CryoLetters **37** (5), 308-317 (2016)
© CryoLetters, businessoffice@cryoletters.org

OPTIMIZATION OF WATER CONTENT FOR THE CRYOPRESERVATION OF *Allium sativum IN VITRO* CULTURES BY ENCAPSULATION-DEHYDRATION

Paul T Lynch[1], Graham R Souch[1], Jiří Zámečník[2] and Keith Harding[3*]

[1] Environmental Sustainability Research Centre, University of Derby, Kedleston Road, Derby, DE22 1GB, UK
[2] Crop Research Institute, Drnovshá 507, CZ 161 06 Prague 6- Ruzyně, Czech Republic
[3] Damar Research Scientists, Damar, Drum Road, Cuparmuir, Fife, Scotland, KY15 5RJ, UK
*Corresponding author email: k.harding-damar@tiscali.co.uk

Abstract

BACKGROUND: There is a general requirement to determine and correlate water content to viability for the standardization of conservation protocols to facilitate effective cryostorage of plant germplasm. **OBJECTIVE:** This study examined water content as a critical factor to optimize the cryostorage of *Allium sativum*. **MATERIALS AND METHODS:** Stem discs were excised from post-harvest, stored bulbs prior to cryopreservation by encapsulation-dehydration and water content was determined gravimetrically. **RESULTS:** Survival of cryopreserved stem discs was 42.5%, with 22.5% exhibiting shoot regrowth following 6 h desiccation. Gravimetric data demonstrated a correlation between water content corresponding with survival / regrowth from desiccated, cryopreserved stem discs. For encapsulated stem discs a 25% residual moisture and corresponding water content of 0.36 g H_2O g^{-1} d.wt correlated with maximal survival following ~6.5 h of desiccation. **CONCLUSION:** The data concurs with the literature suggesting the formation of a stable vitrified state and a ‘window’ for optimal survival and regrowth that is between 6 – 10 h desiccation. Further studies using differential scanning calorimetry (DSC) are suggested to substantiate these findings.

Keywords: Cryopreservation, garlic, regrowth, stem discs, survival, vitrification

INTRODUCTION

The genus *Allium* consisting of vegetables, flowering ornamentals and wild species (20, 41) is of considerable agricultural and horticultural value (22, 31). *Allium* species are cross-fertile, but only a few accessions of *A. sativum* (garlic) display fertility (38) making the bulbs, cloves, basal bulblets and bulbils (topsets) important for vegetative propagation of garlic which does not generally produce seed (44, 46); garlic accessions also show a diversity of clonal characteristics (8, 40).

Allium germplasm is prone to loss, for example due to high susceptibility of species to a range of pests and pathogens and/or adverse weather conditions. *In situ* and *ex situ* conservation is important for the utilization of *Allium* genetic resources (25). *In vitro* storage can reduce some of these problems. However, there is a risk of culture instability *via* somaclonal variation with cost and capacity implications (30).

Cryostorage of vegetatively propagated *Allium* species at ultra-low temperature in liquid nitrogen (LN) can potentially obviate these risks and reduce the cost of maintaining *in vitro* cultures (26, 37). However, long-term conservation of vegetatively propagated *Allium* germplasm depends upon reproducible cryopreservation protocols (21, 29), particularly as cryopreservation of clonally propagated crops such as *Allium* is a complex, multi-component process (3). Development of the cryogenic component of a cryopreservation protocol is not the sole requirement (19) as its success also relies upon the provision of suitable material (46), optimization of tissue culture and an efficient plant regeneration system (26).

The use of vitrification solutions (11, 35, 32, 45, 46) and droplet-vitrification (23, 24) protocols have been described, albeit there are few reports which evaluate the encapsulation-dehydration technique for the cryostorage of garlic germplasm (26). Previously, we have reported: (a) the optimization of post-harvest storage to break dormancy in garlic bulbs; and (b) the use of stem discs as novel explants for the micropropagation of garlic (28) to improve the survival and regrowth of shoots following cryopreservation by encapsulation-dehydration (27). This present study examines water content as a critical factor in the further optimization of encapsulation-dehydration for the cryostorage of *A. sativum*.

MATERIALS AND METHODS

Post-harvest storage of garlic material, sterilization and excision

The conditions of post-harvest storage for garlic cloves, surface sterilization, stem disc excision and encapsulation, sucrose pretreatment, desiccation and cryopreservation are fully described by Lynch *et al.* (27) and briefly outlined as follows: bulbs of *A. sativum* (kindly provided by Dr. Joachim Keller, IPK, Gatersleben, Germany) were harvested in August and stored at 10°C in the dark. A post-harvest storage time of >2 months was required to break bulb dormancy but >6 months storage caused a decline in bulb quality. Clove sheaths were removed and cut to ~0.5 cm to include the basal section. Clove explants were dipped in 70% (v/v) ethanol for 2 s before being sterilized using 3% (v/v) sodium hypochlorite for 30 min. The tissues were washed with sterile distilled water. Stem disc explants (1.5 - 2.0 mm thick) were excised and each stem disc was cut into quarters.

Cryopreservation by encapsulation - dehydration

Stem disc quarters were placed in sterile universal vials containing 3% (w/v) sodium alginate in Murashige and Skoog (MS) medium (34) with 30 g l^{-1} sucrose, supplemented with 1.5 mg l^{-1} 2iP (6-γ-γ-dimethylallylaminopurine) and adjusted to pH 5.8. The alginate-suspended stem discs were then dripped into MS culture medium containing 0.1 M calcium chloride. The encapsulated stem discs were allowed to polymerize for 30 min before removing surface moisture and placed onto semi-solid MS culture medium containing 0.5 M sucrose and 8 g l^{-1} agar for 72 h at 25°C in the dark. Surface moisture was removed from the encapsulated stem discs after their preculture on semi-solid MS culture medium with 0.5 M sucrose followed by transfer onto the surface of a double layer of sterile filter paper in a powder jar containing 70 g sterilized silica gel and sealed. Explants were desiccated at 25°C for up to 14 h in the dark. The desiccated, encapsulated stem discs were placed in cryovials (four encapsulated stem disc quarters from a single clove) and plunged directly into LN and stored for at least 7 d. Cryovials containing encapsulated stem discs were rapidly rewarmed at 40°C for 2 min. The stem disc beads were rehydrated by placing them on semi-solid MS culture medium for 24 h under standard culture conditions (27). Afterwards the encapsulated explants were excised from the alginate and transferred to fresh semi-solid MS culture medium and

maintained as described by Lynch *et al.* (27). Growth was monitored over 6 weeks and survival was defined as explants that exhibited any sign of greening, swelling, and/or regrowth of roots, or the regrowth of one or more shoots similar to the criteria established by Keller (21).

Residual moisture and water content of stem discs

The fresh weight (f.wt) moisture content of non-encapsulated and encapsulated garlic stem disc quarters was determined by weighing stem discs before and after the combined sucrose pretreatment and desiccation by silica gel exposure up to 14 h. At each interval of 0, 2, 4, 6, 8, 10, 12 and 14 h, groups of 20 non-encapsulated and 20 encapsulated stem discs (five replicates of four stem disc quarters per time interval) were re-weighed and placed into glass 9 cm Petri dishes prior to oven drying. Dry weights (d.wt) were determined following drying in an oven at 105°C until a consistent d.wt value was achieved, usually after 16 h. Residual moisture (% RM) content was determined from the f.wt of a sample of beads from each stage in the encapsulation procedure and from the corresponding dry weights of stem discs that were dried at 105°C overnight (4, 18). The % RM for each treatment was calculated from these parameters (i.e. f.wt - d.wt / f.wt x 100 = % RM). The water content is defined by the f.wt / d.wt gravimetric values of non-encapsulated and encapsulated, desiccated stem discs that were used to determine the water content (f.wt - d.wt / d.wt) expressed as grams water per gram dry weight (g H_2O g^{-1} d.wt) as described elsewhere (6, 48, 46).

Experimental design and statistical analysis

The experimental design incorporated control materials which consisted of non-desiccated and non-frozen stem disc quarters and for comparative evaluations, non-encapsulated stem disc quarters were equally treated along with the encapsulated material as described above. Bulbs / cloves were randomly selected from the same batch. Ten cloves were used at each sampling time, where each clove produced four stem disc quarters, for a total of 40 explants per experimental treatment (n = 4 per replicate group with 10 replicated groups per treatment).

Statistical analysis was carried out using SPSS Version 10. Non-parametric tests (Mann-Whitney U on two independent sample test sets and Kruskal-Wallis H on several independent samples) assume unequal variances. One-way analysis of variance (ANOVA) with Dunnett's T3 *post hoc* pairwise comparison test (unequal variances) was used with a 95% Marco Polo confidence limit to determine significance (40).

RESULTS

Desiccation of non-encapsulated and encapsulated, non-frozen stem disc

Fig. 1 illustrates the effects of desiccation over silica gel on precultured (medium containing 0.5 M sucrose), non-encapsulated and encapsulated stem disc quarters observed before plunging in LN. Increasing duration of desiccation resulted in

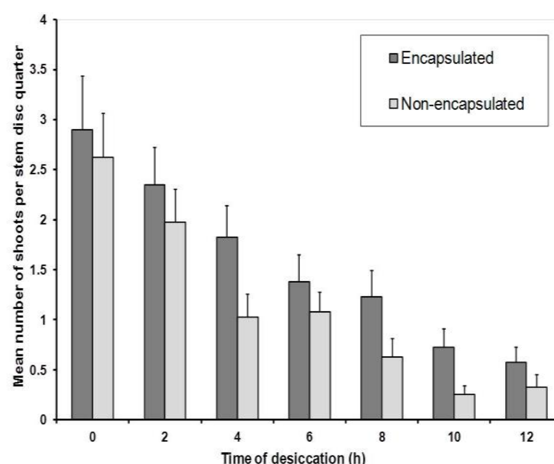

Figure 1. Frequency of shoot formation from non-frozen, non-encapsulated and encapsulated stem disc quarters following increased desiccation time; (means ± SE, n=10 replicates).

a decrease in the mean number of shoots derived from non-encapsulated and encapsulated stem discs. This trend was

evident with significant differences in shoot numbers in non-encapsulated stem disc quarters desiccated for 4 h ($P = 0.04$), 6 h ($P = 0.043$), 8 h ($P = 0.002$), 10 h ($P < 0.001$) and 12 h ($P < 0.001$) compared to non-desiccated controls (data not shown). A significant decrease in mean shoot numbers was also observed with desiccated encapsulated stem disc quarters after 10 h ($P = 0.007$) to 12 h ($P = 0.003$) desiccation compared with non-desiccated controls. Comparatively, the regrowth data for non-encapsulated stem discs exhibited more variability particularly between 4 to 12 h of desiccation. As a general observation the incidence of hyperhydrated shoots and roots increased following longer desiccation periods. The frequency of abnormal growth was higher on non-encapsulated explants as compared to encapsulated stem discs; root nodules and callus growth increased particularly for non-encapsulated explants following longer (10 to 12 h) desiccation periods.

Desiccation of non-encapsulated and encapsulated, non-frozen and frozen stem discs

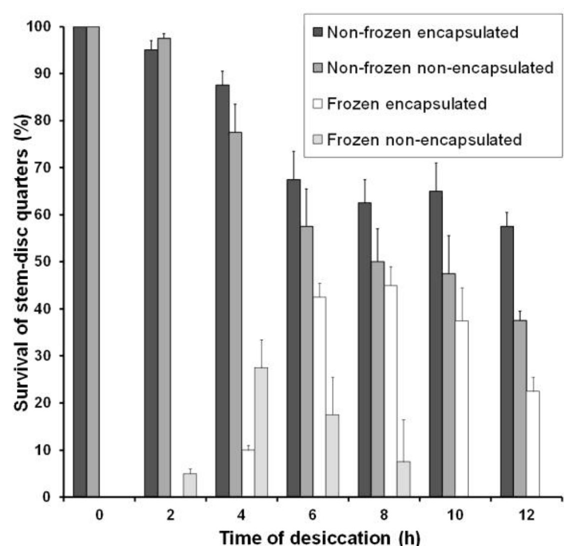

Figure 2. The survival of non-frozen and frozen stem disc quarters following increased desiccation time; values represent the mean of 10 replicates per treatment ± standard error.

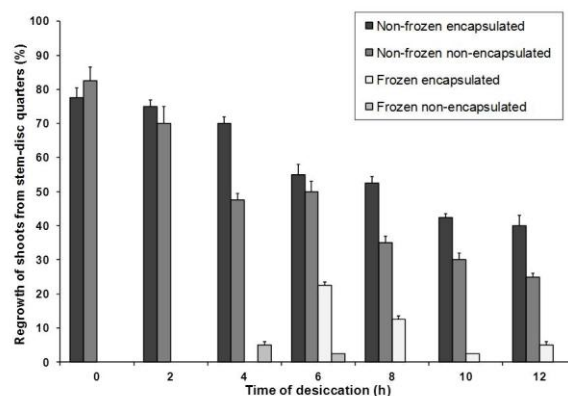

Figure 3. The regrowth of shoots of non-frozen and frozen garlic stem-disc quarters following increased desiccation time; values represent the mean of ten replicates per treatment ± standard error.

The effects of desiccation on survival (%) and shoot regrowth (%) of non-frozen and frozen, non-encapsulated and encapsulated stem disc quarters are shown in Figs. 2 and 3, respectively. A comparison between controls of the non-frozen, non-encapsulated and encapsulated stem disc quarters showed a progressive decline in survival of non-encapsulated stem disc with increasing desiccation over the 12 h period (Fig. 2). This effect was also observed in the encapsulated stem discs up to 6 h; however survival data ranging from ~60 - 70% after 6 to 12 h of desiccation indicates encapsulation reduces the effect of desiccation on stem discs. Following exposure to LN, these trends were similarly observed for survival of frozen, non-encapsulated and encapsulated stem disc quarters with increasing duration of desiccation (Fig. 2). A comparison between frozen, non-encapsulated and encapsulated stem disc quarters showed an increase in survival of non-encapsulated stem discs after 2 h, which peaked after 4 h and continued to decline after 6 h of desiccation. By contrast, frozen, encapsulated stem discs survived after 4 h increasing to 8 h and declining from 10 to 12 h of desiccation. Maximal survival for frozen, non-encapsulated stem discs was 27.5% compared to 42.5% for encapsulated stem disc quarters.

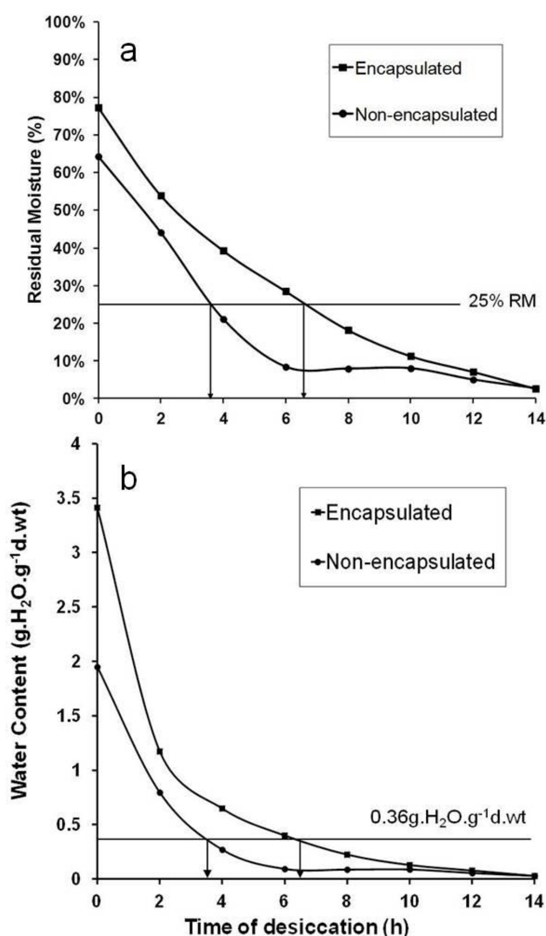

Figure 4. (a) Residual moisture and (b) water content (grams water per gram dry weight) of encapsulated and non-encapsulated stem disc following increased desiccation time; values represent the mean of five replicates per treatment.

Fig. 3 shows an overall reduction in regrowth of shoots in the controls for the non-frozen, non-encapsulated and encapsulated stem disc quarters with increasing desiccation over a 12 h period. Regrowth of shoots derived from non-frozen, non-encapsulated stem discs was lower relative to the encapsulated stem disc counterparts. The regrowth of shoots following exposure to LN was further reduced in frozen, non-encapsulated stem discs after 4 h and 6 h of desiccation. Regrowth was maximal from frozen, encapsulated stem discs after 6 h and declined up to 12 h of desiccation. Overall, the survival of frozen, encapsulated explants was 43% following 6 h and 45% after 8 h desiccation with 23% and 13% regrowth for the same corresponding desiccation times (Figs. 2 and 3). Generally, statistical analysis showed survival and regrowth between encapsulated/non-encapsulated, desiccated/non-desiccated frozen/non-frozen stem discs was significantly different (P <0.05); values for encapsulated stem disc explants after 6 and 8 h desiccation treatments (P = 0.009 and P <0.001 respectively) were significantly different compared to non-encapsulated stem discs.

Desiccation and residual moisture and water content of non-encapsulated and encapsulated, non-frozen and frozen stem discs

Fig. 4a shows % RM values calculated for each desiccation treatment of non-encapsulated and encapsulated stem discs. These two types of samples have distinctly different desiccation characteristics. The desiccation profile of non-encapsulated stem discs showed a lower RM of 65% at time t = 0; this dropped with increased desiccation to <4 h which transected the 25% RM threshold line. In comparison to encapsulated stem disc, time t = 0 of the encapsulated stem disc desiccation started at a higher (78% RM) value, then gradually decreased with increasing desiccation to <7 h which transected the 25% RM threshold line. The threshold line of 25% RM transected the curves at ~3.5 h for non-encapsulated and ~6.5 h for encapsulated stem discs.

Fig. 4b shows the water content (g H_2O g^{-1} d.wt) calculated for each desiccation treatment of non-encapsulated and encapsulated stem discs displaying different curve characteristics. The non-encapsulated stem discs exhibited a lower water content compared to their encapsulated counterparts. As desiccation progressed both non-encapsulated and encapsulated stem disc increasingly lost water and their desiccation curves transected the threshold line approximating at the value 0.36 g H_2O g^{-1} d.wt at ~3.5 h and ~6.5 h, respectively. There was a gradual decrease in the rate of

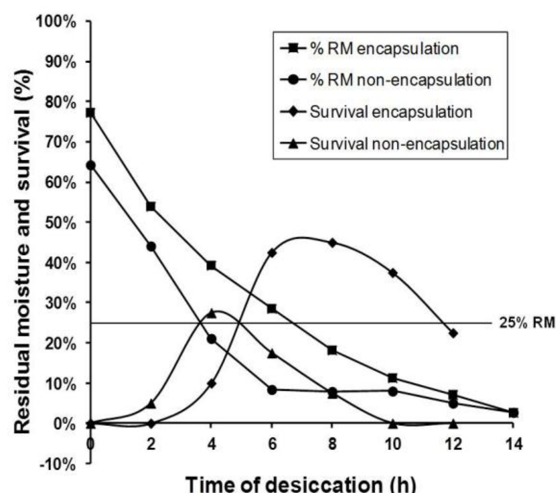

Figure 5. Residual moisture and survival following desiccation of non-encapsulated and encapsulated stem discs after recovery from LN.

water loss below this threshold for both types of stem disc with increasing desiccation until both reached a point close to zero at 14 h.

Fig. 5 shows a composite plot of % RM data (Fig. 4a) and g H_2O g^{-1} d.wt data (Fig. 4b) with the corresponding survival (%) data (Fig. 2) from desiccated and cryopreserved non-encapsulated and encapsulated stem disc samples. Survival for desiccated, cryopreserved non-encapsulated stem discs spanned a 2 to 10 h desiccation interval reaching the highest point after 4 h. Cryopreserved encapsulated stem discs showed extended survival over 2 to 12 h, peaking between 6 - 8 h of desiccation. There were correlations between the 25% RM (Fig. 4a) and 0.36 g.H_2O g^{-1} d.wt (Fig. 4b) with the trend for maximal survival at ~3.5 h for non-encapsulated and ~6.5 h encapsulated stem discs. Data demonstrated that as the water content progressively decreased - survival increased in both cryopreserved non-encapsulated and encapsulated stem discs and that once passed a maximal point of desiccation this caused a gradual decline in stem disc viability.

DISCUSSION

Long-term *in vitro* conservation requires the implementation of standardized protocols for the effective cryopreservation of clonal crop genetic resources (3). The success of any given cryopreservation protocol is dependent on non-cryogenic and cryogenic factors (19). The importance of understanding the seasonal storage behaviour and physiological responses of plant material and its impact on cryopreservation is illustrated by monitoring storage performance and breaking the dormancy phase of garlic bulbs to maximize recovery of *in vitro* shoots (26, 27, 28). The present study underlies the need to optimize the cryogenic components of a protocol, which is central to understanding the critical factors that affect post-storage outcomes (2).

Encapsulation-dehydration is one example of complex, multi-component procedure (14), which requires an assessment of the sensitivity of plant material to a range of extreme osmotic and drying experimental conditions (33). In a previous study (40), osmotic dehydration was optimized by the regrowth of garlic *in vitro* shoots on medium containing 0.5 M sucrose (29). Extending this investigation determined the sensitivity of garlic stem disc to increasing desiccation (Fig. 1). The differential regrowth response of garlic shoots indicates that alginate encapsulation provides a protective “micro-environment” compared to non-encapsulated stem discs during desiccation. This effect was more evident by the display of abnormal growth attributed to non-encapsulated *versus* encapsulated stem discs (40), although the effects of other contributory factors cannot be excluded (7, 42). The reproducibility of this protective response to desiccation by encapsulation was further shown by the control non-frozen stem discs (Figs. 2 and 3). Similar to these findings, Fabre and Dereuddre (12) reported that exposure of encapsulated-dehydrated potato meristems to LN influenced survival and regrowth of shoots compared to the control counterparts. The trend in responses of encapsulated stem

discs was further extended with higher maximal survival indicating tolerance to desiccation compared to non-encapsulated stem discs, which had a greater sensitivity to desiccation (Fig. 5).

Similarly, desiccation profiles for non-frozen, non-encapsulated and encapsulated stem discs (Fig. 1) were determined from graphical plots of % RM against the time of desiccation (Fig. 4a). The curve characteristics showed two distinct responses: (i) the desiccation sensitivity of non-encapsulated stem discs through rapid loss of water ; and (ii) desiccation tolerance of encapsulated stem discs by the gradual delay in loss of water over the duration of desiccation (48). A comparison of these observations illustrates the principle of the encapsulation-dehydration technique in that the respective delayed water loss in encapsulated stem discs (contrary to non-encapsulated) causes a proportionate increase in viability until a critical point is reached when viability declines. These observations demonstrate the variability in viability following recovery from LN (Fig. 5), where water content is critical in order to avoid lethal intracellular ice formation and optimal desiccation removes sufficient water to enable totipotent cells in the stem discs to survive (2, 14). A critical level of water loss is reached that allows the maximal survival of stem discs, after which the further removal of water causes cell damage and loss of viability by excessive desiccation (Fig. 5). This critical point varies according to the type of plant material and technical specifications of encapsulation-dehydration (14, 43); however 25 % RM appears to be a consistent approximation to examine the critically of water status in many species and genotypes (5, 9, 14, 16, 18).

Setting the threshold line at 25 % RM transects the desiccation curves at ~3.5 h for non-encapsulated and ~6.5 h for encapsulated stem discs, indicating that these periods of desiccation correspond to optimal water loss to achieve a predetermined level of survival following recovery from LN (Fig. 5). Water content calculations of the corresponding g H_2O g^{-1} d.wt values for each desiccation treatment provide further insights into the desiccation characteristics, in that the given times of ~3.5 h for non-encapsulated and ~6.5 h for encapsulated stem discs both relate to the threshold line approximating to the value of 0.36 g H_2O g^{-1} d.wt (Fig. 4b). These desiccation times correlate the 25% RM and 0.36 g H_2O g^{-1} d.wt values with the survival of cryopreserved stem discs by encapsulation-dehydration. Similar desiccation and survival characteristics have been observed in other species (17, 18, 33). Together the 25% RM and 0.36 g H_2O g^{-1} d.wt values transect the survival curves approximating to the point of maximal survival for both non-encapsulated and encapsulated stem discs (Fig. 5). Above the critical point indicated by the 25% RM threshold line the data curve displays a 'window' for optimal survival (Fig. 2) and regrowth (Fig. 3) that lies between 6 - 10 h desiccation (Fig. 5). These studies indicate that the 25% RM limit is not an absolute parameter (15, 16) for example decreasing the RM value to 20 % accordingly would further reduce the g H_2O g^{-1} d.wt value and extend the limits of survival across the desiccation range.

The encapsulation-dehydration (12) of plant tissues is a vitrification-based technique (2, 9), which has been routinely applied to a wide range of species/genotypes (14, 18, 36). An intensive study by differential scanning calorimetry (DSC) is usually required to accurately determine the osmotically active (frozen) and inactive (non-freezable) water content of tissues to confirm the vitrified state following encapsulation-dehydration (5, 6, 9, 10, 13, 33, 46, 47). There are several reports, which correlate water status of tissues with the onset of the vitrified state at crucial stages during the desiccation process. DSC analysis of *Ribes nigrum* and *R. ciliatum* encapsulated meristems detected a Tg following sucrose dehydration and 7 h desiccation over silica gel which reduced the bead water content to 0.3 g H_2O g^{-1} d.wt

(39). DSC analysis of *R. sanguineum* confirmed vitrification by detecting the glass transition (Tg) in encapsulated meristems following sucrose dehydration and 4 h air desiccation which corresponded to an osmotically inactive water content of 0.33 g H_2O g^{-1} d.wt (4); a Tg was also detected in encapsulated shoot meristems of *Picea sitchensis* and water content of 0.43 g.H_2O g^{-1} d.wt (13). Comparative studies of alginate beads demonstrated successful vitrification produced a stable glass with water content of ~26% and 0.4 g H_2O g^{-1} d.wt mainly composed of osmotically inactive water (6). This optimal water content 0.4 g H_2O g^{-1} d.wt was found to be important for the cryopreservation of potato, hops and apple to produce high levels of regeneration (46) and similarly for blackberry and raspberry (18).

Although thermal analysis was not conducted, comparatively the 25% RM and 0.36 g H_2O g^{-1} d.wt data values are in good agreement with the reported DSC data suggesting the formation of a stable vitrified state following sucrose dehydration, desiccation and plunging in LN by encapsulation-dehydration of garlic stem discs. Other studies report the cryopreservation of *Allium* species by PVS-vitrification based methodologies (11, 23, 24, 32, 35, 42, 45, 46, 47); however the stem disc approach described by Ayabe and Sumi (1) offers distinct advantages: (i) the potential for higher levels of regeneration from multi-basal plate apices compared to methods that use single primary meristem cultures; and (ii) an alternative to difficult to handle bulb material and *Allium* species that have non-bolting (topsets) genotypes (22, 46). In conclusion, this study confirms the need to correlate viability to water content for the standardization of cryopreservation protocols applied to garlic stem discs and to facilitate the effective implementation of *Allium* cryostorage.

Acknowledgements: Funding: EU COST Action 871 Cryoplanet; EU Crymcept QLK5CT-2002-01279; J. Keller, IPK, Germany for garlic germplasm; K. Harding for the Visiting Professorship at the University of Derby.

REFERENCES

1. Ayabe M & Sumi S (1998) *Plant Cell Reports* **17**, 773-779.
2. Benson EE (2008) *Critical Reviews Plant Sciences* **27**, 141-219.
3. Benson EE, Harding K, Debouck D, Dumet D, Escobar R, Mafla G, Panis B, Panta A, Tay D, Van den houwe I & Roux N (2011) *Refinement and Standardization of Storage Procedures for Clonal Crops - Global Public Goods Phase 2: Part I. Project Landscape and General Status of Clonal Crop* in vitro *Conservation Technologies*. System-wide Genetic Resources Programme, Rome.
4. Benson EE, Johnston J, Muthusamy J & Harding K (2005) in: *Plant Tissue Culture Engineering,* (eds) SD Gupta & Y Ibaraki, Springer, Dordrecht, pp 441-473.
5. Benson EE, Reed BM, Brennan R, Clacher KA & Ross DA (1996) *CryoLetters* **17**, 347–362.
6. Block W (2003) *Cryobiology* **47**, 59-72.
7. Brunakova K, Zamecnik J, Urbanova M & Cellarova E (2011) *Thermochimica Acta* **525**, 62-70.
8. Colmsee C, Keller ERJ, Zanke C, Senula A, Funke T, Oppermann M, Weise S & Scholz U (2012) *Genetic Resources Crop Evolution* **59**, 1407-1415.
9. Dereuddre J, Hassen N, Blandin S & Kaminski M (1991) *CryoLetters* **12**, 135-148.
10. Dumet D, Block W, Worland R, Reed BM & Benson EE (2000) *CryoLetters* **21**, 367–378.
11. Ellis D, Skogerboe D, Andre C, Hellier B & Volk G (2006) *CryoLetters* **27**, 99-106.
12. Fabre J & Dereuddre J (1990) *CryoLetters* **11**, 413-426.
13. Gale S, Benson EE & Harding K (2013) *CryoLetters* **34**, 30-39.

14. Gonzalez-Arnao MT & Engelmann F (2006) *CryoLetters* **27**, 155-168.
15. Gonzalez-Arnao MT, Engelmann F, Urra C, Morenza M & Rios A (1998) *CryoLetters* **19**, 177-182.
16. Gonzalez-Arnao MT, Juarez J, Ortega C, Navarro L & Duran-Vila N (2003) *CryoLetters* **24**, 85-94.
17. Grospietsch M, Stodulkova E & Zamecnik J (1999) *CryoLetters* **20**, 339-346.
18. Gupta S & Reed BM (2006) *CryoLetters* **27**, 29-42.
19. Harding K, Johnston JW & Benson EE (2009) *Agriculture Food Science* 18: 3-16.
20. Keller ERJ (2002) in: *Biotechnology Agriculture Forestry*, Volume 50, *Cryopreservation of Plant Germplasm II*, (eds) LE Towill & YPS Bajaj, Springer-Verlag, Berlin, pp 37-47.
21. Keller ERJ (2005) *CryoLetters* **26**, 357-366.
22. Keller ERJ, Zanke CD, Blattner FR, Kik C, Stavělíková H, Zámečník J, Esnault F, Kotlińska T, Solberg S & Miccolis V (2012) *ISHS Acta Horticulturae* **969**, 319-327.
23. Kim HH, Lee YG, Park SU, Lee SC, Baek HJ, Cho EG & Engelmann F (2009) *CryoLetters* **30**, 291-299.
24. Kim HH, Lee YG, Shin DJ, Ko HC, Gwag JG, Cho EG & Engelmann F (2009) *CryoLetters* **30**, 320-334.
25. Lynch PT, Benson EE & Harding K (2007) *J Hort Sci and Biotechnology* **82**, 157-160.
26. Lynch PT, Souch GR & Harding K (2010) *Cryobiology* **61**, 400-401.
27. Lynch PT, Souch GR & Harding K (2012) *J Hort Sci and Biotechnology* **87**, 588–592.
28. Lynch PT, Souch GR & Harding K (2013) *CryoLetters* **34**, 215-216.
29. Lynch PT, Souch GR & Harding K (2014) Abstract in: *Society for Low Temperature Biology, Freezing Biological Time, 50th Anniversary Celebration, 8–10th October 2014, Jodrell Laboratory*, The Royal Botanic Gardens, Kew, London. p. 63.
30. Lynch PT, Souch GR, Trigwell S, Keller J & Harding K (2011) *Asia Pacific Journal of Molecular Biology and Biotechnology* **18**, 239-242.
31. Maggioni L, Keller J & Astley D (2002) *European Collections of Vegetatively Propagated* Allium. Gatersleben, International Plant Genetic Resources Institute, Rome.
32. Makowska Z, Keller J & Engelmann F (1999) *CryoLetters* **20**, 175-182.
33. Martinez D & Revilla MA (1998) *CryoLetters* **19**, 333-342.
34. Murashige T & Skoog FA (1962) *Physiologia Plantarum* **15**, 473-497.
35. Niwata E (1995) *CryoLetters* **16**, 102-107.
36. Reed BM (2008) *Plant Cryopreservation: a Practical Guide*. Springer, New York.
37. Senula A & Keller ERJ (2002) in: *European Collections of Vegetatively Propagated* Allium, (eds) L Maggioni, J Keller & D Astley, Gatersleben, Germany, International Plant Genetic Resources Institute, Rome, pp. 66-71.
38. Shemesh E, Scholten O, Rabinowitch HD & Kamenetsky R (2008) *Planta* **227**, 1013-1024.
39. Sherlock G, Block W & Benson EE (2005) *CryoLetters* **26**, 45-54.
40. Souch GR (2006) *Long-term Conservation of* Allium *Germplasm Resources: the Cryopreservation of A. sativum Using an Encapsulation / Dehydration Approach*. Ph.D thesis, University of Derby, Derby, UK
41. Stavělíková H (2008) *Hort Sci (Prague)* **35**, 130–135.
42. Teixeira AS, Faltus M, Zámečník J, González-Benito ME & Molina-García AD (2014) *Thermochimica Acta* **593**, 43–49.
43. Verleysen H, Van Bockstaele E & Debergh P (2005) *Scientia Horticulturae* **106**, 402–414.

44. Volk GM, Henk AD & Richards CM (2004a) *J Amer Soc Hort Sci* **129**, 559-569.
45. Volk GM, Maness M & Rotindo K (2004b) *CryoLetters* **25**, 219-226.
46. Zámečník J, Faltus M & Bilavčík A (2012) in: *Current Frontiers in Cryopreservation*, (ed) I Katkov, InTech, Rijeka, pp 333-358.
47. Zamecnik J, Faltus M, Kotkova R & Hejnak V (2011) *Acta Horticulturae* **908**, 33-38.
48. Zamecnikova J, Fernandez E, Viehmannova I, Zamecnik J & Faltus M (2011) *Acta Horticulturae* **908**, 331-338.