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ORIGINAL PAPER

Somatic embryogenesis from whole flowers, anthers and ovaries of grapevine (*Vitis* spp.)

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Abstract A novel method for initiating somatic embryogenesis in grapevine, based on immature whole flower culture, is presented. The embryogenic competence of flowers was compared to that of anthers and ovaries, the most widely used explant types, for five grapevine cultivars. Both the genotype and the explant source affected the differentiation of somatic embryos. The highest percentages of embryogenesis were obtained in ovary-derived calli from all cultivars tested with the exception of Brachetto a grappolo lungo. Whole flowers proved to be suitable material for initiating embryogenic cultures for most tested cultivars, and for 110 R, Chardonnay, and Grignolino they gave similar or better results than anthers. Collection of whole flowers from the inflorescence is easier and faster than excision of anthers and ovaries from the flower itself; it can be done without the use of a stereomicroscope and damage to the explant is unlikely. No morphological difference was noted among embryogenic cultures originated from ovaries, flowers, or anthers.

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	6-Benzyladenine
NOA	2-Napthoxyacetic acid
IAA	Indole-3-acetic acid

Introduction

The development of an efficient regeneration system is a prerequisite for the application of gene technologies to plant breeding when an entire plant must be originated from the manipulated cell. In grapevine (*Vitis* spp.) the most frequently adopted regeneration method is somatic embryogenesis, which has been used not only for genetic engineering (Franks et al. 1998; Gambino et al. 2005), but also for virus eradication (Goussard et al. 1991; Gambino et al. 2006), in vitro mutant isolation (Franks et al. 2002), germplasm cryopreservation (Gray and Compton 1993), and production of synthetic seeds (Das et al. 2006). Somatic embryos have long been obtained from a few grape species and cultivars, but recently improved methodology has increased application to a broader base of germplasm (Martinelli and Gribaudo 2001). However, somatic embryogenesis remains genotype dependent (Perrin et al. 2004; Maillot

et al. 2006). The explant source also influences the efficiency of regeneration protocols. Immature anthers have been the most widely used explant (Martinelli and Gribaudo 2001) with response dependent on genotype, culture medium (Perrin et al. 2001) and microspore developmental stage (Gribaudo et al. 2004). Immature ovaries (Nakano et al. 1997; Martinelli et al. 2001) and stigma-style explants (Carimi et al. 2005) have been reported to be more responsive to embryogenic induction than anthers. Because of the small size of the explants [anthers range from 0.5 mm to 1.3 mm (Gribaudo et al. 2004)] their dissection is usually done under a stereomicroscope. This operation is tedious, time consuming and may damage the explants. To avoid these problems, several authors have attempted to induce somatic embryogenesis from vegetative tissues, such as nodal sections (Maillot et al. 2006), leaf and petiole fragments (Das et al. 2006), and tendrils (Salunkhe et al. 1997) but only a few cultivars yielded promising results.

In this work we present a method based on the culture of immature whole flowers as explants for initiating embryogenic cultures. The suitability of flowers was compared to that of anthers and ovaries.

Materials and methods

Five grapevine cultivars were used for immature whole flower, anther, and ovary culture: *Vitis vinifera* cvs. Chardonnay, Müller Thurgau, Grignolino and Brachetto a grappolo lungo (Brachetto g.l.) and the *V. berlandieri* × *V. rupestris* rootstock 110 Richter (110R).

Over a 3-week period during spring (May 2006), flower clusters were collected from plants in the field (Grugliasco, North-Western Italy); only the basal half of the inflorescence was retained (Fig. 1a). The developmental stage of explants was preliminarily determined by observing the flowers and anthers under a stereomicroscope and examining the stage of microsporogenesis under an optical microscope after anthers were squashed in a drop of acetocarmine. Explants were cultured at stages III, IV and V as described by Gribaudo et al. (2004). The inflorescences were surface sterilized for 10 min with sodium hypochlorite (1.5% available chlorine) containing a few drops of Tween 20 as a wetting agent, rinsed

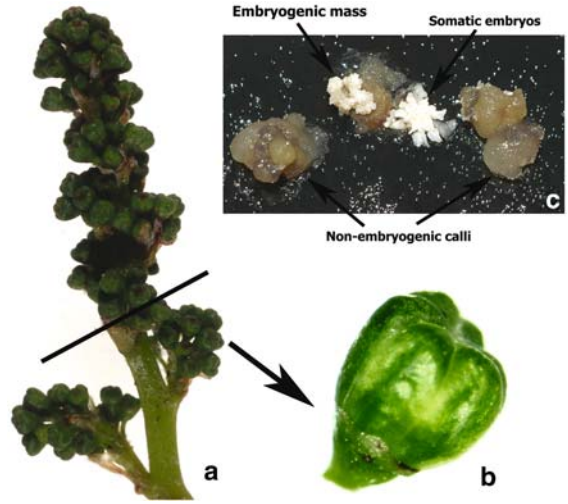

Fig. 1 (**a**) Inflorescence and (**b**) whole flower of *V. vinifera* cv Chardonnay; (**c**) embryogenic and non-embryogenic calli from flowers of Chardonnay after 5 months of culture

several times with sterile distilled water, placed in sterile Petri plates and chilled at 4°C for 4–6 days.

Before explant excision, the inflorescences were subjected to a second sterilization treatment with the above described solution for 15 min. Whole flowers (Fig. 1b) were aseptically removed from the inflorescence by cutting the pedicels. The flowers were then plated with their longitudinal axis parallel to the medium surface. Separately, anthers and ovaries were excised from flowers under a stereomicroscope. Cultured anthers and the attached filaments were plated with the adaxial side in contact with the medium. Explants were initially cultured on callus induction medium (PIV; Franks et al. 1998; Gribaudo et al. 2004) containing: Nitsch and Nitsch (1969) mineral salts, Murashige and Skoog (1962) vitamins, 6% sucrose, 0.3% gelrite. The basal medium was supplemented with 4.5 μM 2,4-D and 8.9 μM BA and the pH adjusted to 5.8 with 0.5 N NaOH before autoclaving (120°C for 10 min). For all cultivars tested, 300 flowers, 375 anthers and 150 ovaries were cultured in 90 mm Petri plates containing 25 ml of medium (25 explants/plate). The cultures were maintained at 26°C in the dark.

Three months after the initiation of the culture, calli were transferred to embryo proliferation medium (GS1CA; Franks et al. 1998; Gribaudo et al. 2004) of similar composition to PIV except the growth regulators were changed to 10 μM NOA,

1 μM BA, 20 μM IAA (the latter was filter sterilized and added after autoclaving), 1% bactoagar instead of gelrite and 0.25% activated charcoal, pH 5.8. The cultures were maintained under the conditions described above.

The number of explants differentiating somatic embryos was recorded 3 months and 5 months after the initiation of the culture. Final percentage data were arcsin transformed and then subjected to analysis of variance (GLM procedure; SAS statistical software, version 8.2, SAS Institute, Cary, NC).

Results and discussion

After 3 months of culture, calli were obtained from all explant types: about 92% of the flowers, 70% of the ovaries, and 22% of the anthers produced calli. As previously reported (Perrin et al. 2004; López-Pérez et al. 2005; Gambino et al. 2006) callus morphology and embryogenic competence were related. In our cultures most granular white or yellow calli that eventually were associated with dark callus differentiated somatic embryos whereas dark and compact or watery and soft callus showed little or no embryogenic competence (Fig. 1c).

After 5 months of culture the percentages of calli differentiating somatic embryos increased for all cultures but at different rates depending on the explant (Table 1). Calli from ovaries and whole flowers often expressed their embryogenic competence later than those from anthers. The percentages of embryogenic explants across the cultivars between the two sampling dates increased from 11.2 to 24.4 for ovaries, from 3 to 9.4 for flowers, and from 9.4 to 11.6 for anthers. The larger explant size of flowers and ovaries may have delayed the effect of growth regulators in solid medium and consequently delayed the appearance of embryogenic masses. Both the genotype and the explant type had a significant effect on the differentiation of somatic embryos. The highest percentages of embryogenic callus were obtained from ovary-derived calli from four of five cultivars tested. Whole flowers gave similar or better results for initiating embryogenic cultures than anthers for 110 R, Chardonnay, and Grignolino.

Both the suitability of ovary as the explant source and the genotype effect were in accordance with previous results (Gribaudo 2001; Martinelli et al. 2001; Nakano et al. 1997; López-Pérez et al. 2005). Whole flower explants were adequate for initiating embryogenic callus of Chardonnay, Müller Thurgau, Grignolino, and 110 R, while flowers of Brachetto g.l. showed a slight embryogenic competence. Flowers have been used in other plant species to induce somatic embryogenesis, e.g., the female flowers of pistachio (*Pistacia vera* L.; Onay et al. 2004) and the male flower buds of banana (*Musa* spp.; Khalil et al. 2002). Whole flowers are suitable explants for establishing grapevine embryogenic cultures because of their ease of collection and excision compared to anthers and ovaries; dissection can be done without a stereomicroscope and damaging the explants during excision is unlikely. The greater number of explants that can be plated in a given time counterbalances their lower embryogenic competence compared to anthers and ovaries. The double sterilization of the inflorescences before culture initiation was successful in reducing contamination, which never exceeded 2% of plated explants.

Table 1 Frequency (%) of somatic embryogenesis in whole flower, ovary and anther-derived calli of *V. vinifera* cvs Chardonnay, Grignolino, Müller Thurgau, Brachetto g.l. and *V. berlandieri* × *V. rupestris* 110R, after 3 and 5 months of culture. For each cultivar 375 anthers, 300 flowers and 150 ovaries were cultivated

Cultivar	Explant type	Embryogenesis (%) after 3 months of culture	Embryogenesis (%) after 5 months of culture[a]
Chardonnay	ovary	5.3	20 a
	anther	7.5	9.6 b
	flower	2.4	15.7 ab
Grignolino	ovary	5.9	21.3 a
	anther	2.4	5.6 b
	flower	1	5.9 b
Müller Thurgau	ovary	20	23.5 a
	anther	16.3	18.4 a
	flower	2	7.5 b
Brachetto g.l.	ovary	4.7	14.7 a
	anther	12.8	16 a
	flower	0	1.3 b
110R	ovary	20.1	42.3 a
	anther	8	8.5 c
	flower	9.6	16.7 b

[a] For each cultivar, means followed by the same letter do not differ significantly at $P \leq 0.05$ as determined by the Duncan's multiple range test

No morphological difference was noted among embryogenic cultures originating from ovaries, flowers, or anthers. In grape, it is generally accepted that anther-derived embryos have a somatic origin from cells of the anther connective tissue (Faure et al. 1996). Srinivasan and Mullins (1980) described the nucellar origin of calli originated from ovules; Nakano et al. (2000) demonstrated that embryogenic calli of grapevine cv Neo Mat was derived from ovary receptacle tissues; and Martinelli et al. (2004) excluded the participation of cells of the sexual lines to the morphogenic process. There are no reports of cellular origin of callus from whole flowers. Detailed ontogenic studies are necessary. Additionally, the ploidy stability and the possibility of somaclonal variation need to be carefully ascertained in regenerated plantlets (Leal et al. 2006).

To our knowledge this is the first report of whole flower culture for grapevine somatic embryogenesis. This method may be tested for other important cultivars of *Vitis* spp. and for otherwise recalcitrant plant species.

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