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SHORT COMMUNICATION

In vitro developmental study of oil palm (*Elaeis guineensis* Jacq.) polyembryoids from cell suspension using scanning electron microscopy

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Abstract In the present study, we report the in vitro development of polyembryoids with identification of a definite stage that can be used for subsequent uniform plantlet regeneration in oil palm (*Elaeis guineensis* Jacq.). Induction and maturation of polyembryoids was accomplished when cell suspension culture was transferred in MS (Murashige and Skoog, Physiol Plant 15:473–497, 1962) semisolid medium consisting of 30 g L^{-1} sucrose and 3.5 g L^{-1} gelrite® devoid of any plant growth regulator. Growth and development of cell suspension culture into polyembryoids were assessed by stereo and scanning electron microscopy (SEM) to identify the sequential events as well as the differentiation that occur during each stage. Observations on the differentiation symptoms showed that the embryos pass through distinct morphological characteristics indicating distinctively varied stages. SEM observations indicated the development of extracellular network at an early stage of differentiation and acts as the structural marker of differentiation leading to the development of polyembryoids via formation of globular proembryo and haustorium. Eventually, a specific developmental stage comprising haustorium and torpedo-shaped structure was identified, for conservation, regeneration or multiplication, based on the embryogenic competence.

Keywords Developmental stage · Dura × Pisifera · Extracellular matrix · Micromorphology · Multicellular suspensor · Rosette like complex · Scanning electron microscopy · Somatic embryo

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ECM	Extracellular matrix
FM	Fibrillar material
GPE	Globular proembryo
IC	Intact cells
MCS	Multicellular suspensor
PT	Protoderm
RLC	Rosette like complex
SEM	Scanning electron microscope
SP	Shoot primordia

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a monocotyledonous plant belonging to the family Arecaceae. It is an economically important source of vegetable oil that has been largely traded in the international market (Corley and Tinker 2003). Oil palm is normally propagated by seeds and today the Dura (D) × Pisifera (P) hybrids, produced by authorized agencies, are being used as the source of seeds. However, to accelerate and to maximize the yield potential, clonal plantlets derived

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from selected ortets have been reported to be the way forward. According to Hardon et al. (1987), the use of clones can result in yields by about 30 % more than the seed-derived D × P standards. Currently, micropropagation via somatic embryogenesis originating from young leaf has become the key method for clonal multiplication of oil palm (Konan et al. 2006). Young leaves are cultured for the induction of embryogenic callus in media containing high auxins, which is then converted into cell suspension culture. Subsequent subculture in auxin free media leads to the transformation of the suspension cells into embryoids. Interestingly, in oil palm, somatic embryogenesis eventually culminates in the production of a typical self-perpetuating structure termed as 'polyembryoid', which is an aggregation of somatic embryos (Suranthran et al. 2012).

The process of embryoid formation from suspension culture has been reported to be a time consuming process requiring circa 2 years. Additionally, in vitro culture of oil palm for conversion into polyembryoids has been reported to produce asynchronous embryoids of different sizes, colors, and developmental stages at any given culture period indicating that the cultures are highly heterogenous. This heterogeneous in vitro behavior of oil palm polyembryoids directly impacts the productivity of plantlets of each clonal line. As a result, management and forecasts of large scale production of clonal plants through somatic embryogenesis still remains difficult (Konan et al. 2006). The ability to identify appropriate stage of polyembryoid maturity which is suitable for direct conversion into plantlet among the various heterogeneous growth stages and isolation of propagules of this stage will allow better exploitation of its regeneration potential. Although tissue culture of oil palm has been conducted for many years, however, no report exists especially on handling and selection of suitable stage, for rapid conversion into uniform plantlet. Hence, an understanding of this developmental phenomenon may help in improving the selection of embryoids at the right stage for rapid conversion to plantlet; thus, uniform seedlings of oil palm can be obtained for large scale production. Furthermore, the propagules of the precise stage have the potential for storage via synthetic seed production, cryopreservation as well as production of large numbers of disease-free planting stock for conservation and restoration (Ma et al. 2012).

Somatic embryogenesis in oil palm begins with the formation of callus, followed by conversion of the callus into suspension culture which often contains two cell types namely, the large, highly vacuolated, and freely dispersed cells, or the small, densely cytoplasmic, and aggregated cell clumps. Selection of the latter is necessary and in recent times, substantial interest is being raised on extracellular matrix (ECM) layer, a fundamental component of ECM-plasma membrane-cytoskeleton continuum which plays an essential task in the reception as well as transduction of signals linked with positional information, recognition, cell fate determination, and plant growth (Popielarska-Konieczna et al. 2008). Šamaj et al. (1999) reported the morphoregulatory significance of ECM during somatic embryogenesis and organogenesis, implying an active role in plant morphogenesis. Later, Bobák et al. (2003) proposed the fundamental role of ECM in cell recognition, cell-to-cell interaction, cell division and differentiation, and also in generation and maintenance of some traits in plant cell populations. Recently, Lai et al. (2011) described the potential of ECM as a structural marker of embryogenic competence.

Therefore, the present study was conducted to determine micromorphological differences which indicate embryogenic competence during development of polyembryoids from cell suspension culture in oil palm. Besides, the knowledge on changes that take place is essential to understand the organization of cells at different developmental stages which aid in the identification of suitable stage for desired use, such as for regeneration into plantlet.

Materials and methods

Plant material

Cell suspension culture induced from young leaf of selected D × P hybrid plants was obtained from Felda Biotechnology Centre, Seremban, Malaysia.

Maintenance of cell suspension culture

Rapidly growing cell suspension culture (0.5 g fresh weight) was transferred to 125 ml Erlenmeyer flasks containing 30 ml of liquid MS (Murashige and Skoog 1962) media supplemented with 30 g L^{-1} sucrose and 0.1 mg L^{-1} 2,4-D. The medium was adjusted to pH 5.7 and sterilized by autoclaving at 1.1 kg cm^{-2} (121 °C) for 20 min. The cell suspensions were subcultured every 2 weeks and maintained at 25 °C on an orbital shaker (80 rpm) under standard cool fluorescent tubes (Philips Lifemax, Phillips, Indonesia) providing 60 µ mol m^{-2} s^{-1} irradiance and a 16 h photoperiod. After 50 days in liquid culture, embryogenic cells were passed through a 500 µm sieve and the flow-through were subcultured again in a cyclic way at 30 days interval to maintain the cell suspension culture, and the sieved cell aggregates were advanced for embryogenesis experiment.

Embryogenesis

Cell aggregates more than 500 µm in size were transferred to MS solid medium which consisted 30 g L^{-1} sucrose,

vitamins, and 3.5 g L^{-1} gelrite®. The cultures were maintained at 25 °C under standard cool fluorescent tubes (Phillips Lifemax, PHILLIPS, Indonesia) providing 60 μ mol m^{-2} s^{-1} irradiance and a 16 h photoperiod. Continuous subcultures of cell aggregates were carried out in MS solid medium until the formation of embryoid was observed. Different morphological stages were identified and collected for observation under the light microscope and for scanning electron microscope (SEM).

Preparation of samples and observation under SEM

Different morphological stages were prefixed with 4 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.8) at 4 °C for 48 h. Samples were then postfixed in 1 % osmium tetroxide at 4 °C for 2 h. Postfixed samples were then rinsed with 0.1 M sodium cacodylate buffer (pH 6.8) thrice for 30 min each. Thereafter, they were gradually dehydrated in a graded acetone series, and dried at the critical point in Leica EM CPD 030 Critical Point Dryer (Leica, Germany), for 30 min (using CO_2 as the transient fluid). Finally, the samples were mounted in stub using colloidal silver followed by coating with gold using Polaron E5100 sputter coater (Polaron, USA) and observed by Leo 1455 variable pressure SEM (Leo, USA).

Results and discussion

This study revealed that the embryogenesis process from the time of initial suspension culture of two-month-old aggregated cells (measuring 200–500 μm) to the appearance of mature polyembryoid is a relatively long process which requires about 21 months. The above findings is supported by the report of Soh et al. (2011), who found that even at the callogenesis stage, induction and growth rate of *E. guineensis* callus was slow compared to those of other species, and responses among genotypes differed even under similar conditions. This study also confirmed that the process was continuous, whereby, a number of mature polyembryoids were present yet others lagged behind and converted themselves into polyembryoids at a later stage resulting in heterogenous growth, and hence, repeated visits to the culture vessel were necessary to obtain the maximum number of polyembryoids per culture vessel.

Upon isolation of structures termed mature polyembryoid, they were able to form plantlet with shoot and root immediately. However, it is the motive of this paper to identify some distinct morphological changes and provide base line information on the changes that occur during the transformation from cell suspension culture into embryoid, polyembryoid, and finally into plantlet as the means of morphological marker for selection and isolation of propagules for immediate and efficient conversion into plantlet. The term polyembryoid refers to a structure which consists of many self-perpetuating embryoids formed in clusters which are unable to develop if disintegrated (Suranthran et al. 2012). The detailed characteristics of each of the stages identified have been accentuated by micromorphology study using stereomicroscopy and SEM.

The initial two-month-old cell suspension culture consisted of tiny nodular cells measuring 200–500 μm (Fig. 1a). The wide range in size shows the presence of loose cells as well as the larger aggregates of cells. Often, the loose cells remain loose and are discarded while the aggregated cells further divide and enlarge in size. The observation of this aggregated nodular structure under SEM revealed that at this initial developmental stage, some regions of the cellular surface were covered with ECM which is known as the supraembryonic network which appeared mostly as a thin membranous layer (Fig. 2a) that holds the aggregates together. The appearance of ECM is a characteristic associated with embryogenesis, described in gymnosperms (Šamaj et al. 2008) and angiosperms (Chapman et al. 2000; Bobák et al. 2003; Pan et al. 2011), including palms (Verdeil et al. 2001; Steinmacher et al. 2012). According to Steinmacher et al. (2012), the presence of the ECM is associated with a specific group of cells from which somatic embryos develop. Similar extracellular matrix surface network structure was also observed by Popielarska-Konieczna et al. (2008) in kiwifruit and they reported that ECM is a distinct heterogeneous layer, up to 4 μm thick, comprising amorphous dark-staining material, osmiophilic granules and reticulated fibers present outside the outer callus cell wall. ECM covered the surface of cells forming morphogenic domains and was reduced or degraded during organ growth, possibly liberating its contents into the culture medium (Steinmacher et al. 2012). This structure is postulated to be linked to acquisition of morphogenic competence and thus may serve as a structural marker which also supports the earlier embryogenic study reports of Chapman et al. (2000) in *Citrus* and *Asparagus*, Namasivayam et al. (2006) in *Brassica napus* spp. *oleifera*. These extracellular materials that appear at the early stages of development seemed to be important for plant regeneration (Bobák et al. 1999). Hence, identification of the presence of ECM in the present study is a potential indicator to ascertain the ability of the aggregated nodular cells at the cell suspension stage to further develop via embryoid initiation.

After periodical subculture in liquid medium for 8 months, aggregated nodular cells in the suspension enlarged (Fig. 1b), resulting in clumps ranging from 500 to 800 μm in size (Fig. 1b). These aggregated clumps were observed to have compact yellowish cauliflower like nodules (Fig. 1c i) which upon observation under SEM

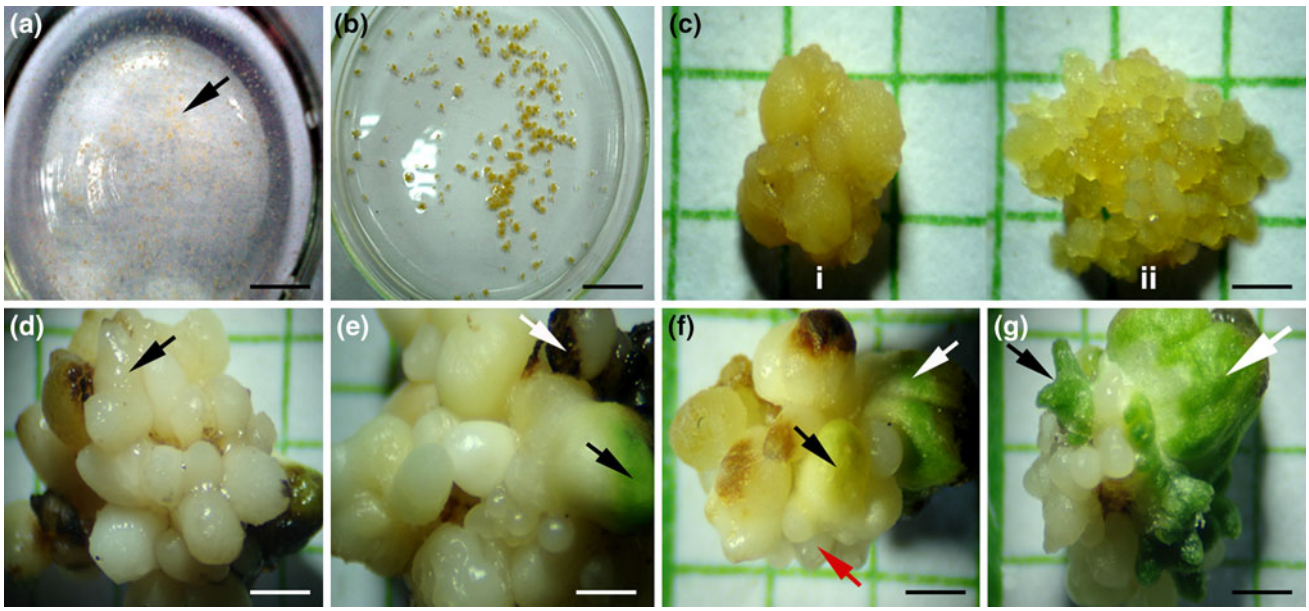

Fig. 1 Micromorphological assessment of oil palm (*Elaeis guineensis* Jacq.) polyembryoid development from cell suspension culture using stereomicroscopy. **a** Two-month-old tiny nodular cell suspension culture (*Bar* = 1.5 cm), *black arrows* shows tiny cells, **b** aggregates of cell suspension culture of 8-months growth stage (*Bar* = 1.5 cm), **c** callus derived from cell suspension at 12-months growth stage (i) compact nodular embryogenic callus and (ii) friable non-embryogenic callus (*Bar* = 0.8 mm), **d** polyembryoids at 16-months growth stage, *black arrow* shows whitish protruding tissues in cluster of embryoids (*Bar* = 0.93 mm), **e** 20-month-old polyembryoids, *black arrow* shows whitish tissue that is slowly turning into green haustorium and *white arrow* shows necrotic tissues (*Bar* = 0.95 mm), **f** 21-month-old polyembryoids, *white arrow* shows presence of distinct haustorium, *black arrow* shows globular embryoids, and *red arrow* shows secondary somatic embryo (*Bar* = 0.75 mm), and **g** 22-month-old differentiating polyembryoids (*Bar* = 1 mm), *white arrow* shows the growing haustorium and *black arrow* shows multiple shoot initiation

revealed the presence of proembryo with globular cells (>200 μm) (Fig. 2b) with a smooth surface. It was also observed that in some places rosette like complex (RLC) was present (Fig. 2b). According to Brett (2000), RLC plays an active role in elongation of cellulose microfibril on the cell surface to form cell wall. The cell wall will eventually undergo breakdown and regeneration due to the character of proembryonic cell that continuously grow and divide to form globular embryo. The presence of globular intact cells on clumps measuring 500–800 μm is an indicator for removal from the liquid culture into solid media for the maturation process.

Upon subsequent transfer to MS solid media devoid of auxins, the globular intact cells developed into compact embryogenic nodular mass (1–2 mm^2) at 12-month growth stage. At this stage mitotic division of globular intact cells resulted in the formation of globular proembryo (GPE) with multicellular suspensor (MCS) (Fig. 2c). The suspensor, based on the structure presented in Fig. 2c, may be the connective pathway for the transfer of nutrient and growth factors to the embryo proper. Often, spontaneous formation of friable non-embryogenic callus (Fig. 1c ii) at least percentage (<3 %) was observed at this stage which can easily be identified due to its loosely aggregated structure. As friable callus is considered to being responsible for the formation of translucent-like structures (appearance of abnormality), it needs to be discarded during continuous subculture process (Duval et al. 1988).

During 16-month culture period, persistent division of dominant globular proembryos resulted in the formation of cluster of embryoids, collectively termed as polyembryoids (3–5 mm^2) (Fig. 1d) which has whitish protruding tissues. In the case of other crops such as *Citrus* and *Asparagus* (Chapman et al. 2000), the embryoids can be separated, but in the case of oil palm, a few embryoids are so intimately attached to each other in such a way that if they are separated death occurs. Due to this strong union between numbers of embryoid, this structure is termed as polyembryoid and should be used as a unit for regeneration. Further maturation took place with the formation of protoderm (PT) followed by deposition of cuticular layer (CL) over the embryoid surface derived due to periclinal division (Fig. 2d). The protoderm will later form the epidermal layer on the embryoids. According to Chen et al. (2003), formation of cuticular layer on the early stage of somatic embryogenesis will act as a protective layer to the embryo.

The advancement of polyembryoid maturation during 20-month culture period progressed with the formation of green tissues from the dorsal portion of the embryoid

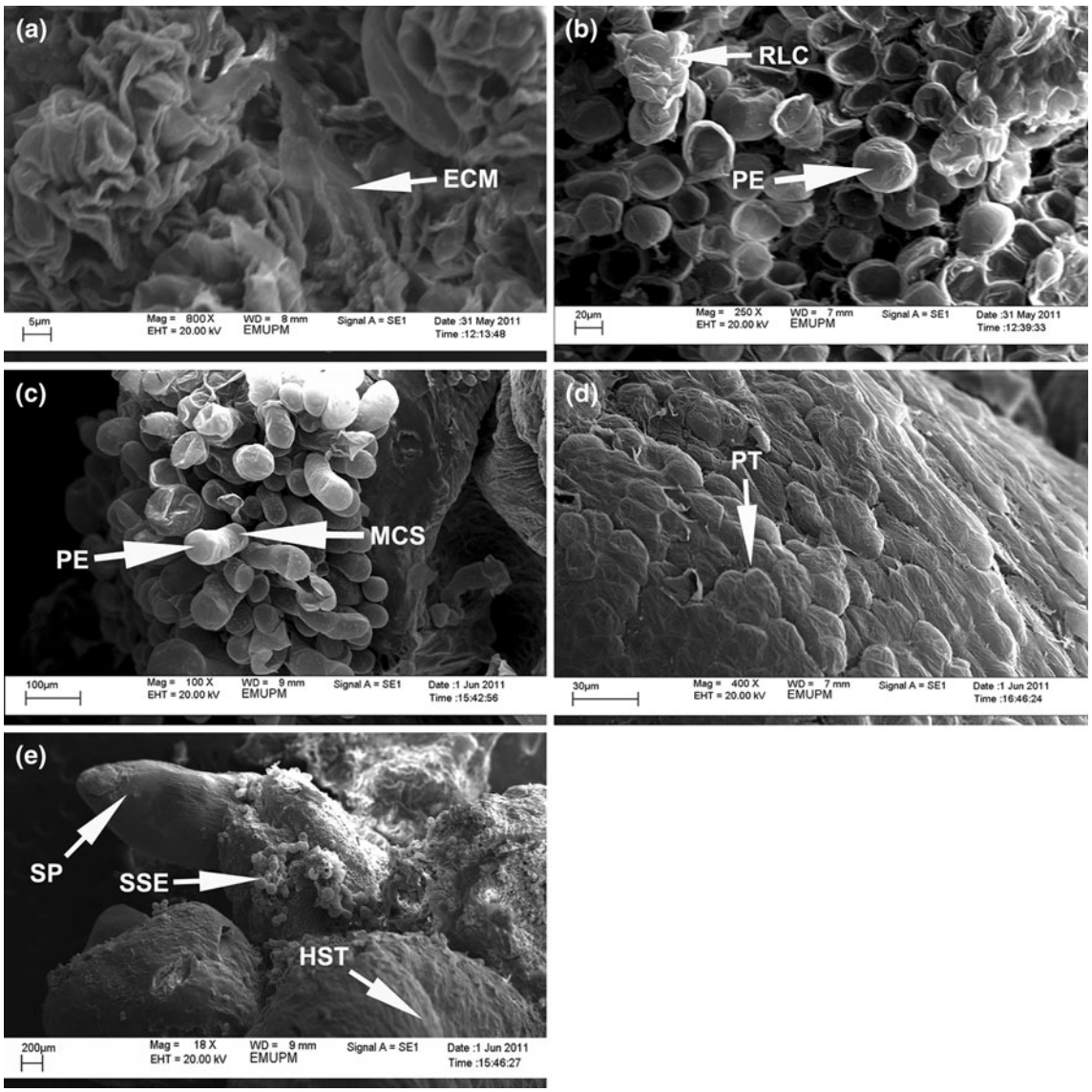

Fig. 2 Scanning electron microscopic assessment of cell suspension and its differentiation to polyembryoids in oil palm (*Elaeis guineensis* Jacq.) **a** cell surface composed of extracellular matrix (ECM), **b** emergence of proembryo (PE), supported by rosette-like structure (RLC), **c** developed globular PE supported by multicellular suspensor (MCS), **d** protoderm (PT) derived from periclinal division polyembryoid formation, and **e** differentiating polyembryoid consisting of haustorium (HST), secondary somatic embryo (SSE), and shoot primordia (SP)

clump (Fig. 1e) termed as haustorium which gradually assumed the role of supporting the embryoids through the supply of nutrients from the media. The other whitish embryoids (Fig. 1e) which were sitting on the haustorium, gradually turned yellow-green color and finally formed torpedo-like structures (Fig. 1f) measuring 3–5 mm^2 in diameter, at 21-month culture period. Additionally, the presence of secondary somatic embryos (SSE) was observed on the polyembryoids using higher magnification at this stage. Isolation of polyembryoids should be carried out at this stage as leaving the polyembryoids beyond this stage will result in rapid changes via asynchronous initiation of shoot primordia from the torpedo-shaped embryoids (Fig. 1g). Often, more than one shoot primordia can be observed depending on the speed of development and the variations among the embryoids on the haustorium. The initiation of shoot primordia (SP) from protruding embryo at this stage has been illustrated in Fig. 2e.

In this study, the morphological observation revealed that polyembryoids with haustorium housing a number of embryoids obtained at 21-month-old stage indicates that it is the right stage prior to plantlet conversion due to its highest morphogenetic potential for conversion into complete plantlet. Hence, the embryoids at the right stage can

be collected and stored for rapid conversion into plantlets as well as to obtain uniform seedlings for large scale production. To obtain the right stage for oil palm polyembryoids for further application it takes almost 2 years from suspension culture. In contrast, in cassava, somatic embryogenesis starts with the culture of leaf explants on solid MS medium supplemented with auxins and mature somatic embryos are formed within 6 weeks (Raemakers et al. 1993). It appears that response to somatic embryogenesis is not only species dependent but also genotype dependent. According to Soh et al. (2011), oil palm exhibits genotype dependent embryogenic growth-phase even under similar conditions. Hence, considering this variable genotypic response of oil palm, this particular polyembryoid stage can be obtained at any given period, presumably close to the observed period in our study.

Conclusion

The present study relates stereo microscopic observation of oil palm embryogenesis to more detailed information concerning the changes during the development using scanning electron microscopy. Aggregated cell suspension culture measuring 500–800 μm with the cauliflower-like structure is a morphological indicator for transfer into solid medium for embryo maturation as the SEM revealed the presence of ECM, RLC, and globular proembryo. Further, development in solid media was slow with the establishment of dominant globular embryos turning into opaque white tissue. An important landmark prior to mature polyembryoid formation was the initiation of haustorium which turned green supporting a number of embryoids. In this study, we have identified the polyembryoids, ideal for rapid conversion into plantlets, as the stage which comprises torpedo-shaped embryoids supported by distinct haustorium. Often, these structures also consisted of SSEs. This indeed aids in identification of synchronized and uniform polyembryoids to facilitate uniform clonal propagation.

Author contribution U.R. Sinniah, S. Subramaniam, and M.A. Aziz—conceived the idea and designed the experiments; S.R. Palanyandy, P. Suranthran, S. Gantait-Executed the experiments; S.R. Palanyandy, S. Gantait, U.R. Sinniah—wrote the manuscript; S.S.R.S. Alwee, S.H. Roowi—contributed explant materials and technology transfer for in vitro culture.

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