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Propagation of *Syzygium malaccense* through seed fractionation technique

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Abstract

Syzygium malaccense belonging to the large family of Myrtaceae produces medicinal plant parts and edible fruits which contain high nutritional values and antioxidants. However, the limited occurrence of this fruit species within Malaysia has hindered its commercialization potential. Large-scale planting is necessary to promote this indigenous fruit. A timely supply of sufficient planting materials is needed for this purpose. To date, this tree species is mainly propagated by seeds while stem cutting and grafting techniques are less successful. Although a whole seed has a single plumule-radicle axis, preliminary work showed that a seed could produce more than one plant with its fragmented portions. The cotyledonary fraction devoid of embryo could become regenerative leading to the formation of an entire plant. This phenomenon in *S. malaccense* could be associated with polyembryony documented in many other members within Myrtaceae. The present study explored seed fractionation as a non-conventional method in the propagation of *S. malaccense*. The seeds cut systematically into separated halves and quarters were studied for in vivo sprouting potential in a moistened sand medium. The experiment was carried out on germination racks in the laboratory. The results indicated that the reduced reserve did not impact the development of full plants from the seed fractions. The quarter seeds were comparable to the half seeds with a full plant regeneration rate of up to 1.5 while the intact whole seeds had a rate of 0.93. Thus, this simple seed fractionation technique is useful for the multiplication of *S. malaccense*. Moreover, most of the remaining seed fragments were rooted simultaneously but their shoots were yet to be visible by the end of the study period of five months. Attempts that facilitate the shoot development after adventitious rooting from the fractions through the manipulation of some environmental factors would be beneficial for the propagation of this fruit species. The genetic fidelity of the plantlets originating from a single seed is another research focus in sourcing an alternative for clonal planting materials.

1. Introduction

Syzygium malaccense is a woody plant species belonging to the family Myrtaceae. It is commonly known as the Malay apple and is native to the Indo-Malayan region. The tree has several economic values. Its barks and leaves have medicinal properties to cure inflammations, treat mouth infections and reduce sore throat (Karioti *et al*., 2007; Lim, 2012; Lim and Rabeta, 2013; Batista *et al*., 2017). Additionally, it bears large-ovoid fruits with high amounts of fibre, vitamins, minerals and antioxidants (Whistler and Elevitch, 2006; Pazzini *et al*., 2021). The fruit is normally consumed fresh or made as a component in a mixed fruit salad. It is also utilized in the production of fruit paste, candied fruit or jam.

Despite having commercial potential, *S. malaccense* remains grown wildly or only domesticated for household consumption. This fruit tree is still an underutilized indigenous plant species in Malaysia (Fernandes and Rodrigues, 2018). Considering its commercial viability, it is necessary to establish the large -scale production of the economic parts, for example the fresh fruits. While this dicotyledonous plant is generally propagated through seeds as cutting and grafting techniques are less successful, the seed-based propagation has its own limitations, particularly when the fruits and seeds are vulnerable to animal predation (Ryadin *et al*., 2014; Yusnita *et al*., 2018). However,

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many seeds have mechanisms to counteract animal consumption in ensuring their population growth in the natural environment. Protecting the embryos within seeds is one of the important strategies to secure the development of seedlings at post-predation. Yet, the loss of the plumule-radicle axis is not necessarily lethal. Past research reported sprouting from some seed fragments devoid of their regenerative axes in the unattended field. Decades ago, the fragments of a large seed of *Gustavia superba* from the family Lecythidaceae were found mostly capable of developing plantlets in the forest (Howe and Richter, 1982; Sork, 1987; Dalling *et al*., 1997; Dalling and Harms, 1999). The large seed of *Idiospermum australiense* from Idiospermaceae could also produce plantlets from its cotyledonary sections in the natural sites of north Queensland, Australia (Edwards *et al*., 2001). Later, accumulating evidence noted that several monoembryonic *Eugenia* species from the family Myrtaceae could also regenerate with their seed fractions in the laboratories (Delgado *et al*., 2010; Prataviera *et al*., 2015; Calvi *et al*., 2017; Alonso *et al*., 2019). Such a unique tolerance to damage in seeds is not well understood. It is hypothesized that the cotyledonary fragments were capable of inducing the embryonic cell development and entire plant formation as an adaptation to face injuries (Kinsman, 1990; Joshi *et al*., 2006; Anto *et al*., 2018). The cellular growth and differentiation responses within cotyledons have been analogous to plant regeneration with cutting and grafting. With *Eugenia* seeds, the multiple plant formation from the fractions could be associated with polyembryony, despite having a single differentiated embryonic axis within a seed, since many species in the family of Myrtaceae bear seeds with multiple embryos each (Thurlby *et al*., 2011; Thurlby *et al*., 2012).

Within the large family of Myrtaceae represented by approximately 3,000 woody species, the genus *Syzygium* is closely related to the genus *Eugenia* although these two genera are separated taxonomically in terms of organography, histology and vasculature (McVaugh, 1968; Schmid, 1972; Parnell, 1999; Mitra *et al*., 2012). Thus, the monoembryonic *Syzygium* could have a high likelihood of producing more than one plantlet when fractionated, especially considering many other members of *Syzygium* that naturally contain multiple distinguishable embryos in each seed (Sivasubramaniam and Selvarani, 2012; Rekha *et al*., 2020). In one of the instances, preliminary study revealed the sprouting potential from the fragments of the monoembryonic *S. malaccense*. Thus, this work aimed to systematically investigate the effect of seed fraction size on the regenerative capability of *S. malaccense* through the reserve effect hypothesis. In this hypothesis, the larger seed fragments have more nutrients in the cotyledons to act as a risk hedge against the exhaustion of energy in the germination or sprouting success (Harms and Dalling, 1997; Perea *et al*., 2011; Loayza *et al*., 2015). Simultaneously, the most appropriate fractionation technique with regards to the regenerative polarity was also assessed with the different types of separated seed portions in this study. In due course, the findings could be beneficial in determining the potential of this non-conventional propagation strategy to provide timely and multiple-fold planting materials.

2. Materials and methods

2.1 Fruit collection and seed preparation

Fully ripe fruits of *S. malaccense* with reddish pink pericarp were collected from a farm located in Tanjung Sepat, Selangor, Malaysia (2°38'55.9"N, 101°35'38.7"E), at the end of November 2021. A total of 250 fruits were collected from a single tree. The harvested fruits were brought to the Plant Science Laboratory of the Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA, Jasin Campus, Malacca.

On the following day, the crispy white fruit flesh was removed carefully using a pair of secateurs. Then, the ovoid seeds measuring approximately 2.5 cm in diameter were extracted from the fruits. Each fruit contained a single seed. The seeds were washed under slow-running tap water to remove the pulp residue. The seeds were pad-dried with a paper towel and air-dried for two hours on the laboratory bench to remove surface water. Seeds with visible damages were eliminated at this point in time, leaving behind 200 intact seeds for experimentation.

2.2 Intact seed weighing

After removal of surface water, the seeds were randomly tagged and numbered in celled plastic containers. Each numbered seed was weighed using an analytical balance.

2.3 Seed fractionation

The tagged seeds were assessed for regeneration potential following four seed fractionation treatments. Seed cutting was carried out using a sharp knife as the seeds were relatively large. The control was the intact whole seed (C0), as shown in Figure 1a. Each treatment was replicated four times with 10 seeds per replicate.

For consistency in fractionation treatments, each seed was first identified for its left, right, proximal and distal parts based on the seed architecture and positions of the hilum and a small pit on the seed. For obtaining the half seeds with longitudinal fractionation (C2L), a seed was cut from the hilum to the base of the seed

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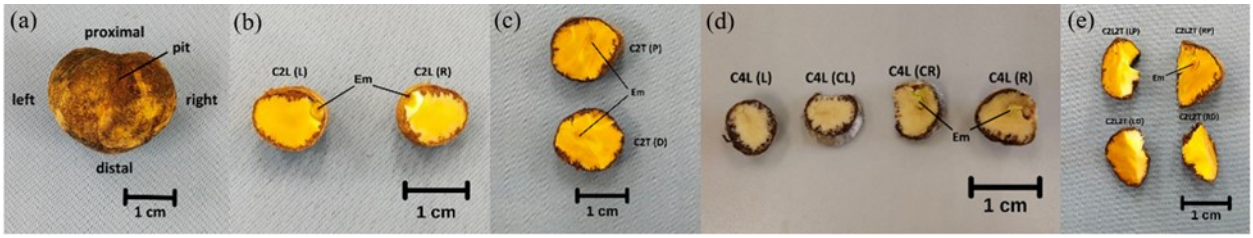

Figure 1. Seed fractionation studied. (a) C0 showing the small pit, left (L), right (R), proximal (P) and distal (D) parts; seed fractions of (b) C2L(L) and C2L(R); (c) C2T(P) and C2T(D); (d) C4L(L), C4L(CL), C4L(CR) and C4L(R); (e) C2L2T(LP), C2L2T(LD), C2L2T(RP) and C2L2T(RD).

(Figure 1b). The right (R) half was named [C2L(R)]. It was the half seed with the small pit. The accompanying half was named C2L(L). The other seed-halving procedure was with transversal cutting of the seed (C2T) (Figure 1c). The half seed with the hilum or the proximal (P) half was named [C2T(P)], while C2T(D) was the basal or distal (D) fragment.

The regeneration potential of the quarter seeds was also studied. To obtain quarters having both the proximal and basal parts, a seed was cut longitudinally into four fractions (C4L) as in Figure 1d. The seed fragments in order from left (L), centre left (CL), centre right (CR) and right (R) were C4L(L), C4L(CL), C4L(CR) and C4L (R), respectively. The other seed fractionation procedure that resulted in four seed fragments was C2L2T. The first cut on the seed was longitudinal halving to obtain C2L (L) and C2L(R). Then, each half seed was cut transversely. Thus, the seed fragments having the left (L), right (R), proximal (P) and distal (D) parts in combination were C2L2T(LP), C2L2T(LD), C2L2T(RP) and C2L2T(RD), respectively (Figure 1e).

The seed was exalbuminous. The two cotyledons were tightly packed within the seed coat despite the full ripeness of the fruit enclosing it. By virtue of the architecture of the seed, the above fractionation treatments did not separate the cotyledons entirely. Thus, each half and quarter seed generally had asymmetrical fractions of both cotyledons.

Each seed fragment was weighed immediately after the fractionation procedure using the analytical balance. Then, whole seeds and seed fractions were tagged accordingly with the aid of celled plastic containers for the next sprouting procedure.

2.4 Regeneration assessment

The regeneration potential of control whole seed and seed fractions was assessed in moist sand-sized 0.2-2.0 mm (International Seed Testing Association (ISTA), 2019). The sand was sieved and washed thoroughly with slow-running tap water a few days before fruit harvesting. The cleaned sand was then dried completely under the sun followed by cooling to room temperature prior to use.

An amount of 500 g sand was moistened evenly with 80 mL tap water in each labelled transparent plastic box measuring 14.5 × 9.5 × 6.5 cm. Then, control whole seeds and seed fragments were sprouted in the medium based on a completely randomized design. Seed fragments were sprouted in vivo without any surface sterilization procedure. The containers were closed with their lids throughout the sprouting assessment except during data collection. The sand medium generally remained moist throughout the experiment while a suitable amount of water was sprayed onto the medium using a hand spray bottle when and as necessary.

Sprouting of whole seeds and seed fractions was monitored on alternate days for a period of 150 days after no new growth of plantlet or rooting was recorded for a consecutive 30 days. Intact seeds and seed fractions were each considered to have sprouted when a root measuring at least 2 mm was visible. A magnifying glass was used to confirm the root emergence when and as necessary. Subsequently, the rooted whole seeds and seed fragments were each counted as plantlet development success when a young shoot of at least 1 cm turned visible.

At the end of the study, the plantlet regeneration rate and the rate of rooting with no shoot development 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 (developed plantlet or rooted, separately) experimental units (whole seeds or seed fractions, separately) on the i^{th} day (not the accumulated number), N = total number of whole seeds and k = last day of the sprouting procedure

Total sprouting for each seed fractionation treatment was the sum of plantlet regeneration rate and rate of rooting with no shoot development with all its seed fractions. For example,

Total sprouting rate for C2L2T = Sum of plantlet regeneration rates with C2L2T(LP), C2L2T(LD), C2L2T (RP) and C2L2T(RD) + Sum of rooting rates with no shoot development with C2L2T(LP), C2L2T(LD),

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C2L2T(RP) and C2L2T(RD)

Each whole seed and seed fraction was also recorded for the time taken for root and plantlet development. Dead seeds were recorded and discarded immediately.

2.5 Statistical analysis

Whole seeds and seed fractions were described by their weight. In the sprouting assessment, independent-samples Kruskal-Wallis tests were carried out for plantlet regeneration rate and total sprouting rate, respectively. Mean separations were performed with Mann-Whitney U-tests at P = 0.05.

3. Results

3.1 Weight of whole seed and seed fractions

The intact seeds applied for experimentation showed homogeneity of variances (P>0.05), as assessed by Levene's test for equality of variances. The mean whole seed weight was 12.5637±0.6779 g with a coefficient of variation (CV) of 5%. With fractionation procedures, half seeds obtained following C2L and C2T had a mean weight of 6.1612±0.6122 g with a CV of 10%. Simultaneously, quarters resulting from C4L and C2L2T were 3.0741±0.3702 g with a CV of 12%. The weight of the seed fractions implied that the seed-cutting treatments were appropriate for the sprouting study.

3.2 Sprouting rate

In the in vivo sprouting assessment in a moistened sand medium, the seed fragments proved their capability to provide entire plants despite reduced reserves incurred by seed fractionation. While C0 whole seeds achieved a plantlet regeneration rate of 0.93, half seeds of C2L(L), C2L(R) and C2T(P) had a full plant development rate of 0.7 to 0.8, respectively (Figure 2a). Nonetheless, the basal halves had a lower ability to establish full plants; C2T(D) had a plantlet development rate of only 0.475 (Figure 2a). In conjunction with the aforementioned sprouting performance, halving a seed into two separated portions is noteworthy for inducing more entire plants. C2L offered a plantlet regeneration rate of above 1; it had a significantly higher rate of 1.5, contributed by both the C2L(L) and C2L(R) fractions, when compared to a rate of 0.93 gained by whole seeds of C0 (Figure 2a). C2T also provided more full plants at a rate of 1.2 in comparison to C0.

A further reduction of reserves following fractionation by means of C4L and C2L2T to produce quarter fragments generally did not impact the entire plant regeneration potential (Figures 2a, 3a and 3b). In all, C4L and C2L2T had plantlet development rates of 1.5 and 1.4, respectively, which were comparable to those subjected to C2L and C2T (Figure 2a). Among the quarter seeds, only the leftmost quarters of C4L(L) were apparently less capable of establishing new plants.

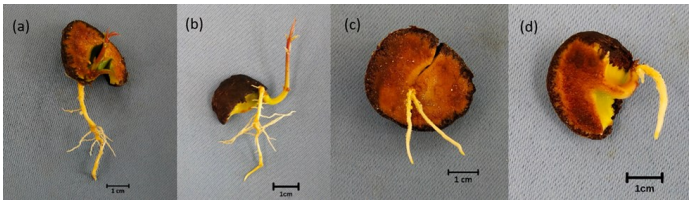

Figure 3. Sprouting from quarter fractions; development of plantlets from (a) C4L(R) and (b) C2L2T(RD); growth of only adventitious roots from (c) C4L(L) and (d) C2L2T (RP).

Despite fractionation treatments, the plantlets developed from the seed fragments had normal root and epicotyl elongation. Both the whole seeds and seed fractions demonstrated hypogeal sprouting whereby the hypocotyl did not elongate and the cotyledons stayed in the medium after sprouting. Only the epicotyl emerged from the sand medium marking the formation of a full plant. The exposed cotyledonary surfaces at post-fractionation were free from microbial contamination during in vivo sprouting. Thus, this study shed light on the potential of multiplication of *S. malaccense* through seed fractionation technique.

On the other hand, the remaining seed fractions showed a high rate of adventitious root development but did not develop their shoots until the end of the study period (Figures 2b, 3c and 3d). When combined with the plantlet regeneration rates mentioned above, C2L and

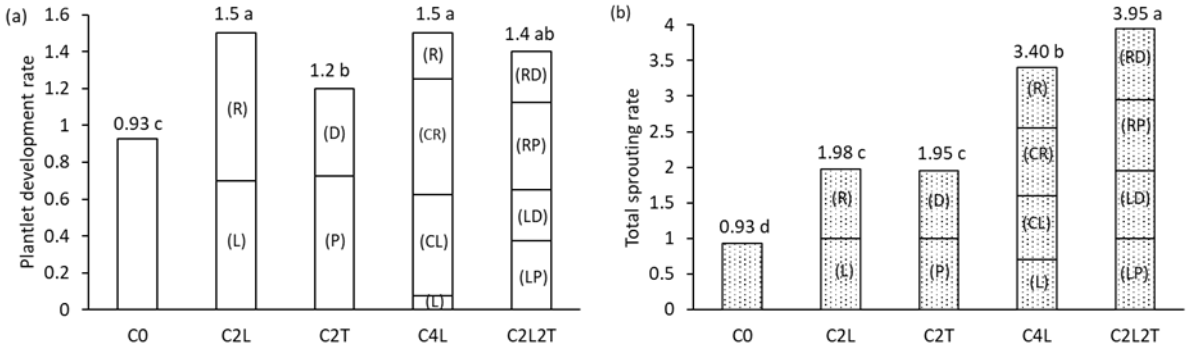

Figure 2. Sprouting rate following seed fractionation: (a) Plantlet development rate, (b) total sprouting rate; means within each chart having similar letter are not significant at P = 0.05.

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C2T gained an almost doubled total sprouting rate of above 1.9 as compared to a rate of 0.93 with C0, since all the sprouted whole seeds developed full plants (Figure 2b). Likewise, C4L had a total sprouting rate of 3.4 while C2L2T had the highest total sprouting rate of 3.95 (Figure 2b). These seed fragments were also free from microbial problems while their adventitious roots utilized the fraction reserve for limited growth.

3.3 Sprouting time

In the event of plantlet development, both the whole seeds and seed fragments generally rooted within three weeks followed by the growth of shoots in the next two to three weeks (Figure 4a). The mean and median values for the time to the first root emergence were 6.79±4.38 days and 6 days, respectively, while those for the shoot development were 20.79±16.35 days and 15 days, respectively. However, a small percentage (6%) of the seed fractions, especially the quarter fragments, took more than six weeks after rooting for shoot formation, as reflected by a high standard deviation for shoot emergence time. One of the seed fragments of C4L(CR) developed its shoot at 114 days, although it had rooted long before at 11 days.

On the other hand, the seed fractions that only sprouted with adventitious roots but did not show any sign of shoot development generally rooted later in comparison to those which formed full plants (Figure 4b). Furthermore, a higher proportion of these cut seeds demonstrated unsynchronized rooting resulting in a higher standard deviation for the rooting time. The mean and median values for the rooting time for these fragments were 21.72±17.62 days and 15 days, respectively.

4. Discussion

The current work showed that a non-conventional propagation method through seed fractionation is applicable for *S. malaccense*. The half-seeds and quarter-seeds proved their capability to develop full plants. Thus, cutting a seed to stipulate separated portions resulted in a plantlet development rate of above perfect 1 in contrast to a rate of 0.93 for the intact seeds. In this context, the full plant formation potential from the seed fragments was unlikely dependent on their reserve or mass according to the reserve effect hypothesis. In contrast, the cotyledonary surfaces of *S. malaccense* were hypothesized as regenerative or embryonic. The quarter seed with very much reduced reserves was still capable of forming an entire plant even at the absence of the differentiated plumule-radicle axis. On account of the sporadic plantlet development from most of the under-studied seed fractions, except for the leftmost quarter seeds, the whole seed could likely be totipotent, in contrast to polarity demonstrated by the differentiated embryonic axis in intact seed germination. In conjunction to such a growth potential from the cotyledons, seed fractionation enabled a higher plantlet development rate of up to 1.5 in the current work. The increased regeneration rate was in accordance with those reported earlier with several *Eugenia* spp. and *Syzygium myrtifolium* from the same family of Myrtaceae (Delgado *et al*., 2010; Teixeira and Barbedo, 2012; Calvi *et al*., 2017; Alonso *et al*., 2019; Tsan, 2023).

Additionally, the mitotic cells in the cotyledons also resulted in the development of adventitious roots from most of the other fractions culminating in a total sprouting rate as high as 3.95 by the seed fractionation treatment of C2L2T. The rooting on the cotyledonary surfaces of these seed fragments was similar to the adventitious root development with the stem cutting propagation. For these rooted seed fractions, some environmental cues of light level, temperature, nutrition or external plant growth regulator applications could be studied in future for regulating the development of their shoots, corroborating seed fractionation as a viable technique for the multiplication of planting materials (Rikiishi *et al*., 2015; Wei *et al*., 2020).

The sprouting time exhibited by the seed fractions further supported the regenerative properties within cotyledons of *S. malaccense*. In the case of plantlet development, some rooted seed fragments took more

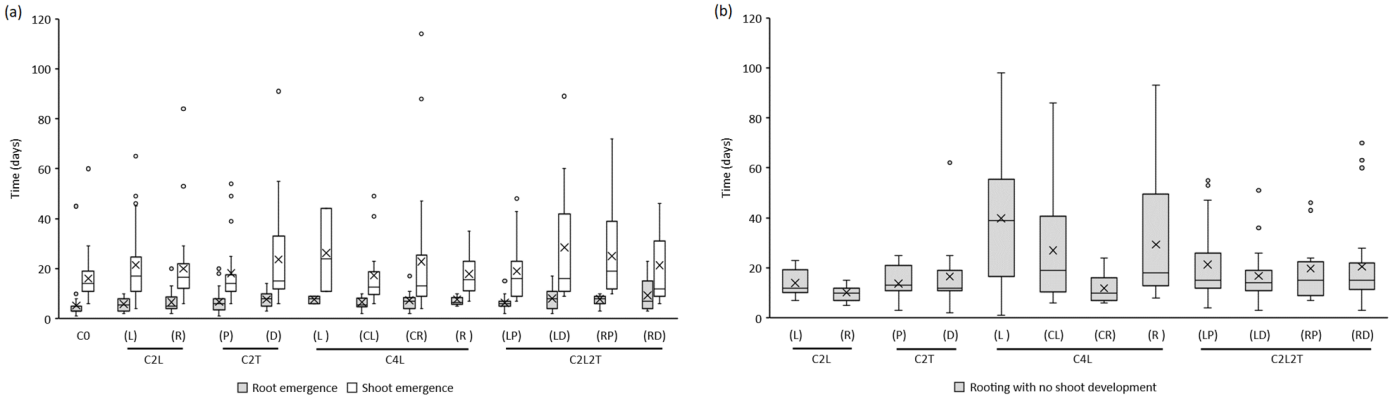

Figure 4. Sprouting time for fractions which developed (a) plantlets and (b) only adventitious roots; all C0 developed plantlets.

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than two months to develop their shoots in opposed to the simultaneous shoot emergence in the next three weeks after radicle protrusion with whole seeds. There were also a few seed fragments which developed the shoots first followed by the growth of their roots from the basal part of their shoots within a few days. Such an unusual growth phenomenon was opposed to seed germination in which the radicle always emerges prior to plumule elongation. In this case, the shoot-root development sequence could be analogous to the in vitro proliferation of shoots. Other evidence with regards to the natural cell division potential in the cotyledons of *S. malaccense* seed was the marked differences in time to the first root development from the seed fractions which only rooted throughout the study period, yet to develop their shoots. The developmental plasticity displayed by the seed fragments suggested the likelihood of a fractionation technique for the propagation of this woody plant species (Gaillochet and Lohmann, 2015; Ikeuchi *et al*., 2016; Landrein *et al*., 2018). The other study subject is the genetic variations among the regenerants originating from the same seed in deducing the clonal planting material production option through this simple propagation technique (Fei *et al*., 2019; Souza Ferreira *et al*., 2019).

5. Conclusion

Propagation of *S. malaccense* is possible through seed fractionation. The seeds gained a higher plantlet regeneration rate of up to 1.5 as compared to intact whole seeds which had a rate of 0.93. In addition, most other seed fractions developed a single or multiple adventitious roots each, which was similar to rooting from the stem cuttings. Future studies for inducing the rapid and uniform development of shoots from the rooted fractions and the genetic fidelity research on the regenerants would be beneficial for the multiplication of this woody fruit species.

Conflict of interest

The authors declare no conflict of interest.

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