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Sterilization Method for Reducing Microbial Contamination and Phenolic Compounds present in Coconut (*Cocos Nucifera* L.) Leaf Culture

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ABSTRACT: The aim of the study was to investigate efficient sterilization methods for reducing microbial contamination and phenolic compound of coconut (*Cocos nucifera* L.) leaf culture. The non-chlorophyllous immature coconut leaves explant used were taken from unopened spear leaves tissue of the coconut seedling, from the apical growing regions close to the meristem of the palm sucker of about 15 months old. Murashige and Skoog (MS medium) supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) at concentration of 30 mg/L and 6-Benzyl amino purine (BAP) at concentration of 1.5 mg/L were used for morphologic responses. Mercuric chloride, ethanol, calcium hypochlorite and sodium hypochlorite were usedto sterilize the explants at concentrations of 0.1 %, 0.2 %, 0.3 % and 0.4 % and 70-95 % of ethanol for 5 minutes. This was followed by rinsing the explants with distilled water four successive times. The sterilized explants were inoculated on MS media and were incubated at 25±2°C in the dark. Results showed that contamination was less in the cultures, particularly in explants sterilized with 70 % ethanol. Although, all the steriliants did well, but ethanol is more preferable than the rest steriliants, in solving both problems.

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Keywords: *Cocos nucifera*, immature leaf, Sterilizing agents, Contamination, Browning.

Coconut palms play an important role in agriculture and tourism. In the tropical Pacific Islands, coconut is grown worldwide in about 14.4 million, hectare of which 87 % are located in the Asia Pacific region (Foale, 2003). This palm grows in more than 80 countries which can be grouped into eight distinct coastal/oceanic regions on four continents (Howard *et al.*, 2001). In the tropics, most parts of the plant are used for food, oil production, construction material, source of energy, and cosmetics (Campbell *et al.*, 2000). Coconut cultivars are generally classified into the tall and dwarf types. The tall palm can grow at a rate of more than 50 cm annually when young and flowers 6 -10 years with an economic life span of 60-70 years. The dwarf type can grow at a rate of 15 to 30 cm annually, with a productive life span of 30 - 40 years but usually start flowering in the third year (Bourdeix *et al.*, 2005). Apart from their usually short height, most of the dwarfs also play an important role in genetic improvement programs. However, this variety is suffering from drastic production constraints, including pests and diseases. In addition, a number of aging coconut plantations are now being uprooted in order to make way for the planting of new ones (Warner *et al.*, 2007). Therefore, there is an urgent need to implement efficient coconut germplasm development via *in vitro* technique of tissue culture that allows germination and conversion into disease free plantlets in a controlled environment. Sterilization is the process of making explants free of contamination before establishment of cultures. Various sterilization agents are used to decontaminate the tissues. The disinfectants usually used are sodium hypochlorite, calcium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide and silver nitrate (Himabindu *et al.*, 2012). Clonal propagation through

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tissue culture is one of the most widely accepted tools for large scale production of disease free and genetically improved plantlets (George *et al.,* 2008). One of the greatest problems in micro-propagation is contamination with fungi and bacteria. Therefore, sterile conditions are essential for successful tissue culture procedures. In order to maintain an aseptic environment, all culture wares, media and instruments used in handling tissues must be sterilized (Goswami and Handiqu, 2013). Apart from contamination, another major problem in coconut leaf tissue culture is phenolization. This term is described as the blackening and browning of explants shortly after inoculation. It is caused by the release of phenolic compounds into the media where they are immediately oxidized by peroxidases (Pirtilla *et al.,* 2008). Thus, the objectives of this study were to determine the best concentration of sterilant to be used in leaf explants sterilization and toidentify the sterilants that can reduce contamination and phenolic compounds without affecting the culture.

MATERIALS AND METHODS

Plant material: The immature coconut leaf explant was obtained from coconut seedling of about 1-2 years old palm, unopened spear leaves tissue taken from the apical growing regions close to the meristem of the palm sucker were used. The explant was harvested from nursery field of Nigerian Institute for Oil Palm Research (NIFOR) Benin City, Edo State, Nigeria.

Preparation of culture medium: The basal medium used for this experiment was the MS medium (Murashige and Skoog, 1962). The basal medium was supplemented with growth hormone 2,4-Dichlorophenoxyacetic acid (2,4-D) at a concentration of 30mg/L and 6- Benzyl amino purine (BAP) at a concentration of 1.5 mg/L for promoting the morphologenic responses, that could have resulted in callus formation with the addition of these hormone. The medium contained 35g/L sucrose plus 2.5 g/L activated charcoal; the pH of the medium was adjusted to 5.7 and solidified with 6 g/L agar. The medium was autoclaved at 121°C for 25 minutes and allowed to cool before use. All experiments were replicated three times.Averages of all the three trials were taken and arranged by completely randomized design (CRD). Data from *in vitro* cultures were subjected to ANOVA and means.

Sterilization and inoculation of explants: The leaf explant (coconut seedling) were chopped or reduced in size, washed with Tween®20 due to dirt and debris from the soil, rinsed under running tap water for 5-10 minutes, before taken to laminar flow chamber for proper sterilization and inoculation. Four sterilants and concentrations namely Mercuric chloride, Sodium hypochlorite, calcium hypochlorite all at 0.1, 0.2, 0.3 and 0.4 % and Ethanol at 70, 85, 90 and 95 % concentration were used for the study to standardize the best sterilization protocol for *in vitro* culture of coconut leaf (Chengalrayan *et al.,* 2005). The leaves were then introduced into the various sterilants solution for 5 minutes with continuous stirring. Later, the disinfectant solution was discarded and explants were rinsed three times with sterile distilled water. Thereafter, the sterilized explants were inoculated on the basal media. Cultures were incubated in dark room at 25±2 ºC. Contaminated cultures were picked and counted on weekly bases and necrotic cultures were also observed and recorded. All trials were replicated three times.

Statistical analysis: The data generated was subjected to ANOVA in complete randomized design using R-software at 5 % level of significance.

RESULTS AND DISCUSSION

The effects of different sterilant on contamination of coconut leaf culture have been presented in Table 1. Among the various concentration (0.1, 0.2, 0.3 and 0.4) % of Mercuric chloride used, 0.4% had the less contamination value with a range of 0.10- 6.40, compared to 0.1 % value which range from 0.06±0.66 - 22.20±0.22. 0.1 % Mercuric chloride was highly significant in terms of contamination compared to other Mercuric chloride concentrations used. Also 70 % Ethanol had the less contamination value with the range of 0.03±0.33 - 0.10±0.00, while other concentrations had a value range of 0.10±0.00 - 13.43±0.33. Calcium hypochlorite, 0.4 % concentration had less contaminated cultures with 0.06±0.66 - 13.50±0.00. While 0.1 % had the highest contamination value with 20.26±0.66 - 33.40±0.33, followed with other concentrations. At 0.3 % Sodium hypochlorite less, contamination was observed with value range of 0.03±0.33 - 6.43±033. 0.1 % had a higher range of 13.13±0.33 - 13.46±0.66, compare with others. There was no browning in all the cultures from concentrations 0.1 – 0.4 % of all the sterilants in the first week (Table 2). Slight browning was observed in mercuric chloride at 0.4 % concentration and ethanol at 95 % concentration after two weeks of culture. At week three slight browning was observed in mercuric chloride at 0.1 % - 0.4 %, ethanol at 95 %. Calcium hypochlorite at 0.3 % and 0.4 % while in sodium hypochlorite cultures were free from browning. At four-week mercuric chloride at 0.1 % to 0.4 % shows high level of browning, while ethanol 70 %, 85 % and 90 % shows no browning but 95 % shows slight browning.

Table 1: Effect of different sterilant on contamination of coconut leaf culture explants

Sterilant	**Concentration (%)**	**Contaminated cultures (%)** 1 WACI	2 WACI	3 WACI	4 WACI
Mercuric chloride	0.1	0.06±0.66	13.23±0.33	22.2±0.22	22.13±0.33
	0.2	0.10±0.00	6.50±0.00	6.50±0.00	13.26±0.66
	0.3	6.53±0.33	13.43±0.33	6.50±0.00	6.46±0.66
	0.4	6.40±0.00	0.10±0.00	0.13±0.33	0.01±0.00
Ethanol	70	0.03±0.33	0.06±0.66	0.06±0.66	0.10±0.00
	85	0.10±0.00	6.60±0.00	6.40±0.00	13.23±0.66
	90	6.46±0.66	0.06±0.66	0.13±0.33	0.06±0.66
	95	0.23±0.33	0.10±0.00	6.50±0.55	13.43±0.33
Calcium hypochlorite	0.1	20.26±0.66	22.2±0.33	33.4±0.33	33.36±0.66
	0.2	6.46±0.66	13.36±0.66	13.26±0.66	26.26±0.66
	0.3	6.53±0.33	22.40±0.00	33.40±0.00	22.20±0.00
	0.4	0.13±0.33	0.06±0.66	13.50±0.00	13.30±0.00
Sodium hypochlorite	0.1	13.26±0.66	13.16±0.66	13.13±0.33	13.46±0.66
	0.2	0.03±0.33	0.06±0.66	22.06±0.66	0.03±0.33
	0.3	0.03±0.33	0.03±0.33	0.03±0.33	6.43±033
	0.4	0.03±0.33	0.06±0.66	0.03±0.33	0.03±0.33

Key: WACI: weeks after culture initiation

Table 2: Effect of different sterilants on browning of coconut leaf culture

Steriliant	**Concentration (%)**	**Contaminated cultures (%)** 1 WACI	2WACI	3WACI	4WACI
Mercuric chloride	0.1	-	-	+	++
	0.2	-	-	+	++
	0.3	-	-	+	++
	0.4	-	+	+	++
Ethanol	70	-	-	-	-
	85	-	-	-	-
	90	-	-	-	-
	95	-	+	+	+
Calcium hypochlorite	0.1	-	-	-	+
	0.2	-	-	-	+
	0.3	-	-	+	+
	0.4	-	-	+	+
Sodium hypochlorite	0.1	-	-	-	-
	0.2	-	-	-	-
	0.3	-	-	-	-
	0.4	-	-	-	+

Key: WACI: weeks after culture initiation, No browning: - , Slight brown: +, Dark brown: ++

Plate 1 Harvested coconut seedling (Leaf explants)

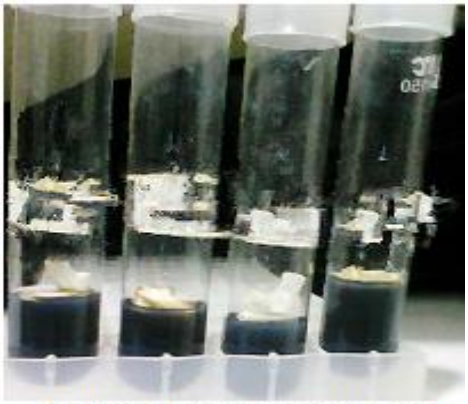

Plate 2 Culture sterilized with ethanol

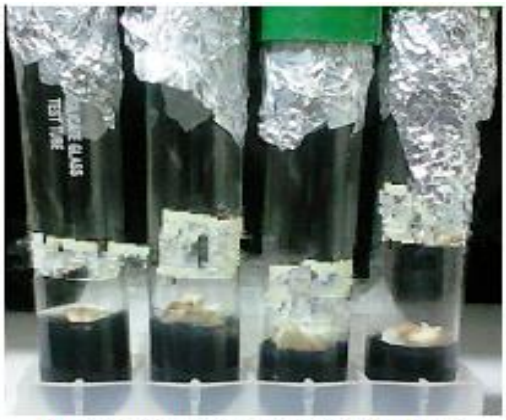

Plate 3 Culture sterilized with Sodium hypochlorite

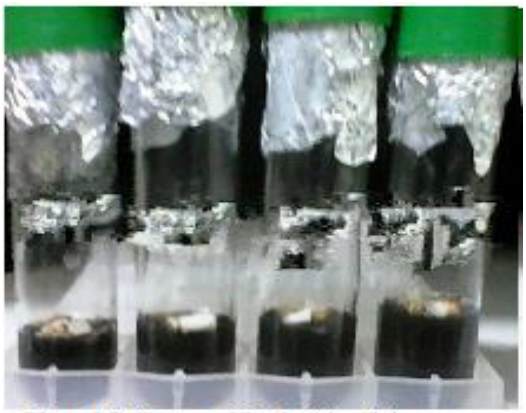

Plate 4 Culture sterilized with calcium hypochlorite

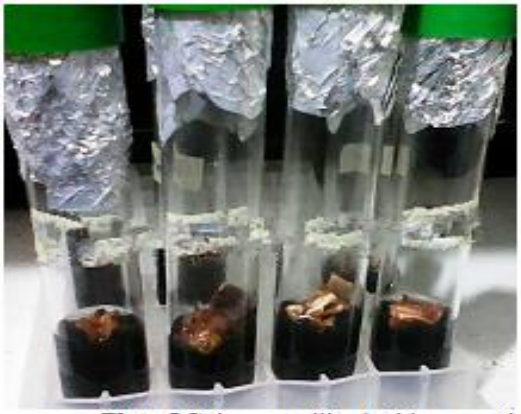

Plate 5 Culture sterilized with mercuric chloride

Calcium hypochlorite at 0.1 % – 0.4 % cultures shows slight browning. Lastly, sodium hypochlorite at 0.1 %, 0.2 % and 0.3 % were free from browning, while 0.4 % had slight browning. The purpose of the experiment was to identify the best sterilizing agent and their concentrations that can control contamination and phenolic compound on immature coconut leaf. In the present study, ethanol happens to be the best sterilizing agents tested with less contamination value of 0.03±0.33, Contamination was noticed in coconut leaf cultures after 4 days of inoculation in calcium hypochlorite with a high value of 20.26±0.66, sodium hypochlorite with 13.26±0.66 after one week followed by the rest of the steriliants. This finding was similar with those of (Hadiuzzaman *et al.*, 2001), where `they reported that contamination was observed in banana shoot tip after 5 days, using divers steriliants before inoculation. Contamination was less in the cultures that were sterilized with ethanol and less browning was observed in sodium hypochlorite than mercuric chloride and calcium hypochlorite in all the concentrations. Various studies, on *in vitro* sterilization of explants, with respect to different sterilizing agents has reported that sodium hypochlorite a very effective killer of bacteria and fungi, (Oyebanji *et al.*, 2009). In this study, ethanol was the best sterilizing agent, followed by sodium hypochlorite in terms of bacteria and fungi control of coconut leaf explants. Apart from contamination, another major problem in coconut tissue culture is the browning of explants shortly after inoculation.

The results obtained in this study showed that browning can be controlled after two weeks of inoculation. Slight browning started at week two, on the cultures that had mercuric chloride with concentration 0.4 % and 95 % ethanol. In sodium hypochlorite and calcium hypochlorite at week two, the cultures were still free from slight browning. At week four, most of the cultures in mercuric chloride at all concentrations shows high rate of browning.

A similar report had been made in solving the problem of browning on date palm inflorescence explants, (El-Shafey *et al.*, 1999). However, it shows that the rate of contamination and browning were observed more at week three and increases in week four. All steriliants did well, but ethanol at 70 % is the best sterilizing agent that can control contamination and browning rate of coconut leaf for the first 4 weeks of inoculation.

Conclusion: This study has shown that all sterilants in this study are capable of reducing or checking contamination in coconut leaf cultures, browning is likely to start occurring after two weeks of inoculation, and can be effectively controlled if the explants is treated with the various sterilants at different concentration. Of all the steriliants used, ethanol was identified as the best steriliants that can control contamination and browning rate in coconut leaf.

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