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BENEFICIAL EFFECT OF POST-THAWING OSMOCONDITIONING ON THE RECOVERY OF CRYOPRESERVED COFFEE (*COFFEA ARABICA* L.) SEEDS

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Summary: Osmoconditioning - controlled rehydration of seeds in a solution with low osmotic potential - has been shown to reinvigorate aged seeds. The present work aimed at investigating the effect of osmoconditioning on the germination of cryopreserved seeds of *Coffea arabica*, whose viability and vigour are drastically affected by cryopreservation. For cryopreservation, seeds were desiccated to 0.21 g H_2O/g dw, cooled at 1°C/min to -50°C, then immersed rapidly in liquid nitrogen. After rapid rewarming, seeds were osmoconditioned for 1 to 6 weeks using solutions with osmotic potentials between -1 and -4 MPa. The time to produce half of the final percentage of normal seedlings, T_{50}, was about three fold lower with osmoconditioned seeds than with non-osmoconditioned seeds (12-14 d *vs* 36 d). Moreover, after a 6-week osmoconditioning treatment with solutions with osmotic potential of -1 and -1.25 MPa, the percentage of seedlings recovered from cryopreserved seeds was 64-74%, against 13-16% only for cryopreserved seeds which were not osmoconditioned.

Key words: *Coffea arabica* - seed - cryopreservation - germplasm conservation - osmoconditioning -PEG - priming.

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

INTRODUCTION

Whatever their water content, seeds of *Coffea arabica* do not withstand direct immersion in LN (2, 7). Normal seedlings can be produced after cryopreservation only if seeds are dehydrated to a very precise water content (0.2 g H_2O/g dw) (5, 7, 8) and are slowly precooled to -50°C before LN exposure (5, 7). However, even if they are desiccated and cooled under optimal conditions, the percentage of cryopreserved seeds which develop into normal seedlings remains low, about 17% in average (6), and the growth rate of these seedlings is dramatically reduced in comparison with unfrozen controls.

Seed osmoconditioning (osmopriming) is a pre-sowing treatment consisting of a controlled rehydration of seeds using solutions with low osmotic potentials, generally between

-1 and -1.5 MPa (10). Osmoconditioning accelerates seed germination and renders it more uniform (3). These beneficial effects are now used on a large scale with many crops, mainly vegetable and flower species, which exhibit slow and erratic seed germination (15). With some species, the benefits of seed osmoconditioning are conserved when seeds are dried back after completion of the osmoconditioning treatment. Moreover, it has been shown that controlled rehydration of seeds using a low osmotic potential solution can also allow partial recovery of the viability and/or vigour lost during natural or accelerated ageing of seed lots (1, 11, 13, 16). Osmoconditioning has thus been described as a reinvigorating treatment.

To our knowledge, the effect of seed osmoconditioning after cryopreservation has been previously investigated with celery seeds only (9). In this study, no reinvigorating effect of osmoconditioning of cryopreserved seeds could be found since cryopreservation affected neither their germination rate nor their mean germination time.

In the present work, we investigated the effect of controlling seed rehydration after thawing, using low osmotic potential solutions, on the germination of cryopreserved seeds of *C. arabica*, whose viability and vigour are drastically affected by cryopreservation.

MATERIALS AND METHODS

Plant material

Fresh mature seeds of two varieties of *C. arabica* L., Bourbon and Typica, were manually harvested in the field collection of CATIE, Costa Rica and partially dehydrated under ambient conditions for two weeks. Seed water content upon receipt in the laboratory (IRD for the experiment with Bourbon seeds ; CATIE for that with Typica seeds) was about 0.5 g H_2O/g dw with both varieties. Seed lots characteristics, which are presented in Table 1 allowed to verify that the tolerance to desiccation and the sensitivity to rapid freezing of whole seeds and zygotic embryos were equivalent to that observed in previous studies (5, 7).

Table 1. Viability (%) of whole seeds and zygotic embryos of *C. arabica* varieties Bourbon and Typica after desiccation to 0.21 g H_2O/g dw and rapid freezing. Zygotic embryos were extracted from the seeds after desiccation and cryopreservation. For each treatment, seed viability was assessed on 50 seeds.

		Viability (%)	
Treatment	Material	Bourbon	Typica
Desiccation	Seeds	98	100
	Embryos	100	100
Desiccation + rapid freezing	Seeds	0	0
	Embryos	86	92

Desiccation and cryopreservation

Upon receipt in the laboratory, the testa (endocarp) was removed from the seeds. Therefore, seeds without testa were employed in all experiments performed.

Seeds were desiccated to 0.21 g H_2O/g dw (s.d.=0.001 and 0.002 g H_2O/g dw with Bourbon and Typica seeds, respectively) by equilibrating them for 3 weeks under 78% RH obtained using an NH_4Cl saturated solution. The water content of seeds (expressed in g H_2O/g dw) was estimated using 3 replicates of 10 seeds and their dry weight measured after 2 d of desiccation in an oven at 105°C.

Before cryopreservation, seeds were hermetically sealed in 15 ml polypropylene tubes

(50 seeds per tube). The initial temperature of the seeds was approximately 25°C (room temperature). Seeds were precooled to -50°C at 1°C/min, then immersed in LN before rewarming. Cryopreserved Bourbon and Typica seeds were stored at -196°C for 8 and 2 months, respectively. Rewarming was carried out by plunging the tubes in a 40°C water-bath for 2 min.

Osmoconditioning and culture conditions

After thawing, seeds were either directly placed under germination conditions as described by Dussert *et al.* (5) or osmoconditioned before their transfer under germination conditions. Bourbon seeds were treated for 1 or 2 weeks using solutions with osmotic potentials of -1, -2 and -4 MPa, while osmotic potentials of -1, -1.25 and -1.5 MPa in combination with osmoconditioning durations of 2, 4 or 6 weeks were tested in Typica seeds. With both varieties, 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 aqueous PEG 6000 solution. PEG concentrations were calculated to achieve osmotic potentials of -1, -1.25, -1.5, -2 and -4 MPa at 27°C using the equation developed by Michel and Kaufmann (12). Since germination conditions (27°C; dark) were identical to those used for osmoconditioning treatments, batches of seeds placed directly under germination conditions (upon water) after thawing were considered as the 0 MPa control.

Survival assessment

The development of normal seedlings was used to assess seed survival in both varieties. Seedlings which stood upright on the medium (soldier stage) were considered normal. In the experiment with Bourbon seeds, the time to reach half of the final percentage (P_f) of normal seedlings, T_{50}, was estimated using the least square regression and the following model where P is the percentage of normal seedlings, T the time in days and A a treatment-dependent variable describing synchronization of seedlings development : $P = P_f / (1 + \exp(A\ (T - T_{50})))$.

For each treatment, seed viability was assessed on 48-50 seeds. Multiple comparison of final percentages of normal seedlings was carried out using the Ryan's test (14). The effect of the osmoconditioning duration on the percentage of seedlings recovered from Typica seeds was tested through χ^2 decomposition (17).

RESULTS

All osmoconditioning treatments tested with Bourbon seeds drastically reduced T_{50} values (Table 2). When cryopreserved seeds were placed under germination conditions immediately after rewarming, the T_{50} value was 36 d whereas it was 12-14 d only with osmoconditioned seeds, independently of the osmotic potential of the solution and of the duration of the osmoconditioning treatment employed.

With Bourbon seeds, two-week osmoconditioning treatments with -1 and -2 MPa solutions significantly improved the final percentage of normal seedlings recovered from cryopreserved seeds (P=0.0054 and P=0.0183, respectively), while all one-week osmoconditioning treatments experimented and the two-week treatment with a -4 MPa solution had no significant effect on seedling development (Fig. 1). The production of normal seedlings after osmoconditioning treatment of seeds with a -1 MPa solution was three fold that of untreated (0 MPa) cryopreserved seeds (39% *vs* 13%).

Table 2. Estimated time to obtain half of the final percentage of normal seedlings (T_{50}) from cryopreserved seeds of *C. arabica* variety Bourbon, without or with 1- or 2-week post-thawing osmoconditioning treatment using solutions with osmotic potential of -1, -2 and -4 MPa. Final proportion of variance explained by the regression, R^2.

Post-thawing treatment	Osmotic potential (MPa)	T_{50}	R^2
Control	0	36.0	1.00
One-week osmoconditioning	-1	12.4	0.99
	-2	13.7	0.96
	-4	12.1	1.00
Two-week osmoconditioning	-1	12.7	1.00
	-2	12.2	0.99
	-4	12.1	0.99

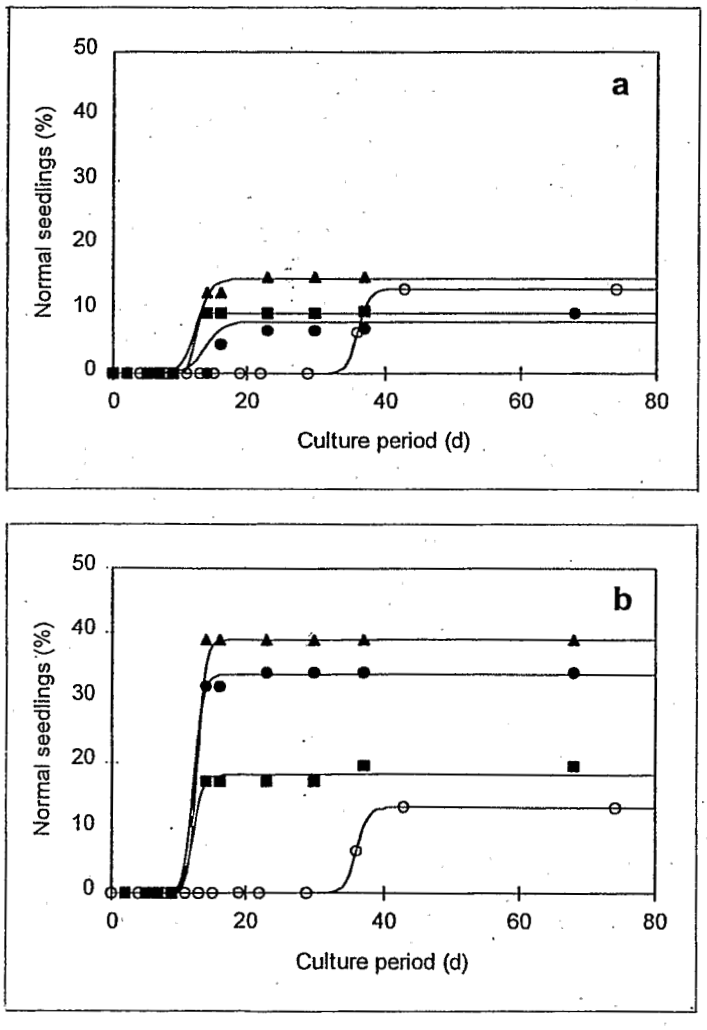

Figure 1. Evolution with time in culture under germination conditions of the percentage of normal seedlings developed from cryopreserved seeds of *C. arabica* variety Bourbon after a 1-(a) or 2-(b) week osmoconditioning treatment with PEG solutions with osmotic potentials of -1 (▲), -2 (●) and -4 (■) MPa or without osmoconditioning treatment (O).

The beneficial effect of osmoconditioning on the percentage of seedlings produced by cryopreserved Typica seeds was significant in all treatments using PEG solutions of -1 and -1.25 MPa: the percentage of seedlings produced from treated seeds ranged from 50 to 74%, against 16% for untreated (0 MPa) seeds (Table 3). Moreover, the percentage of seedlings was significantly ($P_{slope}<0.025$) and linearly ($P_{linearity}<0.05$) correlated with the duration of the osmoconditioning treatment with seeds treated with a -1.25 MPa solution.

Table 3. Percentage of normal seedlings recovered from cryopreserved seeds of *C. arabica* variety Typica after a 2-, 4- and 6- week osmoconditioning treatment with PEG solutions with osmotic potentials of -1, -1.25 and -4 MPa or without osmoconditioning treatment. Percentages of normal seedlings followed by the same letter were not significantly different at the P=0.05 level as tested by the Ryan's test of multiple comparison of proportions.

	Osmotic potential (MPa)	Normal Seedlings (%)
Control	0	16 [a]
2-week osmoconditioning	-1	52 [b]
	-1.25	50 [b]
	-1.5	2 [a]
4-week osmoconditioning	-1	54 [b]
	-1.25	62 [b]
	-1.5	22 [a]
6-week osmoconditioning	-1	64 [b]
	-1.25	74 [b]
	-1.5	10 [a]

DISCUSSION/CONCLUSION

With both Bourbon and Typica varieties, no seedlings could be recovered from seeds desiccated to 0.21 g $H_2O.g^{-1}$ dw and cooled rapidly, while the percentage of seeds developing into seedlings after slow cooling was 13-16%. These results are in agreement with those obtained in several previous studies using various varieties of *C. arabica* (2, 5, 6, 7, 8). The value of the percentage of seedling production observed with Typica seeds cooled slowly (16%) was very similar to that (17%) previously obtained over 7 repeats with the same variety (6).

This study has shown for the first time, using *C. arabica* as a model, that controlled rehydration of cryopreserved seeds by an osmoconditioning treatment can have a dramatic beneficial effect on seedling recovery. This beneficial effect was observed both on the final percentage of seedlings recovered and on their mean germination time, T_{50}. Under optimal post-thawing osmoconditioning conditions (6 weeks over -1.25 MPa), up to 74% of normal seedlings could be recovered from seeds cooled slowly. Moreover, for the first time, some seedlings could be recovered from *C. arabica* seeds cooled rapidly (direct immersion into LN) if those seeds were osmoconditioned after thawing (unpublished results). *C. arabica* seeds proved to be a suitable material to demonstrate this phenomenon since their viability and vigour are severely affected by cryopreservation, without leading to a complete loss of survival.

Reinvigoration of deteriorated seeds by osmoconditioning has been observed in many species (1, 4, 11, 13, 16). The mechanisms by which osmoconditioning allows to partly or completely restore viability and/or vigour in aged seeds are not fully understood but recent

studies provide clear evidence of repair processes taking place during the osmoconditioning treatment (1, 4). Under optimal osmoconditioning conditions, seeds are maintained artificially in phase II (i.e. lag phase) of water uptake. It has been hypothesized that the extension of phase II allows some repair mechanisms to occur (4). Strong evidence has been provided that DNA synthesis and repair, degradation of damaged rRNA and *de novo* synthesis of intact RNA, as well as enhancement of protein synthesis and of free radical scavenging take place during the osmoconditioning treatment (1, 4). One or several of these mechanisms could be involved in the beneficial effect of osmoconditioning treatment observed with cryopreserved seeds of *C. arabica*. Investigations are under way to determine if free radical production and lipid peroxidation occur during rehydration of cryopreserved *C. arabica* seeds and if post-thawing seed osmoconditioning treatments reduce or suppress these phenomena..

In conclusion, osmoconditioning is a simple technology which has allowed to dramatically enhance the efficiency of cryopreservation of *C. arabica* seeds. The results presented in this study open new perspectives for the cryopreservation of seeds of species, including many species of the genus *Coffea*, whose viability and vigour is negatively affected by cryopreservation.

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