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Evaluation of Essential Oils and Their Major Components for Disease Resistance

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EVALUATION OF ESSENTIAL OILS AND THEIR MAJOR COMPONENTS FOR DISEASE RESISTANCE

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Essential oils (EOs) are natural hydrophobic compounds, which are highly complex, typically comprising 20 to 60 constituents, with two or three main components making up 20% to 95% of their composition, alongside other minor constituents. Research has firmly established the potent antibacterial and antifungal properties of EOs and their primary components. To assess their efficacy, one EO, Nutmeg oil, and seven main components, including safrole, toscanol, safraleine, cinnamaldehyde, carvacrol, carvone, and thymol, were tested against four economically significant fungal pathogens (*Alternaria solani*, *Fusarium graminearum*, *Rhizoctonia solani*, and *Sclerotiorum sclerotiorum*), one oomycete pathogen (*Phytium ultimum*), and four bacterial pathogens (*Clavibacter michiganensis subs. nebraskensis*, *Xanthomonas campestris* pv. *campestris*, *Pseudomonas syringae* pv. *tomato* DC3000, and GFP-tagged *P. syringae* pv. *tomato*). In vitro studies were conducted to evaluate the inhibitory effects of these selected EOs and major components on fungal and bacterial growth, determine the Minimum Inhibition Concentration (MIC), Minimum Fungicidal Concentration (MFC), and Minimum Bactericidal Concentration (MBC) of the compounds, and investigate their impact on spore production and germination of *F. graminearum*. Among the tested components,

carvacrol, carvone, cinnamaldehyde, safraleine, and thymol exhibited significant growth inhibition of the pathogens. These five major components were further tested in wheat heads against *F. graminearum* to assess their efficacy in reducing infection, mycotoxin levels, and wheat quality parameters in greenhouse conditions. Similar studies were conducted on tomato seedlings infected with *P. syringae* pv. *tomato* to evaluate the impact on disease severity, agronomic features, and the relative expression levels of genes associated with resistance pathways, including SAR, ISR, and R-genes. The in vitro screening revealed varying efficacy of the main EO components against fungal and bacterial pathogens, depending on the tested components, their concentrations, and the pathogens. Cinnamaldehyde, carvacrol, and thymol demonstrated the strongest growth inhibition against both fungal and bacterial pathogens, with MIC, MFC, and MBC concentrations ranging from 0.25 mg/mL to 5 mg/mL. These components also inhibited spore production and germination at relatively low concentrations. Furthermore, safraleine and carvacrol significantly reduced Fusarium Head Blight (FHB) infection and deoxynivalenol (DON) levels while improving yield and maintaining nutritional properties. Application of cinnamaldehyde and thymol resulted in a significant decrease in chlorotic areas on leaves and the incidence of bacterial speck lesions in tomato seedlings. Additionally, EO application upregulated the expression of defense-related genes PR1, PR2, and PR3 in tomato plants infected with *P. syringae* pv. *tomato* DC3000. The testing of major EO components establishes a foundation for discovering novel alternative biocides for controlling fungal and bacterial diseases.

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Chapter I

Essential Oils: Antimicrobial Potentials, Activities, Testing Methods, and Limitations

1. Introduction

The world's population is rapidly increasing every year and is expected to reach approximately 9.7 billion people by 2050, as reported by United Nations (UN). This population growth is accompanied by an increase in the living standards of many individuals, leading to an increased demand for food supplies (Gupta et al., 2023). To meet the need for food, it is substantial to balance crop productivity with the ecological health of agroecosystems. The rapid expansion of farm mechanization in agriculture has resulted in the use of intensive agronomic methods to achieve immediate crop improvement (Kumar & Singh, 2015).

Agriculture is adversely affected by various pests such as fungi, weeds, bacteria, and insects which cause yield declines and lower quality of the products (Kumar and Singh, 2015). Worldwide, about 40% of agricultural production is lost because of plant diseases, weeds, and pests in total (Mahmood et al., 2016). The practices to reduce the negative effects of pests and diseases have been established in parallel with the beginning of agricultural activities. It is thought that sulfur compounds were regarded as the first recorded pesticides used for the suppression of insect and mite populations in the field around 4500 years ago by the Sumerians (Umetsu & Shirai, 2020).

Pesticides are formulated with active ingredients as well as inactive substances, proprietary substances, contaminants, and impurities (Mahmood et al., 2016). Just about one-third of agronomic products are subject to pesticide application. Without use of

pesticides, crop losses would have reached a great portion of annual production (Mahmood et al., 2016). It is estimated that 78% of fruit, 54% of vegetable, and 32% of cereal production would be lost (Tudi et al., 2021). Even if these compounds promise to be effective against the harmful agents in production, the overreliance on synthetic chemicals within pest control programs has given way to numerous harmful repercussions (Nicholson, 2007). These repercussions range from the contamination of drinking water and the presence of hazardous residues on food, to the acute or chronic detrimental effects on mammals as well as non-target organisms such as birds, bees, predators, and parasitoids. Additionally, this approach has contributed to the emergence of pest resistance, further exacerbating the issue (Ebadollahi et al., 2020).

Despite the fact that synthetic pesticides have been a popular choice for farmers and gardeners for decades (Isman, 2000), recently, the focus of agricultural research has shifted towards sustainable farming practices in an attempt to reduce the pesticide load in soils (Gupta et al., 2023). Numerous studies have provided irrefutable evidence on the harmful effects of pesticides, particularly their carcinogenicity. Synthetic pesticides, including insecticides, herbicides, and fungicides, have been linked to different types of cancers with exposure to these chemical compounds. These reports highlight the serious health risks posed by the use of pesticides in agriculture and other industries, underscoring the need for safer and sustainable alternatives to protect human health and the environment (Chaudhary et al., 2017). There is a growing concern among the public regarding the long-term health and environmental effects of these pesticides, particularly in Europe and North America. In response to this concern bio-pesticides are being widely promoted for managing pests. Bio-pesticides, which are substances derived from living

organisms such as plants, nematodes, bacteria, viruses, and fungi, have been receiving an increasing interest as alternatives to synthetic chemical pesticides. Due to their ability to avoid the use of chemical toxins in food chains, bio-pesticides are becoming popular around the world as vector control agents (Isman et al., 2006; Al-Harbi et al., 2021). Furthermore, such bio-pesticides are increasingly being acknowledged as ecologically friendly alternatives to chemical pesticides, primarily due to their biodegradable nature, quick decomposition, lack of persistence, sustainable agriculture properties, and absence of adverse effects on groundwater (Alliance et al., 2017).

Plants were the primary source of pest control until about 2000 years ago when chemical pesticides became widely used (Gupta et. al. 2023). Plant-based pesticides, including crude extracts, and purified or partially purified products from plants, can be used alone or in combination with other substances to combat various pest vectors (Chaudhary et al., 2017). Based on the prediction done by the research and advisory company "Lux Research" in Boston, MA, USA, the market size of bio pesticides will reach the market size of synthetic (chemical) pesticides by the late 2040s and beginning of 2050s (Olson, 2015; Van de Braak et al., 1999).

Among the different types of plant-based products, plant essential oils have emerged as a preferred alternative for biological pest control owing to their current and historical use in food preparation, natural origin and wide range of bioactivities (Gupta et al., 2023). The application of such biological agents is promoted in Europe by the directive 2009/128/CE to lessen the usage of pesticides to protect sustainability (Villaverde et al., 2014).

2. Essential Oils

"*All the pests that out of earth arise, the earth itself the antidote supplies" (Lithica circa 400 B.C.).*

Plants are remarkable organisms that have evolved to produce an astonishing array of molecules to protect themselves against a variety of threats, including pathogens. These molecules, known as secondary metabolites, play a crucial role in the plant's defense mechanism. Among these, essential oils stand out as a group of volatile compounds with a characteristic aroma and potent biological properties (Raveau et al., 2020; Vergis et al., 2015).

Essential oils (EOs) are hydrophobic, natural compounds synthesized as secondary metabolites in more than 17,500 plant species in a large number of plant families involving *Asteraceae*, *Lamiaceae*, *Myrtaceae*, *Rutaceae*, and *Zingiberaceae* (Mérillon & Rivière, 2018). Aromatic plants and plant products such as anise, fennel, coriander, thyme, frankincense, and myrrh were mentioned in the 20 m long papyrus called Papyrus Ebers coming from antique Egypt (B.C. 1550) containing around 700 formulas and remedies (Elansary & Ashmawy, 2013). Many years later, nearly 200 medicinal and aromatic plants and their benefits were portrayed in the tractate "Corpus Hippocratium" by the ancient Greek physician Hippocrates known as the father of medicine (Ferdes, 2018). It is thought that the term "essential oil" was first named by Paracelsus von Hohenheim, (a Swiss physician and alchemist in the 15th century) in his theory of *Quinta essentia*.

EOs became popular in the Europe after 16th century with their trading of them in the city of London (Crosthwaite, 1998). It was stated that with the colonization of

Australia in the 18th century tea tree oil became used for medical purposes in Europe (Carson & Riley, 1993). ECs are typically extracted through steam or hydro distillation first developed by Arabs during the Middle Ages (Bakkali et al., 2008). The first study was conducted by De la Croix in 1881 to evaluate essential oil vapors for their bactericidal potency (Najafian, 2014). Although EOs were chiefly used for flavor and aroma through the 19th and 20th, the current trend has changed the direction of use toward their antimicrobial properties again.

Essential oils primarily liquid at room temperature although some are solid or semi-liquid (resinous) and possess various color scales ranging from light yellow to beryl green and from pale blue to dark brownish red (Bassolé & Juliani, 2012). They are highly volatile and aromatic compounds produced through the malonic acid, mevalonic acid, and methyl-d-erythritol-4-phosphate (MEP) pathways in the cytoplasm and plastids of plant cells and deposited in the secretory cavities, epidermic cells, glands, and resin channel (Wińska et al., 2019). Being present in different parts of aromatic plants such as flowers, leaves, rhizomes, roots, seeds, fruits, wood, and bark, EOs have an important ecological role in plants, acting as chemical signals to regulate and control the environment. They serve a variety of purposes, including repelling predators, attracting pollinators, inhibiting seed germination, and facilitating communication between plants (Hanif et al., 2019; Frabboni et al., 2019).

Throughout history, EOs have been utilized in traditional medicine for their antiseptic, antioxidant, and anesthetic properties due to their biologically active compounds (Shaaban et al., 2012). In recent years, EOs have drawn attention due to their broad range of biological properties which includes embalming, preserving food,

remedies for various ailments, and antimicrobial activities as antibacterial (Gormez et al., 2016), antifungal (Banani et al., 2018; Amri et al., 2022), antiviral (Ma & Yao, 2020), insecticidal (Oladipupo et al., 2022), nematicidal (Deng et al., 2022; Ferreira Barros et al., 2021), and herbicidal (Amri et al., 2022; Duke et al., 2022).

Lately, there is an increased tendency for naturally originated supplements in the food industry to direct usage of them in recipes or to blend with other components. Diseases including cancer, organoleptic and neurodegenerative disorders release free radicals damaging the other systems in the body (Hale et al., 2008). Natural origin compounds such as EOs have received noticeable interest due to their radical-cleaning activities (de Sousa Barros, 2015). Another current concern closely influencing public health is the development of resistance against antibiotics as a result of the large-scale use of antibiotics. *Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella* spp., *Shigella* sp., *Enterococcus* sp., and *Escherichia coli* are examples of community- and hospital-acquired pathogenic bacteria that are showing multidrug resistance. Increasing instances of multi-drug resistance has resulted in the requirement for new antibiotics and also an increased curiosity for the application of herbal medicinal products with antimicrobial activities (Chouhan et al., 2017). Herbal extracts and EOs could function as effective instruments to decrease the occurrence and spreading of multi-drug resistance among bacteria (Stefanakis et al., 2013).

2.1. The Chemical Composition of Essential Oils

The biological properties of essential oils are primarily determined by their major components, which can be classified into two distinct groups based on their bio-synthetic origin. The first group comprises terpenes and terpenoids, while the second group is

made up of aromatic and aliphatic constituents. They are usually composed of two or three main components ranging from 20% to 95% concentrations as well as other components found at very low levels (Raveau et al., 2020). To illustrate, d-limonene is primarily found in *Citrus* peel oils with over 80%, while *O. compactum* oil contains high levels of carvacrol and thymol with around 30% and 27%, respectively. Menthol and menthone with 59% and 19% in *Mentha piperita* oil, carvone, and d-limonene in *Anethum graveolens* seed oil with 58% and 37%, α-phellandrene, and limonene in *A. graveolens* leaf oil with 36% and 31%, α-/β-thujone and camphor in *Artemisia herba-alba* oil with 57% and 24%,1,8-cineole in *Cinnamomum camphora* oil with 50%, are the examples of main components present in various plant species (Shaaban et al., 2012). The biological potency is considered to be directly associated with the major component profile of the EOs.

2.1.1. Terpenes and Terpenoids

Terpenes are a class of naturally produced chemical compounds by plants with the combining of 5 carbon-base (C_5H_8) units known as isoprenes. Terpene biosynthesis occurs in the cytoplasm of plant cells through the mevalonic acid pathway beginning from the acetyl-CoA. In a variety of terpenes, the isopentenyl diphosphate (IPP) precursor is processed followed by the combination of several IPPs to constitute the prenyl-diphosphate precursor next, the terpene skeleton is formed through the slight modification of allylic prenyl-diphosphate with terpene-specific synthetases, lastly, it is subjected to secondary enzymatic modifications by redox reactions to optimize the functionality of the different terpenes (Chouhan et al., 2017). While monoterpenes ($C_{10}H_{16}$) and sesquiterpene ($C_{15}H_{24}$) are the most common types of terpenes, other types

of it with longer chains including hemiterpenes (C_5H_8), diterpenes ($C_{20}H_{32}$), triterpenes ($C_{30}H_{40}$) and tetraterpenes ($C_{40}H_{64}$) are also present. The monoterpenes ($C_{10}H_{16}$) are the most common type of molecules existing with around 90% in EOs leading to a broad range of structural variation. To illustrate, citronellol, α-pinene, camphor, (e)-β-ocimene, geraniol, limonene, linalool, myrcene, nerol, and terpineol are examples of major monoterpenes in EOs (El Asbahani et al., 2015).

Terpenoids are a type of compound that is formed when enzymes modify terpenes by adding oxygen molecules and moving or removing methyl groups. Terpenoids can be further categorized into different types such as alcohols (menthol), phenols (thymol, carvacrol), esters (linalyl acetate), aldehydes (citronellal), ethers (menthofurane), and ketones (piperitone) and epoxides (Chouhan et. al., 2017).

As an example of monoterpenoid phenols, namely carvacrol or cymophenol are found in the essential oil of oregano, thyme, pepperwort, and wild bergamot. Carvacrol has been found to inhibit the production of diarrheal toxin by *Bacillus cereus* and the growth of vegetative bacteria. Its precursor is p-cymene, which is a monoterpene with a benzene ring and no functional groups on its side chains. When used alone, p-cymene is not an efficient antimicrobial compound, but its activity can be potentiated by compounds like carvacrol and polymyxin B nona peptide (Bagamboula et al., 2004). Studies have shown that p-cymene is hydrophobic in nature and causes greater swelling of the cytoplasmic membrane (Burt et al., 2007). Furthermore, It has been reported that EOs abundant in carvacrol demonstrate high performance in their antimicrobial responses (ÇETİN et al., 2011).

2.1.2. Phenylpropenes

Plants synthesize phenylpropenes from the amino acid precursor phenylalanine, which falls under the phenylpropanoid category. Although phenylpropenes make up a small proportion of essential oils, they are still considered an important organic compound. The most extensively researched phenylpropenes include safrole, eugenol, isoeugenol, vanillin, and cinnamaldehyde. The antimicrobial activity of phenylpropenes depends on several factors including the microbial strains selected, the type and number of substituents on the aromatic ring, and the experimental parameters such as temperature and growth medium used (Heer et al., 2017).

3. The Activities of Essential oils

The natural properties of essential oils, such as their antifungal, antibacterial, and insecticidal activities, make them a popular choice for a variety of industries. With approximately 3000 essential oils known, 300 of them are commercially significant for cosmetics, perfumery pharmaceuticals, sanitary, agronomic, and food as aroma enhancers, perfume additives, medicine, and food flavor and preservatives (Bhavaniramya et al., 2019). While understanding some of their mechanisms of action has improved over time, the fundamental characteristics of essential oils have remained unchanged (Bakkali et al., 2008).

3.1. Antifungal Activity

There have been numerous efforts to determine the mechanism of actions of EOs and their components against a broad range of microorganisms for many years. However, the mechanisms of actions toward a variety of microbes have still not been entirely

figured out till now. A number of mechanisms have been attributed to EOs and their active constituents to unveil their activities. The potential mechanisms of action are related closely to the profile of EOs, functional groups that exist in their main structures, their synergistic activities, and the tested strains of microorganisms.

Increasing awareness regarding the health and environment led to considering alternative methods that be harmless, and more natural. In light of these reasons, the use of plant-derived essential oils has been a notable natural agent as an alternative component in controlling fungi (Nazzaro et al, 2017; Angioni et al., 2006). Based on the current knowledge, it has been reported that essential oils possess various mechanisms that exhibit antifungal properties. Cell walls in fungi are characteristically composed mainly of glucans, chitin, and glycoproteins, which play a crucial role in the viability and expansion of fungi (Nazzaro et al., 2017). One of the primary mechanisms is the inhibition of β-glucans and chitin production, which are essential compounds of the fungal cell wall. This results in the prevention of cell wall formation and ultimately leads to the disruption of cell wall integrity (Hector, 1993). Disturbing the cell membrane by blocking the synthesis of ergosterol, fundamental for cell membrane integrity, leading to a relatively weak cell membrane becoming leaky is another mechanism of antifungal action by Eos (Viuda-Martos et al., 2007). The deficiency of fungal mitochondria is also considered an important mechanism by which Eos cause the inhibition of mitochondrial electron transport lowering the mitochondrial membrane potency through the hindering of the proton pumps in the respiratory chain reducing ATP processing, and eventually occurrence of cell death (Kang et al., 2010). EOs can restrict cell division with the prevention of microtubule polymerization, such that suppression of the mitotic spindle

configuration occurs (Lagrouh et al., 2017). Additionally, EOs can interrupt RNA, DNA, or protein synthesis with penetration of antifungal EOs into cells. They interfere with the RNA causing deficient RNA production and faulty DNA transcription subsequently inhibiting protein synthesis (Raveau et al., 2020). EOs and their components can disrupt the efflux pumps existing in living cells functioning as dischargers of toxic chemicals out of the cells (Kang et al., 2010).

Chitin, a natural polysaccharide, is a linear homopolymer of β-1,4-linked N-acetyl glucosamine and is catalyzed by chitin synthase. This structure is essential for the survival of fungi. Therefore, EOs causing restriction in polymerization may result in damage to the structure, division, and growth of the fungi cell (Wu et al., 2008). It has been pointed out that the essential oil of *Citrus sinensis*, which comprised nearly 84.2% limonene, prevented the growth of *A. niger* and caused constant destructive morphological alterations with damage in the cytoplasm of fungal hyphae, and the hyphal tip (Gogoi et al., 2008). Similarly, the essential oil of black cumin seed, thymoquinone was indicated to be destructive to the cell wall of fungi and cytoplasmic membrane (Iscan et al., 2016). The essential oil extracted from *Litsea cubeba* was tested against *Fusarium moniliforme*, *F. solani*, *Alternaria alternata*, and *A. niger* and it was observed that there was not only damage to the fungi cell wall and membrane, but also DNA, RNA, protein, and peptidoglycan biosynthesis was partially inhibited (Haque et al., 2016).

Terpenoids in EOs impact mitochondrial effectiveness by destroying the action of mitochondrial dehydrogenases, involved in the ATP biosynthesis of fungi (Nazzaro et al., 2017, Haque et. al 2016). A study conducted on antifungal agents revealed that the EOs of *A. graveolens* caused damage to the citric acid cycle and ATP synthesis in the

mitochondria of *C. albicans* (Chen et al., 2013). Likewise, *O. compactum*, *A. herba-alba*, and *C. camphora* essential oils are considered to have a destructive effect on mitochondria leading to cytoplasmic mutations in *Saccharomyces cerevisiae* (Bakkali et al., 2006). For the nutrition intake of fungi, supporting the plasma membrane H+-ATPase, which establishes the electrochemical gradient and promotes cell growth, is essential, as restriction in H+-ATPase results in intracellular acidification and death of the cell (Perlin et al., 2006). A study conducted by Ahmad et al. (2013) showed that eugenol and thymol restrained H+-ATPase activity, and furthermore, the compounds of thyme oil, thymol, and carvacrol limited the over-expression of efflux-pump genes CDR1 and MDR1 in *C. albicans* with an azole antimycotic fluconazole.

The *Fusarium* genus including *F. culmorum* and *F. graminearum* cause serious plant diseases which lead to significant losses in crop yields globally (Avanço et al., 2017). Additionally, human and animal health-threatening mycotoxins are produced by *Fusarium* species; therefore, to ensure food and feed safety, it is essential to control growth and mycotoxin spread by *Fusarium* spp. in crops (Perczak et al., 2019). To find a more natural disease management solution, Perczak et al. (2019) conducted a study using EOs from *C. zeylanicum*, *O. vulgare*, palmarosa leaves, orange peel, verbena leaves and flowers, spearmint leaves, fennel seeds, and rosewood to determine their antifungal effects on *F. graminearum* and *F. culmorum*. It has been stated that cinnamon, oregano, and palmarosa EOs showed significant antifungal activity. Eugenol, A study found eugenol (which is a clear to pale yellow oily liquid, is extracted from clove oil, nutmeg, cinnamon, basil, and bay leaves) to be the antifungal bioactive molecule from *C. tamala*, inhibitory against the fungal plant pathogens *A. alternata* and C*urvularia lunata*.

Likewise, application of thymol caused the cell membrane and vesicles of *Candida* strains to be destabilized as a result of the disruption of ergosterol biosynthesis regulating the membrane fluidity and integrity by the EO (Ahmad et al., 2011). Cinnamaldehyde has been reported as showing a growth inhibition against a destructive soil-borne oomycete pathogen *Phytophthora capsici* by disturbing the fatty acid, polysaccharide, and leucine metabolisms causing cell malfunction or eventually death of cells (Wang et al., 2021). While a 0.1% application of nutmeg oil with 10.8% myristicin has been shown to prevent the growth of two significant storage pathogens, the fungi *A. flavus*, and *A. ochraceus* by 43% and 65%, respectively, whereas a 0.3% application inhibited growth by 84% and 79%, respectively (Valente et al., 2015). Similarly, another comparable study revealed that EOs of *Mikania scandens* inhibited hyphal growth of fungal pathogens *Rhizoctonia solani* with the MIC value of 250 μg/ml, while against *Pythium graminicola* and *F. oxysporum* with MIC value of 500 μg/ml (Siddiqui et al., 2017).

3.2. Antibacterial Activity

Globally, plant diseases caused by bacteria are the reason for a significant economic loss in valuable crops (Bozkurt et al., 2020). In controlling bacterial disease, copper-included synthetic pesticides and antibiotics are broadly used. Even though copper is considered to impede bacterial growth, managing the disease may not always be possible since bacteria are developing copper resistance (Nguyen et al., 2018; Rhouma et al., 2008). Therefore, bioactive ingredients including alternatives are considered as a viral control method.

Several possible mechanisms have been documented to unearth the potential properties of EOs and their main components that cause structural and functional damages to the bacterial cells affecting the internal and external parts of the cells. Hydrophobicity caused by EOs influences the potential level of the EO's toxicity against bacterial colonies. This hydrophobic character provides penetration ability to microbial cells leading to changes in the structure and functions of the cells. EOs, hydrophobic chemicals, can potentially deteriorate the bacterial cell membrane resulting in abnormally increased permeability, lessening the membrane potency and ending in the leakage of cell contents, and eventually cell death. Cell membrane conductivity is a vital function to maintain several cellular activities including the control of turgor pressure, the energy production of the cells, the transportation of substances, and the regulation of metabolic processes. The differentiation in permeability of the membrane and deficiency in the movement of molecules and ions generate an unbalanced environment in the microbial cells, which induces the coagulation of cytoplasm, the distortion of a variety of enzymes and proteins, and metabolites and ions damage (Burt & Reinders, 2003). As a matter of fact, EOs and their primary compounds can degrade bacterial cell walls, damage the cytoplasmic membrane, coagulate the cytoplasm, and precipitate the membrane proteins, and reduce the proton motive force and the intracellular ATP concentration through decrement in ATP synthesis (Nazzaro et al., 2013). The phospholipid bilayer of the bacterial cell wall can be destabilized via EOs, especially rich in phenolics, leading to defects in plasma membrane composition and functionality, and blocking the enzymatic processes (Juven et al., 1994). Furthermore, EOs can destruct the electron transport system affecting membrane permeability (Tassou et al., 2000). Cell cytolysis and even

death can occur as a consequence of physiological alterations such as the prevention of electron transmission to produce energy, disturbing the proton motive force, inhibiting the relocation of proteins, and reduction in the biosynthesis of cellular components (Ben Arfa et al., 2006). The phenomenon called quorum sensing (QS) which is the intercellular communication system used for the coordination of cell-to-cell and cell-to- the external environment of bacteria can be affected by the EOs and their main compounds. It is the mechanism dependent on cell density and synthesis, transport, and sensing of small signaling molecules known as auto-inducers among the population. Small dispersive molecules, *N*-acyl-homoserine lactones (AHLs) as Quorum sensing (QS) signals, are used by Gram-negative bacteria, while modified oligopeptides are exploited by Gram-positive bacteria (Camilli & Bassler, 2006). Quorum sensing (QS) is closely connected with several vital aspects of bacterial life including bioluminescence, conjugation, the expression of virulence factors, and the formation of biofilm (Khan et al., 2009). QS may be an intriguing target for controlling diseases, inhibiting antimicrobial resistance, and limiting food loss due to its fundamental aspects in bacterial life (Faleiro, 2011). One study showed that among 21 EOs tested, four EOs obtained from clove, cinnamon, lavender, and peppermint performed promising anti-QS activity against *Chromobacterium violaceum* (CV12472 and CVO26) and *P. aeruginosa* (PAO1) (Khan et al., 2009). QS was effectively lowered with the application of rose, geranium, lavender, and rosemary oils, however, EOs of orange and juniper did not show anti-QS activities (Szabó et al., 2010).

In general, EOs are more effective, with some exceptions, against Gram-positive bacteria than Gram-negative bacteria due to distinctions in cell membrane features

(Gutierrez et al., 2008). The cell wall of Gram-negative bacteria shows more resistance to EOs and their major constituents by blocking the insertion of hydrophobic substances compared to the Gram-positive bacteria. A rigid complex outer membrane formed of a double layer of phospholipids rich in lipopolysaccharides (LPS) exists in Gram-negative bacteria, which provides the endurance of Gram-negative bacteria to Eos. Whereas, there is no extra complex membrane in Gram-positive bacteria, which are encircled with a thick peptidoglycan wall that is not sufficiently compact to resist small antimicrobial molecules, easing the diffusion into the inner membrane (Chouhan et al., 2017). In addition, the lipophilic ends of lipoteichoic acid found in the cell membrane of Gram-positive bacteria lead to easiness for the entrance of hydrophobic components of EOs into the cells (Cox et al., 2000). The experiment of Sahin et al. (2003), in which the extract obtained from *Satureja hortensis* was tested against 55 bacteria, including plant pathogenic bacteria, resulted in the realization that the extracted EO from this plant can be used as a biocontrol agent. In the study conducted by Elshafie et al. (2019) the antibacterial activity of *Solidago canadensis* essential oil, which consists of germacrene D (34.9%), followed by limonene (12.5%), α-pinene (11.6%), β-elemen (7.1%), and bornyl acetate (6.3%), were tested on some phytopathogenic bacteria such as *B. megaterium, Clavibacter michiganensis, Xanthomonas campestris, P. fluorescens,* and *P. syringae* pv. *phaseolicola*. The result of the study revealed that oil extracted from *S. canadensis*, showed antibacterial features, and the highest activity was seen against *P. fluorescens*, compared to other bacteria, while it displayed moderate activity against *C. michiganensis*.

In research carried out by Bozkurt et al. (2020) several medicinal plant species including Lamiaceae (*Thymbra spicata var. spicata, T. serpyllum, T. sipyleus, O. syriacum, O. majorana, Ocimum basilicum, M. spicata, Melissa officinalis, L. stoechas var. stoechas, Rosmarinus officinalis, Salvia officinalis*), Lauraceae (*Laurus nobilis*) and Apiaceae (*Foeniculum vulgare*) families on gall-forming bacterial disease agents *R. radiobacter, P. savastanoi* pv. *savastanoi* and *P. savastanoi* pv. *Nerri* were tested for antibacterial activity. With the use of the disc diffusion technique, the antibacterial activities of essential oils from each plant species were evaluated by examining inhibition zones that occurred against the 3 pathogens. Based on the results, he most effective EOs belonged to the Lamiaceae family (*T. serpyllum, T. spicata var. spicata, and O. syriacum*). Moreover, for *P. savastanoi* pv. *savastanoi*, the highest inhibitive effect was observed using EOs from *O. syriacum* and *T. serpyllum* whereas the lowest was seen in *F. Vulgare* EOs. In the case of the activity of EOs on *R.radiabacter*, it has been noted that the highest inhibitory effect was obtained from *O. syriacum* and *T. serpyllum*, while the least activity was achieved by *R. officinalis*. According to the study conducted by Carezzano et al. (2017) it was exhibited that the EOs extracted from *T. vulgaris* (thyme) and *O. vulgare* (oregano) possess a great potential to inhibit the bacterial growth of *P. syringae* strains and reduce its virulence factor even in very low concentration. Carvacrol interacts with the outer membrane inducing passive ions transportation over the membrane, also affecting the fluidity of the cells due to alteration in fatty acid profile with the application of it (Di Pasqua et al., 2006). The cytoplasmic membrane is considered the main site of action by carvacrol. The cell size, length, and diameter reduced in gram-negative and gram-positive bacterial strains including *Brochothrix*

thermosphacta 1R2, *Carnobacterium maltaromaticum* 9P, *Lactobacillus plantarum* 48M, *Listeria innocua* 1770, *E. coli* 32, *Hafnia alvei* 53M, *P. fragi* 25P, and *Serratia proteamaculans* 42M under 3.3 mM carvacrol exposure because of the leaking out the cells cytosolic fluids (La Storia et al., 2011). Thymol and thymol-rich EOs are the other natural compounds extensively studied over the last decades. It is thought that the exposure of thymol lessens the recovery ability of cells due to affecting some important energy-generating processes including the citrate metabolic pathway. Thymol with interaction of *S. enterica* at the sub-lethal level caused changes in the accumulation of outer membrane proteins and upregulated the genes that take part in the outer membrane protein synthesis (Di Pasqua et al., 2010).

4. Methods for *In Vitro* Evaluating of Antimicrobial Activity

Over the past decades, a great effort has been taken to find and develop novel antimicrobial compounds from different sources to control pathogen development and growth and inhibit resistance to the chemicals. Currently, extensive research to find alternative sources and discover new antimicrobial molecules has been conducted to reduce the possibility of emerging microbial resistance in field applications. Plant and microbial extracts, essential oils, pure secondary metabolites, and newly synthesized molecules have been primarily investigated as potential antimicrobial agents against various bacterial and fungal pathogens. However, the challenge in determining these naturally produced compounds is due to the difficulties in comparing results, using non-standardized methods, inoculum preparation techniques, size, growth media, incubation conditions, and endpoint detection. Preliminary data associated with the antimicrobial activities of the candidate molecules should be reliable and repeatable to allow

investigators to compare the outcomes. Several laboratory bioassays including disk-diffusion, well diffusion, and broth or agar dilution methods, which are predominantly applied, are used to screen and evaluate *in vitro* antimicrobial effects of the candidate chemicals or compounds against target pathogens. In addition to these methods, flow cytofluorometric and bioluminescent methods are also employed. However, flow cytofluorometric and bioluminescent methods are seldom compared to the diffision approaches due to the requirement of specific equipment and difficulties of repeatability and standardization. Regardless, the diffusion methods produce quick outcomes from the tested agents and in-depth information about the effect of tested chemicals on the microbial cell viability and damage. It is important to improve our knowledge of the presently available methods to screen and quantify the antimicrobial effects of candidate molecules to determine the potential applications of Eos in human health, agriculture, and the environment.

4.1. The Agar Disk-diffusion Method

The Agar disk-diffusion method is a common antimicrobial susceptibility testing method officially applied by many clinical microbiology laboratories, which was developed in 1940 (Heatley, 1944). Currently, the Clinical and Laboratory Standards Institute (CLSI) published several accepted and approved standards both yeasts (CLSI, 2018) and bacteria assays (CLSI, 2018). In this technique, solid agar plates are inoculated with a standardized microbial inoculum of target pathogens, followed by the placement of the filter paper disks approximately 6 mm in diameter immersed with the test molecules at desired concentrations to the surface of Petri plates. Then, the processed sample Petri plates are incubated under optimized conditions for a period of time based

on the growth of the pathogen (around 4-7 days in fungi, 1-4 days in bacteria). Tested antimicrobial agents disperse into the medium and prevent the germination and growth of the target microorganism with the forming of a clear zone around the filter paper disks, if they are functional against the target microorganism. The rating of pathogens' susceptibility toward tested compounds or their effectiveness was measured by the clear zone formation surrounding the placed paper disks.

The disk diffusion method provides qualitative results for the level of microorganism susceptibility which is categorized as susceptible, intermediate, or resistant (Reller et. all, 2009). These qualitative outcomes are derived from the test instead of MIC (Minimum Inhibitory Concentration). It is not an appropriate technique to determine the MIC because it is not possible to quantify the amount of diffusion of the tested compound into the agar medium. However, there are some available commercial zone reader systems claiming to estimate approximate MIC for particular organisms and molecules based on the comparison of clear zones with standard curves in stored algorithms (Reller et. all, 2009). In addition, the growth inhibition of the tested pathogen does not imply its death, so it is not a suitable method for the characterization of the cidal or static effect of a chemical. Moreover, it is an inappropriate method to test lipophilic extracts or their compounds such as essential oils due to insufficient diffusion of the EOs and their components from paper disks to agar medium. EOs and their component mostly perform a small clear zone or do not show any clear zone surrounding the paper disks in the disk diffusion method. However, it is a relatively simple and least costly method comparing the others with no requirement for special equipment, and the outcomes of the assay are also easily explicated and categorized by all researchers. Additionally, it allows

the researchers to examine plenty of microorganisms and candidate agents in an assay (up to six agents in a Petri plate).

The agar disk diffusion method was used to determine the susceptibility of filamentous fungi against posaconazole (López-Oviedo et al., 2006). Additionally, micafungin against *Aspergillus* spp. and caspofungin against *Aspergillus* spp. and *Fusarium* spp. were tested by using this method (Balouiri et al., 2016).

4.2. The Antimicrobial Gradient Method

The antimicrobial gradient method is a technique based on the formation of an antimicrobial concentration gradient in an agar medium to determine the susceptibility of microorganisms against the tested chemicals. The commercial version is available as ETEST® (BioMérieux) on the market. A tiny plastic strip impregnated with an increasing concentration gradient of the tested compound is used. There is a scale from one side to another side on the surface of it showing the examined concentration of the antimicrobial agent. In the process, it is placed on an agar medium previously inoculated with the standardized organism suspension. After overnight incubation, samples are ready for the observation of minimum inhibitory concentration (MIC) from the surface of the plates. The MIC value is the point of intersection on the lower part of the plastic strip and the oval-shaped growth inhibition zone, which can be easily observed and implemented. It is a routinely applied method with intrinsic flexibility to test different molecules the researchers demanded. The cost of each ETEST strip is around $4 to $5. It is a suitable approach in the scenario in which one or two chemicals are tested to see MIC value, and also where to use a fastidious organism with an enriched medium and special incubation condition in the experiment (eg, penicillin and ceftriaxone with pneumococci) (Citron et

al., 1991). The MIC values generated from ETEST outcomes show a generally positive correlation between MICs identified from the broth and agar dilution methods based on the previous studies (Baker et al., 1991). Additionally, either synergistic or antagonistic interactions between the tested two antimicrobial agents can be investigated by using this method (White et al., 1996).

4.3. The Agar Well Diffusion Method

The agar well diffusion method is one of the most applied qualitative assays to investigate the antimicrobial potential of plant extracts and their major components (Valgas et al., 2007). In the assay, the agar plate surface is impregnated by spreading with a certain volume of microbial inoculum in a standardized concentration, and a hole (6 to 8 mm) with a sterilized pipet tip or other apparatus is created on the Petri plate. Then, a volume (10 to 200 ul) of the tested antimicrobial agents at desired concentration is inserted into the well followed by the incubation of samples in appropriate conditions based on the examined pathogens. The antimicrobial agent disperses into the agar medium, and if the antimicrobial agent has activity against the target microbe, a clear zone due to growth inhibition is observed around the well. The clear zone around the well is measured to compare the control group to determine the antimicrobial potential of the tested agents. It is a relatively cost efficient and simple method comparing the other methods. It does not require any specialized equipment in the laboratory to establish an experiment. Also, a large number of different plant extracts or their major components can be screened at the same time. However, the limitation related to this procedure is the differentiation seen in the diffusion level of plant extracts or essential oils from the well to the agar medium. For example, terpenoid-rich essential oils have a low level of

diffusion due to less solubility in the agar medium, meaning that antimicrobial activity cannot be expressed properly, and end up with a wrong observation (Guimarães et al., 2019).

4.4. The Poisoned Food Method

The poisoned food method is one of the most common approaches exploited to screen the antifungal effects of plant extracts or their major compounds. In the process, the antimicrobial agent or plant extract is added into the molten agar medium cooled to 35 °C to 40 °C at desired final concentration, mixed rigorously, and poured into the Petri plates at the same volume. After overnight pre-incubation at room temperature, mycelia discs (6 to 8 mm diameters) obtained from the freshly grown stock culture are placed on the center of the antimicrobial agent-impregnated Petri plates, and samples are left for incubation under the optimum conditions for the target organism. The diameter of fungal growth is measured both in the control group non-treated with an antimicrobial agent and sample plates, then, the antifungal effect of tested agents is calculated with the following formula.

Antifungal activity (%) = ((Dc-Ds)/Dc) x100

Dc: The diameter of hyphal growth in the control group.

Ds: The diameter of hyphal growth in sample plates treated with antimicrobial agents.

In addition to the hyphal growth experiment, the sporulation and spore germination effect of the candidate antimicrobial molecules can also be investigated by comparing the control group.

4.5. Thin-Layer Chromatography (TLC)-Bioautography

Thin-Layer Chromatography (TLC)-Bioautography is the simple, rapid, and cost-effective technique combining TLC with both biological and chemical determination to screen complex mixtures such as plant extracts of their potential activities against fungal and bacterial targets. A large number of samples can be screened at the same time without the requirement of sophisticated equipment. It can be categorized as three different techniques including agar diffusion, direct bioautography, and agar overlay assay.

4.5.1. Agar Diffusion

Agar diffusion, named as an agar contact method, is the least used technique among these three categories. In the procedure, antimicrobial molecules are transferred from the chromatogram (PC or TLC) to the agar plate already inoculated with the target pathogens. After the diffusion of the antimicrobial molecules, the chromatogram is removed and plates are incubated under favorable conditions based on the tested organism. Then the growth inhibition areas become visible on the sites in which the antimicrobial agent touches the agar plate (Marston, 2011).

4.5.2. Direct Bioautography

Direct bioautography is the most exploited technique as the TLC method. In the method, the prepared TLC plate is inoculated with the microbial suspension by dipping or spraying followed by incubation of the bioautogram at 25 °C for 48 h under high humidity (Dewanjee et al., 2015). Tetrazolium salts such as p-Iodoni-trotetrazolium violet are commonly used to visualize microbial growth (Choma & Grzelak, 2011). Bioautogram is treated with these salts by spraying and then left for incubation at 25 °C

for 24 h or 37 °C for 3–4 h (Runyoro et al., 2006), then inhibition zones are observed and measured. It is possible to employ against both fungal and bacterial targets by using less amount of samples comparing the agar disc diffusion method (Runyoro et al., 2006).

4.5.3. Agar overlay Bioassay

Agar overlay bioassay**,** also named immersion bioautography, is the combination of these two methods previously mentioned. In the process, TLC plates are overlaid with molten agar inoculated with the pathogen of interest and then incubated under favorable conditions for the target pathogen. After incubation, staining is processed by tetrazolium salts for the inhibition zone observation.

4.6. Dilution Methods

Dilution methods including agar and broth dilutions are the most suitable techniques for detecting MIC values due to their availability to evaluate several antimicrobial agents at desired concentrations. The *in vitro* effect of tested compounds can be quantitatively measured through both agar and broth dilution methods. MIC (Minimum Inhibitory Concentration) is the lowest concentration of the tested antimicrobial agent inhibiting the visible growth of the examined pathogens, which is generally asserted as ug/ml or mg/ml. Several authorized guidelines, produced by the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), for dilution methods, are available to test the antimicrobial susceptibility of fastidious or non-fastidious bacterial and filamentous fungal pathogens, which offer a consistent approach in practical application. The bioassays can be performed in recognized methodological standards providing an evaluation of the laboratory relevance of the outcomes (Pfaller et al., 2004).

4.6.1. Broth Dilution

Broth dilution (macro or micro dilution) is one of the most common and precise methods applied for antimicrobial susceptibility testing. In the method, the candidate antimicrobial agents are prepared in desired concentrations by two-fold dilutions (eg. 4, 8, 16, 32, 64 ug/ml) in the liquid growth medium, then distributed in 2 ml tubes known as macro-dilution or in 96-well micro titration plates around 200 ul known as microdilution. Each tube or well is inoculated with the target pathogen and mixed vigorously, followed by incubation under favorable conditions for the tested pathogen with suitable agitation. After a certain time of incubation based on the tested microorganism, a sample from the solution is taken out and streaked onto the agar plates to observe microorganism growth for the determination of the MIC and MBC (Minimum Bactericidal Concentration) or MFC (Minimum Fungicidal Concentration) endpoints. MBC is the lowest concentration of antimicrobial compounds required for complete death of the final bacterial inoculum after 24 h incubation at the favorable condition for the tested pathogen. MFC is described as the lowest concentration of antimicrobial agents that kills the targeted fungal microbe compared to the initial inoculum.

Among the dilution methods, macro-dilution is a tedious and more labor-demanding technique than micro-dilution. Also, the risk of errors is relatively higher, especially in the preparation of the antimicrobial solutions for each experiment and it requires a high volume of reagents and room compared to the micro-dilution. Moreover, the main advantage of microdilution is the repeatability of the tests in the less volume of the antimicrobial solutions in intralaboratory and interlaboratory due to being miniaturized experiments. In the micro-dilution method, a spectrophotometer in optical

density (600 nm) can be used for the observation of bacterial growth to determine MIC endpoints. In addition, a number of colorimetric techniques based on the dying reagents including tetrazolium salts and the Alamar blue dye (resazurin) can be used for the identification of MIC breakpoints for both antifungal and antibacterial microdilution (Al-Bakri et al., 2007).

It is known that MIC values are directly affected by the inoculum size, growth media, incubation time, and inoculum preparation techniques (Balouiri et al., 2016). CLSI and EUCAST have developed standards for test parameters similar in principals to examine bacterial and fungal, and yeast pathogens. For example, the inoculum size is adjusted to $0.4x10^4$–$5x10^4$ CFU/mL based on the CLSI standards, while it is (2–5) x 10^5 CFU/mL in EUCAST standards for the testing of the conidium or spores-producing fungi in the microdilution method (Lass-Flörl et al., 2006).

4.6.2. Agar Dilution

Agar dilution is one of the most well-established techniques to identify antimicrobial susceptibility against tested compounds, which is suitable for both bacterial such as *Helicobacter* species and fungi such as *Candida* sp., *Aspergillus* sp., *Fusarium* sp. testing (Imhof et al., 2003). The antimicrobial agents are impregnated into agar plates (molten agar medium) by mostly using serial two-fold dilutions, then a standardized microbial inoculum is applied to the agar surface and incubated under suitable conditions, followed by the observation of MIC endpoints. It is more useful where many isolates are examined against a single compound or the dilution method is not available for MIC determination. Currently, there are commercialized inoculum replicators used for the transfer of up to 60 bacterial inocula to prepared agar plates. Contrary to the advantages,

it is not easy to emulsify the tested plant extracts or essential oils with the agar to determine their antimicrobial activities.

4.7. Time-kill Test

Time-kill test is a simple and appropriate method for the determination of the bactericidal and fungicidal effects of candidate antimicrobial agents. It is an effective tool to collect data about the dynamic interaction among antimicrobial agents and tested organisms, revealing information about the time-dependent or concentration-dependent results. It is a well-standardized method performed in a broth culture medium with three tubes harboring microbial suspension (eg. $5x10^5$ CFU/mL of bacteria). The first and second tubes include the final concentration of tested molecules at the 0.25xMIC and 1xMIC levels, respectively, and the third tube serves as a control group. Followed by counting the living cell number (CFU/mL) from each tube by agar plate count method in varied time intervals (0, 4, 6, 8, 10, 12, and 24 h) under suitable incubation conditions (Konaté et al., 2012). The estimated bactericidal value is usually calculated as a lethality rate at 6 h equal to complete cell death at 24 h of the experiment, the similar method can be used for the determination of the antifungal effect as well as the assessment of synergism and antagonism impact of the candidate molecules (Balouiri et al., 2016).

4.8. ATP Bioluminescence Assay

ATP bioluminescence assay is the method associated with the measurement of adenosine triphosphate (ATP) generated by the target microorganisms. ATP is present in a certain amount of cells as it is the chemical form of energy in all living cells. This process is based on the quantification of the ATP level for the estimation of microbial population in a sample. In the presence of ATP, D-luciferin is converted to light-

producing oxyluciferin by luciferase. The emitted light is quantitatively measured by a luminometer producing results as a relative light unit (RLU) transformed into the RLU/mole of ATP. Indeed, a linear relationship is present between the viability of cells and the level of luminescence. There is a broad range of application potential for it in different purposes including cytotoxicity tests, antibacterial testing, and antifungal test against yeast and molds. The main advantage of the method is the production of quantitative outcomes in a short time around three to four days compared to the traditional dilution approach requiring three to four weeks (Gonzalez-Pastor et al., 2023).

4.9. The flow Cytofluorometric Method

The flow cytofluorometric method has the potential to investigate the antibacterial and antifungal activity of drugs, plant extracts, and essential oils. It is a reproducible rapid detection method (4 to 6 h) of membrane-damaged cells in comparison to the microdilution method (3 to 4 days) depending on the usage of proper dye staining including propidium iodide (PI) and carboxyfluorescein acetate. Not only lysed cells but also dead, viable, and injured cells can be distinguished by using this process. Thus, the flow-cytoflourometric is a beneficial tool to detect antimicrobial resistance against the tested molecule and estimate the effect of the tested molecules on the target microbe. However, the wide application potential for antimicrobial susceptibility testing is relatively low soon due to the requirement of specific flow cytometry equipment which is inaccessible in all labs.

5. Limitations

Although plant essential oils can be effective in controlling pests, they have some limitations when used as a pesticide. Compared to synthetic pesticides, in some cases,

they are less effective and require higher application rates. Additionally, outdoor use may require frequent reapplication. Using plant essential oils as commercial pesticides presents additional challenges that must be addressed.

It is important to consider that the chemical makeup of plant species can differ naturally based on various factors such as geographic location, genetics, climate, and seasonal changes. This means that manufacturers of pesticides must take extra steps to ensure their products will work consistently. This process can be costly and smaller companies may not be able to invest the necessary funds without the possibility of recovering costs through market exclusivity, particularly patent protection (Koul et al., 2008).

Securing regulatory approval is a crucial step for any pesticide seeking to enter the market. It is a significant hurdle that cannot be skipped, as it is the only way to get product sales and generate revenue. However, getting approval for essential oil-based pesticides in other parts of the world has proven to be challenging due to regulatory guidelines being tailored to evaluate synthetic pesticides with a single active ingredient and no uncertainty. Although certain essential oils have been granted exempt active ingredient status in the USA, gaining approval in other parts of the world is difficult. Nonetheless, the EU is developing criteria for "low-risk active substances," and some essential oils may meet these new standards. If successful, it could open the door for more pesticide approvals based on these oils (Villaverde et al., 2014). Regulatory agencies evaluate product safety through a comprehensive analysis of various factors, including chemical composition, environmental impact, and toxicity to laboratory

animals, as well as other living organisms like fish, wildlife, and pollinators. While efficacy data may be required by some agencies, others may not demand it (Isman, 2016).

Essential oils are frequently used for their enticing aroma in consumer goods and cosmetics, as well as their healing properties in aromatherapy. They also serve as popular flavor enhancers in food and beverages. While their chemical makeup and potential toxicity to lab animals are typically well documented, this information is often regarded as confidential and difficult to obtain. Although there is limited research on the impact of plant essential oils on the environment, studies suggest that their high volatility enables them to evaporate quickly, resulting in low persistence in nature, except when stored in enclosed containers (Hu & Coats, 2008).

Due to high competitiveness within the agrochemical industry, even the most eco-friendly pesticide may not be desirable if it is not cost-effective compared to other pesticide products available in the market. This is particularly relevant in the agricultural and pest control sectors, where large quantities are required and application costs can be significant. The reason some plant essential oils have low material costs attractive enough for use in pesticides is that they are internationally traded on a large scale as commodities for the flavoring and fragrance industries. These established uses and supply chains dictate prices, and though these oils have been used in pesticides as an alternative market for producers, they have yet to grow significantly enough to affect prices (Isman, 2016). The prices of major essential oils remain mostly stable due to their well-established supply chains. However, fluctuations in prices can occur due to crop failures caused by abnormal weather patterns in key production regions. Additionally, certain oils have experienced a decrease in price due to production shifting to regions with lower land and

labor costs, such as China, India, and Brazil. In contrast, oils like clove and patchouli largely remain produced in their original cultivation regions, such as Indonesia. It is essential to consider that the pricing of essential oils varies to a large extent based on factors such as quality, source, and volume (Schmidt, 2016).

The use of essential oil-based pesticides for agricultural or home and garden purposes can pose a unique challenge due to phytotoxicity when applied directly to plants. Any oil, whether from a natural or petrochemical source, can be phytotoxic if applied at concentrations exceeding 2% as an aqueous emulsion, and in some cases, even at concentrations as low as 1%. Essential oil-based pesticides currently in use are typically recommended to be used at concentrations ranging from 0.5-1.0%, depending on the crop and pest. Clove oil, for example, can be used as an herbicide at concentrations of 5% or higher (Stoklosa et al., 2012). Given the small margin of error for certain plants, it is highly recommended to conduct empirical testing of any product on the intended crop under realistic conditions. Formulation is also an effective way to mitigate or eliminate phytotoxicity when the risk to a target crop is significant (Isman, 2016).

As mentioned, a number of different laboratory methods are currently present to test candidate compounds against a variety of pathogens for their antimicrobial susceptibility. Among these methods, a number of it was standardized against certain microorganisms by the CLSI and EUCAST, specifying the main points in the field. However, natural extracts consist of a combination of various molecules that may express different performances as predicted in the approach, which creates several challenges when the clinical guideline is used as standardized because there are no certain standards

for natural extracts. Therefore, some minor revisions in these standardized methodologies without changing major steps are required for the testing of natural extracts such as essential oils due to the characteristics of these naturally obtained compounds.

Considering the importance and challenges of EOs, this study mainly focused on identifying pure compounds of EOs that could potentially combat plant fungal and bacterial pathogens, with a particular emphasis on economic significance. Experiments began with in vitro tests to assess the antifungal and antibacterial activities of various compounds. Based on these results, we selected specific pure compounds to undergo planta experiments in both wheat and tomato plants. In the wheat study, the effectiveness of tested compounds in controlling fusarium head blight disease (FHB), caused by the fungal pathogen *F. graminearum*, was evaluated by observing their effect on disease severity, mycotoxin level, and quality parameters. Similarly, in the tomato experiments, the protective potential of the same compounds against bacterial speck disease caused by *P. syringae* pv. *tomato* was evaluated by monitoring disease severity and several agronomic properties, including plant height, weight, and flowering time, as well as assessing the impact of the compounds on the expression of defense-related genes in tomato.

6. References

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Chapter II

Evaluation of Antifungal and Antibacterial Activity of Selected Essential Oils (and Major Components) against Plant Fungal and Bacterial Pathogens *In vitro*

1. Introduction

Crop production is continually exposed to a plethora of disease threats including fungal and bacterial pathogens adversely affecting the sustainability of the food supply (Taheri et al., 2023). Plant diseases are primary and growing risks for global food safety due to causing severe reductions in yield and quality of agricultural products (Singh et al., 2023). Although several strategies including cultural practices (tillage, crop rotation, irrigation management, etc.), use of disease-resistant or tolerant plants, usage of synthetic chemical pesticides (fungicides, bactericides, nematicides, etc.), and biological control are applied for the management of the occurrence, intensity, and severity of the plant diseases (Maloy, 2005), currently the application of synthetic chemical pesticides is still the main approach (Zamani-Zadeh et al., 2013). However, the indiscriminate use of synthetic pesticides may result in a wide array of human health breakdowns such as dermatological, neurological, respiratory, and endocrine disorders, reproductively and developmentally, or acute toxicity (Nicolopoulou-Stamati et al., 2016). Moreover, pesticide residues, not completely removed by washing or peeling, were detected in many daily foods and beverages including cooked meals, vegetables, fruits, refreshments, fruit juices, wine, animal feeds, and most importantly water (Reiler et al., 2015). Furthermore, synthetic pesticides show detrimental implications on the environment including the contamination of soil, water, and air with the addition of toxicity to non-target creatures such as domestic animals, birds, honey bees, fishes, and aquatic invertebrates (Kumar et

al., 2020). In addition, the frequent (or sometimes inadequate) use of synthetic compounds may lead to the development of resistance in the targeted pathogens against the applied chemicals creating difficulties for the management of the plant diseases in agriculture (Clerck et al., 2020). These concerns indicate the need for novel, eco-friendly compounds as alternatives to synthetic chemical pesticides for the sustainable management of plant diseases. Over recent years, there has been an exponentially increasing interest in the new natural molecules, especially essential oils, and their major compounds (e.g., terpenes, terpenoids, and aromatic compounds) for their antimicrobial properties to find novel bio-pesticides against plant pathogens to replace the conventional pesticides. EOs, generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) (Perczak et al., 2019), are relatively less toxic to humans, highly volatile, and degrade rapidly in nature compared to synthetic pesticides (Bajpai et al., 2011).

Essential oils (EOs) are hydrophobic, natural compounds synthesized as secondary metabolites in more than 17,500 plant species in a large number of plant families viz, *Asteraceae*, *Lamiaceae*, *Myrtaceae*, *Rutaceae*, *Myrtaceae*, and *Zingiberaceae* (Mérillon & Rivière, 2018). They are naturally found in different parts of plants including bark, flower, leave, root, twigs, and seeds (Bakkali et al., 2008). Although there are several methods for the extraction of the EOs from the different parts of the plants, steam hydro-distillation is the most common technique (Nazzaro et al., 2017). EOs are highly complex compounds containing around 20 to 60 constituents at relatively different concentrations, usually composed of two or three main components ranging from 20% to 95% concentrations as well as other components found at very low

levels (Raveau et al., 2020). For example, d-limonene is primarily found in *Citrus* peel oils with over 80%, while *Origanum compactum* oil contains high levels of carvacrol and thymol with around 30% and 27%, respectively (Chouhan et al., 2017). The chemical composition of EOs shows differentiation based on several factors such as plant species, plant part used for extraction, plant age, geographic location, climate, soil composition, agricultural practices used for cultivation, and extraction technique (Perczak et al., 2019). The chemical composition of the volatile mixtures is comprised of two distinct bio-synthetical origin groups which are terpenes (e.g., limonene, linalool) and terpenoids (e.g., thymol, carvacrol), and other aromatic compounds (e.g., cinnamaldehyde, safrole) (Pichersky et al., 2006), which are characterized as low molecular weight (Clerck et al., 2020). The complex composition of EOs harbor a diverse range of biological activities against the pathogens reducing the risk of resistance development toward the chemicals (Božović et al., 2023). The bioactivity potency is directly associated with the major component profile of the EOs, functional groups that exist in their main structures, their synergistic activities, and the tested strains of microorganisms (AbouAitah & Lojkowski, 2022). Although the antimicrobial properties of the EOs and their major components are well documented against the phytopathogens, their mechanisms of action have still not been entirely figured out (Parikh et al., 2021). EOs and their major components have a great potential to protect crops including cereals, grains, pulses, fruits, and vegetables from a variety of threats including fungal and bacterial pathogens.

In the last few decades, numerous studies have shown that plant-derived EOs and their major compounds have been notable natural agents as alternative components in controlling plant pathogenic fungi (Nazzaro et al, 2017). Harčárová et al. (2021) found

that the EOs of *Syzygium aromaticum*, *Thymus vulgaris*, and *O. vulgare* performed 100 % radial growth inhibition against the *Fusarium graminearum* species at the concentration of 500 µg/mL and 1000 µg/mL. The study pointed out that EOs of *Mikania scandens* inhibited hyphal growth of fungal pathogens *Rhizoctonia solani* with the MIC (minimum inhibitory concentration) value of 250 µg/mL, while against *Pythium graminicola* and *F. oxysporum* with MIC value of 500 µg/mL (Siddiqui et al., 2017). Seema & Devaki (2010) showed that the EOs of cinnamon (*Cinnamomum zeylanicum* Breyne.) completely suppressed the growth of the *R. solani* with 500 ppm, whereas EOs of clove (*S. aromaticum*) and coriander (*Coriandrum sativum*) achieved 100% growth inhibition at the concentration of 1000 ppm. Amini and his colleagues (2010) conducted a study showing the application of *Zataria multiflora*, *T. vulgaris*, and *T. kotschyanus* essential oils at a concentration of 200 µl/L resulted in 100% suppression of hyphal growth in four economically significant fungal pathogens including *F.graminearum*, *R. solani* (AG4), *Sclerotinia sclerotiorum*, and *P. aphanidermatum*. In addition to inhibiting hyphal growth, the application of essential oils also has an adverse effect on spore formation. This leads to a reduction in spore production and germination, resulting in a significant decrease in the propagation and spread of infections. A study showed that the application of *Curcuma longa* L. EO at a concentration of 0.5% completely inhibits the spore production and germination of *Aspergillus flavus* (Ferreira et al., 2013). Similarly, the EOs of *Ocimum sanctum* L. at a concentration of 1800 µg/ml completely inhibited the germination of *F. graminearum* (Kalagatur et al., 2015). Hou et al. (2020) demonstrated that the application of thymol at 250 µg/mL and carvacrol at 300 µg/mL inhibited the spore germination of *Botrytis cinerea*, while the spore germination was around 20% at

the concentration of 300 µg/mL of *Origanum vulgare* EO. In another study, cinnamaldehyde at 36 mg/L and thymol at 72 mg/L completely blocked the zoospore production of *Phytophthora parasitica* var. *nicotianae*, while 100% germination inhibition of the zoospores was observed at 18 mg/L for both major components (Lu et al., 2013).

Essential oils (EOs) and their primary components are highly effective in managing bacterial diseases in plants due to their potent antibacterial properties. They can cause structural and functional damage to the bacterial cells, which adversely affects the internal and external parts of the cells and eventually cell death. Research has shown that EOs extracted from thyme (*T. vulgaris*) and oregano (*O. vulgare*) have the potential to inhibit the growth of *Pseudomonas syringae* strains and reduce their virulence, even at low concentrations of 1.43 to 11.5 mg/mL and 5.8 to 11.6 mg/mL, respectively (Carezzano et al., 2017). A study by Amini et al. (2018) found that applying *Z. multiflora* EO at a concentration of 232 µg/mL completely inhibited the growth of *Xanthomonas campestris* pv. *campestris* strains. In another study, cinnamon oil completely inhibited the growth of *X. arboricola* pv. *pruni* strain (Xap3) with MIC values of 31.25 µg/mL, while the major components trans-cinnamaldehyde, thymol, and citral had MIC values of 100 µg/mL, 50 µg/mL, and 400 µg/mL, respectively (Kolozsváriné et al., 2023). The EO of *Thuja occidentalis* performed a highly strong antibacterial activity against *Agrobacterium tumefaciens* and *Erwinia carotovora* var. *carotovora* with MIC values of 400 and 350 mg/L, respectively (Badawy & Abdelgaleil, 2014). In general, EOs are more effective, with some exceptions, against Gram-positive bacteria than Gram-negative bacteria due to distinctions in cell membrane features blocking the insertion of hydrophobic substances

through its lipopolysaccharide layer (Burt, 2004). Lambert et al. (2001) presented that the bacterial growth of *P. aeruginosa* was hindered by the application of thymol with a MIC value of 255 mg/L, and carvacrol with a MIC value of 278 mg/L, while the MIC values were 91 mg/L and 105 mg/L, respectively, for the complete bacterial growth prevention of *Staphylococcus aureus*. The study showed that among tested food-borne bacterial pathogens (*S. aureus, Bacillus subtilis, B. cereus, Listeria monocytogenes, Salmonella typhimurium*, and *Escherichia coli* O157:H7), *L. monocytogenes* was the most sensitive bacterial species to the antibacterial effect of *Mentha spicata* EO (78.76% carvone) with MIC value of 2.5 µL/mL (Shahbazi, 2015).

Considering the importance of finding novel green biocides for controlling fungal and bacterial diseases in crop plants, a study was conducted to evaluate the in vitro antifungal and antibacterial activities of one EO, nutmeg oil, and seven different major components, including safrole, toscanol, safraleine, cinnamaldehyde, carvacrol, carvone, thymol, against four economically important fungal pathogens, namely *A. solani*, *F. graminearum*, *R. solani,* and *S. sclerotiorum,* as well as one oomycetes pathogen, *P. ultimum,* and four bacterial pathogens, including *Clavibacter michiganensis* subs. *nebraskensis, X. campestris* pv. *campestris, P. syringae* pv. *tomato DC3000,* and *GFP tagged P. syringae* pv. *tomato.* The main objectives of this work were to assess the inhibitory growth effect of the selected EOs and major constituents on tested fungal pathogens, determine the MIC and MFC (Minimum Fungicidal Concentration) of examined EO and major compounds against examined fungal pathogens, investigate the impact of tested chemicals on spore production and germination of *F. graminearum,* additionally, screen the potential antibacterial activity of the selected EO and major

constituents on tested bacterial pathogens, and identify the MIC and MBC (Minimum Bactericidal Concentration) of examined EO and major compounds. The present study revealed that among the tested chemicals, cinnamaldehyde, carvacrol, and thymol had more potent in vitro antifungal and antibacterial activities at lower concentrations compared to other compounds.

2. Materials and Methods

2.1. *In vitro* Antifungal Bioassays

2.1.1. Microorganisms and Culture Conditions

The effectiveness of the tested compounds was examined against four fungal species including *A. solani* (As), the causal agent of early blight disease, *F. graminearum* (Fg), the causal agent of head blight disease, *R. solani* (Rs), the causal agent of damping-off & root rot diseases, *S. sclerotiorum* (Ss), the causal agent of white mold disease, and one species belonging to the oomycetes, *P. ultimum* (Pu), the causal agent of damping-off & root rot diseases, in in-vitro experiments. As culture media, potato dextrose agar (PDA) (4 g/L potato extract, 20 g/L dextrose, and 15 g/L agar) was employed for the testing of *F. graminearum*, *R. solani*, and *S. sclerotinia*, while 10% handmade potato dextrose agar (10% HPDA)(100 ml/L 100% handmade potato stock solution, 8 g/L dextrose, 16 g/L agar) for *P. ultimum*, and V8 media (160 ml/L V8® juice, 14.4 g/L agar) for *A. solani*. All tested pathogens were freshly grown before the experiments on Petri plates (90 mm in diameter) in appropriate media, and incubated at room temperature (25 ^{o}C) under the light/darkness photoperiod of 12 h for 7 days.

2.1.2. *In vitro* Tested EOs and Major Components

The potential antifungal properties of seven different major components of EOs including safrole (Sigma-Aldrich, S9652), toscanol (Givaudan, 16510-27-3), safraleine (Givaudan, 54440-17-4), cinnamaldehyde (Sigma-Aldrich, W228613), carvacrol (Sigma-Aldrich, W224611), carvone (Sigma-Aldrich, 124931), thymol (Sigma-Aldrich, 16254), and nutmeg oil (Sigma-Aldrich, W279307) as essential oil, and two fungicides, Miravis® Ace (13.3% Pydiflumetofen + 11.4% Propiconazole, Syngenta), and TOPGUARD® EQ (25.3% Azoxystrobin + 18.63% flutriafol, FMC Corp.) were tested in vitro against targeted pathogens mentioned above. A 20% stock emulsion was prepared by dissolving in 10% Dimethyl sulfoxide (DMSO) of each tested chemical to adjust at the desired final concentrations which are 0.1, 0.25, 0.5, 1, 2, 3, 4, and 5 mg/mL.

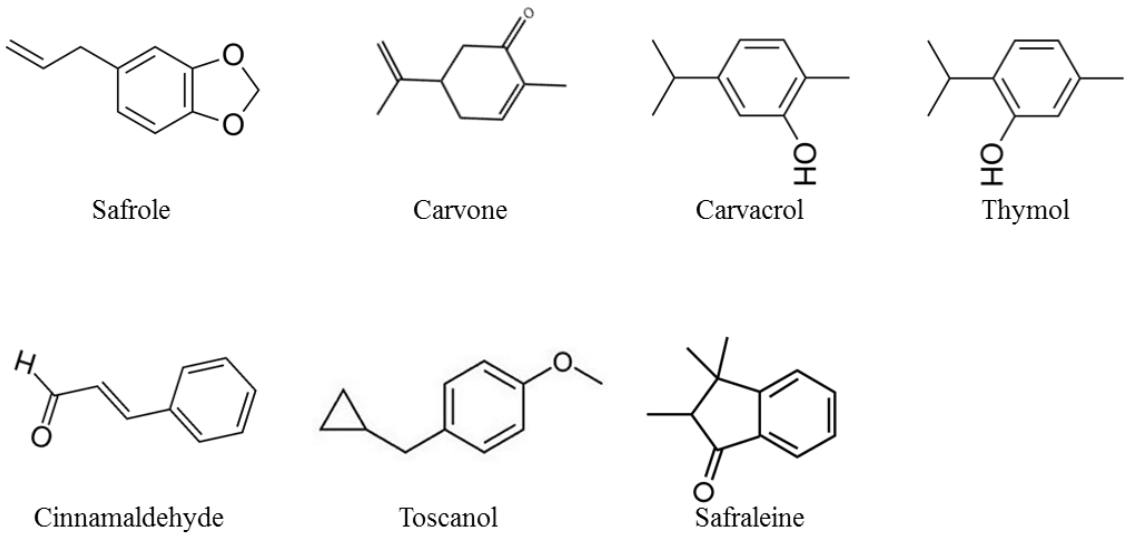

Figure 1. Chemical structure of pure compounds used in the experiments.

2.1.3. The Inhibitory Effect of the EO and Major Components on the Radial Growth of Fungal Pathogens

The inhibitory impact of the EO and major compounds on the mycelial growth of *A. solani*, *F.graminearum*, *R. solani*, *S. sclerotiourum*, and *P. ultimum* was evaluated by using the poisoned food method as described by Balouiri et al. (2014) with minor modifications. The suitable media for the selected pathogens were prepared and sterilized by autoclaving at 121 ^{o}C for 30 minutes. After, cooling down to around 40-45 ^{o}C, the evaluated chemicals were incorporated into the growth media at the desired final concentrations and mixed vigorously. Then, they were immediately poured into the Petri dishes (approximately 20 mL/Petri dishes) and left for the solidification. Following the solidification, eight mm diameter mycelial plugs from the edge of the seven days old cultures were aseptically deposited in the center of the prepared Petri dishes and incubated at room temperature (25 ^{o}C) under the darkness/light photoperiod of 12 h for five days. The mycelial growth diameter of the tested pathogens in the control group, without antimicrobial compounds, and sample plates were quantified by measuring the diameter of the radial growth of the colonies after five days of inoculation. The experiment for each concentration was triplicated with six replications. The percentage of the mycelial growth inhibition (MGI) was determined with the following formula:

$$MGI\ (\%) = ((Dc-Ds)/Dc)\ x100$$

Dc: The diameter of hyphal growth in the control group.

Ds: The diameter of hyphal growth in sample plates treated with examined chemicals.

2.1.4. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of the EO and Major Components

The MIC is the lowest concentration of an antifungal agent necessary to inhibit the visible growth of the tested pathogens. The PDA, 10% HPDA, and V8 media previously mentioned were prepared and amended with the desired final concentrations (0.1, 0.25, 0.5, 1, 2, 3, 4, and 5 mg/mL) of the tested chemicals (safrole, toscanol, safraleine, cinnamaldehyde, carvacrol, carvone, thymol, nutmeg oil, Miravis® Ace, and TOPGUARD® EQ) with their parallel control plates. The eight mm plugs of the fungal and oomycetes pathogens from the edge of the active growing site of the 7-day-old cultures were placed in the center of the suitable media, stated before for each pathogen, incorporated with the examined chemicals. The inoculated plates were stored at room temperature (25 °C) for five days under a 12 h photoperiod of darkness/light and observed for the percentage of radial growth suppression. The MIC value was determined as the lowest concentration of tested chemicals displaying 100% growth inhibition.

The MFC is the minimum concentration of an antifungal agent that results in fungal death. This means that the pathogen completely suppressed by the EOs does not revive when the plugs of the pathogen from the media treated with the EOs are relocated to the EOs-free Petri plates. To determine the MFC, plugs of the pathogens with 100% growth inhibition in the MIC experiment were transferred onto the newly prepared petri plates without EOs, and incubated at room temperature (25 °C) for 7 days under a 12 h photoperiod of darkness/light to observe whether the pathogen regrown on the petri plates without EOs. The MFC value was identified as the lowest concentration of the tested chemicals where there was no pathogen regrowth in the absence of tested chemicals. The

MIC and MFC study was repeated three times with six replications for each concentration.

2.1.5. The Effect of the EO and Major Components on Spore Production

In the study, the effect of the tested chemicals on the sporulation of the *F. graminearum* (NE-16-07) isolate was assessed by using CMC (carboxymethyl cellulose agar) medium specifically used for the spore production of *F. graminearum*. CMC medium with the following ingredients (g/L); 15 g Carboxymethylcellulose sodium salt, 1 g Sodium nitrate (NH_4NO_3), 1 g Monopotassium phosphate (KH_2PO_4), 0.5 g Magnesium (II) sulfate ($MgSO_4$-7 H_2O), 1 g Yeast Extract, and 10 g Bacto-Agar), was prepared and sterilized by autoclaving at 121 ^oC for 30 minutes. Following the autoclaving, when the media cooled down to around 40-45 ^oC, the tested chemicals were added into the media at the desired final concentrations, mixed vigorously, and immediately poured approximately 20 mL into the Petri plates with the size of 90 mm diameter and 15 mm height. The prepared CMC plates were inoculated with the eight mm diameter mycelial plugs of seven days old *F. graminearum* cultures grown in potato-dextrose-agar (PDA) and stored at room temperature (25 °C) under a 12 h photoperiod of darkness/light for 21 days for sporulation. After 21 days of inoculation, five ml of autoclaved distilled water was added to each inoculated Petri dish, and macroconidia were harvested by gently scratching the surface of the medium via a rubber spatula. The harvested solution mixture including macroconidia and mycelial of the tested fungus was through from cheesecloth to remove mycelial fragments and other debris from the solution. Then, the number of microconidia was counted by using a hemocytometer under

the microscope. The experiment for each concentration was repeated three times with six replications. The percentage of the sporulation inhibition (SI) was estimated as follows:

$SI\ (\%) = ((S_c - S_t)/ S_c)\ x100$

S_c: The number of conidia spores counted from the control group.

S_t: The number of conidia spores counted from the sample plates treated with examined chemicals.

2.1.6. The Effect of the EO and Major Components on Spore Germination

In the conidial germination assay, the microconidia of *F. graminearum* (NE-16-07) isolate were obtained from the 21-days-old cultures grown on the CMC media in the Petri dish (90 mm diameter) under the 12 h photoperiod of darkness/light. Five mL of autoclaved distilled water was added to each inoculated Petri dish, and macroconidia were harvested by gently scratching the surface of the medium via a rubber spatula. Then, the harvested solution was through from cheesecloth to remove mycelial fragments and other debris from the solution, and the number of microconidia was adjusted to 1 x 10^5 conidia/mL with the help of a hemocytometer. Four aliquots (25 ul) of microconidia suspension (1 x 10^5 conidia/mL) were equidistantly spotted into the PDA media amended with the tested chemicals at the desired final concentrations, and incubated at room temperature (25 °C) under a 12 h photoperiod of darkness/light. To count germinated microconidia, 100 microconidia were randomly chosen from each of the four replicated spots on each PDA plate and observed under the stereoscope at 40x at 6^{th}, 12^{th}, 24^{th}, 48^{th}, and 72^{nd} h after the inoculation. The microconidia were considered germinated when the germ tube elongation was equal to half of the microconidia length or more. The percentage of spore germination inhibition (SGI) was calculated by using the formula:

$SGI\ (\%) = ((M_c - M_t)/ M_c) \times 100$

M_c: The number of germinated microconidia counted from the control group.

M_t: The number of germinated microconidia counted from the sample plates treated with examined chemicals.

The trial for each concentration was conducted three times with six replications.

2.2. *In vitro* Antibacterial Bioassays

2.2.1. Microorganisms and Growth Conditions

Four plant bacterial pathogens, *C. michiganensis* subs. *nebraskensis* (Cmn225c) causing Goss,s bacterial wilt and leaf blight of maize, *X. campestris* pv. *campestris* (Xcc702d) causing the black rot in crucifers, *P. syringae* pv. *tomato* DC3000 (Pst DC3000) and GFP tagged *P. syringae* pv *tomato* (Pst-GFP) (gift from L. Zeng, University of Nebraska) causing of bacterial speck on tomato were used to screen the antibacterial potential of the EOs and major components (safrole, toscanol, safraleine, cinnamaldehyde, carvacrol, carvone, thymol, nutmeg oil). Bacterial strains Cmn225c and Xcc702d were grown in the Yeast Extract Peptone broth (YEP) (10 g yeast extract, 10 g Bacto peptone, 5 g NaCl L^{-1}, pH 7.0) at 25 °C on a rotary shaker at 180 rpm overnight whereas Pst and Pst-GFP were at the 28 °C and stored as stock bacterial suspension in 25% glycerol in 2 mL Eppendorf tubes at -80 °C.

2.2.2. The Effect of the EO and Major Components on the Bacterial Kinetic Growth

The microtiter plate-based antimicrobial assays were used for the determination of the impact of the EOs and major compounds on bacterial kinetic growth in vitro. Bacterial cultures were overnight grown in YEP broth in suitable conditions mentioned above just before the experiment and inoculum suspension was adjusted to the OD_{600}: 0.4

(approximately 1-5 x 10^6). The EOs and major compounds of EOs were dissolved in 10% DMSO and different stock solutions of EOs in YEP broth were prepared for the adjustment of desired final concentrations of EOs. Following, dilutions were adjusted in the 96-well microtiter plates by adding an equal volume of 100 µl freshly grown bacterial culture and 100 µl of stock solutions of EOs to reach the desired final concentrations (0.025, 0.05, 0.1, 0.25, 0.5, 1, 2, 3, 4, 5 mg/mL) with their parallel negative control (without inoculation) and positive control (with inoculation) wells filled with YEP broth without EOs. The inoculated microtiter plates were incubated at a suitable temperature (25 °C for Cmn225c and Xcc702d, 28 °C for Pst-DC3000 and Pst-GFP) on a rotary shaker at 180 rpm for 24 h. Bacterial growth was evaluated by measuring the optical density at 620 nm with a spectrometer (Thermo Fisher Scientific, Multiskan™ FC Microplate Photometer, MA, USA) at 8^{th}, 16^{th}, and 24^{th} h after inoculation. The antibacterial effectiveness (AE) of the tested chemicals was estimated by following formula;

$$AE\ (n)\ (\%) = [((A' - A_0) - (A_n - A_{n0}))/ (A' - A_0)] \times 100$$

A': the optical density of the growth of control without tested chemicals at time "t".

A_0: the optical density of the growth of the control without tested chemicals at time "0".

A_n: the optical density of tested chemical concentration of "n" at time "t".

A_{n0}: the optical density of tested chemical concentration of "n" at time "0".

All experiments were repeated twice with four replications.

2.2.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the EO and Major Components

The MIC was assessed by using a similar method applied in the determination of the efficacy of tested chemicals on bacterial kinetic growth. The suspension of overnight-grown bacterial cultures was arranged to the OD_{600}: 0.4 (approximately 1-5 x 10^6). The stock solutions of EOs in YEP broth in different concentrations were prepared to adjust the desired final concentrations of EOs which the starting concentration was 0.025 mg/mL. The microtiter plate wells were filled with an equal volume of 100 µl freshly grown bacterial culture and 100 µl of stock solutions of EOs for the achievement of desired final concentrations with their parallel inoculated and non-inoculated control wells filled with YEP broth without EOs, which continued until determining the MIC value for tested EOs. The inoculated microtiter plates were incubated at a suitable temperature (25 °C for Cmn225c and Xcc702d, 28 °C for Pst-DC3000 and Pst-GFP) on a rotary shaker at 180 rpm for 24 h. The minimum concentration inhibiting the visual growth of the microorganism following 24 h of the inoculation was considered the minimum inhibitory concentration (MIC) value.

The MBC is the lowest concentration of an antibacterial compound that results in bacterial death over 99.9%. The same samples examined in the MIC experiment were used to estimate the MBC value of the tested chemicals. A 25 µl from the last wells of EOs concentration showing visible growth and from all the wells of EOs concentrations with no visible bacterial growth were cultured on YEP agar plates with no EOs and incubated at favorable temperatures for 48 h. After incubation, the colony-forming units were counted. The lowest concentration in a subculture with no colony existence or the

presence of not more than three colonies after 48 h incubation was identified as MBC value. All experiments were repeated twice with four replications each.

2.3.5. Statistical Analysis

The experimental data were subjected to analysis of variance using the SAS 9.2 (SAS Institute Inc 2008) software. Significant means were compared with the use of the LSD multiple comparison test using statistical probability $P \leq 0.05$.

3. Results

3.1. *In vitro* Antifungal Activity of Selected EO and Major Components

The inhibitory effect of selected EO and major components was tested in vitro against four phytopathogenic fungi including, *A. solani*, *F. graminearum*, *R. solani*, *S. sclerotiourum*, and one oomycete *P. ultimum*. The tests were conducted in eight different concentrations (0.1, 0.25, 0.5, 1, 2, 3, 4, and 5 mg/mL) as well as control group (non-treated plates). In addition to the EOs, two fungicides, Miravis® Ace and TOPGUARD® EQ, were screened in vitro only against *F. graminearum* in similar eight concentrations. For the tests, bioassay plates were prepared with the desired concentrations, and 8 mm seven-day-old mycelial plugs from each pathogen were inoculated at the center of the plates and stored at room temperature (25 ^{o}C) for five days. After five days of inoculation, the diameter of mycelial radial growth was measured both in the control group (non-treated plates) and treated plates. Growth inhibition rates were calculated based on the formula previously mentioned for each tested chemical in each concentration.

Among the tested essential oils, thymol was found to be the most effective compound against *A. solani*, completely inhibiting its growth at a concentration of 0.25

mg/mL (as shown in Table 1). Safraleine, cinnamaldehyde, and carvacrol also exhibited 100% growth inhibition at a concentration of 0.5 mg/mL, while carvone was effective at 1 mg/mL and toscanol at 2 mg/mL. On the other hand, nutmeg oil showed the lowest efficacy against *A. solani* with only around 73% growth inhibition even at the highest tested concentration of 5 mg/mL.

The major components, carvacrol and thymol provided complete growth suppression against *F. graminearum* at 0.25 mg/mL, followed by safraleine and cinnamaldehyde at 0.5 mg/mL (Table 2). While toscanol and carvone showed 100% growth inhibition at 1 mg/mL, safrole and nutmeg oil still inhibited *F. graminearum* growth by approximately 78% and 71%, respectively, even when applied at 5 mg/mL. Notably, the tested pesticides demonstrated around 80% in vitro growth inhibition against *F. graminearum* even at the highest tested concentration of 5 mg/mL.

As shown in Table 3, 100 % growth inhibitory activities against *R. solani* were observed in all tested essential oils at the concentration of 3 mg/mL and above. Applying safraleine, cinnamaldehyde, carvacrol, and thymol at 0.25 mg/mL or more resulted in an absolute growth inhibition toward the *R. solani*. It is noteworthy that carvone and toscanol had similar antifungal performances, with a 100% growth inhibitory effect on *R. solani* at concentrations of 0.5 mg/mL and above. Additionally, safrole and nutmeg oil also expressed similar trends in suppressing the growth of R. solani, with a 90% reduction in hyphal growth observed at a concentration of 2 mg/mL.

For *S. sclerotiorum*, it was observed that the application of safraleine, cinnamaldehyde, carvacrol, and thymol at a concentration of 0.25 mg/mL or greater had a total inhibitory effect on mycelial growth of *S. sclerotiorum* (Table 4). Carvone treatment

at a concentration of 0.5 mg/mL or higher resulted in a 100% reduction in growth of *S. sclerotiorum*. However, nutmeg oil required a higher concentration of 4 mg/mL or above to achieve the same result. The major components, safrole, and toscanol, were found to be less effective in reducing the growth of *S. sclerotiorum*, even at the highest dose of 5 mg/mL, with only around an 85% reduction compared to the other essential oils tested.

The data presented in Table 5 show that cinnamaldehyde, carvacrol, and thymol exhibited 100 % growth inhibition against *P. ultimum* at a concentration of 0.25 mg/mL or higher. Similarly, safraleine and carvone were able to completely repress the hyphal growth of *P. ultimum* at 0.5 mg/mL, whereas safrole and nutmeg oil demonstrated comparable performance at 3 mg/mL and the highest concentration of 5 mg/mL, respectively. Out of the chemicals tested, it was determined that toscanol was the least effective main compound toward the *P. ultimum*, with only 86% growth inhibition at 5 mg/mL.

Table 1. The *in vitro* mycelial growth inhibition rate (%) of the tested components against *A. solani* after five days of incubation.

Doses (mg/ml)	Essential oils								Means
	Safrole	Nutmeg oil	Toscanol	Safraleine	Cinnamaldehyde	Carvacrol	Carvone	Thymol	
0	0.00^{m}	0.00^{m}	0.00^{m}	0.00^{m}	0.00^{m}	0.00^{m}	0.00^{m}	0.00^{m}	**0.00^{h}**
0.1	0.00^{m}	0.00^{m}	0.00^{m}	4.25^{k}	7.66^{j}	36.32^{i}	0.00^{m}	76.73^{c}	**15.62^{g}**
0.25	0.00^{m}	0.00^{m}	2.54^{l}	79.24^{b}	53.92^{h}	80.30^{b}	4.16^{k}	100.00^{a}	**40.02^{f}**
0.5	0.00^{m}	0.00^{m}	53.15^{h}	100.00^{a}	100.00^{a}	100.00^{a}	77.29^{c}	100.00^{a}	**66.30^{e}**
1	3.45^{kl}	3.97^{k}	68.14^{f}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**71.94^{d}**
2	79.03^{b}	57.11^{g}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**92.02^{c}**
3	100.00^{a}	67.02^{f}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**95.88^{b}**
4	100.00^{a}	69.66^{e}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**96.21^{b}**
5	100.00^{a}	72.70d	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**96.59^{a}**
Means	**42.50^{g}**	**30.05^{h}**	**58.20^{f}**	**75.94^{c}**	**73.51^{d}**	**79.62^{b}**	**64.60^{e}**	**86.30^{a}**	

Means with different letters indicate a statistically significant difference at a 5% level of probability.

Table 2. The *in vitro* mycelial growth inhibition rate (%) of the tested components against *F. graminearum* after five days post-inoculation.

Doses (mg/ml)	Essential oils										Means
	Safrole	Nutmeg oil	Toscanol	Safraleine	Cinnam	Carvac	Carvone	Thymol	Miravis	Topguard	
0	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	**0.00^{i}**
0.1	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	12.80^{s}	48.87^{r}	0.00^{u}	67.47^{kl}	55.10^{p}	51.29^{q}	**23.55^{h}**
0.25	0.00^{u}	0.00^{u}	0.00u	89.67^{b}	52.53^{q}	100.00^{a}	4.00^{t}	100.00^{a}	61.87^{n}	66.26^{klm}	**47.43^{g}**
0.5	0.00^{u}	0.00^{u}	80.04^{cde}	100.00^{a}	100.00^{a}	100.00^{a}	75.94^{gh}	100.00^{a}	65.25^{m}	75.04^{gh}	**69.63^{f}**
1	3.78^{t}	0.00^{u}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	71.72^{j}	80.30^{cd}	**75.58^{e}**
2	72.53^{uj}	58.05^{o}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	74.21^{hı}	80.30^{cd}	**88.51^{d}**
3	75.12^{gh}	65.77^{lm}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	74.98^{gh}	80.30^{cd}	**89.62^{c}**
4	77.09^{fg}	68.26^{k}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	78.12^{ef}	80.30^{cd}	**90.38^{b}**
5	78.18^{def}	71.29^{j}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	80.79^{c}	80.30^{cd}	**91.06^{a}**
Means	**34.08^{h}**	**29.26^{ı}**	**64.45^{f}**	**76.63^{c}**	**73.93^{d}**	**83.21^{b}**	**64.44^{f}**	**85.27^{a}**	**62.45^{g}**	**66.01^{e}**	

Means with different letters indicate a statistically significant difference at a 5% level of probability.
Cinnam: cinnamaldehyde; **Carvac**: carvacrol.

Table 3. The *in vitro* mycelial growth inhibition rate (%) of the tested components against *R. solani* after five days post-inoculation.

Doses (mg/mL)	Essential oils								Means
	Safrole	Nutmeg oil	Toscanol	Safraleine	Cinnamaldehyde	Carvacrol	Carvone	Thymol	
0	0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	**0.00^{f}**
0.1	0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	6.99^{e}	26.26^{d}	0.00^{f}	65.32^{c}	**12.32^{e}**
0.25	0.00^{f}	0.00^{f}	0.00^{f}	100.00^{a}	100.00^{a}	100.00^{a}	0.00^{f}	100.00^{a}	**50.00^{d}**
0.5	0.00^{f}	0.00^{f}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**75.00^{c}**
1	0.00^{f}	0.00^{f}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**75.00^{c}**
2	89.70^{b}	89.50^{b}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**97.40^{b}**
3	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**100.00^{a}**
4	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**100.00^{a}**
5	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**100.00^{a}**
Means	**43.30^{f}**	**43.28^{f}**	**66.67^{e}**	**77.78^{d}**	**78.55^{c}**	**80.70^{b}**	**66.67^{e}**	**85.04^{a}**	

Means with different letters indicate a statistically significant difference at a 5% level of probability.

Table 4. The *in vitro* mycelial growth inhibition rate (%) of the tested components against *S. sclerotiorum* after five days post-inoculation.

Doses (mg/mL)	Essential oils								Means
	Safrole	Nutmeg oil	Toscanol	Safraleine	Cinnamaldehyde	Carvacrol	Carvone	Thymol	
0	0.00^{l}	0.00^{l}	0.00^{l}	0.00^{l}	0.00^{l}	0.00^{l}	0.00^{l}	0.00^{l}	**0.00^{i}**
0.1	0.00^{l}	0.00^{l}	0.00^{l}	2.26^{k}	3.88^{j}	18.57^{h}	0.00^{l}	5.06^{i}	**3.72^{h}**
0.25	0.00^{l}	0.00^{l}	0.00^{l}	100.00^{a}	100.00^{a}	100.00^{a}	3.16^{jk}	100.00^{a}	**50.40^{g}**
0.5	0.00^{l}	0.00^{l}	0.00^{l}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**62.50^{f}**
1	0.00^{l}	3.79^{j}	0.00^{l}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**62.97^{e}**
2	68.44^{g}	80.91^{f}	74.17^{f}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**90.44^{d}**
3	80.15^{e}	86.59^{b}	80.72^{e}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**93.43^{c}**
4	82.47^{d}	100.00^{a}	85.61^{bc}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**96.01^{b}**
5	84.62^{c}	100.00^{a}	86.60^{b}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**96.40^{a}**
Means	**35.08^{g}**	**41.25^{e}**	**36.34^{f}**	**78.03^{c}**	**78.21^{bc}**	**79.84^{a}**	**67.02^{d}**	**78.34^{b}**	

Means with different letters indicate a statistically significant difference at a 5% level of probability.

Table 5. The *in vitro* mycelial growth inhibition rate (%) of the tested components against *P. ultimum* after five days post-inoculation.

Doses (mg/mL)	Essential oils								Means
	Safrole	Nutmeg oil	Toscanol	Safraleine	Cinnamaldehyde	Carvacrol	Carvone	Thymol	
0	0.00^{k}	0.00^{k}	0.00^{k}	0.00^{k}	0.00^{k}	0.00^{k}	0.00^{k}	0.00^{k}	**0.00^{h}**
0.1	0.00^{k}	0.00^{k}	0.00^{k}	0.00^{k}	0.00^{k}	38.03^{j}	0.00^{k}	68.63^{g}	**13.33^{g}**
0.25	0.00^{k}	0.00^{k}	0.00^{k}	82.10^{e}	100.00^{a}	100.00^{a}	0.00^{k}	100.00^{a}	**47.76^{f}**
0.5	0.00^{k}	0.00^{k}	0.00^{k}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**62.50^{e}**
1	0.00^{k}	0.00^{k}	0.00^{k}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**62.50^{e}**
2	95.92^{b}	76.63^{f}	62.49^{h}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**91.88^{d}**
3	100.00^{a}	82.99^{e}	58.54^{i}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**92.69^{c}**
4	100.00^{a}	89.51^{c}	68.38^{g}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**94.74^{b}**
5	100.00^{a}	100.00^{a}	86.14^{d}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	**98.27^{a}**
Means	**43.99^{f}**	**38.79^{g}**	**30.62^{h}**	**75.79^{d}**	**77.78^{c}**	**82.00^{b}**	**66.67^{e}**	**85.40^{a}**	

Means with different letters indicate a statistically significant difference at a 5% level of probability.

3.2. Minimum Inhibitory and Minimum Fungicidal Concentration (MIC and MFC)

MIC and MFC assessments are being exploited as one of the most noteworthy indexes for determining the inhibitory efficacy of antifungal chemicals. In vitro experiments exhibited that cinnamaldehyde, carvacrol, thymol, safraleine, and carvone,

were effective in achieving strong antifungal properties at relatively lower concentrations compared to other molecules tested against fungal pathogens (Table 6). The minimum inhibitory (MIC) and minimum fungicidal concentrations (MFC) values of the fungi species in the tested chemicals were in the range of 0.25 - >5 mg/mL (Table 6). Notably, cinnamaldehyde, carvacrol, and thymol displayed the highest fungistatic and fungicidal activity with MIC and MFC values ranging from 0.25 mg/mL to 0.5 mg/mL toward the tested fungal pathogens. Cinnamaldehyde revealed strong fungicidal properties against *P. ultimum*, *R. solani* and *S. sclerotiorum* with an MFC value of 0.25 mg/mL while with 0.5 mg/mL MFC value for *F. graminearum*, *A. solani*. As well, carvacrol and thymol also displayed a higher antifungal performance toward *R. solani* and *S. sclerotiorum* with a similar MIC and MFC value of 0.25 mg/mL, however, a slightly increased MFC value of 0.5 mg/mL was observed against the *F. graminearum*, *A. solani*, and *P. ultimum*. Interestingly, although safraleine inhibited the hyphal growth of all tested fungal pathogens at lower concentration between 0.25 mg/mL to 0.5 mg/mL, the fungal death was seen in over the 5 mg/mL for *F. graminearum*, *A. solani*, and *S. sclerotiorum*, except for the pathogen *R. solani* and *P. ultimum* with MFC value of 0.5 mg/mL and 2 mg/mL, respectively. It was observed that toscanol, safrole, and nutmeg oil had no fungicidal effect against the all tested fungal pathogens in all examined eight doses. Similarly, pesticides tested against only *F. graminearum* did not show any fungistatic and fungicidal effect even at the highest concentration of 5 mg/mL.

Table 6. The minimal inhibitory and minimal fungicidal concentration (MIC and MFC) values of tested components against *F. graminearum, A. solani, P. ultimum, R. solani,* and *S. sclerotiorum*.

Pathogens	***F. graminearum***		***A. solani***		***P. ultimum***		***R. solani***		***S. sclerotiorum***	
Essential oils (mg/mL)	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Cinnamaldehyde	0.5	0.5	0.5	0.5	0.25	0.25	0.25	0.25	0.25	0.25
Carvacrol	0.25	0.5	0.5	0.5	0.25	0.5	0.25	0.25	0.25	0.25
Thymol	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.25	0.25	0.25
Safraleine	0.5	>5	0.5	>5	0.5	2	0.25	0.5	0.25	>5
Carvone	1	2	1	2	0.5	1	0.5	2	0.5	2
Toscanol	1	>5	2	>5	>5	>5	0.5	>5	>5	>5
Safrole	>5	>5	3	>5	3	>5	3	>5	>5	>5
Nutmeg oil	>5	>5	>5	>5	5	>5	3	>5	4	>5
Topguard	>5	>5	-	-	-	-	-	-	-	-
Miravis	>5	>5	-	-	-	-	-	-	-	-

3.3. The Effect of the EO and Major Components on Spore Production

The effects of EO, main components, and synthetic pesticides in vitro on the spore production of *F. graminearum*, the causal agent of fusarium head blight disease, can be seen in Table 7. The results exhibited that a highly variable sporulation inhibiting performance was determined against the *F. graminearum* based on the tested compounds and concentrations. Among the tested essential oils, carvacrol was identified as the most effective compound, completely suppressing sporulation of *F. graminearum* in vitro even at the tested lowest concentration of 0.01 mg/mL, followed by the safraleine and thymol also exhibiting a significant reduction of 85% and 72% in sporulation at a similar concentration. An absolute spore production inhibition was obtained by applying cinnamaldehyde at a dose of 0.5 mg/mL, whereas it was seen in a slightly higher concentration of carvone with 1 mg/mL. Similarly, *F. graminearum* sporulation was

completely inhibited by safrole and toscanol at the same concentration of 2 mg/mL. Furthermore, a strong sporulation suppression appeared with the application of the synthetic pesticide Miravis with a 100% reduction at 2 mg/mL. However, although topguard also had a relatively strong impact on sporulation, the inhibition rate only reached up to 83 % even in the highest concentration of 5 mg/mL tested.

Table 7. The *in vitro* sporulation inhibitory effectiveness (%) of the tested chemicals on *F. graminearum*.

Doses (mg/mL)	Essential oils										
	Safrole	Nutmeg oil	Toscanol	Safraleine	Cinnam	Carvac	Carvone	Thymol	Miravis	Topguard	Mean
0	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	**0.00^{g}**
0.1	3.78^{t}	0.00^{u}	47.58^{r}	85.02^{kl}	87.86^{ij}	100.00^{a}	54.37^{q}	71.81^{o}	97.14^{cde}	92.33^{h}	**63.99^{f}**
0.25	4.34^{t}	0.00^{u}	38.64^{s}	100.00^{a}	99.73^{ab}	100.00^{a}	89.46^{i}	100.00^{a}	93.20^{gh}	86.81^{jk}	**71.22^{e}**
0.5	52.61^{q}	67.23^{p}	40.20^{s}	100.00^{a}	100.00^{a}	100.00^{a}	97.14^{cde}	100.00^{a}	96.80^{cdef}	93.60^{gh}	**84.76^{d}**
1	98.96^{abc}	94.88^{efg}	$89.39ı$	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	97.96^{abcd}	87.96^{ij}	**96.91^{c}**
2	100.00^{a}	94.70^{fg}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	$89.59ı$	**98.43^{a}**
3	100.00^{a}	96.05^{def}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	81.04^{n}	**97.71^{b}**
4	100.00^{a}	97.40^{bcd}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	82.28^{mn}	**97.97^{ab}**
5	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	83.91^{lm}	**98.39^{a}**
Mean	**62.19^{g}**	**61.14^{h}**	**68.42^{f}**	**87.22^{b}**	**87.51^{b}**	**88.89^{a}**	**82.33^{d}**	**85.76^{c}**	**87.23^{b}**	**77.50^{e}**	

Means with different letters indicate a statistically significant difference at a 5% level of probability.
Cinnam: cinnamaldehyde; **Carvac**: carvacrol.

3.4. The Effect of the EO and Major Components on Spore Germination

The effectiveness of bioactive molecules and two pesticides in inhibiting spore germination of *F. graminearum* was evaluated in vitro at eight different concentrations (ranging from 0.1 to 5 mg/mL). Observations were taken under a stereoscope at 40x magnification at different time intervals (6^{th}, 12^{th}, 24^{th}, 48^{th}, and 72^{nd} hours) after inoculation. The results showed that the inhibitory effectiveness varied widely depending on the tested compounds, their concentrations, and the duration of compound exposure. Among the tested compounds, cinnamaldehyde was determined as the most effective compound, inhibiting spore germination completely at a concentration of 0.01 mg/mL or

higher after 72 hours of treatment. Carvacrol and thymol also exhibited a high level of spore germination inhibition, with rates of up to 100 % and 91 % respectively at a concentration of 0.01 mg/mL within the first 6 hours of treatment. However, complete inhibition was observed at a concentration of 0.25 mg/mL after 72 hours of inoculation. Safraleine significantly reduced spore germination by roughly 87% at a concentration of 0.25 mg/mL during the first 6th hour, with 100% inhibition at a concentration of 0.5 mg/mL or higher after 72nd-hour inoculation. Similarly, carvone also caused a considerable decrement in the spore germination by 64 % at 0.25 mg/mL at the 6th h of treatment, however, an absolute inhibition was observed at a slightly higher concentration of 2 mg/mL or more at 72nd h. Although safrole, nutmeg oil, and toscanol achieved a strong inhibition performance at the first 6th h, the spores of *F. graminearum* were all germinated after 48 hours of treatment, indicating the static effect of these compounds. For the pesticides, Miravis was found to be more effective in inhibiting germination, with 100% suppression observed at a concentration of 2 mg/mL after 72 hours of incubation, compared to Topguard which displayed roughly 73% germination inhibition even at the tested highest concentration at the same period.

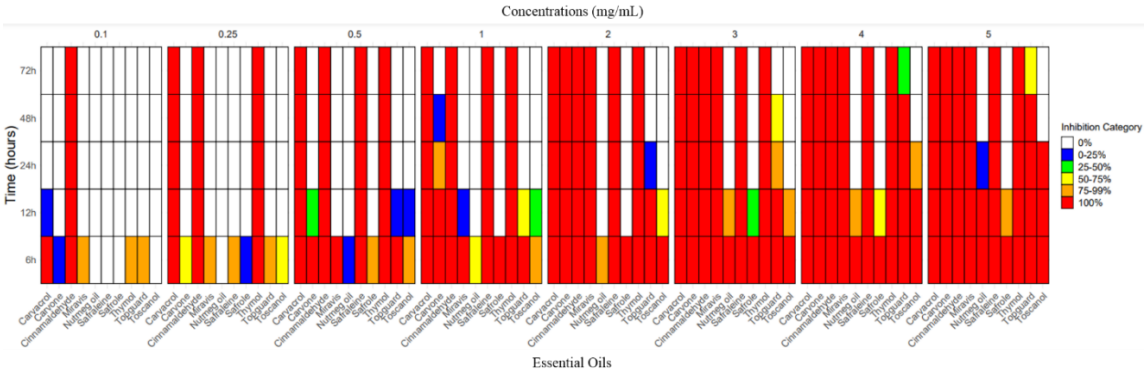

Figure 2. The heat map shows the effectiveness of tested components at eight different concentrations (0.1, 0.25, 0.5, 1, 2, 3, 4, and 5 mg/mL) in inhibiting the spore germination of *F. graminearum*. It measures the inhibition at 6th, 12th, 24th, 48th, and 72nd hour post-inoculation. White squares denote no germination inhibition, blue squares indicate inhibition between 0- 25%, green squares represent 25-50% inhibition, and yellow squares express germination suppression between 50-75%. The efficiency between 75-99% is depicted by orange squares, whereas red squares exhibit complete spore germination inhibition of *F. graminearum*.

3.5. The Effect of the EO and Major Components on the Bacterial Kinetic Growth

The putative antibacterial potency of the major components of essential oils was assessed in vitro against four phytopathogenic bacteria, including *C. michiganensis* subs. *nebraskensis* (Cmn225c), *X. campestris* pv. *campestris* (Xcc702d), *P. syringae* pv. *tomato* DC3000 (Pst DC3000), and GFP tagged *P. syringae* pv. *tomato* (Pst-GFP), by using a microtiter plate-based antimicrobial assay at ten different concentrations (0.025, 0.05, 0.1, 0.25, 0.5, 1, 2, 3, 4, 5 mg/ml). Results showed a highly variable antibacterial activity depending on the type of compound, its concentration, the pathogen, and the duration of exposure. Among the examined compounds, cinnamaldehyde, carvacrol, and thymol were three major components that performed the strongest antibacterial activities against all tested bacterial pathogens in comparison to other tested molecules.

Carvacrol was the most effective major compound toward the Cmn225c causing a significant reduction in growth even at the lowest concentration of 0.025 mg/mL and completely inhibiting bacterial growth at the concentration of 0.05 mg/mL at 24 h.

Thymol and cinnamaldehyde were also highly effective against the Cmn225c with 100 % growth inhibition at 1 and 2 mg/mL, respectively, 24 h after incubation. Although nutmeg oil, safraleine, and carvone achieved potential antibacterial properties against the Cmn225c, the bacterial growth was not completely suppressed even at the highest concentration tested. Toscanol and safrole did not exhibit any antibacterial activity against Cmn225c at the examined concentrations after 24 hours.

It was observed that carvacrol at a concentration of 0.05 mg/mL inhibited Xcc702d completely and this inhibition lasted for 24 hours post-inoculation. Similarly, cinnamaldehyde and thymol also showed 100% growth suppression of Xcc702d at a similar concentration of 0.5 mg/mL after 24 hours of incubation, followed by carvone at 3 mg/mL and safraleine at 4 mg/mL to 100% decrease in growth. Safrole and toscanol were found to have relatively weaker antibacterial properties with less than 50% reduction in the growth of Xcc702d even at a concentration of 5 mg/mL after 24 hours.

As is shown in Figure 3, cinnamaldehyde was observed to have the highest antibacterial activity against Pst-DC3000 and Pst-GFP, with complete and durable inhibition for 24 hours at a concentration of 0.5 mg/mL, followed by carvacrol at 2 mg/mL and 1 mg/mL, respectively. Although carvone, safraleine, nutmeg oil, and thymol were also effective against Pst-DC3000 and Pst-GFP, bacterial growth was not completely suppressed even at the highest concentration after 24 hours of incubation. Safrole was significantly less efficient against both Pst-DC3000 and Pst-GFP, while toscanol did not exhibit any antibacterial activity at any of the tested concentrations.

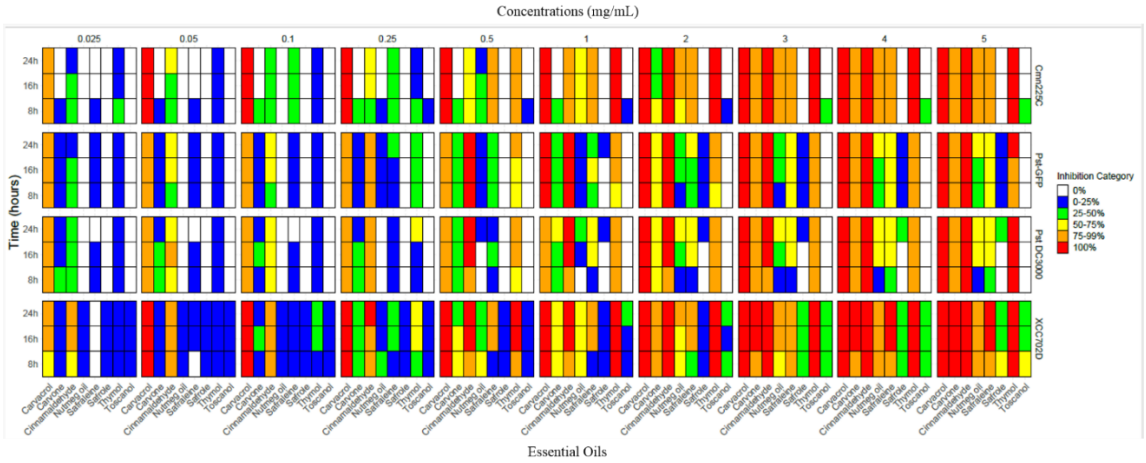

Figure 3. The heat map displays the efficacy of tested components at ten different concentrations (0.025, 0.05, 0.1, 0.25, 0.5, 1, 2, 3, 4, and 5 mg/mL) on the growth of four bacterial pathogens at the 8th, 16th and 24th h after post-inoculation *in vitro*. White squares denote no growth inhibition, blue squares indicate inhibition between 0- 25%, green squares represent 25-50% inhibition and yellow squares express growth suppression between 50-75%. The efficiency between 75-99% is depicted by orange squares, whereas red squares exhibit complete growth inhibition of bacterial pathogens.

3.6. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the EO and Major Components

The minimum inhibitory (MIC) and bactericidal (MBC) concentrations are two prominent parameters used for the estimation of bacteriostatic and bactericidal effectiveness of bioactive substances. This study examined the antibacterial properties of eight essential oils against four bacterial pathogens, including *C. michiganensis* subs. *nebraskensis* (Cmn225c), *X. campestris* pv. *campestris* (Xcc702d), *P. syringae* pv. *tomato* DC3000 (Pst DC3000), and GFP tagged *P. syringae* pv. *tomato* (Pst-GFP), with their respective MIC and MBC values detailed in Table 7. The results of this experiment showed a wide range of MIC and MBC values, varying from 0.025 mg/mL to no effect (-). Cinnamaldehyde and carvacrol, followed by thymol, demonstrated the highest levels of antibacterial activity against the tested bacterial pathogens. Carvacrol was found to be the most effective against Cmn225c and Xcc702d, with MIC and MBC values of 0.025

mg/mL and 0.05 mg/mL, respectively. Cinnamaldehyde showed strong antibacterial properties against Xcc702d, with MIC and MBC values of 0.1 and 0.25 mg/mL, respectively, while slightly increased MIC and MBC values of 0.25 mg/mL and 0.5 mg/mL were observed against Pst-DC3000 and Pst-GFP. The MIC and MBC values of thymol against Cmn225c and Xcc702d were determined as 0.5 and 1 mg/mL, respectively, while they were 5 and 6 mg/mL against Pst-DC3000 and Pst-GFP. The bacterial pathogens examined in the study displayed less sensitivity to safraleine, carvone, toscanol, safrole, and nutmeg oil, as evidenced by their higher MIC and MBC values ranging from 2 mg/mL to no effect (-). Notably, toscanol was identified as the least effective agent tested, with significantly higher MIC and MBC values of 740 and 750 mg/mL against Cmn225c and Xcc702d.

Table 8. The minimal inhibitory and minimal bactericidal concentration (MIC and MBC) values of examined bioactive components against C. *michiganensis* subs. *nebraskensis* (Cmn225c), *X. campestris* pv. *campestris* (Xcc702d), *P. syringae* pv. *tomato* DC3000 (Pst-DC3000) and GFP tagged *P. syringae* pv *tomato* (Pst-GFP).

Pathogens	**Cmn 225c**		**Xcc 702d**		**Pst-DC3000**		**Pst-GFP**	
Essential oils (mg/mL)	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Cinnamaldehyde	1	2	0.1	0.25	0.25	0.5	0.25	0.5
Carvacrol	0.025	0.05	0.025	0.05	1	2	0.5	1
Thymol	0.5	1	0.5	1	5	6	5	6
Safraleine	5	6	3	4	43	44	43	44
Carvone	5	6	2	3	30	31	39	40
Toscanol	740	750	740	750	-	-	-	-
Safrole	16	17	15	16	26	27	41	42
Nutmeg oil	6	7	8	9	240	250	240	250

4. Discussion

In recent years, there has been a growing interest towards finding new and safe sources of antimicrobials. This is aimed at reducing the dependence on synthetic pesticides in crop cultivation, due to concerns over environmental pollution and potential development of resistance to conventional chemical agents. Researchers are actively exploring this area to identify effective, affordable, and readily available natural pesticides. One of the most promising options that has been extensively studied is essential oils and their major components. They have the potential to serve as a viable substitute for synthetic pesticides, thereby mitigating the harmful impact of chemical substances on humans and the environment. In this study, seven pure compounds, including safrole, toscanol, safraleine, cinnamaldehyde, carvacrol, carvone, and thymol, and one EO, nutmeg oil were tested *in vitro* as potential agents against fungal and bacterial pathogens that are economically important in agriculture. The inhibitory effectiveness of these selected compounds on the hyphal growth of four fungal pathogens and one oomycete, the MIC, and MFC concentrations, and their impact on sporulation and spore germination of *F. graminearum* were examined *in vitro*. Additionally, these compounds were evaluated for their potency on bacterial growth, and MIC and MBC values, towards four bacterial pathogens.

The study found that all the compounds performed growth-inhibitory activity with a high variation in efficacy, as measured by their ability to inhibit the hyphal growth of fungi and oomycetes *in vitro*. This variation in the antifungal effectiveness of the tested compounds was possibly caused by the physical, molecular, and chemical characteristics of the compounds, and the sensitivity of the pathogens to the quantity of the compounds

tested. In this study, thymol was found to be the most effective against all fungal and oomycete pathogens, followed by carvacrol, cinnamaldehyde, safraleine, carvone, toscanol, safrole, and nutmeg oil. These findings align with previous research by Koroch et al. 2007; Sadgrova & Jones, 2015; Parick et al. 2021, which demonstrated that the antifungal activity of essential oils' major components following the order of phenols, aldehydes, ketones, alcohols, ethers, hydrocarbons. Elshafie et al. (2015) also reported that carvacrol and thymol exhibited the strongest in vitro antifungal activity towards the three *Manilinia* species (*M. laxa*, *M. fructicola*, and *M. fructigena*). Rahmouni et al. (2019) showed that thymol and carvacrol were highly effective against the *Fusarium oxysporum* f. sp. *albedinis*, causing fungal death at concentrations of 0.94 and 2.08 μl mL^{-1}, respectively.

Carvacrol (2-methyl-5-(1-methylethyl)-phenol) and its isomer thymol (2-isopropyl-5-methylphenol) are small molecules existing in several plant species, mainly oregano (*O. vulgare*) and thyme (*T. vulgaris*). These molecules have lipophilic properties, which allow them to disrupt membrane-catalyzed enzymes and enzymes responsible for energy and protein production. This disruption ultimately leads to the death of the cell (Shirzad et al., 2011). The presence of an aromatic nucleus and one hydroxyl group is crucial for their antimicrobial activity, causing abnormalities in the hyphal morphology and aggregates of microbial pathogens. This results in reduced diameters and lyses of the hyphal wall (Numpaque et al. 2011). The hydrophobic nature of the -OH group in carvacrol and thymol, as well as their ability to exchange protons, play critical roles in their antimicrobial activities. These factors affect the cell wall and membrane integrity of microbial cells. Moreover, carvacrol and thymol can also interfere

with physiological processes within the cell, such as binding to DNA and inhibiting ergosterol biosynthesis, ultimately causing cell death (De Castro et al. 2015; Liu et al. 2019).

The position of the hydroxyl group in the phenol is also crucial in determining the compounds' effectiveness against fungal pathogens. When the hydroxyl group is found in the meta position, it performs a higher antifungal activity than in the ortho position. Thymol is a more potent antifungal agent than carvacrol and this activity difference has also been reported against various fungal pathogens including *B. cinerea, Colletotrichum acutatum, Botryodiplodia theobromae, A. niger* (Numpaque et al. 2011; Moghtader, 2012; Zhang et al. 2019).

Cinnamaldehyde is an α, β-unsaturated aldehyde and a major component of cinnamon oil. Previous studies have shown that it exhibits antifungal properties against various fungal pathogens, including *Geotrichum citri-aurantii, P. capsici*, and *Fusarium sambucinum* (OuYang et al., 2019; Hu et al., 2013; Wei et al., 2019). Our research found that cinnamaldehyde exhibited strong antifungal activity against the tested fungal pathogens, although its potency was slightly lower than that of thymol and carvacrol. Another investigation conducted by Sempere-Ferre et al. (2021) also demonstrated that cinnamaldehyde exhibited potent antifungal effectiveness against *Botryotinia fuckeliana* and *R. solani*, although thymol and carvacrol were more effective. The precise mechanisms through which cinnamaldehyde exerts its antifungal activity are not fully understood, but experimental evidence suggests that it may work by inhibiting cell wall biosynthesis, damaging cell membrane integrity, and blocking ATPases activity. For instance, OuYang et al. (2019) observed that cinnamaldehyde caused deterioration in cell

wall ability and structure of *G. citri-aurantii*. Similarly, treatment of *Penicillium expansum* with cinnamaldehyde resulted in changes in cell folding, disruption of cell wall integrity and plasma membrane, mitochondrial damage, and loss of intracellular organelles. Therefore, it is apparent that the biotoxic action of cinnamaldehyde is a complex process that varies under different conditions.

Carvone is an important monoterpene ketone that is naturally obtained from aromatic and medicinal plants in the *Lamiaceae* and *Asteraceae* families. In a recent study, carvone demonstrated moderate antifungal activity against targeted pathogens. The results showed that carvone was less effective than thymol, carvacrol, and cinnamaldehyde, but more effective than other compounds tested. These findings are consistent with a study by Boonruanga et al. (2017) which found that thymol was more effective against the anthracnose pathogen *C. gloeosporioides* isolated from avocado and citrus. Another study by Marei et al. (2012) reported that thymol was the most effective molecule among twelve pure compounds tested against *R. solani* and *F. oxysporium*, with EC_{50} values of 33.5 mg/L and 50.37 mg/L, respectively, which was comparable to a reference fungicide carbendazim. Conversely, carvone was less effective with higher EC_{50} values. Tsao and Zhou (1999) also showed that among twenty-two monoterpenes tested against the postharvest pathogens *B. cinerea* and *M. fructicola*, while thymol and carvacrol performed a complete suppression toward these pathogens, carvone was not as effective as these two phenolic compounds.

The study evaluated the antifungal properties of safrole, which is a derivative of the aromatic phenol ether found naturally in sassafras essential oils. The research examined safrole's effects on four fungal and one oomycete pathogens and found that it

had a minimal fungistatic impact and no fungicidal effect at the tested concentrations. These results are consistent with a previous study by Vizcaíno-Páez et al. (2015), which demonstrated that safrole was less effective than dillapiole in suppressing the mycelial growth of *B. theobromae* and *C. acutatum*. This type of molecule can cause a reduction in the hyphal growth of some plant pathogens by acting as phytoanticipins (Vizcaíno-Páez et al. 2015). Hossain et al. (2015) also noted that etherified molecules including anethole, methyl chavicol, and safrole have low fungicidal activity. Nevertheless, there is limited understanding of these molecules' direct antimicrobial effects on phytopathogenic fungi.

The nutmeg oil derived from *Myristica fragrans* Houtt (Myristicaceae) was the only crude essential oil tested in the present experiments. Results showed that nutmeg oil is effective against examined pathogens at a concentration of 1 mg/mL or higher, similar to safrole. A similar antifungal activity was determined by Valente et al. (2015) found that nutmeg oil at a concentration of 0.1% reduced the mycelial growth of *A. flavus* and *A. ochraceus* by 43% and 65%, respectively. Increasing the concentration to 0.3% resulted in higher growth inhibition rates of 84% and 79%, respectively. However, when nutmeg oil was applied at the same concentration (300-500 ppm) as thyme oil containing mainly thymol and carvacrol, it did not exhibit any antifungal performance against *A. alternata* (Feng & Zheng, 2007). A study by Seema & Devaki, (2010) demonstrated that nutmeg oil was effective against *R. solani* at the concentration of 2000 ppm, whereas the growth of *R. solani* was completely inhibited by cinnamon oil (source of cinnamaldehyde) at a concentration of 500 ppm.

Two synthetic ingredients commonly used in fragrances, safraleine, and toscanol were examined for the first time for their potential to combat plant fungal and oomycete

pathogens. The results indicated that safraleine demonstrated strong antifungal properties against the target pathogens, even at concentrations as low as 0.01 mg/mL, effectively inhibiting hyphal growth. In contrast, toscanol exhibited relatively weak antifungal activity. Interestingly, safraleine only had a fungicidal impact on two out of the five tested pathogens, namely *P. ultimum* and *R. solani*, at concentrations of 2 and 0.5 mg/mL, respectively.

The study determined the MIC values of various chemicals that were tested for their antifungal performance. The poisoned food method was utilized for experimentation and the results indicated that the compounds had a concentration-dependent suppression effect. (Table 6). Several studies revealed that higher concentrations of EO led to increased inhibition of mycelial growth (Aguiar et al. 2014; Parikh et al. 2021). Additionally, it was observed that the MIC value was closely linked to the specific pathogen being targeted (Table 6). Compounds that could achieve complete growth inhibition at lower concentrations would be advantageous in terms of reducing application volume and cost, as well as avoiding potential phytotoxic effects on plants at higher concentrations.

After conducting MIC experiments, the study evaluated MFC values to determine the fungicidal properties of the screened compounds. As stated by Stević et al. (2014), MFC values were either as similar to the MIC values or very close to the MIC concentrations with some exceptions. In this study, one such exception was safraleine, which exhibited 100% growth inhibition against *F. graminearum*, *A. solani*, and *S. sclerotiorum* at low concentrations ranging from 0.25 to 0.5 mg/mL. However, the inhibition was reversible without safraleine, indicating a fungistatic effect instead of a

fungicidal one. On the other hand, thymol, carvacrol, cinnamaldehyde, and carvone showed fungicidal activity against all tested fungal and oomycete pathogens at concentrations ranging from 0.25 to 2 mg/mL (Table 6), which is consistent with the findings of Stević et al. (2014).

The ability to produce conidia spores is a crucial trait that facilitates the spread and secondary infection of *F. graminearum*. To investigate the potential for inhibiting sporulation, screening was conducted using seven pure compounds, one EO, and two pesticides against *F. graminearum* in vitro. The results revealed that all tested chemicals significantly reduced the amount of spores by varying percentages, depending on the concentration and specific chemical. Carvacrol was found to be the most effective pure compound for sporulation inhibition, with complete sporulation inhibition at a concentration of 0.1 mg/mL or higher, followed by thymol and safraleine with a 100% reduction in sporulation at a concentration of 0.25 mg/mL. Cinnamaldehyde and carvone also performed a strong inhibition on sporulation, however, a complete suppression was observed at a slightly increased concentration of 0.5 and 1 mg/mL, respectively. Interestingly although safrole, toscanol, and nutmeg oil did not demonstrate strong efficacy in suppressing mycelial growth, they displayed remarkable performance in inhibiting sporulation. Among the pesticides, Miravis was found to be more efficient in reducing the number of spores, with a total inhibition of spore production at a dose of 2 mg/mL compared to Topguard. Similarly, a study conducted by Faghih-Imani et al. (2020) reported that thyme essential oil and its major constituent carvacrol significantly reduced the sporulation of *F. pseudograminearum* and *F. culmorum*, limiting the dissemination of these pathogens by lowering the macroconidia number as an inoculum

source. The reduction in sporulation may be due to the effect of highly volatile compounds on the development of fungal mycelia as the site of spore production, and the perception or changes in the signals responsible for the switch from the vegetative to the reproductive phase (Faghih-Imani et al., 2020). Additionally, the aggregation and destruction of mycelial, delayed hyphal growth, or inhibition of hyphal growth could be the reason for incomplete spore formation (Mihai & Popa, 2015). Sun et al (2016) demonstrated that the application of cinnamaldehyde significantly decreased the radial growth, mycelial formation, and sporulation of *A. flavus* in applied concentrations. Mahmoudi et al. (2012) showed that the essential oil of *Z. multiflora* (76.12% carvacrol) was highly effective in reducing spore production of *A. alternata* at a dose of 200-500 ppm.

A study was conducted to investigate the effectiveness of certain chemicals in preventing spore germination in *F. graminearum*. The experiment involved observing macroconidia germination at various intervals (6^{th}, 12^{th}, 24^{th}, 48^{th}, and 72^{nd} hour post-inoculation) in Petri plates that were either treated with the chemicals or used as control groups. The results revealed that the efficacy varied greatly depending on the chemicals, their concentration, and exposure time. It was found that the higher the concentration of the chemical, the better the germination inhibition performance, as seen in the 6^{th}-hour observation, which is consistent with the results of Mihai & Popa, (2015), Moutassem et al. (2019), Faghih-Imani et al. (2019). Among the compounds tested, cinnamaldehyde was found to be the most effective in terms of spore germination inhibition. It exhibited complete spore germination suppression even at the lowest concentration of 0.1 mg/mL or higher for 72 hours post-inoculation. These findings align with the results of a study

conducted by Wang et al. (2022), which showed that cinnamaldehyde totally inhibited the *F. solani* spores at a concentration of 120 µl l−1, indicating that cinnamaldehyde is more effective against the spores than mycelia. The study by Wei et al. (2020) further explained that cinnamaldehyde's potency in inhibiting spore germination could be attributed to its ability to damage the cell wall and cell membrane, accumulate ROS, disrupt mitochondrial function, and hinder ergosterol synthesis, ultimately leading to cell death. In addition, carvacrol and thymol have also shown strong sporicidal properties at a slightly increased concentration of 0.25mg/mL, lasting for 72 hours after inoculation. Gill et al. (2016) & Pei et al. (2020) highlighted that carvacrol and thymol exposure to fungal spores resulted in the prevention of ergosterol production, increased cell membrane permeability, depletion of intracellular components, and ultimately led to cell death in the spore. However, our study found that the germination inhibition performance of safraleine, carvone, and Miravis to possess moderated efficacy with a complete germination inhibition at 0.5, and 2 mg/mL for 72 h after post-inoculation, respectively, compared to the cinnamaldehyde, carvacrol, and thymol. Interestingly, safrole, nutmeg oil, toscanol, and topguard successfully inhibited the spore germination at the beginning of the experiment, however, the effectiveness did not last for 72 h. These differences could be due to variations in the compounds' mode of action and the spores' sensitivity to them. Therefore, suppression of sporulation production and germination are desirable for targeting the pathogens to inhibit or reduce intra-plant and interplant disease propagation.

For bacteria, an experiment was conducted to determine the antibacterial efficacy of seven pure compounds and EO, nutmeg oil *in vitro* via microtiter plate-based dilution technique against four bacterial pathogens (Cmn225c, Xcc702d, Pst-DC3000, and Pst-

GFP). The current study found carvacrol, thymol, and cinnamaldehyde as the most effective major compounds against the tested bacterial compounds, which were the similar ones performing the highest activities for fungal and oomycete pathogens. It was reported by Khoshbakht et al. (2020) & Kolozsváriné Nagy et al. (2023) that three small molecules (carvacrol, thymol, and cinnamaldehyde) could inhibit bacterial growth by disrupting the lipid structure of the bacterial cell wall and penetrating membrane, leading to denaturation of proteins, destruction of the cell membrane, cytoplasmic leakage, blocking the DNA transcription, protein synthesis, and eventually kill bacterial cells. It was stated that gram-positive bacteria are more sensitive to EOs or their major compounds than gram-negative bacteria, with some exceptions. The results demonstrated that carvacrol and thymol were more effective against the Cmn225c (Gram (+)) than Pst-DC3000 and Pst-GFP (Gram (-)), which is in agreement with this phenomenon, except with the efficacy against the Xcc702d (Gram (-)). Gram-negative bacteria possess an outer membrane rich in lipopolysaccharide (LPS) surrounding the cell wall, which provides rigidity and complexity, preventing the diffusion of the hydrophobic molecules through the cell wall, however, gram-positive bacteria, lack an outer membrane, are covered by a thick peptidoglycan wall, which is not dense enough to limit small antimicrobial molecules, causing susceptibility to EOs and their constituents (Kachur & Suntres, 2019). Additionally, the lipophilic ends of lipoteichoic acid exist in the cell membrane of gram-positive bacteria leading to easiness in diffusions of hydrophobic compounds of EOs (Chouhan et al., 2017). Interestingly, contrary to this phenomenon, our results showed that the antibacterial effectiveness of cinnamaldehyde was relatively

higher against tested gram-negative bacterial pathogens (Xcc702d, Pst-DC3000, and Pst-GFP) than gram-positive bacteria Cmn225c.

The study also involved evaluating the ability of the tested compound to inhibit bacterial growth and kill pathogens by measuring MIC and MBC values. Our findings revealed that carvacrol, thymol, and cinnamaldehyde performed significantly well in inhibiting bacterial growth and killing pathogens in low concentrations compared to other compounds. However, the MIC and MBC values varied greatly depending on the bacterial pathogens and compounds tested. This is due to the differences in the mode of action of the compounds and the varying sensitivity of certain species, even for different strains within the same species, to the EOs and pure compounds. For instance, our study found that carvacrol had MIC and MBC values of 1 and 2 mg/mL against Pst-DC3000, respectively, while it exhibited values of 0.5 and 1 mg/mL against Pst-GFP. De Martino et al. (2009) also reported that the susceptibility of two strains of *B. cereus* to the same EOs and their pure compounds was highly variable.

5. Conclusion

Through our investigation, we determined that thymol, carvacrol, and cinnamaldehyde are highly effective compounds with strong fungistatic and fungicidal activity against all tested fungal and oomycete pathogens. We also found that the synthetic pure molecule safraleine exhibited powerful fungistatic properties against all tested pathogens. The effectiveness of these compounds was dependent on the specific pathogen being tested, as well as the concentration of the compound. Carvacrol and thymol were found to be the most effective in inhibiting sporulation, while cinnamaldehyde was the most potent in suppressing spore germination. Overall, the

inhibition of sporulation and spore germination was closely related to the tested chemicals and their concentrations, as well as exposure time. Notably, cinnamaldehyde demonstrated powerful germination inhibition even at its lowest tested concentration, extending up to 72 hours post-inoculation.

The present study revealed that carvacrol, thymol, and cinnamaldehyde also possessed strong bacteriostatic and bactericidal properties towards the examined significant plant pathogenic bacteria. It appears that the efficacy of these compounds varies depending on the type of bacteria, exposure time, and the specific compound tested. These findings, considered together, have greatly contributed to our comprehension of the fungicidal and bactericidal potential of pure molecules, and encourage the possibility of the usage of pure compounds of EOs as natural biocides for the management of plant fungal and bacterial pathogens.

6. References

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Chapter III

Evaluation of Major Components of Essential Oils for Controlling Bacterial Speck Disease in Tomato caused by *Pseudomonas syringae* pv. *tomato*

1. Introduction

The tomato (*Solanum lycopersicum* Mill.) is a highly valued vegetable crop that is grown extensively all over the world, which has been successfully grown in a wide range of climates, including tropical and temperate regions. China is the top producer of tomato with the production of around 65 million tons in the year of 2021, followed by India and Turkey with the production of nearly 21 and 13 million tons, respectively (Tiwari et al., 2022). The production of tomatoes can be influenced by a range of biotic and abiotic factors affecting tomato production and quality. The most challenging among the biotic factors are diseases caused by nematode, fungal, viral, and bacterial agents, which have a detrimental effect on plant health and ultimately result in lessened harvest. Among them, bacterial diseases are particularly problematic, causing substantial reductions in both quality and yield, and resulting in significant economic losses.

Bacterial speck disease is one of the most prevalent and economically significant bacterial disease of tomato. The causative agent of this disease, *Pseudomonas syringae* pv. *tomato* (Pst), which was first determined and identified in Taiwan and the United States in 1933, is a Gram-negative, rod-shaped bacteria that is commonly found in all tomato crop growing areas, and is a member of the *P. syringae* species complex. There are two known races of the pathogen; Race 0 has a global distribution, while Race 1 is found in Canada, Bulgaria, Serbia, the United States, and Italy (Ally et al., 2023). The disease cycle is initiated by natural phenomena, such as rain and wind, water irrigation,

and contaminated seeds. As an epiphyte, *P. syringae* pv. *tomato* can survive and thrive on the surface of leaves and seeds. Under favorable environmental conditions (low temperatures of 18-24°C and high humidity), it can rapidly increase its population and enter the host plant through natural openings or wounds, using its polar flagella (Orfei et al., 2023). Bacterial speck disease is characterized by symptoms that affect various parts of the plant, including leaves, flowers, fruits, and stems. The symptoms include small, dark brown to black necrotic spots typically encircled by a yellow halo (green on fruit) caused by the bacterial toxin coronatine (Pedley et al., 2003). If left untreated, these spots can rapidly spread and increase the necrotic area of the foliage, ultimately leading to plant death. The economic impact of a bacterial speck outbreak can be devastating, with yield losses ranging from 20-25% for tomato seedlings to up to 75% for tomato fruits.

Controlling bacterial diseases is notably challenging due to the requirement for intensive integrated management strategies to reduce severe economic losses. For the management of tomato bacterial speck disease, cultural practices including removing the weeds and plant residues, the destruction of infected plants or pruning of infected leaves, avoiding excessive handling of transplants to prevent the spread of bacteria on tools and workers hands, avoiding overhead and excessive watering, and applying crop rotation with non-host plants are commonly applied approaches to reduce disease incidence and severity in the field. The usage of pathogen free seeds and seedlings as well as following the good sanitation practices such as washing and sanitizing tools, trays, equipment, surfaces, is also critical to prevent the spread of the disease. In addition to these preventative strategies, using resistant plants is the most cost-effective approach for controlling tomato bacterial speck disease. However, host resistance is not consistent in

its durability. Thus, the bacterial colonies can rapidly develop tolerance to the resistance source. Interestingly, the highly virulent resistance braking strains of *P. syringae* pv. *tomato* with a lack of the avirulence gene avrPto, was determined among the natural populations even before the commercialization of the varieties carrying the resistance gene of the *Pto* gene family providing resistance to the Pst in tomato (Louws et al., 2001). The chemical management of Pst is another prevalent method to suppress the disease development in the tomato plants. Copper-based bactericides are the most common group of pesticides (FRAC Group M01) used by growers for controlling Pst in tomato in both non-organic and organic agriculture. Copper ions degrade proteins leading to the denaturation of enzymes, which are essential for the function of bacterial cells (Mohsin et al., 2016). Copper has the ability to eliminate pathogenic cells present on the surface of plant leaves. However, once these bacterial cells enter the host tissue, their sensitivity to copper processing decreases. This means that copper sprays are effective as protective bactericidal during the initial stages of infection, but do not provide extended or consistent protection (Kolomiiets et al., 2019). Also, the multiple applications are required for the efficient protection in a growing season, resulting in detrimental impact on the environment. Most importantly copper-resistant bacterial strains was emerged in the nature as a result of intensive usage of the copper based bactericides. It was reported that copper-resistant, which is encoded by a copper-inducible operon (copABCD), populations of *P. syringae* pv. *tomato* are identified worldwide reducing the effectiveness of the copper-based products for the management of bacterial speck disease, leading to interest in alternative control strategies (Griffin et al., 2017).

Plant pathogens can activate several defense-related pathways, including SAR (Systemic Acquired Resistance), ISR (Induced Systemic Resistance), and R-genes, which enhance the resistance of plants to pathogen infection (Dalio et al., 2020). The pathogenesis-related defense genes *PR1*, *PR2*, and *PR3* are the typical genes associated with the SAR pathways (Zhang et al., 2020), While *JAZ2* is the gene related to the ISR pathways (Dalio et al., 2020).

In recent years, the induction of SAR (Systemic Acquired Resistance), ISR (Induced Systemic Resistance), and related strategies has been considered a new approach to the integrated management of plant bacterial diseases. The successful application of chemical inducers to induce SAR in plants is a noteworthy accomplishment in the transferring of basic research findings into practical disease management techniques. For a substance to qualify as a SAR-inducing agent, it needs to provide immunity against the same range of pathogens as a natural inducer without exhibiting any antimicrobial properties (Graves & Alexander, 2002). Notably, acibenzolar-S-methyl (ASM), a plant elicitor belonging to the benzothiadiazole chemical class, meets the criteria for a SAR-inducer and is marketed as Actigard® 50WG by Syngenta. Acibenzolar-S-methyl exhibits an induction time of 2 to 4 days following application, rendering it a potent preventive measure for disease control. Various mechanisms seem to be triggered simultaneously during Systemic Acquired Resistance (SAR) expression against pathogen establishment. This could potentially minimize the development of SAR-insensitive strains of bacterial pathogens. By pre-conditioning plants through foliar application of ASM, this approach has proven effective in several pathosystems such as bacterial canker and wilt of tomato, fire blight (Sundin et al., 2016),

as well as bacterial spot and speck of tomato (Louws et al., 2001). Nevertheless, ASM alone may not be entirely effective, and incorporating it into an integrated program can lead to the attainment of the desired control levels while minimizing the need for other materials, such as copper-based products (Sundin et al., 2016). These findings indicate the need for novel chemicals as alternatives to current bactericides for controlling bacterial speck disease in tomatoes. These chemicals can be rotated with the current bactericides and also incorporated into the integrated management strategies to suppress the disease occurrence and prevent the development of bactericide-resistant strains in pathogen populations.

In recent times, there has been an increasing focus on natural products, particularly essential oils (EOs) and their major components which are obtained from different parts of plants for their antibacterial properties and their potential to be used as new biocides against plant bacterial pathogens, such as *P. syringae* pv. *tomato*. EOs and their main components have been found to directly impact pathogens through various mechanisms. These mechanisms include disrupting cell membrane integrity, increasing permeability which can lead to the loss of cellular components and ions, inhibiting the synthesis of flagellin a microbial protein necessary for bacterial motility, and inhibiting the cell-cell communication quorum sensing network mediated by various bacterial signal molecules. Furthermore, they can induce host resistance by triggering the production of phytoalexins, increasing pathogenesis-related protein activity, synthesizing structural compounds, and biochemical plant defense (Swamy et al., 2016). A study by Lucas et al. (2012) showed that clove essential oil significantly reduced the severity of tomato bacterial spot caused by *Xanthomonas vesicatoria* and stimulated an increase in

β‑1,3‑glucanase, chitinase, and peroxidase activities in tomato seedlings. Similarly, Da Silva et al. (2014) reported that the application of lemongrass, thyme, and tea tree oils noticeably lowered the disease severity of tomato bacterial speck compared to copper chloride. Another study by Bagy & Abo-Elyousr (2019) found that thyme oil performed the strongest antibacterial activity toward tomato bacterial spot, followed by oleum and then lemongrass, while the highest induction of oxidative enzymes, specifically peroxidase (PO) and polyphenol enzyme was observed in the treatment of oleum oil followed by lemongrass and thyme oils when compared to the control group. A study conducted by Qiao et al. in 2020 indicates that carvacrol at 32 mg/ L was effective in reducing bacterial spot of tomato severity and increased the effectiveness of copper against copper-resistant *X. perforans*. Additionally, a study by Deepa et al. in 2022 revealed that the application of linalool, a small molecule, at a concentration of 80 ppm significantly reduced the infection rate of Pst-DC3000 by suppressing the Quorum Sensing mechanism, inhibiting the production of acyl-homoserine lactone (AHL), and preventing biofilm formation.

Given the potential of EOs and their major components for controlling bacterial diseases, this study aimed to evaluate the effect of the tested pure compounds on the disease severity of bacterial specks of tomato. The compounds, including carvacrol, carvone, thymol, cinnamaldehyde, and safraleine were assessed for impact on the agronomical features on tomato plants exposed to bacterial speck disease. To determine the impact of these examined components on the relative expression of defense-related genes under exposure of Pst-DC3000, the features of shoot height, fresh and dry weight, root fresh and dry weight, and flowering time.

2. Materials and Methods

2.1. Preparation of Bacterial Pathogen Inoculum

The strain of *Pseudomonas syringae* pv. *tomato* DC 3000 (Pst-DC3000), the causal agent of tomato bacterial speck, was used as inoculum for the infection of tomato plants *in planta* experiments. 250 ml Yeast Extract Peptone broth (YEP) (10 g yeast extract, 10 g Bacto peptone, 5 g NaCl L^{-1}, pH 7.0) was put in 500 ml Erlenmeyer flasks and autoclaved. 250 ml autoclaved YEP broth was inoculated with the Pst-DC3000 and then incubated for 18 hours at 28 °C on a rotary shaker at 220 rpm. After incubation, the inoculum suspension was adjusted to the OD_{600}: 0.4 (approximately 1-5 x 10^6).

2.2. Preparation of Treatments

The five different major components of essential oils (EOs) including carvacrol (Sigma-Aldrich, W224611), carvone (Sigma-Aldrich, 124931), cinnamaldehyde (Sigma-Aldrich, W228613), safraleine (Givaudan, 54440-17-4), and thymol (Sigma-Aldrich, 16254), and the natural defense system activator Actigard® 50WG (Syngenta) with an active ingredient (ai) of 50% acibenzolar-S-methyl (ASM), were tested in planta greenhouse experiments. All EOs and Actigard® 50WG were dissolved in the treatment mixture of 96 ml autoclaved distilled water, 0.5 ml TWEEN® 80 (Sigma-Aldrich, P1754), 0.5 ml EOs/Actigard® 50WG/water, and 3 ml 10% DMSO (Dimethyl sulfoxide)(Sigma-Aldrich, 5.89569) per 100 ml at the desired concentration. The concentration of EOs was adjusted at the doses of 3 mg/mL based on in vitro tests for foliar applications. Actigard® 50WG was prepared at the recommended concentration of 0.75 oz/A for aerial application on tomato plants. The control groups (with pathogen and

without pathogen) were sprayed with the same amount of 0.5 ml autoclaved distilled water-added treatment solution.

2.3. *In Planta* Greenhouse Experiments

In planta experiments were conducted with the tomato variety M-82 (LA3474), obtained from the Tomato Genetic Resource Center, Department of Plant Sciences, University of California-Davis, at the Plant Pathology greenhouses located on the North side of the East Campus of the University of Nebraska-Lincoln from September to December in the year of 2022. Plant materials 'M-82' were planted in 15 cm diameter plastic pots filled with steam-pasteurized standard greenhouse mix (5 gallons peat, 3 gallons soil, 2.5 gallons sand, 2.5 gallons vermiculite) and grown in the greenhouse with daily watering at 25°C under 16 h light: 8 h dark photoperiod. The experiment was arranged in the randomized complete block design with three replications. When the tomato seedlings were at the 3 to 5 real leaf stage, treatments were sprayed onto the tomato seedlings by using a hand sprayer. Nine tomato seedlings were used for each treatment. There were three different application cycles in the *in planta* experiments which were pre-application, one-application, and post-application.

In pre-application, the prepared treatment solutions were sprayed onto the tomato seedlings and stored for five days in the greenhouse at 23-25°C under 16 h light: 8 h dark photoperiod. Following the five-day period, the seedlings were resprayed and allowed to dry for approximately eight hours. Once dry, the treated seedlings were dipped into the prepared pathogen inoculum for two minutes. The inoculated seedlings were then incubated for 48 hours in sealed gasket boxes with the dimensions 22 1/2" L x 16" W x

12 3/4" H at 24°C under 16 h light: 8 h dark photoperiod with high humidity (over 90% RH).

In one-application, the tomato seedlings were sprayed with the prepared treatment solutions and allowed to dry for approximately 8 hours. Once dry, they were dipped into the pathogen inoculum for 2 minutes. The inoculated seedlings were incubated for 48 hours in sealed gasket boxes with the dimensions 22 1/2" L x 16" W x 12 3/4" H at 24°C under 16 h light: 8 h dark photoperiod with high humidity, exceeding 90% RH.

In post-application, the prepared treatment solutions were sprayed onto the tomato seedlings and treated tomato seedlings were left for around 8 hours to dry. After eight hours treated seedlings were dipped into the prepared pathogen inoculum for 2 minutes. The inoculated seedlings were incubated for 48 hours in sealed gasket boxes with the dimensions 22 1/2" L x 16" W x 12 3/4" H at 24°C under 16 h light: 8 h dark photoperiod with high humidity (over 90% RH). Five days after the inoculation, the same amount of treatment solution was sprayed onto the tomato seedlings.

In all three application cycles, after 48 hours of pathogen inoculation, treated seedlings were grown in the greenhouse at 25°C under 16 h light: 8 h dark photoperiod for the monitoring of disease development and were watered as needed.

2.4. Disease Severity (0-5)

Disease severity was visually observed based on the chlorotic and necrotic symptoms in the foliar part of the tomato seedlings after ten days of inoculation, which are typical symptoms of bacterial speck disease. A scale based on the symptoms from 0 to 5 was used for rating severity. While 0 means that there was no symptom on the

above-ground part of tomato plants, 5 implies that tomato plants were infected severely and displaying extensive chlorosis and severe necrotic symptoms.

2.5. The Plant Height (cm), Shoot Fresh and Dry Weight (g) of Tomato Plants

To collect data on plant height, the distance from the soil surface to the shoot tip of tomato plants was measured 30 days after inoculation. Once this measurement was taken, the above-ground portion of six tomato plants from each treatment was carefully cut from the surface of the soil and weighed by using precise scales. The tomato shoots were then placed in paper bags and dried in a 65 °C dryer for five days. After thorough drying, their weight was once again measured by using precision scales.

2.6. The Root Fresh and Dry Weight (g) of Tomato Plants

To remove all dirt particles, the roots of six plants from each treatment were washed thoroughly with tap water. They were then left to dry for eight hours. The fresh weight of the roots was then measured using precision scales. Subsequently, the roots from each treatment were placed in paper bags and dried for five days in a 65 °C dryer. Once completely dried, their weight was again measured with precision scales.

2.7. Flowering Time

Three tomato plants were randomly selected from each treatment and grown to observe their flowering. The florescence of the first vein of the treated tomato plants was monitored following inoculation to record their flowering time on specific days.

2.8. RNA Extraction and cDNA Synthesis

Total RNA was extracted from tomato leaf samples by using the TriPure™ Isolation Reagent (Roche, 11667165001) in accordance with the manufacturer's instructions. To synthesize cDNA, reverse transcription of the total RNA was carried out

in a 20 µL reaction volume using the ProtoScript® II Reverse Transcriptase First Strand cDNA Synthesis Kit (New England Biolabs (NEB #M0368)). For each sample, the following components were mixed: 1 µg total RNA, 2 µl of d(T)23VN, 4 µl of 5X ProtoScript II buffer, 2 µl of 0.1 M DTT, 1 µl of ProtoScript II RT (200 U/µl), 1 µl of 10 mM dNTP, 0.2 µl of RNase Inhibitor (40 U/µl), and nuclease-free H_2O to a total volume of 20 µl, and then incubated at 42°C for 1 hour. The synthesized cDNA was further diluted 20 times and stored at -80°C for future experiments.

2.9. Quantitative real-time PCR analysis for Gene Expression Analysis

To conduct a real-time PCR assay, a 20-fold dilution of cDNA was used as the template, and the specific primer sequences can be found in Table X. The relative expression levels were determined through the $2^{(-\Delta\Delta Ct)}$ analysis method (Livak and Schmittgen, 2001), and expression values were normalized using the *β-Actin* gene. A 20 µL reaction volume was used for the qPCR reaction, consisting of 2 µL of (1:20) cDNA, 10 µL of the PowerTrack™ SYBR™ Green Master Mix (Thermo Fisher Scientific), 1 µL of forward (0.5 µL) and reverse primers (0.5 µL) of the gene of interest, and 6.5 µL of nuclease-free water. The quantitative real-time PCR (qPCR) was performed in the CFX96™ Real-Time System (Bio-Rad, USA), in three biological and technical repetitions under the following conditions: 95°C for 2 minutes, followed by 39 cycles of 95°C for 10 seconds and 58°C for 30 seconds. The specificity of the primers used to measure the expression of each gene was verified by conducting a melt curve analysis, wherein the sample temperature was gradually raised from 65°C to 95°C in 0.5°C steps for 5 seconds each. Primers were designed with specific parameters: GC content between 40-60%, length of 18-24 nucleotides, an annealing temperature of 60 ± 2°C, and

amplicon length between 70-200 base pairs by using Primer3 Plus software (http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi).

Table 1. List of primers used in the analysis of gene expression.

ID_GENE	Primer F	Primer R
WRKY	ATCCTCGCCAGCAGTTAGCA	TCGTGGAGCTTTGCAAGGTAG
JAZ2	CCCCACCACCACTCAGACTAA	TATGGCGCTCTAGCCGTGT
ACC	AGCTACGTCAATGGCAGCAC	AGGAAGGGTGGGGACTTCTG
HCR2	GCATGCAAGGACTGGTATGGA	TCTCGAGAAAAGGGAGGGATGA
PR2	TCTAAGTTCTCTCTTAGCTTCCCT	GATAGGCTGCGCTCCTGTTA
NPR1	GGTGCATCGGTGCATTTTGT	AATAGGCGAGCACACTGACC
MYC2	AAGGAAACCACGTAGAGGCG	TACGTTTGGAACCACCTCGC
PR3	ATCACCCGTTGCACTGTCTT	ATGGAACCGGCATTTTGTGC
PR1	TCATATGAGACGTCGAGAAGTTAAA	TCATATTAGCAACATCAAAAGGGAA
Actin	CGGTGACCACTTTCCGATCT	TCCTCACCGTCAGCCATTTT

2.10. Statistical Analysis

A two-way ANOVA was performed to test for significance amongst the tested groups. Once statistical significance is established through the ANOVA, comparisons for all pairs were done using Tukey-Kramer Honestly Significant Difference (HSD) test ($p<0.05$).

3. Results

3.1. The Impact of Tested Pure Compounds on the Disease Severity

Tomato plants exposed to Pst-DC3000 culture suspension exhibited severe necrotic symptoms and extensive chlorosis in their foliar parts after ten days. This is a typical characteristic symptom of bacterial speck disease. However, using the tested compounds in all three application cycles resulted in a significant decrease in the chlorotic area on the leaves, as well as the incidence of bacterial speck lesions in different scales in the tomato seedlings compared to the non-treated tomato seedlings infected with

Pst-DC3000. Among all the compounds tested, cinnamaldehyde was found to be the most effective in reducing the disease severity. Especially dual applications (pre and post) of cinnamaldehyde showed better performance compared to a single application of it. Following cinnamaldehyde, thymol was also highly effective in mitigating the symptoms of bacterial speck diseases, showing similar effectiveness in all three application cycles. Furthermore, safraleine significantly reduced the disease's severity when compared to non-treated tomato plants that were infected with Pst-DC3000. It is noteworthy that safraleine exhibited a similar pattern of effectiveness as cinnamaldehyde, based on the application cycles. Additionally, for carvacrol, the pre-application resulted in a considerable reduction in the disease severity compared to the one and post-application. There was a significant decrease in the disease severity when tomato seedlings were also treated with carvone compared to the control (+). However, the post-application of the carvone displayed the most effectiveness in reducing the disease severity compared to the one and pre-applications. Interestingly, although Actigard treatment resulted in a significant decrease in disease severity compared to the control (+), it was less effective than all pure components tested throughout all three application cycles.

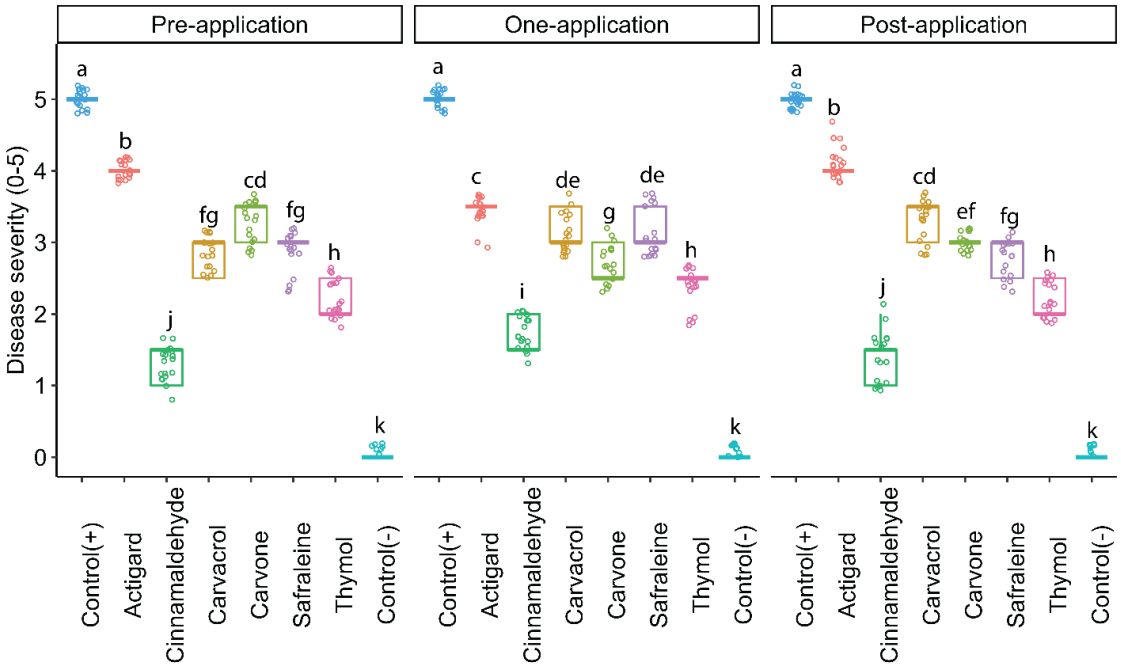


Figure 1. The effect of the treatments on the disease severity (0-5) of bacterial speck disease caused by *P. syringae* pv *tomato* DC3000 on tomato plants after ten days post-inoculation. Control (+) refers non-treated tomato plants infected with the Pst-DC3000. Control (-) refers non-treated tomato plants with no infection. Means with different letters indicate a statistically significant difference at a 5% level of probability.

3.2. The Impact of Tested Pure Compounds on the Shoot Height (cm), Fresh and Dry Weight (g)

Figure 2 displays the impact of treatments on tomato plant height (cm) after thirty days of Pst-DC3000 inoculation. The shoot height was significantly higher in all treatments compared to the non-treated tomato plants infected with Pst-DC3000. However, the maximum shoot height was observed in the treatment of cinnamaldehyde at around 33 cm in the post-application, followed by the pre-application of it, with 31.6 cm, and post-application of thymol with 31.5 cm. There was a significant increase in the plant height of tomato plants treated with carvacrol, carvone, and safraleine compared to the control (+), with similar plant heights observed through the three application cycles.

Actigard treatment resulted in the lowest plant height among all the tested chemicals, with a height of around 26 cm in all three application cycles.

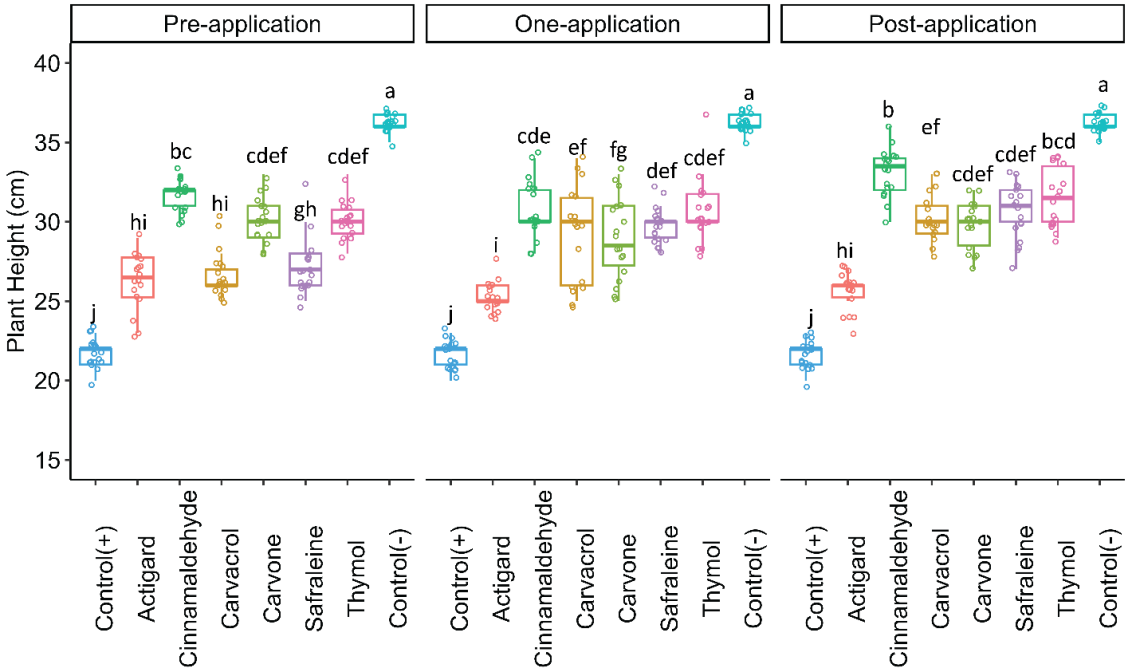


Figure 2. The effect of the treatments on the plant height (cm) on tomato plants after thirty days post-inoculation of *P. syringae* pv *tomato* DC3000. Control (+) refers to non-treated tomato plants infected with the Pst-DC3000. Control (-) refers to non-treated tomato plants with no infection. Means with different letters indicate a statistically significant difference at a 5% level of probability.

The effect of the treatments on the shoot fresh weight (g) on tomato plants after thirty days post-inoculation of Pst-DC3000 was shown in Figure 3. Applying pure components in all three types of cycles resulted in a noteworthy increase in shoot fresh weight compared to control (+) plants. Among the tested compounds, the highest shoot fresh biomass was observed in the pre-application of cinnamaldehyde at around 43.7 g, followed by the post-application at around 40.9 g. Similarly, the pre and post-application of thymol performed a significant enhancement in shoot fresh biomass with around 38.6 g and 37.8 g in comparison to the control (+) plants with 23.5 g. Interestingly, while the treatment of Actigard showed a significant increase in shoot height (Figure 2), the

application of it did not provide any promotion on shoot fresh biomass compared to the control (+) plants (Figure 3).

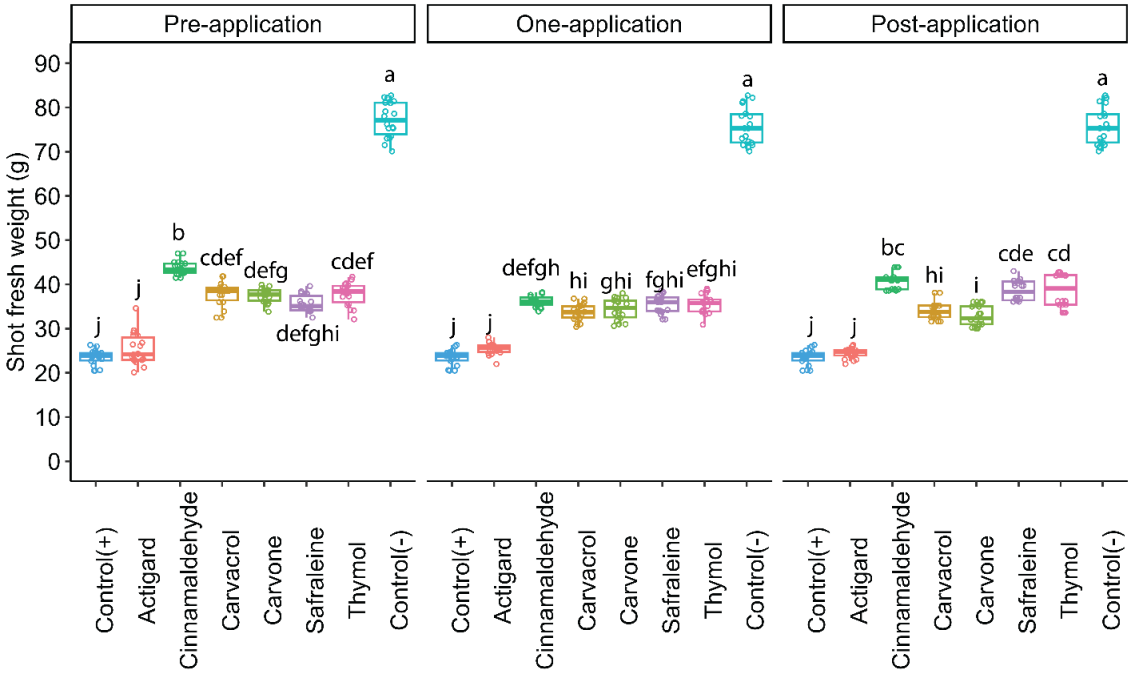


Figure 3. The effect of the treatments on the shoot fresh weight (g) on tomato plants after thirty days post-inoculation of *P. syringae* pv *tomato* DC3000. Control (+) refers to non-treated tomato plants infected with the Pst-DC3000. Control (-) refers to non-treated tomato plants with no infection. Means with different letters indicate a statistically significant difference at a 5% level of probability.

Figure 4 shows the effect of the treatments on the shoot dry weight (g) on tomato plants after thirty days post-inoculation of Pst-DC3000. When comparing the application cycles, the shoot dry matter was higher in the pre-application of the treatments than in the one and post-application of the tested pure components. The tomato plants with pre-application of cinnamaldehyde exhibited the highest shoot dry biomass with 7.3 g, followed by the pre-application of the thymol, safraleine, carvacrol, and carvone with around 6.8 g, 6 g, 5.7 g, and 5.7 g, respectively. For the Actigard application, a similar result was observed in the shoot dry weight as for the shoot fresh biomass. The

application of Actigard did not perform any changes in the shoot dry weight compared to the control (+) plants (Figure 4).

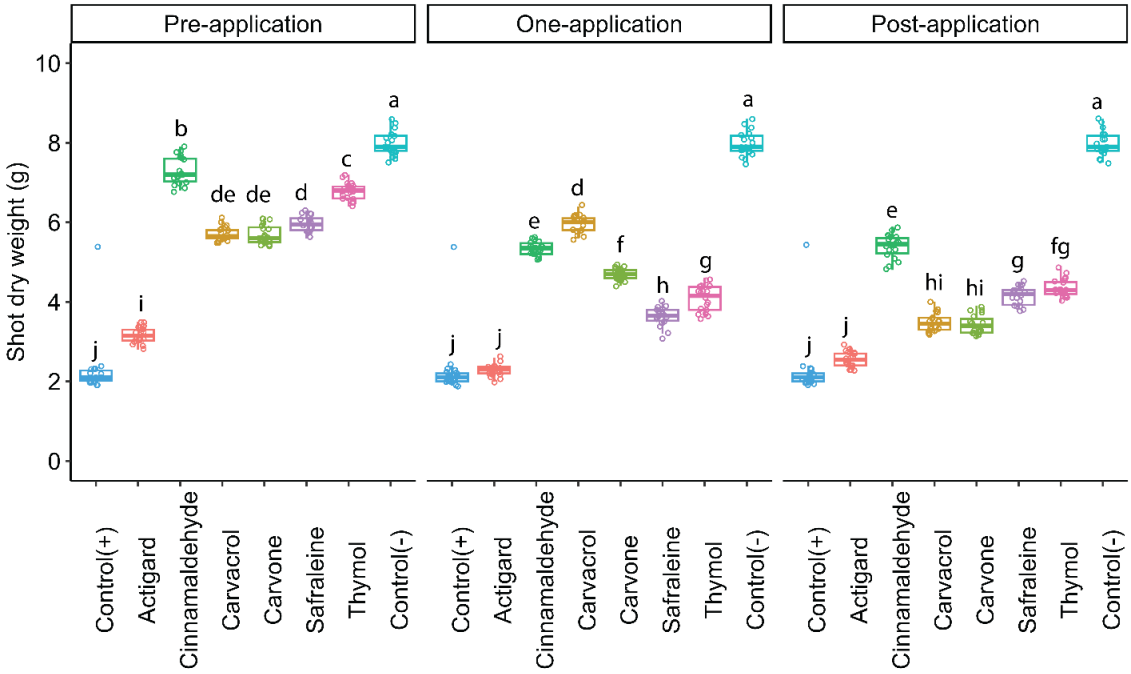


Figure 4. The effect of the treatments on the shoot dry weight (g) on tomato plants after thirty days post-inoculation of *P. syringae* pv *tomato* DC3000. Control (+) refers to non-treated tomato plants infected with the Pst-DC3000. Control (-) refers to non-treated tomato plants with no infection. Means with different letters indicate a statistically significant difference at a 5% level of probability.

3.3. The Impact of Tested Pure Compounds on the Root Fresh and Dry Weight (g)

Figure 5 illustrates the dry root weights of tomato plants after thirty days post-inoculation of Pst-DC3000 under different treatments. Analysis of the estimates of the root fresh weight of the treatments revealed that the highest values were obtained from the pre-application of cinnamaldehyde, with approximately 10.2 g. This was followed by the post and one application of cinnamaldehyde, and pre-application of thymol, with 9 g, 8.9 g, and 8.8 g, respectively. Other treatments of pure components also resulted in a significant increase in fresh root weights, ranging from 7.8 g to 6.9 g, compared to the

control (+) plants, which had a fresh root weight of approximately 4 g. The lowest dry root weight was observed in the one-application of Actigard, at approximately 4.2 g.

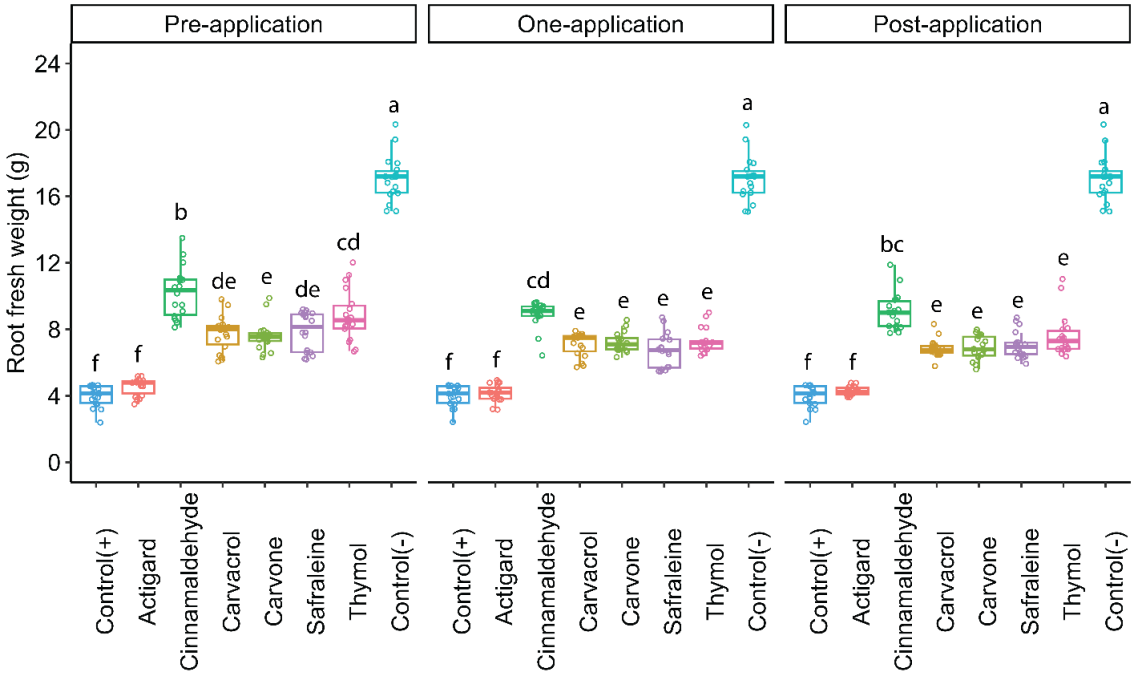


Figure 5. The effect of the treatments on the root fresh weight (g) on tomato plants after thirty days post-inoculation of *P. syringae* pv *tomato* DC3000. Control (+) refers to non-treated tomato plants infected with the Pst-DC3000. Control (-) refers to non-treated tomato plants with no infection. Means with different letters indicate a statistically significant difference at a 5% level of probability.

As seen in Figure 6, the tested pure components with three application cycles significantly increased the root dry weight ranging from 0.95 g to 0.5 g compared to the control (+) plants with 0.23 g. Based on the results, the highest dry root weight was obtained by the pre-application of cinnamaldehyde with 0.95 g, followed by the pre-application of thymol and post-application of cinnamaldehyde with 0.87 g and 0.77 g, respectively. Also, the pre-application of safraleine, carvacrol, and carvone significantly enhanced the root dry weight by around 0.7 g. Among the treatments, one application of Actigard had the lowest value for the root dry weight, with 0.27 g.

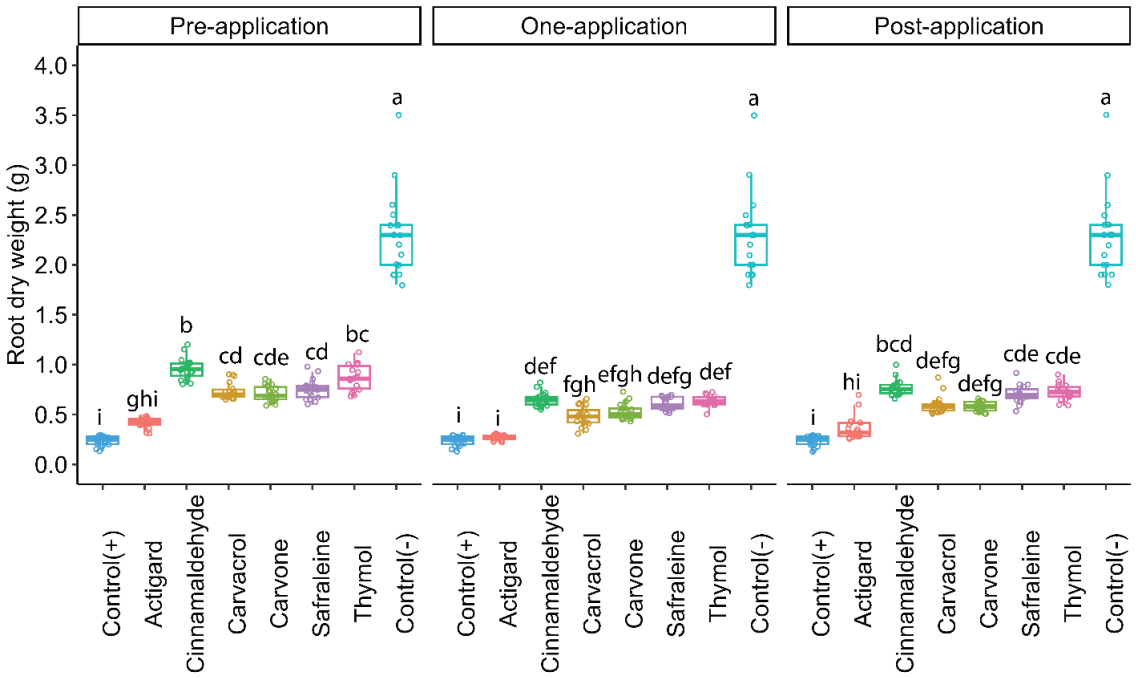


Figure 6. The effect of the treatments on the root dry weight (g) on tomato plants after thirty days post-inoculation of *P. syringae* pv *tomato* DC3000. Control (+) refers to non-treated tomato plants infected with the Pst-DC3000. Control (-) refers to non-treated tomato plants with no infection. Means with different letters indicate a statistically significant difference at a 5% level of probability.

3.4. The Impact of Tested Pure Compounds on the Flowering Time

Figure 7 shows the effect of the pure component treatments on the flowering time of tomato plants exposed to the Pst-DC3000. According to the results, the non-treated tomato plants infected with the bacterial speck disease flowered after around fifty-five days post-inoculation, while flowers emerged in the non-treated tomato plants with no infection around twenty days post-inoculation. Tomato plants from all treatments, except the post-application of Actigard, were significantly flowered earlier than the control (+) plants. The earliest flowered tomato plants were recorded in the one and pre-application of carvone with around thirty-six days post-inoculation of Pst-DC3000, followed by the pre-application of safraleine, thymol, and cinnamaldehyde with around 36, 38, and 38

days post-inoculation, respectively. Among the treatments, the latest flowering was observed in Actigard applications.

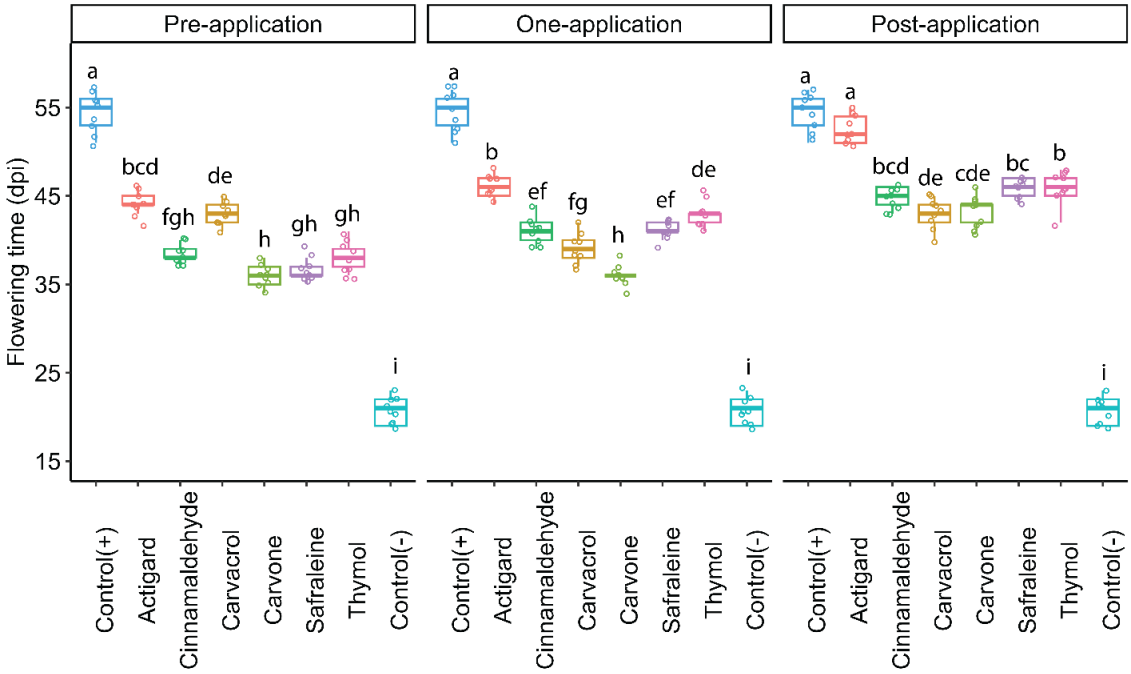


Figure 7. The effect of the treatments on the flowering time (dpi) of tomato plants exposed to *P. syringae* pv *tomato* DC3000. Control (+) refers to non-treated tomato plants infected with the Pst-DC3000. Control (-) refers to non-treated tomato plants with no infection. Means with different letters indicate a statistically significant difference at a 5% level of probability.

3.5. The Impact of Tested Pure Components on the Expression of Defense Related Genes

Five pure components, namely carvacrol, carvone, cinnamaldehyde, safraleine, and thymol, were applied to tomato plants and inoculated with Pst-DC3000. The symptoms of disease in all treatments were observed and recorded. The pure component treatments without inoculation revealed no symptoms or any sign of phytotoxicity. In comparison, the inoculated plants exhibited symptoms and visibly impaired growth due to the infection, as compared to control plants. However, the treatment of pure components significantly reduced the symptoms caused by the bacterial infection.

The relative expression of genes related to the resistance pathways including the SAR pathway (*WRKY*, *NPR1*, *PR1*, *PR2*, and *PR3*), to the ISR pathways (*MYC2*, *JAZ2* and *ACC*), and R-genes (*HCR2*) on leaf samples of tomato plants were analyzed in asymptomatic plants no-treated with pure components (control (-)), pure components treated plants, non-treated symptomatic plants (control (+)) and pure component treated symptomatic plants. The results exhibited that the bacterial infection led to an up-regulation of several defense-related genes including *PR1, PR2, PR3, WRKY*, and *JAZ2*, whereas the ISR pathway gene *ACC* was downregulated at three days post-inoculation (Figure 8). Gene array analysis revealed that pure component application induced expression of genes associated with SAR (*PR1*, *PR2*, and *PR3*) and ISR pathways (*JAZ2*) at three days post-spraying (Figures 2 and 3). Cinnamaldehyde was the most effective small molecule, followed by carvacrol and safraleine, in inducing expression of *PR1*, *PR2*, *PR3*, and *JAZ2* genes at three days post-spraying (Figures 9 and 10). Carvone was also highly effective, increasing the expression of *PR2*, *PR3*, and *JAZ2* genes. Thymol increased expression of *PR2*, *PR3*, and *JAZ2* genes, but was less effective than other tested compounds.

Furthermore, it was observed that the genes *PR1*, *PR2*, and *PR3*, which encode pathogenesis-related proteins, were significantly upregulated following the dual application of the tested compounds on tomato plants infected with Pst-DC3000. Interestingly, the dual application of carvone, cinnamaldehyde, and safraleine led to a notable increase in the expression of *PR1*, whereas the dual application of thymol and carvacrol did not have any impact on its expression level (Figure 11). On the other hand, the dual application of all five tested components induced the expression of *PR2* and *PR3*

(Figures 12 and 13). Notably, carvone was found to be the most effective major component responsible for the overexpression of these three pathogenesis-related genes in tomato plants infected with Pst-DC3000, followed by cinnamaldehyde and safraleine. Although carvacrol increased the expression of *PR2* and *PR3* in dual application, it did not show any significant change in the expression of PR1. However, the expression of the gene *JAZ2* remained unchanged following the dual application of tested components (Figure 14).

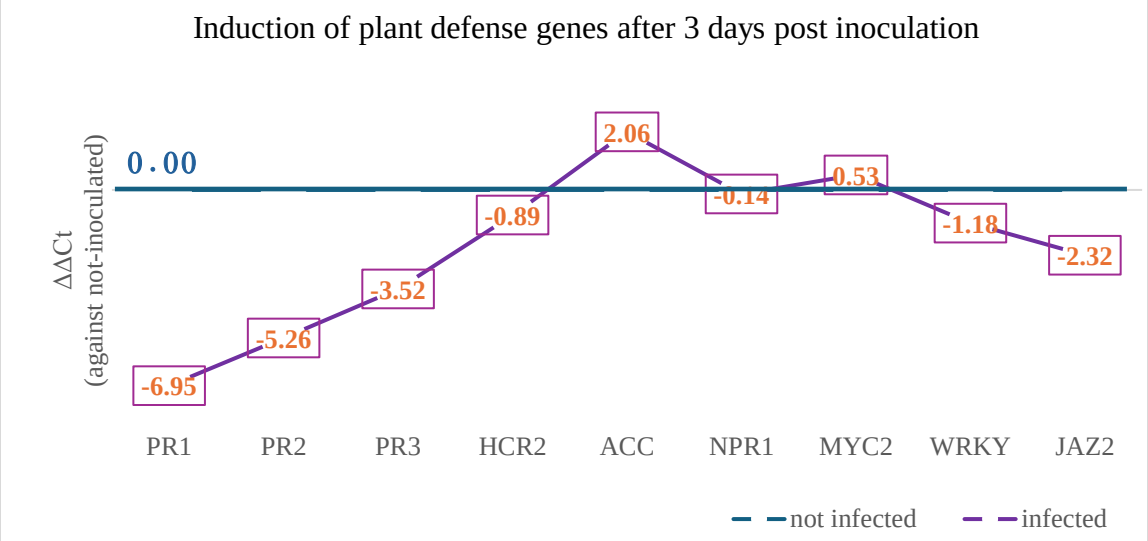


Figure 8. The relative expression level of plant defense genes *PR1*, *PR2*, *PR3*, *HCR2*. *ACC*, *NPR1*, *MYC2*, *WRKY*, and *JAZ2* at three days post-inoculation of Pst-DC3000 in tomato plants.

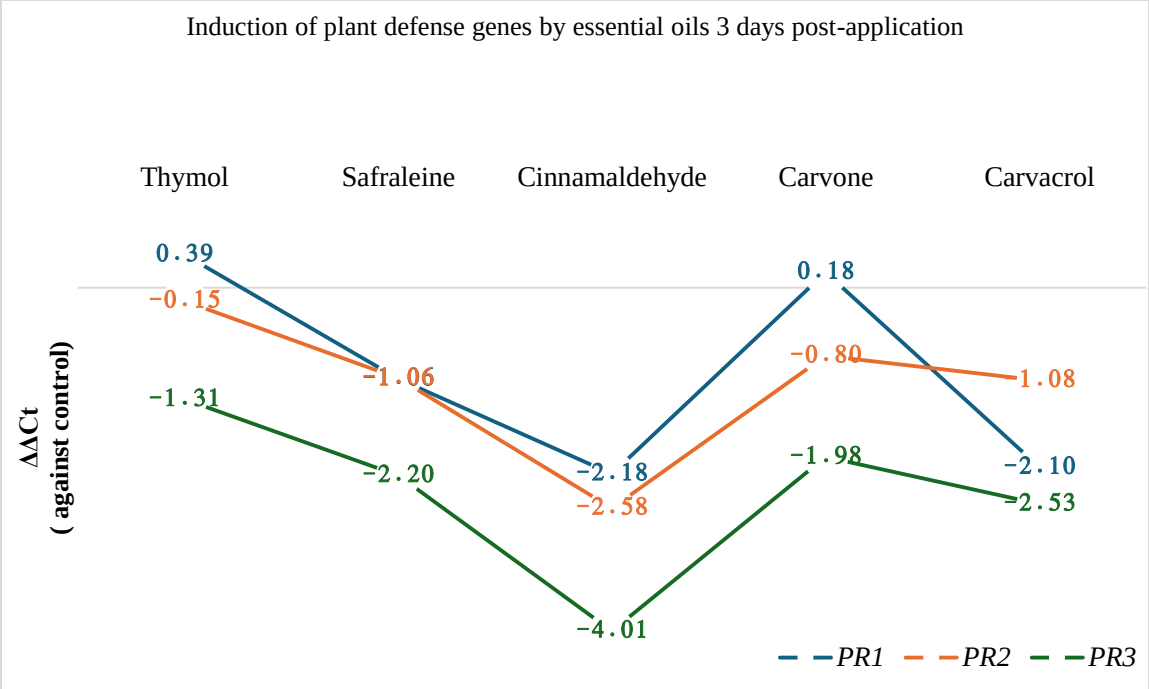


Figure 9. The relative expression level of the pathogenesis-related defense genes *PR1*, *PR2*, and *PR3* in tomato plants after three days post-spraying (dps) of tested pure compounds.

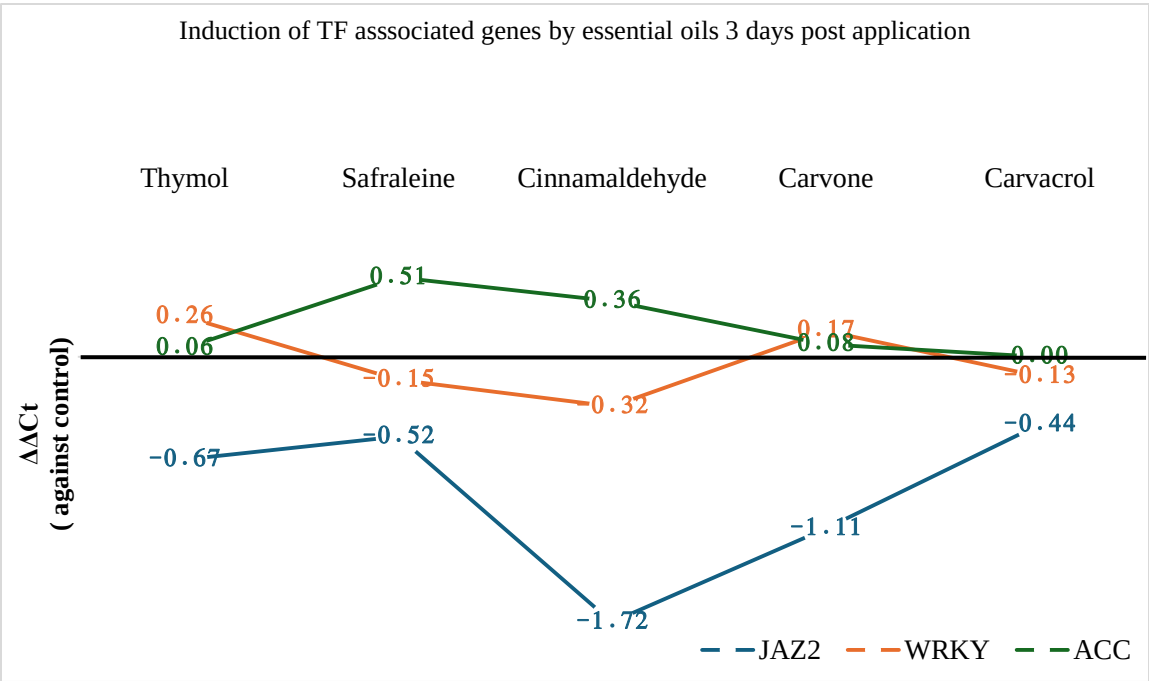
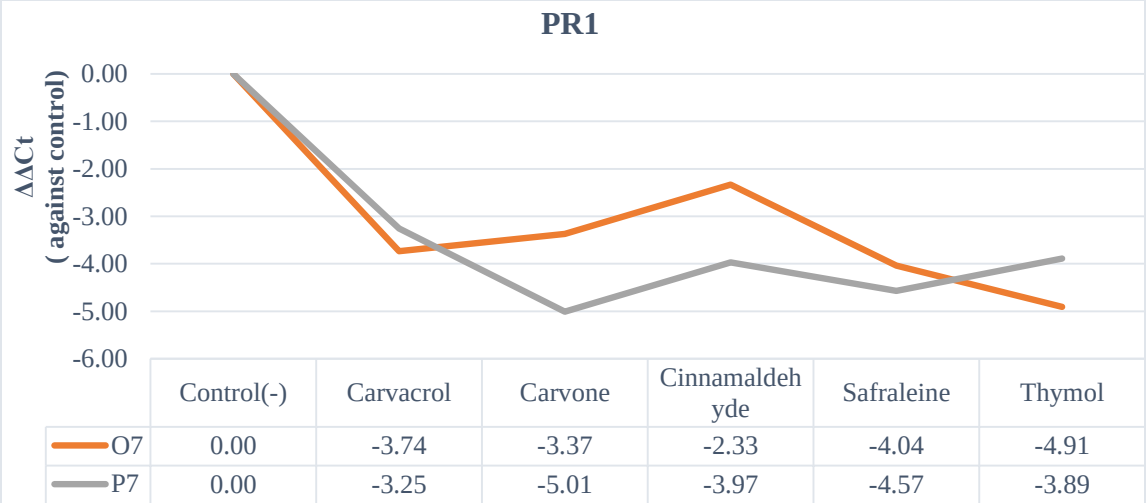
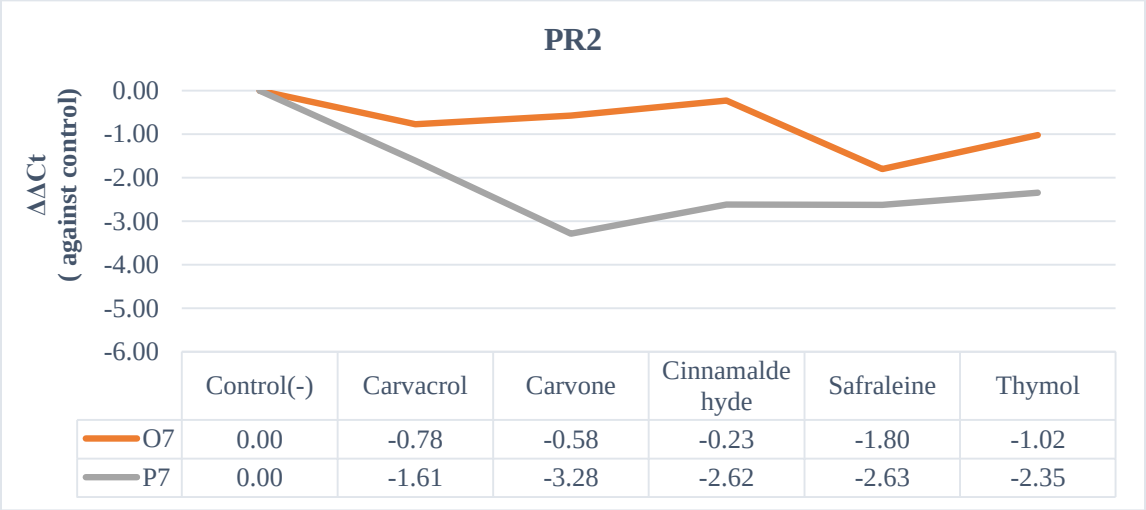


Figure 10. The relative expression level of the transcription factor (TF) associated genes *JAZ2*, *WRKY*, and *ACC* in tomato plants after three days post-spraying (dps) of tested pure compounds.



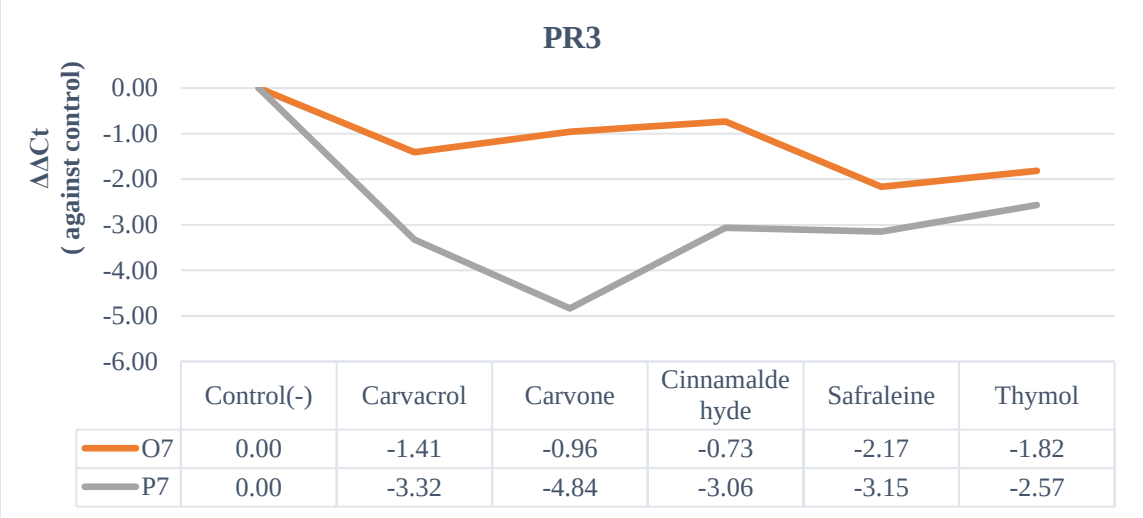
	Control(-)	Carvacrol	Carvone	Cinnamaldehyde	Safraleine	Thymol
O7	0.00	-3.74	-3.37	-2.33	-4.04	-4.91
P7	0.00	-3.25	-5.01	-3.97	-4.57	-3.89

Figure 11. The comparison of the relative expression level of the pathogenesis-related defense gene ***PR1*** in tomato plants after seven days post-inoculation of Pst-DC3000. **O7** represents the samples collected seven days after the application of the tested pure components. **P7** represents the samples collected seven days after the dual application of the pure compounds (on the same day as the Pst-DC3000 inoculation and five days post-inoculation).



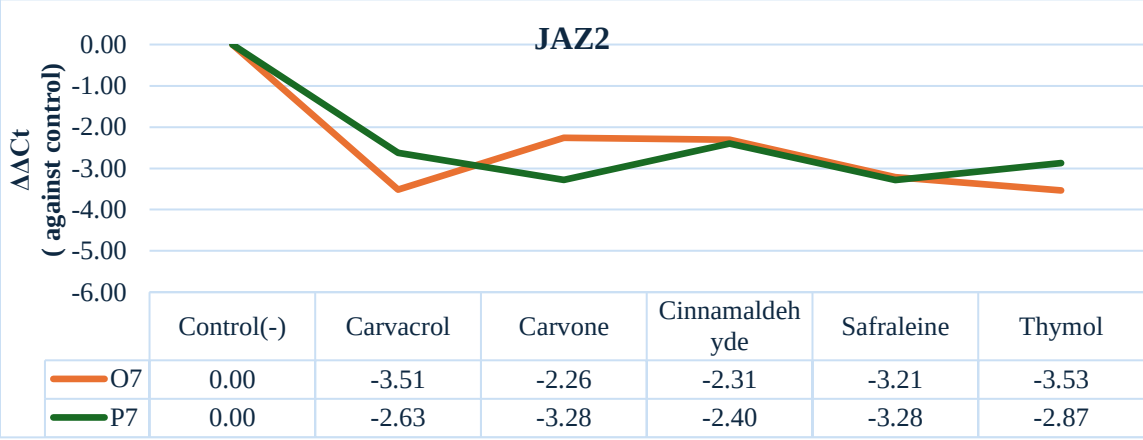
	Control(-)	Carvacrol	Carvone	Cinnamaldehyde	Safraleine	Thymol
O7	0.00	-0.78	-0.58	-0.23	-1.80	-1.02
P7	0.00	-1.61	-3.28	-2.62	-2.63	-2.35

Figure 12. The comparison of the relative expression level of the pathogenesis-related defense gene ***PR2*** in tomato plants after seven days post-inoculation of Pst-DC3000. **O7** represents the samples collected seven days after the application of the tested pure components. **P7** represents the samples collected seven days after the dual application of the pure compounds (on the same day as the Pst-DC3000 inoculation and five days post-inoculation).



	Control(-)	Carvacrol	Carvone	Cinnamaldehyde	Safraleine	Thymol
O7	0.00	-1.41	-0.96	-0.73	-2.17	-1.82
P7	0.00	-3.32	-4.84	-3.06	-3.15	-2.57

Figure 13. The comparison of the relative expression level of the pathogenesis-related defense gene ***PR3*** in tomato plants after seven days post-inoculation of Pst-DC3000. **O7** represents the samples collected seven days after the application of the tested pure components. **P7** represents the samples collected seven days after the dual application of the pure compounds (on the same day as the Pst-DC3000 inoculation and five days post-inoculation).



	Control(-)	Carvacrol	Carvone	Cinnamaldehyde	Safraleine	Thymol
O7	0.00	-3.51	-2.26	-2.31	-3.21	-3.53
P7	0.00	-2.63	-3.28	-2.40	-3.28	-2.87

Figure 14. The comparison of the relative expression level of the transcription factor associated gene ***JAZ2*** in tomato plants after seven days post-inoculation of Pst-DC3000. **O7** represents the samples collected seven days after the application of the tested pure components. **P7** represents the samples collected seven days after the dual application of the pure compounds (on the same day as the Pst-DC3000 inoculation and five days post-inoculation

4. Discussion

The management of bacterial disease in tomato plants is primarily achieved by the use of synthetic pesticides, especially copper-based pesticides, which can be toxic to

animals and humans and accumulate in living systems (Ansari & Malik, 2009). Furthermore, the intensive use of synthetic pesticides leads to the development of resistance to chemical pesticides in plant pathogens (McManus et al., 2002). It is crucial to determine novel compounds for the effective controlling of plant bacterial diseases. Recently, plant extracts, especially essential oils, and their major components have drawn great attention for their potential antibacterial properties. The potential of the tomato bacterial disease management with the plant essential oils or their main components appears of considerable interest due to the limited valid alternatives to copper-based chemicals and lacking commercial *P. syringae* pv. *tomato* resistant cultivars on the market, particularly in organic agriculture. In addition, plant-derived molecules are highly volatile, resulting in less residue in the products and limited adverse effects on human health and on the environment. In fact, a large number of plant natural products have been studied directly or as a basis for developing new agrochemicals, making them a valuable source of inspiration for modern agrochemical research.to determine and develop new agrochemicals (Quattrucci et al., 2013).

The essential oils and their major components exhibited great potential antibacterial activities with several mechanisms of action. Studies have shown that essential oils can have a significant impact on the cellular structure by destabilizing the cell's architecture. This can lead to a breakdown of the membrane's integrity and increased permeability, which in turn can disrupt various cellular activities, including energy production and metabolic regulatory functions. (Oussalah et al., 2006). The effects of essential oils can extend to both the external envelope of the cell and the cytoplasm (Raut & Karuppayil, 2014), due to their lipophilic nature. Researchers have

reported that essential oils derived from various medicinal and aromatic plants can increase bacterial cell membrane permeability, causing the leakage of cellular components and loss of ions (Saad et al., 2013). Additionally, the antibacterial properties of essential oils have been related to the reduction in membrane potentials, the perturbation of proton pumps, and the depletion of ATP (Turina et al., 2006). The structure of fatty acids, phospholipids bilayers, and polysaccharide molecules can be disrupted with the diffusion of the essential oils through the cell wall and cytoplasmic membrane(Raut & Karuppayil, 2014), potentially resulting in the coagulation of inner cellular components in the cytoplasm and destroying the bonds between lipid and protein layers (Burt, 2004).

In the *in planta* assay, the tested five pure components, and Actigard had efficient disease suppression performance. There was a significant decrease in the chlorotic area on the leaves and the incidence of bacterial speck lesions, and an increase in the plant height, fresh and dry weight, and root fresh and dry weight of treated plants compared to non-treated control plants infected with Pst-DC3000. Additionally, the treated tomato seedling flowered earlier than untreated control plants infected with Pst-DC3000. In the present study, cinnamaldehyde had the highest protection performance in three application cycles, with the highest values according to the screened categories compared to the other tested compounds. According to a study by Ooi et al. (2006), cinnamaldehyde has strong bactericidal properties against eight bacterial pathogens, including *Staphylococcus aureus* and several Gram-negative strains. The primary target of cinnamaldehyde is the bacterial membrane, which it destroys by disrupting membrane functions or causing the loss of channel proteins. This ultimately leads to bacterial cell

death (Song et al., 2016). Following the cinnamaldehyde, thymol was also highly effective successfully reducing the disease severity and causing significant enhancement in the observed categories. Thymol is a hydrophobic compound expressing strong antibacterial activities by destruction of the lipid fraction of bacterial plasma membranes, leading to the increase in membrane permeability resulting in the leakage of intracellular materials, and eventual cell death (Trombetta et al., 2005). Although safraleine, carvacrol, and carvone were highly effective in terms of protecting tomato plants from the Pst-DC3000, in general, their effectiveness was less than cinnamaldehyde and thymol applications. However, interestingly, the earliest flowering was observed in the application of carvone.

Our findings suggest that while Actigard provided significant protection against bacterial speck disease compared to untreated control plants infected with Pst-DC3000, it was the least effective compound among the tested chemicals. This contradicts previous studies, such as Da Silva et al. (2014), which reported Actigard as the most effective in controlling tomato bacterial speck disease, with around a 90% reduction in disease severity compared to the applications of essential oils of lemongrass, thyme, tea tree, and cinnamon. Similarly, Lucas et al. (2012) showed that Actigard was more effective than the application of Indian clove essential oil in reducing disease severity in tomato plants infected with *Xanthomonas vesicatoria*, the causal agent of tomato bacterial spot disease.

The potential of tested components on the elicitation of defense responses mediated by the host was examined on tomato plants infected with Pst-DC3000. Elicitors have the ability to activate defense responses in plants by mimicking the interactions of natural microbes, the interactions of natural microbe molecular patterns or defense

signaling molecules with their corresponding plant receptors, or by disrupting other defense signaling components. The term "plant activators" is frequently used to describe these molecules, as they can protect plants from diseases by stimulating immune responses. Prior applications of carvacrol, carvone, cinnamaldehyde, safraleine, and thymol to the asymptomatic tomato plants not-infected with the Pst-DC3000 led to the induction of the expression of genes associated with the SAR pathways *PR1*, *PR2*, and *PR3*, and the gene *JAZ2* related to the ISR pathways after three days post spraying (Figures 9 and 10). Although the overexpression of these resistance-mediating genes was relatively strong, it was slightly less than the non-treated symptomatic plants after three days post-infection with the Pst-DC3000. Interestingly, cinnamaldehyde led to the overexpression of these defense-related genes almost as strongly as in the tomato plants at three days post-infection with Pst-DC3000. This indicates that tested components can activate the defense-related pathways, leading to an increase in the resistance of plants before the infection of the plant pathogens.The inoculated plants with Pst-DC3000 exhibited a strong overexpression of the SAR-associated genes *PR1*, *PR2*, and *PR3* and ISR-related gene *JAZ2* after three days post-inoculation (Figure 8). This implies that pathogens led to induction of the expression of pathogenesis-related genes in the SAR and ISR pathways enhancing the resistance of plants toward the pathogen infection.The expression level of defense-related genes was gradually lowered in symptomatic tomato plants treated with pure components at seven days post-inoculation. This could be attributed to the decrease in infection intensity as well as the impact of the tested compounds on the induction of defense-related genes. Interestingly, a second application of the pure compounds resulted in a significant increase in the expression levels of these

defense-related genes at the same time point. This suggests that dual application of pure components can enhance and extend the resistance period against plant pathogens, thereby providing longer-lasting protection. Similarly, Kaneko et al. (2024) revealed that the application of rose essential oil after three days post-spraying significantly upregulated the expression of *PR1* gene in tomato plants. Also, a second application of rose essential oil 3 days after the initial application sustained the overexpression level of *PR1* for an additional 3 days.

5. Conclusion

The results indicate that all tested pure components had a great potential to protect the tomato plants against the bacterial speck disease. Comparing the tested components, especially cinnamaldehyde, followed by thymol, was the most effective compound to suppress the bacterial speck disease with a significant reduction in disease severity and growth promotion on tomato plants inoculated with Pst-DC3000. Additionally, our findings proved that the tested components (carvacrol, carvone, cinnamaldehyde, safraleine, and thymol), particularly cinnamaldehyde, are highly effective resistance inducers in tomato plants as they up-regulate the expression of defense-related genes in SAR and ISR pathways.

Further studies are necessary to find out their role in antibacterial effectiveness against the bacterial speck disease of tomato and their large-scale application in the management of bacterial diseases. The antibacterial potential of these pure components might open new avenues for controlling the bacterial speck disease of tomatoes by providing novel alternatives to conventional bactericides and promoting plant growth.

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Chapter IV

The Evaluation of Essential Oil Applications on Disease severity, Mycotoxin level and Nutritional Properties of Wheat Grains Infected with Fusarium Head Blight

1. Introduction

Fusarium species represent a significant group of plant pathogens, primarily responsible for Fusarium head blight (FHB) disease in small-grain cereals, including wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), triticale (× Triticosecale Wittmack), and oats (*Avena sativa* L.). Fusarium head blight (FHB) caused by the fungal pathogen *Fusarium graminearum* is one of the most devastating fungal diseases of wheat, one of the world's most produced and widely distributed crops (Schmidt et al. 2016). Although FHB infestation is especially prevalent in temperate climates, it has been discovered in all regions and is a major concern in the grain industry globally (Charmley and Trenholm, 2012). The incidence and severity of FHB are increasing worldwide. (Kautzman et al. 2015).

Fusarium head blight epidemics often cause significant reductions in cereal grain yield and quality, leading to severe economic losses for farmers (Mauler-Machnik and Zahn, 1994). As a negative consequence of FHB infection, significant yield losses occur due to fewer grains per head, smaller grain size, and lower weight of infected grain (Snijders 2004), which often results due to the reduced thousand-grain weight and yield of infected grains (Martin et al. 2017).

In addition to the severe yield and quality losses, *F. graminearum* produces harmful secondary metabolites, called mycotoxins, which pose a severe threat to food and feed safety, when the inflorescences become infected. The deoxynivalenol (DON) is the

primary toxin released by *F. graminearum* associated with FHB infection in wheat and barley, which remains stable even at high temperatures and low pH. Studies have revealed that wheat contaminated with DON can remain toxic for up to 4 years even after storage (Li et al., 2023). This toxin can enter the food chain at any point, from processing to transportation, storage, and consumption. It can contaminate plant-based products both directly and indirectly, leading to its accumulation in the human food supply. This harmful substance inhibits protein synthesis, leading to a disruption of normal cellular function and can have severe consequences on the digestive system of swine and other monogastric animals. The severe gastrointestinal symptoms have earned the toxin DON the colloquial name of vomitoxin. Consuming wheat flour contaminated with DON can cause nausea, fever, headaches, and vomiting in humans. The FDA has established advisory levels for DON to ensure the safety of food and feed. Presently, food products intended for human consumption are advised to contain no more than 1 part per million (ppm). Ruminating beef and feedlot cattle older than four months are recommended to have a maximum of 10 ppm (not exceeding 50% of their diet), while poultry are advised to have no more than 10 ppm (also not exceeding 50% of their diet). Swine are recommended to contain no more than 5 ppm (not exceeding 20% of their diet), while all other animals are advised to have 5 ppm (not exceeding 40% of their diet). Accordingly, managing Fusarium Head Blight (FHB) in wheat is critical to minimize yield loss and reduce mycotoxin contamination.

The mycotoxin DON (3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one) is primarily produced by *F. graminearum* and *F. culmorum*, and can be found predominantly in cereals and cereal-based items. It is categorized as a type B

trichothecene due to a double bond with oxygen at carbon number 8 within its molecular structure (Kamle et al., 2022). In 1970, Yoshizawa et al. were the first to identify and chemically analyze DON after isolating and purifying it from moldy barley grains (Li et al., 2023). Although the presence of DON poses a significant risk to both humans and animals, it plays a crucial role in the infection of plants and fungal survival. This toxin can suppress a plant's natural defense mechanisms or protect the fungus from other microorganisms. *F. graminearum*'s ability to synthesize DON allows it to spread from an infected branch to a healthy branch through hyphae formation, making it more difficult for the plant to defend itself. Additionally, this toxin prevents the thickening of the plant cell wall, which would typically act as a barrier against fungal penetration (Kamle et al., 2022). DON confers a two-fold benefit in the niches for resources on crop residues and organic matter. Moreover, DON exhibits antimicrobial properties that disrupt protein biosynthesis, rendering it potent against other eukaryotic soil organisms (Audenaert et al., 2013). This may also impinge on the metabolite production of other fungi residing in the soil, including *Trichoderma* spp., which are renowned for their aptitude to outmatch their counterparts through mycoparasitism, facilitated by enzymes like chitinases (Audenaert et al., 2013).

Fungal growth can alter the chemical and physical properties as well as the nutritional content of the grain (Hermann et al. 1998), possibly affecting its nutritional value. To establish infection and developing disease, *F. graminearum* uses proteins, lipids, starch, or nucleic acids in host plants (Brown et al. 2012). Researchers have reported that the fungus is an aggressive invader that alters starch granules and storage proteins (Bechtelet et al. 1985). *Fusarium* spp. can produce enzymes that can modify and

break down nutrients stored in the grain, such as proteins and carbohydrates, to make them more accessible for their use (Schmidt et al. 2016). It has been shown that proteases secreted by *Fusarium* spp. have the ability to degrade gluten proteins in an in vitro system (Eggert and Pawelzik 2011). Meye et al. (1986) reported an increase in crude ash content as a result of FHB infection. In addition, significant modifications of host cell walls such as cellulose, xylan, and pectin have been observed in the growth of the pathogen. This led to the idea that the fungus might produce cell wall-degrading enzymes during infection (Kang and Buchenauer, 2000b). Schwarzet et al. (2001) observed that Fusarium proteases continue to hydrolyze endosperm proteins during dough mixing and fermentation, resulting in dough weakening and loaf volume reduction (Bechtel et al. 1985). Similarly, Lancova et al. (2016), stated that viscosity is reduced in infected wheat. Considering the use of wheat as food and feed, it is crucial to examine the effect of Fusaria and its mycotoxins on defined quality parameters, since it decreases grain yield and quality that affects suitability for human and animal consumption (Kautzman et al. 2015; McLaughlin et al. 2021).

For the management of the FHB, the planting of tolerant or resistant varieties and the usage of synthetic pesticides are the primary strategies used by the growers. The cultivation of tolerant or resistant cultivars is the most cost-effective method for the control of FHB disease, however, currently, only a few tolerant cultivars are available on the market due to the quantitative nature of the resistance which slows down the development of the resistance cultivars (Wegulo et al. 2015). Fungicide application is the most popular strategy for the suppression of the FHB and reduction of the disease severity in the field. The demethylation inhibitor (DMI) class (Group 3) fungicides are

the most extensively applied pesticides including Folicur® 3.6 F (Tebuconazole, Bayer CropScience), Prosaro 421 SC (Prothioconazole + Tebuconazole, Bayer CropScience), Caramba 0.75 SL (Metconazole, BASF Corp.), Proline 480 SC (Prothioconazole, Bayer CropScience), TOPGUARD® EQ (Azoxystrobin + flutriafol, FMC Corp.) and Miravis® Ace (Pydiflumetofen + Propiconazole, Syngenta). The effective fungicide application can significantly reduce the incidence and severity of the FHB, and increase the total yield and quality compared to the non-sprayed check. However, FHB is not completely controlled with DMI-class fungicides alone. Most importantly, the pathogen can develop resistance to fungicides as a result of the application of the same group of fungicides annually. Spolti et al. (2014) reported the first DMI class fungicide-resistant *F. graminaerum* isolates in the US in New York in 2014, which showed the potential presence of fungicide-resistant isolates among the fungal populations. In addition to the US, *F. garaminearum* isolates resistant to DMI-class fungicides were also detected in Europe and Asia (Anderson et al. 2020). These findings indicate the need of novel chemicals as alternatives to current fungicides for the control of FHB, which can be rotated with the DMI class fungicides to prevent the development of the fungicide-resistant *F. graminearum* populations. Recently, there has been growing attention to natural products, especially essential oils, and their major compounds (e.g., terpenes, terpenoids, and aromatic compounds) obtained from different parts of the plants for their antifungal properties to be used as new biocides against *F. graminearum* as alternatives to conventional fungicides. Perczak et al. (2019) pointed out that EOs of cinnamon (*C. zeylanicum*), oregano (*O. vulgare*), and palmarosa (*Cymbopogon martini*) expressed significant antifungal activity against *F. graminearum* and *F. culmorum*. Harčárová et al.

(2021) determined that 100% mycelial growth inhibition against the *F. graminearum* CCM 8244 and *F. graminearum* CCM 683 was achieved at the application of the EOs of oregano, *Thymus vulgarus* L. (thyme), and *Syzygium aromaticum* L. (clove) at the concentration of 500 ug/ml *in vitro*. Thymol, a natural plant-derived compound, showed a strong antifungal activity and inhibited the hyphal growth, conidia production, and the conidia germination of *F. graminearum* isolates with an average EC50 value of 26.3 ug/ml (Gao et al., 2016). Cinnamaldehyde, a major component of the cinnamon oil, completely suppressed the hyphal growth and spore germination of *F. solani*, the causal agent of root rot, of *Astragalus mongholicus* Bunge (Mongolian milkvetch) *in vitro* at the concentration of 300 ul/l and 120 ul/l, respectively (Wang et al. 2022). Also, approximately 90% reduction was seen in the disease index of *A. membranaceus* root rot in the application of cinnamaldehyde at the concentration of 600 ul/l under greenhouse conditions (Wang et al. 2022).

Considering the potential of EOs and their major components for the control of fungal diseases, a study was undertaken to assess the effects of tested essential oils (carvacrol, carvone, thymol, cinnamaldehyde, safraleine) and compare them with chemical pesticides (Topguard and ACTIGUARD Plant Activator ®[Acibenzolar-S-methyl]) applications on the disease severity of FHB, the accumulation of DON (deoxynivalenol) in the wheat kernels, the total yield and the nutritional properties of the wheat grains exposed to the FHB disease.

2. Materials and Methods

2.1. Inoculum Preparation

The macroconidia of *F. graminearum* (NE-16-07) isolate were used as inoculum for the infection of wheat plants *in planta* experiments. For the large scale of macroconidia production, CMC medium (carboxymethyl cellulose agar) with following ingredients (g/L); 15 g Carboxymethylcellulose sodium salt, 1 g Sodium nitrate (NH_4NO_3), 1 g Monopotassium phosphate (KH_2PO_4), 0.5 g Magnesium (II) sulfate ($MgSO_4$-7 H_2O), 1 g Yeast Extract, and 10 g Bacto-Agar), was prepared and sterilized by autoclaving. After autoclaving, when the media cooled down, it was poured approximately 20 ml into the Petri dishes with the size of 100 mm diameter and 15 mm height. Eight mm diameter mycelial plugs of seven days old *F. graminearum* cultures grown in potato-dextrose-agar (PDA) were placed in the center of the prepared CMC plates. The inoculated CMC plates were stored at room temperature (25 °C) under a 12 h photoperiod for 21 days for sporulation. Then, five ml of autoclaved distilled water was added to each inoculated CMC media, and macroconidia were harvested by gently scratching the surface of the medium via a rubber spatula. The harvested solution mixture including macroconidia and mycelial of the tested fungus was through from cheesecloth to remove mycelial fragments and other debris from the solution. The macroconidia suspension was determined by using a hemocytometer followed by the preparation of final inoculum suspension in sterile distilled water at the concentration of 1×10^4 conidia/ml for the in-planta application.

2.2. Preparation of Treatments

The five different major components of essential oils (EOs) including carvacrol (Sigma-Aldrich, W224611), carvone (Sigma-Aldrich, 124931), cinnamaldehyde (Sigma-Aldrich, W228613), safraleine (Givaudan, 54440-17-4), and thymol (Sigma-Aldrich, 16254), and two fungicides Actigard® 50WG (Syngenta) with an active ingredient (ai) of 50% acibenzolar-S-methyl (ASM) which is a natural defense system activator, and TOPGUARD® EQ (FMC Corp.) with the active ingredient of 25.3 % azoxystrobin and 18.63% flutriafol were tested in planta greenhouse experiments. All EOs and fungicides were dissolved in the treatment mixture of 96 ml autoclaved distilled water, 0.5 ml TWEEN® 80 (Sigma-Aldrich, P1754), 0.5 ml EOs/fungicides/water, and 3 ml 10% DMSO (Dimethyl Sulfoxide)(Sigma-Aldrich, 5.89569) per 100 ml at the desired concentration. The concentration of EOs was adjusted at the doses of 5 mg/ml based on in vitro tests for foliar applications. The 3mM ASM was made by adding 0.126 g of Actigard into the 100 ml treatment mixture (Walnut, 2019). TOPGUARD® EQ was prepared at the recommended concentration of 10-14 fl oz/A for aerial application on wheat plants. The control groups (with pathogen and without pathogen) were sprayed with the same amount of 0.5 ml autoclaved distilled water-added treatment solution.

2.3. *In Planta* Greenhouse Experiments

In planta experiments were conducted with the hard red spring wheat variety ‘Bobwhite’ which is susceptible to fusarium head blight disease at the Plant Pathology greenhouses located on the North side of the East Campus of the University of Nebraska-Lincoln from April to September in the year of 2022. Plant materials ‘Bobwhite’ were planted in 15 cm diameter clay pots (4-6 plants in each pot) filled with steam-pasteurized

standard greenhouse mix (5 gallons peat, 3 gallons soil, 2.5 gallons sand, 2.5 gallons vermiculite) and grown in the greenhouse with daily watering at 25°C under 16 h light: 8 h dark photoperiod. The experiment was arranged in the randomized complete block design with four replications. When the wheat heads were at the anthesis stage (Feekes 10.51) (yellow anthers are extruded from florets), treatments were sprayed onto the heads (approximately 5 ml/ head) and flag leaves (approximately 2 ml) by using a hand sprayer. Thirty heads and flag leaves, five to six heads and flag leaves in a pot, at the same flowering stage were used for each treatment. There were three different application cycles in the in planta experiments which were pre-application, one-application, and post-application.

In pre-application, the prepared treatment solutions were sprayed onto the wheat heads and flag leaves and stored for five days in the greenhouse at 23-25°C under 16 h light: 8 h dark photoperiod. Following the five-day period, the wheat heads and flag leaves were resprayed again with treatment solution and allowed to dry for approximately eight hours. Once dry, approximately 1 ml prepared conidia suspension (1×10^4 conidia/ml) was applied to each treated head by using a hand sprayer and covered with plastic bags (Ziplock® bags) for 72 hours to keep high humidity around the heads to favor disease development. After 72 hours Ziplock® bags were removed.

One-application, the prepared treatment solutions were sprayed onto the wheat heads and flag leaves and allowed to dry for approximately eight hours. Once dry, approximately 1 ml prepared conidia suspension (1×10^4 conidia/ml) was applied to each treated head by using a hand sprayer and covered with zip lock bags for 72 hours to keep

high humidity around the heads to favor disease development. After 72 hours zip lock bags were removed.

Post-application, the prepared treatment solutions were sprayed onto the wheat heads and flag leaves and allowed to dry for approximately eight hours. Once dry, approximately 1 ml prepared conidia suspension (1×10^4 conidia/ml) was applied to each treated head by using a hand sprayer and covered with zip lock bags for 72 hours to keep high humidity around the heads to favor disease development. Five days after the inoculation, the same amount of treatment solution was sprayed onto the wheat heads and flag leaves again.

In all three application cycles, after pathogen inoculation, treated wheat plants were grown in the greenhouse at 25°C under 16 h light: 8 h dark photoperiod for the monitoring of disease development and were watered as needed.

2.4. The Effect of Tested Components on the FHB Infection in Wheat Heads

A total of thirty heads from each different treatment was used to determine the FHB infection rate in wheat plants. Following 14 days of inoculation, the total number of spikes and symptomatic spikes were tallied for each wheat head. The infection rate was then calculated per wheat head by using the subsequent formula:

Infection rate (%) = (Total number of symptomatic spikes in per head / Total number of spikes in per head) x 100

2.5. Grain yield and One Hundred Seed Weight

Wheat heads (30 spikes/per treatment) were harvested when the grains were at the hard dough stage from each different treatment and dehulled by using a dehulling machine. The total weight of wheat grains harvested from each treatment was weighed

for grain yield. In addition to this, a hundred seeds were randomly selected from the bulk of wheat grains obtained from each treatment with three replications and used for measuring hundred seed weight.

2.6. DON (Deoxynivalenol) Analysis

The quantification of deoxynivalenol (DON) was conducted at the Plant Pathology, Physiology, and Weed Science Department of Virginia Polytechnic Institute and State University (Blacksburg, VA, USA) using gas chromatography-mass spectrometry (GC - MS) with an Agilent 6890/5975 system. To start the process, a 4 g bulk sample of ground grain from each treatment was weighed and placed into a 50 mL screw cap conical tube. Then, 16 mL of acetonitrile: water (86:14) solution was added to each tube and mixed thoroughly with a vortex mixer. The extraction tubes were left to shake horizontally at room temperature for 1 hour at 200 rpm. Afterward, approximately 3ml of the liquid extract was decanted into a C18: Aluminum oxide (1:3) cleanup column, and the flow through was collected in a clean glass tube. The cleaned extract flow through was then transferred to a new glass tube and dried under nitrogen flow at 37°. Next, 100 uL of freshly prepared TMSI: TMCS (100:1) was added to each dried sample and manually vortexed each tube to coat the sidewalls, followed by incubation at room temperature for 10 minutes. To complete the process, 1 mL isooctane + Mirex (0.5 ppm) was added to each tube, followed by 1 mL ddH2O, and vortexed well to mix. The tubes were then incubated at room temperature for 5 minutes or until two distinct layers appeared. Finally, 125 uL of the top layer of each sample was carefully transferred into a clean GC/MS assay vial to quantify the presence of deoxynivalenol (DON). The analyses were carried out concurrently with standards of the toxin, ranging from 0.05 ppm – 10

ppm, on an Agilent 7890B/5977B GC/MS. Throughout each batch run, internal checks were performed for quality control purposes.

2.7. Biochemical Assays

Harvested wheat grains were dried at 65 °C, ground (IKA MF-10.1, Staufen, Germany), and made ready for biochemical analyses. Wheat grain samples were preserved at +4 °C throughout the analysis.

2.7.1. Crude Protein Content

About 1 g of sample was taken and the nitrogen ratio was determined with the use of the macro Kjeldahl method. The resultant nitrogen value was multiplied by 6.25 (N x 6.25) to get sample crude protein content (AOAC, 1990).

2.7.2. Crude Ash Content

About 1 g of sample was ashed in a muffle furnace at 550 ºC for 8 hours to get the crude ash content of the samples.

2.7.3. Non-resistant, Resistant, and Total Starch Content

Megazyme Resistant Starch Assay (catalog number K-RSTAR, Megazyme International Ireland Ltd. Co. Wicklow, Ireland) kit developed in accordance with AOAC Official Method 2002.02 and AACC 32-40 Method was used to determine resistant starch content of 100 mg samples.

2.7.4. Amylose-Amylopectin Content

Amylose and amylopectin fractions of the starch were determined using the Megazyme Amylose/Amylopectin Analysis Kit (K-AMYL, Megazyme International Ireland, Wicklow, Irelan).

2.7.5. Pythic Acid Content

Phytic acid content was detected with myo-inositol assay kit (K-INOSL, Megazyme Intl, Wicklow, Ireland) in accordance with the instructions of the manufacturer. About 1 g sample was used in the analysis.

2.7.6. Mineral Contents

Wheat whole grain samples were subjected to acid digestion in nitric perchloric acid (Mertens, 2005a) and then P, Mg, K, Ca, Fe, Na, S, Mn, Cu Zn, and B contents were determined in an ICP-OES spectrometer (Inductively Couple Plasma spectrophotometer) (Agilent 5800) (Mertens, 2005b).

2.7.7. Pasting Properties

Pasting properties of the samples were analyzed by using Rapid Visco-Analyzer (RVA 4, Perten Inst., Australia) using STD1 pasting profile. In this assay, 3.5 g of samples (14% moisture basis) was mixed with distilled water to make a total mixture weight of 28.0 g. Sample was equilibrated at 50 °C for approximately 1 min, heated to 95 °C for 3 min 42 sec, held at 95 °C for 2.5 min, cooled down to 50 °C for 3 min 48 sec, kept at 50 °C for 2 minutes. The constant rotating speed of the paddle was 160 rpm. Pasting data were acquired by using Thermocline Software (Perten Inst., Australia). Pasting tests were repeated twice for each sample.

2.7.8. Amino Acid Analysis

Samples (0.5 g) were weighed and burned with 20 ml of HCl at 110 °C for 24 hours. 20 ml of distilled water was added and it was dried in an evaporator at 70 °C. It was completed to 25 ml in a volumetric flask with distilled water. Samples passed through a 0.45 µm syringe filter and put into the vials. An LC-MS/MS (SHIMADZU

LCMS-8030 PLUS) system equipped with a prominence fluorescence detector and Zorbax Eclipse/AAA (4.6×150 mm, 3.5µm) was used for the free amino acid composition of the samples. The flow rate was 1ml/min and the colon temperature was 40 °C. Formic acid:water (1:99) was used as elution buffer A formic acid:methanol (1:99) was used as elution buffer B. The amino acids' standard curves were converted into corresponding amino acid concentrations by an external standard method according to the instruction of the manufacturer (Sigma-Aldrich, St Louis, MO, USA).

2.8. Statistical Analysis

The experimental data were subjected to analysis of variance according to the randomized plots experimental design using the SAS 9.2 (SAS Institute Inc 2008) software. Significant means were compared with the use of the LSD multiple comparison test using statistical probability $P \leq 0.05$.

3. Results

3.1. The Effect of Tested Components on the FHB Infection Rate

The effect of the tested components and synthetic pesticides on the infection rate in wheat heads is shown in Figure 1. All treatments exhibited a significant decrease in FHB infection when compared to untreated wheat heads that were inoculated with *F.graminearum*. Topguard was found to be the most effective chemical in reducing FHB infection in wheat grains across all three application cycles, resulting in a similar infection rate to that of untreated wheat plants with no infection. Meanwhile, Actigard was the least effective in all three application cycles. Safraleine, one of the pure components, was highly effective in reducing the infection of FHB, with about 40% infection in all three application cycles. Interestingly, while pre-application of safraleine

resulted in around 38% infection, a slightly increased infection was observed in the post- and one-application, with 40% and 42%, respectively. Carvacrol was also effective in reducing FHB infection in wheat heads compared to untreated wheat plants infected with FHB. Pre- and post-application of carvacrol showed around 40% and 42% infection, respectively, while one application resulted in around 50% infection. Although carvone, cinnamaldehyde, and thymol also reduced FHB infection in wheat heads, they were less effective than safraleine and carvacrol. According to the application cycles, although there were no significant differences in the treatment of Topguard, dual applications (especially pre-application) of the pure components resulted in less infection than the one-application.

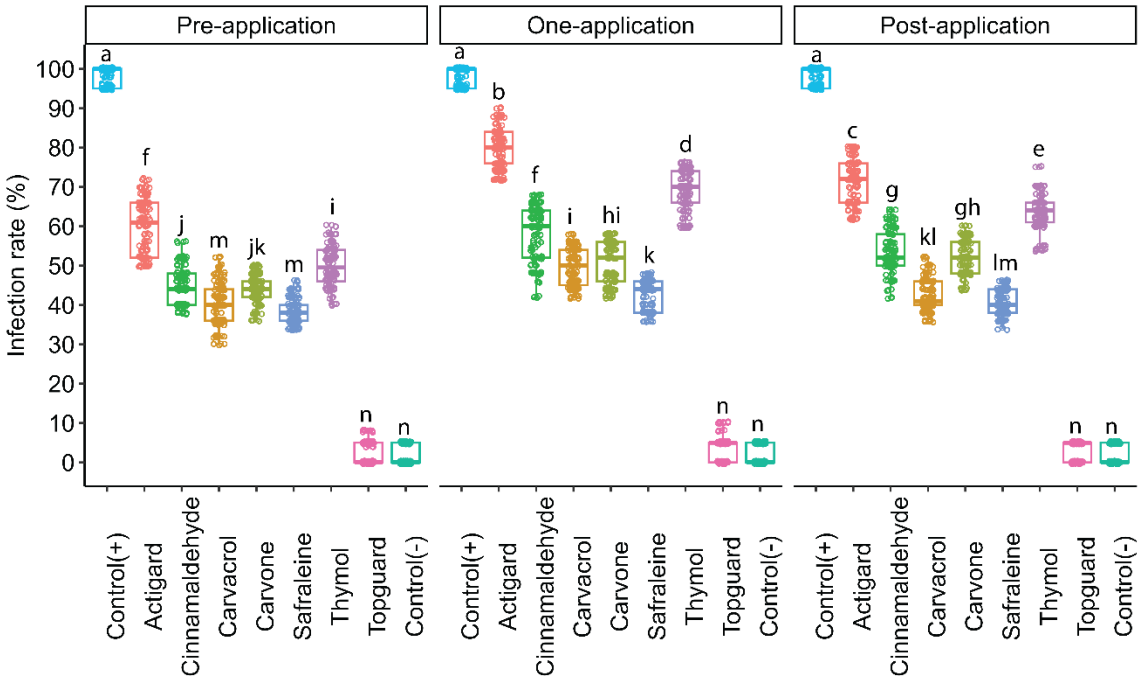

Figure 1. Effect of the tested components and chemical pesticides on the infection rate in wheat heads after 14 days post inoculation of *Fusarium graminearum* in the greenhouse. Control (+) refers to non-treated wheat plants infected with *Fusarium graminearum*. Control (-) refers to non-treated wheat plants with no infection. Means with different letters indicate a statistically significant difference at a 5% level of probability.

3.2. Grain Yield and One Hundred Seed Weight

The grain yield (g/30 spikes) and 100 seeds weight of the wheat samples harvested from the treatments were given in Figures 2 and 3. With the infection of the FHB, the grain yield reduced from around 14.20 g/30 spikes to 2.50 g/30 spikes, while the 100 seeds weight decreased from around 3.55 g to 0.63 g. The infection of FHB caused approximately 82% yield loss. Among the tested chemicals, Topguard showed the closest results to the control group without FHB infection in all three application cycles with a grain yield of 13.5, 13.20, and 13.05 g/30 spikes and 100 seeds weight of 3.47 g, 3.33 g and 3.24 g in pre-, post-, and one-applications, respectively. However, Actigard yielded the lowest amount of total grain and hundred seed weight with 5.76 g/30 spikes and 1.36 g from the one-application. In fact, Actigard treatment resulted in the lowest grain yield and hundred seed weight across all three application cycles. As for the tested major components of EOs, the highest total yield, and hundred seed weight were obtained in the pre-application of safraleine with 11.1 g/30 spikes and 2.91 g, respectively, followed by the pre-application of carvacrol with a total weight of 10.9 g/30 spikes and a hundred seed weight of 2.72 g. Comparing the application cycles in general, the pre-application of the treatments resulted in a higher total grain yield and hundred seed weight, followed by the post-application and one-application of the treatments.

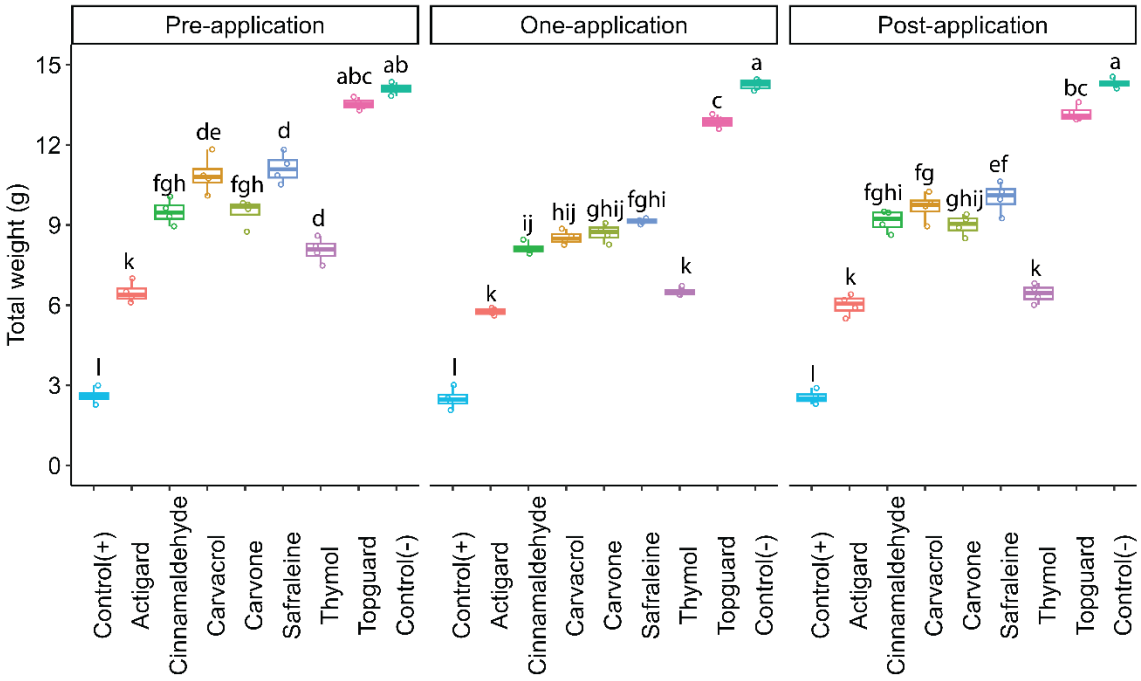

Figure 2. Total weight of wheat grains harvested from the treatments. Control (+) refers to non-treated wheat plants infected with *Fusarium graminearum*. Control (-) refers to non-treated wheat plants with no infection. Means with different letters indicate a statistically significant difference at a 5% level of probability.

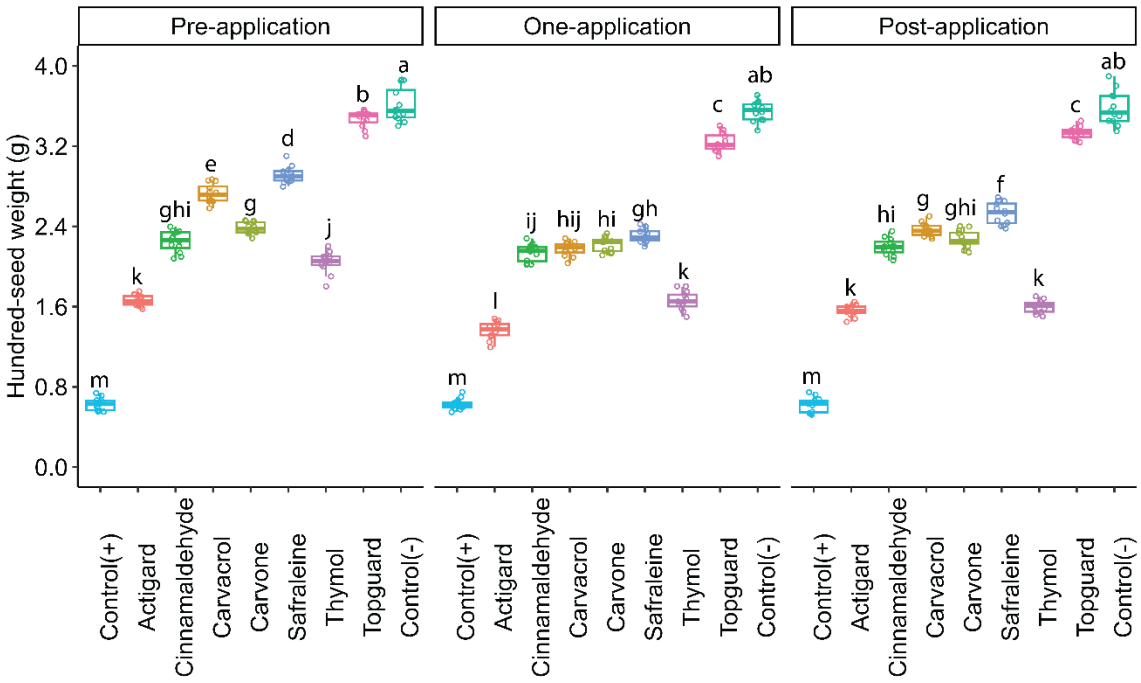

Figure 3. Hundred-seed weight of wheat grains harvested from the treatments. Control (+) refers to non-treated wheat plants infected with *Fusarium graminearum*. Control (-) refers to non-treated wheat plants with no infection. Means with different letters indicate a statistically significant difference at a 5% level of probability.

3.3. The Effect of Tested Components on the Deoxynivalenol (DON) Concentration

The impact of the tested components and synthetic pesticides on the level of deoxynivalenol (DON) in wheat grains is illustrated in Figure 4. All treatments significantly reduced the DON concentration compared to the untreated wheat plants infected with FHB (Figure 4). Topguard proved to be the most effective treatment, leading to a decrease in DON levels, while Actigard was the least effective chemical tested. Topguard showed a DON level similar to that of untreated wheat plants with no infection during all three application cycles. Following Topguard, safraleine significantly reduced the DON concentration in pre- and post-application cycles. Post-application of carvacrol also led to a significant decline in the level of DON, but less than Topguard and safraleine treatments. Cinnamaldehyde, carvone, and thymol were also highly effective in reducing the DON concentration in wheat grains compared to the untreated wheat plants inoculated with FHB; however, the decline in DON levels was relatively less than Topguard and safraleine treatments. In terms of application cycles, the DON level was relatively lower in dual applications (pre and post) than in one-application treatments.

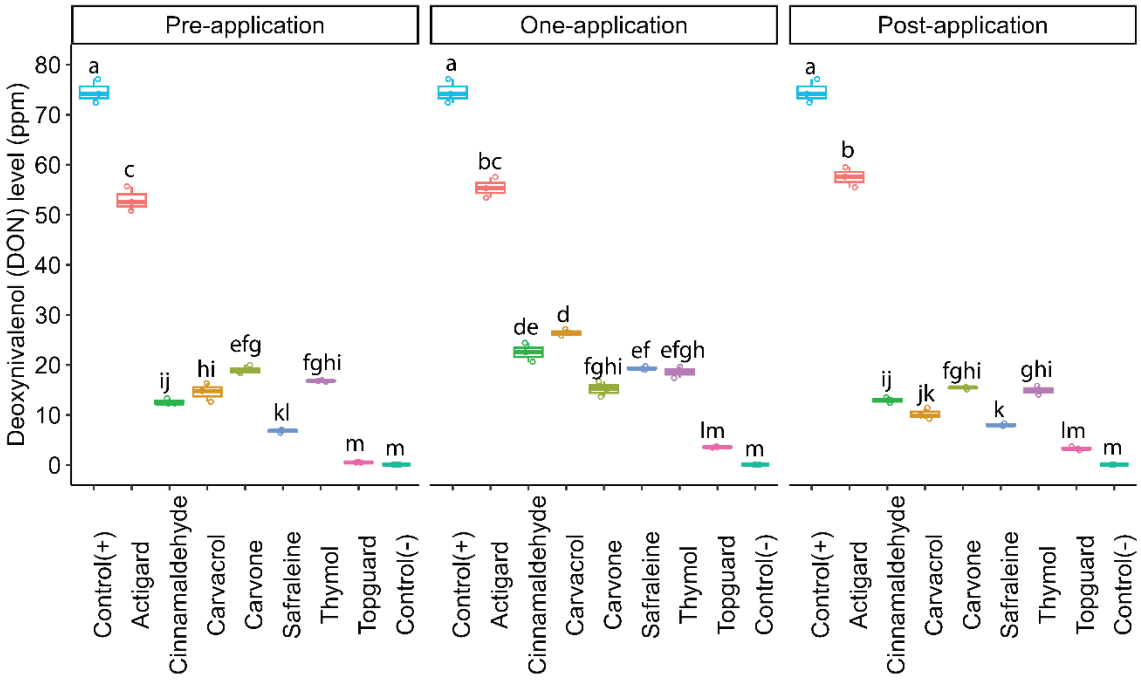

Figure 4. Effect of the tested components and chemical pesticides on the deoxynivalenol (DON) concentration in wheat grains inoculated with *Fusarium graminearum* in the greenhouse. Control (+) refers to non-treated wheat plants infected with *Fusarium graminearum*. Control (-) refers to non-treated wheat plants with no infection. Means with different letters indicate a statistically significant difference at a 5% level of probability.

3.4. Biochemical Assays

Tables 1, 2, and 3 show the average values of biochemical properties of wheat grains harvested from the treatments in pre-, one-, and post-application cycles, respectively. The effect of essential oils and pesticides on the examined properties was found to be statistically significant ($p \leq 0.01$) in all three application cycles.

In the pre-application period, the control treatment produced the highest oil content (2.07%) and ash content (3.71 %), while the lowest was obtained from the cinnamaldehyde treatment with 1.81 % and 2.61% respectively. Additionally, control^{+}, thymol, and Actigard were in the group with the highest ash content.

The crude protein ratios of wheat samples varied from 11.67% to 18.51%. The lowest protein content was obtained from the control^{-} group and cinnamaldehyde applied

plants (11.83 %), while the highest was obtained from the control^{+} group samples. The total dietary fiber content also varied among the applications. The control^{-} group had the lowest value of 2.90%, while the control^{+} group had the highest value of 5.52 %. In terms of resistant starch content, the cinnamaldehyde application had the highest value of 1.79% while Actigard resulted in the lowest resistant starch content (0.12%). On the other hand, the control- application had the highest non-resistant starch content (57.82%) and total starch content (58.94%), whereas safraleine, cinnamaldehyde, and control^{+} applications resulted in the lowest non-resistant content of 51.55%, 51.63%, and 51.84%, respectively and total starch contents of 53.02% in safraleine and 52.58% in control^{-} group.

The phytic acid content of wheat grains ranged from 1.15% (Control^{-} group) to 1.66% (Control^{+} group). The study also found that the lowest amylose and highest amylopectin contents were observed in samples from Control^{+} group, while the highest amylose and the lowest amylopectin amounts were detected in samples from Control^{-} group.

The results of the biochemical analysis of wheat grains harvested from the one-application treatments are presented in Table 2. The effect of the applications of major components and pesticides on the biochemical properties was determined statistically highly significant ($p \leq 0.01$). The wheat grains harvested from the control^{+} (control group with the pathogen) possed the highest values for content of crude ash (3.67%), crude protein (18.51%), crude oil (1.90%), phytic acid (1.66%), and amylopectin (83.09%), however, the highest amount of resistant starch (1.89%), non-resistant starch (59.63%), total starch (61.52%), and amylose (30.57%) were detected in wheat grains obtained from

the control^{-} (control group without the pathogen). The closest amount of the RS, NRS, and total starch content was determined in the Topguard application to the control^{-} whereas the content of amylose and amylopectin was in the safraleine application. In addition, the application of carvacrol, safraleine, and Actigard exhibited the closest results for the content of total dietary fiber with 1.76 % to the control^{-}. Furthermore, the closest results to the control- wheat samples in crude oil content were determined in the wheat grains collected from the applications of cinnamaldehyde, carvone, and safraleine, in crude protein content from the applications of thymol and safraleine, and crude ash content from carvone application.

Based on the post-application results (Table 3), the highest crude oil content of 2.12% was observed in control^{-} group and crude ash contents were obtained from the control^{-} (3.60%) and control^{+} group (3.67%), while the highest crude protein value (18.51 %) was obtained from control^{+} group. Moreover, the lowest crude oil content (1.47%) was obtained from safraleine applied samples, the lowest crude ash content (2.59%) was observed in the carvacrol applied samples, and the lowest crude protein content was seen in the control^{-} group (11.33%), and carvone (11.51%) treatments. Total dietary fiber ratios of wheat grains infected with FHB varied between 2.71-5.52%, the lowest total dietary fiber ratio was obtained from the control^{-} group, whereas the highest ratio was obtained from the control^{+}.

According to the findings related to starch content, the control^{-} group yielded the highest amounts of resistant starch (1.88%), non-resistant starch (61.02%), and total starch (62.90%). On the other hand, the control^{+} group (0.74%) and cinnamaldehyde application (0.72%) had the lowest resistant starch, while the control^{+} group (51.84) and

thymol application (51.68%) had the lowest non-resistant starch (51.68%) and total starch (52.54%). Similarly, the lowest total starch content was observed in control^{+} group (52.58%) and Thymol application (52.54%).

The lowest phytic acid values were recorded in the control^{-} group and thymol treatments, whereas the highest value was found in the control^{+} group. The Amylose content in wheat grains ranged from 16.91 % to 34.42%, with the highest value observed in the Control^{-} group and the lowest in the control^{+} group. The samples' amylopectin ratios ranged from 65.69% (control^{-}) to 83.09% (control^{+}).

Table 1. Biochemical composition of wheat grains harvested from the treatments infected with the FHB disease in pre-application.

Treatments	CA	CP	CO	TDF	RS	NRS	TS	Phy	Amylos	Amylopec
Control^{+}	3.67^{a}	18.51^{a}	1.90^{c}	5.52^{a}	0.74^{e}	51.84^{e}	52.58^{e}	1.66^{a}	16.91^{f}	83.09^{a}
Control^{-}	3.71^{a}	11.67^{f}	2.07^{a}	2.90^{g}	1.12^{d}	57.82^{a}	58.94^{a}	1.15^{e}	33.08^{a}	66.98^{d}
Cinnamaldehyde	2.61^{e}	11.83^{f}	1.81^{d}	3.61^{d}	1.79^{a}	51.63^{e}	53.42^{de}	1.24^{d}	22.78^{e}	77.49^{b}
Thymol	3.61^{ab}	12.75^{d}	2.07^{a}	3.41^{ef}	0.18^{f}	53.33^{cde}	53.51^{de}	1.21^{d}	23.12^{e}	77.15^{b}
Carvacrol	3.50^{bc}	12.57^{d}	2.00^{b}	3.30^{f}	0.17^{fg}	53.67^{c}	53.83^{de}	1.23^{d}	31.30^{b}	68.80^{d}
Carvone	3.48^{c}	12.05^{ef}	1.98^{b}	3.50^{de}	1.57^{b}	53.47^{cd}	55.04^{cd}	1.31^{c}	26.94^{d}	73.11^{c}
Safraleine	3.31^{d}	12.37^{de}	1.87^{c}	3.51^{de}	1.47^{c}	51.55^{e}	53.02^{e}	1.20^{d}	22.57^{e}	77.76^{b}
Topguard	3.51^{bc}	13.89^{c}	1.91^{c}	4.01^{c}	0.16^{fg}	56.01^{b}	56.16^{bc}	1.52^{b}	28.45^{c}	71.82^{c}
Actigard	3.61^{ab}	15.01^{b}	2.00^{b}	4.41^{b}	0.12^{g}	57.43^{ab}	57.55^{ab}	1.34^{c}	26.90^{d}	73.40^{c}
Sg Dg	**	**	**	**	**	**	**	**	**	**
LSD	0.12	0.49	0.06	0.12	0.05	1.79	1.81	0.04	0.83	2.42

Control^{+}: control (with pathogen); **Control^{-}:** control (without pathogen); **CA:** crude ash; **CP:** crude protein; **CO:** crude oil; **TDF:** total dietary fiber; **RS:** resistant starch; **NRS:** non-resistant starch; **TS:** total starch; **Phy:** phytic acid; **Amylos:** amylose; **Amyl:** amylopectin; **Sg Dg:** significant degree; **LSD:** least significant differences; ****:** $p \leq 0.01$.

Table 2. Biochemical composition of wheat grains harvested from the treatments infected with the FHB disease in one-application.

Treatments	CA	CP	CO	TDF	RS	NRS	TS	Phy	Amylos	Amylopec
Control^{+}	3.67^{a}	18.51^{a}	1.90^{a}	5.52^{a}	0.74^{f}	51.84^{d}	52.58^{e}	1.66^{a}	16.91^{g}	83.09^{a}
Control^{-}	2.17^{de}	10.49^{e}	1.12^{g}	1.72^{f}	1.89^{a}	59.63^{a}	61.52^{a}	1.23^{e}	30.57^{a}	69.43^{g}
Cinnamaldehyde	2.35^{cde}	13.93^{b}	1.22^{f}	1.83^{de}	1.06^{d}	53.67^{c}	54.73^{cd}	1.30^{d}	24.66^{d}	75.34^{d}
Thymol	3.08^{b}	11.01^{de}	1.58^{c}	2.13^{b}	0.91^{e}	55.01^{c}	55.92^{cd}	1.23^{e}	24.53^{de}	75.47^{cd}
Carvacrol	2.68^{bc}	11.83^{c}	1.76^{b}	1.76^{ef}	0.72^{f}	53.70^{c}	54.42^{d}	1.49^{c}	23.92^{ef}	76.08^{bc}
Carvone	2.11^{e}	11.33^{cd}	1.18^{f}	1.93^{c}	0.92e	54.72^{c}	56.09^{c}	1.27^{de}	23.38^{f}	76.62^{b}
Safraleine	2.72^{bc}	10.77^{de}	1.22^{f}	1.76^{ef}	1.36^{c}	54.30^{c}	55.22^{cd}	1.52^{bc}	29.27^{b}	70.73^{f}
Topguard	2.54^{cd}	13.54^{b}	1.32^{e}	1.90^{cd}	1.56^{b}	58.04^{b}	59.60^{b}	1.29^{d}	28.47^{c}	71.53^{e}
Actigard	2.70^{bc}	14.06^{b}	1.48^{d}	1.76^{ef}	1.09^{d}	57.68^{b}	58.77^{b}	1.57^{b}	24.54^{de}	75.46^{cd}
Sg Dg	**	**	**	**	**	**	**	**	**	**
LSD	0.42	0.59	0.05	0.08	0.06	1.50	1.53	0.05	0.71	0.71

Control^{+}: control (with pathogen); **Control^{-}:** control (without pathogen); **CA:** crude ash; **CP:** crude protein; **CO:** crude oil; **TDF:** total dietary fiber; **RS:** resistant starch; **NRS:** non-resistant starch; **TS:** total starch; **Phy:** phytic acid; **Amylos:** amylose; **Amyl:** amylopectin; **Sg Dg:** significant degree; **LSD:** least significant differences; ****:** $p \leq 0.01$.

Table 3. Biochemical composition of wheat grains harvested from the treatments infected with the FHB disease in post-application.

Treatments	CA	CP	CO	TDF	RS	NRS	TS	Phy	Amylos	Amylopec
Control^{+}	3.67^{a}	18.51^{a}	1.90^{c}	5.52^{a}	0.74^{f}	51.84^{g}	52.58^{g}	1.66^{a}	16.91^{g}	83.09^{a}
Control^{-}	3.60^{ab}	11.33^{e}	2.12^{a}	2.71^{g}	1.88^{a}	61.02^{a}	62.90^{a}	1.06^{e}	34.42^{a}	65.69^{f}
Cinnamaldehyde	3.03^{d}	12.24^{d}	1.55^{e}	3.34^{e}	0.72^{f}	54.92^{de}	55.65^{de}	1.20^{d}	25.01^{de}	76.19^{bc}
Thymol	3.31^{c}	12.01^{d}	1.67^{d}	3.62^{d}	0.86^{e}	51.68^{g}	52.54^{g}	1.08^{e}	22.42^{f}	78.01^{b}
Carvacrol	2.59^{f}	12.28^{d}	1.55^{e}	3.40^{e}	1.61^{b}	53.53^{ef}	55.14^{ef}	1.31^{b}	22.04^{f}	78.19^{b}
Carvone	2.71^{e}	11.51^{e}	1.87^{c}	3.01^{f}	0.93^{d}	52.71^{fg}	53.63^{fg}	1.18^{d}	24.55^{e}	75.71^{cd}
Safraleine	3.21^{c}	12.25^{d}	1.47^{f}	3.61^{d}	1.29^{c}	58.11^{b}	59.40^{b}	1.20^{d}	25.40^{d}	74.92^{cd}
Topguard	3.50^{b}	13.18^{c}	2.02^{b}	4.00^{c}	0.90^{de}	56.78^{bc}	57.69^{c}	1.22^{d}	26.55^{c}	73.53^{de}
Actigard	3.51^{b}	14.01^{b}	2.07^{ab}	4.91^{b}	0.91^{de}	56.01^{cd}	56.92^{cd}	1.27^{c}	27.62^{b}	72.56^{e}
Sg Dg	**	**	**	**	**	**	**	**	**	**
LSD	0.10	0.46	0.06	0.11	0.05	1.68	1.71	0.04	0.77	2.19

Control^{+}: control (with pathogen); **Control^{-}:** control (without pathogen); **CA:** crude ash; **CP:** crude protein; **CO:** crude oil; **TDF:** total dietary fiber; **RS:** resistant starch; **NRS:** non-resistant starch; **TS:** total starch; **Phy:** phytic acid; **Amylos:** amylose; **Amyl:** amylopectin; **Sg Dg:** significant degree; **LSD:** least significant differences; ****:** $p \leq 0.01$.

3.4.1. Mineral Contents

The mean values of the mineral content of grains obtained from wheat plants treated with pure components and synthetic pesticides in pre-, one-, and post-application

cycles, respectively, are given in Tables 4, 5, and 6. In all three application cycles, the effect of tested pure components and pesticides on mineral matter content was found statistically significant ($p \leq 0.01$). The highest Ca content (999.38 ppm), K content (8059.57 ppm), Mg content (2585.11 ppm), Na content (342.61 ppm), P content (7819.79 ppm), S content (1963.70 ppm), Cu content (6.63 ppm), Mn content (82.09 ppm), Zn content (115.70 ppm), B content (1.70 ppm) and Ni content (1.12 ppm) were obtained from pre-application.

Based on the results, the lowest Ca content was observed in the treatment of Topguard (617.35 ppm), K content in the control$^-$ group (2785.06 ppm), Mg content in the cinnamaldehyde (1340.70 ppm), Na content in control$^-$ group (134.10 ppm), P content in control$^-$ group (4442.44 ppm) and cinnamaldehyde treatment (4279. 66 ppm), S content in safraleine (831.65 ppm) and Topguard treatments (805.10 ppm), Cu content in control$^-$ group (4.92 ppm), Mn content in cinnamaldehyde application (37.33 ppm), Zn content (55.24 ppm) and Boron content (0.40 ppm) in control$^-$ group and Ni content (0.26 ppm) in Safraleine application. The highest Fe content was obtained from control$^-$ group with 105.89 ppm and thymol application with 108.82 ppm, while the lowest values were obtained from Topguard application with 77.66 ppm and Actigard application with 78.42 ppm.

The mineral content of the wheat grains harvested from the treatments in one-application was provided in Table 5. The highest mineral content was observed in the wheat sample of control$^+$. The highest content of Ca, K, Mg, Na, P, and S was determined as 999.38, 8059.57, 2585.11, 342.61, 7819.79, and 1963.70 ppm, respectively, whereas the amount of the Fe, Mn, Zn, B, and Ni were 90.18, 82.09, 115.70, 1.71, and 1.12 ppm,

respectively. Interestingly, although the highest content of Cu was seen in the application of thymol with 17.20 ppm, the minimum amount of Cu was in the application of carvone with 5.56 ppm. The lowest amount of Ca and K were quantified in the wheat sample of control$^-$ with 745.94 ppm and 3465.3 ppm. Additionally, the least amount of Mg was measured in the applications of cinnamaldehyde (1341.10 ppm) and carvone (1380.21 ppm), similarly, the amount of P was lowest in the applications of cinnamaldehyde (4287.36 ppm) and carvone (4252.26 ppm). Furthermore, the lowest amount of Na was found in the applications of carvacrol (246.44 ppm), carvone (241.88 ppm), and Actigard (244.69 ppm), while the amount of S was in the carvone application with 1265.22 ppm. The content of Fe was the lowest in the applications of thymol, carvone, and safraleine with 42.09 ppm, 42.54 ppm, and 42.84 ppm, respectively, the Mn content in the application of cinnamaldehyde with 45.55 ppm, the Zn content in the safraleine application with 63.08 ppm, the B content in the Topguard application with 0.05 ppm, and the Ni content in the application of carvone with 0.38 ppm.

In the post-application, similar to the pre-application, the highest values in all mineral contents except Fe were obtained from the control$^+$ application, while the highest Fe content was obtained from the treatment of carvone. According to findings, the lowest Ca content was observed in the contol$^-$ group (618.72 ppm) and in the treatment of carvone (645.89 ppm), K content in Topguard (2523.41 ppm), Mg content in thymol application (1216. 34 ppm), Na content in control$^-$ group (125.43 ppm), P content in the treatment of thymol (3849.83 ppm). S content was lowest in Topguard application (868. 38 ppm), while Cu content was in Actigard with 4.80 ppm. The content of Fe was lowest in the treatment of Actigard with 81.49 ppm, however, Mn content was in Topguard

application with 35.48 ppm. Interestingly, Zn content was lowest in Thymol application (47.55 ppm), B content (0.41 ppm) and Ni content (0.26 ppm) was in Control^{-} group.

Table 4. Mineral content of wheat grains from the treatments infected with the FHB disease in pre-application.

Treatments	Macro Minerals (ppm)					
	Ca	K	Mg	Na	P	S
Control^{+}	999.38^{a}	8059.57^{a}	2585.11^{a}	342.61^{a}	7819.79^{a}	1963.70^{a}
Control^{-}	742.09^{e}	2785.06^{g}	1469.58^{f}	134.10^{f}	4442.44^{g}	1327.81^{b}
Cinnamaldehyde	881.63^{c}	3653.57^{e}	1340.70^{g}	201.12^{b}	4279.66^{g}	873.58^{e}
Thymol	867.44^{c}	4335.45^{d}	1769.79^{d}	185.87^{c}	5157.40^{e}	997.13^{d}
Carvacrol	963.74^{b}	6228.37^{b}	2170.98^{b}	197.39^{b}	6358.27^{b}	1144.75^{c}
Carvone	792.30^{d}	5020.74^{c}	2029.27^{c}	170.50^{d}	5928.62^{c}	910.02^{e}
Safraleine	653.60^{f}	3335.83^{f}	1627.80^{e}	164.03^{de}	4848.17^{f}	831.65^{f}
Topguard	617.35^{g}	4394.03^{d}	1766.12^{d}	160.82^{e}	5567.52^{d}	805.10^{f}
Actigard	631.21^{fg}	4393.47^{d}	1810.43^{d}	161.89^{e}	5444.10^{d}	905.40^{e}
Sg Dg	**	**	**	**	**	**
LSD	31.28	172.69	66.08	6.96	199.39	39.29
Treatments	Micro Minerals (ppm)					
	Cu	Fe	Mn	Zn	B	Ni
Control^{+}	6.63^{a}	90.18^{c}	82.09^{a}	115.70^{a}	1.70^{a}	1.12^{a}
Control^{-}	4.92^{f}	105.89^{a}	43.62^{f}	55.24^{g}	0.40^{e}	0.28^{g}
Cinnamaldehyde	5.19^{e}	95.20^{b}	37.33^{g}	60.36^{f}	0.44^{bc}	0.35^{f}
Thymol	5.47^{b}	108.82^{a}	47.18^{d}	73.69^{c}	0.42^{cd}	0.44^{e}
Carvacrol	6.12^{b}	97.36^{b}	55.16^{b}	82.53^{b}	0.45^{b}	0.48^{d}
Carvone	6.24^{b}	83.19^{d}	49.75^{c}	80.35^{b}	0.41^{de}	0.52^{c}
Safraleine	5.56^{c}	79.80^{de}	46.61^{de}	70.87^{d}	0.42^{cd}	0.26^{h}
Topguard	5.36^{cde}	77.66^{e}	46.14^{def}	58.45^{f}	0.46^{b}	0.48^{d}
Actigard	5.28^{de}	78.42^{e}	44.97^{ef}	68.12^{e}	0.46^{b}	0.58^{b}
Sg Dg	**	**	**	**	**	**
LSD	0.21	3.45	1.75	2.64	0.02	0.02

Control^{+}: control (with pathogen); **Control^{-}:** control (without pathogen); **Sg Dg:** significant degree; **LSD:** least significant differences; ****:** $p \leq 0.01$.

Table 5. Mineral content of wheat grains from the treatments infected with the FHB disease in one-application.

Treatments	Macro Minerals (ppm)					
	Ca	K	Mg	Na	P	S
Control^{+}	999.38^{a}	8059.57^{a}	2585.11^{a}	342.61^{a}	7819.79^{a}	1963.70^{a}
Control^{-}	745.94^{g}	3465.30^{f}	1447.28^{e}	265.85^{b}	4615.88^{cd}	1317.37^{de}
Cinnamaldehyde	910.74^{c}	3982.62^{e}	1341.10^{f}	264.32^{b}	4287.36^{e}	1367.23^{b}
Thymol	892.28^{cd}	4409.97^{bc}	1593.23^{c}	255.19^{c}	4756.21^{c}	1321.07^{de}
Carvacrol	840.73^{e}	4285.93^{c}	1512.40^{d}	246.44^{d}	4748.54^{c}	1300.22^{ef}
Carvone	785.99^{f}	3592.31^{f}	1380.21^{f}	241.88^{d}	4252.26^{e}	1265.22^{f}
Safraleine	854.79^{e}	4134.73^{d}	1429.20^{e}	262.73^{bc}	4535.27^{d}	1355.38^{cd}
Topguard	880.89^{d}	4092.19^{de}	1604.91^{c}	266.44^{b}	5077.31^{b}	1287.48^{ef}
Actigard	965.27^{b}	4522.15^{b}	1662.90^{b}	244.69^{d}	5043.94^{b}	1420.94^{b}
Sg Dg	**	**	**	**	**	**
LSD	29.24	134.20	47.20	7.82	148.55	40.83
Treatments	Micro Minerals (ppm)					
	Cu	Fe	Mn	Zn	B	Ni
Control^{+}	6.63^{b}	90.18^{a}	82.09^{a}	115.70^{a}	1.71^{a}	1.12^{a}
Control^{-}	5.77^{e}	48.69^{c}	58.34^{b}	74.57^{c}	0.57^{b}	0.39^{e}
Cinnamaldehyde	6.24^{c}	47.85^{cd}	45.55^{g}	63.26^{f}	0.44^{d}	0.39^{e}
Thymol	17.20^{a}	42.09^{e}	51.10^{e}	72.87^{c}	0.18^{g}	0.54^{c}
Carvacrol	5.73^{ef}	49.05^{c}	57.68^{bc}	66.61^{de}	0.23^{f}	0.53^{c}
Carvone	5.56^{f}	42.54^{e}	47.87^{f}	67.37^{d}	0.49^{c}	0.38^{e}
Safraleine	6.21^{cd}	42.84^{e}	48.26^{f}	63.08^{f}	0.41^{e}	1.03^{b}
Topguard	6.01^{d}	46.85^{d}	56.49^{c}	64.91^{ef}	0.05^{h}	0.45^{d}
Actigard	6.37^{c}	51.37^{b}	53.85^{d}	79.27^{b}	0.20^{g}	0.53^{c}
Sg Dg	**	**	**	**	**	**
LSD	0.20	1.57	1.68	2.19	0.02	0.02

Control^{+}: control (with pathogen); **Control^{-}:** control (without pathogen); **Sg Dg:** significant degree; **LSD:** least significant differences; ****:** $p \leq 0.01$.

Table 6. Mineral content of wheat grains from the treatments infected with the FHB disease in post-application.

Treatments	Macro Minerals (ppm)					
	Ca	K	Mg	Na	P	S
Control^{+}	999.38^{a}	8059.57^{a}	2585.11^{a}	342.61^{a}	7819.79^{a}	1963.70^{a}
Control^{-}	618.72^{f}	4084.25^{c}	1601.99^{b}	125.43^{e}	4146.69^{d}	1172.69^{b}
Cinnamaldehyde	678.80^{e}	4103.03^{c}	1513.61^{c}	141.62^{cd}	4866.24^{c}	996.49^{c}
Thymol	724.63^{bcd}	3202.24^{e}	1216.34^{f}	146.64^{c}	3849.83^{e}	910.55^{d}
Carvacrol	739.48^{b}	4172.54^{bc}	1623.91^{b}	160.07^{b}	5169.21^{b}	981.52^{c}
Carvone	645.89^{f}	3430.75^{d}	1397.69^{d}	138.25^{d}	4382.58^{d}	876.89^{d}
Safraleine	706.68^{cde}	4368.46^{b}	1644.51^{b}	154.86^{b}	5082.56^{bc}	991.41^{c}
Topguard	732.59^{bc}	2523.41^{f}	1302.60^{e}	160.12^{b}	4361.95^{d}	868.38^{d}
Actigard	698.64^{de}	3149.39^{e}	1365.04^{de}	143.73^{cd}	5077.87^{bc}	884.96^{d}
Sg Dg	**	**	**	**	**	**
LSD	31.79	197.56	74.81	7.81	236.18	49.90
Treatments	Micro Minerals (ppm)					
	Cu	Fe	Mn	Zn	B	Ni
Control^{+}	6.63^{a}	90.18^{c}	82.09^{a}	115.70^{a}	1.70^{a}	1.12^{a}
Control^{-}	5.02^{ef}	84.92^{d}	49.21^{b}	66.87^{c}	0.41^{c}	0.26^{h}
Cinnamaldehyde	5.31^{cd}	91.71^{bc}	44.84^{c}	65.00^{c}	0.46^{b}	0.40^{f}
Thymol	5.39^{bc}	85.38^{d}	49.86^{b}	47.55^{f}	0.45^{b}	0.42^{ef}
Carvacrol	5.56^{bc}	93.95^{bc}	48.53^{b}	70.53^{b}	0.44^{b}	0.46^{d}
Carvone	5.08^{de}	105.33^{a}	38.17^{d}	59.22^{d}	0.44^{b}	0.49^{c}
Safraleine	5.58^{b}	95.70^{b}	44.08^{c}	57.20^{d}	0.45^{b}	0.32^{g}
Topguard	4.83^{ef}	93.03^{bc}	35.48^{e}	53.61^{e}	0.44^{b}	0.44^{de}
Actigard	4.80^{f}	81.49^{d}	38.88^{d}	57.01^{d}	0.45^{b}	0.51^{b}
Sg Dg	**	**	**	**	**	**
LSD	0.25	4.27	2.23	3.09	0.03	0.02

Control^{+}: control (with pathogen); **Control^{-}:** control (without pathogen); **Sg Dg:** significant degree; **LSD:** least significant differences; ****:** $p \leq 0.01$.

3.4.2. Pasting Properties

The mean values of the pasting properties analyses of samples from the treatments in pre-application are given in Figure 5. Regarding the findings, the peak viscosity values of the flour samples varied between 217 cP and 1171 cP, the lowest peak viscosity was obtained from the samples of the Control^{+} group (with pathogen), while the highest peak viscosity was obtained from the samples of the Control^{-} group (without pathogen). The lowest through viscosity (93 cP), final viscosity (189 cP), and setback (96 cP) values

were obtained from the Topguard application, whereas the highest values were obtained from the Control- group as 797 cP, 1043 cP, and 606 cP, respectively.

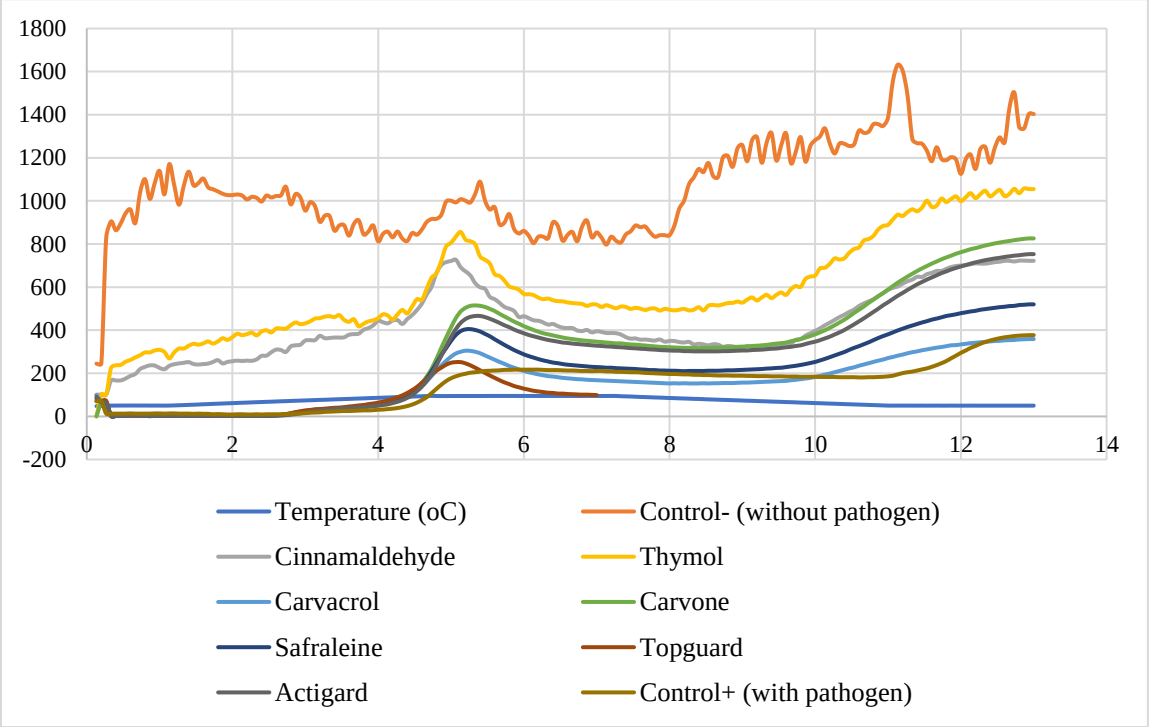

Figure 5. Rapid Visco Analyzer (RVA) pasting curves of wheat grains obtained from the various treatments in pre-applications under exposure to FHB disease.

The pasting properties of the samples and calculated averaged pasting parameters of samples from the treatments in one-application were given in Figure 6. As is seen from the Figure, peak viscosity values were in the range of 217-1235 cP while the highest PV value was recorded for the control sample with no pathogen and the lowest PV was for the control sample infected with pathogen. Trough values of the samples also changed parallelly to the PV of the samples and the lowest trough value (181 cP) was for the infected control sample with pathogen and the highest value was recorded for the sample of control with no pathogen. For the samples treated with the pesticide and essential oils, carvone treated sample showed the lowest trough, and the Topguard-treated sample showed the highest trough value among the samples. Breakdown is the difference

between peak viscosity and holding strength known as also the lowest viscosity. The pathogen-infected control sample had the lowest breakdown (36 cP) value while the highest was in the control sample with no pathogen. The treated samples with essential oil or pesticide exhibited similar breakdown but all treated samples showed higher breakdown values compared to the control group infected with pathogen. Final viscosity values of the samples also ranged between 377-1466 cP and the highest final viscosity value was monitored for the sample treated with carvacrol and the lowest final viscosity was determined for the control sample infected with pathogen. A similar trend was also recorded for the setback values and the setback values were in the range of 196-960 cP. As is seen from the Figure, the control sample infected with the pathogen displayed the lowest setback while the highest setback value was measured for the sample treated with carvacrol.

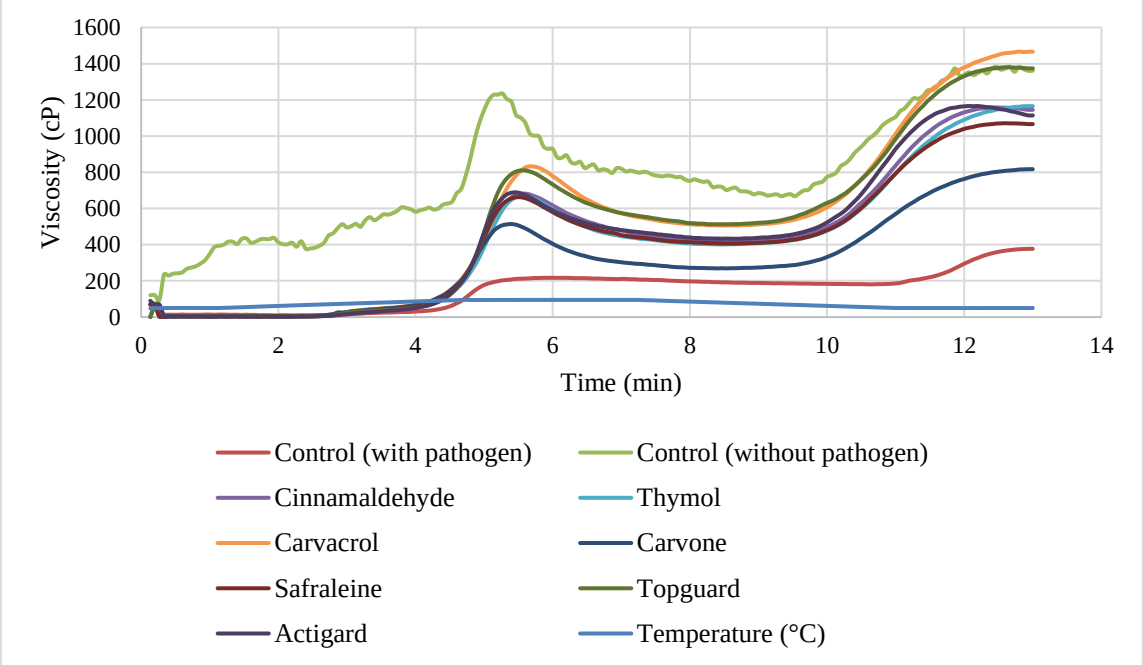

Figure 6. Rapid Visco Analyzer (RVA) pasting curves of wheat grains obtained from the various treatments in one-application under exposure to FHB disease.

The pasting properties of the samples and calculated averaged pasting parameters of samples from the treatments in post-application were given in Figure 7. Based on the results, the peak viscosity values of the samples varied between 217-1046 cP. The lowest pasting properties value was obtained from the Control^{+} group (with pathogen) and the highest value was obtained from the Control^{-} group (without pathogen). The lowest through viscosity value of the samples was observed in the Cinnemaldahyde application with 109 cP and the highest value was observed in the Control^{-} group (without pathogen) treatment with 562 cP.

The sample from the Control^{+} group (infected with FHB) had the lowest breakage value (36 cP), while the highest value was obtained from the Control^{-} group (484 cP). The final viscosity values of the samples varied between 256 cP (Cinnemaldahyde application) and 1097 cP (Control^{-} group). Similarly, the lowest value in Setback was 147 cP for the grains from the Cinnemaldahyde application, and the highest value was 535 cP for the grains from the Control^{-} group.

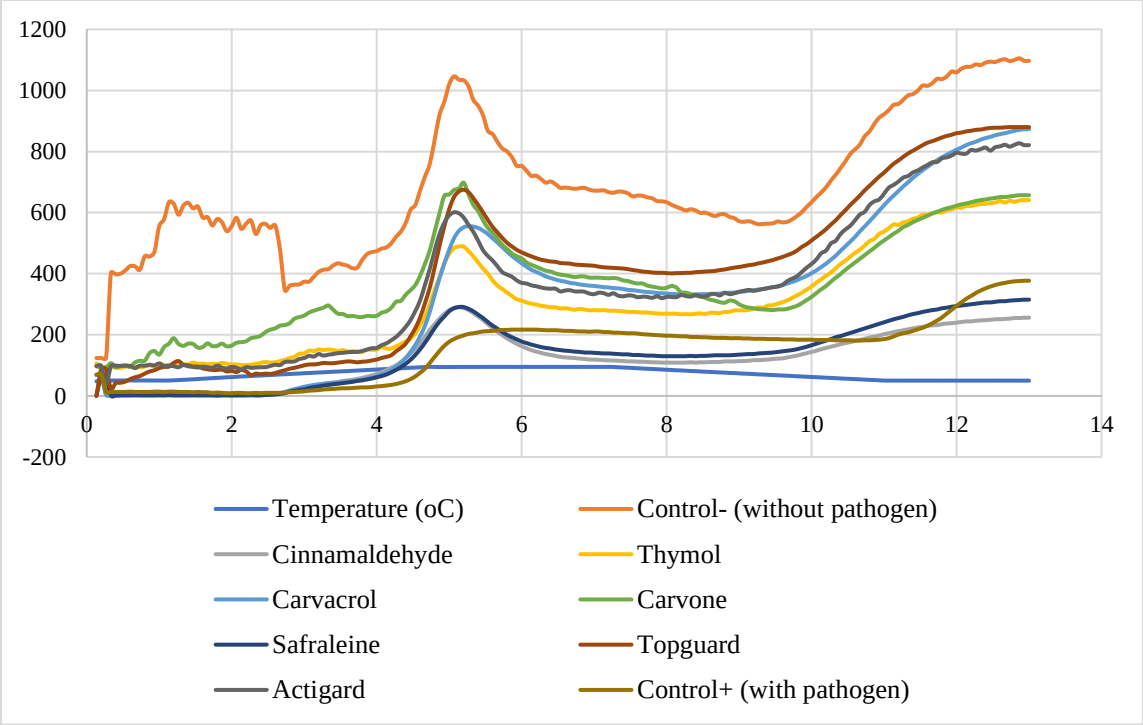

Figure 7. Rapid Visco Analyzer (RVA) pasting curves of wheat grains obtained from the various treatments in post-applications under exposure to FHB disease.

3.4.3. Amino Acid Analysis

Table 7 enlists the amino acid contents of the pre-application treatments of wheat plants infected with FHB. It was observed that the essential oil application had a statistically significant effect ($p \leq 0.01$) on the amino acid content during the pre-application period. The highest alanine (48.33 mg/g), aspartic acid (79.60 mg/g), histidine (14.36 mg/g), lysine (7.69 mg/g), proline (104.63 mg/g), serine (43.22 mg/g), threonine (31.82 mg/g), tyrosine (6.22 mg/g), total amino acid content (790.35 mg/g) were obtained from control^{+} treatment, while the highest glutamine (10.95 mg/g), glutamic acid (441.95 mg/g), histidine (14.74 mg/g) and total amino acid content (803.46 mg/g) were observed in cinnamaldehyde treatment. In addition, the highest leucine (26.75 mg/g), arginine (16.32 mg/g), glutamine (11.25 mg/g), isoleucine (43.32 mg/g), methionine (7.21 mg/g) and valine content (16.67 mg/g) were obtained from carvacrol treatment, while the

highest cysteine (4.39 mg/g), glutamic acid (443.16 mg/g) and total amino acid content (791.73 mg/g) were observed from safraleine treatment. The Topguard treatment showed the highest serine content (42.96 mg/g), whereas the Actigard treatment displayed the highest aspartic acid content (80.63 mg/g) and phenylalanine content (18.02 mg/g). The control^{-} treatment had the lowest total amino acid content.

In the study conducted to examine the effects of different pure components and chemical pesticide treatments in one-application on wheat exposed to FHB disease, the average values of the amino acid contents of wheat grains were demonstrated in Table 8. The effect of applications was identified as statistically highly significant for the amino acid content of the samples ($p \leq 0.01$). The highest content of alanine (48.33 mg/g), arginine (15.31 mg/g), aspartic acid (79.60 mg/g), glutamine (8.51 mg/g), histidine (14.36 mg/g), lysine (7.69 mg/g), methionine (5.64 mg/g), and proline (104.63 mg/g) was seen in the wheat grains of control^{+}, the content of glutamic acid (485.54 mg/g) in the application of carvone, the content of leucine (18.74 mg/g), and threonine (33.09 mg/g) in the application of safraleine, and the content of cysteine (4.70 mg/g), isoleucine (29.66 mg/g), phenylalanine (16.26 mg/g), serine (46.23 mg/g), tyrosine (7.14 mg/g), and valine (12.60 mg/g) in the application of Topguard (Table 8). The lowest amount of total amino acid was obtained in the application of Actigard with 636.59 mg/g although the highest was in the application of safraleine with 900.32 mg/g.

The amino acid contents of wheat plants infected with FHB in post-application treatments are listed in Table 9. The essential oil treatment had a statistically significant effect on amino acid contents ($p \leq 0.01$). Among the treatments, control^{+} had the highest content of aspartic acid (80.34 mg/g), glutamine (8.51 mg/g), glutamic acid (355.40

mg/g), histidine (14.36 mg/g), lysine (7.69 mg/g), methionine (5.64 mg/g), proline (104.63 mg/g), tyrosine (6.22 mg/g), and total amino acid content (791.08 mg/g). On the other hand, control$^-$ had the highest cysteine (4.03 mg/g) and isoleucine (38.98 mg/g) contents. Carvacrol treatment showed the highest leucine (24.40 mg/g), alanine (79.93 mg/g), arginine (15.77 mg/g), aspartic acid (79.93 mg/g), isoleucine (39.53 mg/g), valine (15.91 mg/g), and total amino acid (786.82 mg/g) contents. Moreover, the Carvone treatment had the highest glutamine content (8.57 mg/g), while the Safraleine treatment had the highest phenylalanine (17.55 mg/g) and serine (44.15 mg/g) contents. Topguard treatment had the highest serine (45.08 mg/g) content, while Actigard treatment had the highest arginine (15.89 mg/g) and threonine (35.19 mg/g) contents. The control$^-$ treatment resulted in the lowest total amino acid content of 562.64 mg/g.

Table 7. Amino acid composition of wheat grains collected from the treatments in pre-application exposed to the FHB disease.

Amino Acids (mg/g)	Control^{+}	Control^{-}	Cinnamaldehyde	Thymol	Carvacrol	Carvone	Safraleine	Topguard	Actigard	Sg Dg	LSD
Leucine	17.27^{d}	16.04^{e}	18.25^{c}	19.50^{b}	26.75^{a}	13.98^{f}	16.37^{e}	14.62^{f}	20.04^{b}	**	0.733
Alanine	48.33^{a}	30.45^{ef}	29.28^{f}	31.16^{e}	34.22^{cd}	34.18^{cd}	35.49^{c}	38.05^{b}	33.00^{d}	**	1.326
Arginine	15.31^{b}	10.52^{f}	15.43^{b}	11.07^{de}	16.32^{a}	11.42^{d}	11.32^{d}	10.68^{ef}	11.94^{c}	**	0.486
Aspartic acid	79.60^{a}	62.75^{c}	68.55^{b}	71.30^{b}	55.13^{d}	70.22^{b}	64.03^{c}	71.09^{b}	80.63^{a}	**	2.781
Cysteine	3.55^{c}	2.67^{f}	3.42^{cd}	3.29^{d}	2.94^{e}	3.81^{b}	4.39^{a}	2.48^{g}	2.99^{e}	**	0.133
Glutamine	8.51^{e}	8.07^{f}	10.95^{a}	9.76^{b}	11.25^{a}	9.19^{c}	8.61^{de}	4.85^{g}	8.97^{cd}	**	0.366
Glutamic acid	355.40^{d}	248.73^{h}	441.95^{a}	324.00^{e}	381.58^{c}	395.46^{b}	443.16^{a}	305.09^{f}	290.42^{g}	**	13.8
Histidine	14.36^{a}	8.61^{f}	14.74^{a}	10.09^{d}	12.26^{b}	10.58^{c}	9.43^{e}	9.04^{e}	10.06^{d}	**	0.429
Isoleucine	24.68^{e}	26.57^{d}	32.37^{bc}	31.28^{c}	43.32^{a}	22.60^{f}	26.11^{d}	25.56^{de}	32.82^{b}	**	1.207
Lysine	7.69^{a}	4.24^{f}	5.77^{b}	4.79^{d}	4.51^{e}	5.36^{c}	4.25^{f}	4.52^{e}	4.55^{e}	**	0.192
Methionine	5.64^{cd}	5.03^{f}	5.38^{e}	5.44^{de}	7.21^{a}	4.50^{g}	4.98^{f}	6.35^{b}	5.81^{c}	**	0.221
Phenylalanine	12.22^{de}	11.60^{f}	11.66^{ef}	16.43^{b}	12.49^{d}	14.48^{c}	17.58^{a}	11.47^{f}	18.02^{a}	**	0.599
Proline	104.63^{a}	60.09^{e}	72.86^{c}	67.14^{d}	75.76^{b}	71.88^{c}	74.51^{bc}	67.64^{d}	60.41^{e}	**	2.725
Serine	43.22^{a}	31.33^{e}	28.64^{f}	26.43^{g}	33.74^{d}	31.45^{e}	39.48^{c}	42.96^{a}	40.86^{b}	**	1.357
Threonine	31.82^{a}	24.73^{d}	28.43^{b}	25.85^{c}	21.44^{e}	19.48^{f}	20.52^{e}	19.34^{f}	18.10^{g}	**	0.935
Tyrosine	6.22^{a}	3.89^{d}	4.18^{c}	3.68^{e}	5.59^{b}	3.39^{f}	3.85^{d}	4.27^{c}	4.22^{c}	**	0.164
Valine	11.89^{b}	7.31^{ef}	11.60^{b}	9.47^{c}	16.67^{a}	6.67^{g}	7.65^{e}	7.01^{fg}	8.79^{d}	**	0.384
Total Amino Acids	790.35^{a}	562.64^{e}	803.46^{a}	670.72^{d}	761.18^{b}	728.66^{c}	791.73^{a}	645.01^{d}	651.61^{d}	**	26.005

Control^{+}: control (with pathogen); **Control^{-}:** control (without pathogen); **Sg Dg:** significant degree; **LSD:** least significant differences; ****:** $p \leq 0.01$.

Table 8. Amino acid composition of wheat grains collected from the treatments in one-application exposed to the FHB disease.

Amino Acids (mg/g)	Control^{+}	Control^{-}	Cinnamaldehyde	Thymol	Carvacrol	Carvone	Safraleine	Topguard	Actigard	Sg Dg	LSD
Leucine	17.27^{c}	17.99^{b}	14.58^{f}	15.48^{e}	15.42^{e}	14.97^{f}	18.74^{a}	18.33^{b}	16.14^{d}	**	0.41
Alanine	48.33^{a}	38.11^{e}	38.08^{e}	38.58^{e}	36.54^{f}	44.84^{b}	45.37^{b}	43.52^{c}	39.89^{d}	**	1.02
Arginine	15.31^{a}	13.44^{b}	12.89^{c}	12.61^{cd}	12.30^{d}	12.89^{c}	13.74^{b}	13.68^{b}	12.51^{d}	**	0.33
Aspartic acid	79.60^{a}	45.67^{f}	54.30^{e}	56.95^{d}	45.31^{f}	61.69^{c}	69.34^{b}	58.02^{d}	46.58^{f}	**	1.45
Cysteine	3.55^{e}	3.68^{d}	3.85^{c}	4.02^{b}	3.36^{f}	3.20^{g}	2.73^{h}	4.70^{a}	3.69^{d}	**	0.10
Glutamine	8.51^{a}	6.12^{e}	6.48^{c}	6.27^{de}	5.70^{f}	6.36^{cd}	6.68^{b}	6.41^{cd}	6.44^{cd}	**	0.16
Glutamic acid	355.40^{e}	371.22^{d}	356.64^{e}	387.37^{c}	377.11^{d}	485.54^{a}	483.03^{a}	464.55^{b}	304.92^{f}	**	9.98
Histidine	14.36^{a}	13.22^{b}	11.46^{e}	10.77^{f}	10.86^{f}	11.19^{e}	12.42^{c}	11.99^{d}	10.39^{g}	**	0.30
Isoleucine	24.68^{d}	25.85^{c}	23.01^{g}	24.45^{de}	23.85^{ef}	24.13^{def}	28.50^{b}	29.66^{a}	23.75^{f}	**	0.63
Lysine	7.69^{a}	4.91^{d}	5.10^{c}	4.06^{f}	4.47^{e}	4.17^{f}	5.60^{b}	4.37^{e}	4.35^{e}	**	0.12
Methionine	5.64^{a}	4.93^{e}	5.59^{a}	5.16^{c}	4.15^{f}	5.31^{b}	4.98^{de}	5.35^{b}	5.09^{cd}	**	0.14
Phenylalanine	12.22^{e}	13.46^{c}	11.73^{f}	12.82^{d}	12.91^{d}	12.45^{e}	15.19^{b}	16.26^{a}	12.84^{d}	**	0.33
Proline	104.63^{a}	70.52^{h}	90.02^{c}	78.52^{e}	81.57^{d}	76.22^{f}	95.95^{b}	73.76^{g}	75.08^{fg}	**	2.07
Serine	43.22^{b}	38.36^{d}	35.73^{e}	39.26^{cd}	39.38^{c}	38.87^{cd}	46.10^{a}	46.23^{a}	32.36^{f}	**	0.10
Threonine	31.82^{b}	23.09^{f}	27.04^{d}	29.07^{c}	24.17^{e}	32.96^{a}	33.09^{a}	29.57^{c}	24.74^{e}	**	0.72
Tyrosine	6.22^{c}	6.15^{cde}	6.05^{de}	6.20^{cd}	6.00^{e}	6.13^{cde}	6.57^{b}	7.14^{a}	6.08^{cde}	**	0.16
Valine	11.89^{c}	9.48^{g}	9.19^{h}	10.24^{e}	10.58^{d}	9.90^{f}	12.28^{b}	12.60^{a}	11.72^{c}	**	0.27
Total Amino Acids	790.34^{c}	706.19^{e}	711.76^{e}	741.84^{d}	713.69^{e}	850.83^{b}	900.32^{a}	846.13^{b}	636.59^{f}	**	19.06

Control^{+}: control (with pathogen); **Control^{-}:** control (without pathogen); **Sg Dg:** significant degree; **LSD:** least significant differences; ****:** $p \leq 0.01$.

Table 9. Amino acid composition of wheat grains collected from the treatments in post-application exposed to the FHB disease.

Amino Acids (mg/g)	Control^{+}	Control^{-}	Cinnamaldehyde	Thymol	Carvacrol	Carvone	Safraleine	Topguard	Actigard	Sg Dg	LSD
Leucine	17.27^{e}	23.45^{b}	14.83^{g}	19.78^{d}	24.40^{a}	15.71^{f}	16.71^{e}	15.90^{f}	21.19^{c}	**	0.578
Alanine	48.33^{b}	31.78^{f}	38.90^{d}	26.01^{g}	79.93^{a}	19.94^{h}	33.98^{e}	34.92^{e}	46.18^{c}	**	1.072
Arginine	15.31^{b}	13.22^{e}	13.09^{e}	15.56^{ab}	15.77^{a}	14.44^{c}	13.97^{d}	14.42^{c}	15.89^{a}	**	0.328
Aspartic acid	80.34^{a}	59.67^{e}	77.79^{b}	52.02^{f}	79.93^{a}	59.83^{e}	67.96^{d}	69.84^{cd}	71.69^{c}	**	1.892
Cysteine	3.55^{c}	4.03^{a}	1.09^{h}	1.51^{f}	3.67^{b}	2.09^{e}	1.35^{g}	2.04^{e}	2.31^{d}	**	0.069
Glutamine	8.51^{a}	6.43^{e}	7.97^{b}	7.16^{c}	7.04^{c}	8.57^{a}	6.27^{e}	5.51^{f}	6.67^{d}	**	0.225
Glutamic acid	355.40^{a}	233.68^{f}	313.56^{d}	348.75^{ab}	329.15^{c}	272.64^{e}	318.64^{d}	265.21^{e}	341.58^{b}	**	9.646
Histidine	14.36^{a}	11.65^{f}	12.65^{cde}	13.86^{b}	12.91^{cd}	13.00^{c}	12.33^{e}	12.29^{e}	12.60^{de}	**	0.396
isoleucine	24.68^{f}	38.98^{a}	23.77^{f}	30.79^{c}	39.53^{a}	26.03^{e}	26.21^{e}	27.31^{d}	35.34^{b}	**	0.925
Lysine	7.69^{a}	4.49^{c}	5.21^{b}	4.26^{d}	3.76^{e}	4.29^{d}	3.58^{f}	4.44^{c}	4.45^{c}	**	0.135
Methionine	5.64^{a}	4.11^{d}	3.79^{e}	3.32^{g}	5.14^{b}	3.58^{f}	3.37^{g}	3.75^{e}	4.23^{c}	**	0.113
Phenylalanine	12.22^{e}	6.89^{g}	9.25^{f}	15.86^{c}	16.87^{b}	9.41^{f}	17.55^{a}	14.18^{d}	9.32^{f}	**	0.422
Proline	104.63^{a}	80.85^{b}	43.11^{f}	49.65^{e}	82.25^{b}	44.71^{f}	54.10^{d}	50.42^{e}	70.39^{c}	**	1.735
Serine	43.22^{bc}	29.17^{g}	30.46^{f}	28.68^{g}	33.27^{e}	35.47^{d}	44.15^{ab}	45.08^{a}	42.96^{c}	**	1.055
Threonine	31.82^{b}	28.52^{c}	27.68^{d}	26.69^{e}	31.37^{b}	22.10^{f}	18.16^{g}	25.89^{e}	35.19^{a}	**	0.804
Tyrosine	6.22^{a}	4.52^{c}	3.77^{e}	4.17^{d}	5.91^{b}	3.85^{e}	4.05^{d}	4.55^{c}	4.57^{c}	**	0.130
Valine	11.89^{b}	10.99^{c}	8.32^{f}	10.34^{d}	15.91^{a}	6.99^{g}	9.04^{e}	9.18^{e}	12.06^{b}	**	0.306
Total Amino Acids	791.08^{a}	592.43^{e}	635.25^{d}	658.42^{c}	786.82^{a}	562.67^{f}	651.40^{cd}	604.94^{e}	736.60^{b}	**	19.348

Control^{+}: control (with pathogen); **Control^{-}:** control (without pathogen); **Sg Dg:** significant degree; **LSD:** least significant differences; ****:** $p \leq 0.01$.

4. Discussion

The primary approach for FHB disease management in wheat fields is through the use of demethylation inhibitor (DMI) class (Group 3) fungicides, which are synthetic pesticides. While these pesticides are highly effective in controlling the disease, they cannot provide complete control when used alone. Moreover, the extensive use of synthetic pesticides belonging to the same group can result in the emergence of fungicide-resistant fungal strains in nature and can also be toxic to humans and other living organisms. These challenges urge researchers to find new alternative compounds to replace or rotate with the intensively applied conventional fungicides.

In recent years, the EOs and their major components (e.g., terpenes, terpenoids, and aromatic compounds) have been explored as alternatives to synthetic pesticides for the management of fungal threats due to being eco-friendly bioactive compounds, their low toxicity, and broad spectrum antifungal properties. A plethora of EOs originating from various plants have been reported for their high potential fungistatic and fungicidal activities against numerous plant pathogenic fungi. Amini et al. (2012) stated that in vitro applications of the EOs oils of *Zataria multiflora, Thymus vulgaris*, and *T. kotschyanus* at the concentration of 200 µl/l resulted in 100 % growth inhibition of four species of pytopathogenic fungi including *Pythium aphanidermatum, Rhizoctonia solani* (AG4), *F. graminearum* and *Sclerotinia sclerotiorum*. Krzyśko-Łupicka et al. (2019) showed that among the EOs of lemon, rosewood, geranium, and rosemary, and geranium EOs completely inhibited hyphal growth of the two *F. graminearum* isolates (ZALF 24 and ZALF 339) at the concentration of 0.125%. The essential oils of cinnamon and clove suppressed by 90 % the hyphal growth of *F. culmorum* and *F. verticillioides* at the

dosage of 300 ug/ml. In contrast, the EO of oregano was 100 % effective (Roselló et al., 2015). It was reported that all vegetative traits and total yield of common beans infected with *R. solani* significantly increased compared with the untreated control with the applications of cuminaldehyde, linalool, and carvone at the concentration of 100 ug/ml (Derbalah et al. 2022). The study conducted by Gill et al. (2016) displayed that the FHB index [= (total number of infected florets × Disease severity)/100] was significantly reduced with the application of 0.1% thymol emulsion to wheat heads infected with the FHB.

In our *in planta* assay, the tested five pure components, and two synthetic pesticides had efficient disease suppression performance with a significantly lowered infection compared to the untreated check infected with FHB. Comparing the treatments, Topguard was the most efficient in terms of the reducing the FHB infection in all application cycles, followed by the pure components safraleine, and carvacrol. However, it is worth noting that while the application of Topguard resulted in a similar infection rate to that of untreated wheat plants with no infection, safraleine and carvacrol showed a relatively high infection rate of around 40%. This was the first time safraleine, a synthetic ingredient commonly used in fragrances, was examined for its potential for suppressing plant fungal pathogens, and further studies are needed to understand its mechanism of action. Carvacrol, on the other hand, is a small hydrophobic molecule that performs powerful antifungal activities against various fungal pathogens. Its primary target is the fungal cell membrane, which damages the cell membrane integrity, leading to increased permeability and leakage of cytoplasmic content. Additionally, carvacrol disrupts intracellular mechanisms, including inhibition of ergosterol synthesis, which is a crucial

sterol component of cell and mitochondrial membranes in fungi. Although cinnamaldehyde, carvone, and thymol were highly effective in protecting wheat plants from FHB, their effectiveness was generally less than safraleine and carvacrol applications.

As FHB symptoms intensify, the fungus takes over the developing grain, reducing its size due to the shrinkage of grains and wrinkling within the head. This ultimately leads to significant losses in both yield and quality. Affected kernels typically display a rough and shriveled exterior, with colors ranging from soft gray and pink to light brown. In the present study, total grain weight was decreased over the 80 % in untreated control group inoculated with the FHB compared to the untreated control group with no infection. Topguard exhibited a strongest protection performance among the treatments with having almost similar total yield and hundred seed weight as untreated control group with no infection. Similarly, Paul et al. (2010) pointed out that triazoles, a class of chemical fungicides in the demethylation inhibitor (DMI) fungicide group causing hindrance in sterol biosynthesis, are considered one of the most effective group of fungicides suppressing FHB and leading to an increase in the total yield and decline in the mycotoxin level. According to D'Angelo et al. (2014), tebuconazole + prothioconazole (TEBU+PROT; Prosaro) and metconazole (METC; Caramba) were the two most efficient fungicides to control FHB and inhibit the production of DON when applied within one week of anthesis, resulting in a significant increase in yield. Following the Topguard, pre-application of safraleine and carvacrol for the pure components resulted in a higher total grain yield and hundred seed weight, parallel to the effects on the infection rate compared to the other treatments.

Deoxynivalenol (DON), a mycotoxin belonging to the class of trichothecenes, is produced by several *Fusarium* spp., acts as a virulence factor and is essential for symptom development after initial wheat infection. It can cause acute and chronic health problems in both humans and animals when contaminated grains or products produced from the contaminated grains consumed. In the study, relatively high level of DON was detected in the untreated wheat plants infected with FHB. However, although all treatments significantly decreased the concentration of DON in wheat grains compared to the untreated control group infected with FHB, only wheat samples harvested from Topguard resulted in the DON concentration under the threshold levels determined by the Food and Drug Administration of the United States (FDA). Although safraleine and carvacrol were highly effective in reducing the infection rate of FHB, the concentration of DON in the samples harvested from these treatments was relatively over the threshold limits specified for human consumption. Actigard was the least effective compound, resulting in the highest level of DON among the treatments.

In FHB-sensitive varieties, the overall amount of gluten and gluten subunits decreases while the quantity of DON (mycotoxins, primarily deoxynivalenol) increases. In resistant varieties, the defense mechanism is different because the protein composition is dependent on the activity of peroxidase (POD) as a detoxification agent (Santiago et al. 2019). Furthermore, the period and amount of infection are influenced by FHB's effect on grain quality (Edwards et al. 2017). FHB effects differ according to weather conditions and time of infection (Sforza et al. 2005). A reduction in the physical and chemical quality of the grain is associated with early infection. In particular, the beginning of the flowering and milk stages in wheat are at the highest risk for disease (Del Ponte et al.

2020). Delay in harvest time increases mycotoxin content (Schöneberg et al. 2018). These circumstances lead to disagreements among researchers.

FHB infection adversely affects the components of starch and proteins, deteriorating grain quality (Kreuzberger et al. 2015), and as a result, the quality characteristics of wheat products change. Due to the negative impacts of infection on the paste and cooking properties of wheat flour (Siuda et al. 2010; Lancova et al. 2016), bread quality is reduced. Impaired synthesis of fungal enzymes and grain components is reported to be responsible for changes in the biochemical structure of FHB-infected wheat, resulting in a change in its quality. During FHB infection, they secrete enzymes such as carbohydrases and proteases, thus degrading starch, cell wall components, and gluten proteins (Kreuzberger et al. 2015, Eggert et al. 2004). In moderately infected wheat, the protein amounts in the albumin and acetic acid extractable glutenin fraction are significantly reduced. Therefore, the loss of gluten strength is consistent with the observed decrease in the glutenin fraction. According to Bechtel et al. (1985), *F. graminearum* hyphae remove the protein matrix from aleurone cells and digest the storage protein. In addition, FHB infection can impede the complete accumulation of grain components due to mechanical blockage of vascular bundles caused by fungal mycelium or disruption of grain component synthesis resulting from mycotoxins (Kreuzberger et al. 2015). In this research, significant declines were noticed in nutritional qualities in FHB-infected samples, while those treated with chemicals and pure components retained better nutritional properties.

It has been reported that while there was a slight increase in the amount of protein in moderately infected wheat in FHB, there was a decrease in relatively mildly infected

wheat. In the study, samples infected with FHB were found to have a higher protein content. The *Fusarium* fungus may be partly responsible for the increased protein content, using certain carbohydrate components. Furthermore, the increase may be related to the protein obtained from *Fusarium* since hyphae grown in liquid culture have a crude protein content of approximately 42% (Boyacıloğlu and Hettiarachchy, 1995). It has been reported that the most noticeable effects caused by *F. graminearum* infection are the breakdown of cell walls in the starchy endosperm and certain parts of the endosperm itself. FHB utilizes proteins and starch to spread infection and cause disease, according to Brown et al. (2012).

A decrease in the amylose level of wheat with moderate infection is indicative of starch degradation. Alconada et al. (2021) report that *F. graminearum* can destroy starch granules during the invasion of wheat grains. In the study, a significant decrease in starch content has been observed due to *Fusarium* infection. The increases observed in the oil content in the grain indicate that the fungus does not use lipids as an energy source. Since *F. graminearum* uses some grain components for its growth, increases in lipids are likely to the detriment of the substances used (Boyacıloğlu and Hettiarachchy, 1995).

According to the calculated RVA parameters, the pathogen caused a significant change in the structure of the starch present in the wheat, and a degradation started in the structure of the biological macromolecules in the wheat composition (Cuesta Seijo et al. 2013). It is well known that starch is the basic component of cereals and is responsible for gelatinization which is the characteristic of the starch molecules showing a major effect on the swelling, birefringence, and viscosity of the starch molecules (Juhász and Salgó 2008). Kreuzberger et al. (2015) reported that carbohydrases secreted by *Fusarium*

spp. resulted in a significant degradation in the structure of the starch granules and so pasting characteristics of the wheat started to change significantly. Tang et al. (2012) studied the effect of different pesticide application patterns on grain yield, quality, and profit of wheat and reported that the pasting properties of wheat flour improved by applying the pesticides.

In the experiment, with FHB infection, there was a decrease in the hundred-grain weight and endosperm part of wheat grains, and therefore, the top coat and embryo ratio of the grain increased. As a result of this growing rate, the mineral content and dietary fiber levels in grains harvested from infected plants were increased since wheat germ contains the highest level of elements.

The study found that the control group (with the pathogen), which was severely infected with FHB, had the highest amount of raw ash, indicating the total mineral content. FHB infection inhibits the development of the grain, causing the kernels to become small, wrinkled, and lightweight, and increasing the ash content (Kochiieru et al. 2021). It is also rich in polysaccharides such as cellulose, hemicellulose, inulin, and lignin, which form dietary fiber (Hocaoglu et al. 2020).

Amino acid metabolism has a crucial role in regulating plant growth and development, as well as in providing resistance to biological or abiotic stress. Although some amino acids show an increase in concentration under stressful conditions, many others do not exhibit any such increase in concentration (Zhao et al. 2021). Gardiner et al. (2010) reported that the concentrations of amino acids such as glycine, valine, arginine, alanine, phenylalanine, lysine, and leucine increased during the development of FHB disease. In response to stress, proline levels significantly increase in various plants, acting

as compatible osmolytes to protect subcellular structures and macromolecules (Batista-Silva et al. 2019). It has been found that proline, which provides energy for plant growth, has various protective functions in mitochondria (Welchen et al. 2021). Studies have shown that applying proline externally to ears and leaves *in vitro* can induce resistance to FHB (Zhao et al. 2021). Similarly, according to Halkier and Gershenzon (2006), alaline serves as a precursor of glucosinates and plays a role in disease resistance. Furthermore, cysteine content has been reported to increase with the duration of FBH infection. In this study, the highest alanine, arginine, aspartic acid, glutamine, histidine, lysine, methionine, and proline values were obtained from control^{+}.

5. Conclusion

In this study, the effect of five pure components and two synthetic pesticides on the disease severity, total grain yield, DON concentration, and nutritional properties of wheat grains infected with FHB was investigated in detail. FHB is known to cause significant yield losses with high infection and produce mycotoxin (DON) in wheat while affecting its nutritional properties. Although the applications of pure components significantly reduced the DON concentration, the level of DON in the treatments except Topguard was higher than the threshold concentration. Only the treatment of Topguard kept the concentration of DON under the threshold level.

The study revealed that FHB disease increased the content of crude protein, ash, oil, phytic acid, total dietary fiber, and amylopectin in wheat grain while decreasing the total grain yield, 100-grain weight, resistant, non-resistant, and total starch content. The highest value for all mineral substances except Cu and Fe was obtained from samples harvested from untreated wheat plants infected with FHB. FHB significantly reduced

pasting properties. However, FBH caused an increase in amino acids such as alanine, arginine, aspartic acid, glutamine, histidine, lysine, methionine, and proline.

According to the findings, the tested pure components and pesticides could not completely inhibit the development of FHB, while it has been determined that the development of FHB disease is reduced by the application of the pure components, especially safraleine, and carvacrol in pre-application. This highlights the value that using sustainable and environmentally friendly agriculture practices can contribute to disease control. The pure components are as effective as chemical pesticides in maintaining many nutritional properties. Hence, there is a need to conduct further research on the utilization of various major components of EOs in the future. In this regard, the pure components with high yield and nutritional value, with less DON concentration, such as safraleine and carvacrol have shown promising results.

6. References

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