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CRYOPRESERVATION OF COFFEE (*COFFEA ARABICA* L.) SEEDS: IMPORTANCE OF THE PRECOOLING TEMPERATURE.

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Summary: the effect of the precooling temperature on the survival rate of cryopreserved *Coffea arabica* seeds and excised zygotic embryos was investigated. Seeds were desiccated to 0.2 g $H_2O.g^{-1}$ dw, then cooled at $1°C.min^{-1}$ to various precooling temperatures. The optimal germination rate (70%) of cryopreserved seeds was achieved after precooling to -50°C. After 4 months in culture, 30% of seeds developed into normal seedlings. For embryos extracted after thawing from cryopreserved seeds, maximal survival rate (97%) was observed when seeds were immersed into liquid nitrogen directly after dehydration, without precooling. All viable embryos developed into normal seedlings after 2 months in culture.

Key words: *Coffea* - seed - zygotic embryo - cryopreservation - desiccation - genetic resources conservation- precooling temperature.

Abbreviations

CATIE: Centro Agronomico Tropical de Investigacion y Enseñanza; dw dry weight; LN liquid nitrogen; ORSTOM: Institut français de recherche scientifique pour le développement en coopération; IPGRI: International Plant Genetic Resources Institute; RH: relative humidity.

Introduction

Although more than 100 distinct coffee taxa have been identified to date in inter-tropical forests of Africa and Madagascar (4), commercial coffee production relies on two species only, *Coffea arabica* L. and *C. canephora* Pierre. *C. arabica* is the only tetraploid species (2n=4x=44) in the genus *Coffea* and is autogamous, while other species are diploid and generally self-incompatible (5). Higher quality is associated with *C. arabica* and arabica coffee represents 70% of the world production.

Though *C. arabica* seeds can withstand desiccation down to 0.06-0.08 g $H_2O.g^{-1}$ dw (2, 13), they cannot be considered orthodox because they remain cold-sensitive and desiccation does not improve their longevity (2, 13, 24). *C. arabica* seeds are also characterised by their very short lifespan in the hydrated state (8, 24). Thus, the development of cryopreservation techniques for long-term conservation of coffee genetic resources is an important priority (10, 11).

Whatever their water content, *C. arabica* seeds do not withstand direct immersion into liquid nitrogen temperature (2, 12). However, it has been shown recently that a slow precooling step prior to immersion into LN is the key to successful cryopreservation of

C. arabica seeds (Dussert *et al.*, unpublished). Indeed, 25 % of cryopreserved *C. arabica* seeds could develop into normal seedlings when dehydrated to 0.2 g $H_2O.g^{-1}$ dw and precooled slowly ($2°C.min^{-1}$) down to -50°C prior to immersion into LN. No seedlings were obtained after cryopreservation if seeds were dehydrated to a water content even only slightly different from 0.2 g $H_2O.g^{-1}$ dw. In addition, several precooling rates were tested and it appeared that the lower the precooling rate, the higher the survival rate was after cryopreservation. However, in these experiments, only one precooling temperature was tested (-50°C).

Beside, successful cryopreservation of zygotic embryos, extracted from mature seeds, was achieved for *C. liberica* (19), *C. arabica* (1, 16), *C. canephora* and the interspecific hybrid arabusta (1). With all species, partial dehydration of excised embryos to 0.2 g $H_2O.g^{-1}$ dw was sufficient to obtain high survival rates after direct immersion in liquid nitrogen.

In the present work, we studied the effect of the precooling temperature on the germination rate of *C. arabica* seeds, on the survival rate of excised zygotic embryos and on the subsequent development of seedlings.

Materials and methods

Plant material

Fresh mature seeds of *C. arabica* var. *typica* were provided from CATIE, Costa Rica. Seed water content upon receipt was 0.5 g $H_2O.g^{-1}$ dw.

Desiccation and cryopreservation

After the testa was removed, seeds were desiccated to 0.2 g $H_2O.g^{-1}$ dw by equilibrating them for 3 weeks under 78% RH obtained using an NH_4Cl saturated solution. Water content (expressed in g $H_2O.g^{-1}$ dw) was estimated using 3 replicates of 10 seeds. Dry weight was measured after 2 days of desiccation in an oven at 105°C.

Before cryopreservation, seeds were hermetically sealed in 10 ml polypropylene tubes (50 seeds per tube). The initial temperature of the seeds was 25°C (room temperature). Seeds were precooled to 0°C, -20°C, -50°C and -100°C at $1°C.min^{-1}$, then rewarmed directly (precooling controls) or immersed in LN before rewarming. Some seeds were directly transferred *in vitro* or cryopreserved without precooling. Precooling was carried out using a programmable cooling apparatus (Minicool LC 40, L'Air Liquide, France). Cryopreserved seeds were stored for one week at -196°C before thawing. Thawing was carried out by plunging the cryotubes in a 40°C water-bath for 2 min.

Culture conditions

After freezing, both seeds and zygotic embryos were inoculated and cultured *in vitro* for survival assessment. Before disinfection, seeds were washed with soap and tap water. Disinfection was achieved by soaking seeds in sodium hypochlorite (12%) for 15 min with continuous shaking on a rotary shaker, followed by 5 min under vacuum and 10 min again with shaking. Seeds were rinsed three times with sterile water before inoculation into test tubes (250 x 24 mm) sealed with Parafilm Ribbon on water gel ($3 g.l^{-1}$ agar).

Before excision of zygotic embryos, disinfected seeds were immersed for two days in sterile water for rehydration. Excised zygotic embryos were inoculated on the germination medium (20 ml) defined by Bertrand-Desbrunais and Charrier (3) into test tubes (250 x 24 mm) sealed with Parafilm Ribbon.

All cultures were maintained in the dark until the hypocotyl stood upright. They were

then transferred to light conditions (30 $\mu E.m^{-2}.s^{-1}$, 12h light/12h dark photoperiod).

Four month-old well-developed *in vitro* seedlings were transferred to the greenhouse and grown on an acidophilic peat compost.

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 after 1 and 4 months in culture. Seedlings which stood upright on the medium at those times of observation were considered normal. The ratio of normal seedlings after 1 month in culture to normal seedlings after 4 months was used to estimate the development rate of seedlings. After 3 months in the greenhouse, greening of cotyledonary leaves and development of apical shoot was used as the criterion for assessing normal plants. Excised embryos were considered viable when they stood upright on the culture medium and the first pair of leaves was developed.

Statistical analysis

One sample of 50 seeds was used in each treatment. Multiple comparison of germination rates of precooling controls and cryopreserved seeds and of percentages of normal seedlings produced was carried out using the Ryan's test (21). The effect of the precooling temperature on the viability of zygotic embryos and seedling growth rate was estimated by linear regression.

Results

Effect of desiccation

Equilibrating seeds under 78% RH for 3 weeks consistently produced seeds at the required water content for cryopreservation (mean = 0.20 g $H_2O.g^{-1}$ dw; s.d. = 0.0003 g $H_2O.g^{-1}$ dw) and without any viability loss (95.4% of normal seedlings after 4 months in culture).

Effect of the precooling temperature on seed viability

After 4 months in culture, there was no significant difference in viability between control seeds and precooling controls for precooling temperatures of 0, -20 and -50°C (Figs. 1 and 2). In all cases, around 95% of the seeds germinated and developed into normal seedlings. A drastic decline in viability was observed when seeds were precooled to -100°C, since only 55% of them germinated (Fig. 1) and 24% produced normal seedlings (Fig. 2).

For precooling temperatures above -50°C, viability of seeds immersed in liquid nitrogen after precooling was always significantly lower than that of precooling controls (Figs. 1 and 2). By contrast, when seeds were precooled to -100°C, there was no difference in survival between precooling controls and cryopreserved seeds.

The germination rate of cryopreserved seeds increased with precooling temperatures down to -50°C, from 26% without precooling up to 70% for a precooling temperature of -50°C, and then decreased down to 55% for seeds precooled to -100°C (Fig. 1). The percentage of normal seedlings produced from cryopreserved seeds followed the same trend (Fig. 2) and was highly correlated with the germination rate (F=10.61; P=0.0470). It could be approximated by subtracting 40% to the germination rate : e.g. after precooling to -50°C and immersion in LN, the germination rate was 70% and the percentage of normal seedlings was 30%.

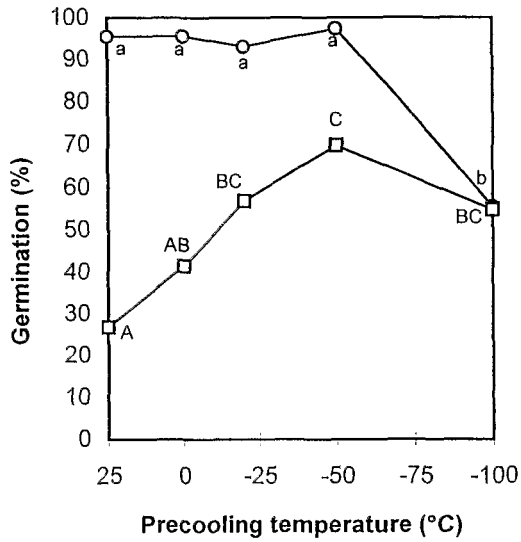

Figure 1. Effect of precooling temperature on the germination rate (%) of *C. arabica* precooling control seeds (**O**) and cryopreserved seeds (□). Points followed by the same letter were not significantly different at the 0.05 probability level as determined by Ryan's test.

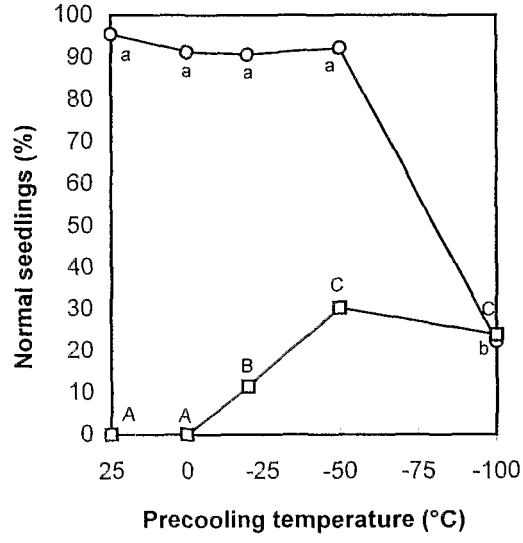

Figure 2. Effect of precooling temperature on the rate of normal seedlings produced (%) from *C. arabica* precooling control seeds (**O**) and cryopreserved seeds (□). Points followed by the same letter were not significantly different at the 0.05 probability level as determined by Ryan's test.

Effect of precooling temperature on zygotic embryo viability

Precooling had a significantly negative effect on the viability of zygotic embryos extracted from precooled (F=35.60 ; P=0.0094) and cryopreserved (F=21.38 ; P=0.0191) seeds (Fig. 3). However, this effect was very low and survival rates ranged from 83 to 97% for precooling controls and from 65 to 93% for cryopreserved seeds. The maximal viability (97%) was obtained with embryos extracted from seeds directly immersed into LN after desiccation, without any precooling treatment. All viable embryos developed into healthy plantlets which could be transferred to greenhouse conditions without any viability loss.

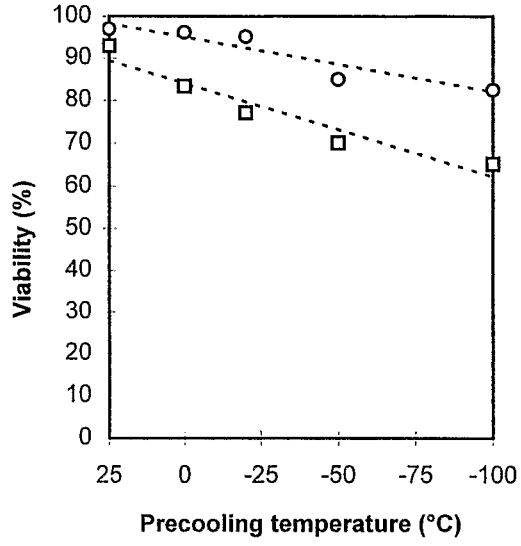

Figure 3. Effect of precooling temperature on the viability (%) of zygotic embryos extracted from *C. arabica* precooling control seeds (**O**) and cryopreserved seeds (□). Dotted lines correspond to linear regressions.

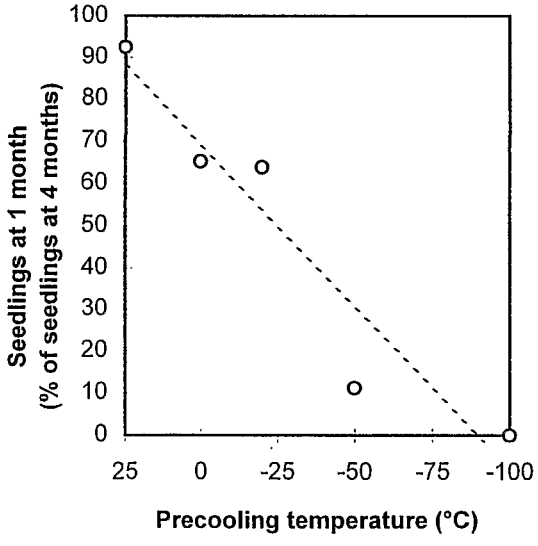

Figure 4. Development rate (%) of normal seedlings obtained from precooling control seeds, expressed by the ratio: seedlings developed after 1 month in culture/seedlings developed after 4 months in culture. Dotted line corresponds to linear regression.

Effect of precooling temperature on seedling development rate

For precooling controls, there was a significant negative effect (F=29.99; P=0.0119) of the precooling temperature on the development rate of seedlings, as estimated by the ratio: normal seedlings after 1 month of culture/normal seedlings after 4 months (Fig. 4). In the case of cryopreserved seeds, the development rate of seedlings could not be measured due to the delayed germination of seeds.

Discussion

This study confirmed that whole seeds of *C. arabica* can be cryopreserved if they are slowly precooled to -50°C prior to their immersion in liquid nitrogen. Even if the percentage of seeds which developed into normal seedlings remained relatively low (30%) in comparison with that obtained from excised zygotic embryos, this protocol could represent a simple and efficient complementary option to field conservation for genebanks which cannot afford *in vitro* culture facilities. However, it should be first verified that cryopreserved seeds can germinate normally under greenhouse or nursery conditions. Moreover, this method might be simplified by using a -80°C freezer for precooling seeds to -50°C and could thus be more easily employed routinely in a large number of genebanks maintaining coffee genetic resources. However, additional research should be undertaken to determine the minimum number of seeds which have to be cryopreserved to guarantee the recovery of a minimum number of plants.

A drastic decline in survival was observed when seeds were precooled below -50°C (without liquid nitrogen exposure) and -50°C appeared to be the optimal precooling temperature for cryopreservation. If the importance of a low cooling rate for seed cryopreservation has been reported for different species (23, 25), it is the first time that the importance of the precooling temperature was clearly demonstrated. A two-step freezing procedure has been widely used for cryopreservation of *in vitro* cultured material including cell suspensions, calluses, somatic embryos (18, 26) but has been employed in a limited number of cases only for zygotic embryos (6, 17, 20). With *in vitro* cultured material, optimal survival rates were generally observed for precooling temperatures between -30°C and -50°C which allow optimal freeze-induced dehydration of the samples (18, 22). With *C. arabica* seeds, the beneficial effect of precooling could not be associated with freeze-induced dehydration, since there was no more freezable water in seed tissues at 0.2 g $H_2O.g^{-1}$ dw (2). Vertucci (25) has indicated that the cooling rate might modify the interactions between bound water and lipids at ultra-low temperatures. The beneficial effect on survival of a precisely defined precooling rate for *C. arabica* seeds could be related to the high lipid content of the endosperm of *C. arabica* seeds (7).

In our study, very high survival rates were obtained with zygotic embryos extracted from seeds cryopreserved under all conditions used in the experiments. By contrast, with whole seeds, high germination and seedling development rates could be obtained for a limited set of conditions only. After precooling to -50°C and cryopreservation, 70% of excised embryos were viable and could develop into normal plantlets; 70 % of seeds germinated, but 30% of seeds only developed into normal seedlings after 4 months in culture. It is thus obvious that immersion into liquid nitrogen caused damages to the endosperm of about 40% of the seeds. In such cases, the endosperm was sufficiently intact to allow germination of all viable embryos but could not play its nutritional function and ensure normal further development of the embryos during the four months of culture.

When seeds were dehydrated under a controlled atmosphere, 97% of zygotic embryos extracted from seeds immersed directly into LN after desiccation survived and developed into normal seedlings. This survival rate was higher than that (30%) obtained in a previous study where seeds were dehydrated using silica gel (10). It has been shown that the range of water contents for which coffee zygotic embryos could withstand cryopreservation is very narrow (1). The higher intra-treatment standard deviation in water contents obtained with silica gel dehydration (0.0334 g $H_2O.g^{-1}$ dw) compared with that obtained with controlled RH (0.0003 g $H_2O.g^{-1}$ dw) could thus be responsible for the large difference in survival of seeds frozen at the same mean water content (0.2 g $H_2O.g^{-1}$ dw). This result indicates the use of controlled

atmospheres for drying seeds before cryopreservation.

Cryopreservation protocols developed for zygotic embryos include their excision from seeds before the successive steps of disinfection, desiccation and freezing (14). Rapid dehydration is generally achieved using the sterile air-stream of a laminar flow cabinet, air-tight containers with silica gel, compressed air stream or vacuum (9). Though the efficiency of these protocols has been demonstrated for numerous plant species (14, 15), including *C. arabica*, *C. canephora* and *C. liberica* (1, 19), they present several constraints and disadvantages: i) the need to work under aseptic conditions before cryopreservation ; ii) difficulty to achieve reproducible desiccation conditions when using the sterile air-stream of a laminar flow cabinet or silica gel: since the desiccation periods are generally very short, they need to be very precise and are highly dependent of the initial moisture content of the samples and of the characteristics of the air-flow or the silica gel used ; iii) impossibility to treat simultaneously large amounts of material since the time needed to extract one embryo (1 to 2 min for a single coffee seed) is very long compared with the optimal desiccation period (e.g. 30 min under the laminar flow for coffee embryos with an initial moisture content of 60% (fresh weight basis), as reported by Abdelnour-Esquivel *et al.* (1)). We propose a new and simple approach which consists of drying seeds under controlled RH and of germinating *in vitro* whole seeds or embryos extracted from the seed after thawing only. This new method should allow to remove many of the problems encountered with traditional zygotic embryo cryopreservation protocols for all coffee species and, possibly, for other species which produce intermediate seeds of relatively small size. Equilibrating coffee seeds under 78% R.H. allowed the seeds to reach optimal water content for cryopreservation without any viability loss, in a very easy and reproducible manner. This also allowed the processing of large amounts of seeds at the same time. Moreover, aseptic conditions were requested after thawing only. This method was very efficient since, when embryos were extracted after freezing, no loss in viability was observed after cryopreservation (97% survival) in comparison with unfrozen controls. The only drawback of this approach, compared with classical protocols, is that it would require a larger volume for storing seeds in liquid nitrogen containers, in comparison with excised embryos.

If whole seeds are germinated after cryopreservation, particular attention should be given to the possible occurrence of intra-accession genetic drift. Indeed, if the biochemical composition of the seeds is implicated in their tolerance to LN exposure and if this characteristic is genetically controlled, a 30% survival rate could lead to a genetic selection for 'LN exposure adapted' genotypes. When embryos are extracted from seeds after thawing and grown *in vitro*, no genetic deviation should be observed, in view of the very high survival rates obtained.

In conclusion, depending on the laboratory's or genebank's facilities, one of the two protocols proposed in this study could be easily applied for the establishment of a *C. arabica* germplasm cryobank. The application of this cryopreservation protocol to other coffee species is currently under investigation.

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