South African Journal of Botany 134 (2020) 225–229

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Diversity of endophytic fungi associated with *Carapichea ipecacuanha* from a native fragment of the Atlantic Rain Forest

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ARTICLE INFO

Article History:
Received 29 July 2019
Revised 22 December 2019
Accepted 26 December 2019
Available online 11 January 2020

Edited by JF White

Keywords:
Carapichea ipecacuanha
Taxonomy
Diversity
Fungal endophytes

ABSTRACT

We focused on the taxonomy and diversity of endophytic fungi associated with the threatened medicinal plant *Carapichea ipecacuanha*, present in a native fragment of the Atlantic Rain Forest in Brazil. One hundred and seventy-six fungal isolates were recovered from leaf, stem, and root tissues of *C. ipecacuanha*. The isolates comprised 28 taxa of *Colletotrichum, Ceratobasidium, Fusarium, Trichoderma, Diaporthe, Pochonia, Calonectria*, and *Xylaria*. *Colletotrichum* was the dominant genus. *Colletotrichum gigasporum* and *Colletotrichum* sp. 1 were the most dominant taxa, which occurred systematically in all plant tissues. In contrast, *Calonectria lateralis, Fusarium delphinoides, Xylaria* sp., and *Diaporthe* spp. occurred as singlets. We detected a rich and diverse endophytic fungal community in the different tissues of *C. ipecacuanha* dominated by genera recognised as phytopathogens and decomposers. The findings indicate that tropical plants are a rich reservoir of fungal diversity, which is also threatened by the devastation of the natural tropical rainforest environments.

1. Introduction

Evaluation of microbial diversity is a major challenge for modern microbiology, given the large number of species already known or believed to exist (Gamboa et al., 2002). Plant tissues represent a promising reservoir of microbial diversity (Aly et al., 2011, Bhardwaj and Agrawal, 2014). Among them, endophytic microorganisms, which reside asymptomatically within plant tissues for at least one period of their life cycle, may have different effects on the ecology of the host plant, its healthy state, and its evolution (Petrini, 1991).

Despite the great diversity that likely exists, relatively few of these microorganisms have been characterised and the endophytic diversity of many biomes is unknown. In addition, many endophytes produce bioactive secondary metabolites (Smith et al., 2008) that are valuable in applications that include the pharmaceutical and agricultural industries. Endophytic microorganisms have been used for the biological control of pests and plant diseases, and for the production of enzymes, vitamins, antibiotics, and antitumor compounds. In Brazil, for example, a current focus is on the characterization of this microbial community and investigation of its biotechnological applications (Azevedo, 2014). Thus, it is important to study the diversity and ecology of endophytes in plants from unknown environments.

Carapichea ipecacuanha (Brot.) L. Andersson (Rubiaceae), popularly known as poaia, is a medicinal flowering plant that is distributed in Brazil, Colombia, and Central America (Rossi et al., 2008; Oliveira et al., 2010a, 2010b; Otoni et al., 2015). According to Oliveira et al. (2010a, 2010b), roots of *C. ipecacuanha* have been used as expectorant, amoebicidal, and emetic properties. The widespread pharmacological use of *C. ipecacuanha* is due to the bioactive alkaloids found in its roots (Garcia et al., 2005). The main alkaloids are emetine and cefepime, which are used to combat fever and treat malaria (Agra et al., 2008).

C. ipecacuanha is threatened by genetic erosion and is included on a list of species threatened with extinction as a result of the re-collection of its roots and the drastic reduction of its habitat in sub-forested areas (Oliveira et al., 2010a, 2010b, Otoni et al., 2015). In the present study, we investigated the taxonomy, diversity, and ecology of the endophytic fungal community associated with plant tissues of *C. ipecacuanha*.

2. Materials and methods

2.1. Plant collection and isolation of fungal endophytes

Twenty-five specimens of *C. ipecacuanha* were collected in July 2017 at Rio Doce State Park (19°42′19″ S, 42°30′45″ W), a protected area in the state of Minas Gerais, Brazil, which represents the largest (3597 km^2) and a threatened native fragment of the Atlantic Rain Forest. Identification of *C. ipecacuanha* was based on comparisons with the voucher specimen deposited at the herbarium of the Institute of Biological Science (BHCB) of the Federal University of Minas Gerais, Brazil (http://sciweb.nybg.org/science2/IndexHerbariorum.asp) under the code BHCB 184,744. The collection of the plant material was carried out according to the Brazilian biological diversity rules. Twenty-five *C. ipecacuanha* specimens were sampled. Three healthy leaves, three stems,

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https://doi.org/10.1016/j.sajb.2019.12.031
0254-6299/

and three roots obtained from each plant were placed in sterile plastic bags and stored at 10 °C until the isolation of endophytic fungi (<24 h). Five fragments (approximately 0.5 cm long and 0.5 cm wide) of each leaf, stem, and root were cut using a flame-sterilised blade in a laminar flow hood. The tissue fragments were surface disinfected by immersion in 70% ethanol for 1 min and 2% sodium hypochlorite for 3 min, followed by washing with sterile distilled water for 2 min (Carvalho et al., 2012). The fragments were plated onto Petri dishes containing potato dextrose agar (PDA; Difco, USA) supplemented with 200 mg L^{-1} chloramphenicol. The plates were incubated at 25 °C for 60 days. To test the effectiveness of the surface sterilization, 100 mL of the final rinse water was plated on PDA medium and incubated under the same conditions. Hyphal growth was monitored over an 8-week period. Endophytes were aseptically transferred to PDA contained in 60-mm Petri plates and photographed after the completion of growth. The long-term preservation of filamentous fungal colonies was carried out in cryotubes containing 15% sterile glycerol at −80 °C, and in sterile distilled water at 25 °C. All pure cultures of the endophytic fungal isolates were deposited in the Culture Collection of Microorganisms and Cells of the Federal University of Minas Gerais.

2.2. Fungal identification

The protocol for DNA extraction was described previously by Rosa et al. (2009). The internal transcribed spacer (ITS) region was amplified with the universal primers ITS1 and ITS4 (White et al., 1990). Amplification of the ITS region was performed as described by Rosa et al. (2009). Amplification of the b-tubulin (Glass and Donaldson, 1995) was performed with the Bt2a/Bt2b primers, according to protocols established by Godinho et al. (2013). The obtained sequences were analyzed with SeqMan P with Lasergene software (DNASTAR Inc., Madison, WI, USA), and a consensus sequence was obtained using Bioedit v. 7.0.5.3 software (Carlsbad, ON, Canada). Representative consensus sequences of fungal taxa were deposited into GenBank (Table 1). To achieve species-rank identification based on ITS and b-tubulin data, the consensus sequence was aligned with all sequences from related species retrieved from the NCBI GenBank database using BLAST (Altschul et al., 1997). Taxa that displayed query coverage and identities ≥98% or an inconclusive taxonomic position were subjected to phylogenetic ITS and b-tubulin analysis in comparison with sequences of type species deposited in the GenBank database, with estimations conducted using MEGA Version 6.0 (Tamura et al., 2013). The maximum composite likelihood method was employed to estimate evolutionary distances with bootstrap values calculated from 1000 replicate runs. The information about fungal classification generally follows the dictionary of Kirk et al. (2008) and MycoBank (http://www.mycobank.org) and Index Fungorum (http://www.indexfungorum.org) databases.

2.3. Ecological analysis

To quantify species diversity, richness and dominance, we used Fisher's α, Margalef's, and Simpson's indices, respectively. The rarefaction curve was calculated using the Mao Tao index. All of the results were obtained with 95% confidence, and the bootstrap values

Table 1
Molecular identification of endophytic fungi associated with the medicinal plant *Carapichea ipecacuanha* (Rubiaceae) from fragment of the Atlantic Rain Forest of Brazil.

UFMGCB[a]	Tissue[b]	N° of isolates	cTop BLAST search results (GenBank accession number)	Query cover (%)	Identity (%)	N° of bp analyzed	Proposed taxa (GenBank acc. n°)
15,191	L, S, R	30	*Colletotrichum gigasporum* (NR145380)[d]	100	100	476	*Colletotrichum gigasporum* (MN206836[f])
15,163	L, S, R	24	*Colletotrichum jasminigenum* (NR144789)[d]	100	95	420	*Colletotrichum* sp. 1 (MN206837[f])
15,066	S, R	23	*Ceratobasidium ramicola* (NR138368)[d]	96	96	503	*Ceratobasidium* sp. (MN206838[f])
15,039	L, S	15	*Colletotrichum citri-maximae* (KX943582)[d,e]	100	99	416	*Colletotrichum* sp. 2 (MN206839[f], MN205553[g])
15,068	L, S	15	*Colletotrichum phyllanthi* (NR111698)[d]	99	100	505	*Colletotrichum phyllanthi* (MN206840[f])
15,232	L, R	13	*Fusarium chlamydosporum* var. *fuscum* (AY213655)[d]	100	100	441	*Fusarium chlamydosporum* (MN206841[f])
15,252	L	12	*Colletotrichum brevisporum* (NR111637)[d]	100	99	425	*Colletotrichum brevisporum* (MN206842[f])
15,093	S, R	6	*Trichoderma spirale* (NR077177)[d]	100	99	453	*Trichoderma spirale* (MN206843[f])
15,130	S	5	*Colletotrichum vietnamense* (NR132058)[d]	100	98	320	*Colletotrichum* cf. *vietnamense* (MN206844[f])
16,234	L, R	3	*Colletotrichum plurivorum* (MG600718)[d]	95	100	470	*Colletotrichum* sp. 3 (MN206845[f])
15,138	S	3	*Colletotrichum theobromicola* (NR111512)[d]	100	99	387	*Colletotrichum theobromicola* (MN206846[f])
15,222	L	3	*Fusarium keratoplasticum* (NR130690)[d]	96	100	410	*Fusarium* sp. 1 (MN206847[f])
15,088	S	3	*Trichoderma caerulescens* (NR134432)[d]	100	99	441	*Trichoderma caerulescens* (MN206848[f])
15,099	S	3	*Trichoderma hispanicum* (NR138451)[d]	99	100	438	*Trichoderma* cf. *hispanicum* (MN206849[f])
15,170	R	2	*Diaporthe endophytica* (NR111847])[d]	100	97	428	*Diaporthe* sp. 1 (MN206850[f])
15,133	S	2	*Diaporthe novem* (NR111855)[d]	100	99	421	*Diaporthe novem* (MN206851[f])
15,286	S	2	*Fusarium petersiae* (NR156397)[d]	91	91	410	*Fusarium* sp. 2 (MN206852[f])
15,182	R	2	*Pochonia chlamydosporia* var. *spinulospora* (AB709857)[d]	100	99	455	*Pochonia chlamydosporia* (MN206853[f])
15,230	L	1	*Calonectria lateralis* (KY653258)[d]	100	99	365	*Calonectria lateralis* (MN206854[f])
15,278	L	1	*Colletotrichum limonicola* (NR152312)[d]	99	98	507	*Colletotrichum* sp. 4 (MN206855[f])
15,161	R	1	*Diaporthe acutispora* (NR152466)[d]	100	94	406	*Diaporthe* sp. 2 (MN206856[f])
15,160	R	1	*Diaporthe hongkongensis* (NR111848)[d]	100	95	438	*Diaporthe* sp. 3 (MN206857[f])
15,143	S	1	*Diaporthe masirevicii* (NR147534)[d,e]	100	97	429	*Diaporthe* sp. 4 (MN206858[f], MN205552[g])
15,087	S	1	*Diaporthe miriciae* (NR147535)[d]	100	98	360	*Diaporthe* cf. *miriciae* (MN206859[f])
15,141	S	1	*Diaporthe sclerotioides* (NR111069)[d]	99	96	454	*Diaporthe* sp. 5 (MN206860[f])
15,006	L	1	*Diaporthe sojae* (NR147542)[d]	98	96	429	*Diaporthe* sp. 6 (MN206861[f])
15,162	R	1	*Fusarium delphinoides* (NR130680)[d]	100	100	340	*Fusarium delphinoides* (MN206862[f])
15,144	S	1	*Xylaria bambusicola* (NR153200)[d]	100	94	462	*Xylaria* sp. (MN206863[f])

[a] UFMGCB = Culture of Microorganisms and Cells from the Federal University of Minas Gerais.
[b] Tissue: L = leaf, S = stem and R = root. [c]Top BLAST search results represent the first sequences matched with the endophytic fungi sequences of *Carapichea ipecacuanha*. Taxa subjected to sequencing and phylogenetic analysis based on the [d]ITS1-5.8S-ITS2
[e] β-tubulin. Sequences codes deposited in GenBank: [f]ITS1-5.8S-ITS2, and [g]β-tubulin.

were calculated from 1000 iterations. The diversity indices and rarefaction curve were performed using the computer program PAST, version 1.90 (Hammer et al., 2001). Venn diagram was prepared according to Bardou et al. (2014) to illustrate the comparison among fungal assemblages of the different plant tissues.

3. Results

3.1. Taxonomic and diversity analyses

One hundred seventy-six fungal isolates were recovered as endophytes of *C. ipecacuanha*. They comprised 28 taxa belonging to *Colletotrichum, Ceratobasidium, Fusarium, Trichoderma, Diaporthe, Pochonia, Calonectria*, and *Xylaria* genera (Table 1). *Colletotrichum* was the dominant genus with 108 (61%) endophytic isolates. *Co. gigasporum, Colletotrichum* sp. 1, *Ceratobasidium* sp., *Colletotrichum* sp. 2, *C. phyllanthi, Fusarium chlamydosporum*, and *C. brevisporum* were the most dominant taxa. In contrast, *Calonectria lateralis, F. delphinoides, Xylaria* sp., and *Diaporthe* spp. occurred as singlets.

The diversity indices indicated that the endophytic fungal community was rich (Margalef = 5.22) and diverse (Fisher-α = 9.39) but dominated by *Colletotrichum* and *Diaporthe* (Simpson = 0.91). The distribution of the endophytic taxa in the different tissues of *C. ipecacuanha* was depicted in a Venn diagram (Fig. 1). *C. gigasporum* and *Colletotrichum* sp. 1 were the most dominant taxa, which occurred in all plant tissues. *F. chlamydosporum* and *Colletotrichum* sp. 3 colonised leaf and root tissues and *Ceratobasidium* sp. and *Trichoderma spirale* colonised stems and roots. However, 20 fungal taxa occurred only in one kind of tissue. Sample coverage was assessed using a rarefaction curve (Fig. 2), which continued to rise, indicating that not all of the diversity had been recovered.

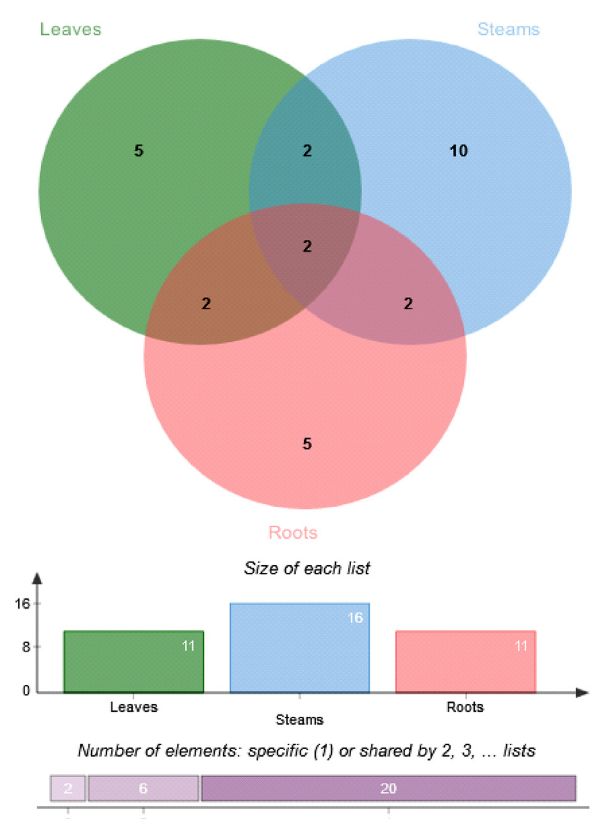

Fig. 1. Similarity among the fungal assemblages present in leaf, steam, and root tissues of *Carapichea ipecacuanha* using the Venn diagram. The *Y* axis representes the number of taxa.

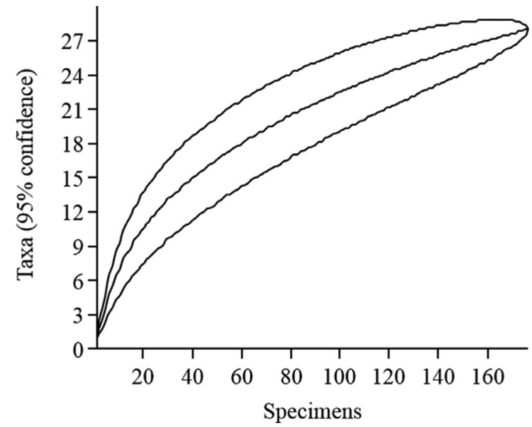

Fig. 2. A species accumulation curve (Mao Tau = solid lines) of the endophytic fungi associated with *Carapichea ipecacuanha. Y* axis repesent the number of taxa and *X* axis the number of samples.

4. Discussion

4.1. Taxonomic and diversity analyses

C. ipecacuanha tissues shelter a rich and diverse fungal community dominated by *Colletotrichum* taxa. *Colletotrichum* includes approximately 190 recognised species (Jayawardena et al., 2016), which are commonly isolated as phytopathogens and foliar endophytes of different plants (Farr and Rossman, 2009; Rojas et al., 2010), but also as epiphytes, saprobes, and human pathogens (Hyde et al., 2009). *C. gigasporum* was originally reported from healthy leaves of *Centella asiatica* in Madagascar and *Stylosanthes guianensis* in Mexico, as well as from *Coffea arabica* in Colombia (Rakotoniriana et al., 2013). It has an endophytic growth habit and could be isolated from various host plants occurring in geographically distant areas (Liu et al., 2014). However, recent studies have reported that *C. gigasporum* as causal agent of anthracnose in host like avocado (*Persea americana* L.) and *Dalbergia odorifera* (Hunupolagama et al., 2015; Wan et al., 2018).

Co. brevisporum as well as C. *gigasporum* are described as endophyte microorganisms in several plants, but are also commonly described as a cause of anthracnose, including *Neoregelia* sp. from Thailand (Noireung et al., 2012), papaya fruits (Vieira et al., 2013), *Lycium chinense* Mill (Paul et al., 2014), chayote fruits (Bezerra et al., 2016), pepper (Liu et al., 2016) and others. *Co. phyllanthi* is known only from the original collection taken from leaves of *Phyllanthus acidus* in India in 1970 (Pai, 1970; Damm et al., 2012). It has already been described as endophytic of several plants, including mango (*Mangifera indica* L.) (Vieira et al., 2014a, 2014b), *Bauhinia variegata* and *Bougainvillea* sp. (Sharma and Shenoy, 2013).

Ceratobasidium ramicola (*Basidiomycota*) was recovered from the stem and roots of *C. ipecacuanha. C. ramicola* was originally described from epiphytic growth on the leaves of *Pittosporum tobira* in Florida (Tu et al., 1969). However, it has been widely described as an endomycorrhizal fungus in association with several plant species, such as orchids and cocoa (Samuels et al., 2012; Senthilkumar et al., 2018).

In addition to be an endophyte of *C. ipecacuanha*, the genus *Fusarium* is reported in several types of natural ecosystems, such as tropical, temperate and desert forests, as well as in polar environments (Leslie and Summerell, 2006). Many species are found as saprobes in the soil or as secondary colonisers of plant roots. Other species have already been described as plant pathogens capable of causing different diseases. Many *Fusarium* species have also been reported as endophytic but may also become pathogenic when their hosts are subjected to strong environmental stresses (Walsh et al., 2010). *F. chlamydosporum* represents a well-defined morphospecies of both phytopathological and clinical importance (Lombard et al., 2019), commonly isolated from soils and grains in arid and semi-arid regions (Sangalang et al., 1995), from plant material displaying disease symptoms that include crown rot (Du et al., 2017), blight (Satou et al., 2001), damping off (Lazreg et al., 2013) and stem canker (Fugro 1999). *F. chlamydosporum* has been reported as an endophyte from stems of *Tylophora indica* (Chaturvedi et al., 2014) and *Crataeva magna* (Nalini et al., 2005).

The second dominant fungal genera present in tissues of *C. ipecacuanha* was *Diaporthe*. The *Diaporthe/Phomopsis* genus represents a complex of anamorph/teleomorph fungi, respectively, which have already been isolated as saprobes, endophytes and pathogens from different host plants. They are considered to cause several diseases, some of great importance in agriculture (Santos et al., 2010; Ko et al., 2011). *Phomopsis* (anamorph) contains approximately 1000 species often considered predominant in their hosts and approximately 95 species of *Diaporthe* (teleomorph) have been described (Gomes et al., 2013).

Within *Sordariomycetes*, four other taxonomic groups were found in low frequency: *Calonectria, Pochonia, Trichoderma*, and *Xylaria*. The genera found in association with the tissues of *C. ipecacuanha* represent taxa commonly reported as endophytic in different plants of tropical environments (Carvalho et al., 2012; Rosa et al., 2012; Vieira et al., 2014a, 2014b; Ferreira et al., 2015; Silva-Hughes et al., 2015, 2017).

We detected in the different tissues of *C. ipecacuanha* a rich and diverse endophytic fungal community dominated by genera recognised as phytopathogens and decomposers. The findings demonstrate that tropical plants represent a rich reservoir of fungal diversity that is also threatened by devastation of natural tropical environments.

Declaration of Competing Interest

None.

Acknowledgments

We are grateful for the financial support from CAPES, CNPq, and FAPEMIG.

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