Evaluation of the phosphomannose isomerase-based selection system for gene transfer in grape

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Summary

The suitability of the PMI-based system for efficient gene transfer in grape was assessed on *V. vinifera* 'Brachetto' and 'Chardonnay' and the rootstock '110 Richter'. The effect of mannose on non-transformed tissues was evaluated during a long culture period in the crucial stages of morphogenesis from callus to plantlet. Grape tissues of the genotypes used were affected by mannose as the carbohydrate source, damage, however, appeared after extremely long culture times. In addition, plantlets were regenerated from embryogenic calli after co-culture with *Agrobacterium* LBA 4404 carrying the *manA* gene in the PMI-GUS-Intron plasmid based on the pNOV2819 vector by Syngenta (Positech® system). Plants recovered after selection in the presence of mannose were found to be non-transgenic for the manA gene. Accordingly, PMI seems to be an unsuitable alternative to traditional marker gene selection for successful gene transfer in grape.

K e y   w o r d s : *V. vinifera,* positive selection, mannose, PMI, marker genes.

A b b r e v i a t i o n s : 2,4-D = 2,4-dichlorophenoxyacetic acid; BA = 6-benzyladenide; Fw = forward primer; GUS = β-glucuronidase; IAA = indole-3-acetic acid; NAA = 2-naphtoxyacetic acid; PMI = phosphomannose isomerase; Rv = reverse primer.

Introduction

Successful gene transfer into plants requires effective selection to be made between cells that have inserted the foreign gene and those that have not. Genes coding for antibiotic and herbicide resistance have been the most widely used selection markers so far. However, marker genes have become one of the controversial issues in the debate concerning GMO safety (Martinelli and Marin 2008) and their impact has been the subject of European regulation (European Parliament and Council 2001) and of opinion by the European Food Safety Authority (2004). Thus, alternative strategies are being studied in the development of reliable selection systems (Puchta 2003, Miki and McHugh 2004).

The approach known as "positive selection" is based on marker genes that confer to the transgenic cells a metabolic advantage enhancing their ability to survive in the presence of the selective agent (Wenck and Hansen 2005). Among these genes, the *manA* from *Escherichia coli* coding for the phosphomannose isomerase (PMI) enzyme [EC 5.3.1.8] has been proposed.

Once mannose has been taken up by the plant cells, it is readily phosphorylated to mannose 6-phosphate by the hexokinase enzymes. PMI catalyzes reversible interconversion between mannose 6-phosphate and fructose 6-phosphate, an intermediate of glycolysis (Reed *et al.* 2001). In mannitol-metabolising plants, mannose is an important substrate for carbohydrate translocation and PMI is expressed at very high levels (Stoop *et al.* 1996); in most other species, however, PMI is expressed at very low or undetectable levels (Herold and Lewis 1977). As a result, mannose 6-phosphate accumulates in the cells with lethal consequences, such as the blocking of glycolysis by depletion of fructose 6-phosphate and the sequestration of the phosphates required for ATP synthesis (Weiner *et al.* 1992). Increased levels of mannose 6-phosphate are also reported to affect the glycolysis by inhibiting the phosphoglucose isomerase (Goldsworthy and Street 1965).

Plant cells expressing the *manA* gene are expected to be able to convert effectively mannose 6-phosphate to fructose 6-phosphate. This allows the regular flow of glycolysis and provides this pathway with an increased amount of its substrate.

Safety assessments of the PMI-based selection system have proved that the exogenous expression of the PMI protein in plants is safe for humans and animals (Delaney *et al.* 2008).

The suitability of the *manA* gene as a selection marker has been proved in a wide range of plant species, such as *Arabidopsis thaliana* (Todd and Tague 2001), sugar beet (Joersbo *et al.* 1998), maize (Ahmadabadi *et al.* 2007), rice and golden rice (Datta *et al.* 2003; He *et al.* 2004), orange (Boscariol *et al.* 2003), hemp (Feeney *et al.* 2003), pearl millet (O'Kennedy *et al.* 2004), sorghum (Gao *et al.* 2005), papaya (Zhu *et al.* 2005), cabbage (Ku *et al.* 2006), onion (Aswath *et al.* 2006), wheat (Gadaleta *et al.* 2006), apple (Degenhardt *et al.* 2006), almond (Ramesh *et al.* 2006), cucumber (He *et al.* 2006), flax (Lamblin *et al.* 2007), *Torenia fournieri* (Li *et al.* 2007), sugarcane (Jain *et al.* 2007), tomato and potato (Sigareva *et al.* 2004, Bříza *et al.* 2008).

In grapevine, the gene transfer technique is a crucial tool for functional studies and as a means of improving established cultivars. The process of gene transfer in the *Vitis* genus is mainly based on the co-culture of embryogenic calli or somatic embryos with *Agrobacterium tumefaciens*

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(Martinelli and Mandolino 2001). Successful applications of the biolistic technology have been obtained (Bouquet *et al.* 2008), while agro-infiltration methods (Zottini *et al.* 2008, Santos-Rosa *et al.* 2008) have been developed for the transient expression of exogenes. The selection of transgenic cells is usually based on marker genes conferring resistance to antibiotics (*nptII* – neomycin phosphotransferase II, *hpt* – hygromycin phosphotransferase) or herbicides (*bar* – phosphinothricin acetyltransferase, *tfdA* – 2,4-D monooxygenase), *nptII* being the most widely applied (Bouquet *et al.* 2008).

To obtain marker-free grapevines, the few literature reports on evaluation of strategies based on co-transformation (Dutt *et al.* 2008), marker gene removal (Dalla Costa *et al.* 2009) and positive selection systems, such as PMI (Reustle *et al.* 2003, Kieffer *et al.* 2004) and D-xylose ketol-isomerase (Kieffer *et al.* 2004).

As for the PMI-based selection, Kieffer *et al.* (2004) focused on the evaluation of a preliminary, yet crucial, aspect of the strategy, *i.e.* the cytotoxic effects of mannose on non-transgenic embryogenic cultures of 'Chardonnay'. Results obtained during short-term observation (9 weeks) and restricted to the early stages of morphogenesis highlighted the ability of these cultures to grow in the presence of mannose as unique carbohydrate source.

Reustle and co-workers (2003) transferred the β-glucuronidase (GUS) and the *manA* genes into pro-embryogenic masses of *V. vinifera* 'Merlot', 'Seyval blanc' and *V. berlandieri x riparia* rootstock and assessed different sucrose and mannose ratios. Histochemical GUS assays showed the chimeric or non-transgenic nature of the differentiated embryos, although no molecular analysis of *manA* insertion and expression nor data on plant regeneration were provided. Nevertheless, in order to fully assess the applicability in grape of *manA* as a selection marker, the authors note the need for further optimisations of the mannose-based selection management.

In this framework, our research was aimed to perform an extensive assessment of the applicability of the PMI-based system for the selection of transgenics to be evaluated throughout an already established protocol for gene transfer and plantlet regeneration in grape. The research was conducted on two important *V. vinifera* cultivars ('Brachetto' and 'Chardonnay') and on the rootstock '110 Richter' (*V. berlandieri x rupestris*). First, we focused on the effect of mannose on non-transformed cultures during a long culture period in the crucial stages of morphogenesis from callus to plantlet. Then we performed the gene transfer experiments and the molecular evaluation of the regenerants.

Material and Methods

P l a n t   m a t e r i a l : Embryogenic calli of *Vitis vinifera* 'Brachetto' and 'Chardonnay' and the rootstock '110 Richter' (*V. berlandieri x rupestris),* obtained and cultured as described in Martinelli *et al.* (2001), were employed. Long-term cultures were preserved by alternating the media formulated for callus proliferation (PIV) and for embryo differentiation (GS1CA) every two months (Franks *et al.* 1998). Both media contained NN (Nitsch and Nitsch 1969) major elements, MS (Murashige and Skoog 1962) minor elements and Fe-EDTA, B5 (Gamborg *et al.* 1968) vitamins, 0.1 g·l^{-1} myoinositol, 60 g·l^{-1} sucrose and 0.4 % phytagel. The GS1CA medium was obtained by the addition of 1 μM 6-benzyladenide (BA), 20 μM indole-3-acetic acid (IAA), 10 μM 2-naphtoxyacetic acid (NAA) and 0.25% activated charcoal and pH fix at 6.2. The PIV medium was added with 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 8.9 μM BA and pH adjusted at 5.7. The cultures were kept in the dark at $24 \pm 1°C$ and the media were renewed monthly.

E v a l u a t i o n   o f   t h e   e f f e c t s   o f   m a n n o s e   o n   t h e   p l a n t   m a t e r i a l : The effect of mannose was evaluated in three crucial phases of the morphogenesis, i.e. embryogenic callus proliferation, somatic embryo differentiation, germination and plant micropropagation.

E m b r i o g e n i c   c a l l u s   p r o l i f e r a t i o n : The mannose effect on embryogenic callus proliferation was assessed in the PIV medium where the sucrose was replaced with 40 g·l^{-1} mannose; the positive and the negative controls were propagated on sucrose 60 g·l^{-1} and 0 g·l^{-1} respectively. For each genotype and sugar formulation, 4 replicas were set, each one consisting in a 90 mm Petri dish in which 7 calli of 5 mm diameter were plated. Cultures were maintained in the dark at 24 ± 1 °C for 10 months, monthly subcultured on the same substrate and visually evaluated. During the monthly subcultures, callus proliferation and aspect (colour, consistency and hydration) were qualitatively evaluated.

S o m a t i c   e m b r y o   d i f f e r e n t i a t i o n : The efficiency of somatic embryo regeneration on mannose was assessed on embryogenic calli deprived of visible embryos cultured on the GS1CA medium containing 40 g·l^{-1} mannose. The same substrate containing either sucrose 60 g·l^{-1} or no carbohydrates was used for the positive and negative controls, respectively. For each genotype and sugar formulation, 4 replicas were set, each one consisting in a 90 mm Petri dish containing 7 calli of 5 mm diameter. Cultures were maintained for 10 months in the dark at $24 \pm 1°C$ and monthly subcultured. Starting from the 4th of the monthly subcultures, the number of differentiated embryos was counted every 120 d.

G e r m i n a t i o n   a n d   p l a n t   m i c r o p r o p a g a t i o n : Conversion into plant in the presence of mannose was observed during embryo germination and further rooting and elongation of the plantlets. Somatic embryos at the torpedo stage were collected from the above-described calli cultured for 10 months on GS1CA medium. Embryos were planted at a rate of 10 embryos/plate on germination medium (Martinelli *et al.* 2001) containing either mannose 20 g·l^{-1} or sucrose 15 g·l^{-1}, according to the preceding carbohydrate source. For 'Brachetto', 'Chardonnay' and '110 Richter', 50, 50 and 60 embryos respectively were selected from the calli grown in the presence of mannose. For the positive controls, 40, 52 and 55 embryos, respectively, of 'Brachetto', 'Chardonnay' and '110 Richter' were planted on medium supplemented with sucrose. Further micropropagation was induced by placing the first

emerging shoot of each embryo on NN medium free of growth regulators and supplemented with mannose 20 g l^{-1} or sucrose 15 g·l^{-1}, according to the preceding media. For both steps, cultures were kept at 24 ± 1°C with a 16 h photoperiod, 70 μmol·m^{-2}·s^{-1} cool white lights and the media renewed every 2 months.

G e n e   t r a n s f e r : The *manA* gene from *Escherichia coli* (MILES *et al.* 1984) coding for the phosphomannose isomerase enzyme (PMI) was transferred into the embryogenic cultures *via Agrobacterium tumefaciens* (HOEKEMA *et al.* 1983) LBA 4404 carrying the pNOV2819 plasmid (Syngenta Positech® system, www.positech-marker.com). In this construct, *manA* is under the control of the constitutive CMPS promoter from *Cestrium* Yellow Leaf Curling Virus (STAVOLONE *et al.* 2003) and the NOS terminator from *A. tumefaciens*. This plasmid was modified in the PMI-GUS-Intron plasmid by cloning into the *Hind*III site an expression cassette from p35SGUSint (VANCANNEYT *et al.* 1990) containing the *E. coli* gene for β-glucuronidase (GUS) between the 35-S promoter and terminator from Cauliflower Mosaic Virus. According to our protocol (DALLA COSTA *et al.* 2009), *Agrobacterium tumefaciens* cultures were initiated at 28 °C on LB-Lennox solid medium (Lennox 1955; tryptone 10 g·l^{-1}, NaCl 5 g·l^{-1}, yeast extract 5 g·l^{-1}, MgSO$_4$ 2mM, bactoagar 15 g·l^{-1}, pH 7.0) supplemented with 15 mg·l^{-1} rifampicin, 200 mg·l^{-1} spectinomycin and 200 mg·l^{-1} streptomycin. A single colony was picked, inoculated in the same liquid medium and grown at 28°C shaking continuously at 170 rpm until the suspension reached an OD$_{600}$ of 0.5.

Established embryogenic cultures of 'Chardonnay', 'Brachetto' and '110 Richter' were used in the experiment. Calli were submerged for 5-7 minutes with 10 ml of bacterial suspension in 90 mm sterile Petri dishes, and gently shaken before syphoning off the *Agrobacterium*. Co-culture was performed on PIV medium in the dark at 24 ± 1 °C for 48 h. After 3 rinses in sterile water supplemented with 300 mg·l^{-1} cefotaxime, cultures were dried on sterile filter paper and grown for 1 month on PIV medium supplemented with 300 mg·l^{-1} cefotaxime. Calli were subcultured according FRANKS *et al.* (1998), replacing sucrose with 40 g·l^{-1} mannose. The medium was refreshed monthly, progressively reducing the cefotaxime concentration to 50 mg·l^{-1}. After 1 year, 100 embryos of each genotype at the torpedo stage were planted at a rate of 10 embryos/90 mm Petri dish on germination medium (MARTINELLI *et al.* 2001) with mannose 20 g·l^{-1}. Within 10 months, the first emerging shoot from each embryo was cut and micropropagated on NN medium supplemented with mannose 20 g·l^{-1}. Germination and plant propagation were performed at 24 ± 1 °C with a 16 h photoperiod (70 μmol·m^{-2}·s^{-1} cool white lights) and the medium was refreshed every two months.

M o l e c u l a r   a n a l y s i s : Total genomic DNA was extracted from leaves of putatively transgenic plants according to DOYLE and DOYLE (1990), adding polyvinylpyrrolidone (PVP) 1 g·l^{-1} to the isolation buffer. PCR amplifications were performed in a total reaction volume of 25 μl using the GoTaq® Green Master Mix (Promega). Each reaction contained 100 ng of template DNA, 1 U of Taq DNA polymerase, 200 μM of each dNTP and 1.5 mM

MgCl$_2$. Amplifications were performed in a Biometra Thermal Cycler under the following conditions: a 2 min denaturation step at 95 °C, 30 cycles of [95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min] and a 5 min extension step at 72 °C. The PCR products were stained with Sybr Green (Invitrogen) and observed on 1.5 % agarose gels.

Primer set Fw: 5'- ACAGCCACTCTCCATTC -3' and Rv: 5'- GTTTGCCATCACTTCCAG -3' [Syngenta instructions] was used for the specific amplification of a 532-bp fragment of the *manA* gene. In addition, possible *Agrobacterium* contamination was checked with the primers Fw: 5'-AAAAACAAACTCGCATCCGTCA-3' and Rv: 5'-CTCGGTCGTTTCCATGTTCTTA-3', designed by the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi); these are expected to amplify a 492-bp sequence of the LBA4404 gene *dps* [DQ078256], coding for the decaprenyl diphosphate synthase. Positive control reactions were set using 10 μl of LBA4404 pNOV2819 lysate (95 °C, 5 min) as template. Negative control reactions were prepared using as a template 100 ng of genomic DNA extracted from non-transgenic plants of 'Chardonnay', 'Brachetto' and '110 Richter'. In addition, a blank reaction (bi-distilled sterile water) was analyzed.

Results and Discussion

Numerous studies carried out in various plant species have proved the suitability of the PMI-based strategy for a proper selection of transgenic cells during gene transfer. Very little literature is, however, available on grape (REUSTLE *et al.* 2003, KIEFFER *et al.* 2004) and the results reported so far need to be reliably confirmed. In particular, there are no data concerning long-term evaluation of the mannose effect on grape tissues and no gene transfer experiments leading to molecular analysis of regenerated plants.

Our evaluation has been performed during the gene transfer protocol we are successfully using in our laboratory (DALLA COSTA *et al.* 2009). Our aim was to consider how mannose would effect the main stages of morphogenesis from callus to plant. This meticulous work has been essential for further evaluation of the PMI-based system on plants putatively containing the *manA* exogenous gene, in particular during the selection stage where putatively transgenic from non transgenic tissues have to be discriminated.

E v a l u a t i o n   o f   t h e   e f f e c t s   o f   m a n n o s e   o n   p l a n t   m a t e r i a l : The effect of mannose used instead of sucrose was compared with the standard medium compositions containing sucrose (positive controls) and with the complete lack of sugars (negative controls). In previous experiments, various mannose/sucrose ratios (REUSTLE *et al.* 2003) or 20 g·l^{-1} mannose (KIEFFER *et al.* 2004) were employed. Accordingly we performed preliminary observations (VACCARI *et al.* 2007) where various mannose concentrations as well as their combinations with sucrose (unpublished) have been evaluated, and set up the critical concentrations of this sugar for the present study at 40 g·l^{-1} for the callus cultures and at 20 g·l^{-1} for the embryo and the plant cultures. Worth stress-

ing, compared to literature, both concentrations are higher than those previously tested and thus adequately elevated for a conclusive assessment. Finally, mannose supply was optimised after a realistic evaluation of the maintenance costs of the cultures, in which we considered the extremely high price of mannose and the long-term selection required for an efficient transgenic plant recovery.

E m b r y o g e n i c   c a l l u s   p r o l i f e r a t - i o n: The effect of mannose on embryogenic callus proliferation was observed during 10 month cultures grown on PIV medium supplied with either mannose or sucrose, or sugar free. Cell proliferation, resulting in the production of callus mass, and culture quality – the latter determined in terms of colour, consistency and hydration – were visually estimated.

Positive controls exhibited the growth features usually observed over several years of maintenance in our laboratory, in terms of both quality and quantity. Negative controls turned black and gradually died over 3 months after progressive reduction of proliferation ability. Calli cultured on mannose showed moderate proliferation, browning and reduction of morphogenic masses only after an extended culture period (1 year). However, no clear evidence of a lethal outcome could be unambiguously reported. Moreover, among the three genotypes, differing degrees of sensitivity to mannose were documented, 'Chardonnay' being the most susceptible and '110 Richter' the most tolerant. In Fig. 1, 'Brachetto' cultures have been chosen as the representative case with respect to these findings, being the intermediate example among the three genotypes.

S o m a t i c   e m b r y o   d i f f e r e n t i a t i o n: During the subcultures performed on the differentiation medium (GS1CA), the gradual embryo development became visible (Fig. 2). The persistence of callus morphogenic competence was evaluated over 10 months by considering the quality and quantity of the somatic embryos differentiated on GS1CA medium supplemented with either mannose or sucrose or sugar free. Within a 10 month-period, all the calli grown on mannose and sucrose continued to differentiate somatic embryos, while negative controls produced a negligible amount of embryos and progressively extinguished within 3 months.

The efficiency evaluation was assessed by keeping count of the number of embryogenic events progressively regenerated for 'Chardonnay', 'Brachetto' and '110 Richter' in the presence of sucrose, mannose or in the sugar-free medium. Starting from the 4th month of culture, the production of somatic embryos was scored three times (at 4, 7 and 10 months), by means of the removal with a sterile forceps of all visible embryos. For each genotype, morphogenesis efficiencies were calculated on the mean of the total number of somatic embryos progressively produced per plate replica in the three carbohydrate sources, and were expressed as percentages (Tab. 1).

In the presence of both carbohydrates, all three genotypes tested were found to maintain their morphogenic competence since the calculated efficiencies were high. The highest levels were found in the positive controls, *i.e.* in the presence of sucrose, with '110 Richter' performing best (69 %). Different degrees of sensitivity to the mannose
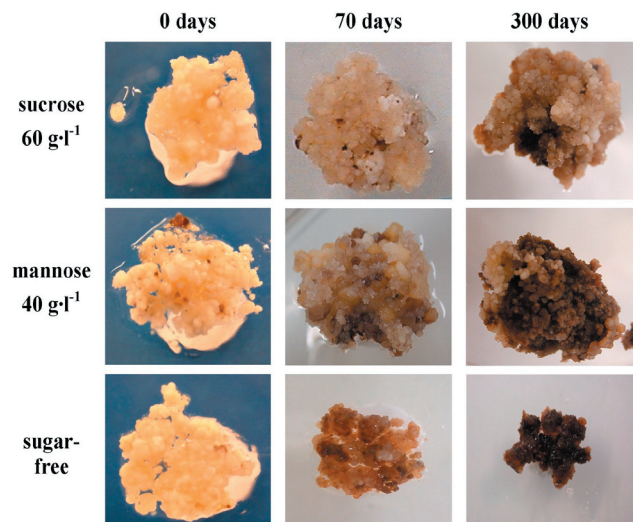

Fig. 1: Effect of carbohydrate source on the proliferation of 'Brachetto' embryogenic calli cultured on PIV medium at 0, 70, and 300 d. The callus cultured on sucrose (positive controls) shows active proliferation and morphogenic masses at early embryogenic stages are visible. In the sugar-free medium (negative controls) cultures died out within 3 months, while in the presence of mannose a moderate proliferation and progressive browning and reduction of morphogenic masses were observed starting from 70 d. Scale: ——— 1 cm.
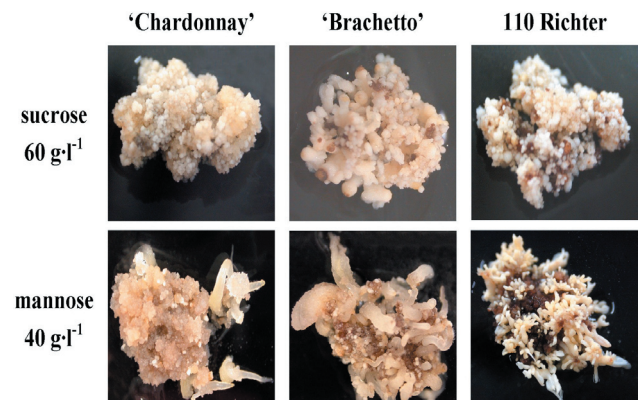

Fig. 2: Effect of mannose on somatic embryo of 'Chardonnay', 'Brachetto' and '110 Richter' induced to differentiate on GS1CA medium for 250 d. In the medium formulated for differentiating and developing somatic embryos, in the presence of both carbohydrates, morphogenic competence is maintained in all genotypes and embryo differentiation at various developmental stages (from globular to cotyledonary embryo) is visible. However, in the mannose-containing medium, while '110 Richter' developed embryos with canonical shape (polarization of the root and shoot axes and the presence of a hypocotyl and two cotyledons), 'Chardonnay' and 'Brachetto' regenerated abnormal somatic embryos with various teratologies (fused cotyledons, giant size and vitrification). Scale: ———1 cm.

were found when the results obtained from the three genotypes were compared, *i.e.* 46 %, 38 % and 30 % respectively for 'Brachetto', 'Chardonnay' and '110 Richter'.

These results are based on a progressive score of the embryos produced within a 10 month period. In addition, interesting observations were made by calculating at 4, 7 and 10 months the mean number of embryos differentiated in the 4 plate replicas set for each genotype cultured

Table 1

Effect of mannose on the morphogenic efficiencies of non-transgenic cultures. Within a 10 month culture, numbers of somatic embryos differentiated were scored for each genotype and sugar treatment (Total embryos N). The mean numbers of somatic embryos produced in 4 plate replicas (N mean) supplemented with either sucrose 60 g·l⁻¹ (positive control), mannose 40 g·l⁻¹ or no sugar (negative control) were calculated for 'Chardonnay', 'Brachetto' and '110 Richter'. During culture progression, at 4, 7 and 10 months, the percentages (%) of embryos produced were calculated out of the mean of the total embryos of each genotype (mean tot.), *i.e.* 2115 for 'Chardonnay', 2004 for 'Brachetto' and 1418 for '110 Richter'

Genotype	Carbohydrate source	Total embryos N	4 months		7 months		10 months	
			N mean	%	N mean	%	N mean	%
Chardonnay	sucrose	4992	288	14	748	35	1248	59
	mannose	3216	290	14	595	28	804	38
	no sugar	256	64	3	64	3	64	3
						mean tot.	2115	
Brachetto	sucrose	4224	136	7	556	28	1056	53
	mannose	3732	188	9	486	24	933	46
	no sugar	60	15	1	15	1	15	1
						mean tot.	2004	
110 Richter	sucrose	388	342	24	478	34	978	69
	mannose	1728	33	2	198	14	432	30
	no sugar	32	8	1	8	1	8	1
						mean tot.	1418	

on mannose-containing medium (Fig. 3). Whilst 'Brachetto' and '110 Richter' showed a positive trend in embryo production, the gradual decrease in embryo number found with 'Chardonnay' confirmed the higher sensitivity to mannose of this genotype.

Moreover, different morphological features were found in the embryos produced in the mannose-containing medium. 'Chardonnay' and 'Brachetto' regenerated somatic embryos with teratologies, such as fused cotyledons, giant size and vitrification, that were already dramatically evident from the torpedo stage (Fig. 2). Conversely, '110 Richter' developed embryos showing the canonical shape, *i.e.* polarization of the root and shoot axes and the presence of a hypocotyl and two cotyledons (MARTINELLI and GRIBAUDO 2009). In addition to this morphogenic capability and despite the lowest efficiency, the embryos of this genotype cultured on mannose were characterized by a faster development compared with its positive control on sucrose (Fig. 2).
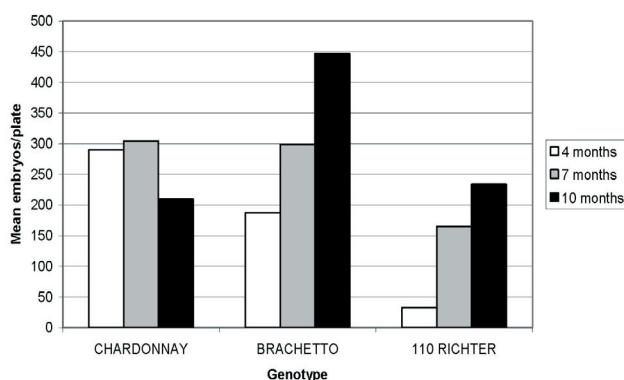

Fig. 3: Numbers of embryos of 'Chardonnay', 'Brachetto' and '110 Richter' differentiated at 4, 7 and 10 months on GS1CA medium containing mannose. The regenerated embryos are reported as mean numbers of 4 plate replicas (Mean embryos/plate).

G e r m i n a t i o n a n d p l a n t m i c r o - p r o p a g a t i o n : The ability of the embryos to convert into plantlets was assessed on individual embryos at the torpedo stage from the GS1CA medium described above, planted on germination medium (MARTINELLI *et al.* 2001) and supplemented with either mannose or sucrose according to the nature of the preceding carbohydrate source. The germination efficiencies were obtained by calculating the percentages of the embryos giving rise to at least one shoot. The percentages of dead embryos were also considered, whilst alive embryos failing to germinate were kept in cultures until the end of the experiment and were not included in these scores (Tab. 2). This first step of embryo conversion into plants was obtained on both mannose- and sucrose-containing medium in all the three genotypes. In the presence of mannose, however, 'Chardonnay' and 'Brachetto' exhibited the lowest efficiencies of first shoot regeneration and the highest percentage of dead embryos, while '110 Richter' was less affected by this sugar.

The ability of the seedlings to develop whole plantlets during micropropagation was also determined by considering root production. The first emerging shoot of each germinating embryo was cut and planted on NN medium containing either mannose or sucrose. For each genotype and carbohydrate source, the efficiencies of plant recovery were calculated as the percentage of rooted plantlets on the plated germinating embryos. Among the three genotypes, only '110 Richter' plantlets proved to be capable of rooting on mannose (Tab. 3).

In this part of the work, we have carried out a long-term evaluation of the mannose effect on the grape tissues of three genotypes during crucial stages of morphogenesis such as embryogenic callus proliferation, embryo regeneration and plant recovery. During the steps leading from callus to plant, the three genotypes reacted differently to mannose, '110 Richter' being the least affected in all the stages

Table 2

Germination efficiencies of non-transgenic and putatively transgenic somatic embryos. The percentages (%) of germinated and dead embryos were calculated out of the total numbers of embryos planted (N) for each genotype and carbohydrate source within a 10-month culture. Some embryos, even if not died out, failed to germinate and their number were not reported in the table, as in the case of the samples labelled with (*)

Genotype	Carbohydrate source	N	Germinated embryos (%)			Dead embryos (%)		
			4 months	7 months	10 months	4 months	7 months	10 months
non-transgenic								
Chardonnay	sucrose	52	15	27	27	38	42	73
	mannose	60	0	0	10	32	62	90
Brachetto	sucrose	40	3	13	18	15	48	82
	mannose	50	0	4	6	40	82	94
110 Richter	sucrose	55	13	24	27 (*)	42	58	82 (*)
	mannose	51	22	43	45	14	33	55
putatively transgenic								
Chardonnay	mannose	100	16	18	18 (*)	53	69	76 (*)
Brachetto	mannose	100	1	1	3 (*)	56	70	84 (*)
110 Richter	mannose	100	32	32	40 (*)	50	58	46 (*)

Table 3

Rooting capabilities of non-transgenic and putatively transgenic germinated embryos. For each genotype and carbohydrate source, the number of germinated embryos (N) and the number of plant-lets rooted (N) from these latter during a 10-months micropropagation is reported

Genotype	Carbohydrate source	Germinated embryos	Rooted plants
		N	N
non-transgenic			
Chardonnay	sucrose	14	7
	mannose	6	0
Brachetto	sucrose	7	4
	mannose	3	0
110 Richter	sucrose	14	5
	mannose	23	11
putatively transgenic			
Chardonnay	mannose	18	8
Brachetto	mannose	3	0
110 Richter	mannose	40	19

considered. Conversely, 'Chardonnay' and 'Brachetto' somatic embryos exhibited teratologies and no plant-rooting capabilities. However, no clear evidence of lethal effect was generally observed in the embryogenic calli grown on mannose, even after long-term exposure (10 months) to the selective substrate.

Although non-transgenic cultures were found able to grow on mannose, we considered that this result may not fully exclude the suitability of the PMI-based selection strategy. For this reason, we felt it necessary to complete the present study with gene transfer experiments with the aim of comparing the behaviour of non-transgenic and putatively transgenic cultures in the presence of mannose as a sugar source and as selective agent as well. Cells with *manA*, in fact, are expected to have a selective advantage over the wild-type tissues due to the expression of PMI.

Thus, regeneration of transgenic plantlets should be expected.

Production and evaluation of putatively transgenic plants: After gene transfer via *Agrobacterium*, the embryogenic calli were subcultured for 12 months according to Franks *et al.* (1998) in the presence of mannose as exclusive carbohydrate source. At least one-year selection on mannose was carried out on the basis of the results obtained during the previously described assays. Proliferation of the putative transgenic cultures was found to be quantitatively and qualitatively very similar to the non-transformed ones (not shown). Further somatic embryo germination and conversion into plants was obtained following persistent selection on mannose-containing media, according to the plant regeneration protocol adopted for the non-genetically modified cultures.

Putatively transgenic embryos at the torpedo stage were then set on mannose-containing regeneration medium for germination induction. The three genotypes showed different efficiencies, germinated embryos being 18 %, 3 % and 40 % respectively of the embryos planted from 'Chardonnay', 'Brachetto' and '110 Richter' (100 plated embryos per genotype) (Tab. 2).

The first shoot produced from these embryos was planted on NN medium containing mannose. While no plants were obtained from 'Brachetto', both 'Chardonnay' and '110 Richter' somatic embryos converted into plants with high efficiencies (Tab. 3) originating respectively 8 and 18 potentially distinct lines, which were used for the PCR assays.

The primer sets designed for amplifying the *manA* gene and the *Agrobacterium dps* gene proved to be suitable for screening the putatively transgenic plants. In fact, no aspecific product was found where DNA extracted from the non-transgenic plants was used as template. Furthermore, analysis of the bacterial lysate gave both the expected bands.

Plant recovery from 'Chardonnay' and '110 Richter' would lead us to expect successful transfer of the *manA* gene. Molecular assays preformed on all the regenerated plants of both genotypes, however, excluded this possibility. In fact, no exogenous sequences were amplified from the grape genomic DNA. No PCR products were obtained for the *dps* gene, thus excluding the persistence of *Agrobacterium* contamination, neither for the *manA* gene, thus proving the non-transgenic nature of the plants obtained.

Conclusion

The present research was based on a thorough assessment of the suitability of the PMI-based system for efficient gene transfer in grape. The effect of mannose on crucial stages of non-transgenic tissue cultures from embryogenic callus to plant recovery and rooting, and the mannose selection of cultures during *manA* gene transfer experiments have been evaluated.

The results obtained with 'Chardonnay', 'Brachetto' and '110 Richter' show that grape tissues are affected by mannose as carbohydrate source. Mannose damage, however, appears after extremely long culture times, and in particular during embryo germination. Moreover, the degree of tolerance to this sugar was found to be related to the genotype. As a consequence, the ambiguous results arising during embryogenic callus propagation may lead to erroneous conclusions since evaluation of the mannose effect may be prone to operator error and not based on objective assessment of the callus appearance. This fact is particularly relevant considering that embryogenic callus is the morphogenic stage most commonly adopted for gene transfer.

Above all, the plants regenerated after the *manA* gene transfer and selected in the presence of mannose were found to be non-transgenic. We already pointed out some disadvantage of the application in grape of the mannose-based selection protocol, such as the need of time-consuming and expensive maintenance. However, it is the non-transgenic nature of the regenerants to provide the conclusive demonstration of the failure of the mannose selection strategy. In fact, the crucial requisite of a proficient selection step is to prevent proliferation and morphogenesis of wild-type tissues while on the same time allowing transgenic cells to undergo proliferation and regeneration.

In light of our results and considering the high cost of mannose compared to cheaper selective agents, PMI seems to be an unsuitable alternative to traditional marker gene selection for successful gene transfer in grape.

Acknowledgements

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