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Seed germination of the wild banana *Musa ornata* (Musaceae)

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Summary

Musa ornata is the only wild species representing the Musaceae family in Mexico. It has ornamental value and is an important phytogenetic resource. Nevertheless, their populations are threatened. To provide tools for its conservation and management, we evaluated the effect of different treatments on seed germination *in vitro*. Fresh seeds and seeds that were stored for two or four months were used. Treatments were: exposure of endosperm, mechanical scarification, immersion in gibberellic acid, excised zygotic embryos and chemical scarification with sulphuric acid (H_2SO_4) for different immersion periods. Germination occurred only with excised zygotic embryos or following chemical scarification. In the case of excised zygotic embryos, germination was ≥ 90% after 21 days, with significant seedling development in both fresh and stored seeds. Seed immersion in H_2SO_4 for five minutes resulted in 16% germination after 90 days. Plants obtained *in vitro* were cultivated *ex vitro* in a greenhouse. There was 100% survival. Factors affecting seed germination included fungal contamination, hardness and resistance of testa, and storage.

Introduction

Musa L. comprises about 65 species known as plantains and bananas, important global food crops (Simmonds, 1966; Berrie, 1997). Bananas are the fourth main staple globally and the principal food for 400 million people (Nyine and Pillay, 2007). However, due to the low genetic diversity of cultivated varieties, they are susceptible to pests and diseases (e.g. Black Sigatoka) (Orozco-Santos and Orozco-Romero, 2004). This has caused production to diminish at an accelerating rate (Nyine and Pilay, 2007; Pearce, 2008). Consequently, banana producers are under increasing pressure to produce disease-resistant varieties and fertile seed germplasm is required for breeding programmes to improve edible bananas. Despite this, most studies focus on edible varieties (Wong *et al*., 2002; Ude *et al*., 2003), whereas the wild species have attracted little attention, as is the case of *Musa ornata* Robx. (Cheesman, 1931; Cheesman, 1949; Sundararaj

and Balasubramanyam, 1971; Burgos-Hernández *et al*., 2013) and only two deal with *in vitro* propagation (Cronauer-Mitra and Krikorian, 1988; Kottackal, 2005). It has been observed that this species is relatively free of diseases that affect edible varieties (Kottackal, 2005). This characteristic gives it great importance, because it represents a gene pool for breeding new commercial varieties and improved landraces.

In the case of the genus *Musa*, *in vitro* propagation methods have been mainly asexual (e.g. Gübbük and Pekmezci, 2004; Martin *et al*., 2006; Suárez-Castella *et al*., 2012; Rahman *et al*., 2013), thus, cultivars are produced quickly whilst preserving commercially advantageous features. However, the use of seeds is preferable for conserving genetic diversity (Fay, 1992). Little is known about the factors affecting seed germination in *Musa*; however, it appears to be extremely variable and relatively difficult to achieve (Stotzky *et al*., 1962; Chin, 1996). It has also been observed that germination of intact *Musa* seeds either fails to occur, is variable, erratic or of low percentage (Cox *et al.,* 1960; Purseglove, 1972; De Langhe, 2009). For example, seeds from *M. violascens* Ridl. that were sown immediately after being harvested, registered 96% germination (Chin, 1996), whereas germination of *M. balbisiana* Colla. seeds in greenhouse ranged between 90% in the summer months to near 0% in winter (Stotzky *et al*., 1962). Other studies have demonstrated high values for germination using zygotic embryos (Cox *et al*., 1960; Afele and De Langhe, 1991; Asif *et al*., 2001). Yet, in other cases, the use of embryos has not been successful (Vuylsteke *et al*., 1990). Clearly, it is not yet fully understood what factors are involved in seed germination of the *Musa* genus, and it has not been possible to standardise a protocol. The present study is aimed to contribute to the knowledge of *Musa* seed germination by investigating the effect of different treatments on the germination of *M. ornata* seeds sown *in vitro* and their subsequent survival in greenhouse conditions.

Materials and methods

Seed collection

Seeds were collected from a conserved population of *M. ornata* in the municipality of Teapa in the southern state of Tabasco, Mexico (N 17° 31'30.5"; W 92° 53'42.8"; 400 masl) during the months of April and May 2011. Intact fruits were collected (Gold *et al.*, 2004) and morphological indicators such as pericarp colour, texture and pulp odour were used to identify the dispersion phase of the plant (Brown and Briggs, 1991). A third of the collected seeds were processed immediately after harvest. The remainder was dried at room temperature (22 ± 2°C) for two days. After this period, half of the seeds were stored for two months and the rest for four months, at 10% relative humidity (RH) and 4 ± 2°C.

Seed sterilisation

Seeds were washed using distilled water and commercial soap for 15 minutes. They were then sterilised by immersion in 3% hydrogen peroxide (pharmaceutical grade; 1 L oxygen volumes; Kurak®) for 10 minutes, 70% ethanol (v/v) for two minutes, followed by 30 minutes in 30% commercial chlorine (v/v) (sodium hypochlorite, 6% active chlorine) supplemented with Tween®80 (two drops per 100 ml) for 30 minutes. Seeds were then

placed in a fungicide solution (Captan® 3 g L^{-1}) for 24 hours. After this period, there was further sterilisation with 30% commercial chlorine. Finally, the seeds were rinsed three times with sterile distilled water under aseptic conditions to remove any chemical residue (Mata-Rosas *et al*., 2011).

Culture medium

The basal culture medium used for the *in vitro* germination of *M. ornata* seeds consisted of MS medium at 50% of its components (MS 50%) (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} sucrose. The pH was adjusted to 5.8 (± 0.1) with 0.1 M HCl and 0.1 M NaOH prior to the addition of 6 g L^{-1} agar (Plant TC/micropropagation grade; Phytotechnology Laboratorios®). 25 ml of medium was poured into 125-ml baby food jars which were then sterilised in an autoclave at 121°C for 20 minutes.

Treatments

Based on the protocols from other studies carried out on *Musa* (Stotzky *et al*., 1962; Pancholi *et al*., 1995), the following treatments were applied to fresh seeds as well as those that had been stored for two and four months:

1) Control. Surface sterilisation of intact seeds only.
2) Excised zygotic embryos. Seeds were cut longitudinally under aseptic conditions to remove the embryo.
3) Endosperm exposure. A lateral portion of the testa, equivalent to 2 mm, was removed (length of *M. ornata* seeds ranged from 4 to 5 mm).
4) Mechanical scarification. Rubbing of the seed testa with fine grade sandpaper.
5) Chemical scarification. Seeds were immersed in concentrated sulphuric acid (H_2SO_4) for 0, 1, 2, 5, 10, 15, 20, 25, 30, 40, 50 or 60 minutes. Seeds were subsequently rinsed three times with distilled water.
6) Immersion in gibberellic acid (GA_3). Seeds were submerged for 24 hours in 0, 50, 100, 150, 200 or 250 mg L^{-1} GA_3 solutions, previously sterilised by filtration.

All treatments consisted of 30 repetitions of five seeds each. The jars were placed in a growth chamber at 25 ± 1°C with a photoperiod of 16 hours light (white fluorescent lamps; 50 µmol m^{-2} s^{-1}), 8 hours dark. A seed was considered germinated once the radicle was visible. Weekly records of seed germination were taken.

Seeds that did not germinate after four months were observed under the microscope and assessed for viability in order to identify the causes of germination failure. For this, embryos were immersed in a solution of 0.5% 2,3,5-triphenyltetrazolium chloride (TTC) for 24 hours at room temperature (22 ± 2°C) in the dark (Wharton, 1955)

Data about *in vitro* seedling growth were recorded one month after registering the highest germination percentage for each treatment. These data included: number of leaves, height (mm), diameter (mm), number and length (mm) of roots.

Ex vitro cultivation

Twenty seedlings germinated *in vitro* were taken at random, 10 from each treatment and rinsed with tap water to remove any adhering medium. The seedlings were transplanted into sterile soil mix consisting of black soil, leaf mould, earthworm compost (vermicompost®) and tepezil (volcanic pumice gravel) at a ratio of 1:1:1:1 held in a plastic tray 320 mm L × 450 mm W × 100 mm D and placed in a greenhouse with an average temperature of 30°C.

During the first 20 days, with the aid of a translucent plastic cover, RH was maintained at 80 to 90%. Subsequently, the lid was gradually removed to reduce RH to between 50 and 60%. Finally, the plants were placed individually in black plastic nursery bags and cultivated in the greenhouse.

Throughout one year, monthly data were recorded taking into account number of leaves, height (mm) and stem diameter (mm). Additionally, at the beginning and during the final period of observation, data on number and length (mm) of roots were recorded.

Data analysis

The percentage seed germination was obtained and the development of seedlings *in vitro* and *ex vitro* (height, diameter, number and length of roots), was analysed by one-way ANOVA. Normality of data was assessed by the Shapiro-Wilk test, using the statistical XLSTAT complement to Excel v. 2007. Data that did not conform to perceived normality were transformed by logarithm and square root to validate the ANOVA. For percentages, arcsine transformation was used. Means were discriminated by applying a Tukey multiple range, using the statistical package Statistical v. 6. Data referring to plants cultivated *ex vitro* were analysed using descriptive statistics.

Results

Seed germination

Although carefully disinfected and treated with fungicide, seeds treated by mechanical scarification, exposure of endosperm or immersion in GA_3 were infected with a very persistent fungal species. The morphological characteristics of the fungus, especially the spores, made it possible to identify this as a species of *Botryodiplodia* Sacc. Infection always occurred during the first week after cultivation, both in the case of fresh and stored seeds. Because of this, it was not possible to evaluate the effectiveness of these treatments for germination. 95% of the control treatment was contaminated, whereas when chemical scarification was used, contamination was only 10% (data not shown).

Seeds from the control treatment, which were not contaminated, did not germinate (table 1). In contrast, in the treatment with excised zygotic embryos, germination was evident seven days after cultivation for both fresh seeds and those that had been stored for two months (figure 1A). The first sign was a change in embryo colour from white to yellowish and after nine days, the radicle began to develop. The maximum germination was at 21 days, with 92% germination for fresh seeds and 90% for stored seeds.

Table 1. Effect of different treatments on the germination of *Musa ornata* seeds and *in vitro* development of the seedlings after one month of the maximum germination percentage in each treatment.

Treatments	Germination (%)	Number of leaves†	Height (mm)†	Stem diameter (mm)†	Number of roots†	Root length (mm)†
Control	0d	0d	0c	0.0d	0d	00.0d
Excised zygotic embryo (SF)	92[a]	4 ± 0.7a	60 ± 3.0a	5.0 ± 0.2a	5 ± 0.6a	50.0 ± 2.4b
Excised zygotic embryo (S2)	90[a]	4 ± 0.7a	58 ± 1.0a	5.0 ± 0.1a	4.5 ± 0.6a	50.0 ± 1.4b
H_2SO_4 (1)	8c	2 ± 0.6c	18 ± 6.6c	3.0 ± 0.1c	0d	00.0d
H_2SO_4 (2)	12b	2 ± 0.6c	24 ± 4.9c	3.0 ± 0.0c	2 ± 0.6c	10.0 ± 0.8c
H_2SO_4 (5)	16b	3 ± 0.7b	48 ± 1.7bc	4.0 ± 0.1b	3 ± 0.4b	25.0 ± 1.2b
H_2SO_4 (10)	4c	3 ± 0.6b	50 ± 1.7b	4.0 ± 0.2b	3 ± 0.3b	25.6 ± 5.2b
H_2SO_4 (15)	4c	2 ± 0.6c	40 ± 1.0c	3.5 ± 0.3bc	3 ± 0.4b	18.0 ± 1.6bc
Total chemical scarification	9	2 ± 0.7	30 ± 8.8	3.0 ± 1.7	2 ± 0.4	20.0 ± 5.1

SF (fresh seeds), S2 (seeds with two months in storage). †Mean ± standard deviation.
* Different letters within columns indicate significant difference $P \leq 0.05$.

In the case of seeds treated with H_2SO_4, only those that had been stored for two months germinated. The first evidence of germination was elongation of the radicle which was followed by hypocotyl emergence. With this treatment, maximum values of germination were after 12 weeks of cultivation (figure 1B). The highest germination (16%) was obtained after immersion in H_2SO_4 for five minutes. Shorter treatments resulted in lower germination (8 or 12% for seeds treated for 1 or 2 minutes, respectively). Immersion times between 20 to 60 minutes did not result in any seed germination. Seedlings from the chemical scarification treatments showed a 100% survival rate.

The first conspicuous organ of the seedlings was the radicle, followed by the emergence of adventitious roots that appeared with the first leaf. These structures took three weeks to appear in the case of the excised zygotic embryos treatment and three months in the case of seeds chemically scarified with H_2SO_4.

Fresh seeds that were subjected to chemical scarification immediately after collection did not germinate. According to the TTC test, embryos were not viable after treatment. Observing seeds microscopically, they showed cracks in the seed coat, the embryo presented brown colouration and in some cases the endosperm was absent. The same features were observed in stored seeds treated with H_2SO_4 for a period of 20 to 60 minutes. The H_2SO_4-treated 4-month stored seeds did not germinate, nor were they viabile after treatment, but unlike the fresh seeds, they presented no morphological abnormalities. Control seeds did not germinate after four months, did not manifest any anomalous features and the zygotic embryos showed a positive reaction to the TTC test (intense red coloration of the embryo), indicating that they were viable.

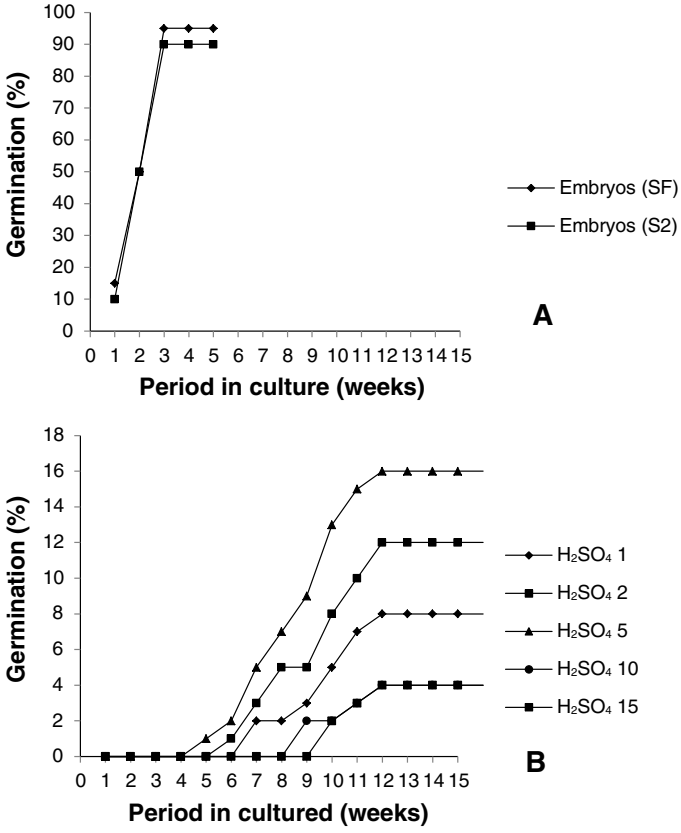

Figure 1. Germination of *Musa ornata* seeds following different treatments. **A**, germination of excised zygotic embryos from fresh seeds (SF) and from seeds that were stored for two months (S2). **B**, germination following chemical scarification with H_2SO_4. Numbers on the right indicate the periods of immersion in minutes.

Considering all variables tested, there were significant differences in germination ($F_{7, 30} = 3.56$, $P < 0.001$) between treatments for excised zygotic embryos and immersion of seeds in H_2SO_4 for 5 and 10 minutes (table 1). Although five minutes immersion in H_2SO_4 gave the highest germination, this treatment did not significantly influence further development of the seedlings (table 1). While the treatment involving excised zygotic embryos did not result in significant differences in germination between fresh seeds and those that had been stored for two months, it did result in significant differences ($P < 0.001$) in number of leaves, plant height and number and length of roots, compared with the other treatments (table 1).

Ex vitro cultivation

Seedlings transplanted into soil under greenhouse conditions showed a survival rate of 100% after 12 months. Furthermore, there was no significant difference in *ex vitro* plant growth between treatments.

According to the descriptive analysis (figure 2), increase in plant size was slow during the first four months after transplanting, corresponding to the end of the wet season and beginning of the cold season in the northern hemisphere (August-December). Growth accelerated to an average of 250 mm per month during the last four months, which corresponded to the months of highest temperature and humidity (May-August).

Leaf growth manifested linear behaviour in time, generating one leaf every two months. Plant diameter increased by about 2 mm per month during the first eight months (September to April) and 7 mm in the last four months (May-August).

At the end of the observation period (12 months), plants reached a height of 1800 ± 28 mm (mean ± s.d.), with10 leaves and a stem diameter of 50 ± 1.8 mm. The number of roots was 17 ± 2.3, with a length of 400 ± 103 mm.

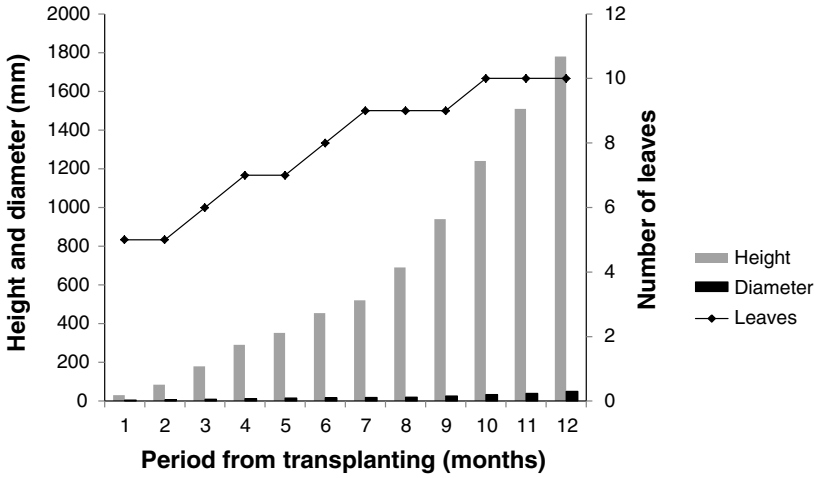

Figure 2. *Ex vitro* growth of *Musa ornata* plants: height, number of leaves and diameter. Data recorded over a period of 12 months (for this study, one month was equivalent to four weeks).

Discussion

Only the excised zygotic embryos treatment did not become infected with *Botryodiploidia* fungus. Treatments where the testa was intact and where seeds were not chemically scarified became infected. According to studies by Goss *et al.* (1961), the fungus grows in the coat and in the micropyle of the seed, but not in the endosperm and embryo. It is likely that the warty ornamentation of the testa serves as anchorage and protection for the *Botryodiploidia* sp. However, the reasons why the fungus does not grow in the endosperm and the embryo have not yet been defined. Only 10% of the cultures of seeds treated with this H_2SO_4 were affected by this fungus. Goss *et al*. (1961) reported that the growth of the fungus in seeds treated with strong acids is lower than in those treated with dilute acids, indicating that high concentrations of strong acids can kill the fungus on the surface of seeds. In contrast, other reagents used for sterilisation (e.g. 70% ethanol, H_2SO_4, NaClO) did not effectively remove it, permitting its subsequent development.

Germination resulting from zygotic embryos of fresh seeds (92%) and 2-month stored seeds (90%) of *M. ornata* was similar to that reported for *M. balbisiana* (94%), although the authors do not specify whether those seeds had been stored previously (Afele and De Langhe, 1991). High germination was also recorded for embryos from fresh seeds of *M. acuminata* ssp. *malaccensis* (Ridl.) Nasution (90.8%; Asif *et al*., 2001). However, these values contrast with those reported by Vuylsteke and Swennen (1993) for *M. acuminata* Colla., where only 15% of zygotic embryos germinated, even when they were cultured 3-5 days after harvesting, whereas for *M. velutina* seeds, 69.9% germination was achieved using the same treatment (Pancholi *et al.,* 1995).

In the case of chemical scarification, overall germination of 9% (across all H_2SO_4 treatments) for *M. ornata* seeds was lower than that reported by Stotzky *et al*. (1962) where a total of 25% was achieved with seeds from *M. balbisiana* that had been stored for 3-18 months. In contrast, the use of H_2SO_4 on seeds of the closely related genus *Ensete ventricosum* (Welw.) Cheesman for one minute did not have a positive effect on germination compared with intact seeds (Karlsson *et al*., 2013).

It should be noted that when freshly harvested seeds of *M. ornata* were treated with H_2SO_4, the testa was ruptured, allowing acid to penetrate, thus damaging the embryo. This may occur because these have not lost humidity, so the testa is smoother and more permeable. However, when the seeds are dried, the testa becomes less permeable and harder, enabling it to resist immersion in H_2SO_4 for prolonged periods. In this regard, Vuylsteke *et al*. (1990) mention that banana seeds tend to dormancy once they have been dried for storage (i.e. secondary dormancy; Chin, 1996) and therefore germination does not occur. In contrast, it has been reported that the seeds of some unidentified species from the genus, do not germinate when freshly harvested, but show 65% germination after three months of storage (Chin, 1996). Similarly, it was reported that storage of *M. balbisiana* seeds for three months, increases germination to 73-95% (Simmonds, 1952). Likewise *M. acuminata* seeds showed 28-86% germination after six months storage (Simmonds, 1959). These contrasting results may be due to differences in the width of the water channel in banana seeds. It has been reported that the water channel is narrower in fresh seeds than dry seeds of *M. acuminata*, therefore water uptake is higher for dry seeds (Puteh *et al*., 2011). Slow but steady water uptake, with and without scarification of seeds of *M. acuminata* has been reported by Puteh *et al*. (2011) and of seeds of *E. venticosum* by Karlsson *et al*. (2013). These observations contrast with our results, since intact seeds of *M. ornata* did not germinate. Germination only occurred when the embryos were excised or the seeds were chemically treated, in both cases using seeds stored for two months. Seeds stored for four months lost their viability and freshly harvested seeds germinated only when the coat seed was removed.

Previous studies have shown that banana seeds can be stored up to two years without problem (Simmonds, 1952). Nevertheless, other authors have reported a germination failure in *Musa* seeds that had been stored for the same period of time (Stotzky and Cox, 1962).

The differences in seed germination of *Musa* species may be a reflection of different degrees of dormancy as defined by Baskin and Baskin (2004) and it is even possible that some seeds of the genus do not present any dormancy. For example, Baskin and Baskin

(1998) reported that some members of the Musaceae have physical dormancy (i.e. water-impermeable palisade or palisade-like layer(s) of cells in seed (or fruit) coat). However, later Baskin *et al*. (2000) based on the work of Humphrey (1896), Stotzky *et al.* (1962) and Graven *et al.* (1996), reported that Musaceae seeds do not have physical dormancy. Similarly, Simmonds (1959) reported that seeds of *M. acuminata* and *M. balbisiana* do not manifest dormancy. In contrast, recent studies report physical dormancy in *Musa* seeds (Asif *et al.*, 2001; Finch-Savage and Leubner-Metzger, 2006; Uma *et al*., 2011). In this regard, Chin (1996) mentioned that while most *Musa* species present some degree of dormancy, a few do not.

These differences, together with the results from this study suggest that viability and the presence and degree of dormancy appears to be species characteristic. Puteh *et al*. (2011) showed that morphological variation of seeds among ecotypes of the same species is attributable to differential rates of water uptake. Specific studies on anatomy of *M. ornata* seeds are therefore needed to understand processes of germination, otherwise generalising any of these factors at genus or family level, as has been done in previous studies (Baskin and Baskin, 1998; Baskin *et al*., 2000), would be speculative.

It is likely that factors that affect the germination of *M. ornata* are present in the testa made evident by the apparent lack of germination in intact seeds (control treatment), as well as the slower and less effective germination following chemical scarification. Guan (1991) reported that the cell walls of the mesotesta contain water repellent substances, which may inhibit imbibition. This, coupled with the presence of plant polyphenolic substances is likely to act as a hindrance to oxygen diffusion through the seed coat. Conversely, removing the testa (e.g. excised zygotic embryo treatment) permits water absorption and gas exchange, facilitating the germination process.

Conclusion

Of the treatments evaluated, the most effective was the use of excised zygotic embryos, promoting greater germination in less time and better seedling development. By contrast, chemical scarification with concentrated H_2SO_4 showed low rates of germination and seedling development was slow. Infected seeds prevented the evaluation of germination response in other treatments. It should be emphasised that seed extraction should be performed aseptically, taking care in removing all the mucilage from the surface, to avoid contamination of plants.

Seedlings obtained through *in vitro* germination did not present problems for *ex vitro* establishment, registering 100% survival and successful development. However, it is important to supplement the information generated here with that from other studies providing data on the establishment and development of plants introduced *in vivo,* in order to promote specific actions towards a conservation strategy for the species. Studies on anatomy and physiology of *M. ornata* seeds, as well as further germination studies in this and different species of *Musa* are essential to better understand the processes involved in germination, as it is clear that what is effective for one species is not necessarily effective for another.

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