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Germination of stored and scarified seeds of Passiflora caerulea L. (Passifloraceae)

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Abstract
This work evaluates the influence of storage and scarification in the germination of Passiflora caerulea L., in order to acquire the necessary knowledge to cultivate this economically important species. Seeds stored one month under different conditions (no storage, fermentation, desiccation) were submitted to various pre-treatments (aril removal, mechanical and chemical scarification or combinations of these). Emergence was recorded periodically for 11 months in a greenhouse. Germination time, germination percentage and germination speed were calculated. Histochemical tests were applied to seeds maintained under the three storage conditions, for observing possible changes in the integument and storage tissue. Viability was maintained at least for the storage period tested, as germination occurred after that time. Because the seeds stored under desiccation germinated, the species is orthodox. Chemical scarification prevented germination in most cases. Although germination levels were low, they were higher in stored seeds (fermented and desiccated) than in fresh ones. Fermented seeds (which exhibited no storage tissue and less lignin in the integument) devoid of the aril germinated earlier, faster and in greater quantity. The type of dormancy of this species is discussed.

Keywords: Dormancy, germination, histochemistry, Passiflora caerulea, scarification, storage

Several factors affect seed germination. First of all, seeds must be viable, i.e. they must be capable of germination. Seeds must be placed in adequate environmental conditions (moisture, temperature and oxygen). Proper storage of seeds also influences germination. Finally, seeds must be able to overcome dormancy. If these conditions are met, germination may occur.

The seed is often well equipped to survive extended periods of unfavourable conditions, as the embryo is protected by one or several layers of different tissues. These include storage tissue, seed coats and fruit tissues; these surrounding layers play an important part in the regulation of germination (Smith et al. 2002). Many weed seeds possess dormancy mechanisms that may be responsible for long-term survival in the soil, and that allow germination only when conditions are favourable for establishment of a new plant (Ghersa & Roush 1993).

Seed dormancy could be considered as a block to the completion of germination of an intact viable seed under favourable conditions (Bewley 1997). Seed dormancy may be due to physical, physiological, and morphological causes or combinations of these (Finch-Savage & Leubner-Metzger 2006). Physical dormancy prevents imbibition due to the impermeability of the seed coat to water. Water impermeability, in turn, may be due to different physical and chemical characteristics of the seed coat (Werker 1997). To overcome this type of dormancy, scarification must be accomplished (Baskin & Baskin 1998). In nature, this includes desiccation, freezing–thawing, fire, highly fluctuating temperatures and passage through an animal's digestive tract (Baskin et al. 2000). Microbial attack also seems to soften

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hard coats (Rolston 1978). Reduction of the seed coat impermeability due to pectins by pectinase from fungi was reported for Gymnocladus dioica (Werker 1997).

Some Passiflora species exhibit dormancy (ISTA 1985; Delanoy et al. 2006). Based on seed coat anatomy studied by other authors, Baskin et al. (2000) suggested that physical dormancy may occur in this family. Usually, untreated seeds of various passion vines require from two weeks to several months to germinate, depending on the species (Purseglove 1979; Correa 1996; Maciel et al. 1997; Vanderplank 1997; McGuire 1998). Treatments applied to seeds of Passiflora species include aril removal, desiccation, storage for several months (Purseglove 1979), soaking in water for different numbers of hours (McGuire 1998; Delanoy et al. 2006), various constant temperature and light conditions (Benvenuti et al. 2001), fermentation (Duarte & Marín 2002), and immersion in gibberellin (Ferreira et al. 2005). Correa (1996) reported that Passiflora spp. seeds remain viable for 12 months, and that germination of P. mollissima is enhanced when the seeds are stored for two months.

Impermeability to water and, consequently, the general seed coat structure, is usually specific of each family (Werker 1997). However, there may be differences within even lower taxonomic levels. Galusi et al. (2005) encountered morpho-physiological differences (including organic and inorganic components and testa anatomy) between varieties of the same species. Hence, the need to study and compare species from a single genus and infra-species categories.

A previous study in Passiflora caerulea L. evidenced low germination levels, slow germination speed and an imbibition period of two to five months, irrespective of the pre-treatments applied to fresh seeds, which suggested the existence of dormancy in this species (Mendiondo & Amela García 2006). Despite its edible (Arenas 1983), foraging (Alonso 2004) and ornamental (Bailey 1941) values, the most important use of P. caerulea is medicinal (Alonso 2004). Several trademarks of herbal teas and dietary supplements employ this species as raw material. Information about cultivation is hard to get, as most laboratories do not supply information regarding the source of their products, which suggests that it is harvested directly from the wild; as far as it is known one laboratory does so (Ferres, personal communication). Considering that the number of laboratories devoted to products based on this species is increasing, natural populations, abundant in the past at least in the Buenos Aires area, may be threatened. Attempts to cultivate the species have been initiated (Traverso, personal communication), and showed slow germination.

This work examines the effects of storage and scarification on the germination pattern of this promising crop, as well as the type (or combination of types) of dormancy it possesses.

Material and methods

Germination assays

A germination phenology study, as the one described by Baskin and Baskin (1998), was performed. The experiment was undertaken between November 2002 and December 2003. Eighty-nine physiologically ripe fruits were collected from 18 healthy plants that naturally grow on the campus of the Facultad de Ciencias Exactas y Naturales of Universidad de Buenos Aires, Argentina. The homogeneous lot of seeds was divided in three sets: the first one was sown immediately (fresh, no storage), the remaining two were stored for one month under different conditions (fermentation in air-tight containers in a refrigerator and desiccation at room temperature). Before sowing, all seeds were treated as explained in Table I, to simulate the corresponding possible natural outcomes in this ornithocorous species (Mendiondo & Amela García 2006).

Mechanical scarification was performed with sandpaper for wood and chemical scarification was

Table I. Treatments applied to P. caerulea seeds.

	Treatment	Simulation
A	With aril	Fallen seed
B	Without aril	Aril removed by a bird
C	Without aril + mechanical scarification	Aril removed by a bird + seed coat damage by bill or grid
D	Without aril + chemical scarification 10 min	Aril removed by a bird + retention in digestive tract (considering time)
E	Without aril + chemical scarification 10 min + hot water 15 min	Aril removed by a bird + retention in digestive tract (considering time and temperature)
F	Without aril + mechanical scarification + chemical scarification 10 min + hot water 15 min	Aril removed by a bird + seed coat damage by bill or grid + retention in digestive tract (considering time and temperature)

carried out with pure hydrochloric acid. Water was heated to 36 °C. The seeds were sown in plastic divided trays filled with soil:perlite (3:1) with one seed per cell. The treatment distribution was randomly assigned. The trays were placed inside a greenhouse, under natural photoperiod, where ambient temperature and relative humidity (recorded with a digital thermohygrometer), varied from 12 to 42 °C and 43 to 99%, respectively. The soil was watered periodically with tap water. Seedling emergence was registered daily. The germination technological criterion recommended by ISTA (International Seeds Testing Association) cited in Labouriau (1983) was used: "the emergence or development, from the seed embryo, of the essential structures that, for the seed species in question, indicate its capacity for producing normal plants in favourable conditions". The following parameters were obtained for each treatment: T_0 (germination starting time): days from sowing to germination of the first seedling (Labouriau 1983); P (germinability): germination percentage; V (vigour): estimated by the germination speed, which is defined by V = (a/1 + b/2…x/n)*100/S, where 'a, b, …, x' represent the quantity of seeds that germinated after '1, 2, …n' days since imbibition and 'S' is the total of the seeds sown. The resultant values of P and V were assigned to the categories that appear in López et al. (1999).

A frequency analysis with contingency tables was applied to the data. Posteriori contrasts were performed as needed, using the partition method by the degrees of freedom in r × 2 tables (Siegel & Castellan 1988). Percentages are shown in the text, for comparative purposes.

Histochemical assays

To determine the main storage substances in the storage tissue, longitudinal sections were performed to fresh seeds, and iodine solution, sudan IV and picric acid were applied to test for starch, lipids and proteins, respectively (Johansen 1940).

To look for possible changes in the seed coat composition, histological slides were prepared with the seeds that had been subjected to different storage conditions. In all cases a cut was made in the seed coat, after which the seeds were submerged in pure hydrochloric acid for four days, with the aim to soften the integument, and to extract most of the storage components present, and thus to be able to perform the sections. Afterwards, they were rinsed and fixed in alcohol 70%. Two types of inclusions were carried out, one in resin and the other in paraffin.

Inclusion in resin. The seeds were refixed in 20% glutaraldehyde in a phosphate buffer (pH 7.2) for 24 h, and post-fixed in OsO_4 at 2 °C in the same buffer for 3 h, dehydrated in an ethanol series and embedded in Spurr's resin. Sections (1 μm thick) were made with an ultramicrotome, stained with toluidine blue, and observed with an optical microscope to search for differences in cell wall composition: primary pectic walls stain purplish-pink; secondary lignified walls stain blue.

Inclusion in paraffin. The seeds were dehydrated in an ascendant alcohol series (ethanol–xylol) and slowly infiltrated with paraffin. Sections (10 μm thick) were made with a Minot type rotative microtome; the sections were mounted on slides and paraffin was removed with a descendant alcohol series (xylol–ethanol–water). Microchemical tests were performed to detect the following compounds related to water impermeability (Werker 1997): lignin, fats and oils, and tannins with floroglucine, sudan IV, 10% ferric chloride and sodium carbonate, respectively (D'Ambrogio de Argueso 1986). Lignin stains violet red, fats and oils stain red, and tannins stain greenish blue. The reactions were compared with control sections mounted in water.

Results

Germination assays

Values of the recorded parameters are shown in Table II. The germination percentage was (1) low with the aril (A) under all storage conditions, (2) moderate without the aril (B), and with the aril and mechanical scarification (C) (except for fresh seeds in B, in which it was low), (3) null without the aril plus chemical scarification for 10 min (D), without the aril plus chemical scarification plus hot water (E), and without the aril plus mechanical scarification plus chemical scarification + hot water (F) (except for fermentation, in which it was low) (Table II).

In fresh seeds, removal of the aril and mechanical scarification (C) resulted in a significantly higher germination percentage than with the other treatments (Table II).

After fermentation, higher germination was observed in seeds without the aril, while no significant differences were observed in seeds with the aril (A) and in seeds without the aril plus mechanical scarification (C) (Table II). Germination percentage without the aril plus mechanical scarification plus chemical scarification for 10 min plus hot water (F) was significantly different from the rest of the treatments, but it was still low.

After desiccation, the germination percentages of treatments without the aril (B), and without the aril and mechanical scarification (C) were significantly greater than with the aril (A) (Table II).

Table II. Germination values obtained after the different pre-treatments and storage conditions.

Storage condition	No storage			Fermentation			Desiccation		
Treatment	P	T_0	V	P	T_0	V	P	T_0	V
A	6 a	82	0.07	25 c	20	1.57	25 b	20	1.92
B	2 a	92	0.02	60 d	15	9.16	50 c	19	4
C	34 b	59	0.26	33 c	17	5.20	46 c	19	2.2
D	0 a	0	0	0 a	0	0	0 a	0	0
E	0 a	0	0	0 a	0	0	0 a	0	0
F	0 a	0	0	2 b	22	0.38	0 a	0	0
χ_i^2		21.36			99.70			50.89	

P, Germinability (germination %); T_0, Germination starting time; V, Vigour (germination speed). Values with different letters differ significantly within each storage condition ($p < 0.01$). N = 732 seeds: fresh = 300, fermented = 288, dried = 144 (50, 72 and 72 seeds, respectively, for each treatment).

The highest germination percentage was obtained with the seeds after fermentation and without the aril (Table II).

The earliest germination starting time (T_0) occurred 15 days after sowing in fermented seeds without the aril (Table II), while the latest occurred 92 days after sowing in fresh seeds without the aril. For all the successful storage treatments T_0 took place in the third week after sowing, in contrast to fresh seeds, which began germination after two, or even three, months.

The germination speed was low for all types of storage in treatment A and for fresh and dry seeds in treatments B and C, moderate for fermented seeds in treatments B and C, null for all types of storage in treatments D–F, except for fermented seeds in treatment F in which it was slow (Table II).

All the cumulative germination curves showed an imbibition phase of at least two months followed by a gradual increase until a steady speed was reached (Figure 1). The imbibition phase was delayed by one month for treatments A and B in fresh seeds. The increase lasted one or two months, except in fresh seeds without the aril and mechanical scarification, in which it was extended for eight months.

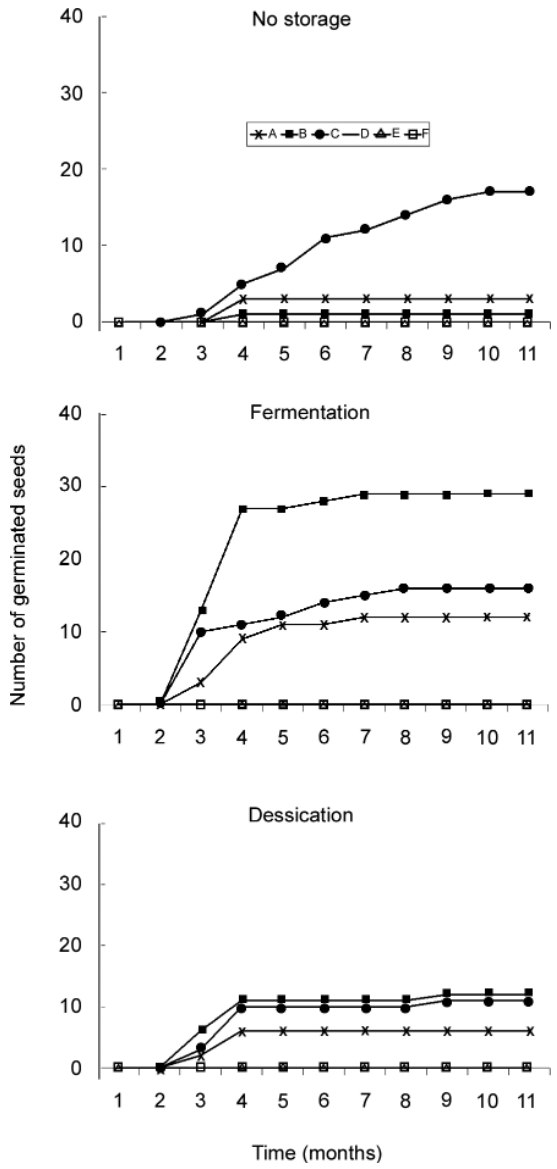

Figure 1. Cumulative germination according to treatments: A = with aril; B = without aril; C = without aril + mechanical scarification; D = without aril + chemical scarification 10 min; E = without aril + chemical scarification 10 min + hot water; F = without aril + mechanical scarification + chemical scarification 10 min + hot water.

Histochemical analyses

The storage tissue stained positive mainly for proteins and only slightly for lipids. In spite of the cut performed to the seed coat and the immersion in hydrochloric acid fresh seeds still exhibited a certain amount of storage substances, whereas the stored ones (either fermented or desiccated) had no such cellular content (Figure 2(A), 2(C) and 2(E)).

The seed integuments under different storage conditions showed different staining reactions with toluidine blue. The fresh ones were blue; the fermented ones were purplish-pink in the basal zone and blue in the rest; the dried ones showed a lesser

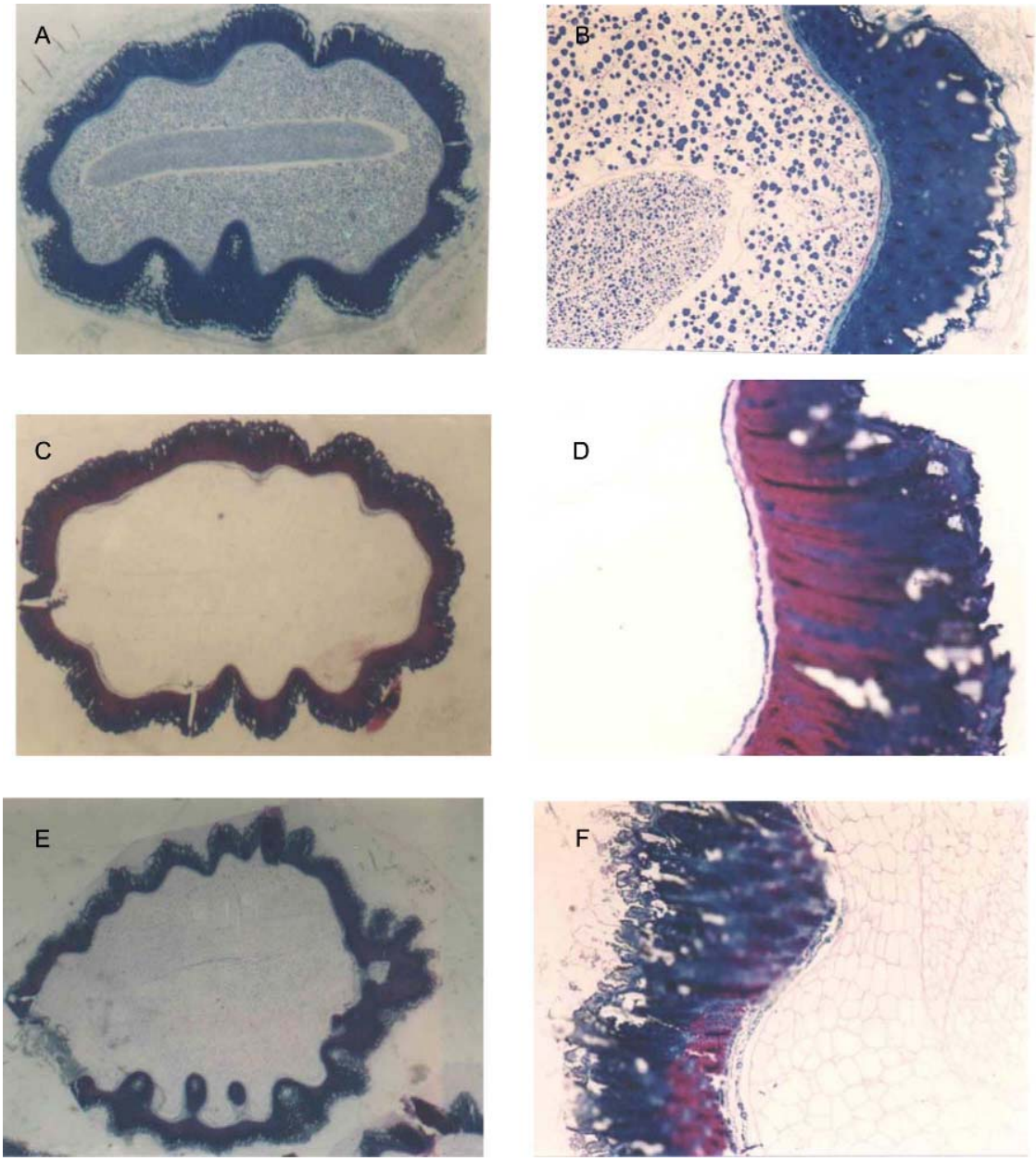

Figure 2. Transverse sections of seeds subjected to different storage conditions and stained with toluidine blue: (A, B) no storage; (C, D) fermentation; (E, F) desiccation; (A, C, E) view of storage tissue; (B, D, F) detail of seed coat.

proportion of purplish-pink colour than the fermented ones in the basal region and blue in the rest. This indicated that the fresh ones had more lignified material than the dried seeds, and these more than the fermented ones; concomitantly, the disappearance of lignin made the pectic substances visible (Figure 2(B), 2(D) and 2(F)).

The histochemical reactions showed a lower amount of lignin in the cell walls of the integument of the fermented seeds than in those of the fresh and dried ones, but the opposite occurred for tannins and fats (Table III).

Discussion

Appearance time of digesta varies between avian taxa and diet. As P. caerulea berries are eaten at least by Tyranidae, Turdidae and Mimidae (Mendiondo & Amela García 2006), we simulated the retention time in the digestive tract of berry-eating birds based on Hoyo et al. (1992) and Montaldo (2005); the latter author recorded the time in two of the three species involved in P. caerulea dispersion. Nevertheless, nearly null germination was obtained by chemical scarification, which may be due to

Table III. Results from the histochemical tests applied to the integument of seeds stored under different conditions.

Tested substance (testing method)	No storage	Fermentation	Desiccation
Lignin (toluidine blue)	+++	+	++
Lignin (floroglucin)	+++	+	+++
Fats (sudan IV)	+	+++	+
Tannins (ferric chloride 10%, sodium carbonate)	+	+++	+

failure in choosing the appropriate acid concentration. No data could be found on the degree of acidity of avian digestive tracts. On the contrary, Schoniger (1974–76) obtained germination for P. mollissima, whose rotten fruits are ingested by a bird (Lewin & Lewin 1984), with a more diluted solution of the same acid. The immersion time employed resembled the retention time for granivorous Passeriforms that were given cereals reported by Karasov (1990). Thus, acidity seems to be a more important factor than the time spent inside the digestive tract.

Considering that germination was obtained from dried seeds, it seems that the diaspores of P. caerulea are orthodox, a condition also referred for P. edulis, P. ligularis, P. maliformis and P. quadrangularis (ISTA 1985).

Control treatments made to P. caerulea yielded low germination percentages, as reported by Delanoy et al. (2006) for three Passiflora species. In both works, a single satisfactory treatment could not be encountered. This may be related to the fact that more than one factor is responsible for the primary dormancy mechanism (see below).

Ferreira et al. (2005) obtained higher germination levels when the aril was removed and gibberellin was added to the substrate. Mendiondo and Amela García (2006) obtained the highest germination percentages and the lowest T_0 when the seeds were devoid of the aril and soaked in water at room temperature for 24 h. In this work, germination was enhanced when the aril was removed in seeds stored under fermentation for two months. The aril might act like the mucilage in other seeds, in which it constitutes a barrier against oxygen (Werker 1997). Another possibility might be the inhibition of germination that the osmotic potential of this tissue could impose to the seeds. Welbaum and Bradford (1990) and Berry and Bewley (1992) demonstrated that precocious germination is prevented by low osmotic potential of the surrounding fruit tissues in musk melon and tomato. This may be a common phenomenon in berries (which is the case of tomato and most passion vines), a type of fruit in which the seeds, in spite of being immersed in a liquid medium propitious for germination, do not germinate.

The significant difference between the germinability of the fresh seeds in which the aril was removed and mechanical scarification was applied (treatment C), in contrast to seeds with and without aril (A and B), suggests that the restriction to germination is in the seed coat. Nevertheless, if attention is paid to the stored seeds, germination levels were significantly different from the controls (arilate seeds) when treatment B was applied, for both storage conditions, and also in treatment C (desiccation). It is thus apparent that in stored seeds mechanical scarification would not be necessary. Germination levels of the stored seeds were higher than the ones of the seeds without storage, both with and without aril (treatments A and B), suggesting that the storage time or storage conditions made possible changes of certain factors in the integument or in the storage tissue. This is apparent in seeds tolerant to desiccation, although the changes that take place during after-ripening have not been understood yet (Bewley & Black 1994). The fact that the differences were not so great between fresh and stored seeds after mechanical scarification (treatment C) would constitute an additional indication that the restriction is in the seed coat of P. caerulea. Poljakoff-Mayber et al. (1992) found that the release from dormancy with increasing storage time in a Malvaceae species was not accompanied by decreased seed coat resistance to pressure.

The presence of lignin, fats and oils and tannins would confirm that these seeds have a physical dormancy, as these substances impregnating the palisade cells are responsible for water impermeability (Baskin et al. 2000). The palisade cells are present in most hard-seeded species (Baskin et al. 2000). Although the seed anatomy of P. caerulea has not been studied yet, the co-generic species so far examined exhibit these kinds of cells, and the genus seems rather uniform in this aspect (Corner 1976). Besides, a frequent correlation between the dark colour of seeds and impermeability (Werker 1997) would support the water repellence in this obscure seed-coated species.

The permanence of part of the storage compounds in the non-stored seeds and their complete disappearance in the stored ones (either fermented or desiccated) suggests that the immersion in acid only partially removed them. The consumption of the rest in the stored seeds might be due to the fungal activity in the fermented ones, and

to the maturation-drying process in the desiccated ones.

Cardoso et al. (2001) sowed arilated seeds that had been kept under fermentation, although only for three to six days, obtaining earlier germination and greater vigour than from non-fermented ones. Pereira and Dias (2000) also obtained better results from fermented seeds. According to Morton (1987) germination speed increases if the aril is left to ferment for some days. In P. caerulea, the varied colourations with toluidine blue staining in the seeds stored under different conditions would indicate a differential presence of compounds in the integument cell walls, which was corroborated by the lower reaction for lignin in the fermented seeds. Fermentation, then, seems to make the seed coat more permeable, by degradation of certain cell walls substances (depending on the trophic characteristics of the attacking fungi), thus allowing imbibition and, consequently, germination.

If only the storage time is considered, Salinero Corral et al. (1993) reported that the highest germination percentages of P. edulis are obtained when the seeds are sown immediately after collection; thus, these seeds would be non-dormant. Nevertheless, data are contradictory for this species (ISTA 1985). In P. caerulea, in general, germination percentages increased when the seeds were stored for 60 days (this work) or 30 days (Mendiondo 2004), even without mechanical scarification, both under fermentation and desiccation conditions. After-ripening in dry storage can break non-deep physiological dormancy (Finch-Savage & Leubner-Metzger 2006). It is then possible that dormancy would also be due to the hardness of the integument plus immaturity of the embryo, in which case, it would be physiological, as the embryo seemed to be completely developed in the sections observed. The values of T_0 would also suggest embryo immaturity, as if the storage time is added to the T_0 values of stored seed, similar figures are obtained to the ones of the fresh diaspores. Thus, P. caerulea would possess combinational dormancy (physical plus physiological), one of the types reviewed by Finch-Savage and Leubner-Metzger (2006). To probe this, excision of the embryo must be carried out and the production of normal seedlings must be verified.

Although the present experiment lasted more months than the one of Delanoy et al. (2006), the germination curves of the three species tested by those authors were sigmoid, as the ones obtained here, under all three storage conditions, and for most of the treatments in both works. This general pattern was observed in other experiments involving P. caerulea (Mendiondo & Amela García 2006). Nevertheless, a slight variant appeared: in the present experiment (which lasted 11 months), a simple sigmoid pattern became evident, while in the one of Mendiondo and Amela García (2006) (which lasted 16 months) an oscillating sigmoid pattern was revealed. This may be due to various natural germination flushes, as occurs in most plants in temperate climates (Rathcke & Lacey 1985). Hardseededness is related to germination by degrees in seed banks (Leck et al. 1989, cited in Devesa et al. 1998). Hence, there is the need to perform longer studies if the ecological pattern is to be established.

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