Somatic embryogenesis and plant regeneration of Vitis vinifera cultivars 'Macabeo' and 'Tempranillo'

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Somatic embryogenesis and plant regeneration of *Vitis vinifera* cultivars 'Macabeo' and 'Tempranillo'

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Summary

Different experimental conditions have been compared to achieve a high efficiency in embryogenic calli initiation from 'Macabeo' and 'Tempranillo' anthers. Specifically, two stages of anther development were tested (corresponding to tetrad cells or uninucleate pollen), and direct culture of anthers was compared to culture after a cold treatment of inflorescences (4 °C during 48 h). In addition, two induction media (C_1^P and B2), mainly differing by microelement and cytokinin levels, were evaluated. Experiment repeatability was also examined with a repetition of anther culture one week later. Callus initiation was similar in all media and treatments for both cultivars, usually starting from the anther filament. A simple protocol for efficient induction of embryogenesis in 'Macabeo' and 'Tempranillo' consisted in: (i) selecting the first inflorescence from hardwood cutting, (ii) excising anthers at uninucleate pollen stage without cold treatment of the inflorescences, (iii) incubating anthers on C_1^P medium. The procedure used for embryo germination and plant regeneration, allowed to obtain a conversion rate up to 75 % in 'Macabeo' and 60 % in 'Tempranillo'. The protocol proposed represents the first regeneration system developed for the Spanish cultivars 'Macabeo' and 'Tempranillo'.

K e y w o r d s : grapevine, fruiting cuttings, *in vitro* culture, somatic embryos, regeneration.

Introduction

Somatic embryogenesis can be a very useful tool for grapevine genetics and breeding (Martinelli and Gribaudo 2001). It can be used to develop *in vitro* selection systems for obtaining resistant plant to different kinds of stress (Bouquet and Torregrosa 2003), to eliminate viruses in infected plant material (Goussard *et al.* 1991, Gambino *et al.* 2006), to produce hypocotyls highly compatible for micrografting (Torres-Viñals *et al.* 2004) or to introduce genes by genetic transformation (Kikkert *et al.* 2001). The somatic embryogenesis process in grapevine is highly dependent on the interaction among genotype, explants source and culture medium, so each *Vitis vinifera* cultivar requires an adapted protocol (for review see: Martinelli and Gribaudo 2001). Although several protocols have been developed to induce somatic embryogenesis from different *Vitis vinifera* cultivars, only one has been established in a Spanish cultivar, *i.e.* 'Don Mariano' (López-Pérez *et al.* 2005). Among cultivars originating from Spain, 'Macabeo' and 'Tempranillo' are two of the most cultivated (respectively 7th and 2nd in terms of area), so the establishment of a protocol to obtain a highly efficient initiation of embryogenic calli and subsequent regeneration could be of great importance to future breeding programs.

Two previous studies proposed culture media, whose composition has been improved to reduce genotypic differential responses to somatic embryogenesis induction and plant regeneration (Torregrosa 1998, Perrin *et al*. 2004). Additionally, immature anthers or anther filaments appear to be the most receptive tissues for induction of embryogenic calli in grapevine, so anthers with filaments were chosen as explants to test on induction media proposed. Nevertheless, even in these media the variability of embryogenesis among genotypes was important, so different experimental conditions have to be tested to optimise this process for specific cultivars. For the first time, an efficient protocol is set up to obtain 'Macabeo' and 'Tempranillo' embryogenic calli, able to differentiate into somatic embryos and to regenerate plants with a very high conversion rate.

Material and Methods

P l a n t m a t e r i a l : Woody canes of *Vitis vinifera* L. 'Macabeo' (cl. 630) and 'Tempranillo' (cl. 771) were obtained from the Etablissement National Technique pour l'Amélioration de la Viticulture (Grau-du-Roi, France) and processed following the method described by Mullins and Rajasekaran (1981) to obtain immature inflorescences from fruiting cuttings (Fig. 1 a).

E m b r y o g e n i c c a l l u s i n d u c t i o n a n d s t a b i l i z a t i o n. When the first inflorescences showed separate flowers (6-8 weeks after cuttings culture), the stage of anther development was daily tested by acetic carmine staining. Tetrad stage was chosen as development stage D1 and uninucleate pollen as D2 (Fig. 1 b and c). Sets of 8-10 inflorescences were surface-sterilized for 10 min in a solution of sodium hypochlorite (2.6 % active chlorine) and 1 % (v/v) Tween 20, rinsed three times with sterile distilled water and divided into two groups. One group was dedicated to direct culture of anthers, whereas the other was maintained at 4 °C during 48h before stamen excision

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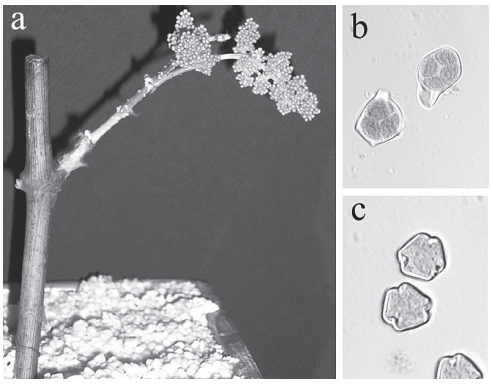

Fig. 1: Inflorescences emerging from hardwood cutting and anther development stage. (**a**) General view of 'Tempranillo' inflorescence, (**b**) D1, tetrad cells and (**c**) D2, uninucleate pollen.

and culture. Anthers were extracted with their filament and placed with the abaxial side on C_1^P (Torregrosa 1998) or B2 (Perrin *et al.* 2004) induction media. The pH value of both media was adjusted to 5.8 with KOH before incorporating 0.5 % w/v Sigma® Phytagel. They were autoclaved 30 min at 110 °C. The experimental unit was represented by a Petri dish of 55x10 mm, with 10 ml culture medium, 25 anthers, sealed with scellofrais® polyethylene wrap and incubated at 26 ± 1 °C in darkness. Each treatment was represented by 10 plates (250 anthers). The experiment was repeated one week later, with different inflorescences from the same fruiting cuttings. During induction, explants were maintained on the same medium for 45 d. Afterwards, all embryogenic calli were subcultivated on C_1^P medium (pH adjusted to 6) every 4-5 weeks to achieve stabilization and long-term conservation of embryogenic tissues (Torregrosa 1998).

Embryo development and plant regeneration: Embryogenic calli were cultured on GS1CA medium (Franks *et al.* 1998) without IAA until torpedo-heart stage single embryos were obtained (1-2 months depending on the genotype). Single embryos (2-4 mm) were individually and horizontally cultivated on MS medium with half-strength macroelements (Murashige and Skoog 1962), with 2 % sucrose and without plant growth regulators for 10-15 d, at 26 °C ± 1°C in darkness. Then, cultures were transferred under attenuated light (15 h photoperiod at 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity, 26 ± 1°C, 70 ± 10 % humidity) until embryos started to germinate. Embryos with emerging epicotyls were planted in 150 mm-long culture tubes (15 ml of MS medium as described above) and grown under 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ intensity light until they developed into plantlets (Torregrosa 1998). Regenerated plants were then subcultured every 8-10 weeks using standard micropropagation procedures.

Statistical analysis: Results were subjected to analysis of variance (ANOVA) using the SPSS statistical software, version 12.0; SPSS inc. Chicago, Illinois.

Results and Discussion

Fig. 2 shows anther response in term of callogenesis and embryogenesis to the different conditions evaluated. A high percentage of anthers developed calli after 45 d of culture with differences between the cultivars as observed by other authors (Bouquet *et al*. 1982, Gribaudo *et al*. 2004, López-Pérez *et al.* 2005). 'Macabeo' exhibited a much higher capacity to produce callus from anther than 'Tempranillo'. Development stage of anthers, medium and cold treatment were found to have little influence on callogenesis.

The browning of explants was more intense with C_1^P induction medium than with B2 medium, particularly with

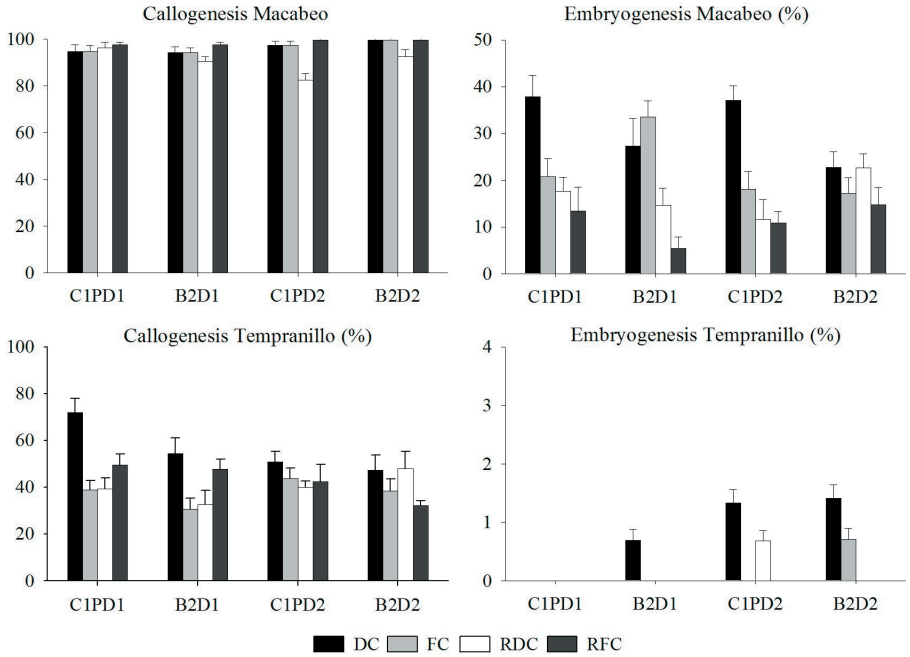

Fig. 2: Effects of different induction media (C_1^P or B2) and cold treatment on callogenesis and embryogenesis of 'Macabeo' and 'Tempranillo'. Anther development stages: D1 or D2; DC: Direct culture after anther disinfection; FC: Anther culture after 48 h at 4 °C. RDC and RFC: Repetition of DC and FC culture, one week later. Values represent the mean of 10 plates ± SD.

Macabeo. However, browning affected anther locules but not emerging callus, as reported by Torregrosa (1998). The evolution of callus mainly varied with the cultivar and the development stage of anther. Four different types of calli were obtained from 'Macabeo' anthers after three subcultures: a small cotton-like callus (Fig. 3 a), a hyperhydric callus (Fig. 3 b), a translucent compact callus (Fig. 3 c) and a yellowish granular callus (Fig. 3 d) which was found the only able to produce embryogenic structures. Only two types of calli were obtained from 'Tempranillo' anthers: A translucent compact callus (Fig. 3 c) and a granular white embryogenic callus (Fig. 3 e).

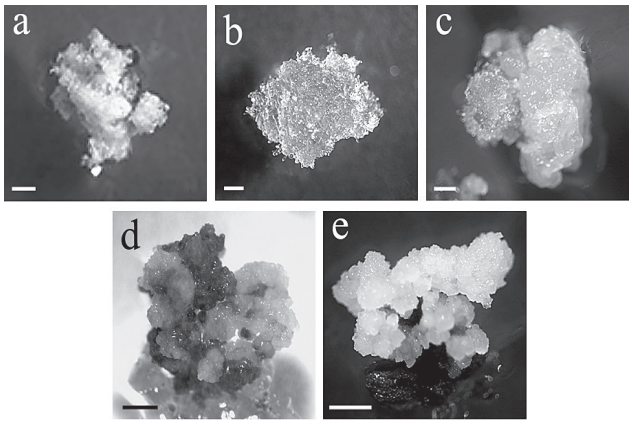

Fig. 3: Different types of calli developed from 'Macabeo' (a, b, c and d) or 'Tempranillo' anthers (c and e). Bar represents 1 mm.

Embryogenic calli most frequently emerged from filaments with no obvious correlation with callogenesis, as described for other cultivars (Torregrosa 1998, Perrin *et al*. 2004, López-Pérez *et al.* 2005). 'Tempranillo' appeared more recalcitrant than 'Macabeo' to produce embryogenic calli. Selecting the most appropriate conditions appeared important to obtain 'Tempranillo' embryogenic callus (Fig. 2), the stage of anther development being most critical. The influence of anther development stage on grapevine somatic embryogenesis has been extensively studied by Gribaudo *et al.* (2004) demonstrating a strong interaction with the genotype.

Chilling treatment was proposed by several authors to improve embryogenesis from inflorescence explants (Martinelli *et al*. 2001, Perrin *et al.* 2004). Here, when a cold treatment was applied, the best responses were observed with B2 induction medium for both genotypes (33,5 % for 'Macabeo' and 0,7 % for 'Tempranillo'). However, cold treatment did not improve either callogenesis or embryogenesis, and in some conditions drastically decreased embryogenesis (Fig. 2). López-Pérez *et al.* (2005) reported similar observations with several other cultivars of *Vitis vinifera*. A higher percentage of embryogenic calli was obtained by plating directly the anthers on C_1^P induction medium (37,9 % for 'Macabeo' and 1,3 % for 'Tempranillo'). Compared to B2 medium, C_1^P was found more convenient as it is easy to prepare and suitable for both embryogenesis induction and long-term conservation (Torregrosa 1998).

Surprisingly, repeated experiments provided different results. While callogenesis percentages did not significantly change with the experiment, embryogenesis decreased, especially for 'Tempranillo'. In the second experiment we used new inflorescences arising one week later from the same fruiting cuttings, we can assume that the physiological changes during the culture (carbon reserve, plant growth regulator balance…) could negatively affect the regeneration ability of flower explants. A similar effect of the physiological status of the explants sampled from the field has been suggested to explain interannual variations of anther response (Perrin *et al.* 2004).

From a practical point of view, to obtain embryogenic cultures of 'Macabeo' and 'Tempranillo' we suggest to: (i) only use the first inflorescences arising from fruiting cutting buds, (ii) select flowers with pollen at the uninucleate pollen development stage and (iii) culture anthers immediately after disinfection without cold storage. For both cultivars, long-term conservation and propagation of embryogenic cultures can be achieved by subculturing small fragments of embryogenic tissues every 4-5 weeks onto C_1^P medium.

For plant regeneration, we successfully used a protocol adapted from Torregrosa (1998) and Franks *et al.* (1998). Embryogenic calli of both cultivars favourably responded to the culture on GS1CA without IAA. 'Macabeo' embryogenic callus developed embryos at the torpedo stage in 4-5 weeks, while 'Tempranillo' embryo development required an extra 2-3 weeks on fresh medium. Torpedo stage embryos were subcultured on MS/2 medium, with similar duration of germination for both cultivars. Embryo-to-plant conversion rates reached 75 % in 'Macabeo' and 60 % in 'Tempranillo', with regenerated plants displaying when established a normal *in vitro* phenotype.

This study developed an efficient procedure to produce 'Macabeo' and 'Tempranillo' tissue cultures suitable for technologies dealing with grapevine somatic embryogenesis, *i.e.* virus sanitation or *Agrobacterium*-mediated transformation.

Acknowledgments

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