Research Paper

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International Journal on Agricultural Sciences

International Journal on Agricultural Sciences 12 (1) : 8-12 ***January - June 2020***
ISSN No.: **0976-450X**; NAAS Rating **2.60**
https://doi.org/10.53390/ijas.v11i1.2

BACTERIAL AND FUNGAL CONTAMINANTS OF TISSUE-CULTURED 'LAKATAN' BANANA

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Received: **20.05.2020** Revised: **10.06.2020** Accepted: **15.06.2020**

ABSTRACT

This study aimed to characterize the bacterial and fungal contaminants of tissue-cultured 'Lakatan' banana (*Musa acuminata*) during initiation stage. This was conducted at the University of Southeastern Philippines, Tagum-Mabini Campus from October 2015 to February 2016. The sterilized banana explant was placed in a bottle of solidified nutrient agar medium. Detectable bacterial and fungal contaminants were isolated and sub-cultured into the fresh medium two to four days after incubation. The bacterial isolates were cultivated by streaking into fresh culture medium and incubated at 30-32^{0}C for three days and fungi culture disk was transferred to fresh culture medium and incubated at 30-32^{0}C for three to five days. Both bacterial and fungal contaminants were identified and characterized and assessed for extent of contamination. Results showed that the different contaminants occurred during the initiation stage of tissue-cultured 'Lakatan' banana meriplants were composed of *Rhizopus* sp., unidentified fungus and Gram-negative bacterium. Generally, 35% contamination was observed on this stage.

Keywords: Bacteria, Fungi, Contaminants, Tissue-culture.

INTRODUCTION

Banana (*Musa sapientum*) is the fourth most important crop worldwide for developing countries, where they provide an important starch source (PCARRD 1992). They are one of the most essential staples in tropical areas and their production for sale in local market (Frison and Sharrock 2000). According to World Trade Statistics, Ecuador, Philippines, and Costa Rica are the top 3 major exporters of dessert bananas in 2006.

'Lakatan' is the most highly priced cultivar in the Philippines and is traded locally. It is mainly used as food supplement. Nowadays, 'Lakatan' is considered as one of the most important banana cultivars in domestic and export market, moreover, it is the leading fruit crops in terms of volume, area and value of production with the national average yield of 9.4 tons/ha (PCARRD 2004).

The tissue cultured technique developed by Damasco and Barba (1984) was further modified to sustain and become economically viable in vitro propagation system for banana. This technology was developed for the purpose of producing a high and disease-free plant for wide areas. Furthermore, the tissue-cultured derived plants perform much better in terms of growth, vigor and yield. Hence, 'Lakatan' banana plantlets are now being mass produced through tissue culture to increase its planting materials and meet the increasing demand.

Plant in vitro micropropagation is an aseptic technique for rapid multiplication of pest-free plant materials from organs, tissue and cells of desirable plants (Vuylsteke and De Langhe 1985). Microbial contamination is one of the major challenges facing plant in vitro propagation during different stage of culture processes such as culture

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initiation and sub-culturing. Sub-culture process is a major source of contamination with about 5-15% of contaminants being introduced for every sub-culture (Leifert 1991; Leifert and Cassels 2001). The major causes of the microbial contamination are insufficient sterilization of explants, growing media, working tools and operators' hand (Omamor *et al.,* 2007). The principal microbial contaminants frequently reported in plant in vitro cultures are bacteria and fungi (Cassels 1996; 2001).

Hence, this study was conducted to identify and characterize the contaminants of tissue-cultured 'Lakatan' banana during initiation stage.

MATERIALS AND METHODS

Duration and Location of the Study

The study was conducted at the Tissue Culture Laboratory and Crop Research Laboratory of the University of Southeastern Philippines, Tagum-Mabini Campus, Mabini Unit, Pindasan, Mabini, Compostela Valley Province from October 2015 to February 2016.

Identification and Characterization of Microbial Contaminants

The suckers of 'Lakatan' were collected from the University of Southeastern Philippines, Mabini, Compostella Valley Province. Each sucker was cut into one to three inches' quarters. For each treatment, four to five explants (20 culture media) were used. The isolation room was maintained in aseptic condition or free from any contamination. The laminar floor was sprayed with 70% ethanol. The UV light was turned on for 30 minutes before use of the isolation room.

The media and glass wares were sterilized for 15 minutes using pressure cooker at 15 psi. The modified MS media was used and added with the different rates of antibiotics depending on the treatments used and pH was adjusted to 5.7 (Damasco and Barba 1985) before sterilization for 15 minutes.

Establishment and Stabilization of Explants in Culture

This was done by selecting healthy suckers of 'Lakatan' used for tissue culture. Young suckers (50 cm-100 cm) that are disease-free were collected. The suckers collected from the field were washed in tap water and air-dried. The upper middle portion and the outer bracts of the suckers were removed with sharp knife and the remaining basal portion was washed with commercial bleach solution. The next layers of leaves and excess corm tissues were removed to obtain a block measuring six to eight cm long, three to five cm in diameter and soaked in commercial bleach solution for 20 minutes.

Under aseptic condition inside the laminar flow, superfluous tissues were removed by trimming away the tightly overlapping leaf sheaths and bases, exposing the meristemic cells in between the leaf bases. The shoot tip is decapitated and a block of tissue about 1.5 cm 3 was excised, divided into four quarters and inoculated unto the multiplication medium. The cultures were labeled, transferred to the growing culture room and incubated at 26-28 0C with 16-hour light/dark cycle for four weeks. During incubation in the growth room, cultures were inspected for contamination and mortality of explant tissues.

Isolation of Bacterial and Fungal Contaminants

Each of the sterilized explant was placed in a bottle of solidified nutrient agar medium. Detectable bacterial and fungal contaminants were isolated and sub-cultured into the fresh medium two to four days after incubation. The bacterial isolates were cultivated by streaking into fresh culture medium and incubated at 30-32 0C for three days. For fungi, culture disk was transferred to fresh culture medium and incubated at 30-32 0C for three to five days.

Purification of Bacterial and Fungal Contaminants

Detected fungal contaminants with highest frequency of occurrence were inoculated unto Potato Dextrose Agar (PDA) while bacteria were inoculated in Potato Sucrose Agar. The isolates were purified by series of transfers to fresh culture medium. Identification of fungal contaminants was done four to seven days after transferring into fresh medium when pure cultures were obtained. Bacterial contaminants were preceded to Gram staining after two to three days to identify the bacteria that are gram positive or gram negative.

Gram Staining Procedure

This was done by placing the slide with heat-fixed smear on staining tray. The smear was flooded gently with crystal violet and let stand for one minute, and the slide were tilt slightly and gently rinsed with tap water or distilled water using a wash bottle. The smear was gently flooded with Gram's iodine and let stand for one minute. The smear appears and purple circle on the slide, was decolorized using 95% ethyl alcohol or acetone. The slide was tilted slightly and applied with alcohol drop by drop for five to ten seconds until the alcohol ran almost clear. Care was done not to over-decolorize, then immediately rinsed with water. It was gently flooded with safranin to counter-stain and let stand for 45 seconds. The slide was tilted slightly and gently rinsed with tap water or distilled water using a wash bottle. Then slides were blot dried with bibulous paper. Lastly, smear was viewed using a light-microscope

under oil-immersion. Gram-negative bacteria appeared red or pink following a Gram stain procedure due to the effects of the counterstain (for example safranin) and gram positive bacteria appeared blue-violet.

Data Gathered

Number of days to appearance of microbial contaminants was taken by counting the number of days that the fungal and bacterial contaminants appeared on the test medium.

Percentage of contaminated growing media was computed by counting the contaminated culture media from the total culture and computed using the following formula:

Percent Contamination = No. of contaminated culture media / Total no. of culture media x 100

Frequency of Occurrence of Contaminants in Culture medium was determined by the number of times a contaminant appeared on the culture medium. Fungal contaminants were identified according to their genera while bacterial contaminants were identified either as gram positive or gram negative.

Bacterial contaminants were described on the bases of their colony form, colony color, colony texture, colony elevation, colony margin or edge, and colony color. Colony form maybe circular, irregular, filamentous and rhizoid or curled. Colony texture maybe dry, moist, mucoid, brittle, viscous, butyrous (buttery) etc. Colony elevation was described on the side view of a colony as elevated, convex, concave, umbonate/umbilicate. Colony margin or edge maybe entire, undulate, crenated, fimbriate or curled. Colony color could be yellow, white, pink, green etc. Colony structure as opaque, translucent or transparent.

Fungal contaminants were described based on their colony form, colony elevation, colony margin, colony surface, and colony texture and colony color. Colony form maybe described as circular, irregular, filamentous, and rhizoid. Colony elevation maybe raised, convex, flat, and crater form. Colony margin maybe described as entire, undulate, filiform, curled and lobate. Colony surface as smooth, glistening, rough, wrinkled or dull and colony texture as cottony, dry and etc. Colony color could be white, buff, red, black, purple and etc.

The data were analyzed using Analysis of Variance (ANOVA) and the differences among the treatment means were compared using Honest Significant Difference (HSD) test when significant findings were obtained from ANOVA.

RESULTS

Bacterial and fungal contaminants were observed during the initial stage of micropropagation of tissue-cultured 'Lakatan' banana (Table 1). After 11 days from initiation, the fungal contaminant appeared was suspected as Rhizopus sp. in two culture media.

Table 1 also revealed the percentage of contaminated growing media of tissue cultured 'Lakatan' banana. After 30 days of initiation stage of tissue-cultured 'Lakatan' banana, the result showed 10% of suspected Rhizopus sp., 15% of unidentified fungus and 10% of bacterial contaminants for a total of 35% of contamination.

Suspected Rhizopus sp. was observed twice, while the unidentified fungus appeared thrice in 20 culture media. It was also noticed that bacterial contaminant occurred twice in 20 culture media.

The first . (Figure 1). The unidentified fungal contaminant

Table 1: Percentage, Occurrence, and Days appeared of microbial contamination by different microorganism during initiation stage of Tissue-cultured 'Lakatan' Banana in 20 Culture Media.

Suspected Contaminants	Percentage of Contaminated Culture Media	Frequency of Occurrence	Days Appeared
Rhizopus sp.	10%	2/20	11th day
Unidentified Fungus	15%	3/20	13th day
Gram Negative Bacteria	10%	3/20	13th day
Total	35%	7/20	-

in tissue- cultured 'Lakatan' banana has almost the same morphological appearance as Penicillium sp. (Figure 2). The bacterial contaminants appeared in tissue-cultured 'Lakatan' was characterized as a gram-negative bacterium (Figure 3).

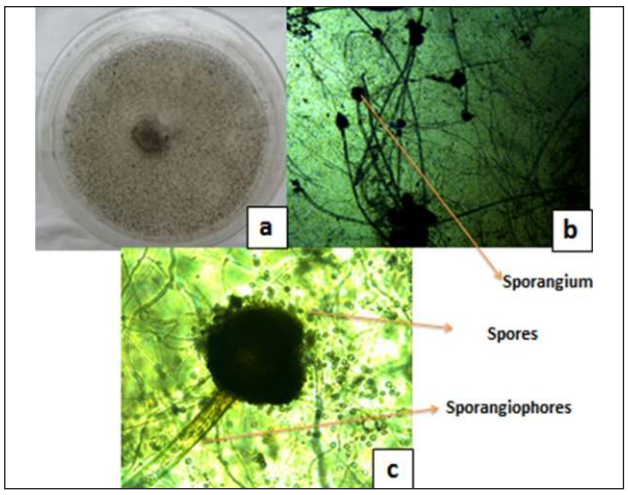

Fig.1: Photomicrograph of *Rhizopus* sp. (a) Pure culture growing on PSA (b) 40X c) 400X.

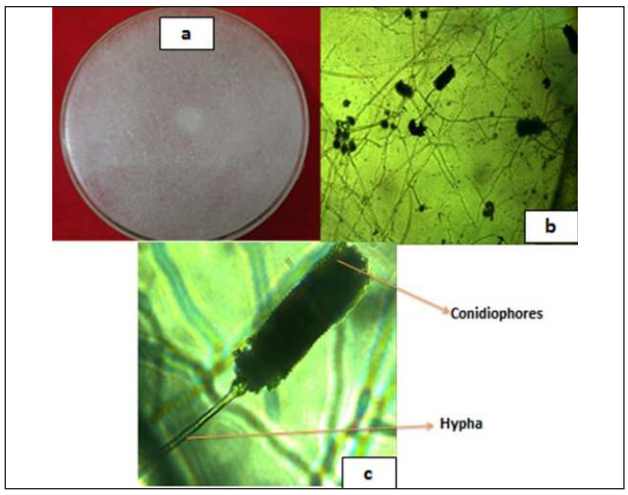

Fig. 2: Photomicrograph of Unidentified fungus, (a) Pure culture growing in PSA (b) 40X (c) 400X.

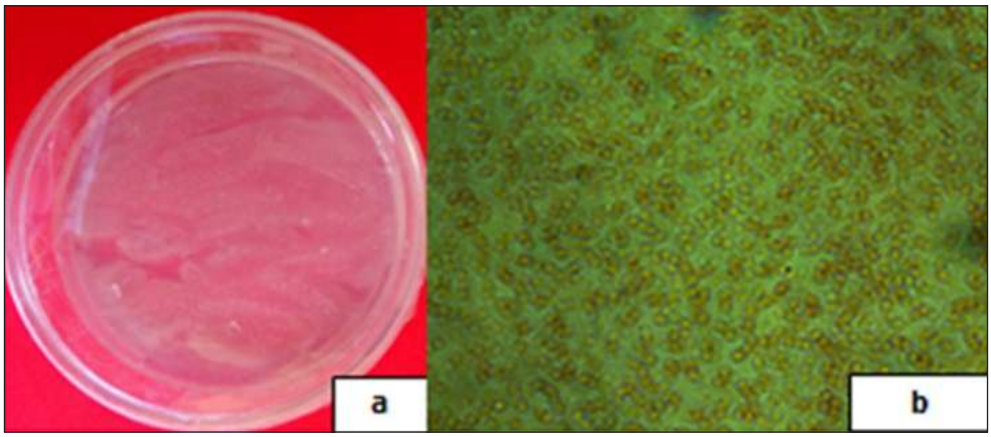

Fig. 3: Gram Negative Bacterium (a) Pure culture growing on PSA (b) Stained Cells (400X).

DISCUSSION

The species of Rhizopus are very common laboratory contaminants and are weak parasites as well as saprophytes to various common substrates (O'Donnel and Kerry 1979). At 13 days after micropropagation, unidentified fungus appeared in three bottles and also bacterial contaminants appeared in two bottles with white/transparent morphological appearance.

As observed, 10% of suspected Rhizopus sp., 15% of unidentified fungus and 10% of bacterial contaminants for a total of 35% of contamination occurred after 30 days of initiation. This observation coincides with the encountered problem of the Tissue Culture Laboratory of the University of Southeastern Philippines, based on personal communication with the laboratory technicians.

Sub-culture process is a major source of contamination with about 5-15% of contaminants being introduced for every subculture (Leifert 1991; Leifert and Cassels 2001). The major cause of the microbial contamination is insufficient sterilization of explants, growth media, working tools and operators' hands (Omamor et al. 2007). According to the study of Leggatt et al. (1994) approximately 31 micro-organisms from 10 different plant cultivars growing in micro-propagation have been isolated identified and characterized, with Yeasts, Coryne-bacterium spp. and Pseudomonas spp. being predominant.

The Rhizopus sp. mycelia were aerial and creepin type. Stolons developed rhizoids at certain points or upon contact with solid base. Sporangiospores were long and upright. The sporangia are spherical containing minute aplanospores which are easily dispersed with the breaking of the sporangiosphores envelop (O'Donnel and Kerry 1979).

The unidentified fungal contaminant has conidiophores arising from the mycelium or less often synnemata. It is branched near the apex to form a brush-like conidia-bearing apparatus, ending in phialides which pinch off conidia celled, mostly globose or ovoid and produced basipetally.

The bacterial contaminants that appeared are Gram-negative bacteria which are bacteria that do not retain the crystal violet dye in the Gram stain protocol. Gram-negative bacteria appeared red or pink following a Gram stain procedure due to the effects of the counterstain (for example safranin).

The principal microbial contaminants frequently reported in plant in vitro cultures are bacteria and fungi (Cassels 1996). Pseudomonas syringae, Bacillus licheniformis, Bacillus subtilis, Cornebacterium sp. and Erwinia spp. have been reported to be the major bacterial contaminants in plant tissue cultures (Odutayo et al. 2004) while the main fungal contaminants frequently observed in plant tissue cultures are Alterneria tenius, Aspergillus niger, Aspergillus fumigatus and Fusarium culmorum (Odutayo et al. 2004; Odutayo et al. 2007).

CONCLUSION

The fungal and bacterial contaminants appeared from 10th to 13th day at initiation stage of micropropagation with a 35% of contamination. The identified contaminants were Rhizopus sp., un-identified fungus and a gram negative bacterium.

CONFLICT OF INTEREST

"The authors declare no conflicts of interest".

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