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Research

Regeneration of *Syzygium myrtifolium* Walp. from Seed Fragments – Evidence of Polyembryony?

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ABSTRACT

Intact *Syzygium myrtifolium* seed produces a single seedling although most species within the same genus are polyembryonic. Following the earlier work that found the potential of more than one plantlet development from the fractionated seeds of some monoembryonic *Eugenia* spp. belonging to the same family, the present work assessed the sprouting of *S. myrtifolium* seed fractions on moistened paper towel pads. This study was carried out in the enclosed plastic boxes at ambient temperature in the laboratory. The results obtained indicated that the different parts of the cotyledons could develop plantlets spontaneously following incision of a seed into separated fractions. Despite the reduced mass, the seed fragments revealed the potential of developing more than one plantlet in vivo from a seed. Injury or seed incision has been suggested to trigger the development of embryonic cell in the cotyledon leading to the formation of an entire plant in contrast to polarity committed by whole seed. For the fragmented seeds that did not regenerate full plants, more than 80% of them exhibited unsynchronized adventitious root development. In the re-growth evaluation, more than 70% of the seed fragments formed new adventitious roots on the new cotyledonary surfaces after the removal of plantlets and the attached cotyledons. This phenomenon suggests the spread of meristematic tissues within the cotyledons. Future work should look into the intrinsic signals and external cues that regulate the cellular differentiation and development in the seed fractions in revealing the polyembryony in *S. myrtifolium*.

Key words: Adventitious, cotyledon, in vivo, plantlet, spontaneous

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INTRODUCTION

Syzygium myrtifolium Walp. is a member of the family Myrtaceae. With its local names of kelat paya, ubah laut, Chinese red wood, wild cinnamon or Australian brush cherry, it is acknowledged as a multi-purpose woody plant. Its leaves have been used in traditional medicine as a stomachic. Recent research has confirmed the presence of many antitumor and anticancer compounds in this plant part (Memon *et al*., 2014, Farsi *et al*., 2016). Besides medicinal values, this plant species is also a primary choice for the beautification of urban areas for its striking reddish young foliage (Ahmad Nazarudin *et al.*, 2014).

Within the genus of *Syzygium* which consists of possibly 1,200 shrubs and trees in the tropical and subtropical countries in Africa, and throughout the Southeast Asia region, the fruit and seed characters are among the important evidences in the cladistic studies and reviews of this large genus (Lughadha & Proença, 1996; Landrum & Kawasaki, 1997; Staggemeier *et al*., 2010; Sivasubramaniam & Selvarani, 2012; Nair *et al*., 2020, WCSP, 2021). Many seeds of *Syzygium* contain multiple embryos within their seed coat (Thurlby *et al*., 2011, Blando *et al*., 2013; Shareef *et al*., 2013, Sujanapal *et al*., 2013, Mohamed *et al*., 2014, Karuppusamy & Ravichandran, 2016, Snow *et al*., 2016). However, *S. myrtifolium* seed has no visible embryonic axis when it is shed from tree (Tsan & Awang, 2021). The ovoid to pea-like shaped seed measuring less than 6 mm in breadth is exalbuminous. The absence of differentiated embryo within seed at full ripeness is a less common phenomenon while it has been described earlier for some *Eugenia* species from the same family of Myrtaceae (van Wyk & Botha, 1984). According to the researchers, the regenerative tissues are spread in the cotyledons of the eugenoid seeds, instead of differentiated shoot-radicle axes. In terms of regeneration from seed, each *S. myrtifolium* seed produces only a single seedling after it is shed from tree under natural condition. This suggests that there must

be a series of cellular events after detachment from tree which leads to the development of an embryo and an entire plant.

In the past decade, some researchers have found the potential growth of full plants from the fragmented seeds of monoembryonic *Eugenia* spp. (Delgado *et al*., 2010, Amador & Barbedo, 2015, Calvi *et al*., 2017a). An unknown mechanism promotes the development of embryonic or regenerative cell randomly from the cotyledon after incision, leading to the formation of a full plant. Such physiological response has been demonstrated in more than one fraction of a seed revealing its ability to form more than one plant while intact seed develops a single seedling. Inspired by the polyembryony in most *Syzygium* species, this study was carried out to corroborate the meristematic properties in the cotyledons of *S*. *myrtifolium*. Despite taxonomically different from *Eugenia* spp., some preliminary work found that the cotyledonary fractions of *S*. *myrtifolium* could also regenerate entire plants. Preceding work also revealed the formation of a plantlet in each separated half of a seed suggesting the regenerative capability within cotyledons and potential polyembryony. Sequel to plantlet formation, this study also explored the resprouting potential of the seed fragments to validate the existence of meristematic tissues within whole cotyledons. Such a unique biological feature could be studied further with regards to the natural adaptation of this woody plant, and probably also its significance in genetic algorithm.

MATERIALS AND METHODS

Seed preparation

Fully ripe *S*. *myrtifolium* fruits in black colour were collected from a single tree in Shah Alam, Selangor, Malaysia (3°5'51.9"N, 101°26'41.1"E), in October 2020. On the following day, the fruits were brought to the laboratory and the seeds were carefully extracted from the fruits by rubbing off the juicy pericarp manually. The seeds were then cleaned with slow running tap water followed by pat drying with paper towel. A total of 180 seeds of uniform ovoid shape and size of 5.0-5.5 mm in breadth were used for experimentation.

Seed fractionation

Within the same day after seed preparation as described above, the seeds were randomly subjected to five fractionation treatments, respectively, based on a completely randomized experimental design. Each was replicated trice with 12 seeds per replicate. The control was whole seed (C0) weighing 0.0652±0.0119 g. To cut a seed into approximate halves, the less convex side of the seed was laid down on a glass Petri dish, and the part having the hilum was taken as the proximal side of the seed as shown in Figure 1a. For longitudinal halving (C2L), the seed was cut from hilum to the base of the seed along Plane A using a scalpel (Figure 1b). The left (L) part was named C2L(L) whilst the half on the right side (R) was C2L(R). With Levene's test and independent t-test, C2L(L) which weighed 0.0338±0.0102 g was not significantly different from C2L(R) (0.0317±0.0090 g) ($P>0.05$). For transversal cutting along Plane B (C2T), the proximal (P) portion with hilum was C2T(P) and the distal (D) half was named C2T(D) (Figure 1c). As whole seed is ovoid with larger dimension at proximal side as shown in Figure 1a, C2T(P) was significantly larger ($P<0.05$) than its complement C2T(D); the fragment weight was 0.0395±0.0129 g and 0.0301±0.0086 g, respectively.

To obtain smaller approximate quarters, the seeds were either cut longitudinally into four (C4L) or halved along Plane A followed by complete incision along Plane B (C2L2T) (Figures 1d and 1e). In the procedure to cut a seed longitudinally into four fractions (C4L) with cutting parallel to Plane A, the quarters in order from left (L), centre left (CL), centre right (CR) and right (R) were named as C4L(L), C4L(CL), C4L(CR) and C4L(R), respectively (Figure 1d). By weight, C4L seed fractions were not significantly different from one another ($P>0.05$); the fractions in order from left to right as described above weighed 0.0199±0.0065 g, 0.0202±0.0061 g, 0.0171±0.0050 g and 0.0183±0.0056 g, respectively.

With C2L2T, a seed was halved longitudinally from hilum to the base of the seed along Plane A first. Then, each halved seed was further cut transversely. In total, C2L2T also provided four fractions from a seed. The quarters were C2L2T(LP), C2L2T(LD), C2L2T(RP) and C2L2T(RD) for those having the left (L), right (R), proximal (P) and distal (D) seed part in combination, respectively (Figure 1e). By virtue of the larger proximal side with the ovoid whole seed, the proximal quarters of C2L2T(LP) and C2L2T(RP) (0.0208±0.0057 g and 0.0191±0.0053 g, respectively) were significantly larger than their counter quarters of C2L2T(LD) and C2L2T(RD) (0.0170±0.0047 g and 0.0136±0.0052 g, respectively).

After fractionation, all the seed fragments remained in a firm condition with the cotyledon(s) tightly packed and inseparable. All the seed fractions were tagged accordingly for the immediate next sprouting procedure.

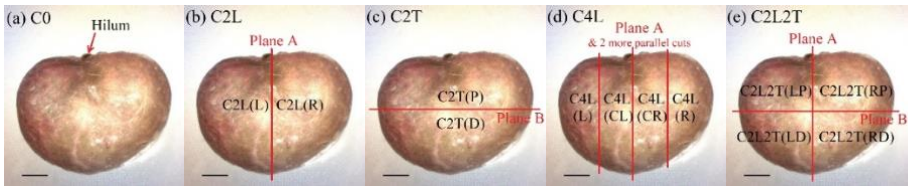

Fig. 1. (a) C0 – whole seed with the less convex side laid down; illustration for fractionation through (b) C2L; (c) C2T; (d) C4L; (e) C2L2T; bar = 1 mm

Sprouting assessment

Sprouting of tagged whole and fractionated seeds was assessed on moistened paper towel pads according to International Seed Testing Association (ISTA, 2019). Each tagged whole seed or seed fraction remained identifiable throughout the experiment with the aid of a labelled aluminium foil boat sized ≈ 15 mm in diameter and ≈ 10 mm in height. A moistened paper towel pad was placed in each aluminium boat for sprouting of the whole seed or seed fraction. The experimental units were placed randomly in numbered transparent plastic containers according to a completely randomized design as mentioned. Tap water was added to the pads using a dropper when and as necessary to ensure moist condition throughout the sprouting evaluation period. The boxes were kept closed throughout experimentation except during data collection procedure. The whole seeds and seeds fractions were sprouted at ambient temperature of 25±2 °C and with diffused light reaching the seed germination rack near the window in the laboratory.

Daily or on alternate days, the boxes were uncovered for examining the growth of roots and shoots from whole seeds and seed fractions. Rooting was noted when a root of ≥2 mm in length emerged. A stereo microscope was used to confirm the root emergence when and as necessary. Then, each rooted unit was followed up for growth of hypocotyl and epicotyl. In view of epigeal sprouting of the whole and fragmented seeds, plantlet development was only recorded after the epicotyl had elongated to ≥5 mm in length. The first two tiny true leaves of the plantlet were distinguishable at this stage. Sprouting assessment was terminated after 240 days when no new root or shoot development was noted for 30 consecutive days. Rate of plantlet development and rate of rooting with no shoot emergence for each type of seed fraction were calculated separately using the formula below.

$$Rate = \left(\frac{\sum_{i=1}^{k} n_i}{N}\right)$$

where,

n_i = number of sprouted (rooted or developed plantlet, respectively) experimental units (whole seeds or seed fractions, respectively) on the i^{th} day (not the accumulated number)

N = total number of experimental units

k = last day of sprouting procedure

Thus, the total sprouting rate for each seed fractionation treatment was sum of plantlet development rate and rate of rooting with no shoot growth for all its seed fractions. For example, total sprouting rate for C2L = rate of plantlet development for [C2L(L) + C2L(R)] + rate of rooting with no shoot emergence for [C2L(L) + C2L(R)].

Re-growth assessment

Following the growth of epicotyl, the entire plantlet and a portion of cotyledon connected to it were cut off carefully using a scalpel. Then, the seed fragment was evaluated for the potential of re-sprouting. For C0, both the separated cotyledons obtained after the detachment of plantlet and portions of cotyledons connected to it were used in the re-growth assessment. Re-growth from cotyledon was also studied on moistened paper towel pad as described above but both the pad and aluminium foil boat were renewed for this purpose. Re-growth was counted when a new root of ≥2 mm in length was formed from the cotyledon. The re-rooted units were also monitored for subsequent shoot development. Assessment was terminated at the same time as mentioned after no re-growth was recorded for more than 30 consecutive days. Re-growth rate was calculated using the above formula.

Statistical analysis

The rate of plantlet regeneration as well as the rate of total sprouting as impacted by seed fractionation treatment were subjected to analysis of variance (ANOVA), respectively. Treatment means were compared using the least significant difference (LSD) test at 5% level of significance. To describe the time to root and shoot emergence, a box plot was generated according to the type of seed fraction. In the re-growth assessment, bar chart and scatter plot were employed to describe the re-growth rate and time, respectively. Statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) version 25.

RESULTS AND DISCUSSION

The fully ripe fruits provided highly germinative seeds. Each whole seed (C0) germinated and provided a seedling with a robust tap root and a shoot (Figure 2a; Table 1). Thus, C0 had a perfect regeneration rate of 1. C0 showed polarity in germination. Within one to two weeks after radicle protrusion, the hypocotyl elongated and uplifted both cotyledons from the moistened paper towel pad. Then, an epicotyl developed from the hypocotyl, which was always connected to the two cotyledons near the distal part of the seed (Figure 2a).

Halving a seed did not affect its ability to develop an entire plant. In the current work, as high as 97% of the seeds following longitudinal halving (C2L) produced plantlets with either half fraction, respectively (Table 1). The right side [C2L(R)] of the seed had a relatively higher tendency (61%) for entire plant development. However, cell proliferation and differentiation on the cotyledonary surfaces of their complementary halves brought about the formation of a single or a few adventitious roots each while no growth of shoot was seen. In total, the sprouting rate for C2L was 1.94, which was significantly ($P<0.05$) higher than the sprouting rate of 1 with C0 (Table 1).

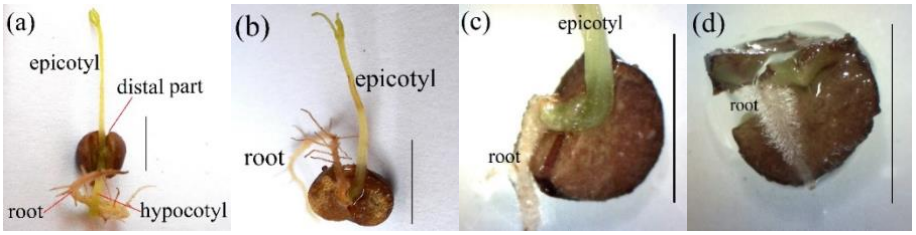

Fig. 2. Regeneration of plantlet from (a) C0; (b) C2T(D); (c) C4L(L); (d) growth of adventitious root from C2L2T(LD); bar = 5 mm

Table 1. Sprouting rates of whole seeds and seed fractions

Fractionation treatment	Seed fraction	Rate of plantlet development (Rate of rooting with no shoot emergence)	Total sprouting rate
C0 (whole seed)	-	1 (0)	1
C2L	L + R	0.36 + 0.61 = 0.97 (0.61 + 0.36 = 0.97)	1.94
C2T	P + D	0.19 + 0.81 = 1 (0.66 + 0.17 = 0.83)	1.83
C4L	L + CL + CR + R	0.14 + 0.31 + 0.22 + 0.25 = 0.92 (0.69 + 0.61 + 0.64 + 0.69 = 2.63)	3.55
C2L2T	LP + LD + RP + RD	0.08 + 0.14 + 0.25 + 0.28 = 0.75 (0.72 + 0.69 + 0.69 + 0.67 = 2.77)	3.52

LSD (P=0.05) for rate of plantlet development is 0.2153.
LSD (P=0.05) for total sprouting rate (rate of plantlet development + rate of rooting with no shoot emergence) is 0.3203.

With transversal cutting on the seeds (C2T), one of the halves of each seed developed a plantlet (Figure 2b). Thus, C2T was equivalent to C0 with respect to entire plant development rate, which was 1. The distal region of the seed [C2T(D)] had a higher plantlet development rate of 0.81 compared to C2T(P). For their complementary halves, 83% of them rooted. In total, C2T had a sprouting rate of 1.83, which was comparable to that achieved by C2L.

When a seed was cut longitudinally into four fractions (C4L), 83% of the seeds could still provide plantlets with at least one of their quarters despite having much reduced mass (Table 1). The centre quarters of C4L(CL) and C4L(CR) as well as the right fractions of C4L(R) (Figure 1c) had plantlet regeneration rates of above 0.22 while 14% of the left quarters [C4L(L)] developed plantlets (Figure 2c). Interestingly, C4L interrupted the polarity in seed and induced the haphazard development of embryonic cell in the cotyledons. One of the seeds yielded two plantlets from two of its separated fractions. In this case, the two plantlets were produced by the left [C4L(L)] and centre left [C4L(CL)] portions, respectively. In other word, C4L revealed the potential of polyembryony in this species. On the other hand, more than 80% of their complementary cut seeds developed adventitious roots from the cotyledonary surfaces. Therefore, C4L had the highest total sprouting rate of 3.55 (Table 1).

C2L2T further supported the spread of meristematic tissues within both cotyledons of *S. myrtifolium*. Although this seed incision procedure significantly reduced the plantlet regeneration rate, being only 0.75, the rooting rate of the fractions was high leading to a total sprouting rate of also 3.5, which was comparable to that for C4L (Figures 2d; Table 1). Like C4L, C2L2T also recorded an incident of double plantlet regeneration from a seed; its proximal fractions, being [C2L2T(LP)] and [C2L2T(RP)], developed plantlets in this case.

In terms of sprouting time, whole seeds (C0) and fractionated seeds that developed plantlets generally exhibited radicle protrusion simultaneously within three weeks (Figure 3). Then, their shoots emerged within the next one to three weeks culminating the development of full plants. However, the rooted seed fractions that did not regenerate any shoot had marked differences in time to rooting (Figure 3). Some rooted within a few days while others took much longer time of up to 209 days to form their first roots. The seed fragments were free from microbial infestation despite the moist environment within plastic boxes throughout the sprouting study.

After the removal of the plantlet alongside a portion of cotyledon attached to it, the seed fragment showed high possibility for new adventitious root development from the cotyledon but no shoot regeneration could be achieved in the re-growth stage. Re-rooting rates were mostly above 0.7 (Figure 4). Likewise, seed fragments showed irregular re-rooting time (Figure 4). Cut seeds were also mostly free from microbial problem in the re-growth study.

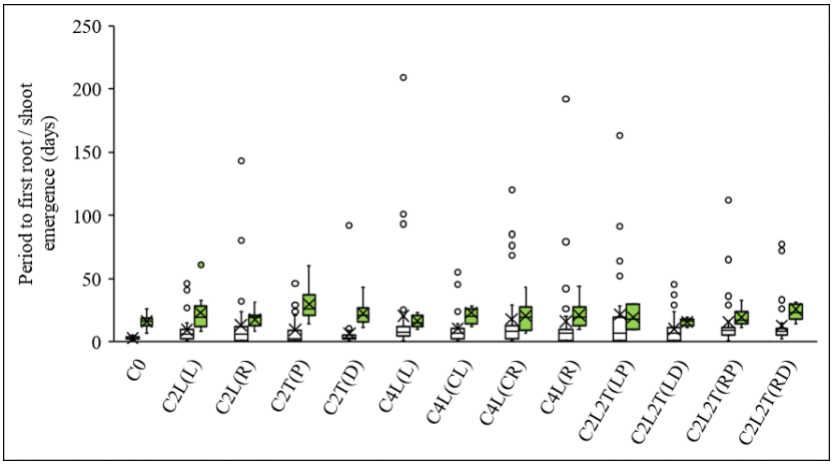

Fig. 3. Quartiles and range (whiskers) for rooting (blank bars) and shoot (green bars) emergence periods, respectively; x indicates mean value; blank dot (○) indicates outlier for rooting period; green dot (●) with C2L(L) indicates outlier for shoot emergence period.

The above results imply the spread of meristematic tissues in the two cotyledons of *S*. *myrtifolium* seed. In general, complete incision(s) on a seed to stipulate separated halves and even smaller quarter fractions did not affect its capability of developing a full plant. However, the way how a seed was fractionated could jeopardize its polarity and trigger the development of an embryonic or regenerative cell in other part of the cotyledons. While whole seeds had consistently germinated and developed seedlings near their basal side away from hilum, dividing a seed into fractions revealed the possibility for regeneration of full plant from other region of the cotyledons. At a substantially reduced size after a seed was sectioned into four portions, the patchiness of embryonic cell development turned obvious. It was even possible for developing more than one plant from the separated fractions of a seed. The self-inhibition effect that hinders the concurrent growth of more than a plantlet must have masked the expression of polyembryony in intact seed of *S*. *myrtifolium* (Amador & Barbedo, 2015). The injuries and reserve reduction resulted from cutting a seed into fractions could have impacted its structural, physiological and biochemical dynamics in culminating the formation of an entire plant (Amador & Barbedo, 2015, Calvi *et al*., 2017b). The transcriptional control and molecular signals involved in the regulation of cellular differentiation followed by certain tissue and organ formation from the cotyledons are other subjects of research (Sampathkumar *et al*., 2014, Li *et al*., 2020). The genotype of the individual plants of *S*. *myrtifolium* as induced by seed sectioning is yet to be validated as the adventitious true-to-type for future phylogenetic systematics.

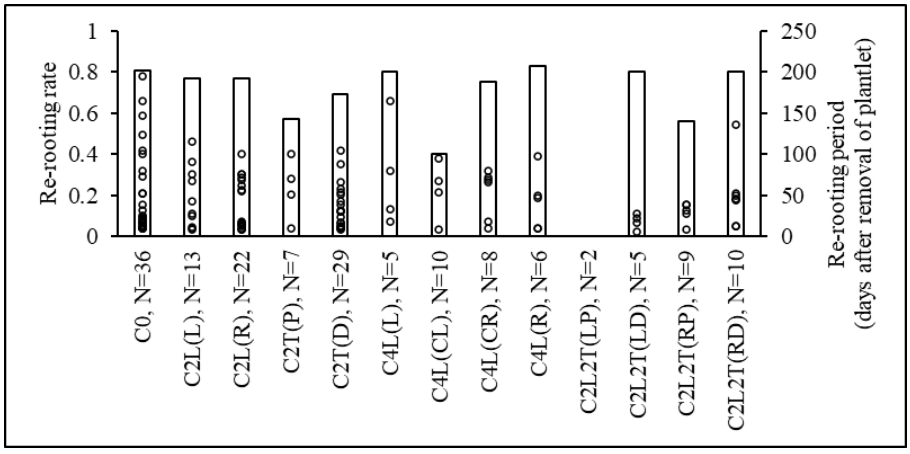

Fig. 4. Re-rooting rate (bar) and time (○) after removal of plantlet and a portion of cotyledon connected to it

However, the reasons for shoot development failure in the complementary fractions, and during the re-growth stage, are yet to be investigated. Internally, the availability and balance among the organic compounds, hormones and minerals in the underlying cells that differ by seed development phase may affect their developmental abilities (Chickarmane *et al*., 2012, Pierre-Jerome *et al*., 2018). Environmental cues, for example lighting and nutrition during sprouting, may also have a role to play in interaction with certain genes in determining the growth of shoots after root formation from the cotyledons (Gaillochet & Lohmann, 2015, Gambhir *et al*., 2017, Zimik & Arumugam, 2017).

CONCLUSION

Whole seed of *S. myrtifolium* exhibited polarity but sectioning a seed into fractions induced the development of regenerative cell haphazardly in other regions of the cotyledons. Thus, a seed could possibly produce more than one entire plant from its fractionated portions suggesting that the regenerative cotyledons could be liable for the polyembryony potential in this plant species.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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