VI. Cryopreservation of coffee genetic resources

Stéphane Dussert,[1] *Nelly Vasquez,*[2] *Karol Salazar,*[3] *François Anthony*[4] *and Florent Engelmann*[5]

[1] Seed Conservation Biology & Technology, Institut de recherche pour le développement (IRD), UMR DIA-PC, 911 avenue Agropolis, BP 64501, F-34394 Montpellier cedex 5, France
[2] Biotechnology, Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), 7170 Turrialba, Costa Rica
[3] Tissue Culture, Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), 7170 Turrialba, Costa Rica
[4] Plant Genetics & Genomics, Institut de recherche pour le développement (IRD), UMR RPB, 911 avenue Agropolis, BP 64501, F-34394 Montpellier cedex 5, France
[5] In vitro and cryopreservation, Institut de recherche pour le développement (IRD), UMR DIA-PC, 911 avenue Agropolis, BP 64501, F-34394 Montpellier cedex 5, France, and Honorary Research Fellow, *In vitro* and Cryopreservation, Bioversity International, Via dei Tre Denari 472/a, 00057 Maccarese, Rome, Italy

Introduction

Because of the non-orthodox storage behaviour of their seeds (van der Vossen 1977; Ellis et al. 1990), coffee (*Coffea* spp.) genetic resources are conserved in field collections. However, since coffee field collections are costly to maintain (Charrier et al. 1989) and leave the material exposed to biotic and abiotic hazards (see Chapter 3), research for alternative methods to field conservation has become a priority (see Chapter 1).

Cryopreservation, i.e. storage at ultra-low temperature of biological material (-196°C, in liquid nitrogen), is the only technique available to ensure safe and cost-effective long-term conservation of coffee germplasm (see Chapter 1). For *C. arabica*, which is autogamous and seed propagated, attention has to be given to seed cryopreservation. Despite early pessimistic reports on the feasibility of coffee seed cryopreservation (Becwar et al. 1983; Stanwood 1985), considerable efforts have been made since 1997 at IRD-Montpellier (France) to investigate the basis of the high sensitivity of coffee seeds to liquid nitrogen (LN) temperature, which research led to the development of a cryopreservation procedure providing satisfactory survival percentages.

Through several years of research using seeds of two well-characterized types (Bourbon and Typica) as models, it was shown that tolerance to LN exposure of whole seeds of *C. arabica* is a complex phenomenon. The interval of water contents allowing seed survival is very narrow, i.e. 0.20-0.23 g $H_2O.g^{-1}$ dw (Dussert et al. 1997, 2003a), and the optimal water status corresponds to the unfreezable water content of seeds (Dussert et al. 2001). After desiccation to the optimal water content and exposure to LN, a high proportion of seeds show hypocotyl and radicle extrusion but a very low percentage of them develop into normal seedlings (Dussert et al. 1997; Eira et al. 1999). If embryos are extracted from frozen seeds after thawing, and then cultivated *in vitro*, a very high proportion of them produce a normal seedling, indicating a different sensitivity to LN exposure between the endosperm and the embryo (Dussert et al. 1997). The percentage of seeds developing into normal seedlings after LN exposure is partly improved by slow cooling of seeds (Dussert et al. 1997). Moreover, controlled rehydration of seeds, through a 6-week osmoconditioning treatment in a –1.25 MPa polyethylene glycol solution, after thawing and before culture under germination conditions, also increases the percentage of seeds developing into normal seedlings (Dussert et al. 2000).

In this way, the percentage of seeds surviving after cryopreservation was improved step by step, reaching values around 40% and 70% in Typica and Bourbon, respectively, in 2000. These percentages of seedling recovery were judged sufficiently high to consider the transfer of the procedure developed at IRD to a coffee genebank and to test whether it could be used as standard protocol for cryopreservation of *C. arabica* genetic resources. It is in this framework that the Bioversity, IRD and CATIE collaborative project on cryopreservation of wild coffee was initiated. The main objective of this study was to test the effects of immersion in LN and post-thaw osmoconditioning on seed and embryo viability within a set of 30 accessions of the CATIE field genebank. This set of 30 accessions is a subset of the 74 accessions of the CATIE coffee core collection (see Chapter 5).

The aim of this chapter is to present the results obtained in the Bioversity-IRD-CATIE collaborative project, whose results have been published recently (Vasquez et al. 2005). Their application to the choice of the standard protocol for the CATIE coffee core collection is also described. The need for optimizing some of the steps is then discussed, taking into account new progress made at IRD subsequent to the project described here.

Choice of the core subset of 30 accessions

The thirty accessions of *C. arabica* used in this study are presented in Table 6.1. They were selected among the 74 accessions of the CATIE coffee core collection (see Chapter 5), which were themselves selected from the CATIE field genebank on the basis of their molecular and agronomic evaluation (see Chapter 4). Twenty-seven accessions were randomly chosen to represent the genetic diversity of the wild material collected by FAO (Fernie et al. 1968) and ORSTOM (Guillaumet and Hallé 1978) in the centre of origin of *C. arabica* (south-west Ethiopia). Two accessions were selected to represent Ethiopian cultivated varieties collected in the same area. The variety Caturra (Bourbon type) was included into the core subset as a comparative cross-reference to the plant material used in previous studies (Dussert et al. 1997, 2000).

Table 6.1. The 30 accessions from the CATIE field genebank analysed for their seed response to cryopreservation.

Type	Origin	Accession number
Wild	Kefa Province	T.4495; T.4621; T.4661; T.4664; T.4665; T.4900; T.16689; T.16697; T.16723; T.16724; T.16726; T.16729; T.16737; T.16733
	Ilubabor Province	T.4824; T.4837; T.4857; T.4863; T.4865; T.16695; T.17177; T.16694; T.16700; T.16702; T.16706; T.16707; T.16712
Cultivated in Ethiopia	Var. Cioiccie	T.2710
	Var. Loulo	T.4007
Cultivated worldwide	Var. Caturra	

Seed preparation, desiccation and cryopreservation

For each accession of the core subset, fresh fruits were harvested manually from the field collection. After wet-process depulping, seeds were partially dehydrated in the shade at ambient temperature for two weeks. Seed water content upon receipt in the laboratory was 0.3-0.4 g $H_2O.g^{-1}$ dw for all accessions. Seeds were desiccated by equilibration for 3 weeks under 78% relative humidity, obtained using an NH_4Cl saturated solution, as described in Dussert et al. (2000, 2001). Seed water content at equilibrium varied between 0.21 and 0.23 g $H_2O.g^{-1}$ dw, depending on the genotype.

The water content of seeds was estimated using 10 replicates of one seed and their dry weight measured after 2 days of desiccation in an oven at 105°C. Before cooling, seeds were hermetically sealed in 15 ml polypropylene tubes (50 seeds per tube). Seeds were precooled to -50°C at 1°C/min using a Cryomed © programmable freezing apparatus, then immersed in LN. Seeds were stored at -196°C for at least one week before thawing. Thawing was carried out by plunging the tubes in a 40°C water-bath for 4 minutes.

After thawing, seeds were either placed directly in germination conditions, or osmoconditioned for 6 weeks before their transfer to germination conditions. Seed culture was carried out according to the method described by Dussert et al. (1997). Osmoconditioning was carried out at 27°C in the dark by placing batches of ten seeds in Petri dishes sealed with Parafilm™ Ribbon on a thin layer of cotton wool imbibed with 20 ml of a -1.25 MPa aqueous PEG 6000 solution, as described in Dussert et al. (2000). Zygotic embryos were extracted from desiccated or desiccated and frozen seeds after disinfection, and cultured *in vitro* for survival assessment. Disinfection, extraction and culture were performed as described in Dussert et al. (1997).

Seed and embryo viability after desiccation

Very high variability was observed within the 30 accessions studied for the viability of seeds after desiccation, since it ranged from 11 to 100%, as estimated by the percentage of seeds developing into normal seedlings when placed in germination conditions (Figure 6.1). In contrast, viability of embryos extracted from desiccated seeds showed little variation and was always very high, since it ranged from 72 to 100%.

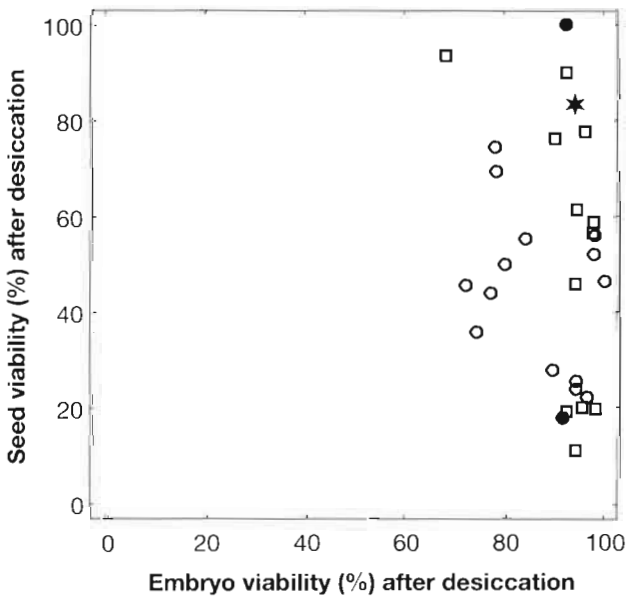

Figure 6.1. Relationship between seed viability (normal seedling development) after desiccation and viability of embryos extracted from desiccated seeds within the core subset studied. The subset included 27 wild accessions originating from Kefa (○) and Ilubabor (□) provinces, two Ethiopian varieties (●) and one commercial variety (✶).

There was no significant correlation between viability of seeds after desiccation and that of zygotic embryos extracted from desiccated seeds. Variability for seed viability after desiccation was not significantly correlated to seed size, nor to seed water content after desiccation. No association was found between the origin of the plant material and seed response to desiccation.

The possibility that this variability is associated with variability for desiccation tolerance cannot be excluded. However, previous studies have shown that the intraspecific variability for seed desiccation tolerance was very low in *C. arabica*, with a loss of seed viability occurring always below 0.12 g $H_2O.g^{-1}$ dw (Ellis et al. 1990; Eira et al. 1999).

From our results, we can rule out that this variability is due to differences in the ability of embryos to develop into normal seedlings, since this was high, with a mean value of 90.1%, independent of seed viability after desiccation. It can thus be speculated that this variability is associated with differences in endosperm quality at harvest. The nature of these differences in endosperm quality are unknown, but they might be related to differences between accessions in the time to achieve complete maturation, or to differences in response of seeds to the post-harvest process, which has been developed for traditional varieties and might be inappropriate for wild forms of *C. arabica*.

Seed viability after cryopreservation and rapid rehydration

Throughout four years of work with Typica and Bourbon seeds (Dussert et al. 1997, 2000, 2001), very low variability was observed among repetitions for the proportion of seeds developing into normal seedlings after cryopreservation under the same conditions, which was always about 15% of the desiccation control. Similarly, with seeds of four other varieties, Eira et al. (1999) observed viability percentages between 10 and 30% after desiccation to the same water content and LN exposure.

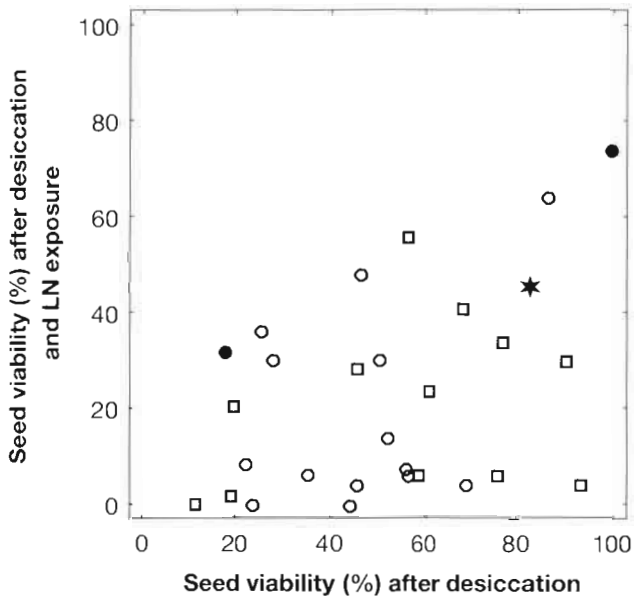

Figure 6.2. Relationship between seed viability (normal seedling development) after desiccation and LN exposure and viability of seeds after desiccation only, within the core subset studied. The subset included 27 wild accessions originating from Kefa (○) and Ilubabor (□) provinces, two Ethiopian varieties (●) and one commercial variety (★).

In contrast, in the present study, a very high variability for seed sensitivity to LN exposure was observed within the 30 accessions studied. Seed survival after cryopreservation varied from 0 to 74% (0 to 100% when expressed as a percentage of the desiccation control). It is also illustrated in Figure 6.2 by the fact that the points are widely spread between the y = x line (the most LN-tolerant accessions) and the y = 0 line (the most LN-sensitive accessions).

When expressed as the percentage of the desiccation control, seed sensitivity to LN exposure was not correlated to seed size, seed water content after desiccation, or seed and embryo viability after desiccation. There was no apparent association between the origin of the plant material and seed sensitivity to LN exposure. The causes for the variability to LN exposure observed in the present work thus remain to be identified.

This variability could also be expressed through the non-parametric analysis of viability percentages, which showed that, in 8 of the 30 accessions studied, seed viability after desiccation and LN exposure was not significantly different from viability of desiccated seeds, while, in the 22 other accessions, there was a negative effect of LN exposure on seed viability (Table 6.2). The decrease in viability observed in frozen var. Caturra seeds was equivalent to that observed previously in another Bourbon-derived variety (Dussert et al. 2000).

Table 6.2. Multiple comparison of the percentages of seeds developing into normal seedlings after (i) desiccation, (ii) desiccation and LN exposure, and (iii) desiccation, LN exposure and post-thaw osmoconditioning: number of accessions showing, or not, a significant difference for each pair of proportions compared according to the Ryan's test (Ryan 1960).

	Number of accessions
Cooling effect	
Negative effect on seed viability	22
No effect on seed viability	8
Osmoconditioning effect	
Beneficial effect on seed viability	23
No effect on seed viability	7

Seed viability after cryopreservation and controlled rehydration

The post-thaw osmoconditioning treatment resulted in an overall beneficial effect on viability (normal seedling development) of frozen seeds, as illustrated by the fact that all points were located very close to or above the y = x line in Figure 6.3. However, a very high variability for the beneficial effect of seed osmoconditioning was observed within the 30 accessions studied (Figure 6.3). For each accession, this effect could be estimated by the ratio between viability of frozen and osmoconditioned seeds to that of frozen seeds. This variable varied from 1 to 25 among the studied accessions and was not correlated to seed size, seed water content after desiccation, or seed and embryo viability after desiccation. No association was found between the origin of the accessions studied and the viability percentage of osmoconditioned frozen seeds. The beneficial effect of post-thaw seed osmoconditioning observed in Caturra seeds was similar to that observed previously in another Bourbon-derived variety (Dussert et al. 2000). Non-parametric analysis of viability percentages showed that, in 7 of the 30 accessions studied, viability of osmoconditioned frozen seeds was not significantly higher than viability of frozen seeds, while in the 23 other accessions, there was a beneficial effect on seed viability from the post-thaw osmoconditioning treatment (Table 6.2).

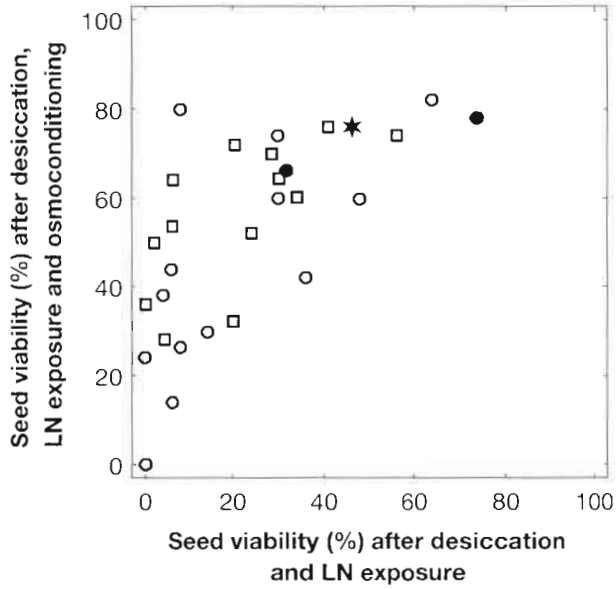

Figure 6.3. Relationship between seed viability (normal seedling development) after desiccation, LN exposure and post-thaw osmoconditioning, and viability of seeds after desiccation and LN exposure only, within the core subset studied. The subset included 27 wild accessions originating from Kefa (O) and Ilubabor (□) provinces, two Ethiopian varieties (●) and one commercial variety (✶).

The beneficial effect of post-thaw osmoconditioning on viability of cryopreserved seeds has been shown for the first time with coffee seeds (Dussert et al. 2000). The present study confirms that this treatment improves the proportion of seeds that develop into seedlings after LN exposure, with mean values of 22 and 52% without and with osmoconditioning, respectively. Recent results showing the importance of slowing down the rate of water uptake after cryopreservation suggest that the beneficial effect of post-thaw seed osmoconditioning could be associated with the reduction of imbibitional membrane injury (Dussert et al. 2003a).

Viability of embryos extracted from frozen seeds

Viability (development into normal seedlings) of embryos extracted from frozen seeds was always very high, with a mean value of 76% (Figure 6.4). Moreover, embryo viability after LN exposure was significantly (P=0.034) correlated with that of embryos extracted from desiccated seeds. The slope of the line of regression was close to 1, suggesting that the negative effect of LN exposure on embryo viability was low in comparison with that observed in whole seeds.

In contrast to the results described above with whole seeds, the present results establish clearly that there is no intraspecific variability for the tolerance of embryos to LN exposure. Differences in results obtained with whole seeds and embryos have already been reported in two *C. arabica* varieties (Dussert et al. 1997, 2000) and in other coffee species (Dussert et al. 2001). The very high tolerance of coffee embryos to LN exposure, when seeds are desiccated to their unfreezable water content before cooling, suggests that the decline or the loss of seed viability observed after LN exposure with 22 out of the 30 accessions

studied is due to damage to the endosperm only. In coffee seeds, the transition from the germinated (radicle and hypocotyl emerged) stage to the normal seedling stage appears thus to be strictly dependent on endosperm integrity. The present results suggest thus that the endosperm should be studied to uncover the causes for the intraspecific variability for seed sensitivity to LN exposure.

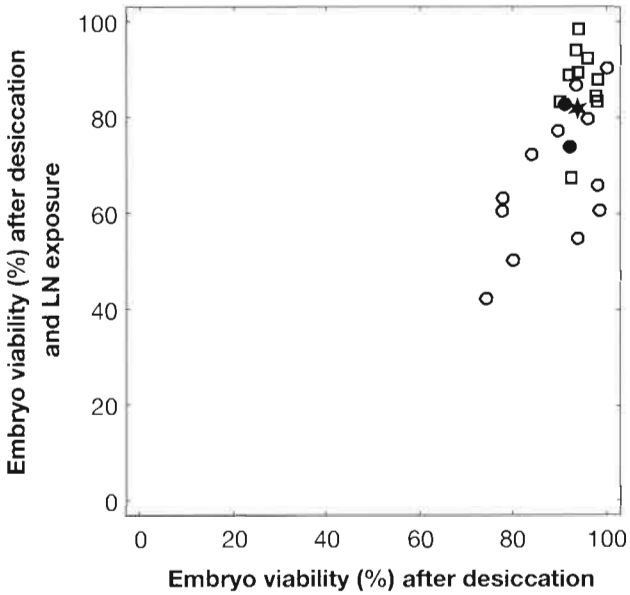

Figure 6.4. Correlation between viability (normal seedling development) of embryos extracted from seeds desiccated and exposed to LN and viability of embryos extracted from desiccated seeds, within the core subset studied. The subset included 27wild accessions originating from Kefa (○) and Ilubabor (□) provinces, two Ethiopian varieties (●) and one commercial variety (✶).

Application to the CATIE cryopreserved coffee core collection

As foreseen in previous reports (Dussert et al. 1997, 2000), two cryopreservation strategies can be employed for routine use in coffee genebanks (for details see Boxes). Each protocol presents its own advantages and drawbacks.

Box 1. Strategy 1

- Desiccation of seeds
- Cooling at 1°C/min to –50°C
- Immersion in liquid nitrogen
- Rapid rewarming (40°C, 4 minutes)
- 6-week osmoconditioning treatment with a –1.25 MPa PEG solution
- Germination in greenhouse

Box 2. Strategy 2

- Desiccation of seeds
- Immersion in liquid nitrogen
- Rapid rewarming (40°C, 4 minutes)
- Seed disinfection
- Extraction of embryos and inoculation in vitro
- 6-week culture period in vitro
- Acclimatization of plantlets
- Transfer to greenhouse

The main advantages of Strategy 1 are that it does not require the use of tissue culture and that seedlings recovered from frozen seeds can be transferred directly to greenhouse conditions. However, it also presents two drawbacks: mean survival is moderate (52%) and a programmable freezer is required. The first drawback is however counterbalanced by the fact that, according to a recent probabilistic study (Dussert et al. 2003b), with a sample size of 50 seeds, the probability to recover at least one plant from the cryopreserved sample is higher than 0.95 for all samples showing a recovery percentage higher than 12%. In the present study, this was the case for 29 of the 30 accessions studied. In order to recover at least five plants, the recovery percentage must be higher than 30%, which was not the case for only five accessions. However, one recovered plant could be considered as being enough to represent the diversity of a given accession because species self-compatibility has contributed to produce homozygous genotypes.

The principal advantages of Strategy 2 are that it enables one to achieve high survival percentages (74% mean) and that it does not require the use of a programmable freezer (direct immersion in LN). However, tissue culture is more time consuming than the standard germination procedure and is associated with additional problems, such as the risk of contamination, which caused the loss of two accessions in the present study, and the acclimatization of *in vitro* plantlets recovered from frozen embryos, which is a second source of plantlet loss. However, these problems should appear less important in the future because the rewarming and the use of a cryobank sample should remain very occasional, allowing samples to be treated very carefully.

Because the second protocol allows the freezing of a higher number of samples simultaneously (direct immersion in liquid nitrogen), it has been chosen by the team of CATIE in charge of this project for the establishment of a cryobank of coffee seeds. In 2002, the 74 accessions of the core collection were cryopreserved according to Strategy 2.

Two very important additional points should also be considered. Firstly, the cryopreserved collection should be duplicated in a secure place other than CATIE, for safety reasons. Secondly, it is essential to cryopreserve a sufficient amount of seeds per accession to ensure their regeneration. The number of seeds to be stored by accession should be calculated as a function of their survival to freezing and of their future utilization. A paper dealing with these issues has already been published (Dussert et al. 2003b) which provides tools for such calculations. In this paper, a simple method, based on the binomial distribution, is proposed to calculate the probability of recovering at least one (or any other fixed number of) plant(s) from a cryobank sample using four given parameters: the percentage of plant recovery observed from a control sample, $p_{obs;}$ the number of propagules used for this control, n_1; the number of propagules in the cryobank sample, n_2; and a chosen risk for the calculation of a confidence interval for the observed plant recovery, α. Using this method, it is possible to assess the number of propagules that should be rewarmed immediately after freezing in order to estimate the plant recovery percentage as a function of the total number of propagules available. It also allows the calculation of the minimum plant recovery percentage to ensure that the probability of recovering at least one (or A, with $A>1$) plant(s) is higher than a fixed probability level, as a function of the control and the cryobank sample sizes. Reciprocally, once the plant recovery percentage has been estimated, it is possible to assess the minimum size of the cryobank sample to obtain a probability to recover at least one (or A, with $A>1$) plant(s) higher than some fixed level.

Prospects

This collaborative project between Bioversity, IRD and CATIE on cryopreservation of coffee germplasm was very fruitful regarding many issues. Not only did it lead to the establishment of the first world coffee cryobank, but also demonstrated the feasibility of transferring proce-

dures set up in a laboratory located out of the coffee growing area to a centre in charge of the conservation of coffee genetic resources located in a developing country.

Secondly, this study highlighted that some of the procedures employed need further optimization. In particular, the unexpected variability observed for seed viability after desiccation clearly showed that the harvest and post-harvest processes have to be re-examined. The fact that the requirement for a programmable freezer disqualified Strategy 1, despite its simplicity, also demonstrated the need for a simpler and low-tech procedure to perform the slow cooling step. Recent trials (unpublished results) have shown that it can achieved by an optimized exposure in a –80°C freezer or in a dry-ice bath.

Finally, considerable progress has been made in the understanding of the mechanisms involved in coffee seed sensitivity to desiccation and LN exposure since the achievement of the project described in the present document (Dussert et al. 2001, 2003a, 2003b, 2004). In particular, the rewarming and rehydration protocols have been significantly improved, allowing achievement of full (100%) survival of frozen seeds. We have indeed shown that pre-heating (soaking seeds in a 40°C water bath for at least 30 minutes) and pre-humidification (placing seeds in water-saturated air at 25 or 37°C (warm pre-humidification) for 24 or 48 h) of seeds after cryopreservation were more efficient procedures than the post-thawing osmoconditioning treatment used in the present study. This improvement will have to be included in future applications of cryopreservation for long-term conservation of coffee germplasm.

References

Becwar, M.R., Stanwood, P.C. & Lehonardt, K.W. 1983. Dehydration effects on freezing characteristics and survival in liquid nitrogen of desiccation-tolerant and desiccation-sensitive seeds. Journal of the American Society for Horticultural Science 108:613–618.

Charrier, A., Berthaud, J. & Anthony, F. 1989. Coût et valorisation des ressources génétiques caféières. pp. 429–437 *in:* Proceedings of the 13th International Scientific Colloquium on Coffee. Paipa, Colombia, 21–25 August 1989. ASIC, Montpellier, France.

Dussert, S., Chabrillange, N., Engelmann, F., Anthony, F. & Hamon, S. 1997. Cryopreservation of coffee (*Coffea arabica* L.) seeds: importance of the precooling temperature. CryoLetters 18:269–276.

Dussert, S., Chabrillange, N., Vasquez, N., Engelmann, F., Anthony, F., Guyot, A. & Hamon, S. 2000. Beneficial effect of post-thawing osmoconditioning on the recovery of cryopreserved coffee (*Coffea arabica* L.) seeds. CryoLetters 21:47–52.

Dussert, S., Chabrillange, N., Rocquelin, G., Engelmann, F., Lopez, M. & Hamon, S. 2001. Tolerance of coffee (*Coffea* spp.) seeds to ultra-low temperature exposure in relation to calorimetric properties of tissue water, lipid composition and cooling procedure. Physiologia Plantarum 112:495–504.

Dussert, S., Chabrillange, N., Montillet, J.L., Agnel, J.P., Engelmann, F. & Noirot, M. 2003a. Basis of coffee seed sensitivity to liquid nitrogen exposure: oxidative stress or imbibitional damage? Physiologia Plantarum 119:534–543.

Dussert, S., Engelmann, F. & Noirot, M. 2003b. Development of probabilistic tools to assist in the establishment and management of cryopreserved plant germplasm collections. CryoLetters 24:149–160.

Dussert, S., Engelmann, F., Louarn, J. & Noirot, M. 2004. Inheritance of seed desiccation sensitivity in a coffee inter-specific cross: evidence for polygenic determinism. Journal of Experimental Botany 55:1541–1547.

Ellis, R.H., Hong, T.D. & Roberts, E.H. 1990. An intermediate category of seed storage behaviour? I. Coffee. Journal of Experimental Botany 41:1167–1174.

Eira, M.T.S., Walters, C., Caldas, L.S., Fazuoli, L.C., Sampaio, J.B. & Dias, M.C. 1999. Tolerance of *Coffea* spp. seeds to desiccation and low temperature. Revista Brasileira de Fisiologia Vegetal 11:97–105.

Fernie, L.M., Greathead, D.J., Meyer, F.G., Monaco, L.C. & Narasimhaswamy, R.L. 1968. FAO coffee mission to Ethiopia, 1964-65. FAO, Rome, Italy. 204 p.

Guillaumet, J.-L. & Hallé, F. 1978. Echantillonnage du matériel récolté en Ethiopie. Bulletin IFCC 14:13–18.

Ryan, T.A. 1960. Significance tests for multiple comparison of proportions, variances and other statistics. Psychological Bulletin 57:318–328.

Stanwood, P.C. 1985. Cryopreservation of seed germplasm for genetic conservation. pp. 199–226, *in:* K.K. Kartha (editor). Cryopreservation of Plant Cells and Organs. CRC Press, Boca Raton, USA.

van der Vossen, H.A.M. 1977. Methods of preserving the viability of coffee seed in storage. Kenya Coffee 45:31–35.

Vasquez, N., Salazar, K., Anthony, F., Chabrillange, N., Engelmann, F. & Dussert, S. 2005. Variability in response of seeds to liquid nitrogen exposure in wild coffee (*Coffea arabica* L.). Seed Science and Technology 33:293–301.

Dussert Stéphane, Vasquez N., Salazar K., Anthony François, Engelmann Florent (2007)

Cryopreservation of coffee genetic resources

In : Engelmann Florent (ed.), Dulloo M.E. (ed.), Astorga C. (ed.), Dussert Stéphane (ed.), Anthony François (ed.). Conserving coffee genetic resources : complementary strategies for ex situ conservation of coffee (*Coffea arabica* L.) genetic resources : a case study in CATIE, Costa Rica

Rome (ITA) ; Paris : Biodiversity International ; IRD, 49-58

ISBN 978-92-9043-752-9