MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO RIO GRANDE
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EXTRATOS DE COGUMELOS COMO POTENCIAIS BIOCONTROLADORES AGRÍCOLAS E SUAS IMPLICAÇÕES TOXICOLÓGICAS

Lisiane Martins Volcão

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Orientador: Prof. Dr. Flávio Manoel Rodrigues da Silva Júnior

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“Mesmo com todas as adversidades do meu dia a dia, consegui alcançar a felicidade na forma que imaginei, não sei se tô vivendo ou sonhando “

Agradecimentos

Em primeiro lugar a **Deus**, por colocar exatamente aquilo que preciso no meu caminho.

A **minha família**, pelo amor e apoio incondicional em todos os momentos. Pelos ensinamentos de honestidade, bondade, empatia, e igualdade. E que todos tenham a oportunidade de aprender sobre equidade ao longo da vida.

Ao **meu companheiro** e coorientador, por segurar minha mão em TODOS os momentos desse doutorado e sempre. Estar sempre por perto para me segurar caso eu caísse, foi essencial.

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Aos meus **amigos** mais próximos, por entenderem as minhas muito ausências, e por sempre arranjarem um "jeitinho" de me trazer um conforto.

Aos colegas do **LEFT**, gostaria de ser mais informal, mas vou manter a categoria. Vocês são demais, continuem batalhando juntos, assim todos crescem juntos. Só orgulho dessa gente.

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A todos que de alguma forma contribuíram direta ou indiretamente para a realização deste estudo.

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A urbanização desordenada das cidades, associada à falta de controle ambiental urbano, rural e silvestre eficientes, vem criando grandes dificuldades e desafios na relação homem/ambiente. Entre estes desafios está o controle eficaz e seguro de pragas na agricultura, os quais geram perdas significativas no rendimento de culturas. O controle convencional destes organismos é feito através de aplicações preventivas e corretivas de produtos químicos, porém o seu uso indiscriminado gera danos ao meio ambiente e à saúde humana. Produtos naturais têm sido pesquisados como alternativas no controle biológico de microrganismos, como, por exemplo, extratos de cogumelos. Estes extratos, além de antimicrobianos têm demonstrado serem indutores dos mecanismos naturais de defesa da planta. O estudo investigou o efeito *in vitro* de alguns extratos de cogumelos, provenientes da região Sul do Rio Grande do Sul, frente à patógenos de plantas, assim como, aspectos tóxicos para a própria planta, toxicidade celular *in vitro*, toxicidade *in vivo* para a minhoca californiana *Eisenia andrei*, e efeitos na estrutura funcional da microbiota do solo. Em um primeiro estudo, foi demonstrada a capacidade antibacteriana de extratos etanólicos dos cogumelos *Russula xerampelina* e *Suillus granulatus* em *Pseudomonas aeruginosa*, sem apresentar fitotoxicidade significativa na germinação de alface e tomate. O segundo estudo apresentado, mostrou inibição do crescimento e modificações no crescimento micelial do fungo fitopatogênico *Monilinia fructicola* exposto ao extrato hidroalcoólico de *Lactarius deliciosus*. Somado a isso, o extrato de *L. deliciosus* não apresentou citotoxicidade em células VERO, e moderada fitotoxicidade em *Brassica oleracea* (couve). O último estudo desta tese, apresentou o efeito do extrato hidroalcoólico de *Laccaria laccata* na inibição do crescimento de *Fusarium solani*, bem como o seu efeito estimulante na estrutura funcional da microbiota do solo. Não houve toxicidade celular *in vitro* para esse extrato, assim como foi demonstrada a não toxicidade *in vivo* no modelo terrestre escolhido, *E. andrei*. Com o exposto ao longo da referida tese, demonstramos as potencialidades de um grupo de organismos pouco explorados para pesquisas em controle biológico de "pragas" agrícolas. Além do seu potencial antibacteriano e antifúngico, nenhum dos extratos apresentou citotoxicidade nas concentrações utilizadas, e aqueles testados em organismos do solo, demonstraram um potencial para bioestimulação.

Palavras-chaves: basidiomicetos; controle biológico; fitotoxicidade; citotoxicidade; fauna edáfica.

ABSTRACT

VOLCÃO, L.M. MUSHROOM EXTRACTS AS AGRICULTURAL BIOCONTROLLERS AND THEIR TOXICOLOGICAL IMPLICATIONS. Tese de Doutorado – Universidade Federal do Rio Grande – Rio Grande, 2020.

The disorderly urbanization of cities, associated with the lack of efficient urban, rural and wild environmental control, has created great difficulties and challenges in the relationship between man and the environment. Among these challenges is the effective and safe control of pests in agriculture, which generate significant losses in crop yields. The conventional control of these organisms is done through preventive applications of chemical products, but their indiscriminate use generates damage to the environment and human health. Natural products have been researched as alternatives in the biological control of microorganisms, such as, for example, mushroom extracts. These extracts, in addition to antimicrobials, have been shown to induce the plant's natural defense mechanisms. The study presents the in vitro effect of certain mushroom extracts, from the Southern of Rio Grande do Sul, against plant pathogens, as well as toxicological aspects for the plant itself, in vitro cell toxicity, in vivo toxicity to the Californian earthworm *Eisenia andrei*, and effects on the structural microbiome of the soil. In a first study, the antibacterial capacity of ethanolic extracts of *Russula xerampelina* and *Suillus granulatus* mushrooms was demonstrated in *Pseudomonas aeruginosa*, without showing significant phytotoxicity in the germination of lettuce and tomatoes. The second study presented, showed growth inhibition and changes in mycelial growth of the phytopathogenic fungus *Monilinia fructicola* exposed to the hydroalcoholic extract of *Lactarius deliciosus*. In addition, the *L. deliciosus* extract did not show cytotoxicity in Vero cells, and moderate phytotoxicity in *Brassica oleracea* (cabbage). The last study of this thesis, presented the effect of the hydroalcoholic extract of *Laccaria laccata* in inhibiting the growth of *Fusarium solani*, as well as its stimulating effect on the functional structure of the soil microbiota. There was no cellular toxicity in vitro for this extract, as well as non-toxicity in vivo was demonstrated in the chosen terrestrial model, *E. andrei*. With the exposed throughout the referred thesis, we demonstrate the potential of a group of organisms little explored for researches in biological control of agricultural "pests". Since, in addition to their antibacterial and antifungal potential, none of the extracts showed cellular toxicity, and those tested on soil organisms, demonstrated a potential for biostimulation

Keywors: basidiomycetes; biological control; phytotoxicity; cytotoxicity; edaphic fauna.

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LISTA DE ABREVIATURAS

IBGE – Instituto Brasileiro de Geografia e Estatística

PIB – Produto Interno Bruto

OECD-FAO – Organization for Economic Co-operation and Development - Food and Agriculture Organization of the United Nations (Organização para a Cooperação e Desenvolvimento Econômico – Organização da Nações Unidas para Alimentação e Agricultura)

DDT – Dicloro-difenil-tricloetano

MAPA – Ministério da Agricultura e Pecuária

IBAMA – Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis

DA – Dose Diária Admissível

PA – Princípio Ativo

CBm – Carbono de Biomassa Microbiana

EthE – Extrato Etanólico

EtOH – Etanol

TPC – Total Phenol Content (Conteúdo Total de Fenóis)

HPLC – High Performance Liquid Chromatography (Cromatografia Líquida de Alta Eficiência)

DPPH• - radical 2, 2-diphenyl- 1 – picrylhydrazy

MIC – Minimal Inhibitory Concentration (Concentração Mínima Inibitória)

CFU – Colony Unit Forming (Unidade Formadora de Colônia)

MHB – Muller Hinton Broth (Caldo Muller Hilton)

DMSO – Dimetilsulfóxido

FICI – Fractional Inhibitory Concentration Index (Índice de Concentração Fracional Inibitória)

GS – Germination Speed (Velocidade de Germinação)

GSI – Germination Speed Index (Índice de Velocidade de Germinação)

RI – Índice de Efeito Alelopático

HydE – Extrato hidroalcoólico

CLSI – Clinical & Laboratory Standard Institute

MGRI – Mycelial Growth Rate Index (Velocidade de Inibição do Crescimento Micelial)

MGI% - Mycelial Growth Inhibition Percentage (Porcentagem de Inibição do Crescimento Micelial)

ABMGC – Area Below Mycelial Growth Curve (Área Abaixo da Curva de Crescimento Micelial)

IC50 – Inibição de 50% do Crescimento

PDA – Potato Dextrose Agar (Ágar Batata Dextrose)

SAT – Solo Artificial Tropical

1. INTRODUÇÃO

As transformações ambientais e sociais ocorridas pelo uso da terra para agricultura se intensificaram no século XX. Mais precisamente, a agricultura no Brasil tem passado por importantes transformações desde a década de 1960 (VIEIRA FILHO & GASQUES, 2016).

O uso da terra para agricultura e pecuária, no modelo atual de produção, necessita de uma área relativamente ampla. A área precisa estar limpa, ou seja, desmatada. O processo como um todo cria diversos desafios na relação homem/meio ambiente, como o controle eficaz e seguro de "pragas", como artrópodes e microorganismos, os quais geram perdas significativas no rendimento de culturas agrícolas (YORINORI & SARTORATO, 2011).

O controle convencional de organismos nocivos à planta é realizado através de aplicações preventivas de produtos químicos. Entretanto, o uso indiscriminado destas substâncias pode gerar danos ao meio ambiente, à saúde humana, e ainda provocar casos de resistência, onde a planta torna-se predisposta a uma maior incidência de determinada doença (GUINI & KIMATI, 2000; RICHTER et al., 2015).

O uso de pesticidas tem aumentado consideravelmente desde o século passado (LAMBROPOULOU et al., 2015). Estima-se que tenham sido utilizados ao redor do mundo em 2014, cerca de 450.000 toneladas de herbicidas, ≅ 210.000 toneladas de fungicidas/bactericidas, e em uma terceira maior quantidade, diferentes tipos de inseticidas (≅ 100.000 toneladas). Dentre estes, os principais tipos de substâncias químicas utilizadas foram os organofosfatos, ditiocarbamatos, amidas e hormônios fenoxi- (ZHANG, 2018).

Apesar dos benefícios oriundos da utilização de pesticidas, como o aumento no rendimento das culturas e a redução nos preços de produtos agrícolas, a detecção de seus

resíduos em diferentes esferas do ambiente e nos alimentos tem levantado preocupações (AKOTO et al., 2016; FANG et al., 2017). Dentre estas, pode-se citar o seu uso excessivo, contínuo e incorreto, gerando muitas vezes efeitos tóxicos aos seres humanos, assim como o aumento do seu potencial de risco ecológico (CURL et al., 2020; JIAO et al., 2020).

Produtos naturais têm sido pesquisados como alternativa no controle de organismos e microorganismos fitopatogênicos, como por exemplo extratos produzidos a partir de cogumelos (ARRUDA et al., 2012; DEGENKOLB & VILCINKAS, 2016; SAHLI et al., 2018). Junto a isso, é importante compreender que os cogumelos (Basidiomycota/Ascomycota) coevoluem com insetos e microorganismos, tornando-se assim, fontes de substâncias inseticidas e antimicrobianas naturais que são produzidas em resposta ao ataque destes organismos (CHRISTENSEN, 1989). Diversos estudos avaliam os efeito toxicológicos de extratos provenientes de cogumelos, entretanto, a maiorias dessas pesquisas não são realizadas com espécies comumente encontradas na região sul do Brasil (LAU et al. 2013; GUISSOU et al. 2015; ZHUK et al., 2015).

Frente ao número limitado de estudos acerca do potencial inibitório de extratos de basidiomicetos em microorganismos fitopatogênicos, e a escassa caracterização de espécies de cogumelos no sul do país, o presente estudo teve como tese a presença de atividade antifúngica e antibacteriana em extratos de cogumelos coletados na região sul do Rio Grande do Sul, Brasil.

2. REVISÃO DE LITERATURA

2.1 Brasil e a Produção Agrícola

O Brasil é considerado uma potência mundial na produção de alimentos, assim como, um importante exportador agrícola no mercado internacional. O país está entre os maiores fluxos econômicos do mundo, com um PIB de R$ 6,9 trilhões em 2018, sendo o Rio Grande do Sul, o quinto estado com maior PIB no país em 2017 (IBGE, 2019).

Pode-se dizer que a produção agrícola em massa teve seu início com a chamada "Revolução Verde", no início da década de 60. Esta caracterizava-se por um conjunto de práticas tecnológicas, como o uso de organismos geneticamente modificados, o uso de fertilizantes químicos, agrotóxicos, irrigação e motomecanização (EHLERS, 1994). Este pacote tecnológico tinha como intuito a maximização dos rendimentos dos cultivos, elevando ao máximo a capacidade potencial destes, por meio da criação de condições ecológicas "ideias" (BARROS, 2010).

Com o crescente aumento da agricultura, o Brasil tornou-se o segundo maior exportador de produtos agrícolas e agroalimentares, atrás apenas dos Estados Unidos (OECD-FAO, 2015). As Américas em particular, ao longo dos anos 2000, fortaleceram sua posição como fornecedores de mercadorias agrícolas como milho, soja e carne (OECD-FAO, 2019). Estima-se ainda que, a partir do ano de 2020, a América Latina e Caribe tenham um aumento em suas exportações quando em comparação com a América do Norte e outras regiões do mundo (Figura 1).

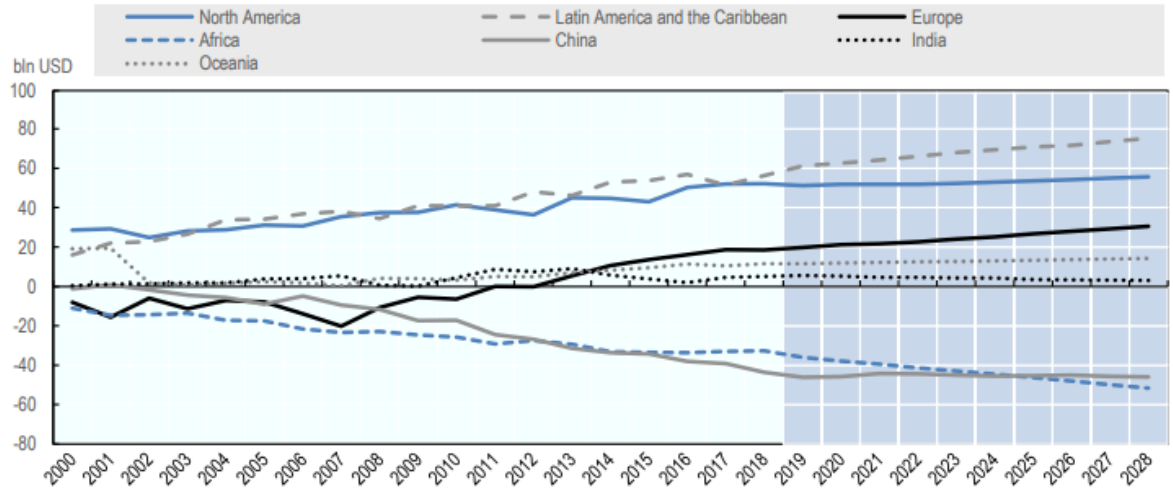

Figura 1: Crescimento dos saldos comerciais agrícolas por região em valor constante (USD), com destaque para América Latina + Caribe e América do Norte.

Fonte: (OECD/FAO, 2019).

2.1.1 Consequências da Grande Produção Agrícola

Apesar do nome, a "Revolução Verde" apresentou como principal objetivo dominar os processos da natureza, como por exemplo, o uso de agentes químicos para afastar predadores naturais do seu próprio habitat.

Com isso, a percepção dos problemas desta nova forma de produzir, foi quase simultânea aos primeiros efeitos da Revolução Verde, podendo-se citar alguns destes, como a erosão e a perda de fertilidade do solo, danos à biodiversidade, contaminação dos solos, água, do campo e dos alimentos com produtos químicos (ALTIERI, 2002). É notável também, que os sistemas de irrigação possam expandir o espaço para vetores de doenças, principalmente ao redor de corpos d'água (ROGER et al., 1991). Somado a isto, as monoculturas podem servir como fontes sazonais de alimentos para animais selvagens, os quais podem estar em um status de hospedeiro para agentes patogênicos zoonóticos (PATZ et al., 2005; RICHTER et al., 2015).

Outra problemática do mecanismo de produção extensiva refere-se à aplicação de produtos químicos para o controle de “pragas”, uma vez que esta pode resultar na proliferação de populações de consumidores primários, como larvas de dípteros e/ou caracóis vetores (PATZ et al., 2005). Mesmo com alertas ao longo de décadas acerca dos riscos ecológicos e a saúde humana sobre o uso de pesticidas (ENGELHARD et al., 1979; AXELSON, 1987; REPETTO & BALIGA, 1997; BROCK et al., 2006; ZHANG et al., 2018), o consumo de pesticidas aumentou ao redor do mundo durante a primeira década do século XXI (Figura 2).

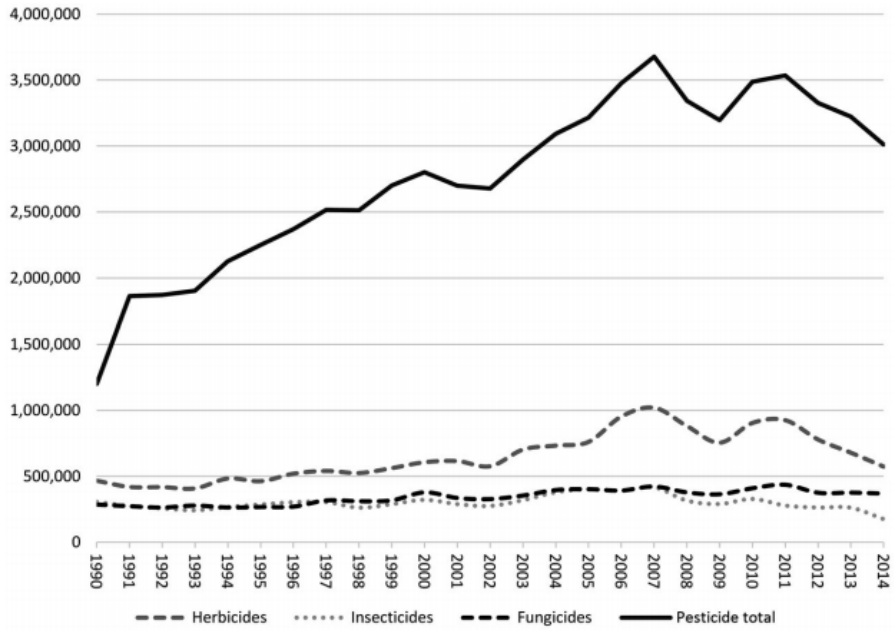

Figura 2: Valores totais globais do uso de pesticidas em variáveis dependentes* (herbicidas, inseticidas, fungicidas) de 1990-2014 em toneladas.

*os dados para rodenticidas e desinfetantes não estão explícitos no gráfico, exceto no número total de toneladas.

Fonte: (HEDLUND et al., 2019).

O Brasil é um dos principais consumidores de pesticidas no mundo, com uma forte expansão nas últimas quatro décadas, com um aumento de 700%. O Rio Grande do Sul ganha

destaque, ocupa a 4° posição no consumo de agrotóxicos no país (CHIARELLO et al., 2017). Com isso, é importante salientar a contaminação de determinadas áreas e sua relação com as condições climáticas naturais de uma região. Um exemplo disto são estudos que demonstram, que pesticidas regulamentados na América do Sul, incluindo os organoclorados e bifenilos, possuem a capacidade de se transportar via atmosfera para regiões montanhosas mais remotas de clima tropical e subtropical boliviana e chilenas, assim como à terras brasileiras mas altas (ESTELLANO et al., 2008; MEIRE et al., 2012).

2.2 Pesticidas e Efeitos Tóxicos

2.2.1 Aspectos Legais

A obra Primavera Silenciosa de Rachel Carson (CARSON, 1962) foi o primeiro relato sobre os efeitos nocivos dos pesticidas utilizados na agricultura, e culminou com a proibição do DDT (dicloro-difenil-tricloetano), inseticida utilizado em plantações e na prevenção de vetores em diversos países.

A proibição do uso deste composto químico para fins agrícolas foi feita há várias décadas em países como Suécia, Estados Unidos, Dinamarca e França (MAPA, 2009). No Brasil a proibição para fins agrícolas ocorreu em 1985, e somente em 2009 o DDT foi banido para qualquer outro fim no país (BRASIL, 2009). Por ser um dos grandes exportadores agrícolas do mundo, o Brasil tornou-se dependente de uma quantidade significativa de insumos químicos, notando-se a necessidade de uma legislação atualizada para sua utilização (HANDFORD et al., 2015). Estados Unidos, China, Japão, Europa e Brasil são exemplos de regiões chave no desenvolvimento de legislações sobre o uso de pesticidas (Figura 3 e 4).

O Brasil sendo um dos maiores consumidores de pesticidas do mundo, possui uma ampla cobertura legal, tanto em relação à sua toxicidade quanto à sua escala de uso no

território nacional. Para serem produzidos, exportados, importados, comercializados e utilizados, estes devem primeiramente ser registrados em um órgão federal, de acordo com normas e diretrizes de órgãos responsáveis pelos setores da saúde, meio ambiente e agricultura. O Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA), é o responsável pela avaliação do potencial de periculosidade ambiental de todos os agrotóxicos registrados no país (BRASIL, 2017).

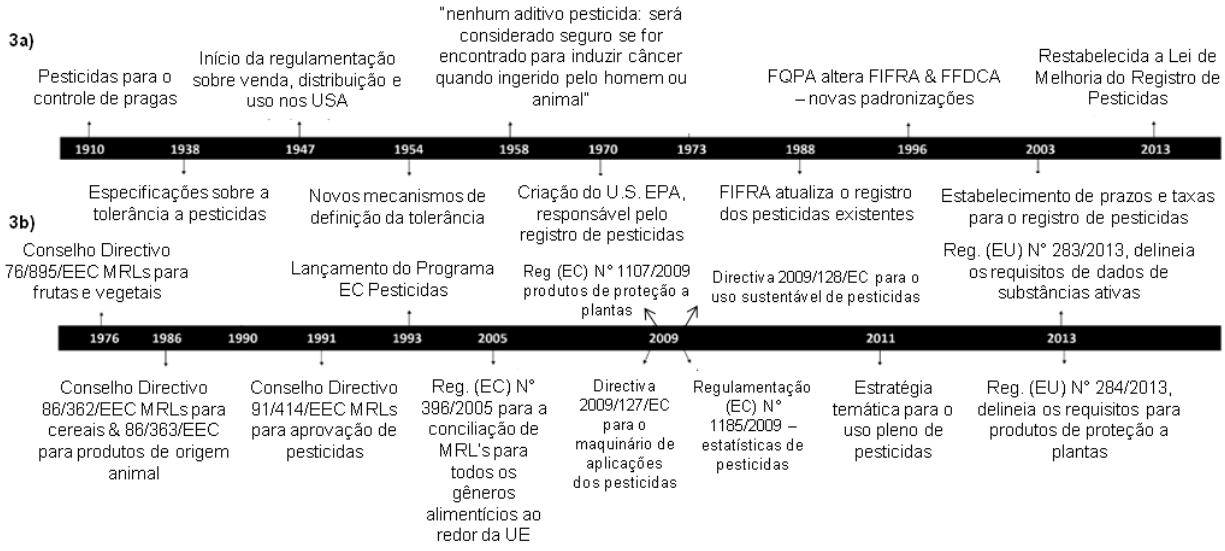

Figura 3: Linha do Tempo sobre o desenvolvimento das legislações para pesticidas nos Estados Unidos da América (3a) e na União Europeia (3b).

Legenda: U.S. EPA – Environmental Protection Agency; FIFRA – Federal Inseticide, Fungicide & Rodenticide Act; EC – European Comission; EU – European Union; FQPA – Food Quality Protection Act; MRL‟s – Maximum Residues Limits.

Fonte: Adaptado de Handford et al. (2015).

Neste contexto, é importante salientar que os pesticidas podem conter adjuvantes, os quais podem e são muitas vezes mantidos em sigilo pelas empresas de manufatura, além de um Princípio Ativo (PA) que é declarado e o único avaliado mais intensivamente ao longo dos testes toxicológicos. Com isso será calculada a Dose Diária Admissível (IDA), ou seja, o

nível de exposição aparentemente seguro para seres humanos a longo prazo, entretanto estes dados não vão incluir os adjuvantes presentes na formulação, os quais também podem ter efeitos tóxicos nas células (MESNAGE et al., 2014).

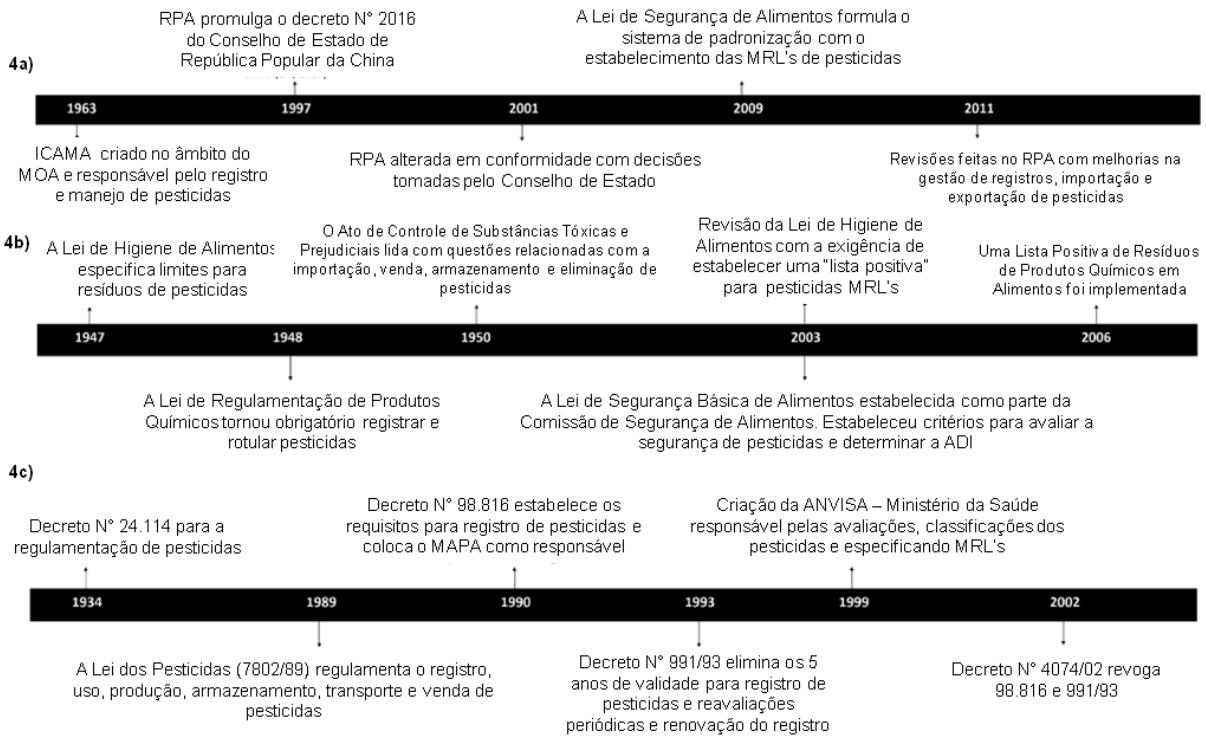

Figura 4: Linha do Tempo sobre o desenvolvimento das legislações para pesticidas na China (4a), Japão (4b) e no Brasil (4c).

Legenda: U.S. EPA – Environmental Protection Agency; FIFRA – Federal Inseticide, Fungicide & Rodenticide Act; EC – European Comission; EU – European Union; FQPA – Food Quality Protection Act; MRL"s – Maximum Residues Limits.

Fonte: Adaptado de Handford et al. (2015).

2.2.2 Pesticidas *versus* Saúde Humana

O envenenamento acidental com pesticidas tem sido resultado do seu manuseio e exposição diária em plantações. O riscos iniciam-se desde a exposição dos agricultores durante a aplicação, incluindo técnicas incorretas de manejo e armazenamento, além do

contato dos trabalhadores com campos tratados, sendo que a exposição dos alimentos e da água aos resíduos destes químicos também se tornou uma grande preocupação (RIVERA et al., 2016; REED et al., 2020).

A exposição aos pesticidas e fertilizantes químicos tem sido associada ao desenvolvimento de diversos problemas de saúde. Um importante grupo frequentemente exposto a estes produtos químicos são os trabalhadores rurais. Estudos que avaliaram agricultores do Rio Grande do Sul, demonstraram a associação entre a exposição a pesticidas e danos genéticos, como danos aos telomêros e aumento na metilação do DNA no grupo exposto (KAHL et al., 2015; BENEDETTI et al., 2018). Somado a isso, as consequências da exposição materna e de crianças a pesticidas têm sido relatadas em diversos estudos (ESKENAZI et al., 2004; ESKENAZI et al., 2010; ROCA et al., 2014; LIU et al., 2015). Sendo importante salientar que o feto e neonatos são mais vulneráveis devido ao rápido crescimento, diferenciação celular, imaturidade das vias metabólicas; e crianças pelo fato da baixa atividade e níveis de enzimas detoxificantes para certos tipos de pesticidas (ESKENASI et al., 1999; HOLLAND et al. 2006).

2.2.3 Pesticidas e Meio Ambiente

Outra exposição danosa em consequência ao uso de pesticidas é aquela sofrida pelo meio ambiente. Este contato do ambiente, principalmente, ao redor das plantações com produtos químicos tóxicos, pode gerar danos tanto aos organismos ali presentes, quanto para a própria planta.

Os pesticidas podem apresentar grande impacto nos aspectos biológicos do solo. Um exemplo disso é a influência de pesticidas e fertilizantes na diversidade estrutural e funcional da microbiota do solo (PRASHAR & SHAH, 2016), sendo importante salientar que a interação do pesticida com as propriedades do solo depende da complexidade química do

composto (KAUR et al., 2017). FERNANDES et al. (2020) em estudo bibliográfico, demonstraram a grande diversidade de classes de pesticidas em solos agrícolas, entretanto, observaram que nos solos residenciais ocorria uma menor variedade de produtos químicos, porém em uma maior concentração (Figura 5).

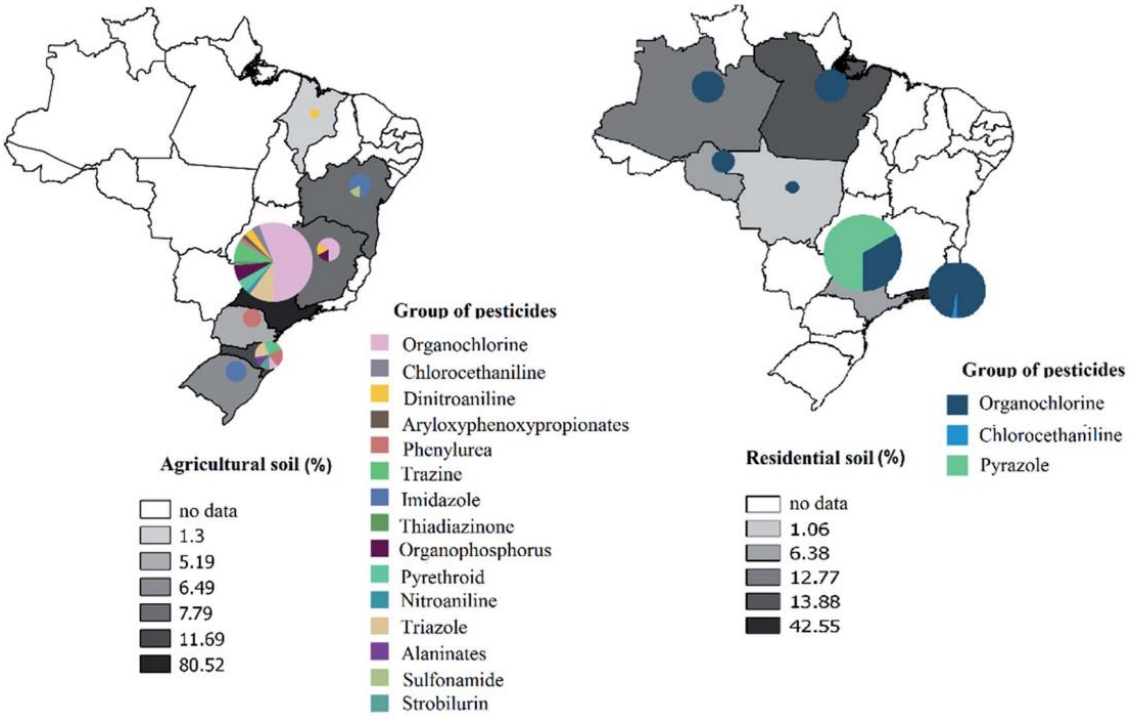

Figura 5: Resíduos totais (%) de diferentes grupos de pesticidas detectados em solos agrícolas e residenciais, por grupo químico e por estado brasileiro.

Fonte: (Adaptado de FERNANDES et al., 2020).

Pesquisas que avaliam os efeitos da contaminação do meio ambiente com pesticidas empregados na agricultura, demonstram desde a resistência de fitopatógenos (MARCIN et al., 2013; OLIVEIRA et al., 2015), resultante da pressão seletiva sofrida como consequência do seu uso contínuo, até mesmo o rápido aumento na prevalência de deformidades nos membros de anfíbios expostos à escoamentos agrícolas (SPARLING et al., MACÁT et al., 2015; JAYAWARDENA et al., 2016).

Somado as problemáticas descritas, uma das alterações que vem ganhando destaque ao longo dos anos são os efeitos individuais e a nível de colônia observados em abelhas quando em contato com pesticidas (GILL et al., 2012), sendo importante salientar que este grupo é responsável por aproximadamente 80% da polinização por insetos (POTTS et al., 2010). Diversos estudos ao redor do mundo observaram a presença de grupos químicos de pesticidas no pólen e no mel, estimando associações entre a exposição e o colapso nas colônias (BLASCO et al., 2003; RISSATO et al., 2007; CHOUDHARY et al., 2008; PETTIS et al., 2013; NAGGAR et al., 2015), demonstrando ser este não um fenômeno local, mas sim global.

2.3 Interação Planta e Microbioma

2.3.1 Microbioma Vegetal

O termo microbioma vegetal, ou *plant microbiome*, tem sido utilizado há poucos anos (LAKSHMANAM et al., 2014). Este termo demonstra toda a interação ecológica entre solo, planta e os microorganismos ao seu redor, como fungos e bactérias. A figura 6 demonstra os fatores do solo que influenciam os processos da planta e dos microorganismos ao seu redor, que consequentemente irão modificar o ambiente do próprio solo (CHAPARRO et al., 2012).

A população bacteriana do solo varia entre 10^8 a 10^9 microorganismos por grama de solo. Cada célula bacteriana possui um tamanho relativamente pequeno em comparação com os outros microorganismos do solo, devido a isso, compõem menos da metade do Carbono de Biomassa Microbiana (CBm), podendo atingir aproximadamente 4.000 $Kg.ha^{-1}$ (GRISI et al., 1988). Um importante grupo bacteriano são as espécies com capacidade de esporulação, as quais podem ser utilizadas, como componentes, na avaliação de impactos e alterações causadas no solo, resultantes de adubação ou até mesmo derramamento de produtos químicos (PIMENTEL et al., 2016).

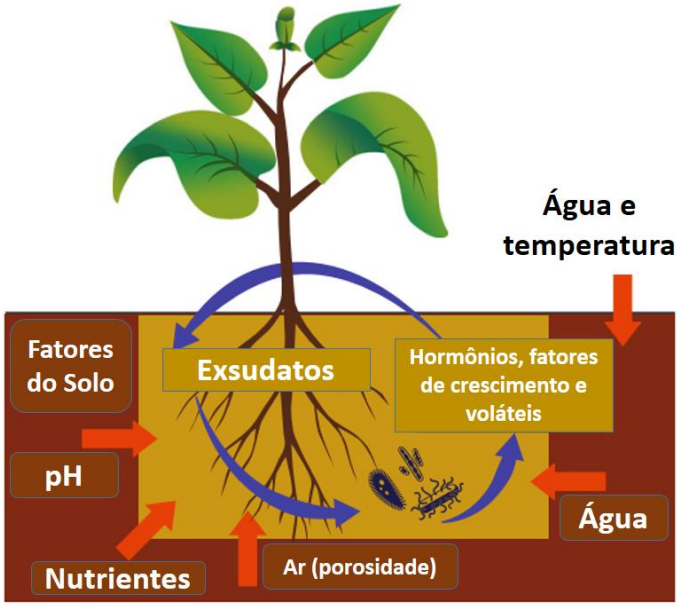

Figura 6: Ilustração de alguns fatores físicos, químicos e biológicos que influenciam na dinâmica solo – planta – biomassa microbiana.

Fonte: (Adaptado de CHAPARRO et al., 2012).

Os organismos fúngicos contribuem com a maior parte da biomassa microbiana do solo, em torno de 70% e 80%, podendo atingir 5 toneladas.ha^{-1}. Apesar de baixa densidade populacional, possuem hifas de elevado comprimento e diâmetro, as quais elevam a sua biomassa (ALEXANDER, 1980; BRANDÃO, 1992). Os fungos possuem no solo, atividade quimioheterotrófica sobre restos vegetais, sendo organismos importantes na indicação da qualidade do solo (PIMENTEL et al., 2016) e candidatos em potencial para o tratamento de áreas contaminadas por metais pesados (DA SILVA Jr. et al., 2018). As actinobactérias do solo, antes denominadas como Actinomycetos, possuem uma alta diversidade morfológica, além de uma grande variedade fisiológica e na produção de metabólitos (PIMENTEL et al., 2016). Estes microorganismos, principalmente os fungos, continuam a ter um papel

fundamental na busca por produtos farmacêuticos e agroquímicos, inclusive como base para a geração de novos compostos através da biologia sintética (ALBERTI et al., 2017).

2.3.2 Principais Grupos de Fitopatógenos

Plantas e microorganismos desenvolveram inter-relações que os permitiram coexistir em um mesmo espaço (NIHORIMBERE et al., 2011). Entretanto, o microbioma vegetal pode vir a ter influências negativas na planta, assumindo caráter de fitopatógenos (figura 7).

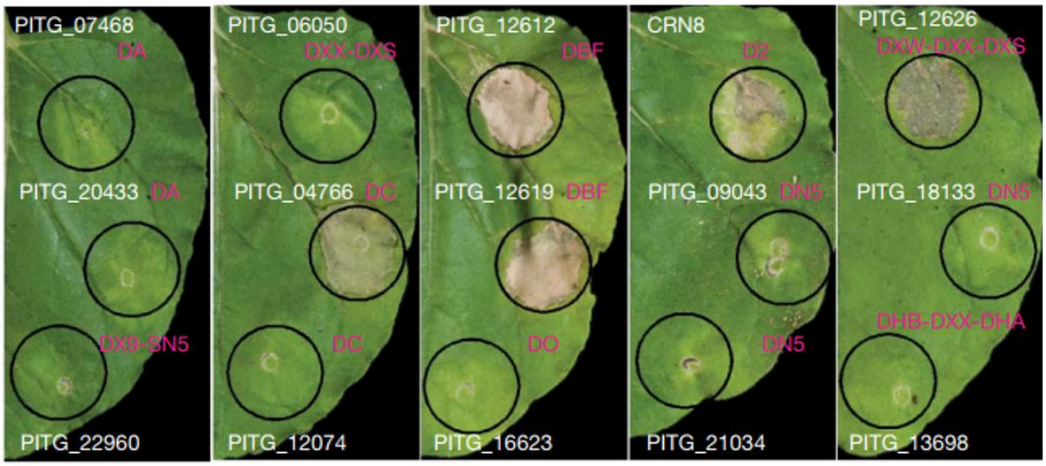

Figura 7: Folha de batata apresentando sintomas de infecção por diferentes linhagens (círculos pretos) de *Phytophthora* spp.

Fonte: (Adaptado de HAAS et al., 2009).

As doenças que afetam os organismos vegetais são inúmeras, causadas tanto por fungos, bactérias ou vírus. Pode-se citar alguns fungos e oomicetos fitopatogênicos de grande importância para a agricultura, como *Phytophtora infestans* na infestação da batata e responsável pelo episódio da Grande Fome Irlandesa do século XIX (GOSS et al., 2014). Outro fungo fitopatogênico, *Monilinia fructicola*, é um importante microorganismo causador da podridão-parda que afeta frutas com caroço. É comum nas Américas, África e Austrália, sendo listado como um organismo de quarentena europeu (LARENA et al., 2005).

Muitas espécies bacterianas também possuem considerável importância na fitopatologia. *Ralstonia solanacearum* é atribuída a cerca de 400 diferentes espécies de plantas (WICKER et al., 2007), como berinjela, tomate e pimenta. Além desta espécie, cultivos de tomate também estão suscetíveis a infestação por *Pseudomonas* sp., bactéria intimamente ligada ao microbioma do solo e um hospedeiro natural deste fruto (SETUBAL et al., 2005). Diferentemente de fungos e bactérias, apenas uma pequena parcela de actinobactérias são patogênicas às plantas, cerca de 10 espécies dentro do gênero *Streptomyces* sp. (ZHANG et al., 2016). A capacidade de colonizar tecidos vivos é extremamente rara, e entre as espécies com essa capacidade está o *Streptomyces scabiei*, causador da "sarna da batata" e de nodulações na vagem do amendoim (LORIA et al., 1997; BIGNELL & CHENG, 2017).

Por serem organismos obrigatoriamente intracelulares, os vírus necessitam da maquinaria celular do hospedeiro para se replicar (WANG, 2015). Para uma transmissão e infecção eficiente, o vírus deve ter a capacidade de atravessar a parede celular vegetal. Esse movimento ocorre através dos plasmodesmas, principalmente nas células epidérmicas e mesofílicas (MAULE, 2008). O vírus do mosaico amarelo em leguminosas, o vírus X da batata, o vírus do mosaico do pepino, o vírus da ampla necrose do feijão, são exemplos de fitopatógenos virais que afetam muitas culturas básicas pelo mundo (HONDA & SAITO, 1983; TAKAHASHI et al., 1994; LÓPEZ et al., 2003; COLLINS et al., 2009).

2.4 Controle Alternativo de "Pragas"

A busca pelo consumo de alimentos seguros e de qualidade tem sido o principal motivo para a utilização de métodos de controle alternativo de fitopatógenos. Alguns destes métodos são conhecidos desde a antiguidade, por civilizações no Egito, Grécia, Índia e China,

através da utilização de inseticidas botânicos e derivados de plantas, aplicados diretamente nos grãos a serem cultivados (Thacker, 2002; El-Wakeil, 2013).

Na Europa e América do Norte, o uso de pesticidas botânicos registra-se a cerca de 150 anos. Um exemplo deste tipo de produto, ainda utilizado atualmente é a nicotina, um alcaloide originado das folhas de tabaco (*Nicotiana tabacum*) com atividade inseticida (El-Wakeil, 2013). Yeo e Tham (2012), ao estudarem plantas da medicina tradicional chinesa, observaram o potencial destas em interromper o mecanismo de *Quorum Sensing*, comunicação química entre células bacterianas, importante para o estabelecimento da infecção na planta. A pesquisa com extratos de plantas é também direcionada ao controle de insetos, como por exemplo, no controle de larvas de dípteros e lepidópteros (Arshad et al., 2019; Hamad et al., 2019).

Além de produtos de origem vegetal, tem-se investido no uso de microorganismos antagonistas ao crescimento de fitopatógenos. Em estudo de Yun et al., (2017), fungos endofíticos (*Penicillium simplicissium* e *Leptosphaeria* sp.) isolados de raízes de algodão foram utilizados no controle da doença *Verticillium* do algodão, sendo demonstrada a capacidade *in vitro* e *in vivo* destes em reduzir os sintomas da doença. Outra abordagem na aplicação de microorganismos antagonistas para o controle de doenças em plantas é a combinação de diferentes espécies bacterianas. Thangavelu e Gopi (2015) demonstraram uma supressão de 100% do fitopatógeno *Fusarium oxysporum* f.sp. *cubensi* em banana (cv. Cavendish) com a combinação de bactérias rizosféricas e endofíticas, além de observarem uma aumento significativos nos parâmetros de crescimento da planta.

2.5 Basidiomicetos e Atividade Biológica

Dentre todos os grupo de organismos da Terra, o reino Fungi é o mais diversificado (Tedersoo et al., 2014). Sendo importante salientar que, a dificuldade de isolamento e a não

aplicação de métodos moleculares para identificação, podem contribuir para o número subestimado de espécies relatadas (Abdel-Azee, 2010; Blackwell, 2011).

A divisão Basidiomycota, onde encontram-se a maioria dos cogumelos, corresponde a maior diversidade dentro do reino. Há muito, cogumelos selvagens têm sido coletados e utilizados como alimento, assim como, uma quantidade significativa tem colaborado para a medicina humana (Martins, 2017). Sua importância é indiscutível, sendo tema de diversos estudos e aplicações:

> "Cogumelos têm sido valorizados em todo o mundo como alimento e remédios por milhares de anos. Em todo o mundo, muitas pessoas gostam de caçar cogumelos selvagens, deliciando-se na variedade de suas formas, tamanhos e cores exibidas por estas 'flores do outono' (Hobbs, 2002)"

Há mais de 1000 anos, os cogumelos têm sido empregados na medicina popular da Ásia, para prevenir e curar diferentes doenças. Os exemplos mais tradicionais são *Ganoderma lucidum* (Curtis) P. Karst, *Phellinus linteus* (Berk. & M.A. Curtis) Teng, *Cordyceps sinensis* (Berk.) Sacc., Tramates versicolor (L.) Lloyd, e Inonotus obliquus (Ac. Ex Pers.) Pilát. (Soković et al., 2017). Estudos recentes demonstram propriedades antioxidantes de diferentes espécies de cogumelos, além de anti-inflamatórias, antifúngicas, antibacterianas e antitumorais em diferentes espécies de cogumelos (Knežević et al., 2015; Choi et al., 2019; Volcão et al., 2019; Owaid et al., 2020) (Figura 8). Os cogumelos possuem diversas moléculas bioativas, como terpenóides, esteroides, fenóis, nucleotídeos, derivados de glicoproteínas e polissacarídeos (Soković et al., 2017).

Figura 8: Algumas espécies de cogumelos encontradas na região sul do Rio Grande do Sul Brasil em estudo desenvolvido por Volcão et al., 2019.

Fonte: autor.

Além do uso para fins medicinais, tem-se pesquisado o possível emprego de microorganismos endofíticos e/ou seus produtos para o controle de fitopatógenos (Figuereido & Castro e Silva, 2014; Barneche et al., 2016; Yuan et al., 2017; Chang & Wasser, 2018). Diversos estudos avaliam as propriedades toxicológicas e a segurança de extratos provenientes de espécies de cogumelos, entretanto a maioria destes estudos foi realizada com espécies exóticas, que em sua maioria não pertencem à região Sul do Brasil (Lau et al., 2013; Guissou et al., 2015; Zhuk et al., 2015). Um exemplo de cogumelo com conhecida toxicidade, é *Amanita muscaria*, entretanto já foi demonstrado que em pequenas frações, o extrato deste cogumelo é suficiente para o controle biológico de artrópodes, como mosquitos da espécie

Culex quiquefasciatus e de *Musca domestica* (Rosa et al., 2006; Carcamo et al., 2006; Wood et al., 2013).

Poucos estudos na literatura o potencial de cogumelos e compostos para o controle de fitopatógenos. Entre alguns estudos, já foi avaliado o efeito de extratos de cogumelos do gênero *Russula* sp. e *Boletus* sp. contra larvas de *Anopheles gambiae*, *Aedes aegypti* e *Culex quinquefasciatus* (Chelela et al., 2014). Metabólitos provenientes de cogumelos já foram também muito pesquisados como herbicidas, fungicidas, bactericidas e nematocidas (Barsehyyan et al., 2016). Em estudo de Wu et al. (2016), os autores observaram o benefício do uso de substratos de cogumelos comestíveis para otimizar a produção de *Bacillus thuringiensis*, uma importante bactéria utilizada para o controle biológico de fitopatógenos.

3 JUSTIFICATIVA

Comparado à pesquisas utilizando extratos de vegetais para o controle de doenças em plantas, o número de estudos aplicando extratos de cogumelos para esse fim é relativamente limitado (Stangarlin et al., 2011; Degenkolg & Vilcinskas, 2016). Este fato torna-se mais evidente, em relação a espécies de basidiomicetos comumente encontradas naturalmente na região Sul do Rio Grande do Sul, Brasil.

Em vista disso, e considerando a importância do desenvolvimento de novas alternativas no controle de fitopatógenos, que possam ser utilizadas sem risco de dano ao meio ambiente e à saúde humana, existe a necessidade da avaliação da toxicidade de extratos que apresentem eficácia no controle do crescimento de micro-organismos causadores de patologias de culturas agrícolas.

4 OBJETIVOS

4.1 Objetivo Geral

O estudo teve como objetivo determinar a capacidade antimicrobiana de extratos produzidos a partir dos corpos de frutificação de basidiomicetos coletados na região Sul do Rio Grande do Sul, Brasil, bem como analisar seus aspectos toxicológicos com vistas a toxicidadade celular, fitotoxicidade, e toxicidade *in vivo*.

4.2 Objetivos Específicos

Detecção de ácidos fenólicos e potencial antibacteriano dos extratos etanólicos de *Russula xerampelina* e *Suillus granulatus* contra *Pseudomonas aeruginosa*;

Avaliar a toxicidade dos extratos etanólicos de *Russula xerampelina* e *Suillus granulatus* em *Lactuca sativa* e *Solanum lycopersicum*;

Definir a concentração de ácidos fenólicos no extrato hidroalcoólico de *Lactarius deliciosus;*

Determinar o potencial do extrato hidroalcoólico de *Lactarius deliciosus* em inibir e afetar o crescimento radial do fungo fitopatogênico *Monilinia fructicola*;

Avaliar a citotoxicidade e a toxicidade do extrato hidroalcólico *Lactarius deliciosus* em *Brassica oleraceae*.

Caracterizar os ácidos fenólicos e a atividade antifúngica contra *Fusarium solani* do extrato hidroalcoólico de *Laccaria laccata*;

Determinar a toxicidade celular e a toxicidade *in vivo* do extrato hidroalcoólico de *Laccaria laccata*.

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5 MANUSCRITOS

ARTIGO 1

Bioactive mushrooms extracts of *Russula xerampelina* and *Suillus granulatus* in the in vitro control of *Pseudomonas aeruginosa* phytopathogenic

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Bioactive mushrooms extracts of *Russula xerampelina* and *Suillus granulatus* in the in vitro control of *Pseudomonas aeruginosa* phytopathogenic

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Abstract

New products, including natural extracts, have been researched for use in controlling phytopathogenic organisms. This study aims to evaluate the antibacterial potential of ethanolic extracts from mushrooms, followed by an evaluation of their allelopathic potential. The characterization of phenolic acids in *Russula xerampelina* and *Suillus granulatus* extracts was evaluated by high-performance liquid chromatography, followed by the assessment of their antibacterial potential, only and combined, against *Pseudomonas aeruginosa*. The allelopathic effects in lettuce and tomato were measured in terms of germination and root length parameters. The results demonstrate an additive profile of the combined extracts against *Pseudomonas aeruginosa*. Both extracts showed a decrease in the percent germination of lettuce and tomato at higher concentrations. The seeds were positively influenced when analyzing the allelopathic index. These results indicate the potential of these extracts for the control of phytopathogens of the genus Pseudomonas, mainly because they do not cause phytotoxicity in terms of the evaluated parameters.

Keywords: *Lactuca sativa*; *Solanum lycopersicum*; mushrooms; biological control; additivity.

Introduction

Mushrooms are ancient organisms widely used as traditional Chinese Medicine in Asia, and they have attracted attention in the field of agriculture research. [1] These organisms contain many bioactive molecules, such as terpenoids, steroids, phenols, glycoprotein, derivates and polysaccharides [2] (Soković et al. 2017), which have antibacterial, antifungal, antiparasitic and antioxidant properties. [3-5]

Numerous studies in the biological control field have shown alternatives to the massive use of agricultural pesticides. [6,7] This fact is a result of the growing concern with toxicological aspects of pesticides and the implications for human health and the environment. Some of these alternatives are based on the application of an active microorganism or their metabolic products, such as the use of entomopathogenic fungi and the application of extracts. [8,9]

The apparent problems of the current form of agricultural production, including the contamination of soil and water, damage to biodiversity and chemical residuals in foods that reach the population, are remarkable. [10-12] The toxicological characteristics of these agrochemicals, along with the uncertainties of long-term damage, demonstrate the need for viable and nontoxic alternatives to pest control.

The genus Pseudomonas consists of bacteria that can affect various agricultural crops. [13] *Pseudomonas aeruginosa* is a common cause of diseases in vegetables, such as lettuce, while *Pseudomonas syringae* is a classic phytopathogen in tomato fruits. [14] The first specie has a set of virulence factors that make it efficient in plant infection. [Ausubel, 2005; Starkey and Rahme, 2009]

In addition, is an important contaminant of fresh vegetables, and which might be a source of infection for susceptible persons. [Allidyce 2012]

The chemical control of *Pseudomonas* sp. in tomato predominantly involves copper-based bactericides. However, as with any chemical, ecological and evolutionary processes can reduce the efficacy of these products for pathogen control. [15]

In this study, we performed the chemical characterization of two ethanolic extracts (EthE) from wild mushrooms and evaluated the control potential against *Pseudomonas aeruginosa*, and the allelopathic effects of this extracts on *Lactuca sativa* and *Solanum lycopersicum*.

Materials and Methods

Mushroom collection and extraction

Mushroom samples were collected from an area belonging to the Universidade Federal de Pelotas. *Russula xerampelina* and *Suillus granulatus* identification was performed with the aid of identification guidelines. [16] The fruiting bodies were dried in an oven at 50 °C. The mushroom samples were prepared by maceration; the EthE as prepared with 25 g of each mushroom species in 95% EtOH (1:4 w/v) by ultrasonication at 40 °C for 120 min. [17] The final mixtures of the extracts were filtered through Whatman® No. 1 filter paper in order to eliminate particulate matter.

Preliminary phytochemical evaluation

The total phenol content content (TPC) in the EthE were analyzed using the method described by Roesler et al. [17]. For the colorimetric reaction employed in TPC analysis, a methanol extract solution (1:20 v/v) was used and the TPC was expressed as milligram gallic acid equivalent per gram of extract (mg GAE/g).

High-Performance Liquid Chromatography

The identification of phenolic acids was determined by the retention time of reference standards obtained from Sigma Aldrich (gallic, protocatechuic, chlorogenic, p-hydroxybenzoic, caffeic, syringic, vanilli, p-coumaric and ferulic acids). Aliquots of the extracts were previously lyophilized and resuspended in the mobile phase using methanol and acidified water (1% acetic acid) (1:1). The mixture was injected into a liquid chromatography system coupled to a DAD detector using a C18 column (4.6 × 250 mm, 5 mg; Discovery, USA). HPLC-DAD was run at a flow rate of 0.7 mL/min at 35 °C using methanol and acidified water (1% acetic acid) as the mobile phase at a ratio of 20:80 (v/v) for 25 min, with readings at 280 and 320 nm. [18]

Antioxidant activity of ethanolic extracts

The free radical scavenging activities of *R. xerampelina* and *S. granulatus* EthE were measured according to the method reported by Roesler et al. [17] using the radical 2, 2-diphenyl- 1 – picrylhydrazy (DPPH•). In this study, the mushroom extracts were diluted in methanol (1:10), and the samples were subjected to ultrasound for 30 min at 10 °C and subsequently diluted to different concentrations. The standards and samples were incubated for 30 min at room temperature protected from light. The absorbance of the mixture was

measured at λ = 517 nm, and the amount of DPPH consumed was expressed as µmol Trolox equivalent per mL of extract (µmol Trolox/mL). The ability to scavenge free radicals was expressed as percent inhibition (% inhibition) according **Equation 1.**

$$Scavenging\ effect\ (\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) * 100 \quad (1)$$

Where: $A_{control}$ is the DPPH solution absorbance and A_{sample} is the absorbance of the sample.

Effect of *R. xerampelina* and *S. granulatus* treatment on a Pseudomonas aeruginosa control

Pseudomonas aeruginosa ATTC 15442 was treated with *R. xerampelina* and *S. granulatus* EthE through the broth microdilution test [19], where the minimal inhibitory concentration (MIC) capable of inhibiting bacterial growth was established. After establishing the MIC for each extract, the fractional inhibitory concentration index (FICI) for the combination of extracts was evaluated by the checkerboard method. [20]

Before antibacterial analysis, the EthE was evaporated to dryness under vacuum, and the extracts were dissolved in a 5% DMSO aqueous solution (10 mg/mL). The MIC was determined using serial dilutions (5 – 0.625 mg/mL) performed in Mueller Hinton broth. For the test, *Pseudomonas aeruginosa* inoculum was prepared in saline solution (0.85 %) at a density adjusted to the 0.5 MacFarland turbidity standard, obtaining a final concentration of 1.5 x 10^5 CFU/mL per microplate well. Sterility controls of the culture medium and pure extracts were performed, as well as a growth control using the culture medium MHB + DMSO/Tw80 solution at the concentrations used to dissolve the extracts. The microplate was

incubated for 24 h at 37 °C, and resazurin reagent (0.02%) was used for the analysis of cell viability. The change in reagent color from blue to pink indicates bacterial growth, while the absence of a color change of the reagent indicates a bacteriostatic effect of the extract at a given concentration.

The FICI was determined using 20 different combined serial concentrations (1/2) of the EthE (5 – 0.625 µg/ml). The test was performed in Mueller Hinton broth, and inoculum of Pseudomonas sp. was prepared in saline solution (0.85%) at a density adjusted to the 0.5 MacFarland turbidity standard, obtaining a final concentration of 1.5 x 10^5 CFU/mL per microplate well. The plate was incubated for 24 h at 37 °C, and the cell viability was analyzed using resazurin reagent (0.02%). The FICI of the mixture was analyzed according to **Equation 2**.

$$FICI = \frac{MIC_a\ in\ combination}{MIC_a\ alone} + \frac{MIC_b\ in\ combination}{MIC_b\ alone} \quad (2)$$

Where: FICI is the fractional inhibitory concentration index; MIC_a is the minimal inhibitory concentration of extract a; and MIC_b is the minimal inhibitory concentration of extract b. The FICI was interpreted as follows: FICI ≤ 0.5 indicates a synergistic effect; 0.5 < FICI ≤ 1 indicates an additive effect; 1.0 < FICI ≤ 2.0 indicates an indifferent effect; and FICI > 2.0 indicates an antagonistic effect.

Germination and allelopathic index bioassays

The assays for analyzing the effects on plant species were performed as described in OECD [21] with some modifications. To evaluate germination, seeds of the target species lettuce and

tomato were used, these were exposed to crude extract (250 mg/mL). We tested four extract concentrations (0.125, 0.25, 0.5 and 1%) of the separate extracts and two concentrations of an extract mixture [0.5% of *R. xerampelina* (1.25 µg/mL) + 0.06% of *S. granulatus* (0.156 µg/mL), and 1% of *R. xerampelina* (2.5 µg/mL) + 0.12% of *S. granulatus* (0.312 µg/mL)]. For the control treatment, only distilled water and two other solvents were used for extract preparation (0.125 and 1% ethanol).

Seeds were placed to germinate in Petri dishes on filter paper moistened with 2.5 times the mass of the paper of distilled water or according to their respective treatment. Then, the plates were placed in BOD at 22 °C. The germination speed (GS) and the germination speed index (GSI) were determined by counting the number of germinated seeds (radicular protrusions of 3 to 4 mm) after 72, 120 and 168 h at the beginning of the test. The results were expressed by the mean of the indices of replicates (four) and calculated according to **Equations 3** and **4**.

$$GS = \frac{(N1 * G1 + N2 * G2 + N3 * G3)}{(G1 + G2 + G3)} \qquad (3)$$

$$GSI = \frac{G1}{N1} + \frac{G2}{N2} + \frac{G3}{N3} \qquad (4)$$

Where: G, the number of germinated seeds with primary root emission; G1, seeds counted at the first count; G2, seeds counted at the second count; G3, seeds counted at the third count; N, days number of seeding; N1, days number of seeding of the first count; N2, of the second count; N3, of the third count. [22]

The allelopathic effect index (RI) was estimated according to Gao et al. [23] by **Equation 5**, where C is the control germination speed and T is the treatment germination speed.

$$RI = 1 - \frac{C}{T}\ (T \geq C)\ or\ RI = \frac{T}{C} - 1\ (T\ < C) \qquad (5)$$

At the end of the germination test, the initial growth, measured by the length of the aerial part obtained by the distance between the insertion of the basal portion of the root to the apex of the aerial part, was measured, while the root length was determined by measuring the distance between the apical and basal parts. The results are expressed in mm seedlings.

Statistical analysis

The data of the germination and allelopathic analysis were submitted to analysis of variance and had 5% significance ($p \leq 0.05$). The results of initial seedling growth were submitted to ANOVA followed by Tukey's test (GraphPad Prism 4 program).

Results and Discussion

The analysis of TPC showed a value of 1.85 µg of galic acid equivalent/µl of sample in *R. xerampelina* EthE, and 2.04 µg of galic acid equivalent/µl of sample in *S. granulatus* EthE. The scavenging effect was 29.2% and 38.9% to *R. xerampelina* (1.7 µmol of Trolox equivalent/µl of sample) and *S. granulatus* (2.6 µmol of Trolox equivalent/µl of sample) EthE, respectively. Analysis of the mushroom EthE revealed the presence of some phenolic acids; in the *R. xerampelina* EthE, the presence of 3.0 µg/g gallic acid was detected, and in the *S. granulatus* EthE, the presence of 54.0 µg/g protocatechuic acid and 23.9 µg/g *p*-hydroxybenzoic acid was detected.

In the literature, there are few studies evaluating the composition of phenolic acids in *R. xerampelina*. The studies that evaluated the phytochemical composition of *Russula delica* and *Russula brevipes*, edible species of this genus, showed the presence of gallic acid [24,25], which is also found in the *R. delica* EthE produced from this mushroom. [26] As in the present study, the presence of protocatechuic acid and *p*-hydroxybenzoic acid in *S. granulatus* is commonly observed in other studies. [24, 25, 27]

The antioxidant activity as well as the chemical composition in the same mushroom species can vary according to several factors including, for example, the geographic location of the mushroom specimen; [28] the structure (pileus or stipe or both) used for extraction of the compounds; [29] and especially the type of solvent used during the extraction process. [30] The last factor will directly determine the type of compound that will be extracted from the mushroom and consequently its antioxidant potential. Some studies already use plant extracts containing antioxidant compounds in tomato conservation. Natural antioxidants may prove to be an alternative in protecting against environmental stress on these fruits. [31,32]

The antimicrobial effect of *R. xerampelina* and *S. granulatus* EthE was tested against *Pseudomonas* sp. The *R. xerampelina* EthE showed better results than did the *S. granulatus* EthE, MIC = 2.5 and MIC = 5.0 mg/mL, respectively. After demonstrating the bacteriostatic effects of the tested extracts at these concentrations, with regard to the fractional inhibitory concentration index (FICI), our results indicate an additive effect between the compounds. The lowest combined EthE concentrations were 2.5 mg/mL for *R. xerampelina* and 0.31 mg/mL for *S. granulatus*, resulting in an FICI of 0.62.

Few studies have evaluated the antimicrobial activity of *R. xerampelina*; Singdevsachan et al. [33] showed the antibacterial activity of *Russula vesca* and *R. delica* ethanolic, methanolic and aqueous extracts against two gram-negative bacteria (*Escherichia coli* and *Vibrio cholerae*) and demonstrated better activity than that of the negative control, a standard antibiotic. The solvent used in the extraction processes is a crucial factor for the antibacterial potential of the final extract. Reis et al. [34] showed a better antibacterial activity of the methanolic mushroom extract of *S. granulatus* against *Pseudomonas aeruginosa* than that shown in this study. Methanol and ethanol have different polarities, so they can extract compounds of different polarities or even different amounts of the same compound. These same authors also achieved higher values of p-hydroxybenzoic acid than that demonstrated in our study, and its antimicrobial potential has already been shown. [35,36]

Volcão et al. [37] showed the antibacterial activity of *R. xerampelina* aqueous extracts against gram-negative and gram-positive bacteria; however, the *S. granulatus* aqueous extract did not achieve the same performance. The synergistic effects resulting from the combination of different extracts have been documented in the literature. [38,39] The mechanisms responsible for the additive effect between the EthE under study are still unknown. There are some pathways that cause inhibition of bacterial growth, such as disturbing the permeability of the cytoplasm membrane that facilitates the influx of antibacterial compounds, disrupting peptidoglycans in the cytoplasm membrane, inhibiting resistance enzyme activity and inhibiting efflux pumps. [39-41] In this case, the action of one of our extracts seems to favor the action of the other, each acting on different action sites.

This study showed a small decrease in the percentage of lettuce seed germination at a maximum concentration of both extracts (figure 1a), whereas for tomato seed, there was a

decrease in this percentage at all concentrations of both extracts, except with 0.25% *S. granulatus* (figure 1b). Despite differences in the seed germination percentage, no significant difference was observed between the treatments (table 1). The germination rate indicates the increase in the number of days required for seed germination. A significant increase ($p \leq 0.05$) in the germination rate of lettuce treated with both ethanolic extracts was observed (figure 2a), and an increase the germination rate of tomato seeds was also noted, even though this showed a less promising increase in the *S. granulatus* EthE treatment (figure 2b).

At the maximum concentration of both extracts, a decrease in the rate of germination in the lettuce seeds was observed (figure 3a), while in the tomato treatment, all the concentrations of both extracts caused a decrease in this index (figure 3b). The reduction in germination rate values may indicate, according to the definition, a decrease in seed vigor. [22] In the present study, the trend of the index was inversely related to the germination rate, both with significant differences between the treatments. Some mechanisms can cause a decrease in the germination percentage and germination rate, including increased membrane deterioration, mainly by lipid peroxidation, inhibition of photosynthesis, and alterations in phytohormones and nutritional elements. [42,43] Thus, the increase in the number of days for germination may reflect the emergence of seedlings in the substrate, influencing their initial establishment.

The values of the allelopathic index with respect to the germination of lettuce seeds demonstrate a positive relation in the quadratic model (figure 4a) for both EthEs. In the tomato treatment, there was a positive increase in the allelopathic effect in the 0.25, 0.5 and 1% *S. granulatus* EthE (figure 4b). Exposure to the *R. xerampelina* EthE caused a decrease in the allelopathic effect, even though it was still at a positive level at the highest concentration (1%). The differences among the EthE treatments for *L. sativa* and *S. lycopersicum* are shown

in **Table 2**. The results revealed that the tomato seeds were more sensitive to treatments with the EthEs than were the lettuce seeds. The *R. xerampelina* EthE at a concentration of 1% appears to interfere with root growth and with the initial growth of both test species. According to the statistical analysis, the results of most EthE concentrations of both extracts did not differ from those of the control (distilled water).

The secondary metabolites excreted by mushrooms, such as phenolic acids, represent a way of surviving environmental stress conditions. These and/or other secondary metabolites may be responsible for the positive response of lettuce and tomato seeds to the extracts as observed in this study. A management strategy that already happens in soybean production is the coverage of the soil by oat straw, which releases substances such as coumaric acid and ferulic acid, thus reducing the emergence of several weed species. [44,45]

R. xerampelina and *S. granulatus* are arbuscular mycorrhizal fungi that establish symbiotic relationships with *Pinus* species. In evolutionary terms, several metabolites are produced as a result of this association, and these compounds are mainly responsible for the effects on other organisms. [46,47] Analysis of the results regarding the initial growth and allelopathic effects revealed a positive response in germination, growth and/or development stimulus. The use of mushroom compost for organic fertilization in horticulture has already been carried out. [48,49] The importance of mushrooms in most diverse areas of food is unquestionable, and the data of the present study demonstrate that these organisms can contribute to sustainable agriculture, especially in reducing pest control by pesticide use.

Conclusion

Our study indicates the potential of *Russula xerampelina* and *Suillus granulatus* ethanolic extracts for the control of *Pseudomonas aeruginosa*. The use of such products originating from organisms is promising, since no phytotoxicity was observed on lettuce and tomato seeds in terms of the parameters evaluated in this study. With further studies, mushrooms can provide an alternative for the control of plant pathogens.

Declaration of interest statement

Not applicable

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FIGURE CAPTIONS

Figure 1. Percentage of germination of *Lactuca sativa* (a) and *Solanum lycopersicum* (b) treated with *Russula xerampelina* (R.x.) and *Suillus granulatus* (S.g.).

Figure 2. Germination rate (days) of *Lactuca sativa* (a) and *Solanum lycopersicum* (b) treated with *Russula xerampelina* (R.x.) and *Suillus granulatus* (S.g.).

Figure 3. Germination rate index of *Lactuca sativa* (a) and *Solanum lycopersicum* (b) treated with *Russula xerampelina* (R.x.) and *Suillus granulatus* (S.g.).

Figure 4. Allelopathic index of *Russula xerampelina* and *Suillus granulatus* ethanolic extract in *Lactuca sativa* (a) and *Solanum lycopersicum* (b).

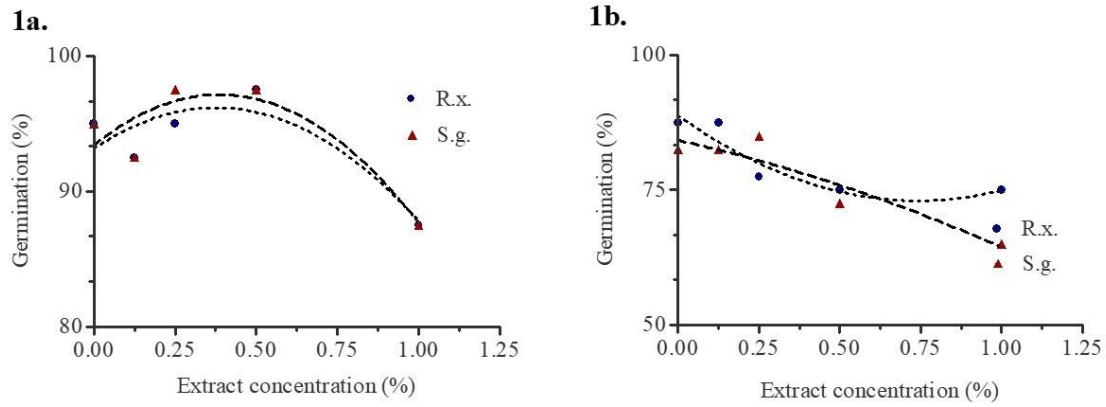

Fig. 1

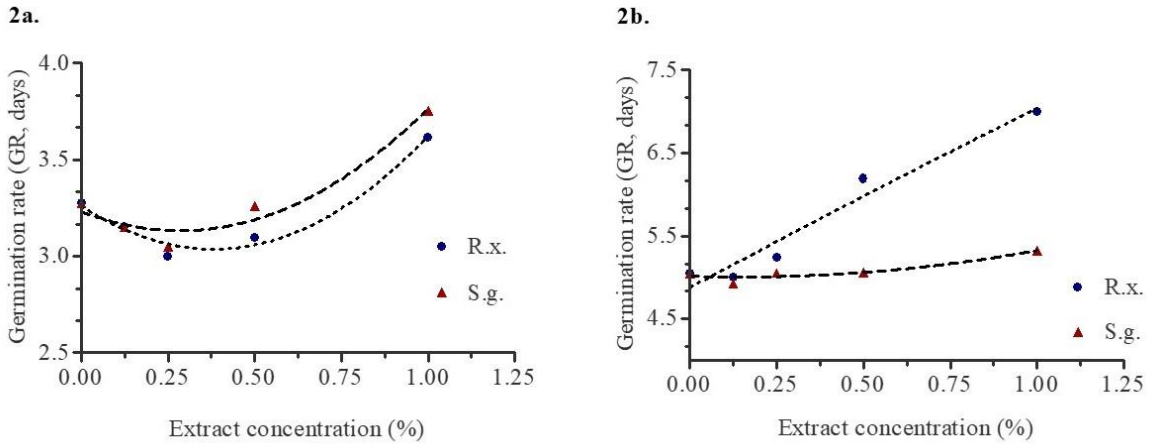

Fig. 2

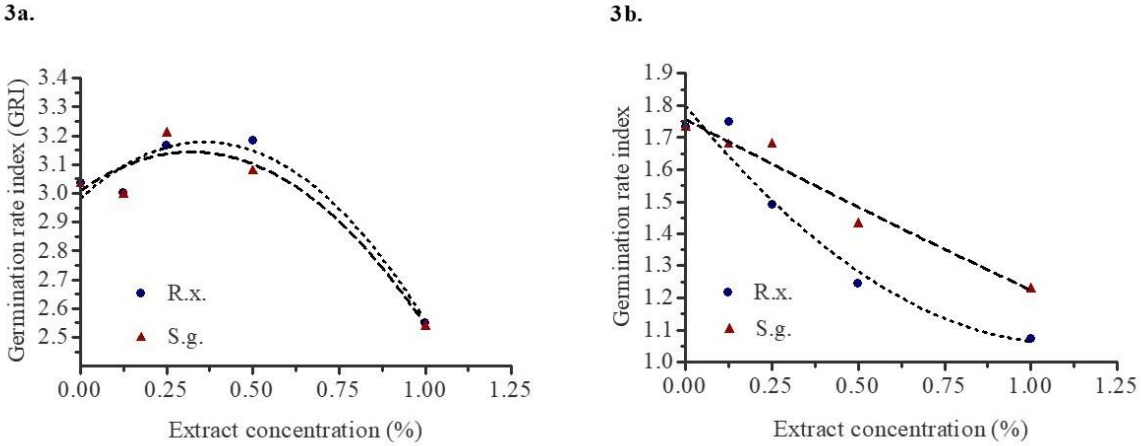

Fig. 3

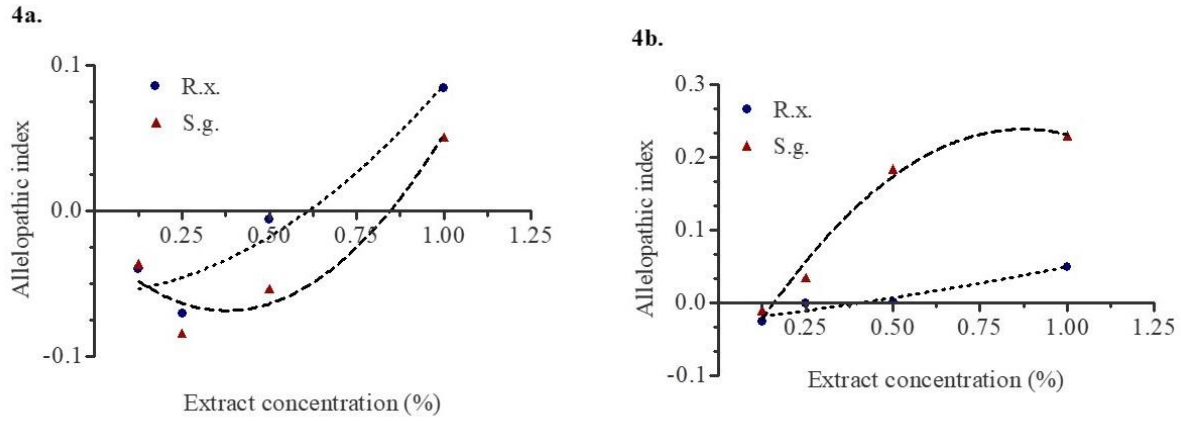
4a.
0.1
0.0
-0.1
R.x.
S.g.
Allelopathic index
0.25
0.50
0.75
1.00
1.25
Extract concentration (%)
4b.
0.3
0.2
0.1
0.0
-0.1
R.x.
S.g.
Allelopathic index
0.25
0.50
0.75
1.00
1.25
Extract concentration (%)

Fig. 4

Table 1. Values of early germination (%), germination rate (GR, days), and germination rate index (GRI) of *Lactuca sativa* exposed to *Russula xerampelina* and *Suillus granulatus* ethanolic extracts.

Treatment	Mean ± SE*		
	Germination (%)	GR (days)	GRI
T_1	95.0 ±5.00^{a}	3.2 ±0.16^{ab}	3.1 ±0.07^{ab}
T_2	92.5 ±4.78^{a}	3.1 ±0.09^{ab}	3.0 ±0.15^{ab}
T_3	97.5 ±2.50^{a}	3.0 ±0.05^{b}	2.7 ±0.16^{ab}
T_4	92.5 ±4.78^{a}	3.1 ±0.09^{ab}	3.0 ±0.14^{ab}
T_5	95.0 ±2.88^{a}	3.0 ±0.00^{b}	3.1 ±0.09^{ab}
T_6	97.5 ±2.50^{a}	3.1 ±0.05^{ab}	3.1 ±0.06^{ab}
T_7	87.5 ±4.78^{a}	3.6 ±0.12^{ab}	2.5 ±0.10^{b}
T_8	92.5 ±4.78^{a}	3.1 ±0.15^{ab}	3.0 ±0.13^{ab}
T_9	97.5 ±2.50^{a}	3.0 ±0.05^{b}	3.2 ±0.07^{a}
T_{10}	97.5 ±2.50^{a}	3.2 ±0.10^{ab}	3.0 ±0.12^{ab}
T_{11}	87.5 ±2.50^{a}	3.7 ±0.08^{a}	2.5 ±0.08^{b}
T_{12}	97.5 ±2.50^{a}	3.1 ±0.15^{ab}	3.1 ±0.09^{ab}
T_{13}	92.5±2.50^{a}	3.5 ±0.32^{ab}	2.7 ±0.23^{ab}
p-value	0.4618	0.0031	0.0024

Legends: SE - standard deviations; *Similar letters within the same column indicate statistically similar results at 5% significance level. T_1) control; T_2) ethanol 0.12%; T_3) ethanol 1%; T_4) *R. xerampelina* 0.12%; T_5) *R. xerampelina* 0.25%; T_6) *R. xerampelina* 0.5%; T_7) *R. xerampelina* 1%; T_8) *S. granulatus* 0.12%; T_9) *S. granulatus* 0.25%; T_{10}) *S. granulatus* 0.5%; T_{11}) *S. granulatus* 1%; T_{12}) *R. xerampelina* 0.5% + *S. granulatus* 0.06%; T_{13}) *R. xerampelina* 1% + *S. granulatus* 0.12%.

Table 2. Values of early germination (%), germination rate (GR, days), and germination rate index (GRI) of *Solanum lycopersicum* exposed to *Russula xerampelina* and *Suillus granulatus* ethanolic extracts.

Treatment	Mean ± SE*		
	Germination (%)	GR (days)	GRI
T_1	87.5 ±7.50^{a}	5.0 ±0.13^{c}	1.6 ±0.10^{ab}
T_2	85.0 ±2.88^{a}	5.3 ±0.11^{c}	1.2 ±0.17^{ab}
T_3	62.5 ±4.78^{a}	5.0 ±0.00^{c}	1.6 ±0.08^{ab}
T_4	87.5 ±6.29^{a}	5.0 ±0.00^{c}	1.7 ±0.12^{a}
T_5	77.5 ±10.31^{a}	5.2 ±0.10^{c}	1.4 ±0.19^{ab}
T_6	75.0 ±8.66^{a}	6.1 ±0.18^{b}	1.2 ±0.14^{ab}
T_7	75.0 ±8.66^{a}	7.0 ±0.00^{a}	1.0 ±0.12^{b}
T_8	82.5 ±7.50^{a}	4.9 ±0.07^{c}	1.6 ±0.13^{ab}
T_9	85.0 ±6.45^{a}	5.0 ±0.05^{c}	1.6 ±0.12^{a}
T_{10}	72.5 ±8.53^{a}	5.0 ±0.06^{c}	1.4 ±0.16^{ab}
T_{11}	65.0 ±8.66^{a}	5.3 ±0.11^{c}	1.1 ±0.16^{ab}
T_{12}	85.0 ±2.88^{a}	6.0 ±0.11^{c}	1.4 ±0.05^{ab}
T_{13}	85.0 ±5.00^{a}	6.7 ±0.08^{a}	1.2 ±0.09^{ab}
p-value	0.2156	< 0.0001	0.0073

Legends: SE - standard deviations; *Similar letters within the same column indicate statistically similar results at 5% significance level. T_1) control; T_2) ethanol 0.12%; T_3) ethanol 1%; T_4) *R. xerampelina* 0.12%; T_5) *R. xerampelina* 0.25%; T_6) *R. xerampelina* 0.5%; T_7) *R. xerampelina* 1%; T_8) *S. granulatus* 0.12%; T_9) *S. granulatus* 0.25%; T_{10}) *S. granulatus* 0.5%; T_{11}) *S. granulatus* 1%; T_{12}) *R. xerampelina* 0.5% + *S. granulatus* 0.06%; T_{13}) *R. xerampelina* 1% + *S. granulatus* 0.12%.

Table 3. Values of early seedling growth (mm) of *Lactuca sativa* and *Solanum lycopersicum* exposed to *Russula xerampelina* and *Suillus granulatus* ethanolic extracts.

Treatment	Early seedling growth (mm) - Mean ± SE*			
	Lactuca sativa		*Solanum lycopersicum*	
	Initial growth	Root length	Initial growth	Root length
T_1	26.5 ±1.26^{ab}	29.6 ±9.39^{ab}	30.0 ±1.21^{a}	31.6 ±3.16^{abc}
T_2	27.0 ±1.17^{ab}	36.0 ±3.13^{a}	22.0 ±1.11^{abc}	40.6 ±1.73^{a}
T_3	17.1 ±1.95^{cde}	28.4 ±1.46^{ab}	3.1 ±0.84^{f}	36.6 ±2.56^{ab}
T_4	25.2 ±1.36^{abc}	33.9 ±1.63^{a}	25.7 ±1.74^{a}	36.5 ±3.46^{ab}
T_5	28.7 ±1.23^{a}	34.6 ±1.90^{a}	17.0 ±1.72^{cd}	22.6 ±3.81^{bcd}
T_6	25.4 ±1.39^{ab}	33.0 ±1.46^{a}	11.7 ±1.02^{de}	22.5 ±3.86^{bcd}
T_7	15.8 ±1.63^{de}	24.6 ±3.63^{ab}	5.0 ±1.60^{ef}	12.1 ±3.51^{d}
T_8	26.6 ±0.74^{ab}	32.2 ±2.83^{a}	25.3 ±1.44^{ab}	31.0 ±4.45^{abc}
T_9	28.1 ±1.35^{ab}	34.9 ±2.08^{a}	24.6 ±1.39^{abc}	35.5 ±3.68^{ab}
T_{10}	20.4 ±1.42^{bcd}	23.8 ±2.00^{ab}	17.5 ±1.83^{bcd}	31.1 ±2.98^{abc}
T_{11}	10.5 ±1.26^{e}	11.5 ±1.59^{b}	13.8 ±2.65^{d}	21.5 ±4.88^{bcd}
T_{12}	21.4 ±2.59^{abcd}	32.4 ±3.86^{a}	9.9 ±1.20^{def}	17.3 ±1.36^{cd}
T_{13}	16.5 ±2.91^{de}	24.0 ±5.82^{ab}	5.7 ±1.53^{ef}	13.4 ±3.36^{d}
p-value	< 0.0001	0.0032	< 0.0001	< 0.0001

Legends: SE - standard deviations; *Similar letters within the same column indicate statistically similar at 5% significance level. T_1) control; T_2) ethanol 0.12%; T_3) ethanol 1%; T_4) *R. xerampelina* 0.12%; T_5) *R. xerampelina* 0.25%; T_6) *R. xerampelina* 0.5%; T_7) *R. xerampelina* 1%; T_8) *S. granulatus* 0.12%; T_9) *S. granulatus* 0.25%; T_{10}) *S. granulatus* 0.5%; T_{11}) *S. granulatus* 1%; T_{12}) *R. xerampelina* 0.5% + *S. granulatus* 0.06%; T_{13}) *R. xerampelina* 1% + *S. granulatus* 0.12%.

ARTIGO 2

Effect of mycorrhizal mushroom extract of *Lactarius deliciosus* on brown rot control

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Effect of mycorrhizal mushroom extract of *Lactarius deliciosus* on brown rot control

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Abstract

Monilinia fructicola (Wint.) Honey is a plant pathogenic fungus that infects stone fruits such as peach, nectarine and plum, high demand cultivars in Brazil. This pathogen may remain latent in the host, showing no signs of disease, and consequently spreading among different countries. The aim of this study was to evaluate the activity of *Lactarius deliciosus* mushroom hydroalcoholic extract (HydE) against *M. fructicola*, and its effect on mycelial growth, as well as its toxicological aspects. The *L. deliciosus* HydE was able to inhibit fungal growth and cause alterations in the phytopatogen mycelial development at a concentration of 1.25 mg/mL. As indicated by the phytotoxicity tests, the extract showed moderate phytotoxicity (interference on germination percentage and rate index, root and initial growth measures, and on the fresh weight of the seedlings) and no cytotoxicity in Vero line cells. Thus, our results highlight the use of mushrooms extracts for biological control, taking into account the efficiency and environmentally friendly feature.

Keywords: mushrooms; phytopatogen; *Monilia fructicola*; mycelial growth; natural products, phytotoxicity.

Introduction

Brown rot, which has as its etiological agent the fungus *Monilia fructicola* (Wint.) Honey, is one of the main disease affecting stone fruit cultivars such as nectarine, plum and peach. This species is commonly found in Australia, South Africa, North and South America, and is listed as a European quarantine organism (European and Mediterranean Plant Protection Organization, 1992; Larena et al., 2005). The first report of *M. fructicola* in Europe occurred in 2001 in France, followed in the Czech Republic, and in 2009 in Spain (Lichou et al., 2002; Duchoslavová et al., 2007; De Cal et al., 2009).

The main losses in fruit yield are due to flower infection and fruit decay in the harvest and postharvest stages (Emery et al., 2000), being the disease-causing microorganisms able to hibernate in certain plant organs. For this reason, methods for prophylaxis are important in controlling brown rot (Garcia-Benitez et al., 2016). Pereira et al. (2019) demonstrate that species of *Monilinia* spp. isolated from imported fruits, can adapt to the conditions of peach producing regions in Brazil, and put at risk the management of the disease. In cultivation regions where the damage is most significant, chemical control is necessary before fruiting. This control is performed in Brazil by pulverization of the cultivars with triforine, vinclozolin, iprodione, flusilazole or benzimidazoles and the treatment may also occur pre and post harvest (Moreira and May-De Mio, 2009; Pavanello et al., 2015).

However, in the current scenario, there has been constant concern about pesticides use and its effect on the human and environmental health. With this, numerous studies have been conducted to biological control field, showing environmental friendly alternatives to the massive use of agricultural pesticides (Cotes et al., 2018; Nahar et al., 2019). Some of these alternatives are based on active organism or microorganism and some of its metabolites, as the use of entomopathogenic fungi and the application of natural extracts (Yánez-Mendizábal et al., 2012; Pazolini et al., 2016).

Compared to research using plant extracts for biological control, the number of studies using mushroom extracts to plant pathogens control is relatively limited (Stangarlin et al., 2011; Degenkolh and Vilcinskas, 2016). Thus, the overall objective of the present study was to determinate the effect of *Lactarius deliciosus* hydroalcoholic extract in the in vitro control of the *M. fructicola* and to analyze some toxicological effects of the extract, such as the response of a plant organism after exposure to this extract, and the *in vitro* cytotoxicity.

Methodology

Mushroom collection and extraction

Mushrooms samples were collected from an area belong to the Universidade Federal de Pelotas (Capão do Leão Campus – 31º48'06" S, 52º24'52"W) and the *L. deliciosus* identification was performed using an Identification Guides (Wright and Albertó 2002). Mushrooms were dried in an oven at 50 °C and the samples were prepared by maceration. The hydroalcoholic extract (HydE) was prepared with 25 g of each mushroom specie with EtOH 50% (1:4 w/v) by ultrasonic processes (SB – 5200 DTDN Ultrasonic Cleaner) at 40 °C for 120 min (Roesler et al. 2007). The final mixtures of the extract was filtered through Whatman® No. 1 filter paper in order to eliminate the precipitates.

Phytochemical evaluation

Total phenol content evaluation

HydE was analyzed for total phenol content (TPC) using the method described by Roesler et al. (2007) For colorimetric reaction of TPC analysis, a methanol extract solution (1:20 v/v) was used. TPC was expressed as microgram of gallic acid equivalent per milligram of extract (µg GAE/mg).

High Performance Liquid Chromatographic

The identification of phenolic acids was determined by the retention time of reference standards obtained from Sigma Aldrich (gallic, protocatechuic, chlorogenic, p-hydroxybenzoic, caffeic, syringic, vanilli, *p*-coumaric and ferulic acids). Aliquots of the extracts were previously lyophilized and resuspended in the mobile phase using methanol and acidified water (1% acetic acid) (1:1). The mixture was injected into a liquid chromatography system coupled to a DAD detector using a C18 column (4.6 × 250 mm, 5 mg; Discovery, USA). HPLC-DAD was run at a flow rate of 0.7 mL/min at 35 °C using methanol and acidified water (1% acetic acid) as the mobile phase at a ratio of 20:80 (v/v) for 25 min, with readings at 280 and 320 nm (Scaglioni et al., 2014).

Antioxidant activity

The free radical scavenging activities of *L. deliciosus* HydE were measured according to the DPPH• method reported by Roesler et al. (2007) In this study, the mushroom extracts were diluted in methanol (1/10), and the samples were subjected to ultrasound for 30 min at 10 °C and subsequently diluted to different concentrations. The standards and samples were incubated for 30 min at room temperature protected from light. The absorbance of the mixture was measured at λ = 517 nm, and the amount of DPPH consumed was expressed as µmol Trolox equivalent per mL of extract (µmol Trolox/mL). The ability to scavenge free radicals was expressed as percent inhibition (% inhibition) according **Equation 1**.

$$\%\ inhibition = \left(A_{DPPH} - \frac{\frac{A_{Am}}{trolox}}{A_{DPPH}} \right) * 100 \qquad (\mathbf{1})$$

Where: A_{DPPH}, DPPH solution absorbance; A_{Am}/Trolox, sample or standard in solution absorbance.

Antifungal assay

Broth microdilution test

Preliminary antifungal activity analysis of *L. deliciosus* HydE was performed according the Broth microdilution test (CLSI 2015), where the Minimal Inhibitory Concentration (MIC) capable inhibiting the fungal growth was established. The target fungal species, *Monilinia fructicola* IOC 4630, were cultivated in potato dextrose agar (PDA – Sigma®) plates for 5 to 7 days. The spore suspension was prepared from a PDA culture by adding 5 ml of saline solution (0.85% v/v). A sterile Drigalski spatula was used in the agar surface to obtain a spore suspension and the concentration of this spore solution was determined by counting the conidia in a Neubauer chamber. Before antifungal analysis, the HydE were evaporated to dryness under vacuum, and the extracts were dissolved in DMSO 5% aqueous solution (10 mg/mL). The serial dilutions (2500, 1250 and 625 µg/mL) were performed in RPMI-1640 medium, in a 96-well plate and the final spore inoculum used was 6 × 10^5 per well. For the colorimetric reaction were added 10 µl of resazurin (0.002% w/v) per well, used as an indicator of cell growth. Each microplate contained positive control of fungal growth, and two negative controls (for the medium and for the extract). The plates were subsequently incubated for 24 h at 37° C, and the experiment was performed in triplicate (Monteiro et al., 2012).

Agar dilution method

Antifungal activity of the *L. deliciosus* HydE on fungal colony development was performed by a dilution method of extract in PDA. The extract was added to 10 ml of PDA before solidification in a Petri dish, in three different concentrations (0.625, 1.25 and 2.5 mg/mL). A control containing distilled water plus DMSO 5% was used. One disc (10 mm diameter) of mycelial plugs, taken from the edge of a 5-7 day fungal culture, was placed into

the Petri dish and incubated at 25°C ±2. Evaluations were based on daily measurements of colony diameter on four orthogonal axes (mean of the two diametrically opposite measurements) until the treatment reached the edges of the plate. The radial growth of the mycelium was recorded and the efficacy of the HydE was evaluated by the mycelium growth rate (mm/days). The experiment was performed in four replicates (Mahanta et al., 2007).

These data were also used to calculate the mycelial growth rate index (MGRI), according to the **Equation 2** described by Oliveira (1991), and the percentage of mycelial growth inhibition (MGI %) according to the **Equation 3** (Pandey et al., 1982).

$$MGRI = \sum \frac{(d - dp)}{N} \quad (\mathbf{2})$$

$$MGI\ (\%) = \frac{(dc - dt)}{dc} \times 100 \quad (\mathbf{3})$$

Where: d, is the current average diameter of colony; dp, average diameter of previous day colony; N, the number of days after inoculation; and dc, is the diameter in control; dt, diameter of the treatment. Lastly, it was calculated from the area below the mycelial growth curve (ABMGC), according the **Equation 4** (Campbell and Madden, 1990).

$$ABMGC = \sum (\frac{yi + 1 + yi}{2}) \times (ti + 1 + ti) \quad (\mathbf{4})$$

Where: yi and yi + 1, are the colony growth values observed in two consecutive evaluations; ti + 1 and ti, are the evaluation periods.

Toxicological analysis

Germination and seedling analysis

The assays for the analysis of effects on plant species were performed as described in OECD (2006) with some modifications. To evaluate the germination, seeds of the target specie cabbage (*Brassica oleracea* L.) was used. We tested four extract concentrations (625, 1250, 2500 and 5000 µg/mL) and for the control treatment was used only distilled water.

Seeds were placed to germinate in Petri dishes on filter paper humidified with 2.5 times the mass of paper with distilled water (for control group) or according to their respective treatment. After, the plates were placed in BOD at 22 °C. The germination speed (GS) and the germination speed index (GSI) were determined by counting the number of germinated seeds daily (radicular protrusions of 3 to 4 mm). The result was expressed by the mean of the indices of replicates (four) and calculated according to **Equation 5** and **6**.

$$GS = \frac{(N1 * G1 + N2 * G2 + \cdots + Nn * Gn)}{(G1 + G2 + \cdots + Gn)} \quad (\mathbf{5})$$

$$GSI = \frac{G1}{N1} + \frac{G2}{N2} + \frac{Gn}{Nn} \quad (\mathbf{6})$$

Where: G, the number of germinated seeds with primary root emission; G1, seeds counted at the first count; G2, seeds counted at the second count; Gn, seeds counted at last count; N, days number of seeding; N1, days number of seeding of the first count; N2, of the second count; Nn, last count (Maguire 1962). The allelopathic effect index (RI) was estimated according to Gao et al. (2009) by **Equation 7**. Where: C, control germination speed; T, treatment germination speed.

$$RI = 1 - \frac{C}{T}\ (T \geq C)\ or\ RI = \frac{T}{C} - 1\ (T\ < C) \quad (\mathbf{7})$$

At the end of the germination test, the initial growth, measured by the length of the aerial part obtained by the distance between the insertion of the basal portion of the root to the apex of the aerial part, was measured, while the root length was determined by measuring the distance between the apical and basal parts. The results are expressed in mm seedlings.

Cytotoxicity assay

The cytotoxicity of the HydE was evaluated on adhered cells of VERO lineage. In a 96-well plate, 200 µl of a fibroblast suspension was cultivated in DMEM medium (Vitrocell Embriolife) supplemented with 10% of fetal bovine serum and maintained for 24 h at 37°C (humid atmosphere with 5% CO_2, at the 3.4×10^5 cells/mL concentration) (Snewin et al., 1999). After this period, adherent cells were exposed to serial HydE concentrations (2500 to 312.5 µg/mL), the plate was incubated for 24 h at 37°C (5% CO_2). To determinate the concentration of the extract capable of maintaining the viability of 50% of the cells (IC_{50}) was added 30 µl of 0.01% resazurin reagent, and the plates was incubated for 6 h until the fluorescence measurement at 620 nm (Pavan et al., 2010).

Statistical analysis

Statistical analyses were performed using one-way of variance followed by Tukey's test ($p < 0.05$) via GrapPad Prism 4 (GraphPad Software, La Jolla, California, USA). The results of antifungal test, germination, and allelopathic analyses are expressed as mean and standard deviation.

Results and Discussion

Phenolic composition and antioxidant activity

By phenol analysis, the *L. deliciosus* HydE showed 2.4 µg of GAE equivalent/mg of sample. Among the researched phenolic acids, we found 5,656.7 µg/g of gallic acid, 1,425.9 µg/g of protocatecoic acid, 6,801.6 µg/g of hydroxybenzoic acid, and 3,927.0 of coumaric acid. Because they are products of the secondary metabolism of mushrooms, TPC production is subject to environmental variations and stress, such as temperature, humidity, and injuries by other organisms (Stadler and Sterner, 1998; Oke et al., 2011), may vary in mushrooms of the same species.

Phenolic acids have an important role in the antioxidant function of plant and mushroom extracts, mainly due to their redox properties which allow them to act as reducing agents, hydrogen donors and metal chelators (Rice-Evans et al., 1997; Oke et al., 2011). The antioxidant potential of some compounds may prove to be an alternative in protecting the fruits against environmental stress, avoiding deterioration of different varieties of stone fruits in the field and in storage.

In this study, we observed a 57.8% antioxidant capacity of the extract, corresponding to a value of 0.903 µmol/mL of Trolox equivalent. Aqueous solvents and hydroalcoholic mixtures have similar polarities; however, Volcão et al. (2019) demonstrated that in the same extraction technique and mushroom species, aqueous extracts had lower amounts of TPC and antioxidant activity than the HydE of the present study.

Antifungal analyzes

In our study, it was observed in the microdilution broth test, that 1.25 mg/mL was sufficient for growth inhibition of *M. fructicola*. Villarino et al. (2011) reported that the presence of phenolic acids in immature peaches contributed to the reduction of *Monilinia taxa* infection. It is important to note that the oxidation of phenolic compounds, even present in the pathogen exudate, contributes to the synthesis of melanin (Wang et al., 2018). The melanin is an essential factor of the susceptibility of phytopatogen to natural mechanisms of plant defense (Abo Ellil, 1999), in the development of sclerotia (a resistance structure), a strong cell wall, and aspects related to pathogen hibernation (Wang et al., 2018). Possibly, the antagonistic relationship between pathogen and compounds present in the evaluated extract causes the suppression of melanin synthesis, and the probable decrease in conidia production and consequent decrease in mycelium vigor (figure 1).

Some aspects of antifungal activity were evaluated, such as mycelial growth, mycelial growth inhibition rate, and mycelial growth inhibition percentage. Our results indicates a metabolic stability in the fungal growth by the continuous growth of the colonies in all treatments (figure 2). The increase in mycelial growth (diameter, mm) was directly proportional to the increase of the colony as well as mycelial growth rate inhibition (figure 3a); where the control (39.94c mm/day) did not differ from the lowest extract concentration (51.14c mm/day) by the Tukey's test. The mycelia growth index can be observed in **Figure 3b**.

Despite these results, the observed growth pattern may indicate an "exploratory" behavior by the pathogen. This type of comportment has already been reported in *Streptomyces* bacteria, where these, in the presence of inhibitors or nutrient deficits, begin to acquire an exploratory behavior in the culture medium (Richards et al., 2012; Jones et al., 2017). This may still be accompanied by the growth of exploratory cells such as unbranched hyphae. In addition, because the filamentous cycle is extremely effective in the soil, the absence of sporulation would mean a lack of ability to colonize new environments (Jones and Elliot, 2017).

The Area Below the Mycelial Growth Curve (ABMGC) is a variable that explains the mycelial behavior of the microorganism during the experiment (figure 4). The curve obtained in the study demonstrates a possible search for a favorable environment for mycelia development and spore production, where there is no interference of mushroom metabolites. The mycelial growth values and the ABMGC were positively correlated, where the increase of mycelial growth led to the increase of the ABMGC.

Toxicological experiments

According the **Table 1**, we observed a decrease in germination percentage, but not statistically significant between treatments and control (figure 5) in the Tukey's test. The two highest concentrations of the HydE, 2.5 and 5 mg/mL, were able to affect significantly the growth rate (figure 6a). However, regarding to the germination rate index (figure 6b), only the lowest concentration of the extract did not differ from the control. The results demonstrate that the HydE from *L. deliciosus* negatively influences in some aspects of the germination process. Some substances from the secondary metabolism of mushrooms may cause an allelopathic effect (figure 7), such as p-hydroxybenzoic acid, caffeic acid, chlorogenic acid, ferulic acid, and others (Wang et al., 2017).

In the seedling parameters growth, we can observe that for growth of root, initial growth and fresh weight, only the treatment carried out with the lowest extract concentration (0.625 mg/mL) did not differ from the control. The increase of days for germination may reflect the emergence of seedlings, influencing their initial establishment. Studies have shown that protocatechuic acid influences root knot formation and root growth (Mandal et al., 2009; Shang et al., 2017). However, it has been shown that ferulic and hydroxybenzoic acid, can decrease stomatal density and inhibit the accumulation of biomass (Fung et al., 2019).

Only in the analysis of fresh weight a significant difference was observed between treatments T3 (1.25 mg/ml) and T4 (2.5 mg/ml) with the control (figure 8). The application of the biocontroller as well as the growing season of the plant species receiving the compound will be of important influence on its antimicrobial and toxicological action. Concentrations that exhibit toxicity in early growth-related tests may not be toxic when applied to the established plant.

Cytotoxicity analyzes show an IC_{50} > 2.5 mg/mL, with an inhibition percentage of 34.4% at the highest concentration (2.5 mg/mL), followed by 3.21% inhibition at a concentration of 1.25 mg/mL and 0.15% at 0.625 mg/mL of HydE. There was no inhibition of

VERO cells at the lowest extract concentration (0.312 mg/mL). The reduced toxicity of this extract demonstrates its potential and its cellular safety level for application in food products, such as stone fruits, both preharvest and postharvest. In addition to the non-toxicity of these extracts, studies demonstrate the immunostimulatory and immunomodulatory potential of polysaccharides extracted from *L. deliciosus* (Hou et al 2013; Cheng et al 2019). These points may be responsible for better consumer acceptance, especially in fruits that are commonly consumed with peel.

Conclusions

Our study showed that, under controlled conditions, the inhibitory potential and the effectiveness of *L. deliciosus* hydroalcoholic extract interfere negatively with the mycelial development of *M. fructicola* phytopathogen. The interpretation of the toxicological results, suggests that hydroalcoholic extract are a potential source of bioactive compounds with moderate phytotoxicity for plant models. Added for this, the extract present no to tested cells, and can also be considered an environmentally friendly product.

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Conflicts of interest

Not applicable.

Availability of data and material

Not applicable.

Code availability

Not applicable.

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Tables

Table 1 Values of germination (%), germination rate (GR, days), germination rate index (GRI) and early seedling growth (mm) of *Brassica oleracea* L. exposed to *Lactarius deliciosus* hydroalcoholic extract. Data are means ± SD (n= 4).

Treatment	Mean ±SD*				
	Germination (%)	GR (per day)	GRI	Initial growth	Root growth
T_1	63.75 ±13.1^{ab}	3.15 ±0.2^{c}	4.88 ±0.9^{a}	26.45 ±2.8^{a}	25.07 ±2.8^{a}
T_2	71.00 ±18.4^{a}	3.74 ±0.2^{bc}	3.69 ±0.6^{ab}	19.70 ±3.2^{ab}	21.40 ±3.4^{ab}
T_3	51.25 ±13.7^{ab}	3.97 ±0.3^{bc}	2.70 ±0.6^{bc}	16.02 ±1.7^{bc}	13.82 ±10.1^{bc}
T_4	37.50 ±15.8^{b}	4.10 ±0.2^{ab}	1.87 ±0.7^{c}	16.92 ±4.0^{bc}	10.85 ±1.7^{bc}
T_5	35.00 ±17.5^{ab}	4.89 ±0.6^{a}	2.20 ±0.7^{bc}	9.27 ±6.2^{c}	5.47 ±2.6^{c}
p-value	0.0218	0.0002	0.004	0.0004	0.0005
C.V. (%)	29.34	9.70	26.89	21.26	28.03

Legends: SD - standard deviations; *Similar letters within the same column indicate statistically similar bond strength results at 5% significance level. T_1) control; T_2) *L. deliciosus* hydroalcoholic extract 0.625 mg/mL; T_3) *L. deliciosus* hydroalcoholic extract 1.25 mg/mL; T_4) *L. deliciosus* hydroalcoholic extract 2.5 mg/mL; T_5) *L. deliciosus* hydroalcoholic extract 5.0 mg/mL.

Figures

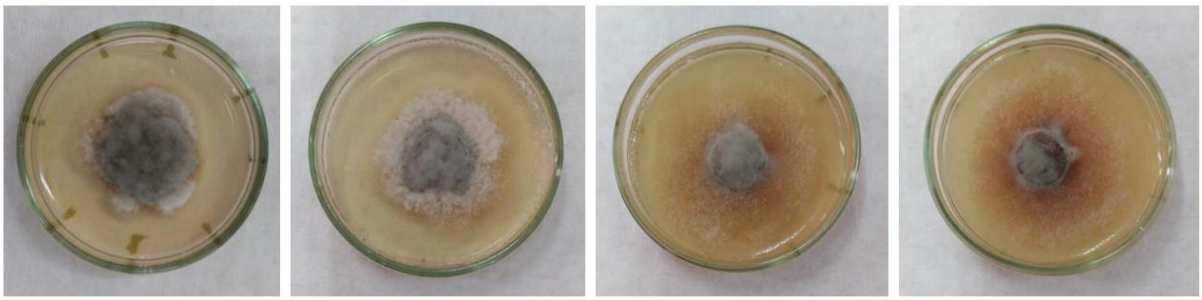

Fig. 1. Mycelial growth of the *Monilinia fructicola* in the presence (mg/mL) or absence (control) of *Lactarius deliciosus* hydroalcoholic extract. Treatment from left to right: control, 0.625 mg/ml, 1.25 mg/ml and 2.5 mg/ml.

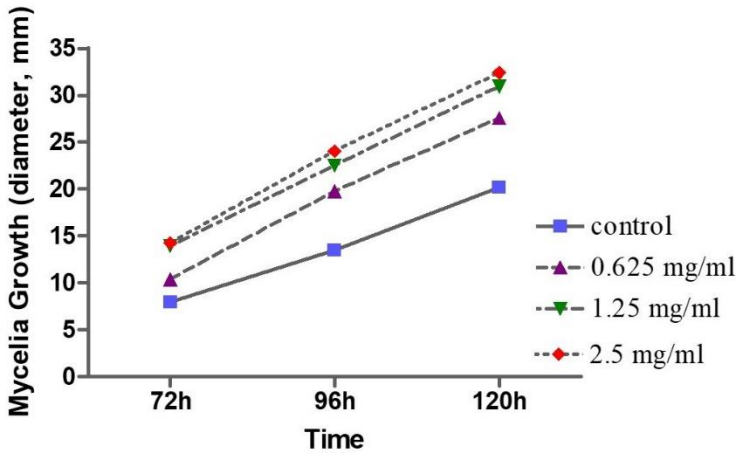

Fig. 2. Mycelial radial growth of *Monilinia fructicola* colony (diameter, mm) exposed to different concentrations extract of *Lactarius deliciosus*. The growth was calculated after 5 days of cocultivation.

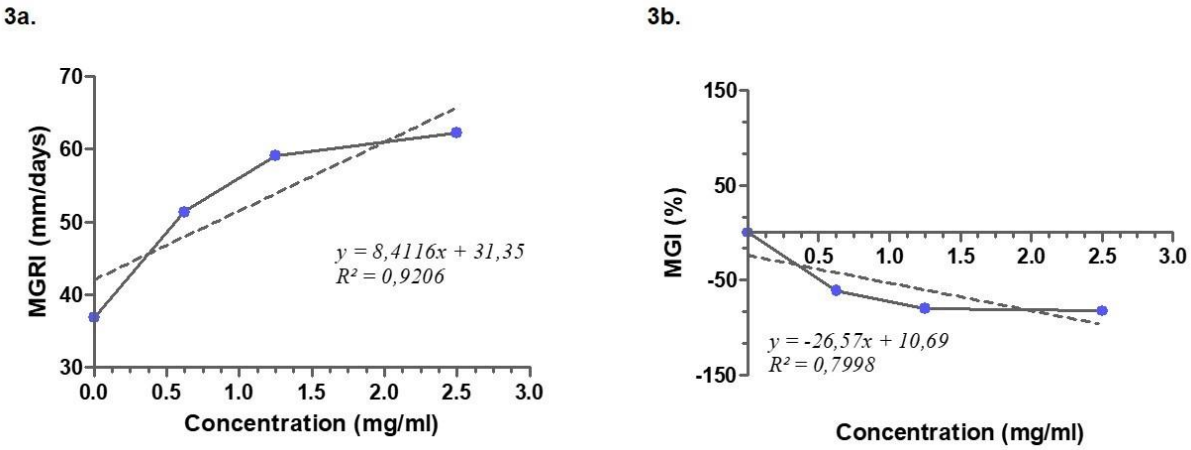

Fig. 3. Determination of mycelial growth rate inhibition (3a) and mycelial growth inhibition (3b) of the *Monilinia fructicola* exposed to different concentrations extract of *Lactarius deliciosus*.

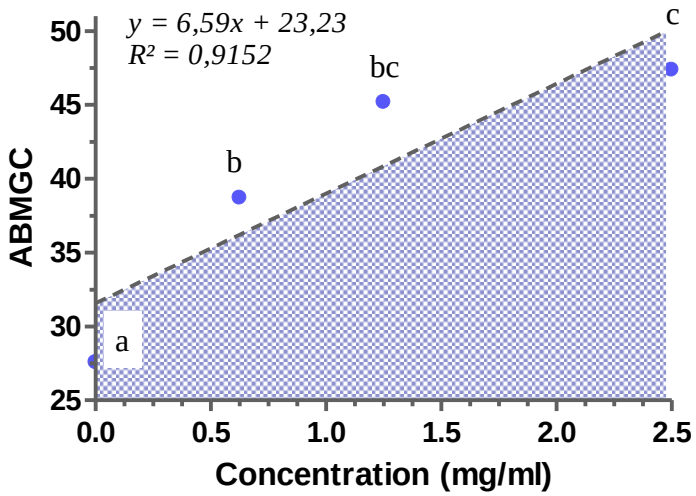

Fig. 4. Effect of increasing concentrations of *Lactarius deliciosus* extract (mg/ml) on *Monilinia fructicola* growth, represented by the Area Below the Mycelia Growth Curve (ABMGC). Inclination coefficient of the line was significant at 5% probability, by the Tukey's test. Similar letters indicate statistically similar bond strength results.

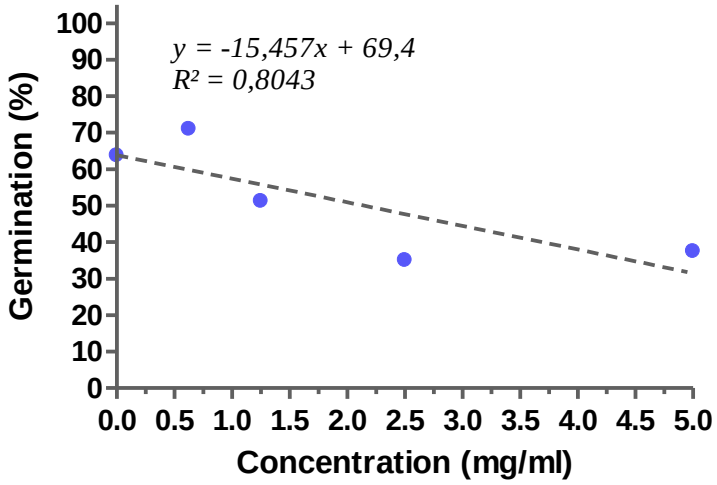

Fig. 5. Percentage germination of *Brassica oleracea* L. seeds treated with *Lactarius deliciosus* hydroalcoholic extract. Each point is the mean of four replicates.

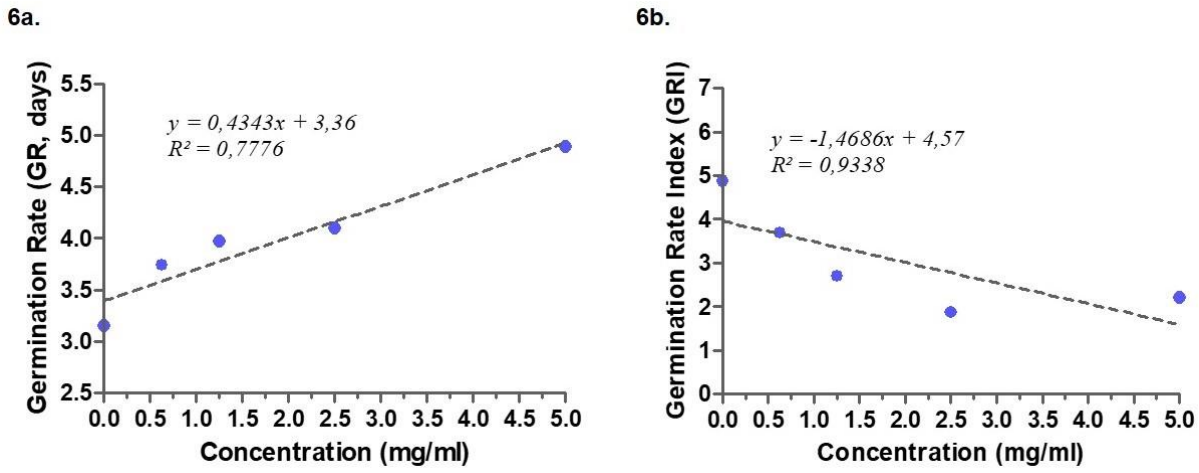

Fig. 6. Germination rate (GR, days) (6a) and Germination rate index (GRI) (6b) of *Brassica oleracea* L. seeds treated with *Lactarius deliciosus* hydroalcoholic extract. Each point is the mean of four replicates.

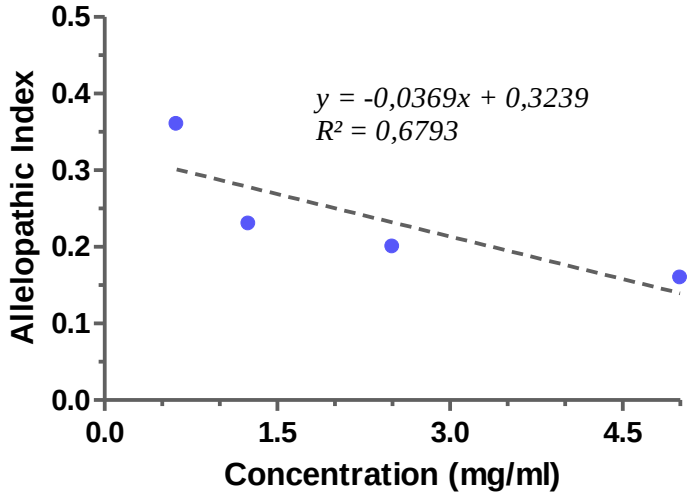

Fig. 7. Allelopathic Index of *Brassica oleracea* L. seeds treated with *Lactarius deliciosus* hydroalcoholic extract. Each point is the mean of four replicates.

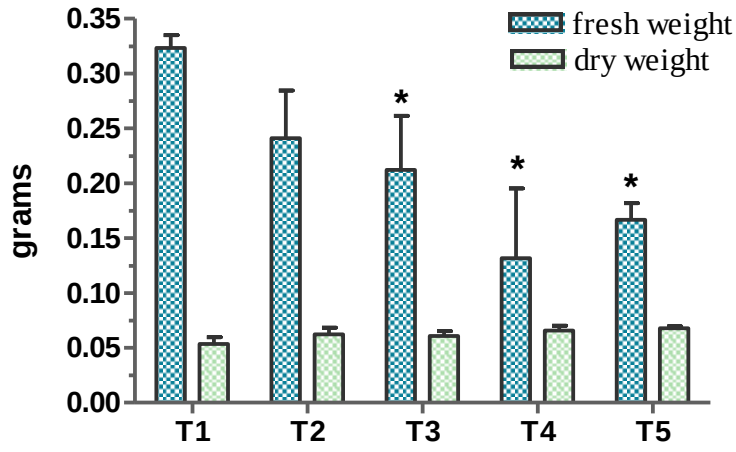

Fig. 8. Changes in average seedlings fresh and dry weight (grams) of *Brassica oleracea* L. treated with *Lactarius deliciosus* hydroalcoholic extract. T_1) control; T_2) *L. deliciosus* hydroalcoholic extract 0.625 mg/mL; T_3) *L. deliciosus* hydroalcoholic extract 1.25 mg/mL; T_4) *L. deliciosus* hydroalcoholic extract 2.5 mg/mL; T_5) *L. deliciosus* hydroalcoholic extract 5.0 mg/mL. Data are means ± SD (n= 4). *treatments with statistically significant difference (p<0.05).

ARTIGO 3

Artigo 3

Efficacy of *Laccaria laccata* mushroom extract in the control of *Fusarium solani* and Soil Toxicological Analyses

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Efficacy of *Laccaria laccata* mushroom extract in the control of *Fusarium solani* and Soil Toxicological Analyses

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Abstract

Fungal contamination is closely associated with agriculture, and due to the large territorial area needed for it, a large volume of chemical inputs are used, mainly in conventional crops. Studies in biological control of *Fusarium* sp., an important genera that affect soybean, has been observed by the use of different microorganism and plant extracts. However, few studies employed mushroom extracts in the agricultural field, and analyzing its impact on the environment. Thus, the objective of the study was to determinate the effect of *Laccaria laccata* mushroom hydroalcoholic extract in the in vitro control of the *Fusarium solani*, and analyze some toxicological aspects of this extract. The hydroalcoholic extract was effective to inhibit *F. solani* at a 0.625 mg/ml concentration. The in vitro toxicity test on VERO cells showed non-toxic effect on any tested concentrations. According to in vivo toxicity test, we can observe an increase in the survival of earthworms in artificial soils treated with different concentration of the extract in relation to the group control. As well, in the test that evaluates the functionality of the microbiota in an exposed natural soil, we observed a significant increase in the parameters of diversity and equability in the groups treated with the hydroalcoholic extract. This research demonstrated the efficacy of *Laccaria laccata* extracts in control of *Fusarium solani*. The lack of in vitro and in vivo toxicity in the evaluated parameters, make this extract a promising product for use in research on biostimulation in soils and substrates in agriculture.

Keywords: fusariosis, basidiomycete; antibacterial; biopesticides; mushrooms.

1. Introduction

During the 1960s, wheat was the main crop produced in Southern Brazil, with soybeans only a summer option. However, with efforts to increase the production of pigs and poultry, which generated the demand for soybean meal, and the explosion of its price on the world market, there was a need for investments for commercial production of this culture (EMBRAPA, 2020).

The agricultural production requires a relatively large territorial area, consequently, in conventional crops a large volume of chemical inputs are used. The chemical used in plantations are mainly for the prevention and treatment of plant disease that causes loss in production, such as sudden death syndrome, charcoal rot, and fusariosis (Allen et al., 2017). Fungal contamination is closely associated with agriculture, with *Fusarium solani* infection being significant in several crops. Conidia can colonize the host, while chlamydospores, and in addition to direct culture colonization, can overwhinter and restart the cycle when conditions are favorable (Dweba et al., 2017).

In addition to soybeans, *Fusarium solani* species complex has phytopathogenic potential in several agricultural crops, such as potatoes (Schroers et al., 2017), chickpea (Egamberdieva et al., 2017), ervilha (Šišić et al., 2018), and in another 100 crops (Kolattukudy et al., 1995). Studies in biological control of *Fusarium* sp. has been observed the use of different microorganism (Pastrana et al., 2016; Báez-Vallejo et al., 2020), and the use of plant extracts on the control of this disease (Kumar and Singh, 2016; Abdel et al., 2019).

However, research using mushroom extracts to control phytopathogenic organisms are scarce. Thus, the overall objective of this study was to determinate the effect of *Laccaria laccata* mushroom hydroalcoholic extract in the in vitro control of the *Fusarium solani*, and analyze toxicological aspects in vitro and in vivo.

2. Methodology

2.1. Collection and extraction mushrooms

Mushroom samples were collected from an area belonging to the Universidade Federal de Pelotas. *Laccaria laccata* identification was performed with the aid of identification guidelines (Wright and ALbertó 2002). Mushrooms were dried in an oven at 50 °C. The mushroom samples were prepared by maceration, and the hydroalcoholic extract (HyE) was prepared with 25 g of each mushroom species in 50% EtOH (1:4 w/v) by ultrasonication at 40 °C for 120 min (Roesler et al. 2007). The final mixtures of the extracts were filtered through Whatman® No. 1 filter paper in order to eliminate particulate matter

2.2. Determination of total phenol content and antioxidant activity

The total phenol content (TPC) in *the L. laccata* EtOH50 were analyzed using the method described by Roesler et al. (2007). For the colorimetric reaction employed in TPC analysis, a methanol extract solution (1:20 v/v) was used and the TPC was expressed as milligram gallic acid equivalent per gram of extract (mg GAE/g).

In order to evaluate the antioxidant activity of the EtOH50 was performed the analyses of free radical scavenging using the radical 2, 2-diphenyl- 1 – picrylhydrazyl (DPPH). The capacity to scavenge the 'stable' free radical DPPH was performed using the method described by Roesler et al. (2007). For the test, DPPH solution (0.0004% m/v) was prepared on the day of the assay, and the absorbance was measured at intervals between 0.8 and 1.2 abs (λ = 517 nm). In this study, the mushrooms extracts were diluted in methanol (1/10), and the samples were subjected to ultrasound for 30 min at 10°C, and subsequently diluted to different concentrations. The standard curves (Trolox equivalent)

and samples were incubated for 30 min at room temperature protected from light. The absorbance of the mixture was measured at λ = 517 nm, and DPPH was expressed as µmol de Trolox equivalent per mL of extract (µmol Trolox/mL). The ability to scavenge free radicals was expressed as percent inhibition (% inhibition) according to **Equation 1**.

$$Scavenging\ effect\ (\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) * 100 \qquad (\mathbf{1})$$

2.3. Effect of *Laccaria laccata* extract in *Fusarium solani* control

Antifungal activity of *L. laccata* EtOH50 extract was performed according the Broth microdilution test (CLSI 2015), where the Minimal Inhibitory Concentration (MIC) capable inhibiting the fungal growth was established. Before analysis, the extracts were evaporated to dryness under vacuum, and dissolved in DMSO 5% aqueous solution (10 mg/mL). The target fungal species, *Fusarium solani* IOC 2163, were cultivated in potato dextrose agar (PDA – Sigma®) plates for 5 to 7 days. The spore suspension was prepared from a PDA culture by adding 5 ml of saline solution (0.85% v/v). A sterile Drigalski spatula was used in the agar surface to obtain a spore suspension. The concentration of this spore solution was determined by counting the conidia in a Neubauer chamber. The assay was performed in 96-well plate, used serial dilutions of each extracts (2500, 1250 and 625 µg/mL) performed in RPMI-1640 medium, and the final spore inoculum used was 4×10^7 per well. For the colorimetric reaction 10 µl of resazurin (0.002% w/v) per well were used as an indicator of cell growth. Each microplate contained positive control of fungal growth, and two negative controls (for the medium and for the extract). The plates were subsequently incubated for 24 h at 37° C, and the experiment was performed in triplicate (Monteiro et al., 2012).

2.4. In vitro analyze

The cytotoxicity of the EtOH50 extract was evaluated on adhered cells of VERO lineage. In a 96-well plate, 200 µl of a fibroblast suspension was cultivated in DMEM medium (Vitrocell Embriolife) supplemented with 10% of fetal bovine serum and maintained for 24 h at 37°C (humid atmosphere with 5% CO_2, at the 3.4×10^5 cells/mL concentration) (Snewin et al., 1999). After this period, adherent cells were exposed to serial EtOH50 concentrations (2500 to 312.5 µg/mL), the plate was incubated for 24 h at 37°C (5% CO_2). To determinate the concentration of the extract capable of maintaining the viability of 50% of the cells (IC_{50}) was added 30 µl of 0.01% resazurin reagent, and the plates was incubated for 6 h until the fluorescence measurement at 620 nm (Pavan et al., 2010).

2.5 Toxicological in vivo analyses

2.5.1 Soil characterization and samples preparation

The tropical artificial soil (SAT) modified from the OECD artificial soil (OECD, 2004) according to Garcia et al. (2004), was used in the lethality test. Its composition consists of 75% fine sand, 20% kaolinitic clay and 5% coconut fiber. The water holding capacity has been adjusted to 50% and calculated according to ISO 15799 (ISO, 2003). The SAT samples were exposed to different concentrations of *L. laccata* EtOH50 extract (5.0, 2.5, and 1.25 mg/ml), in addition to the group control (sterile distilled water) and treatment with the solvent used in the production of the extract (EtOH 1%). Each replicate (five) consisted of 250 grams of SAT treated with 100 ml of solution.

The natural soil used in the microbial functional analyze was obtained from the Campus of the Federal University of Pelotas (UFPel). After collection, soil was sieved (32 mesh) to remove rocks, roots, and other large particles. Physical and chemical characteristics of this soil are shown in **Table 1**. To the soil, solutions of *L. laccata* EtOH50 extract were

added separately to target the following concentrations 5.0, 2.5, and 1.25 mg/ml, as well as control with sterile distilled water and solvent (EtOH 1%). Each replicate (three) consisted of 200 grams of natural soil treated with 50 ml of solution. After homogenization, the soil samples were kept at room temperature for 14 days.

2.5.2 Lethality test

Earthworms (*Eisenia andrei*) were used in the test. The adults clitellated were obtained from Biotério de Invertebrados Terrestres of Instituto de Ciências Biológicas/FURG. The cultures reared under a photoperiod light-dark at 25 °C and humidity of 40-50%. The animals were not fed during the experiment. Earthworms were maintained at 20 ± 1 °C (10 per replicate), and a light-to-dark of 12:12 h for 14 days. The number of surviving *E. andrei* was recorded after 7 and 14 days of exposure (OECD 2004).

2.5.3 Analysis of *L. laccata* exposure on the microbial functional profile

To analyze changes in the microbial functional profile of the contaminated soil, the Biolog EcoPlate™ system was used. After the exposure time (14 days), 5 g of each soil treatment and control/solvent was shacked with 45 mL of sterile 0.85% NaCl solution for 30 min. Each solution was diluted 150 times, and 150 µl of sediment suspension was added into each well of Eco microplate. The plates were incubated at 28º C, and the color development was measured with a microplate reader at 590 nm, in 24h, 120h, and 144h of incubation. Microbial activity in each treatment was expressed as average well color development (AWCD) according **Equation 2**. Substrate richness values (*R*) were calculated as the number of utilization substrates, diversity (*H'*) (Equation 3) and evenness (*E*) (Equation 4) were calculated according to Zak et al. (1994).

$$AWCD = \sum \frac{OD_i}{31} \qquad Equation\ (\mathbf{2})$$

$$Shannon\ Index\ (H') = \sum pi(lnpi) \qquad Equation\ (\mathbf{3})$$

$$Shannon\ Evenness\ (E) = \frac{H}{lnS} \qquad Equation\ (\mathbf{4})$$

In which pi = proportional color development of the well over the total color development of all wells of a plate; H' = Shannon index of diversity; S = number of wells with color development (substrate utilization richness).

2.6 Data analysis

Survival earthworms (%), richness (R), evenness (E) and Shannon index (H') were investigated by analysis of one-way analysis of variance (ANOVA). The data about assimilation substrates groups was performed using two-way analysis of variance (GraphPad Prism 4 Software, La Jolla, California, USA). The results are expressed as mean and standard deviation, with a significant level of 95% ($p < 0.05$).

3. Results and Discussion

The analysis of TPC in *L. laccata* EtOH50 extract showed a value of 3.6 µg of galic acid equivalent/µl of sample, and the scavenging effect was 80.26% in a 250 mg/ml of extract concentration, a value of 6.72 µmol of Trolox equivalent/µl of sample).

From the data of analysis of TPC and DPPH of EtOH50 extract, we observed higher value compared to our previous study using mushrooms aqueous extracts, including the same species (Volcão et al., 2019). The polarity of the solvent used for the extraction is determining factor for the TPC values. Aroso et al. (2017) when analyzing extracts with different polarities (water, ethanol, and water: ethanol 1:1) obtained from *Quenus suber* L. cork, observed that higher TPC are achieved for extractions performed with hydroalcoholic solvent, in a

percentage of 50%. In addition, they demonstrated that the extracts radical scavenging performance decreasing with the higher ethanol percentages (> 50% of EtOH).

As with the amount of phenols and the antioxidant activity, the solvent used in the extraction process determines the antimicrobial activity of an extract. Research on functional foods based on extracts or compounds derived from mushrooms are classical (Reis et al., 2017). However, studies using these extracts in agriculture as biopesticides are still few. The antifungal potential of the *L. laccata* EtOH50 extract in this study was 0.625 mg/ml (MIC) against *F. solani*. Some authors already observe the effectiveness of some plant extracts in biological control of *F. solani* (Goss et al., 2017; Kareen and Matloob, 2019), as well as, Soltanzadeh et al. (2016) by studying the production of extracellular lytic enzymes by actinomycetes from the soil to inhibiting this phytopatogen.

Cytotoxicity analyzes show an IC_{50} > 2.5 mg/ml in Vero cells exposed to EtOH50 extract, with an inhibition percentage of 3.21% at the highest concentration (2.5 mg/ml), indicating that these compound showed no cytotoxicity activity to fibroblasts. Wang et al. (2018) demonstrated that the protective effect of compounds from *Hizilia fusiforme* mushroom against reactive oxygen species (ROS) in Vero cells, possible reducing apoptosis or cell death by scavenging of intracellular ROS.

According to the 14 days toxicity data using *E. andrei*, we do not observe lethality effect in solvent, 2.5 and 5.0 mg/ml concentration (figure 1). There are no papers in the literature for analyzing the impact of mushrooms extracts on earthworms. However, there are many studies using species of fungi, including mushrooms substrates, for use in bioremediation processes (Delgado et al., 2015; da Silva Jr., 2018). In addition, and taking into account the antioxidant activity and the non-cytotoxicity in vitro of the extract, we can observe a possible “protective” effect of the *L. laccata* ETOH50 extract.

According assimilation date, this study suggests that there are some changes in functionality if the soil microbioma exposed to *L. laccata* EtOH50 extract. The **figure 2** showed few changes in carbon sources oxidation between the treatment and the control. However, it is important to note that metabolic variations can be influencing in the genetic structure of microorganisms (Souza et al., 2012), which could be better explained in the long-term of exposure.

In total, the system analyzes 31 carbon sources, which were dived into five groups. When these groups were analyzed individually, we can see that the control group sowed a better amino acids assimilation in relation to the treatments (figure 3). The lack of considerable changes in the assimilation of carbon sources can be an indicator of soil quality. Changes in the soil microbiota are directly responsible for changes in the soil meso and macrofauna, since the interaction of soil fauna-microorganisms-plant biomass is a fundamental axis in carbon cycling (Stechauner and Madriñán, 2013).

Indices like H' (Shannon Index), S (equability) and R (richness) calculated based on results measured the OD are useful to describe activity and diversity of microorganisms population. The changes at physiological level can be a good indicator of reflecting changes on versatility of microbial communities exposed to certain conditions (Gryta et a., 2014).

No studies in the literature assessing the exposure of soil microbiome to mushroom extracts for the purpose of biological control. We observed that in all treatments, there was assimilation of all carbon sources. In addition, there was an increase ($p<0.001$) in diversity and equability in the assimilation of these substrates by the microbiota of the soils treated with *L. laccata* EtOH50 extracts (table 2). This fact makes the extracts promising for the control of *Fusarium solani* and other pathogens that affect soybean and others crops. Since, in addition to their antioxidant activity, antifungal potential, no in vitro and in vivo toxicity,

these extracts may also prove to be promising biostimulators in the soil or in substrates on which grains and cereals will be grown.

4. Conclusion

This research demonstrated the efficacy of *Laccaria laccata* EtOH50 extracts on the control of *Fusarium solani*. The lack of in vitro and in vivo toxicity in the evaluated parameters, coupled with a significant increase in the diversity and equability of the exposed soil microbiota, also make this extract a promising product for use in research on biostimulation in soils and substrates in agriculture.

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Tables

Table 1. Physical and chemical characteristics of natural soil collected.

Parameter	**Measurement**
pH	5.0
Organic matter (%)	1.66
Sand (%)	66.57
Silt (%)	25.33
Clay (%)	8.10
Calcium ($cmol_c/dm^3$)	2.9
Magnesium ($cmol_c/dm^3$)	1.2
Aluminum ($cmol_c/dm^3$)	1.0
Aluminum H^+ ($cmol_c/dm^3$)	4.4
Sulfur (mg/dm^3)	10.1
Potassium (mg/dm^3)	108.0
Copper (mg/dm^3)	0.4
Zinc (mg/dm^3)	2.7
Manganese (mg/dm^3)	21.0
Sodium (mg/dm^3)	10.0
Iron (%)	0.19

Table 2. Mean values of Shannon's evenness (*E*), Shannon's diversity (*H*), and richness of soil microbiota based on Biolog EcoPlate™ 144-h incubation.

Treatments	Means ±SD*		
	Shannon's evenness (*E*)	Shannon's diversity (*H'*)	Richness (*R*)
Control	0.95 ±0.007[e]	3.26 ±0.025[b]	31
Solvent	0.98 ±0.001[d]	3.39 ±0.004[a]	31
1.25 mg/ml	1.00 ±0.004[c]	3.37 ±0.014[a]	31
2.5 mg/ml	1.03 ±0.001[b]	3.39 ±0.002[a]	31
5.0 mg/ml	1.08 ±0.005[a]	3.41 ±0.014[a]	31
p-value	>0.001	>0.0001	-

Means (n = 3); SD – standard deviation; *similar letters within the same column indicate statistically similar bond strength results at 5% significance level.

Figures

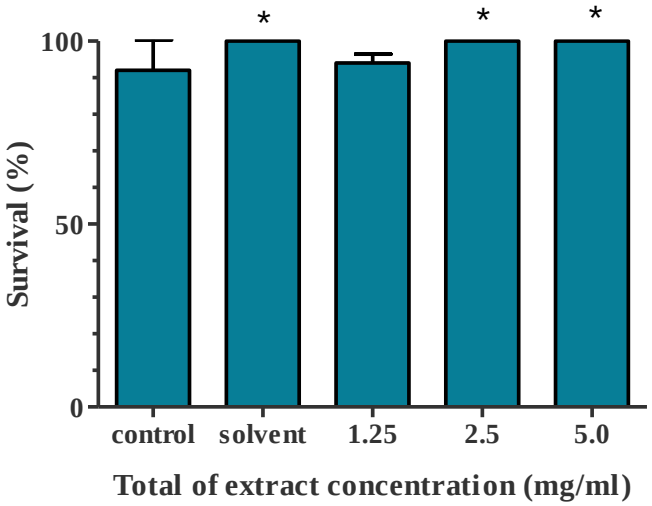

Figure 1. *Eisenia andrei* survival at the end of 14 days exposure to *Laccaria laccata* HyE. *Statistical difference between the treatments compared to control. Values are expressed as mean ±SD (Standart deviation), n = 5.

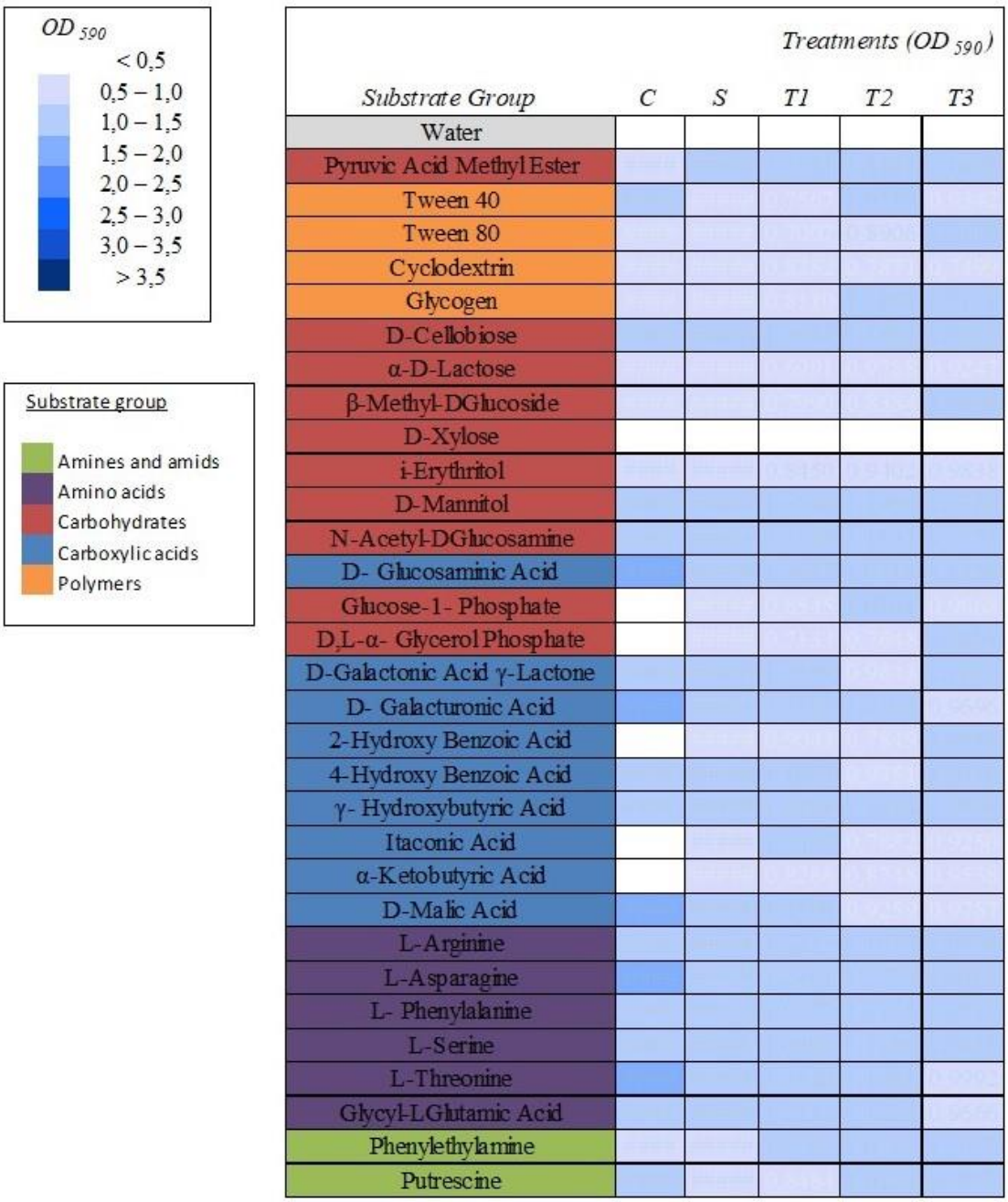

Figure 2. Cluster analysis of microorganism present in soil samples exposed to different extract concentration of *Laccaria laccata* EtOH50, depending on utilization carbon substrate in Biolog EcoPlate®.

Legend: OD_{590} – optical density in 590 nm; *C* – control; *S* – solvent EtOH 1%; *T1* – 1.25 mg/mL; *T2* – 2.5 mg/mL; *T3* – 5 mg/mL.

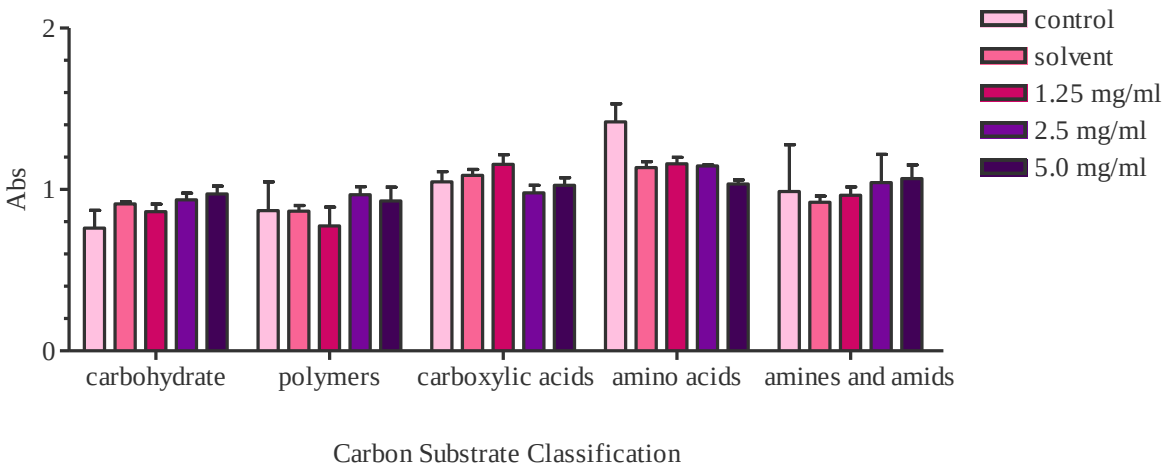

Figure 3. Means utilization substrates by soil microbiome exposed to different concentrations of *Laccaria laccata* EtOH50, based on 144-h incubation (n=3).

6 CONCLUSÃO

Com o exposto ao longo do estudo, demonstramos em condições controladas, o promissor potencial de extratos de cogumelos para aplicação como biocontroladores agrícolas. Foi possível observar a inibição *in vitro* de micro-organismos que afetam diversas culturas de plantas. Além disso, os extratos analisados no presente estudo não apresentaram toxicidade celular, bem como, foi possível observar a estimulação da diversidade funcional da microbiota do solo exposta ao extrato de *Laccaria laccata*. Não foi possível observar toxicidade deste mesmo extrato no organismo terrestre modelo *Eisenia andrei*. Os extratos avaliados apresentaram modificações em determinados parâmetros do crescimento vegetal.

A importância dos cogumelos na alimentação é inquestionável, e os dados da presente tese demonstram que este grupo pode contribuir para o desenvolvimento da agricultura sustentável, principalmente na redução dos insumos agrícolas. Em suma, a tese fornece informações pouco disponíveis na literatura, e que poderão ser utilizadas como base para o desenvolvimento de novos estudos na área do controle biológico.