *HortScience,* 41: 832-836.

Bretagnolle, F., J.D. Thompson and R. Lumaret, 1995. The influence of seed size variation on seed germination and seedling vigour in diploid and tetraploid *Dactylis glomerata* L. *Ann. Bot.,* 76: 607-615.

Burger, D.W. and W.P. Hackett, 1986. Gradients of adventitious bud formation on excised epicotyl and root sections of citrus. *Plant Sci.,* 43: 229-232.

Cancino, G.O., M.I.S. Gill, P. Anthony, M.R. Davey, J.B. Power and K.C. Lowe, 2001. Pluronic F-68 enhanced shoot regeneration in a potentially novel citrus rootstock. *Artificial Cells, Blood Substitutes, and Immobilization Biotechnology,* 29: 317-324.

Cervera, M., J. Juárez, A. Navarro, J.A. Pina, N. Durán-Vila and L. Navarro, 1998. Genetic transformation and regeneration of mature tissues of woody fruit plants bypassing the juvenile stage. *Transgenic Res.,* 7: 51-59.

Cervera, M., A. Navarro, L. Navarro and L. Peña, 2008. Production of transgenic adult plants from clementine mandarin by enhancing cell competence for transformation and regeneration. *Tree Physiology,* 28: 55-66.

Cervera, M., J.A. Pina, J. Juárez, L. Navarro and L. Peña, 1998. *Agrobacterium*-mediated transformation of citrange: Factors affecting transformation and regeneration. *Plant Cell Rep.,* 18: 271-278.

Chandramu, C.R., D. Manohar Rao and V. Dashavantha Reddy, 2003. High frequency induction of multiple shoots from nodal explants of *Vitex negundo* L. using sodium sulphate. *J. Plant Biotech.,* 5: 107-113.

Costa, M., W. Otoni and G. Moore, 2002. An evaluation of factors affecting the efficiency of *Agrobacterium* -mediated transformation of *Citrus paradisi* (Macf.) and production of transgenic plants containing carotenoid biosynthetic genes. *Plant Cell Rep.,* 21: 365-373.

Costa, M.G.C., V.S. Alves, E.R.G. Lani, P.R. Mosquim, C.R. Carvalho and W.C. Otoni, 2004. Morphogenic gradients of adventitious bud and shoot regeneration in epicotyl explants of Citrus. *Sci. Hortic.,* 100: 63-74.

Curtis, I.S. and T.E. Mirkov, 2011. Influence of surfactants on growth and regeneration from mature internodal stem segments of sweet orange (*Citrus sinensis*) cv. Hamlin. *Plant Cell, Tissue Organ Culture,* 108: 345-352.

da Silva, R.P., M.A.P. De Carvalho Costa, A. Da Silva Souza and E.W.A. De Almeida, 2005. Regeneration of 'Pera' sweet orange plants through *in vitro* organogenesis. *Regeneração de plantas de laranja 'Pêra' via organogênese in vitro,* 40: 1153-1159.

da Silva, R.P., A.J. de Souza, B.M.J. Mendes and F.A.A. Mourão Filho, 2010. Sour orange bud regeneration and *in vitro* plant development related to culture medium composition and explant type. *Regeneração de gemas de laranja-azeda e desenvolvimento in vitro de plantas em função da composição do meio de cultura e tipo de explante,* 32: 1-8.

Davey, M.R., G.O. Cancino, M.I.S. Gill, P. Anthony, J.B. Power and K.C. Lowe, 2003. Micropropagation of tropical fruits: Beneficial effects of non-ionic surfactants. p. 353-358.

Duran-Vila, N., V. Ortega and L. Navarro, 1989. Morphogenesis and tissue cultures of three citrus species. *Plant Cell Tiss. Org. Cult.,* 16: 123-133.

Dutt, M. and J.W. Grosser, 2009. Evaluation of parameters affecting *Agrobacterium*-mediated transformation of citrus. *Plant Cell, Tissue Organ Culture,* 98: 331-340.

Eriksson, O. 1999. Seed size variation and its effect on germination and seedling performance in the clonal herb *Convallaria majalis*. *Acta Oecol.,* 20: 61-66.

García-Luis, A., Y. Bordón, J.M. Moreira-Dias, R.V. Molina and J.L. Guardiola, 1999. Explant orientation and polarity determine the morphogenic response of epicotyl segments of Troyer citrange. *Ann. Bot.,* 84: 715-723.

García-Luis, A., R.V. Molina, V. Varona, S. Castelló and J.L. Guardiola, 2006. The influence of explant orientation and contact with the medium on the pathway of shoot regeneration *in vitro* in epicotyl cuttings of Troyer citrange. *Plant Cell Tiss. Org. Cult.,* 85: 137-144.

Gupta, S.K., P.K. Singh, S.V. Sawant, R. Chaturvedi and R. Tuli, 2000. Effect of light intensity on *in vitro* multiple shoot induction and regeneration of cotton (*Gossypium hirsutum* L. cv Khandawa-2). *Indian J. Exp. Biol.,* 38: 399-401.

Gutiérrez-E, M.A., D. Luth and G.A. Moore, 1997. Factors affecting *Agrobacterium*-mediated transformation in Citrus and production of sour orange (*Citrus aurantium* L.) plants expressing the coat protein gene of citrus tristeza virus. *Plant Cell Rep.,* 16: 745-753.

He, Y., S. Chen, A. Peng, X. Zou, L. Xu and T. Lei, 2011. Production and evaluation of transgenic sweet orange (*Citrus sinensis* Osbeck) containing bivalent antibacterial peptide genes (Shiva A and Cecropin B) via a novel *Agrobacterium*-mediated transformation of mature axillary buds. *Sci. Hortic.,* 128: 99-107.

Helenius, A. and K. Simons, 1975. Solubilization of membranes by detergents. *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes,* 415: 29-79.

Herman, E.B. 1995. Recent Advances in Plant Tissue Culture III - Regeneration and Micropropagation: Techniques, Systems and Media 1991-1995. Agritech Consultants, Inc., Shrub Oak, NY.

Hsia, C.N. and S.S. Korban, 1998. Effect of growth regulators, dark treatment and light intensity on shoot organogenesis from leaf tissues of evergreen azalea. *Journal Horticultural Science Biotechnology,* 73: 53-60.

Keddy, P.A. and P. Constabel, 1986. Germination of ten shoreline plants in relation to seed size, soil particle size and water level: an experimental study. *J. Ecol.,* 74: 133-141.

Khatun, A., M.R. Davey, J.B. Power and K.C. Lowe, 1993. Stimulation of shoot regeneration from jute cotyledons cultured with non-ionic surfactants and relationship to physico-chemical properties. *Plant Cell Rep.,* 13: 49-53.

Khatun, A., L. Laouar, M.R. Davey, J.B. Power, B.J. Mulligan and K.C. Lowe, 1993. Effects of Pluronic F-68 on shoot regeneration from cultured jute cotyledons and on growth of transformed roots. *Plant Cell Tiss. Org. Cult.,* 34: 133-140.

Khawale, R.N., S.K. Singh, G. Garg, V.K. Baranwal and S.A. Ajirlo, 2006. *Agrobacterium*-mediated genetic transformation of Nagpur mandarin (*Citrus reticulata* Blanco). *Current Science,* 91: 1700-1705.

Khurana, E. and J.S. Singh, 2004. Germination and seedling growth of five tree species from tropical dry forest in relation to water stress: Impact of seed size. *J. Trop. Ecol.,* 20: 385-396.

Li, D.D., W. Shi and X.X. Deng, 2003. Factors influencing *Agrobacterium*-mediated embryogenic callus transformation of Valencia sweet orange (*Citrus sinensis*) containing the pTA29-barnase gene. *Tree Physiology,* 23: 1209-1215.

Luth, D. and G. Moore, 1999. Transgenic grapefruit plants obtained by *Agrobacterium tumefaciens*-mediated transformation. *Plant Cell Tiss. Org. Cult.,* 57: 219-222.

Maggon, R. and B. Deo Singh, 1995. Promotion of adventitious bud regeneration by ABA in combination with BAP in epicotyl and hypocotyl explants of sweet orange (*Citrus sinensis* L. Osbeck). *Sci. Hort.,* 63: 123-128.

Magyar-Tábori, K., J. Dobránszki, J.A.T. da Silva, S.M. Bulley and I. Hudák, 2010. The role of cytokinins in shoot organogenesis in apple. *Plant Cell Tiss. Org. Cult.,* 101: 251-267.

Marques, N.T., G.B. Nolasco and J.P. Leitão, 2011. Factors affecting *in vitro* adventitious shoot formation on internode explants of *Citrus aurantium* L. cv. Brazilian. *Sci. Hort.,* 129: 176-182.

Marutani-Hert, M., K.D. Bowman, G.T. McCollum, T.E. Mirkov, T.J. Evens and R.P. Niedz, 2012. A dark incubation period is important for *Agrobacterium*-mediated transformation of mature internode explants of sweet orange, grapefruit, citron, and a citrange rootstock. *PLoS ONE* 7(10): e47426. doi: 10.1371/journal.pone.0047426.

Molina, R.V., S. Castelló, A. García-Luis and J.L. Guardiola, 2007. Light cytokinin interactions in shoot formation in epicotyl cuttings of Troyer citrange cultured *in vitro*. *Plant Cell Tiss. Org. Cult.,* 89: 131-140.

Moore, G.A., C.C. Jacono, J.L. Neidigh, S.D. Lawrence and K. Cline, 1992. *Agrobacterium*-mediated transformation of Citrus stem segments and regeneration of transgenic plants. *Plant Cell Rep.,* 11: 238-242.

Moreira-Dias, J.M., R.V. Molina, Y. Bordón, J.L. Guardiola and A. García-Luis, 2000. Direct and indirect shoot organogenic pathways in epicotyl cuttings of Troyer citrange differ in hormone requirements and in their response to light. *Ann. Bot.,* 85: 103-110.

Moreira-Dias, J.M., R.V. Molina, J.L. Guardiola and A. García-Luis, 2001. Daylength and photon flux density influence the growth regulator effects on morphogenesis in epicotyl segments of Troyer citrange. *Scientia Hort.,* 87: 275-290.

Mudge, K., J. Janick, S. Scofield and E.E. Goldschmidt, 2009. A History of Grafting. *Horticultural Reviews*. p 437-493.

Murashige, T. and D.P.H. Tucker, 1969. Growth factor requirements of citrus tissue culture. *Proc. First Intl. Citrus Symp.,* 3: 1155-1161.

Niedz, R.P. and T.J. Evens, 2006. A solution to the problem of ion confounding in experimental biology. *Nat. Methods,* 3: 417.

Niedz, R.P. and T.J. Evens, 2011. Mixture screening and mixture-amount designs to determine plant growth regulator effects on shoot regeneration from grapefruit (*Citrus paradisi* macf.) epicotyls. *In vitro Cell. Dev. Biol. Plant,* 47: 682-694.

Niedz, R.P., S.S. Smith, K.B. Dunbar, C.T. Stephens and H.H. Murakishi, 1989. Factors influencing shoot regeneration from cotyledonary explants of *Cucumis melo*. *Plant Cell Tiss. Org. Cult.,* 18: 313-319.

Orbović, V., E.M. Göllner and P. Soria, 2012. The effect of arabinogalactan proteins on regeneration potential of juvenile citrus explants used for genetic transformation by *Agrobacterium tumefaciens*. *Acta Physiologiae Plantarum,* 35: 1409-1419.

Özgen, M., M. Yildiz, N. Koyuncu and S. Önde, 2007. The effect of seed size on tissue culture response of callus from endosperm-supported mature embryos of barley (*Hordeum vulgare* L.). *Cereal Research Communications,* 35: 1415-1425.

Peña, L., M. Cervera, J. Juárez, A. Navarro, J.A. Pina and L. Navarro, 1997. Genetic transformation of lime (*Citrus aurantifolia* Swing.): Factors affecting transformation and regeneration. *Plant Cell Rep.,* 16: 731-737.

Pena, L., M. Cervera, J. Juarez, C. Ortega, J.A. Pina and N. Duran-Vila, 1995. High efficiency *Agrobacterium*-mediated transformation and regeneration of citrus. *Plant Sci.,* 104: 183-191.

Pérez-Molphe-Balch, E. and N. Ochoa-Alejo, 1997. *In vitro* plant regeneration of Mexican lime and mandarin by direct organogenesis. *HortScience,* 32: 931-934.

Sengar, K., R.S. Sengar and S.K. Garg, 2011. The effect of *in vitro* environmental conditions on some sugarcane varieties for micropropagation. *Afr. J. Biotechnol.,* 10: 17122-17126.

Sim, G.E., C.J. Goh and C.S. Loh, 1989. Micropropagation of citrus mitis blanco-multiple bud formation from shoot and root explants in the presence of 6-Benzylaminopurine. *Plant Sci.,* 59: 203-210.

Soltani, A., S. Galeshi, E. Zeinali and N. Latifi, 2002. Germination, seed reserve utilization and seedling growth of chickpea as affected by salinity and seed size. *Seed Science Technology,* 30: 51-60.

Srivastava, S., R. Sinha and D. Roy, 2004. Toxicological effects of malachite green. *Aquat. Toxicol.,* 66: 319-329.

Stephen, A.M., G.O. Phillips and P.A. Williams, 2006. *Food Polysaccharides and Their Applications*. Second ed. CRC Press, Boca Raton, FL.

U.S. Environmental Protection Agency, 1997. METHOD 300.1-Determination of inorganic anions in drinking water by ion chromatography. http: //water.epa.gov/scitech/methods/cwa/bioindicators/upload/2007_07_10_methods_method_300_1.pdf.

Van Le, B., N. Thanh Ha, L.T. Anh Hong and K. Trân Thanh Vân, 1999. High frequency shoot regeneration from trifoliate orange (*Poncirus trifoliata* L. Raf.) using the thin cell layer method. *Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie,* 322: 1105-1111.

Yu, C., S. Huang, C. Chen, Z. Deng, P. Ling and F.G. Gmitter Jr, 2002. Factors affecting *Agrobacterium*-mediated transformation and regeneration of sweet orange and citrange. *Plant Cell Tiss. Org. Cult.,* 71: 147-155.

Zammit, C. and P.H. Zedler, 1990. Seed yield, seed size and germination behaviour in the annual *Pogogyne abramsii. Oecologia,* 84: 24-28.

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