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Original Article

Cryopreservation of *Vasconcellea quercifolia* St.-Hil (Caricaceae) Zygotic Embryos

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Abstract

Vasconcellea quercifolia A. St.-Hil. (Caricaceae) is a tropical fruit species native to Brazil, with a great importance in plant breeding programs. The *V. quercifolia* has a resistance to the main diseases of Caricaceae, Papaya Ringspot Virus (PRSV). Considering its potential, cryopreservation becomes a tool for the conservation of this species. The objective of this paper was to study the cryopreservation of *V. quercifolia* zygotic embryos through dehydration in silica gel. Excised zygotic embryos were dehydrated in silica at 0, 20, 40, 80 and 100 minutes and then inoculated in MS medium. The percentage of germinated and recovered embryos, and growth analysis were evaluated, besides water content. Subsequently, they were acclimatized in a growth room with temperature controlled. For cryopreservation, the embryos were excised and dehydrated in silica for 0, 20, 40 and 60 minutes, immersed in Liquid Nitrogen (LN) for 1 hour, thawed in Recovery Solution (RS) and inoculated in MS medium. After 30 days, the percentage of germinated and recovered embryos was evaluated. The silica gel promotes a fast dehydrate of embryos. The results showed that embryo dehydration affected seedling development, and dehydration for over 20 minutes showed a reduction in all evaluated parameters. The plantlets regenerated from embryos dehydrate survive the acclimatization. It was possible to cryopreserve the *V. quercifolia* zygotic embryos when the dehydration time of 20 minutes by silica gel was used.

Keywords: Caricaceae; dehydration, *in vitro* conservation; silica gel; tropical fruit

Introduction

Vasconcellea quercifolia A. St.-Hil. is a native Brazilian species, belonging to the family Caricaceae. This tropical species presents small fruits with smooth skin and firm pulp, with potential use in the food and pharmaceutical industry (Alamery and Drew, 2014; Faccio *et al*., 2015). *V. quercifolia* produces large amount of papain, that is used as a debridant, acts as an anti-inflammatory, and can be used in several healing stages (Falanga, 2002; Torres *et al*., 2012). Also, *V. quercifolia* has a great importance for its resistance to the Papaya Ringspot Virus (PRSV), one of the main diseases of Caricaceae. This makes it a potential species for use in breeding programs of Caricaceae species, as well as a rootstock (Caetano *et al*., 2008; Alamery and Drew, 2014; Chaves-Bedoya and Ortiz-Rojas, 2015; Kanchana-Udomkan, *et al*., 2016).

Considering the characteristics of economic interest of *Vasconcellea quercifolia*, their *in vitro* conservation is essential for the preservation of the species, as well as a complementary tool for this species multiplication. Among the conservation techniques, cryopreservation, which consists in the preservation of biological material at ultra-low temperature (-196 °C) in liquid nitrogen, has been the most outstanding. This technique allows for long-term storage, maintaining all the characteristics of explants indefinitely. Cryopreservation is a promising technique for the preservation of plant diversity of tropical plants (Engelmann *et al*., 2011; Engelmann and Dussert, 2013; Elliott *et al*., 2017; Lambardi *et al*., 2018).

Most plant materials used in cryopreservation contain high amounts of intracellular water being extremely sensitive to freezing injury caused by the formation of ice crystals when exposed to low temperatures (Porto *et al*., 2014; Wesley-Smith *et al*., 2014; Pérez-Rodríguez *et al*.,

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2017). So, for the successful of cryopreservation, it is essential that the plant material has been dehydration prior cryopreservation. A way to efficiently reduce the water content in the plant material is with the use of the silica gel dehydration that can be used for different material to physical desiccation (Pinto *et al*., 2016; Pérez-Rodríguez *et al*., 2017; Carmona-Martín *et al*., 2018). Since the silica gel technique is simple it becomes more reproducible. This technique shows advantages such as the promotion of uniform desiccation of explants (between and within sample), an accurate dehydration (Pains *et al*., 2001; Sherlock *et al*., 2005; Sisunandar *et al*., 2010). However, the time of desiccation need to be evaluated among species to obtain a high survival rate, besides maintenance of biochemical, physiological and genetic characteristics (Fonseca and Freire, 2003; Michalak *et al*., 2015; Pinto *et al*., 2016).

Cryopreservation appears as a technique for the conservation of *V. quercifolia*, with the objective of avoiding the genetic erosion of this material and preserving the species. Given the above, this study has the objective to cryopreserve *V. quercifolia* zygotic embryos using silica gel dehydration.

Materials and Methods

Dehydration test

Ripe *V. quercifolia* fruits were collected in Lavras, Southern Minas Gerais, Brazil (21° 14 'S and 45° 00' W Gr. and 918 m altitude). The fruits were washed and taken to a laminar flow chamber for asepsis, where they were immersed in 70% v/v alcohol for one minute, then placed in a 1% sodium hypochlorite solution with 1% active chlorine for 10 minutes and washed three times in sterile water. After asepsis, the fruits were depulped, the seeds were immersed in 70% v/v alcohol for 30 seconds and then immersed in a sodium hypochlorite solution with 1% active chlorine for 5 minutes. After asepsis, the embryos were excised.

Excised zygotic embryos were dehydrated in silica gel for different periods (0, 20, 40, 60, 80 and 100 minutes) and then inoculated in MS medium (Murashige and Skoog, 1962), supplemented with 30 g L^{-1} sucrose and solidified with 7g L^{-1} agar and previously autoclaved at 121 °C for 20 minutes. The pH of the medium was adjusted to 5.8. The material was kept in the absence of light for seven consecutive days, and then transferred to a growth room at 25±2 °C, with a 16-hour photoperiod and a luminous flux density of 48 μmol m^{-2} s^{-1} for 45 days. After 45 days, the following parameters were evaluated: (i) germination (%), recovery (embryos that developed shoots and roots) (%), (ii) number of green leaves, (iii) seedling length (cm), (iv) root number (v) and length of the largest root (cm). The water content of the zygotic embryos was also determined in the different dehydration periods (0, 20, 40, 60, 80 and 100 minutes). After determination of initial fresh matter, the embryos were dehydrated and their weight was determined; subsequently, they were completely oven dried at 70 °C for 72 hours. After this period, the final dry matter of the embryos was verified. With the values of fresh and dry matter at the different dehydration times, the water content of the embryos was determined.

Acclimatization

After the dehydration process, germinated seedlings were transferred to tubes with commercially available Plantmax® substrate with clear plastic bag cover. The seedlings were kept in a growth room at an average temperature of 25 ± 2 °C. The plastic bag was gradually removed, weekly, until complete withdrawal after 30 days. After the acclimatization period, survival (%), leaf and root number, plant length and main root length (cm), besides fresh and dry matter (mg), were evaluated.

Cryopreservation

Zygotic embryos were extracted and dehydrated in periods of 0, 20, 40 and 60 minutes. The dehydration periods were chosen taking into account the results found in previous dehydration tests. The embryos were placed in 2-mL cryotubes containing one drop of PVS2 [(Plant Vitrification Solution 2), solution consisting of 30% glycerol (w/v), 15% ethylene glycol (w/v) and 15% DMSO (w/v)] (Sakai *et al*., 1990). Subsequently, the embryos were immersed in Liquid Nitrogen (LN) at -196 °C for at least 30 minutes. The materials were thawed in Recovery Solution [(RS), 0.4 mol L^{-1} sucrose in MS medium] (Sakai *et al*., 1990) for 45 minutes at room temperature. After thawing, the cryopreserved embryos were inoculated in MS medium supplemented with sucrose (0.3 M) for 24 hours and kept in a chamber in the absence of light, then transferred to MS medium supplemented with sucrose (0.09 M) and maintained in the absence of light for six days. After this period, the embryos were transferred to the presence of light at a 16-hour photoperiod at 25±2 °C and luminous flux density of 48 μmol $m^{-2}s^{-1}$ for 30 days. The evaluated parameters were percentage of embryos germinated and recovered (complete seedling formation with shoot and root).

Statistical analysis

For the dehydration test, three replicates with ten embryos per Petri dish were used per treatment; for the determination of water content, 10 embryos were used per treatment and, for acclimatization, 10 normal plants. For cryopreservation, 30 zygotic embryos were used for each treatment.

The design was completely randomized (CRD), the data were submitted to analysis of variance (ANOVA) and the means were compared by the Tukey test at 5% significance, using the statistical software SISVAR (Ferreira, 2014).

Results and Discussion

Dehydration test

The *V. quercifolia* zygotic embryos had an initial moisture content of 80%. After 20 minutes of dehydration, the zygotic embryos showed a drastic fall to values close to 40% water, showing not statistical significance from the other dehydration times (Fig. 1).

V. quercifolia embryos showed a fast dehydration during first 20 min and stabilized after this period. Similar results have been reported for *Haemanthus montanus* (Sershen *et al*., 2011), *Cocos nucifera* L. (Sisunandar *et al*., 2010), *Byrsonima intermedia* (Silva *et al*., 2014), *Physalis angulata*

(Faria *et al*., 2016), *Coffee* zygoticos embryos (Pinto *et al*., 2016).

Even though there was no deep decrease in water content after 20 min of dehydration, with the increase in dehydration time, it was verified a decrease in embryo germination and recovery, opposite from observed in control. The control showed 100% of germination and recovery. After 40 min of dehydration, germination and recovery were lower than 50%, which is considered low (Fig. 2).

The decline in germination and recovery percentage in embryos exposed to the highest periods in silica gel (over 20 minutes) could have occurred due to fast water loss, with a drop of approximately 50% water content in only 20 minutes.

The low embryo survival from 40 minutes of dehydration possibly occurs due to the stress caused by dehydration, and changes induced in the embryo physiology, biochemistry and genetic stability (Urbanova *et al*., 2006). Also in *H. montanus* zygotic embryos was observed that dehydration over a water content optimum induce a decrease in vigour and recover of the seedling (Nadio *et al*., 2010).

After 45 days from each dehydration time the zygotic embryos kept on *in vitro* culture were evaluated. It was observed that the different dehydration times in silica gel affected shoot length, leaf and root number, besides the length of the largest *V. quercifolia* root.

The plants resulting from zygotic embryos which showed values close to 40% water after dehydration was carried out for 20 minutes showed a decreased number of roots and a decrease in root and shoot length, however, for leaf number, the statistical difference was only observed when the embryos were dehydrated from 40 minutes (Fig. 3).

As observed in Fig. 3C and D, the seedlings from zygotic embryos exposed for 20 minutes in silica gel had a larger plant length, and root formation compared to the other dehydration treatments. It can also be observed that the leaf number decreased as the dehydration time increased.

These results demonstrate that not only did the dehydration of *V. quercifolia* zygotic embryos cause a decrease in germination and recovery, but also in seedling growth and development, thus leading to lower seedling growth in the shoot and root as dehydration time increased.

Similar results were also obtained in a study with *Quercus robur* plumules, in which the authors observed the effect of dehydration in silica gel, finding that plumules that had been dehydrated for 50 minutes showed a reduction in survival rate from 18% to about 0%, after drying for 70 minutes (Chmielarz *et al*., 2011). The decrease in survival rate was also reported by Scocchi *et al.* (2007), who obtained only 27% survival of *Melia azedarach* L. somatic embryos, which were kept in silica gel for four hours.

After *in vitro* culture, the plants where acclimated and 30 days after acclimatization, it was observed that 100% plant survival in all treatment. Plants from embryos that did not undergo dehydration showed higher growth during acclimatization when compared with plants originally obtained from previously dehydrated embryos. In general, among the dehydration treatments, embryos dehydrated for 20 minutes had plants with the best characteristics for the analyzed variables, when compared to plants with greater dehydrated embryos time (Fig. 4).

In relation to shoot length, plants from embryos that were not dehydrated had a better result, 7.8 cm (Fig. 4A). Regarding leaf number, there was no significant difference among the treatments with 0, 20, 40 and 80 minutes dehydration, with a mean of 3.1 leaves per plant (Fig. 4B). As for fresh and dry matter, plants from the dehydration periods of 20 and 40 minutes showed higher mean fresh (0.34 and 0.28, respectively) and dry matter (0.05 and 0.03, respectively). In relation to root number and length, plants from embryos dehydrated for 0 and 20 minutes had the best means (Fig. 4D).

Acclimatization is a delicate stage and the embryos were submitted to a dehydration process. Therefore, in addition to being sensitive, the seedlings from the *in vitro* culture under study are possibly under stress due to water loss. This stress during acclimatization is also due to the non-development of a thick cuticle, which allows a high cuticular transpiration rate, considering that the cell walls of plants grown *in vitro* do not have the necessary rigidity to support the new plant, sometimes compromising a successful acclimatization (Silva *et al*., 2015; Faria *et al*., 2016). But, the plants regenerated from *V. quercifolia* zygotic embryos dehydrated survived to acclimatization stage e presented good development. This demonstrates the rusticity and developmental plasticity of this species.

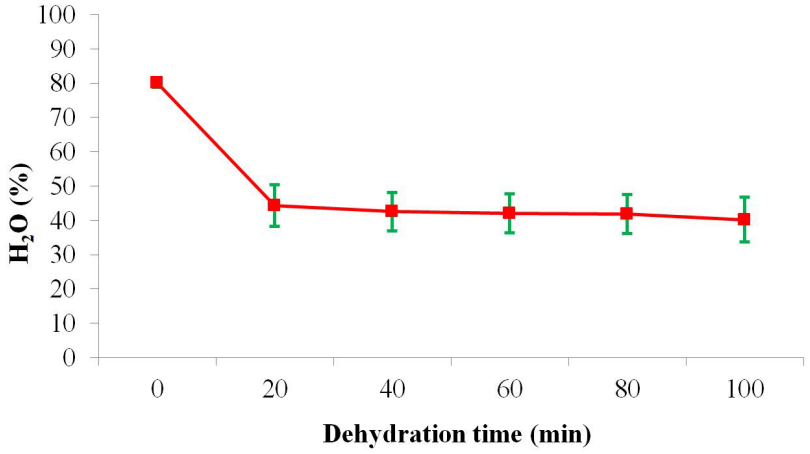


Fig. 1. Moisture content of *V. quercifolia* embryos after dehydration for different periods (min) in silica gel

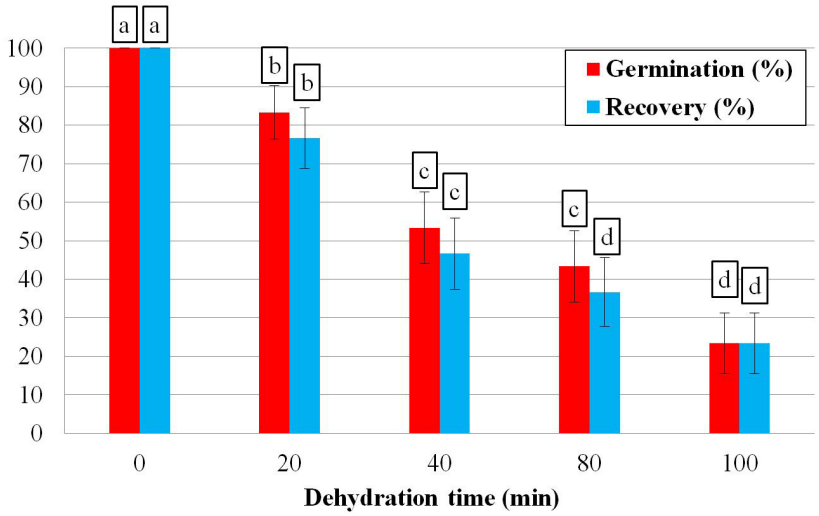
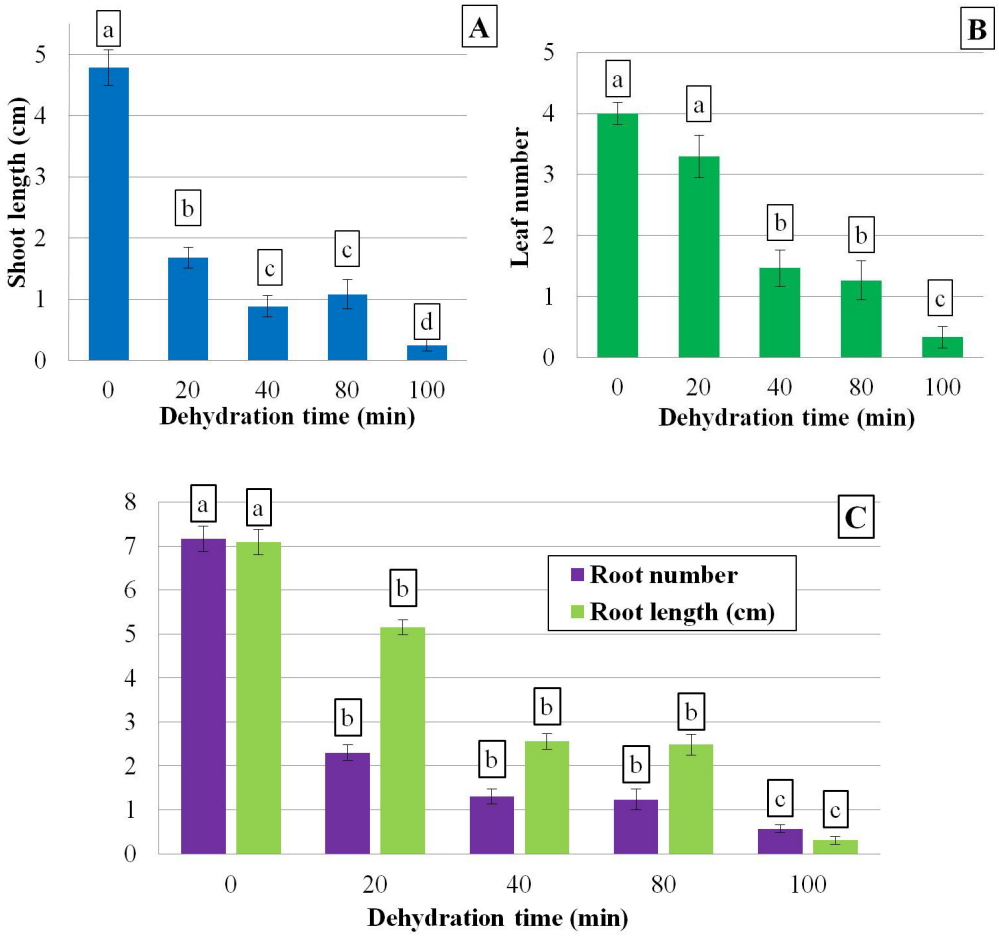


Fig. 2. Germination and recovery percentage of zygotic embryos at different dehydration periods in silica gel



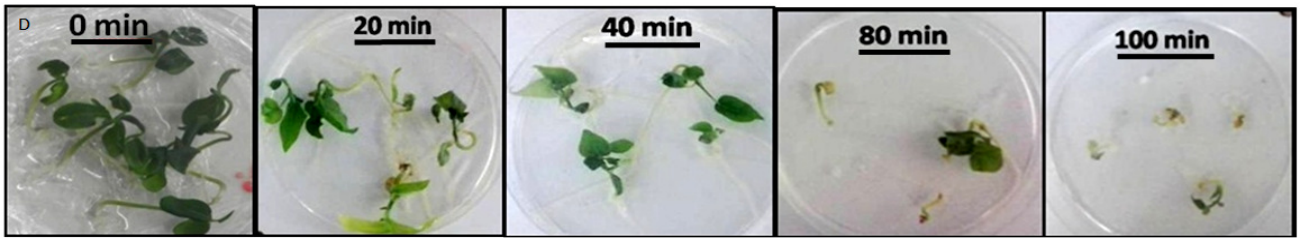


Fig. 3. *V. quercifolia* plants from embryos dehydrated in silica gel for different periods after 45 days of acclimatization. Shoot length (A), leaf number (B), root number and length (C); seedlings from embryos dehydrated in silica gel after 30 days

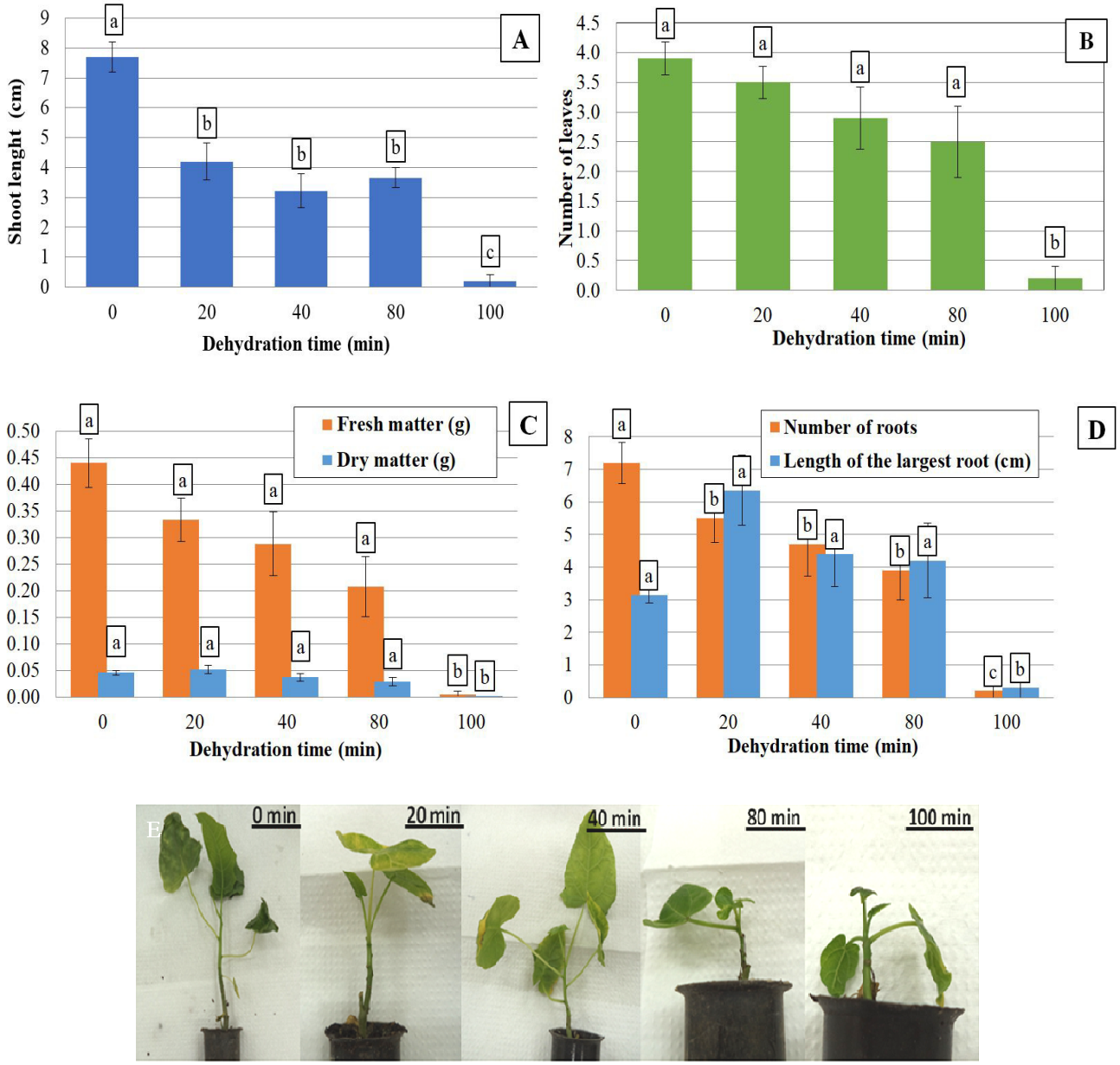


Fig. 4. *V. quercifolia* plants from embryos dehydrated for different periods in silica gel after 30 days of acclimatization. Shoot length (A), leaf number (B), fresh and dry matter (C), root number and length (D); plant appearance

Cryopreservation

The dehydration of *V. quercifolia* zygotic embryos is a crucial step before cryopreservation, since embryos that were not dehydrated did not survive liquid nitrogen exposure.

The highest germination rate was observed in the treatment in which the embryos were cryopreserved after dehydration for 20 minutes in silica gel, resulting in a germination rate of 26% (Fig. 5), followed by the treatment using 40 minutes of dehydration, with 16%. However, for recovery, only the treatment with 20 minutes of dehydration presented recovered embryos after cryopreservetion (6.7%).

In general, embryos contain high amounts of intracellular water and are, therefore, extremely sensitive to freezing injury caused by the formation of ice crystals when exposed to low temperatures (Porto *et al*., 2014; Wesley-Smith *et al*., 2014; Pérez-Rodríguez *et al*., 2017). Exposure to these low temperatures is also highly stressful for these explants, and may cause metabolic changes; thus, favourable conditions after cultivation are necessary.

The low recovery rate possibly occurred due to the complexity and sensitivity of the tissues that make up the embryo, according to Engelmann (2011), these are the factors that limit germination and recovery of zygotic embryos from species sensitive to dehydration and freezing in liquid nitrogen. As an example, these changes occur in several organelles such as mitochondria, endoplasmic reticulum and Golgi complex, compromising embryo metabolism (Sershen *et al*., 2012).

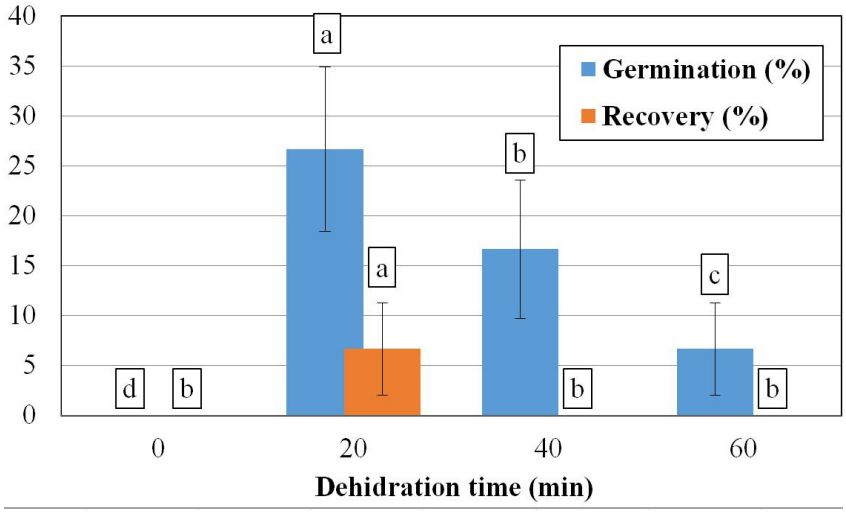


Fig. 5. Percentage of zygotic embryos germinated and recovered after cryopreservation using different dehydration periods in silica gel

The dehydration process has to be sufficient to avoid the formation of ice crystals during the contact of the plant material with ultra-low temperatures. Therefore it is important to avoid the excessive water efflux, which can cause cell death during the dehydration process, and find the minimum of water needed to seedling formation takes place (Freitas *et al*., 2016).

Conclusions

It was possible to cryopreserve the *V. quercifolia* zygotic embryos when the dehydration time of 20 minutes by silica gel was used. When dehydration was carried out for more than 20 minutes, although germination was obtained, seedling recovery was not observed after cryopreservation. Due to the decline in the evaluated parameters after cryopreservation process, other studies are suggested to optimize the dehydration time or conditions after cryopreservation of *V. quercifolia* embryos, aiming to improve the rate of recovered embryos after cryopreservation.

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