In Vitro Plant Regeneration of Mexican Lime and Mandarin by Direct Organogenesis

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Abstract. An efficient system for in vitro regeneration by organogenesis starting from internodal stem segments from seedlings of Mexican lime (*Citrus aurantifolia* Christm. Swing.) and mandarin (*C. reticulata* Blanco cv. Monica) was developed. The best results were obtained when the wounded edges of internodal stem segments cut longitudinally were placed downward on the surface of the culture medium. The optimal culture medium from both species was Murashige and Skoog with vitamins from B₅ medium, 5% sucrose, 33.3 μM BA and 5.4 μM NAA. The best response was obtained when the segments were incubated at 25 ± 2 °C for 21 d in darkness, followed by 29 d on a 16/8-h light/dark cycle (fluorescent light, 54 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The best regeneration system tested allowed the attainment of adventitious shoots from 96% and 88% of the explants in Mexican lime and mandarin, respectively. In Mexican lime an average of 7.8 well-differentiated shoots per explant was obtained, and in mandarin the yield was 5.1. Rooting of 70% of the shoots was achieved in culture medium with NAA (2.7–5.4 μM) or IBA (2.5–4.9 μM). Of the rooted plants, 85% adapted well to soil conditions. Chemical names used: 6-benzylaminopurine (BA), α -naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA).

Citrus species are cultivated in most tropical and subtropical regions of the world. In spite of their importance, several factors exist that limit the cultivation of citrus fruits. Among these are their low resistance to chilling injury and their susceptibility to phytopathogens and pests. Breeding has been limited due to several factors: 1) the reproductive biology (partial and complete pollen and ovule sterility, gametophytic systems of self- or cross-incompatibility, apomixis from nucellar cells); 2) long juvenile periods; 3) other genetic factors, since the genetic nature and mode of inheritance of most important traits are unknown (Gmitter et al., 1992).

Plant biotechnology appears to be a viable option for the improvement of the citrus species by means of plant tissue culture and genetic transformation. The establishment of an efficient in vitro plant regeneration system suitable for genetic transformation is the first step in this approach. In vitro citrus plant regeneration via organogenesis has been de-

scribed from several tissues, including roots (Bhat et al., 1992; Sauton et al., 1982; Sim et al., 1989), leaf sections (Chaturvedi and Mitra, 1974), and stem internodes (Barlass and Skene, 1982; Duran-Vila et al., 1989; Moore, 1986). Previous studies have shown differences in the response of each species to in vitro culture and in intraspecific responses to culture conditions.

The objective of this study was to determine the optimal conditions for in vitro plant regeneration via organogenesis of Mexican lime and mandarin cv. Monica using explants taken from in vitro cultured seedlings.

Materials and Methods

Plant material

In vitro germinated seedlings of Mexican lime and mandarin cv. Monica were used as tissue sources. Seeds were washed 5 times with 0.1% Extran (Merck, Darmstadt, Germany), disinfected for 1 min in 70% ethanol and 25 min in 2% sodium hypochlorite, then rinsed 4 times with sterile distilled water. Seeds were germinated in Phytacon vessels (Sigma Chemical Co., St. Louis) containing half-strength Murashige and Skoog (1962) medium with 1.5% sucrose, 0.8% agar (Sigma Chemical Co.), pH 5.7. The cultures were maintained on a 16/8 h-light/dark cycle under fluorescent light (54 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, daylight lamps) at 25 ± 2 °C for 3 months.

Culture media

The inorganic salts of Murashige and Skoog (1962) with the vitamins of Gamborg's B₅ (1968) were used as the basal medium. The medium was supplemented with 2-(*N*-morpholino)ethanesulfonic acid (MES, Sigma) at 0.5 g·L⁻¹ and with 10 concentrations of growth regulators and 3%, 4%, 5%, or 6% sucrose. Growth regulators and sucrose were added before autoclaving. The media were solidified by adding agar (Sigma) at 8 g·L⁻¹ and the pH was adjusted to 5.7 with 0.1 N NaOH. Aliquots of 30 mL of the media were dispensed into baby food jars, capped with polypropylene caps (Magenta Corp., Chicago) and autoclaved at 121 °C for 15 min.

Adventitious shoot induction

Growth regulators (Expt. 1). The effects of various combinations and concentrations of growth regulators on adventitious shoot formation from leaf, stem, and root explants of Mexican lime and Mandarin were tested. Experiments were carried out using basal medium containing 3% sucrose and supplemented with several combinations of BA, KIN, or Thidiazuron (TDZ) plus NAA (Table 1). These treatments were selected according to the results of previous experiments with these species (data not shown).

Explant source and culture conditions. In vitro germinated seedlings with four to six internodes were used as explants source. Leaves longer than 8 mm were excised from in vitro shoot cultures and the petiole and the midrib were removed. The leaf segments were placed with their adaxial side in contact with the surface of the regeneration medium. Roots were cut into 10-mm segments and placed horizontally on the medium. Internodal stem pieces (10 mm) were inoculated vertically onto the medium with the apical end of the segment protruding 6 to 7 mm. Cultures were kept under fluorescent light (54 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, daylight lamps) on a 16/8-h light/dark cycle at 25 ± 2 °C, for 50 d. The number of adventitious shoots was recorded periodically. At least 50 explants of each kind from each species were used per treatment and the entire experiment was carried out twice. The best treatment and explant of this first experiment were then used to determine the influence of sucrose concentrations, dark incubation, and explant culture position and wounding on adventitious shoot induction.

Effect of sucrose concentration (Expt. 2). To determine the effect of sucrose on adventitious shoot formation, internodal stem pieces were cultured as in the first experiment on basal medium with 33.3 μM BA plus 5.4 μM NAA and supplemented with 3%, 4%, 5%, or 6% sucrose. The number of explants per treatment, number of replications, and culture conditions were similar to those described for Expt. 1.

Influence of light/dark on regeneration (Expt. 3). A third series of experiments was conducted to investigate the influence of incubation in several light conditions. Internodal

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Table 1. Effect of various combinations and concentrations of the growth regulators 6-benzylaminopurine (BA), thidiazuron (TDZ), kinetin (KIN), and α -naphthaleneacetic acid (NAA) on adventitious shoot proliferation in internodal stem pieces of Mexican lime and mandarin. Data pooled from two independent experiments.

Species and growth regulators (μM)				Explants with adventitious shoots ^a (%)	Adventitious shoots per explant ^b (no. ± SE)
BA	TDZ	KIN	NAA		
Mexican lime					
11.1	---	---	5.4	19	1.77 ± 0.28 a ^x
22.2	---	---	5.4	47	2.22 ± 0.23 a
33.3	---	---	5.4	75	3.38 ± 0.25 b
33.3	---	---	2.7	59	2.38 ± 0.21 a
44.4	---	---	5.4	55	2.56 ± 0.23 ab
33.3	---	---	---	55	2.95 ± 0.26 ab
---	0.45	---	5.4	22	1.09 ± 0.09 a
---	0.90	---	5.4	18	2.00 ± 0.24 a
11.1	0.23	---	5.4	23	2.54 ± 0.43 ab
---	---	46.5	5.4	22	1.09 ± 0.09 a
Mandarin					
11.1	---	---	5.4	21	1.26 ± 0.29 a
22.2	---	---	5.4	52	1.89 ± 0.24 a
33.3	---	---	5.4	71	2.96 ± 0.30 b
33.3	---	---	2.7	66	2.79 ± 0.21 b
44.4	---	---	5.4	57	2.23 ± 0.32 ab
33.3	---	---	---	59	2.16 ± 0.31 ab
---	0.45	---	5.4	20	1.12 ± 0.34 a
---	0.90	---	5.4	16	1.28 ± 0.34 a
11.1	0.23	---	5.4	26	1.98 ± 0.36 ab
---	---	46.5	5.4	20	1.20 ± 0.14 a

^aValues refer to the percentage of explants that produced adventitious shoots.

^bMean of the number of shoots per explant was calculated by dividing the total number of shoots by the number of explants with shoots.

^cMean separation according to the Tukey Kramer multiple comparison test ($P \leq 0.01$).

segments were cultured on a basal medium amended with 33.3 μM BA, 5.4 μM NAA, and 5% sucrose. Fifty explants were kept under fluorescent light as described in Expt. 1, another 50 were kept under continuous light, a third group of 50 explants were kept in darkness at 28 °C, and the last 50 explants were kept for 21 d in the darkness at 28 °C and then transferred to fluorescent light (54 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, daylight lamps) in a 16/8-h light/dark cycle. Data for all treatments were collected after 50 d of culture in vitro. The experiment was carried out twice.

Explant position and wounding (Expt. 4). These experiments were conducted for two reasons: first, the shoots appeared to originate from the internal tissues of the explants, so that, if these remain more exposed, the production of adventitious shoots could increase. On the other hand, when the shoots originate in a site of the explant far from the culture medium surface, this hinders the use of the regeneration system for genetic transformation purposes. Contact between the selective

agent in the medium and the area consisting of the buds that give origin to adventitious shoots is necessary for an efficient selection of transgenic shoots. The influence of the explant position and wounding on adventitious shoot formation was investigated using explants cultured on basal medium amended with 5% sucrose, 33.3 μM BA, and 5.4 μM NAA. The explants tested were internodal stem segments that were a) inserted vertically into the medium with the apical end of the segment protruding 6 to 7 mm, b) inserted vertically into the medium with the basal end of the segment protruding 6 to 7 mm, c) inserted vertically into the medium with the apical end of the segment protruding 6 to 7 mm and with several longitudinal cuts, d) placed horizontally on the medium surface, e) cut longitudinally and cultured with the cut surface in contact with the medium, f) placed horizontally on the medium with several transverse cuts. At least 30 explants per treatment were used for each species. Cultures were kept for 21 d in the darkness at 28 °C and then incubated under fluorescent light

(54 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) in a 16/8-h light/dark cycle at 25 $\pm$ 2 °C for 29 d.

Data analysis. The data were tested by analysis of variance (ANOVA). When significant differences were found, the values were compared according to Tukey Kramer multiple comparison test ($P \leq 0.01$).

Rooting of in vitro generated shoots (Expt. 5)

Adventitious shoots longer than 8 mm were excised and transferred onto a half-strength basal medium amended with 2% sucrose, 0.2% activated charcoal (Sigma) and supplemented with the following auxins: 1) 2.7 μM NAA, 2) 5.4 μM NAA, 3) 2.5 μM indole-3-butyric acid (IBA), 4) 4.9 μM IBA. The cultures were maintained for 40 d in a 16/8-h light/dark cycle at 25 $\pm$ 2 °C with fluorescent light (54 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, daylight lamps). Rooted shoots were transplanted into plastic pots containing a mixture of 1 vermiculite : 1 peatmoss. The lid was loosened 1 week be-

Table 2. Effect of light conditions on adventitious shoot proliferation in internodal stem pieces of Mexican lime and mandarin. Data pooled from two independent experiments.

Species and light conditions	Explants with adventitious shoots ^a (%)	Adventitious shoots per explant ^b (no. $\pm$ SE)
Mexican lime		
16:8-h light : dark, 50 d	85	3.73 $\pm$ 0.26 a ^c
24-h light	85	3.68 $\pm$ 0.34 a
Dark, 50 d	82	2.20 $\pm$ 0.25 b
Dark, 21 d—16:8-h light : dark, 29 d	80	4.55 $\pm$ 0.26 a
Mandarin		
16:8-h light : dark, 50 d	78	3.37 $\pm$ 0.23 a
24-h light	74	3.24 $\pm$ 0.30 a
Dark, 50 d	78	1.98 $\pm$ 0.29 b
Dark, 21 d—16:8-h light : dark, 29 d	76	3.79 $\pm$ 0.28 a

^aValues refer to the percentage of explants that produced adventitious shoots.

^bMean of the number of shoots per explant was calculated by dividing the total number of shoots by the number of explants with shoots.

^cMean separation according to the Tukey Kramer multiple comparison test ($P \leq 0.01$).

Fig. 1. Adventitious shoot production in stem explants of Mexican lime cultured (a) in the vertical position with the apical end protruding, (b) in the vertical position with the apical region wounded, (c) in stem segments cut longitudinally and cultured with the cut surface in contact with the culture medium. Adventitious shoot production in stem explants of mandarin cultured (d) in the vertical position with the apical end protruding, (e) in the vertical position with the apical region wounded, (f) wounded and cultured horizontally. (Bar = 1 cm except in 1f, where bar = 1 mm.)

fore the plantlets were removed from the in vitro culture.

Results and Discussion

Effect of growth regulators on adventitious shoot induction. None of the cultures of leaf and root explants had produced adventitious shoots after 50 d on the tested media. Callus formed on some explants and most became necrotic. Internodal stem explants of Mexican lime and mandarin produced adventitious shoots in all treatments (Table 1). The number of shoots per explant ranged from 0 to 6. The best treatments, in terms of the response fre-

quency and mean number of shoots per explant, were $33.3 \mu\text{M}$ BA with $5.4 \mu\text{M}$ NAA for Mexican lime and $33.3 \mu\text{M}$ BA with 5.4 or $2.7 \mu\text{M}$ NAA for mandarin (Table 1). BA requirements for regeneration of adventitious shoots were similar for the two species; however, the number of shoots per explant was mostly higher in Mexican lime than in mandarin. Adventitious shoots originated on the protruding apical end of the stem explant, except in the media containing TDZ, in which the shoots were produced on the basal end of the stem explant immersed in the culture medium. The adventitious shoots generated in the presence of TDZ also exhibited an abnormal leaf mor-

phology. These shoots did not form roots in any treatment. In this first experiment, root, leaf, and internodal stem segments inoculated vertically with the apical end protruding from the culture were used as explants, as previously reported for other citrus species (Moore et al., 1992; Sim et al., 1989).

Effect of sucrose level on adventitious shoot formation. The yield of adventitious shoots was nonsignificantly higher with 5% than with 3% sucrose in both species. A significant negative effect was observed by increasing the concentration of sucrose up to 6% (data not shown).

Effect of light on adventitious shoot production. The highest yield of adventitious shoots expressed as the mean number of shoots per explant from both species was obtained with 21 d of incubation in darkness followed by 29 d of incubation under fluorescent light in a 16/8-h light/dark cycle (Table 2). However, there was no statistically significant difference between this treatment and results obtained after constant incubation in a 16/8-h light/dark cycle or 24-h light. Incubation in darkness during the whole experiment significantly decreased the yield of adventitious shoots. Other work has demonstrated that, in some fruit crops, the rate of in vitro regeneration by organogenesis increases notably upon giving a period of induction in darkness (Leblay et al., 1991).

Influence of the explant culture position and wounding on adventitious shoot formation. In the previous experiments, internodal stem pieces inserted in the vertical position with the apical end protruding were used as explants (Fig. 1 a and d). In this experiment, placing longitudinally cut explants horizontally, or wounding them, increased the yield of adventitious shoots significantly (Table 3). Yields of shoots were higher after wounding the apical region of the vertical explants (Fig. 1 b and e). The explants placed horizontally on culture medium produced adventitious shoots from both cuts and also from the wounds (Fig. 1f). The best response was obtained with internodal stem segments cut longitudinally with the cut surface in contact with the culture medium. With these conditions, the adventitious shoots originated from the whole wounded surface of the explant; this procedure significantly increased the yield of adventitious shoots in both species (Fig. 1c). With this treatment, 7.8 ± 0.8 and 5.1 ± 0.4 shoots per each explant were obtained in Mexican lime and mandarin, respectively. These experiments indicate that increasing the area of the wounded surface also increases the production of adventitious shoots.

Rooting and establishment in soil of in vitro regenerated shoots. There were no significant statistical differences in the growth of adventitious roots among the four tested treatments. An average of 2.9 ± 1.0 roots was produced by each shoot, and the length of the roots fluctuated between 10 to 40 mm. Roots formed on 70% of the treated shoots. Plants obtained in vitro were adapted and transferred to soil with a survival rate of 85%.

Table 3. Effect of the explant position and wounding on adventitious shoot proliferation in internodal stem pieces of Mexican lime and mandarin. Data pooled from two independent experiments.

Species and explant position and preparation	Explants with adventitious shoots ^a (%)	Adventitious shoots per explant ^b (no. $\pm$ SE)
Mexican lime		
Vertical with the apical end protruding	82	4.39 $\pm$ 0.30 a ^c
Vertical with the basal end protruding	73	3.09 $\pm$ 0.47 a
Vertical with the apical end protruding and wounded	86	2.96 $\pm$ 0.28 a
Horizontal	75	6.61 $\pm$ 0.39 b
Split longitudinally with the cut surface in contact with the medium	96	7.77 $\pm$ 0.83 b
Horizontal wounded	57	3.50 $\pm$ 0.47 a
Mandarin		
Vertical with the apical end protruding	74	3.42 $\pm$ 0.42 a
Vertical with the basal end protruding	42	2.74 $\pm$ 0.53 a
Vertical with the apical end protruding and wounded	76	3.96 $\pm$ 0.44 ab
Horizontal	68	4.06 $\pm$ 0.61 b
Split longitudinally with the cut surface in contact with the medium	88	5.12 $\pm$ 0.38 b
Horizontal wounded	72	3.78 $\pm$ 0.44 ab

^aValues refer to the percentage of explants that produced adventitious shoots.^bMean of the number of shoots per explant was calculated by dividing the total number of shoots by the number of explants with shoots.^cMean separation according to the Tukey Kramer multiple comparison test ($P \leq 0.01$).

Conclusions

The regeneration system we developed allowed adventitious shoots formation in 96% and 88% of the explants in Mexican lime and mandarin, respectively. In Mexican lime, an average of 7.8 well-differentiated shoots per explant was obtained, which is similar to that reported by Duran-Vila et al. (1989) with this species and who were capable of inducing 8.2 buds per explant, but only in the 27% of the cultured explants. In mandarin, we obtained 5.1 shoots per explant. Another advantage of our regeneration system is that it is suitable for genetic transformation assays. Our regeneration system for these crop species can be used for the introduction of reporter genes and agronomically important genes by means of *Agrobacterium tumefaciens* infection.

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