UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM FITOTECNIA
DOUTORADO EM FITOTECNIA

CYNTHIA PATRICIA DE SOUSA SANTOS ALVES

ROOT PATHOGENS IN CUCURBITS: SPECIES DIVERSITY, PATHOGENICITY AND CHEMICAL CONTROL

MOSSORÓ
2023

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Tese apresentada ao Doutorado em Fitotecnia do Programa de Pós-Graduação em Fitotecnia da Universidade Federal Rural do Semi-Árido como requisito para obtenção do título de Doutora em Agronomia: Fitotecnia.

Linha de Pesquisa: Proteção de Plantas

Orientador: Prof. Dr. Rui Sales Júnior

Coorientadora: Prof.ª Dra. Andréia Mitsa Paiva Negreiros

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2023

A474 Alves, Cynthia Patricia de Sousa Santos.
ROOT PATHOGENS IN CUCURBITS: SPECIES DIVERSITY, PATHOGENICITY AND CHEMICAL CONTROL / Cynthia Patricia de Sousa Santos Alves. - 2023.
85 f. : il.

Orientador: Rui Sales Júnior.
Coorientador: Andréia Mitsa Paiva Negreiros.
Tese (Doutorado) - Universidade Federal Rural do Semi-árido, Programa de Pós-graduação em Fitotecnia, 2023.

1. Macrophomina spp.. 2. Lasiodiplodia spp.. 3. Cucumis melo. 4. Citrullus lanatus. 5. Fungicidas. I. Sales Júnior, Rui , orient. II. Negreiros, Andréia Mitsa Paiva , co-orient. III. Título.

Ficha catalográfica elaborada por sistema gerador automáto em conformidade com AACR2 e os dados fornecidos pelo) autor(a).
Biblioteca Campus Mossoró / Setor de Informação e Referência
Bibliotecária: Keina Cristina Santos Sousa e Silva
CRB: 15/120

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Defendida em: 28/07/2023.

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Àqueles que me inspiram a ser uma pessoa melhor a cada dia: meu esposo, meus pais e irmãos. Agradeço por me ensinarem a persistir, mesmo perante as dificuldades, convicta de que com dedicação, disciplina e humildade tudo pode ser conquistado. É com grande admiração e gratidão que dedico este trabalho a todos vocês.

AGRADECIMENTOS

À Deus e à Nossa Senhora, pela presença divina que me guiou e fortaleceu, grata pela proteção e sabedoria que recebi, e por tudo o que me foi concedido ao longo desta jornada.

Ao Programa de Pós-Graduação em Fitotecnia da Universidade Federal Rural do Semi-Árido – UFERSA, pelo conhecimento adquirido e pelas habilidades desenvolvidas, e por me proporcionar uma formação de excelência com o apoio de professores capacitados.

Aos meus orientadores, Professor Dr. Rui Sales Júnior, Professora Dra. Andréia Mitsa Paiva Negreiros e Professora Dra. Kamila Câmara Correia, pela orientação, paciência e generosidade em compartilhar conhecimento, recursos e aconselhamento durante a realização deste trabalho. Suas contribuições foram fundamentais para o sucesso desta tese e para o meu desenvolvimento acadêmico e profissional.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, pela disponibilização da bolsa de pesquisa durante o doutorado.

À equipe do laboratório de Fitopatologia II da UFERSA: Naama Melo, Allinny Cavalcante, Elisângela Maniçoba, Dariane Viana, Fernanda Santos, Moisés Tavares, Sara Hellen, Gilmara Vitória, Hiago Souza, Abner Reurison, Juliana Oliveira, Breno Holanda e Márcio Thalison pela colaboração inestimável ao desenvolvimento desta tese. Sem o trabalho incansável e a dedicação de cada um de vocês, esta pesquisa não seria possível. Foi uma honra trabalhar ao lado de uma equipe tão comprometida e talentosa.

Ao Laboratório de Fitopatologia da Universidade Federal do Cariri – UFCA e ao Professor Dr. Sami Jorge Michereff pelo suporte e contribuição durante o desenvolvimento deste trabalho.

À minha amiga Izabelle, que desde está comigo desde a graduação somando forças para chegar até o doutorado.

E a todos que de alguma forma colaboraram para minha formação acadêmica.

Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota.

Madre Teresa de Calcutá

RESUMO

Os gêneros *Macrophomina* e *Lasiodiplodia* estão associados à podridão de raízes e declínio de ramas em plantios de cucurbitáceas na região semiárida do Nordeste brasileiro, provocando perdas econômicas. Pouco se conhece sobre a diversidade, patogenicidade e manejo das espécies fúngicas presentes nessas áreas de produção. Para abordar essas questões, foram realizados três estudos, com o objetivo de identificar a diversidade genética e patogenicidade de fungos estabelecidos em áreas produtoras de melancia para exportação e estudar o efeito de diferentes ingredientes ativos e doses no crescimento micelial *in vitro* e *in vivo* de *Macrophomina.* spp. em meloeiro. No primeiro estudo foram coletadas 30 plantas de melancieira sintomáticas em cada um dos 16 campos comerciais de produção de melancia avaliados, nos estados do Rio Grande do Norte e Ceará. De cada planta coletada, foram isoladas amostras da parte radicular para isolamento fúngico e posterior identificação em nível de gênero. Um total de 156 isolados foi identificado molecularmente por meio de extração de DNA e amplificação por reação em cadeia da polimerase, utilizando primers específicos (MpTefF e MpTefR para *M. phaseolina*, MsTefF e MsTefR para *M. pseudophaseolina*, e MeTefF e MeTefR para *M. euphorbiicola*). A fim de confirmar a patogenicidade, foram selecionados aleatoriamente 50 isolados de *M. phaseolina* e 50 de *M. pseudophaseolina*. A incidência e severidade da doença, o comprimento aéreo e radicular e o peso seco de todas as plantas do ensaio foram avaliados. Foram observados e confirmados como patogênicos para melancieira todos os isolados de *Macrophomina* testados, sendo que *M. phaseolina* apresentou maior incidência (97%) e severidade (3,21) da doença em relação à *M. pseudophaseolina* (70% e 1,15, respectivamente). Este foi o primeiro relato patogênico de *M. pseudophaseolina* à melancieira no mundo. Melancieiras infectadas com *M. phaseolina* corresponderam a um maior comprimento aéreo (74,89 cm) e radicular (17,96 cm), e menor peso seco (2,12 g) em comparação à *M. pseudophaseolina* (66,61 cm, 17,42 cm e 2,22 g, respectivamente). O segundo estudo avaliou o efeito *in vitro* e *in vivo* de boscalida, carbendazim, ciprodinil, fluazinam e fludioxonil em cinco doses (0,01; 0,10; 1; 10; e 100 mg/L i.a.) no crescimento micelial diário, na porcentagem de inibição e na concentração efetiva para reduzir 50% do crescimento micelial de nove isolados de *Macrophomina* (*M. phaseolina*: CMM1556, CMM4748 e CMM4764; *M. pseudophaseolina*: CMM2163, CMM4815 e CMM4767; e *M. euphorbiicola*: CMM2158, CMM4868 e CMM4867). Os resultados dos testes *in vitro* mostraram que o fluazinam e o fludioxonil foram altamente tóxicos (CE_{50} = 0,03 mg/L i.a.) à *Macrophomina.* Nos testes *in vivo*, o fluazinam foi capaz de reduzir os danos da patogenicidade de *M. phaseolina* e *M. pseudophaseolina* no meloeiro, em pelo menos 21,43%. No entanto, os isolados de *M. euphorbiicola* provocaram menor incidência (28,57%) e severidade (0,29) da doença em plantas tratadas com fludioxonil. O terceiro estudo relatou pela primeira vez no Brasil e no mundo a ocorrência de *Lasiodiplodia brasiliensis* em melancia. Testes de patogenicidade confirmaram que os isolados causaram sintomas característicos de podridão de raízes e declínio em melancieira.

Palavras-chave: *Macrophomina* spp.; *Lasiodiplodia* spp.; *Cucumis melo*; *Citrullus lanatus*; Fungicidas.

ABSTRACT

The genus *Macrophomina* and *Lasiodiplodia* are associated with root rot and vine decline in cucurbit crops in the semiarid region of Northeastern Brazil, causing economic losses. Little is known about the diversity, pathogenicity and management of fungal species in these production areas. To address these issues, three studies were conducted, with the aim of identifying the genetic diversity and pathogenicity of fungi established in areas producing watermelon for export and studying the effect of different active ingredients and doses on the *in vitro* and *in vivo* mycelial growth of *Macrophomina* spp. in melon plants. In the first study, 30 symptomatic watermelon plants were collected in each of the 16 commercial watermelon production fields evaluated, in the states of Rio Grande do Norte and Ceará. From each plant collected, samples of the root part were isolated for fungal isolation and subsequent identification at the genus level. A total of 156 isolates were molecularly identified using DNA extraction and polymerase chain reaction amplification, with specific primers (MpTefF and MpTefR for *M. phaseolina*, MsTefF and MsTefR for *M. pseudophaseolina*, and MeTefF and MeTefR for *M. euphorbiicola*). To confirm the pathogenicity, 50 isolates of *M. phaseolina* and 50 of *M. pseudophaseolina* were randomly selected. Disease incidence and severity, shoot and root length and dry weight of all plants in the trial were evaluated. All *Macrophomina* isolates tested were observed and confirmed as pathogenic for watermelon, with *M. phaseolina* showing higher incidence (97%) and severity (3.21) of the disease compared to *M. pseudophaseolina* (70% and 1.15, respectively). This was the first pathogenic report of *M. pseudophaseolina* on watermelon in the world. Watermelon trees infected with *M. phaseolina* corresponded to greater shoot (74.89 cm) and root (17.96 cm) length, and lower dry weight (2.12 g) compared to *M. pseudophaseolina* (66.61 cm, 17.42 cm and 2.22 g, respectively). The second study evaluated the *in vitro* and *in vivo* effect of boscalid, carbendazim, cyprodinil, fluazinam and fludioxonil in five doses (0.01; 0.10; 1; 10; and 100 mg/L a.i.) on daily mycelial growth, in percentage of inhibition and effective concentration to reduce 50% of mycelial growth of nine *Macrophomina* isolates (*M. phaseolina*: CMM1556, CMM4748 and CMM4764; *M. pseudophaseolina*: CMM2163, CMM4815 and CMM4767; and *M. euphorbiicola*: CMM2158, CMM4868 and CMM4867). Results of *in vitro* tests showed that fluazinam and fludioxonil were highly toxic (EC_{50} = 0.03 mg/L a.i.) to *Macrophomina*. In *in vivo* tests, fluazinam was able to reduce the pathogenic damage of *M. phaseolina* and *M. pseudophaseolina* in melon, by at least 21.43%. However, *M. euphorbiicola* isolates caused lower disease incidence (28.57%) and severity (0.29) in plants treated with fludioxonil. The third study reported for the first time in Brazil and in the world the occurrence of *Lasiodiplodia brasiliensis* in watermelon. Pathogenicity tests confirmed that the isolates caused characteristic symptoms of decline in watermelon.

Keywords: *Macrophomina* spp.; *Lasiodiplodia* spp.; *Cucumis melo*; *Citrullus lanatus*; Fungicides.

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LIST OF ABBREVIATIONS AND ACRONYMS

µl	Microliter
µm	Micrometre
a. i.	Active ingredient
BA	Bahia
CE	Ceará
CFC	Culture Collection of Phytopathogenic Fungi from Cariri
cm	Centimeter
CMM	Coleção Maria Menezes
CV	Coefficient of variation
cv.	Cultivar
DAI	Days after inoculation
DAT	Days after transplanting
DNA	DeoxyriboNucleic Acid
DW	Dry weight
EC_{50}	Effective concentration
FAO	Food and Agriculture Organization
FRW	Fresh root weight
FSW	Fresh shoot weight
g	Gram
ha	Hectare
IBGE	Instituto Brasileiro de Geografia e Estatística
ITS	Internal Transcribed Spacer
L	Liter
LSD	Least Significant Difference
mg	Miligram
mi	Million
min	Minute
ml	Milliliter
MP	*Macrophomina phaseolina*
MPS	*Macrophomina pseudophaseolina*
NaOCl	Sodium hypochlorite

PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PGI	Percentage of growth de inhibition
ppm	Parts per million
RL	Root length
RN	Rio Grande do Norte
RRVD	Root rot and vine decline
SC	Suspension concentrate
SL	Shoot lenght
t	Ton
UFRPE	Universidade Federal Rural de Pernambuco
UV	Ultraviolet radiation
WG	Water Dispersible Granules

LIST OF SYMBOLS

©	Copyright
®	Trademark
%	Percentage
°C	Celsius degree

SUMMARY

CHAPTER I

1 GENERAL INTRODUCTION

Cucurbits are crops belonging to the Cucurbitaceae family, distributed in 95 genera, with approximately 1000 species (Christenhusz; Byng, 2016). Of which, watermelon (*Citrullus lanatus* (Thunb.) Mat. & Nak.), cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.) and pumpkins (*Cucurbita* spp.) stand out (Grumet, *et al*., 2021; Pan *et al*., 2019). In 2021 a world production of 101 mi t of watermelon was recorded, followed by 93 mi t of cucumber, 28 mi t of melon and 23 mi t of pumpkins (FAO, 2023). Together, these species account for 70% of the world's vegetable crop (FAO, 2023; Grumet *et al*., 2021).

Brazil has the fifth and 11th largest production of watermelon and melon in the world, registering in 2021 production values of 2,141,970 t and 607,047 t, respectively (FAO, 2023; IBGE, 2023; Kist *et al*., 2022). The Northeast region of Brazil is the main producer of watermelon, with its largest producers being the states of Rio Grande do Norte (RN) (340 t) and Bahia (BA) (213 t) (IBGE, 2023). As well, more than half of the national melon crop is also concentrated in this region, in the states of RN (361,649 t), BA (86,866 t) and Ceará (CE) (70,665 t) (Andrade *et al*., 2022; IBGE, 2023; Kist *et al*., 2022; Silva *et al*., 2024).

Because they present greater adaptability to edaphoclimatic conditions, watermelon trees of Japanese and American origin, as well as melon trees of the *inodorus* and *cantalupensis* groups are the most cultivated in Brazil (Melo *et al*., 2021; Vendruscolo *et al*., 2018).

The main characteristic of the development of these crops in the Brazilian Northeast is that commercialization is mostly directed to the international market. About 80% of watermelon and melon fruits are destined for export to European markets and the Arab world (Kist *et al*., 2022). As a result, the market has demanded the adoption of cultivation techniques aimed at increasing production in quantity and quality of the fruits produced (Campos *et al*., 2019).

Among the cultivation technologies adopted by producers, we highlight the use of high-yield inputs, hybrid seeds, mulching, increased population density, repeated cultivation in the same soil/year (Figueirêdo *et al*., 2017; Lambert *et al*., 2017). It is known that the erroneous adoption of this management has limited the production of cucurbits and decreased the quality of the fruits, precisely because it interferes with the soil microbiota and plant health (Sales Júnior *et al*., 2019). These disturbances cause diseases caused by a complex of

pathogens that cause wilting, collapse, sudden plant death, as well as root rot and vine decline (RRVD) (Cohen *et al*., 2012a; Infantino *et al*., 2021; Porto *et al*., 2019).

Typical symptoms of RRVD consist of discoloration and necrotic lesions on the roots, in addition to brown watery spots on the plant neck. As the disease progresses, these spots tend to darken, as well as the appearance of longitudinal cracks (Pereira *et al*., 2012). In the leaves, there is a reduction in size, yellowing, followed by wilting and death, due to the production of fungal toxins and blockage of xylem vessels (Dhingra; Sinclair, 1978; Manjunatha; Saifulla, 2018; Martyn; Miller, 1996; Sales Júnior *et al*., 2019). These symptoms manifest themselves close to the fruit harvest period when the plant's water demand is highest. Root rot results in a reduced capacity for water absorption and transport, causing wilting and decline of the plant (Martyn; Miller, 1996).

Fungi of the Botryosphaeriaceae family are among the pathogens that cause this disorder, among them those of the genus *Macrophomina* and *Lasiodiplodia*, which have already been identified in cucurbits causing RRVD, wilt and vascular gummosis, and are characterized by the wide range of hosts with reports worldwide (Mello *et al*., 2021; Pisani *et al*., 2021; Sarr *et al*., 2014; Silva *et al*., 2023).

Macrophomina spp. belongs to the phylum Ascomycota, class Dothideomycetes and order Botryosphaeriales. It is a polyphagous and mitosporic fungus, which can form two types of structures: pycnidia (asexual) and microsclerotia (resistance) (Babu *et al*., 2011; Kumar *et al*., 2023; Marquez *et al*., 2021). *Macrophomina* microsclerotia are the primary inoculum, observed in the germination, penetration, parasitic and saprophytic phases, through the association of individual hyphae cells (multicellular) linked by a melanin material (Bruton *et al*., 1987; Kaur *et al*., 2012; Reis *et al*., 2014). In the parasitic phase, these structures block the vascular system of the host, preventing the transport of water and nutrients and causing root discoloration, decline and death of the plant (Basandrai *et al*., 2021; Lodha; Mawar, 2020).

In the saprophytic phase, microsclerotia have the ability to survive for long periods (2 to 15 years) and can germinate throughout the growing season (Baird *et al*., 2003; Gupta *et al*., 2012; Short *et al*., 1980). In this same phase, in addition to the production of microsclerotia and hyphae, the formation of larger, globose structures, grayish to black in color, called pycnidia (Basandrai *et al*., 2021). They remain in the host tissue and rupture when mature, dispersing the conidia (Kaur *et al*., 2012).

To date, five species belonging to this genus are known: *Macrophomina phaseolina* (Tassi) Goid.; *Macrophomina pseudophaseolina* Crous, Sarr & Ndiaye; *Macrophomina*

euphorbiicola A.R. Machado, D.J. Soares & O.L. Pereira; *Macrophomina vaccinii* Y. Zhang & L. Zhao; and *Macrophomina tecta* Vaghefi, B. Poudel & R.G. Shivas (Goidànich, 1947; Machado *et al*., 2019; Poudel *et al*., 2021; Sarr *et al*., 2014; Zhao *et al*., 2019).

Macrophomina phaseolina is the oldest species of the genus, described in 1947 in Italy on *Phaseolus*, and in 1935 in Brazil on common bean (*Phaseolus vulgaris* L.), based only on the morphological characteristics of the fungus (Bitancourt, 1935; Babu *et al*., 2007; Dhingra; Sinclair, 1978). Since then, reports of hosts of this pathogen have been recurrent, reaching more than 100 plant families, in more than 800 plant species (Farr; Rossman, 2023). Only in 2014, after morphological and molecular analyses, *M. pseudophaseolina* was described causing diseases in cowpea (*Vigna unguiculata* (L.) Walp), peanut (*Arachis hypogaea* L.), sorrel (*Hibiscus sabdariffa* L.), and okra (*Abelmoschus esculentus* L.) in Senegal (Mbaye *et al*., 2015; Sarr *et al*., 2014). In Brazil, this species has been found since 2018 attacking peanut, cotton (*Gossypium hirsutum* L.), castor bean (*Ricinus communis* L.), cassava (*Manihot esculenta* Crantz) and other crops (Brito *et al*., 2019; Machado *et al*., 2019). In 2018, *M. euphorbiicola* was reported for the first time in Brazil on castor bean and jatropha (*Jatropha gossypiifolia* L.), with subsequent reports on sweet potato (*Ipomoea batatas* (L.) Lam.) and stevia (*Stevia rebaudiana* [Bertoni] Bertoni) (Machado *et al*., 2019; Mello *et al*., 2021; Sanabria-Velazquez *et al*., 2023). *Macrophomina vaccinii* was described in 2019, only on blueberry (*Vaccinium* spp.) in China (Zhao et al., 2019) and *M. tecta* in 2021, on sorghum (*Sorghum bicolor* L.) and mungbean (*Vigna radiata* L.) in Australia (Poudel *et al*., 2021).

Two species of *Macrophomina* are known so far to be associated with cucurbits. *Macrophomina phaseolina* is pathogenic to cucumber, melon and watermelon, and *M. pseudophaseolina* to melon, watermelon and was found in weeds (*Trianthema portulacastrum* L. and *Boerhavia diffusa* L.) associated with these crops present in the states of RN and CE (Basandrai *et al*., 2021; Egel *et al*., 2020; Negreiros *et al*., 2022; Negreiros *et al*., 2019; Sales Júnior *et al*., 2012).

According to Sarr et al. (2014), it is known that the genus *Macrophomina* presents good adaptation to tropical and subtropical conditions. However, this characteristic has become a concern due to the damage caused by this fungus. Due to the high adaptability capacity, the pathogen has been verified in new crops, as well as its occurrence in several countries (Farr; Rossman, 2023). Therefore, constant monitoring on the possible hosts, species diversity and survival conditions of *Macrophomina* are important (Poudel *et al*., 2021).

The advancement of molecular techniques and technology based on polymerase chain reaction (PCR) has contributed to a better understanding of the genetic and pathogenic variability of *Macrophomina* (Machado *et al*., 2019; Marquez *et al*., 2021; Sarr *et al*., 2014). The study of these aspects is faster, especially with the use of specific primers in PCR, which offer speed in the detection of species, simplicity in execution and low cost, compared to multilocus techniques (Cota-Barreras *et al*., 2022; Santos *et al*., 2020). Recently, specific primers amplifying the translation elongation factor gene region (TEF1- α) have been developed for *M. phaseolina*, *M. pseudophaseolina* and *M. euphorbiicola* species (Santos *et al*., 2020). They have assisted in rapid decision making on the management to be adopted in the field (Marquez *et al*., 2021; Santos *et al*., 2020).

Management of diseases caused by soil-inhabiting fungi involves strategies that prevent the introduction of the pathogen into new areas or reduce its severity once it is established (Lodha; Mawar, 2020). These approaches are achieved by using resistant cultivars, applying fungicides and employing biological products (Lodha; Mawar, 2020). Management of *Macrophomina* in cucurbits crops is mainly based on cultural practices aimed at decreasing the survival of the inoculum in the field. Once the pathogen is established, control options are limited due to the absence of resistant varieties and chemicals registered for the specific control of this fungus in these crops in Brazil (AGROFIT, 2023; Linhares *et al*., 2020).

In the literature there are studies with *in vitro* and *in vivo* evaluations, in seeds and plants, with some active ingredients such as boscalid, carbendazim, cyprodinil, fluazinam and fludioxonil in the control of pathogens that cause RRVD in cucurbits (Athira, 2017; Cavalcante *et al*., 2021; Cohen *et al*., 2012b; Iqbal; Mukhtar, 2020; Kumari *et al*., 2015; Lokesh *et al*., 2020).

Boscalid, carbendazim and cyprodinil are active ingredients that have a systemic action on plants and differ in their mode of action on fungi. Boscalid acts on complex II respiration and inhibits the enzyme succinate dehydrogenase (FRAC, 2023). Its high fungitoxicity to *Macrophomina* was verified by Cohen et al. (2012b), in melon, associated with pyraclostrobin. Carbendazim acts on the mitosis and cell division of pathogens (FRAC, 2023). It is known that this fungicide is capable of inhibiting the mycelial growth of *Macrophomina in vitro*, as well as reducing the incidence and severity of the disease in *in vivo* trials (Iqbal; Mukhtar, 2020; Lokesh et al., 2020). Like the previous ones, cyprodinil is systemic but acts on the synthesis of amino acids and proteins of the fungi (FRAC, 2023).

This fungicide, although there are no studies on the control of *Macrophomina*, Cavalcante *et al*. (2021) found it to be moderately toxic to *Monosporascus* spp., which causes RRVD.

Fluazinam and fludioxonil are contact active ingredients, which act on respiration and osmotic signal transduction, respectively (FRAC, 2023). Among these, fludioxonil is the only fungicide registered for *Macrophomina* control in Brazil, however, it is limited to bean seed treatment (AGROFIT, 2023). In the United States, this active ingredient is released for use in the *Monosporascus* x watermelon pathosystem for vine decline (Cannonball, 2023). Despite this, little is known about the effect of these fungicides on mycelial inhibition and reduction of disease incidence and severity for different *Macrophomina* species.

Another fungal genus of importance for cucurbits is *Lasiodiplodia*, formed by 48 species, present in tropical and subtropical regions, associated with a wide host range, causing diseases such as stem and fruit rot, gummosis, canker and death (El-Ganainy *et al*., 2022; Pisani *et al*., 2021; Santos *et al*., 2022; Suwannarach *et al*., 2020). This pathogen produces pycnidia, conidia and has white colored mycelium that turns greenish-gray (Santos *et al*., 2022). The morphophysiological identification of these species is not secure, since the characteristics may vary depending on environmental and climatic conditions (Coutinho *et al*., 2016). Thus, multilocus molecular techniques based on the amplification of the internal transcribed spacer (ITS) region and fragments of the translation elongation factor 1-α (TEF) and β-tubulin 2 (TUB) genes have been widely used for the phylogenetic characterization of *Lasiodiplodia* (Coutinho *et al*., 2016; El-Ganainy *et al*., 2022).

Polyphagous and cosmopolitan in nature, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. is the most common species of the genus, and is associated with diseases in cucurbits, causing postharvest damage in melon fruits and stem rot in watermelon plants (Keinath *et al*., 2017; Pisani *et al*., 2021; Suwannarach *et al*., 2020). *Lasiodiplodia brasiliense* MSB Netto, MW Marques & AJL Phillips is also reported in these crops, causing gummosis (Farr; Rossman, 2022) and decline in watermelon, which is a characteristic symptom of RRVD also caused by *Macrophomina* spp. (Santos *et al*., 2022). The similarity of these symptoms makes diagnosis challenging (Keinath *et al*., 2017; Santos *et al*., 2022).

In this sense, the occurrence of phytopathogens that cause RRVD in cucurbits production areas has caused significant losses, which reinforces the realization of a constant monitoring of the species present in the areas, as well as the development of studies that evaluate active ingredients for their management. For this reason, this work aimed to identify the genetic diversity and pathogenicity of fungi established in watermelon producing areas for

export and to study the effect of different active ingredients and doses on *in vitro* and *in vivo* mycelial growth of *Macrophomina* spp. in melon.

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CHAPTER II

DIVERSITY AND PATHOGENICITY OF *Macrophomina* spp. ASSOCIATED WITH WATERMELON PLANTS

ABSTRACT: This study explored the diversity and pathogenicity of *Macrophomina* spp. in watermelon-producing regions of Ceará and Rio Grande do Norte States, Brazil. From 16 commercial fields across both states, we collected 30 watermelon plants exhibiting RRVD symptoms. Fungal growth was identified from root isolates based on morphological characteristics. The species of each isolate were further determined using specific primers: MpTefF and MpTefR for *M. phaseolina*, MsTefF and MsTefR for *M. pseudophaseolina*, and MeTefF and MeTefR for *M. euphorbiicola*. Out of 156 confirmed *Macrophomina* isolates, 75 were identified as *M. phaseolina* and 81 as *M. pseudophaseolina*. We assessed the pathogenicity and virulence of 50 isolates from each species on the watermelon variety 'Crimson Sweet' using infested husk rice grain inoculation. Parameters such as disease incidence and severity, as well as shoot and root length, and dry weight, were evaluated. The experiment employed a completely randomized design with 101 treatments (100 *Macrophomina* isolates and control) and 8 replications. All isolates proved pathogenic to watermelon. Notably, plants inoculated with *M. phaseolina* showed greater disease incidence (97%) and severity (3.21) than those colonized by *M. pseudophaseolina* (70% and 1.15, respectively). Moreover, *M. phaseolina*-inoculated plants had longer average shoot (79.89 cm) and root lengths (17.96 cm) but lower dry weight (2.22 g). In conclusion, our findings present the first global report on the pathogenicity of *M. pseudophaseolina* to watermelon plants and highlight its predominant presence in the surveyed regions. Still, *M. phaseolina* exhibited greater aggressiveness toward watermelon plants.

Keywords: *Citrullus lanatus*; Specific primers; Root rot; Disease severity.

1 INTRODUCTION

Watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] is a globally cultivated fruit with over four million hectares dedicated to farming and a production surpassing 160 million tons in 2021 (FAO 2023). Ranking fifth in watermelon cultivation, Brazil focuses approximately 37% of its production in the Northeast, especially in Rio Grande do Norte (RN) and Bahia (BA) (FAO 2023; IBGE 2023). Productivity has consistently risen (FAO 2023), which has been spurred by advanced technologies like hybrid seeds, drip irrigation, and mulching (Sales Júnior et al. 2019).

However, the combination of these technologies with conventional monoculture planting, featuring multiple crops per area annually, has intensified disease issues. Prominent among them is the "root rot and vine decline" (RRVD) syndrome. Caused by various soil-root

pathogens, this syndrome damages plant root systems, creating water imbalances and leading to vine decline (Martyn and Miller 1996; Boughalleb et al. 2010; Sales Júnior et al. 2010).

RRVD has significantly impacted cucurbits, such as melons and watermelons, causing symptoms ranging from reduced stands and yellowing to wilting and death (Martyn and Miller 1996; Sales Júnior et al. 2004; Cohen et al. 2012; Wu et al. 2022). The disease can decimate up to 40% of watermelon productivity (Sinclair and Backman 1989; Bianchini et al. 1997; Kaur et al. 2012; Athayde Sobrinho 2016; Cohen et al. 2016; Gomes-Silva et al. 2018; Porto et al. 2019; Wu et al. 2022).

The fungal genus *Macrophomina* (Botryosphaeriaceae, Ascomycota), which affects over 800 plant species (Farr and Rossman 2023), is a chief contributor to RRVD (Bruton 1998; Cohen et al. 2016; Negreiros et al. 2019). Recent discoveries of new *Macrophomina* species and their diverse host range underline the need for ongoing research (Negreiros et al. 2019; Farr and Rossman 2023). To date, five *Macrophomina* species have been identified globally, namely: *Macrophomina phaseolina* (Tassi) Goid., *M. pseudophaseolina* Crous, Sarr & Ndiaye, *M. euphorbiicola* A.R. Machado, D.J. Soares & O.L. Pereira, *M. vaccinii* Y. Zhang ter & L. Zhao, and *M. tecta* Vaghefi, B. Poudel & R.G. Shivas (Goidanich 1947; Sarr et al. 2014; Machado et al. 2018; Zhao et al. 2019; Poudel et al. 2021).

In cucurbit contexts, *M. phaseolina* has been reported in crops like watermelon, melon, and cucumber (Andrade et al. 2005; Dantas et al. 2013; Egel et al. 2020; Kim, Kim and Lee 2021; Silva et al. 2022; Wu et al. 2022). Studies by Negreiros et al. (2019 and 2022) identified *M. phaseolina* and *M. pseudophaseolina* isolates as pathogenic to watermelon and melon plants. The diversity and economic significance of *Macrophomina* highlight the importance of studying isolate variation within similar areas (Sarr et al. 2014; Machado et al. 2018; Basandrai et al. 2021).

Fast pathogen detection is crucial for timely disease management and epidemic prevention (Santos et al. 2020; Marquez et al. 2021). The advent of specific primers has expedited the differentiation of *Macrophomina* species by targeting the TEF1- α I gene fragment (Santos et al. 2020). In Brazil, precision-oriented primers for *M. phaseolina*, *M. pseudophaseolina*, and *M. euphorbiicola* have been developed, with successful applications both nationally and internationally (Santos et al. 2020; Cota-Barreras et al. 2022; Dell'Olmo et al. 2022).

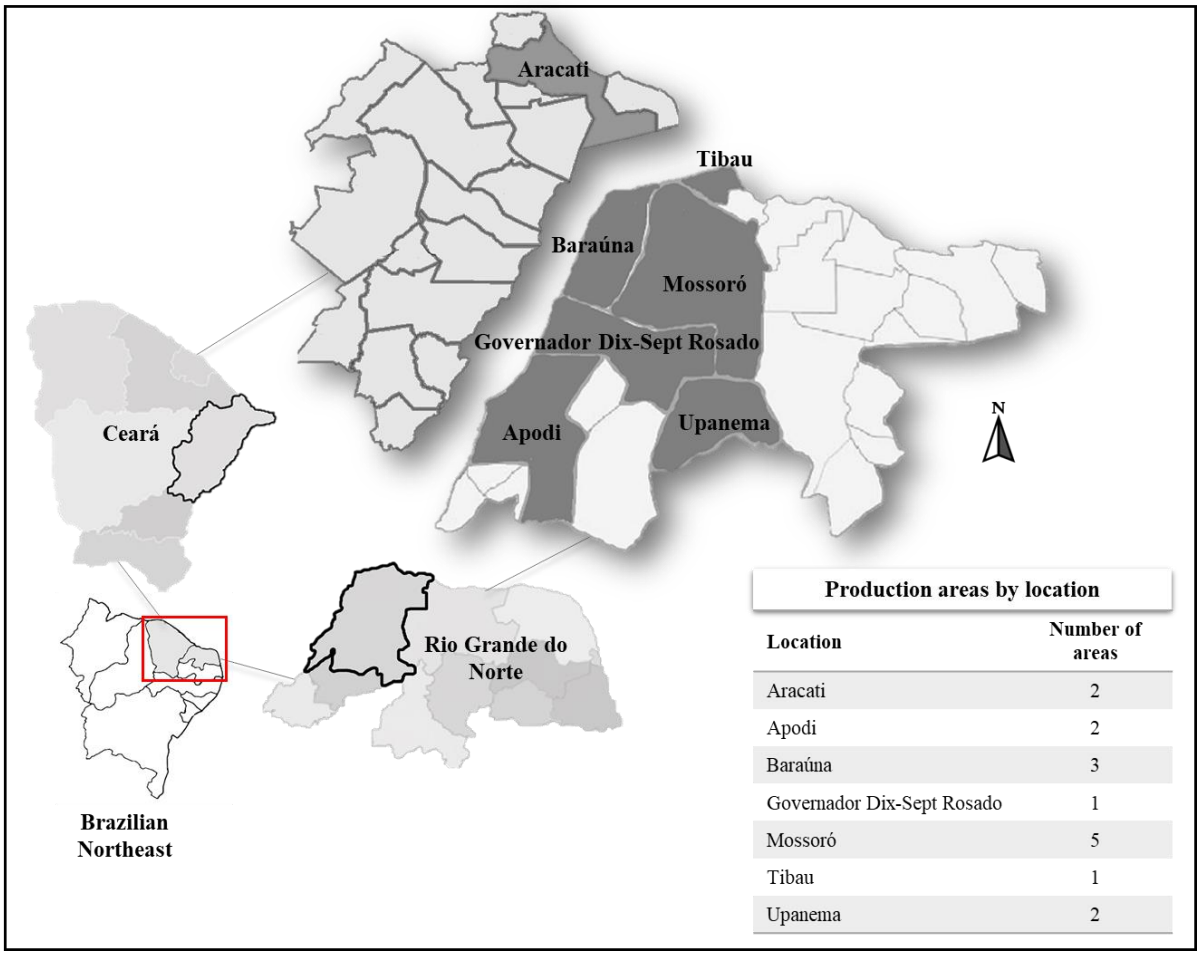
Given the potential significant diversity of *Macrophomina* species in watermelon farming regions and varying pathogenicity levels among isolates, understanding disease dynamics is pivotal. Thus, this study aimed to assess the species diversity and pathogenicity

of *Macrophomina* spp. isolates from watermelon fields in Ceará and Rio Grande do Norte States, Brazil.

2 MATERIAL AND METHODS

2.1 Sample Collection

From August to December 2019, 30 watermelon plants displaying RRVD symptoms were sampled from each of the 16 commercial fields assessed. These fields were situated in Aracati in Ceará State (CE) and in Apodi, Baraúna, Governador Dix-Sept Rosado, Mossoró, Tibau, and Upanema in Rio Grande do Norte State (RN), Brazil (Figure 1).



Production areas by location

Location	Number of areas
Aracati	2
Apodi	2
Baraúna	3
Governador Dix-Sept Rosado	1
Mossoró	5
Tibau	1
Upanema	2

Fig. 1 Locations of watermelon fields where plants with RRVD symptoms were identified and collected.

2.2 Fungal Isolation

From August to December 2019, watermelon roots underwent a cleaning and disinfection process. They were first rinsed with tap water and then disinfected by immersing

them in 1.5% hypochlorite for a minute, followed by two immersions in sterile distilled water for the same duration. Next, small root fragments, measuring 4-5mm, were placed on Petri dishes filled with Potato Dextrose Agar (PDA, Merck KGaA, Darmstadt, Germany) fortified with 0.5 g L^{-1} streptomycin sulfate (PDAS). These dishes were then stored in the dark at a temperature of 28 ± 1 ºC for 72 hours.

Colonies resembling *Macrophomina* were transferred to new Petri plates with PDA and again incubated under the same conditions until their growth spanned the diameter of the plate. The identity of these isolates was confirmed through microscopic examination, checking for hyaline mycelia, brown staining, and the presence of microsclerotia, as described in prior studies (Goidanich 1947; Sarr et al. 2014; Machado et al. 2018; Zhao et al. 2019; Poudel et al. 2021).

Pure fungal cultures were procured using the mono-hyphal technique. For long-term preservation, both the 'Castellani' method and the organic-sandy substrate method were employed (Medeiros et al. 2015; Alfenas and Mafia 2016). These isolates were then cataloged in the fungal collection at the Phytopathology Laboratory II of the Universidade Federal Rural do Semi-Árido (UFERSA).

2.3 DNA extraction and PCR amplification

For genomic DNA extraction, the isolates were cultivated on a PDA culture medium covered with a semipermeable cellophane membrane for a week. The Wizard® DNA extraction kit (Promega Corporation, Madison, USA) was employed, strictly adhering to the provided instructions.

The PCR setup had a final volume of 12.5 µL: 6.25 µL of GoTaq® Green Master Mix (2X) from Promega Corporation, Madison, USA; 4.25 µL of Milli-Q® Water; 0.5 µL of both forward and reverse primers, and 1 µL of DNA (concentration: 25ng/µL). The translation elongation factor-1alpha (TEF1- α) region was targeted to differentiate between *Macrophomina* species using the specific primers for *M. phaseolina* (MpTefF-AAACACACTTTTCGCACTCCTGC, MpTefR-TATGCTCGCAGAGAAGAACACGA), *M. pseudophaseolina* (MsTefF-GCACACTTTTCGCGCTTCTGTA, MsTefR-TGTGCTCGCTGGGAAGAACATGA), and *M. euphorbiicola* (MeTefF-AAGCATACTTTTCGTGCTCCTGC, MeTefR- AAAGGAACATGAGTGGCCAAAAA) (Santos et al. 2020). Water substituted for DNA in the negative control, while for the positive

controls, previously identified isolates using the same specific primers were used: CMM228 for *M. phaseolina*, CMM208 for *M. pseudophaseolina*, and CMM136 for *M. euphorbiicola*.

PCR was executed in a thermocycler under the following protocol: initial denaturation at 94°C for two minutes, succeeded by 30 cycles comprising denaturation at 94°C for one minute, annealing at 63°C for 30 seconds, and extension at 72°C for one minute. This was rounded off with a final extension at 72°C for ten minutes (Santos et al. 2020). Post PCR, the amplified products were subjected to electrophoresis on a 1% agarose gel, treated with GelRed2X ™ (Biotium 28 Inc., Hayward, USA) added directly to the sample, and then examined under ultraviolet light.

2.4 Pathogenicity and virulence on watermelon

Inocula were prepared following the method outlined by Songa et al. (1997) and Souza et al. (2022), with certain alterations. Specifically, 35g husked rice grains were placed in 50 mL Falcon tubes, moistened with distilled water, and autoclaved three times at 24-hour intervals. These tubes were then inoculated with five 8mm PDA plugs colonized by *Macrophomina*, distributed as 50 *M. phaseolina* and 50 *M. pseudophaseolina* isolates. The tubes were incubated for 15 days at about 30°C and stirred daily.

Under a greenhouse environment, the pathogenicity of *Macrophomina* isolates on 'Crimson Sweet' watermelon plants was evaluated. Following the direct sowing in a mixture of sterilized soil and commercial substrate Tropstrato HT® (3:1 v/v in polyethylene pots), six husked rice grains colonized with the fungal isolate were deposited. Control pots received pathogen-free rice grains. Regular watering was ensured till the experiment concluded.

The pathogenicity test was performed 60 days after sowing. Disease prevalence was gauged by calculating the percentage of infected plants. Using a visual grading scale from Ravf and Ahmad (1998), isolate virulence was ascertained. The scale is as follows: 0 for uninfected tissues, 1 for <3% infected hypocotyl tissues, 2 for 3-10% infection, 3 for 11-25% infection, 4 for 26-50% infection, and 5 for over 50% infection. Plant metrics included shoot length (SL, cm), root length (RL, cm), and dry weight (DW, g).

The experiment adopted a completely randomized design with 101 treatments: 100 *Macrophomina* isolates (50 each of *M. phaseolina* and *M. pseudophaseolina*) and one control, with each having eight replications. This study was repeated twice. Preliminary ANOVA was used to check for significant variance between the two repetitions and to decide if data pooling was feasible. The Kruskal-Wallis non-parametric test ($p \leq 0.05$) was used to assess disease incidence and severity for each isolate using ASSISTAT software v. 7.7 (Silva and

Azevedo 2016). For metrics like shoot and root length and plant dry weight, ANOVA and the Scott-Knott test were employed for analysis, again using ASSISTAT software v. 7.7 (Silva and Azevedo 2016). Combined data for the *Macrophomina* species, concerning incidence and severity, were scrutinized with the Mann-Whitney test at 5% significance. Meanwhile, the Student's t-test, at a 5% significance level, was utilized for the plant's SL, RL, and DW measurements using STATISTIX software v. 9.0.

3 RESULTS

3.1 Fungal Isolation

From the roots of watermelon plants exhibiting RRVD symptoms, 156 *Macrophomina* spp. isolates were retrieved. These exhibited morphological features consistent with the genus (Figure 2). These isolates were sourced from 16 production fields across various locations: Aracati in CE (20 isolates), Apodi (20 isolates), Baraúna (28 isolates), Gov. Dix-Sept Rosado (10 isolates), Mossoró (49 isolates), Upanema (20 isolates), and Tibau (9 isolates) in RN, Brazil.

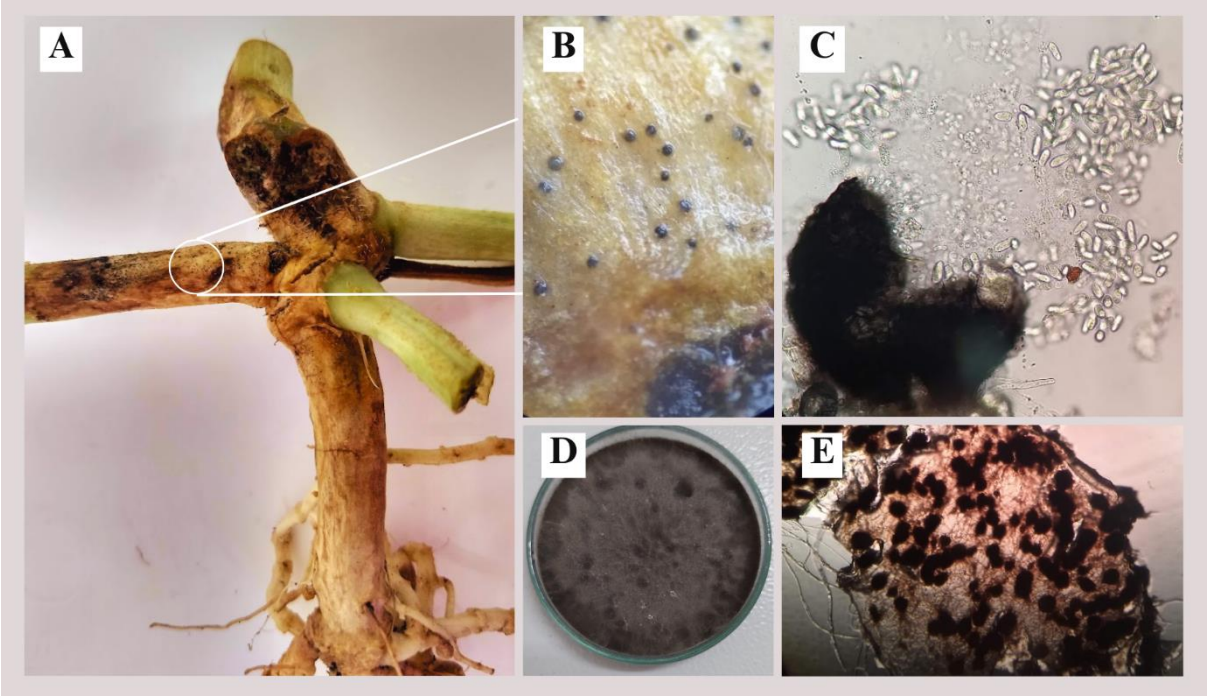


Fig. 2 Morphological traits of *Macrophomina*. A: Stem of a watermelon plant displaying signs of infection and RRVD manifestations. B: Pycnidia embedded within the host tissue. C: Distinctive pycnidia and conidia formations of *Macrophomina*. D: Characteristic dark mycelium. E: Microsclerotia of *Macrophomina*

3.2 PCR amplification and species frequency

All 156 isolates that were morphologically characterized produced an amplification with one of the primer pairs specifically designed for either *M. phaseolina* (MpTefF/MpTefR) or *M. pseudophaseolina* (MsTefF/MsTefR). Amplification frequencies varied among the municipalities. Notably, none of the isolates were amplified with the primer specific for *M. euphorbiicola* (MeTefF/MeTefR) (Table 1).

Table 1 Frequency of *Macrophomina* species identified with specific primers by county in 16 watermelon-producing fields in Ceará and Rio Grande do Norte States, Brazil

County	State	*M. phaseolina*	*M. pseudophaseolina*
Aracati	CE[1]	6	14
Apodi	RN[2]	0	20
Baraúna	RN[2]	22	6
Gov. Dix-Sept Rosado	RN[2]	0	10
Mossoró	RN[2]	37	12
Tibau	RN[2]	8	1
Ipanema	RN[2]	2	18
Total		75	81

[1]CE = State of Ceará, [2]RN = State of Rio Grande do Norte.

Macrophomina phaseolina appeared to be the least prevalent species among the sampled locations. Specifically, 78.7% of the *M. phaseolina* isolates originated from Mossoró (37 isolates) and Baraúna (22 isolates) in RN (Table 1). The remaining 21.3% were distributed among Aracati (6 isolates) in CE, and Tibau (8 isolates), Upanema (2 isolates), Apodi (0 isolates), and Gov. Dix-Sept Rosado (0 isolates) in RN (Table 1).

Conversely, *M. pseudophaseolina* emerged as the dominant species across the locations, with a total of 81 isolates. The highest numbers were recorded in Apodi (20 isolates), Upanema (18 isolates), Mossoró (12 isolates), and Gov. Dix-Sept Rosado (10 isolates) in RN, as well as Aracati (14 isolates) in CE. Among the remaining locations, Baraúna in RN reported six isolates of *M. pseudophaseolina*, while Tibau in RN had only one isolate of this species (Table 1).

3.3 Pathogenicity and virulence on watermelon

In all tests conducted, experimental repetitions did not yield a significant effect (ANOVA, $p > 0.05$), leading to a combination of the data. Every watermelon plant that was inoculated with *Macrophomina* isolates displayed RRVD symptoms. There were notable statistical differences in both the incidence and severity of the disease caused by various

Macrophomina species. This highlights the susceptibility of watermelon plants to *Macrophomina* spp. (Table 2).

Table 2 Incidence and severity of *Macrophomina phaseolina*- and *Macrophomina pseudophaseolina*-induced disease on watermelon.

M. phaseolina						*M. pseudophaseolina*					
Isolates	**Location**	**Incidence**		**Severity**		**Isolates**	**Location**	**Incidence**		**Severity**	
		Rank[1]	**Mean(%)**	**Rank[1]**	**Mean**			**Rank[1]**	**Mean(%)**	**Rank[1]**	**Mean**
A1P1	Mossoró, RN	214.50	100.00	164.81	2.80	**A2P16**	Tibau, RN	268.50	100.00	205.50	1.00
A1P3	Mossoró, RN	214.50	100.00	138.94	2.40	**A3PL3**	Aracati, CE	192.00	62.50	152.62	0.63
A1P6	Mossoró, RN	214.50	100.00	112.38	2.00	**A3PP2**	Aracati, CE	217.50	75.00	267.62	1.63
A1P9	Mossoró, RN	214.50	100.00	321.75	4.60	**A3PL2**	Aracati, CE	166.50	50.00	165.12	0.75
A1P14	Mossoró, RN	214.50	100.00	146.25	2.50	**A3PL6**	Aracati, CE	192.00	62.50	197.81	1.00
A2P6	Tibau, RN	214.50	100.00	146.25	2.50	**A3PL23**	Aracati, CE	115.50	25.00	99.75	0.25
A2P14	Tibau, RN	214.50	100.00	254.94	3.90	**A5P9**	Mossoró, RN	268.50	100.00	323.87	2.25
A2P15	Tibau, RN	163.50	75.00	77.50	1.40	**A5P16**	Mossoró, RN	217.50	75.00	200.37	1.00
A2P18	Tibau, RN	214.50	100.00	300.75	4.40	**A5P26**	Mossoró, RN	192.00	62.50	152.62	0.63
A2P19	Tibau, RN	214.50	100.00	143.00	2.40	**A7P6**	Upanema, RN	268.50	100.00	326.00	2.00
A3PP7	Aracati, CE	163.50	75.00	45.50	0.90	**A7P13**	Upanema, RN	217.50	75.00	230.50	1.25
A4P1	Mossoró, RN	214.50	100.00	244.62	3.80	**A7P15**	Upanema, RN	268.50	100.00	335.75	2.63
A4P4	Mossoró, RN	214.50	100.00	245.31	3.80	**A7P35**	Upanema, RN	192.00	62.50	197.81	1.00
A4P9	Mossoró, RN	214.50	100.00	119.69	2.00	**A7P37**	Upanema, RN	268.50	100.00	284.62	2.00
A4P11	Mossoró, RN	214.50	100.00	202.62	3.30	**A8P15**	Upanema, RN	141.00	37.50	117.37	0.38
A4P20	Mossoró, RN	189.00	87.50	50.00	1.00	**A8P3**	Upanema, RN	166.50	50.00	157.06	0.75
A5P2	Mossoró, RN	214.50	100.00	104.37	1.90	**A8P9**	Upanema, RN	141.00	37.50	162.56	0.75
A5P4	Mossoró, RN	214.50	100.00	319.31	4.60	**A8P13**	Upanema, RN	166.50	50.00	165.12	0.75
A5P6	Mossoró, RN	138.00	62.50	65.94	1.10	**A8P34**	Upanema, RN	141.00	37.50	117.37	0.38
A5P15	Mossoró, RN	214.50	100.00	288.69	4.30	**A9P11**	Mossoró, RN	217.50	75.00	185.31	0.88
A5P18	Mossoró, RN	214.50	100.00	131.87	2.30	**A9P21**	Mossoró, RN	268.50	100.00	328.75	2.50
A5P19	Mossoró, RN	214.50	100.00	226.75	3.50	**A9P29**	Mossoró, RN	141.00	37.50	117.37	0.38
A6P5	Mossoró, RN	214.50	100.00	312.81	4.50	**A9P50**	Mossoró, RN	268.50	100.00	331.19	2.63
A6P8	Mossoró, RN	214.50	100.00	172.12	2.80	**A9P7**	Mossoró, RN	217.50	75.00	244.50	1.50
A6P16	Mossoró, RN	214.50	100.00	245.31	3.80	**A10P2**	Apodi, RN	166.50	50.00	165.12	0.75
A6P20	Mossoró, RN	214.50	100.00	220.94	3.40	**A10P16**	Apodi, RN	268.50	100.00	333.31	2.50
A6P25	Mossoró, RN	214.50	100.00	307.25	4.50	**A10P45**	Apodi, RN	115.50	25.00	99.75	0.25
A7P8	Upanema, RN	163.50	75.00	62.44	1.10	**A10P44**	Apodi, RN	243.00	87.50	218.00	1.13
A7P23	Upanema, RN	214.50	100.00	211.56	3.40	**A10P30**	Apodi, RN	217.50	75.00	170.25	0.75
A9P20	Mossoró, RN	214.50	100.00	163.19	2.60	**A11P8**	Baraúna, RN	243.00	87.50	187.87	0.88
A11P1	Baraúna, RN	214.50	100.00	321.75	4.60	**A12P3**	Aracati, CE	243.00	87.50	233.06	1.25
A11P2	Baraúna, RN	214.50	100.00	331.37	4.80	**A12P5**	Aracati, CE	115.50	25.00	99.75	0.25
A11P16	Baraúna, RN	214.50	100.00	258.06	3.90	**A12P12**	Aracati, CE	192.00	62.50	152.62	0.63
A11P17	Baraúna, RN	214.50	100.00	331.37	4.80	**A12P14**	Aracati, CE	192.00	62.50	182.75	0.88

A11P22	Baraúna, RN	214.50	100.00	229.87	3.50	**A12P25**	Aracati, CE	141.00	37.50	117.37	0.38
A12P2	Aracati, CE	214.50	100.00	280.69	4.10	**A13QP1**	Gov. Dix- Sept Rosado, RN	192.00	62.50	197.81	1.00
A12P10	Aracati, CE	214.50	100.00	194.62	3.10	**A13LP2**	Gov. Dix- Sept Rosado, RN	268.50	100.00	309.87	2.00
A12P9	Aracati, CE	214.50	100.00	239.50	3.60	**A13LP3**	Gov. Dix- Sept Rosado, RN	166.50	50.00	180.19	0.88
A12P21	Aracati, CE	214.50	100.00	152.62	2.50	**A13QP5**	Gov. Dix- Sept Rosado, RN	268.50	100.00	306.69	2.25
A12P22	Aracati, CE	214.50	100.00	309.69	4.50	**A13LP10**	Gov. Dix- Sept Rosado, RN	192.00	62.50	167.69	0.75
A15P3	Baraúna, RN	163.50	75.00	79.37	1.40	**A14P3**	Apodi, RN	217.50	75.00	200.37	1.00
A15P4	Baraúna, RN	214.50	100.00	275.94	4.10	**A14P10**	Apodi, RN	217.50	75.00	215.44	1.13
A15P9	Baraúna, RN	214.50	100.00	297.62	4.40	**A14P13**	Apodi, RN	217.50	75.00	215.44	1.13
A15P17	Baraúna, RN	214.50	100.00	160.87	2.60	**A14P22**	Apodi, RN	192.00	62.50	212.87	1.13
A15P19	Baraúna, RN	214.50	100.00	260.50	3.90	**A14P28**	Apodi, RN	217.50	75.00	200.37	1.00
A16P8	Baraúna, RN	214.50	100.00	248.44	3.80	**A15P12**	Baraúna, RN	192.00	62.50	167.69	0.75
A16P11	Baraúna, RN	214.50	100.00	258.06	3.90	**A15P15**	Baraúna, RN	166.50	50.00	180.19	0.88
A16P3	Baraúna, RN	214.50	100.00	220.50	3.50	**A15P16**	Baraúna, RN	268.50	100.00	296.19	2.13
A16P25	Baraúna, RN	214.50	100.00	181.75	2.90	**A15P22**	Baraúna, RN	268.50	100.00	282.19	1.88
A16P26	Baraúna, RN	214.50	100.00	239.50	3.60	**A16P6**	Baraúna, RN	268.50	100.00	205.50	1.00
Control*		10.50	0.00	10.50	0.00	**Control***		64.50	0.00	64.50	0.00
χ^2		219.76		226.56		χ^2		118.58		159.03	

χ 2 = significant chi-square values; values followed by the same letter in the columns show no statistical difference between them by Kruskal-Wallis test ($p \leq 0.05$), [1]Average of the ranks for all observations within each sample. Data are mean values from two experiments, each with eight repetitions (pots) per treatment and one.

The watermelon variety 'Crimson Sweet' is a host for *M. phaseolina* and *M. pseudophaseolina*. Disease incidence caused by *M. phaseolina* was 100% for most treatments, except for isolates A2P15 (75.00%), A3PP7 (75.00%), A4P20 (87.50%), A5P6 (62.50%), and A15P3 (75.00%) (Table). Notably, the control (non-inoculated plants) showed no disease incidence. Treatments with 100% disease incidence were isolates collected from production fields in Mossoró and Baraúna in RN, and Aracati in CE (Table 2).

Plants inoculated with *M. pseudophaseolina*, specifically isolates A2P16, A5P9, A7P6, A7P15, A7P37, A9P21, A9P50, A10P16, A13LP2, A13QP5, A15P16, A15P22, and A16P6 from locations such as Tibau, Mossoró, Upanema, Apodi, Governador Dix Rosado, and Baraúna in RN, exhibited a disease incidence of 100% (Table 2). The lowest disease incidence for plants inoculated with this fungus was recorded at 25% (A3PL23). Again, the non-inoculated plants (control) showed no disease (Table 2).

As for the severity of RRVD in plants infected by *M. phaseolina*, it is clear that this pathogen is highly virulent to watermelon, with damage percentages ranging from 11% to over 50% in plant tissues (Table 2). Severity scores ranged from 0.90 (A3PP7) to 4.80 (A11P2 and A11P17). Treatments with average severity scores above 4 included isolates A1P9, A2P18, A5P4, A5P15, A6P5, A6P25, A11P1, A11P2, A12P2, A12P22, A15P4 and A15P9 obtained from production fields in Mossoró, Tibau, and Baraúna in RN, and Aracati in CE (Table 2).

The average severity scores for *M. pseudophaseolina* on watermelon ranged between 0.25 (A3PL23, A10P45, and A12P5) and 2.63 (A7P15 and A9P50) (Table 2). Treatments with average severity scores above 2 included isolates A5P9, A7P6, A7P15, A7P37, A9P21, A9P50, A10P16, A13LP2, A13QP5 and A15P16 sourced from Mossoró, Upanema, Apodi, Gov. Dix Rosado and Baraúna in RN. Controls for both sets of trials displayed no severity (Table 2).

Statistical analysis revealed significant differences in both disease incidence and severity among Macrophomina species, according to the Mann-Whitney test at a 5% probability (Figure 3).

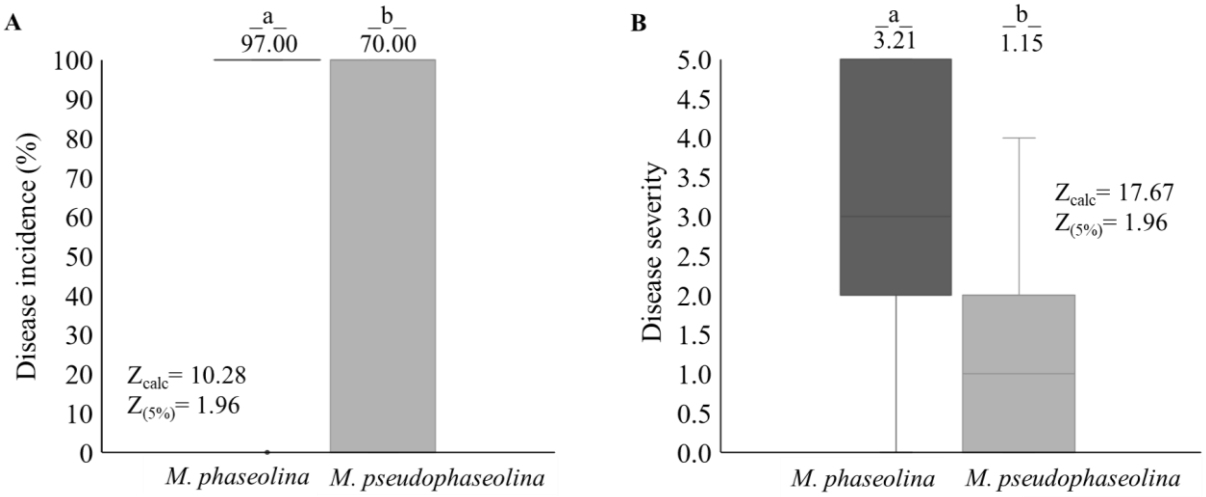


Fig. 3 Boxplots depicting (A) Incidence and (B) Severity of disease caused by *Macrophomina* species on watermelon plants. The height of the rectangle represents quartiles. The line inside the rectangle indicates the group's median. Lines extending above and below the rectangle denote the maximum and minimum values of the dataset, respectively. Different lowercase letters indicate significant differences as per the Mann-Whitney test ($p \leq 0.05$). Zcalc represents the calculated "Z score" value, while Z refers to the tabulated "Z score" value at a 5% probability.

When data from isolates of the same species were combined, it was evident that both pathogens induced RRVD symptoms in watermelons. *Macrophomina phaseolina* exhibited a 97% disease incidence, while *M. pseudophaseolina* showed a 70% incidence, with the latter displaying a wider interquartile range (Figure 3A).

The severity of disease in watermelon was higher following inoculation with *M. phaseolina*, which had a severity score of 3.21. In contrast, *M. pseudophaseolina*, with a score of 1.15, demonstrated lower virulence compared to the former species (Figure 3B).

The parameters of shoot and root length, as well as dry weight in watermelon, were affected by the inoculation of both *M. phaseolina* and *M. pseudophaseolina*. Notably, there were significant statistical differences among the isolates based on the Scott-Knott test ($p \leq 0.05$) (Table 3).

Table 3 Pathogenicity of *Macrophomina phaseolina* and *Macrophomina pseudophaseolina* isolates on the length and dry weight of watermelon.

M. phaseolina					*M. pseudophaseolina*				
Isolates	**Locations**	**SL[1](cm)**	**RL[2](cm)**	**DW[3](g)**	**Isolates**	**Locations**	**SL[1](cm)**	**RL[2](cm)**	**DW[3](g)**
A1P1	Mossoró, RN	66.62 b	17.37 b	2.16 a	**A2P16**	Tibau, RN	38.37 e	17.75 b	1.45 d
A1P3	Mossoró, RN	62.37 c	19.44 a	2.25 a	**A3PL3**	Aracati, CE	62.37 b	16.50 c	2.26 a
A1P6	Mossoró, RN	81.67 a	19.07 a	2.10 a	**A3PP2**	Aracati, CE	58.37 c	15.25 c	2.27 a
A1P9	Mossoró, RN	58.25 c	17.96 b	1.97 b	**A3PL2**	Aracati, CE	67.37 b	17.37 c	2.22 a
A1P14	Mossoró, RN	75.90 a	15.32 b	1.76 b	**A3PL6**	Aracati, CE	62.62 b	19.37 b	2.22 a
A2P6	Mossoró, RN	73.25 b	19.75 a	1.91 b	**A3PL23**	Aracati, CE	68.00 b	19.12 b	2.28 a
A2P14	Tibau, RN	80.90 a	20.37 a	2.22 a	**A5P9**	Mossoró, RN	49.50 d	15.37 c	1.57 d
A2P15	Tibau, RN	65.62 c	21.60 a	1.99 b	**A5P16**	Mossoró, RN	46.50 d	17.50 c	1.63 d
A2P18	Tibau, RN	75.07 a	19.50 a	2.05 b	**A5P26**	Mossoró, RN	51.37 c	18.00 b	1.80 c
A2P19	Tibau, RN	80.50 a	20.87 a	2.41 a	**A7P6**	Upanema, RN	52.87 c	20.50 a	2.09 b
A3PP7	Tibau, RN	72.52 b	18.95 a	2.23 a	**A7P13**	Upanema, RN	77.75 a	20.87 a	2.17 b
A4P1	Aracati, CE	80.62 a	16.55 b	2.06 b	**A7P15**	Upanema, RN	69.75 b	14.25 d	2.21 a
A4P4	Mossoró, RN	73.12 b	18.50 a	2.19 a	**A7P35**	Upanema, RN	71.50 a	16.87 c	2.12 b
A4P9	Mossoró, RN	82.42 a	18.72 a	1.75 b	**A7P37**	Upanema, RN	60.00 c	12.12 d	2.21 a
A4P11	Mossoró, RN	68.12 b	16.37 b	2.25 a	**A8P15**	Upanema, RN	72.50 a	18.25 b	2.30 a
A4P20	Mossoró, RN	70.25 b	18.04 b	2.00 b	**A8P3**	Upanema, RN	56.87 c	19.50 a	1.90 c
A5P2	Mossoró, RN	80.12 a	19.47 a	2.21 a	**A8P9**	Upanema, RN	66.87 b	17.37 c	2.28 a
A5P4	Mossoró, RN	81.75 a	16.15 b	2.11 a	**A8P13**	Upanema, RN	68.25 b	20.25 a	2.29 a
A5P6	Mossoró, RN	71.27 b	16.40 b	1.86 b	**A8P34**	Upanema, RN	63.75 b	19.25 b	2.09 b
A5P15	Mossoró, RN	80.87 a	16.35 b	1.84 b	**A9P11**	Mossoró, RN	68.75 b	17.75 b	2.24 a
A5P18	Mossoró, RN	66.75 b	18.12 b	2.16 a	**A9P21**	Mossoró, RN	69.87 b	16.37 c	1.99 b
A5P19	Mossoró, RN	69.75 b	17.50 b	2.13 a	**A9P29**	Mossoró, RN	73.37 a	20.62 a	2.25 a
A6P5	Mossoró, RN	62.00 c	20.31 a	1.95 b	**A9P50**	Mossoró, RN	76.87 a	18.37 b	2.23 a
A6P8	Mossoró, RN	84.00 a	16.84 b	2.26 a	**A9P7**	Mossoró, RN	64.62 b	20.00 a	2.29 a
A6P16	Mossoró, RN	71.97 b	18.12 b	2.05 b	**A10P2**	Apodi, RN	76.00 a	16.12 c	2.20 a
A6P20	Mossoró, RN	75.00 a	17.75 b	2.15 a	**A10P16**	Apodi, RN	64.50 b	15.50 c	1.76 c
A6P25	Mossoró, RN	80.57 a	14.80 b	1.86 b	**A10P45**	Apodi, RN	78.12 a	17.00 c	2.40 a
A7P8	Mossoró, RN	80.24 a	19.80 a	2.36 a	**A10P44**	Apodi, RN	66.50 b	16.75 c	2.27 a
A7P23	Upanema, RN	69.00 b	19.72 a	2.12 a	**A10P30**	Apodi, RN	73.62 a	16.00 c	2.23 a
A9P20	Upanema, RN	69.37 b	16.75 b	2.17 a	**A11P8**	Baraúna, RN	71.25 a	16.50 c	2.19 a
A11P1	Mossoró, RN	73.50 b	14.55 b	1.89 b	**A12P3**	Aracati, CE	69.75 b	18.12 b	1.94 c
A11P2	Baraúna, RN	85.65 a	24.10 a	2.42 a	**A12P5**	Aracati, CE	66.87 b	16.00 c	1.92 c
A11P16	Baraúna, RN	75.25 a	15.31 b	2.05 b	**A12P12**	Aracati, CE	75.62 a	20.25 a	2.41 a
A11P17	Baraúna, RN	80.62 a	15.91 b	2.15 a	**A12P14**	Aracati, CE	74.12 a	16.12 c	2.27 a

A11P22	Baraúna, RN	77.00 a	17.16 b	2.01 b	**A12P25**	Aracati, CE	53.37 c	18.37 b	2.03 b
A12P2	Baraúna, RN	80.50 a	16.61 b	2.36 a	**A13QP1**	Gov. Dix- Sept Rosado, RN	72.75 a	18.75 b	2.10 b
A12P10	Aracati, CE	84.27 a	20.72 a	2.11 a	**A13LP2**	Gov. Dix- Sept Rosado, RN	72.75 a	19.00 b	2.11 b
A12P9	Aracati, CE	67.17 b	16.37 b	1.94 b	**A13LP3**	Gov. Dix- Sept Rosado, RN	73.37 a	15.87 c	2.13 b
A12P21	Aracati, CE	76.87 a	20.60 a	2.19 a	**A13QP5**	Gov. Dix- Sept Rosado, RN	66.87 b	14.25 d	2.06 b
A12P22	Aracati, CE	81.12 a	19.75 a	2.26 a	**A13LP10**	Gov. Dix- Sept Rosado, RN	65.62 b	17.25 c	2.33 a
A15P3	Aracati, CE	82.00 a	17.62 b	2.11 a	**A14P3**	Apodi, RN	76.87 a	16.12 c	2.07 b
A15P4	Baraúna, RN	60.37 c	21.07 a	2.06 b	**A14P10**	Apodi, RN	66.25 b	17.50 c	2.38 a
A15P9	Baraúna, RN	80.50 a	18.87 a	2.29 a	**A14P13**	Apodi, RN	63.00 b	17.00 c	2.23 a
A15P17	Baraúna, RN	79.12 a	18.85 a	2.13 a	**A14P22**	Apodi, RN	66.87 b	18.00 b	2.27 a
A15P19	Baraúna, RN	74.95 a	15.95 b	2.26 a	**A14P28**	Apodi, RN	68.75 b	15.75 c	2.27 a
A16P8	Baraúna, RN	71.87 b	14.62 b	2.06 b	**A15P12**	Baraúna, RN	76.50 a	16.75 c	2.24 a
A16P11	Baraúna, RN	71.00 b	16.37 b	2.12 a	**A15P15**	Baraúna, RN	75.37 a	20.25 a	2.39 a
A16P3	Baraúna, RN	77.60 a	14.17 b	1.77 b	**A15P16**	Baraúna, RN	48.12 d	17.37 c	1.64 d
A16P25	Baraúna, RN	87.12 a	16.52 b	2.47 a	**A15P22**	Baraúna, RN	73.37 a	13.62 d	2.06 b
A16P26	Baraúna, RN	68.25 b	15.12 b	1.87 b	**A16P6**	Baraúna, RN	75.87 a	18.12 b	2.04 b
Control		76.57 a	21.80 a	2.41 a	**Control**		76.57 a	21.80 a	2.41 a
CV (%)		11.82	16.52	13.51	CV (%)		11.16	13.90	11.41

CV (%) = coefficient of variation; values followed by the same letter in the columns do not present statistical differences from each other by the Scott-Knott test ($p \leq 0.05$). Data comprise mean values from two experiments, each with eight repetitions (pots) per treatment and one plant per repetition, [1]Shoot length, [2]Root length, [3]Dry weight.

In *M. phaseolina*-inoculated plants, shoot lengths varied significantly across treatments, ranging from 58.25 cm (A1P9) to 85.65 cm (A11P2) (Table 3). Similarly, significant differences were observed in root lengths; the control exhibited a length of 21.80 cm, while lengths for *M. phaseolina*-inoculated plants spanned from 14.17 cm (A16P3) to 24.10 cm (A11P2). After drying, the minimum plant dry mass was 1.75 g (A4P9), which was distinct from the control (2.41 g) and the maximum of 2.47 g (A16P25) for the inoculated plants (Table 3).

For *M. pseudophaseolina*-inoculated plants, shoot lengths also varied, with a minimum of 38.37 cm (A2P16) — notably different from the control (76.57 cm) — and a maximum of 78.12 cm (A10P45) (Table 3). Likewise, root lengths demonstrated significant disparities, as they ranged from 12.12 cm (A7P37) to 20.87 cm (A7P13), compared to the control (21.80 cm). The dry weight of these watermelon plants varied significantly among treatments, extending from 1.45 g (A2P16) to 2.41 g (A12P12); the latter was statistically on par with the control (2.41 g) (Table 3).

In comparing *Macrophomina* species, there were notable differences in shoot and root lengths, as well as plant dry weight, as determined by the Student's t-test at a 5% probability level (Figure 4).

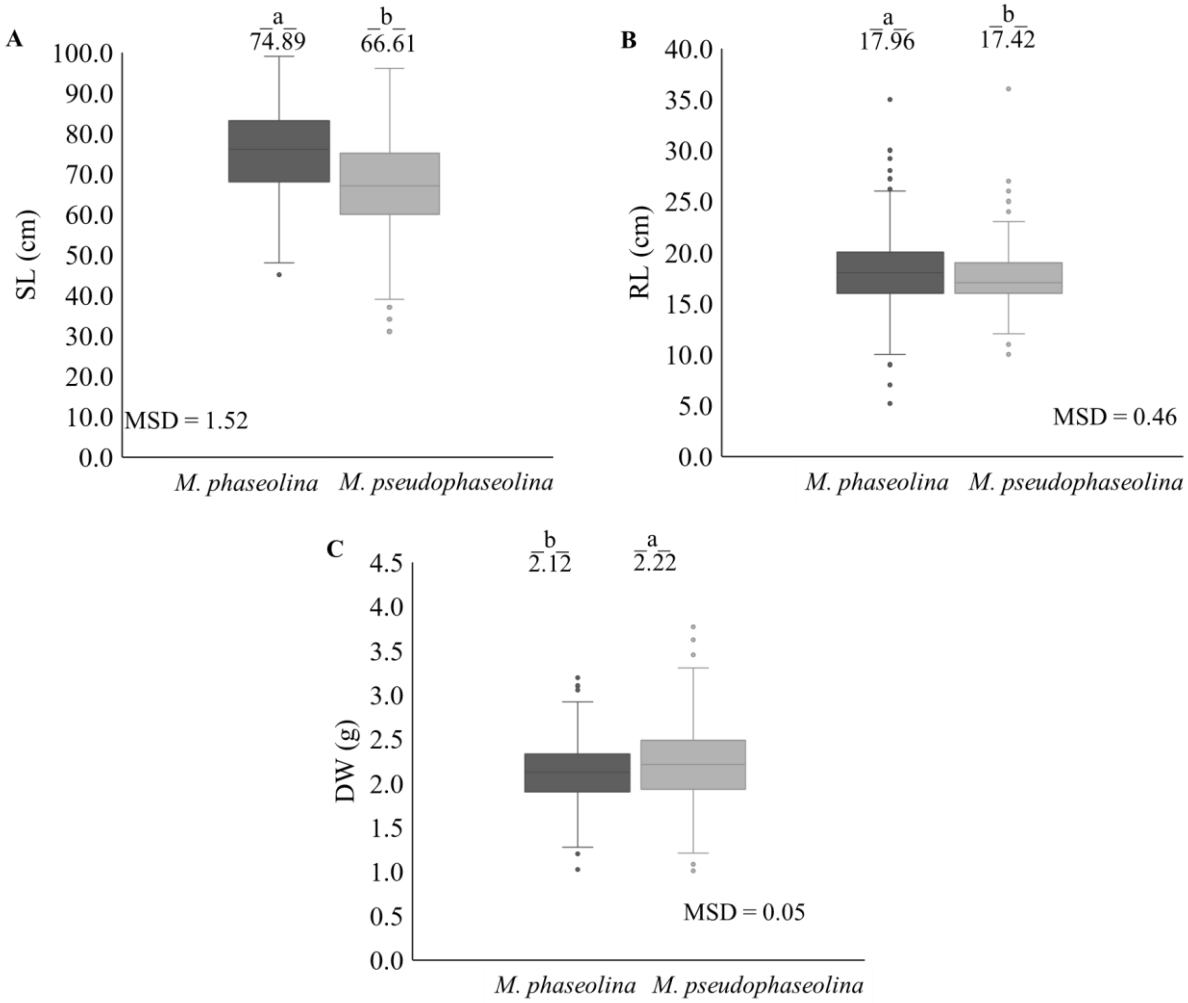


Fig. 4 Boxplots depicting (A) Shoot Length (SL), (B) Root Length (RL), and (C) Dry Weight (DW) of watermelon plants inoculated with *Macrophomina* species. The height of the rectangle represents quartiles. The line inside the rectangle indicates the group's median. Lines extending above and below the rectangle denote the maximum and minimum values of the dataset, respectively. Outliers are highlighted with painted dots. Different lowercase letters indicate significant differences as per the Student's t-test ($p \leq 0.05$). MSD represents the minimum significant difference between treatments.

Adjusted data between same-species isolates revealed shoot lengths of 74.89 cm for *M. phaseolina* inoculated plants, compared to 66.61 cm for *M. pseudophaseolina* (Figure 4A). Root lengths followed suit and were longer in *M. phaseolina* (17.96 cm) than in *M. pseudophaseolina* (17.42 cm) (Figure 4B). Conversely, plants infected by *M. pseudophaseolina* had a slightly higher dry weight (2.22 g) than those with *M. phaseolina* (2.12 g) (Figure 4C).

4 DISCUSSION

Using specific primers to amplify the TEF1-α gene, isolates from watermelon roots in the CE and RN states were characterized. Results indicated that *M. phaseolina* and *M. pseudophaseolina* are common fungi in these regions, with the latter being more dominant in the watermelon production areas. Amplifying the TEF1-α gene region with specific primers is now a common method to differentiate *Macrophomina* species (Sarr et al. 2014; Machado et al. 2018; Santos et al. 2020). Due to their efficiency and quick detection, these primers have been invaluable in characterizing and tracking the occurrence of this pathogen both in Brazil and globally (Cota-Barreras et al. 2022; Santos et al. 2020).

The prevalence of *M. pseudophaseolina* in watermelon fields in CE and RN suggests that this species has adapted well to the environmental conditions of Northeast Brazil. This adaptation is supported by Negreiros et al. (2019), who found a high prevalence of *M. pseudophaseolina* in weeds within melon production fields in the same producing areas. Intriguingly, both identified species were found in five distinct watermelon production locations in CE and RN states, supporting the theory that multiple *Macrophomina* species can coexist in the same field, as previously hypothesized by Sarr et al. (2014).

Both species proved pathogenic to watermelon. Notably, this is the world's first report linking *M. pseudophaseolina* to this host. The species was first documented in RN's oilseeds in 2018 and later in weeds from melon crops across RN and CE in 2019 (Machado et al. 2018; Negreiros et al. 2019). On the other hand, *M. phaseolina* has recently been associated with watermelon in both Brazil and China (Silva et al. 2022; Wu et al. 2022), but its presence in RN can be traced back to 2002 (Marinho et al. 2002). *Macrophomina phaseolina* might be more aggressive towards watermelons, as seen by more pronounced symptoms like root discoloration and collar cracking. Past research has highlighted aggressiveness variability among *Macrophomina* species, and this could be influenced by the diversity of isolates (Ndiaye et al. 2015; Ramos et al. 2016; Khan et al. 2017). *Macrophomina phaseolina* is usually more aggressive and causes greater damage to crops (e.g., cucurbits and cowpea) than *M. pseudophaseolina* (Ndiaye et al. 2015; Negreiros et al. 2019).

Interestingly, despite the pathogenicity of *M. phaseolina* evidenced here, watermelon plants inoculated with this species showcased longer shoot and root lengths compared to those inoculated with *M. pseudophaseolina*. This might suggest that the infection process activated defense responses in the plant, redirecting energy toward tissue growth and tapping into its genetic survival potential (Santos et al. 2022).

In short, our findings highlight the species diversity and pathogenicity of *Macrophomina* isolates present across various watermelon production sites in the Northeast of Brazil and shed light on the epidemiology and dynamics of fungal infestation in this crop. They emphasize the need for ongoing surveillance of these production zones and tracking *Macrophomina* occurrences to detect emerging species. This surveillance aims to reduce the detrimental effects of RRVD on watermelon, ensuring both the quality and yield of the fruits.

ACKNOWLEDGMENTS

This study was funded by the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) – Financial Code 001.

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CHAPTER III

EFFICACY OF FUNGICIDES FOR CONTROLLING *Macrophomina* spp. IN MELON PLANT

ABSTRACT: This study assessed the efficacy of five active ingredients (boscalid, carbendazim, cyprodinil, fluazinam, and fludioxonil) at five concentrations (0.01, 0.10, 1.00, 10.00, and 100.00 mg/L a.i.) against nine *Macrophomina* isolates (*M. phaseolina*: CMM1556, CMM4748, and CMM4764; *M. pseudophaseolina*: CMM2163, CMM4815, and CMM4767; and *M. euphorbiicola*: CMM2158, CMM4868, and CMM4867). Therefore, we evaluated daily mycelial growth, growth inhibition percentage, and the effective concentration that inhibits 50% of the radial mycelial growth (EC_{50}). Additionally, the *in vivo* impact of fluazinam and fludioxonil on the incidence and severity of root rot and vine decline, as well as melon plants biometrics, were determined. Increasing fungicide dose resulted in a higher percentage of mycelial growth inhibition, with the most favorable outcomes observed at 100.00 mg/L a.i. for all tested products. Overall, the *Macrophomina* isolates exhibited greater tolerance to boscalid (EC_{50} = 13.40 mg/L a.i.), followed by cyprodinil (EC_{50} = 1.18 mg/L a.i.), carbendazim (EC_{50} = 0.05 mg/L a.i.), fluazinam (EC_{50} = 0.03 mg/L a.i.), and fludioxonil (EC_{50} = 0.03 mg/L a.i.). The latter two demonstrated high efficacy *in vitro*. While none of the products achieved complete control of the fungus *in vivo*, the lowest incidence and severity of root rot and vine decline in melon plants were 28.57% and 0.29, respectively, in plants inoculated with *M. euphorbiicola* and treated with fludioxonil. For the other species and isolates, fluazinam exhibited better control, resulting in reduced mass loss and root length.

Keywords: Active ingredient; Chemical control; *Cucumis melo*; Growth inhibition.

1 INTRODUCTION

Melon (*Cucumis melo* L.) is a commercially significant vegetable-fruit crop in Brazil, ranking eleventh in global melon production. The country is renowned for exporting fruits during the off-season in northern hemisphere countries (Vargas et al. 2013; FAO 2023). Among the leading melon-producing states, Rio Grande do Norte (RN) held the highest production and exportation figures in Brazil in 2021 (IBGE 2023).

Despite high productivity facilitated by favorable conditions and cultivation techniques such as elevated temperature, low humidity, monoculture, and mulching, the prevalence of diseases caused by thermotolerant root pathogens, specifically root rot and vine decline (RRVD), has been observed (Bruton et al. 1998; Gomes Silva et al. 2018; Basandrai et al. 2021). RRVD leads to yellowing, wilting, and eventual death of melon plants in the field, particularly close to harvest time (Sinclair and Backman 1989; Kaur et al. 2012; Cohen et al. 2016; Porto et al. 2019). The soil-borne pathogen *Macrophomina* is one of the causal agents of RRVD.

The genus *Macrophomina* (Ascomycota, Botryosphaeriaceae) comprises five polyphagous and necrotrophic species: *Macrophomina phaseolina* (Tassi) Goid (Goidanich 1947), *Macrophomina pseudophaseolina* Crous, Sarr & Ndiaye (Sarr et al. 2014), *Macrophomina euphorbiicola* A.R. Machado, D.J. Soares & O.L. Pereira (Machado et al. 2018), *Macrophomina vaccinii* Y. Zhang ter & L. Zhao (Zhao et al. 2019), and *Macrophomina tecta* Vaghefi, B. Poudel & R.G. Shivas (Poudel et al. 2021). These species collectively affect over 750 plant species worldwide (Negreiros et al. 2022; Farr and Rossman 2023).

In Brazil, only *M. phaseolina*, *M. pseudophaseolina*, and *M. euphorbiicola* have been reported (Machado et al. 2018; Brito et al. 2019; Negreiros et al. 2019). These fungi cause diseases at various stages of plant growth (Gupta et al. 2012) and have a long lifespan in the soil due to the formation of resistant structures (microsclerotia) in plant tissue. These structures function as primary inoculum in the field after crop residue decomposition (Machado et al. 2018; Jaber and Fayyadh 2019). Consequently, managing RRVD caused by *Macrophomina* species remains challenging in Brazil (Negreiros et al. 2019).

Cultural control measures such as crop rotation are difficult to implement due to the substantial number of hosts involved (Romero Luna et al. 2017). Although several studies have sought melon cultivars resistant to *M. phaseolina* in Brazil (Ambrósio et al. 2015; Lima et al. 2021; Melo et al. 2021), currently, there are no commercially available melon cultivars resistant to this pathogen, ruling out genetic control as an option. The simplest and most immediate management approach would be chemical control; however, no fungicides are registered for the *Macrophomina* x melon pathosystem in Brazil (AGROFIT 2023).

Given the economic importance of melon in Brazilian fruit production and the common occurrence of RRVD caused by *Macrophomina*, active ingredients that can reduce the harm caused by these pathogens on melon plants must be assessed. Previous studies have demonstrated promising outcomes with fungicides containing fluazinam, fludioxonil, and carbendazim as active components, effectively controlling diverse root pathogens in melon both *in vitro* and *in vivo* (Medeiros et al. 2006; Cohen et al. 2012; Iqbal and Mukhtar 2020).

Therefore, this study aimed to evaluate the *in vitro* effects of five active ingredients (boscalid, carbendazim, cyprodinil, fluazinam, and fludioxonil) on the inhibition of mycelial growth percentage and the effective concentration required to reduce mycelial growth by 50% in *Macrophomina* (*M. phaseolina*, *M. pseudophaseolina*, and *M. euphorbiicola*). Additionally, the *in vivo* effects of fluazinam and fludioxonil on the pathogenicity, virulence of these fungi, and the biometric characteristics of melon plant were examined.

2 MATERIAL AND METHODS

2.1 Fungal isolates

Three isolates representing each *Macrophomina* species were included in this study, identified by Negreiros et al. (2019) (CMM4748, CMM4764, CMM4815 and CMM4767), and Negreiros et al. (2022) (CMM4868 and CMM4867), except CMM1156, CMM2163 and CMM2158 (unpublished data). These isolates are stored in the fungal collection Professor Maria Menezes (CMM) at the Federal Rural University of Pernambuco, UFRPE, Recife, Pernambuco (Table 1).

Table 1 *Macrophomina* isolates evaluated in the study.

Macrophomina species	Code (CMM)[1]	Host	Location[2]	GenBank accession numbers[3]
M. phaseolina	CMM1556	*Cucumis melo*	Brazil, RN, Mossoró	MN355981
	CMM4748	*Trianthema portulacastrum*	Brazil, CE, Icapuí	MH373438
	CMM4764	*Boerhavia diffusa*	Brazil, RN, Mossoró	MH373455
M. pseudophaseolina	CMM2163	*Cucumis melo*	Brazil, RN, Mossoró	MN356011
	CMM4815	*Boerhavia diffusa*	Brazil, RN, Mossoró	MH373522
	CMM4767	*Trianthema portulacastrum*	Brazil, CE, Icapuí	MH373513
M. euphorbiicola	CMM2158	*Cucumis melo*	Brazil, RN, Mossoró	MN264619
	CMM4868	*Boerhavia diffusa*	Brazil, RN, Assú	MH712510
	CMM4867	*Trianthema portulacastrum*	Brazil, RN, Assú	MH712509

[1]CMM = Culture Collection of Phytopathogenic Fungi "Prof. Maria Menezes" of the Federal Rural University of Pernambuco (Recife, PE, Brazil); [2]CE = state of Ceará, and RN = state of Rio Grande do Norte; [3]Identified from a fragment of the translation elongation factor 1-α (TEF) gene

2.2 *In vitro* test

The *in vitro* evaluation involved assessing mycelial growth of the fungal isolates when treated with five fungicides: boscalid (Cantus WG, 50% a.i., systemic, BASF S.A., São Paulo, Brazil), carbendazim (Carbendazim, 99.9% a.i., systemic, Syngenta Produção de Cultivos Ltda., São Paulo, Brazil), cyprodinil (Unix 750 WG, 75% a.i., systemic, Syngenta Produção de Cultivos Ltda., São Paulo, Brazil), fluazinam (Frowncide 500 SC, 50% a.i., contact, ISK Biosciences do Brazil Defensivos Agrícolas Ltda., São Paulo, Brazil), and fludioxonil (Maxim, 25% a.i., contact, Syngenta Produção de Cultivos Ltda., São Paulo, Brazil) at different concentrations: 0.01, 0.10, 1.00, 10.00, and 100.00 mg/L a.i. (Cavalcante et al. 2020). Each isolate was obtained as an eight-mm-diameter mycelial plug, seven days old, and placed at the center of Petri dishes containing Potato Dextrose Agar (PDA; Merck, Darmstadt, Germany) along with the respective fungicide concentrations. The dishes were then incubated

at 28°C in darkness. The control treatment comprised Petri plates with PDA but without fungicides.

The *in vitro* test used five plates (replicates) per treatment, and the experiment was replicated. After 24 hours of incubation, the radial mycelial growth (cm) of each isolate was measured using a pachymeter in two perpendicular directions. The evaluation period was determined based on when the control treatment reached the edge of the Petri plate or, in the case of slow growth of the isolate, seven days after plating. Subsequently, the percentage of growth inhibition (PGI%) was calculated using the formula described by Nascimento et al. (2013): PGI (%) = (mean daily diameter of the control – mean daily diameter of the treatment) / (mean daily diameter of the control) x 100.

The effective concentration that inhibits 50% of the radial mycelial growth (EC_{50}) was determined. The fungitoxicity of the active ingredients was classified based on the EC_{50} values using the classification system of Edgington et al. (1971), wherein: $EC_{50} \geq 50$ mg/L a.i. indicates no fungitoxicity, EC_{50} between 10 and 50 mg/L a.i. suggests low toxicity, EC_{50} between 1 and 10 mg/L a.i. denotes moderate toxicity, and $EC_{50} < 1$ mg/L a.i. means high toxicity.

A preliminary ANOVA was conducted to assess significant differences between the two experiments and the possibility of combining the data. For qualitative data, means were compared by Tukey's test at 5% probability using ASSISTAT software version 7.7 (Silva and Azevedo 2016), while regression analysis and Log-Probit values were employed for quantitative data, determining the EC_{50} of each a.i. and isolate using TableCurve 2D v. 5.01 (Systat Software, Inc., San Jose, CA, USA) (Systat 2002).

2.3 *In vivo* test

The same isolates (Table 1) and two fungicides that yielded the best results in the in vitro test (fludioxonil and fluazinam) were employed for the subsequent experiment. The treatments consisted of nine *Macrophomina* isolates, two active ingredients, the control and seven replicates per treatment. This experiment was also replicated.

Soil was infested using the paddy rice grain method, adapted from Songa et al. (1997). It involved placing 35 g of rice grains in 50 mL Falcon tubes, moistened with distilled water (1:1), and autoclaved three times at 24-hour intervals. Subsequently, five eight-mm-diameter plugs of PDA colonized with the pathogen were placed inside each tube. These tubes were maintained in an incubator at about 30°C for 15 days, with daily stirring to ensure

colonization uniformity. Ten days before transplanting the seedlings, six grains of rice colonized with each isolate were deposited in the pots.

Seeds of melon plant 'Natal' RZ F1 were initially planted in polyethylene trays filled with commercial substrate ('Tropstrato' HT Hortaliças®). After ten days, the seedlings were transplanted into 2.8 L pots containing a mixture of substrate and autoclaved sandy-clay soil in a 3:1 v/v ratio, respectively.

The fungicides were applied to the plant stems following the recommended guidelines for pathogens associated with RRVD. The volume applied was equivalent to 0.29 L ha^{-1} fludioxonil (Cannonball 2023) and 1 L ha^{-1} fluazinam (Guimarães et al. 2008). The first application (fluazinam and fludioxonil) occurred seven days after transplanting (DAT), followed by additional applications at 14, 21, and 28 DAT. The control group comprised plants inoculated with the isolates but without fungicide treatment.

At 50 DAT, disease incidence and severity were assessed by counting the number of symptomatic plants for RRVD and classified according to the scale described by Ravf and Ahmad (1998). It consists of: 0 = asymptomatic tissues, 1 = less than 3% of shoot tissues infected, 2 = 3 to 10% of shoot tissues infected, 3 = 11 to 25% of shoot tissues infected, 4 = 26 to 50% of shoot tissues infected, and 5 = more than 50% of shoot tissues infected. Additionally, the length and fresh weight of shoot and root tissues of all plants were measured.

To determine if there were significant differences between the two repetitions of the experiment, a preliminary ANOVA was performed. The data for disease incidence and severity were analyzed using the Kruskal-Wallis non-parametric test at a 5% probability level ($p < 0.05$) using ASSISTAT software version 7.7 (Silva and Azevedo 2016). The data obtained for the length and fresh weight of shoot and root tissues were subjected to ANOVA, and the means were compared using the LSD test at a 5% probability level using ASSISTAT software version 7.7 (Silva and Azevedo 2016).

3 RESULTS

For all *in vitro* and *in vivo* tests, there was no significant effect of the experiment repetitions (ANOVA, $p > 0.05$), thus the data were combined.

3.1 *In vitro* test

The mycelial development of *Macrophomina* exhibited statistically significant differences (ANOVA, $p \leq 0.05$) among the isolates and species at each evaluated dose, as per the concentrations of the fungicides. The percentage of growth inhibition (PGI%) of the treatments compared to the control (0.00 mg/L a.i.) increased proportionally with the concentration of the tested products. Notably, the highest level of mycelial control was observed at a dose of 100 mg/L a.i. (Table 2).

Table 2 Percentage growth inhibition (PGI%) of *Macrophomina phaseolina*, *Macrophomina pseudophaseolina*, and *Macrophomina euphorbiicola* by different fungicides.

Fungicide	Species	Isolate	PGI (%) *				
			Dose (mg/L a.i.)				
			0.01	0.10	1.00	10.00	100.00
Boscalid	*M. phaseolina*	CMM1556	0.00 e	33.33 bc	71.94 a	79.24 a	83.75 a
		CMM4748	20.00 b	41.34 b	51.22 b	61.67 bc	72.78 bc
		CMM4764	15.78 cd	38.89 b	54.22 b	65.33 b	67.22 cd
	M. pseudophaseolina	CMM2163	40.00 a	57.95 a	60.14 b	62.24 bc	64.19 d
		CMM4815	18.97 bcd	26.91 cd	29.08 c	53.51 cd	75.66 b
		CMM4767	15.00 d	20.80 d	22.00 c	54.00 cd	74.80 b
	M. euphorbiicola	CMM2158	0.00 e	0.00 e	0.00 d	0.00 e	0.00 f
		CMM4868	17.11 bcd	26.91 cd	35.90 c	46.33 d	50.80 e
		CMM4867	19.34 bc	24.56 cd	35.20 c	45.80 d	51.80 e
	CV (%)		**12.32**	**17.08**	**13.11**	**8.27**	**5.99**
Carbendazim	*M. phaseolina*	CMM1556	33.33 b	84.63 a	89.91 b	93.72 b	93.27 bc
		CMM4748	0.43 c	78.72 ab	83.72 de	86.38 d	87.53 d
		CMM4764	0.00 d	72.56 bc	82.11 e	100.00 a	100.00 a
	M. pseudophaseolina	CMM2163	40.00 a	71.53 c	83.43 de	92.71 bc	93.91 b
		CMM4815	0.00 d	82.33 a	85.33 cd	87.78 cd	88.55 cd
		CMM4767	0.00 d	72.33 bc	86.11 cd	87.78 cd	88.67 cd
	M. euphorbiicola	CMM2158	0.00 d	70.67 c	93.67 a	95.05 ab	97.38 ab
		CMM4868	0.00 d	82.33 a	87.45 bc	90.78 bcd	100.00 a
		CMM4867	0.00 d	58.47 d	84.89 cde	90.78 bcd	93.00 bc
	CV (%)		**0.62**	**4.58**	**1.78**	**2.76**	**2.53**
Cyprodinil	*M. phaseolina*	CMM1556	0.00 c	0.00 c	57.65 a	82.60 a	82.79 bc
		CMM4748	0.00 c	1.28 c	43.61 b	74.03 b	82.50 bc
		CMM4764	0.00 c	1.00 c	45.67 b	65.22 c	82.56 bc
	M. pseudophaseolina	CMM2163	40.00 a	40.00 a	58.90 a	87.71 a	90.52 a
		CMM4815	0.00 c	0.00 c	58.22 a	81.89 a	86.22 ab
		CMM4767	5.25 b	5.51 b	44.33 b	62.13 c	82.42 bc
	M. euphorbiicola	CMM2158	0.00 c	0.00 c	57.57 a	62.76 c	82.62 bc
		CMM4868	0.00 c	0.00 c	57.67 a	72.89 b	83.67 bc
		CMM4867	0.00 c	0.00 c	24.00 c	61.67 c	80.33 c
	CV (%)		**37.68**	**26.54**	**7.59**	**4.70**	**3.06**
Fluazinam	*M. phaseolina*	CMM1556	43.18 bc	76.38 abc	87.94 bc	90.67 ab	92.64 bc
		CMM4748	12.92 d	81.53 ab	88.28 b	90.36 ab	100.00 a
		CMM4764	37.00 c	76.06 abc	87.22 bc	89.44 b	100.00 a
	M. pseudophaseolina	CMM2163	59.38 ab	60.24 d	83.29 e	92.19 ab	95.00 b
		CMM4815	14.89 d	74.00	86.56 cd	90.56 ab	88.67 c
		CMM4767	74.78 a	86.11 a	88.00 b	88.56 b	88.67 c
	M. euphorbiicola	CMM2158	0.00 d	61.90 cd	90.10 a	93.86 a	94.86 b
		CMM4868	5.33 d	74.00	85.56 d	88.22 b	91.33 bc
		CMM4867	9.33 d	71.00 bcd	87.33 bc	89.44 b	92.45 bc
	CV (%)		**27.27**	**9.51**	**0.78**	**2.11**	**2.48**
Fludioxonil	*M. phaseolina*	CMM1556	51.24 a	81.33 ab	90.79 ab	93.72 a	94.86 a
		CMM4748	33.45 b	75.44 bc	86.78 bc	97.22 a	100.00 a
		CMM4764	36.44 b	78.33 abc	87.78 bc	97.78 a	100.00 a
	M. pseudophaseolina	CMM2163	60.19 a	77.57 bc	87.81 bc	88.62 a	90.09 a
		CMM4815	26.33 b	81.22 ab	87.44 bc	88.67 a	100.00 a
		CMM4767	12.08 c	74.11 c	87.33 bc	92.15 a	94.44 a

	M. euphorbiicola	CMM2158	50.00 a	84.62 a	95.57 a	96.43 a	100.00 a
		CMM4868	28.78 b	81.22 ab	84.00 c	90.56 a	100.00 a
		CMM4867	25.89 b	48.44 d	92.22 ab	100.00 a	100.00 a
	CV (%)		**16.14**	**4.43**	**3.28**	**8.00**	**4.90**

[c]CV (%): Coefficient of variation. *Values sharing the same letter within a column are not deemed statistically significant by Tukey's test at 5% probability

At the lowest dose of 0.01 mg/L a.i. of boscalid, a mycelial inhibition was observed for most isolates except CMM1556 (*M. phaseolina*) and CMM2158 (*M. euphorbiicola*), with the highest recorded PGI of 40.00% for CMM2163 (*M. pseudophaseolina*) (Table 2). Increasing the concentration to 0.10 mg/L a.i. reduced mycelial development in most isolates except CMM2158 (*M. euphorbiicola*), which showed no PGI. At this dose, the highest PGI was 57.95% for CMM2163 (*M. pseudophaseolina*). At 1.00 mg/L a.i. of boscalid, there was no inhibition of CMM2158 (*M. euphorbiicola*), but the other isolates showed mycelial PGI, with the maximum rate of 71.94% observed in CMM1556 (*M. phaseolina*). Similar results were observed at 10.00 and 100.00 mg/L a.i., where the growth of CMM2158 (*M. euphorbiicola*) was not inhibited. However, mycelial PGI was observed for the other isolates and both concentrations, with CMM1556 (*M. phaseolina*) being the most affected, showing mycelial inhibition rates of 79.24 and 83.75%, respectively (Table 2).

Compared to the control, carbendazim at 0.01 mg/L a.i. reduced the growth of CMM1556 (*M. phaseolina*), CMM4748 (*M. phaseolina*), and CMM2163 (*M. pseudophaseolina*) by 33.33, 0.43, and 40.00%, respectively (Table 2). The remaining isolates showed no mycelial PGI. However, fungal development was inhibited in all treatments starting from the concentration of 0.10 mg/L a.i. At this dose, the highest PGI values were recorded for isolates CMM1556 (*M. phaseolina*, 84.63%), CMM4815 (*M. pseudophaseolina*, 82.33%), CMM4868 (*M. euphorbiicola*, 82.33%), and CMM4748 (*M. phaseolina*, 78.72%), while the lowest PGI was 58.47% for CMM4867 (*M. euphorbiicola*). At 1.00 mg/L a.i. of carbendazim, all isolates showed PGI above 82.00%, ranging from 82.11% for CMM4764 (*M. phaseolina*) to 93.67% for CMM2158 (*M. euphorbiicola*). From 10.00 mg/L a.i. onwards, the maximum PGI was observed for isolate CMM4764 (*M. phaseolina*) at 100.00%, while the lowest rate was 86.38% for CMM4748 (*M. phaseolina*). Finally, fungal growth was totally inhibited for isolates CMM4764 (*M. phaseolina*) and CMM4868 (*M. euphorbiicola*) at 100.00 mg/L a.i., while the other treatments showed mycelial PGI of at least 87.53%, corresponding to CMM4748 (*M. phaseolina*) (Table 2).

In the case of cyprodinil, at 0.01 mg/L a.i., mycelial PGI was absent for most treatments except for isolates CMM2163 and CMM4767, both species *M. pseudophaseolina*,

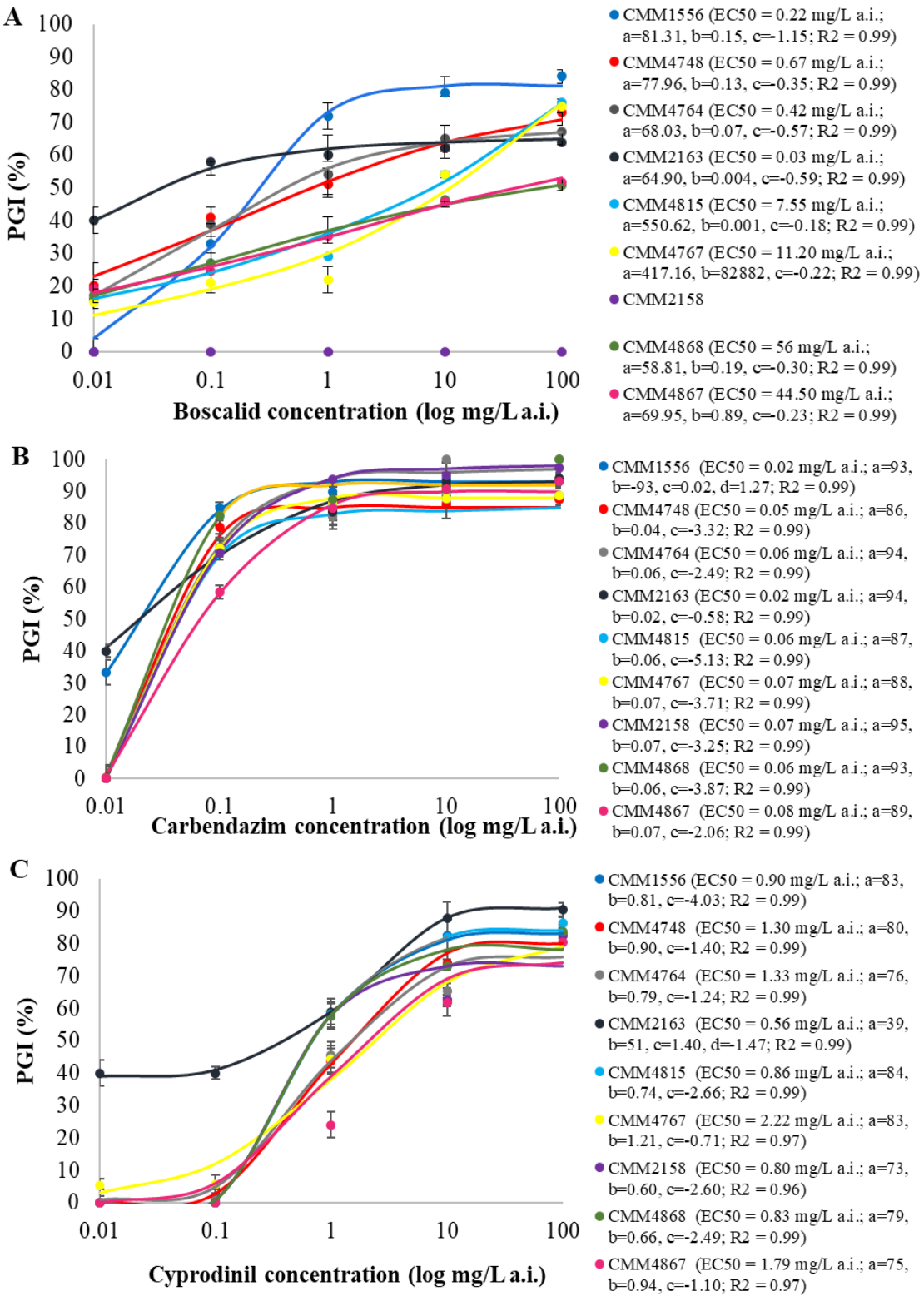
with inhibition rates of 40.00% and 5.00%, respectively (Table 2). At 0.10 mg/L a.i., most isolates still showed no mycelial PGI, except for CMM4748 (*M. phaseolina*, 1.28%), CMM4764 (*M. phaseolina*, 1.00%), CMM2163 (*M. pseudophaseolina*, 40.00%), and CMM4767 (*M. pseudophaseolina*, 5.51%). Only at 1.00 mg/L a.i. of cyprodinil, mycelial PGI was observed for all isolates, ranging from 24.00% for CMM4867 (*M. euphorbiicola*) to 58.90% for CMM2163 (*M. pseudophaseolina*). Fungal growth in all treatments was reduced by at least 61.67% (CMM4867, *M. euphorbiicola*) compared to the control, with a maximum inhibition of 82.60% observed in isolate CMM1556 (*M. phaseolina*). At the highest concentration (100.00 mg/L a.i.), the growth of all isolates was limited, with the lowest mycelial PGI being 80.33% for CMM4867 (*M. euphorbiicola*) and the highest being 90.52% for CMM2163 (*M. pseudophaseolina*) (Table 2).

Fluazinam inhibited fungal growth of most isolates at the lowest dose of 0.01 mg/L a.i., except for CMM2158 (*M. euphorbiicola*) with no effect (Table 2). The highest mycelial PGI recorded at this concentration was 74.78% for CMM4767 (*M. pseudophaseolina*). Increasing the dose to 0.10 mg/L a.i. resulted in reduced mycelial growth for all isolates. The lowest PGI was 60.24% for CMM2163, while the highest was 86.11% for CMM4767, both isolates of *M. pseudophaseolina*. At 1.00 mg/L a.i., the growth of *Macrophomina* isolates was reduced by at least 83.00%, with the lowest PGI being 83.29% for CMM2163 (*M. pseudophaseolina*) and the highest being 90.10% for CMM2158 (*M. euphorbiicola*). At 10 mg/L a.i. of the product, all treatments showed mycelial PGI ranging from 88.22% for isolate CMM4868 (*M. euphorbiicola*) to 93.86% for CMM2158 (*M. euphorbiicola*). Fluazinam completely inhibited the growth of isolates CMM4748 and CMM4764 (*M. phaseolina*) at 100 mg/L a.i., and mycelial PGI was at least 88.67% (CMM4815 and CMM4767, *M. pseudophaseolina*) for the other isolates (Table 2).

Fludioxonil was the only active ingredient that reduced mycelial growth in all isolates at the lowest dose. The lowest mycelial PGI observed at 0.01 mg/L a.i. was 12.08% for CMM4767 (*M. pseudophaseolina*), while the highest was 60.19% for CMM2163 (*M. euphorbiicola*) (Table 2). The mycelial PGI increased at 0.10 mg/L a.i., ranging from 48.44% for CMM4867 to 84.62% for CMM2158, both for *M. euphorbiicola* isolates. At 1.00 mg/L a.i., the lowest mycelial growth inhibition was 84.00% for CMM4868 (*M. euphorbiicola*), while the highest was 95.57% for CMM2158 (*M. euphorbiicola*). The 10.00 mg/L a.i. concentration of fludioxonil resulted in a range of mycelial PGI rates, with the lowest being 88.62% for CMM2163 (*M. pseudophaseolina*) and the highest being 100.00% for CMM4867 (*M. euphorbiicola*). Similar to the previous dose, at 100.00 mg/L a.i., most treatments

exhibited complete inhibition of fungal growth, except for isolates CMM1556 (*M. phaseolina*, 94.86%), CMM2163 (*M. pseudophaseolina*, 90.09%), and CMM4767 (*M. pseudophaseolina*, 94.44%) (Table 2).

By adjusting the doses, the EC_{50} values for each product used to inhibit the growth of the studied fungi were calculated (Figure 1).



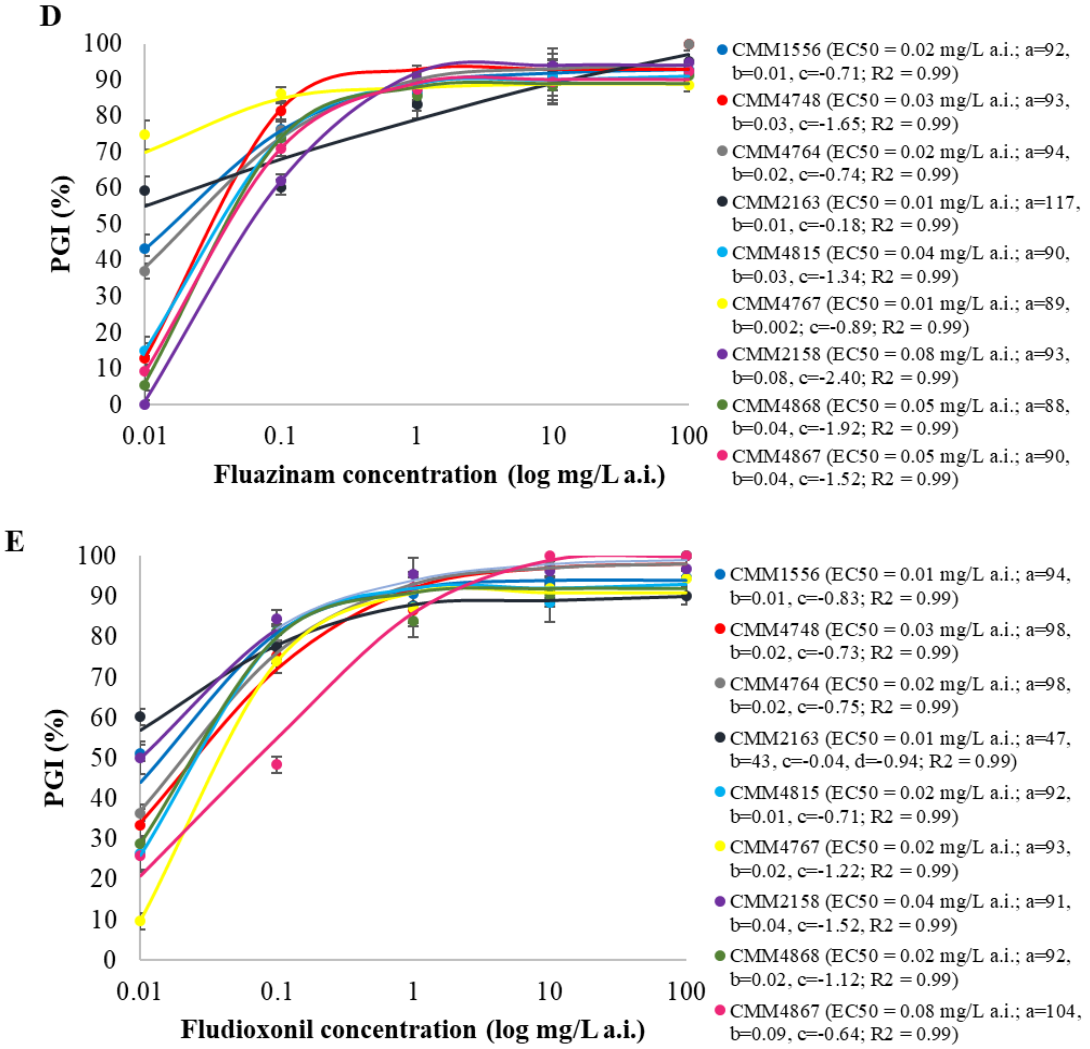


Fig. 1 Regression equation, coefficient of determination (R2), and EC_{50} values for each *Macrophomina* spp. isolate treated with the fungicides: (A) boscalid, (B) carbendazim, (C) cyprodinil, (D) fluazinam, and (E) fludioxonil. The equation is fitted with the PGI values (=PGI) at doses of 0.01, 0.10, 1.00, 10.00, and 100.00 mg/L a.i. per fungicide per isolate. EC_{50} represents the effective concentration inhibiting mycelial growth by 50%, calculated using the regression equation.

The fungitoxic effect of boscalid varied among the *Macrophomina* isolates, with EC_{50} values ranging from 0.03 mg/L a.i. for CMM2163 (*M. pseudophaseolina*) to 56.00 mg/L a.i. for CMM4868 (*M. euphorbiicola*) (Figure 1). According to the classification of Edgington et al. (1971), boscalid exhibited high toxicity for all *M. phaseolina* isolates and for CMM2163 (*M. pseudophaseolina*), moderate toxicity for CMM4815 (7.55 mg/L a.i., *M. pseudophaseolina*), low toxicity for CMM4767 (11.20 mg/L a.i., *M. pseudophaseolina*) and CMM4867 (44.50 mg/L a.i., *M. euphorbiicola*), and no toxicity for CMM4868 (56.00 mg/L

a.i., *M. euphorbiicola*). In terms of *Macrophomina* species, the fungitoxicity of boscalid was high for *M. phaseolina* (0.44 mg/L a.i.), moderate for *M. pseudophaseolina* (6.26 mg/L a.i.), and low for *M. euphorbiicola* (33.50 mg/L a.i.) (Figure 1).

All isolates tested showed high sensitivity to carbendazim, with EC_{50} values ranging from 0.02 mg/L a.i. (CMM1556 - *M. phaseolina* and CMM2163 - *M. pseudophaseolina*) to 0.08 mg/L a.i. (CMM4867 - *M. euphorbiicola*) (Figure 1). In terms of fungitoxicity, cyprodinil exhibited a range from 0.56 to 2.22 mg/L a.i., with high toxicity for CMM1556 (0.90 mg/L a.i., *M. phaseolina*), CMM2163 (0.56 mg/L a.i., *M. pseudophaseolina*), CMM4815 (0.86 mg/L a.i., *M. pseudophaseolina*), CMM2158 (0.80 mg/L a.i., *M. euphorbiicola*), and CMM4868 (0.83 mg/L a.i., *M. euphorbiicola*), and moderate toxicity for isolates CMM4748 (1.30 mg/L a.i., *M. phaseolina*), CMM4764 (1.33 mg/L a.i., *M. phaseolina*), CMM4767 (2.22 mg/L a.i., *M. pseudophaseolina*), and CMM4867 (1.79 mg/L a.i., *M. euphorbiicola*) (Figure 1).

Fluazinam and fludioxonil demonstrated high toxicity to all isolates, with EC_{50} values ranging from 0.01 (CMM2163 - *M. pseudophaseolina* and CMM4767 - *M. pseudophaseolina* for fluazinam; CMM1556 - *M. phaseolina* and CMM2163 - *M. pseudophaseolina* for fludioxonil) to 0.08 mg/L a.i. (CMM2158 - *M. euphorbiicola* for fluazinam; CMM4867 - *M. euphorbiicola* for fludioxonil) (Figure 1).

In general, *Macrophomina* species exhibited higher tolerance to boscalid (EC_{50} = 13.40 mg/L a.i.) and cyprodinil (EC_{50} = 1.18 mg/L a.i.), requiring higher effective concentrations to inhibit 50% of mycelial growth compared to the other tested fungicides (Figure 2). All isolates showed higher sensitivity to carbendazim (EC_{50} = 0.05 mg/L a.i.), followed by fludioxonil and fluazinam (EC_{50} = 0.03 mg/L a.i.) (Figure 2).

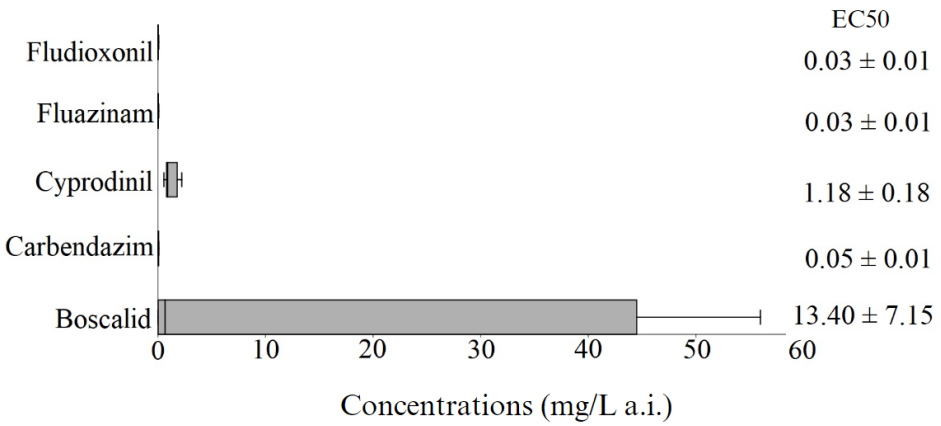


Fig. 2 Effective concentration to inhibit 50% of the mycelial growth of *Macrophomina* spp. (EC_{50}), [mg/L a.i. ± SD (standard deviation)] of the fungicides tested.

3.2 *In vivo* test

Significant differences ($p \leq 0.05$) were observed in the action of fungicides on the incidence and severity of melon plants infected with different isolates of *Macrophomina* spp. (Table 3).

Table 3 Incidence and severity of disease caused by *Macrophomina* spp. in melon treated with fludioxonil and fluazinam

Species	Treatment	Incidence		Severity	
		Rank[1]	Mean (%)	Rank[1]	Mean
M. phaseolina	CMM1556 - FD	19.50 ab	57.14	16.10 a	1.00
	CMM1556 - FZ	16.50 a	42.86	13.18 a	0.64
	CMM1556 - TS	28.50 b	100.00	35.21 b	3.42
	χ^2	10.88		28.24	
	CMM4748 - FD	20.00 ab	64.28	18.25 a	1.43
	CMM4748 - FZ	17.00 a	50.00	12.25 a	0.64
	CMM4748 - TS	27.50 b	100.00	34.00 b	3.71
	χ^2	8.88		24.47	
	CMM4764 - FD	20.50 a	78.57	16.64 a	1.36
	CMM4764 - FZ	19.00 a	71.43	16.14 a	1.36
	CMM4764 - TS	25.00 b	100.00	31.71 b	3.14
	χ^2	4.35		15.22	
M. pseudophaseolina	CMM2163 - FD	24.00 b	92.86	21.82 b	1.79
	CMM2163 - FZ	15.00 a	50.00	10.61 a	0.64
	CMM2163 - TS	25.50 b	100.00	32.07 b	2.86
	χ^2	12.96		22.83	
	CMM4767 - FD	22.00 b	92.86	22.61 b	2.57
	CMM4767 - FZ	19.00 a	78.57	11.53 a	1.29
	CMM4767 - TS	23.50 b	100.00	30.36 b	3.57
	χ^2	3.77		17.27	
	CMM4815 - FD	24.50 b	100.00	31.21 b	2.71
	CMM4815 - FZ	15.50 a	57.14	9.43 a	0.71
	CMM4815 - TS	24.50 b	100.00	23.86 b	2.00
	χ^2	13.67		25.54	
M. euphorbiicola	CMM2158 - FD	13.00 a	28.57	9.86 a	0.29
	CMM2158 - FZ	23.50 b	78.57	22.43 b	1.64
	CMM2158 - TS	28.00 b	100.00	32.21 b	2.71
	χ^2	17.18		24.83	
	CMM4868 - FD	20.50 ab	78.57	16.75 a	1.50
	CMM4868 - FZ	19.00 a	71.43	18.68 ab	1.64
	CMM4868 - TS	25.00 b	100.00	29.07 b	2.71
	χ^2	4.35		8.71	
	CMM4867 - FD	17.00 a	57.14	16.25 a	1.36
	CMM4867 - FZ	21.50 ab	78.57	19.39 ab	1.71
	CMM4867 - TS	26.00 b	100.00	28.86 b	2.86
	χ^2	7.45		8.47	

FD = fludioxonil, FZ = fluazinam, TS = control treatment, and χ 2 = significant chi-square values. Values sharing the same letter within a column are not deemed statistically significant between each other by Kruskal-Wallis test at 5% probability. [1]Average ranks for all observations within each sample. These mean values were obtained from two experiments, with each experiment consisting of seven replicates (pots) per treatment

Treating melon plants with fluazinam (FZ) reduced by 57.14% the incidence of disease caused by *M. phaseolina* in plants infested with CMM1556 (42.86%) and by 50% plants infested with CMM4748 (50%), compared to the control (TS - 100%) (Table 3). The effect of fludioxonil (FD) on the two mentioned isolates showed no statistically significant difference between the fluazinam (FZ) treatment and the control (TS).

Regarding the effect of fungicides on the incidence of disease caused by CMM4764, there was a reduction of at least 21.43% in diseased plants, and the average incidence was statistically similar between the two active ingredients used, fludioxonil (78.57%) and fluazinam (71.43%), differing only from the control (TS, 100%). There was no statistically significant difference between fluazinam (FZ) and fludioxonil (FD) for disease severity, except when compared to the control (TS). Therefore, the application of either fungicide will decrease the percentage of tissues covered by symptoms (Table 3).

The incidence of disease caused by isolates of *M. pseudophaseolina* in melons was lower with the treatment of fluazinam (FZ). It reduced the percentage of diseased plants by 50% in infections caused by CMM2163 (50%), 21.43% for CMM4767 (78.57%), and 42.86% for CMM4815 (57.14%), which was statistically different from the control (TS - 100%) (Table 3). The effect of fludioxonil (FD) on the incidence of disease caused by isolates of *M. pseudophaseolina* was statistically similar to the control (TS). The severity of the disease in melons for all isolates was lower with fluazinam (FZ), showing a statistically significant difference between the application of fludioxonil (FD) and the control (TS) (Table 3).

The incidence of disease caused by CMM2158 (28.57%) of the *M. euphorbiicola* species was reduced by 71.43% with the application of fludioxonil (FD). This reduction was statistically different from the treatment with fluazinam (FZ - 78.57%), which only reduced the incidence by 21.43%, and the control (TS - 100%) (Table 3). There was no statistical difference between fludioxonil (FD) and fluazinam (FZ) for isolates CMM4868 (78.57% and 71.43%, respectively) and CMM4867 (57.14% and 78.57%, respectively), except when compared to the control (TS - 100%). The same trend was observed for the severity of the disease. Plants infested with CMM2158 (0.29) and treated with fludioxonil showed a lower mean severity, which was statistically different from the treatment with fluazinam (FZ - 1.64) and the control (TS - 2.71). The data for the other isolates (CMM4868 and CMM4867) were statistically similar between the evaluated products, but fludioxonil (FD - 1.50 and 1.36, respectively) showed a statistically significant difference when compared to the control (TS - 2.71 and 2.86, respectively) (Table 3).

The lengths and weights of plants evaluated in each treatment exhibited statistically significant differences ($p \leq 0.05$) with the use of the studied active ingredients (Table 4).

Table 4 Effect of fludioxonil and fluazinam on length and fresh weight of shoot and root of melon plants for *Macrophomina* spp. control

Species	Treatment	SL[1] (cm)	RL[2] (cm)	SFW[3] (g)	RFW[4](g)
M. phaseolina	CMM1556 - FD	86.78 b	20.86 b	50.73 b	2.26 b
	CMM1556 - FZ	94.07 a	28.46 a	60.63 a	4.00 a
	CMM1556 - TS	53.00 c	25.00 ab	21.75 c	1.23 c
	CV (%)	11.99	28.63	10.63	14.06
	CMM4748 - FD	81.93 b	19.28 b	48.91 b	1.69 b
	CMM4748 - FZ	90.78 a	24.28 a	54.61 a	2.85 a
	CMM4748 - TS	63.14 c	19.57 b	21.49 c	1.02 c
	CV (%)	3.32	13.70	7.64	25.80
	CMM4764 - FD	85.86 a	19.43 b	53.61 a	1.89 b
	CMM4764 - FZ	82.28 b	24.86 a	52.29 a	2.76 a
	CMM4764 - TS	60.00 c	22.86 a	23.87 b	1.01 c
	CV (%)	4.20	17.11	15.34	20.60
M. pseudophaseolina	CMM2163 - FD	91.21 a	20.93 a	49.26 a	1.43 b
	CMM2163 - FZ	81.07 b	20.21 a	50.11 a	2.22 a
	CMM2163 - TS	52.14 c	15.71 b	19.80 b	0.52 c
	CV (%)	4.86	12.71	9.69	15.70
	CMM4767 - FD	80.28 a	20.86 a	45.04 b	1.51 b
	CMM4767 - FZ	79.64 a	21.34 a	54.14 a	3.77 a
	CMM4767 - TS	60.00 b	18.78 a	22.09 c	0.55 c
	CV (%)	10.56	22.91	12.05	14.59
	CMM4815 - FD	87.07 a	21.71 b	45.23 b	1.32 a
	CMM4815 - FZ	80.07 b	25.78 a	50.74 a	3.26 b
	CMM4815 - TS	55.00 c	12.57 c	20.00 c	0.47 c
	CV (%)	5.59	14.14	10.94	12.21
M. euphorbiicola	CMM2158 - FD	94.50 a	27.64 a	52.18 a	3.84 a
	CMM2158 - FZ	86.57 b	25.32 a	44.86 b	2.26 b
	CMM2158 - TS	61.00c	19.57 b	21.89 c	0.63 c
	CV (%)	5.06	18.06	12.48	15.11
	CMM4868 - FD	83.36 b	21.57 b	44.72 b	1.73 b
	CMM4868 - FZ	89.93 a	25.11 a	50.30 a	2.02 a
	CMM4868 - TS	59.86 c	14.71 c	22.85 c	0.65 c
	CV (%)	7.05	16.05	11.14	23.49
	CMM4867 - FD	95.93 a	22.28 a	53.71 a	2.44 a
	CMM4867 - FZ	85.43 b	20.93 a	46.05 b	2.05 b
	CMM4867 - TS	58.71 c	12.28 b	17.77 c	0.44 c
	CV (%)	9.27	15.11	8.01	10.44

FD = fludioxonil, FZ = fluazinam, TS = control treatment, and CV (%) = significant coefficients of variation. Values sharing the same letter within the columns show no statistical difference from each other by LSD test at 5% probability. The data presented are mean values derived from two experiments, with seven repetitions (pots) per treatment and one plant per repetition. [1]Shoot length. [2]Root length.[3]Shoot fresh weight. [4]Root fresh weight

Melon plants infested with isolates of *M. phaseolina* showed increased shoot length when treated with fungicides compared to the control (TS). Treatment with fluazinam (FZ) to mitigate infection caused by CMM1556 and CMM4748 resulted in shoot lengths of 94.07 cm

and 90.78 cm, respectively, compared to the control (53.00 cm and 63.14 cm, respectively) (Table 4). Plants infested with CMM4764 exhibited greater shoot length with the application of fludioxonil (FD - 85.86 cm) compared to the control (60.00 cm).

The root length of melon plants infected with CMM1556 was statistically similar between the control (TS) and the other fungicides. In the case of infection caused by CMM4748, plants treated with fluazinam (FZ) showed greater root length (24.28 cm). For plants inoculated with CMM4764, root size was larger with the use of fluazinam (FZ - 24.86 cm), and this did not differ from the control (22.86 cm).

Regarding shoot fresh weight, once again fluazinam (FZ) provided the best results for plants infected with CMM1556 (60.63 g) and CMM4748 (54.61 g) compared to the control (21.75 g and 21.49 g, respectively). There was no statistical difference between fludioxonil (FD - 53.61 g) and fluazinam (FZ - 52.29 g) in the case of CMM4764, but both differed from the control (23.87 g).

Regarding root fresh weight, plants infected with CMM1556 (4.00 g), CMM4748 (2.85 g), and CMM4764 (2.76 g) obtained higher values with the application of the same active ingredient, statistically differing from the control (TS - 1.23 g, 1.02 g, and 1.01 g, respectively) (Table 4).

The response of plants infested with *M. pseudophaseolina* and treated with fungicides varied for each trait and evaluated isolate. Plants infected with CMM2163 (91.21 cm) and CMM4815 (87.07 cm) and treated with fludioxonil (FD) exhibited greater shoot lengths compared to the control (TS - 52.14 cm and 55.00 cm, respectively) (Table 4). The effects of fludioxonil (FD) and fluazinam (FZ) on melon plants were similar for the CMM4767 isolate (80.28 cm and 79.64 cm, respectively), differing from the control (TS - 60.00 cm).

When it came to root length, the use of fludioxonil (FD - 20.86 cm) and fluazinam (FZ - 21.34 cm) on melon plants infested with CMM2163 resulted in a gain of at least 4.50 cm compared to the control (TS - 15.71 cm). For plants infested with CMM4767, all treatments showed similar results for this trait. The application of fluazinam (FZ - 25.78 cm) led to greater root length in melon plants infested with CMM4815, differing from the fludioxonil treatment (FD - 21.71 cm) and the control (TS - 12.57 cm).

Plants infected with CMM2163 and treated with the fungicides exhibited a gain in shoot fresh weight of at least 29.46 g compared to the control (TS - 19.80 g). For the other isolates, CMM4767 (54.14 g) and CMM4815 (50.74 g), higher fresh masses were obtained with the use of fluazinam (FZ).

Regarding root fresh weight, plants inoculated with CMM2163 (2.22 g) and CMM4767 (3.77 g) showed higher mass with the use of fluazinam (FZ), resulting in a gain of 1.70 g and 3.22 g, respectively, compared to the control (TS). For CMM4815 (1.32 g), the best result was obtained with fludioxonil (FD) (Table 4).

Plants infested with *M. euphorbiicola* (CMM2158 and CMM4867) exhibited greater shoot lengths (94.50 cm and 95.93 cm, respectively) when treated with fludioxonil (FD), resulting in a gain of 33.5 cm and 37.22 cm in length, respectively, compared to the control (TS) (Table 4). For the isolate CMM4868, the highest value was obtained with fluazinam (FZ - 89.93 cm).

The root length of plants inoculated with CMM2158 and CMM4867 showed no significant difference among the tested active ingredients but differed from the control (TS - 21.89 cm and 17.77 cm, respectively). However, for the treatment with CMM4868, the root length was greater with the application of fluazinam (FZ - 25.11 cm).

In terms of shoot fresh weight, plants infected with CMM2158 and CMM4867 exhibited greater mass with fludioxonil (FD - 52.18 g and 53.71 g, respectively), while for CMM4868, the weight was higher with fluazinam (FZ - 50.30 g). In these treatments, the plants experienced a gain in shoot fresh weight of 30.29 g, 27.45 g, and 35.94 g, respectively, compared to the control. The same trend was observed for root fresh weight, which was higher in the treatments with fludioxonil (FD) for isolates CMM2158 (3.84 g) and CMM4867 (2.44 g), while for CMM4868, this mass was higher with fluazinam (FZ - 2.02 g) (Table 4).

Visually, melon plants exhibited improved size and tissue coloration when treated with fluazinam (FZ) for all three *Macrophomina* species (Figure 3).

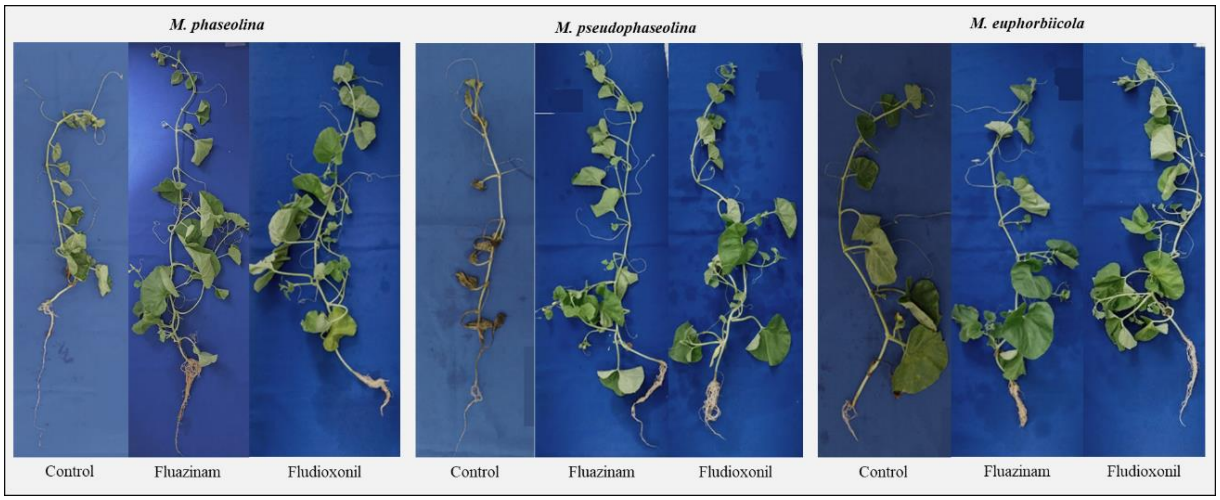


Fig. 3 Visual aspects of melons infested with *M. phaseolina*, *M. pseudophaseolina*, and *M. euphorbiicola* and treated with fluazinam and fludioxonil.

4 DISCUSSION

In Brazil, there are no registered fungicides for the control of *Macrophomina* in melon (AGROFIT 2023). However, the *in vitro* tests conducted in this study with five active ingredients (boscalid, carbendazim, cyprodinil, fluazinam, and fludioxonil) indicate the potential for controlling different *Macrophomina* species based on the evaluation of PGI (percent growth inhibition) and EC_{50} values. *In vitro* tests are valuable for predicting the behavior of active ingredients in field conditions (Cohen et al. 2012).

Among the five active ingredients tested *in vitro*, boscalid demonstrated the lowest PGI, even at the highest tested dose (100.00 mg/L a.i.), and low toxicity (EC_{50} = 13.40 mg/L a.i.) against the *Macrophomina* species evaluated. It is likely that boscalid needs to be combined with another active ingredient to enhance its toxicity against the fungus. Preliminary studies conducted by Cohen et al. (2012) have shown satisfactory results when combining boscalid with pyraclostrobin to control *Macrophomina* in melon plants. Additionally, boscalid is widely used in various crops due to its broad spectrum of action and systemic properties, targeting the mitochondrial succinate dehydrogenase enzyme complex II in multiple pathogens (Qian et al. 2018; Sun et al. 2022).

The high toxicity of carbendazim against the tested *Macrophomina* isolates was expected, as it is commonly used in seed treatment to mitigate root rot damage (Basandrai et al. 2021). A concentration of 100.00 mg/L a.i. inhibited the mycelial growth of the isolates by at least 87.53%. Iqbal and Mukhtar (2020) found that carbendazim, at a concentration of 150 ppm (mg/L), could inhibit the growth of *M. phaseolina* by 79.11% and control charcoal rot, a disease that causes similar damage to RRVD

The moderate toxicity of cyprodinil (EC_{50} = 1.18 mg/L a.i.) in inhibiting the mycelial growth of *Macrophomina* has also been reported by Cavalcante et al. (2020) in their evaluation of fungicides for the control of *Monosporascus* spp. Additionally, chemical management of RRVD caused by *M. phaseolina* with systemic products is challenging since they are not efficiently transported to the roots, which is the site of entry and infection by *Macrophomina*. (Marquez et al. 2021)

Fluazinam and fludioxonil, as contact fungicides, showed high toxicity (EC_{50} = 0.03 mg/L a.i.) against *Macrophomina* isolates. They function as a barrier, preventing pathogens from penetrating the plant (Leite and Lopes 2018). The high toxicity of fluazinam has been demonstrated against root pathogens causing plant root diseases (Medeiros et al. 2006;

Guimarães et al. 2008; Cohen et al. 2012). Fludioxonil was the only active ingredient able to completely inhibit the mycelial growth of isolates from all three *Macrophomina* species at the highest tested dose (100 mg/L a.i.). There is also a report on the efficacy of fludioxonil in controlling *Monosporascus cannonballus* on *C. melo*, with complete inhibition of the pathogen being observed at a concentration of 0.01 µg/ml (Pivonia et al. 2010). These characteristics of the active ingredients were considered when selecting them for the *in vivo* assays.

Based on the evaluated aspects in the *in vivo* study, we observed that the incidence and severity of RRVD caused by *Macrophomina* isolates were lower when treated with fluazinam, resulting in a reduction of up to 57.14% in incidence and 82.75% in severity. Fluazinam has previously been evaluated by Yogev et al. (1997) under field conditions for managing soil pathogens, with a recorded disease incidence of 4.00% when applied via drip irrigation. Additionally, the *in vivo* application of fluazinam and fludioxonil resulted in a significant increase in shoot and root lengths, as well as weights of melon plants, which are important physiological characteristics. The stimulation of root growth observed in response to application of the active ingredients is favorable and relevant since enhanced root development, which can reduce the severity of diseases caused by root pathogens (Martyn 2007).

Typically, active ingredients are chosen based on the genus of the pathogen causing the disease in the field (AGROFIT 2023). However, differences have been observed in the effects of active ingredients both *in vitro* and *in vivo* between isolates of the same species, suggesting the need for further research and the development of more specialized products, as well as continuous monitoring of production areas to identify the specific species present and provide appropriate management strategies.

The economic losses and lack of registered products for effectively controlling RRVD caused by *M. phaseolina* in melon underscore the importance of chemical management approaches. Among the fungicides tested, fluazinam and fludioxonil showed promising results in inhibiting the mycelial growth of *Macrophomina* species and reducing the incidence and severity of the disease. However, further research is necessary to evaluate the effects of these active ingredients in field conditions, validate their efficacy, and hence obtain product registrations for practical use.

ACKNOWLEDGEMENTS

This study received partial funding from the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) under Financial Code n° 001, and by the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq).

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CHAPTER IV

FIRST REPORT OF *Lasiodiplodia brasiliensis* CAUSING ROOT ROT ON WATERMELON IN BRAZIL

Watermelon (*Citrullus lanatus* (Thumb.) Matsum. & Nakai) is an important crop in Brazil both for export and domestic consumption. In October 2019, watermelon plants showing decline and root rot symptoms were surveyed in 16 commercial fields in Baraúna's municipality (Rio Grande do Norte, Brazil). The disease prevalence was 12.5%, and the average incidence was 5%. Affected root segments were cut into small pieces and surface-disinfected with 70% ethyl alcohol and 1.5 % NaOCl for 1 and 2 min, respectively. Tissues were plated onto potato dextrose agar (PDA) and incubated at 25°C for 7 days. Fungal colonies developed from the infected tissues were dark or greyish, and pure cultures were obtained by hyphal tip isolation technique. Six fungal isolates with the same morphology were obtained. Two of them were selected for morphological and molecular characterization (CFC-1123 and CFC-1124). Isolates grew rapidly in PDA, covering the entire surface of the Petri dishes within 3 days. The aerial mycelium was initially white, turning dark greenish-gray after 4 to 5 days of incubation at 25°C in the dark. Isolates produced pycnidia and conidia in water-agar medium with sterilized pine needles after 30 days of incubation at 25°C under near-UV light. The conidia were initially hyaline and brown with central transverse septum and longitudinal streaks when mature. Conidia were ellipsoid to oval (22.83 ± 3.1 µm long and 11.58 ± 1.5 µm wide). Based on morphological features, the isolates were initially identified as *Lasiodiplodia* sp. (Phillips et al. 2013). To confirm the identification, genomic DNA was extracted and the internal transcribed spacer (ITS) region as well as fragments of the translation elongation factor 1-α (TEF) and β-tubulin 2 (TUB) genes were amplified using the primer pairs ITS1/ITS4 (White et al. 1990), EF1-728F/EF1-986R (Carbone and Kohn 1999) and Bt2a/Bt2b (Glass and Donaldson 1995), respectively. The sequences were deposited in GenBank under accession numbers OL841380, OL865376 and OL890691 for CFC-1123, and OL841381, OL865377 and OL890692 for CFC-1124. Maximum likelihood phylogenetic analysis of the concatenated sequences of ITS, TEF and TUB gene regions of some reference sequences and ex-types of *Lasiodiplodia* spp. was performed. Phylogenetic analysis revealed that the isolates grouped in the *L. brasiliensis* clade (Netto et al. 2014) with 80/79% of bootstrap. The isolates were deposited in the Culture Collection of Phytopathogenic Fungi from Cariri (CFC) at the Universidade Federal do Cariri (Crato,

Brazil). Pathogenicity of the two isolates was determined using colonized wheat grains as inoculum source. One watermelon seed (cv. Crimson Sweet) was placed in a sterile plastic pot (500-mL) filled with 6 cm layer of a substrate composed of soil and Tropstrato® (5:1 w/w). Three wheat grains (50 mg) colonized with each isolate were placed 10 mm above the seed and covered with the substrate. Control pots were inoculated only with sterile wheat grains. There were five replicates for each isolate. The pots with seedlings were maintained in a greenhouse at 28 ± 2°C under natural light conditions. The inoculated seedlings showed poor growth, withering and drying leaves 45 days after inoculation (DAI), and subsequently root rot symptoms and death at 60 DAI. Control seedlings remained asymptomatic. The pathogen was re-isolated from all inoculated seedlings and identified by conidia morphology to fulfill Koch's postulates. *Lasiodiplodia brasiliensis* has been reported to cause postharvest rot and gummosis of watermelon (Farr and Rossman 2022). However, to our knowledge, this is the first report of watermelon decline caused by this fungus in Brazil and worldwide. This finding must be considered for developing efficient control strategies for the disease.

KEYWORDS phylogeny; vine decline; watermelon.

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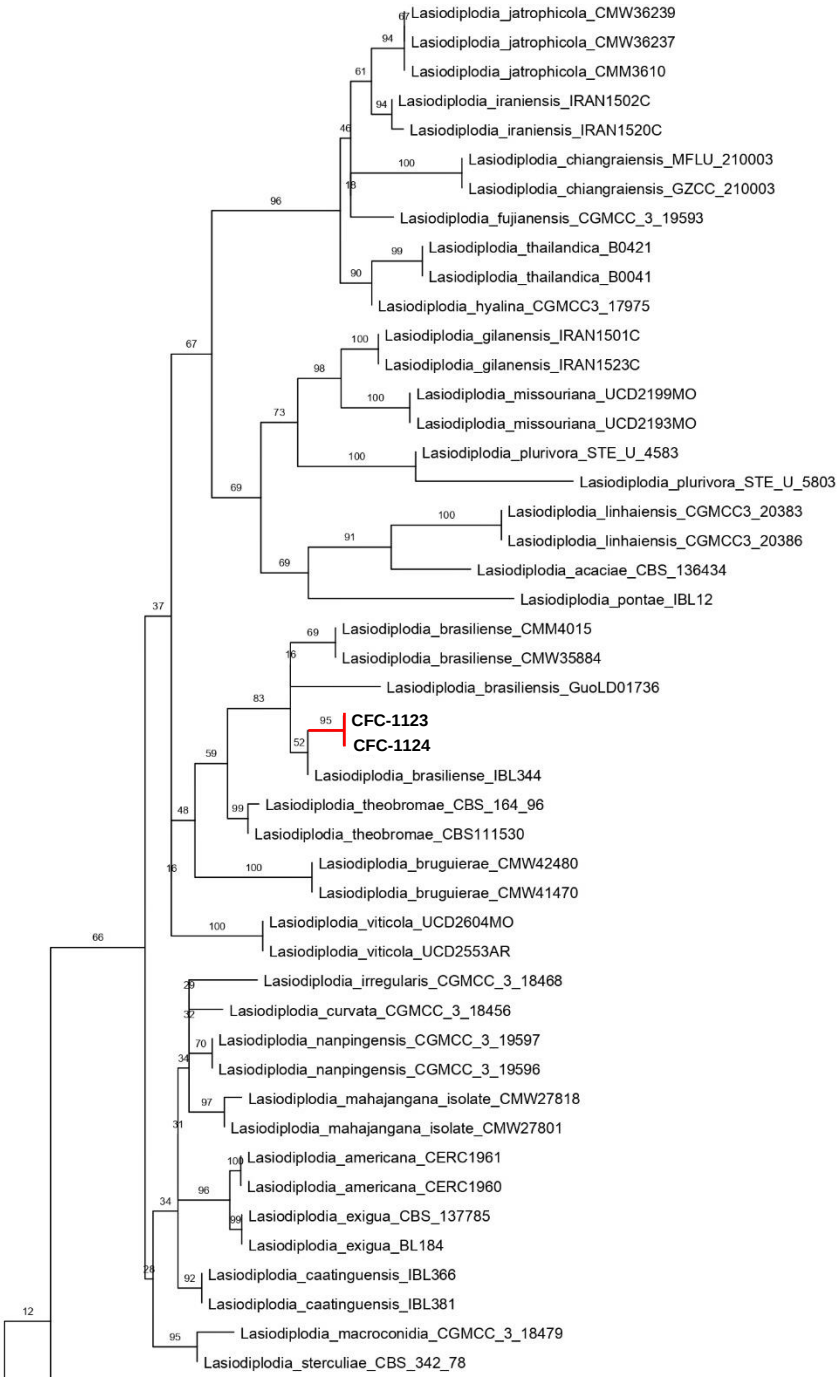
ATTACHMENT - Supporting Information



Cont.

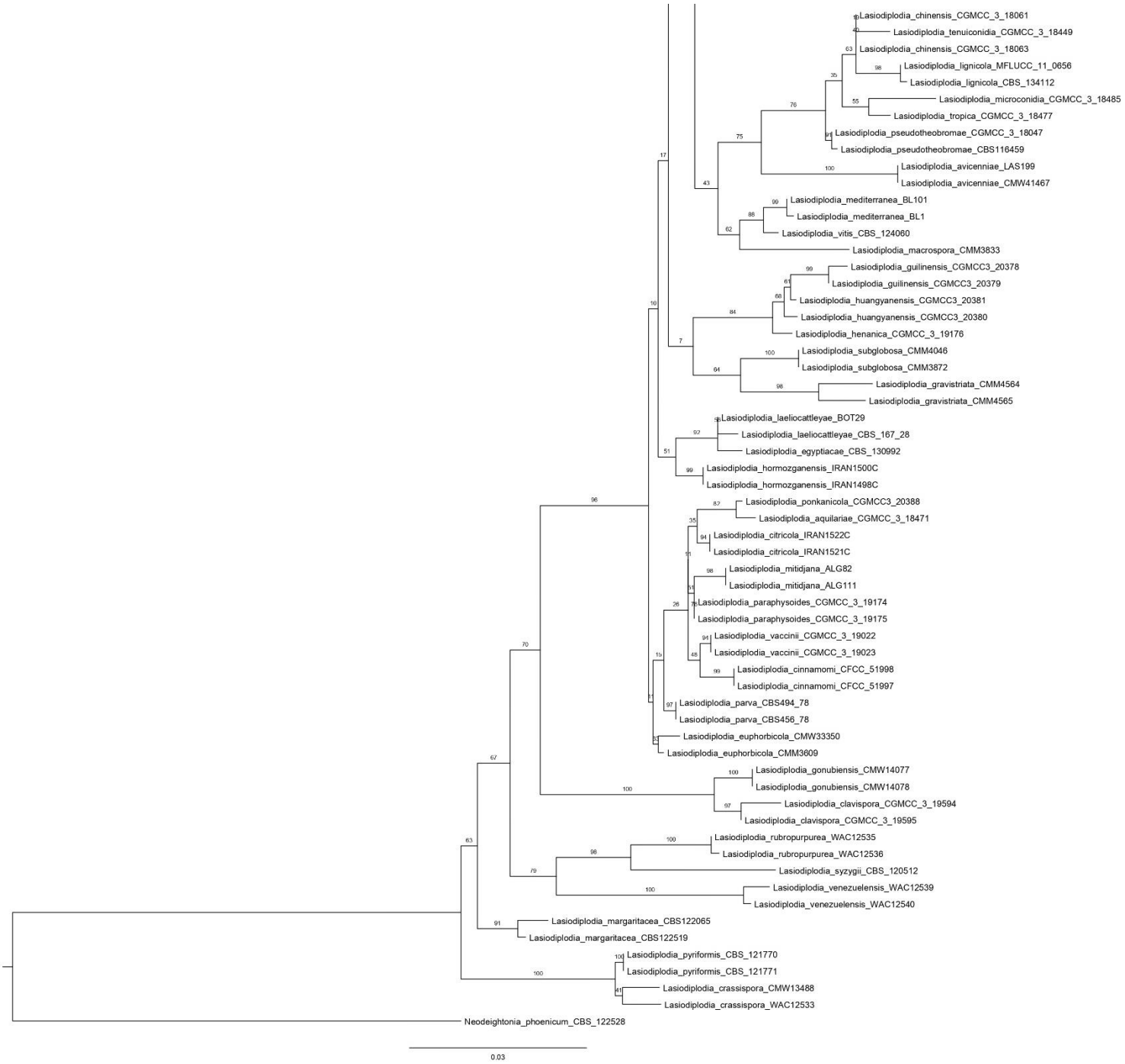


Fig. 1 Maximum likelihood phylogeny inferred from the combined internal transcribed spacer (ITS) regions and fragments of the translations elongation factor 1- α (*TEF*) and β-tubulin 2 (*TUB*) sequence alignments used to infer the relative position of isolates inside the *Lasiodiplodia* spp. Support values [Maximum Likelihood (ML) bootstrap] are given at the nodes. The tree was midpoint rooted.