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Factors affecting somatic embryogenesis in eight Italian grapevine cultivars and the genetic stability of embryo-derived regenerants as assessed by molecular markers

Angela Carra[a,1], Maurizio Sajeva[b,1], Loredana Abbate[a,1], Mirko Siragusa[a,1], Ranjith Pathirana[c,1], Francesco Carimi[a,*,1]

[a] Institute of Biosciences and BioResources, National Research Council (CNR), Research Division of Palermo, Palermo, Italy
[b] Department of Biological Chemical and Pharmaceutical Science and Technologies, University of Palermo, Palermo, Italy
[c] The New Zealand Institute for Plant & Food Research Limited, Palmerston North, New Zealand

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ABSTRACT

Embryogenic cultures have been used in cryopreservation, genetic transformation, propagation, virus elimination, induced mutagenesis and in many other biotechnological applications, providing excellent opportunities for biotechnology advances in grapevine. Unfortunately the efficiency of somatic embryogenesis (SE) is genotype-dependent in addition to showing interaction with explant type used and the plant growth regulator (PGR) composition. In order to identify the interaction of these parameters in SE, we tested eight wine grapevine cultivars, three explant types (ovary, anther/filament and stigma/style) and four PGR combinations in a statistically designed experiment. The genotype was the major determining factor, with embryogenic response varying from 0.1 to 5.1% (about 50-fold difference). For PGR composition of the medium, embryogenesis ranged between 0.5 and 3.3% (a ≈7-fold difference). The explant type was the least important factor with embryogenesis ranging between 0.8% (anther/filament) and 2.3% (ovary)—only a ≈3-fold change. Anther/filament, that had generally been considered to be the most promising explant, surprisingly gave the lowest embryogenesis percentage. Genetic homogeneity of plants developed from *in vitro* SE was assessed in comparison to mother plants using six inter-simple sequence repeat and ten random amplified polymorphic DNA primers that produced reproducible and clear bands ranging from 150 to 3500 bp. The amplification products were monomorphic across all the regenerated plants and their respective mother plants confirming the genetic homogeneity of the regenerants and demonstrating the suitability of SE for *in vitro* grapevine germplasm conservation and propagation.

1. Introduction

Grapevine (*Vitis* spp.) is one of the most economically important fruit crops cultivated worldwide, covering about 7.16 million hectares (FAOSTAT 2013). Cultivation and selection over many centuries under different climatic conditions has resulted in an enormous diversity of local grapevine cultivars (10,000–14,000). Natural and human selection have led to countless typologies of wine, strictly related to a specific area of origin and a multitude of table grape cultivars and rootstocks (Carimi et al., 2012).

The rapid erosion of genetic diversity of wild and cultivated plants has highlighted the importance of biodiversity conservation and, as a consequence, several techniques are used for germplasm conservation and utilization. To maintain genetic diversity of grapevine the germplasm is usually conserved in field collections where each genotype is replicated with a limited number of plants, but the management of plants in the field is expensive and carries high risks of loss. In particular, the risk of disease transmission through vegetative multiplication and insects is high and difficult to avoid (Bouquet and Torregrosa, 2003; Rao, 2004). To deal with these problems, tissue culture techniques are important in the conservation, propagation and storage of plant germplasm at reduced costs (Carimi et al., 2012). In particular, somatic embryogenesis (SE) has been proven to be highly effective for germplasm conservation and for eliminating viruses from grapevine

* Corresponding author.
E-mail address: francesco.carimi@ibbr.cnr.it (F. Carimi).
[1] These authors contributed equally to this work.

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(Gribaudo et al., 2006). Therefore *in vitro* conservation of germplasm regenerated through SE is preferred because it is an inexpensive technique that provides new individuals potentially virus-free. Nevertheless, SE in *Vitis* is influenced by several parameters, which makes application of the technique difficult. Successful induction of embryogenic cultures and their maintenance depend on a range of factors such as culture conditions, genotype and explant type. However, these influences are not so clear for many grapevine genotypes, in which the requirements can vary greatly.

The aim of this research was to identify the most important factors influencing SE in major Italian grapevine cultivars. Genetic stability was evaluated for the two most important varieties for wine production among those tested; one ('Nerello cappuccio') is typical for wine production in eastern Sicily, the other ('Perricone') for wine production in western Sicily. Three explant types (ovary, anther/filament and stigma/style) were compared in eight diverse grapevine varieties cultured on four different plant growth regulator (PGR) combinations. The genetic fidelity of regenerated plants of the two selected varieties was detected by two different DNA-based techniques, inter-simple sequence repeats (ISSR) and random amplified polymorphic DNA (RAPD).

2. Materials and methods

2.1. Plant material, media and culture conditions

Cultures were initiated as described in Carimi et al. (2005) from explants (anther/filament, stigma/style and ovary) dissected from immature flowers (collected 15 days before anthesis) from field-grown plants of eight Italian Vitis *vinifera* cultivars used for wine production, namely 'Albanello', 'Corinto nero', 'Damaschino', 'Frappato', 'Malvasia delle Lipari', 'Nerello cappuccio', 'Perricone' and 'Zibibbo'. Information on the varieties used in the study is reported in Carimi et al. (2010). Explants were cultured on full strength MS (Murashige and Skoog, 1962) medium supplemented with the following combinations of PGR: 1) VV-4 medium, 5 μM *N*-(2-chloro-4-pyridyl)-*N*-phenylurea (4-CPPU) + 5 μM 2,4-dichlorophenoxy acetic acid (2,4-D) (Matsuta and Hirabayashi, 1989); 2) VV-5 medium, 20 μM β-naphthoxyacetic acid (NOA) + 4 μM *N*-phenyl-*N*'-1,2,3-thiadiazol-5-ylurea (TDZ) (Harst, 1995); 3) VV-12 medium, 5 μM NOA + 4.4 μM N^6-benzylaminopurine (BA) (Stamp and Meredith, 1988), and 4) VV-16 medium, 10 μM NOA + 4.4 μM BA (Carimi et al., 2005). The choice of these factors (optimised parameters: explant-type/PGR-combination/culture-conditions) was influenced by our own experience on *in vitro* grapevine regeneration in two laboratories in Italy and New Zealand and our recent review of the literature (Carimi et al., 2012, 2013). All chemicals used in tissue culture experiments were purchased from Duchefa Biochemie, Netherland. Media were solidified with 6 g/L plant agar and supplemented with 88 mM sucrose. The pH of media was adjusted to 5.7–5.8 with 1 N NaOH before autoclaving (Carimi et al., 2005).

Explants and calli were subcultured at 60-d-intervals and maintained in a climatic chamber at 26 °C with a 16 h photoperiod (40 μmol m^{-2} s^{-1} at shelf level provided by Osram Cool White 18 W fluorescent lamps). Those explants showing embryogenic responses were transferred to basal MS medium [that consisted of macronutrients, micronutrients and vitamins as described by Murashige and Skoog (1962) without the addition of PGR] supplemented with 88 mM sucrose and cultured for a further 4 weeks to allow embryo proliferation and development.

Individual germinated somatic embryos (about 1–2 cm in length) were transferred to Magenta™ vessels to allow further growth using one embryo/Magenta™ vessel containing 100 ml of basal solid MS medium.

For acclimatization, plantlets (10–15 cm) were transferred to autoclaved Jiffy peat pellets and maintained on a heating bench at 25 °C and at high relative humidity (95–98%). After 4–5 weeks, rooted plants were transferred into pots containing sterile soil and maintained in the greenhouse under natural daylight at 22/27 °C (night/day).

2.2 Assessment of genetic stability of somatic embryo-derived regenerants in two varieties

Twenty plants per treatment of 'Nerello cappuccio' and 'Perricone' coming from different embryogenic events randomly chosen from three different explants cultured on different media were selected for the analyses of genetic stability. Regenerated plants were considered coming from different embryogenic events when somatic embryos were regenerated from different explants or from clearly defined, distinct region of the same explant. Genetic stability of regenerated plants was determined by ISSR and RAPD analysis as described by Siragusa et al. (2007). A total of six ISSR primers – i.e., $(AG)_8$YC [Annealing Temperature (T_a) 52.6 °C], $(AC)_8$YT (T_a 50.3 °C), $(AG)_8$YT (T_a 50.3 °C), $(GT)_8$YG (T_a 52.1 °C), $(GA)_8$YC (T_a 52 °C) and $(CA)_8$RG (T_a 51 °C) – and ten 10-mer RAPD primers – i.e., OPH04 (5′-GGAAGTCGCC-3′), OPAT14 (5′-GTGCCGCACT-3′), OPH15 (5′-AATGGCGCAG-3′), OPM04 (5′-GGCGGTTGTC-3′), UBC219 (5′-GTGACCTCAG-3′), UBC234 (5′-TCCACGGACG-3′), UBC237 (5′-CGACCAGAGC-3′), UBC239 (5′-CTGAAGCGGA-3′), UBC247 (5′-TACCGACGGA-3′) and UBC251 (5′-CTTGACGGGG-3′) – were used to amplify the DNA. The primers were purchased from Life Technologies, Gaithersburg, MD. DNA amplification reactions were performed with Platinum *Taq* polymerase (Life Technologies). To confirm the reproducibility of the banding patterns, all analyses were repeated twice.

2.3. Statistical analysis

All experiments were carried out in a randomized complete block design. Each treatment comprised of ten replicates. Five explants each for stigma/style and ovary, and twenty-five explants for anther/filament were used per Petri plate representing a replicate, making a total of 11,200 explants in the experiment. The number of embryogenic explants was recorded after 6 months from culture initiation. Embryo germination data were collected 2 months from the incubation of somatic embryos on PGR-free medium. Effects of genotype, PGR combination and type of explant on percentages of embryogenic explants, and percentages of embryo germination were tested by ANOVA ($P \leq 0.05$) and the differences among means were tested by Tukey's test. Prior to analysis, percentage data were arcsin-square root transformed. Statistical analysis was performed using SigmaStat 3.5 for Windows.

3. Results

Callus formation and proliferation from explants was observed 10–30 days after culture initiation under all the PGR combinations tested. Cultures from all the cultivars produced embryogenic callus and somatic embryos appeared after 3–4 months from culture initiation on the surface of callus (Figs. 1A and B). Table 1 shows the embryogenic responses after 6 months of culture initiation in different treatments tested in eight cultivars. The percentages of embryogenic explants varied greatly (0–25%) depending on cultivar, explant type and PGR combination used.

During induction of SE, differences were observed among cultivars in the efficiency of embryogenesis. Percentage of embryogenic