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Seasonality affects the community of endophytic fungi in coconut (*Cocos nucifera*) crop leaves

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ABSTRACT

The diversity of endophytic fungi in healthy coconut palm leaves (*Cocos nucifera*) was assessed by analyzing fungal isolates from three coconut cultivars (yellow dwarf, green dwarf, and a hybrid cultivar PB121) used agriculturally in Brazil. The influence of season (rainy or dry) on the endophytic fungal community was also analyzed. Overall, 318 fungal isolates were obtained from 972 coconut leaf fragments. The rDNA ITS region was sequenced from representative species of the isolated endophytic fungi and the most common species identified were *Nigrospora oryzae*, *Pestalotiopsis* sp., and *Zasmidium musae*. The alpha diversity of the endophytic fungi was also calculated. Nonmetric multidimensional scaling (NMDS) ordination and permutational analysis of variance (PERMANOVA) revealed significant seasonal effects on the composition of the endophyte community. However, practically no influence was observed on the fungal communities for the different cultivars of coconut.

Keywords: Arecaceae, coconut palm, *Cocos nucifera,* endophytic fungi, internal transcribed

Introduction

Coconut (*Cocos nucifera*) belongs to the palm family Arecaceae and is one of the most important tropical crops in the world (Lamdande *et al.* 2018). It is commonly used to produce coconut oil, husk, fiber, and water, but it also has non-food uses in various industries, such as for brick, furniture, and coir production, as well as in the adsorption industry (Roopan & Elango 2015). Additionally, coconuts are important for health promotion and disease prevention (DebMandal & Mandal 2011), and they have been studied for their potential to prevent and treat Alzheimer's disease (Fernando *et al.* 2015), as well as for their anti-inflammatory activity (Silva 2013).

Endophytic fungi are microorganisms that inhabit plant tissues without causing disease symptoms (Azevedo & Araujo 2007). They are helpful to many different plants by acting as biocontrol agents (Mejía *et al.* 2008; Larran *et al.* 2016; Saad *et al.* 2019; NI Silva *et al.* 2019), promoters of antimicrobial activity (Malhadas *et al.* 2017), and contributing to abiotic stress tolerance (Hubbard *et al.* 2014).

Several new species of endophytic fungi have been described (Bezerra *et al.* 2017a; b; 2018; Oliveira *et al.* 2016; Silva *et al.* 2019a; b) and many studies have been carried out on the endophytic fungal communities of important crop plants, such as coffee, rice, grapes, and soybeans (Pimentel *et al.* 2006; Sette *et al.* 2006; Naik *et al.* 2009; Lima *et al.* 2014; Oliveira *et al.* 2014a; Vaz *et al.* 2014; Varanda *et al.* 2016). Initial

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studies of endophytic fungi in palm species (Arecaceae) were carried out by Rodrigues & Samuels (1990) in Australia, who isolated fungi from the leaves of *Licuala ramsayi*. Further studies were performed by Taylor *et al.* (1999), who characterized the endophytic mycobiota of *Trachycarpus fortunei*; Rodrigues (1994), who isolated endophytic fungi from the Amazonian palm (*Euterpe oleracea*); and Mariano *et al.* (1997), who compared the epiphytic and endophytic communities of *Cocos nucifera*. In addition, other studies on endophytic fungal isolates from palm species have been carried out on the Bermudian palmetto (*Sabal bermudana*), the Chinese fan palm (*Livistona chinensis*), and *Wallichia caryotoides* (Southcott & Johnson 1997; Lumyong *et al.* 2009). Many studies have already investigated the influence of seasonality on endophytic fungal communities (Arnold & Lutzoni 2007; Mishra *et al.* 2012; U'Ren *et al.* 2012; Ek-Ramos *et al.* 2013; Yadav *et al.* 2016). However, little is known about the influence of seasonality on endophytic fungi in species of palm crops (such as coconut).

Studies on the composition of endophytic fungal communities, in relation to the influence of seasonality and the differences between plant cultivars of the same species, are poorly understood, especially in palm crops. We hypothesize that the endophytic fungal communities of different cultivars of *Cocos nucifera* are influenced by both seasonal factors and cultivar type. The aim of the present study was to evaluate the influence of both seasonality and coconut cultivar on the community of endophytic fungi in healthy leaves of plants cultivated in Northeastern Brazil.

Materials and methods

Sampling area

The study was conducted in three coconut crops located in the Instituto Agronômico de Pernambuco (IPA) in Goiana, Pernambuco state, Northeastern Brazil (7°33'45" S, 35°0'0" W). The precipitation and temperature for the area are shown in Figure 1.

Leaf sampling

Between May 2012 and April 2013, six leaf collections were performed (three in the dry season, September to March, and three in the rainy season, April to August). Samples were collected from three subareas, each of which corresponded to the location of a specific coconut cultivar. The cultivars used were as follows: 1. Yellow dwarf, 2. Green dwarf, and 3. A hybrid cultivar (PB121). In each subarea, three random points were delimited. Three individuals of coconut were chosen at each point, from which three leaves were collected, giving a total of 27 leaves from each subarea. Eighty-one leaves were obtained per collection from across all of the subareas, giving an overall total of 486 leaves for the study (243 leaves from the dry season and 243 from the rainy season). The samples were processed within a maximum of 24 h from collection.

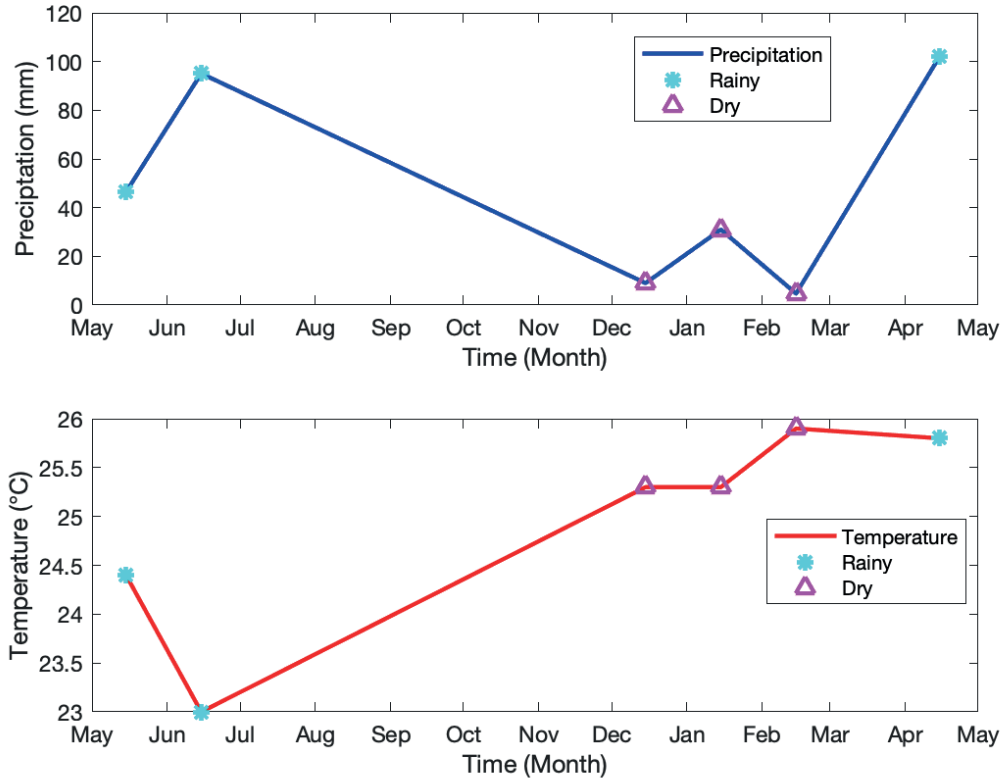

Figure 1. Climatic characteristics (precipitation and temperature) of the collecting area located inPernambuco, Brazil. The collection dates are represented by circles and triangles (source: http://www. inmet.gov.br).

Isolation of endophytic fungi

In the laboratory, the collected leaves were first washed carefully with running water and soap. Leaf discs (6 mm diameter) were then cut at random from all parts of the leaves. The discs were then decontaminated with 70 % ethanol for 1 min and 2 % sodium hypochlorite (NaOCl) for 2.5 min. Finally, they were washed again with 70 % alcohol for 30 s to remove excess hypochlorite. To complete the sterilization process, the material was rinsed with sterilized distilled water (Araújo *et al.* 2002).

The leaves from each collection point were mixed to create composite samples. In total, 972 leaf fragments were obtained (equating to 324 fragments per cultivar or 486 fragments per season). Six leaf discs were transferred to each of three Malt Extract Agar (MEA) Petri dishes supplemented with chloramphenicol (50 mg L-1), which were incubated at room temperature (28 ± 2 °C) and observed daily for 15 days to record the development of fungal colonies from each sample. As an aseptic control, 50 µL of the final rinsing water was plated on MEA as evidence of surface disinfection (Pereira *et al.* 1993). Isolates were first grouped into morphotypes based on morphological characteristics, and then confirmed by DNA sequencing.

Extraction, amplification, and sequencing of rDNA

Biomass was obtained from cultures grown on MEA after 7 days at 25 °C. DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method based on the protocol described by Oliveira *et al.* (2016). To amplify the internal transcribed spacer (ITS) sequence of nuclear rDNA, the primer pair ITS1/ITS4 (White *et al.* 1990) were used, following the PCR protocol described by Oliveira *et al.* (2014b). Sequencing was performed by the Plataforma Multiusuária de Sequenciamento de DNA, Centro de Biociências, Universidade Federal de Pernambuco, Recife, Brazil. The sequences obtained were deposited in the National Center for Biotechnology Information (NCBI), under accession numbers MK508790 to MK508819, and were compared by percentual of identity, with homologous sequences of several fungi taxa (by BLASTn) already deposited in the NCBI (Tab. 1). Species sequences with multiple coincidences or ambiguous results were only classified to the genus level. *Cladosporium dominicanum* (KJ000287) and *Phaeosphaeria nodulispora* (KR092904) were identified from previous studies (Oliveira *et al.* 2014c; Oliveira *et al.* 2016).

Data analysis

The sampling efficacy was assessed using individual-based rarefaction (interpolation) and extrapolation analyses with 95 % unconditional confidence intervals in the *iNEXT* R software package (Hsieh *et al.* 2016). The rarefied and extrapolated alpha diversities of taxa were displayed in relation to the sample unit. The general linear model (GLM) was used to test for significant differences in fungal endophyte diversity (alpha diversity and effective alpha diversity as the exponential of the Shannon index) for each cultivar and season (Jost 2006). A general linear mixed model (GLMM) was used to test for cultivar specificity of endophytic species abundance, with plant species as fixed factors and plots as random factors, using the *lme4* software package (Bates *et al.* 2014). The generalized additive model (GAM) was used to test for the influence of sampling time on endophytic species diversity, using the *mgcv* R software package (Wood 2017). Post hoc multiple comparison tests were conducted using the *agricolae* software package with the Bonferroni-adjusted kruskal function (Mendiburu 2016). The diversity and overlap of endophytic taxa across the three cultivars were visualized by assembling a bipartite network, implemented with the bipartite package in R (Dormann *et al.* 2008).

The endophytic community data were standardized according to the Hellinger method, using the decostand function of the *vegan* R software package, to avoid variable sampling intensity due to variation in the number of species. A variety of other statistical tests were also performed using various functions of the *vegan* software package, as described below. The variation in endophytic fungal communities between host cultivars was determined using multivariate analysis (Oksanen *et al.* 2015), and differences in fungal community composition between the samples were calculated using the Bray–Curtis dissimilarity method. Nonmetric multidimensional scaling (NMDS) was used to graphically visualize the organization of the samples in two-dimensional space using the *metaMDS* function. To test the overall effects of host identity and seasonality on the endophytic fungal community, permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001; McArdle & Anderson 2001) was performed using the adonis function (9,999 permutations). The turnover of species composition among communities (beta diversity) was evaluated using the betadisper function to perform analyses of multivariate homogeneity of group dispersion (Anderson 2006). Post hoc pairwise PERMANOVA analyses were used to test for significant differences in each of the factors.

Results

Composition of fungal endophytes

In the 972 leaf fragments, 318 fungal endophyte specimens were found, with an isolation frequency of 32.7 %. Overall, 32 species belonging to 14 orders were identified. The rDNA ITS region was sequenced from representatives of each endophytic fungal species isolated from *Cocos nucifera*, except for *Syncephalastrum racemosum* and *Xylaria* sp. (for which it was not possible to amplify the ITS region). The maximum identity of each species was then calculated using the BLASTn program from the NCBI platform (Tab. 1).

Table 1. Endophytic fungi isolated from three cultivars of coconut crop in Brazil.

Species	Yellow dwarf D/R	Green dwarf D/R	Hybrid PB121 D/R	Total number of isolates D/R	Total Ra (%)	GenBank accession number	Order	BLAST match (GenBank accession number and species name)	Id (%)
Alternaria sp.	0/1	0/0	0/0	0/1	0.31	MK508801	Pleosporales	MH865506, *Alternaria burnsii*	100
Arthrinium sp.	0/0	0/1	0/0	0/1	0.31	MK508790	Xylariales	NR_120274, *Arthrinium xenocordella*	97.2
Ascotricha sp.	0/1	2/0	0/0	2/1	0.94	MK508791	Xylariales	MF380809, *Ascotricha sinuosa*	100
Aureobasidium sp.	1/0	0/0	3/1	4/1	1.57	MK508797	Dothideales	MT119460, *Aureobasidium melanogenum*	100
Cercospora apii	0/1	0/0	0/0	0/1	0.31	MK508802	Capnodiales	NR_147294, *Cercospora musigena*	100
Cladosporium dominicanum	0/1	0/0	0/0	0/1	0.31	KJ000287*	Capnodiales	MF472970, *Cladosporium dominicanum*	100
Cladosporium sp.	3/4	3/7	1/1	7/12	5.97	MK508793	Capnodiales	MH864840, *Cladosporium tenuissimum*	100
Curvularia sp.	0/0	1/1	0/0	1/1	0.62	MK508794	Pleosporales	MH857943, *Curvularia fallax*	100
Diaporthe sp. 1	0/0	0/0	1/0	1/0	0.31	MK508808	Diaporthales	NR_111841, *Diaporthe anacardii*	97.7
Diaporthe sp. 2	0/1	1/0	1/0	2/1	0.94	MK508809	Diaporthales	NR_111843, *Diaporthe arengae*	99.8
Diaporthe sp. 3	0/0	0/0	1/0	1/0	0.31	MK508810	Diaporthales	MK432965, *Diaporthe endophytica*	100
Diaporthe sp. 4	1/0	0/0	0/0	1/0	0.31	MK508811	Diaporthales	MK650376, *Diaporthe* sp.	99.8
Fusarium sp. 1	0/0	0/7	0/0	0/7	2.20	MK508807	Hypocreales	MK611678, *Fusarium proliferatum*	100
Gelasinospora sp.	0/0	1/0	0/0	1/0	0.31	MK508817	Sordariales	MH856623, *Gelasinospora retispora*	99.3
Nectria pseudotrichia	0/0	0/0	0/1	0/1	0.31	MK508803	Hypocreales	MK047644, *Nectria pseudotrichia*	98.3
Nigrospora sp.	5/12	20/11	8/15	33/38	22.32	MK508792	Trichosphaeriales	MN341462, *Nigrospora lacticolonia*	99.7
Occultifur externus	1/0	1/0	0/0	2/0	0.62	MK508800	Cystobasidiales	KY104389, *Occultifur externus*	100
Paraphaeosphaeria arecacearum	0/1	0/0	0/0	0/1	0.31	MK508804	Pleosporales	NR_145166, *Paraphaeosphaeria arecacearum*	100
Penicillium sp.	1/0	0/2	0/8	1/10	3.45	MK508805	Eurotiales	MH864240, *Penicillium citrinum*	100
Pestalotiopsis sp.	0/12	1/7	0/18	1/40	12.57	MK508806	Amphisphaeriales	NR_147553, *Pestalotiopsis papuana*	100
Phaeosphaeria nodulispora	0/0	1/0	0/0	1/0	0.31	KR092904*	Pleosporales	KR092904, *Phaeosphaeria nodulispora*	100
Phyllosticta capitalensis	0/1	0/0	0/0	0/1	0.31	MK508796	Botryosphaeriales	MH865128, *Phyllosticta capitalensis*	100
Pseudozyma hubeiensis	0/0	0/5	0/1	0/6	1.88	MK508798	Ustilaginales	KY828936, *Pseudozyma hubeiensis*	99.8
Purpureocillium lilacinus	0/0	0/0	1/3	1/3	1.25	MK508812	Hypocreales	MN634691, *Purpureocillium lilacinum*	99.8
Pyrenochaetopsis indica	0/0	0/0	1/0	1/0	0.31	MK508813	Pleosporales	NR_160058, *Pyrenochaetopsis indica*	100
Pyrenochaetopsis microspora	0/0	0/0	0/3	0/3	0.94	MK508814	Pleosporales	NR_160059, *Pyrenochaetopsis microspora*	100
Radulidium apiculatum	0/0	1/0	0/0	1/0	0.31	MK508815	incertae sedis	KC986372, *Ramichloridium apiculatum*	99.6
Sarocladium sp.	1/0	0/1	0/0	1/1	0.62	MK508816	Hypocreales	NR_145044, *Sarocladium bactrocephalum*	99
Symmetrospora marina	1/0	0/0	0/0	1/0	0.31	MK508799	incertae sedis	NR_073272, *Symmetrospora marina*	98.4
Syncephalastrum racemosum	0/1	0/0	0/0	0/1	0.31	-	Mucorales	-	-
Xylaria sp.	0/0	0/0	1/0	1/0	0.31	-	Xylariales	-	-
Zasmidium musae	10/12	17/15	52/17	79/44	38.67	MK508818	Capnodiales	EU514291, *Stenella musae*	99.7
Total	24/48	50/56	74/66	148/170	100				

Ra = relative abundance; D = dry season; R = rainy season; Id = percentual of identity.

*Accession number provided by previous studies (Oliveira *et al*. 2014c; Oliveira *et al*. 2016).

- DNA amplification was not successful, and the identification was performed with morphological analysis.

Variation in the diversity of endophytic fungi between cultivars

The abundance of endophytic species differed between the cultivars (X^2 = 7.39, P = 0.02), with the highest number of species found in the PB121 hybrid cultivar (Fig. S1A in the supplementary material). The most abundant taxa were *Cladosporium* sp., *Pestalotiopsis* sp., *Nigrospora oryzae*, and *Zasmidium musae*, which presented similar distribution patterns in the different coconut cultivars throughout the sampling period (Fig. S1B in the supplementary material). The alpha diversity and effective number of species (exponential of Shannon index) of endophytic fungi were similar in the three cultivars (X^2 = 0.40, P = 0.81 and X^2 = 0.56, P = 0.75, respectively) (Fig. S2 in the supplementary material). The endophytic fungi rarefaction curves indicated that the overall number of endophytic fungi was higher in the yellow dwarf cultivar (mean and SE per plot; 2.74 ± 0.39), followed by the green dwarf (2.56 ± 0.33) and the PB121 hybrid (2.5 ± 0.25) (Fig. S3 in the supplementary material). The extrapolation curves suggest that additional sampling may allow the detection of more endophytic fungi.

The alpha diversity of endophytic fungi varied significantly with the sampling season (edf = 5, F = 2.67, P = 0.03). The alpha diversity of endophytic fungi associated with the green dwarf cultivar was lower (X^2 = 11.3, P = 0.04) at the end of both the rainy and dry seasons, while the alpha diversity of the yellow dwarf (X^2 = 3.2, P = 0.66) and PB121 hybrid cultivars (X^2 = 1.53, P = 0.9) did not differ over time (Fig. 2). Only six fungal taxa were common to the three cultivars, with the highest number of shared taxa (eight) found between the yellow and green dwarf cultivars (Fig. S4 in the supplementary material). According to a network analysis, the PB121 hybrid presented the highest specificity index (0.29), followed by green dwarf (0.25), with yellow dwarf (0.24) being the most generalist host among the coconut cultivars.

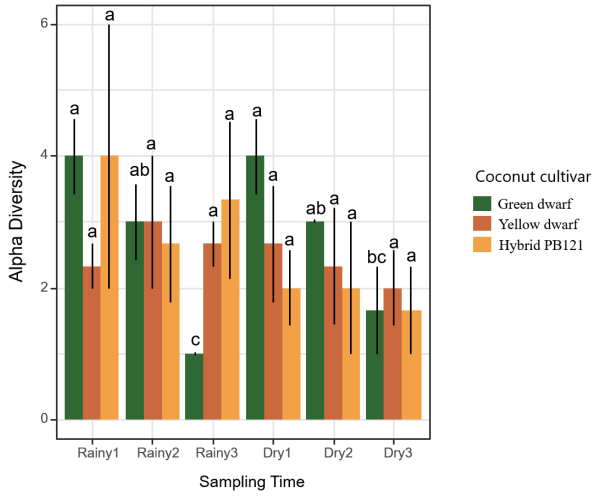

Figure 2. The alpha diversity of endophytic fungi associated with the three coconut cultivars throughout the sampling timeframe. The letters compare the alpha diversity of endophytic fungi associated with a host (mean ± SE) at different sampling times. Different letters represent significant differences identified using the generalized additive model (GAM) and post hoc Kruskal-Wallis test for multiple comparisons (p<0.05).

Occurrence and community composition of endophytic fungi according to season

NMDS ordination and PERMANOVA analyses revealed significant effects of the season on endophytic fungal community composition (Pseudo-F = 4.38, R^2 = 0.08, P = 0.003). The standardized deviation ellipses showed clear separation of the fungal communities between the rainy and dry seasons (Fig. 3). In the dry season, 21 taxa from 10 orders were recorded, compared to 22 species from 11 orders in the rainy season. The greatest number of orders was associated with the yellow dwarf cultivar (12), followed by the green dwarf (11) and PB121 hybrid (10) cultivars. Capnodiales and Tricosphaeriales were the richest orders found in the three cultivars. The order Mucorales occurred only in the yellow dwarf cultivar. The order Amphisphaeriales was mostly present during the rainy season, with the highest abundance at the beginning of the season in all the cultivars. Cystobasidiales was present only in the yellow dwarf and green dwarf cultivars, and only two isolates of this order occurred during the dry season. Conversely, the order Ustilaginales occurred only during the rainy season (Tab. 1).

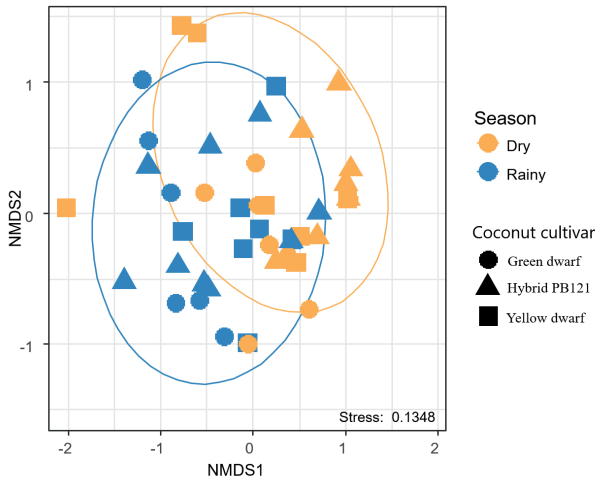

Figure 3. Nonmetric multidimensional scaling (NMDS) ordination plot of the endophytic fungal communities associated with the three coconut cultivars based on Bray-Curtis dissimilarities between samples. Ellipses represent confidence regions based on the SD from the centroid for each ecological unit.

Discussion

The isolation frequency (32.7 %) of endophytic fungi was similar to that found in other studies of the Arecaceae family. Rodrigues (1994) isolated endophytic fungi from *Euterpe oleracea*, obtaining a 21-30 % isolation rate, and Southcott

& Johnson (1997) reported an isolation frequency of 20.3% in two species of palm (*Sabal bermudana* and *Livistona chinensis*). However, Taylor *et al*. (1999) and Lumyong *et al*. (2009) reported higher isolation frequencies of endophytic fungi, of 60.9% and 68.7%, from *Trachycarpus fortunei* and *Wallichia caryotoides*.

In this study, we found that alpha diversity was similar between the three cultivars, and the most abundant taxa were the same for all the cultivars. The genetic and physiological differences between the coconut cultivars are insufficient to greatly influence the species richness of the endophytic fungal communities. Mariano *et al*. (1997), also working with *Cocos nucifera,* similarly reported that very common species were not specific to any cultivar. Pancher *et al*. (2012) suggested that differences between grape cultivars (Merlot and Chardonnay) may drive only a minor shift in endophyte composition when compared to crop management. In addition, the composition of the endophytic fungal communities was not found to be significantly different between the Syrah, Cabernet Sauvignon, and Aragonez grape cultivars (Varanda *et al*. 2016). On the other hand, the endophytic communities were found to be statistically different between two cultivars of pepper (at the seedling stage) in open field and greenhouse trials (Halász *et al*. 2016). In olive cultivars, endophytic fungi richness and diversity are influenced by changes in season, site, and cultivar (Materatski *et al*. 2019). The reports above suggest that the differences in endophytic fungal community composition between cultivars depend on the host species.

Although the endophytic fungal community composition was not significantly different between cultivars, NMDS ordination and PERMANOVA analyses revealed significant effects of the season on the fungal community composition. This suggests that environmental factors can influence the fungal community more than the choice of cultivar. It is possible that differences in the environmental humidity favor some groups of endophytic fungi, or that, depending on the season, the plant physiology changes to give an advantage to some taxa of fungi over others. In *Eugenia jambolana,* the diversity of fungal species was different in summer compared to winter (Yadav *et al*. 2016). According to Martins *et al*. (2016), seasonal differences in the endophytic fungal communities were detected in the olive tree, but other factors, such as different tissues of the host, are also important. The fungal communities in the Indian medicinal plant *Tinospora cordifolia* were more strongly affected by season when compared to geographic location (Mishra *et al*. 2012). Similarly, according to Yadav *et al*. (2016), the diversity and colonization frequency of endophytic populations are more influenced by factors such as season.

In the present study, some species were detected only in the rainy season, while others were present only in the dry season. The order Cystobasidiales, for example, was present only during the dry season, while Ustilaginales occurred only during the rainy season. According to Sadeghi *et al*. (2019), the species richness of endophytic fungi in mandarin trees is probably higher in the autumn than in the spring, due to the higher rainfall. Factors such as precipitation and temperature can impact the release of inoculum and the germination of fungal spores, subsequently influencing the establishment of the fungi. Under favorable conditions (depending on the season), a greater number of fungal morphotypes can be isolated from the foliar tissue (Singh *et al*. 2017).

In this study, we observed that seasonal differences have an influence on the community of endophytic fungi. However, practically no influence was observed on the fungal communities for different cultivars of coconut. Thus, our hypothesis was only partially confirmed. As coconut is an important crop, studying the diversity of endophytic fungi in different cultivars and seasons is important for better understanding the dynamics of these fungal communities. Environmental factors (*i.e.*, the season) can alter the communities of endophytic fungi in coconut leaves. Considering that some of these endophytic fungi can be latent pathogens, knowledge about their ecology, pertaining to differences between seasons and cultivars, can contribute towards future work on phytopathology in order to assist farmers with the management of this plant. However, more studies are necessary to better understand these relationships and how they can benefit crop systems.

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