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BRIEF COMMUNICATION

Callus sieving is effective in improving synchronization and frequency of somatic embryogenesis in *Citrus sinensis*

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Abstract

Somatic embryogenesis has been obtained in many citrus cultivars. However, culture synchronization is yet to be achieved and in the present work we evaluate the effect of desiccation, cold and size of cell clusters on embryo production efficiency from callus cultures of *Citrus sinensis* L. Osbeck, cv. Valencia. The results showed that sieving was effective in promoting somatic embryo synchronization, whether or not it was followed by cold or desiccation treatments. Histological and histochemical analyses are presented to characterize the structure of cell aggregates and protein accumulation.

Additional key words: cell aggregates, cold, desiccation, histology, proteins.

Somatic embryogenesis shows promise of practical application for clonal propagation and improvement of plants and it is an excellent tool for basic studies of the molecular and biochemical events during embryogenesis. However, the underlying mechanisms behind this phenomenon remain to be determined (Ikeda-Iwai *et al.* 2003).

In citrus, the traditional methods of genetic improvement are seriously limited because of its complex biology (Grosser and Gmitter 1990). Somatic embryogenesis is an important alternative for the regeneration of genetically improved genotypes, and has been induced successfully for many plant species, besides citrus species and cultivars, with variable results in terms of conversion into plants (Tomaz *et al.* 2001, Duan *et al.* 2007, Shekhawat *et al.* 2009, Yadav *et al*. 2009). Nevertheless, the effectiveness of *in vitro* culture protocols varies according to the genotypes, explants, and incubation conditions (Tavano *et al*. 2009).

The quality of somatic embryos and the maturation frequency are the most important criteria for the optimization of a somatic embryogenesis protocol (Maruyama *et al.* 2007). Both quality and quantity of somatic embryos produced are dependent not only on genetic and physiological factors, but also on the culture conditions in which the callus is cultivated (Grosser and Gmitter 1990). Mingozzi and Morini (2009) showed that somatic embryogenesis is also significantly affected by the donor plant growth conditions. The absence of synchrony, occurrence of embryo abnormality and the low frequency of development of citrus somatic embryos have already been reported (Pérez *et al.* 1998, Tomaz *et al.* 2001, Duan *et al.* 2007). In this way, Hussain *et al.* (2009) suggested that culture manipulations focusing high frequency production of quality embryos is necessary. Several methodologies have been used in attempts to improve the production of citrus somatic embryos: treatments with auxins and cytokinins (Ling and Iwamasa 1994, Vila *et al*. 2009), gibberellin (Sim *et al.* 1988), osmoticum (Kobayashi *et al.* 1985) or different

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Abbreviation: EME - medium according to Grosser and Gmitter (1990).
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sugars (Pérez *et al.* 1998, Tomaz *et al.* 2001). Stress treatments during *in vitro* cultivation of several species have been shown to increase the induction rates, maturation and germination of somatic embryos (Janeiro *et al.* 1995, Lee *et al.* 2001, Luo *et al.* 2003, Zhang *et al.* 2006). Previous studies have showed that the separation of cell aggregates also improve the synchronization and normalization of somatic embryogenesis (Anandarajah and McKersie 1990, Merkle *et al.* 1990, Niedz *et al.* 2002, Niedz 2006).

The aim of the present study was to improve a citrus somatic embryogenesis system by evaluating the effect of the size of cell aggregates and of stress treatments such as desiccation and cold in the induction, synchronization and frequency of somatic embryogenesis in *Citrus sinensis*.

Callus cultures of sweet orange [*Citrus sinensis* (L.) Osbeck, cv. Valencia] were initially obtained from nucellar tissue (Oliveira *et al.* 1994) and maintained at 27 ± 1 °C in the dark, on callus proliferation medium, which was EME medium (Grosser and Gmitter 1990) supplemented with 50 g dm^{-3} sucrose, 500 mg dm^{-3} malt extract and 8.0 g dm^{-3} agar. After several monthly subcultures, approximately 50 mg of callus was transferred to a Petri dish containing somatic embryogenesis induction medium (EME medium with 13.4 g dm^{-3} maltose) for somatic embryo development. To facilitate uniform spreading of the cultures on the culture medium, 1 cm^3 of liquid medium was added. The control was cultivated at 26 ± 1 °C, 16-h photoperiod and irradiance of 40 μmol m^{-2} s^{-1} following the protocol by Tomaz *et al.* (2001). The stress and sieving treatments were prepared as follows. Callus cultured on solidified proliferation medium was transferred to liquid prolife-ration medium and cultured under agitation at 200 rpm during 24 h, and then sieved. Sieves with screen mesh sizes of 150 μm (S150), 300 μm (S300) and 600 μm (S600) were used to separate the initial cultures in more uniform cluster-size cultures. After sieving, approximately 50 mg of cultures retained in each sieve and the same amount of the original culture (non-sieved) was collected and transferred, each to a separate Petri dish containing EME medium supplemented with 13.4 g dm^{-3} maltose (Tomaz *et al.* 2001). In addition to the effect of cluster size, stress pre-treatments were applied to sieved and non-sieved callus, consisting of: *1*) cold: callus cultured in Petri dishes containing somatic embryogenesis induction medium at 4 °C for 4 weeks in the dark, and 2) desiccation: callus placed in Petri dishes in absence of medium and in the dark at 26 ± 1 °C for 6 d. Subsequently, all cultures were grown in Petri dishes containing induction medium at 26 ± 1 °C, 16-h photoperiod and irradiance of 4 μmol m^{-2} s^{-1}. Thus, treatments were: no sieving cultured at 26 ± 1 °C (T1), cold (T2), desiccation (T3); sieving with S150 and cultured at 26 ± 1 °C (T4), cold (T5), desiccation (T6); sieving with S300 and cultured at 26 ± 1 °C (T7), cold (T8), desiccation (T9); sieving with S600 and cultured at 26 ± 1 °C (T10), cold (T11), desiccation (T12). Callus samples were collected from each treatment at 0, 7, 15 and 30 d and processed for light microscopy according to Rodriguez and Wetzstein (1998). Sections were stained with acid fucsin (0.1 %) and counter stained with toluidine blue (0.05 %) for structural observations and with Comassie blue (0.25 % in 3 % acetic acid) for the detection of proteins, rinsed and immersed in glacial acetic acid (3 %) for 24 h (Fisher 1968), then mounted with *Entellan* mounting medium, covered with cover slips, observed and photographed under an inverted microscope (*Zeiss Axiovert*, *Carl Zeiss*, Jena, Germany).

The experiment was designed in a 4 × 3 factorial design resulting in 12 treatments with four replications per treatment, totaling 48 completely randomized plots in each block. Contamination occurred and some plots were missed. Before the analysis of the data, the arcsin transformation was necessary. Tukey's test was used for means comparison. Evaluations of frequency of embryo formation and of the developmental stages of somatic embryos after 30 and 60 d in culture was done under the stereomicroscope. Somatic embryo frequency was roughly determined by the percent area of callus showing the development of somatic embryos.

Control cultures showed non-synchronized somatic embryo development, with embryos in various developmental stages in the same culture after about 30 d in EME medium with maltose. Sieving of the cultures with a series of three sieves placed in a sequence of larger to smaller mesh size was efficient to separate the callus cultures in different types of cell aggregates (Fig. 1). Histological analysis of callus evidenced the presence of three types of cellular aggregates. Type 1 aggregates (Fig. 1*A*) were the most frequent in callus sieved with S150 (Fig. 1*B*). The cells were small, round, with evident nucleus and presented high frequency of cell divisions. Type 2 aggregates (Fig. 1*C*) were formed by slightly larger cells, with larger vacuoles, less evident nucleus and lower frequency of cell divisions, and were observed more frequently in callus sieved with S300 (Fig. 1*D*). Type 3 aggregates (Fig. 1*E*), most frequently observed in callus sieved with S600 (Fig. 1*F*), presented mostly large cells with prominent vacuoles, mostly non-evident nuclei, and with low frequency of cell divisions.

Initiation of embryo development, at the globular stage was easily detected by a green color in the callus, which is characteristic of the citrus somatic embryo as early as the globular stage. Higher synchronization of somatic embryo initiation was visually detected in sieved cultures, compared to the control (Fig. 1*B*,*C*,*D*).

In cultures that received the desiccation treatment, we observed that the embryogenic callus, which was cream colored before treatment, became brownish after 2 - 3 d under desiccation. After transfer to medium for embryo induction/development the callus slowly recovered and

after approximately 15 d the initiation of somatic embryos was observed, by the presence of green dots in the callus. During the cold treatment (4 °C for four weeks) somatic embryo formation was not observed, however soon after transfer of the callus cultures to 26 ± 1 °C, somatic embryos began to form.

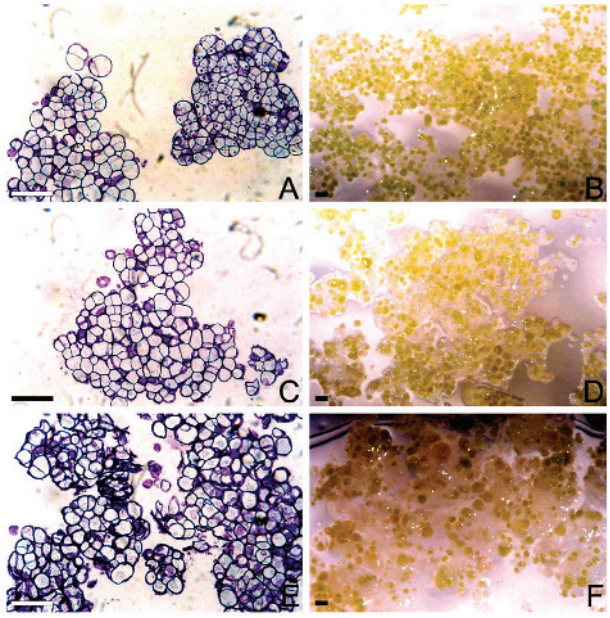

Fig. 1. Callus cultures of sweet orange cv. Valencia 60 d after sieving. Histological sections stained with acid fucsin and toluidine blue of callus types 1 (*A*), 2 (*C*) and 3 (*E*), corresponding to callus most frequently observed after sieving with 150, 300 and 600 µm mesh sizes, respectively. Characteristics of the callus in culture showing the green dots, corresponding to the formation of somatic embryos in representative cultures sieved with different screen mesh size: 150 µm (*B*), 300 µm (*D*) and 600 µm (*E*). *Bars* = 100 µm (*A*, *C*, *E*) and 1000 µm (B, *D*, *F*).

The analysis of somatic embryo frequency (Table 1) in all treatments and control showed that sieving with S300 without any further treatment (T7) gave the highest percentage (99.8 %), however not differing significantly from other six treatments (T4, T5, T6, T8, T9, T10). These treatments showed frequencies between 74.4 % (T10) and 92.9 % (T6). All of these treatments involved sieving with S150 (T4, T5, T6), S300 (T7, T8, T9) and S600 (T10), leading to the conclusion that sieving is important for a better somatic embryo frequency, probably due to a more synchronized somatic embryo initiation. Desiccation and cold were effective only in cultures sieved with S150 and S300. Somatic embryo frequency in cultures sieved with S150, S300 and S600 did not differ statistically. Non-sieved cultures had lower frequencies either without further treatments or after a subsequent cold or desiccation treatment. Cultures sieved with S600, which consisted of larger aggregates, when submitted to cold (T11) or desiccation (T12) gave the lowest somatic embryo frequency results, 36.7 and 27.8 %, respectively. The statistical analysis showed no significant differences between the control cultures (T1, T2, T3) and cultures sieved with S600 (T10, T11, T12).

Lee *et al.* (2001) observed that desiccation of cell cultures inhibits normal undifferentiated cell proliferation, stimulating the production of somatic embryos and the synchronization of embryo development. Wetzstein *et al.* (1990) observed that desiccation followed by cold in pecan cultures improved the development of somatic embryos.

The application of a cold-treatment increased somatic embryogenesis, embryo germination, embryo maturation and subsequent plant conversion (Janeiro *et al.* 1995, Luo *et al.* 2003, Zhang *et al.* 2006, Li *et al.* 2008). Ikeda-Iwai *et al.* (2003) tested several stress treatments (cadmium chloride, osmotic stress using sorbitol, mannitol or sucrose and dehydration) and frequency of somatic embryo formation in *Arabidopsis* callus was dependent on the type and duration of the stress treatment.

In citrus embryogenic cultures, a change in the carbon source, from sucrose to maltose, is known to be sufficient for somatic embryo initiation (Tomaz *et al.* 2001). However, not all the cells respond to the sugar change, creating a non-synchronous culture. Histologically, different types of cells are observed from highly mitotic aggregates, as observed in type 1 callus to more vacuolated cells as seen in type 3 callus. These structural characteristics were also observed by other authors (Shekhawat *et al.* 2009, Yadav *et al.* 2009). The embryogenic or regenerative callus was characterized by small cells with densely staining cytoplasm and prominent nucleous.

Table 1. Effect of sieving and stress treatments on the frequency of somatic embryos from callus cultures of sweet orange cv. Valencia. Means ± SE, n = 4 - 8. Means with the same letter are not significantly different according to Tukey´s test (α = 0.05).

Treatment	Frequency [%]	Treatment	Frequency [%]
T1	49.8±0.13abc	T7	99.8±0.11e
T2	69.2±0.10abcd	T8	87.0±0.12cde
T3	47.3±0.09ab	T9	78.6±0.09bcde7
T4	86.1±0.10cde	T10	74.7±0.12abcde
T5	86.1±0.10cde	T11	36.7±0.12ab
T6	92.9±0.11de	T12	27.8±0.10a

The accumulation of proteins is evidently higher in highly mitotic cell groups, as observed in type 1 callus. The early embryogenic cell cycle is accompanied by synthesis of several new proteins (Nomura and Komamine 1995). During embryogenesis many genes are activated. Pan *et al.* (2009) have recently reported 24 differentially expressed proteins identified during the process of somatic embryogenesis in Valencia sweet orange.

When somatic embryos are induced from embryogenic callus cells, embryos at different stages of development coexist in a given culture. A synchronized culture, however, would facilitate physiological, biochemical, and molecular investigations of somatic embryogenesis. In this research, the results showed that the use of sieves of 150 and 300 μm was effective in promoting somatic embryo synchronization, followed or not by cold or desiccation treatments. As observed in the histological analysis, these sieves separated most of the smaller aggregates, which consisted of cell groups more tightly organized, with smaller highly mitotic cells. Cultures composed of higher aggregates, which were collected by the 600 μm sieve, gave the lowest frequency of somatic embryos, statistically similar to the control, where cultures were not sieved.

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