DOI: https://doi.org/10.24297/jbt.v10i.9195

Response of haustorium tissues and coconut water in somatic embryos induction for the coconut palm (*Cocos nucifera* L.) variety PB121

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Abstract

The coconut tree (*Cocos nucifera* L.) is a fruit plant that contributes significantly to improved nutrition, food security, job creation, and household income in Benin. However, its production is suffering from the unavailability of certified seedlings. The present work aimed to optimize the propagation of coconut trees through the somatic embryogenesis technique. Zygotic embryos were cultured *in vitro* on Y3 medium supplemented with 0.7% Agar; 2.5 g/l activated charcoal, 5% sucrose to obtain haustorium, and the radicle explants for somatic embryogenesis. Three months after, callus and somatic embryos were induced from haustorium and radicle on medium Y3 supplemented with different doses of 2,4-D (0.3 and 0.35 mM) and coconut water (0, 50, 100 and 150 ml/l). 80% of callus was induced of induced with haustorium explant on Y3 medium supplemented with 0.7 mM 2,4-D. The combination of 2,4-D and coconut water resulted in the highest average number of somatic embryos with 59 and 63 embryos obtained respectively on Y3 medium enriched with 150 ml/l coconut water and supplemented with 0.3 mM and 0.35 mM 2,4-D. Using haustorium explant for mass propagation through somatic embryogenesis remains an exploring way for *in vitro* seedling of coconuts.

Keywords: *Cocos nucifera*, Seedling improvement, Somatic embryogenesis, Haustorium tissues

Introduction

Coconut palm (*Cocos nucifera* L.) is a fruit plant cultivated in many countries worldwide that generates important economic value to producers [1]. It contributes significantly for food security, employment generation, and household incomes [2]. In the last 15 years, the commercial importance of coconut has grown rapidly due to its high-value products such as packaged coconut water, coconut oil, coconut milk, coir-biodiesel, fibber derivatives for the automotive industry and horticulture ([3,4]. However, the loss of coconut trees due to phytosanitary threats, especially phytoplasma diseases such as lethal yellowing disease and aging of plantations constitutes the main constraint of coconut production [5]. In Benin, the multiplication and extension of selected (hybrid) coconut plants was carried out solely by the National Institute of Agricultural Research of Benin (INRAB). Unfortunately, the intensive felling of coconut seedlings and non-operation of the Centre over a long period have led to difficulties in making hybrid seedlings available to farmers for intensive production. To achieve this objective and meet the growing market demand, it is imperative to rejuvenate most of the plantations. Indeed, micropropagation by embryogenesis is being considered to overcome this shortcoming due to its exceptional multiplication capacity [1]. However, *in vitro* regeneration of coconut plants is limited by the low regenerative capacity of coconut tissues [6] (Fernando and Gamage, 2000). During the 1970s and early 1980s, various works were carried out on somatic embryogenesis of coconut from different types of explants: shoot apical meristem [7], endosperm ([8,9], leaves [10,11], roots [12], zygotic embryos [13,14]. Most of the early progress was made using rachis explants obtained from inflorescence ([15,16]. So far, the rates of embryogenic callus formation, somatic embryos and their germination remained very low [17]. In the mid-1990s, the Scientific Research Centre of Yucatan (CICY, Mexico), in collaboration with Wye College, tried to test different parts of the zygotic embryos,

including the plumula, which was most sensitive to the formation of embryogenic callus, somatic embryos and subsequent conversion to seedlings with low yield [18]. A mass propagation protocol has recently been developed, using plumula explants through the production of embryogenic callus and its multiplication, with yields estimated at 100,000 somatic embryos per explant [19]. This protocol, currently considered semi-commercial, suffers from enormous embryogenic callus multiplication steps and requires secondary somatic embryogenesis to maintain the regenerative capacity of the callus. Thus, in order to optimize yield and reduce the numerous embryogenic callus multiplication steps, different plant growth regulators and compounds such as abscisic acid and osmotic agents [20], brassinosteroids [21], gibberellic acid [22], polyamines [23] have been tested but without any real improvement. The plumula explant is collected from zygotic embryos directly excised from the nuts but other explants such as haustorium and radicle can be used for somatic embryogenesis of coconut after germination of zygotic embryos. Obtaining new explants capable of regenerating plantlets by somatic embryogenesis would significantly optimize somatic embryos yield. *In vitro* culture of these embryos still having their plumula after excision of these two explants would also have an advantage on the propagation of coconut trees because plantlets can be regenerated. Furthermore, for micropropagation plants, positive effects on callus induction, embryos formation, survival, and growth have been observed on medium supplemented with coconut water in several species such as spinach [24], date palm [25], hybrids of calanthe [26], banana [27] and olive [28]. Coconut water can therefore be used to optimize somatic embryogenesis in coconut. The present study aims to (i) determine the effect of 2, 4-D, and coconut water combinations on callus induction of haustorium and radicle tissues; (ii) evaluate the aptitude of haustorium and radicle tissues on callus conversion to somatic embryos.

Materials and Methods

Plant material collection

The plant material consisted of 12 to 14 months of mature fruits collected from PB121 hybrid (Yellow Dwarf Malay × Great West African) at Sèmè-kpodji station of the National Institute of Agricultural Research of Benin (INRAB). The experimentation was carried out on the PB121 hybrid variety because it is widely cultivated in Benin with an annual production of between 120 and 200 fruits by plant a year, and it is a better adaptation to high water deficits. It produces high yields and tolerates both acidic (3.5-4) and basic (8.0) pH and is resistant to the main pests.

Zygotic embryos extraction and disinfection

Fruits were selected and cut transversely with a cutlass, showing zygotic embryos, surrounded by the solid endosperm. The part of solid endosperm containing the embryos was excised by using a sterilized knife. Thereafter, the zygotic embryos were disinfected with 6% sodium hypochlorite (NaClO) solution for 20 min [29]. After three successive rinsing with sterile distilled water, the zygotic embryo explants were directly placed on the germination medium under a horizontal laminar flow hood. The haustorium and radicle from the germinated zygotic.

Induction of embryogenic callus

Rootlet and haustorium explants excised from germinated zygotic embryos and then grown for 90 days on callus induction medium with Y3 basal medium [30] supplemented with 7 g/L agar, 2.5 g/L activated charcoal, 5% sucrose, and different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and different volumes of coconut water (Table I). Coconut water was collected from immature shelled coconuts by drilling holes in two of the micropylons and sterilized at 100°C for 10 minutes with continuous stirring to precipitate proteins, fats, and other compounds. The precipitated water was filtered and stored at -20°C for medium preparation. The pH of the media was adjusted to 5.7 ± 0.1 and a volume of 25 ml was dispensed into each tube.

Table I: Different media for embryogenic callus induction

Culture Media	Basal Medium	2,4-D mM)	Coconut water (ml/L)
M1	Y3	0.5	0
M2	Y3	0.5	50
M3	Y3	0.5	100
M4	Y3	0.5	150
M5	Y3	0.6	0
M6	Y3	0.6	50
M7	Y3	0.6	100
M8	Y3	0.6	150
M9	Y3	0.7	0
M10	Y3	0.7	50
M11	Y3	0.7	100
M12	Y3	0.7	150

Induction and regeneration of somatic embryos

The embryogenic callus was transferred to four different somatic embryo induction media (medium II) with the same content as callus induction media with differences in 2, 4 -D, and coconut water contents (Table II). Finally, somatic embryos induced were transferred to a germination medium (medium III) consisting of Y3 basal medium supplemented with 6.10^{-3} mM of 2,4-D, 0.3 mM 6- benzylaminopurin (BAP), and 28.10^{-4} mM gibberellic acid (GA3), 5% Sucrose and 0.7 % agar.

Table II: Different medium of somatic embryos induction

Media tested	Basal medium	2,4-D (mM)	Coconut water (ml/L)
MIE1	Y3	0.3	0
MIE2	Y3	0.3	150
MIE3	Y3	0.35	0
MIE4	Y3	0.35	150

Study parameters

The variables measured in the study consist to:

- The percentage of explants producing callus: number of explants with callus per the total number of explants.

- The percentage of embryogenic callus produced: number of explants with embryogenic callus per total number of explants induced callus.

- The percentage of somatic embryos: number of somatic embryos produced per embryogenic callus counted.

Data collection and analysis

Callus and somatic embryos number were recorded each week for three months and data were analyzed using the test of regression. The continuous data were subjected to analysis of variance (ANOVA) to reveal differences between treatments and the test of comparison means was done using Tukey's or Duncan's test at the 5% threshold. XLStat version 14 software was used for all analyses.

Results

Effect of 2, 4. D / Coconut water combination on callus induction of different explant.

Combination 2, 4. D and coconut water in the media significantly influenced the callus induction rate ($p < 0.0001$) in haustorium explants used. Whatever the combination of 2, 4. D and coconut water in the media, there is no callus formed with radicle explants. 60% - 100% of haustorium explants were initiated callus on the media M2; M7; M9; M10; M11 and M12 after one month of culture (Fig 1). Of the three concentrations of 2,4-D used, the 0.7 mM concentration was the most suitable for callus induction with haustorium explants as it reproducibly produced callus (Figure 1).

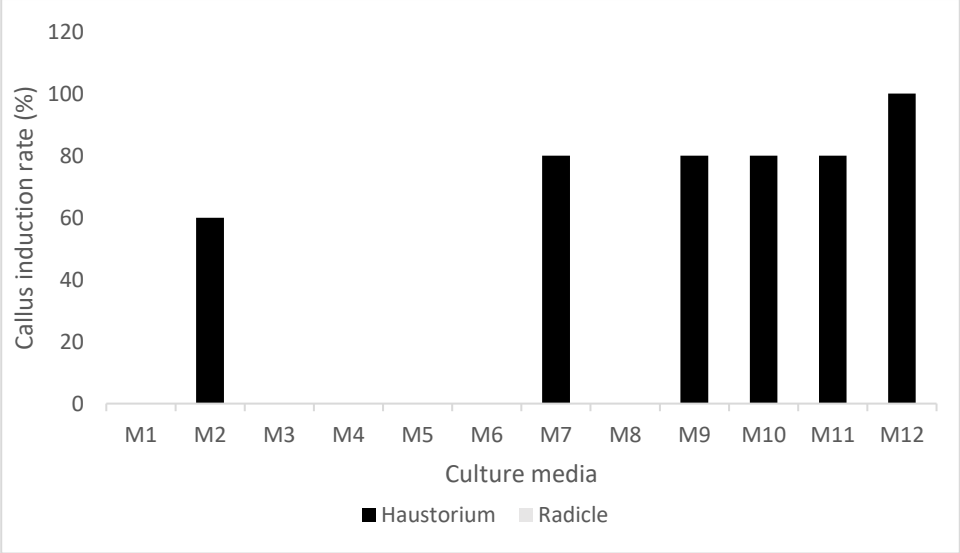


Figure 1: Influence of 2, 4-D and coconut water on callus induction rate of different explants.

The coconut water (150 ml/L) improved callus production. However, the interaction of the medium M12 with M7, M9, M10, and M11 was not significant on callus induction rate. (Table IV).

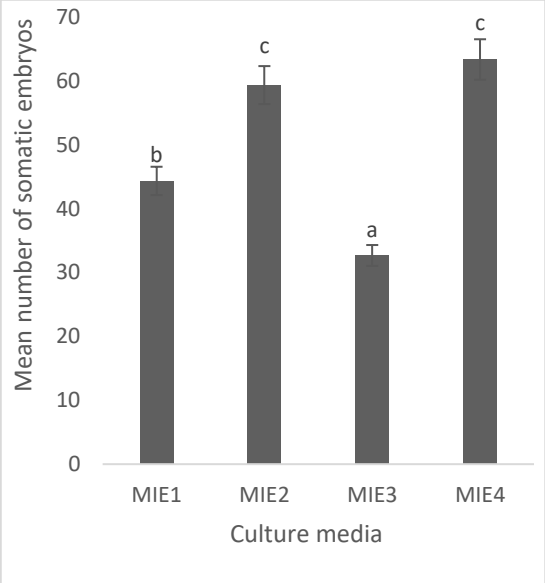
Table III: Differences in the growing medium factor

Interaction	Difference	Standardized difference	Critical value	Pr > Diff	Alpha (Modified)	Significant
M12 vs M8	0.500	3.772	2.376	0.013	0.431	Yes
M12 vs M1	0.500	3.772	2.361	0.011	0.401	Yes
M12 vs M3	0.500	3.772	2.344	0.010	0.370	Yes
M12 vs M4	0.500	3.772	2.325	0.008	0.337	Yes
M12 vs M5	0.500	3.772	2.303	0.006	0.302	Yes
M12 vs M6	0.500	3.772	2.277	0.005	0.265	Yes

M12 vs M2	0.200	1.509	2.245	0.659	0.226	No
M12 vs M10	0.100	0.754				No
M12 vs M11	0.100	0.754				No
M12 vs M7	0.100	0.754				No
M12 vs M9	0.100	0.754				No

Effect of 2, 4-D/coconut water combination on callus conversion to somatic embryos.

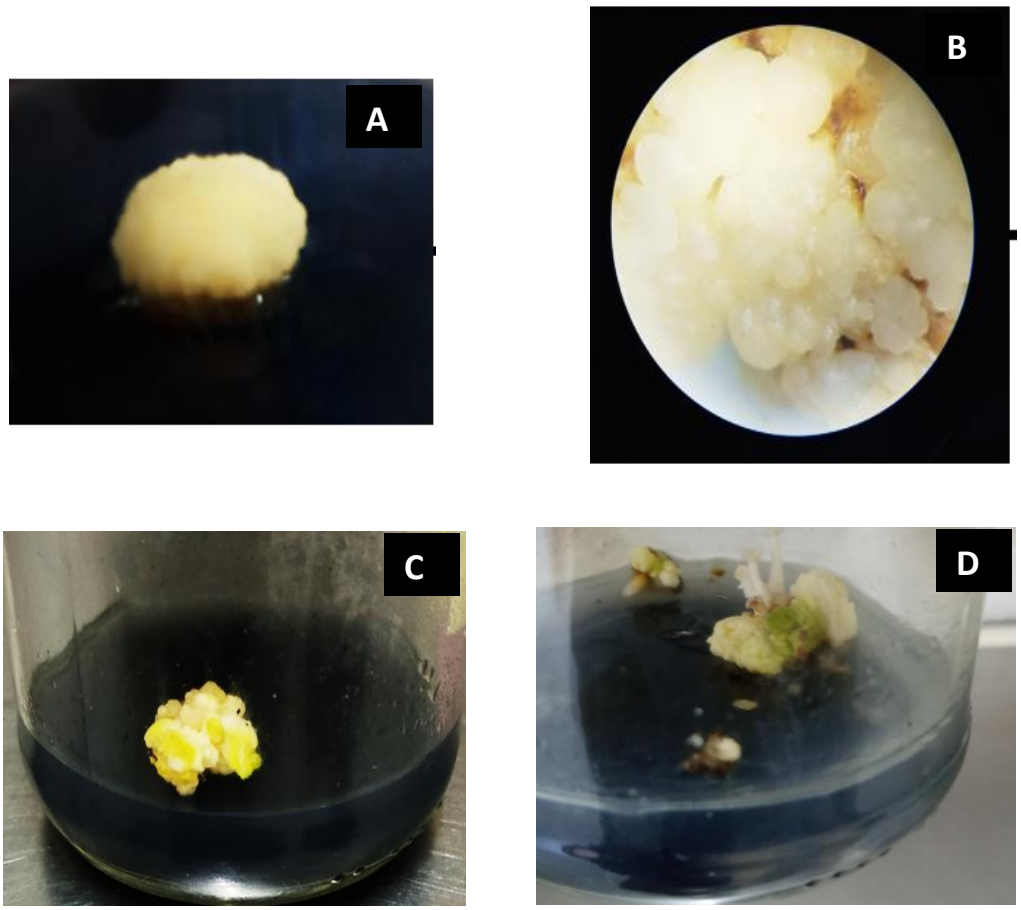
The number of somatic embryos induced per explant revealed that the concentration of 2,4-D and the addition or not of coconut water to the culture medium significantly influenced the number of embryos induced per callus ($P < 0.0001$). The lowest number of somatic embryos was induced on the MIE3 medium (31) and the highest on the MIE4 medium (67). The lower concentration of 2,4-D (0.3 mM) induced the highest average number of somatic embryos (44,33) while a higher concentration of 2,4-D (0.35 mM) recorded about 32,66 somatic embryos. Adding 150ml/L coconut water significantly improved the number of embryos. An average of 59,33 somatic embryos per explant was noted on the media MIE2 while 63,33 somatic embryos were induced on MIE4 medium (Fig 2). A significant difference was recorded on the average number of somatic embryos induced between the media without coconut water and those containing coconut water.



M1= Y3 + 0,3 mM 2,4-D; M2= Y3 + 0,3 mM 2,4-D + 150ml/L coconut water; M3=Y3 + 0,35 mM 2,4-D; M4 =Y3 + 0,35 mM 2,4-D + 150ml/L coconut water

Figure 2: Number of somatic embryos induced in the different mediums used.

The haustorium explants have shown the only explant susceptible to embryogenic callus induction. One month after culture the haustorium explants induced callus that was converted to embryogenic callus after the formation of translucent structures. The callus was characterized by compact, pearly white globular structures after three months of culture (Fig 3A). The callus with the globular structure was subcultured on a somatic embryo induction medium and developed torpedo-shaped embryos (Fig 3B). The torpedo-shaped embryos were germinated and started to green up (Fig 3C).



(A) Embryogenic callus obtained after 90 days of culture on medium I; (B) Somatic embryos obtained on medium II; (C) Germinated embryos (in green color) obtained on medium III and the (D) first step of plantlet regeneration.

Figure 3: Morphological changes in haustorium explants during somatic embryogenesis

Discussion

In vitro propagation of coconut using plumule explants was reported by [19]. Plumule explants produced the embryogenic callus and their multiplication is currently the only method for propagating the progenies of selected coconut trees. This technique can be optimized if other explants can be used for regenerating plantlets. Thus, this work aimed to determine whether haustorium or radicle explants can be used for the somatic embryogenesis of coconut. In the first trial, the efficacy of 12 different treatments (different combinations of 2,4-D and coconut water) to induce callus with embryogenic structures was evaluated on haustorium and radicle explants of the hybrid PB121. After three months of culture, only haustorium explants induced upper than 80% embryogenic callus whereas plumule and radicle explants induced embryogenic callus at a respective rate from 40% to 60% [1] and lower than 20% [31]. The lowest rate of callus induced with radicle explants may be related to the absence of meristematic cells in the explants. Coconut water used during the callus initiation did not significantly improve the rate of embryogenic callus but it did optimize callus mass. The best results were obtained by combining 2,4-D (0.7 mM) with 150ml/L (coconut water) in the M12. Similar results were obtained in date palm using coconut water [25]. The average number of embryos per callus was 38 embryos when the medium was devoid of coconut water and 61 embryos per callus when the medium was enriched with 150ml/l coconut water. This means that coconut water optimized the conversion of embryogenesis callus to somatic embryos. This average is higher than those obtained with plumule explants (3.8-10.8 embryos) per callus when

explants were pre-treated with brassinosteroids [21] and (5-16 embryos) when gibberellic acid is added to the embryo's induction medium [22]. The increase of embryos number obtained after the addition of coconut water to the somatic embryo's induction medium can be explained by the presence of heavy metal ions (iron, aluminum, zinc, copper, or selenium) and osmotic compounds (mannitol and sorbitol). Indeed, high concentrations of salt, heavy metal ions, or osmotic stress positively influence the induction of somatic embryos in various plant species [32]. The presence of abscisic acid in coconut water is also a factor for improving the number of somatic embryos induced as described by [6] who reported that decreasing the concentration of 2, 4-D and adding abscisic acid to the medium improved the number of somatic embryos induced in coconut. On the germination medium, by decreasing the concentration of 2,4-D in the somatic embryos induction medium and adding BAP (0.3 mM), and GA3 (28.10-4 mM) to the medium allowed the germination of somatic embryos [33]. The results confirm the possibility of mass propagation of coconut by somatic embryogenesis from haustorium explants.

Conclusions

Through this study, it is demonstrated that haustorium tissues can express embryogenic capacities. The embryogenic callus was developed after three months of culture and was converted into embryos. The highest number of embryos was recorded on the medium supplemented with 0.3 mM 2, 4-D but combined with 150ml/L coconut water. Coconut water optimized the number of somatic embryos by almost 61%. Low concentrations of 2, 4-D, BAP, and GA3 in the medium improve the germination of somatic embryos. Using haustorium explant for mass propagation through somatic embryogenesis remains an exploring way for coconuts multiplication.

Conflits of Interest

The authors declare that they have no conflict of interest in the publication.

Acknowledgments

The authors acknowledged the staff of the Central Laboratory of Biotechnology and Breeding Plant of the University of Abomey-Calavi for the technical support. We also thank Dr. Fayalo, the head of CRAPP at Sèmè-kpodji station of the National Institute of Agricultural Research of Benin (INRAB)at this period for his full collaboration during the identification of the variety and the collection of coconut ripe fruits.

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