In vitro regeneration of coconut plantlets from immature inflorescence

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Clonal propagation of elite, disease-free coconut palms is a promising technique for producing uniform planting material with high yield and disease resistance. Over the past few decades cloning of coconut has been attempted in a number of laboratories worldwide; however, success has been limited. In the present study, immature inflorescences of 2–12 cm size were collected from West Coast Tall variety and the rachilla segments were cultured on four different media combinations in dark condition. White translucent outgrowths were maximum in Y3 medium supplemented with 4.54 µM 2,4-dichlorophenoxyacetic acid (92%) followed by medium 72 with 41.4 µM picloram, 61.8 µM putrescine and 4.54 µM thidiazuron (TDZ) (87%). After eight weeks in dark, shoot-like outgrowth was noticed more in Y3 III (65%) followed by Y3 I. After eight months dark incubation, the cultures were transferred to 1/2 Murashige and Skoog (MS) with two hormone combinations and high frequency of multiple shoot formation was noticed in 1/2 MS with 5.37 µM naphthalene acetic acid (NAA) and 4.44 µM 6-benzylaminopurine (BAP). Maximum shoot development was observed Y3 medium fortified with 5 µM 2-isopentenyl adenine (2ip) and 5 µM BAP. The individual shoots after development of 3–4 leaves were transferred to 1/2 Y3 medium supplemented with 5.37 µM NAA and 24.6 µM indole-3-butyric acid (IBA), and root initiation was observed in 39.28% plantlets. Start codon targeted (SCoT) profiling based on banding pattern of PCR-amplified products confirmed the clonal fidelity of *in vitro* regenerated coconut plantlets. The study indicates the possibility of developing an *in vitro* regeneration protocol for coconut using immature inflorescence explants.

Keywords: Clonal fidelity, coconut, *in vitro* regeneration, rachillae.

THE coconut palm is one of the most important cultivated palms in the world, and is popular for its industrial and commercial applications in the tropics and subtropics. The crop faces many challenges and among these, the incidence of debilitating phytoplasmal diseases is one of the major problems faced by coconut farmers all over the world. This has resulted in significant yield reduction in many coconut-growing countries. The use of high-yielding and disease-resistant varieties is a viable option to manage the situation. Unfortunately, the disease resistant genotypes are scarce and conventional seed propagation technique alone is not sufficient to meet the rapid growing demand of planting materials. Through seed propagation a maximum of only 40–50 quality coconut seedlings can be produced from a single mother plant and its uniformity is not assured on account of the cross-pollinating nature of coconut. Tissue culture seems to be the only possible clonal propagation method to achieve this objective in a crop like coconut. Therefore, mass multiplication of coconut is the need of the hour for large-scale production of elite and disease-resistant coconut palms for replanting the senile and diseased-gardens.

Over the past few decades, cloning coconut has been attempted in a number of research laboratories worldwide[1]. However, the landmark research achievement with regard to coconut tissue culture has not been attained. Some of the reasons for the slow progress in tissue culture include lack of reproducibility, slow growth of these explanted tissues *in vitro*, and their further lack of vigour when planted *ex vitro*[2]. Protocols for micropropagation of coconut have been developed in various laboratories using different explant sources, viz. tender leaf[3–5], immature inflorescence[6–9], embryo and plumular tissue[10–15]. However, the available protocols lack reliability and repeatability. Any progress in achieving the clonal propagation of this highly recalcitrant palm will be of great advantage for crop improvement of the crop and also for the prosperity of coconut farmers worldwide. Among the different explants, rachilla explants from immature inflorescence[16] and plumule explants from zygotic embryo[17] were the most extensively studied. In the case of plumule explants, it is possible to propagate the progeny of selected parents at zygotic embryo stage without assessment of its performance. However, if the aim is to propagate adult palms of known traits such as productivity or disease resistance, other explants from these palms must be used[18]. In this context, rachilla explants from immature inflorescence seem to the ideal candidates. The advantage of rachillae explants from immature inflorescence is that it enables multiplication of adult-bearing palms of known performance.

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Therefore, the study was conducted to standardize the size of explants, inoculation media, culture conditions and subsequent media combinations for *in vitro* regeneration from immature inflorescence of coconut, and also to assess the clonal fidelity of *in vitro* raised plantlets.

Materials and methods

The study was conducted at ICAR-Central Plantation Crops Research Institute, Regional Station, Kayamkulam, Kerala, during April 2013–March 2018.

Standardization of inoculation media

Immature inflorescences with outer spathe length of 2–12 cm were collected by destructive sampling from 25 to 30-year-old palms belonging to West Coast Tall variety. The inflorescences were surface-sterilized in 0.1% $HgCl_2$ solution for 10 min followed by washing in 5–6 changes of sterile water. The outer spathes were removed under laminar air-flow chamber. In order to define suitable developmental stage, the inflorescences were categorized as those with outer spathe length up to 4, 4–7, 7.5–10 cm and more than 10 cm. After removal of spathe, the rachillae were sliced into 1–1.5 mm long bits and inoculated in test tubes containing 20 ml culture media. Two basal media, i.e. Y3 (ref. 19) and medium 72 formulated by Karunaratne and Periyapperuma[10] were used for inoculation. These two basal media were supplemented with two hormone combinations, one with 41.4 μM picloram, 61.9 μM putrescine and 4.54 μM thidiazuron (TDZ), and another with 4.54 μM 2,4-dichlorophenoxyacetic acid (2,4-D). The basal media also contained 30 g l^{-1} sucrose, 1 g l^{-1} charcoal and agar 6 g l^{-1}. The pH of the media was adjusted to 5.8 using 0.1 N NaOH/1N HCl before autoclaving for 20 min at 121°C. The cultures were incubated in dark at $27° \pm 2°C$ for a period of 16 weeks. Each treatment had 10 cultures and 5 replications. The cultures were monitored at weekly intervals. During the dark incubation, sub-culturing was done to the same medium as and when it showed symptoms of nutrient depletion.

Regeneration

After four months, sub-culturing was done to Y3 medium supplemented with three hormone combinations, i.e. Y3 I with 4.54 μM 2,4-D, Y3 II with half the concentration of 2,4-D (2.27 μM), and Y3 III with 4.54 μM 2,4-D and 4.14 μM picloram, and again incubated in dark.

Multiple shoot induction

After eight months of dark incubation, sub-culturing was done to 1/2 Murashige and Skoog (MS) medium[20] supplemented with two sets of hormone composition. One set (1/2 MS I) was supplemented with 30.7 μM picloram, 30.9 μM putrescine and 4.54 μM TDZ, and the other set (1/2 MS II) supplemented with 5.37 μM naphthalene acetic acid (NAA) and 4.44 μM 6-benzylaminopurine (BAP). The cultures were initially kept in diffused light for one month followed by incubation in light condition. After 4–6 months in light, the cultures were observed for multiple shoot formation and abnormal growth.

In vitro shoot growth and multiplication

The multiple shoots were separated from the parental clump and transferred to shoot regeneration medium containing Y3 with two hormone combinations. One shoot regeneration medium used was Y3 + 5 μM 2-isopentenyl adenine (2ip) + 5 μM BAP, and the second was Y3 medium with 5.37 μM NAA and 4.44 μM BAP.

Root regeneration

For *in vitro* rooting, each plantlet consisting of 3–4 leaves was transferred to full-strength Y3 medium supplemented with 5.37 μM NAA, 24.6 μM indole-3-butyric acid (IBA) and 4.44 μM BAP as well as half-strength Y3 medium fortified with different concentrations of auxins (NAA and IBA), alone or in combination with cytokinins.

Histological studies

The tissue samples were fixed in a standard fixative Carnoy's 'B' solution (chloroform 30 ml; absolute alcohol 60 ml and glacial acetic acid 10 ml) for 24 h. The materials were dehydrated serially using different concentrations of alcohol alone (70% < 80% < 90% < 100%) and different concentrations of alcohol along with butanol (3 : 1, 1 : 1, 1 : 3). For infiltration and embedding, a mixture of paraffin wax and bee wax of melting point 58–60°C was used. Paraffin blocks were prepared by fixed tissues and histological sections were taken using the microtome (Leica RM 2145 country, Germany). Slides were de-paraffinized using xylene. Subsequent dehydration was done with butanol and alcohol. The slides were transferred to 100% alcohol and subjected to histochemical staining, either directly or after hydration depending on the stain used for tissues. Periodic acid–Schiff's reagent and toluidine blue were used to staining the sections. After subjecting the sections for histochemical staining, they were dehydrated subsequently using butanol. Dehydrated sections were mounted on DPX and observed under Wild Heerburg stereo (Switzerland) and Leitz Diaplan (Germany) binocular microscope. Photographs were taken and analysed using Leica Application Suite.

Assessment of clonal fidelity

In order to assess clonal fidelity, total genomic DNA was extracted from 100 mg of leaf tissue of the *in vitro* regenerated plant using a DNeasy Plant Maxi Kit (Qiagen, Valencia, CA, USA). The quality of DNA was checked on a 1% (w/v) agarose gel and concentration was measured using a spectrophotometer. Tissues from mother palm and six *in vitro* raised plantlets were tested for clonal fidelity using a set of 25 start codon targeted (SCoT) primers (SCoT1–SCoT25) described by Collard and Mackill[21]. All SCoT primers were synthesized from SIGMA (India) and initially screened for polymorphism and reproducibility. PCR amplification of these primers was carried out according to the protocol developed for coconut by Rajesh *et al.*[22]. Gels were visualized in a gel documentation system (Bio-Rad).

Results and discussion

Standardization of inoculation media

In order to standardize inoculation media, four media–hormone combinations were evaluated. Tiny swellings were observed from the ends of rachillae bit explants after 1–2 weeks of culture. The swellings gradually developed into white translucent outgrowths within 2–4 weeks. Observations taken after one month revealed that maximum response for white translucent outgrowths was observed in Y3 medium supplemented with 4.54 µM 2,4-D (92%), followed by 87% in M72 medium supplemented with 41.4 µM picloram, 61.9 µM putrescine and 4.54 µM TDZ. Figure 1 shows the percentage response of rachillae explants in different inoculation media. Browning was found to be maximum in Y3 medium with 41.4 µM picloram, 61.9 µM putrescine and 4.54 µM TDZ (55%), followed by M72 medium with 4.54 µM 2,4-D. In the present study, Y3 medium supplemented with 4.54 µM 2,4-D gave the best result for inoculation with maximum white translucent outgrowth and minimum browning. For coconut callus culture, the most preferred media are Y3 and M72, whereas MS and B5 (ref. 23) have been found to be less effective[3,24]. Although auxins like 2,4-D in high concentration are generally used for callus formation, the working concentration of 2,4-D was found to vary for different cultivars and also with the type of explant. In Sri Lankan Tall, a low 2,4-D (24 µM) treatment was found to be optimal to initiate callus production using zygotic embryos[25], whereas higher concentration (125 µM) initiated callus formation in Malayan Yellow Dwarf and Buta Layar Tall[26,27]. Similarly, very high concentration of 2,4-D (450–600 µM) was required for callus production on immature inflorescence and plumular tissue[7]. Vidhanaarachchi and Weerakoon[28] reported that in

modified Y3 medium supplemented with 200 µM 2,4-D and 0.2% activated charcoal, translucent outgrowths developed directly from the rachillae bits within six weeks of culturing. Shoot regeneration was observed in some of these outgrowths upon culturing in a medium containing 8 µM 6-BAP and 10 µM 2,4-D. However, the frequency of shoot regeneration was very low. However, Raju[9] has reported the use of half-strength modified MS medium fortified with 41.41 µg picloram and 4.54 µg TDZ for inoculation of rachillae bits for direct *in vitro* shoot development.

Regeneration

Sub-culturing was done to Y3 medium containing 2,4-D at monthly intervals, or as and when the medium showed symptoms of nutrient depletion. After four months, sub-culturing was done to Y3 medium with three hormone combinations. Table 1 shows the response of rachillae bits. At this stage, development of shoot-like outgrowth was 42% in Y3 medium supplemented with 4.54 µM 2,4-D, 17% in Y3 medium with 2.27 µM 2,4-D and 66.5% in Y3 medium with 4.54 µM 2,4-D and 4.14 µM picloram. Similarly, abnormal growth was 24% in Y3 medium supplemented with 4.54 µM 2,4-D, 49% in Y3 medium with 2.27 µM 2,4-D and 22% in Y3 medium with 4.54 µM
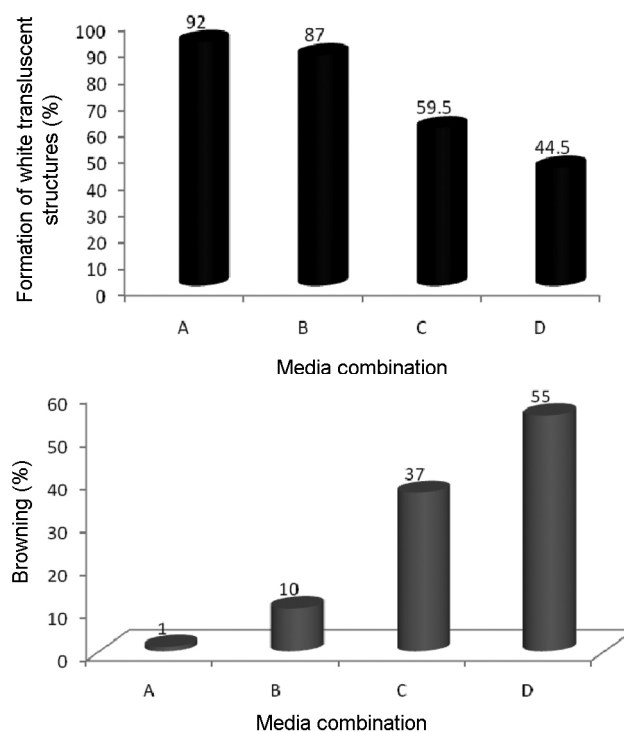

Figure 1. Response of rachille explants to different media combinations. A, Y3 + 2,4-dichlorophenoxyacetic acid (2,4-D) (4.54 µM); B, M 72 + picloram (41.4 µM), putrescine (61.2 µM) and thidiazuron (TDZ) (4.54 µM); C, M 72 + 2,4-D (4.54 µM); D, Y3 + picloram (41.4 µM), putrescine (61.2 µM) and TDZ (4.54 µM).

Table 1. Response (%) of rachillae bits to different media (after four months in dark)

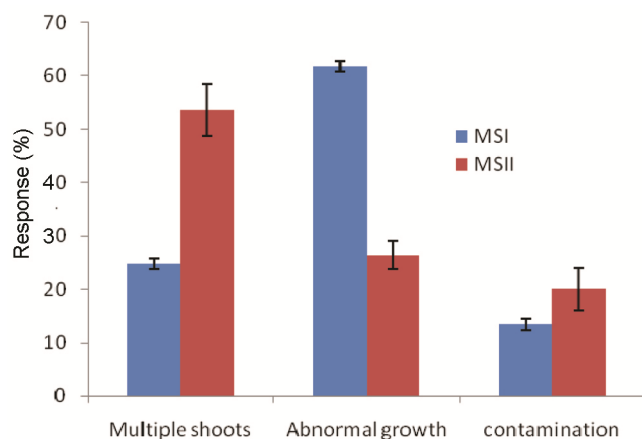| Response	Y3 I	Y3 II	Y3 III	CD ($P = 0.05$)
Development of shoot-like outgrowth	42 (40.33)	17 (24.11)	66 (54.39)	5.09
Abnormal/retarded growth	24 (29.14)	49 (44.40)	22 (27.63)	5.58
Browning/contamination	34 (35.43)	34 (35.54)	12 (19.94)	8.48

Numbers in the parenthesis are transformed values.

Figure 2. Response of rachillae bits in different media combination under light condition. Murashige and Skoog MS I – 1/2 MS + 30.7 µM picloram, 30.9 µM putrescine and 4.5 4 µM TDZ; MS II – 1/2 MS + 5.37 µM naphthalene acetic acid (NAA) and 4.44 µM 6-benzylaminopurine (BAP).
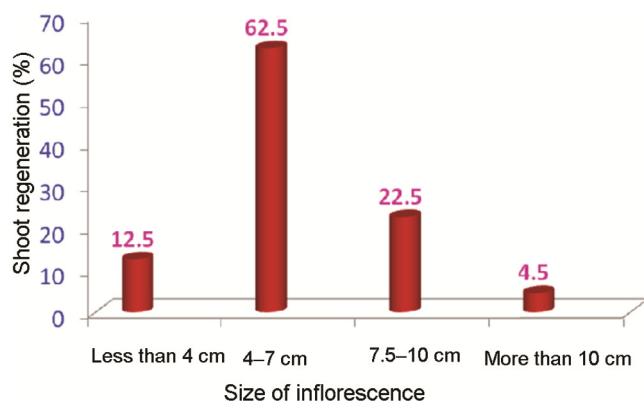

Figure 3. Effect of size of inflorescence on *in vitro* plant regeneration.

2,4-D and 4.14 µM picloram. Thus sub-culturing in Y3-III (Y3 medium supplemented with 4.54 µM 2,4-D and 4.14 µM picloram) resulted in maximum shoot-like structures and minimum abnormal growth. The shoot-like structures were transferred to 1/2 MS medium with two hormone combinations and kept under diffused light for a period of one month followed by incubation in light condition. During the cycle of maintenance under light, multiple shoot formation from individual cultures could be seen. When the cultures were transferred from diffused light to light condition, they gradually developed chlorophyll and the white shoot-like structures turned green. At the end of this cycle of incubation, the shoots were quite distinct and multiple shoots were formed from rachillae bits. The proportion of multiple shoot formed was recorded after the cultures were kept in light for 4–6 months (Figure 2). Observations revealed that 1/2 MS with 5.37 µM NAA and 4.44 µM BAP recorded 53.5% multiple shoot formation, whereas only 25% multiple shoot formation was noticed in 1/2 MS medium with 30.7 µM picloram, 30.9 µM putrescine and 4.54 µM TDZ. The abnormal or retarded growth was maximum (62%) in 1/2 MS medium with 30.7 µM picloram, 30.9 µM putrescine and 4.54 µM TDZ, whereas it was only 26.5% in 1/2 MS medium with 5.37 µM NAA and 4.44 µM BAP. During incubation of cultures in light, the maximum proportion of multiple shoots and minimum abnormal growth were observed in 1/2 MS medium supplemented with 5.37 µM NAA and 4.44 µM BAP. The abnormal structures were characterized by the presence of fused structures and sometimes with roots alone or with buds. The number of shoots developed from rachillae bit explants varied from 1 to 17. However, the size of individual shoots was not uniform. Some of them were very small and few had developed 2–3 leaves. Emergence of shoots (ranging from 1 to 14) from floral primodia in rachillae bit cultures maintained for 16 weeks on modified 1/2 MS medium with 8.28 µM picloram and 4.54 µM TDZ has already been reported[9].

In vitro shoot growth and multiplication

From the multiple shoots, individual shoots could be easily separated and cultured in two different media combination for a shoot development. Shoot regeneration was achieved in both the media combinations and resulted in the emergence of leaves. However, elongation and formation of vegetative shoots was maximum and faster in Y3 medium supplemented with 5 µM 2ip + 5 µM BAP compared to Y3 medium with 5.37 µM NAA and 4.44 µM BAP. The rachillae explants which responded to shoot development with minimal abnormalities were recorded and it was found that immature inflorescence with outer spathe length 4–7 cm resulted in maximum, i.e. 62.5% shoot regeneration (Figure 3). According to Vidhanaarachchi and Weerakoon[28], the frequency of shoot regeneration from immature inflorescence explants was very low.

Table 2. Evaluation of rooting media combinations

Medium composition	% Rooting	SD
1/2 Y3 + NAA (13.44 µM) + IBA (2.46 µM)	7.67	2.52
Y3 + NAA (5.37 µM) + IBA (24.6 µM) + BAP(4.44 µM)	15.55	8.55
1/2 Y3 + NAA (2.68 µM) + IBA (12.3 µM)	31.67	24.66
1/2 Y3 + NAA (5.37 µM) + IBA (24.6 µM)	39.28	31.23

NAA, naphthalene acetic acid; IBA, indole-3-butyric acid , BAP, 6-benzylaminopurine.
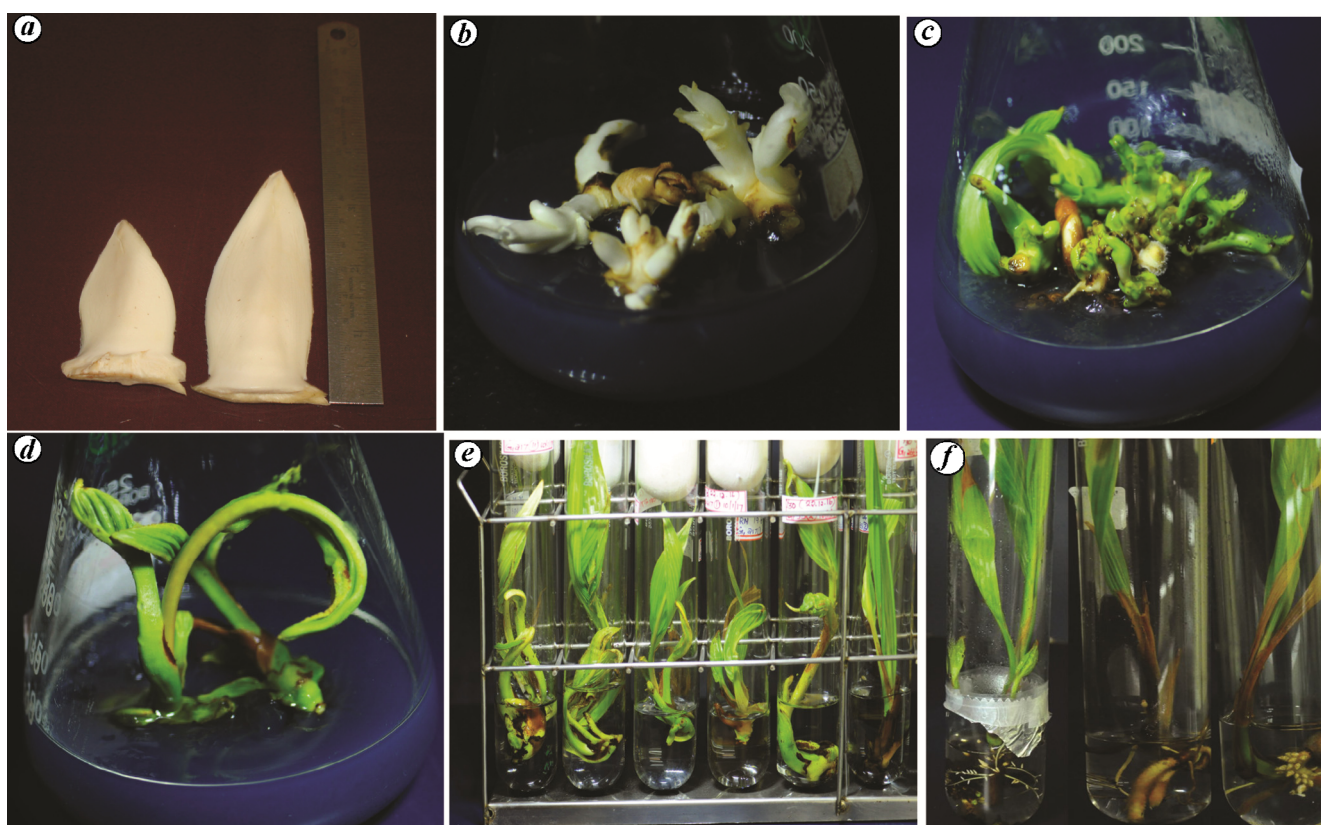

Figure 4. Stages of *in vitro* plant regeneration from immature inflorescence.

The present study revealed that the right stage of the explant for *in vitro* shoot regeneration was immature inflorescence with outer spathe length of 4–7 cm. In Y3 medium some of the shoots grew to about 15 cm and most of them had 3–4 leaves. However, majority of the cultures did not develop roots at the base. A few shoots had developed roots at the base. In order to develop roots, the cultures were subsequently transferred to rooting medium.

Root regeneration

Only very few plantlets cultured in shoot regeneration medium developed roots naturally at the base. In some cases, profuse rooting was observed and in such plantlets further shoot growth was retarded. For inducing roots, plantlets with 3–4 leaves were transferred to various rooting media combinations and it was observed that

medium containing 1/2 Y3 supplemented with 5.37 µM NAA and 24.6 µM IBA resulted in root initiation in 39.28% of the plantlets. Table 2 shows the response of cultures to various rooting media combinations. Various stages of *in vitro* regeneration of coconut from immature inflorescence are shown in Figure 4. Vidhanaarachchi and Weerakoon[28] reported that rooting was induced by dipping the shoots in 100 µM indole acetic acid solution for three days and subsequently transferring to an auxin-free medium[29].

Histology of organogenesis

Histological studies reveal that individual and multiple shoot formation occurs directly from rachillae bit explants (Figure 5 *a* and *b*). However, multiple shoot formation from proliferating callus can also be implicated considering the fact that there is delayed vasculature in
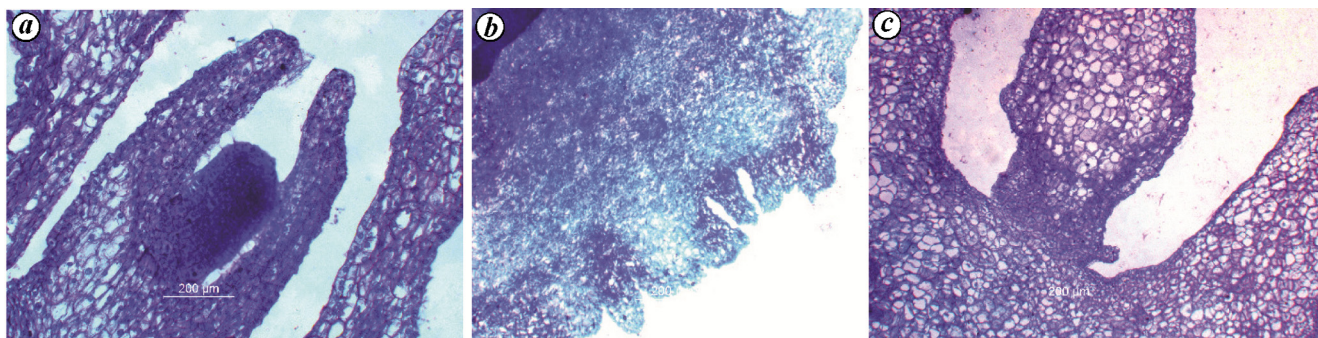
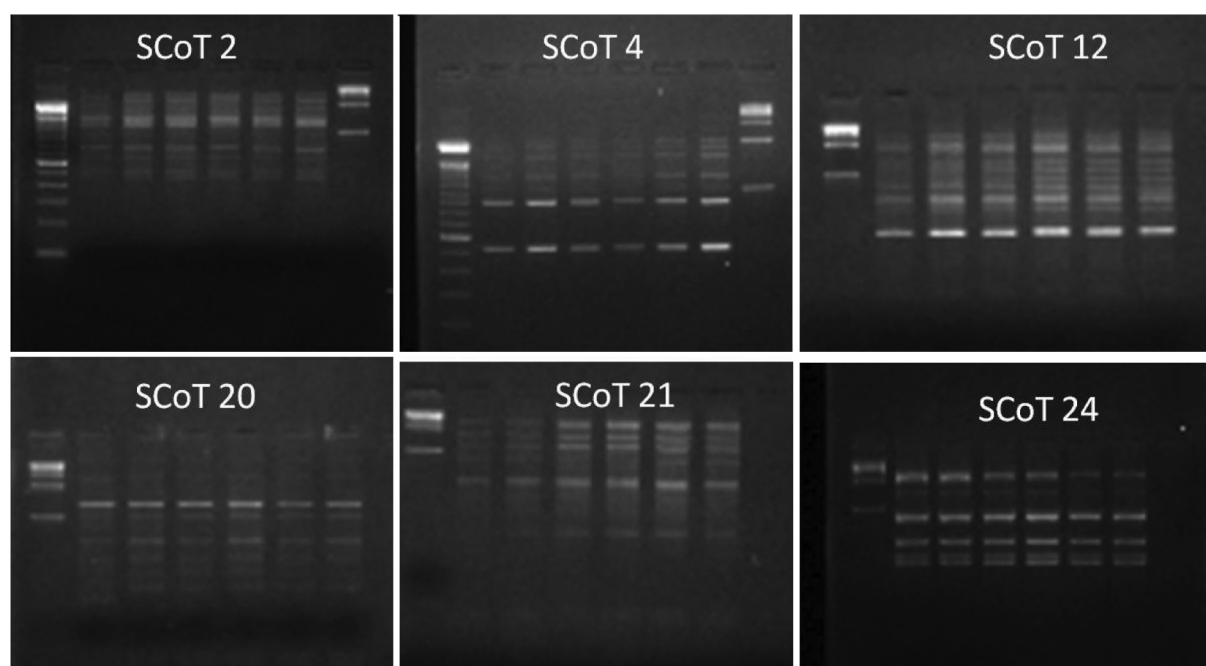**Figure 5.** Histology of organogenesis from rachillae explants of coconut.

Figure 6. Assessment of clonal fidelity of *in vitro* raised plantlets using start codon targeted marker. Lane 1 – 1 KB marker; Lane 2 – mother palm; Lanes 3–7 – *in vitro* raised plants.

few of the sections examined (Figure 5 c). Further studies are necessary to confirm the path of organogenesis (direct or indirect) obtained. The translucent outgrowths noticed before multiple shoot formation must be subjected to detailed histological studies to clear such ambiguity.

Assessment of clonal fidelity

Plants regenerated through tissue culture need to be phenotypically and genetically identical to the progenitor plant. However, in some cases, such as oil palm, the regenerants exhibit deviations from the parental type[30], defined as somaclonal variation[31]. Therefore, evaluation of *in vitro* regenerants in the field is essential prior to mass multiplication. DNA markers are a reliable means of detecting somaclonal variations as they are more informative and developmentally stable.

Twenty-five SCoT primers were used to screen the clonal fidelity, out of which 20 primers could amplify the genomic DNA. SCoT primers amplified 66 distinct and scorable bands, and the number of bands ranged from 3 to 12 with an average of 5.8 bands. The band size ranged between 240 and 1455 bp. No polymorphic bands were observed across the *in vitro* raised plantlets as well as the parental plant analysed. Figure 6 depicts a representative of SCoT profile. The monomorphic banding profile among the *in vitro* raised plantlets and mother palm confirms the clonal fidelity of *in vitro* raised plantlets indicating that these plantlets were devoid of somaclonal variation. Fernando *et al.*[32] have successfully utilized SSR markers to confirm the clonal fidelity of *in vitro* raised plantlets of coconut. The present study reveals that there was no somaclonal variation among the plantlets, even though they were under *in vitro* conditions with different hormone and chemical levels for more than two and a half years. Somaclonal variations occur due to the cell-cycle disturbance caused by the exogenous hormones applied in the culture process. Bandupriya *et al.*[33] found

that the level of hormones, especially 2,4-D and BAP, is sufficient to retain the genetic integrity of regenerated plants, which indicates that the above hormone levels are not deleterious to induce variation in callus-mediated coconut cultures.

Conclusion

Coconut is propagated exclusively by seeds due to the recalcitrant nature of the crop. Coconut tissue culture has been attempted in many laboratories worldwide and various tissues, including tender leaf, immature inflorescence, shoot tip, immature embryo and plumule have been used as explants. Among these, immature inflorescences are the most extensively studied, and they can also be considered an ideal source of explant since the performance of parental palm is already known. In the present study, immature inflorescence with outer spathe length of 4–7 cm was found to be the right developmental stage for *in vitro* shoot regeneration. Rachillae bits of size 1.0–1.5 mm obtained from the immature inflorescences were inoculated in four media combinations. Y3 medium fortified with 4.54 μM 2,4-D gave maximum response of white translucent outgrowth with minimum browning. After four months incubation in complete darkness, subculturing to Y3 medium with 4.54 μM 2,4-D and 4.14 μM picloram resulted in maximum shoot-like outgrowth and minimum abnormal growth. During the third cycle, transfer of these shoot-like outgrowths to 1/2 MS medium with 5.37 μM NAA and 4.44 μM BAP under light condition resulted in maximum multiple shoot formation. After maintaining the cultures for 4–6 months in 1/2 MS medium with 5.37 μM NAA and 4.44 μM BAP, the individual shoots were separated and transferred to Y3 medium containing 5 μM 2ip and 5 μM BA for shoot regeneration. The rooting of *in vitro* raised plantlets was found to be difficult and medium containing 1/2 Y3 supplemented with 5.37 μM NAA and 24.6 μM IBA resulted in root initiation in plantlets. The clonal fidelity of the plantlets was tested using SCoT markers, and all the plantlets were found to be uniform. The present study has demonstrated the possibility of inducing direct *in vitro* plant regeneration under appropriate culture conditions using the right developmental stage of inflorescence. This study forms the basis for developing an *in vitro* regeneration protocol using rachillae bit explants, which will ultimately enable large-scale multiplication of disease-free and high-yielding coconut palms.

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