Isolation, Identification and Classification of Contaminating Microbes at an Ibadan-Based Plant Tissue Culture Laboratory

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***Abstract:-*Microbial contamination in laboratories is a serious problem worldwide and characterization of these contaminants is imperative for achievement of successful *in vitro* research activities for immeasurable and unquantifiable agro-economic benefits. Literatures have shown that there is an ongoing effort(s) to identify the various groups of microbial contaminants within the plant cultures in mosttissue culture laboratories. Thus, this study has been designed to isolate, identify and classify the contaminating microbes at an Ibadan-based Plant Tissue Culture Laboratory. Established but contaminated cultures of different crop species were collected over a period of four weeks, from February 1st to 28th, 2019. And from these, isolation of pure microbial cultures was carried out based on their morphological differences and where colony form, elevation, pigmentation and size were used to distinguish bacteria and fungi contaminants. Also, specific microbes were further authenticated using differential and selective media and isolated fungi were identified using microscopic observations of size, and shape. The results showed that the contaminating microbes are of different types. Themacroscopic and microscopic observations of fungi confirmed presence of *Cladosporium* sp, *Penicillium* sp, *Aspergillus* sp and *Alternaria* sp while the persistent bacteria identified were *Shigella* sp, *Pseudomonasaeruginosa*, *Corynebacteria* sp, *Bacillus* sp and *Staphylococci* aureus. The contaminants were similar to standard strains but there was a significant difference in contamination. It is concluded that despite disinfection with sodium hypochlorite, the bacterial and fungal contaminants persist in micropropagation culture media and there is need to either increase the concentration of the disinfectant or change the disinfectant to a different one.**

Keywords: **micro-propagation, plant tissue culture, bacteria, fungi, contaminants.**

I. INTRODUCTION

Preventing or avoiding microbial contamination in plant tissue culture laboratory is critical to successful experiments. The practice of plant tissue culture has contributed towards the propagation of large numbers of plant from small pieces of stock plant in relatively short period of time (Daniel, 1998).

Basically, the technique consists of taking a piece of a plant (such as a stem tip, node, meristem, embryo, or even a seed) and placing it in a sterile, (usually gel-based) nutrient medium where it multiplies. In most of the cases, the original plant is not destroyed in the process, a factor of considerable importance to the owner of a rare or unusual plant. Micro-propagation has also been used extensively in the improvement of selections of plants with enhanced stress or pest resistance, production of pathogen-free plants and somatic hybridizations. The formulation of the growth medium depends upon whether it is intended to produce undifferentiated callus tissue, multiply the number of plantlets, grow roots, or multiply embryos for artificial seed and so on (Daniel,1998).

The nutrient media in which the plant tissue is cultivated is a good source of nutrient for microbial growth. These microbes compete adversely with plant tissue culture for nutrient. The presence of these microbes in any plant tissue cultures usually results in increased culture mortality, the presence of latent infections that can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Kane, 2003).

Although, the tissue culture techniques usually involve growing stock plants in ways that will minimize infection, treating the plant materials with disinfecting chemicals to kill superficial microbes and sterilizing materials/tools used for dissection along with the vessels and media in which culture are grown is imperative (Kane, 2003). However, contamination has been reported as constant problem, which can compromise development of all *in-vitro* techniques (George 1993). About thirty-one micro-organisms from ten different plants cultivators growing in micro-propagation have been isolated, identified and classified; with Yeasts, *Corynebacterium* sp. and *Pseudomonas* sp. being predominant (Leggatt *et al.*, 1994). *Bacillus* sp., Corynebacterium sp. and an Actinomycete have also been found contaminating the vitro culture of apple rootstocks (Hennerty et al., 1994). Oduntayo*et al.* (2004) had also reportedly associated the following bacteria namely *Pseudomonas*

*syringe,*pv*phaseolicoli*, *Bacillus licheniformis*, *Bacillus subtilis*, *Corynebacterium* sp. and *Erwinia* sp. with the contamination of *Hibiscus cannabinus* and *Telfariaoccidentalis* in Nigeria.

II. MATERIALS AND METHODS

Sample Collection and Preparation: The sample collection for this research study was carried out at the plant tissue culture laboratory of NACGRAB, where vessels containing varieties of microbial contaminants of plant cultures were collected biweekly (every Tuesday and Friday) from the PTC laboratory over a period of four weeks, from February 1st 2019 to February 28th 2019. Fifteen cultures of different species of plants were collected per visit. The plant culture samples collected over the period of four weeks contains yam varieties (*Dioscoreaalata*, *Dioscorearotundata*), tiger nut (*Cyperusesculentus*), rice (*Oryza sativa*), pineapple (*Ananascomosus*), cassava (*Manihot esculenta*), banana (*Musa* sp), eucalyptus (*Eucalyptus camadulensis*), pepper (*Caspicum* sp), plantain (*Musa paradisiaca*), and neem plants (*Azadirachtaindica*) respectively.

After collection, the contaminated cultures were taken to the microbiology laboratory of the Institute of Agricultural Research And Training (IAR&T), where the microbial contents in each of the plant cultures were extracted and analyzed.

Microbial Analytical and Nutrient Media Preparation: All media used for this study were purchased from soil microbiology laboratory of the Institute of Agricultural Research And Training (IAR&T), Ibadan. Most media used in microbial cultures usually differs from each other based on the requirements of the growth of the specimen that it supports. The specific media used for bacteria isolation and growth is called "Nutrient Agar" and this was purchased from IAR&T in powdered form. It is made by suspending 5g of digest of animal tissue, 1.5g of beef extract, 1.5g of yeast extract, 5g of sodium chloride (NaCl) and 15g of agar constituted in 1litre/500ml of distilled water (Tokuyasu*et al.*, 2012). It is usually prepared by weighing 28g/L or 14g/500ml and constituting it using distilled water. The prepared agar mixture was then dissolved in water by boiling at 100^{o}C, and then the molten agar was sterilized by autoclaving at a pressure of 15 pascals and a temperature of 121^{o}C for 15 minutes. Potato dextrose agar (PDA) was used for the fungi isolation and growth. The agar in powdered form was weighed, prepared and reconstituted using distilled water. The potato dextrose agar (PDA) was made using 39 g/L or 19.5 g/500ml. The agar mixture was dissolved in water by boiling at 100^{o}C, and then the agar was sterilized by autoclaving at a pressure of 15 pascals and a temperature of 121^{o}C for 15 minutes. All the media supported growth as per the requirement. Therefore, they were used in this study.

Isolation of Bacterial and Fungal Contaminants from Plant Cultures: In microbiology, the term isolation is often used and it refers to the separation of a strain from a natural, mixed population of living microbes, as present in the environment. Using a wire/inoculating loop, the swabs were removed from the tubes and inoculated onto fifteen (15) plates containing NA and PDA. The PDA plates were then incubated at a temperature of 25^{o}C for 72 h while the NA plates were incubated at 37^{o}C for 24 h for fungal growth and bacterial growth respectively. All samples were analyzed using plate-count method specifically surface-spread or spread-plate technique.

Isolation of Bacteria: The method used for inoculating the solid media depends upon the purpose of inoculation, which is either to have isolated colonies that reveals the bacterial load of the sample known as quantitative analysis or otherwise. For obtaining the isolated colonies, streaking method/serial dilution method is usually used. The most common method of inoculating on agar plate is streaking. Below are the steps used in this study to isolate the bacteria contaminants.9 ml of distilled water was put into the test/eppendorf tubes using a syringe. Thereafter, the water was sterilized in an autoclave for 15 minutes and was allowed to cool. Using a wire/inoculating loop, the contaminants from the collected samples were isolated and put into the sterilized water. Then, a syringe was used to extract 1ml from the sterilized water containing the contaminants and this was put into the petri dishes. After this, the Nutrient Agar (NA) was poured into the petri dishes and allowed to solidify. This was then left for 72 hours before being used in identifying and classifying the microbes.

*Isolation of Fungi - Pour plate method***:** Pour plate method is the method often used for the isolation of fungi contaminants. The procedure below was used to isolate fungi in this study: The potato dextrose agar media (PDA) was prepared and sterilized. Then, this was allowed to cool. The PDA media was poured into the petri dishes and allows to solidify. Using inoculating wire loop, the contaminants were extracted from the collected samples and streaked on the petri dishes. This was then left for 24 hours before identification and classification was done. The fungi and bacteria that grew on the media were identified using procedures outlined above.

Identification of Pure Colonies Using Morphological Characteristics: According to Del *et al.* (2014), colonies were isolated from their axenic culture containing only a single species of microorganisms based on the morphological differences. Petri-dishes were divided into quadrants and sub-culturing was carried out by streaking. Streaked plates were incubated as outlined in section 3.4. Colony morphology observations formed a major identifying criterion for bacteria. The characteristics observed included; circular, irregular, spreading; elevation - flat, slightly, raised or markedly raised; pigmentation - red, white, pink, colorless; size - pinpoint, small, medium, large; and finally, texture. The fungal isolates were observed under the microscope and identified using cultural characters, morphology and by examining spore arrangements according to Barnett and Hunter (1972).

Identification of Bacterial Contaminants using Biochemical Methods: Bacteria identification was carried out by morphology and biochemical reaction to specific media to obtain pure isolates. Isolation of pure cultures was carried out based on morphological differences where colony form, evaluation, pigmentation and size were the distinguishing factors for both bacteria and fungi contaminants.

Data Analysis: The data on bacterial and fungal contamination in the tissue culture laboratory was then analyzed using ANOVA with statistical GENESTAT version 6 computer software. The means of bacterial and fungi occurrence were also separated.

III. RESULTS AND ANALYSIS FINDINGS

Isolation and Identification of Contaminants in Plant Tissue Culture Laboratory: A total of six bacterial isolates were obtained and identified from the different samples. Some of the contaminants vary while some were the same. The bacterial contaminants include; *Pseudomonas aeruginosa, Escherichia coli, Streptococcus pneumoniae, Staphylococcus aureus, Proteus* spand *Bacillus cereus*. Also, a total of seven fungal segregates were also isolated. They included *Aspergillus niger, Aspergillustamarii, Aspergillusflavus, Rhizopus nigricans, Eurotium amstelodami,penicillium chrysogenum*and *Fusarium* sp. On separating the means of occurrence of bacteria and fungi, the occurrence of fungal isolates was higher than that of bacteria isolates.

Identification of Bacteria Using Colonial Growth on Selective Media

The test for specific microbes carried out using selective media showed the growth of single, pairs and irregular clusters of *Pseudomonas aeruginosa, Escherichia coli, Streptococcus pneumonia, Staphylococcus aureus, Proteus* spand *Bacillus aureus.*

Frequency of Occurrence of Bacterial and Fungal Contaminants

The rates of occurrence of fungal isolates were higher than that of bacteria. It was observed that the fungi were most found because they may be associated with tissue culture cabinets.

Table 1: Different Strains of Microbes Found in Plant Culture for Week One (1)

	WEEK ONE (A)		**WEEK ONE (B)**		
CULTURE SAMPLE CODE	**BACTERIA**	**FUNGI**	**SAMPLE CODE**	**BACTERIA**	**FUNGI**
Banana 1	*Proteus sp* *E. coli*	*A.tamarii* *Fusarium sp*	**Banana** 1	*Proteus sp*	*Penicillium sp* *A.niger*
Banana 2	*Streptococcus sp* *E. coli* *Bacillus sp*	*Penicillium sp* *A. niger*	**Banana 2**	*Streptococcus sp* *E. coli* *Proteus sp*	*Eurotium amstelodami*
Banana 3	*E. coli* *Proteus sp*	*Eurotium sp*	**Banana 3**	*Staphylococcus sp* *E. coli* *Proteus sp*	*Aspergillus versicolor*
Cassava	*Micrococcus sp* *E. coli* *Bacillus sp*	*A. tamarii* *Fusarium sp*	**Cassava 1**	*E. coli* *Staphylococcus sp*	*Penicillium chrysogenum*
Cassava 2	-	-	**Cassava 2**	*No growth (NG)*	*Penicillium citrinum*
Eucalyptus	*Proteus sp* *E. coli* *Bacillus sp*	*A. niger* *Penicillium sp*	**Eucalyptus**	*E. coli* *Proteus sp*	*Penicillium citrinum*
Pineapple 1	*Bacillus sp* *Proteus sp* *Pseudomonas sp*	*A.niger* *Fusarium sp*	**Pineapple 1**	*E. coli* *Proteus sp*	*Aspergillus sp* *Penicillium sp*
Pineapple 2	*Proteus sp* *Pseudomonas sp* *Bacillus sp*	*A. flavus* *Fusarium compactum*	**Pineapple 2**	*Bacillus sp*	*Yeast*
Pineapple 3	*Pseudomonas sp* *Proteus sp*	*A. niger*	**Pineapple 3**	*E. coli*	*A. niger*
Pineapple 4	*Proteus sp* *Pseudomonas sp* *E.coli*	*A. tamarii*	**Pineapple 4**	-	-
Rice 1	*Proteus sp* *E.coli*	*A.tamarii* *A. flavus* *Fusarium sp*	**Rice 1**	*E. coli* *Staphylococcussp*	*Fusarium sp*
Rice 2	*E. coli* *Proteus sp*	*Rhizopus sp* *A. niger* *Penicillium sp*	**Rice 2**	*E. coli* *Bacillus sp* *Staphylococcus sp*	*Aspergillus sp*
Yam 1	*Micrococcus sp* *Pseudomonas sp*	*Penicillium sp* *A. niger*	**Yam 1**	*Bacillus sp* *Streptococcus sp* *Proteus sp*	*Aspergillus sp* *A. niger*

Yam 2	*Pseudomonas sp* *Micrococcus* *Bacillus sp*	*Aspergillus sp*	**Yam 2**	*Streptococcus sp* *Micrococcus sp*	*Penicillium citrinum*
Yam 3	*Bacillus sp* *Pseudomonas sp*	*fusarium*	**Yam 3**	*Bacillus sp* *Micrococcus*	*A. niger* *A. flavus*
Yam 4	*Micrococcus sp* *Bacillus sp*	*Yeast*	**Yam 4**	*Bacillus sp* *Proteus sp* *Micrococcus sp*	*Penicillium sp*

IV. DISCUSSION

The results of this study has shown that from the isolation of microbial contaminants in this plant tissue culture laboratory, the extent of microbial contamination in tissue culture laboratory was high. The bacterial occurrence found showed an indication that the bacterial contaminations may be sourced from sources relating tofaecal contamination within the tissue culture laboratories.

According to Cheng *et al*. (2014), the laboratory walls, tables and cabinets may have been the source of most of the contaminating microbes. Also, these microbes are living biological contaminants, which can be transmitted by infected people, plants and indoor air, and can also travel through the air and get inside buildings. Bacteria species like *Staphylococcus* sp are mostly found on human skin (Del *et al*., 2014). *S. aureus* is emitted from the nasopharynx of normally healthy individuals when the person talks and is commonly found in air, water, and the skin (Ewing and Green, 2011). *Pseudomonassp* has been reportedly associated with wet surfaces of air- conditioning systems, cooling coils drain pans and sump pumps (Tokuyasu, *et al.,* 2012). Poppert, *et al.* (2015) reported the isolation and characterization of thirty-one microorganisms from different laboratory locations including walls, tables and floor.*Corynebacterium sp* and *Pseudomonassp* were predominant. Libert*oet al.* (2011), identified *Bacillus sp, Corynebacterialsp* and *Actinomycete* fungi as contaminant in the laboratory cabinets.

The results of this study pointed to the fact that most of these microbial population could have originated from the preparatory rooms rather than the incubating rooms. This might be attributed to the fact that more people enter the preparation room. Mitsuko *et al.* (2005) discovered that presence of bacteria in a room with larger presence of people and he further stated that their levels may get high when the building is heavily populated. Fungal contaminants were also found associated with the tables, walls and humans gloves. Typically, fungi made up two-thirds of all of airborne, living micro-organisms (Saglani*et al*., (2005). Miller *et al*. (2007) had isolated *Cladosporium, Penicillium, Aspergillus* and *Alternaria* as the most common fungi in biosafety cabinets. Regularly used furniture has been reposted as a major source of fungal spores (Zoumat*et al*.,2006). Shade around the house has also been reported to increase indoor fungi counts fivefold (Sakai, 2004). Fungi usually grow anywhere indoor especially where there is moisture and a food source. Many building material consist of cellulose materials that are particularly suitable for fungi growth when they are wet. Other materials that also support fungi growth include dust and paints.

Through microscopic observations, different species of fungi were identified from the studied laboratory cultures. These include *Cladosporiumsp, Penicillium* sp, *Aspergillus* sp and *Alternaria* sp. Odutayo*et al*. (2004) isolated *Pseudomonas syringaepvphaseolicoli, Bacillus licheniformis,* B *subtilis, Corynebacterium sp* and *Erwin sp* from biosafety laboratory, some of which is similar to what was found in this study. The fungal isolation in this study therefore confirms that it is a common contaminant. The fungus has also been reported to cause opportunistic pathogens, which is associated with pulmonary infection in immune-compromised host (Zoumat*et al.,* (2006). *Cladosporium* and *Alternaria* were also isolated in the study by Odutayo*et al.* (2004). Although some of his findings have been associated with infection in immune-compromised hosts (Zoumat*et al*., 2006). However, the majority of fungal organisms identified in this study are environmental in origin with little or no clinical relevance. After disinfection of most explants to be cultured with a disinfectant, it has been found out that *Shigella* sp, *Pseudomonasaeruginosa, Bacillus* sp and *Saphylococci* sp were resistant in the tissue culture laboratories.

Penicillium which is associated with infections including pneumonia especially in immune-compromised individuals was also isolated in this study. Whitewell*et al*., (2008) have also reported fungal contaminants of plant tissue culture. Presence of large number of selected pathogenic bacteria in this study shows that the laboratory staff/personnel do not follow safety rules.

V. CONCLUSION

In conclusion, it was observed that each sample collected contained more than one microbial contaminants and the isolated bacteria / fungi contaminants are similar based on morphological and biochemical characteristics among the plant tissue culture laboratories. Also, the bacteria persistent to disinfection isolated were *Pseudomonas* sp, *Staphylococcus aureus,* and *Shigella* sp.

Thus, this study has shown that the Ibadan-based plant tissue culture laboratory is contaminated with a wide variety of potentially pathogenic bacteria and harmful fungus. Therefore, the hypothesis is accepted. Bacterial and fungal contamination remain a continuing threat in plant tissue culture laboratories although several techniques for reducing contaminations are available. It is therefore imperative to

intensify efforts to get rid of these incessant contaminants for a successful micropropagation activities in the studied laboratory.

CONFLICT INTEREST

The author declares no conflict of interest.

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