In Vitro Adventitious Shoot Regeneration from Sweet Orange Using Thin Epicotyl Sections

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ABSTRACT

A system for *in vitro* plant regeneration from thin transversal epicotyl sections of sweet orange (*Citrus sinenis* L Osbeck cvs Pera, Valencia and Folha Murcha) was developed. Thin section explants (1-2 mm) from seedlings produced adventitious shoot buds on MS medium supplemented with BA (0.5-5 µM). Optimal shoot induction was achieved on 2-5 µM BA. Explants with buds were transferred to elongation media in order to improve the recovery of normal shoots. Higher number of elongated shoots was obtained on medium with 0.5 µM BA. Elongated shoots were rooted on half strength MS medium without growth regulators and transferred to soil for acclimatization. The advantages of this system for transformation are discussed.

KEY WORDS: *Citrus sinensis*, Adventitious buds, Shoot multiplication.

INTRODUCTION

Citrus breeding is limited by several factors: 1) certain aspects of the reproductive biology, 2) long juvenile periods and 3) genetic factors (Gmitter et al., 1992). Plant improvement *via* genetic transformation is an alternative to reduce the time necessary for releasing new cultivars. For developing a routine genetic transformation protocol, an efficient system for *in vitro* regeneration is required.

Transgenic citrus plants have been obtained by direct DNA transfer into protoplasts (Vardi et al., 1990), co-cultivation of internode or epicotyl segments with *Agrobacterium* (Moore et al., 1992; Kaneyoshi et al., 1994; Peña et al., 1995a,b; Gutiérrez et al., 1997; Peña et al., 1997; Cervera et al., 1998), and particle bombardment of nucellar embryogenic cell suspensions (Yao et al., 1996). At present, the most widely used method of gene transfer in citrus is the *Agrobacterium*-mediated transformation of 1cm internode or epicotyl segments. Using this system, transgenic plants of *C. sinensis* (Peña et al., 1995a; Cervera et al., 1998), *C. aurantifolia* (Gutiérrez et al., 1997), *C. aurantium* (Peña et al., 1997), *C. sinensis* x *Poncirus trifoliata* (Moore et al., 1992; Peña et al., 1995b) and *P. trifoliata* (Kaneyoshi et al., 1994) have been obtained. However, one limitation of this system is the high number of non-transformed and chimeric plants. Probably, the large size of the initial explants allowed the growth of escape shoots during the selection phase due to the protective effect of the underlying mass of tissue.

In order to improve transgenic plant recovery in citrus it is important to have explants with reduced size and high regeneration competence to reduce non-transformed and chimeric plants. The objective of this study was to develop a regeneration system for sweet orange suitable for transformation using *Agrobacterium* and particle bombardment. We describe a simple and efficient method for *in vitro* regeneration of sweet orange from thin epicotyl transversal sections. Factors affecting induction, elongation and rooting of adventitious shoots were analyzed.

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MATERIALS AND METHODS

Preparation of explants

Seeds of sweet orange (*Citrus sinensis* (L.) Osbeck) cvs. Pera, Valencia and Folha Murcha were peeled removing both seed coats, surface disinfected with 2.5% (v/v) sodium hypoclorite solution for 10 min and rinsed three times with sterile distilled water. Seeds were sown individually in polypropylene-capped 150 x 25 mm test tubes containing 25 ml of basal medium consisting of MS salt solution (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose and solidified with 2 g l^{1} Phytagel™, without plant growth regulators (PGR). The pH of the medium was adjusted to 5.7 before autoclaving for 20 min at 121°C. The cultures were maintained in darkness at 23±1°C for 4 weeks, and then at 27±1°C under a 16 h-photoperiod provided by cool white fluorescent lights to give 30 µE m^{-2} s^{-1} photosynthetically active radiation for 3 weeks.

The epicotyls from 7-week-old seedlings were removed, hand cut transversally into thin sections (1-2 mm) and inoculated with the basal end of the explant in contact with the shoot induction medium.

Shoot induction

Shoot induction media contained MS salts, 30 g l^{-1} sucrose, 2 g l^{-1} Phytagel™ and different concentrations 6-benzyladenine (BA :0, 0.5, 1, 2 or 5 µM). The pH of the medium was adjusted to 5.7 with 0.1 N NaOH and autoclaved at 121°C for 20 min. Aliquots of 25 ml of media were dispensed into Petri dishes (90 x 15 mm). Five Petri dishes with 15 explants each were used per treatment. Cultures were kept under dark at 27±1°C. The number of buds was recorded after four weeks of culture initiation. Means and standard errors were calculated for each BA concentration at each variety. Experiments were arranged in a randomized design.

Growth regulators for elongation of adventitious shoots

Explants with shoot buds, formed under optimal condition for each cultivar (Table 1), were transferred to elongation medium. For shoot elongation in cv. Pera, explants were cultured on MS basal media without plant growth regulator or with BA (0.5 µM), gibberelic acid (GA_3: 1 µM) and BA + GA_3 (0.5 + 1.0 µM). For cultivars Valencia and Folha Murcha, elongation was only done in MS medium with 0.5 µM BA. For this experiment, 200 ml flasks containing 50 ml of medium, closed with hard polypropylene caps, were used. Cultures were kept at 27±1°C under a 16-photoperiod, provided by cool white fluorescent lights to give 30 µE m^{-2} s^{-1} PAR. Two explants were placed in each flask and there were 5 flasks per treatment. The number of elongated shoots (≥ 1 cm) were counted after 4 weeks of culture. Individual shoots were excised from the proliferating basal shoot mass and transferred to the rooting medium. The basal mass with shoot primordia was left on the same medium for a second cycle of regeneration (another 4 weeks). Means and standard errors were calculated for each treatment after 4 and 8 weeks.

Rooting formation and acclimatization

Elongated shoots were placed vertically in half strength MS without growth regulators in polypropylene-capped 150 x 25 mm test tubes containing 15 ml of medium. One elongated shoot was placed in each test tube for rooting and there were 10-12 tubes from the respective elongation treatment. The cultures were kept at 27±1°C under a 16 h-photoperiod, 30 µE m^{-2} s^{-1} PAR. After 50 days, shoots were washed to remove the medium from their roots and transplanted into pots containing a commercial substrate (Plantmax[tm]) with organic matter and vermiculite and placed on a mist chamber for acclimatization.

RESULTS

Shoot induction

Epicotyl transversal thin sections (1-2 mm) of the sweet orange cultivars produced adventitious shoots in media with all levels of BA (0.5-5.0 µM) (Figure 1a). On PGR free medium a white friable callus developed over the cut surface of the explant, but buds did not develop. On the BA

supplemented media, organized meristematic structures developed directly from the cut surface of the epicotyl sections within 7 days of culture. Callusing at all levels of BA was much less visible than in the PGR free medium. After 2 weeks of initiation, these meristematic structures differentiated into shoots. The number of shoot buds per explant ranged from 0 to 12. The best treatments for mean number of adventitious shoot buds per explant were 2 and 5 µM BA for cv. Pera and 1 and 2 µM BA for Valencia and Folha Murcha (Table 1). Buds on lower concentrations of BA (0.5-1 µM) started to elongate on the same medium. However buds induced on 2-5 µM BA required to be transferred to an elongation medium for normal development.

Table 1 - Induction of adventitious shoot buds in thin sections of epicotyl of sweet orange cultured in media supplemented with different concentrations of BA .

Cultivars	BA (µM)	Explants with shoot buds (%)[1/]	Shoot buds per explant[2/]
	0	0a	0
	0.5	66.6b	3.0±0.2
Pera	1.0	66.6b	2.5±0.2
	2.0	63.3b	4.7±0.4
	5.0	62.3b	4.6±0.4
	0	0a	0
	0.5	53.3b	2.9±0.3
Valencia	1	55.9b	5.3±0.6
	2	77.3c	5.9±0.5
	5	43.8b	4.7±0.3
	0	0a	0
	0.5	31.6b	3.3±0.3
Folha Murcha	1	32.3b	3.7±0.4
	2	20b	4.7±0.7
	5	18.9b	3.6±0.4

[1/] Percentages followed by the same letter in the same variety are not significantly different at 5% probability (Chi-square test).
[2/] Values are mean ± standart error from 5 replicates with 15 explants/replicate.

Shoot elongation

Because of the short size of buds induced by shoot induction medium with 2-5 ?M BA, an elongation phase was necessary. For cv. Pera, the highest number of elongated shoots (mean = 4.3 per explant) were produced on medium containing BA (0.5 µM) plus GA_3 (1.0 µM) and the second highest number (mean = 3.1 per explant) occurred on medium containing BA (0.5 µM) after 4 weeks in culture. In the second culture period, medium containing only BA produced the highest number of elongated shoots, followed by BA+ GA_3 and no PGR medium (Table 2). GA_3 supplemented medium produced only 2 shoots from 10 explants. Cultivars Valencia and Folha Murcha, that were elongated only on 0.5 ?M BA supplemented medium, produced 6.3 and 4.2 elongated shoots/ explant on the first culture period and 3.0 and 2.8 elongated shoots/explant on the second culture period, respectively. Elongated shoots ranged from 1 to 3 cm (Figure 1b). Shoots elongated in all media did not show any abnormalities such as hyperhydricity, deformed leaves or fasciated stems.

Rooting of elongated shoots

At the final stage of the *in vitro* development, elongated shoots were transferred to a half strength basal MS medium lacking growth regulators for rooting. Rooting was initiated within twelve days after inoculation on the medium (Figure 1c). For cv. Pera, the shoots that elongated a on medium with no PGR had the highest root formation (90%), followed by GA_3, BA and GA_3+BA. The average number of roots per shoot ranged from 1.1 to 1.4. Shoots that elongated on media containing BA (BA and BA+GA_3) showed two weeks delay on root formation compared to shoots elongated on a medium without BA. For the Valencia and Folha Murcha cultivars, which were elongated on a BA supplemented medium, the percentage of rooting was similar (Table 2).

Table 2 - Effect of growth regulators on number of elongated shoots formed from thin sections of sweet orange after exposure to 5.0 mM BA (Pera) and 2.0 mM BA (Valencia and Folha Murcha).

Cultivar	PGR (■M)	Number of elongated shoots per explant after 1 month *in vitro* ±SE	Number of elongated shoots per explant after 2 month *in vitro* ±SE	Rooting %
	no PGR	2.2[1/] ± 0.4	1.5 ± 0.5	90a[2/]
Pera	GA_3 (1.0)	2.7 ± 0.9	0.2 ± 0.2	81.8a
	BA (0.5)	3.1 ± 0.9	3.2 ± 0.6	66.6a
	BA(0.5) + GA_3 (1.0)	4.3 ± 0.6	1.9 ± 0.5	63.6a
Valencia	BA (0.5)	6.3 ± 1.2	3.0 ± 0.7	83.3a
F.Murcha	BA (0.5)	4.2 ± 0.8	2.8 ± 0.7	84.6a

[1/] Values represent mean ± standard error of the number of shoots (³1 cm) of 5 replicates with 2 explants/ replicate.

[2/] Percentages followed by the same letter are not significantly different at 5% probability (Chi-square test)

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Acclimatization

Rooted shoots from cvs. Pera, Valencia and Folha Murcha were successfully acclimated (100%) under mist conditions. Regenerated shoots were phenotypically normal after grown for 3 months in the glasshouse (Figure 1d).

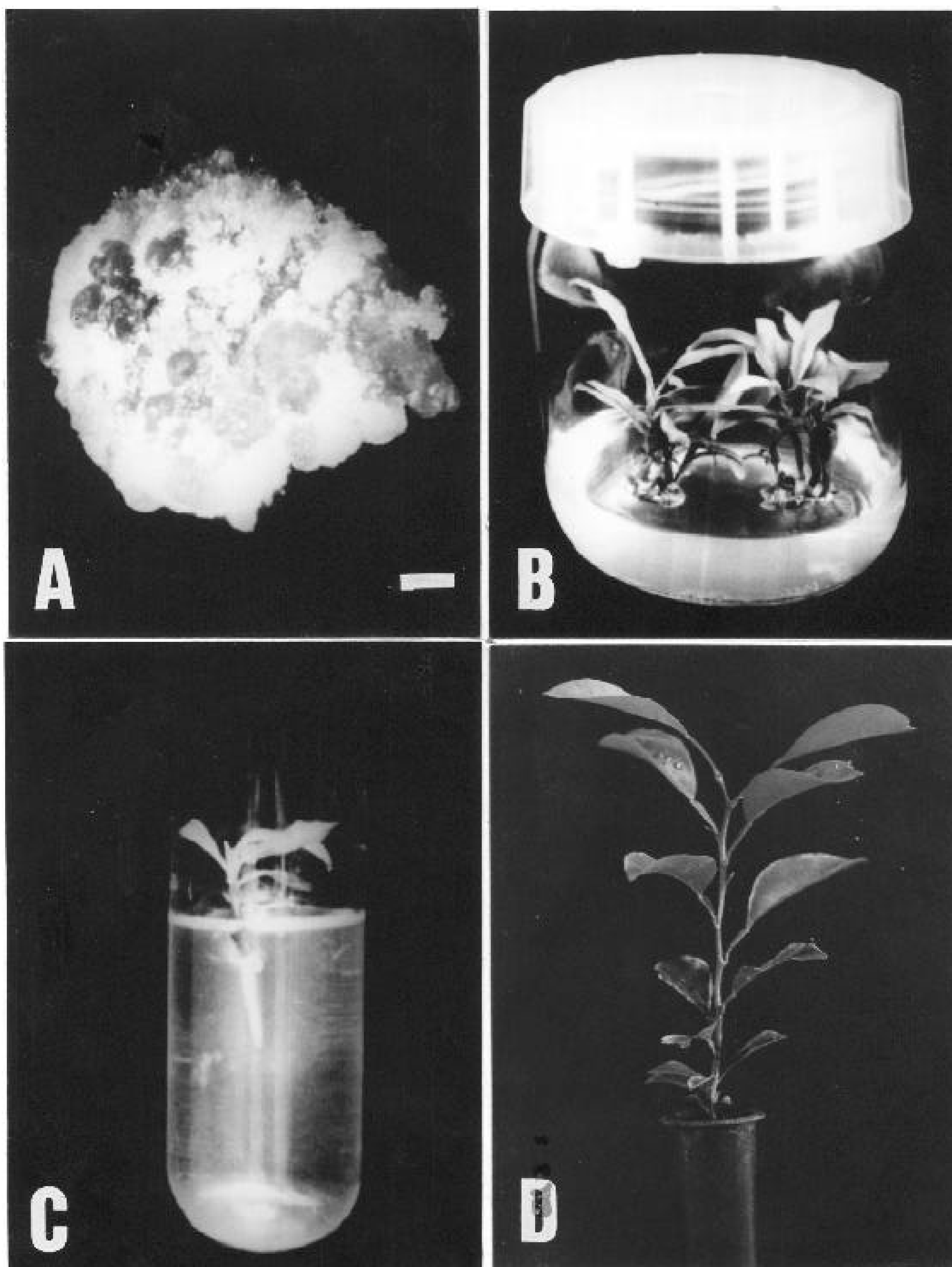

Figure 1 - Regeneration of sweet orange cv. Pera from thin epicotyl sections: a) thin sections cultured on 5 µM BA showing various buds (bar = 1 mm); b) shoots elongating on 0.5 µM BA; c) shoot rooting on half strength MS medium; d) acclimated plant.

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DISCUSSION

A new technique for citrus regeneration using thin epicotyl sections was developed. In the preliminary experiments, using 1 cm epicotyl segments, we observed that shoots were formed on the cut edges of the explants only. A similar result was found in the Mexican lime and mandarin internode segments culture (Balch and Alejo, 1997). With this information, we reduced the explant size, trying to obtain shoot regeneration from thinner sections. In our cultures, transversal epicotyl sections of 1 mm were the thinnest explants capable of adventitious shoot regeneration.

Optimal shoot regeneration from thin epicotyl sections in the tested genotypes was achieved on media supplemented with 2-5 µM BA, followed by incubation in an elongation medium with 0.5 µM BA or 0.5 µM BA plus 1.0 µM GA_3. Much higher doses of BA (13.3-33.3 µM) were necessary for optimal shoot induction when 1 cm internodes segments were used in various *Citrus* species (Moore et al., 1992; Balch and Alejo, 1997).

Addition of BA (alone or with GA_3) to the elongation medium improved the number of elongated shoots obtained in two subculture periods. A possible explanation for this is that lower concentrations of BA continued to stimulate shoot initiation while reducing the dominance of newly-formed buds, whereas GA_3 promoted the elongation of shoot buds initiated on an induction medium. Bhagwat et al. (1996) obtained similar results with adventitious shoots of cassava. Omura and Hidaka (1992) found that addition of cytokinin and GA_3 was necessary for a successful shoot tip culture of citrus. When 1 cm stem segments of citrus were used as explant for shoot proliferation an elongation phase was not necessary (Duran-Vila et al., 1989; Moore et al., 1992).

Improving the rate of adventitious shoot production and plant regeneration increases the probability of obtaining transgenic plants from desirable genotypes. Considering that one seedling of cv. Pera can give 50 thin sections with 60% shoot induction efficiency and 6 elongated shoots/explant, 180 shoots per seedling can be regenerated in a 5 months using epicotyl thin sections. For cvs. Valencia and Pera, the potential of regeneration is 358 and 112 shoots per seedling respectively. In all three genotypes, plants with well developed root systems were obtained in an efficient and repeatable manner.

Regeneration using thin epicotyl explants was also obtained in citrange carrizo (data not shown). This suggests that transversal thin section explants can probably be used with success in other citrus species which have been regenerated from epicotyl segments.

The choice of an explant tissue which is suitable for transformation with different protocols is a critical factor in the development of transgenic citrus plants. *Agrobacterium*-mediated genetic transformation of 1 cm epicotyl segments is the most used system in *Citrus*. In the present procedure, shoot regeneration is higher enough to increase the transformation efficiency compared to that using larger stem segments.

Also, this system is especially appealing to microprojectile bombardment. In *Citrus* the use of bombardment in transformation has been limited to cell suspension cultures derived from nucellar embryogenic callus (Yao et al., 1996). However, the use of nucellar tissue as explant for initiating somatic cell cultures and for plant regeneration is time consuming; and nucellar explants are available only for a short period during the year (Gill et al., 1995). Therefore, thin epicotyl section explants can be used with advantages over the nucellar tissue in genetic transformation via bombardment.

A close contact between the selective agent in the medium and the tissue that will originate the adventitious shoots is necessary for an efficient selection of transgenic plants. Previous reports on citrus transformation using 1 cm internode segments showed a high rate of non-transformed and chimeric plants (Moore et al., 1992; Cervera et al., 1998). In such tissue culture systems, the frequency of escapes can be high, especially when

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shoots grow out of the top of large masses of the heterogeneous proliferating tissue. Use of thin transversal sections (1-2 mm) may reduce this problem, as the whole explant is effectively exposed to the selective agent in the culture medium.

We are currently using transversal thin epicotyl section explants to developed a transformation protocol for sweet orange through particle bombardment. Furthermore, we are trying to regenerate citrus plants from mature tissues using thin section explants.

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RESUMO

Regeneração de Gemas Adventícias de Laranja Doce A Partir de Segmentos Finos de Epicótilo

Um sistema foi desenvolvido para a regeneração *in vitro* de laranja doce (*Citrus sinensis* cvs Pera, Valência e Folha murcha) a partir de segmentos transversais de epicótilo. Segmentos finos (1-2 mm) de epicótilo de plântulas produziram gemas adventícias em meio MS acrescido de BA (0,5-5 µM). Os melhores tratamentos foram 2,0 e 5,0 µM BA. Explantes com gemas foram transferidos para meio de alongamento para melhorar a obtenção de brotos. O maior número de brotos alongados foi obtido em meio com 0,5 µM BA. Brotos alongados foram enraizados em meio com metade da concentração de sais do MS e transferidos para solo para aclimatização. As vantagens desse sistema para a transformação genética são discutidas.

REFERENCES

Balch, E.P.M. and Alejo, N. O. 1997. *In vitro* plant regeneration of Mexican lime and mandarim by direct organogenesis. Hortscience. 32:931-934.

Bhagwat, B.; Vieira, L. G. E. and Erickson, L. R. 1996. Stimulation of *in vitro* shoot proliferation from nodal explants of cassava by thidiazuron, benzyladenine and gibberelic acid. Plant Cell Tissue and Organ Culture. 46:1-7.

Cervera, M.; Juárez, J.; Navarro, A.; Pina, J.; Navarro, L. and Peña, L. 1998. Genetic transformation of woody fruit plants bypassing the juvenile stage. Transgenic Research. 7:51-59.

Duran-Vila, N.; Ortega, V. and Navarro, L. 1989. Morphogenesis and tissue culture of three citrus species. Plant Cell Tissue and Organ Culture. 16:123-133.

Gill, M. I. S.; Singh, Z.; Dhillon, B. S. and Gosal, S. S. 1995. Somatic embryogenesis and plantlet regeneration in mandarin (*Citrus reticulata* Blanco). Scientia Horticulturae. 63:167-174.

Gmitter Jr, F. G.; Grosser, J. W. and Moore, G. A. 1992. Citrus. p.335-369. In: Hammerschlag FA and Litz RE (Eds). Biotechnology of perennial fruit crops. Biotechnology in Agriculture n.8. CAB International, Cambridge.

Gutiérrez-E, M. A.; Luth, D. and Moore, G. A. 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 Reports. 16:745-753.

Kaneyoshi, J.; Kobayashi, S.; Nakamura, Y.; Shigemoto, N. and Doi, Y. 1994. A simple and efficient gene transfer system of trifoliate orange (*Poncirus trifoliata* Raf.). Plant Cell Reports. 13: 541-545.

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

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum. 15: 473-479.

Omura, M. and Hidaka, T. 1992. Shoot tip culture of Citrus. Bulletin of the Fruit Tree Research Station. 22:23-35.

Peña, L.; Cervera, M.; Juárez, J.; Navarro, A.; Pina, J. A. and Durán-Vila, N. 1995a. *Agrobacterium*-mediated transformation of sweet orange and regeneration of transgenic plants. Plant Cell Reports. 14:616-619.

Peña, L.; Cervera, M.; Juárez, J.; Navarro, A.; Pina, J. A. and Navarro, L. 1995b. High efficiency *Agrobacterium*-mediated transformation and regeneration of citrus. Plant Science. 104:183-191.

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

Vardi, A.; Bleichman, S. and Aviv, D. 1990. Genetic transformation of *Citrus* protoplasts and regeneration of transgenic plants. Plant Science. 69:199-206.

Yao, J-L.; Wu, J-H.; Gleave, A. P. and Morris, B. A. M. 1996. Transformation of citrus embryogenic cells using particle bombardment of transgenic embryos. Plant Science. 113:175-183.

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