

Original Article

Diversity of culturable endophytic fungi vary through time in *Manihot esculenta* Crantz

A diversidade de fungos endofíticos cultiváveis varia ao longo do tempo em *Manihot esculenta* Crantz

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Abstract

Endophytic fungi are a ubiquitous group that colonize all plant species on earth. Studies comparing the location of endophytic fungi within the leaves and the sampling time in *Manihot esculenta* Crantz (cassava) are limited. In this study, mature leaves of *M. esculenta* from Panama were collected in order to compare the cultivable diversity of endophytic fungi and to determine their distribution within the leaves. A total of one hundred sixty endophytes belonging to 97 species representing 13 genera and 8 morphospecies determined as mycelia sterilia that containing 63 isolates were isolated. *Cladosporium*, *Nigrospora*, *Periconia*, and mycelia sterilia 1 and 3 were the most predominant isolated endophytes. We detected that endophytes varied across the sampling time, but not amongst locations within leaves. The endophytes composition across sampling and the location of endophytes within leaf was similar, except for *Periconia* and mycelia sterilia 3 and 7. The data generated in this study contribute to the knowledge on the biodiversity of endophytic fungi in Panama, and establish the bases for future research focused on understanding the function of endophytes in *M. esculenta* crops.

Keywords: ascomycota, endophytes, biodiversity, cassava.

Resumo

Os fungos endofíticos são um grupo ubíquo que colonizam todas as espécies de plantas na terra. Os estudos que comparam a localização dos fungos endofíticos dentro das folhas de *Manihot esculenta* Crantz (mandioca) e o tempo de amostragem são muito escassos. Neste estudo, folhas maduras de *M. esculenta* foram coletadas do Panamá com a finalidade de comparar a diversidade cultivável de endófitos e determinar sua distribuição dentro das folhas. Um total de 170 endófitos foram isolados de 97 espécies que representam 13 gêneros e 8 morfoespécies determinadas como micélios esterilizados contendo 63 isolados. Os fungos *Cladosporium*, *Nigrospora*, *Periconia* e mycelia sterilia 1 e 3 foram os isolados mais predominantes. Também detectamos que os endófitos variaram ao longo do tempo de amostragem, mas não entre os locais dentro das folhas. A composição de endófitos na amostragem e localização de endófitos dentro da folha foi semelhante, exceto para *Periconia* e mycelia sterilia 3 e 7. Os dados gerados neste estudo contribuem para o conhecimento da biodiversidade de fungos endofíticos no Panamá e estabelecem as bases para pesquisas sobre o entendimento da função de endófitos em culturas de *M. esculenta*.

Palavras-chave: ascomycota, endófitos, biodiversidade, mandioca.

1. Introduction

The fungal diversity has been estimated at up to 5.1 to 12 million based on high-throughput sequencing methods and culture-dependent culture-independent survey (O'Brien et al., 2005; Wu et al., 2019). Approximately 100,000 fungal species have been described, and countless are uncover every year (Blackwell, 2011). Many of these fungi have been associated with plants and divided into five main functional groups: mycorrhizal, pathogenic, saprotrophic, epiphytic, and endophytic fungi (Porrás-

Alfaro and Bayman, 2011). Of these, endophytic fungi are special interest in the agricultural area.

Fungal endophytes are inhabiting plant tissue without causing apparent harm to the host (Petri, 1991). They have a symbiotic-mutualistic relationship with plants, colonizing different above-ground tissues such as leaves, stems, seeds, bark, petioles, and reproductive structures, which differentiates them from mycorrhizal groups that inhabit roots (Faeth and Fagan, 2002; Shearin et al., 2018). Every

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Received: June 10, 2021 – Accepted: September 27, 2021



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plant species on earth has a community of endophytes, e.g., leaf segments of 2mm² in cacao (*Theobroma cacao*) trees and two understory (*Heisteria concinna* and *Ouratea lucens*) trees harbor naturally abundant, dense, and diverse endophyte infections ranged from 82% to 100% (Arnold et al., 2000, 2003). However, such proportion and diversity of fungi in plants could be dependent upon the host, age, nutrient, the environment, season, or the interaction with other microorganisms that surround them (Porrás-Alfaro and Bayman, 2011). Because fungal endophytes are everywhere, their uses as biological control agents against pathogens, and their pharmaceutical potential, are undeniable reasons for their study (Mejía et al., 2008; Sridhar, 2019; Adeleke and Babalola, 2021). In agricultural crops, endophytes play crucial roles by protecting plants against many biotic (conferring pest and disease resistance) and abiotic stresses (including impacts of climate change), increasing their resilience, and for improved management of post-harvest control (Lugtenberg et al., 2016). Hence, understanding their diversity in plants of agricultural importance, such as *Manihot esculenta* Crantz (cassava) is a first step to understand its functionality in that host.

Manihot esculenta (Phanerogama, Euphorbiaceae) is an ancient plant that has been extensively cultivated in tropical and subtropical regions in the world (Liu et al., 2014). Its worldwide production ended in approximately 277 million tons in 2015 with the main production areas being Africa (55%), Asia (33%), and Latin America (12%) (FAO, 2017). The domesticated species *M. esculenta* is grown for its starch-rich storage roots, providing food and energy security for about 800 million people (Burns et al., 2010; FAO 2013; Parmar et al., 2017).

In this study, two questions were addressed: (1) Are culturable endophytic fungi distinctly diverse across sampling time and the position of endophytes within leaves. Because communities of endophytic fungi can vary across time and within leaf parts (Suryanarayanan and Thennarasan, 2004; Porrás-Alfaro and Bayman, 2011), we hypothesized that fungal endophytes differ between sampling time and surveyed endophytes within the leaves. (2) How is the mycobiota of *M. esculenta* distributed spatially? The distribution of endophytes within leaves and across time is typically heterogeneous (Gamboa and Bayman, 2001; Cannon and Simmons, 2002; Arnold and Herre, 2003; Suryanarayanan and Thennarasan, 2004). We hypothesized that some endophytic fungi are found in all location within the leaf parts and sampling time; however, some endophytes will be limited to a specific location within the leaf or sampling time in *M. esculenta*.

These questions would be valid and novel for any plant. *M. esculenta* was used because is naturalized in Panama, produced in small farms, and the relationships between fungal endophytes have important implications for fungal biodiversity and plant health. For example, endophytes may potentially be useful for biocontrol of pathogens (Arnold et al., 2003; Ezra et al., 2009), and several cassava pathogens are very devastating (Lozano and Booth, 1974; Legg and Alvarez, 2017).

2. Material and Methods

2.1. Collection of *Manihot esculenta* Crantz leaves and isolation of fungal endophytes

Healthy leaves of *M. esculenta* were collected during the summer season (Jan to Mar) in a plantation in Ocú, Herrera (Panama). Five trees of ~10 months old were randomly selected, and from each tree, three mature leaves (5–7 lobes) were chosen randomly but visually healthy and free of damage by herbivores or pathogens. Samples were packaged and preserved at 4 °C, and same trees were collected five times during 2 ½ months with 15 days of interval.

The isolation method was carried out in the Mycological laboratory of the Instituto de Investigación Agropecuaria de Panama (I.D.I.A.P.), located in Divisa, Herrera (Panama). The collected leaves samples were divided as lower, middle and top parts within 24 h of collection and washed under running tap water for 1 min. Sixteen segments of 2 mm² were cut from each leaf part and surface-sterilized through sequential immersion in 70% alcohol (1 min), 0.5% sodium hypochlorite (3 min), 70% ethanol (30 s), and another 40% alcohol (30–40 s) (Bethancourt, 2000). The leaf samples were soaked and shaken with sterile distilled water three times for each 3 min. The sterilized samples were dried with sterile tissue paper for ~30 min and plated on 2% potato dextrose agar (PDA) medium supplemented with 50 mg of Vancomycin Hydrochloride and an infusion of 30 g/L of *M. esculenta* leaves. PDA is a common and nonselective media made from potato infusion, and dextrose as a carbohydrate source that provides enough nutrients to encourage the growth of a range of fungi; the infusion of *M. esculenta* leaves could increase the growth of fungal endophytes while the Vancomycin Hydrochloride avoid bacterial contamination. Also, PDA has been used extensively for the growth of different fungal endophytes in other tropical and crop plants (Gamboa and Bayman, 2001; Santamaría and Bayman, 2005; Hartanti et al., 2021). Uninoculated plates were used as controls. Endophytes were incubated at 25–28 °C for 2 weeks in 12 h of light-dark cycles. Mycelium from distinct colonies emerging from leaf segments were subcultured on new PDA plates to obtain pure colonies. Endophytes colonies were submitted to microculture on glass slides for examination of reproductive structures (Riddell, 1950). For sporulating fungi, we used morphology-based taxonomy (Barnett and Hunter, 1972). Pure colonies were transferred to a 2 mL glass vial with 2% of malt extract agar (MEA) supplemented with the antibiotic above described and grouped by morphospecies based on morphological characteristics in the mycelium growing in tubes (Ramírez-Camejo et al., 2017).

2.2. Fungal diversity analysis

Diversity analysis were performed using the endophyte species (morphospecies) isolated based on two criteria: 1) sampling time and 2) position in the lobed leaf (lower vs middle vs top). Species diversity was calculated using the sample size- and coverage-based rarefaction and extrapolation (R/E) of the Hill numbers of species, i.e.,

richness ($q = 0$), Shannon diversity ($q = 1$, the exponential of Shannon entropy), and Simpson diversity ($q = 2$, the inverse of Simpson concentration), as implemented in the R package Interpolation and Extrapolation for species diversity (iNext) v2.0.20 (Chao and Jost, 2012; Hsieh et al., 2016).

PAleontological STatistics (PAST) v4.04 was used to compare fungal diversity in both datasets using one-way ANOVA and Kruskal-Wallis tests followed by Tukey's Pairwise to test sample means and medians, respectively (Hammer et al., 2001). Additionally, Mann-Whitney pairwise and Dunn's post hoc tests were used to identify which means were significantly different from the others. The composition of the communities of fungal endophytes within leaves and between sampling time was performed using Whittaker index. Chi square (χ^2) tests were used to compare differences between endophytes collected in a different position within leaves and sampling time.

3. Results

One hundred-sixty endophytes were isolated; all in the phylum Ascomycota except by one isolate (Mucoromycota), and grouped into 21 morphospecies (Table 1). Cultural

characteristics of some morphospecies are represented as shown in Figure 1. Of these, 63 isolates (39%) remained unidentified. The most abundant genus identified was *Cladosporium*, which represented 14% of identified isolates, followed by *Nigrospora* (10%), *Periconia* (9%), *Corinespora* (7%), *Histoplasma* (6%), *Fusarium* (6%), and *Aspergillus* and *Penicillium* (4%). *Mycelia sterilia* 1 (11%) and 3 (11%) were the most notorious morphospecies. No fungi grew on control plates.

The proportion of endophytes isolated between sampling time was higher in the four (34%) and third (22%) sampling but lower in the rest of sampling that ranged from 13% to 16%. The proportion of fungi isolated on the lobed leaves was high in the lower part (44%) than in the middle and top part with 33% and 23%, respectively.

Estimated species richness was 13 species for sampling 1, 12 species for sampling 2, 14 species for sampling 3, 23 species for sampling 4 and, 12 species for sampling 5 (Figure 2A). Shannon's index estimated 13 fungal species in sampling 1 followed by 11, 10, 16, and 12 species for sampling 2–5, respectively (Figure 2A). Simpson's index estimated 11, 9, 9, 13, and 11 species for sampling 1–5, respectively (Figure 2A). When samples were grouped based on leaf positions, the estimated species richness was 16, 25, and 18; 13, 17, and 14 for Shannon's index; and

Table 1. Fungal endophyte species from different leaf location and sampling time of *Manihot esculenta* Crantz.

Species	Leaf position				Sampling time					
	Top	Middle	Lower	P value	S1	S2	S3	S4	S5	P value
<i>Aspergillus</i> sp.	2	1	3	0.607	0	1	1	2	2	0.675
<i>Candida</i> sp.	1	1	0	0.607	0	0	1	1	0	0.558
<i>Cladosporium</i> sp.	5	8	10	0.438	5	3	6	6	3	0.736
<i>Corinespora</i> sp.	4	3	4	0.913	2	4	3	2	0	0.406
<i>Curvularia</i> sp.	0	2	0	0.135	0	0	0	2	0	0.092
<i>Embellisia</i> sp.	0	1	0	0.368	1	0	0	0	0	0.406
<i>Fusarium</i> sp.	4	1	5	0.273	1	0	4	4	1	0.136
<i>Nigrospora</i> sp.	5	3	8	0.305	3	1	6	5	1	0.165
<i>Penicillium</i> sp.	1	2	4	0.368	0	2	0	3	2	0.273
<i>Periconia</i> sp.	2	9	3	0.046*	1	1	0	9	3	0.001*
<i>Phomopsis</i> sp.	0	1	1	0.607	0	0	0	1	1	0.558
<i>Rhizopus</i> sp.	0	1	1	0.607	1	0	0	1	0	0.558
<i>Trichoderma</i> sp.	0	1	0	0.368	0	0	0	1	0	0.406
<i>Mycelia sterilia</i> 1	6	4	7	0.662	2	2	5	6	2	0.346
<i>Mycelia sterilia</i> 2	0	0	2	0.135	0	0	0	0	2	0.092
<i>Mycelia sterilia</i> 3	1	6	11	0.016*	0	4	4	5	5	0.311
<i>Mycelia sterilia</i> 4	1	2	2	0.819	2	2	0	1	0	0.406
<i>Mycelia sterilia</i> 5	2	1	1	0.779	2	0	1	1	0	0.478
<i>Mycelia sterilia</i> 6	1	2	0	0.368	2	0	0	1	0	0.255
<i>Mycelia sterilia</i> 7	0	2	3	0.247	0	0	4	1	0	0.017*
<i>Mycelia sterilia</i> 8	2	1	6	0.097	2	1	0	3	3	0.437

*Asterisks represents significant differences by Chi square test. S1 to S5 represent the sampling time in this study.

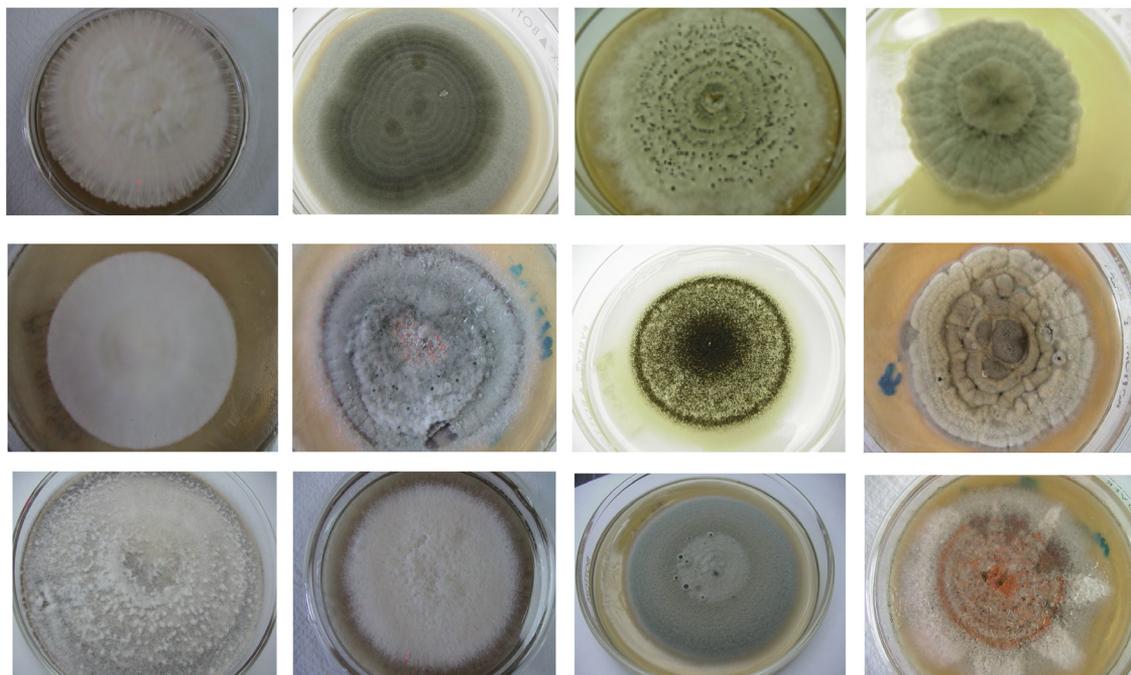


Figure 1. Colony morphology of some endophytic fungi isolated from *Manihot esculenta* Crantz grown on PDA at 25–28 °C.

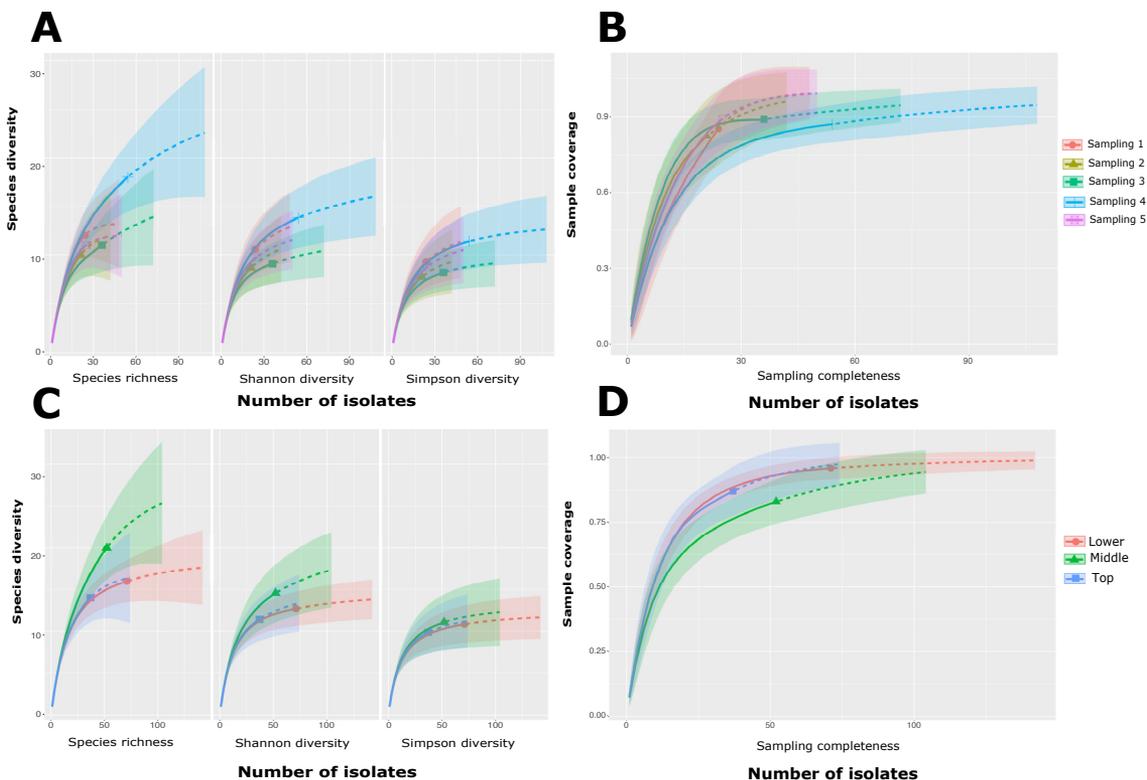


Figure 2. Diversity of fungal endophytes isolated from *Manihot esculenta* Crantz among sampling days and location of fungi within leaves, following Chao and Jost (2012): A-C: Sample-size-based rarefaction and extrapolation (R/E) curves. Plots shown three measures of species diversity: species richness, Shannon diversity (the exponential of Shannon entropy), and Simpson diversity (the inverse of Simpson concentration). Solid parts of the curves represent rarefaction while dashed curves represent extrapolation beyond observed samples. B-D: Sampling completeness curves. Shaded areas, 95% confidence intervals (bootstrapped).

11, 12, and 12 for Simpson's index between top, middle, and lower position, respectively (Figure 2C).

The sampling completeness curve increases as the number of fungal isolates increases (Figure 2B, D). The sample completeness was highest for sampling 3 and 5 (0.89%) followed by sampling 4 (0.87%), and decreasing on sampling 1 and 2 (0.85% and 0.82%) (Figure 2B). Lower sampling contributed 0.96% of sampling completeness while 0.87% and 0.83% was for top and middle sampling (Figure 2D).

Species diversity differed significantly among samplings (ANOVA, $F = 2.597$, $df = 4, 99$, $P = 0.041$) but not between the position of fungal endophytes within the leaf (ANOVA, $F = 1.93$, $df = 38.32$, $P = 0.159$). A significant difference was found between sampling 2 and 4 (Tukey's pairwise, 0.045; Mann-Whitney pairwise tests, 0.011, and Dunn's post hoc tests, 0.010). No significant differences were showed among the position of fungal endophytes within the leaf during Tukey's pairwise tests, Mann-Whitney pairwise tests and Dunn's post hoc tests ($P \geq 0.05$).

The species composition did not differ significantly between sampling (Whittaker = 0.693) or position of endophytes in the leaf (Whittaker = 0.26). Only the fungus *Periconia* ($\chi^2 = 4.7$, $P < 0.046$) and mycelia sterilia 3 ($\chi^2 = 6$, $P < 0.016$) was significantly more common in some part of the leaf than in other leaf parts (Table 1). Also, *Periconia* ($\chi^2 = 2.8$, $P < 0.001$) and mycelia sterilia 7 ($\chi^2 = 1$, $P < 0.017$) was significantly more common in some sampling than others (Table 1). The remaining endophytes did not differ significantly in frequency where endophytic fungi was isolated by sampling or within leaves ($P > 0.05$).

4. Discussion

This study explores the culturable endophytic fungi isolated from *M. esculenta* leaves and omitted nonculturable fungi or those that require special culture media or cultural conditions (Bayman, 2006). This implies a portion of the total fungal diversity present in *M. esculenta* are revealed. On the other hand, most endophytes are culturable and grow well with the method used here. No endophytic fungi grew in control plates during the culturing procedure, indicating that the manipulation during the field trip and isolation techniques were effective in preventing contamination.

Cladosporium, *Nigrospora*, *Periconia*, and mycelia sterilia 1 and 3 were the most representative endophyte taxa identified in *M. esculenta* leaves and these genera have also been shown to be the dominant genera in endophytes of other plants such as *Camellia sinensis*, *Mentha* sp., and palm trees (Table 1) (Song et al., 2016; Zakaria and Wan Aziz, 2018; Wu et al., 2020). Interestingly, the first three genera and mycelia sterilia 1 were found across all sampling days and in all locations within leaves, suggesting that common taxa may come from a similar source (e.g., airborne spores and soil spores) or leaves are favorable substrates for colonization of certain endophytes than others.

In this study, species of *Candida*, *Curvularia*, *Embellisia*, *Phomopsis*, *Rhizopus*, and *Trichoderma*, were the least abundant endophytes (Table 1). This result could reflect

differences in the culture media used (PDA + Vancomycin Hydrochloride) or methods of sampling. Furthermore, it could represent that every sampling event is different, and the abundance and diversity of endophytes in the leaf is driven by the tree or environmental factors such as temperature and water stress.

Our results suggest that the overall species richness of endophytes in *M. esculenta* among sampling time ranged from 12 and 23 species, with Shannon diversity between 10–16 species, and Simpson diversity from 9–13 species (Figure 2A). These values increased from sampling 1 and 4 and decreased from sampling 2, 3, and 5, reflect the fungal succession that occurred as the leaves receive new endophytes across time. Fungi isolated from the top part of the leaf in *M. esculenta* had a similar estimated diversity of fungal species than middle and lower parts for Richness, Shannon diversity, and Simpson diversity, respectively (Figure 2C). This indicated the persistence of the fungi associated with adult leaves within *M. esculenta*.

Overall, species diversity was similar across sampling but with significant differences on Sampling 2 vs Sampling 4 (Figure 2A), but not for fungi isolated between locations within leaves. This supported the hypothesis that communities of endophytes vary across sampling days of *M. esculenta*, but not all the time (Porrás-Alfaro and Bayman, 2011). This is possibly due to the physiological changes that commonly occur in the plant as it progresses in its biological cycle (Alves, 2002; El-Sharkawy, 2004). For example, in different stages of plant development, changes occur in the distribution of photosynthates and energetic compounds demanded by the different organs of the plant, as well as by changes in the climatic patterns and that occur as the growing season progresses (Yoshioka, 1986; Gray and Brady, 2016). This study was accomplished during the summer season where lack of water and high-temperature stresses can decrease the rate of leaf formation in *M. esculenta* (El-Sharkawy, 2004), which affect directly the abundance and diversity of fungi (Talley et al., 2002). Also, we cannot discard the possibility that differences in fungal diversity on sampling time could be due to leaf nutrient availability as showed in *Pinus muricata* and *Vaccinium ovatum* plants (Oono et al., 2020).

The highest number of isolates were recorded from the lower part of the *M. esculenta* lobe, but such abundance was not significant across other parts of the leaf. Possibly the proximal leaf part close to the base provides favorable conditions for the germination of spores, since the moisture may be higher than other parts. Similar results were found in *Betula pubescens* var. *tortuosa* where a greater number of endophytes (over 30%) was shown in the basal part of the leaf (Helander et al., 1993). In contrast, tropical forests reported that the largest endophytic population is found in the midrib and tip region compared to the base of the leaf (Cannon and Simmons, 2002; Suryanarayanan et al., 2002). These reports indicate that the pattern described for simple leaves (a single lobe) does not fit in the case of palm-split leaves such as *M. esculenta*.

Further analysis on increasing the number of samples per time and per leaf segment would improve the strength of the inferences that can be drawn, as suggested by the sampling completeness curves for *M. esculenta*

(Figure 2B, D). This is true especially for sampling days 1, 2, and 5, and for endophytes located in the top and middle part within the leaf where the sampling did not reach the asymptotic curve (Figure 2D).

Periconia and mycelia sterilia 3 and 7 ($P < 0.05$) differ in their abundance present amongst sampling time and its location within leaves. A similar pattern was documented in the composition of endophytic fungi of *Bauhinia brevipes* and the tea tree *Camellia sinensis* where differences between different part of leaves were significant (Hilarino et al., 2011; Wu et al., 2020). This result not only highlights those endophytic fungi can coexist in the same leaf depending on the part of tissue we select but also demonstrates a temporal variation in species abundance in leaves. Apart from *Periconia* and mycelia sterilia 3 and 7, species composition amongst sampling and location of the endophytes within leaf were similar; suggesting that the contribution of each endophyte species to the *M. esculenta* are homogeneous established across time and within leaves.

Morphological features carried out for the identification of endophytic fungi from *M. esculenta* were successfully assigned at genera level for 13 of 21 species. However, at the species level it has challenging to identify, so, 39% of fungal endophytes were categorized as mycelia sterilia since reproductive structures in anamorphic fungi were absent in the culture. Further molecular analysis is necessary to determine the species level of these 21 endophytes; for example, using amplification of the nuclear ribosomal Internal Transcribed Spacer (White et al., 1990). We cannot rule out that these 13 genera may be pathogens. For example, in Kenya *Cladosporium* was identified as the causative agents of brown leaf spot in *M. esculenta* leaves with a relative prevalence of 24%. In our study, *Cladosporium* was the most important genera isolated, and its abundance may reflect either the presence of many avirulent endophytes or dormant pathogens and the lack of the environmental conditions that stimulate signs of diseases as suggested in another endophyte (Ek-Ramos et al., 2013).

In conclusion, the sampling strategy used for the isolation of endophytes using leaf segments of 2 mm² was successful for estimating the diversity of endophytic fungal communities based on a culture dependent approach. Different endophyte species were found to dominate in *M. esculenta* leaves, which varied across sampling but not amongst location within leaves. This study is a preliminary step towards understanding functional relationships between *M. esculenta* and their endophytes and towards a more comprehensive picture of what *M. esculenta* endophytes do in the agricultural crop system. The information on fungal endophyte of *M. esculenta* leaves also contributes to the knowledge on the biodiversity of endophytic fungi in Panama.

Acknowledgements

This paper is dedicated to the memory of the late Dr. Kilmer Von Chong-Sanchez. The work was supported by Secretaría Nacional de Ciencia, Tecnología e Innovación

of Panamá and the Sistema Nacional de Investigación of Panamá. I am grateful to Armando González for technical support during the development of this work. Special thanks to Marlene V. Castillo-Bultrón who helped in the field and in the laboratory.

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