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In vitro regeneration of coconut (*Cocos nucifera* L) through indirect somatic embryogenesis in Kenya

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Regeneration of the coconut (*Cocos nucifera* L) through indirect somatic embryogenesis using 9 to 12 months embryos explants was established in Y3 medium supplemented with 100 to 250 µM 2,4 - dichlorophenoxyacetic acid (2, 4-D) alone and in combination with gibberellic acid (GA_3), 6-benzylaminopurine (BAP) and thiadiazuron (TDZ). The highest callus induction (100%) was observed in medium containing 150 and 250 µM 2, 4-D + 5 µM BAP while the least was in 2, 4-D alone (16.7%) and in combination with 0.35 µM GA_3 (0). The highest embryogenic calli were observed in medium with 125 µM 2, 4-D + 5 µM BAP (100%) and the least was in medium with 2, 4-D alone (16.7) and in combination with 0.35 µM GA_3 (0). Multiple shoot induction was observed in medium supplemented with 10 µM kinetin, 10 µM BAP, 0.5 µM GA3, and 200 µM NAA. Maximum shoot elongation (1.667 cm) was in medium containing 10 µM BAP and the least (0.811 cm) was in medium with 5 µM BAP. Rooting was done in Y3 medium containing three levels of indole-3-butyric acid (IBA). The highest response was observed in Y3 medium supplemented with 5 µM IBA+ 0.5 µM GA_3 both with respect to the number of roots 8.33 and root length 5.10 cm while the least was with 10 µM IBA+ 0.5 µM GA_3 with regard to the number of roots 3.33 and root length 2.83 cm. Acclimatization was achieved in media prepared with soil: sand: manure ratio (3:1:1) with 25% survival rate and vermiculate medium with 8.3% survival rate. Hence, *in vitro* regeneration of coconut through somatic embryogenesis is a viable alternative for mass propagation.

Key words: *Cocos nucifera* L., somatic embryogenesis, zygotic embryos.

INTRODUCTION

Cocos nucifera L. belongs to family Arecaceae and lone species in the genus *Cocos*. It is a large palm growing to a height of up to 30 m tall with leaves 4to 6 m long. The life span of coconut varies depending on the variety; the tall variety can last even between 80 and 90 years while the dwarf variety can last to 50 years. In Kenya, coconut is almost exclusively found in the coastal areas which include Mombasa, Kwale, Kilifi, Lamu Tana River, and Taita Taveta counties. However, recent studies have shown that there is potential for coconut farming in other parts of the country. Piloting trials have indicated encouraging results in Tharaka Nithi, Makueni, Rift valley, Nyanza and western regions which do not belong to the coastal regions. Coconut is both heterozygous (tall variety) and homozygous (dwarf variety) and is so far propagated mainly through seeds (Bandupriya et al., 2016).

Coconut is an important palm in tropics and it provides

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a source of livelihood directly and indirectly to more than 50 million smallholder farmers worldwide. It is grown in more than 12 million ha in 90 countries mainly in the Asian Pacific regions where Indonesia has been ranked as the largest coconut growing region followed by the Philippines and thirdly India (Exposomics, 2017). It has a variety of traditional uses such food, beverage, oil, fiber, timber, thatch, mats, fuel, and domestic utensils (Batugal and Oliver, 2005). The copra and its products produce food and non-foods which are environmentally friendly and are used locally or exported hence becoming a source of foreign exchange. The virgin coconut oil is a very rich source of vitamins and enzymes which are used in the cosmetic and pharmaceutical industries (Mansor et al., 2016).

Despite all these, there are also challenges facing the coconut industry which include poor infrastructure, industrialization, utilization of coconut farms for other high-value cash crops, natural disasters, droughts, lack of quality planting material pest and diseases. In Kenya, according to the report released by the Agricultural Business Development (ABD) program of the Danish International Development Agency (DANIDA) which was carried out in collaboration with the Coast Development Authority (CDA) and others on a survey of coconut trees, the main challenge facing farmers at the production level was inaccessibility of quality planting materials and also pest and diseases which hence hindered the realization of its full potential. Farmers generally rely on their current crop to obtain seed nuts for raising seedlings. This conventional breeding approach using the nuts to replant land is very expensive due to the slow multiplication rate of seeds and even when elite germplasm is available it takes decades to multiply. Furthermore, the linkage between the farmer and the processor has not been fully exploited (Muhammed et al., 2013), because the existing aged orchards are poorly managed, high pest infestations and disease and inadequate technologies for mass production (Baudouin et al., 2018; Saha and Mat, 2018; Sathana, 2018).

Considering the economic importance of coconut, crop improvement is of high priority. Tissue culture of coconut is of prime importance for rapid multiplication and distribution of the best genotypes obtained through conventional breeding such as selected parental exhibiting resistance to biotic and/or abiotic stresses and for increasing the yield (Muhammed et al., 2013).

Direct and indirect organogenesis using plumules has often be reported in coconut as compared to somatic embryogenesis (Das et al., 2014). Direct organogenesis has been reported to be more effective in many plant species. This technique is preferred due to minimal soma clonal variation and also cost effective. However, regeneration of coconut through tissue culture technique is still a major challenge because it is a recalcitrant species. On the other hand, SE is a process by which somatic cells develop callus from an explant under induction. Bipolar structures are formed without connection with the original tissue. Then somatic embryos developed from the callus can convert into whole plantlets (Pérez-Núñez et al., 2006). Different coconut explants have been used and each giving diverse results whereas immature inflorescence and plumule have shown the ability of callogenesis, formation of embryos, maturation and plantlet formation (Pérez-Núñez et al., 2006). Somatic embryogenesis allows rapid multiplication of genotypes with the abiotic and abiotic resistant traits, high yielding and fast-growing nature. Through this technique, high quality and quantity of plantlets obtained can be used for establishing uniform orchards which are easy to maintain and even harvesting

Hence, the objective of this study was to develop an efficient protocol using 2,4D in combination with other plant growth regulators for effective *in vitro* regeneration of Kenya coconut and hence can be used for producing improved planting materials for mass production.

MATERIALS AND METHODS

Plant

Plant materials used in this study were obtained from mature nuts (10-11 months old) harvested from the clean coconut mother-plants which had been identified to be highly productive by the Kenya Coconut Development Authority, a Kenya Government Authority in charge of the coconut sector. By using a purposive sampling method, the nuts were collected from South Coast including Msambweni Majikuko Village (4°28'11.34''S, 39°28'46.68''E) and the North Coast including Birini, (3°45'04.14''S, 39°35'27.27''E), Mmleka (3°58'24.36''S, 39°44'00.95''E), and Kinarani (3°43'35.35''S, 39°34'21.66''E) region of Mombasa.

The nuts were packaged in netting bags containing 60 to 100 nuts and transported to Jomo Kenyatta University of Agriculture and Technology (JKUAT), Institute of Biotechnology Research (IBR).

Embryo extraction

The nuts were first dehusked and cleaned thoroughly by gently brushing under running tap water. A cylinder of endosperm surrounding an embryo was carefully extracted from each mature nut using a 2 mm diameter sterile cork borer (Sukendah and Cedo, 2005) and then washed with distilled water (Molla et al., 2004).

Surface sterilization of endosperms extracts and embryo excision

The endosperms containing embryos were soaked in 10% savlon with 100 µl of Tween® 20 for 30 min and rinsed with double distilled water. The endosperm portions with embryos were then placed in 95% ethanol with gentle swirling for 1 min and rinsed with double distilled water then soaked in 10% sodium hypochlorite containing 100 µl of Tween® 20 for 20 min and then rinsed with double distilled water.

Under aseptic conditions inside the clean bench, the embryos were excised from the endosperm cylinders using a sterile scalpel. The embryos were finally sterilized using 1% sodium hypochlorite for 1 min and rinsed thrice with double distilled water.

Effect of plant growth regulators on callus induction from coconut embryo explants

The sterilized embryos were initiated on the Y3 medium (Eeuwens, 1976) containing Morel and Whites vitamins (White, 1951) for callus induction. Six concentrations of 100-250 µM 2,4-dichlorophenoxyacetic acid (2, 4-D) were tested alone (A) and in combination with 0.35 µM gibberellic acid (GA_3) (B), 0.5 µM GA3 (C), 5 µM 6-benzylaminopurine (BAP) (D) and 9 µM thiadiazuron (TDZ) (E) which were combined in 5 different treatments and a control (without plant growth regulators) (Perera et al., 2009; Vidhanaarachchi et al., 2013). All the media were supplemented with 4% (w/v) sucrose, 1 g/l activated charcoal and 0.28% (w/v) gel rite.

The cultures were kept for 12 weeks in dark at 25±2°C for callus induction.

Effect of plant growth regulators somatic embryo formation

Callus formed were transferred to embryo formation media containing half 2, 4-D (50-125 µM) auxin concentrations alone (*A), and in combination with other PGRs including 0.35 µM GA_3 (*B), 0.5 µM GA_3 (*C), 5 µM BAP (*D) and 9 µM TDZ (*E). The cultures were kept in dark for 2 months at 25±2°C for embryo formation (Antonova, 2009; Vidhanaarachchi et al., 2013).

Effect of plant growth regulators on shoot induction and elongation

The embryos formed were transferred to a shoot induction media containing 5 µM BAP+ 0.5 µM GA_3 during 4 weeks and then subcultured into Y3 medium supplemented with 10 µM kinetin + 10 µM BAP + 0.5 µM GA_3 and 200 µM NAA (Vidhanaarachchi et al., 2016) for 8 weeks and, after that, the induced shoots were subcultured on Y3 medium supplemented with three concentrations of BAP (5, 10 and 15 µM) in combination with 0.5 µM GA_3 for shoot elongation. All the cultures were then kept under the light with a photoperiod of (16 h/8 h) provided by cool white fluorescent tubes in the culture room at 25±2°C for 8 weeks (Perera et al., 2008).

Effect of plant growth regulators rooting induction

The elongated shoots (5-6 cm long) were transferred to rooting media containing Y3 medium supplemented with 5, 10 and 15 µM IBA in combination with 0.5 µM GA_3. All the media was supplemented with 4% (W/V) sucrose, 1 g/l activated charcoal and 0.28% (w/v) gel rite. The cultures were incubated in the culture room under a photoperiod of16/8 h of light and darkness and the temperatures were maintained at 28±2°C for 8 weeks to induce roots.

Acclimatization in the greenhouse

The fully developed plantlets with three leaves and at least 5 roots which were at least 3 cm were de-flasked, rinsed with double distilled water and drenched in a mixture of Y3 nutrients and a mixture of BAP and NAA hormones for 1 min before transfer to clear polypropylene bags containing sterilized potting mixtures. Two different substrates that were tested were sterilized soil: sand: manure (3:1:1) (A) and a vermiculate (B). The plantlets were acclimatized at 28±2°C and relative humidity ranging from 70 to 80%. The plants were watered regularly and data on the survival rate was recorded every 2 weeks for 3 months.

Experimental design, data collection, and analysis

Experiments on callus induction, somatic embryo formation shooting, rooting and acclimatization were all set up in a completely random design in 3 replicates. Parameters taken on callus and somatic embryo formation were color texture and period took for callus and embryo induction. All these were done by observation and counting the explants which formed callus and embryos and each was estimated to be 100%. For the shoot induction and elongation, the parameters taken were period taken for shoots to form and the color change which was done by observation, counting the number of leaves and measuring the height of the plantlets (cm). The rooting parameters were root length taken using a ruler (cm) and the numbers of roots formed were counted. The length and number of roots were analyzed using the statistical software to give the means. All the data recorded were analyzed using Minitab 17 Statistical software. Analysis of variance (ANOVA) was used to test the significant differences between the various means (%) and Fisher's test at $P<0.05$ was used to separate means. The variability in data was expressed as the percentage mean ± standard error (SE).

RESULTS

Effect of plant growth regulators on callus induction

The first signs of callusing which include swelling of the embryo and the color change were observed from the eight week. When the explants were subcultured into the same media for an additional four weeks, the callus became more profound and could easily be differentiated from the swollen embryos (Figure 1). The texture of the calli became more visible after 12 weeks where both friable and compact calli were seen and also the color changed from white to cream-white was visible. The calli formed in media containing 2,4-D alone and in combination with GA_3 and some in BAP were compact while those formed in a media containing 2,4-D in combination with TDZ were friable. Data analysis on callus induction after 12 weeks revealed that all the embryos in Y3 media supplemented with 150D and 250D formed calli (100%). Both compact and friable textures of callus were observed in the treatments which were supplemented with BAP (D). To check the optimum hormone combination for callus induction (Table 1 on % frequency) combinations of 2,4-D plus 5 µM BAP (D), the highest % frequency of 61.1%, 2,4-D plus 0.5 µM GA_3 (C) with a % frequency of 50% and those in 2,4-D plus 9 µM TDZ (E) with a % frequency of 47%. While the least frequency was recorded in 2,4-D alone with 16.7% (A) (Table 1).

However, some of the embryos remained dormant even after 16 weeks in the callus induction media.

Effect of PGRs on the formation of embryos

When the formed calli were subcultured into embryo formation media, the majority of the calli became embryogenic after 8 weeks. Data analysis after 8 weeks

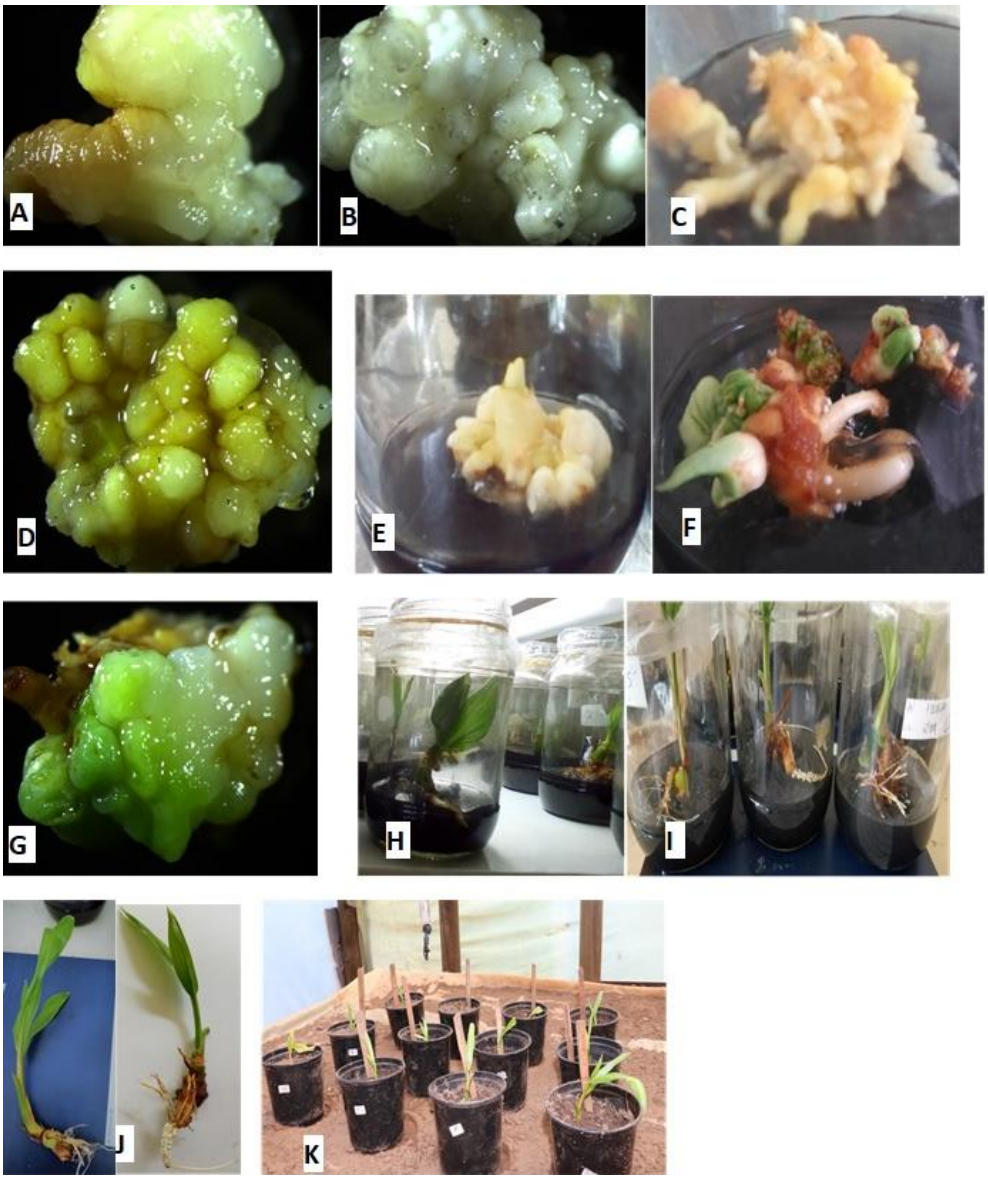

Figure 1. (A) Primary callus formed after 12 weeks in callus induction media; B and C, Compact and friable embryogenic calli, respectively formed after 8 weeks in embryo formation media; D, Embryogenic calli with all stages of embryogenic callus development that is g-globular, H-heart shape and T-torpedo stages; E shows shoot induction from compact embryogenic calli after 8 weeks in maturation media containing BAP and GA_3; F, Multiple shoot induction from friable embryogenic calli in media containing 10 µM kinetin, 10 µM BAP, 200 µM NAA and 0.5 µM GA_3; G, Elongated shoots from green embryogenic calli; H, I and J, Rooted after 8 weeks in root induction media; and K shows the acclimatization of plantlets in the greenhouse.

of subculture in embryo formation media revealed that all the calli in media which were supplemented with 125 µM 2, 4-D+5 µM BAP became embryogenic (100%).

To check the optimum hormone combination and concentration for embryogenic callus induction, (Table 2 on % frequency) a combination of 2, 4-D plus 5 µM BAP (*D) was the highest with 58.3% and the least embryo induction frequency was recorded in the hormonal combination of 2,4-D alone with 13.9% (*A). Just as in callus induction experiments, all the embryogenic calli formed in treatment with 24-D alone and in combination with GA_3 (*B and *C) and some in BAP (*D) were compact while for treatment containing TDZ (*E) and some in BAP media (*D) were friable Table 2.

Also to note is that, embryogenic calli induced after 8 weeks in embryo formation media were visible under the microscope (Figure 1B). Different stages of embryogenic calli development were observed in the same explants

Table 1. Effect of 2,4-D alone and in combination with 0.35 µM GA_3, 0.5µM GA_3, 5 µM BAP and 9 µM TDZ on callus induction from embryo explants after 12 weeks.

Concentration of 2,4-D (µm)	Plant growth regulators (µM)				
	A	B	C	D	E
Control	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}
100	33.3 ± 0.21^{bcd}	16.7 ± 0.17^{cd}	50.0 ± 0.22^{abcd}	33.3 ± 0.21^{bcd}	66.7 ± 0.21^{abc}
125	16.7 ± 0.17^{cd}	16.7 ± 0.17^{cd}	66.7 ± 0.21^{abc}	$50.0\pm0.22a^{bcd}$	33.3 ± 0.21^{bcd}
150	16.7 ± 0.17^{cd}	16.7 ± 0.17^{cd}	83.3 ± 0.17^{ab}	100^{a}	66.7 ± 0.21^{abc}
175	16.7 ± 0.17^{cd}	16.7 ± 0.17^{cd}	33.3 ± 0.21^{bcd}	$50.0\pm0.22a^{bcd}$	$50.0\pm0.22a^{bcd}$
200	16.7 ± 0.17^{cd}	50.0 ± 0.22^{abcd}	33.3 ± 0.21^{bcd}	33.3±0.21bcd	33.3 ± 0.21^{bcd}
250	16.7 ± 0.17^{cd}	0^{d}	33.3 ± 0.21^{bcd}	100^{a}	33.3 ± 0.21^{bcd}
% Frequency	16.7 ± 0.63^{b}	19.44 ± 0.67^{b}	16.7 ± 0.17^{cd}	61.11 ± 0.82^{a}	47.22 ± 0.84^{a}

Means (± SE) followed by the same alphabets in each column were not significantly different at P≤0.05 using Fisher's test (n=186). A = (100-250 µM 2,4-D alone); B=(100-250 µM 2,4-D +0.35 µM GA_3); C=(100-250 µM 2,4-D+0.5 µM GA_3); D=(100-250 µM 2,4-D +0.5 µM BAP) and E=(100-250 µM 2,4-D +9 µM TDZ), e.g. 100A will be Y3 media supplemented with 100 µM 2,4-D alone and 125E will be Y3 media supplemented with 125 µM 2,4-D +9 µM TDZ. % Frequency =average frequency % for hormone combinations (A B C D and E).

Table 2. Effect of 2,4-D alone and in combination with 0.35µM GA_3, 0.5µM GA_3, 5µM BAP and 9µM TDZ on embryo formation after 8 weeks.

Concentration	Plant growth regulators in (µM)				
	*A	*B	*C	*D	*E
Control	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}
50	0^{c}	16.7 ± 0.17^{c}	50.0 ± 0.22^{abc}	33.3 ± 0.21^{bc}	50.0 ± 0.22^{abc}
62.5	16.7 ± 0.17^{c}	16.7 ± 0.17^{c}	50.0 ± 0.22^{abc}	50.0 ± 0.22^{abc}	33.3 ± 0.21^{bc}
75	16.7 ± 0.17^{c}	16.7 ± 0.17^{c}	83.3 ± 0.17^{ab}	83.3 ± 0.17^{ab}	33.3 ± 0.21^{bc}
87.5	16.7 ± 0.17^{c}	16.7 ± 0.17^{c}	33.3 ± 0.21^{bc}	50.0 ± 0.22^{abc}	33.3 ± 0.21^{bc}
100	16.7 ± 0.17^{c}	33.3 ± 0.21^{bc}	33.3 ± 0.21^{bc}	33.3 ± 0.21^{bc}	16.7 ± 0.17^{c}
125	16.7 ± 0.17^{c}	0^{c}	33.3 ± 0.21^{bc}	100^{a}	16.7 ± 0.17^{c}
% Frequency	13.89 ± 0.59^{c}	16.7 ± 0.63^{c}	47.22 ± 0.84^{ab}	58.33 ± 0.83^{a}	30.56 ± 0.78^{bc}

Means (± SE) followed by same alphabets in each column were not significantly different at P ≤0.05 using Fisher's test (n=186). *A = (50-125 µM 2,4-D alone);*B= (50-125 µM 2,4-D +0.35 µM GA_3); *C= (50-125 µM 2,4-D +0.5 µM GA_3);*D=(50-125 µM 2,4-D +0.5 µM BAP) and *E=(50-125 µM 2,4-D +9 µM TDZ), e.g. 50*A will be Y3 media supplemented with 50 µM 2,4-D alone and 125*E will be Y3 media supplemented with 125µM 2,4-D +9 µM TDZ. % Frequency = average frequency % for hormone combinations (A B C D and E).

which include the globular stage was round and shiny protrusions, torpedo and heart shape stages (Figure 1D).

Effects of PGRs on shoot induction

The compact embryogenic calli matured to form shoots after 4 weeks in Y3 media containing 5 µM BAP and 0.5 µM GA_3. The shoots were also white cream in color since they were still in darkness (Figure 1E). When all the embryogenic explants were further subcultured into a fresh media containing 10 µM kinetin, 10 µM BAP, 200 µM NAA and 0.5 µM GA_3 in light, multiple shoots were induced from friable embryogenic calli after 5 weeks in and the color changed from cream white to pale green (9 days) and finally to green (18 days) (Figure 1F). Due to the long juvenile stage of coconut development, some embryogenic calli had not induced shoots but were green and some white cream visible embryos (Figure 1G). Also, to note is that all explants which were initially initiated in Y3 media supplemented with 2.4-D alone eventually matured to form shoots, though at a low rate. It was also very important to note that most explants which were first initiated in Y3 media supplemented with 2,4-D and 0.5 µM GA_3 formed shoots after various subcultures in the shooting media.

Effect of PGRs on shoot elongation

The green, well germinated shoots with an average height of 2.5 cm successfully elongated when they were subcultured in Y3 medium supplemented with BAP in combination with 0.5 µM GA_3 (Figure 1H). The explant

Table 3. Shoot elongation of coconut on Y3 medium supplemented with BAP and GA_3, after 8 weeks.

Parameter	BAP (µm)			
	5	10	15	Control
Shoot height (cm)	1.178±0.19[b]	1.667±0.22[a]	0.811±0.19[c]	0.1±0.03[c]
Number of leafs	2.67±0.33[a]	2.67±0.33[a]	2.67±0.33[a]	0[b]

Means (± SE) followed by the same alphabets in each column were not significantly different at P ≤0.05 using Fisher's test n=36.

which exhibited the highest mean length were those in 10 µM BAP with a significant mean of 1.67 cm and the lowest mean among the three concentrations was at 15 µM BAP with a mean of 0.81 cm. However, the control experiment showed the least mean length of 0.1 cm (Table 3).

Effect of PGRs on the root formation

The shoots with at least 5 cm in height and 2 developed leaves were evident in Y3 media supplemented with all three concentrations of IBA in combination with GA_3 after 8 weeks (Figure 1I). The highest number of roots induced was in the treatment containing 5 µM IBA + 0.5 µM GA_3 while the treatment with 10 µM IBA + 0.5 µM GA_3 had the least number of roots induced as shown in Table 4. However, the control did not induce roots. To check the effect of different PGRs and their concentration on root length, analysis of data revealed that the concentration with significant longer roots were 5 µM IBA + 0.5 µM GA_3 with 5.10 cm while the lowest among the three concentrations was at 10 µM IBA with 2.83 cm (Table 4).

Notably, 5 µM IBA + 0.5 µM GA_3 recorded the highest mean both in length and number of roots induced and the media supplemented with 10 µM IBA + 0.5 µM GA_3 recorded the least mean both in the number of roots induced and root length. Nonetheless, all three levels of IBA were able to induce roots with no difference in their morphological appearances Figure 1I and J.

Effect of different media on acclimatization of coconut plantlets in the greenhouse

After 1 week of acclimatization in the greenhouse, there was 100% survival of all the plantlets with no change in both plant height and number of leaves. The mean height of plantlets in soil media was 8.25 and 6.9 cm in vermiculate with 100% survival. However, during the first 4 weeks of greenhouse acclimatization, there was no or minimal changes in the plantlets. Some died while others experienced a very slow growth rate. To also note is that the majority of the plantlets which were lost began to rot from the roots upwards irrespective of the media. By the end of 12 weeks, the mean height of plantlets in soil media was 9.20 and 7.63 cm in vermiculate. The survival rate for plantlets in both media was 25 and 8.3% in soil and vermiculate media respectively, and since then the remaining plantlets successfully acclimatized with a continued increase in height and new leaves had not formed at that early stage of acclimatization (Table 5).

DISCUSSION

Somatic embryogenesis enables mass production of plants all year round and can be used for genetic improvement of all plants irrespective of the species (Bhansali, 1990). Many trials have been attempted since 1954 by different authors to propagate *C. nucifera in vitro* (Cutter Jr and Wilson, 1954), but still, none of the protocols was found to be efficient. This is because coconut is one of the recalcitrant species to tissue culture and the existing protocols at times cannot be repeated (Solís-Ramos et al., 2012). For somatic embryogenesis to be achieved in coconut there must be a callus intervening stage where callus is first formed and then the regeneration of plantlets from the embryogenic callus (Fernando et al, 2004).

The plant growth regulators concentration, explant source, age, and the variety determines the regeneration efficiency of plants (Kumar et al., 2010). Though callusing can be achieved with or without cytokinins, 2,4-D which is an endogenous auxin is very critical for callogenesis to be achieved and also its concentration varies depending on the type of explants (Solís-Ramos et al., 2012). However, there are several factors including *in vitro* conditions, genotype and mother plant maturity and these may affect the *in vitro* performance (Hernandez-Fernandez and Christie, 1989; Basra, 1995). The gradual reduction in endogenous auxin with a corresponding increase in cytokinins level permits initiation and further development of somatic embryos.

The mature zygotic embryos explants showed better callusing and embryo development, as compared to immature inflorescence which was reported by various authors (Hornung, 1995). Dormancy was also experienced in some embryos and other studies by (Cutter Jr and Wilson, 1954), dormancy was attributed to

Table 4. Effect of IBA in combination with 0.5µM GA_3 on root induction after 8 weeks.

Parameter	IBA (µm)			
	5	10	15	Control
Root length height (cm)	5.10±1.99[a]	2.83±0.72[ab]	3.27±0.43[ab]	0[b]
Number of roots	8.33±2.0[a]	3.33±0.88[ab]	7.33±2.18[a]	0c

Means (± SE) followed by the same alphabets in each column were not significantly different at P≤0.05 using Fisher's test n=36.

Table 5. Effect of media on greenhouse acclimatization of coconut plantlets after 3 months (n=20).

Media	Media components	Shoot height (cm)	Survival rate (%)
A	Soil: Sand: Manure (3:1:1)	9.20±1.5[a]	25
B	Vermiculate	7.63±0.5[a]	8
C	Control	0[b]	0

Means (± SE) followed by the same alphabets in each column were not significantly different at P≤0.05 using Fisher's test.

the use of nuts with mature embryos and endosperm which are likely to contain inhibitory substances leading to dormant cells. The use of young/juvenile explants has also been reported in palms to be more responsive for callus formation (Steinmacher et al., 2007) because their cells are rapidly dividing unlike the mature explants which causes dormancy when subjected to *in vitro* propagation. On subjecting the explants to various combinations and concentrations of plant growth regulators, profuse callus induction was achieved after 8 weeks. And at 12 to 20 weeks, this callus became more profound and even developed embryos. The use of the Y3 medium (Eeuwens, 1976) has been reported to be more favorable for tissue of coconut as compared to the use of MS medium (Murashige and Skoog, 1962). The ammonium and nitrate nitrogen content are half in the Y3 medium as compared to MS medium while the concentrations of microelements such as cobalt copper and iodine are tenfold greater in Y3 medium as compared to MS. This thus reflects those found in the coastal soil providing a favorable habitat for coconut germination (Nguyen et al., 2015).

However, the culture conditions and culture media may vary from one species to another. The addition of 0.4% (w/v) sucrose was essential for callusing and even to germination of the embryos *in vitro.* 0.28% (w/v) gel rite was used as a solidifying agent in this study. Callus induction and regeneration of coconut plantlet *in vitro* was done in darkness at 25 to 28°C. These conditions are required to optimize the germination of the embryo and also reflects the *ex vitro* requirements.

Another component that has shown to be essential in initiating coconut somatic embryos is the presence of activated charcoal (Fernando et al., 2004; Perera et al., 2007; Pérez-Núñez et al., 2006). The role of the activated charcoal is that it has the adsorptive properties hence the ability to adsorb phenols and other growth inhibitory substances. In coconut zygotic embryo culture, the activated charcoal also reduces callus necrosis. It has been known to remove toxic substances released by non-reactive tissues and hence permitting more embryogenic cells to grow (Kumar et al., 2010).

For best callusing to be achieved, it requires the ideal combination of 2, 4-D and activated charcoal in the tissue culture medium. The optimum concentration of 2,4-D in the tissue culture medium may vary with the adsorption capacity of the activated charcoal which also adsorbs 2,4-D (Perera et al., 2007).

Though the specific mechanism involved is less certain. The elimination of growth-inhibitory chemicals such as those produced on autoclaving the medium including 5-hydroxymethylfurfural or from dehydration of sucrose, or by the explanted tissue as toxic metabolites has also been reported. Ethylene gas accumulation especially in closed culture jars/vessels in response to wounding either by forceps or scalpels used during extraction and surface sterilization and also elevated concentrations of exogenous auxin can also be lessened by the activated charcoal and finally controlling browning. Besides the absorption properties of the activated charcoal, in the process of coconut somatic embryogenesis it acts as an "auxin slow-release agent" (Antonova, 2009; Hornung and Verdeil, 1999). Hence, it is an essential component for the successful formation of coconut somatic embryos.

Callusing was evident in all the experiments performed in the Y3 medium (Euwens, 1976) supplemented with 2,4D alone, GA_3 BAP which were compact and TDZ which formed friable calli. The explants in medium

supplemented with various levels of 2,4D and 0.5 µM BAP yielded an average percentage of 61% and the least was those in Y3 media supplemented with 2,4D alone with 16.7%. In previous studies, various authors have also reported different callusing frequencies for example (Hornung, 1995) with 75% (Chan et al., 1998) with 60% and (Fernando et al., 2004) and with 54.3% all-in Euwens Y3 media.

GA_3 was added to the culture medium to promote the germination of somatic embryos. However, the effect of GA_3 has not been tested on the formation of somatic embryos (Perera et al., 2009).

In this study, the addition of 0.5 µM GA_3 proved to be better in the induction of both callus (50%) and embryogenic calli (47.2%) as compared to 0.35 µM GA_3 which resulted in callus induction frequency of 16.7% and embryogenic calli frequency of 13.9%. Higher callus induction in GA_3 containing medium was also reported using coconut ovary explants (Perera et al., 2009). This phytohormone also was used for breaking dormancy at every stage of embryo development hence improving the performance of coconut micropropagation.

TDZ on the other hand as compared to other plant growth regulators when incorporated in the initiation media also revealed to be a potential plant growth hormone for somatic embryogenesis (Perera et al., 2009) reported the use of 9 µM TDZ to be effective in callusing frequency using the ovary explants which resulted in a callusing frequency of 76.4%. The callus induction frequency in this study from embryo explants in the media supplemented with 9 µM TDZ was 47% and the frequency of embryogenic calli was 30.6% which is lower compared to the previous studies. However, the friable and creamy white translucent mass of globules developed in TDZ containing media later induced multiple shoots when subcultured into shooting media (Figure 1F). Similarly, Mweu et al., (2016) in their study also revealed that TDZ is vital for high-frequency induction of callus and multiple shoots which was in agreement for this study.

However, the explants initiated in hormone-free media which were used as a control did not induce callus or embryogenic callus. The hormone-free media without phytohormonal additives serves occasionally in germination of shoots but not in the whole process of tissue culture because in one way or another a plant established *ex vitro* uses the endogenous hormones produced in different pathways aided by the natural environment and hence the *in vitro* plants produced in the lab also requires the phytohormones in their pathways for a complete plantlet formation and also the rapid growth for normal tissues.

Substances that stimulate growth can be applied in form of natural fluids such as coconut water which contains various hormones that break its dormancy, auxin, and cytokinins, and also various vitamins (Fernando and Gamage, 2000) and sugars or chemical compounds required by plants.

Successful initiation of roots is one key factor that determines the survival rate of the tissue culture plantlets upon acclimatization and field transfer. Successful rooting was achieved by supplementing Y3 media with IBA and GA_3. The use of a high concentration of IBA induced rooting in micropropagation of sandalwood which is also one of the recalcitrant plants as well as coconut palm (Solís-Ramos et al., 2012) and for this study, 5 µM IBA induced the highest rooting as compared to 15 µM IBA though not significantly different. No rooting was observed in the treatment without the hormones meaning that the IBA hormone was solely responsible for root induction. This study also revealed that a combination of plant growth regulators in tissue culture media has more affirmative results as compared to using only one hormone (Vidhanaarachchi et al., 2013). GA_3 has been reported by other researchers in various topics to promote normal growth, root development, and axis development and also elongation which were also evident in this study. The quantity of IBA auxin reaching the cambial activity is adequate for initiating root primordia. The maximum number of roots formed could also be due to the hormonal effect resulting to the buildup of internal substances and their downward movement which result in more cell division and hence success in root induction (Muthan et al., 2006).

Tissue cultured plantlets were able to acclimatize in soil: sand: manure (ratio 3:1:1) and vermiculate after 12 weeks though with low survival rate. When tissue cultured plantlets were transplanted, their growth rate in the greenhouse was slow and this may be contributed by many factors one being the leaves. Normally when *in vitro* raised plants are taken for acclimatization their leaves are not fully developed and hence a low level of the photosynthesis and undeveloped cuticular wax impairs stomatal mechanisms. Despite the addition of minerals and hormones to aid in successful acclimatization, yellowing of leaves was still observed after 3 months and the plantlet started withering gradually. This is also in agreement with Rival (2000) who attributed the same to the photosynthetic mechanism and/or poor nutrition in the potting media used in the acclimatization process. Due to the aforementioned characteristics, the nature of the tissue-cultured plantlets during the *ex vitro* transfer causes them prolonged acclimatization duration and the same was also reported by Fernando et al., (2004) and Gunathilake et al. (2004) using plumule as the explants. Also, in some explants rotting from the roots upwards was evident. The same was reported by Steinmacher et al. (2007) and this might have been a result of a poor formation of the root system resulting in a low rate of survival. Acclimatization is a key challenge in most of the tissue cultured plants due to the shock and stress experienced upon transfer from the *in vitro* environment to the *ex vitro* conditions in the greenhouse. In spite of the recalcitrance of coconut palms to tissue culture, a high rate of plantlet loss upon acclimatization

hinders mass production. After the successful hardening of the plantlets, they steadily overcome the morphological stresses and adapted to *ex vitro* conditions which were also in agreement with Fernando et al., (2004).

Conclusion

The present study indicated the viability of regeneration of *C. nucifera* L using embryo explants via indirect somatic embryogenesis where zygotic embryos were initiated in 2,4D (100 to 250 µM) in combination with GA_3, BAP, and TDZ. Lowering the concentration of 2,4D to half while retaining the concentrations of other growth regulators induced embryogenic calli and finally subculturing them to a shoot induction media containing kinetin, BAP and NAA caused multiple shoot induction. This is a key milestone of coconut regeneration in Kenya using Eeuwens Y3 medium with distinct callus becoming embryogenic and eventually developing multiple shoots. This protocol, however, is reproducible and can be used in the mass propagation of coconut.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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