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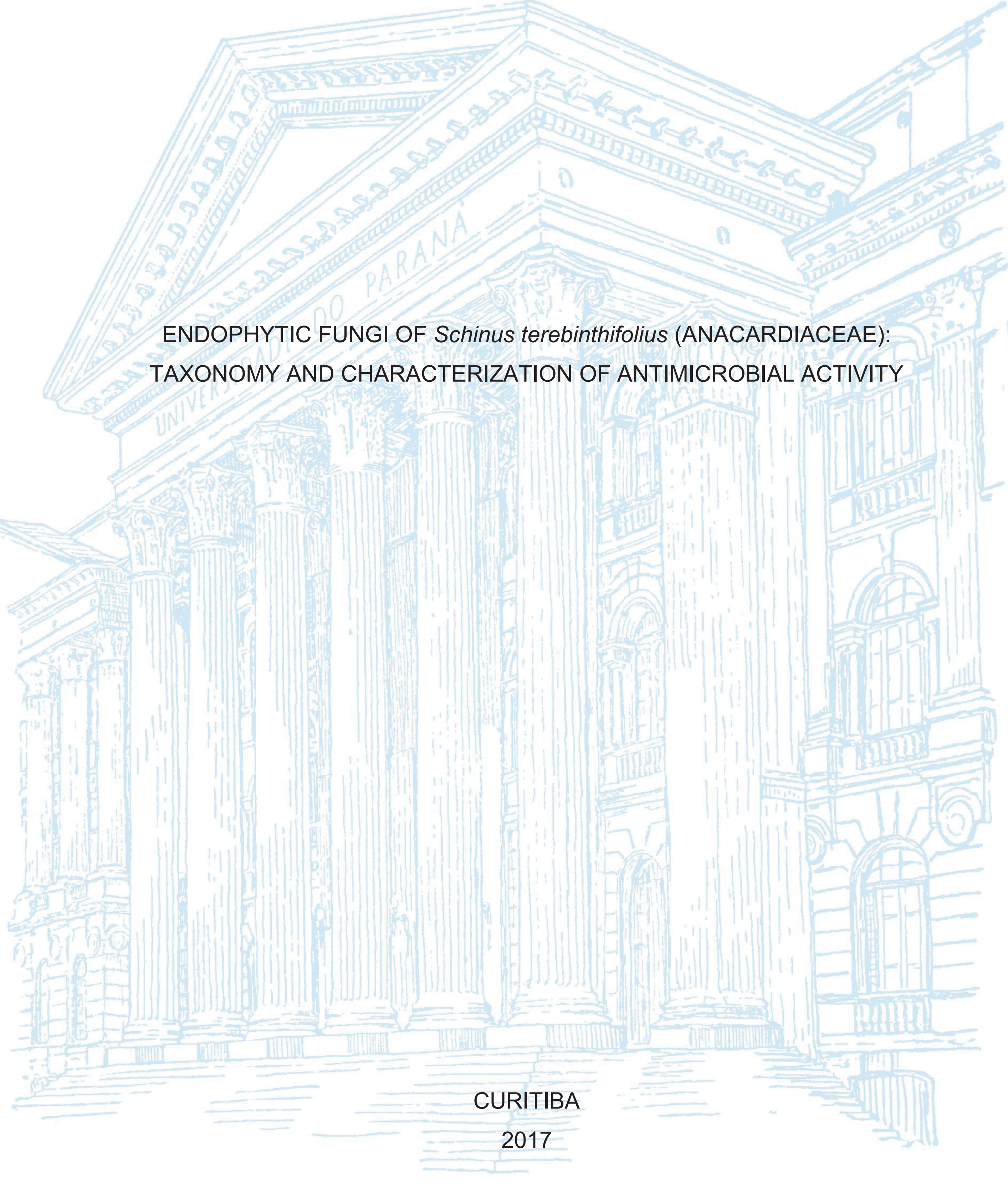

ENDOPHYTIC FUNGI OF *Schinus terebinthifolius* (ANACARDIACEAE): TAXONOMY AND CHARACTERIZATION OF ANTIMICROBIAL ACTIVITY

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Tese apresentada como requisito parcial à obtenção do título de Doutor, curso de Pós-Graduação em Microbiologia Parasitologia e Patologia, Setor de Ciências Biológicas, Universidade Federal do Paraná

Orientador(a): Prof(a). Dr(a). Vania Aparecida Vicente
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"ENDOPHYTIC FUNGI OF *Schinus terebinthifolius* (ANACARDIACEAE): TAXONOMY AND CHARACTERIZATION OF ANTIMICROBIAL ACTIVITY"

por

Germana Davila dos Santos

Tese aprovada como requisito parcial para obtenção do grau de Doutor no Curso de Pós-Graduação em Microbiologia, Parasitologia e Patologia, pela Comissão formada pelos professores:

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Curitiba, 31 de outubro de 2017.

“Science without religion is lame,
religion without science is blind”
Albert Einstein

Dedico

Aos meus amados pais, Carmem e Elmo.
Muito obrigado por tudo!

I dedicate

To my beloved parents, Carmen and Elmo.
Thank you so much for everything!

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RESUMO

As plantas medicinais são fontes relevantes de novos compostos antimicrobianos e são colonizadas por fungos endofíticos, que parecem exercer um papel importante na produção dos metabolitos pela planta, uma vez que os extratos de plantas utilizadas na medicina tradicional podem ter parte de sua atividade como resultado desta interação. Da mesma forma, os fungos representam uma rica fonte de moléculas bioativas funcionais, sendo que as espécies endofíticas se destacam quanto a esta propriedade. Sendo assim, o objetivo central desta tese foi compreender a relação entre fungos endofíticos e o potencial antimicrobiano da planta *Schinus terebinthifolius*. Neste contexto, os fungos endofíticos isolados de folhas de *S. terebinthifolius* foram inicialmente identificados. Um total de 232 endofíticos fúngicos foram obtidos, e caracterizados por macro e micro morfologia, seguido por métodos moleculares, que permitiram a identificação oito tipos diferentes de gêneros *Alternaria, Colletotrichum, Diaporthe, Epicoccum, Phyllosticta, Fusarium, Pestalotiopsis* e *Cryptococcus* sp., com a introdução de um novo taxon descrito como *Diaporthe foliar* (CMRP1438). A avalição do potencial antimicrobiano destes isolados revelou que os extratos brutos de *Diaporthe terebinthifolii* (CRMP1430 e CRMP1436) apresentaram a melhor ação contra patógenos de origem bacteriana e fúngica. Além disso, as linhagens *Diaporthe malorum* (CRMP1321 e CRMP1330) e *Diaporthe foliar* (CRMP1429 e CRMP1438) foram as que apresentaram atividade antifungica expressiva frente as leveduras *Candida albicans*. A partir da identificação das linhagens com maior potencial antimicrobiano foram realizados diversos bioensaios com o objetivo de induzir e otimizar a produção de metabólitos secundários entre esses fungos endofiticos. Para isto, foram adotadas estratégias OSMAC (uma linhagem vários compostos), utilizando dois tipos de modificadores epigenéticos selecionados para induzir a produção de metabólitos. Os extratos *D. terebinthifolii* e *D. malorum* gerados apresentaram um perfil de atividade antimicrobiana similar aos extratos obtidos na ausência dos indutores. Uma caracterização química destesextratos foi conduzida visando identificar as alterações decorrentes da indução. Em síntese, um banco biológico de dados foi construído e após a purificação e identificação dos extratos um novo espectro de dados tanto de origem natural e ou originados por indução da produção de metabólitos podem ser elucidados.

Palavras-chave: Endofíticos, metabólitos fungicos e bioprospecção

ABSTRACT

Medicinal plants are relevant sources of new antimicrobial compounds and are colonized by endophytic fungi which appear to play an important role in the production of the plant metabolites., since that, Plant extracts used in traditional medicine may have part of their activity as a result of this interaction. Likewise, fungi represent a rich source of functional bioactive molecules, and the endophytic species stand out for this property. Thus, the main objective of this thesis is to understand the relationship between endophytic fungi and the antimicrobial potential of the *Schinus terebinthifolius* plant. In this context, endophytic fungi isolated from leaves of the *S. terebinthifolius* plant were initially identified. A total of 232 endophytic fungal specimens were obtained, characterized by macro and micro morphology followed by molecular methods allowing identification of eight different genera: *Alternaria, Colletotrichum, Diaporthe, Epicoccum, Phyllosticta, Fusarium, Pestalotiopsis* and *Cryptococcus* sp., with introduction of a new taxon described as *Diaporthe foliar* (CRMP1438). The antimicrobial potential evaluation of these isolates revealed that the crude extracts of *Diaporthe terebinthifolii* (CRMP1430 and CRMP1436) presented the best action against pathogens of bacterial and fungal origin. In addition, *Diaporthe malorum* strains (CRMP1321 and CRMP1330) and *Diaporthe foliar* (CRMP1429 and CRMP1438) were the ones that presented expressive activity against yeast *Candida albicans*. From the identification of the strains with greater antimicrobial potential, a variety of bioassays were performed to induce and optimize the production of secondary metabolites; among these is endophytic fungi. OSMAC strategies approach (one strain many compounds) was adopted, using two types of epigenetic modifiers selected to induce the production of metabolites. The new extracts generated in large scale from *D. terebinthifolii* and *D. malorum* presented a similar profile of antimicrobial activity in comparison with the previous extract obtained in the inducers absence. A chemical characterization of these new extracts was conducted, aiming to identify the changes resulting from the induction. In synthesis, a biological database was built. Therefore, after the purification and identification of the extracts, a new spectrum of natural data and or originated by induction of the production of metabolites can be elucidated.

Key words: Endophytic, fungal metabolites and bioprospecting

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CHAPTER II

LIST OF ACRONYMS

5-aza- 5-azacitidine
BT2 - β-tubulin gene BT2
CAPES - Coordination for the Improvement of Post-Graduate Education
CBS - Fungal Biodiversity Centre, Centraalbureau voor Schimmelcultures
CDC- Cell Division Cycle
CFU- Colony-forming unit
CMRP - Microbiological Collections of Paraná Network
CNPq - Brazilian Agency for Scientific and Technological Development
CTAB - Cetyltrimethylammonium Bromide
DMSO- Dimethyl sulfoxide
DNA - Deoxyribonucleic Acid
ITS - Internal Transcribed spacer region
LSU - Large ribosomal subunit
MFLUCC - Mae Fah Luang University Culture Collection, Thailand
PCR- Polymerase chain reaction
PDB- Potato Dextrose Broth
PDT- Photodynamic Therapy
pH- Potential of hydrogen
rDNA- ribosomal Deoxyribonucleic Acid
Sodium: sodium butyrate
TEF1 - Translation Elongation Factor 1-alpha
UFPR - Federal University of Parana
OSMAC- one strain many compounds

SUMMARY

CHAPTER I- OUTLINE OF THE THESIS

CHAPTER I: OUTLINE OF THE THESIS

1 GENERAL INTRODUCTION

Brazil has a rich biodiversity of medicinal plant species related to a long tradition of herbal medicine, these plants are used in the form of crude extracts, infusions, or plasters to treat disease (VIEIRA et al., 2012). New drugs were discovered through products bioprospection, via the isolation of bioactive metabolites from living organisms (DOS SANTOS et al., 2015; MARTINEZ-KLIMOVA; RODRÍGUEZ-PEÑA; SÁNCHEZ, 2017).

Approximately 22,000 bioactive secondary metabolites of microorganisms were already described (KUSARI; SINGH; JAYABASKARAN, 2014). Around 8,600 (38%) are obtained from fungi (CARTER, 2011), which highlight the biochemical richness of these organisms. The discoveries of new metabolites of biologically active fungi have been the focus on producing new antibiotics, chemotherapeutic agents, and pesticides. These fungus-produced metabolites are generally effective, have low toxicity and have a lower impact on the environment (MARTINEZ-KLIMOVA; RODRÍGUEZ-PEÑA; SÁNCHEZ, 2017).

Several metabolites products, synthesized and secreted by plants, assumed as promising new pharmaceutical molecules are associated to microorganisms, called endophytic (GUTIERREZ; GONZALEZ; RAMIREZ, 2012). Endophytic fungi are fungi that grow inside plant tissues without cause immediate damage (PETRINI, 1991). In addition, the metabolites produced by endophytic fungi can transform the main compounds of plants of pharmaceutical interest, altering the therapeutic value of the plant (GONDA et al., 2013).

Medicinal plants have played an important role in the search for new bioactive strains of endophytic fungi, as it is possible that their beneficial characteristics are a result of the metabolites produced by their endophytic community (KUSARI; HERTWECK; SPITELLER, 2012). Moreover, extracts of plants used in traditional medicine can have their activities influenced by the endophytic microorganisms present (RADU; KQUEEN, 2002; SANTOS et al., 2017). In addition, endophyte fungi can regulate biochemical routes, leading to the production of substances common to their hosts or vice versa, which may have applications outside the plant in which they reside (STIERLE et al., 1995).

Some endophytic fungi provide benefits to their drought tolerance (HUBBARD; GERMIDA; VUJANOVIC, 2012), pathogen protection (ARNOLD et al., 2003), improve of plant growth (REN; LI; GAO, 2017), and herbivorous defense. (BORGES et al., 2009). These characteristics, combined with its immense diversity, led scientists to classify endophytic fungi as potential producers of interesting metabolites (STROBEL, 2003).

The *Schinus terebinthifolius* as well as the plants in general, presents colonization by endophytic microorganisms (TONIAL et al., 2016; SANTOS et al., 2017). This plant is a perennial tree belonging to the family Anacardiaceae and native to South America (FARIZEL; GAGLIANONE, 2008). Which presents ample use within popular medicine, which allows choice in biological, clinical, pharmacological and chemical studies (SILVA et al., 2017). The extracts of *S. terebinthifolius* and their endophytic fungi presented high potential for bioprospecting (TONIAL et al., 2017). Due to the medicinal properties of *S. terebinthifolius*, this species was the focus in the present study of a search for endophytic fungi able to produce bioactive substances with antimicrobial activity, the thesis was presented in four chapters:

The **Chapter I** provides a general overview about endophytes fungal in medicinal plants *S. terebinthifolius.* In **Chapter II** presents isolate and identify endophytic fungi from the same tree *S. terebinthifolius* and investigate the antimicrobial potential of the endophytic species isolates in order to elucidate the relationship of endophytic in compounds production by this host plant. **Chapter III** Screening and isolation of antifungal compounds from endophytes fungal *Schinus terebinthifolius.* A final consideration highlighting the main scientific findings is in the **Chapter IV**.

2 OBJECTIVES

2.1 GENERAL

The main objective of this study was characterization of endophytic fungi of *Schinus terebinthifolius* with potential antimicrobial activity.

2.2 SPECIFIC

- Carry out taxonomic studies based on the molecular characterization of endophytic isolates from previously selected *S. terebinthifolius* plants with relevant antimicrobial potential;
- To study the production of the fungal metabolites of these isolates through the production of crude and fractionated extracts under different growing conditions;
- To purify the compounds with antimicrobial activity from the endophytes of the "Brazilian pepper tree".

3 LITERATURE REVIEW

3.1 INTRODUCTION

Medicinal plants have been used by humans for the treatment of many diseases since the olden times. Plants constitute the first medicinal products available in nature to treat, cure or alleviate the symptoms of diseases (DIAS; URBAN; ROESSNER, 2012). Antimicrobials obtained from plants have been receiving attention due to a necessity to discover new antimicrobials agents with different chemical structures and novel mechanisms of action for the treatment of new and reemerging infectious diseases (SHARMA et al., 2013).

In popular medicine, *Schinus terebinthifolius* has been used for treatment of respiratory problems, wounds, rheumatism, as an anti-inflammatory and hemostatic agent, for the treatment of urinary and respiratory infections, as well as an aid for prevention of cancer through its antioxidant properties (DE MELO JÚNIOR et al., 2002; EL-MASSRRY et al., 2009).

The vast majority of crude drugs originated from medicinal plants. The quality and the quantity of these drugs can be affected by several factors such as the genetic background, ecological habitat, and soil nutrients (JIA et al., 2016). Nowadays, it is gradually recognized that endophytic microorganisms have played a very important role in affecting the qualitative and quantitative properties of this metabolites because of their particular fungus-host interaction. Therefore, it is more important to understand the particular relationship between endophytic fungi and medicinal plants that are required for promoting crude drug production (NAIR; PADMAVATHY, 2014). In this chapter we reviewed the studies of *S. terebinthifolius* properties, a medicinal plant known as “aroeira” and the endophytic fungi, focusing in the most frequent genera isolated from this plant.

3.2 *Schinus terebinthifolius*

Schinus terebinthifolius Raddi, commonly known as “aroeira” in Brazil, belongs to the Anacardiaceae family being largely distributed along the Brazilian territory, mainly found in the Atlantic Forest (WHEELER; MASSEY; ENDRIES, 2001).

The fruits, commonly known as rose pepper, foster great interest in the food industries because of their flavor and aroma. In addition, *S. terebinthifolius* has been widely used in folk medicine especially as a natural anti-inflammatory agent (DE MELO JÚNIOR et al., 2002; EL-MASSRRY et al., 2009). Studies with extracts of 'aroeria' obtained from leaves have corroborated this antioxidant, anti-allergic, antimicrobial, anti-inflammatory, antiulcer and antiadherent properties as well as wound-healing properties (BARBIERI et al., 2014; ULIANA et al., 2016; SILVA et al., 2017).

The biological applications of this species have been described since the first edition of the Brazilian Pharmacopoeia in 1926. A Brazilian gel-based aqueous bark extract of *S. terebinthifolius* has been marketed since 1999 for the treatment of vaginitis and cervical vaginitis (LEITE et al., 2011). Given the therapeutic interest and its pharmacological properties, in 2009 the plant was included in the National Program of Medicinal Plants and Phytotherapy (Programa Nacional de Plantas Medicinais e Fitoterápicos) of Interest to Unified Health System (SUS- Sistema Único de Saúde (SOARES, 2010).

3.2.1 PHARMACOLOGICAL AND BIOLOGICAL ACTIVITIES OF *S. terebinthifolius*

The phytochemical profile of *S. terebinthifolius* is characterized by high content of tannins, gallic acid, and flavonoids (QUEIRES et al., 2006; CERUKS et al., 2007). The chemical constituents of the methanolic extract from the leaves of *S. terebinthifolius* revealed the presence of important bioactive components. These compounds have antioxidant, anti-inflammatory, healing, antimutagenic and antitumor properties. They are also considered chemical inhibitors of carcinogenesis (CARVALHO et al., 2013).

The activity of the essential oil of *S. terebinthifolius* has been reported in several studies. The essential oil of *S. terebinthifolius* leaves can be used as an effective repellent and a hunger inhibitor in *Sitophilus oryzae* L., popularly known as weevils (BENZI; STEFANAZZI; FERRERO, 2009). It also has insecticidal and repellent activity against *Trogoderma granarium*, commonly known as the rice insect pest, and *Tribolium castaneum*, the brown beetle, which attacks all types of ground cereals (PATOCKA; ALMEIDA, 2017).

Uliana et al. (2016) identified 32 compounds that resemble 97.9% of the composition of the essencial oil from *S. terebinthifolius*. The major compound identified in the essential oil was δ-3-caene (68.78%), followed by the e-caryophyllene (8.22%), myrcene (6.78%), and α-pinene (4.05%). Likewise, Silva et al. (2010) identified δ-3-carene (55.43%), α-pinene (16.25%), sylvestrene (10.67%), germacrene-d (2.17%), β-myrcene (1.99%), and isoter-pinolene (1.40%) as the main compounds in the fruits (rose pepper) of those collected in Vitória (southeast of Brazil). The basic components of the essential oils isolated from *S. terebinthifolius* fruits collected in Reunion Island were limonene, α-phellandrene, α-pinene and germacrene-D (PÉRINO-ISSARTIER et al., 2010). Additionally α -pinene, α-phellandrene, β-phellandrene and limonene were reported as the main components in specimens from German (RICHTER; VON REUSS; KÖNIG, 2010). The variations in the composition of the essential oil and ethanol extracts are related to many factors such as the season of harvesting, temperature, humidity, soil, plant part, time of collection, among others factors (ULIANA et al., 2016).

Queires et al. (2006) reported antiproliferative and antitumor activity on cancerous prostate cells by the polyphenol fraction of the leaf extract of *S. terebinthifolius*, likely due to the fact that these compounds act on lysosomal enzymes of the cells and, consequently, induce apoptosis. Matsuo et al. (2011) reported that α-pinene, isolated from fruits of *S. terebinthifolius,* may be an important component of melanoma therapy, given its high potential for inducing apoptosis in cancer cells. In rats treated systemically with α-pinene, there was a marked reduction in lung tumor nodules, indicating important activity against metastatic melanoma. Santana et al. (2012) described, for the first time, the presence of gallic acid in the active extract of *S. terebinthifolius* with antitumor activity.

Fedel-Miyasato et al. (2014) suggest that the methanolic extract from *S. terebinthifolius* leaves has therapeutic potential. They showed that *S. terebinthifolius* presents significant wound-healing, anti-inflammatory, and chemopreventive properties, which can be correlated with the prevention and/or treatment of degenerative diseases related to inflammation and mutagenic processes. This study also confirms that the extract of *S. terebinthifolius* can prevent and/or repair DNA damage by desmutagenic and bioantimutagenesis processes. Recently, two derivatives of gallic acid and four flavonoids were isolated and exhibited considerable antioxidant activity. The extract and its compounds showed this property selectivity towards ovarian cancer cells (SILVA et al., 2017).

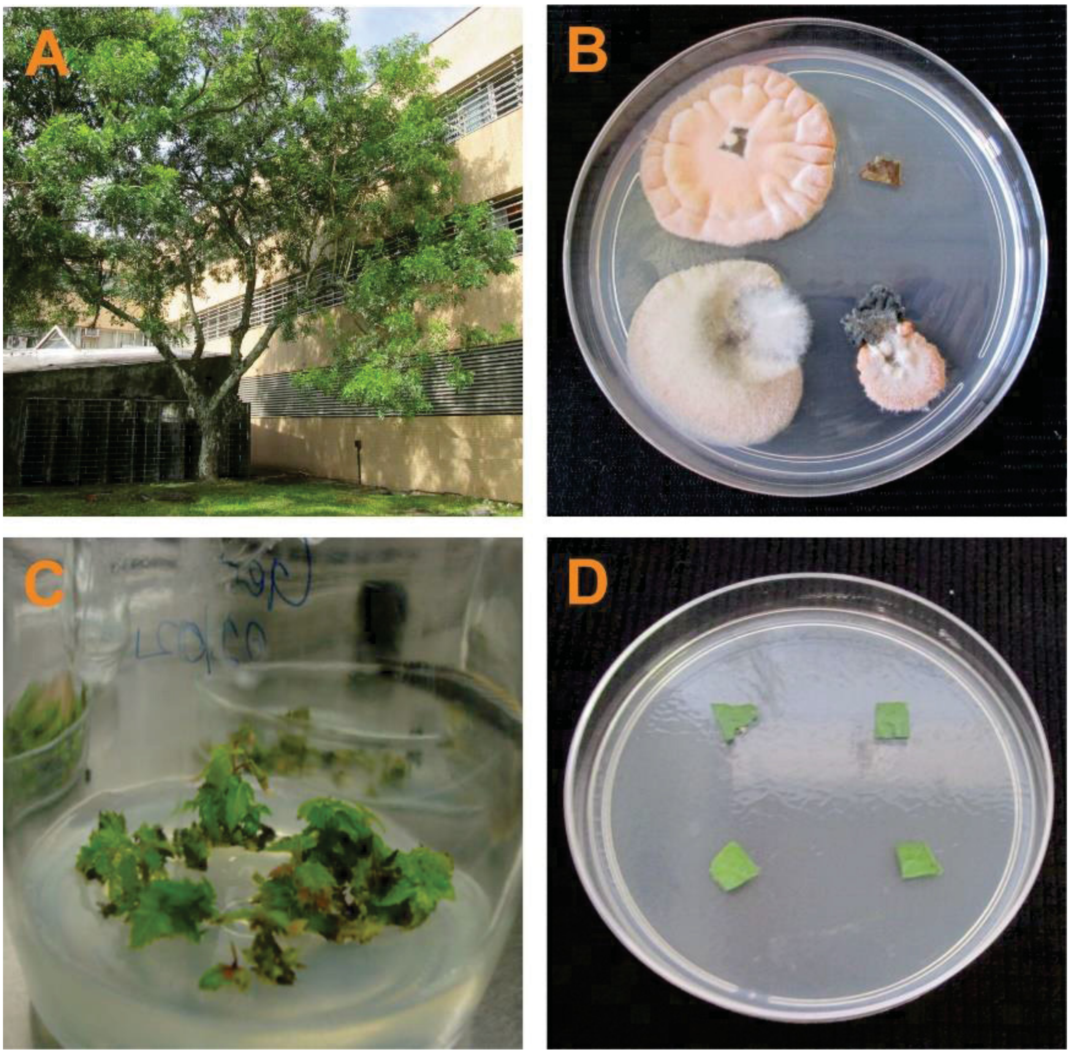
The relationship between the quantity of phenolic compound and antioxidant activity was confirmed by several studies (KOSANIĆ et al., 2013; ZHANG et al., 2013; SCHERER; GODOY, 2014; LEMOS et al., 2015). Uliana et al. (2016) observed a direct relationship between the antioxidant activity and the total phenolic content. Rosas et al. (2015) demonstrated the ability of *S. terebinthifolius* hydroalcoholic extracts to inhibit cell migration in arthritis, suggesting a putative use of this plant for the development of phytomedicines to treat inflammatory diseases, such as joint inflammation.

Several studies have reported antimicrobial potential of *S. terebinthifolius*. The alcoholic extract obtained from fruits of *S. terebinthifolius* presented a significant amount of flavone apigenin and ellagic acid that showed inhibitory effects on the growth of *Staphylococcus aureus* and *Bacillus* cereus, these extracts (DEGÁSPARI; WASZCZYNSKYJ; PRADO, 2005). In another study, the aqueous extract of *S. terebinthifolius* showed activity against *S. aureus, S. epidermidis, B. cereus, Pseudomonas aeruginosa, Trichophyton rubrum, Microsporum canis, Epidermophyton floccosum,* and *Candida albicans* (LIMA et al., 2004). The authors concluded that this antifungal activity is possibly associated with the presence of certain chemical compounds, especially tannins, in addition to compounds present in lesser amounts, such as alkaloids, steroids, chalcones, and urundeuvins (LIMA et al., 2004). Moura-Costa et al., (2012) reported that the leaf extract of *S. terebinthifolius* was not effective against gram-positive and gram-negative bacteria. However, it exhibited good results against *Candida* species.

The best antimicrobial activity of extracts obtained by ultrasound was correlated to a higher concentration of flavonoids, such as quercetin. Braga et al. (2007) investigated the antimicrobial activity of extracts and fractions of leaves of *S. terebinthifolius* and correlated their action with the presence of phenolic compounds and flavonoids. El-massrry et al. (2009) reported very similar results for the ethanol extract and essential oil from leaves of *S. terebinthifolius,* cultivated in Egypt. Thus, the antibacterial activity of extracts of *S. terebinthifolius* may be consider a good medicinal alternative to treat infections in Intensive Care Unit (UCI). Moreover, further chemical investigations may be done to isolate and identify minor chemical constituents in the leaves and to screen other potential bioactivities. Uliana et al., (2016) showed that extracts obtained by maceration or ultrasound exhibited higher activity against *S. aureus* compared to norfloxacin, which is widely used for treatment in Intensive Care Unit (UCI).

Many drugs have been originated from medicinal plants, and several factors such as the genetic background, ecological habitat, and soil nutrients can affect the quality and the quantity of these compounds (JIA et al., 2016). Some studies suggest that endophytes microorganisms have played a very important role in affecting the qualitative and quantitative properties of these metabolites because of their particular fungus-host interaction (FIGURE 1) (SANTOS et al., 2017).

FIGURE1-ENDOPHYTIC FUNGI ISOLATED FROM OUTDOOR-GROWN AND MICROPROPAGATED SAMPLES OF *S. terebinthifolius*.



A-outdoor-grown *S. terebinthifolius*; B- Isolation of endophytic fungi from outdoor-grown *S. terebinthifolius*; C–*n vitro S. terebinthifolius*; D- Isolation of endophytic fungi from *in vitro S. terebinthifolius*. (SANTOS et al., 2017)

The endophytic fungi confer fundamental implications on their host plants such as enhancing their growth, increasing their fitness, strengthening their tolerances to abiotic and biotic stresses, and promoting their accumulation of secondary metabolites (NAIR; PADMAVATHY, 2014; JIA et al., 2016). Therefore, it is important to understand the relationship between endophytic fungi and medicinal plants to improve production antimicrobial drugs.

3.3 ENDOPHYTIC FUNGI

It is estimated that 1.5 million species of fungi are present on our planet (HAWKSWORTH, 2001). Among these are the endophytic fungi, which colonize tissues of plants in an intracellular or extracellular form, in a relationship that varies from symbiosis to the pathogenicity borderline (SAIKKONEN et al., 1998). Nowadays the most commonly used definition for endophyts is from Petrini, 1991. In the broadest sense, endophytic fungi are fungi that colonize living plant tissue without causing any immediate or overt negative effects (HIRSCH; KAPULNIK, 1998).

The symbiotic relationship between the plant and the endophytic microorganisms is beneficial to the microorganism because it provides protection and nutrition. Moreover, the substances produced by the microorganism can increase plant growth, reproduction, and resistance to environmental stressors (SAIKKONEN et al., 2004). Many of these microorganisms are important in the process of adaptation and selection of various plant species because the fungi may produce compounds that develop an unpleasant taste in the plant, preventing insect attacks or even promoting growth in hostile environments (CARROL, 1988).

Endophytic fungi may regulate biochemical routes and produce substances common to their hosts or vice versa, which may have applications outside the host plant (STROBEL, 2003). Paclitaxel (taxol®) was the first report of isolation from the *Taxomyces andreanae* fungus. This fungus was isolated from *Taxus brevifolia* (STIERLE et al., 1995). Taxol® or paclitaxel is a potent antineoplastic therapeutic agent which was initially identified in *Taxus brevifolia* extracts (WANI et al., 1971). Other examples of endophytic microorganisms that produced the same metabolites as the host plants are *Fusarium* sp. and *Myrothecium* sp. fungi (TRAPP et al., 1998).

They are reported as producers of macrocyclic trichothecenes which were isolated from *Baccharis megapotamica* and *B. coridifolia* plants (RIZZO et al., 1997).

Kusari et al., (2014) reported that the endophytic-plant interaction is beyond the balance between virulence and defense, and this interaction is complex and precisely controlled. One example is camptothecin, which is found in the shell of *Camptotheca acuminata*, and shows cytotoxic and anticancer activity due to inhibition of the enzyme topoisomerase I, involved in DNA transcription and replication processes and which is highly expressed in tumors (KUSARI; HERTWECK; SPITELLER, 2012). The fungus *Fusarium solani* isolated from the tissues of *C. acuminata*, also reported as a camptothecin producer, guarantees its protection against this compound through specific alterations in the catalytic domains of its topoisomerase I (KUSARI; ZUHLKE; SPITELLER, 2011). Similarly, topoisomerase I encoded by *Ophiorrhiza japonica*, another endophyte isolated from the same host, which exhibits partial resistance to camptothecin *in vivo*. Although it does not produce this compound itself, *O. japonica* contains the same changes to make it resistant to camptothecin action. It suggests that evolutionary pre-adaptation similar to endophytes infecting the same plant, regardless of their biosynthetic capacity (SIRIKANTARAMAS; YAMAZAKI; SAITO, 2009).

The presence of endophytic fungi and some metabolites produced by them can modify the metabolic pathway of plants. Wang et al. (2011) investigated the effects of the *Gilmaniella* sp., an endophytic fungus, and its metabolites in the defense and metabolic responses of *Atractylodes lancea* plant to understand how to best use endophytic fungi and their elicitor resources. Both fungus and elicitor enhanced defense-related enzyme activities. In fungus-inoculated groups, phenylalanine ammonia lyase and polyphenol oxidase activities increased slowly, and reached a maximum level during the later stages, whereas peroxidase activity peaked in the first few days. The results indicate that fungal metabolites could substantially improve the total volatile oil content, while the fungus could more effectively improve the quality of herbal medicines.

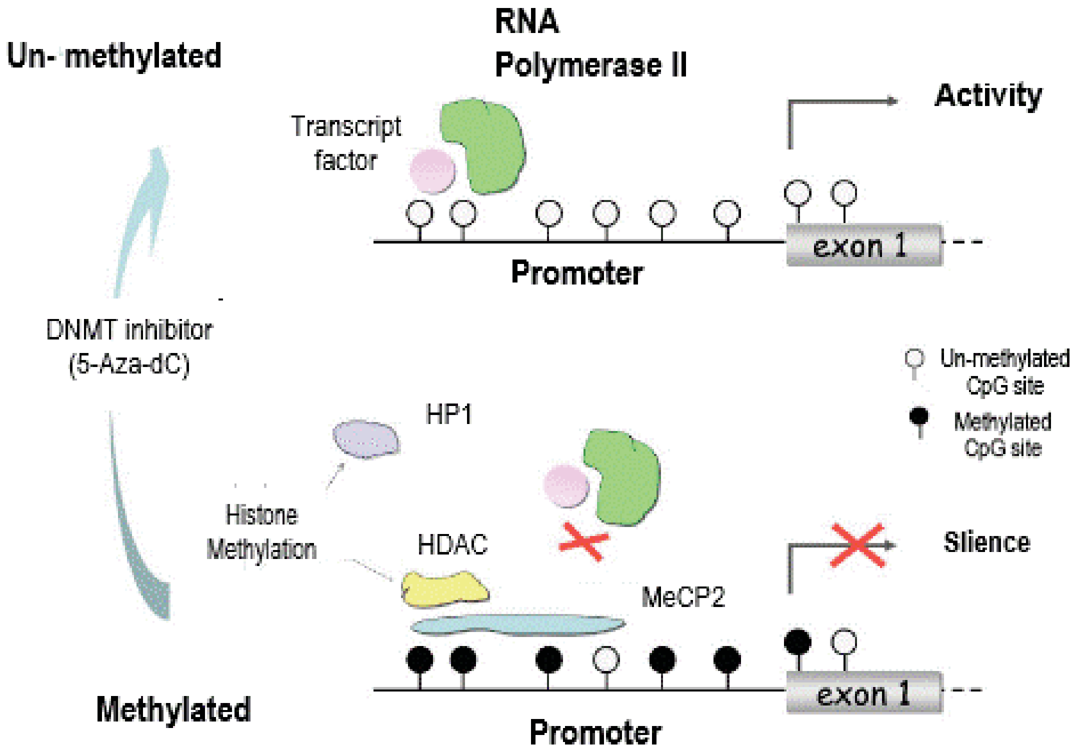
The endophytes are increasingly becoming the object of research efforts, especially when the source plant is traditionally used for healing (DENG; CAO, 2017). The endophytes produce molecules which are less likely to be toxic for eukaryotes, as they do not harm the plant host. However, it is also possible that the compounds do not harm the plant host because the plant host produces the same or similar compounds, and therefore is tolerant to them. Whether the same principle applies to mammalian cells requires testing (ALVIN; MILLER; NEILAN, 2014).

Secondary metabolites from microorganisms have a wide range of biological activities due to their structural diversity and have been proved a major source of compounds. Moreover, many microbial genes are apparently not transcribed under standard laboratory conditions which remain silent. Several strategies exist that try to overcome these limitations during fermentation of microbes (MARMANN et al., 2014). In in this context, one of the tools for promote compounds discovery from microorganisms involves the application of the OSMAC (One Strain Many Compounds) method that attempts to induce silent biogenetic clusters and hence may lead to accumulation of compounds not detected during conventional fermentations (PÉREZ HEMPHILL et al., 2017). Examples of such culture variations for filamentous fungi include the use of different liquid or solid media, as applied in a previous study with *Fusarium tricinctum* fermented on solid beans and liquid Wickerham medium versus cultivation on solid rice medium, which demonstrated an enhanced production of metabolites on beans (PÉREZ HEMPHILL et al., 2017).

Epigenetic small-molecule modifiers of DNA methyltransferase (DNMT) and histone deacetylase (HDAC) activities are being used to perturb the fungal secondary biosynthetic mechanisms (GONZÁLEZ-MENÉNDEZ et al., 2016). Two of the major epigenetic signals of chromatin regulation are acetylation of histones, which results mainly in activation of gene transcription, and methylation of histones and DNA (FIGURE 2), which is mainly linked to silencing of transcription (BROSCH; LOIDL; GRAESSLE, 2008).

These molecules can lead to the induction of the silent metabolite pathways expression. For example, DNMT inhibitors such as 5-azacitidine (FIGURES 3 AND 4) and hydralazine hydrochloride showed potential to reduce DNA-methylation-mediated silencing of different resistance genes and cellular processes in a wide variety of fungal species (GONZÁLEZ-MENÉNDEZ et al., 2016).

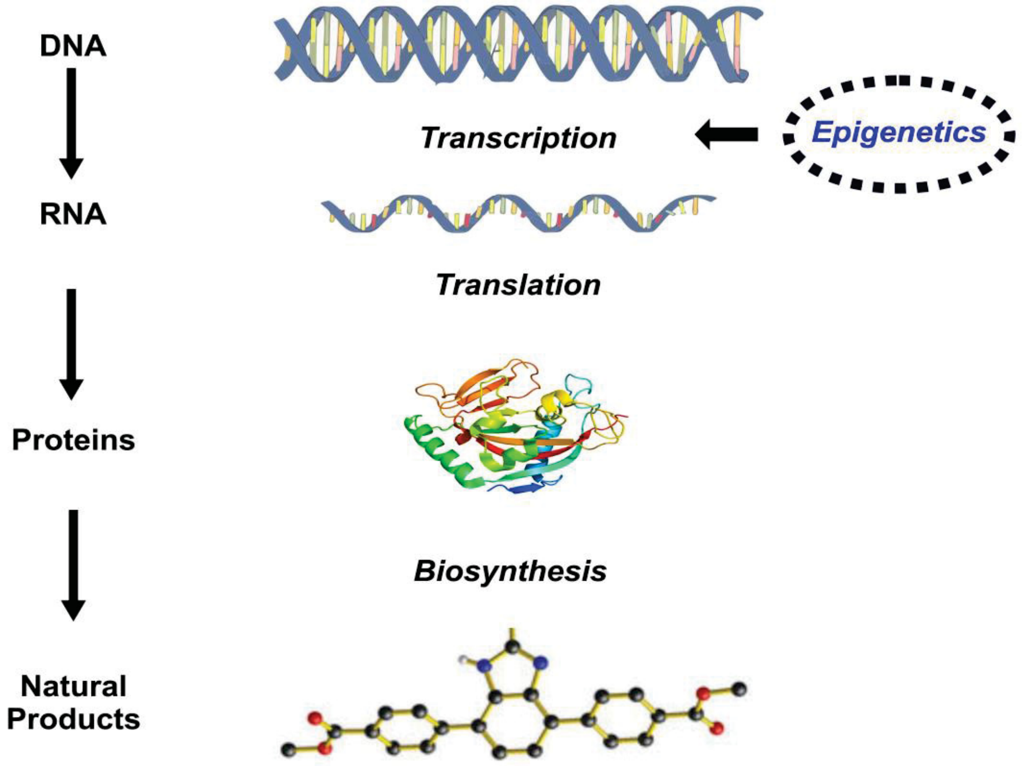
FIGURE 2- DNA METHYLATION INHIBITS GENE TRANSCRIPTION



Adapted from Zhu et.al., 2014

Three classes of histone deacetylases (HDAC) (I, II and III) have been identified in fungi (BROSCH; LOIDL; GRAESSLE, 2008). They are NAD-dependent protein involved in a wide variety of biological processes, including transcriptional silencing, apoptosis, fat mobilization, and stress resistance (PREYAT; LEO, 2013). For example, sodium butyrate is a potent inhibitor of histone deacetylases on fungi. (FIGURE 3). Zutz et al. (2014) demonstrated that the *Penicillium restrictum* grown in the presence of sodium butyrate produced extracts which revealed significant higher antimicrobial activity against *S. aureus* and multiresistant *S. aureus* strains and displayed no cytotoxicity against human cells.

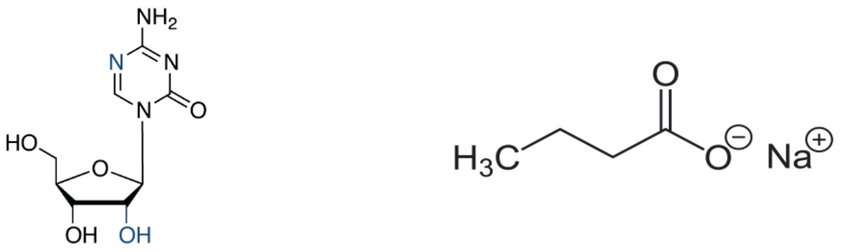
FIGURE 3- OUTLINE ILLUSTRATING THE FLOW OF GENETIC INFORMATION LEADING TO SECONDARY METABOLITE BIOSYNTHESIS IN FUNGI



Adapted from Cichewicz, 2011

More and more information is becoming available in the literature on the biodiversity and biosynthetic potential of endophytes. The section below included exclusively information about the main endophytes reported as isolated from *S. terebinthifolius* and produce metabolites capable of inhibiting the growth of bacteria and fungi (SANTOS et al., 2016; TONIAL et al., 2017).

FIGURE 4- EPIGENETIC SMALL-MOLECULE MODIFIERS



5-azacitidine

Sodium butyrate

3.4 ENDOPHYTIC FUNGI FROM S*chinus terebinthifolius*

Species composition may vary according to the host, geographical distribution, plant age, and ecological conditions including altitude and precipitation. One or two species are considered as predominant as endophytic in a given host, while all other isolates are rare (CARROL, 1988). Few studies were carried out regarding the *S. terebinthifolius* endophytic community. Strapasson; Santos; Medeiros (2002) isolated fungi belong to the genus *Fusarium, Alternaria, Pestalotiopsis, Penicillium, Aspergillus, Trichoderma, Chaetomium, Nigrospora, Goetrichum* and *Mucor* reported as *S. terebinthifolius* endophytic. Lima (2008) isolated endophytes fungal from the leaves of *S. terebinthifolius* fungi of the genus *Colletotrichum, Phomopsis/Diaporthe, Pestalotiopsis* and *Phyllosticta*. In a bioprospecting study, endophytes isolated from “aroeira” leaves belong to three different species of the genus *Colletotrichum* and showed great genetic diversity. These species included *C. gloeosporioides*, *C. boninense,* and *C. simmondsii* (LIMA et al., 2012).

Gomes et al. (2013) identified a collection of endophytic *Diaporthe* strains isolated from several medicinal hosts in Brazil. Thus, *D. schini, D. terebinthifolii, D. infecunda* and *D. oxe* were introduced as new endophytes species obtained from *S. terebinthifolius*. Tonial et al., 2016 reported the antimicrobial activity of extracts obtained from endophytic fungi isolated from *Schinus terebinthifolius* which belonged to the genera *Alternaria, Bjerkandera, Colletotrichum, Diaporthe, Penicillium* and *Xylaria*. In another study, a strain of *D. terebinthifolii* from *S. terebinthifolius* leaves. This strain presented metabolites which inhibited *P. citricarpa* spore germination. These findings suggested that *D. terebinthifolii* can be used as an effective alternative for directional Citrus Black Spot disease control (SANTOS et al., 2016; TONIAL et al., 2017). Therefore, the endophytes of *S. terebinthifolius* may comprise a promising source of antimicrobial compounds, especially the most frequent genus isolated *Diaporthe*.

3.5 *Diaporthe* GENUS

The genus *Diaporthe*, anamorph *Phomopsis* belongs to the Ascomycota phylum, Pezizomycotin subphylum, the Sordariomycetes class, the Sordariomycetidae subclass, located at the Diaporthales order in the Diaporthaceae family. Based on the deletion of Art. 59 from the International Code of Nomenclature for algae, fungi, and plants (ICN), asexual and sexual names of fungi receive equal status (GOMES et al., 2013). Therefore, as the name *Diaporthe* (1870) predates *Phomopsis* (1905), *Diaporthe* was adopted for this group of fungi (GOMES et al., 2013).

Previous taxonomic studies in *Diaporthe* genus, primarily based on morphology, which showed to be unnatural to reflect evolutionary history due to the simple and plastic morphological characters. Many Diaporthe species that are morphologically similar proved to be genetically distinct, and several isolates that were formerly identified based on their host, were shown to represent different taxa (GOMES et al., 2013). In addition, some species of *Diaporthe* are host specific, and a great number occur on more than one host (GOMES et al., 2013; GAO et al., 2015). The taxonomy of *Diaporthe* is far from being settled, since numerous taxa are only known from depauperate herbarium specimens, and/or the type material has apparently been lost (DAYARATHNE et al., 2016).

Udayanga et al. (2012) re-evaluated the phylogenetic species recognition in *Diaporthe* using a multi-locus phylogeny based on a combined data matrix of ITS, and partial sequences from the translation elongation factor 1-α, β tubulin, and calmodulin molecular markers. They included DNA sequences of available 26 ex-type cultures, providing a multi-locus backbone tree. Gomes et al. (2013) provided detailed descriptions of 54 species of *Diaporthe* that were linked to the types. In addition, Hyde et al. (2014) provided a backbone phylogenetic tree of 72 species, also derived from the types.

Recently, Dissanayake et al. (2017) reported that the genus has 171 species known from culture or from direct sequencing that is linked to their holotype, epitype, isotype or neotype and that can be recognized with molecular data (DISSANAYAKE et al., 2017). However, defining the boundaries of species remains a major challenge in *Diaporthe* (HUANG et al., 2015). This may be a consequence of limited sampling or the use of DNA loci with insufficient phylogenetic resolution (GAO et al., 2017). It has therefore been proposed that new species in the genus should be introduced with caution, and that multiple strains from different origins should be subjected to a multi-gene phylogenetic analysis to determine intraspecific variation (GAO; LIU; CAI, 2016).

The genus *Diaporthe* is distributed worldwide and has a great variety of hosts. Species of this genus may be phytopathogens, saprophytes, or endophytic symbionts (GOMES et al., 2013). Studies reported that *Diaporthe* may cause diseases on a wide range of plants hosts, some of which are economically important, as causing root and fruit rots, dieback, cankers, leaf spots, blights, decay, and wilt (UECKER, 1988; MOSTERT et al., 2001; GOMES et al., 2013). Curiously, some species of *Diaporthe* can be either pathogenic or harmless endophytes depending on the host and its health. For example, *D. phaseolorum* is pathogenic to soybean (SANTOS et al., 2011), but endophytic in *Laguncularia racemosa* mangroves (SEBASTIANES et al., 2012).

Among *Diaporthe* pathogens, *D. ampelina* is one of the pathogenic species which is best known as the causal agent of *Phomopsis* cane, leaf spot and grape yield losses in temperate regions (LAWRENCE; TRAVADON; BAUMGARTNER, 2015). The *D. phaseolorum* and *D. longicolla* are pathogenic to soybean (SANTOS et al., 2011). Besides some isolates of *Diaporthe* spp. have also been reported as pathogens of humans and other mammals (UDAYANGA et al., 2012). For instance, *Phomopsis leptostromiformis* was reported to cause lupinosis, a type of mycotoxicosis in sheep following consumption of diseased plants (VAN WARMELO; MARASAS, 1972). Human cutaneous infections have been reported in an immunosuppressed renal transplant recipient caused by *P. longicolla* and human phaeohyphomycotic osteomyelitis caused by a species of *Phomopsis* (SUTTON et al., 1999; GARCIA-REYNE et al., 2011).

Diaporthe species have been isolated as endophytic fungi from a wide variety of plants, both temperate and tropical climates (PEREIRA; VIEIRA; AZEVEDO, 1999; BOTELLA; JAVIER DIEZ, 2011; GONZÁLEZ; TELLO, 2011; ROCHA et al., 2011), and agricultural crops with high economic value, such as cocoa (RUBINI et al., 2005), coffee (VEGA et al., 2010), soybean (DE SOUZA LEITE et al., 2013), common bean (DOS SANTOS et al., 2016), and citrus (HUANG et al., 2015).

The genus has also frequently been recognized as a producer of interesting enzymes and secondary metabolites, which may have antibiotic or anticancer activity (ISAKA et al., 2001; KOBAYASHI et al., 2003; ZHANG et al., 2014). Furthermore, species of *Diaporthe* have in the past been noted to deter herbivory (VESTERLUND et al., 2011), have lignocellulolytic activities (JORDAAN; TAYLOR; ROSSENKHAN, 2006), and have been applied as bioherbicides (ASH et al., 2010).

The number of known endophytic *Diaporthe* species has increased rapidly in recent years (HUANG et al., 2015; GAO; LIU; CAI, 2016). Wang et al. 2014 concluded that our current knowledge of the ecology and biology of endophytic *Diaporthe* species is just the "tip of the iceberg". Gomes et al. (2013) introduced ten new endophytic species from medicinal plants: *D. endophytica, D. terebinthifolii, D. schini, D. infecunda, D. mayteni, D. oxe, D. paranensis, D. raonikayaporum, D. brasiliensis* and *D. inconspicua.* The research on Citrus conducted by Huang et al., (2015) recorded seven undescribed endophytic *Diaporthe* species, namely *D. biconispora, D. biguttulata, D. discoidispora, D. multigutullata, D. ovalispora, D. subclavata*, and *D. unshiuensis*. Inspection of *Diaporthe* species on *Camellia sinensis* resulted in the description of four new and five known species, all occurring as endophytes (GAO; LIU; CAI, 2016). Because some of these plant *Diaporthe* species are encountered as sterile endophytes and the recently reclassification of the genus, a multigene DNA database using the legitimate names will be essential to correct identification.

Therefore, DNA sequences analysis with combined rDNA-internal transcribed spacer (ITS), partial translation elongation factor 1-alpha (TEF1), beta tubulin (TUB), histone (HIS) and calmodulin (CAL) data matrix are employed to infer the species relationships (GOMES et al., 2013). Gao et. al. (2016) related that the pathogenic and endophytic isolates often clustered in the same clade and do not show recognizable segregation in the multi-locus phylogenetic tree. They suggested that the factors related to different life modes do not appear to play an essential role in driving the evolution of this group of fungi, and provide further support to the speculation that endophytes might be latent pathogens (PHOTITA et al., 2001; LIU et al., 2015).

Diaporthe is a creative genus with diverse bioactive secondary metabolites which have been widely isolated and characterized for industrial purposes. Liu et al. (2013) discovered 8 compounds including fungal toxins from one endophytic *Diaporthe* sp. of *Camptotheca acuminate*. Antimicrobial activities of endophytic *D. helianthi* isolated from *Luehea divaricata* were ensured by Specian et al. (2012). Two new fatty acid derived metabolites Diapolic acid A–B (1–2), were elucidated from the *D. terebinthifolii* crude extract. This fungal endophyte was isolated from the rhizome of *G. glabra*, India. The compounds were found to exhibit potential cytotoxic activity against the breast cancer cell line and moderate antifungal activity against *C. albicans* (YEDUKONDALU et al., 2017).

Many of the metabolites recently reported from *Diaporthe* were isolated from endophytic strains of medicinal plants, presumably following the rationale that the endophytic fungi may produce some of the active plant ingredients (RAJAMANIKYAM et al., 2017). A review presented by Chepkirui; Stadler (2017) reported 200 active compounds obtained from *Diaporthe*. Moreover, they did not detect a single molecule that has also been concurrently isolated from the respective host plant of the respective *Diaporthe* species. Rather, many metabolites, and the polyketides, are also known from other classes and families of Ascomycota, and some are particularly widespread. Therefore, *Diaporthe* is a good source of metabolic diversity, and it would certainly be worthwhile to further explore the genus for novel secondary metabolites. However, future studies should be directed towards the role of all these metabolites in the ecological relationships of the host plants with the endophytes.

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1 ABSTRACT

Endophytic fungi from *Schinus terebinthifolius* were isolated and evaluated for their antimicrobial activity. Two hundred thirty-four endophytic fungal isolates were obtained and identified by molecular methods into 12 different taxa of the genera *Alternaria*, *Colletotrichum, Diaporthe, Epicoccum, Phyllosticta, Fusarium, Pestalotiopsis* and *Cryptococcus* sp. Moreover, *Diaporthe foliar* was introduced as new species; The crude extracts from *Diaporthe terebinthifolii* (CRMP1430 and CRMP1436) showed the best action against pathogens. Moreover, the strains *Diaporthe malorum* (CRMP1321 and CRMP1330) and *Diaporthe foliar* (CRMP1429 and CRMP1438) inhibited *Candida albicans*. The antimicrobial activity of these endophytic microorganisms could be exploited in the biotechnological, medicinal, and agricultural industries.

Keywords: Fungal endophytes, *Schinus terebinthifolius*, *Diaporthe foliar*

2 INTRODUCTION

Endophytic fungi are microorganisms that spend all or part of their life cycles inter- and/or intracellularly colonizing healthy tissues of host plants and cause no apparent disease symptoms to their hosts (PETRINI, 1991; YANG et al., 2015). Their ecological significance has been well-studied but remains not completely understood. Furthermore, endophytic fungi can protect plants against the effects of herbicides, herbivore, and phytopathogens, and they may also act to stimulate growth through induction of morphological, physiological and biochemical changes in their hosts (BAYAT; MIRLOHI; KHODAMBASHI, 2009; GUNDEL et al., 2010). The species' composition can vary according to the hosts, geographic distribution, age of the plant, and abiotic conditions, including altitude and precipitation. For instance, one or two species are predominant such as endophytes, in a determined host, while other isolates are less frequent (PETRINI, 1991; BERNARDI-WENZEL et al., 2013).

Endophytic fungi supply a vast reservoir of secondary metabolites because of their diversity, and therefore are viewed as an outstanding source of novel and viable drugs (SURYANARAYANAN; THIRUNAVUKKARASU; GOVINDARAJULU, 2009). The ability of endophytic microorganisms to produce bioactive metabolites or molecules as precursors to chemical and pharmacological applications makes them a great exploration niche, since practically all plants have at least one of this coumponds (STROBEL, 2003). Considering this, medicinal plants stand out in this field since they have the ability to produce specific bioactive molecules, generating a complex metabolic habitat for the development of endophytic microorganisms (ALVIN; MILLER; NEILAN, 2014).

A study carried out by Pandey et al. (2016) strongly suggested that the presence of some endophytes is able to promote the biosynthetic potential of the plant for some key secondary metabolites. These findings emphasize that the presence of natural endophytes in the plant, probably plays an important role in promoting the growth of the host plant by increasing photosynthetic pigment synthesis, photosynthetic rate, stomatal conductance, and transpiration rate, which results in increased starch accumulation and biomass. Accrding to the literature, the absence of natural endophytes resulted in reduced *in vitro* plant growth. Furthermore, different studies reported that plants colonized by endophytic fungi represent an important repository of the fungal diversity and new species of fungi (CARVALHO et al., 2012; JIANG et al., 2013).

Schinus terebinthifolius Raddi (Anacardiaceae) is a native plant from South America, which has been used in folk medicine in teas, infusions or tincture such as anti-inflammatory, febrifuge, analgesic, and depurative agent; and to treat urogenital system illnesses (CARVALHO et al., 2013). It is commonly known as Brazilian pepper tree, it is likewise used in biological, clinical, pharmacological and chemical studies (ROSAS et al., 2015). Previous work determined the chemical composition of essencial oils from *S. terebinthifolius*, identifying predominance of monoterpenes such as α-pinene, β-Pinene, myrcene and limonene, followed by a lower concentration of sesquiterpenes such as D-germacrene (GOIS et al., 2016). Other studies have verified the *in vitro* antimicrobial activity of the extracts from brazilian pepper tree fruits (AUMEERUDDY-ELALFI; GURIB-FAKIM; MAHOMOODALLY, 2015). The antioxidant activity also has been evaluated in essential oils from brazilian pepper tree leaves (ULIANA et al., 2016).

Gundidza et al. (2009) related chemical and antibacterial profile differences among extracts of *S. terebinthifolius* originally from Zimbabwe, South Africa and from Brazil. Likewise, Tonial et al. (2016) reported fungal endophytes isolated from *S. terebinthifolius* with antimicrobial activity. In our previous study, it was demonstrated that the plant produced *in vitro* and the wild tree with endophytes microorganisms present a different antimicrobial activity profile which suggested that the endophytic community could influence the *S. terebinthifolius* metabolites production. (SANTOS et al., 2017). Moreover, the relationship of endophytic in compounds production by this host plant should investigate in order to elucidate this profile activity.

Therefore, this study aimed to isolate and identify endophytic fungi from the same tree *S. terebinthifolius* used by Santos et al. (2017) and investigate the antimicrobial potential of the endophytic species isolates.

3 MATERIALS AND METHODS

3.1 PLANT MATERIAL

The Brazilian pepper tree (*Schinus terebinthifolius*) leaves were collected from a tree found at latitude - 25º26.827S, longitude – 49º13.997O. The botanical identification has been made at the Department of Botany Herbarium at Federal University of Paraná (UFPR – UPCB).

3.2 ISOLATION OF ENDOPHYTIC FUNGI

Two hundred leaves were collected, placed in sterile plastic bags, and stored for less than 24 h at 10 ºC before isolating the endophytic fungi. Four fragments (approximately 0.5 cm long and 0.5 cm wide) of each leaf were cut by using a flame-sterilised blade in a laminar flow hood to prevent the air from contaminating the tissues. The surface of the leaves was sterilized (ARAUJO et al., 2002) by immersion in 70% ethanol for 1 min, followed by sodium hypochlorite (2 e 2.5% active chlorine) for 4 min, then in 70% ethanol 30s, and washed three times with sterile distilled water. The fragments were transferred aseptically to the Petri dishes containing Potato Dextrose Agar (PDA medium: potato 200 g/L, dextrose 20 g/L and agar 15 g/L supplemented with chloramphenicol (100 mg/mL) to inhibit the growth of bacteria. Efficient surface disinfection was confirmed by placing 1 mL of water from the last rinse onto the PDA. The Petri dishes were incubated at 25 ± 2°C for 30 days, observed daily and any fungal colonies present were isolated, purified and identified.

3.3 PRE-SELECTION

Fungal isolates were clustered into different groups according to their macromorphology: colony color and texture, border type, and radial growth rate on PDA agar. Based on the preliminary grouping, one isolate of each morphotype was selected for further molecular identification and antimicrobial activity. The endophytic fungus strain was deposited at Microbial Collections of Paraná Network- TAX online at Federal University of Paraná, the register in TABLE 1.

3.4 MOLECULAR CHARACTERIZATION

DNA extraction was performed using glass beads (Sigma G9143) according to protocols described previously (VICENTE et al., 2014). The rDNA Internal Transcribed Spacer (ITS) of the isolates was amplified using primers ITS1, ITS4 and ITS5 (WHITE et al., 1990). Also, isolates of the *Diaporthe* group which showed the best antimicrobial activity were selects for multi-locus phylogenetic analysis, using in addition to the ITS, the partial genes: β-tubulin (TUB), calmodulin (CAL), translation elongation factor 1-α (TEF1) and histone (HIS). All available type sequences of *Diaporthe* species were included in multigene phylogenetic analysis to identify the close relatives of the strains included in this study (Supplementary material- TABLE S1). The Partial large subunit nrDNA (LSU) was analysed to infer the generic relationships within Diaporthaceae.

Amplicons were amplified according to Gomes et al. 2013 and purified with Exonuclease I and Shrimp Alkaline Phosphatase (SAP) according to manufacturer's instructions (USB). Amplicons were sequenced with BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) and sequences were analyzed on an ABI Prism 3500 DNA Sequencer (Perkin-Elmer, Norwalk, Foster City, CA, USA).

3.5 PHYLOGENETIC ANALYSIS

Sequences were edited with the BioEdit software (HALL, 1999) and compared to the reference sequences at GenBank (NCBI) data sets (Supplementary material-

TABLE S1). The alignment was performed with MAFFT (KATOH; STANDLEY, 2013) and the visual inspection by the MEGA v.7 version software (KUMAR; STECHER; TAMURA, 2016). The best evolutionary model for each dataset was estimated using the program MEGA v.7 The phylogenetic trees were constructed with 1000 bootstrap replicates using the Maximum Likelihood Implemented in Mega v.7 software. New sequences generated in this study were deposited in the NCBIs GenBank nucleotide database (www.ncbi.nlm.nih.gov; TABLE 1) and the alignment and phylogenetic tree in TreeBASE (Data not showed during this submission will be available after acceptation).

3.6 MORPHOLOGY AND PHYSIOLOGY

Isolates which represented a new species fungus were identified based on macro and micromorphological features, and physiological tests were performed when necessary. The fungal sporulation was stimulated by several techniques: one based on Crous et al. (2009) which included exposure to 12 h near-ultraviolet light (400–315nm); incubation in the dark on 2% water agar supplemented with sterile pine needles -PNA (SMITH et al., 1996); autoclaved leaf pieces of *Schinus terebinthifolius* and culture in PDA, oatmeal agar (OA), and 2% malt extract agar (MEA). In addition, we used a protocol adapted from Araújo et al. (2002) which consisted of 500 mL of the aqueous extract of leaves of *S. terebinthifolius* diluted in 1L of water with aga/agar (WA) 15 g/L. In addition, tomato agar was tested (tomato juice 50 mL, distilled water 50 mL, agar 1.6 g. The samples were incubated at 20°C during at least one month.

Cardinal growth temperatures were determined in PDA medium. Plates were incubated in the dark for 3 weeks at a 21–36°C temperature range with intervals of 3°C; the growth was also recorded at 37 and 40°C. Experiments consisted of three simultaneous replicates for each the averages of three measurements were calculated.

3.7 SCREENING OF ENDOPHYTIC FUNGI FOR ANTIMICROBIAL ACTIVITY

The endophytic fungi were screened using the agar diffusion method, which allows for the rapid and qualitative identification of bioactive micro-organisms. One representative of each taxon of the endophytic fungal isolates was subjected to an antimicrobial essay using the modified agar block method (DE SIQUEIRA et al., 2011). The endophytic fungi were cultivated on solid PDA media at 28°C over 7 days. Plugs (6 mm in diameter) of the media plus the adhering mycelia were subsequently added to media and previously inoculated with *Staphylococcus aureus* (ATCC 27213), *Escherichia coli* (ATCC 35219), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231). The specific culture media used for bacteria was nutrient agar (Difco) and the pathogenic fungi were cultivated Sabouraud agar medium (SAB: peptone 10 g/L, dextrose 40 g/L, agar 16 g/L. The Petri dishes were incubated at 37°C for 24 hr for bacteria and at 30°C for 72 hr for fungi. The zones of inhibition were measured using a ruler. The test was performed in triplicate (NASCIMENTO et al., 2015).

3.8 FUNGAL CULTURE AND CRUDE EXTRACT PREPARATION

The endophytic fungal isolates were cultured on PDA solid media as follows: PDA, containing (g/L): agar 15, potato 200 and dextrose 20; pH 6.0. The fresh mycelia of different endophytic fungi were grown on plates at 28 °C for more than 7 days. Five plugs (6 mm in diameter) of media plus the adhering mycelia were subsequently added to 500-mL Erlenmeyer flasks containing 250 mL of potato dextrose broth. All liquid cultures were kept at 28°C for 14 days with shaking (120 rpm). The fermentation product of each fungus was filtered to separate the mycelia from the filtrates. The liquid were extracted with ethyl acetate (EtOAc) to obtain the bioactive extracts (HORMAZABAL; PIONTELLI, 2009).

To evaluate the activities of the extracts we utilized adapted protocol standardized by the National Committee for Clinical Laboratory Standards. For these analyses, wells were made by cutting out 6-mm sections of Mueller Hinton Agar (MHA; HiMedia, Mumbai, India) in Petri dishes. The inoculum (10^8 CFU/mL of *S. aureus* or *P. aeruginosa, E. coli* or 10^6 CFU/mL of *C. albicans*) was then spread homogeneously over the surface of culture medium. Each well was filled with 50 µL of the test solution, and dishes were incubated at 35°C for 18 h. Chloramphenicol (1 mg/mL for *S. aureus* and 10 mg/mL for *P. aeruginosa*) and nystatin (100000 IU/mL for *C. albicans*) were used as positive controls, and methanol and saline solutions were used as negative controls. The presence of a growth inhibition halo around a well was considered a positive result. The experiment was carried out in duplicate.

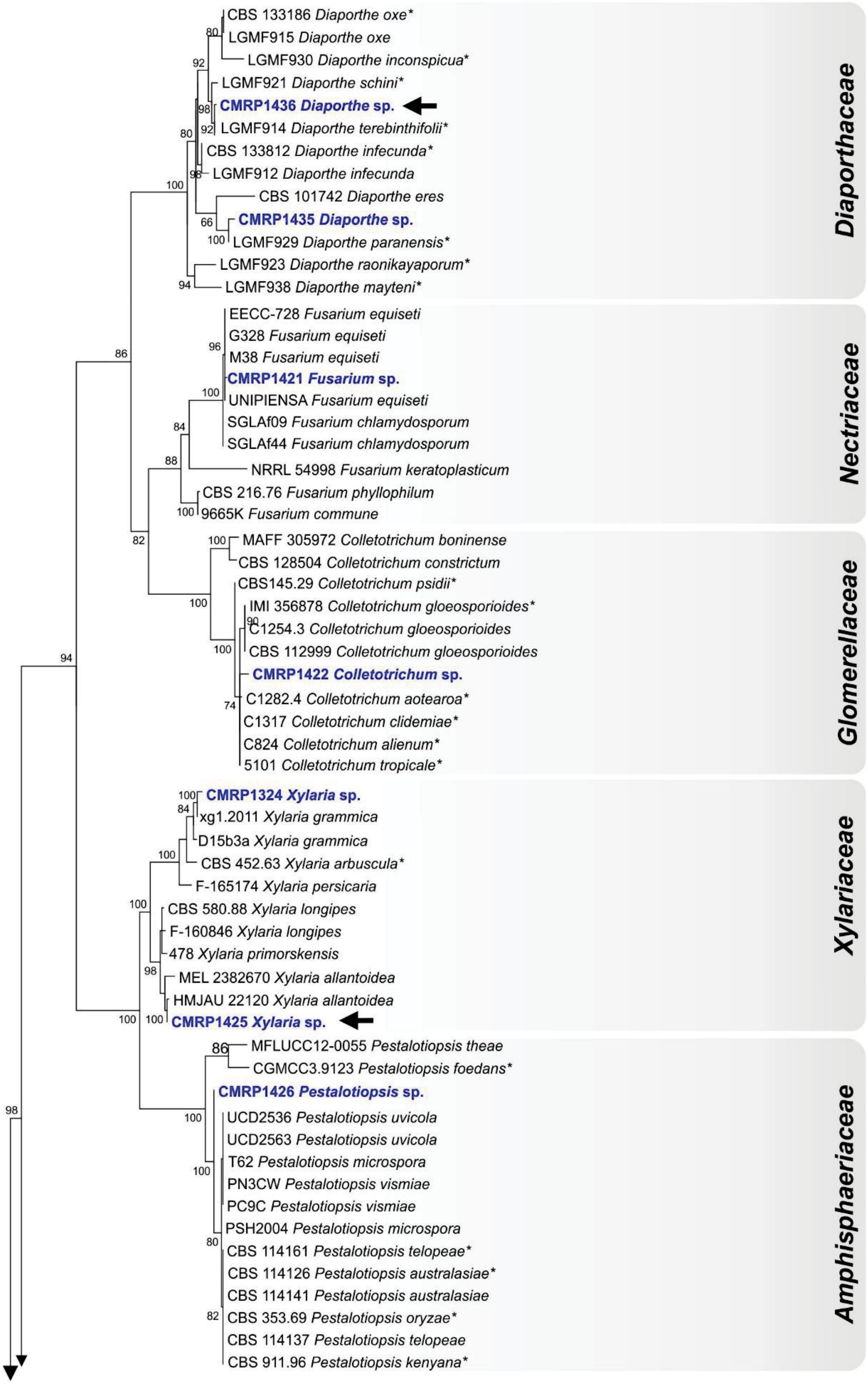
4 RESULTS

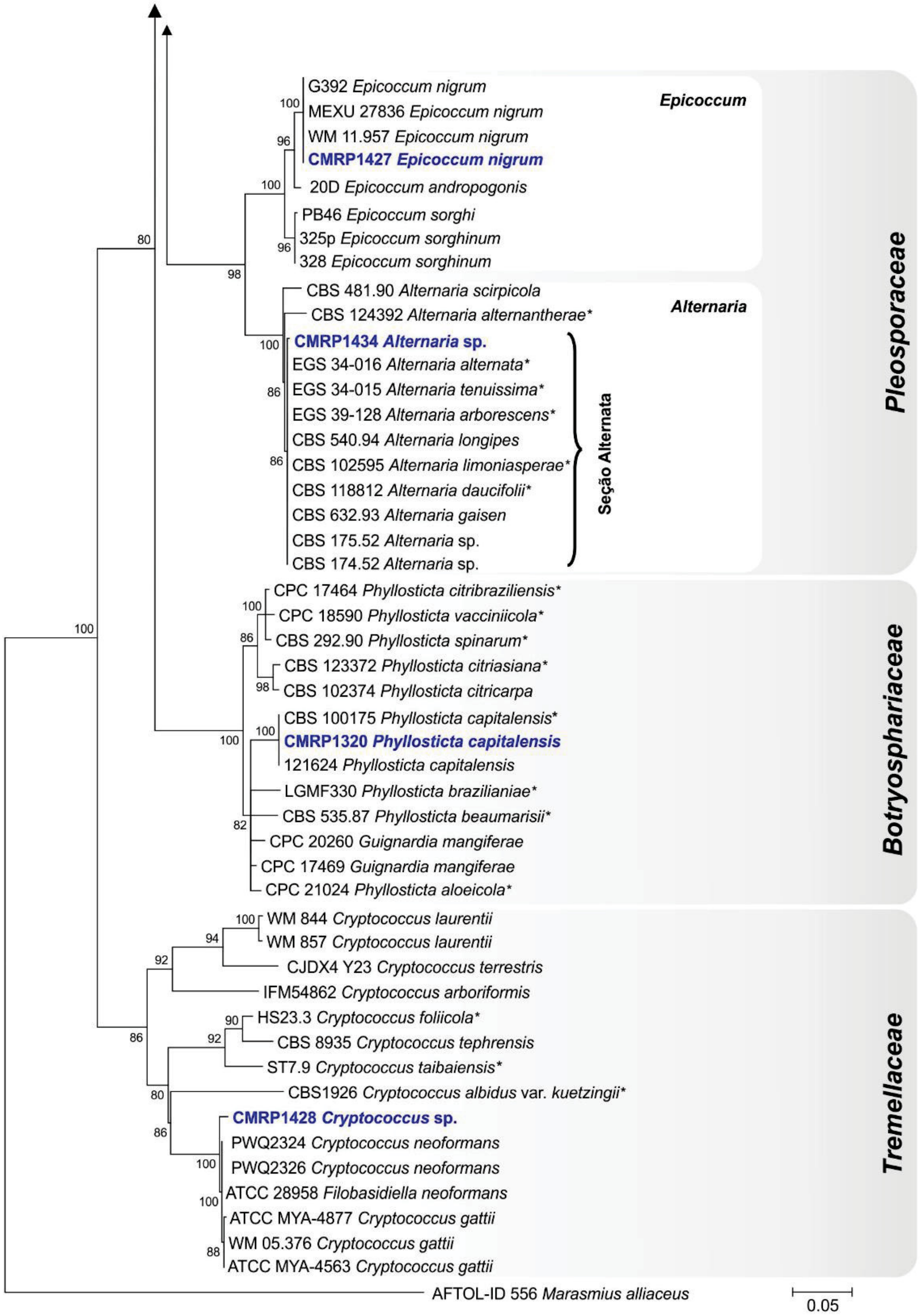
4.1 PHYLOGENETIC AND ANTIMICROBIAL ACTIVITY ANALYSIS OF ENDOPHYTIC FUNGI IN *Schinus terebinthifolius*

A total of 234 endophytic isolates were obtained from the leaf fragments *Schinus terebinthifolius*. Based on their morphological characteristics, these were initially classified into 11 groups with were identified by ITS1-5.8S-ITS2 sequences and compared with reference strains (TABLE S1). A selection of these strains was used to build a tree with Maximum Likelihood implemented in MEGA v. 7 using the substitution model Kimura 2-parameter. A total of 445 sites were evaluated, and the empirical base frequencies were pi (A): 0.02528, pi(C): 0.02435, pi(G): 0.02426, pi(T): 0.02609, with 100 bootstrap inferences. Judging from ITS sequences isolates could be attributed to the genus *Alternaria, Colletotrichum, Cryptococcus, Diaporthe*, *Epicoccum*, *Fusarium*, *Pestalotiopsis*, *Phyllosticta* and *Xylaria* (FIGURE 1).

Among the endophytic fungi recovered, the dominant genus was *Diaporthe* (Morphotypes IV and V), which represented 32.05% of the total isolates. Moreover, *Phyllosticta capitalensis* and *Xylaria* sp. (Morphotypes X and XI) were found in the *S. terebinthifolius* leaf, with a similar frequency of 13.68%, and 13.25% respectively. The remaining fungal endophytes isolated from *S. terebinthifolius* leaves were *Pestalotiopsis* sp. (8.55%) and *Eppicoccum nigrum* (8.55%), *Fusarium* sp. (8.12%), *Alternaria* sp., *Colletotrichum* sp. and *Cryptococcus* sp. were also recovered found in 6.41%, 5.13% and 4.27%, respectively. Most of the phylotypes belong to the Ascomycota, except *Cryptococcus* sp., which belongs to the Basidiomycota (FIGURE 1; TABLE 1).

FIGURE 1- PHYLOGENETIC RELATIONSHIPS OF FUNGAL ENDOPHYTES ISOLATED FROM *Schinus terebinthifolius*





NOTE: The parsimonious tree was constructed based on ITS1-5.8S-ITS2 sequences. ML/MP bootstrap values 80% are displayed above or below each branch. In blue case are isolate codes of fungal endophytes from leaf of *Schinus terebinthifolius*. The tree is rooted with *Marasmius alliaceus* (AFTOIL-ID) * Ex-type strain.

TABLE 1- ENDOPHYTIC STRAINS ISOLATED FROM LEAF OF *Schinus terebinthifolius*, ANACARDIACEAE AND ANTIMICROBIAL ACTIVITY

Morphotypes	**Frequency (%)**	**Biological activity**[1]	**Id – Name**	**Strain**	**GenBank accession numbers (ITS; TEF1; TUB; HIS; CAL; LSU)**[2]
I (*Alternaria*) n=15	6.41	ND	*Alternaria* sp.	CMRP1434	KY461084
II (*Colletotrichum*) n=12	5.13	ND	*Colletotrichum* sp.	CMRP1422	KY461082
III (*Cryptococcus*) n=10	4.27	ND	*Cryptococcus* sp.	CMRP1428	KY461083
IV (*Diaporthe*) n=17	7.26	*E. coli* (14.3) *P. aeruginosa* (17.7) *S. aureus* (18.1) *C. albicans* (ND)	*Diaporthe terebinthifolii*	CMRP1436	KY461099; KY461101; KY461100
			Diaporthe terebinthifolii	CMRP1430	KY461102; KY461103; KY461128
			Diaporthe foliar ***sp. nov.***	CMRP1438	KY461201; KY461202; KY461226; KY461211; KY461212; KY461213;
			Diaporthe foliar	CMRP1429	KY461205; KY461206; KY461227; KY461214; KY461215; KY461216;
			Diaporthe foliar	CMRP1585	KY461305; KY1306; KY461228
			Diaporthe infecunda	CMRP1325	KY461104; KY461105
			Diaporthe infecunda	CMRP1432	KY461097; KY461098; KY461130
			Diaporthe infecunda	CMRP1433	KY461106; KY461127
			Diaporthe infecunda	CMRP1437	KY461096; KY461132; KY461131
			Diaporthe infecunda	CMRP1439	KY461107; KY461108
			Diaporthe infecunda	CMRP1440	KY461093; KY461095; KY461094
			Diaporthe malorum	CMRP1321	KY461109; KY461111; KY461110
			Diaporthe malorum	CMPR1330	KY461112; KY461114; KY461113
			Diaporthe oxe	CMRP1327	KY461120; KY461129
			Diaporthe oxe	CMRP1418	KY461121; KY461123; KY461122
			Diaporthe oxe	CMRP1419	KY461124; KY461126; KY461125
			Diaporthe oxe	CMRP1420	KY461117; KY461119; KY461118
V (*Diaporthe*) n=58	24.79	ND	*Diaporthe* sp.	CMRP1435	KY461115; KY461116
VI (*Epicoccum*) n= 20	8.55	ND	*Epicoccum nigrum*	CMRP1427	KY461091
VII (*Fusarium*) n=19	8.12	ND	*Fusarium* sp.	CMRP1421	KY461081
VIII (*Pestalotiopsis*) n=20	8.55	ND	*Pestalotiopsis* sp.	CMRP1426	KY461085
IX (*Phyllosticta*) n= 32	13.68	ND	*Phyllosticta capitalensis*	CMRP1320	KY461092
X (*Xylaria*) n=15	6.41	*E. coli* (11); *P. aeruginosa* (ND); *S. aureus* (13.5); *C. albicans* (ND).	*Xylaria allantoidea*	CMRP1424	KY461088
XI(*Xylaria*) n=16	6.84	ND	*Xylaria grammica*	CMRP1425	KY461090

NOTE: ND means no activity detected;[1]means Diameter of inhibition zone (mm) of pathogen; [2]ITS: internal transcribed spacer regions of the nrDNA and intervening 5.8S nrDNA; HIS: partial histone H3 gene; TEF1: partial translation elongation factor 1-alpha gene; TUB: partial beta-tubulin gene.

4.2 ANTIMICROBIAL ACTIVITY

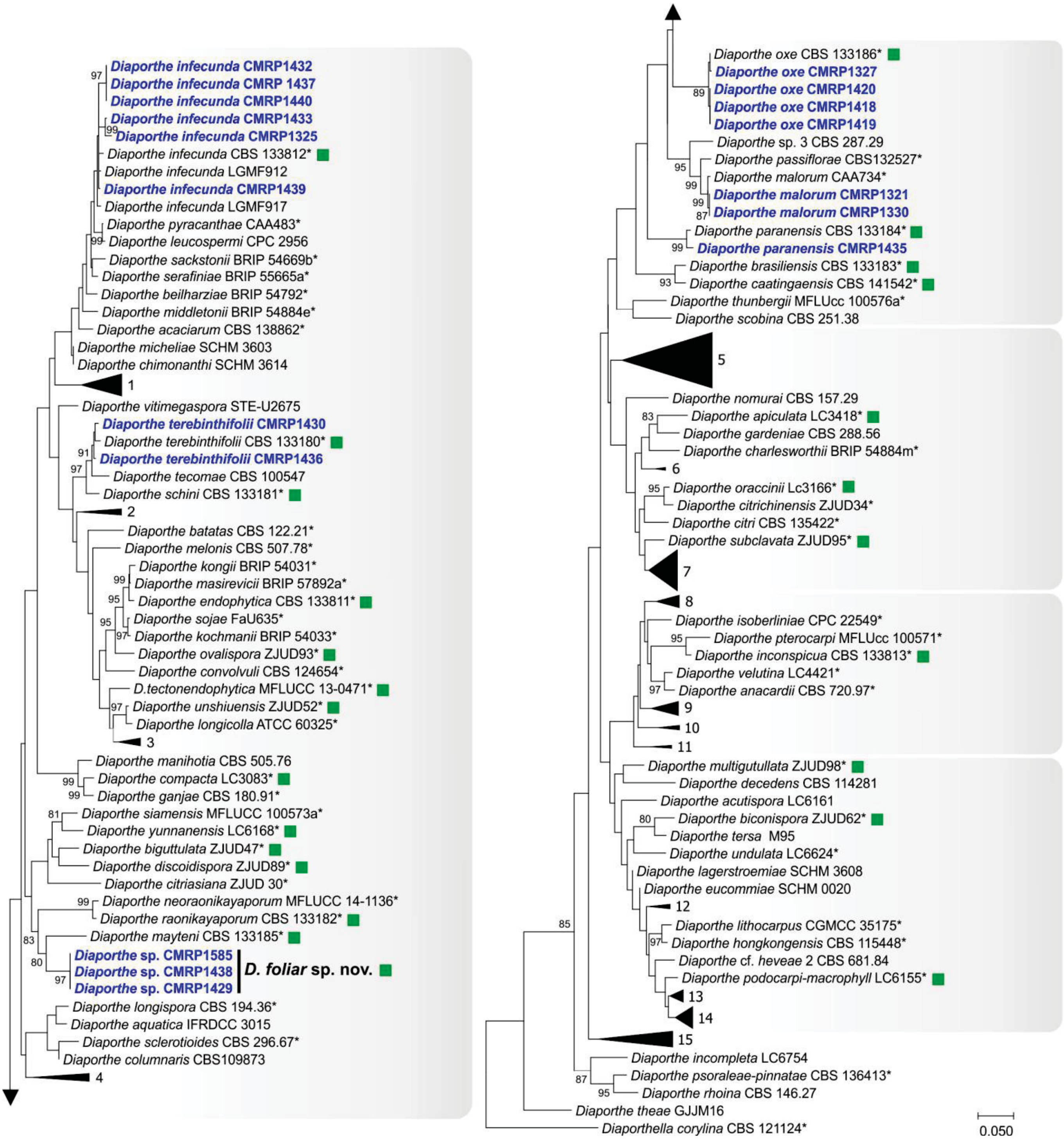
In order to evaluate the antimicrobial activity of these endophytic fungi, an agar diffusion assay was employed to search for bioactive endophytic strains, against the clinical bacteria *E. coli*, *P. aeruginosa*, *S. aureus* and yeast *C. albicans*. Of the

11 morphotypes evaluated, two had inhibitory effects against the tested pathogenic microorganisms (TABLE 1), *Xylaria* sp. (CRMP1424) was antagonic against *E. coli* and *S. aureus* with 11 and 13.5 mm diameter of inhibition zone respectively. *Diaporthe* sp. (CRMP1463) showed the best antimicrobial activity, which inhibited the growth of the three bacteria evaluated, *E. coli* (14.3 mm), *S. aureus* (18.1mm) and *P. aeruginosa* (17.7 mm). Hence, all *Diaporthe* isolates of the morphotype IV were selected for further study.

4.3 PHYLOGENETIC ANALYSIS OF *Diaporthe* STRAINS

Due to bioactive showed by strains *Diaporthe* sp. (morphogroup 4) in screening we performed sequence analysis for defining these species. The Phylogenetic analysis of *Diaporthe* strains was first analysed separately by single ITS1-5.8S-ITS2 of rDNA, partial β-tubulin, calmodulin, histone and translation elongation factor 1-α genes to investigate relationships among 249 strains (trees /not shown). Multilocus sequence analyses using these five informative loci were performed for identification and for elucidation of species identities including all sequences of *Diaporthe* strains known to be ex-type strains or are considered to be authentic/legitimate for the species are used (FIGURE 2; TABLE S1). All isolates of the genus *Diaporthe* (morphotype IV) obtained from S. terebinthifolius were distribute in five well-supported clades and the isolate CMRP1435 of the morpphotype 5 was identified as *D. paranensis*. The topology and branching order were essentially similar for the phylogenetic trees conducted. However, only the multi-locus analysis provided a more robust phylogenetic relationship and the species identification may be inferred.

FIGURE 2- PHYLOGENETIC TREE OF DIAPORTHE SPECIES GENERATED FROM THE COMBINED ANALYSIS OF FIVE GENE REGIONS (ITS, CAL, HIS, TEF1 AND TUB)



NOTE: The tree was constructed with Maximum likelihood, implemented in MEGA 7. Bootstrap support was calculated from 100 replicates; supported branches ith values >80 % are displayed above or below each branch. The tree is rooted with *Diaporthella corylina* (CBS 121124). Isolates from *Schinus terebinthifolius* obtained in thisstudy are indicated in blue. The new species *D. foliar* are indicate as sp. nov. Green boxes indicate the *Diaporthe* species described only as harmlessendophytes. Compressed/collapsed clades are numbered from 1 to 15, and the strains of these are indicate in the Supplementary material- TABLE S1. * Type strain.

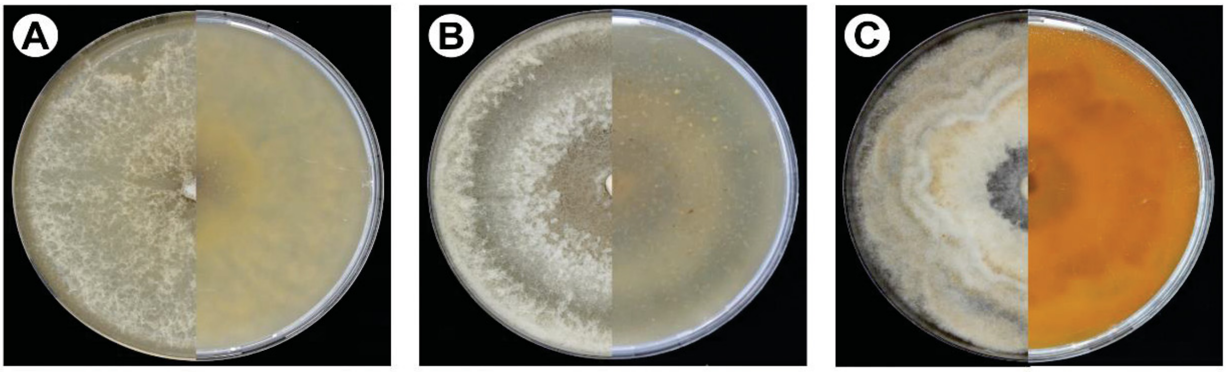
Among the strains previously identified as morphotype IV (n=17), six strains (CMRP1325, CMRP1432, CMRP1433, CMRP1437, CMRP1439 and CMRP1440) were grouped as *Diaporthe infecunda*; four (CMRP1327, CMRP1418, CMRP1419 and CMRP1420) were assigned to *D. oxe*; two strains (CMRP1430 and CMRP1436) were identified as *D. terebenthifolii* and two strains (CMRP1321; CMPR1330) as *D. malorum*. Three isolates (CMRP1429, CMRP1438 and CMRP1585) identified as *Diaporthe* sp. were distinct from all known species. The unnamed specie was clearly separate from the taxa already known, and it will be introduced below as a new species:

Diaporthe foliar G.D. Santos, R.R. Gomes & V.A. Vicente, ***sp. nov.*** - Mycobank MB (in progress)
Etymology. Named after the host tissue from which it was isolated, leaf.
Cultures sterile. *Diaporthe foliar* differs from its closest phylogenetic neighbors, clade *D. mayteni*, *D. raonikayaporum* and *D. neoraonikayaporum* by unique fixed alleles in five loci based on alignments of the separate loci deposited in TreeBase (in progress): ITS positions 13(T), 27(T), 29(A), 112(T), 316(C), 317(T), 318(G), 319(T), 322(Indel), 325(A), 327(G), 329(C); TEF1 positions 386(T), 460(C), 474(T), 480(T), 508(A), 511(G), 522 (G), 531(T), 533(C), 536(A), 546(A), 547(C), 548(G), 562(C), 567(T), 569(A), 579(T), 583(A), 587(C); CAL positions 657(T), 688(T), 698(G), 702(C), 712(T), 729(G), 730(T), 732(T), 782(G), 783(T), 784(C), 792(C), 835(T), 863(T), 898(C), 973(T), 975(G), 980(C), 984(Indel), 985(Indel), 1000(C), 1001(T), 1002(T), 1004(G), 1005(C), 1006(G), 1013-1016(Indel), 1020(T), 1021 (G); HIS positions only compared as *D. mayteni*, *D. raonikayaporum* 1129(A), 1213(C), 1257(G), 1266(T), 1278(T), 1279(T), 1282(Indel) 1286(T), 1297(G), 1396(G), 1423(T), 1515(G) and TUB positions 1532(A), 1537(G), 1539(G), 1558(T), 1574(T), 1583(A), 1659(C), 1678(A), 1709(C), 1776(A), 1789(G), 1896 (G).

Description of *Diaporthe foliar* CMRP1438 strain after 2 wk incubation in the dark at 25°C (FIGURE 3). Cultural Characteristics: Colonies on PDA showing growth rate 2.0 cm diameter, covering the dishes after 2 weeks, it was flat presenting entire edge, surface mycelium smooth in the center of the colony and felty in periphery, amber, straw, honey to isabelline, with reverse amber and ochreous. On OA showing growth rate 1.8 cm diameter, the colony was flat, entire edge, growth of the cottony aerial mycelium, grey and buff olivaceous, reverse olivaceous buff and smoke grey.

On MEA it presented raised, with an entire edge, surface mycelium cottony, buff, ochreous and patches amber, with a growth rate of 1.3 cm diameter. Reverse ochreous.

FIGURE 3- MACROMORFOLOGY *DIAPORTHE FOLIAR* HOLOTYPE: CRMP 1438 STRAIN. (A) PDA; (B) OA AND (C) MEA



(author personal collection)

The novel *Diaporthe* species showed optimal development at 25°C, while growth was observed in the entire range between 16−30°C. The maximum growth temperature of all strains analyzed was found to be 37°C. No growth was observed at 37°C. Diaporthe foliar represents a distinct species, containing three sterile isolates originating from Brazil. Isolates could not be induced to sporulate on any of the media used in this study, neither on the sterilized *S. terebinthifolius* tissues placed on WA and PDA. Descriptions based on DNA characters are provided. The phylogeny analysis based on the large subunit of the nuclear ribosomal RNA gene (LSU) supported the D. foliar as for its phylogenetic position in Diaporthaceae (tree /not shown).

Note: *Species examined.* Brazil, Paraná, Curitiba, endophytic species isolated from leaf of *Schinus terebinthifolius* (popular name “aroeira”), July 2011, G. Santos. Holotype: dried holotype UPCB (In progress) at Department of Botany Herbarium at Federal University of Paraná (UPCB); type strain CMRP1438= LMICRO469. Additional material examined listed in TABLE 1.

In addition, the antimicrobial activity of the crude extracts of this strain were analyzed. Of the 17 endophytes evaluated eight produced active metabolites against at least one pathogen. On the other hand, nine *Diaporthe* spp. failed to produce antimicrobial extracts under the conditions tested (TABLE 2).

TABLE 2- ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACTS FROM ENDOPHYTES FUNGAL OF *Schinus terebinthifolius*

Endophytic fungi	**Id – Name**	**Inhibition diameter zone (mm)**			
		S. aureus	***E. coli***	***P. aeruginosa***	***C. albicans***
Diaporthe foliar	CMRP1429	ND	ND	ND	28±2
Diaporthe foliar	CMRP1438	ND	ND	ND	26±2
Diaporthe foliar	CMRP1585	ND	ND	ND	ND
Diaporthe infecunda	CMRP1325	11±3	ND	ND	ND
Diaporthe infecunda	CMRP1432	ND	ND	ND	ND
Diaporthe infecunda	CMRP1433	ND	ND	ND	ND
Diaporthe infecunda	CMRP1437	ND	ND	ND	ND
Diaporthe infecunda	CMRP1439	ND	ND	ND	ND
Diaporthe infecunda	CMRP1440	ND	ND	ND	ND
Diaporthe malorum	CMRP1321	ND	ND	ND	27±3
Diaporthe malorum	CMRP1330	ND	ND	ND	23±2
Diaporthe oxe	CMRP1327	ND	ND	ND	ND
Diaporthe oxe	CMRP1418	12±2	ND	ND	ND
Diaporthe oxe	CMRP1419	ND	ND	ND	ND
Diaporthe oxe	CMRP1420	ND	ND	ND	ND
Diaporthe terebinthifolii	CMPR1430	29±2	21±2	28±2	19±2
Diaporthe terebinthifolii	CMPR1436	32±2	24±2	26±2	16±2

ND means no activity detected

The crude extracts from *Diaporthe terebinthifolii* (CRMP1430 and CRMP1436 strains) showed the best action against pathogens. Moreover, the strains *Diaporthe malorum* (CRMP1321 and CRMP1330 strains) and *Diaporthe foliar* (CRMP1429 and CRMP1438 strains) inhibited *Candida albicans* used as reference strain.

5 DISCUSSION

Endophytic fungi have a long-lasting evolutionary relationship with their host plants. So, many of them have the same or similar bioactive components as their host plants (SOLTANI; HOSSEYNI MOGHADDAM, 2015). Furthermore, endophytic fungi produce a broad diversity of beneficial metabolites which confer major ecological adaptation capabilities to their host plants (KUSARI; SINGH; JAYABASKARAN, 2014). In our previous study, which evaluated the antimicrobial activity in of the *S. terebinthifolius* extracts obtained from outdoor-grown specimen and micropropagated in aseptic conditions (*in vitro*). We have noted that the plant with endophytes microorganisms presents a different profile of the antimicrobial activity which suggested that the endophytic fungi influence in plant metabolites production with antimicrobial activity (SANTOS et al., 2017). Due to this finding, we identified the endophytic fungi from *S. terbinthifoius* and evaluated their bioactive potential the same tree *S. terebinthifolius* used by Santos et al. 2017. Therefore, a total of 234 isolates were obtained from leaves of this plant.

In this study, like in previous studies on the isolation and identification of endophytic fungi from medicinal plants, genera such as *Alternaria*, *Colletotrichum*, *Diaporthe*, *Epicoccum*, *Fusarium*, *Pestalotiopsis*, *Phyllosticta* and *Xylaria* were isolated. Among these genera, the species of the genus *Alternaria* were also present among the isolates of leaf endophytic fungi in several plant species including plants with medicinal qualities (WANG et al., 2014b; ZHANG et al., 2016). In addition, *Colletotrichum, Diaporthe, and Xylaria* were common isolates from a wide variety of plant families, different altitudes and different years, indicating that these genera could be truly generalist endophytes of plants (ROJAS-JIMENEZ et al., 2016).

The most genera identified belong to the phylum Ascomycota in this study which were attributed to the genera *Alternaria, Colletotrichum, Diaporthe*, *Epicoccum, Phyllosticta, Fusarium*, *Pestalotiopsis* and *Xylaria.* The Ascomycota are often isolated from plants as endophytic (WANG et al., 2015). Similarly, the endophytic fungi obtained from wild rice from were all authenticated in phylum Ascomycota (WANG et al., 2015). These findings are highly consistent with those of previous studies that Ascomycota fungi have been proven to be relatively easy to cultivate *in vitro* (DING et al., 2011).

There are few reports of endophytic fungi belonging to the phylum Basidiomycota (DE SOUZA LEITE et al., 2013). In this study, *Cryptococcus* genus was the unique which belong of the phylum Basidiomycota. This genus has already been reported as endophytic in other plants such as *C. flavescens* isolated from *Citrus sinensis* and from *Opuntia humifusa*, a species of cactus (GAI et al., 2009; HERRERA et al. 2010). In another study, the species *C. victoriae* was reported as endophytic of *Cephalotaxus hainanensis* a known medicinal plant used in China for treating leukemia and leprosy (YANG et al., 2015). These reports indicate that although less frequent, *Cryptococcus* has been reported as an endophytic fungus.

The other purpose of the present study was to investigate the antimicrobial activity of strains which was obtained from this plant. According to Martinez-Klimova et al. (2017) microorganisms residing within plants are increasingly becoming the object of research efforts, especially when the source plant is traditionally used for healing. Among the 11 representative endophytic strains which were utilized for antimicrobial screening, two isolates have shown antimicrobial potential against clinical strains which belong *Diaporthe* and *Xylaria* genera. The strain *Xylaria* sp. (CRMP1424) was active against reference strains of *E. coli* and *S. aureus*. This finding corroborates with studies which related that *Xylaria* metabolites have shown promising compounds for applications in agriculture for plague control as biopesticides, and biocontrol agents; and in medicine, for example as drugs for the treatment of infectious and noninfectious diseases (MACÍAS-RUBALCAVA; SÁNCHEZ-FERNÁNDEZ, 2017).

In addition, the strains *Diaporthe* genus exhibited greater antimicrobial activity. The genus *Diaporthe* is a rich source of many novel bioactive compounds, moreover different strains and species seem to produce different metabolites (CHEPKIRUI; STADLER, 2017). Additional analyses this morphotype revealed at least six different species of *Diaporthe* as endophytic from *S. terebinthifolius.* At least one strains, of the five species (*Diaporthe infecunda*, *D. oxe*, *D. terebenthifolii*, *D. malorum* and the new species *D. foliar*) referent to the morphotype IV, produced active metabolites against at the minimum one pathogen. Both strains of *D. terebinthifolii* showed the best antimicrobial activity against fungi and bacterial. Moreover, the strains *Diaporthe malorum* (CRMP1321 and CRMP1330) and *Diaporthe foliar* (CRMP1429 and CRMP1438) inhibited *Candida albicans xxx used as a reference strins*.

According to Gomes et al. (2013) species delimitation in *Diaporthe* based on morphological characters is challenging, as most taxa in culture do not produce all spore states of the asexual (alpha, beta and gamma conidia) or the sexual morph. The description of novel taxa in this genus in the absence of molecular data should thus be strongly discouraged. In this study, *Diaporthe foliar* was introduced as new species, and all isolates studied could not be induced to sporulate under any laboratory conditions evaluated, probably due to several factors such as age, repeated sub culturing or as an intrinsic feature of this species, authought the new microculture are being carried out. Therefore, following the approach of Gomes et al. (2013), they were here distinguished from closest phylogenetic neighbors clades according to single nucleotide polymorphisms (FIGURE 2).

Based on the phylogenetic analysis the new species *D. foliar* is closely related to the *D. mayteni* (distances of 8.6%), *D. raonikayaporum* (distances of 11.2%) both are also isolated as endophytic from Brazil (GOMES et al. 2013). However, *D.*

neoraonikayaporum (distances of 12.1%) is a pathogenic species described causing dieback lesion of *Tectona grandis* from Thailand (DOILOM et al., 2016).

In this study we found three known species, which reported as endophytes fungal in medicinal plants *D. infecunda, D. oxe* and *D. terebinthifolii.* (GOMES et al., 2013). However, we found *D. malorum*, which was aggressive species on apple fruit. (GAO; LIU; CAI, 2016). In addition, the result reveals that the pathogenic and endophytic isolates often clustered in the same clade and do not show recognizable segregation in the multilocus phylogenetic tree. These findings accord to Gao et al. (2016) that suggested that the factors related to different life modes do not appear to play an essential role in driving the evolution of this group of fungi and provide further support to the speculation that endophytes might be latent pathogens.

Therefore, it is important to observe that the findings of this study provide a strong platform for the isolation and purification of novel natural antimicrobial agents from endophytic fungi of *S. terebinthifolius* which may comprise a promising source of antimicrobial compounds. Moreover, *Diaporthe foliar* was introduced as new species of endophytic fungi demonstrating that *S. terebinthifolius* is an important repository of the fungal diversity and new species.

6 ACKNOWLEDGMENTS

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CHAPTER III- Antimicrobial compounds from endophytic fungi

CHAPTER III: ANTIMICROBIAL COMPOUNDS FROM ENDOPHYTIC FUNGI

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1 ABSTRACT

In this study, we analyzed the antimicrobial activity of extracts harvested from 4 endophytic fungi isolated from the medicinal plant *Schinus terebinthifolius*: *Diaporthe terebinthifolii* CRMP1436, *Diaporthe malorum* CRMP1321 and *Xylaria* sp. CRMP1425 wereobtained from Microbial Collections of Paraná Network-TAX online at Federal University of Paraná. The liquid media (Potato Dextrose Broth-PDB) and Cheerios® breakfast cereal supplemented with a 0.3% sucrose solution was used for fermentation. The extraction was performed using ethyl acetate. The extracts demonstrated activity against *S. aureus* and only two fungi-produced-extracts inhibited the growth of *C. albicans*. *D. terbinthifolii* extracts showed the best inhibitory action against bacteria. *Diaporthe malorum* showed the best activity against *C. albicans.* Based on these results, we selected *D. terebinthifolii* and *D. malorum* strains for production of large-scale metabolites for purification and identification of these compounds. In addition, two classes of epigenetic small-molecule modifiers were utilized to induce metabolite production. After purification, identification, and action these compounds produce, we will evaluate the strains potentials for application in the pharmaceutical industry and the effect of epigenetic small-molecule modifiers in metabolites production.

Keywords: OSMAC, fungal endophytes, epigenetic modifiers; secondary metabolites

2 INTRODUCTION

The resistance developed by pathogenic bacteria and fungi to commercial drugs is an important problem faced by health services and a serious concern around the world (DOS SANTOS et al., 2015). This resistance is a demonstration of evolution in “real time” in response to the chemical warfare waged against microbes through the therapeutic and non-therapeutic uses of antimicrobial agents. Natural products or their derivatives represented about 52% of the new chemicals introduced into the market between 1981 and 2002 (NEWMAN et al., 2007).

Besides plants, microorganisms are a major source of natural products with desirable bioactive properties (DOS SANTOS et al., 2015). Fungi are among the most important eukaryotic organisms that are being explored for active metabolite production. Drugs of fungal origin include b-lactam antibiotics, griseofulvin, cyclosporine A, taxol, ergot alkaloids, and lovastatin (SURYANARAYANAN; THIRUNAVUKKARASU; GOVINDARAJULU, 2009). Several studies on endophytes over the last decades have shown that these organisms are a rich source of functional bioactive molecules, including compounds mimetic to the host plant biosynthetic (STIERLE et al., 1995; RAJAMANIKYAM et al., 2017). Endophytes fungal are an ecological group of fungi mostly belonging to the Ascomycota that resides in the aerial tissues and roots of plants without inducing any visual symptoms of their presence (PETRINI, 1991).

Bioactive molecules are secondary metabolites which are produced by organisms to help them to proliferate and to protect them against predators. In sterile laboratory conditions, many genes are not activated and secondary metabolites are not produced (TONIAL et al., 2016). In many cases, the chemical diversity of cultured fungi increases when using more complex culture media. High amounts of sucrose and glucose, together with yeast extract or malt extract, and added minerals and trace metals in culture media usually give highly varied metabolites at a high yield. (FRISVAD; ANDERSEN; THRANE, 2008). In this context, systematic alteration of cultivation parameters of a given microorganism, known as the OSMAC (one strain many compounds) approach, greatly increases the likelihood of finding new metabolites from a single strain (ALY et al., 2008).

Challenging the cells with external signals, so called “elicitors”, is a recognized strategy to generate novel, biologically active metabolites (LUTI; MAVITUNA, 2011). Small molecule chemical elicitors can promote different responses in microorganisms such as the increased production of metabolites (ROMERO et al., 2007), biofilm formation induction, and modulation of virulence expression (EVEN et al., 2009; NOUAILLE et al., 2009; BAJAJ et al., 2014; ABDELMOHSEN et al., 2015).

Medicinal plants represent an important role in the search for new antimicrobial compounds obtained from their endophytic fungi. (RAJAMANIKYAM et al., 2017). A repertoire of medicinal plants remains to be studied regarding their endophytic composition, for example, *S. terebinthifolius* (SANTOS et al., 2017). This is a well-known Brazilian medicinal plant whose leaves have been proven to have anti-inflammatory, antimicrobial, antiadherent and antioxidant proprieties. (BARBIERI et al., 2014; PATOCKA; ALMEIDA, 2017). In previous studies in our group, we observed that the endophytic fungi of *S. terebinthifolius* may be a strong platform for the isolation and purification of novel natural antimicrobial agents (SANTOS et al., 2016; TONIAL et al., 2017). Therefore, we selected endophytes fungal from *S. terebinthifolius* for a search for bioactive substances with antimicrobial activity through OSMAC strategies.

3 MATERIAL E METHODS

3.1 ENDOPHYTIC FUNGI

Diaporthe terebinthifolii CRMP1436, *Diaporthe malorum* CRMP1321 and *Xylaria* sp. CRMP1425 were obtained from Microbial Collections of Paraná Network-TAX online at the Federal University of Paraná. The endophytic species was isolated from the leaves of *Schinus terebinthifolius*.

3.2 SMALL-SCALE CULTURES – FERMENTATION

For the fermentation in the liquid medium PDB, the endophytic fungi were grown on PDA, at 25°C, for 5 days. Mycelia discs were extruded from the “Transfer Tube” and crushed in the bottom of inocula tubes (25 X150 mm), containing 12 mL potato dextrose broth (PDB; Himedia, Mumbai, India) and medium composed of 1,5 g Cheerios® breakfast cereal supplemented with a 0.3% sucrose solution. Tubes were incubated on an orbital shaker (160rpm) at 28°C, after three days. Tubes were then incubated for seven days (FIGURE 1).

3.2 LARGE-SCALE CULTURES – FERMENTATION

For the fermentation in the liquid medium PDB, the endophytic fungi were grown on PDA, at 25°C, for 5 days. Six mycelia discs (6 mm) of mycelial agar plugs were inoculated into 500 mL Erlenmeyer flasks containing 250 mL potato dextrose broth (PDB; Himedia, Mumbai, India) and medium composed of 100g Cheerios® breakfast cereal supplemented with a 0.3% sucrose solution (FIGURE 1).

Epigenetic modifiers were added to each tube to attain a final concentration of 100µM. Three epigenetic modifiers were selected for its addition into inoculum and fungal fermentations sodium butyrate (303410, Sigma-Aldrich), 5-azacitidine (A2385, Sigma-Aldrich, St. Louis, MO, USA) and DMSO.

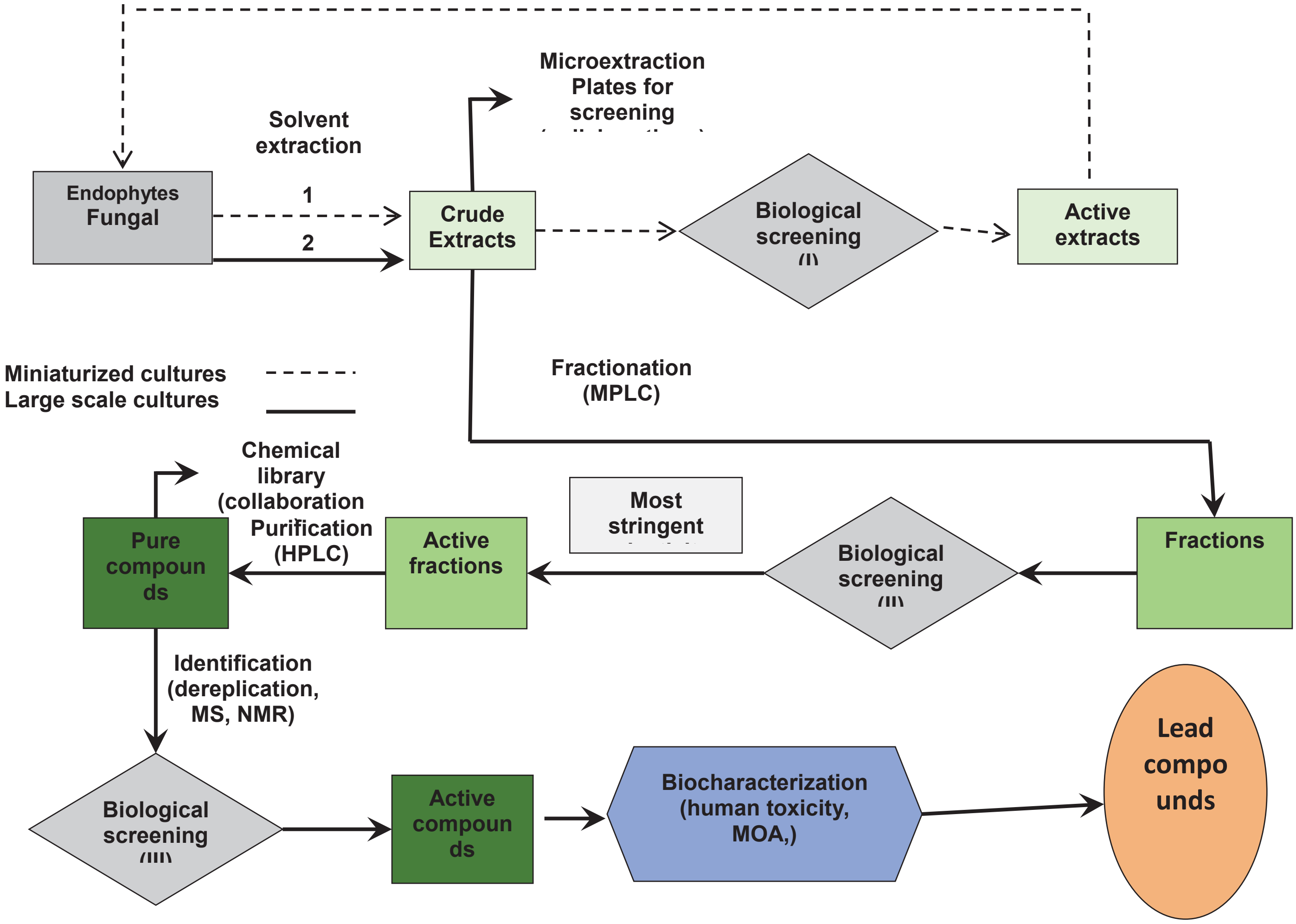
3.4 PREPARATION OF EXTRACTS

After fermentation, the mycelium was separated from the fermented liquid by Whatman n°4 filtration paper. The extraction was performed using ethyl acetate (EtOAc) (Merck). Solvent evaporation was carried out using a rotaevaporator at 45°C. The final extract was weighed and diluted in DMSO at a concentration of 10 mg/mL (FIGURE 1).

3.5 ANTIMICROBIAL BIOASSAYS

Microbial susceptibility testing was performed using the microbroth dilution assay with the following strains: methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231 (FIGURE 1). The positive control antibiotic for the antibacterial assays was tetracycline (50 µg/mL for *P. aeruginosa* and 10 µg/mL for all other strains). The control antifungal compound was amphotericin B (2 µg/mL).

FIGURE 1- EXPERIMENTAL DESIGN

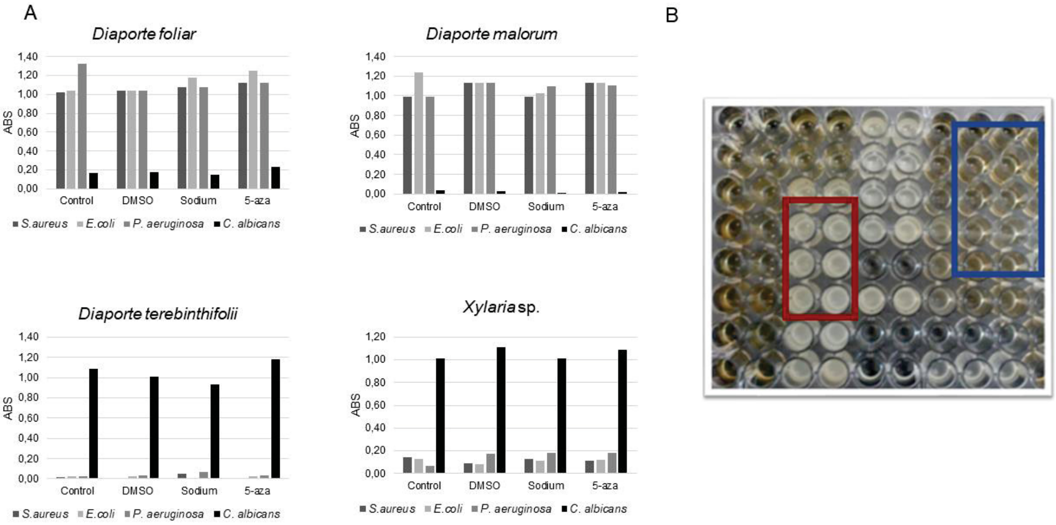


4 RESULTS

Small-scale cultures fermentation in 12 mL after seven days showed that any of the extracts exert antimicrobial bioactivity, while the fermentation into 500 mL Erlenmeyer flasks containing 250 mL PDB. The majority of the active extracts inhibited the growth of *S. aureus* MRSA, while only two fungi produced extracts inhibited the growth of *C. albicans*. *Diaporthe terbinthifolii* extracts showed the best inhibitory activity against bacteria and *D. malorum* showed the best activity against *C. albicans* (TABLE 1). Based on these results, we selected *D. terebinthifolii* and *D. malorum* strains for production of large-scale metabolites aimed the purification and identification these compounds.

In order to characterize active compound and evaluate the epigenetic small-molecule modifiers, we selected the strains *D. terebinthifolii* and *D. malorum* which showed strong activity against bacterial and *C. albicans* (FIGURE 2). So far, we have obtained large-scale fermentation *D. terebinthifolii* (200mg) in PDB (FIGURE 2).

FIGURE 2 – ANTIMICROBIAL SCREENING OF ENDOPHYTES FUNGI FROM *Schinus terebinthifolius*



NOTE- A- Inhibition graph B- PDB Medium (potato dextrose broth) Control - in absence elicitor; DMSO- Dimethyl sulfoxide; Sodium: sodium butyrate (303410, Sigma-Aldrich); 5-aza:5-azacitidine (A2385, Sigma-Aldrich), red squares- growth; profile blue squares- growth inhibition profile.

5 DISCUSSION

The microorganisms residing within plants are increasingly becoming the object of research, especially when the source plant is traditionally assumed as medicinal. Therefore, the biodiversity and biosynthetic potential of endophytes has been extensively explored (MARTINEZ-KLIMOVA; RODRÍGUEZ-PEÑA; SÁNCHEZ, 2017). Endophytes of *S. terebinthifolius* may comprise a promising source of antimicrobial compounds, including anti-MRSA compounds. These fungi and the compounds they produce warrant further investigation (SANTOS et al., 2016; TONIAL et al., 2016).

In this study, we selected four strains which have been reported as producers of active metabolites (SANTOS et al. 2017 in press), with antimicrobial activity selected by liquid fermentation using both a reduce (12mL) and large volume (250mL) of medium in the submerged culture. According to previous reports, several authors have been screening strains of fungal species in a reduce volume of medium ensuring the generation of enough biomass for this evaluation (BILLS et al., 2008; GONZÁLEZ-MENÉNDEZ et al., 2016). However, in this study the strains produced activity only in a volume of 250mL, demonstrating that the volume seems to influence the activity. According to the Tonial et al. 2016, the production of secondary metabolites by microorganisms is a strain-specific characteristic that depends on the growth conditions.

Among the strains evaluated, *D. terebinthifolii* showed activity against the methicillin resistant *S. aureus* (MRSA). The MRSA comprise the most common cause of community acquired antibiotic-resistant infections. Although MRSA was first described more than 50 years ago, the challenges in obtaining and implementing new drugs make this pathogen a continuing global health problem (MOELLERING, 2012).

Secondary metabolites from microorganisms have a wide range of biological activities due to their structural diversity and have been proved a major source of drug lead compounds (KUSARI; HERTWECK; SPITELLER, 2012). Moreover, the traditional method of a single culture restricts the metabolic pathways of microorganisms and, as a result, many metabolites cannot be formed (WEI et al., 2010).Therefore, another purpose of this study is the induction of new secondary metabolites by the OSMAC (One Strain MAny Compounds) strategy. OSMAC terms the modulating effect that altered culture conditions and consequently the secondary metabolite production of microorganisms (WEI et al., 2010; PÉREZ HEMPHILL et al., 2017).

Examples of such culture variations for filamentous fungi include the use of different liquid or solid media, as applied in a previous study with *Fusario tricinctum* fermented on solid beans and liquid Wickerham medium versus cultivation on solid rice medium, leading to an enhanced production of enniatins on beans (PÉREZ HEMPHILL et al., 2017). Another example is the mimicry of extreme habitats by cultivating at colder temperatures or using highly saline media (for example, growing *Gymnascella dankaliensis* on either NaCl or NaBr (enriched rice medium) that led to the induction of chlorinatedor brominated metabolites (WANG et al., 2016).

Fungal endophytes are known to produce a wide variety of secondary metabolites (SMs) involved in their adaptation and survival within higher plants. The plant-microbe interaction may influence the expression of some biosynthetic pathways.

Several reports have described the variable effects observed in natural product profiles in fungi treated with small molecule histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors which are commonly used to perturb the production of fungal metabolites, that can lead to the induction of the expression of silent metabolite pathways (GONZÁLEZ-MENÉNDEZ et al., 2016). The biosynthetic potential of most secondary metabolite produced by fungi remains to be determined. Two main challenges limit the access to these potentially new compounds: the inability to cultivate diverse and potentially interesting producers of natural products in the laboratory and the perception that the majority of secondary metabolite biosynthesis gene clusters is silent under standard laboratory conditions (HERTWECK, 2009).

In this study, two class of epigenetic small-molecule modifiers was utilized to induce metabolite production. We used 5-azacitidine classified as DNMT inhibitors which have already demonstrated their ability to reduce DNA-methylation-mediated silencing of different resistance genes and cellular processes in a wide variety of fungal species. In addition, sodium butyrate was used due to several reports that have described the variable effects of HDAC inhibitors on fungi. The new extracts generated in large-scale for *D. terebinthifolii* and *D. malorum* in presence and absence of the epigenetic small-molecule modifiers (described above) presented a similar antimicrobial activity profile to the previous extract obtained without these inducers. Several authors demonstrate that the use of epigenetic modifiers is extremely effective for isolating novel compounds from nature (BRAKHAGE; SCHROECKH, 2011; ABDELMOHSEN et al., 2015; ASAI et al., 2016). Previous reports demonstrated that novel compounds of acid fusaric 1,6- dihydropyridine-2-carboxylic acid and 1,6-dihydropyridine-2-carboxylic acid) by *Fusarium oxysporum* and *F. verticillioides* were detected by the species treated with epigenetic modifier, supplying SBHA to the culture although no additional antimicrobial activity hasn't been reported in these strains (CHEN et al., 2013). Therefore, even the new extracts didn’t present additional activity, and after purification and identification, we could provide the new data spectral by the induction metabolite production.

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CHAPTER IV- FINAL CONSIDERATIONS

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Thus, this thesis is part of a project that aims at understanding the relationship between endophytic fungi and the antimicrobial potential of *Schinus terebinthifolius*. The previous publishing indicated the properties of extracts of this plant may vary. Santos et al. (2017) has observed through micropropagation techniques that the extract of plants grown *in vitro* presented antimicrobial activity in a different concentration than *S. terebinthifolius* extracts collected from the wild environment, which suggest that population of endophytic fungi could be related to production extracts of this plant. In addition, the selection of fungal endophytes and their extracts with antimicrobial activity associated with micropropagation protocol of *S. terebinthifolius,* already established by previous work, may represent a strong tool for a better understanding of the relationship between endophytic microorganisms and the activity of *S. terebinthifolius.* Therefore, these findings encourage the investigation of the relationship between endophytic microorganisms for a better understanding of the activity of *S. terebinthifolius* metabolites and its medicinal profile.

In this context, the first objective of this thesis was to identify endophytic fungi present in the plant of *S. terebinthifolius*. Based on the results, it was possible to observe that *S. terebinthifolius* represents a vast repository of endophytic fungi, including reports of new species, as well as species already known as metabolites producers with antimicrobial activity. Our results indicate that endophytic fungi from leaves of *S. terebinthifolius* have pharmaceutical potential as they produce antimicrobial compounds. However, further studies are now needed to identify the active compounds produced to discover new molecules with antibacterial and/or antifungical activity.

In the last chapter of this study, it was evaluated that two class of epigenetic small-molecule modifiers were utilized for induction metabolite production with a screening of two *Diaporthe* species. The new extracts generated in large-scale for *D. terebinthifolii* and *D. malorum*, presented a similar antimicrobial activity profile in comparison to the previous extract obtained without these inducers. Therefore, even though the new extracts apparently seem to have a similar activity of the previous extract produced without inducers, they represent a new source of research efforts, since, after purification and identification, it may provide the new data spectral obtained by the induction of metabolites production.

In conclusion, this work resulted in the characterization of 234 endophytic fungi isolates obtained from the *S. terebinthifolius* medicinal plant, which presents a natural anti-inflammatory and antimicrobial activity, with the description of the new species *D. foliar* CMRP1438 type strain. The endophytes fungal were evaluated for their antimicrobial potential, including *in vitro* induction tests with 04 selected strains, among them the new taxa of *Diaporthe* presented extracts with antibacterial profile and a prominent antifungal activity as well. This work provided a biological database from an inexhaustible bioprospecting studies conducted, which explore different plant sources and conditions for the production of metabolites. Therefore, the extend the studies of the interaction between plant and microorganisms, and an investigation of other potentialities of the vast biomolecules spectrum is mandatory in order to elucitated propely this antimicrobial activity potential.

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APENDIX

LIST OF PUBLICATIONS (2013-2017)

NASCIMENTO, MARIANA M.F. ; VICENTE, V. A. ; BITTENCOURT, JULIANA V.M. ; GELINSKI, JANE MARY L. ; PRENAFETA-BOLDÚ, FRANCESC X. ; ROMERO, MAYCOLL ; FORNARI, GHENIFFER ; GOMES, RENATA R. ; **SANTOS, G. D.** ; GERRITS VAN DEN ENDE, A.H.G. ; SILVA DE AZEVEDO, CONCEIÇÃO D.M.P. ; DE HOOG, G.SYBREN . Diversity of opportunistic black fungi on Babassu coconut shells, a rich source of esters and hydrocarbons. Fungal Biology^{JCR}, v. 121, p. 488-500, 2017.

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TABLE S1- SOURCES OF ISOLATES AND GENBANK ACCESSION NUMBERS USED IN THIS STUDY

Species names	**Culture collection no.**	**Isolation sources**	**Host family**	**GenBank Accession Numbers ITS; TEF1; TUB; HIS; CAL; LSU**
Diaporthe acaciarum	CBS 138862*	*Acacia tortilis*	*Mimosaceae*	KP004460; -; KP004509; KP004504; -; -
Diaporthe acaciigena	CBS 129521*	*Acacia retinodes*	*Mimosaceae*	KC343005; KC343731; KC343973; KC343489; KC343247; -
Diaporthe acericola	MFLUCC 17-0956*	*Acer negundo*	*Aceraceae*	KY964224; KY964180; KY964074; -; KY964137; -
Diaporthe acerina	CBS 137.27	*Acer saccharum*	*Aceraceae*	KC343006; KC343732; KC343974; KC343490; KC343248; -
Diaporthe acutispora	CGMCC 3.18285*; LC6161	*Coffea* sp., endophyte	*Rubiaceae*	KX986764; KX999155; KX999195; KX999235; KX999274; -
Diaporthe alleghaniensis	CBS 495.72*	*Betula alleghaniensis*, branches	*Betulaceae*	KC343007; KC343733; KC343975; KC343491; KC343249; -
Diaporthe alnea	CBS 146.46*	*Alnus* sp.	*Betulaceae*	KC343008; KC343734; KC343976; KC343492; KC343250; -
Diaporthe ambígua	CBS 114015*	*Pyrus communis*	*Rosaceae*	KC343010; KC343736; KC343978; KC343494; KC343252; -
Diaporthe ampelina	CBS 114016*	*Vitis vinifera*	*Vitaceae*	AF230751; AY745056; JX275452; -; AY745026; AF439635
Diaporthe amygdali	CBS 126679*	*Prunus dulcis*	*Rosaceae*	KC343022; KC343748; KC343990; KC343506; KC343264; -
Diaporthe anacardii	CBS 720.97*	*Anacardium occidentale*	*Anacardiaceae*	KC343024; KC343750; KC343992; KC343508; KC343266; -
Diaporthe angelicae	CBS 111592*	*Heracleum sphondylium*	*Apiaceae*	KC343027; KC343743; KC343995; KC343511; KC343269; -
Diaporthe apiculata	CGMCC 3.17533*; LC 3418	*Camellia sinensis*, leaf, endophyte	*Theaceae*	KP267896; KP267970; KP293476; KP293550; -; KY011852
Diaporthe aquática	IFRDCC 3051*	Aquatic		JQ797437; -; -; -; -; -
Diaporthe aquática	IFRDCC 3015	Aquatic habitat		JQ79743; ; ; ; ;
Diaporthe arctii	CBS 136.25*	*Arctium* sp.	*Arecaceae*	KC343032; KC343758; KC344000; KC343516; KC343273; -
Diaporthe arecae	CBS 161.64*	*Areca catechu*, fruit	*Arecaceae*	KC343032; KC343758; KC344000; KC343516; KC343274; -
Diaporthe aseana	MFLUCC 12–0299a*			KT459414; KT459448; KT459432; -; KT459464; -

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Diaporthe asheicola	CBS 136967*	*Vaccinium ashei*	*Ericaceae*	KJ160562; KJ160594; KJ160518; -; KJ160542; -
Diaporthe aspalathi	CBS 117169*	*Aspalathus linearis*	*Fabaceae*	KC343036; KC343762; KC344004; KC343520; KC343278; -
Diaporthe australafricana	CBS 111886*	*Vitis vinifera*	*Vitaceae*	KC343038; KC343764; KC344006; KC343522; KC343280; -
Diaporthe averrhoae	SCHM 3605	*Averrhoa carambola*	*Oxalidaceae*	AY618930; -; -; -; -; -
Diaporthe azadirachtae	TN 01	*Azadirachta indica*	*Meliaceae*	KC631323; -; -; -; -; -
Diaporthe baccae	CBS 136972*	*Vaccinium corymbosum*	*Ericaceae*	KJ160565; KJ160597; -; -; -; -
Diaporthe batatas	CBS 122.21*	*Ipomoea batatas*	*Convolvulaceae*	KC343040; KC343766; KC344008; KC343524; KC343282; -
Diaporthe beckhausii	CBS 138.27	*Viburnum* sp.	*Caprifoliaceae*	KC343041; KC343767; KC344009; KC343525; KC343283; -
Diaporthe beilharziae	BRIP 54792*	*Indigofera australis*	*Papilionaceae*	JX862529; JX862535; KF170921; -; -; -
Diaporthe benedicti	BPI 893190*	Salix sp.	*Salicaceae*	KM669929; KM669785; ; ; KM669862;
Diaporthe betulae	CFCC 50469*	*Betula platyphylla*	*Betulaceae*	KT732950; KT733016; KT733020; KT732999; KT732997; -
Diaporthe betulicola	CFCC 51128*	*Betula albosinensis*	*Betulaceae*	KX024653; KX024655; KX024657; KX024661; KX024659; -
Diaporthe bicincta	CBS 121004*	*Juglans* sp., dead wood	*Juglandaceae*	KC343134; KC343860; KC344102; KC343618; ; -
Diaporthe biconispora	ZJUD62*; IcMP20654	*Citrus grandis*	*Rutaceae*	KJ490597; KJ490476; KJ490418; KJ490539; -; -
Diaporthe biguttulata	ZJUD47*	*Citrus limon*	*Rutaceae*	KJ490582; KJ490461; KJ490403; KJ490524; -; -
Diaporthe biguttusis	CGMCC 3.17081*	*Lithocarpus glabra*	*Fagaceae*	KF576282; KF576257; KF576306; -; -; KY011878
Diaporthe bougainvilleicola	SCHM 3006	-	-	AY601920; -; -; -; -; -
Diaporthe brasiliensis	CBS 133183*	*Aspidosperma tomentosus*	*Apocynaceae*	KC343042; KC343768; KC344010; KC343526; KC343284; -
Diaporthe caatingaensis	CBS 141542*	*Tacinga inamoena*	*Cactaceae*	KY085927; KY115603; KY115600; KY115605; KY115597; -

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Species names	**Culture collection no.**	**Isolation sources**	**Host family**	**GenBank Accession Numbers ITS; TEF1; TUB; HIS; CAL; LSU**
Diaporthe camptothecae	SCHM 3611	*Camptotheca acuminate*	*Cornaceae / Nyssaceae*	AY622996; -; -; -; -; -
Diaporthe canthii	CBS 132533*	*Canthium inerme*	*Rubiaceae*	JX069864; KC843120; KC843230; -; KC843174; -
Diaporthe carpini	CBS 114437	*Carpinus betulus*	*Corylaceae*	KC343044; KC343770; KC344012; KC343528; KC343286; -
Diaporthe cassines	CPC 21916*	*Cassine peragua*	*Celastraceae*	KF777155; KF777244; -; -; -; -
D. castaneae-mollisimae	DNP 128*	*Castanea mollissima*	*Fagaceae*	JF957786; JX275401; JX275438; -; JX197430; -
Diaporthe caulivora	CBS 127268*	*Glycine max*	*Fabaceae*	KC343045; KC343771; KC344013; KC343529; KC343287; -
Diaporthe celastrina	CBS 139.27*	*Celastrus scandens*	*Celastraceae*	KC343047; KC343773; KC344015; KC343531; KC343289; -
Diaporthe chamaeropis	CBS 454.81*	*Chamaerops humilis*, dead part of leaf	*Arecaceae*	KC343048; KC343774; KC344016; KC343532; KC343290; -
Diaporthe charlesworthii	BRIP 54884m*	*Rapistrum rugostrum*	*Brassicaceae*	KJ197288; KJ197250; KJ197268; -; -; -
Diaporthe chimonanthi	SCHM 3614	*Chimonanthus praecox*	*Calycanthaceae*	AY622993; -; -; -; -; -
Diaporthe cichorii	MFLUCC 17-1023*	*Cichorium intybus*	*Compositae*	KY964220; KY964176; KY964104; -; KY964133; -
Diaporthe cissampeli	CPC 27302 = CBS 141331*	*Cissampelos capensis*	*Menispermaceae*	KX228273; -; KX228384; KX228366; -; -
Diaporthe citriasiana	ZJUD 30*	*Citrus unshiu*, dead wood	*Rutaceae*	JQ954645; JQ954663; KC357459; -; KC357491; -
Diaporthe citrichinensis	ZJUD34*	*Citrus* sp.	*Rutaceae*	JQ954648; JQ954666; -; -; KC357494; -
Diaporthe columnaris	35 (CBS109873)	*Vaccinium vitis-idaea*	*Ericaceae*	AF439625; -; -; -; -; -
Diaporthe compacta	LC3083*	*Camellia sinensis*, leaf, endophyte	*Theaceae*	KP267854; KP267928; KP293434; KP293508; -; KY011840
Diaporthe cinerascens	CBS 719.96	*Ficus carica*	*Moraceae*	KC343050; KC343776; KC344018; KC343534; KC343292; -
Diaporthe citri	CBS 135422*	*Citrus sp*	*Rutaceae*	KC843311; KC843071; KC843187; -; KC843157; -
Diaporthe convolvuli	CBS 124654*	*Convolvulus arvensis*	*Convolvulaceae*	KC343054; KC343780; KC344022; KC343538; KC343296; -

TABLE S1- SOURCES OF ISOLATES AND GENBANK ACCESSION NUMBERS USED IN THIS STUDY

Species names	**Culture collection no.**	**Isolation sources**	**Host family**	**GenBank Accession Numbers ITS; TEF1; TUB; HIS; CAL; LSU**
Diaporthe cotoneastri	CBS 439.82	*Cotoneaster* sp	Rosaceae	FJ889450; GQ250341; JX275437; -; -; -
Diaporthe crataegi	CBS 114435	*Crataegus oxyacantha*	*Rosaceae*	KC343055; KC343781; KC344023; KC343539; KC343297; -
Diaporthe crotalariae	CBS 162.33*	*Crotalaria spectabilis*	*Fabaceae*	KC343056; KC343782; KC344024; KC343540; KC343298; -
Diaporthe cucurbitae	DAOM42078*	*Cucumis sativus*	*Cucurbitaceae*	KM453210; KM453211; KP118848; KM453212; -; -
Diaporthe cuppatea	CBS 117499*	*Aspalathus linearis*	*Fabaceae*	KC343057; KC343783; KC344025; KC343541; KC343299; -
Diaporthe cynaroidis	CBS 122676*	*Protea cynaroides*	*Proteaceae*	KC343058; KC343784; KC344026; KC343542; KC343300; -
Diaporthe cytosporella	AR 5149	*Citrus sinensis*	*Rutaceae*	KC843309; KC843118; KC843222; -; KC843143; -
Diaporthe decedens	CBS 114281	*Corylus avellana*	*Corylaceae*	KC343060; KC343786; KC344028; KC343544; KC343302; -
Diaporthe detrusa	CBS 109770	*Berberis vulgaris*	*Berberidaceae*	KC343061; KC343787; KC344029; KC343545; KC343303; -
Diaporthe diospyricola	CPC 21169*	*Diospyros whyteana*	*Ebenaceae*	KF777156; -; -; -; -; -
Diaporthe discoidispora	ZJUD89*	*Citrus unshiu*	*Rutaceae*	KJ490624; KJ490503; KJ490445; KJ490566; -; -
Diaporthe dorycnii	MFLUCC 17-1015*	*Dorycnium hirsutum*	*Fabaceae*	KY964215; KY964171; KY964099; -; -; -
Diaporthe elaeagni	CBS 504.72	*Elaeagnus* sp., twig	*Elaeagnaceae*	KC343064; KC343790; KC344032; KC343548; KC343306; -
Diaporthe eres	CBS 13859*	*Ulmus laevis*	*Ulmaceae*	KJ210529; KJ210550; KJ420799; KJ420850; KJ434999; -
Diaporthe elaegni-glabrae	CGMCC 3.18287*; LC 4802	*Elaeagnus glabra, pathogen*	*Elaeagnaceae*	KX986779; KX999171; KX999212; KX999251; KX999281; KY011885
Diaporthe ellipicola	CGMCC 3.17084*	*Lithocarpus glabra, diseased leaves*	*Fagaceae*	KF576270; KF576245; KF576291; -; -; KY011873
Diaporthe endophytica	CBS 133811*	*Schinus terebinthifolius*	*Anacardiaceae*	KC343065; KC343791; KC344033; KC343549; KC343307; -
Diaporthe eucalyptorum	CBS 132525*	*Eucalyptus sp*	*Myrtaceae*	NR120157; -; -; -; -; -

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Species names	**Culture collection no.**	**Isolation sources**	**Host family**	**GenBank Accession Numbers**
Diaporthe eucommiae	SCHM 0020	*Eucommia ulmoides*	*Eucommiaceae*	AY601921; -; -; -; -; -
Diaporthe eucommicola	SCHM 3607	*Eucommia ulmoides/Styrax hypoglauca*	*Eucommiaceae/Styracaceae*	AY578071; -; -; -; -; -
Diaporthe eugeniae	CBS 444.82	*Eugenia aromatica*, leaf	*Mrytaceae*	KC343098; KC343824; KC344066; KC343582; KC343340; -
Diaporthe fibrosa	CBS 109751	*Rhamnus cathartica*	*Rhamnaceae*	KC343099; KC343825; KC344067; KC343583; KC343341; -
Diaporthe foeniculina (ex-type of P. theicola)	CBS 187.27*	*Camellia sinensis*, leaves and branches	*Theaceae*	KC343107; KC343833; KC344075; KC343591; KC343349; -
Diaporthe fraxini-angustifoliae	BRIP 54781*	*Fraxinus-angustifolia* subsp. Oxycapa	*Oleaceae*	JX862528; JX852534; KF170920; -; KT459462; -
Diaporthe fusicola	CGMCC 3.17087*	*Lithocarpus glabra*	*Fagaceae*	KF576281; KF576256; KF576305; -; KF576233; KY011836
Diaporthe fukushii (P. fukushii)	CBS 116953	*Pyrus pyrifolia*	*Roseceae*	KC343147; KC343873; KC344115; KC343631; KC343389; -
Diaporthe ganjae	CBS 180.91*	*Cannabis sativa*, dead leaf	*Cannabaceae*	KC343112; KC343838; KC344080; KC343596; KC343354; -
Diaporthe gardeniae	CBS 288.56	*Gardenia florida*, stem	*Rubiaceae*	KC343113; KC343839; KC344081; KC343597; KC343355; -
Diaporthe garethjonesii	MFLUCC 12-0542a*	*Folha em decomposição desconhecida*	-	KT459423; KT459457; KT459441; -; KT459470; -
Diaporthe glabrae	SCHM 3622	*Bougainvillea glabra*	*Nyctaginaceae*	AY601918; -; -; -; -; -
Diaporthe goulteri	BRIP 55657a*	*Helianthus annuus*	*Asteraceae*	KJ197289; KJ197252; KJ197270; -; -; -
Diaporthe gulyae	BRIP 54025*	*Helianthus annuus*	*Asteraceae*	JF431299; JN645803; KJ197271; -; KY964107; -
Diaporthe helianthi	CBS 592.81*	*Helianthus annuus*	*Asteraceae*	KC343115; KC343841; KC344083; KC343599; KC343357; -
Diaporthe helicis	AR5211*	*Hedera helix*	*Araliaceae*	KJ210538; KJ210559; KJ420828; KJ420875; KJ435043; -
Diaporthe hickoriae	CBS 145.26*	*Carya glabra*	*Juglandaceae*	KC343118; KC343844; KC344086; KC343602; KC343360; -
Diaporthe hordei	CBS 481.92	*Hordeum vulgare*	*Poaceae*	KC343120; KC343846; KC344088; KC343604;

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Species names	**Culture collection no.**	**Isolation sources**	**Host family**	**GenBank Accession Numbers**
Diaporthe impulsa	CBS 114434	*Sorbus aucuparia*	*Rosaceae*	KC343121; KC343847; KC344089; KC343605; KC343363; -
Diaporthe incompleta	CGMCC 3.18288*; LC 6754	*Camellia sinensis*, pathogen	*Theaceae*	KX986794; KX999186; KX999226; KX999265; KX999289; -
Diaporthe inconspícua	CBS 133813*	*Maytenus ilicifolia*, endophytic in petiole	*Celastraceae*	KC343123; KC343849; KC344091; KC343607; KC343365; -
Diaporthe infecunda	CBS 133812*	*Schinus terebinthifolius*	*Anacardiaceae*	KC343126; KC343852; KC344094; KC343610; KC343368; -
Diaporthe infecunda	LGMF908	*Schinus terebinthifolius*	*Anacardiaceae*	KC343127; KC343853; KC344095; KC343611; KC343369; -
Diaporthe infecunda	LGMF912	*Schinus terebinthifolius*	*Anacardiaceae*	KC343128; KC343854; KC344096; KC343612; KC343370; -
Diaporthe infecunda	LGMF917	*Schinus terebinthifolius*	*Anacardiaceae*	KC343129; KC343855; KC344097; KC343613; KC343371; -
Diaporthe infecunda	LGMF918	*Schinus terebinthifolius*	*Anacardiaceae*	KC343130; KC343856; KC344098; KC343614; KC343372; -
Diaporthe infecunda	LGMF920	*Schinus terebinthifolius*	*Anacardiaceae*	KC343131; KC343857; KC344099; KC343615; KC343373; -
Diaporthe infecunda	LGMF933	*Maytenus ilicifolia*	*Celastraceae*	KC343132; KC343858; KC344100; KC343616; KC343374; -
Diaporthe infecunda	LGMF940	*Maytenus ilicifolia*	*Celastraceae*	KC343133; KC343859; KC344101; KC343617; KC343375; -
Diaporthe isoberliniae	CPC 22549*	*Isoberlinia angolensis*	*Fabaceae*	KJ869133; -; KJ869245; -; -; -
Diaporthe juglandicola	CFCC 51134*	*Juglans mandshurica*	*Juglandaceae*	KU985101; KX024628; KX024634; KX024622; KX024616; -
Diaporthe juniperivora	CBS 186.27			DQ116555; ; ; ; ; -
Diaporthe kochmanii	BRIP 54033*	*Helianthus annuus*	*Asteraceae*	JF431295; JN645809; -; -; -; -
Diaporthe kongii	BRIP 54031*	*Helianthus annuus*	*Asteraceae*	JF431301; JN645797; KJ197272; -; -; -
Diaporthe litchicola	BRIP 54900*	*Litchi chinensis*	*Sapindaceae*	JX862533; JX862539; KF170925; -; -; -
Diaporthe lagerstroemiae	SCHM 3608	*Lagerstroemia indica*	*Lythraceae*	AY622994; -; -; -; -; -
Diaporthe leucospermi	CBS 111980	Leucospermum sp.	Proteaceae	JN712460; KY435632; KY435673; KY435653;; -

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Species names	**Culture collection no.**	**Isolation sources**	**Host family**	**GenBank Accession Numbers**
Diaporthe liquidambari	SCHM 3621	*Liquidambar formosana*	Altingiaceae	AY601919; ; ; ; ; -
Diaporthe lithocarpus	CGMCC 3.15175*	*Lithocarpus glabra*	*Fagaceae*	KC153104; KC153095; KF576311; ; ; -
Diaporthe longicicola	CGMCC 3.17089*	*Lithocarpus glabra*	*Fagaceae*	KF576267; KF576242; KF576291; -; -; -
Diaporthe longicolla	FAU 599; ATCC 60325*	*Glycine max*	*Fabaceae*	KJ590728; KJ590767; KJ610883; KJ659188; -; -
Diaporthe longispora	CBS 194.36*	*Ribes* sp.	*Grossulariaceae*	KC343135; KC343861; KC344103; KC343619; KC343377; -
Diaporthe lonicerae	MFLUCC 17-0963*	*Lonicera* sp	Caprifoliaceae	KY964190; KY964146; KY964073; -; KY964116; -
Diaporthe loropetali	SCHM 3615	*Loropetalum chinense*	Hamamelidaceae	AY601917; ; ; ; ; -
Diaporthe lusitanicae	CBS 123212*	*Foeniculum vulgare*	*Apiaceae*	KC343136; KC343862; KC344104; KC343620; KC343378; -
Diaporthe macinthoshii	BRIP 55064a*	*Rapistrum rugostrum*	*Brassicaceae*	KJ197290; KJ197251; KJ197269; -; -; -
Diaporthe manihotia	CBS 505.76	*Manihot utilissima*, leaves	*Euphorbiaceae*	KC343138; KC343864; KC344106; KC343622; KC343380; -
Diaporthe mahothocarpus	CGMCC 3.15181*	*Lithocarpus glabra*	*Fagaceae*	KC153096; KC153087; KF576312; -; -; KY011871
Diaporthe malorum	CAA734*	*Malus domestica*	*Rosaceae*	KY435638; KY435627; KY435668; KY435648; KY435658; -
Diaporthe marítima	NB 365-71I*	*Picea rubens needle*	*Pinaceae*	KU552025; KU552023; KU574615; -; -; -
Diaporthe masirevicii	BRIP 57892a*	*Helianthus annuus*	*Asteraceae*	KJ197277; KJ197239; KJ197257; -; -; -
Diaporthe mayteni	CBS 133185*	*Maytenus ilicicolia*	*Celastraceae*	KC343139; KC343865; KC344107; KC343623; KC343381; -
Diaporthe maytenicola	CPC 21896*	*Maytenus acuminata*	*Celastraceae*	KF777157; -; KF777250; -; -; -
Diaporthe megalospora	CBS 143.27	*Sambucus canadensis*	*Caprifoliaceae*	KC343140; KC343866; KC344108; KC343624; KC343382; -
Diaporthe melonis	CBS 507.78 *	*Cucumis melo*	*Cucurbitaceae*	KC343142; KC343868; KC344110; KC343626; KC343384; -
Diaporthe micheliae	SCHM 3603	*Michelia alba*	Magnoliaceae	AY620820; -; -; -; -; -
Diaporthe middletonii	BRIP 54884e*	*Rapistrum rugostrum*	*Brassicaceae*	KJ197286; KJ197248; KJ197266; -; -; -
Diaporthe miriciae	BRIP 54736j*	*Helianthus annuus*	*Asteraceae*	KJ197282; KJ197244; KJ197262; -; -; -
Diaporthe multigutullata	ZJUD98*; IcMP20656	*Citrus grandis*	*Rosaceae*	KJ490633; KJ490512; KJ490454; KJ490575; -; -

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Species names	**Culture collection no.**	**Isolation sources**	**Host family**	**GenBank Accession Numbers**
Diaporthe musigena	CBS 129519*	*Musa* sp., leaves	*Musaceae*	KC343143; KC343869; KC344111; KC343627; KC343385; -
Diaporthe momicola	MFLUCC 16-0113*	*Prunus persica*	*Rosaceae*	KU557563; KU557631; KU557587; -; KU557611; -
Diaporthe neilliae	CBS 144. 27*	*Spiraea* sp.	*Rosaceae*	KC343144; KC343870; KC344112; KC343628; KC343386; -
Diaporthe nobilis Complex	CBS 200.39	*Laurus nobilis*, stem	*Lauraceae*	KC343151; KC343877; KC344119; KC343635; KC343393; -
Diaporthe neoarctii	CBS 109490*	*Ambrosia trifida*	*Asteraceae*	KC343145; KC343871; KC344113; KC343629; KC343387; -
Diaporthe neoraonikayaporum	MFLUCC 14–1136*	*Tectona grandis*	*Verbenaceae*	KU712449; KU749369; KU743988; -; KU749356; -
Diaporthe nothofagi	BRIP 54801*	*Nothofagus cunninghamii*	*Fagaceae*	JX862530; JX862536; KF170922; -; -; -
Diaporthe novem	CBS 127270*	*Glycine max*	*Fabaceae*	KC343156; KC343882; KC344124; KC343640; KC343398; -
Diaporthe nomurai	CBS 157.29	*Morus* sp.	*Moraceae*	KC343154; KC343880; KC344122; KC343638; KC343396; -
Diaporthe oncostoma	CBS 100454	*Robinia pseudoacacia*, leaf spot	*Fabaceae*	KC343160; KC343886; KC344128; KC343644; KC343402; -
Diaporthe ocoteae	CBS 141330*	*Ocotea obtusata*	*Lauraceae*	KX228293; -; KX228388; -; -; -
Diaporthe oraccinii	CGMCC 3.17531*; Lc3166*	*Camellia sinensis*, leaf, endophyte	*Theaceae*	KP267863; KP267937; KP293443; KP293517; -; KY011843
Diaporthe ovalispora	ZJUD93*	*Citrus limon*	*Rosaceae*	KJ490628; KJ490507; KJ490449; KJ490570; -; -
Diaporthe ovoicicola	CGMCC 3.17093*	*Citrus sp*	*Rosaceae*	KF576265; KF576240; KF576289; -; KF576223; -
Diaporthe oxe	CBS 133186*	*Maytenus ilicifolia*	*Celastraceae*	KC343164; KC343890; KC344132; KC343648; KC343406; -
Diaporthe padi var. padi	CBS 114200	*Prunus padus*	*Rosaceae*	KC343169; KC343895; KC344137; KC343653; KC343411; -
Diaporthe paranensis	CBS 133184*	*Maytenus ilicifolia*	*Celastraceae*	KC343171; KC343897; Kc344139; KC343655; KC343413; -
Diaporthe parapterocarpi	CPC 22729*	*Pterocarpus brenanii*	*Fabaceae*	KJ869138; -; KJ869248; -; -; -
Diaporthe pascoei	BRIP 54847*	*Persea americana*	*Lauraceae*	JX862532; JX862538; KF170924; -; -; -

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Diaporthe passiflorae	CBS 132527*	*Passiflora edulis*	*Passifloraceae*	JX862532; JX862538; KF170924; -; KY435664; -
Diaporthe passifloricola	CBS 141329*	*Passiflora edulis*	*Passifloraceae*	JX069860; -; -; -; -; -
Diaporthe penetriteum	LC 3353	*Camellia sinensis*, leaf	*Theaceae*	KP714505; KP714517; KP714529; KP714493; -; KY011850
Diaporthe phaseolorum	CBS 139281; AR4203*	*Phaseolus vulgaris*	*Fabaceae*	KJ590738; KJ590739; KJ610893; KJ659220; KJ612135; -
Diaporthe perjuncta	CBS 109745*	*Ulmus glabra*	*Ulmaceae*	KC343172; KC343898; KC344140; KC343656; KC343414; -
Diaporthe perniciosa	CBS 124030	*Malus pumila*, bark	*Rosaceae*	KC343149; KC343875; KC344117; KC343633; KC343391; -
Diaporthe pescicola	MFLUCC 16-0105*	*Prunus persica*	*Rosaceae*	KU557555; KU557623; KU557579; -; KU557603; -
Diaporthe perseae	CBS 151.73*	*Perseae gratissima*, young fruit	*Lauraceae*	KC343173; KC343899; KC344141; KC343657; KC343415; -
Diaporthe phragmitis	CBS 138897*	Phragmites australis	*Poaceae*	KP004445; -; KP004507; KP004503; -; -
Diaporthe phyllanthicola	SCHM 3680	*Phyllanthus emblica*	Phyllanthaceae	AY620819; -; -; -; -; -
Diaporthe podocarpi-macrophylli	CGMCC 3.18281*; LC 6155	*Podocarpus macrophyllus*, endophyte	*Podocarpaceae*	KX986774; KX999167; KX999207; KX999246; KX999278; -
Diaporthe pseudophoenicicola	CBS 462.69*	Phoenix dactylifera, dead tops of green leaves	*Anacardiaceae*	KC343184; KC343910; KC344152; KC343668; KC343426; -
Diaporthe pseudomangiferae	CBS 101339*	*Mangifera indica*	*Anacardiaceae*	KC343181; KC343907; KC344149; KC343665; KC343423; -
Diaporthe pseudotsugae	MFLU 15-3228	*Pseudotsuga menziesii*	*Pinaceae*	KY964225; KY964181; KY964108; -; KY964138; -
Diaporthe psoraleae	CBS 136412*	*Psoralea pinnata*	*Fabaceae*	KF777158; KF777245; KF777251; -; -; -
Diaporthe psoraleae-pinnatae	CBS 136413*	*Psoralea pinnata*	*Fabaceae*	KF777159; -; KF777252; -; -; -
Diaporthe pterocarpi	MFLUCC 10-0571*	*Pterocarous indicus*	*Papilionaceae*	JQ619899; JX275416; JX275460; -; JX197451; -
Diaporthe pterocarpicola	MFLUCC 10-0580a*	*Piterocarpus indicus*	*Papilionaceae*	JQ619887; JX275403; JX275441; -; JX197433; -
Diaporthe pulla	CBS 338.89*	*Hedera helix*	*Araliaceae*	KC343152; KC343878; KC344120; KC343636; KC343394; -

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Diaporthe pustulata	CBS 109760	*Acer pseudoplatanus*	*Aceraceae*	KC343186; KC343912; KC344154; KC343670; KC343428; -
Diaporthe pyracanthae	CAA483*	*Pyracantha coccinea*	*Rosaceae*	KY435635; KY435625; KY435666; KY435646; KY435656; -
Diaporthe raonikayaporum	CBS 133182*	*Spondias mombin*	*Anacardiaceae*	KC343188; KC343914; KC344156; KC343672; KC343430; -
Diaporthe ravennica	MFLUCC 15–0479*	*Tamarix sp*	*Tamaricaceae*	KU900335; KX365197; KX432254; -; -; -
Diaporthe rhoina	CBS 146.27	*Rhus toxicodendron*	*Anacardiaceae*	KC343189; KC343915; KC344157; KC343673; KC343431; -
Diaporthe rhusicola	CBS 129528*	*Rhus pendulina*	*Anacardiaceae*	JF951146; KC843100; KC843205; -; KC843124; -
Diaporthe rostrata	CFCC 50062*	*Juglans mandshurica*	*Juglandaceae*	KP208847; KP208853; KP208855; KP208851; KP208849; -
Diaporthe rudis	CBS 113201*	*Vitis vinifera*	*Vitaceae*	KC343234; KC343960; KC344202; KC343718; KC343476; -
Diaporthe saccarata	CBS 116311*	*Protea repens, cankers*	*Proteceae*	KC343190; KC343916; KC344158; KC343674; KC343432; -
Diaporthe sackstonii	BRIP 54669b*	*Helianthus annuus*	*Asteraceae*	KJ197287; KJ197249; KJ197267; -; -; -
Diaporthe salicicola	BRIP 54825*	*Salix purpurea*	*Salicaceae*	JX862531; JX862537; KF170923; -; -; -
Diaporthe schini	CBS 133181*	*Schinus terebinthifolius*, endophytic in leaf	*Anacardiaceae*	KC343191; KC343917; KC344159; KC343675; KC343433; -
Diaporthe schoeni	MFLU 15-1279*	*Schoenus nigricans*	*Cyperaceae*	KY964226; KY964182; KY964109; -; KY964139; -
Diaporthe sclerotioides	CBS 296.67*	*Cucumis sativus*	*Cucurbitaceae*	KC343193; KC343919; KC344161; KC343677; KC343435; -
Diaporthe scobina	CBS 251.38	*Fraxinus Excelsior*, living and dead twig	*Oleaceae*	KC343195; KC343921; KC344163; KC343679; KC343437; -
Diaporthe serafiniae	BRIP 55665a*	*Helianthus annuus*	*Asteraceae*	KJ197274; KJ197236; KJ197254; -; -; -
Diaporthe siamensis	MFLUCC10-573a*	*Dasymaschalon* sp.	*Annonaceae*	JQ619879; JX275393; JX275429; -; -; -
Diaporthe sojae	FAU635*	*Glycine max*	*Fabaceae*	KJ590719; KJ590762; KJ610875; KJ659208; KJ612116; -
Diaporthe sennicola	CFCC 51634*	*Senna bicapsularis*	*Fabaceae*	KY203722; KY228883; KY228889; -; KY228873; -

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Species names	Culture collection no.	Isolation sources	Host family	GenBank Accession Numbers
Diaporthe sennae	CFCC 51636*	*Senna bicapsularis*	*Fabaceae*	KY203724; KY228885; KY228891; -; KY228875; -
Diaporthe sterilis	CBS 136969*	*Vaccinium corymbosum*	*Ericaceae*	KJ160579; KJ160611; KJ160528; -; KJ160548; -
Diaporthe stewartii	CBS 193.36	-	-	FJ889448; GQ250324; ; ; ; -
Diaporthe stictica	CBS 370.54*	*Buxus sampervirens*, dead twig	*Buxaceae*	KC343212; KC343938; KC344180; KC343696; KC343454; -
Diaporthe subclavata	ZJUD95*	*Citrus unshiu*	*Rosaceae*	KJ490630; KJ490509; KJ490451; KJ490572; -; -
Diaporthe subordinaria	CBS 464.90*;	*Plantago lanceolata*	*Plantaginaceae*	Kc343214; KC343940; KC344182; KC343698;
Diaporthe tecomae	CBS 100547	*Tabebuia* sp.	*Bignoniaceae*	KC343215; KC343941; KC344183; KC343699; KC343457; -
Diaporthe spartinicola	CBS 140003*	*Spartium junceum*	*Fabaceae*	KR611879; -; KR857695; KR857696; -; -
Diaporthe taoicola	MFLUCC 16-0117*	*Prunus persica*	*Rosaceae*	KU557567; KU557635; KU557591; -; -; -
Diaporthe tectonae	MFLUCC 12–0777*	*Tectona grandis*	*Verbenaceae*	KU712430; KU749359; KU743977; -; KU749345; -
Diaporthe tectonendophytica	MFLUCC 13–0471*	*Tectona grandis*	*Verbenaceae*	KU712439; KU749367; KU743986; -; KU749354; -
Diaporthe tectonigena	MFLUCC 12–0767*	*Tectona grandis*	*Verbenaceae*	KU712429; KU749371; KU743976; -; KU749358; -
Diaporthe terebinthifolii	CBS 133180*	*Schinus terebinthifolius*	*Anacardiaceae*	KC343216; KC343942; KC344184; KC343700; KC343458; -
Diaporthe ternstroemia	CGMCC 3.15183*	*Ternstroemia gymnanthera*	*Pentaphylacaceae*	KC153098; KC153089; -; -; -; KY011874
Diaporthe tersa	M95			LC041027; ; ; ; ; -
Diaporthe theae GJJM16	GJJM16			JN638438; ; ; ; ; -
Diaporthe thunbergii	MFLUCC10-576a*	*Thunbergia laurifolia*	*Acanthaceae*	JQ619893; JX275409; JX275449; -; JX197440; -
Diaporthe thunbergiicola	MFLUCC 12–0033*	*Thunbergia laurifolia*	*Acanthaceae*	KP715097; KP715098; -; -; -; -
Diaporthe torilicola	MFLUCC 17-1051*	*Torilis arvensis*	*Apiaceae*	KY964212; KY964168; KY964096; -; KY964127; -
Diaporthe toxica	CBS 534.93*	*Lupinus angustifolius*, stem	*Fabaceae*	KC343220; KC343946; KC344188; KC343704; KC343462; -
Diaporthe tulliensis	BRIP 62248a*	*Theobroma cacao*	*Sterculiaceae*	KR936130; KR936133; KR936132; -; -; -
Diaporthe ueckerae	FAU656*	*Cucumis melo*	*Cucurbitaceae*	KJ590726; KJ590747; KJ610881; KJ659215; KJ612122; -

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Species names	Culture collection no.	Isolation sources	Host family	GenBank Accession Numbers
Diaporthe undulata	CGMCC 3.18293*; LC 6624	Unknown host, pathogen	-	KX986798; KX999190; KX999230; KX999269; -; KY011858
Diaporthe unshiuensis	ZJUD52*; CGMCC 3.17569	*Citrus unshiu*	*Rosaceae*	KJ490587; KJ490466; KJ490408; KJ490529; -; -
Diaporthe vexans	CBS 127.14	*Solanum melongena*	*Solanaceae*	KC343229; KC343955; KC344197; KC343713; KC343471; -
Diaporthe vaccinii	CBS 160.32*	*Oxycoccus macrocarpos*	*Ericaceae*	KC343228; KC343954; KC344196; KC343712; KC343470; -
Diaporthe vangueriae	CPC 22703* CBS 137985	*Vangueria infausta*	*Rubiaceae*	KJ869137; -; KJ869247; -; -; -
Diaporthe vawdreyi	BRIP 57887a*	*Psidium guajava*	*Sterculiaceae*	KR936126; KR936129; KR936128; -; -; -
Diaporthe velutina	CGMCC 3.18286*; LC 4421	*Neolitsea* sp., pathogen	*Lauraceae*	KX986790; KX999182; KX999223; KX999261; ; KY011884
Diaporthe virgilia	CMW 40755*	*Virgilia oroboides*	Unknown	KP247573; -; KP247582; -; -; -
Diaporthe viticola	CBS 114011	*Vitis vinifera*	*Vitaceae*	KC343235; KC343961; KC344203; KC343719; KC343477; -
Diaporthe woodii	CBS 558.93	*Lupinus* sp.	*Fabaceae*	KC343244; KC343970; KC344212; KC343728; KC343486; -
Diaporthe woolworthii	CBS 148.27	*Ulmus americana*	*Ulmaceae*	KC343245; KC343971; KC344213; KC343729; KC343487; -
Diaporthe vitimegaspora	STE-U2675	*V. vinifera*	-	AF230749; -; -; -; -; -
Diaporthe xishuangbanica	CGMCC 3.18282*; LC 6707	*Camellia sinensis*, pathogen	*Theaceae*	KX986783; KX999175; KX999216; KX999255; -; KY011860
Diaporthe yunnanensis	CGMCC 3.18289* ; LC6168	*Coffea* sp., endophytes	*Rubiaceae*	KX986796; KX999188; KX999228; KX999267; KX999290; KY011867
Diaporthe cf. heveae 1	CBS 852.97	*Hevea brasiliensis*	*Euphorbiaceae*	KC343116; KC343842; KC344084; KC343600; KC343358; -
Diaporthe cf. heveae 2	CBS 681.84	*Hevea brasilliensis*, leaf	*Euphorbiaceae*	KC343117; KC343843; KC344085; KC343601; KC343359; -
Diaporthe / P. conorum	CBS 587.79	*Penus pentaphylla*	*Pinaceae*	KC343153; KC343879; KC344121; KC343637; KC343395; -
Diaporthe/ P. emicis	BRIP 45089a*	*Emex australis*	*Polygonaceae*	JF957784; JX275414; JX275458; -; JX197449; -
Diaporthe sp.	LGMF947	*Glycine max*	*Fabaceae*	KC343203; KC343929; KC344171; KC343687; KC343445; -
Diaporthe sp.	CBS 287.29	*Pseudotsuga menziesii*	*Pinaceae*	KC343205; KC343931; KC344173; KC343689; KC343447; -

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Species names	Culture collection no.	Isolation sources	Host family	GenBank Accession Numbers
Diaporthe sp.	CBS 125575	*Acer opalus*	*Aceraceae*	KC343207; KC343933; KC344175; KC343691; KC343449; -
Diaporthe sp.	CBS 115584	*Maesa perlarius*	*Myrsinaceae*	KC343208; KC343934; KC344176; KC343692; KC343450; -
Diaporthe sp.	CBS 115595	*Maesa perlarius*	*Myrsinaceae*	KC343209; KC343935; KC344177; KC343693; KC343451; -
Diaporthe sp.	CBS 458.78	*Anacardium occidentale*	*Anacardiaceae*	KC343210; KC343936; KC344178; KC343694; KC343452; -
Diaporthe sp.	LGMF925	*Aspidosperma tomentosum*	*Apocynaceae*	KC343211; KC343937; KC344179; KC343695; KC343453; -
Diaporthella corylina	CBS 121124*	*Corylus sp.*, dying stems	*Corylaceae*	KC343004; KC343730; KC343972; KC343488; KC343246; -
Mazzantia napelli	AR 3498	*Aconitum vulparia*	*Ranunculaceae*	; -; -; -;-; -; AF408368
Ophiodiaporthe cyatheae	BCRC 34961	*Cyathea lepifera*	*Cyatheaceae*	; -; -; -;-; -; JX570891
Ph. megalosporum	CBS 284.65	*Rice-feld soil*	-	; -; -; -;-; -; FR748103
Ph. plurivorum	CBS 113835	*Helianthus annuus*	*Asteraceae*	; -; -; -;-; -; FR748104
Ph. sacchari	CBS 275.34	-	-	; -; -; -;-; -; FR748105
Phaeocytostroma ambiguum	CPC 17071	*Zea mays*	*Poaceae*	; -; -; -;-; -; FR748036
Pustulomyces bambusicola	MFLUCC 11-0436	*on dead culm of bamboo*	-	; -; -; -;-; -; KF806753
S. maydis	CBS 117558	*Zea mays*	*Poaceae*	; -; -; -;-; -; DQ377936
Stenocarpella macrospora	CBS 117560	*Zea mays*	*Poaceae*	; -; -; -;-; -; DQ377934
Valsa ambiens	CFCC 89894	*Pyrus bretschneideri*	Rosaceae	; -; -; -;-; -; KR045699