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Effect of Essential Oils in the Control of Fungal Diseases in Small Grains

Esneider Mahecha Bojaca
South Dakota State University, esneider.mahechabojaca@jacks.sdstate.edu

Effect of Essential Oils in the Control of Fungal Diseases in Small Grains

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Esneider Mahecha Bojaca

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Shaukat Ali
Advisor Date

David Wright
Department Head Date

Nicole Lounsbery, PhD
Director, Graduate School Date

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ABBREVIATIONS

% v/v = Percent Volume/Volume

%DI= Percentage of disease

^{0}C= Centigrade grades

ANOVA=Analysis of variance

BPS= *Bipolaris sorokiniana*

Bt= *Bacillus thuringiensis*

bu= bushels

Cm= Centimeters

Dat= Days after treatment

DMI=Demethylation inhibitors

DON= Deoxynivalenol

DSI = Disease severity index

EOs=Essential oils

EPA=Environmental protection agency

FDK= Fusarium-damaged kernels

FHB= Fusarium head blight

FL oz= Fluid ounce

FRAC= Fungicide resistance action committee

GC-MS= Gas chromatography with mass spectrometry

Ha= Hectares

IPM= Integrated pest management

Kg= Kilogram

Kg/ha= Kilograms per hectare

LSD= Least significant difference

m= meters

MFC= minimum fungicide concentration

MGI %= Mycelial growth inhibition percent

MIC= minimum inhibitory concentration

ml= milliliter

ml/acre= milliliters per acre

mm = millimeters

NDVI= Normalized difference vegetation index

OMRI= Organic Materials Review Institute

PDA= Potato dextrose agar

Ppm= Parts per million

PSN= *Parastagonospora nodorum*

PTR= *Pyrenophora tritici-repentis*

PIPs= Plant-Incorporated Protectants

QoI= Quinone outside inhibitor

SD= Standard deviation

SDHI= Succinate dehydrogenase inhibitors

TW= test weight

U.S.= United states

ZEA= Zearalenone

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ABSTRACT

EFFECT OF ESSENTIAL OILS ON THE CONTROL OF FUNGAL DISEASES IN SMALL GRAINS.

ESNEIDER MAHECHA BOJACA

2022

Spring wheat (*Triticum aestivum* L.*)* and oats (*Avena sativa* L.*)* are important crops due to their high consumption in our daily diet worldwide. They are the main food of consumption per capita due to the high amino-acid content as well of vitamins. However, grain production is limited due to the negative impact of the diseases that cause significant yield loss. Diseases in oats, such as crown rust caused by *Puccinia coronata f. sp. avenae (Pca)*, and in wheat such as Fusarium head blight (FHB) caused by the fungus *Fusarium graminearum.* Foliar diseases such as tan spot incited by the fungi (*Pyrenophora tritici-repentis*), Septoria nodorum blotch (*Parastagonospora nodorum*), and spot blotch (*Bipolaris Sorokiniana*) are known to compromise grain yield and seed quality.

The integrated pest management for these diseases is by using tolerant/resistant plant varieties, crop rotation, and mostly fungicide applications due to their high efficacy controlling fungal diseases. However, the overuse of synthetic fungicides has led to the development of pathogen resistance, and concerns in the environment and human health. Therefore, it is necessary to explore new products with lower toxicity and less negative impact on human health and the environment. Biopesticides such as essential oils (EOs) and pure bioactive compounds present in the EOs are substances with known antifungal

properties, multiple mechanisms of action, and low toxicity in humans and environment. However, little is known about their effect on the fungal pathogens that affect small grains. The objectives of this study were: **(1)** To determine the potential EOs in the control of oat crown rust *in vivo*, **(2)** To determine the potential effect of EOs and bioactive compounds in the control of three important pathogens that cause foliar diseases in wheat *in vitro*, and **(3)** To determine *in vitro* and *in vivo* the potential as fungicide of two EOs, two commercial products with EOs, and two bioactive compounds presents in EOs, in the control of the fungal pathogen *Fusarium graminearum*.

For the first objective, a two-year field study experiment, was conducted testing two EOs, neem and karanja, at two concentrations (52 and 72 %) and three rates (3.7, 7.5, 11.25 L/ha). The synthetic commercial fungicide Headline (Pyraclostrobin) and a non-treated (water) were used as checks. Applications of the above treatments were made at the flag leaf stage (Zadok's- 37) in two oats cultivars, Horsepower and Goliath. Percentage of disease incidence (%DI), stem lodging percentage, normalized difference vegetation index (NDVI), test weight (TW), and yield were assessed for each year. In both years, the two essential oils showed a higher yield and a reduced %DI at the highest concentration and rate compared with the untreated check. The synthetic fungicide, Headline, showed a significantly higher yield ($P < 0.05$) and lowest disease and lodging. This study showed the potential efficacy of using the EOs neem and karanja controlling crown rust.

For the second objective, four essential oils were assessed: tea tree, lemongrass, neem and karanja oil and three compounds present in the EOs: carvacrol, thymol, and

linalool. Mycelial growth inhibition, spore germination, and volatile activity were evaluated for *Pyrenophora tritici-repentis* (PTR), *Parastagonospora nodorum* (PSN), and *Bipolaris sorokiniana* (BPS). One hundred percent of mycelial growth inhibition occurred in PTR, PSN, and BPS respectively, when using these concentrations; lemongrass (500, 1000 ppm), carvacrol and thymol (200, 500 ppm). Spore germination inhibition was observed with the concentration of 1000 ppm for lemongrass and 500 ppm for carvacrol and thymol. Lemongrass, carvacrol, and thymol generally showed fungicidal and fungistatic properties for the pathogens PTR, BPS, and PSN. This *in vitro* study showed the potential use of lemongrass and two bioactive compounds, thymol and carvacrol, to control three pathogens that cause leaf spotting diseases in wheat.

The third objective was to evaluate the efficacy of two pure EOs (tea tree and lemongrass), two commercially available EOs with proven antifungal properties (Thymox® and Timorex®), two bioactive compounds present in EOs (carvacrol and thymol), a synthetic fungicide Prosaro® (Prothioconazole + Tebuconazole), and untreated water control in the management of FHB.

The study was conducted *in vitro* using inhibitory disk diffusion method to detect the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). Additionally, conidia germination inhibition was evaluated, and volatile activity was determined. *In vivo,* the EOs and their constituents were tested under greenhouse and field conditions. FHB disease index, yield, FDK, and DON were recorded. There was 100% mycelial growth inhibition (MIC) in *F. graminearum* with lemongrass at 1000 ppm, Thymox® at 500 ppm, carvacrol and thymol at 200 and 500 ppm, respectively. One

hundred percent spore germination inhibition was observed at the rate of 1000 ppm for lemongrass, tea tree, and Thymox®, and 500 ppm in carvacrol and thymol. The MFC was tested in the concentrations that showed the MICs for each treatment. In this study, all the concentrations of the EOs showed an MFC equal to the MIC except carvacrol, which showed the MFC at 500 ppm, where fungal regrowth was observed. Volatility, fungistatic activity of EOs was observed in lemongrass, Thymox®, and Thymol in a range of 6.25 to 38.44% of mycelial growth inhibition compared with the untreated.

In greenhouse and field studies, the spring wheat, FHB susceptible cultivar "Select" was inoculated at anthesis by spraying *F. graminearum* spore suspension and then treated with the EOs. Spikes were evaluated 21 days after inoculation for FHB disease index. The plants treated with lemongrass oil, carvacrol, thymol, and Thymox® showed significantly lower FHB-disease index ($P<0.05$) relative to the untreated control. In the field trial, an increase in the kilograms per hectare for 10.4 and 13.2% compared with the untreated control was observed. DON concentration decreased between 36 and 59% for all treatments with EOs relative to the untreated control

In this study, *in vitro*, we observed the fungicidal and fungistatic potential of the EOs lemongrass, carvacrol, and thymol and the commercial EOs Thymox® in the control of *F. graminearum*. Under greenhouse conditions, the EOs decreased 38% the disease in the spikes compared with the untreated control. Under field conditions, the EOs mixed as emulsions and sprayed once, showed an increase in yield, and reduction in the FHB index and DON accumulation compared to the untreated control. In general, the fungicide Prosaro was the most effective in the control of the disease.

Overall, this study provides information about the use of EOs and bioactive compounds as new alternative biopesticides in managing important diseases in small grains.

Keywords: Biopesticides, essential oils, thymol, carvacrol, lemongrass oil, fungicide.

CHAPTER I : Literature Review

Effect of essential oils on the control of fungal diseases in small grains

1.1 Introduction

The small grains are an important source of amino acids, vitamins, and minerals, since the first civilizations., and have served as a basis of livelihood and food. Cereals are highly important due to their nutrient value and high consumption worldwide, where more than 50% of the world populations daily caloric intake is derived directly from cereal grain consumption (Awika, 2011).

Wheat is one of the main crops that contribute about 20% of the total dietary calories and proteins worldwide (Shiferaw et al., 2013), It is the most widely cultivated cereal in the world, with more than 220 million hectares planted annually under wide ranges of climatic conditions and numerous geographic regions. Wheat present a total average production of 670 million tons annually (Shiferaw et al., 2013), and ranks as the third largest crop planted behind corn and soybean in the USA. The average wheat production in the USA totaled 1.6 billion bushels in 2021/2022 from a harvested area of 37.2 million acres (USDA, 2021), with an estimated yield of 47.3 bushels per acre (USDA, 2021). Wheat is planted in South Dakota mostly as a spring crop and typically planted in late March and April. Production is severely reduced if planting continues into May, with production generally declining up to one bushel per acre per day if planting takes place after May ten (Adviento., 2020). Usually, the crop is alternated with soybean

and corn in a three-year rotation or following fallow or a non-small grain in other production systems (Ruden et al., 1999).

Oats (*Avena sativa*), sometimes called the common oat, are suitable for human consumption as oatmeal and rolled oats, but the most common use is for livestock feed. (Chartsbin., 2017). Oat is the sixth largest cereal crop based on worldwide production (FAO, 2018), with Russia, Canada, the European Union, Australia, and the USA as the major producers. Oat ranks fourth among the cereals after wheat, rice, and corn based on human consumption (Nazareno et al., 2018). Oat production in the USA was estimated at 39.8 million bushels with an estimated yield of 61.3 bushels per acre with a harvested area of 560,000 acres (USDA, 2021).

1.2 Diseases in Small Grains

Projections made by the food and agriculture organization (FAO) indicate that by 2050, agriculture, in general, will need to produce 50% more food to meet the nutritional needs of a constantly growing population; however, increasing production also increases the pressure of plant diseases (FAO., 2018).

A study by Savary et al., (2019) assessed the percentage of yield losses in different agricultural centers worldwide, where the diseases in wheat were estimated in 21.5% yield losses. including the fungal diseases Fusarium head blight, Stagonospora nodorum blotch, tan spot, and spot blotch among the ten most important diseases reported. A similar estimate was shown by Murphy (2017) showing the Fungal diseases FHB as one of the most important economic diseases in wheat production. For the oats

production, crown rust is the most important disease that affects the crop (Nazareno et al., 2018).

1.3 Pathogens and Diseases

Crown Rust

Crown rust, caused by *Puccinia coronata f.* sp. *Avenae (Pca)*, is the most important disease in oats affecting crops from the Dakotas to New York (Smith., 2021). The pathogen is spread from leaf to leaf when pustules release spores into the air. When the disease affects the flag leaf, this results in the reduction of the photosynthetic capacity of the plant, interfering with the translocation of photo-assimilates to fill the grain. Affecting negatively the kernel weight and the forage quality.

The major epidemic caused by crown rust in the U.S. occurred in 2014, resulting in a loss of 13 million bushels; roughly 18.7% of the oat production, where South Dakota and Minnesota, reported yield losses of 35% and 50%, respectively (Nazareno et al., 2018). Recently in South Dakota, losses were reported around 3% or equivalent to 333.4 bu/acre in 2020 (Fajolu., 2020).

Puccinia coronata (*Pca*) is heteroecious and a basidiomycete pathogen that produces five spore types: basidiospores, pycniospores (spermatia), aeciospores, urediniospores (uredospores), and teliospores. The primary inoculum of crown rust in North America comes from the alternate host buckthorn (*Rhamnus spp*). Teliospores in the debris from the previous summer germinate in the spring, producing basidiospores that infect young leaves of *Rhamnus*. These infections produce aecia, which releases

aeciospores that can infect oat plants. The fungus grows as dikaryotic, where infection on the leaves produces uredinium filled with brick-red, spiny urediniospores (Nazareno et al., 2018). Another source of inoculum is the uredinio spores that come from infected oats crops from the southern states that are blown by the wind.

This pathogen is an r-strategist, which means that its strategy is to produce a high number of spores to survive due to its biotrophic behavior. Just one uredinium can produce at least 100,000 urediniospores (Schumann and Leonard., 2000). The symptoms include small, oval pustules that contain masses of orange-yellow spores, after two or three weeks, the postulates turn black indicating the appearance of teliospores. The favorable environmental conditions for the development of the disease are warm temperatures of 20-25^0 C and humidity conditions.

Tan Spot

Tan spot, also known as yellow spot, is caused by the pathogen *Pyrenophora tritici-repentis* (PTR) (Syn: *Drechslera tritici-repentis*; (Died.) Drechsler 1923). PTR is a stubble-borne necrotrophic fungal pathogen, in epidemic situation, tan spot disease can cause up to 70% of yield reduction (Shabeer, 1988; Rees et al., 1982). In South Dakota, 5% grain yield loss has been attributed to tan spot, but individual field grain yield loss can be higher, up to 30% (Buchneau et al. 1983). Tan spot is one of the most important foliar diseases in South Dakota and North Dakota (Liu et al., 2015). PTR infects the leaves, affecting photosynthetic areas causing reduction in grain quality and quantity hence lower test weight, kernel shriveling, and reduced kernel numbers per head (De Wolf et al., 1998; Geeta and Mishra, 2018; Shabeer, 1988; Singh et al., 2011). *P. tritici-*

repentis survives on debris, and under moist conditions the pseudothecia produce ascospores, which initiate the disease on wheat leaves during rain periods. After the establishment of tan spot in the field the plants develop necrotic symptoms. The primary infection gives rise in a conidiophore with spores called conidia that are disseminated by the wind to other foliage wheat fields. The symptom of tan spot initially appears as small, brown spots on the leaves. Spots enlarge and develop into tan necrotic spots with a yellow halo where a pinhead size black spot may be present at the infection site (Liu et al., 2015).

The expanding halo will give the tan, oval spots a diamond-shaped appearance, and lesions can coalesce to form large areas of blotchy, tan, necrotic regions. Favorable weather conditions for the infection are temperatures above 10^0C and high relative humidity (about 78-100%). Spores produce the germ tube on the leaf which later produces the appressorium from which the penetration peg develops that enter in the through epidermal cells or via stomates (Hosford, et al. 1987). In PTR there are pathogenic variations due to the production of a range of host-specific toxins (HST), including ToxA, ToxB, and ToxC, that induce symptoms such as tan necrosis or extensive chlorosis on differential hosts (Lamari and Bernier, 1989; Orolaza et al., 1995), where each HST interacts with the corresponding host-sensitive locus Tsn1, Tsc2, and Tsc1, respectively (Dinglasan et al., 2016).

Septoria nodorum blotch

Parastagonospora nodorum (syn. *Septoria/stagonospora/Phaeosphaeria,* Berk. Castell. and Germano) is the pathogen causing Septoria nodorum blotch, glume blotch

(SNB), Infection with Parastagonospora nodorum cause similar level of losses in grain yield as PTR, ranging from 18 to 31% (Bhathal et al., 2003). In the United States, Septoria nodorum blotch is a frequent disease that can occur solely or co-occur with other foliar diseases. In the Dakotas, Stagonosopora nodorum blotch co-occurs with Septoria tritici blotch (STB) and tan spot. However, in the Pacific Northwest and areas near the Great Lakes, it may co-occurs with STB, and in the southeastern United States, from Georgia to Delaware, SNB occurs solely (Cowger et al., 2020).

Parastagonospora nodorum survives on infested stubble and produces ascospores from pseudothecia under moist conditions (Laubscher et all.,1966), and air currents spread these ascospores to other plants (Bathgate and Loughman., 2001). On the plant, the fungus produces asexual spores called pycnidiospores within brown pycnidia in the dead leaf tissue. Pycnidiospores spread to new crop foliage through rain splashes (Shipton et al., 1971; Ficke et al., 2018). The optimal temperature for infection and spread is between 5 and 30°C (Ficke et al., 2018).

Disease symptoms first appear as small chlorotic lesions on the lower leaves of the plant. Meanwhile, symptoms in older leaves appear as small necrotic brown to dark brown lesions without the distinct yellow border typical of tan spot lesions on leaf sheaths, and glumes (Friskop and Zhaohui, 2021). Later, these lesions expand to form lens-shaped/elliptical structures with a grayish-white center. A mature lesion consists of numerous brown to dark brown pycnidia distributed randomly around the center (Brennan et al., 1986). Epidemics of Stagonosopora nodorum affect the leaves and the

glumes resulting in high yield losses and a reduction in grain quality (King et al., 1983; Eyal and Ginkel., 1987).

Spot Blotch

Bipolaris sorokiniana (Syn: *Cochliobolus sativus* (Sorokin) Shoemaker, 1959) is the causal agent of spot blotch, also called leaf spot disease (Kumar et al., 2002). Reports indicated that the average yield loss worldwide due to spot blotch can be estimated to be 15–20% but can reach 40–70% in susceptible cultivars (Acharya et al., 2011; Chand et al., 2003; Chowdhury et al., 2013; Sharma and Duveiller., 2004). Spot blotch is the most widespread disease in cereal crops (especially wheat and barley) in the subtropics. The disease is mainly present in south Asia and some parts of South America (Chowdhury et al., 2013). In North America, the disease is present in the states of Indiana, Kansas, Minnesota, Montana, North Dakota, South Dakota, Utah, and Virginia (Acharya et al., 2011). The symptoms appear as brown lesions with yellow halos in the first stages of the infection, which enlarge with time to cover larger areas of the leaf, the lesions then change in color to olive brown (Gupta et al., 2018). There is no sign of the chlorotic margin at the initial stage of infection (Acharya et al., 2011). Spot blotch is a seed transmitted disease, and most of the spores survive in soil debris. The primary inoculum includes mycelium from infected seed, conidia in the soil, or conidia on the kernel surface (Neupane et al., 2007; Chand et al., 2003). Spot blotch affects first the lower leaves and progresses to the upper parts of the plants. Where high humidity and a moderate to warm temperature range (18˚C to 32˚C) favor the growth of *B. sorokiniana* (Al-Sadi., 2021).

Fusarium Head Blight

Fusarium Head Blight (FHB), commonly called scab, is one of the most serious diseases affecting wheat worldwide. It is caused primarily by the pathogen *Fusarium graminearum* (Syn: *Gibberella zeae* (Schwein.)., Schwabe, 1839)**.** Some other *Fusarium* species such as *F***.** *culmorum* (W. G. Smith) Sacc. *F*. *avenaceum* (Fr.) Sacc. and *F*. *poae* have also been reported associate with FHB.(Haile et al., 2019; Osborne and Stein., 2007). The fungus is a facultative parasite, that normally exists as a saprophyte, but can live as a parasite in plants during flowering (Wegulo et al., 2008). The disease results in direct economic losses, including reduced grain yield and quality due to aborted or shriveled seed and reduced seed size, and indirect loss due to contamination by mycotoxins deoxynivalenol (DON), nivalenol, HT2/T2, and zearalenone (ZEA) (Bottalico and Perrone, 2002). Annually, losses globally are in the billions of dollars, with common bread wheat and durum wheat accounting for a major portion of these losses (Powell and Vujanovic, 2021). FHB affected over 10 million acres of wheat in Minnesota, North Dakota, South Dakota, and the Canadian province of Manitoba, causing over $1 billion in yield losses in 1993 (McMullen et al., 1997). FHB continues to be a persistent problem in wheat production with increased mycotoxin levels in harvested grains (Osborne and Stein, 2007; Horevaj et al., 2011).

The fungus overwinters as a saprophyte in the plant debris of small grains and corn, which serves as primary inoculum under field conditions when temperatures are between 25 to 30° C and wet conditions exist (Miedaner et al., 2003; Parry et al., 2007). Infections occur mostly during anthesis, the stage at which anthers rupture and shed

pollen during flowering (Osborne and Stein., 2007). The symptoms appear on immature heads, showing a whitened or bleached discoloration in the head. As symptoms progress later in the season, there is a visible formation of pinkish, orange colored. Asexual fruiting structures (sporodochia) in the spikelet which produce tombstones. The infected kernels have a rough, shriveled appearance ranging in color from pink, soft-gray, to light-brown (Schmale III., 2003).

1.4 Disease Management

Disease management in small grains is influenced by the type of pathogen, distribution of the disease, favorable environmental conditions and cropping practices. Integrated disease management utilizes prevention, avoidance, monitoring, and suppression strategies (Kirby et al., 2017).

.Selecting varieties with resistance to diseases such as crown rust, tan spot, and Septoria nodorum blotch, can be an effective management strategy. Wheat cultivars that have good resistance to some of these fungal leaf spot pathogens are available (Phuke et al., 2020; Francki., 2013). However, wheat and oats cultivars may range from susceptible to moderately resistant to common fungal pathogens (Simón et al., 2021). Planting resistant cultivars is one of the least expensive and most effective management strategies for controlling tan spot and spot blotch in wheat and crown rust in oats.

Cultural management: Management of foliar and head diseases in wheat through cultural practices include, crop rotation, appropriate use of fertilizers, controlled irrigation, proper land preparation, non-host crops and timely harvesting. Practices such as crop rotation with non host-crops such as mustard, soybean, corn, and flax, reduce

resource of inoculum of leaf diseases in wheat. However, the effectiveness of cultural practices depend on the pressure of the pathogen in each crop season and the historical amount of diseases in the field, It is necessary to bear in mind which diseases are more prevalent and what kind of rotation is the right one; i.e., planting wheat into the residue of oats, millet, and barley reduces the risk of fungal leaf spotting diseases (Friskop and Zhaohui., 2021), but those residue may be a potential inoculum source for other diseases of wheat, such as Fusarium head blight, which is capable of surviving as a saprophyte on crop debris (Schmale III, 2003; Wagacha Muthomi., 2007).

Foliar and head diseases induced by fungal pathogens can reduce the yield significantly in small grains. Fungicides are most effective and economical when used in conjunction with a scouting program. One of the major aims of a foliar fungicide program is to keep the flag leaf free from disease for the reason that in this stage in the plant is crucial for filling the grain.

Fungicides have shown 77 and 89% effectiveness reducing disease severity of FHB and mycotoxin content, respectively (Haidukowski et al., 2005). Fungicides with active ingredients of metconazole, prothioconazole, tebuconazole, and propiconazole have shown extremely effective against FHB. On the other hand, fungicides in the strobilurins and triazole classes are effective in wheat leaf diseases, tan spot, stagonospora nodorum blotch, and spot blotch management. They include azoxystrobin, pyraclostrobin, metconazole, propiconazole, prothioconazole, and tebuconazole (Sundin et al., 1999 ; ;Bhathal et al., 2003).

The recommended fungicide application timing in controlling leaf disease and head disease from FHB is between Feekes growth stage 10 (boot) and Feekes 10.5 (fully headed to flowering) However, an earlier application of fungicide may be necessary if the leaf spotting diseases pressure is high early in the growing season due to cultivar susceptibility and availability of source of inoculum such as infested residue from the previous year crop.

1.5 Biopesticides

Biopesticides are formulations from naturally occurring substances derived from plants, bacteria, and certain minerals (EPA, What are Biopesticides? | US EPA) that can control agricultural pests and pathogens. The biofungicides are classified into three major groups by the United States Environmental Protection Agency (EPA):

1. Microbial fungicides: consist of microorganisms (bacteria, fungi, viruses, or protozoans) as the active ingredient. One of the most known is the entomopathogen bacteria, *Bacillus thuringiensis* or Bt, which is a potential controller of insects.

2. Plant-Incorporated Protectants (PIPs): PIPs are pesticidal substances produced by plants by incorporation an example is the first-generation of insecticidal PIPs that were introduced into genetically modified crops contained transgenes from the soil bacterium *Bt.*

3. Biochemical fungicides: Naturally occurring, as bioactive compounds that control pests, some examples in this group include essential oils, semiochemicals, plant growth-promoting regulators, insect growth regulators, secondary metabolites, and natural minerals.

Some of the advantages of the use of biopesticides are that they are generally less toxic than synthetic pesticides, present a broad spectrum in the control of pathogen microorganisms, and no residual effects in the crops; hence, presenting fewer risks to human health (Kumar and Singh, 2014).

Biopesticides represent just 5% of the global market in crop protection (Seiber et al., 2014). However, biopesticide use globally is increasing by almost 10% every year. Just in the United States there are 299 registered biopesticides and the interest in in their use has grown in recent years due to their effectiveness in small amounts and quickly decomposition (Damalas and Koutroubas, 2018).

1.6 Essential Oils

Essential oils (EOs) are products derived from different parts of the plant and are formed as secondary metabolites with different chemical substances belonging to over 20 groups of chemical compounds with different chemical families, including terpenes; aldehydes, alcohols, esters, and phenolics (Bakkali et al., 2008; Perczak et al., 2019; Burt, 2004; Bhavaniramya et al., 2019). These volatile oils are generally liquid and colorless at room temperature, characterized by volatile and low molecular weights, powerful aroma and soluble in alcohol, ether, and other oils but mostly insoluble in water.

The term EOs has been used since the 16th century as derived from the Latin name ‘‘Quinta Essentia,” named by Paracelsus von Hohenheim of Switzerland (Guenther and Althausen, 1948). The benefits of the EOs applications on plants have been well recognized since prehistoric times and widely used by all civilizations throughout the

millennia; where plant-derived therapeutic oils are mentioned in clay findings of cuneiform writing (2600 B.C) in Mesopotamia (Sakkas and Papadopoulou., 2017). EOs were later used as merchandise in the great empires, and later moved to the monasteries where the monks characterized them and studied their medicinal properties. The role of the EOs in the plants are for protection and defense against pests. However, in some species, those are used as an attractant for pollinating (Jugreet et al., 2020).

Essential oils are extracted from plants using distillation, fermentation, crushing, extraction, hydrolysis, or airing, where the most frequent method is by steam distillation (Djilani and Dicko., 2012). Three thousand EOs have been identified and about 300 types of EOs are being used commercially in the market (Maurya et al., 2021).

Essential oils have a high composition variability in qualitative and quantitative terms depending on the geographic location, climate, soil composition **(Table 1.1)** , plant part used (leaves, roots, flowers, etc.), maturity of the plant or extrinsic factors related to the extraction method and the environment where growth the plants (Dhifi et al., 2016).

1.6.1 Constituents and Compounds in EOs

EOs are synthesized in the cytoplasm of certain plants, mainly found in secretory cells, trichomes (hairs), epidermis cells, and secretory pockets. In the EOs, only a few compounds appear in the vast majority and are responsible for their bioactive nature. These major groups of substances are divided into two chemical groups based on the metabolic pathway of their synthesis (Anna., 2014; Pavela and Benelli, 2016): 1) terpenes/terpenoids and 2)aromatic/aliphatic component (phenylpropanoid synthesis) (Turek and Stintzing, 2013).

Terpenes/ terpenoids are mainly synthesized from isopentenyl diphosphate precursors, followed by modification in prenyl diphosphate by terpene synthetase enzymes. The distribution of the major groups are hemiterpenes (C5), sesquiterpenes (C15), monoterpenes (C10), triterpenes (30), diterpenes (C20), and tetraterpenes (C40). Alcohol, aldehyde, ketone, ester, ether, and phenols. Terpenoids constitute about 90% of the essential oils and allow a great variety of structures, where some of the most studied terpenoids are thymol, carvacrol, linalyl acetate, linalool, piperitone, citronellal, geraniol, and menthol (Nazzaro et al., 2017), (**Figure 1.1).** On the other hand, the aromatic components of EOs are the derivative of phenylpropane and less frequently observed than terpenes (Maurya et al., 2021). Common aromatic aldehyde, alcohols, phenols, and methoxy derivatives of EOs are cinnamaldehyde, cinnamic alcohol, eugenol, elemicin, and estragol (Maurya et al., 2021). Most of the EOs are composed of two or three major compounds (20-70%), while other compounds are present in trace amounts (Bilia et al., 2014).

Table 1.1. Examples of plants and parts used for the essential oil extraction and main compounds found in these.

Common plant name	**Essential oil name**	**Plant part used**	**Principle compound**	**References**
Oregano	Origanum vulgare	Leaves and stem	Monoterpene: Thymol (45.43%) γ-terpene (23.69%)	(Vinciguerra et al., 2019)
Korean perilla	Perilla frutescens	Leaves and Stems	Monoterpene: Linalool (46.55%) 2-hexanoylfuran (30.79%) Sesquiterpene	(Luo et al., 2019)

			hydrocarbon: β-Caryophyllene (5.34%)	
Rosemary Thyme	Rosmarinus officinalis Thymus vulgaris	Leaves and stem	1,8-cineole (45.27%), Borneol (12.94%) p-cymene (39.18%), Thymol (25.05%)	(Iseppi et al., 2019)
Tea tree	Melaleuca alternifolia	Leaves	Terpinen-4-ol(30%) γ-Terpinene (23.0%) α-Terpinene (10.4%)	(Carson et al., 2006)
Oregano	Origanum vulgare	Flowers leaves	Carvacrol (83.37%) p-cymene(9.87)	(Béjaoui et al., 2013)
Lemongrass	Cymbopogon flexuous	Leaves	Citral (22.94%) Neral (19.88%)	(Devi et al., 2021)
Neem tree	Neem oil	Seed Kernel, Fruits	Azadirachtin(30%) Azadiradione 30%	(Kurose and Yatagai, 2005; Siddiqui et al., 2009)
Karanja Oil	Pongamia oil Karanja oil	Bark	1.25% karanjin	(Mandal et al., 1984; Nimesh et al., 2021)

1.6.2 Mode of Action of Essential Oils

The antifungal activity of the EOS and compounds might be caused by the properties of terpenes/terpenoids, which due to their highly lipophilic nature and low molecular weight, can disrupt the cell membrane causing cell death (Nazzaro et al., 2017). Their lipophilic nature and low molecular weight allow EOs to easily penetrate through the cell membrane. Where these compounds are able to cause structural and functional damage in the cell disrupting the membrane permeability, interrupting the electron flow and active transport (Kalagatur et al., 2015; Burt, 2004; Prakash et al., 2015; Swamy et al., 2016).

Raveau et al., (2020) highlight six different mechanisms of the EOs in controlling the fungal pathogens, summarized below:

• Inhibiting the fungi cell wall formation.

• Disrupting the cell membrane by inhibiting ergosterol synthesis.

• Affecting the fungal mitochondria by inhibiting the mitochondrial electron transport.

• Inhibiting cell division.

• Interfering with either RNA or DNA synthesis and/or inhibiting protein synthesis.

• Inhibiting efflux pumps.

1.6.3 Essential Oils and Assays *in vitro*

The fungicidal assays of EOs include different methods with either *in vitro* or *in vivo* assessments. The results are mostly expressed as 1) half maximal inhibitory concentration (IC50), 2) minimum inhibitory concentration (MIC), 3) minimum fungicidal concentration (MFC), and 4) zone of inhibition (ZOI). Some commonly studied traits are mycelial growth inhibition, conidial germination, dry hyphal mass weight, germ tube elongation, and hyphal morphology. Some of the observations were done by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Balouiri et al., 2016).

1.6.4 EOs in the Control of Plant Pathogens

Effective plant disease management results from the combination of several activities such as crop rotation, planting date, predictive model of the diseases, and planting tolerant varieties. However, in general, the fungicide application combined with

the use of tolerant varieties has led to better disease control and lower mycotoxin contaminations in the case of fusarium head blight diseases (Mesterházy et al., 2018). Fungicides used in the control of fungal diseases in crops account for < 10% of the total pesticides used in the United States (Zubrod et al., 2019).

In addition, due to the limited genetic resources for host resistance and the wide host range of plant pathogens, the application of fungicides is a primary tool for controlling fungal diseases (Duan et al., 2018). For example, FHB has been mainly controlled with chemical fungicides with active molecules belonging to the demethylase inhibitors (DMI), such as triazoles and imidazoles, which are the most effective fungicides currently available against *Fusarium* species (Paul et al., 2007; Paul et al., 2008; Sun et al., 2014). Application of DMI fungicides have shown reduced DON concentrations by 46% and 48% compared with other fungicides (Ioos et al., 2005).

On the other hand, the application of synthetic fungicides to control diseases in crops increases the yields and keeps the quality of the harvests. However, the overuse of fungicides has resulted in the tolerance of pathogens and environmental hazard contamination (Piel et al., 2019; Zubrod et al., 2019; Damalas and Eleftherohorinos., 2011). Thus, there is a need to find alternative products with known antifungal properties for add-in disease management, such as a plant derivative essential oil (EOs).

EOs have been shown to possess anti-microbial and antifungal properties against various pathogens in agriculture (Prakash et al., 2015) (**Table 1.2).** in the case of *Fusarium graminearum*, the EOs have shown fungal inhibition and reduction in the

production of mycotoxins (Matusinsky et al., 2015; Kalagatur et al., 2015). However, the available literature provides limited data concerning comprehensive studies on the effect of EOs on the inhibition of both *Fusarium* growth and mycotoxin biosynthesis in field studies.

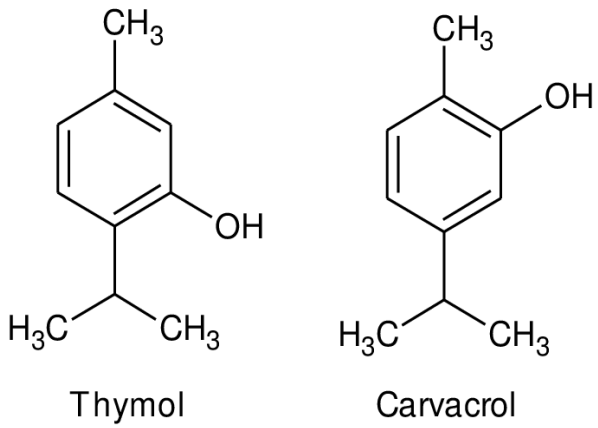

Figure 1.1. Thymol structural monoterpenoid phenol compound present in the essential oils, and their isomer carvacrol, typically compounds extracted from thyme plants.

Table 1.2. Essential Oils and compounds with antifungal properties reported in the literature as controllers of important disease that affects crops in the agriculture.

EOs and compound	Crop/Disease	Pathogens	Studied	Reference
Tea Tree (*Melaleuca alternifolia*),	Strawberry (Post-harvest)	*Alternaria spp*	*In vitro*	(Bishop and Thornton, 1997)
	Cucumber	*powdery mildew*	*In vivo*	(Reuveni, Sanches, et al.,

				2020)
	Strawberry (Post-harvest)	*Botrytis cinerea*	*In vitro*	(Yu et al., 2015)
	Wheat/ fusarium head blight	*Fusarium graminearum*	*In vitro*	(Terzi et al., 2007)
	Banana Black Sigatoka	*Mycosphaerella fijiensis*	*In vivo*	(Reuveni, Barbier, et al., 2020)
Lemongrass (*Cymbopogon flexuosus*)	Postharvest pathogens	*Colletotrichum coccodes, Botrytis cinerea, Cladosporium herbarum, Rhizopus stolonifer Aspergillus niger*	*In vitro*	(Tzortzakis and Economakis, 2007)
Cymbopogon citratus,nardus , and schoenanthus	Fusarium Head blight Wheat	*Fusarium avenaceum*	*In vitro*	(Zhang et al., 2022)
Lemongrass (*Cymbopogon citratus*)	Postharvest diseases	*Aspergillus flavus*	*In vitro*	(Sawadogo et al., 2022)
	Wheat, rape	*Rhizoctonia solani.*	*In vitro*	(Lee et al., 2020)
	Squash	*Stagonosporopsis cucurbitacearum and Alternaria alternata*	*In vitro*	(Moumni et al., 2021)
Pongamia pinnata Karanja oil	Northern leaf blight in corn Tomato/ early	*Helminthosporium turcicum* *Alternaria solani*	*In vitro* *In vitro*	(More and Baig, 2013)

	blight Strawberry / grey mold	 *Botrytis cinerea*		(Jabeen et al., 2014)
Neem oil (*Azadirachta indica*)	Postharvest Fruits Stored grains cereals	*Aspergillus carbonarius* *Fusarium sp. Rhizopus sp. Curvularia sp. and Aspergillus sp*	*In vitro*	(Rodrigues et al., 2019) (Adepoju et al., 2014)
Carvacrol	(Tomato/damping -off)	*Rhizoctonia solani*	*In vivo*	(Gwinn et al., 2010)
	Apricot fruit/brown and gray mold rot	*Monilinia fructicola, Botrytis cinereal fungus*	*In vivo*	(Sun et al., 2014 ; Bouchra et al., 2003)
	Tomato	*Sclerotinia sclerotium*	*In vitro and in vivo*	(Soylu et al., 2010)
Thymol	Wheat/FHB Maize kernel /Aspergillus Ear rot Wheat/FHB	*Fusarium graminearum* *Aspergillus flavus* *Fusarium graminearum*	*In vitro* and *in vivo* *In vitro* *In vitro*	(Gill et al., 2016) (Shen et al., 2016) (Gao et al., 2016)
Linalool	Wheat/FHB Fabaceaes Damping-off,	*Fusarium spp* *Sclerotinia sclerotiorum.* *Sclerotium cepivorum, and Fusarium oxysporum f. sp. Dianthi.* *Rhizoctonia*	*In vitro* *In vitro* *In vitro*	(Kordali et al., 2007) (Pitarokili et al., 2002) (Marei et

	vascular mold, green mold, and black mold	*solani and Fusarium oxysporum, Penecilliu m digitatum and Aspergil lus niger*		al., 2012)

1.7 Research Justification

Fungal diseases such as crown rust in oat, leaf spotting diseases, and Fusarium head blight in wheat caused significant grain yield losses annually, and the application of synthetic fungicides is recommended every season in crops to combat diseases and guarantee yield due to their high efficacy (Poole and Arnaudin, 2014). However, it is known that the overuse of these fungicides can cause resistance by the pathogens to the different fungicide molecules (Avozani et al., 2014; Lucas et al., 2015). On the other hand, environmental problems due to fungicide residual levels and toxicity (Piel et al., 2019), Begs for alternatives to inorganic synthetic fungicides, biopesticides derived from plants - essential oils and compounds present in EOs have been shown to have antifungal properties to plant pathogens *in vitro* and *in vivo,* being those of great interest due to their low negative impact on the environment and human health (Lengai and Muthomi, 2018). These are highly attractive in the production of organic crops.

For this reason, essential oils look like a promising alternative lead to integrated pest management disease control. The main goal of our study was to determine the efficacy of several essential oils and bio compounds in the control of fungal pathogens in small grains, oat, and wheat.

In this work the specific objectives were:

(1) Determine the potential of two essential oils in the control of oat crown rust in a two-year field experiment, **(2)** Determine the potential effect of essential oils and bioactive compounds in the control of three pathogens (PTR, PSN, BPS) that cause foliar diseases in wheat *in vitro*, and **(3)** determine *in vitro* and *in vivo* the potential as a fungicide of two EOs, two commercial products with EOs, and two bioactive compounds in the control of the fungal pathogen *Fusarium graminearum*.

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Chapter II : Efficacy of Essential Oils on the Management of Crown Rust in Oat

2.1 ABSTRACT

Crown rust, caused by *Puccinia coronata,* sp. *avenae,* is one of the most devastating diseases after stem rust, causing significant yield and quality reduction in oats worldwide. The management of this disease is through using resistant varieties, eradication of alternate hosts, and the application of synthetic fungicides. Where Fungicides play a fundamental role mitigating the negative impacts of the diseases and promoting higher yields; however, the frequent application of fungicides with similar modes of action can eventually result in its diminished efficacy due to fungicide insensitivity of the pathogen. In addition, it is known that the excessive use of fungicides can generate harmful and serious issues for human health and ecosystems. Thus, it is necessary to develop different alternatives such as plant derived biopesticides with known antifungal activity and environment friendly. Essential oils (EOs) are secondary metabolites derived from plants with known antifungal properties that can be used as a potential fungicide in agriculture and complement integrated disease management. Neem (*Azadirachta indica)* and Karanja (*Millettia pinnata*) oils are traditional biopesticides with antifungal properties that have been tested for crop pathogens and may be usable as an alternative fungicide for the control of crown rust with the advantage of low residuality and low negative impact on the environment. A two-year field study was conducted to test the efficacy of two EOs, neem and karanja, against crown rust of oats. EOs were evaluated at two concentrations (52 and 72%) and three rates (3.7, 7.5, 11.25 L/ha) in Brookings South Dakota. The treatments were applied at flag leaf emergence

stage in two oats cultivars, Horsepower and Goliath in 2019 and 2020, respectively. A synthetic commercial fungicide, Headline (Pyraclostrobin), and a non-treated (water) served as untreated check. Percentage of diseased leaf area, stem lodging percentage, normalized difference vegetation index (NDVI), test weight (TW), and yield were assessed for each year.

In both years, the two essential oils karanja and neem at higher concentration and rate showed reduced Disease severity (%) ($P < 0.05$) and a high yield in the 2020 compared with the untreated check. The synthetic fungicide, Headline, showed a significantly higher yield, lower crown rust severity and lower lodging compared to the essential oils and untreated check. This study showed the efficacy of use the EOs neem and karanja, in the control of crown rust but also warrant further research for the application rate and timing. The two EOs have potential to be used in organic oat production.

Keywords: Essential oils, Crown rust, Alternatives Fungicides, Biopesticides .

2.2 Introduction

Crown rust, caused by the fungus *Puccinia coronata* f. sp. *avenae*, is the most widespread and damaging disease of oat. It has been reported that moderate to severe epidemics can reduce yield by 10 to 40% worldwide (Behnken et al., 2012; Leonard and Martinelli, 2005). In the United states an epidemic of crown rust in 2014 caused 18.7% loss in oat production, where the states of Minnesota and South Dakota were the most affected by the disease (Nazareno et al., 2018).

Crown rust affects the foliar surface area where the symptoms appear as orange–yellow round to oblong uredinia (pustules), which produce a new set of urediniospores after 7–10 days. These structural alterations affect the photosynthesis in the plant and consequently the grain development. Additionally, this disease causes a decrease in test weight and severe lodging (May et al., 2014; Nazareno et al., 2018). Factors that can increase the severity of this disease include: 1) the susceptibility level of the cultivar, 2) favorable weather conditions such as warm temperatures (between 20–25 °C) and high humidity, and 3) the presence of the alternative host plant *Rhamnus* spp (Fulcher et al., 2020).

Management of this disease usually involves the eradication of the alternative host, the use of resistant cultivars, or the application of foliar fungicides. However, the genes of resistance in the cultivars are rapidly lost due to development of new pathogen races as a result of recombination in the sexual stage of the pathogen that occurs in the alternative host (Nazareno et al., 2018; Lucas et al., 2015). On other hand, the number of fungicides available for controlling crown rust is limited, where the QoI-fungicide

(Quinone outside Inhibitors) group is the most used e.g. pyraclostrobin, and azoxystrobin (Behnken., 2012).

Despite the benefits of increased yield and effective disease control attributed to synthetic fungicides (May et al., 2014; Poole and Arnaudin., 2014), the application of fungicides also produces concerns for their detrimental effects on the environment, and to wildlife and human health (Damalas and Eleftherohorinos., 2011).

On other hand, organic oat crop markets have grown in the last years to fulfill a growing demand for products with a friendly approach to the environment, a high biodegradability, and minimal risk to humans. For example, in 2019, 54,000 acres of organic oat were harvested in the U.S. (NASS, 2019), where products such as biopesticides fulfill these needs for this market.

Biopesticides are defined as pesticides derived from natural materials such as animals, plants, bacteria, and certain minerals (https://www.epa.gov/ingredients-used-pesticide-products/what-are-biopesticides). The essential oils (EOs), such as neem (*Azadirachta indica*) and karanja (*Millettia pinnata*) oils are classified as plant extracts. Biopesticides as plant extract essential oils have become relevant in agriculture due to their antimicrobial capacity, low hazard to humans, and high biodegradability in the environment (Fierascu et al., 2020).

EOs are hydrophobic liquids derived from mostly plant secondary metabolites that contain a wide range of volatile molecules; some with antifungal properties (Maurya et al., 2021; Raveau et al., 2020). The modes of action of EOs have been studied but are

not fully known. Various studies of essential oil and bioactive compounds such as carvacrol or thymol, have shown to produce membrane disruption, efflux inhibition, increase of membrane permeability, and a decrease in intracellular ATP (reviewed by Aljaafari et al., 2021; Maurya et al., 2021). Antifungal effects of EOs have been reported from *in vitro* studies for several pathogens that cause diseases in cereals and oilseeds (De Clerck et al., 2020; Perczak et al., 2020; Stević et al., 2014). For example, several EOs exhibit antifungal properties against pathogens that affect wheat such as *Fusarium graminearum, Bipolaris sorokiniana* (Harčárová et al., 2021; Naz et al., 2018).

Among the EOs available, karanja and neem oils have exhibited insecticidal, antibacterial, and antifungal properties *in vitro* for several fungal pathogens (Dubey et al., 2009; Pulipati, 2018; Campos et al., 2016). Neem oil is commercially available and has been evaluated in the control of several pests in the agriculture crops (Kumar et al., 2022), however, this EO has not been tested in field application for the control of crown rust in oats.

Karanja (*Millettia pinnata*, syn. P. glabra; Family: *Leguminosae*) present over 200 plant species distributed in tropical Africa, Asia, and Australasia (Chen et al., 2018). Traditionally it is used as a potent medicine with antibacterial, anti-tumor, insecticidal properties, (reviewed by Jena et al., 2020). Compounds such as flavonoids, terpenoids, phenols, saponins, alkaloids, and alkyd resin are extracted from the kernel (Bora et al., 2014), where the major active compounds isolated in *Milletia pinnata* is karanjin, a furanoflavonoid named pongapinnols (Katekhaye et al., 2012). Karanja oil is reported to have antifungal properties on crop pathogens *in vitro* such as B*otrytis cinerea* (Jabeen et

al., 2014) and Sc*lerotium rolfsii* (Shaheen et al., 2017), but, thus far this EO has not been evaluated *in vivo* in the control of crown rust.

Neem oils are extracted from the neem tree, *Azadirachta indica*, a member of the *Meliaceae* family. Neem oil presents at least 100 biologically active compounds. Among them, the major constituents are triterpenes known as limonoids (e.g., azadirachtin, meliantriol, salannin, diacetyl salannin, nimbin, desacetyl nimbin, and nimbidin). The most important of these is azadirachtin, which appears to cause 90% of the repellent effects on most pests (Laxmishree and Singh, 2017; Campos et al., 2016). Neem oil is mainly obtained from seeds, kernels, and fruits of the neem tree (Kumar et al., 2022). Several studies have shown the *in vitro* efficacy of this essential oil in the control of pathogenic fungi such as *Alternaria alternata, Fusarium moniliform, Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, Drechslera hawiinesis, Fusarium semitectum,* and *Fusarium nivali* (Kumar et al., 2022). An *in vivo* study also has reported the efficacy of neem oil in controlling wheat leaf rust disease caused by *Puccinia triticina* (Shabana et al., 2017).

The high evolutionary capacity of the crown rust pathogen due to sexual recombination, the difficulty eradicating of alternative host, and the increasing use of fungicides in the crop has increased the prospect of fungicide insensitivity in the pathogen. Plant extracts from karanja and neem, with a known antifungal potential in other crops, might be an important alternative for controlling crown rust disease in organic oats production. The objective for this study was to determine the viability of using neem and karanja oils against crown rust disease.

2.3 Materials and Methods

Products: Karanja and Neem essential oils used in this study are commercially available, twelve treatments between karanja and neem oil were applied at two concentrations of 52 and 72% v/v with three rates of application (3.7, 7.5, 11.25 L/ha), concentrations were tested along with an untreated (water) and a commercial fungicide Headline® as checks. The study was conducted at the South Dakota State University Research Farm near Brookings for two years in 2019 and 2020 Two crown rust susceptible and moderately susceptible variety, Horsepower and Goliath, was planted in 2019 and Goliath cultivar in 2020 due non-availability of Horsepower seed.

2.3.1 Field Experiment

Planting, rating, and harvest date are shown in (**Table 2.1).** Weed control was manually done in all trials for both years. Treatments were applied at the flag leaf emergence (Feekes scale 9 to 10.1) with a CO^2 backpack sprayer. Plots for each year were arranged in a randomized complete block design with four replications. The experimental units consisted of plot sizes of 1.5 by 4.5 meters. The crown rust disease development was from the natural inoculum.

Crown rust severity was visually estimated as the percent of flag leaf covered with uredinial pustules (Fulcher et al., 2020). Where ten flag leaves were assessed on each plot using a modified Cobb Scale (Peterson et al., 1948). A Green Seeker Handheld Crop Sensor® was used to measure each plot (with three readings per plot). The sensor displays the measured value in terms of NDVI reading (ranging from 0.00 to 0.99), where

values approaching zero mean a high severity caused by the disease and values approaching 0.9 indicating healthy plants. Additionally, lodging was rated in each plot at physiological maturity using a 1 to 100 modified scale by (May et al., 2014; Gutierrez and Conley, 2016). Harvest of the plots was done with a mechanical Wintersteiger combine and the yield was determined with 13% moisture at harvest time.

Table 2.1. Flow chart of activities in both field years.

Year	Cultivar	Planting date	Fungicide Application, Feekes scale (9-10)	Rating date	Green Seeker date	Harvesting date
2019	Horsepower	April 29	July 20	August 6	August 13	September 19
2020	Goliath	April 18	July 1	July 17	July 24	September 22

2.3.2 Statistical Analysis

Crown rust severity, lodging and yield data were analyzed separately for each year due to the high differences presented by the cultivars. The variables were subjected to normality test and log transformation was done to meet assumptions of normality. Data were then subjected to ANOVA, followed by a Fishers Least Significant Difference test at ($P < 0.05$). using the statistical program Rstudio 2022.02.0.

2.4 Results

2.4.1 Effect of Essential Oils on Crown Rust severity and Yield

Crown rust severity varied across the two years in the untreated check, though two different varieties were used. The 2019 had relatively higher level of crown rust severity at 85% whereas crown rust on the variety Goliath in 2020 was 71% on the untreated check. Neem oil generally reduced disease severity and lodging compared to karanja in both years, there was no difference between the treatments in the green seeker lecture and test weight results between the treatments in 2019 and 2020.

In 2019, treatments with neem oil generally showed a higher yield compared with the karanja oil treatments; furthermore, the neem oil concentration of 72%, at the high rate of 11.25 L/h, presented the highest yield 1827.88 kg/h compared with the rest of treatments with essential only surpassed by the commercial fungicide Headline (**Figure 2.3),** The concentration of 72% showed the highest yield compared to the lower concentration of 52% for this year. The fungicide Headline presented the highest performance with a yield of 4725.06 kg/h, and a test weight of 424.88. And showed significant lower disease severity at (25%) and lodging (16.25%) compared with the neem and karanja oil treatments (**Figure 2.1, Figure 2.2.)**. Overall, when the EOs rate increased, there was observed a decrease in percentage of the crown rust disease severity **(Table 2.2)**.

In 2020, the yield was significantly higher (P>0.05) at 72% for karanja oil in all application rates over the untreated check. The yield in neem oil at 52% at the 7.5 L/ha concentration and neem at 72% at the 11.25 L/h concentration, showed a significantly

higher yield compared with the untreated check (**Table 2.2)**. Lodging and test weight did not significantly differ from the untreated check. karanja oil at the concentration of 72% in the rates 7.5 and 11.25 L/h showed the lowest percentage of disease severity (**Figure 2.4)**.

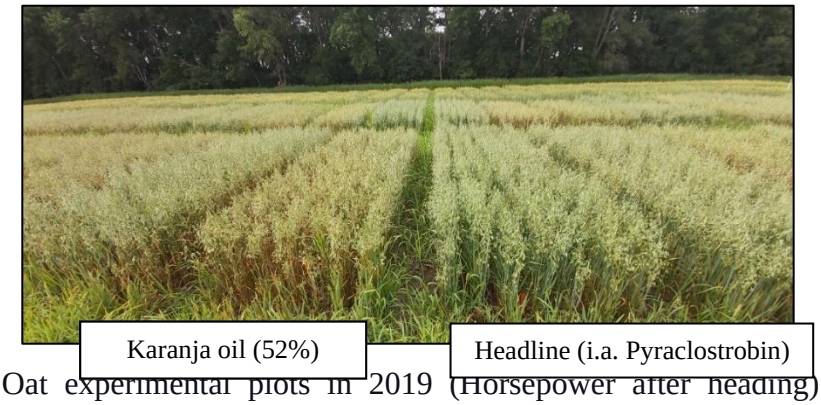

Figure 2.1. Oat experimental plots in 2019 (Horsepower after heading). Plots with orange-yellow color in the figure showed high crown rust severity(left) compared with the commercial fungicide Headline (right).

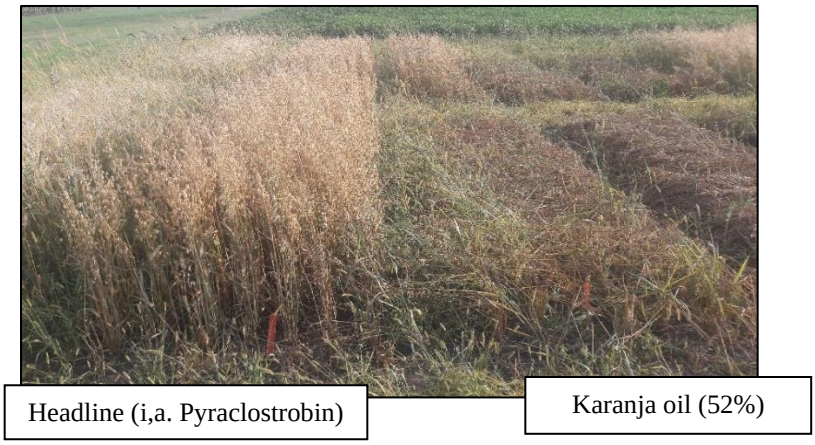

Figure 2.2. Lodging in the first year of experiment 2019 with the cultivar Horsepower. Comparing the plot treated with the commercial fungicide Headline with a 10 % of lodging and the karanja oil at 52% using the lower rate, showing the 100 % lodging.

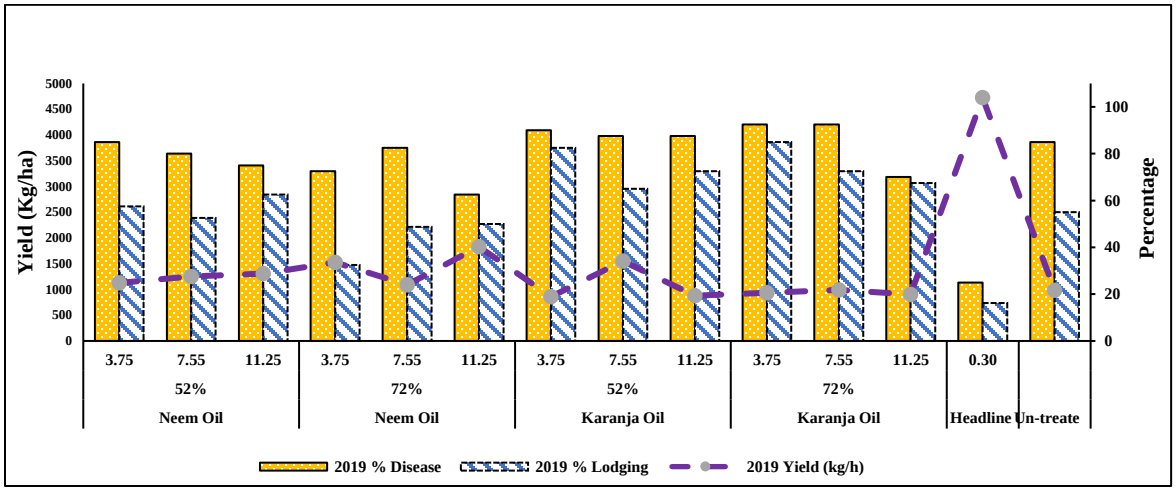

Figure 2.3. Summary results year 2019,Percentage of disease and lodging (bars) and yield (Purple dot line), at the different concentrations tested 52 and 72 %, and the rates of application in the field (3.7, 7.5, 11.25 L/ha).

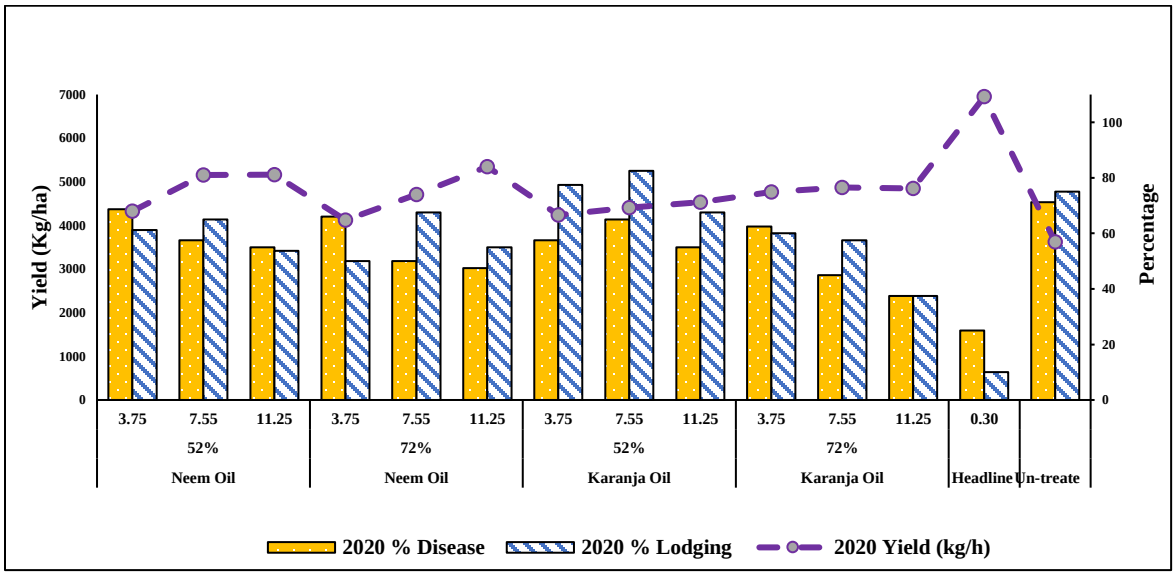

Figure 2.4. Summary results in the 2020, percentage of disease and lodging (bars) and yield (Purple dot line), at the different concentrations tested 52 and 72 %, and the rates of application in the field (3.7, 7.5, 11.25 L/ha).

2.5 Discussion

Under field conditions was evaluated for two years the effect of neem and karanja essential oils in the control of crown rust in oats, lodging, test weight, and yield. In general, in 2019, the application of neem oil at 72% v/v and highest rate 11.25 L/h showed an increase in yield of 53.7% compared with the untreated, also for the 2020 season, neem oil at the highest concentration and rate showed an increased yield of 76.6% compared with the untreated check. Additionally in this concentration and rate of application, neem oil decreased the disease severity in 2019 a 26.4% and in 2020 a 26.6%. compared with the untreated check. This agrees with the study realized by Shabana et al. (2017) that in a two year field experiment with wheat with leaf rust (*Puccinia triticina*), treated with eight plant extracts included neem extracts at 2%v/v and showed that plants treated with neem significantly reduced the infection of 66.67% with one application and 94.44% with two spray applications, and increased 3.35% test weight compared with the untreated plot. Leaf rust control may have been superior to crown rust due to the purity of the neem extract. On other hand, in the 2020, karanja oil at 72% oil also showed a significant ($P < 0.05$) increase in yield at the rates of application (7.55 and 11.25 L/ha) and a significant reduction in the percentage of the disease severity compared with the untreated check. However, these results were not similar in 2019. It is possible that karanja oil could be used as a bio-fungicide for organic production of oats if it is applied to moderately resistant varieties.

Another study by Uwineza et al. (2019) that used the EOs pennyroyal (*Mentha pulegium*) in the control of yellow rust (*Puccinia striiformis*), the EOs increased grain

yield by 23% when it was compared to the untreated control. Similarly, in a two-year field experiment by Kader et al. (2021) used three wheat cultivars to evaluate the ability of three EOs to control stem rust caused by *Puccinia graminis f. sp. Tritici*. Where, thyme oil, cinnamon, and garlic oil, at a concentration of 1ml/L, showed a reduction in the average disease severity by 84.02%, 88.41%, and 91.79% respectively compared with the untreated controls. Additionally, Tohamey and El-Sharkawy (2014) evaluated the control in wheat leaf rust caused by *Puccinia triticina*, in the field, with the use of the EOs chamomile, thyme, cumin, basil, eucalyptus, and garlic at 20 ml/L, and reported concentration showing a decreased leaf rust severity and also had increase grain weight compared with the untreated.

Based upon results from the present study, neem and karanja oils are potential alternative fungicides when moderately resistant varieties are being used, although the efficacy is still low for field applications, when contrasted with a synthetic inorganic fungicide. This may be due to their high volatility, the inability to standardize the extraction process. However, the use of new technologies such as nanoencapsulation are improving the use of EOs applications in the field. With this study, we measured the effectiveness of these two products in the control of crown rust severity. In future studies, it will be necessary to evaluate the effects of varying the number of applications in the field, the cost of the EOs, the effectiveness of other EOs and compounds, and in tank mix with other pesticides.

The efficacy of the EOs in controlling crown rust in field application is likely limited because this is a non-penetrating and protective product. This may require

increased rate of application, which may reduce the profitability due to the need to be applied repeatedly. The EOS are more efficient as protectants in controlling crown rust severity rather than curatives or eradicating fungicides. To ensure high yields in the organic production of oats, , our results suggest using moderate resistant cultivars sowed early in the season and repeated applications of EOs should be made depending on the pressure of the rust inoculum.

2.6 Conclusion

The loss of resistance in the oat cultivars due to the high recombination in the lifecycle of *Puccinia coronata*, the few new classes of fungicides available on the market for the control of crown rust, the outbreaks of the new races, the hazard concerns with the use of pesticides, and the need in the market for new products for organic oat crop production require the development of new biofungicides such as essential oils. In this study, we found that karanja and neem oils improved the yield and decreased crown rust severity at the highest concentration and rate compared with the untreated check. The use of EOs as fungicides against crown rust seems to be promising. More studies on the timing of application, numbers of application for an acceptable control of the diseases are still needed.

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Table 2. Analysis of variance (ANOVA) for the variables ev

Year		2019							
nts (V/V)	Rate (L/ha)	ield g/ha)	Test weight (kg/m^3)	% Disease severity	% Lodging	Green Seeker NDVI	Yield (Kg/ha)	Test weight (kg/m^3)	Lo
(52%)	3.75	.46 bc	284.20 cde	85 ab	57.50 ab	0.63 a	4324.24 bcd	431.92 b	25
(52%)	7.55	.85 bc	266.56 de	80 abc	52.50 abc	0.69 a	5157.48 bc	418.53 b	00
(52%)	11.25	.36 bc	272.61 de	75 bc	62.50 ab	0.67 a	5163.38 bc	428.70 b	75
(72%)	3.75	.91 bc	287.16 de	72.5 bc	32.50 bc	0.70 a	4121.15 cd	423.29 b	00
(72%)	7.55	.16 bc	264.89 cde	82.5 abc	48.75 abc	0.69 a	4706.23 bc	431.66 b	50
(72%)	11.25	.88 b	323.58 de	62.5 c	50.00 abc	0.68 a	5349.82 b	438.87 b	00
Oil(52%)	3.75	.80 c	266.18 b	90 ab	82.50 a	0.68 a	4238.16 cd	435.13 b	.50
Oil(52%)	7.55	.46 bc	297.19 de	87.5 ab	65.00 ab	0.66 a	4407.64 bcd	415.31 b	.50
Oil(52%)	11.25	.32 c	269.14 bc	87.5 ab	72.50 a	0.69 a	4535.41 bcd	418.66 b	50
Oil(72%)	3.75	.10 c	282.66 de	92.5 a	85.00 a	0.65 a	4764.74 bc	436.42 b	00
Oil(72%)	7.55	.92 bc	257.57 bcd	92.5 a	72.50 a	0.66 a	4870.99 bc	427.67 b	50
Oil(72%)	11.25	.51 bc	264.27 e	70 bc	67.50 ab	0.66 a	4847.46 bc	434.11 b	50
trobin)	0.30	.06 a	424.88 a	25 d	16.25 c	0.73 a	6949.73 a	474.52 a	.00
l check		.21bc	285.47 cde	85 ab	55.00 ab	0.66 a	3620.13 d	427.41b	.00

alues are least squared means of 4 replications separated by the two years of experiment. Different letters in the same column for each treatment present significant differences according to Fishers Least Significant Difference test ($P \leq 0.05$). Treatments with the same letters are not significantly fferent from each other. Lodging scored as percentage: 0%= no lodging to 100% = plot entirely lodged. Crown rust disease scored as a percentage: %= no pustule to 100%= leaves completely covered with pustules, green seeker, NDVI reading score ranging from 0.00 to 0.99

Chapter III: Evaluation of Essential Oils for Leaf Spotting Disease Management in Wheat.

3.1 ABSTRACT

Wheat is one of the main crops cultivated and consumed worldwide, However, wheat production is under constant threat due to multiple pests and diseases, which include tan spot (*Pyrenophora tritici-repentis* (PTR)), Septoria/Stagonospora nodorum blotch (*Parastagonospora nodorum* (PSN)), and spot blotch (*Bipolaris sorokiniana* (BPS)) that impact crop productivity. Managing these leaf spot diseases typically involves using resistant cultivars and/or fungicide applications. However, the overuse of fungicides can lead to fungicide resistance in the pathogen population and can lead to human health and environmental concerns. Consequently, this creates a need to find natural alternatives to incorporate into integrated disease management. Plant extracts, such as essential oils and derivatives from plants that possess antifungal properties, may have potential in the control of leaf spotting disease in wheat. In this study, four essential oils: tea tree, lemongrass, neem, and karanja, and three constituents present in essential oils: carvacrol, thymol, and linalool, were evaluated against PTR, PSN, and BPS based on mycelial growth and spore germination inhibition *in-vitro*. Lemongrass at 500 and 1000 ppm, carvacrol and thymol at 200 and 500 ppm, showed 100% inhibition of mycelial growth in PTR, PSN, and BPS. One hundred percent of spore germination for the three pathogens was inhibited by lemongrass at 1000 ppm and carvacrol and thymol

at 500 ppm. The results indicate that lemongrass, carvacrol, and thymol have fungicidal and fungistatic properties *in vitro* against PTR, BPS, and PSN.

3.2 Introduction

Wheat is the most cultivated cereal worldwide, where the global production for 2022 is estimated at 770.3 million tons (FAO, 2022). The United States is expected to contribute about 6% of the worldwide production by 2030 (OECD, 2021). However, cereal production is constantly affected by diseases that negatively impact the yield and quality of the grain. A study by Oerke et al. (2006) estimated that the potential yield loss in wheat by pathogens could reach 6% worldwide.

Fungal leaf diseases such as; tan spot caused by *Pyrenophora tritici-repentis* (PTR), Stagonospora nodorum blotch caused by *Parastagonospora nodorum* (PSN), and spot blotch caused by *Bipolaris sorokiniana* (BPS) affect the grain yield. Savary et al. (2019) in a survey classified the most important diseases that cause yield loss in different agriculture centers around the world, showing that PTR, PSN, and BPS are in the top 11 of 31 present diseases in field crops. These three pathogens belong to a complex of leaf spotting diseases in wheat that can infect the crop simultaneously, where their main source of inoculum comes from crop residues of previous small grain crops. Infection of these diseases induces necrotic areas in the leaf and causes an average of 30 to 50% yield reduction in susceptible cultivars, (Bhathal et al., 2003; Duveiller et al., 2005; Friesen et al., 2018; Gupta et al., 2018).

The symptoms of tan spot are, tan elliptical lesions with a distinctive yellow border, while SNB results in lens-shaped necrotic lesions and yellow borders without an

obvious halo in the lesion. However, in field conditions the two diseases are not easily distinguishable in the late growing season when the lesions coalesce (Liu et al., 2015). On the other hand, BPS leaf spotting disease shows small brown lesions less than one centimeter in diameter, which may coalesce into large, elongated blotches of necrotic tissue (Creswell., 2014) .

PTR, PSN, and BPS first colonize the lower leaves at the base of the wheat plant and then progress upward to the flag leaf. High relative humidity, wet weather, and warm temperatures between (18 – 33°C) are favorable for the infection (Mehta., 1988) . The leaf spotting complex pathogens also are the causal agent of other diseases in wheat **(Table 3.1)**, causing reduction in kernel weight and decreasing grain weight per head (Fernandez et al., 2016). Additionally, BPS has been shown to possess the virulence gene to produce the proteinaceous necrotrophic effector *ToxA,* also found in the pathogens PSN and PTR. (Friesen et al., 2018; McDonald et al., 2018).

The management of the complex leaf spotting disease is through the use of moderate resistant cultivars, tillage practices, crop rotation (Fernandez et al., 1999), and mainly with the use of synthetic fungicides such as demethylation inhibitors (DMIs), Quinone outside inhibitors (QoI) and succinate dehydrogenase inhibitors (SDHIs). Examples in the group of QoI are the Strobilurins include; azoxystrobin, pyraclostrobin, and trifloxystrobin; while examples in DMI are the triazoles which include metconazole, propiconazole, prothioconazole, and tebuconazole (Lopez et al., 2015; Wegulo et al., 2012). Fungicide application often guarantees an optimal yield at harvest, but problems with fungicide resistance in the control of these complex pathogens already

exists, and the control is compromised by resistance to the major fungicide classes, DMIs and SDHIs (Blixt et al., 2009; Sierotzki et al ., 2007; Dominguez et al., 2021; Sautua and Carmona, 2021). Moreover, synthetic fungicides cause concerns in the environment and human health (Costa et al., 2008; Damalas and Eleftherohorinos, 2011). Biopesticides enter in the scene as an alternative, safer for humans and benign to the environment (Kumar and Singh, 2015).

Biopesticides are naturally occurring substances in organisms or compounds that kill, hinder, or suppress the growth and proliferation of pests by multiple mechanisms of action. They are categorized into three groups: microbial biopesticides (i.e., *Bacillus thuringiensis* (Bt)), plant-incorporated protectants (i.e., transgenic plants, Bt Cry proteins), and biochemical biopesticides the last one, defined as a compound of natural origin through different mechanisms of action. (i.e., the essential oils (EOs)) (Fenibo et al., 2021). EOs are an important group in biopesticides due to the high diversity of plants and compounds with antimicrobial properties.

The EO, is from the Latin “Quinta essentia “ (quint essence) meaning the efficient part of every drug (Guenther., 1949), which owes its name for the medical reformer Bombastus Paracelsus von Hohenheim (1493-1541). The EOs are complex mixtures of volatile compounds and can be extracted from the plant leaves, bark, flowers, bud, seed, root, stem, and wood. The essential oils in the plants have several ecological roles, such as attracting pollinating insects and as repellents to predators. Additionally, EOs also have shown antifungal or insecticidal properties. (D’agostino et al., 2019; Dhifi et al., 2016). It is important to mention that the amount of these compounds in the EOs depends

on the plant species, geographical location, physiological stage, and mode of obtention (hydrodistillation, steam distillation, dry distillation) (Ferhat et al., 2007; Hussain et al., 2008). Essential oils are soluble in alcohol, ether, and other oils and non-soluble in water.

Essential oils are composed of a mix of bioactive compounds categorized in different classes: alcohols, ethers or oxides, aldehydes, ketones, esters, amines, amides, phenols, heterocycles, and mainly monoterpenes (Dhifi et al., 2016). An example is a study by Střelková., (2021), assessed by gas chromatography mass spectrometry (GC-MS), found that the main compounds in thyme oil were thymol (58%), p-cymene (22%), and linalool (3%). Oregano oil was composed of carvacrol (70%) followed by p-cymene (11%) and thymol (3%). The lemongrass oil contained geranial (42%) and neral (28%), and geraniol (5%). And the major components in cajeput oil were terpinen-4-ol (44%), γ-terpinene (20%), and p-cymene (14%); α-terpineol (4%) and 1,8-cineole (3%). Krzyśko et al., (2020) also reported that the composition of thyme, lemongrass, verbena, and cajeput have a high content of monoterpenoids between 59.02–87.17%. where the monoterpenes are important in the EOs for their antifungal activity (Fierascu et al., 2020). The most studied terpenoids for antimicrobial properties are eugenol, eucalyptol, germacrene, carvacrol, limonene, and thymol, (Burt, 2004; Lambert et al., 2001; Shen et al., 2016). The EO possesses multiple modes of action due to the diversity of its compounds; one of the mechanisms of action of the EOs is the interruption in the ergosterol synthesis in the plasma membrane affecting the integrity and cell wall synthesis, causing disorders in the mitochondrial membrane potential, and disrupting the DNA and gene expression of the pathogens reviewed by Maurya et al., (2021).

Tea tree (*Melaleuca alternifolia*), lemongrass (Cy*mbopogon flexuosus*), karanja oil (*Millettia pinnata*), neem oil (*Azadirachta indica*), and the bioactive compounds; carvacrol, thymol and linalool have shown antifungal properties (Hou et al., 2020; Jiménez et al., 2019; Krzyśko et al., 2020), in a high number of pathogens including BPS (Bahadar and Munir, 2016).

Due to the known antifungal potential of essential oils and bioactive compounds and the efficacy in the control of different pathogens that cause yield loss in agriculture, EOs fill the need to find alternatives that are safer for humans and friendly to the environment. This study aimed to test *in vitro* antifungal potential of four EOs and three compounds in the control of PTR, PSN, and BPS that cause leaf spotting diseases in wheat. To the best of our knowledge, this is the first study to determine the potential of these EOs and compounds *in vitro* for leaf spotting diseases management in wheat.

Teleomorph	**Anamorph**	**Leaves**	**Seeds**	**Roots**	**Spikelet**
Pyrenophora tritici-repentis (Died)	*Drechslera tritici-repentis (Died)*	yellow leaf spot, Tan Spot	red smudge		
Phaeosphaeria nodorum (E. Müller)	*Stagonospora nodorum [Berk.] Castellani and E.G. Germano)*	Stagonospora Nodorum Blotch			- Septoria nodorum blotch -glume blotch
Cochliobolus sativus (S. Ito and Kurib) Drechsler ex Dastur	*Bipolaris sorokiniana*	Spot Blotch	Black point	Common root rot and crown rot	

Table 3.1. Spotting diseases caused by three pathogens that also affect distinct parts in wheat plants.

3.3 Materials and Methods

3.3.1 Fungal Isolates

Fungal pathogens PTR, PSN and BPS were obtained from the plant science laboratory and seed tech laboratory building at South Dakota State University the three isolates are rutinary used for screening wheat germplasm.

3.3.2 Materials Compounds Essential Oils

In this study, four 99.9% pure essential oils were evaluated; tea tree (*Melaleuca alternifolia*), Lemongrass (*Cymbopogon flexuous*) from doTERRA®, karanja oil (*Millettia pinnata*), and neem oil (*Azadirachta indica*), and three compounds at 99.9% purity; carvacrol, thymol, and linalool from sigma Aldrich®. Both the essential oils and the compounds were mixed with 0.1% Tween-20 in each experiment to get them into emulsions. Carvacrol, thymol, and linalool were diluted in acetone before of each experiment.

3.3.3 Determination of Minimum Inhibitory Concentration (MIC)

For the determination of MIC, which represents the concentration that completely inhibits the growth of the fungus, agar dilution method was made by mixing PDA amended with each EO, along with an untreated control of PDA without EOs was assessed. Concentrations of 100, 200, 500, 1000, 1500, and 2000 ppm were assessed for tea tree and lemongrass; concentrations of 150, 300, 700, 1200, 1600, and 2500 ppm for

karanja and neem oil; and finally, concentrations of 50, 100, 200, 500, and 700 ppm were tested for carvacrol, thymol, and linalool. Each concentration was prepared in sterilized, molten, then cooled PDA medium which was then poured in fifty-millimeter petri dishes (50 x 20 mm; Falcon, USA). Mycelial disc plugs of each culture pathogen; PTR, BPS, and PSN were placed in the center of the plate with a sterilized 5mm, cork borer. Plates were then incubated at 25±2°C for four days and the radial growth was measured with a caliper in cross shape and when the fungus reached maximum growth in control plates. All treatments consisted of three replicates laid out in a complete randomized design, the experiment was realized twice, and the averages of the experimental results were determined with the percentage of growth inhibition:

MGI% = (Dc − Dt)/(Dc − D0) × 100. **Equation (1)**

Mycelial growth inhibition percent (MGI %), where D0: is the diameter of the initially inoculated plug, Dc: is the diameter of control treatment and Dt: is the diameter of each treatment.

In this study, the MIC was defined as the lowest EO or compound concentration at which no mycelium growth was visually evaluated after four days of incubation.

3.3.4 Determination of the Minimum Fungicidal Concentrations (MFC)

The minimum fungicidal concentration (MFC) is the lowest concentration of an EO that kills the fungus or shows fungicidal activity. To determine the minimum fungicidal concentration (MFC), the MFC was evaluated by taking a plug of the fungus that showed the complete inhibition from the MIC test and transfer to a fresh PDA plate

without the amended EOs or constituents. After four days, the petri plates were measured as “positive growth” where the growth was observed or “negative growth” where the fungus did not show growth. The MFC was defined as the lowest concentration of EOs where there was no observed regrowth on fresh PDA.

3.3.5 Fungistatic Effect of Volatile Essential Oils

The fungistatic volatility activity of EOs was evaluated in PTR, PSN, and BPS in the highest concentration used in the MIC or the next concentration above in the case of tea tree **(Table 3.4).** Media plates of PDA were amended with EOs and compounds, and PDA plates without EOs were used in the same way as the control. After 12 hours, when the agar amended with the EOs had solidified, four 6 mm plugs were removed with a cork borer from each mix of EOs amended PDA and transferred onto a new, sterile V8-PDA plates (150 ml V8 juice, 10 g PDA, 3 g $CaCO_3$, 10 g agar, and 1-liter distilled H_2O) (Lamari and Bernier, 1989) without treatments at 20 mm from the center. Then, a 5 mm plug of a 7-day old, actively growing culture of each pathogen in the center was placed, and petri plates were sealed with parafilm (Bemis, laboratory films). The fungistatic activity of the EOs was assessed with the radial mycelial growth and calculated fungal mycelial inhibition with **Equation 1.**

3.3.6 Efficacy of Plant Essential Oils on Conidia Germination

Conidia germination inhibition assays were tested in petri dishes (50 mm diameter) having PDA amended and non-amended (control) with EOs. Petri plates with the amended treatments were inoculated with a suspension of 200 spores/ml adjusted by

hemocytometer, using four drops of 100 microliters each one obtained from sporulated mycelia of 7-day-old cultures grown on V8-PDA media.

After a 12 h incubation at 22 ± 2°C, lactophenol cotton blue was used for spore staining where germinated and non-germinated spores were counted under microscope as seen under 40X (Leica microsystem). At least 100 conidia were counted for each drop deposited in the petri dishes and an average per each petri plate was obtained. Conidia were considered germinated when the germ tube length was at least half the length of the diameter of the conidia. Three replicates (petri dishes) were used for each treatment and the experiments were repeated twice.

Percent germination was determined with the formula:

% Spore germination = (Number of spore germinated/ 100 spores) *100

3.3.7 Statistical analysis

Data was analyzed using a generalized linear mixed model (GLIMMIX, SAS version, SAS institute, 2016) to examine the effect of EOs with appropriate link function where necessary. A post-hoc test by Tukey's HSD was used to determine significant differences at a level of ($P < 0.05$). All experiments consisted of three replicates per treatment and each experiment was performed twice. Data were transformed as necessary and was presented as the means of the original data (non-transformed).The repeated experiment was compared and there was no significant variability between them, therefore data was combined across repeats.

3.4 Results

3.4.3 Minimum Inhibitory Concentration (MIC) in Tested EOs and Compounds

Of the four essential oils tested, (tea tree, lemongrass, neem and karanja) lemongrass showed MIC against PTR and PSN. This essential oil inhibited 100% of the mycelium growth for PTR at 1000 ppm and PSN at 500 ppm. Additionally, among the EOs, lemongrass showed the highest level of inhibition for BPS at 84% MGI. The EO tea tree showed a MIC in PTR at 2000 ppm, while for this same concentration it showed mycelial growth inhibition of 71% in BPS and 81% in PSN. Karanja and neem oils at the highest concentration showed the lowest or null mycelial inhibition in the three pathogens.

The compounds carvacrol at 200 ppm showed MIC in PTR, while for PSN and BPS, MIC was found at 500 ppm (**Table 3.2.1 and 3.2.2, Figure 3.1)**.For the treatment thymol in the fungal pathogens PTR and PSN, MIC was observed at 200 ppm. Meanwhile in BPS was observed at 500 ppm. Linalool showed the lowest inhibition of the three pathogens. In general, BPS was the fungus with the smallest fungal inhibition showed by all treatments **(Appendix.3.1).**

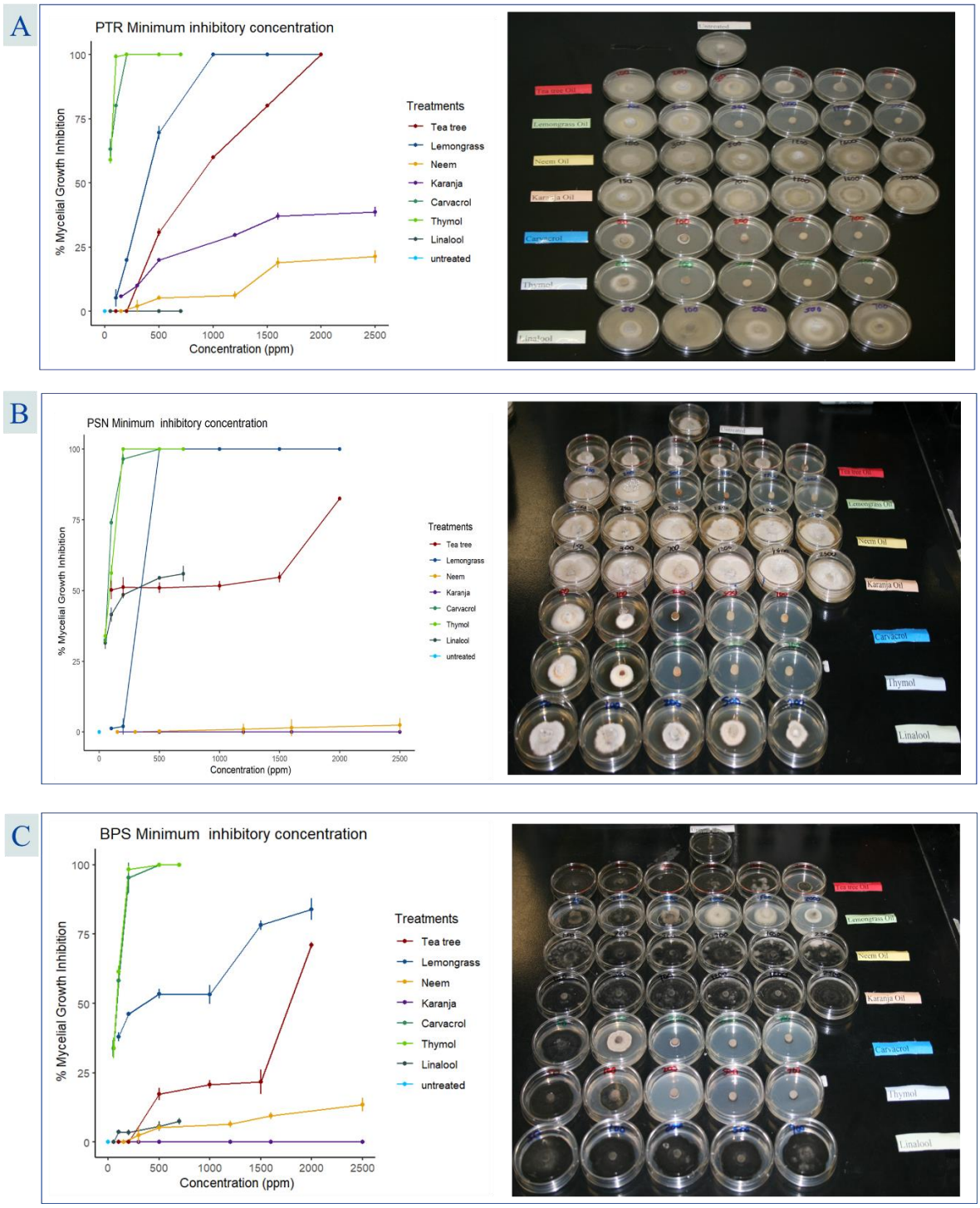

Figure 3.1. Mycelial growth inhibition of PTR, PSN, and BPS. 100% of inhibition represents the MIC for each treatment. **A)** The pathogen PTR tested at different

concentrations showed 100 % of mycelial growth inhibition at 1000 ppm for lemongrass, and 2000 ppm for tea tree and compounds thymol, and carvacrol at 200 ppm, respectively. **B)** The 100 % MGI growth of PSN was observed at 500 ppm for lemongrass, 500 ppm for the compound carvacrol, and 200 ppm for thymol; **C)** In the case of BPS, carvacrol and thymol inhibited 100% growth at 500 ppm.

Table 3.2.1. Means (± SD) of the mycelial growth inhibition for *Pyrenophora tritici-repentis* (PTR), *Parastagonospora nodorum* (PSN)), and *Bipolaris sorokiniana* (BPS). Tested with four essential oils. at different concentrations. Where the 100% of mycelial growth inhibition is taking as the minimum inhibitory concentration (MIC).

Essential oils		**Mycelial growth inhibition %**		
	Concentration (ppm)	**PTR**	**PSN**	**BPS**
Tea Tree	100	0.00 ± 0.00	50.25 ± 3.30	0.00 ± 0.00
	200	0.00 ± 0.00	51.25 ± 3.50	0.00 ± 0.00
	500	30.75 ± 1.50	51.00 ± 1.82	17.25 ± 2.21
	1000	60.00 ± 0.00	51.75 ± 1.70	20.75 ± 1.50
	1500	80.00 ± 0.00	54.75 ± 1.89	21.75 ± 4.50
	2000	100.00 ± 0.00	81.50 ± 1.00	81.50 ± 0.00
Lemongrass	100	5.25 ± 3.40	1.25 ± 0.50	38.00 ± 1.63
	200	20.00 ± 0.00	2.00 ± 2.82	46.25 ± 0.50
	500	69.50 ± 2.64	100.00 ± 0.00	53.50 ± 1.73
	1000	100.00 ± 0.00	100.00 ± 0.00	53.25 ± 3.40
	1500	100.00 ± 0.00	100.00 ± 0.00	78.25 ± 1.70

	2000	100.00 ± 0.00	100.00 ± 0.00	84.00 ± 4.00
Neem	150	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	300	2.00 ± 2.44	0.00 ± 0.00	2.50 ± 2.88
	500	5.25 ± 0.95	0.25 ± 0.50	5.25 ± 0.95
	1200	6.25 ± 1.25	1.00 ± 2.00	6.50 ± 1.29
	1600	19.00 ± 2.00	1.50 ± 3.00	9.50 ± 1.29
	2500	21.25 ± 2.50	2.50 ± 2.38	13.50 ± 2.38
Karanja	150	5.75 ± 0.50	0.00 ± 0.00	0.00 ± 0.00
	300	10.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	500	20.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	1200	29.75 ± 0.50	0.00 ± 0.00	0.00 ± 0.00
	1600	37.00 ± 1.41	0.00 ± 0.00	0.00 ± 0.00
	2500	38.75 ± 1.89	0.00 ± 0.00	0.00 ± 0.00

Table 3.2.2. Means (± SD) of the mycelial growth inhibition for *Pyrenophora tritici-repentis* (PTR), *Parastagonospora nodorum* (PSN)), and *Bipolaris sorokiniana* (BPS). Tested with three compounds presents in the essential oils.at different concentrations. Where the 100% of mycelial growth inhibition is taking as the minimum inhibitory concentration (MIC).

Compounds		**Mycelial growth inhibition %**		
	Concentration (ppm)	**PTR**	**PSN**	**BPS**
Carvacrol	50	63.25 ± 3.94	32.50 ± 1.29	33.75 ± 2.98
	100	80.00 ± 0.00	74.00 ± 1.41	58.25 ± 0.50

	200	100.00 ± 0.00	90.00 ± 0.00	95.25 ± 5.50
	500	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	700	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Thymol	50	59.00 ± 1.41	34.00 ± 2.58	34.00 ± 3.55
	100	96.00 ± 0.00	56.25 ± 2.75	61.50 ± 2.08
	200	100.00 ± 0.00	100.00 ± 0.00	96.50 ± 0.00
	500	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	700	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Linalool	50	0.00 ± 0.00	31.50 ± 2.08	0.00 ± 0.00
	100	0.00 ± 0.00	41.50 ± 2.51	3.75 ± 0.50
	200	0.00 ± 0.00	48.50 ± 1.29	3.50 ± 1.00
	500	0.00 ± 0.00	54.50 ± 0.57	5.75 ± 1.70
	700	0.00 ± 0.00	56.00 ± 2.70	7.50 ± 1.00
Untreated		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

3.4.4 Minimum Fungicidal Concentrations (MFC)

MFC was found at the same concentration of the MIC in lemongrass.100 and 500 ppm for PTR and PSN, respectively. The MFC test in thymol and carvacrol showed regrowth at 200 ppm for PTR, however, at 500 ppm, the growth was reported as "negative" **(Figure 3.2).** In general, at 500 ppm, the compounds thymol and carvacrol showed the MFC for the three pathogens **(Table 3.3).** For this experiment, treatments with neem, karanja oil, and linalool were not tested because the MIC was not found at the concentration tested.

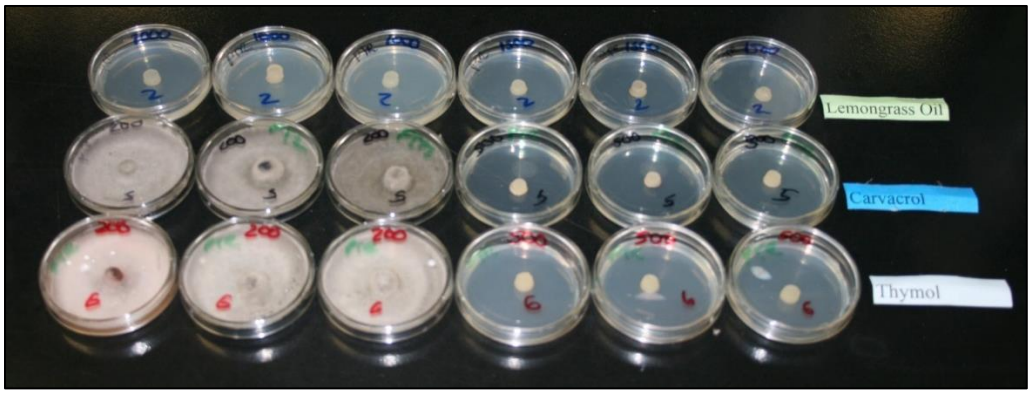

Figure 3.2. Minimal fungicidal concentration (MFC), tested against *Pyrenophora tritici-repentis* showing three replicates per treatment, where the MFC in lemongrass was found at 1000 ppm and in carvacrol and thymol at 500 ppm, respectively.

Table 3.3 Treatments showing the minimum fungicide concentration, for; *Pyrenophora tritici-repentis* (PTR), *Parastagonospora nodorum* (PSN) and *Bipolaris sorokiniana* (BPS). The MFC was defined as the lowest concentration that inhibit the fungus development in the plug. It was tested by putting the completely inhibited fungus found in the MIC into a new fresh PDA(without EOs). Reported as positive or negative growth.

EOs, and compounds	PTR	PSN	BPS
Tea Tree	**Positive (2000 ppm)**		
Lemongrass	Negative (1000 ppm) Negative (1500 ppm)	Negative (500 ppm) Negative (1000 ppm)	
Carvacrol	**Positive (200 ppm)** Negative (500 ppm)	Negative (500 ppm) Negative (700 ppm)	Negative (500 ppm) Negative (700 ppm)
Thymol	**Positive (200 ppm)** Negative (500 ppm)	**Positive (200 ppm)** Negative (500 ppm)	Negative (500 ppm) Negative (700 ppm)

3.4.5 Effect of EOs on Spore Germination

The effect of tea tree and lemongrass when were tested on PTR, PSN and BPS showed a complete inhibition in the germination (0% of spore germination). In manner similar to the compounds carvacrol and thymol at 200 ppm. The essential oils neem and karanja at 2000 ppm and the constituent linalool at 200 ppm did not show any sporicidal properties (100% of germination). This was equal to the untreated group that showed 100% of spore germination **(Table 3.4).**

Table 3.4. Treatments EOs and compounds were tested at different concentration (ppm). Percentage of spore germination, different letters in the same column for each treatment represent significant differences (P≤ 0.05). Treatments with the same letters are not significantly different from.

EOs and compounds		PTR	PSN	BPS
Tea tree	(2000)	0 c	0 c	0 c
Lemongrass	(1000)	0 c	0 c	0 c
Neem	(2500)	95 b	99.25 ab	99.66 a
Karanja	(2500)	100 a	99.67 a	100 a
Carvacrol	(200)	0 c	0 c	0 c
Thymol	(200)	0 c	0 c	0 c
Linalool	(200)	100 a	99.50 a	100 a
Untreated		100 a	97.50 b	100 a

3.4.6 Volatile Fungistatic Activity of EOs

The volatile activity was tested in tea tree at the concentration of 3000 ppm, lemongrass at 1500 ppm, and compounds carvacrol and thymol at 700 ppm.

The mycelium growth percent inhibition measured for the volatile compounds was observed in carvacrol between 51 and 58% and in thymol between 62 and 73% inhibition for the three pathogens at 700 ppm. Due to the different groupings, each pathogen was compared separately, (**Table 3.5, Figure 3.3).**

Table 3.5. Percentage mycelial growth inhibition means (± SD) in the volatile fungistatic test volatility test. Treatments and rates were chosen if they showed fungistatic effect or complete inhibition in the high rates tested in the MIC

Treatment	ppm	PSN	BPS	PTR
Untreated		0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
Tea tree	(3000)*	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
Lemongrass	(1500)	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
Carvacrol	(700)	51.16 ± 0.88 b	57.75 ± 3.20 b	56.68 ± 2.35 b
Thymol	(700)	61.91 ± 3.19 a	68.73 ± 3.14 a	72.82 ± 0.81 a

Different letters in the same column for each treatment represent significant differences (P≤ 0.05). Treatments with the same letters are not significantly different from.

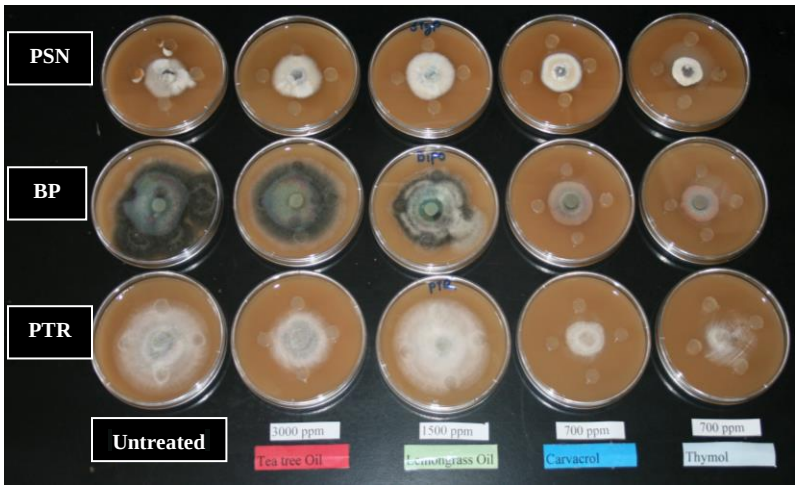

Figure 3.3. The volatile activity of two EOs and two compounds, four days after plugging. Fungal inhibition was recorded between (51.16 to 72.82 %) for carvacrol and thymol at 700 ppm.

3.5 Discussion

Among all the treatments tested, the essential oils lemongrass, the compound carvacrol, and thymol showed the lowest minimum inhibitory concentration. Therefore, lemongrass carvacrol and thymol were the most effective EOs and bioactive compounds inhibiting the mycelium growth of the three pathogens at a concentration of 200 ppm and fungicidal activity at 500 ppm. This agrees with a study by Pérez et al., (2012) which tested carvacrol and thymol *in vitro* to control *Penicillium digitatum* and *Penicillium italicus,* which showed 100% mycelial growth inhibition in concentrations of 250 to 500 ppm. Also, our results agree with an *in vitro* study by Saghrouchni et al., (2021), who tested carvacrol ability to control *Fusarium oxysporum, Neocosmospora solani, and Microdochium nivale* spores showing sporicidal and 100% of fungal reduction at 250 and 500 ppm respectively.

In the spore germination test in our study, carvacrol and thymol showed 100% inhibition at 200 ppm; Several studies have shown that essential oils and their main components have a broad spectrum of antifungal activity, showing fungistatic and fungicidal properties against plant pathogens. Some works have described the extensively antifungal power *in vitro* of thymol and carvacrol (Chen et al., 2022). In this study,

thymol and carvacrol also showed a broad-spectrum activity in the control of PTR, PTN, and BPS.

In the case of the treatments of tea tree, karanja, neem oil, and the compound linalool, the inhibition was minimum or null. In neem oil, the mycelial inhibition was 13.50% at 2500 ppm. This result agrees with studies by Debsharma et al., (2021), who tested in vitro the efficacy of ginger, clove, eucalyptus, tilt, and neem oil in the mycelial growth inhibition, and found on the day four after inoculation similarly inhibition of 16.27%. BPS showed inhibition of 71% with a concentration of 2000 ppm using *Eucalyptus erythrocorys* essential oils (Ben et al., 2013). In the same way, karanja and neem at 2000 ppm did not show inhibition in the spore germination for any of the tested fungal pathogens.

The volatile fungistatic activity of EOs in this study was observed in thymol and carvacrol at 700 ppm. In this study, we evaluated the activity of pure EOs such as tea tree, lemongrass, neem, and karanja that contain a complex mixture of compounds. The mixture of these compounds can cause a synergistic influence on the antifungal activity. However, our results showed that the individuals monoterpenes, carvacrol, and thymol were more effective as sporicidal and inhibiting the mycelial growth and combining thymol and carvacrol has shown increased antifungal activity (Lambert et al., 2001). Phenolic monoterpenes such as carvacrol and thymol have shown antifungal properties in different fungal pathogen species (Saghrouchni et al., 2021). Carvacrol and its isomer thymol cause alterations in hyphal morphology and spore inhibition. Thymol and carvacrol change the morphology and structure of fungus (Bhavaniramya et al., 2019;

Ranjbar et al., 2022), affecting the cellular metabolic targets in pathogenic fungi, disrupting cell walls or membrane integrity, and interfere with ergosterol biosynthesis (De Lira et al., 2012; Zhang et al., 2019; Santra and Banerjee, 2020).

Additionally, thymol has been tested as a chemosensitizing agent (as synergists that affect the target pathogen) mixed with azoles and strobilurins fungicides in vitro, which showed that the mix of fungicides with thymol produced much higher growth inhibition of *Bipolaris sorokiniana*, *Phoma glomerata,* Alternaria sp. and *Stagonospora nodorum* than the fungicide alone (Dzhavakhiya et al., 2012). Additionally, in a study by Kartashov et al., (2019), the use of thymol as a chemosensitizer agent decreased the resistance of the PNm1 strain of *Stagonospora nodorum* to difenoconazole enhancing sensitivity to the difenoconazole.

Lemongrass at 1000 ppm showed the MIC for PTR and PSN. In a similar study conducted by Sharma et al., (2017), lemongrass (*Cymbopogon citrus*) used in vitro by microdilution, showed the MIC of lemongrass essential oil against a pathogenic strain of *Fusarium oxysporum*.

Where the complete inhibition of spore germination and mycelial growth was at a concentration of 250 ppm, this change in the minimum inhibitory concentration compared with our study can be attributed to the species and the method of extraction of the EOs; to our knowledge, this is the first time that lemongrass (*Cymbopogon flexuosus*) has been tested in the control of these three important pathogens.

Lemongrass EO is considered a potential antifungal agent (Al-Ghanayem, 2022; Wan et al., 2019a) that inhibits the mycelial growth by interacting with cellular lipid components in the cell membranes and thereby inhibiting fungal enzymes required for its growth and metabolism (Singh et al., 2016). The major components in most lemongrass species include neral, isoneral, geranial, isogeranial, geraniol, geranyl acetate, citronellal, citronellol, germacrene-D, and elemol that make up about 60–80% of its oil. (reviewed by Mukarram et al., (2022)). The antimicrobial activity of lemongrass is extensively attributed to the citral (aldehyde) and geranial (Wan et al., 2019) present in its oil where this EO also causes plasma membrane disruption and mitochondrial structure disorganization (Wan et al., 2019).

3.6 Conclusion

The use of EOs and bioactive compounds in the control of fungal diseases has gained interest and is currently used in the control of different pathogens that affect agriculture; In this study, we found promising antifungal activity in the EOs for lemongrass and the bioactive compounds thymol and carvacrol in the control of this three important pathogens that cause spotting diseases in wheat. Essential oils bioactive compounds could be used as an effective alternative or complement of synthetic fungicides in agriculture, without inducing environmental problems.

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Appendix 3.1 Mycelial growth inhibition, result multiple comparison between the treatments per each pathogen Means with the same letters in each column are not statistically significantly different (P<0.05).

Treatment EOS and Constituents		**Mycelial growth inhibition**		
	Concentration (ppm)	**PTR**	**PSN**	**BPS**
Tea Tree	**100**	0.00 L	50.25 GH	0.00 N
	200	0.00 L	51.25 FGH	0.00 N
	500	30.75 G	51.00 FGH	17.25 IJ
	1000	60.00 DE	51.75 EFGH	20.75 I
	1500	80.00 B	54.75 DEF	21.75 I
	2000	100 A	81.50 B	71.00 D
Lemongrass	**100**	5.25 JK	1.25 K	38.00 H
	200	20.00 H	2.00 K	46.25 G
	500	69.50 C	100 A	53.50 F
	1000	100 A	100 A	53.25 F
	1500	100 A	100 A	78.25 C
	2000	100 A	100 A	84.00 B
Neem	**150**	0.00 L	0.00 K	0.00 N
	300	2.00 KL	0.00 K	2.50 MN

	500	5.25 JK	0.25 K	5.25 LMN
	1200	6.25 J	1.00 K	6.50 LM
	1600	19.00 H	1.50 K	9.50 KL
	2500	21.25 H	2.50 K	13.50 JK
Karanja	**150**	5.75 J	0.00 K	0.00 N
	300	10.00 I	0.00 K	0.00 N
	500	20.00 H	0.00 K	0.00 N
	1200	29.75 G	0.00 K	0.00 N
	1600	37.00 F	0.00 K	0.00 N
	2500	38.75 F	0.00 K	0.00 N
Carvacrol	**50**	63.25 D	32.50 J	33.75 H
	100	80.00 B	74.00 C	58.25 EF
	200	100 A	90 AB	95.25 A
	500	100 A	100 A	100 A
	700	100 A	100 A	100 A
Thymol	**50**	59.00 E	34.00 J	34.00 H
	100	96.00 AB	56.25 D	61.50 E
	200	100 A	100 A	96.50 A
	500	100 A	100 A	100 A
	700	100 A	100 A	100 A
Linalool	**50**	0.00 L	31.50 J	0.00 N
	100	0.00 L	41.50 I	3.75 MN
	200	0.00 L	48.50 H	3.50 MN
	500	0.00 L	54.50 DEFG	5.75 LM
	700	0.00 L	56.00 DE	7.50 LM
Untreated		0.00 L	0.00 K	0.00 N

Chapter IV : Efficacy of Essential Oils in the Management of Fusarium Head Blight in Spring Wheat

4.1 ABSTRACT

Fusarium head blight (FHB), caused by *Fusarium graminearum* is one of the most important diseases of wheat. FHB results in grain yield loss, seed quality reduction, and the accumulation of mycotoxins such as zearalenone (ZEA) and deoxynivalenol (DON). Fusarium head blight disease management is mainly with the use of partially resistant cultivars and the application of synthetic fungicides to help guarantee a higher yield.

However, the constant application of synthetic fungicides could cause negative impacts on humans and in the environment. Furthermore, fungicides used to control *F. graminearum* belong mainly to one class of fungicides, the triazoles, increasing the risk of loss of the pathogen sensitivity to the fungicides. Therefore, there is a need to test and implement new biological and eco-friendly alternatives for managing FHB. Essential oils (EOs) are volatile secondary metabolites extracted from plants, characterized by their aromatic and lipophilic nature, and composed of a mix of bioactive compounds such as terpenes, phenols, aldehydes, alkaloids, and flavonoids with known antifungal properties.

Therefore, the objective of this study was to evaluate the efficacy of two pure EOs (tea tree and lemongrass), two commercially available EOs with proven antifungal properties (Thymox® and Timorex®), and two bioactive compounds present in EOs (carvacrol and thymol), in the management of FHB.

The study was conducted *in vitro* using inhibitory disk diffusion to detect the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). Additionally, conidia germination inhibition and volatile activity evaluated. I*n vivo*, the EOs and constituents were tested under greenhouse and field conditions. Disease FHB index, yield, FDK, and DON were evaluated. There was 100% MIC in *F. graminearum* with lemongrass at 1000 ppm, Thymox at 500 ppm, and carvacrol, and thymol at 200 and 500 ppm, respectively.

One hundred percent spore germination inhibition was observed at 1000 ppm of lemongrass, tea tree, and Thymox®, and at 500 ppm of carvacrol and thymol. In this study, all the EOs concentrations showed a MFC equal to the MIC except, with carvacrol where fungal regrowth was observed, which showed the MFC at 500 ppm. Volatility fungistatic activity of EOs was observed in lemongrass, Thymox®, and thymol with 6.25 to 38.44% of mycelial growth inhibition compared with the untreated.

For the greenhouse and field studies, spring wheat susceptible cultivar “Select” was inoculated at anthesis by spraying *F. graminearum* spore suspension and then treated with the EOs. Spikelets were evaluated 15 days after inoculation for FHB disease index (DI). Plants treated with lemongrass, carvacrol thymol, and Thymox® showed a significantly lower DI ($P<0.05$) relative to the untreated control. In the field trial treatments the EOs in emulsions showed an increase in yield between 10.4 and 13.2% relative to the untreated control. The DON concentration decreased between 36 and 59% for all the treatments with EOs compared to the control.

In vitro and *in vivo* experiments, we reported the fungicidal and fungistatic potential of the EOs lemongrass, carvacrol, thymol and the commercial EO Thymox® in the control of *F. graminearum.*

4.2 Introduction

Fusarium head blight (FHB) is one of the most damaging diseases that affects wheat worldwide. This disease is caused by the pathogen *Fusarium graminearum* along with *F. culmorum, F. avenaceum,* and other related fungi (Haile et al., 2019; Osborne and Stein., 2007; Sun et al., 2014). The disease results in indirect economic losses, including reduced grain yield and quality (aborted or shriveled kernels, and reduced seed size) and indirect loss due to contamination by mycotoxins deoxynivalenol (DON) and zearalenone (ZEA) (Desjardins., 2006). There are no effective controls, against FHB such as fully resistant cultivars or cultural controls such as tillage and crop rotation. However, the management of the disease is primarily by the use of fungicides and moderately resistant varieties (Angelo et al., 2014; Comby et al., 2017).

Fungicides are synthetic molecules that disrupt the metabolic process of the fungal cells, inhibiting biochemical processes and affecting the fungus, i.e., nucleic acid metabolism, cellular respiration, or cell wall stability (ergosterol biosynthesis). Most of the fungicides used in controlling FHB are demethylation inhibitors (DMI) of the chemical group triazoles such as tebuconazole, metconazole, propiconazole, and prothioconazole or the mix prothioconazole plus tebuconazole (Tini et al., 2020; Wegulo et al., 2015). However, pathogens such as *F. graminearum* have shown a loss of

sensitivity due to the overuse of fungicides belonging to the same group (Chaves et al., 2022; Spolti et al., 2014; Tini et al., 2020). In addition, it is well known that synthetic fungicides use implies hazardous effects to humans and can negatively impact the environment (Nicolopoulou et al., 2016; Zubrod et al., 2019).

Therefore, it is urgent to find alternatives that are environment and user friendly, capable to avoid pathogen resistance, with relatively low toxicity to humans, and with the potential to be added to integrated pest management programs (IPM). In recent years, the study of natural alternatives for the control of crop diseases has gained attention. Extracts from plants essential oils (EOs), are one of the most promising sources to develop new safe products compounds with antifungal properties.

The EOs are defined as volatile plant secondary metabolites and classified as biopesticide derivates from plant extracts, generally composed of a complex mixture of bioactive compounds such as monoterpenes, esters, sesquiterpenes, phenols, and aldehydes. Where most of them are hydrophobic compounds soluble in alcohol (Gato et al., 2021). The chemical composition of EOs depends on different factors such as the physiological stage of the plant, climate characteristics, soil conditions, environment, and harvest time (Dassanayake et al., 2021). The essential oils are potential fungicides with a different mechanism of action, having a wide spectrum of compounds with the advantage that they mostly are non-toxic to humans and are highly biodegradable in the environment (Medina-romero et al., 2022).

EOs extracted from lemongrass (*Cymbopogon flexuosus*) are mainly composed of geraniol, and from tea tree (*Melaleuca alternifolia*) mainly composed of terpinen-4-ol, γ-terpinene, p-cymene, α-terpinene, 1,8-cineole, α-terpineol, and α-pinene. These EO extracts have shown antifungal properties against pathogens that cause disease in crops (Devi et al., 2021; Kalagatur et al., 2018; Lee et al., 2021; Moumni et al., 2021; Terzi et al., 2007). On other hand, bioactive compounds such as the monoterpenes, phenol, thymol, and carvacrol are present in large amounts in aromatic plants such as oregano (O*riganum vulgare*) and thyme (T*hymus vulgaris*) (Lambert et al., 2001; Morshedloo et al., 2018; Sakkas and Papadopoulou., 2017) which have shown efficient fungicidal and bactericidal activity against several fungal pathogens including *F. graminearum* (Shen et al., 2016; Chen et al., 2022; Pérez et al., 2012; Zhang et al., 2019). Currently on the market are biopesticides that contain EOs and are registered in the Organic Materials Review Institute (OMRI) list (https://www.omri.org/omri-lists) which evaluates products allowed for use in organic production, i.e., Timorex® (tea tree oil…12.5 %), and Thymox® control (Thyme oil… 27%).

Essential oils and bioactive compounds have exhibited antifungal properties against *F. graminearum* reported by mycelial growth inhibition (Krzysko et al., 2019; 2020), spore germination inhibition (Gao et al., 2016), and also in the reduction of mycotoxins such as deoxynivalenol (DON) (Perczak et al., 2019) and zearalenone (ZEA) (Kalagatur et al., 2015). However, little information is known about the efficacy of EOs in the control of FHB under field conditions.

In this study, we explore the efficacy of two pure EOs; tea tree, and lemongrass, two EOs with a market label Thymox® and Timorex®, and two monoterpenes-phenols bioactive compounds (carvacrol and thymol) that have shown *in vitro* to have efficacy in the control of *F. graminearum* **(Figure 4.1)** and also are listed in the fungicidal resistance action committee (FRAC) and are classified as biologicals with multiple modes of action: Plant extracts, carvacrol and thyme (FRAC List, 2022).

4.3 Materials and Methods

4.3.1 Chemical Products and Fungal Pathogen Isolation

Essential oils, tea tree (*Melaleuca alternifolia*) and lemongrass (*Cymbopogon flexuosus*), were obtained from doTERRA® at 99.9% purity. Two commercially labeled essential oils were used: Timorex ACT® (tea tree… 12.5%) from Summit AgroUSA and Thymox Control® (Thyme oil… 27%) from Kemin, and finally two constituents of essential oils, carvacrol, and thymol at 99.9 % of purity from Sigma-Aldrich, Acetone 99.5% VWR chemicals, Tween-80, and glycerol 99% FisherThermo Scientific.

4.3.2 Fungal Pathogen

The *F. graminearum* (*Fg)* strain used for this experiment was obtained from the strain collection of the extension plant pathology laboratory at South Dakota State University. A fresh culture of the isolate ***Fg*** was initiated by plating the dry isolate plugs from storage onto PDA (Potato dextrose agar = 19.5 g; agar = 12.5 g; distilled water =

950 ml) plates and incubated for 5–6 days at room temperature. Plugs of this culture were then multiplied for each experiment.

4.3.3 Determination of the Minimum Inhibitory Concentration (MIC)

Volumes of the EOs were mixed with 0.05% Tween-80 and added to PDA medium to reach the desired concentrations. Essential oils were tested at different concentrations depending on their origin and previous *in vitro* experiment results. Lemongrass and tea tree were tested at rates of; 100, 200, 500, 1000, and 1500 ppm; commercially available products Thymox® at 100, 200, 500, 1000, and 1500 ppm, Timorex ACT® at 100, 200, 500, 1000,1500, 2000, 2500, 3000 and 4000 ppm; and the components of the EOs thymol and carvacrol at the rates of 50,100, 200, 500, 1000 and 1500 ppm; along with an untreated check control of distilled water.

The different concentrations of essential oils were added to the cooled liquid PDA before pouring into sterile plates (50 mm diameter), petri plates were then left overnight at room temperature. Plugs of the cultured *Fg* isolate were cut with a sterile, 5 mm cork borer from the margin of the actively growing region of a seven-day old culture and placed in the center of each new petri plate containing the PDA-EOs media. The inoculated plates were incubated at 20±2 ^{0}C for five days with intervals of 12 h light and 12 h dark. Radial growth measurements were made with a caliper (Vernier 0.001"/0.02mm) and the average mycelial inhibition was determined from three replicate (petri plates). The experiment was repeated twice, and the percentage of fungal inhibition was calculated using the following equation 1:

MGI%= = (Dc − Dt)/(Dc − D0) × 100 **(1)**

mycelial growth inhibition percent (MIG %); where D0: is the diameter initially of the inoculation plug, Dc: is the diameter of control and Dt: is the diameter of each treatment. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the EOs where 100% of fungal inhibition was present.

4.3.4 Effect of EOs in Spore Germination

Conidia germination assays were tested *in vitro* on petri dishes (50 mm diameter) containing PDA amended with the EOs lemongrass, tea tree, and Thymox® at the concentration of 1000 ppm, Timorex® at 2000 ppm, and the bioactive compounds thymol and carvacrol at 500 ppm. PDA without the essential oils was used as the untreated control.

Petri plates with the amended treatments were inoculated with *F.graminearum* obtained from sporulating mycelia of 7-day-old cultures using four drops of a 100 microliters suspension containing 200 spores/ml adjusted using a hemocytometer.

After the 12 h incubation at 22±2°C, lactophenol cotton blue was used for spore stain, and the germinated spores were observed under a microscope (Leica microsystem) under 40X magnification. At least 100 conidia were counted for each drop deposited in the prepared petri dishes and the average per each petri plate was obtained. Conidia were considered germinated when the germ tube length was at least half the length of the diameter of the conidia. Three replicates were used for each treatment and the experiments was repeated twice.

The percent of germination was determined with the formula:

% Spore germination = (Nº spore germinated/ 100 spores) *100

4.3.5 Minimum Fungicide Concentration

Minimum fungicide concentration (MFC) is defined as the lowest concentration that kills the fungus (Parikh et al., 2020; Talibi et al., 2012). The MFC was tested by taking a plug of the fungus that showed the complete inhibition from the MIC test and then transferred to a fresh PDA plate without the amended EOs or bioactive compound. After four days, the petri plates were evaluated as "positive growth" where regrowth was observed or "negative" where the fungus did not grow. The MFC was defined as the lowest concentration of plugs with EOs where no regrowth was observed on fresh PDA.

4.3.6 Antifungal Activities of Volatile Essential Oils

Most antifungal tests *in vitro* are defined by the direct contact between the anti-biological products and the fungal pathogen or avoiding direct contact between them. EOs possess a high volatility activity due to their structural composition (phenols, terpenoids). This last test is an important indicator of the fungistatic potential of the EOs. Where the fungistatic effect is defined as the reversible growth in the absence of the volatile compound.

The fungistatic volatility activity of EOs was tested in *F. graminearum* in the highest concentration used in the MIC test and the concentration above this **(Table 4.5).**

The media PDA was amended with the EOs and a control (without EOs) was prepared in the same way as mentioned above. After 24 hours, 6mm diameter plugs were removed with a cork punch from the PDA amended with EOs and transferred to petri plates (90 mm diameter) containing fresh sterile PDA (without treatments). Four plugs of the PDA amended with EOs were evenly placed 20 mm from the center on a new, sterile PDA plate. Following this, in the center of each plate, was placed a 5mm plug of *F. graminearum* extracted from an actively growing seven-day old culture **(Figure 4.5),** and the petri plate was then sealed with parafilm (Bemis, laboratory films). The fungistatic activity of the EOs and radial mycelial growth was measured with a caliper five days after inoculation and the fungal mycelial inhibition was calculated using Equation 1.

4.3.7 Essential Oils in Greenhouse and Field Tests

Studies to establish the efficacy of EOs in managing FHB were conducted in the greenhouse and the field. A greenhouse experiment was accomplished in the South Dakota State University Plant Science greenhouse complex and in the field at the South Dakota State University SDSU Volga Research Farm, where the trial was planted on April 30, 2021.

4.3.7.1 Essential oils, Emulsion Formulation

EOs are subject to various criticisms as a fungicide due their high volatility and degradability in the environment and their hydrophobic and generally lipophilic characteristics (Kalagatur et al., 2018). To overcome these issues, the applications in the greenhouse and in the field the EOs lemongrass, tea tree, and the bioactive compounds

carvacrol and thymol were prepared as nanoemulsions, which offer the advantages of decreasing the volatility and increasing the stability and water dispensability (Giunti et al., 2019). Nanoemulsions can be obtained by two main processes categorized as high-energy or low-energy. High-energy methods use mechanical devices that disrupt the oil (hydrophobic molecules) and aqueous phase (distilled water) into nano droplets dispersed in the water. Low-energy, which does not need an external mechanical force for its formation is created by its chemical nature; for example, spontaneous emulsification where an organic phase containing oil is mixed with the aqueous phase and results in spontaneous oil droplets.

In this experiment, a high-energy method was carried out using sonication following the methodology of Campolo et al., (2020) with few modifications. The compounds used for nanoemulsion are summarized in **(Table 4.1).** EOs and substances were mixed in a 50 mL beaker where the EO and the surfactant "Tween-80" were first mixed with the glycerol and the aqueous phase was then slowly mixed with the oil mix under magnetic stirring at 800 rpm for 30 minutes. The bioactive compounds: carvacrol and thymol were dissolved in acetone and later added with the other ingredients to produce the emulsion then formulations were sonicated for 30 min using a sonicator (The Virtis Company, inc. Gardiner, NY. 12525 40 USA) at 100 W power (frequency: 18 kHz). Where the process was carried out in an ice bath to avoid the EO degradation due to the heat generated by the sonicator. The physical stability of the nanoemulsion was evaluated visually (Chang et al., 2013). After seven days the formulation that did not sowing separation were chosen for the applications (**Figure 4.2).**

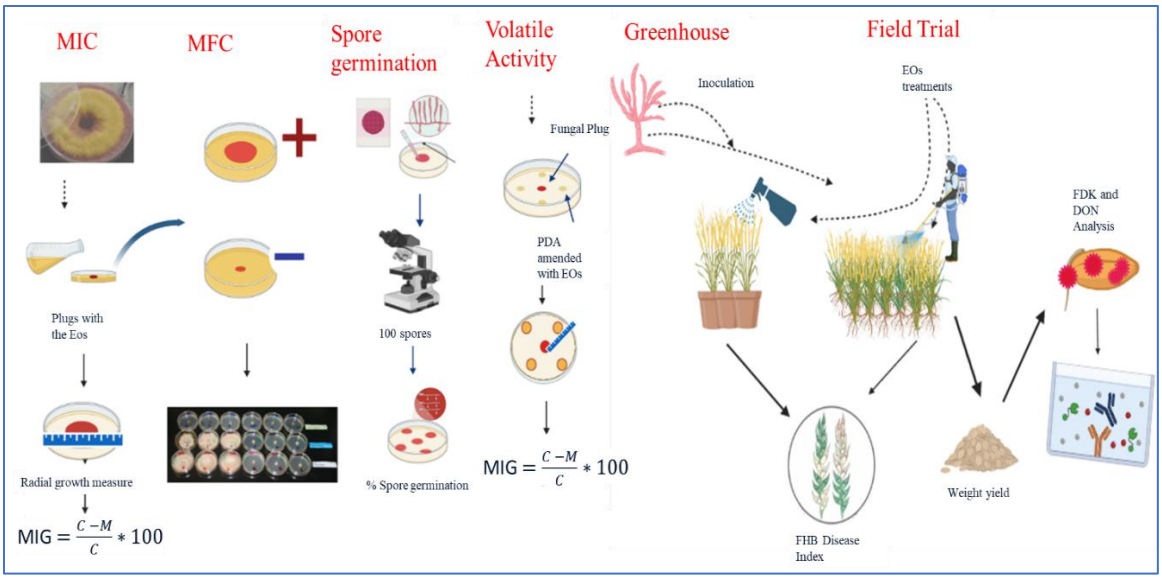


Figure 4.1. Methodology and experiments summary *in vitro* and *in vivo*. Where *in vitro* four tests were used to evaluate the efficacy of the *EOs* in the control of *Fusarium graminearum*, and *In vivo* where EOs efficacy was tested in the control of FHB under greenhouse and field conditions.

Figure 4.2 Emulsion visual stability test after 24 hours of mixed showing three different concentrations of oil-water used to stabilize the emulsion. From left to right: 4, 8, and 12 % of the surfactant Tween -80.

Table 4.1. Final mix of compounds and essential oils (tea tree, lemongrass, carvacrol, and thymol) combined used to spray in greenhouse and field experiments.

Compound mixed	Amount (v/v) %
EOs	15
Tween (80)	4
Glycerol	5
Distilled water	75
Acetone	1

4.3.7.2 Effect of Essential Oils in the Control of FHB in Greenhouse

Susceptible wheat variety "Select" was sown into 5 cm diameter x 20 cm depth containers and filled with the soil mix, Pro-Mix® BX mycorrhizae (Greenhouse Megastore, Danville, IL). Three seeds per cone were planted, and three cones per treatment were used as replicates in a completely randomized design. Where the experiment was executed twice. Along with the EO treatments, the synthetic fungicide Prosaro, and an un-treated check used as control, treatments were sprayed at anthesis (Feekes growth stage 10.5.1) in different concentrations depending on the EOs **(Appendix .4.2).** After 12 hours of the treatment application, plants were inoculated with *F.graminearum* at a concentration of 6x10^4 spores/ml previously determined with a hematocytometer and adjusted with distilled water. Spikelets were then covered with plastic Ziploc bags to keep the humidity around the heads for twelve hours. FHB disease index (DI) was calculated following the equation proposed by Gorczyca et al., (2018).

Where DI is equal to the disease severity multiplied by the disease incidence divided by 100. The rating was taken 16 days after the inoculation.

4.3.7.3 Effect of Essential Oils in the Control of FHB in the Field Trial

The same susceptible variety "Select" was planted in field plots using a 7-row tractor-mounted small grain planter fitted with cone units at a seeding rate of 323 seeds/m2. The plot size was 1.5m wide by 4.6m long and each treatment had four replicates planted in a randomized complete block (RCBD). Field plots were under mist irrigation to increase the incidence of the disease. Plant extract EOs were sprayed at anthesis (Feekes growth stage 10.5.1) at the rates of application listed in (**Appendix 4.3).** Commercial synthetic fungicide Prosaro (i.a prothioconazole + tebuconazole) and an untreated check without treatment was used as control. Sixteen hours after the application of the EOs, plots were inoculated with *Fusarium graminearum* using a CO_2-powered backpack sprayer with 6x10 ^4 spore/ml concentration. FHB disease was assessed 15 days after treatment (Dat). Fifty spikes were randomly chosen in each plot and using the scale modified by Gorczyca et al., (2018), FHB disease index (DI) was calculated. Plots were harvested using a Wintersteiger combine. And yield weight, test weight, and protein content were recorded per each plot.

4.3.7.4 Effect of EOs in the Control of Fusarium-Damaged Kernels and DON Concentration

After the weighing of seeds to determine the yield, 20 grams of seeds were collected from each experimental unit (field plot) for Fusarium-damaged kernels (FDK)

and deoxynivalenol (DON) assessment. FDK was analyzed following the methodology by Ackerman et al. (2022), where twenty grams of seeds per plot were randomly collected and three subsample units of 100 seeds were visually analyzed then counted the FDK in each set and the average reported.

Immunochemical tests such as enzyme-linked immunosorbent assay (ELISA) is a quick and accurate method to estimate mycotoxins in wheat samples. In this experiment, the AgraQuant® Deoxynivalenol Assay 0.25/5.0 (AQ) from Romer Labs® (Tulln, Austria) was used to analyze the samples for DON. Samples were ground with a coffee bean grinder (Hamilton Beach®) To guarantee the homogeneity and coarseness the flour samples were passed through a 20-mesh sieve, according to manufacturer instructions, and 20 grams was mixed in jelly jars with a 100 ml of distilled water. Samples were vigorously shaken for 15 minutes and then were filtered via a Whatman number one filter paper. The samples were analyzed following the kit instructions found in : (https://www.romerlabs.com) then wells were analyzed spectrophotometrically at 450/650 nm absorbance (Zachariasova et al., 2008). The absorbance measurements were performed using the Molecular Devices FilterMax F5 microplate reader, where each extraction was analyzed in triplicate per each sample (20 grams of seeds) and the mean was reported per each treatment.

4.3.8 Statistical Analysis

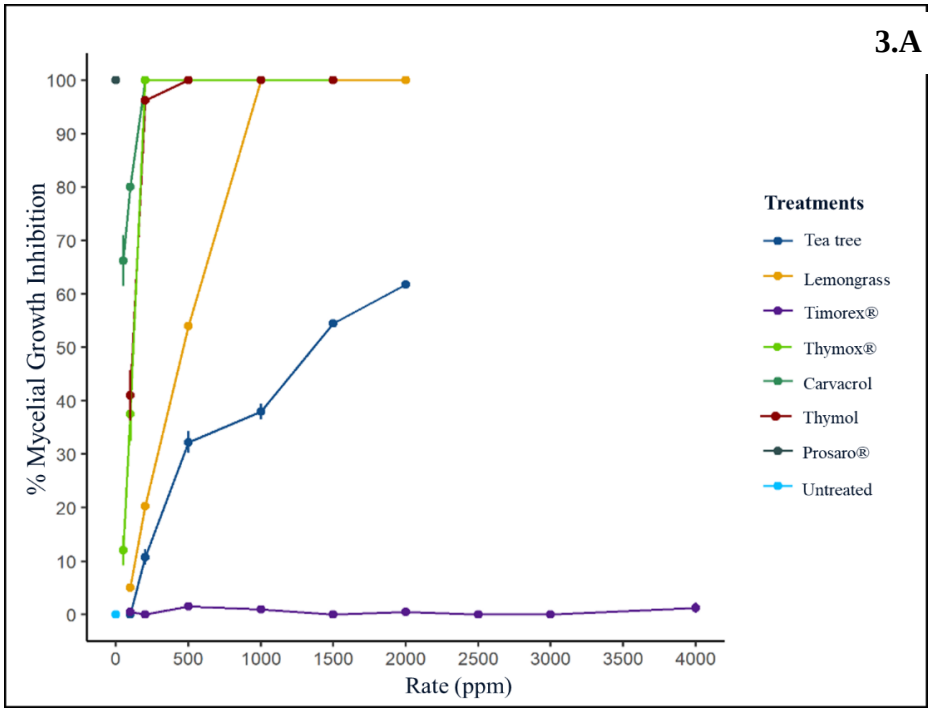
Data was analyzed using a generalized linear mixed model (GLIMMIX, SAS version, SAS institute, 2016) to examine the effect of Eos with appropriate link function where

necessary. A post-hoc test by Tukey's HSD was used to determine significant differences at a level of ($P < 0.05$). 4.4 Results

4.4 Results

4.4.1 Minimum Inhibitory Concentration

The minimum inhibitory concentration was defined as the lowest concentration that inhibits 100 % of mycelium growth. The efficacy of EOs in the control of *Fusarium graminearum* were evaluated with the fungal growth mycelial inhibition percent. In this study the percent of mycelia inhibition was higher in the bioactive compounds carvacrol at 200 ppm and thymol at 500 ppm respectively, followed by the EO with the commercial label Thymox® (derived from thyme oil) at 200 ppm and the pure essential oil lemongrass at 1000 ppm. The commercial EO Timorex® (derived from tea tree oil) did not show mycelial inhibition at the highest rate of 4000 ppm. When the concentration of the EOs or constituent were tested in the higher concentration. The treatments carvacrol, thymol, Thymox®, and lemongrass, showed a significant higher inhibition in the MIC ($P<0.05$) when were compared with the other EOs in the lower concentrations (**Appendix 4.1, Figure 4.3 and 4.4).**



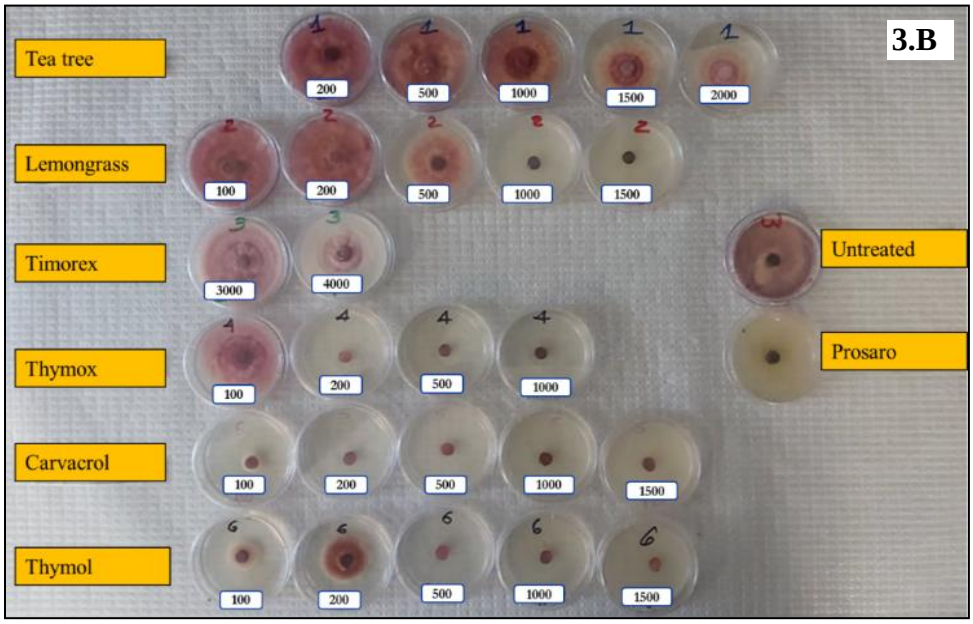


Figure 4.3. A) Essential oils in culture are showing mycelial growth inhibition, rating after four days of plating, **B)** The minimum inhibitory concentration was defined by the mycelial growth inhibition and plates that showed 100% inhibition were defined as MIC.

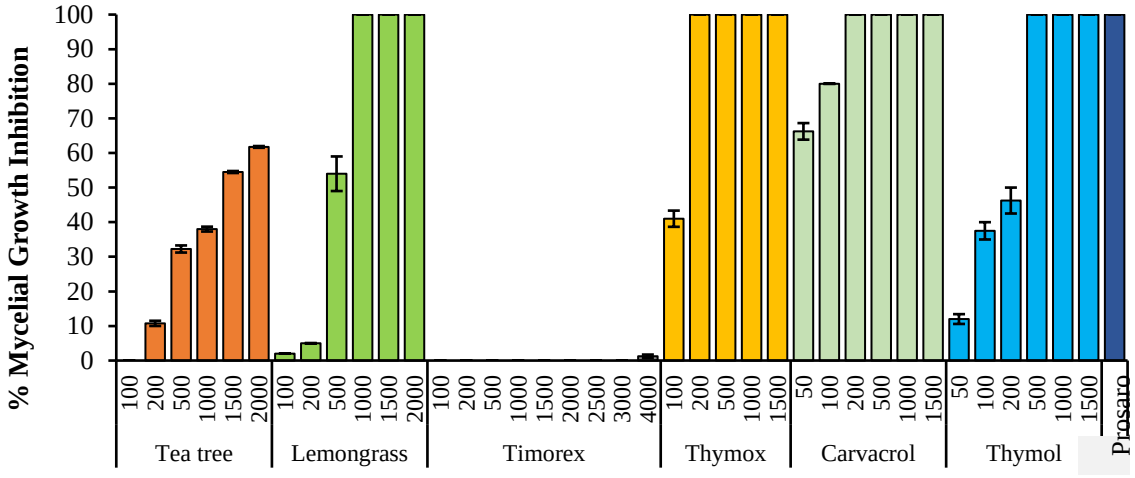


Figure 4.4. Mycelial growth inhibition for the EOs.100% mycelial inhibition was found in lemongrass, Thymox®, carvacrol, and thymol. Treatments of tea tree and Timorex® did not show complete inhibition in the tested concentrations.

4.4.2 Effect of Essential Oils in Spore Germination

The concentrations tested in this test were based on the concentration where was visually identified the MIC, however, to test the potential rate for greenhouse and field applications the concentration finally used was the next concentration of the MIC **(Table 4.2).** Where all the treatments did not show germination in the tested concentrations excepting the treatment Timorex® and the un-treatment that showed 95 and 100 % of spore germination **(Table 4.2).**

Table 4.2. Spore germination of *F. graminearum*, where 100% denotes no inhibition, and zero values indicate complete spore inhibition. means with the same letters representing values which are not significantly different according to Tukey test ($P<0.05$).

EOs compound	Concentration (ppm)	%Spore germination
Tea tree	1000	0% b
Lemongrass	1000	0% b
Timorex®	2000	95% a
Thymox®	1000	0% b
Carvacrol	500	0% b
Thymol	500	0% b
Control	-	100% a

4.4.3 Minimum Fungicidal Concentration

The minimum fungicidal concentration is the lowest concentration that can kill the fungal pathogen. For this experiment the were used the treatments lemongrass, Thymox®, carvacrol, and thymol that showed MIC then plugs with the fungus of this treatments were taking and transferred to new fresh PDA plates. without the amended EOs. The EOs lemongrass, Thymox®, and thymol in the two concentrations tested for each treatment did not show growth s, indicating that the MFC in this experiment was the same MIC. However, in the case of the compound carvacrol, that showed a MIC at 200 ppm did not show the MFC in this concentration and was found at 500 ppm, and the treatment evaluated as “negative growth” **(Table 4.3).**

Table 4.3. Minimum fungicide concentration test plugs of *F. graminearum*, where the fungal growth was estimated as "positive" or "negative" five days after placing the plug onto the culture plate Without treatments.

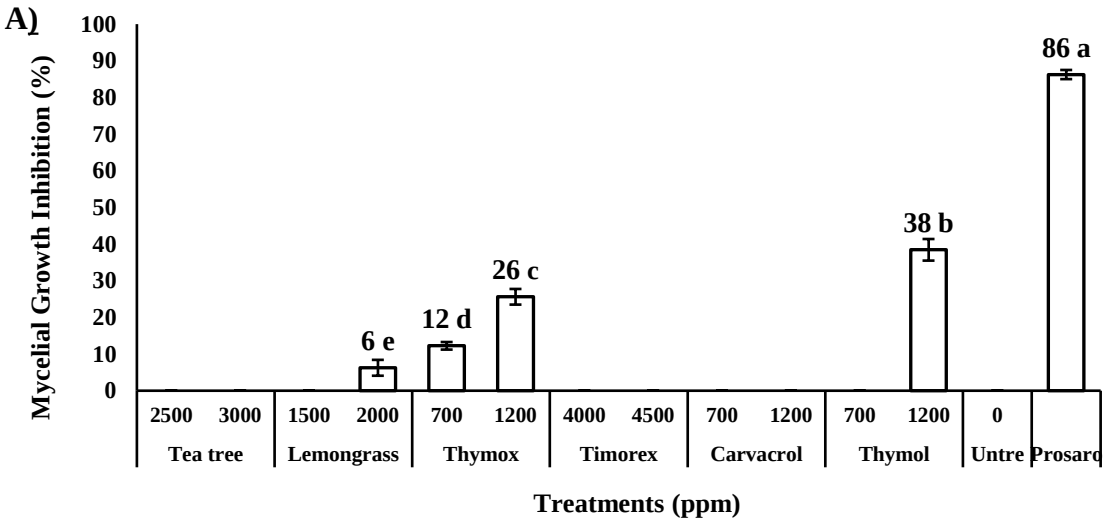
Treatments	Concentration	Growth
Lemongrass	1000 ppm	Negative
	1500 ppm	Negative
Thymox®	200 ppm	Negative
	500 ppm	Negative
Carvacrol	200 ppm	Positive
	500 ppm	Negative
Thymol	500 ppm	Negative
	1000 ppm	Negative

4.4.4 Effect of Volatile Essential Oils and Compounds in the Control of *F. graminearum*

Four plugs amended with the EOs were arranged around a plug of actively growing of *F. graminearum* that was placed in a new fresh PDA petri plate. The fungistatic effect of the EOs was measured with the mycelial growth inhibition percent using the Equation 1.

The Concentrations that showed a MIC and the next of this were tested in this experiment. Where the EO Lemongrass did not show inhibition at 1500 ppm, but showed inhibition of 6.25% at 1200 ppm, in the case of the compound thymol, this treatment only showed antifungal activity at 1200 ppm. Showing a 38% of fungal inhibition, the

commercial EO Thymox® showed a mycelial growth inhibition between 12 and 26% in the two-concentration tested. The Eos Tea tree and Timorex® did not show any control in the fungal growth of *F.graminearum*, in this experiment **(Figure 4.5).**



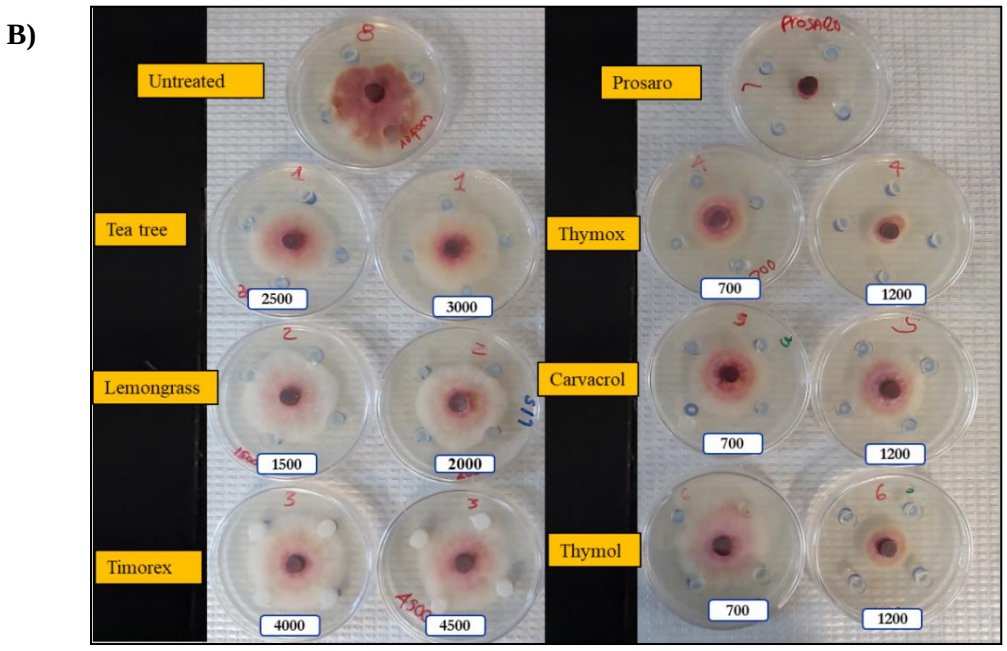


Figure 4.5. A) Mycelial growth inhibition of *F. graminearum* measure were realized after four days of placing the fungal plugs. **B)** Volatile activity test, where around the fungal plug were put the plugs with the amended

4.4.5 Effect in FHB Disease Index Caused by Essential Oils and Compounds, Greenhouse experiment

Under greenhouse conditions the essential oils and bioactive compounds showed a reduction of the disease index. Treatments Thymox® and thymol showed reduction in the FHB disease index, between 55.45 and 61.17% respectively, followed by lemongrass with a 48%. Of reduction, In general the treatments lemongrass, carvacrol, thymol, and Thymox® showed a significantly lower FHB DI ($P<0.05$) compared with the un-treated control **(Table 4.4).**

Table 4.4. Greenhouse evaluation of FHB disease Index, The %DI reduction are values getting of compare the treatments with the un-treated control, where the rating of the diseases was made 15 days after treatment, Values represented in DI means (± SE) with the same letters in each column are not statistically significantly different (≤ 0.05).

Treatment	% DI Reduction
Tea tree	33.17
Lemongrass	48.00
Timorex®	15.62
Thymox®	61.17
Carvacrol	47.80
Thymol	55.45
Prosaro®	93.00
Untreated	

Table 4.5. Yield and percentage of FHB Disease Index (DI) for the greenhouse and field trials, plots treated with different concentrations of EOs in the summer of 2021; Location Volga SD. Means (± SD) with the same letters in each column are not statistically significantly different (P<0.05).

Treatments	Yield (Kg/ha)	Disease Index field	FDK	DON (ppm)
Tea tree	1726.33 ± 193.01 b	11.96 ± 3.61 b	14.50 ± 2.89 b	5.42 ± 3.74 b
Lemongrass	1956.33 ± 73.30 ab	10.76 ± 4.79 b	10.25 ± 8.65 b	4.41 ± 3.14 b
Timorex®	1815.11 ± 55.82 b	14.62 ± 6.41 ab	10.50 ± 5.44 b	6.23 ± 2.68 ab
Thymox®	1979.87 ± 176.20 ab	11.32 ± 6.18 b	8.75 ± 4.85 b	4.02 ± 1.54 bc
Carvacrol	2008.79 ± 143.24 ab	10.93 ± 8.91 b	8.50 ± 1.91 b	5.86 ± 1.64 ab
Thymol	1967.77 ± 103.57 ab	12.41 ± 3.84 b	18.00 ± 4.53 b	5.30 ± 2.63 b
Prosaro®	2199.11 ± 170.82 a	1.43 ± 1.11 c	1.25 ± 0.95 c	2.09 ± 1.40 c
Control	1737.77 ± 200.41 b	20.10 ± 4.63 a	32.50 ± 13.22 a	9.87 ± 2.48 a

4.4.6 Effect of Essential Oils on Yield.

The weight yield significantly increased *(P < 0.05)* with the fungicide Prosaro® (2199.11 Kg/ha) compared with the EOs tea tree, Timorex®, and the untreated check. No significant differences were observed in yield between the synthetic fungicide Prosaro® and the EOs lemongrass, Thymox®, carvacrol, and thymol. The Disease Index showed that the synthetic fungicide Prosaro® proved the best performance in the control of FHB, with a DI of 1.43. compared with the EOs treatments where the range was between 10.76 and 12.4. Additionally, all the EOs with the exception of Timorex® showed significantly lower (*P<0.05*) DI compared with the control. (**Table 4.5).**

4.4.7 Effect of EOs in FDK and DON Accumulation in Harvested Grain.

All the essential oils and compounds showed a significant reduction ($P < 0.05$) in the FDK compared with the un-treated control. The synthetic fungicide Prosaro® showed the lower FDK with 1.25%. The highest reduction in DON was observed with the treatments Prosaro® and Thymox® compared with the untreated ($P<0.05$), where the synthetic fungicide Prosaro® showed a reduction of 78% , followed by the EO Thymox® with 59% and lemongrass with 55.3 %. The range of the EOs concentration was between 4.02 and 5.42 ppm and lower than the untreated with 9.87 ppm (**Figure 4.6, Table 4.5).**

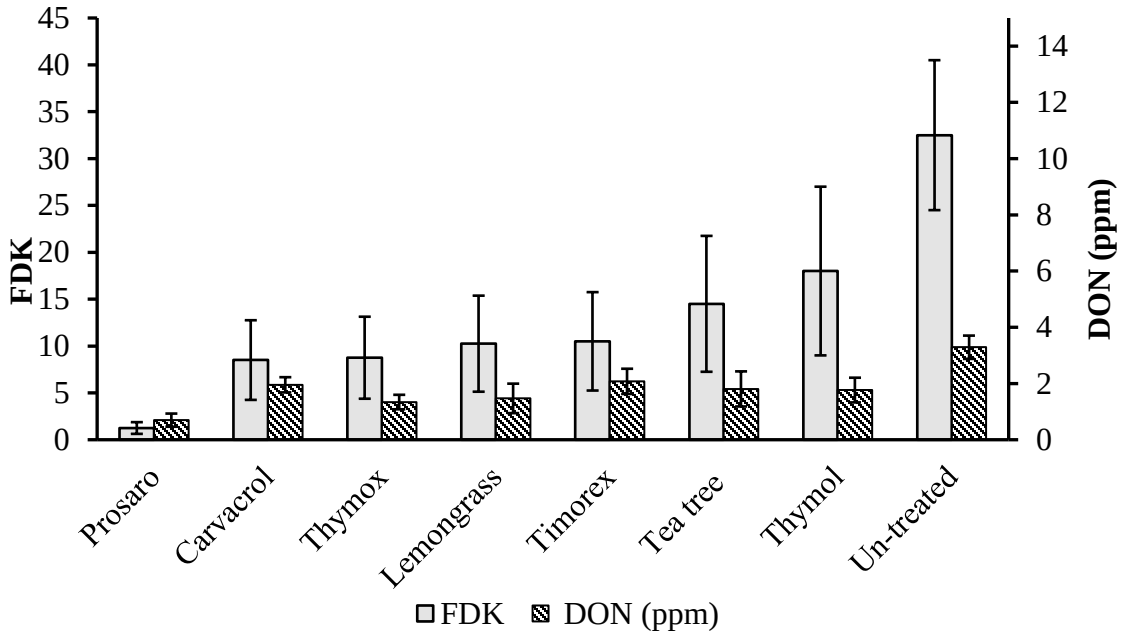


Figure 4.6. Numbers of kernels infected, and Don concentration presented in each treatment. Columns whiskers indicate the standard error (SE). DON = deoxynivalenol content in harvested grains.

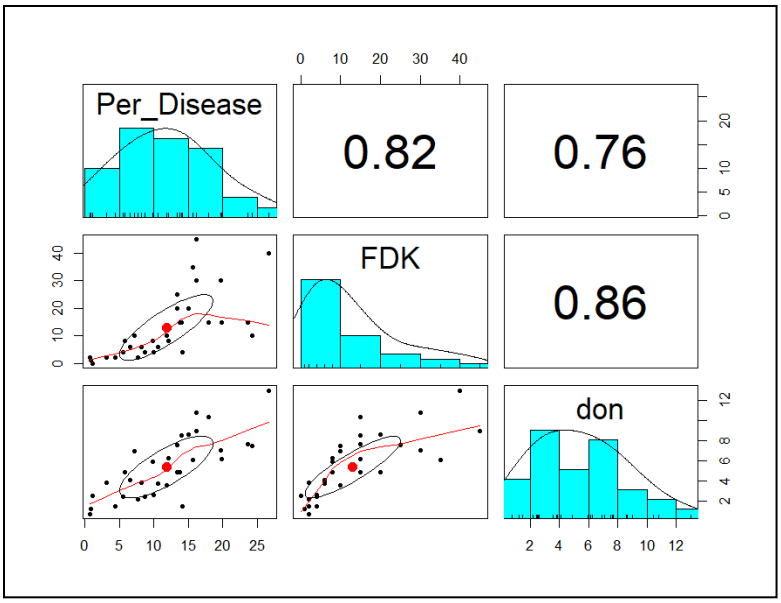


Figure 4.1. Spearmen correlation, FHB disease index (Per_Dise) showed a high positive correlation with the FDK and DON concentration. In the same way, a high significant correlation was observed between FDK and DON. Indicating that the visual rating can be used as in indicator of DON mycotoxin concentration. The inhibition of fungal growth is often related to a decrease in mycotoxin production.

4.5 Discussion

The search for new environment and friendly alternatives to control plant diseases has gained attention over the years and it is manifested by the increased demand for organic products for plant protection (Damalas and Koutroubas, 2018). Essential oils have become an important alternative due the antifungal properties they seem to elicit.

In this study the EO Lemongrass showed a MIC of 1000 ppm. In agreement with this, Seseni et al. (2015) tested the efficacy of ten EOs in the control of four *Fusarium* species, and reported 100% of mycelial inhibition at 1000 ppm for all the

fusarium species included *F. graminearum* using lemongrass. Another microdilution study by Lee et al. (2021) showed that lemongrass at 800 ppm produced 100% of mycelial inhibition against the fungus *Aspergillus brasiliensis,* an important postharvest pathogen in wheat. Also, in a study by Kalagatur et al., (2018) by micro-well dilution method the effect of the EO Lemongrass *(Cymbopogon martini),* an aromatic plant of the same genus *Cymbopogon,* in the control of *F. graminearum* showing a MIC at 421 ppm and a MFC of 618 ppm for this treatment.

The mode of action of lemongrass has been studied by Kalagatur et al., (2018) showing that lemongrass induces the death of fungi by elevating intracellular reactive oxygen species that destroy cellular components such as DNA, RNA, proteins, lipids.

The EO lemongrass contain the compound citral at the highest concentrations compared with other components that have shown antifungal properties. In a study by Zhang et al., (2022). Lemongrass *(Cymbopogon flexuosus),* and the main compounds, were tested in the control of *F. avenaceum,* finding that the main compound, citral, increased the permeability in the cell membrane of the pathogen, resulting in increased conductivity, protein and soluble sugar leakage.

Based on the MIC, carvacrol, thymol, and Thymox® (derived from the thyme plant) were the most effective at inhibiting mycelial growth and showed a MIC between 200 and 500 ppm. The antifungal power of the EOs, carvacrol and thymol derivatives, in the control of fusarium species has been previously reported by Saghrouchni et al., (2021) which tested carvacrol at different concentrations *in vitro* in the control of *F. oxysporum* where the MIC was at 250 ppm. These results are consistent with those

previously reported by Hamdani et al., (2017) in liquid media, where carvacrol and thymol showed a MIC of 250 ppm for both bioactive compounds in the control *F. oxysporum*. However, when the MFC was tested with this methodology, it was found at 1000 ppm. Three times superior to the MIC found in this study, where thymol and carvacrol showed an MFC of 500 and 200 ppm respectively. However, these results do not agree with Matusinsky et al., (2015) where they tested *in vitro* five EOs in the control of *F. graminearum*, and the EOs thyme (*Thymus vulgaris*), which its main compound is thymol ≈ 60% thymol (Seseni et al., 2015) showed complete fungal inhibition at 1000 ppm. In addition to this, Harčárová et al., (2021),tested *Origanum vulgare* and *Thymus vulgaris in vitro* using the broth microdilution method, where two isolates *F. graminearum* CCM F-683 and CCM 8244 showed an of MIC 400 ppm. We assumed that the difference in MICs can be due to the different methods used to identify the MIC (liquid or agar medium), the different culturing techniques, or the solvents used in each test which can affect antifungal activity results.

On the other hand it is known that the bioactive compounds, carvacrol and thymol, cause damage to the cell membrane integrity and interferes with ergosterol biosynthesis (Chavan and Tupe, 2014; Hou et al., 2020; Nazzaro et al., 2017; Shcherbakova et al., 2021). Gao et al., (2016) tested the inhibitory effect of thymol on the growth of fifty-nine isolates of *F. graminearum*, showing the efficacy of the bioactive compound on the mycelial growth and spore germination inhibition.

In the present study, specific concentrations of EOs and compounds were tested on spore germination **(Table 4.2),** where the spore germination was rated 12 hours after

plate inoculation. All the EOs were found to cause 100% spore inhibition except for Timorex®, which produced only 5% of spore inhibition. The commercial EO Timorex®, used as a fungicide (contains tea tree), appeared to be inefficient on mycelial growth inhibition (higher than 4000 ppm) compared with the other EOs tested, Although, the antifungal power of the tea tree is known and has been reported for other fungal pathogens, for example Li et al., (2017) tested a concentration of 1000 ppm of tea tree oil *in vitro* and they observed that it inhibited spore germination of *B.cinerea* by inducing the formation of reactive oxygen species (ROS) and decreased enzymatic activity related to mitochondrial function. In the current study we assumed that the low sporicidal potentials of tea tree oil -Timorex® was due to the emulsion formulation, which could have caused drastic changes in the main components of tea tree oil thus losing the antifungal power in the control of *F. graminearum.*

In our study carvacrol and thymol at 500 ppm showed complete spore inhibition which is consistent and previously reported by Saghrouchni et al., (2021), where carvacrol was tested *in vitro* and a complete inhibition in the germination of *F. oxysporum* spores at 200 ppm was observed. In another study by Hamdani et al., (2017), it was observed that the sporicidal effect of carvacrol on strains of spores using a liquid medium that reached 100% spore inhibition at a rate of 250 ppm. Additionally, Shen et al., (2016) confirmed the positive roles of ROS in the fungicidal activity of thymol against *A*spergillus *flavus.*

In our experiment the treatment lemongrass showed fungal and spore inhibition Is known that the EO lemongrass increases the production of ROS in the cell which leads to

apoptotic death of the fungal spores (Gao et al., 2020). Additionally pure compounds as carvacrol or thymol showed inhibition in the mycelial growth of *F. graminearum* in lower concentrations, have been reported that these compounds caused significant damage to the hyphal morphology and affect the spore production and germination of *Fusarium spp*. (Medina et al., 2022).

The volatile action in this experiment showed inhibitory effects from the treatments of lemongrass, Thymox®, thymol, and the synthetic fungicide Prosaro. Most of the methodologies to evaluate the volatility of EOs consist of adding the undiluted EOs (pure EOs) to an inverted lid. In this experiment, plugs were solidified with the amended EOs. Using this test was observed the potential of the volatile compounds in the control of the mycelial growth on *F. graminearum.* Proving to be a reliable methodology to test the volatility of the EOs.

The essential oil lemongrass, with the label market Thymox®, and compounds (carvacrol thymol) showed a higher weight yield compared with the untreated control. Although no significant differences were observed between the EOs treatments, EO treatments increased yield between 10.5 and 13% compared to the control, where carvacrol presented the highest efficacy among the EOs in the control of the disease. This may indicate that to certain point, the EOs lemongrass and compounds carvacrol and thymol could control FHB disease

In general, the high disease FHB index showed an increased DON concentration **(Figure 4.6)**. In a study carried out by Gill et al., (2016), using thymol + emulsion tested in the greenhouse two susceptible varieties of wheat, "Apogee" and "Bobwhite". where plants were inoculated and then applied with thymol plus the emulsion after 30 minutes of the inoculation. showing a FHB Index significantly lower compared with the untreated after ten days of treatment. Additionally, a study by Drakopoulos et al., (2020), three botanical based on mustard were tested *in vitro,* and in field studies in the control of *F. graminearum* in wheat. where the Botanicals used did not showed significant effects relative to the control.

F. graminearum decrease quality and yield of cereals and also, generates diverse mycotoxins, including deoxynivalenol (DON) and acetylated derivatives, nivalenol, zearalenone, fusarin C (Desjardins and Proctor, 2007) being DON the most frequently found.

All the EOs tested in this experiment showed reduction in FDK and DON concentrations compared with the untreated control. This agrees with a previous study performed by Lahooji and Mirabolfathy, (2010) where EOs *Zataria multiflora* and *Satureja hortensis* as well the biocompounds thymol and carvacrol, (derivatives from the same EOs) showed to decrease DON concentration by from *F. graminearum* isolated at 84, 89.1, 95 and 86.6% compared with the untreated. *in vitro* Experiments by Kalagatur et al., (2018) have shown the effect of lemongrass on DON concentration, showed a decrease in the concentration of DON mycotoxins as the

concentration of the EO lemongrass increased, showing values between 0 and 2 ppm in a concentration of 800 ppm.

In this study, was found a positive strong correlation ($r=0.78$; $P < 0.05$). between DON and FDK, it is known that FDK is a consequence of mycelium present within the grain, which leads to mycotoxin contamination of the grain, increasing the deoxynivalenol (DON)

Literature is scarce on measuring the effect of EOs on the concentration of DON in field conditions; most studies are carried out *in vitro* under controlled conditions (Elhouiti et al., 2017; Perczak et al., 2019, 2020). This experiment indicates the effect of EOs in mycotoxins production in field conditions, where treated plots of wheat with EOs showed a mycotoxin reduction of 45-59% compared to control plots.

4.6 Conclusion

In vitro studies shown*;* EO lemongrass Thymox®, and compounds carvacrol, and thymol may result effective in the control of *F. graminearum* at lower concentrations. In experiments performed *in vivo,* essential oils showed to reduce FHB disease index, DON, and FDK, which suggests that t essential oils and bioactive compounds could be added to integrated pest management programs for the control of FHB. Moreover, they may require somewhat greater application rates and may require frequent reapplication in the field conditions. The EOs found with antifungal properties against FHB can be used in future studies to produce chemosensitization with other EOs or synthetic fungicides.

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Appendix 4.1 Percent mycelial growth inhibition of *F. graminearum* in different concentrations. means with the same letters in each column are not statistically significantly different (≤ 0.05).

Treatment	Concentration	% Mycelial inhibition
Tea tree	100	0.00 ± 0.00 **k**
	200	10.75 ± 1.50 **ij**
	500	32.25 ± 2.00 **g**
	1000	38.00 ± 1.40 **fg**
	1500	54.50 ± 0.59 **d**
	2000	61.75 ± 0.50 **c**
Lemongrass	100	5.0 ± 5.50 **jk**
	200	20.25 ± 0.50 **h**
	500	54.00 ± 0.00 **d**
	1000	100 ± 0.00 **a**
	1500	100 ± 0.00 **a**
	2000	100 ± 0.00 **a**
Timorex	100	0.00 ± 0.00 k
	200	0.00 ± 0.00 **k**
	500	0.00 ± 0.00 **k**
	1000	0.00 ± 0.00 **k**
	1500	0.00 ± 0.00 **k**
	2000	0.00 ± 0.00 **k**
	2500	0.00 ± 0.00 **k**
	3000	0.00 ± 0.00 **k**
	4000	0.00 ± 0.00 **k**
Thymox	100	41.00 ± 4.69 **fe**
	200	100 ± 0.00 **a**
	500	100 ± 0.00 **a**
	1000	100 ± 0.00 **a**
	1500	100 ± 0.00 **a**
Carvacrol	50	66.25 ± 4.78 **c**
	100	80.00 ± 0.00 **b**
	200	0.00 ± 0.00 **a**
	500	0.00 ± 0.00 **a**
	1000	0.00 ± 0.00 **a**
	1500	0.00 ± 0.00 **a**
Thymol	50	12.00 ± 0.00 **i**
	100	37.50 ± 5.00 **fg**
	200	46.25 ± 7.50 **e**
	500	100 ± 0.00 **a**

	1000	100 ± 0.00 **a**
	1500	100 ± 0.00 **a**
Untreated		0.00 ± 0.00 k
Prosaro		100 ± 0.00 **a**

Appendix 4.2 Greenhouse rates of applications, The rates were adjusted at concentrations that showed inhibition in the *in vitro* test and did not show phytotoxicity in a greenhouse trail(data not shown).

Treatment	Concentration (ppm)	mL/acre	FL oz /acre	Dose (L ha^{1})
Tea tree *(Melaleuca alternifolia)*	2500	2362.02	79.88	2.36
Lemon grass (*Cymbopogon flexuosus*)	1500	1417.21	47.93	1.42
Timorex ACT Tea tree oil……. 12.5%	3000	5895.60	199.38	5.90
Thymox ……. 27%	700	661.37	22.37	0.66
Carvacrol (pure)	700	687.82	23.26	0.69
Thymol (pure)	700	687.82	23.26	0.69
Prosaro (Prothioconazole +Tebuconazole)	93	187.85	6.35	0.19
Control water	-	-	-	-

Appendix 4.3 Field rates applications the volume of water sprayed was 1.8 liters in four plots with a total of 27.7 mt^2.Where the rates were adjusted by the label recommendations and *in vitro* test results.

Treatment	**Concentration (ppm)**	**mL/acre**	**FL oz/ acre**	**Dose (L ha^{1})**
Tea tree *(Melaleuca alternifolia)*	3000	2834.42	95.85	2.83
Lemongrass (*Cymbopogon flexuosus*)	2500	2362.02	79.88	2.36
Timorex ACT Tea tree oil……. 12.5%	4000	4913.00	166.15	4.91
Thymox ……. 27%	1200	1133.77	38.34	1.13
Carvacrol (pure)	1200	1179.12	39.88	1.18
Thymol (pure)	1200	1179.12	39.88	1.18
Prosaro (Prothioconazole +Tebuconazole)	93	187.85	6.35	0.19
Control (water)	-	-	-	-

Chapter V: A Brief Review, Essential Oils, Use as Biofungicides, Perspectives, and Future.

5.1 ABSTRACT

The use of essential oils (EOs) and bioactive compounds have gained significant importance as biofungicides due to their known antifungal properties and wide range of control in plant pathogens, with a minimum impact in the environment and human health. The EOs are potential alternatives to synthetic fungicides in agriculture. However, at present, the production of biofungicides with EOs is limited in field applications due several concerns. On this brief review we will discuss the advantages and disadvantages of using essential oils, specifically in field crops, where we will mention some commercially available products with EOs registered in the market, some new technologies, and future perspectives of the EOs as biofungicides in the control of fungal plant diseases.

5.2 EOs as a Biopesticide

Crops are under constant attack from plant diseases that cause reduction in crops yields worldwide. Savary et al., (2019) estimated the yield loses caused by diseases in important field crops around the world. Where, crops such as wheat showed a 21.5%, maize 22.5%, and soybean 21.4% of yield loss. Diseases control management usually is by several practices such as resistant varieties, crop rotation or the application of fungicides, being the last one fundamental to guarantee the yield and the grain quality for most agriculture crops (Jampilek., 2016; Poole and Arnaudin, 2014.; Zubrod et al., 2019).

Worldwide it is estimated that about 2.5 million tons of pesticides are used as crop protectants every year (Koul et al., 2008), where the application of fungicides and bactericides cover the 12.0 % and herbicides the 25.1% of the total pesticides applied. However, pesticide dependence to guarantee yields has shown several disadvantages, including hazardous effects such as; pesticide residues, resistant phytopathogen strains, and threats to human health (Lucas et al., 2015; Piel et al., 2019). So, there is a need to search for new friendly biodegradable pesticides. Currently, the biopesticides cover a small part of the total crop protection market worldwide, with a value of about $3 billion, reporting just 5% of the total crop protection pesticides in the market (Olson., 2015). The production and use of biopesticides on a global scale are increasing by almost 10% every year, and it is expected that biopesticides will line up with traditional pesticides in terms of market size, between the late 2040s and the early 2050s (Damalas and Koutroubas, 2018).

Biopesticides are naturally occurring substances in organisms or compounds that kill, delay or suppress the growth and proliferation of pests by multiple mechanisms of action. And are categorized into three groups: microbial biopesticides (i.e., *Bacillus thuringiensis* (Bt)), plant-incorporated protectants (i.e., transgenic plants, Bt Cry proteins), and biochemical biopesticides, the last group defined as compounds of natural origin that control pests through different mechanisms of action (i.e., the essential oils (EOs)) (Fenibo et al., 2021; Kumar et al., 2021).

The essential oils are an important group in the biochemical biopesticides due to the high diversity of plant resources and known compounds with antimicrobial properties.

Just in the last 20 years, the publications on the use of EOs have increased by about 13.2 % per year **(Figure 5.1).**

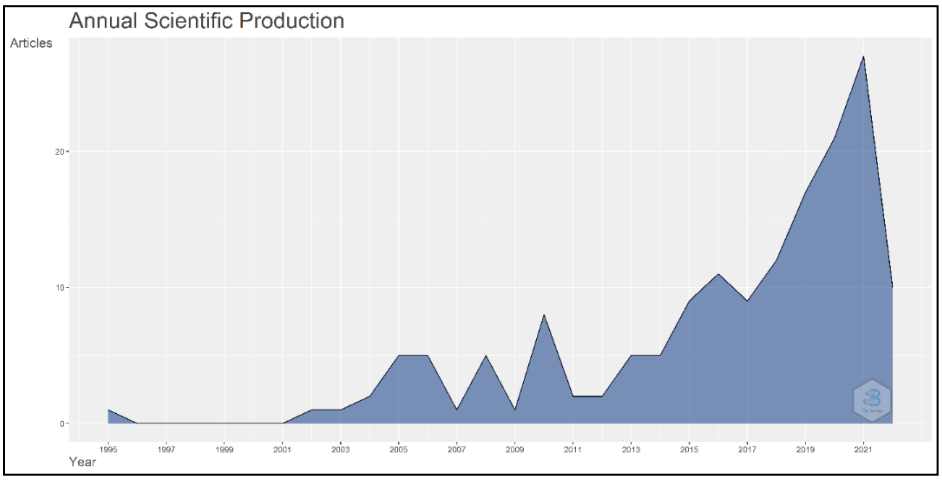


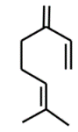
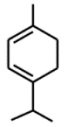
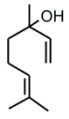
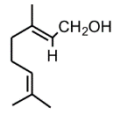
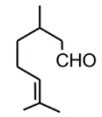
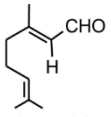
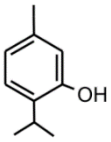
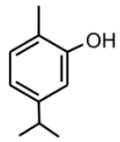
Figure 5.1. Plot realized with the program library "bibliometrix" of R, where the filter in the article research "SCOPE" of Elsevier Publishing company, with the search words "essential oils," "antifungal," and "agriculture" getting a total of 218 articles since 1995 to 2022.

5.2.1 Essential Oils

Essential oils are complex mixtures of volatile compounds derived from aromatics plants. And are extracted from several parts of the plants such as; leaves, stems, fruits, flowers, roots, and wood. The amount of essential oil found in the plants is around 1 to 2% but can contain highest amounts ranging from 2 to 10%, depending on the species (Koul et al., 2008). The composition of EO compounds depends on variables such as the age of the plant, the environment, and the type of obtention of the EOs (i.e., steam distillation).

Essential oils are mixtures of more than 100 compounds, where the terpenoids, also called isoprenoids, are mostly about 60% of the total composition. The major part of the molecules in the EOs are monoterpenes and sesquiterpenes, for example, the compound 1,8-cineole is present in a high concentration in rosemary and eucalyptus oils; eugenol from clove oil; and thymol from thyme oil (Isman., 2020a; Koul et al., 2008), some elements found in the EOs are showed in (**Table 5.1).**

Table 5.1. Different structures and compounds present in the essential oils.

Terpenes/terpenoids	**Compounds**	**Chemical structures**
Hydrocarbons	myrecene pinene terpinene limonene pcymene	 Myrcene  α-Terpinene
Oxygen containing hydrocarbons	geraniol	 Linalool  Geraniol
	linalool	
Aliphatic aldehydes	citral citronellal	 Citronellal  Citral (Geranial)
	perillaldehyde	
Phenols	carvacrol thymol safrole eugenol	 Thymol  Carvacrol

5.3 The Essential Oils Have Shown Antifungal Activity in Laboratory Tests, However, in the Field is not the Same History

Essential oils and its derivatives have shown antifungal properties, *in vitro* experiments, against plant pathogens in numerous reviews (Alonso-Gato et al., 2021; De Clerck et al., 2020; Hou et al., 2020). Previous work has shown that EOs and similar compounds may be excellent alternatives to synthetic fungicides showing a reducing the negative impact on human health and the environment.

Today, products containing EOs and bioactive compounds are registered in the fungicide resistance action committee (FRAC). Plant extracts such as those obtained from *Melaleuca alternifolia,* known as tea tree oil, and the bioactive compounds eugenol, geraniol, and thymol classified in the biological group with multiple modes of actions present in the FRAC Code List, 2022. In addition the United States environmental protection agency (EPA) in the 2022 has registered 390 biopesticide ingredients including: citronella, bergamot, lemongrass, garlic, eucalyptus, cedarwood, mint, thyme, black pepper, oregano, neem, and tea tree oil. Also, in the market some biopesticides with EOs are utilized as disease controllers (**Table 5.2).**

Table 5.2. Commercial products with essential oils present in the market and used in the control of fungal diseases.

EOs source	Essential oil	Commercial name
Allium sativum	Garlic oil	GC-3™ Garlic Barrier®
Azadirachta indica	Neem oil	Trilogy™

		Monterey Fruit Tree Spray Plus Triact® 70EC
Gossypium hirsutum	Gossypium oil	GC-3™
Melaleuca alternifolia	Tea tree oil	Timor™, Timorex™
Mentha piperita	mentha oil	Fungastop™
Reynoutria sachalinensis	Reynoutria sachalinensis oil	Milsana™
Rosmarinus officinalis	Rosemary oil	Sporan™
Rosemary, thyme, and clove oils	Rosemary, thyme, and clove oils	Sporatec™
Sesame	Sesame oil	Organocide™
Simmondsia californica	Jojoba oil	E-Rase™
Thymus vulgaris	Thyme oil	Proud 3 ™ Promax™ Thyme Guard™ Thymox™

The efficacy of some essential oils in the control of diseases in greenhouses and postharvest is known. However, little is known about the efficacy of EOs in field applications (Chang et al., 2022; Maurya et al., 2021; J. Zhang et al., 2019). **So, are essential oils useful as fungicides for field applications?**

Most of the studies reported on the efficacy of EOs have shown *in vitro* and few documents appear on the use or efficacy of EOs in field environments or as field tests (Parikh et al., 2020). And mainly of the field tests are performed by companies with promissory products to launch on the market, where few information of the development of the product is available, showing a disconnection between the development of academic research and the industry in the production of bioinsecticides, Isman (2020)

mentioned this problem in the development of bioinsecticides with EOs, in addition, the results of negative results using EOs in field applications is non-documented.

On other hand, Pavela and Benelli (2016) argue that there are four reasons why EOs are not used in field applications similar to commercial products which are (i) many published studies *in vitro* but few practical results; (ii) strict legislation for the registration; (iii) low persistence of effects (volatilization); and (iv) quality and quantities of the resources (plants for extraction) for affordable prices.

EOs as biofungicides present a series of disadvantages for application in the field, some of which are summarized in the (**Table 5.3)** following the references from these authors Alonso-Gato et al., 2021; Campos et al., 2016; Fenibo et al., 2021; Isman, 2020a; Koul et al., 2008; Lahlali et al., 2022; Liao et al., 2021; Napoli and Di Vito, 2021; Pavela and Benelli, 2016,who have worked with EOs constantly made publications looking for solutions to these limitations in the use of EOs as a biopesticide.

Go back to the proposed question ,The answer could be yes and no because EOs show a certain control but not to the point of comparison with synthetic fungicides. They are currently not the magic solution for pathogen control, and in the event that an acceptable solution for disease control comes from essential oils, the resources to extract these are currently limited. However, with improved extraction methods EOs could become part of the solution when added to an IPM program to control diseases.

Table 5.3. Disadvantages of the use of EOs in field applications.

Rapid evaporation and degradation of the active components (volatilization and light sensibility).
It is difficult to get the essential oils' standard chemical composition (uniformity); it depends on several factors in the crop development such as: clime physiological stage, kind of soil, and biological and non-biological stress.
Most experiments on antifungal properties are *in vitro* and not *in vivo.*
Higher application is necessary, which results in more labor expense (Higher cost of the product for the farmers).
Little knowledge about the adverse effects EOs may have on the application equipment.
Due to the complex composition of the EOs it is difficult to understand the mode of action.
Limited data on the efficacy of biopesticides which could be considered their main disadvantage.
The efficacy of essential oils toward fungal diseases is not as apparent or obvious as that seen with synthetic fungicides.
Some essential oils with antimicrobial properties are difficult to extract due to a lack of resources.

Despite the disadvantages mentioned above, a key advantage is that EOs present a lower level of risk to the environment than current synthetic fungicides. The volatile

nature of EOs makes them highly compatible in integrated pest management programs, this volatility also makes essential oils safer for the pollinator and natural predators. Additionally, due to the complex mixture of compounds within essential oils they present multiple modes of action which cause the resistance of pathogens to develop more slowly. On the other hand, Isman (2020b) lists the development of bioinsecticides with essentials oils and bioactive compounds. Showing the different pathways that can be used in the formulation of the fungicides depending on the genesis of the product. For example, products whose active ingredients consist of :

(1) a mixture of essential oils such as thyme oil and clove oil; (2) a single essential oil, or a single terpenoid constituent such as tea tree oil; (3) a blend of terpenoids that are synthetically produced and that emulate those in a plant essential oil such as α-terpinene, p-cymene, and d-limonene, each of which are available through synthesis.; (4) a novel, non-natural blend of terpenoids such as carvacrol plus thymol.

5.4 Future and Perspectives of the Essential Oils as Fungicides in Field Crops.

Some challenges that research should focus on in the use of EOs for field applications are listed :

1. Development of efficient stabilization processes (e. g., microencapsulation/nanoencapuslation).

Nanoencapsulation is a technique in which a membrane encloses the active ingredient, protecting this from environmental conditions and avoiding the volatility of the active ingredient along with technology that produces a gradual release of the essential etheric oils and natural components.

2. Simplification of the complex and costly extraction of the EOs and authorization of these requirements for the development of the product placed under more flexible legislation.

3. Optimization of plant growing conditions and the extraction processes leading to EOs with more homogeneous chemical compounds (Pavela and Benelli, 2016).

4. Plant genetic manipulation to increase the amount of the EOs or a specific terpenoid produced within the plant (Yu and Utsumi, 2009).

5. Chemosensitization of the essential oils and synthetic fungicides to control diseases in field crops (Dzhavakhiya et al., 2012; Shcherbakova et al., 2021).

5.5 Conclusions

Despite the disadvantages that Essential oils present, more scientific works and interest by companies are promoting the study of these resources and the solution to these problems, promoting the use of EOs as a key component to use in the control of fungal diseases.

Essential oils as biopesticides may in the future become a valuable part in the development of new alternatives in plant protection products due to the advantages of being safer to humans and non-target organisms, thus making EOs an option to integrate into IPM strategies.

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Chapter VI. General Conclusions

To manage fungal diseases in small grains such as crown rust in oats or Fusarium head blight, tan spot, spot blotch, and stagonospora and leaf blotch in wheat, strategies used in integrated pest management programs consist in the use of resistant varieties, cultural practices and mostly the use of synthetic fungicides. Due to overuse and concerns regarding synthetic fungicides such as the high residuality or the negative impact in different ecosystems.

There is a need to search and find new molecules more friendly to the environment. The use of biopesticides, such as essential oils, is taking relevant importance in plant diseases management. Essential oils are derived from plants with known antifungal properties that could be a friendly alternative in controlling fungal diseases in small grains. The objectives of this dissertation were: (**1)** Determine the potential of two EOs neem and karanja in the control of oat crown rust in a two-year field experiment, **(2)** Determine the potential effect of essential oils and bioactive compounds in the control of three pathogens (PTR, PSN, BPS) that cause foliar diseases in wheat *in vitro*, and **(3)** determine *in vitro* and *in vivo* the potential as fungicide of two EOs, two commercial products with EOs, and two bioactive compounds in the control of the fungal pathogen *Fusarium graminearum*.

In our field experiment testing the essential oils karanja and neem against crown rust disease, karanja and neem oil showed a significant reduction($P<0.05$) % disease severity and a high yield compared with the un-treated check at high concentration 72%

and rate of application 11.22 L/h. However, in this two year experiment the synthetic fungicide Headline showed the best performance in controlling the disease and yield. Due to the lowest % of disease severity and a higher yield in EOs at the highest application rates treatments, we can infer from this study that using EOs as fungicides for the control of crown rust seems promising. More studies on the timing of application, price in the market, and the number of applications or new technologies such as nanoemulsions are necessary to determine the optimal rate and make Eos use economically viable.

In the second study testing *in vitro* the essential oils and biocompounds, four essential oils: tea tree, lemongrass, neem, and karanja oil, and three constituents present in essential oils: carvacrol, thymol, and linalool, were evaluated on PTR, PSN, and BPS based on mycelial growth and spore germination inhibition *in-vitro*. Lemongrass at 500 and 1000 ppm, carvacrol and thymol at 200 and 500 ppm, showed 100% inhibition of mycelial growth in PTR, PSN, and BPS. One hundred percent of spore germination for the three pathogens was inhibited by lemongrass at 1000 ppm and carvacrol and thymol at 500 ppm. We found in this *in vitro* study as promising antifungal EOs lemongrass and the bioactive compounds thymol and carvacrol in the control of three important pathogens that cause spotting diseases in wheat.

The third research objective was to evaluate *in vitro* and *in vivo* the efficacy of two pure EOs (tea tree and lemongrass), two commercially available EOs with proven antifungal properties (Thymox® and Timorex®) and two bioactive compounds present in EOs (carvacrol and thymol), in the management of FHB. In the *in vitro* experiment, we reported the fungicidal and fungistatic potential of the EOs lemongrass, carvacrol, and

thymol and the commercial EO Thymox® in the control of *F. graminearum.* In the *in vivo,* a greenhouse study, EOs decreased the disease in the spikelet compared with the untreated control. Under field conditions, the EOs in emulsions showed an increase in yield with reduction in the FHB index and DON accumulation compared with the untreated control. In general, the fungicide Prosaro was the most effective in controlling the disease.

EOs and bioactive compounds could be added to integrated pest management programs for the control of FHB. Moreover, EOs require greater application rates and may require frequent reapplication in the field conditions. The EOs found with antifungal properties against FHB can be used in future studies to produce chemosensitization with other EOs or synthetic fungicides.

EOs in the control of *fusarium graminearum* showed a superior control compared to the un-treated. *In vitro* in general; thymol, carvacrol, and thymol showed a high efficiency in the control of leaf spotting pathogens and *Fusarium graminearum.* In our experience, applying the EOs in the field taught us that they should not be used the same way as traditional fungicides. It is necessary to make several applications and follow an IPM program strictly.

We recommended more testing in vivo mixing compounds such as carvacrol and thymol and more field experiment with repeated applications in moderately resistant varieties.

This work, in general, could help farmers and researchers have a basic idea of the potential of some EOs and compounds for controlling diseases that affect small grain.