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Toxic effect of antibiotics in grapevine (*Vitis vinifera* 'Albariño') for embryo emergence and transgenic plant regeneration from embryogenic cell suspension

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Summary

Regeneration of grapevine (*Vitis vinifera* L.) from embryogenic cultures after gene transfer is traditionally linked to a selection procedure using antibiotic containing media. The neomycin phosphotransferase II (npt-II) and hygromycin phosphotransferase (hpt) genes that confer resistance to the antibiotics kanamycin and hygromycin, respectively, have been the selectable marker genes most frequently used for selection of transgenic grapevines. In this work, the phytotoxic effects on embryo development and plant growth of these two antibiotics were examined in 'Albariño'. Embryogenic cell suspensions were evaluated based on a two-step strategy using untransformed and transformed tissues. The phytotoxic effect was significantly different at 20 mg·L^{-1} (and higher) for kanamycin and at 5 mg·L^{-1} (and higher) for hygromycin. Minimal killing concentrations of kanamycin and hygromycin for 'Albariño' cell suspensions were 50 and 12.5 mg·L^{-1}, respectively. Embryogenic cell suspensions were bombarded using the biolistic system with the construct pBI426, harboring the selectable npt-II gene, and incubated on kanamycin containing media to determine the best inhibitory concentration allowing embryo and shoot development of only transgenic events. Only 20 % of PCR-positive transgenic embryos and 20 % of plant regeneration resulted from embryos emerged on 30 mg·L^{-1}. However, 80 % of PCR-positive transgenic embryos but only 10 % of plant regeneration were obtained from embryos emerged on 40 mg·L^{-1}. The method described, based in untransformed and transformed plant material, could be used to determine the optimal antibiotic concentration for other *V. vinifera* cultivars for efficient selection and regeneration of transgenic events.

K e y w o r d s : Antibiotic selection, biolistics, embryogenic cell suspensions, genetic transformation, hygromycin, kanamycin.

Introduction

Genetic transformation of grapevine (*Vitis sp.*) focused on regeneration of transgenic plants with improved traits has been achieved using the *Agrobacterium*-mediated method (Bouquet *et al.* 2006) and the biolistic system (Kikkert *et al.* 2004). With both methods, recombinant DNA with the gene of interest is usually transferred to embryogenic cell suspensions, which are initiated from embryogenic calli (Kikkert *et al.* 2005, Martinelli and Gribaudo 2009) and are considered to be the optimal target plant material for gene transfer to grapevine (Jayasankar *et al.* 1999). The gene of interest is usually linked or co-transferred with a selectable marker gene for the selection procedure of transgenic events, mainly on antibiotic containing media (Colova-Tsolova *et al.* 2009).

The neomycin phosphotransferase II (*npt*-II) gene that confers resistance to the aminoglycoside antibiotic kanamycin has been the selectable gene most frequently used in grapevine (Martinelli and Mandolino 1994, Mauro *et al.* 1995, Franks *et al.* 1998, Bouquet *et al.* 2008); the hygromycin phosphotransferase (*hpt*) gene that confers resistance to the aminoglycoside antibiotic hygromycin has been used to a lesser extent (Le Gall *et al.* 1994, Perl *et al.* 1996). In plants, including grapevine, different genotypes within the same species usually show a different response to antibiotics. In general, grapevine is highly sensitive to antibiotics. Péros *et al.* (1998) reported different effects of kanamycin and hygromycin on growth inhibition and necrosis; compared to hygromycin, that kills grapevine plantlets at very low selection regimes, kanamycin has less toxic effects. It was suggested that approaching regeneration of transgenic plantlets is a compromise that has to be found between effective selection of transformed cells and the inhibitory effect on embryo development (Mullins *et al.* 1990). A high antibiotic concentration could kill or damage cells and a low one could give rise to many escapes.

Given the toxic effects of antibiotics on grapevine cells, other selectable marker genes such as the phosphomannose-isomerase (*pmi*) gene (Kieffer *et al.* 2004) and the phosphinothricin amino-transferase (*pat*) gene were assayed on grapevine cell suspensions without success (Reustle and Buchholz 2009). Therefore, nowadays, resistance to antibiotics is still the main strategy for selection of transgenic events and plant regeneration after genetic transformation of grapevine cell suspensions, via either *Agrobacterium* or the biolistics system (Vidal *et al.* 2010). Moreover, a recent statement of the European Food Safety Authority (EFSA 2009) indicates that adverse effects on human health and the environment associated with use of genetically modified plants containing the *npt*-II gene are unlikely, in addition to previous statements of the U.S. Food and Drug Administration regulatory authority (U.S. FDA 1998).

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Previous studies on sensitivity of grapevine to antibiotics by using axillary buds as plant material revealed a high phytotoxic effect of kanamycin and hygromycin on root and stem length killing plants at low-pressure regimes (Péros *et al.* 1998). Although embryogenic cultures are much less sensitive to antibiotics than axillary buds, the need persists to adequately balance the selection requirement against the inhibitory effects of the antibiotic on shoot development (Torregrosa *et al.* 2000). The same authors pointed out that the response of non-transformed tissue could be a starting point to determine the optimal selection level.

In this paper, two antibiotics (kanamycin and hygromycin) were assayed to determine the minimal killing concentration that inhibits embryo emergence from untransformed embryogenic cell suspensions of the Spanish cultivar *Vitis vinifera* 'Albariño' (Vidal *et al.* 1998, 1999). Also, we report a precise determination of the best level of kanamycin to be applied, allowing embryo emergence of only transgenic events from embryogenic cell suspension after biolistics. Inhibitory concentration was evaluated by PCR screening of Albariño embryos emerged on different selection levels after particle bombardment of cell suspensions with the pBI426 construct containing the *npt*-II gene.

Material and Methods

Embryogenic cell suspensions and culture media: Pro-embryogenic calli of *Vitis vinifera* 'Albariño' were induced on semi-solid media from floral explants as previously described (Vidal *et al.* 2009). Embryogenic cell suspensions were initiated and established from embryogenic calli and maintained over time by refreshing on a weekly basis as described in De la Torre *et al.* (2012). Cell suspensions were filtered and quantified, and cell density was adjusted to 0.1 of packed cell volume (PCV) prior to plating on filter paper support and then transferred to MS/2CA selection medium [hormone-free half-strength Murashige and Skoog (1962) with 30 $g \cdot L^{-1}$ of sucrose, 3 $g \cdot L^{-1}$ of activated charcoal, 7 $g \cdot L^{-1}$ Bacto-agar (Difco) and the selectable agent] or PAGAR bombardment medium [Paper-Agar containing hormone-free half-strength MS with 0.125 M mannitol, 0.125 M sorbitol, 30 $g \cdot L^{-1}$ sucrose and 2.5 $g \cdot L^{-1}$ Phytagel (Sigma)] as described elsewhere (Kikkert *et al.* 2004). Some embryos emerged on selective media were transferred to MS/2Phy germination medium [MS/2CA with 2.5 $g \cdot L^{-1}$ Phytagel instead of Bacto-agar and without selection] and incubated as described in Kikkert *et al.* (2004).

Antibiotic sensitivity of untransformed cell suspension: The toxic effect of kanamycin and hygromycin on embryogenic cell suspensions of 'Albariño' was determined. Cell suspensions (5 mL at 0.1 of PCV) from independent lines (originated from separated floral explants of the same plant or different clones) that were plated on filter paper support were incubated on 10 different concentrations of kanamycin (0, 10, 20, 30, 40, 50, 60, 70, 80, and 100 $mg \cdot L^{-1}$) or hygromycin (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 and 25 $mg \cdot L^{-1}$) in selection media (Table). Five plates per antibiotic concentration and culture line were assayed. Plates containing cell suspensions were incubated at 27ºC during 3 months. The average number of emerged embryos was determined for each antibiotic concentration during this 3-month period. Every month, emerging embryos (≥5 mm) were counted and filter

Table

Antibiotic toxicity: Grapevine untransformed embryos (≥ 5 mm) emerged on selection media during a three-month incubation

Embryogenic cell suspension line[1)]			Number of embryos[3)] emerged per Petri plate on increased concentration ($mg \cdot L^{-1}$) of antibiotic								
	Calli Induction[2)]	Biomass Growth	Kanamycin								
			0	10	20	30	40	50	60	70	80
Albariño 6	A.II.NN	Slow	99 ± 17	112 ± 23	110 ± 27	10 ± 0.9*	0.3 ± 0.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Albariño 10	A.II.NN	Slow	94 ± 12	60 ± 12	53 ± 15	6.2 ± 3.2*	0.2 ± 0.2	0 ± 0	0 ± 0	0 ± 0	Nt
Albariño 14	A.II.NN	Slow	81 ± 16	61 ± 19	21 ± 10*	5.6 ± 2.1	0.2 ± 0.2	0 ± 0	0 ± 0	0 ± 0	Nt
Albariño 25	A.II.NN	Fast	256 ± 38#	189 ± 40	61 ± 12*	5.8 ± 2.0	4.8 ± 2.4	0 ± 0	0 ± 0	0 ± 0	Nt
Albariño 26	A.II.NN	Fast	169 ± 47	80 ± 18	56 ± 2.5	11 ± 5.0*	0 ± 0	0 ± 0	Nt	Nt	Nt
			Hygromycin								
			0	2,5	5	7,5	10	12,5	15	17,5	20
Albariño 5	A.II.MS	Slow	110 ± 50	122 ± 38	103 ± 12	20 ± 2.5*	4.4 ± 1.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Albariño 25	A.II.NN	Fast	315 ± 53#	283 ± 60	60 ± 11*	1 ± 0.4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	Nt
Albariño 26	A.II.NN	Fast	154 ± 25	216 ± 31	109 ± 13	11 ± 1.5*	8.2 ± 2	0 ± 0	0 ± 0	0 ± 0	Nt

[1)] Cell suspension lines showing slow or fast biomass growth in liquid medium during a week period. Suspension with slow growth were only tested in an antibiotic-containing medium either kanamycin or hygromycin. Each line was assayed at least twice. Data from the most representative experiment are shown.

[2)] Induction of the calli that were used as inoculum to initiate embryogenic cell suspensions. Floral explant (Anther [A]), floral stage (II) and induction medium MS (Murashige and Skoog) and NN (Nitsch and Nitsch) as reported in Vidal *et al.* (2009).

[3)] Data are average +/- standard error of embryos emerged on filter paper supports on selection media. The symbol * indicates that this group at x $mg \cdot L^{-1}$ antibiotic concentration and higher showed a significant difference (Dunnett´s Method, $P < 0.05$) compared to the control group (antibiotic free media). The symbol # indicates that this 'Albariño' line showed a significant difference (Student-Newman-Keuls Method, $P < 0.05$) among other 'Albariño' lines for the control group (antibiotic-free medium).

Nt means not tested.

paper supports were transferred to fresh selection medium. During the first experiments (Table 1), it was found that no embryo emerged in cell suspensions of Albariño (line 6) at 80 mg·L^{-1} of kanamycin (and higher) and of Albariño (line 5) at 20 mg·L^{-1} of hygromycin (and higher); therefore, these concentrations were avoided in subsequent experiments. Data were analyzed and compared using SigmaStat statistical software version 3.0 (Systat Softwsare Inc).

Gene transfer and embryo emergence under selection pressure: The effect of kanamycin concentration on transgenic events selection was evaluated. 'Albariño' cell suspensions (line 14) were bombarded using the PDS-1000/He biolistic device (BioRad). Plating of cell suspensions on filter paper support, coating of gold particles with plasmid DNA, particle bombardment and GUS assays were performed as described previously (Kikkert *et al.* 2004). An experiment was designed to compare the selection pressure efficiency between non-bombarded (NB) and bombarded cell suspensions with two separated constructs. Plasmid pBI221 (Clontech®), containing the reporter *uid*A (β-glucuronidase, *gus*) gene [under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (nos) terminator from *Agrobacterium tumefaciens*] and without selectable marker gene, was used during the bombardment procedure as a control of cell damage (mainly due to helium shock wave and vacuum pressure), to ensure that the effects on development and regeneration were caused by the selection regime and not by the transformation technique. Plasmid pBI426 (Kikkert *et al.* 1996), containing the reporter *uid*A gene fused to the selectable *npt*-II gene [under the control of a double CaMV 35S promoter, the alfalfa mosaic virus leader sequence and the nos polyadenylation sequence], was used for selection on increasing kanamycin pressure. A total of 45 filter paper supports containing cells suspensions (15 per treatment: non-bombarded (NB), pBI221 and pBI426) were prepared. After bombardment with pBI221 or pBI426, both NB and bombarded cell suspensions were randomly incubated in four kanamycin concentrations (10, 20, 30 and 40 mg·L^{-1}) in addition to kanamycin-free medium. A total of 3 plates per treatment and concentration were evaluated and the experiment was repeated twice. Plates were incubated at 27 °C. Every month during a 3-month period, emerged embryos (≥ 5 mm) were counted and either stored at -20 °C until their use for DNA extraction, or transferred to antibiotic-free MS/2CA medium for plant regeneration (Kikkert *et al.* 2004).

Testing of putative transgenic embryos of Albariño: Genomic DNA was isolated from emerged embryos and screened by PCR for positive signals of the exogenous *npt*-II gene. DNA extraction was carried out using the DNeasy Plant Mini Kit® (Qiagen, Spain). The PCR reaction (25 µl) contained 22 µl of ReddyMix PCR MasterMix 1.1X (ThermoScientific, Spain), 2 µl of genomic DNA (2.5 ng/µl), and 0.5 µl of each primer (10 µM). The primers used for amplification of a 750-bp fragment of the *npt*-II gene were 5'-CAAGATGGATTGCACGCAGG-3' and 5'-TAGAAGGCGATGCGCTGCGA-3'. PCR amplification was carried out in a Mastercycler Pro S (Eppendorf, Spain) thermal cycler, which was programmed for one initial step of 7 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1.5 min at 72 °C, and finally 10 min at 72 °C. PCR products were separated by electrophoresis in 1 % (w/v) agarose gels containing ethidium bromide. Profiles were recorded with a Quantum ST4 camera and program (Vilber Lourmat, France).

Results

Toxic effect of antibiotics on embryo emergence from cell suspensions: All cell suspension lines assayed had a fine to medium clump size (visual observation) after filtering (see material and methods) prior to toxicity experiments. The emergence and development of embryos occurred mainly during the second and third month of incubation, both on antibiotic-free medium and on selection medium. The toxic effect of different antibiotic concentrations on embryos emergence was determined (Table). The analysis of variance (Dunnett´s Test) within each 'Albariño' line showed that cell suspensions incubated on kanamycin-containing medium at 20-30 mg·L^{-1} and higher significantly ($P < 0.05$) reduced the emergence of embryos as compared with the control group on kanamycin-free medium (Table). The ANOVA (Student-Newman-Keuls Test) among 'Albariño' lines for the control group (kanamycin-free medium) showed that line 25 developed more embryos and differs significantly ($P = 0.003$) from the other lines. In some cases, more embryos emerged on low antibiotic pressure than in antibiotic-free medium due to saturation and competence effects (Table). In cell suspensions of 'Albariño' incubated on 50 mg·L^{-1} of kanamycin, the emergence of embryos was completely inhibited during the three months of culture, but this antibiotic concentration did not completely kill all cells or clusters (Fig. 1a). Depending on the 'Albariño' cell line, 30 or 40 mg·L^{-1} of kanamycin was the maximum concentration that allowed the emergence of some embryos. Necrosis started at 20 mg·L^{-1} of kamamycin, increasing gradually from mild to severe at 80-100 mg·L^{-1}. When compared with kanamycin, the toxic effect of hygromycin on the emergence of Albariño embryos was more intense (Table). The analysis of variance (Dunnett´s Test) within each 'Albariño' line showed that cell suspensions incubated on hygromycin-containing medium at 5-7.5 mg·L^{-1} and higher significantly ($P < 0.05$) reduced the emergence of embryos as compared with the control group on hygromycin-free medium (Table). The ANOVA (Student-Newman-Keuls Test) among Albariño lines for the control group (hygromycin-free medium) showed again that line 25 developed more embryos and was significantly different ($P = 0.003$) from the other lines. The minimal hygromycin concentration that inhibited the emergence of Albariño embryos from cell suspensions was 12.5 mg·L^{-1}; emergence of some embryos was observed at 10 mg·L^{-1} of hygromycin during the three months of incubation on selection medium. Higher hygromycin concentrations completely killed cell suspensions. A significant necrosis at 5 mg·L^{-1} and higher was observed.

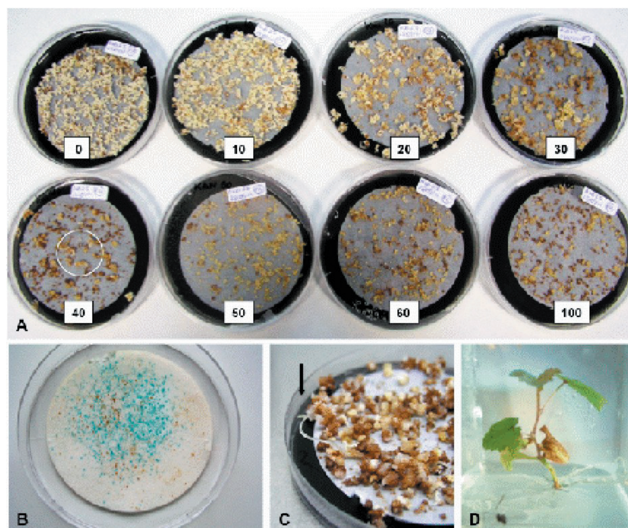

Fig. 1: Embryo development and plant regeneration from embryogenic cell suspensions. Inhibitory concentration of kanamycin for emergence of 'Albariño' embryos derived from competent embryogenic cell suspensions: (**a**) Maximum antibiotic concentration (mg·L^{-1}) allowing emergence of non-transgenic embryos as determined on kanamycin-containing media with increases of 10 mg·L^{-1} at three months of incubation (see Material and Methods). The circle shows emergence of some embryos on 40 mg·L^{-1} of kanamycin-containing medium. (**b**) Competence of cell suspensions for gene transfer based on GUS assay (blue spots) 2 d after bombardment of cells with pBI426. (**c**) The arrow points out the emergence and development of a putative transgenic embryo on 40 mg·L^{-1} kanamycin-containing medium. (**d**) Shoot development of PCR-positive plantlet selected on 40 mg·L^{-1} kanamycin inhibitory medium.

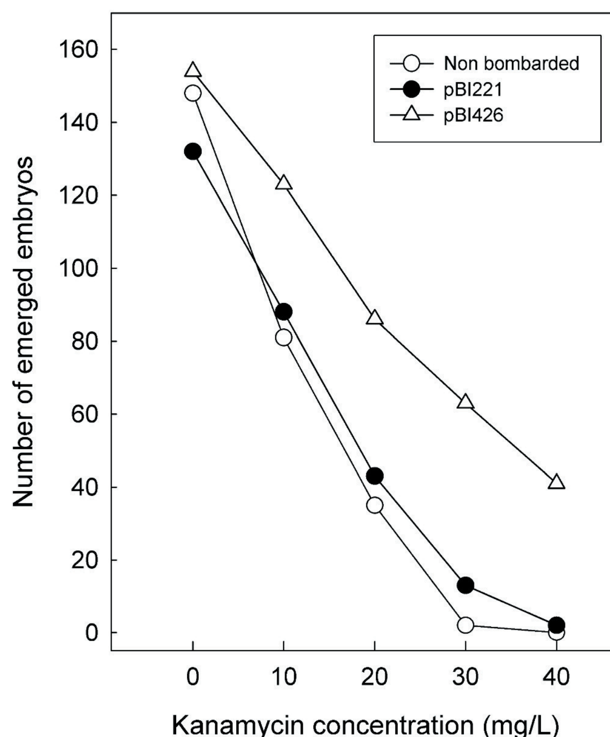

Fig. 2: Emerged embryos on different kanamycin concentrations. Number of embryos emerged from 'Albariño' cell suspensions (line 14) incubated on five concentrations (mg·L^{-1}) of kanamycin-containing selection medium. Data from non-bombarded and bombarded cell suspensions with two separated constructs, pBI221 [harbouring the *uid*A gen (without selectable gene)] or pBI426 [harbouring the *uid*A/*npt*-II gene fusion] of the most representative experiment are represented. Graphic representation for each treatment is the total number of emerged embryos per kanamycin concentration.

Efficiency of kanamycin pressure on embryo emergence from transformed cell suspensions: The toxic effect of kanamycin on cell suspensions bombarded with pBI221 or pBI426 was evaluated by incubating Albariño cells on four antibiotic concentrations in the range of 10 to 40 mg·L^{-1}. Non-bombarded cells were incubated as control (Fig. 2). Additional cell suspensions were also bombarded with pBI426 and two days later were evaluated for GUS expression as a measure of transient transformation efficiency; an average of 3645 ± 958 blue spots per filter paper were obtained (Fig. 1b). The number of emerged embryos decreased with the increment of kanamycin concentration during the three months of incubation on selection media (Fig. 2), in agreement with the data obtained previously and showed in the Table. For each kanamycin concentration, the number of embryos emerged from suspensions bombarded with pBI221 (without selectable marker gene) and from NB suspensions was similar. As expected, the emergence of embryos from suspensions bombarded with pBI426 (with selectable *npt*-II gene) was higher. A total of 63 and 41 embryos emerged from pBI426-bombarded suspensions compared to 13 and 2 embryos emerged from pBI221-bombarded suspensions incubated on 30 and 40 mg·L^{-1} kanamycin containing medium, respectively, were obtained (Fig. 2). Of the total embryos emerged on 30 and 40 mg·L^{-1} kanamycin-containing medium after pBI426 bombardment, 30 embryos from each concentration were randomly selected and grounded for obtaining genomic DNA to determine the efficiency of the selection pressure on the emergence of transgenic embryos. The screening by PCR of the selectable *npt*-II gene resulted in 80 % (24 out 30 embryos) of positive signals of the expected length from embryos emerged on 40 mg·L^{-1} of kanamycin and only 20 % from embryos emerged on 30 mg·L^{-1} of kanamycin. Other embryos (33 and 11 embryos emerged from 30 and 40 mg·L^{-1} of kanamycin, respectively) were saved (Fig. 1c) and transferred into MS/2Phy regeneration medium to determine the correlation of selection pressure on shoot development (Fig. 1d). The conversion rate of emerged embryos into plantlets was 20 % from embryos emerged on 30 mg·L^{-1}, and less than 10 % from embryos emerged on 40 mg·L^{-1}.

Discussion

Selection is a crucial step for successful regeneration of transgenic grapevine plants after gene transfer experiments and has been a major bottleneck for the application of this biotechnology to *Vitis* sp. (BOUQUET *et al.* 2008).

Very few assays on the toxic effect of antibiotics on grapevine growth are available in the literature. A different killing effect between kanamycin and hygromycin (Torregrosa *et al.* 2000) and different toxicity for each antibiotic among few grapevine cultivars (Colby and Meredith 1990, Péros *et al.* 1998, Wang *et al.* 2005) were reported. Here we evaluated for first time the sensitivity of 'Albariño' cultivar to these two antibiotics based on untransformed and transformed tissues. As expected, it was demonstrated that hygromycin has a more toxic effect than kanamycin on embryogenic cell suspensions of this cultivar, in agreement with previous studies with other *V. vinifera* cultivars (Reustle and Buchholz 2009).

We focused our study in the emergence of embryos (≥ 5 mm) on a range of antibiotic concentrations from untransformed and transformed embryogenic cell suspensions of 'Albariño'. The higher emergence of embryos from line 25 when compared to lines 10 y 14 **(**Table**)** could be due to the different biomass growth of the cell suspensions (De la Torre *et al.* 2012) as well as size and physiologic stage of the suspension grain, as previously reported in 'Chardonnay' (Vidal *et al.* 2003). High density of cell suspensions has been reported to inhibit embryo development in many plant species including *V. vinifera* (Wang *et al.* 2005). This phenomenon was also observed in our lab, as occasionally more embryos emerged on 10 $mg \cdot L^{-1}$ kanamycin-containing medium than in antibiotic-free medium **(**Table**)**.

In previous studies, the antibiotic concentration ($mg \cdot L^{-1}$) used for selection of putative transgenic events of grapevine varied between 15 to 100 for kanamycin (Franks *et al.* 1998, Vidal *et al.* 2003, Zhijian *et al.* 2008) and between 1 to 25 for hygromycin (Le Gall *et al.* 1994, Perl *et al.* 1996, Torregrosa *et al.* 2000), using mainly embryogenic calli as plant target and *Agrobacterium* as transformation system. However, a previous determination of the optimal antibiotic concentration for efficient selection was not reported. It was suggested that a fast induction of cell death during selection improves transformation efficiency (Perl *et al.* 1998, Wang *et al.* 2005). In our work, hygromycin produced a faster killing effect, accompanied by necrosis on surviving plant material, when compared to kanamycin. Necrosis is not suitable because of the risk of exuding phenolic compounds in the medium (Torregrosa *et al.* 2000). The killing effect of hygromycin on 'Albariño' untransformed suspensions was detected at low concentrations; embryo emergence was inhibited as low as 10 $mg \cdot L^{-1}$.

On the other hand, the minimal kanamycin concentration inhibiting embryo emergence was 40-50 $mg \cdot L^{-1}$ for 'Albariño'. Because of the severe necrosis caused by hygromycin in Albariño cultures, we further studied the effects of four kanamycin concentrations (10 to 40 $mg \cdot L^{-1}$) on transformation efficiency from pBI426-bombarded cell suspensions of 'Albariño'. In this work, the efficiency in the emergence of transgenic Albariño embryos on selection medium after gene transfer was confirmed by PCR from embryos picked at 30 and 40 $mg \cdot L^{-1}$ of kanamycin pressure. Selection was operative at both evaluated concentrations, whereas embryo emergence was compromised at 40 $mg \cdot L^{-1}$ of kanamycin with a very low conversion rate (10 %) from embryo into plants. Previous studies in our lab with embryogenic cell suspensions of 'Albariño' resulted in only a 5 % efficiency of transgenic events from embryos picked at 20 $mg \cdot L^{-1}$ of selection pressure but with a higher conversion rate (29.3 %) from embryos to plantlets (Rama 2009). However, 15 $mg \cdot L^{-1}$ of kanamycin was the optimal selection pressure for transgenic 'Chardonnay' plants, with a 54 % of conversion rate (Vidal *et al.* 2003, 2006). The inhibitory effect of high kanamycin concentration on shoot development was initially reported by Mullins *et al.* (1990). *Torregrosa et al.* (2000) also pointed that the main problem in transforming grapevine is to balance the selection requirement against the inhibitory effects of the antibiotic on shoot development. Therefore, for each genotype, and perhaps for each individual embryogenic line, a preliminary experiment to determine the minimal inhibitory concentration of antibiotic allowing the emergence and development of transgenic embryos is suggested.

Based in our experiments with untransformed and transformed plant material, we could suggest a step-wise procedure of selection pressure for further transformation of 'Albariño' cell suspension. Depending of the 'Albariño' cell line, selection pressure should be initiated at 20 or 30 $mg \cdot L^{-1}$ of kanamycin during the first month and then cell suspensions should be transferred to 30 or 40 $mg \cdot L^{-1}$ of kanamycin during the second month and further. However, selection pressure using hygromycin could be started at 5 $mg \cdot L^{-1}$ during the first month before transferring the cells to 7,5 or 10 $mg \cdot L^{-1}$ of hygromycin during the second month and further. In any case, definitive suggestions for 'Albariño' and antibiotic hygromycin should be confirmed with transformed tissue.

In conclusion, besides providing a selection method for cultivar Albariño, our results confirm the need of a fine tuning of the selection system with a cultivar-specific approach. Antibiotic concentration has to be investigated thoroughly; in 'Albariño', a modest increment in antibiotic concentration on selection media implies a higher percentage of positive embryos but a huge drop in plant regeneration. Thus, a balance between selection and toxicity, to allow transgenic embryo emergence and plant development, has to be precisely defined for each cultivar of grapevine. This two-step strategy based on untransformed and transformed cell suspensions may be applied to determine in a precise way the minimal antibiotic concentration for efficient transgenic selection of other *Vitis vinifera* cultivars.

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