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CRYOPRESERVATION OF KIWI SHOOT TIPS

Yongjie Wu[1]*, Yanhua Zhao[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]IPGRI, Via dei Tre Denari, 472/a, 00057 Maccarese (Fiumicino), Rome, Italy.
[3]IRD, BP 5045, 34032 Montpellier Cedex 01, France (present address).
[4]IPGRI, East Asia Office c/o CAAS, 30 Bai Shi Qiao Road, Beijing100081, China.

Summary

In this study, the effect of various factors, including the physiological state of mother-plants and parameters of the encapsulation-dehydration protocol (cryoprotective treatment, freezing protocol) on the regrowth of shoot tips of kiwi *in vitro* plantlets was studied. The optimal protocol established was the following: shoot tips were sampled on mother-plants after a one month cold-acclimation period at 5°C. For preculture, which was performed at 5°C, encapsulated shoot tips were transferred at 24-hour intervals on solid media with increasing sucrose concentrations, from 0.5 to 1.0M. Beads were then desiccated to 26% moisture content (fresh weight basis), prefrozen from 0°C to -40°C at 0.2°C/min and immersed rapidly in liquid nitrogen. No differences were noted between *in vitro* plantlets produced from cryopreserved and control shoot tips as regards leaf color, average height, multiplication rate and peroxidase zymogram pattern. Apices of 3 kiwi accessions were frozen using the above protocol with regrowth percentages ranging between 22 and 56%.

Keywords: *Actinidia* spp., cryopreservation; shoot tip; encapsulation-dehydration; preculture; freezing protocol.

INTRODUCTION

China is the center of origin of the genus *Actinidia* which comprises around 60 different species, including the kiwifruit. Kiwi germplasm is usually conserved as whole plants in field collections. *In vitro* conservation protocols have been developed for slow growth storage (3, 7). Cryopreservation was first attempted by Jian and Sun (5) with stem segments of *A. chinensis*, but only callus regrowth was achieved. Recently, Bachiri *et al.* (1) used the encapsulation-dehydration technique for freezing kiwi shoot tips. Their study focused more specifically on the importance of the nature and concentration of growth regulators present in the culture medium, especially during the post-thaw recovery step.

At the Changli Institute of Pomology, 7 kiwi accessions are currently maintained in the field collection, 4 of which are duplicated *in vitro*. The institute has established a cryopreservation unit, which performs research on the development and application of cryopreservation protocols for fruit tree species. Such a research project has been initiated recently on kiwi. The objectives of the current work are to study some of the factors that

affect the cryopreservation of kiwi shoot tips, including several parameters of the cryopreservation protocol and the physiological state of donor plants; to check the genetic stability of plants recovered from cryopreservation using isozymes and several *in vitro* growth parameters; and to test the cryopreservation protocol developed on several kiwi accessions.

MATERIALS AND METHODS

Plant material

The plant material employed in this study consisted of *in vitro* plantlets of *A. deliciosa* 'Tomuri M' and 'Tomuri F' and of *A. chinensis* var. *chinensis*. These cultures were established by introducing *in vitro* buds sampled on accessions from the kiwi germplasm field collection maintained by the Changli Institute of Pomology and multiplied *in vitro* according to the protocol established by Guo and Li (4).

Methods

In vitro culture

Plantlets and shoot tips were cultured on a Murashige and Skoog medium (MS, 8) containing 0.5mg/l benzyladenine (BA), 0.05mg/l naphtalene acetic acid (NAA), 30g/l sucrose and 5g/l agar. Plantlets were grown on 50 ml medium in 100 ml glass flasks at 25°C, under white fluorescent light (52 $\mu mol\ m^{-2}\ s^{-1}$) with a 12 h light/12 h dark photoperiod.

Cryopreservation

In vitro mother-plants were kept without subculture under the above culture conditions for 50 days, then cold-hardened, or not, for 1 month at 5°C under an 8 h light/16 h dark photoperiod. Shoot tips (approx. 2 mm long) were excised from the mother-plants and encapsulated in 3% calcium alginate beads of approximately 5 mm of diameter, containing 0.5M sucrose. For preculture, beads were placed at 5°C either directly on solid media with sucrose concentrations between 0.5 to 1.2M for 1 to 5 days, or transferred every 12 or 24 hours on media with increasing sucrose concentrations (between 0.5 and 1.2M). One experiment compared the effect of encapsulation of shoot tips during preculture and another one the efficiency of preculture with liquid and solid medium. Beads were then desiccated with silica gel (250 ml flasks with 60 g silica gel for 20 beads) to various moisture contents. Dried beads were placed in 2 ml cryotubes and cryopreserved either rapidly by direct immersion of the cryotubes in liquid nitrogen or slowly by two-step freezing (prefreezing to temperatures between -20 and -40°C at 0.2°C/min followed by immersion in liquid nitrogen). Samples were rewarmed for 2-3 min in a water-bath at 25°C and transferred for regrowth on solid medium under standard culture conditions.

Regrowth was defined as the percentage of shoot tips that produced normal shoots 40 days after rewarming. Three replicates of 20 apices were used per experimental condition. Results are presented with their standard deviation.

Characteristics of *in vitro* cultures recovered from cryopreserved shoot tips

The shoots produced from control and cryopreserved shoot tips of *A. deliciosa* 'Tomuri M' were subcultured under standard conditions at 40 days intervals and observed for the following characteristics: color and shape of leaves produced, growth of shoots and multiplication rate. 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 apices were used per experimental condition. Results are presented with their standard deviation.

The peroxidase zymograms of control and cryopreserved *in vitro* plantlets of *A. deliciosa* 'Tomuri M' were compared. Leaves were sampled on plantlets originating from control and cryopreserved shoot tips at the end of a subculture. One gram of leaves was homogenized for 2 min in 2 ml of Tris-Gly buffer (pH 8.3) at 0°C. The homogenate was filtered through 4 layers of cheesecloth and centrifuged for 10 min at 1000 g. Fifty µl of supernatant were used for polyacrylamide gel electrophoresis, with a 7.5% separation gel (pH 8.9) and a 4% stacking gel (pH 6.7). Electrophoresis was performed for 5 hours at 30 mA. The gels were then stained with benzidine for observation.

RESULTS

Effect of preculture duration and sucrose concentration on regrowth of shoot tips

The higher sucrose concentrations (1.0 and 1.2M) and longer (3 and 5 days) preculture periods tested were toxic to shoot tips (Table 1). Indeed, regrowth of shoot tips after one day of preculture decreased from 100% with 0.5M sucrose to 60% with 1.0 and 1.2M sucrose. After 3 days of preculture, regrowth of shoot tips dropped to 15 and 30% with 1.0 and 1.2M sucrose, respectively, and was nil after 5 days with these two sucrose concentrations.

Table 1. Effect of preculture duration and sucrose concentration of preculture medium on regrowth (%) of *Actinidia deliciosa* 'Tomuri M' shoot tips. Shoot tips were excised from plantlets 50 days after the last subculture, then encapsulated in alginate beads containing 0.5M sucrose. Preculture was performed at 5°C.

Sucrose concentration (M)	Preculture duration (days)		
	1	3	5
0.5	100	90±11	70±2
0.7	80±1	60±3	50±6
1.0	60±1	15±1	0
1.2	60±6	30±3	0

Effect of encapsulation and of sequence of media employed during preculture on regrowth of shoot tips

Encapsulated shoot tips withstood high final sucrose concentrations much better when the increase in sucrose concentration was progressive during preculture than when they were precultured directly with a high sucrose concentration. Shoot tips displayed 95% regrowth when the final sucrose concentration reached was 1M (Table 2), compared to only 15% when they were precultured directly with this sucrose concentration (Table 1). Regrowth of non-encapsulated shoot tips decreased very rapidly with increasing final sucrose concentrations (Table 2), from 100% (final sucrose concentration of 0.5M) to 0% (final sucrose concentration of 1.2M).

Table 2. Effect of encapsulation and of sequence of media employed during sucrose preculture on regrowth (%) of *Actinidia deliciosa* 'Tomuri M' shoot tips. Shoot tips were excised from plantlets 50 days after the last subculture, then encapsulated in alginate beads containing 0.5M sucrose. Encapsulated shoot tips were transferred to a new preculture medium with higher sucrose concentration every 24 hours. Preculture was performed at 5°C.

Sequence of preculture media	Regrowth (%)	
	With encapsulation	Without encapsulation
0.5M	100	100
0.5M/0.7M	100	80±2
0.5M/0.7M/1.0M	95±6	20±10
0.5M/0.7M/1.0M/1.2M	50±5	0

Effect of temperature of preculture and sequence of transfers on preculture media on regrowth of shoot tips

Regrowth of shoot tips was much lower with both preculture sequences tested when preculture was performed at 25°C in comparison with 5°C (Table 3). At 5°C, regrowth was very high (95-100%) with both preculture sequences.

Table 3. Effect of temperature of preculture and sequence of transfers on preculture media with increasing sucrose concentrations on regrowth (%) of *Actinidia deliciosa* 'Tomuri M' shoot tips. Shoot tips were excised from plantlets 50 days after the last subculture, then encapsulated in alginate beads containing 0.5M sucrose. Encapsulated shoot tips were transferred to a new preculture medium with higher sucrose concentration every 12 or 24 hours.

Preculture sequence	Temperature of preculture	
	25°C	5°C
0.5M 12 h/0.7M 12 h/1.0M 12 h	60±3	100
0.5M 24 h/0.7M 24 h/1.0M 24 h	40±5	95±1

Effect of dehydration duration on bead moisture content and regrowth of cryopreserved shoot tips

The moisture content of beads decreased from 78% without dehydration to 18% after 6 hours of dehydration (Table 4). Regrowth of control shoot tips remained high (80%) until 26% bead moisture content, but dropped drastically afterwards, to 20% for a bead moisture content of 18%. No regrowth was achieved with cryopreserved shoot tips, whatever the moisture content tested.

Table 4. Effect of dehydration duration on bead moisture content (%, fresh weight basis) and on regrowth (%) of control (-LN) and cryopreserved (+LN) *Actinidia deliciosa* 'Tomuri M' shoot tips. Shoot tips were excised from plantlets 50 days after the last subculture, then encapsulated in alginate beads containing 0.5M sucrose. Encapsulated shoot tips were precultured at 5°C on media with daily increasing sucrose concentrations following the sequence: 0.5/0.7/1.0M. After dehydration with silica gel, beads were immersed rapidly in liquid nitrogen.

Dehydration duration (hours)	Bead moisture content (%)	Regrowth (%)	
		-LN	+LN
0	78±5	100	0
1	64±3	100	0
2	40±3	80±1	0
4	26±2	80±6	0
6	18±3	20±10	0

Effect of cold acclimation and of preculture procedure on regrowth of cryopreserved shoot tips

Regrowth of cryopreserved shoot tips was possible only after cold acclimation of mother-plants (Table 5). The preculture procedure had no effect on regrowth of control shoot tips. By contrast, regrowth of cryopreserved shoot tips was much higher after preculture on solid medium.

Table 5. Effect of cold acclimation of *in vitro* mother-plants and of preculture procedure on regrowth (%) of control (-LN) and cryopreserved (+LN) *Actinidia deliciosa* 'Tomuri M' shoot tips. Shoot tips were excised from plantlets 50 days after the last subculture, then submitted, or not, to a 30-day cold acclimation period. They were then encapsulated in alginate beads containing 0.5M sucrose and precultured at 5°C, either on solid media with daily increasing sucrose concentrations (0.5/0.7/1.0M) or in liquid medium with 0.5M sucrose for one day, then on solid media with daily increasing sucrose concentrations (0.7/1.0M). After dehydration with silica gel to 26% moisture content, beads were prefrozen from 0°C to -40°C at 0.2°C/min, then immersed rapidly in liquid nitrogen.

	Regrowth (%)			
	Without cold acclimation of mother-plants		With cold acclimation of mother-plants	
Sucrose preculture	-LN	+LN	-LN	+LN
Solid medium	100	0	100	54±5
Liquid medium	100	0	100	22±6

Effect of cold acclimation of in vitro mother-plants and of prefreezing temperature on regrowth of cryopreserved shoot tips

Without cold acclimation of mother-plants, regrowth of prefrozen shoot tips decreased dramatically with decreasing prefreezing temperatures and no regrowth was achieved after cryopreservation (Table 6). With cold acclimation of mother plants, regrowth of prefrozen shoot tips remained high for both prefreezing temperatures tested. After cryopreservation, regrowth of shoot tips increased from 35% for a prefreezing temperature of -20°C to 55% for a prefreezing temperature of -40°C.

Table 6. Effect of cold acclimation of *in vitro* mother-plants and of prefreezing temperature on regrowth (%) of prefrozen (-LN) and cryopreserved (+LN) *Actinidia deliciosa* 'Tomuri M' shoot tips. Shoot tips were excised from plantlets 50 days after the last subculture, then submitted, or not, to a 30-day cold acclimation period. They were then encapsulated in alginate beads containing 0.5M sucrose and precultured at 5°C on solid media with daily increasing sucrose concentrations (0.5/0.7/1.0M). After dehydration with silica gel to 26% moisture content, beads were prefrozen at 0.2°C/min to various temperatures, then immersed rapidly in liquid nitrogen.

	Regrowth (%)			
	Without cold acclimation of mother-plants		With cold acclimation of mother-plants	
Prefreezing temperature	-LN	+LN	-LN	+LN
0°C	100	0	100	0
-20°C	16±3	0	78±3	35±5
-40°C	0	0	72±4	55±3

Characteristics of in vitro cultures recovered from cryopreserved shoot tips

No differences were noted between *in vitro* plantlets regenerated from control and cryopreserved shoot tips for the three characteristics studied, leaf color, average height of plantlets at the end of a subculture and multiplication rate (Table 7). The patterns of the peroxidase zymograms were similar between control and cryopreserved material (data not shown).

Table 7. Leaf color, average height of *in vitro* plantlets and multiplication rate of cultures regenerated from control (-LN) and cryopreserved (+LN) shoot tips of *Actinidia deliciosa* 'Tomuri M' and 'Tomuri F' shoot tips. Shoot tips were excised from plantlets kept for 50 days without subculture, then submitted to a 30-day cold acclimation period. They were then encapsulated in alginate beads containing 0.5M sucrose and precultured at 5°C on solid media with daily increasing sucrose concentrations (0.5/0.7/1.0M). After dehydration with silica gel to 26% moisture content, beads were prefrozen at 0.2°C/min to -40°C, then immersed rapidly in liquid nitrogen.

	Leaf color	Average height of plantlets (cm)	Multiplication rate
-LN	Dark green	2.5±2.0	9.5
+LN	Dark green	2.4±0.8	10

Effect of freezing procedure on regrowth of shoot tips of different kiwi accessions

No (*A. deliciosa* 'Tomuri M' and *A. chinensis* var. *chinensis*) or only limited regrowth (Tomuri F) was achieved after freezing using a rapid freezing procedure (Table 8). Regrowth was higher with all three accessions when shoot tips were frozen using a two-step freezing protocol, ranging between 22 (*A. deliciosa* 'Tomuri F') and 56% (*A. deliciosa* 'Tomuri M').

Table 8. Effect of freezing procedure on regrowth (%) of cryopreserved shoot tips of *Actinidia deliciosa* 'Tomuri F' and 'Tomuri M' and *A. chinensis* var. *chinensis*. Shoot tips were excised from plantlets after 50 days without subculture and a 30-day cold acclimation period at 5°C. They were then encapsulated in alginate beads containing 0.5M sucrose and precultured at 5°C in liquid medium with 0.5M sucrose for one day, then on solid media with daily increasing sucrose concentrations (0.7/1.0/1.2M). After dehydration with silica gel to 26% moisture content, beads were either frozen rapidly by direct immersion in liquid nitrogen (rapid freezing) or prefrozen from 0°C to -40°C at 0.2°C/min, then immersed rapidly in liquid nitrogen (two-step freezing).

Freezing procedure	Regrowth (%)		
	A. deliciosa 'Tomuri F'	*A. deliciosa* 'Tomuri M'	*A. chinensis*
Rapid freezing	12±1	0	0
Two-step freezing	22±5	56±3	38±8

DISCUSSION

The optimal protocol developed in this study included a preculture of encapsulated kiwi shoot tips at 5°C on solid medium with daily increasing sucrose concentration from 0.5 to 1.0M followed by desiccation to 26% moisture content (fresh weight basis), prefreezing from 0°C to -40°C at 0.2°C/min and rapid immersion in liquid nitrogen. The experiments performed allowed to highlight the importance of the physiological state of mother-plants and of parameters of the encapsulation-dehydration protocol (cryoprotective treatment, freezing protocol) on the regrowth of shoot tips of kiwi *in vitro* plantlets.

As already indicated by Bachiri *et al.* (1), kiwi shoot tips proved to be sensitive to sucrose and only preculture with progressively increasing sucrose concentrations led to high survival. A comparable preculture treatment has been employed for freezing grape (9, 10) and coffee (6) apices which were highly sensitive to high sucrose concentrations. Toxicity of sucrose to kiwi shoot tips increased when preculture was performed at higher temperature (25°C *vs.* 5°C) and when the duration of culture with each sucrose concentration was extended.

The positive effect of cold acclimation of kiwi mother-plants, which has already been demonstrated when cryopreserving apices of cold tolerant species (2, 11, 13) was also highlighted in this study. In our experiments, one set of conditions only (5°C for one month, under an 8 h light/16 h dark photoperiod) has been experimented for the cold acclimation treatment. It is possible that modifications in the conditions, including the duration, temperature and photoperiod employed during the cold treatment could further improve the results, as demonstrated notably for the temperature and duration with pear shoot tips (12).

In contrast with the protocol developed by Bachiri *et al.* (1) which employed rapid freezing, slow, controlled prefreezing of apices dramatically improved regrowth in our experiments. This might be due to the fact that in the former protocol, beads were desiccated

to around 17% MC (fresh weight basis) before freezing, whereas, in our case, beads were frozen at 26% MC. An additional freeze-induced dehydration during slow prefreezing was thus necessary to decrease bead moisture content before immersion of samples in liquid nitrogen. This difference in the freezing protocol might explain the generally lower regrowth percentages obtained in our study.

The positive results obtained with peroxidase patterns and the *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 (2).

In conclusion, the encapsulation-dehydration technique can be successfully employed for freezing kiwi apices despite the intermediate regrowth percentages achieved. However, it might be worth experimenting other cryopreservation techniques such as vitrification or encapsulation-vitrification, which have produced very good results with an increasing number of species (2).

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