Cryopreservation of coffee (*Coffea arabica* L.) seeds: toward a simplified protocol for routine use in coffee genebanks

S. Dussert[1], Nathalie Chabrillange[1], Florent Engelmann[2], F. Anthony[3] and S. Hamon[1]

[1] *ORSTOM, 34032 Montpellier Cedex 1, France*
[2] *IPGRI, 00145 Rome, Italy*
[3] *Centro Agronómico Tropical de Investigación y Enseñanza, Apartado 59, 7170 Turrialba, Costa Rica*

Introduction

Though *Coffea arabica* seeds can withstand desiccation down to 0.06-0.08 g H_2O/g dw (Becwar *et al.* 1983; Ellis *et al.* 1990), they cannot be considered orthodox because they remain cold-sensitive and desiccation does not improve their longevity (Van der Vossen 1977; Ellis *et al.* 1990). *Coffea arabica* seeds are also characterized by their very short lifespan in the hydrated state (Couturon 1980).

Whatever their water content, *C. arabica* seeds do not withstand direct immersion in liquid nitrogen (Becwar *et al.* 1983). However, successful cryopreservation of zygotic embryos extracted from mature seeds has been achieved with *C. liberica* (Normah and Vengadasalam 1992), *C. arabica* (Abdelnour-Esquivel *et al.* 1992; Florin *et al.* 1993), *C. canephora* and the interspecific hybrid arabusta (Abdelnour-Esquivel *et al.* 1992). With all species tested, partial dehydration of excised embryos to 0.2 g H_2O/g dw was sufficient to obtain high survival rates after their direct immersion in liquid nitrogen.

Even if cryopreservation of excised zygotic embryos represents an interesting alternative strategy for the long-term preservation of *C. arabica* genetic resources, this technique presents some drawbacks for routine use in coffee genebanks: (i) in the case of coffee seeds, embryo extraction is very time-consuming and labour-intensive, (ii) low reproducibility was observed when desiccation of zygotic embryos used classical desiccation methods (airflow or silica gel), and (iii) all stages of the cryopreservation procedure have to be performed under aseptic conditions, which does not allow avoiding the use of *in vitro* culture techniques. Cryopreservation of whole seeds, instead of zygotic embryos, would allow elimination of these drawbacks.

With this aim, the effects of several parameters of the cryopreservation protocol (desiccation, cooling, thawing and post-treatment) were investigated with *C. arabica* seeds to define conditions which ensure survival of both the endosperm and the embryo (Dussert *et al.* 1997, 1998). It was shown that: (i) the optimal water content for cryopreserving whole coffee seeds was 0.2 g H_2O/g dw, (ii) a two-step freezing procedure including precooling at 1°C/min to –50°C was imperatively required to recover normal seedlings after cryopreservation, and (iii) there was no effect of the thawing rate on survival of cryopreserved seeds. Under these conditions, the maximal percentage of normal seedlings produced after

cryopreservation was about 30% (Dussert *et al.* 1997). In addition, it was observed that, whatever the cooling process, the survival rate of zygotic embryos extracted from cryopreserved seeds after thawing was always very high (80-90%).

In this study, the reproducibility of seedling recovery was investigated by carrying out several cryopreservation experiments using the same optimal conditions. In addition, the effect of various post-thawing treatments was studied in order to improve the production of normal seedlings after cryopreservation of whole seeds.

Materials and methods

Plant material, desiccation and cryopreservation

Fresh mature seeds of *C. arabica* var. Typica were provided from CATIE, Costa Rica. After the testa was removed, seeds were desiccated to 0.2 g H_2O/g dw by equilibration for 3 weeks under 78% RH obtained using a saturated NH_4Cl solution (Dussert *et al.* 1997). The different cryopreservation procedures (rapid cooling, two-step cooling, slow and rapid rewarming) were carried out following the protocols described by Dussert *et al.* (1997, 1998).

Osmopriming

After thawing, some seeds were primed for 2 weeks at 27°C in the dark using PEG solutions. Osmopriming was carried out by placing batches of 10 seeds in Petri dishes sealed with Parafilm Ribbon, on a thin layer of cotton wool imbibed with 20 ml of aqueous PEG 6000 solution. PEG concentrations were calculated to achieve osmotic potentials of –1, –2 and –4 MPa at 27°C using the equation given by Michel and Kaufmann (1973).

Culture conditions

After thawing and/or osmopriming, seeds were cultured *in vitro* for survival assessment. Disinfection and *in vitro* culture were performed as described by Dussert *et al.* (1997). Extraction of zygotic embryos and *in vitro* culture were carried out according to the method of Bertrand-Desbrunais and Charrier (1989).

Survival assessment

Both germination *sensu stricto* and development of normal seedlings were used to assess seed survival. Emergence of the hypocotyl and radicle was used as the criterion for estimating the germination rate. Seedlings which stood upright on the medium were considered normal. Excised embryos were considered viable when they stood upright on the culture medium and when their first pair of leaves was developed. The time to reach half of the final proportion (P_1) of normal seedlings, T_{50}, was estimated using the least square regression and the following model where P is the proportion of normal seedlings, T the time in days and A a treatment-dependent variable describing the synchronization of seedling development: $P = P_1 / \{1 + \exp[A\ (T - T_{50})]\}$.

Results

Effects of slow and rapid cooling

When cooled rapidly (200°C/min) by direct immersion in LN, none of the cryopreserved seeds produced normal seedlings. By contrast, viability of zygotic embryos extracted from rapidly cooled seeds was always very high. If seeds were precooled to –50°C at 1°C/min before immersion in LN (slow cooling), over seven repeats, a mean value of 17% of cryopreserved seeds developed into normal seedlings. Under these conditions, some normal seedlings were always recovered after germination of whole seeds, but high variability in the final survival rate was observed (6–29%) (Table 1).

Table 1. Number of experiments (n) and percentage (mean, minimal and maximal values) of normal seedlings recovered from whole seeds or extracted zygotic embryos after desiccation of seeds to 0.2 g H_2O/g dw, followed by rapid cooling (direct immersion in LN; 200°C/min) or slow cooling (1°C/min to –50°C prior to immersion in LN)

	Whole seeds		Zygotic embryos	
	n	Seedlings (%) [min-max]	n	Seedlings (%) [min-max]
Rapid cooling	4	0	3	91 [83-98]
Slow cooling	7	17 [6-29]	1	70

Effect of osmopriming of seeds after rewarming

Osmopriming of seeds after rewarming improved the final proportion of normal seedlings recovered from cryopreserved seeds but the gain in survival decreased in line with decreasing osmotic potential of the PEG solution used for the osmopriming treatment (Fig. 1). Production of normal seedlings under optimal priming conditions (–1 MPa) was 3-fold higher than that of unprimed cryopreserved seeds. Post-thawing seed osmopriming drastically reduced T_{50}, the time to reach half of the final proportion of seedlings: when cryopreserved seeds were placed under germination conditions directly after thawing, T_{50} value was 36 d, while with osmoprimed seeds, it was about 12-13 d, independently of the osmotic potential of the priming solution.

Discussion

When seeds of *C. arabica* at 0.2 g H_2O/g dw were cooled rapidly (200°C/min), none of them developed into normal seedlings. This result is consistent with those of Becwar *et al.* (1983) who showed that *C. arabica* seeds did not survive after immersion in LN, even if all freezable water had been removed from the seeds. By contrast, when seeds were slowly precooled to –50°C at 1°C/min before immersion in LN, an average value of 17% (over seven repeats) of cryopreserved seeds developed into normal seedlings. It is thus clear that slow precooling of *C. arabica* seeds had a dramatic effect on their survival and their capacity to develop normally (Dussert *et al.* 1998). However, in view of the high variability observed for the survival rate over seven experiments, improvement to the method appeared necessary before routine use and various post-thawing treatments were investigated.

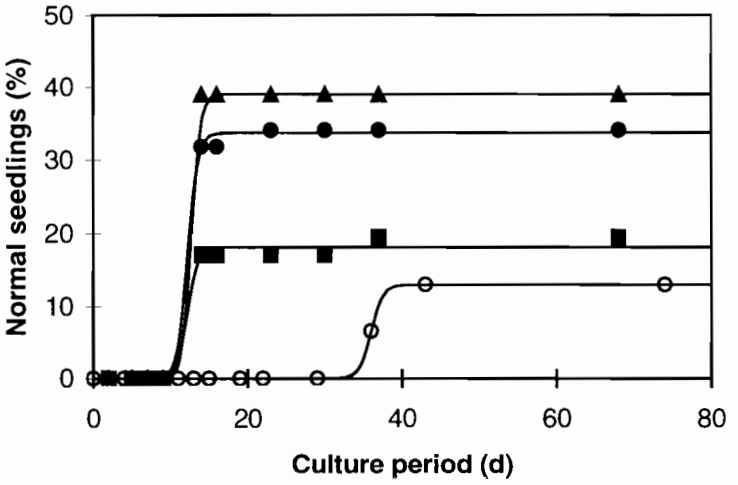

Fig. 1. Evolution with time in culture under germination conditions of the percentage of normal seedlings recovered from cryopreserved seeds after a 2-week osmopriming treatment on PEG solutions at –1 (▲), –2 (●) and –4 (■) MPa or without osmopriming treatment (O).

It was shown for the first time that seed osmopriming (osmoconditioning) carried out after thawing had a dramatic beneficial effect on the proportion of normal seedlings recovered after cryopreservation and on seedling growth rate: under optimal osmoconditioning conditions (–1 MPa for 2 weeks), the percentage of cryopreserved coffee seeds which developed into normal seedlings was 3-fold that of unprimed cryopreserved seeds (39% vs. 13%) and the time to reach half of final percentage of normal seedlings (T_{50}) was about 3-fold lower (13 vs. 36 d). To our knowledge, the effect of seed osmopriming after cryopreservation has been investigated previously in one study on celery seeds only (Gonzales-Benito *et al.* 1995). In this study, no effect of osmopriming could be found since cryopreservation did not affect germination rates and T_{50} values. Osmopriming was first employed to improve the rate and uniformity of germination (Heydecker *et al.* 1975). Since then, a beneficial effect of osmopriming after seed ageing has been observed both on germination percentage and on germination rate with numerous species (Bewley and Black 1994; Bray 1995).

Thus, even if the percentage of seeds which developed into normal seedlings remained relatively low in comparison with that obtained from excised zygotic embryos, a combination of slow cooling and osmopriming treatment could represent a simple and efficient complementary option to field conservation for genebanks which cannot afford *in vitro* culture facilities. Moreover, this method might be simplified by using a –80°C freezer for precooling seeds to –50°C and could thus become more easily employed routinely in a larger number of genebanks maintaining coffee genetic resources. However, additional research should be undertaken to optimize osmopriming conditions and to carry out direct germination tests under greenhouse or nursery conditions.

In cases where very high survival rates are required for routine use, we propose a second alternative approach based on the extraction of zygotic embryos after rewarming of seeds, which would avoid most of the drawbacks of cryopreservation protocols developed for zygotic embryos. Equilibrating coffee seeds under 78% RH allowed seeds to reach optimal water content for cryopreservation in a very easy and reproducible manner. This method also allows the processing of large amounts of seeds at the same time. Moreover, aseptic culture conditions are required only after thawing.

In conclusion, depending on the genebank's facilities, one of the two protocols proposed in this study could be easily applied for the establishment of *C. arabica* germplasm cryobanks.

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