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Conservation of almond germplasm by cryopreservation

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SUMMARY – Almond is an important nut crop in Australia. The industry is steadily growing due to an increase in tree numbers and average tree productivity. A major research programme is aiming to further increase both productivity and quality, and it is also necessary to systematically conserve the almond germplasm in Australia. However, the costs of maintaining the gene pool by traditional methods are high and an alternative approach is necessary. This paper reports the results of storing almond germplasm by cryopreservation. Shoot tips of two almond scion cultivars, 'Ne Plus Ultra' and 'Nonpareil 15-1', and an almond/peach hybrid rootstock were successfully cryopreserved using a one-step vitrification technique. Shoot tips, 2-2.5 mm long, were excised, cold-hardened, precultured, and dehydrated in a vitrification solution adopted from Sakai *et al.* (1990). They were then stored under liquid nitrogen (LN) for up to 6 months. After thawing and culturing the shoot tips, shoot regeneration was usually observed within 2-3 weeks. Shoot survival was 77, 52, and 78% for 'Ne Plus Ultra', 'Nonpareil 15-1' and the hybrid rootstock respectively. All shoots had the same morphological appearance as the initial *in vitro* cultures. However, the possibility of genetic change during long-term preservation is being investigated.

Key words: Almond, cryopreservation, vitrification.

RESUME – *"Conservation du germoplasme d'amandier par cryopréservation". La récolte des noyaux d'amande est une production agricole importante en Australie. L'industrie augmente fermement car le nombre des arbres devient plus nombreux et la productivité moyenne des arbres monte. Un programme de recherches majeur a pour but l'augmentation de la productivité et de la qualité et il est aussi nécessaire de conserver systématiquement le germoplasme de l'amande en Australie. Pourtant, les coûts de l'entretien du pool de gènes par les méthodes traditionnelles sont élevés et une approche alternative est nécessaire. Cet article rapporte les résultats du stockage de germoplasme d'amande par cryopréservation. Les bouts de pousses de deux cultivars de scions d'amande, 'Ne Plus Ultra' et 'Nonpareil 15-1', et un porte-greffe hybride d'amandier/pêcher ont été cryopréservés avec succès, par l'emploi de la technique de vitrification en un seul pas. Des bouts de pousses, 2-2,5 mm de longueur, ont été découpés, durcis au froid, précultivés, et déshydratés dans une solution de vitrification adoptée de Sakai* et al. *(1990). Ensuite, ils étaient stockés dans de l'azote liquide (LN) jusqu'à six mois. Après décongélation les bouts de pousses ont été cultivées et la régénération a été observée ordinairement en moins de 2-3 semaines. La survie des pousses était de 77, 52, et 78% respectivement pour 'Ne Plus Ultra', 'Nonpareil 15-1' et l'hybride sauvage. Toutes les pousses avaient la même apparence morphologique que les cultures initiales* in vitro. *Pourtant, la possibilité de changement génétique pendant la préservation de longue durée est en investigation.*

Mots-clés : *Amande, cryopréservation, vitrification*.

Introduction

Many different varieties of almond scions and rootstocks are used by growers, but genetic resources have not been conserved systematically. Preservation of germplasm as mature trees requires extensive space and labour (Sakai and Nishiyama, 1978), and the material is open to attack by pests and pathogens. Storage as seeds suffers from viability loss (Kartha *et al*., 1979), and furthermore, seeds of almond do not breed true because they are derived from cross-pollination (Micke and Kester, 1978).

With the increasing interest in genetic engineering of plants, the preservation of cultured cells and meristems with unique characters is assuming greater importance (Matsumoto *et al.*, 1995), and will require the development of techniques to successfully store this material (Niino *et al.*, 1992b). *In vitro* cell culture has been used for germplasm preservation, but spontaneous mutations (Stephonkus, 1985; Elleuch *et al*., 1998) and changes in methylation (Kaeppler and Phillips, 1993; Harding *et al*., 1996) have been reported to occur. Cryopreservation offers the possibility for long-term storage with maximal phenotypic and genotypic stability using a minimum of space and maintenance (Stephonkus, 1985; Takagi *et al.*, 1997), and is therefore an

appropriate strategy for long term preservation of germplasm.

The vitrification method for preparing plant tissues for cryopreservation has been applied to woody species, such as apple, pear, cherry and sweet cherry (Niino *et al.*, 1992a,b, 1997). This paper reports on cryopreservation of shoot tips from *in vitro* cultures of two almond scions and one hybrid rootstock.

Materials and methods

Plant material

In vitro-cultured shoots of *Prunus dulcis* cvs 'Ne Plus Ultra' and 'Nonpareil 15-1' and an almond/peach hybrid (*P. dulcis* cv. 'Titan' × *P. persica* cv. 'Nemaguard') rootstock were used in this study.

Stock cultures of 'Ne Plus Ultra' and the hybrid rootstock were maintained on MS medium (Murashige and Skoog, 1962) supplemented with 0.049 µM indole-3-butyric acid (IBA), 4.44 µM 6-benzyl-aminopurine (BAP), 0.088 M sucrose, and 0.7% (w/v) agar (Difco Bitek). Stock cultures of 'Nonpareil 15-1' were maintained on AP medium (Almehdi and Parfitt, 1986) supplemented with 0.049 µM IBA, 3.1 µM BAP, 0.058 M sucrose, and 0.7% (w/v) agar. The pH of both media was adjusted to 5.7 prior to adding agar and autoclaving at 121°C for 20 min. All cultures were maintained in 250 mL polypropylene pots containing 50 mL of culture media under metal halide lights (40 µmol/m^2/s) with a 16 h photoperiod at 25 ± 3°C and subcultured every 4-5 weeks.

Cold-hardening and preculture

Three-week-old shoot cultures were exposed to 4°C for up to 42 days under 15 µmol/m^2/s cool white fluorescent lights with a 10 h photoperiod. Shoot tips with 3-5 leaf primordia (2-2.5 mm long, 1-1.5 mm base diameter) were dissected from cold-hardened shoots and precultured on the appropriate medium supplemented with 0.7 M sucrose and 0.7% (w/v) agar for 1 day under the same conditions.

Vitrification

Following preculture, groups of ten shoot tips were transferred to 2 mL cryotubes (Nalgene) containing 1 mL of a vitrification solution (PVS2) (Sakai *et al.*, 1991) and incubated at 25°C for various period of time. PVS2 contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide prepared in either liquid MS for 'Ne Plus Ultra' and the hybrid rootstock, or AP for 'Nonpareil 15-1', supplemented with 0.4 M sucrose, at pH 5.8. After replacing the PVS2 once during the incubation period, the shoot tips were finally suspended in 0.5 mL of fresh PVS2 and the cryotubes were stored under liquid nitrogen (LN) at –196°C.

Regeneration

Shoot tips were rapidly thawed from LN in a water bath at 30°C, and washed twice with either liquid MS or AP medium as appropriate, supplemented with 1 M sucrose. They were then transferred to either MS medium with 0.008 M sucrose and 0.9% (w/v) agar but excluding NH_4NO_3 (Kuriyama *et al.*, 1990) for 'Ne Plus Ultra' and the hybrid rootstock, or AP medium with 0.058 M sucrose and 0.9% (w/v) agar but excluding $(NH_4)_2SO_4$ for 'Nonpareil 15-1', and cultured with a 16-h photoperiod (40 µmol/m^2/s) at 25 ± 3°C. Survival was recorded as the percentage of shoot tips that produced at least 1 new shoot 4 weeks after thawing. Shoots were then transferred to either standard MS or AP medium as appropriate, excluding plant growth regulators, for a further 1 or 2 weeks before maintenance as stock cultures as described above.

Statistical analysis

Each treatment was replicated 4 times with 10 shoot tips per replicate, and the results were analysed by Duncan's new multiple range test using PlotIT (Scientific Programming Enterprises, Haslett, MI, US, version 3.20l).

Results

Shoot tips of 'Ne Plus Ultra' were used to determine both the optimum cold-hardening period at 4°C, and the optimum dehydration time in PVS2 at 25°C before storing under LN. For cold-hardening, the highest survival of 87.5% occurred after 21 days, although there was no significant difference between 7 and 21 days. After 28 days, survival was significantly decreased (Fig. 1).

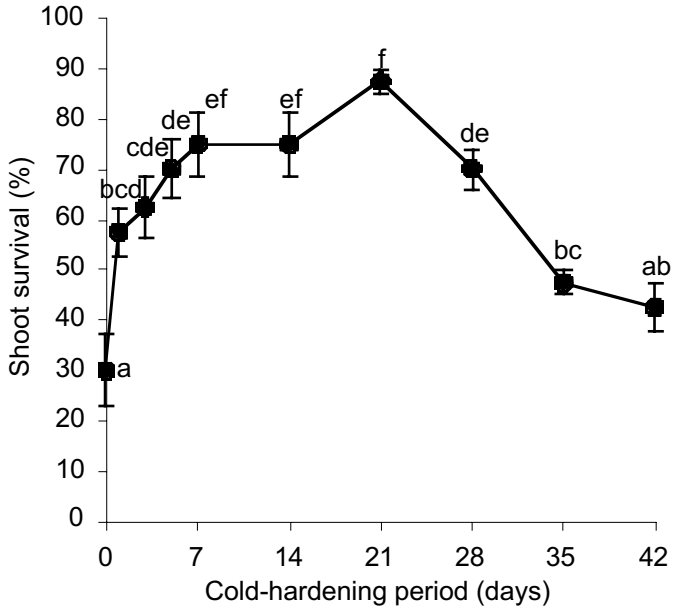

Fig. 1. Effect of cold-hardening period of shoot cultures at 4°C on shoot survival of 'Ne Plus Ultra' stored in LN by vitrification. Excised shoot tips from cold-hardened shoots were precultured at 4°C for 1 day on MS agar medium supplemented with 0.7 M sucrose. The shoot tips were dehydrated using PVS2 for 45 min before storage under LN. Data were recorded after 3 days of storage in LN. The bars represent mean ± SE. Means with the same letter are not significantly different at the 5% level using Duncan's new multiple range test.

The survival of shoot tips, after removal from LN, increased with the time of incubation in PVS2. The highest survival of 87.5% occurred after 45 min (Fig. 2). Control shoot tips, treated with PVS2, but not immersed in LN showed survival of between 85-100% (Fig. 2). Longer periods of incubation in PVS2 decreased the survival of both LN-treated and control shoot tips.

For 'Nonpareil 15-1' and the hybrid rootstock, using the same vitrification procedures as for 'Ne Plus Ultra', the highest survival was 60.0% at 45 min incubation time in PVS2 and 72.5% at 60 min incubation time in PVS2 respectively (Table 1).

For long-term preservation, up to 180 days, the survival of cryopreserved shoot tips was between 51.7-77.5%. The survival of 'Ne Plus Ultra' was slightly decreased by the time of preservation from 87.5% at 3 days of storage to 76.7% at 180 days of storage. There was no significant decrease in survival after 180 days of storage for 'Nonpareil 15-1' and the hybrid rootstock (Table 2).

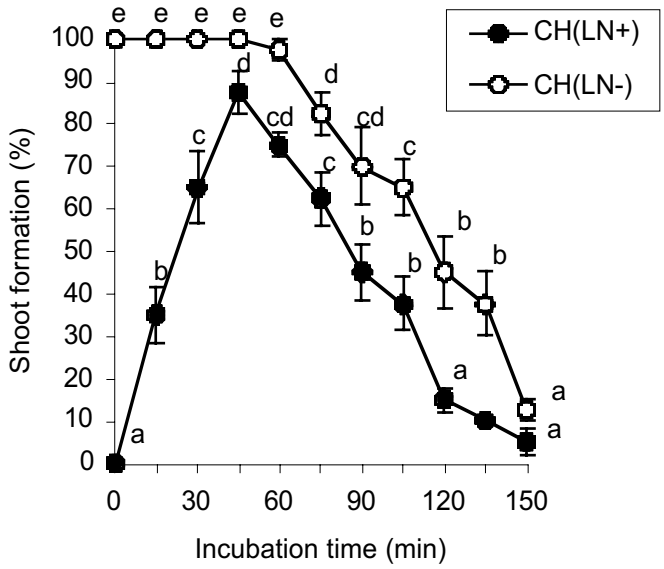

Fig. 2. Effect of time in PVS2 on shoot survival of 'Ne Plus Ultra'. Shoot cultures were cold-hardened at 4°C for 21 days. Shoot tips were excised, precultured at 4°C for 1 day on MS agar medium supplemented with 0.7 M sucrose, and stored for 3 days ± LN. Values are the mean of four replicates, each with 10 shot tips. The bars represent ± standard error (SE). Means with the same letter on the same graph line are not significantly different at the 5% level using Duncan's new multiple range test. LN+ = stored in LN; LN- = stored at 25°C.

Table 1. Effect of dehydration time on survival of shoot tips. Shoot tips of 'Nonpareil 15-1' and the hybrid rootstock were cold-hardened at 4°C for 21 days, precultured at 4°C for one day on appropriate agar media (see text) supplemented with 0.7 M sucrose, dehydrated in PVS2 for different periods of time, and subsequently stored in LN for at least 3 days before thawing

Cultivars	Shoot survival (%) ± SE† Incubation time in PVS2 (min)			
	30	45	60	75
'Nonpareil 15-1'	47.5 ± 6.29	60.0 ± 7.07	37.5 ± 4.78	37.5 ± 8.53
Hybrid rootstock	60.0 ± 65.77	65.0 ± 6.45	72.5 ± 7.5	57.5 ± 8.53

†Four replicates were used with 10 shoot tips for each replicate. SE = standard error.

Discussion

Most woody plant species require cold hardening to improve shoot survival (Niino *et al.*, 1997). This appears to ameliorate any harmful effects of the cryoprotectants and osmotic stress during dehydration (Yamada *et al.*, 1991). For example, the preculture of excised shoot tips at 5°C for 1 day in agar medium supplemented with 0.7 M sucrose was effective for improving survival of cryopreserved shoots in many fruits such as apple, mulberry and cherry (Niino *et al.*, 1992a,b, 1997) and *Prunus* rootstock (Brison *et al.*, 1995). In the present study, preculturing for 1 day at 4°C was a successful prerequisite to the cryopreservation of two almond cultivars and a hybrid rootstock.

Both the time and temperature of incubation in vitrification solution are important to avoid the

damaging effects of crystallisation, chemical toxicity of cryoprotectants, and excess osmotic stress during dehydration (Niino *et al.*, 1992b, 1997). The size of excised shoot tips also seems to be an important factor (Takagi *et al*., 1997). In the present study, optimum incubation times for the almond cultivars and the hybrid rootstock were 45 and 60 min respectively.

Table 2. Survival of cryopreserved shoot tips. Shoot tips were stored for up to 180 days in LN, and then thawed at 30°C and cultured on appropriate media (see text) without ammonium ions

Period of preservation	Shoot survival (%) ± SE[†]		
	'Ne Plus Ultra'	'Nonpareil 15-1'	Hybrid rootstock
3 days	87.5 ± 2.5 b	60.0 ± 3.7 ab	72.5 ± 3.9 a
90 days	78.3 ± 3.7 ab	49.2 ± 7.3 a	82.5 ± 2.2 b
180 days	76.7 ± 3.6 a	51.7 ± 3.4 ab	77.5 ± 3.3 ab

[†]Four replicates were used with 10 shoot tips for each replicate. SE = standard error.
[a,b]Means followed by the same letter are not significantly different.

Rapid thawing at 25-40°C in a water bath after storage under LN prevents recrystallisation (Sakai *et al.*, 1990) and produces higher survival rates compared to a slow warming (Matsumoto *et al.*, 1995; Takagi *et al.*, 1997). In this study, the plant material was rapidly warmed in a water bath at 30°C.

The medium used for recovery of cryopreserved shoot tips is also very significant, especially the concentration of ammonium ions (Kuriyama *et al*., 1990). After cryopreserved shoot tips were recultured in the appropriate medium, excluding both ammonium ions and growth regulators, new shoots readily developed that could be successful transferred to a multiplication medium.

Conclusion

Vitrification is a relatively simple method for cryopreserving plant materials that results in a high survival rate of the tissues after their removal from LN compared to other techniques, such as stepwise freezing and simple freezing (Towell and Jarret, 1992). The procedure reported here has eliminated the need to vary the composition and concentrations of different reagents. After three days storage in LN, shoot survival was 60.0-87.5% compared to 52-78% after 180 days. Shoot survival for unfrozen control shoot tips was 85-100%. Shoots regenerated from cryopreserved shoot tips did not show any apparent morphological abnormalities, but the possibility of genetic change is being investigated.

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