American Journal of Plant Sciences, 2013, 4, 917-921
http://dx.doi.org/10.4236/ajps.2013.44113 Published Online April 2013 (http://www.scirp.org/journal/ajps)

Endophytic Mycobiota Characterization of the Amazonian Mistletoe *Cladocolea micrantha* Hosted in Cashew Tree

Anderson C. Guimarães[1], Antonio C. Siani[2*], José L. Bezerra[3], Antonia Q. Lima de Souza[4], Maria Inez M. Sarquis[5]

[1]Instituto de Ciências Exatas e Tecnologia de Itacoatiara, Universidade Federal do Amazonas, Itacoatiara, Brazil; [2]Departamento de Produtos Naturais, Farmanguinhos, Fiocruz, Rio de Janeiro, Brazil; [3]Departamento de Ciências Agronômicas e Ambientais, Universidade Estadual de Santa Cruz, Ilhéus, Brazil; [4]Laboratório de Genética, Escola Superior de Ciências da Saúde, Universidade Estadual do Amazonas, Manaus, Brazil; [5]Laboratório de Taxonomia Bioquímica e Bioprospecção de Fungos, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil.
Email: [*]siani@far.fiocruz.br

Received March 5th, 2013; revised April 3rd, 2013; accepted April 12th, 2013

ABSTRACT

Endophytic fungi were identified from different parts of the medicinal parasitic mistletoe *Cladocolea micrantha* and from its host *Anacardium occidentale*, suggesting a strict host-parasite relationship. Eight fungal endophytes were isolated and morphologically characterized. The ascomycete *Guignardia mangiferae* and strains of *Mycelia sterilia* were prevalent in the isolations. The unequivocal identification of *Guignardia mangiferae* at a probabilistic degree close to 100% was carried out by DNA extraction followed by PCR analyses of the ITS-1 and ITS-2 regions and comparison of the genetic sequence with the NCBI database.

Keywords: *Cladocolea micrantha*; Endophytic mycobiota; Loranthaceae; *Guignardia mangiferae*; *Anacardium occidentale*

1. Introduction

The Loranthaceae mistletoes, popularly known in Brazil as "erva-de-passarinho" [1] play an important and complex part in the biological system they inhabit, interacting with insects, birds, fishes and mammals [2]. These epiphytic and hemiparasitic plants adhere to the branches and twigs of trees by means of haustoria, penetrating the host to absorb water and nutrients [3]. Species of this family are used all over the world in traditional medicine to treat a wide range of diseases, such as arthritis, diabetes, general inflammation, breathing and nervous problems and some types of cancer [4]. In the Amazonian region, the leaves of the species *Cladocolea micrantha* (Eichler) Kuijt are widely used in the non-conventional therapy of several types of tumors and inflammatory processes [5], with a preference for specimens growing on the cashew tree *Anacardium occidentale* (Anacardiaceae) [6]. The genus *Cladocolea* comprises approximately 40 species and occurs from central to southern Mexico and in some localities of Latin America. The most important morphological feature of this genus is the occurrence of a determinate inflorescence and single lateral ebracteolate flowers or a simple derivative thereof; this characteristic serves to differentiate *Cladocolea* from other similar mistletoe genera [7]. Chemical studies on this genus are restricted to *Cladocolea micrantha* which describes the isolation of diverse triterpenes as well as kaempferol and quercetin derivatives presenting an uncommon interglycosidic O-(1→3) linkage [8]. Complementarily, the triterpenes *α*-amyrin, *β*-amyrin and their respective ketones were remarkably characterized from the chloroform extracts of mycelia of *G. mangiferae*, *P. clavisporus* and *P. guepinii* 7-d to 14-d-old colonies, isolated from this mistletoe [9].

Endophytic fungal communities occupy intercellular organs in plants, usually inducing an associative relationship that ranges from mutualism to host plant parasitism. Such a symbiosis may confer some protection against insects, herbivores or phytopathogenic microorganisms by producing toxins, antibiotics, specific enzymes or growth-promoting agents and other substances [10]. Endophytic fungi have also been found to be even-

[*]Corresponding author.

tually responsible for the bioactive compounds produced by host plants. A corollary to this assumption would be the production of the anti-cancer agent taxol by the endophytic fungi *Taxomyces andreanae*, *Pestalotiopsis microspora* and *P. guepinii* isolated from the Pacific *Taxus brevifolia*. Similarly, the fungus *Phialocephala fortinii* isolated from *Podophyllum peltatum* may produce the antitumoral podophyllotoxin [11,12]. Endophytes have been identified on species associated with many botanical families, and currently, a significant amount of research is focused on obtaining active metabolites from endophytes, aiming to utilize them in medicine and biological control [11,13,14]. The present study aimed to identify and to morphologically and molecularly characterize the endophytic fungi from different parts of the parasite mistletoe *Cladocolea micrantha* and from its host *Anacardium occidentale*.

2. Material and Method

2.1. Plant Material

Leaves and stems of *Cladocolea micrantha* (Eichler) Kuijt and *Anacardium occidentale* L. for the host-parasite mycobiotic analyses were collected on the campus of the Federal University of Amazonas (UFAM), Manaus, Brazil, S 03˚07.785', W 058˚26.631', each from a single individual host specimen 8 m tall. Both species were identified, and vouchers were deposited in the Herbarium of UFAM under No. 6213.

2.2. Surface-Disinfecting and Inoculation of the Botanic Material

Endophytic fungi were isolated according to the procedure by Petrini (1992) [15,16] using fresh botanical material from both *C. micrantha* and *A. occidentale*. The plant material was carefully washed with a neutral detergent and running water. After drying, the leaves and stems were sliced into pieces of approximately 8 cm^2 and were soaked for 1 min in a 500 ml beaker containing 70% ethanol in water. The fragments were then transferred to another beaker containing a 3% solution of sodium hypochlorite for a period of 4 min. The pieces of leaves and stems were then returned to the beaker containing the ethanol/water solution for 30 s and were finally transferred to a flask with sterilized water for 1 min. After this procedure, fragments of leaves and stems from both plants were cultured on eight Petri plates (six fragments per plate) containing malt-agar extract (MAE) (Difco) and chloramphenicol (100 μg/ml MAE) with the aid of a Drigalski loop, resulting in 192 inocula distributed onto 32 plates. A negative control was run by applying 0.05 ml of the distilled water from the last sterilization step onto Petri dishes using the same medium. Cultured samples were incubated at room temperature (approximately 25˚C in the laboratory) and observed daily.

2.3. Endophytic Fungi Isolation and Identification

After eleven days, the plates contained 160 growing colonies that were singly prepared on slides and carefully inspected under a microscope. Identical fungal strains were separately pooled into eight different representative groups according to their macro- and micromorphological similarities, and their purity was certified by macro- and microscopic evaluation. For genus and species assignments, the fungal strains were separately grown on the following media: PDA, malt agar extract (Difco), Czapek-dox agar (Merck) and oatmeal agar (60.0 g oatmeal and 18.0 g agar). Nutrient-poor synthetic medium agar [1.0 g KH_2PO_4, 1.0 g KNO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose and 18.0 g agar (all Merck) in 1000 ml distilled water] and sunflower seed extract agar were also used according to classical methodologies [17]. The microscopic characteristics were evaluated in microcultures on coverslips in a drop of Amann's lactophenol [20.0 g phenol (Quimibras), 20.0 ml lactic acid (Merck), 20.0 ml distilled water, 40.0 ml glycerine (Merck)] with 0.05 g cotton blue (Sigma). The filamentous fungi were classified based on standard procedures reported for morphological characterization [18-20]. The fungi were then transferred to test tubes containing MAE for successive growth. Samples were lyophilized and stored under mineral oil in the Fungi Culture Collection Filamentous Fungi of the Oswaldo Cruz Institute, Rio de Janeiro, under Nos. IOC4553 (*Aspergillus* sp.), IOC 4548 (*G. mangiferae*), IOC4555 (*M. sterilia*), IOC4549 (*Myrothecium* sp.), IOC4550 (*P. clavisporus*), IOC4551 (*P. guepinii*), IOC4552 (*T. pseudoviridae)* and IOC4554 (*Drechslera* sp.).

2.4. DNA Extraction and PCR Conditions

G. mangiferae identification was confirmed by sequencing the ITS-1 and ITS-2 rDNA and comparing it with sequences from GenBank. For ITS-1 and ITS-2 rDNA analyses, the strain was stirred at room temperature for 12 days (120 rpm) on a potato-dextrose medium enriched with 0.2% PDY (potato dextrose yeast) extract. From 200 mg of mycelium separated by filtration, genomic DNA was extracted using the cetyltrimethylammonium bromide method [21]. Prior to PCR, the DNA concentration was visually estimated at 100 ng/μl by comparing it with the 1 kb marker on a 1% agarose gel. Each 25 μl of reaction contained 10.2 μl of water, 2.5 μl of 10× buffer, 3.0 μl of 25 mM $MgCl_2$, 3.0 μl of 1.25 mM dNTP, 2.0 μL each of ITS-1 and ITS-2 primers at 10 mM, 0.3 μl of Taq

DNA polymerase (5.0 U) and 2.0 µl of genomic DNA. Four reactions were carried out with an initial denaturing step of 94˚C for 4 min, followed by 40 cycles of 2 min denaturing at 94˚C, 2 min annealing at 55˚C and 2 min elongation at 72˚C. The amplification was terminated for 10 min at 72˚C. PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide, compared with a DNA ladder of 100 bp and estimated at 50 ng/µl. In all, 2 µl of the PCR products were sequenced on an acrylamide gel, and the sequences obtained were matched with those deposited in the NCBI (National Center for Biotechnology Information) database.

3. Results and Discussion

A total of 160 fungal strains (from the 192 total fragments of plant material) comprising 8 groups of endophytic fungi were isolated from both mistletoe and the plant host. Stems and leaves of the mistletoe *Cladocolea micrantha* led to the isolation of the following fungal species and genera: *Aspergillus* sp. (Trichocomaceae, Order Eurotiales), *Guignardia mangiferae* (Botryosphaeriaceae, Order Botryosphaeriales) *Mycelia sterilia* (deuteromycetes, Order Agonomycetales), *Myrothecium* sp. (Incertae sedis, Order Hypocreales), *Paecilomyces clavisporus* (Trichocomaceae, Order Eurotiales), *Pestalotiopsis guepinii* (Amphisphaeriaceae, Order Xylariales) and *Trichoderma pseudoviridae* (Hypocreaceae, Order Hypocreales). The following species and genera were identified in the tissues of *Anacardium occidentale*: *Pestalotiopsis guepinii*, *Drechslera* sp. (Pleosporaceae, Order Pleosporales), *Guignardia mangiferae*, *Mycelia sterilia*, and *Trichoderma pseudoviridae*. The three species/genera in boldface were common to both the parasite and the host plant (**Table 1**). All of these occurrences are described here for the first time, especially for the *Cladocolea micrantha* mycobiota, for which, as far as we know, this is the first report.

The non-sterile ascomycetes *G. mangiferae* and *Mycelia sterilia* were found as the most frequent fungal endophytes in both the parasite and the host plants in the experimental sampling. Identification of *G. mangiferae* was based on macroscopic characteristics and on the microscopic measurements of individuals from the 7-d-old colony on MAE, which presented the following dimensions: pseudothecia [(640 - 1232) × (112 - 424) µm]; ostioles [(80 - 400) × (80 - 200) µm]; asci [(40 - 68) × (10 - 12) µm; ascospores [(15 - 16) × (5 - 6) µm] [22]. Similar results for the ascospore sizes [(13 - 17) × (5 - 8) µm] were observed for *G. mangiferae* isolated from *Anacardium giganteum* [23]. The trusted pairwise comparisons revealed 99% identity with *G. mangiferae* and 100% reliability for the 576 DNA base pairs (data not shown). Such a high probabilistic correlation provides additional relevant data on tropical endophytes, given that the *Guignardia* species present wide intraspecific genetic variability [24,25]. Species of *Guignardia* are usually recognized as endophytic fungi and have been isolated from asymptomatic tissues from a diversity of Myrtaceae and Anacardiaceae species as well as citrus plants [25]. However, some species such as *G. citricarpa* and *G. psidii* cause serious diseases to agriculture by infesting fruit and leaves post-harvest. General studies of endophytes are more prevalent in temperate regions [26] and should be extended to tropical species when considering the pharmaceutical potential of tropical medicinal plants [27].

4. Conclusion

The findings reported here reinforce the cosmopolitan occurrence of the endophytic *G. mangiferae* in tropical plants [23,28,29]. The non-sterile mycobiota of the leaves of both parasitic and host plants is characterized by the common presence of *G. mangiferae* as the most frequently occurring fungus. This presence was not observed for the

Table 1. Endophytic fungi characterized in the tissues of *Cladocolea micrantha* and *Anacardium occidentale*.

Fungus species	CM leaf	CM stem	AO leaf	AO stem
Aspergillus sp. [2]	n	n	2/10	n
Drechslera sp. [1]	n	n	1/10	n
Guignardia mangiferae [15]	8/15	n	7/10	n
Myrothecium sp. [2]	1/15	1/18	n	n
Mycelia sterilia [133]	31/15	38/18	35/10	29/3
Paecilomycesclavisporus [2]	2/15	n	n	n
Pestalotiopsis guepinii [2]	n	2/18	n	n
Trichoderma pseudoviridae [3]	1/15	n	1/10	1/3

CM = *Cladocolea micrantha*; AO = *Anacardium occidentale*. In brackets = total of similar pooled strains. Results (X/Y) indicate the ratio of culture plates (X) growing positively over the total cultured plates (Y), including replication experiments; n = no growth observed.

stems, suggesting that some horizontal fungal contamination (leaf-leaf) cannot be excluded [30]. This is the first description of *G. mangiferae* as an endophyte in *C. micrantha*. Moreover, its presence on *A. occidentale* suggests a close host-parasite relationship, a fact that may be relevant to the medicinal properties of the parasite plant and to the popular preference for *G. mangiferae* grown on this host plant.

5. Acknowledgements

The authors wish to thank Dra. Lilian A. Procópio for the plant identification, and plant collectors Silo Silva and Rosalba Bilby for their help.

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