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Screening sterilizing agents and antibiotics for the elimination of bacterial contaminants from oil palm explants for plant tissue culture

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ABSTRACT

The demand for oil palm tissue culture seedlings is on the increase. The use of oil palm as a source of explants material for the production of clean in vitro plantlets to meet the demand, presents a major challenge of endophytic bacterial contamination. To avoid contamination from explants before being used in plant tissue culture, different sterilizing agents were used. The best for surface sterilization was 50% Chlorox plus 0.1% mecuric chloride with 6% contaminants, followed by 50% chlorox (7.5%), 0.1% Mecuric chloride (12.0%) and 70% Ethanol (27%). Color browning of the explants was noticed. Sterile distilled water was less supported with 85%. Six types of bacterial species were isolated from the explants inoculated in the medium without antibiotics. These were Proteus vulgarius, Bacillus subtilis, Pseudomonas flourescens. Erwinia sp., Staphylococcus aureus and Corynebacterium sp. Seven antibiotics were evaluated to determine their potentials to inhibit these bacterial species. Antibiotics such as Gentamycin and Ampicilin best supported inhibition of mycelial growths with 90% each. These were followed by Streptomycin with 80%, Rifampicin (80%), Tetracycline (60%), Cefotaxime (55%), Penicillin (45%) and Sterile distilled water (10%) ten days after incubation in antibiotics Nutrient agar medium. The poor performance of surface sterilization using sterile distilled water was an indication that, the host plant was a source of bacterial contaminants. This study has shown that some bacteria survived surface sterilization of oil palm explants before being used for tissue culture.

Key words: Antibiotics, Bacteria, Contaminant, Explant, Oil palm, Tissue culture

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INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a perennial crop that originated from the tropical rain forest of West Africa [1]. It can however be said to be a native of Africa covering a total area of about 2 million hectares in Nigeria and endemic to the south eastern states of Nigeria. Later, it spread to South America in the 16th century and to Asia in the 19th century [2].

The main advantage of tissue culture technology lies in the production of high quality and uniform planting material that can be multiplied on a year-round basis under disease free conditions. Aseptic conditions are usually implied but many plant cultures do not stay aseptic *in vitro* and contamination by microorganism, especially bacteria is a continuing problem for commercial and research plant propagators [3].

The major challenge that oil palm tissue culture is facing using tissue culture techniques are that of endophytic bacterial contamination. *Proteus vulgarius, Bacillus subtilis, Pseudomonas flourescens. Erwinia sp., Staphylococcus aureus* and *Corynebacterium* sp. are the major bacteria contaminants implicated in Oil Palm Tissue Culture at the Nigerian Institute for Oil Palm Research (NIFOR), Nigeria. Some of these bacteria have been found to be soil and oil palm associated bacteria (4; 5].

Bacterial contamination is a major threat in plant tissue culture. Plant tissue cultures could habour in a totally unsuspecting manner, either externally in the medium / plant or endophytically [6]. Epiphytic bacteria may lodge in plant structures where disinfectants cannot reach [7] while endophytic bacteria may be localized within the plant at cell junctions and the intercellular spaces of cortical parenchyma [8]. Bacterial contaminants found at explants initiation, present in explants from collection dates and resistant to surface disinfection are likely to be endophytic. Both surface sterilization-resistant microorganisms [9] may survive in the plant material for several subculture cycles and over extended periods of time without expressing symptoms in the tissue or visible signs in the medium.

Contamination of plant tissue culture by different microorganisms, such as bacteria and fungi, reduces their productivity and can completely prevent their cultivation. Successful tissue culture protocols start with effective explants sterilization [10]. Several different methods are used to eliminate fungal and bacterial contamination, including the use of antibiotics and fungicides, as well as inactivation by heat and light [10]. Many sterilants are also toxic to the plant tissues, and hence optimum concentrations of sterilants, duration of exposure of explants to sterilants, the sequences of sterilants used etc. need to be determined to minimize explants injury and to achieve better survival [11].

Little is known about using sterilizing agents and antibiotics to eliminate bacterial contaminants of oil palm explants before being used for plant tissue culture. Antibiotics, such as carbenicillin and cefotaxime, are the most commonly used in plant transformation protocols, since they have a broad spectrum of activity against bacteria and a low toxicity to eukaryotes [12; 13]. The objective of this study was to screen sterilizing agents and antibiotics with a view of identifying the best one that can eliminate bacterial contaminants of the oil palm explants before being used in tissue culture..

MATERIALS AND METHODS

Plant Materials

A four year old tenera palm from NIFOR field 54 extension was used as source of plant material in this study. The plant materials (explants) used were leaf explants obtained by decapitating the tenera palm, 5cm just above the apical meristem. The explants were divided into 5 sets. Each set was sterilized using the following sterilizing agents.

Sterilization Procedure

1. The first set of leaf explants was sterilized by soaking in 50% chlorox (5.25% NaOCL) containing two drops/100ml solution of tween-20) polyoxyyethy lenesorbitan monolaurate) as wetting agent for 5 mins. They were then rinsed three times in sterile distilled water followed by soaking in 0.1% $HgCl_2$ for another 5 mins. These were finally rinsed three times in sterile distilled water before use.
2. The second set was sterilized by soaking the explants in 50% chlorox (5.25% NaOCL) containing 2 drops/100ml solution of tween-20 for 10 mins, followed by rinsing three times in sterile distilled water.
3. The third set was sterilized by soaking in 0.1% $HgCl_2$ containing 2 drops/100ml of tween 20 for 5 mins followed by rinsing thrice in sterile distilled water.
4. The fourth set was sterilized using 70% ethanol containing 2 drops/100ml tween-20 for 10 mins. They were then rinsed three times in sterile distilled water.
5. The control set was soaked in sterile distilled water containing 2 drops/100ml tween-20 for 10 mins followed by three rinses in sterile distilled water.

Five plates for each treatment was repeated twice, they were incubated and monitored daily for ten days under aseptic condition at 30 – 32ºC

Isolation of Bacterial Contaminants

Each of the sterilized explants was placed in each of the Petri dishes of solidified Nutrient agar medium without antibiotic. Detectable bacterial contaminants were individually isolated and subcultured onto the fresh medium 2 to 4 days after incubation. The isolates were cultivated by streaking onto Nutrient agar and incubated at 30 – 32 ^{0}C for ten days. Percentages of contaminations were recorded.

Biochemical Tests

Different tests were carried out to confirm the identity of the isolates gram negative or positive. These tests were catalase, oxidase, coagulase, citrate utilization, urease and indole production. Also sugar fermentation test according to cheesebrough [14].

Bacterial Isolates in Antibiotics Medium

We selected seven antibiotics, Gentamycin, Ampicilin, Streptomycin, Rifampicin, Tetracycline, Cefotaxime and Penicillin. To test their potentials in eliminating bacterial contamination, each was added to the Nutrient agar

medium, i.e., 1mg / 250ml. A 2mm disc from each of the bacterial isolates was transferred onto the centre of solidified Nutrient agar medium. The control treatment was similar with disc without antibiotics. The experiment was conducted in five replicates repeated twice. The cultures were recorded daily for ten days by measuring colony diameter according to Lilly and Barnett [15]. The Petri dishes were incubated at 30 – 32^{0}C. The inhibition percentage was obtained using the formula 1% = [(C^2-C^1)/C^3)] x 100 [16].

RESULTS AND DISCUSSION

Various sterilizing agents were used at the same concentration and different durations to determine the most efficient for the elimination of bacterial contaminants. It was revealed from the results (table 1) that all the sterilizing agents on oil palm explants reduced bacterial contaminants. The best sterilizing agents of explants was 50% Chlorox plus 0.1% mecuric chloride, followed by 50% chlorox, 0.1% Mecuric chloride and 70% Ethanol. The sterile distilled water was the least effective. The browning colors were seen on explants treated with 50% Chlorox plus 0.1% mecuric chloride, 50% chlorox and 0.1% Mecuric chloride from two days during the ten days incubation period. This could be as a result of long minutes of exposure. This is in agreement with other reports [10]; Johnson et al., [17]. The poor performance of Ethanol was the non-inclusion of other sterilizing agents. This was supported by Bloomfield [18], Ethanol is generally used prior to treatment with other compounds. It has been reported that alcohols are rapidly bactericidal rather than bacteriostatic against vegetative forms of bacteria, but do not destroy bacterial spores. Their cidal activity drops sharply when diluted below 50%. The optimum bacterialcidal is 60 to 90% solution in water [19].

Table 1. Effects of Surface Sterilization in Culture Medium on Oil Palm Explants for Plant Tissue culture Ten Days after Incubation

Surface sterilization	Duration of exposure	Contamination %	Explants color
50% Chlorox plus 0.1 $HgCl_2$ with 2 drops / 100ml Tween 20	5 mins	6.0	Browning
50% Chlorox with 2 drops / 100ml Tween 20	10 mins	7.5	Browning
0.1% $HgCl_2$ with 2 drops / 100ml Tween 20	5 mins	12.0	Browning
70% Ethanol with 2 drops / 100ml Tween 20	10 mins	27.0	Light browning
Sterile distilled water with 2 drops / 100ml Tween 20	10 mins	85.00	Normal

Table 2. Cultural, Morphological and Biochemical Characteristics of the Bacterial Contaminants from Oil Palm Explants for Plant Tissue Culture

Characteristics	1	2	3	4	5	6
Cultural						
Elevation	Flat	Flat	Low convex	Low convex	Convex	Convex
Margin	Serrated	Serrated	Entire	Entire	Entire	Entire
Colour	Cream	Cream	Dirty white	Dirty white	Yellow	Cream
Shape	Irregular	Irregular	Circular	Circular	Circular	Circular
Morphological						
Gram staining	-	+	-	-	+	+
Cell type	Rod	Rod	Rod	Rod	Cocci	Rod
Cell arrangement	Single	Chains	Single	Single	Cluster	Single
Spore staining	-	+	-	-	-	-
Biochemical						
Catalase	+	+	+	+	+	+
Oxidase	-	-	+	-	-	-
Coagulase	-	-	-	-	-	-
Urease	-	+	-	-	+	+
Indole	-	-	-	-	-	-
Citrate	+	+	+	+	+	+
Glucose	+	+	+	+	+	+
Lactose	-	-	-	-	-	-
Isolates	*Proteus vulgarius*	*Bacillus subtilis*	*Pseudomonas flourescens*	*Erwinia* sp.	*Staphylococcus aureus*	*Corynebacterium* sp.

Six bacterial contaminants or isolates obtained from yet to be used oil palm explants for tissue were identified. The cultural, morphological and biochemical characteristics of the six bacterial isolates were *Proteus vulgarius, Bacillus subtilis, Pseudomonas flourescens. Erwinia sp., Staphylococcus aureus* and *Corynebacterium* sp. (table 2). Similar bacterial isolates had been reported [4].

The results of inhibition of bacterial isolates in antibiotic medium are presented in table 3. In this study all the antibiotics showed inhibition activity against the bacterial isolates or contaminants. The most effective in the inhibition of mycelial growths were Gentamycin and Ampicilin with 90% each. These were followed by Streptomycin with 80%, Rifampicin (80%), Tetracycline (60%), Cefotaxime (55%), Penicillin (45%) and Sterile

distilled water (10%) ten days after incubation in antibiotic Nutrient agar medium. This result is in agreement with Reed and Traprasert [20], knowledge of the effect of the antibiotics on both bacteria and the plants is essential for the recovery of healthy plants. No single antibiotics had 100% inhibition of mycelial growth of any of the isolates. This was supported by Van den Houwe and Swennen [21], single antibiotic treatments were ineffective against banana tissue cultures. According to Young et al. [22], combination of antibiotics are used against bacteria from plant tissue cultures and may be more effective than single antibiotics in killing contaminants and reducing the risk of antibiotic resistance developing in the microbial populations.

Table 3. Inhibition of Bacterial Isolates of Oil Palm Explants in Antibiotic Medium

Inhibition rates of mycelial growth (%)								
Bacteria Isolates	**Gentamycin**	**Ampicilin**	**Streptomycin**	**Rifampicin**	**Tetracycline**	**Cefotaxime**	**Penicillin**	**Sterile Distilled Water**
Proteus vulgarius	90.0 ± 0.07a	90.0 ± 0.03a	80.0 ± 0.01b	80.0 ± 0.08b	60.0 ± 0.12c	55.0 ± 0.7d	45.0 ± 0.14e	0.0 ± 00f
Bacillus subtilis	90.0 ± 0.07a	88.0 ± 0.08a	80.0 ± 0.01b	65.0 ± 0.05c	65.0 ± 0.05c	60.0 ± 0.11d	60.0 ± 0.11d	0.0 ± 0.0e
Pseudomonas flourescens	86.0 ± 0.08a	80.0 ± 0.01b	64.0 ± 0.02c	70.0 ± 0.08d	45.0 ± 0.12e	60.0 ± 0.11d	40.0 ± 0.06f	0.0 ± 0.0g
Erwinia sp.	90.0 ± 0.07a	85.0 ± 0.04b	70.0 ± 0.08c	70.0 ± 0.08c	60.0 ± 0.11d	50.0 ± 0.6e	60.0 ± 0.11d	0.0 ± 0.0f
Staphylococcus Aureus	80.0 ± 0.08b	85.0 ± 0.04a	80.0 ± 0.01b	85.0 ± 0.07a	70.0 ± 0.08c	80.0 ± 0.08b	70.0 ± 0.08c	0.0 ± 0.0d
Corynebacterium Sp.	90.0 ± 0.07a	86.0 ± 0.08b	85.0 ± 0.07b	80.0 ± 0.01c	70.0 ± 0.08d	65.0 ± 0..05e	60.0 ± 0.08f	0.0 ± 0.0g

The same letters indicate non-significant difference while different letters in the same column indicate significant difference in regard to % inhibition at p = 0.05 using student test (test), to test for standard error of mean (SEM).

CONCLUSION

The study has shown that after the surface sterilization of oil palm explants before being used in tissue culture laboratory, some bacterial isolates survived the sterilization techniques. With the rising rates of bacterial contaminants causing high mortality in plant tissue culture, antibiotics will reduce the contaminants there by increase survival rate of the plantlets. Pytotoxicity study is required to determine the effect of the sterilizing agents and antibiotics on the explants before being used in tissue culture.

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