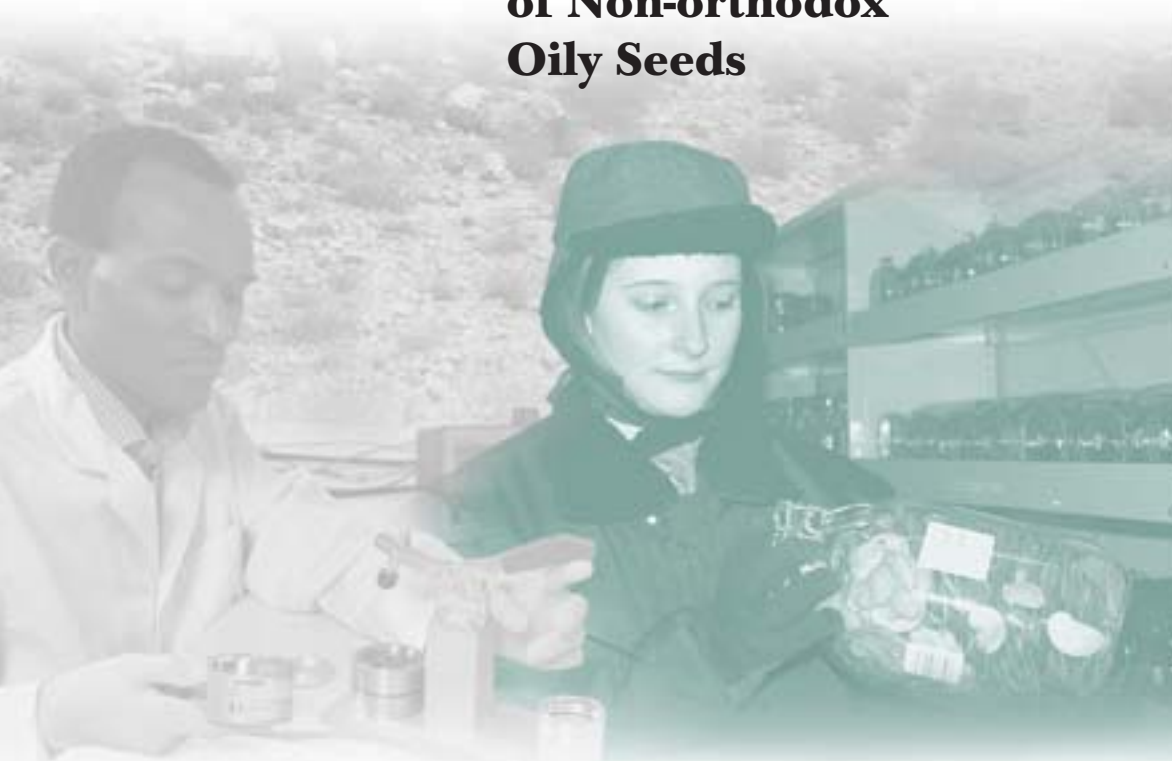


## **Determination of the Hydration Window for Cytopreservation of Non-orthodox Oily Seeds**



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### Summary

In order to determine the limits of the hydration window for cryopreservation of non-orthodox oily seeds, the effect of exposure to liquid nitrogen temperature on viability of seeds desiccated to various water contents was investigated in nine coffee species. When expressed in terms of water content, the higher limit (*HL*) of the hydration window was highly variable across species but corresponded always to the seed unfreezable water content, suggesting that seed survival strictly depended on avoidance of intracellular ice formation. The unfreezable water contents were negatively correlated with seed lipid contents. When expressed in terms of water activity, the interspecific variability in *HL* was very low, suggesting that desiccating seeds under 75–86% RH ensures an acceptable hydration level for the cryopreservation of non-orthodox oily seeds. In coffee seeds, the lower limit of the hydration window for cryopreservation was always much higher than the critical water content for seed desiccation sensitivity.

### Introduction

In some species, the range of seed water contents conducive to tolerance to liquid nitrogen (LN) exposure, when using low to high cooling rates (1 to 200°C min<sup>-1</sup>), is limited on the one side by desiccation sensitivity – the lower limit (*LL*) – and on the other by the onset of intracellular ice formation – the higher limit (*HL*) (Becwar *et al.*, 1983; Kuranuki and Yoshida, 1996). However, this simple rationale for seed tolerance to LN exposure has been questioned by the demonstration that intracellular ice formation in seed tissues during cooling to LN temperature is not always a lethal event. In pea seeds, the unfreezable water content, as determined from DSC heating thermograms, was found to be lower than the *HL*, i.e., 0.26 and 0.33 g H<sub>2</sub>O g<sup>-1</sup> dw, respectively (Vertucci, 1989). However, for seeds of some species, survival to LN exposure seems to be unconditionally dependent on the avoidance of intracellular ice formation. In soybean seeds, the *HL* was shown to coincide with the unfreezable water content (Vertucci, 1989).

In orthodox seeds, the *LL* of the hydration window for tolerance to LN exposure is generally not taken into account from a practical standpoint, because orthodox seeds are tolerant to desiccation to very low moisture contents. With non-orthodox seeded species, the potential hydration window for cryopreservation is very narrow because of their sensitivity to desiccation. It is therefore necessary to optimize the moisture content of such seeds before cryopreservation (Becwar *et al.*, 1983; Kuranuki and Yoshida, 1996; Dussert *et al.*, 2001). It is generally accepted that the *LL* corresponds to the water content at which desiccation sensitivity is detected at room temperature.

However, to our knowledge, this has never been verified quantitatively. Moreover, according to the thermodynamic model of the interactions between water content and temperature on seed freezing and desiccation damage developed by Vertucci *et al.* (1994), the *LL* of the hydration window for seed cryopreservation should be higher than the hydration level at which seeds are damaged by desiccation, as quantified in terms of water content at room temperature.

This chapter considers the limits of the hydration window for cryopreservation of non-orthodox oily seeds and their biophysical basis. For this purpose, we investigated the effect of exposure to LN temperature on the viability after desiccation to various water contents of seeds of nine coffee species previously shown to display a high variability in seed desiccation tolerance (Dussert *et al.*, 1999). We also measured the lipid content, and unfreezable water content of these seeds using DSC. The value of using DSC analysis in seed cryopreservation studies is explained in Box 44.1.

#### **Box 44.1 The value of using DSC analysis in seed cryopreservation studies**

Based on the results obtained with soybean, pea (Vertucci, 1989) and coffee seeds, when using low to high cooling rates (1 to 200°C min<sup>-1</sup>), the higher limit (*HL*) of the hydration window for cryopreserving whole seeds of lipid-rich species corresponds to their unfrozen water content. In non-orthodox oily seeds, the *HL* may also correspond to the optimal hydration level for whole seed cryopreservation, as shown with all coffee species studied, and, in some species (e.g., *Coffea arabica*), it is the only hydration level at which seedlings can be recovered from cryopreserved seeds. Thus, determining the unfrozen water content of seeds of a given species by DSC analysis prior to cryopreservation trials maximizes the chances of achieving successful cryopreservation. For pea, soybean and coffee seeds, the water activity corresponding to the seed unfreezable water content is between 0.75 and 0.86. Therefore, if non-orthodox seeds are desiccated in equilibrium with 75–86% RH, the seeds should be at their optimal hydration level for whole seed cryopreservation. It has been shown that the unfreezable water content of coffee seeds is negatively correlated with their lipid content. Therefore, measuring the lipid content of seeds to be cryopreserved constitutes an alternative method to estimate, in terms of water content, the hydration level at which seeds are amenable to cryopreservation. It should be noted that the fact that seeds tolerate desiccation to their unfrozen water content is not a sufficient condition for them to tolerate exposure to LN temperature after desiccation to this water content.

## Materials and Methods

Fresh mature seeds of *Coffea arabica* L. were provided from CATIE, Costa Rica. Bults of seeds of *C. brevipes* Hiern, *C. canephora* Pierre, *C. eugenoides* Moore, *C. liberica* Hiern, *C. pseudozanguebariae* Bridson, *C. racemosa* Lour., *C. sessiliflora* Bridson and *C. stenophylla* G. Don. were obtained from the field collections of CNRA-IRD, Divo and IRD, Man, both in Côte-d'Ivoire. Desiccation, cooling, thawing and culture conditions have been described in Dussert *et al.* (1999 and 2001). Desiccation sensitivity was quantified by the water content at which 50% of the initial viability was reached,  $WC_{50}$ , using the desiccation sensitivity model developed in a previous study (Dussert *et al.*, 1999). Seed survival was assessed using the criterion of normal seedling development. Total lipids were extracted from seeds and quantified as described in Dussert *et al.* (2001). The unfrozen water content,  $WC_u$ , of seeds was determined from DSC heating thermograms after desiccation to a broad range of seed water contents as described in Dussert *et al.* (2001). The water activity,  $a_{wu}$ , corresponding to  $WC_u$  was calculated using the water sorption model and sorption data reported in Dussert *et al.* (1999).

## Results and Discussion

### 1. Tolerance to Liquid Nitrogen Exposure

Three groups of species could be distinguished as regards seedling recovery after LN exposure (Table 44.1, Figure 44.1). In species of the first group, *C. brevipes*, *C. canephora*, *C. liberica* and *C. stenophylla*, no seedling production could be obtained after LN exposure, irrespective of the seed water content and of the cooling procedure (Figure 44.1). In contrast to these species, seedlings could be recovered after LN exposure when seeds of the five other species (groups 2 and 3) were sufficiently dehydrated (Table 44.1, Figure 44.1). In the two species of group 2, *C. arabica* and *C. eugenoides* (Figure 44.1), recovery was very low or nil after rapid cooling and only moderate (17–19%) after slow cooling (Table 44.1). Seeds of species of group 3, *C. pseudozanguebariae* (Figure 44.1), *C. racemosa* and *C. sessiliflora*, showed very high percentages of seedling development after both rapid and slow cooling (Table 44.1).

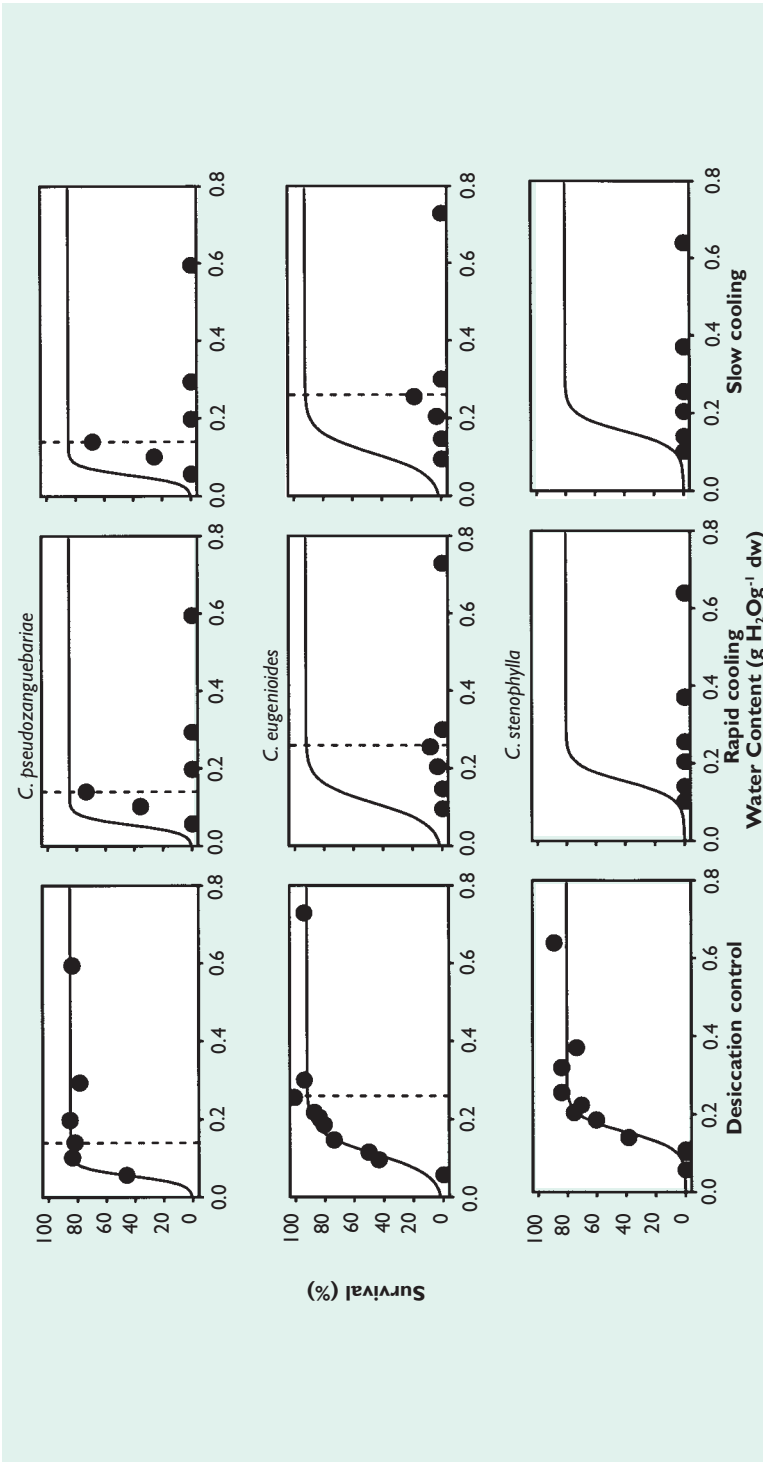
**Table 44.1 Desiccation sensitivity and freezing responses in seeds of nine coffee species**

Species (Group)	WC <sub>50</sub> (g g <sup>-1</sup> )	HL (g g <sup>-1</sup> )	Normal seedlings (%)		WC <sub>u</sub> (g g <sup>-1</sup> )	a <sub>wu</sub>
			Rapid cooling	Slow cooling		
<i>C. brevipes</i> (1)	0.20	-	0	0	ND	ND
<i>C. canephora</i> (1)	0.17	-	0	0	0.28	0.86
<i>C. liberica</i> (1)	0.29	-	0	0	0.26	0.85
<i>C. stenophylla</i> (1)	0.16	-	0	0	ND	ND
<i>C. arabica</i> (2)	0.11	0.21	0	17	0.21	0.78
<i>C. eugenioides</i> (2)	0.11	0.26	8	19	0.26	0.86
<i>C. pseudozanguebariae</i> (3)	0.06	0.14	73	68	0.14	0.75
<i>C. racemosa</i> (3)	<0.14 <sup>a</sup>	0.23	67	73	0.24	0.83
<i>C. sessiliflora</i> (3)	<0.14 <sup>a</sup>	0.19	81	76	0.18	0.79

Desiccation sensitivity, as estimated by the water content at which 50% of the initial viability was reached, WC<sub>50</sub>, higher limit of the hydration window for seed cryopreservation, HL, percentages of seedlings recovered after desiccation to HL and direct immersion into LN (rapid cooling) or by a precooling to -50°C at 1°C min<sup>-1</sup> prior to immersion in LN (slow cooling), seed unfrozen water content, WC<sub>u</sub>, and corresponding water activity, a<sub>wu</sub>, of nine coffee species classified in three groups according the percentages of seedling recovery after LN exposure (g g<sup>-1</sup> = g H<sub>2</sub>Og<sup>-1</sup> dw; ND = not determined). <sup>a</sup> The desiccation sensitivity model could not be applied because no decline in seed viability was observed at the lowest water content tested, 0.14 g H<sub>2</sub>Og<sup>-1</sup> dw. Data are from Dussert *et al.* (2001) with permission of Blackwell Publishing.

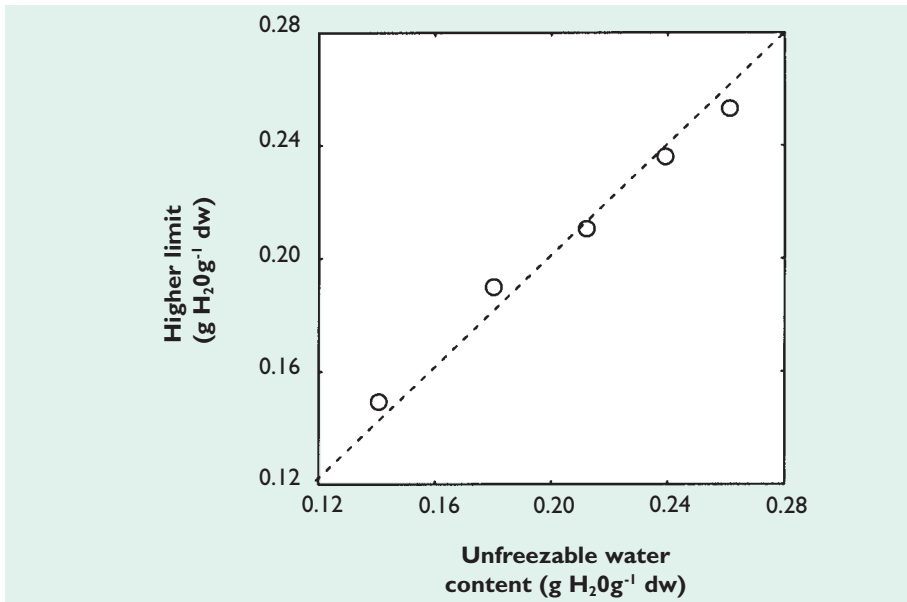
## 2. The Higher Limit of the Hydration Window for Seed Cryopreservation

A high interspecific variability was observed within the five coffee species of groups 2 and 3 (Table 44.1) for the *HL*, which ranged from 0.14 in *C. pseudozanguebariae* to 0.26 g H<sub>2</sub>Og<sup>-1</sup> dw in *C. eugenioides*. In these species, *HL* corresponded to the unfrozen water content (Table 44.1). With *C. pseudozanguebariae* and *C. eugenioides* seeds, seedlings could be recovered after both rapid and slow cooling and the *HL* value was the same for the two cooling protocols (Figure 44.1). A very highly significant correlation was found between WC<sub>u</sub> and *HL* values within the five species of groups 2 and 3 (Figure 44. 2). The slope of the regression line was not significantly different from one, showing that *HL* was not significantly different from WC<sub>u</sub> in these five coffee species. Presumably, seeds of these species were not able to withstand intracellular ice formation during the freezing/thawing process. Whole seed cryopreservation studies involving determination of the



**Figure 44.1**

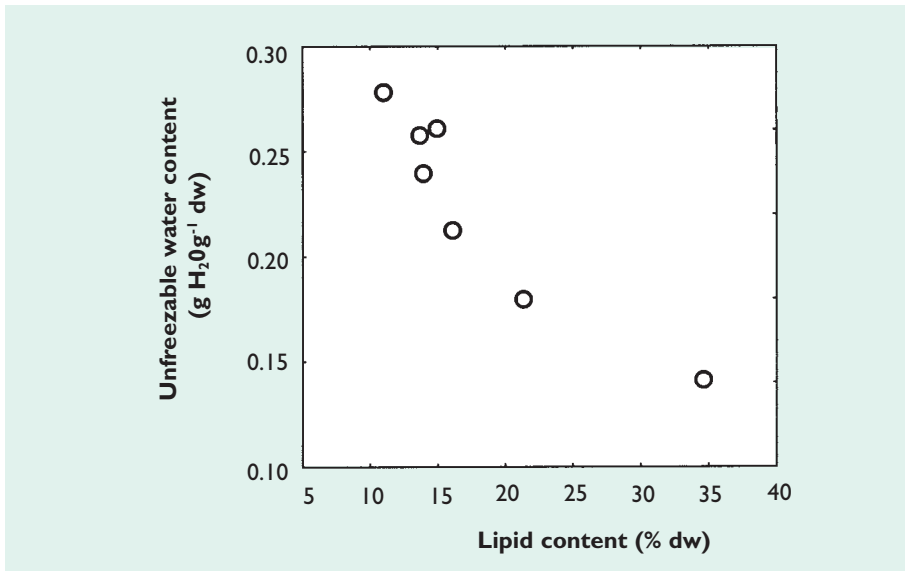
Survival, according to normal seedling development criterion, of *C. pseudozanguebariae*, *C. eugenioides* and *C. stenophylla* seeds after desiccation to various water contents (desiccation control) followed by either direct immersion into LN (rapid cooling) or by a precooling to -50°C at 1°C min<sup>-1</sup> prior to immersion in LN (slow cooling). The higher limit of the hydration window for seed cryopreservation, HL, is indicated by a vertical dashed line. The solid line represents the pattern of the desiccation sensitivity model fitted to desiccation control data. (From Dusserre et al. (2001) with permission of Blackwell Publishing).



**Figure 44.2** Relationship between the unfrozen water content, as determined from DSC analysis, and the higher limit of the hydration window for seed cryopreservation, *HL*, of seeds of *C. arabica*, *C. eugenoides*, *C. pseudozanguebariae*, *C. racemosa* and *C. sessiliflora*, all of which produced normal seedlings after cooling to LN temperature. (From Dussert *et al.* (2001) with permission of Blackwell Publishing).

unfreezable water content from DSC analysis are scarce (Vertucci, 1989, Dussert *et al.*, 2001). However, it could be established that seeds of some species can withstand the presence of a limited quantity of freezable water during exposure to sub-freezing temperatures, while others cannot. The present results are consistent with the earlier study of Vertucci (1989) with orthodox soybean seeds, and confirm that intracellular ice formation seems to be lethal in lipid-rich seeds, independent of their desiccation sensitivity level. Therefore, the unfreezable water content does correspond to the upper limit of the hydration window allowing the successful cryopreservation of such seeds.

The interspecific variability observed in coffee seeds for the water activity corresponding to the unfreezable water content was very low, since  $a_{wu}$  ranged between 0.75 and 0.86 (Table 44.1). These values of  $a_{wu}$  are remarkably consistent with those observed in orthodox seeds of pea and soybean (Vertucci, 1990). Moreover, in contrast to  $WC_u$ ,  $a_{wu}$  was found to be independent of the seed lipid content. Because *HL* represented the optimal



**Figure 44.3** Relationship between the lipid content and the unfreezable water content, as determined from DSC analysis, of seeds of seven coffee species. (From Dussert *et al.* (2001), with permission of Blackwell Publishing).

hydration level for coffee seed cryopreservation and, because  $HL$  corresponded to  $WC_{in}$ , we propose that the optimum water content for the cryopreservation of non-orthodox oily seed can be achieved by drying to 75–86% RH at room temperature.

A negative non-linear relationship was found between the unfrozen water content and the lipid content of seeds of the seven coffee species for which DSC analysis was performed (Figure 44.3). Additional data, previously reported by other authors, fit remarkably well with the relationship shown in Figure 44.3. The unfreezable water contents of seeds of *Quercus robur* and *Azadirachta indica* were of c. 0.30 and 0.14 g H<sub>2</sub>Og<sup>-1</sup> dw, respectively, whereas their lipid contents were 2 and 43% dw, respectively (Pritchard and Manger, 1998; Sacandé *et al.*, 2000). If the relationship observed with coffee species is validated with other oily seeds, then measuring the lipid content of seeds of a given species could allow an estimate to be made of their unfrozen water content. This could constitute a simple alternative to DSC analysis for determining the  $HL$  of the hydration window for cryopreservation of non-orthodox oily seeds.

### 3. The Lower Limit of the Hydration Window

In *C. arabica*, *C. canephora* and *C. eugenioides*, since desiccation sensitivity was observed at hydration levels much lower than the unfreezable water content of their seeds ( $WC_{50} < WC_u$ , Table 44.1), one could have expected very high seed survival after desiccation to  $WC_u$  and exposure to LN temperature. However, seed survival was very low or nil after such treatments in those species (Table 44.1, Figure 44.1), indicating clearly that the *LL* does not correspond to desiccation sensitivity, as quantified in terms of water content. The same phenomenon was observed in *C. pseudozanguebariae*, *C. racemosa* and *C. sessiliflora* (group 3), where the *LL* of the hydration window for seed cryopreservation was always much higher than the critical hydration level for desiccation tolerance (data not shown). A theoretical basis for this phenomenon has been proposed by Vertucci *et al.* (1994). Assuming that a single critical water activity determines the desiccation sensitivity of seeds of a given species, this result can be explained by the decrease in seed water activity with decreasing temperatures. Therefore, even if, at room temperature, the water activity of seeds desiccated to  $WC_u$  is higher than the critical water activity for desiccation sensitivity, when cooled to  $-196^\circ\text{C}$ , it is possible that the water activity of seeds is decreased to a value lower than the critical water activity for desiccation sensitivity, thus inducing injuries in cryopreserved seeds.

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