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Caracterização morfo-molecular e patogenicidade dos isolados de *Phytophthora* e *Lasiodiplodia* causando doenças em cacaueiro no Equador

Sergio Miguel Vélez Zambrano

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Tese apresentada ao Programa de Pós-graduação em Fitopatologia do Instituto de Ciências Biológicas da Universidade de Brasília como parte dos requisitos necessários para obtenção do título de doutor.

Orientador

Prof. Danilo Batista Pinho

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Sergio Miguel Vélez Zambrano
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CARACTERIZAÇÃO MORFO-MOLECULAR E PATOGENICIDADE DOS ISOLADOS DE *Phytophthora* E *Lasiodiplodia* CAUSANDO DOENÇAS EM CACAUEIRO NO EQUADOR.

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VÉLEZ, Sergio Miguel. **Caracterização morfo-molecular e patogenicidade dos isolados de *Phytophthora* e *Lasiodiplodia* causando doenças em cacaueiro no Equador**. 2024. Tese de Doutorado em Fitopatologia. Universidade de Brasília, Brasília, DF.

O cacaueiro (*Theobroma cacao* L.) é a espécie mais importante do gênero *Theobroma* devido à sua ampla distribuição geográfica e comercialização mundial do chocolate produzido a partir das amêndoas dos frutos. O Equador ocupa a sétima posição mundial entre os países produtores de cacau. O litoral é a principal região produtora de cacau do Equador, embora a cultura seja cultivada em 16 das 24 províncias. O cacaueiro cultivado na região litorânea possui alta produtividade e incidência de doenças como a podridão parda e a morte descendente. A podridão parda é causada por espécies de *Phytophthora* enquanto um complexo de espécies de *Lasiodiplodia* causam a morte descendente. A identificação precisa desses patógenos tem sido realizada por comparação de características morfológicas em combinação com dados moleculares de diferentes regiões genômicas. Essa abordagem tem revelado a descoberta de novas espécies e/ou novos relatos de patógenos em várias culturas agrícolas. Baseado nessas informações, acredita-se que a podridão parda e a morte descendente do cacaueiro no Equador sejam causadas por diferentes espécies de *Phytophthora* e *Lasiodiplodia*, respectivamente. Portanto, os objetivos desse trabalho são: (i) identificar as espécies de *Phytophthora* associadas com a podridão parda, (ii) identificar as espécies de *Lasiodiplodia* associadas com a morte descendente, (iii) determinar a patogenicidade e agressividade dos isolados de *Phytophthora* e *Lasiodiplodia*, e (iv) atualizar a lista de patógenos associados ao cacaueiro no Equador. Frutos com sintomas de podridão parda e tecidos de plantas com sintomas da morte descendente foram coletados nas províncias de Esmeraldas, Guayas, Los Ríos, Manabí, Morona Santiago, Pichincha e Santo Domingo de los Tsáchilas para isolamento indireto. Para identificação prévia, a região parcial do gene β-tubulina (*Phytophthora* spp.) ou fator de elongação (*Lasiodiplodia* spp.) foi amplificada e sequenciada. Posteriormente, isolados representativos foram selecionados para o sequenciamento adicional das regiões genômicas ITS e *COX2* para *Phytophthora* spp., e ITS, *β-TUB* e *RPB2* para *Lasiodiplodia* spp. As sequências nucleotídicas foram comparadas e adicionadas ao banco de dados do GenBank para análises de Inferência Bayesiana (IB). Entre os 181 isolados de *Phytophthora* coletados de frutos e ramos do cacaueiro, somente *P. palmivora* foi identificada. As análises filogenéticas confirmam que os 166 isolados de *Lasiodiplodia* pertencem às espécies *L. theobromae*, *L. pseudotheobromae*, *L. brasiliensis* e *L. laeliocattleyae*, sendo que as duas últimas são relatadas pela primeira vez como agente causal da morte descendente do cacaueiro. Todas as espécies encontradas foram patogênicas no cacaueiro. O esclarecimento da etiologia da podridão parda e morte descendente é fundamental para o direcionamento dos programas de melhoramento e controle eficiente das doenças do cacaueiro no Equador.

Palavras-chave: Botryosphaeriaceae, doenças de plantas, fungos, Peronosporales, taxonomia.

Orientador – Dr. Danilo Batista Pinho – Universidade de Brasília

GENERAL ABSTRACT

VÉLEZ, Sergio Miguel. **Morpho-Molecular characterization and pathogenicity of *Phytophthora* and *Lasiodiplodia* isolates causing cocoa tree diseases in Ecuador**. 2024. Thesis of Doctorate in Plant Pathology - Universidade de Brasília, Brasília, DF**.**

The cocoa tree (*Theobroma cacao* L.) is the most important species of the genus *Theobroma* due to its wide geographical distribution and global marketing of chocolate produced from the pods. Ecuador occupies the seventh position in the world among cocoa producing countries. The Coast region is the main cocoa producing region of Ecuador, with its culture cultivated in 16 of 24 provinces. Cocoa grown in the coastal region has high productivity and incidence of diseases such as black pod rot and dieback. Black pod rot and canker are caused by *Phytophthora* spp., while a complex of *Lasiodiplodia* spp. causes dieback. The precise identification of these pathogens has been carried out by comparison of morphological characteristics in combination with molecular data from different genomic regions. This approach reveals the discovery of new species and/or new of pathogens in various agricultural cultures. Based on this information, it is believed that black pod and dieback of cacao in Ecuador are caused by different species of *Phytophthora* and *Lasiodiplodia*, respectively. Therefore, the objectives of this work are: (i) identify the species of *Phytophthora* associated with the black pod, (ii) identify the species of *Lasiodiplodia* associated with dieback, (iii) determine the pathogenicity and aggressiveness of the isolates of *Phytophthora* and *Lasiodiplodia* and (iv) update the list of pathogens associated with cocoa in Ecuador. Pods with symptoms of black pod and plant tissues with symptoms of dieback were collected in the provinces of Esmeraldas, Guayas, Los Ríos, Manabí, Morona Santiago, Pichincha, and Santo Domingo de los Tsáchilas for indirect isolation. For prior identification, the partial region of the β-tubulin gene (*Phytophthora* spp.) or elongation factor (*Lasiodiplodia* spp.) was amplified and sequenced. Subsequently, representative isolates were selected for additional sequencing of the genomic regions ITS and *COX2* for *Phytophthora* spp., and ITS, *β -TUB,* and *RPB2* for *Lasiodiplodia* spp. The nucleotide sequences were compared and added to the GenBank database for Bayesian Inference (BI) analyses. Among the 181 isolates of *Phytophthora* collected from pods and stems, only *P. palmivora* was identified. Phylogenetic analyses confirm that the 166 isolates of *Lasiodiplodia* belong to the species *L. theobromae, L. pseudotheobromae, L. brasiliensis,* and *L. laeliocattleyae,* being that the last two were only reported for the first time as the causal agent of dieback of cacao. All species found were pathogenic. The clarification of the etiology of black pod rot, canker, and dieback is essential for the direction of the programs of improvement and efficient control of cacao diseases in Ecuador.

Keywords: Botryosphaeriaceae, plant disease, fungi, Peronosporales, taxonomy.

Advisor – Dr. Danilo Batista Pinho – Universidade de Brasília

CAPÍTULO 1

REVISÃO DE LITERATURA

INTRODUÇÃO

O cacaueiro (*Theobroma cacao* L.) é uma espécie arbórea pertencente à família Malvaceae Bhattacharjee & Akoroda, 2018). Originário da América do Sul, o cacaueiro é nativo da bacia hidrográfica do rio Amazonas (Bayer & Kubitzki 2003; Bartley 2005; Zhang & Motilal, 2016). Devido à sua ampla adaptação edafoclimática e importância econômica, o cacaueiro é cultivado em todas as zonas tropicais úmidas, movimentando bilhões de dólares anualmente com a comercialização das amêndoas na bolsa de valores (Ploetz, 2016).

Em 2022 foram produzidas 5,87 milhões de toneladas de amêndoas de cacau. A Costa do Marfim é o maior produtor mundial com uma produção de dois milhões de toneladas enquanto o Equador ocupa a sétima posição (FAOSTAT, 2024). Aproximadamente 75% do cacau produzido no Equador é destinado para a comercialização do cacau de aroma fino (Afoakwa et al., 2008).

A produção de cacau no Equador é feita em 16 das 24 províncias em uma área de 527.327 ha. As províncias de Guayas, Los Ríos, Manabí, Esmeraldas e El Oro representam 80% da produção, enquanto as províncias de Azuay, Bolívar, Cotopaxi, Orellana, Pichincha, Napo, Sucumbíos e Zamora Chinchipe complementam a produção (INEC, 2020).

A ocorrência de doenças reduz drasticamente a produtividade do cacaueiro, e consequentemente, o retorno econômico da cultura (Bailey & Meinhardt, 2016; . Os principais patógenos da cultura variam de acordo com a localização geográfica dos plantios (Marelli et al., 2019). No Equador, as principais doenças do cacaueiro são: monilíase causada por *Moniliophthora roreri* (Cif.) H.C. Evans, Stalpers, Samson & Benny; vassoura de bruxa causada por *M. perniciosa* (Stahel) Aime & Phillips-Mora; e murcha de Ceratocystis causada por *Ceratocystis cacaofunesta* Engelbr. & T.C.Harr.

Nos últimos anos duas doenças têm se destacado: podridão parda causada por diferentes espécies de *Phytophthora* e a morte descendente causada por *Lasiodiplodia theobromae* .

A podridão parda é a principal doença que afeta o cacaueiro mundialmente. Além dos sintomas nos frutos, também são observados cancros nos ramos e troncos do cacaueiro .

Diferentes espécies de *Phytophthora* têm sido relatadas como causadoras da podridão parda, destacando-se *P. megakarya*, *P. palmivora*, *P. capsici, P. heveae* e recentemente *P. theobromicola* (Akrofi, 2015; Akrofi et al., 2003; S. S. Ali et al., 2016; Erwin & Ribeiro, 1996; Shahin S. Ali et al., 2017; Bahia et al., 2015; Decloquement et. al., 2021). *Phytophthora megakarya* não foi relatada nas Américas, mas é considerada a espécie mais destrutiva na cultura por ocasionar perdas que variam de 60 a 100% da produção (Ali et al. 2017a.

A morte descendente reduz significativamente a produção de cacau em plantas debilitadas devido principalmente a ocorrência de ferimentos, estresse hídrico e deficiência nutricional (Oliveira e Luz, 2005; Bailey & Meinhardt, 2016). O agente causal da doença foi identificado como *Lasiodiplodia theobromae* (Pat) Griffon & Maubl. (Botryosphaeriaceae).

Esse patógeno ocorre em uma ampla gama de hospedeiros de forma endofítica, saprofítica e/ou patogênica . Enquanto o fungo *L. theobromae* causa perdas elevadas no Brasil, Venezuela e Peru, os prejuízos causados pela morte descendente do cacaueiro no Equador ainda são desconhecidos .

A utilização de caracteres morfológicos de forma isolada para identificação de fungos é imprecisa e não recomendável devido à sobreposição de características morfológicas, a variação durante o desenvolvimento do fungo e a ocorrência de espécies crípticas, sendo necessária a inclusão de uma abordagem polifásica para a identificação acurada .

A identificação precisa dos fungos associados a diversos hospedeiros têm sido realizada por comparação de um conjunto de características morfológicas em combinação com dados moleculares de diferentes regiões genômicas .

Essa abordagem demonstra que existe a possibilidade de que várias espécies de fungos estejam associadas a doenças que anteriormente foram relatadas como sendo causadas somente por um fitopatógeno .

A crescente importância econômica do cacaueiro e a ausência de estudos usando uma abordagem polifásica para identificação dos fitopatógenos de plantas no Equador demonstram a importância desse estudo. Portanto, esse trabalho tem como objetivo esclarecer a etiologia da podridão parda e da morte descendente do cacaueiro visando o direcionamento dos programas de melhoramento e controle eficiente das doenças do cacaueiro no Equador.

OBJETIVOS

- Identificar as espécies de *Phytophthora* e *Lasiodiplodia* associadas com podridão parda, cancro e morte descendente do cacaueiro;
- Determinar a patogenicidade das espécies de *Phytophthora* e *Lasiodiplodia*;
- Atualizar a lista de patógenos associados ao cacaueiro no Equador.

Revisão de Literatura

A cultura do cacaueiro

O cacaueiro (*Theobroma cacao* L.) é nativo da região nordeste da América do Sul com evidências arquelógicas há 5300 anos na província de Zamora Chinchipe no sudeste do Equador (Zarrillo et al., 2018). Devido a sua ampla importância econômica se encontra distribuído globalmente, sendo produzido em diferentes ambientes o que tem contribuído ao desenvolvimento de vários materiais genéticos que possuem uma ampla tolerância a diversas condições climáticas (Jaimez et al., 2008).

O futuro da economia mundial do cacau depende significativamente da utilização de germoplasma com uma ampla base genética para criar novas variedades com resistência a doenças e pragas, características de qualidade desejáveis e capacidade de adaptação a ambientes em mudança (Jaimez et al., 2022; Zhang & Motilal 2016).

Doenças do cacaueiro

A distribuição das doenças do cacaueiro está fortemente relacionada à localização geográfica, por exemplo: *Moniliophthora roreri* esta distribuída desde a América do Sul até a América do Norte , *Moniliophthora perniciosa* está presente principalmente na América do Sul ; *Ceratocystis cacaofunesta* Engelbr. & TC Harr. na América Central e do Sul .

Entre as diferentes espécies de *Phytophthora* que causam a podridão parda, *P. megakarya* é a mais agressiva e se encontra restrita ao continente africano (Fig. 1) . A virose causada por *Cocoa swollen shoot virus* é a principal doença que afeta a produção de cacau na África .

A monilíase é a doença mais destrutiva do cacaueiro na América Latina, sendo inicialmente relatada em 1915 no Equador e em seguida no resto da América. No entanto, a a

origem da doença foi considerada no noroeste da Colômbia . Esta doença altamente destrutiva é causada pelo fungo basidiomiceto *Moniliophthora roreri* Cif. e par. (Evans et al., 1978).

Esse fungo fitopatogênico tem a habilidade de afetar os frutos do cacau em diferentes estágios de desenvolvimento, causando sintomas internos e externos . Em relação aos sintomas externos, se observa o aparecimento de manchas aquosas e necróticas, maturação precoce, deformação e inchaço dos frutos enquanto uma massa de esporos é observada nos estágios avançados da doença. Internamente ocorre uma podridão aquosa das amêndoas de cacau .

Outra doença que afeta o cacaueiro é a vassoura de bruxa, causada pelo fungo basidiomiceto hemibiotrófico *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora. Esse fungo possui uma alta variabilidade genética e ocasiona uma das doenças mais devastadoras que afetam o cacaueiro , causando perdas entre 50 a 90% da produção . A doença foi descoberta pela primeira vez no Suriname em 1895 e se espalhou pela Guiana, Equador, Trinidade, Colômbia e Granada nos anos seguintes, até chegar ao Brasil em 1989, causando uma redução drástica da produção brasileira de cacau .

Esse fitopatógeno possui um ciclo de vida composto por duas fases, uma fase saprofítica e uma fase parasítica . A fase parasítica é caracterizada por um micélio monocariótico sem grampos de conexão e com crescimento intercelular. O micélio dicariótico que desenvolve intra e intercelularmente é geralmente mais espesso do que na fase saprofítica e possui grampos de conexão Oliveira & Luz, 2005). No final do ciclo, o fungo produz basidiomas nos tecidos mortos de plantas que foram previamente infectadas.

O basidioma é capaz de produzir milhões de basidiósporos que podem infectar tecidos jovens das plantas . A penetração do fungo ocorre através da base dos tricomas, estômatos,

aberturas naturais, cutícula superficial e o desenvolvimento nos tecidos está amplamente relacionado ao genótipo do hospedeiro, sendo que os sintomas em plantas suscetíveis se desenvolvem rapidamente em relação aos genótipos que possuem alguma resistência .

Figura 1. Sintomas de doenças do cacaueiro no Equador. A) Fruto com podridão parda; B) Cancro em caule e podridão em fruto causados por *Phytophthora* sp.; C) Fruto com monilíase; D) Brotação com vassoura de bruxa; E) Ramos secos causados pela morte descendente; F) Planta morta causada pelo mal do facão.

Fungos da família Botryosphaeriaceae

Os fungos botriosferiaceos podem manter diferentes tipos de relações com as plantas, por exemplo, alguns podem ser endofíticos, ou seja, infectam a planta sem causar sintomas e outros podem causar doenças em uma ampla gama de hospedeiras . Esses fungos causam doenças em vários hospedeiros em ambientes naturais ou áreas cultivadas, e embora sejam mais frequentes em regiões tropicais, com exceção das regiões polares, são relatados mundialmente .

Além disso, em uma mesma planta são encontrados isolados patogênicos e endofíticos de uma mesma espécie fúngica. Essa ampla ocorrência tem estimulado o levantamento preciso dos gêneros e espécies nas principais culturas cultivadas mundialmente .

Esses fungos podem ser considerados patógenos de plantas, pois causam cancros e a morte descendente em uma ampla gama de espécies perenes. Embora sejam fitopatógenos extremamente agressivos em plantas lenhosas, também podem infectar hospedeiros herbáceos de importância agrícola . Em muitos casos, esses fungos sobrevivem endofiticamente nas plantas e em situações de estresse como ferimentos, escassez de água ou nutrientes, se tornam patógenos ao colonizar os tecidos do hospedeiro e causar doenças em frutos e ramos .

Taxonomia de Botryosphaeriales

Botryosphaeriales é um grupo de fungos cosmopolitas com uma ampla distribuição geográfica associados com uma extensa variedade de plantas monocotiledôneas, dicotiledôneas e gimnospermas e que causam doenças em galhos lenhosos, folhas herbáceas, caules, talos de líquens e frutos. No entanto, muitos espécies podem existir endofíticamente em tecidos vegetais sem causar sintomas no hospedeiro .

Com base nas diferenças existentes entre os membros do grupo, do ponto de vista morfológico, filogenético e ecológico, a ordem foi dividida em 9 famílias, compostas principalmente por um ou dois gêneros. Entretanto, a família Botryosphaeriaceae se destaca pela existência de 23 gêneros .

A ordem Botryosphaeriales foi introduzida a partir de um estudo abrangente da classe Dothydeomycetes (Schoch et al. 2006), onde todos os gêneros existentes foram colocados na família Botryosphaeriaceae. Apesar dessa classificação, a família Botryosphaeriaceae sofreu várias alterações ao longo do tempo, principalmente após o surgimento de técnicas moleculares que permitiram uma identificação mais precisa e acurada .

Essa família foi introduzida por Theissen & Sydow em 1918 devido às diferenças existentes entre o pletênquima que forma o ascoma e as bordas dos lóculos (Miller 1928; Miller 1938). Lutrell (1955) estudou os tipos de centrum e a importância taxonômica dos filamentos interascais, propondo a substituição do nome Pseudosphaeriales para Pleosporales. Portanto, o gênero *Botryosphaeria* foi alocado nessa ordem devido à semelhança no desenvolvimento do *centrum* (AJL Phillips et al., 2013).

Posteriormente, outras dúvidas taxonômicas surgiram e a família foi definida pela formação de ascostromas uni ou multiloculares com paredes multicamadas, isolados ou agregados em um estroma pulvinado, frequentemente formando um conidioma inserido no hospedeiro a partir de um estroma basal que se abre na maturidade (Miller, 1928, Luttrell, 1951, 1955; Eriksson, 1981; Sivanesan, 1984).

As formas assexuadas da família Botryosphaeriaceae incluem vários gêneros, entre os quais podemos citar: *Aplosporella, Diplodia, Dothiorella, Fusicoccum, Lasiodiplodia,*

Macrophomina, *Microdiplodia*, *Neofusicoccum*, *Neoscytalidium*, *Pseudofusicoccum* e *Sphaeropsis* . Esses gêneros são morfologicamente reconhecidos a partir da cor, forma, ausência e presença de septos e estrias nos conídios maduros.

No passado, a principal forma de identificar esses fungos era por meio de características morfológicas como a forma (ovóides ou oblongos) e a pigmentação (hialinos ou pigmentados) dos ascósporos (Luttrell, 1955; Eriksson, 1981; Sivanesan, 1984). Em algumas situações, a identificação morfológica causava confusão taxonômica devido à semelhança de características entre gêneros diferentes. Os fungos que possuíam ascósporos escuros foram classificados em três gêneros distintos a partir de uma árvore filogenética da família Botryosphaeriaceae .

Os gêneros da família Botryosphaeriaceae foram propostos principalmente a partir da observação das características morfológicas em um período que não se armazenava as culturas fúngicas . A existência de holótipos deteriorados em herbários e a plasticidade morfológica de espécies de acordo com o ambiente e a hospedeira limitam os estudos taxonômicos devido à dificuldade de comparação dos espécimes . Para corrigir esse problema, espécimes referência ou ex-types podem ser designados desde que as coletas sejam realizadas na mesma hospedeira e região geográfica do holótipo e a morfologia seja semelhante.

A caracterização molecular dos fungos botriosfaeriaceos revelou a existência de gêneros desconhecidos e espécies crípticas . Alguns estudos propuseram a ocorrência de híbridos entre as espécies de *Lasiodiplodia*, mas as informações não foram suficientes para confirmar essa hipótese (Cruywagen et al., 2017; Rodríguez-Gálvez et al., 2017).

Um dos primeiros trabalhos utilizando sequências da região ITS para inferir o relacionamento filogenético das formas assexuais de *Botryosphaeria* comparou os grupos filogenéticos com características morfológicas de culturas e conídios (Denman et al., 2000). A partir desse estudo houve um aumento elevado de sequências de DNA de fungos botriosfaeriaceos depositados no GenBank.

No último levantamento mais de 10.000 sequências de diferentes regiões genômicas foram utilizadas em estudos sobre a sistemática e taxonomia da ordem Botryosphaeriales (Slippers et al., 2017). Esse avanço permitiu a segregação de *Botryosphaeria* em outros gêneros da família Botryosphaeriaceae, sendo que anteriormente todos espécimes sem fase sexual eram classificados nesse gênero (Phillips et al., 2019).

Caracterização molecular da ordem Botryosphaeriales

O uso de sequências do espaçador interno transcrito (ITS) para a identificação dos gêneros da família Botryosphaeriaceae foi essencial para esclarecer conflitos taxonômicos baseados em características morfológicas (Slippers et al., 2014; White et al., 1990). A utilização dessa região genômica permitiu separar *B. corticola* e *B. stevensii* associadas com a morte descendente e o cancro do carvalho (Alves et al., 2004). Além disso, novas espécies e novas relações entre o fungo e a hospedeira foram possíveis com a utilização da região ITS (Alves et al., 2013). Apesar da recomendação da região ITS para a caracterização molecular dos isolados da ordem Botryosphaeriales, a sua utilidade é limitada, uma vez que o sinal filogenético dessa região não é suficiente para separar espécies filogeneticamente próximas .

A identificação precisa das espécies da ordem Botryosphaeriales é feita por meio da análise multigênica das regiões ITS, *β-tubulina* e *TEF1*–α . No entanto, para alguns gêneros são necessárias outras regiões genômicas para a identificação precisa das espécies .

Para *Diplodia* Fr, *Neofusicoccum* Crous e *Saccharata* Denman & Crous é recomendado a combinação das regiões LSU-*RPB2* e *β-TUB*. Portanto, as regiões genômicas recomendadas para a identificação de espécies variam de acordo com o gênero em estudo, sendo que, pelo menos, cinco regiões genômicas são necessárias para identificação precisa de espécies da ordem Botryosphaeriales .

Distribuição Geográfica dos fungos da família Botryosphaeriaceae

A distribuição geográfica dos fungos da família Botryosphaeriaceae varia de acordo com as espécies. Existem espécies com ampla distribuição mundial enquanto outras são restritas a determinadas áreas ou países. O fungo *Lasiodiplodia theobromae* é altamente cosmopolita e associado a uma ampla gama de hospedeiros, como endófito ou fitopatógeno .

Os fungos *Diplodia* e *Neofuscicoccum* são encontrados associados com plantas de diferentes famílias botânicas que ocorrem em amplitudes variadas . *Botryoshaeria dothidea*, *Lasiodiplodia pseudotheobromae* e *Saccharata viticola* possuem uma ampla distribuição geográfica e têm a capacidade de mover entre diferentes hospedeiros.

Contrariamente, algumas espécies são limitadas a poucos hospedeiros e possuem uma distribuição geográfica limitada, ocorrendo em áreas específicas. Essas condições favorecem a hipótese na qual a localização geográfica contribui significativamente para a diversidade de espécies fúngicas (Slippers et al., 2014).

Lasiodiplodia

O fungo *Lasiodiplodia* é classificado na classe Dothideomycetes, da ordem Botryosphaeriales, família Botryosphaeriaceae (Sutton, 1980). Este gênero foi introduzido em 1894 por Ellis considerando *L. tubericola* como a espécie-tipo, embora Ellis não a tenha descrito.

A fase sexual de *L. theobromae* era considerada *Botryosphaeria rodina* Berk e MA Curtis. No entanto, trabalhos recentes afirmam que a ausência de observação da fase sexual em estudos taxonômicos indicam a necessidade de comprovar essa associação .

Botryodiplodia theobromae foi sinonimizado com *Lasiodiplodia theobromae* devido à presença de paráfises nos picnídios, sendo que *L. theobromae* foi considerado a espécie tipo do gênero apesar da inexistência do holótipo (Phillips et al., 2013). Da mesma forma foi proposto a sinonimização com *Diplodia* (Denman et al., 2000), mas estudos filogenéticos utilizando a região ITS comprovaram que esses fungos são distintos. Além disso, os conídios de *Lasiodiplodia* possuem estrias longitudinais que os diferenciam de *Diplodia* .

Aproximadamente 20 espécies desse gênero foram descritas com base nas características morfológicas dos conídios e das parafíses . *Lasiodiplodia* produz micélio imerso ou superficial, ramificado, septado, marrom escuro a preto; Ascoma de cor marrom escuro a preto, uniloculado com parede pseudoparenquimatosa espessa, ostiolada, imerso no substrato e parcialmente eruptivo; Pseudoparáfises hialinas, septadas; Asca bitunicada com endotúnica espessa e câmara apical bem desembrulhada, pregada, estipulada, de 90 a 120 μm de comprimento; Ascósporo irregularmente bisseriado, inicialmente hialino, ficando marrom escuro; Conidioma estromático, imerso ou superficial, separado ou aglomerado, confluente,

globoso, castanho escuro, uni ou multiloculado, parede marrom escuro, com paredes grossas de textura angular, pálido e muito fino na direção da região da conidiogênese, freqüentemente com hifas marron escuras na superfície; Ostíolo central, solitário, papilado .

Os conidióforos são hialinos, simples, raramente ramificados e muitas vezes reduzidos a células conidiogênicas cilíndricas que podem ser ramificadas, hialinas, holoblásticas, lisas, cilíndricas a sub-obpiriforme, de paredes espessas, com um ou dois anéis ou células em proliferação no mesmo nível, dando origem ao espessamento periclinal, formado principalmente por células que revestem a parede interna dos conidiomas. Conídio inicialmente hialino e de parede espessa que forma um septo na região mediana e possui coloração escura e estrias longitudinais com a maturidade. Os conídios maduros possuem paredes grossas e são oblongos à elipsoides; Paráfises hialinas, cilíndricas e septadas .

Distribuição geográfica de *Lasiodiplodia*

Nas Américas, várias espécies de *Lasiodiplodia* têm sido descritas, como: *L. brasiliensis, L. caatinguensis, L. crassispora, L. euphorbicola, L. gonubiensis, L. gravistriata, L. iraniensis, L. jatrophicola, L. laeliocattleyae, L. macrospora, L. marypalmiae, L. parva, L. pontae, L. pseudotheobromae, L. subglobosa, L. theobromae, L. venezuelensis* e *L. viticola.*.

O Brasil é o país com o maior número de estudos sobre a identificação das espécies de *Lasiodiplodia* em hospedeiros pertencentes a diferentes famílias botânicas, como: *Anacardium occidentalis, Annona* spp., *Carica papaya*, *Citrullus lanatus*, *Cucumis melo*, *Citrus aurantium* L., *C. aurantifolia*, *C. sinensis*, *Cocos nucifera*, *Hibiscus sabdarifa* L., *Jatropha curcas*, *Malus domestica*, *Malpighia glabra*, *Manilkara zapota*, *Mangifera indica*, *Nephelium lappaceum* L.,

Passiflora edulis, *Persea americana*, *Pinus pseudostrobus*, *Pouteria sapota*, *Ricinus comunis*, *Tectona grandis*, *Theobroma cacao*, *Zea mays* e outras espécies silvestres .

No passado, a maioria das identificações foram feitas por meio da caracterização morfométrica, na qual a identificação acurada não é possível devido à existência de espécies crípticas. Para reduzir as identificações errôneas, comparações morfológicas são realizadas em combinação com comparações moleculares (Alves et al., 2008). Essa nova abordagem tem revelado a descoberta de novas espécies. Por exemplo, *L. gonubiensis* foi descrita infectando árvores nativas de *Syzigium cordatum* na África do Sul por meio da comparação de sequências da região ITS do rDNA (Draginja Pavlic et al., 2004).

A região ITS do rDNA também foi utilizada para confirmar *L. theobromae* como agente causal da morte descendente do cacau nas Filipinas (Alvindia & Gallema, 2017) e infectando sementes de *Pinus* sp. no estado do Rio Grande do Sul, no Brasil .

A identificação acurada das espécies de *Lasiodiplodia* é feita por comparações morfológicas combinadas com análises filogenéticas de duas ou mais regiões genômicas . Entre as diferentes regiões genômicas, o gene fator de elongação tem o melhor sinal filogenético para a distinção das espécies de *Lasiodiplodia* (Machado et al., 2014).

As espécies *L. crassispora*, *L. rubropurpurea* e *L. venezuelensis* foram descobertas por análises filogenéticas das regiões ITS e *EF1*-α (Burgess et al., 2006). A análise multigênica das regiões ITS, *EF1*-α e beta-tubulina permitiram identificar *L. brasiliense*, *L. caatinguensis*, *L. euphorbicola*, *L. pontae*, *L. pseudotheobromae* e *L. theobromae* em diferentes espécies frutíferas, incluindo *Annona* spp., *Citrus sinensis*, *Spondias purpurea* e *Tamarindus indica* na

região nordeste do Brasil, sendo que *L. caatinguensis* e *L. pontae* foram propostas como duas novas espécies (Coutinho et al., 2017).

A diversidade de espécies de *Lasiodiplodia* causando podridão peduncular no mamoeiro foi estudada por meio de comparações morfológicas e análises filogenéticas das regiões ITS e *EF1*-α. Nesse estudo, *L. brasiliense*, *L. hormozganensis*, *L. marypalme*, *L. pseudotheobromae* e *L. theobromae* foram identificadas, sendo que *L. brasiliense* e *L. marypalme* foram propostas como novas espécies (Netto et al., 2014). Um estudo semelhante realizado para a determinação das espécies associadas à morte descendente de uvas de mesa revelou oito espécies de *Lasiodiplodia*, dentre as quais *L. brasiliense*, *L. laeliocattleyae* (=*L. egyptiacae*), *L. euphorbicola*, *L. hormozganensis* e *L. jatrophicola* foram relatadas pela primeira vez causando a morte descendente da uva no mundo (Correia et al., 2016).

Os marcadores moleculares SSR (single sequence repeat) foram utilizados para compreender a variabilidade inter e intraespecífica da família Botryosphaeriaceae (Mohali et al., 2008). A diversidade e o fluxo gênico entre as populações de *L. theobromae* da Venezuela, México e África do Sul revelaram 64 alelos em 8 locus analisados, evidenciando diferenças entre as populações de cada país, sendo que a diversidade genotípica das populações da Venezuela e África do Sul é relativamente baixa.

O estudo de isolados de *L. theobromae* obtidos de *Pinus* sp. no México, Indonésia e África do Sul usando 8 marcadores SSR confirma uma maior semelhança em relação à hospedeira do que a origem geográfica (Burgess, Wingfield, Wingfield 2003). Adicionalmente, as populações de *Diplodia pinea* da Austrália, Argentina, Etiópia e África do Sul confirmam

essa hipótese, uma vez que foi observada uma alta taxa de recombinação entre as diferentes populações (Bihon et al., 2012).

As informações sobre a taxonomia de *Lasiodiplodia* confirmam a necessidade da utilização de comparações morfológicas com análises filogenéticas de diferentes regiões genômicas para identificação acurada das espécies crípticas . A distinção de *L. theobromae* e *L. pseudotheobromae* foi comprovada após análises filogenéticas de diferentes regiões genômicas e diferenças entre as populações obtidas de *Theobroma cacao* e *Terminalia* spp. nos Camarões . Após a descoberta de *L. hormozganensis* a partir de amostras de diferentes hospedeiras e regiões geográficas descobriu-se que vários isolados previamente identificados como *L. theobromae* pertencem a essa espécie .

Apesar da importância das ferramentas moleculares, o sequenciamento das regiões genômicas recomendadas para cada gênero da família Botryosphaeriaceae é fundamental para a identificação acurada. Para o gênero *Neofusicoccum*, a região ITS não é suficiente para a discriminação de espécies enquanto a subunidade maior da RNA polimerase II (*RPB2*) possui o melhor sinal filogenético (Pavlic et al., 2009).

Alguns estudos propuseram a ocorrência de espécies híbridas de *Lasiodiplodia* devido inconsistências nas árvores filogenéticas individuais das diferentes regiões genômicas (ITS, *EF1*-α, *β-TUB, RPB2* e *CmdA*) analisadas (Cruywagen et al., 2017; Rodríguez-Gálvez et al., 2017). No entanto, essa hipótese não foi aceita pela comunidade científica e uma análise concatenada de quatro regiões genômicas (ITS, *EF1*-α, *β-TUB* e *RPB2*) foi proposta para a identificação acurada das diferentes espécies de *Lasiodiplodia* (Rahim et al., 2022; Rathnayaka et al., 2023).

***Lasiodiplodia* em Cacau**

A espécie *L. theobromae* é a mais frequente e distribuída mundialmente em associação com o cacaueiro , na forma endofítica , ou causando sintomas de morte descendente, cancro no caule e/ou podridão do fruto (Fig. 2) Bailey & Meinhardt, 2016; Serrato-Díaz, 2020). No continente africano, essa espécie foi relatada causando a morte descendente em Camarões enquanto que em Gana, *L. pseudotheobromae* e *L. theobromae* foram relatadas .

Lasiodiplodia theobromae também foi relatada em vários países do continente Asiático, como Índia , Indonésia e Filipinas . Nesse último país, o sintoma observado foi uma clorose nas folhas do cacaueiro denominada morte descendente vascular.

A etiologia da podridão negra dos frutos e cancro no caule do cacaueiro na Malásia foi confirmada como *L. theobromae* por meio de comparações morfológicas e análises filogenéticas *utilizando* as regiões genômicas ITS, *tef1*-α, *tub2* e *rpb2* . Em Porto Rico, *L. pseudotheobromae* foi identificada causando a podridão negra por análises filogenéticas das regiões ITS, *tef1*-α e *tub2* (Serrato-Díaz, 2020).

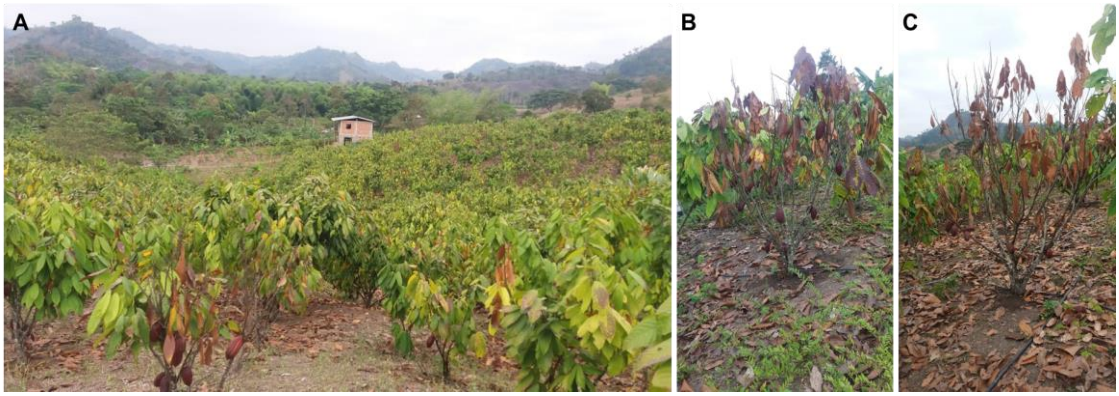

Figura 2. Plantas com sintomas de morte descendente provocada por *Lasiodiplodia* spp. A) Plantas de cacau sintomáticas em plantio com 4 anos de idade; B) Planta com morte parcial C) Planta com sintomas avançados

As informações existentes sobre *Lasiodiplodia* causando sintomas de morte descendente e podridão do fruto no Equador são escassas. Os estudos existentes relatam a associação endofítica de *L. theobromae* em *Theobroma gileri* (Evans et al., 2003) ou causando morte descendente (Solis, et al., 2021), podridão negra em frutos (Iniap, 2010) e morte de mudas enxertadas de cacaueiro .

Apesar dos estudos existentes, a caracterização morfo-molecular e os testes de patogenicidade foram realizados para poucos isolados. No Equador, apenas *L. theobromae* e *L. pseudotheobromae* foram identificadas causando à morte descendente em *Schizolobium parahyba* por análises filogenéticas das regiões ITS, EF1-α e BT2 (Dissanayake et al., 2016). Embora *L. theobromae* seja a espécie predominante no Equador, *L. pseudotheobromae* e *Neofusicoccum parvum* também foram relatadas causando morte descendente em plantas .

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CAPÍTULO 2

Molecular characterization and pathogenicity of *Phytophthora palmivora* isolates causing cocoa diseases in Ecuador

Molecular characterization and pathogenicity of *Phytophthora palmivora* isolates causing cocoa diseases in Ecuador

Abstract

Black pod disease and stem canker of cocoa are major diseases that affect crops in Ecuador and are caused by *Phytophthora* species. This study aimed to identify the *Phytophthora* species associated with black pod rot and stem canker and to determine the pathogenicity and aggressiveness of *Phytophthora* isolates on pods and seedlings. Isolates were collected from five provinces, then, amplified and sequenced the partial region of the *β-tubulin* gene for initial identification and subsequently sequenced the ITS and *COX1* regions of representative isolates. Pathogenicity and aggressiveness tests were conducted on pods and seedlings of the CCN51 clone. Out of the 181 isolates collected from different locations, only *P. palmivora* was identified. In this study, all tested isolates were found to produce lesions on pods and stems. According to molecular data, *P. palmivora* is the causal agent of black pod rot and cacao canker in Ecuador. This discovery provides critical information for pathogen understanding and can aid in integrated disease management.

Keywords: Ecuador, black pod rot, canker, Peronosporales, taxonomy.

Introduction

Cocoa is a plant species native to the tropical rainforests of South America. It belongs to the Malvaceae family and is widely cultivated across many continents (Asigbaase et al., 2019; Wessel & Quist-Wessel, 2015). Cocoa production in Ecuador is mainly concentrated in the provinces of Los Ríos, Manabí, and Guayas, situated along the coast. In 2022, the country produced 337,149 tons of cocoa, which were grown on 509,179 hectares (INEC, 2020). Ecuador is positioned in the seventh world ranking of cocoa-producing countries (FAOSTAT 2024).

Various factors can affect the yield of cocoa crops, one of which is the presence of diseases caused by plant pathogens (Díaz-Valderrama et al., 2022; Espinal et al., 2023; Huda-Shakirah et al., 2022; Lisboa et al., 2020), like the phytopathogen oomycete *Phytophthora*, which is the causal agent of black pod rot and stem canker in cocoa plants (Abad, Burgess,

Bourret, et al., 2023; Ali, Shao, Lary, Kronmiller, Shen, Strem, Amoako-Attah, et al., 2017; Appiah Ab et al., 2004; Morales-Cruz et al., 2020; Puig, Quintanilla, et al., 2021; Marelli et al., 2019). These diseases that affect cocoa production worldwide and causes significant losses in various areas. In addition, *Phytophthora* spp. infect the pods and can also cause infections in branches and stems (Perrine-Walker, 2020; Saul Maora et al., 2017).

Several species of *Phytophthora* are known to cause those diseases, including *P. megakarya*, which is the most aggressive one and can cause yield losses of 60 to 100%. *P. megakarya* is widely distributed in Africa (Akrofi, 2015; Ali, Shao, Lary, Kronmiller, Shen, Strem, Amoako-Attah, et al., 2017; Mbarga et al., 2014; Morales-Cruz et al., 2020; Ndoungué Djeumekop et al., 2021), while in America, *P. capsici* Leonian, *P. citrophthora* Leonian (Kellam & Zentmyer 1986), *P. megasperma* Dreschler, and *P. hevea* Thompson are described (Erwin & Ribeiro, 1996; Lozano & Romero, 1974; Reyes & Capriles, 2000). In Asia, *P. hevea* in Malaysia and *P. katsurae* in India and Sri Lanka have been reported (Stamps et al., 1990; Ko & Wang, 2006). Recently, *P. tropicalis* was described in México (Chávez-Ramírez et al., 2021) and a new species called *P. theobromicola* was discovered in Bahia state, Brazil (Decloquement et al., 2021).

In the past, identifying *Phytophthora* species used to be done only through the analysis of the morphological characteristics, like the size and shape of the sporangium, oospores and chlamydospores, and cultural characteristics (Kaosiri et al., n.d.; Levin et al., 2001; Lg et al., 2012). However, this method of identification has its limitations and may lead to incorrect identification (Saul Maora et al., 2017; Yang et al., 2017). To avoid this, researchers now use a phylogenetic analysis of sequences of several gene regions of *Phytophthora* DNA for more precise identification and these molecular techniques have been used in various studies (Abad, Burgess, Redford, et al., 2023; Appiah Ab et al., 2004; Bawage et al., 2013; Bivi et al., 2013; Maizatul-Suriza et al., 2019; Pandey et al., 2015).

The use of both morphological characteristics and the analysis of specific genomic regions (such as internal transcribed spacer (ITS), beta-tubulin (*β-TUB)*, elongation factor (*TEF-1α)*, and Cytochrome oxidase I and II (*COXI* and *COXII*) can accurately diagnose species of the *Phytophthora* that cause diseases worldwide in different crops (Barboza et al., 2020;

Decloquement et al., 2021; Ghaderi & Habibi, 2021; Rodríguez-Polanco et al., 2020; Tabima et al., 2021).

In Ecuador, cocoa production is economically important, however, the black pod rot disease causes significant damage to the crop, unfortunately, there is not much detailed information available on the phylogeny, diversity, and virulence of *Phytophthora*, the causal agent of the disease (Abad et al., 2018; Freile-Almeida et al., 2018; Ordoñez et al., 2016). For this reason, it is crucial to conduct research to correctly identify the species involved in causing these diseases (Burgess et al., 2021; Chen et al., 2023; Puig, Keith, et al., 2021; Rêgo et al., 2023).

This research will help to develop more precise control strategies and genetic improvement programs to enhance the crop's resistance to the disease in the future. In this context, this study aimed to identify the species of *Phytophthora* found in Ecuador's cocoa-producing regions through the analysis of their morphological features and sequencing of the ITS, *COXI*, and *beta-tubulin* regions. Additionally, the research establishes the genetic diversity of this pathogen and determines the pathogenicity and aggressiveness of selected isolates on both cocoa pods and plants.

Material and Methods

Sample Collection

From January to May 2018, 2019, and 2020, cocoa pods and stems with symptoms of black pod disease and stem canker caused by *Phytophthora* spp. were collected in five provinces in Ecuador: Guayas, Esmeraldas, Manabí, Los Ríos, and Pichincha. The pods and stems were collected using pruning shears that were disinfected with 70% alcohol or 3% v/v sodium hypochlorite to prevent contamination between samples. The samples were placed in paper bags, stored in coolers, and transported to the Molecular Biology Laboratory of the Escuela Superior Politécnica Agropecuaria de Manabí Manuel Félix López, located in Calceta, Manabí province, Ecuador.

Isolation

The pods and stems showing symptoms, were washed with tap water and detergent for about 3 minutes to remove any impurities from the field. They were then placed on paper towels

to dry. The intersection area between the healthy and symptomatic tissue was cut into fragments of size 1 x 1 cm. These fragments were disinfected with 70% alcohol for 1 minute, followed by 2% sodium hypochlorite for 1.5 minutes. They were then rinsed with distilled water 3 consecutive times and placed on sterile paper towels to dry. The fragments were carefully placed in Carrot Agar medium (CA), enriched with ampicillin, gentamicin and Pentachloronitrobenzene fungicide at concentrations of 1, 10, and 100 mg/L. The fragments were kept at 25°C for 48 hours.

DNA extraction

To obtain genetic purity of the isolates, a hypha tip was performed for the growth of *Phytophthora* isolates. The isolates were then placed on agar carrot (CA) covered with cellophane previously sterilized at 121 ° C for 20 minutes (Scanu et al., 2014). The monohyphae culture was let to grow in BOD chamber at 25 ± 2 ° C for 3-5 days. Using a sterile toothpick, part of the mycelial growth was removed and placed in 1.5 ml microtubes containing 50 ml of Tris-EDTA (TE) buffer, four metal beads (2.8 mm), and 600 ml of Nuclei Lysis Solution (Promega).

The total DNA was extracted using the protocol of Wizard Genomic DNA Purification Kit (Promega) according to the methodology described by manufacturers. The quality of the DNA was assessed by visualizing them on a 1 % agarose electrophoresis gel, stained with GelRed (Biotium), and visualized under UV light. The samples were stored at -20°C for further use.

Amplification and sequencing

The Polymerase Chain Reaction (PCR) was performed to amplify the genomic regions. Initially, a partial region of the *β-tubulin* gene was amplified and sequenced using the primer set BTub_F1/TUBUR1 (Blair et al., 2008). To confirm identification, representative isolates from each location were then selected for amplification and sequencing of the internal transcribed spacers of the nuclear ribosomal DNA (ITS) and cytochrome c oxidase subunit I (*COXI*). The ITS and *COXI* were amplified using the primers set DC6/LR0 (Choi et al., 2006) and OomCoxILevup/Fm85mod (Robideau et al., 2011).

The PCR amplifications were performed in a final volume of 12.5µL, containing 6.25 ul of MyTaq MasterMix 2x, 0.3 ul of each primer, 4.25 ul ultrapure water (Milli-Q), and 1 ul of

template DNA (25 ng/μL). The PCR cycles were carried out at initial denaturation at 95°C for 1.5 min, followed by 35 cycles at 95°C for 20 s, 60°C (*β-tubulin*) for 45 s, and 72°C for 1 min, and final extension at 72°C for 5 min. The annealing temperature varied according to the genomic region to be amplified: 50°C (*COXII*), and 58°C (ITS).

Additionally, the extension times were different. The PCR products were then analyzed through agarose electrophoresis gel of 1% stained with GelRed (Biotium) and visualized under UV light. Finally, the PCR products were purified and bidirectionally Sanger sequenced at Macrogen Inc., (South Korea), and the sequences obtained were assembled and manually edited with DNA Dragon.

Sequence analysis and phylogeny

Electropherograms were visualized using DNA Dragon software (www.sequentix.de). Representative isolates were then selected to create a concatenated phylogenetic tree with the *COX* and ITS regions. Additionally, sequences of reference isolates of *Phytophthora* species were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov). The sequence of the isolate P3980 (*Phytopythium vexans*) was used as an outgroup and aligned with the new sequences generated using the MUSCLE program (Edgar, 2004), implemented in the MEGA 7 (Molecular Evolutionary Genetics Analysis) software (Tamura et al., 2011).

The program MrModeltest 2.3 (Posada & Buckley, 2004) was used to determine the nucleotide substitution patterns for each partition. The selected patterns were based on the Akaike Information Criterion (AIC) (Guindon & Gascuel, 2003). Next, the Bayesian analysis was performed using MrBayes v. 3.1.2, which was provided by The Cipres web portal (Miller et al., 2010), through the Markov Chain Monte Carlo (MCMC) method. Initially, the analysis was done with each genomic region separately, and then with the concatenated sequences that were defined earlier, with a total of 1000000 generations, sampling every 1000 generations.

The maximum likelihood analysis (ML) was reconstructed with the RAxML 8.2.9 algorithm (Stamatakis, 2014). The bootstrap support values were calculated using 1000 replicates. The phylogenetic trees were visualized and processed with Figtree V1.4 (Rambaut 2018) and Inkscape.

Pathogenicity tests

Representative isolates (n=18) that were previously identified as *P. palmivora* were selected from each location and tissue (pod and stem) to evaluate the pathogenicity and aggressiveness on susceptible cocoa clone CCN51. The experiment was designed entirely randomized. Asymptomatic pods that were 4-5 months old were washed with a stream of tap water and liquid soap, and then dried with a paper towel. The pods were wounded at points equidistant to 5 cm approximately from each peduncle and tip using a sterile needle. Then, a mycelial plug (5 mm) that had grown for 5 days on CA at 27 ±1 °C was placed on the wounded areas. The treatments consisted of 4 replicates of each isolate, and for the mock treatment, a CA plug without growing mycelium was used. The pods were then placed in a humid chamber and maintained at 25°C ± 1°C for 7 days after inoculation. The progress of the lesion was evaluated daily for 5 days after an incubation period of 48 hours. Each pod was considered an experimental unit.

To assess the pathogenicity on 3-month-old seedlings of clone CCN51, 18 isolates were used. The seedlings were wounded on the stem base and 5 mm mycelial plugs, grown on CA at 27 ± 1 °C for 5 days, were placed on the wounded areas. The treatments included 4 replicates of each *Phytophthora* isolate and a mock treatment with a CA plug without growing mycelium. Cotton fragments moistened with sterilized distilled water were placed over the mycelial plugs, and the seedlings were kept under greenhouse conditions at 28 °C ± 2°C, for two months after inoculation. Each seedling was considered an experimental unit.

For the evaluation of the aggressiveness of the isolates on cacao pods from the CCN51 clone, a completely random design and variance analysis were used; for the means separation procedure, Tukey was used at 5% of the error probabilities.

Results

Obtaining *Phytophthora* isolates

In this study, pods and trunks with symptoms of black pod rot were identified based on their visual characteristics, such as brown spots and white mycelial production. The identification was later confirmed through molecular analysis of partial sequences of *beta-tubulin*, which revealed the presence of *Phytophthora* sp. A total of 181 isolates were collected

from various provinces between 2018 and 2020, with the majority coming from Guayas (89), Esmeraldas (37), Manabí (27), Los Ríos (26), and Pichincha (2). Out of these, 171 isolates were obtained from pods, while the remaining 10 were collected from stems. (Figure 2).

Phylogenetic Relationship

The initial identification was conducted using partial sequences of *beta-tubulin* of each of the 181 isolates obtained, each sequence generated was submitted the Blastn algorithm that grouped those isolates inner the *Phytophthora* genus. The aligned sequences had a length of 1150 bp, of which 806 and 250 were classified as conserved and parsimony sites, respectively. A Bayesian inference was performed using a GTR+ I+ G substitution model and the generated phylogenetic tree showed that all *Phytophthora* isolates belonged to a single clade, corresponding to *P. palmivora*.

Subsequently, representative isolates of *Phytophthora* sp. were selected to compose the multilocus analysis. All new sequences generated were deposited in Genbank. For multilocus analysis, other partial sequences of *β-tubulin*, COXI, and ITS were downloaded from Genbank and included to the dataset. The dataset was composed by 37 isolates where concatenated alignment resulted in a sequence of 2054 characters, with 1434 and 568 conserved and variable sites, respectively. Furthermore, 190 sites were considered to be phylogenetically informative.

Table 1. List of accessions from species of Phytophthora used for the phylogenetic analysis of BTUB, ITS, and COX1 regions. Accessions identified as the same species are not obligatory *from the same isolate*

***Phytophthora* species**	**Isolate code**	**Host**	**Location of origin**
P. alticola	CBS141718	*Eucaliptus grandis*	South Africa
P. alticola	CMW34279	*Eucaliptus dunni*	South Africa
P. boodjera	VHS26806	*soil dump*	Australia
P. boodjera	VHS27017	*Eucaliptus* sp.	Australia
P arenaria	P19599	*E. drummondii (*soil samples*)*	United States
P arenaria	CBS 127950	*E. drummondii*	Australia
P. quercetorum	CBS 121119	*Quercus rubra*	United States
P. quercetorum	P15555	*Quercus rubra*	United States
P. litchi	P19950	*Litchi chinensis*	Taiwan
P. litchi	P15218	*Litchi chinensis*	Taiwan
P. megakarya	P1664	*Theobroma cacao*	Nigeria
P. megakarya	P8516	*Theobroma cacao*	Sao Tome and Principe
P. cathayensis	CP29	*Carya cathayensis*	China

P. cathayensis	CP30	*Carya cathayensis*	China
P. palmivora	P0633	*Areca catechu*	India
P. palmivora	P3738	*Cocos nucifera*	Indonesia
P. palmivora	P0255	*Theobroma cacao*	Costa Rica
P. palmivora	2547	*Theobroma cacao*	Ecuador
P. palmivora	2584	*Theobroma cacao*	Ecuador
P. palmivora	2588	*Theobroma cacao*	Ecuador
P. palmivora	2594	*Theobroma cacao*	Ecuador
P. palmivora	3440	*Theobroma cacao*	Ecuador
P. palmivora	3448	*Theobroma cacao*	Ecuador
P. palmivora	3456	*Theobroma cacao*	Ecuador
P. palmivora	3459	*Theobroma cacao*	Ecuador
P. palmivora	3460	*Theobroma cacao*	Ecuador
P. palmivora	3462	*Theobroma cacao*	Ecuador
P. palmivora	3463	*Theobroma cacao*	Ecuador
P. palmivora	3469	*Theobroma cacao*	Ecuador
P. palmivora	3471	*Theobroma cacao*	Ecuador
P. palmivora	3479	*Theobroma cacao*	Ecuador
P. palmivora	3486	*Theobroma cacao*	Ecuador
P. palmivora	3546	*Theobroma cacao*	Ecuador
P. palmivora	3549	*Theobroma cacao*	Ecuador
P. palmivora	3555	*Theobroma cacao*	Ecuador
P. palmivora	3561	*Theobroma cacao*	Ecuador

The best nucleotide substitution model for each partition in the concatenate data was considered to generate the phylogenetic tree, GTR+I+G (*BTUB*, *COXI*, ITS). The *Phytophthora* isolates obtained here belonged to only one phylogenetic clade, which was grouped with *P. palmivora* (clade 4).

Pathogenicity test

All inoculated isolates of *P. palmivora* caused initial symptoms of brown spots within 48 hours of inoculation with mycelial plugs in CCN51 pods. The presence of *P. palmivora* in pods inoculated artificially was confirmed by re-isolating in selective culture media CA agar. The isolates CCUB3471, CCUB2584, and CCUB3463 were found to have the highest aggressiveness averages, inducing the more extended diameters of lesions in pods with 143; 143.9, and 143.7 mm respectively. In contrast, CCUB3546 and CCUB3469 induced smaller lesions with 122.2 and 119 mm in diameter, at the seventh day after inoculation. Generally, all

remaining isolates of *P. palmivora* showed values of aggressiveness with statistical differences on pods of CCN51.

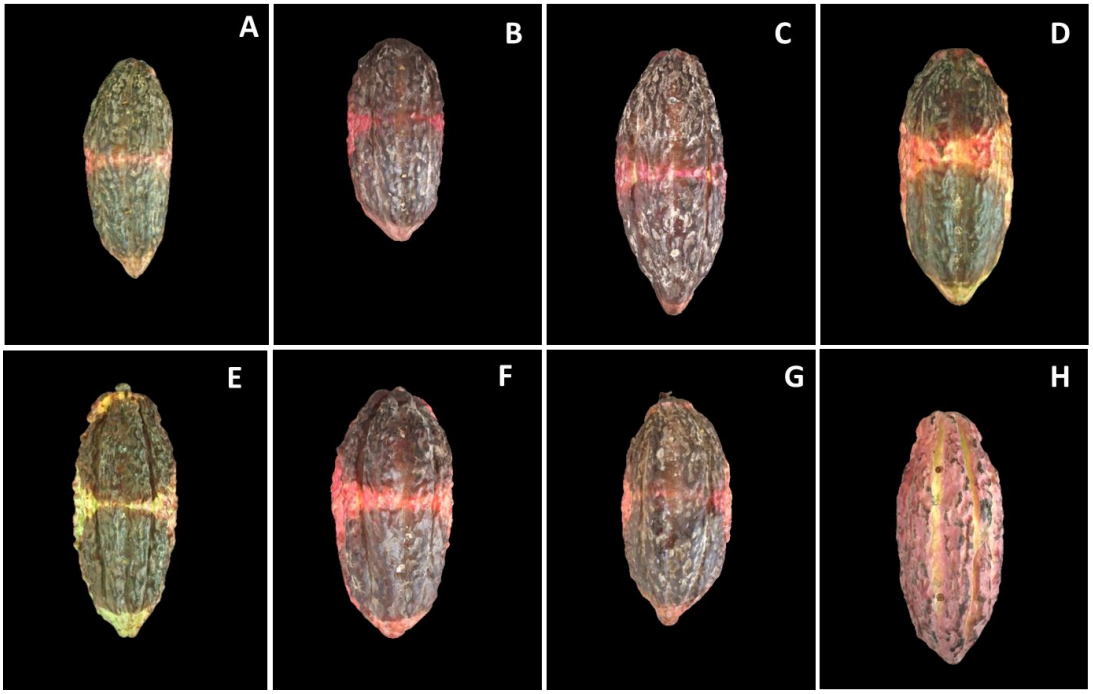

Figure 1. Artificial inoculation of *Phytophthora palmivora* on detached, asymptomatic cocoa pods at 6 days after inoculation: **A**, 2584. **B**, 2588. **C**, 2594. **D**, 3459. **E**, 3462. **F**,3464. **G**, 3546. **H**, uninoculated (control) pods.

All *P. palmivora* isolates inoculated on CCN51 seedlings showed pathogenicity. Symptoms such as yellowing and loss of turgency in the leaves were observed 10 days after inoculation in 10% of the seedlings. Later, symptoms such as stem rot, wilt, and death were noticeable between 14 and 60 days. The mock treatment, which used seedlings with CA discs without mycelium, showed no visible symptoms until 90 days after inoculation.

Figure 2. Artificial inoculation of *Phytophthora palmivora* on CCN-51 seedlings. **A.** Wilted plant 45 days after inoculation. **B.** Canker symptons. **C.** Plant showing internal symptoms 15 days after inoculation. **D-E.** Plants showing internal symptons 60 days after inoculation. **F.** Uninoculated seedling (control).

Discussion

The main goal of this study was to investigate the diversity of *Phytophthora* causing black pod rot and cocoa canker, in the provinces of Guayas, Esmeraldas, Manabí, Los Ríos, and Pichincha, considered the most significant cocoa-producing areas in Ecuador (INEC, 2020). Black pod rot disease is one of the most harmful pathologies for cocoa productivity, causing a 20 to 30% decrease in yield (Guest, 2006). Among the principal species that cause this disease, which has been described in other countries that produce cocoa, are *P. palmivora*, *P. megakarya*, *P. citrophthora*, *P. capsici*, *P. heveae* (Akrofi, 2015; Ali, Shao, Lary, Kronmiller, Shen, Strem, Amoako-attah, et al., 2017; Appiah et al., 2004; Luz et al., 2018; Morales-Cruz et al., 2020; Puig, Quintanilla, et al., 2021), *P. tropicalis* (Chávez-Ramírez et al., 2021) and *P. theobromicola* (Decloquement et al., 2021).

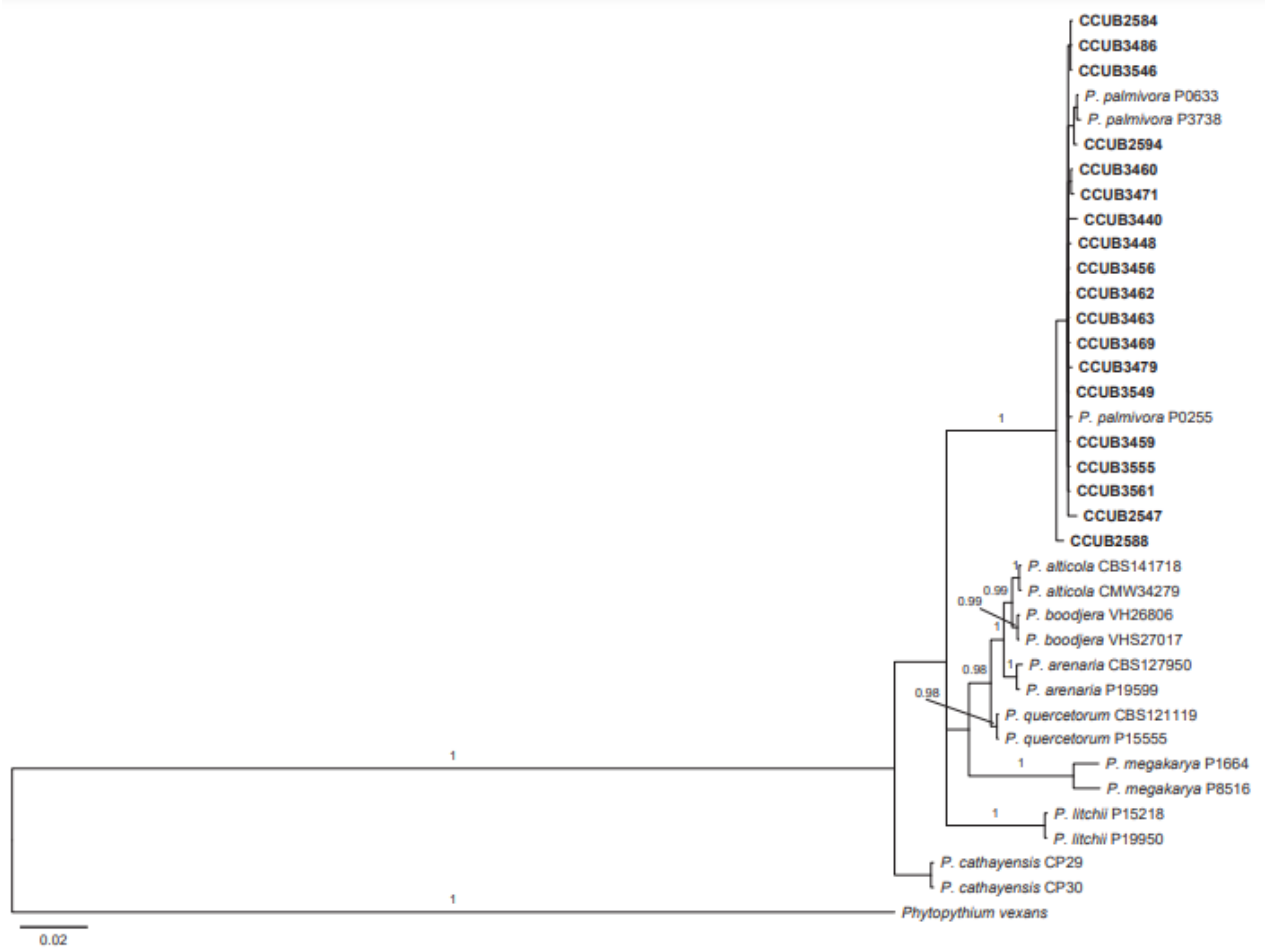

Figure 3. Bayesian phylogenetic tree based on concatenated sequences (BTUB, COXI and ITS) of Phytophthora clade 4. *Phytopythium vexans* used as an outgroup.

However, in all the samples of pods and stems processed in our research, regardless of the year and location of collection, we identified only *P. palmivora*. This suggests that the only species causing black pod rot and canker in cocoa-producing areas in Ecuador is *P. palmivora*. The prevalence of *P. palmivora* affecting cocoa plantations has been described in Hawaii (Puig et al., 2021), Papua New Guinea (Saul Maora et al., 2017) and Colombia (Rodríguez-Polanco et al., 2020; Gil et al., 2020; Suriza et al., 2019). Other species of *Phytophthora* may exhibit the same pattern of behavior as *P. nicotianae*, which affects citrus in Brazil, South Africa, and Egypt (Ahmed et al., 2012.; Meitz-Hopkins et al., 2014; Urashima et al., 2016).

It is worth mentioning that in Ecuador, the current studies on black pod rot and canker of cocoa are superficial, limited to morphological and molecular studies with no in-depth analysis

of multilocus genetics associated with pathogenicity data. Therefore, this study is among the first ones to be conducted on a large scale in the country (Suárez, 1993; Paredes et al., 2022; Pico et al., 2012; Moreira et al., 2020). The accuracy of the identification of *P. palmivora* was verified by a phylogenetic analysis using partial regions of *β-tubulin*, ITS, and *COX1*, which resulted in the formation of a single clade (4).

The use of these 3 genomic regions is supported by the 7 loci recommended for the phylogenetic analysis of *Phytophthora* species (ITS rDNA, *COI*, *YPT1*, β-*tub*, *EF1α*, *L10*, and *HSP90*) (Abad et al. 2023a), having as reference the source of sequence data consisting of over 1320 publicly available nucleotide accessions in the nucleotide collection of GenBank and most of those sequences were generated during the implementation of the IDphy online resource (Abad et al. 2023b). This information indicates the precision of these 3 genomic regions for the identification of oomycetes (Barboza et al., 2020; Blair et al., 2008; Céspedes et al., 2013; Decloquement et al., 2021; Kroon et al., 2012; Türkölmez et al., 2015).

The aggressiveness in cocoa pods from 18 selected isolates was partially variable. However, the isolates from the province of Guayas (CCUB3471, CCUB3463, and CCUB3440), Esmeraldas (CCUB2584), and Manabí (CCUB2594) induced the largest diameters in the lesions in contrast with the isolate (CCUB3469) of Guayas, which showed the lowest diameter. This suggests that aggressiveness is closely associated with the pathogenic characteristics of each isolate rather than the locality (Chávez-Ramírez et al., 2021; Klotioloma et al., 2018; Perrine-Walker, 2020).

The aggressiveness data found in this investigation has a close relationship with other trials developed on cacao pods artificially inoculated with *P. palmivora* in Colombia (Rodríguez-Polanco et al., 2020) and Brazil (Decloquement et al., 2021). However, it is known that isolates from other species, such as *P. megakarya* and *P. theobromicola*, can demonstrate higher levels of aggressiveness on pods of different cocoa genotypes (Bailey & Meinhardt, 2016; Decloquement et al., 2021; Efombagn et al., 2011; Onomo et al., 2017).

It has been shown that *P. megakarya* is present in the main cocoa-producing countries in Africa, causing considerable economic losses in cultivation (Paulin et al., 2008; Onomo et al., 2017; Akrofi, 2015). In addition, *P. theobromicola*, a recently discovered species in northeastern Brazil, has higher aggressiveness values than *P. palmivora* (Decloquement et al.,

2021). Therefore, it is important to consistently review the genetic material arriving in Ecuador from cocoa-producing countries and this can be done by implementing and improving plant quarantine services and laboratories for the molecular detection of plant pathogens (Gao et al., 2016; Hariharan & Prasannath, 2021; Hyun & Choi, 2014; Martin et al., 2016). These measures can help prevent the arrival of the mentioned species that could cause severe damage to cocoa plantations in the country (Acebo-Guerrero et al., 2012; Gao & Zhang, 2013; Shin et al., 2012).

There are various methods to manage black pod rot in cacao crops (Adejumo, 2005; Akrofi et al., 2003; Anzules-Toala et al., 2022; Deberdt et al., 2008; Sims et al., 2019; Toala et al., 2019). Fungicides are commonly used, but they are expensive and can be harmful to people and the environment (Kalyabina et al., 2021; Syromyatnikov et al., 2020). Developing resistant cocoa varieties is a better strategy due to its effectiveness and cost-effectiveness (Andersen et al., 2018; Iwaro et al., 2003, 2006; Pokou et al., 2008). Knowing the distinct species of a particular plant pathogen in a region can help improve genetic programs (Bahia et al., 2015; Andersen et al., 2018).

Due, cocoas clones may react differently to *Phytophthora* inoculation, for example, the APA 5, PA 30, PA 285, PA 294, and SC6 clones were proved to be black pod rot resistant (de Souza et al., 2021), and the evaluation of genotypes of the cocoa progeny segregating in the F1 generation (TSH 1188 x CCN 51) are very useful in studies aimed at increasing cocoa resistance to black pod disease (Barreto et al., 2015). In the cocoa-growing areas of Ecuador, *P. palmivora* seems to be the only cause of black pod rot and stem canker. This presents an opportunity to focus on developing *P. palmivora* resistant cocoa varieties as it is the only species in the country. By doing so, effective cocoa disease management strategies can be established.

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CAPÍTULO 3

PHYTOPHTHORA IN COCOA: A REVIEW

Phytophthora in cocoa: a review

Summary

Cacao is one of the world's most important commercial crops, as chocolate is produced from its seeds. However, black pod and canker, caused by various species of *Phytophthora*, are the primary factors reducing cacao's productivity in cultivation regions. *Phytophthora palmivora* has been regarded as the predominant species causing disease in cacao due to its global distribution. In contrast, *P. megakarya* is primarily found in Africa and has been identified as an emerging species, contributing to significant revisions in the taxonomy of this genus. The typical symptoms of black pod manifest as small necrotic spots either at the center or edges of fruits at various phenological stages. In advanced stages, these lesions may exhibit mycelial growth. In contrast, canker is characterized by necrotic spots accompanied by the exudation of a yellowish, resinous liquid, which can result in branch dieback and, in severe cases, the death of the entire plant. This review explores the species of *Phytophthora* that are pathogenic to cacao, along with key aspects such as symptomatology, epidemiology, distribution, identification, phylogeny, classical and molecular diagnostics, and disease management. Additionally, it highlights how advances in comparative genomics can enhance our understanding of the molecular mechanisms involved in pathogenicity, paving the way for the development of improved management strategies for *Phytophthora*-induced diseases in cacao.

Keywords: *Theobroma cacao*, Plant disease complex, Diagnosis, Peronosporales, taxonomy.

Introduction

Cacao (*Theobroma cacao* L.) is a plant species native to South America. Thanks to its broad edaphoclimatic adaptability and significant economic value, it is cultivated across all humid tropical regions worldwide, generating billions of dollars annually through the trade of its beans for chocolate production (Ploetz, 2016). In 2020, global cacao production reached 5.7 million tons, with the majority concentrated in Ivory Coast, Ghana, and Indonesia (FAOSTAT, 2024). However, the occurrence of diseases significantly reduces cacao productivity, with black pod disease caused by *Phytophthora* being one of the most serious threats to cacao cultivation

Black pod disease is recognized as the most significant disease affecting cacao worldwide, with *Phytophthora palmivora* being the most commonly encountered species in production areas (Puig et al., 2021). Depending on the geographical region, other species have also been identified as causal agents of this disease, including: *P. megakarya*), *P. capsici* (AA Appiah et al., 2004), *P. citrophthora* (Luz et al., 2018), *P. megasperma* (Rumbos et al., 2007), *P. heveae* *P. tropicalis* (Chávez, et al., 2021) and *P. theobromicola* .

Phytophthora species can cause lesions at various developmental stages of cacao plants (Vanegtern et al., 2015). The primary symptoms include brown spots on fruits that coalesce and infect the seeds, as well as cankers affecting the trunk and branches (Drenth and Guest, 2013). Less common symptoms include foliar and floral infections (Sriwati & Muarif, 2012). Under favorable environmental conditions, these diseases can reduce production by 20–30%, though losses may exceed 90% in some cacao-producing regions (Acebo-Guerrero et al., 2012a). Additionally, plant mortality due to canker can reach approximately 10% (Appiah et al., 2004).

Traditionally, *Phytophthora* species were identified based on morphological characteristics of the colony, sporangia, and oospores. However, relying solely on these traits may be insufficient to distinguish cryptic species and presents certain limitations (Appiah et al., 2004; Ho, 2018). To enhance the sensitivity, specificity, and reliability of the identification process, it is essential to combine morphological characteristics with phylogenetic analysis of different genomic regions. (Barboza et al., 2020; Decloquement et al., 2021; Rodríguez-Polanco et al., 2020).

The diseases caused by *Phytophthora* in cacao are attributed to several species, each of which may require a distinct management strategy. Therefore, accurate identification of the causal agent is essential for effective control. Key management strategies for black pod and trunk canker include the use of resistant genotypes (Barreto et al., 2018; Joseph et al., 2016), phytosanitary practices such as the removal of infected pods, improvement of drainage conditions, and proper plant spacing (Akrofi, 2015; Isaac Y. Opoku et al., 2007), the use of potential antagonistic microorganisms, the application of protective and systemic fungicides (Acebo-Guerrero et al., 2012b; Deberdt et al., 2008), and trunk injection with phosphonates (McMahon et al., 2010).

Given the devastating impact of *Phytophthora* spp. on cacao, numerous international studies have focused on understanding plant-microorganism interactions, pathogen diagnosis and identification, and the development of effective management strategies (Marelli et al., 2019; Perrine-Walker, 2020). In this context, the aim of this review is to provide insights into key aspects of the biology, distribution, taxonomy, molecular diagnosis, symptoms, and control measures for the *Phytophthora* species responsible for black pod and trunk canker in cacao.

Morphology

Phytophthora species are characterized by the presence of coenocytic, hyaline, and branched mycelium. The primary morphological structures used for characterization are the sporangia, which can be ovoid, obpyriform, or lemon-shaped. These sporangia may be produced in a sympodial and branched manner and can germinate either directly via a germination tube or indirectly through the release of zoospores (Alves et al., 2019; Reyes-Tena et al., 2021; Bailey & Meinhardt, 2016).

The sporangia can vary in form, being papillate, semi-papillate, non-papillate, deciduous, or persistent (Bush et al., 2006; Bawage et al., 2013). Zoospores are hyaline, ovoid, and biflagellate. They are notable for their motility and serve as the primary infective propagules of this plant pathogen. Zoospores are dispersed by water and attach to their host, leading to subsequent infections (Yan Wang et al., 2011; Ho, 2018; Tashiro et al., 2012).

Chlamydospores are resistant structures capable of surviving for extended periods. These structures are not unique to *Phytophthora* species but are also produced by other oomycetes and fungi. Chlamydospores can vary in shape, ranging from spherical to ellipsoid, and in color, from hyaline to yellowish. Their walls may be thin or thick (Nath et al., 2015; Ho, 2018; Perrine-Walker, 2020).

The spores involved in sexual reproduction are called oospores. They are spherical, hyaline, and occasionally yellowish, with very thick and robust walls. Oospores can overwinter in extremely low temperatures but are more sensitive to high temperatures (Turkensteen et al., 2000; Donahoo & Lamour, 2008; Kaosiri et al., 1980). The species *Phytophthora palmivora* is heterothallic, and oospores are produced only when A1 and A2 mating types are grown together

on agar plates or in infected plants. Interestingly, in *P. palmivora*, the A2 mating type is predominant in cacao worldwide.

Biology and Phylogeny of *Phytophthora* sp.

Phytophthora was initially considered a true fungus due to its ability to acquire essential nutrients for survival through mycelia, a trait similar to that of true fungi (Rossman & Palm, 2006; Bush et al., 2006). However, subsequent studies on members of Oomycota revealed that it is more closely related to algae and diatoms (Surujdeo-Maharaj et al., 2016). Evidence suggests that oomycetes evolved from holocarpic marine parasites and likely developed the ability to form mycelium and reproduce sexually as they adapted to terrestrial environments (André Lévesque, 2011; Beakes & Glockling, 2012).

Members of the Oomycota are classified within the Stramenopila kingdom (Cavalier-Smith, 2003; Cavalier-Smith et al., 2014; Cavalier-Smith, 2018) and are distinct from true fungi due to key differences. These include the presence of cellulose and β-glucans in their cell walls, as well as the lack of ergosterol synthesis, a predominant characteristic of fungi (Latijnhouwers et al., 2003; Bailey & Meinhardt, 2016; Rossman & Palm, 2006; Weizhen Wang et al., 2021).

Identification

Species of *Phytophthora* are traditionally classified based on their morphological, cytological, and biochemical characteristics (Appiah et al., 2004). The species *P. palmivora* can be divided into four categories based on the characteristics of the sporangia pedicel: (i) Sporangia with a round base and thick pedicels compressed to less than 5 μm; (ii) Sporangia with a round base and thin pedicels compressed to approximately 10 μm; (iii) Elongated sporangia with a platform-like structure, featuring predominantly thick and long pedicels compressed to more than 10 μm; and (iv) Isolates with persistent sporangia. In addition, cultural

characterization is considered a viable alternative for identifying *Phytophthora* species (Zentmyer, 1976; Islam et al., 2005; Wibowo et al., 2019).

Due to the high intra- and interspecific variability among *Phytophthora* species (Chee & Newhook, 1965; Ochoa-Fuentes et al., 2007; Gil et al., 2020), the results of morphological analyses are often inaccurate and prone to identification errors. Therefore, this information should be used cautiously, particularly when dealing with very similar species (Awasthi, 2015). To achieve more precise identification, alternative techniques have been developed. These include the study of total proteins and isoenzyme systems, such as esterases, dihydrogen malate, and superoxide dismutases, for analyzing *Phytophthora* species. These approaches have revealed minimal intraspecific variability among confirmed species (Old et al., 1984; Nwaga et al., 1990; Mchau & Coffey, 1994)."

Serological tests, such as ELISA, have been used to detect *Phytophthora* isolates in pure culture media and plant tissues infected by the pathogen (Amouzou-Alladaye, Dunez & Clerjeau, 1988; Oudemans & Coffey, 1991). In addition, intraspecific karyotype variations have been analyzed using electrophoresis. Beyond these biochemical approaches, studies on DNA polymorphisms have also been conducted (Martin & Tooley, 2004). Using RFLP, researchers have successfully identified *Phytophthora* species. Moreover, specific studies employing the RFLP technique have been carried out to identify *P. parasitica* and *P. citrophthora* (Ersek, Schoelz & English, 1994).

Molecular Identification

Phenotypic taxonomic characterizations can lead to identification challenges, often causing confusion between different species and isolates. To address these issues, more specific

methods focused on DNA analysis have been developed for microorganism identification (Badali & Nabili, 2013; Hariharan & Prasannath, 2021; Tsui et al., 2011).

Molecular methods offer greater reliability and accuracy in identifying pathogens and plant diseases (Manawasinghe et al., 2021; Vincelli & Tisserat, 2008). Moreover, they are faster, more precise, specific, and sensitive compared to traditional identification approaches (Kumar et al., 2015; Alemu, 2014; McCartney et al., 2003).

Traditionally, the genus *Phytophthora* has been morphologically associated with the genus *Pythium*. Due to these similarities, both were historically classified as members of the Pythiaceae family (Agrios, 2005; Ho, 2018). However, more in-depth analyses, including studies of the major and minor subunits of ribosomal DNA and the cox2 gene, have shown that *Phytophthora* is more closely related to other genera within the order Peronosporales (Beakes & Sekimoto, 2009; Petersen & Rosendahl, 2000; Thines & Choi, 2016). Despite these findings, a more detailed multigenic study that includes other members of the Oomycota is still necessary to clarify the phylogenetic relationships among these genera (Kong et al., 2003; McCarthy & Fitzpatrick, 2017; Yang et al., 2017).

Members of the *Phytophthora* genus have traditionally been classified into eight phylogenetic clades using ITS analysis (Cooke et al., 2000) and a multigenic approach (Kroon et al., 2004). However, a study utilizing genomic data to identify 225 markers with potential for phylogenetic analysis provided sufficient evidence to support the division of the genus into ten clades (Blair et al., 2008).

Various molecular techniques have been employed to assess intra- and interspecific variation in *Phytophthora* species (Appiah et al., 2004). For instance, the ITS (Internal

Transcribed Spacer) region of ribosomal DNA has been extensively utilized as a dataset for identifying *Phytophthora* species (Martin et al., 2012; Hussain et al., 2005; Zhang et al., 2008). This approach has been applied to various species and hosts, including: *P. nicotianae* in strawberry, jojoba, and cacao; *P. palmivora* in cherry, olive, tangerine, cacao, and kiwi; *P. capsici*, *P. citrophthora,* and *P. megakarya* in cacao; *P. ramorum* in forest trees; *P. cinnamomi* in blackberry, and *P. theobromicola* in cacao (Kurbetli et al., 2018; Lucero et al., 2007; Tashiro et al., 2012; Türkölmez et al., 2015; Appiah et al., 2004; Çiftçi et al., 2015; Huarhua et al., 2018; Andres et al., 2021). However, when phylogenetically closely related species, such as *P. rubi* and *P. fragariae*, are analyzed, the ITS region alone is not recommended as the sole method for differentiation (Martin et al., 2012).

For these reasons, a multigenic approach has been widely adopted, utilizing genomic regions such as cytochrome c oxidase subunit II (COXII), β-tubulin (β-tub), ITS, elongation factor 1α (EF1α), and heat shock protein 90 (HSP90). This approach has enabled more precise identification of various *Phytophthora* species (Blair et al., 2008; Kroon et al., 2004; Martin et al., 2014; Chang et al., 2017; Gloria & Gallup, 2016). To facilitate the accurate identification of *Phytophthora* species, the Tree-Based Alignment Selector Toolkit (T-BAS) was developed to construct a phylogeny of 192 formally described species and 33 informal taxa within the genus, using sequences from nine loci: 28S, 60SL10, Btub, EF1α, Enl, HSP90, TigA, ITS, and CoxI (Coomber et al., 2023).

The molecular identification of *Phytophthora* species is not standardized across studies, as seen in the case of *P. palmivora*, which is identified using both the ITS and COX regions. (Barboza et al., 2020; Rodríguez-Polanco et al., 2020). However, in other cases, more detailed approaches have been applied for this species, utilizing ITS, β-tub, EF1α, COXI, and COXII

(Suriza et al., 2019; Alsultan et al., 2021). For the identification of a new species in Brazil, *P. theobromicola*, an integrated approach was also employed, using ITS, β-tub, EF1α, COXI, COXII, and HSP90 (Decloquement et al., 2021). A similar situation occurred with *P. tropicalis*, identified in Mexico, where the ITS, β-tub, EF1α, and COXII regions were used for its identification (Chávez et al., 2021).

Molecular diagnosis

Due to the significance of *Phytophthora* in cacao cultivation, rapid and precise identification of this phytopathogen is crucial. To address this need, various molecular techniques are available, differing in complexity based on the procedures and equipment involved (Martin et al., 2012). Initially, the nuclear ribosomal region was the most commonly used for identifying Oomycetes and phytopathogenic fungi, as it provided a substantial amount of information on this locus. Over time, the use of additional genomic regions, such as β-tubulin (β-tub) and mitochondrial DNA (COX1 and COXII), has increased to achieve greater specificity (Barboza et al., 2020; Martin et al., 2014).

In this context, due to its advantages such as requiring a small amount of initial DNA, as well as its speed, efficiency, and specificity for detecting this phytopathogen, PCR has become one of the preferred techniques for diagnosing *Phytophthora*. Furthermore, modifications of this technique have been developed to enhance its effectiveness in achieving this goal.

Real-time PCR is a technique that offers higher sensitivity and the ability to process a larger number of samples compared to conventional PCR (SA Deepak et al., 2007). This method has been successfully used in diagnosing *Phytophthora* species (*Phytophthora* spp.) (Bilodeau et al., 2009; Hayden et al., 2004; Kunadiya et al., 2019; Legavre et al., 2015;

McDougal et al., 2021; Minerdi et al., 2008). A qPCR approach was proposed using a Beacon® P-PHYTO-PB-1 probe with 30 ng/µL to distinguish isolates of *P. palmivora*, confirming the detection of the phytopathogen (Palacios et al., 2021). For the diagnosis of *P. capsici*, a qPCR was developed targeting the ITS region, utilizing SYBR Green and specific primers, achieving successful identification with just 10 pg of *P. capsici* (Silvar et al., 2005), underscoring the sensitivity and effectiveness of this technique.

Nested PCR is a modified version of conventional PCR that involves two sequential amplification steps, with each reaction using a different pair of primers (Hariharan & Prasannath, 2021). This technique has been used with varying diagnostic success for *Phytophthora* in different crops (Grote et al., 2002; Ying Wang et al., 2007; Williams et al., 2009; ZG Zhang et al., 2006). A nested PCR assay was developed targeting the ITS region, using primers for the ITS1 and ITS2 regions, to differentiate *Phytophthora* species. This method successfully detected *P. palmivora* among the analyzed samples (Tsai et al., 2006).

Multiplex PCR is a technique that uses multiple pairs of primers, enabling the amplification of several DNA fragments in a single reaction (Sint et al., 2012). This approach, designed to broaden the detection of multiple *Phytophthora* species simultaneously, has been successfully employed in various diagnostic tests (Bi et al., 2019; G. Bilodeau et al., 2009; GJ Bilodeau et al., 2014; Ippolito et al., 2004). For instance, a multiplex PCR assay was developed for the detection of *P. palmivora* and successfully identified isolates infesting cocoa fruits in Indonesia (Masanto et al., 2019). A highly sensitive multiplex PCR targeting the ITS1 and ITS2 genes was developed to *P. palmivora* in rubber trees, demonstrating the efficacy of the procedure using the Pal1s/Pal2a primers .

The application of new molecular techniques for detecting *Phytophthora* DNA in samples from various hosts has been steadily increasing. One such technique, LAMP (Loop-Mediated Isothermal Amplification), is a novel method designed to amplify genetic material, producing multiple copies of DNA with high specificity in a short time, all under isothermal conditions (Notomi et al., 2000). Numerous assays employing this technique have been proposed for the detection of various phytopathogens within the Peronosporales order (Feng et al., 2019; Hansen et al., 2016; Htun et al., 2020; Khan et al., 2017; X. Kong et al., 2016; Ristaino et al., 2020; T. Wang et al., 2021). While this procedure has not yet been extensively explored for *Phytophthora* species affecting cacao, its remarkable potential indicates that it could become a highly effective tool for the accurate and reliable diagnosis of these pathogens.

Another promising technique is the use of biosensors, which consist of a recognition component that binds to the target analyte and a transducer that converts the molecular interaction into measurable signals. This approach offers several advantages, including high sensitivity, ease of use, and low cost (Chen et al., 2021; B. Li et al., 2015; Velusamy et al., 2010). Due to these benefits, biosensors have been successfully applied in the detection of plant pathogens (Cesewski & Johnson, 2020; Khater et al., 2017; M. Park et al., 2013). Recognizing the potential of this methodology, a nanosensor was developed for the electrochemical detection of *P. palmivora* by targeting the ITS region. Using only 0.30 ng of DNA, this nanosensor demonstrated high reliability, sensitivity, and selectivity in detecting phytopathogens in cocoa pods (Franco et al., 2019).

Given the extensive damage caused by phytopathogenic fungi, rapid and accurate detection is essential. In this context, progress in databases such as GenBank from the National Center for Biotechnology Information (NCBI), the International Nucleotide Sequence

Database Collaboration (INSDC) at the European Bioinformatics Institute (EBI), MycoBank, and others has been invaluable. These platforms provide access to nucleotide sequences, taxonomic updates, and other critical information about phytopathogenic fungi (Acland et al., 2014; Hariharan & Prasannath, 2021; Prakash et al., 2017; Robbertse & Tatusova, 2011; Vu et al., 2019).

Additionally, a specialized platform—the *Phytophthora* database—has been developed to organize diverse data, including morphological characteristics, geographic distribution, moleculares tools, and phylogenetic relationships of *Phytophthora* species. This database serves as an excellent resource for advancing knowledge and supporting research efforts (B. Park et al., 2013; J. Park et al., 2008).

Genomics tools

The advent of advanced nucleic acid sequencing technologies has enabled the generation of numerous sequences, providing deeper insights into the genes that constitute phytopathogens by facilitating whole-genome analyses through techniques such as next-generation sequencing (Espindola et al., 2015; Hariharan & Prasannath, 2021). These techniques are marked by superior efficacy, sensitivity, and efficiency in detecting phytopathogens compared to conventional methods (Díaz-Cruz et al., 2019). Moreover, they contribute significantly to a more comprehensive understanding of the epidemiological, phylogenetic, and taxonomic relationships that remain insufficiently clarified, making them invaluable tools for developing effective management strategies to mitigate the damage caused by *Phytophthora* spp. (Capote et al., 2012; Martinelli et al., 2015; Sankaran et al., 2010).

Advances in understanding the distribution of various non-genomic genetic markers have facilitated research on the biology of *Phytophthora* populations (Lanaud et al., 2009;

Mucherino Muñoz et al., 2021). For instance, these markers have been instrumental in identifying genes involved in host-phytopathogenic fungi interactions, with techniques such as genome-wide association studies (GWAS) and quantitative trait locus (QTL) mapping playing a significant role (Asekova et al., 2021). These methodologies have been widely applied to detect resistance genes to black pod disease in cacao, offering critical insights that could contribute to genetic improvement programs (Barreto et al., 2018; Brown et al., 2007; Joseph et al., 2016; Risterucci et al., 2003).

The genomes of two *Phytophthora* species associated with cacao, *P. palmivora* and *P. megakarya*, have been fully sequenced (Shahin S. Ali, Shao, Lary, Kronmiller, et al., 2017; Morales-Cruz et al., 2020). Additionally, the genomes of *P. capsici* have been sequenced from isolates obtained from *Capsicum* spp. and *Cucurbita pepo* (Reyes-Tena et al., 2019; Shi et al., 2021).

For instance, the genome of *P. megakarya* is approximately the same size (222 Mbp) as two genomes of other species found in cacao but is nearly twice as large as the genome of *P. palmivora* (135 Mbp) and significantly larger than the average genome size (approximately 100 Mbp) of other *Phytophthora* species (Morales-Cruz et al., 2020). The assembly of these genomes has provided valuable insights into key biological processes, including the production of toxins, elicitors, and effector proteins (Shahin S. Ali, Shao, Lary, Kronmiller, et al., 2017; Perrine-Walker, 2020; Pettongkhao et al., 2020).

The understanding of key genes, such as effectors including crinklers (CRNs), necrosis-inducing proteins (NPPs), RxLR (synonymous with Avr and Avh), and elicitins, has proven critical for studying pathogenicity and host specificity (Mafurah et al., 2015; Masanto et al., 2021; S. Wang et al., 2019; Wenjing Wang & Jiao, 2019). Additionally, the role of repetitive elements (transposable elements) associated with the evolutionary processes of *Phytophthora*

has provided valuable insights into its infection mechanisms (Ayala-Usma et al., 2021; Kasuga et al., 2016; Morales-Cruz et al., 2020). Together, these advances deepen our understanding of the complex processes underlying *Phytophthora* infections.

Phytophthora in cacao, origin and distribution

Initially, *Phytophthora palmivora* was identified as the causal agent of cacao black pod disease. However, this identification has undergone significant revisions due to the efficient application of various diagnostic techniques. These include studying colony characteristics, sporangia morphology, the presence or absence of chlamydospores (Kellam & Zentmyer, 1996; Erwin & Ribeiro, 1996; Torres, 2016; Masanto et al., 2019), enzyme patterns, and physiological traits (Saul Maora et al., 2017). Most notably, the use of molecular markers, such as ITS, β-tubulin, COX II, and HSP90 regions, has proven instrumental in refining species identification (SS Ali et al., 2016; Blair et al., 2008; Decloquement et al., 2021; Marelli et al., 2019; Puig et al., 2021).

As a result of these advancements, additional *Phytophthora* species associated with cacao black pod disease have been identified, including *P. megakarya*, *P. capsici*, *P. citrophthora*, *P. theobromicola*, and *P. tropicalis* (Appiah et al., 2004; Blair et al., 2008; Decloquement et al., 2021; Chavez et al., 2021). These species are known to cause significant economic losses in cacao plantations worldwide. Other species, such as *P. botryosa*, *P. megasperma*, and *P. heveae*, have been associated with milder reductions in crop productivity (Appiah et al., 2004).

Among these species, *P. palmivora* has the broadest geographic distribution, occurring in all cacao-producing regions globally. It is considered the dominant species responsible for black pod and canker in cacao (Aguilar-Anccota et al., 2020; Brasier & Griffin, 1979; Klotioloma et al., 2018; Perrine-Walker, 2020; Rodríguez-Polanco et al., 2020). *P. capsici* is

primarily found in India, Central, and South America, while *P. citrophthora* has been reported in Brazil and India, causing economic losses in cacao plantations (Faleiro et al., 2004). In Malaysia, *P. heveae* was documented by Turner (1961), and in Venezuela, *P. megasperma* was reported to affect cacao (Rumbos et al., 2007; Molina et al., 2016).

Other unique records include *P. parasitica* (syn. *P. nicotianae*) in Cuba (Lacourt et al., 1994) and *P. botryosa* in Sri Lanka (Adikaram & Yakandawala, 2020). The most aggressive species, *P. megakarya*, is predominantly distributed in the western and central regions of Africa, where it has become the primary limitation to cacao production. Its damage far exceeds that caused by *P. palmivora* (Akrofi, 2015; SS Ali et al., 2016; Ali et al., 2017a; Ali et al., 2017b; Appiah et al., 2004; Bae et al., 2005; Bailey et al., 2005; Marelli et al., 2019).

Table 1. *Phytophthora* species associated with black pod disease in cacao, including their geographical distribution, host range, phylogenetic clade, and genome size (adapted from Perrine-Walker, 2020).

Species name	**Geographical distribution**	**Host**	**Clade**	**Genome size (Mb)**
Phytophthora capsici (Leonian)	Brazil, El Salvador, Guatemala, India, Jamaica, Mexico, Trinidad, Venezuela	Wide range	2	64
P. citrophthora (RE Smith and EH Smith)	Brazil, India, Mexico	Wide range	2	48.5
P. heveae (Thompson)	Malaysia	Wide range	5	

P. megakarya (Brasier and Griffin)	Cameroon, Côte d'Ivoire, Fernando Poo, Gabon, Ghana, Nigeria, São Tomé (islands of Principe and São Tomé), and Togo	*Theobroma cacao*	4	126.88
P. megasperma (Dreschler)	Venezuela	Wide range	6	62
P. nicotianae	Cuba	Wide range	1	76.5
P. palmivora (Butler)	Pantropical	Wide range	4	151.23
P. tropicalis (Aragaki & J.Y. Uchida 2001)	Mexico	*T. cacao*	2 b	
P. theobromicola Pinho, Ramos-Sobrinho & Marelli 2021	Brasil	*T. cacao*	2 b	

Symptoms and epidemiology

Oomycetes are microorganisms that share similar characteristics with true fungi but differ significantly in their phylogenetic aspects. Among them, *Phytophthora* spp. stands out as a major phytopathogen, causing severe damage to the productivity of various cultivated plants and natural ecosystems (Grünwald et al., 2008; Kurbetli, Aydoğdu & Çetinel, 2018; Roese & Goulart, 2014; Türkölmez et al., 2015; Colangelo et al., 2018; DW Li et al., 2019). One of the most well-known examples of epidemics caused by this genus is potato late blight, attributed to *Phytophthora infestans* (Haas et al., 2009; Goss et al., 2014).

The disease cycle begins with the recognition of the host by zoospores. This stage is typically marked by the activity of zoospores, which germinate, penetrate the plant's internal tissues, and colonize them to acquire essential nutrients (Hardham, 2001).

Motile zoospores play a pivotal role in initiating infection by forming host-attached cysts, which later give rise to intracellular hyphal growth (Kong et al., 2010; Perrine-Walker, 2020).

These spores are capable of locating their host through chemotaxis (movement toward chemical signals) or electrotaxis (movement in response to electrical signals), facilitating precise host recognition (Kong & Hong, 2010; Fawke et al., 2015).

Black pod disease in cocoa can be caused by various *Phytophthora* species, all of which produce similar symptoms. While the pods are the most susceptible parts of the plant, the pathogen can also infect other tissues, including leaves and stems (Ali et al., 2017; Kuswinanti et al., 2020; Decloquement et al., 2021).

Symptoms begin with spots that can appear on different parts of the fruit at any stage of development. This happens as the pathogen penetrates the cuticle and reaches the epidermis, leading to the formation of brown lesions at the infection points. These spots are often found either at the center or the extremities of the pods (Guest, 2007; Akrofi, 2015; Vanegtern et al., 2015).

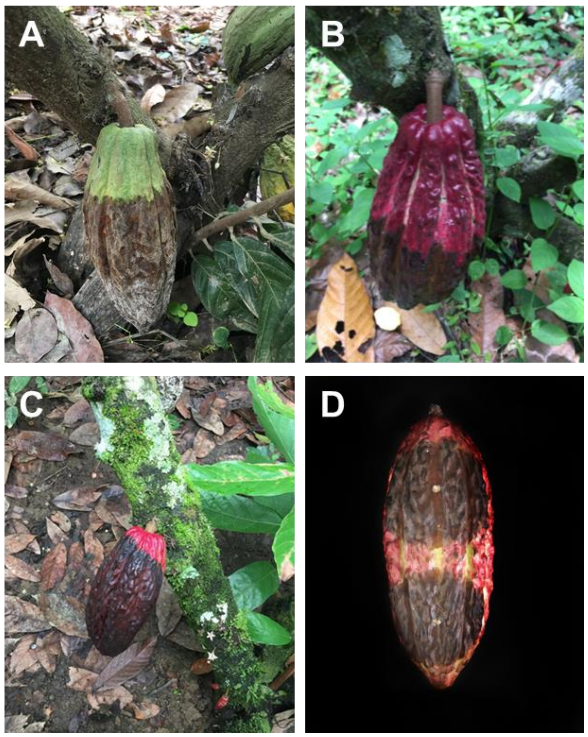

Figure 1. Symptoms of black pod disease observed in a field in Esmeraldas, Ecuador (A, B, C), and an artificially inoculated pod (D).

Mycelium originating from infected pods can spread along the trunk and reach deeper tissues. However, the infection is often not immediately detected because the symptoms develop beneath the bark and gradually progress to the internal tissues. In advanced stages, dark spots may appear on the wood, accompanied by a yellow gummy secretion (Firman & Vernon, 1970; Perrine-Walker, 2020). In cases of severe infection, the pathogen can spread to the branches, potentially leading to plant death (Vanegtern et al., 2015; Guest, 2007; Appiah et al., 2004).

Generally, one of the characteristic symptoms for recognizing the presence of the pathogen is leaf death, even in the absence of direct pathogen presence (Aguilar-Anccota et al., 2020). In many cases, the canker caused by *Phytophthora* is often underestimated; however, it can lead to reduced vigor, decreased pod yield, and act as a significant source of inoculum (A. Appiah et al., 2004; Oliveira & Luz, 2005; Guest, 2007).

When weather conditions are favorable, the lesions progress rapidly, covering the entire surface of the pod, affecting internal tissues and the beans. The disease's progression depends on the susceptibility of the cacao variety (Perrine-Walker, 2020). Over time, the mummified pods may produce sporangia, becoming an inoculum source for several years (Guest, 2006; Puig et al., 2021; Taylor & Grünwald, 2021).

Once the beans are infected, they deteriorate and rot rapidly, particularly when the infection occurs during the early stages of fruit development (Vanegtern et al., 2015). Under optimal conditions, a single infected pod can produce up to 4.000 sporangia, which can be dispersed by wind, rain, animals, insects, and contaminated materials or tools (Guest, 2007; Evans, 1978).

In addition to causing black pod and cankers in cacao, *Phytophthora* can also lead to other, less studied symptoms in its hosts, such as floral and foliar infections. These infections

are closely related to fruit and stem diseases (Sriwati & Muarif, 2012; Guest, 2007; Marelli et al., 2019). It is important to note that when environmental conditions are unfavorable, such as during the dry season, propagules can persist in flower buds until conditions improve. This helps them spread quickly in the field, significantly impacting the production of infected pods (Ortega et al., 2017; Delgado-Ospina et al., 2021; Ndoumbe Nkeng et al., 2017; Leandro-Muñoz et al., 2017; Vanegtern et al., 2015).

Management

Adequate knowledge of *Phytophthora* species is crucial for a better understanding of cacao disease dynamics, as insufficient knowledge about epidemiology, life cycle, and host-pathogen interactions can lead to ineffective disease management (Mpika et al., 2009; Pratama et al., 2013; Volynchikova & Kim, 2022). Therefore, it is essential to identify which species are involved in the disease etiology in the field to gain a clearer understanding of the phytosanitary issue.

The integration of various control methods—such as quarantine, cultural, biological, genetic, and chemical approaches—is a key strategy for reducing the economic impact caused by *Phytophthora* spp. in cacao (Sims et al., 2019; Giachero et al., 2022; Saltos et al., 2022; Santos et al., 2023; He et al., 2023).

The distribution of *Phytophthora* species is not uniform across all cocoa-producing regions of the world. For instance, *P. megakarya* is primarily found in the central and western regions of Africa and has not been reported in the Americas and Asia (Akrofi, 2015; Marelli et al., 2019). *P. theobromicola* has only been reported in Brazil and Colombia. Both species are considered more aggressive than *P. palmivora*, which is the most widely distributed species in

all cocoa-producing areas (Akrofi et al., 2003; Decloquement et al., 2021; Ramirez Martinez et al., 2021).

Given the significant exchange of cacao genetic material between producing countries, it is crucial to enhance and refine techniques for evaluating and assessing plant genetic material through plant quarantines (Ali et al., 2017; Gao et al., 2016; Hyun & Choi, 2014; RR Martin et al., 2016). This is necessary to prevent the introduction of pathogens into regions where they are not yet present, thereby avoiding a potentially severe impact on cocoa production (Cho et al., 2016; Parke et al., 2019).

Cultural control is a primary strategy to mitigate the damage caused by *Phytophthora* spp. in cacao plantations, especially for small- and medium-sized farmers, due to its ease of application and low costs (Akrofi, 2015; Ndoumbe-Nkeng et al., 2004), while also boosting production (Abdulai et al., 2018; Yeo et al., 2017). Cultural practices are also employed to create unfavorable conditions for the disease's development (Bailey et al., 2018; Soberanis et al., 1999).

These practices include pruning, which opens the cocoa canopy and allows light to penetrate, reducing relative humidity and decreasing disease incidence (Acebo-Guerrero et al., 2012a; Krauss & Soberanis, 2001), and the removal of diseased pods, which helps reduce sporangia numbers and stem canker formation. In African countries, pod removal, combined with other control techniques, has proven to be highly effective in reducing black pod incidence (Maddison & Idowu, 1981; Tondje et al., 1993; Adejumo, 2005; Ndoumbe-Nkeng et al., 2004). In Peru, weekly pod removal reduced field damage by 35–66% (Soberanis et al., 1999).

Numerous microorganisms have shown antagonistic potential against *Phytophthora* spp. both in vitro and in field conditions (Mpika et al., 2009; Tondje et al., 2007; Stephen et al., 2020; Jiang et al., 2016; Hung et al., 2015). Endophytic fungi such as *Colletotrichum*, *Clonostachys*, and *Botryosphaeria* have reduced black pod disease losses in Panama (Mejía et al., 2008).

There is further evidence of antagonistic fungi in laboratory settings; for example, isolates of *Paecilomyces* and *Rhizopus* demonstrated strong antagonistic potential against *P. palmivora* (Amadi, 2010), while *Aspergillus* spp. has been effective in reducing pathogen development in culture media (Adebola & Amadi, 2010; Kang et al., 2005; Stephen et al., 2020).

Among various antagonistic fungi, *Trichoderma* is the most studied. For instance, *Trichoderma* spp. inhibits the growth of *P. palmivora* (Mpika et al., 2009) and *P. megakarya* (Adedeji et al., 2008) in culture media. *T. asperellum* has shown significant potential as a mycoparasite against *Phytophthora* spp. (Tondje et al., 2007) and as a resistance inducer in plants (Tchameni et al., 2017). Furthermore, secondary metabolites produced by *Trichoderma* spp. inhibit mycelial growth and sporangial germination of *P. palmivora*, *P. megakarya*, and *P. capsici* (Pakora et al., 2018).

Various bacteria have also shown promising results against *Phytophthora* spp., both in vitro and under field conditions (Koranteng & Awuah, 2011; Maryam et al., 2019). Genera like *Pseudomonas fluorescens*, *Bacillus subtilis* (Pratama et al., 2013; Bhusal & Mmbaga, 2020), *Acinetobacter calcoaceticus*, *Serratia marcescens*, *P. protegens*, and *P. veronii* have

demonstrated effectiveness against *Phytophthora* spp. The chitinolytic ability of these bacteria plays a key role in this antagonistic effect (Melnick et al., 2011).

The use of fungicides remains the most traditional method for controlling *Phytophthora* damage in cacao, with various trials conducted in cocoa-producing regions worldwide (Akrofi et al., 2003; Isaac Y. Opoku et al., 2007; Puig et al., 2021; Rodriguez Polanco et al., 2021; Chi et al., 2020). The success of this method depends on environmental conditions and correct application, considering the farmers' economic situation and factors such as fungicide dosage, frequency, form, and application timing (Opoku et al., 2000; Chi et al., 2020; Sonwa et al., 2008; Bolaños-Carriel et al., 2020).

Fungicides that have shown effectiveness in controlling *Phytophthora* spp. include cupric hydroxide, cuprous oxide, and systemic metalaxyl, fosetyl-Al (Acebo-Guerrero et al., 2012a; Adejumo, 2005; Groves & Ristaino, 2000; Matheron & Porchas, 2000; Reis et al., 2005; Hao et al., 2019; Rodriguez Polanco et al., 2021). Their effectiveness can be influenced by pathogen susceptibility and the geographical origin of isolates (Belisle et al., 2019; Puig et al., 2021). Another promising approach is the use of inducers like phosphonates, which control *Phytophthora* disease indirectly (Daniel & Guest, 2006; Guest et al., 1994; Holderness, 1992; McMahon et al., 2010; Manghi et al., 2021; Brandano et al., 2023).

Although chemical control is effective when used alone, achieving optimal results in managing black pod disease and stem canker caused by *Phytophthora* spp. requires integrating various management strategies to minimize the impact of this phytopathogen

Conclusions

Black pod and stem canker caused by *Phytophthora* spp. are indeed among the most significant challenges to sustainable cocoa production worldwide. A thorough understanding and accurate identification of the pathogen species responsible for these diseases, facilitated by molecular biology, is critical for developing effective management strategies. These strategies could encompass biological, cultural, chemical, and genetic improvement approaches.

This research has confirmed the presence of at least six *Phytophthora* species associated with cacao. However, the majority of existing studies have primarily concentrated on *P. palmivora*. This observation suggests the potential presence of other species linked to cacao, underscoring the pressing need for further studies on the epidemiology, identification, distribution, and control strategies for all the reported species.

In this regard, future research should focus on employing advanced, sensitive techniques, such as genome sequencing (Shahin S. Ali, Shao, Lary, Kronmiller, et al., 2017; Morales-Cruz et al., 2020). These approaches would enhance our understanding of essential aspects, including diagnosis, genetic diversity, and plant-microorganism interactions. Additionally, they can help identify genes related to pathogenicity and infection processes, which would significantly contribute to a more comprehensive understanding of the various factors associated with *Phytophthora* spp. in cacao.

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CAPÍTULO 4

Caracterização de *Lasiodiplodia* causando à morte descendente do cacaueiro no Equador

Resumo

O cacaueiro, *Theobroma cacao* L., é uma cultura de grande importância econômica em várias províncias do Equador. Entre as doenças mais proeminentes do cultivo encontra-se a morte descendente. Este estudo avaliou a diversidade e patogenicidade de espécies de *Lasiodiplodia* associadas com a morte descendente em cacaueiro no Equador. Os isolados foram coletados e identificados a partir de características morfológicas e análises filogenéticas da região parcial do gene fator de elongação 1-alpha (*TEF-1α*), β- tubulina (*TUB2*) e RNA polimerase II (*RPB2*). Testes de patogenicidade foram realizados com isolados representativos de cada província em mudas de cacaueiro CCN51 com 4 meses de idade. As análises filogenéticas confirmaram a existência de quatro espécies de *Lasiodiplodia*: *L. theobromae, L. pseudotheobromae, L. brasiliensis* e *L. laeliocattleyae*, sendo que, *L. brasiliensis* e *L. laeliocattleyae* são relatadas pela primeira vez como causadoras da morte descendente do cacaueiro no mundo. Os 20 isolados de *Lasioplodia* spp. que foram inoculados artificialmente, induziram sintomas nas mudas do clone CCN51. Identificar patógenos emergentes que podem causar perdas na cacauicultura é crucial para o Brasil e outros países com diferentes condições climáticas, pois isso ajuda a compreender a distribuição geográfica desses fitopatógenos e as condições que favorecem seu desenvolvimento.

Palavras-chave: fungos, cancro, taxonomia, complexo de doenças de plantas, diagnose

Abstract

The cacao tree, *Theobroma cacao* L., is a crop of significant economic importance in several provinces of Ecuador. Among the most prominent diseases affecting this crop is dieback. This study evaluated the diversity and pathogenicity of *Lasiodiplodia* species associated with dieback in cacao in Ecuador. Isolates were collected and identified based on morphological

characteristics and phylogenetic analyses of the partial region of the elongation factor 1-alpha (TEF-1α) gene, β-tubulin (TUB2), and RNA polymerase II (RPB2). Pathogenicity tests were conducted with representative isolates from each province on 4-month-old CCN51 cacao seedlings. Phylogenetic analyses confirmed the presence of four *Lasiodiplodia* species: *L. theobromae*, *L. pseudotheobromae*, *L. brasiliensis*, and *L. laeliocattleyae*, with *L. brasiliensis* and *L. laeliocattleyae* being reported for the first time as causes of dieback in cacao worldwide. The twenty *Lasiodiplodia* spp. isolates that were artificially inoculated induced symptoms in CCN51 seedlings. Identifying emerging pathogens that may cause losses in cacao production is crucial for Brazil and other countries with different climatic conditions, as it helps to understand the geographic distribution of these phytopathogens and the conditions that favor their development.

Keywords: fungi, taxonomy, canker, plant disease complex, diagnosis

Introdução

O Equador se destaca como um dos principais produtores e exportadores de cacau (*Theobroma cacao* L.) no mundo, com a produção concentrada nas províncias de Los Ríos, Manabí e Guayas (INEC, 2020). Em 2022, a produção de cacau atingiu 337.149 toneladas em uma área de 509.179 hectares, posicionando o país como o sétimo maior produtor mundial de cacau (FAOSTAT, 2024).

O potencial produtivo do cacaueiro pode ser reduzido por diversos fatores, sendo as doenças causadas por fungos fitopatogênicos um dos mais impactantes (Bowers et al., 2001; Marelli et al., 2019). Embora os fungos da família Botryosphaeriaceae causem morte

descendente em várias culturas, sua importância no cacaueiro ainda é subestimada (Hernández et al., 2023; Pornsuriya et al., 2023).

Fatores como temperaturas elevadas e escassez hídrica provocam estresse na planta, favorecendo a colonização por espécies de *Lasiodiplodia* (Agustí-Brisach et al., 2020; Calvo-Garrido et al., 2021; Slippers et al., 2017). Além disso, a presença de ferimentos e a deficiência nutricional favorecem a ocorrência da morte descendente, resultando em perdas significativas na produção de cacau (Alvindia & Gallema, 2017; Hendra et al., 2019; Kannan et al., 2010a; Mbenoun et al., 2008; Oliveira & Luz, 2005; Rahim et al., 2022).

No Equador, o cultivo do cacaueiro a pleno sol predomina nas principais regiões produtoras, intensificando o estresse das plantas (Dinis et al., 2022; Fernandez et al., 2023) e aumentando a incidência de doenças bióticas, como a morte descendente (Antony et al., 2023; Galarneau et al., 2019; Hrycan et al., 2020; Qiu et al., 2016; Rodríguez-Gálvez et al., 2021).

O fungo *Lasiodiplodia* é frequentemente relatado como o principal agente causal da morte descendente do cacaueiro (Bezerra et al., 2021; Phillips et al., 2013, 2019; Rahim et al., 2022). Embora seja saprófita e/ou endofíto, esse fungo causa doenças em uma ampla gama de hospedeiros em regiões tropicais e subtropicais (Mehl et al., 2017; Salvatore et al., 2020). Os sintomas associados com as infecções de *Lasiodiplodia* são: podridão de frutos, seca de ponteiros, cancro no caule e morte das plantas (Ismail et al., 2012; Linaldeddu et al., 2015; Marques et al., 2013; Netto et al., 2014a; Saeed et al., 2017).

Em alguns países da América do Sul, como Brasil, Chile, Peru e Venezuela, *Lasiodiplodia* tem sido relatada em uma ampla gama de hospedeiros (Fischer et al., 2017; Gonçalves et al., 2016; Machado et al., 2014; Marques et al., 2013; Ramos et al., 2023). No

Equador, esse fungo foi relatado causando à morte descendente em *Schizolobium parahyba* var. *amazonicum* e o cancro em teca (Vélez-Zambrano et al., 2023; Mehl et al., 2014; Morrillo et al., 2021). No entanto, não existe um estudo abrangente sobre a identificação de espécies de *Lasiodiplodia* no cacaueiro.

Historicamente, a identificação dos fungos da família Botryosphaeriaceae era baseada na comparação de características morfológicas como forma e tamanho de conidióforos e conídios (Lima et al., 2013; Phillips et al., 2013). No entanto, a identificação morfológica tornou-se limitada em nível de gênero devido à descoberta de espécies crípticas (Alves et al., 2008; Rosado et al., 2016; Ko et al., 2023).

A combinação de comparações morfológicas e análises filogenéticas de diferentes regiões genômicas permite uma identificação acurada em nível de espécie (Batista, 2022; Cruywagen et al., 2017; Jami et al., 2012; Netto et al., 2014b; Pavlic et al., 2009; Slippers et al., 2013). As regiões genômicas recomendadas para a identificação das espécies de *Lasiodiplodia* são espaçador interno transcrito (ITS), beta-tubulina (β-TUB), fator de elongação (TEF-1α) e RNA polimerase II (RPB2; Batista et al., 2020; End et al., 2017; Kazemzadeh Chakusary et al., 2019; Li et al., 2018; Phillips et al., 2008; Pillay et al., 2013; Valencia et al., 2019).

O aumento de doenças causadas por fungos da família Botryosphaeriaceae, aliado à escassez de estudos abrangentes sobre a etiologia da morte descendente, sugere a possível presença de um complexo de espécies de *Lasiodiplodia* infectando o cacaueiro no Equador. Diante disso, esse estudo teve como objetivo caracterizar morfo-molecularmente as espécies de *Lasiodiplodia* e avaliar a patogenicidade dos isolados.

MATERIAL E MÉTODOS

Coleta e Isolamento

Entre 2019 e 2021, ramos com sintomas típicos da morte descendente foram coletados em plantios comerciais das províncias de Manabí, Guayas, Los Ríos, Esmeraldas e Santo Domingo, localizadas no litoral, e em Morona Santiago, na região Oriente do Equador (Fig. 1).

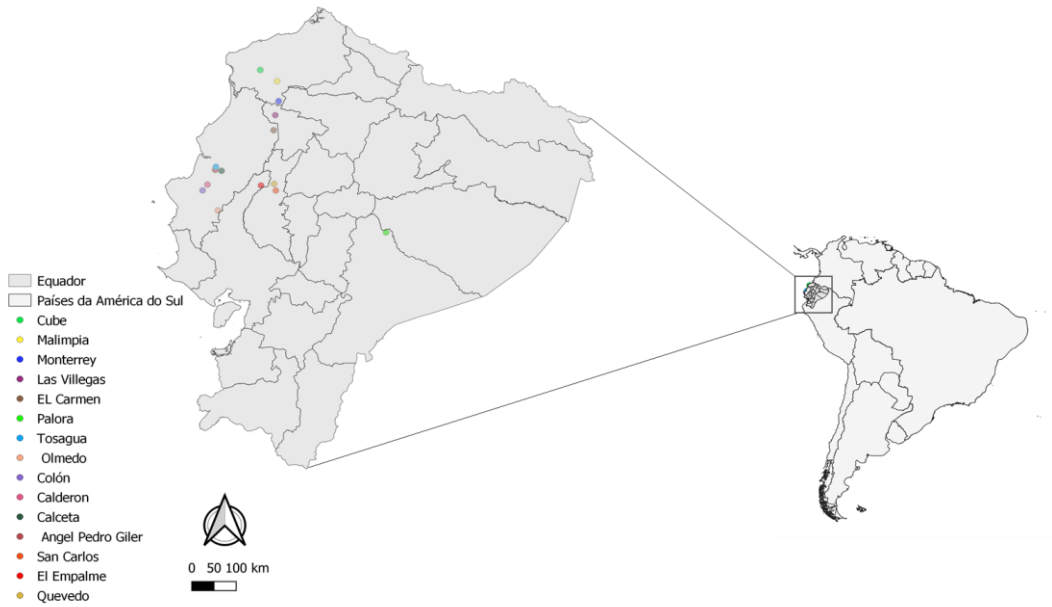


Figura 1. Mapa contendo os pontos de coleta dos isolados de *Lasiodiplodia* spp. obtidos do cacaueiro no Equador.

O isolamento foi realizado conforme a metodologia de Rodríguez-Gálvez et al. (2017). Fragmentos de aproximadamente 5 mm foram retirados da área de transição entre o tecido doente e o tecido assintomático. Em seguida, foram desinfestados em etanol 70% por 30 segundos, imersos em hipoclorito de sódio 2% por três minutos e lavados três vezes consecutivas com água destilada esterilizada. Todos os fragmentos foram depositados sobre

papel toalha para reduzir a umidade. Posteriormente, cinco fragmentos foram transferidos para placas de Petri contendo meio Batata Dextrose Agar (BDA) (Difco), suplementado com ampicilina (250 mg/L) e gentamicina (10 mg/L).

As placas foram mantidas a 25 °C sob um fotoperíodo de 12 horas de luz e 12 horas de escuro. Após cinco dias de incubação, colônias típicas de fungos da família Botryosphaeriaceae foram transferidas para novas placas de Petri contendo meio extrato de malte Agar (MEA) e mantidas a 25 °C no escuro. Para garantir a pureza genética dos isolados, culturas foram obtidas pela remoção de uma pequena porção da ponta de uma hifa. Após 7 dias de incubação a 25 °C no escuro, as culturas foram depositadas na Coleção de Culturas da Universidade de Brasília.

Extração, Amplificação e Sequenciamento

Aproximadamente 40 mg de micélio fúngico foi removido da superfície das colônias com cinco dias de crescimento, utilizando um palito de madeira esterilizado e depositado em tubos de microcentrifuga de 1,5 mL contendo 30 µL de tampão Tris-EDTA (TE). A extração do DNA genômico foi realizada utilizando o Kit Wizard Genomic DNA Purification (Promega Corporation, WI, U.S.A) conforme as recomendações do fabricante.

As reações de PCR foram realizadas em um volume total de 12,5 mL, distribuídos em 6,25 µL de MyTaq TM Mix 2x (Bioline EUA Inc., Taunton, Inglaterra), 0,3 µL de cada primer (senso e anti-senso), 4,65 µL de água ultrapura e 1 µL de DNA genômico total (amostra).

A identificação prévia de todos os isolados foi realizada por meio do sequenciamento de uma região parcial do gene Fator de elongação (*TEF-1α*). Posteriormente, isolados representativos foram selecionados para inferir a árvore filogenética concatenada com as regiões genômicas Beta - tubulina (*β-TUB*) e RNA polimerase II (*RPB2*). Os primers EF1F/

EF2R (Alves et al., 2008), 2a/2b (Glass & Donaldson, 1995) e 5F2/7cR (Sung et al., 2007). foram utilizados para amplificar uma região parcial dos genes *TEF-1α, β-TUB* e *RPB2*, respectivamente.

Os parâmetros utilizados durante o ciclo da PCR foram: Desnaturação inicial a 95 ºC por 1 minuto e 30 segundos, seguida por 35 ciclos de desnaturação a 95 ºC por 20 segundos, anelamento a 54 ºC (*rpb2*), 55 ºC (*β-Tub*), ou 56°C (*tef1*–α) por 45 segundos, extensão a 72 ºC por 45 segundos, e uma extensão final a 72 °C por 5 minutos.

Os produtos de PCR foram analisados por eletroforese em gel de agarose 1 % corados com corante fluorescente GelRedTM (Biotium, Hayward, California, USA) e visualizados sob luz ultravioleta para verificar o tamanho e a pureza dos fragmentos amplificados. Os produtos de PCR foram purificados usando Exosap IT conforme as recomendações do fabricante e enviados para o seu sequenciamento.

Análises filogenéticas

As sequências foram editadas utilizando o software DNA Dragon (www.sequentix.de) e alinhadas manualmente. O arranjo dos nucleotídeos em posições ambíguas foi corrigido por comparação das sequências senso e anti-senso. As sequências das regiões *TEF1–α*, *β-TUB*, *RPB2* foram utilizadas para as análises filogenéticas.

Além disso, sequências representativas de espécies pertencentes ao gênero *Lasiodiplodia* (Tabela 2) foram obtidas do GenBank (http://www.ncbi.nlm.nih.gov) e alinhadas manualmente com as novas sequências usando o programa MUSCLE (Edgar, 2004), implementado no software MEGA 6 (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2011). O fungo *Diplodia mutila* isolado CMW7060 foi utilizado como outgroup.

Para cada região genômica foram determinados os modelos de substituição de nucleotídeos utilizando o programa MrModeltest v. 2.3 (Posada & Buckley, 2004) de acordo com o Akaike Information Criterion (AIC) (Guindon & Gascuel, 2003). As análises de Inferência Bayesiana (IB) foram realizadas com MrBayes v. 3.1.2 (Rannala & Yang, 1996; Ronquist & Huelsenbeck, 2003) por meio do método de Markov Chain Monte Carlo (MCMC), inicialmente com cada região genômica separadamente e posteriormente com as sequências concatenadas (*TEF1*–α, *β-TUB*, *RPB2*).

O MrBayes foi executado no servidor CIPRES Science Gateway 3.1 (Miller et al., 2010). A árvore foi visualizada no software FigTree (http://tree.bio.ed.ac.uk/software/figtree/) e exportada para programas gráficos.

Patogenicidade dos isolados

A patogenicidade de 20 isolados representativos foi avaliada em mudas de cacaueiro CCN51 com 4 meses de idade. As mudas foram mantidas em embalagens de polietileno contendo substrato composto de solo e areia (3:1) com irrigação a cada 3 dias. A epiderme do caule foi removida a 15 cm da base da planta com auxílio de um furador cilíndrico de 5 mm de diâmetro e 1 mm de profundidade. Em seguida, um disco (5 mm diâmetro) de MEA com cinco dias de crescimento micelial foi colocado sobre o ferimento e um pequeno pedaço de algodão umedecido em água destilada e esterilizada foi colocado no lado oposto ao ponto de inoculação. A área inoculada foi envolvida com parafilme para preservar a umidade. Nas plantas controle, apenas discos de MEA foram colocados sobre os ferimentos.

As mudas inoculadas foram mantidas em câmara climática a 28 °C por 35 dias. Os primeiros sintomas começaram a ser observados cinco dias após a inoculação. Ao final de 35

dias, apenas os isolados que induziram sintomas de morte descendente com diâmetro superior a 5 mm foram considerados patogênicos. O experimento foi repetido duas vezes para garantir a reprodutibilidade dos resultados.

Resultados

Obtenção dos isolados de *Lasiodiplodia* spp.

Os 166 isolados de *Lasiodiplodia* foram obtidos nas províncias de Guayas, Manabí, Santo Domingo, Los Ríos, Esmeraldas e Morona Santiago no Equador (Tabela 1) a partir de 265 plantas de cacaueiro com sintomas de amarelecimento e queda de folhas, seca de ramos e ponteiros, e morte parcial ou total das árvores (Fig. 2).

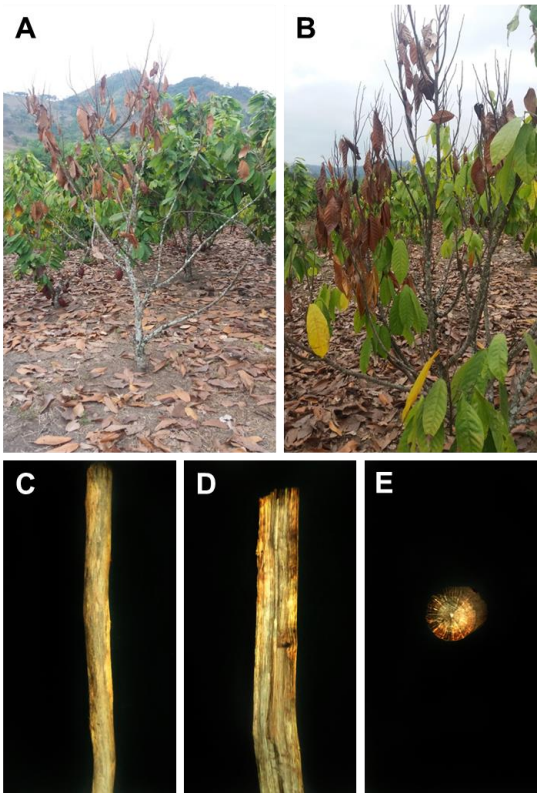


Figura 2. Sintomas de morte descendente em cacau (EET-103). **A)** Planta com sintomas severos de morte descendente **B)** Ramos com sintomas da morte descendente **C)** Ramo sem a casca **D)** Sintomas internos de morte descendente afetando tecidos vasculares; **E)** Corte horizontal de ramo afetado por *Lasiodiplodia* sp.

Identificação e análises filogenéticas

A comparação de sequências do gene fator de elongação no banco de dados do GenBank revelou quatro espécies de *Lasiodiplodia: L. theobromae, L. pseudotheobromae, L. brasiliensis* e *L. laeliocattleyae* (Tabela 2).

Tabela 1. Número de isolados obtidos em relação a quantidade de amostras coletadas em cada área.

Província	**Localidade**	**Número de amostras**	**Número de isolados**
Manabí	Angel Pedro Giler	35	19
	Calceta	40	28
	Calderón	10	4
	Colón	15	8
	El Carmen	15	5
	Olmedo	40	28
Los Ríos	San Carlos	15	8
	Quevedo	15	9
Guayas	El Empalme	20	9
Santo Domingo de los Tsáchilas	Monterrey	10	6
	Las Villegas	10	5
Esmeraldas	Cube	15	8
	Malimpia	30	23
Morona Santiago	Palora	15	6
Total		285	166

As análises filogenéticas utilizando sequências do gene fator de elongação dos 166 isolados em comparação com 88 sequências representativas das diferentes espécies de *Lasiodiplodia* confirmam os resultados da ferramenta BLAST do GenBank. O alinhamento possui 779 caracteres, dos quais 548 foram conservados, 215 foram variáveis e 141 foram informativos para parcimônia.

A árvore obtida por Inferência Bayesiana agrupa os isolados obtidos nesse estudo em quatro clados filogenéticos. Os 166 isolados de *Lasiodiplodia* (Fig. 3) foram identificados

como *L. theobromae* (n= 106), *L. pseudotheobromae* (n= 29), *L. brasiliense* (n= 23), e *L. laeliocattleyae* (n= 7).

A identificação acurada dos isolados representativos utilizando sequências dos genes *TEF1*–α, *β-TUB* e *RPB2* confirma a identificação prévia realizada por meio da comparação de sequências do gene *TEF1-α*. A espécie mais frequente em ordem decrescente foi *L. theobromae* (64%), *L. pseudotheobromae* (17%), *L. brasiliense* (14%), e *L. laeliocattleyae* (5%).

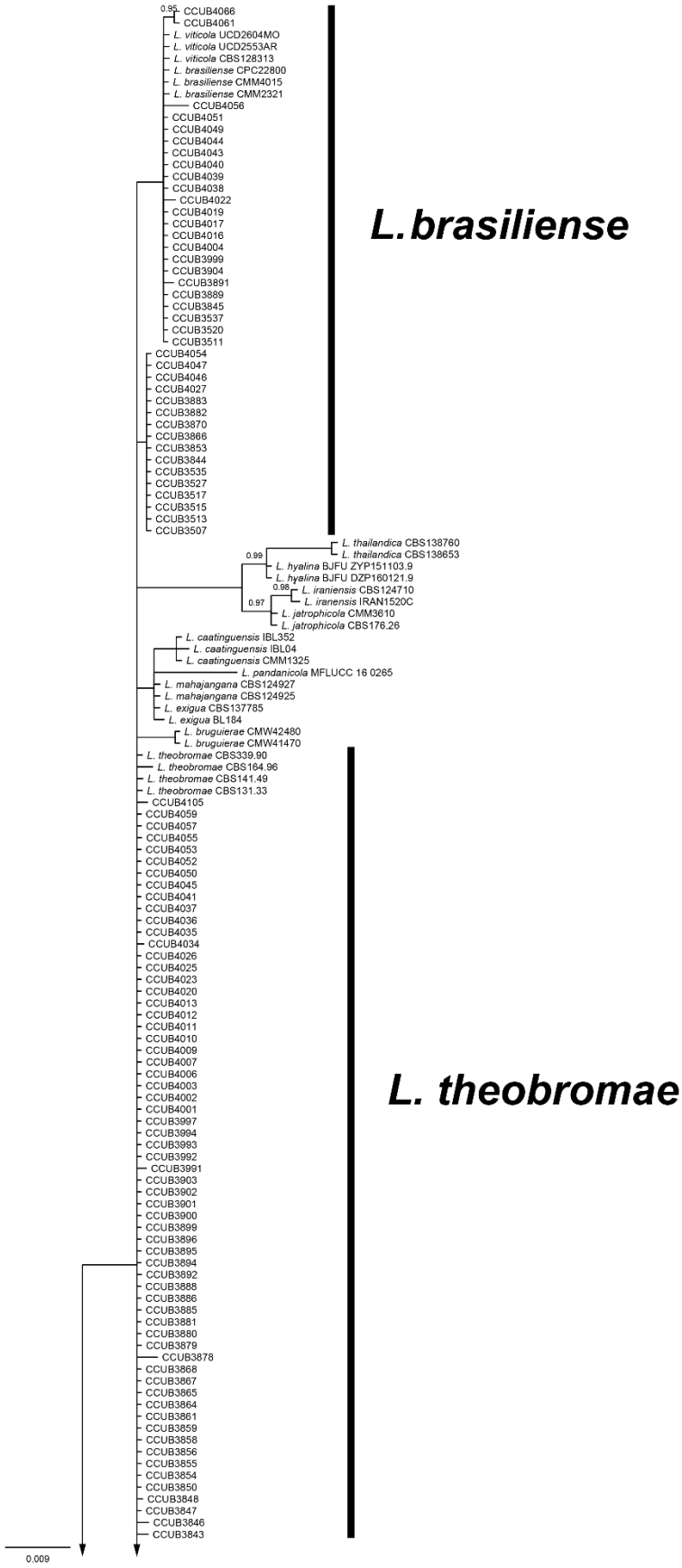
Tabela 2. Código de acesso das sequências obtidas no GenBank para identificação das espécies de *Lasiodiplodia*.

Fungo	**Isolado**	**Hospedeiro**	**Acceso do Genbank região (*tef-1α*)**
L. avicenniae	LAS 158		KU587946.1
L. avicenniae	LAS 199	*Avicennia marina*	KU587947.1
L. avicenniae	CMW 41467	*Avicennia marina*	KP860680
L. brasiliensis	CMM2321	*Carica papaya*	KC481528.1
L. brasiliensis	CMM4015	*Mangifera indica*	JX464049.1
L. brasiliensis	CPC 22800	*Mangifera indica*	KJ193687.1
L. bruguierae	CMW41470	*Bruguiera gymnorrhiza*	KP860678.1
L. bruguierae	CMW42480	*B. gymnorrhiza*	KP860677.1
L. caatinguensis	CMM1325	*Citrus sinensis*	KT008006
L. caatinguensis	IBL 04	*Anacardium occidentale*	KT154752.1
L. caatinguensis	IBL 352	*Anacardium occidentale*	KT154753.1
L. chinensis	BJFU_ZYP151106.36	*Canarium parvum*	KX499929.1
L. chinensis	CGMCC3.18061	unknown	KX499927
L. citrícola	CBS124707		

L. citricola	IRAN1521C	*Citrus* sp	GU945339.1
L. crassispora	CBS 118741	*Santalum album*	DQ103557
L. crassispora	CMW 13488	*Eucalyptus urophylla*	DQ103559.1
L. egyptiacae	BOT10	*Mangifera indica*	JN814424.1
L. egyptiacae	BOT29	*Mangifera indica*	JN814428.1
L. egyptiacae	CBS 130992	*Mangifera indica*	JN814424
L. euphorbicola	CBS356.59	*T. cacao*	EF622062.1
L. euphorbicola	CMM3609	*Jatropha curcas*	KF226689.1
L. exigua	CBS 137785	*Retama raetam*	KJ638336
L. exigua	BL 184	*Retama raetam*	KJ638337.1
L. gilanensis	CBS 124,705	desconhecido	GU945341
L. gilanensis	CBS 124,704	desconhecido	GU945342
L. gonunbiensis	CBS 115,812	*Syzigium cordatum*	DQ458877.1
L.gonunbiensis	CMW41236	*B. gymnorrhiza*	KP860685.1
L. gonunbiensis	CMW43762	*B. gymnorrhiza*	KU587943.1
L. gravistriata	CMM4564	*Anacardium humile*	KT250950.1
L. gravistriata	CMM4565	*Anacardium humile*	KT266812.1
L. hormozganensis	CBS 124,709	*Olea* sp	GU945343
L. hormozganensis	CCMF CNPA0603	*Ricinus communis*	MG806913.1
L.hormozganensis	IRAN1498C	*Mangifera indica*	GU945344.1
L. hyalina	BJFU DZP160121-9	Unknown	KY676796.1
L. hyalina	BJFU ZYP151103.9	Unknown	KX499917.1
L. iraniensis	IRAN 1520C	*Salvadora persica*	GU945336.1
L. iraniensis	IRAN 1502	*Juglans* sp	GU945335
L. jatrophicola	CBS176.26		KM066118.1
L. jatrophicola	CMM3610	*Jatropha curcas*	KF226690.1
L. laeliocattleyae	CBS 167.28	*Laeliocattleya*	KU507454.1
L. laeliocattleyae	LAREP1	*M. indica*	KU507451.1
L. lignicola	CBS 134,112	Madeira morta	KU887003.1

L. lignicola	MFLUCC11_0435	Madeira morta	KP872375
L. macrospora	CMM3833	*Jatropha curcas*	KF226718.1
L. mahajangana	CBS 124,925	*Terminalia catappa*	FJ900641
L. mahajangana	CBS 124,927	*Terminalia catappa*	FJ900643
L. margaritacea	CBS 122,065	*Adansonia gibbosa*	EU144066.1
L. margaritacea	CBS 122,519	*Adansonia gibbosa*	EU144065.2
L. marypalme	CMM 2275	*C. papaya*	KC481567.1
L. marypalme	CMM 2289	*C. papaya*	KC481564.1
L. mediterranea	CBS137783	*Quercus ilex*	KJ638331
L. mediterranea	CBS137784	*Vitis vinifera*	KJ638330
L. missouriana	CBS 128,311	*Vitis* sp	HQ288267
L. missouriana	CBS 128,312	*Vitis* sp	HQ288268
L. pandanicola	MFLUCC160265	Unknown	MH412774.1
L. parva	CBS 456.78	Solo de mandioca	EF622063.1
L. parva	CBS 494.78	Solo de mandioca	EF622064.1
L. plurivora	CBS 121,103	*Vitis vinifera*	EF445396.1
L. plurivora	STE-U 5803	*P. salicina*	EF445395
L. pontae	CMM1277	*Spondias purpurea*	KT151791
L. pontae	IBL 14	*Anacardium occidentale*	KT151793
L. pontae	IBL 18	*Anacardium occidentale*	KT151793
L. peudotheobromae	CBS116459	*Gmelina arborea*	EF622057
L. peudotheobromae	IBL266	*Anacardium occidentale*	KT247484
L. peudotheobromae	CMM3887	*Jatropha curcas*	KF226722
L. peudotheobromae	CMM2288	*C. papaya*	KC481553
L. peudotheobromae	IRAN 1518C	*Citrus* sp	GU973866
L. peudotheobromae	CBS374.54	*Coffea* sp.	EF622059
L. pyriformis	CBS 121,770	*Acacia mellifera*	EU101352

L. pyriformis	CBS 121,771	*Acacia mellifera*	EU101353
L. rubropurpurea	CBS 118,740	*Eucalyptus grandis*	DQ103571
L. rubropurpurea	WAC 12,536	*Eucalyptus grandis*	DQ103572
L. sterculiae	CBS342.78	*Sterculia oblonga*	KX464634
L. sterculiae	CBS447.62		
L. subglobosa	CMM 3872	*Jatropha curcas*	KF226721
L. subglobosa	CMM 4046	*Jatropha curcas*	KF226723
L. thailandica	CBS 138,760	*Mangifera indica*	KJ193681
L. thailandica	CBS 138,653	*Phyllanthus acidus*	KM006464
L. theobromae	CBS131.33		
L. theobromae	CBS141.49		
L. theobromae	CBS164.96	Fruit along coral reef coast	KM006464
L. theobromae	CBS339.90	phaeohyphomycotic cyst	EF622052
L. theobromae	CMW41214	*Barringtonia racemosa*	KU666547
L. theobromae	CMW41222	*B. racemosa*	KU666549
L. venezuelensis	WAC 12539	*Acacia mangium*	DQ103568
L. venezuelensis	WAC 12,540	*Acacia mangium*	DQ103569
L. viticola	CBS 128,313	*Vitis sp*	HQ288269
L. viticola	UCD2553AR	*Vitis vinifera*	HQ288269
L. vitis	CBS 124,060	*Vitis vinifera*	KX464642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L. viticola CBS128313
L. brasiliense CPC22800
L. brasiliense CMM4015
L. brasiliense CMM2321
CCUB4056
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CCUB3866
CCUB3853
CCUB3844
CCUB3535
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CCUB3515
CCUB3513
CCUB3507
L. brasiliense
L. thailandica CBS138760
L. thailandica CBS138653
0.99
L. hyalina BJFU ZYP151103.9
L. hyalina BJFU DZP160121.9
0.98
L. iraniensis CBS124710
L. iraniensis IRAN1520C
0.97
L. jatrophicola CMM3610
L. jatrophicola CBS176.26
L. caatinguensis IBL352
L. caatinguensis IBL04
L. caatinguensis CMM1325
L. pandanicola MFLUCC 16 0265
L. mahajangana CBS124927
L. mahajangana CBS124925
L. exigua CBS137785
L. exigua BL184
L. bruguierae CMW42480
L. bruguierae CMW41470
L. theobromae CBS339.90
L. theobromae CBS164.96
L. theobromae CBS141.49
L. theobromae CBS131.33
CCUB4105
CCUB4059
CCUB4057
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L. theobromae
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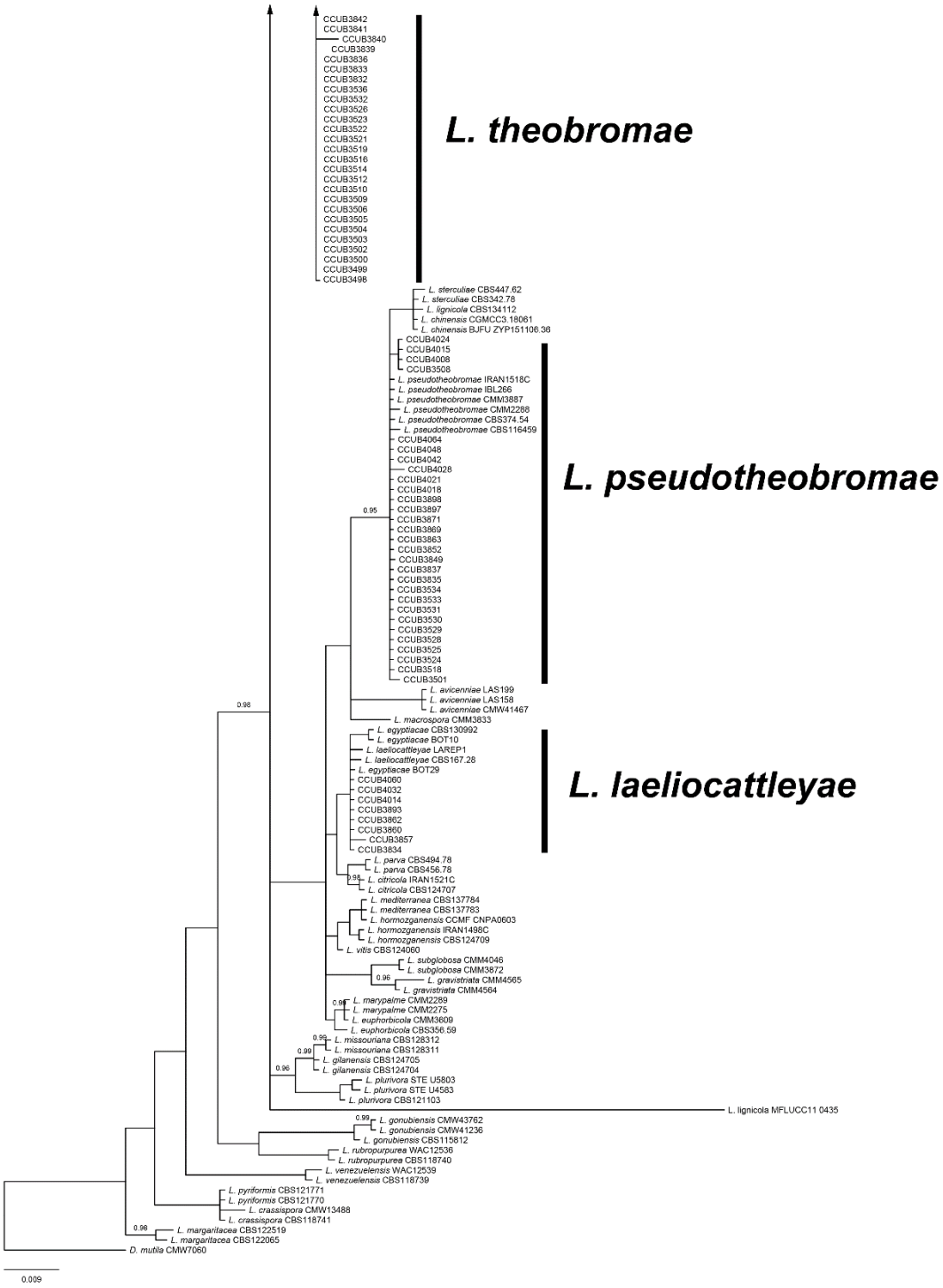

Figura 3. Árvore filogenética da região fator de elongação, Beta-tubulina e RNA Polimerase 2 contendo 254 isolados de *Lasiodiplodia*, incluindo 166 isolados obtidos nesse estudo e 88 isolados representativos das diferentes espécies de *Lasiodiplodia.* A espécie *Diplodia mutila* foi utilizada como outgroup.

Distribuição

As quatro espécies (*L. theobromae*, *L. pseudotheobromae*, *L. brasiliensis* e *L. laeliocattleyae*) foram encontradas nas províncias de Esmeraldas, Guayas e Manabí enquanto somente *L. theobromae* e *L. brasiliensis* foram relatadas em Morona Santiago. Nas províncias de Los Rios e Santo Domingo somente *L. pseudotheobromae* não foi encontrada (Fig. 4).

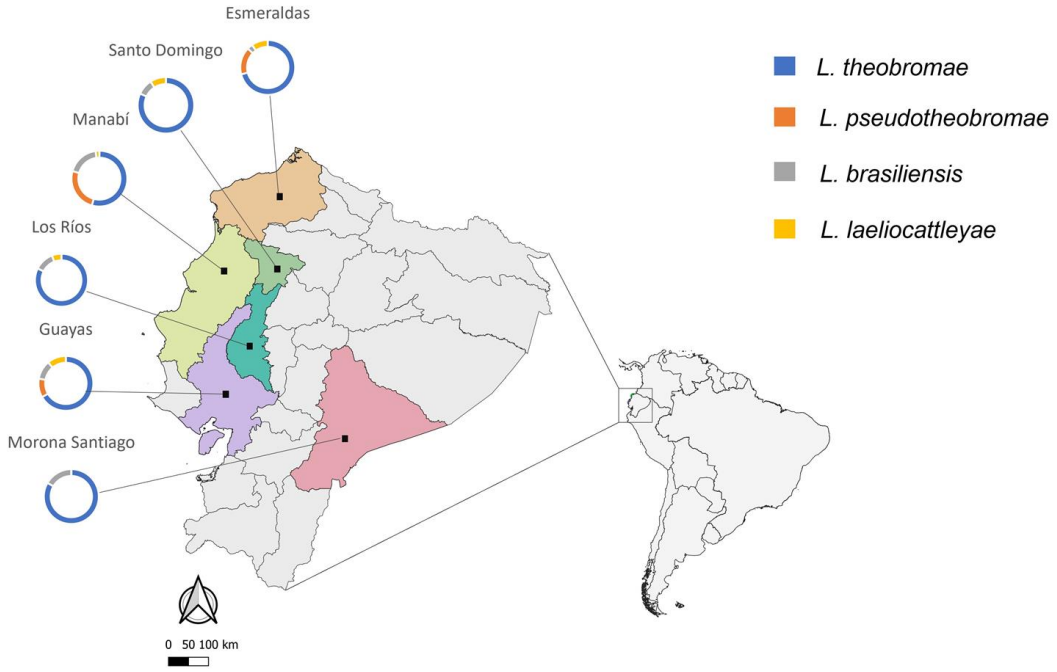

Figura 4. Mapa ilustrando a distribuição dos isolados de *Lasiodiplodia* spp. nas províncias do Equador.

Patogenicidade

Os isolados de *L. theobromae* (CCUB2627, CCUB2933, CCUB2966, CCUB2983, CCUB2997, CCUB3000), *L. pseudotheobromae* (CCUB2653, CCUB2937, CCUB3005), *L. brasiliensis* (CCUB2643, CCUB2930, CCUB2974, CCUB2987, CCUB2996, CCUB3001) e *L. laeliocattleyae* (CCUB2919, CCUB2947, CCUB2976, CCUB3035, CCUB3087) foram capazes de causar lesões no ponto de inoculação e reproduziram os sintomas da morte descendente em mudas de cacaueiro.

Os primeiros sintomas foram observados entre a primeira e a terceira semana após a inoculação. Lesões necróticas e escuras foram observadas no caule e, em seguida, um amarelecimento gradual das folhas do ápice em direção as folhas mais velhas. Posteriormente, ocorreu o enrolamento, murcha e necrose do tecido foliar. O tecido dos ramos e do caule também se tornaram necróticos. Ao realizar o corte transversal do caule, se observou o escurecimento do tecido interno. Em algumas mudas foi observado a necrose total e morte das plantas. A formação de lesões deprimidas e necróticas (cancros) ao redor do ponto de inoculação concomitante com a formação abundante de picnídios foram observadas nas mudas que não foram mortas. As plantas utilizadas como testemunha mantiveram assintomáticas. As diferentes espécies de *Lasiodiplodia* foram re-isoladas em todas as mudas inoculadas.

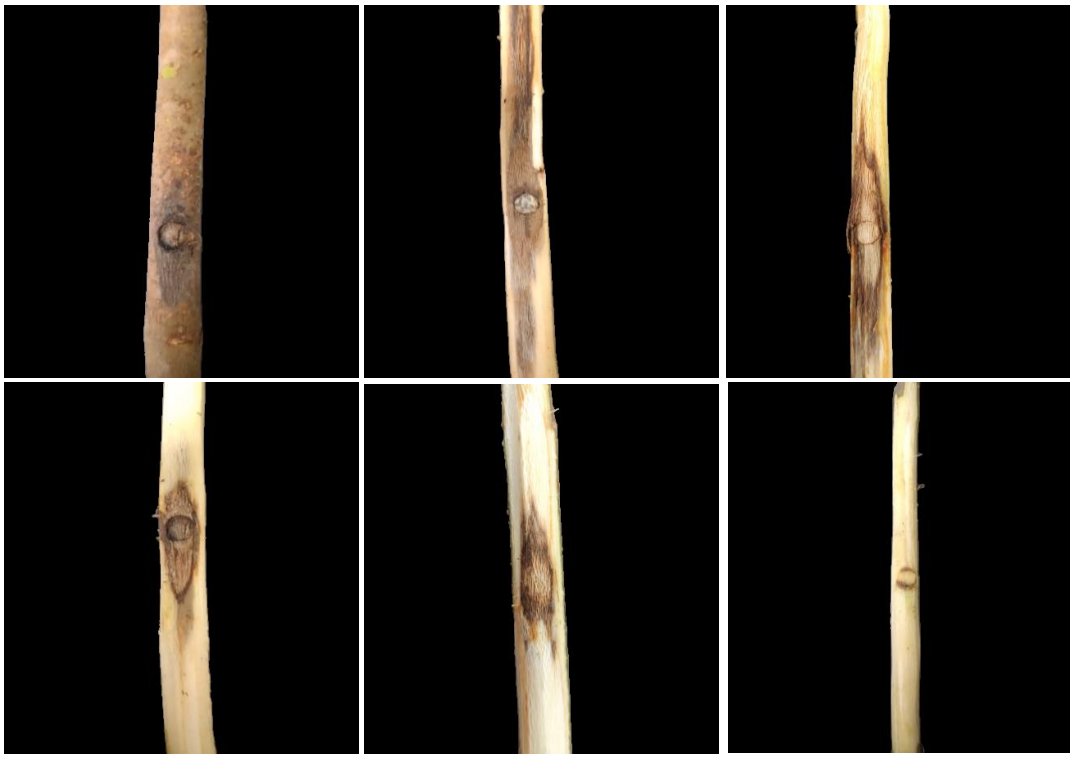

Figura 5. Inoculação Artificial de *Lasiodiplodia* spp em plántulas de cacao clone CCN-51. **A.** Sintomas externos. **B, C, D, E.** Síntomas internos de em isolados de *L. theobromae* (CCUB2983), *L. pseudotheobromae* (CCUB2937), *L. brasiliensis* (CCUB2974), e *L. laeliocattleyae* (CCUB2947) aos 60 dias após inoculação**. F.** Testemunha.

Discussão

Plantas com sintomas iniciais ou avançados da morte descendente foram encontradas em todas as propriedades amostradas. Portanto, a eliminação de ramos doentes é essencial para reduzir a disseminação da doença na área (Alvindia & Gallema, 2017; Kannan et al., 2010a).

Lasiodiplodia theobromae foi a espécie predominante em todas as províncias do Equador. Embora a maioria das identificações foram realizadas por comparações morfológicas, essa espécie também foi predominante em causar podridão do fruto, cancro, e morte descendente nas demais regiões produtoras de cacau (Kannan et al., 2010b; Mbenoun et al., 2008; Rahim et al., 2022; Serrato-Díaz et al., 2020b). Além disso, esse fungo possui uma ampla gama de hospedeiras em regiões tropicais e subtropicais (Burgess et al., 2006; Marques et al., 2013; Rodríguez-Gálvez et al., 2017, 2021; Tiznado et al., 2018; Úrbez-Torres et al., 2008; Vélez et al., 2023).

Na América do Sul esse fungo foi anteriormente relatado em fruteiras como abacate, anonáceas, caju, coco, mamão, mandioca, maçã, manga e uva (Rodríguez-Gálvez et al., 2015; Rodríguez-Gálvez et al., 2017; Alama et al., 2006; Rodríguez-Gálvez et al., 2021; Brito et al., 2020; Halfield vieira 2005; Coelho et al., 2020; Rosado et al., 2020; Santos et al., 2020; Marques et al., 2013; Netto et al., 2014; Netto et al., 2017; Machado et al., 2019; Coutinho et al., 2017; Úrbez-Torres 2008; Aguilera-Cogley et al., 2022; Díaz et al., 2022; Al Sadi et al., 2013; Arjona et al., 2019; Biju et al., 2021; Ponsuriya et al., 2023; Li et al., 2019).

Lasiodiplodia pseudotheobromae foi anteriormente relatado causando podridão de frutos de cacau em Porto Rico (Serrato et al., 2020), mas essa é a primeira observação desse fungo causando a morte descendente no cacaueiro. Além disso, esse fungo é relatado causando

podridão de frutos e morte descendente em outras fruteiras como abacate, caju, ciriguela, citros, coco, manga, mirtilo, pitaya, rambutão e tamarindo (Bautista-Cruz et al., 2019; Chen et al., 2021; Valle-De la Paz et al., 2019; Ismail et al., 2012; Marques et al., 2013; Rodríguez-Gálvez et al., 2017; Sakalidis et al., 2011; Rodríguez-Gálvez et al., 2020; Serrato-Diaz et al., 2020; Navarro et al., 2022; Rodríguez-Gálvez et al., 2021; Coutinho et al., 2017b; Conforto et al., 2019; Coelho et al., 2022; Phillips et al., 2013).

A espécie *L. brasiliensis* foi encontrada pela primeira vez causando doença no cacaueiro embora seja relatada causando podridões, gomose e morte descendente em anonáceas, caju, ciriguela, coco, mamão, manga, rambutão e sapoti (Coelho et al., 2022; Serrato-Diaz et al., 2020; Machado et al., 2019; Netto et al., 2017; Netto et al., 2014b Coutinho et al., 2017b).

Lasiodiplodia laeliocattleyae (=*L. egyptiaceae*) também foi encontrada pela primeira vez causando doença no cacaueiro. Essa espécie foi relatada causando doenças no abacate, coco, mandioca, manga, mirtilo, pinhão manso, tangerina e uva (Marques et al., 2013; Rosado et al., 2016; El-Ganainy et al., 2022; Correia et al., 2016; Rodríguez-Gálvez et al., 2017, 2020, 2021).

A abordagem morfo-molecular para a identificação das espécies de *Lasiodiplodia* têm revelado um complexo de espécies causando doenças em uma mesma hospedeira (Santos et al., 2020; Machado, Pinho & Pereira; Rosado et al., 2016; Coelho et al., 2022; Coutinho et al., 2017b; Machado et al., 2019). Essa informação, aliada ao número limitado de estudos sobre a caracterização morfo-molecular dos fungos da família Botryosphaeriaceae no Equador, evidencia a necessidade urgente de uma identificação acurada desses fungos.

A identificação precisa do agente causal da morte descendente do cacaueiro é fundamental para impulsionar estudos sobre a epidemiologia da doença e avaliar seu impacto

na produção de cacau. Esses estudos orientam programas de melhoramento genético e auxiliam os produtores no desenvolvimento de estratégias de manejo eficazes para reduzir a incidência da doença. Além disso, a detecção de patógenos emergentes é crucial para os países produtores de cacau que enfrentam condições climáticas distintas, pois permite compreender a distribuição geográfica desses fitopatógenos e os fatores que favorecem seu desenvolvimento.

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Considerações finais

Somente *Phytophthora palmivora* foi encontrada causando podridão parda e cancro em frutos e caules de diferentes clones de cacaueiro criolo e CCN51 em 5 províncias do Equador com diferentes condições de precipitação e altitude. Todos os isolados proveniente de frutos e caule foram patogênicos em frutos e mudas do clone CCN51, confirmando a importância da remoção de frutos e tecidos doentes da área para redução da fonte de inóculo da podridão parda e cancro do cacaueiro.

A morte descendente do cacaueiro é ocasionada por quatro espécies de *Lasiodiplodia* nas províncias do Oriente e Litoral do Equador. *Lasiodiplodia theobromae* (n=106) foi a espécie predominante em mais da metade das amostras, seguida por *L. pseudotheobromae* (n=29), *L. brasiliensis* (n=23) e *L. laeliocattleyae* (n=7). Enquanto *L. theobromae* e *L. pseudotheobromae* já tinham sido relatadas no cacaueiro, esse é o primeiro relato mundial de *L. brasiliensis* e *L. laeliocattleyae* causando doença no cacaueiro.

A caracterização morfo-molecular das espécies de *Phytophthora* e *Lasiodiplodia* causando a podridão parda e a morte descendente elucidou a diversidade de espécies de dois fitopatógenos de importância econômica na cadeia produtiva e certamente auxiliará no direcionamento dos programas de melhoramento e nas estratégias de controle eficiente de doenças do cacaueiro no Equador.