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# Endophytic fungi in *Billbergia pyramidalis:* A preliminary survey

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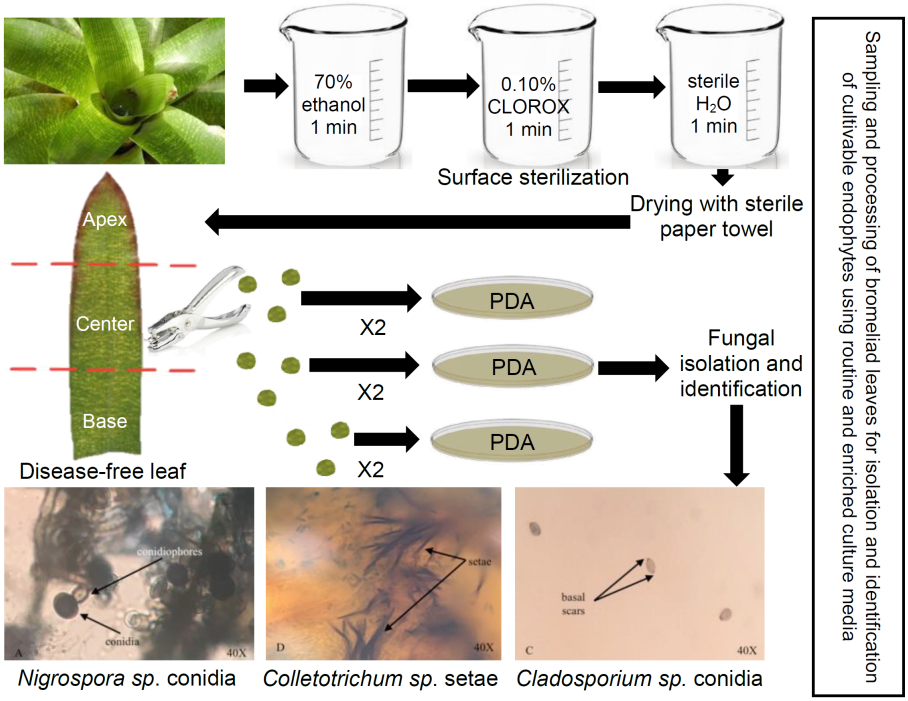
Despite the high value of bromeliads as ornamental plants, as well as habitat for invertebrates and amphibians in their juvenile stages, the occurrence of fungal endophyte communities in the leaves of these plants has not been studied in Puerto Rico. Endophytic fungi may provide their host plant defense against biotic and abiotic factors, through the production of bioactive compounds and enzymes that compete for space within the infected plant cells. Bioactive compounds produced by endophytic fungi also increase the plant's defenses and its ability to avoid pathogens. Three samples were collected to study the endophytic fungi community associated with *Billbergia pyramidalis,* an ornamental bromeliad. The identity and frequency of endophytic fungi were determined by evaluating sterilized superficial tissue fragments from three individuals free of visible symptoms of disease or damage. The rate of fungal infection per sampled leaf and the frequency of the morphotypes isolated from the plant tissue were calculated. The most prevalent endophytes were *Colletotrichum sp,* morphotype M05*,* and *Nigrospora sp.*




## Introduction

Bromeliads are epiphytic plants with approximately 2,900 identified species (Frank et al., 2010). Some bromeliads contain water at the base of the leaves, forming a pool-like structure that accumulates water. Bromeliads make up tropical forests and arboreal perches, although climatic conditions can cause them to grow in the soil of these environments. The species cultivated on land grow by roots that give them support and capture nutrients such as atmospheric nitrogen through cells in the sub axillary part of its tissue. Bromeliads have the ability to reproduce in soils while their leaves are not in a state of deterioration, and as long as it receives sufficient light (Pett-Ridge and Silver, 2002). An indication of the plant's state of deterioration or sickness is the presence of foliar spots on its leaves. Bromeliads are usually free of pathogens, but unbalanced environments promote sickening of the leaves (Leahy, 1999).

The term endophyte (*endon*: inside, *phyton*: plant) has been known since the 19th century and applies to fungi that live asymptomatically inside a plant. Endophytes have been recognized as organisms (fungus, bacteria, protest or invertebrates) that during their life cycle invade living plant tissues and cause asymptomatic infections within them (Gamboa-Gaitán, 2006), developing a characteristic and complex symbiotic relationship with their host. Endophytic fungi are a polyphyletic group that inhabits various parts of plants. Although little is known about the fungal distribution in plant tissue, they can be distributed through the plant depending on its biochemical need to coexist (Gamboa-Gaitán, 2006). Fungi-plant relationships can produce potentially toxic secondary metabolites (Sánchez et al., 2013). Environmental factors and the growth stage dictate the availability of the plant and the fungus to coexist in a balanced antagonistic relationship. When

there is a degree of balance, it is called an endophytic relationship. This relationship can contribute to the protection of the host against abiotic and biotic factors directly, indirectly or ecologically. The fungus offers protection by directly producing enzymes and secondary anti-pathogenic metabolites, while indirectly increasing the expression of chemical defense mechanisms in the plant. Although endophytic fungi can benefit the state of its host while maintaining a balance, when it fades in a stressed host, the fungus becomes pathogenic, causing disease to the plant, as seen in bromeliads (Sánchez-Fernández et al., 2013).

Bromeliads can be affected by different types of pathogens, including several fungi. According to Hernández-Romero (2018), five fungal genera have been found predominantly in bromeliad including *Aspergillus sp.*, *Fusarium sp.*, *Penicillium sp.*, *Neopestalotiopsis sp.,* and *Colletotrichum sp.* Between August and October of 2018, three samples were collected from three leaves of *Billbergia pyramidalis* growing in front of the Celis Building at the University of Puerto Rico Mayagüez Campus to study their endophytic fungi community. The methods and results of these preliminary evaluations are summarized below.

## Materials and Methods

*Collection and handling of bromeliad leaves*

Bromeliad leaves from *Billbergia pyramidalis* free of visible symptoms of disease or damage caused by predators or pests were chosen from the garden in front of the Celis Building at the University of Puerto Rico, Mayagüez Campus. *Billbergia pyramidalis*, or the flaming torch, is a species of bromeliad naturalized in Puerto Rico, that can grow terrestrially, creating large clumps or, epiphytically. amples were collected for three leaves: one from a single plant on August 24, 2018, and two from different plants on September 14, 2018. Each leaf was rinsed with tap water to proceed with the surface sterilization protocol; one rinse in 70% ethanol for one minute, then in 0.10% Clorox for one minute and finally in distilled water for another minute. Following Rivera-Méndez & Maldonado-Ramírez (2016) each leaf was then blotted dry with a sterile paper towel.

*Sampling*

After sterilization, each leaf was divided by section: apex, center, and base. With a sterilized puncher, two samples of three fragments were collected per area, for a total of six fragments per area. Three fragments from a single area were placed equidistantly in a Petri dish with Potato Dextrose Agar (PDA) supplemented with lactic acid. The procedure was repeated for each area for a total of 18 fragments/sample (Rivera-Méndez & Maldonado-Ramírez, 2016).

*Sample incubation*

Petri dishes containing the fragments were incubated at 25 ± 2°C. Colony growth was documented at 48 and 72 hours, and observed daily. Control plates were also incubated as culture media control and hood control.

*Colony transferring*

After documenting the growth of colonies from each dish, different colonies were chosen and transferred individually to Petri dishes with PDA supplemented with lactic acid to isolate pure cultures. The dishes were sealed with parafilm and incubated again at 25 ± 2°C. Pure colonies were characterized by color, texture, and pigmentation for easy identification.

*Semi-permanent slide preparation*

As described, from each pure culture, semi-permanent slides were prepared using lactophenol or lactophenol cotton blue. Non-sporulating cultures were exposed to photoperiods (alternate periods of light and dark) to help the sporulation process. Undifferentiated cultures were transferred to specialized media, such as Malt Extract Agar (MEA), Cornmeal Agar (CMA), V8 Agar (V8A) and Oatmeal Agar (OA) or to wet chambers with carnation leaves after being exposed to photoperiods to promote sporulation (Su et al., 2012).

*Identification of fungi*

Mycelial fungi were identified considering microscopic morphological characteristics such as shape and arrangement of phial-

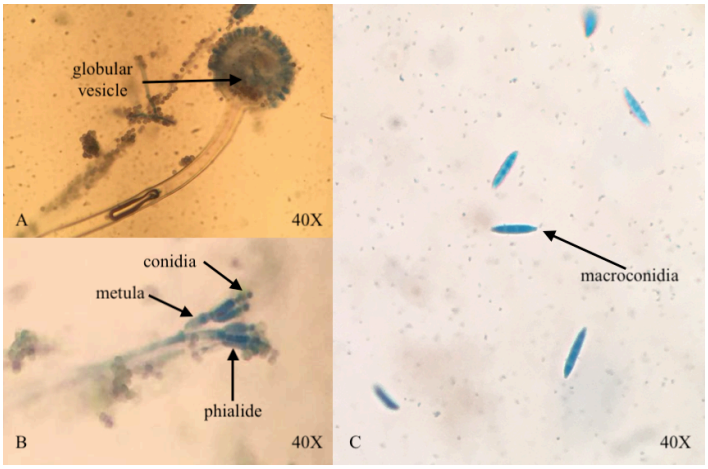



Figure 1: Fungal conidia of *Aspergillus sp.* as endophyte (A), *Penicillium sp.* as hood/control contaminant (B), and *Fusarium sp.* as endophyte (C).

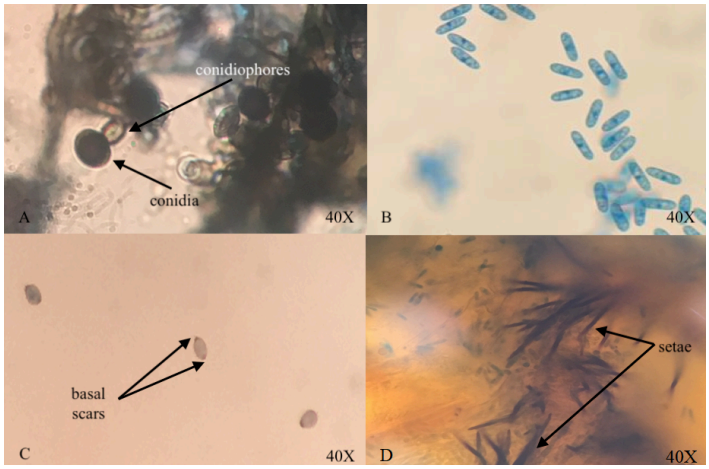



Figure 2: Fungal endophytes from *B. pyramidalis: Nigrospora sp.* (A), *Colletotrichum sp.* Conidia (B), *Cladosporium sp.* (C), and *Colletotrichum sp.* setae (D).

ides, as well as the presence and type of conidia, conidiophores or chlamydospores (Barnett and Hunter 1998). Macroscopic characteristics of the cultures, namely, shape, color, texture, and presence of exudate were determined using Munsell Soil Color Charts (2000). Fungal cultures without any reproductive structures were assigned a number based on their particular morphological characteristics and designated as morphotype.

*Statistical analysis*

Colonization rates were calculated as the percentage of fragments incubated that showed fungus infection, as described by Frolich et al., (2000). The relative frequency of the fungus was calculated as the rate between the colonization rate of the fungus and the most frequently isolated species (Petrini et al., 1992).

## Results

While sampling, controls for the hood and the culture media were prepared. While there was no growth of any contaminant in the medium, two contaminants were found on the hood: *Penicillium sp.*, and another unidentified fungus. From the sampled leaves, seven distinct morphotypes were isolated, but none were identified because they lack any reproductive structures. After growing them on various specialized media (MEA, CMA, V8 Agar, and OA) following Su et al., (2012) to promote sporulation no reproductive structures were observed. Figures 1 and 2 show morphological characteristics of the fungi identified. The total fungal infection rate was 0.30.

Table 1. Frequencies of cultivable fungi from sampled leaves

| Sample | Identified organism | Frequency |
|---|---|---|
| Apex 1 | *Colletotrichum sp* | 0.27 |
| Center 1 | M01 | 0.03 |
| Center 2 | *Aspergillus sp* | 0.05 |
| P12 and P13 | *Fusarium sp* | 0.05 (each) |
| P22 | *Cladosporium sp* | 0.01 |
| P23 A | M02 | 0.05 |
| P23 B | *Aspergillus sp* | 0.05 |
| P1 Apex (1 A and 1 B) | *Colletotrichum sp* | 0.27 (each) |
| P1 Apex 2 A | *Nigrospora sp* | 0.11 |
| P1 Apex 2 B | M03 | 0.03 |
| P1 Apex 3 A | M04 | 0.03 |
| P1 Apex 3 B | *Colletotrichum sp* | 0.27 |
| P1 Center (2 A and 2 B) | M05 | 0.16 (each) |
| P1 Center 2 C | *Cladosporium sp* | 0.11 |
| P1 Center 2 D | *Colletotrichum sp* | 0.27 |
| P1 Center 3 A | M06 | 0.03 |
| P1 Center 3 B | M05 | 0.16 |
| P1 Base 3 A | M02 | 0.05 |
| P1 Base 3 B | *Colletotrichum sp* | 0.27 |
| P2 Apex (1 A and 1 C) | *Nigrospora sp* | 0.11 (each) |
| P2 Apex (1 B and 2 B) | M05 | 0.16 (each) |
| P2 Apex 2 A and P2 Center (1 A, 1 B, 2B) | *Colletotrichum sp* | 0.27 (each) |
| P2 Center 2 A | M05 | 0.16 |
| P2 Center 2 C | M07 | 0.03 |
| P2 Base (2 A, 2 B and 2 C) | *Cladosporium sp, Cladosporium sp and Nigrospora sp* | 0.11 (each) |

Tables 1 and 2 summarize the relative frequencies calculated by genera and the total frequency of fungi identified per leaf section for all the samples evaluated.

Table 2. Frequencies of cultivable fungi by genera and leaf section

| Fungi identified | Frequency | | |
|---|---|---|---|
| | Apex | Center | Base |
| *Aspergillus sp* | 0 | 0.08 | 0 |
| *Cladosporium sp* | 0 | 0.08 | 0.40 |
| *Colletotrichum sp* | 0.42 | 0.31 | 0.20 |
| *Nigrospora sp* | 0.25 | 0 | 0.20 |
| M01 | 0 | 0.08 | 0 |
| M02 | 0 | 0 | 0.20 |
| M03 | 0.08 | 0 | 0 |
| M04 | 0.08 | 0 | 0 |
| M05 | 0.17 | 0.31 | 0 |
| M06 | 0 | 0.08 | 0 |
| M07 | 0 | 0.08 | 0 |

## Discussion

The objective of this research was to identify and determine the most frequent endophytic fungi from sterilized superficial tissue of *Billbergia pyramidalis*. As shown in Table 1, the genera *Colletotrichum sp.*, *Nigrospora sp.*, and *Cladosporium sp.* were identified in higher frequency. Also, a non-identifiable isolate, morphotype 05 (M05) showed a high frequency and was present in two of the leaf sections. Overall, the most commonly observed morphotypes can be seen in Figure 3.

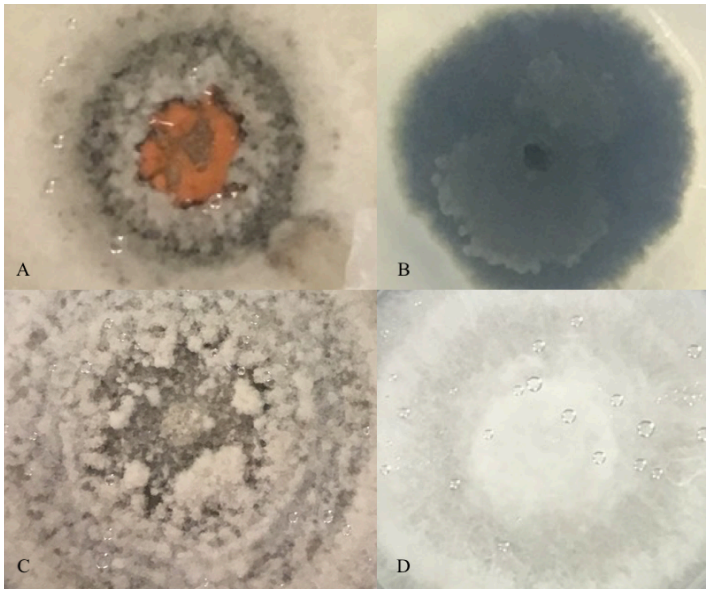



Figure 3: Most common morphotypes isolated from *B. pyramidalis*: *Colletotrichum sp*. (A), M04 (B), *Nigrospora sp*. (C), and M05 (D).

As seen in Table 2, the total frequencies of cultivable fungi per area of *B. pyramidalis* leaves were calculated. Overall, the isolate in highest frequency in the apex and center was *Colletotrichum sp.* While in the base, the isolate in highest frequency was *Cladosporium sp.*, though *Colletotrichum sp.* was present. Also, worth

noting is that the leaf area with the highest cultivable fungi was located at the center, with 64% of the total endophytic community studied.

The different fungi identified have been reported previously as plant pathogens in other ornamental or cultivable plants. If the plant is under unfavorable conditions, it is possible that these fungi will infect them. An infection may result in potential limitation of plant growth and development (Sharma, 2012), as has been reported for plants infected with *Aspergillus sp.*, *Fusarium sp.*, *Colletotrichum sp.*, and *Nigrospora sp.*

## Conclusion

After completing this research, we can conclude that *B. pyramidalis* hosts common genera of endophytic fungi previously reported, including *Colletotrichum*, *Cladosporium*, and *Nigrospora*. Also, we were able to identify more fungi after using culture media, such as OA and V8A, which had nutrients similar to the ones provided by the bromeliad. Nonetheless, the four genera identified represent the first report of mycelial endophytic fungi associated with *B. pyramidalis*.

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