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Rhizoctonia como patógeno em batata: influência de grupos de anastomose na adaptabilidade e controle com óleos essenciais

Avanor Cidral da Costa Junior

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AVANOR CIDRAL DA COSTA JUNIOR

RHIZOCTONIA COMO PATÓGENO EM BATATA: INFLUÊNCIA DE GRUPOS DE ANASTOMOSE NA ADAPTABILIDADE E CONTROLE COM ÓLEOS ESSENCIAIS

Tese apresentada ao Programa de Pós-Graduação em Fitopatologia da Universidade Federal Rural de Pernambuco, como parte dos requisitos para obtenção do título de Doutor em Fitopatologia.

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AVANOR CIDRAL DA COSTA JUNIOR

COMITÊ DE ORIENTAÇÃO:

Prof. Dr. Sami Jorge Michereff (UFRPE) – Orientador

Prof. Dr. Marco Aurélio Siqueira da Gama (UFRPE) – Coorientador

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ORIENTADOR:

_____

Prof. Dr. Sami Jorge Michereff (UFRPE)

EXAMINADORES:

_____

Prof. Dr. Alexandre Reis Machado (UFPE)

_____

Profa. Dra. Rejane Rodrigues da Costa e Carvalho (UFRPE)

_____

Profa. Dra. Elineide Barbosa de Souza (UFRPE)

_____

Prof. Dr. Delson Laranjeira (UFRPE)

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SUMÁRIO

RESUMO GERAL

A rizoctoniose é uma importante doença da batata no Brasil, causada principalmente pelos grupos de anastomose AG-3 PT, AG-4 HGI e AG-R de *Rhizoctonia*. Este estudo teve como objetivo comparar a adaptabilidade de 15 isolados de cada AG em relação a temperatura, pH, potencial hídrico, salinidade, fungicidas e agressividade em diversas espécies de plantas, bem como selecionar óleos essenciais com atividade antifúngica a isolados pertencentes a AG-3 PT e AG-R. Isolados pertencentes ao mesmo AG não diferiram entre si em relação ao crescimento micelial nos diferentes experimentos. A temperatura ótima para o crescimento de AGR-R (29,5° C) foi superior aos demais e AG-4 HGI apresentou o maior crescimento micelial (96,6 mm). Os níveis de pH testados (5, 6 e 7) não influenciaram no crescimento micelial dos três AGs. AG-4 HGI demonstrou menor sensibilidade à deficiência hídrica, enquanto AG-3 PT apresentou maior sensibilidade à salinidade. Os AGs diferiram de sensibilidade somente em relação ao fungicida fluazinam, com AG-3 PT apresentando a maior redução no crescimento. Todos os isolados induziram sintomas nas 14 espécies de plantas inoculadas e AG-4 HGI foi o mais agressivo. Em todas as situações, os isolados de AG-4 HGI evidenciaram maior potencial adaptativo saprofítico e patogênico que os isolados de AG-3 PT e AG-R. A atividade antifúngica de trinta óleos essenciais de diferentes plantas foi avaliada na inibição do crescimento micelial de dois isolados de *Rhizoctonia* pertencentes aos AG-3 PT e AG-R, respectivamente, pelo método de disco-difusão em ágar. Sete óleos essenciais inibiram completamente o crescimento micelial do fungo, dos quais os óleos de *Mentha rotundifolia*, *Thymus zygis* e *Satureja montana* foram selecionados. GC-MS identificou 26 compostos químicos no óleo essencial de *M. rotundifolia* e *T. zygis* e 14 compostos químicos no óleo essencial de *S. montana*. Óxido de piperitona (36,97%) e piperitona (20,77%) foram os principais componentes no óleo de *M. rotundifolia,* timol (60,27%), óxido de piperitona (6,56%) e piperitona (5,18%) foram os principais componentes no óleo de *T. zygis* e carvacrol (49,38), p-cimeno (28,08) e timol (8,30) foram os principais componentes do óleo de *S. montana.* TBARS revelou maiores valores de ROS (127,4 nmol g^{-1}) e MDA (5,9 nmol g^{-1}) para AGR-R com o tratamento com óleo *de S. montana* a 5 mg mL^{-1} e maiores valores de ROS (131,9 nmol g^{-1}) e MDA (5,4 nmol g^{-1}) para AG-3 PT no tratamento com óleo de *M. rotundifolia* a 5 mg mL^{-1}. Os dados comprovam que os óleos essenciais são promissores no manejo de *Rhizoctonia*.

Palavras-chave: *Rhizoctonia*; *Solanum tuberosum*; grupos de anastomose; adaptabilidade saprofítica; adaptabilidade patogênica; óleos essenciais; biopesticida.

GENERAL ABSTRACT

Rhizoctoniosis is an important potato disease in Brazil, caused mainly by the AG-3 PT, AG-4 HGI and AG-R anastomosis groups of *Rhizoctonia*. The objective of this study was to compare the adaptability of 15 isolates of each AG in relation to temperature, pH, water potential, salinity, fungicides and aggressiveness in several plant species, as well as to select essential oils with antifungal activity in AG-3 isolates PT and AG-R. Isolates belonging to the same AG did not differ in relation to mycelial growth in the different experiments. The optimum temperature for AGR-R (29.5° C) growth was higher than the others and AG-4 HGI had the highest mycelial growth (96.6 mm). The pH levels tested (5, 6 and 7) did not influence the mycelial growth of the three AGs. AG-4 HGI demonstrating less sensitivity to the water deficit, while AG-3 PT presented the highest sensitivity to the salinity. The AGs differed only in relation to the fungicide fluazinam, with AG-3 PT showing the greatest reduction in growth. All isolates induced symptoms in 14 inoculated plant species and AG-4 HGI was the most aggressive. In all situations, AG-4 HGI isolates showed greater saprophytic and pathogenic adaptive potential than AG-3 PT and AG-R isolates. The antifungal activity of 30 essential oils from different plants was evaluated in the inhibition of mycelial growth of two *Rhizoctonia* isolates belonging to AG-3 PT and AG-R, respectively, by disc-diffusion method in agar. Seven essential oils completely inhibited the mycelial growth of the fungus, of which the oils of *Mentha rotundifolia, Thymus zygis* and *Satureja montana* were selected. GC-MS identified 26 chemical compounds in the essential oil of *M. rotundifolia* and *T. zygis* and 14 chemical compounds in *S. montana* essential oil. Piperitone oxide (36.97%) and piperitone (20.77%) were the major components in the oil of *M. rotundifolia*, thymol (60.27%), piperitone oxide (6.56%) and piperitone 18%) were the main components in the oil of *T. zygis* and carvacrol (49,38), p-cymene (28,08) and thymol (8,30) were the main components of *S. montana* oil. TBARS showed higher values of ROS (127.4 nmol g^{-1}) and MDA (5.9 nmol g^{-1}) for AGR-R with *S. montana* oil treatment at 5 mg mL^{-1} and higher ROS values (131.9 nmol g^{-1}) and MDA (5.4 nmol g^{-1}) for AG-3 PT in the treatment with *M. rotundifolia* oil at 5 mg mL^{-1}. The data demonstrate that the essential oils are promising in the management of *Rhizoctonia*.

Keywords: *Rhizoctonia*; *Solanum tuberosum*; anastomosis groups; saprophitic fitness; pathogenic fitness; essential oils; biopesticide.

Capítulo I

Introdução Geral

RHIZOCTONIA COMO PATÓGENO EM BATATA: INFLUÊNCIA DE GRUPOS DE ANASTOMOSE NA ADAPTABILIDADE E CONTROLE COM ÓLEOS ESSENCIAIS

INTRODUÇÃO GERAL

1. A cultura da batata

A batata (*Solanum tuberosum* L.), pertencente à família Solanaceae, é propagada vegetativamente. A nova planta é considerada clone genético da planta mãe, e pode produzir até 20 novos tubérculos. Contudo, as plantas também podem produzir flores e bagas, e conter de 100 a 400 sementes, que ao serem cultivadas, produzirão plantas geneticamente diferentes da planta mãe. O rendimento de um hectare cultivado com batata é de duas a quatro vezes o rendimento da mesma área cultivada com cereais e a eficiência no uso da agua é ate sete vezes maior (CIP, 2018).

A batata teve sua origem nas terras altas do Peru, na região ao redor do lago Titicaca. Sua domesticação aconteceu provavelmente há 7.000 anos, quando comunidades desenvolveram tecnologia para a preservação, ao expor os tubérculos às condições de congelamento seco da montanha, conhecido como "Chuno". Grandes civilizações, como a Huari e Inca, desenvolveram-se tendo a batata como seu alimento principal. Em 1532, à medida que os espanhóis chegavam ao continente americano e o Império Inca era destruído, os espanhóis tomaram o controle das terras e assimilaram os costumes da população nativa, dentre eles, o cultivo da batata. A introdução da batata na Europa ocorreu antes de 1570, pela Espanha. Em 1573, a batata foi incluída na dieta hospitalar em Sevilla e em 1587 deu-se a primeira descrição botânica pelo austríaco Clusius (CHOISEUL; DOHERTY; ROE, 2008).

Do comércio local com a Espanha, o cultivo de batata se espalhou pela Europa. Em diversos países, por questão de segurança alimentar, a batata se tornou a principal planta cultivada (CHOISEUL; DOHERTY; ROE, 2008). Contudo, em 1845 houve um surto de doença, conhecida como "praga da batata", que foi estudada por muitos cientistas, e dentre eles, Anton de Bary, que em 1861 provou que a doença era causada pelo fungo chamado *Phytophthora infestans* (Mont.) de Bary. A doença contaminou em larga escala as plantações de batata por toda a Europa, em especial a Irlanda, no qual sua população dependia

exclusivamente da batata para a alimentação. A fome provocou a morte de cerca de um milhão de pessoas e forçou mais de um milhão a emigrar da ilha (MOURA, 2002). A doença também ocasionou a perda de quase todas as variedades de batata que eram cultivadas. Atualmente, existem mais de 5.000 variedades de batata que ainda são cultivadas nos Andes, de diferentes formatos e tamanhos, e que constituem a principal fonte de diversidade e resistência genética a pragas e doenças (CIP, 2018).

Além da importância histórica, em particular com a Fitopatologia, a cultura da batata possui uma importância econômica. Seu cultivo esta presente em mais de 100 países, sendo que, diariamente um bilhão de pessoas sobrevivem graças ao cultivo de batata (FAO, 2018). A produção mundial de batata ultrapassa os 330 milhões de toneladas anuais, sendo a terceira cultura alimentar mais importante no planeta, atrás apenas do arroz e do trigo. Mais da metade da produção mundial vem de países em desenvolvimento (FAO, 2018).

No Brasil, a produção de batata é estimada em 3,9 milhões de toneladas anuais, sendo que 96,1% da produção está distribuída entre os estados das regiões Sul e Sudeste. Os maiores produtores são Minas Gerais (32,3% do total), Paraná (22,6%), São Paulo (20,5%), Rio Grande do Sul (9,6%), Goiás (5,9%) e Santa Catarina (4,2%) (IBGE, 2018).

Existe um problema político-econômico com a comercialização da batata nacional, e reflete diretamente na produção e geração de empregos. Devido às importações desnecessárias, e, muitas vezes impostas ao Brasil, grande parte da batata consumida é importada (ABBA, 2018). Os dados mostram que 70% da batata palito consumida internamente é importada, quase que exclusivamente da Europa, e apenas 25% do total consumido é produzido no Brasil. A comercialização interna é realizada basicamente *in natura*, onde os atacadistas e varejistas optam pela aparência do tubérculo ao invés da aptidão culinária. Dezenas de variedades são cultivadas, dentre elas, Cupido, Ágata e Asterix. Pela preferência do consumidor brasileiro, 95% da batatas são disponibilizadas na forma lavada (ABBA, 2018).

2. Doenças da batata e rizoctoniose

As doenças são responsáveis por elevadas perdas na produção de batata e mais de uma centena de doenças já foi registrada na cultura. Muitas doenças são devastadoras que, quando não adequadamente controladas, causam a perda total da produção ou afetam a qualidade do produto, cuja aparência é muito valorada pelo consumidor brasileiro. Essas doenças podem

ser de natureza biótica, causadas por fungos, oomicetos, bactérias, vírus e nematoides (doenças transmissíveis), bem como de natureza abiótica ou não transmissíveis, principalmente decorrentes de distúrbios fisiológicos (LIMA; LOPES; REIS, 2015).

No Brasil, muitas doenças bióticas afetam a batata, mas a rizoctoniose, causada por *Rhizoctonia solani* (Kühn) [teleomorfo *Thanatephorus cucumeris* (Donk)] e Rhizoctonia binucleada (BNR) [teleomorfo *Ceratobasidium*] tem destacada importância (DIAS; IAMAUTI; FISCHER, 2016; LIMA; LOPES; REIS, 2015; ZAMBOLIM; VALE; COSTA, 2000).

A rizoctoniose em batata é mais comum da emergência à amontoa, período que se estende dos 25 aos 40 dias após o plantio, e os sintomas são podridões e cancros de caules e raízes, tombamento de pré e pós-emergência, queima, morte de plantas, podridões em tubérculos, manchas, queima das folhas e brotos e formação de escleródios na superfície dos tubérculos, também conhecido como crosta preta (BANVILLE, 1989; LIMA; LOPES; REIS, 2015; REIS; LOPES, 2011; TSROR, 2010).

Os dados sobre o efeito da rizoctoniose no rendimento dos cultivos de batata são limitados, porém são estimadas perdas de 10 a 30% (BANVILLE, 1989; BANVILLE; CARLING; OTRYSKO, 1996; LEHTONEN, 2009; WOODHALL et al., 2007). Além do ataque direto do patógeno à planta, causando morte de brotos, folhas, desuniformidade de crescimento e redução na produção, as perdas podem ser qualitativas, como produção de tubérculos pequenos e deformados, com aspecto de sarna, fatores estes que prejudicam a comercialização (BANVILLE, 1989; TSROR, 2010; YANG et al., 2015a).

Rhizoctonia habita naturalmente os solos e possui uma ampla gama de plantas hospedeiras, formada por mais de 500 espécies, distribuídas entre as famílias *Asteraceae, Brassicaceae, Fabaceae, Poaceae, Solanaceae,* bem como plantas ornamentais e florestais (OGOSHI, 1987).

Como saprófito, *Rhizoctonia* possui uma grande habilidade competitiva e pode sobreviver por vários anos na forma de escleródios ou hifas com paredes espessas melanizadas, em tubérculos, restos culturais e no solo (BANVILLE, 1989; TSROR, 2010). O tubérculo de batata infectado com escleródios ou micélio pode disseminar o fungo a longas distâncias (BANVILLE, 1989; MUZHINJI et al., 2015). No solo, os propágulos não são distribuídos uniformemente e são encontrados em baixas densidades, variando de 0,1 a 63 propágulos/g de solo (OGOSHI, 1996). *Rhizoctonia* também possui a capacidade de utilizar outras fontes de carbono quando os recursos são limitados, como celulose, no qual é raramente aproveitada por outros microrganismos (DEACON, 1996).

Em condições favoráveis, como alta umidade relativa do ar e do solo, solos argilosos e mal drenados, matéria orgânica não decomposta, excesso de adubação nitrogenada, plantios profundos e presença de exsudatos radiculares liberados pelo sistema radicular de plantas hospedeiras, os escleródios germinam e atacam o tubérculo, caule e brotações da planta. O processo infeccioso é iniciado após o contato primário com a superfície da planta, no qual as hifas em crescimento direcional as células epidérmicas, formam almofadas de infecção, que se fixam fortemente a epiderme do hospedeiro (KEIJER, 1996). Ferimentos auxiliam a penetração ativa do fungo (KEIJER, 1996; REIS; LOPES, 2011). Por outro lado, a penetração passiva é limitada a infecções foliares e ocorre raramente (KEIJER, 1996; WEINHOLD; SINCLAIR, 1996). O processo de penetração envolve enzimas, como cutinases, pectinases e xilanases (WEINHOLD; SINCLAIR, 1996) e pressão hidrostática (DEMIRCI; DÖKEN, 1998). Internamente nas células vegetais, as hifas crescem e degradam tecidos celulares, causando morte das células e formação de lesões necróticas no tecido epidérmico de folhas, caule, raízes e estolões (DEMIRCI; DÖKEN, 1998).

O controle de rizoctoniose em batata é muito difícil, pois o patógeno sobrevive no solo, em tubérculos e restos culturais sob a forma de hifas ou escleródios, possui ampla gama de plantas hospedeiras, elevada agressividade e grande diversidade genética (TSROR, 2010; MUZHINJI et al., 2015). Além disso, cultivares de batata com níveis aceitáveis de resistência à doença não são disponíveis (Tsror 2010) e a eficácia dos fungicidas disponíveis é variável (CAMPION et al., 2003; LEHTONEN et al., 2008; ÖZER; BAYRAKTAR, 2015).

3. *Rhizoctonia* e grupos de anastomose

Rhizoctonia é um grupo heterogêneo de fungos filamentosos, que não produzem esporos assexuais e que possuem características comuns em seu estado anamórfico, como: ramificação em ângulo reto próximo ao septo distal em hifas jovens, presença de um septo na ramificação da hifa próximo do seu ponto de origem, presença de septos do tipo doliporo, ramificações de hifas que são concêntricas em sua extremidade basal, ausência de grampos de conexão, ausência de conídios, tecido esclerocial não diferenciado em membrana, córtex e medula, ausência de rizomorfas (GARCÍA; ONCO; SUSAN, 2006; MOORE, 1996; OGOSHI, 1987, 1996).

O gênero *Rhizoctonia* foi proposto por De Candolle, em 1815, e revisado por Parmeter e Whitney em 1970 (GARCÍA; ONCO; SUSAN, 2006). Esse gênero é composto por espécies

binucleadas (*R. callae* E. Castell, *R. cerealis* Van Der Hoeven, *R. endophytica* Saksena & Vaartaja, *R. fragariae* S. Husain & W.E. McKeen, *R. fumigata* S. Gunnell & R.K. Webster, *R. ramicola* W.A. Weber & D.A. Roberts, *R. oryzae-sativae* (Sawada) Mordue, *R. repens* Bernard e *R. anaticula* Currah) e multinucleadas (*R. oryzae* Ryker e Gooch, *R. solani* Kühn e *R. zeae* Voorhees) (SNEH et al., 1996). Os teleomorfos de *Rhizoctonia* pertencem o filo Basidiomycota, classe Agaricomycetes, tendo *Thanatephorus* Donk., *Ceratobasidium* Rogers, *Waitea* & Talbot e *Tulasnella* Schröter, os principais teleomorfos (GARCÍA; ONCO; SUSAN, 2006).

Rhizoctonia solani [teleomorfo = *Thanatephorus cucumeris* Frank (Donk)] é a espécie mais importante, e foi descrita por Julius Kühn em 1858 (ANDERSON, 1982; OGOSHI, 1987). Os isolados de *R. solani* diferem em características fenotípicas e genotípicas, e tradicionalmente têm sido organizados em grupos geneticamente relacionados baseados na capacidade da hifa de sofrer anastomose (AGs) com isolados testadores, além de sintomas do hospedeiro, distribuição geográfica, padrões bioquímicos, composição de ácidos graxos e sequência de DNA nuclear (CARLING; KUNINAGA; BRAINARD, 2002; CUBETA; VILGALYS; GONZALEZ, 1996; GONZÁLEZ; CUBETA; VILGALYS, 2006; OGOSHI, 1987). Alguns AGs têm sido divididos em subgrupos, denominados de grupos intraespecíficos (ISGs), baseados em características aditivas à reação de anastomose, como patogenicidade, morfologia, requerimento de vitaminas, taxa de crescimento micelial, temperatura ótima de crescimento, tipo de escleródios produzidos e gama de hospedeiros (GARCÍA; ONCO; SUSAN, 2006; OGOSHI, 1987; SHARON et al., 2007; TSROR, 2010). Até o momento foram identificados 14 AGs (AG-1 a AG-13, e AG-BI) (AJAYI-OYETUNDE; BRADLEY, 2018; CARLING; KUNINAGA; BRAINARD, 2002; CUBETA; VILGALYS; GONZALEZ, 1996; GARCÍA; ONCO; SUSAN, 2006; SHARON et al., 2008; YANG et al., 2017). Em *R.solani* são relatados quatro ISGs dentro do AG-1 (AG-1-IA, AG-1-IB, AG-1-IC e AG-1-ID), oito dentro do AG-2 (AG-2-1, AG-2-2-IIIB, AG-2-2-IV, AG-2-2-HB, AG-2-2-LP, AG-2-3, AG-2-4, AG-2-BI), dois dentro do AG-3 (PT e TB), três dentro do AG-4 (HG-I, HG-II e HG-III), dois dentro do AG-6 (HG-I e GV) e dois dentro do AG-9 (TP e TX) (CERESINI, 2014; SHARON et al., 2007, 2008; YANG; LI, 2012; YANG et al., 2015b, 2017).

Até o momento foram descritos 23 AGs de *Rhizoctonia* binucleada (BNR) (AG-A a AG-W) (YANG et al., 2015b), porém apenas 17 AGs são reconhecidos (AG-A a AG-V) (DONG et al., 2017). São relacionados três ISGs dentro de AG-B (AG-Ba, AG-Bb, AG-B(o)) e três dentro de AG-D (I, II e III) (YANG; LI, 2012; YANG et al., 2017). Os AGs foram

propostos para representar uma unidade evolucionaria independente ou filoespécies, dentro do complexo de espécies de *R. solani* (ANDERSON, 1982; GARCÍA; ONCO; SUSAN, 2006; VILGALYS; CUBETA, 1994). Cada AG representa populações geneticamente isoladas e não intercruzáveis (ANDERSON, 1982).

Tradicionalmente, os AGs de *Rhizoctonia* são atribuídos pela determinação da capacidade das hifas de sofrerem anastomose com isolados testadores. Os isolados pertencentes ao mesmo AG realizam fusão de hifas, enquanto isolados de diferentes AG não sofrem anastomose (CARLING, 1996; SNEH; BURPEE; OGOSHI, 1991). No entanto, essa metodologia é demorada e tem alguns problemas, como em situações onde é impossível determinar o AG do isolado, uma vez que o isolado não apresenta afinidade para anastomose com o isolado testador, o isolado perde a capacidade de sofrer anastomose ou quando o isolado possui a afinidade de anastomose com isolados de outros AGs (SHARON et al., 2006).

É de extrema importância a determinação dos AGs dos isolados com precisão (PATIL; SOLANKI, 2016) e várias ferramentas moleculares têm sido empregadas para melhorar a identificação dos AGs e ISGs de *Rhizoctonia* (AGARWAL, 2010; GARCÍA; ONCO; SUSAN, 2006; SHARON et al., 2006). Nesse contexto, a análise filogenética da região espaçadora transcrita interna (ITS) do DNA ribossômico (rDNA) é a técnica mais utilizada e possibilita a diferenciação da maioria dos AGs e ISGs (AGARWAL, 2010; ARAKAWA; INAGAKI, 2014; CERESINI, 2014; CUBETA; VILGALYS, 2000; DAS et al., 2014; GARCÍA; ONCO; SUSAN, 2006; KUNINAGA, 2002; LÜBECK, 2004; MUZHINJI et al., 2015; PATIL; SOLANKI, 2016; SHARON et al., 2006, 2008).

Diversos AGs têm sido associados à rizoctoniose da batata em nível mundial, com predomínio de AG-3 PT (TSROR, 2010). No Brasil, poucos trabalhos sobre a diversidade de AGs de *Rhizoctonia* em batata foram publicados. Em um trabalho pioneiro, foram avaliados 10 isolados obtidos no estado de São Paulo e dois obtidos no estado de Goiás. Pelo pareamento de hifas com isolados testadores, 10 isolados foram classificados como AG-3 e dois isolados como AG-4 (BOLKAN; RIBEIRO, 1985). Posteriormente, 16 isolados de *Rhizoctonia* obtidos em um campo no estado do Paraná foram caracterizados pelo pareamento com isolados testadores e análise de sequências ITS, sendo classificados como AG-4 HGI, AG-4 HGII e AG-7, respectivamente com sete, sete e dois isolados (ROSA et al., 2005). Recentemente, 66 isolados de *Rhizoctonia* obtidos nas principais regiões produtoras de batata do Brasil tiveram os AGs caracterizados pela análise de sequências ITS. Vinte e cinco isolados (37,3%) foram classificados como AG-3 PT, 20 isolados (29,9%) como AG-4 HGI,

15 isolados (22,4%) como AG-R, três (4,5%) isolados como AG-2-1, três (4,5%) isolados como AG-A, e um isolado (1,5%) como AG-1 IB (INOKUTI, 2016).

O conhecimento dos grupos e subgrupos de anastomose tem grande importância no entendimento da diversidade genética de *Rhizoctonia* e das possíveis influências epidemiológicas dessa diversidade e para a implementação de medidas de controle das doenças (AGARWAL, 2010; AJAYI-OYETUNDE; BRADLEY, 2018; ANDERSON, 1982; OGOSHI, 1987; GODOY-LUTZ et al., 2008; SHARMA-POUDYAL et al., 2015; TSROR, 2010). Isolados de diferentes AGs podem reagir de maneira diferente às medidas de controle (CAMPION et al., 2003; KATARIA; GISI, 1999; ÖZER; BAYRAKTAR, 2015).

4. Coevolução e adaptabilidade de fungos fitopatogênicos

Os ecossistemas naturais são complexos e diversificados, e a diversidade é verificada em todos os aspectos, em especial, ao genético. Os ecossistemas agrícolas ou agroecossistemas, baseados no modelo de agricultura convencional, têm modificado as interações, visando simplificar e homogeneizar o que naturalmente é diversificado. A agricultura convencional tem contribuído para a perda da diversidade genética em agroecossistemas, devido à substituição de variedades locais por genótipos melhorados; pelo uso exagerado de pesticidas e em consequência a indução de resistência em populações de patógenos, e pelo uso da mecanização em larga escala, do plantio a colheita, que contribui para a perda da estrutura e fertilidade dos solos (MIKABERIDZE; MCDONALD, 2015).

No mesmo sentido que os agroecossistemas modernos altamente mecanizados são fábricas extremamente produtivas de alimentos, são também incubadoras altamente eficazes de evolução de patógenos (STUKENBROCK; MCDONALD, 2008). Como resultado de tecnologias agrícolas como o aumento da densidade de plantio e da uniformidade genética das plantas (hospedeiro), o tamanho da população de patógenos aumentou, gerando maior diversidade genética para a seleção natural atuar, maior número de mutações disponíveis na escala de tempo, ao passo que é reduzido os efeitos da deriva genética. Essas mudanças têm contribuído para o potencial evolutivo de patógenos agrícolas, tornando-os domesticados e adaptados ao ambiente do agroecossistema (MIKABERIDZE; MCDONALD, 2015).

Na patologia de plantas, uma raça é definida pela virulência de um isolado de patógeno em cultivares diferenciais da mesma espécie hospedeira (MILGROOM, 2015). O reconhecimento de que nem todos os indivíduos da mesma espécie do patógeno são

igualmente virulentos para o mesmo genótipo do hospedeiro significa que a variação genética da população de patógenos pode ter um impacto significativo no manejo da doença e na quebra da resistência genética (MILGROOM, 2015; MIKABERIDZE; MCDONALD, 2015). Esse impacto ficou evidente quando cultivares resistentes perderam a eficácia por causa da evolução de novas raças virulentas. A ruptura repentina da resistência nas culturas agrícolas causada pelo surgimento de raças virulentas motivou uma compreensão mais ampla da evolução dos patógenos das plantas e coevolução entre hospedeiros e patógenos (MILGROOM, 2015).

Coevolução é um termo utilizado para designar uma mudança na composição genética de uma espécie (ou grupo) em resposta a uma mudança genética em outra (JANZEN, 1980). De forma axiomática, na interação parasítica entre fungo e planta, hospedeiros e patógenos estão em constante corrida armamentista, de um lado a genética de resistência e de outro a da virulência. Populações de plantas são polimorfas para a resistência e os patógenos são polimorfos para a virulência. O polimorfismo é mantido pelos contínuos ciclos de coevolução patógeno-hospedeiro, somado o efeito ocasional do fluxo gênico de novos genes de resistência e virulência vindos de populações distantes (MACKEY, 1986).

Três fatores que precisam ocorrer para haver coevolução no sistema patógeno-planta: i) existência de variação genética para a resistência e virulência na população de hospedeiros e patógenos, repectivamente (a variação fenotípica é relevante, uma vez que a coevolução é conduzida pela seleção); ii) efeitos recíprocos da resistência sob a adaptabilidade do patógeno e da virulência sob a adaptabilidade do hospedeiro; iii) o resultado de qualquer interação depende da combinação dos genótipos dos hospedeiros e genótipos dos patógenos (WOOLHOUSE et al., 2002).

A seleção natural é uma força poderosa que age na evolução dos organismos e ocorre por conta da quantidade de descendentes capazes de sobreviver. Esta sobrevivência não é aleatória, sendo muitas vezes dependente da adaptabilidade (ou fitness", em inglês) dos indivíduos (MILGROOM, 2015). A adaptabilidade biológica de um patógeno de planta é descrita como a habilidade relativa para persistir em um ambiente por um longo período de tempo (NELSON, 1979), sendo constituída de adaptabilidade saprofítica e adaptabilidade patogênica (HARTEVELD et al., 2014). A combinação desses atributos leva a um incremento na reprodução e sobrevivência pelo crescimento micelial, esporulação, patogenicidade e agressividade dos fungos fitopatogênicos (LEACH et al., 2001; PRINGLE; TAYLOR, 2002).

A capacidade competitiva e a adaptabilidade relativa de um isolado ou espécie de patógeno de planta são determinadas por suas propriedades biológicas intrínsecas, pela

resistência e heterogeneidade da população hospedeira, pela densidade populacional e relação genética dos isolados concorrentes, e pelo ambiente físico (ZHAN; MCDONALD, 2013).

A capacidade competitiva pode ser inferida indiretamente por componentes de adaptabilidade. Como a adaptabilidade é relativa, deve ser estimada pela mensuração de caracteres que propiciam alguma vantagem adaptativa entre os indivíduos. Vários componentes de adaptabilidade podem ser mensurados em patógenos de plantas, dependendo da sua biologia e da facilidade de obtenção por métodos experimentais (MILGROOM, 2015). Marcadores fenotípicos, como taxa de crescimento micelial, potencial reprodutivo, sensibilidade a fungicidas e agressividade tem sido úteis para avaliar a adaptabilidade em patógenos das plantas (ANTONOVICS; ALEXANDER, 1989; ALLEN; LENNÉ; WALLER, 1999; BROWN, 2006; LANNOU, 2012; MILGROOM, 2015).

A análise da adaptabilidade pode fornecer informações valiosas sobre como as populações de fungos fitopatogênicos evoluem em resposta à perturbação ou estresse (ZHAN; MCDONALD, 2013). Apesar da importância da rizoctoniose da batata em nível mundial, até o momento não foi comparada a adaptabilidade dos diferentes AGs de *Rhizoctonia* associadas à doença, o que pode gerar informações importantes para o desenvolvimento de estratégias de manejo da doença.

4. Óleos essenciais como alternativa no manejo de fungos fitopatogênicos

Em diversos cultivos agrícolas, os fungos são considerados os mais destrutivos fitopatógenos e causam expressivas perdas econômicas. Dentro desse grupo de patógenos, estão aqueles que causam doenças radiculares e têm recebido menos atenção que patógenos foliares, pela dificuldade de controle e pela complexidade das relações patógeno-hospedeiro-ambiente (MICHEREFF et al., 2001). Os fungos que causam doenças radiculares passam a maior parte do seu ciclo de vida no solo, infectam órgãos subterrâneos ou caules das plantas, possuem uma ampla gama de plantas hospedeiras; podem sobreviver no solo por longos períodos de tempo na ausência de hospedeiros, como saprófitos, com elevada capacidade de competição, e, basicamente, seus estádios de disseminação e sobrevivência são confinados no solo, embora alguns produzam esporos que são disseminados pelo ar ou água. Muitos fungos que habitam naturalmente os solos produzem estruturas como agregados miceliais, esclerócios, oósporos, clamidósporos ou outros tipos de esporos, que resistem às condições

ambientais adversas e permanecem viáveis quando as plantas hospedeiras não estão presentes (AGRIOS, 2005; HILLOCKS; WALLER, 1997).

A eficácia do controle químico às doenças fúngicas, em especial, aos patógenos que sobrevivem nos solos, é variável, e a má gestão do uso de pesticidas tem levado a sérios problemas de saúde pública e ambiental (SALER JR. et al., 2005).

Ao longo dos últimos 50 anos, o manejo integrado de pragas e doenças, visto como a principal estratégia holística global para a proteção fitossanitária tem incentivado a busca por alternativas de controle de doenças, cujo principal objetivo tem sido a redução do uso de pesticidas. A busca por biopesticidas despertou muito interesse da comunidade científica devido à expansão da agricultura orgânica, regulamentos mais restritivos aos pesticidas químicos e à demanda por produtos mais saudáveis e seguros (POPP; PETŐ; NAGY, 2013).

Os óleos essenciais (OEs), incluídos no grupo dos biopesticidas de origem botânica, são misturas complexas de voláteis, principalmente produtos do metabolismo secundário das plantas, que compreendem terpenos (principalmente mono-, sesqui e alguns diterpenos) e compostos fenólicos (fenilpropanóides), embora outros grupos de compostos também possam ocorrer em quantidades relevantes (ARYA; PERELLÓ, 2010; EL-MOHAMEDY, 2017). Esses voláteis possuem componentes aromáticos que fornecem odor, sabor ou aroma, distintos de cada planta e fazem parte do mecanismo de defesa da planta para o ataque de micro-organismos. A maioria das espécies de plantas tem 1 a 2% de EOs, mas em algumas espécies esse valor pode atingir 10%, como em manjericão (*Ocimum basilicum* L.) (LAWERENCE; REYNOLDS, 2001).

Os benefícios dos OEs como pesticidas são reconhecidos há milhares de anos, antes mesmo, dos benefícios à saúde humana. Há relatos do uso de OEs como pesticidas na história chinesa, grega, romana e indiana, e muitas relacionadas ao uso do azeite de neem (*Azadirachta indica* A. Juss.) (EL-MOHAMEDY, 2017). A atividade antifúngica dos OEs é atribuída pelos seus componentes, como carvacrol, acetato alfa-terpineol, cimene, timol, pinene e linalol, dos quais a atividade antimicrobiana é bem conhecida (ANDRÉS et al., 2012; EL-MOHAMEDY, 2017). Na literatura são descritos OEs de plantas das famílias Asteraceae, Liliaceae, Apocynaceae, Solanaceae, Caesalpiniaceae, Rutaceae, Piperaceae, Sapotaceae, com atividade antifúngica. EOs de plantas da família *Lamiaceae* apresentaram resultados positivos no controle de diversos fungos fitopatogênicos. Os OEs de orégano (*Oreganum vulgare* L.) e tomilho (*Thymus vulgaris* L.) demonstraram efetividade sobre vários fungos fitopatogênicos habitantes do solo, incluindo *Fusarium oxysporum* (Schlecht.) Snyder & Hansen, *F. solani* (Mart.) Sacc., *Macrophomina phaseolina* (Tassi) Goid., *R. solani*, *Sclerotinia sclerotiorum*

(Lib.) de Bary e *Sclerotium rolfsii* Sacc. (EL-MOHAMEDY, 2017). Em adição, os OEs de *Bunium persicum* (Boiss.) B. Fedtsch. (KHALEDI; TAHERI; TARIGHI, 2015), *Calocedrus macrolepis* var. *formosana* Florin (CHANG et al., 2008), *Cinnamomum zeylanicum* Breyne (SEEMA, M.; DEVAKI, 2010) *Salvia fruticosa* Mill. (PITAROKILI et al., 2003), *Mentha piperita* L. (KHALEDI; TAHERI; TARIGHI, 2015; ZAMBONELLI et al., 2004) e *Monarda fistulosa* L. (GWINN et al., 2010) mostraram atividade inibitória sobre *R. solani*.

As espécies multinucleadas de *Rhizoctonia solani* e as espécies binucleadas de *Rhizoctonia* sp. causam sérios sintomas de rizoctoniose em muitas espécies de plantas e em especial a batata (*Solanum tuberosum*). O fungo é de difícil controle e o complexo de espécies possui elevada variabilidade genética. Poucos trabalhos estão presentes na literatura sobre o comportamento de espécies multinucleadas e binucleadas de *Rhizoctonia* em batata frente às variáveis condições ambientais, dos quais, quando mensuradas podem proporcionar informações importantes sobre a adaptabilidade evolutiva do fungo e direcionar as medidas de controle. Na busca por medidas de controle mais eficientes, a obtenção de óleos essenciais pode representar uma potencial ferramenta de proteção fitossanitária. Essa tese teve o objetivo de analisar a adaptabilidade saprofítica e patogênica dos principais AGs de *Rhizoctonia* associadas à batata no Brasil e investigar a atividade antifúngica de óleos essenciais ao fungo.

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Capítulo II

Comparative adaptability of *Rhizoctonia* anastomosis groups associated with stem canker and black scurf of potatoes in Brazil

Comparative adaptability of *Rhizoctonia* anastomosis groups associated with stem canker and black scurf of potatoes in Brazil

Avanor C. Costa Junior[1] • Gleiciellen S. R. Santos[1] • Fábio J. A. Silva[1] • Gabriel S. Michereff[1] • Lucas C. S. Amâncio[1] • Ailton Reis[2] • Sami J. Michereff[1]

[1]Department of Agronomy, Federal Rural University of Pernambuco, Recife, PE 52171-900, Brazil

[2]Laboratório de Fitopatologia, Embrapa Hortaliças, Brasília, DF 70359-970, Brazil

✉ Sami. J. Michereff

smichereff@gmail.com

ORCID

Sami J. Michereff

http://orcid.org/0000-0002-2156-3502

Abstract

Rhizoctoniosis is an important potato disease in Brazil, caused mainly by the AG-3 PT, AG-4 HGI and AG-R anastomosis groups of *Rhizoctonia*. This study aimed to compare the saprophytic adaptability of 15 isolates of these AGs in relation to mycelial growth under

different conditions of temperature, pH, water potential, salinity and fungicides, as well as pathogenic adaptability in relation to aggressiveness in several plant species. The optimum temperature for AGR-R (29.5° C) growth was higher than the others and AG-4 HGI had the highest mycelial growth (96.6 mm). The pH levels tested (5, 6 and 7) did not influence the mycelial growth of the three GAs, as well as the interaction between AGs and pH levels was not significant. For the three AGs the mycelial growth reduced with decreasing water potential. AG-4 HGI and AG-R showed the highest growths at -0.8 MPa, while the former also stood at -1.6 and -3.2 MPa. No isolates of *Rhizoctonia* grew at 6 and 8% salinity. There was a significant difference in the mycelial growth between the AGs and between the salinity levels. AG-3 PT showed the lowest mycelial growth in the presence of salinity (43.6 mm) and the growth of the isolates reduced significantly with the elevation of the salinity level. The isolates of the three AGs showed a reduction in mycelial growth in the presence of fungicides Fluazinam, Fludioxonil, Pencicurom and Procymidone, but differed in sensitivity only in relation to Fluazinam, with AG-3 PT showing the greatest reduction of growth (95.9%). All isolates induced symptoms in the 14 inoculated plant species and AG-4 HGI was the most aggressive, except in castor bean, which did not differ from AG-R. In all analyzed situations, the AG-4 HGI isolates showed a greater saprophytic and pathogenic adaptive potential than the AG-3 PT and AG-R isolates.

Keywords: *Rhizoctonia solani;* Binucleated *Rhizoctonia*; *Solanum tuberosum*; temperature; water potential; aggressiveness.

Introduction

Potato (*Solanum tuberosum*) is the third most important food crop in the world, with production exceeding 330 million tonnes per year (FAO 2018). In Brazil, production is estimated at 3.9 million tons per year, 96.1% of production distributed between the states of the South and Southeast regions (IBGE 2018).

In Brazil, many diseases affect potato, but rhizoctoniosis, caused by the fungus *Rhizoctonia* spp., has a significant importance (Lima et al. 2015; Dias et al. 2016). Data on yield losses in potato due to rhizoctoniosis are limited worldwide but are estimated to be up to 30% of production (Banville et al. 1996; Woodhall et al. 2007).

Potato rhizoctoniosis is more common from emergence to heap, a period extending from 25 to 40 days after planting, and the symptoms are rot and stem and root cancers, pre and post-emergence tipping, burning and plant death, tuber rot, spotting and burning of leaves and shoots and formation of sclerotia on the surface of tubers, also known as black crust (Banville et al. 1996; Tsror 2010; Lima et al. 2015). The control of rhizoctoniosis in potatoes is very difficult, because the pathogen survives in the soil, in tubers and cultural remains in the form of hyphae or sclerotia, it has a wide range of host plants, high aggressiveness and great genetic diversity (Tsror 2010; Muzhinji et al. 2015). In addition, potato cultivars with acceptable levels of disease resistance are not available (Tsror 2010) and the efficacy of available fungicides is variable (Campion et al. 2003; Lehtonen et al. 2008; Özer & Bayraktar 2015).

The genus *Rhizoctonia* is composed of multinucleated and binucleate species, as well as by genetically related intraspecific groups and subgroups capable of self-recognition by fusion of hyphae, called anastomosis groups (AGs) (Ogoshi 1987; Carling 1996; González

García et al. 2006; Sharon et al. 2006; Ajayi-Oyetunde & Bradley 2018). *Rhizoctonia solani* Kühn (the teleomorph *Thanatephorus cucumeris* (Donk)) is the most studied multinucleate species and 14 AGs (AG-1 to AG-13, and AG-BI) are recognized, as well as subgroups (Yang et al. 2017; Ajayi-Oyetunde & Bradley 2018). Among the binucleate species (teleomorph *Ceratobasidium* Rogers), 17 AGs (AG-A to AG-V) are recognized, including subgroups (Dong et al. 2015).

Several AGs have been associated with potato rhizoctoniosis worldwide, with a predominance of AG-3 PT (Tsror 2010). In Brazil, few papers on the diversity of Rhizoctonia AGs in potatoes have been published. In a pioneer work, 10 isolates were evaluated by hypophysial pairing with test isolates, with AG-3 and AG-4 being identified (Bolkan & Ribeiro 1985). Afterwards, 16 isolates were characterized by the pairing with isolated isolates and sequence analysis of the internal transcribed spacer region (ITS) of the ribosomal DNA (rDNA), being classified as AG-4 HGI, AG-4 HGII and AG-7 (Rosa et al. 2005). Recently, 66 isolates obtained in the major producing regions of Brazil were characterized by the analysis of ITS sequences and classified as AG-3 PT (37.3% of the isolates), AG-4 HGI (29.9%), AG-R (22.4%), AG-2-1 (4.5%), AG-A (4.5%) and AG-1B1 (1.5%) (Inokuti 2016).

The importance of determining the anastomosis groups is due to the fact that each group can be considered an evolutionary unit, since they represent genetically isolated and non-interconnected populations (Anderson 1982; Carling 1996; González García et al. 2006). Therefore, the knowledge of the anastomosis groups and subgroups has great importance in understanding the genetic diversity of *Rhizoctonia* and the possible epidemiological influences of this diversity in disease control measures (Anderson 1982;

Ogoshi 1987; Godoy-Lutz et al. 2008; Tsror 2010, Sharma-Poudyal et al. 2015, Ajayi-Oyetunde et al. 2018).

Natural selection is a powerful force that acts on the evolution of organisms and occurs because of the number of descendants capable of surviving. This survival is not random, and is often dependent on the "fitness" of individuals (Milgroom 2015). The biological adaptability of a phytopathogen is described as the relative ability to persist in an environment for a long period of time (Nelson 1979), being composed of saprophytic adaptability and pathogenic adaptability (Harteveld et al. 2014). The combination of these attributes leads to an increase in reproduction and survival due to mycelial growth, sporulation, pathogenicity and aggressiveness of phytopathogenic fungi (Leach et al. 2001; Pringle & Taylor 2002).

The competitive capacity and relative adaptability of an isolate or plant pathogen species are determined by their intrinsic biological properties, host resistance and heterogeneity, population density and genetic relationship of competing isolates, and the physical environment (Zhan & McDonald 2013). As adaptability is relative, it must be estimated by the measurement of characters that provide some adaptive advantage among individuals. Several adaptability components can be measured in plant pathogens, depending on their biology and the ease of obtaining them by experimental methods (Milgroom 2015). In this context, phenotypic markers such as mycelial growth rate, reproductive potential, sensitivity to fungicides and aggressiveness have been useful to evaluate the adaptability in plant pathogens (Antonovics & Alexander 1989; Lannou 2012; Milgroom 2015).

Despite the importance of potato rhizoctoniosis worldwide, so far, the adaptability of different *Rhizoctonia* AGs has not been compared, which may generate important

information for the development of disease management strategies. The objective of this study was to compare saprophytic adaptability in relation to the mycelial growth of the isolates belonging to the main *Rhizoctonia* AGs associated with potato in Brazil under different environmental conditions (temperature, pH, water potential and salinity) and fungicides, as well as pathogenic adaptability in relation to aggressiveness in several plant species.

Material and methods

Fungi isolates: In all experiments, 15 isolates of *Rhizoctonia* obtained from potato with symptoms of black scurf belonging to the AG-3 PT, AG-4 HGI and AG-R anastomosis groups (Table 1) were used, the most prevalent in Brazil (Inokuti 2016). The isolates were identified by phylogenetic inference based on the complete sequence of the rDNA ITS region (Inokuti 2016) and are deposited in the "Prof. Maria Menezes" (CMM) Collection of Phytopathogenic Fungi Cultures of the Federal Rural University of Pernambuco (Recife, Pernambuco Brazil). Stock cultures were maintained in test tubes with potato-dextrose-agar medium (PDA; Acumedia, Lansing, USA) tilted at 5 ° C in the dark.

Effect of temperature on mycelial growth Mycelial: Discs (5 mm diameter) were removed from the colony margin of each isolate with seven days growth and transferred to the center of Petri dishes (100 mm diameter) containing PDA. Three plates of each isolate were placed in incubators with controlled temperatures at 10, 15, 20, 25, 30, 35 and 40º C in the dark. Prior to use, incubators were equilibrated at respective temperatures for at least 12 hours. The experimental design was completely randomized, in a factorial arrangement

15x7, with three replicates (plates) by combination of isolates and temperature. The diameter of each colony was measured with 48 hours of incubation in two perpendicular directions and the mean (mm) was obtained.

Effect of pH on mycelial growth: Mycelial (5 mm) discs were removed from the margin of the colony of each isolate with seven days of growth in PDA and transferred to the center of Petri dishes containing PDA adjusted to pH 5, 6 and 7 by use of 1M solutions of NaOH and HCl. The plates were incubated in the dark at 25° C. The experimental design was completely randomized, in a factorial arrangement 15x3, with three replicates (plaques) by combination of isolates and pH level. The diameter of the colonies was evaluated as previously described.

Effect of water potential (Ψs) on mycelial growth: Mycelial discs (5 mm) were removed from the colony margin of each isolate with seven days of growth in PDA and transferred to the center of Petri dishes containing PDA supplemented with KCl to obtain (Ψs) of -0.8, -1.6 and -3.2 MPa, according to Michel & Radcliffe (1995). The plates were incubated in the dark at 25° C. The experimental design was completely randomized, in a 15x3 factorial arrangement, with three replications per combination of isolates and water potential level. The diameter of the colonies was evaluated as previously described.

Effect of salinity on mycelial growth: Mycelial (5 mm) discs were removed from the margin of the colony of each isolate with seven days of growth in PDA and transferred to the center of Petri dishes containing PDA supplemented with 1, 2, 4, 6 and 8% (w/v) NaCl.

The plates were incubated in the dark at 25° C. The experimental design was completely randomized, in a 15x5 factorial arrangement, with three replicates (plaques) by combination of isolates and salinity levels. The diameter of the colonies was evaluated as previously described.

Effect of fungicides on mycelial growth: The commercial formulations of Fluazinam fungicides (Frowncide 500 SC, 500g active ingredient (i.a)/L, ISK Biosciences Ltda., São Paulo - SP), Fludioxonil (Maxim, 25g i.a/L, Syngenta Proteção de Cultivos Ltda., São paulo – SP), Pencicurom (Monceren 250 SC, 250g i.a/L, Bayer SA, São Paulo - SP) and Procymidone (Sumilex 500 WP, 500g a.i/L, Sumitomo Chemical Ltda., São Paulo - SP), registered in the Ministry of Agriculture for the control of *R. solani* in potato (MAPA 2018), representing different chemical groups and modes of action. The fungicides were dissolved in sterile distilled water and added to the PDA flux medium (45° C) to reach the concentrations of 10 µg i.a./mL (Fluazinan) and 1 µg i.a./ml (Fludioxonil, Pencicurom and Procymidone). Mycelial (5 mm diameter) discs were removed from the colony margin of each isolate with seven days of growth in PDA and transferred to the center of Petri dishes containing PDA supplemented with each fungicide. Plates containing PDA without fungicide were used as controls. The experimental design was completely randomized, in a 15x4 factorial arrangement, with three replicates (plaques) by combination of isolates and fungicides. The diameter of the colonies was evaluated as previously described and the percentage inhibition of mycelial growth against the control without fungicide was calculated.

Aggressiveness in several plant species: In this study, aggressiveness is defined as a quantitative component of pathogenicity, that is, the amount of disease induced by a

pathogenic organism in a susceptible host (Agrios 2005). The inoculum of *R. solani* was standardized using rice grains infested by the fungus as a source of infection. For the inoculum production, 50 g of parboiled rice grains and 30 mL of distilled water were placed in each 250 mL glass vial. After autoclaving (120º C, 1 atm, 30 minutes) and cooling, in each vial were placed three disks (5 mm diameter) of fungus culture, previously grown in PDA medium for seven days. After seven days of incubation (25° C and photoperiod of 12 hours), the substrate colonized by the pathogen was packed in paper bags and placed to dry for 48 hours at 40° C. The aggressiveness of the isolates was evaluated in potato cv. Agata (*Solanum tuberosum* L.) broccoli cv. Ramoso Piracicaba (*Brassica oleracea* L. var. *itálica*), okra cv. Santa Cruz 47 (*Abelmoschus esculentus* L.), tomato var. IPA 6 (*Solanum lycopersicum* L.), cowpea var. IPA 207 (*Vigna unguiculata* L.), fava beans cv. Boca de Moça *(Phaseolus lunatus* L.), soybean cv. BRS Pérola (*Glycine max* L.), castor bean cv. BRS Energia (*Ricinus communis* L.), maize cv. BRS Caatingueiro (*Zea mays* L.), sorghum cv. BRS 610 (*Sorghum bicolor* L.), brown hemp cv. IAC 1 (*Crotalária Juncea* L.), jack bean cv. Comum (*Canavalia ensiformis* L.), pigeon pea cv. BRS Mandarim (*Cajanus cajan* L.) and mucuna cv. Preta (*Mucuna pruriens* L.). In potato inoculation, certified, disease-free minitubers were germinated at 22° C in the dark until the shoots were 3 mm in length. A germinated tuber was placed in each plastic vial (500 mL) filled with a 3 cm substrate layer consisting of soil mixture and cow manure (5: 1 v/v, sterilized by dry heat at 161° C for 3 hours , on two consecutive days). A grain of rice colonized with each isolate was placed 10 mm above the budding point and covered with the substrate. In the inoculation of the other plant species, the seeds were planted in polypropylene trays of 128 wells (pits) containing the same substrate used in planting potatoes. The inoculation was carried out by deposition of a grain of rice colonized by the fungus 1 cm above each seed

and then covered with the substrate. Witches were inoculated with rice grains not colonized by the fungus. The plant species were evaluated separately and for each species the experimental design was completely randomized, with 15 treatments (isolated), four replications per treatment and nine replication plantings containing one seed potato or seed per pit. The pots and trays were kept in a greenhouse and the plants irrigated daily to maintain field capacity. The aggressiveness of the potato isolates was evaluated at 28 days after inoculation, based on the relative size of the necrotic area in the subterranean stems, with the following scale: 0 = no symptoms, 1 = less than 10%, 2 = 10-50%, 3 = 50-100%, and 4 = dead plant (Yang et al., 2014). The aggressiveness of the isolates in the other plant species was evaluated at 12 days after inoculation, by quantifying the severity of the disease using the following diagrammatic scale: 0 = no symptoms, 1 = hypocotyl with small lesions, 2 = hypocotyl with (3) = totally constricted hypocotyl, showing tipping, and 4 = non-germinated seeds and / or non-emerged seedlings (Noronha et al. 1995). With the data of the evaluations the disease severity index was calculated in each repetition, according to McKinney (1923).

Statistical analyzes: The experiments were repeated to confirm the observations. Data from the two independent replicates for each experiment were pooled after performing the variance homogeneity analysis using the Levene test and no heterogeneity was detected (P> 0.05). In the temperature experiment, the data were submitted to linear and non-linear regression analysis, having temperature as independent variable and mycelial growth as dependent variable. The optimum temperature (temperature that provided the greatest mycelial growth) and maximum mycelial growth (at the optimum temperature) were estimated using the regression model and the numerical summary (second derivative) with

the aid of the TableCurve ™ 2D 5.01 program (Systat Software Inc Chicago, USA). The choice of model was determined by the coefficient of determination (R2), distribution of residues and mean squared error. The significance of the regressions was verified by the F test ($P < 0.05$) and by the t test ($P < 0.05$). The values of the variables estimated in the temperature experiment and the data obtained in the other experiments were submitted to analysis of variance (ANOVA) and the means compared by Fisher's least significant difference test (LSD) ($P = 0.05$). The Levene and t tests, analyzes of variance and the comparisons of means were performed using Statistix 9.0 software (Analytical Software, Tallahassee, USA).

Results

Effect of temperature on mycelial growth: All *Rhizoctonia* isolates belonging to AG-3 PT, AG-4 HGI and AG-R were grown at 10° C and did not grow at 40° C, whereas AG-PT isolates also did not grow at 35° C. The temperatures influenced the mycelial growth of the isolates of the three AGs. The fifth-degree polynomial regression model ($y = a + bx2 + cx4 + dx6 + ex8 + fx10$) provided an excellent adjustment to the mycelial growth data as a function of temperature for all isolates, with R2 values ranging from 0.984 to 0.998. With the calculation of the derivatives of the parameters of this regression it was possible to estimate the optimal temperature for mycelial growth and maximum mycelial growth. Isolates belonging to the same AG did not differ among themselves ($P = 0.3411$) in relation to mycelial growth at different temperatures. The AGs differed in relation to the optimum temperature for growth ($P = 0.0216$), with AG-R requiring the highest temperature (29.5° C) and the lowest AG-3 PT (24.4° C). At the optimum temperature, AG-4 HGI presented

the highest mycelial growth (96.6 mm) and AG-3 PT the lowest growth (74.4 mm) (Table 2).

pH effect on mycelial growth: The pH levels tested (5, 6 and 7) did not influence the mycelial growth of *Rhizoctonia* isolates belonging to the three AGs (P = 0.2291), as well as the interaction between AGs and pH levels was not significant (P = 0.1762). Isolates from the same AG did not differ among themselves (P = 0.2738) in relation to mycelial growth at different pH levels. However, there was a significant difference (P <0.0001) between the AGs in relation to mycelial growth, especially AG-4 HGI, which presented higher growth (97.6 mm), followed by AG-R (90.9 mm) and AG-3 PT (72.5 mm).

Effect of water potential (Ψs) on mycelial growth: *Rhizoctonia* isolates belonging to the three AGs increased at the levels of Ψs tested. The Ψs significantly influenced the mycelial growth of the isolates from the three AGs, as well as the interaction between AGs and Ψs levels was significant (P <0.0001). Isolates of the same AG did not differ among them (P = 0.1193) in relation to the mycelial growth in the evaluated levels. On the other hand, AGs differed (P = 0.02519) in relation to mycelial growth in the Ψs levels. For the three AGs the growth reduced with the decrease of Ψs. AG-4 HGI and AG-R showed the highest growths at -0.8 MPa, without differing between them, while the former also stood out at -1.6 and -3.2 MPa, differing from the other AGs (Table 3) .

Effect of salinity on mycelial growth: No *Rhizoctonia* isolate grew at 6 and 8% salinity levels. Salinity levels influenced the mycelial growth of *Rhizoctonia* isolates belonging to

283 the three AGs, but the interaction between AGs and salinity levels was not significant (P =
284 0.2481). There was a significant difference in mycelial growth between the AGs (P
285 <0.0001) and between the salinity levels (P <0.0001). AG-3 PT showed the lowest mycelial
286 growth in the presence of salinity (43.6 mm), while AG-4 HGI and AG-R showed lower
287 sensitivity and did not differ from each other. Regardless of AG, mycelial growth
288 significantly reduced with elevation of salinity level (Table 4).
289
290 **Effect of fungicides on mycelial growth:** The fungicides were evaluated separately and
291 the isolates of the three AG showed a great reduction in mycelial growth in the presence of
292 fungicides Fluazinan, Fludioxonil, Pencicurom and Procymidone. However, only in
293 relation to the fungicide Fluazinam the AGs differed from sensitivity (P <0.0001). AG-3 PT
294 showed greater sensitivity to this fungicide, with 95.9% reduction in mycelial growth,
295 while AG-4 HGI and AG-R showed lower sensitivities (78.9% and 78.8%, respectively)
296 and did not differ among themselves (Table 4).
297
298 **Aggressiveness in several plant species:** The 14 plant species were evaluated separately
299 and all *Rhizoctonia* isolates induced symptoms in these species, including stem and root
300 cancers, seed rot and seedling tipping. Isolates belonging to the same AG did not differ
301 among themselves (P = 0.1932) in relation to the aggressiveness, represented by the
302 severity of the induced disease. AGs differed from aggression in all evaluated plants (P
303 <0.0001). AG-4 HGI was the most aggressive in all plant species, except for castor bean,
304 which did not differ from AG-R. The highest levels of disease severity were recorded in
305 cowpea (98.8%), pigeon pea (93.9%), fava beans (88.5%) and soybean (82.3%), while
306 maize (6%) and sorghum (9.4%) were the species less affected by *Rhizoctonia* (Table 5).

Discussion

The results obtained in this study show that the adaptability attributes of the main *Rhizoctonia* AGs associated with potato in Brazil are highly variable. In addition, they confirm the information that the adaptability of any phenotype is directly related to the environmental factors that involve the individual, being important the understanding of these environmental influences to make predictions about the ecological tolerance of the studied individual (Balodi et al. 2017). Some phenotypic features such as mycelial growth and aggressiveness tend to reflect much of the genome, and more importantly, they are often adaptive traits with implications for the organism's broader ecological strategy. These characteristics may be sensitive indicators of differences in adaptability between individuals or populations (Brasier 1999).

Analysis of mycelial growth is of particular importance for *Rhizoctonia*. The fungus does not produce conidia and the production of sex spores is rare, so the mycelium represents the main form of fungus reproduction. In addition, the mycelium is extremely sensitive to environmental variations (Sherwood 1970). Mycelial growth is an important component of adaptability and easy measurement. It is also a good indicator of the ability to compete and adapt to the environment (Brasier 1999).

Many environmental factors have been postulated as drivers of the distribution, growth and survival of *Rhizoctonia*, with emphasis on temperature (Sherwood 1970; Harikrishnan & Yang 2004). In this study, the three main AGs associated with potatoes in Brazil demonstrated mesophilic behavior, most of them capable of growing in a temperature range between 10 and 35° C, similar to those observed in other studies

involving different *Rhizoctonia* AGs (Burpee & Martin 1992, Hide & Firmager 1989, Harikrishnan & Yang 2004, Mihan & Aghajani 2016, Yildirim & Erper 2017). The differential influence of temperature on the mycelial growth of AGs was evident, as AGR-R was shown to be more adapted to higher temperatures, while AG-3 PT at milder temperatures. In field conditions, temperature is an important factor linked to the geographical distribution of AGs, since some are more adapted to certain temperatures and indirectly adapted to host plants that predominate at these temperatures (Harikrishnan & Yang 2004).

The similarity in the mycelial growth presented by the AGs in the range of pH 5-7 indicates that the concentration of hydrogen ions in the culture medium did not affect the fungal growth, although this chemical element could indirectly influence the availability of nutrients or directly by the action on the surfaces cell phones (Deacon 2006). The results also indicate the similar adaptability of the AGs to the pH levels of soils that predominate in the potato producing regions of Brazil (Fernandes et al. 2016). At the field level, this may mean little or no liming influence on acid soils on the reduction of the adaptability of the isolates, which could negatively affect the saprophytic capacity of the fungus in the soil (Papavizas 1970; Okubara et al. 2014) and decrease pathogenic activity (Baker & Martinson 1970, Watanabe et al. 2011, Naseri & Hemmati 2017).

The reduction of the mycelial growth of the AGs with the decrease of Ψs resembles that registered for fungi (Deacon 2006), as well as in particular for *Rhizoctonia* (Sherwood 1970; Dubé et al. 1971; Sterne & McCarver 1978; Ritchie et al. 2006). Water availability is one of the most important environmental factors that affect fungal activity (Deacon 2006, Palacios et al. 2014). The fungi need to maintain the internal Ψs lower than the total Ψs of

the environment to maintain the turgidity of the cells and allow the continuation of mycelial growth (Cook 1973; Eamus & Jennings 1986). The internal Ψs of the fungus is thus determined by the surrounding environment, with exposure to changes in water availability within the matrix itself (matrix potential) and the presence of dissolved substances (osmotic potential) (Cook 1973). Mycelial growth at all levels of Ψs tested may indicate the ability of isolates to withstand water deficit conditions. However, the lower sensitivity to the reduction of Ψs presented by AG-4 HGI indicates greater adaptability to the water stress condition, compared to the other AGs.

The reduction of the mycelial growth of the *Rhizoconia* isolates with the elevation of the salinity levels observed in this study is common in most fungi, considering NaCl toxicity to the fungal cell (Deacon 2006). As salinity negatively affects the pathogenicity and aggressiveness of *R. solani* by the reduction in the activity of cell wall degrading enzymes (El-Abyad et al. 1992), the lower sensitivity to salinity presented by AG-4 HGI may be an important factor of competitiveness in relation to the other AGs in salinized soils, being less affected in the saprophytic growth in the soil and in the enzymatic activity (pathogenesis) on the host plant.

The high sensitivity of *Rhizoctonia* AGs to Fluazinan, Fludioxonil, Pencicurom and Procymidone, representing different chemical groups and modes of action, indicates that fungus populations are still poorly exposed to these fungicides in potato cultivation, with no risk of emergence of resistance to molecules in the short term. However, the variation in the sensitivity between the AGs observed in relation to Fluazinam can be a concern factor, considering that this fungicide can act as a directional selection factor (Milgroom 2015),

that is, selecting populations of AG isolates -4 HGI and AG-R with lower sensitivity, which may predominate in the long term to the detriment of more sensitive AGs.

Aggressiveness is an important component of pathogenic adaptability, since it indicates the ability of a pathogen to cause severe epidemics, in view of the greater competence to induce the disease in a smaller scale of time and/or greater damage to the host population (Sacristan & Garcia- Arenal 2008; Pariauld et al. 2009; Lannou 2012). In this study, the pathogenic adaptive advantage of the AG-4 HGI isolates in relation to the other AGs was evident, demonstrating greater aggressiveness in 13 of the 14 inoculated plant species, including vegetables, food and industrial crops, and species used as green manures. The highest aggressiveness of the AG-4 HGI isolates in potato was reported only in Brazil (Inokuti 2016) and Peru (Anguiz & Martin 1989), because in other countries the isolates of AG-3 PT were the most aggressive to this Solanacea (Campion et al. 2003; Lehtonen et al. 2009; Fiers et al. 2011; Das et al. 2014; Muzhinji et al. 2015; Özer e Bayraktar 2015; Yang et al. 2017). Isolates of AG-4 have been highlighted as the most aggressive in other plant species (Agarwal 2010; Yildirim & Erper 2017).

This study identified differences in adaptability among the three main *Rhizoctonia* AG associated with potato in Brazil, when comparing the saprophytic adaptability of the isolates under the influence of temperature, salinity, pH and fungicides, as well as the pathogenic adaptability based on aggressiveness in several species of plants. In all analyzed situations, the AG-4 HGI isolates showed a greater saprophytic and pathogenic adaptive potential than the AG-3 PT and AG-R isolates. However, this greater adaptability of AG-4 HGI does not reflect the greater distribution and prevalence in the areas of potato production in Brazil, in which AG-3 PT stood out, as in other parts of the world. The

predominance of AG-3 PT is associated with factors unrelated to saprophytic or pathogenic adaptability.

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Compliance with Ethical Standards

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Research involving human and / or animal participants - Not applicable.

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595

596 **Table 1** Isolates from three groups of *Rhizoctonia* anastomosis obtained from potato with
597 symptoms of black crust collected in Brazil and used in the study.

598

Group of anastomosis[1]	Isolated[2]	City (State)[3]	GenBank accession (ITS)
AG-3 PT	CMM-1806	Serra Salitre (MG)	KT366803
	CMM-2099	São Gotardo (MG)	KT366807
	CMM-4152	Canoinhas (SC)	KT366808
	CMM-4257	Papanduva (SC)	KT366812
	CMM-4424	Cristalina (GO)	KT366819
AG-4 HGI	CMM-1350	Água Fria (GO)	KT366791
	CMM-4212	Cristalina (GO)	KT366782
	CMM-4236	Cristalina (GO)	KT366786
	CMM-4404	Casa Branca (SP)	KT366788
	CMM-4408	Casa Branca (SP)	KT366794
AG-R	CMM-1337	Mucugê (BA)	KT366826
	CMM-1340	Mucugê (BA)	KT366833
	CMM-1829	Brasília (DF)	KT366835
	CMM-2098	São Gotardo (MG)	KT366824
	CMM-4235	Cristalina (GO)	KT366825

599

600 [1] AG-3 PT e AG-4 HGI = *Rhizoctonia solani*, AG-R = *Rhizoctonia* binucleada

[2] CMM = Collection of Phytopathogenic Fungi Cultures "Prof. Maria Menezes" from the Federal Rural University of Pernambuco (Recife, Pernambuco, Brazil)

[3] BA = Bahia, DF = Distrito Federal, GO = Goiás, MG = Minas Gerais, SP = São Paulo, SC = Santa Catarina

Table 2 Effect of temperature on mycelial growth of three groups of *Rhizoctonia* anastomosis obtained from potato with rhizoctoniosis symptoms collected in Brazil.

Group of anastomosis	Optimum temperature[1] (ºC)	Maximum mycelial growth[2] (mm)
AG-3 PT	24,4 c[3]	74,4 c
AG-4 HGI	28,7 b	96,6 a
AG-R	29,5 a	90,5 b

[1,2] Values estimated by the adjustment of the polynomial regression model: $y=a+bx^2+cx^4+dx^6+ex^8+fx^{10}$, where y = mycelial growth; a, b, c, d, e, f are regression parameters; x = temperature (*AG-3 PT:* $y=-10,91+0,31x^2-0,0006x^4+(7,87e^{-7})x^6-(6,13e^{-10})x^8+(1,82e^{-13})x^{10}$, $R^2=0,984$, $p>F=0,039$; *AG-4 HGI:* $y=-46,34+0,74x^2-0,0022x^4+(3,67e^{-6})x^6-(2,82e^{-9})x^8+(7,63e^{-13})x^{10}$, $R^2=0,994$, $p>F=0,014$; *AG-R:* $y=-44,33+0,76x^2-0,0029x^4+(5,25e^{-6})x^6-(4,15e^{-9})x^8+(1,13e^{-12})x^{10}$, $R^2=0,998$, $p>F=0,004$)

[3] Mean values in the column followed by the same letter did not differ significantly from each other by Fisher's LSD test (P = 0.05).

614 **Table 3** Effect of water potential on mycelial growth of three groups of *Rhizoctonia*
615 anastomosis obtained from potato with rhizoctoniosis symptoms collected in Brazil.

Group of anastomosis	Water potential/Mycelial growth (mm)		
	-0,8 MPa	-1,6 MPa	-3,2 MPa
AG-3 PT	75,4 bA[1]	38,3 bB	16,0 cC
AG-4 HGI	94,9 aA	76,6 aB	43,6 aC
AG-R	89,5 aA	46,7 bB	31,6 bB

616

617 [1] Means followed by the same lowercase letter in the column and upper case in the row did not differ
618 significantly from each other by Fisher's LSD test (P = 0.05).

Table 4 Effect of salinity on mycelial growth of three groups of *Rhizoctonia* anastomosis obtained from potato with rhizoctoniosis symptoms collected in Brazil.

Group of anastomosis	Mycelial growth (mm)	Salinity	Mycelial growth (mm)
AG-3 PT	43,6 b[1]	1%	82,3 a[1]
AG-4 HGI	64,3 a	2%	56,2 b
AG-R	57,8 a	4%	22,3 c

[1] Averages followed by the same letter in the column did not differ significantly from each other by Fisher's LSD test (P = 0.05).

624 **Table 5** Effect of fungicides on the mycelial growth of three groups of *Rhizoctonia*
625 anastomosis obtained from potato with symptoms of rhizoctoniosis collected in Brazil.

Group of anastomosis	Fungicide[1] / Inhibition of mycelial growth (%)			
	Fluazinam	Fludioxonil	Pencicuron	Procimidona
AG-3 PT	95,9 a[2]	93,9 a	100,0 a	83,9 a
AG-4 HGI	78,9 b	95,7 a	96,4 a	89,5 a
AG-R	78,8 b	99,4 a	91,3 a	87,4 a

626

627 [1] Concentrations of 10 µg de i.a./mL (Fluazinam) and 1 µg de i.a./mL (Fludioxonil, Pencicuron and
628 Procimidona).

629 [2] Averages followed by the same letter in the column did not differ significantly from each other by Fisher's
630 LSD test (P = 0.05).

631 **Table 6** Aggressiveness (severity) in several plant species of three *Rhizoctonia* anastomosis
632 groups obtained from potato with rhizoctoniosis symptoms collected in Brazil.
633

Group of anastomosis	Plant species/Severity (%)						
	Broccoli	Brown hemp	Cowpea	Jack bean	Fava beans	Pigeon pea	Potato
AG-3 PT	37,8 b	42,8 b	11,5 c	13,3 c	19,3 c	76,4 b	29,3 c[1]
AG-4 HGI	74,6 a	79,9 a	98,8 a	66,7 a	88,5 a	93,9 a	56,9 a
AG-R	32,4 b	41,1 b	31,2 b	23,7 b	29,8 b	77,2 b	40,9 b

Group of anastomosis	Plant species/ Severity (%)						
	Castor bean	Maize	Mucuna	Okra	Sorghum	Soybean	Tomato
AG-3 PT	26,6 b	5,6 c	43,9 b	27,8 b	9,4 b	40,9 c	20,1 c
AG-4 HGI	51,7 a	22,3 a	71,7 a	43,1 a	28,3 a	82,3 a	52,2 a
AG-R	48,2 a	11,2 b	40,5 b	30,2 b	11,2 b	57,5 b	39,7 b

634

635 [1] Means followed by the same letter in the column within each host do not differ significantly from each other
636 by Fisher's LSD test (P = 0.05).

Capítulo III

Selection of essential oils with antifungal activity *in vitro* and *in vivo* against aggressive isolates of *Rhizoctonia* sp.

Antifungal activity and TBARS of essential oils against aggressive isolates of *Rhizoctonia* associated with stem canker and black scurf of potatoes

A. C. Costa Junior[a], R. A. Contreras[b], P. A. Bermúdez-Cañete[d], A. González-Coloma[c*], M. F. Andrés Yeves[c], S. J. Michereff[a].

[a]Laboratory of Epidemiology of Plant Diseases, Federal Rural University of Pernambuco (UFRPE), Recife, PE, Brasil; [b]Faculty of Chemistry and Biology, University of Santiago de Chile (USACH), Alameda 3363, Estación Central, Santiago, Chile; [c]Instituto de Ciencias Agrarias, Consejo Superior de Investigaciones Científicas (ICA-CSIC), Serrano 115-b, 28006 Madrid, Spain; [d]E.T.S. de Ingenieros de Montes, Polytechnic University of Madrid (UPM).

*Corresponding author e-mail: azu@ica.csic.es

Abstract

This study aimed to select essential oils with antifungal activity against aggressive isolates of *Rhizoctonia* belonging to AG-3 PT and AG-R. Thirty essential oils from different plants and their chemical components were tested at three concentrations, 0.1, 0.5 and 1.0 mg mL^{-1}, by the agar diffusion method. Seven essential oils with antifungal activity to *Rhizoctonia* were found. Of these, *Mentha rotundifolia, Thymus zygis* and *Satureja montana* oils were selected and presented low values of IC 50 (0 - 0.1 mg mL^{-1}) and low MIC values (0.1-0.5 mg mL^{-1}). GC-MS identified 26 chemical compounds in the essential oil of *M. rotundifolia*

and *T. zygis* and 14 chemical compounds in the essential oil of *S. montana*. Piperitone oxide (36.97%) and piperitone (20.77%) were the major components in the oil of *M. rotundifolia,* thymol (60.27%), piperitone oxide (6.56%) and piperitone (5.18%) were the major components in the oil of *T. zygis* and carvacrol (49.38), p-cymene (28.08) and thymol (8.30) were the major components in *S. montana* oil. When evaluated separately, treatments with carvacrol at concentrations of 0.01 and 0.05 mg mL^{-1} significantly inhibited the fungus ($p < 0.0001$) with 28 and 29.8% inhibition of mycelial, respectively. The TBARS assay revealed that the *Rhizoctonia* isolates reacted differently to the tested oils. In the treatment with *S. montana* oil at 5 mg mL-1, AG-R presented higher values of ROS (127.4 nmol g^{-1}) and MDA (5.9 nmol g^{-1}) and in the treatment with *M. rotundifolia* oil at 5 mg mL^{-1} AG-3 PT presented higher ROS (131.9 nmol g^{-1}) and MDA (5.4 nmol g^{-1}). The results reveal essential oils that can be tested in field assays as well as application technology studies and open a window on the management of diseases caused by *Rhizoctonia*.

Keywords: *Mentha rotundifolia. Satureja Montana. Thymus zygis.* ROS. TBARS. GC-MS analysis.

1 Introduction

Potato (*Solanum tuberosum*) is the third most important agricultural crop on the planet and its cultivation is present in more than 100 countries (FAO, 2018). Many potato plants are attacked by different soil pathogens including *Rhizoctonia solani* (Kuhn), in which they are among the most the most aggressive soil phytopathogens (Anderson, 1982; Banville, 1989; Banville *et al.*, 1996; Tsror, 2010). Potato rhizoctoniosis is most common

in the 20 to 40 days after planting and the symptoms are stem and root cankers; damping off; plant burning and death; tuber rot, spotting, leaf burning, sprouting and formation of sclerotia on the surface of tubers, also known as black scurf (Banville, 1989; Carling *et al.*, 1989; Banville *et al.*, 1996; Tsror, 2010).

Rhizoctonia multinucleate isolates identified as *R. solani* (Kühn) [teleomorph *Thanatephorus cucumeris* (Donk)] and binucleated *Rhizoctonia* (BNR) isolates [teleomorph *Ceratobasidium*] are commonly found causing rhizoctoniosis in potatoes (Yang *et al.*, 2014, 2015a,b, 2017; Muzhinji, N., Truter, M., Woodhall, J. W., van der Waals, 2015). *Rhizoctonia* isolates differ in phenotypic and genotypic characteristics and are arranged in genetically related intraspecific groups (AGs) and subgroups capable of self-recognition through hyphae fusion (Carling, 1996; Carling *et al.*, 2002; Sharon *et al.*, 2006, 2007, 2008). Currently, 14 AGs (AG-1 to AG-13 and AG-BI) from *R. solani* are recognized, with AG-3 being the most widely distributed in potato crops worldwide. Among BNR 17 AGs (AG-A to AG-V) are recognized, with recent attention being given to the AG-R group due to the prevalence and high aggressiveness in potato production fields (Yang *et al.*, 2014, 2015a,b; Muzhinji *et al.*, 2015).

Many pesticides and fumigants, such as Benomyl and methyl bromide, have been used as the primary control measure of *Rhizoctonia* (Sneh *et al.*, 1996; Jeger *et al.*, 1996). Currently, due to the variable efficiency of available fungicides and serious social and environmental problems, such as the appearance of resistance to fungicides and rigid regulations for food production, new measures of fungus control are being studied (Campion *et al.*, 2003; Woodhall *et al.*, 2007; Tsror, 2010; Popp *et al.*, 2013; Özer & Bayraktar, 2015).

The use of biopesticides, such as Essential Oils (EOs), showed antifungal potential and, because they have multiple compounds, decrease the risk of developing fungal resistance (González-Coloma *et al.*, 2010). EOs are very complex natural mixtures containing from 20 to 60 components in different concentrations and low molecular weight. They are characterized by containing 2 to 3 major components of volatile molecules such as terpenoids, aromatic phenolic derivatives and aliphatic components (Bakkali *et al.*, 2008). To date, 3000 EOs are known, of which 300 are commercially important for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfumery industries (Bakkali *et al.*, 2008).

Some studies have demonstrated the antifungal activity of EOs to *R. solani*. Khaledi *et al.*, 2015) studied the antifungal activity of EOs of *Mentha piperita, Bunium persicum* and *Thymus vulgaris* against *R. solani* and *Macrophomina phaseolina* and verified that in the treatments in which the growth of *R. solani* was inhibited, the hyphae underwent structural modifications and there was less activity of pectinase, which is an important virulence attribute in *R. solani*. Plodpai *et al.* (2013) reported that the foliar application of *Desmos chinensis* reduced the intensity of the disease caused by *R. solani* in rice. Arici & Sanli (2014) studied the efficiency of EOs of *Cuminum cyminumum, Anethum graveolens, Salvia officinalis, Origanum onites, Rosmarinus officinalis* and *Lavandula intermedia* against *R. solani* and *Streptomycetes scabies* on potato and found that the *S. officinalis* EO reduced *R. solani* infection in 4,2%, and oregano's EO reduced the disease severity caused by *S. scabies* to 1.8%. Positive results have also been found with *Piper Chaba* Oils (Rahman *et al.*, 2011).

The aggressive isolates of *Rhizoctonia solani* and *Rhizoctonia* sp. pose a threat to the durability of current fungus control measures. Advances in the discovery and development

of biofungicides such as EOs have become an important tool for the holistic control of *Rhizoctonia.* This study aimed to study the antifungal properties of several EOs against AG-3 and AG-R of *Rhizoctonia*, identifying the main chemical components and quantifying oxidative stress. In the field, preliminary studies aimed at visualizing the results obtained *in vitro*.

2 Materials and methods

2.1 Plant pathogenic fungi

Two isolates of *Rhizoctonia* were used in this study obtained from the Collection of Phytopathogenic Fungi Cultures "Prof. Maria Menezes" (CMM) of Rural Federal University of Pernambuco (UFRPE). Isolate 2099 identified as *Rhizoctonia solani* multinucleate from the anastomosis AG-3 PT group and isolate 1829 identified as *Rhizoctonia* spp. binucleate of AG-R. Isolates were found causing stem canker and black scurf in potatoes in Brazil. The isolates were kept in potato-dextrose-agar medium (PDA) at 4^{o}C and subcultured at monthly intervals.

2.2 Plant materials

Aromatic plants with the exception of 1 and 18 (Table 1) were collected from experimental fields located in the Comarca of Castilla-La Mancha region of the Alcarria (Cuenca and Guadalajara), Serranía Alta (Cuenca), Señorío de Molina-Alto Tajo (Guadalajara) and Campo del Cariñena, Aguarón, Zaragoza, Spain, during the period of full

flowering. From each species were collected the aerial part (inflorescences, leaves and stems) by subjecting them to drying at room temperature for 5 days until the EO was extracted.

2.3 Extraction of EOs

The EOs were extracted by hydrodistillation using a Clevenger type apparatus and organic extraction using a Sohxlet type apparatus, according to the method recommended by the European Pharmacopoeia. Pilot plant vapor pressure extraction was carried out in a stainless steel distillation plant equipped with a 100 Kg distillation chamber, a 500 L vessel and a pressure range of 0.5-1.0 bar. The water collected after the EO was decanted (1.16 L) was filtered to give an acidic water residue (WR, 4.5 mg/mL of organic extract, pH 3.2). 155 mL of WR were extracted with dichloromethane (DCM, 150 mL x 3) to give an organic fraction (WROE) (230 mg, 0.15 % yield). 50 mL of WR were neutralized at pH 6.62 with NaOH 2N and lyophilized to give a dry residue (WRNL, 36.7 mg, 0.073% yield). The organic extractions (hexane, H; ethyl acetate, EtOAc and ethanol, EtOH) were carried out in a Soxhlet for 12 h (131g, 1.2%; 0.76 % and 12.5 % yield respectively). The insoluble material from the H extraction (1.60 g) was filtered, and the solution obtained was washed with 2N NaOH solution. The aqueous layer was acidified with 2N HCl at pH 2 and extracted with t-butylmethylether (E). The oil was dried over anhydrous Na_2SO_4 and preserved in sealed glass bottles. It was protected from light by wrapping in aluminum foil and stored at 4^{o}C until used.

2.4 Determination of sensitivity of *Rhizoctonia* isolates to EOs

144

145 The antifungal activity was analyzed as mycelial growth inhibition by a modified
146 PDA dilution method, which included the addition of 0.05 mg / mL of methyltetrazolium
147 (MTT) salts and extract, tested in three different concentrations: 1; 0.5 and 0.1 mg extract
148 per ml of PDA medium. Control was performed by the same procedure using EtOH as the
149 sample. The assay was performed in 12-well plates. Each well was inoculated in the center
150 with a 10 mm diameter mycelial disc taken from the periphery of the *Rhizoctonia* colony
151 grown in PDA at 25°C for 72 h. The positive control was inoculated following the same
152 procedure. Six replicates were performed for each concentration and the assay was
153 performed in duplicate. The plates were incubated at 27^{o}C for 48 h and the colonies were
154 digitized and measured by Image-J version 1.43. The percent inhibition (% I) was
155 calculated as: % I = (C-T/ C) × 100, where C is the diameter of the colonies. The data were
156 used to estimate IC 50 (concentration capable of reducing the fungus population by 50%)
157 and MIC (minimum inhibitory concentration). At the end of the test three EOs were
158 selected.

159

160 2.5 Analysis of EOs

161

162 The constituents of the EOs were determined by GC-MS using an Agilent 6890N gas
163 chromatograph (Agilent Technologies, Palo Alto, California, USA) coupled to an Agilent
164 5973N mass detector (electron ionization, 70 eV) (Agilent Technologies, Palo Alto,
165 California, USA) and equipped with a 25 m × 0.2 mm id HP-1 (methyl polisiloxane, 0.2
166 µm film thickness) and a 30 m × 0.25 mm id Carbowax (polyethylene glycol, 0.25 µm film
167 thickness) capillary columns (Hewlett-Packard). Working conditions were as follows:

injector temperature, 260°C; temperature of the transfer line connected to the mass spectrometer, 280°C; column temperature 70–190°C, 5°C min^{-1}. EI mass spectra and retention data were used to identify compounds by comparing them with those of standards or found in the Wiley Mass Spectral Database (2001). Quantitative data were obtained from the TIC peak areas without the use of response factors.

2.6 Antifungal activity of main constituents of the selected EOs

Based on the results of GC-MS analysis, the main constituents of the selected EOs were tested at four different concentrations: 0.5; 0.1; 0.05 and 0.01 mg of pure compound per ml of medium by the same procedure described in item 2.5.

2.7 Total Reactive Oxygen Species (ROS)

Total ROS was analyzed using fluorimetric quantitation of dichlorodihydrofluorescein-diacetate (DCDHF-DA) that was oxidized by ROS. For each fungal isolate, the mycelium was cultured at EO concentrations 1, 5, 25 and 50 mg mL^{-1} in PDA medium. The fresh mycelium from each treatment was incubated in 10 µM of DCDHF-DA in Tris-HCl (50 mM, pH 8.0) for 1 h at room temperature in the proportion of 100 mg of fresh micelia per mL of solution, then the tissue was washed with 50 mM EDTA to remove the incubation solution and ground to a fine powder as above and extracted in 1 mL of Tris-HCl (50 mM, pH 8.0). The supernatant obtained from this mixture after centrifugation at 10,000 rpm for 10 min was filtered in Miracloth. The fluorescence (488

nm excitation wavelength, 535 nm emission wavelength, TECAN infinite 200 pro, Austria) was determined for the filtered supernatant (Ross *et al.*, 2008).

2.8 Membrane Peroxidation (MDA)

For each fungal isolate, the mycelium was cultured at EO concentrations 1, 5, 25 and 50 mg mL^{-1} in PDA medium. Fresh tissue was ground to a powder as above and suspended in 1% of trichloroacetic acid (TCA) in the proportion of 50 mg of mycelium per mL. The resultant mixture was centrifuged at 8,000 rpm for 5 min. To the supernatant (250 mL), 1 mL of 0.5% of thiobarbituric acid in 20% TCA was added and the mixture was boiled for 30 min. This was then allowed to cool to room temperature and the adduct formed by TBA-malondialdehyde (MDA) was quantified at 532 and 600 nm using Δ532-600 and 155 mM^{-1} cm^{-1} (Ederli *et al.*, 2004).

2.9 Statistical analysis

The data were analyzed with one-way ANOVA (one-way ANOVA) with Tukey multicomparison analysis using a $P < 0.05$ to assign significant differences.

3 Results

3.1 *In vitro* antifungal activity of EOs and their main chemical components

The study revealed that the two isolates of *Rhizoctonia* belonging to different anastomosis groups behaved differently from the 30 EOs tested (Figure 1). The ANOVA identified significant differences ($p < 0.0001$) for the main effects and also for their interaction in 18 treatments in A and 12 treatments in B. The isolate of *R. solani* (B) was sensitive to few EOs, but these EOs inhibited 100% the mycelial growth of the fungus at the lowest concentration tested, at 0.1 mg mL^{-1}. The EOs that showed this effect were *S. montana* (25) and *T. vulgaris* (26 and 27).

The isolate of *Rhizoctonia* sp. (A) was shown to be more sensitive to a larger spectrum of EOs, but only when tested at a concentration greater than or equal to 0.5 mg mL^{-1}. No EO was able to completely inhibit the mycelial growth of *Rhizoctonia* sp. (A) at 0.1 mg mL^{-1}. The results of the screening counted seven EOs that were efficient in inhibiting the mycelial growth of *Rhizoctonia*, *Mentha rotundifolia* (16), *Oreganum vulgare* (21), *Salvia officinalis* (24), *Satureja montana* (25), *Thymus vugaris* (26 and 27) and *Thymus zygis* (29). Regarding the availability of EOs, EOs 16, 25 and 29 were chosen.

MICs and IC 50 of the selected EOs are presented in Table 2. The three EOs presented low values of IC50 and MIC. The IC 50 were within the range of 0-0.1 mg m^{-1} and the MICs were within the range 0.1-0.5 mg mL^{-1}. The EO of *S. montana* showed the lowest IC 50, and this value in practice may present high phytotoxicity.

Table 3 shows the chemical components identified by mass gas chromatography (GC-MS). Twenty-six chemical compounds were identified in the EO of *M. rotundifolia* and *T. zygis*, piperitone oxide (36.97%) and piperitone (20.77%) were found in greater quantities in the EO of *M. rotundifolia* and Thymol (60.27%), followed by piperitone oxide (6.56%), piperitone (5.18%) and other compounds in a proportion of less than 5% were found in the *T. zygis* EO. In the EO of *S. montana* were identified 14 chemical components,

238 with carvacrol (49.38), p-cymene (28.08) and thymol (8.30) in greater amounts.

239 The antifungal activity of pure compounds in the mycelial growth of *Rhizoctonia*
240 isolates is shown in Table 4. The results showed that only Carvacrol treatment at the
241 concentration 0.01 and 0.05 mg mL^{-1} were significant ($p < 0.0001$) at 28 and 29.8 %
242 inhibition of mycelial growth of *R. solani* (CMM-2099 isolate), respectively. Binucleated
243 Rhizoctonia isolate (CMM-1829) showed no significant sensitivity to any specific pure
244 chemical compound, but it interacted with more treatments when compared to the *R. solani*
245 isolate.

246

247 3.2. Total Reactive Oxygen Species (ROS) and Membrane Peroxidation (MDA)

248

249 Figure 2 shows the quantification of Total reactive oxygen species (ROS) in the
250 treatments tested with the EOs of *M. rotundifolia, T. zygis* and *S. montana*. In *Rhizoctonia*
251 sp. (A), treatment with *S. montana* 5 mg mL^{-1} showed the highest ROS value (127.4 nmol g
252 $^{-1}$) and in *R. solani* (B) treatment with *M. rotundifolia* 5 mg mL^{-1} presented the highest
253 value of ROS (131.9 nmol g^{-1}). All other treatments were significant ($p < 0.0001$) with the
254 exception of the treatment *M. rotundifolia* 1 mg mL^{-1} (B).

255 Figure 3 shows the malondialdehyde content (MDA) of the treatments tested. In
256 *Rhizoctonia* spp. (A) treatment with *S. montana* 5 mg mL^{-1} presented the highest value of
257 MDA (5.9 nmol g^{-1}) and in *R. solani* (B) treatment with *M. rotundifolia* 5 mg mL^{-1}
258 presented the highest value of MDA (5.4 nmol g^{-1}). ANOVA revealed that all treatments
259 were significant ($p < 0.0001$) for the main effects and their interaction.

260

261 **4. Discussion**

In this study, the antifungal activity of thirty EOs obtained from different plants was studied against aggressive *Rhizoctonia* isolates in *in vitro* and *in vivo* assay (data not shown). The two *Rhizoctonia* isolates used in this study belong to distinct AGs and represent the AGs most found in potato production fields with rhizoctoniosis. Isolates from different *Rhizoctonia* AGs may react differently to control measures (Campion *et al.*, 2003; Özer & Bayraktar, 2015) and provide valuable information on how fungal populations evolve in response to disturbance or environmental stressors.

A larger number of EOs had an effect on the mycelial growth of the AG-R isolate, but only completely inhibited the fungus at a concentration higher than that observed for AG-3 PT isolate. In contrast, the isolate AG-3 PT showed sensitivity to a restricted group of EOs, but in a lower concentration. Brasier (1999) discusses the presence of environmental tolerance genes in phytopathogenic fungi. These genes confer specific host virulence, resistance to fungicides, toxins, and heavy metals. Understanding this behavior may provide future tools to avoid problems of resistance or overpopulation.

Phenotypic mycelial growth assays are important for the fungus *Rhizoctonia*. Due to the absence of conidia and the lack of sexual spores, together with the sclerotia, the mycelium represents the main form of reproduction of the fungus. The mycelium is extremely important for the fungus in general, because it supports all fungal activity, from the germination of the spores to the formation of the fruiting body. It is very sensitive to small variations of the environment such as chemical constituents, temperature and light and is also extremely sensitive to the internal environment related to the cellular level of the hyphae. If the mycelium is viable, its relative genetic ability to colonize and use trade-off, multiply and reproduce is greater, making the population of the fungus more competitive,

consequently more adapted to the environment. Mycelial growth is an important component of fitness and is easy to measure. The fitness of the isolates tends to be the ability of these isolates to survive and reproduce in a given environment (Antonovics & Alexander, 1989; McDonald, 1997; Brasier, 1999). (Antonovics & Alexander, 1989) argue that the whole fungus needs to combine its fitness genome to different stages of its life cycle. Some combine more of a lifestyle (saprophytic and parasitic), as is the case with *Rhizoctonia*.

The screening revealed seven EOs that were effective in the mycelial inhibition of the aggressive isolates of *Rhizoctonia, M. rotundifolia, O. vulgare, S. officinalis, S. montana, T. zygis* and *T. zygis.* In the literature there are many studies, mainly *in vitro* studies, showing the antifungal activity of *R. solani* from *T. vulgaris* EOs (Zambonelli *et al.*, 1996), *Salvia Fruticosa* (Pitarokili *et al.*, 2003), *M. piperita* (Zambonelli *et al.*, 2004; Lee *et al.*, 2007; Khaledi *et al.*, 2015), *Monarda* sp. (Gwinn *et al.*, 2010), *Calocedrus macrolepis* var. *formosana* (CHANG *et al.*, 2008) and *Bunium persicum* (Khaledi *et al.*, 2015).

Our results showed that *S. montana* (25) and *T. vulgaris* (26 and 27) oils when tested at the concentration of 0.1 mg mL^{-1} (100 ppm) and the oils of *M. rotundifolia* (16), *O. vulgare* (21), *S. officinalis* (24), *S. montana* (25), *T. vulgare* (26 and 27) and *T. zygis* (29) when tested at the concentration of 0.5 mg mL^{-1} (500 ppm) were effective in completely inhibiting mycelial growth of the fungus. Seema & Devaki (2010) also observed that when cinnamon oil (*Cinnamomum zeylanicum* Breyne) was used at the 500 ppm concentration it completely inhibited the mycelial growth of *R. solani*. Muchembled *et al.* (2017) studied some EOs against *Venturia inaequalis* strains of apples with different sensitivities to Tebuconazole compared to the application of copper sulphate and emphasized the effectiveness of clove EOS (*Syzygium aromaticum*), eucalyptus (*Eucalyptus citriodora*), mint (*Mentha spicata*) and savory (*S. montana*) with priority components such as eugenol

and carvacrol. They also found that each strain of the fungus reacted differently to each treatment, indicating that each strain of the pathogen had different survival mechanisms.

GC-MS identified many chemical components present in the oils of *M. rotundifolia*, *T. zygis* and *S. montana*. Concentrations of piperitone and piperitone oxide found in *M. rotundifolia* oil were similar to those found by Brada *et al.* (2006). Carvacrol and thymol found in high concentrations in the oils of *T. zygis* and *S. montana* and p-cymene and y-terpinene have proven antimicrobial activity, being the last biosynthetic precursors of thymol and carvacrol (Nhu-Trang *et al.*, 2006).

In our study, we also verified that the great antifungal efficiency of *S. montana* oil is mainly due to carvacrol. Carvacrol tested at concentrations 0.01 and 0.05 mg mL^{-1} inhibited the mycelial growth of the AG-3 PT isolate significantly, confirming this hypothesis. Fraternale *et al.* (2007)observed that *S. montana* oil with 18% carvacrol tested at MIC of 250 µl/ml was effective against nine species of phytopathogenic fungi, including *R. solani.* The presence of carvacrol, p-cymene, thymol and γ-terpinene in the oil were associated with mycelial inhibition of other fungi such as *Penicillium expansum* and *Botrytis cinerea* (Lopez-Reyes *et al.*, 2010) and *Aspergillus niger* (Moghtader, 2012).

The plants of the Labiatae and Laminaceae families are rich in terpenes. The quality and quantity of the EOs depend not only on the variety of the plant, but also on the location, harvesting time and plant phenological status. These parameters alter the composition of the volatile terpenoids and their concentration (Gwinn *et al.*, 2010). In *S. Montana* oil, for example, in summer there is a higher concentration of carvacrol and in winter a higher concentration of p-cymene (Mastelić & Jerković, 2003).

Our results showed that the inhibition of mycelial growth of *Rhizoctonia* by other chemical components was variable. Gwinn *et al.* (2010) studied the composition, antifungal

activity and suppression of the disease caused by *R. solani* in 13 EOs of *Monarda* sp. The EOs that were effective in reducing mycelial growth of the fungus had a functional group containing oxygen. Thymol and carvacrol (phenolic monoterpenes) reduced mycelial growth by more than 65% when tested at the highest concentration (5 μmol/dish). For alcohols, such as 1-octen-3-ol and borneol, the reduction of growth was lower. The authors also observed that EOs that did not have an oxygen-containing functional group did not inhibit the growth of *R. solani* (limonene, mirrene and a-pinene) or the reduction of mycelial growth was less than 20% (β-pinene, thyroquinone , γ-terpinene and cymene). In addition, they found that all compounds that reduced *R. solani* growth by more than 25% were oxygenated monoterpenes.

The TBARS tests identified that *S. montana* oil at 5 mg mL^{-1} caused greater oxidative stress in the AG-R isolate and *M. rotundifolia* oil at 5 mg mL^{-1} caused greater oxidative stress to the AG-3 PT isolate. In the case of the dichlorodihydrofluorescein (DCF) assay the total amount of ROS may be radicals such as hydroxyl, as well as singlet oxygen, superoxide anion, but mainly hydrogen peroxide. The quantification of malondialdehyde (MDA) in the TBARS test indicates an estimate of membrane lipoperoxidation. Rellán (2013) studied different species of *Satureja* and observed that *S. montana* oil showed to be more efficient in the inhibition of *R.solani* and greater antioxidant activity. The antioxidant activity was attributed to a higher amount of total phenolics. Fenois provide plants with a defense mechanism to neutralize reactive oxygen species (ROS), for survival and to prevent molecular damage and by microorganisms or insects (Dzamic *et al.*, 2016). Phenols have redox properties by the mechanisms of free radical scavenging, transition-metal-chelating activity and singlet oxygen-quenching capacity, stabilization of lipid peroxidation and inhibition of oxidizing enzymes (Serrano *et al.*, 2011). Mihajilov-Krstev *et al.* (2014)

358 reported that the extract of *S. montana* showed a better ability to reduce the stable DPPH
359 radical, which is linked to a higher concentration of active components such as thymol,
360 carvacrol and thymoquinone, as well as by the inhibition of the cholinesterase enzyme.
361
362 **Conclusion**
363
364 Our study identified the oils of *M. rotundifolia, O. vulgare, S. officinalis, S. montana,*
365 *T. vulgare* and *T. zygis* with antifungal activity to *Rhizoctonia* AG-3 PT and AG-R and we
366 proved to be carvacrol the main chemical component associated with antifungal activity
367 against fungus. The study also revealed that isolates from different AGs responded
368 differently to the concentration tested and the efficiency of the selected oils was confirmed
369 by the measurement of oxidative stress. The difference in the response of the isolates to the
370 oils suggests the identification of the AG of the fungus and better targeting of the control
371 measure. The study with essential oils is a promising window for the management of
372 *Rhizoctonia* and suggests future studies of field oil efficiency and application technology.
373
374 **Acknowledgment**
375
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379
380 **References**
381

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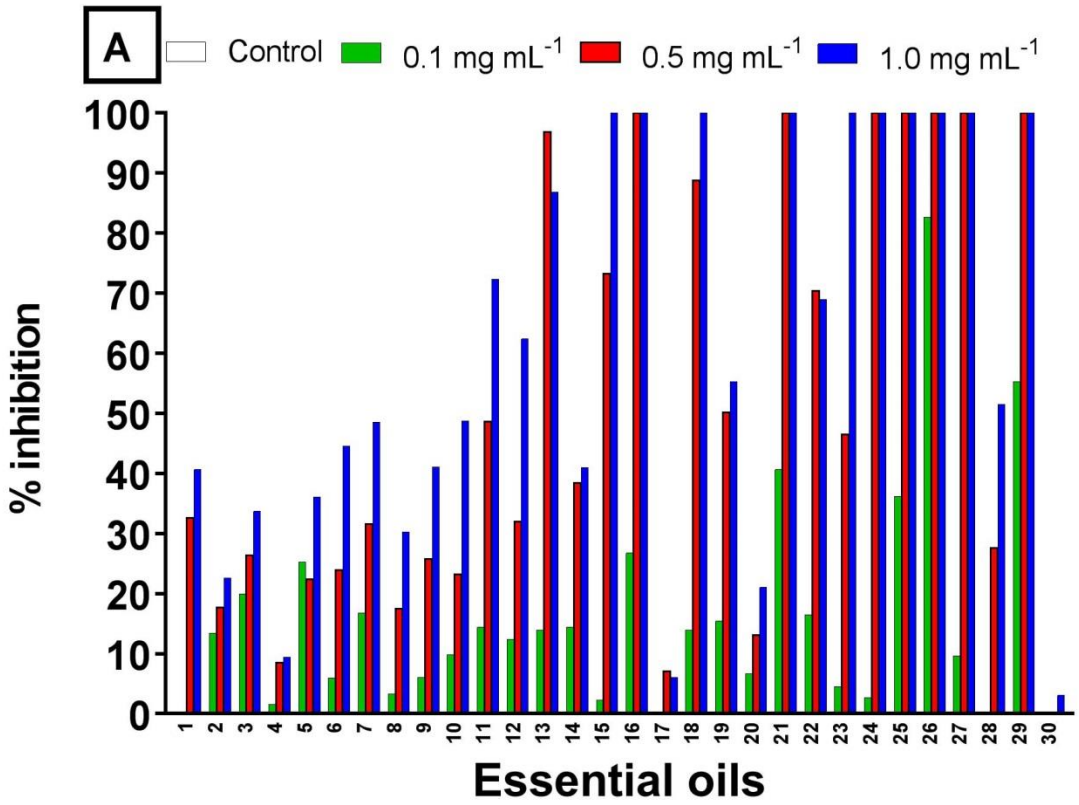
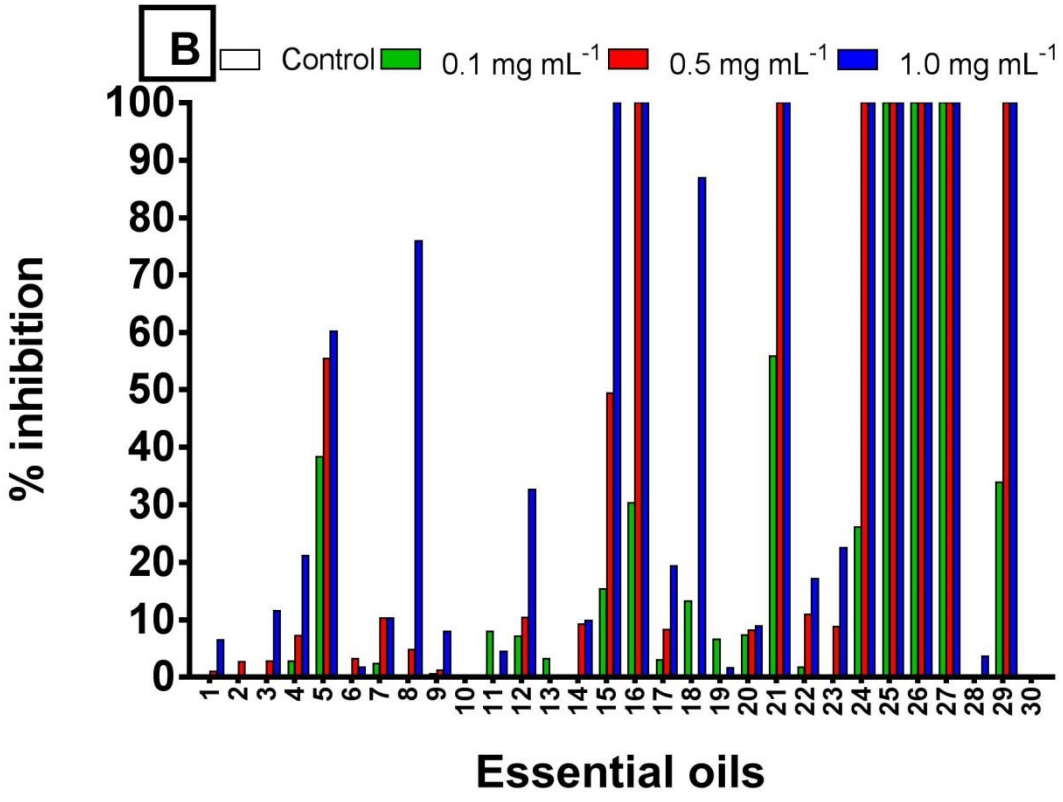
Table 1. List of plant species, common names, family and part used in the screening for antifungal properties.

Nº	Plant species	Family	Plant part used
1	*Porlieria chilensis*	Zygophyllaceae	Leaves and flowers
2	*Lippia alba*	Verbenaceae	Leaves and flowers
3	*Mentha rotundifolia*	Labiatae	Leaves and flowers
4	*Mentha longifolia*	Labiatae	Leaves and flowers
5	*Mentha spicata*	Labiatae	Leaves and flowers
6	*Mentha arvensis*	Labiatae	Leaves and flowers
7	*Mentha piperita*	Labiatae	Leaves and flowers
8	*Thymus vulgaris*	Labiatae	Leaves and flowers
9	*Thymus mastichina*	Labiatae	Leaves and flowers
10	*Thymus zygis*	Labiatae	Leaves and flowers
11	*Tanacetum vulgare*	Asteraceae	Leaves and flowers
12	*Artemisia dracunculus*	Asteraceae	Leaves and flowers
13	*Artemisia absinthium*	Asteraceae	Leaves and flowers
14	*Lavandula angustifolia*	Labiatae	Leaves and flowers
15	*Lavandula lanata*	Labiatae	Leaves and flowers
16	*Lavandula luisieri*	Labiatae	Leaves and flowers
17	*Dittrichia graveolens*	Asteraceae	Leaves and flowers
18	*Aloysia fiebrigii*	Verbenaceae	Leaves and flowers
19	*Elettaria cardamomum*	Zingiberaceae	Leaves and flowers
20	*Hyssopus officinalis*	Labiatae	Leaves and flowers
21	*Salvia officinalis*	Labiatae	Leaves and flowers
22	*Satureja montana*	Labiatae	Leaves and flowers
23	*Oreganum vulgare*	Lamiaceae	Leaves and flowers


A
Control
0.1 mg mL-1
0.5 mg mL-1
1.0 mg mL-1
% inhibition
Essential oils

537


B
Control
0.1 mg mL-1
0.5 mg mL-1
1.0 mg mL-1
% inhibition
Essential oils

538

Figure 1. Effect of different concentrations of essential oils on the mycelial growth of *Rhizoctonia* from different anastomosis groups. The data were analyzed statistically by the program Graphpad prism version 7.0. (A) AG-R (*Rhizoctonia* sp.) and (B*)* AG-3 PT (*Rhizoctonia solani*). Essential oils: *Aloysia fiebrigii* (1); *Artemisia dracunculus* (2); *Artemisia absinthium* (3); *Artemisia absinthium* (4); *Artemisia tridentada* (5); *Dittrichia graveolens* (6); *Dittrichia graveolens* (7); *Elettaria cardamomum* (8); *Hyssopus officinalis* (9); *Lavandula angustifólia* (10); *Lavandula luisieri* (11); *Lavandula luisieri* (12); *Lippia alba* (13); *Lippia alba* (14); *Mentha rotundifolia* (15); *Mentha rotundifolia* (16); *Mentha longifolia* (17); *Mentha spicata* (18); *Mentha arvensis* (19); *Mentha piperita* (20); *Oreganum vulgare* (21); *Porlieria chilensis* (22); *Salvia officinalis* (23); *Salvia officinalis* (24); *Satureja montana* (25); *Thymus vulgaris* (26); *Thymus vulgaris* (27); *Thymus mastichina* (28); *Thymus zygis* (29); *Tanacetum vulgare* (30).

563 **Table 2.** *In vitro* antifungal activity of the selected essential oils against the mycelial
564 growth of *Rhizoctonia* from different anastomosis groups.

Essential oils	AG-R		AG-3 PT	
	IC 50	MIC	IC 50	MIC
Mentha rotundifolia (sample 16)	0.1	0.5	0.1	0.5
Satureja montana (sample 25)	~ 0.0	0.1	0.1	0.5
Thymus zygis (sample 29)	0.1	0.5	0.1	0.5

565

566 MIC: minimum inhibitory concentration (mg mL^{-1}); IC50: inhibitory concentration with
567 50% inhibitory effect on the fungal growth (mg mL^{-1}).
568 (~) Value approximately zero. AG-R (*Rhizoctonia* sp.); AG-3 PT (*Rhizoctonia solani*).
569 Essential oils chosen due to the availability of the tests.
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581 **Table 3.** Chemical components of essential oils of *Mentha rotundifolia* (A), *Thymus zygis* (B) and *Satureja Montana* (C).

A

	Mentha rotundifolia	
Nº	Compound name	Percentage (%)
1	Piperitone	20,77
2	Piperitone oxide	36,97
3	germacrene-d	7,39
4	Limonene	6,63
5	trans-caryophyllene	6,07
6	trans-caryophyllene	6,07
7	endobornyl	1,92
8	farnesol	1,85
9	β-pinene	1,65
10	1-octen-3-yl acetate	1,24
11	β-myrcene	1,21
12	α-pinene	0,94
13	epi-bicyclosesquiphellandrer	0,84
14	α-humulene	0,83
15	cis-ocimene	0,63
16	viridiflorol	0,51
17	p-cymen-8-ol	0,51
18	β-elemene	0,46
19	β-cubenene	0,41
20	endoborneol	0,39
21	cis jasmone	0,35
22	β-bourbonene	0,31
23	4-terpineol	0,25
24	gama-terpinene	0,25
25	Thymol	t
26	Carvacrol	t
	Total identified	98,45

B

	Thymus zygis	
Nº	Compound name	Percentage (%)
1	Thymol	60,27
2	piperitenone	5,18
3	piperitone oxide	6,56
4	endoborneol	4,60
5	Linalol	3,90
6	trans-caryophyllene	2,81
7	germacrene-d	2,46
8	Cinerolone	2,29
9	Caryophyllene ox,	1,35
10	viridiflorol	1,07
11	delta-cadinene	1,06
12	Camphor	0,99
13	trans sabinene hydrate	0,94
14	p-Cymene	0,87
15	β-farnesene	0,81
16	germacrene-d	0,66
17	Terminen-4-ol	0,55
18	epi-bicyclosesquiphellandrene	0,54
19	α-humulene	0,53
20	Spathulenol	0,49
21	γ-Terpinene	0,44
22	γ-cadinene	0,43
23	t-muurolol	0,34
24	γ-Muurolene	0,30
25	1,8-cineole	0,30
26	1,10-epi-Cubenol	0,26
	Total identified	100,00

C

	Satureja montana	
Nº	Compound name	Percentage (%)
1	Carvacrol	49.38
2	p-Cymene	28.08
3	Thymol	8.30
4	Thymoquinone	2.81
5	1-Octen-3-ol	1.74
6	l-Limonene+1,8-Cineol	1.64
7	Borneol	1.25
8	trans-Sabinene hydrate	1.22
9	(-)-α-Pinene	0.93
10	Linalool	0.93
11	β-bisabolene	0.90
12	α-Thujene	0.76
13	Terpinen-4-ol	0.75
14	α-Terpinene	0.47
	Total identified	99.16

582

583 t = trace (< 0,1)

584 **Table 4.** Behavior of *Rhizoctonia* isolates from different anastomosis groups the different

585 concentrations of pure chemical constituents.

	Inhibition of mycelial growth (%)							
	AG-R (*Rhizoctonia* sp.)				AG-3 PT (*Rhizoctonia solani*)			
Treatment	Concentration (mg mL^{-1})				Concentration (mg mL^{-1})			
	0.01	0.05	0.1	0.5	0.01	0.05	0.1	0.5
Control	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a
cis-Ocimene	0.0 ± 0.0a	0.8 ± 0.9a	5.6 ± 1.3a	3.3 ± 1.7a	0.0 ± 0.0a	0.0 ± 0.0a	2.6 ± 1.9a	2.8 ± 2.7a
Linalool	5.3 ± 1.0a	2.8 ± 1.7a	1.8 ± 1.0a	1.6 ± 1.6a	0.0 ± 0.0a	1.3 ± 1.9a	4.0 ± 1.5a	0.7 ± 1.2a
γ -Terpinene	5.8 ± 1.6a	7.6 ± 2.4a	1.8 ± 1.5a	0.9 ± 1.0a	0.0 ± 0.0a	0.7 ± 1.3a	0.0 ± 0.0a	2.2 ± 0.9a
Borneol	10.5 ± 2.8a	5.6 ± 1.1a	4.9 ± 2.2a	4.1 ± 1.2a	0.2 ± 1.2a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a
Caryophyllene	7.0 ± 1.6a	5.6 ± 1.0a	8.3 ± 2.8a	0.0 ± 0.0a	0.0 ± 0.0a	2.5 ± 2.9a	0.0 ± 0.0a	0.1 ± 1.5a
Carvone	4.1 ± 1.9a	1.5 ± 1.4a	2.6 ± 2.4a	4.0 ± 2.8a	6.5 ± 1.8a	5.7 ± 1.4a	13.3 ± 1.6a	17.6 ± 4.1a
Camphor	7.5 ± 2.0a	6.6 ± 1.9a	4.8 ± 1.6a	1.9 ± 1.9a	4.4 ± 2.0a	1.6 ± 1.8a	3.1 ± 1.1a	0.0 ± 0.0a
Limonene	6.4 ± 2.3a	3.9 ± 1.2a	1.1 ± 1.8a	4.8 ± 1.6a	3.6 ± 1.4a	4.1 ± 1.5a	15.5 ± 4.1a	7.4 ± 1.7a
Thymol	6.8 ± 1.7a	5.0 ± 1.1a	3.3 ± 1.2a	3.6 ± 1.9a	5.6 ± 1.7a	3.5 ± 2.9a	1.7 ± 1.8a	0.8 ± 1.6a
Carvacrol	4.6 ± 1.0a	3.0 ± 1.6a	4.4 ± 1.7a	8.4 ± 1.9a	**28.0 ± 5.9b**	**29.8 ± 4.5b**	1.5 ± 1.3a	10.2 ± 2.6a
Piperitenone	7.0 ± 1.3a	10.2 ± 2.3a	5.5 ± 1.4a	4.9 ± 1.3a	3.3 ± 0.9a	5.8 ± 1.2a	15.8 ± 2.8a	18.1 ± 5.4a
α/β-Thujone	4.8 ± 1.1a	3.1 ± 1.5a	4.4 ± 1.0a	1.1 ± 0.5a	1.4 ± 1.4a	0.0 ± 0.0a	0.0 ± 0.0a	2.6 ± 1.1a
Cineol	3.3 ± 1.6a	0.2 ± 1.5a	7.6 ± 2.5a	0.1 ± 1.1a	0.5 ± 1.2a	0.4 ± 2.7a	0.9 ± 1.6a	1.0 ± 0.6a
β-Pinene	1.4± 1.8a	2.6 ± 1.7a	9.5 ± 1.7a	0.0 ± 0.0a	0.0 ± 0.0a	2.1 ± 1.8a	0.0 ± 0.0a	4.0 ± 1.2a
α-Terpinolene	0.0 ± 0.0a	8.1 ± 1.4a	8.9 ± 3.8a	3.5± 2.1a	2.2± 2.6a	0.9 ± 1.9a	3.2 ± 2.5a	0.2 ± 1.2a

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587 The results are means ± standard errors of six replications. Means within a column

588 indicated by same letter were not significantly different according to Tukey´s multiple test

589 at the level $P < 0,05$.

590 Chemical components available and tested.

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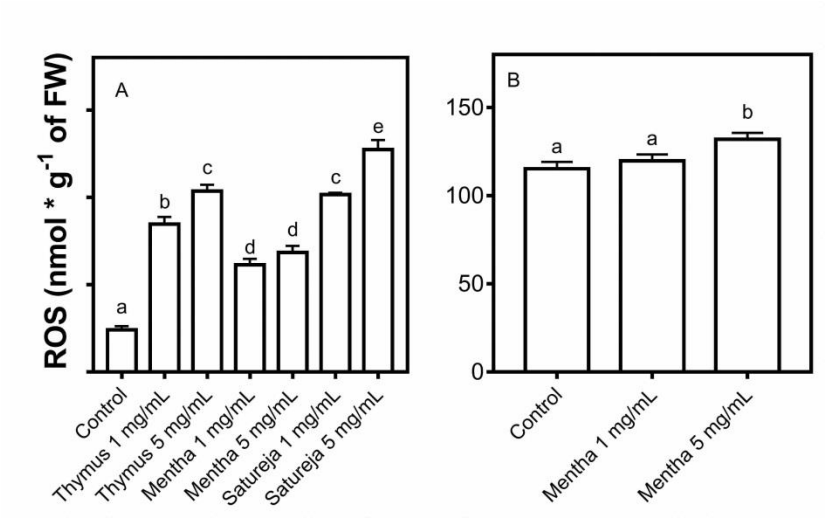

Figure 2. Reactive oxygen species (ROS) from the essential oils of *Mentha rodundifolia*, *Thymus zygis* and *Satureja montana* to *Rhizoctonia*. (A) AG-R (*Rhizoctonia* sp.) and (B) AG-3 PT (*Rhizoctonia solani*). Means within a column indicated by same letter were not significantly different according to Tukey´s multiple test at the level $P < 0,05$. Treatments not shown in the graph did not show fungal growth.

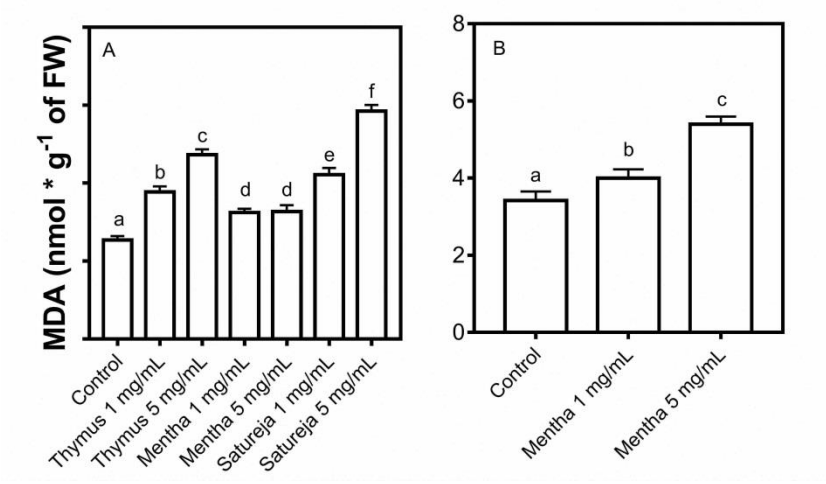

Figure 3. Quantification of malondialdehyde (MDA) from the essential oils of *Mentha rodundifolia*, *Thymus zygis* and *Satureja montana* to *Rhizoctonia*. (A) AG-R (*Rhizoctonia* sp.) and (B) AG-3 PT (*Rhizoctonia solani*). Means within a column indicated by same letter

were not significantly different according to Tukey´s multiple test at the level $P < 0,05$. Treatments not shown in the graph did not show fungal growth.

Conclusões Gerais

CONCLUSÕES GERAIS

1. Há diferenças de adaptabilidade saprofítica e patogênica entre os três principais grupos de anastomose (AGs) de *Rhizoctonia* associados à batata no Brasil, quando comparados sob a influência da temperatura, potencial hídrico, salinidade, pH e agressividade em diversas espécies de plantas;

2. Isolados de AG-4 HGI possuem maior potencial adaptativo saprofítico e patogênico que os isolados de AG-3 PT e AG-R;

3. Óleos essenciais de *Mentha rotundifolia*, *Oreganum vulgare*, *Salvia officinalis*, *Satureja montana*, *Thymus vugaris* e *Thymus zygis* possuem atividade antifúngica a *Rhizoctonia* do AG-3 PT e AG-R associados à rizoctoniose da batata;

4. Tratamentos com óleo de *Mentha rotundifolia* apresentou maiores valores de ROS e MDA a AG-3 PT e os tratamentos com *óleo de Satureja montana* revelou maiores valores de ROS e MDA a AG-R.