CryoLetters **35** (3), 188-196 (2014)


# CRYOPRESERVATION OF QUINCE (*CYDONIA OBLONGA* MILL.)

Paul T. Lynch[1*], Ayesha Siddika[1], Aradhana Mehra[1], Carla Benelli[2] and Maurizio Lambardi[2]

[1]Biological Sciences Research Group, University of Derby, Derby, UK. [2]Istituto per la Valorizzazione del Legno e delle Specie Arboree, (IVALSA), National Research Council, (Firenze) Italy.
*Corresponding author email: P.T.Lynch@derby.ac.uk

## Abstract

**BACKGROUND:** Quince (*Cydonia oblonga* Mill.) has great potential for utilisation in pharmaceutical and food industries. **OBJECTIVE:** The study was to develop an efficient cryopreservation approach for quince. **METHODS:** Factors on the survival and regrowth such as cold acclimation, explant type and recovery media composition were assessed. The effectiveness of the resultant protocols for a number of quince cultivars was determined. **RESULTS and CONCLUSION:** Quince shoot tips and nodal sections are successfully cryopreserved. Sustained regrowth of quince 'Angers A' was observed after encapsulation-osmoprotection/dehydration, encapsulation-dehydration and PVS2 vitrification. The highest regrowth rate (80%) was obtained from explants excised from cold hardened shoots and cryopreserved using encapsulation-osmoprotection/dehydration and vitrification protocols. The optimised vitrification protocol in combination with shoot cold hardening and a MS recovery medium without activated charcoal and auxin resulted in satisfactory regrowth of shoots from six quince cultivars. The morphology of acclimatised plants derived from cryopreserved shoots was comparable with non-cryopreserved plants.

**Keywords:** quince, shoot tips, nodal sections, cold hardening, recovery medium

## INTRODUCTION

Currently the main commercial use of quince (*Cydonia oblonga* Mill.) is as rootstocks of pear, particularly in continental Europe (35). As a result of its strong fragrance, quince is used as food flavouring and in deodorisers (14). Phytochemicals isolated from quince have been shown to have a range of bioactivities, including cytotoxic effects on human cancer cell lines (19, 27). The greatest potential for future utilisation of quince is in the pharmaceutical and food industries (6). Conservation of improved cultivars and wild types of this underutilised crop is essential if its future potential is to be realised. Indeed, attempts have been made in Europe to conserve germplasm of quince within the project 'Conservation of Minor Fruit Tree Species' (5). In common with other temperate fruits, quince germplasm is conserved primarily in field collections, where it remains at risk due to pests and diseases and environmental stresses (5). Cryopreservation, successfully used for other temperate fruit species such as apple, pear and *Prunus* (5), offers an alternative approach for long-term quince germplasm conservation.

The aim of this study was to develop an efficient cryopreservation approach for quince. Towards this, cryopreservation approaches were compared and the effects of factors on the survival and regrowth such as cold acclimation, explant type and recovery media composition were assessed. Subsequently the effectiveness of cryopreservation on a number of quince cultivars was determined.

## MATERIALS AND METHODS

### *Quince cultivars*

Unless otherwise stated quince 'Angers A' obtained from the Institut National de la Rescherche Agronomique, Angers (France) was used in this study. Plants of the cultivars 'Champion', 'Isfahan', 'Siberian Gold' (also

known as 'Lescovacz'), 'Vranja' and 'Aromatrya' were obtained from Reads Nursery, Norfolk (UK) and used in a validation of the optimised cryopreservation protocol.

***In vitro culture and cold hardening***

*In vitro* quince shoot cultures were initiated from glasshouse grown plants. Shoots 2-3 cm in length and with axillary buds were dissected, washed and surface sterilised with 0.1% (w/v) of mercuric chloride solution supplemented with two drops of Domestos (Lever Brother Ltd, UK) for 2.5 min with gentle shaking. After removing the cut edges, explants were washed with sterile distilled water and placed in 28 $cm^3$ glass vials containing 10 $cm^3$ QL medium composed of 3.6 g/l QL salts (28), 1 ml/l MS vitamins (23), 30 g/l sucrose and 7 g/l Bactoagar (Difco Laboratories, USA) supplemented with 1 mg/l 6-benzylaminopurine (6-BAP), 0.5 mg/l gibberellic acid ($GA_3$) and 0.1 mg/l indole butyric acid (IBA). Explants were incubated at 20 ± 1°C under a 16 h photoperiod at 60-65 $\mu mol/m^2/s$ photon flux (standard growth conditions). After 3 weeks incubation on QL medium, shoots were transferred into 250 $cm^3$ jars containing 50 $cm^3$ MS medium (23) with 30 g/l sucrose, 1 mg/l 6-BAP, 0.1 mg/l IBA and 8 g/l Bactoagar, (MS–AC+IBA medium). Shoot cultures were maintained under standard growth conditions by monthly subculture.

Shoot cultures, 7 days after subculturing, were cold hardened by maintenance at 5°C for 4 weeks under continuous light at photon flux 8.6 $\mu mol/m^2/s$. Nodal sections 1.0 - 2.5 mm in length consisting of one or two lateral buds from both cold hardened and non-cold hardened *in vitro* grown shoots were excised and used for cryopreservation studies.

***Cryopreservation***

Encapsulation-osmoprotection/dehydration: Nodal sections were encapsulated and cryopreserved using the encapsulation-dehydration method described by Sakai *et al.* (30). Excised nodal sections were precultured on MS–AC+IBA medium containing 0.3 M sucrose for 24 h under standard growth conditions. Precultured nodal sections were placed into calcium free liquid MS–AC+IBA medium containing 3% (w/v) sodium alginate solution, 2 M glycerol and 0.4 M sucrose. After gentle mixing, suspended nodal sections were drawn up by 5 $cm^3$ automatic pipette into tips which were cut back to make a bore of 4.0 mm. The alginate solution was dispensed drop by drop, each containing a nodal section, into MS–AC+IBA medium with 0.1 M $CaCl_2$, 2 M glycerol and 0.4 M sucrose. Encapsulated nodal sections were left in this medium for 45-60 min, at room temperature, to allow the beads to polymerise. Beads were then separated from the calcium rich medium and excess moisture removed by rolling beads on sterile filter paper (Whatman No. 1). Encapsulated nodal sections (8 beads per jar) were dehydrated over sterile silica gel (80 g per 250 $cm^3$ jar) for up to 8 h. The moisture content of the beads was determined by measuring the fresh and dry weight of beads containing nodal sections. After dehydration beads were either transferred into 9 cm Petri dishes containing 25 $cm^3$ of MS–AC+IBA medium (8 beads per dish) and incubated under standard growth conditions as non-cryopreserved controls or transferred into 2 ml cryovials (Sarstedt Ltd., Germany) (8 beads per vial) and plunged into liquid nitrogen (LN). Beads were rewarmed after at least 1 h of storage in LN by placing into a 40°C water bath for 2 min.

To investigate the effect of activated charcoal (AC) and growth factors on shoot regrowth, nodal sections were maintained on one of four recovery media, specifically: (1) MS–AC+IBA medium with 0.01% (w/v) activated charcoal, designated MS +AC+IBA; (2) MS–AC+IBA medium without activated charcoal or IBA, designated MS–AC– IBA; (3) MS–AC+IBA medium with activated charcoal, but without IBA, designated MS+AC–IBA; (4) MS–AC+IBA medium. After 7 d of incubation at 20 ± 1°C in the dark, nodal sections were excised from the beads and transferred onto MS–AC+IBA medium and incubated under standard growth conditions.

Encapsulation-dehydration: Nodal sections were cryopreserved by the encapsulation-dehydration approach based on the protocol of Fabre and Dereuddre (16). Excised nodal sections were precultured in MS–AC+IBA medium containing progressively higher sucrose (0.25 M, 0.5 M and 0.75 M) for 24 h at each concentration. After 3 d preculture, nodal sections were encapsulated as described above for encapsulation-osmoprotection/dehydration, except that the 2 M glycerol was omitted from the encapsulation solution. Subsequently the encapsulated nodal sections were precultured on 9 cm Petri dishes containing 25 $cm^3$ of MS–AC+IBA medium containing 1 M sucrose (8 beads per dish) for 24 h. Beads were dehydrated

and moisture content determined as described for encapsulation-osmoprotection/ dehydration. Dehydrated beads were transferred to either MS–AC+IBA or MS-AC-IBA medium and incubated under standard growth conditions (non-cryopreserved control), or plunged into LN. After at least 1 h of storage in LN, beads were rewarmed by plunging into a 40°C water bath for 2 min and transferred either onto MS–AC+IBA medium MS-AC–IBA medium and incubated in the dark at 20 ± 1°C. After 7 days nodal sections were excised from the beads, transferred on to fresh MS–AC+IBA medium and incubated under standard growth conditions.

PVS2 Vitrification: Nodal sections from cold hardened and non-cold hardened *in vitro* shoots were cryopreserved using a Plant Vitrification Solution 2 (PVS2) approach based on the procedure described by Sakai *et al.* (29). Excised nodal sections were cryoprotected in liquid MS–AC+IBA medium containing 2 M glycerol and 0.4 M sucrose (8 nodal sections per ml in a cryovial) for 20 min at 25°C and then transferred into PVS2 (8 nodal sections per ml) on ice for 0–3 h. Nodal sections were then either rinsed 3 times with liquid MS–AC+IBA or MS-AC-IBA medium containing 1.2 M sucrose for 20 min periods and transferred onto a double layer of filter paper (Whatman No. 1), overlaying 9cm Petri dishes containing MS–AC+IBA or MS-AC-IBA media and incubated in dark at 20± 1°C (non-cryopreserved control) or transferred into fresh PVS2 (8 nodal sections per vial) and plunged into LN. After at least 1 h storage in LN, cryovials were rewarmed by plunging into a 40°C water bath for 30 s. PVS2 was removed and nodal sections were rinsed three times with liquid MS–AC+IBA or MS-AC-IBA medium containing 1.2 M sucrose for 20 min and cultured as the non-cryopreserved controls. After 7 days, cryopreserved and non-cryopreserved nodal sections were transferred to MS–AC+IBA medium and incubated under standard growth conditions.

***Comparison: shoot tips and nodal sections***

Shoot tips (approx. 1.5 mm long and containing two pairs of leaf primordia) and nodal sections from cold hardened *in vitro* shoot cultures were cryopreserved using the optimum protocols developed for nodal sections and stored in LN for at least 1 h. Cold-hardened cryopreserved shoot tips and nodal sections after rewarming were cultured using MS–AC+IBA and MS-AC-IBA media as described above.

***Influence of quince genotype***

Nodal sections of cold hardened shoot cultures of quince cultivars 'Angers A', 'Champion', 'Isfahan', 'Siberian Gold' (also known as 'Lescovacz'), 'Vranja' and 'Aromatrya' were cryopreserved using the PVS2 approach described previously. The nodal sections were incubated for 2 h in PVS2 solution. After rewarming they were cultured using MS–AC+IBA and MS-AC-IBA media as described above.

***In vitro rooting of regenerated shoots and transfer of rooted shoots to compost***

Regenerated shoots from cryopreserved, non-cryopreserved nodal sections were transferred to jars containing 50 $cm^3$ rooting medium (RM1) which consisted of 2.2 g/l of MS salts, 30 g/l sucrose and 9 g/l Bactoagar and supplemented with either 0.5 mg/l naphthalene acetic acid (NAA) or 0.5 mg/l IBA and incubated in the dark at 20 ± 1°C for 7 d. After dark incubation, shoots were either maintained on the same medium or transferred to growth factor free medium (RM2) and incubated under standard growth conditions for 4 to 6 weeks.

Plantlets were acclimatised by loosening culture jar lids for 48 hours. Plantlets were then removed from jars, residual medium washed from the roots, dipped into systemic fungicide (Doff Portland Ltd, Nottingham, UK) potted into 6 cm pots containing sterile Levington F1 compost (Levington, Ipswich, UK) and placed in a plant propagator. The propagator lid was sealed onto the base with Whatman Lab Sealing Film and placed in a glasshouse under a 16 h photoperiod at 20°C. Over a 4 week period the vents on the propagator were opened to reduce humidity and finally the top of the propagator was removed completely. After a further 3 weeks the plants were transferred to 13 cm pots containing a compost mix of 1/3 perlite and 2/3 F1 compost and maintained under the same glasshouse conditions.

***Experimental design***

Each experiment consisted of a minimum of three replicate Petri dishes containing a minimum of 5 nodal sections or shoot tips. Nodal section or shoot tip regrowth after each treatment was assessed after a 4 weeks cultivation period. Regrowth was defined as a percentage of cryopreserved and non-cryopreserved nodal sections or shoot tips exhibiting bud development and shoot growth. Data based on percentages were transformed by

$log_{10}$ before analysis by one way ANOVA and general linear model univariate by SPSS, Version 10 (SPSS Inc. Illinois, USA).

## RESULTS

***Encapsulation-osmoprotection /dehydration***

The frequency of regrowth after dehydration of non-cryopreserved nodal sections decreased significantly ($p$<0.05) from 93% to 25% as the moisture content of the beads fell from 73% to 14% (Table 1). Non-cryopreserved nodal sections were green after dehydration and exhibited regrowth within 3 days of transfer onto MS–AC+IBA medium. For cryopreserved nodal sections, the highest frequency (39%) of regrowth after rewarming was obtained after 6 h dehydration (25% moisture content). Nodal sections were green immediately after rewarming then turned to pale green after 1 d in culture. Rewarmed shoots exhibited regrowth after 2-3 weeks incubation.

The rate frequency of regrowth of nodal sections after rewarming maintained on MS+AC+IBA or MS-AC-IBA medium was higher as compared with nodal sections maintained on MS–AC+IBA or MS+AC-IBA medium (Table 2). The highest frequency of regrowth was obtained from nodal sections maintained on MS-AC-IBA medium. The regrowth after rewarming was significantly higher ($p$<0.05) from the nodal sections dehydrated for 6 h compared to 4 or 8 h when maintained on MS–AC+IBA, MS+AC-IBA or MS-AC-IBA medium. However, no significant differences were observed in regrowth of nodal sections maintained on MS+AC+IBA medium. Regrowth was generally observed 2 weeks after rewarming. However regrowth of nodal sections incubated on MS+AC-IBA medium was only observed 3 weeks after rewarming and the regrowth remained slow as compared with the shoots maintained on the other recovery media.

After rewarming the leaf primordia on 72% of the regrowing shoots developed a callus-like appearance (Table 3). These leaf primordia became brown after 6-8 weeks culture, but subsequently the shoots showed sustained normal regrowth. The callus-like appearance initially observed in leaf primordia derived from nodal sections maintained on MS–AC+IBA was significantly ($p$<0.05) reduced on recovery medium without IBA (MS-AC-IBA). The frequency of regrowth after rewarming was not

**Table 1.** Encapsulation-osmoprotection/dehydration: the effect of dehydration on the frequency of regrowth of non-cryopreserved and cryopreserved nodal sections maintained on MS–AC+IBA medium 4 weeks after rewarming.

| Dehydration duration (h) | Moisture content (%) | Regrowth of nodal sections (%) | |
|---|---|---|---|
| | | Non-cryopreserved | Cryopreserved |
| 0 | 73 | 92.9 ± 7.1[a] | 0.0 |
| 2 | 57 | 7.5 ± 0.0[a] | 0.0 |
| 4 | 41 | 53.8 ± 11.1[b] | 15.1 ± 1.8[a] |
| 6 | 25 | 45.8 ± 11.0[b] | 39.1 ± 8.5[b] |
| 8 | 14 | 25.0 ± 0.0[c] | 12.5 ± 0.0[a] |

Values represent the mean regrowth frequency ± standard error (non-cryopreserved nodal sections n = 4 and cryopreserved nodal sections n = 5). Values within a column with different superscripts are statistically different ($p$<0.05)

**Table 2.** Encapsulation-osmoprotection/dehydration: the effect of recovery media on the frequency of regrowth of nodal sections 4 weeks after rewarming

| Dehydration duration (h) | Regrowth of nodal sections (%) | | | |
|---|---|---|---|---|
| | MS–AC+IBA | MS+AC+IBA | MS+AC-IBA | MS-AC-IBA |
| 0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 2 | 0.0 | 2.1 ± 2.0[a] | 0.0 | 0.0 |
| 4 | 15.0 ± 1.8[a] | 34.1 ± 4.6[b] | 4.2 ± 4.2[a] | 56.5 ±7.0[a] |
| 6 | 39.0 ± 8.5[b] | 37.5 ± 7.2[b] | 38.3 ± 0.1[b] | 59.3 ± 19.8[b] |
| 8 | 12.5 ± 0.0[a] | 43.7 ± 6.2[b] | 12.5 ± 0.0[c] | 46.6 ±6.6[a] |

Values represent the mean regrowth frequency ± standard error, n = 4. Values within a column with different superscripts are statistically different ($p$<0.05)

**Table 3.** Encapsulation-osmoprotection/dehydration: the frequency of shoot regrowth from rewarmed nodal sections maintained on MS-AC+IBA or MS-AC-IBA medium and the relative proportion of shoots with leaf primordia exhibiting a callus-like appearance 4 weeks after rewarming

| Media | Total regrowth (%) | Shoots + callus (%) | Shoots - callus (%) |
|---|---|---|---|
| MS-AC+IBA | 39.1 ± 8.5[a] | 72.0 ± 4.2[a] | 27.6 ± 3.7[a] |
| MS-AC-IBA | 59.3 ± 19.8[a] | 10.4 ± 8.2[b] | 89.6 ± 8.2[b] |

Values represent the mean ± standard error, n = 4. Values within a column with different superscripts are statistically different ($p$<0.05)

**Table 4.** Encapsulation-osmoprotection /dehydration: Effect of cold hardening and recovery media on the frequency of regrowth of nodal sections, which were dehydrated over silica gel for 6 h, 4 weeks after rewarming

| | Regrowth of nodal sections (%) | |
|---|---|---|
| Recovery media | Non-cold hardened | Cold hardened |
| MS-AC+IBA | 39.0 ± 8.5[a] | 53.3 ± 9.4[a] |
| MS-AC-IBA | 59.3 ± 19.8[a] | 60.0 ± 14.1[a] |

Values represent the mean regrowth ± standard error, n = 4. Values within a column with different superscripts are statistically different ($p$<0.05)

higher ($p$>0.05) from cold hardened nodal sections maintained either on MS–AC+IBA or MS-AC-IBA medium (Table 4). However, the frequency of regrowth from non-cold hardened nodal sections after rewarming was higher after culture on MS-AC-IBA medium as compared with MS–AC+IBA medium.

***Encapsulation-dehydration***

Regrowth of non-cryopreserved nodal sections was 80% after 6 h dehydration (Table 5). Regrowth of cryopreserved nodal sections was observed only after 6 h dehydration over silica gel (moisture content 21%). Regrowth of nodal sections was observed 3-4 weeks after rewarming with shoots developing from buds of nodal sections without any callus growth.

Incubation on recovery medium MS-AC-IBA did not significantly ($p$>0.05) increase the frequency of regrowth after rewarming (Table 6), but the frequency of regrowth was increased when MS-AC-IBA recovery medium was used in combination with cold hardening.

***PVS2 vqitrification***

The frequency of regrowth of non-cryopreserved nodal sections decreased significantly ($p$<0.05) after incubated for more than 1 h in PVS2 (Table 7), while the frequency of regrowth of cryopreserved nodal sections increased, although not significantly ($p$>0.05), after more than 1.5 h incubation in PVS2. The highest frequency of regrowth after rewarming (43%) was observed from nodal sections incubated for 2 h in PVS2. Shoots from nodal sections started growing within 3 to 7 d after rewarming. The rate of regrowth was faster after PVS2 vitrification as compared with encapsulation-osmoprotection/dehydration or encapsulation-dehydration methods. However, after PVS2 vitrification leaf primordia of some regenerated shoots appeared hyperhydrated. The hyperhydrated leaf primordia became brown and died, but the subsequently the shoots showed sustained normal regrowth.

The frequency of regrowth after rewarming of cold hardened nodal sections did not significantly ($p$>0.05) increase as compared with non-cold hardened nodal sections when MS–AC+IBA was used as the recovery medium (Table 8). However, regrowth after rewarming

**Table 5.** Encapsulation-dehydration: the effect of dehydration on the moisture content and the frequency of regrowth of non-cryopreserved and cryopreserved nodal sections 4 weeks post-treatment.

| Dehydration duration (h) | Moisture content (%) | Regrowth nodal sections (%) | |
|---|---|---|---|
| | | Non-cryopreserved | Cryopreserved |
| 0 | 68.2 ± 0.3[a] | 91.6 ± 8.3[a] | 0.0 |
| 2 | 54.2 ± 1.4[a] | 91.5 ± 8.5[a] | 0.0 |
| 4 | 37.6 ± 0.6[b] | 85.0 ± 7.6[a] | 0.0 |
| 6 | 21.3 ± 1.2[b] | 80.0 ± 8.6[a] | 2.5 ± 10.2 |

Values represent the mean ± standard error, n = 3. Values within a column with different superscripts are statistically different ($p$<0.05)

**Table 6.** Encapsulation-dehydration: Effect of cold hardening and recovery media on regrowth of nodal sections, which were dehydrated over silica gel for 6 h, 4 weeks after rewarming.

| | Regrowth nodal sections (%) | |
|---|---|---|
| Recovery media | Non cold hardened | Cold hardened |
| MS-AC+IBA | 12.5 ± 10.2[a] | 18.6 ± 5.1[a] |
| MS-AC-IBA | 6.7 ± 4.7[a] | 20.8 ± 1.9[a] |

Values represent the mean regrowth ± standard error, n 4. Values within a column with different superscripts are statistically different ($p$<0.05)

**Table 7.** Effect of PVS2 exposure on the regrowth of cryopreserved and non-cryopreserved nodal sections 4 weeks post-treatment.

| PVS2 exposure time (h) | Regrowth of nodal sections (%) | |
|---|---|---|
| | Non-cryopreserved | Cryopreserved |
| 0 | 100.0 ± 0.0[a] | 0.0 |
| 0.5 | 75.0 ± 0.0[b] | 0.0 |
| 1.0 | 72.2 ± 27.7[b] | 16.2 ± 3.8[a] |
| 1.5 | 29.0 ± 4.0[c] | 32.9 ± 0.4[b] |
| 2.0 | 32.5 ± 10.3[c] | 43.3 ± 6.7[b] |
| 2.5 | 33.3 ± 3.3[c] | 35.8 ± 12.1[b] |
| 3.0 | 33.7 ± 21.2[c] | 37.7 ± 25.2[a/b] |

Values represent the mean regrowth ± standard error, n = 3. Values within a column with different superscripts are statistically different ($p$<0.05).

**Table 8.** PVS2 vitrification: Effect of cold hardening and recovery media on the regrowth of nodal sections, which were incubated for 2 h in PVS2 solution, 4 weeks after rewarming.

| Recovery media | Regrowth of nodal sections (%) | |
|---|---|---|
| | Non-cold hardened | Cold hardened |
| MS-AC+IBA | 43.3 ± 6.7[a] | 50.8 ± 10.4[a] |
| MS-AC-IBA | 4.2 ± 5.9[b] | 55.0 ± 25.0[a] |

Values represent the mean regrowth ± standard error, n = 4. Values within a column with different superscripts are statistically different ($p$<0.05)

was enhanced significantly ($p$<0.05) when the recovery medium, MS-AC-IBA was used in combination with cold hardening.

***Regrowth of shoot tips and nodal sections***

Shoot tips were cryopreserved using the optimised protocols developed for nodal sections. With exception of PVS2 vitrification, a significantly higher ($p$<0.05) frequency of regrowth after rewarming was observed from shoot tips as compared with the nodal sections maintained on either MS–AC+IBA or MS-AC-IBA media (Table 9). As compared with shoot tips PVS2 treated nodal sections showed a higher frequency of regrowth after rewarming when cultured on MS–AC+IBA medium.

***Influence of quince genotype***

Shoot regrowth was observed from nodal sections of each of the quince cultivars investigated (Table 10). The frequency of regrowth was significantly ($p$>0.05) lower from nodal sectors of cultivar 'Champion' as compared with the other cultivars tested. The frequency of regrowth of the other cultivars was not significantly different ($p$>0.05). Leaf primordia of some regenerated shoots appeared hyperhydrated, but in all cases subsequent shoot growth exhibited a normal morphology. The frequency of hyperhydricity did not appear to differ between the different cultivars.

***In vitro rooting of cryopreserved and non-cryopreserved shoots***

The frequency of rooting of cryopreserved and non-cryopreserved shoots was not significantly different ($p$>0.05) when maintained on RM1 medium (Table 11). Some thickened roots developed from shoots maintained on RM1 medium, but not RM2 medium. All plantlets survived after transfer to compost. Under glasshouse conditions the morphology of plants derived from non-cryopreserved and cryopreserved plantlets was comparable with mother plants from which *in vitro* cultures were derived.

## DISCUSSION

***Encapsulation-osmoprotection/dehydration***

The frequency of regrowth of nodal sections cryopreserved by encapsulation-osmoprotection /dehydration protocols was higher as compared with the encapsulation /dehydration protocol. This could be due to the combined effect of glycerol and sucrose included in alginate solution as a part of encapsulation-osmoprotection /dehydration protocols. Glycerol is a colligative cryoprotectant, which can penetrate cells and interact with membrane phospholipids and proteins by replacing water and so reducing the osmotic stress caused by dehydration and

**Table 9.** Frequency of regrowth of cold hardened shoot tips and nodal sections 4 weeks after rewarming: comparison of different cryopreservation protocols.

| Method | Regrowth (%) | | | |
|---|---|---|---|---|
| | MS-AC+IBA | | MS-AC-IBA | |
| | Shoot tips | Nodal sections | Shoot tips | Nodal sections |
| Encapsulation-osmoprotection/dehydration | 73.3 ± 9.4[a] | 33.3 ± 9.4[a] | 80.0 ±16.3[a] | 40.0 ±16.3[a] |
| Encapsulation-dehydration | 20.0 ± 10.0[b] | 0.0 | 30.5 ± 9.3[b] | 5.5 ± 7.8[b] |
| Vitrification in PVS2 | 35.0 ± 12.2[b] | 66.6 ± 9.4[b] | 80.0 ± 0.0[a] | 30.0 ± 8.3[a] |

Values represent the mean regrowth frequency ± standard error, n = 4. Values within a column with different superscripts are statistically different ($p<0.05$

**Table 10.** The regrowth of nodal sections from different quince cultivars cryopreserved using the optimised PVS2 vitrification protocol

| Cultivar | Regrowth of nodal sections (%) |
|---|---|
| 'Champion' | 36.7 ± 13.7[a] |
| 'Isfahan' | 60.0 ± 11.6[b] |
| 'Siberian Gold' | 63.3 ± 13.7[b] |
| 'Aromatrya' | 53.3 ± 22.1[b] |
| 'Vranja' | 60.0 ± 21.3[b] |
| 'Angers A' | 56.7 ± 21.3[b] |

Values represent the mean regrowth ± standard error, n = 5. Values within a column with different superscripts are statistically different ($p<0.05$)

**Table 11.** The effect of different growth regulators added to RM1 medium on the rooting of shoots derived from cryopreserved and non-cryopreserved nodal sections

| Shoots derived from | *In vitro* rooting (%) | |
|---|---|---|
| | 0.5 mg/l IBA | 0.5 mg/l NAA |
| Non-cryopreserved nodal sections | 83.3 ± 16.6[a] | 66.6 ± 16.6[a] |
| Cryopreserved nodal sections | 70.0 ± 20.0[a] | 80.0 ± 12.3[a] |

Values are the mean of cryopreserved and non-cryopreserved shoots ± standard error (n=10 and 6 respectively). Values in a column with different superscripts are statistically different ($p<0.05$)

cooling (1). Glycerol is a smaller molecule compared to sugar alcohols and has OH groups along one side of the molecule, which enable effective 'packing' of membrane head groups (33). Furthermore, the pretreatment with and encapsulation in sucrose may induce uptake of sucrose into the cells by osmotic responsiveness and reduce freezable water, resulting in cooling tolerance (21).

The frequency of survival after rewarming is also dependent on the moisture content of the cryopreserved tissue. The optimal water content for regrowth of nodal sections after rewarming was 24.6%; at this water content the glass transition probably occurred during cooling. However, the water content leading to optimal regrowth after rewarming, depends on genotype; for example, for shoot apices of black chokeberry and citrus species water content was 19% and 18% respectively (2, 18) while in the case of 'Troyer' citrange shoot tips it was 20% (34). Crowe *et al.* (11) suggested that water content below 21% induces lipid phase transition of membrane from liquid crystaline to gel phase which is lethal for tissue.

***Encapsulation-dehydration***

The regrowth of quince following the encapsulation-dehydration protocol was lower as compared with the encapsulation-osmoprotection/dehydration. Niino and Sakai (24) using encapsulation/dehydration protocol, obtained 80 % post-thaw regrowth in cold hardened shoots of apple, pear and mulberry. However in the present study, the frequency of regrowth is much lower (12.5%), but higher than in *Prunus* species (13). Santos and Stushnoff (31) suggested that although sucrose is a compatible osmolyte, sucrose concentration in preculture medium needs to be optimised to increase dehydration tolerance and cooling and to maintain tissue integrity. Concentrations of sucrose between 0.3 M and 0.75 M are optimum in most cases to induce dehydration tolerance and subsequently chilling tolerance (17).

***PVS2 vitrification***

The frequency of regrowth following PVS2 vitrification was higher than the encapsulation-

dehydration protocol, but similar to the encapsulation-osmoprotection/dehydration protocol. Nodal sections were cryoprotected in 2 M glycerol and 0.4M sucrose prior to dehydration in concentrated PVS2 which may provide protection of cell against severe dehydration by PVS2. For most plant species such as apple, pear and *Prunus* 60-90 min dehydration in PVS2 is sufficient to exert protection and produce vitrified state during cooling (12, 25, 37). However, 120 min exposure in PVS2 was needed to obtain a better frequency of regrowth. In the former cases apices were precultured in sucrose prior to cryoprotection and PVS2 treatments, which may be required prior to exposure to PVS2 to ensure glass formation during cooling. However, in this study sucrose preculture prior cryoprotection and PVS2 dehydration was detrimental for quince as no regrowth was observed after such treatment (data not reported).

***Recovery media, cold hardening and regrowth***

Addition of activated charcoal in MS+AC+IBA recovery media increased the frequency of regrowth. Similar positive growth responses have been reported, for example with the regrowth of redwood *in vitro* buds after cryopreservation (26). Such effects may have been due to absorption of the potentially damaging substances, such as phenols produced form the injured tissues (13). Activated charcoal also maintains pH levels of the media during culture and increases nitrogen uptake, which may enhance regrowth (15). However in the present study, incubation on recovery medium, MS+AC-IBA resulted in lower regrowth, this could be due to the absorption of residual growth factors by activated charcoal (20).

The type and concentration of growth regulators in the recovery medium is important for the sustained growth of shoots without any intermediary callus (34). Callus growth from shoots after rewarming is associated with cryoinjury, normally caused by stress imposed by dehydration and chilling (32). In the present study callus appearance, a possible indication of cryoinjury, is reduced by removal of auxin from the recovery medium. Reduction of callus formation associated with shoots after rewarming by using recovery media free of IBA has been reported for example in *Rubus spp.* (8).

Regrowth of quince nodal sections after rewarming was increased by cold hardening. Use of cold hardening is not an uncommon requirement of effective cryopreservation (4). The improved tolerance to cryopreservation may be due to a number of factors including changes in the endogenous abscisic acid, proline, and antioxidant activity (3, 7, 10). Reports on cryopreservation of temperate fruit tree species suggest that cold acclimation for 3-4 weeks is required to attain maximum chilling tolerance by inducing balance in lipid composition of plasma membrane (36).

Hyperhydricity has been previously reported in plant tissue recovering from cryogenic storage (22). Following cryopreservation by the PVS2 protocol hyperhydricity was observed in the regrowing explants. PVS2 vitrified non-cryopreserved explants were not hyperhydrated, hence indicating that, hyperhydricity of cryopreserved explants could be a result of desiccation and chilling stress.

***Shoot tips and nodal sections***

Shoot tips from cold hardened shoots generally showed significantly higher regrowth than nodal sections. The lower regrowth from the nodal sections may be a result of the more dormant character of axillary buds compared with apical buds (9). Although the frequency of regrowth of nodal sections is lower, their greater availability from *in vitro* shoots and easier excision as compared with shoot tops make them a more practicable explant for routine quince cryopreservation.

Encapsulation-osmoprotection/dehydration and vitrification protocols are efficient for the routine cryopreservation of quince nodal sections and shoot tips. Although hyperhydricity and callusing was observed in leaf primordia of shoots, all shoots exhibited a normal morphology within 4 weeks after rewarming. However, after rewarming, shoots developed only from the original apical or lateral buds, no regeneration from callus was observed. Importantly, the optimised PVS2 vitrification protocol was shown to be effective in cryopreserving a number of cultivars of quince, indicating the applicability of cryopreservation as complementary approach to the traditional approaches for long-term *ex situ* conservation of quince germplasm.

**Acknowledgements:** Ayesha Siddika is grateful to the University of Derby for the research studentship. The authors acknowledge the Commission of European Union for funding

support through CRYMCEPT (Establishing Cryopreservation Methods for Conserving European Plant Germplasm Collections, Quality of Life and Management of Living Resources, QLK5-CT-2002-01279) and CRYOPLANET, COST Action 871 (Establishing Cryopreservation of Crop Species in Europe ). Maurizio Lambardi thanks the Ente Cassa di Risparmio of Florence for the support for this study (project POLICENTRO).

## REFERENCES

1. Akhoondi M, Oldenhof H, Sieme H & Wolkers WF (2012) *Mol. Mem. Biol.* **29**, 197 – 206.
2. Al-Ababneh S, Karam N & Shibli R (2002) In Vitro *Cell. and Devel. Biol, Plant* **38**, 602 – 607.
3. Benelli C, De Carlo A & Englemann F (2013) *Biotech. Advan.* **31**, 175 – 185.
4. Bachiri Y, Song GQ, Plessis P, Shoar-Ghaffari A, Rekab T & Morisset C (2001). *CryoLetters* **22**, 61 – 74.
5. Benelli C, De Carlo A & Engelmann F (2013) *Biotech. Advan*. **31**, 175 – 185.
6. Bellini E & Giordani E (2000) *Acta Hort.* **522**,165-173.
7. Bilavcik A, Zamecnik J, Grospietsch M, Faltus, M & Jadrna P (2012) *Trees Struct. Funct.* **26**, 1181 – 1192.
8. Chang Y & Reed B (1999) *CryoLetters* **20**, 371-376.
9. Chatfield SP, Stirnberg P, Forde BG & Leyser O (2000) *Plant Journal* **24**, 159-169.
10. Chetverikova EP (1999) *Russian J. Plant Physiol.***46**, 721 – 727.
11. Crowe JH, Crowe LM & Carpenter JF (1987) *Biochem. J.* **242,** 1-10.
12. De Carlo A, Benelli C & Lambardi M (2000) *CryoLetters* **21**, 215-222.
13. Dominguez F, Chavez M, Luisa Garduno-Ramirez M, Chavez-Avila VM, Mata M & Cruz-Sosa F (2010) *Natural Prods. Comms.* **5**, 235 – 240.
14. Erdoğan T, Gönenç T, Hortoğlu ZS1, Demirci B, Başer KHC & Kıvçak B (2012) *Med Aromat Plants* **1**, 1 – 3.
15. Eymar E, Alegre J, Toribio M & Lopez-Vela D (2000) *Plant Cell, Tiss. Org. Cult.* **63**, 57-65.
16. Fabre J & Dereuddre J (1990) *CryoLetters* **11,** 413-426.
17. Gonzalez-Arnao MT & Engelmann F (2006) *CryoLetters* **27**, 155 – 168.
18. Kami D, Uenohata M, Suzuki T and Oosawa K (2008) *CryoLetters* **29**, 209-216.
19. Khoubnasabjafari M & Jouyban A (2011) *J. Med. Plants Res.* **5**, 3577 – 3594.
20. Lynch PT (2002) *Recent Res. Devel. Plant Biol* **2**, 261-275.
21. Lynch PT, Sidika A, Johnston JW, Mehera A, Lambardi M, Benelli C & Benson EE (2011). *Plant Sci.* **181**, 47 – 56.
22. Maslanka M, Panis B & Bach A (2013) *CryoLetters* **34**, 1 – 9.
23. Murashige T & Skoog F (1962) *Physiol. Plant.* **15**, 473-497.
24. Niino T & Sakai A (1992) *Plant Sci.* **87**, 199-206.
25. Niino T, Sakai A, Yakuwa H & Nijiri K (1992) *Plant Cell, Tiss. Org. Cult.* **28**, 261-266.
26. Ozudogru EA, Kirdok E, Kaya E, Capuana M, Benelli, C & Engelmann, F (2011) *CryoLetters* **32**, 99 – 110.
27. Pacifico S, Gallicchio M, Fiorentino A, Fischer A, Meyer U & Stintzing FC (2012) *Food Chem. Tox.* **50**, 4130 – 4135.
28. Quoirin M & Lepoivre P (1977) *Acta Hort.* **78**, 437-442.
29. Sakai A, Kobayashi S & Oiyama I (1990) *Plant Cell Rep.* **9,** 30-33.
30. Sakai A, Matsumoto T, Hirai D & Niino T (2000) *CryoLetters* **21,** 53-62.
31. Santos IRI & Stushnoff C (2002) *Plant Gen. Res. Newslett.***131**, 36-41.
32. Touchell DH, Chiang VL & Tsai C-J (2002) *Plant Cell Rep.* **21**, 118-124.
33. Turner S, Senaratna T, Touchell D, Bunn E, Dixon K & Tan B (2001) *Plant Sci.***160**, 489-497.
34. Wang QC, Batuman O, Bar-Joseph P Li. M & Gafny R (2002) *Plant Cell Rep.* **20**, 901-906.
35. Wertheim SJ, (2002) Rootstocks for European pear: a review. *Acta Hort.* **596**, 299-309.
36. Wu Y, Zhao YH, Engelmann F, Zhao MD, Zhang DM & Chen SY (2001) *CryoLetters* **22**, 375-380.
37. Zhao YH, Chen SY Wu Y, Chang Y & Zhang D (1995) in *China Association for Science and Technology, Second Academic Annual Meeting of Youths Proceedings* (Horticulture Sciences). Beijing Agriculture University Press, Beijing, 406-409.