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BRIEF COMMUNICATION

Morphological and histological changes during the somatic embryogenesis of mangosteen

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Abstract

Induction of somatic embryogenesis in leaf explants from young mangosteen seedlings using different concentrations and combinations of 6-benzylaminopurine (BAP) and thidiazuron (TDZ) was investigated. The best medium inducing the formation of globular structures (40 %) was Murashige and Skoog medium with 0.7 mg dm^{-3} BAP and 0.7 mg dm^{-3} TDZ. For their further development, subculturing onto different maturation media was carried out, but these globular structures did not develop futher stages of somatic embryogenesis. However, they developed shoots after 90 d of culture on the original medium. Morphological and histological analyses were performed, and showed that the globular structures resembled closely the undifferentiated structure of the mangosteen seed. We propose that the development of mangosteen somatic embryos does not follow the typical course of somatic embryogenesis, but the course of development that is natural for mangosteen seed, where procambium is the only structure observed and there is no differentiated embryo.

Additional key words: Garcinia mangostana, globular embryo, seed structure, undifferentiated structure.

Somatic embryogenesis has been successfully applied in the tissue culture of hundreds of plants. The specification and requirements (*e.g.* combination of plant growth regulators, media, pretreatments and culture environments) differ according to the species, the genotype and the culture environment (Feher 2008, Jalil *et al*. 2008). The process of somatic embryogenesis is expected to follow the stages in the zygotic embryogenesis process, where the globular, heart and torpedo shape stages are successcively observed (Zimmerman 1993, Evans 2003).

The mangosteen (*Garcinia mangostana* L.), is one of the most well known tropical fruits but also among the least studied ones. Commonly, propagation is performed through seed germination, but only a limited number of seeds can be obtained from one fruit (Almeyda and Martin 1976) and the recalcitrant nature of mangosteen seed causes it to lose viability quickly. A better understanding of the fundamental mechanisms underlying somatic embryogenesis of mangosteen will be important in the development of methods for mass propagation of this species.

In mangosteen, organogenesis has been successfully achieved (Goh *et al*. 1988, Normah *et al*. 1992, Normah *et al*. 1995, Te-Chato *et al*. 1995, Te-Chato and Lim 2000), but there has been no successful report on the achievement of somatic embryogenesis.

Mangosteen seeds are formed through agamospermy (Richard 1990). Mangosteen has a unique seed structure, with no differentiated embryo formation. Sprecher (1919) described the seed as having a not well-differentiated plumule at one end and radicle at the other, which are

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Abbreviations: ABA - abscisic acid; BAP - 6-benzylaminopurine; IBA - indole-3-butyric acid; IAA - indole-3-acetic acid; FAA - formalin + acetic acid + alcohol; MS - Murashige and Skoog; PEG - polyethylene glycol; SEM - scanning electron microscope; TDZ - thidiazuron; WPM - Woody plant medium.

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connected by two thin procambial strands. During germination, the shoot formed from one end of the procambium while the radicle emerged from the other end. A similar structure has been described for seeds of *Symphonia globulifera* (Corbineau and Come 1986) and *Rheedia acuminata* (Do Nascimento *et al*. 2002) which belong to the same *Clusiaceae* family as the mangosteen.

We investigated the *in vitro* requirements for somatic embryogenesis of mangosteen and we performed morphological and histological analysis of the structures obtained and compared them to those of the mangosteen seed in the search of a solution to the puzzle of the unusual somatic embryogenesis of mangosteen.

Reddish young mangosteen leaves (3 cm in length) from four-month-old seedlings were used as explants. Leaf sterilization was performed by cleaning the surface of the leaves with distilled water, continuing with sterilization (80 % ethylalcohol for 5 min and then 20 % *Clorox* with a few drops of *Tween 20* in sterile distilled water for 20 min) under the laminar air flow. The explants were rinsed with sterile distilled water and cultured abaxial side down on Murashige and Skoog (1962; MS) medium with a 6-benzylaminopurine (BAP; 0, 0.1, 0.3, 0.5, 0.7 mg dm^{-3}), thidiazuron (TDZ; 0, 0.1, 0.3, 0.5, 0.7 mg dm^{-3}), 6 % sucrose and 2.5 g dm^{-3} *Gelrite* (*Duchefa*, The Netherlands). They were incubated in the dark until globular structures formed (about 21 d), after which cultures were transferred to a growth chamber with 12-h photoperiod (irradiance of 22.26 μmol m^{-2} s^{-1}) and temperature of 23 ± 2 °C. To stimulate further growth in the next stages of somatic embryogenesis (heart shape, torpedo and cotyledonary), such globular structures were subcultured onto various maturation media based on previous reports on maturation of somatic embryos (*e.g*. Litz 1988, Su *et al.* 1997, Te-Chato and Lim 1999): *1*) MS medium without plant growth regulators, *2*) Woody plant medium (WPM; Llyod and Mc Cown 1980) with 1 mg dm^{-3} indole-3-butyric acid (IBA) and acid abscisic (ABA; 0, 1, 2, 3 mg dm^{-3}), *3*) WPM with 3.64 mg dm^{-3} BAP and 1 mg dm^{-3} kinetin, *4*) MS with 0.5 mg dm^{-3} indole-3-acetic acid (IAA), 2 mg dm^{-3} BAP and 50 g dm^{-3} sucrose, *5*) MS with 100 mg dm^{-3} polyethylene glycol (PEG) 6000, *6*) MS with 10 mg dm^{-3} ABA, *7*) MS with 90 g dm^{-3} sucrose, and *8*) MS with 30 g dm^{-3} sucrose and 5 g dm^{-3} *Gelrite.*

Globular structures were fixed in a 100 cm^3 solution of formalin, acetic acid and alcohol (FAA), which contains 10 cm^3 of 37 % formaldehyde, 5 cm^3 glacial acetic acid, 50 cm^3 of 95 % ethanol and 35 cm^3 distilled water. Samples were dehydrated in serial grades of alcohol and tertiary butylalcohol and subsequent xylene steps; samples were solidified in paraffin wax. Paraffin blocks were sectioned 6 μm thick using a microtome and stained with Safranin O and Alcian green (*Sigma*, St. Louis, USA). The stained samples on microscope slides were then observed under light microscope (*Carl Zeiss*, Jena, Germany). Scanning electron microscopy (*Philips XL 30*, The Netherlands) was also performed to further analyze the morphological features of the globular structure. A histological study of the mature mangosteen seed before and during germination was also performed for comparison.

Table 1. Percentage of globular structure formation after 25 d culture on MS medium with different combinations and concentrations of TDZ and BAP. No globular structures were formed on medium without TDZ. Means followed by different letters within columns differ significantly by Duncan multiple range test ($P \leq 0.05$).

TDZ [mg dm^{-3}]	BAP [mg dm^{-3}]	Globular structures [%]
0.1	0	26.67 ^{bc}
	0.1	23.33 ^{bc}
	0.3	30.00 ^{ab}
	0.5	20.00 ^{bcd}
	0.7	20.00 ^{bcd}
0.3	0	13.33 ^{de}
	0.1	16.67 ^{cde}
	0.3	10.00 ^{ef}
	0.5	0 ^{h}
	0.7	26.67 ^{bc}
0.5	0	10.00 ^{ef}
	0.1	6.67 ^{fg}
	0.3	16.67 ^{cde}
	0.5	30.00 ^{ab}
	0.7	3.33 ^{gh}
0.7	0	13.33 ^{de}
	0.1	23.33 ^{bc}
	0.3	26.67 ^{bc}
	0.5	30.00 ^{ab}
	0.7	40.00 ^{a}

The leaf explants started to swell soon after culturing in the induction media, and globular structures were first induced after 25 d of culture. The highest percentage (40 %) explants producing globular structures were obtained from those cultured on MS with 0.7 mg dm^{-3} BAP and 0.7 mg dm^{-3} TDZ (Table 1). Culturing in the dark, the globular structures formed were creamy and translucent (Fig. 1*A*), and were easily separated from one another and individually developed (Fig. 1*A*). Each globular structure seemed to adhere to neighbouring ones only because of the high frequency of globular structure formation from explants. Prolonged culture in a dark environment was found to increase the development of secondary globular structures (Fig. 1*A*). These growth regulators have been successfully used to induce organogenesis and also to promote the development of embryogenic masses in several species, including longan (Litz 1988), geranium (Visser *et al*. 1992) and rice (Gairi and Rahid 2003). Earlier reports suggest that in dicots, TDZ can be used for high-frequency somatic embryogenesis, for example, in the *Arachis hypogaea* (Murthy *et al*. 1995) and coffee (Giridhar *et al*. 2004).

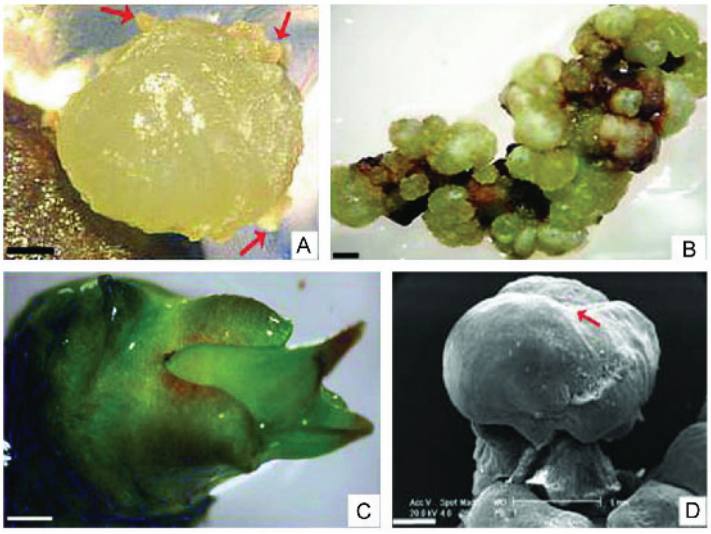

Fig. 1. Globular structure development from leaf explants. *A* - Morphology of the globular structure after 50 d of culture. Formation of secondary globular structure (*arrow*) when cultures were kept in dark. *B* - Growth of the globular structures after transfer to light. *C* - Shoot arising after 90 d of culture. *D* - The globular structure seems to have a shoot apex after 65 d (*arrow*) as observed under scanning electron microscope. *Bar* = 500 μm.

The globular structures turned green during culture under light (Fig. 1*B*). They were transfered to maturation medium to stimulate subsequent stages of somatic embryogenesis. The globular structures were subcultured on several maturation media, combining various plant growth regulators (IBA, BAP, kinetin and ABA), increasing water stress environment (addition of sucrose, *Gelrite* and PEG), and adding ABA, involved in dormancy process. However, no changes occured in the globular structures after 60 d of culture for all the media tested, even on basal MS medium widely used to achieve somatic embryogenesis. Nevertheless, globular structures subcultured onto the same induction medium (MS with 0.7 mg dm^{-3} BAP and 0.7 mg dm^{-3} TDZ) developed into shoots after 90 d of culture (Fig. 1*C*).

Scanning electron microscopy (SEM) analysis was used to verify the morphological observations. The individuality of globular structures was ascertained. Under SEM observation, the shoot apex appeared to start to develop on the top of the globular structure (Fig. 1*D*).

For histological analysis, the meristematic region and protoderm were observed in the early formation of the globular structure. After three months of culture the shoot apical meristem was observable. Subsequently, the globular structure's independent vascular tissue (not connected to the mother explants) was identified (Fig. 2*A1*). Lastly, the shoot arose (Fig. 2*B1*). The presence of a well-defined protoderm and vascular tissue provided evidence for the individuality of the globular structure.

The histology of the globular structures (Fig. 2*A1*,*B1*,*C1*) was then compared with mangosteen seed structure (Fig. 2*A2*,*B2*,*C2*). In the seed structure (Fig. 2*C2*), only the procambium strand with no polarity of shoot or root apex formation was observed. This lack of polarity and presence of only a procambium strand in the mature mangosteen seed was found to be similar to that of the globular structure, which also exhibits no polarity and has only a procambium strand (Fig. 2*C1*).

In both globular and seed structures (early stage of germination), the meristematic region of actively dividing cells and the vascular system were observed; a plumule could be seen to emerge from the seed and then to start to differentiate as one end of the procambium elongated (Fig. 2*A1,A2*). Further during seed germination, a radicle developed at the opposite end from the plumule. However, it is not predictable which part of the procambium will develop into a plumule or a radicle. In the globular structure as well, only one end of the procambium strand developed into a plumule (Fig. 2*B1*), but no radicle was found to develop from the opposite end (from the plumule). A unique feature of the mangosteen seed is the emergence of an adventitious root. A radicle formed at the opposite end of the seed from the plumule as the seed germinated (Fig. 2*B2*). However, the radicle was not functioning and did not further develop into the main root. Instead, an adventitious root emerged from the same end of the seed as the plumule and developed into the main root (Fig. 2*D*).

In our mangosteen globular structures, several embryogenic cell structure characteristics were also observed through histological analysis. The presence of the meristematic region in the early development of globular structures indicated that cells are actively dividing; the reprogramming of cell division in somatic plant cells is required for dedifferentiation and the establishment of embryogenic competence (Nagata *et al.* 1994, Dudits *et al.* 1995). A well-defined protoderm was

also observed in the early development of the globular structure confirming the individuality of the structure. Several studies (West and Harada 1993, Von Arnold *et al.* 2002, Blazquez *et al.* 2009) have reported that the protoderm was the first tissue that could be identified in the analysis of somatic embryogenesis. Similarly, Thorpe and Stasolla (2001) have proposed that the presence of the protoderm is one of the unique features of somatic embryo development. Kiran Ghanti *et al*. (2010) showed similar direct globular structure formation in chickpea.

We found that the globular structures of mangosteen further developed to form a shoot apex after 65 d of culture. The use of 0.7 mg dm^{-3} TDZ may have contributed to this, as the concentration is rather high and more suitable for shoot formation. Individual vascular tissue was also observed during shoot development, whose histological identification confirmed the isolation of globular structures from each other.

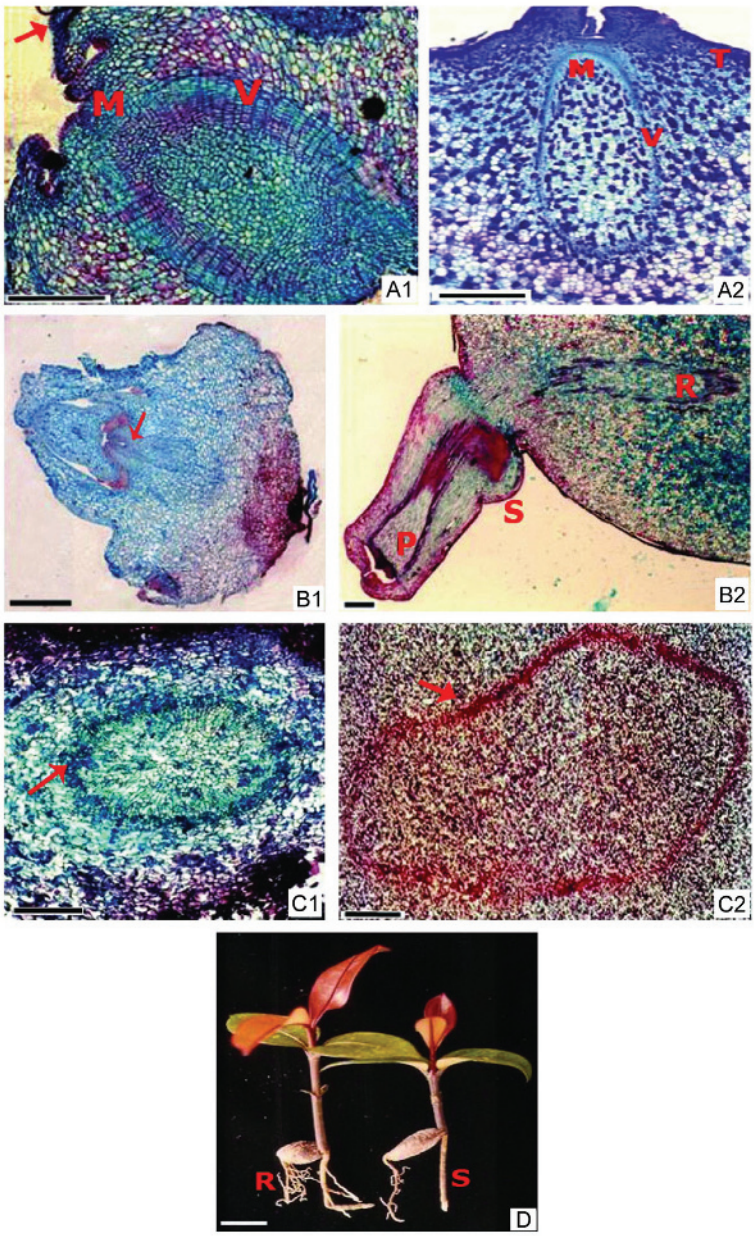

Fig. 2. A comparison of the globular (*A1, B1, C1*) and seed (*A2, B2, C2*) structures. *A1, A2* - Elongated vascular tissue (V), meristematic region (M) and protoderm (*arrow*) in both globular structure and seed (T = seed testa). *B1, B2* - Plumule (*arrow*) without radicle in globular structure, but plumule (P) and radicle (R) in the seed with adventitious root (S). *C1, C2* - No polarity of the procambium strand (*arrow*) is apparent in both globular structure and seed. *Bar* = 500 µm. *D* - Mangosteen seed after germination. Radicle (R) does not develop as the main root. The emergence of the adventitious root (S) from the same end of plumule. *Bar* = 10 mm.

In this study, we also found an absence of bipolarity in the mangosteen globular structures. Several possible explanations exist to account for the observed atypical trajectory of the somatic embryogenesis process in mangosteen as referred to the somatic embryogenesis process observed commonly, for examples, cotton (Hussain *et al*. 2009), *Cedrela fissilis* (Vila *et al*. 2009) and *Azadirachta indica* (Shekhawat *et al*. 2009). Bipolarity is also absent in the mangosteen seed. Unlike other seeds, the procambium strand is the only structure observed in the seed; the plumule and the radicle develop from opposite ends of the procambium strand. Furthermore, it is not clear which part of the procambium will develop into a shoot or a root before the seed germinates. According to Lim (1984) the mature embryo of mangosteen acts as the cotyledons, while Vestal (1937) and Ha *et al*. (1988) reported that the seed of mangosteen is a hypocotyl and does not have cotyledons, based on its structure and germination characteristics. Sprecher (1919) refererred to the whole seed structure as 'hypocotyl-tubercle', later confirmed by Horn (1940). The same characteristics were reported in other *Garcinia* species such as *G. malaccensis* and *G. parvifolia* (Ha *et al*. 1988). This characteristic of the seed may account for the observations described here for *in vitro* development of the embryo/seed/hypocotyl-tubercle, thus not following the general stages of somatic embryogenesis observed in other species. Our observations may be in line with Zimmerman (1993), who reported that the somatic embryogenesis process resembles the stages in typical zygotic embryogenesis.

In this study, the globular structures formed from the mangosteen leaf explant and the structure of the mangosteen seed were analyzed and compared to study the achievement of the somatic embryogenesis of mangosteen; similar characteristics were observed in both structures. We conclude that the globular structures obtained from mangosteen leaf explants are indeed a similar structure to the seed, albeit with unusual characteristics from what have been considered typical of somatic embryogenesis. We propose that, while not exactly following all the stages of the somatic embryogenesis observed in other species, the somatic embryogenesis of mangosteen actually follows the natural development of its seed.

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