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Zygotic embryo *in vitro* culture of *Cocos nucifera* L. (sv. East African Tall variety) in the coastal lowlands of Kenya

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***In vitro* embryo culture of *Cocos nucifera* L. was carried out with the aim of determining whether it can be applied as an alternative coconut propagation tool to address the lack of planting materials in Kenya. Zygotic embryos excised from mature healthy pyrenes of high yielding *Cocos nucifera* L. (sv. East African Tall, a coconut variety from Kenya) were cultured using Murashige and Skoog (MS) culture medium supplemented with plant growth regulators (PGRs) namely 6-benzyl aminopurine (BAP) (0.5 mg/l), naphthalene acetic acid (NAA) (0.5 mg/l), 2,4 dichlorophenoxyacetic acid (2,4-D) (1.7 mg/l) and indole butyric acid (IBA) (1.7 mg/l). Germination of 84 and 27% embryos were recorded in liquid and semi-solid MS medium, respectively. Embryo cultured in liquid medium and incubated in darkness during the initial four weeks resulted in a germination percentage of 80% while incubation in light resulted in only 30% germination. MS medium was also supplemented with 100, 25, 15 and 0% (v/v) coconut water (CW). Embryo germination was 60% in medium supplemented with 15% (v/v) coconut water although 0% (v/v) gave the highest germination rate at 67%. Medium supplementation by 1.7 mg/l 2,4-D PGR resulted in germination of 30% when plant growth regulator was co-autoclaved and 84% when plant growth regulator was sterilized by microfiltration. The results presented in this study indicate that *in vitro* micropropagation of the Kenyan variety of *Cocos nucifera* L. is a feasible alternative.**

Key words: *Cocos nucifera* L., *in vitro* embryo culture, east African tall (EAT) Kenyan variety, zygotic embryo.

INTRODUCTION

The coconut palm is grown throughout the tropic regions and is usually termed as a “tree of life” (Waaijenberg, 1994) as virtually all parts of the coconut trees are utilized; from the nuts, husks, inflorescences, stems and even the roots. In Kenya, coconut wine (“*mnazi*”) is the crop's main used (60%) product, other products include nuts, leaves for roofing (*“Makuti”*), brooms, coco wood, and copra which are processed into oil mainly for the soap industry, cosmetics, and candle wax. Coconut trunks are used for house construction. Coconut water contains sugar, proteins, antioxidants, vitamins and minerals and provides an isotonic electrolyte balance while the coir obtained from the fiber husk of the coconut, can be used for making ropes, mats, brushes, sacks, and stuffing fiber for mattresses (Gachanja et al., 2007).

The embryo culture technique in coconut has been successfully applied in propagating the Makapuno coconut (De Guzman and Del Rosario, 1964; Del Rosario and De Guzman, 1976). Makapuno is an important coconut mutant from Philippines with a high commercial value due to

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the accumulation of galactomannan in the maturing nut. This accumulation leads to many novel elite applications in the food, medical and pharmaceutical industries and in personal health (Nguyen et al., 2010). The excised embryos were found to be capable of germinating when cultured on solid White's medium resulting in high number of quality plantlets which were reported to adapt well to the prevailing field conditions (De Guzman and Del Rosario, 1964). The possibility of regenerating plantlets from plumules resulting from germinating embryos has been investigated. The results reveal that callus can be initiated from plumule explants where about 50% of the regenerated plantlets could be successfully acclimatized in the field (Fernando et al., 2004). Trials in *in vitro* embryo culture of the Sri Lankan Tall coconut variety in Bangladesh resulted in 89% germination (Molla et al., 2004).

In Kenya, lack of access to quality planting materials is one of the challenges facing the coconut sub sector (Mwachiro and Gakure, 2011) where farmers generally rely on their current crop to obtain seedlings. Furthermore, the linkage between the farmer and the processor has not been fully exploited due to low quality coconut seeds (raw materials) productivity (Muhammed et al., 2012).

In addition, there are no well established technologies to mass propagate coconut in Kenya and dissemination (Gachanja et al., 2007). Embryo culture micropropagation has several advantages over conventional propagation methods, for instance; the production of disease-free, high quality planting material and the rapid production of many uniform plantlets in a limited space area. It also increases the amount of quality planting material to facilitate distribution and large scale planting. The technique has not been tried for the Kenyan variety. The *in vitro* production of young plantlets from excised embryos might be a tool for a large number of quality individuals' yield in a limited space; to revive the coconut sector in Kenya. This paper thus reports *in vitro* coconut embryo culture of the East African Tall (EAT) variety of *Cocos nucifera L*. for the first time in the coastal lowlands of Kenya.

MATERIALS AND METHODS

Embryos were obtained from 10 to 12 month old nuts harvested from mother plants of the East African Tall (EAT) variety coconut palms which had been identified to be highly productive by the Kenya Coconut Development Authority, a Kenya Government Authority in charge of the coconut sector. Nuts were collected from the following sites within the coastal region of Kenya: Roka (1° 36' 0" S, 39°12'0"E), Chumani (3°28'01.96"S, 39°53'47.95"E), Lamu (2°16'10.41"S, 40°54'02.31"E) and Kaloleni (3°48'53.43"S, 39°37'42.91"E).

Embryo extraction

The collected nuts (10 to 12 months old) were split transversely using a machete and endosperm surrounding embryo was excised from the split nuts using a clean knife (Molla et al., 2004).

Surface sterilization of endosperm extracts

Surface sterilization of endosperm extracts was carried out as described (Sukendah and Cedo, 2005) and immediately after excision of embryo extracts were placed in 20% (v/v) sodium hypochlorite commercial bleach (Jik regular bleach form Reckitt Benckiser East Africa Limited) for 20 min. They were subsequently sterilized for 1 to 2 min in 70% (v/v) ethanol and rinsed thrice with distilled water. The endosperms were washed for 10 min in 20% (v/v) bleaching agent and 70% (v/v) ethanol for 10 min under aseptic conditions. They were finally rinsed thrice in sterile distilled water. The embryos were then excised from endosperm, using sterile scalpel inside the clean bench. The scalpel was frequently dipped in ethanol and flamed to ensure thorough sterilization. Excised embryos were surface sterilized and cultured in liquid medium.

Culture medium

Embryos were cultured in a 50 ml glass vial bottles containing growth medium. The components of the media were 4.4 g/l MS medium (Highveld biological (PTY) limited); 1 g/l activated charcoal (Lobal Chemie) as detailed herein (Ashburner et al., 1995). Medium was prepared with or without 8 g/l agar (Duchefa Biochemie) resulting in semi-solid medium or liquid medium respectively. The pH was adjusted to 5.7 before autoclaving at a temperature of 121°C and 103.42 Kpa pressure for 15 min.

Culture conditions

Cultured embryos were cold treated at 4°C for three days after which they were incubated in darkness (Ranasinghe et al., 1999) at 29°C and 65% humidity for four weeks. After four weeks, the liquid medium was decanted out and the embryos irrigated with fresh medium. After germination, embryos were transferred to 300 ml glass jars containing 70 ml liquid medium and incubated in darkness for additional two weeks before transferring to light. All subsequent *in vitro* culture was carried out at a photoperiod of 15/9 h (dark/light). After six weeks, embryos that had started forming plumules were transferred onto semi-solid medium supplemented with the following plant growth regulators, naphthalene acetic acid (NAA) (0.5 mg/l), indole butyric acid (IBA) (7 mg/l) (Duchefa Biochemie) and 6-benzyl aminopurine (BAP) (0.5 mg/l) (Duchefa Biochemie.) Plantlets were transferred onto fresh medium every four weeks until they developed three to five leaves and three primary roots.

Effect of sterilization method of plant growth regulators

Experiments were carried out in which, the plant growth regulator stock solutions NAA (0.5 mg/l), BAP (0.5 mg/l) and 2,4-D (1.7 mg/l) were co-autoclaved with the medium and then embryos inoculated into the media after being dispensed into glass jars. Alternatively, the plant growth regulator stock solutions were filter sterilized using microfilters of 0.2 µM pore size and added to sterilized MS medium (Pech et al., 2007).

Effect of coconut water (CW) concentration on embryo development

Experiments were conducted whereby culture medium was sup-

Table 1. Effect of culture MS media on the embryo germination.

Media	Total number of embryos	Number of embryos germinated (%)	Mean number of days to plumule initiation	Mean number of days to radicle initiation
Semi-solid	30	27	33	-
Liquid	30	84	70	47

Table 2. Effect of sterilization method on cultured embryos to co-autoclaved and microfiltered sterilization of 2, 4-D PGR.

Treatment type	Germinated embryo (%)	Conversion to plantlet (%)	Mean number of days to plumule initiation	Loss due to contamination(%)
Co-autoclaving	30	40	70	54
Microfiltration	84	42	30	51

100% corresponds to 50 embryos initiated for each experimental set up.

plemented with coconut water (CW). During the process of embryo extraction, nuts (10 to 12 months old) were split open and the coconut water was collected. To determine the effect of coconut water on embryo development, coconut water was collected from 10 to 12 month old nuts. The coconut water was filtered through a sieve to remove any particulate matter. MS media was supplemented with varying concentrations of coconut water from 0 to 100%. Coconut water (CW) was co-autoclaved with MS media and addition of plant growth regulators were added after they were microfiltered to the media containing CW.

Effects of light exposure on embryo germination

Embryos were inoculated into culture medium as explained above; 15 embryos in glass jars were cultured under 15/9 h (dark/light) photoperiods while 15 others were cultured in darkness. Both sets of embryos were incubated at 29°C and 65% humidity for 4 weeks and then the germination percentage was determined.

RESULTS AND DISCUSSION

Effect of culture medium and culture conditions

Embryos cultured on liquid initiation medium resulted in a higher germination percentage (84%) as compared to semi-solid medium (27%) (Table 1). Therefore, liquid medium was preferred to semi-solid medium on the initiation stage until after germination when the plantlets were transferred onto semi-solid medium.

Growth of the embryos was observed by swelling although some of the embryos also showed other signs of growth such as cracking on the surface and colour change from cream to pale green when transferred to light. Embryos were considered germinated when the plumule sprouts and the radicle shows signs of emergence as reported by Danson et al. (2009). The embryos initiated in liquid medium were observed to grow by enlarging in size, and plumule formation was observed after four to five weeks. Embryos that were initiated on semi-solid medium had not been observed to grow by the 14th week. However, after being transferred to liquid medium, they were observed to swell after one week: by the 2nd week after transfer onto liquid medium, the embryos had started forming plumules and hence indicating that liquid medium was effective in initiation of cultures as compared to semi-solid medium. Embryos on liquid medium took 33 days to develop radicles as compared to 70 days on the semi-solid medium. These results indicate that liquid medium provides optimum uptake of nutrients by the germinating embryos and also improved gaseous exchange than in semi-solid medium has been reported earlier (Areza-Ubaldo et al., 2003).

Plant growth regulator supplementation

An experiment was conducted where; the 1.7 mg/l 2, 4-D plant growth regulator was co-autoclaved with the growth medium. Embryos cultured in this medium showed reduced germination rate of 30% as opposed to microfiltered plant growth regulator where 84% germination was recorded (Table 2). Plumule and radicle formed faster on growth plant growth regulators that were microfiltered than those that were co-autoclaved. Since plant growth regulators are thermolabile in nature, and may decompose during autoclaving, the use of filter-sterilized plant growth regulator proved more effective in embryo germination (Table 2). It also resulted in im-proved root and shoot development in the subsequent embryo culture procedure. The levels of contamination when using microfiltered plant growth regulators was 51% as compared to 54% when using co -autoclaved plant growth regulator. Systemic contaminants within the explants could have been the factor contributing to the elevated loss of embryos, as it was observed that during embryo excision, some nuts contained rotten embryos.

Table 3. Effect of coconut water on embryo development .

Media	Media component	Germination (%)	Mean number of days to swollen embryos
A	CW only	-	-
B	MS media constituted with 100% CW	27	21
C	MS media constituted with 25% CW	27	17
D	MS media constituted with 15% CW	60	14
E	MS media constituted with 0% CW	67	14

100% corresponds to 15 embryos set up for each media type.

Table 4. Effect of light exposure on embryo germination.

Incubation condition	Total embryos	Number of embryos germinated (%)	Number of embryos forming plumule only (%)
Light	15	33	13
Darkness	15	80	26

Effect of coconut water on embryo germination

Embryo germination was observed to be highest from MS medium without CW (67%), followed by MS medium supplemented with 15% CW where 60% germinated embryos was recorded (Table 3). On the contrary, coconut water has been reported to effectively enhance plantlet development (Nasib et al*.,* 2008; Mohammad et al., 2010), hence leading to its extensive supplementation in tissue culture medium. Coconut water was found to contain PGRs which included auxins mainly indole-3-acetic acid, cytokinins such as N^6-isopentenyladenine, dihydrozeatin and kinetin. Coconut water was also found to have gibberellins and abscisic acid ABA (Jean et al., 2009). Coconut water stimulates callus growth in date palm (*Phoenix dactylifera* L.), growth responded to CW concentrations from as low as 5% and reaching maximum growth at 10 to 15% depending on the cultivar (Al-Khayri, 2010).

Effect of light exposure on embryo germination

Incubation of embryos in darkness during the initial stages of culture (four weeks) resulted in improved germination (80%) as compared to the embryos incubated in presence of light (33%). Results are summarized in Table 4. During the study, it was postulated that incubating the embryos in darkness mimics the dark natural status of the embryo in the seed nut. This condition was reported to have been efficient in the ORSTOM and UP Los Banos protocols for *in vitro* coconut embryo culture (Batugal and Engelmann, 1998).

The general survival percentage of the embryos that had successfully converted into plantlets was observed to be 63%. Secondary roots development was observed in 33% of the surviving plantlets, while the remaining 69% had developed an enlarged haustorium with poorly developed secondary roots were also observed as illustrated in Figure 1a and b, respectively.

Conclusions

The results of this study indicate that embryo culture is a viable option for coconut micropropagation of the Kenyan variety of *C. nucifera* L. by initiating the embryos in liquid media supplemented with 2,4-D growth regulator and then incubating in darkness for four to six weeks until germination occurred. Germinated embryos should then be transferred onto semi solid media supplemented with a combination of BAP and 2, 4-D hormone for a 15/9 (dark/light) h photoperiod time. The plantlets should then be transferred onto fresh medium at monthly interval until they develop three to four leaves whereby they will be exposed to natural light in the green house but still in the plant growth medium for 1 to 2 weeks.

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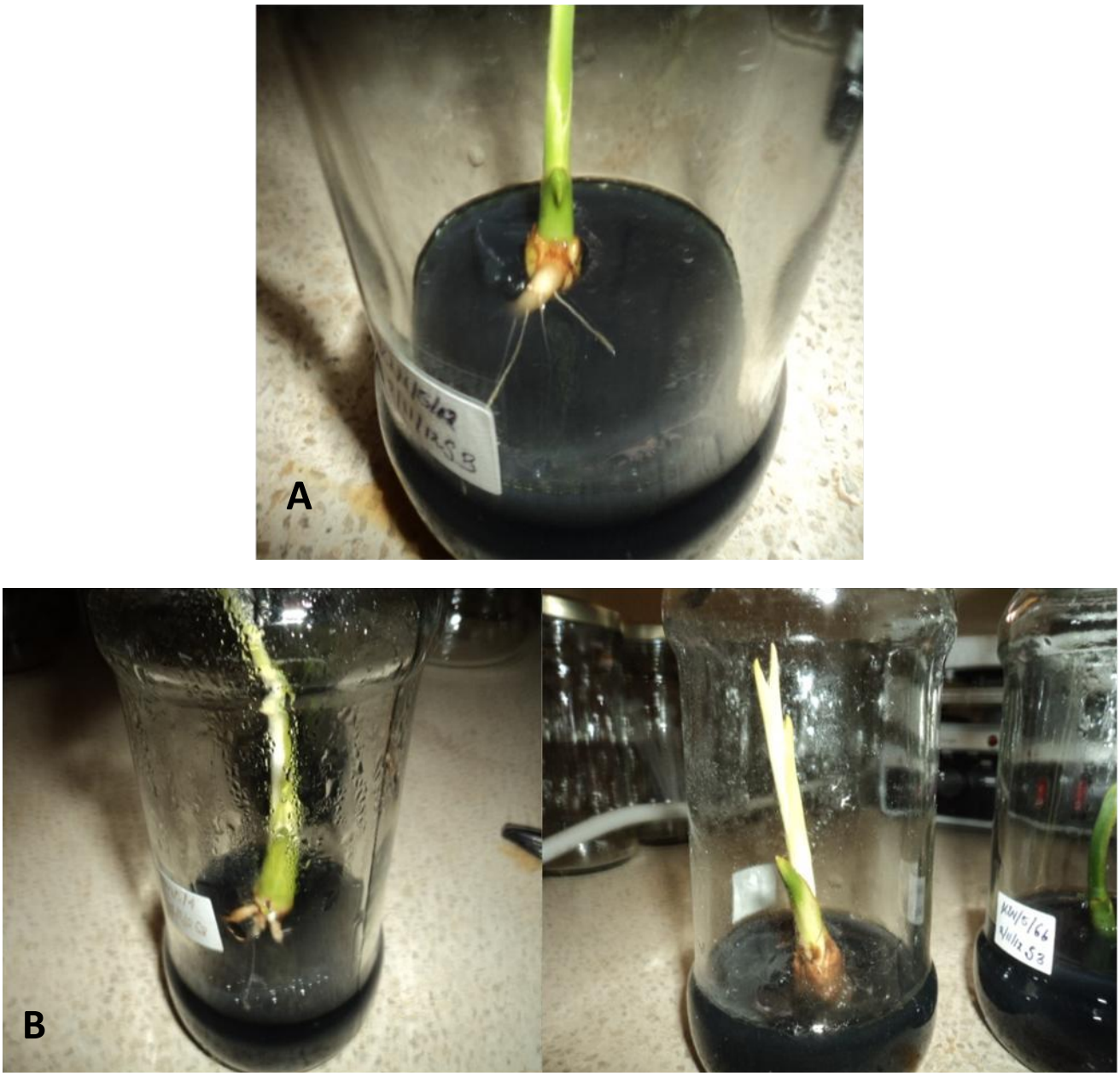

Figure 1. (a) Secondary root development**. (b)** Plantlets with enlarged haustorium and secondary root development.

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