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Thermochimica Acta 349 (2000) 147–151

thermochimica acta

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Differential scanning calorimetry applied to the storage at ultra low temperatures of olive and hop in vitro grown shoot-tips

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Received 6 August 1999; received in revised form 27 October 1999; accepted 1 November 1999

Abstract

Differential scanning calorimetry was used for monitoring the survival conditions of olive in vitro grown shoot-tips under cryopreservation (−196°C), as well as for establishing the storage conditions at temperatures higher than liquid nitrogen. Olive survival is related to the absence of formation and growing of ice crystals during the cooling process. These results are compared with those obtained in hop. The final storage temperature is given as a function of the shoot tip survival to the dehydration step and the absence of ice crystal growth during cooling and warming. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Differential scanning calorimetry; *Olea europaea* L.; *Humulus lupulus* L.; Liquid nitrogen; Cryopreservation

1. Introduction

Thermal analysis techniques [1,2] have been found to be suitable for the characterization of a wide variety of systems: synthetic compounds [3,4]; natural rocks [5,6]; foods [7–9]; and biological materials [10]. Most of the applications of thermal methods to biological materials have been for identification and characterization. However, perhaps this is to be expected because of the complexity and heterogeneity of these materials. The DSC curves are frequently quite broad and are devoid, in many cases, of narrow endothermic and exothermic peaks.

One of the main barriers to survival of plant tissues in cryopreservation is avoiding the formation and growth of ice glasses [10]. Numerous plant species have been successfully cryopreserved to temperatures of liquid nitrogen (−196°C) [11,12]. However, specific protocols for different plant species, sophisticated freezing equipment and a source of liquid nitrogen are generally necessary. Recently, the development of direct dehydration methods make possible the storing of plant samples at temperatures higher than liquid nitrogen [13,14].

A direct dehydration method has been described for in vitro grown shoot-tips of olive [15] and hops [16]. It involved pre-culturing the merismatic dome with 1 or 2 pairs of leaf primordia for 2 days in culture medium enriched with sucrose, followed by dehydration in an air current cabinet, transfer to cryovials, plunging into liquid nitrogen for storage and rewarming at room temperature. The present study shows that differential scanning calorimetry is a powerful tool to measure plant survival from cryopreservation. Thermal

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PII: S0040-6031(99)00508-0

changes during cooling and rewarming cycles are used to study the ability of storing at temperatures higher than liquid nitrogen.

2. Experimental

Micropropagated shoots of olive (*Olea europaea* L. var. Arbequina), originating from embryo material [17] were grown on a medium previously described for walnut (DKW) with 3% sucrose, pH 5.8 [18]. Hops shoots (*Humulus lupulus* L. var Nugget), were grown on a medium containing the mineral salts of Murashige and Skoog [19] and the vitamins of Wetmore and Sorokin [20] (MSWS) with 3% glucose and 0.4% activated charcoal, pH 5.2. Both media were supplemented with 4.4 μM benzylaminopurine and 0.5 μM indole-3-butyric acid, 0.75% agar. Growth conditions were 25°C and 16 h photoperiod (40 μmol/m^2/s). Transfers of the micro-shoots to fresh media were made every 40 days.

For cryopreservation experiments, apical and axillary hops shoot tips (0.5–2 mm) from cold-acclimated plants previously encapsulated in alginate beads [16], and apical olive shoot tips (2–5 mm), were excised from 40-day-old micro-shoots under a stereo microscope. Both types of samples comprising the meristematic dome with 1 or 2 pairs of leaf primordia were precultured for 2 days on solid medium supplemented with 0.75 M sucrose and dehydrated for different duration. The dehydration step was performed at 20°C under the air current of a flow cabinet placing beads entrapping hop shoot tips or naked olive shoot tips on top of a filter paper in an open Petri dish containing 30 g of silica gel (previously activated at 100°C for 2 days) and weighing over 8 h at intervals of 30 min. Dry weights were determined by oven-drying the samples for 16 h at 110°C. The difference between initial fresh weight and final dry weight was considered 100% of water content.

To study survival percentage after cryopreservation, samples were introduced into 2 ml cryovials (10 hop beads or 20 naked olive shoot tips per cryovial) and frozen by direct immersion in liquid nitrogen for at least 1 h. Afterwards, they were thawed in a flow cabinet at room temperature for 15 min and cultured on the respective proliferation medium. After six weeks of culture under the same growth conditions described above, survivals were measured as percentage of shoot tips that regenerated new buds and leaves from frozen and control samples (sucrose preculture plus dehydration).

Differential scanning calorimetry was performed using a Mettler TA 4000 system (TC 11 PC and DSC 30). Sample fresh masses were between 0.2 and 1 mg, as a function of the water content, remaining the dry weight constant. Samples were placed in aluminum pans. DSC profiles were recorded using a scanning rate of 10°C/min from +20 to −150°C for cooling and warming. Analysis was repeated at least six times to ensure reproducibility.

3. Results and discussion

Both cryopreservation methods described in this paper for hop and olive are dependent upon the removal of water from plant tissues using osmotic dehydration and air desiccation. Fig. 1 shows water content of the olive shoot tips as a function of the dehydration time in the air of a flow cabinet at 20°C. The water content remained constant at 22% after 4 h of dehydration. Shoot tips that were non-dehydrated and non-cooled in liquid nitrogen had very high survival (Fig. 2a). Dehydration appeared to cause a decrease in the survival percentage and after 4 h no survival was observed. When samples were frozen, maximum recovery was observed after 1.5–2.5 h of

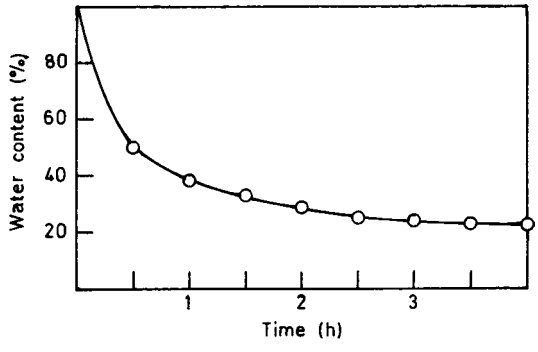

Fig. 1. Changes in water content of olive apical shoot tips after 2-day-preculture with 0.75 M sucrose, expressed as a function of air-drying time (0–4 h) under the air current of a flow cabinet. Dates expressed relative differences of fresh and dry weights and represent the media of five replicates of 15 shoot tips per replicate.

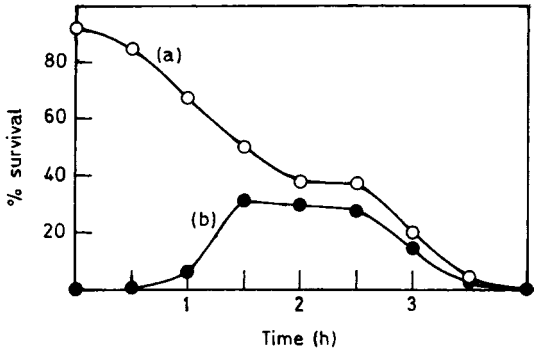

Fig. 2. Effect of dehydration time on the percentage of survival from olive apical shoot tips: (a) precultured in 0.75 M sucrose and dehydrated (control samples); and (b) subsequently cooled in liquid nitrogen (frozen samples).

dehydration (Fig. 2b). Considering survival of the frozen samples with respect to survival of dehydrated samples for the same period, an increase in the relative survivals is obtained as dehydration degree of the sample increased (Fig. 3).

The success of these cryopreservation methods are largely dependant upon desiccation tolerance and the ability to circumvent ice nucleation during cooling. Fig. 4 shows typical DSC profiles for these systems. Shoot tips at 100% water content showed an exothermic peak (Q=93.5±18.2 J/g) resulting from ice formation (Fig. 4a). Samples dehydrated for 1 h (Fig. 4b), also showed this signal but with less heat,

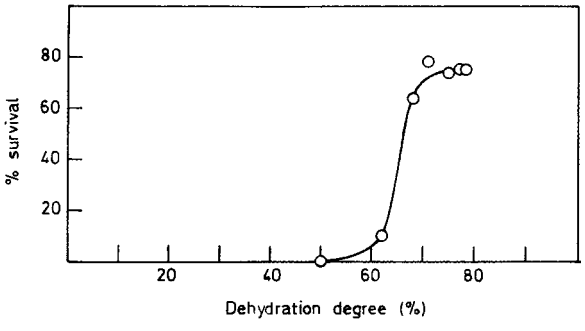

Fig. 3. Effect of dehydration degree on the relative survival (survival of frozen samples with respect to survival of control samples, non-frozen) from olive apical shoot tips in the cryopreservation process. Samples were previously 2-day-precultured in 0.75 M sucrose and dehydrated under the air of a flow cabinet.

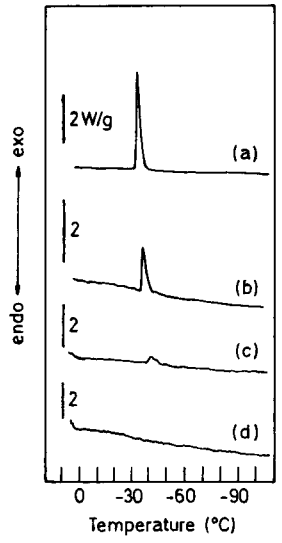

Fig. 4. Cooling process: DSC profiles of olive apical shoot tips with different water contents (a) 100%, (b) 38%, (c) 32%, and (d) 28%. Six different samples for each water content were analyzed. Ordinate in watts per gram of tissue.

Q=50.4±14.7 J/g, in accordance with a lower content of water (38%). For samples dehydrated for 1.5 h (32% water content), a further decrease of the exothermic peak is recorded (Q=6.7±3.5 J/g), (Fig. 4c). Tissue with longer dehydration time (2 h) had no ice formation signal (Fig. 4d) and glass transition occurs at −40, −60°C, without heat changes. Fig. 5 shows the correlation between the heat of ice formation and the relative survival rate after cryopreservation. The results are qualitatively similar to the

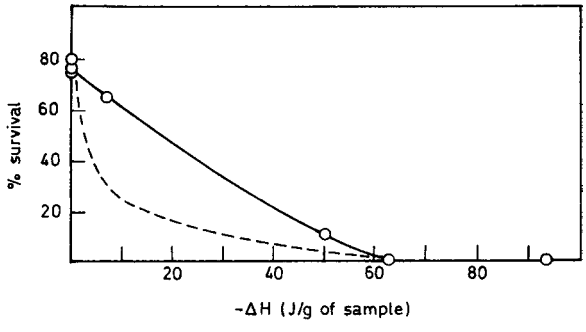

Fig. 5. Olive apical shoot tips survival rate in the cryopreservation process vs. heat of ice formation. The dashed line shows the results from hop shoot tips cryopreservation [10].

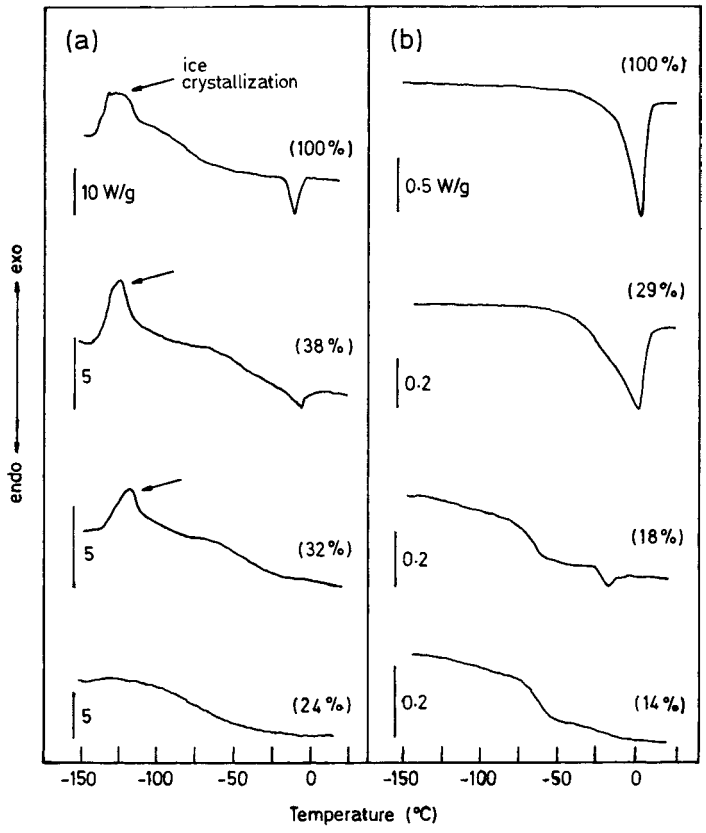

Fig. 6. Warning process: DSC profiles of (a) olive apical shoot tips, and (b) hop shoot tips. Sample water content in parenthesis.

previously described in the study of *Humulus lupulus* L. cryopreservation [10], relating high survival rates after cryopreservation with absence of ice nucleation and enthalpy variations.

In addition to ice damage during freezing, plant tissue can suffer damage during thawing due to crystallization of the ice [21,22]. Ice crystallization associated with a melt peak were observed in olive samples (Fig. 6a) when the dehydration was low (water content >30%). After 3 h of dehydration (24% water content) only glass transition was observed. However, in hops crystallization of ice was not observed, at any stage of hydration (Fig. 6b). These results indicate that it is important to determine in cryopreserved plant tissues the critical point of desiccation to avoid ice nucleation during cooling and also during warming. The stabilization of the glassy state on re-warming is clearly of major importance in terms of circumventing cellular damage due to ice nucleation. The absence of ice nucleation during the cooling and warming indicate that it may be possible to store plant tissues at temperatures above liquid nitrogen without the risk of crystallization.

4. Conclusions

These findings suggest that thermal behaviors of plant tissues (hops or olive) and the cryoprotectant systems may be different. While glass transition events are reproducible on cooling, warming can incur devitrification or ice crystallization, which is largely dependent upon achieving a critically low moisture content.

Survival during cryopreservation is directly related to the absence of lethal ice crystals. Differential

scanning calorimetry can be used to detect ice formation in plant samples with different water contents. Therefore, thermal profiles during cooling and warming can be used for studying the ability of storage at temperatures higher than liquid nitrogen.

Acknowledgements

S.A.E. de Fomento del Lúpulo and Universidad de Oviedo (Project FC-TA-96-517-5) supported this work.

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