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EM FITOPATOLOGIA**

Tese de Doutorado

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

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**Recife – PE
2018**

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***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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minha mãe Sueli Nazareth Christ da
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força, honestidade e humildade.*

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“Tenho a impressão de ter sido uma criança brincando à beira-mar,
divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais
bonita que as outras, enquanto o imenso oceano da verdade continua
misterioso diante de meus olhos”.
(Isaac Newton)

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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; saprophytic 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 água é até 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 está 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% das 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 cancrios 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 (Tsrör 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 rizomorfias (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. zae* 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 evolucionária 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, respectivamente (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 saprofitica 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 manjeriço (*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

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

3

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18

19 **Abstract**

20

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

24 different conditions of temperature, pH, water potential, salinity and fungicides, as well as
25 pathogenic adaptability in relation to aggressiveness in several plant species. The optimum
26 temperature for AGR-R (29.5° C) growth was higher than the others and AG-4 HGI had the
27 highest mycelial growth (96.6 mm). The pH levels tested (5, 6 and 7) did not influence the
28 mycelial growth of the three GAs, as well as the interaction between AGs and pH levels
29 was not significant. For the three AGs the mycelial growth reduced with decreasing water
30 potential. AG-4 HGI and AG-R showed the highest growths at -0.8 MPa, while the former
31 also stood at -1.6 and -3.2 MPa. No isolates of *Rhizoctonia* grew at 6 and 8% salinity.
32 There was a significant difference in the mycelial growth between the AGs and between the
33 salinity levels. AG-3 PT showed the lowest mycelial growth in the presence of salinity
34 (43.6 mm) and the growth of the isolates reduced significantly with the elevation of the
35 salinity level. The isolates of the three AGs showed a reduction in mycelial growth in the
36 presence of fungicides Fluazinam, Fludioxonil, Pencycurom and Procymidone, but differed
37 in sensitivity only in relation to Fluazinam, with AG-3 PT showing the greatest reduction of
38 growth (95.9%). All isolates induced symptoms in the 14 inoculated plant species and AG-
39 4 HGI was the most aggressive, except in castor bean, which did not differ from AG-R. In
40 all analyzed situations, the AG-4 HGI isolates showed a greater saprophytic and pathogenic
41 adaptive potential than the AG-3 PT and AG-R isolates.

42

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

45

46 **Introduction**

47

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

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

56 Potato rhizoctoniosis is more common from emergence to heap, a period extending
57 from 25 to 40 days after planting, and the symptoms are rot and stem and root cankers, pre
58 and post-emergence tipping, burning and plant death, tuber rot, spotting and burning of
59 leaves and shoots and formation of sclerotia on the surface of tubers, also known as black
60 crust (Banville et al. 1996; Tsror 2010; Lima et al. 2015). The control of rhizoctoniosis in
61 potatoes is very difficult, because the pathogen survives in the soil, in tubers and cultural
62 remains in the form of hyphae or sclerotia, it has a wide range of host plants, high
63 aggressiveness and great genetic diversity (Tsror 2010; Muzhinji et al. 2015). In addition,
64 potato cultivars with acceptable levels of disease resistance are not available (Tsror 2010)
65 and the efficacy of available fungicides is variable (Campion et al. 2003; Lehtonen et al.
66 2008; Özer & Bayraktar 2015).

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

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

76 Several AGs have been associated with potato rhizoctoniosis worldwide, with a
77 predominance of AG-3 PT (Tsrer 2010). In Brazil, few papers on the diversity of
78 *Rhizoctonia* AGs in potatoes have been published. In a pioneer work, 10 isolates were
79 evaluated by hypophysial pairing with test isolates, with AG-3 and AG-4 being identified
80 (Bolkan & Ribeiro 1985). Afterwards, 16 isolates were characterized by the pairing with
81 isolated isolates and sequence analysis of the internal transcribed spacer region (ITS) of the
82 ribosomal DNA (rDNA), being classified as AG-4 HGI, AG-4 HGII and AG-7 (Rosa et al.
83 2005). Recently, 66 isolates obtained in the major producing regions of Brazil were
84 characterized by the analysis of ITS sequences and classified as AG-3 PT (37.3% of the
85 isolates), AG-4 HGI (29.9%), AG-R (22.4%), AG-2-1 (4.5%), AG-A (4.5%) and AG-1B1
86 (1.5%) (Inokuti 2016).

87 The importance of determining the anastomosis groups is due to the fact that each
88 group can be considered an evolutionary unit, since they represent genetically isolated and
89 non-interconnected populations (Anderson 1982; Carling 1996; González García et al.
90 2006). Therefore, the knowledge of the anastomosis groups and subgroups has great
91 importance in understanding the genetic diversity of *Rhizoctonia* and the possible
92 epidemiological influences of this diversity in disease control measures (Anderson 1982;

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

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

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

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

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

123

124 **Material and methods**

125

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

134

135 **Effect of temperature on mycelial growth Mycelial:** Discs (5 mm diameter) were
136 removed from the colony margin of each isolate with seven days growth and transferred to
137 the center of Petri dishes (100 mm diameter) containing PDA. Three plates of each isolate
138 were placed in incubators with controlled temperatures at 10, 15, 20, 25, 30, 35 and 40° C
139 in the dark. Prior to use, incubators were equilibrated at respective temperatures for at least
140 12 hours. The experimental design was completely randomized, in a factorial arrangement

141 15x7, with three replicates (plates) by combination of isolates and temperature. The
142 diameter of each colony was measured with 48 hours of incubation in two perpendicular
143 directions and the mean (mm) was obtained.

144

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

152

153 **Effect of water potential (Ψ s) on mycelial growth:** Mycelial discs (5 mm) were removed
154 from the colony margin of each isolate with seven days of growth in PDA and transferred
155 to the center of Petri dishes containing PDA supplemented with KCl to obtain (Ψ s) of -0.8,
156 -1.6 and -3.2 MPa, according to Michel & Radcliffe (1995). The plates were incubated in
157 the dark at 25° C. The experimental design was completely randomized, in a 15x3 factorial
158 arrangement, with three replications per combination of isolates and water potential level.
159 The diameter of the colonies was evaluated as previously described.

160

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

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

168 **Effect of fungicides on mycelial growth:** The commercial formulations of Fluazinam
169 fungicides (Frowncide 500 SC, 500g active ingredient (i.a)/L, ISK Biosciences Ltda., São
170 Paulo - SP), Fludioxonil (Maxim, 25g i.a/L, Syngenta Proteção de Cultivos Ltda., São
171 paulo – SP), Pencicuirom (Monceren 250 SC, 250g i.a/L, Bayer SA, São Paulo - SP) and
172 Procymidone (Sumilex 500 WP, 500g a.i/L, Sumitomo Chemical Ltda., São Paulo - SP),
173 registered in the Ministry of Agriculture for the control of *R. solani* in potato (MAPA
174 2018), representing different chemical groups and modes of action. The fungicides were
175 dissolved in sterile distilled water and added to the PDA flux medium (45° C) to reach the
176 concentrations of 10 µg i.a./mL (Fluazinan) and 1 µg i.a./ml (Fludioxonil, Pencicuirom and
177 Procymidone). Mycelial (5 mm diameter) discs were removed from the colony margin of
178 each isolate with seven days of growth in PDA and transferred to the center of Petri dishes
179 containing PDA supplemented with each fungicide. Plates containing PDA without
180 fungicide were used as controls. The experimental design was completely randomized, in a
181 15x4 factorial arrangement, with three replicates (plaques) by combination of isolates and
182 fungicides. The diameter of the colonies was evaluated as previously described and the
183 percentage inhibition of mycelial growth against the control without fungicide was
184 calculated.

185

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

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

212 and then covered with the substrate. Witches were inoculated with rice grains not colonized
213 by the fungus. The plant species were evaluated separately and for each species the
214 experimental design was completely randomized, with 15 treatments (isolated), four
215 replications per treatment and nine replication plantings containing one seed potato or seed
216 per pit. The pots and trays were kept in a greenhouse and the plants irrigated daily to
217 maintain field capacity. The aggressiveness of the potato isolates was evaluated at 28 days
218 after inoculation, based on the relative size of the necrotic area in the subterranean stems,
219 with the following scale: 0 = no symptoms, 1 = less than 10%, 2 = 10-50%, 3 = 50-100%,
220 and 4 = dead plant (Yang et al., 2014). The aggressiveness of the isolates in the other plant
221 species was evaluated at 12 days after inoculation, by quantifying the severity of the
222 disease using the following diagrammatic scale: 0 = no symptoms, 1 = hypocotyl with
223 small lesions, 2 = hypocotyl with (3) = totally constricted hypocotyl, showing tipping, and
224 4 = non-germinated seeds and / or non-emerged seedlings (Noronha et al. 1995). With the
225 data of the evaluations the disease severity index was calculated in each repetition,
226 according to McKinney (1923).

227

228 **Statistical analyzes:** The experiments were repeated to confirm the observations. Data
229 from the two independent replicates for each experiment were pooled after performing the
230 variance homogeneity analysis using the Levene test and no heterogeneity was detected ($P >$
231 0.05). In the temperature experiment, the data were submitted to linear and non-linear
232 regression analysis, having temperature as independent variable and mycelial growth as
233 dependent variable. The optimum temperature (temperature that provided the greatest
234 mycelial growth) and maximum mycelial growth (at the optimum temperature) were
235 estimated using the regression model and the numerical summary (second derivative) with

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

245

246 **Results**

247

248 **Effect of temperature on mycelial growth:** All *Rhizoctonia* isolates belonging to AG-3
249 PT, AG-4 HGI and AG-R were grown at 10° C and did not grow at 40° C, whereas AG-PT
250 isolates also did not grow at 35° C. The temperatures influenced the mycelial growth of the
251 isolates of the three AGs. The fifth-degree polynomial regression model ($y = a + bx^2 + cx^4$
252 $+ dx^6 + ex^8 + fx^{10}$) provided an excellent adjustment to the mycelial growth data as a
253 function of temperature for all isolates, with R² values ranging from 0.984 to 0.998. With
254 the calculation of the derivatives of the parameters of this regression it was possible to
255 estimate the optimal temperature for mycelial growth and maximum mycelial growth.
256 Isolates belonging to the same AG did not differ among themselves (P = 0.3411) in relation
257 to mycelial growth at different temperatures. The AGs differed in relation to the optimum
258 temperature for growth (P = 0.0216), with AG-R requiring the highest temperature (29.5°
259 C) and the lowest AG-3 PT (24.4° C). At the optimum temperature, AG-4 HGI presented

260 the highest mycelial growth (96.6 mm) and AG-3 PT the lowest growth (74.4 mm) (Table
261 2).

262

263 **pH effect on mycelial growth:** The pH levels tested (5, 6 and 7) did not influence the
264 mycelial growth of *Rhizoctonia* isolates belonging to the three AGs ($P = 0.2291$), as well as
265 the interaction between AGs and pH levels was not significant ($P = 0.1762$). Isolates from
266 the same AG did not differ among themselves ($P = 0.2738$) in relation to mycelial growth at
267 different pH levels. However, there was a significant difference ($P < 0.0001$) between the
268 AGs in relation to mycelial growth, especially AG-4 HGI, which presented higher growth
269 (97.6 mm), followed by AG-R (90.9 mm) and AG-3 PT (72.5 mm).

270

271 **Effect of water potential (Ψ s) on mycelial growth:** *Rhizoctonia* isolates belonging to the
272 three AGs increased at the levels of Ψ s tested. The Ψ s significantly influenced the mycelial
273 growth of the isolates from the three AGs, as well as the interaction between AGs and Ψ s
274 levels was significant ($P < 0.0001$). Isolates of the same AG did not differ among them ($P =$
275 0.1193) in relation to the mycelial growth in the evaluated levels. On the other hand, AGs
276 differed ($P = 0.02519$) in relation to mycelial growth in the Ψ s levels. For the three AGs the
277 growth reduced with the decrease of Ψ s. AG-4 HGI and AG-R showed the highest growths
278 at -0.8 MPa, without differing between them, while the former also stood out at -1.6 and -
279 3.2 MPa, differing from the other AGs (Table 3) .

280

281 **Effect of salinity on mycelial growth:** No *Rhizoctonia* isolate grew at 6 and 8% salinity
282 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, Pencicuum 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).

307

308 **Discussion**

309

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

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

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

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

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

349 The reduction of the mycelial growth of the AGs with the decrease of Ψ_s resembles
350 that registered for fungi (Deacon 2006), as well as in particular for *Rhizoctonia* (Sherwood
351 1970; Dubé et al. 1971; Sterne & McCarver 1978; Ritchie et al. 2006). Water availability is
352 one of the most important environmental factors that affect fungal activity (Deacon 2006,
353 Palacios et al. 2014). The fungi need to maintain the internal Ψ_s lower than the total Ψ_s of

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

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

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

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

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

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

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

401

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403

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406

407 **Compliance with Ethical Standards**

408

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413

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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*

- 601 ² CMM = Collection of Phytopathogenic Fungi Cultures "Prof. Maria Menezes" from the Federal Rural
602 University of Pernambuco (Recife, Pernambuco, Brazil)
- 603 ³ BA = Bahia, DF = Distrito Federal, GO = Goiás, MG = Minas Gerais, SP = São Paulo, SC = Santa Catarina

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

Group of anastomosis	Optimum temperature ¹ (°C)	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

606

607 ^{1,2} Values estimated by the adjustment of the polynomial regression model: $y=a+bx^2+cx^4+dx^6+ex^8+fx^{10}$,
 608 where y = mycelial growth; a, b, c, d, e, f are regression parameters; x = temperature (AG-3 PT: $y=-$
 609 $10,91+0,31x^2-0,0006x^4+(7,87e^{-7})x^6-(6,13e^{-10})x^8+(1,82e^{-13})x^{10}$, $R^2=0,984$, $p>F=0,039$; AG-4 HGI: $y=-$
 610 $46,34+0,74x^2-0,0022x^4+(3,67e^{-6})x^6-(2,82e^{-9})x^8+(7,63e^{-13})x^{10}$, $R^2=0,994$, $p>F=0,014$; AG-R: $y=-$
 611 $44,33+0,76x^2-0,0029x^4+(5,25e^{-6})x^6-(4,15e^{-9})x^8+(1,13e^{-12})x^{10}$, $R^2=0,998$, $p>F=0,004$)

612 ³ Mean values in the column followed by the same letter did not differ significantly from each other by
 613 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).

619 **Table 4** Effect of salinity on mycelial growth of three groups of *Rhizoctonia* anastomosis
 620 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

621

622 ¹ Averages followed by the same letter in the column did not differ significantly from each other by Fisher's

623 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.**

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

3

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15

16 **Abstract**

17

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

25 and *T. zygis* and 14 chemical compounds in the essential oil of *S. montana*. Piperitone
26 oxide (36.97%) and piperitone (20.77%) were the major components in the oil of *M.*
27 *rotundifolia*, thymol (60.27%), piperitone oxide (6.56%) and piperitone (5.18%) were the
28 major components in the oil of *T. zygis* and carvacrol (49.38), p-cymene (28.08) and thymol
29 (8.30) were the major components in *S. montana* oil. When evaluated separately, treatments
30 with carvacrol at concentrations of 0.01 and 0.05 mg mL⁻¹ significantly inhibited the fungus
31 (p <0.0001) with 28 and 29.8% inhibition of mycelial, respectively. The TBARS assay
32 revealed that the *Rhizoctonia* isolates reacted differently to the tested oils. In the treatment
33 with *S. montana* oil at 5 mg mL⁻¹, AG-R presented higher values of ROS (127.4 nmol g⁻¹)
34 and MDA (5.9 nmol g⁻¹) and in the treatment with *M. rotundifolia* oil at 5 mg mL⁻¹ AG-3
35 PT presented higher ROS (131.9 nmol g⁻¹) and MDA (5.4 nmol g⁻¹). The results reveal
36 essential oils that can be tested in field assays as well as application technology studies and
37 open a window on the management of diseases caused by *Rhizoctonia*.

38

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

41

42 **1 Introduction**

43

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

49 in the 20 to 40 days after planting and the symptoms are stem and root cankers; damping
50 off; plant burning and death; tuber rot, spotting, leaf burning, sprouting and formation of
51 sclerotia on the surface of tubers, also known as black scurf (Banville, 1989; Carling *et al.*,
52 1989; Banville *et al.*, 1996; Tsrer, 2010).

53 *Rhizoctonia* multinucleate isolates identified as *R. solani* (Kühn) [teleomorph
54 *Thanatephorus cucumeris* (Donk)] and binucleated *Rhizoctonia* (BNR) isolates
55 [teleomorph *Ceratobasidium*] are commonly found causing rhizoctoniosis in potatoes
56 (Yang *et al.*, 2014, 2015a,b, 2017; Muzhinji, N., Truter, M., Woodhall, J. W., van der
57 Waals, 2015). *Rhizoctonia* isolates differ in phenotypic and genotypic characteristics and
58 are arranged in genetically related intraspecific groups (AGs) and subgroups capable of
59 self-recognition through hyphae fusion (Carling, 1996; Carling *et al.*, 2002; Sharon *et al.*,
60 2006, 2007, 2008). Currently, 14 AGs (AG-1 to AG-13 and AG-BI) from *R. solani* are
61 recognized, with AG-3 being the most widely distributed in potato crops worldwide.
62 Among BNR 17 AGs (AG-A to AG-V) are recognized, with recent attention being given to
63 the AG-R group due to the prevalence and high aggressiveness in potato production fields
64 (Yang *et al.*, 2014, 2015a,b; Muzhinji *et al.*, 2015).

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

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

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

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

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

101

102 **2 Materials and methods**

103

104 2.1 Plant pathogenic fungi

105

106 Two isolates of *Rhizoctonia* were used in this study obtained from the Collection of
107 Phytopathogenic Fungi Cultures "Prof. Maria Menezes" (CMM) of Rural Federal
108 University of Pernambuco (UFRPE). Isolate 2099 identified as *Rhizoctonia solani*
109 multinucleate from the anastomosis AG-3 PT group and isolate 1829 identified as
110 *Rhizoctonia* spp. binucleate of AG-R. Isolates were found causing stem canker and black
111 scurf in potatoes in Brazil. The isolates were kept in potato-dextrose-agar medium (PDA) at
112 4°C and subcultured at monthly intervals.

113

114 2.2 Plant materials

115

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

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

123

124 2.3 Extraction of EOs

125

126 The EOs were extracted by hydrodistillation using a Clevenger type apparatus and
127 organic extraction using a Soxhlet type apparatus, according to the method recommended
128 by the European Pharmacopoeia. Pilot plant vapor pressure extraction was carried out in a
129 stainless steel distillation plant equipped with a 100 Kg distillation chamber, a 500 L vessel
130 and a pressure range of 0.5-1.0 bar. The water collected after the EO was decanted (1.16 L)
131 was filtered to give an acidic water residue (WR, 4.5 mg/mL of organic extract, pH 3.2).
132 155 mL of WR were extracted with dichloromethane (DCM, 150 mL x 3) to give an
133 organic fraction (WROE) (230 mg, 0.15 % yield). 50 mL of WR were neutralized at pH
134 6.62 with NaOH 2N and lyophilized to give a dry residue (WRNL, 36.7 mg, 0.073% yield).
135 The organic extractions (hexane, H; ethyl acetate, EtOAc and ethanol, EtOH) were carried
136 out in a Soxhlet for 12 h (131g, 1.2%; 0.76 % and 12.5 % yield respectively). The insoluble
137 material from the H extraction (1.60 g) was filtered, and the solution obtained was washed
138 with 2N NaOH solution. The aqueous layer was acidified with 2N HCl at pH 2 and
139 extracted with t-butylmethylether (E). The oil was dried over anhydrous Na₂SO₄ and
140 preserved in sealed glass bottles. It was protected from light by wrapping in aluminum foil
141 and stored at 4°C until used.

142

143 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°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: $\% I = (C-T/ C) \times 100$, 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 polysiloxane, 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:

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

173

174 2.6 Antifungal activity of main constituents of the selected EOs

175

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

179

180 2.7 Total Reactive Oxygen Species (ROS)

181

182 Total ROS was analyzed using fluorimetric quantitation of
183 dichlorodihydrofluorescein-diacetate (DCDHF-DA) that was oxidized by ROS. For each
184 fungal isolate, the mycelium was cultured at EO concentrations 1, 5, 25 and 50 mg mL⁻¹ in
185 PDA medium. The fresh mycelium from each treatment was incubated in 10 µM of
186 DCDHF-DA in Tris-HCl (50 mM, pH 8.0) for 1 h at room temperature in the proportion of
187 100 mg of fresh micelia per mL of solution, then the tissue was washed with 50 mM EDTA
188 to remove the incubation solution and ground to a fine powder as above and extracted in 1
189 mL of Tris-HCl (50 mM, pH 8.0). The supernatant obtained from this mixture after
190 centrifugation at 10,000 rpm for 10 min was filtered in Miracloth. The fluorescence (488

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

193

194 2.8 Membrane Peroxidation (MDA)

195

196 For each fungal isolate, the mycelium was cultured at EO concentrations 1, 5, 25 and
197 50 mg mL⁻¹ in PDA medium. Fresh tissue was ground to a powder as above and suspended
198 in 1% of trichloroacetic acid (TCA) in the proportion of 50 mg of mycelium per mL. The
199 resultant mixture was centrifuged at 8,000 rpm for 5 min. To the supernatant (250 mL), 1
200 mL of 0.5% of thiobarbituric acid in 20% TCA was added and the mixture was boiled for
201 30 min. This was then allowed to cool to room temperature and the adduct formed by TBA-
202 malondialdehyde (MDA) was quantified at 532 and 600 nm using $\Delta 532-600$ and 155 mM⁻¹
203 cm⁻¹ (Ederli *et al.*, 2004).

204

205 2.9 Statistical analysis

206

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

209

210 **3 Results**

211

212 3.1 *In vitro* antifungal activity of EOs and their main chemical components

213

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

221 The isolate of *Rhizoctonia* sp. (A) was shown to be more sensitive to a larger
222 spectrum of EOs, but only when tested at a concentration greater than or equal to 0.5 mg
223 mL^{-1} . No EO was able to completely inhibit the mycelial growth of *Rhizoctonia* sp. (A) at
224 0.1 mg mL^{-1} . The results of the screening counted seven EOs that were efficient in
225 inhibiting the mycelial growth of *Rhizoctonia*, *Mentha rotundifolia* (16), *Oreganum*
226 *vulgare* (21), *Salvia officinalis* (24), *Satureja montana* (25), *Thymus vulgaris* (26 and 27)
227 and *Thymus zygis* (29). Regarding the availability of EOs, EOs 16, 25 and 29 were chosen.

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

232 Table 3 shows the chemical components identified by mass gas chromatography
233 (GC-MS). Twenty-six chemical compounds were identified in the EO of *M. rotundifolia*
234 and *T. zygis*, piperitone oxide (36.97%) and piperitone (20.77%) were found in greater
235 quantities in the EO of *M. rotundifolia* and Thymol (60.27%), followed by piperitone oxide
236 (6.56%), piperitone (5.18%) and other compounds in a proportion of less than 5% were
237 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

262

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

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

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

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

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

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

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

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

318 In our study, we also verified that the great antifungal efficiency of *S. montana* oil is
319 mainly due to carvacrol. Carvacrol tested at concentrations 0.01 and 0.05 mg mL⁻¹ inhibited
320 the mycelial growth of the AG-3 PT isolate significantly, confirming this hypothesis.
321 Fraternali *et al.* (2007) observed that *S. montana* oil with 18% carvacrol tested at MIC of
322 250 μ l/ml was effective against nine species of phytopathogenic fungi, including *R. solani*.
323 The presence of carvacrol, p-cymene, thymol and γ -terpinene in the oil were associated
324 with mycelial inhibition of other fungi such as *Penicillium expansum* and *Botrytis cinerea*
325 (Lopez-Reyes *et al.*, 2010) and *Aspergillus niger* (Moghtader, 2012).

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

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

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

344 The TBARS tests identified that *S. montana* oil at 5 mg mL⁻¹ caused greater oxidative
345 stress in the AG-R isolate and *M. rotundifolia* oil at 5 mg mL⁻¹ caused greater oxidative
346 stress to the AG-3 PT isolate. In the case of the dichlorodihydrofluorescein (DCF) assay the
347 total amount of ROS may be radicals such as hydroxyl, as well as singlet oxygen,
348 superoxide anion, but mainly hydrogen peroxide. The quantification of malondialdehyde
349 (MDA) in the TBARS test indicates an estimate of membrane lipoperoxidation. Rellán
350 (2013) studied different species of *Satureja* and observed that *S. montana* oil showed to be
351 more efficient in the inhibition of *R. solani* and greater antioxidant activity. The antioxidant
352 activity was attributed to a higher amount of total phenolics. Fenols provide plants with a
353 defense mechanism to neutralize reactive oxygen species (ROS), for survival and to prevent
354 molecular damage and by microorganisms or insects (Dzamic *et al.*, 2016). Phenols have
355 redox properties by the mechanisms of free radical scavenging, transition-metal-chelating
356 activity and singlet oxygen-quenching capacity, stabilization of lipid peroxidation and
357 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

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375

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379

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

528

Nº	Plant species	Family	Plant part used
1	<i>Porlieria chilensis</i>	Zygophyllaceae	Leaves and flowers
2	<i>Lippia alba</i>	Verbenaceae	Leaves and flowers
3	<i>Mentha rotundifolia</i>	Labiatae	Leaves and flowers
4	<i>Mentha longifolia</i>	Labiatae	Leaves and flowers
5	<i>Mentha spicata</i>	Labiatae	Leaves and flowers
6	<i>Mentha arvensis</i>	Labiatae	Leaves and flowers
7	<i>Mentha piperita</i>	Labiatae	Leaves and flowers
8	<i>Thymus vulgaris</i>	Labiatae	Leaves and flowers
9	<i>Thymus mastichina</i>	Labiatae	Leaves and flowers
10	<i>Thymus zygis</i>	Labiatae	Leaves and flowers
11	<i>Tanacetum vulgare</i>	Asteraceae	Leaves and flowers
12	<i>Artemisia dracunculus</i>	Asteraceae	Leaves and flowers
13	<i>Artemisia absinthium</i>	Asteraceae	Leaves and flowers
14	<i>Lavandula angustifolia</i>	Labiatae	Leaves and flowers
15	<i>Lavandula lanata</i>	Labiatae	Leaves and flowers
16	<i>Lavandula luisieri</i>	Labiatae	Leaves and flowers
17	<i>Dittrichia graveolens</i>	Asteraceae	Leaves and flowers
18	<i>Aloysia fiebrigii</i>	Verbenaceae	Leaves and flowers
19	<i>Elettaria cardamomum</i>	Zingiberaceae	Leaves and flowers
20	<i>Hyssopus officinalis</i>	Labiatae	Leaves and flowers
21	<i>Salvia officinalis</i>	Labiatae	Leaves and flowers
22	<i>Satureja montana</i>	Labiatae	Leaves and flowers
529 23	<i>Oreganum vulgare</i>	Lamiaceae	Leaves and flowers

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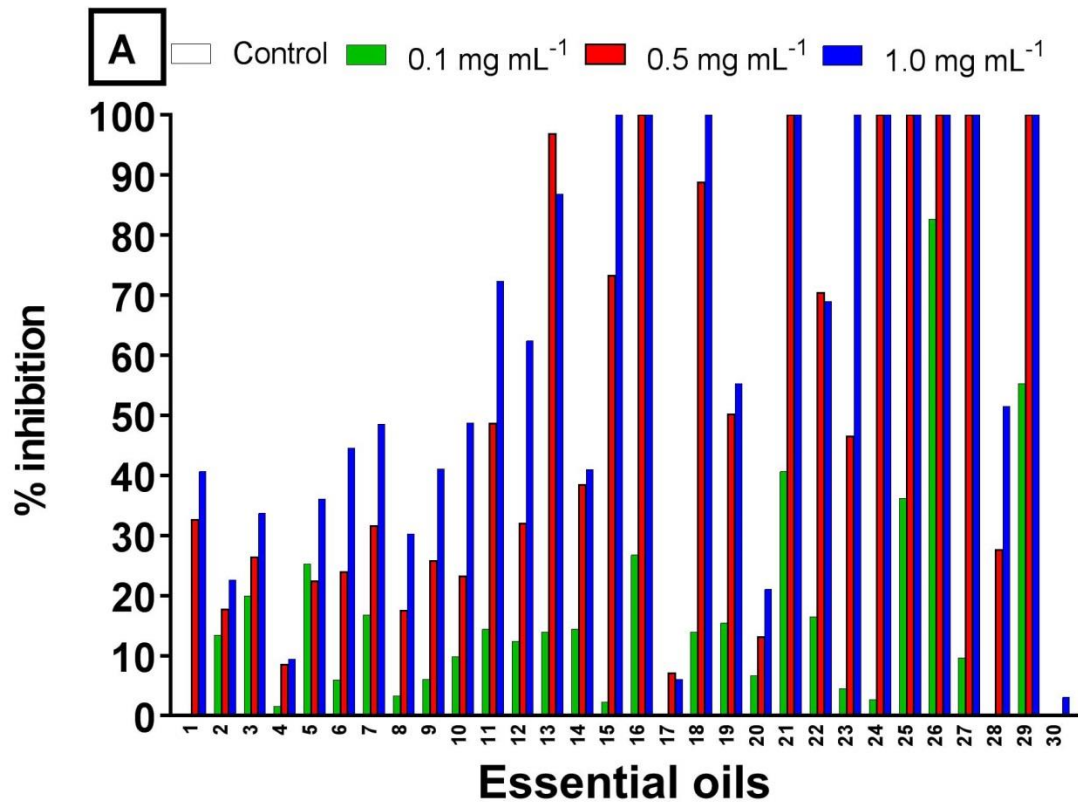
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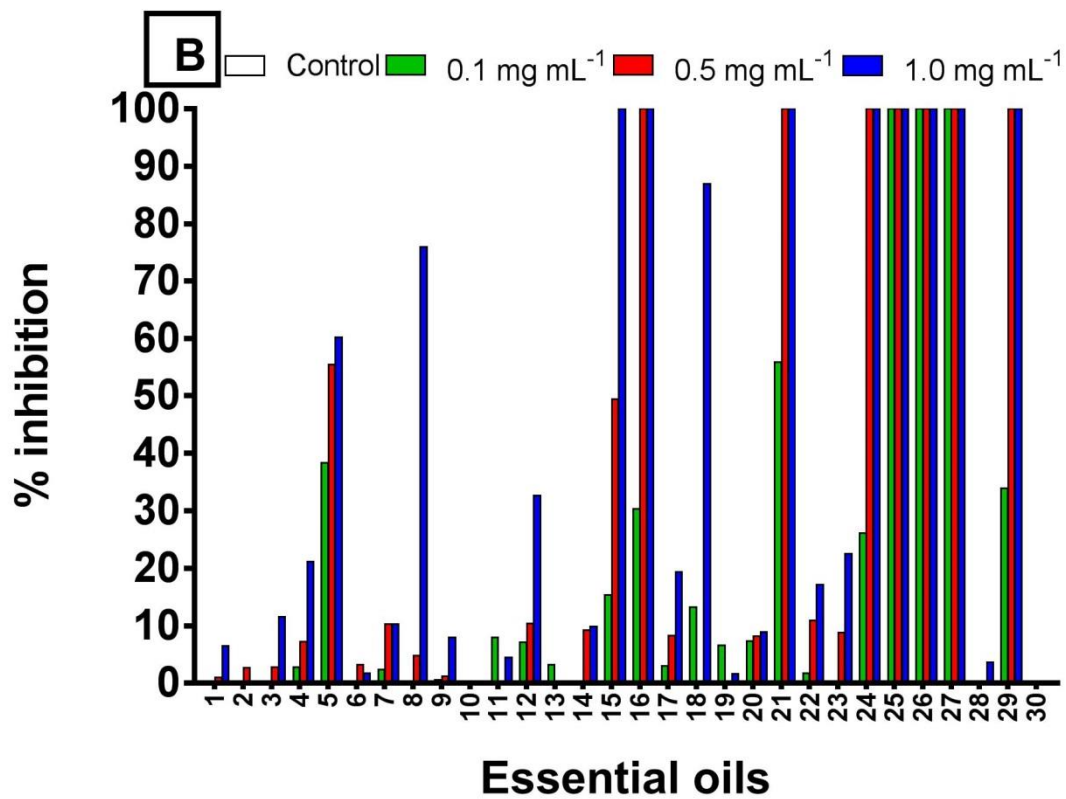
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539 **Figure 1.** Effect of different concentrations of essential oils on the mycelial growth of
540 *Rhizoctonia* from different anastomosis groups. The data were analyzed statistically by the
541 program Graphpad prism version 7.0. (A) AG-R (*Rhizoctonia*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

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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
<i>Mentha rotundifolia</i> (sample 16)	0.1	0.5	0.1	0.5
<i>Satureja montana</i> (sample 25)	~ 0.0	0.1	0.1	0.5
<i>Thymus zygis</i> (sample 29)	0.1	0.5	0.1	0.5

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 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			B			C		
<i>Mentha rotundifolia</i>			<i>Thymus zygis</i>			<i>Satureja montana</i>		
Nº	Compound name	Percentage (%)	Nº	Compound name	Percentage (%)	Nº	Compound name	Percentage (%)
1	Piperitone	20,77	1	Thymol	60,27	1	Carvacrol	49.38
2	Piperitone oxide	36,97	2	piperitenone	5,18	2	p-Cymene	28.08
3	germacrene-d	7,39	3	piperitone oxide	6,56	3	Thymol	8.30
4	Limonene	6,63	4	endoborneol	4,60	4	Thymoquinone	2.81
5	trans-caryophyllene	6,07	5	Linalol	3,90	5	1-Octen-3-ol	1.74
6	trans-caryophyllene	6,07	6	trans-caryophyllene	2,81	6	l-Limonene+1,8-Cineol	1.64
7	endobornyl	1,92	7	germacrene-d	2,46	7	Borneol	1.25
8	farnesol	1,85	8	Cinrolone	2,29	8	trans-Sabinene hydrate	1.22
9	β -pinene	1,65	9	Caryophyllene ox,	1,35	9	(-)- α -Pinene	0.93
10	1-octen-3-yl acetate	1,24	10	viridiflorol	1,07	10	Linalool	0.93
11	β -myrcene	1,21	11	delta-cadinene	1,06	11	β -bisabolene	0.90
12	α -pinene	0,94	12	Camphor	0,99	12	α -Thujene	0.76
13	epi-bicyclosquiphellandrer	0,84	13	trans sabinene hydrate	0,94	13	Terpinen-4-ol	0.75
14	α -humulene	0,83	14	p-Cymene	0,87	14	α -Terpinene	0.47
15	cis-ocimene	0,63	15	β -farnesene	0,81		Total identified	99.16
16	viridiflorol	0,51	16	germacrene-d	0,66			
17	p-cymen-8-ol	0,51	17	Terminen-4-ol	0,55			
18	β -elemene	0,46	18	epi-bicyclosquiphellandrene	0,54			
19	β -cubenene	0,41	19	α -humulene	0,53			
20	endoborneol	0,39	20	Spathulenol	0,49			
21	cis jasmone	0,35	21	γ -Terpinene	0,44			
22	β -bourbonene	0,31	22	γ -cadinene	0,43			
23	4-terpineol	0,25	23	t-muurolol	0,34			
24	gama-terpinene	0,25	24	γ -Muurolene	0,30			
25	Thymol	t	25	1,8-cineole	0,30			
26	Carvacrol	t	26	1,10-epi-Cubenol	0,26			
	Total identified	98,45		Total identified	100,00			

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583 t = trace (< 0,1)

584 **Table 4.** Behavior of *Rhizoctonia* isolates from different anastomosis groups the different
 585 concentrations of pure chemical constituents.

Treatment	Inhibition of mycelial growth (%)							
	AG-R (<i>Rhizoctonia</i> sp.)				AG-3 PT (<i>Rhizoctonia solani</i>)			
	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
586 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

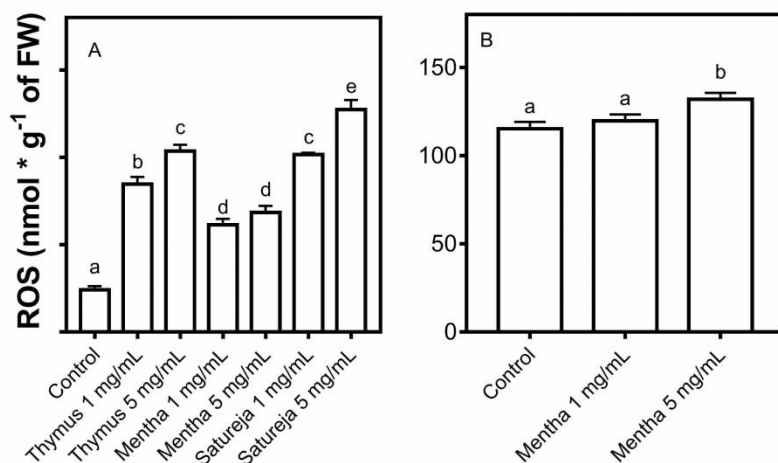
587 The results are means ± standard errors of six replications. Means within a column
 588 indicated by same letter were not significantly different according to Tukey's multiple test
 589 at the level $P < 0,05$.

590 Chemical components available and tested.

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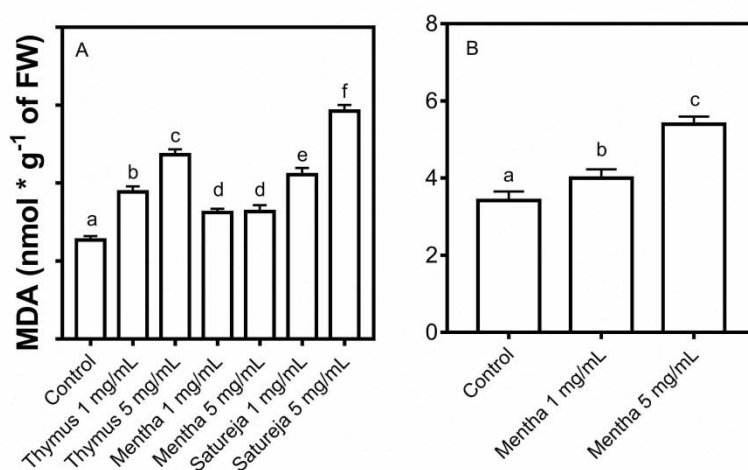
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595 **Figure 2.** Reactive oxygen species (ROS) from the essential oils of *Mentha rotundifolia*,
 596 *Thymus zygis* and *Satureja montana* to *Rhizoctonia*. (A) AG-R (*Rhizoctonia* sp.) and (B)
 597 AG-3 PT (*Rhizoctonia solani*). Means within a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

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

605 were not significantly different according to Tukey's multiple test at the level $P < 0,05$.

606 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 vulgaris* 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.