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

ALLINNY LUZIA ALVES CAVALCANTE

ADAPTABILITY AND PATHOGENICITY OF *Monosporascus* SPECIES IN CUCURBITS

MOSSORÓ
2021

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Dissertação apresentada ao Mestrado em Fitotecnia do Programa de Pós-Graduação em Fitotecnia da Universidade Federal Rural do Semi-Árido como requisito para obtenção do título de Mestre em Fitotecnia.

Linha de Pesquisa: Fitopatologia

Orientador: Rui Sales Júnior, Prof. D. Sc.

Co-orientador: Andréia Mitsa Paiva Negreiros, Profa. D. Sc.

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Dados Internacionais de Catalogação na Publicação (CIP)
Biblioteca Central Orlando Teixeira (BCOT)
Setor de Informação e Referência (SIR)

C376a Cavalcante, Allinny Luzia Alves.
Adaptability and pathogenicity of Monosporascus species in cucurbits / Allinny Luzia Alves Cavalcante. - 2021.
47 f. : il.

Orientador: Rui Sales Júnior.
Coorientadora: Andréia Mitsa Paiva Negreiros.
Dissertação (Mestrado) - Universidade Federal Rural do Semi-árido, Programa de Pós-graduação em Fitotecnia, 2021.

1. Fungicides. 2. Mycelial growth. 3. Pathogenicity. 4. Soilborne fungi. 5. Virulence. I. Sales Júnior, Rui, orient. II. Negreiros, Andréia Mitsa Paiva, co-orient. III. Título.

Bibliotecário-Documentalista
Nome do profissional, Bib. Me. (CRB-15/10.000)

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Defendida em: 08 / 03 / 2021.

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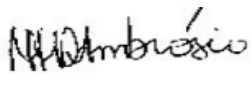

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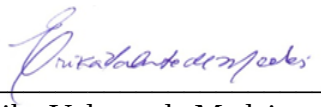

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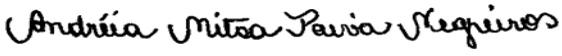

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À minha família, que sempre teve todo o cuidado e esforço necessários para que eu pudesse levar meus estudos adiante e ter as oportunidades que a eles não chegaram.

AGRADECIMENTOS

Agradeço primeiramente à Deus, que me permitiu chegar até aqui e sempre se mostrou presente nos momentos difíceis, quando pensei em desistir.

À Universidade Federal Rural do Semi-Árido (UFERSA) e ao Programa de Pós-Graduação em Fitotecnia pela oportunidade de ingresso e por fornecer a estrutura/insumos necessários para a realização deste trabalho.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de estudos.

Ao meu comitê de orientação, Professores D. Sc. Rui Sales Júnior, orientador, e Andréia Mitsa Paiva Negreiros, co-orientadora, pela atenção, dedicação, paciência e por todos os ensinamentos transmitidos desde a minha chegada ao Laboratório de Fitopatologia II.

Ao Professor D. Sc. Josep Armengol Fortí, do Instituto Agroforestal Mediterrâneo (Universidad Politécnica de Valencia, Espanha), pela contribuição na elaboração deste trabalho, sendo parte importante na publicação do mesmo.

Ao pessoal integrante do Laboratório de Fitopatologia II, Moisés Tavares, Cynthia Santos, Andrezza Araújo, Naama Melo, Érica Barreto, Sara Oliveira, Elisandra Bento e Matias de Souza, pela ajuda no desenvolvimento deste trabalho.

Aos amigos que estiveram presentes, me ajudando nos dias longos e cansativos de análises, Anne Caroline, Fernanda Santos e Virton Rodrigo, ainda que seus afazeres diários diferissem dos meus. Obrigada por disponibilizarem seus horários e dias livres para mim!

A todos que direta ou indiretamente contribuíram para a conclusão deste trabalho e para a minha formação profissional.

A jornada de mil quilômetros começa com o primeiro passo.

Tom Rogers – **O Rei Leão 3**, 2004.

RESUMO

Neste estudo, cinco novas espécies recentemente descritas de *Monosporascus*, *M. brasiliensis*, *M. caatinguensis*, *M. mossoroensis*, *M. nordestinus* e *M. semiaridus*, que foram encontradas em plantas daninhas coletadas em campos de cultivo de cucurbitáceas no Nordeste do Brasil foram caracterizadas em relação ao crescimento micelial *in vitro* em diferentes níveis de pH e concentrações de salinidade (NaCl), sua patogenicidade em espécies selecionadas de meloeiro, melancieira, pepineiro e aboboreira, e sua sensibilidade *in vitro* aos fungicidas boscalida, carbendazim, cyprodinil, fluazinam e fludioxonil. Os resultados revelaram uma grande variabilidade entre os isolados representativos de cada espécie de *Monosporascus*. Todos eles mostraram uma ampla faixa de tolerância aos diferentes níveis de pH, com o O_{pH} das espécies variando entre 5,72 e 8,05. As concentrações de NaCl reduziram significativamente o crescimento micelial *in vitro*, com EC_{50} acima de 900 mM para todas as espécies, embora nenhuma concentração tenha sido capaz de inibi-las completamente. Nos testes de patogenicidade, todas as cucurbitáceas avaliadas, foram suscetíveis às cinco espécies de *Monosporascus* em experimento em casa de vegetação, usando inoculação artificial de raízes. Além disso, todas as espécies de *Monosporascus* foram altamente suscetíveis aos fungicidas fludioxonil e fluazinam, exibindo valores de EC_{50} abaixo de 1 mg/L i.a. Nossos resultados fornecem informações relevantes sobre a resposta dessas novas espécies de *Monosporascus* a fatores ambientais, genótipos de plantas e fungicidas.

Palavras-chave: Crescimento micelial. Fungicidas. Fungos radiculares. Patogenicidade. pH. Salinidade. Virulência.

ABSTRACT

In this study, five new recently described *Monosporascus* species, *M. brasiliensis*, *M. caatinguensis*, *M. mossoroensis*, *M. nordestinus* and *M. semiaridus*, which were found on weeds collected from cucurbits cultivation fields in northeastern Brazil, were characterized regarding in vitro mycelial growth at different pH levels and salinity (NaCl) concentrations, their pathogenicity to selected species of melon, watermelon, cucumber and pumpkin, and their in vitro sensitivity to the fungicides boscalid, carbendazim, cyprodinil, fluazinam, and fludioxonil. The results revealed great variability among the representative isolates of each *Monosporascus* species. All of them showed a wide range of tolerance to different pH levels, with the O_{pH} of the species ranging from 5.72 to 8.05. NaCl concentrations significantly reduced in vitro mycelial growth, with EC_{50} above 900 mM for all species, although no concentration was able to inhibit them completely. In pathogenicity tests, all the cucurbits evaluated, were susceptible to the five *Monosporascus* species in a greenhouse experiment using artificial inoculation of roots. Moreover, all *Monosporascus* species were highly susceptible to the fungicides fludioxonil and fluazinam, exhibiting EC_{50} values below 1 mg/L a.i. Our results provide relevant information about the response of these new *Monosporascus* species to environmental factors, plant genotypes and fungicides.

Keywords: Fungicides. Mycelial growth. Pathogenicity. pH. Salinity. Soilborne fungi. Virulence.

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LIST OF ABBREVIATIONS AND ACRONYMS

µg	Microgramme
µm	Micrometre
a.i.	Active ingredient
BA	Bahia
$CaCl_2$	Calcium chloride
CE	Ceará
CFU	Colony-forming unit
cm	Centimeter
CMM	Coleção Maria Menezes
CV	Coefficient of variation
cv.	Cultivar
d	Day
DRW	Dry root weight
DSW	Dry shoot weight
EC_{50}	Half-maximal effective concentration
FRW	Fresh root weight
FSW	Fresh shoot weight
g	Gram
h	Hour
ha	Hectare
HCl	Hydrochloric acid
ITS	Internal Transcribed Spacer
kg	Kilogram
L	Liter
mg	Milligram
MGR	Mycelial growth rate
min	Minute
mm	Millimeter
mM	Millimolar
MRRVD	*Monosporascus* root rot and vine decline
NaCl	Sodium chloride

NaOH	Sodium hydroxide
PDA	Potato-dextrose-agar
PE	Pernambuco
PGI	Percentage of growth inhibition
PGPB	Plant growth promoting bacteria
pH	Hydrogen potential
RL	Root length
RN	Rio Grande do Norte
RRVD	Root rot and vine decline
SL	Shoot length

LIST OF SYMBOLS

°C	Celsius degree
%	Percentage
®	Trademark

SUMMARY

CHAPTER I

1 GENERAL INTRODUCTION

The Cucurbitaceae family includes numerous plant species of economic importance, among them, watermelon (*Citrullus lanatus* [Thunb.] Matsum. & Nakai), cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.) and pumpkin (*Cucurbita moschata* [Duchesne] Duchesne et Poir) (FAO, 2021). In Brazil, the Northeast region stands out for its high potential for the production of these species, especially melon and watermelon crops, which are responsible for most of the country's fruit and vegetable production (ANUÁRIO, 2020; IBGE, 2020).

The main melon producing states in Brazil are Rio Grande do Norte - RN (356,705 tons; 12,680 ha) and Ceará - CE (68,866 tons; 2,072 ha), which represent 60.7 and 11.7% of Brazilian production, respectively (IBGE, 2020). The main producing states in Northeast Brazil for watermelon are RN (351,997 tons; 15,268 ha) and Bahia - BA (166,046 tons; 13,239 ha), which together represent 66.8% of production in the region (IBGE, 2020). It should be noted that the state of RN is the largest national producer of melons and watermelons in Brazil (IBGE, 2020).

The technological inputs used in cultivation of melon and watermelon such as: use of hybrid cultivars, plastic mulch, transplanting of seedlings, drip irrigation and increase in plant density, have allowed the intensification of the cultivation of these cucurbits, with monoculture being carried out in two or more repeated cycles in the same soil/year (FIGUEIRÊDO *et al*., 2017). However, wrong practices in the use of these inputs, as well as in the phytosanitary management of these crops can put this agricultural activity at risk. According to Bruton (1998), the use of these inputs may be related to a higher incidence and severity of root diseases, among which “root rot and vine decline” (RRVD) stand out.

The RRVD is one of the main root problems affecting the cultivation of melons and watermelons in Brazil (SALES JÚNIOR *et al*., 2003; 2010; NEGREIROS *et al*., 2019a) and worldwide (MARTYN & MILLER, 1996). It is a complex syndrome where several pathogens are involved, such as: *Acrocalymma vagum* Crous & Trakunyingcharoen (FARR *et al*., 1998; ARMENGOL *et al*, 2003), *Fusarium oxysporum* f. sp. *melonis* Snyder & Hansen (COHEN *et al*., 2012a; 2016), *Fusarium solani* (Mart.) Sacc. f. sp. *cucurbitae* Snyder & Hans (CHAMPACO *et al*., 1993; ANDRADE *et al*., 2005), *Macrophomina phaseolina* (Tassi) Goid. (CARTER, 1979; COHEN *et al*., 2012a; 2016), *Monosporascus cannonballus* Pollack

& Uecker (SALES JÚNIOR *et al*., 2004; COHEN *et al*., 2012b), *Paramyrothecium roridum* (Tode) L. Lombard & Crous (CARTER, 1980; NORONHA *et al*., 2008; CHEN *et al*., 2018), *Plectosphaerella melonis* (Watan & Sato) Phillips, Carlucci & Raimondo (BRUTON *et al*., 1995; ARMENGOL *et al*., 1998), *Rhizoctonia solani* Kühn (AL-SADI *et al*., 2011) and *Stagonosporopsis cucurbitacearum* (Fr.) Aveskamp, Gruyter & Verkley (NUNES *et al*., 2004; BASIM *et al*., 2016). All these pathogens can attack the roots of cucurbits alone or in interaction with each other at the same time, causing symptoms ranging from yellowing and drying of leaves to premature death of the plant (MARTYN & MILLER, 1996).

Among the pathogens associated with the RRVD, *M. cannonballus* deserves mention for being present in the main producing and exporting areas of cucurbits in Northeast region of Brazil, affecting roots of melon and watermelon plants (SALES JÚNIOR *et al*., 2004, 2010). In addition, it has been reported in several countries causing the disease "*Monosporascus* root rot and vine decline" (MRRVD) (SALES JÚNIOR *et al*., 2003; 2004; 2010; COHEN *et al*., 2012b; AL-MAWAALI *et al*., 2013; YAN *et al*., 2016; MARKAKIS *et al*., 2018).

Monosporascus cannonballus is an ascomicete, a natural inhabitant of soils, which presents thermophilic characteristics, adapting well to arid and semi-arid conditions (MEDEIROS *et al*., 2006). It is characterized by the formation of perithecia, which generally appear at the end of the crop cycle, on affected roots, and are easily visible to the naked eye (SALES JÚNIOR *et al*., 2002; COHEN *et al*., 2012b). Perithecia have the ability to survive in soil for long periods, producing asci that contain a large ascospore (rarely two). Besides *M. cannonballus*, another species of the genus, *Monosporascus eutypoides* (Petrak) von Arx has also been reported to cause MRRVD in watermelon and cucumber crops in Tunisia (BEN SALEM *et al*., 2013).

The damage caused by *M. cannonballus* reduces the plant's capacity to absorb water and nutrients, impairing the fruit's ripening period, and in some cases can cause the total loss of the crop (MARTYN & MILLER, 1996). Studies by García-Jimenez *et al*. (2000) have shown that melon production in Spain has decreased by about 40% in only 15 years due to this disease. Therefore, the MRRVD continues to be a great challenge for researchers and melon producers in Brazil and worldwide.

Recently, phylogenetic studies conducted by Negreiros *et al*. (2019b) on occurrence of weeds prevalent in cucurbitaceous fields as alternative hosts for RRVD-causing fungi, identified five new species of the genus *Monosporascus*, present in roots of *Boerhavia diffusa* L. and *Trianthema portulacastrum* L., named as: *M. brasiliensis* A. Negreiros, M. León, J.

Armengol & R. Sales Júnior, *M. caatinguensis* A. Negreiros, M. León, J. Armengol & R. Sales Júnior, *M. mossoroensis* A. Negreiros, M. León, J. Armengol & R. Sales Júnior, *M. nordestinus* A. Negreiros, M. León, J. Armengol & R. Sales Júnior, and *M. semiaridus* A. Negreiros, M. León, J. Armengol & R. Sales Júnior, species reported so far only in Brazilian territory.

All recently reported new species have optimal growth range at temperatures above 30 °C and the environmental conditions required are similar to those described for other species of the genus (NEGREIROS *et al*., 2019b). According to the results obtained in the study, *M. nordestinus* presents 1-3 ascospores per asci and is closely related to *M. mossoroensis*, based on phylogenetic inference, with morphology close to *M. eutypoides*, distinguished by its optimal growth rate being at higher temperature (32.4 °C). *Monosporascus semiaridus* presents one ascospore per asci and is closely related to *M. brasiliensis*, based on phylogenetic inference, with morphology close to *M. cannonballus*, distinguished by its slightly smaller asci (50.2 to 77 µm). The other species did not present sexual morphological characteristics, because they did not sporulate in the different culture media and temperatures tested (NEGREIROS *et al*., 2019b).

In terms of adaptability, *M. cannonballus* is the most reported species of the genus. It has an optimum growth temperature ranging from 25 to 35 °C, being inhibited at temperatures above 40 °C and below 15 °C (MARTYN & MILLER, 1996). *Monosporascus eutypoides* presents the same optimal growth temperature variation, 25-35 °C, being inhibited at temperatures above 45 °C (SIVANESAN, 1991). According to Pivonia *et al*. (2002a), this thermophilic character predicts that the fungus is pathogenic only in hot regions, being saprophytic in colder regions. The range of pH that provides better in vitro growth of *M. cannonballus* oscillates between 6 and 7. However, studies have confirmed that the fungus can also grow at pH 9. This growth is reduced at pH 5, and totally inhibited at pH below 4 (MARTYN, 2002). In this understanding, *M. cannonballus* prefers a pH that goes from neutral to slightly basic. This optimal pH is generally found in alkaline soils, which largely occur in arid climate zones (MARTYN & MILLER, 1996). With regard to salinity, *M. cannonballus* shows high tolerance. In vitro tests have indicated that this species is capable of tolerating moderately high concentrations of sodium chloride (NaCl) and calcium chloride ($CaCl_2$), in solutions that can range from 8-10% of these solutions (MARTYN & MILLER, 1996).

Pathogenicity studies of *M. cannonballus* to cucurbits have been carried out all over the world, with the first reports being made in Israel (REUVENI & KRIKUN, 1983), Japan

(UEMATSU *et al*., 1985; UEMATSU & SEKIYAMA, 1990) and United States of America (MERTELY *et al*., 1991; AEGERTER *et al*., 2000), evidencing its pathogenicity to melons. Crops such as pumpkin, summer squash (*Cucurbita pepo* L.), loofah (*Luffa aegyptiaca* Mill), bottle gourd (*Lagenaria siceraria* [Molina] Standl.), watermelon, winter squash (*Cucurbita máxima* Duch.) and cucumber have been reported with symptoms of RRVD associated with *Monosporascus*, being watermelon, melon, and cucumber, the main crops affected (MERTELY *et al*., 1993; COHEN *et al*., 2012b). Sales Júnior *et al*. (2018a) studied the behavior of pumpkin, watermelon, melon, and cucumber, with two cultivars for each species tested, against artificial inoculation with *M. cannonballus*. The results indicated that all species tested showed damage to the root system, being possible to visualize perithecia of the pathogen. *Monosporascus eutypoides* has also reported pathogenicity in melon, watermelon, and cucumber (BEN SALEM *et al*., 2013). Species of *Monosporascus* can also be observed in plants that do not belong to the cucurbitaceae family. However, Mertely *et al*. (1993) reports that in species other than those of the cucurbitaceae family may not be of agricultural importance, contributing only to the persistence of the pathogen during short or long periods of crop rotation.

Numerous control methods against *M. cannonballus* have already been proven in isolation, with variable results. However, the use of integrated management, combining different control techniques, seems to be the best alternative to control this disease (COHEN *et al*., 2012b). Among the most commonly used techniques, solarization combined with fumigants, crop rotation, destruction of crop residues and incorporation of green fertilizers, and biological, genetic and chemical controls can be mentioned (SALES JÚNIOR *et al*., 2018b).

The use of soil solarization is a widely used technique to control or reduce the attack of different soil-dwelling pathogens and some pests. It is a non-chemical method, which uses the incidence of solar radiation on moist soil with a transparent plastic covering for a period of 45 to 60 days, aiming to increase soil temperature to eliminate microorganism propagules, weed seeds, nematode eggs, pests, etc. (KATAN, 1996; KANAAN *et al*., 2017). Because they are thermophilic microorganisms, capable of surviving at higher temperatures, the solarization method itself is not efficient in the management of *Monosporascus* (REUVENI & KRIKUN, 1983; COHEN *et al*., 2000; PIVONIA *et al*., 2002b). However, the use of combined methods between solarization and fumigants at reduced doses results in generally effective control of the pathogen (COHEN *et al*., 2000; GAMLIEL *et al*., 2000; TJAMOS *et al*., 2000).

The adoption of crop rotation is also a practice widely used by producers, aiming to reduce the attack of pests and diseases. The effect of different combinations of crop rotation was previously verified on the population dynamics of *M. cannonballus* ascospores in the soil and on the incidence of RRVD, concluding that the planting of a non-preferential crop of the pathogen, such as tomato, reflects in reduction of the incidence of disease in the second year of planting (BEN SALEM *et al*., 2015).

The destruction of crop residues is one of the most recommended plant disease control practices, especially for those produced by soil-dwelling root pathogens (BAIRD *et al*., 2003). Sales Júnior *et al*. (2017), studying the incorporation of green fertilizer as a way to induce suppressivity to RRVD in soil naturally infested by *M. cannonballus* observed that the most efficient treatment in reducing the severity, and that presented, numerically, a greater number of commercial melon fruits was the treatment with *Stilozobium aterrimum* Piper & Tracy.

The use of antagonistic agents is used as a biological control route for RRVD. Zhang *et al*. (1999) studying the potential of *Trichoderma virens* (Miller, Giddens & Foster) Arx in controlling *M. cannonballus* in a greenhouse, suggested its potential use in RRVD management, according to the results obtained. In Brazil, Sales Júnior *et al*. (2007) reported the efficiency of *Chaetomium* sp. against *M. cannonballus* in melon Pele de Sapo (cv. PS 1430) in pots in greenhouse. One of the most current lines of study in biocontrol is the use of plant growth promoting bacteria (PGPB). Antonelli *et al*. (2013), studying the behavior of PGPB isolated from solarized soil, and inoculating them on seeds, highlighted the potential synergistic effect of *Bacillus subtilis* and *Pseudomonas putida* in integrated RRVD management.

Several studies related to genetic control have been performed against *M. cannonballus*, among them: host resistance test, pathogenicity test of melon germplasm, use of rootstocks and studies of root architecture and its influence on RRVD control, being the use of melon rootstocks over other cucurbits species one of the most promising techniques found so far (COHEN *et al*. 2000; SALES JÚNIOR *et al*., 2018b).

The efficiency of pesticide use in controlling *M. cannonballus* has so far shown inconsistent results with respect to in vitro and field trials. Cohen *et al*. (1999) evaluated several fungicides for their ability to inhibit the vegetative growth of *M. cannonballus* in the laboratory, with the active agents fluazinam and kresoxim methyl, being the ones that completely inhibited the growth of the pathogen. Guimarães *et al*. (2008), conducting greenhouse studies, concluded that small doses of fluazinam (1.0 L/ha) can be recommended to control RRVD by *Monosporascus* in melon crops. Doses of 10 µg/L of fluazinam were also

efficient in in vitro control of 57 Brazilian *M. cannonballus* isolates (CORREIA *et al*., 2014). In a field experiment conducted in Israel, Pivonia *et al*. (2010) concluded that azoxystrobin, prochloraz, and pyraclostrobin + boscalid have similar high efficiencies in controlling RRVD. In Brazil, to date, there is no product registered for this pathogen. However, in the United States, the company Syngenta Crop Protection registered in 2005 the commercial product Cannonball®, whose active ingredient is fludioxonil. However, in tests carried out in Israel, this fungicide was less effective than azoxystrobin and, in certain cases, showed some phytotoxicity on melon plants (PIVONIA *et al*., 2010).

So far, due to the recent discovery of new species of *Monosporascus*, *M. brasiliensis*, *M. caatinguensis*, *M. mossoroensis*, *M. nordestinus*, and *M. semiaridus*, there is no information about their adaptability components. What is known until now is that their ideal growth temperatures range from 30 to 33 °C (NEGREIROS *et al*., 2019b). It is therefore important to study the behavior of these new fungal species reported in the region, so that preventive management measures can be adopted, in order to minimize losses resulting from the attack of these root pathogens, as well as to generate subsidies for future plant breeding work with a view to implementing new technologies used in the cultivation of cucurbits.

Thus, the objective of this study is to obtain new phenotypic and pathogenic information of new *Monosporascus* species (*M. brasiliensis*, *M. caatinguensis*, *M. mossoroensis*, *M. nordestinus*, and *M. semiaridus*) from cucurbitaceae fields in Northeast region, evaluating: 1) their mycelial growth at different pH levels and salinity concentrations; 2) their pathogenicity in melon, watermelon, cucumber and pumpkin seedlings; and 3) their sensitivity to the fungicides boscalid, carbendazim, cyprodinil, fluazinam, and fludioxonil.

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CHAPTER II

CHARACTERIZATION OF FIVE NEW *Monosporascus* SPECIES: ADAPTATION TO ENVIRONMENTAL FACTORS, PATHOGENICITY TO CUCURBITS AND SENSITIVITY TO FUNGICIDES

Abstract

In this study, five new recently described *Monosporascus* species, *M. brasiliensis*, *M. caatinguensis*, *M. mossoroensis*, *M. nordestinus*, and *M. semiaridus*, which were found on weeds collected from cucurbit cultivation fields in northeastern Brazil, are characterized regarding mycelial growth at different pH levels and salinity (NaCl) concentrations, their pathogenicity to selected cucurbit species, and their sensitivity to fungicides with different modes of action. Our results reveal great variability among the representative isolates of each *Monosporascus* spp. All of them showed a wide range of tolerance to different pH levels, and NaCl significantly reduced their in vitro mycelial growth, although no concentration was able to inhibit them completely. In pathogenicity tests, all seedlings of cucurbits evaluated, melon, watermelon, cucumber, and pumpkin, were susceptible to the five *Monosporascus* spp. in greenhouse experiments using artificial inoculation of roots. Moreover, all *Monosporascus* spp. were highly susceptible to the fungicides fludioxonil and fluazinam. Our findings provide relevant information about the response of these new *Monosporascus* spp. to environmental factors, plant genotypes and fungicides.

Keywords: Fungicides. Mycelial growth. Pathogenicity. pH. Salinity. Soilborne fungi. Virulence.

1 INTRODUCTION

Technological advances employed in cucurbits cultivation such as the use of hybrid cultivars, transplanted seedlings, plastic mulch, drip irrigation, and increased plant density have allowed the intensification of melon (*Cucumis melo* L.) and watermelon crops (*Citrullus lanatus* [Thunb.] Matsum. and Nakai). However, this has been also directly related to an increasing incidence of root diseases, such as *Monosporascus* root rot and vine decline (MRRVD), which is one of the major factors currently limiting the production and expansion of melon and watermelon crops worldwide (MARTYN & MILLER, 1996; BRUTON, 1998;

COHEN *et al*., 2012). The main symptoms of MRRVD on cucurbits manifest close to harvest, when yellowing, wilting and drying of the leaves occur, followed by a sudden vine decline, which causes the death of the plants and important economic loss (COHEN *et al*., 2012).

Monosporascus root rot and vine decline is caused by two soilborne ascomycetes belonging to the genus *Monosporascus*: *M. cannonballus* Pollack and Uecker and *M. eutypoides* (Petrak) von Arx, which colonize the roots of cucurbits, causing extensive necrotic and rotted areas, in which black perithecia, corresponding to the sexual reproductive structures of these pathogens, can be observed (COHEN *et al*., 2012; BEN SALEM *et al*., 2013). These fungal species were suggested to be conspecific, but it was demonstrated that they are distinct, with *M. cannonballus* being the most widespread (BEN SALEM *et al*., 2013).

Monosporascus cannonballus is a cosmopolitan pathogen which, to date, has been associated with MRRVD of cucurbits in 22 countries worldwide (SALES JÚNIOR *et al*., 2004; 2010; COHEN *et al*., 2012; AL-MAWAALI *et al*., 2013; YAN *et al*., 2016; MARKAKIS *et al*., 2018). However, *M. eutypoides* has been unequivocally reported only in Tunisia from roots of watermelon and cucumber crops (*Cucumis sativus* L.) (BEN SALEM *et al*., 2013). Recently, Negreiros *et al*. (2019) conducted a survey in northeastern Brazil to investigate the role of prevalent weeds present in cucurbit fields as alternative hosts for fungal root pathogens. These authors found five new *Monosporascus* species on roots of *Boerhavia diffusa* L. and *Trianthema portulacastrum* L., which were described based on morphology and multilocus DNA sequence analyses: *M. brasiliensis* A. Negreiros, M. León, J. Armengol and R. Sales Júnior, *M. caatinguensis* A. Negreiros, M. León, J. Armengol and R. Sales Júnior, *M. mossoroensis* A. Negreiros, M. León, J. Armengol and R. Sales Júnior, *M. nordestinus* A. Negreiros, M. León, J. Armengol and R. Sales Júnior, and *M. semiaridus* A. Negreiros, M. León, J. Armengol and R. Sales Júnior. *Monosporascus* has been also reported as a common root endophyte in surveys of grasses, shrubs and herbaceous plants located in the southwestern United States using both molecular and culturing methods (PORRAS-ALFARO *et al*., 2008; HERRERA *et al*., 2010; DEAN *et al*., 2015; ROBINSON *et al*., 2020).

Monosporascus cannonballus is the most well-known species of the genus. There is some knowledge about the adaptability of *M. cannonballus* populations to different environmental factors such as temperature, hydrogen potential (pH) and salinity (MARTYN & MILLER, 1996; KWON *et al*., 2001; MARTYN, 2002; PIVONIA *et al*., 2002; WAUGH *et al*., 2003; YASUAKI *et al*., 2005; HAMZA *et al*., 2007; SENHOR *et al*., 2009; SILVA *et al*., 2010; ARMENGOL *et al*., 2011; BEN SALEM *et al*., 2011; COHEN *et al*., 2012; CORREIA

et al., 2014; RHOUMA *et al*., 2019). In fungi, these factors profoundly affect their growth rate but also can act as triggers in development pathways (DEACON, 2006). This information has been inferred from in vitro studies conducted with collections of *M. cannonballus* isolates obtained in areas where this fungus is a prevalent cucurbit pathogen.

Monosporascus cannonballus is considered a thermophilic fungus, well adapted to arid and semi-arid conditions, with optimal growth temperature for mycelial growth ranging from 25 to 35 °C; some isolates have been reported to grow at temperatures above 40 °C, and to be inhibited at temperatures below 15 °C (MARTYN & MILLER, 1996; COHEN *et al*., 2012). Yasuaki *et al*. (2005) showed that *M. cannnonballus* can tolerate an immersion in hot water at 50 °C for up to three days, but it died after 90 min immersion in hot water at 60 °C. Its optimal pH for mycelial growth ranges between 6 and 7; however, it was confirmed that this fungus can also tolerate a pH of 9, but its mycelial growth is highly reduced at pH 5, and it is totally inhibited at a pH below 4 (MARTYN, 2002). Regarding salinity, *M. cannonballus* shows a high tolerance. In vitro tests showed that mycelium of this fungus can tolerate relatively high concentrations of NaCl and $CaCl_2$ ranging from 8-10% of these compounds (MARTYN & MILLER, 1996). Recent studies conducted by Rhouma *et al*. (2019) corroborated these previous studies, demonstrating that soils with high salt content may be favorable for MRRVD disease.

Monosporascus cannonballus is a melon and watermelon pathogen, but its pathogenicity to other Cucurbit species has also been explored. Cucumber, summer squash (*Cucurbita pepo* L.), pumpkin (*C. moschata* [Duch.] Duch. ex Poir), winter squash (*C. maxima* Duch.), bottle gourd (*Lagenaria siceraria* [Molina] Standl.), and loofah (*Luffa aegyptiaca* Mill.) have already been described as being susceptible to this pathogen in host range studies with artificial inoculation (MERTELY *et al*., 1993; KWON *et al*., 2001; SALES JÚNIOR *et al*., 2018).

Control of *M. cannonballus* is difficult and the management programs against this pathogen integrate different management strategies, including the use of fungicides (MEDEIROS *et al*., 2008; AWAD, 2016; SALES JÚNIOR *et al*., 2017). The fungicides fluazinam and kresoxim methyl completely inhibited the vegetative growth of *M. cannonballus* in vitro and fluazinam was able to suppress MRRVD in the field, with results ranging from 87 to 32% in disease control (COHEN *et al*., 1999). Guimarães *et al*. (2008) conducted greenhouse studies with fluazinam, concluding that this active ingredient (a.i.) can be recommended in small doses (1.0 L/ha) to control MRRVD in melon crops. Doses of 10 µg/L a.i. of fluazinam were also effective in inhibiting the in vitro mycelial growth of 57

Brazilian isolates of *M. cannonballus* (CORREIA *et al*., 2014). Azoxystrobin, prochloraz, and pyraclostrobin + boscalid exhibited high and similar efficacies in the control of *M. cannonballus* in field experiments, but fludioxonil was less effective than azoxystrobin and, in certain cases, showed some phytotoxicity to melon plants (PIVONIA *et al*., 2010).

Much less information is available for *M. eutypoides*. This fungus has a mean optimum growth temperature of 29.43 ± 0.03 °C and only its pathogenicity to melon, watermelon and cucumber has been confirmed (BEN SALEM *et al*., 2013).

Regarding the five new *Monosporascus* species recently described in Brazil, *M. brasiliensis*, *M. caatinguensis*, *M. mossoroensis*, *M. nordestinus*, and *M. semiaridus*, what it is known so far is that their optimal growth temperatures range from 30 to 33 °C, and none of them was able to grow in potato-dextrose-agar (PDA) at temperatures of 10 and 40 °C (NEGREIROS *et al*., 2019). Thus, the objective of this study was to obtain new phenotypic and pathogenicity information for these *Monosporascus* species by evaluating: (i) their mycelial growth at different pH levels and salinity concentrations; (ii) their pathogenicity to different cucurbits; and (iii) their sensitivity to several fungicides with different modes of action. In all these experiments, *M. cannonballus* was included as the reference species for the genus *Monosporascus*.

2 MATERIALS AND METHODS

2.1 *Monosporascus* spp. Isolates

Six *Monosporascus* spp. isolates were used in this study (Table 1). Their identity was previously confirmed by molecular techniques (NEGREIROS *et al*., 2019), and all of them were deposited in the Collection of Phytopathogenic Fungi “Prof. Maria Menezes” (CMM) at the Universidade Federal Rural de Pernambuco (Recife, PE, Brazil). Prior to use, these isolates were grown in Petri dishes with PDA (Merck KGaA, Darmstadt, Germany) at 25 °C in darkness for 7–10 days.

Table 1. Isolates of the *Monosporascus* spp. used in this study.

***Monosporascus* Species**	**Code (CMM) [1]**	**Host**	**Location [2]**	**GenBank ITS Region [3]**
M. brasiliensis	4839	*Trianthema portulacastrum*	Brazil, RN	MG 735234
M. caatinguensis	4833	*Boerhavia diffusa*	Brazil, CE	MG 735228
M. cannonballus	2429	*Cucumis melo*	Brazil, RN	JQ 762366
M. mossoroensis	4857	*Trianthema portulacastrum*	Brazil, RN	MG 735252
M. nordestinus	4846	*Trianthema portulacastrum*	Brazil, RN	MG 735241
M. semiaridus	4830	*Trianthema portulacastrum*	Brazil, CE	MG 735222

[1] CMM = Culture Collection of Phytopathogenic Fungi "Prof. Maria Menezes" of the Universidade Federal Rural de Pernambuco (Recife, PE, Brazil). [2] CE = Ceará state and RN = Rio Grande do Norte state. [3] Sequence of the Internal Transcribed Spacer Region (ITS) of the isolates deposited at GenBank.

2.2 Effect of pH on the Mycelial Growth Rate of *Monosporascus* spp.

The effect of pH on the mycelial growth rate of all isolates was determined using cultures grown on PDA. Mycelial plugs (8 mm in diameter) obtained from the growing edge of 10-day-old colonies were transferred to the center of PDA plates (one plug per plate) which were adjusted to pH 5, 6, 7, 8, and 9 with the addition of 1.5 N hydrochloric acid (HCl) or 1 N sodium hydroxide (NaOH). Plates were incubated in the dark at 28 °C. There were five replicates for each isolate and pH combination. The diameter of each colony was measured twice perpendicularly when it reached at least two thirds of the plate or at 7 days of growth, and the data were used to calculate the mycelial growth rate (MGR) as cm per day (cm/d). The experiment was conducted twice and was set up with a completely randomized design. One-way analysis of variance (ANOVA) was performed with data obtained from MGR, and the optimum pH (O_{pH}) for MGR of each isolate was plotted against pH and a curve was fitted by a cubic polynomial regression ($y = a + bx + cx^2 + dx^3$) using TableCurve 2D v. 5.01 (Systat Software, Inc., San Jose, CA, USA).

2.3. Effect of Salinity on the Mycelial Growth of *Monosporascus* spp.

The effect of salinity on the mycelial growth of all isolates was determined using cultures grown on PDA. Mycelial plugs (8 mm in diameter) obtained from the growing edge of 10-day-old colonies were transferred to the center of PDA plates (one plug per plate) with the following concentrations of NaCl: 0, 250, 500, 750, and 1000 mM (CERVANTES-GARCÍA *et al*., 2003). Plates were incubated in the dark at 28 °C. The experiment was conducted twice and was set up with a completely randomized design, with five replicates for each isolate. Mycelial growth rate was evaluated as described before and used to calculate the percentage of growth inhibition (PGI). The PGI of each isolate at different NaCl concentrations were subjected to a regression analysis using TableCurve 2D v. 5.01 (Systat Software, Inc., San Jose, CA, USA) and half-maximal effective concentration (EC_{50}) was determined.

2.4 Pathogenicity of *Monosporascus* spp. to Cucurbits

The pathogenicity of *Monosporascus* spp. isolates to cucurbits was evaluated in a pot assay. Fungal inoculum was prepared following the method described by Ben Salem *et al*. (2015), with some modifications. Wheat seeds were autoclaved in flasks three times at 120 °C for 1 h, with an interval of 24 h. Mycelial plugs (8 mm in diameter) of each isolate were used for inoculation of the seeds, which were incubated at 25 °C for four weeks until complete colonization by the pathogens. Flasks containing the inoculated seeds were agitated manually once a week to avoid inoculum clustering. The substrate used in the pots was composed of a mixture of sandy-clay soil passed through a 2 mm mesh and Tropstrato HT Hortaliças® (Vida Verde, Brazil), at a proportion of 2:1. This mixture was autoclaved twice at 120 °C for 1 h, with an interval of 24 h. For soil infestation, approximately 12 g of the seeds colonized with each isolate were added in pots containing 2 kg of the sterile soil-substrate mixture. Only autoclaved noncolonized wheat grains were added to the substrate in the controls. One week after inoculation, 10-day-old seedlings of cucumber cv. ‘Racer’, melon cv. ‘Titannium’, pumpkin cv. ‘Mírian’ and watermelon cv. ‘Manchester’, were transplanted to pots containing infested soil. There were five pots per *Monosporascus* spp. and cucurbit species combination, with one seedling each. The pots were arranged in a complete randomized experimental design in a greenhouse at an average temperature of 35 °C, under natural daylight conditions, and watered thrice a week at field capacity. The experiment was conducted twice.

Disease evaluation was performed 50 days after the transplant. Plants were carefully removed from the pots, and the root systems were gently washed with tap water. Disease incidence in each cucurbit species was determined as the total number of infected plants from each *Monosporascus* spp. and expressed as a percentage. Disease severity was assessed using a diagrammatic scale adapted from Aegerter *et al*. (2000), where: 0 = symptomless; 1 = less than 10% of the roots with weak discoloration or lesions; 2 = moderate discoloration or rot, with lesions reaching 25 to 35% of the roots; 3 = lesions converging to 50% of the roots and death of secondary roots; and 4 = generalized necrosis of the roots or dead plant. For isolation, roots were surface sterilized for 1 min in a 2.0% sodium hypochlorite solution and washed twice with sterile water. Seven root segments per plant from apparently affected areas were transferred to PDA supplemented with 500 mg/L of streptomycin sulphate.

The results of incidence and severity were analyzed with the non-parametric Kruskal–Wallis test at the probability level of 5% ($p < 0.05$) using the software Assistat, version 7.7 (SILVA & AZEVEDO, 2016).

In addition, root and shoot lengths (RL and SL) of each plant, and the fresh and dry root (FRW and DRW) and shoot (FSW and DSW) weights were also measured. Dry weights were obtained by placing plant parts individually in paper bags, which were introduced in a forced circulation oven at 70 °C until a constant dry weight was reached. Data were submitted to ANOVA and means compared by Tukey at 5% probability using the Assistat software, version 7.7 (SILVA & AZEVEDO, 2016).

2.5 Sensitivity of *Monosporascus* spp. to Fungicides

The effect of fungicides on mycelial growth of *Monosporascus* spp. was determined in vitro as described by Tonin *et al*. (2013). The treatments included five a.i. — boscalid (Cantus WG, 50% a.i., systemic, BASF S.A.), carbendazim (Carbendazim, 99.9% a.i., systemic, Syngenta Proteção de Cultivos Ltda), cyprodinil (Unix 750 WG, 75% a.i., systemic, Syngenta Proteção de Cultivos Ltda), fluazinam (Frowncide 500 SC, 50% a.i., contact, ISK Biosciences do Brasil Defensivos Agrícolas Ltda), and fludioxonil (Maxim, 25% a.i., contact, Syngenta Proteção de Cultivos Ltda) — and five concentration levels: 0.01, 0.1, 1, 10 and 100 mg/L a.i. PDA plates without fungicide were used as controls. Mycelial plugs (8 mm in diameter) obtained from the growing edge of 10-days-old isolates of each *Monosporascus* spp. were transferred to the center of PDA plates containing the different fungicide concentration combinations and were incubated at 28 °C in darkness during 7 days. A completely

randomized experimental design was used with five replicates per fungicide, concentration and *Monosporascus* spp. Colony diameters (cm) were measured in two perpendicular directions. The experiment was conducted twice. A preliminary ANOVA was performed to determine whether there were significant differences between the two repetitions of the experiment and whether the data could be combined. The TableCurve 2D v. 5.01 (Systat Software, Inc., San Jose, CA, USA) was used to determine the EC_{50} of PGI for each fungicide and *Monosporascus* spp. combination, using a four-, and three-parameter logistic model by plotting Probit transformed values of fungicide concentration and PGI, respectively.

3 RESULTS

3.1 Effect of pH on Mycelial Growth of *Monosporascus* spp.

There was no significant effect of the experiment repetitions (ANOVA, $p > 0.05$), thus the data were combined. The cubic polynomial regression ($y = a + bx + cx^2 + dx^3$) selected to describe the mycelial growth at different pH levels, adjusted MGR data with $R^2 > 0.95$ for all *Monosporascus* spp. isolates (Figure 1). All *Monosporascus* spp. grew at all pH levels. O_{pH} for mycelial growth varied between 5.72 (*M. caatinguensis*) and 8.05 (*M. cannonballus*). *Monosporascus brasiliensis*, *M. mossoroensis*, *M. nordestinus*, and *M. semiaridus* had optimal pH values of 7.53, 7.99, 6.52 and 6.85, respectively.

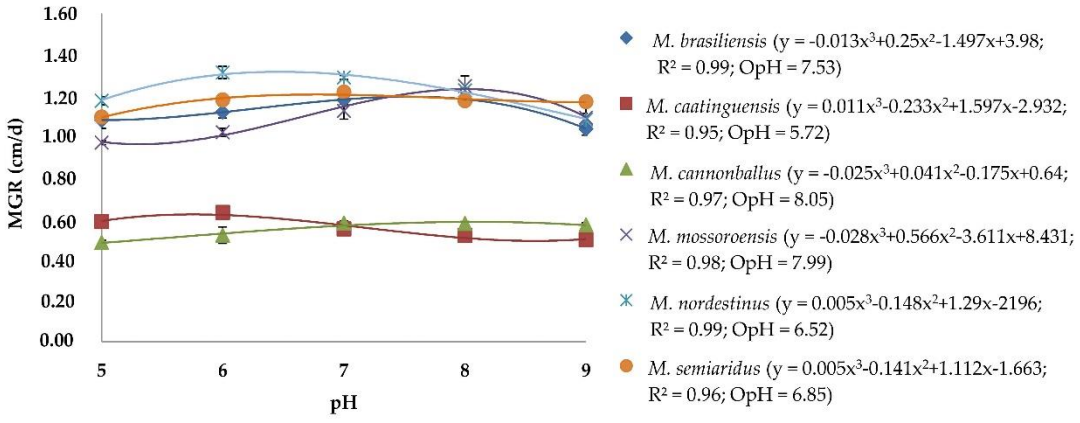


Figure 1. Regression equation, coefficient of determination (R^2) and optimal pH for mycelial growth (O_{pH}) of *Monosporascus* spp. isolates. y = adjusted with the values of the mycelial growth rate (MGR) at pHs 5, 6, 7, 8, and 9. O_{pH} = optimal hydrogen ion potential for mycelial growth of *Monosporascus* spp. calculated from the regression equation.

3.2 Effect of Salinity on Mycelial Growth of *Monosporascus* spp.

There was no significant effect of the experiment repetitions (ANOVA, $p > 0.05$), thus the data were combined. The adjusted means of NaCl concentrations, subjected to a regression analysis, showed significant positive correlations with $R^2 > 0.98$ for all *Monosporascus* spp. isolates (Figure 2). All *Monosporascus* spp. grew in all NaCl concentrations, but this growth was reduced at the highest concentrations evaluated. The salinity concentrations that inhibit 50% of mycelial growth of *Monosporascus* spp. varied between 903.69 (*M. cannonballus*) and 994.41 mM (*M. mossoroensis*). The remaining species, *M. brasiliensis*, *M. caatinguensis*, *M. nordestinus*, and *M. semiaridus*, had EC_{50} values of 971.88, 961.02, 975.49 and 950.64 mM, respectively.

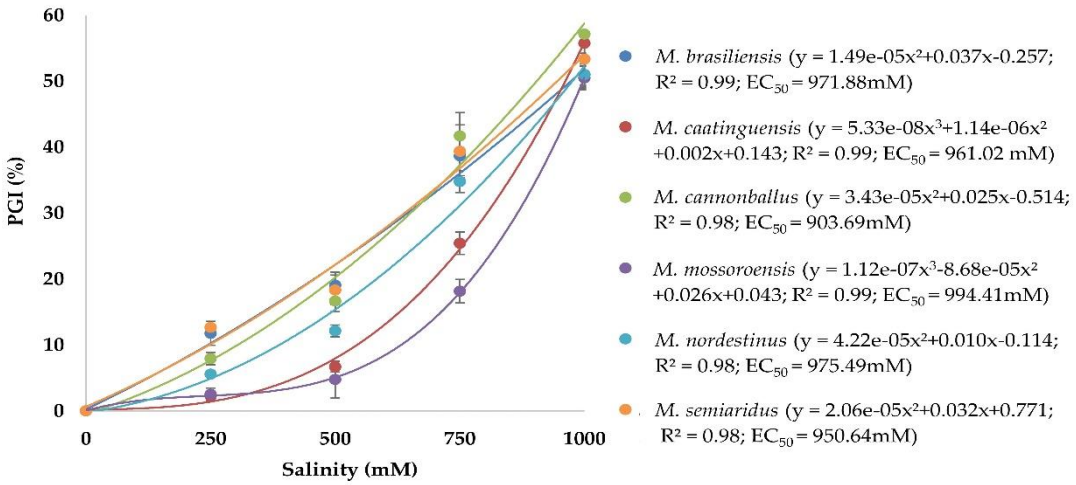


Figure 2. Regression equation, coefficient of determination (R^2) and salinity half-maximal effect concentration (EC_{50}) of *Monosporascus* spp. isolates. y = adjusted with the values of the percentage of growth inhibition (PGI) at NaCl concentrations of 0, 250, 500, 750, and 1000 mM. EC_{50} = salinity concentration that inhibits 50% of mycelial growth of *Monosporascus* spp. calculated from the regression equation (mM).

3.3 Pathogenicity of *Monosporascus* spp. to Cucurbits

There was no significant effect of the experiment repetitions (ANOVA, $p > 0.05$) for all variables, thus the data were combined. The inoculation of cucurbits with *Monosporascus* spp. caused significant statistical effect on disease incidence by *Monosporascus* spp. in cucumber ($\chi^2 = 39.73$; $p < 0.05$), pumpkin ($\chi^2 = 46.64$; $p < 0.05$), melon and watermelon seedlings ($\chi^2 = 69.00$; $p < 0.05$) (Table 2). In melon and watermelon, all the species inoculated

caused a disease incidence of 100%, with all plants infected. In cucumber, the highest incidence (100%) was caused by *M. caatinguensis*, *M. cannonballus* and *M. semiaridus*, while in pumpkin, the highest incidence was caused by *M. caatinguensis*, *M. cannonballus*, *M. mossoroensis* and *M. nordestinus*.

Significant statistical effect was also observed for disease severity in cucumber (χ^2 = 35.87; $p < 0.05$), melon (χ^2 = 32.57; $p < 0.05$), pumpkin (χ^2 = 42.23; $p < 0.05$) and watermelon seedlings (χ^2 = 31.66; $p < 0.05$) (Table 2). In cucumber, the highest mean disease severity (3.30) was caused by *M. semiaridus*, while in melon, *M. brasiliensis*, *M. cannonballus*, *M. mossoroensis*, and *M. semiaridus* produced the highest mean disease severity (1.60). In pumpkin, the highest mean disease severity (1.80) was caused by *M. mossoroensis*, while in watermelon, *M. nordestinus* produced the highest mean disease severity (1.90). Thus, these were considered the most virulent *Monosporascus* spp. to each corresponding cucurbit species. The lowest mean disease severity was caused by *M. mossoroensis* in cucumber (0.40), *M. nordestinus* in melon (1.20), *M. brasiliensis* in pumpkin (0.60), and *M. semiaridus* in watermelon (1.30), thus these were considered the least virulent *Monosporascus* spp. to each corresponding cucurbit species.

Table 2. Incidence and severity of the disease in cucumber, melon, pumpkin, and watermelon seedlings by *Monosporascus* spp.

Treatments	Cucumber				Melon			
	Disease Incidence		Disease Severity		Disease Incidence		Disease Severity	
	Rank [1]	Mean (%)	Rank [1]	Mean (%)	Rank [1]	Mean (%)	Rank [1]	Mean (%)
M. brasiliensis	32.50 ab	60	39.35 bc	2.10	40.50 b	100	41.90 b	1.60
M. caatinguensis	46.50 b	100	37.75 abc	1.30	40.50 b	100	36.75 b	1.30
M. cannonballus	46.50 b	100	42.55 bc	1.60	40.50 b	100	43.45 b	1.60
M. mossoroensis	25.50 ab	40	20.90 ab	0.40	40.50 b	100	43.45 b	1.60
M. nordestinus	39.50 b	80	39.00 bc	1.60	40.50 b	100	34.00 b	1.20
M. semiaridus	46.50 b	100	57.45 c	3.30	40.50 b	100	43.45 b	1.60
Control	11.50 a	0	11.50 a	0.00	5.50 a	0	5.50 a	0.00
χ^2	39.73		35.87		69.00		32.57	
	Pumpkin				Watermelon			
M. brasiliensis	29.50 b	60	25.60 ab	0.60	40.50 b	100	38.00 b	1.70
M. caatinguensis	43.50 b	100	47.65 bc	1.60	40.50 b	100	41.20 b	1.70
M. cannonballus	43.50 b	100	37.00 bc	1.00	40.50 b	100	42.80 b	1.80

M. mossoroensis	43.50 b	100	48.30 bc	1.80	40.50 b	100	44.80 b	1.80
M. nordestinus	43.50 b	100	50.15 c	1.70	40.50 b	100	44.40 b	1.90
M. semiaridus	36.50 b	80	31.30 abc	0.80	40.50 b	100	31.80 b	1.30
Control	8.50 a	0	8.50 a	0.00	5.50 a	0	5.50 a	0.00
χ^2	46.64		42.23		69.00		31.66	

χ^2 = significant chi-square values; values followed by the same letter in the columns do not present statistical difference between them by the non-parametric Kruskal–Wallis test ($p < 0.05$). [1] Average of the ranks for all observations within each sample. Data are mean values of two experiments, each with five replications (pots) per treatment and one plant per replication.

In cucumber, the shorter RL (17.40 cm) was observed in the inoculation with *M. semiaridus*, which also caused the smallest FRW (7.94 g), SL (28.55 cm), FSW (11.94 g) and DSW (2.00 g) (Table 3). For DRW, all species differed statistically from the control. Melon presented shorter RL after the inoculation with *M. cannonballus* (19.44 cm), followed by *M. semiaridus* (19.90 cm) (Table 3). For FRW and DRW, all species differed statistically from the control, and shorter SL was observed the inoculation with *M. mossoroensis* (73.80 cm). The FRW and DSW did not differ statistically, with a coefficient of variation (CV) of 22.66 and 29.03%, respectively. In pumpkin, all species differed statistically from the control to RL and DRW, and the lowest FRW values were obtained in *M. caatinguensis*, *M. cannonballus* and *M. nordestinus* (8.69, 9.47, and 10.11 g, respectively) (Table 3). For SL, a lowest value was observed for *M. cannonballus* inoculation (13.62 cm), which was also observed for FSW (32.00 g). DSW showed the lowest values after the inoculation with *M. cannonballus*, *M. semiaridus* and *M. brasiliensis* (3.40, 3.49, and 3.63 g, respectively). Finally, the shorter RL value in watermelon was observed in the inoculation with *M. brasiliensis* (25.40 cm), which also showed lower FRW with *M. mossoroensis*, *M. caatinguensis* and *M. cannonballus* (4.39, 4.17, 4.01, and 3.94 g, respectively) (Table 3). All species differed statistically from the control to DRW and did not differ from each other to SL, FSW and DSW, with a CV of 31.14, 33.00 and 20.85%, respectively.

Table 3. Effect of *Monosporascus* spp. inoculation on root and shoot length, fresh root and shoot weight, and dry root and shoot weight, of cucumber, melon, pumpkin and watermelon seedlings.

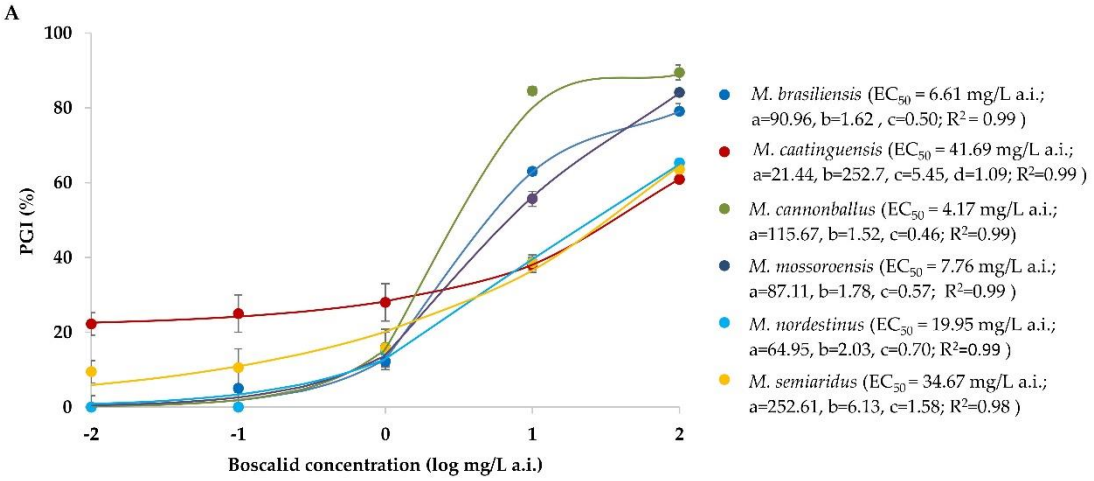
Treatments	RL [1] (cm)	FRW [2] (g)	DRW [3] (g)	SL [4] (cm)	FSW [5] (g)	DSW [6] (g)
			Cucumber			
M. brasiliensis	21.30 c	14.24 ab	0.37 b	46.90 b	27.47 a	2.95 bc
M. caatinguensis	35.40 a	12.65 ab	0.62 b	60.80 a	36.26 a	4.88 a
M. cannonballus	30.80 ab	9.90 bc	0.47 b	64.40 a	31.33 a	4.37 ab
M. mossoroensis	32.08 ab	12.27 abc	0.56 b	62.90 a	33.79 a	4.03 ab
M. nordestinus	24.60 bc	10.50 bc	0.44 b	68.00 a	29.84 a	3.91 ab
M. semiaridus	17.40 c	7.94 c	0.45 b	28.55 c	11.94 b	2.00 c
Control	39.80 a	17.52 a	1.00 a	67.12 a	37.19 a	4.90 a
CV (%)	23.78	27.04	37.67	16.37	28.72	30.39
			Melon			
M. brasiliensis	27.00 ab	3.84 b	0.28 b	97.20 a	46.51 a	6.06 a
M. caatinguensis	25.46 abc	7.08 b	0.36 b	97.22 a	46.73 a	6.86 a
M. cannonballus	19.44 c	4.32 b	0.35 b	89.82 ab	50.60 a	6.49 a
M. mossoroensis	24.70 abc	5.68 b	0.31 b	73.80 b	38.47 a	5.21 a
M. nordestinus	22.90 abc	6.43 b	0.40 b	106.75 a	51.72 a	7.67 a
M. semiaridus	19.90 bc	4.91 b	0.36 b	85.00 ab	47.31 a	5.99 a
Control	28.80 a	12.44 a	1.32 a	108.00 a	43.05 a	6.12 a
CV (%)	22.07	38.89	35.21	18.05	22.66	29.03
			Pumpkin			
M. brasiliensis	36.50 b	11.48 bc	0.67 b	16.10 ab	32.41 b	3.63 c
M. caatinguensis	37.80 b	8.69 c	0.66 b	18.75 a	38.24 ab	4.76 ab
M. cannonballus	35.73 b	9.47 c	0.67 b	13.62 b	32.00 b	3.40 c
M. mossoroensis	36.11 b	10.70 bc	0.69 b	16.76 ab	32.22 b	3.82 bc
M. nordestinus	31.78 b	10.11 c	0.66 b	17.18 ab	36.35 ab	4.23 abc
M. semiaridus	35.80 b	15.11 b	0.81 b	17.80 ab	34.40 b	3.49 c
Control	50.60 a	20.02 a	2.57 a	18.70 a	45.78 a	4.91 a
CV (%)	22.32	26.58	34.20	18.57	21.83	18.81
			Watermelon			

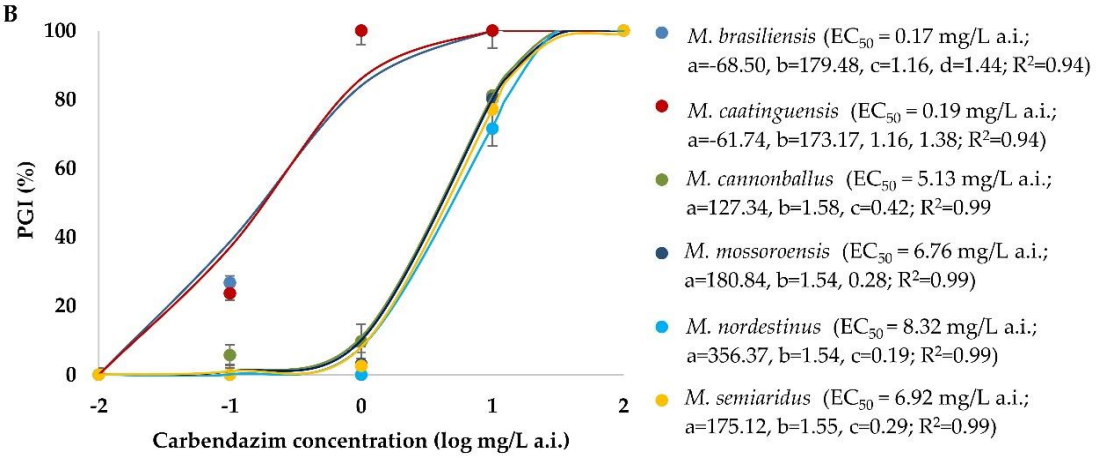
M. brasiliensis	25.40 b	4.39 c	0.30 b	96.90 a	32.72 a	4.55 a
M. caatinguensis	28.96 ab	4.01 c	0.27 b	100.05 a	31.04 a	3.94 a
M. cannonballus	26.50 ab	3.94 c	0.29 b	102.80 a	35.87 a	5.04 a
M. mossoroensis	27.50 ab	4.17 c	0.30 b	109.24 a	36.50 a	4.45 a
M. nordestinus	29.80 ab	7.26 b	0.36 b	120.20 a	38.58 a	4.84 a
M. semiaridus	28.85 ab	5.50 bc	0.38 b	103.70 a	37.48 a	5.20 a
Control	34.11 a	11.67 a	1.57 a	103.08 a	36.44 a	5.25 a
CV (%)	19.62	35.32	36.64	31.14	33.00	20.85

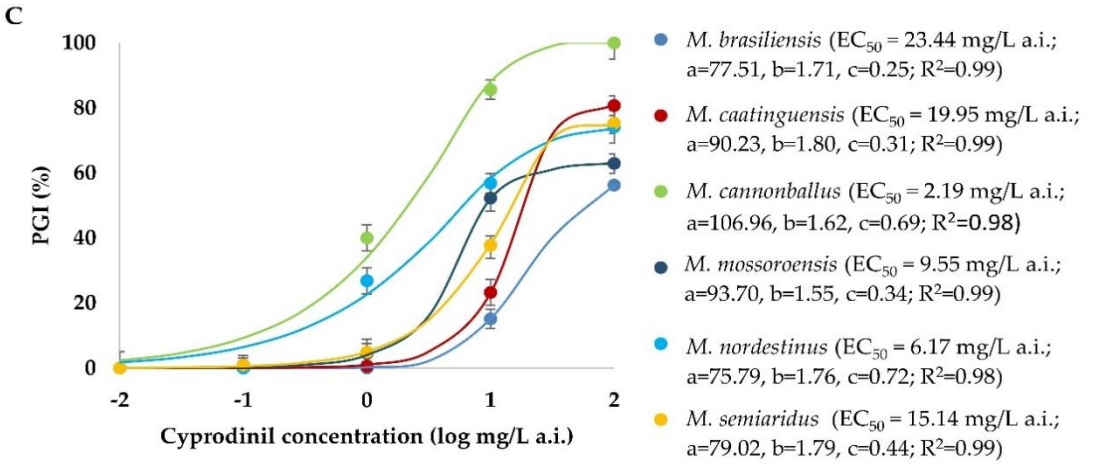
CV (%) = significant coefficient of variation values; values followed by the same letter in the columns do not present statistical difference between them by the Tukey test ($p < 0.05$). Data are mean values of two experiments, each with five replications (pots) per treatment and one plant per replication. [1] Root length. [2] Fresh root weight. [3] Dry root weight. [4] Shoot length. [5] Fresh shoot weight. [6] Dry shoot weight.

3.4 Sensitivity of *Monosporascus* spp. to Fungicides

There was no significant effect of the experiment repetitions (ANOVA, $p > 0.05$) for each fungicide, thus the data were combined. The effects of different fungicides on mycelial growth of *Monosporascus* spp. isolates are shown in Figure 3. Four-parameter logistic equations were adjusted and the EC_{50} values were calculated. The coefficients of determination ranged from 0.83 to 0.99. The mean EC_{50} for boscalid was 19.14 mg/L a.i. and the values of this variable ranged from 4.17 (*M. cannonballus*) to 41.69 mg/L a.i. (*M. caatinguensis*). For carbendazim, the mean EC_{50} was 4.58 mg/L a.i. and the values ranged from 0.17 (*M. brasiliensis*) to 8.32 mg/L a.i. (*M. nordestinus*). For the fungicide cyprodinil, the mean EC_{50} was 12.74 mg/L a.i. and the values ranged from 2.19 (*M. cannonballus*) to 23.44 mg/L a.i. (*M. brasiliensis*). For fluazinam, the mean EC_{50} was 0.34 mg/L a.i. and the values ranged from 0.04 (*M. cannonballus*) to 0.95 mg/L a.i. (*M. brasiliensis*). The mean EC_{50} for fludioxonil was 0.035 mg/L a.i., and the values of this variable ranged from 0.01 (*M. cannonballus*, *M. mossoroensis*, *M. nordestinus*, and *M. semiaridus*) to 0.09 mg/L a.i. (*M. brasiliensis*). Of the fungicides evaluated, those with lower EC_{50} for all *Monosporascus* spp. studied were fludioxonil and fluazinam (0.03 and 0.34 mg/L a.i., respectively).


A
PGI (%)
Boscalid concentration (log mg/L a.i.)
M. brasiliensis (EC50 = 6.61 mg/L a.i.; a=90.96, b=1.62 , c=0.50; R2 = 0.99)
M. caatinguensis (EC50 = 41.69 mg/L a.i.; a=21.44, b=252.7, c=5.45, d=1.09; R2=0.99)
M. cannonballus (EC50 = 4.17 mg/L a.i.; a=115.67, b=1.52, c=0.46; R2=0.99)
M. mossoroensis (EC50 = 7.76 mg/L a.i.; a=87.11, b=1.78, c=0.57; R2=0.99)
M. nordestinus (EC50 = 19.95 mg/L a.i.; a=64.95, b=2.03, c=0.70; R2=0.99)
M. semiaridus (EC50 = 34.67 mg/L a.i.; a=252.61, b=6.13, c=1.58; R2=0.98)


B
PGI (%)
Carbendazim concentration (log mg/L a.i.)
M. brasiliensis (EC50 = 0.17 mg/L a.i.; a=-68.50, b=179.48, c=1.16, d=1.44; R2=0.94)
M. caatinguensis (EC50 = 0.19 mg/L a.i.; a=-61.74, b=173.17, 1.16, 1.38; R2=0.94)
M. cannonballus (EC50 = 5.13 mg/L a.i.; a=127.34, b=1.58, c=0.42; R2=0.99
M. mossoroensis (EC50 = 6.76 mg/L a.i.; a=180.84, b=1.54, 0.28; R2=0.99)
M. nordestinus (EC50 = 8.32 mg/L a.i.; a=356.37, b=1.54, c=0.19; R2=0.99)
M. semiaridus (EC50 = 6.92 mg/L a.i.; a=175.12, b=1.55, c=0.29; R2=0.99)


C
PGI (%)
Cyprodinil concentration (log mg/L a.i.)
M. brasiliensis (EC50 = 23.44 mg/L a.i.; a=77.51, b=1.71, c=0.25; R2=0.99)
M. caatinguensis (EC50 = 19.95 mg/L a.i.; a=90.23, b=1.80, c=0.31; R2=0.99)
M. cannonballus (EC50 = 2.19 mg/L a.i.; a=106.96, b=1.62, c=0.69; R2=0.98)
M. mossoroensis (EC50 = 9.55 mg/L a.i.; a=93.70, b=1.55, c=0.34; R2=0.99)
M. nordestinus (EC50 = 6.17 mg/L a.i.; a=75.79, b=1.76, c=0.72; R2=0.98)
M. semiaridus (EC50 = 15.14 mg/L a.i.; a=79.02, b=1.79, c=0.44; R2=0.99)

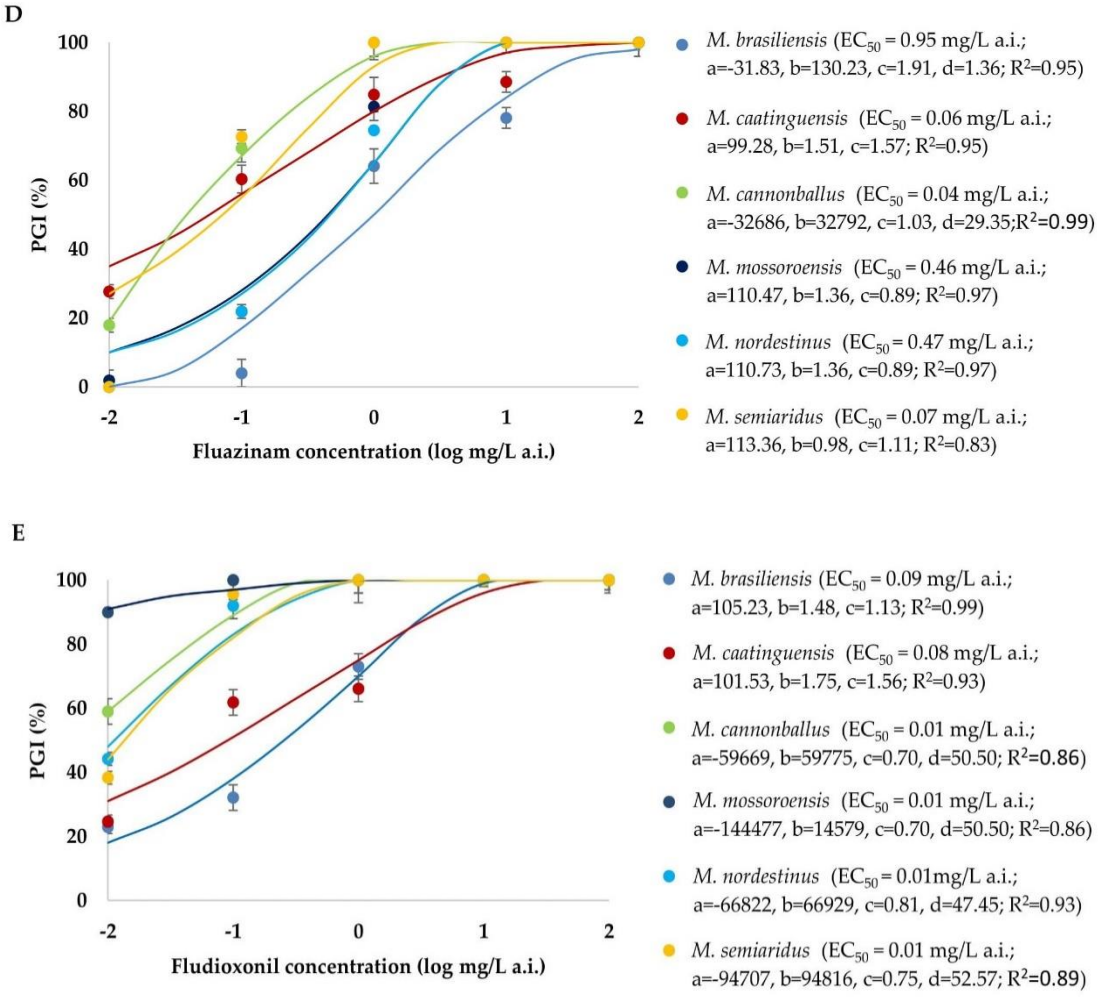


Figure 3. Regression equation, coefficient of determination (R^2) and half-maximal effect concentration (EC_{50}) of each *Monosporascus* spp. for the fungicides (**A**) boscalid, (**B**) carbendazim, (**C**) cyprodinil, (**D**) fluazinam, and (**E**) fludioxonil. y = adjusted with the values of percentage of growth inhibition (PGI) at concentrations of 0.01, 0.1, 1, 10 and 100 mg/L a.i. per fungicide. EC_{50} = 50% mycelial growth inhibition concentration calculated from the regression equation (mg/L).

4 DISCUSSION

The main objective of this research was to obtain new biological information about five recently described *Monosporascus* species, regarding mycelial growth at different pH levels and salinity concentrations, their pathogenicity to cucurbits, and their sensitivity to fungicides with different modes of action.

Our results reveal great variability among the representative isolates of each species included in this study.

The optimal pH for mycelial growth of *Monosporascus* spp. ranged from 5.72 to 8.05, showing a wide range of tolerance, in agreement with previous studies conducted with *M.*

cannonballus (MARTYN & MILLER, 1996; ARMENGOL *et al*., 2011; BEN SALEM *et al*., 2011). These values correspond with those indicated as a suitable soil pH range for cucurbits cultivation (MENDES *et al*., 2008).

The presence of NaCl significantly reduced the in vitro mycelial growth of all *Monosporascus* spp. studied, and although no concentration was able to completely inhibit its growth, EC_{50} was above 900 mM for all species, indicating a moderately high tolerance to this substance. Previous studies have shown that *M. cannonballus* tolerates a high salinity content of NaCl or $CaCl_2$ solutions when evaluated in vitro (MARTYN & MILLER, 1996). Reduction in mycelial growth in vitro by NaCl has also been observed in other cucurbit root fungal pathogens such as *Macrophomina phaseolina* (Tassi) Goid. and *M. pseudophaseolina* Crous, Sarr and Ndiaye (CERVANTES-GARCÍA *et al*., 2003; TIJERINA-RAMÍREZ *et al*., 2014; NEGREIROS *et al*., 2020). The salinity stress is a major environmental constraint in semi-arid cucurbit-growing regions such as northeastern Brazil, where these new *Monosporascus* species were found. Exposure of the fungal cells to saline stress implies both exposure to specific osmotic stress that restricts water availability, and ion toxicity due to their ability to inhibit specific metabolic pathways (THANGAVELU *et al*., 2006).

Monosporascus root rot and vine decline caused by *M. cannonballus* and *M. eutypoides* occurs mainly on melon, watermelon, and cucumber crops, although other cucurbit species have been shown to be susceptible in artificial inoculation studies (MERTELY *et al*., 1993; KWON *et al*., 2001; COHEN *et al*., 2012; BEN SALEM *et al*., 2013; SALES JÚNIOR *et al*., 2018). In a similar way, in our study, the seedlings of all cucurbits evaluated were susceptible to *M. brasiliensis*, *M. caatinguensis*, *M. mossoroensis*, *M. nordestinus*, and *M. semiaridus*. It is interesting to note that to date, these new *Monosporascus* species have only been found as being associated to weed roots in cucurbit cultivation fields (NEGREIROS *et al*., 2019). Although pot experiments cannot be used to predict the results in the field, our results suggest that more attention should be paid to these fungal species as potential cucurbit pathogens. Bruton (1998) indicated that inadequate crop rotation contributes more than other factors to increase the inoculum of cucurbit soilborne pathogens, thus determining the emergence of new diseases or increasing disease incidence and severity of the already existing ones. Moreover, in the specific case of *Monosporascus* spp., Robinson *et al*. (2020) commented that there is no evidence that isolates of *Monosporascus* from the roots of plants in natural ecosystems cause disease symptoms, despite their broad host association, thus raising questions regarding whether presumed endophytic lineages differ from pathogenic lineages with respect to specific genes or groups of genes. These authors compared the

genomes of endophytic and pathogenic isolates within the genus *Monosporascus* and also across genera within the Xylariales with respect to genes for carbohydrate-active enzymes, genes known to be involved in pathogenicity in certain fungi, and genes for effector proteins that facilitate the colonization of plant tissues. Their results show that endophytic *Monosporascus* isolates from New Mexico contain more predicted genes associated with pathogenesis and host plant interactions than their agricultural relatives.

Our pathogenicity tests were conducted using an inoculum density (12 g of the seeds colonized/2 kg of the sterile soil-substrate mixture) lower than the one recommended by Ben Salem *et al*. (2015) (200 g of inoculum/kg of peat). Andrade *et al*. (2005) studied the influence of inoculum density of 44 isolates of *M. cannonballus* on the severity of MRRVD to melon. These authors concluded that low inoculum densities (0.1, 0.5, and 1.0 colony-forming unit (CFU)/g soil) produced high levels of disease, and this severity level did not increase when densities were increased. More recently, Castro *et al*. (2020) evaluated the response of different melon genotypes to inoculation with *M. cannonballus* and *M. eutypoides* in greenhouse experiments in three different years, by using an inoculum dose of 200 g of inoculated wheat seeds/kg of substrate. They found a strong influence of temperature conditions in the different years of experiments on the incidence and severity of the disease caused by these pathogens on melon roots. This could have also influenced our results, because of the high average temperature in the greenhouse (35 °C) and the general thermophilic nature of *Monosporascus* spp. that could have favored root infection (PIVONIA *et al*., 2002; BEN SALEM *et al*., 2013; NEGREIROS *et al*., 2019).

For *M. semiaridus* and *M. brasiliensis* in cucumber, the severity of the disease was highly correlated with a reduction in shoot and root length, and also for fresh and dry weights of roots and shoots, being therefore considered the most aggressive species for this cucurbit species. These variables were previously shown as being useful to evaluate the severity of the disease caused by *M. cannonballus* in cucurbits (BEN SALEM *et al*., 2015; CASTRO *et al*., 2020). Differences in the root system, shoot length and dry weights were also observed in pumpkin, with emphasis on infection by *M. cannonballus*. Sales Júnior *et al*. (2018) studied the reactions of cucurbits such as cucumber, melon, pumpkin and watermelon, after artificial inoculation with *M. cannonballus*. These authors observed that in all cucurbit species, there were root lesions, and it was also possible to observe perithecia of the pathogen. In our experiment, despite the severe damage caused to the root system in melons and watermelons, the dry weight of the plants was not affected, different from what was previously reported by

several authors, and it was not possible to observe perithecia of the pathogens (MERTELY *et al*., 1991; LOVIC *et al*., 1994).

Monosporascus spp. sensitivity to fungicides was measured by EC_{50}, which is specific and constant for a given a.i. and pathogen, and a low EC_{50} value represents a high fungicidal power (REIS *et al*., 2010; TONIN *et al*., 2013). All *Monosporascus* spp. were highly susceptible to fludioxonil and fluazinam fungicides, exhibiting EC_{50} values below 1 mg/L a.i. Fludioxonil and fluazinam also showed good in vitro efficacy against *M. cannonballus* in experiments performed by Pivonia *et al*. (2010). Contact fungicides such as fludioxonil and fluazinam do not have the ability to penetrate the tissues, acting as a barrier that protects the propagating structures from infection, being able to eradicate the pathogens found on the surface, and have been widely used in the management of root pathogens (LEITE & LOPES, 2018). Boscalid, carbendazim and cyprodinil, with systemic modes of action, were moderately fungitoxic to *Monosporascus* spp., according to the criteria proposed by Edgington *et al*. (1971) to frame substances with respect to their fungitoxicity. Boscalid and cyprodinil were moderately fungitoxic to all *Monosporascus* spp., and carbendazim, although moderately fungitoxic to all the species in general, showed high fungitoxicity to *M. brasiliensis* and *M. caatinguensis.*

To date, *M. brasiliensis*, *M. caatinguensis*, *M. mossoroensis*, *M. nordestinus*, and *M. semiaridus* have been only found in northeastern Brazil associated to weeds growing in cucurbit fields. But, managing soil-borne fungal diseases is a matter of understanding complex species interactions with the soil and host plant microbiome, and their response to environmental factors, plant genotypes and different control measures. The findings of this study provide relevant information about the behavior of these new *Monosporascus* spp.

ACKNOWLEDGEMENTS

This study was financed in part by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior —Brazil (CAPES)— Finance Code 001 and, by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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