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CRYOPRESERVATION OF AXILLARY BUDS OF GRAPE (*Vitis vinifera*) *IN VITRO* PLANTLETS

Yanhua Zhao[1], Yongjie Wu[1]*, Florent Engelmann[2,3] and Mingde Zhou[4]

[1]Changli Institute of Pomology, Hebei Academy of Agricultural and Forestry Sciences, Hebei 066600, Changli Town, China. (Email: zhouxm@qh-user.he.cninfo.net).
[2]International Plant Genetic Resources Institute (IPGRI), Via dei Tre Denari, 472/a, 00057 Maccarese (Fiumicino), Rome, Italy.
[3]Institut de recherche pour le développement (IRD), BP 5045, 34032 Montpellier Cedex 01, France (present address).
[4]International Plant Genetic Resources Institute (IPGRI), East Asia Office c/o CAAS, 30 Bai Shi Qiao Road, Beijing100081, China.

Summary

Axillary buds sampled from *in vitro* plants of four grape varieties could withstand cryopreservation using the encapsulation-dehydration technique. Regrowth percentages ranged between 15 and 40%. Buds were encapsulated in alginate beads containing 0.5M sucrose, precultured at 5°C on media containing daily increasing sucrose concentrations from 0.1 to 1.0M, then desiccated to 21% moisture content (fresh weight basis) and frozen slowly (prefreezing to -40°C at 0.2°C/min followed by immersion in liquid nitrogen). The physiological state of the *in vitro* mother-plants and the freezing procedure employed dramatically influenced the results. Regrowth after cryopreservation was achieved only when buds were sampled from mother-plants that had been kept without subculture for 3 to 4 months. An additional one-month period of cold-acclimation of mother-plants at 5°C and a two-step freezing procedure improved growth recovery. The multiplication rate of shoots produced from cryopreserved buds was lower than that of controls during the first subcultures after thawing. However, the rooting ability of control and cryopreserved plantlets became similar from the fourth subculture onwards.

Keywords: grape; *Vitis vinifera*; cryopreservation; encapsulation-dehydration; cold-acclimation; freezing rate.

INTRODUCTION

Grape (*Vitis* spp.) genetic resources are traditionally conserved *ex situ* as plants in field collections. New *ex situ* conservation techniques, including *in vitro* conservation and cryopreservation have also been developed. Slow growth storage of *in vitro* plants is routinely used in various genebanks as a back up to grape field collections (see 14 for a review). As regards cryopreservation, preliminary experiments performed with dormant buds have resulted in limited survival after freezing (6). Apices sampled from *in vitro* plantlets have been successfully cryopreserved using the encapsulation-dehydration (9, 10) and vitrification techniques (8). Embryogenic cell suspensions have also been cryopreserved using a two-step

freezing protocol (3, 4).

The Changli Institute of Pomology holds 106 grape accessions in its field collection, 29 of which are duplicated *in vitro*. The Institute has established a cryopreservation unit that performs research on the development and application of cryopreservation for some species including apple (15) and kiwi (17). Research on grape cryopreservation has been initiated recently. This paper reports experiments examining some of the factors that affect the cryopreservation of grape shoot tips (parameters of the cryopreservation protocol; physiological state of donor plants); assessing the genetic stability of plants recovered from cryopreservation using isozyme patterns and several *in vitro* growth parameters; and testing the freezing protocol developed on several accessions.

MATERIALS AND METHODS

Plant Material

The plant material employed in this study consisted of *in vitro* plants of grape (*Vitis vinifera*) cultivars Cabernet franc, Chardonnay, Fengh 51 and rootstock LN 33. These cultures were established from accessions of the grape germplasm field collection maintained by the Changli Institute of Pomology (1).

Methods

In vitro culture

Grape *in vitro* plants were cultured on B5 medium (7) containing 0.2 mg/l indole acetic acid (IAA), 20 g/l sucrose and 5 g/l agar. Plantlets were grown on 50 ml medium in 100 ml glass flasks at 25±2°C, under white fluorescent light (52 µmol m^{-2} s^{-1}) with a 12 h light/12 h dark photoperiod.

Cryopreservation

In vitro mother-plants were kept without subculture for various durations and cold hardened, or not, for 1 month at 5°C under an 8 h light/16 h dark photoperiod. Axillary buds (approximately 2 mm long) were excised from these plantlets and encapsulated in 3% calcium alginate beads of approximately 5 mm of diameter, containing 0.5M sucrose. For preculture (performed at 5°C), encapsulated buds were transferred on media with daily increasing sucrose concentrations (0.1/0.3/0.7/1.0M). Beads were desiccated with silica gel (20 beads above 60 g of silica gel in 250 ml flasks) to various moisture contents, placed in 2 ml cryotubes and cryopreserved either rapidly by direct immersion in liquid nitrogen or slowly by two-step freezing (cooling at 0.2°C/min to various temperatures followed by immersion in liquid nitrogen) using a programmable freezer (HIT-4, China). After storage at -196°C for 24 h, samples were rewarmed for 2-3 min in a water-bath at 25°C and transferred onto solid medium under standard conditions for regrowth.

For measurement of moisture content, beads were placed in an oven at 85°C for 24 h. Moisture content was expressed on a fresh weight basis. Three replicates of twenty beads were employed to measure the water content. The moisture content of beads after desiccation durations between 0 and 6 h was measured in one experiment only (Table 1). The moisture content values thus obtained were used in further experiments, as the desiccation method employed consistently gave very reproducible results.

The regrowth percentage was defined as the percentage of treated buds that produced normal shoots 40 days after rewarming. Three replicates of twenty buds were used per experimental condition. Regrowth percentages are presented with their standard deviation.

Characteristics of *in vitro* cultures recovered from cryopreserved buds

The shoots produced from control buds (*i.e.* buds pretreated, desiccated but not cryopreserved) and cryopreserved buds were subcultured at 30 day intervals. They were observed for the color and shape of leaves produced and for their rooting ability, and their multiplication rate was measured. The multiplication rate corresponds to the number of new microcuttings that could be produced from each shoot at the time of a new subculture. Three replicates of 20 explants were used for each experimental condition.

RESULTS

Effect of desiccation duration on regrowth of buds

The regrowth percentage of buds decreased with decreasing bead moisture contents (Table 1). There was a dramatic drop in regrowth percentage between 5 and 6 hours of dehydration, corresponding to bead moisture contents of 21 and 18%, respectively. In further experiments, beads were desiccated for 5 hours to 21% moisture content.

Table 1. Effect of desiccation duration (hours) on bead moisture content (%, fresh weight basis) and regrowth (%) of grape (*Vitis vinifera* var. Cabernet franc) *in vitro* axillary buds. Buds were excised from *in vitro* mother plants which had been kept for 2 months without subculture. After encapsulation in alginate beads with 0.5M sucrose, buds were precultured at 5°C on media with daily increasing sucrose concentrations (0.1/0.3/0.7/1.0M).

Desiccation duration (h)	Bead moisture content (%)	Regrowth (%)
0	66±0.5	100
1	54±0.2	72±3
2	40±0.7	67±1
4	26±0.4	60±2
5	21±0.8	66±1
6	18±1.2	34±5

Effect of treatment of in vitro mother-plants and of cryopreservation procedure on regrowth of buds

No regrowth was achieved whatever the cryopreservation procedure employed with buds sampled from mother-plants kept without subculture for 2 months (Table 2). With buds sampled on mother-plants kept for 4 months without subculture, regrowth was noted only with those frozen using a two-step freezing procedure. When mother-plants were cold hardened and placed under a modified photoperiod for one month after a 4-month period without subculture, regrowth was obtained after both rapid and slow freezing but was much higher with slow freezing. After slow freezing, regrowth was considerably higher than with buds that had not been submitted to a cold-hardening treatment.

Table 2. Effect of treatment of *in vitro* mother-plants and of cryopreservation procedure on regrowth (%) of cryopreserved grape (*Vitis vinifera* var. Cabernet franc) *in vitro* axillary buds. Mother-plants were kept at 25°C without subculture for 2 or 4 months, followed, or not, by 1 month cold-hardening at 5°C. After encapsulation in alginate beads with 0.5M sucrose, buds were precultured at 5°C on media with daily increasing sucrose concentrations (0.1/0.3/0.7/1.0M), desiccated to 21.0±0.8% moisture content, then cooled rapidly by direct immersion in liquid nitrogen, or slowly (cooling to -40°C at 0.2°C/min followed by immersion in liquid nitrogen).

Treatment of *in vitro* mother-plants	Regrowth (%)	
	Rapid freezing	Slow freezing
2 months without subculture	0	0
4 months without subculture	0	29.3±0.8
4 months without subculture + 1 month cold-hardening at 5°C	12.5±1.2	40.9±0.2

Effect of treatment of in vitro mother-plants and of prefreezing temperature on regrowth of buds

When buds were sampled on mother-plants which had not been subcultured for 2 months, no regrowth was noted for prefreezing temperatures lower than -20°C and no buds withstood cryopreservation (Table 3). When mother-plants were kept for 3 months without subculture, regrowth of prefrozen buds was obtained for all prefreezing temperatures tested. After cryopreservation, regrowth increased progressively from 12.5% without prefreezing to 40.9% for a prefreezing temperature of -40°C.

Table 3. Effect of treatment of *in vitro* mother-plants and of prefreezing temperature on regrowth of control (-LN) and cryopreserved (+LN) grape (*Vitis vinifera* var. Cabernet franc) axillary buds. Mother-plants were kept at 25°C without subculture for 2 or 3 months followed by 1 month cold-hardening at 5°C. After encapsulation in alginate beads with 0.5M sucrose, buds were precultured at 5°C on media with daily increasing sucrose concentrations (0.1/0.3/0.7/1.0M), desiccated to 21.0±0.8% moisture content, then cooled slowly to various temperatures at 0.2°C/min followed by immersion in liquid nitrogen.

Prefreezing temperature (°C)	Regrowth (%)			
	2 months without subculture + 1 month cold-hardening		3 months without subculture + 1 month cold-hardening	
	-LN	+LN	-LN	+LN
0	80±2	0	80.0±0.3	12.5±0.1
-10	70±8	0	70.0±1.3	15.0±0.8
-20	57±3	0	57.0±2.0	34.5±0.7
-40	0	0	56.0±1.8	40.9±1.2
-60	0	0	-	-

Characteristics of in vitro cultures recovered from cryopreserved buds

The shape and color of leaves produced by control and cryopreserved buds were similar (data not shown). However, the multiplication rate and rooting ability were different between control and cryopreserved materials (Table 4). As for non-cryopreserved control shoots, the multiplication rate increased progressively from 3 after the first subculture to reach a value of 8 from the third and fourth subcultures. By contrast, it is only at the end of the third subculture that shoots produced from cryopreserved buds reached a multiplication rate of 2. It increased during the fourth subculture, reaching a value of 3, *i.e.* the value observed with control shoots during the first subculture. From the sixth subculture onwards, depending on the accession, the multiplication rate of shoots originating from cryopreserved material became equivalent to that of non-cryopreserved controls. As regards rooting ability, 100% of control plantlets could produce roots from the first subculture onwards, whereas, with cryopreserved material, this was achieved only after the fourth subculture.

Table 4. Effect of the number of subcultures on the multiplication rate of grape (*Vitis vinifera* cv. LN33) *in vitro* plantlets originating from control (-LN) and cryopreserved (+LN) buds. Mother-plants were kept at 25°C without subculture for 3 months followed by 1 month cold-hardening at 5°C. After encapsulation in alginate beads with 0.5M sucrose, buds were precultured at 5°C on media with daily increasing sucrose concentrations (0.1/0.3/0.7/1.0M), desiccated to 21.0±0.8% moisture content, then cooled slowly at 0.2°C/min to -40°C followed by immersion in liquid nitrogen.

Number of subcultures	Multiplication rate		Rooting (%)	
	-LN	+LN	-LN	+LN
1	3.0±0.3	1.0±0.2	100	0
2	5.0±2.0	1.0±0.2	100	0
3	8.0±2.6	2.0±0.2	100	0
4	8.5±2.5	3.0±0.3	100	100
6	8.5±2.0	8.5±2.5	100	100

Cryopreservation of buds of additional grape varieties

In addition to Cabernet Franc, three additional accessions were cryopreserved using the protocol established. Regrowth percentages, based on three independent experiments with 20 buds/condition, ranged between 15 and 40% (Table 5).

Table 5. Regrowth (%) of control (-LN) and cryopreserved (+LN) buds of different grape varieties. Mother-plants were kept at 25°C without subculture for 3 months followed by 1 month cold-hardening at 5°C. After encapsulation in alginate beads with 0.5M sucrose, buds were precultured at 5°C on media with daily increasing sucrose levels (0.1/0.3/0.7/1.0M), desiccated to 21.0±0.8% moisture content, then cooled slowly at 0.2°C/min to -40°C followed by immersion in liquid nitrogen.

	Regrowth (%)	
Variety	-LN	+LN
Cabernet franc	100	15±2
Chardonnay	100	35±2
LN33	100	40±1
Fengh-51	100	26±1

DISCUSSION

In this study, a cryopreservation protocol using the encapsulation-dehydration technique was established for axillary buds sampled on *in vitro* grape plantlets, which included the following successive steps: mother-plants were kept without subculture for 3 to 4 months, then cold acclimated for one month at 5°C. Axillary buds sampled on the plantlets were encapsulated in alginate with 0.5M sucrose, precultured on media with daily increasing sucrose concentrations from 0.1 to 1.0M, desiccated to 21% moisture content (fresh weight basis), then prefrozen slowly (0.2°C/min) to -40°C and immersed in liquid nitrogen. Regrowth percentages achieved with the four accessions tested ranged between 15 and 40%.

This study underlined the importance of the physiological state of the mother-plants and of various parameters of the freezing protocol on cryopreservation success. Concerning the physiological state of the mother-plants, regrowth after cryopreservation was achieved only when buds were sampled on plants which had been kept without subculture for 3 to 4 months. A similar observation has already been made during cryopreservation of apple (16, 18) and kiwi (17) apices. The positive effect of an extended period without subculture may be related notably to a decrease in water content of the apices. Indeed, Wu *et al.* (16) showed that the water content of apple apices decreased from about an initial 85-88% (fresh weight basis) to around 75% after 12 weeks without subculture.

Another important parameter contributing to improved regrowth of cryopreserved apices of cold tolerant species is the cold acclimation of mother-plants (5, 11, 13). In the case of grape, a one month cold acclimation with a shortened day-length of the photoperiod, following the period without subculture, greatly improved the results by allowing limited regrowth after rapid freezing and ensuring high regrowth after slow freezing. One set of conditions only (5°C for one month, under an 8 h light/16 h dark photoperiod) was used in the current study for cold acclimation treatment. Modifications in the conditions, including the duration, temperature and photoperiod employed during the cold treatment could further improve the results, as demonstrated notably with pear shoot tips, for which survival after cryopreservation increased from 0 to 80% after exposure of mother-plants at 0°C under constant illumination for 0 and 8 weeks, respectively (12).

Our observations concerning some of the parameters of the cryopreservation protocol confirmed those of Plessis *et al.* (9, 10). Grape axillary buds are sensitive to high sucrose concentrations, thus necessitating a progressive increase in sucrose level during preculture. They are also sensitive to desiccation, since desiccation of beads from 66 to 21% MC resulted in a drop in regrowth percentage from 100 to 66%. Two-step freezing resulted in much higher

regrowth percentages than rapid freezing, suggesting that some freeze-induced dehydration during slow prefreezing was still necessary. In our experiments, buds were prefrozen to -40°C only. Lower prefreezing temperatures should be examined, since Plessis *et al.* (9) demonstrated a positive effect of prefreezing temperatures of -80 or -100°C on regrowth of grape buds.

The results obtained with *in vitro* growth parameters studied confirm the observations concerning the stability of cryopreserved material made by numerous authors using various techniques on an increasing number of plant materials (5). The markedly slower regrowth of cryopreserved buds observed in our experiments could be a consequence of cryopreservation being stressful such that there is the need for a period of recovery before outgrowth. A systematic histological study of the buds during the whole cryopreservation process would allow the identification of the critical steps in the procedure, thus facilitating subsequent improvements to be made (2).

In conclusion, additional experiments should be performed to improve the cryopreservation protocol established in this study before it can be routinely applied for long-term conservation of grape germplasm. Other cryopreservation techniques should also be tested, including the two- and three-step vitrification protocols proposed by Matsumoto and Sakai (8) which produced relatively high recovery rates with the first few grape accessions employed.

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