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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

Irailton Prazeres dos Santos

ISOLAMENTO E CARACTERIZAÇÃO DOS METABÓLITOS PRODUZIDOS PELO FUNGO *Nigrospora sphaerica* (Sacc.) Mason ENDOFÍTICO DE *Indigofera suffruticosa* Mill. E AVALIAÇÃO DO POTENCIAL BIOTECNOLÓGICO

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Orientadora: Profa. Dra. Vera Lúcia de Menezes Lima

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DEDICATÓRIA

A minha família:
O bem mais precioso que uma pessoa pode ter.

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A Deus

Hoje, mais do que nunca, compreendo a existência de uma força maior...
Sei que essa força me ajudou a seguir por este caminho que chegou ao fim. Sei também que será essa mesma força que me fará seguir sempre em frente por qualquer caminho! Mesmo que Dele, eu tenha por algum momento, esquecido.

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“O que fizemos apenas por nós mesmos morre conosco. O que fizemos pelos outros e pelo mundo permanece e é imortal”.

Albert Pike

RESUMO

Fungos endofíticos vêm despertando grande interesse biotecnológico em virtude da aplicabilidade de seus metabólitos secundários na medicina, indústria e agricultura. A espécie *Indigofera suffruticosa* é uma planta encontrada no Brasil, conhecida por apresentar várias atividades biológicas. Nesse trabalho fungos endofíticos foram isolados de folhas de *I. suffruticosa* na Mata Atlântica e na Caatinga, do estado de Pernambuco, durante as estações seca e chuvosa, taxonomicamente caracterizados e avaliados quanto a produção de metabólitos com atividade antimicrobiana. Todos os fungos isolados foram submetidos a um *screening* preliminar, e os que apresentaram melhores resultados foram fermentados em diferentes meios de cultura líquidos e semi-sólidos, e tiveram sua atividade antimicrobiana análisada através do método de difusão em disco. O extrato metanólico de *Nigrospora sphaerica* (NsME) obtido a partir do clutivo em meio de cultura de arroz, e o extrato de acetato de etila de *N. sphaerica* (NsEAE) obtido a partir do sobrenadante após o cultivo no meio líquido Batata Dextrose, tiveram suas concentração mínima inibitória (CMI) e concentração mínima bactericida (CMB) determinadas contra bactérias Gram-negativas e bactérias Gram-positivas. O extrato NsEAE foi submetido a colunas cromatográficas e as frações e subfrações obtidas foram analizadas através de ressonância magnética nuclear, resultando na identificação do ácido indólico-3-carboxílico, o qual foi avaliado quanto a sua atividade anti-*Candida*. Um total de 107 fungos foram isolados e identificados de acordo com as características morfológicas e chaves taxonómicas. Entre os nove táxons identificados, *Colletotrichum gloeosporioides* (13,4%), *Pseudocochliobolus pallescens* (8,3%), *Nigrospora sphaerica* [*Khuskia oryzae*] (7,4%), e *Pestalotiopsis maculans* (6,9%) foram os mais frequentes. *Curvularia australiensis* e *Chaetomella raphigera* foram isolados apenas na Caatinga durante períodos seco e chuvoso, respectivamente e são relatadas como a primeira vez que foram isoladas a partir de uma planta na Caatinga. *Lasiodiplodia theobromae* foi encontrada apenas na Mata Atlântica no período de seco, floresta em que a diversidade de fungos isolados foi maior. Entre os fungos endofíticos isolados, 18 fungos mostraram atividade contra pelo menos um microorganismo teste em um *screening* preliminar, e os melhores resultados foram obtidos com a espécie *N. sphaerica* (URM-6060) e *P. maculans* (URM-6061). Após a fermentação, apenas *N. sphaerica* apresentou atividade antibacteriana quando fermentada meio de cultura líquido Batata Dextrose e no meio semi-sólido arroz. Os extrato NsME e NsEAE foram ativos contra bactérias Gram-negativas e Gram-positivas, com CMI variando entre 1.56 e 6.25 mg/mL e CMB variando entre 6.25 e 50 mg/mL. O melhor resultado foi observado contra *Staphylococcus aureus*, com CMI 1,56 mg/ml e CMB 6,25 mg/ml para NsME e CMI 0,39 mg/ml e CMB 3,12 mg/ml com o extrato NsEAE. O ácido indólico-3-carboxílico apresentou atividade fungicida contra todas as espécies de *Candida* testadas, com CMI variando entre 0.125 e 0.0039 mg/mL e concentração mínima fungicida (CMF) variando entre 0.125 e 0.0039 mg/mL, sendo os melhores resultados observados contra *Candida guilermondii*, *C. haemulonii* and *C. albicans*. Estes resultados representam o primeiro relato sobre a micobiota endofítica de *I. suffruticosa* demonstrando que as características da Mata Atlântica favorecem uma maior colonização de fungos endofíticos, em comparação com a Caatinga, em que o fungo *N. sphaerica* demonstrou ser capaz para produzir agentes bioativos com potencial uso farmacêutico na busca de novas fontes biológicas de candidatos a fármacos.

Palavras-chave: diversidade fúngica, Caatinga, atividade antimicrobiana, *Nigrospora sphaerica*, *Staphylococcus aureus*, ácido indólico-3-carboxílico, atividade anti-*Candida*.

ABSTRACT

Endophytic fungi have gained great biotechnological interest due to the applicability of its secondary metabolites in medicine, industry and agriculture. The *Indigofera suffruticosa* species is a plant found in Brazil, known to have various biological activities. In this work endophytic fungi were isolated from *I. suffruticosa* leaves in the Atlantic Forest and Caatinga, in the state of Pernambuco, during the dry and rainy seasons, taxonomically characterized and evaluated for the production of metabolites with antimicrobial activity. All fungal isolated were submitted to a preliminary screening, and those with best results were fermented in different liquid culture media and semi-solids, and had their antimicrobial activity analyzed using the disk diffusion method. The methanolic extract of *Nigrospora sphaerica* (NsME) obtained from clutivo in rice culture medium, and the ethyl acetate extract of *N. sphaerica* (NsEAE) obtained from the supernatant after culture in liquid medium Potato Dextrose, had their minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) evaluted against Gram-negative and Gram-positive bacteria. The NsEAE extract was subjected to chromatography columns and the fractions and subfractions were analyzed by nuclear magnetic resonance, resulting in the identification of indole-3-carboxylic acid, which was evaluated for its anti-*Candida* activity. A total of 107 fungal were isolated and identified according to the morphological features and taxonomic keys. Among the nine taxa identified, *Colletotrichum gloeosporioides* (13.4%), *Pseudocochliobolus pallescens* (8.3%), *Khuskia oryzae* (formerly *Nigrospora sphaerica*) (7.4%), and *Pestalotiopsis maculans* (6.9%) were the most frequent. *Curvularia australiensis* and *Chaetomella raphigera* were isolated only in the Caatinga during dry and rainy periods, respectively, and are reported as the first time we were isolated from a plant in the Caatinga. *Lasiodiplodia theobromae* was found only in the Atlantic Forest in the dry period, the forest in which the diversity of fungi isolated was higher. Among the endophytic fungi isolated, 18 fungi showed activity against at least one microorganism in a preliminary screening test, and the best results were obtained with the species *Nigrospora sphaerica* (URM-6060) and *Pestalotiopsis maculans* (URM-6061). After fermentation, only *N. sphaerica* presented antibacterial activity when fermented liquid culture medium Potato Dextrose and semi-solid rice. The extract NsEAE and NsME were active against Gram-negative and Gram-positive bacteria with MIC ranging between 1.56 and 6.25 mg/ml and MBC varying between 6.25 and 50 mg/ml. The best result was observed against *Staphylococcus aureus* with MIC 1.56 mg/ml and CMB 6.25 mg/ml for NsME and MIC 0.39 mg/ml and MBC 3.12 mg/ml with the NsEAE extract. The indole-3-carboxylic acid showed fungicidal activity against all *Candida* species tested, with MIC ranging between 0.125 and 0.0039 mg/ml and minimum fungicidal concentration (MFC) between 0.125 and 0.0039 mg/ml, being the best results observed against *Candida guilermondii*, *C. haemulonii* and *C. albicans*. These results represent the first report of *I. suffruticosa* endophytic mycobiota of demonstrating that the properties of the Atlantic Forest favor greater colonization of endophytic fungi, as compared to the Caatinga, wherein the fungus *N. sphaerica* shown to be able to produce bioactive agents with potential pharmaceutical use, an attractive feature in the search for new biological sources of drug candidates.

Keywords: Fungal diversity, Caatinga, antimicrobial activity, *Nigrospora sphaerica*, *Staphylococcus aureus*, indole-3-carboxylic acid, anti-*Candida* activiry.

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SUMÁRIO

1. INTRODUÇÃO

O uso de plantas medicinais no tratamento e prevenção de doenças está fortemente presente na cultura popular, e no Brasil a utilização desse recurso natural tem grande influência das culturas indígena e africana (COSTA E SILVA, 1994; FREYRE, 2003; GUARIM-NETO et al., 2000).

Entre essas plantas encontra-se a espécie *Indigofera suffruticosa* que, apesar de ser uma planta exótica originária das Antilhas e América Central, introduzida no Brasil para extração do pigmento indigo blue (ALZUGARAY AND ALZUGARAY, 1988), é conhecida na medicina popular por apresentar várias propriedades medicinais, além de apresentar relatos na literatura de várias atividades biológicas (ALMEIDA et al. 2013; LEITE et al., 2004; LUIZ-FERREIRA et al, 2011; VIEIRA et al. 2007), inclusive atividade antimicrobiana (LEITE et al., 2006).

I. suffruticosa adaptou-se a diferentes biomas do Brasil, e no estado de Pernambuco pode ser encontrada na Caatinga, bioma que cobre uma vasta área da região Nordeste, caracterizado pela deficiência hídrica originada da baixa pluviosidade, alta evapotranspiração e distribuição irregular das chuvas (BASSO et al., 2005; LEAL et al., 2005), bem como na Mata Atlântica, que devido a proximidade com o Oceano Atlântico fornece uma fonte estável de umidade, permitindo alta densidade de vegetação (BASSO et al., 2005).

O crescimento e o desenvolvimento de plantas medicinais em diferentes locais que apresentam fatores climáticos distintos, pode ocasionar alterações no teor e na diversidade dos princípios ativos produzidos por esses vegetais (BORELLA et al., 2001; BOTREL et. al., 2010; SANTOS et. al., 2009), e esse sucesso adaptativo em ambientes completamente diferentes pode esta relacionado com a capacidade de interação desses vegetais com outros seres vivos incluíndo os microrganismos, os quais têm o matabolismo fortemente influênciado por variações do clima e nas condições de cultivo do vegetal (SIMÕES-AMBROSIO et al., 2010; ZALAMEA et al., 2013).

Esses microrganismos interagem de maneiras distintas em diferentes áreas do vegetal, sendo denominados de endofíticos aqueles microrganismos que passam todo ou uma parte do seu ciclo de vida colonizando inter e/ou intracelularmente os tecidos da planta hospedeira, sem causar sintomas aparentes de doença (BANCON; WHITE, 2000; TAN; ZOU, 2001).

Os fungos endofíticos estão presentes em todas as espécies vegetais analisadas até o momento (ARAÚJO et al., 2010; NAIR; PADMAVATHY, 2014), e atualmente têm sido apontados como fontes promissoras na descoberta de novos compostos bioativos com grande potencial para a exploração numa ampla variedade de áreas (TENGURIA et al., 2011). Entre esses fungos, a espécie *Nigrospora sphaerica* [*Khuskia oryzae*] tem sido investigada por ser

descrita como uma fonte de metabolitos secundários biologicamente ativos de diferentes classes, incluindo os diterpenos (TURNER; ALDRIDGE, 1983), Dicetopiperazinas (CUTLER et al., 1991), lactonas (KIM et al., 2001), nigrosporolides (ZHANG et al., 2009) e esteroides (METWALY et al. 2014).

Entre os metabolitos secundários investigados, os que apresentam atividade antimicrobiana despertam bastante interesse, uma vez que os antibióticos estão entre os medicamentos mais prescritos no mundo inteiro, porém sua eficácia clínica tem enfrentado sérias preocupações especialmente devido ao aparecimento de microrganismos resistentes (TAKAHASHI et al., 2008). Por esse motivo, a descoberta de novos antibióticos se faz necessária devido ao aparecimento desses microrganismos resistentes, bem como a toxicidade de alguns produtos que já estão no mercado.

Considerando-se que seis entre vinte dos medicamentos mais comumente prescritos são de origem fúngica e que, em comparação com fungos obtidos de outros substratos poucos metabólitos têm sido isolados de endofíticos (SCHULTZ et al., 2002), a investigação química e biológica dos metabólitos produzidos pelos fungos endofíticos de que apresentam atividade antagônica contra bactérias e fungos patogênicos ao homem fundamenta-se e, atrelado ao caráter medicinal de *I. suffruticosa*, oferecem uma fonte potencial para desenvolvimento de novos produtos.

Assim, este trabalho visou à obtenção de metabólitos secundários a partir da fermentação dos fungos endofíticos de *I. suffruticosa*, para serem avaliadas quanto à sua atividade antimicrobiana, uma vez que essa espécie vegetal apresenta várias atividades biológicas descritas na literatura, incluindo atividade antimicrobiana, e esse caráter medicinal pode está relacionado com os fungos endofíticos que a colonizam.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. Plantas medicinains

O emprego de recursos vegetais está fortemente presente na cultura popular que é transmitida de pais para filhos no decorrer da existência humana, tornando-se uma tradição entre os povos contemporâneos. Este conhecimento geralmente é encontrado em povos tradicionais que tende à redução ou mesmo ao desaparecimento, quando sofre a ação inexorável da modernidade (GUARIM-NETO et al., 2000).

O uso dessas plantas no tratamento e prevenção das enfermidades é tão antigo quanto a espécie humana, e sua utilização no tratamento de várias patologias, ocorre há séculos. O homem faz uso dessas alternativas por meio de observação e experimentação, possibilitando assim a descoberta das atividades farmacológicas de cada planta com tal finalidade (IOANNIDES-DEMOS et al., 2011).

A colonização das Américas estabeleceu uma via de mão dupla na troca de informações acerca das plantas para fins medicinais, pois muitas espécies utilizadas pelos povos nativos das colônias passaram a ser utilizadas também pelos europeus, bem como várias espécies foram introduzidas no novo mundo, trazendo consigo a orientação secular do seu uso como medicinal. Durante todo o período escravista, Brasil e África estiveram em contanto constante através do oceano, por onde os escravos que chegavam traziam plantas de suas nações, e os marinheiros, os mercadores e os ex-escravos, no retorno, levavam novas plantas do Brasil (COSTA; SILVA, 1994).

Além disso, o conhecimento dos curandeiros indígenas assim como as rezas, benzeduras e ervas da senzala influenciaram os médicos europeus que passaram a receitar plantas em vez de medicamentos sintéticos (FREYRE, 2003). Os africanos, juntamente com os índios e europeus, foram responsáveis pela formação da base do conhecimento cultural e biológico acerca das plantas úteis no Brasil. Os escravos desempenharam dois papéis neste processo histórico, transplantado um sistema de classificação botânica africano e introduzindo plantas nativas brasileiras em sua própria cultura através de seus efeitos medicinais simbólicos (ALMEIDA, 2011).

Segundo o último levantamento, planta medicinal é o nome dado às espécies vegetais utilizadas com propósitos terapêuticos, sejam elas cultivadas ou não (WHO, 2003), podendo ser consideradas ainda como plantas medicinais aquelas espécies que têm uma história de uso tradicional como agente terapêutico (BRASIL, 2006).

O crescente interesse pelo uso de plantas na atualidade está relacionado a vários fatores como: o alto custo dos medicamentos industrializados, a crise econômica, a falta de

acesso da população à assistência médica e farmacêutica e uma tendência dos consumidores em utilizar produtos de origem vegetal (BASTOS, 2007).

Segundo a Organização Mundial de Saúde (OMS), cerca de 80% da população mundial faz uso de algum tipo de erva para tratamento de enfermidades, demonstrando assim que essa prática ainda é bem significativa e tradicional entre os povos do mundo todo (MARTINS, 2003). No Brasil, a utilização de plantas para fins medicinais pela população é uma prática tradicional (RITTER et al., 2002; MAIOLI-AZEVEDO; FONSECA-KRUEL, 2007), sendo muitas vezes o único recurso utilizado na atenção básica de saúde (VEIGA JUNIOR et al., 2005).

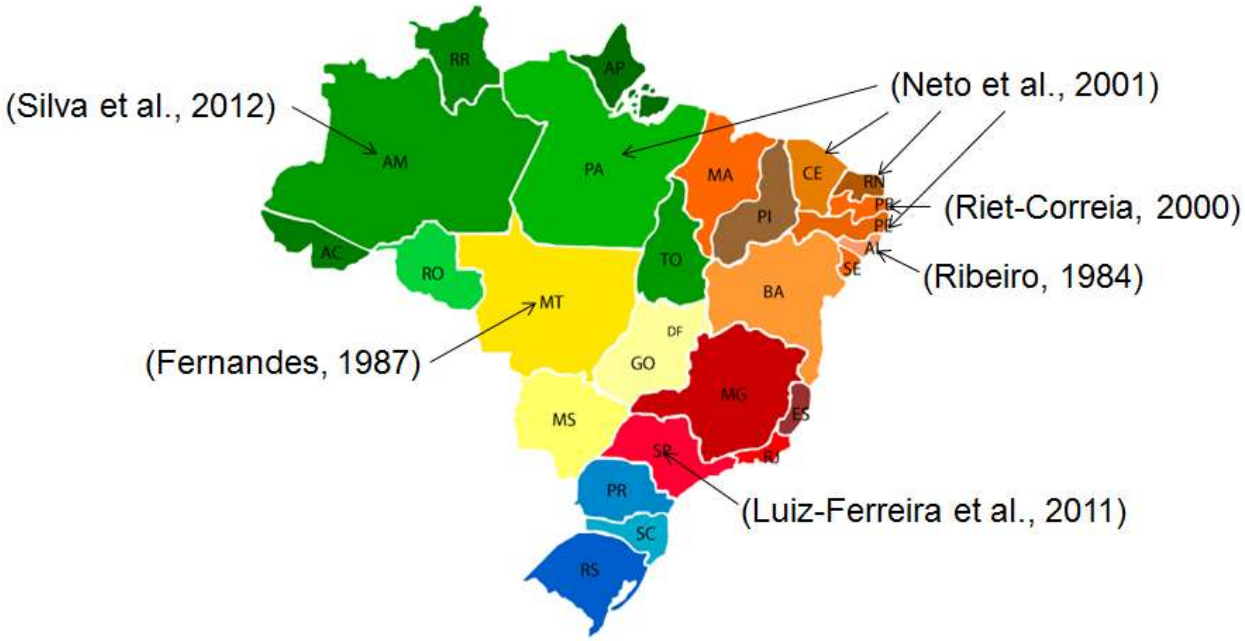
2.2. *Indigofera suffruticosa* Miller

A espécie *I. suffruticosa* (Figura 1) é uma planta originária da Antilha e América Central (ALMEIDA, 1993). Popularmente é conhecida como anil do campo, anileira, anileira-da-índia, anileira verdadeira, caá-chica, caá-chira, índigo, timbó-mirim, timbozinho, indigueira, indigófera, e encontra-se distribuída por toda a América tropical (CESÁRIO DE MELO, 1980).

Figura 1 – Aspecto geral de *Indigofera suffruticosa* em Igarassu – PE

No Brasil há registro da espécie nos estados do Mato Grosso (FERNANDES, 1987), Alagoas (RIBEIRO; SILVA; RANGEL, 1984), Paraíba (RIET-CORREA, 2000), Ceará , Rio Grande do Norte, Pará e Pernambuco (NETO et al., 2001), São Paulo (LUIZ-FERREIRA et al., 2011) e no Amazonas (SILVA et al., 2012), conforme mostrado na Figura 2.

Figura 2 - Registro na literatura da ocorrência de *Indigofera suffruticosa* no Brasil.



De modo geral, caracterizam-se como ervas anuais e perenes, eretas, prostadas, difusas ou escandentes, subarbusto, arbustos eretos e árvores de pequeno, médio e grande porte, com sistema radicular bem desenvolvido e predominância da raiz principal sobre suas ramificações. São pouco exigentes e crescem em solos de baixa fertilidade (BARROSO, 1984).

Segundo Braga (1976), *I. suffruticosa* é um arbusto de 1-2 m de altura e com ramos pubescentes. Suas folhas são pínadas, com 7-15 folíolos oblongos ou ovais, glabros na face e no verso. As flores são miúdas, numerosas, albo-róseas ou amareladas, em racemos axilares. Possui pequena vagem com 6-10 sementes parecida com feijão (Figura 3).

Figura 3 –*Indigofera suffruticosa*: A- folhas. B- Infrutescência e C- Inflorescência

O uso mais conhecido e antigo uso do gênero *Indigofera* sp. é na produção do pigmento azul. No século XVI, o pigmento atingiu preço equivalente a 220 dólares/kg e era mais importante para os europeus do que o cravo da Índia. Com a colonização, as Américas passaram a ser grandes fornecedores do pigmento. A indústria usou o índigo até o início do nosso século quando a produção sintética de anilina substituiu o pigmento natural. Ainda hoje, as comunidades do interior do Brasil fabricam e usam o pigmento para colorir roupas de lã e algodão. A tecnologia de produção consiste em fermentar as folhas para liberar o pigmento, produzindo um macerado esverdeado que é coado e alcalinizado com soda ou cinza, obtendo-se assim o pigmento azul (RODRÍGUES-KÁBANA et al., 1988).

Informações etnofarmacológicas indicam que espécies do gênero *Indigofera* são utilizadas para o tratamento de afecções gastrointestinais. Outros atributos significantes, os quais espécies arbustivas como *I.spinosa*, valiosa como planta forrageira, é o seu ciclo de vida perene, palatabilidade, resistência para herbívoros e habilidade para resposta a pequenos eventos de chuvas (COPPOCK; ELLIS; SWIFT, 1986, 1988; BAMBERG, 1986; COUGHENOUR et al., 1990).

Diversos usos medicinais são registrados para *I. suffruticosa* destacando-se o uso das folhas como agente antiespasmódico, sedativo, estomático, diurético e purgativo, antiepiléptico, anticonvulsivante e antiinflamatório e da raiz na icterícia (BHASKAR et al., 1982; LEITE et al., 2003; ROIG e MESA, 1974). Efeitos embriotóxicos e atividade antimicrobiana foram relatados (LEITE et al., 2003, 2004, 2006) e recentemente, atividades anticâncer (VIEIRA et al., 2007) e anticonvulsivo (ALMEIDA et al., 2013) foram descritas, além de estudo realizado por Luiz-Ferreira et al. (2011) apontar esta espécie como agente gastroprotetor. Esta planta também é empregada nas mordeduras de cobras e como insetífuga, sendo esta última propriedade extensiva às sementes após serem pulverizadas (ROIG; MESA, 1974; ALEJO; MIRANDA; RODRIGUES, 1996). Possui propriedades medicinais nos tratamentos de febres, parasitoses, doenças de pele e problemas cardíacos (ALLEN; ALLEN, 1981).

2.3. Influência dos fatores climáticos nas substâncias produzidas pelos vegetais

O Brasil, dada sua vasta extensão territorial, de dimensões continentais, possui uma tipologia climática variada. Além de sua extensão, outros fatores influentes nos diversos climas brasileiros são as condições de temperatura, altitude, pressão e proximidade com o oceano. Esta grande diferenciação climática do país resulta, por sua vez, em paisagens vegetais bastante variadas, o que faz do Brasil um dos países detentores do ecossistema mais variado e complexo no mundo (SILVA et al., 2007).

Essas variações dos fatores climáticos afetam diretamente o crescimento e o desenvolvimento das plantas sob diferentes formas e nas diversas fases do ciclo da cultura. Assim sendo o conhecimento acerca de elementos climatológicos como: precipitação e disponibilidade hídrica, são indispensáveis para o planejamento da escolha de épocas para o plantio e o manejo do solo com fins conservacionistas (SILVA et al., 2007).

Deve-se considerar que essas variações podem afetar o teor e a diversidade de princípios ativos de plantas medicinais, sendo por isso um importante fator a ser estudado para o estabelecimento de critérios de qualidade na coleta dessas plantas (BORELLA et al., 2001; BOTREL et. al., 2010; SANTOS et. al., 2009).

Alguns estudo têm demonstrado de forma clara essas alterações no metabolismo vegetal, como o que constatou variações sazonais significativas na composição química das folhas de *Maytenus aquifolium*. Estes resultados indicam que a utilização de *M. aquifolium*

como matéria-prima de medicamentos fitoterápicos requer a padronização do teor de flavonóides e de triterpenos (YARIWAKE et. al., 2005).

Os componentes do óleo essencial de *Foeniculum vulgare* var. *vulgare* variaram quantitativamente e qualitativamente de acordo com a fenologia e época do ano em que se coletou a planta. O composto majoritário encontrado tanto nas folhas como nos frutos foi o *trans*anetol, destacando, que o mesmo composto comportou-se de forma diferente em relação ao teor no óleo das folhas entre o final do inverno e da primavera. Destaca-se ainda que as folhas coletadas no final do inverno, apresentaram uma maior composição em constituintes químicos (SOUSA, 2005).

Uma espécie vegetal que sofre bastante influência dos fatores climáticos é a *I. suffruticosa*, uma planta originária das Antilhas e América Central que foi introduzida no Brasil onde foi cultivada em larga escla para extração do indigo blue, um pigmento natural muito utilizado pela indústria têxtil, mas que na década de 80 foi substituído por um pigmento artificial (ALZUGARAY; ALZUGARAY, 1988).

Apesar de ser uma planta exotica, *I. suffruticosa* se adaptou muito bem as diferentes regiões do Brasil, e no estado de Pernambuco essa planta pode ser encontrada em biomas extremamente diferentes como é o caso da Caatinga e da Mata Atlântica.

A Caatinga é floresta tropical seca do Brasil, que apresenta o clima marcado por altas temperaturas com chuvas esparsas e irregulars, e precipitação média anual que varia entre 250 e 500 mm, com solos de diferentes origens, e como regra, quimicamente fértil, bem drenado, e oxigenado mas que têm enfrentado degradação intensiva devido à introdução deliberada de plantas exóticas para dar apoio às atividades agrícolas (BASSO et al., 2005; LEAL et al., 2005).

A Mata Atlântica que se caracteriza por uma elevada biodiversidade, e a proximidade com o Oceano Atlântico fornece uma fonte estável de umidade, permitindo alta densidade de vegetação, revelando muitas espécies de interesse econômico, e muitas dessas espécies apresentam propriedades medicinais (BASSO et al., 2005).

Provavelmente, o sucesso adaptativo de *I. suffruticosa* a ambientes tão destintos está relacionado com sua capacidade de iteragir com outros seres vivos, entre esses os microrganismos, uma vez que estudos demonstraram que a colonização por microrganismos endofíticos específicos confere uma maior resistência em relação às plantas não colonizadas por essas espécies (CLARKE et al., 2006) e, embora este mecanismo de resistência ainda não seja totalmente compreendido, alguns estudos demonstraram que o matabolismo sofre forte influência do clima e de variação nas condições de cultivo do vegetal (SIMÕES-AMBROSIO

et al., 2010; ZALAMEA et al., 2013), e que a variação sazonal e a localização geográfica pode afetar a colonização endofítica (MARTÍN et al., 2004;. GORE; BUCAK, 2007; GUO et al., 2008), e consequentemente a produção de metabólitos secundários por esses edofíticos.

2.4. Microrganismos endofíticos

A palavra endofítico vem do grego (*éndon* + *phytón*), significa "dentro da planta", abrangendo bactérias, fungos, algas, vírus e insetos que convivem em simbiose com a planta hospedeira (SCHULZ; BOYLE, 2005). A utilização do termo endofítico se dá principalmente para bactérias e fungos (GUNATILAKA, 2006; JOHRI, 2006; OWEN; HUNDLEY, 2004; TAN; ZOU, 2001).

São considerados endofíticos, aqueles microrganismos que passam todo ou uma parte do seu ciclo de vida colonizando inter e/ou intracelularmente os tecidos da planta hospedeira, sem causar sintomas aparentes de doença (BANCON; WHITE, 2000; CABRAL; STONE; CARROLL, 1993; PETRINI, 1991; TAN; ZOU, 2001).

Outra definição sugere endófitos como microrganismos que podem ser isolados de tecidos vegetais, após desinfecção externa e que não causam, aparentemente, danos as plantas (HALLMANN et al., 1997). Azevedo e Araújo (2007) definiram endófitos como sendo todo microrganismo, cultivável ou não, que coloniza o interior de tecidos vegetais sem causar danos aparentes ao hospedeiro e sem produzir estruturas externas visíveis. Mais tarde, a definição anterior foi ampliada considerando, adicionalmente, a divisão de microrganismos endófiticos em dois tipos, sendo: Tipo I, os que não produzem estruturas externas à planta; e Tipo II, os que produzem estruturas externas à planta (MENDES; AZEVEDO, 2007).

Os endofíticos estão presentes em todas as espécies vegetais analisadas até o momento (ARAÚJO et al., 2010; NAIR; PADMAVATHY, 2014). Em geral, dezenas a centenas de endofíticos podem ser isolados de uma única planta e, a partir deste único hospedeiro, pelo menos uma espécie se mostra específica, confirmando a importância dos endofíticos como componente da diversidade microbiana (STROBEL; DAISY, 2003; TAN; ZOU, 2001).

Microrganismos endofíticos já foram relatados interagindo com diversos tipos de vegetais como plantas herbáceas (KIM et al, 2012; MUNIF et al, 2012; TAECHOWISAN; PEBERDY; LUMYONG, 2003), plantas de florestas tropicais (SURYANARAYANAN; KUMARESAN, 2000; STROBEL, 2002; BEZERRA et al., 2012, 2013), plantas cultivadas (MELNROY; KLOEPPER, 1995; BACILIO-JIMÉNEZ et al, 2003), plantas medicinais (HUANG et al., 2001; SIQUEIRA et al., 2011; BASHA et al., 2012) e plantas aquáticas

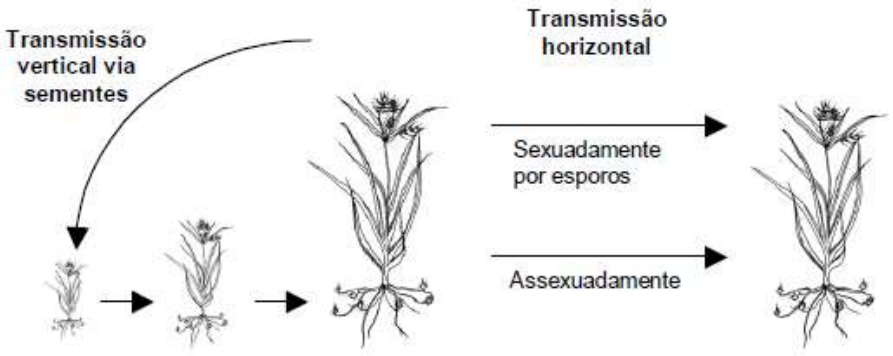
(CHEN et al., 2003; CHEN et al., 2012). Frequentemente, de dezenas a centenas de isolados podem ser obtidos de um único vegetal e, a partir deste único hospedeiro, pelo menos uma espécie se mostra específica, reforçando o fato que os endofíticos são um importante componente da diversidade microbiana (TAN; ZOU, 2001; STROBEL; DAYSE, 2003).

Além disso, a presença de microrganismos endofíticos pode ser observada em todos os tecidos e órgãos vegetais incluindo, entre outros, raízes, caules, folhas, flores e frutos (KOBAYASHI; PALUMBO, 2000; MCINROY; KLOEPPER, 1995; MELNICK et al., 2008; PICCOLO et al., 2010; SIQUEIRA et al., 2011; BEZERRA et al., 2012, 2013; VAZ et al., 2014; NAIR; PADMAVATHY, 2014). Alguns são mais frequentes em determinado tipo de vegetal, designados dominantes, em contraposição há outros mais raros, chamados de secundários (PILEGGI, 2006).

Para penetrar no vegetal hospedeiro os endófiticos podem viver como epifíticos por certo tempo, bem como, um microrganismo endofítico pode tornar-se patogênico em um vegetal em certas condições de estresse (ARAÚJO et al., 2010). Dessa maneira, a diferenciação dos termos tem função meramente didática, e entre eles existe um gradiente que é intrínseco à Biologia de forma geral (AZEVEDO et al., 2002).

Os endófitos, com exceção dos transmitidos pelas sementes (transmissão vertical) penetram primariamente através da zona radicular, embora microrganismos do ar possam utilizar aberturas naturais como estômatos e hidatódios presentes nas partes aéreas da planta como folhas, caule, cotilédones, flores e frutos (transmissão horizontal) (Figura 4) (ARAÚJO et al., 2010).

Figura 4. Formas de transmissão dos fungos endofíticos para os hospedeiros



Fonte: GUIMARÃES et al., 2006.

Segundo Neto, Azevedo e Araujo (2002), os endófitos podem desempenhar importante função no que se refere a sanidade vegetal, o que permitiu a associação mutualística com os microrganismos (RODRIGUEZ; REDMAN, 2008). Atualmente, acredita-se que todas as espécies de plantas que vivem em ecossistemas naturais estabelecem algum tipo de associação simbiótica com esses microrganismos (RODRIGUEZ et al., 2009), proporcionando alguns benefícios para o hospedeiro, como, melhoria da tolerância à seca (HUBBARD et al., 2012), proteção contra patógeno (ARNOLD et al., 2003), maior crescimento (REN et al., 2011), defesa contra herbivoria (SULLIVAN et al., 2007; ZHANG et al., 2009); e ainda podem ser empregados em práticas agrícolas, como por exemplo, no controle biológico de pragas e doenças de plantas, ou seja, são potenciais substitutos de produtos químicos, como inseticida e fungicida, e ainda podem ser usados como vetores genéticos (AZEVEDO et al, 2000; SOUZA et al, 2004; NAIR; PADMAVATHY, 2014).

2.5. Fungos endofíticos

O último levantamento realizado apontou que existe cerca de 1,5 milhões de espécies de fungos, dos quais apenas 5% estão descritos (HAWKSWORTH, 2001). Em contrapartida, estima-se que das 300 mil espécies de plantas que existem no planeta, cada uma abrigue no interior de seus tecidos um ou mais fungos endofíticos, podendo-se talvez chegar a um milhão de espécies de fungos, considerando somente os endofíticos (STROBEL; DAISY, 2003). Dessa forma, são grandes as chances de se encontrar novos fungos endofíticos dentre os milhares de plantas nos diferentes ecossistemas, constituindo-se numa fonte promissora de diversidade genética, como também, de novos produtos naturais (STROBEL; DAISY, 2003).

Na literatura, os fungos endofíticos são divididos em dois grupos ecológicos: *balansiaceous* e não-*balansiaceous*. O primeiro grupo é composto por fungos Ascomicetos pertencentes aos gêneros *Epichloe* e *Balansia* (anamorfos *Neotyphodium* e *Ephelis*) (SCHULZ; BOYLE, 2005). Segundo Petrini (1996), são proximamente relacionados e apresentam necessidades ecológicas e adaptações distintas de todos os outros fungos endofíticos. Além disso, desenvolvem sistêmica e intercelulamente e são transmitidos verticalmente através de sementes (SCHULZ; BOYLE, 2005).

Em contraste, endófitos não-*balansiaceous* são diversos, tanto filogeneticamente quanto em relação à sua estratégia de vida. São transmitidos horizontalmente, tendo sido isolados na maioria das plantas (ARNOLD et al., 2003). A colonização pode ser inter ou intracelular, localizada ou sistêmica (SCHULZ; BOYLE, 2005).

As espécies isoladas com maior frequência pertencem ao filo Ascomicota, incluindo os anamorfos (Hyphomycetes e Coelomycetes) (SCHULZ; BOYLE, 2005). Espécies de Basidiomycota e Zigomycota também são isoladas como endofíticos, porém, representam um número menor (SCHULZ; BOYLE, 2005). Na vegetação brasileira, os gêneros *Ascochyta, Cladosporium, Colletotrichum, Fusarium, Glomelera, Guignardia, Mucor, Nodulisporium, Pestalotia, Phomopsis, Phyllosticta, Rhizopus* e *Xylaria* são os mais frequêntes (PEIXOTO-NETO et al., 2002).

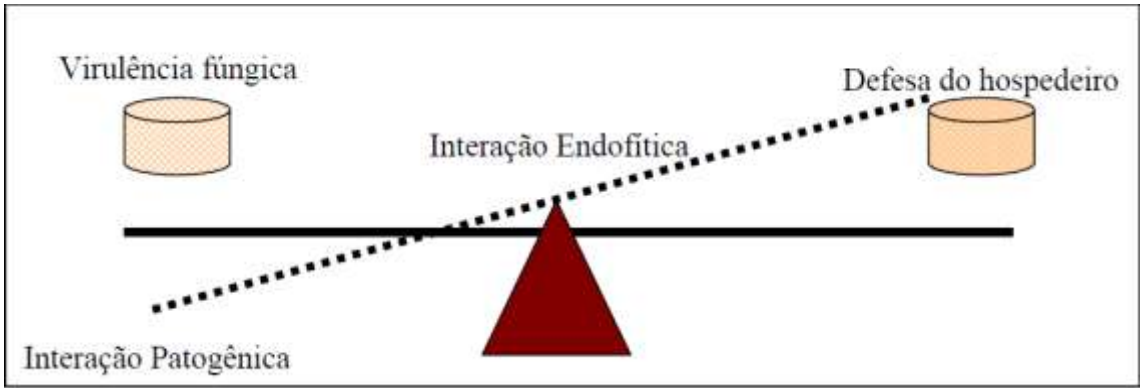
A associação entre fungos endofíticos e seus hospedeiros é extremamente complexa e seu completo entendimento ainda é prematuro (ARAÚJO et al., 2010). Sabe-se que, em geral, essa íntima associação envolve vários processos, que por sua vez, podem ser influenciados pelo genótipo, estágio de crescimento, condições fisiológicas e tipo de tecido vegetal, além de condições ambientais e práticas agrícolas (COMPANT et al., 2010; FIRÁKOVÁ et al., 2007; FORCHETTI et al., 2007; HARDOIM et al, 2008; SIEBER, 2007). Neste contexto, têm sido estudadas duas hipóteses principais acerca da interação endofíticos/hospedeiros: a da simbiose mutualística e a do equilíbrio antagônico (FAETH, 2002; SELOSSE et al., 2004; RUDGERS et al., 2004; SAIKKONEN et al., 2004; MÜLLER; KRAUSS, 2005; SCHULZ; BOYLE, 2005; KOGEL; FRANKEN, 2006; MAHESHWARI, 2006).

A primeira hipótese sugere que os endofíticos co-evoluíram com seus hospedeiros, apresentando uma íntima relação mutualística, onde os endofíticos recebem nutrientes e proteção da planta hospedeira, da mesma maneira, a planta também é beneficiada com essa interação. Dentre os benefícios destacam-se uma maior resistência em ambientes com intenso estresse causado por fatores abióticos como temperatura, umidade, luz, contaminantes, etc (SAIKKONEN et al., 1998). Este tipo de interação também possibilita, em alguns casos, resistência frente à patógenos (HALLMANN et al., 1997; M'PIGA et al., 1997; RYU et al., 2006; SENTHILKUMAR et al., 2007), aumento do crescimento vegetal (HALLMANN et al., 1997; BENT; CHANWAY, 1998; TSAVKELOVA et al., 2007), estímulo e indução do processo de germinação de sementes (HOLLAND, 1997; FREYERMUTH et al., 1996).

Sabe-se que numa relação sintomática metabólitos de defesa são produzidos pela planta hospedeira em resposta aos metabólitos tóxicos produzidos pelos fungos (AGRIOS, 1997). Segundo Peters et al. (1998), após análises *in vitro* foi verificado que tanto o endofítico como a planta hospedeira produziam e secretavam metabólitos tóxicos para ambos, resultado não esperado em uma relação assintomática. Neste contexto, Schulz e Boyle (2005) concluíram que a colonização assintomática é conseqüente da interação antagônica balanceada entre a planta hospedeira e o fungo. Caso este equilíbrio seja afetado, tanto por

uma diminuição na defesa da planta, através da síntese de metabólitos tóxicos, como por um aumento da virulência fúngica, há o desenvolvimento de doença, conforme demonstrado na Figura 5 (SCHULZ et al., 2002).

Figura 5 - Hipótese do equilíbrio antagônico.



Fonte: SCHULZ et al., 2002.

A hipótese do equilíbrio antagônico não exclui a possibilidade do endofítico exercer uma função benéfica para seu hospedeiro. Nesta interação, os fungos endofíticos influenciam na produção ou inibição de metabólitos com função de defesa, conferindo aos vegetais vantagens como resistência ao ataque de insetos, produção de antimicrobianos contra microrganismos fitopatogênicos (AZEVEDO et al., 2000; ARNOLD et al., 2003; MUCCIARELLI et al., 2002; SELOSSE et al., 2004; BANDARA et al., 2006).

A complexidade dessas interações tem direcionado estudos com endofíticos não só para os focos tradicionais, como promoção de crescimento vegetal e controle biológico de pragas, como, principalmente na obtenção de produtos de interesse biotecnológico (ARAÚJO et al., 2010).

2.6. Antibióticos produzidos por fungos

Uma das maiores causas de morte em todo mundo é o surgimento de doenças infecciosas decorrentes da alta taxa de microrganismos patogênicos resistentes a diversos antibióticos sendo necessário, portanto, a busca por novos antimicrobianos mais eficazes e de baixa toxicidade (LEVY; MARSHALL, 2004; APPELBAUM; JACOBS, 2005; YONEYAMA; KATSUMATA, 2006; BUTLER; BUSS, 2006).

Os primeiros relatos do uso de antibióticos pelo homem são muito antigos, como a descrição do uso de sapatos mofados por chineses para curar feridas infeccionadas nos pés

(3000 anos a.C.), porém, o primeiro metabólito fúngico de notória eficácia foi, sem dúvida, a penicilina, substância produzida pelo fungo *Penicillium notatum,* cuja capacidade de inibir o crescimento bacteriano foi descoberta acidentalmente por Fleming, em 1928. Seu emprego em larga escala no início da década de 40, fruto dos esforços dos pesquisadores ingleses Forey e Chain, levou à redução do índice de mortandade de soldados de 39% durante a Primeira Guerra Mundial para 3,9%, na Segunda Guerra. O grande impacto do uso da penicilina motivou sua produção industrial, sendo este o primeiro medicamento produzido em grande escala, originando, portanto, a indústria dos antibióticos (KOROLKOVAS; BURKHALTER, 1988).

Segundo Bettiol e Ghini (1995), a antibiose é definida como a interação entre organismos na qual um ou mais metabólitos produzidos por um organismo têm efeito danoso sobre o outro. Nesse contexto, muitos fungos e bactérias inibem fitopatógenos, competindo por nutrientes, parasitando e/ou produzindo metabólitos secundários como enzimas líticas e antibióticos. O resultado dessas interações antagônicas, tais como antibiose, competição, indução de defesa e parasitismo leva ao controle biológico de doenças de plantas, assim como pode desenvolver um papel muito importante na medicina com a descoberta de novos fármacos (MELO, 1998).

Antibióticos estão entre os medicamentos mais prescritos no mundo inteiro, porém sua eficácia clínica tem enfrentado sérias preocupações especialmente devido ao aparecimento de bactérias resistentes (TAKAHASHI et al., 2008), como por exemplo, a resistência exibida por *S. aureus* à meticilina e à vancomicina (ANTHONY et al., 2009). Em último levantamento realizado, foi constatado que mais de 70% das espécies das bactérias causadoras de infecções, são resistentes a pelo menos um dos antibióticos comumente utilizados (OVERBYE; BARRETT, 2005). Porém, o tempo estimado desde o descobrimento até a comercialização de uma nova droga é de aproximadamente 10 anos, acarretando um gasto superior a 800 milhões de dólares (REICHERT, 2003; DICKSON; GAGNON, 2004). Além disso, apenas um entre 5000 compostos testados consegue chegar à triagem clínica e ser aprovado para aplicação terapêutica (BALUNAS; KINGHORN, 2005).

Mesmo havendo grandes investimentos, a resistência a fármacos continua sendo um grave problema de saúde pública. Produtos naturais têm prevalecido como a maior fonte de novas drogas e muitos são isolados de sistemas simbióticos microrganismos-plantas. (RAMOS et al., 2010).

Dentre os microrganismos, em especial os fungos, são conhecidos pela sua capacidade de produzir uma grande diversidade de moléculas bioativas. Por sua vez, estas substâncias

podem ser altamente tóxicas, como as micotoxinas ou serem bastante úteis por poderem ser utilizadas como fármacos de uso em várias patologias. Esta dicotomia de funções ocorre em consequência da grande diversidade de compostos químicos que os fungos produzem (PINTO et al., 2002).

Dos vinte medicamentos mais vendidos no ano de 1994, o que representou um mercado de aproximadamente 6,7 bilhões de dólares, seis deles foram obtidos *in natura* ou por transformação química de metabólitos provenientes de fungos. Dentre os medicamentos de maior repercussão terapêutica para doenças infecciosas destacam-se os antibióticos penicilinas e cefalosporinas como os exemplos mais conhecidos de produtos de fungos (SINGH; BARRETT, 2006).

A produção de metabólitos secundários por fungos é um processo complexo, estes produtos secundários pertencem a diversos grupos estruturais, como esteroides, xantonas, compostos fenólicos, isocumarinas, quinonas, terpenos, citocalasanas, alcaloides e diversos outros grupos estruturais (RAMOS, 2008).

2.7. Metabólitos secundários com atividade antimicrobiana isolados de fungos endofíticos

O estudo dos microrganismos endofíticos tem aumentado substancialmente na última década, pois foi observado que essa comunidade apresenta um importante papel no desenvolvimento da planta hospedeira. Algumas espécies de microrganismos endofíticos são produtoras de fármacos, por exemplo antitumorais e antibióticos, e outras produzem fatores de crescimento do vegetal, toxinas e enzimas (STIERLE; STROBEL; STIERLE, 1993; HELLWING et al., 2002; SANTOS; RODRIGUEZ, 2003; EZRA et al., 2004; BERDY, 2005; REDKO et al., 2006; CHANDRA PAUL et al., 2007; BORGES, 2008; RALPHS; CREAMER; BAUCOM, 2008; KUSARI et al., 2008; FAVARO, 2009; GU, 2009; KJER et al., 2009).

Os fungos endofíticos se destacam como grandes produtores de metabólitos secundários (GUNATILAKA, 2006), sendo esses metabólitos pertencentes as mais variadas classes como alcaloides, terpenoides, esteroides, quinonas, isocoumarinas, lignanas, fenilpropanoides, fenois e lactonas (YU et al., 2010; ZHOU et al., 2010), e descritos como antimicrobianos, antinseticida, anticancerígenos, antidiabeticos e compostos imunosupressores (KHARWAR et al., 2011; ZHAO et al., 2011). Além disso, esses microrganismos se apresentam fonte importante na pesquisa dos metabólitos quando

comparados com fungos obtidos de outros substratos, pois muitos metabólitos têm sido isolados de fungos endofíticos, além do que, 51% das substâncias já estudadas se tratam de compostos inéditos, enquanto que da microbiota do solo apenas 38% eram novas. A procura por novos habitats e a diversidade fúngica são fatores importantes na busca por produtos naturais e, consequentemente, no desenvolvimento de novas drogas (SCHULZ et al., 2002).

Diversos compostos obtidos a partir do cultivo de fungos endofíticos apresentam atividade antimicrobiana, conforme apresentado na Tabela 1, onde alguns exemplos de metabólitos secundários com atividade antimicrobiana isolados de fungos endofíticos são destacados.

Tabela 1 - Metabólitos secundários com atividade antimicrobiana isolados de fungos endofíticos

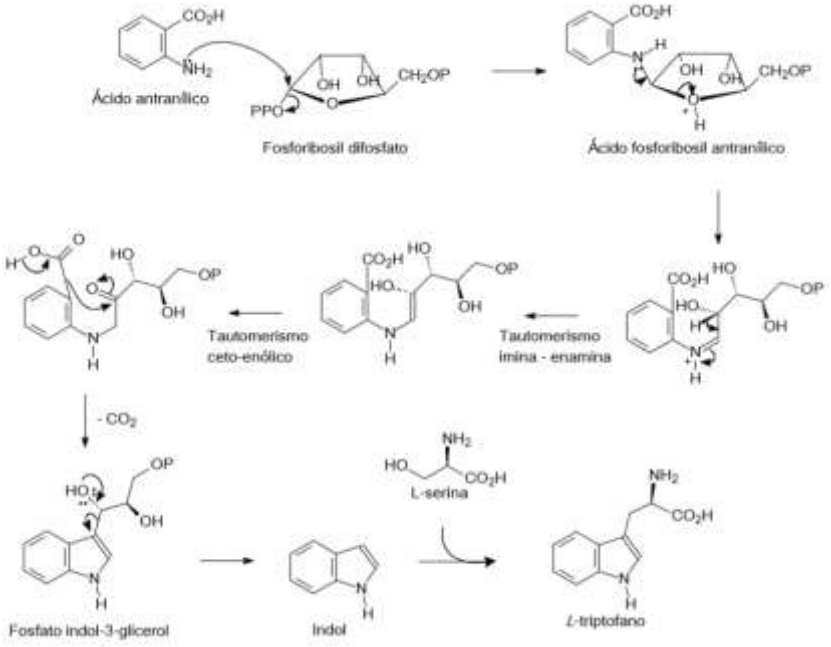
Fungo endofítico / Planta hospedeira	**Metabólito secundário**	**Atividade Biológica**
Colletotrichum gloeosporioides endofítico de *Cryptocarya mandioccana*	(-)-cis-4-hidroxi-6-eoxiscitalona (INÁCIO et al., 2006).	Atividade antifúngica contra *Cladosporium cladosporioides* e *Cladosporium sphaerospermum*
Penicillium sp. endofítico de *Mauritia flexuosa*	Glandicolina B (FERREIRA KOOLEN et al., 2012)	Atividade antibacteriana contra *Staphylococcus aureus*, *Micrococcus luteus* e *Escherichia coli*
Aspergillus tamarii endofítico de *Ficus carica*	Fumitremorgina B (ZHANG et al., 2012)	Atividade antifúngica contra os fitopatógenos *Fusarium graminearum*, *Botrytis cinérea*, *Phytophthora capsici* e *P. oryzae*
Fungo CR115 não identificado endofítico de *Daphnopsis americana*	Guanacastepeno (HUGHES et al., 2003)	Atividade antibacteriana contra *Staphylococcus aureus* e *Enterococcus faecium*
Edenia gomezpompae endofítico de *Callicarpa acuminata*	Preussomerina EG_1 (MACÍAS-RUBALCAVA et al., 2008)	Atividade antifúngica contra *Phythophtora capsici*, *Phythophtora parasitica*, *Fusarium oxysporum* e *Alternaria solani*
Chaetomium globosum endofítico de *Ginkgo biloba*	Chaetomugilina D (QIN et al., 2009)	Atividade antifúngica contra *Mucor miehei*
Pestalotiopsis fici endofítico de uma planta não identificada coletada em Hangzhou, República Popular da China	Pestalofona C (LIU et al., 2009)	Atividade antifúngica contra *Aspergillus fumigatus*, *Candida albicans* e *Geotrichum candidum*
Morinia pestalozzioides endofítico de *Santolina rosmarinifolia*	Moriniafungina (BASILIO et al., 2006)	Atividade antifúngica contra *Candida glabrata*, *Candida krusei*, *Candida lusitaniae* e *Crytpococcus neoformans*
Phomopsis sp. endofítico de *Costus sp.*	Fomoxantona A (ELSÄSSER et al., 2005)	Atividade antifúngica contra *Phythophtora infestans*, *Botrytis cinérea*, *Pyricularia oryzae* e *Ustilago violacea*

Cabe destacar que uma das grandes vantagens que representa o conhecimento sobre metabólitos secundários isolados de fungos endofíticos e descritos com atividade antimicrobiana, é evitar a necessidade de cultivar e coletar espécies vegetais silvestres, assim como reduzir o custo da produção dos princípios ativos (DEBBAB et al., 2009). Da mesma forma, se tem proposto que estes metabólitos poderiam ser utilizados no desenvolvimento de agroquímicos de origem natural, os quais seriam menos prejudiciais para o homem e para o meio ambiente quando comparados aos compostos químicos sintéticos que atualmente se utiliza na agricultura, já que os produtos naturais podem interagir com as plantas de forma específica, afetar processos fisiológicos específicos e apresentar menores índices de bioacumulação (STROBEL et al., 2004).

2.8. Considerações sobre o ácido indolico-3-carboxilico

Alcalóides indólicos são os alcalóides derivados do aminoácido L-triptofano, que é originado a partir da rota do chiquimato via ácido antranílico (Figura 6) e apresentam um amplo espectro de atividades farmacológicas, tais como analgésica, antiinflamatória, bactericida, estrogênica, estimulante e depressora do sistema nervoso central, dentre outras (FUMAGALI et al., 2008).

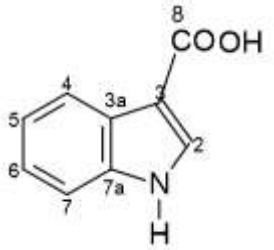
Figura 6 - Biossíntese do L-triptofano



Fonte: DEWICK, 2002.

Dentre essa classe de compostos, encontra-se o ácido indolico-3-carboxilico (Figura 7), o qual já foi encontrado em extratos de plantas, tais como de raízes do arroz (RIMANDO et al., 2001), paredes celulares de folhas e raízes da *Arabidopsis thaliana* (TAN et al., 2004) e folhas de *Bauhinia tarapotensis* (BRACA et al., 2001).

Figura 7 - Estrutura química do ácido indol-3-carboxilico



Este ácido também já foi isolado a partir de cultura fermentada de um fungo endofítico, EM-22, derivado da alga vermelha marinha Polysiphonia urceolata (ZHAO et al., 2009), e a partir da fermentação dos fungos *Lasiodiplodia theobromae* (ALDRIDGE et al. 1971) e *Aporpium caryae* (LEVY et al., 2000).

Foi observado que sua concentração nas folhas é aumentada pela infecção destas com *Pseudomonas syringae* (HAGEMEIRER et al., 2001) e, nas raízes, pela contaminação do fungo *Pythium silvaticum* (TAN et al., 2004). As atividades biológicas descritas para o ácido indolico-3-carboxilico são a antiinflamatória e anti-pirética, sendo utilizado no tratamento da artrite e desordens dermatológicas (SHEN, 1967), atividade contra replicação de HIV (WU et al., 2004), inibindo a proliferação de células HL-60 (NISHIO et al., 2012), atividade contra os fungos fitopatogênicos *Cladosporium cucumerinum* (LEVY et al., 2000) e *Alternaria brassicicola* (PEDRS; ABDOLI, 2013), e contra p petogeno humano *Candida sp*. (KAVITHA et al., 2010).

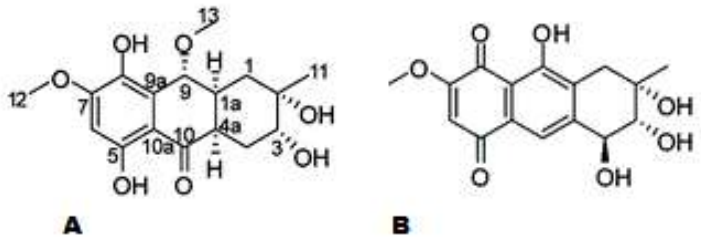
Além disso, este alcalóide é matéria prima para a preparação de inúmeros derivados apresentando atividade biológica, tais como ésteres ativos contra vírus da hepatite e influenza A (VASIL'EVICH et al., 2009), alcalóides marinhos inibidores da topoisomerase II (POUILHES et al., 2003) e derivados piridínio-ilamidas, que apresentaram alta afinidade e seletividade para o receptor 5-HT2C (receptores serotonínicos) e D2-D4 (receptores dopamínicos) (PARK et al., 2008).

2.9. O gênero *Nigrospora* como uma fonte de metabolitos secundários bioativos

Atualmente, os fungos têm sido apontados como fontes promissoras na descoberta de novos compostos bioativos com grande potencial para a exploração numa ampla variedade de áreas (TENGURIA et al., 2011). Entre esses fungos, os pertencentes ao gênero *Nigrospora*, atualmente sua fase sexual (teleomorfo) está incluída no gênero *Khuskia*, têm sido descritos como uma fonte rica de metabolitos secundários bioativos, tais como nigrosporolides que mostrou capacidade de inibir o crescimento de plantas parasitas, e fomalactonas com efeito antifúngico (KIM et al., 2001), lactonas fitotóxicas (FUKUSHIMA et al., 1998), além de nigrosporinas, epoxydonas e pironas com atividade antibacteriana (TANAKA et al., 1997; TRISUWAN et al., 2008).

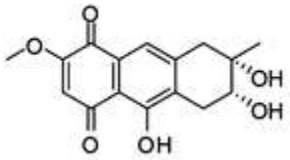
Fungos desse gênero têm sido isolados das mais diversas fontes e a partir desses novas moléculas bioativas têm sido isoladas. Como exemplo podemos citar a investigação química do extrato obtido a partir do fungo *Nigrospora sp*. derivado de uma anêmona do mar, que levou à descoberta de dois novos derivados de hidro antraquinonas bioativos, 4a-*epi*-9α-methoxydihydrodeoxybostrycina e 10-deoxybostrycina (Figura 8), além de sete análogos conhecidos de antraquinona, como a Nigrosporina B (Figura 9) que, juntamente com o 10-deoxybostricina, apresentou forte atividade citotóxica contra células A549, e mostrou considerada atividade antibacteriana contra *Bacillus subtilis* e *B. cereus*, com valores iguais ou mais fortes do que o do antibiótico Ciprofloxacina®, utilizado como controle positivo. (YANG et al., 2012).

Figura 8 - A - 4a-*epi*-9α-methoxydihydrodeoxybostrycina e B - 10-deoxybostrycina



Fonte: YANG et al., 2012

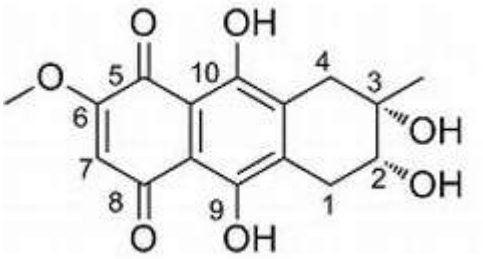
Figura 9 - Nigrosporina B



Fonte: YANG et al., 2012

O gênero *Nigrospora* também têm se sido isolado como endofítico e se destacado como produtor de metabólitos conhecidos por apresentarem atividades biológicas. Um bom exemplo disso é o composto Deoxybostricina (Figura 10), o qual foi obtido por Xia et al. (2011) a partir do fungo endófito *Nigrospora* sp. isolado de mangue coletado do Mar do Sul da China, o qual é relatado na literatura científica por apresentar diversas propriedades biológicas como fitotoxicidade (CHARUDATTAN et al., 1982), atividades citotóxicas (GE et al., 2005), atividade contra malária (SOMMART et al., 2008) e atividade antibacteriana (XIA et al., 2011).

Figura 10 - Deoxybostricina



Fonte: XIA et al., 2011

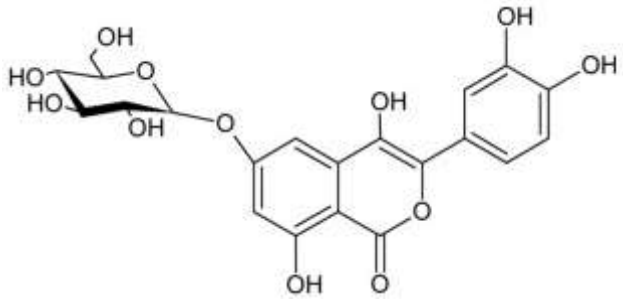
Além da Deoxybostricina , Quatro metabolitos secundários foram isolados a partir de culturas líquidas do fungo endófito *Nigrospora* sp. isolado a partir da raiz de *Moringa oleifera* coletado em Xiamen, província de Fujian, República Popular da China. Suas estruturas químicas foram determinadas e avaliadas quanto a presença de atividade antifúngica. Entre eles, griseofulvina exibiu uma atividade forte contra *Botrytis cinerea* e *Colletotrichum orbiculare* e uma atividade moderada contra outros fungos de teste. (ZHAO et al., 2012).

Dentre o gênero *Nigrospora*, a espécie *Nigrospora sphaerica* [*Khuskia oryzae*] tem sido relatada como endofítico de várias plantas e organismos marinhos (ZHANG et al., 2009). *N. sphaerica* tem sido bastante investigado por ser descrito como uma fonte de metabolitos

secundários biologicamente ativos de diferentes classes, incluindo os diterpenos (TURNER; ALDRIDGE, 1983), dicetopiperazinas (CUTLER et al., 1991), lactonas (KIM et al., 2001), nigrosporolides (ZHANG et al., 2009) e esteroides (METWALY et al., 2014).

Também foi realizada investigação química e biológica do fungo endófito *N. sphaerica*, que conduziu ao isolamento da Nigrosphaerina A (Figura 11), um novo isochromeno derivado, juntamente com outros dezenove compostos conhecidos. Este composto apresentou atividade leishmanicida, boa atividade antileucêmica contra células HL60 (aguda), e atividade antifúngica moderada contra *Cryptococcus neoformans* (METWALY et. al., 2014).

Figura 11 - Nigrosphaerina A



Fonte: METWALY et. al., 2014

Dessa forma podemos evidenciar que o gênero *Nigrospora* se destaca como fonte promissora para investigação obtenção de compostos bioativos, os quais podem ser utilizados como agentes agroquímicos naturais e como fármacos úteis para humanidade.

2.10. Infecções causadas por microrganismos patogênicos

O sucesso dos primeiros antibióticos na cura de doenças até então consideradas letais acarretou uma intensa busca por novas drogas. De fato, as décadas de 40, 50 e 60 do século passado foram marcadas pela imensa quantidade de antibióticos produzidos e rapidamente incorporados às práticas clínicas (BUTLER; BUSS, 2006). No entanto, nas décadas posteriores houve um aumento significativo das infecções causadas por microrganismos. De acordo com Luzhetskyy e colaboradores (2007), as doenças infecciosas estão em segundo lugar como causa de morte em todo mundo. Infecções bacterianas causam 17 milhões de mortes globais, sendo as crianças e os idosos os mais atingidos (BUTLER; BUSS, 2006).

O aumento do número de infecções bacterianas e fúngicas se devem a vários fatores como, por exemplo, a resistência dos microrganismos às drogas, muitas vezes devido ao uso

extensivo ou inapropriado; más condições de higiene; fluxo contínuo de viajantes; aumento de pacientes imunocomprometidos com doenças como a AIDS, doença aguda do sistema respiratório e doenças autoimunes; demora no diagnóstico das infecções (VON NUSSBAUM et al., 2006); tratamentos prolongados como a hemodiálise e procedimentos invasivos (uso de cateteres) são fatores intrínsecos para o surgimento de resistência dos microrganismos (BUTLER; BUSS, 2006).

Aproveitando este contexto, é possível citar alguns microrganismos causadores de infecções em humanos. Neste trabalho serão abordadas seis bactérias de importância clínica, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* e *Staphylococcus aureus*, além das leveduras de importância médica *Candida albicans*, *C. krusei*, *C. tropicalis*, *C. guilliermondii*, *C. parapsilosis* e *C. haemulonii*.

Bacillus subtilis - são bacilos Gram positivos de solo, não colonizador de tecidos, naturalmente transformável (a capacidade de capturar DNA exógeno faz parte do seu ciclo de vida), formadores de esporos e não patogênicos, porém, considerados modelo de estudo de bactérias Gram positivas (PACCEZ, 2007) como, por exemplo, o *B. cereus* considerado similar ao *B. subtilis* (SOUZA, et al., 2004) e produtor de duas toxinas: emética e diarréica (FORSYTHE, 2002).

Escherichia coli - classificam-se como bastonetes Gram negativos, anaeróbios facultativos, comumente denominados de entéricos, pertencentes à família Enterobacteriacea. O nome se refere o fato da bactéria ser encontrada no trato intestinal de humanos e outros animais (TORTORA et al., 2005). Todas as linhagens patogênicas possuem fímbrias especializadas que permitem a ligação da bactéria às células do epitélio intestinal. Três síndromes clínicas resultam da infecção causada por *E. coli* patogênica: infecções do trato urinário, septicemias seguidas de meningites e gastrenterites (TRABULSI; ORDONEZ, 2004). Essa bactéria é o principal coliforme fecal encontrado em águas contaminadas, sendo responsável por 75% dos casos de pielonefrite (SANTIAGO et al., 2005).

Klebsiella pneumoniae - é um bacilo Gram negativo, anaeróbio facultativo, pertencente à família Enterobacteriacea, está presente em indivíduos saudáveis, colonizando principalmente o trato intestinal e nasofaringe (PODSCHUN; ULLMANN, 1998). No entanto, pode atuar como patógeno de grande importância nas infecções hospitalares em todo o mundo (SADER et al., 2002). *K. pneumoniae* pode causar infecções comunitárias, como pneumonia ou infecção do trato urinário (PODSCHUN; ULLMANN, 1998).

Pseudomonas aeruginosa - é um bacilo Gram negativo, não fermentador e taxonomicamente encontra-se na família Pseudomonadaceae. É um dos microrganismos mais

ubiquitários, pois é encontrado no solo, água, vegetais, animais e nos mais diversos ambientes hospitalares (FRANCO; LANDGRAF, 2003). No Brasil, de acordo com um estudo sobre resistência aos antimicrobianos, *P.aeruginosa* foi a causa mais frequente de infecções do trato respiratório, a segunda causa mais frequente de infecções urinárias e infecções de feridas cirúrgicas e o sexto agente associado a infecções sistêmicas (SADER et al., 2001).

Staphylococcus aureus - ocorrem em grupos que se assemelham a cachos de uva. São anaeróbicos facultativos e crescem bem sob alta pressão osmótica e pouca umidade (TORTORA et al., 2005). São responsáveis por doenças com baixa morbidade e mortalidade, como por exemplo, foliculite e intoxicação alimentar, mas também por doenças severas e fatais como a endocardite e a síndrome do choque tóxico (KUEHNERT et al., 2006). O aumento da resistência deste microrganismo às drogas e o aparecimento de estafilococos meticilina-resistente, tem se tornado um grave problema nos centros de saúde. Apesar de não ser causa de doenças negligenciadas, doenças causadas por *S. aureus* são graves que afetam toda população mundial (CAMPOS, 2009).

***Candida* spp.** - as leveduras do gênero *Candida* são aeróbicas ou anaeróbicas facultativas, pertencentes à família Saccharomycetaceae. são patógenos oportunistas frequentemente isolados das superfícies mucosas de indivíduos normais (MORAGUES et al., 2003), entretanto, o delicado balanço entre o hospedeiro e esse fungo comensal pode-se transformar em uma relação parasitária, com o desenvolvimento de infecções denominadas candidíases (LAJEAN et al., 1998). Espécies de *Candida* são responsáveis por causar infecções oportunistas em pacientes imunocomprometidos, desde infecções superficiais até infecções sistêmicas (SHAO et al., 2007). A maioria destas infecções é causada pela levedura *C. albicans*, no entanto, outras espécies do gênero *Candida*, incluindo *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. dubliniensis*, e *C. parapsilosis* tem sido cada vez mais responsáveis por causar infecções generalizadas (PFALLER; DIEKEMA, 2007, ANDES et al., 2012), e são responsáveis por quase 50% das infecções sistêmicas ocasionadas pelo gênero *Candida* (SOBEL, 2006).

Além dessas, *C. guilliermondii* é responsável por de 1-3% de todos os casos de candidemia, e a maioria desses casos estão associados a pacientes de oncologia (GIRMENIA et al., 2006; PFALLER et al., 2006). Além disso, *C. guilliermondii* tem demonstrado ser mais resistente a agentes antifúngicos do que outras espécies de *Candida* (ARENDRUP, 2010; PFALLER et al., 2006; BEYDA et al., 2012) e também pode causar osteomielite, artrite séptica, endocardite e lesões na pele (GIRMENIA et al., 2006, SAVINI et al., 2010).

C. haemulonii frequentemente não causam infecções humanas (GARGEYA et al., 1991), mas mostra que é resistente à anfotericina B e outros agentes anti-fúngicos tais como azoles (GARCÍA-MARTOS et al., 2001), e têm sido frequentemente associadas com falha de tratamentos clínicos (KHAN et al., 2007, RUAN et al., 2010).

Esses microrganismos estão cada vez mais resistentes aos antifúngicos disponíveis no mercado (BARD et al., 2005), pois as classes de antifúngicos apresentam baixa eficácia e frequentemente levam à resistência (ANDERSON, 2005).

3. OBJETIVOS

3.1. Objetivo Geral

Identificar os fungos endofíticos de *I. suffruticosa*, avaliar o potencial antimicrobiano dos fungos isolados e dos metabólitos produzidos pelo fungo endofítico *N. sphaerica*.

3.2. Objetivos Específicos

- ✓ Isolar fungos endofíticos de folhas saudáveis de *I. suffruticosa* coletadas na Mata Atlântica e na Caatinga, nos períodos seco e chuvoso;
- ✓ Purificar os fungos isolados;
- ✓ Identificar as espécies de fungos isolados;
- ✓ Avaliar a atividade antimicrobiana dos metabólitos totais produzidos por fungos endofíticos de *I. suffruticosa*;
- ✓ Purificar e identificar metabólito(s) secundário(s) produzido(s) pelo fungo endofítico *N. sphaerica*;
- ✓ Avaliar a atividade antimicrobiana do(s) metabólito(s) isolado(s).

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5. PRIMEIRO CAPÍTULO

5.1. Endophytic mycobiota from leaves of *Indigofera suffruticosa* Miller (Fabaceae): the relationship between seasonal change in Atlantic Coastal Forest and tropical dry forest (Caatinga), Brazil.

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O objetivo deste trabalho foi isolar e caracterizar taxonomicamente fungos endofíticos da planta medicinal *Indigofera suffruticosa*, coletada na Mata Atlântica e na Caatinga, durante os períodos seco e chuvoso, em Pernambuco, Brasil. Um total de 107 microrganismos foram isolados, a partir de 216 fragmentos de folhas, e identificados de acordo com as características morfológicas e chaves taxonómicos. Todos os isolados fúngicos pertenciam ao filo Ascomycota, e os grupos identificados foram Pleosporales, Sordariomycetidae, Xylariales, Diaporthales, Leotiomycetes e Bryosphaeriales. Entre os nove táxons identificados, *Colletotrichum gloeosporioides* com freqüência relativa (fr) 27,1%, *Pseudocochliobolus pallescens* com fr 16,82%, *Khuskia oryzae* com fr 14,95%, e *Pestalotiopsis maculans* com fr 14,02% foram os mais frequentes. As espécies *Curvularia australiensis* e *Chaetomella raphigera* foram isoladas apenas na Caatinga durante períodos seco e chuvoso, respectivamente; estas espécies, são relatadas pela primeira vez como isoladas a partir de uma planta na Caatinga. *Lasiodiplodia theobromae* foi encontrada apenas em Mata Atlântica no período seco, e de acordo com Simpson (D ') e Shannon-Wiener (H') índices de diversidade de fungos foram considerados mais significantes nesta floresta. Além disso, foi observada uma maior semelhança entre fungos isolados na Mata Atlântica e Caatinga na estação seca, sugerindo a predominância de sazonalidade, em vez de fator geográfico. Este estudo é o primeiro relato sobre fungos endofíticos de *I. suffruticosa*, e os resultados representam uma base importante para futuros estudos nos campos da ecologia e da biotecnologia, uma vez que estes fungos endofíticos podem ser fontes importantes para futuros estudos na busca de novos compostos naturais com atividades biológicas.

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Endophytic mycobiota from leaves of *Indigofera suffruticosa* Miller (Fabaceae): the relationship between seasonal change in Atlantic Coastal Forest and tropical dry forest (Caatinga), Brazil.

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ABSTRACT

Endophytic fungi were isolated from leaves of the medicinal plant *Indigofera suffruticosa* collected at the Atlantic Coastal Forest and tropical dry forest (Caatinga), Pernambuco, Brazil, during the dry and rainy seasons. A total of 107 fungal isolates, representing 9 fungal taxa, were obtained and classified as Ascomycota, among them *Colletotrichum gloeosporioides* with relative frequency (fr) 27.1% and *Pseudocochliobolus pallescens* with fr 16.82% were the most frequent. *Curvularia australiensis* and *Chaetomella raphigera* were isolated only in Caatinga during rainy and dry seasons, respectively, and for the first time they were isolated from a Caatinga plant. *Lasiodiplodia theobromae* was found only in Atlantic Coastal Forest in dry season, and according to Simpson (*D'*) and Shannon-Wiener (*H'*) indices fungal diversity were considered statistically significantin this forest. Besides, a greater similarity was observed between fungi isolated in Atlantic Coastal Forest and Caatinga in the dry season, suggesting the predominance of seasonality rather than geographical factor. This study is the first report on endophytes fungi from *I. suffruticosa*, and the results represent an important basis for further studies in the fields of ecology and biotechnology, since these endophytic fungi may be important source for future study in searching for new natural compounds with biological activities.

Keywords: Ascomycota; Caatinga; endophytic mycobiota; fungal diversity; *Indigofera suffruticosa*; seasonal predominance.

INTRODUCTION

Endophytic fungi are microorganisms that, during part or all of their life cycle, colonize inter and/or intracellularly healthy plant tissues, in an asymptomatic manner, without causing any apparent damage to their host (Tan and Zou, 2001), many of which are able to produce secondary metabolites that may offer protection against different phytopathogens and herbivores (Rivera-Orduña et al., 2011). The endophytic fungi represent a wide diversity of microbial adaptations that have developed in special and unusual environments, making them a great source of study and research for new chemicals for medicinal, industrial and agricultural uses (Aly et al., 2011; Kusari and Spiteller, 2011; Rajulu et al., 2011; Li et al., 2012; Kusari et al., 2013; Lou et al., 2013; Pharamat et al., 2013; Teiten et al., 2013). Furthermore, the production by the endophytic fungi of a variety of secondary compounds, such as alkaloids, terpenoids, steroids and aromatic compounds that are repellent or toxic to their enemies, gives greater competitive ability to colonized plants due to this symbiosis (Redman et al., 2002; Arnold et al., 2003; Rodriguez and Redman, 2008; Porras-Alfaro and Bayman, 2011).

Clarke et al. (2006) demonstrated that plants colonized by endophytes have greater resistance when compared to non-colonized plants, and that endophytic fungi may be used to suppress plant diseases in various environments. Although this resistance mechanism is not fully understood, some studies have shown that climate variation and cultivation conditions influence vegetable matabolism (Simões-Ambrosio et al., 2010; Zalamea et al., 2013) and that seasonal variation and geographical location affect endophytic colonization (Martín et al., 2004; Göre and Bucak, 2007; Guo et al., 2008). Therefore, studies on endophytic fungi are of great importance for providing essential information for assessing fungal diversity under the influence of geographical and seasonal factors.

Few studies have been conducted with the communities of endophytic fungi in leaves of plants of the same species growing in different locations with distinct ecological characteristics (Collado et al., 1999; Martín et al., 2004; Göre and Bucak, 2007; Vaz et al., 2014). However, the composition of the endophytic mycobiota from different locations is very important for the understanding of the relationship between endophytic fungal populations and the establishment of plants subjected to distinct selection pressures in different ecosystems, mainly considering that endophytes may also increase the resistance of plants against biotic and abiotic stresses and produce compounds of biotechnological interest (Azevedo, 2014).

Indigofera suffruticosa Mill. is a shrub 1-2 meters high originally from the Antilles and Central America, which was introduced and cultivated in Brazil on a large scale for the extraction of natural indigo dye for the textile industry, but in the 1980s this natural dye was replaced by an artificially produced pigment

(Alzugaray and Alzugaray, 1988). *I. suffruticosa* is found in different biomes of Brazil, among these tropical dry forests (Caatinga) and Atlantic Coastal Forest. This plant is used in the folk medicine as a febrifuge, purgative, sedative, (Almeida, 1993; Hasting, 1990), and to treat epilepsy, infection and inflammation such as gastrointestinal pain (Roig, 1988; Matos, 1999; Agra 2007). Previous studies have shown that the leaves of *I. suffruticosa* have embryotoxic effects (Leite et al., 2004), antimicrobial (Leite et al., 2006; Bezerra dos Santos et al., 2015), and anticonvulsant (Almeida et al., 2013) activities, and act as gastroprotective agent (Luiz-Ferreira et al., 2011).

Almost half of the world's tropical forests are represented by tropical dry forests. In Brazil the tropical dry forests is named Caatinga due to the predominant type of vegetation (Albuquerque et al., 2012), and climate marked by high temperatures with sparse and irregularly distributed rains, with annual average precipitation ranging between 250 and 500 mm (Basso et al., 2005). The Caatinga soils are of different origins, and as a rule, are chemically fertile, well drained, and oxygenated. Water bodies are rarely permanent, drying completely during the summer (Basso et al.,2005), but has faced intensive degradation from exhaustion due to deliberate introductions of exotic plants for giving support to farming activities (Leal et al., 2005). The rapid reduction of forests in tropical areas of Brazil is a major problem since this situation could result in the extinction of many endophytic fungi with the loss of potentially important products for use in agricultural, pharmaceutical, environmental, and other fields of interest (Azevedo, 2014).

Another important biome of Brazil is the Atlantic Coastal Forest one of the most widely distributed tropical forests in Southern America, occupying almost all Brazilian Eastern coast besides inland areas and is marked by the occurrence of three important forest types (Oliveira-Filho and Fontes, 2000). That is characterized by a high biodiversity, and by proximity to the Atlantic Ocean, which provides a stable source of humidity, allowing high vegetation density. A floristic survey of the southern limit of Atlantic Forest area has been carried out, revealing several species of economic interest, many of which exhibit medicinal properties (Basso et al., 2005). Ribeiro et al. (2009) reported that due to the fragmentation process only 11.73% of the Atlantic Coastal Forest remains in Brazil, of which 12.1 % is in Pernambuco State, where the sugar-cane plantations is among one of the main factors responsible for the fragmentation (Lôbo et al. 2011).

The isolation and identification of endophytic mycobiota is necessary, since the medicinal properties of a plant may be due to the ability of their endophytic microorganisms to produce biologically active secondary metabolites of medical and industrial interest, e.g. the taxol, which an anticancer agent produced by *Taxus*

brevifolia Nutt., and by its endophyte fungus *Taxomyces andreanae* Strobel, A. Stierle, D. Stierle & W.M. Hess (Stierle et al., 1993; Bhardwaj and Agrawal, 2014).

In this context, the results of this research may contribute to future search on bioactive compounds derived from endophytic fungi species, since this is the first study on endophytic fungi colonization associated with *I. suffruticosa*. Thus, the objective of this study was to evaluate the relative geographical influence of two different locations and seasonality on the endophytic mycobiota associated with *I. suffruticosa*.

MATERIALS AND METHODS

Plant material and Study area

The collection of the plant material was made at natural growing in two areas studied in the rainy season (June) of 2009 and dry season (January) of 2010, at two different ecosystems. One collection site is located in the municipality of Caatinga (08°19'33"S, 36°04'21"W) in semiarid region of Pernambuco State, with average annual precipitation of 526.2 mm and a dry season that typically lasts nine months per year. The other site is in the municipality of Atlantic Coastal Forest (07°50'00"S, 34°54'30"W) in the coast of the Pernambuco State, with average annual precipitation of 1709.2 mm and a dry season that typically lasts three months per year (Rodal et al., 2008; APAC, 2013). Leaves of three health specimens of *I. suffruticosa* were randomly collected, in three different points of each areas studied and were put in plastic bags, transported to laboratory, processed on up to 48 h for isolation and characterization of endophytic fungi according to methodologies established previously (Araújo et al., 2002). The plant material was authenticated by the Biologist Marlene Barbosa from the Botanic Department, Universidade Federal de Pernambuco (UFPE). A voucher specimen number 45217 has been deposited at the UFP Geraldo Mariz Herbarium of UFPE.

Isolation of endophytic fungi

The plant material was subjected to a surface sterilization process in accordance with the methodology described by Araújo et al. (2002), where healthy leaves of *I. suffruticosa* were washed with running water, followed by immersion in 70% ethanol for one minute, sodium hypochlorite (2-2.5% active chlorine) for four minutes and washed three times in sterilized, distilled water. After surface sterilization, the samples were cut into fragments of 0.5 cm^2 and transferred aseptically to Petri dishes containing Potato Dextrose Agar (PDA) culture medium supplemented with chloramphenicol (50 mg L^{-1}) to suppress bacterial growth. The Petri dishes were

inoculated each with 6 leaf fragments from different points of each area studied, in triplicate, were incubated at room temperature (28 ± 2°C) for 30 days, analyzed daily and any fungal colony present was isolated, purified and kept in PDA medium for subsequent identification. The control of efficiency of the sterilization method was confirmed by seeding 1 mL of the last washing in Petri dishes containing PDA medium.

Identification of endophytic fungi

The morphological identification of endophytic fungi from *I. suffruticosa* was performed at the Micoteca URM, Department of Mycology, Federal University of Pernambuco, Recife, Brazil. The macro and micro morphological characteristics were observed based in technics and literature specific (Morton and Smith, 1963; Ellis, 1971,1976; Sutton, 1980; Barnett and Hunter, 1987; Hanlin, 2000). After morphological identification, the representative fungi cultures were preserved in the Culture Collection – Micoteca URM (WDCM604), Department of Mycology, Federal University of Pernambuco, Brazil.

Data analysis

The frequencies of isolation of endophytic fungi were calculated. The absolute frequency (f) was estimated as the total number of endophytes isolates, and the relative frequency (fr) was the number of endophytes of each species divided by the total number of endophytic fungi. The rate of colonization was estimated as the total number of fragments of leaves colonized by fungi, divided for total number of fragments used for isolation of endophytes, as reported by Larran et al. (2002).

The number of isolates obtained was used to calculate the components of diversity: richness (S), number of different species found at each site and in each periods of the year, and evenness (*J'*), the Simpson (*D'*) and Shannon-Wiener (*H'*) diversity indices, as described by Magurran (1988) and the similarity matrix was constructed from the Sørensen index, wich grouped using UPGMA as clustering algorithm.

$$\text{Shannon-Wiener index } (H') = -\sum_{i=1}^{s} pi \ln pi$$

$$\text{Simpson index } (D') = 1 / \sum_{i=1}^{s} pi^2$$

$$\text{evenness } (J') = H' / \ln N$$

In the Shannon index, **p** is the proportion (n/N) of fungi of one particular species found (n) divided by the total number of fungi found (N), **ln** is the natural log, **Σ** is the sum of the calculations, and **s** is the number of species.

In the Simpson index, **p** is the proportion (n/N) of individuals of one fungal species found (n) divided by the total number of fungi found (N), **Σ** is still the sum of the calculations, and **s** is the number of species.

The evenness was the ratio of observed diversity to maximum diversity (ln N), N is the total number of fungi found.

Data were analyzed using Bioestat v. 5.0 by one-way analysis of variance (ANOVA) and Tukey test to determine statistical significance. A *p*-value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

From 216 fragments analyzed, of which 54 were collected at each site and in different periods of the year, a total of 107 fungal isolates, representing 9 fungal taxa, were obtained from isolations. The isolates were identified as Ascomycota and belonging the groups Pleosporales, Sordariomycetidae, Xylariales, Diaporthales, Leotiomycetes and Bryosphaeriales. Among them, the *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. was the taxon most frequent (27,41%), followed by *Pseudocochliobolus pallescens* Tsuda & Ueyama (16.82%), *Khuskia oryzae* H.J. Hunds (14.95%) and *Pestalotiopsis maculans* (Corda) Nag Raj (14.02%). The isolates that did not develop reproductive structures in medium culture were grouped as sterile mycelia (Table 1).

The species *P. pallescens*, *Phomopsis archeri* B. Sutton and *Colletotrichum dematium* (Pers.) Grove were detected in both sites and in particular only during the rainy season. Also *Colletotrichum gloeosporioides* and *Khiskia oryzae* were detected in both sites, but not season association was observed. The species *P. maculans* presented locality specificity, since this species was isolated only in collections made in the Atlantic forest in both periods of the year (Table 1). Moreover, *Chaetomella raphigera* Swift, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. and *Curvularia australiensis* (Tsuda & Ueyama) Manamgoda, L. Cai & K.D. Hyde exhibit specificity regarding the locality and period of the year. Specifically, *C. australiensis* and *C. raphigera* were isolated only in Caatinga during the rainy season and in the dry season, respectively, whilst *L. theobromae* was found only in Atlantic forest during the dry season (Table 1).

Incidental species are frequently observed in studies with endophytic fungi and represent those which have been isolated in a small number, as described by Siqueira et al. (2011). Incidental species were observed in the present study and included *C. australiensis*, *C. raphigera* and *L. theobromae*. The genera *Curvularia* were also found as incidental species in *Lippia sidoides* Cham. (Siqueira et al., 2011), and in the study of Bezerra et al. (2013) from the analysis of the endophytic fungi isolated from cactus *Cereus jamacaru* DC. growing in Caatinga, Brazil. The species *L. theobromae* has been isolated as endophytes associated with *Piper hispidum* Sw. (Piperaceae) leaves (Orlandelli et al., 2012) and *C. raphigera* were also found as incidental species in

Catharanthus roseus (L.) G. Don and *Cassia tora* L., herbaceous medicinal plants from the Malnad region, southern India (Krishnamurthy et al., 2008). Investigation of fungi isolated from plants that live in extreme environments is of paramount importance, since some studies have raised the hypothesis that endophytic fungi can alter the levels of the plant hormones that control stomatal behavior and osmotic adjustment (Malinowski and Belesky, 2000; Mandyam and Jumpponen, 2005). Given that plants are exposed to environmental stress due to factors such as low water availability, high salinity, high irradiance and nutrient deprivation, the association with these microorganisms can be the determining factor in the adaptation of plants to environmental conditions adverse.

In our study, the properties of the Atlantic Coastal Forest and Caatinga ecosystems present differences in several aspects. In terms of altitude, the municipality of Atlantic Coastal Forest is approximately 19 m a.s.l. (above sea level) and Caatinga approximately 552 m a.s.l.. Comparing the type of soil, in Atlantic Coastal Forest there is a predominance of floodplain type soil, while in Caatinga clay types predominate. In terms of the vegetation around the site, the Caatinga is generally described as a woody vegetation with discontinuous canopy, variable in both height (3–9 m) and density, composed mostly of succulent (cacti essentially) and nonsucculent shrubs and trees, most of which are armed with either thorns or prickles and bear microphyllous foliages, though they are leafless during the long-lasting periods of drought; the ground layer is rich in bromeliads annual herbs, and geophytes (Ab'Saber, 1974; Prado, 2003; Cardoso and Queiroz, 2011). According to data shown in Table 1, it can be seen that the environmental conditions found in Atlantic Coastal Forest may have favored more endophytic colonization than those found in Caatinga, since the number of fungi isolated in Atlantic Coastal Forest in the two seasons studied, was higher than the number of isolates from Caatinga in the same periods. Differences related to the number of species and specimens of endophytic fungi, in studies conducted in different locations and periods of the year, can be explained by the fact that the species involved can vary according to the host, geographic distribution, host plant age, ecological and seasonal conditions, including altitude and precipitation, and differences in the vegetation around the local where the plant was collected (Arnold et al., 2003; Helander et al., 2007).

Our results are in agreement with those observed in other studies, where it is reported that the population of endophytic fungi is directly related to the difference in intensity and period of exposure of these organisms to solar radiation, which generally occurs in areas that have differences in climatic factors (Collado et al., 1999; Martínet al., 2004; Martín Pinto et al., 2006; Hashizume et al., 2010). In addition, other environmental

parameters such as soil, amount of available water, plant physiological condition at the time of collection and management, can also influence endophytic colonization (Hashizume et al., 2008).

The dominant endophytic fungi differed according to the collection sites and the period of the year (Table 1). In the municipality of Atlantic Coastal Forest it was observed that the dominant endophytes differed according to the period of the year in which the collections were made. The fungus *C. gloeosporioides* was the dominant species in Caatinga in both periods of the year, unlike Atlantic Coastal Forest where *P. pallescens* was the dominant species in the rainy season and sterile mycelia was dominant in the dry season.

These results are in accordance with other studies of endophytes where it has been reported that many species of fungi can be isolated from a certain plant, but only a few are frequently found. This was the case, for example, in the study by Xing et al. (2010), which investigated the distribution and diversity of endophytic fungi in different tissues and ages of American ginseng. Among the 27 taxa isolated, *Glomerella* sp. was the dominant fungus in most tissues. In addition, Siqueira et al. (2011) analysing the endophytic mycobiota of leaves and stems of *L. sidoides* observed that *C. gloeosporioides* was the most frequent species in the leaves, while *Alternaria alternata* (Fr.) Keissl. was most frequent in stems. In a recent study, Vaz et al. (2014) observed that *Pseudocercospora basintrucata* Crous and *Xylaria* sp. were the most frequent taxa isolated from the *Luma apiculata* (DC.) Burret in Andean Patagonian forest, while *Colletotrichum* sp. was the most frequent fungal species isolated from *Eugenia neomyrtifolia* M. Sobral in Atlantic rainforest, Brazil.

Studies in the semiarid region of the Brazilian northeast showed the endophytic associations of *Opuntia ficus-indica* (L.) Mill. and *Cereus jamacaru* with isolates of *K. oryzae* and species of the genera *Curvularia* (Bezerra et al 2012; 2013). The genera *Curvularia* was also isolated by Oliveira et. al. (2013) in a study on filamentous fungi diversity obtained from the soil of the semiarid area (Caatinga), Pernambuco, Brazil.

Although the species *C. australiensis* and *C. raphigera* of endophytic fungi have been reported in isolates from other plant species, our study is the first report of these fungi isolated from a plant of the semiarid region of Brazil. However, *C. australiensis* and *C. raphigera* were isolated as endophytic fungi in seed of *Withania somnifera* (L.) Dunal collected in a semiarid region of Pakistan (Khan et al., 2010). These species have also been isolated from leaves of *Ziziphus* sp. collected in the mountains of arid regions located at the South of the Arabian Gulf (El-Nagerabi et al., 2013). Nevertheless, in the semiarid region of Pernambuco, Brazil, the specie *C. australiensis* was found from soil (Oliveira et al., 2013), and the genera *Drechslera* (nowadays known as Curvularia) was also isolated as endophyte in seeds of Cowpea (*Vigna unguiculata* (L.) Walp.), a plant of the Fabaceae family (Rodrigues and Menezes, 2002).

In our work there was an occurrence of fungi that not possible to sporulate in culture after a certain incubation period and they were classified as sterile mycelia. This was not a surprise since sterile mycelia were prevalent in several studies with endophytes (Xing et al., 2010; Siqueira et al., 2011; Sun et al., 2012; Bezerra et al., 2013). We hypothesize that the difficulty that some fungi have to develop reproductive structures is probably related to the fact that artificial culture media do not offer the same set of conditions that these fungi encounter in their host plants. These fungi have been identified in some studies with the aid of molecular biology (Guo et al., 2003; Giordano et al., 2009; Lacap et al., 2003), and the species classified as sterile mycelia in this study. Will also be identified through these tools in future studies.

Table 2 shows the diversity indices of endophytic fungal species isolated at different study sites and season, which compose three groups in accordance to the component of diversity that express richness (S), evenness (J') and dominance (D). The results obtained indicate that the richness of fungal species at the different study sites was S=5 during the rainy season, but showed variations in the dry season with S=4 in the Atlantic Coastal Forest and S = 3 in the Caatinga. The diversity was analyzed by Shannon-Wiener (H') and Simpson (D') indices, and showed distinct variation of fungi, without repetition of the results at any of the study sites or period of the year. The highest diversity indices and evenness (J') was found in Atlantic Coastal Forest in the dry season. Based on the Shannon-Wiener (H') and Simpson (D') indices, is possible to see that there is a dominance of species during the rainy season compared to the dry season in Caatinga was significant, while during the dry season in relation to the rainy season in Atlantic Coastal Forest it was not significant. The differences in fungal endophytic assemblages observed in this study, indicate that variations in the study site and period of the year influence the species colonization and distribution in *I. suffruticosa* leaves, since the differences observed in the rainy and dry seasons in the different study sites were considered statistically significant. However, other factors may also influence endophytic colonization, such as the age of the plant collected, the differences in nutritional supply of host tissue and the differences in climatic conditions e.g. relative humidity and intensity of light exposure (Talley et al., 2002; Vieira et al., 2011).

Moreover, some studies have indicated that the endophytic diversity in dry areas is low due to enviromental factors, such as reduced rainfall and low vegetation density (Arnold et al., 2003; Suryanarayanan et al., 2002,2003,2005). For instance, Tejesvi et al. (2005) found only five species in a study with endophytic fungal assemblages from inner bark and twig of *Terminalia arjuna* (Roxb.) Wight & Arn. In a study on leaves and stems of plants from desert areas in China, Sun et al. (2012) found values for Shannon's index varying from 0.29 to 4.78, and for Simpson's index from 1.00 to 6.60. However, by contrast to our results, some studies have

reported high endophytic diversity in plants of Caatinga, Pernambuco. A total of 71 fungi species involving 23 genera was found within four hundred seeds of Cowpea (*Vigna unguiculata* (L.) Walp) collected in Caruaru and Serra Talhada counties (Rodrigues and Menezes, 2002). Bezerra et al. (2012) studing the cactus *Opuntia ficus-indica* (L.) Mill. obtained 44 endophytic fungi, belonging to 12 genera and 13 species. In another survey with cactus *C. jamacaru*, Bezerra et al. (2013) reported values for Shannon's index varying from 2.273 to 2.597, and for Simpson's index from 0.8127 to 0.9008.

According to the Dendrogram shown in Figure 1 it is possible to perceive that there is a greater similarity between fungi isolated in Atlantic Coastal Forest and Caatinga in the dry season when compared to the rainy season, suggesting that it may be occurring the predominance of seasonality rather than geographical factor.

The results presented in this work are the first study of endophytic fungi from leaves of *I. suffruticosa* growing in Atlantic Coastal Forest and Caatinga in Brazil. This study documents that the properties of the Atlantic Coastal Forest biome associated to increased rainfall seem to favor greater endophytic colonization of *I. suffruticosa*, in comparison to the Caatinga. In this study it is reported for the first time the isolation of endophytic fungi *Curvularia australiensis* and *Chaetomella raphigera* from a plant of the Brazilian Caatinga. Finally, the results indicate that there is a diversity of the endophytic fungi from *I. suffruticosa*, which are of ecological importance for plant growing in different areas studied, and also that these fungi may be important source for future study in searching for new natural compounds with potential antimicrobial.

Conflict of Interest

The authors declare that no conflict of interest exists.

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Table 1. Absolute (f) and relative (fr) frequency of endophytic fungi isolated from *Indigofera suffruticosa* at Atlantic Coastal Forest and Caatinga (tropical dry forest) in rainy and dry season (Brazil).

Endophytic fungi	Atlantic Coastal Forest				Caatinga				Total	
	Rainy		Dry		Rainy		Dry			
	f	fr	f	fr	f	fr	f	fr	f	fr
Colletotrichum gloeosporioides (Penz.) Penz. & Sacc.			8	23.55	6	37.5	15	57.7	29	27.10
Colletotrichum dematium (Pers.) Grove (URM-6063)	4	12.9			2	12.5			6	5.60
Pseudocochliobolus pallescens Tsuda & Ueyama (URM-6064)	14	45.17			4	25.0			18	16.82
Phomopsis archeri B. Sutton (URM-5630)	2	6.45			2	12.5			4	3.74
Curvularia australiensis (Tsuda & Ueyama) Manamgoda, L. Cai & K.D. Hyde					2	12.5			2	1.87
Pestalotiopsis maculans (Corda) Nag Raj (URM-6061)	9	29.03	6	17.64					15	14.02
Khuskia oryzae H.J. Huds. (URM-6060)	2	6.45	6	17.64			8	30.77	16	14.95
Chaetomella raphigera Swift							3	11.53	3	2.80
Lasiodiplodia theobromae (Pat.) Griffon & Maubl.			3	8.82					3	2.80
Sterile mycelia			11	32.35					11	10.30
Total of endophytic fungi	**31**	**100**	**34**	**100**	**16**	**100**	**26**	**100**	**107**	**100**

Table 2. Diversity, evenness and species richness of endophytic fungi isolated from *Indigofera suffruticosa* at Atlantic Coastal Forest and Caatinga (tropical dry forest) in rainy and dry season (Brazil).

Site of Collection	***H'***	***D'***	***J'***	**S**
Atlantic Coastal Forest				
Rainy season	1.334	3.215	0.388	5
Dry season	2.328	8.000	0.660	4
Caatinga				
Rainy season	1.490	4.048	0.537	5
Dry season	0.926	2.272	0.284	3

Shannon-Wiener (*H'*) and Simpson (*D'*) diversity indices, evenness (*J'*) and richness (S)

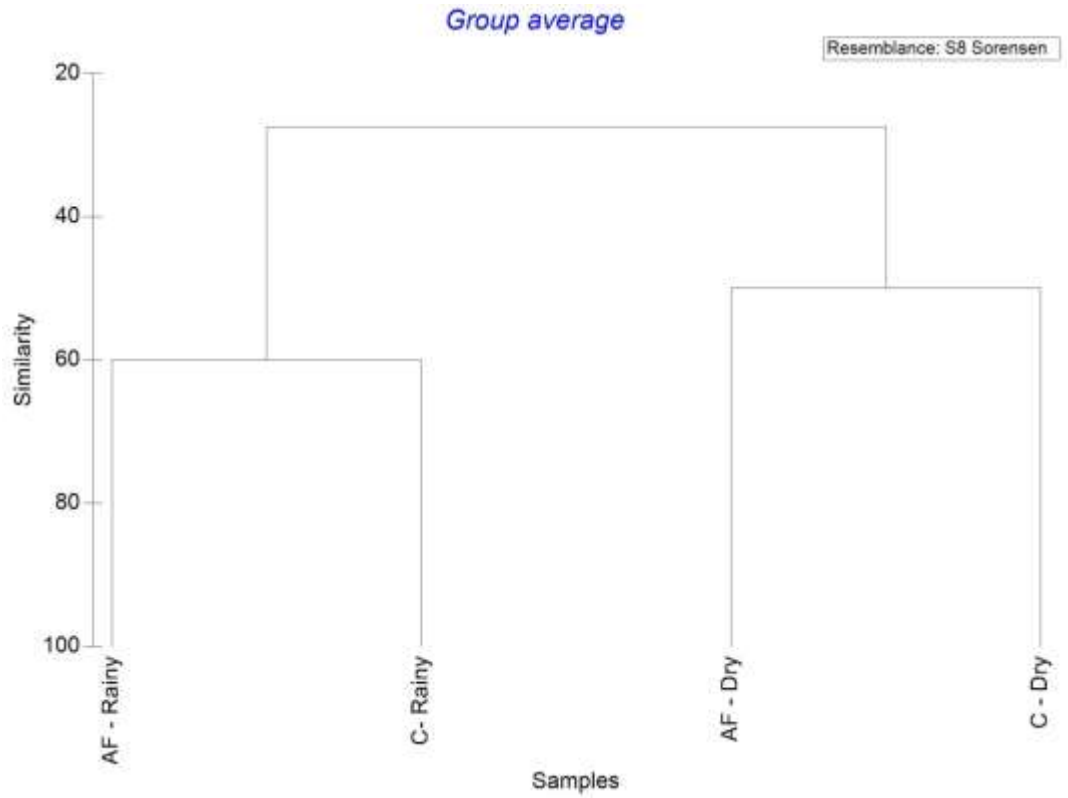

Figure 1. Dendogram showing the relationship of communities of endophytic fungi isolated from *Indigofera suffruticosa* at Atlantic Coastal Forest and tropical dry forest (Caatinga) in rainy and dry season (Brazil) from Sørensen similarity index. AF, Atlantic Forest; C, Caatinga.

6. SEGUNDO CAPÍTULO

6.1. Antimicrobial activity of endophytic fungi from leaves of *Indigofera suffruticosa* Mill (Fabaceae)

Artigo publicado no periódico Frotiers in Microbiology

Fungos endofíticos foram isolados a partir de folhas saudáveis de *Indigofera suffruticosa* Mill., Uma planta medicinal encontrada no Brasil, e utilizada na medicina popular para o tratamento de várias doenças. Entre os 65 fungos endofíticos isolados, 18 mostraram atividade contra pelo menos um microrganismo testado no *screening* preliminar, e os melhores resultados foram obtidos com a espécie *Nigrospora sphaerica* (URM-6060) e *Pestalotiopsis maculans* (URM-6061). No entanto, após fermentação em meio líquido (Caldo Batata Dextrose, de Sabouraud, Caldo Extrato Malte e Meio de Produção de Eurimicina), e em meios de cultura semi-sólido de arroz ou milho, apenas *N. sphaerica* apresentou atividade antibacteriana (em Caldo Batata Dextrose e no meio de cultura semi-sólido arroz), tal como indicado por análise de difusão em disco. Extrato metanólico de *N. sphaerica* (NsME) a partir do cultivo no meio de cultura semi-sólido arroz e extrato de acetato de etila de *N. sphaerica* (NsEAE) obtido a partir do sobrenadante da fermentação em Caldo Batata Dextrose exibiu antimicrobiana contra bactérias Gram-negativas e Gram-positivas. O melhor resultado foi observado contra *Staphylococcus aureus*, com MIC 1,56 mg/mL e MBC 6,25 mg/mL para NsME e MIC 0,39 mg/mL e MBC 3,12 mg/mL para NsEAE. Este estudo é o primeiro relato sobre a atividade antimicrobiana de fungos endofíticos isolados a partir de folhas de *I. suffruticosa*, no qual o fungo *N. sphaerica* demonstrou capacidade de produzir agentes bioativos com potencial farmacêutico, e pode ser utilizado como uma fonte promissora na busca de novas candidatos a fármacos.

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Antibacterial activity of endophytic fungi from leaves of *Indigofera suffruticosa* Miller (Fabaceae)

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Endophytic fungi were isolated from healthy leaves of *Indigofera suffruticosa* Miller, a medicinal plant found in Brazil which is used in folk medicine to treat various diseases. Among 65 endophytic fungi isolated, 18 fungi showed activity against at least one tested microorganism in preliminary screening, and the best results were obtained with *Nigrospora sphaerica* (URM-6060) and *Pestalotiopsis maculans* (URM-6061). After fermentation in liquid media and in semisolid media, only *N. sphaerica* demonstrated antibacterial activity (in Potato Dextrose Broth-PDB and in semisolid rice culture medium). In the next step, a methanolic extract from rice culture medium (NsME) and an ethyl acetate extract (NsEAE) from the supernatant of PDB were prepared and both exhibited antimicrobial activity against Gram-negative and Gram-positive bacteria. The best result was observed against *Staphylococcus aureus*, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 1.56 mg/mL and 6.25 mg/mL, respectively, for NsME and MIC and MBC values of 0.39 mg/mL and 3.12 mg/mL, respectively, for NsEAE. This study is the first report about the antimicrobial activity of endophytic fungi residing in *I. suffruticosa* leaves, in which the fungus *N. sphaerica* demonstrated the ability to produce bioactive agents with pharmaceutical potential, and may provide a new lead in the pursuit of new biological sources of drug candidates.

Keywords: antibacterial activity, endophytic microorganisms, minimum inhibitory concentration, *Nigrospora sphaerica*, *Staphylococcus aureus*

Introduction

The development of resistance by existing pathogenic bacteria and fungi to commercial drugs is a relevant problem faced by health services (Costelloe et al., 2010) and has become a serious concern around the world (Aksoy and Unal, 2008). Several factors have favored this scenario, such as extensive and often inappropriate use of antibiotics, poor hygienic conditions, continuous movement of travelers, increased numbers of immunocompromised patients, and delay in diagnosis of infections (von Nussbaum et al., 2006). As a result, an intensive search for new, effective antimicrobial agents is necessary, which is facilitated by exploring new niches and habitats (Xing et al., 2011; Zhao et al., 2011a).

A range of microbial species are known to be endophytic, colonizing inter- and intracellular spaces of plant tissues without causing apparent damage and appearing to be associated with all plants in natural ecosystems (Rodriguez et al., 2009). Among endophytic microorganisms, fungi have an intimate relationship with host plants and can produce compounds that promote vegetative growth, competitiveness and protection of the host against herbivores and pathogens (Porras-Alfaro and Bayman, 2011). Endophytic fungi represent a wide diversity of microbial adaptations that have evolved in special and unusual environments, making them a great source of study and research for new drugs for medical, industrial, and agriculture uses (Yu et al., 2010; Li et al., 2012; Teiten et al., 2013; Mapperson et al., 2014). These microorganisms are well known to produce bioactive secondary metabolites such as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols, and lactones (Radić and Štrukelj, 2012; Deshmukh et al., 2014).

Plants used in traditional medicine have played a very 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 (Kaul et al., 2012; Kusari et al., 2013). Despite this potential, a repertoire of medicinal plants remains to be studied regarding their endophytic composition, for example *Indigofera suffruticosa* Miller. This is a well-known Brazilian medicinal plant whose leaves have been proven to have anti-inflammatory, anticonvulsant antimicrobial, and wound-healing properties (Leite et al., 2004, 2006; Carli et al., 2010; Luiz-Ferreira et al., 2011; Almeida et al., 2013; Chen et al., 2013; Bezerra dos Santos et al., 2015). Due to the medicinal properties of *I. suffruticosa*, this species was the focus in the present study of a search for endophytic fungi able to produce bioactive substances with antimicrobial activity.

Materials and Methods

Plant Samples

The collection of plant samples was performed in Igarassu (07°50′00″S 34°54′30″W), Atlantic Coastal Forest, Pernambuco, Brazil. Samples were immediately processed in the Mycology Department, Federal University of Pernambuco (UFPE), Recife, Brazil.

Isolation of Endophytic Fungi

In order to eliminate epiphytic microorganisms, plant samples were superficially sterilized by the method described by Araújo et al. (2002). Healthy leaves of *I. suffruticosa* were washed in running tap water, followed by immersion in 70% ethanol for 1 min, in sodium hypochlorite (2.0–2.5% available chlorine) for 4 min, in 70% ethanol for 30 s and washed three times with sterilized, distilled water. The efficiency of sterilization was confirmed by inoculating water from the last washing into Petri dishes containing potato dextrose agar (PDA; containing potato (200 g/L), dextrose (20 g/L), and agar (15 g/L), pH 6.0). After surface sterilization, the samples were cut into 0.5 cm^2 pieces and aseptically transferred to Petri dishes containing PDA culture medium supplemented with chloramphenicol (100 μg/mL) to suppress bacterial growth. The Petri dishes were incubated at 30°C for 30 days and checked daily, and all fungal colonies found were isolated, purified and maintained in PDA for later testing.

Screening of Antimicrobial Acitivity

Tested Microorganisms

Among the microorganisms used for antimicrobial tests, five were fungi pathogenic to humans (*Candida albicans,* URM-5852; *Epidermophyton flocosum,* URM-5510; *Malassezia furfur,* URM-5389; *Microsporum gypsseum,* URM-5478; and *Trichophyton mentagrophytes,* URM-5589) URM Culture Collection (WDCM604) of the Federal University of Pernambuco, Recife, Brazil. The other five microorganisms were bacteria obtained from the Culture Collection UFPEDA, Department of Antibiotics, UFPE, Recife, Brazil, two of which were Gram-positive bacteria (*Staphylococcus aureus*, UFPEDA-02; and *Bacillus subtilis*, UFPEDA-16), and three were Gram-negative (*Escherichia coli*, UFPEDA-224; *Klebsiella pneumoniae*, UFPEDA-396; and *Pseudomonas aeruginosa*, UFPEDA-39).

Antimicrobial Assay

The endophytic fungi were subjected to an antimicrobial assay using a solid medium (Ichikawa et al., 1971) which permits a rapid and qualitative selection of the bioactive microorganisms. Each endophytic strain was cultivated on the surface of PDA in Petri dishes, at 30°C, for 7 days. Then, disks were cut from the PDA plate (6 mm diameter) and transferred to the surface of Petri dishes previously spread with bacteria (Müller-Hinton agar, MHA) and fungi [Sabouraud dextrose agar (SDA) and SDA supplemented with 0.5% olive oil for *M. furfur*]. The Petri dishes were incubated at 37°C during 24 h for bacterial growth, and at 30°C during 48 h for fungal growth. Antimicrobial activity was assayed by the measurement of any inhibition diameter zones (IDZ).

Identification of Endophytic Fungi

The identification of endophytic fungi was performed at the Mycology Department, UFPE, Recife, Pernambuco, Brazil, by means of analysis of macroscopic and microscopic characteristics of colonies. After identification, fungi that showed antimicrobial activity were stored in the URM Culture Collection (WDCM604) of the Federal University of Pernambuco, Recife, Brazil.

Fermentation Assay in Liquid Medium

Strains that showed the best results in the antimicrobial screening were further examined using a diffusion assay, in order to provide a way to select the best medium and incubation time for the production of the bioactive metabolites. Pre-inoculum were prepared in 250 mL Erlenmeyer flasks by adding five plugs (6 mm of diameter) of growing culture and 50 mL of PDB (potato: 200 g/L; dextrose: 20 g/L; pH 6.0). All cultures were incubated at 28 ± 2°C, on a rotary shaker, at 180 rpm. An aliquot (10 mL) of each pre-inoculum was transferred to 500 mL Erlenmeyer flasks containing 90 mL of the following media: PDB, Sabouraud broth (SAB), Malt Extract

Broth (MEB; malt extract: 20 g/L), or Eurimycin Production Medium (EPM; soy flour: 20 g/L; glucose: 20 g/L; $CaCO_3$: 2 g/L; and NaCl: 5 g/L). The flasks were incubated under the same conditions (28 ± 2°C, on a rotary shaker, at 180 rpm for 96 h). Every 24 h, samples of 1 mL from the fermentation broth were centrifuged for 15 min (Siqueira et al., 2011). For the fermentation in the liquid medium PDB, the endophytic fungi were grown on PDA, at 25°C, for 5 days. Three pieces (0.5 cm × 0.5 cm) of mycelial agar plugs were inoculated into 500 mL Erlenmeyer flasks containing 300 mL of potato dextrose broth and incubated at room temperature for 4 weeks. The cultures were filtered and the wet mycelia were discarded (Trisuwan et al., 2008). For the antimicrobial activity test, 30 μL of each supernatant obtained was utilized according to the disk diffusion method proposed by Bauer et al. (1966).

Fermentation Assay in Semisolid Medium

The endophytes that showed the largest IDZ against the largest number of test microorganisms were cultured in the center of Petri dishes containing the medium PDA at 28 ± 2°C for 7 days, and from these colonies, five blocks of 5 mm diameter were transferred to Erlenmeyer flasks (1000 mL) containing the rice or corn semisolid media.

The preparation of semi-solid media was performed according to the methodology described by Aly et al. (2008), where 100 g of commercially available rice or corn and 100 mL of distilled water were added to Erlenmeyer flasks, autoclaved three times on alternate days and cultivated with the fungi *N. sphaerica* and *P. maculans* in static conditions at room temperature for 30 days. After the incubation period, methanol (300 mL) was added to each Erlenmeyer flask, followed by maceration. After 24 h, each sample was subjected to gravity filtration. The filtrate was concentrated on a rotary evaporator under reduced pressure at 50°C to obtain the methanolic extracts (NsME). The extract was kept at −20°C, and dissolved in dimethyl sulfoxide (DMSO) when ready for use.

Preparation of Ethyl Acetate Extract From Fermentation Assay in Liquid Medium (PDB)

The method described by Trisuwan et al. (2008) was used, where after the fourth week, the culture fermented by *N. sphaerica* was filtered in vacuum filter using a no.3 Buchner funnel. The culture filtrates were extracted with ethyl acetate (2 × 300 mL) by partitioning in a separating funnel (solvent–solvent extraction). The culture filtrates (ethyl acetate extract –NsEAE) was concentrated on a rotary evaporator under reduced pressure at 50°C. The extract was kept at −20°C, and dissolved in dimethyl sulfoxide (DMSO) when ready for use.

Phytochemical Analysis

Phytochemical analytical tests were performed to detect the presence of steroids, saponins, alkaloids, flavonoids, tannins, reducing compounds, terpenoids, cinnamic derivatives, and anthracene derivatives, according to the method described by Kokate (1994) and Harborne (1998).

Determination of Minimum Inhibitory and Minimum Bactericidal Concentrations

A broth microdilution susceptibility assay was used for the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), as recommended by the National Committee for Clinical Laboratory (National Committee for Clinical Laboratory Standards [NCCLS], 2009). All tests were performed in Muller-Hinton broth. Bacteria were cultured overnight at 37°C. The test samples of the extracts were dissolved in 10% DMSO. Dilutions were prepared in 96-well microtiter plates to get final concentrations ranging from 0 to 50 mg/mL. After this step, each well received 10 μL of the suspension of microorganisms and 100 μL of liquid culture media. Plates were incubated at 37°C for 24 h; 15 μL of 0.01% resazurin was added as a colorimetric indicator of oxide reduction to characterize cell viability. Then the microplates were re-incubated for 4 h, and the lowest concentration of the extract that inhibited microbial growth was recorded as the MIC. Using the results of the MIC assay, the concentrations showing a complete absence of visual growth of bacteria were identified, and 50 μL of each culture broth were transferred onto the agar plates and incubated for the specified time and temperature as mentioned above. The complete absence of growth on the agar surface at the lowest sample concentration was defined as the MBC. Each assay in this experiment was replicated three times.

Statistical Analysis

Data were analyzed using GraphPad Prism by one-way analysis of variance (ANOVA) and Tukey to determine statistical significance. A p-value of < 0.05 was considered to be statistically significant. The correlation index was calculated using the Pearson coefficient (ρ).

Results

Antimicrobial Activity Screening of Endophytic Fungi From Leaves of *I. suffruticosa*

A total of 65 endophytic strains were isolated from leaves of *I. suffruticosa* and subsequently submitted to a preliminary antimicrobial screening on solid medium. A total of 18 endophytic isolates showed activity against at least two of the tested bacteria, thus 33.6% of the isolates were found to be active and the majority of these endophytic strains (except for *N. sphaerica* ISEF 13) also showed a wide spectrum, inhibiting both Gram-positive, and Gram-negative organisms (**Table 1**). On the other hand, none of the endophytic isolates showed any ability to inhibit the growth of any of the five fungi tested in this study. From those 18 active strains, all of them inhibited *B. subtilis,* while *E. coli* was not inhibited by only one of the four isolated strains of *N. sphaerica*, *S. aureus,* and *K. pneumoniae* were inhibited by 77.78% (14/18) and 72.22% (13/18) of the active endophytic strains, respectively. On the other hand, *P. aeruginosa* was only inhibited by one strain of *N. sphaerica*.

The strains *N. sphaerica* (URM-6060) and *Pestalotiopsis maculans* (URM-6061) showed the best action, and no significant

TABLE 1 | Antibacterial activity of endophytic fungi isolated from leaves of *Indigofera suffruticosa*.

Endophytic fungi		Inhibition diameter zone (mm)				
		Staphylococcus aureus	***Bacillus subtilis***	***Escherichia coli***	***Klebsiella pneumoniae***	***Pseudomonas aeruginosa***
ISEF 1	*Colletotrichum gloeosporioides*	26 ± 2^{a}	15 ± 0^{a}	22 ± 1^{a}	22 ± 2^{a}	0 ± 0^{a}
ISEF 2	*C. gloeosporioides*	16 ± 1.73^{b}	24 ± 1^{b}	15 ± 1.73^{b}	28 ± 2^{b}	0 ± 0^{a}
ISEF 3	*C. gloeosporioides*	26 ± 1^{a}	13 ± 1.73^{c}	10 ± 1^{c}	26 ± 0^{b}	0 ± 0^{a}
ISEF 4	*C. gloeosporioides*	22 ± 2^{c}	12 ± 0^{c}	10 ± 0^{c}	26 ± 1^{b}	0 ± 0^{a}
ISEF 5	*Colletotrichum dematium*	0 ± 0^{d}	20 ± 1^{d}	11 ± 1.73^{c}	24 ± 1^{a}	0 ± 0^{a}
ISEF 6	*Curvularia pallescens*	0 ± 0^{d}	11 ± 0^{c}	10 ± 0^{c}	0 ± 0^{c}	0 ± 0^{a}
ISEF 7	*C. pallescens*	0 ± 0^{d}	12 ± 1^{c}	12 ± 0^{c}	0 ± 0^{c}	0 ± 0^{a}
ISEF 8	*Lasiodiplodia theobromae*	14 ± 0^{e}	$15 \pm 1.73^{a,c}$	21 ± 1.73^{a}	23 ± 1.73^{a}	0 ± 0^{a}
ISEF 9	*L. theobromae*	32 ± 2^{f}	13 ± 1.73^{c}	20 ± 1^{a}	12 ± 1^{d}	0 ± 0^{a}
ISEF 10	*Mycelia sterilia*	22 ± 1^{c}	$22 \pm 1^{b,d}$	14 ± 2.64^{b}	26 ± 1^{b}	0 ± 0^{a}
ISEF 11	*M. sterilia*	18 ± 1^{b}	32 ± 1.73^{e}	13 ± 1^{b}	21 ± 1.73^{a}	0 ± 0^{a}
ISEF 12	*M. sterilia*	$14 \pm 1.73^{b,e}$	15 ± 1.73^{a}	10 ± 0^{c}	15 ± 1	0 ± 0^{a}
ISEF 13	*Nigrospora sphaerica*	16 ± 1.73^{b}	14 ± 0^{c}	0 ± 0^{d}	0 ± 0^{c}	0 ± 0^{a}
ISEF 14	*N. sphaerica*	12 ± 1^{e}	12 ± 1^{c}	14 ± 1^{b}	0 ± 0^{c}	0 ± 0^{a}
ISEF 15	*N. sphaerica*	13 ± 1.73^{e}	16 ± 0^{a}	14 ± 0^{b}	12 ± 0^{d}	0 ± 0^{a}
ISEF 16	*N. sphaerica* (URM-6060)	36 ± 1^{f}	28 ± 2^{f}	34 ± 1.73^{e}	28 ± 1^{b}	12 ± 1.73^{b}
ISEF 17	*Pestalotiopsis maculans* (URM-6061)	34 ± 1^{f}	28 ± 1^{f}	26 ± 1^{f}	27 ± 1^{b}	0 ± 0^{a}
ISEF 18	*Phomopsis archeri*	0 ± 0^{d}	$14 \pm 1.15^{a,c}$	14 ± 1^{b}	0 ± 0^{c}	0 ± 0^{a}

The same superscript letter$^{(a-f)}$ indicates no significant difference ($p > 0.05$) between inhibition diameter zones (IDZ) values from different endophytic fungi against each pathogen (same column).

TABLE 2 | Antimicrobial activity *N. sphaerica* (URM-6060) isolated from leaves of *I. suffruticosa* cultivated in different growth media.

Culture medium	Inhibition diameter zone (mm)				
	S. aureus	***B. subtilis***	***E. coli***	***K. pneumoniae***	***P. aeruginosa***
SAB	0 ± 0^{a}	0 ± 0^{a}	20 ± 1^{a}	0 ± 0^{a}	0 ± 0^{a}
MEB	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{b}	0 ± 0^{a}	0 ± 0^{a}
EPM	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{b}	0 ± 0^{a}	0 ± 0^{a}
PDB	30 ± 1^{b}	36 ± 1^{b}	32 ± 2^{c}	26 ± 1^{b}	28 ± 1^{b}
Rice	15 ± 1.73^{c}	18 ± 2^{c}	16 ± 1^{d}	11 ± 1.73^{c}	12 ± 1^{c}
Corn	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{b}	0 ± 0^{a}	0 ± 0^{a}

The same superscript letter$^{(a-d)}$ indicates no significant difference ($p > 0.05$) between IDZ values from strain URM-6060 cultivated in different growth media against each pathogen (same column).

differences ($p > 0.05$) were observed between their IDZ against most of the tested pathogens (except to *E. coli* and *P. aeruginosa*, which were more efficiently inhibited by the strain URM-6060) These two strains were stored in the URM Culture Collection (WDCM604) and submitted to the other assays.

Fermentation Assays in Liquid and Semisolid Medium

The antimicrobial activity of the two most active strains (*N. sphaerica* URM-6060 and *P. maculans* URM-6061) was evaluated by liquid and semisolid fermentation assays using different growth media. *N. sphaerica* URM-6060 showed antimicrobial activity against all tested bacteria, but only when it was grown in rice and PDB media. The best activity was observed in PDB medium against all pathogens ($p < 0.05$) with inhibition zones of 36, 32, 30, 28, and 26 mm against *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa,* and *K. pneumoniae*, respectively, (**Table 2**). When cultivated in SAB, the strain URM-6060 only inhibited the growth of *E. coli* (20 mm). In semi-solid media, only the methanol extract obtained from rice medium (NsME) showed antimicrobial activity with IDZ ranging from 18 (*B. subtilis*) to 11 mm (*K. pneumoniae*). Interestingly, a strong correlation could be observed between the IDZ values obtained with PDB and rice media ($\rho = 0.97$). On the other hand, the strain *P. maculans* URM-6061 was not able to produce extracellular antimicrobial compounds in any of the tested media.

Minimum Inhibitory and Minimum Bactericidal Concentrations of the NsME and NsEAE Extracts of the Endophytic Fungus *N. sphaerica* (URM-6060)

The NsME (methanolic extract obtained from rice culture medium) and NsEAE (ethyl acetate extract obtained from the filtrate of PDB medium) were subjected to the microdilution test to determine the MIC and MBC, as shown in **Table 3**. NsME was more active against *S. aureus*, *B. subtilis,* and *P.* aeruginosa (MIC of 1.56 mg/mL for all), followed by *E. coli* and *K. pneumoniae* (MIC of 6.25 mg/mL). Furthermore, the MBC values of NsME ranged from 6.25 to 50 mg/mL, and it predominantly showed bacteriostatic actions (MBC/MIC ≥ 4; Pankey and Sabath, 2004; except for *K. pneumoniae*; MBC/MIC ratio of 2).

The best antimicrobial action was found with NsEAE as its MIC values ranged from 0.39 to 3.12 mg/mL and MBC values

TABLE 3 | Minimum Inhibitory and Minimum Bactericidal Concentrations of extracts of *N. sphaerica* (URM-6060) isolated from leaves of *I. suffruticosa* against human pathogens.

Extract	Concentration (mg/mL)									
	S. aureus		*B. subtilis*		*E. coli*		*K. pneumoniae*		*P. aeruginosa*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
NsME	1.56	6.25	1.56	12.5	6.25	50	6.25	12.5	1.56	25
NsEAE	0.39	3.12	1.56	6.25	1.56	25	3.12	12.5	1.56	12.5

NsME – methanolic extract obtained from rice culture medium.
NsEAE – ethyl acetate extract obtained from the filtrate of the culture in Potato Dextrose Broth (PDB) medium.

TABLE 4 | Phytochemical analysis of the extracts from *N. sphaerica* (URM-6060) isolated from leaves of *I. suffruticosa*.

Extract	Compound								
	Steroid	Saponin	Alkaloid	Flavonoid	Tannin	Sugar	Terpenoids	Cinnamic derivatives	Anthracene derivatives
NsME	+	−	+	−	+	+	+	+	−
NsEAE	+	−	+	−	+	+	+	+	+

NsME – methanolic extract obtained from rice culture medium.
NsEAE – ethyl acetate extract obtained from the filtrate of the culture in PDB medium.

from 3.12 to 50 mg/mL, respectively. In fact the strongest activity was found against *S. aureus* (MIC of 0.39 mg/mL and MCB of 3.12 mg/mL), followed by *B. subtilis* (MIC of 1.56 mg/mL and MCB of 6.25 mg/mL) and *P. aeruginosa* (MIC of 1.56 mg/mL and MCB of 12.5 mg/mL), *E. coli* (MIC of 1.56 mg/mL and MCB of 25 mg/mL), and *K. pneumoniae* (MIC of 3.12 mg/mL and MCB of 12.5 mg/mL). This extract also showed bacteriostatic actions (MBC/MIC $\geq$ 4).

Phytochemical Screening of the NsME and NsEAE Extracts of the Endophytic Fungus *N. sphaerica* (URM-6060)

Phytochemical analysis revealed the presence of terpenoids, steroids, hydrolyzable tannins, alkaloids, and cinnamic derivatives (**Table 4**). Both extracts showed the same chemical constitution, except for the presence of anthracene derivatives in NsEAE. All these classes of compounds have been reported as antimicrobial agents.

Discussion

One of the most important properties of endophytic microorganisms, especially fungi, is linked to their metabolic potential to produce a large variety of bioactive molecules that can protect the plant against pathogens (Tan and Zou, 2001; Strobel, 2003). For example, natural compounds synthesized by endophytic fungi have been reported as inhibitors of a wide variety of animal and plant pathogens (Wiyakrutta et al., 2004; Gunatilaka, 2006; Zhao et al., 2011b). The isolation and identification of endophytic mycobiota is necessary, since the medicinal properties of a plant can be a consequence of the capacity of its endophytic microorganisms to produce biologically active secondary metabolites (Kaul et al., 2012; Kusari et al., 2013). This was the case in the classic example of taxol, an anticancer agent produced by *Taxus brevifolia* Nutt., and its endophyte *Taxomyces andreanae* (Stierle et al., 1993).

In the present work 65 endophytic strains were isolated from the medicinal plant *I. suffruticosa* and the antimicrobial activity of them was evaluated. A total of 18 (33.6%) strains showed antibacterial activity, most of them (17/18; 94.44%) with wide spectrum. The percentage of endophytic fungi isolated from leaves of *I. suffruticosa* showed that antimicrobial activity was comparable and even exceeding some results reported by other authors in similar studies, revealing the enormous capacity of production of bioactive compounds with antimicrobial potential by these microorganisms. For example, only 8.3% of the strains isolated from *Dracaena cambodiana* and *Aquilaria sinensis* showed antimicrobial activity (Gong and Guo, 2009), whereas 27.6% of strains isolated from *Camptotheca acuminata* displayed antimicrobial activity against some pathogens (Lin et al., 2007).

The two most active strains (*N. sphaerica* URM-6060 and *P. maculans* URM-6061) were further examined using liquid and semi-solid fermentation assays. Only *N. sphaerica* URM-6060 showed antibacterial activity (only in PDB, SAB, and rice media), and the best results were found using PDB medium. Similar results were observed by Siqueira et al. (2011) in a study of the antimicrobial activity of endophytic fungi from *Lippia sidoides* Cham., where 16 out of 203 endophytic isolates showed antimicrobial activity in an assay on solid medium, and of the 16 endophytic fungi which were submitted to the fermentation assay, 10 displayed antimicrobial activity. The production of a bioactive compound by an endophyte can be stimulated in the host plant or by a host plant extract. When grown *in vitro*, an endophyte may continue to produce bioactive material, or this may cease after a certain time. Research is needed to discover what factors could encourage endophytes to continue synthesizing compounds *in vitro* (Owen and Hundley, 2004).

Endophytic fungi from the genus *Nigrospora* have been reported as rich sources of bioactive secondary metabolites with applications in various fields. Such secondary metabolites include herbicidal phomalactone (Kim et al., 2001), phytotoxic and antibacterial nigrosporins (Tanaka et al., 1997), phytotoxic lactones (Fukushima et al., 1998), and activity against plant pathogenic fungi (Zhao et al., 2012). In this work the best antimicrobial action was found with NsEAE, this extract was most active against *S. aureus*. This extract is composed of terpenoids, steroids, hydrolyzable tannins, alkaloids, cinnamic derivatives, and anthracene derivatives, all of them reported to be antimicrobial agents (Funatogawa et al., 2004; Yu et al., 2010; Du et al., 2012; Sova, 2012; Mousa and Raizada, 2013).

Specifically, the production of terpenoids by endophytic fungi and their biological activities were reported in a recent review (Souza et al., 2011). The steroid ergosta-7,9 (14), 22-triene-3β-ol, produced by the endophytic fungus *N. sphaerica* isolated from leaves of *Vinca rosea*, showed antifungal activity against *Cryptococcus neoformans* with an IC50 value of 14.81 μg/mL (Metwaly et al., 2014). The incubation temperature, medium composition, and degree of aeration affect the amount and kinds of compounds that are produced by an endophytic fungus (Strobel et al., 2004). The culture medium, agitation, and temperature can increase or reduce the production of the bioactive compounds by fungi. For this reason, further tests are needed to evaluate the biological activity of the strains that showed inhibition on solid media but did not produce bioactive compounds during the fermentation assay. The production of a bioactive compound by an endophyte can be stimulated in the host plant or by a host plant extract. Other explanations for this may be the presence of some inhibitory compound in the extract, the range of concentration of extract tested and the pathogenic fungi selected for the test (Pawle and Singh, 2014).

Furthermore, our observations indicate that endophytic fungi from leaves of *I. suffruticosa* have pharmaceutical potential as they produce antimicrobial compounds, and that the medicinal properties of this plant may be a consequence of the capacity of its endophytic microorganisms to produce biologically active secondary metabolites. Further studies are now needed to identify the active compounds produced in order to discover new drugs with antibacterial activity.

Author Contributions

Conceived and designed the experiments: IPS, VLML, and MSC. Performed the experiments: IPS and MSC. Analyzed the data: VLML, MSC, LCNA, and JMA. Contributed reagents/materials/analysis tools: VLML, MSC, and JMA. Wrote and enriched the literature: IPS, LCNA, VLML, and MVS.

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7. TERCEIRO CAPÍTULO

7.1. Production of Indole-3-carboxylic acid by endophytic fungus *Nigrospora sphaerica* URM-6060 isolated from *Indigofera suffruticosa* Miller and evaluation of anti-*Candida* activity

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O composto ácido indólico-3-carboxílico é um agente citotóxico potente e conhecido como antifúngico e inibidor da replicação do HIV. O objetivo deste estudo foi o isolamento de metabólitos secundários produzidos pelo fungo *N. sphaerica*, a identificação química destes compostos e avaliação de sua atividade anti-*Candida*, no qual fomos capazes de extrair este composto a partir do fungo endofíticos *Nigrospora sphaerica* URM-6060, isolado de *Indigofera suffruticosa*. Para realizar estes objetivos, o fungo foi cultivado no meio de cultura caldo batata dextrose (BD), à temperatura ambiente, durante 4 semanas, e, em seguida, o caldo foi separado do micélio e o filtrado obtido foi extraído com AcOET, e depois foi submetido a coluna cromatográfica (CC), utilizando como fase estacionária Sephadex LH-20 e fase móvel MeOH, e CC utilizando como fase estacionária sílica flash (70-230 Mersh) e como fase móvel Hexano, AcOET e MeOH, em ordem crescente de polaridade. As frações e subfrações obtidas foram analisadas por ressonância magnética nuclear (RMN) para elucidar os componentes químicos. Este procedimento resultou na identificação de ácido indólico-3-carboxílico. Avaliação da actividade anti-*Candida* deste composto foi realizada, e os resultados indicam uma actividade fungicida contra todas as espécies de *Candida* testadas, com os melhores resultados contra *Candida guilermondii*, *C. haemulonii* e *C. albicans*, respectivamente. Os resultados obtidos neste estudo mostraram que *N. sphaerica* URM-6060, isolado a partir de folhas de *I. suffruticosa* produz o metabólico bioativo ácido indólico-3-carboxílico, um importante composto com a aplicação anti-*Candida*.

Production of Indole-3-carboxylic acid by endophytic fungus *Nigrospora sphaerica* URM-6060 isolated from *Indigofera suffruticosa* Miller and evaluation of anti-*Candida* activity

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Abstract The compound Indole-3-carboxylic acid is a potent citotoxic agent and known as a antifungal and inhibitor of HIV replication. The aim of this study was the isolation of secondary metabolite produced by *N. sphaerica*, the chemical identification of these compound and evaluation of their anti-*Candida* activity, in which we were able to extract this compound from the endophytic fungus *Nigrospora sphaerica* URM-6060, isolated from *Indigofera suffruticosa*. To accomplish these goals, the fungus was cultured in BD broth at room temperature for 4 weeks, and then the broth was separated from the mycelium, and the filtrate was extracted with AcOET. After was subjected to CC over Sephadex LH-20 and CC over silica Flash (70-230 Mersh), the fractions and subfraction were analyzed by nuclear magnetic resonance to elucidate the chemical components. This procedure resulted in the identification of Indole-3-carboxylic acid in the subfraction a. Evaluation of the anti-*Candida* activity of this compound has been accomplished, and the results indicate fungicidal activity against all Candida species tested, with the best results against *Candida guilermondii*, *C. haemulonii* and *C. albicans*, respectively. The results obtained in this study showed that *N. sphaerica* URM-6060, isolated from *I. suffruticosa* produces the bioactive metabolic Indole-3-carboxylic acid, an important compound with anti-*Candida* application.

Keywords Chemical characterization Bioactive compound Secondary metabolite *Candida guilermondii C. haemulonii* and *C. albicans*

Introduction

Endophytic fungi are microorganisms that, during part or all of their life cycle, colonize inter and/or intracellularly healthy plant tissues, in an asymptomatic manner, without causing any apparent damage to their host (Tan and Zou 2001), and represent a great source of study and research for new chemicals for medicinal, industrial and agricultural use (Aly et al. 2011; Kusari and Spiteller 2011; Rajulu et al. 2011; Li et al. 2012; Kusari et al. 2013; Teiten et al. 2013).

These microorganisms are considered a significant reservoir of novel bioactive secondary metabolites could classified as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols and lactones (Yu et al. 2010; Zhou et al. 2010), and describeds as anti-microbial, anti-insect, anti-cancer, antidiabetic and immunosuppressant compounds (Kharwar et al. 2011; Zhao et al. 2011).

Endophytic fungi from the genus *Nigrospora* have been reported as rich sources of bioactive secondary metabolites with applications in various fields. Such secondary metabolites include herbicidal phomalactone (Kim, 2001), phytotoxic and antibacterial nigrosporins (Tanaka, 1997), phytotoxic lactones (Fukushima, 1998) and activity against plant pathogenic fungi (Zhao et al. 2012).

Indigofera suffruticosa Miller is a plant found in the North and Northeast of Brazil, and several medicinal properties are attributed by the population to this specie. Previous studies have shown that the leaves of *I. suffruticosa* have embryotoxic effects (Leite et al. 2004), antimicrobial (Leite et al. 2006), anticancer (Vieira et al. 2007) and anticonvulsant (Almeida et al. 2013) activities, and act as gastroprotective agent (Luiz-Ferreira et al. 2011).

The isolation and identification of endophytic mycobiota is necessary, since the medicinal properties of a plant can be a consequence of the capacity of its endophytic microorganisms to produce biologically active secondary metabolites (Kaul et al. 2012; Kusari et al. 2013). This is the case in the classic example of taxol, an anticancer agent produced by *Taxus brevifolia*., and its endophyte *Taxomyces andreanae* (Stierle et al. 1993).

Although numerous antifungal compounds have been discovered or synthetized in chemical synthesis programmes, new products applicable to humam therapy are needed owing to a drastic increase in the number of immunocompromised patients coupled with the development of resistence among clinically relevant fungi against several of the limited number of antimycotics in current use (Weber et al. 2007).

Candida species are opportunistic pathogens that inhabit the human body as commensal microorganisms and have been considered the major cause of fungal infections in humans (Monge et al. 2006). Among these species, *Candida albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei*, are the more found in humans infections, and cause more than 90% of infections, and the arising of *C. guilliermondii*, *C. kefyr*, *C. rugosa*, *C. dubliniensis* and *C. famata* as pathogens has also been reported worldwide (López-Martínez, 2010; Miceli et al. 201; MacCallum, 20121).

One of the most important virulence factors of Candida species is their ability to form the biofilm, which has repercussions in the clinical context, because it is associated with increased resistance to antimicrobial agents (Mukherjee and Chandra 2004; Ramage et al. 2005; Henriques et al. 2006; Thein et al. 2006).

Given the evident growth in the number of pathogens resistant to antibiotics currently used in the clinics, there is a clear and emerging need to introduce novel antimicrobial agents to the therapeutic arsenal (Khan et al. 2008).

Thus, this study we performed the isolation and subsequent identification of chemical component of the ethyl acetate extract obtained from cultive in PDB medium of the endophytic fungus *N. sphaerica* and evaluated the anti-Candida activity of the molecule isolated.

Materials and methods

Fungal material

The endophytic fungus *N. sphaerica* URM-6060 was isolated from healthy leaves of *I. suffruticosa* by the method described by Araújo et al. (2002). After the isolation, the identification of endophytic fungi was performed at the Mycology Department, UFPE, Recife, Pernambuco, Brazil, by means of analysis of macroscopic and microscopic characteristics of colonies. After identification, fungi that showed antimicrobial activity were stored in the URM Culture Collection (WDCM604) of the Federal University of Pernambuco, Recife, Brazil.

Fermentation and isolation

The endophytic fungus *N. sphaerica* URM-6060 was grow on potato dextrose agar at 25 °C for 5 days. Three pieces (0.5 x 0.5 cm^2) of mycelial agar plugs were inoculated into 500 mL Erlenmeyer flasks containg 300 mL of dextrose potato broth at room temperature for 4 weeks. The filtrate was extracted three times with AcOET to afford a broth extract as a brown gum after evaporation to dryness under reduced pressure. The crude extract (474.5 mg) was subjected to CC over Sephadex LH-20, using MeOH as eluent, to obtain four fractions (1A-1D). Fraction 1A (237.1 mg) was further purified by CC over silica Flash (70-230 Mersh), using hexane, MeOH and AcOET, in increasing order of polarity, as eluent, to afford two subfractions (A and B). Subfraction A (41.4 mg), was purified by CC over silica Flash (70-230 Mersh), using hexane, MeOH and AcOET, in increasing order of polarity, as eluent, and the subfraction a (20 mg) obtained was analyzed by nuclear magnetic resonance (NMR) 1H (500 MHz) and 13C (125 MHz), using CD_3OD as eluent, to elucidate the chemical structures.

Target microorganisms

Candida species (*C. albicans* ATCC, *C. tropicalis* 43, *C. parapsilosis* 346, *C. haemulonii* 347, *C. albicans* 12680, *C. krusei* 14206 and *C. guiliermondii* 14495) were isolated from patients met at the Medical Mycology Laboratory of the Federal University of Pernambuco, Recife, Brazil, was used for antifungal test.

Determination of Minimum Inibitory and Minimum Fungicidal Concentrations

A broth microdilution susceptibility assay was used for the determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC), as recommended by the NCCLS (2009). All tests were performed in Sabouraud broth. *Candida* species were cultured at 37°C during 48 hours. The test samples of the compound was dissolved in DMSO. Dilutions were prepared in 96-well microtiter plates to get

final concentrations ranging from 0 to 0.5 mg/mL. After this step, each well received 10 µL of the suspension of microorganisms and 100 mL of liquid culture media. Plates were incubated at 37°C, for 48 hours, 15 µL of 0.01% resazurin was added as a colorimetric indicator of oxide reduction to characterize cell viability. Then the microplates were reincubated for 4 h, and the lowest concentration of the extract that inhibited microbial growth was recorded as the MIC. Using the results of the MIC assay, the concentrations showing a complete absence of visual growth of bacteria were identified, and 50 µL of each culture broth were transferred onto the agar plates and incubated for the specified time and temperature as mentioned above. The complete absence of growth on the agar surface at the lowest sample concentration was defined as the MFC. Each assay in this experiment was replicated three times.

Results and discussion

Analysis of subfraction a was performed using thin layer chromatography (TLC), which was relatively pure, the ^{1}H NMR spectroscopy in conjunction with the analysis of the ^{13}C NMR spectroscopy (Table 1) and a comparison with published data (Hagemeier et al. 2001), we identified the compound as Indole-3-carboxylic acid (Fig. 1).

This compound has been isolated from extracts of plants, such as rice roots (Rimando et al. 2001), leaves from *Bauhinia tarapotensis* (Braca et al. 2001) and cell walls of leaves and roots from *Arabidopsis thaliana* (Tan et al. 2004). This acid was also isolated from culture filtrates from the fungus *Lasiodiplodia theobromae* (Aldridge et al. 1971), from a culture of the fungus *Aporpium caryae* (Levy et al. 2000) and from culture of the endophytic fungus EM-22, derived from marine red alga *Polysiphonia urceolata* (Zhao et al. 2009), but, is the first time that this compound is isolated from the fungus *N. sphaerica*.

Among the biological activities described, indole-3-carboxylic acid demonstrated significant activity against HIV replication in H9 lymphocyte cells (Wu et al. 2004), inhibited the proliferation of HL-60 cells (Nishio et al. 2012), presented antifungal activity against the phytopathogenic fungi *Cladosporium cucumerinum* (Levy et al. 2000) and *Alternaria brassicicola* (Pedras and Abdoli 2013), and activity against the human pathogenic yeast *Candida sp*. (Kavitha et al. 2010).

The Indole-3-carboxylic acid was subjected to the microdilution test to determine the MIC and MFC, as shown in Table 2. Indole-3-carboxylic acid showed MIC values ranging from 125.00 and 3.90 µg/mL, this compound was more active against *C. guilermondii*, (MIC of 3.90 µg/mL), followed by *C. haemulonii* (MIC of 7.81 µg/mL), and *C. albicans* (MIC of 62.50 µg/mL). The MFC values of Indole-3-carboxylic acid ranged from 125.00 and 3.90 µg/mL, and it showed a bactericidal action (MBC/MIC < 4; Pankey and Sabath, 2004), against all *Candida* species tested with the MBC/MIC ratio ranging from 1 and 2.

The best anti-*Candida* activity was found against *C. guiliermondii*, as its MIC and MFC values was 3.90 µg/mL, followed by *C. haemulonii* (MIC of 7.81 µg/mL and MFC of 15.60 µg/mL) and *C. albicans* (MIC of 62.50 µg/mL and MFC of 125.00 µg/mL). In this study Nystatin was used as positive control, and showed MIC and MFC values similar or best that Indole-3-carboxylic acid, range to 3.90 at 31.20 µg/mL against all *Candida* strains tested, except against *C. haemulonii* where our results showed that Indole-3-carboxylic acid presente fungicidal activity, while Nystatin presente only fungistatic activity (Table 2).

The indole-3-carboxylic acid has shown good results when evaluated about their antifungal activity, but no specific study was carried out against *Candida* species, there are few studies investigating anti-*Candida*

activity in the literature. Indole-3-carboxylic acid was isolated from the EtOAc extract of the culture broth of the actinomycete *Acrocarpospora sp*., And tested by disc diffusion method against *C. albicans* (BCRC-21538), but not displayed activity (Cheng et al. 2009), while this compound isolated from *Streptomyces sp.* from the soil, showed MIC value of 10 µg/mL against *C. albicans* (Kavitha et al. 2010).

Natural products from endophytic have been observed to inhibit or kill a wide variety of microorganisms, but a few study showed endophytic with hability for production of secundary metabolites with activity against espécies do gênero *Candida*. Screening of Antimicrobial Activities of the Endophytic Fungi Isolated from Sesbania grandiflora showed that 7 (10.14%) isolates inhibited C. albicans (Powthong et al. 2013), while the metabolites cerulanin, arundifungin, spharopsidin A, 5-(1,3-butadiene-1-yl)-2-(5H)-furanone, ascosteroside A and B, from endophytic fungi, were tested by disc diffusion method against *C. albicans, C. glabrata, C. krusei* and *C. parapsilosis*, and present fungicidal action against all *Candida* species tested, at concentration of 5 µg by disc, with diameter of inhibition zones range at 9 to 22 mm (Weber et al. 2007).

Davis et al. (2005) also tested antimicrobial activity of endophytic fungi. After chemical analysis of endophytic fungi cultures, *Eupenicillium sp*. Isolated from an endemic plant in Australia, *Glochidium ferdinandi* (Euphorbiaceae), the authors verified the presence of the following compounds: phomoxin B e C, eupenoxide e phomoxin. The isolated compounds were tested against plenty of microorganism associated to nosocomial infections, and according to the authors, the isolated substances showed antimicrobial activity against these microorganisms including *Candida albicans*.

In our study, the Indole-3-carboxylic acid presented fungicidal action against all *Candida* species tested, with the best results against *C. guilermondii, C. haemulonii* and *C. albicans*, respectively, and was best that the results presented by Nystatin against *C. haemulonii*. Nystatin is a synthetic antifungal agents used as controls of *Candida* inffections and have been used as reference for the treatment of superficial fungal infections caused by *Candida* species. Castro and Lima (2013) evaluated the susceptibility to the antifungal nystatin of strains of *C. albicans, C. tropicalis* and *C. krusei*, found MIC values ranged from 0.032 to 0.064 mg/mL and MFC of 0.064 mg/mL. Wingeter et al. (2007) assessed the sensitivity of *C. albicans* strains and *Candida* non-albicans isolated from the patients with denture stomatitis. They observed MIC values between 0.002 and 0.064 mg/mL. These findings were similar to those found in this study, in which for Nystatin MIC and MFC values ranged from 0.0039 at 0.0312 mg/mL.

These results are of paramount importance, since *C. guilliermondii* accounts for 1–3% of all candidemia and that most cases of *C. guilliermondii* infection are associated with oncology patients (Girmenia et al. 2006; Pfaller et al. 2006). In addition, *C. guilliermondii* has been shown to be more resistant to antifungal agentes than other Candida species (Pfaller et al. 2006; Arendrup 2010; Beyda et al. 2012) and can also cause osteomyelitis, septic arthritis, candiduria, endocarditis and skin lesions (Girmenia et al. 2006; Savini et al. 2010).

Candida albicans remains the most common *Candida* species, and infections caused by this specie are increasing (Tortorano et al. 2004). Currently, *Candida parapsilosis* has been recognized as the second or third most frequently isolated *Candida* species that causes bloodstream infection (Klingspor et al. 2004; Tortorano et al. 2004; Maganti et al. 2011). *C. parapsilosis* infections particularly affect neonates (Trofa et al. 2008; Spiliopoulou et al. 2012) and patients surgical of intensive care unit (ICU) atients (Pfaller et al. 2011).

The presence of Non-albicans *Candida* species (NACS) including *C. tropicalis, C. glabrata, C. krusei, C. dubliniensis, C. haemulonii* and *C. parapsilosis* has increasingly been responsible for causing nosocomial

bloodstream infections (Pfaller and Diekema 2007; Andes et al. 2012) and accounts for almost 50% of non-superficial *Candida* infections (Sobel, 2006). Mortality rates of 40 to 70% have been associated with the presence of *C. tropicalis* in the bloodstream (Wingard, 1995; Gottfredsson et al. 2003).

Furthermore, *C. haemulonii* does not frequently cause human infections (Gargeya et al. 1991), but shows that it is resistant to amphotericin B and other antifungal agents such as azoles (García-Martos et al. 2001), which have often been associated with clinical treatment failure (Khan et al. 2007, Ruan et al. 2010), and mortality rates among such patients with *C. krusei* fungemia are unacceptably high, ranging from 60 to 80% (Abbas et al. 2000), and ranked in fifth place among 22 different species of *Candida*, accounting for 3.3% of all *Candida* isolates both in Europe and North America (Muñoz et al. 2005; Pfaller et al. 2008).

After a literature review, we understood that Indole 3-carboxylic acid is a strong anticancer agent. However, there is only limited information regarding its antifungal activity, especially against *Candida* species. Therefore, the inhibitions by Indole 3-carboxylic acid of the *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. haemulonii*, *C. krusei* and *C. guiliermondii* are novel and important results that have not previously appeared in the literature. Therefore, *N. sphaerica* as an endophyte of *I. suffruticosa* produces in its secondary metabolism an important compound with biotechnological application.

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Table 1 - Spectral data for ^{13}C NMR (CD_3OD, 125 MHz) and ^{1}H (CD_3OD, 500 MHz)

	Indole-3-carboxylic acid		Indole-3-carboxylic acid*	
	δ ^{1}H	**δ ^{13}C**	**δ ^{1}H**	**δ ^{13}C**
2	7.95 (s)	133.40	7,9 (s)	133,40
3		108.73		108,70
3[a]		127.51		127,60
4	8.07 (m)	122.38	8,1 (dd, *J* = 7,7; 1,3)	122,00
5	7.16 – 7.22 (m)	123.59	7,2 (ddd, *J* = 7,7; 7,7; 1,3)	122,40
6	7.16 – 7.22 (m)	122.00	7,2 (ddd, *J* = 7,7; 7,7; 1,3)	123,60
7	7.44 (m)	112.90	7,4 (dd, *J* = 7,7; 1,3)	112,90
7[a]		138.17		138,20
8		169.45		**

*(Hagemeier et al., 2001); ** not detected signal; Coupling constants (*J* in Hz).

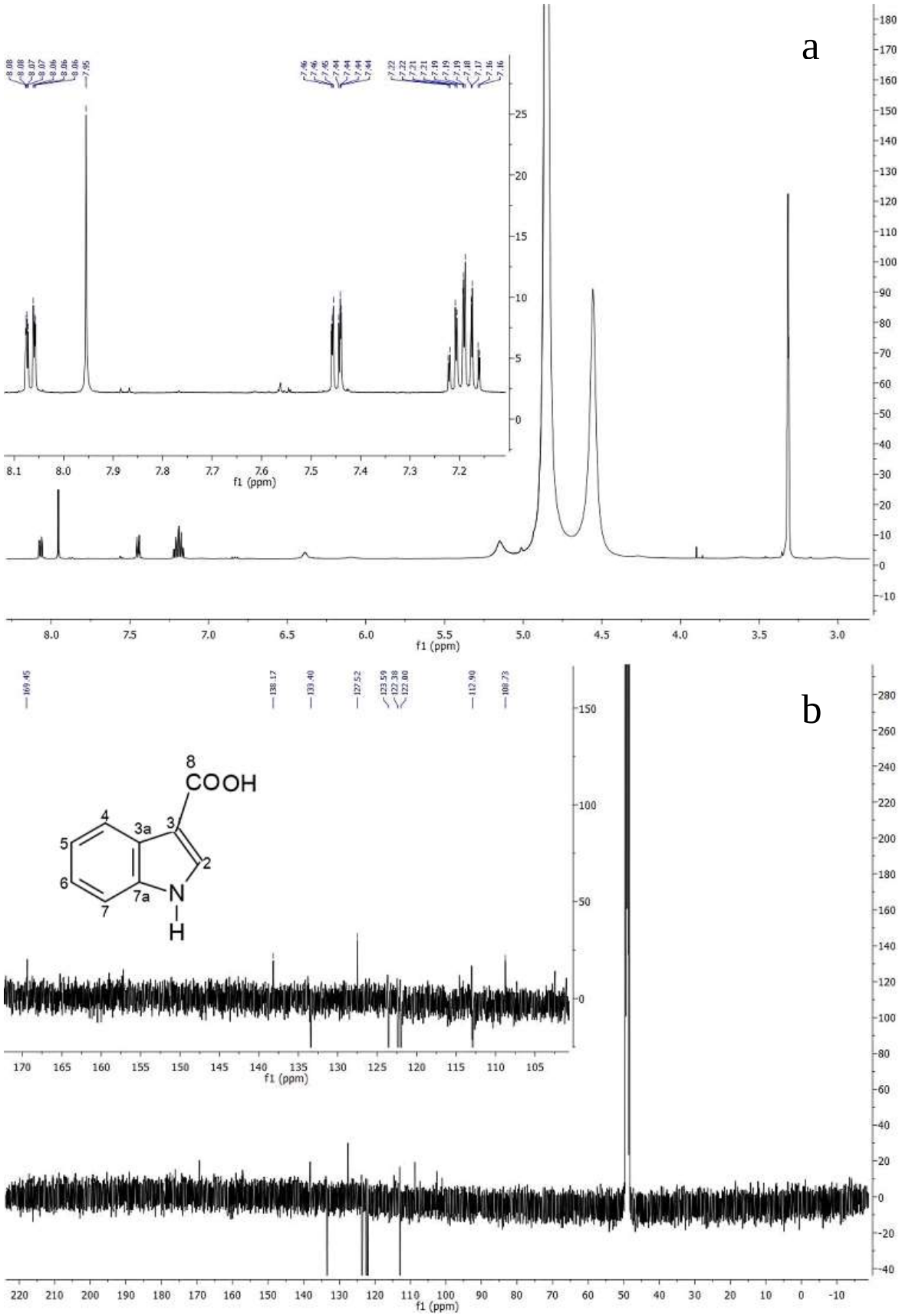

Fig. 1 NMR spectroscopy of ^{1}H (a) and ^{13}C (b) of the Indole-3-carboxylic acid produced by the endophytic fungus *Nigrospora sphaerica* (URM-6060) isolated from leaves of *Indigofera suffruticosa*.

Table 2 - Minimum Inhibitory and Minimum Fungicidal Concentrations of Indole-3-carboxylic acid produced by the endophytic fungus *Nigrospora sphaerica* (URM-6060) isolated from leaves of *Indigofera suffruticosa* and Nystatin against *Candida* species.

***Candida* species**	**Concentration (µg/mL)**					
	Indole-3-carboxylic acid			**Nystatin**		
	MIC	**CMF**	**MFC/MIC**	**MIC**	**CMF**	**MFC/MIC**
C. albicans (ATCC)	62.50	125.00	2	31.20	31.20	1
C. tropicalis (43)	125.00	125.00	1	31.20	31.20	1
C. parapsilosis (346)	125.00	125.00	1	7.81	15.60	2
C. haemulonii (347)	7.81	15.60	2	3.90	15.60	4
C. albicans (12680)	62.50	125.00	2	31.20	31.20	1
C. krusei (14206)	125.00	125.00	1	31.20	31.20	1
C. guilermondii (14495)	3.90	3.90	1	3.90	3.90	1

8. CONCLUSÕES

- Os resultados apresentados nesse estudo representam o primeiro relato da micobiota endofítica associada à planta medicinal *I. suffruticosa* coletada na Mata Atlântica e na Caatinga, no Brasil, e os resultados obtidos sugerem que a mcobiota endofítica de *I. suffruticosa* é diferente quanto ao período do ano e o Bioma estudados, sendo o período seco na Mata Atlântica o que apresentou uma micobiota endofítica mais diversificada.
- As espécies fúngicas *P. pallescens*, *Phomopsis archeri* e *Colletotrichum dematium* foram e isoladas de ambos períodos do ano e Biomas, enquanto *L. theobromae* foi encontrada apenas na Mata Atlântica na estação seca, e as espécies *Curvularia australiensis* e *Chaetomella raphigera* foram isoladas apenas na Caatinga durante as estações chuvosa e seca, respectivamente, e é a primeira vez que são isoladas de uma planta da Caatinga.
- Fungos endofíticos de *I. suffruticosa* são produtores de metabólitos bioativos de potencial uso terapêutico contra bactérias e fungos patogênicos ao homem.
- Entre os fungos endofíticos isolados de *I. suffruticosa*, *N. sphaerica* demonstrou capacidade de produzir o ácido indólico-3-carboxílico, o qual apresentou considerável atividade anti-*Candida* frente as as espécies *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. haemulonii*, *C. krusei* and *C. guiliermondii*, sendo apontado como candidato na descoberta de novas drogas com atividades antifúngica.