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BRIEF COMMUNICATION

In vitro organogenesis of *Citrus volkameriana* and *Citrus aurantium*

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Abstract

In vitro organogenesis of *Citrus volkameriana* and *C. aurantium* was studied considering three explant types: epicotyl segment, internodal segment, and hypocotyl segment with attached cotyledon fragment. The explants were cultured in medium according to Grosser and Gmitter (EME) supplemented with 0, 0.5, 1.0, 1.5, and 2.0 mg dm^{-3} 6-benzylaminopurine (BAP), incubated firstly in darkness for 4 weeks, and then transferred to 16-h photoperiod for 2 weeks. Comparing epicotyl and internodal segments, a higher percentage of responsive explants and a higher number of shoots per explant were obtained with epicotyl segments, regardless of the BAP concentration. For *C. volkameriana* the highest percentage of responsive epicotyl segments (42 %) was obtained in EME with 1.0 mg dm^{-3} BAP, while for *C. aurantium* (59 %) in EME with 0.5 mg dm^{-3} BAP. The organogenesis efficiency was the best with the use of the hypocotyl segment with attached cotyledon fragment (77 % for *C. volkameriana* and to 75 % for *C. aurantium*). With this explant the morphogenesis occurred only in the hypocotyl region. The *in vitro* organogenesis was characterized by histological analyses showing that the morphogenic process started in the cambium region near the explant cut end.

Additional key words: adventitious bud, BAP, epicotyl, hypocotyl, internodal segment, sour orange, volkamer lemon.

Since the first report of citrus genetic transformation (Kobayashi and Uchimiya 1989), transgenes have been introduced in many citrus cultivars (Peña *et al.* 2001, Boscariol *et al.* 2006). Among the rootstock cultivars, transgenic plants can easily be obtained for Carrizo citrange (Peña *et al.* 1995, Yu *et al.* 2002), however, other rootstock cultivars, such as *C. limonia*, *C. volkameriana*, and *C. aurantium*, have shown to be recalcitrant (Gutiérrez. *et al.* 1997, Azevedo *et al.* 2006). These difficulties may be related not only to gene transfer process but also to the efficiency of *in vitro* organogenesis. Most citrus *in vitro* organogenesis protocols are based on epicotyl or internodal segment explants cultured in media supplemented with cytokinins (Bordón *et al.* 2000, Moreira-Dias *et al.* 2001, García-Luis *et al.* 2006). However, in some cultivars the number of responsive explants is small even when young and highly responsive explants are used. This low *in vitro* organogenesis rate reflects in low genetic transformation efficiency.

Genetic transformation of *C. volkameriana* and *C. aurantium* has proven to be very difficult. In order to investigate if difficulties could be related to the efficiency of *in vitro* organogenesis process we studied the adventitious bud development in these two recalcitrant rootstock cultivars using three different explant types cultured in media supplemented with several cytokinin concentrations. Besides the standard explants (epicotyl and internodal segments) a hypocotyl segment with attached cotyledon fragment was also tested. The morphogenic process was characterized by histological analyses

Volkamer lemon (*Citrus volkameriana* Ten. & Pasq.) and sour orange (*C. aurantium* L.) explants (epicotyl segments, hypocotyl segments with attached cotyledon fragment, and internodal segments) were collected from *in vitro* germinated seedlings or plants cultivated in the greenhouse. For *in vitro* germination, seeds were extracted from mature fruits and dried at room temperature (24 h). The seed coat was removed, and the seeds were treated with sodium hypochlorite solution (0.5 %) for 15 min, followed by three rinses with sterile distilled

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Abbreviations: BAP - 6-benzylaminopurine; EME medium - medium according Grosser and Gmitter; MS - Murashige and Skoog.
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water. The seeds were cultured on solid Murashige and Skoog (1962; MS) medium and incubated at 27 ºC. Epicotyl segment explants (0.8 - 1.0 cm) were extracted from seedlings (12 to 15 cm in height) germinated in the dark (3 - 4 weeks) and transferred to 16-h photoperiod (irradiance of 65 μmol m^{-2} s^{-1}) for 10 d. Hypocotyl segments with attached cotyledon fragment (Fig. 1*e*) were extracted from seeds germinated in the dark for 20 d. Internodal segments (0.8 - 1.0 cm) were collected from plants grown in the greenhouse.

The explants were cultured in Petri dish (100 × 15 mm) containing the medium according to Grosser and Gmitter (1990; EME) supplemented with 6-benzylaminopurine (BAP - 0, 0.5, 1.0, 1.5, and 2.0 mg dm^{-3}), incubated firstly in the dark for 30 d and then transferred to 16-h photoperiod. Evaluation was performed after 45 d in culture to determine both the number of responsive explants and the number of shoots per explant with the help of a stereomicroscope. In order to compare epicotyl and intermodal segment explants the experiments followed a complete factorial design with 5 replicates. Each replicates consisted of a Petri dish with 10 explants each. The experiments with hypocotyl segments with attached cotyledon fragment contained 4 to 5 replicates (Petri dishes with 6 to 8 explants). The data were analyzed by *ANOVA* and the means compared by Tukey's test. Experiments were repeated at least twice.

For histological analyses explants cultured in EME + BAP (0.5 - 1.5 mg dm^{-3}) in darkness were sampled after 0 - 24 d. Samples were fixed under refrigeration in para-formaldehyde (4 %). Dehydration was made at room temperature in a series of 100 % methylcellosolve, ethanol, propanol, and butanol, followed by infiltration in butanol + infiltration medium (*Historesin*, *Leica*, Heidelberg, Germany; 1:1) at 4 ºC overnight. Infiltration was completed with 100 % infiltration medium. For polymerization the material was incubated at room temperature for 24 to 48 h. Serial sections (5 μm) were prepared in a rotary microtome. The sections were floated in water drops and dried on a hot plate (40 ºC). The material was stained with acid fuchsin (1 %), rinsed in distilled water and counter-stained with toluidine blue (0.05 %) for general observations.

In vitro organogenesis occurred in both species *C. volkameriana* and *C. aurantium*, using epicotyl and internodal segment as explants (Fig. 1*a*,*c*) and BAP supplementation was essential for adventitious bud development. Considering both the percentage of responsive explants and the number of shoots developed per explant, the best results for *C. volkameriana* were obtained with 1.0 and 1.5 mg dm^{-3} BAP (Table 1). However, *C. aurantium* apparently required lower concentrations of cytokinin as the best results were obtained with 0.5 and 1.0 mg dm^{-3} BAP (Table 1). Regardless of the species studied and the cytokinin concentration tested, the efficiency of *in vitro* organogenesis was higher when epicotyl segments were used as explants with values of 42 and 59 % of responsive explants for *C. volkameriana* and *C. aurantium*, respectively, with the development of one shoot per explant on average.

The efficiency of *in vitro* organogenesis could be improved for both species studied by using hypocotyl segments with attached cotyledon fragment (Table 1, Fig. 1*e*). The values of explant responsiveness increased to 77 and 75 % for *C. volkameriana* and *C. aurantium*, respectively. Moreover, the number of shoots developed per explant was higher than the values obtained with the use of the standard explants, with the development of three shoots per explant on average.

The histological analyses performed in epicotyl and internodal segments cultured for 10 d in BAP supplemented media showed that the morphogenic process started with periclinal cell divisions in the cambium region near the explant cut end. The process evolved to callus formation, and meristematic regions could be detected after 20 d of culture. Adventitious buds could be visualized in both explants after 20 - 30 d in culture (Fig. 1*b*,*d*). Regarding hypocotyl segment with attached cotyledon fragment, the morphogenic process occurred

Table 1. *In vitro* organogenesis from epicotyl, internodal segments and hypocotyl segment with attached cotyledon fragment from *Citrus volkameriana* and *Citrus aurantium*. Means ± SE, n = 50 (epicotyl, internodal segment) or 40 (hypocotyl). Means with the same lowercase letter in the column, for each species, are not significantly different by Tukey's test ($P \leq 0.05$).

Species	BAP [mg dm^{-3}]	Responsive explants [%]			Number of shoots [$explant^{-1}$]		
		epicotyl	internodal	hypocotyl	epicotyl	internodal	hypocotyl
C. volkameriana	0.0	6.0 ± 9.8b	0.6 ± 2.5c	10.4 ± 12.4b	0.06 ± 0.11c	0.06 ± 0.25b	0.20 ± 0.26c
	0.5	24.0 ± 20.6ab	11.0 ± 13.5bc	60.4 ± 25.1a	0.52 ± 0.47bc	0.19 ± 0.26ab	2.94 ± 1.00b
	1.0	42.0 ± 25.6a	24.0 ± 17.2ab	77.0 ± 19.8a	1.04 ± 0.64a	0.45 ± 0.37a	4.56 ± 1.87a
	1.5	37.0 ± 20.5a	30.0 ± 21.5a	75.0 ± 15.5a	0.92 ± 0.58ab	0.43 ± 0.41a	4.45 ± 0.81ab
C .aurantium	0.0	23.0 ± 26.3c	0.0 ± 0.0c	0.0 ± 0.0b	0.26 ± 0.32c	0.00 ± 0.00c	0.00 ± 0.00c
	0.5	59.0 ± 17.0a	39.0 ± 19.4ab	75.0 ± 19.7a	1.07 ± 0.45a	0.67 ± 0.43ab	3.70 ± 1.14a
	1.0	51.0 ± 17.2ab	48.0 ± 20.3a	67.5 ± 14.2a	0.80 ± 0.39ab	0.78 ± 0.40a	2.80 ±1.08ab
	1.5	37.0 ± 13.3bc	35.0 ± 18.4ab	52.5 ± 24.0a	0.58 ± 0.24bc	0.68 ± 0.37ab	1.50 ± 0.88b
	2.0	36.0 ± 22.6bc	22.0 ± 19.0b	-	0.59 ± 0.42bc	0.40 ± 0.38b	-

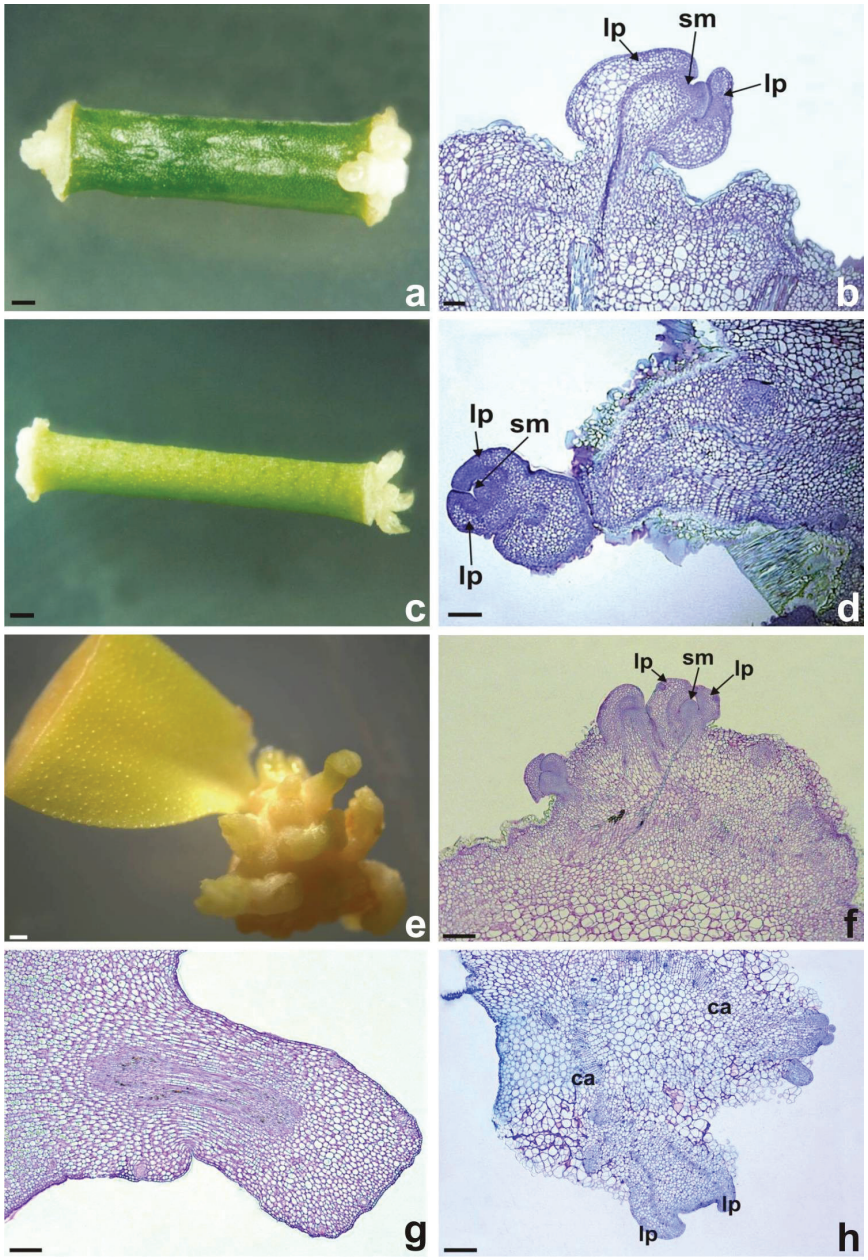

Fig. 1. *In vitro* organogenesis of *C. volkameriana* and *C. aurantium*. Internodal segment explants of *C. volkameriana* (*a*) and epicotyl segment explants of *C. aurantium* (*c*) showing adventitious shoots developed after 30 d of incubation in darkness; longitudinal section of *C. volkameriana* (*b*) and *C. aurantium* (*d*) after 20 - 24 d in culture, showing adventitious shoot with meristem (sm), leaf primordium (lp), and well defined epidermis; hypocotyl fragment attached to cotyledon segment explant of *C. volkameriana* (*e*) showing adventitious shoot developed at hypocotyl region; histological section of hypocotyl fragment attached to cotyledon segment initial explant (*g*); longitudinal section of *C. aurantium* (*f*) after 13 d in culture, showing adventitious shoot with meristem (sm), and leaf primordium (lp); *C. volkameriana* histological section (*h*) after 13 d in culture, showing initial cell divisions at cambium region (ca) and adventitious shoot development. *Bars* = 50 µm (*b*), 100 µm (*d*), 200 µm (*a*, *c*, *f*, *g*, *h*), 500 µm (*e*).

only in the hypocotyl region. No structural alterations and adventitious bud could be detected in the cotyledon region. Similarly to standard explants, cell divisions started at cambium region with the development of callus, meristematic regions, and adventitious buds characterizing an indirect organogenic process (Fig. 1*f*-*h*).

The use of tissue culture techniques in citrus genotypes started with the *in vitro* culture of ovules and nucelli tissue aiming to recover virus free plants (Ranga Swamy 1958, Kochba *et al*. 1972). Biotechnology development including the description of new tissue culture media mixtures and supplements (Murashige and

Tucker 1969) makes it possible both to culture many citrus tissues and organs *in vitro* and to regenerate plants from most cultivars. However, the efficiency of *in vitro* culture protocols varies according to the genotypes, explants, and incubation conditions.

Epicotyl and internodal segments are successfully used as explants for several citrus scion and rootstock cultivars. The responsiveness of these explants ranged from 95 % for Troyer and Carrizo citrange (Moreira-Dias *et al.* 2001, Bordón *et al.* 2000) and 90 % for grapefruit (Costa *et al.* 2004) to values around 70 % for sweet orange (Duran-Vila *et al.* 1992). However, some genotypes show a low regeneration efficiency with a percentage of explants developing adventitious buds ranging from 60 % for *C. sinensis* cv. Pera, 45 to 60 % for *C. limonia* (Moura *et al.* 2001, Costa *et al.* 2004) to the extreme low responsiveness of 4 % for *C. aurantium* (Bordón *et al.* 2000).

Although it has been reported that some buds differentiate and shoots grow in citrus explants cultured on basal MS medium (Costa *et al.* 2004, García-Luis *et al.* 2006) the culture media supplemented with cytokinins especially BAP improves the efficiency of the organogenic process (Moreira-Dias *et al.* 2001, Molina *et al.* 2007). In our work, the number of responsive explants and the number of shoots developed per explants increased with BAP concentration upto 1 or 1.5 mg dm^{-3}. The inhibitory effect of high concentrations of BAP has also been reported for other citrus genotypes (Moreira-Dias *et al.* 2000, Silva *et al.* 2005, Molina *et al.* 2007, Cervera *et al.* 2008).

Comparing the use of epicotyl with intermodal segments we detected a higher efficiency in organogenesis when epicotyl segments were used, for both genotypes studied. Although it was possible to obtain plants for both species studied, the responsiveness rate (around 50 %) may not be considered adequate for genetic transformation. It is well known that high efficiency in shoot regeneration is very important for genetic transformation as it increases the chances to obtain transgenic plants.

Ananthakrishnan *et al.* (2003) described an efficient explant for squash *in vitro* organogenesis consisting of a proximal segment of cotyledon attached to hypocotyl fragment. Hypocotyl segments have been described as very responsive explants in other species such as passion fruit (Fernando *et al.* 2007), cotton (Divya *et al.* 2008), and *Eucommia ulmoides* (Chen *et al.* 2008). Also, Moreira-Dias *et al.* (2001) described that the capacity of epicotyl segments regeneration decreases markedly by the cotyledonary node distancing. Therefore, the hypocotyl segments with attached cotyledon fragment were used as explants in order to improve the rate of adventitious bud development in these recalcitrant genotypes. In fact, the percentage of responsive explants increased to 77 % for *C. volkameriana* and 75 % for *C. aurantium*. Moreover, the number of shoots per explant increased to 3 shoots per explant. The adventitious bud development occurred only in the hypocotyl end, contrasting with the results obtained for squash where the regeneration occurred at the junction of cotyledon and hypocotyl fragment (Anathakrishman *et al.* 2003). The histological analyses showed that the regeneration process starts with the cells division at the cambium region evolving to an indirect organogenesis process, as already described for citrus with other explants (García-Luis *et al.* 1999, Bordon *et al.* 2000, Almeida *et al.* 2006). To the best of our knowledge this is the first report on shoot regeneration *via* organogenesis from hypocotyl segments with attached cotyledon fragments in *Citrus*.

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