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The Fungicidal Effect of Essential Oils of Fennel and Hops against Fusarium Disease of Pea

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Abstract: Modern integrated farming systems encourage the search for alternative (non-chemical), highly effective methods of plant protection. In this study, the fungistatic effect of fennel essential oil (FEO) and hop essential oil (HEO) on fungal growth and their ability to treat Fusarium wilt was investigated. The study was conducted *in vitro* and in pot experiments. The severity of infection was assessed by disease index (DI), presence of *Fusarium culmorum* gDNA (qPCR) and anatomical analyses of infected plant tissue using an optical (OM) and scanning electron microscope (SEM). Laboratory analyses showed that FEO inhibits mycelial growth of *Fusarium* fungi (*F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. oxysporum*, *F. poae*, *F. solani*, *F. sporotrichioides*, *F. tricinctum*), *Botrytis cinerea* and *Cylindrocarpon destructans* more effectively than HEO. FEO at a concentration of 2000 ppm completely inhibited the growth of *F. culmorum*, *F. poae* and *B. cinerea*. Both essential oils reduced the severity of Fusarium wilt caused by *F. culmorum* in pea plants (DI, OM, SEM). The qPCR shows that both essential oils are also able to reduce the synthesis of trichothecenes in the tissues of infected pea plants. The results of the study suggest that FEO and HEO represent a broad spectrum bio-fungicidal agent that can be applied directly to plants at a concentration of 500 ppm, greatly reducing the level of infection.

Keywords: fungicidal activity; *Humulus lupulus*; *Foeniculum vulgare*; qPCR; SEM

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1. Introduction

The pea (*Pisum sativum* L., Fabaceae) is widely cultivated worldwide, mostly as an edible plant. Pea seeds contain biologically valuable protein with a desirable amino acid composition [1]. In 2021, global pea production reached about 12.4 million tons, of which 48% was produced in Europe, 21% in North America and 21% in Asia. In Europe, peas are the leading protein crop, accounting for 55.6% of total legume production. Outside Europe, soybeans are the most important protein crop, with global production exceeding that of pulses by a factor of four. Average pea yields are generally low, equivalent to about 65–70% of global soybean yields (1.72–2.04 versus 2.67–2.86 t/ha) [2]. Pea yields

are limited by: (i) low temperatures and soil dryness during seedling emergence; and (ii) biotic factors causing plant diseases, including fungi of the genus *Fusarium* such as *F. avenaceum*, *F. culmorum*, *F. solani*, *F. oxysporum* and others, which cause seedling blight and fusarium root and stem rot in the early stages of plant development and fusarium wilt in peas during flowering [3]. These pathogens colonise the seeds and are subsequently transmitted to the plants [4]. Fungi of the genus *Fusarium* cause the most devastating plant diseases around the world [5–7]. Almost all *Fusarium* species produce mycotoxins that have adverse health effects on humans and animals. Mycotoxins determine the progression of the disease in the early stages of infection, they accumulate in the seeds during maturation and thus affect their quality [8–10]. The following *Fusarium* species are most frequently isolated from cultivated plants in Poland: *Fusarium oxysporum*, *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. dimerum*, *F. solani*, *F. sporotrichioides*, *F. proliferatum* and *F. verticillioides* [3,11]. *Fusarium* pathogens are mainly controlled by the use of fungicides, which, however, can have potentially harmful effects on the environment and human and animal health [8,12]. *Fusarium* species are characterised by high variation within and between species. The widespread use of fungicides has led to their accumulation in food, sometimes beyond the permissible residue levels [13], and it has also triggered mutations that lead to changes in the morphology, physiology and biochemical properties of the fungi. This has contributed to the development of new, highly resistant fungal races with different host plant preferences [14]. Therefore, to protect the environment and consumers, a sustainable approach to crop production is needed that includes natural alternatives that are as effective as chemical fungicides. The secondary metabolites produced by plants, such as essential oils, are generally considered safe, easy to extract, environmentally friendly, biodegradable and largely non-toxic to mammals [15]. From a chemical point of view, essential oils (EOs) are multi-component mixtures of volatile aromatic hydrophobic compounds, mostly monoterpenes, sesquiterpenes and their derivatives [16–18]. Due to the considerable differences in their chemical composition, essential oils possess a wide range of physicochemical, sensory, biological and antimicrobial properties [19,20]. Research has shown that some essential oils are as effective or even more effective than fungicides in inhibiting fungal growth [21,22]. The efficacy of essential oils in inhibiting plant pathogens has been confirmed by numerous *in vitro* studies [23,24]. It is believed that the antifungal activity of essential oils is related to the unique properties of terpenes/terpenoids. Due to their high lipophilicity and low molecular weight, these compounds are able to damage cell walls, lead to cell death or inhibit sporulation and germination of various fungal species [17,25,26]. In recent years, numerous studies (mostly based on *in vitro* tests) have investigated the antifungal potential of essential oils against various pathogens, including *Fusarium* species. Recent research has demonstrated the fungistatic effect of rosemary (*Rosmarinus officinalis* L.) and grain mint (*Mentha arvensis* L.) essential oils on *Fusarium* fungi [27], the antifungal activity of clove essential oil (*Syzygium aromaticum* (L.) Merr. and L.M. Perry) against *F. oxysporum* f. sp. *radicis lycopercisi*, *F. redolens* and *F. commune* [28,29], the antifungal activity of essential oils from the stems/leaves and flowers of French lavender (*Lavandula stoechas* L.) against *F. oxysporum* [30] and the antifungal potential of myrrh essential oil (*Commiphora molmol* (Engl.) Engl. ex Tschirch) against *F. solani*, *F. oxysporum*, *Alternaria alternata*, *Aspergillus flavus* and *Cladosporium* sp. [31]. Sarkhosh et al. [32] found that peppermint (*Mentha* × *piperita* L.), savoury (*Satureja khuzistanica* Jamzad), thyme (*Thymus daenensis* Celak.), cinnamon (*Cinnamomum verum* J. Presl., *Cinnamomum zeylanicum* Blume.), true lavender (*Lavandula angustifolia* Mill.), eucalyptus (*Eucalyptus globulus* Labill), myrtle (*Mytrus communis* L.) and tea tree essential oils are effective inhibitors of *F. solani* and *Phytophthora palmivora*.

Hops (*Humulus lupulus* L., Cannabaceae) and fennel (*Foeniculum vulgare* Mill., Apiaceae/Umbelliferae) are valuable sources of biologically active compounds, including essential oils. Hops have long been used in traditional medicine [33] and in beer brewing [34]. The female flowers of hop plants (*Lupuli flos*) are rich in secondary plant metabolites, resins (α- and β-acids) and essential oils [35]. Niknejad [36] demonstrated *in vitro* the

fungistatic and fungicidal (dose-dependent) effects of ethanol extracts from hop flowers on the mycelial growth of five strains of the major food spoilage moulds.

Fennel is an aromatic plant used for both culinary and medicinal purposes [37]. Its biological activity can be attributed to the presence of active compounds in the seeds (*Foeniculi fructus*), including essential oils containing trans-anethole, fenchone and estragole [38]. Zellagui et al. [39] described the high antimicrobial potential of crude fennel extracts against G(−) and G(+) bacteria and against the fungi *Aspergillus versicolor*, *Aspergillus fumigatus* and *Penicillium camemberti*. Fennel essential oil (FEO) was also characterised by strong antifungal activity against *Cladosporium cladosporioides* and *Puccinia helianthi* plant pathogens as well as *Trichophyton mentagrophytes* dermatophytes [39]. According to Zeng et al. [40], FEO has potent antidermatophytic activity and is more effective than the widely used fluconazole and amphotericin B. Soylu et al. [41] found that FEO inhibits grey mould rot of tomato (*Lycopersicon esculentum* L.) caused by *B. cinerea*. According to Perczak et al. [25], FEO reduces the content of ergosterol, a characteristic component of fungal cell walls, and significantly lowers the concentrations of zearalenone and group B trichothecenes (mycotoxins).

The aim of this study was to evaluate the antifungal properties of essential oils from fennel fruits and female hop flowers against selected pathogens of pea under *in vitro* conditions and against Fusarium wilt disease of pea plants artificially inoculated with *F. culmorum* spores (isolated in a previous study) in a pot experiment.

2. Materials and Methods

2.1. Plant Materials and Essential Oils Extraction

Essential oils were extracted from fennel fruits (*Foeniculum vulgare*) and the female flowers of hop plants (*Humulus lupulus*) cv. Lubelski. The plant material came from the organic farm "Dary Natury" (Korycin, Poland) in north-eastern Poland. Representative plants of *H. lupulus* and *F. vulgare* were deposited in the collection of the Department of Agricultural Technology and Agribusiness of the Faculty of Agriculture and Forestry of the University of Warmia and Mazury (Syg. WR/H- II/Z/38 and Syg. WR/H- II/Z/50 for fennel and hop plants, respectively).

The essential oils of fennel and hops (FEO and HOE, respectively) were extracted using the hydrodistillation method described by Deryng [42]. The ratio of plant material to solvent was 1:10 (*w*/*w*) for both extraction methods. The water was heated in a heating jacket with temperature control. The extraction time was 4 h from the time the water reached boiling point. The extracted oils were placed in tight containers under a nitrogen atmosphere and were stored at a temperature of 4 °C until analysis.

2.2. Microorganisms

Single-spore fungal cultures (*Botritis cinerea*, *Cylindrocarpon destrurctans*, *Fusarium avenaceum*, *Fusarium culmorum*, *Fusarium equiseti*, *Fusarium oxysporum*, *Fusarium poae*, *Fusarium solani*, *Fusarium sporotrichioides*, *Fusarium tricinctum*) from the collection of the Department of Entomology, Phytopathology and Molecular Diagnostics of the Faculty of Agriculture and Forestry of the University of Warmia and Mazury in Olsztyn were used for the analyses.

2.3. Chemicals

All chemicals used for the analyses described in Sections 2.4–2.8 were purchased from Merck KGaA (Darmstadt, Germany). The reagents for molecular analysis were purchased from the Thermo Fisher Scientific (Waltham, MA, USA) portfolio. The primers and probes for qPCR analysis were synthesised at Merck KGaA (Darmstadt, Germany).

2.4. Evaluation of Antifungal Activity In Vitro

The influence of FEO and HEO on the linear growth of fungal pathogens on pea was determined *in vitro*. The fungistatic activity of natural biological extracts were evaluated in a food poisoning method by Ngono et al. [43].

The essential oils were prepared as aqueous extracts (10 mg/mL) containing 1% Tween-80 (polyoxyethylene sorbitan monooleate) to facilitate incorporation of the oil into the culture medium. A solution of EO was added to a sterile potato dextrose agar (PDA) medium at a temperature of 40 °C to obtain a final concentration of 125, 250, 500, 1000 and 2000 ppm essential oil in the medium. Twenty millilitres of the prepared growth medium was poured into Petri dishes with a diameter of 85 mm. After 24 h, discs containing 10-day-old mycelial cultures of each fungal species (with a diameter of 5 mm) were placed in the centre of each plate. Fungal cultures grown on media without FEO or HEO served as controls. To compare the effect of EOs, tebuconazole was used as a control. Fungi were cultured at a temperature of 22 °C for 10 days and linear growth of fungal colonies was measured daily in two perpendicular directions. The fungistatic activity (percentage inhibition of mycelial growth, % MGI) of the essential oils tested was evaluated after 10 days, as the experiment was established [44], with the use of a scale: +30 < MGI- medium growth promotion; +30 < MGI < 0- weak growth promotion; 0–30-weak inhibition; 31–50-medium inhibition; 51–80- strong inhibition; 81–100- bio-fungicide effect. The analyses were carried out in four replicates for each experimental treatment (essential oil vs. fungal species) in two independent series.

2.5. Determination of Antifungal Activity Ex Vitro

Seeds of *Pisum sativum* cv. Grot were disinfected with 70% ethanol (30 s) and 1% sodium hypochlorite solution (15 min), soaked in distilled water for 12 h and sown 1 cm deep in pots filled with sterilised horticultural soil and sand in a 2:1 ratio. The pots were placed in a climate chamber (GC 600 Nueve, Switzerland) with controlled temperature and light conditions (day 22 °C/night 10 °C; 16 h light/8 h dark cycle). Fourteen-day-old pea seedlings were sprayed with FEO or HEO solution or with water containing 1% Tween-80. For the pot experiment, solutions of FEO and HEO with a concentration of 500 ppm and 2000 pm were used. After 24 h, pea seedlings were inoculated in the soil by spreading *F. culmorum* spores (111/Fc/2015) at a concentration of 1×10^6/mL. Pea seedlings sprayed with water containing 1% Tween-80 and supplied with water instead of the spore suspension formed the negative control. Experimentally inoculated seedlings that had been sprayed with water instead of FEO or HEO solution 24 h before inoculation constituted the positive control. The experiment was carried out in three replicates in two independent series.

2.6. Disease Index

The health status of pea plants was estimated 14 days after infection using the modified scale of Hillstrand and Auld [45]: 0—no disease symptoms, 1—infection rate (IR) of 1–10%, 2—IR of 11–20%, 3—IR of 21–30%, 4—IR of 31–40%, 5—IR of 41–60%, 6—IR of 61–80%, 7—IR of 81–90%, 8–9—IR of 91–100%. The results were used to calculate the disease index (DI) [46].

2.7. DNA Extraction and qPCR Analysis

DNA was isolated from pure cultures as well as from stems and roots of pea plants. Genomic DNA was extracted from pea tissue (stored at −80 °C prior to analysis) using Maxwell 16 kits (Promega GmbH, Madison, WI, USA) for DNA isolation from plant tissue. Pure cultures of F. culmorum (OlH2014/75) were used for the qPCR assay. The quality and quantity of DNA was determined using the Nanodrop ND 2000C (Thermo Fisher Scientific, Waltham, MA, USA) and the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA was rinsed in TE buffer and stored at 4 °C until further analysis.

High quality gDNA (A260/280 ratio of 1.8 to 2.0) was used for the qPCR assay. The following primers and probes were used to quantify the severity of Fusarium wilt disease in pea plants in the pot experiment: FC _Tri5 forward: TCTTAACAC-TAGCGTGCGCCTTC, FC _Tri5 reverse: CATGCCAACGATTGTTTGGAGGGA, FC _Tri5 probe: fam- AACAAG-GCTGCCCACCACTTT GCTCAGCCT—Tamra [47]. The qPCR reactions were performed

using the ABI Prism 7500 Fast System (Thermo Fisher Scientific, Waltham, MA, USA). All qPCR reactions were performed in a total volume of 20 µL containing 3 µL genomic DNA solution, 1 × TaqMan Universal PCR Master Mix with ROX (Thermo Fisher Scientific, Waltham, MA, USA), 4 µL of primer mix (160 nM) and 2 µL of probe (100 nM). The following DNA amplification cycle was programmed: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing 60 °C for 15 s and extension 72 °C for 60 s. Each sample was loaded in triplicate onto a single qPCR plate. DNA was quantified using a standard curve generated from samples with known concentrations of *F. culmorum* gDNA obtained by serial (10-fold) dilution ranging from 10 to 0.001 ng/µL using sterile, deionised water.

2.8. Light Microscopic and Scanning Electron Microscopic Analyses

Control plants and plants treated with FEO, HEO and *F. culmorum* were viewed under a stereoscopic microscope (M205 C, Leica Microsystems GmbH, Wetzlar, Germany) (magnification 1.5–2x). Samples of stem and root surfaces and tissue sections were also analysed (Eclipse 80i, Nikon Instruments Inc., Tokyo, Japan). Fragments of the root–stem transition area were prepared for analysis using a scanning electron microscope (5310- LV, JEOL Ltd., Akishima, Japan). Fresh tissue samples were fixed overnight at 4 °C (2.5% glutaraldehyde in PBS, pH = 7.4) and dehydrated at room temperature in a graded series of ethanol concentrations. The dehydrated samples were dried at the critical point of CO_2 (CPD 030, BAL-TEC AG, Balzers, Liechtenstein) and sputter-coated with gold in an argon atmosphere (JCF-1200, JEOL Ltd., Akishima, Japan) [48]. The prepared samples were viewed with SEM at 15 kV.

2.9. Statistical Analysis

Results were processed in Dell Statistica v. 13 (Dell Inc., Round Rock, TX, USA, 2016) using Tukey's HSD test for multiple comparisons at $p \leq 0.01$. Standard deviation and significant differences between means of homogeneous groups (A, B, C) were determined (means labelled with the same letter are not significantly different at $p = 0.01$). Linear regression coefficients were calculated to determine the strength of the relationships between the amount of *F. culmorum* DNA (pg) and the disease index of Fusarium wilt in pea (%). A significant R-value of *p*-0.05 was assumed.

3. Results and discussion

3.1. Determination of Antifungal Activity In Vitro

The antifungal activity of EOs has been investigated in numerous *in vitro* studies [32,40,49]. In the present study, *in vitro* analysis of the linear growth of the fungal species studied showed differences in the antifungal potential of the essential oils investigated. Mycelial growth of all pathogens studied was significantly inhibited only by the two highest concentrations of FEO and HEO (1000 ppm and 2000 ppm) (Figures S1 and S2).

When applied at the highest concentration (2000 ppm), HEO inhibited the growth of fungal pathogens by 60.7–91.5% and FEO by 79.31–100% (Tables 1 and 2). Tebuconazole inhibited mycelial growth of all species tested in the trial. *Fusarium poae* (91.5% growth inhibition), followed by *F. culmorum* (86.6%) and *B. cinerea* (81.3%) were the most susceptible to HEO. *Fusarium tricinctum* (76.2%), *F. equiseti* (76.0%), *F. sporotrichioides* (73.0%) and *F. avenaceum* (71.7%) were also effectively inhibited by HEO. Hop essential oil was least effective against *C. destructans*, *F. solani* and *F. oxysporum*, whose mycelial growth was reduced by 65.8%, 61.9% and 60.7%, respectively, at the highest concentration of HEO (2000 ppm) (Table 2).

Table 1. MGI of selected pea pathogens with different concentration of fennel essential oil [ppm].

Treatment	Fungal Species									
	1*	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
125 ppm	1.66	+2.1	+6.7	2.9	+7.1	0.7	8.7	+9.3	1.8	9.5
250 ppm	0.23	+15.4	+13.5	7.9	+22.6	+20.8	10.8	+29.4	+2.87	1.6
500 ppm	2.25	18.4	+7.59	23.5	+6.2	7.2	14.8	+1.0	12.5	19.8
1000 ppm	24.76	31.3	+7.3	49.0	12.1	25.1	31.7	17.6	29.6	55.8
2000 ppm	100	89.3	83.8	100	91.2	99	100	79.31	99	95.7
Tebuconazole	100	100	100	100	100	100	100	100	100	100

1*—*Botrytis cinerea*; 2—*Cylindrocarpon destructans*; 3—*Fusarium avenaceum*; 4—*Fusarium culmorum*; 5—*Fusarium equiseti*; 6—*Fusarium oxysporum*; 7—*Fusarium poae*; 8—*Fusarium solani*; 9—*Fusarium sporotrichioides*; 10—*Fusarium tricinctum*.

Legend	+30 < MGI	+30 < MGI < 0	0–30	31–50	51–80	81–100

Table 2. MGI of selected pea pathogens on different concentrations of hop essential oil [ppm].

Treatment	Fungal Species									
	1*	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
125 ppm	0.7	+19.5	+8.9	6.1	4.5	+0.2	27.9	+18.5	15.6	19.8
250 ppm	0.4	+27.9	+6	18.5	5.5	+12.9	32.8	+32.3	15.8	29.5
500 ppm	4.4	14.1	23.4	41.3	39.6	21.9	45.3	10.6	23.5	34.7
1000 ppm	42.4	35.5	35.3	47.3	56.5	28.0	63.1	24.0	31.1	47.8
2000 ppm	81.3	65.8	71.7	86.6	76.0	60.7	91.5	61.9	73.0	76.2
Tebuconazole	100	100	100	100	100	100	100	100	100	100

1*—*Botrytis cinerea*; 2—*Cylindrocarpon destructans*; 3—*Fusarium avenaceum*; 4—*Fusarium culmorum*; 5—Fusarium equiseti; 6—*Fusarium oxysporum*; 7—*Fusarium poae*; 8—*Fusarium solani*; 9—*Fusarium sporotrichioides*; 10—*Fusarium tricinctum*.

Legend	+30 < MGI	+30 < MGI < 0	0–30	31–50	51–80	81–100

The present *in vitro* study showed that higher concentrations of FEO and HEO inhibited mycelial growth of all fungal species studied. The percentage of mycelial growth inhibition (MGI) varied between the essential oils and pathogens tested. Similar observations were made by Palfi et al. [21] who analysed the fungistatic activity of 12 essential oils, including FEO, against *F. oxysporum* and *B. cinerea*. The cited authors reported significant differences in MGI between the essential oils studied and their concentrations. In 8-day-old cultures of *F. oxysporum*, mycelial growth was not significantly inhibited by the four lowest concentrations of FEO (3, 5, 7, 9 and 15 µL/10 mL PDA).

Fusarium solani was one of the least susceptible pathogens, with 79.31% of its growth inhibited by both essential oils. The other *Fusarium* pathogens were more sensitive to FEO, which reduced fungal activity by 83.8% in *F. avenaceum*, 89.3% in *C. destructans*, 91.2% in *F. equiseti* and 95.7% in *F. tricinctum*. The antifungal activity of FEO against *F. oxysporum* and *F. sporotrichioides* was very high at 99%. Similar to HEO, FEO was most effective against *B. cinerea*, *F. culmorum* and *F. poae*, whose growth was completely (100%) suppressed (Table 1).

The study by Palfi et al. [21] showed that only the higher concentrations of FEO strongly (15, 30, 50 µL/10 mL) or completely (70 µL/10 mL) inhibited the growth of *F. oxysporum*. Mycelial growth of *B. cinerea* was not affected by FEO concentrations of 3 to 30 µL/10 mL, and a significant reduction was only observed at FEO concentrations of 50 and 70 µL/10 mL. The highest FEO concentration (70 µL/10 mL) inhibited *B. cinerea* growth by 100%. Perczak et al. [25] demonstrated *in vitro* that FEO is an effective antifungal agent against *F. culmorum* and *F. graminearum*.

The opposite results were obtained by Wodnicka et al. [49], who investigated the antifungal properties of dill seed essential oil at a concentration of 10 ppm. The analysed

oil reduced the mycelial growth of *F. graminearum* by 48.8%, of *F. avenaceum* by 13.3% and of *F. culmorum* by only 6.2%. These results contrast strongly with the results of the current study, in which HEO, which was considered less effective than FEO, was a stronger inhibitor of *F. avenaceum* (35.3%) and *F. culmorum* (47.3%) at a concentration of 1000 ppm.

The differences in the antifungal activity of FEO and HEO could be attributed to the specific properties of the pathogenic strains, such as their metabolic capabilities, as well as differences in the chemical composition of the essential oils. The physical and biological properties of essential oils are determined by their composition. Many authors have postulated that the antimicrobial activity of essential oils is closely related to the content of various constituents, especially the major components [50,51]. According to Wodnicka et al. [49], the differences in antifungal activity of essential oils isolated from fennel seeds can be attributed to their chemical polymorphism. The essential oil obtained from the seeds of fennel plants grown in Poland contained the highest levels of trans-anethole (69.95%) and fenchone (18.14%) and showed strong antifungal activity. In contrast, the oil of fennel seeds from Egypt contained mainly estragole (87.49%) and limonene (8.63%) and were characterised by low antifungal activity. The essential oil of fennel seeds grown in Poland completely inhibited the growth of *S. sclerotiorum* and reduced the growth of *R. solani* and *B. cinerea* by 80% and 60%, respectively. In contrast, oil from fennel seeds grown in Egypt had no effect on *S. sclerotiorum* and inhibited the growth of *R. solani* and *B. cinerea* by only 16.6% and 20.0%, respectively. At a concentration of 1000 ppm, both oils had different effects on the growth of three *Fusarium* species.

The oil extracted from the fruits of *F. vulgare* grown in Poland was most effective against *F. graminearum* (MGI of 48.8%), followed by *F. avenaceum* (13.3%) and *F. culmorum* (6.2%) [49]. Egyptian fennel essential oil was also most effective against *F. graminearum*, but the reduction in mycelial growth was only 28.8%. Egyptian fennel oil inhibited the growth of *F. culmorum* by 14.0% and had no effect on *F. avenaceum* [49]. According to the literature, the percentage content of essential oil constituents is influenced by: (i) variety, (ii) chemotype, (iii) ontogeny, (iv) plant organs, (v) harvest time, (vi) latitude and environmental conditions, (vii) nutritional status of plants, (viii) harvest date and drying conditions, and (ix) extraction methods and conditions [30,38,52,53]. Essential oils extracted from seeds of 16 wild populations of *F. vulgare* in Tunisia (subhumid, middle and upper semi-arid and humid climates) contained mainly phenylpropanoids with a dominance of estragole (66.09–85%), but they differed considerably in the content of phenolic compounds [54]. Essential oils from *F. vulgare* populations growing in different regions of Iran were characterised by different contents of trans-anethole (46.5–84%) and fenchone (9.1–23.8%) [55]. Senatore et al. [56] demonstrated that essential oils isolated from fruits of *F. vulgare* with different phenological patterns and different origins can show significant differences in the content of trans-anethole, estragole, fenchone and α-phellandrene. Kovačevič and Kač [57] found that the variety has a greater influence on the chemical composition of HEO than the growing conditions or the processing and storage methods. Similar conclusions were formulated by Almaguer et al. [58], who observed correlations between *H. lupulus* cultivars and plant age as well as hydrocarbon and oxidised compounds. Essential oils extracted from the fruits of three organically grown fennel cultivars (*F. vulgare* var. *azoricum*, *F. vulgare* var. *dulce* and *F. vulgare* var. *vulgare*) in Egypt contained 18 monoterpenoids, with trans-anethole, estragole and fenchone dominating [59]. In the above study, FEO was characterised by similar antimicrobial activity against gram positive and gram negative bacteria, *A. niger* and *C. albicans*, despite differences in the chemical composition of the oils extracted from the different varieties. Research has also shown that the overall antifungal activity of essential oils significantly exceeds the individual effects of even the most potent compounds [30,60]. The biological activity of essential oils seems to be determined by the synergistic effect of all ingredients [61,62]. Bocquet et al. [63] found that HEO strongly reduced (85–100%) the mycelial growth of *Zymoseptoria tritici*, while the three main constituents of HEO (α-humulene, myrcene and trans-caryophyllene) with proven antimicrobial potential showed no significant activity in single tests. These results confirm

that the synergistic interactions between the essential oil components significantly enhance the biological activity of the oil. In the current study, the overall effect of the essential oils was investigated rather than their individual components. The plants studied (*F. vulgare* and *H. lupulus*) were organically grown in northeastern Poland, which is characterised by a low level of urban and industrial development.

According to Wójcik-Stopczyńska et al. [64], the differences in the sensitivity of *Fusarium* species to essential oils could be due to the unique properties of these pathogens. The pathogenicity of *Fusarium* species differs in different hosts and has been linked to the molecular patterns of specific fungal proteins [65]. The metabolic activity of the pathogens could influence their susceptibility to antifungal agents, regardless of the plant species from which the essential oils are derived. An analysis of genome size and the number of coding genes in the genus *Fusarium* based on NCBI data revealed significant individual differences within the species analysed. These differences could be reflected in the pathogenic potential of isolates of the same species, including their ability to synthesise various mycotoxins and metabolise essential oil components. Watanabe [66] used the maximum likelihood method to show that *F. solani* is genetically the most distant from the other *Fusarium* species. *Fusarium solani* belongs to the clade III, while *F. oxysporum* belongs to clade V, *F. avenaceum* and *F. tricinctum*—to clade VI and *F. culmorum*, *F. equiseti*, *F. poae* and *F. sporotrichioides*—to clade VII.

3.2. Determination of Antifungal Activity Ex Vitro

3.2.1. Results of the Pot Experiment

The results of the pot experiment in which pea plants were artificially inoculated with *F. culmorum* spores showed that both FEO and HEO provide protection against Fusarium wilt (Figure 1). The subsequent test was carried out with *F. culmorum*, as it is an important pathogen under Polish conditions, causing late blight of pea, as well as stem base rot, root rot and Fusarium wilt of pea plants. Disease progression was inhibited in pea plants sprayed with both essential oils 24 h before artificial inoculation. An assessment of the health status of the pea plants 14 days after the fungal spores were applied to the soil also confirmed that both essential oils reduced the severity of Fusarium wilt compared to the positive control. Fennel essential oil was a more potent antifungal than HEO. When applied at a lower concentration (500 ppm), FEO reduced infection by 40.4% (DI = 37.3%) and HEO by 25% (DI = 52%) (Figure 1).

At a concentration of 2000 ppm, HEO inhibited the progression of Fusarium wilt by 43% (DI = 34%) and FEO by 60.4% (DI = 17.3 %), relative to the positive control (DI = 77.7%) (Figure 1). Plants that were not inoculated with *F. culmorum* were healthy. In other studies, fennel essential oil also inhibited the progression of fungal diseases. Kalleli et al. [67] reported that application of 1 mL FEO at a concentration of 500 µL/mL suppressed Fusarium wilt in tomato plants both one week before and two weeks after artificial inoculation with *Fusarium oxysporum* f. sp. *lycopersici* spores. An *ex vitro* experiment conducted by Soylu et al. [68] showed that essential oils of fennel and Syrian oregano (*Origanum syriacum* subsp. *bevanii* (Holmes) Greuter and Burdet) promoted the survival of tomato seedlings grown on soil infected with *S. sclerotinum*. In the *in vitro* experiment, FEO was a more effective antifungal agent, while in the *ex vitro* experiment Syrian oregano oil protected the tomato seedlings better against fungal infection. The survival rate of tomato seedlings increased from 26.6% to 69.8% and 63.3%, respectively, after treatment with Syrian oregano oil and FEO at the highest concentration (3.2 µg/mL) compared to control plants treated with *S. sclerotinum* spores. The antiviral effect of FEO was also confirmed against courgette yellow mosaic virus and its vector *Aphis gossypii* [69].

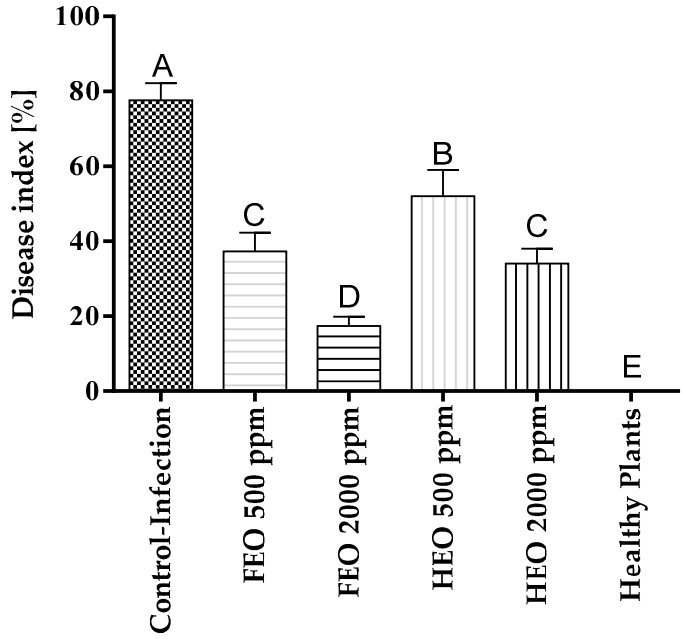

Figure 1. Reduction in Fusarium wilt (*Fusarium culmorum*) by use different concentrations of essential oils from fennel and hops—disease index (%) (A,B,C,D,E—significant at *p*-0.01 by Tukey's HSD test).

The antifungal activity of essential oils extracted from other plant species has been investigated in numerous studies [49,70–75]. Different ways of applying essential oils to plants are also used, e.g., dipping the seeds in the tested essential oils for 24 h reduces the Fusarium wilt and seedling rot and improved the survival of pea plants [76]. Seeds preserved with cumin oil had better aroma and flavour than those treated with a synthetic fumigant [77]. In artificially inoculated wheat grain (*Triticum aestivum* L.), herbal application of essential oils of fennel, cinnamon tree (*Cinnamomum* Scheffer), oregano (*Origanum vulgare* L.), palmarosa leaves (*Cymbopogon martini* (Roxb.) W. Watson), spearmint (*Mentha viridis* L.), bitter orange peel (*Citrus* × *aurantium* L.), thyme hyemalis leaves and flowers (Lange) and rosewood (*Aniba rosaeodora* Ducke) inhibited ergosterol production by almost 100% and thus strongly inhibited the growth of *F. graminearum* and *F. culmorum*. The essential oils studied also significantly reduced the concentrations of fungal mycotoxins, zearalenone and group B trichothecenes, in wheat grains [71]. The inhibitory effect of essential oils on the biosynthesis of mycotoxins was also described by Xing et al. [78], Kalagatur et al. [79] and Perczak et al. [80].

Mycotoxin contamination was not investigated in the present study, but a significant decrease in the concentration of *F. culmorum* DNA was observed in pea plants treated with both FEO and HEO. The gDNA of *Fusarium culmorum* was amplified in the qPCR assay by quantification of the *TRI5* gene (Figure 2), which encodes the trichodia synthase that has been shown to catalyse the first step in the trichothecene pathway of *Fusarium* [81].

The use of the qPCR assay based on the quantification of the *TRI5* gene can be used both as a tool to assess the level of infection of plant material, as shown by other studies [8,9], and according to our results in this study, to indicate that the application of FEO and HEO reduces the concentration of DON, a group B trichothecene. The degradation of DON *in vitro*, with essential oils from other plants was presented in a study by Perczak et al. [82].

The severity of Fusarium wilt was highest in control plants, where the amount of *F. culmorum* DNA reached 645 pg. The amount of *F. culmorum* DNA in pea plants grown at a lower concentration (500 ppm) of FEO and HEO was determined to be 48.16 and 108.74 pg, respectively. Both oils had a stronger antifungal effect when applied at a concentration of 2000 ppm, and relatively low levels of *F. culmorum* DNA were detected in the treated plants. The average amount of *F. culmorum* DNA was determined to be 13.5 pg in the plants sprayed with FEO, while it was higher at 34.27 pg in the plants treated with HEO (Figure 2).

Linear regression analysis showed that the results of the qPCR test were consistent with the disease index of Fusarium wilt. In the greenhouse experiment, a positive correlation was found between the symptoms of Fusarium wilt and the results of the qPCR quantification of *F. culmorum* (*TRI5* gene), which was confirmed by the linear relationship between the variables (y = 0.76x+ 25.5) and the value of Pearson's R (0.76) (Figure 3).

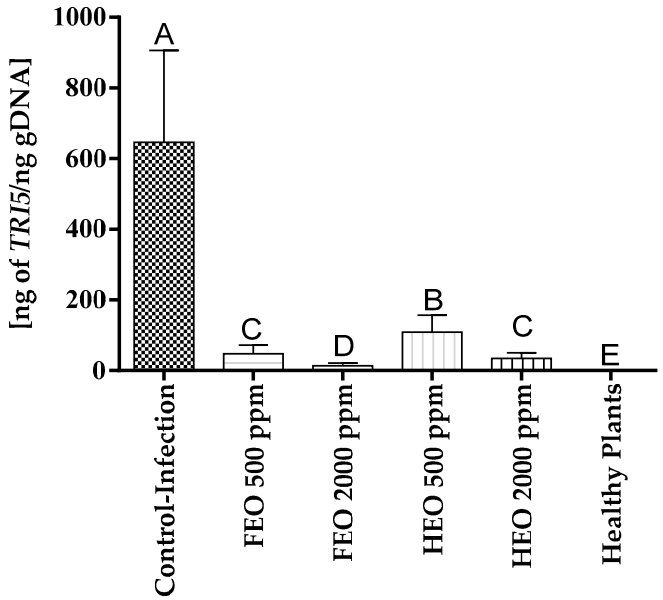

Figure 2. The qPCR quantification of gDNA *Fusarium culmorum* (*TRI5* gene) in pea plants treated with different concentrations of essential oils from fennel and hops (pg DNA), (A,B,C,D,E—significant at *p*-0.01 by Tukey's HSD test).

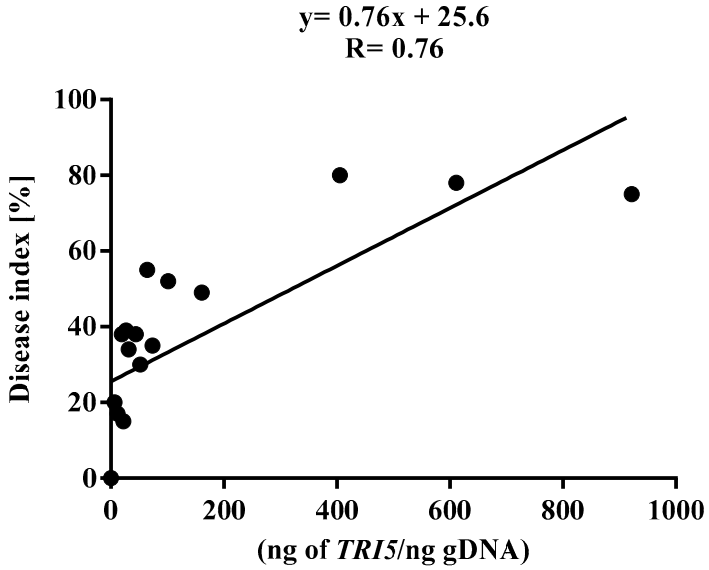

Figure 3. Linear regression analysis between symptoms of fusarium wilt and results of qPCR quantification of *TRI-5* gene (R significant at *p*-0.05).

3.2.2. Microscopic Analysis

The results of microscopic analysis confirmed the effectiveness of HEO and FEO in reducing Fusarium wilt in pea plants. An analysis of the root-stem transition zone showed that the plant tissue was less penetrated by *F. culmorum* mycelia in seedlings treated with HEO and FEO than in control plants that were not sprayed with essential oils and irrigated with a spore suspension of *F. culmorum* (Figure 4).

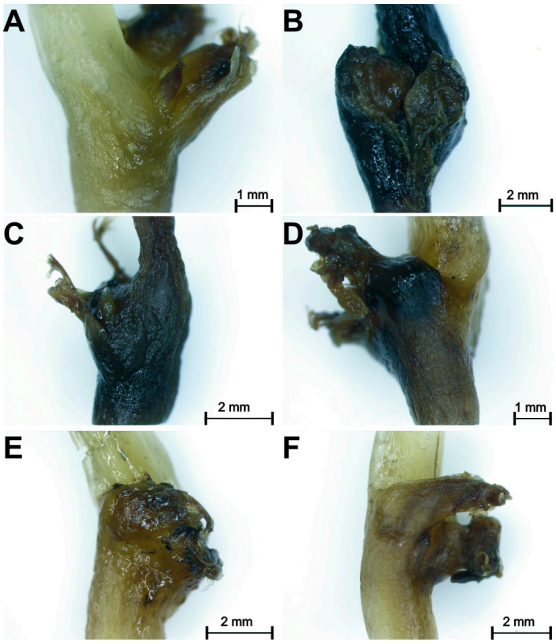

Figure 4. Transition region between stem and root of the pea plants: non-treated (**A**) and treated with *Fusarium culmorum* (**B**), treated *Fusarium culmorum* with HEO (500 ppm—(**C**), 2000 ppm—(**D**)) and FEO (500 ppm—(**E**), 2000 ppm—(**F**)). Scale bar: 1 and 2 mm.

An anatomical analysis (Figures 5 and 6) revealed significantly fewer fungal mycelia in plants sprayed with HEO and FEO (Figures 5C–F, 6C–F and 6C′–F′) than in control plants not treated with essential oils (Figures 5B,B′ and 6B,B′). The severity of pathogenic changes on the surface of roots (Figures 4 and 5) and stems (Figures 4 and 6) was influenced by the applied oil and its concentration. Fennel essential oil was more effective than HEO in reducing the symptoms of infection (Figures 4–6), which was confirmed by the disease index of Fusarium wilt. The anatomical analysis of stems demonstrated that FEO was a more potent antifungal agent than HEO (Figure 6).

Analysis of root (Figures 4 and 5) and stem (Figure 4) surfaces and anatomical analysis of stems (Figure 6) confirmed that HEO at a concentration of 2000 ppm strongly inhibited mycelial growth of *F. culmorum*. The most severe symptoms of fungal infection, including visible mycelia and brown discolouration indicative of tissue damage, were observed in the epidermis, cortex and vascular bundles of the stem (Figure 6C, arrows) in plants treated with 500 ppm HEO. In plants sprayed with 2000 ppm HEO, symptoms of fungal infection were only observed in the epidermis and peripheral vascular bundles (Figure 6D, arrows). The higher concentration of FEO had a similar antifungal effect. Mycelial growth was more effectively inhibited when FEO was applied at a concentration of 2000 ppm than at a concentration of 500 ppm (Figure 6E,F). Although infection symptoms were similar on the surface of the plant tissue (Figure 6E,F), cross-sections of the stems showed infected vascular bundles in plants treated with FEO at a concentration of 500 ppm (Figure 6E, arrow), whereas in plants sprayed with 2000 ppm FEO, symptoms of fungal infection were observed only on the surface of the stems (Figure 6F, arrows). The direct effects of essential oils on the structure of fungal mycelia have been widely reported in the literature [17,25,26], while their contribution to reducing the severity of plant diseases has never been confirmed in anatomical analyses of plant tissues. Essential oils alter the texture, colour and sporulation properties of fungal mycelia [61]. The disadvantage of essential oils is their volatility, which can lead to a loss of their efficacy when used under field conditions [83]. The widespread use in agriculture is opposed by the fact that the approval and registration procedures are very costly, as the evaluation of toxicity and environmental suitability is associated with high costs [84,85]. The number of registered biological control agents based on EO is significantly lower in Europe than in the United

States [86]. In Europe, an increasing number of Eos have been approved as biocides for use in agriculture in recent years. Current research shows the potential for using FEO and HEO to protect peas against *Fusarium* fungi that cause root necrosis, stem base and Fusarium wilt. However, further trial studies need to be conducted to assess their efficacy under natural infection conditions as well as their environmental safety.

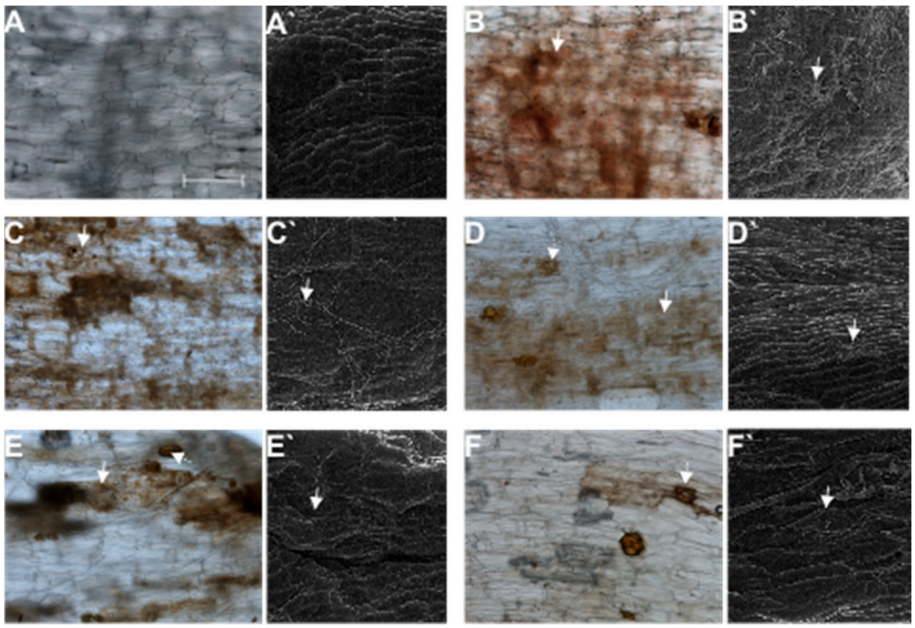

Figure 5. The surface of the pea root: non-treated (**A**,**A′**) and treated with *Fusarium culmorum* (**B**,**B′**), treated *Fusarium culmorum* with HEO (500 ppm—**C**,**C′**, 2000 ppm—**D**,**D′**) and FEO (500 ppm—**E**,**E′**, 2000 ppm—**F**,**F′**). Brown colour indicates infected cells (arrow) and possible place of fungus penetration (dotted arrows). Scale bar: 100 µm.

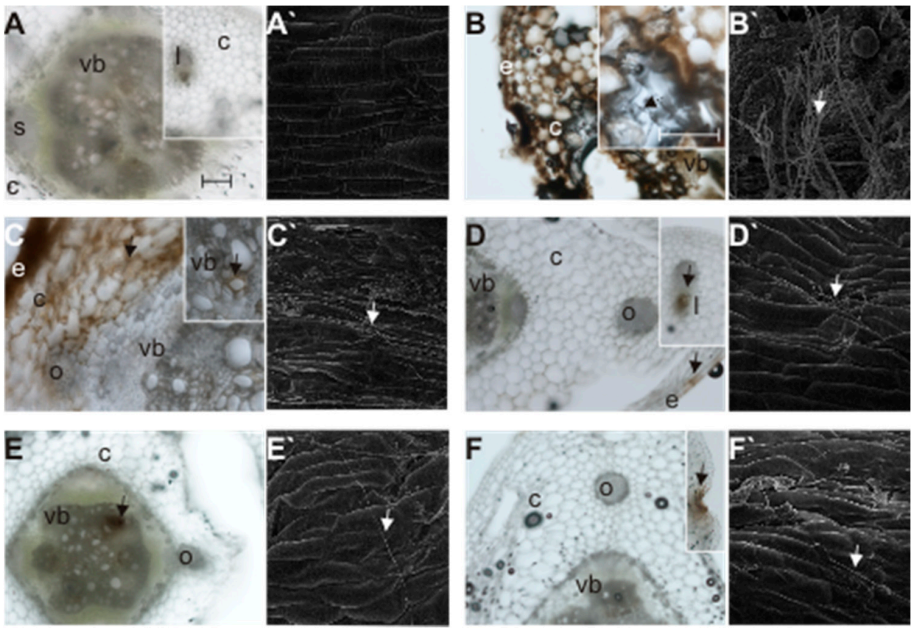

Figure 6. Cross section of the pea stem: non-treated (**A**,**A′**) and treated with *Fusarium culmorum* (**B**,**B′**), treated *Fusarium culmorum* with HEO (500 ppm—**C**,**C′**, 2000 ppm—**D**,**D′**) and FEO (500 ppm—**E**,**E′**, 2000 ppm—**F**,**F′**). Abbreviations: e—epidermis, c—cortex, vb—vascular bundle of the stele, o—outer part of a phloem split strand, l—phloem lateral strand. Scale bar: 100 µm, Arrows—description in the text.

4. Conclusions

The results of this study clearly indicate that FEO is a broad-spectrum antifungal agent that is effective in reducing the severity of infections caused by many *Fusarium* species as well as *B. cinerea* and *C. destructans.* The results of the *ex vitro* trial confirmed the results of the *in vitro* tests and showed that HOE is a less potent inhibitor of pea pathogens than FEO. The qPCR studies for the detection of the *TRI5* gene show that the use of essential oils can probably also reduce the synthesis of trichothecenes. These results suggest that FEO, a natural product, can effectively replace synthetic fungicides and provide an alternative approach for the prevention and treatment of Fusarium wilt in pea. In the future, FEO may be used in integrated pest management programs to control multiple plant pathogens simultaneously.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13106282/s1, Figure S1. The effect of FEO at different concentrations (125–2000 ppm) on the linear growth of plant pathogenic fungi (A): *B. cinerea*; (B): *C. destructans*; (C): *F. avenaceum*; (D): *F. culmorum*; (E): *F. equiseti*; (F): *F. oxysporum*; (G): *F. poae*; (H): *F. solani*; (I): *F. sporotrichiodes*; (J): *F. tricinctum*, (A,B,C—significant at *p*-0.01); Figure S2. The effect of HEO different concentrations (125–2000 ppm) on linear growth of plant pathogenic fungi (A): *B. cinerea*; (B): *C. destructans*; (C): *F. avenaceum*; (D): *F. culmorum*; (E): *F. equiseti*; (F): *F. oxysporum*; (G): *F. poae*; (H): *F. solani*; (I): *F. sporotrichiodes*; (J): *F. tricinctum*, (A,B,C—significant at *p*-0.01).

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