In Vitro Techniques for Grapevine Germplasm Conservation

D. Dal Bosco, I. Sinski, V. Comachio, J.D.G. Maia, P.S. Ritschel and V. Quecini
Embrapa Uva e Vinho
Bento Gonçalves RS
Brazil

Keywords: germplasm, in vitro, tissue culture, micropropagation, phytosanitary improvement

Abstract

Plant genetic resources hold significant phenotypic variation resultant from the presence of allelic diversity, which is maintained by evolutionary processes or by artificial selection. Therefore, plant germplasm encompasses the huge genotypic diversity found in wild and cultivated species and constitutes a source of interesting traits for breeders. Although extremely valuable, biodiversity conservation has high demand for funding, physical space and labor. In vitro conservation is an interesting alternative for the maintenance of highly-heterozygous, vegetatively propagated, perennial species, such as grapevine. It also contributes to the plants' phytosanitary conditions. The current work aimed to develop effective and feasible means toward in vitro establishment and conservation of grapevine germplasm. Woody stakes were obtained from the field collection of the Grapevine Germplasm Bank, at Embrapa, and were surface disinfected, planted in a mixture of autoclaved soil and vermiculite (1:1), and kept under controlled temperature (23±5°C) and relative humidity (70%). Young apical shoots were excised and superficially disinfected in the presence of 1% (w/v) polyvinylpirrolidone. Explants were transferred to tubes containing Galzy medium with active charcoal, under aseptic conditions. Established plants were propagated and maintained in vitro as duplicates. For long-term conservation, the effectiveness of two cryopreservation techniques; vitrification and encapsulation-dehydration, was compared for 11 grapevine genotypes, including *Vitis vinifera*, *V. labrusca* and hybrids *V. berlandieri* × *V. rupestris*, and *V. riparia* × *V. berlandieri*. Shoot induction from treated stakes under protected greenhouse conditions significantly reduced environmental contamination and, along with the use of anti-oxidant agents, allowed in vitro establishment of approximately 1200 (85% of the accessions held by the bank) grapevine accessions. The establishment of the remaining accessions is underway. Plants free of ectophytes were produced for 900 (64.3%) accessions. Cryogenic protocols require further adjustments to allow acceptable recovery rates. High-scale in vitro conservation of grapevine germplasm is feasible and may safeguard valuable biodiversity. Although promising, cryopreservation requires further studies for protocol optimization.

INTRODUCTION

The availability of well-characterized and secured genetic variation is essential for the success of grapevine breeding programs (Myles, 2013). As a self-incompatible and cross-pollinated crop, grape seeds do not represent the true genotype; thus, the storage of genetic variation in seed banks is not feasible. Typically, grapevine germplasm banks are maintained as whole-plant field collections. The maintenance and propagation of grapevine accessions is highly determined by climatic conditions and the occurrence of diseases and pests. Thus, field maintenance of *Vitis* and its related species is costly and time-consuming, due to the requirement of large extensions of experimental land, application of management practices and control measures, such as pesticides, fertilizers, fungicides, etc. In the case of extraneous species, the need of intensive management significantly adds to the costs of the maintenance and conservation of foreign germplasm. In vitro culture techniques have allowed the development of biotechnological strategies for plant genetic resources conservation as practical alternatives to field collections

(Börner, 2006; Khoury et al., 2010). Moreover, in vitro conservation and propagation of grapevine genetic resources promotes the removal of epiphytic organisms, allowing the production of healthy, uniform plants in a timely manner. The establishment of in vitro plants is also instrumental for the development of virus-free plants employing meristem culture, thermo- and chemotherapy and the combination of these techniques (Baránek et al., 2010; Hu et al., 2013). A large number of grape genotypes have been successfully cultured and propagated in vitro, however, the efficiency of shoot multiplication and rooting is highly genotype-dependent and methods for large-scale genetic resources maintenance and propagation remain scarce (Alizadeh et al., 2010). Cryopreservation employs liquid nitrogen, at the temperature of approximately -200°C, to store plant material without modification or alteration for extended periods (Engleman, 2011). The technology allows protection of the explants from contaminations and requires limited maintenance, however, its application requires extensive protocol optimization depending on the species and even genotype. The current work describes in vitro establishment, propagation and conservation of grapevine germplasm and a comparison of plant recovery efficiency for two cryopreservation methodologies, encapsulation-dehydration and vitrification, for twelve grapevine cultivars.

MATERIAL AND METHODS

Woody stakes were obtained from the field collection of the Grapevine Germplasm Bank at Embrapa, and were surface disinfected, planted in a mixture of autoclaved soil and vermiculite (1:1), and kept under controlled temperature (23±5°C) and relative humidity (70%). Young apical shoots were excised and superficially disinfected in 70% (v/v) ethanol followed by immersion in 1% (v/v) bleach supplemented with 1% (w/v) polyvinylpirrolidone. Explants were transferred to tubes containing 12 ml of Galzy medium (Galzy, 1964) supplemented with 0.25% (w/v) active charcoal, under aseptic conditions. Established plants were propagated and maintained in vitro as duplicates, in growth chamber under 16-h photoperiod (75 μmol m^{-2} s^{-1}), provided by warm and cold white LED sources, and temperature of 23±3°C. After rooting (45 to 60 days), plants were replicated to fresh Galzy medium, without active charcoal, and maintained as duplicates under the previously described growth conditions.

For long-term conservation, the effectiveness of two cryopreservation techniques; vitrification (Shatnawi et al., 2011) and encapsulation-dehydration (Wang et al., 2003), was compared for 11 grapevine genotypes, including *Vitis vinifera*, *V. labrusca* and hybrids *V. berlandieri* × *V. rupestris*, and *V. riparia* × *V. berlandieri* (Table 1).

RESULTS AND DISCUSSION

The in vitro establishment of a wide range of grapevine accessions from a germplasm bank was successfully achieved employing agents to reduce phenolic browning during explant surface disinfection (polyvinylpirrolidone) and initial in vitro propagation (active charcoal) (Fig. 1). Explant contamination was reduced by surface disinfection of woody stakes from the field and induced shooting under controlled conditions in the greenhouse (Fig. 1). The current approaches have allowed the establishment and in vitro maintenance of more than 1200 distinct genotypes of grapevine, including wild species (Table 1). New plants, with improved phytosanitary conditions by the removal of ectophytes, were produced from the in vitro plants and are currently kept as a backup bank in a dedicated greenhouse.

The efficiency of plant recovery was tested after treatment with two cryopreservation methods, namely encapsulation-dehydration (Wang et al., 2003) and modified vitrification (Shatnawi et al., 2011), for twelve grapevine genotypes, including table, wine and juice grapes and rootstocks (Table 2). Encapsulation-dehydration produced slightly higher rates of plant recovery after cryopreservation in comparison to vitrification (Table 2, Fig. 2), however, re-growth was erratic and plants exhibited shoot scalding and bleaching (Table 2, Fig. 2).

CONCLUSIONS

Critical factors affecting in vitro plant establishment, contamination and phenolic browning, were bypassed by the surface disinfection of woody stakes, induction of shooting under controlled greenhouse conditions and addition of antioxidant agents to explants' surface disinfection and initial in vitro growth steps. The employed conditions allowed the establishment and propagation of a wide range of grapevine accessions, including cultivars, wild species and *Ampelopsis*. The removal of ectophytes by in vitro propagation has allowed the production of a backup germplasm bank with improved phytosanitary conditions. The tested cryopreservation protocols, encapsulation-dehydration and vitrification, were not effective to provide consistent plant recovery to allow germplasm safeguard for twelve investigated cultivars, including table, wine and juice grapes and rootstocks. Moreover, recovered plants exhibit leaf scalding and bleaching symptoms for the vast majority of the tested cultivars. Although, promising results are available in the literature, the effectiveness of cryopreservation of grapevine is likely to be cultivar dependent and to require thorough optimization to be routinely used for germplasm maintenance.

ACKNOWLEDGEMENTS

The work is financed by a Macroprograma 2 grants to P.S.R and V.Q. and AgroVerde - Grapevine Germplasm Bank from Embrapa to P.S.R.

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Tables

Table 1. In vitro conservation of grapevine germplasm bank. Accessions are maintained as duplicates, sub-cultured at 100-day intervals, under 16-h photoperiod (75 µmol m^{-2} s^{-1}) and 23±2°C, in tubes containing 18 ml of Galzy medium. Ectophyte-free plants were rooted and ex vitro acclimated to a greenhouse-based backup bank.

Accession type	Number	In vitro (%)	Ex vitro acclimated
Vitis vinifera	755	660 (87.4)	660 (87.4)
Hybrids	661	545 (82.4)	545 (82.4)
Other species*	40	27 (67.5)	27 (67.5)
Total	1456	1232 (84.6)	1232 (84.6)

* Including species of *Vitis* distinct from *V. vinifera* and *Ampelopsis* species.

Table 2. Summary of cryopreservation results for 12 grapevine cultivars using the encapsulation-dehydration (Wang et al., 2003) and vitrification (Shatnawi et al., 2011) methodologies. The results correspond to average values (± standard deviation) for three independent experiments.

Cultivar	Species	Cryopreservation survival (%)	
		Encapsulation - dehydration	Vitrification
Cabernet sauvignon	*Vitis vinifera*	0	0.33333±0.58
Chardonnay	*V. vinifera*	9.37±0.76	0
Crimson Seedless	*V. vinifera*	7.35±1.25	0
Italian Riesling	*V. vinifera*	1.43±0.44	0
Merlot	*V. vinifera*	0	0
BRS Clara	hybrid	0	0
BRS Morena	hybrid	1.50±0.51	0
Isabella	*V. labrusca*	5.33±1.02	0
Riparia Gloire	*V. riparia*	8.45±1.87	1.33333±0.58
Paulsen P1103	*V. berlandieri* × *V. rupestris* (hybrid)	0	0
R110	*V. berlandieri* × *V. rupestris* (hybrid)	0	0
SO4	*V. berlandieri* × *V. riparia* (hybrid)	1.37±0.31	1±0.73

Figures

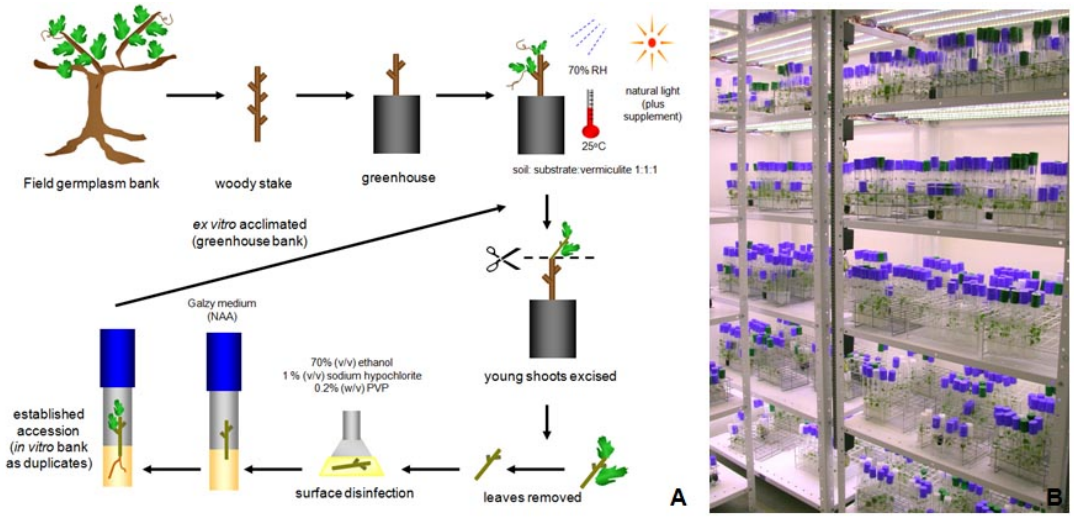

Fig. 1 In vitro conservation of grapevine germplasm. (A) Schematic representation of workflow for in vitro conservation of grapevine germplasm and production of plants with improved phytosanitary condition. (B) In vitro germplasm bank, maintained as duplicates, in Galzy medium, under 16-h photoperiod (75 µmol m^{-2} s^{-1}), provided by LED sources, and temperature of 23±3°C.

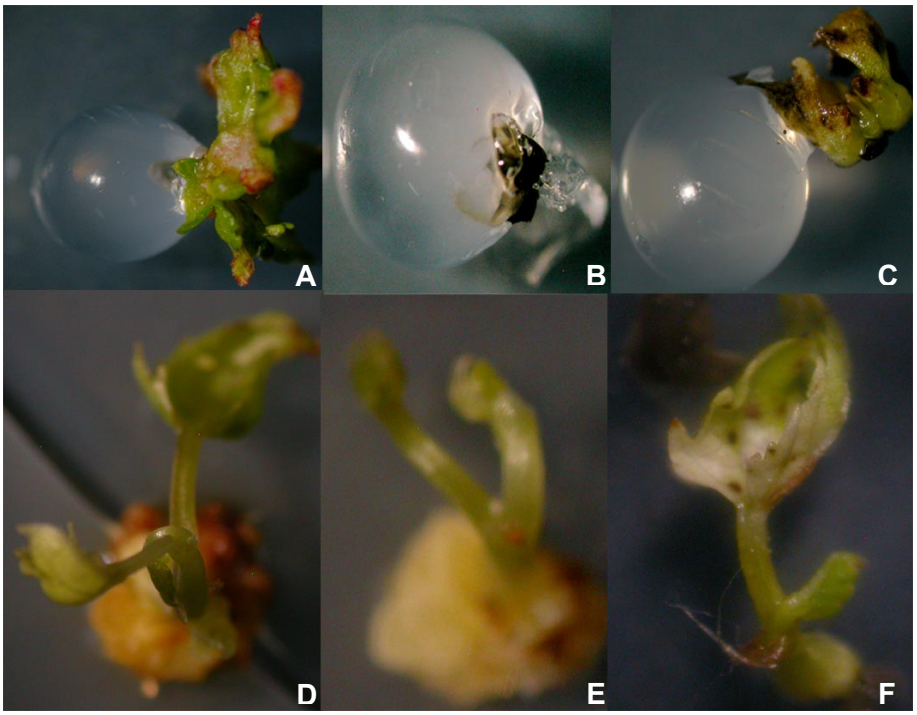

Fig. 2. Plant regeneration from grapevine shoot apices, after cryopreservation by encapsulation-dehydration (A, B, C) and vitrification (D, E, F). The cultivars correspond to 'Isabella' (A), 'Cabernet Sauvignon' (B), 'Chardonnay' (C), SO4 (D), 'Cabernet Sauvignon' (E) and 'Riparia Gloire' (F). Magnification of 40× (A, B, C) and 60× (D, E, F).