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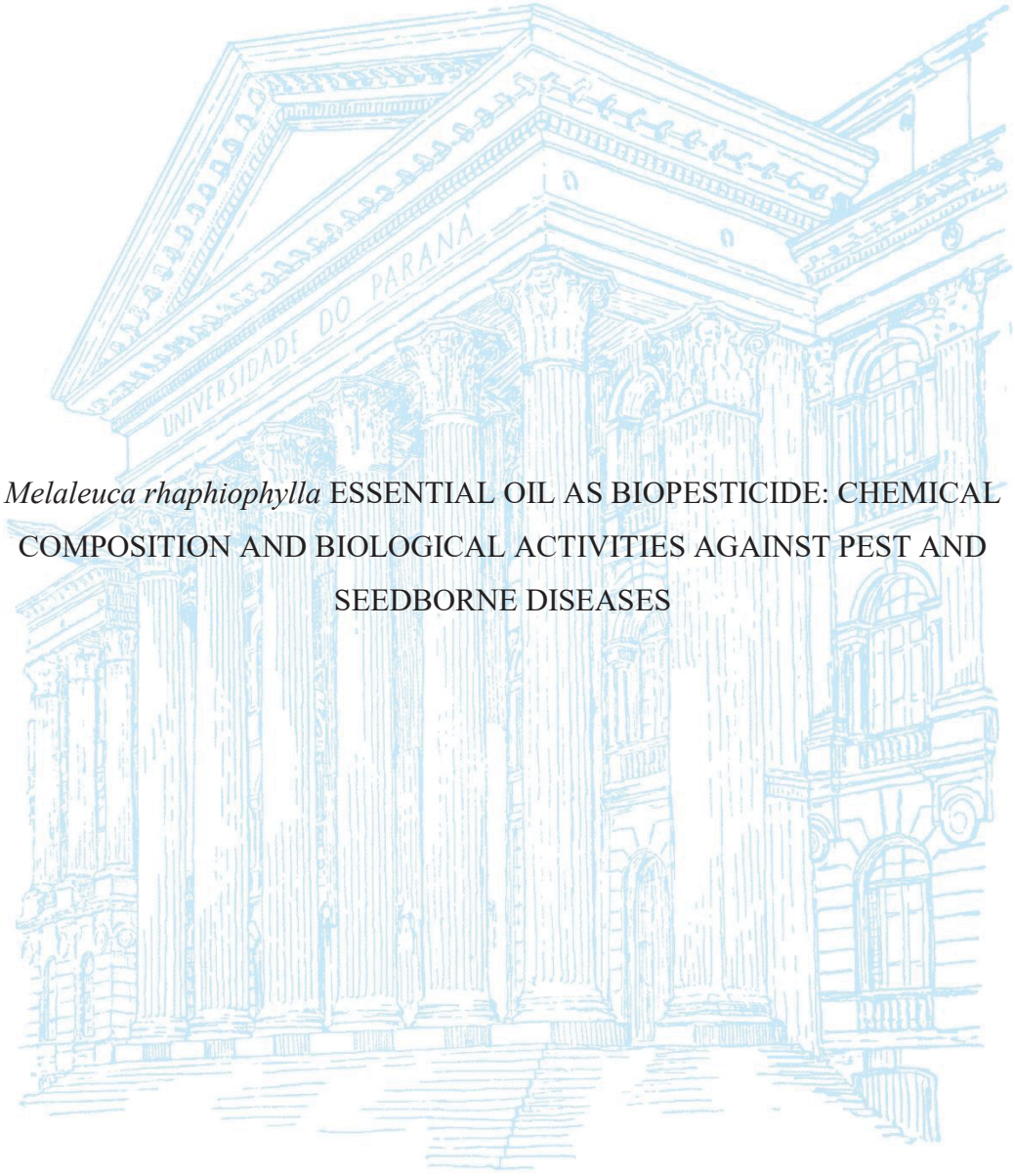

Melaleuca rhaphiophylla ESSENTIAL OIL AS BIOPESTICIDE: CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES AGAINST PEST AND SEEDBORNE DISEASES

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Tese apresentada ao Programa de Pós-Graduação em Agronomia, Área de Concentração em Produção Vegetal, Universidade Federal do Paraná, como parte das exigências para obtenção do título de Doutor em Agronomia.

Advisor: Prof. Dr. Henrique da Silva Silveira Duarte

Co-advisors: Dra. Alessandra Benatto
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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação AGRONOMIA (PRODUÇÃO VEGETAL) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **RUBENS CÂNDIDO ZIMMERMANN** intitulada: ***Melaleuca rhaphiolphylla* ESSENTIAL OIL AS BIOPESTICIDE: CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES AGAINST PEST AND SEEDBORNE DISEASES**, sob orientação do Prof. Dr. HENRIQUE DA SILVA SILVEIRA DUARTE, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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Ao meu pai e minha avó

Rubens Zimmermann e Celina Andrade Cândido *in memoriam*

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“Can you hear me when I sing?

You're the reason I sing

You're the reason why the opera is in me”

Bono

RESUMO

O uso recorrente e indiscriminado de pesticidas ocasionou o surgimento de insetos e linhagens fúngicas resistentes, além da presença de resíduos nos grãos, afetando a saúde humana. Diversas pesquisas demonstram a eficácia do uso de óleos essenciais (EOs) no controle de insetos e patógenos de armazenamento. Diante do exposto, o objetivo da pesquisa foi analisar e avaliar a eficiência do EO de *Melaleuca rhaphiophylla* (Myrtaceae) (MREO) no manejo de pragas (*Sitophilus zeamais* e *Sitophilus oryzae*) e fitopatógenos (*Aspergillus* spp. e *Fusarium* spp.) de armazenamento. O MREO foi extraído pelo método de arraste a vapor em dorna, e analisado por cromatografia gasosa acoplado a espectrometria de massas. No capítulo 01, foi avaliado o efeito inseticida contra os gorgulhos pelo método de contato e fumigação. Em seguida, a concentração letal (CL), tempo letal (TL) e o tempo médio de sobrevivência (TMS) foram estimados pelo método de fumigação. A atividade fungicida contra *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nomius* e *Fusarium graminearum* foi avaliada pelo método de contato e volatilização. O capítulo 02 objetivou avaliar a atividade inseticida e o efeito em marcadores bioquímicos do MREO e sua emulsão (MREM) contra *S. zeamais* e *S. oryzae*. Enquanto, o capítulo 03 objetivou avaliar o efeito do MREO sobre o crescimento micelial, estimatimar concentração mínima inibitória (CIM) e a concentração inibitória média (CI_{50}), determinar se o efeito é fungistático ou fungicida, avaliar o efeito do MREO na germinação de conídios, esporulação e no metabolismo respiratóriodo contra *A. flavus*, *A. niger*, *A. nomius*, *Fusarium culmorum* e *F. graminearum*, além do efeito na germinação de sementes de trigo. Foram identificados 22 compostos na composição química do MREO e os compostos majoritários foram α-terpineno (6,46%), 1,8-cineol (11,54%), γ-terpineno (13,2%), terpinoleno (28,72%) and terpinen-4-ol (19,82%). O método de fumigação ocasionou maior mortalidade para ambos os insetos (> 80%). Os valores da CL_{50} (90,55 e 72,88 de substância por L^{-1} de ar), TL_{50} (0,92 e 1,23 horas) e TMS (92,17 e 92,67 horas) foram similares entre as espécies (*S. zeamais* e *S. oryzae*, respectivamente). O método de volatilização demonstrou baixa atividade fungicida (< 30% de inibição) contra todos os isolados. O método de contato ocasionou uma inibição superior a 90%, com alta toxicidade para *Aspergillus*. O MREO ocasionou taxa de mortalidade acima de 25%, com aumento na síntese enzimática da acetilcolinesterase (AChE) para ambas as espécies de gorgulhos. Houve dano de peroxidação lipídica (LPO) apenas para *S. zeamais* e inibição da glutathione s-transferase (GST), α-esterase, e superoxido dismutase (SOD) para *S. oryzae*. O tratamento MREM causou taxa de mortalidade inferior a 1.5%, com aumento das enzimas GST, SOD e LPO para ambas as espécies e um aumento na atividade enzimática de esterase-β para *S. zeamais* e esterase-α para *S. oryzae*. Na concentração de 1,5%, o MREO ocasionou inibição do crescimento micelial acima de 95%, com o valor do CIM sendo 1% para todos os isolados. O valor da CI_{50} variou entre nos isolados expostos ao MREO, com o menor valor de 0,07% para com *A. niger* e o maior em 0,33% para *F. culmorum* (NRRL3288). O MREO inibiu a taxa de germinação dos conídios de todos os isolados e reduziu a capacidade de esporulação dos fungos, especialmente do gênero *Fusarium*. O aumento na concentração do MREO ocasionou maior inibição da atividade respiratória para os isolados de *Fusarium*. A taxa de germinação das sementes de trigo após o tratamento com o MREO foi de 96%, e no controle negativo 98%. Concluí-se que o MREO demonstra elevada atividade inseticida e fungicida, enquanto o MREM ocasiona uma toxicidade crônica contra os gorgulhos.

Nossos resultados apontam que o MREO apresenta potencial como um produto alternativo ao controle de pragas e fungos de armazenamento, e para o tratamento de sementes de trigo, visando a produção orgânica da cultura.

Palavras-chave: Óleos Essenciais. Biopesticidas. Produtos Naturais. *Sitophilus*. *Aspergillus*. *Fusarium*.

ABSTRACT

The recurrent and indiscriminate use of pesticides has led to the emergence of resistant insects and fungal strains, in addition to the presence of residues in grains, affecting human health. Several studies demonstrate the effectiveness of using essential oils (EOs) in controlling insects and storage pathogens. In view of the above, the objective of this research is to analyze and evaluate the efficiency of *Melaleuca rhaphiophylla* (Myrtaceae) essential oil (MREO) in the management of storage pests (*Sitophilus zeamais* and *Sitophilus oryzae*) and phytopathogens (*Aspergillus* spp. and *Fusarium* spp.). The EO was extracted in a vat using the steam drag and analyzed by gas chromatography–mass spectrometry. In chapter 01, the insecticidal effect against weevils was evaluated through contact and fumigation. Then, the lethal concentration (LC), lethal time (LT), and mean survival time (MST) were estimated by fumigation. The antifungal activity against *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nomius* and *Fusarium graminearum* was tested through volatilization and direct contact. The chapter 02 aimed to evaluate the insecticidal activity and effect of MREO and its emulsion (MREM) on biochemical markers against *S. zeamais* and *S. oryzae*. The chapter 03 aimed to evaluate the effect of MREO on mycelial growth, estimate minimum inhibitory concentration (MIC) and mean inhibitory concentration (IC_{50}), determine whether the effect is fungistatic or fungicidal, evaluate the effect of MREO on conidial germination, sporulation and respiratory metabolism against *A. flavus*, *A. niger*, *A. nomius*, *Fusarium culmorum* and *F. graminearum*, in addition to the effect on wheat seed germination. Twenty-two compounds were identified in the chemical composition of MREO, and the major compounds were α-terpinene (6.46%), 1,8-cineole (11.54%), γ-terpinene (13.2%), terpinolene (28.72%) and terpinen -4-ol (19.82%). Regarding the insects, the application using fumigation caused the highest mortality in both species (> 80%). The values for LC_{50} (90.55 and 72.88 of substance L^{-1} of air), LT_{50} (0.92 and 1.23 hours) and MST (92.17 and 92.67 hours) were similar between species (*S. zeamais* and *S. oryzae*, respectively). The volatilization method showed low fungicidal activity (< 30% inhibition) to all isolates. The contact method showed an inhibition greater than 90% with higher toxicity for *Aspergillus*. The MREO caused a mortality rate above 25%, with increased enzymatic synthesis of acetylcholinesterase (AChE) for both weevils. There was lipid peroxidation (LPO) damage only for *S. zeamais* and inhibition of GST, α-esterase, and superoxide dismutase (SOD) activity for *S. oryzae*. The MREM caused a mortality rate lower than 1.5%, with an increase in glutathione s-transferase (GST), SOD, and LPO enzymes for both species and an increase in the enzymatic activity of esterase-β for *S. zeamais* and esterase-α for *S. oryzae*. MREO at 1.5% caused growth inhibition above 95%, with the MIC value of 1% for all isolates. The IC_{50} value varied among isolates exposed to MREO, with lower value of 0.07% for *A. niger* and higher at 0.33% for *F. culmorum* (NRRL3288). MREO inhibited the germination rate of conidia of all isolates, and reduced the sporulation capacity of fungi, especially species of the genus *Fusarium*. The increase in the concentration of MREO caused greater inhibition of respiratory activity for all isolates of *Fusarium* spp.. The germination rate of wheat seeds after treatment with MREO was 96%, while negative control was 98%. We concluded that MREO demonstrated higher insecticidal and fungicidal activity, while MREM caused chronic toxicity against weevils. Our results showed that MREO has potential as an alternative

product to control storage pests and fungi, for the treatment of wheat seeds, aiming at the organic production of the crop.

Key words: Essential oils. Biopesticides. Natural Products. *Sitophilus*. *Aspergillus*. *Fusarium*.

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LIST DE ACRONYMS

AChE = acetylcholinesterase

AF = aflatoxin

ATC = iodidetoacetylcholine

BHT = butylated hydroxytoluene

BSA = bovine serum albumin

CAPES = coordination for the improvement of higher education personnel

CDNB = chloro-dinitrobenzene

DON = deoxynivalenol

DTNB = 5,5-Dithio-bis-2nitro-benzoate

EDTA = ethylenediamine tetraacetic acid tris buffer

EST-α = esterase-α

EST-β = esterase-β

GABA = gamma-aminobutyric acid

GC-FID = gas chromatography with flame ionization detection

GC-MS = gas chromatography coupled to mass spectrometry

GST = glutathione s-transferase

HPMC = hydroxypropyl methylcellulose

IC_{50} = medium inhibitory concentration

LC = lethal concentration

LPO = lipid peroxidation

LT = lethal time

MIC = minimum inhibitory concentration

MREM = *Melaleuca rhaphiophylla* emulsion

MREO = *Melaleuca rhaphiophylla* essential oil

MST = mean survival time

OTA = ochratoxin A

PDA = potato dextrose agar

RH = relative humidity

RI = retention indices

RI cal = experimental retention index

RI lit = literature retention index

ROS = reactive oxygen species

SD = standard deviation

SNA = synthetic nutrient agar

SOD = superoxide dismutase

ZEN = zearalenone

SUMÁRIO

GENERAL INTRODUCTION

Brazil stands out as one of the largest grain producers in the world (FAO, 2022). However, the Brazilian storage system is deficient both in relation to its spatial distribution and in the management of these warehouses, with losses of up to 17% of total grain production (ARAÚJO et al., 2019). Among the main causes are insect pests and fungi (PIMIENTA-RAMÍREZ et al., 2016; TANG et al., 2018).

During the storage period, grains and seeds are susceptible to attack by pests, with arthropods being the most relevant, notably *Sitophilus zeamais* and *Sitophilus oryzae* (Coleoptera: Curculionidae) (ATHANASSIOU et al., 2017; HADDI et al., 2018; HONG et al., 2018). These species are considered polyphagous primary pests and are cross-infested (ARAÚJO et al., 2019). The damage caused by the genus *Sitophilus* occurs both in the adult stage, when piercing healthy seeds and grains for food and oviposition, and in the larval stage that feeds on the endosperm, damaging the quality of the product (LORINI et al., 2015). These pests, when damaging the grains, favor the emergence of secondary pests, and contribute to increasing the humidity and temperature of the grain or seed mass, allowing the proliferation of fungi, and altering the metabolic activity of the seeds (SOUJANYA et al., 2016;PESCHIUTTA et al., 2017).

The main genera of fungi responsible for the contamination of grains and seeds during the harvest and storage period are *Aspergillus* and *Fusarium* (ANŽLOVAR et al., 2017). These groups of microorganisms are toxigenic, that is, they are capable of producing toxic secondary metabolites that persist in food and feed (AMZA, 2018; RÓŻEWICZ et al., 2021). Globally, it is estimated that between 25% and 50% of food is contaminated by these substances (MOGHADAM et al., 2016). In addition to contamination by mycotoxins, fungi cause changes in the appearance, flavor and texture of grains and other stored products. They can also cause the loss of seed viability (DANIA et al., 2019).

Due to the losses in productivity caused by these phytosanitary problems, it is extremely necessary to control them in storage units, aiming to minimize the economic impacts (PIMIENTA-RAMÍREZ et al., 2016). The control of these pests and phytopathogens is carried out using chemical insecticides and fungicides in a preventive and mainly curative manner (PIMIENTA-RAMÍREZ et al., 2016), as it presents high efficiency and ease of use compared to other control strategies (KHANI et al., 2017). However, the indiscriminate use and incorrect application of these products have led to

the emergence of resistant breeds and strains, making their control difficult, in addition to the generation of chemical residues in grains (PESCHIUTTA et al., 2017; PERCZAK et al., 2019; SOUZA et al., 2020).

Within this situation, there is a worldwide interest in the search for alternative substances to synthetic products, in an attempt to minimize the problems caused by insect pest and fungi. Therefore, it is necessary to discover new molecules that present less risk to human and animal health, have less environmental impact, and have less potential to cause resistance.

In this context, the use of bioactive plants represents a possible viable alternative to synthetic insecticides and fungicides to be integrated into health management in storage systems (PERCZAK et al., 2019). These plant species contain several substances, originating from secondary metabolism, with phytosanitary potential, which can be used mainly in the form of essential oils (EOs) (SOUJANYA et al., 2016).

EOs can be defined as a liquid compound of lipophilic nature, with an aromatic and volatile odor, stored in different plant organs, and extracted through distillation techniques (CRESCENTE et al., 2023). Among the main advantages over synthetic molecules is a lower risk of causing resistance in insects and fungi (KIRAN and PRAKASH, 2015; KHANI et al., 2017; TOMAZONI et al., 2018), and can also be used within organic agriculture (SPADARO et al., 2017).

Several studies prove the effect of EOs on controlling pests and fungi (DHIFI et al., 2016; PAVELA, 2016; BORGES et al., 2018; BASAID et al., 2020; WERRIE et al., 2020; CHANG et al., 2022; HOU et al., 2022; ZENOOZI et al., 2022). Studies suggest that EO of species of the genus *Melaleuca* (Myrtaceae) have insecticidal and fungicidal action against insects (LIAO et al., 2016; SONG et al., 2016; HUANG et al., 2018; ZIMMERMANN et al., 2021) and storage phytopathogens (RICCIONI and ORZALI, 2011; PUJIARTI et al., 2017; DA ROCHA NETO et al., 2019; CHAUDHARI et al., 2022; ZIMMERMANN et al., 2023), demonstrating the possibility of using these substances as an alternative to synthetic chemical molecules in phytosanitary management for storage systems (AMOABENG et al., 2019). In view of the above, the objective of the research was to analyze the chemical composition of *Melaleuca rhaphiophylla* (Myrtaceae) EO, in addition to its insecticidal, fungicidal activity, toxicity and its mechanism of action against pests (*S. zeamais* and *S. oryzae*) and storage fungi

(*Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nomius*, *Fusarium culmorum* and *Fusarium graminearum*).

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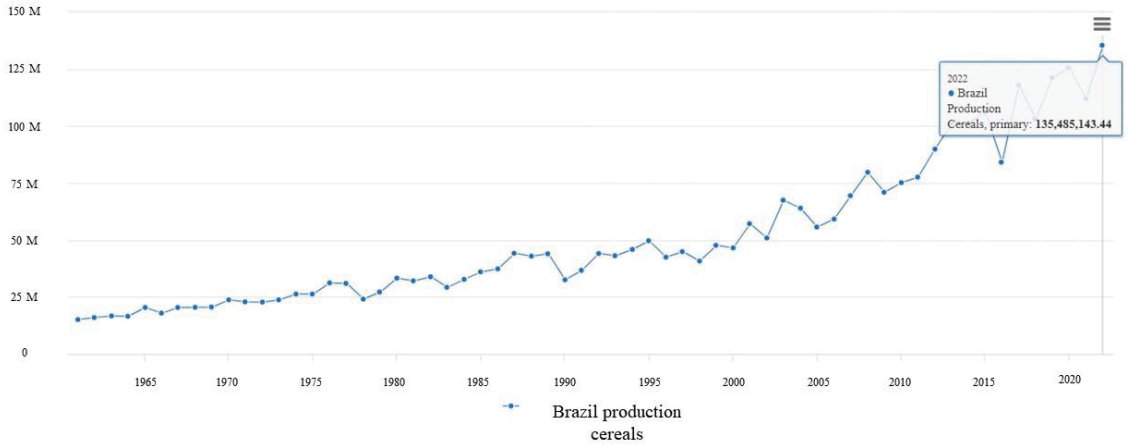
2.1 Stored grain production

2.1.1 Importance of cereals

Among crops of agricultural interest, cereals are the main basic food source for human and animal consumption, with emphasis on species from the Poaceae family (SARWAR, 2013; KAUR et al., 2014). These crops are of great importance because they have numerous nutrients necessary for basic physiological processes (PAL and MOLNÁR, 2021). Due to its relevance, during the domestication process of these plants, the aim was to improve three main criteria: productivity (considering the cultivar's resistance to diseases, its adaptation to climatic conditions and productivity), grain composition (nutritional value and absence of toxic substances) and its technological characteristics (suitability for a given final product) (ABBO et al., 2014; FULLER et al., 2010)

Aiming for global supply, high productivity becomes necessary, which causes an economic impact on essentially agricultural countries (TANG et al., 2018). The main cereal producing countries are China, India, the United States and Brazil (FAO, 2022). Brazil stands out as one of the largest food producers in the world (FAO, 2022), due to its favorable climatic conditions and vast availability of agricultural land. In the Brazilian agricultural sector, grain production stands out the most in terms of productivity, which has increased in recent years (Figure 01).

Figure 01. World cereal production, in tons, in Brazil.



Source: FAO, 2024.

Because they are widely exploited, these crops are subject throughout their entire cycle to attack by pests and diseases that can cause losses in production in the field and

post-harvest (AWADALLA et al., 2017; PIMIENTA-RAMÍREZ et al., 2016). However, storage is one of the most critical stages in the production chain, as at this stage the grains are stored for long periods, and are consequently exposed to insect infestation and fungal proliferation (KUMAR and KALITA, 2017; MESTERHÁZY et al., 2020).

2.1.2 Post-harvest losses

The occurrence of phytosanitary problems causes losses in productivity (PIMIENTA-RAMÍREZ et al., 2016), affecting the world economy (SOUZA et al., 2020), with economic losses being greater during storage (TANG et al., 2018). At a global level, post-harvest losses can account for 60% of the total produced annually (MESTERHÁZY et al., 2020). In underdeveloped or developing countries, losses can vary between 15 and 50% of annual grain production (RIBEIRO et al., 2018), and in the case of Brazil, these values are approximately 17% per year (ARAÚJO et al., 2019).

For the food industry, both quantitative post-harvest losses (RAJKUMAR et al., 2019), as well as qualitative ones, they are also of great economic importance, as they result in the depreciation of the product, with lower added value or resulting in low quality final products (PIMIENTA-RAMÍREZ et al., 2016; FOUAD and DA CAMARA, 2017). Among the main causes of these losses are physical and nutritional degradation, in addition to the sanity of seeds and grains (FRANZ et al., 2011).

Pest infestation can occur in the pre-harvest phase, due to the favorable climatic conditions for the emergence and development of these insects (NENAAH and IBRAHIM, 2011), with the greatest proliferation occurring in the post-harvest stage. During the storage period, temperature and humidity are controlled, aiming to avoid environmental conditions conducive to these agents (NESCI et al., 2011), as its presence causes the depreciation of the final product (NENAAH, 2014).

Storage pests have the ability to penetrate grains in the field (ARAÚJO et al., 2019)and continue the attack on grain in storage units. The existence of these insects causes damage to stored products (TAMGNO et al., 2019), which are caused during your diet (KIRAN and PRAKASH, 2015), in addition to being vectors of fungal spores (NESCI et al., 2011).

The high infestation of these animals causes an increase in metabolic activity, and as a consequence, there is a gradual increase in temperature and humidity inside the silos (NENAAH, 2014). These environmental conditions favor the germination of

conidia, and subsequently, the proliferation of fungi, and contamination with mycotoxins (MAEDEH et al., 2012). Thus, the association between insects and fungi is the main factor that contributes to the high post-harvest loss of grains and seeds (KIRAN and PRAKASH, 2015).

2.2 Storage pests and pathogens

2.2.1 Storage Pests

During the storage period, grains and seeds are subject to attack by vertebrate and invertebrate animals (NENAAH and IBRAHIM, 2011). Among invertebrates, insects are of greater importance, due to the environmental conditions of storage systems being favorable to their development (JAYAKUMAR et al., 2017). Worldwide, insects are considered the major pests of stored products (PANDEY et al., 2018), due to the quantitative and qualitative damage they cause to cereals (PUGAZHVENDAN et al., 2012)

It is estimated that insect attacks cause losses of approximately 30% of the total cereals stored (STATHERS et al., 2020; SINGH et al., 2021). In the United States of America, it is estimated that the economic losses by these pests cause annual losses of 1.25 to 2.5 billion dollars (PANDEY et al., 2018), and in India, these damages can reach up to 42.66 million dollars annually. According to Saeidi and Pezhman (2018), in Iran these losses can reach up to 80% of the total produced. While in Brazil, it is estimated that these pests cause losses of up to 17% of grain production (ARAÚJO et al., 2019).

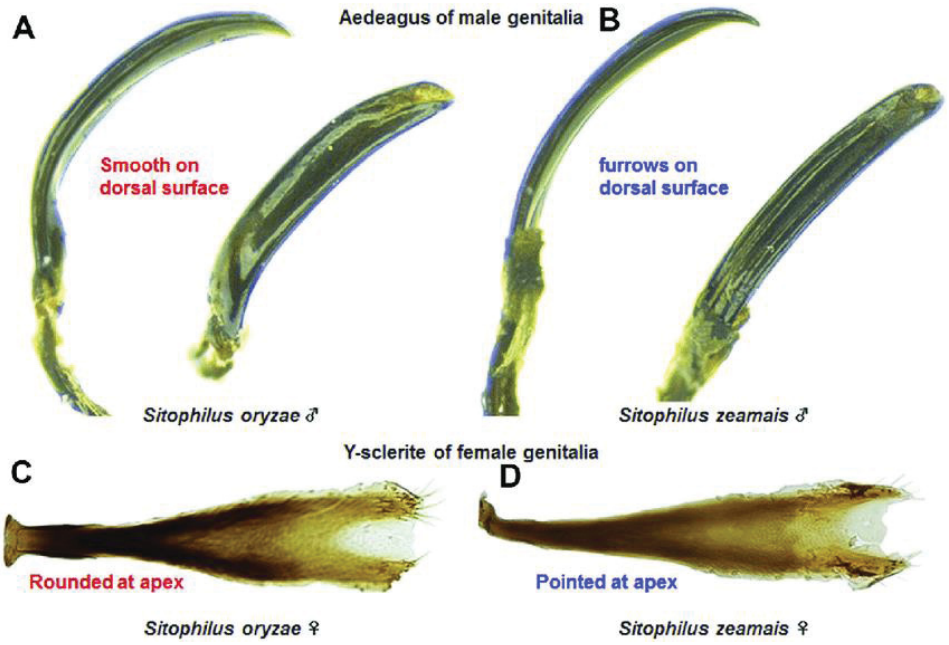
The main storage pests are beetles (Coleoptera) and moths (Lepidoptera) (HU et al., 2019). There are approximately 600 species of coleoptera responsible for losses in stored products (HU et al., 2019), and it is estimated that these pests cause damage between 10 and 40% (RAJKUMAR et al., 2019). Due to its economic importance by the damage caused to stored products, this order is among the most studied for its control (85.41%) (CAMPOLO et al., 2018).

In the tropics, the genus *Sitophilus* stands out as the main pest of stored grains (TAMGNO et al., 2019), which can cause losses ranging between 30 and 40% of total production (JAYAKUMAR et al., 2017). The maize weevil (*S. zeamais*) and the rice weevil (*S. oryzae*) have greater economic importance (JAWALKAR and ZAMBARE, 2020). These species stand out for being widespread throughout the world (EBADOLLAHI and JALALI SENDI, 2015), being primary pests with multiple hosts

(ARAÚJO et al., 2019), present cross-infestation (SOUJANYA et al., 2016) and high biotic potential (ATHANASSIOU et al., 2017).

These two species belong to the Curculionidae family, and are very similar in morphological characters, differing only in genitalia (PEREIRA and ALMEIDA, 2001; HONG et al., 2018) (Figure 02). They can occur singly or together in any grain mass (ATHANASSIOU et al., 2017).

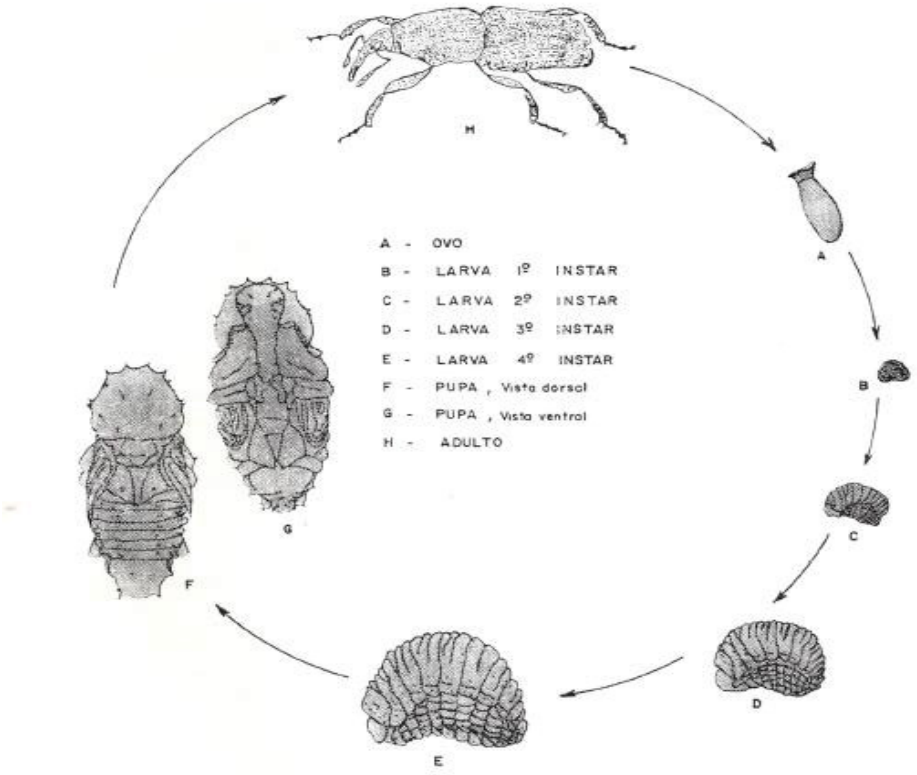
Figure 02. Identification of species of the genus *Sitophilus* by the morphological differentiation of the genitalia.



Source: Hong et al. (2018)

Adult insects measure 2 to 3.5 cm in length, are dark brown in color, and have lighter spots on the elytra. The larvae have a light yellow body and a dark brown head, with the pupae being white. The ideal conditions for their development are a temperature of 28 ºC and 60% relative humidity, where the longevity of the adult female is 142 days and that of the male 140 days. The incubation period varies between 3 and 6 days and the total life cycle is approximately 34 days (Figure 03) (LORINI et al., 2010).

Figure 03. Life cycle of the maize weevil (*S. zeamais*).



Source:Santos (1981)

The damage caused by the insect occurs both in the adult and larval stages (PESCHIUTTA et al., 2017), and cause direct and indirect damage. Direct damages are weight loss of grain mass due to pest feeding (JAYAKUMAR et al., 2017; PESCHIUTTA et al., 2017), and related to odor, appearance, flavor (KUMAR et al., 2008), nutritional changes (KUMAR et al., 2011) and contamination with fragments and dead insects (NENAAH, 2014). Indirect damage is associated with the emergence of secondary pests (PESCHIUTTA et al., 2017; ATHANASSIOU et al., 2017) and providing conditions for the proliferation of fungi (SOUJANYA et al., 2016).

2.2.2 Storage fungi

Cereals are of great importance to humanity, as they are the main source of food (MORCIA et al., 2017), in addition to its influence on the world economy (TANG et al., 2018). Despite the relevance of these grains, phytopathogenic fungi can reduce the productivity of the main crops by up to 20% (ROSELLÓ et al., 2015). During the development of these crops, plants are subject to attack by various diseases caused by fungi (MORCIA et al., 2017).

In the phase pre-harvesting, fungal spores spread, which infect healthy grains and seeds (BOŽIK et al., 2017), when environmental conditions are favorable, conidia germinate, and consequently the pathogen colonization process (SAHAB et al., 2014). However, the most critical period is post-harvest, especially when grains or seeds are stored for long periods (SUMALAN et al., 2013). Fungi are the main pathogenic agents responsible for storage losses, as their spores are carried from the field to storage units via insects (NESCI et al., 2011).

During the storage period, insect attacks cause changes in temperature and humidity inside the silos, creating favorable environmental conditions for the germination process of these microorganisms (NENAAH, 2014). Primary pests contribute to the development of fungi, as they damage healthy grains (SOUJANYA et al., 2016), provide substrate for the growth and proliferation of these pathogens (RASOOLI et al., 2006).

The main genera responsible for post-harvest contamination are *Aspergillus* and *Fusarium* (SAHAB et al., 2014; ANŽLOVAR et al., 2017). These pathogens cause quantitative damage through deterioration and consequent weight loss of the grains (MOHAPATRA et al., 2017). While qualitative damage is related to the visibility of mycelial growth causing product depreciation (SAHAB et al., 2014), reduction of germination power and vigor of seed (ISHAQ et al., 2017), decreased nutritional value of the grain (MOHAPATRA et al., 2017). However, the most serious damage is contamination by the presence of mycotoxins (BOŽIK et al., 2017).

Mycotoxins are compounds originating from the secondary metabolites of fungi (GARCÍA-DÍAZ et al., 2019). These substances are extremely toxic to humans and animals, even in low concentrations, and can cause poisoning problems (MORCIA et al., 2012), with the main toxic effects being hepatotoxic, carcinogenic and mutagenic. The species *A. niger* (KUMAR et al., 2017), *A. nomius* (NESCI et al., 2011), *A. flavus* (MOGHADAM et al., 2016), *F. culmorum* and *F. graminearum* (Perczak et al., 2019), as they stand out among storage fungi for producing these compounds.

Contamination of fungi and mycotoxins in grains makes their consumption unfeasible (VILELA et al., 2009), and according to Moghadam et al. (2016), it is estimated that approximately 25 to 50% of the world's production of agricultural products is contaminated with mycotoxins. Second López et al. (2004), mycotoxin contamination has already been reported in products from several countries, especially grains, causing

economic losses worldwide (KALAGATUR et al., 2015). The impact caused by the presence of mycotoxins in food is significant, as these products cannot be sold (GARCÍA-DÍAZ et al., 2019) due to presenting a risk to human and animal health (BOŽIK et al., 2017).

2.3 Control methods

2.3.1 Synthetic insecticides and fungicides

The importance of insect control is related to the impact of damage (NENAAH and IBRAHIM, 2011), in addition to their association with fungi (PESCHIUTTA et al., 2017). While fungal control is necessary due to contamination with mycotoxins, as even in small quantities these are substances that are extremely toxic to human and animal health (MORCIA et al., 2012).

The synergistic attack of these bioagents causes a reduction in global productivity (PIMIENTA-RAMÍREZ et al., 2016). Due to these factors, its control in storage units becomes a priority in order to avoid possible losses. According to Kiran and Prakash (2015), pest control is mainly carried out using synthetic insecticides. These products are used preventively or more frequently in a curative manner (CARDOSO, 2009).

In preventive control, liquid insecticides are applied along the conveyor belt or in the flow pipe (LORINI, 2010), with its mode of action being contact (OBOH et al., 2017). To control weevils in maize and wheat, the most commonly used chemical groups are organophosphates and pyrethroids (KIRAN and PRAKASH, 2015), being represented by the active ingredients pirimiphos-methyl and deltamethrin, respectively (AGROFIT, 2020). The pirimiphos-methyl insecticide has toxicological class III, with environmental classification II (AGROFIT, 2019).

The purge curative method (fumigation) is the most used in storage systems around the world (PIMIENTA-RAMÍREZ et al., 2016). The purge aims to eliminate any pest infestation through the use of gases, using aluminum or magnesium phosphide tablets (AGROFIT, 2022), and is popularly known as phosphine (FREITAS et al., 2016). The fumigation method is the most used because it is more economical compared to other synthetic insecticides (BACHROUCH et al., 2010), as it has action against a wide variety of pests (KHANI et al., 2017) and because it has a quick effect (OGENDO et al., 2008). Phosphine is an extremely volatile and toxic molecule to insects and has toxicological classification I, with environmental classification I (AGROFIT, 2022).

Fungus control is carried out with the use of synthetic fungicides, applied preventively to avoid contamination with mycotoxins (SUMALAN et al., 2013). These products are applied in the field, immediately after harvest, with the aim of protecting the grains or seeds from possible contamination before being stored (SOUZA et al., 2020).

Among the main chemical groups used are benzimidazoles (SREENIVASA et al., 2011), which can be used separately or in mixture (MORCIA et al., 2017). One of the main active ingredients used in the control of storage pathogens is thiophanate-methyl and tebuconazole (GALVÃO et al., 2003), whose toxicological classification is II and IV, and its environmental classification is III and II, respectively (AGROFIT, 2022).

2.3.2 Use of synthetic insecticides and fungicides and their impacts

The simultaneous control of storage pests and fungi is essential in order to avoid quantitative and qualitative losses of agricultural products in storage units. Therefore, the use of synthetic insecticides and fungicides is the widely used control measure (PIMIENTA-RAMÍREZ et al., 2016).

Maintaining current high levels of agricultural productivity would not be possible without the use of synthetic insecticides and fungicides, which demonstrates that they will continue to play an important role in integrated pest and disease management programs (MARANGONI et al., 2012). However, due to their efficiency and ease of use, these products are used intensively, aiming to increase productivity, but their intensive, incorrect and indiscriminate use has caused several problems (PIMIENTA-RAMÍREZ et al., 2016).

The main impact caused by the recurrent use of synthetic products is the increase in selection pressure, causing resistance in insect populations (RIBEIRO et al., 2003; PIMENTEL et al., 2008; PIMENTEL et al., 2009) and fungal strains (ANŽLOVAR et al., 2017; SOUZA et al., 2020), as these products have only one mode of action (KIRAN and PRAKASH, 2015). The emergence of resistant agents leads to the need for doses above those recommended, aiming to control them (KIRAN and PRAKASH, 2015; MORCIA et al., 2017). The increase in doses of synthetic insecticides and fungicides causes an increase in the amount of residues in grains (BLUMA et al., 2008; KIRAN and PRAKASH, 2015), which can cause problems in human and animal health (PIMIENTA-RAMÍREZ et al., 2016; KAGUCHIA et al., 2018; PERCZAK et al., 2019). The increase in recommended dosages generates higher production costs for small farmers (TAMGNO

et al., 2019). Associated with these problems, the application of synthetic fungicides in storage units influences the quality of the grains (ANŽLOVAR et al., 2017).

Associated with risks to human and animal health, the indiscriminate use of these chemical substances causes several environmental impacts (KAGUCHIA et al., 2018), such as contamination of water, soil and air (PERCZAK et al., 2019;SOUZA et al., 2020), and negative effects on non-target organisms (PESCHIUTTA et al., 2017; PERCZAK et al., 2019). In addition to these factors, according to Tamgno et al. (2019), these synthetic products have a high cost, economically affecting small producers, as they do not have technical knowledge about their application and use, and are therefore the most harmed.

Given this scenario, there is worldwide interest in the search for alternative substances to synthetic products. Therefore, it is necessary to discover new molecules that present less risk to human and animal health, less environmental impact, and are low cost.

2.4 Natural products from bioactive plants

In recent years, the problems caused by the use of synthetic molecules have highlighted the need to seek alternative products to these substances (JAYAKUMAR et al., 2017). Integrated pest management (IPM) advocates the adoption of different methods, which can be used simultaneously or not, to control pests and diseases (PERCZAK et al., 2019). In this context, bioactive plants have the potential to be used as an alternative to synthetic molecules (BACHROUCH et al., 2010), which can be used within the IPM (UPADHYAY et al., 2018).

Bioactive plants co-evolved with insects/pests, in a system called host/pest interaction (JAYAKUMAR et al., 2017), and during this process they developed defense mechanisms (SAEIDI and PEZHMAN, 2018). The synthesis of compounds with biological activity is one of the main methods of defending plants against abiotic and biotic stresses (BENTO, 2016).

Compared to synthetic products, these chemical substances have the following advantages: they are biodegradable (KUMAR et al., 2011), have low toxicity to mammals (JAYAKUMAR et al., 2017), do not cause environmental impact (SAEIDI and PEZHMAN, 2018), have lower production costs (PIMIENTA-RAMÍREZ et al., 2016).

Secondary metabolites with biological activity can be obtained from bioactive plants (ISMAN, 2000)and used in the form of powders (PROCÓPIO et al., 2003;

RIBEIRO et al., 2008; NENAAH, 2014), botanical extracts and essential oils (EOs) (SOUJANYA et al., 2016). However, studies showed the greater efficiency of EOs compared to botanical extracts (EL KHOURY et al., 2017; SIDDIQUI et al., 2017; BENELLI et al., 2019).

2.4.1 Essencial oils

Medicinal, aromatic and condimentary plants contain a high production of secondary metabolites, which are complex organic compounds that are not directly involved in the processes of plant growth, development and reproduction (ISMAN, 2000;ISMAN, 2019). Second Regnault-Roger and Philogène (2008), bioactive plants contain semiochemicals, which are defined as chemical substances that cause physiological or behavioral changes in other species (EBADOLLAHI and JALALI SENDI, 2015). Within this classification are pheromones, botanical extracts and essential oils (EOs) (TRIVEDI et al., 2017).

In its definition, EOs are a complex set of volatile liquid chemical compounds, with an aromatic odor (PERCZAK et al., 2019), and which have biological activity (KIRAN and PRAKASH, 2015). These substances are used by plants as a defense mechanism against bioagents (CHANG et al., 2022). These secondary metabolites are lipophilic in nature and insoluble in water (CAMPOLO et al., 2018), being produced in different types of structures, such as external and internal glands (OGENDO et al., 2008). Eos can be extracted from different organs such as seeds, stems, leaves, flowers (OLIVEIRA et al., 2018) and roots (SOUZA et al., 2020).

EOs are obtained through the distillation process (MAEDEH et al., 2011), with hydrodistillation, steam distillation, dry distillation and mechanical processes being the main distillation methods (HOU et al., 2022; CRESCENTE et al., 2023). In the hydrodistillation method, the plant material is immersed in boiling water, while in the steam distillation method, the water used only comes into contact with the plant material in its vapor form, and in both methods the EO is separated from the water by decantation. Due to the simple equipment and ease of use, both methods are the most used (CAMPOLO et al., 2018).

EOs are widely used by the industrial sector (RAJKUMAR et al., 2019), which can be used to manufacture medicines and cosmetics (EBADOLLAHI and JALALI SENDI, 2015), in addition to being exploited by the food industry (BENZI et al., 2014).

Some botanical families, highlighting Asteraceae, Lamiaceae, Myrtaceae and Poaceae, (RAJENDRAN and SRIRANJINI, 2008; EBADOLLAHI and JALALI SENDI, 2015; SOUJANYA et al., 2016; CAMPOLO et al., 2018), have chemical compounds with biological properties that function as insecticides (REGNAULT-ROGER and PHILOGÈNE, 2008) or fungicides (SOUZA et al., 2020).

As insecticides, EOs can act to inhibit oviposition, affect feeding, development and emergence of adults (AWADALLA et al., 2017). Second Oboh et al. (2017), EOs can act as repellents and insecticides. Regarding their insecticidal action, EOs can be applied via different exposure routes, and toxicity depends on how this substance will come into contact with the insect (PEIXOTO et al., 2015), among them, ingestion, fumigation (OBOH et al., 2017) or absorbed through the skin (contact) (NENAAH, 2014).

As microbial agents, EOs have fungicidal and/or fungistatic action (TOMAZONI et al., 2018), through inhibition of mycelial growth, conidial germination, spore production and inhibition of mycotoxin production (PIMIENTA-RAMÍREZ et al., 2016; SOUZA et al., 2020). Therefore, these substances can act as protective fungicides and/or curatives for stored seeds and grains (MARÍN et al., 2003).

The insecticidal action of EOs may be associated with the presence of monoterpenes and sesqueterpenes (OBOH et al., 2017), capable of penetrating the cuticle, causing physiological changes in insects and consequently their death (KHANI et al., 2017). Second Medjdoub et al. (2019), these compounds are also responsible for the fungicidal activity of EOs, which can cause physiological damage and damage to the structures of the fungal cell wall (SOUZA et al., 2020).

EOs from different plants demonstrate toxic or repellent activity against different types of pests (KHANI et al., 2017), in addition to the fungicidal effect in vitro (PIZZOLITTO et al., 2020) and in vivo against pathogens (SOUZA et al., 2020). However, the biological activity and toxicity of EOs depends on the chemical constitution, the proportion of compounds present in their composition, the mode of action and the target organism (EBADOLLAHI et al., 2014; KIRAN and PRAKASH, 2015; PERCZAK et al., 2019).

To identify compounds and their proportion in the chemical composition of EOs, it is necessary to use techniques and equipment, such as thin-layer chromatography, gas

chromatography, high-performance liquid chromatography, and gas chromatography coupled to mass spectrometry (GC-MS), the latter being the most used technique (HOU et al., 2022; CRESCENTE et al., 2023).

Variability in chemical composition is related to genetics (PEIXOTO et al., 2015; AWADALLA et al., 2017), geographical conditions, harvest time (OGENDO et al., 2008; BACHROUCH et al., 2010), part of the plant used, climatic season (KIRAN and PRAKASH, 2015), type of soil, nutritional status (MOSSI et al., 2011), chemotype (BACHROUCH et al., 2010), time and extraction method (PEIXOTO et al., 2015), species and cultivation system (PERCZAK et al., 2019). This set of factors causes changes in the qualitative and quantitative aspect of the chemical composition of EOs (SAEIDI and PEZHMAN, 2018).

The biological activity of these substances may be related to the major compounds (SENDI and EBADOLLAHI, 2014). Nevertheless, the effectiveness of EOs cannot be solely attributed to them. They often exhibit a synergistic or antagonistic effect among themselves, or with other minor compounds present in the chemical composition, which affect their biological activity (BACHROUCH et al., 2015). Therefore it is necessary to identify all the compounds (KIRAN and PRAKASH, 2015).

Due to its chemical composition containing different active compounds (BENZI et al., 2014), the probability of causing resistance is lower, as EOs have different modes of action (BACHROUCH et al., 2015; KIRAN and PRAKASH, 2015), which is the main advantage over synthetic products. However, there is a lack of research aimed at identifying the mode of action of these substances, especially in controlling stored grain pests (CAMPOLO et al., 2018). Another advantage is that EOs are quickly degraded, due to their high volatilization (MAEDEH et al., 2012), and therefore cause less impact on the environment (KHANI et al., 2017), and can be used in organic seed production (SPADARO et al., 2017)

2.4.2 Genus *Melaleuca*

It is estimated that there are approximately more than 17 thousand plant species that demonstrate the ability to synthesize and store EOs in different plant structures and organs, with the vast majority belonging to the Angiosperm, especially in the Lamiaceae, Rutaceae, Myrtaceae, Zingiberaceae and Asteraceae families (ZABKA and PAVELA, 2018).

The Myrtaceae family, with around 121 genera and approximately 3,800 to 5,000 species with a predominantly tropical and subtropical distribution, concentrated in the neotropical region and Australia (ANJOS DA SILVA et al., 2023). The family has a large number of species that produce of essential oils with enormous volume of liters (FILOMENO et al., 2020), which present different biological activities (ANJOS DA SILVA et al., 2023). The Myrtaceae family is divided into two large subfamilies, Myrtoideae (occurring in tropical and subtropical America) and Leptospermoideae (mainly distributed in Australia and Asia). The main difference between both subfamilies is in the distribution of leaves and the type of fruit (MOHAMED et al., 2023)

The genus *Melaleuca* stands out among the main genera belonging to the subfamily Leptospermoideae, with approximately 300 species described in Oceania and Southeast Asia (BROPHY et al., 2013), with a predominance of occurrence in coastal regions with high humidity (SHARIFI-RAD et al., 2017). Botanically, the species are shrubby or small trees; with opposite or scattered leaves, entire, flat, concave or rarely with recurved margins; the flowers may be white, yellowish or purple-red and are closely sessile, each subtended by a small deciduous bract, which may occur in a scattered form or in a spike, very small and deciduous bracteoles or absent (CARRICK and CHORNEY, 1979; SHARIFI-RAD et al., 2017).

Species of this genus have high economic value, as they are widely used for landscaping as ornamental plants for gardens and streets, recovery of degraded areas or areas that suffer from salinization processes, in addition to their potential use in the wood industry as a source of fiber (BROPHY et al., 2013). However, the trade in EOs of this type is the main market for the pharmaceutical industry due to their medicinal properties (SHARIFI-RAD et al., 2017).

In Australia, the species *Melaleuca alternifolia*, *Melaleuca cajuputi* subsp. *cajuputi* and *Melaleuca quinquenervia*, popularly known as tea tree, are the most cultivated for this purpose, as they have well-stabilized and defined chemotypes (ISO standard no. 4730; ISO 2004) with different biological activities, including antifungal action (BROPHY et al., 2013; SHARIFI-RAD et al., 2017). However, it is noteworthy that the environmental conditions in which these plants are located can cause variations both quantitatively and qualitatively in the chemical composition present in EOs and determine their bioactivity (CHANG et al., 2022; HOU et al., 2022). In this context,

despite the wide diversity of species within the genus, there is a lack of research that seeks to evaluate the chemical composition and bioactivity of other species.

The species *Melaleuca rhaphiophylla* is characterized as a tall shrub 5 m tall (in southern Australia), with whitish to light gray branches. The leaves are scattered, ascending or narrow-linear, somewhat plano-convex, straight or curved on the upper part especially when young, wedge-shaped on a 1 mm petiole, acute with short mucus, soft, straight or curved, glands on the lower surface conspicuous when young, 1 to 2 cm long and 0.5 to 1 mm wide. The inflorescence has a cylindrical tip, the axis growing before anthesis, glabrous rachis; calyx tube narrowly sessile, more or less cylindrical, expanded and truncated at the base, glabrous, about 1.5 mm long and 2.5 mm in diameter, lobes triangular-oval, acute, scarred margin, glabrous outside and inside, about 0.5 mm long and 1 mm wide; corolla white, petals circular, concave, glabrous, about 2.5 mm in diameter; whitish stamens 4 to 5 mm long and 15 to 20 filaments 2 to 3 mm long; style about 6 mm long, slightly expanded at the apex, and almost flat stigma. The fruits are close to each other, not compacted, partially embedded in the axis, subcylindrical, about 2.5 mm long and 4 to 6 mm in diameter, constricted at the apex, opening about 2 mm in diameter (Figure 04) (Carrick; Chorney (1979).

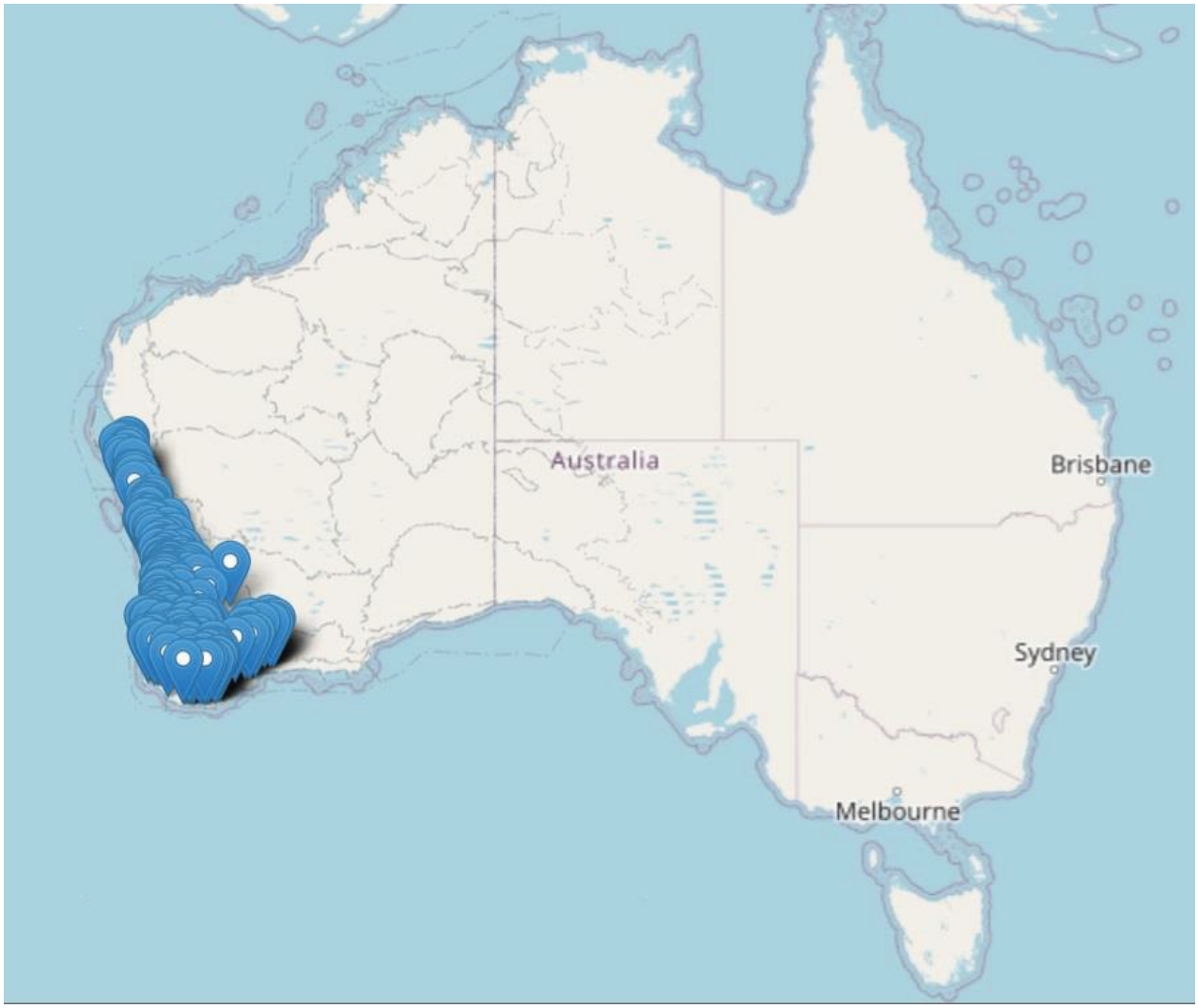
Figure 04. Leaves, flowers and fruits of *Melaleuca rhaphiophylla*

Fig. 17. *M. rhaphiophylla*. A, twig, x ½; B, leaf, x 2; C, flower, x 4; D, stamen bundle, x 5; E, fruits, x 2/3.

Source: Carrick and Chorney (1979).

Its geographic distribution occurs mainly in western and southern Australia (Figure 05). To date, the study conducted by Brophy and Lassak (1992) is the only one on the yield and chemical composition of this species, whose results demonstrated that the species presented a yield of 1.28% by the steam drag method in a vats and with the following major compounds 1, 8-cineole (48.9%), γ- terpinene (6.8%) and terpinen-4-ol (12.2%).

Figure 05. Geographic distribution of *Melaleuca rhaphiophylla*



Source: : https://florabase.dbca.wa.gov.au/browse/profile/5959

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CHAPTER 01: INSECTICIDAL AND ANTIFUNGAL ACTIVITIES OF *Melaleuca rhaphiophylla* ESSENTIAL OIL AGAINST INSECTS AND SEED-BORNE PATHOGENS IN STORED PRODUCTS*

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Abstract

Essential oils (EOs) are substances with biological properties that can be used to inhibit insects and fungi in storage systems. The EOs from plants of the genus *Melaleuca* (Myrtaceae) show insecticidal and antifungal activities. However, so far, there are no reports regarding *Melaleuca rhaphiophylla* (Myrtaceae) EO. Therefore, we sought to investigate the insecticidal and antifungal activities of *M. rhaphiophylla* EO against storage pests and fungi. The plant's EO was extracted in a vat using the steam drag method and analyzed by gas chromatography–mass spectrometry. The insecticidal effect against *Sitophilus zeamais* and *Sitophilus oryzae* (Coleoptera: Curculionidae) was evaluated through contact and fumigation methods in order to select the optimized exposure route. Then, the lethal concentration (LC), lethal time (LT), and mean survival time (MST) were estimated. Antifungal activity against *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nomius* and *Fusarium graminearum* was tested through volatilization and direct contact. Twenty-two compounds were identified in the chemical composition of *M. rhaphiophylla* EO and the major compounds were α-terpinene (6.46%), 1,8-cineole (11.54%), γ-terpinene (13.2%), terpinolene (28.72%) and terpinen-4-ol (19.82%). The fumigation method of application caused the highest mortality in both insects. The values for LC_{50} (90.55 and 72.88 of substance L^{-1} of air), LT_{50} (0.92 and 1.23 hours) and MST (92.17 and 92.67 hours) were similar between species (*S. zeamais* and *S. oryzae*, respectively). The volatilization method showed low fungicidal activity (< 30% of inhibition) against all isolates. The contact method showed an inhibition greater than

90%with higher toxicity for *Aspergillus*. Our results showed that *M. rhaphiophylla* EO has potential as an alternative product to control storage pests and fungi.

Keywords: Essential oils, Chemical composition, *Sitophilus*, *Aspergillus*, *Fusarium*

1. Introduction

Cereals are among the most important crops for humanity due to their nutritional aspects, versatility in the food industry, and, consequently, their impact on the world's economic market (Mlyneková et al., 2014). Of the steps that comprise the grain production process, storage stands out for its importance in preserving these products in the post-harvest stage. However, it is during this period that the majority of post-harvest losses occur, generating economic losses that correspond to up to 17% of the total annual Brazilian production (Araújo et al., 2019).

One of the main causes of productivity losses is pest attacks, associated with the proliferation of microorganisms (Pimienta-Ramírez et al., 2016; Brito et al., 2020). Insects of the genus *Sitophilus* are responsible for causing quantitative and qualitative damage due to their high biotic potential, cross-infestation, and because they are primary pests (Araújo et al., 2019). In addition to these factors, these pests are the main vectors of *Aspergillus* spp. and *Fusarium* spp. spores (Brito et al., 2020), phytopathogenic fungi that cause quantitative and mainly qualitative damage, and, just as with most species, is a mycotoxin producer (Sahab et al., 2014).

Chemical control is the main control measure against pests and storage pathogens and involves the preventive use of synthetic insecticides and fungicides (Pimienta-Ramírez et al., 2016). However, in recent years, studies have shown a growing resistance of these organisms to main pesticides used for their control (Karaağaç and Konuş, 2015; Yörük et al., 2018; Chen et al., 2019; Masiello et al., 2019; Attia et al., 2020; Masiello et al.,2020; Sakka and Athanassiou, 2021). Furthermore, these substances cause environmental impacts and represent a risk to human and animal health due to residues

present in grains and foods (Pimienta-Ramírez et al., 2016; Nayak et al., 2020; Souza et al., 2020).

Substances obtained through the secondary metabolites of plants have been shown to be an alternative to the use of synthetic products. Essential oils (EOs) are a complex set of volatile compounds with biological properties (Ni et al., 2021; Singh et al., 2021), including insecticidal or fungicidal activity, and have potential in the control of storage pests and fungi (Basaid et al., 2020). The advantages of EOs over synthetic pesticides include a lower risk of promoting the selection of resistant organisms, lower toxicity towards mammals, and rapid degradation in the environment (Souza et al., 2020; Singh et al., 2021).

The genus *Melaleuca* (Myrtaceae) originates from Australia, where the largest species are found (Brophy et al., 2013). EOs of *Melaleuca* species contain high concentrations of EOs in leaves, flowers, and stems (Farag et al., 2004) and boasts biological properties, such as insecticidal or fungicidal activity (Polatoğlu and Karakoç, 2016; Sharifi-Rad et al., 2017). Nevertheless, there are no studies regarding the bioactivity of EOs of some of the species within this genus. Because of this, we sought to evaluate the bioactivity of *Melaleuca rhaphiophylla* Schauer EO in the control of stored product pests (*Sitophilus zeamais* (Motschulsky) and *Sitophilus oryzae* (L.)) and fungi (*Aspergillus niger* (Van Tieghem), *Aspergillus flavus* (Link), *Aspergillus nomius* (Kurtzman et al. 1987) and *Fusarium graminearum* (Schwabe)).

2. Material and Methods

2.1. Weevil rearing

The weevils (*S. zeamais* and *S. oryzae*) were obtained from the stock breeding carried out in the Professor A. M. da Costa Lima laboratory of agricultural entomology. The insects were kept and bred following the methodology proposed by Zimmermann et al. (2021). Unsexed adult weevils aged 5 to 10 days old were used for all of the experiments.

2.2. Fungal strains

Isolates of *A. niger*, *A. flavus*, *A. nomius* were obtained from the Microbiological Collections of Paraná Network (Taxonline), and *F. graminearum* was isolated from wheat grains (Tralamazza et al., 2016). *Aspergillus* isolates were cultivated in Potato Dextrose Agar (PDA – ION), and *F. graminearum* was kept in Synthetic Nutrient Agar (SNA) (Beyer et al., 2004) and then incubated in Biochemical Oxygen Demand (BOD) at 28º C, 12h photophase for 14 days.

2.3. Plant Material and Isolation of *Melaleuca rhaphiophylla* EO

A collection of *M. rhaphiophylla* plant material was carried out in November 2019, in Araucária (25º31′00.81″ S and 49º26′18.24″ W), Paraná, Brazil. A sample of this material was deposited at the Curitiba Botanical Museum, under the number 289706. The EO was extracted from the green aerial part, that is, branches with leaves. A steam drag in a vat for 2 h was the distillation method employed; water was used as an extraction solvent. The EO was transferred to vials and refrigerated until its analysis. The EO was diluted to 1% (w/v) using ultrapure hexane.

2.4. Analysis of *Melaleuca rhaphiophylla* EO

The analysis of EO was performed using a Shimadzu GCMS-TQ8040 system (gas chromatography coupled to mass spectrometry (GC-MS)), and it was conducted under the following conditions: Rtx-5MS fused silica capillary column (30m x 0.25mm x 0.25μm). Helium was used as a carrier gas, with a flow rate of 1.02 mL min^{-1}, in split mode 1:90, injector temperature of 250 ºC, and ionization system at 70 eV. Sample (1 μL) was inserted into a heating ramp ranging from 60 ºC to 250 ºC and heating at a rate of 3ºC.min^{-1}.

For quantification, the EOs was analyzed using an Agilent 7890A system (gas chromatography with flame ionization detection (GC-FID)) operated at 280 ºC. The column and analytical conditions were the same as those described above. Helium was used as the carrier gas with a constant flow rate of 1.5 mL/min.

The components of the EOs were identified by comparing retention indices and their mass spectra to literature data available at the Adams and NIST 02 mass libraries. Experimental retention indices (RI) were calculated with the aid of the Van den Dool and Kratz equation represented by homologous series of *n*-alkanes ranging from C8 to C19.

2.5 Insecticidal activity of *Melaleuca rhaphiophylla* EO

2.5.1. Insecticidal activity and selection of exposure method

The insecticidal activity of *M. rhaphiophylla* EO was evaluated against *S. zeamais* and *S. oryzae* through two different exposure routes: surface contact and fumigation. The EO concentration was evaluated at 205.5 μL L^{-1} air (Bhavya et al., 2018; Zimmermann et al., 2021), acetone was used as a solvent. Pirimiphos-methyl insecticide (Actellic® 500 EC) was used as a positive control at a concentration of 16 mL L^{-1} (AGROFIT 2021); water was used as a solvent. Negative controls consisted of acetone and water. The experiments

were conducted in a completely randomized design, with three replicates per treatment and four replicates over time.

The application methodology for both routes followed the model proposed by Zimmermann et al. (2021) with some modifications. A 0.198 mL solution sample was applied to the filter paper (a diameter of 7.5 cm) inside of 145 mL plastic vessels. For both methods, it was used 20 adult non-sexed insects per replicate, aged between three and eight days. Following the application, treated samples were stored in a controlled environment (25 ± 2 ºC, 70 ± 10% RH). The evaluation of insect mortality was performed after 48 h.

2.5.2. Toxicity of *Melaleuca rhaphiophylla* EO against weevils

The exposure method, which showed 80% mortality or higher for any of the species, was used to determine the lethal concentration (LC). The EO was diluted into eight concentrations, ranging from 13.7 μL L^{-1} to 205.5 μL L^{-1} air (Bhavya et al., 2018). The 27.4 μL L^{-1} air interval between concentrations had been stipulated in previous experiments (data not shown). Acetone was used as a negative control. The statistical design and the experimental methodology used were the same described from Section 2.5.1., and the results were used to stipulate LC_{25}, LC_{50}, and LC_{90}.

2.5.3. Lethal time and mean survival time

Lethal time (LT_{50}) and the mean survival time (MST) of weevils exposed to *M. rhaphiophylla* EO was estimated based on the LC_{90} to which they were exposed during fumigation (Reyes et al., 2019; Zimmermann et al., 2021). The negative and positive

controls were acetone and pirimiphos-methyl (16 mL L^{-1}). An entirely randomized design with ten replicates was used for each treatment, and with three replicates over time. Mortality was assessed at 1 h, 2 h, and 4 h, and then at 4h intervals during the first 24 h and then again 48 h after applying the treatments.

2.6. Antifungal activity of *Melaleuca rhaphiophylla* EO

2.6.1. Fungicidal activity by volatilization

The fungicidal action of *M. rhaphiophylla* EO against *A. flavus*, *A. niger*, *A. nomius,* and *F. graminearum* through volatilization was evaluated at a concentration of 100%. The experimental design was entirely randomized, performed in triplicate, with three replicates over time.

The methodology proposed by Cardiet et al. (2012) was employed using some modifications. Disks measuring 8 mm in diameter were removed from seven-day-old inocula and placed in the center of a Petri dish containing the respective medium. The dishes were inverted, and a 10 mm filter paper disk (Waterman® #1) immersed in 10 µL of pure EO was placed in each lid. The same volume of sterilized distilled water was used in the negative control. This procedure was carried out to avoid direct contact of the EO with the fungus. Then, the plates were sealed with polyethylene film to prevent the escape of volatiles and incubated in BOD at 28 ± 1 ºC with a 12 h photophase. Two perpendicular measurements of the colony diameter were performed seven days later using a digital caliper (Souza et al., 2020).

2.6.2. Fungicidal activity by contact

The fungicidal effect of *M. rhaphiophylla* EO by contact in the control of *A. niger*, *A. nomius*, *A. flavus,* and *F. graminearum* was evaluated at a 5% concentration. The experimental design was entirely randomized. A triplicate was made for each treatment, with three replicates in time, totaling nine replicates for each treatment.

The inocula were obtained by scraping the surface of the culture medium using a sterilized spatula and transferring it into sterilized 2 mL microtubes containing RPMI 1640 medium (Arikan et al., 2001). The flasks were vortexed and the suspensions filtered through a porous tissue to remove mycelium. Subsequently, conidia suspension was standardized at a 2 x 10^4 conidia mL^{-1} concentration (Gorran et al., 2013) using a Neubauer chamber.

The microdilution methodology proposed by Kalagatur et al. (2015) was used with some modifications to conduct the experiment. The technique was recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2002). The suspension of conidia and the EO were inoculated in a 96-well microtiter plate with a flat bottom to obtain a final concentration of 10^4 conidia mL^{-1}, equivalent to 5 %, totaling 200 µL per well. The same procedure was adopted for RPMI 1640 medium as a negative control, and the synthetic fungicide thiophanate-methyl (Cercobin® 875 WG) 700 mg L^{-1} (AGROFIT, 2021) was employed as a positive control. Then, the plates were kept in BOD at 28 ± 1 ºC and 12 h photophase for 48 h (Moghadam et al., 2016). Mycelial growth was determined by absorbance at 600 nm using the EPOCH microplate spectrophotometer (Biotek Instrument Inc, Winooski, USA).

2.6.3. Data analysis

Inhibition data for all experiments with fungal isolates were calculated in comparison to the negative control (Vilela et al., 2009):

$$Inhibition\ (\%) = \frac{(C - T)}{C} \times 100$$

Where:

C = Absorbance or growth of the negative control

T = Absorbance or growth of each treatment

2.7. Statistical analysis

Insect mortality rates were corrected based on Abbott (1925) and submitted to their respective analyses. Insecticidal and fungicidal data were adjusted by generalized linear models (GLM) using a binomial distribution and were then submitted to an analysis of variance. The means were compared using Tukey's test at a 5% probability threshold. LC and LT data were submitted to Logit analysis using the ecotox package. The survival curves were estimated using the Kaplan-Meier test and compared using the log-rank test using the Survival R package. All analyses were performed with R software (R Core Team 2019).

3. Results

3.1. Analysis of *Melaleuca rhaphiophylla* EO

Twenty-two compounds were identified in *M. rhaphiophylla* EO. The main compounds identified were α-terpinene, 1,8-cineole, γ-terpinene, terpinolene, and terpinen-4-ol, representing approximately 79.8% of the total chemical composition (Table 1).

Table 1 Essential Oil composition (% ± Standard deviation) of *Melaleuca rhaphiophylla*

N⁰	RI cal[a]	RI lit[b]	Compounds	Concentration[c] %	SD[d]	Error
1	931	932	**α-Pinene**	2.158	±0.007	0.001
2	970	969	**Sabinene**	0.191	±0.001	0.000
3	974	974	**β-Pinene**	0.583	±0.002	0.000
4	989	988	**Myrcene**	1.315	±0.003	0.001
5	1004	1001	**δ-2-Carene**	1.619	±0.003	0.001
6	1015	1014	**α-Terpinene**	6.468	±0.013	0.003
7	1022	1020	**ρ-Cymene**	1.499	±0.002	0.000
8	1026	1024	**Limonene**	1.363	±0.008	0.002
9	1028	1026	**1,8-Cineole**	11.540	±0.030	0.006
10	1055	1054	**γ-Terpinene**	13.208	±0.019	0.004
11	1087	1086	**Terpinolene**	28.721	±0.021	0.004
12	1099	1095	**Linalool**	0.407	±0.000	0.000
13	1175	1174	**Terpinen-4-ol**	19.822	±0.033	0.007
14	1187	1186	**α-Terpineol**	2.739	±0.008	0.002
15	1403	1409	**α-Gurjunene**	0.432	±0.003	0.001
16	1412	1417	**(*E*)-Caryophyllene**	0.481	±0.003	0.001
17	1431	1430	Not identified	1.479	±0.010	0.002
18	1453	1458	***allo*-Aromadendrene**	0.467	±0.004	0.001
19	1467	1471	**Dauca-5,8-diene**	0.269	±0.003	0.001
20	1488	1489	**β-Selinene**	1.351	±0.008	0.002
21	1518	1511	**δ-Amorphene**	1.375	±0.010	0.002
22	1525	1522	**δ-Cadinene**	0.172	0.001	0.000
			Total	96.17	0.010	

[a]RI cal Experimental Retention Index. [b]RI lit Literature Retention Index. [c]Concentrations are means of three determinations. [d]Standard deviation.

3.2. Insecticidal activity and exposure method selection

Melaleuca rhaphiophylla EO displayed insecticidal activity against *S. zeamais* and *S. oryzae*. The mortality rate of weevils exposed to EO was similar to the positive control and varied by comparison to the negative controls. We found a difference in mortality between the exposure methods for both species, and fumigation was more effective in controlling weevils. The Pirimifos-methyl insecticide was effective in the control of *S. zeamais* and *S. oryzae* regardless of the method of exposure (Fig. 1).

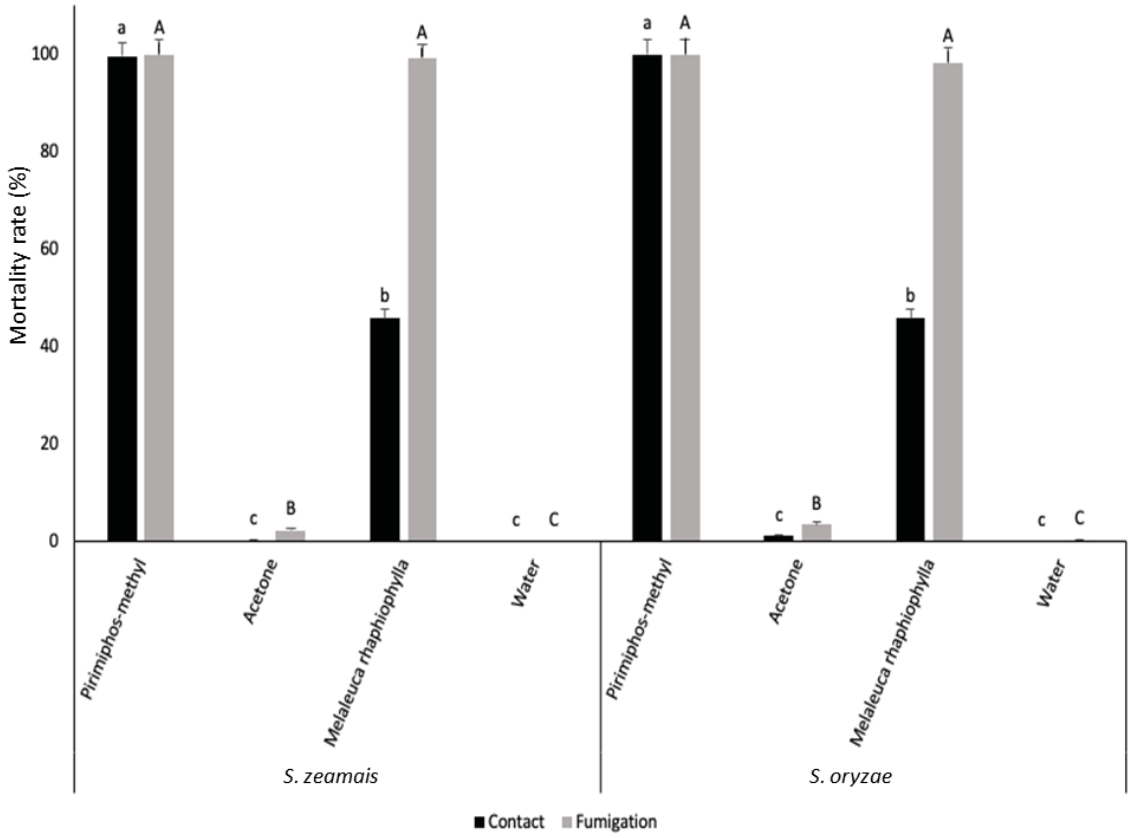

Fig. 1 Mortality rate (mean ± standard deviation) of *Sitophilus zeamais* and *Sitophilus oryzae* after 48 h of exposure via contact and fumigation to 205.5 µL of substance L^{-1} of air of *Melaleuca rhaphiophylla* EO. Acetone and water were used as negative controls, and pirimiphos-methyl was used as a positive control. Columns with the same letter are not significantly different according to Tukey's test analysis ($P < 0.05$).

3.3. Toxicity of *Melaleuca rhaphiophylla* EO against weevils

Melaleuca rhaphiophylla EO had a high toxic effect against weevils via fumigation. However, sensitivity varied among species, with a greater level of toxicity against *S. oryzae*. The mortality caused by the negative control was lower than 5% (Table 2).

Table 2 Toxicity of the *M. rhaphiophylla* EO against adults of *S. zeamais* and *S. oryzae* via fumigation after 48 h of exposure.

Species	N	slope	LC_{25}[a] (CI[b] 95%)	LC_{50} (CI 95%)	LC_{90} (CI 95%)	chi-square	P value
S. zeamais	2.152	0.88	39.18 (34.11-43.97)	90.55 (84.66-95.9)	184.26 (181.25-187.27)	2.24	0.89
S. oryzae	2.153	0.73	25.61 (21.37-29.72)	72.88 (66.58-78.5)	176.73 (173.99-179.47)	4.45	0.61

[a]LC: Lethal concentration (µL of substance L^{-1} of air); [b]95% CI: confidence interval (95%).

3.4. Lethal time and mean survival time

Melaleuca rhaphiophylla EO caused mortality of 50% of the weevil population faster than when using pirimifos-methyl. The LT50 was 0.92 h for *S. zeamais* and 1.23 h for *S. oryzae*. The LT_{50} for the insecticide was approximately 6 h for both species. We found no mortality in the negative control (Table 3).

Table 3 Time required for *Melaleuca rhaphiophylla* EO and pirimiphos-methyl to cause 50% mortality in *Sitophilus zeamais* and *Sitophilus oryzae*.

Species	Treatments	N	LT_{50}[a] (CI[b] 95%)	chi-square	P-value
S. zeamais	*M. rhaphiophylla*	600	0.92 (0.75-1.09)	19	0.0082
	pirimiphos-methyl	600	3.24 (3.09-3.4)	143.57	0.0001
S. oryzae	*M. rhaphiophylla*	600	1.23 (1.07-1.41)	24.66	0.0009

	pirimiphos-methyl	600	3.14 (2.94-3.25)	141.7	0.0001

[a]LT: Lethal time (hours); 95% [b]CI: Confidence interval (95%).

We found a difference in the MST values between weevil exposure via fumigation to *M. rhaphiophylla* EO and pirimifos-methyl (Table 4). Both species showed similar sensitivity, with a lower survival expectancy in the first hours following the exposure to *M. rhaphiophylla* EO (Fig. 2).

Table 4 Estimated mean survival time of *Sitophilus zeamais* and *Sitophilus oryzae* subjected to *Melaleuca rhaphiophylla* EO and pirimiphos-methyl.

Species	Treatments	N	Mortality rate (%)	MST*	Kaplan-Meier
S. zeamais	*M. rhaphiophylla*	600	92.17	4.04	A
	pirimiphos-methyl	600	100	6.79	B
S. oryzae	*M. rhaphiophylla*	600	92.67	4.03	A
	pirimiphos-methyl	600	100	6.68	B

* MST: Mean survival time (hours); 95% CI: Confidence interval (95%).

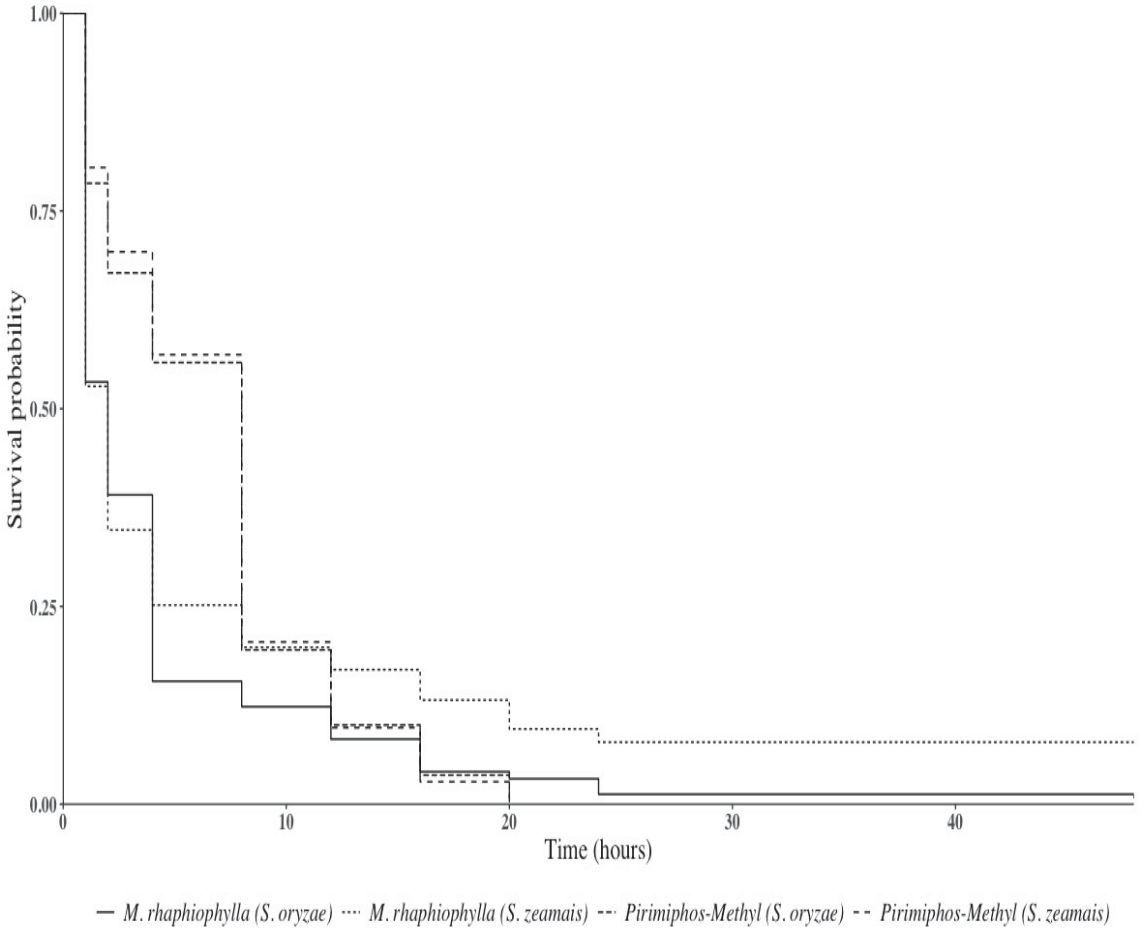

Fig. 2 Survival curves (hours) of *Sitophilus zeamais* and *Sitophilus oryzae* exposed the EOs of *Melaleuca rhaphiophylla* at LC_{90} levels using the Kaplan-Meier test. The pirimiphos-methyl insecticide was used as a positive control.

3.5. Fungicidal activity by volatilization

Melaleuca rhaphiophylla EO did not present fungicidal activity against the fungi tested; inhibition was lower than 30 % for all isolates. The fungi showed different sensitivity to the EO of this plant (Fig. 3).

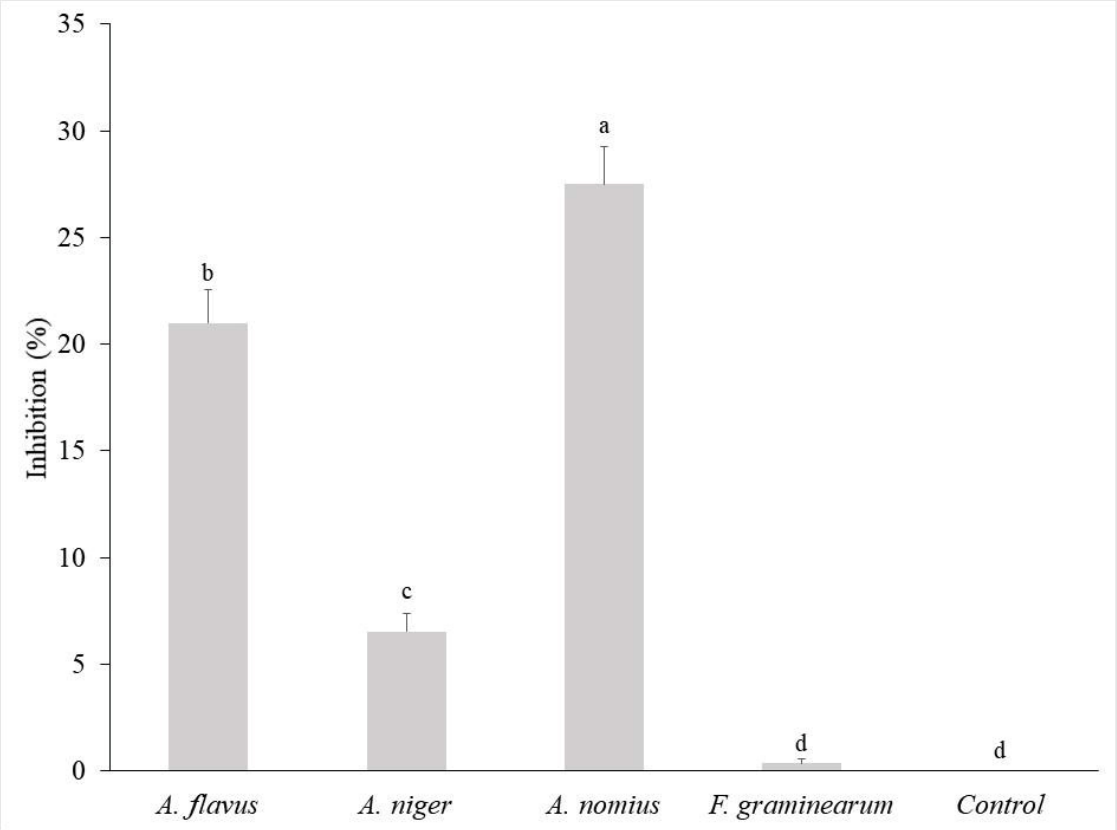

Fig. 3 Inhibition (mean ± standard deviation) of *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nomius,* and *Fusarium graminearum* subjected to *Melaleuca rhaphiophylla* EO by volatilization, after seven days of incubation at 28º C. Sterile distilled water was used as a negative control. Columns with the same letter for each isolate are in the same group according to Tukey's test analysis (P <0.0001).

3.6. Fungicidal activity by contact

Melaleuca rhaphiophylla EO showed fungicidal activity by contact similar to thiophanate-methyl against *A. niger*, *A. nomius,* and *A. flavus*. However, the EO of this plant was more effective in controlling *F. graminearum* (Fig. 4).

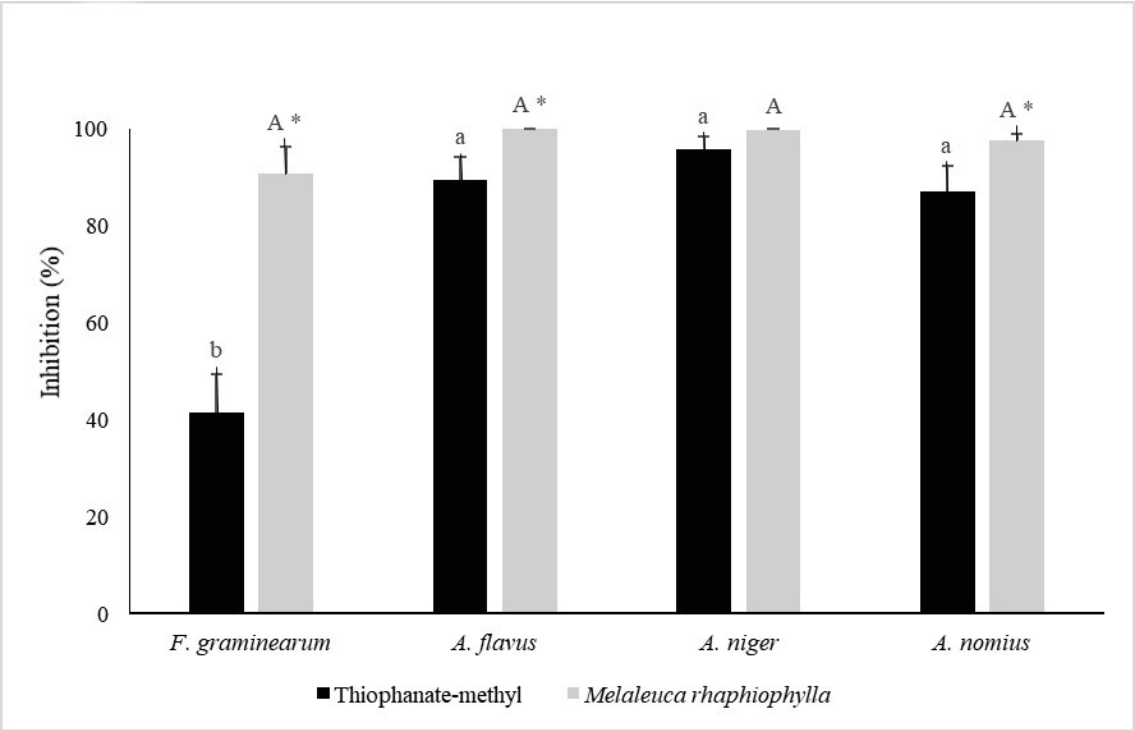

Fig. 4 Inhibition (mean ± standard deviation) of *Fusarium graminearum, Aspergillus flavus*, *Aspergillus niger,* and *Aspergillus nomius* subjected to *Melaleuca rhaphiophylla* EO by contact, after 48 h of incubation at 28º C. RPMI 1640 medium and fungicide thiophanate-methyl were used as negative and positive controls, respectively. Columns with the same letter for each isolate are in the same group according to Tukey's test analysis (P <0.0001).

4. Discussion

This is the first report concerning the bioactivity of *M. rhaphiophylla* EO against insects and fungi. The main compounds identified in the EO of this plant used in our study were α-terpinene, 1,8-cineole, γ-terpinene, terpinolene and terpinen-4-ol. Until now, there is only one report regarding the chemical composition of this species in Australia (Brophy and Lassak, 1992); in that case, the main compounds were also 1,8-cineole, γ-terpinene and terpinen-4-ol, although in different amounts. It could be that the chemical composition of EOs could vary both quantitively and qualitatively due to geographic location, genetics, soil type, and others (Khani et al., 2017). This difference is one of the

main characteristics of EOs and is related to the biological activities that these compounds are capable of presenting (Basaid et al., 2020).

Due to the chemical compounds present in EOs and their multi-site action characteristic, these products are promising in the control of storage pests when applied through fumigation, as contact or as repellent insecticides (Brito et al., 2020). Our results demonstrated that *M. rhaphiophylla* EO showed insecticidal activity against *S. zeamais* and *S. oryzae*. However, mortality was higher (greater than 80%) when weevils were exposed by the extract through fumigation, which indicates that the insecticidal effect of *M. rhaphiophylla* EO is associated with the method of exposure (Spochacz et al., 2018). Fumigation using different EOs was also more effective than contact application against the coleopterans *Hypothenemus hampei* (Ferrari) (Curculionidae) (Reyes et al., 2019), *Oryzaephilus surinamensis* (L.) (Silvanidae), *Tribolium castaneum* (Herbst) (Tenebrionidae) (Bachrouch et al., 2015), *S. oryzae,* and *S. zeamais* (Zimmermann et al., 2021). EOs are formed by volatile chemical compounds, whose lower molecular weight molecules volatilize more rapidly (Khani et al., 2017), increasing their concentration in confined environments. Therefore, these substances are inhaled in greater quantities by insects, causing a higher level of mortality (Bachrouch et al., 2015).

In our results, different toxicity was observed among weevils when subjected to *M. rhaphiophylla* EO, and an increase in concentration caused higher mortality for both species. Similar results were reported using EOs of *Mentha arvensis* (L.) (Lamiaceae) and *Tagetes minuta* (L.) (Asteraceae) in the control of *S. zeamais* and *S. oryzae* (Zimmermann et al., 2021). Toxicity of EOs may vary depending on the species due to factors such as the thickness of the cuticle or its chemical composition (Stefanazzi et al., 2011), in addition to each species' ability to detoxify these substances (Peixoto et al., 2015).

The toxicity of *M. rhaphiophylla* EO against weevil species may be associated with the large number of monoterpenes in its chemical composition. Since these substances are volatile, they can be inhaled through the spiracles or penetrate the insect's cuticle, causing physiological changes and leading to death (Brito et al., 2020). Studies conducted by Peixoto et al. (2015), A Fouad et al. (2021) and Abdelgaleil et al. (2021) evaluated the insecticidal effect of monoterpenes against *S. zeamais* and *S. oryzae*, proving that the major compounds identified in our *M. rhaphiophylla* EO present insecticidal activity and may be responsible for the high mortality observed among these pests.

The presence and synergistic interaction of these molecules are factors responsible for determining the biological and multi-site activities of EOs (Basaid et al., 2020). Our results demonstrate that *M. rhaphiophylla* EO caused high mortality among weevils within a short period, with an LT_{50} inferior to that of the synthetic insecticide. EOs with high levels of monoterpenes cause rapid mortality due to the high volatility of these compounds, which cause insect mortality by asphyxia (Brito et al., 2020). Monoterpenes can also inhibit acetylcholinesterase, affecting nerve transmission and causing paralysis and death (Jankowska et al., 2018; Abdelgaleil et al.,2021). However, the MST results that were observed indicate that there is a synergism between components of *M. rhaphiophylla* EO, especially among the monoterpenes (Bachrouch et al., 2015; Pimienta-Ramírez et al., 2016), which indicates that these substances can act simultaneously in several sites, reducing the insect's chances of survival (Peixoto et al., 2015; Abdelgaleil et al., 2021) .

Some EOs present insecticidal activity and are also natural fungicides against different phytopathogens (Basaid et al. 2020). The antifungal activity of EOs depends on the compounds present in their chemical composition, especially monoterpenes, which

are the main substances responsible for antimicrobial activity (Kumar et al., 2017; Nerilo et al., 2020).

As they are composed of volatile substances, such as monoterpenes, EOs can be applied as a smoking fungicide against stored product pathogens (Kim and Park, 2012). Nonetheless, our results demonstrate that *M. rhaphiophylla* EO does not present effective antifungal activity via volatilization against the tested isolates; inhibition was lower than 30 %. Farag et al. (2004) and Kim and Park (2012) corroborate these findings using EOs from different species of *Melaleuca* via volatilization against *Aspergillus*. However, the studies conducted by Basak and Guha (2015) and Perumal et al. (2016) sustained that EOs might have antifungal activity when applied through the volatilization method against post-harvest and storage pathogens, causing inhibition in the growth of these fungi. Therefore, the antifungal activity of these substances in the volatilization method may be associated with the presence and high amounts of monoterpenes (Kulkarni et al., 2022).

In the present study, *M. rhaphiophylla* EO showed high fungicidal activity via contact through the microdilution technique. In a study with results similar to ours, the EO of *Melaleuca alternifolia* displayed antifungal action against *A. niger* and *A. flavus* (Sevik et al., 2021) and different species of *Fusarium* (Terzi et al., 2007; Sahab et al., 2014; Palfi et al., 2019) via contact.

Terpinen-4-ol, 1,8-cineole, and y-terpinene, the main compounds identified in the present study, demonstrate fungicidal activity against *Aspergillus* and *Fusarium* (Morcia et al., 2012; Terzi et al., 2007). However, our results suggest that the fungicidal activity of EOs is not only linked to monoterpenes but to the relationship of the synergistic effect between the major and minor compounds which act together to potentiate the antifungal effect (Pimienta-Ramírez et al., 2016; Baldim et al., 2018).

Due to the physicochemical characteristics of EOs, the fungicidal effect of these products can be affected by the method employed, which is, therefore, an important factor when determining the antifungal potential of the extract (Basak and Guha, 2015; Hossain et al., 2016). In addition to these factors, other variables such as the strains and species that are used, the chemical composition, and the mode of action of EOs should be considered as determinants when assessing their capacity as a fungicide (Hossain et al., 2016; Manssouri et al., 2016; Kumar et al., 2017; Kulkarni et al., 2022).

5. Conclusions

EOs are promising natural chemical substances for the control of pests and fungi. Therefore, they can be used as an alternative strategy for managing the increased resistance of these pests. Our results demonstrate that *M. rhaphiophylla* EO showed insecticidal and fungicidal activity against the main storage pests and pathogens, demonstrating its potential for use in storage units and systems. However, more studies are required to evaluate formulations, application technologies, and the effect of *M. rhaphiophylla* EO in silo or warehouse conditions in order to estimate their bioactivity in applied field situations, in addition to investigating the impact of these products on seeds and grains.

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CHAPTER 02: TOXICITY AND BIOCHEMICAL EFFECTS OF *Melaleuca rhaphiophylla* ESSENTIAL OIL AND EMULSION AGAINST WEEVILS*

*These results will be submitted in International Journal of Biological Macromolecules

Abstract

The insecticidal action of essential oils (EOs) helps to control species of genus *Sitophilus*. Nevertheless, there are currently no publications on the delivery system's use of biopolymer-based emulsion technologies nor on the mechanism of action regarding the biochemical parameters of *Melaleuca rhaphiophylla* EO (MREO) in insects. Therefore, the present study aimed to evaluate the insecticidal activity and effect on biochemical markers of MREO and its emulsion (MREM) against *Sitophilus zeamais* and *Sitophilus oryzae*. The emulsion was prepared by combining hydroxypropyl methylcellulose (HPMC) (2 wt.%) with MREO to concentrations of 39.18 and 25.61 μL of substance L^{-1} of air for *S. zeamais* and *S. oryzae*, respectively. The rheological characteristics of HPMC-based emulsions with EO were examined to confirm the possible use of these formulations as structured systems for EO delivery. Microscopic images revealed emulsions formed by spherical and well-separated EO droplets measuring 0.8–1.6 μm diameters. Furthermore, the insecticidal activity of MREO and MREM was evaluated by the fumigation method. Then, the effect of the treatments on the activity of enzymes Glutathione S-Transferase (GST), esterase-α, esterase-β, superoxide dismutase (SOD), acetylcholinesterase (AChE), and lipid peroxidation (LPO) were evaluated. The MREO caused a mortality rate above 25%, with increased enzymatic synthesis of AChE for both species. There was LPO damage only for *S. zeamais* and inhibition of GST, esterase α-, and SOD activity for *S. oryzae*. The MREM treatment caused a mortality rate of less than 1.5%, with an increase in GST, SOD, and LPO enzymes for both species and an increase

in the enzymatic activity of esterase-β for *S. zeamais* and esterase-α for *S. oryzae*. We concluded that MREO demonstrated higher insecticidal activity, while MREM caused chronic toxicity against weevils.

Keywords: *Sitophilus*, Essential oil, Hydroxypropyl methylcellulose, Insecticidal, Enzymatic activity

1. Introduction

The genus *Sitophilus* has high sanitary relevance in storage systems, as they are primary pests that have as main characteristics cross infestation, high biotic potential, and attack multiple hosts (Garrido-Miranda et al., 2022; Zimmermann et al., 2022, 2021). In Brazil, the most common species reported are *Sitophilus zeamais* and *Sitophilus oryzae* (Coleoptera: Curculionidade), which cause direct and indirect damage to grains and seeds, causing significant economic losses (Brito et al., 2020; Haddi et al., 2018; Kim et al., 2019; Pimentel et al., 2022). The main method of control is the use of synthetic insecticides, especially those with fuming action (Santana et al., 2022), whose major classes include pyrethroids, organophosphates, and phosphine (Peschiutta et al., 2021; Pimentel et al., 2022).

Intensive and indiscriminate use of these chemicals has resulted in several negative effects, such as an increase in production costs and levels of toxic residues in food, risks to human health, and environmental impacts (Chaudhari et al., 2021; Santana et al., 2022). Furthermore, the biggest problem in the use of insecticides is the selection of insects resistant to the active ingredients (Haddi et al., 2018; Kim et al., 2019; Nayak et al., 2020; Sakka and Athanassiou, 2021; Santana et al., 2022), as these products have only one site of action, acting mainly on the nervous system (Jankowska et al., 2018; Sparks et al., 2020). Faced with the problems caused by the use of synthetic insecticides, it is necessary to search for substances that are effective in controlling storage pests, with less impact on human and environmental health, and that allow the resistance management of these insects in storage systems (Brito et al., 2020; Garrido-Miranda et al., 2022).

A promising alternative is the use of essential oils (EOs), which are defined as a complex set of volatile chemical substances with low molecular weight, synthesized from the secondary metabolism of certain plants (Brito et al., 2020; Lucia and Guzmán, 2021). These compounds have different biological properties of agricultural interest, and due to their ability to volatize, they can act as fuming insecticides in storage systems (Peschiutta et al., 2021).

EOs have several advantages compared to synthetic insecticides, and because they are made up of different chemical molecules, they act on different sites of action, having a lower possibility of selecting resistant individuals (Chaudhari et al., 2021; Garrido-Miranda et al., 2022; Peschiutta et al., 2021). However, few researchers have been investigating the mechanism of action of these products on storage pests (Campolo et al., 2018), which may hinder the regulation and commercialization of EOs as bioinsecticides (Chaudhari et al., 2021; Kiran and Prakash, 2015; Palermo et al., 2021).

Despite the benefits, the use of EOs for pest management in storage systems is limited due to the yield and physicochemical characteristics of these substances, such as high volatilization and the influence of external factors that can affect their effectiveness (Garrido-Miranda et al., 2022; Lucia and Guzmán, 2021; Moura et al., 2021). A strategy to address this adversity is by forming emulsions (Lucia and Guzmán, 2021; Palermo et al., 2021; Santos et al., 2022) through the use of biopolymers, which can prolong the insecticidal effect of these substances and preserve the molecules from agents that can cause their degradation (Menossi et al., 2021; Oliveira et al., 2018).

Among the plants with potential use, EOs of *Melaleuca* species have proven insecticidal action in the control of *S. zeamais* and *S. oryzae*, causing mortality rates in a short period of exposure time (Zimmermann et al., 2022, 2021). In addition, there are only reports on the effect of these substances on biochemical mechanisms in insects for

Melaleuca alternifolia EO (Gómez-Rincón et al., 2014; Liao et al., 2017, 2016). Previous studies demonstrate that MREO has insecticidal activity with high toxicity against *Sitophilus* (Zimmermann et al., 2022). However, to date, there are no studies on the combination of MREO with biopolymers to formulate an emulsion with insecticidal activity and on the effect of these substances on enzymatic parameters of weevils. In this context, this study aimed to evaluate the enzymatic response of *S. zeamais* and *S. oryzae* species when exposed to *M. rhaphiophylla* EO (MREO) and its emulsion (MREM). In addition to the physicochemical characterization of the emulsion formed by combining *M. rhaphiophylla* EO with hydroxypropyl methylcellulose (HPMC).

2. Material and Methods

2.1. Weevil rearing

Adult specimens of *S. zeamais* and *S. oryzae* were obtained from stock reared in the laboratory for five years without exposure to synthetic insecticides. The insects were placed in plastic containers with a capacity of 750 mL, containing a mixture of 500 g of maize for *S. zeamais* and wheat for *S. oryzae*, both from organic farmers, 50 g of cornmeal bran, and 10 g of wheat germ. Subsequently, the containers were kept in a controlled environment at 26ºC and 65% RH. Adult individuals aged between 5 and 8 days, unsexed, were used for the bioassays (Zimmermann et al., 2021).

2.3. Acquisition of *Melaleuca rhaphiophylla* EO

Plant materials were collected in November 2019 in Araucária (25°31′00.81″ S and 49°26′18.24″ W), Paraná, Brazil (Zimmermann et al., 2022). A sample of this material was deposited at the Museu Botânico de Curitiba under the number 289706. EOs were

extracted from the green aerial part, that is, branches with leaves, by steam distillation for 2 h. Water was used as an extraction solvent. EOs were transferred to vials and refrigerated until their analysis. They were then diluted to 1% (w/v) using ultrapure hexane, and their chemical composition was previously reported by Zimmermann et al. (2022).

2.4 Formulation and characterization of *M. rhaphiophylla* emulsion

2.4.1. Preparation of *M. rhaphiophylla* emulsion

The MREO was mixed with 2 wt% HPMC using ultrasound during 60 s, with on/off cycles of 10 s. Emulsions using HPMC for experiments with *S. zeamais* (SZ) and *S. oryzae* (SO) contained 2.86 and 1.87 wt% of MREO(Zimmermann et al., 2022), respectively. The emulsions were named HPMC-SZ and HPMC-SO, and the vehicle was named HPMC.

2.4.2. Rheological measurements

Rheological analyses were performed using a TA Instruments HR-10 rheometer fitted with cone and plate geometry (40 mm in diameter and 350 mm gap). The temperature of the systems was measured using a Peltier system. Flow curves were performed at a shear rate range of 1-100 s^{-1}, for 300 s, at 20°C. Oscillatory frequency sweeps were obtained from a frequency range of 0.01–10.0 Hz under stress in the linear viscoelastic region (γ = 1.0 Pa), obtained by oscillatory stress sweeps between 0.001 and 100 Pa at a constant frequency of 10.0 Hz.

Temperature variation was achieved using a Peltier system with heating (25–75˚C) and cooling gradients (75–25˚C) at a rate of 5.0°C min^{-1}, constant frequency (1.0 Hz), and constant stress (0.1 Pa). A solvent trap was used to permit efficient temperature regulation and prevent evaporation.

2.4.3. Confocal laser microscopy

The microstructures of the emulsions were observed and photographed using a Nikon laser confocal microscope (model AR1+) with 20× and 60× objectives. For this analysis, the EO phase was stained with fluorescein (10 ppm, excitation of green fluorescence at 495 nm and emission at 525 nm). All images obtained were processed using Image J (version 18.0).

2.5. Insecticidal activity

The experiment was conducted in a completely randomized design in a factorial scheme (5 treatments x 2 pests), with 3 replications for each treatment. The treatments were: 1) MREO, 2) MREM, 3) negative control acetone at 100% concentration (for essential oil), 4) negative control hydroxypropyl methylcellulose at 2% concentration (for emulsion), and 5) positive control - synthetic insecticide Pyrimifos-methyl (Actellic 500 EC) at a concentration of 16 mL L^{-1} (AGROFIT, 2022). Each replication per treatment consisted of 20 insects of each species, and the experiment was repeated five times (n=300 insects/treatment).

The treatments were diluted in the respective negative controls up to concentrations 39.18 (2.86%) and 25.61 µL (1.87%) of substance L^{-1} of air to *S. zeamais* for *S. oryzae*, respectively, , which correspond to the lethal concentrations (LC25) determined by Zimmermann et al. (2022). Subsequently, 200 µL of each treatment were pipetted onto filter paper disks with a diameter of 8 cm and placed in a plastic container with a capacity of 145 mL containing the insects. Then, the MREO was kept at room temperature for 7 minutes for the acetone to evaporate and then sealed and conditioned under controlled conditions (25 ± 1ºC, 65% RH, and 12 h of photophase). The insect mortality was evaluated after 48 h of application of treatments (Zimmermann et al., 2021),

and and the live insects were separated individually for the biochemical response experiments described in topic 2.6.

2.6. Effect of *Melaleuca rhaphiophylla* EO and emulsion on biochemical responses in *Sitophilus zeamais* and *Sitophilus oryzae*

The enzymes activity and lipid peroxidation (LPO) were measured using ten pools with four *S. zeamais* and *S. oryzae* of each treatment stored individually in 1.5 mL centrifuge microtubes. Each pool was homogenized in 620 µL of ultrapure water (milli-Q water) and subsequently centrifuged [except the samples for measuring acetylcholinesterase (AChE) activity] at 12000×g at 4 °C for one min and then stored at -80°C. Assays were carried out on a 96-well microplate, and absorbances were measured using spectrophotometry.

2.6.1 Glutathione S-Transferase

The protocol by Keen et al. (1976) was used to determine Glutathione S-Transferase (GST) activity. Fifteen (15) µL of samples were added (for the blank, it was replaced by 15 µL of ultrapure water (milli-Q water)), and 195 µL of solution composed of reduced glutathione (10 mM GSH) and chloro-dinitrobenzene (CDNB; 21 mM). The reading was performed at a wavelength of 340 nm for 20 minutes every minute. The results were expressed in µmoles/mg ptn/min.

2.6.2. Esterases

Esterase-α and esterase-β activities were analyzed following the protocol by Valle and Montella (2006). For both, 10 µL of sample was added (the blank was replaced by 10 µL of milli-Q water). Then, 200 µL of alpha-naphthyl acetate/Naphosphate (0.3 mM) was added for esterase-α, using alpha-naphthol (0.3 mg/L^{-1}) as a positive control. For esterase-β, 200 µL of beta-naphthyl acetate/Naphosphate (0.3 mM) was added, using

beta-naphthol (0.3 mg/L^{-1}) as a positive control. The plates were incubated for 15 minutes at room temperature and light, followed by adding 50 µl of Fast Blue dye (0.3%) and a second incubation for 5 minutes. Then, an endpoint reading was performed with a wavelength of 570 nm. The results were expressed in nmol α-naphthol/ mg ptn /min for esterase-α and nmol β-naphthol/mg ptn/min for esterase-β. A standard curve was performed to convert absorbance values into alpha/beta-naphthol content. For the curve, the masses of 0 µg, 1 µg, 2 µg, 3 µg, 4 µg, and 5 µg of alpha/beta-naphthol were used. For this, volumes of 0 µL, 2 µL, 4 µL, 6 µL, 8 µL, and 10 µL of alpha/beta-naphthol were added in duplicate and ultrapure water (milli-Q water) was added to complete a total volume of 10 µL of sample. Subsequently, the same procedure for preparing and reading the microplates was followed.

2.6.3. Superoxide dismutase

Superoxide dismutase (SOD) activity was analyzed according to the protocol proposed by Gao et al. (1998). For this, 40 µL of samples (blank: 40 µL of Milli-Q water) were aliquoted, in duplicate, in 1.5 mL microcentrifuge tubes. Then, 885 µL of ethylenediamine tetraacetic acid tris buffer (EDTA; pH 8.0; 5 mM) and 50 µL of pyrogallic acid were added. The microtubes were incubated for 30 minutes, and 25 µL of HCl (1 N) was added, which partially interrupted the reaction. Control microplates were prepared, following the same procedures except for the incubation process (absent). Then, 300 µL of the solution was added to the plate in triplicate, and the reading was performed at 440 nm of wavelength. The results were expressed in units (U) of SOD/mg of protein.

2.6.4. Lipid peroxidation

LPO amount was determined following the protocol by Jiang et al. (1992). Seventy (70) µL of sample and 70 µL of methanol were added into 1.5 mL

microcentrifuge tubes. Samples were centrifuged at 5000xg for 5 minutes at 4 °C. 100 µL of the supernatant (100 µL of methanol was substituted for the blank) were transferred to a new microtube, in which 900 µL of Fox 2 reagent was added (methanolic solution (Xylenol Orange 0.1 mM; crystalline butylated hydroxytoluene (BHT) 4 mM; PA methanol); 2500 µM ferrous ammonia sulfate solution (H_2SO_4; ultrapure water (milli-Q water). The centrifuge microtubes were homogenized and incubated in the dark for 30 minutes vortexed every 3 minutes. Afterward, the solution was transferred to a 96-well microplate in triplicate, with a volume of 300 µL each. Reading was performed at 560 nm. The results were expressed in nmol of hydroperoxides/mg of protein.

2.6.5. Acetylcholinesterase

AChE activity was analyzed following the protocol by Ellman et al. (1961), which was modified for microplate by Silva de Assis (1998). Twenty-five (25) µL of samples (the blank was replaced by 25 µL of ultrapure water (milli-Q water)), 200 µL of 5,5-Dithio-bis-2nitro-benzoate (DTNB; 0.75 mM), and 50 µL of iodidetoacetylcholine (ATC; 10 mM). Once pipetted, the plate was incubated at room temperature and light for 30 minutes. The reading was performed every 30 seconds for 5 minutes, with a wavelength of 405 nm. The results were expressed in µmoles/mg ptn/min.

2.6.6. Protein assay

The amount of total protein was determined following the Bradford protocol (1976). Ten (10) µL of samples, 10 µL of Bovine Serum Albumin (BSA) for the positive control, and 250 µL of Bradford dye (1 Bradford: 5 ultrapure water (milli-Q water)) were added. The reading was obtained at a wavelength of 620 nm. For the BSA standard curve, the amounts of 0 µg, 2.5 µg, 5 µg, 7.5 µg, 10 µg and 15 µg of BSA were serially diluted in milli-q water (highest concentration: 150 µL of the stock solution (2 mg /ml BSA) + 50 µL milli-Q water and blank 200 µL ultrapure water (milli-Q water). Then, 10 µL of

the sample was pipetted in quadruplicates, by concentration, in a microplate. The absorbance reading followed the same protocol as that for total protein.

2.7. Statistical analysis

The insect mortality data were corrected by Abbott (1925). Insecticidal data and data of effect on biochemical responses were adjusted by generalized linear models (GLM) using Poisson distribution, and then analysis of variance (ANOVA) was performed for each species. Subsequently, the means were compared using Tukey's test with a 5% probability of error, using R (R CORE TEAM, 2023). A joint analysis was carried out with repetitions of the experiments.

3. Results

3.1. Physicochemical analysis of *Melaleuca rhaphiophylla* essential oil emulsions

In order to evaluate the influence of the addition of EO to HPMC-based emulsions on flow behavior, viscosity curves were analyzed as a function of the shear rate for HPMC, HPMC-SO, and HPMC-SZ (Fig. 1.). HPMC at a concentration of 2 wt% presented a shear thinning behavior, as apparent viscosity decreased with the increase of the shear rate. However, after adding essential oil, the viscosity decreased for HPMC-SO and HPMC-SZ emulsions. These emulsions had almost the same viscosity across the entire range analyzed (0.41–0.49 Pa s at 1 s^{-1}) with no dependence on the shear rate (Newtonian behavior).

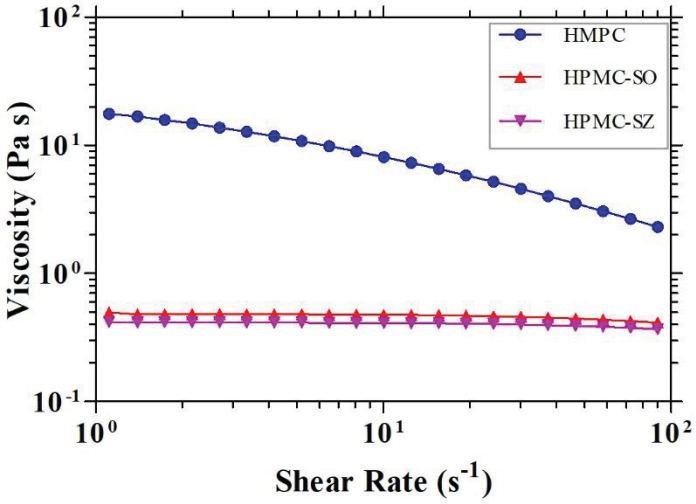

Fig. 1. Viscosity as a function of the shear rate for HPMC, HPMC-SO, and HPMC-SZ at 20°C.

Dynamic measurements were also performed to characterize the viscoelastic behavior of emulsions. Samples were subjected to a frequency sweep at 20°C and a strain of 1.0 Pa (Fig. 2.). Prior to this analysis, the amplitude value was selected by an amplitude sweep test within the linear viscoelastic region.

For all emulsions containing EO, viscous (G″) moduli values were higher than elastic (G′) moduli over the entire frequency range analyzed, indicating a fluid-like behavior. In addition, both moduli increased with increasing frequency, demonstrating a frequency dependency. Compared to HPMC alone (without EO), both moduli decreased after adding EO to emulsions, displaying a more liquid character. At 1 Hz, the G′ modulus of emulsions is 75–350 times lower than for HPMC alone.

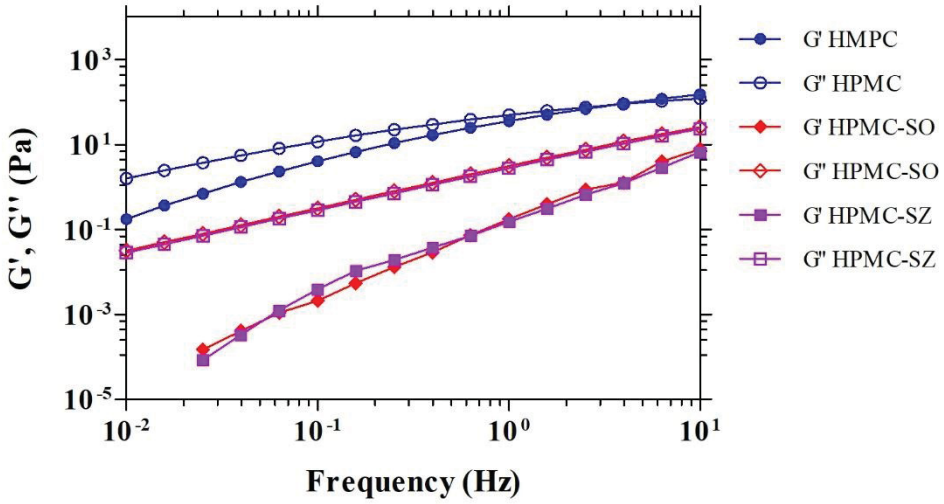

Fig. 2. Elastic (G′) and viscous (G″) moduli as a function of the frequency for HPMC, HPMC-SO, and HPMC-SZ at 20°C for an applied oscillatory stress (τ) = 1.0 Pa. Full dots correspond to elastic modulus G′ (Pa) and open dots to the viscous modulus G″ (Pa).

Then, to evaluate the thermal stability of the emulsions after adding the EO, temperature sweeps at a constant frequency (1 Hz) and stress (0.1 Pa) were performed. Fig. 3 shows the evolution of elastic (G′) and viscous (G″) moduli with heating (25–75˚C) and cooling (75–25˚C) for all emulsions.

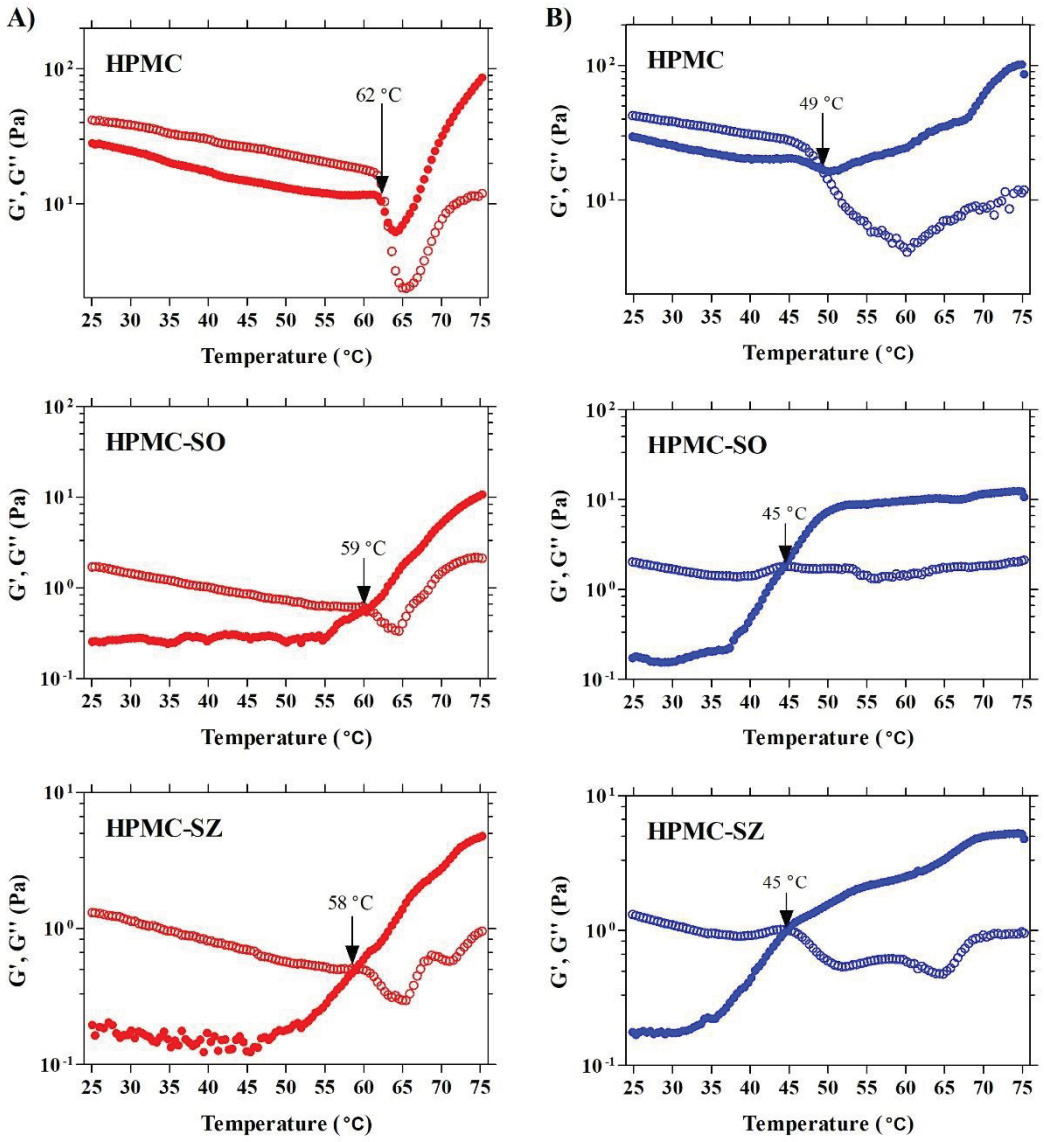

Fig. 3. Temperature dependence of elastic (G′) and viscous (G″) moduli at 1.0 Hz and 0.1 Pa for A) heating (25–75˚C) and B) cooling (75–25˚C) cycles of HPMC, HPMC-SO, and HPMC-SZ. Full dots correspond to G′ and open dots to G″.

For HPMC, at the temperature range of 25 to 61°C, G″ is greater than G′, indicating a viscoelastic behavior dominated by the viscous component. A sol-gel transition (G′=G″) occurred at 62°C, with a drastic decrease in both moduli. Then, at 65–75°C, HPMC exhibited a gel-like behavior (G′>G″), and G′ and G″ progressively increased as temperature increased (Fig. 3. A).

On cooling, from 75–60°C, G′ and G″ showed a slight reduction. Then, G″ started to rise, and at 49°C, dissolution was observed (thermo-reversible), and G" reverted to being greater than G' (Fig. 3. B).

Adding EOs to the emulsions reduced the sol/gel transition temperature from 62 to 58°C for HPMC-SZ and to 59°C for HPMC-SO samples during heating. Changes in the dissolution temperatures were also observed, reducing the temperature from 49 to 45°C for HPMC-SO and HPMC-SZ.

These results indicate that EOs may facilitate heat-induced gel formation by increasing hydrophobic interactions in emulsions.

3.2. Size and morphology of *Melaleuca rhaphiophylla* essential oil emulsions

Fig. 4 depicts the microstructure of the emulsions on confocal microscopy images. The droplets were spherical and remained well separated for the three emulsions analyzed, which further confirmed that an O/W emulsion was formed when HPMC was utilized as an emulsifier. Furthermore, there was no evidence of any flocculation or droplet coalescence. No macroscopic phase separation was observed when the samples were stored quiescently for six months at 4 °C, indicating that all emulsions containing MREO and HPMC were stable. Average droplet diameters were 1.5 μm (±0.7) and 0.8 μm (±0.25) for HPMC-SO and HPMC-SZ emulsions, respectively.

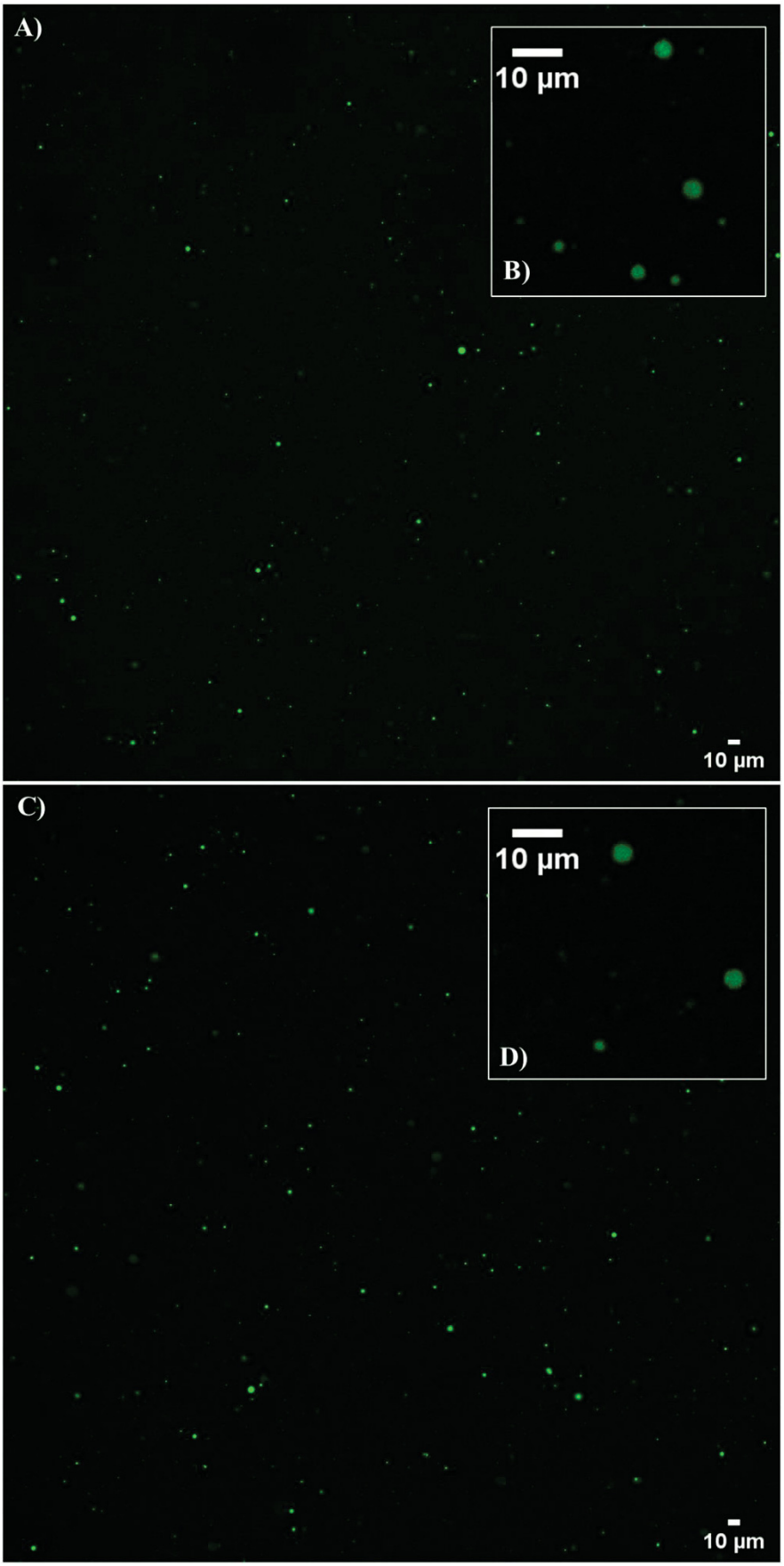
A)
10 µm
B)
10 µm
C)
10 µm
D)
10 µm

Fig. 4. Confocal fluorescence microscopic images of emulsions containing *Melaleuca rhaphiophylla* essential oil A) HPMC-SO (20× objective), B) HPMC-SO (60× objective), C) HPMC-SZ (20× objective), and D) HPMC-SZ (60× objective). Scale bar = 10 μm.

3.3. Insecticidal activity

Mortality for both species of *Sitophilus* was greater than 25% when exposed to MREO, but the mortality rate caused by MREM treatment was less than 1.5%. *Sitophilus oryzae* showed higher mortality rates for the treatments. There was no mortality in the negative control with HPMC.

Table 1. Mortality rate (%) of *Sitophilus zeamais* and *Sitophilus oryzae* after 48 h of exposure via fumigation at the concentration 39.18 and 25.61 μL of substance L^{-1} of air for *S. zeamais* and *S. oryzae*, respectively, of *Melaleuca rhaphiophylla* essential oil (MREO) and its emulsion (MREM). Acetone and hydroxypropyl methylcellulose (HPMC) were used as a negative control. Pirimifos-methyl was used as a positive control.

Species	Treatments	No.[o]	Mortality rate (%)[a] (SD)[b]
S. zeamais	MREO	300	26.25 ± 0.9 bB
	MREM	300	0.84 ± 0.56 cA
	Pyrimifos-methyl	300	100 aA
	Acetone	300	0.41 ± 0.41 cA
	HPMC	300	0 cA
S. oryzae	MREO	300	30.83 ± 1.83 bA
	MREM	300	1.2 ± 0.65 cA
	Pyrimifos-methyl	300	100 aA
	Acetone	300	2.5 ± 0.75 cA
	HPMC	300	0 cA

[a] Averages followed by capital letters compare different insects among the same product, and lowercase letters compare each pest species among the substances tested by Tukey's test at 5%. [b] Standard error.

3.4. *Melaleuca rhophiophylla* essential oil on biochemical responses

For *S. zeamais,* MREO caused a significant change only in AChE, with an increase in enzymatic activity and LPO after acute exposure compared to the control group. As for the biotransformation enzymes (GST, esterase-α, and esterase-β) and antioxidant enzyme SOD, we observed that none differed in their activity in relation to the control (acetone).

For *S. oryzae*, MREO increased AChE enzymatic activity as well as the *S. zeamais*. We found that the oil altered the activity of important biotransformation and oxidative-stress fighting enzymes by inhibiting the activity of GST, esterase-α, and SOD. There was no lipo-oxidative damage caused by the oil and no change in β-esterase activity (Fig. 5).

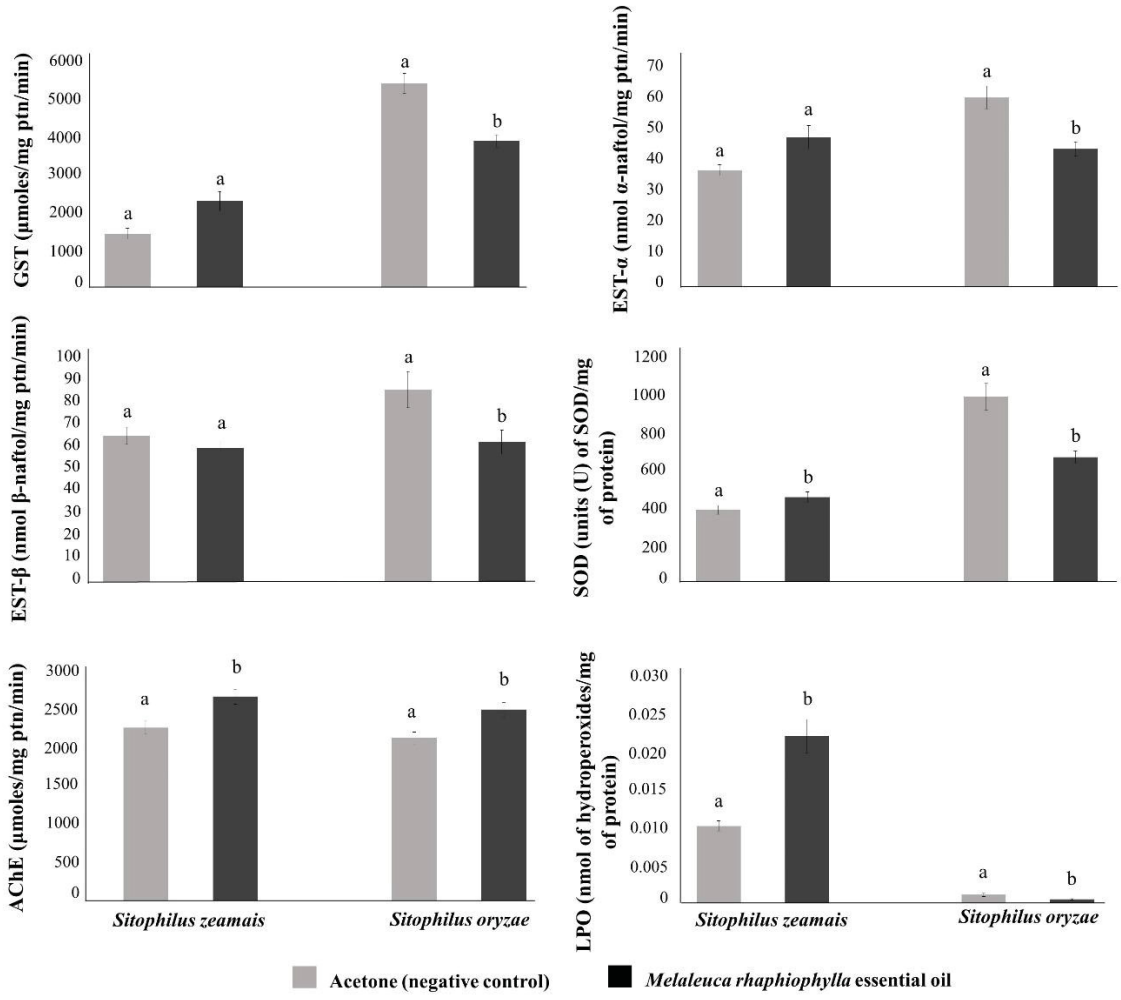

Fig. 5. Effect of acetone (negative control) and *Melaleuca rhaphiophylla* essential oil on GST, Esterase-α (EST-α), esterase-β (EST-β), SOD, LPO, and AChE against *Sitophilus zeamais* and *Sitophilus oryzae*. The means followed by the same letter in the bar diagram are not significantly different according to ANOVA and Tukey's multiple comparison tests.

3.5 *Melaleuca rhophiophylla* emulsion on biochemical responses

For *S. zeamais,* MREM caused a significant change in different enzymatic activities after acute exposure compared to the control group. GST and esterase-β biotransformation enzymes significantly increased activity compared to the control group. There was an increase in SOD activity, an important antioxidant enzyme. Despite this, LPO was observed in organisms exposed to the emulsion in relation to the control. There was no alteration regarding the enzymatic activity of AChE and esterase-α.

For *S. oryza*e, MREM increased enzymatic activity and altered the activity of important enzymes for biotransformation and combating oxidative stress with an increase in the activity of GST, esterase-α, and SOD. As with the other species, the emulsion caused lipid peroxidation damage, and there was no change in AChE activity (Fig. 6).

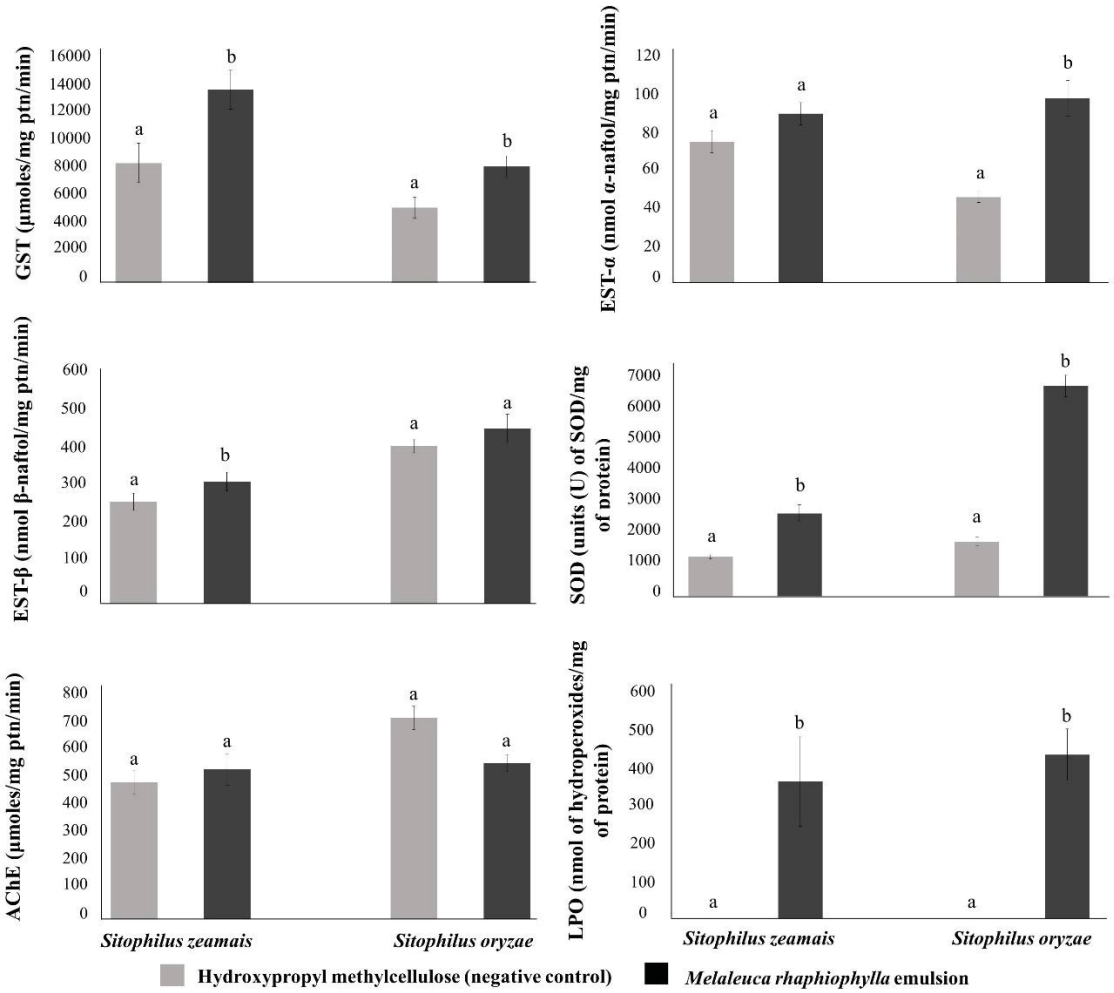

Fig. 6. Effect of hydroxypropyl methylcellulose (negative control) and *Melaleuca rhaphiophylla* emulsion on GST, Esterase-α (EST-α), esterase-β (EST-β), SOD, LPO, and AChE against *Sitophilus zeamais* and *Sitophilus oryzae*. The means followed by the same letter in the bar diagram are not significantly different according to ANOVA and Tukey's multiple comparison tests.

4. Discussion

Controlling storage pests is essential for avoiding major economic losses in the grain and seed production chain. EOs are an effective alternative solution to chemical-synthetic products in the sustainable management of these insects (Dimetry et al., 2019; Menossi et al., 2021; Opiyo et al., 2022). The present study is the first report on the

combination of MREO with HPMC and the effect of these substances on the enzymatic response of weevils. In this study, MREO was stabilized and protected through HPMC-based emulsions.

Through rheological characterization, we observed that the incorporation of EO made the emulsions less viscous than pure HPMC. This behavior is in line with the literature. According to Sánchez-González et al. (2011), HPMC molecules can adsorb on the surface of the oil droplet, which reduces its viscous contribution in the continuous phase. In addition, the droplets become more stable and less sensitive to changes promoted by shear forces. This characteristic was confirmed by the frequency sweep at room temperature, where emulsions containing EO showed a more fluid-like behavior, as demonstrated by the reduction of G′ and G″ moduli.

On the other hand, despite increasing the fluidity of the emulsions, the addition of EO contributed to the reduction of sol/gel transition temperature, as demonstrated in temperature sweeps during heating and cooling ramps. The HPMC biopolymer in aqueous solution are well known to form thermoreversible gels in concentrations of 1–10% (Sarkar, 1979; Sannino et al., 2009; dos Santos Carvalho, Rabelo, & Hubinger, 2022).

At low temperatures (below the sol/gel transition point), there were HPMC chains in solution with water cages surrounding hydrophobic clusters. The water cages are broken as the temperature rises, and the methyl groups are exposed to the aqueous environment. Then, at the transition temperature from the liquid to gel state, the hydrophobic groups of the polymeric chains associate with each other, forming a reticulated network, which leads to gelation (Haque & Morris, 1993; Haque et al., 1993; Wang et al., 2022). Therefore, this biopolymer can interact with non-polar substances (Goff & Guo, 2019), such as EOs.

Thus, we can assume that heating-induced hydrophobic interactions between HPMC and the EOs in HMPC-SO and HPMC-SZ formulations promote a reduction of these biopolymers' gelation temperature

Regarding the biological activity, our results demonstrated the efficacy of MREO as a botanical insecticide against *S. zeamais* and *S. oryzae* using the fumigation method, as described by Zimmermann et al. (2022). Fumigant insecticidal activity expressed by EOs is due to the volatilization capacity of compounds present in their chemical composition (Cao et al., 2018; Dimetry et al., 2019). In storage systems, which are confined and hermetic environments, these substances can be inhaled by the spiracles, interrupting the respiratory chain, or absorbed by the integument through damage to the insect cuticle (Cao et al., 2018; Plata-Rueda et al., 2018; Sahu et al., 2021; Zenoozi et al., 2022; Zimmermann et al., 2021, 2022).

EOs are mainly made up of monoterpenes, which are lipophilic substances; that is, they have affinity for and are soluble in lipids. In this way, they are able to penetrate the insect's body and affect different systems (Palermo et al., 2021; Chauhan et al., 2022; Sahu et al., 2021; Zenoozi et al., 2022), causing disorders and morphophysiological changes in insects (Sahu et al., 2021; Renoz et al., 2022).

The present study is the first regarding insecticidal activity of MREM. However, the emulsion formed with the HPMC biopolymer showed low insecticidal action for both species since the mortality rates were lower than 1.5%. The low mortality may be related to the type of polymer used in the emulsion preparation and the EO chemical composition, which may affect the biological activity presented by these products (Oliveira et al., 2018; Ahsaei et al., 2020; Muturi et al., 2020; Menossi et al., 2021; Barros et al., 2022). However, other research on insecticidal activity of EO and its emulsion with different polymers against different pests demonstrated no toxicity difference between both

products. This fact is related to the size of the EO particle associated with the physicochemical characteristics, which increased efficiency and uniformity of product application, in parallel with the gradual release of the major chemical compounds, which are generally responsible for the insecticidal activity (Lucia et al., 2020; Muturi et al., 2020; Santos et al., 2022).

The mortality rate by MREM may be correlated with the amount of product that was inhaled or absorbed by the insects, since the purpose of the emulsion is to gradually release the EO, allowing for an increase in the duration of the insecticidal effect, and consequently, longer exposure to these compounds (Barros et al., 2022; Santos et al., 2022). However, this change in the concentration of active substances released can cause an adverse effect on the insecticide action through the biotransformation, degradation, and excretion of these toxic compounds (Menossi et al., 2021; Palermo et al., 2021).

EOs comprise different chemical molecule groups with different biological activities (Lucia and Guzmán, 2021). Due to this characteristic, in terms of their mechanism of action, EOs are categorized as multisite, since they act on different target sites within insect organisms and consequently cause different types of morphophysiological disorders (Sahu et al., 2021; Gad et al., 2022a). These disorders can be caused by damage to organ structures as well as biochemical changes (Santos et al., 2022), and regarding the toxicity of these products, lethal and sublethal doses can alter or inhibit enzymatic activities, causing morphophysiological disturbances and death of the insects (Rajkumar et al., 2020; Santos et al., 2022). In this context, the present study is the first report on the effect of MREO and emulsion on biochemical markers of *S. zeamais* and *S. oryzae*.

In our investigation, MREO inhibited antioxidant and biotransformation enzymes, critical for the organism's detoxification. It also caused LPO and induced an increase in

the production of an enzyme important for regulating the nervous system. In comparison, MREM increased the activity of biotransformation and antioxidant enzymes and showed damage with LPO.

The insecticidal activity and toxicity of EOs can be affected by other factors, such as their chemical composition, target organism, method of exposure, and environmental conditions during exposure (Moura et al., 2021; Barros et al., 2022; Chauhan et al., 2022), in addition to the insect's ability to metabolize these toxic substances and excrete them from its body (Renoz et al., 2022). Studies conducted with *Mentha arvensis* (Lamiaceae) EO against *Sitophilus granaries* (Coleoptera: Curculionidae) demonstrated that only a small amount of the compounds present in this EO penetrates the body of the pest and that the duration of these molecules tends to decrease due to the insect detoxification process (Renoz et al., 2022).

During their evolutionary process, plants have developed chemical strategies through secondary metabolic routes to prevent insect herbivory. These substances can cause different physiological, behavioral, and biological effects on these organisms (Plata-Rueda et al., 2018; Zenoozi et al., 2022). On the other hand, insects improved their defense system through the synthesis of enzymes responsible for the detoxification of chemical substances adverse to their metabolism (Plata-Rueda et al., 2018)

In insects, the main enzymes responsible for detoxification are esterases, GST, and SOD which act by biotransforming chemical compounds and combating reactive oxygen species by preventing oxidative stress, blocking the formation of free radicals, and consequently acting in the maintenance of cellular homeostasis (Akami et al., 2019; Chaudhari et al., 2021; Ercan et al., 2022). Our results showed that only MREM caused an increase in GST and SOD enzymes for the weevil species, an increase in esterase-α activity in *S. oryzae,* and esterase-β in *S. zeamais,* indicating that there was chronic

toxicity due to the gradual release of compounds present in the EO. In contrast, MREO inhibited esterase-α, GST, and SOD activity in *S. oryzae*, reducing the ability to inactivate the toxicity of these compounds. Therefore, the role of these enzymes is to degrade and transform toxic molecules into more water-soluble inactive substances that will be excreted, allowing the insect to survive after exposure to insecticides, whether natural or synthetic (Akami et al., 2019; Hu et al., 2019).

Esterases are among the groups of enzymes that perform different vital physiological functions for insects (Campolo et al., 2018; Hu et al., 2019), and studies show that esterase enzymes (esterase-α and esterase-β) act in the detoxification process of chemical molecules from EOs (Campolo et al., 2018; Hu et al., 2019; Piri et al., 2020; Yang et al., 2021), including insecticides from the group of organophosphates, carbamates and pyrethroids (Julio et al., 2017). Our results showed that after exposure to MREO, there was only a change in the activity of these enzymes, with esterase-α inhibition in *S. oryzae*, indicating that the toxicity caused by this EO was acute. However, MREM caused an increase in esterase-α and esterase-β enzymes, suggesting the gradual and slow release of EO compounds in the emulsion allows the toxicity to occur progressively. Low concentrations of EO lead to an increase in the synthesis of these enzymes (Haddi et al., 2020), and consequently, insects can perform biotransformation of these compounds by hydrolyzing the ester chains of chemical compounds present in EOs into molecules that will be excreted by the organism (Marschall and Jiang, 2018; Piri et al., 2020; Shahriari et al., 2020). Furthermore, the increased activity of esterase enzymes is normally associated with resistance to synthetic insecticides (Julio et al., 2017; Piri et al., 2020).

GST is important in phase II of the biotransformation of organic compounds, catalyzing the conjugation of tripeptide glutathione with electrophilic groups of

xenobiotic compounds, aiming to make them less toxic. As with esterases, the increased activity of GST enzyme is associated with the ability to detoxify and survive. In *S. oryzae,* there was a reduction in the activity of this enzyme by MREO, and a higher percentage of mortality was verified. The percentage of mortality was low in exposure to MREM, where there was an increase in enzymatic activity for both species.

The antioxidant enzyme SOD is an important insect defense mechanism against oxidative stress toxicity caused by reactive oxygen species (ROS) (Akami et al., 2019; Kiran and Prakash, 2015; Liao et al., 2016). The increase in the production of this enzyme with emulsion indicates ROS accumulation, which makes it impossible for the insect defense system to neutralize toxic compounds for cells (Chaudhari et al., 2021; Ercan et al., 2022). Inhibition of activity by EO was another detoxification mechanism affected by the oil, as well as the biotransformation enzymes.

In addition to altering enzymatic synthesis, EOs can also act to disrupt cellular homeostasis through the formation and release of free radicals, increasing the level of ROS in cells, resulting in LPO (Olmedo et al., 2015; Shahriari et al., 2020; Moustafa et al., 2021). Our results demonstrate that MREO caused an increase in LPO in *S. zeamais*, and MREM caused an increase in LPO in both species. The main consequence of the high activity of LPO is damage to the cell plasma membrane, increasing permeability and, favoring the release of intracellular content in addition to increasing oxidative stress, resulting in cell death (Gultekin and Ozturk, 2000; Shahriari et al., 2020; Moustafa et al., 2021; Ercan et al., 2022).

The nervous system of insects comprises neurons that conduct chemical signals through neurotransmitters in the synaptic clefts, with acetylcholine as the main and fastest neurotransmitter. In order to interrupt the nerve impulse, AChE is produced, responsible for binding to acetylcholine and degrading the molecule, preventing its accumulation in

the synaptic cleft and consequently avoiding neurotoxicity (Oboh et al., 2017; Piri et al., 2020; Rajkumar et al., 2020).

In our study, MREO caused an increase in the enzymatic synthesis of AChE for both weevil species after 48 h of exposure, indicating an insect attempt to accumulate acetylcholine in the synaptic cleft. In general, the insecticidal effect of EOs is associated with the presence of monoterpenoid compounds that induce damage to the nervous system, mainly by inhibiting neurotransmitters, especially AChE, generating an accumulation of acetylcholine in the synaptic cleft, continuing the uncontrolled transmission of nerve impulses (Cao et al., 2018; Jankowska et al., 2018; Piri et al., 2020; Chaudhari et al., 2021; Ercan et al., 2022) causing hyperexcitability, resulting in paralysis of the muscular and respiratory systems, and insect death (Rajkumar et al., 2020; Moura et al., 2021; Barros et al., 2022; Gad et al., 2022b). In this case, the activity increment may indicate an increase in information passed through the nervous system, as other detoxification systems have been affected.

Meanwhile, MREM did not cause any change in AChE activity. AChE inhibition is extremely fast, as the enzyme-substrate relationship with the degradation of acetylcholine is an instantaneous process due to its reaction speed (Jankowska et al., 2018). Due to the gradual release of compounds present in the emulsion, it is assumed that there was time for detoxification without needing to increase AChE synthesis, justifying the low mortality in both insects.

Because they are formed by different chemical compounds, EOs can also act on other target sites within the nervous system, altering or inhibiting the activity of octopamine, gamma-aminobutyric acid (GABA) and sodium channels (Oboh et al., 2017; Jankowska et al., 2018; Chaudhari et al., 2021; Prakash et al., 2022; Renoz et al., 2022). However, it is noteworthy that the factors concentration and time of exposure to these

products can cause an adverse effect on toxicity in insects, in which low concentrations of EO can induce an increase in AChE activity while high concentrations result in inhibition of AChE (Kiran and Prakash, 2015; Hu et al., 2019; Chaudhari et al., 2021).

Monoterpenes can interfere with the insect detoxification process by inhibiting the production of enzymes responsible for the processes of biometabolization of these compounds (Gad et al., 2022b). However, it is necessary to investigate whether this mechanism of action occurs due to the presence of a compound or by the interaction of all compounds present in the chemical composition of EO, where synergism between the compounds occurs, and therefore, more than one molecule can have affinity for the same target site (Jankowska et al., 2018; Hu et al., 2019).

5. Conclusions

The present study demonstrated that MREO and emulsion have insecticidal activity even at low concentrations and, therefore, are promising alternative products for controlling *S. zeamais* and *S. oryzae*. O/W emulsions were formed comprising MREO stabilized with HPMC. These emulsions demonstrated good stability both by microscopic and rheological techniques. All emulsions containing EO presented a fluid-like behavior ($G''>G'$). Thermal stability was also evaluated, and it was found that the addition of EO reduced the sol-gel transition temperatures of the HPMC-based emulsions, which suggested that EOs may be facilitating heat-induced gel formation by increasing hydrophobic interactions. MREO caused an increase in the enzymatic activity of AChE, while its emulsion caused an increase in the main enzymes responsible for detoxification, in addition to causing LPO, for both species. Despite its efficacy, further research on this EO's effects, in addition to other biopolymers, is required to raise the mortality rate,

evaluate the product's ability to be produced at the nanoscale, and examine the technology for using these products in storage systems.

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CHAPTER 03: *Melaleuca rhaphiophylla* ESSENTIAL OIL AS SEED TREATMENT AGAINST SEEDBORNE DISEASES *

*These results will be submitted in Food Chemistry Journal

Abstract

The *Melaleuca* genus is widely known for producing essential oil (EO) with fungicidal activity against phytopathogens. In this context, this study aimed to evaluate the effect *Melaleuca rhaphiophylla* (MREO) against *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nomius*, *Fusarium culmorum* and *Fusarium graminearum* by using the microdilution method (contact method) on inhibition mycelial growth, through minimum inhibitory concentration (MIC) and medium inhibitory concentration (IC_{50}), evaluate fungistatic or fungicidal activity, effect on conidia germination, sporulation, respiratory metabolism, and in addition their effect on germination of wheat seeds. At a concentration of 1.5%, MREO caused growth inhibition above 95%, with MIC value of 1% for all isolates. The IC_{50} value varied among isolates exposed to MREO, with lower value of 0.07% for *A. niger* and higher at 0.33% for *F. culmorum* (NRRL3288). MREO decreased conidia germination rate for all isolates, and reduced the sporulation for *A. flavus*, *F. culmorum* (NRRL25475) and *F. graminearum*. The increase in the concentration of MREO reduced the respiratory metabolism for all isolates of *Fusarium*. The germination rate of wheat seeds after treatment with MREO was 96%. MREO proves to be an alternative and promising natural substance for the treatment of wheat seeds, aiming at the organic production of the crop.

Keywords: Natural products, *Aspergillus*, *Fusarium*, Antifungal activity, Sporulation, Germination

Highlights

- MREO at 1% showed high toxicity against *Aspergillus* and *Fusarium* isolates.
- MREO reduced germination rate for all isolates.
- MREO reduced sporulation rate for *A. flavus*, *F. culmorum* (NRRL25475) and *F. graminearum*.
- The increase in the concentration of MREO inhibited respiratory metabolism for all *Fusarium* isolates.
- MREO was not phytotoxic to wheat seeds.

1. Introduction

Cereals have several nutritional properties and are part of the basic human diet, which is why they stand out among the main agricultural products of greater economic relevance (Fleurat-Lessard, 2017). Due to high productivity in recent decades (FAO, 2021), the storage stage is fundamental in the distribution and storage logistics of these products (Mesterházy et al., 2020; Prakash et al., 2022). However, during this period, the presence of fungi are one of the main causes of post-harvest grain loss (Anžlovar et al., 2017; Moghadam et al., 2016).

Several phytopathogens are associated with losses in storage, being responsible for causing quantitative and qualitative losses in grains. Quantitative losses are related to deterioration and reduction in the weight of the grain mass, while qualitative losses are associated with changes that cause depreciation in the economic value of the product (Prakash et al., 2022). However, the main problem regarding the occurrence of these fungi in stored grains is the contamination with mycotoxins (Fleurat-Lessard, 2017; Haque et al., 2020).

Mycotoxins are substances from secondary metabolism produced by some groups of fungi, through all the grain production chain because they cause acute and chronic poisoning in humans and animals (Chaudhari et al., 2021). Even small quantities can lead to the disposal of the batch, making trade unfeasible and causing great economic losses (Fleurat-Lessard, 2017; Haque et al., 2020; Perczak et al., 2020). Additionally, these substances have high physicochemical stability, remaining even after food processing steps (Chaudhari et al., 2021; Císarová et al., 2016; Jiang et al., 2023; Wan et al., 2018).

The genera *Aspergillus* and *Fusarium* have great phytosanitary importance, as they produce several types of highly carcinogenic mycotoxins in the main consumed cereals (Krzysko-Łupicka et al., 2019; Krzyśko-Łupicka et al., 2020; Nada et al., 2022).

The main mycotoxins produced are aflatoxin (AF) by *Aspergillus flavus* and *Aspergillus nomius*, ochratoxin A (OTA) by *Aspergillus niger*, deoxynivalenol (DON), and zearalenone (ZEN) by *Fusarium culmorum* and *Fusarium graminearum* (Chelaghema et al., 2022; Perczak et al., 2020).

Preventive control with the use of fungicides is the main strategy for controlling these phytopathogens to avoid contamination with mycotoxins in the field and especially during the storage period (Chaudhari et al., 2021; Císarová et al., 2016; Nada et al., 2022; Perczak et al., 2020). However, the recurrent and intensive use of these substances has caused impacts on human and animal health, on the environment and mainly on the selection of resistant strains (Anžlovar et al., 2017; Chelaghema et al., 2022; Caterina Morcia et al., 2017).

In the search for more sustainable agricultural production models, the use of products derived from bioactive plants is a promising alternative (Anžlovar et al., 2017; Jiang et al., 2023). Researches have shown that essential oils (EOs) are among the substances derived from secondary metabolism of plants that a the great potential as a biopesticide for the control of phytopathogens in stored products (Chaudhari et al., 2021; Zimmermann et al., 2022, 2023). They can act as seed treatment for many cereal crops (Perczak et al., 2019). These products have several advantages, as they are biodegradable with a low residue in food, have low toxicity to mammals (Anžlovar et al., 2017; Chaudhari et al., 2021). Because they are made up of different chemical molecules, they have different mechanisms of action with a fungicidal effect (da Rocha Neto et al., 2019; Perczak et al., 2019; Tang et al., 2018), in addition to demonstrating an effect on the inhibition of mycotoxin synthesis (Jiang et al., 2023; Neme & Mohammed, 2017).

Melaleuca spp. (Myrtaceae) is widely recognized for having secondary metabolites with high antifungal effect in the control of storage fungi (da Rocha Neto et

al., 2019; Moumni, Allagui, et al., 2021; Moumni, Romanazzi, et al., 2021; Pujiarti et al., 2017; Zimmermann et al., 2023), indicating the fungicidal potential of this genus.

Among the species in this genus, *Melaleuca rhaphiophylla* EO (MREO) presents antifungal activity against *Aspergillus flavus*, *Aspergillus niger*, *Aspergillius nomius* and *Fusarium graminearum* isolated from wheat grains by the contact method with inhibition of mycelial growth greater than 90% (Zimmermann et al., 2022). However, to evaluate its potential use as a botanical fungicide for agricultural purposes, more studies are necessary to investigate its effect on other variables related to fungicidal activity, in addition to its influence on the germination of agricultural crop seeds. Therefore, the objective of this research was to evaluate the effect of MREO by contact method on mycelial growth, their minimum inhibitory concentration and medium inhibitory concentration, evaluate fungistatic or fungicidal activity, their effect on conidia germination, sporulation, respiratory metabolism, and on germination of wheat seeds.

2. Material and Methods

2.1. Fungal strains

The mycotoxigenic strains of *A. flavus*, *A. niger* and *A. nomius* were obtained from the Microbiological Collections of Paraná Network (Taxonline), *F. culmorum* (strains NRRL3288 and NRRL25475) and *F. graminearum* (NRRL66031) were obtained from Agricultural Research Service (ARS) Culture Collection. The culture media Potato Dextrose Agar (PDA – SIGMA) and Synthetic Nutrient Agar (SNA) (KH_2PO_4 1g, KNO_3 1g, $MgSO_4$ 0.5g, KCL 0.5g, Glucose 0.2g, Sacarose 0.2g, and Agar 20g with distilled water 1L) were used for the cultivation of *Aspergillus* and *Fusarium* isolates, respectively, then fungal plates were kept in an incubator chamber at 28 ± 1º C, 12 h of photoperiod for 7 days before use (Zimmermann et al., 2022).

2.2. Melaleuca rhaphiophylla essential oil

A collection of plant material was carried out in November 2019, in Araucária (25◦31′00.81″ S and 49◦26′18.24″ W), Paraná, Brazil. A sample of this material was deposited at the Curitiba Botanical Museum (deposit number 289706). The EO was extracted from the green aerial part, that is, branches with leaves by a steam distillation for 2 h. Water was used as an extraction solvent. The EO was transferred to vials and refrigerated until its analysis. The EO was diluted to 1% (w/v) using ultrapure hexane, and the chemical composition was reported by Zimmermann et al. (2022).

2.3. Effect of MREO on micellal growth

To evaluate the inhibition of micellal growth, the experiment was conducted in a completely randomized design in a factorial scheme (4 treatments x 6 fungi), with 3 replications. The treatments were: 1) MREO at a concentration of 1.5%, 2) negative control using only RPMI 1640 culture medium (INLAB), 3) positive control Thiophanate methyl 700 mg L^{-1} (Cercobin® 700 WP), and 4) positive control Tebuconazole 0.75 mL L^{-1} (Folicur® 200 EC). The fungi were: 1) *A. flavus*, 2) *A. niger*, 3) *A. nomius*, 4) *F. culmorum* (NRRL3288), 5) *F. culmorum* (NRRL25475) and 6) *F. graminearum* (NRRL66031). The repetition consisted of one well for each treatment, in microtiter plates with 96 flat bottom wells, one plate per treatment. The experiment was repeated 3 times in time.

A 7-day inoculum were scraped off and diluted in RPMI 1640 culture medium to a concentration of $4x10^4$ conidia.mL^{-1}. The MREO was diluted in the RPMI 1640 culture medium to a concentration of 3%. Subsequently, 100 µL of the fungus and each treatment were pipetted into 96-well microtiter plates with a flat bottom, obtaining final concentrations of $2x10^4$ conidia.mL^{-1} for the isolates and 1.5% for the treatments. Subsequently, the plates were kept in an incubator chamber at 28 ± 1º C and 12 h of

photophase, for 48 h. The evaluation of fungal growth was determined by the absorbance method at 600 nm using the EPOCH microplate spectrophotometer (Biotek Instrument Inc, Winooski, USA) (Zimmermann et al., 2022).

2.4. Determination of minimum inhibitory concentration (MIC) and medium inhibitory concentration (IC_{50})

The minimum inhibitory concentration (MIC) and the medium inhibitory concentration were evaluated as described in topic 2.3, in a completely randomized design with 3 replications. The treatments were: 1) MREO evaluated at 6 concentrations (0.02, 0.04, 0.1, 0.2, 0.4 and 1%% (v:v), in serial dilutions) and 2) negative control with only the RPMI 1640 culture medium. The experiment was repeated 3 times in time. The MIC was determined as the lowest concentration at which there was no change in medium color and IC_{50} was the concentration that will initiate mycelial growth by 50% for each isolate (Zimmermann et al., 2023).

2.5. Effect fungistatic or fungicidal activity of MREO

The fungistatic or fungicidal effect of MREO was evaluated in a completely randomized design with 3 replications. The treatments were: 1) MREO at a concentration of 1, 1.5 and 2% and 2) negative control with only the RPMI 1640 culture medium. The fungi were: 1) *A. flavus*, 2) *A. niger*, 3) *A. nomius*, 4) *F. culmorum* (NRRL3288), 5) *F. culmorum* (NRRL25475) and 6) *F. graminearum* (NRRL66031). The experiment was repeated 3 times in time.

The methodology used was adapted from Krzyśko-Łupicka et al. (2020). A 7-day inoculum were scraped off and diluted in RPMI 1640 culture medium to a concentration of $4x10^4$ conidia.mL^{-1}. The MREO was diluted in the RPMI 1640 culture medium to an initial concentration of 2, 3 and 4%. Subsequently, 100 µL of the fungus and each treatment were pipetted into 96-well microtiter plates with a flat bottom, obtaining final

concentrations of $2x10^4$ conidia.mL^{-1} for the isolates and 1, 1.5 and 2% for the treatment. Subsequently, the plates were kept in an incubator chamber at 28 ± 1º C and 12 h of photophase, for 48 h. After this period, 100 µL aliquots were pipetted and spread using a sterile Drigalski loop in Petri dishes (9 cm in diameter), containing the respective culture media for each fungus. Evaluation of the fungal growth occurred every 48 h, for a period of 6 days, where the absence (fungicide) or presence (fungistatic) of mycelial growth was observed. Each replicate consisted of a petri dish.

2.6. Effect of MREO on conidia germination and sporulation

The the effect of MREO on conidia germination and sporulation was evaluated in a completely randomized design in a factorial scheme (2 treatments x 6 fungi) with 3 repetitions for each experiment. Both experiments were conducted individually. The treatments were: 1) MREO at a concentration of 0.25%, which corresponds to the value of ¼ of MIC (Riccioni & Orzali, 2011; Romoli et al., 2022), and 2) negative control with Distilled water sterilized only for germination, and RPMI 1640 culture medium for sporulation. The fungi were: 1) *A. flavus*, 2) *A. niger*, 3) *A. nomius*, 4) *F. culmorum* (NRRL3288), 5) *F. culmorum* (NRRL25475) and 6) *F. graminearum* (NRRL66031). For each experiment, each repetition was considered as 2-mL centrifuge microtubes. The experiment was repeated 3 times over time.

For conidia germination, the methodology used were proposed by Jiang et al. (2023) and Zimmermann et al. (2023) for sporulation, with modifications. A 7-day inocula were scraped with a sterilized loop and diluted in 0.85% NaCl solution containing 0.01% Tween 80® to an initial concentration of $4x10^5$ conidia.mL^{-1}. The MREO treatment was diluted in 0.85% NaCl solution containing 0.01% Tween 80® (for germination), and RPMI 1640 medium (for sporulation), to an initial concentration of 0.5%. Then, the fungi and the MREO were mixed into 2-mL centrifuge microtubes until a final concentration

of $2x10^5$ conidia.mL^{-1} for the isolates and 0.25% for the treatment. To evaluate conidia germination, after 20 h, a total of 200 conidia (for each repetition) were counted using Neubauer chamber, and classified as germinated (from the emission of the germ tube) and ungerminated conidia (absence of germ tube). To evaluate sporulation, after 48 h of incubation, the conidia were counted using a Neubauer chamber.

2.7. Effect of MREO on respiratory metabolism

The effect of MREO on respiratory metabolism was evaluated by the microdillution method, in a completely randomized design in a factorial scheme (2 treatments x 6 fungi) with 3 replications for each experiment. The treatments were: 1) MREO at concentration 0.1 and 0.2% (Riccioni & Orzali, 2011; Romoli et al., 2022), and 2) negative control with RPMI 1640 culture medium only. The fungi were: 1) *A. flavus*, 2) *A. niger*, 3) *A. nomius*, 4) *F. cumulum* (NRRL3288), 5) *F. cumulum* (NRRL25475) and 6) *F. graminearum*. The repetition consisted of one well for each treatment, in microtiter plates with 96 flat bottom wells, one plate per treatment. The experiment was repeated 3 times over time for each experiment.

The methodology was proposed by Vega et al. (2012), with modifications. A 7-day inocula were scraped and diluted in RPMI 1640 culture medium to a concentration of $5x10^4$ conidia.mL^{-1}. The MREO was diluted in the RPMI 1640 culture medium to a concentration of 0.2 and 0.4%. Subsequently, 100 µL of treatment, 80 µL of fungi and 20 µL of resazurin were pipetted into 96-well microtiter plates with a flat bottom, obtaining final concentrations of $2x10^4$ conidia.mL^{-1} for isolates and 0.1 and 0.2%% for MREO (Riccioni & Orzali, 2011). Subsequently, the plates were kept in an incubator chamber at 28 ± 1º C and 12 h of photophase, for 48 h. The evaluation of respiratory activity was determined by difference in absorbance method between 570 and 600 nm using the EPOCH microplate spectrophotometer (Biotek Instrument Inc, Winooski, USA).

2.8. Effect of MREO germination of wheat seeds

To evaluate the effect of MREO on the germination of wheat seeds (cv. *Tbio Toruk*, 2022 harvest), a completely randomized experiment was conducted, with two treatments and five replicates. The treatments were: 1) MREO at 100%, and 2) distilled water sterilized as negative control. Each replication consisted of a filter paper (Germitest paper 28 x 9 x 38 cm, A3034-8, Germilab, Brazil) containing 20 seeds.

For this, 100 µL of each treatment was pipetted into Petri dishes (9 cm in diameter) containing a filter paper disc (Whatman No. 1), and then 25 g of wheat seeds were placed in each dish. Subsequently, the plates were sealed with polyethylene film and kept in an incubator chamber at 28 ± 1° C and a 12-hour photophase for 24 h. After this period, the 20 seeds/replication were placed on filter paper (Germitest paper 28 x 9 x 38 cm, A3034-8, Germilab, Brazil) moistened with 60 mL of distilled water. After five days, seed germination was evaluated (Remesh et al., 2022). The experiment was repeated four times in time.

2.9. Data and statistical analysis

Inhibition growth or conidia germination inhibition data for all experiments for fungicidal activity were calculated compared to the negative control (Jiang et al., 2023; Vilela et al., 2009):

$$Inhibition\ (\%) = \frac{(C - T)}{C} \times 100$$

Where:

C = Absorbance or Number of germinated conidia of the negative control

T = Absorbance or Number of germinated conidia of each treatment

All inhibition data were analyzed for fungal species and EO using analysis of variance in a generalized linear model, assuming Poisson distribution. The means were

compared using Tukey test at 5% probability. Since the mean square of the residue showed a ratio of less than 7:1 (Banzatto and Kronka, 2013) between the repetitions of the experiments, a joint analysis was carried out with the repetitions of the experiments. The IC_{50} data were submitted to the Probit analysis method using the "ecotox" package (Zimmermann et al., 2023). All analyses were conducted in R (R Core Team 2023).

3. Results

3.1. Fungicidal activity of MREO

There was an effect only in treatments ($P < 0.001$). There was no difference in the inhibition rate of MREO and the fungicide Thiophanate-methyl, with inhibition greater than 95%, being higher than the inhibition rate of the fungicide tebuconazole.

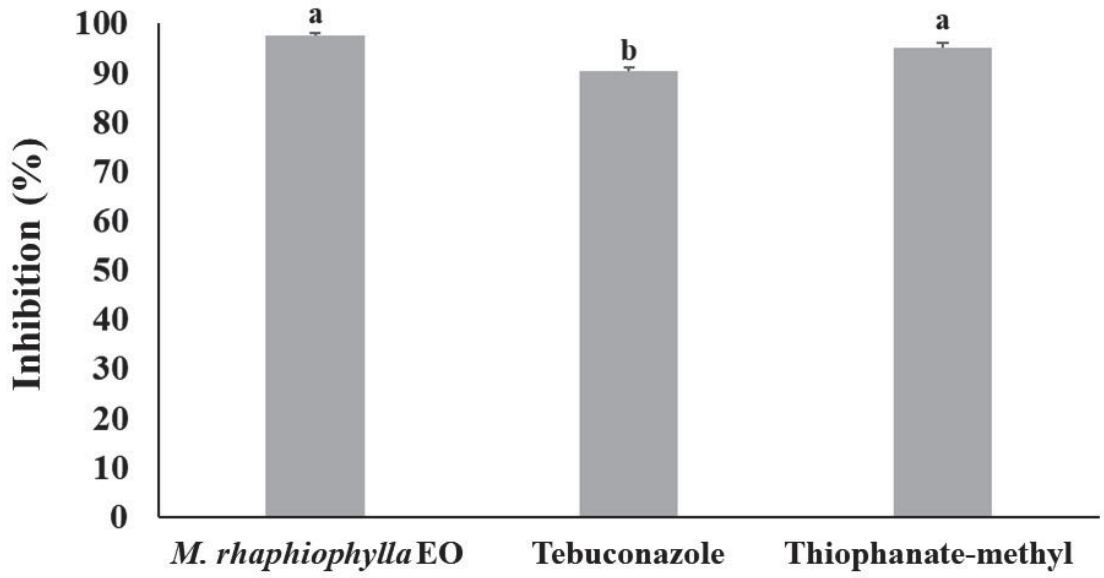

Fig. 1. Inhibition (mean ± standard deviation) of isolates of *Aspergillus* sp. and *Fusarium* sp. treated with *Melaleuca rhaphiophylla* essential oil, tebuconazole and thiophanate-methyl after 48 h of exposure at 28 ± 1 °C and 12-h photophase. The same letters do not differ significantly from each other using Tukey test ($P < 0.001$).

3.2. Determination of minimum inhibitory concentration (MIC) and medium inhibitory concentration (IC_{50})

The MIC for all isolates was 1%. However, there was variation between isolates in IC_{50} values calculated, with *A. niger* and *F. culmorum* (NRRL3288) being more and less sensitive to MREO, respectively (Table 1).

Table 1. Medium inhibitory concentration (IC_{50}) (%) of *Melaleuca rhaphiophylla* essential oil against *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nomius*, *Fusarium culmorum* (NRRL25475), *Fusarium culmorum* (NRRL3288), and *Fusarium graminearum* (NRRL66031) incubated at 28 ± 1 °C and 12-h photophase for 48 h.

Fungus	IC_{50} (%)
A. flavus	0.11
A. niger	0.07
A. nomius	0.18
F. culmorum (NRRL25475)	0.21
F. culmorum (NRRL3288)	0.33
F. graminearum (NRRL66031)	0.27

3.3. Fungistatic or fungicidal activity of MREO

MREO caused a fungicidal effect at a concentration of 1% for isolates of *F. culmorum* (NRRL25475) and *F. graminearum* (NRRL66031), and 1.5% for *F. culmorum* (NRRL3288). For all *Aspergillus* species, MREO concentrations demonstrated only a fungistatic effect, and for the fungicidal effect the concentration is greater than 2%.

3.4. Effect of MREO on conidia germination and sporulation

There was a statistical difference between MREO and negative control in the germination rate of *Aspergillus* and *Fusarium* conidia ($P < 0.001$). The reduction in germination rate ranged from 5.12% for *A. niger* to 18.31% for *F. graminearum*. In the negative control with distilled water, conidial germination was greater than 99% for all isolates (Fig. 2).

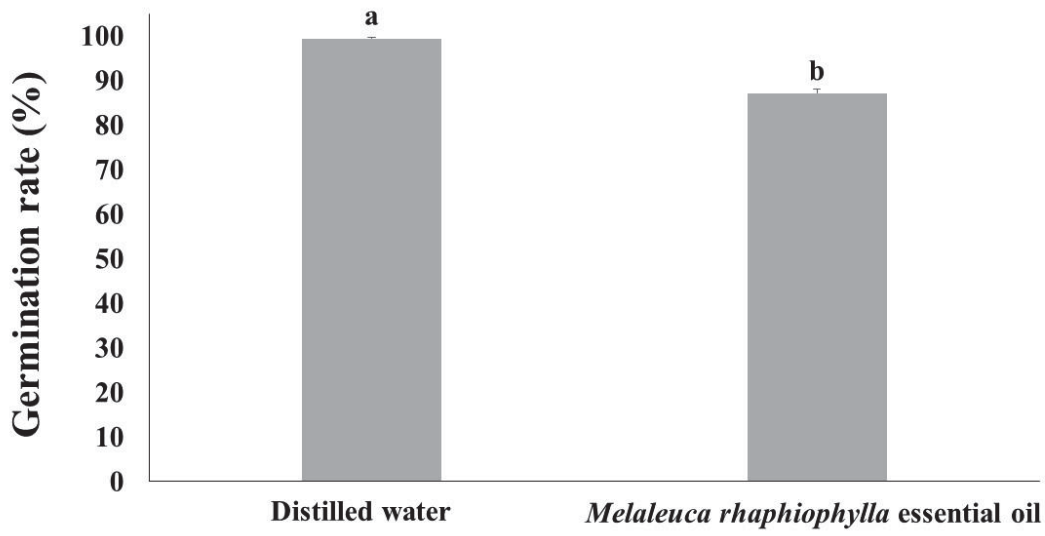

Fig. 2. Germination rate (mean ± standard deviation) of isolates of *Aspergillus* sp. and *Fusarium* sp. treated with *Melaleuca rhaphiophylla* essential oil, and distilled water after 20 h of exposure at 28 ± 1 °C and 12-h photophase. The treatment with asterisk differs significantly from each other using Tukey test ($P < 0.001$).

There was a statistical difference between treatments and fungi ($P < 0.001$). The MREO reduced the sporulation of *A. flavus*, *F. culmorum* (NRRL25475), and *F. graminearum* (NRRL66031), differing statistically from the negative control. Among all the fungi tested, *F. graminearum* (NRRL66031) showed lower sporulation rate when exposed to MREO, followed by the *F. culmorum* isolates. There was no statistical difference to *A. niger* and *A. nomius*, these species being the ones that presented the highest sporulations (Fig. 3).

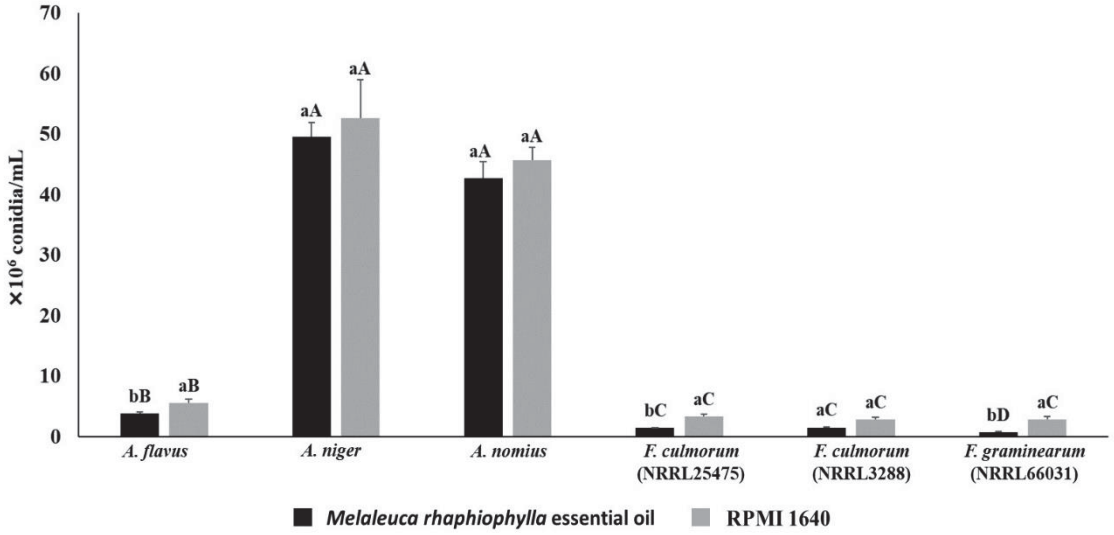

Fig. 3. Conidia/mL (mean ± standard deviation) of isolates of *Aspergillus* and *Fusarium* treated with *Melaleuca rhaphiophylla* essential oil, and RPMI 1640 medium after 48 h of exposure at 28 ± 1 °C and 12-h photophase. Capital letters differentiate the isolates for each treatment, and lowercase letters differentiate the treatments within each isolates using Tukey test ($P < 0.05$).

3.5. Effect of MREO on respiratory chain

There was a statistical difference between treatments and fungi ($P < 0.001$). Both concentrations of MREO inhibited fungi growth, but there was a statistical difference between concentrations only for isolates of *Fusarium* spp., being observed increase in the concentration of MREO reduced the respiratory metabolism. At a concentration of 0.2%, inhibitions greater than 72% were observed, except for the species *A. flavus* differed statistically from the other isolates, with growth inhibition of 36%. While at a concentration of 0.1%, isolates of *A. niger*, *A. nomius*, *F. culmorum* (NRRL25745), and *F. culmorum* (NRRL3288) had a higher inhibition rate, with values of 78.6, 80.9%, 65.9, and 62.7%, respectively (Fig. 4).

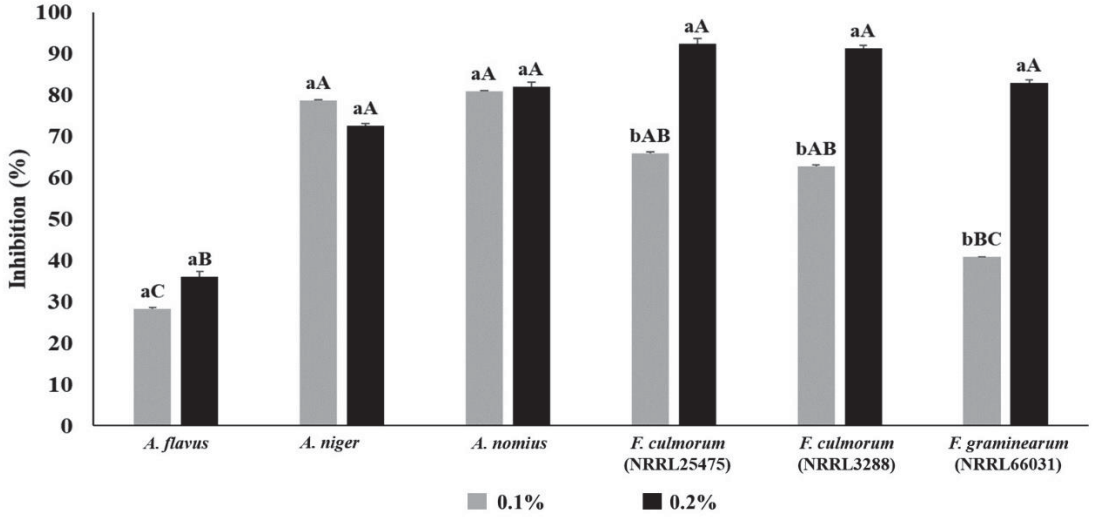

Fig. 4. Inhibition (mean ± standard deviation) of isolates of *Aspergillus* and *Fusarium* treated with *Melaleuca rhaphiophylla* essential oil at concentrations 0.1 and 0.2%, after 48 h of exposure at 28 ± 1 °C and 12-h photophase. Capital letters differentiate the isolates for each treatment, and lowercase letters differentiate the treatments within each isolates using Tukey test ($P < 0.05$).

3.6. Effect of MREO on germination of wheat seeds

The germination rate of wheat seeds exposed to MREO was 96%, statistically different of the negative control, which presented a rate of 98.3%.

4. Discussion

Among the different biological properties of EOs, the antifungal action against different phytopathogens (Arraiza et al., 2018; Chang et al., 2022; Redondo-Blanco et al., 2020) and phytotoxicity against weeds (Ibáñez & Blázquez, 2018; Werrie et al., 2020) present greater relevance for its use for agronomic purposes. Results presented by Zimmermann et al. (2022) demonstrated that MREO presented the following major compounds α-terpinene, 1,8-cineole, γ-terpinene, terpinolene, and terpinen-4-ol, and fungicidal action against storage fungi, with inhibition of mycelial growth above 90% for

the contact method. However, there are no studies on the effect of MREO on the parameters that influence the development and growth including new strains of storage fungi. In this context, this is the first report of the antifungal activity of MREO against *F. culmorum*, in addition its effect on the germination rate of conidia, inhibiting the sporulation capacity and respiratory metabolism of storage fungi, and its effect on wheat seed germination.

Our results demonstrate the effectiveness of MREO in inhibiting the growth of isolates of *Aspergillus* sp. and *Fusarium* sp. in concentrations lower than 1.5%, with inhibition similar to the main commercial fungicides used to control these pathogens. Similar results were described for the species *Melaleuca alternifolia* (Zimmermann et al., 2023), *Melaleuca cajuputi* (Chaudhari et al., 2022), *Melaleuca leucadendron* (Farag et al., 2004; Pujiarti et al., 2017), *Melaleuca ericifolia*, *Melaleuca armillaris* and *Melaleuca styphelioides* (Farag et al., 2004) against *Aspergillus* spp. and *Fusarium* spp., but with quantitatively and qualitatively variation in chemical composition of the EOs.

EOs are synthesized by different metabolic routes in plants, and therefore there is a wide diversity of chemical groups with different bioactivities (Arraiza et al., 2018). Therefore, the chemical composition of EOs can be affected by intrinsic and extrinsic factors of each plant, which can result in a qualitative and quantitative variation of the substances present in the sample (Chaudhari et al., 2022; Chelaghema et al., 2022; Yang et al., 2023).

The chemical composition of the EOs is the most relevant factor in determining the biological activity that these substances may present, including fungicidal action against phytopathogens (Sharma et al., 2017; Yang et al., 2023). In general, the fungicidal activity of EOs may be associated with the major compounds, as these molecules, being in greater proportion, may be responsible for the biological activity that these products

may present (Ni et al., 2021; Sawadogo et al., 2022; Yang et al., 2023). Among the main classes of chemical compounds, there is a predominance of mono and sesquiterpenes, which are responsible for the biological activity that EOs can present (Arraiza et al., 2018; Nazzaro et al., 2017). In the present sample of MREO, the major compounds (> 5%) were α-terpinene (6.46%), 1,8-cineole (11.54%), γ-terpinene (13.20%), terpinolene (28.72%), and terpinen-4-ol (19.82%) (Zimmermann et al., 2022). Studies demonstrated that these compounds have fungicidal action against phytopathogens (An et al., 2019; C Morcia et al., 2012; Pujiarti et al., 2017; Vilela et al., 2009; Yu et al., 2015; Zhang et al., 2022), with different mechanisms of action.

In addition to the chemical composition, is important to determine the antifungal potential these compounds. In the present study, results demonstrated that the increase in the concentration of MREO caused greater inhibition of fungal growth, as the increase in the concentration of the EOs implies a greater proportion of compounds with antifungal activity, which consequently results in greater inhibition of fungal growth (Jiang et al., 2023; Reyes-Jurado et al., 2015; Zimmermann et al., 2023). Our results also indicated that the EOs effects depends on the target organism (genus, species and isolate), and this difference may be related to genetic, biological, and biochemical factors, such as detoxification mechanisms of each species or isolate, and the interaction among the chemical compounds present in the EOs (Hu et al., 2021; Jiang et al., 2023; Ni et al., 2021).

The EOs can have fungistatic or fungicidal effects against storage phytopathogens (Redondo-Blanco et al., 2020). Our results demonstrate that the concentration and target organism affected the bioactivity of MREO, with a prevalence of fungistatic effect for *Aspergillus* sp., and fungicidal effect for *Fusarium* sp.. Similar results were described for different EOs for isolates of *A. flavus* (Anžlovar et al., 2017), *A. niger* (Kumar et al.,

2017), *Fusarium proliferatum*, *Fusarium verticillioides* (Kumar et al., 2016), *Fusarium avenaceum* (Chakroun et al., 2021), *F. graminearum* (Luchesi et al., 2022), *Fusarium oxysporum* and *Fusarium equiseti* (Chacón et al., 2021).

Usually, the molecules present in EOs act in synergism, and the minor compounds can act as agents that enhance the fungicidal activity of the major compounds (Kumar et al., 2016; Sawadogo et al., 2022). This interaction between the substances intensifies the antifungal activity of the EOs, having the potential to cause the death of the pathogen (Nazzaro et al., 2017; Ni et al., 2021; Redondo-Blanco et al., 2020), being more effective when compared to the effect of the major compounds alone (Brito et al., 2021).

The fungistatic effect may be associated with the sensitivity of the isolate to the compounds present in the EO, having the direct effect of reducing or stopping fungal growth after the interaction of the fungus with the EO, while there is direct contact with EO. While the fungicidal effect is related to the complete inhibition of the isolate's development, after exposure to the EO (Krzysko-Łupicka et al., 2019; Krzyśko-Łupicka et al., 2020). However, both effects are dependent on the concentration and type of interaction between the substances present in the chemical composition of MREO (Redondo-Blanco et al., 2020).

Because they are made up of different chemical molecules, EOs can act on more than one target site simultaneously, accentuating their fungitoxicity, this characteristic being considered one of the main advantages of these products compared to synthetic fungicides (Chelaghema et al., 2022; Nazzaro et al., 2017). EOs can cause morphophysiological, biochemical and genetic changes in different fungal structures (An et al., 2019; Hu et al., 2021; Jiang et al., 2023; Ni et al., 2021; Yang et al., 2023). However, there is a lack of studies that seek to elucidate the mechanisms of action of these substances on fungi (Li et al., 2017).

Mycelium and spores are directly involved in the pathogen-host cycle, being responsible for the process of infection, colonization and dissemination of the pathogen in plant tissue (Arraiza et al., 2018), in addition to playing the role of a resistance structure against adverse situations in the environment (Boukaew et al., 2017; Jiang et al., 2023). Our results demonstrated that MREO reduced the conidial germination rate and sporulation of *Aspergillus* and *Fusarium* isolates.

Due to their lipophilic nature, the chemical compounds present in EOs can easily penetrate the lipid bilayer of the fungal membrane, which can cause deformation and damage to the cell wall and cell membrane of fungi (Ni et al., 2021; Sharma et al., 2017). In this circumstance, the inhibition of the synthesis of ergosterol or chitin, and changes in the permeability of the plasma membrane constitute the main changes, resulting in cellular lesions in the hyphae and conidia, having as a direct consequence the inhibition of germination and sporulation (Aisyah et al., 2021; Jiang et al., 2023; Nazzaro et al., 2017; Ni et al., 2021).

According to Hou et al. (2022), EOs can also cause biometabolic changes in fungi, especially in mitochondrial activity, with an increase in reactive oxygen species (ROS) within cells (Arraiza et al., 2018; Li et al., 2017). Our results suggest that the antifungal activity of MREO may have been caused by the reduction in the respiratory activity of *Aspergillus* and *Fusarium* isolates, as with increased concentration these substances may have caused morphological changes and respiratory rates, preventing germination of conidia or mycelial growth.

Damage to mitochondria is related to the inhibition of mitochondrial dehydrogenases, with the direct consequence of inhibiting ATP synthesis and preventing the generation of energy for the cell (Li et al., 2017; Nazzaro et al., 2017; Sawadogo et al., 2022). The effect of EOs on the osmotic permeability of the cell causes an imbalance

in cellular content, and in association with changes in the respiratory chain, results in greater production and accumulation of ROS inside the cell (Hu et al., 2021; Ni et al., 2021). When the increase in ROS exceeds the antioxidative capacity of the cell, the consequence is oxidative stress with the possibility of lipid peroxidation occurring (Kumar et al., 2016; Sawadogo et al., 2022; Yang et al., 2023), which involves damage to DNA, RNA, lipids and proteins (Hou et al., 2022).

In addition to fungicidal activity, EOs can also have a phytotoxic effect, having herbicide functionality by inhibiting the germination of seeds of weeds or crops of agricultural interest (Ibáñez & Blázquez, 2018; Raveau et al., 2020). Our results indicate that MREO were not phytotoxic to wheat seeds. Nonetheless, Agnieszka et al. (2016) emphasizes that there are differences in the phytotoxic action of EOs between mono and dicotyledonous seeds, in addition, factors such as chemical composition and tested concentration can also influence the toxicity of these substances in seeds (Al-Rowaily et al., 2020; Hazrati et al., 2017). As with fungi, the main effect of the herbicidal activity of EOs is to increase the production of ROS within cells, resulting in changes in the respiratory rate and the generation of energy necessary for the seeds to begin their germination process (Raveau et al., 2020; HP Singh et al., 2009).

5. Conclusion

MREO has fungistatic and fungicidal activity against isolates of *Aspergillus* and *Fusarium*, reducing the germination rate of conidia and the sporulation capacity of these fungi. The fungicidal activity is related to the increase in concentration, causing changes in the mitochondrial respiratory chain resulting in cell death. As there are no studies on the effect of phytotoxicity of MREO in seeds, the present results indicate its potential for use as an alternative treatment for wheat seeds.

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FINAL CONSIDERATIONS

The use of essential oils (EOs) proves to be a promising strategy in the integrated management of storage pests and fungi, as an alternative to synthetic molecules. As they are natural substances, they have lower impacts on the environment and human health, and in addition to the possibility of use aimed at the organic production of grains and seeds market.

Due to the variability in chemical composition, essential oils have different mechanisms of action against insects and fungi, minimizing the risk of selecting resistant breeds and strains. *Melaleuca rhaphiophylla* EO extracted in Brazil showed a similar chemical composition to that obtained in Australia, and to other species of the genus that are widely commercialized, indicating the possibility of this species being used for commercial cultivation purposes for the essential oil industry.

Melaleuca rhaphiophylla EO demonstrates high toxicity in controlling pests and storage fungi, demonstrating potential for use as a commercial product. However, more studies are needed to standardize the chemical composition, evaluate its compatibility with other biopolymers, its effect on seeds of other crops of agricultural interest, and develop technologies for large-scale application.

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