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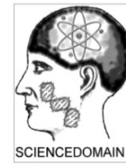

# Common Fungi Contamination Affecting Tissue-cultured Abaca (*Musa textiles* Nee) during Initial Stage of Micropropagation

**Jojine S. Cobrado[1] and Alminda M. Fernandez[1*]**

*[1]University of Southeastern Philippines, Tagum-Mabini, Mabini Unit, Compostela Valley Province 8807, Philippines.*

***Authors' contributions***

*This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.*

***Article Information***

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## ABSTRACT

Philippines is the world's largest producer of abaca fiber, accounting for about 85% share of the global production in 2013. Plant tissue culture technology is being widely used for a large scale plant multiplication. Even though it is possible to produce a large number of plants by micro popagation, the greatest problem in this technique is contamination. A wide range of microorganisms (filamentous fungi, yeast, bacteria, viruses and viroids) and micro-arthropods (mites and thrips) have been identified as contaminants in plant tissue cultures. Contaminants may be introduced with the explant, during manipulation in laboratory, by micro-arthropods vectors.

The study was conducted at the Tissue culture laboratory and Research laboratory of the University of Southeastern Philippines, Mabini Unit, Pindasan, Mabini, Compostela Valley Province, Philippines to identify and characterize the fungal contaminants of abaca *in vitro* cultures. The plant tissue culture used MS medium sterilized using pressure cooker at 15 psi for 20 minutes. The explants were excised and surfaced sterilized with Sodium hypochlorite (Sigma-Aldrich Chenire) for three minutes. The excised explants were aseptically transferred to the culture medium in 20 culture bottles, labeled and incubated at 24℃ for th ree weeks at the growth room. Fungi

*Corresponding author: E-mail: alminda.fernandez@usep.edu.ph;

contaminants were immediately transferred and pure cultured unto PDA (Potato Dextrose Agar) at 28℃ for three to seven days. Microscopic fungi were identified using diagnostic keys. For identification purposes, slide cultures were prepared on malt extract agar and stained with lactophenol-blue.

Two species of fungi were identified as fungal contaminants of the tissue-cultured abaca in the initial stage of micropropagation. Of these genera, *Chrysosporium* sp. like fungus occurred most frequently (10%), followed by *Aspergillus* sp. (5%). These fungal species were found to cause death of the culture material by some probable sources of contaminations such as handling of plant materials, culture vessels and the laboratory.

*Keywords: Fungi; contamination; abaca; tissue culture; Musa textiles.*

## 1. INTRODUCTION

Philippines is the world's largest producer of abaca fiber, accounting for about 85% share of the global production in 2013. In the Philippines, abaca plants are cultivated across 130 thousand hectares of land by over 90 thousand farmers. Abaca fiber is primarily used as a raw material by end industries such as pulp and paper, fiber craft, cordage etc.

Abaca plantlet propagation is now practiced worldwide by cloning through tissue culture. Plant tissue culture technology is being widely used for a large scale plant multiplication. The commercial technology is primarily based on micro propagation, in which rapid proliferation is achieved from tiny stem cuttings, axillary buds and to limited extents from somatic embryos, cell clumps in suspension cultures and bioreactors. The primary advantage of micro propagation or tissue culture is rapid production of high quality, disease-free and uniform planting materials. The plants can be multiplied under controlled environment, anywhere, irrespective of the season of the reason and weather, on a year round basis. Even though it is possible to produce a large number of plants by micro popagation, the greatest problem in this technique is contamination. A wide range of microorganisms (filamentous fungi, yeast, bacteria, viruses and viroids) and micro-arthropods (mites and thrips) have been identified as contaminants in plant tissue cultures. Contaminants may be introduced with the explant, during manipulation in laboratory, by micro-arthropods vectors [1-3].

Frequently encountered bacterial and fungal contamination especially in laboratories of commercial micropropagation posed a considerable problem [4]. Tissue cultures can become contaminated at any stage of tissue culturing process [1]. Hence, this study was conducted for the first time to identify and characterize the fungal contaminants of abaca *in vitro* cultures.

## 2. MATERIALS AND METHODS

### 2.1 Plant Material

The suckers of *Musa textiles* were collected from the 'Abaca' area of the University of Southeastern Philippines, Mabini, Compostella Valley Province. Each sucker was cut into 1-3 inches quarters. For each treatment, 4-5 explant (20 media culture) was used.

### 2.2 Laboratory Site Preparation

The isolation room was maintained in aseptic condition or free from any contamination. The laminar floor was sprayed with 10% zonrox/ ethanol. The UV light was turned on for 30 minutes before use of the isolation room.

### 2.3 Culture Media

The media and glass wares were sterilized for 15 minutes using pressure cooker at 15 psi. The modified MS media [5] was used and added with the different rates of antibiotics depending on the treatments used and pH was adjusted to 5.9 before sterilization for 15 minutes.

### 2.4 Establishment and Stabilization of Explants in Culture

This was done by selecting healthy suckers of abaca hybrid 7 for tissue culture. Young suckers (50 cm-100 cm) that are diseased-free were collected. Abaca 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 explants were excised and surfaced sterilized with Sodium hypochlorite (Sigma-Aldrich Chenire) for three minutes [6]. The next layers of leaves and excess corm tissues were removed to obtain a block measuring 6 to 8 cm long, 3 to 5 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 $cm^3$ is excised, divided into four quarters and inoculated unto the multiplication medium. The meriplant cultures were labeled, transferred to the growing culture room and incubated at 26-28℃ with 16 hours light/ dark cycle for four weeks. During incubation in the growth room, cultures were inspected for contamination and mortality of explant tissues.

### 2.5 Isolation of Fungal Contaminants

Each of the sterilized explant was placed in a bottle of solidified nutrient agar medium without antibiotic. Detectable fungal contaminants were isolated and subcultured into the fresh medium two to four days after incubation. The fungal culture disks were transferred to fresh culture medium and incubated at 30-32℃ for three to five days.

### 2.6 Purification of Fungal Contaminants

Detected fungal contaminants with highest frequency of occurrence were inoculated unto Potato Dextrose Agar (PDA). The isolates were purified by series of transfers to fresh culture medium. Identification of fungal contaminants was done 4-7 days after transferring into fresh medium when pure cultures were obtained.

### 2.7 Number of Days to Microbial Colonies Appearance

This was taken by counting the number of days that the fungal contaminants appeared on the test medium.

### 2.8 Percentage of Contaminated Culture Media

This was done by counting the contaminated culture media from the total culture and computing the percentage.

### 2.9 Frequency of Occurrence of Contaminants

This was determined by the number of times a contaminant appeared on the culture medium.

### 2.10 Identification and Characterization of Microbial Contaminants

Microscopic fungi were identified using the diagnostic keys [7,8]. The technique adopted for identification of the unknown isolated fungi used cotton blue in lactophenol stain [9]. The identification was achieved by placing a drop of the stain on clean slide with the aid of mounting needle, where a small portion of mycelium from the fungal cultures was removed and placed in a drop of lactophenol. The mycelium was spread very well on the slide with the aid of the needle. A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted and observed with x10, x40 and x100 objective lenses respectively.

## 3. RESULTS AND DISCUSSION

### 3.1 Number of Days to Microbial Colonies Appearance

Microbial colonies of contaminants were observed during micro propagation of tissue-cultured abaca hybrid 7 (*Musa textiles* Nee). Microbial symptoms were observed based on their colony shape, colony margin/ elevation and colony color in 20 bottle test media during one month (Initial stage) observation period. The bacterial and fungal contaminants in laboratories of commercial micro-propagation posed a considerable problem [3]. Moreover, the tissue culture laboratory of the University of Southeastern Philippines also encountered both bacterial and fungal contaminants of tissue-cultured abaca hybrid 7 (Musa textiles Nee). Suspected *Aspergillus* spp. (Fig. 1) appeared after 10th days from initiation stage of tissue-cultured abaca hybrid 7. In addition, *Chrysosporium* spp. (Fig. 2) was observed on the 12th day and 14th day from initiation stage and gram negative bacteria was observed during the fourth day from initiation stage. In addition, tissue-cultured meriplants can become contaminated at any stage of tissue culture process [10,11]. Fungus may arrive with an explant, or airborne, or enter a culture [12]. The presence of people and their levels may get high when the building is heavily populated.

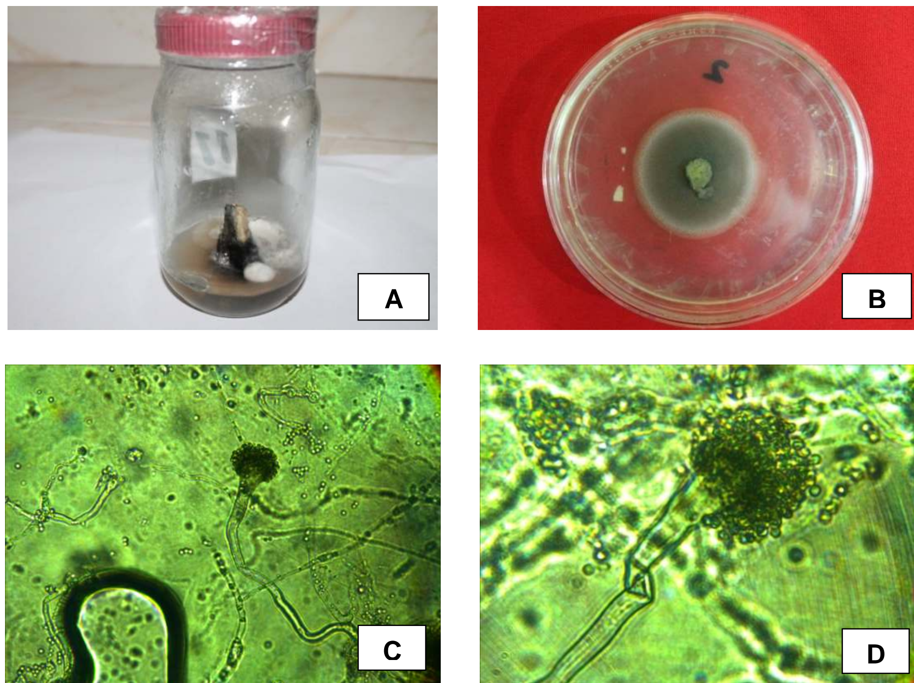


**Fig. 1. Fungal contaminant, *Aspergillus* sp. (A) in Murashige and Skoog culture medium; (B) Pure culture in Potato Dextrose Agar (PDA); (C) Photo micrograph 80x magnification; (D) 400x magnification**

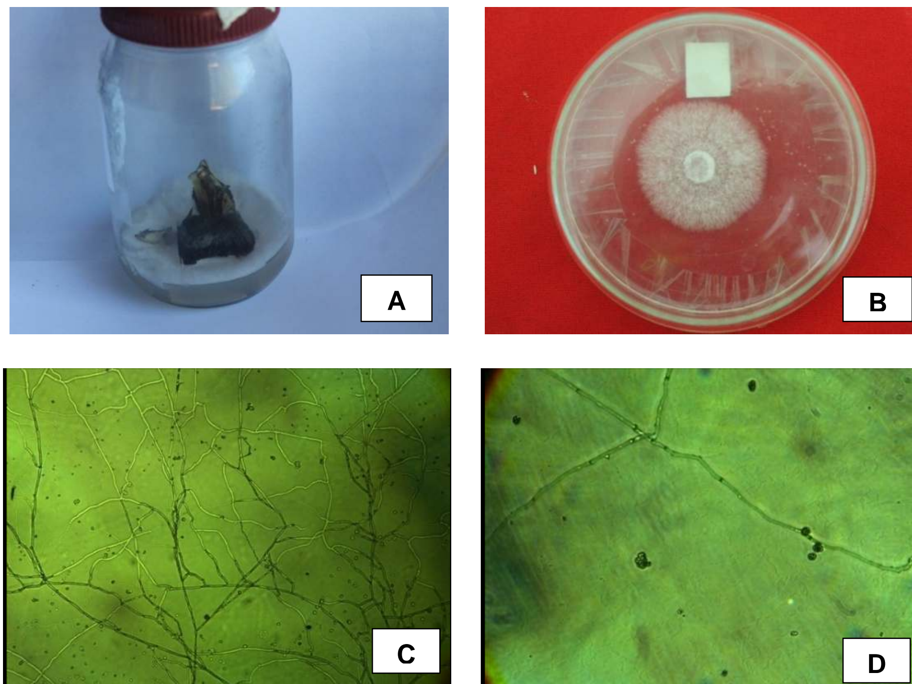


**Fig. 2. Fungal contaminant, *Chrysosporium* sp. (A) in Murashige and Skoog culture medium; (B) Pure culture in Potato Dextrose Agar (PDA); (C) Photo micrograph 80x magnification; (D) 400x magnification**

### 3.2 Percentage of Contaminated Culture Media

The percentage of contaminated media culture of tissue-cultured abaca hybrid 7 (*Musa textiles* Nee) during initial stage revealed that, in every 20 culture media, 10 percent of fungal contaminants appeared. Hence, 10 percent of fungal contamination naturally occurred during initial stage of micropropagation. Based on the observation of the tissue-cultured laboratory technicians of University of Southeastern Philippines, they encountered approximately 8.6% of fungal contaminants of tissue-cultured *Musa* spp. during initial stage. The sub-culture process is a major source of contamination with about 5-15% of contaminants being introduced for every subculture [11]. The major cause of the microbial contamination is insufficient sterilization of explants, growth media, working tools and operators' hands [13].

### 3.3 Frequency of Occurrence of Contaminants

Two fungal contaminants were found associated with abaca hybrid 7 tissue-cultured plants at the University of Southeastern Philippines tissue-culture laboratory. The fungi were suspected *Aspergillus* sp and *Chrysosporium* sp. Despite the fact that some of these contaminants might be endogenously embedded in the plant tissues [14], some might also have emanated from contaminated tools, which were not investigated. Tissue culture vessels are always closed with loose fitting caps in order to allow gaseous exchange with the external environment. However, mites and thrips carrying fungal spores and bacteria in and on their bodies, often gain entry through this loose fittings and travel from one vessel to another thereby contaminating the cultures. The fungal contamination of cultures is usually the first sign of a mite or thrip infestation [15]. Hence, proper sanitation and effective use of appropriate pesticides to control mites and thrips in tissue-cultured laboratories will be desirable. Thorough disinfections and strict hygiene in the laboratory have achieved effective control of microbial contaminants. Movement of people within the preparatory and incubating rooms in tissue culture laboratory should be reduced significantly to avoid the spread of contaminants.

### 3.4 Characterization and Identification of Fungal Contaminants

The principal microbial contaminants frequently reported in plant *in vitro* cultures are bacteria and fungi [16]. The main fungal contaminants frequently observed in plant tissue cultures are *Alterneria tenius*, *Aspergillus niger, Aspergillus fumigatus and Fusarium culmorum* [17,18].

The fungal contaminants of tissue-cultured abaca hybrid 7 (*Musa textiles* Nee) *in vitro* cultures at tissue culture laboratory of University of Southeastern Philippines were identified as *Aspergillus* sp. (Fig. 1) and fungal *Chrysosporium* sp. (Fig. 2). Table 1 shows the cultural and morphological characteristics of both fungi.

**Table 1. Characterization and identification of fungal contaminants of tissue-cultured abaca (*Musa textiles* Nee)**

| Contaminants | Cultural characteristics | Morphological characteristics |
|---|---|---|
| *Aspergillus* sp. | Colonies are flat, circle, filamentous, velvety, wolly or cottony texture. Colony color is gray to green at center with a white border. The reverse is yellow to pail yellow. | Conidiophores bear heads, long and hyaline that terminates in bubous heads while conidia are globose to subglobose and usually yellowish green and dark brown. |
| *Chrysosporium* sp. | Colonies are semi-elevated, circle, fairly rapid grower, smooth. Colony color is white to off-white. The reverse is white to off-white color. | Produced septate, hyaline hyphae. Conidia often appeared to be minimally differentiated from the hyphae and may appear to form directly on the hyphae. Conidia more often formed at the ends of simple or branched conidiophores of varying lengths. Conidiophores were ramified, forming tree-like structures. |

*Aspergillus sp.* with colonies that are flat, circle, filamentous, velvety, wolly or cottony texture. Colony color is gray to green at center with a white border. The reverse is yellow to pail yellow. Conidiophores bear heads, long and hyaline that terminates in bubous heads while conidia are globose to subglobose and usually yellowish green and dark brown. These are exogenously found in soils, water and plant surfaces [16] but are also endophytes in some plant species and also were found in internal tissues of mallow plants [19]. Moreover, *Aspergillus* spp. is one of the major contaminant in tissue -culture of *Lilium candidum, Vigna* sp., *Musa* sp., *Manihot* sp., *and Hibiscus* sp. [18].

*Chrysosporium spp* have semi-elevated, circle, fairly rapid grower, smooth colonies. Colony color is white to off-white. The reverse is white to off-white color. It produced septate, hyaline hyphae. Conidia often appeared to be minimally differentiated from the hyphae and may appear to form directly on the hyphae. Conidia more often formed at the ends of simple or branched conidiophores of varying lengths. Conidiophores were ramified, forming tree-like structures. It is a keratinophilic filamentous fungus commonly isolated from soil where it lives on remains of hairs and feathers [19]. While *Chrysosporium* sp. are occasionally isolated from nail scrapings, skin, especially from feet, and may cause skin infections and onychomycosis in humans, but because they are common soil saprophytes they are usually considered as contaminants. They are also occasional contaminants of respiratory samples. Furthermore, fungal contaminants has associated in human skin and can be transmitted during micro-propagation process [18].

Microbes are living, biological contaminants that can be transmitted by infected people, animals and indoor air, and they can also travel through the air and get inside homes and buildings. The occurrence of exogenous fungal and bacterial contaminants in abaca hybrid 7 (*Musa textiles* Nee) *in vitro* cultures in this study was possibly due to an inadequate surface sterilization. Several studies have also associated the incidence of exogenous fungal contaminants in plant *in vitro* cultures with an insufficient sterilization [16]. Therefore, contamination were possibly due to an insufficient aseptic places among workers during tissue culture operations.

## 4. CONCLUSION

Two species of fungi were identified as fungal contaminants of the tissue-cultured abaca in the initial stage of micropropagation. Of these genera, *Chrysosporium* sp. like fungus occurred most frequently (10%), followed by *Aspergillus* sp. (5%). These fungal species were found to cause death of the culture material by some probable sources of contaminations such as handling of plant materials, culture vessels and the laboratory.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

Approval for this research was given by the head of department.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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