

## DESICCATION SENSITIVITY OF WILD *CASTANEA MOLLISSIMA* BLUME DETERMINED BY DIFFERENTIAL SCANNING CALORIMETRY

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### Abstract

Thermal properties of wild *Castanea mollissima* seeds embryos with different moisture contents, and the optimal moisture contents for cryopreservation were studied. Seeds of similar size and weight ( $3.5 \pm 0.3$ g) were put in silica gel at 0 to 7 days, embryo moisture content, viability and thermal properties were measured everyday. The results showed that the onset temperature and the crystallization peak of the mean enthalpy decreased with the decrease of embryo moisture content. Exothermic peak disappeared when the seeds were dried for 4 days, with an embryo moisture content of  $0.45 \pm 0.15$  gH<sub>2</sub>O/g dw. The unfrozen water content (WC<sub>u</sub>) was 0.247 gH<sub>2</sub>O/g dw. The optimal water content was found to be  $0.45 \pm 0.15$  gH<sub>2</sub>O/g dw, with the survival of 76.2%. *Castanea mollissima* embryonic axes dehydration needs comprehensive consideration of exothermic peak, unfrozen water content, onset temperature and viability loss.

### Introduction

Recalcitrant seeds usually attain maturity with relatively high moisture contents and they are sensitive to dehydration, and cannot be stored in conventional seed banks for long term (Li and Pritchard 2009, Walters *et al.* 2012, Xia *et al.* 2013). Cryopreservation is a viable strategy for embryo storage of some recalcitrant species (Corredoira *et al.* 2004, Xia *et al.* 2014). However, recalcitrant seeds have desiccation sensitive embryos which cannot tolerate sub-zero temperatures at high moisture contents. Hence, in order to do cryopreservation, it is important to know the features of seeds desiccation tolerance, viability and induction of glassy stage.

Differential scanning calorimetry (DSC) is a useful tool for the non-invasive observation of seed tissues, particularly in terms of their thermal behaviour (Walters *et al.* 2005, Lehner *et al.* 2006). DSC can be used in determining 'freezable' free water or unfrozen water content (Zoubi and Normah 2015), free water frozen lead to freezing injury during embryo cryopreservation, if desiccation sensitive seeds remove 'freezable' free water, freezing injury will be avoided during cryopreservation.

*Castanea mollissima* Blume (Chinese chestnut) is a widely distributed species in northern hemisphere (Serdar *et al.* 2011). In China, chestnut is distributed in 24 provinces, covering an area over  $2 \times 10^7$  ha, and it is an important economic forest tree, million farmers rely on chestnuts as their main economic source in mountainous areas (Zou *et al.* 2015). However, Chinese chestnut has desiccation sensitive seeds (Zong and Cai 2010), which makes its germplasm conservation difficult. In the present study, DSC analysis was used to analyze the thermal properties at various moisture contents after the seeds dehydration in silica gel, and to investigate the desiccation sensitive features and the optimal moisture content for cryopreservation.

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## Materials and Methods

In September, wild *C. mollissima* seeds were collected from the mountain area ( N 34°5'41", E 107°39'50", altitude 884.6 m. ) of Baoji City, Shanxi Province. After harvest, the seeds were sent to seed bank. The seeds with similar size and weight ( $3.5 \text{ g} \pm 0.3 \text{ g}$ ) were dried in silica gel for various periods (0, 1, 2, 3, 4, 5, 6, 7 day) at ambient temperature ( $25 \pm 2^\circ\text{C}$ ). Silica gel was dried and changed everyday. After each desiccation period, 23 embryonic axes of the seeds (in triplicates) were randomly sampled and measured. Ten for viability assessment, 3 for DSC and 10 for moisture content determination, the experiment were repeat three times.

Moisture content of the seeds and embryonic axes ( $\text{gH}_2\text{O/g dw}$ ) were gravimetrically determined at the end of each desiccation period, using 10 seeds and 10 embryonic axes, replicated three times and dried to a constant weight in oven at  $103^\circ\text{C}$  for 17 hrs.

Seed viability test was carried out using TTC test. The seeds were cut to seed tissue with embryonic axes inside, 10 seed tissues in 1% TTC were incubated at  $30^\circ\text{C}$  for 3 hrs. After that, the embryonic axes were excised under anatomic microscope and embryos viability was scored and repeated three times.

The thermal analysis of different embryo moisture contents was undertaken using the DSC of TA company D250 (USA), which incorporates a liquid nitrogen cooling system. 3 embryonic axes samples of different moisture contents were placed in aluminium pans, sealed and weighed on a microbalance. The samples were kept at  $30^\circ\text{C}$  for 1 min, cooled at the rate of  $10^\circ\text{C}/\text{min}$  to  $-150^\circ\text{C}$  for 2 min, and then re-warmed to room temperature (Zoubi and Normah 2015).

The embryonic axes survival and DSC data were analysed by an ANOVA with a least significance difference (Tukey test) post-hoc test, difference at the 0.05 level is considered to be significant. For thermograms analysis, DSC equipment TRIOS V4 software (TA, USA) was used.

## Results and Discussion

Silica gel drying resulted in considerable water loss, but the moisture content decreased in different ratios for different dried days (Table 1). For example, dried for 1 day, the embryo moisture content reduced from  $1.59 \text{ gH}_2\text{O/g dw}$  to  $0.73 \text{ gH}_2\text{O/g dw}$ , which decreased 54.1%. However, the seed moisture content only decreased 34.7%, less than the embryo moisture content. The embryo moisture content decrease ratio were 54.1, 15.1, 24.2, 4.3, 15.6, 10.5 and 14.7% at 1 to 7 days, it shows that embryonic axes water loss was in different ratio from the endosperm.

In the first 4 days, silica gel drying not resulted in remarkable embryonic axes viability loss. After dried for 4 days, the embryo moisture content was reduced from  $1.59 \text{ gH}_2\text{O/g dw}$  to  $0.45 \text{ gH}_2\text{O/g dw}$  (Table 1), which decreased 71.7%, however, viability of desiccated embryonic axes just reduced from 90.48 to 76.19%, which decreased 15.8%. Survival of dried embryo showed a significant decline after the moisture content reduced to  $0.38 \text{ gH}_2\text{O/g dw}$ , with the survival of 57.14%. The embryonic axes dried for 7 days showed 47.62% survival with moisture content of  $0.29 \text{ gH}_2\text{O/g dw}$ , which proved that the embryonic axes of *C. mollissima* was desiccation sensitive.

Table 1 shows that the onset temperature, peak temperature and enthalpy decreased as the moisture content of the embryo was decreased. The highest value for the mean enthalpy of fresh embryonic axes was  $125.02 \text{ J/g dw}$ , at embryo moisture content of  $1.59 \text{ gH}_2\text{O/g dw}$ . After desiccation 3 days, mean enthalpy decreased to  $0.6 \text{ J/g dw}$  and the onset temperature decreased to  $-28.95^\circ\text{C}$ , at a moisture content of  $0.47 \text{ gH}_2\text{O/g dw}$  (Table 1). After desiccation for 4 days, the moisture content of embryo decreased to  $0.45 \text{ gH}_2\text{O/g dw}$ , no peak was observed (Table 1 and Fig. 1).

Freezable water leads to crystallization. Fig.1 (0 - 3 days) shows that at relatively higher moisture contents, embryo showed larger exothermic peak, presumably from the crystallization of

free water. Crystallization peak was observed when the mean moisture content was 0.47 gH<sub>2</sub>O/g dw or greater, however, it disappeared when the moisture content was below 0.45 gH<sub>2</sub>O/g dw (Fig. 1, 4 day).

The onset temperature is considered to be the starting point of free water crystallization. Table 1 shows that the mean onset temperature decreased from -14.38 to -28.95°C, and the mean peak temperature decreased from -14.78 to -35.09°C as the moisture content of the embryo was decreased from 1.59 gH<sub>2</sub>O/g dw to 0.47 gH<sub>2</sub>O/g dw. The onset temperature and peak temperature decreased means the embryonic axes free water became harder to freeze, it is beneficial to seeds (embryos) freezer storage.

**Table 1** Moisture content, survival and thermal properties of embryonic axes of *Castanea mollissima* seed dried in silicone.

Seed desiccation time (day)	Seed moisture content (gH <sub>2</sub> O/g dw)	Embryo moisture content (gH <sub>2</sub> O/g dw)	Survival (%)	Thermal properties		
				Onset (°C)	Enthalpy (J/g dw)	Peak temp. (°C)
0	1.01 ± 0.09	1.59 ± 0.24	90.48 <sup>a</sup>	-14.38 ± 1.28	125.01 ± 6.74	-14.78 ± 0.39
1	0.66 ± 0.08	0.73 ± 0.12	90.48 <sup>a</sup>	-19.10 ± 5.79	53.89 ± 39.80	-19.75 ± 6.64
2	0.64 ± 0.08	0.62 ± 0.07	85.71 <sup>a</sup>	-22.05 ± 6.00	53.25 ± 10.37	-23.80 ± 5.55
3	0.46 ± 0.05	0.47 ± 0.06	80.95 <sup>b</sup>	-28.95 ± 12.88	0.60 ± 0.54	-35.09 ± 17.39
4	0.41 ± 0.06	0.45 ± 0.15	76.19 <sup>a</sup>	No peak	No peak	No peak
5	0.36 ± 0.10	0.38 ± 0.09	57.14 <sup>c</sup>	"	"	"
6	0.34 ± 0.07	0.34 ± 0.09	61.90 <sup>b</sup>	"	"	"
7	0.30 ± 0.05	0.29 ± 0.08	47.62 <sup>b</sup>	"	"	"

DSC can be used to determine the unfrozen water content and the optimal moisture content for cryopreservation (Hamilton *et al.* 2009). The unfrozen water content of *C. mollissima* embryo is the x-intercept of the regression lines of best fit of the embryo at the moisture contents, with the presence of the exothermic peak. Fig. 2 shows that the unfrozen water content of *C. mollissima* was determined as 0.247 gH<sub>2</sub>O/g dw ( $R^2 = 0.897$ ). Theoretically, at the embryo moisture content of 0.247 gH<sub>2</sub>O/g dw, the embryo freezable water is no longer present.

Recalcitrant seeds conservation is a great challenge, the main reason is recalcitrant seeds are desiccation sensitive, removal of bound water will lead viability loss (Xia *et al.* 2012, Jin *et al.* 2018). Xia (2014) compared four *Quercus* species recalcitrant embryonic axes and reported that avoid of freezing was more important than drought for increase desiccation tolerance. The present study showed that *C. mollissima* has desiccation sensitive seeds, 0.29 gH<sub>2</sub>O/g dw embryos moisture content with survival percentage dropping to 47.6, however, the unfrozen water content was 0.247 gH<sub>2</sub>O/g dw. This means dehydration of the embryos to unfrozen water content will kill most of the embryonic axes. Corredoira (2004) reported European chestnut (*C. sativa* Mill.) embryonic axes rendering damage (only root growth) after cryostorage when moisture contents was less than 19%, it is similar with *C. mollissima*, their embryos semi-lethal moisture content was below 0.29 gH<sub>2</sub>O/g dw.

This study showed that the embryos moisture content decreased in different ratios when dried for different days (Table 1), presumably due to different types of water in the embryonic axes. As

Zoubi (2015) used DSC analyzed *Fortunella polyandra* embryonic axes water and named four types of water in the embryonic axes, which were sharp peak water, broad peak water, unfrozen water and non-freezable/unfreezable water. However, some researchers thought that sharp peak water has commonly been found in immature axes (Wesley-Smith *et al.* 1992, Farrant and Walters 1998). Wolfe (2002) considered unfrozen and unfreezable water to be in one category. This study showed that *C. mollissima* embryonic axes also have different types of water, sharp peak water and broad peak water. The peak water produced water phase transition (freezing of ice) in the cooling thermograms, which lead to freezing injury to cell and tissue. Wild *C. mollissima* embryonic axes have peak water at the embryo moisture content of 0.47 gH<sub>2</sub>O/g dw or greater, with embryonic axes dried in silica gel for 3 days or less. Removal of all the peak water is necessary for *C. mollissima* seeds and embryonic axes cryopreservation. Cooling rate will also affect the thermogram, probably faster cooling will be able to eliminate the exothermic peak. And cryo-protectants can also be considered for cryopreservation of embryonic axes if seed viability is not so satisfactory below a certain moist content.

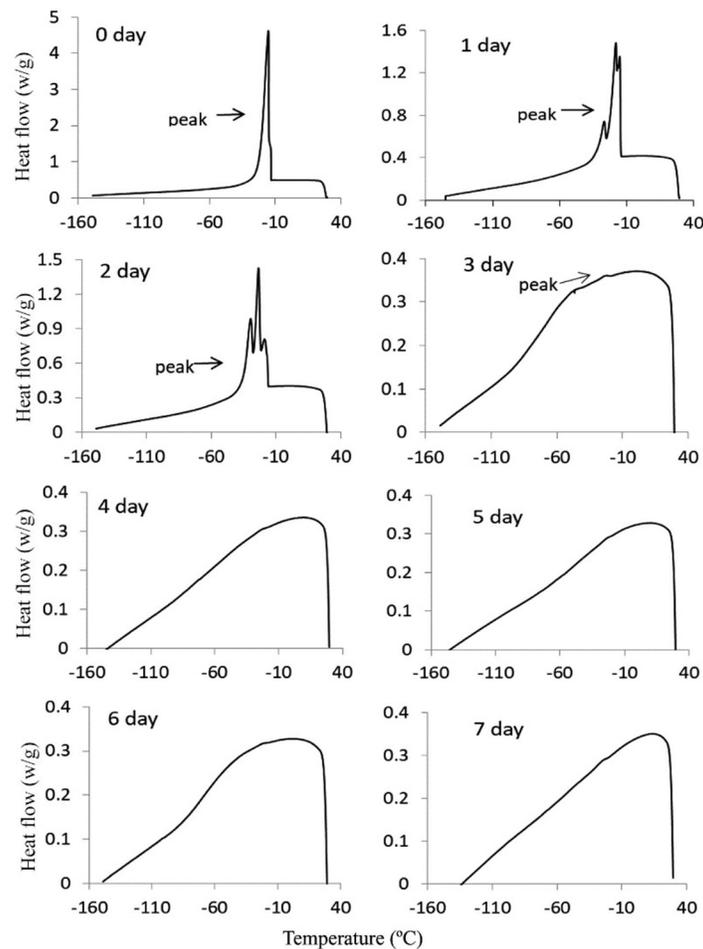


Fig. 1. Thermograms of *Castanea mollissima* embryonic axes desiccated different days. (0, 1, 2, 3, 4, 5, 6 and 7 day). The cooling crystallisation was from right to left.

Differential scanning calorimetry (DSC) is a useful tool to analyze embryo water thermodynamic change (Hor *et al.* 2005, Nadarajan *et al.* 2008). From the DSC analyses of *C. mollissima* embryonic axes, there are three key points for the embryonic axes desiccation: (a) exothermic peak; (b) unfrozen water content, and (c) onset temperature. Crystallization peak during the cooling process of *C. mollissima* means freeze injury to its embryonic axes tissue, so wild *C. mollissima* embryonic axes need to be dried to less than 0.47 gH<sub>2</sub>O/g dw moist content, in

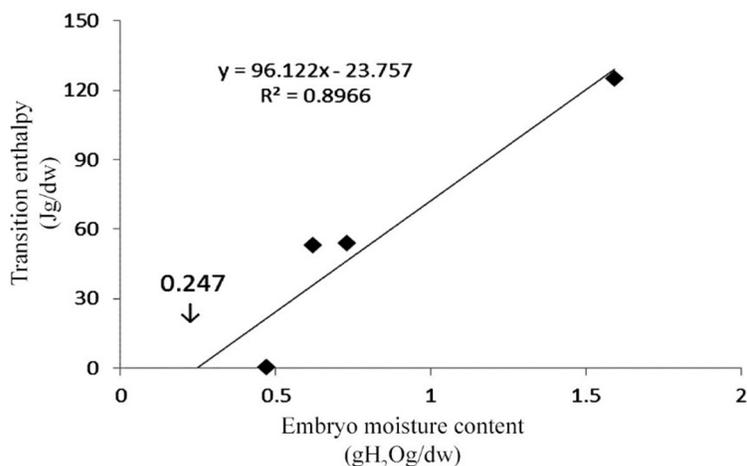


Fig. 2. The mean enthalpies of the freezing transition in *Castanea mollissima* embryonic axes at different moisture content. "x" intercept = 0.247 gH<sub>2</sub>O/g dw.

order to avoid the crystallization peak. Although dried *C. mollissima* embryonic axes to unfrozen water content is logically ideal condition, it means the embryonic axes viability loss to some extent. In conclusion, *C. mollissima* embryonic axes desiccation need comprehensive consideration of crystallization peak, unfrozen water content, onset temperature and viability loss. With 76.19% embryonic axes survival rate, 0.45 gH<sub>2</sub>O/g dw mc is considered to be the optimal embryo moisture content for seeds of wild *C. mollissima*. Above 0.45 gH<sub>2</sub>O/g dw embryo moisture content, it is also potential freezer storage of *C. mollissima* seeds and embryos, which need storage the seed in higher temperature environment than the onset temperature to avoid freezing injury.

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