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Relationship among growth curve, nutrient consumption and genetic transformation efficiency of 'Albariño' (*Vitis vinifera*) cell suspensions

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Summary

Embryogenic cell suspensions of grapevine (*Vitis* sp.) have been proposed as the best target to approach genetic transformation challenges. However, optimal phase and growth period of cell suspensions for successful gene transfer have not been investigated. Here, a step by step protocol to initiate and establish cell suspensions of 'Albariño' (*V. vinifera*) in only 4 weeks is presented. Growth kinetics, cell viability and nutrient consumption (phosphates and nitrates) as well as the number of transient transgenic events (using the uidA reporter gene) were studied in 'Albariño' cell suspensions grown for an 18-days period. Based on biomass growth, the exponential phase of cell suspensions was reached between days 3 to 6. Nutrient uptake results point to the exhaustion of phosphate in the culture medium at day 6. Moreover, the highest number of transgenic events after biolistic bombardment was obtained from cell suspensions grown for 6 days (4032 ± 695 blue spots), compared to 12 and 18 days of continuous culture. Plant regeneration percentage varied depending on the age of the culture and the selected embryo type. In conclusion, this paper shows for the first time the relationship between growth curve and nutrient consumption of embryogenic cell suspension with efficiency in genetic transformation and plant regeneration of grapevine.

Key words: Embryogenic cell suspensions, genetic transformation, growth kinetics, nutrient consumption, plant regeneration, *Vitis vinifera*.

Introduction

Grapevine (*Vitis* sp.) is a fruit crop that has expanded in temperate areas worldwide due to the economical and health benefits of its products, mainly wine and juice (Bouquet *et al.* 2008). Genetic transformation was initially proposed as a tool to approach the molecular breeding of grapevine traits without modifying the identity of the original cultivar (Colova-Tsolova *et al.* 2009). Nowadays, in the post-genomic era, gene transfer is also an indispensable technology to approach functional analysis of this species by over-expressing or silencing specific genes with the goal of determining their function (Vidal *et al.* 2010). However, to successfully apply gene transfer technology to grapevine, an optimal plant target for genetic transformation and plant regeneration is needed.

Embryogenic calli were suggested as suitable target for both genetic transformation and plant regeneration (Perl *et al.* 1996). Somatic embryogenesis can be induced from floral explants and has been obtained from several *Vitis* genotypes (Martinelli and Gribaudo 2009). The initiation and establishment of embryogenic cell suspensions from embryogenic calli were first described by Coutos-Thévenot *et al.* (1992). Later on, Jayasankar *et al.* (1999) reported cell suspensions as the best plant target to approach the genetic engineering of grapevine; this has been confirmed in several studies (Vidal *et al.* 2010 and references therein). However, establishment and maintenance of embryogenic cell suspensions in woody plants, including grapevine, are difficult as well as time and labour consuming (Reustle and Buchholz 2009). Grapevine cell suspensions growth curves and nutrient consumption in the culture medium have been scarcely studied. For practical reasons cell suspensions are generally maintained by subculturing every 1 or 2 weeks (Pepin *et al.* 1995). The analysis of growth kinetics and nutrient uptake of cell suspension for specific genotypes would help to determine the optimal conditions of cells to approach gene transfer experiments.

Grapevine genetic transformation has been approached using the biological *Agrobacterium*-mediated method (Bouquet *et al.* 2006) and the physical biolistic system (Kikkert *et al.* 2004, Vidal *et al.* 2006). Each method has advantages and disadvantages (de la Torre *et al.* 2010) and both use embryogenic cell suspensions as plant target for gene transfer.

In this paper, we describe a step by step protocol for the initiation and maintenance of embryogenic cell suspensions of *Vitis vinifera* 'Albariño', an elite Spanish cultivar of Galician origin (Vidal *et al.* 1999). Cell suspensions were routinely refreshed every 1 or 2 weeks with the goal of avoiding deficit of nutrients in the culture medium. However, for how long cell suspensions are competent for transformation and regeneration without refreshing was ignored. Here, both nutrient consumption and growth curve were examined during an 18-day period, and the best timing for obtaining the highest percentage of transient transgenic events as well as plant regeneration was determined.

Material and Methods

Initiation and establishment of cell suspensions: Pro-embryogenic calli of 'Albariño' cultivar were induced from anthers or ovaries in MS (Murashige and Skoog 1962) or NN (Nitsch and Nitsch

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1969) derived semi-solid media as described in Vidal *et al.* (2009). Pro-embryogenic masses (PEM) of callus from three independent 'Albariño' lines (6, 10 and 14) were used as inocula to initiate and establish embryogenic cell suspensions. Several independent experiments were performed. Cell suspensions were initiated in liquid GM + NOA medium [MS salts and vitamins with 0.37 % (v/v) glycerol, 1.8 % (w/v) maltose and 5.0 µM β-naphthoxyacetic acid (NOA); Kikkert *et al.* 2005] from fine PEM of callus grown on solid medium. Initially, PEM (approx. 200 ± 50 mg) were transferred to 125 mL Erlenmeyer flasks that contained 15 mL of GM + NOA medium and placed on a orbital shaker at 120 rpm and 25 ± 1 °C in the dark. After two weeks, once the initial cell density had doubled, fresh medium was added to a final volume of 30 mL in the same flask.

Two weeks later, when the cell density had doubled again, cultures were transferred to 250 mL Erlenmeyer flasks and fresh medium was added to a final volume of 60 mL for establishment of cell suspensions. During this four-week period, cell suspensions were refreshed every week either replacing one half of the culture medium or doubling as stated above. All reagents used in tissue culture were purchased from Duchefa Biochemie BV.

Maintenance of cell suspensions: After the establishment of cell suspensions, one-half of the medium was replaced with fresh one on a weekly basis. When cell density increased, suspensions (60 mL) were either divided in two 250 mL flasks (30 mL of suspension plus 30 mL of fresh medium) or brought to a final volume of 120 mL in a 500 mL Erlenmeyer flask by adding 60 mL of fresh medium. Cell suspensions were filtered as needed through a mesh (1.0 mm^2 pore size) in order to eliminate large clumps and to synchronize the stage of the culture. Cell suspensions were evaluated for cell viability and contamination routinely.

Cell viability was determined by using the triphenyltetrazolium chloride (TTC) reduction assay (Steponkus and Lanphear 1967). One mL of suspension was allowed to settle in a 1.5-mL tube, the supernatant was removed and 200 µl of TTC solution were added and incubated for 1 h at 24 °C. Viability was evaluated following a visual colour four-category index: from 0 (none) to 3 (intense) red color. In parallel, 0.1 mL of suspension was incubated at 37 °C on Luria Bertani Agar (LBA) and at 28 °C on Potato Dextrose Agar (PDA) specific media (Sigma-Aldrich) for bacteria and fungi, respectively.

Growth curves and nutrient uptake: Five days after subculturing, cell suspensions from different flasks were filtered through a mesh (1.0 mm^2 pore size) in a 1-liter Erlenmeyer flask, and cell density of the pooled culture (approximately 600 ± 50 mL) was adjusted to 0.3 mL of packed cell volume (PCV) per 10 mL suspension using a graduated conical tube (Kikkert *et al.* 2004). Twenty five mL of the pooled cell suspension were added to twenty one 250-mL Erlenmeyer flasks, each containing 25 mL of GM + NOA fresh medium. All 21 flasks, containing a final volume of 50 mL of cell culture, were placed on a gyratory shaker at 120 rpm and 24 ± 1°C in the dark for an 18-d period. Starting day and every three days thereafter, three flasks were randomly selected and removed from incubation to determine both biomass growth and nutrient uptake. Three separated experiments were performed. Every sampling day, cell cultures from three randomly picked flasks were filtered using a Büchner funnel, a side-arm flask and a vacuum pump onto three separate disks of Whatman® No. 2 filter paper. The cellular mass was weighed to determine the fresh weight (average ± standard deviation) and then the disks were placed in an oven at 60 °C for 24 h previous to dry weight determination.

Filtered medium (above) from cell suspensions was used to determine the nutrient uptake of the cells every three days during the 18-d period evaluated. A 10-mL sample from each three filtered media was stored a -20 °C until analysis. The amount of phosphate (PO_4^{3-}), and nitrate (NO_3^-) ions in the culture medium was determined with the H38061 and H38050 test kits (Hanna® Instruments), respectively, following the manufacturer instructions.

Transient expression of β-glucuronidase in cell suspensions: Plating of target cells on filter paper support, coating gold particles with plasmid DNA and particle bombardment using the PDS-1000/He biolistic device (BioRad) were performed as described previously (Kikkert *et al.* 2004). Transient expression of β-glucuronidase (GUS) in cell suspensions grown without refreshing during 6, 12 and 18 d was used to evaluate the gene transfer efficiency after biolistic bombardment with plasmid pBI221 (containing the uidA gene under control of the CaMV 35S promoter and the NOS terminator; Vidal *et al.* 2009). Cell suspension density was adjusted to 0.2 mL of PCV per 10 mL of suspension, and 5 mL of suspension were spread as a fine layer onto a filter paper support in a Petri plate containing bombardment medium (Kikkert *et al.* 2004). A total of 6 plates per treatment (6-, 12- and 18-d growth time) were bombarded and at least two repetitions were made. Transient GUS expression assay was performed as described elsewhere (Vidal *et al.* 2003) and the number of blue spots per plate was counted with a stereomicroscope. The results were analyzed with the SPSS software package (SPSS Inc.).

Embryo development and plant regeneration: Cell suspensions from 6, 12 and 18 d of culture without refreshing were adjusted to 0.2 mL of PCV per 10 mL of suspension and 5 mL of cells were plated on a filter paper support in Petri plates containing MS/2CA medium [half strength MS macro- and micro-elements, MS vitamins, 0.3 % (w/v) active charcoal and 0.25 % (w/v) Phytagel®, pH 5.8] and incubated in the dark at 27 ± 1 °C during two months, to allow embryo development as described elsewhere (Vidal *et al.* 2009). Emerging embryos at two different stages, early stage (< 0.5 cm size, embryo without radicle) and later stage (1 ± 0.5 cm size, embryo with radicle), were picked out and transferred to fresh MS/2CA regeneration medium in Petri plates. Plates were wrapped with Parafilm® and incubated one month in indirect light (15 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and then under direct light (25 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for an additional month. Four months after suspension plating, the percentage of plant regeneration was determined. Plant regeneration experiments were repeated at least twice.

Results

Initiation and maintenance of cell suspension: Embryogenic cell suspensions of 'Albariño' from three independent lines were successfully initiated and established (Fig. 1). Depending on the inoculum stage on solid medium and the subsequent increase of biomass density, one or two additional weeks may be required for the successful establishment of cell suspensions; on average, suspensions were initiated in 15 mL and established in 60 mL in only 6 ± 2 weeks prior to biomass proliferation for gene transfer experiments. Suspension appearance depends on the original PEM callus, which varied in grain size and colour. Suspension colour varies from whitish to yellowish, sometimes with brown appearance (mainly at the beginning of the suspension). Suspension grain size was a mixture of fine, medium and large (> 1 mm diameter) clumps, the latter being removed by filtration as stated above. Biomass growth of the suspension was moderated at the beginning and increased over time (Fig. 2).

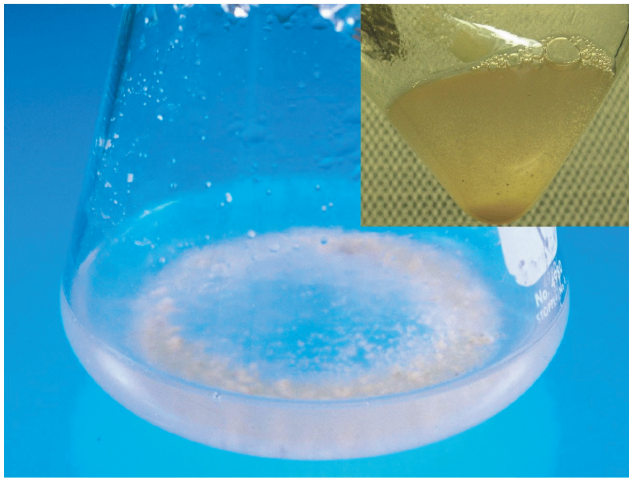

Fig. 1: Cell suspensions derived from proembryogenic masses of 'Albariño' (*V. vinifera*) and established in GM + NOA medium, after 8 weeks of culture. The settling of the fine grain of the suspension is shown in the inset.

Growth kinetics: A total of 21 cell suspension cultures were withdrawn from a homogeneous culture of 'Albariño', and grown for 18 d to determine the growth curve. Fig. 2 represents the average growth curves of cell suspensions for fresh and dry weight. Biomass increment from day 0 to day 18 of culture was approximately 260 % for both fresh (from 432 ± 117 mg to 1542 ± 52 mg) and dry (from 62 ± 19 mg to 225 ± 39 mg) weight. Both growth curves showed a progressive increase of biomass from day 0 to day 18 that could be divided in three phases of growth: exponential (from day 0 to 6), linear (from day 6 to 15) and moderate deceleration (after day 15). Biomass growth from days 0 to 6 was on exponential phase, with cellular weight almost doubling the previous sampling day, followed by a decelerated increase at day 9 and thereafter (Fig. 2). Cell viability of the cell suspensions as assayed using the TTC test was always higher than 95 % during the 18 d of culture (data not shown).

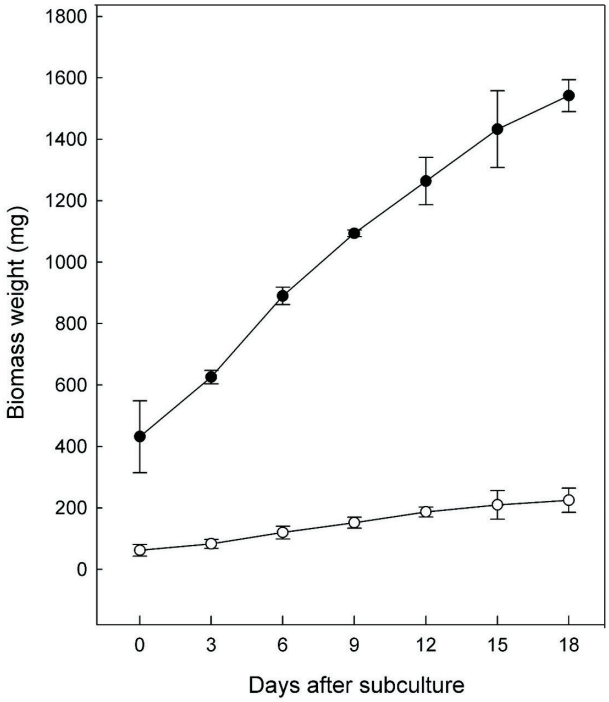

Fig. 2: Growth curves corresponding to fresh (closed symbols) and dry (open symbols) weight of embryogenic cell suspensions of 'Albariño' (*V. vinifera*) cultured during 18 d without refreshing. Results are the means ± SD of three independent experiments.

Nutrient consumption: Filtered medium of each cell suspension (3 samples per each sampling day and experiment) was evaluated to determine the nutrient consumption over time. The progressive biomass proliferation after day 3 entails a consequent uptake of two main macronutrients: phosphates and nitrates (Fig. 3). Consumption of phosphates was drastic from the beginning of the culture, with an accelerated decrease (66 %) from day 0

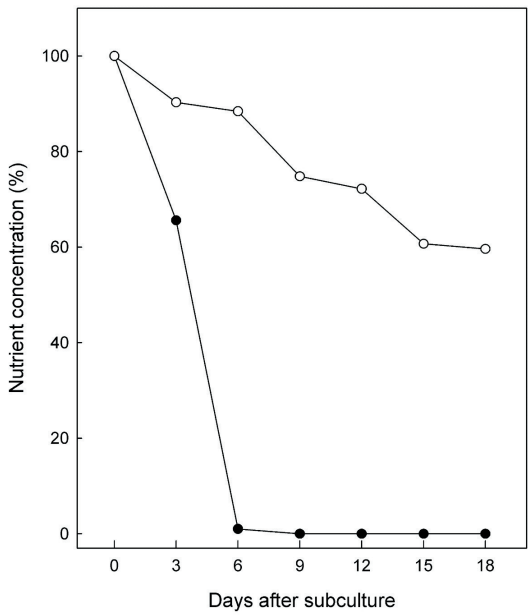

Fig. 3: Percentage of phosphates (closed symbols) and nitrates (open symbols) in the culture medium of cell suspensions of 'Albariño' (*V. vinifera*) grown during 18 d without refreshing.

(84,5 mg·L^{-1}) to day 3 (55,4 mg·L^{-1}) and reaching almost exhaustion (0,9 mg·L^{-1}) at day 6 (Fig. 3), pointing phosphate as a limiting nutrient for the division of cells in suspension. By contrast, consumption of nitrates decreased progressively over the 18 d of culture. Nitrate was not a limiting nutrient as its amount in the medium of culture at day 18 (1634 mg·L^{-1}) was still 60 % compared with day 0 (2739 mg·L^{-1}). These results point to the convenience of refreshing the cell suspension of Albariño with fresh medium every week in order to provide necessary nutrients (especially phosphates) for maintaining exponential growth and cellular viability.

Transient expression of β-glucuronidase gene: Gene transfer experiments were performed using cell suspensions of 6-, 12- and 18-d continuous cultures, corresponding to the theoretical exponential, linear and progressive deceleration phases. A total of 18 plates (6 for each culture day) were bombarded per experiment. The highest number of transient transgenic events per bombarded plate was obtained at day 6 of culture (4032 ± 695 blue spots). The number of transgenic events decreased significantly thereafter at day 12 (2605 ± 777 blue spots) and at day 18 (2329 ± 488 blue spots) of culture without refreshing (Fig. 4). These results showed that cell suspensions from 6 to 18 d old are all competent for gene transfer, although the preferred target material for a more efficient genetic transformation of 'Albariño' cell suspensions are those from 6-d old cultures.

Plant regeneration: Cell suspensions from three continuous culture periods (6, 12 and 18 d) were also evaluated for plant regeneration. Two months after incubation on regeneration medium, embryos started to emerge on the filter paper support; embryos with and without radicle were selected and transferred to a new regeneration medium. Between 30 and 60 embryos per culture time period and embryo type were randomly selected and transferred to light. After two months, percentage of regeneration was determined. Most of the selected embryos from both embryo types germinated. However, the percentage of regeneration was higher from embryos selected with radicle (51 %, [46/90]) compared to without radicle (18 %, [20/110]) considering the three-culture time period (Fig. 5). The percentage of regeneration varied between 60 [18/30] and 33 % [10/30] at day 6 and 18, respectively, from embryos with radicle and between 24 [12/50] and 13 [4/30] at day 6 and 18, respectively, from embryos without radicle. The percentage of regeneration decreased with the age of the culture (Fig. 5).

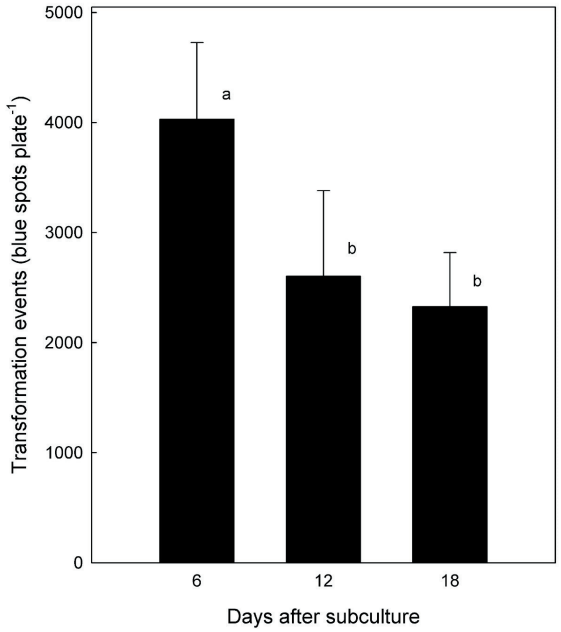

Fig. 4: Number of transient transgenic events after transformation by biolistics of 'Albariño' cell suspensions cultured during 6, 12 and 18 d without refreshing. Results are the means ± SD of three independent experiments, and different letters indicate statistically significant differences ($P < 0.05$).

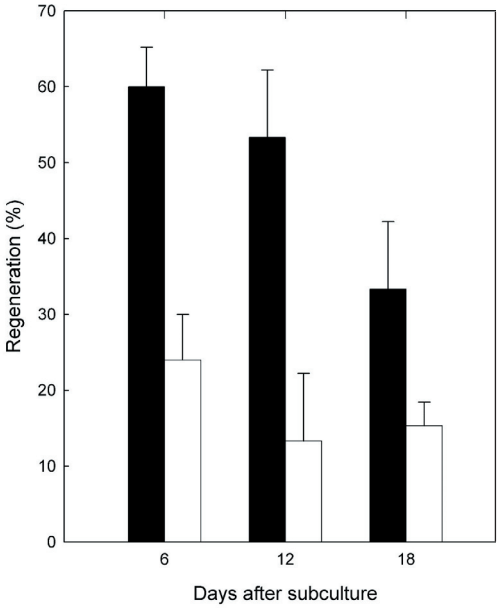

Fig. 5: Percentage of plants regenerated from embryos with radicle (black bars) or without radicle (white bars) derived from 'Albariño' (*V. vinifera*) cell suspensions cultured for 6, 12 and 18 d without refreshing.

Discussion

Nowadays, cell suspension cultures are an adequate plant target to approach proteomic (Sharathchandra *et al.* 2011) and functional (Vidal *et al.* 2010) analysis of grapevine. Embryogenic cell suspensions of 'Albariño' were initiated from fine PEM callus and established in only 4 weeks, with two additional weeks needed to maintain a stable growth prior to biomass proliferation. At that stage, cell biomass doubled almost every week. To keep a high biomass growth rate, in each subculture, high PEM density in the suspension had to be maintained (data not shown). In fact, sensitivity to culture density for several grapevine genotypes was previously reported (Jayasankar *et al.* 1999). 'Albariño' suspensions have to be routinely refreshed with new medium every week in order to provide necessary macro-element, and filtered when big aggregates (≥ 1 mm diameter) are observed to keep fine cell suspensions. Additionally, suspensions could be cryopreserved

without losing embryogenic capacity (González-Benito 2009) when long periods of inactivity are required. After several weeks of biomass proliferation, in case of biomass saturation, the culture medium usually developed a slight pink colour, probably due to the presence of arabinogalactan proteins or other glycoproteins, the former being indicative of cell proliferation and embryogenesis (Ben Amar *et al.* 2007). Browning of the medium was observed sometimes likely due to the secretion of phenolic compounds from dying cells (Pepin *et al.* 1995, Colova-Tsolova *et al.* 2007). In this work, 'Albariño' somatic embryos development was totally arrested by the auxin (NOA) in the maintenance medium during the biomass proliferation.

Biomass proliferation of *V. vinifera* cell cultures was found to be not completely associated with cell division (Pépin *et al.* 1995). In our work, biomass growth showed the model kinetic curve (Chawla 2009), but with a more progressive growth. However, still three growth phases (exponential, linear and deceleration) were easily observed (Fig. 2). Jayasankar *et al.* (1999) reported that suspension of *V. vinifera* 'Chardonnay' proliferated exponentially during 12 weeks with subculturing at 14-d intervals. Later, Colova-Tsolova *et al.* (2007) obtained a stable growth rate (> 100 %) every week for established suspensions of muscadine (*Vitis rotundifolia*) initiated from a 2-gram inoculum. Here, the growth curve for an 18-d period without subculturing of 'Albariño' suspensions that were initiated with approx. 5 % (v/v) of PEM was studied. Although there was a gradual increase of both fresh and dry weight during the 18-d period, the increase of biomass was gradually lower as suspension reaches the stationary phase. Thus, the increase of fresh weight at day 18 is 8 %, lower than the increase at day 15 (13 %). As the phosphate in the medium became almost exhausted at day 6, cell division could stop, and thereafter the increase in biomass weight could be rather due to cell elongation by an increase of cell wall and cytosolic organelles. Similar declines in nutrient uptake for nitrates and phosphates were shown in a previous work on *V. vinifera* 'Gamay' suspensions reported by Pepin *et al.* (1995). In this early work, the exhaustion of phosphates took place at day 4 and nitrates depletion was more important probably because cell suspensions were initiated with higher inoculums levels. These authors suggest that the rapid depletion of phosphates took place due to accumulation into cells for later redistribution through cell division.

The most competent target cells for transient transformation were those from 6-d old cultures, which was in agreement with the consumption of nutrients observed, as the phosphate was exhausted in the medium after the sixth day of incubation. These results suggest that is strongly recommendable to refresh 'Albariño' cell suspensions every week to provide necessary nutrients for an optimal cell division as well as to keep suspensions in optimal conditions for efficient gene transfer experiments. To our knowledge, this is the first report of a comparative study on transformation efficiency using grapevine suspensions at different time periods of a continuous culture. In a previous work, Vidal *et al.* (2003) obtained a higher number of transient transformants using cell suspensions of 'Chardonnay' cultured during a 7-d period. In a more recent work with 'Albariño', Vidal *et al.* (2009) obtained a similar number of blue spots for 7-d culture time. Differences in transient transgenic events within grapevine could be due to use different genotypes, embryogenic lines and the age and/or the physiological stage of the cell suspensions.

Withdrawal of the auxin in the regeneration solid medium appears to be sufficient to promote embryo development, as reported in other grape cultivars (Coutos-Thévenot *et al.* 1992, Jayasankar *et al.* 1999). Most of the embryos with and without radicle germinated, but only those with radicle evolved to plantlets with a significant percentage of regeneration (60 %). Using our procedure plants were regenerated from cell suspensions in only 5 months (one month from initiation to establishment of cell suspensions, two months from incubation of suspensions to embryo emergence and two months from embryo incubation to plantlets regeneration). This result is an important advance to shorten the period required for plant regeneration from cell suspension of *Vitis vinifera* cultivars, as normally a longer period of incubation is necessary to develop plantlets from cell suspension (Ben Amar *et al.* 2007, Vidal *et al.* 2009). In previous works, the percentage of plant regeneration of different grapevine cultivars varied between 10 and 85 % (Kikkert *et al.* 1996, Jayasankar *et al.* 1999, Ben Amar *et al.* 2007, Vidal *et al.* 2009); however, in these works all embryos were selected with radicle and incubated longer to allow plant regeneration.

To our knowledge, this is the first work on growth kinetics and nutrient uptake in embryogenic cell suspensions of 'Albariño'. A clear relationship among growth phase, nutrient availability, transgenic events efficiency and plant regeneration from 'Albariño' suspensions is shown. Phosphate was almost exhausted in culture medium at day 6, during the exponential phase. In conclusion, refreshing cell suspensions on a weekly basis is suggested to provide nutrients for cell division as well as to obtain efficient gene transfer and expression. Moreover, based on our data, the use of cell suspensions between 3 to 6 d after subculturing for efficient genetic transformation followed of plant regeneration is recommended.

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