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Short-term storage of seeds and cryopreservation of embryonic axes of *Lepisanthes fruticosa*

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Author contributions

Suryanti Bustam designed the study, performed the laboratory work, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Mohd Shukri Mat Ali assisted in planning the study and drafting the manuscript. Uma Rani Sinniah assisted in drafting the manuscript. Nur Atisha Shamsuddin and Abdul Muhaimin Abdul Kadir assisted in the laboratory work. All authors read and approved the final manuscript.

Abstract This work highlights short-term storage of recalcitrant *Lepisanthes fruticosa* seeds and long-term conservation attempts of its embryonic axes (EAs) through cryopreservation. Short-term storage was carried out using fresh seeds at 54 % moisture content and stored at 8 ±1 °C and 25 ±2 °C for 7 weeks. Three variations to sterilization were attempted to optimize survival while keeping contamination low for cryopreservation. Cryopreservation using two different methods were tested, namely vitrification and the encapsulation vitrification method. Vitrification technique involved the pre-culturing of EAs overnight in different sucrose pre-culture concentrations (0, 0.2, 0.4 and 0.6 M) prior to, loading, dehydration with plant vitrification solution (PVS2), rapid immersion into liquid nitrogen (-196 °C), rapid warming, unloading and recovery. While, encapsulation vitrification involved encapsulation of the EAs using 3 % sodium alginate followed by exposure to different duration (0, 10, 20, 30, 40 and 50 minutes) of PVS2 prior to cryopreservation. *L. fruticosa* seeds can be safely stored for short-term with no loss in germination up to 7 weeks of storage either at 8 ±1 °C or 25 ±2 °C. This study also showed that EA of *L. fruticosa* was amenable to cryopreservation, 13.0 – 66.67% of viability was obtained when the EAs were cryopreserved using the vitrification technique while

the best result was obtained (66.67 % viability) when the EAs were pre-cultured with 0.4 M sucrose prior to exposure to PVS2 and liquid nitrogen. Cryopreservation of EAs using the encapsulation-vitrification method was unsuccessful.

Keywords: Recalcitrant seeds · seed storage · cryopreservation · embryonic axes

Declarations

Not applicable

Abbreviations

EAs Embryonic axes

MS Murashige & Skoog

PVS2 Plant vitrification solution 2

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Introduction

Ceri Terengganu or *Lepisanthes fruticosa* is an underutilised tropical fruit tree from the family *Sapindaceae*. It can be found in Southeast Asia regions such as Malaysia, Thailand, Indonesia, Myanmar, Indo-china and Borneo. In Malaysia, the species grows naturally in forest mainly in the state of Johor and Terengganu (Abd. Latif et al. 2016). The trees are rarely cultivated and predominantly used as ornamental tree due to its esthetic values that lies on the attractive tree shape, light purple young foliage and long purple inflorescence which then turns into clusters of remarkable shiny bright red fruits (Rukayah 2006). Based on the ethnobotanical studies, this species is used in traditional medicine by rural folks. The seeds are consumed roasted, the roots are used in a compound for treating itchiness and to reduce body temperature during fever. Fruits are rich in antioxidants and the antioxidants activity value is reportedly higher compared to that of popular commercialized fruits such as guavas, oranges and apples by several folds (Mirfat and Salma 2015). The fruit can be consumed fresh when fully ripened (Abd. Latif et al. 2016) or blended into juices. This species has future prospect to be exploited for commercial production by farmers in Malaysia due to its bioactivity, nutritional values and uses.

In view of its importance and potential, storage and conservation methods of the genetic materials should be developed to prevent the species from being threatened by extinction. For the purpose of germplasm, often ex-situ conservation is practised. One of the popular ex-situ conservation methods is seed storage in genebanks, whereby seeds are dried to low moisture content and kept at low temperature (5 % water content and –18 °C) in order to achieve long storage life. In addition, determination of a suitable method to store the seeds will be useful for researchers and growers who are working with this species. Despite the simplicity of the above-mentioned storage method, it is only applicable to orthodox seeds (Hong and Ellis 1996). However, seeds can also fall into the intermediate (can tolerate some desiccation but is chilling sensitive) or recalcitrant (drying and chilling sensitive) categories. Intermediate seeds can be stored for short-term (1 to 3 years) while recalcitrant seeds have extremely limited storability of just a few weeks and the germination percentage will decrease rapidly. Therefore, their

germplasm cannot be maintained for a long period through seeds such as orthodox seed using traditional genebanking at -18 to -20 °C (Bajaj 1995). To date, little information is available on seed storage of *L. fruticosa*. Previous study conducted at our laboratory, revealed that seeds of this species had low tolerance to desiccation where drastic reduction in the germination percentages was observed from 100 % (fresh seeds) to 90, 83 and 17 % as the seeds were dried to the moisture content of 50, 40 and 30% respectively (Suryanti et al. 2019). Hence, it is clear that *L. fruticosa* does not belong to the orthodox type, and desiccation below 40 % is deleterious.

Species with recalcitrant seeds generally originate from tropical or subtropical region. They normally possess high moisture content at maturity, cannot withstand intensive desiccation and most of them are also sensitive to chilling (King and Roberts 1979). Storing recalcitrant seeds is difficult, dry storage is impossible due to the sensitivity towards desiccation and wet storage is also difficult because the temperature must be low enough to prevent germination, but not too low causing chilling injury. The success of recalcitrant seed storage is highly influenced by the technique used and the species characteristics. A universal method for long-term storage has yet to be established for large majority of recalcitrant seeds. Hence, it is essential to identify feasible storage method for every recalcitrant seed species as response to storage is species-specific (Wen 2009). Therefore, it is of utmost importance that storage related research is attempted to establish both short-term as well as long-term storage methods. Since preliminary studies with *L. fruticosa* seeds (Suryanti et al. 2019) shows sensitivity to desiccation, the first part of this research looks into the establishment of short-term storage based on the ability of maintaining seed viability at high moisture content without causing it to germinate in storage. This was done by looking at the effect of storing fresh seeds with high moisture content at two different temperatures on its storability and germination. Seeds stored under the above-mentioned conditions are still metabolically active and metabolism-related deterioration will unavoidably accumulate and adversely affect seed viability. Therefore, recalcitrant seeds cannot be stored for long-term and cannot meet the purpose of long-term conservation of genetic resources (Wen 2009). Generally, genetic resources conservation of species with recalcitrant seeds is carried out by maintaining live plants at field germplasm or arboretum. In addition to the requirement of large space and high labour cost, field collection is exposed to loss due to deforestation and natural disasters such as pests and diseases, flash floods, droughts, heat waves and extreme weather (Malik et al. 2011). In vitro tissue culture method can be used however, they are also at risk of loss due to microbial contamination, fungi, somaclonal variations and human error during subculture (Sen-Rong and Ming-Hua 2013).

Cryopreservation of plant material is currently the only option available for long-term germplasm conservation of species with recalcitrant seeds (Engelmann 2011). Through this technique, theoretically samples can be kept viable for an unlimited period due to storage below the freezing temperature which is -196 °C provided by liquid nitrogen. At this temperature, cellular divisions and metabolic process in the conserved tissues are halted thus reducing deterioration (Chaudhury 2003). According to Reed (2008), cryopreservation technique has been established in various fields such as in medical and reproductive biology of animal. Cryopreservation application for plant tissue is usually to preserve in vitro cultures in the horticultural industry. Several studies on successful cryopreservation of species with recalcitrant seeds namely *Hevea brasiliensis* Muell. (Normah et al. 1986), *Fortunella polyandra* (Al-Zoubi and Normah 2009), *Artocarpus heterophyllus* (Chandel et al. 1995), *Averrhoa carambola* (Normah 2000), *Citrus jambhiri* (Malik et al. 2000), *Litchi sinensis* (Chaudhury et al. 2000) and *Cocos nucifera* (Assy-Bah and Engelmann 1992) have been reported.

Recalcitrant seeds cannot be cryopreserved as they are normally large and are shed at high water content. Therefore, excised embryos or embryonic axes are commonly used as explants (Devi et al. 2019). In India, embryonic axes of *Madhuca indica*, *Manilkara hexandra* and *Salvadora oleoides* and embryos of *A. marmelos* have been successfully cryopreserved in the Cryogenebank at the National Bureau of Plant Genetic Resources (NBPGR) New Delhi (Malik et al. 2011). There are several protocols that have been used for cryopreserving seeds, embryonic axes and zygotic embryos including desiccation-freezing, pregrowth desiccation, encapsulation-dehydration, vitrification and encapsulation-vitrification (Devi et al. 2019; Rohini et al. 2016; Wen et al. 2013; Rajae et al. 2012)

For successful cryopreservation, it is essential to avoid intracellular ice formation in the tissue during the freezing stage which can cause damage to the cell membrane. Intracellular ice formation can be avoided by removal of freezable water in the tissue. Although earlier studies focused on direct desiccation and controlled freezing methods (Wesley-smith et al. 1992; Makeen et al. 2005) recent studies have shown that vitrification-based procedure which involves chemical dehydration before freezing by exposure of samples to highly concentrated cryoprotectant solutions (Engelmann 2009) is more successful. Vitrification is the physical process where transition of a highly concentrated aqueous solution occurs directly from the liquid phase into an amorphous or glassy state during cooling, avoiding ice crystal formation (Fahy et al. 1984). One popular example of vitrification solution that is widely used is plant vitrification solution 2 (PVS2), which is a solution containing a combination of very high concentrations of glycerol, ethylene glycol, and DMSO in basal culture medium containing 0.4 M sucrose (Sakai et al. 1990). The vitrification technique involves several important steps including pre-culture, loading, dehydration with vitrification solution (PVS2), rapid immersion into liquid nitrogen (-196 °C), rapid warming, unloading and recovery (Engelmann 2009). Example of successful cryopreservation of recalcitrant species through vitrification-based technique were *Parkia speciosa* Hassk. (Sinniah and Gantait 2013), *Hevea brasiliensis* (Nakkanong and Nualsri 2018), *Elaeis guineensis* (Periasamy et al. 2011), *Garcinia mangostana* L. (Ibrahim and Normah 2013), *Nephelium ramboutan-ake* (Chua and Normah 2011). The encapsulation-vitrification method has been reported as having advantages over traditional cryopreservation methods, and may have wider applications for germplasm conservation (Hirai and Sakai 1999). It involves a combination of 2 procedures which are encapsulation and vitrification. The sodium alginate beads containing explants produced through encapsulation are subjected to freezing through vitrification. It has been observed that recovery rate of apices frozen using this technique was 30 % higher than with the encapsulation-dehydration technique (Malik and Chaudhury 2019).

This study on *L. fruticosa* aimed to establish a short-term storage method using seeds and a long-term conservation method using EAs via cryopreservation. Specifically, it looked into the feasibility of using vitrification and encapsulation-vitrification methods on cryopreservation of *L. fruticosa* EAs. To support the cryopreservation study, preliminary experiment on the development of the tissue culture techniques for EAs excised from seeds of *L. fruticosa* was conducted to establish a suitable protocol for recovery after cryopreservation.

Materials and methods

Plant Materials

Fruits of *L. fruticosa* were obtained throughout the year from a 4-year-old plant at the Malaysian Agriculture Research and Development Institute (MARDI), Serdang Field

Germplasm. Seeds (Fig.1.a) were extracted from the ripe fruits (Fig.1.b) at maturity indexes 7 – 8 (~5 weeks after flower bloom). The seeds were then washed under running tap water to remove traces of flesh prior to disinfection using Dettol solution for 30 seconds. The seeds were rinsed with distilled water and subsequently blotted dry using tissue paper and were left to surface dry in the air-conditioned laboratory (25 ± 2 °C, 60 % relative humidity) for 30 minutes.

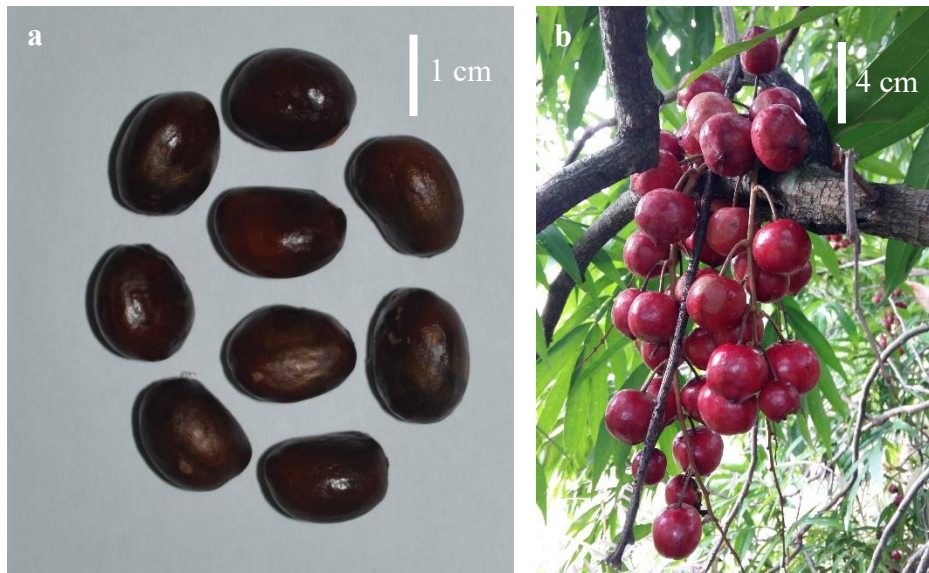


Fig. 1 Seeds and fruits of *L. fruticosa*. **a** Seeds, **b** Fruits

Effect of storage temperature (8 ± 1 °C and 25 ± 2 °C) on germination of seeds

Seeds extracted from freshly harvested fruits with a moisture content of 54 % were kept in an air tight container and stored in a commercial refrigerator (8 ± 1 °C) and air-conditioned room (25 ± 2 °C). Seeds were taken out weekly until the 7th week of storage. Ten seeds were removed at every storage duration and sown on sterilized sand media to determine the ability to germination. The germination test was done in an air-conditioned room (25 ± 2 °C, 60 % relative humidity) in three replications. Non-stored seeds (0 week) were used as a control treatment. Germination percentage of seeds was recorded after 5 weeks.

Sterilization treatments prior to the culture of embryonic axes (EAs)

Seeds extracted from the ripe fruits were rinsed with Dettol solution followed by sterilization with distilled water. Each seed was cut into half using a scalpel blade and the seed coats peeled. This was followed by washing under running tap water for 30 minutes, a quick wash with Dettol solution and rinse with distilled water. The seeds were then brought into the laminar air flow cabinet. Three variations were tested in this experiment, 1) seeds were surface sterilized with 30 % Clorox mixed with 3 drops of Tween 20 for about 30 minutes prior to the excision of EAs measuring 3 mm (length and width) from the seeds (Fig. 2). The seeds were washed with sterilized distilled water thrice and the embryonic axes (EAs) were then carefully excised from the seed without any injury. The EAs were placed in a 50 ml conical flask and washed with 70 % ethanol for 30 second followed by 10 % Clorox mixed with 3 drops of Tween 20 for 5 minutes, 2) EAs were excised first from the prior to surface sterilized with 30 % Clorox mixed with 3 drops of Tween 20 for about 30 minutes. The EAs were washed with 70 % ethanol

for 30 second followed by 10 % Clorox mixed with 3 drops of Tween 20 for 5 minutes, and 3) same technique was applied as in technique 1 except seeds were surface sterilized with 50 % Clorox mixed with 3 drops of Tween 20 for about 15 minutes and the treatment of EAs using 10 % Clorox was done for only 2 minutes. For all techniques tested, the EAs were then rinsed with sterilized distilled water thrice and blotted dry on a sterilized filter paper in a Petri dish. Ten EAs were then singly cultured into culture tubes containing full strength basal Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with myo-inositol (100 mg/L), glycine (2.0 mg/L), nicotinic acid (0.5 mg/L), pyridoxine HCL (0.5 mg/L), thiamine HCL (0.1 mg/L), ferrous sulfate (27.8 mg/L), Na₂ EDTA (37.3 mg/L) and sucrose (20 g/L). The pH of the medium was adjusted at 5.7 – 5.8 with 0.1 M NaOH and 0.1 M HCL and autoclaved at 121 °C and 15 p.s.i for 15 minutes. Experiment was replicated thrice and cultures were kept under 18/6 hour photoperiod (60 $\mu\text{mole m}^{-2} \text{s}^{-1}$), 60 % relative humidity at 25 ± 2 °C. Viability percentage (EAs expand and turn green) was recorded after 2 weeks while clean culture and survival percentage (EAs formed complete plantlet) was recorded after 5 weeks of culture.

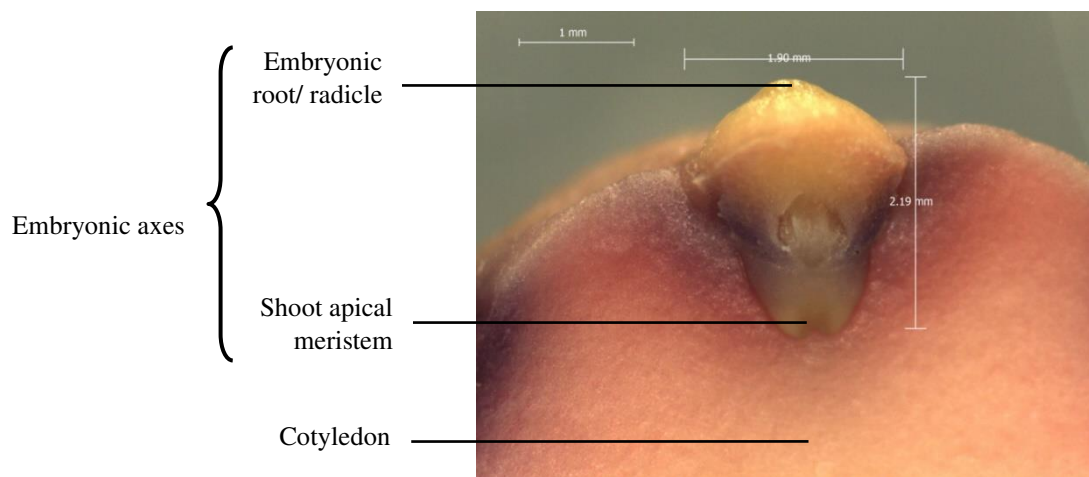


Fig. 2 View of embryonic axes (EA) through stereomicroscope upon removal of one cotyledon

Effects of embryonic axes (EAs) cutting sizes on regeneration

Seeds were surface sterilized using sterilizing treatment 1. The EAs were cut into 4 different diameter sizes namely 7 – 8 mm, 4 – 5 mm, 3 mm and 2 mm. Ten EAs were then singly cultured onto basal MS medium prepared in culture tubes and this was replicated thrice. Cultured EAs were placed in a culture racks in a room with 25 ± 2 °C temperature with 16/8 hours of dark and light photoperiod at 60 $\mu\text{mole m}^{-2} \text{s}^{-1}$ provided by LED light. Survival percentage of EAs was recorded after 5 weeks.

Effect of sucrose pre-culture concentrations on cryopreservation of EAs through vitrification technique

EAs measuring 3 mm (length and width) were used as the explant. The EAs were pre-cultured overnight in the dark in MS medium supplemented with 0, 0.2, 0.4 and 0.6 M sucrose. The pre-cultured EAs were then placed into the cryotubes filled with Loading solution (2 M glycerol + 0.4 M sucrose) for 20 minutes and subsequently dehydrated with PVS2 solution (2 ml: 30 % (w/v) glycerol + 15 % (w/v) ethylene glycol + 15 % (w/v) DMSO-dimethyl sulfoxide + 0.4 M sucrose, pH 5.8) for 5 minutes and then PVS2 solution was replaced with 2 ml fresh PVS2 and

held for 20 min at 0 °C (ice bucket). The EAs in the cryotube were suspended in 0.5 ml PVS2 solutions, tied with cryocane and were directly plunged into Liquid Nitrogen (LN)(cryopreservation) for 1 hour. Non-LN exposed EAs were kept as control and processed in the same way as LN-exposed samples except for the exposure to LN. After LN exposure, rewarming was performed by rapidly immersing the cryotubes containing EAs in a water bath at 40 °C for 90 seconds. PVS2 solution was replaced with washing solution (MS + 1.2 M sucrose) twice at 10 minutes interval, blotted on filter paper layer on MS basal medium for one day and then transferred to recovery [MS medium + 0.5 mg/L BAP (6-Benzylaminopurine) + 0.1 mg/L GA₃ (gibberellic acid)] in dark. After 7 days of incubation in the dark, the EAs were transferred to standard illumination conditions. The EAs were transferred after 4 weeks to medium supplemented with 1.0 mg/L BAP (6-Benzylaminopurine) and 0.5 mg/L NAA (1-Naphthaleneacetic acid). Each sucrose concentration treatment consisted of 10 samples and was replicated 5 times. Viability percentage of EAs was recorded after 12 weeks.

Effect of different duration of PVS2 exposure on cryopreservation of EAs through encapsulation vitrification technique

EAs measuring 3 mm were used as the explant. The EAs were encapsulated with sodium alginate and calcium chloride solution to form calcium-alginate beads. The encapsulated EAs were pre-cultured overnight in the dark in MS medium supplemented with 0.4 M sucrose. Following which, the same process as for vitrification was used except on variations on the duration of PVS2 exposure namely 0, 10, 20, 30, 40 and 50 minutes. Each duration treatment consisted of 10 samples and was replicated 5 times. Viability percentage of EAs was recorded after 12 weeks.

Statistical analysis

All data were analyzed by analysis of variance (ANOVA) using Statistical Analyses System Software (SAS) release 9.4. This study adopted the Completely Randomized Design (CRD). Means was differentiated at $P \leq 0.05$ level of significance using Tukey's Studentized Range (HSD) Test.

Results and discussion

Effect of storage temperature (8 ± 1 °C and 25 ± 2 °C) on germination of seeds

Germination percentage of fresh seeds stored up to 7 weeks at 8 ± 1 °C and 25 ± 2 °C were 100 % (Fig. 3). The results obtained is unusual for recalcitrant seed whereby no reduction in germination was observed over 7 weeks of storage. The response of *L. fruticosa* seeds to storage at low temperature of 8 ± 1 °C contradicted previous report for most tropical recalcitrant seeds which stated that storage temperatures below 15 °C were lethal (Bedi and Basra, 1993). Our study proved that *L. fruticosa* seeds were not sensitive to chilling at 8 ± 1 °C. In this study, although seeds stored at 25 ± 2 °C gave comparable results with storage at 8 ± 1 °C, however, precocious germination and fungal contamination occurred during second week of storage (data not shown). In order to reduce contamination, the seeds were disinfected using Dettol solution prior to germination (Eggers et al. 2007). Incidence of precocious germination and microbial contamination in seeds when stored in ambient conditions beyond 18 days was also reported for other recalcitrant species namely *Garcinia indica*, *Syzygium cumini* and *Madhuca*

indica (Malik et al. 2011). As recalcitrant seeds are not tolerant to desiccation, the seeds must be stored at their shedding water content, since even mild dehydration badly affects viability (Eggers et al. 2007). According to Berjak and Pammenter (2004), fungal contamination is one of the constraints of storing seeds in hydrated form and this can be reduced by cleaning and decontaminating the seeds prior and during storage. Hydrated seeds remain metabolically active even during storage and this often results in the precocious germination in stored recalcitrant seeds (Berjak et al. 1989; Pammenter and Berjak 2014). To reduce the rate of metabolism and germination in hydrated recalcitrant seeds, they could be stored at low temperatures (but above zero). However, to avoid chilling injury, storage must be carried out at the lowest temperature with no adverse effects on vigor and viability (Pammenter and Berjak 2014). Our results suggested that optimum conditions for short-term storage for *L. fruticosa* seeds is to store fresh (hydrated) seeds at low temperature of 8 ± 1 °C as they did not show reduction on germination ability even up to 7 weeks of storage, with no occurrence of precocious germination and microbial contamination. Hydrated storage of recalcitrant seeds is strictly for short-term, either a few days for seeds of many tropical species or possibly a year or more for seeds of chilling-tolerant temperate species that can be maintained at temperatures close to zero (Pammenter and Berjak 2014). Since *L. fruticosa* seeds recorded 100 % viability after storage for 7 weeks, it has the potential to survive in storage for longer duration. The current study only tested for 7 weeks of storage; therefore, the critical storage duration was not obtained. It is suggested that prolonged storage under this condition is carried out to obtain maximum duration that the seeds can maintain their viability.

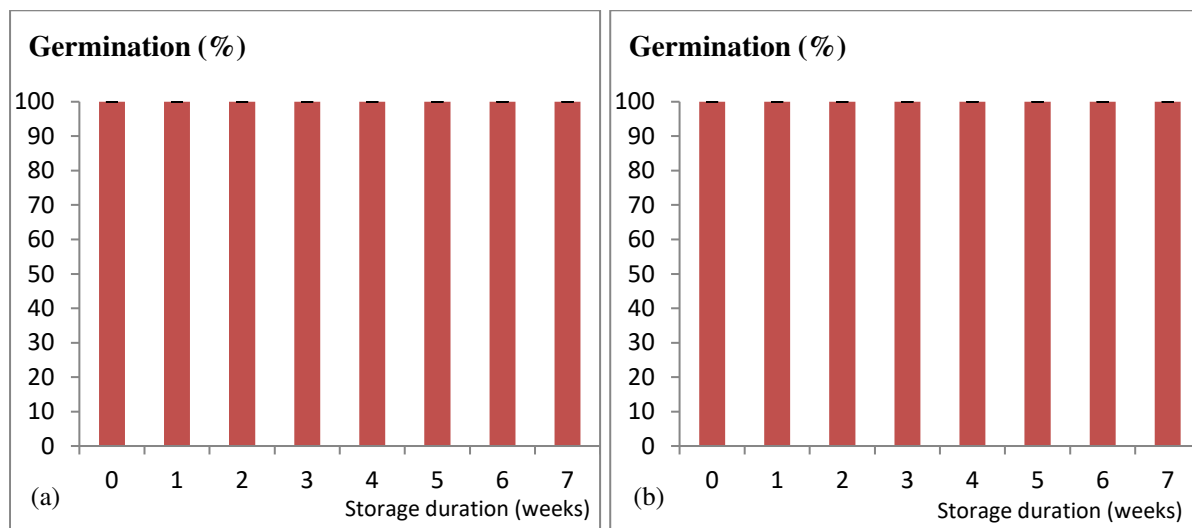


Fig. 3 Germination % of fresh *L. fruticosa* seeds stored for 7 weeks at different temperature. **a** 8 ± 1 °C, **b** 25 ± 2 °C

means with different letters between germination percentages are significantly different at $p \leq 0.05$

Sterilization treatments prior to the culture of embryonic axes (EAs)

A previous study on sensitivity towards desiccation in *L. fruticosa* seeds (Suryanti et al. 2019) made us conclude that they belong to the recalcitrant category. Thus, long-term storage for germplasm conservation via seed storage is not an option. Cryopreservation is the only feasible option to ensure longevity in storage especially for seeds that are recalcitrant in nature. It has been established that big seeds are not suitable for cryopreservation, while the zygotic embryos or embryonic axis are recommended as due to its size, desiccation can occur evenly (Chin 1988). The ability to surgically dissect out the growing portion of the seed (embryonic axis)

and germinate it in vitro was a critical aspect that led to successful cryopreservation of recalcitrant species (Normah et al. 1986). However, the growth and development of the embryonic axis in culture is influenced by sterilization process to obtain clean culture, the types and size of explant and the media. The use of different concentrations of sterilant and variations in the sterilization protocol on *L. fruticosa* had no effect on the percentage of clean culture with all treatment giving 100 % clean cultures (Fig. 4). However, the ability to produce clean culture alone cannot be a satisfactory factor to decide that a particular technique is optimum. The technique must allow the EAs to grow into complete plantlet bearing healthy shoot and root. Although technique 2 recorded full clean culture however, the explants were unable to continue growing which it recorded zero in viability and survival percentages. This might due to the injuries caused by the direct exposure of EAs to high concentration of Clorox (30 %). As the EAs have been excised from the cotyledon prior to the exposure to Clorox solutions, thus there were no mechanisms which can protect them from injuries caused by Clorox. This study also proved that 50 % Clorox is harmful when directly exposed to the seeds as in technique 3 which there was no survival recorded. Treatment 1 could be considered as optimum as it resulted in 100 % viability (EAs expanded and turned green) and 87 % survival (EAs formed complete plantlet with normal shoot and root). Clorox contains sodium hypochlorite which act as the disinfectant agent which has a strong oxidizing property which makes it highly reactive with amino acids, nucleic acids, amines, and amides, it is highly effective against all kinds of bacteria, fungi, and viruses. However, care must be taken during sterilization processes as direct contact of the tissue with disinfectant during the sterilization process may have a severe effect on the viability and regeneration capacity depending on concentration, temperature and application periods of disinfectant (Yildiz et al. 2012).

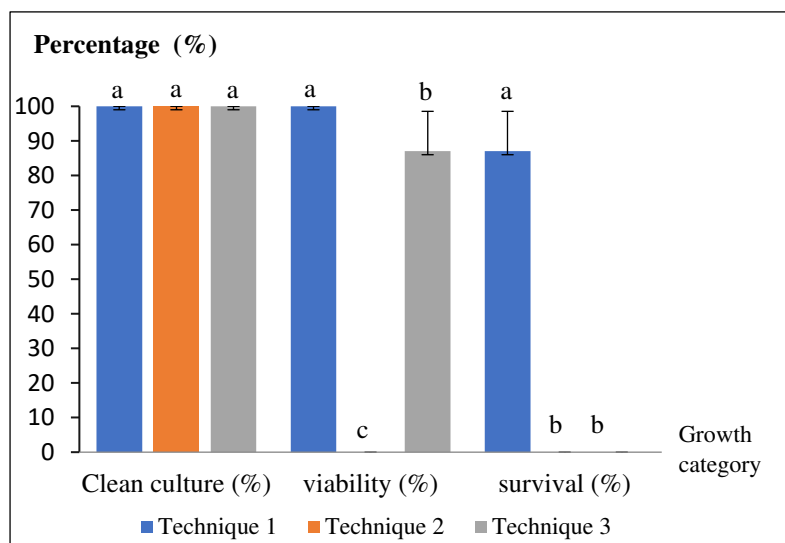


Fig. 4 The effects of variations in sterilization on percentage of clean cultures, viability and survival of embryonic axes (EAs) of *L. fruticosa* cultured on MS medium
means with different letters within each category (clean culture, viability or survival) are significantly different at $p \leq 0.05$

Effects of embryonic axes (EAs) cutting sizes on regeneration

Recalcitrant seeds are mostly too large to permit the rates of drying and cooling required for vitrification, thus the use of excised embryonic axes (EAs) as suggested by Pammenter and Berjak (2014), as the suitable explants to use. Excised embryonic axes are the preferred explants for cryopreservation mainly because of high composition of meristematic tissues with high morphogenetic potential (Malik et al. 2003), in addition to the small size. In some species,

the zygotic embryo is detachable from the cotyledon while in others a portion of the cotyledon has to be excised together with the embryonic axis. In this study, the use on EAs varying in sizes resulted in EAs with bigger size (> 4 mm) having 100 % survival (Fig. 5) and produced complete plantlet with root and shoot (Fig. 6). EA measuring 3 mm had 87 % survival while anything smaller was not able to survive. According to Chin (1988), the smaller the size, the more responsive it is to desiccation, a process highly critical for cryopreservation, however it should not be too small as to disrupt normal growth and development. Hence, the use of EAs measuring 3mm is recommended in this study for the following experiments.

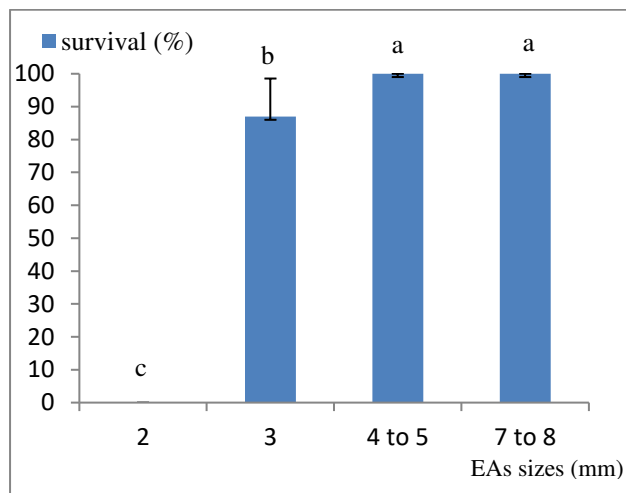


Fig. 5 The effects of embryonic axes (EA) sizes on regeneration (survival percentage). means with different letters between the survival percentages are significantly different at $p \leq 0.05$

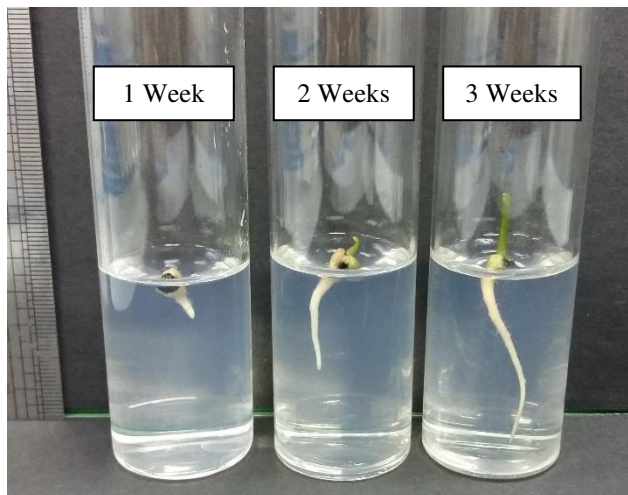


Fig. 6 The normal developmental stages of embryonic axes (EAs) of *L. fruticosa* in tissue culture to form complete plantlet (survival) after 3 weeks

Effect of sucrose pre-culture concentrations on cryopreservation of EAs through vitrification technique

The key for successful cryopreservation by vitrification is to optimize the steps involved in the vitrification technique. The experiments performed with *L. fruticosa* in EAs allowed comparison of two different vitrification procedures. The current study, was performed to obtain suitable concentration of sucrose as pre-treatment prior to cryopreservation (Liquid nitrogen exposure/+LN) through vitrification with PVS2 solution. Based on the results in Table

1, EAs from all concentration had 100 % viability without exposure to liquid nitrogen (-LN). After exposure to liquid nitrogen, the best results were obtained (66.67 %) by overnight preculture with 0.4 M sucrose prior to dehydration using PVS2 solution and cryopreservation. The addition of sucrose to the pregrowth medium has proved to be the key to success in the cryopreservation method described here. The optimal sucrose concentration for *L. fruticosa* (0.4 M) is very similar to that determined for *Musa* spp. meristem culture by Panis et al. (1996) but preculture of *Musa* spp. took 3 weeks instead of only 24 h for *L. fruticosa*. Sucrose plays an important role for success in cryopreservation where it could potentially reduce moisture content due to osmotic action. As a result, it lowered down the freezing point and the amount of freezable water present in tissues (Uragami et al. 1993; Panis et al. 1996). In addition, an indirect effect of sucrose could be the accumulation of endogenous compounds such as Proline induced in immature embryos of *Z. mays* by a mild osmotic stress, which then offer protection against further water stress and cryopreservation Delvallée et al. (1989). Based on Fig. 7, the growth of EAs after vitrification treatment before and after cryopreservation (-LN/+LN) were unable to produce plantlet with shoot and root but produced multiple globular embryos. This is a common phenomenon faced in cryopreservation studies where abnormal seedlings were produced in many species following cryopreservation and the conversion of these seedlings into normal plantlets remains a major challenge for many cryopreservation protocols (Peran et al. 2006, Steinmacher et al. 2007). In coconut, apart from the 20 % soil-established coconut seedlings after cryopreservation, they produced a further 23 % of recovered embryos which were viable. However, the embryos lacked roots or had stunted shoot and unable to produce normal plantlets (Sisunandar et al. 2010). Similar trend was found by Normah (1986) where various abnormalities in seedling growth after cryopreservation were observed in some of the treatments, hence, refinement of the technique is needed. The abnormal growth produced in the current study might be due to the toxic effect of the vitrification solution at full strength (100 %) and therefore, according to Mandal (2003) care must be taken to standardize the time and temperature of treatment as precisely as possible in order to get the normal growth after cryopreservation.

Table 1 The effect of different sucrose concentration (0, 0.2, 0.4 and 0.6 M) on the viability after 12 weeks of culture of post-cryopreserved embryonic axes through vitrification technique

Concentration of sucrose pre-culture	Viability (%)	
	Without cryopreservation (-LN)	After cryopreservation (+LN)
0 M	100 ± 0a	20 ± 0c
0.2 M	100 ± 0a	13.33 ± 5.77c
0.4 M	100 ± 0a	66.67 ± 5.77a
0.6 M	100 ± 0a	40 ± 0b

Means values with different letters within the same column are significantly different at $P \leq 0.05$

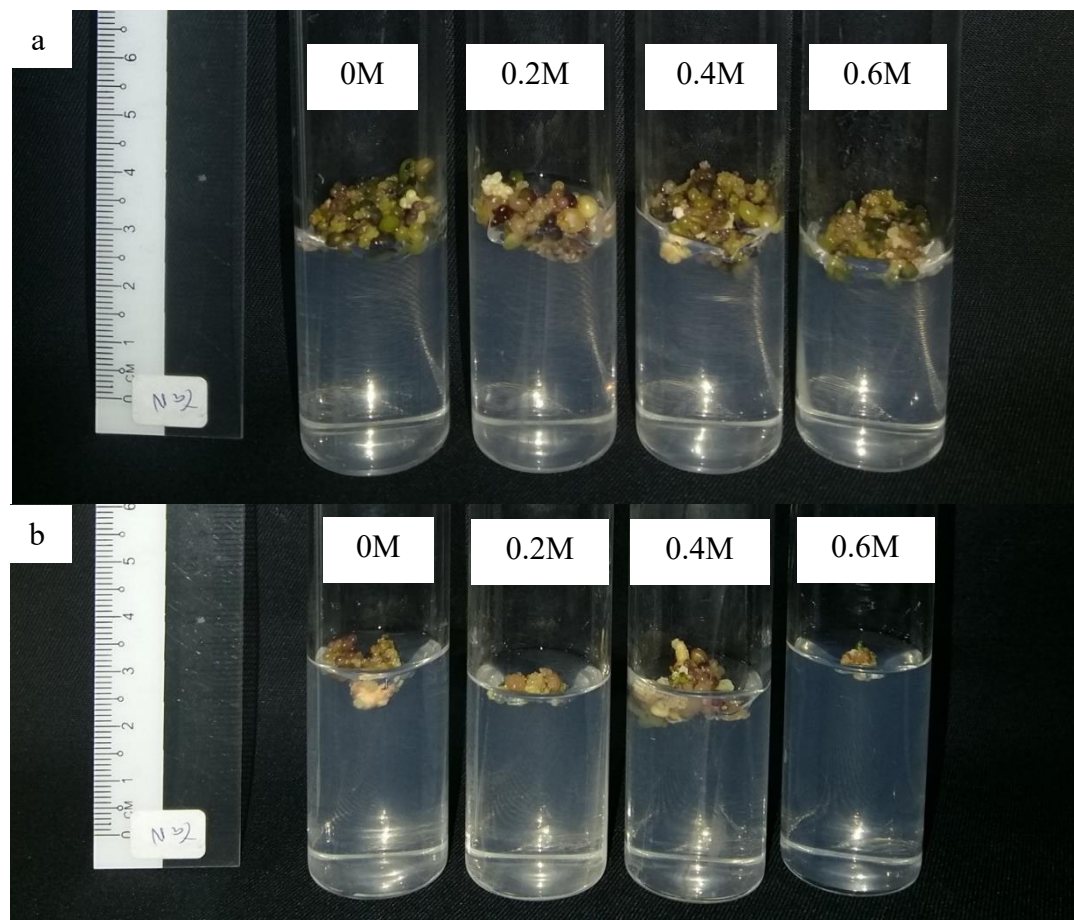


Fig. 7 The growth of EAs after vitrification treatment **a** without, **b** with cryopreservation (-LN/+LN) after 20 weeks

Effect of different duration of PVS2 exposure on cryopreservation of EAs through encapsulation vitrification technique

Based on Table 2, control treatment through the encapsulation-vitrification of *L. fruticosa* EAs without cryopreservation recorded 100 % viability for all PVS2 exposure treatment producing multiple globular embryos. However, cryopreservation of through the encapsulation-vitrification was unsuccessful as all of the EAs tested on different durations of vitrification solution (PVS2) exposure lost their viability after cryopreservation. This was most likely due to the high moisture content in the encapsulated propagules in order survive cryopreservation without the formation of intracellular ice crystals that can be lethal to the tissue during freezing by liquid nitrogen. Previous work by Wu et al., (2003) revealed that encapsulation has slowed down the dehydration processes in mango EMs and they recorded zero recovery after cryopreservation. Pammenter and berjak, (2014) stated that some studies have shown that EAs require protection in the form of coating to avoid direct exposure to toxic chemical and liquid nitrogen. However, the use of such treatment is not always successful and sometimes can be detrimental.

Table 2 The effect of different PVS2 exposure durations (0, 10, 20, 30, 40 and 50 minutes) on the viability after 12 weeks culture of post-cryopreserved embryonic axes through encapsulation-vitrification technique

PVS2 exposure durations	Viability (%)	
	Without cryopreservation (-LN)	After cryopreservation (+LN)
0 Minutes	100 ± 0a	0 ± 0a
10 Minutes	100 ± 0a	0 ± 0a
20 Minutes	100 ± 0a	0 ± 0a
30 Minutes	100 ± 0a	0 ± 0a
40 Minutes	100 ± 0a	0 ± 0a
50 Minutes	100 ± 0a	0 ± 0a

Means values with different letters within the same column are significantly different at $P \leq 0.05$

Conclusion

L. fruticosa seeds can be stored for at least 7 weeks when freshly collected seeds are kept in air-tight container either at 8 ± 1 °C or 25 ± 2 °C with 10 0% germination. Despite retaining high germination percentage, seeds stored 25 ± 2 °C were amenable to fungal contamination. Cryopreservation for conservation of *L. fruticosa* is possible as 66.67 % viability was obtained using freshly excised EAs precultured on 0.4 M sucrose for 24 hours prior to following the standard vitrification process. However, as this is the first study on *L. fruticosa*, further refinement to the protocol is necessary to ensure that the viable EAs can be successfully converted into healthy plantlets after exposure to liquid nitrogen.

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Figures



Figure 1

Seeds and fruits of *L. fruticosa*. a Seeds, b Fruits

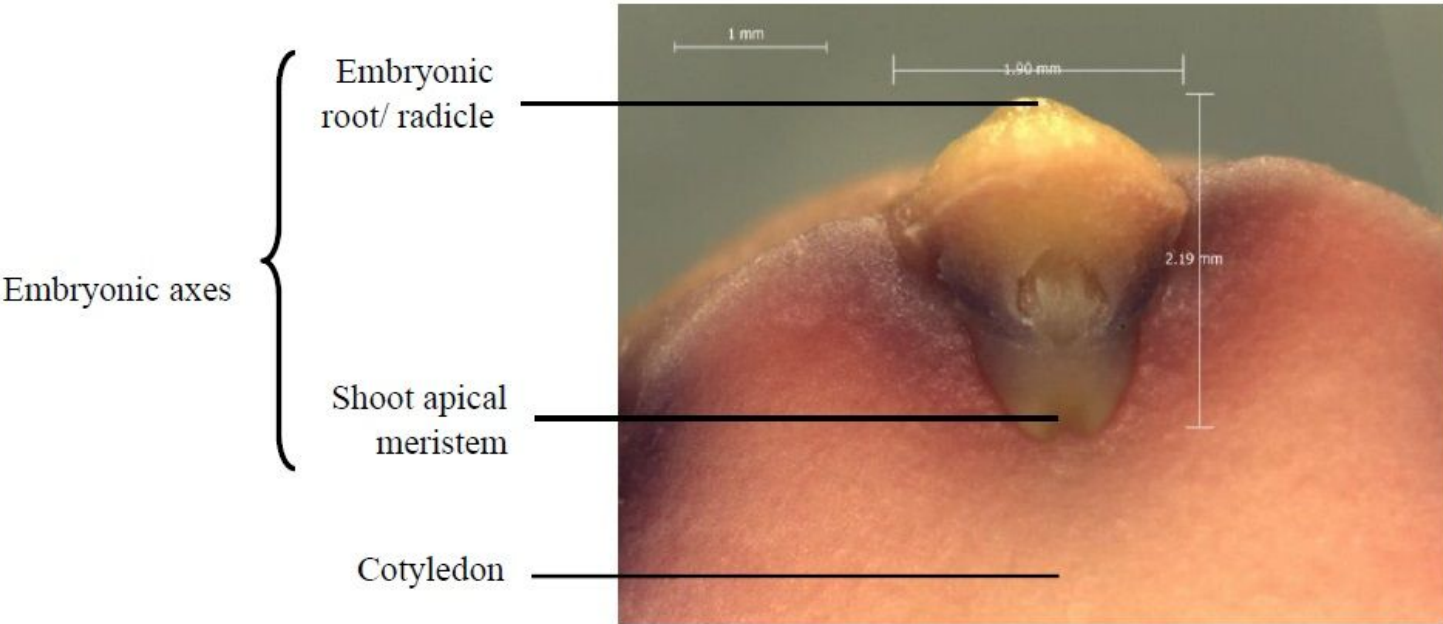


Figure 2

View of embryonic axes (EA) through stereomicroscope upon removal of one cotyledon

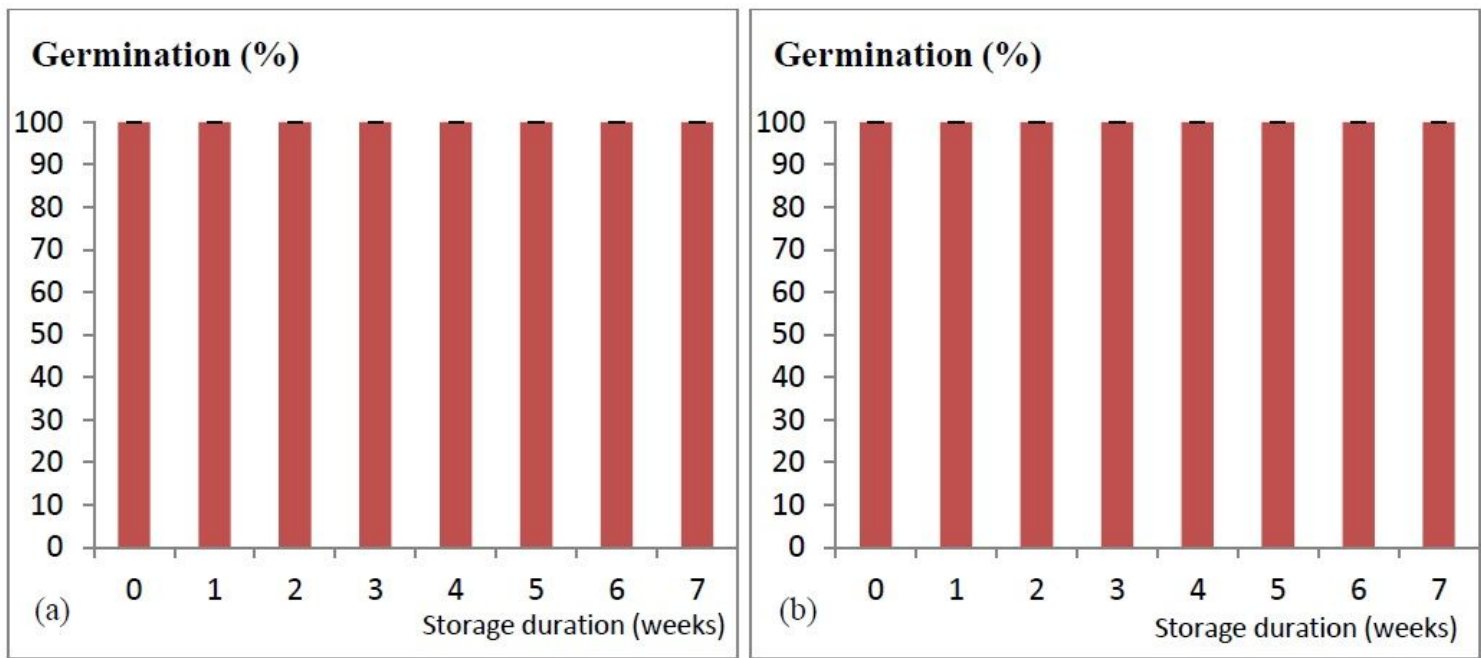


Figure 3

Germination % of fresh *L. fruticosa* seeds stored for 7 weeks at different temperature. a 8 ±1 °C, b 25± 2 °C means with different letters between germination percentages are significantly different at $p \leq 0.05$

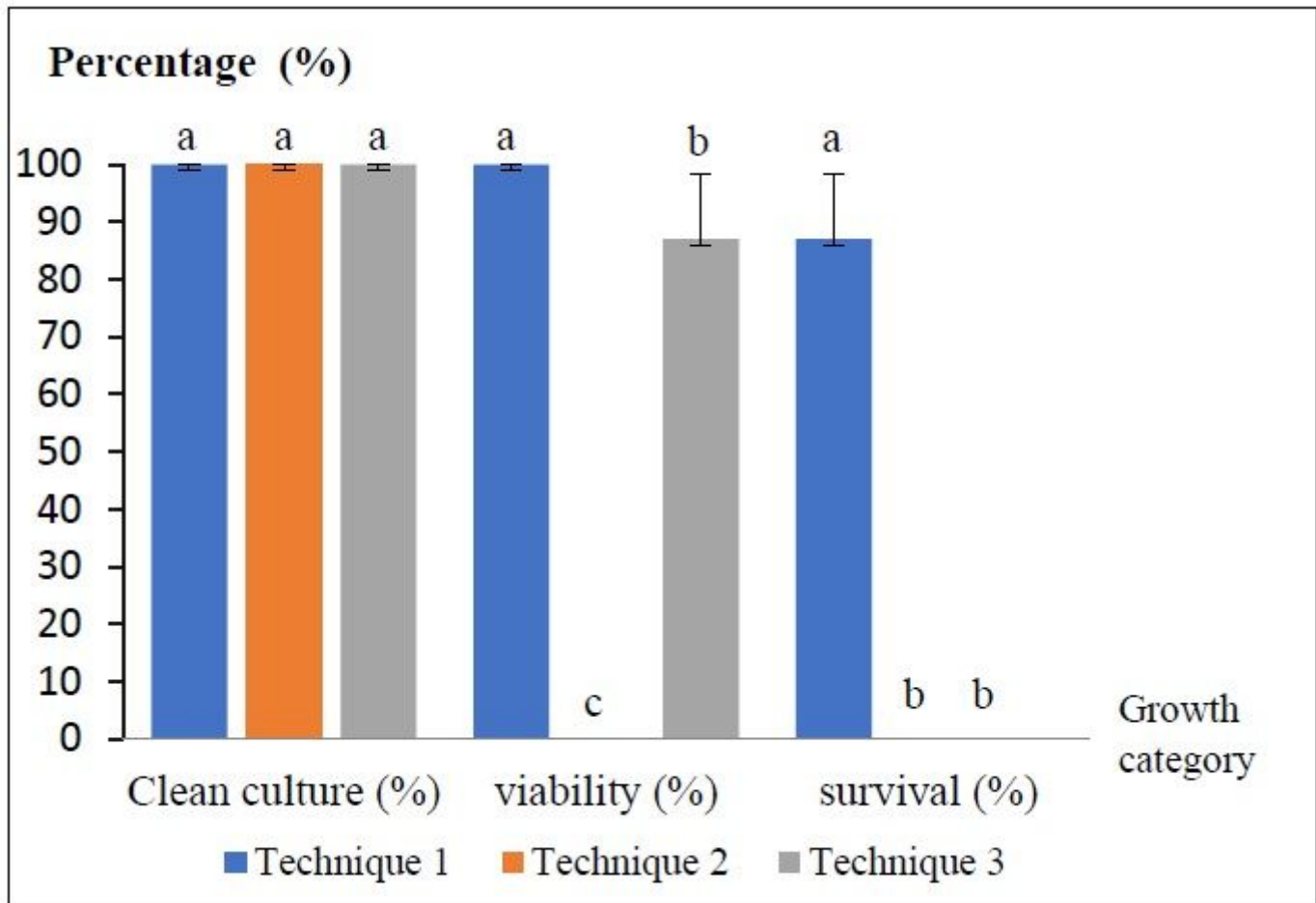


Figure 4

The effects of variations in sterilization on percentage of clean cultures, viability and survival of embryonic axes (EAs) of *L. fruticosa* cultured on MS medium means with different letters within each category (clean culture, viability or survival) are significantly different at $p \leq 0.05$

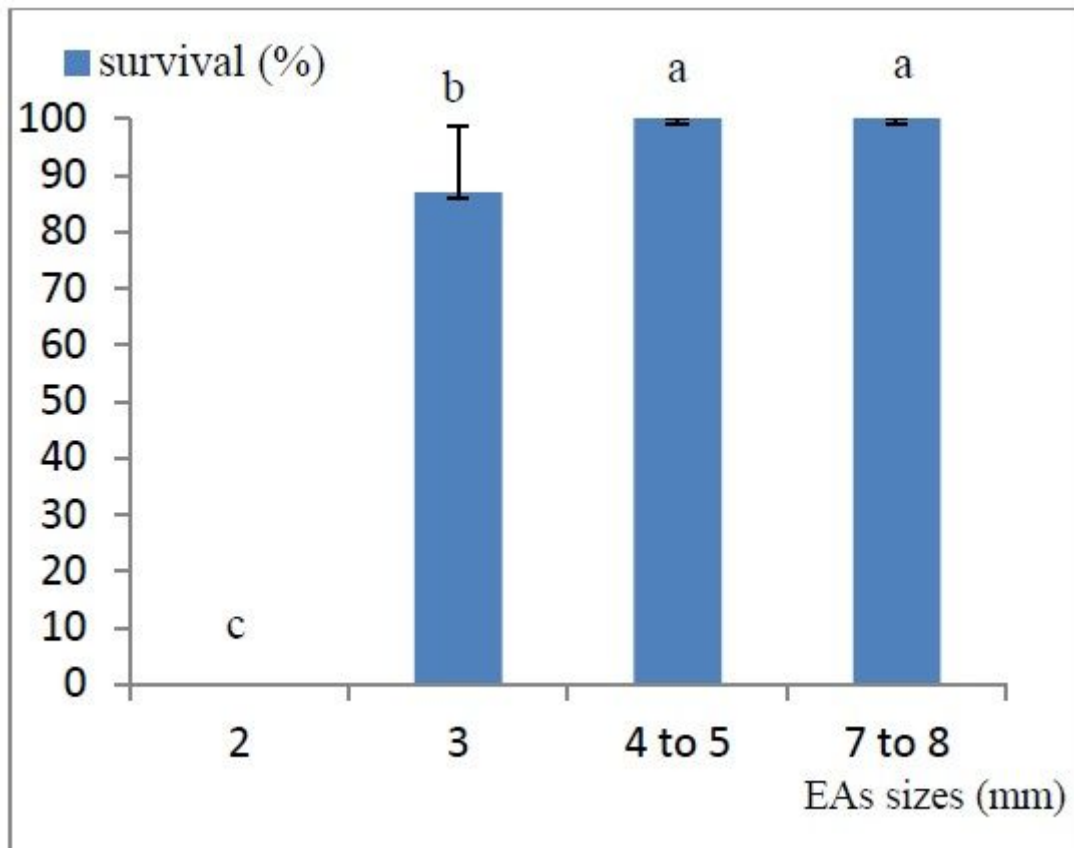


Figure 5

The effects of embryonic axes (EA) sizes on regeneration (survival percentage). means with different letters between the survival percentages are significantly different at $p \leq 0.05$

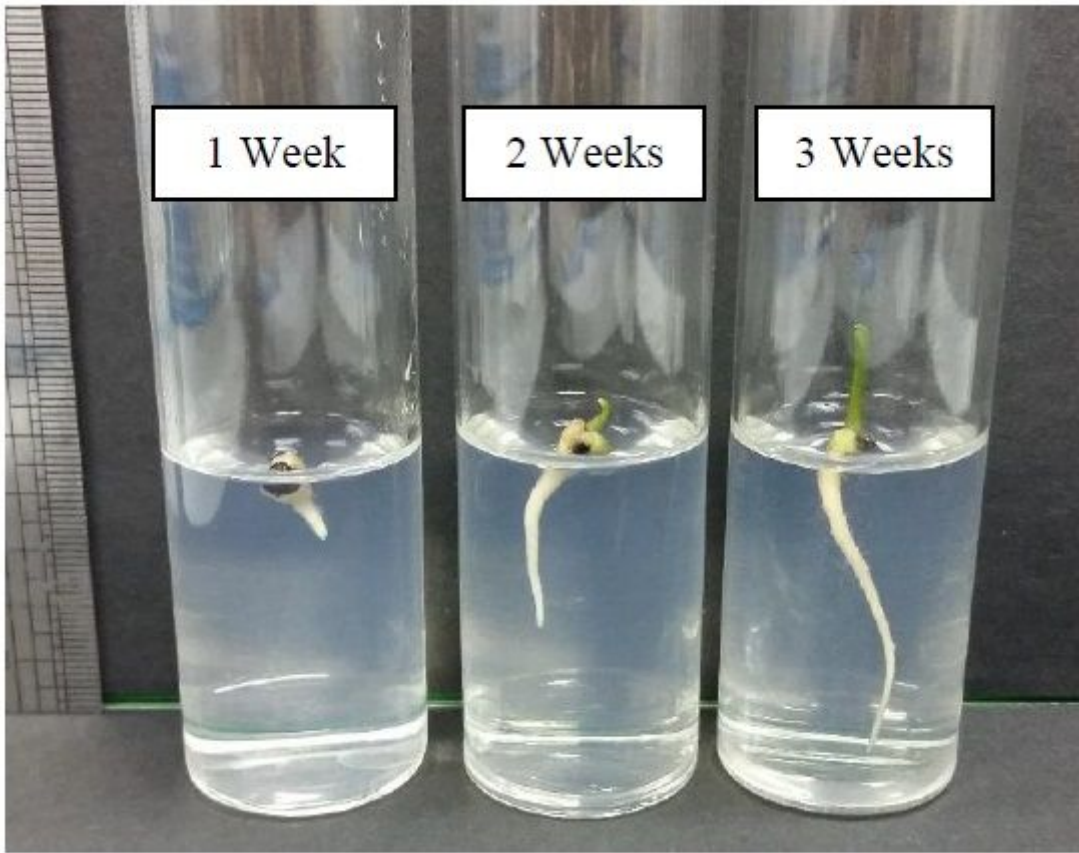


Figure 6

The normal developmental stages of embryonic axes (EAs) of *L. fruticosa* in tissue 430 culture to form complete plantlet (survival) after 3 weeks

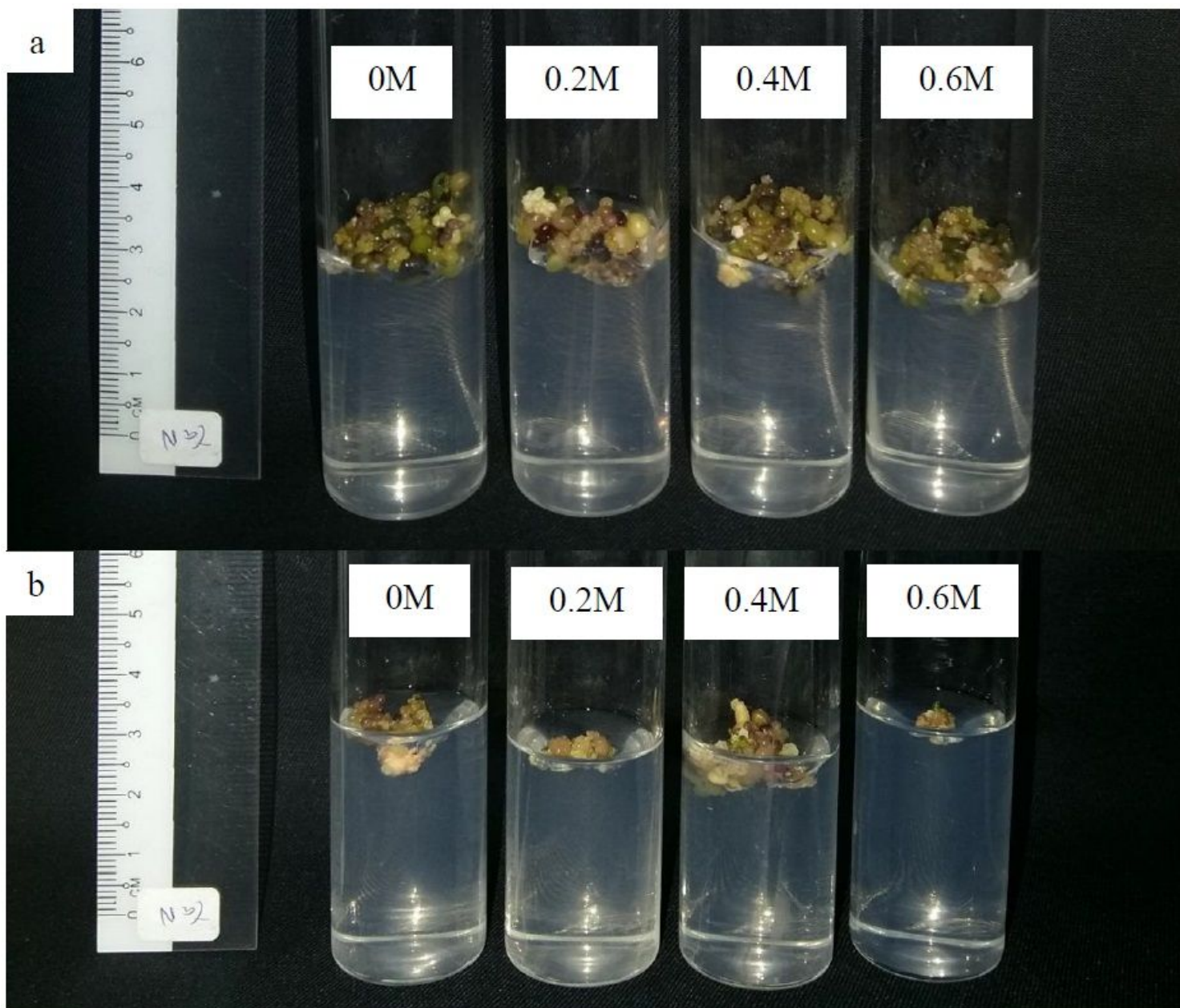


Figure 7

The growth of EAs after vitrification treatment a without, b with cryopreservation (-LN/+LN) after 20 weeks