Nig. J. Biotech. Vol. 41(1): 139-147 (June 2024)
ISSN: 0189 1731
Available online at
http://www.ajol.info/index.php/njb/index
and https://bsn.org.ng
DOI: https://dx.doi.org/10.4314/njb.v41i1.15

NIGERIAN JOURNAL OF BIOTECHNOLOGY

Assessment of Sterilants and their Combined Effect on Surface Sterilization of *Musa* Spp

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Abstract

***Musa* spp accessions were assembled from the Department of Crop Production & Landscape Management, Faculty of Agriculture, Ebonyi State University germplasm, and taken to the culture laboratory where they were washed and trimmed to a size of 1.0 – 1.5 cm. This block of tissue was surface-sterilized with different sterilants which included detergent, ethanol, NaOCl, benlate, $HgCl_2$, UV light, and cefotaxime. The sterilants were applied following 15 different treatment protocols. Only 5 treatments protocols (T3: 70 % for 30 sec, 8 % NaOCL for 5 min, 500 mg/L cefotaxime; T4: 70 % for 30 sec, 8 % NaOCL for 5 min; T5: 70 % for 30 sec, 8 % NaOCL for 5 min, 1.2 g/L $HgCl_2$ for 10 min, 500 mg/L cefotaxime; T6: 70 % for 30 sec, 8 % NaOCL for 5 min, 5 % benlate for 5 min, 1.2 g/L $HgCl_2$ for 10 min, 500 mg/L cefotaxime, and T7: 70 % for 30 sec, 8 % NaOCL for 5 min, 5 % benlate for 5 min, 1.2 g/L $HgCl_2$ for 10 min, U.V. light for 5 min, 500 mg/L cefotaxime) produced clean cultures with variations in the health of the cultures. T5 and T6 produced very healthy plantlets with 75 and 100 percent survival, respectively. T3 and T7 produced healthy plantlets with 100 percent survival, while T4 produced healthy plantlets with 50 % survival. Different sterilants react differently to living tissue, either alone or combined. Therefore, this work has produced standardized protocols for using the sterilants for surface sterilization of 'owom' and 'efol'.**

Keywords: Sterilant, Micro-propagation, *Musa* spp, Microorganism

Introduction

Achieving success in micropropagation depends primarily on getting a sterile culture. This is because any contamination with micro-organisms will most likely negate the progress of micropropagation. Such contaminating bacteria can be harboured in human skin, specimens, laboratory desks, air, reagents, and laboratory apparatus. A report by Odutayo *et al.* (2007) stated that *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, and *Staphylococcus coli* were found to be higher (ranging from 36-46 %) in human skin than all other sampled materials, the laboratory walls, tables, and laboratory indoor air. When these microbes are found in media, they compete with the cultured tissue for available nutrients, leading to the death of tissues. According to Hassen *et al.* (2022), contamination can also lead to tissue necrosis, variable tissue growth, decreased shoot proliferation, and rooting.

Contamination is high when tissue samples are obtained from field-grown plants. *Musa* spp grows in backyards and dirty environments, hence, they carry a high bacterial load. Sterilizing such plant tissue is difficult. According to Deepak and Virk (2022), extreme application of sterilant can damage the plant tissue. Growth media and plantlet contamination are the main causes of loss in large-scale micro-propagation facilities (Okoroafor, 2022). According to Tewelde *et al.* (2020), autoclaving easily controls bacterial contamination of media, whereas controlling plantlet contamination is very difficult, especially contamination caused by endophytes. This is because sterilization is done to kill the surface contaminant and the internal

contaminant while leaving the tissue in a healthy state to survive *in vitro* challenges. All the materials used in the plant tissue culture must be sterilized to kill the microorganisms that are present by using appropriate sterilizing agents (Sessou *et al.,* 2020), and protocol. When appropriate sterilant is applied wrongly, the efficiency of the sterilant is altered. Therefore, for every plant species, sterilant and protocol should be examined and established.

Research has provided effective means of sterilizing different plant species. The report by Magaia (2015) stated that the highest clean explants (87 explants) were achieved when nodal explants from the greenhouse were treated with 70 % ethanol for 2 minutes, proceeded with treatment of 0.05 % mercuric chloride ($HgCl_2$) for 2 minutes. A report by Nwite *et al.* (2022) stated that the coconut leaf explant soaked in 70 % ethanol for 5 minutes had minimal contamination. Sequential sterilization has been reported to give a better response than a single sterilization step. According to Goswami and Handique (2013), a treatment combination of Sodium hypochlorite (1.0 %) for 15 minutes followed by $HgCl_2$ (0.1 %) on banana for 7 minutes gave the highest percentage of aseptic culture establishment in *in vitro* experiment.

Therefore, this work was aimed at determining the efficiency of different sterilants, and their combinational ability in surface sterilization of *Musa* accessions.

Materials and Method

Shoot tips from young suckers of preferred accessions, 'owom' and 'efol' (40 – 100 cm height) growing in the *Musa* field germplasm at the Faculty of Agriculture & Natural Resources Management of Ebonyi State University were used as explants. Suckers collected were thoroughly washed, trimmed to a size of 1.0 – 1.5 cm. This block of tissue was surface-sterilized under different aseptic conditions as shown in Table 1. Final trimming reduced the shoot tips to about 3 × 5 mm consisting of the apical dome, a few leaves primordia, and a thin layer of corm tissue following the procedure of Strosse *et al.* (2003). The explants were placed directly on MS-based culture medium as provided by Murashige and Skoog, (1962). The medium was contained in reusable Magenta boxes (or 125 mL flask) with each containing 50 mL of culture medium (Plate 1a & 1b). The MS-basal medium was supplemented with commercial-grade sugar as reported by Ganapathi *et al.* (1995), at a concentration of 30 g/L. Also, a 6-benzylaminopurine (BAP) concentration of 0.004 g/L was applied as a shooting regulator to generate clean cultures. The treatments that caused less damage to the cultured tissue were identified and studied.

The health status of the cultured plantlets was determined by grouping the cultures into three and representing them with varied number of asterisk (*). A single asterisk (*) represented culture that was not healthy, but rather has dead or decaying tissue. , Healthy cultures that had living tissues, green pigmentation, but no evidence of emerging leaves were represented with two asterisk (**). Whereas, very healthy cultures with evidence a living shoot that is showing emerging leaves were represented with three asterisk (***).

Result

Across the treatments examined, only 5 treatments (T3, T4, T5, T6, and T7) protocols produced clean cultures with variations in the health status of the cultures (Table 2). T5 (represented by Plate 2a), and T6 produced very healthy plantlets with 75 and 100 percent survival, respectively. T3 and T7 (represented by Plate 2b) produced healthy plantlets with 100 percent survival, while T4 produced healthy plantlets with 50 % survival. T1 (as represented by Plate 2c), T2, T8, T9, T10, T11, T12, T13, T14 and T15 produced non-healthy plantlets.

Table 1: Pre-treatment of explants for decontamination, before induction

Treatments (T)	Detergent	70 % Ethanol	90 % Ethanol	5 % NaOCL	8 % NaOCL	10 % NaOCL	5 g/L Benlate	1.2 g/L $HgCl_2$	U. V. Light	500 mg/L Cefotaxime
T 1	-	Explants were socked for 5 min.	-	Explants were socked for 5 min and rinsed.	-	Explants were socked for 10 min and rinsed	-	-	-	-
T 2	-	Explants were socked for 5 min.	-	Explants were socked for 5 min and rinsed.	-	Explants were socked for 10 min and rinsed	-	-	-	After proper rinsing, explants were cultured in media with cefotaxime
T 3	-	Explants were socked for 30 sec.	-	-	Explants were socked for 5 min and rinsed.	-	-	-	-	After proper rinsing, explants were cultured in media with cefotaxime
T 4	-	Explants were socked for 30 sec.	-	-	Explants were socked for 5 min and rinsed.	-	-	-	-	-
T 5	-	Explants were socked for 30 sec.	-	-	Explants were socked for 5 min and rinsed.	-	-	Explants were socked for 10 min and rinsed.	-	After proper rinsing, explants were cultured in media with cefotaxime
T 6	-	Explants were socked for 30 sec.	-	-	Explants were socked for 5 min and rinsed.	-	Explants were socked for 5 min.	Explants were socked for 10 min and rinsed.	-	After proper rinsing, explants were cultured in media with cefotaxime

T 7	-	Explants were socked for 30 sec.	-	-	Explants were socked for 5 min and rinsed.	-	Explants were socked for 5 min.	Explants were socked for 10 min and rinsed.	Explants were exposed for 5 min.	After proper rinsing, explants were cultured in media with cefotaxime
T 8	-	Explants were socked for 30 sec.	-	-	-	-	-	Explants were socked for 10 min and rinsed.	-	-
T 9	-	Explants were socked for 30 sec.	-	-	-	-	-	Explants were socked for 10 min and rinsed.	-	After proper rinsing, explants were cultured in media with cefotaxime
T 10	-	Explants were socked for 30 sec.	-	-	-	-	-	Explants were socked for 15 min and rinsed.	-	-
T 11	-	-	Explants were socked for 10 min and rinsed.	-	-	-	-	Explants were socked for 15 min and rinsed.	-	-
T 12	-	Explants were socked for 30 sec.		-	-	Explants were socked for 10 min and rinsed	-	-	-	-
T 13	-	-	Explants were socked for 5 min and rinsed.	-	-	Explants were socked for 10 min and rinsed	-	-	-	-

T 14	-	Explants were socked for 30 sec.	-	-	Explants were socked for 10 min and rinsed.	-	Explants were socked for 5 min.	-	-	-
T 15	Explants were washed and rinsed.	Explants were socked for 30 sec.	-	-	-	-	-	Explants were socked for 10 min and rinsed.	-	-

T 1 = Treatment 1; T 2 = Treatment 2; T 3 = Treatment 3; T 4 = Treatment 4; T 5 = Treatment 5; T 6 = Treatment 6; T 7 = Treatment 7; T 8 = Treatment 8; T 9 = Treatment 9; T 10 = Treatment 10; 11 = Treatment 11; T 12 = Treatment 12; T 13 = Treatment 13; T 14 = Treatment 14; T 15 = Treatment 15

Table 2: Surface Sterilization Treatment's Effect on Explants after 5 Weeks

Treatment (T)	% Survival	Health Status
Treatment 1 (T 1)	0	*
Treatment 2 (T 2)	0	*
Treatment 3 (T 3)	100	**
Treatment 4 (T 4)	50	**
Treatment 5 (T 5)	75	***
Treatment 6 (T 6)	100	***
Treatment 7 (T 7)	100	**
Treatment 8 (T 8)	0	*
Treatment 9 (T 9)	0	*
Treatment 10 (T 10)	0	*
Treatment 11 (T 11)	0	*
Treatment 12 (T 12)	0	*
Treatment 13 (T 13)	0	*
Treatment 14 (T 14)	0	*
Treatment 15 (T 15)	0	*

Legend: * = Non-healthy, ** = healthy and *** = Very healthy

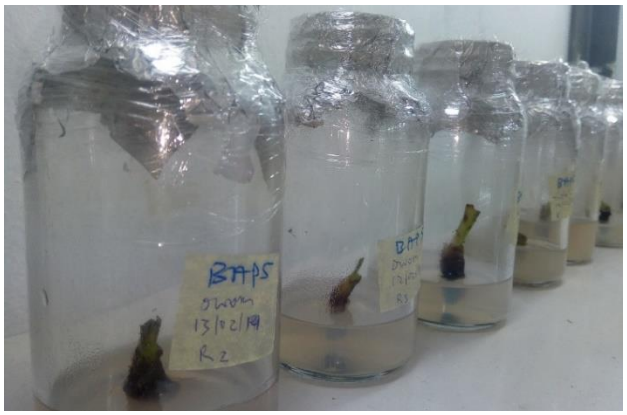
a  b 

Plate 1: Initiation of culture (a & b)

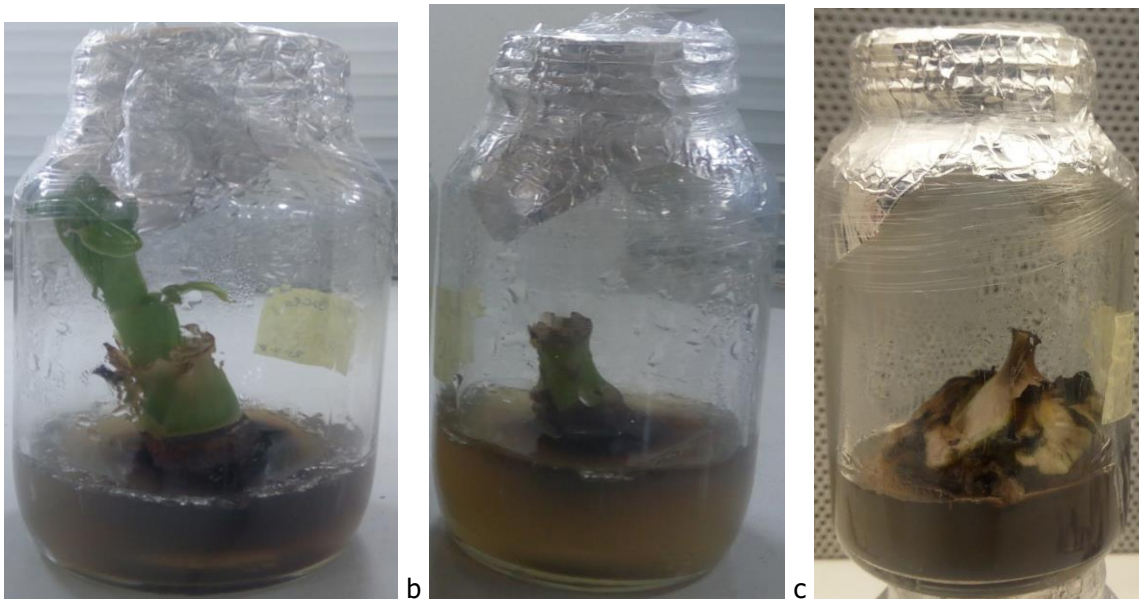


Plate 2: Pictorial View of Explants Treated with Treatment 5 (T5), Treatment 7 (T7), & Treatment 1 (T1).

T 5, T 7, and T1 = a, b, and c respectively

Discussion

Bananas and plantain are planted in environments that are highly prone to various microbial activities, and getting samples that are free for micro-propagation is difficult. Contamination has been reported as an unstoppable problem that can affect the development of all *in vitro* micro-propagation techniques (Enjalric *et al.,* 1998; Odutayo *et al.,* 2004). These micro-organisms may not be harmful to the plant but while in culture, they are not helpful. As reported by Habib *et al.* (2002), bacterial contamination is one of the major problems in *Musa* spp. micro-propagation.

Different sterilization protocols aimed at achieving aseptic and healthy cultures were tried (Table 1). The majority of the treatments applied killed the tissues (T1, T2, and T8 to T15) but Treatment 6 produced very healthy cultures with 100 % survival of all the tissues treated. This could be as a result of the adverse effects of the sterilants to living tissues which vary with their nature, and specific duration when applied individually or in combination (Bharti *et al.,* 2018).

Explants were socked for 30 sec in 70 % ethanol, after which they were soaked for 5 min in 8 % NaOCl and rinsed with distilled water before being socked in 5 g/L benlate for 5 min, and 1.2 g/L $HgCl_2$ for 10 min. After proper washing, they were cultured in a medium supplemented with 500 mg/L Cefotaxime. Shorter protocols have been used to get clean cultures. In a report by Farzinebrahimi *et al.* (2013), 70 % Clorox for five minutes, and ethanol for three minutes, respectively followed by rinsing and washing by sterilized distilled water was employed to achieve 99 % survival of *Musa* spp while a longer protocol achieved 69 % survival rate. This could be attributed to damage done to the tissue by sterilants. Among the fifteen protocols assessed, only 20 % gave 100 % survival, with 66.7 % resulting in a 0 % survival rate. While we attribute the poor survival rate to damage resulting from long sequential protocol, it can also be attributed to improper sterilization which could not kill surface microorganisms and endophytes.

The source of the specimen for culture may affect the simplicity of protocol as that could determine the weight of the bacterial load on the specimen. Previous reports by Titov *et al.* (2006), have shown that aseptic cultures were simply obtained

by soaking explant with 0.1 % $HgCl_2$ for 6 min, followed by several washes in sterile water. Conversely, the optimum % culture survival and the highest percentage of aseptic culture establishment with reduced necrosis was recorded by Singh *et al.* (2022), when the specimens were treated with the combination of 0.1 % Bavistin for 30 seconds, followed by 0.1 % $HgCl_2$ for 5min, 5 % NaOCl for 5min and 70 % EtOH for 30 sec.

However, Jaisy and Ghai (2011) inferred that treatment with 0.1 % of $HgCl_2$ for six minutes was the most effective. A similar report was given by Yadav *et al.,* (2021), whereby 0.1 % mercuric chloride and 70 % ethanol were best for generating contamination-free banana plants. But, Muhammad et *al.* (2004) stated that sodium hypochlorite is the most commonly used disinfectant for surface sterilization of different explants. In a work by Zinabu *et al.* (2018), sterilized explants were cultured on 500 mg/L cefotaxime containing MS medium to evaluate the response of explants sterilization to different NaOCl concentrations. Results showed that 2 % NaOCl was significantly superior to other concentrations. No work has shown the combinational effect of all the sterilants employed in this study, and the concerted effects of the sterilants could be the reason for the very healthy state observed for all the cultures that were sterilized using Treatment 6.

In conclusion, an attempt to micro-propagate *Musa* spp, directly from the field, generating clean cultures was difficult being that they are sourced from a microbial-thriving environment. These organisms could be helpful in the field, but in culturing, they are detrimental to tissues. This work has shown that not all sterilants are favourable to *Musa* spp tissue during culturing and the mode of application also determines the survival and health of the cultures.

Acknowledgement

I appreciate the sincere support of my supervisor, Prof. H. O. Oselebe during the execution of this work. I wholly appreciate Dr. Ihuoma Chizaram Okwuonu, the kind-hearted Coordinator of the Biotechnology Department and Head of the Tissue Culture section of the National Root Crops Research Institute, Umudike, Nigeria, who selflessly gave in her endowed intelligence, experience, and patience in seeing to the success of this research. I appreciate my laboratory instructor, Ijeoma Ihezie Carol who gave additional time and effort to progress the research, and the laboratory assistants, Onuegbu Kelechi Promise and Okoro Tobechukwu Innocent for all their tireless support. Finally, I commend TETFund, Ebonyi State University, and the Academic Staff Union of Universities for their financial assistance during my laboratory work.

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