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RESEARCH NOTE

Morphological and histological analysis of anther-derived embryos of coconut (*Cocos nucifera* L.)

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Abstract

Doubled-haploids are a great source of material for heterozygous coconuts to shorten the time taken for crop improvement through hybridization programs. However, low frequency of embryo conversion and formation of weak plantlets are the major limitations in the protocol. In the present study anther-derived embryos were analysed at cellular level through the histological observations to understand the occurrence of shoot differentiation in those embryos. Attempts were also made to optimise the 6-Benzylaminopurine (BAP) concentration in the regeneration medium. Among the tested BAP levels (5, 15, 25, 35, 45, 55 µM), the regeneration medium containing 25 and 35 µM gave rise to 51.5% sprouted embryos against the control that gave only 22.7%. Among the analysed anther-derived embryos sequential events of differentiation including the formation of provascular strands followed by vascular bundle, differentiation in to the growing point, giving rise to the secondary embryos and polarization in to the shoot and root pole were identified. The blunt embryos devoid any morphological sign of sprouting showed different cellular arrangement viz. haustorial structure without any meristematic point (47%), bipolar with shoot and root meristems (8%) and unipolar with either of the pole (45%). Thick haustorial cover lead to the physical dormancy inhibiting further development of the polar structures. The germinating embryos containing a single shoot gave rise to the healthy plants where as double, multiple or fused shoots formed the week plantlets. The findings can be used for further optimisation of the protocol to achieve a greater plant regeneration frequency.

Key message

1. Culture media supplemented with increased levels of 6-Benzylaminopurine (BAP) enhanced the regeneration frequency of anther-derived embryos.
2. Thick haustorial tissue created an obstacle effect on further development of the differentiated shoot apical meristem.
3. Formation of clustered plants through secondary embryogenesis or shoot multiplication at the early stage of plant regeneration caused to give rise to the week plants.

Keywords Anther culture · Anther-derived embryo · Coconut · Cytokinin · Histology

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	Benzylamino purine
GA_3	Gibberellic acid
MS	Murashige and Skoog

The coconut plam (*Cocos nucifera*) is a versatile tree grown throughout the tropics for culinary and commercial purposes. All forms of coconuts known to date are having diploid chromosome complement (2n = 2x = 32; Arunachalam 2012). The Tall coconut varieties are cross-pollinated while Dwarfs are self-pollinated having monoecious unisexual flowers, few pistillate and numerous staminate flowers.

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Sri Lanka Tall coconut is highly heterozygous with a great diversity among the individual palms (Batugal and Bourdeix 2005). Homozygous lines will assist the breeding in improving the crop overcoming the limitations associated with its long life span. A quick approach of producing 100% homozygous lines is the simple anther culture technique where inoculation of extracted anthers in to the androgenesis induction culture medium is involved. Homozygous plants are produced from the calli or embryos derived from the trigged haploid microspores enclosed in the anthers into the sporophytic pathway followed by chromosome doubling (Ascough et al. 2006; Murovec et al. 2007; Yuan et al. 2015). In a number of crop species a significant progress has been made via anther culture producing doubled haploids, after its discovery by Guha and Maheshwari (1964).

Initial attempts reported a limited success on induction of embryogenesis in the cultured anthers of coconut (Thanh-Tuyen and De Guzman 1983; Monfort 1985). A successful regeneration protocol from cultured anthers was reported by Perera et al. (2008, 2009, 2011) defining the factors including pollen developmental stage (Perera 2003), conditions for triggering the sporophytic pathway, culture medium composition etc. Despite the higher production of embryos/calli, the plant regeneration was not efficient (Perera et al. 2009). Cytokinin level is one of the major factors affecting the regeneration efficiency (Hill and Schaller 2013). Thus the present study was conducted to optimize the concentration of 6-benzylamino purine (BAP) in the regeneration medium in order to obtain enhanced plant regeneration efficiency with a vigorous growth.

Collection of the rachille containing staminate flowers at the correct maturity stage, preparation of the material, induction of microspore embryogenesis, embryo maturation and culture maintenance were done as described by Perera et al. (2008). The culture media reported in Perera et al. (2009) was used as the basal medium. A triplicate experiment with different BAP concentrations (5, 15, 25, 35, 45, 55 µM) in combination with 0.35 µM gibberelic acid (GA_3) in the Murashige and Skoog (MS, Murashige and Skoog 1962) medium was conducted using the anther-derived mature embryos equally distributed among the treatments. After subculturing two times into the above media at monthly intervals the subsequent subculturings were done continuously in to the medium containing 5 µM BAP for further growth. Maximum Likelihood Analysis of Variance was conducted using the Proc CatMod procedures of PC-SAS. Treatment means were compared using orthogonal contrast coefficients at 95% confidence intervals.

Results indicated that the BAP concentration in the regeneration medium is significantly effect for the efficiency of embryo conversion ($X^2 = 19.92$; $p < 0.001$; Fig. 1a). Greatest conversion rate was obtained in the media supplemented with 25 (50%) and 35 µm (53%) BAP whereas only 23% embryos were sprouted in the control medium supplemented with 5 µm BAP. However, further increase of BAP concentration gave a reduction in the conversion rate. A variation in terms of the plant morphology and the vigour was observed. A vigorous growth was observed in the plantlets with single (Fig. 1b) or double shoots whereas the multiple shoots gave rise to the weak plantlets with a slow growth (Fig. 1c). However, the level of BAP did not effect on determining the differentiation of the meristem into the single or multiple shoots. The root course for formation of multiple shoots is yet to be studied.

The embryos produced were analysed histologically to understand development pattern and to suggest evidence for above limitations. The embryos with different morphologies were categorized as immature embryos and secondary

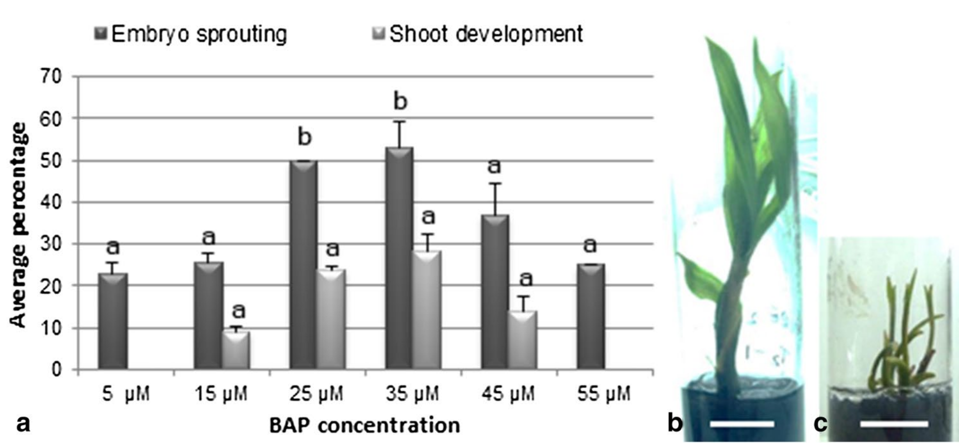

Fig. 1 **a** Effect of the BAP concentration in germination medium on plant regeneration of anther-derived embryos of *Cocos nucifera* L. Average percentages with different letters of each parameter are significantly different at $p < 0.05$ at 95% confidant level. **b**, **c** Plants regenerated from anther-derived embryos of *Cocos nucifera* L. A single plant growing with a vigorous growth (**b**) and multiple shoots with extremely slow growth (**c**). Note that both plants are at the same age (Bar = 2 cm)

embryos from the induction medium, mature embryos containing a germination point, converted solitary and fused embryos with shoot meristem and non-converted/blunt embryos maintained long term in the regeneration medium after exposing to BAP treatments. Fifteen embryos were analysed from each group. Sample preparation, dehydration, wax embedding and staining was done according to Perera et al. (2011).

Immature embryo Two distinct cell types, empty parenchyma cells in the periphery of the embryo and the small meristematic cells in the middle of the embryo were identified. The provascular strands have been formed in the central part of the embryo (Fig. 2a–c) as the first event of embryo differentiation.

Secondary embryo The origination of the secondary embryos from the primary embryos was identified. Initially the parenchyma cells with very low meristematic activity presented in secondary embryos and then formation of provascular strands as in the primary immature embryos could also be observed (Fig. 2d–f). Secondary embryo formation is an added advantage for an efficient plant regeneration protocol.

Non-converted/blunt embryos Nearly half (47%) of the embryos analysed in this study did not contain any sign of presenting either a root or shoot meristem. Even though these embryos were morphologically absent with any sign of regeneration the histological analysis revealed that cellular differentiation has occurred to form the meristematic growing points within the haustorial tissue (Fig. 2g–i). Presence of a thick houstorial tissue imposes a physical barrier for their further development. Among the analysed embryos 8% consisted of the bipolar arrangement indicating the complete embryos which have the capacity to give rise to complete plantlets. By making a physical damage to the haustorial tissue of these embryos further development of the shoot and root can be induced. Among the blunt embryos 13% contained only the shoot meristem, where the rooting can be induced even at the later stages of plant regeneration. Altogether 21% of the blunt embryos were potentially regenerative, thus, it is worth to take necessary actions to break the physical dormancy of these embryos. However, most of the embryos (32%) contained only the root pole in the embryos indicating the termination of their regeneration potential. The reason could be attributed with the hormonal signalling to induce morphogenesis. The critical stage of the immature embryo to be exposed to the correct type of cytokinin, it's level and the duration will affect for regulating the morphogenesis and plant regeneration (Miroshnichenko et al. 2017). Therefore, incomplete embryo formation can be avoided by selective subculturing.

Embryos with germination points The embryos containing a depressed point on the haustorial surface showed the differential cellular arrangement with regards to the meristematic activity (Fig. 2j–l). Meristamatically active cells characterized by a high nucleus to cytoplasmic ratio were surrounded by the large inactive parenchyma cells forming the depression on the surface of the embryo. This structural change is an advantage for easy protrusion of the growing shoot out of the haustorial tissue. Therefore, formation of a germination point is a good indicator of the future conversion of the embryo. Early exposure of these embryos into the media containing cytokinin would be beneficial for regenerating the healthy plants.

Converted embryos Four types converted embryos with shoot meristem covered by leafy scales were identified (Fig. 2m–r). Among the converted embryos tested 53% contained a single shoot that is fully enclosed by the leaf sheaths. These embryos gave rise to the vigorously growing healthy plants. About 33% embryos consisted of double shoots enclosed by the common leaf sheaths. At a low frequency, multiple shoots that contained more than two shoot points and the fused shoots that connected at the centre were also identified each at a frequency of 7%. This cluster of shoots gave rise to the weaker plantlet. Even though, the embryos initially contained a single shoot meristem, it can be multiplied by the continuous exposure of those embryos into the cytokinins rich media. By retaining the number of shoot meristems as one per embryo, higher frequency of vigorously growing healthy plants can be obtained. Therefore, the regeneration protocol has to be refined in order to retain more embryos at a single shoot level.

Present study revealed that the plant regeneration efficiency could be increased by 29% adjusting the BAP concentration of the regeneration medium. Among the analysed anther-derived embryos a sequential event of cellular differentiation including the formation of provascular strands followed by vascular bundle, differentiation in to the growing point, giving rise to the secondary embryos and polarization in to the shoot and root pole were identified. The blunt embryos devoid any morphological sign of sprouting showed a different cellular arrangement viz. bipolar with shoot and root meristems, unipolar with either of the pole or haustorial structure etc. The generated information is useful in subjecting the embryos to a correct maturing and vigorous plant regeneration process. Signalling at correct time is critical for inducing the shoot formation during the embryo developmental pathway. By exposing the embryos at correct maturity stage for most appropriate cytokinin level would therefore enhance the regeneration frequency.

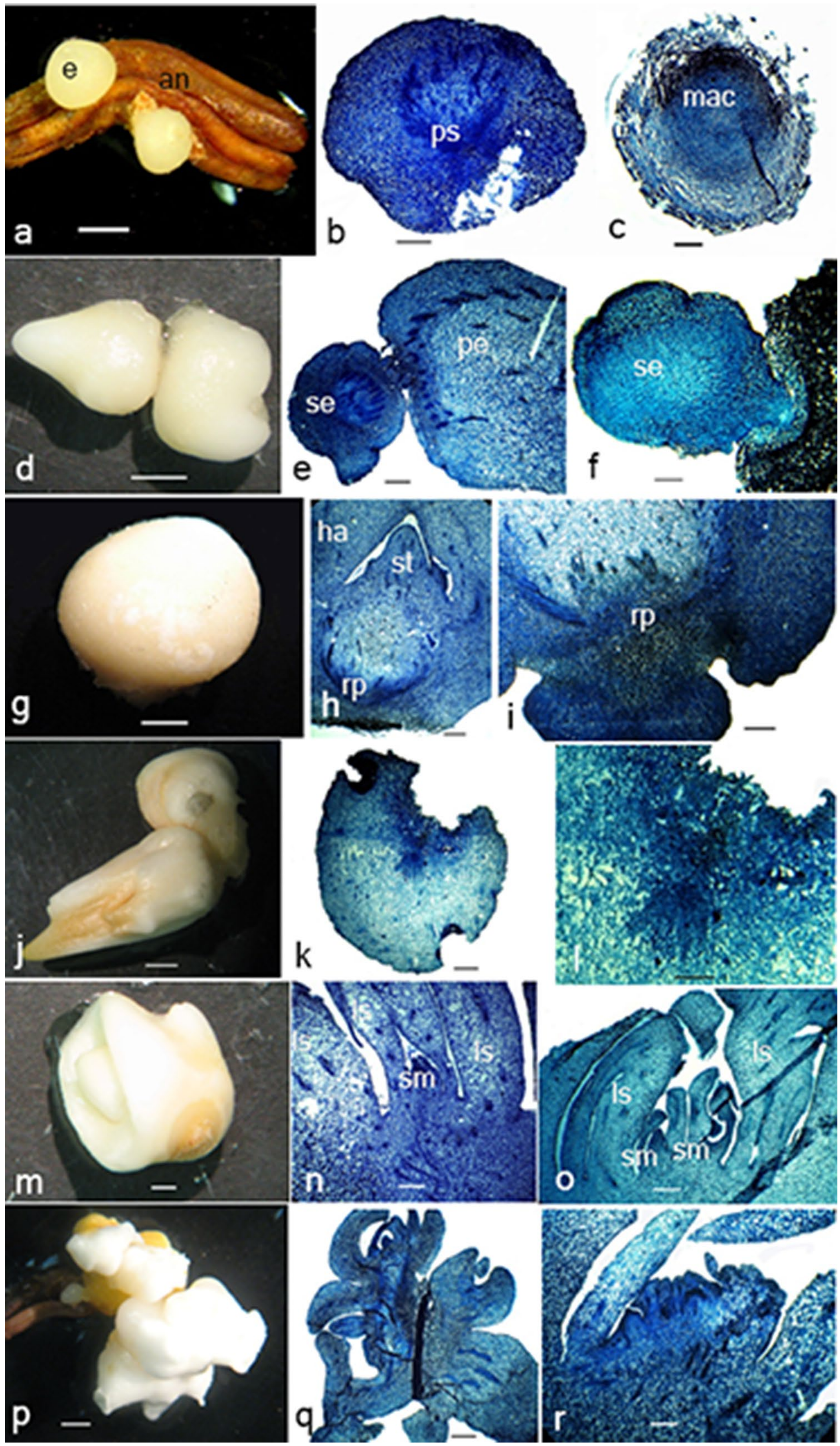

Fig. 2 Morphological (**a**, **d**, **g**, **j**, **m**, **p**) and histological (other figures) aspects of the anther-derived embryos of *Cocos nucifera* L. **a**–**c** Immature embryos (*e*) derived from an anther (*an*) (**a**), the meristematically active cells (*mac*) in the middle part of the embryo indicating the initiation and differentiation of the embryo (**b**), formation of provascular strands (*ps*) and the M zone in the developing embryo (**c**) in induction medium (Bar **a** = 1.43 mm; **b** = 429 µm; **c** = 250 µm). **d–f** Secondary embryogenesis from a primary embryo (*pe*), provascular cell (*pv*) differentiation in maturation medium (Bar **d** = 3 mm; **e** = 300 µm; **f** = 214 µm). **g–i** Non-converted blunt embryos without any sign of shoot development, a complete bipolar embryo with shoot (*st*) and root pole (*rp*) enclosed in the haustorium (*ha*). An incomplete embryo enclosing the developing shoot (Bar **g** = 1.6 mm; **h** = 714 µm; **i** = 727 µm). **j–l** Mature embryos with germination point. Note the depression formed in the haustorium, Longitudinal section across the germination point, Close view of the germination point. Note the differentiating shoot meristem in the haustorium (Bar **j** = 3 mm; **k** = 333 µm; **l** = 250 µm). **m–o** Converted embryos Normal shoot development with a single shoot meristem(*sm*) and double shoots enclosed by the spear leaf sheaths (Ls) (Bar **m** = 1 mm; **n** = 833 µm; **o** = 555 µm). **p–r** Miltiple shoot development, note the differentiation of three visible shoot meristems with in the spear leaf sheaths. Fused shoot development. Three individual shoots are fused by the central haustorial tissue (Bar **p** = 2 mm; **q** = 1.05 mm; **r** = 625 µm)

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