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Desiccation Tolerance in *Phaleria macrocarpa* Embryonic Axes

(Toleransi Terhadap Pengeringan pada Paksi Embrio *Phaleria macrocarpa*)

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ABSTRACT

Phaleria macrocarpa *seeds are rapidly killed with desiccation to moisture content (MC) below 20%. Desiccation tolerance of their embryonic axes was studied for storage and germplasm conservation purposes. Embryonic axes were extracted aseptically from fresh seeds obtained from fully ripe fruits in a horizontal laminar air flow cabinet. They were then desiccated under aseptic condition for periods ranging from 0-8 h. For each desiccation treatment, embryonic axes were drawn randomly for the determination of MC according to ISTA, electrolyte leakage and proliferation on Murashige and Skoog (MS) media supplemented with 1 mg/l 6-benzylaminopurine (BAP) and 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). The results obtained from this study indicated that the embryonic axes could tolerate dehydration down to 13.6% with desiccation for 8 h while retaining relatively high viability of 76.7%. This was supported by only gradual increment of electrolyte leakage with the desiccated embryonic axes. All non-desiccated embryonic axes with MC of 52.5% were capable to grow into normal plantlets* in vitro *but dehydration to MC of 36.0% and further down to 13.6% generally resulted in callus formation with up to 16.7% of the embryonic axes while at least 60.0% of the other embryonic axes were still capable to proliferate as normal plantlets* in vitro.

Keywords: Callus; electrolyte leakage; moisture content; seed; survival

ABSTRAK

Biji benih Phaleria macrocarpa *mati dengan pengeringan ke kandungan kelembapan (MC) di bawah 20%. Ketahanan pengeringan pada paksi embrio dikaji untuk tujuan penyimpanan dan konservasi germplasm. Paksi embrio diekstrak secara aseptik daripada biji benih segar yang diperoleh daripada buah masak dalam kebuk aliran udara laminar. Paksi embrio kemudiannya dikeringkan dalam keadaan aseptik untuk jangka masa 0-8 jam. Untuk setiap rawatan pengeringan, paksi embrio dikeluarkan secara rawak untuk penentuan MC mengikut tatacara ISTA, kebocoran elektrolit dan pertumbuhan di atas media Murashige dan Skoog (MS) yang diperkayakan dengan 1 mg/l 6-benzilaminopurin (BAP) dan 1 mg/l 2,4-diklorofenosiasitik asid (2,4-D). Keputusan yang diperoleh daripada kajian ini menyatakan bahawa paksi embrio dapat bertoleransi terhadap pengeringan ke MC 13.6% dengan pengeringan selama 8 jam sementara masih mengekalkan kemandirian 76.7% yang tinggi. Ini disokong dengan hanya peningkatan kebocoran elektrolit secara perlahan-lahan daripada paksi embrio yang dikeringkan. Semua paksi embrio yang tidak dikeringkan pada MC 52.5% dapat tumbuh kepada planlet yang normal secara* in vitro *tetapi pengeringan ke MC 36.0% dan seterusnya ke 13.6% telah menyebabkan pembentukan tisu kalus sebanyak 16.7% dengan paksi embrio manakala sekurang-kurangnya 60.0% paksi embrio yang lain masih dapat tumbuh kepada planlet yang normal secara* in vitro.

Kata kunci: Biji benih; kalus; kandungan kelembapan; kebocoran elektrolit; kemandirian

INTRODUCTION

Phaleria macrocarpa (Scheff.) Boerl., commonly referred to as Crown of God or Mahkota Dewa (PROSEA 1992), is gaining importance in medical product development in the region of Indonesia and Malaysia. It is used for a wide range of health care purposes from the control of cancer, impotency, diabetes, hemorrhoids, allergies, liver and heart diseases, mellitus, kidney disorders, blood diseases, acne, migraine, stroke to various skin diseases (Harmanto 2005; Tate 2002; Winarto 2004; Zhang et al. 2006). Its therapeutic effects are directly related to the chemical and bio-active compounds in it (Effendy et al. 2011; Hendra et al. 2011; Kusuma et al. 2011; Sher 2009). With the increasing interest in planting this species as one of the prioritized medicinal plants, especially among the rural folks for increased household income, more research on cultivation and postharvest handling of the fruits has also been recently conducted besides more intensive work conducted on its pharmaceutical properties.

Phaleria macrocarpa is primarily propagated using seeds. Among the various environmental factors that play a dominant role in postharvest physiology of fruits and seeds, desiccation tolerance has received the widest attention for seed storage as planting materials and for germplasm conservation purposes. The specific relationships between dehydration and rate of deterioration vary considerably between species (Asomaning 2009; Faria 2006; Roberts 1973; Roberts & King 1980). However, it is generally

known that the best drying procedure to slow down deterioration of seeds is one of the lowest moisture content (MC) that can safely retain seed germinability. Among the adverse consequences of improper seed desiccation include toxin production, growth of fungi, discoloration and eventually loss of viability.

Preliminary studies indicated that *P. macrocarpa* seeds were generally killed with drying to MC below 20% (Ahmed Asrity, unpublished data). For a number of desiccation sensitive or recalcitrant seeds, their embryonix axes have been recorded to survive further drying to certain extent as compared to their whole seeds (Liang & Sun 2000; Normah & Makeen 2008; Obendorf et al. 1998; Spanò et al. 2011). *In vitro* zygotic embryo culture technology provides a useful tool for studying factors affecting seed growth, maturation, desiccation tolerance and germination. However, it is essential that the embryonic axis desiccation process should be safe and simple to operate while avoiding deterioration and fatal damage. Air desiccation by means of sterilized in-air flow in a horizontal laminar air flow cabinet is normally applied for drying of seeds and embryonic axes. This is the simplest method and should always be tried first before going on to other techniques. However, fast and ultra-rapid desiccation procedures may be preferred for achieving desiccation tolerance in some plant tissues as such drying processes minimize the time of intermediate water content in the metabolically active tissues and hence, reduce or hinder deleterious degradative processes in these tissues (Normah & Makeen 2008). This study was carried out to determine the desiccation tolerance in *P. macrocarpa* embryonic axes as an alternative storage means to the desiccation sensitive seeds. Desiccated embryos can be further studied for storage of planting materials and germplasm storage for future genetic manipulation or other future purposes.

Materials and Methods

Seed Procurement and Handling

The fully ripe *P. macrocarpa* fruits were harvested from a farm in Kamunting, Perak, Malaysia (4°51'N, 100°44'E) in October 2011. On the following day, the fruits were brought back to the laboratory at Universiti Teknologi MARA (3.08°N, 101.53°E). Uniform fruits with length ranging from 35 to 45 mm and diameter ranging from 30 to 40 mm were randomly selected for extraction of their seeds, from which their embryonic axes were excised for experimentation.

The fibrous pulp of fruit was first removed carefully from the seed using a pair of scateurs. The seeds were then cleaned with running tap water for 30 min followed by surface sterilization using 95% ethanol for 20 min in a horizontal laminar air flow cabinet in the laboratory (average temperature and average relative humidity (RH) of 25±2°C and 55±5%, respectively). Then, these seeds were rinsed three times with autoclaved distilled water and allowed to air dried for 1 h on sterilized paper towel in the same horizontal laminar air flow cabinet prior to excision of their embryonic axes for desiccation experimentation.

Experimental Procedure and Data Collection

Excision and Desiccation of Embryonic Axes Embryonic axes were excised carefully from surface sterilized seeds using a scalpel and a pair of forceps. There was no further surface sterilization of the embryonic axes conducted. These embryonic axes were then placed as mono-layers on sterile filter paper in several sterilized Petri dishes with their covers removed. A total of 375 embryonic axes were subjected to varying desiccation periods of 0 (control), 2, 4, 6 and 8 h, respectively, in the horizontal laminar air flow cabinet. All standard working procedures with a horizontal laminar air flow cabinet were strictly adhered to ensure extraction and desiccation of embryonic axes were carried out safely under aseptic condition.

Data Collection For each desiccation period, a total of 75 embryonic axes in three replicates were drawn randomly from different Petri dishes for the determination of MC, electrolyte leakage and proliferation on Murashige and Skoog (MS) media (Murashige & Skoog 1962), respectively. The MC entailing the amount of water in the embryonic axes was expressed in percentage on wet weight basis. In this procedure, the weight of embryonic axes was recorded immediately using an analytical balance when each desiccation period elapsed. The MC of embryonic axes was determined gravimetrically as oven drying procedure at 103±1°C for 17±1 h according to ISTA (Sivritepe et al. 2008). MC determinations were made based on ten embryonic axes per replicate for each desiccation treatment.

With each desiccation period, the electrolyte leakage of the embryonic axes was also determined using five embryonic axes per replicate. The embryonic axes were soaked in 25 mL distilled water for 24 h and electrolyte leakage was then measured using an electrical conductivity meter.

For each desiccation period, the remaining ten embryonic axes of each replicate were immediately transferred to autoclaved MS media supplemented with 1 mg/l 6-benzylaminopurine (BAP) and 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) in the horizontal laminar air flow cabinet. The embryonic axes were placed horizontally on the media, sealed and then incubated under cool fluorescent light of 1000 lux light intensity at 16 h photoperiod daily. The average temperature of the incubation room was 25±2°C. The proliferation of each embryonic axis was scored daily based on any visible elongation of radicle or plumule which was eventually observed as normal plantlet. Callus tissue formation was also recorded. Data collection on proliferation of embryonic axes lasted for three weeks. The normal plantlet proliferation index, as an indicator of proliferation speed and vigor of embryonic axes, was then calculated as

$\sum[Dn/D]$, where D*n* was the number of embryonic axes with normal elongated radicles or plumules on day D, and D was the number of days after culture.

Data Analysis

The experimentation was based on a completely randomized design with three replicates. Data were subjected to analysis of variance (ANOVA) and treatment means were compared using Tukey's Honestly Significant Difference (HSD) Test. Percentage data were transformed to arc-sine values before ANOVA. Correlation analysis of parameters was also carried out to determine the relationship between viability of embryonic axes and the other measurements recorded in this study.

Results and Discussion

MC of the embryonic axes of *P. macrocarpa* was reduced significantly and linearly from 52.48 to 13.59% over a desiccation period of 8 h in the horizontal laminar air flow cabinet (MC=49.2-4.88H; r^2 = 0.92, p<0.001) (Table 1). MC was strongly and significantly correlated to proliferation of normal plantlets (r = 0.88) and normal plantlet proliferation index (r = 0.84) (Table 2). Desiccation for 4 h to MC of 30.2% resulted in significant reduction in growth of normal plantlets and normal plantlet proliferation index, where these two growth parameters were strongly and significantly correlated to the increased electrolyte leakage (r = -0.79 and r = -0.81, respectively) (Tables 1 and 2). With dehydration treatment, some small percentage of embryonic axes, up to 17%, showed proliferation of callus tissues with failure to develop into normal plantlets (Table 1; Figure 1). However, the under study desiccation treatments down to relatively low MC of 16.1% still allowed viability of embryonic axes of above 80%, being the total proliferation of normal plantlets and callus tissues, while further drying procedure to MC of 13.6% reduced total proliferation to 76.7% (Tables 1 and 2; Figure 2). Desiccation of embryonic axes to MC below 13% was not carried out as it was rather impossible with the ambient RH of approximately 55% in the laboratory.

The results mentioned above proved that the embryonic axes of *P. macrocarpa* were more desiccation tolerant than the seeds that hardly survived MC below 20% as mentioned. The phenomenon of higher desiccation tolerance with embryonic axes as compared to their whole seeds was reported to be related to the rapid drying that can be achieved with smaller plant materials, thus reduced the cell membrane degradation associated with the homogenous dehydration with slow drying (Nedeva & Nikolova 1997; Pammenter et al. 1998; Panza et al. 2007). It is generally known that cell membrane is susceptible to degradation with homogeneous slow drying as degradative process is an aqueous-based process and oxidative in nature. Although *P. macrocarpa* embryonic axes showed significant reduced proliferation of normal plantlets and normal plantlet proliferation index with dehydration to MC ≤ 30% after 4

TABLE 1. Mean comparison of MC, electrolyte leakage, proliferation of normal plantlet, normal plantlet proliferation index and callus tissue development

Desiccation period (h)	MC (%)	Electrolyte leakage (mS/m)	Proliferation of normal plantlets (%)	Normal plantlet proliferation index	Callus tissue development (%)
0	52.48±2.39 a	132.92±5.76 c	86.67±2.71 a	2.32±0.05 a	0.00±0.00 a
2	35.97±1.84 b	172.92±9.81 bc	76.67±2.22 ab	1.92±0.07 ab	16.67±2.01 a
4	30.17±1.74 b	187.08±12.58 b	70.00±3.66 b	1.73±0.20 bc	13.33±3.93 a
6	16.10±1.02 c	215.16±8.30 ab	66.67±2.01 b	1.70±0.09 bc	16.67±5.39 a
8	13.59±0.41 c	240.67±16.29 a	60.00±0.00 b	1.29±0.08 c	16.67±2.01 a

Mean±SE; means with the same letter within the same column are not significantly different at 5% level of significance

TABLE 2. Pearson's correlation coefficients of measurements

Measurement	MC (%)	Electrolyte leakage (mS/m)	Normal plantlet proliferation (%)	Normal plantlet proliferation index (%)	Callus tissue development (%)
MC (%)	1.00				
Electrolyte leakage (mS/m)	-0.93***	1.00			
Normal plantlet proliferation (%)	0.88***	-0.79***	1.00		
Normal plantlet proliferation index (%)	0.84***	-0.81***	0.91***	1.00	
Callus tissue development (%)	-0.55*	0.47^{ns}	-0.63*	-0.66**	1.00

^{ns} indicates p>0.05, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001

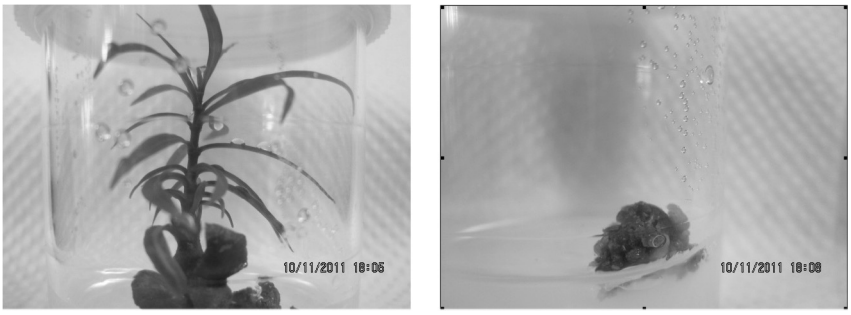

FIGURE 1. Development of normal plantlet (left) and callus tissues (right) with desiccation for 4 h

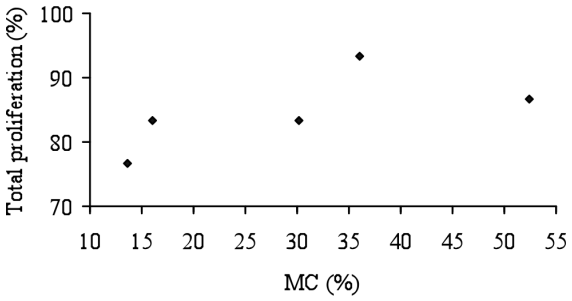

FIGURE 2. MC-total proliferation curve

h in the horizontal laminar air flow cabinet, there was still acceptable above 75% survival with MC down to 13.6%, in which ≥ 60% of them developed into normal plantlets while the smaller percentages of them demonstrated callus tissue proliferation with dehydration treatments under study. The control embryonic axes (no desiccation treatment), on the other hand, were all capable to grow into normal plantlets and no callus tissue formation was recorded with these control embryonic axes. The high viability of desiccated embryonic axes was generally supported by relatively low increment in electrolyte leakage from that of 133 mS/m with the controls to that of 241 mS/m with the 8 h desiccated embryonic axes, indicating that deterioration of the tissues was not at a too drastic damaging level (Table 1), despite the strong negative relationship between MC and electrolyte leakage (r = -0.93) (Table 2). In another word, most parts of the dehydrated embryonic axes were still intact, probably due to rapid drying with smaller materials, as compared to their wholes seeds and therefore they retained their viability even at MC as low as 13.6%. Electrolyte leakage has been the most sensitive indirect test for cell membrane integrity and identification of seed and embryonic axis deterioration in desiccation or storage trials. The development of callus tissues, on the other hand, may indicate some deleterious reactions of the desiccation procedure under study. More rapid dehydration method may be sought after and the application of suitable plant growth regulators may be necessary to get rid of the development of callus tissues (Daffalla et al. 2011).

In view of the relatively acceptable survival of the embryonic axes with desiccation up to 8 h as studied or MC down to 13.6%, further storage of the embryonic axes can be studied as planting materials but more importantly, such findings allow further investigation on the possibility to store this species for germplasm conservation, e.g. cryopreservation, for future genetic manipulation and plant pharming purposes (Normah & Makeen 2008; Radha et al. 2010; Tzec-Sima et al. 2006). Genetic storage of this species is not achievable with the conventional seed storage protocol currently practiced in gene banks due to the desiccation sensitive behavior with its seeds. With the low percentage of the callus tissues developed as a result of desiccation treatment, further studies on the embryogenesis behavior of such callus tissues may also be studied for the mentioned genetic conservation purposes (Kaur & Gosal 2009; Mathews et al. 1993; Mng'omba et al. 2008).

CONCLUSION

In vitro desiccation of *P. macrocarpa* embryonic axes up to 8 h to relatively low MC of 13.6% was concluded viable to retain their viability. There was at least 60% proliferation into normal plantlets and 16.7% callus tissue proliferation. Such viability level was far better than that could be achieved with seed storage, where seeds were generally killed with MC below 20%. The embryonic axes of this medicinal plant can also be categorized to be intermediate, presenting their ability to tolerate dehydration down to MC of 13.6%. Such relatively low MC that retained viability of the embryonic axes generally allows these materials to be further studied for storage even at sub-zero temperatures and with cryopreservation procedure for germplasm conservation.

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