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Research Note

An efficient buds culture method for the regeneration via somatic embryogenesis of table grapes 'Red Globe' and 'Flame Seedless'

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K e y w o r d s : Table grapes, somatic embryogenesis, growth regulators, genetic transformation.

A b b r e v i a t i o n s : NOA = 2-naphthoxiacetic acid; NAA = α-naphthalene acetic acid; BA = 6-benzyladenine; 2,4-D = 2,4-dichlorphenoxyacetic acid; IBA = indolbutyric acid; SE, somatic embryogenesis.

Introduction: Chile is the leading table grape exporter from the Southern Hemisphere and this industry is mainly based on 'Thompson Seedless', 'Red Globe' and 'Flame Seedless' ('Flame'), covering more than 75 % of the table grape planted area in the country. With the aim of improving the productivity and quality of these and other cultivars, a genetic transformation program was initiated by 1999, mainly focused in the introduction of genes related to biotic stress tolerance (Hinrichsen *et al*., 2005). With this goal in mind, somatic embryogenesis (SE) was settled for these cultivars, as this has become the most efficient procedure for the generation of *in vitro* cultures prone to genetic transformation (Stamp and Meredith 1988, Scorza *et al*. 1996, Martinelli *et al*. 2001, Iocco *et al*. 2001, Torregrosa *et al*. 2002). However, grapevine SE is not a routine procedure that can be easily and efficiently reproduced in different cultivars (Martinelli *et al.* 2001). There exists a genotype-specific response even when using the same culture medium in the induction process (Srinivasan and Mullins 1980, Stamp and Meredith 1988, Torregrosa *et al.* 2002). In this regard, most of the advances published on SE has been focused on wine cultivars (Stamp and Meredith 1988, Iocco *et al*. 2001) as well as in 'Thompson Seedless' (Scorza *et al*. 1996). Very few additional work has been directed to optimize SE in other table grape cultivars such as 'Red Globe' (Perl *et al.* 1996) and 'Flame Seedless'. In the case of 'Flame', there are no reports specifically related to the optimization of the production of somatic embryos. Taking these antecedents into consideration, the purpose of this note is to describe the best combination of growth regulators for these two table grape cultivars, in order to optimize the induction of somatic embryos.

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Material and Methods: Apical and axial buds with 2 to 4 leaves were cut from *in vitro* grown plants of 'Red Globe' and 'Flame'. Plants were grown in C2D medium (Cheé and Pool 1987) supplemented with 4 µM 6-benzyladenine (BA) to induce multiple budding, in a period of about 30 d. Buds were excised using a stereoscopic lens and later incubated for callus induction in a modified basal medium [NB® (*Phyto*Technology Labs., Shawnee Mission, KS) plus 3 % sucrose, 1 $g \cdot l^{-1}$ myoinositol and 0.7 % (p/v) TC-agar® (*Phyto*Technology) and 5 µM 2,4-dichlorphenoxyacetic acid (2,4-D)] plus a range of concentrations of α-naphthalene acetic acid (NAA) and BA (see Table for specific conditions). Media were adjusted to pH 5.8 and sterilized by autoclaving at 121°C for 20 min before pouring in 90 x 15 mm Petri dishes. Five explants per plate were incubated for 30 d in darkness at 24 ± 2 °C. After this time, calli formed were kept at 16 h light/8 h darkness, at the same temperature for 150 d.

T a b l e

Concentrations and combinations of growth regulators added to the modified NN medium for the induction of somatic embryogenesis from apical and axilar buds of the 'Red Globe' and 'Flame Seedless' cultivars

Medium	Concentration of growth regulators (µM)		
	2,4 – D	BA	NAA
NB1	5	1	---
NB2	5	5	---
NB3	5	2.5	2.5
NB4	5	2.5	5
NB5	5	5	5

S o m a t i c e m b r y o i n d u c t i o n a n d d e v e l o p m e n t : Pro-embryogenic masses were transferred to X6 medium (Li *et al.* 2001) and kept at 24 ± 2 °C under the same photoperiod for 90 d. Somatic embryos were picked up from masses according to their developmental stage and maintained on the same medium. To induce their development, embryos at cotyledonary stage were transferred to C2D medium supplemented with 0.4 µM BA, 0.25 µM indolbutyric acid (IBA), 2 $g \cdot l^{-1}$ glycine and 0.5 $g \cdot l^{-1}$ activated charcoal and kept under the same conditions of temperature and photoperiod for 20 d. The total time involved in the whole induction and development of embryos was at least 110 d.

S t a t i s t i c a l a n a l y s i s : A completely randomized design with sub sampling was used. The experimental unit was conformed by five bud explants maintained in a single Petri dish, and each treatment consisted of 20 replicates. To stabilize the variance, data were transformed according to $\sqrt{(x + 0.5)}$. Means were separated using the Duncan multiple test ($p = 0.01$).

Results and Discussion: Most commonly used explant and growth regulator combinations for the induction of SE on grapes include the use of leaves and anthers and low doses of cytokinins plus high doses of auxins, such as medium NB1 (Table) (Srinivasan and Mullins 1980, Stamp

and Meredith 1988, Torregrosa *et al.* 2002). Although NB1 has led to the induction of somatic embryogenesis in 'Thompson Seedless' (Scorza *et al.* 1996), its effectiveness in the cultivars 'Red Globe' and 'Flame' produced too low or null yielding, respectively.

As a first approach to the induction of SE in these cultivars, the use of stamens and buds as explants was conducted. Preliminary results showed that stamens never produced embryogenic calli from 'Flame' and very rarely in 'Red Globe' (less than 0.5 % yield; data not shown), even when using NOA plus BA, as it is described for other cultivars (Stamp and Meredith 1988). In the same line, results using young leaves as explants were also deficient in their ability to induce SE.

Because of this, next trials were carried out on axial and apical buds from *in vitro* plantlets, and testing them to different combinations of NAA and BA, with a constant 2,4-D concentration (5 µM; Table). Although the different treatments produced very diverse results, successful media leading to SE yielded at least 25 % more embryo cells in 'Red Globe' than in 'Flame'. (Figure). For both cultivars, the best result was obtained with NB4, that combined 2.5 µM BA plus 5 µM NAA. In every case, globular embryos appeared by budding on the surface of embryogenic calli and later gradually emerged; embryogenic cells were of large size and heart-shaped, characteristics of this stage. By the opposite, when using an equimolar proportion of 2,4-D and BA (medium NB2, 5 µM each), explants of both cultivars became necrotic within three weeks of culture. However, if this hormonal condition was supplemented with another auxin (5 µM NAA; medium NB5), embryogenic response was 10 % and 2 % for 'Red Globe' and 'Flame', respectively (Figure). Reasons for this behaviour are unknown, but most probably it reflects the required balance between auxins and cytokinins and their known effect in terms of cell division *vs.* ellongation, and so, when BA is higher than 2.5 µM, cell division could be affected in these cultivars. A more careful comparison between treatments reveals that in the case of 'Red Globe' medium NB4 was statistically similar to NB3 but superior to NB1 and NB5, these last two with a different level of response in SE.

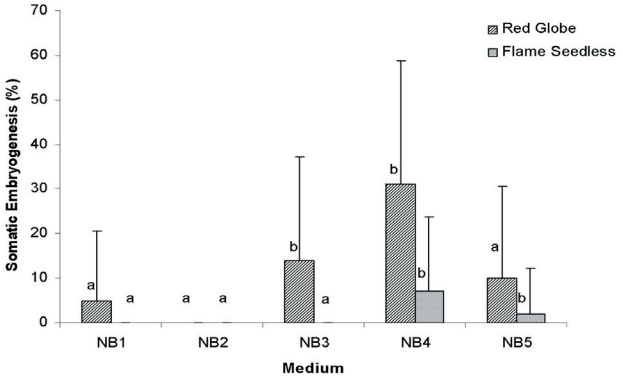

Figure: Induction rate (%) of somatic embryogenesis from apical and axilar buds of 'Red Globe' (diagonal stripes) and 'Flame Seedless' (dots) using different growth regulator concentrations. Medium NB1 serves as "Control" (see Table). Differences in variation coefficients are shown. Same letters means no significant differences between media.

Something similar was observed for 'Flame', but in its case it was required a higher concentration of NAA (5 µM) to observe any response in SE (medium NB4 and NB5). High concentration of BA (5 µM) had a negative effect on both cultivars, reducing to one third SE induction, from 7 % to 2 % in 'Flame' and from 31 % to 10 % in 'Red Globe'. According to these results, the best combination of auxins and cytokinins to induce embryogenic callus from 'Red Globe' and 'Flame' buds as explants was 2.5 µM BA and 5 µM NAA, maintaining 2,4-D at 5 µM (medium NB4), achieving 31 % and 7 % for 'Red Globe' and 'Flame', respectively (Figure). Elongation of embryos was considered normal in the transition from heart-shaped to the torpedo shape and then to advanced developmental stages, with radicle emergence and whole plant formation under light conditions, with the described photoperiod.

In conclusion, there is a positive correlation between response to SE and total concentration of auxins, with an optimum of 7.5-10 µM. It was also evident that BA was deleterious when raising to 5 µM, what had already been described by Srinivasan and Mullins (1980). At the same time, it was confirmed there is a genotype-specific response to SE, Red Globe giving a better yield of SE under every medium assayed. Finally, plantlets regenerated from embryos of both cultivars had a normal appearance, remaining to be evaluated their agronomic performance, to be tested during next seasons.

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