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Research Note

Somatic embryogenesis and efficient regeneration of *Vitis vinifera* L. 'Carménère' plants

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K e y w o r d s : *Vitis vinifera* L., Carménère**,** *In vitro* culture, somatic embryogenesis.

A b b r e v i a t i o n s : 2,4 D (2,4-dichlorophenoxyacetc acid), BA (6-benzyladenine), IAA (indole-3-acetic acid), GA_3 (gibberellic acid).

Introduction: The 'Carménère' cultivar of *Vitis vinifera* L. is of French origin and was renowned as a prestigious grape cultivar during the second half of the XIX century. However, this cultivar practically disappeared in Europe in 1860 due to the attack of a phylloxera plague. The 'Carménère' cultivar was introduced in Chile in 1850 where the agroclimatic and phytosanitary conditions of the country, including the noteworthy absence of phylloxera, favoured the expansion of this culture. 'Carménère' is currently deemed an emblematic and exclusive variety and the wine industry's efforts are focused on optimizing its management and on developing high quality wines. Agronomical, physiological and molecular studies to conserve and improve 'Carménère' cultures have been promoted because homogeneous and high quality fruit is the starting point for obtaining good quality wines. Selection programs and clonal propagation of elite plants are being complemented with the development of *in vitro* regeneration systems. There are several reports of regeneration systems via somatic embryogenesis in *Vitis* (Martinelli and Gribaudo 2001) showing that a series of factors such as the genotype, the developmental stage of explants, the physiological status of the donor plant and the culture media and growth regulators are important variables in determining the efficiency of the method. In the present work, we report for the first time the establishment of an *in vitro* regeneration system for *V. vinifera* cv. 'Carménère', *via* somatic embryogenesis.

Material and Methods: To initiate embryogenic cultures, immature inflorescences (stage 15 as described by Lebon *et al.* 2005) of *Vitis vinifera* L. 'Carménère' were collected from an experimental field, protected in wet paper towels and maintained under refrigeration during transport (Figure, stage 1). Inflorescences were washed with water, placed in Petri dishes and stored at 4 °C for 48 h. After disinfection, anthers and ovaries were isolated from flowers under sterile conditions using a stereomicroscope (Figure, stage 2). A sample of 300 anthers with the filaments and 100 ovaries were placed on PIV medium (Franks *et al.* 1998) containing 4.5 µM 2,4-D and 8.9 µM BA. Additionally, 200 anthers were cultured on B solid medium (Perrin *et al.* 2004). The cultures were kept at 25 °C in the dark for 6 months. The embryogenic calli were maintained by alternating PIV medium with embryo proliferation medium (GS1CA; Franks *et al.* 1998) every two months. To differentiate embryos, 15 clusters of globular embryos (approximately 1 cm^2) derived from anther embryogenic calli were selected from the cultures and placed on differentiation medium (ED) containing activated charcoal and no growth regulators, as described by López–Pérez *et al.* (2005). The cultures were kept at 25 °C in the dark. Subsequently, somatic embryos were cultured for a month on embryo germination medium (EG), which is ED medium supplemented with 10 µM IAA and 1 µM GA_3 (López–Pérez *et al.* 2005). Germinated embryos were developed into plants on Driver and Kuniyuki Walnut medium (Driver and Kuniyuki 1984) modified in our laboratory. This modified medium DKWm contains half the concentration of $Ca(NO_3)_2$ and half the concentration of $CaCl_2$ of the original DKW. Cultures were kept at 25 °C, with a 16:8 h (125 $\mu\varepsilon \cdot m^2 \cdot s^{-1}$) photoperiod.

Results and Discussion: After one month in PIV medium, 85 % of the ovaries and 92 % of the anthers initiated visible growing callus. Two discernible types of tissues originated from both ovary and anther cultures. The first type was a light-brown pre-embryogenic tissue which was morphologically identical to those described as Type I in the literature (Perrin *et al.* 2004). This tissue is characterized by the presence of compact and white globular embryos (Figure, stage 4). The second tissue type had a transparent appearance and generated light-yellow non-embryogenic callus and corresponded to the tissue previously described as Type II (Perrin *et al.* 2004). After six months in PIV medium, 9 % of the ovaries and 19 % of the anthers developed embryogenic calli. It is noteworthy that promising results have been obtained regarding morphogenesis from ovaries, as this explant material proved particularly suitable for the efficient induction of somatic embryogenesis in grapes (Martinelli *et al.* 2004). On the other hand, the 200 anthers cultured on B medium only gave origin to non-embryogenic calli, phenotypically similar to Type II calli.

Embryos were differentiated on ED medium (López–Pérez *et al.* 2005) and after a month, asynchronous development of embryos was initiated (Figure, stage 5). Globular, heart and torpedo stage embryos could be distinguished at the same time. The proliferation process from somatic embryos was obtained in 'Carménère' both through direct and indirect secondary somatic embryogenesis. The former occurred in the root-shoot transition zone of somatic embryos

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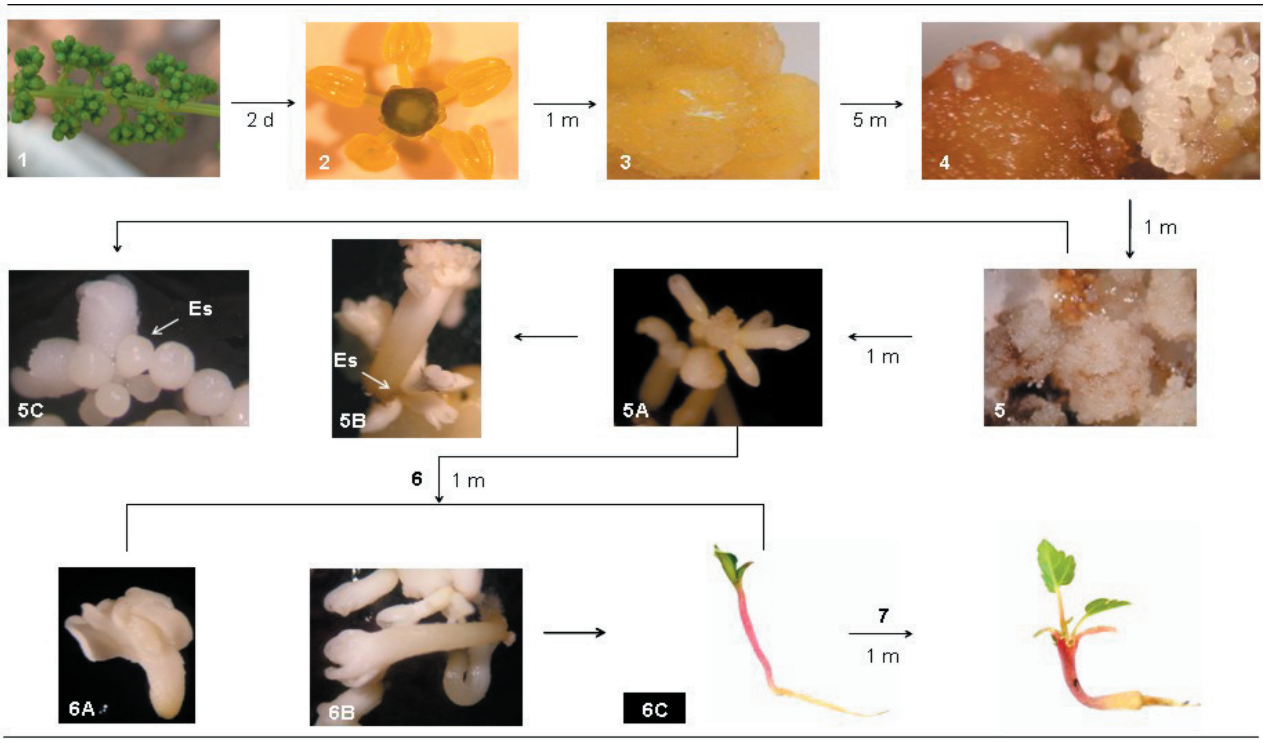

Figure: Crucial stages and timing of the process of anther-derived somatic embryogenesis from explant to plantlet in *Vitis vinifera* cv. 'Carmenère'. (**1**) Inflorescence at the collection and sterilization steps, and (**2**) flower isolation for anther dissection and culturing, (**3**) callus induction, (**4**) embryogenic callus induction, (**5**) embryogenic culture stabilization: (**5 a**) somatic embryo maturation and differentiation, (**5 b-c**) followed by the two alternative ways of secondary embryogenesis, (**6**) germination of embryos: (**6 a-b**) cotyledonary embryos, (**6 c**) embryo germination, (**7**) plantlet at first bud at emerging stage. d = day; m = month; SE = secondary embryogenesis.

at cotyledon stages (Figure, stage 5 b), whilst the latter occurred in globular somatic embryos (Figure, stage 5 c).

To continue embryo development, somatic embryos at the torpedo stage (ranging from 1 to 3 mm in length; Figure, stage 5 a) were cultured for a month on EG medium (López-Pérez *et al.* 2005). From 126 torpedo embryos, 95 % (120) germinated according to the canonic process, passing through the different stages of germination (Figure, stage 6 a-c) acquiring a clear root-shoot axis, and undergoing hypocotyl elongation, the development of green cotyledons and the emergence of the first bud (Figure, stage 6 c). Of particular note is that all 120 canonically-germinated embryos developed into plants by propagation on DKWm. This medium was originally designed to culture 'Carménère' shoot explants *in vitro* after a multiple medium assay (Toro 2003) and resulted appropriate for the development of embryos. Between two to four weeks, all germinated embryos developed into plantlets with an average length of 2 cm and possessing two vegetative leaves (Figure, stage 7).

Conclusion: Our results demonstrate that it is feasible to regenerate 'Carménère' through somatic embryogenesis. This protocol represent a basic material for developing molecular breeding experiment (Kikkert *et al.* 2001), as well as for assessing interesting genes.

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