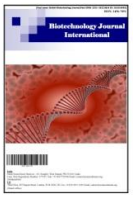

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Effects of Surface Sterilization Protocol and Haustorium Removal on Germination and Growth of Hybrid PB121 Coconut Palm (*Cocos nucifera* L.) Zygotic Embryos Cultured *in vitro*

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AA and FE designed the study. Author SSH performed the statistical analysis. Authors ERS and AA wrote the protocol and the first draft of the manuscript. Authors CA improve the manuscript. Author ERS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aims to identify the best surface sterilization and evaluate the effect of haustorium suppression on *in vitro* germination of coconut palm (*Cocos nucifera* L.) zygotic embryos.
Study Design: Survival rate and contamination rate of zygotic embryos after different surface sterilization treatments, regeneration rate and organogenesis through the number of leaves and the length of shoots after haustorium suppression were determined. For data processing, the Analysis of Variance was used to compare the means which were separated according to Tukey test (P = 0.05).
Place and Duration of Study: Coconut fruits (hybrid PB121) were collected 12 to 14 months after

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controlled pollination from CRAPP (Centre de Recherches Agricoles Plantes Pérennes), station of Sèmè-kpodji in Benin. Experiments were done in Central Laboratory of Plant Biotechnology and Plant Improvement, University of Abomey-Calavi and conducted from june to december in 2019.
Methodology: For the zygotic embryos surface sterilization, four treatments combining three concentrations (3%, 6% and 15%) of commercial bleach (Javel la Croix© containing 12° active chlorine) and immersion durations (5 min, 10 min and 20 min) were tested and the survival rate were determined for each treatment after two months culture. The zygotic embryos were then divided in two sets (haustorium excised embryos set and the whole embryos set) and cultured in modified Y3 medium supplemented with 7 g L^{-1} agar, 2.5 g L^{-1} activated charcoal, 5% sucrose, 6.10^{-3} mM 2.4 D (2.4-dichlorophonoxyacetic acid), gibberellic acid and 0.3 mM BAP(6-benzylaminopurine). After five months culture, the regeneration rate, the number of leaves and the length of shouts were recorded.
Results: The high survival rate (80%) was obtained with 6% of bleach and 20 min for the immersion duration without pre-disinfection. The suppression of haustorium have significantly increased the number of leaves (4.3 ± 0.02) and the length of shoots (16.2 ±0.7cm) compared to the whole zygotic embryos.
Conclusion: This protocol can help to ensure better surface sterilization of zygotic embryos before their *in vitro* culture and the development of vigorous plantlets in order to improve the slow growth of plantlets, when transferred to the greenhouse or field.

Keywords: Cocos nucifera; zygotic embryos; in vitro germination; disinfection protocol improvement; slow growth reduction; Benin.

1. INTRODUCTION

The coconut palm (*Cocos nucifera* L.) is an economic value species cultivated in nearly 90 countries on 12 million hectares [1- 3]. Its commercial importance has increased very rapidly over the last fifteen years [4-6]. In Benin, coconut palm is not highly valued despite the existence of an important export market in Nigeria. The multiplication of hybrid coconut genotypes (elite) at the Sèmè-Kpodji station of the National Agricultural Research Institute of Benin has led to requests for plants from countries such as Nigeria, Senegal, Niger and Lebanon. Unfortunately, exchange of germplasm is difficult because of the high risk of introducing pests and diseases between countries if whole nuts are exchanged, and also because of the high cost of transporting whole nuts [7,8]. To overcome this deficiency, protocols for the *in vitro* culture of zygotic embryos have been advanced in the developing countries. The efficiency of these protocols is feasible by the successful germination of the embryos, their conversion into seedlings and their acclimatization and transfer in *ex-vitro* conditions. The first attempts to isolate and culture zygotic embryos from coconuts back to the 1950s [9,10]. However, it took another decade before *in vitro* seedlings could be regenerated and converted into viable plants [11,12]. The media used for embryo germination and seedling growth varied in the various studies undertaken. Although, many types of culture media have been used for the germination and growth of zygotic embryos, the most commonly used is the Y3 medium developed by Eeuwens [13]. Compared to MS medium [14], the ammonium and nitrate nitrogen content in Y3 medium is half, while microelements such as iodine, copper and cobalt are ten times more concentrated. These changes better reflect the conditions of a coastal soil, a favorable habitat to coconut germination [15]. The survival rate of *ex vitro* seedlings has been improved by the suppression of haustorium on embryos having developed a 2 to 4 cm gemmule [16]. The impact of haustorium suppression has only been evaluated during the acclimatization process of coconut seedlings and could also improve the process of coconut zygotic embryo culture in *in vitro* conditions. Despite these findings, the successful protocol for surface sterilization during the establishment of coconut zygotic embryos and the role of haustorium in plantlets growth are not clearly identified. For this purpose, the objectives of this present work are (i) toidentify the concentration of bleach and immersion duration that improve surface sterilization of zygotic embryos during their initiation and (ii) to study the impact of haustorium removal on growth of coconut zygotic embryos.

2. MATERIALS AND METHODS

2.1 Plant Materials

The plant material consisted of ripe fruits 12 to 14 months after controlled pollination of hybrid PB121 (Malay Yellow Dwarf × Great West Africa) from the Sèmè-kpodji station of the CRAPP (Centre de Recherches Agricoles sur les Plantes Pérennes) used as a source of zygotic embryos.

2.2 Sampling and Disinfection of Explants

The ripe fruits were selected and soaked in water containing 15% bleach concentration during 60 min for their disinfection. After that, the cleaned ripe fruits were cutted transversely with a disinfected machete, revealing the embryo, surrounded by the solid endosperm. The cylinder of solid endosperm containing the embryo was excised using a sterilized corkscrew. Immediately after excision, four different disinfection treatments were tested using sodium hypochlorite (NaClO) provide by the commercial bleach (Javel la Croix©) containing 12°active chlorine (Table 1). After disinfection, the zygotic embryos were excised from the endosperm cylinders under laminar flow and were placed directly on the germination medium. After one month culture, the germinated embryos were divided into sets. The haustorium of zygotic embryos were excised from one set while the whole (non-excised) germinated embryos are using in second set as control test.

2.3 Preparation of Culture Media for Direct Organogenesis

The basic medium Y3 modified by Weerakoon et al [17] and Sáenz et al [18] was used as germination medium for zygotic embryos. The sprout formation medium was prepared using the Y3 medium formulation, with the addition of 7 g/L agar, 2.5 g/L activated charcoal, 5% sucrose, 6.10-3 mM 2,4-D and 0.3 mM BAP and a volume of 25 ml was dispensed into each of the 150 ml jars for tissue culture. The growth medium was prepared using the Y3 medium formulation with the addition of 2.5 g/L activated charcoal and 5% sucrose and a volume of 100 ml was dispensed into each of the 500 ml jars for tissue culture. The pH was adjusted to 5.75 ± 0.1 for all of media which were then autoclaved for 20 minutes at 121°C.

2.4 Growing Conditions

The excised embryos were grown on the germination medium for 30 days in total darkness at 27 ± 2°C. After one month culture, the embryos were transferred to sprouting medium for 60 days under a 12 h photoperiod at 27 ± 2°C. Finally, the shoots were transferred to growth medium for 60 days under a 12 h photoperiod at a temperature of 27 ± 2°C for seedling formation.

2.5 Data Analysis

Analysis of variance was performed using XLStat version 14 software. The treatments means were separate by the Tukey test at 5% significance level.

3. RESULTS

3.1 Survival Rate of Zygotic Embryos after Different Disinfection Treatments

The zygotic embryos survival is highly influenced by the concentration of bleach, the immersion duration and the combination of pre-disinfection and disinfection. The high survival rate (80%) is recorded by the treatment using only disinfection phase with 6% of concentration of bleach and 20 Min immersion duration (T2) while the less survival rate (20%) is presented with T3 treatment when the embryos are exposed for pre-disinfection with 3% concentration of bleach with 5 Min immersion duration and 15% of bleach and 10 Min for immersion in second time disinfection phase (Fig. 1 A). Significant difference in the survival rate is noted between T2 and T3. In contrast, no difference is noted with the treatments T1 and T4 which presented the same survival rate (50%). For the contamination rate (Fig. 1 B), the high level is recorded by treatment T2 (only one time disinfection with 6% concentration of bleach) while the lowest on T3 treatment (double disinfection with 15% concentration of bleach).

3.2 Effect of Haustorium Suppression on the Organogenesis of Germinated Zygotic Embryos

Shoot length is influenced by the type of explant used ($p<0.0001$). It varied between 14.5± 0.2 cm and 16.2 ± 0.7 cm respectively regenerated by whole zygotic embryos and haustorium excised embryos (Fig. 2).

Haustorium suppression significantly influenced the number of leaves per vitroplant ($p=0.019$). The lowest number of leaves (2.4 ± 0.09) was regenerated by whole embryos while the excised embryos regenerated plantlet with the highest number of leaves (4.3 ± 0.02).

Table 1. Different disinfection treatments tested for zygotic embryos surface sterilization

Treatments	Concentration of bleach (NaClO) (%)		Immersion duration (min)	
	Pre-disinfection	Disinfection	Pre-disinfection	Disinfection
T1	3	6	5	20
T2	-	6	-	20
T3	3	15	5	10
T4	-	15	-	10

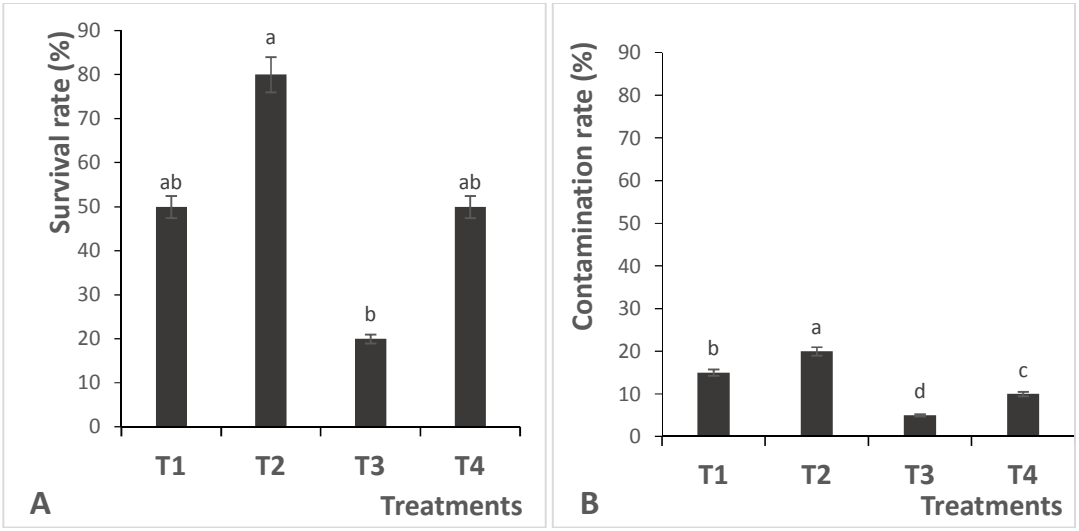

Fig. 1. Effect of bleach concentrations and immersion durations on the zygotic embryos survival (A) and contamination (B) after one month culture

T1= 3% NaClO for 5 min (pre- disinfection step) + 6% NaClO for 20 min (disinfection step); T2=6% NaClO for 20 min (disinfection step); T3= 3% NaClO for 5 min (pre-disinfection step) + 15% NaClO for 10 min (disinfection step); T4= 15% NaClO for 10 min (disinfection step)

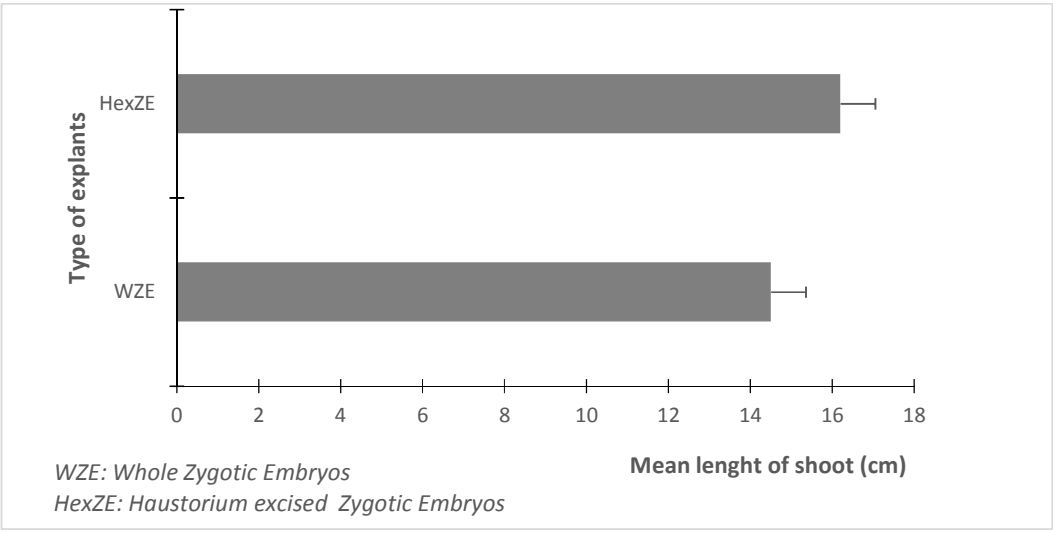

Fig. 2. Average length of regenerated shoots as a function of explant types

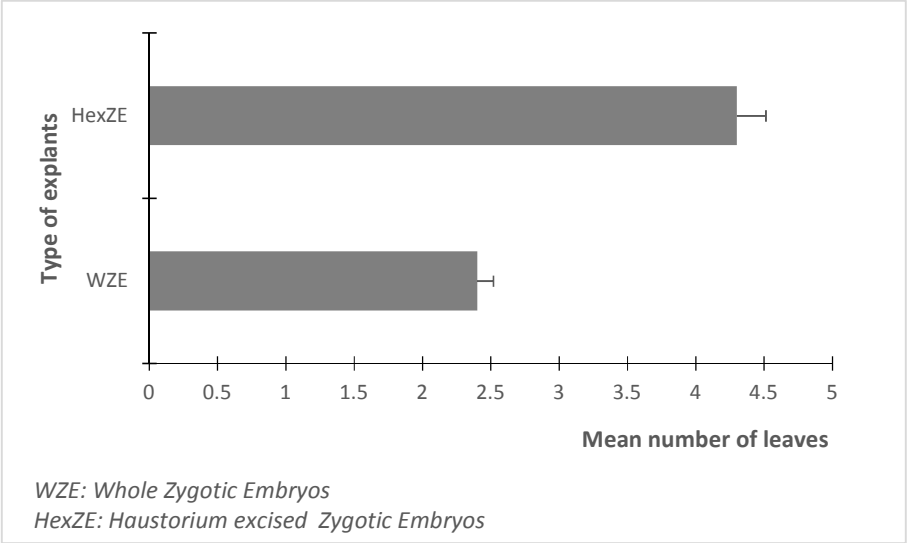

Fig. 3. Average number of leaves produced per vitroplant as a function of explant type after 5 months of culture

After 15 days culture, the embryos have initiated the plumule and the emergence of the radicle was noted (Fig. 4B). At the end of the first month of culture on the germination medium, the embryos had produced gemmules and a well-developed main root. On the shoot formation medium, sprouting is optimized in embryos that their haustorium were removed (Fig. 4C') compared to whole embryos (Fig. 4C). After two weeks of culture, the shoots are then transferred to growth medium for another two months, after which acclimatisable plantlets are obtained (Figs. 4D and 4D'). After five months of culture, the plantlets were removed to their container and cleaned in water (Fig. 5 A) and transferred in polyethylene bags containing sterilised soil (Figs. 5 B and B') for their acclimatization in greenhouse.

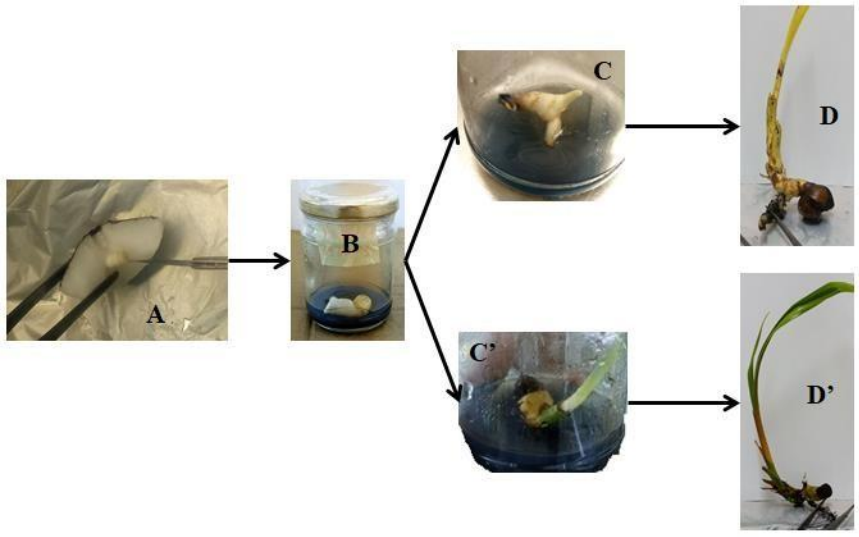

Fig. 4. Stages of coconut tree development during *in vitro* culture of zygotic embryos
(A) embryo extracted from the nut; (B) embryo having initiated plumule; (C) whole embryo having regenerated root and gemmule; (C') haustorium excised embryo having regenerated shoot; (D) seedling regenerated from whole embryo; (D') seedling regenerated from haustorium excised embryo

Fig. 5. Acclimatization of coconut plantlets in greenhouse after five months of culture
A: cleaning of plantlet before planting in polyethylene bag containing sterilised soil. B: plantlet providing to the whole embryo, B': plantlet providing to haustorium excised embryo

4. DISCUSSION

The high demand for coconut (*Cocos nucifera* L.) seedlings necessitates clonal propagation of elite materials [19] which could be increased by the large potential of *in vitro* tissue culture. This process is currently used for the safe exchange of coconut palm germoplasma between countries through the use of zygotic embryos [20,21,12]. Nevertheless, explants surface sterilization is the main challenge for the success of *in vitro* culture establishment. In addition, slow growth of plantlets providing to *in vitro* culture during the acclimatization phase is specially the limited factor for coconut species [20]. Thus, the present protocol tested in this study is to optimize the disinfection protocol of coconut zygotic embryos and to reduce plantlets growth duration after germination. The first trial evaluates the influence of four different disinfection treatments on the germination and regeneration of zygotic embryos. These treatments differed in the presence or absence of pre-disinfection (3% NaClO) and in the concentration of sodium hypochlorite (6% or 15% NaClO) using for disinfection. Treatment without pre-disinfection and with a sodium hypochlorite concentration of 6% gave the best results. The high concentration of sodium hypochlorite (15%) inhibited embryo germination. In addition, pre-disinfection of the embryos with sodium hypochlorite (3%) increased the rate of infection and this can be explained by the low concentration of NaClO which, unable to kill the microorganisms, would provide spore-forming conditions making these microorganisms difficult to eliminate later. These results are contrary to those obtained by Adkins et al. [12] who propose a pre-disinfection with 3% NaClO and those of [22] who propose a disinfection with 20% sodium hypochlorite (NaClO).

From the results obtained after evaluation of the effect of haustorium removal on the *in vitro* development and growth of embryos, it is noted that excised embryos compared to whole embryos regenerated larger and more leaves of seedlings after 5 months of culture. The number of leaves per seedling regenerated after 5 months of culture from excised embryos is at least 4. Similar results were obtained by Molla et al. [23] on three coconut varieties after 6 months of culture. According to Engelmann et al. [7] seedlings can be acclimatized when they display 3 to 4 unfolded green leaves and reach the acclimatization stage 6 to 7 months after initiation on MS basal medium. However, the use of Y3 medium combined with the suppression of

haustorium resulted in acclimatized seedlings after five months of culture. This suppression thus improved the shoots and leaves formation of the coconut. The embryo is more dependent on the nutrients in the culture medium. The absence of the radicle on the first step of regeneration also delays roots formation and permit to the embryos to use all the resources available for the development of shoots. Roots formation is obtained when the shoots regenerated by the haustorium excised embryos are transferred to the growth medium, which is the Y3 medium devoid of growth regulator. This study has thus proved the effectiveness of the Y3 medium developed by Eeuwens et al. [13] for the culture of coconut zygotic embryos which better reflects the conditions of a coastal soil, a habitat favourable to coconut germination. In addition, it demonstrated the advantage of the suppression of the haustorium on the development and *in vitro* growth of coconut zygotic embryos in term of reduction of seedling production duration and well growth of plantlets.

For further approaches, others explants source such as shoot tips and inflorescence which are currently used to micro-propagate coconut with highly efficient [24] will be tested to compare their performance with somatic embryos.

5. CONCLUSION

This study, which is part of an optimization of coconut zygotic embryos *in vitro* culture, has led to conclusive and exploitable results. The zygotic embryos were successfully disinfected with sodium hypochlorite at a dose of 6% without pre-disinfection. Furthermore, the removal of haustorium carried out significantly improved the process of direct organogenesis in the coconut tree. This protocol can help to ensure better surface sterilization of zygotic embryos before their *in vitro* culture and the regeneration of vigorous plantlets in order to improve the slow growth of plantlets, when transferred to the greenhouse or field.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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