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Induction of tolerance to desiccation and to subzero temperatures in embryos of recalcitrant seeds of inga[1]

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ABSTRACT – Drying, widely used for storing orthodox seeds for prolonged periods, cannot be applied to recalcitrant seeds, which are sensitive to desiccation. Thus, inclusion of species with recalcitrant seeds, like inga, in reforestation programs or even for commercial use has been hindered by the lack of technology that would allow storage of these seeds. The remaining option, cryopreservation, is a method of high cost that requires a high level of technology. Knowledge of the processes involved in sensitivity to desiccation continues to be a great challenge for the seed sector. The aim of this study was to analyze the effects of osmotic treatments on tolerance to desiccation and storage capacity of recalcitrant seeds of inga. Embryos were subjected to osmotic stresses with PEG solution and subjected to progressive drying processes. In another experiment, the effects of these solutions on embryo conservation during storage were analyzed. From the results, it may be concluded that incubation of embryos in a solution with -2.0 MPa increases their tolerance to desiccation.

Index terms: osmotic stress, *Inga vera*, drying, recalcitrant seeds.

Indução de tolerância à dessecação e à temperatura subzero em embriões de sementes recalcitrantes de ingá

RESUMO - A secagem, amplamente utilizada para armazenar sementes ortodoxas por períodos prolongados, não pode ser aplicada às recalcitrantes, que são sensíveis à dessecação. Assim, a inclusão, em programas de reflorestamento ou mesmo de uso comercial, de espécies com sementes recalcitrantes, como o ingá, tem sido dificultada pela falta de tecnologia que permita armazenar suas sementes, restando a conservação de embriões criopreservados, que é tecnologia de alto custo e requer elevado nível tecnológico. O conhecimento dos processos envolvidos na sensibilidade à dessecação continua sendo um grande desafio para a área das sementes. Neste trabalho, objetivou-se analisar os efeitos de tratamentos osmóticos sobre a tolerância à dessecação e capacidade de armazenamento de sementes recalcitrantes de ingá. Embriões foram submetidos a estresses osmóticos com solução de PEG e submetidos a secagens progressivas. Em outro experimento, analisaram-se os efeitos dessas soluções sobre a conservação dos embriões durante seu armazenamento. Os resultados permitiram concluir que a incubação de embriões em solução com -2,0 MPa aumenta sua tolerância à dessecação.

Termos para indexação: estresse osmótico, *Inga vera*, secagem, sementes recalcitrantes.

Introduction

The conventional method of seed conservation in germplasm banks, which includes dehydration to extremely low moisture levels (from 3% to 7%) and cold storage at temperatures below zero (near -20 °C), is based on a methodology developed for seeds of agricultural species wich are tolerant to desiccation, called orthodox seeds. It is evident that such a method is not suitable to seeds sensitive to desiccation, called recalcitrant seeds, and species with such seeds have been conserved by cryopreservation of embryos (Barbedo et al., 2013; Hay and Probert, 2013; Van Treuren et al., 2013; Walters et al., 2013).

Tolerance (or sensitivity) to desiccation has been intensely studied not only in seeds, but in diverse organisms (Alpert, 2005). However, up to now, studies have not been successful in the use of drying as a form of storage for recalcitrant seeds for prolonged periods (Barbedo et al., 2013). Studies also showed that a reduction in storage temperature may be important for conservation of these seeds. However, because of the need to maintain a high degree of hydration, these seeds are also intolerant to low temperatures (Hellmann et al., 2006;

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Bonjovani and Barbedo, 2008). Therefore, conservation of seeds sensitive to desiccation continues to be a great challenge and requires, moreover, better knowledge of the physiology of these seeds, both before and after they are dispersed.

Inga vera Willd. subsp. *affinis* (DC.) T. D. Penn., commonly known as inga, is a species whose seeds are among those most sensitive to desiccation and with the lowest capacity of storage, making them an interesting model for studies of this nature (Bilia et al., 2003). Initially, Bilia et al. (1998) observed that when these seeds have small reductions in moisture content (up to values of around 50%) and are stored in polyethylene bags at 10 °C, it is possible to conserve them for up to 60 days, a period four times greater than that observed under natural conditions. After that, Barbedo and Cicero (2000) observed that the application of ABA on these seeds may extend their storage capacity, even when their moisture content is not reduced. Andréo et al. (2006), for their part, observed that the maintenance of hydration of the embryos of these seeds at water potential of around -2.4 MPa allows their conservation, also at 10 °C, for at least 90 days. Also, Bonjovani and Barbedo (2008) observed that inga embryos subjected to controlled drying, bringing their water potential to values near -4.0 MPa, made them more tolerant to reduction in temperature. Nevertheless, Faria et al. (2006) showed that the responses of the inga seeds to small reductions in moisture content and to the applications of osmotic solutions or ABA are not always positive and, therefore, require more studies for an understanding of the mechanisms involved.

Studies on re-induction of tolerance to desiccation of germinating orthodox seeds also provide an interesting perspective for recalcitrant seeds. When orthodox seeds are placed to germinate, they proceed to lose tolerance to desiccation up to a point at which they become totally sensitive, similar to the behavior of recalcitrant seeds. However, studies have shown that some seeds may reacquire such tolerance if they are subjected to water stresses, normally carried out through polyethylene glycol (PEG) solutions of pre-established osmotic potential (Faria et al., 2005). This technique was also successfully used in induction of tolerance to desiccation in orthodox seeds of brazilwood (*Caesalpinia echinata* Lam.) in the stages in which they are sensitive to desiccation (Leduc et al., 2012). According to Barbedo et al. (2013), recalcitrant seeds may, in fact, be orthodox seeds that did not complete their maturation. Therefore, they could also have their tolerance to desiccation and to low temperatures increased, which was the goal of the present study.

Material and Methods

Obtaining the plant material: inga (*Inga vera* Willd. subsp. *affinis* (DC.) T.D. Pennington) fruits were collected in the months of February and March from around 20 mother plants planted in the area belonging to the Centro de Exposições Imigrantes, São Paulo, SP, Brazil (23°38'S, 46°37'W, 778 m altitude), and taken to the Laboratory of seeds of the Instituto de Botânica, São Paulo, SP, Brazil (23°37'S, 46°32'W), where they were manually opened to remove the seeds. They were separated into two stages of maturation (hereinafter called III and IV), corresponding to the stages described in Bonjovani and Barbedo (2008) and Caccere et al. (2013). After that, the entire sarcotesta (seed coat whose external epidermis forms a pulpy layer, according to Oliveira and Beltrati, 1993) was removed, obtaining the embryos used in the experiments. The embryos were then evaluated in regard to their moisture content, water potential, electrical conductivity of the imbibition solution (EC), germinative embryos, and germination, as described below.

Physical and physiological evaluations: the moisture content of the embryos (wet basis) was evaluated by the laboratory oven method at 103 ± 2 ºC for 17 h (Brasil, 2009). The water potential of the embryos was evaluated in a WP4 potentiometer (Dewpoint PotentiaMeter, Decagon). The sample is placed in a hermetically sealed chamber until it enters in hygroscopic equilibrium with the air. Then the temperature of the system is reduced until it reaches the dew point (identified by the deviation of a beam of ligth falling on a mirror), at which time the temperature of the air chamber is measured, obtaining the water potential of the system (Decagon Devices, 2000). To check the water potential, samples were incubated in PEG 6000 solutions with different osmotic potentials (Michel and Kaufmann, 1973).

The EC was evaluated in a benchtop conductivity meter Marconi MA-150 by immersion of 15 embryos in 75 mL of deionized water in 200 mL plastic cups. These immersed embryos remained within BOD incubator chambers with temperature regulated at a constant 20 ºC for 24 hours to later take readings (Barbedo and Cicero, 1998).

The germination test, with 20 embryos per replication, was carried out in a roll of paper, with two sheets for the base and another to cover, pre-moistened at the proportion of two and a half times the weight of the paper (Brasil, 2009), and placed in germination chambers (Marconi MA400), with internal water circulation, regulated to a constant temperature of 25 ºC and constant light (Lamarca et al., 2013). Evaluations were made every two days up to 20 days, with calculation of germinative embryos (i.e., those that exhibited primary root growth of at least 1 cm) and germination (embryos that developed seedlings, with a developed root system and eophylls without apparent defects). As the species produces

polyembryonic seeds, the embryos contained in each seed were kept together in all the evaluations. Even when there was protrusion of more than one primary root or the emergence of more than one seedling per embryo, only one root and/or seedling per embryo were registered (Bonjovani and Barbedo, 2008).

Water stress and tolerance to desiccation: stage IV embryos were used. After removal of the control sample (non-incubated embryos), the embryos were subjected to incubation in osmotic solutions at -1.5 and -2.0 MPa. To this end, PEG 6000 solutions were used, calculating the quantities by the formula of Michel and Kaufmann (1973). Incubation was carried out in plastic trays (26.5 x 19.5 x 5.0 cm) with a transparent base and opaque lid, with two sheets of germination paper for a base and one to cover. These sheets were previously moistened with 250 mL of the solutions. For incubation, the embryos were kept half submerged.

Lids were duly placed on the trays and the trays were kept within the BOD incubator chambers with constant temperature regulated to 20 ± 1 ºC for 24 h. At the end of incubation, the embryos were washed in running water for removal of the PEG solution from the surface and then dried.

After removal of a sample without drying, the rest of the embryos were subjected to six levels of drying in an air circulation laboratory oven at 30 ± 1 ºC, seeking to bring the embryos to moisture contents of 20%. To do so, based on the initial moisture content values of the embryos, samples were periodically removed and weighed until reaching fresh matter values near those desired. At the end of each stress and drying level, the moisture content of the embryos was checked gravimetrically by the laboratory oven method at 103 ± 2 ºC for 17 h, as described above.

Storage of embryos in osmotic solutions: embryos in stages III and IV were used. After removal of the control sample (without incubation in osmotic solution), the remaining embryos were divided into three groups and incubated in water and in osmotic solutions of -1.6 and -2.4 MPa, based on the results of the previous experiment. For this purpose, PEG 6000 solutions were used, calculating the quantities of PEG according to the formula described by Michel and Kaufmann (1973).

The embryos were incubated in a plastic tray (20.0 x 15.0 x 3.3 cm), with a transparent base and lid. For incubation, the embryos were kept submerged in 200 mL of solution, except for the embryos stored without solution. The embryos of the control were also placed in trays, but without water or osmotic solution. The trays of all the treatments were placed in 5 L transparent polyethylene bags (35.0 x 45.0 x 0.2 cm) and closed with adhesive tape. The trays were then kept in chambers with constant temperature regulated to -5, -2, and 6 ± 1 ºC for 30 days. At the end, the embryos were acclimated in the laboratory environment (25 ± 2 °C) for 24 h, for a total of 31 days of storage, and then washed in running water for removal of the PEG solution from the surface. The embryos were then once more evaluated as described above.

Experimental design and statistical analysis of the data: a completely randomized experimental design was used for the physiological evaluations, with four replications for all the tests of all the experiments in a 3 x 5 (incubation x drying) factorial arrangement for the first experiment, and a 2 x 4 x 3 (maturation stage x storage condition x storage temperature) factorial arrangement for the second experiment. The results obtained were analyzed by the F test, and the mean values were compared among themselves by the Tukey test at the level of 5% (Santana and Ranal, 2004).

Results and Discussion

Water stress and tolerance to desiccation: the embryos initially showed rather high moisture content (55.2%, Table 1). Embryos showed significant reduction in moisture content from the second to the last drying, initially to values near 40-45%, and then arriving at values near 20% in the last drying (Table 1). Drying of the embryos previously subjected to water stresses resulted in moisture contents near those not subjected to water stresses, except for those of the 2nd and 5th dryings subjected to -1.5 MPa. Through the water potential values (Table 1), these dryings led to modifications in the water properties, passing from level 5 hydration (Vertucci, 1993) to levels 4 (already in the first drying), 3 (after the 2nd, 3rd, and 4th dryings), and arriving at level 2 after the 5th and 6th dryings. In these last two dryings, apparently the embryos incubated at -1.5 MPa dried more than those not incubated or those incubated at -2.0 MPa. In the 5th drying, both the moisture content and the water potential of the former embryos were significantly lower than the latter; and in the 6th drying, although the moisture content was not significantly different, moisture was also more strongly retained in the embryos incubated at -1.5 MPa.

Embryos not subjected to stresses exhibited progressive reductions on germinability starting at the 2nd drying (Figure 1), i.e., when the moisture content decreased to 45% and the water potential to -4 MPa (Type 3 water, according to Vertucci, 1993). The embryos completely lost germinating ability after the 5th drying when the moisture content reached 31.2%, confirming the sensitivity of the seeds of this species, described in previous studies (Bilia et al., 1999; Bonjovani and Barbedo, 2008). At this level of drying, all the Type 3 water (< -11 MPa, Table 1) had been removed, corroborating the assertion of Vertucci (1993) that recalcitrant seeds frequently do not tolerate removal of this water. Curiously, though, the first drying resulted in a substantial increase in the germinating ability of the embryos, practically doubling the

number of germinative embryos and tripling the germination figures. This fact suggests that this mild drying led to an increase in the vigor of the embryos. This increase in vigor through mild drying resembles that observed by Bonjovani and Barbedo (2008) for embryos of the same species.

Table 1. Moisture content (% wet basis) and water potential (-MPa) of *Inga vera* ssp. *affinis* embryos subjected to different water stresses and different levels of drying.

Drying levels	Levels of stress*		
	Without stress	-1.5 MPa	-2.0 MPa
	Moisture content (%)		
Initial	55.2 aA	56.6 aA	53.8 aA
1st	52.1 aA	50.3 bA	53.7 aA
2nd	44.8 bAB	41.8 cB	45.7 bA
3rd	38.7 cA	36.0 dA	39.6 cA
4th	34.2 cdA	33.0 dA	34.9 cA
5th	31.2 dA	24.3 eB	29.3 dA
6th	22.4 eA	19.0 fA	22.6 eA
C.V. (%) = 5.6			
	Water potential (-MPa)		
Initial	1.5 eA	1.2 dA	1.3 dA
1st	3.3 deA	4.0 cdA	3.9 dA
2nd	6.1 cdeA	7.7 cdA	5.6 cdA
3rd	8.9 bcdA	9.7 cA	5.5 cdA
4th	10.9 bcA	9.0 cA	11.9 bcA
5th	15.7 bB	23.3 bA	13.9 bB
6th	30.1 aB	38.0 aA	25.2 aB
C.V. (%) = 29.8			

*Mean values followed by the same letter (lowercase letters in the columns, among drying levels; and uppercase letters in the rows, among stress levels) do not differ among themselves by the Tukey test at 5%.

The results of EC (Figure 1), which evaluate the integrity of the membranes Marcos–Filho (2005), showed the damages brought about by continuous removal of water. Up to the 2nd drying, when the highest values of germinative seeds and of germination were observed, the EC remained near 25 $\mu S.cm^{-1}.g^{-1}$, which characterizes high vigor embryos (Barbedo and Cicero, 1998). When there was the first fall in the percentage of germinative seeds (2nd drying), the EC rose to values near 50 $\mu S.cm^{-1}.g^{-1}$, characterizing seeds of medium physiological quality (Barbedo and Cicero, 1998). As of that point, the EC increased up to the last level of drying.

The initial stresses (-1.5 and -2.0 MPa) also led to an increase in the germinating ability of the embryos (Figure 1), similar to what occurred at the first level of drying, however, without there being a significant reduction in their moisture content or in their water potential (Table 1). Such results suggest that the increase in the vigor of the embryos is probably not exclusively due to removal of water, but to some physiological process of maturation. It is also noteworthy that the first level of drying of the embryos initially subjected to osmotic solutions also led to an increase in their germinating ability, especially in the production of normal seedlings (Figure 1), broadening the effect of the initial water stress.

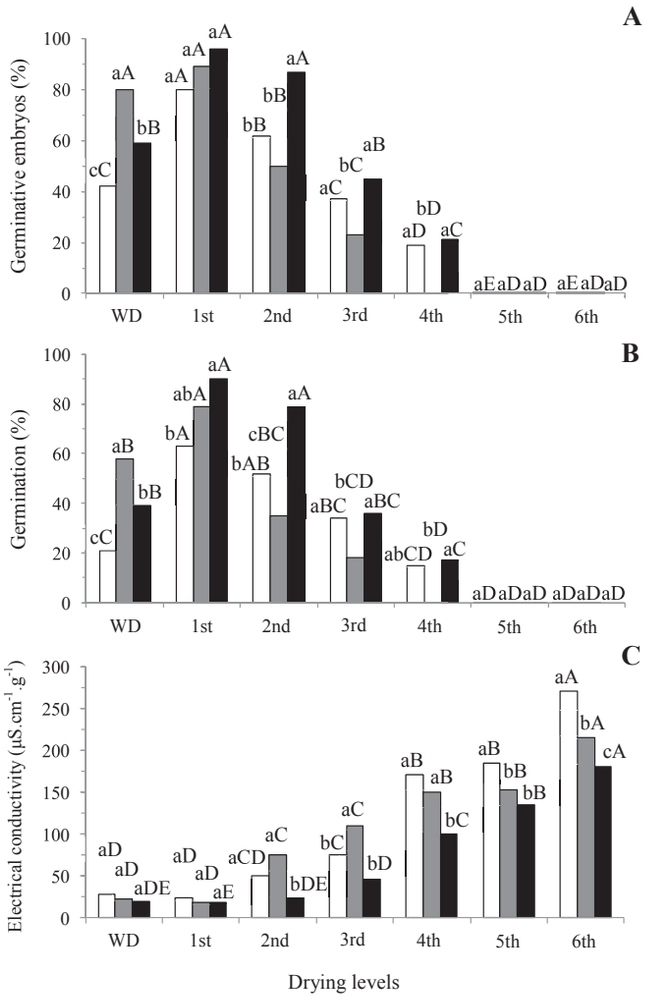

Figure 1. Germinative embryos (A), germination (B), and electrical conductivity (C) of *Inga vera* ssp. *affinis* embryos subjected to different water stresses (white column, without stress; gray column, -1.5 MPa; black column, -2.0 MPa) and different drying levels (WD: without drying). Mean values followed by the same letter (lowercase among stress levels and uppercase among drying levels) do not differ among themselves by the Tukey test at 5%.

Beginning at the 2nd drying, the embryos incubated at -1.5 MPa exhibited a fall in germinating ability in a similar or even more intense way than the non-incubated ones, also

with increasing values of EC (Figure 1). Nevertheless, the same result was not observed for embryos incubated at -2.0 MPa, which did not lose germinating ability in this second drying, when they reached 45.7% moisture and still exhibited 80% germination (90% germinative seeds). They began to lose viability only in the 3rd drying. The EC of the embryos incubated at -2.0 MPa remained practically unchanged up to the 2nd drying, showing that the embryos also maintained high viability. However, the incubation of the embryos at -2.0 MPa granted a small, but real, increase in their tolerance to desiccation. In germinating seeds, it was seen that the more advanced the germination process, the less efficient the re-induction of desiccation tolerance is (Faria et al., 2005).

Storage of embryos in osmotic solutions: the inga embryos used in this experiment, at the time of harvest, exhibited 58.7% and 54.7% moisture, corresponding to the two stages of maturation (stages III and IV) described for the species by Bonjovani and Barbedo (2008), with values similar to those of the final maturation stages of seeds of other species of *Inga*, like *I. striata* (Mata et al., 2013) and *I. laurina* (Shulz et al., 2014). After storage, the stage IV embryos, incubated without solution (control), exhibited variation of 5% in moisture content at temperatures from -2 to -5 °C, whereas the stage III embryos were lower when incubated at -5 °C (Table 2). Incubation in water at 6 °C, attempting to maintain the embryos hydrated, led to an increase in their moisture content, in contrast with those incubated in osmotic solutions (-1.6 MPa and -2.4 MPa) which, in general, showed a reduction the greater and the more negative the incubation solution (except for the stage IV embryos incubated at -1.6 MPa). Storage at negative temperatures, in general, led to reductions in moisture content, which were greater the more negative the temperature and the more negative the osmotic potential of incubation. Interestingly, though, water potential did not exhibit great variations or, at least, not from the magnitude observed in the previous experiments. For the embryos incubated in osmotic solutions, water potential remained between -2.4 and -4.2 MPa, i.e., within the range of level 4 water hydration (Vertucci, 1993). In other words, although the quantity of water of the embryos was significantly reduced, its activity did not change very much during storage. Andréo et al. (2006) observed that the incubation of inga embryos in PEG solutions of -1.6 and -2.4 MPa allowed maintenance of their moisture content with minimal variations, i.e., with small water mobilization between the embryos and the medium, which is probably what occurred to the embryos of the present study.

Table 2. Moisture content (%, wet basis) and water potential (-MPa) of *Inga vera* ssp. *affinis* embryos of two stages of maturity, incubated under different conditions (control, water, osmotic solution at -1.6 and at -2.4 MPa) and stored for 31 days at different temperatures (6, -2, and -5 °C).

Embryo maturity	Storage temperature (°C)	Storage conditions*			
		Control	Water	-1.6 MPa	-2.4 MPa
		Moisture content (%)			
III	6	60.2 aB	70.8 aA	56.7 aB	47.5 aC
	-2	58.9 aA	57.2 bAB	53.6 abB	46.9 aC
	-5	49.6 bB	58.0 bA	51.5 bB	42.1 bC
IV	6	54.5 abB	63.3 aA	56.2 aB	52.8 aB
	-2	57.1 aA	60.4 aA	52.7 aB	46.1 bC
	-5	52.1 bA	53.9 bA	40.5 bB	38.1 cB
C.V. (%) = 4.2					
		Water potential (-MPa)			
III	6	1.8 bC	1.4 aC	2.4 aB	3.2 aA
	-2	1.0 cB	1.3 aB	2.9 aA	3.2 aA
	-5	2.6 aA	1.4 aB	2.4 aA	2.7 aA
IV	6	2.6 aB	2.6 aB	3.0 aB	3.6 bA
	-2	2.6 aB	1.9 bC	2.9 aB	4.2 aA
	-5	2.4 aB	1.7 bC	3.2 aA	3.3 bA
C.V. (%) = 12.8					

*Mean values followed by the same letter (lowercase letter among storage temperatures and uppercase letters among incubation solutions) do not differ among themselves by the Tukey test at 5%.

The EC of the stage III embryos was initially 8.5 $\mu S.cm^{-1}.g^{-1}$. After storage for 31 days, it increased to values from 20 to 90 $\mu S.cm^{-1}.g^{-1}$. However, such an increase was significantly lower for embryos kept in water and in the PEG solution at -1.6

MPa at 6 °C, and in the PEG solution at -1.6 MPa at negative temperatures (Figure 2). As there was generalized death of these embryos (Figure 2A), the EC was no longer a good indicator of the physiological quality of the embryos. Yet the stage IV embryos maintained low values of EC at the temperatures of 6 and -2 °C (except for those kept in water at 6 °C). The temperature of -5 °C led to an increase in the EC of the embryos in all the treatments, probably as a result of the death of the embryos (Figure 2B).

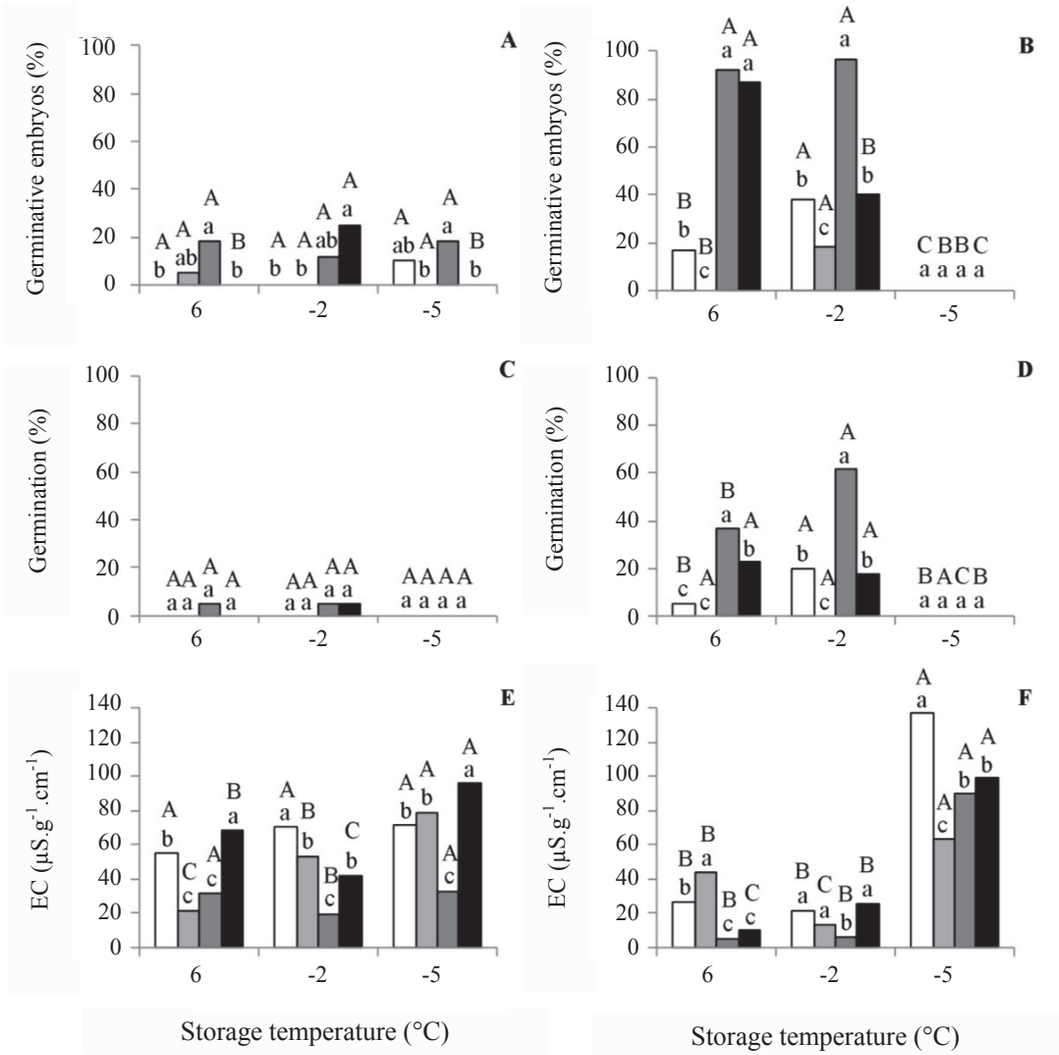

Figure 2. Germinative embryos (A and B), germination (C and D), and electrical conductivity - EC (E and F) of *Inga vera* ssp. *affinis* embryos of two stages of maturity, stage III (A, C, and E) and stage IV (B, D, and F), incubated under different conditions (white column, control; gray column, water; lead column, osmotic solution at –1.6 MPa; and black column at -2.4 MPa) and stored for 31 days at different temperatures (6, -2, and -5 °C). Mean values followed by the same letter (lowercase among incubation solutions, and uppercase among storage temperatures) do not differ among themselves by the Tukey test at 5%.

The stage III embryos in all the treatments exhibited substantial reduction in the values of germinative seeds and of germination after storage. These values that, initially, were 100% and 98%, respectively, decreased to less than 30% and 10% (Figure 2), once more showing the low storage capacity of immature seeds. The stage IV embryos depended on the storage treatment and on the temperature. The embryos without incubation or incubated in water exhibited expressive reduction in germinating ability at any temperature, but those incubated at -1.6 MPa maintained high values of germinative seeds and of germination, especially at -2 °C (Figure 2). Those incubated at -2.4 MPa exhibited intermediate values between

those incubated at -1.6 MPa and the others. Bonjovani and Barbedo (2008) observed that storage at -2 °C was lethal for the stage IV embryos, even after drying; therefore, different from what was observed in the present study. This fact may be related to the maintenance of the moisture content and water activity brought about by incubation in the osmotic solution of -1.6 MPa (Table 2).

The results obtained in the present study and those described in the literature suggest the possibility of obtaining better results in conservation of recalcitrant seeds of inga. For example, obtaining seeds in their more advanced degree of maturity, as suggested by Barbedo et al. (2013), associated with previous treatments of induction of tolerance to desiccation (described in the first experiment of this study) and mild drying (described by Bonjovani and Barbedo, 2008), followed by incubation of inga embryos in osmotic solutions (described by Andréo et al., 2006) and storage at -2 °C (described in the second experiment of this study) may be a useful combination for conservation of these embryos for periods greater than those obtained up to now.

These results evidently do not yet allow the consideration that recalcitrant seeds can achieve the same levels of desiccation tolerance of the orthodox ones. They also do not indicate that, in the near future, such seeds may be stored in germplasm banks, passing from the scale of weeks to that of years, as occurs with orthodox seeds. These seeds are dispersed with a quite accentuated metabolism and directed to rapid germination (Caccere et al., 2013) and, by their high moisture content, susceptible to the action of microorganisms (Parisi et al., 2013), i.e., conditions contrary to those desired for a more prolonged storage period. Once more, we see the need for comparative studies of maturation between recalcitrant and orthodox seeds, especially the immature phases of the latter, seeking to clarify the mechanisms involved in the process of separation of the seeds from the mother plant.

Conclusions

The incubation of embryos of recalcitrant seeds of *Inga vera* in a PEG solution at -2.0 MPa increases their tolerance to desiccation.

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