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Cryopreservation of *Coffea liberica* Seeds and Embryos Following Desiccation and Freezing Treatments

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ABSTRAK

Pengeringan biji benih Coffea liberica *selama 6 hari di dalam bilik berhawa dingin mengurangkan kelembapan biji benih dan embrio tersambung daripada 52.58% dan 47.49% kepada masing-masing 14.58% dan 12.56%. Percambahan biji benih dan kemandirian embrio sambungan kekal pada tahap sederhana, iaitu masing-masing 66% dan 38%. Walau bagaimanapun, semua benih dan embrio sambungan yang telah di keringkan mati selepas pembekuan di dalam nitrogen cecair. Embrio terpotong yang dikeringkan di dalam "lamina flow cabinet" hilang kelembapannya dengan cepat, iaitu daripada 36.8% kepada 9.27% dalam masa 1.5 jam. Lebih daripada 70% embrio ini masih hidup selepas pengeringan. Malahan, pengeringan separa embrio kepada kelembapan 17.17% atau kurang mengakibatkan ketahanan embrio terhadap pembekuan. Di antara 35%-50% embrio tahan kepada pembekuan perlahan sehingga -38°C, tetapi kadar kemandirian ini berkurangan kepada 30% apabila ianya terus dimasukkan ke dalam nitrogen cecair. Pembekuan serta merta dengan memasukkan terus ke dalam nitrogen cecair mengakibatkan 10%-35% kemandirian. Pengeringan embrio kopi selama 0.5 jam kepada kelembapan 17.17% adalah optimum untuk kriopenyimpanan bagi sebarang kadar penyejukan. Analisis terma pembeza tisu biji benih menunjukkan bahawa ketiadaan air "freezable" merupakan satu faktor penting bagi menentukan kejayaan kriopenyimpanan embrio kopi. Walau bagaimanapun, kepentingan kecergasan awal, variasi kelembapan dan medium pemulihan juga dibincangkan.*

ABSTRACT

Desiccation of Coffea liberica *seeds for 6 days in an air-conditioned room reduced seed and attached embryo moisture from 52.58% and 47.49% to 14.58% and 12.56% respectively. Seed germination and viability of the attached embryo were maintained at moderate levels of 66% and 38% respectively. However, none of the desiccated seeds or embryos survived freezing in liquid nitrogen. Excised embryos desiccated in the lamina flow cabinet lost their moisture very rapidly from 36.8% to 9.27% within 1.5 hours. More than 70% of these embryos survived the desiccation. Moreover, partially desiccated embryos at 17.17% moisture or less survived subfreezing temperatures. Between 35% to 50% survived slow freezing to -38°C, but this was reduced to approximately 30% when they were subsequently plunged into liquid nitrogen. Fast freezing by direct plunge into liquid nitrogen also resulted in 10% to 35% survival. Desiccation of excised coffee embryos for 0.5h to 17.17% moisture was optimal for cryopreservation, irrespective of the speed of freezing. Differential thermal analyses of seed tissues suggest that the absence of freezable water is an important factor for successful cryopreservation of excised coffee embryos. However, the importance of initial vigour, moisture variation and recovery media is also discussed.*

INTRODUCTION

Coffea liberica Bull ex Hiern is one of more than twenty species of the genus *Coffea* (Leroy 1967). It is one of the species grown commercially in the tropics as a beverage. Coffee originates from tropical Africa, but as in many other crops the genetic resources of this crop are rapidly eroded through increased land development and deforestation in the tropics. Although pockets of germplasm are scattered in museums and

arboreta, these resources are also endangered by diseases, natural disasters and in some cases, inadequate management. These dangers were recognised by the International Board for Plant Genetic Resources (IBPGR) which listed coffee as one of the priority crops for research to provide more information on techniques for genetic conservation (IBPGR 1983).

However, literature on the storability of coffee seeds is conflicting especially on the effects of moisture and temperature. Owing to this, coffee was classified earlier as a recalcitrant seed and thereby a poor storer (King and Roberts 1979), but was later suggested to be orthodox (Roberts *et al.* 1984). More recently, Ellis *et al.* (1990) made a critical study of the effects of moisture and temperature on storage of coffee seeds and suggested that they should be classified as an intermediate between recalcitrant and orthodox. They further emphasised that the recommended conditions for long-term genetic conservation for seeds at 5% moisture and -18°C are not feasible for coffee.

Although presently there is no practical method for coffee seed conservation using conventional methods, cryopreservation can be an alternate means of genetic conservation. The method has been demonstrated to be successful for a number of orthodox seeds (Stanwood 1984) and even some recalcitrant seed species (Chin and Hor 1989; Hor, Chin and Murugaiah 1990). This study investigates the potentiality of cryopreservation as a means of conserving the genetic resources of coffee by assessing the effects of desiccation and freezing rate on seed and embryo survival in liquid nitrogen.

MATERIALS AND METHODS

Effects of Seed Desiccation

Seeds of *Coffea liberica* were extracted from ripening yellowish red berries and soaked in 0.1% w/w of a 1:1 benomyl-thiram solution for 15 minutes. The seeds were surface dried on clean towels and desiccated in an air-conditioned room at 22°C and 55% relative humidity. After 0, 2, 4 and 6 days of drying, batches of seeds were randomly removed to index seed germination and embryo viability before and after direct plunge into liquid nitrogen. Further samples of seeds were used to measure seed and embryo moisture.

Seed germination was carried out with 20 seeds per replicate using the in-sand method recommended by the International Seed Testing Association (ISTA 1985). For embryo viability, a further 20 seeds were surface sterilised for 10 min in 1% v/v chlorine before the embryos were aseptically excised and cultured in Murashige and Skoog (Murashige and Skoog 1962) medium. The medium was incorporated with 0.2% activated charcoal and 1mg/l each of kinetin, indoleacetic acid and gibberellic acid (GA_3). The embryos were cultured in a 22°C room supplied with 12 hours of light (2000 lux).

Seed and embryo moistures were measured separately by drying ten seeds or embryos in a 103°C oven for 16 h. All treatments were replicated three times.

Effects of Embryo Desiccation and Prefreezing Protocol

Seeds from ripening yellowish red berries of *Coffea liberica* were extracted as described above and surface sterilised for 10 min in 1% w/w chlorine. The sterilised seeds were rinsed twice in sterile water and the embryos excised aseptically in a lamina flow cabinet. The excised embryos were then desiccated on sterile filter papers in the flow cabinet for 0, 0.5, 1.0, and 1.5 h. After desiccation, the embryos were exposed to various freezing protocols. These included slow freezing in an alcohol bath (Julabo circulator, model F40-HC) to -38°C at a rate of -1°C/min, prefreezing in the alcohol bath to - 38°C followed by plunging into liquid nitrogen at -196°C, and fast freezing by direct plunge into liquid nitrogen. For control, desiccated embryos were also cultured directly in Murashige and Skoog (MS) medium without prefreezing or liquid nitrogen exposure. Survival was measured by the percentage of embryos that turned green and expanded in the modified MS medium described above. The experiment was factorial with a randomised complete block design and four replicates of ten embryos per treatment.

Differential Thermal Analysis of Coffee Seeds

The availability of freezable water in coffee seeds dried to different moisture contents was monitored by differential thermal analysis as described before (Hor, Stanwood and Chin 1990). Seeds were dried to various moisture contents in the air-conditioned room before they were wrapped individually with aluminium foil around a thermocouple. The seeds were then frozen at -1°C/min to -70°C after which their moisture content was measured.

RESULTS AND DISCUSSION

Seed Desiccation and Survival

Fresh coffee seeds and their enclosed embryos have similar high moistures of more than 47%. When desiccated in the air-conditioned room, both lost their moisture gradually (*Fig. 1*). However, unlike many other tropical seeds, the moisture contents of the embryonic axes were only slightly higher (2%-8% only) than the moisture content of the whole seed. The differential was further reduced with increasing hours of desiccation. At the end of six days the seed and embryo moisture contents were 14.58% and 12.56% respectively.

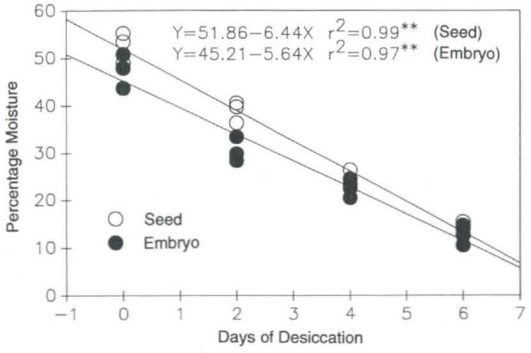

Fig. 1: Percentage moisture of seed and attached embryo after different days of seed desiccation in an air-conditioned environment.

Germination of fresh coffee seeds was above 90%, but with desiccation there was a gradual decrease in germination (*Fig. 2*). By the sixth day of drying when the moisture had decreased to 14.58%, germination was reduced to approximately 66%. The moderate survival at low moisture content of 14.58% confirms that coffee seeds are non-recalcitrant. However, none of the seeds survived exposure to liquid nitrogen within the moisture range of 52.58% to 14.58%.

Compared with seeds, embryos excised from dried seeds had lower viability especially with increased seed desiccation (*Fig. 2*). Embryos from fresh seeds had high viability (more than 90%), but after 6 days desiccation to moisture contents of 12.56%, viability was reduced to 38%. As in seeds, none of these embryos survived exposure to liquid nitrogen, irrespective of their moisture content.

Excised Embryos Desiccation and Survival

In contrast to attached embryos in seeds, excised embryos dried very rapidly in the lamina flow cabinet (*Fig. 3*). This was especially so in the first half hour when nearly 19% of moisture was lost (36.80% to 17.17%). After that there was a more gradual decrease to 9.27% moisture after 1.5 hours of drying.

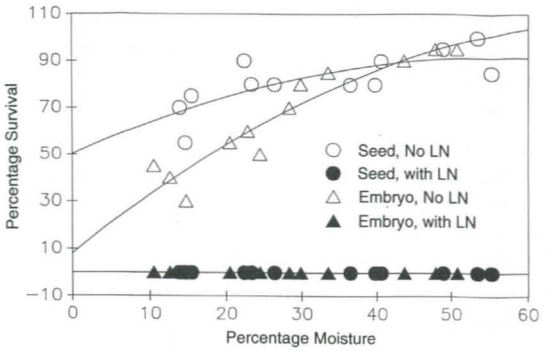

Fig. 2: Percentage survival of desiccated seeds and attached embryos with and without exposure to liquid nitrogen. The appropriate regression equations are :
$Y=50.14+1.50X-0.01X^2$ $r^2=0.59^{**}$ (SEED, NO LN)
$Y=7.89+2.68x-0.02x^2$ $R^2=0.90^{**}$ (EMBRYO, NO LN)

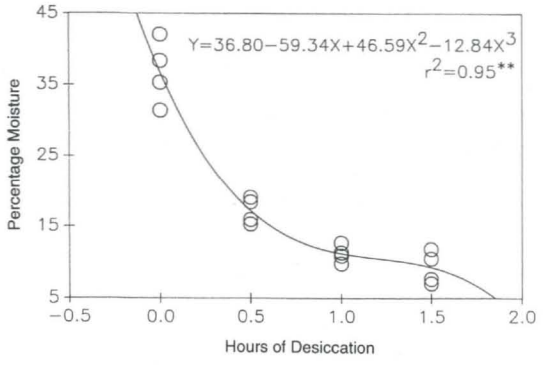

Fig. 3: Percentage moisture of excised embryo desiccated for different hours in the lamina flow cabinet.

Excised embryos desiccated to as low as 9.27% moisture had a good survival rate of more than 70% when they were not subjected to freezing temperatures (*Fig. 4*). Of greater significance is that many of these excised embryos were able to survive exposure to liquid nitrogen, indicating that excised desiccated embryos have potential for cryopreservation of coffee genetic resources. Survival of excised embryos in liquid nitrogen was dependent on their moisture content and freezing rate (*Fig. 4*). Fresh embryos at a high moisture of 36.8% did not survive freezing, but approximately 10% to 50% of the em-

bryos survived when they were dried to 17.17% moisture or less. Embryos cooled only to -38°C in the alcohol bath had a higher survival of 35% to 50%, but this was reduced to approximately 30% when the embryos were subsequently plunged into liquid nitrogen at -196°C. In both cases, survival increased with increased desiccation. Survival was also maintained between 10% to 35% when fast freezing by direct plunge into liquid nitrogen was used. However, at lower moistures of less than 10%, survival was low. In general, desiccation of excised embryos for 0.5 hour to 17.17% moisture was optimal for preservation in liquid nitrogen irrespective of the speed of freezing.

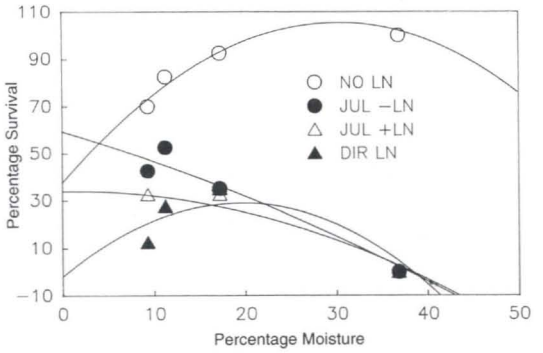

Fig. 4: Percentage survival of desiccated excised embryos after different freezing treatment. (NO LN = no freezing; JUL - LN = only alcohol bath; JUL = LN = alcohol bath and LN freezing; DIR LN = direct LN freezing). (Only the means are shown in the plot). The appropriate regression equations are :

$Y=37.54+4.52X-0.08X^2$ $r^2=0.77^{**}$ (NO LN)
$Y=59.29-1.19X-0.01X^2$ $r^2=0.56^{**}$ (JUL-LN)
$Y=33.93+0.02X-0.02X^2$ $r^2=0.47^{*}$ (JUL +LN)
$Y=3.16X-1.81-0.08X^2$ $r^2=0.49^{*}$ (DIR LN)

Differential Thermal Analysis of Seed Tissues

Seed tissues with moisture content ranging from 26.74% to 52.97% exhibited two exothermal peaks when cooled to -70°C at a rate of -1°C per minute (*Fig. 5*). The first exotherm was broad and minor and occurred at a temperature of approximately -4°C. The size of the exothermal peak is independent of the seed moisture suggesting that it is non-aqueous in origin. Becwar *et al.* (1983) reported similar peaks in *Coffea arabica* and suggested that they were caused by phase transition of the oils or lipids present in the seed tissues. The second peak was sharply spiked and occurred at temperatures between -12° to -31°C. The exotherm rose sharply at the higher temperature, suggesting that freezing commenced instantaneously and progressed homogeneously. As the size of the peak was directly related to the seed moisture, the exotherm was caused by the freezing of water in the seed tissues. This is further supported by the exothermal temperatures (-12°C to -31°C) which were similar to those reported for freezable water in other seeds (Hor, Stanwood and Chin 1990). In general there was a 0.62°C drop in exothermal temperature with every 1% decrease in seed moisture, resulting in lower exothermal

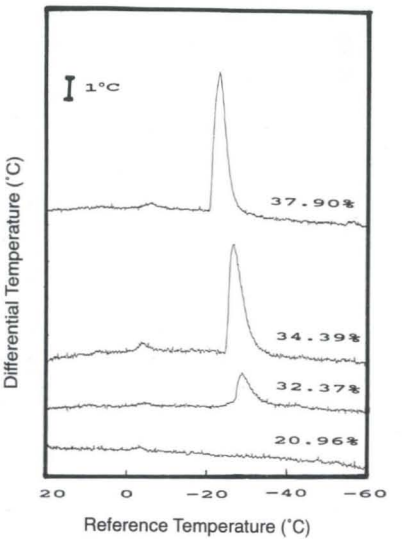

Fig. 5: DTA cooling profiles of coffee seeds dehydrated to different moisture contents.

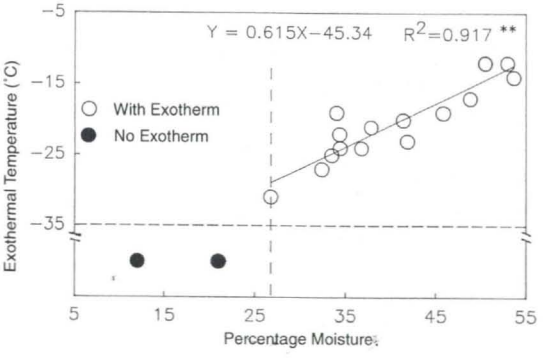

Fig. 6: Exothermal temperatures of Coffea liberica *seed as a function of seed moisture. (The vertical dotted line denotes the threshold moisture below which freezable water is absent).*

temperatures for tissues subjected to greater desiccation (*Fig. 6*). Seed tissues dried to less than 26.74% moisture did not exhibit an exotherm as freezable water within the tissues were removed.

Differential thermal analysis of seed tissues can provide a partial explanation for the survival of excised coffee embryos in liquid nitrogen. Freshly excised embryos did not survive liquid nitrogen exposure because their moisture content (36.80%) was higher than the threshold moisture (26.74%) below which all freezable water was removed from the seed. Freezable water remaining in the freshly excised embryos froze at approximately -12°C causing lethal injury to the tissues. However, excised embryos desiccated for 0.5 hour or longer had moisture contents less than 17.17% which is below the threshold moisture of 26.74%. Such embryos were devoid of freezable water and were therefore able to avoid freezing injury. Many of these embryos were able to survive exposure to liquid nitrogen.

However, the above postulate does not explain why many of the excised embryos dried to moisture content below the threshold levels did not survive liquid nitrogen exposure. Neither does it explain why embryos dried within the seeds to sub-threshold levels (after 6 days drying) did not survive in liquid nitrogen. It does emphasise, however, that although freezable water must be removed from tissues before they can be frozen, other factors are also important for survival in liquid nitrogen. One important factor may be the vigour of the excised embryos. Within a seedlot, vigour can vary depending on the size and maturity of seeds (Chin and Hor 1989). Smaller and more immature embryos may not have sufficient vigour to survive and recover from the liquid nitrogen treatment, although they can grow well in the absence of freezing. This can result in a proportion of the excised, partially desiccated embryos being killed in liquid nitrogen even though they were dried below the threshold level. Other factors, such as variation in moisture between individual embryos and different media requirement of cryopreserved embryos, may also lead to their reduced survival in liquid nitrogen.

Compared with excised embryos, embryos dried in the seeds can have their vigour further reduced. Desiccation of the embryo was slow within the confined, moist environment of the perisperm which can encourage microbial infection and inhibit aerobic respiration. The resulting decreased vigour coupled with the increased resistance of the partially dried perisperm can also prevent many of the embryos from emerging through the perisperm. Further, embryos from seeds dried for a few days were difficult to excise and many may have been injured. Collectively, these factors can be the cause for non-survival of embryos dried within the seeds.

Excising embryos is a useful method for conserving genetic resources of many tropical species in liquid nitrogen. Many of these species produce large seeds, which are impractical to conserve; they are likely to be killed by the freezing. On the other hand excised embryos survive better and are conveniently conserved in small cryovials as reported for other tropical species (Normah *et al.* 1986). Although in the present study the survival of excised coffee embryos in liquid nitrogen was found to be only moderate, further studies are in progress to evaluate the effects of other factors in increasing their survival levels. These studies hopefully will lead to a practical technique for cryopreservation of their genetic resources.

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