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# *IN VITRO* EMBRYO CULTURE OF RARELY ENDANGERED *MUSELLA LASIOCARPA* (MUSACEAE) WITH EMBRYO DORMANCY

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**Abstract**

*Musella lasiocarpa* (Musaceae) is an ornamental annually producing many viable seeds, but seldom recruited by seeds in the wild. One mature *Musella* seed has a small mushroom-shaped embryo without discernible organ differentiation. Therefore, freshly-harvested mature seeds are dormant. When the seeds gradually finished differentiation during warm stratification at 23ºC, they germinated to 82%. Besides, extracted embryos from fresh seeds did not germinate on the basal medium of Murshige and Skoog medium (MS) supplemented with 3% sucrose and 0.8% agar, but they were induced to form calli and root by media. The optimum medium for inducing calli was MS + 1.0 mg/L 6-BA + 0.05 mg/L NAA + 100 mg/L Vc with the highest proliferation coefficient (7.3) in 35 days. Moreover, the embryos from the 6-month warm stratified seeds could proliferate on the suitable medium. The optimal medium for rooting was MS + 0.5 mg/L 2, 4-D + Vitamin C 100 mg/L. The results confirmed that both the embryo developmental stage and appropriate combination of chemicals significantly affected seed germination and *In vitro* embryo culture of this species.

**Key words:** Regeneration; Morphological dormancy; Warm stratification; *In vitro* Embryo culture; Musella

## Introduction

*Musella* (Musaceae) is a monotypic genus with a single species *Musella lasiocarpa* (Franch.) C. Y. Wu ex H. W. Li, endemic to Southwestern China (Liu *et al*., 2002; Wu & Kress, 2000). In addition to being used as medicine, fodder, food, and soil and water erosion control, perennial and succulent *Musella* is an important ornamental with rhizome and erect pseudostem (Qin *et al*., 2006; Xue *et al*., 2007). To date, this species has been widely used as an ornamental for attractive morphological characteristics, such as long-lived, bright yellow (sometimes, red in the wild) inflorescences and broad, green leaves. Recently, besides Chinese gardeners and breeders, some horticulturalists from the United States, European Union, Singapore and Japan have been increasingly interested in the commercial development of this resource as an ornamental. However, *M. lasiocarpa* is an endangered species with less than 1,500 discontinuously left individually in remote mountainous areas in southwest China (based on our field investigation). *Musella lasiocarpa* has been in cultivation for a long time in various sites, which is from vegetative propagation depending on long-lived hypogeotropic, stem-derived rhizomes, because this species is seldom recruited by seeds (Tian & Tang, 2012). Although different explants including leaves and auxiliary buds were induced into seedlings (Guan, 2002; Zeng *et al*., 2007) but embryo culture has not been reported. Moreover, little information on sexual reproduction is available.

Like *Musa* taxa, one *Musella lasiocarpa* seed is with a small mushroom-shaped embryo at collection and hardly germinated under various conditions (Graven *et al*., 1996; Tian & Tang, 2012). Especially, Xue *et al*. (2007) believed that *Musella* seeds are inviable due to female gametophyte and seed development. Previously, Nagano *et al*. (2008) found that seeds of *Musa velutina* had immature mushroom-shaped embryos that could gradually develop at 25°C in moist vermiculite with 75% moisture content, and then germinated well. In order to confirm if *Musella* seeds could germinate, we further investigated embryo differentiation and germinatbility under experimental conditions. Thus, in the present study, embryo differentiation and *In vitro* embryo culture were investigated to effectively utilize valuable seeds to promote sexual seedling establishment.

*In vitro* culture of embryo is usually species-specific. At present, little is known about the factors affecting embryo culture of *Musella*. Moreover, the increasing emphasis on breeding programs (such as breeding new horticultural varieties) of this ornamental and medicinal plant requires a large number of sexual seedlings. Thus, the aim of this study was to screen effective methods for *In vitro* culture of embryo as well as embryo differentiation under different conditions.

## Materials and Methods

**Seed collection and pre-treatment:** A large number of mature fruits of *M. lasiocarpa* were collected on 12 November 2009 from a wild population (101°58´061"E, 25°39´755"N, altitude, 1620 m) distributed in Yuanmou county in central Yunnan Province in Southwestern China. Seeds were extracted by hand from mature fruits and cleaned in running tap water. Subsequently, these seeds were air dried and open stored at an incubator (15°C and 70% relative humidity) until used.

**Germination test of freshly-harvested seeds:** To determine if freshly-harvested seeds could germinate, some seeds were incubated at 15, 20, 30, and 30/20°C (day/night) with a 12-h photoperiod (approximately 90 $\mu mol\ m^{-2}\ s^{-1}$). Four replicates of 100 seeds each were placed on a moist filter paper in glass Petri dishes. The criterion of germination was the radicle emergence from the operculum. The observation was daily conducted. The germination experiments were terminated after 30 d.

**Observation of embryo differentiation and germination test:** Embryo differentiation in *M. lasiocarpa* seeds was studied during warm stratification at 23°C in plastic bags in dark. The stratification medium was river sand with 60% moisture content. The embryos were extracted from 20 seeds stratified for 0, 1, 3 and 6 months, respectively, and fixed in 5:5:90 (v/v/v) of formaldehyde: acetic acid: ethanol (70%) (FAA). The embryo differentiation in these seeds was checked by paraffin sectioning (Huiwo M3500, Shenzhen Technology co. Ltd., Shenzhen, China). Sections (8 μm) were stained with Delafield′s hematoxylin (Sun *et al*., 2000) and observed under a microscope (Zeiss, Axioskop 40 FL, Germany). The warm stratified seeds were incubated under the conditions similar to those of freshly-harvested seeds.

***In vitro* embryo culture of seeds:** As for *In vitro* embryo culture, extracted embryos were from 3 types of seeds: (a) fresh seeds, used as controls; (b) seeds stratified in moist river sands (60% moisture content) at 23°C for three months; and (3) seeds stratified at 23°C for 6 months. To compare their potential of embryo initiation (germination), the excised embryos were inoculated onto the basal medium, which consisted of Murashige & Skoog (1962) mineral salts (MS) plus 3% (w/v) sucrose and 0.8% (w/v) agar. Secondly, some embryos of these three types of seeds were cultured on media supplemented with plant growth regulators and vitamin C ($V_c$).

Especially, a range of media were used to screen suitable media for immature embryos from freshly harvested mature seeds. The media contained different plant growth regulators except for sucrose (3%) and agar (0.8%). Firstly, five types of media including ① MS, ② MS + 1.0 mg/L 6-BA + 1.0 mg/L NAA + 50 mg/l Vc, ③ MS+ 1.0 mg/L 6-BA + 0.05 mg/L NAA + 100 mg/L Vc, ④ MS + 1.0 mg/L 6-BA + 0.02 mg/l NAA + 100 mg/L Vc, ⑤ MS + 2.0 mg/Ll 6-BA + 0.1 mg/L NAA + 150 mg/L Vc were used to induce proliferation. Subsequently, five kinds of media with different combinations of 2,4-D and Vc were used for rooting (Table 1).

In the process of *In vitro* culture, intact seeds were firstly soaked in 1.5% (v/v) sodium hypochlorite solution for 10 min, followed by a quick wash with 70% (v/v) ethanol. After three washes with sterile distilled water under aseptic conditions, seeds were cut longitudinally, and then the immature mushroom-shaped embryos were separated. The excised embryos were aseptically inoculated onto different media (Table 1). All media were adjusted to pH 5.8 and autoclaved for 20 min at 121°C. While still molten, aliquots of the medium were supplemented with filter sterilized NAA to produce different treatments (Table 1). Thirty ml of medium were aseptically dispensed into a 150 ml conical flask which was sealed with plastic film. All conical flasks with excised embryos were placed in a growth room, where the photosynthetic photo flux density (PPFD) and temperature were approximately 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 28 ± 2°C, respectively. During cultivation, the light/dark period is 16 h/ 8 h with light supplied by white fluorescent lamps.

Twenty replications per treatment were carried out. Each replication contained 2-3 embryos. Only the group with the highest multiplication coefficient was used for a successive transfer culture. Data on percentage of embryo germination (if embryo length doubled initial length, the embryo was considered normally germinated), number of shoots, and the onset time to root emergence were recorded during observation.

**Data analysis:** Data were subjected to analysis of one-way variance (ANOVA) ($p = 0.05$) (SPSS 15.0, SPSS Inc., Chicago, Illinois, USA), followed by a Duncan's multiple comparison test.

## Results and Discussion

**Germination of fresh seeds:** Fresh seeds did not germinate under given conditions although the seed coat was water-permeable (data not shown). However, seeds that were warm stratified for 6 months easily germinated to 82 ± 1.2%. This phenomenon indicated that *M. lasiocarpa* seeds were dormant due to an underdeveloped embryo which was further confirmed in Fig. 1. According to Bewley's opinion (1997), we thought that the species had innate embryo dormancy. Further, based on the seed dormancy system proposed by Baskin & Baskin (2004), the seeds of *M. lasiocarpa* have morphological dormancy.

**Table 1. Effects of media on embryo germination, multiplication and rooting in the process of *In vitro* embryo culture of *Musella lasiocarpa* seeds warm stratified at 23°C for 6 months.**

| Plant growth regulator in basal media (mg/L) | | | Days of embryo germinating onset (mean ± SD) | Days to root emergence (mean ± SD) | Proliferation coefficient |
|---|---|---|---|---|---|
| **6-BA** | **NAA** | **Vc** | | | |
| 0 | 0 | 0 | 17.5 ± 0.1 | | 1 |
| 1.0 | 0.1 | 50 | 10.2 ± 0.2 | | 3.5 |
| 1.0 | 0.05 | 100 | 8.6 ± 0.1 | | 7.3 |
| 1.0 | 0.02 | 100 | 11.2 ± 0.3 | | 5.6 |
| 2.0 | 0.1 | 150 | 8.3 ± 0.2 | | 4.7 |
| **2, 4-D** | **Vc** | | | | |
| 0 | 50 | | | 22.5 ± 1.2 | |
| 0.5 | 50 | | | 16.3 ± 0.7 | |
| 0.2 | 100 | | | 20.2 ± 1.2 | |
| 0.5 | 100 | | | 14.3 ± 0.7 | |
| 0.3 | 150 | | | 17.1 ± 0.7 | |

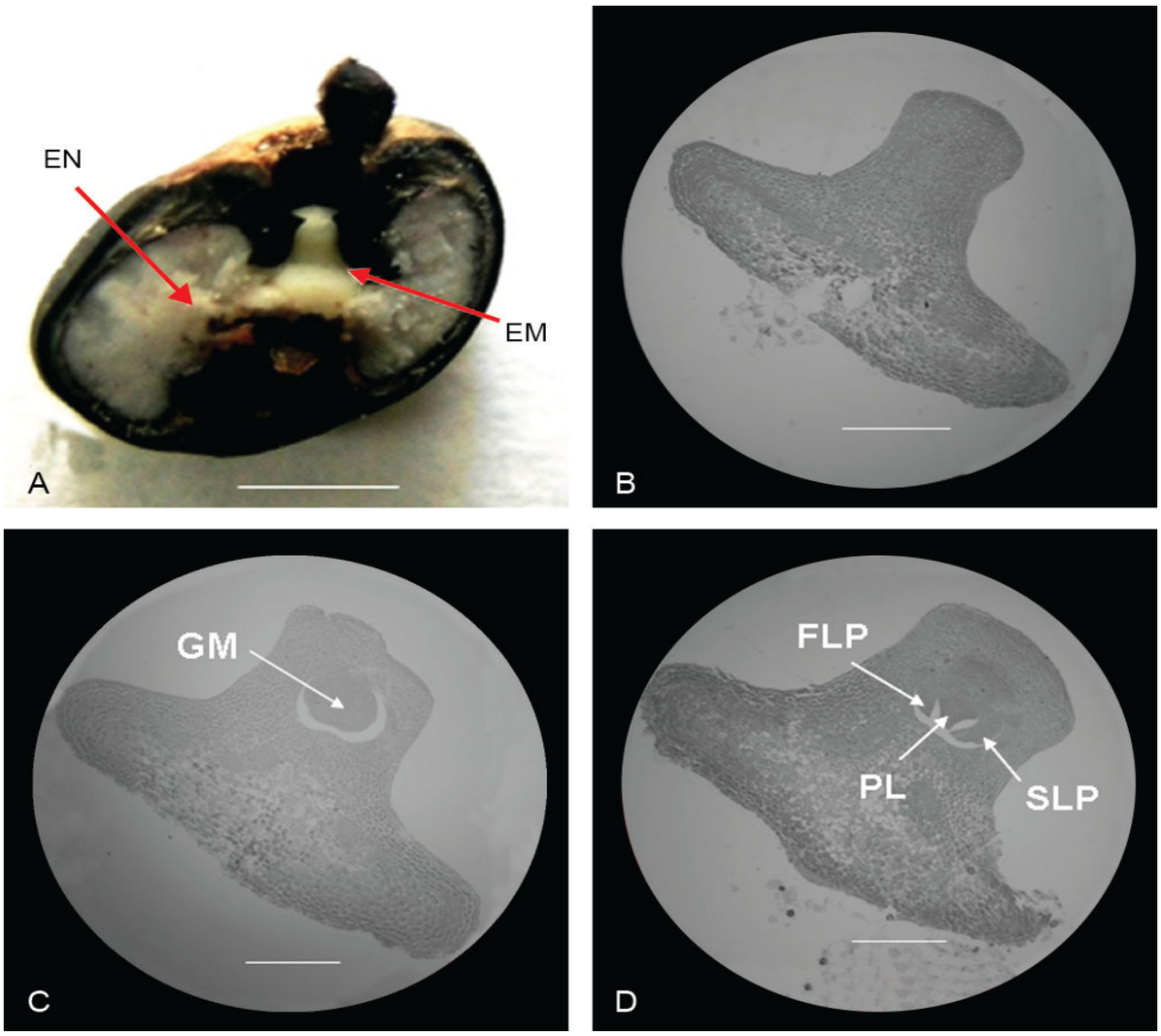


Fig. 1. Longitudinal section of seed and changes in embryo differentiation of *Musella lasiocarpa* seeds during warm stratification (23°C) in moist river sands with 60% moisture content. A, longitudinal section of a fresh seed; B, 0 month, C, 3 months; D, 6 months; EN, endosperm; EM, embryo; GM, global meristem; PL, plumule; FLP, first leaf primordium; SLP, second leaf primordium. Scale bars: (A) 5 mm; (B, C, D) 0.6 mm.

**Embryo differentiation during stratification:** *Musella lasiocarpa* seed has a small mushroom-shaped embryo at collection (Fig. 1A). During warm stratification at 23°C, the embryo differentiation gradually occurred (Fig. 1). The initial embryos from freshly-harvested mature seeds did not show visible organ differentiation (Fig. 1B). After 3-month stratification, the embryo displayed an obvious pro-organ region (Fig. 1C). At the end of 6th month, two leaf primordia and the plumule were discernible (Fig. 1D). Similarly, Nagano *et al*. (2008) previously found that freshly-matured *Musa velutina* seeds had a mushroom-shaped immature stage 0 embryo. Embryos that developed 0, 1, 2 and 3 leaf primordia were defined as Stage 0, Stage 1, 2 and 3, respectively. During stratification at 10, 20, 25 and 30°C for 8 months, some embryos reached stage 3 at 25°C and 20°C; however, most embryos remained at Stage 2 at 10°C or at 30°C. Thus, similar to *M. velutina* seeds, changes of the anatomic structures of the embryo of *M. lasiocarpa* seeds shown by Fig. 1 illustrated that the embryo development (i.e., organ differentiation) was a continuous and modular process, which was triggered by environmental cues.

***In vitro* embryo culture**: The extracted embryos from fresh seeds did not germinate on the basal medium (i.e., MS plus 3% sucrose and 0.8% agar). However, they could proliferate by producing calli except for those on MS. The optimum was MS + 1.0 mg/L 6-BA + 0.05 mg/L 2,4-D + 100 mg/L Vc, followed by MS + 1.0 mg/L 6-BA + 0.02 mg/L NAA and MS + 1.0 mg/L 6-BA + 0.1 mg/L NAA + 150 mg/L Vc. Their proliferation coefficient was 7.3, 5.6 and 4.7, respectively, in 35 days. Furthermore, all the shoots from calli could normally root on rooting media.

Also, these embryos from the seeds stratified for 6 months could rapidly germinate and produced shoots at a range of media (Table 1). After subculture, the shoots could root and normally develop into plantlets (Fig. 2). By comparison with undifferentiated embryos, the fully differentiated embryos germinated more easily. As was

reported before, the embryo developmental stage affects the germination and growth of cultured embryos and excised seeds (Hu, 2005). Previously, Raghayan (1966) identified two phases of embryo development. In the heterotrophic phase, the young embryo depends on the endosperm and the surrounding maternal tissues, and requires a more complex medium and higher osmotic pressure than older embryos. The continued development of developing embryos requires complex media supplemented with combinations of vitamins, amino acids, growth hormones, and, in some cases, natural extracts, such as tomato juice and coconut milk, to support development. During the autotrophic phase, the second stage of embryo growth, the embryo is metabolically capable of synthesizing substances required for its growth. The embryos at the autotrophic stage can germinate and grow on a simple inorganic medium supplemented with a carbon source, such as sucrose. In this study, based on data embryo differentiation and the two-stage opinion quoted above, we evaluated the potential of *In vitro* embryo culture only by using the embryos from fresh seeds and ones warm stratified for 6 months. Similarly, Choi *et al*. (1998) also reported that competence was variable, which was related to the developmental stage of immature zygotic embryos of Chinese cabbage (*Brassica campestris* subsp. *napus pekinensis*). Therefore, this study confirmed that the embryo developmental stage plays a crucial role in embryo culture, at least, for this species.

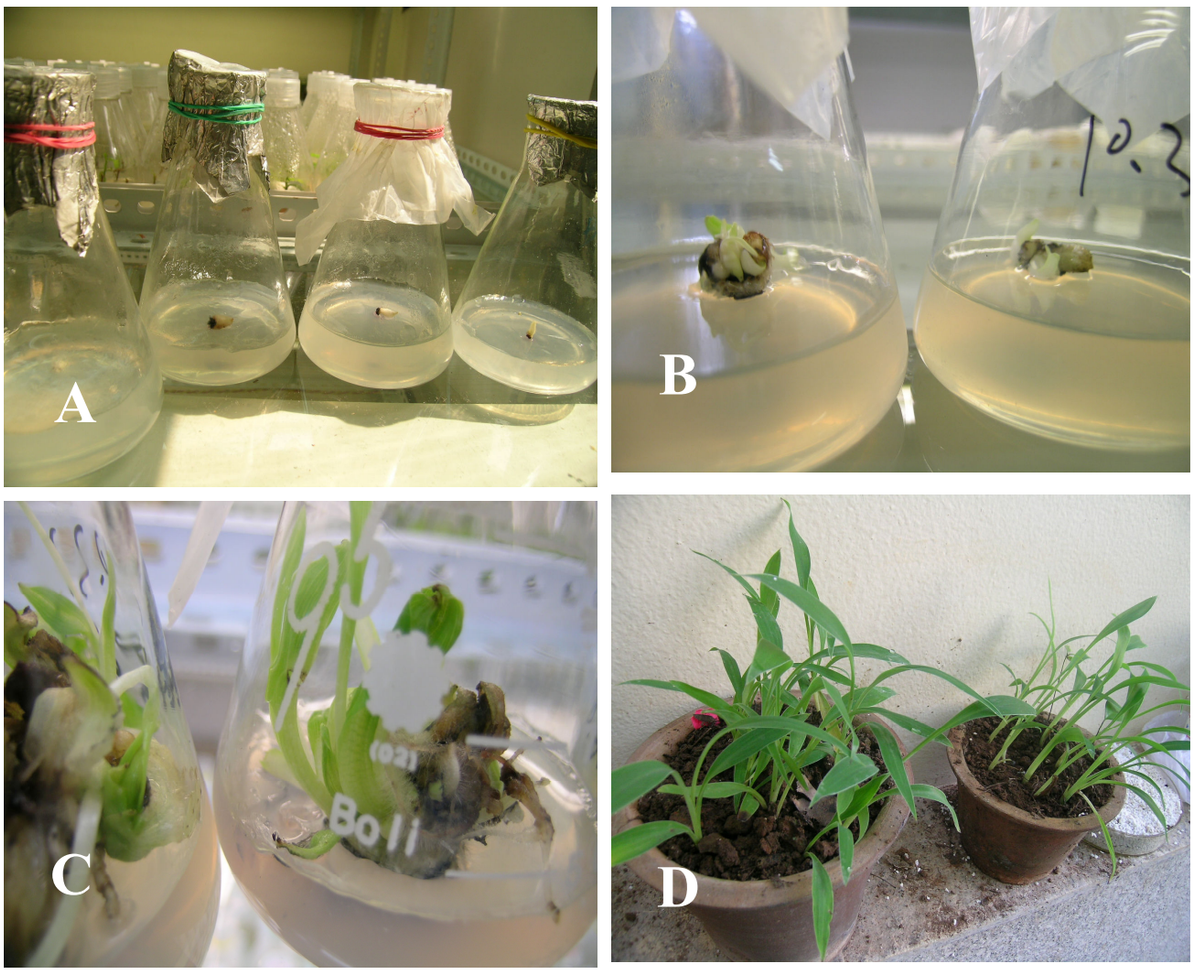


Fig. 2. *In vitro* embryo culture of *Musella lasiocarpa* and plantlets. The embryos were extracted from the seeds stratified at 23**°C** for 6 months. (**A**) proliferation culture for 7 days; (**B**) proliferation culture for 16 days; (**C**) rooting culture for 10 days; (**D**) plantlets grew for 38 days.

**Effects of medium on embryo culture**: As for embryos from fresh seeds, except for MS, other four types of media could induce calli. The best medium for inducing calli was the combination of MS + 1.0 mg/L 6-BA + 0.05 mg/L NAA + 100 mg/L Vc , followed by MS + 1.0 mg/L 6-BA + 0.02 mg/L 2,4-D + 100 mg/L Vc. The obvious difference among the media was the ratio of cytokinin/auxin. These results suggested that the ratio of cytokinin/auxin significantly influence calli inducation of embryos of this species. Similarly, Abbsai *et al*. (2011) found that the concentration of the plant growth regulators, in particular BA, significantly affected *In vitro* regeneration of *Sinapis alba* (Brassicaceae). Yasmin *et al*. (2011) found that Callus proliferation was influenced by genotype and media interaction for immature embryos of wheat (*Triticum aestivum*).

During *In vitro* embryo culture, the media affected initiation of embryo germination, multiplication and rooting although developed embryos were able to grow without exogenous plant growth substance and Vc (Table 1), which took the longest time for the controls to show evidence of embryo germination. The addition of 1.0 mg/L 6-BA + 0.05 mg/L NAA and 100 mg/L Vc was the optimal effective (8.6 ± 0.1 days) with the highest multiplication coefficient (i.e., 7.3), followed by the association of 2.0 mg/L 6-BA + 0.1 mg/L NAA + 150 mg/L Vc (8.3 ± 0.2) with 4.7 of multiplication coefficient. Subsequently, the group with 7.3 was selected as successive rooting material. The application of 0.5 mg/L 2,4-D and 100 mg/L Vc was the most effective for rooting (14.3 ± 0.7 days), the percentage of which was 100%. The results indicated that the appropriate combination of different chemicals strongly affected shoot multiplication and rooting.

According to real-time observation, differentiated zygotic embryos of *M. lasiocarpa* seeds produced shoots with intervening calli (Fig. 2). In addition, Zhang *et al*. (2004) reported that the haustorial buds of *Musella lasiocarpa* could induce proliferation on medium of MS + 6-BA 1.0 mg/L + IBA 0.1 mg/L after 30 days; the proliferation medium was MS + 6-BA 0.3 mg/L + IBA 0.1 mg/L. The reproduction coefficient was more than 2.83. The optimal medium for rooting was MS + NAA 1.0 mg/L and the rooting percentage was 100%. Zeng *et al*. (2007) also reported that suitable combination of phytohormones were critical for micropropagation by using auxiliary buds of *Musella* as explants. Therefore, the experiments also illustrated that different phytohormone combinations/ concentrations significantly influenced *In vitro* embryo culture.

To conclude, *In vitro* embryo culture of 6-month warm stratified seeds of *Musella* was highly feasible, which indeed accelerated seedling establishment in practice. Moreover, the differentiated seeds could germinate under suitable environmental conditions when seeds would be stratified at 23°C up to 6 months in substrates with 60% moisture content, suggesting that it is feasible to produce sexual seedlings for breeding projects.

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