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ARTICLE

Designing ex-situ conservation strategies for seeds storage of *Piper aduncum* and *P. hispidinervum* through cryopreservation and low-temperature techniques

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ABSTRACT

Piper aduncum and *Piper hispidinervum* are species found primarily in the Amazon region, and have been highlighted by the fact that they contain, in their essential oils, significant amounts of dillapiol and safrole. The aim of this work was to assess the seed behavior of *P. aduncum* and *P. hispidinervum* and to develop strategies for medium—to long-term storage of the species at sub-zero and cryogenic temperatures. In the first experiment, seeds were desiccated and their water content was determined. Then, the seeds were kept at −20°C for up to 90 days. In the second experiment, seeds were stored for up to 24 h in liquid nitrogen (LN; −196°C), with and without the use of cryoprotectants. Finally, for assessing the efficiency of these techniques, seeds were stored at −20°C and in LN for up to 360 days. The germination rate was more than 90% for both species when stored at −20°C or LN, without compromising their viability and development. The results suggest that *P. aduncum* and *P. hispidinervum* have seeds with orthodox behavior, which can be stored for the medium to long term at sub-zero and cryogenic temperatures without the need for use of cryoprotectants.

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Piperaceae; germplasm conservation; cryopreservation; desiccation; vitrification

Introduction

The high rate of erosion of genetic resources, combined with the loss of components of biodiversity, has led several countries to conduct research geared toward conservation of their biological resources (Khoury et al. 2010). In Brazil, there is an urgent need to take initiatives to enable rapid advancement of scientific knowledge on the composition and conservation of Amazon species, before they begin to disappear, or even before they have even been cataloged and studied. Among these species, *Piper aduncum* and *Piper hispidinervum*, popularly known in the Amazon region as "pimenta-de-macaco" and "pimenta-longa", respectively, stand out due to the secondary compounds that are extracted from the essential oil present in their leaves and branches in stages. These species also stand out because they can be commercially exploited without requiring their complete destruction (Souza Filho et al. 2010; Abreu et al. 2015).

The essential oil of *P. aduncum* contains significant quantities (up to 80%) of dillapiol, a phenylpropanoid compound responsible for fungicidal, molluscicidal, larvicidal, and insecticidal activity (Brazão et al. 2014). Moreover, several studies also report the effect of this compound in the control of fungi and insects that attack species of economic importance (Araujo et al. 2012; Guerrini et al. 2009; Piton et al. 2014), as well as parasites that cause diseases to humans, such as *Trypanosoma cruzi* (Batista et al. 2008), *Plasmodium falciparum* (Céline et al. 2009), and *Aedes aegypti* (Santana et al. 2015), the etiological agents of Chagas disease, malaria and dengue, and zika, respectively.

The essential oil of *P. hispidinervum* contains high levels (up to 90%) of a phenyl ether known as safrole, which currently presents a serious risk of not being able to meet the growing international demand in the medium- to long-term period (Sauter et al. 2012). Heliotropine is obtained from this compound, which is widely used as a fragrance, and piperonyl butoxide, an essential ingredient for the production of pyrethrum-based biodegradable insecticides (Barbosa et al. 2012). In view of the economic potential that these species have, coupled with the fact that as there are yet no preservation programs to assure these species will be maintained, it is essential to develop conservation strategies to prevent them from becoming endangered species. According to Silva and Scherwinski-Pereira (2011), *P. aduncum* and *P. hispidinervum* are included in the list of priority species for conservation. According to the authors, in these Amazon species, seed preservation is a preventive measure that should be taken to assure the conservation of their genetic resources.

Conservation in seed banks is a methodology aimed at the medium- to long-term preservation of plant genetic resources. But for the success of this technique, it is necessary to know the physiological behavior of the seed to be stored (Hay and Probert 2013). According to Walters (2015), orthodox seeds, which have tolerance to desiccation, have good resistance to sub-zero temperatures. On the other hand, recalcitrant seeds—sensitive to decreased water content—do not survive when exposed to low temperatures.

In light of the foregoing, the classification of the physiological behavior of seeds of a particular species as orthodox or recalcitrant is of the utmost importance to define the conservation strategy to be used, given that only orthodox seeds can be maintained in the medium to long periods in cold-storage facilities (Engelmann 2011). Additionally,

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cryopreservation has also emerged as a rather promising alternative for seeds preservation (Walters et al. 2013). Cryopreservation is the storage of biological material in liquid nitrogen (LN) at a temperature around −196°C. Under these conditions, the plant material can be stored for long periods, because the cell metabolism becomes practically paralyzed, thereby avoiding genetic variability and deterioration of the stored propagule (Kaviani 2011).

Given the potential of *P. aduncum* and *P. hispidinervum* for the production of dillapiol and safrole, together with the lack of studies on the physiological behavior and preservation of their seeds, this study aimed at analyzing the tolerance of these propagules when submitted to a decrease in moisture content and assessing strategies to promote their medium- to long-term conservation using sub-zero and cryogenic temperatures.

Material and methods

The seed lots used for the experiments were obtained from the germplasm bank of *P. aduncum* and *P. hispidinervum* belonging to Embrapa Acre, located in the city of Rio Branco, in the state of Acre, Brazil.

Morphological characterization, determination of moisture content, and tolerance to desiccation

For the morphological characterization, 50 seeds of each species were evaluated for length, width, and thickness; the length was measured from the base to the apex, and the width and thickness were measured at the central portion of the seeds. In both cases, the measurements were taken with the aid of a digital caliper (Mitutoyo). In this phase, we also evaluated the weight of 10 sub-samples with 100 seeds each, to obtain a weight of 1,000 seeds of each species.

As for the determination of the desiccation curve, the *P. aduncum* and *P. hispidinervum* seeds were divided into three lots of 400 seeds each, which were maintained for 0 (control sample), 24 and 48 h in a desiccator containing silica gel. Subsequently, 200 seeds from each lot were subjected to determination of their water content through the oven method at 105 ± 5°C until constant weight was obtained. In this analysis, the moisture contents were calculated using the following equation:

$$MC\ (\%) = \frac{(Iw - Dw)}{Dw} x\ 100$$

where: *MC* (%) = moisture content as a wet basis percentage, *IW* = weight of the subsample prior to desiccation, and *DW* = weight of the subsample after the desiccation period.

Determining a protocol for storing seeds at −20°C

Once the moisture content of the *P. aduncum* and *P. hispidinervum* seeds were determined, after drying for 0, 24, and 48 h, the remaining 200 seeds from each lot were then placed in cryogenic vials of 1.5 mL capacity, inserted into trifoliolate aluminum envelopes, and then stored in a cold room at −20°C for two different time periods: 0 (control) and 90 days.

At the end of the cold-storage period, the seeds were subjected to a disinfection process, consisting of a 2-min immersion in 70% alcohol, followed by 15-min immersion in sodium hypochlorite (NaOCl) at 1.25% of active chlorine, and triple washing in distilled water, then autoclaved to remove excess disinfectant substances.

After disinfection, the seeds were divided into two sub-lots of 100 seeds each, where one was inoculated in glass flasks (250 mL capacity) containing 30 mL of MS medium (Murashige and Skoog 1962) supplemented with 30 g L^{-1} sucrose and 2.5 g L^{-1} Phytagel (Sigma), and the other in gerbox boxes under germitest paper, moistened with distilled water every 48 h. After 30 days of cultivation, the percentage of germinated seeds was determined. In this evaluation, germinated seeds were considered as those that exhibited a radicle protrusion.

Determining a protocol for seed cryopreservation

After the conservation test in a cold room at −20°C, the tolerance of *P. aduncum* and *P. hispidinervum* seeds on immersion in LN (−196°C) was assessed. Because of the small size of the seeds, the need for the use of cryoprotectants was also assessed in this experiment.

Therefore, seeds from these two species were divided into six lots of 100 seeds each. These lots were then placed in cryogenic vials (1.5 mL capacity), which after undergoing the treatments, were placed in trifoliolate aluminum envelopes for cryopreservation. To fine-tune the cryopreservation protocol, the seeds were submitted to the respective treatments: no immersion in LN (control), direct immersion in LN, immersion in LN using PVS2 cryoprotectant, immersion in LN using cooled PVS2 cryoprotectant, immersion in LN using modified PVS2 cryoprotectant, and immersion in LN using cooled and modified PVS2 cryoprotectant.

The PVS2 vitrification solutions (Sakai et al. 1990) were composed of 30% glycerol (v/v), 15% ethylene glycol (v/v), 15% DMSO (v/v), and 40% MS medium. For the modified PVS2 vitrification solutions, the concentration of ethylene glycol was changed from 15 to 30% (v/v), in detriment to the concentration of MS medium, which fell from 40 to 25% (v/v). For the cooling process of the cryoprotectants, they were subjected to a 30-min ice bath. Finally, prior to adding the vitrification solutions in the cryogenic vials containing the seeds, the solutions were sterilized with the aid of a Millipore® 0.22-μm filter.

After 24 h of immersion in LN, the seeds were exposed to room temperature for 30 min, in order to thaw. Next, these propagules underwent the disinfestation process, and then were inoculated in flasks with MS nutrient medium in order to determine their germination percentage after 30 days of cultivation.

Medium- to long-term assessment of the protocols developed for the ex-situ conservation of seeds

Finally, to get a better understanding of the efficiency of the protocols for the medium- to long-term conservation of *P. aduncum* and *P. hispidinervum* seeds, lots containing 100 seeds of each species were then stored in cold rooms at −20°C and LN at −196°C for 0, 180, and 360 days. In this test, due to the fact that the treatments tested in preliminary experiments did not differ statistically among themselves, no period of desiccation was carried out, in the case of

conservation in a cold room at −20°C, and no cryoprotectants were used, in the case of cryopreservation in LN.

After medium- to long-term storage, the seeds of both species were also disinfected and then cultured for 30 days in flasks containing MS medium to promote germination. However, in this stage, in addition to the percentage of seed germination, the height of the germinated plants was also evaluated after 30 days of cultivation.

Statistical analysis

In all of the conservation experiments, the experimental design was totally randomized, with at least four repetitions per treatment, each containing 25 seeds. For germination, the seeds were kept in a growth chamber at a temperature of 25 ± 2°C, with a 16-h photoperiod and light intensity of 38 μmol m^{-2} s^{-1} provided by white-cold fluorescent lamps.

For the statistical analysis, the data were submitted to variance analysis using the statistical analysis system for computers—Sisvar 4.4 (Ferreira 2011), and the means were compared by the Tukey test at 5% probability. Data expressed in percentage (x) were transformed according to arcsine of the square root of $x/100$.

Results and discussion

Morphological characterization, determination of moisture content, and tolerance to desiccation

Table 1 shows the dimensions of the seeds and the values of moisture content of the *P. aduncum* and *P. hispidinervum* seeds—non-desiccated (0 h) and desiccated for 24 and 48 h. In general, it was observed that the *P. aduncum* seeds were larger than the *P. hispidinervum* seeds. For this species, the averages relating to length × width × thickness were 0.84 × 0.71 × 0.44 mm, respectively, and the average weight of 1,000 seeds was 0.232 g. On the other hand, in *P. hispidinervum*, the averages observed for length × width × thickness were 0.74 × 0.53 × 0.32 mm, respectively, and average weight of 1,000 equivalent to 0.165 g. Regarding desiccation, for both species studied, as the desiccation period increased there was a decrease in moisture content. In *P. aduncum*, the moisture content of the non-desiccated seeds and the seeds desiccated for 24 and 48 h were 18.1, 11.8, and 10.6%, while in *P. hispidinervum*, the values were 12.7, 9.7, and 6.7%.

These results demonstrate that the amount of water found in *P. aduncum* and *P. hispidinervum* seeds at the time of dispersion is significantly reduced. According to Walters (2015), at the end of the development period of the seeds classified as orthodox, there is a rapid decrease in moisture content to values close to 10%, varying depending on the species, making the environment of these seeds generally unsuitable for germination. In such a physiological state, these propagules remain quiescent and the germination process is started only when enough water is supplied to the seeds so that they can resume their metabolic activities.

These results are in agreement with Tweddle et al. (2003), who assert that the physiological behavior of the seeds is strongly related to their environmental aspects, such as their successional stage. For the authors, pioneering species—adapted to high solar radiation and having extremely small seeds, such as *P. aduncum* and *P. hispidinervum*—are usually classified as orthodox.

Determining a protocol for storing seeds at −20°C

It was found that the germination percentage of the seeds that had the water content decreased did not differ statistically from the non-desiccated seeds, which on average also showed values around 90%, regardless of the germination environment used (gerbox with germitest paper or flask containing MS culture medium) (Table 2).

Studying the germination of *P. aduncum*, Wen et al. (2015) also reported that the species has small black seeds, with fresh weight of 1,000 seeds around 0.180 g, as well as the fact that they have an initial moisture content of approximately 13.5%. Furthermore, this study also showed that with a similar water content, the seeds of the species also germinated rapidly when incubated at 25°C on filter paper moistened with deionized water, having the first germination documented after 96 h and most seeds having germinated within 2 weeks, with a mean percentage of germination of 89.6% after 30 days of cultivation.

As for the process of preservation in a cold rooms at −20°C for 90 days, we observed that the seeds of both

Table 1. Dimensions, mean weight and moisture content, after desiccation on silica gel, of *Piper aduncum* and *P. hispidinervum* seeds.

Parameters	Species	
	Piper aduncum	*Piper hispidinervum*
Length (mm)	0.84	0.74
Width (mm)	0.71	0.53
Thickness (mm)	0.44	0.32
Weight of 1000 (g)	0.232	0.165
Moisture content (%) after seed desiccation (h)		
0	18.1	12.7
24	11.8	9.7
48	10.6	6.7

For the morphological characterization, 50 seeds of each species were evaluated. To obtain a weight of 1000 seeds of each species, it was evaluated the weight of 10 sub-samples with 100 seeds each. For the determination of the desiccation curve, seeds were divided into three lots of 400 seeds each, which were maintained for 0 (control sample), 24 and 48 h in a desiccator containing silica gel. Subsequently, 200 seeds from each lot were subjected to determination of their water content through the oven method at 105 ± 5°C until constant weight was obtained.

Table 2. Germination of *Piper aduncum* and *P. hispidinervum* seeds desiccated for 0, 24, and 48 h over silica gel and kept for 0 and 90 days in cold rooms at −20°C.

Conservation and drying time	Germination (%)[a]			
	Piper aduncum		*Piper hispidinervum*	
0 days	Gerbox	Flask	Gerbox	Flask
Not desiccated	90.0	90.0	92.0	97.0
Desiccated for 24 h	93.0	89.0	86.0	96.0
Desiccated for 48 h	86.0	82.0	92.0	95.0
90 days				
Not desiccated	88.0	92.0	87.0	90.0
Desiccated for 24 h	86.0	93.0	98.0	93.0
Desiccated for 48 h	98.0	91.0	96.0	98.0
CV (%)	11.1		10.5	

[a]Not significant by the *F* test. The seeds were divided into two sub-lots of 100 seeds each, where one (four repetitions with 25 per plot) was inoculated in glass flasks (250 mL capacity) containing 30 mL of MS medium (Murashige and Skoog 1962) supplemented with 30 g L^{-1} sucrose and 2.5 g L^{-1} Phytagel (Sigma), and the other (four repetitions with 25 per plot) in gerbox boxes under germitest paper, moistened with distilled water every 48 h. After 30 days of cultivation, the percentage of germinated seeds was determined.

species—when compared to non-preserved seeds, regardless of the drying time—also exhibited no significant differences regarding germination percentage (Table 2). In such circumstances, *P. aduncum* and *P. hispidinervum* seeds also had average germination rates close to 90%, thus confirming that the physiological behavior is of the orthodox type.

In the study of water content and ideal storage temperatures for the preservation of germplasm of *Populus nigra*, Suszka et al. (2014) also reported that regardless of whether or not the seeds have been subjected to a previous drying process, they showed no significant loss of germinability when subjected to up to 12 months storage at a temperature of −20°C. Furthermore, this analysis showed that when these propagules were stored at temperatures higher than 3°C, their germination percentages decreased from roughly 85–40% at the end of this storage period. For the authors, these results are most likely related to the fact that the lower temperatures provided greater reduction of metabolism and biochemical reactions of the embryos, thereby reducing the consumption and degradation of reserve compounds of these seeds, which allowed them to be preserved for longer periods of time.

However, for several other plant species, a favorable relationship was observed between the decrease in moisture content and viability of the preserved seeds. According to Hirano et al. (2009), during storage of *Phaius tankervilleae* seeds in a cold room at 4°C for a period of 90 days, the reduction of moisture content from 13.2 to 5.4% significantly increased the viability of stored propagules, which in that case increased from around 50 to around 75%.

According to Ellis and Hong (2006), in some plant species, reducing the moisture content of the seeds to values generally below 20% is a key procedure to enable the medium- to long-term preservation thereof. To these authors, the high moisture content of the seeds is also one of the major causes of loss of germination of these propagules during the storage process, a fact usually caused by the high-respiratory rate of the embryos and the increased action of deteriorative microorganisms.

Determining a protocol for seed cryopreservation

As for testing the tolerance of *P. aduncum* and *P. hispidinervum* seeds to 24-h immersion in LN (−196°C), it was also found that the germination rate of the propagules was around 90% in all of the evaluated treatments (Table 3), thus demonstrating that the seeds of the species studied were also tolerant to cryopreservation, even when cryoprotectants were not used in the process.

Table 3. Tolerance of *P. aduncum* and *P. hispidinervum* seeds to cryopreservation for 24 h in liquid nitrogen (LN; −196°C).

Treatments	Germination (%)[a]	
	Piper aduncum	*Piper hispidinervum*
Control – without conservation	88.7	97.1
(–) Cryoprotectant	88.4	87.1
(+) PVS2	85.1	94.2
(+) PVS2 cooled	85.1	92.2
(+) PVS2 modified	90.2	98.5
(+) PVS2 modified and cooled	86.3	93.8
CV (%)	6.2	9.8

[a]Not significant by the *F* test. PVS2 vitrification solution (Sakai et al. 1990) were composed of 30% glycerol (v/v), 15% ethylene glycol (v/v), 15% DMSO (v/v), and 40% MS medium. For the modified PVS2 vitrification solution, the concentration of ethylene glycol was changed from 15 to 30% (v/v), in detriment to the concentration of MS medium, which fell from 40 to 25% (v/v). For the cooling process of the cryoprotectants, they were subjected to a 30-min ice bath. Each treatment was formed by four repetitions with 25 seeds per plot.

Results different from those obtained by Galdiano et al. (2012), in the study on cryopreservation efficiency of *Dendrobium* hybrid seeds and protocorms, whereby—in the absence of PVS2 cryoprotectant and derivatives—it was found that propagules lost 100% of their viability, with 0% germination at the end of storage in LN. However, this fact is significantly reduced by using cryoprotectants, whereby seed viability only decreased from 85.7 to 77.6%, in the case of the most efficient cryopreservative.

According to Sakai and Engelmann (2007), these results are mainly due to the fact that the cryoprotectants are chemical compounds that cause water to pass from the liquid phase to a vitreous phase, thereby avoiding the damaging effects caused by the ice crystals formed inside and outside the plant cells during the process for freezing in LN. Thus, depending on the species in question, their use may be responsible for significantly increasing the germination rates of seeds after storing at −196°C.

For Engelmann (2011), during seed cryopreservation, the decrease in the moisture content thereof can also result in a higher percentage of germination. According to this author, removing water from within the propagules is often one of the most critical phases of the process, because this water—when frozen—can form ice crystals, which can cause severe damage to the cell membrane system of the embryos, causing them to collapse and in many cases resulting in the inviability of the seeds.

In the study on cryopreservation of *Bletilla formosana* seeds, Wu et al. (2013) found that when the diaspores of the species were stored in LN without drying, with roughly 50% moisture, the germination percentage fell from 76.8 to only 1.8%. But when the seeds were dried for 24 h in a forced-air circulation oven or on desiccants with silica gel, reaching roughly 10% moisture, their germination percentage was reduced only 8% on average, reaching values near 70% at the end of cryopreservation.

However, the results obtained in this study suggest that the initial moisture content of the *P. aduncum* and *P. hispidinervum* seeds (18.1 and 12.7%, respectively) also does not constitute a limiting factor for the cryopreservation thereof in LN. Decruse and Seeni (2003), studied the feasibility of cryopreservation of seeds of six different *Piper* species (*P. mullesua, P. attenuatum, P. argyrophyllum, P. trichostachyon, P. galeatum*, and *P. nigrum*), also reported that with moisture contents from 10.2 to 13.9%, the diaspores of these species also showed high germination, after a week of storing at −196°C, with averages ranging from 60.0 to 73.3%.

Medium- to long-term assessment of the protocols developed for the ex-situ conservation of seeds

Finally, in the experiment involving medium- to long-term preservation of *P. aduncum* and *P. hispidinervum* seeds at sub-zero temperatures (cold room; −20°C) and cryogenic temperatures (LN; −196°C), also did not show the existence of statistical differences between different storage times (0, 180, and 360 days) for the germination variable, which presented averages of around 82.4% at the end of this process (Table 4).

Table 4. Storage of *Piper aduncum* and *P. hispidinervum* seeds at sub-zero temperature (cold room; −20°C) and cryogenic temperature (LN; −196°C) for up to 360 days.

Conservation period (days)	Conservation method	Germination (%)*	
		Piper aduncum	*Piper hispidinervum*
0	–	73.2	85.0
180	LN	80.6	85.0
	−20ºC	84.0	89.0
360	LN	86.0	74.0
	−20ºC	88.0	80.0
CV (%)		21.6	12.4
		Seedlings height (cm)**	
0	–	1.3	0.7 ab
180	LN	1.2	0.8 ab
	−20ºC	1.2	0.9 a
360	LN	1.2	0.6 b
	−20ºC	1.1	0.5 b
CV (%)		13.1	14.7

*Not significant by the *F* test. **Means followed by different letters are significantly different by the Tukey's test at 5%. Each treatment was formed by four repetitions with 25 seeds per plot.

In this test, it was also found that in all treatments employed, seeds of both species resulted in normal seedlings (Figure 1), with mean heights of 1.2 cm in *P. aduncum* and 0.7 cm in *P. hispidinervum*, after 30 days. However, in the case of the latter species, it was found that after 360 days of preservation, regardless of the method used, there was a small reduction in height of the germinated plants, which exhibited a decrease of up to 0.2 cm in the case of storage in a cold room at −20°C; however, such decrease did not affect the subsequent development of these cultures.

Similar results to those reported by Souza et al. (2016) in the cryopreservation of *Physalis peruviana* seeds with up to 11% water content, whereby it was also observed that—after 12 months of storing in LN—that around 90% of the preserved diaspores showed normal germination process, resulting in healthy seedlings suitable for development in a nursery.

After 12 months of storing in a cold room at −20°C, Mello et al. (2013) also observed nearly 90% germination rates in *Caesalpinia echinata* seeds with moisture content near 10%, and such values remained statistically constant for periods up to 48 months.

These results demonstrate that the preservation of seeds at sub-zero temperatures (cold room; −20°C) and cryogenic temperatures (LN; −196°C) are viable and efficient strategies for medium- to long-term storage of plant genetic resources of the species studied in our experiment.

Conclusions

P. aduncum and *P. hispidinervum* seeds are tolerant to desiccation and low temperatures, indicating that their physiological behavior is the orthodox type, allowing them to be preserved in the medium to long term at subzero temperature (cold room;

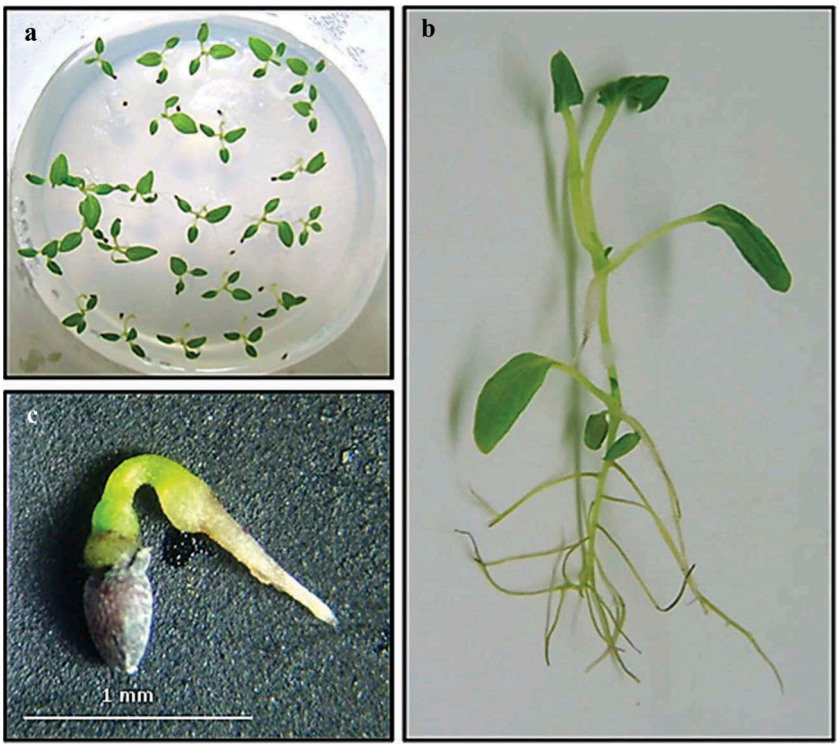

Figure 1. Appearance of *Piper aduncum* and *P. hispidinervum* seed kept for 360 days in a cold room at 20°C and in LN. (a) *P. hispidinervum* seeds germinated after storing in a cold room at −20°C. (b) Seedling of *P. aduncum* with normal development after storing its seed in LN. (c) *P. hispidinervum* seed showing emergence of the radicle.

−20°C) and cryogenic temperature (LN; −196°C) without the use of cryoprotectants and preferably with a moisture content no higher than 12%.

Disclosure statement

No potential conflict of interest was reported by the authors.

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