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Antifungal and Virulence-Modulating Effects of Thyme Essential Oil against *Fusarium* spp., Causing Wheat Diseases

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Abstract: Wheat (*Triticum aestivum* L.) is one of the important cereals and a major source of human food worldwide. *Fusarium pseudograminearum* and *F. culmorum* are the causal agents of important diseases on wheat, including head scab, crown and root rot. Control of Fusarium diseases on wheat is very difficult and important because of not only no complete resistance in any wheat cultivar and long-term survival of the soil-borne pathogen, but also production of carcinogenic mycotoxins in wheat tissues, which are harmful for consumers. Nowadays, to avoid the ecological and environmental damage caused by chemical pesticides and fungicides, there is a great interest to use essential oils as plant-protective agents. Therefore, the aim of this study was to find an alternative to synthetic fungicides currently used in the control of these destructive wheat diseases. We investigated the antifungal activities of the essential oils obtained from 4 different plant species and the Thyme (*Thymus vulgaris*) essential oil (TEO) revealed the highest level of antifungal effect against two mentioned phytopathogens. Contact and volatile phase effects of different concentrations of TEO were found to inhibit the growth of both fungi. Spore germination and production were also strongly inhibited by the TEO. Light microscope scanning of vegetative growth of the fungi revealed destructive changes in the hyphae. The TEO, also showed a high seed protection capability against fungal infection with no negative effect on seed germination. In the *in-vivo* assay, which was performed under greenhouse conditions using susceptible wheat plans treated with the TEO, significant plant protection was observed against the pathogens. Investigating effect of the main components of the TEO revealed that cymene had the highest effect in decreasing both disease incidence (DI) and disease severity index (DSI) on wheat plants inoculated by *Fusarium* species. Therefore, the TEO and its ingredients could be used as environmentally friendly biofungicides in the protection of wheat plants against these *Fusarium* species. To our knowledge, this is the first report on the *in vivo* and *in vitro* inhibitory effects of the TEO and cymene, as its main ingredient, against destructive Fusarium spp. pathogenic on wheat.

***Keywords*:** crown and root rot, *Fusarium culmorum, Fusarium pseudograminearum*, *Thymus vulgaris*, *Triticum aestivum*

1. Introduction

Wheat is the most important cereal worldwide, with highest level of consumption among crop plants [1]. This plant plays a fundamental role in human civilization at the global and regional levels and provides about 19% of the calories and 21% of protein needs of daily human requirements [2]. Many of the developing countries, which depend on wheat as a staple crop, are not self-sufficient in wheat production, and accordingly, wheat is their single most important imported product [3].

Wheat plants can be attacked by several phytopathogens, including fungi. From seed germination to harvest, soil-borne, seed-borne, and air-borne fungal diseases can reduce the quantity and quality of wheat yield [4]. *Fusarium* spp. are among the most economically important phytopathogenic fungi, which can infect small grain cereals (wheat, barley, oat). Various species of *Fusarium* cause important wheat diseases, such as head blight, crown and root rot. Infected plants are stunted, with a reduced number of tillers producing empty or partially filled heads with shriveled grains with lightweight, dull greyish color and chalky or opaque appearance [5] with losses in grain yield and reductions in baking and seed quality. More importantly, the major risk due to *Fusarium* spp. is contamination of the crop with toxic fungal secondary metabolites known as mycotoxins, which have several harmful effects on humans and animals as consumers [6].

DOI: https://doi.org/10.37256/amtt.112020132

Many of the *Fusarium* species including *F. culmorum* and *F. pseudograminearum* (teleomorph; *Gibberella coronicola*; formerly known as *F. graminearum* group 1) can cause not only head blight, but also crown and root rot diseases [7, 8, 9]. These pathogens can be distributed by soil [10], air and water splash [11, 12, 13], leading to infection of adjacent healthy plants.

Different methods have been adopted to control the mentioned pathogens, including crop rotation, tillage practices, resistant cultivars and fungicides application. However, efficacy of these control methods was incomplete [14, 15, 16]. Use of semi-tolerant or partially resistant cultivars is unavailable in some regions and only leads to limited control [17]. Application of fungicides is not effective in controlling these pathogens and especially in reducing formation of fungal mycotoxins, such as deoxynivalenol (DON), nivalenol (NIV), and zearalenon (ZEA) [17, 18]. These mycotoxins are not only fungal virulence factors that accelerate plant infection by the pathogens, but also are extremely harmful for the health of consumers [19]. Moreover, the use of synthetic fungicides in crop protection against pathogens can result in problems such as environmental pollution, phytotoxicity and selection of resistant pathogen populations [20]. Consequently, utilization of these hazardous compounds is limited in various countries [21]. For these reasons, the global demand for natural and socially more acceptable preservatives has been increased in recent years [22].

Essential oils (EOs) are naturally occurring metabolites isolated from various parts of plants [23] and their antifungal effects against various pathogens and mycotoxigenic fungi have been previously examined and demonstrated by several researchers [24, 25, 26]. The most attractive aspect of using EOs as fungicides is that these natural compounds are 'eco-friendly' without hazardous effects in the environment [27].

Therefore in the present study, we examined the effect of various medicinal plants against two important species of *Fusarium*, including *F. culmorum* and *F. pseudograminearum* as the causal agents of crown and root rot and also head blight of wheat not only *in vitro*, but also *in vivo* conditions. The objectives of this work were to (i) select the most effective plant essential oil on vegetative growth of the fungal pathogens and investigating its effects on spore production and germination, (ii) determining its fungicide and/or fungistatic effects, (iii) determining ingredients of the most effective EO and investigating effect of this EO and its main components on production of mycotoxins by the pathogens, (iv) investigate its potential as a preservative for storage of wheat seeds and (v) its potential in protecting wheat plants against the pathogens in greenhouse conditions.

2. Materials and methods

2.1 Plant material

All plant species were collected from Khorasan Razavi province in Iran (Table 1). The species were identified by Institute of Plant Sciences in Ferdowsi University of Mashhad, Iran, and voucher specimens were deposited in the Laboratory of Botany at Ferdowsi University of Mashhad. Plant tissues were air dried at room temperature in the shade for about one week (depending on the species of plants) before using for extraction of the essential oil (EO).

Table 1. List of plant species, common names, family and part used in the comparative assay of essential oils for their antifungal properties

Plant species	Family	Common name	Plant part used
Mentha piperita	Lamiaceae	Peppermint	Leaves
Juniperus sabina	Cupressaceae	Savin juniper	Seeds
Artemisia scoparia	Asteraceae	Redstem wormwood	Seeds
Thymus vulgaris	Lamiaceae	Thyme	Leaves

2.2 Preparation of the essential oils

Extraction of the EOs was carried out using hydro-distillation method as described by Tripathi et al. [28], by adding 100g of powdered plant materials mixed with distilled water (1:3) to a clevenger apparatus (Model: Tef2, Code 238029, Zimax, Germany) for 4h. Distilled water was heated to produce steam that carried the most volatile fractions of the aromatic material with it. The steam was then chilled with a condenser and the essential oils were separated from the resulting distillate. The oils were dehydrated over anhydrous sodium sulfate to remove traces of moisture and stored in dark conditions at 4°C until use.

2.3 Fungal isolates

Two phytopathogenic fungi used in this study, including *F. culmorum* isolate FH9 [19] and *F. pseudograminearum* isolate Ar1 [29], were obtained from Culture Collection of Laboratory of Phytopathology in Ferdowsi University of Mashhad, Iran. Both fungal isolates were highly virulent on wheat plants. The isolates were maintained on Potato Dextrose

Agar (PDA) medium at 4°C.

2.4 Investigating antifungal activity of the EOs *in vitro* using poisoned food (PF) assay

The PF assay was carried out according to the method described by Feng & Zheng [30] with some modifications in two sections. The EOs suspensions were prepared by dissolving required amount of the EOs in Tween 20 (0.1% v/v) solution to obtain (i) 1000µL/L concentration for comparative screening the efficacy of the EOs and to select the most effective ones and (ii) 100, 150, 200, 250, 300, 500 and 750µL/L concentrations for supplementary test, only with the most effective EO. The EOs suspensions were added to the melting PDA medium, and poured to petri dishes. Control plates were prepared in the same way, except that sterile distilled water was used instead of the EOs. After solidification of media, a fungal disc (5 mm in diameter), was cut from the periphery of a 7 days old culture, and placed in the center of each petri dish. The plates were incubated at 28°C until the growth in the control plates reached to the edge of the plates. Growth inhibition was calculated as the percentage of inhibiting radial growth compared to the control using the following formula [31]:

$$\text{Percentage of mycelial growth inhibition} = [(a - b)/a] \times 100 \quad (1)$$

Where, "a" is the mean colony diameter for the control sets and "b" is the mean colony diameter for the treatment sets. Each concentration had three replications and the experiment was repeated three times. Since the Thyme essential oil (TEO) was the most effective EO among others, it was used for further experiments.

2.5 Determination of MIC, MFC and IC_{50} values

The nature of toxicity (fungistatic and/or fungicidal effect) of the TEO against the pathogens was determined as described by Thompson [32] using the PF assay with 100, 150, 200, 250, 300, 500 and 750µL/L concentrations. The minimum inhibitory concentration (MIC) values were determined as the lowest concentration of the TEO, which completely prevented visible fungal growth. To determine the minimum fungicidal concentration (MFC) values, inhibited fungal isolates in the PF assay were transferred into fresh PDA and incubated for 5 days at 28°C, and the lowest concentration of the TEO in which no mycelial growth was observed, was determined as the MFC. The inhibitory concentration 50 (IC_{50}) value (which is a concentration that causes 50% growth inhibition) was calculated by drawing the concentration-inhibitory plot and calculating the linear regression equation for each fungus, using Microsoft Office Excel (ver. 2016).

2.6 Volatile activity (VA) assay

The VA assay was done as described by Singh et al. [33]. Petri dishes were filled with 20ml of PDA and a mycelial plug (5mm diameter) obtained from the edge of a 7 days old fungal culture was placed on PDA in each petri dish. Then, sterile filter paper discs (Whatman No. 1, 10 mm diameter) soaked with 100, 150, 200, 250, 300µl/L concentrations of the TEO were placed in the center of each plate's lid (previously poured 0.5ml PDA on each lid to prevent falling of filter paper). The petri dishes were then wrapped with parafilm to prevent release of volatile components, and incubated at 28°C for 5 days. Each test was replicated three times and fungal sensitivity was calculated in terms of percentage of mycelial zone inhibition as previously mentioned for the PF assay.

2.7 Spore production assay

Spore production assay was carried out using the method of Tzortzakis & Economakis [34], with some modifications. Spores from 10 to 14 days (until spore formation in control) of the PF assay plates of the tested fungi were harvested by adding 2mL of sterile distilled water containing 0.1% (v/v) Tween 20 to each petri dish and gently scraping the mycelial surface to release the fungal spores. Then, the spore numbers of treated plates were scored using a haemocytometer and compared with spore numbers in the control.

2.8 Spore germination assay

Spore germination was tested using the method of Sharma et al. [35], with some modifications. Five concentrations of the TEO (100, 150, 200, 250, and 300µL/L) were tested for determining their effect on spore germination of the tested fungi. Fungal spores were obtained from 14 days old cultures and added to eppendorf tubes containing the TEO suspensions in triplicate. The tubes were incubated at 28°C for 12 to 24h. For each treatment, 100 spores were counted under the light microscope to investigate spore germination, and the number of spores germinated were estimated to calculate the percentage of spore germination.

2.9 Effect of the TEO on hyphal morphology

To investigate effects of the TEO on fungal morphology, morphological changes caused by the TEO at IC_{50} concentration after 4 days on hyphal growth were studied using a light microscope (Olympus DP12, Japan).

2.10 Effect of the TEO on wheat grain conservation

This section was performed to determine potential use of the TEO as a disinfectant against fungal contamination in wheat seeds, using the methods of Roselló et al. [26] and Anžlovar et al. [36] with some modifications.

Healthy wheat grains (*Triticum aestivum* cv. Rushan) were obtained from agricultural research center, Isfahan, Iran. The seeds were soaked in sodium hypochlorite (1%) for 2 min, followed by washing three times with distilled water and air-dried on filter paper. Then, the seeds were sprayed with fungal spore suspension at 5×10^5 concentration (5ml for each 100 seeds), and air-dried in sterile conditions. The seeds were treated with the TEO by indirect treatment method, where sterile filter paper soaked with 250, 300, 400 and 500µL/L concentrations of the TEO solutions or just Tween 20 (0.1%) (in control plates) was placed into inner side of each plate's lid as well as the method used for the VA assay. The grains were distributed on each petri plate and the plates were sealed with parafilm and incubated under shaking (50 rpm) for 24h at 25°C. Then, five seeds from each treatment of the TEO concentrations and fungal suspensions, were placed on a petri dish containing 2% PDA in triplicate and incubated at 25°C (Direct plating method). After 72h, inhibition percent of fungal development on wheat seeds was calculated using following formula (Kumar et al. 2017):

$$\text{Percentage of wheat seed protection} = (D_C - D_T) / Dc \times 100 \quad (2)$$

Where D_C is percent of infected seeds by fungi in the control, and D_T is percent of infected seeds in the treated plates. At the same time, to evaluate possible negative effects of the TEO on seed germination, an assessment was carried out by counting the number of germinated seeds in each treatment and the results were expressed as percentage of germinated seeds.

2.11 Gas Chromatography - Mass Spectrometry (GC-MS) analysis

GC-MS analysis was carried out by using Agilent 7000 mass spectrometer interfaced with a gas chromatograph equipped with fused silica capillary DB-5 column (60m × 0.25mm, film thickness 0.25µm). Oven temperature program was set as gradient from 60 to 250°C at the rate of 4°C/min and finally held at 250°C for 10min. Helium was used as carrier gas with ionization voltage of 70 eV. Components of the TEO were identified based on calculating their retention times on the DB-5 capillary column relative to C_6-C_{24} n-alkanes with those reported in the literature (Adams, 2007), and by comparison of their mass spectral fragmentation patterns with those of similar compounds in the reference mass spectra library (NIST 02. LIB).

2.12 Effect of the TEO and its main ingredients on mycotoxin production by the pathogens

Efficacy of the TEO and its main constituents, at very low concentrations (0.01 IC50) without any effect on the fungal growth, in reducing production of mycotoxins (such as nivalenol, NIV; deoxynivalenol, DON; and zearalenone, ZEA) by the fungal isolates *in vitro* was investigated. Briefly, 25g of rice grains were soaked in 100mL of sterile distilled water for 6 h. Water was drained and the soaked rice was autoclaved twice. Five mL of inoculum suspension (1 × 105 conidia mL−1) from each isolate was added to each flask and incubated at 26 ± 1°C in darkness for 3 weeks. The rice-fungus mixtures were ground in a mortar and then dispensed in an Erlenmeyer flask with 75mL of acetonitrile: methanol:water (80:5:15, v/v/v). The HPLC analysis was carried out on a Waters Alliance 2695 separations module coupled to a Waters 474 scanning fluorescence detector (Waters Corp, Milford, Massachusetts, USA) that was set at 365nm excitation and 440nm emission. To perform choromatographic separation, 500µL of water: methanol (86: 14, v/v) was added to extracts and cleaned up with a C18 Spherisorb 5µm (250* 4.6mm, Merck, Germany). The mobile phase was water:acetonitrile:methanol (78:12:10 v/v/v) with a flow rate of 2.5mL/min. The NIV, DON and ZEA production was measured in µg/kg of sample. A standard of the DON, NIV and ZEA was used to construct a five-point calibration curve of peak areas versus concentration. The injection volume was 50µL for both the standard solution and sample extracts.

2.13 Effect of the TEO on disease development *in vivo*

A greenhouse trial was conducted to study the effect of the TEO on progress of the disease caused by *F. pseudograminearum* and *F. culmorum*. Wheat seeds (*Triticum aestivum* cv. Rushan) were disinfected with sodium hypochlorite 1% for 2 min, rinsed three times with sterile distilled water and then the seeds were sown in 15cm diameter plastic pots containing a mixture of sand, clay and leaf compost (1:1:1 v/v) which had been autoclaved at 121°C for 30 min on 2 successive days. The plants were grown at 25°C under 12 h photoperiod and about 70% relative humidity in the greenhouse. Different concentrations of the TEO (150, 200 and 250 µL/L) were prepared by dissolving the required amounts of the TEO in sterile Tween 20 (0.1% v/v) solution. Four weeks old plants were used for spraying these emulsions until run-off. The plants were inoculated at 2 days after spraying the TEO, using the spore suspension (10^5 spores/mL)

of each pathogenic fungus [38] which was prepared by growing the fungi on carnation leaf agar medium for 14 days. The positive control plants were sprayed with sterile Tween 20 solution (0.1% v/v) and inoculated with spore suspension. Negative control plants were just sprayed with the 0.1% Tween 20 solution. Plants were incubated in the dark at constant 100% relative humidity and 25°C temperature for 24 h and then, the temperature, light and humidity were reset to the above mentioned conditions. Controls and the TEO treated plants were evaluated 21 days after inoculation and each plant was carefully pulled out of the soil and washed. Percentage of the disease incidence was determined using the following formula [38]:

$$\text{Disease incidence (DI)} = (\text{PI} / \text{PT}) \times 100 \quad (3)$$

Where "PI" is the number of infected plants and "PT" is total number of plants. Disease severity was rated using a scale of 0 to 5 based on the root and crown tissues infected, in which: 0 = no infection, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75% and 4 = 76 to 100% infection. Finally, the disease severity index (DSI) was calculated as described by Taheri and Tarighi [39].

In addition, the possibility of phytotoxic effect of the TEO on wheat plants was examined by spraying the plants with different concentrations of the EO until run off and evaluating the plants after 21 days in comparison to controls sprayed with sterile Tween 20 solution (0.1% v/v). The experiments were arranged in completely randomized design with three replicates for each concentration of the TEO and three repetitions of each assay.

2.14 Statistical analysis

At least three replications for each treatment in an assay and three independent repetitions were carried out for each assay. All statistical analyses were performed by Excel and SPSS (version 22). The data were analyzed by one-way analysis of variance (ANOVA) and comparison of the means was done using the Duncan's Multiple Range Test at the level of $P \leq 0.05$.

3. Results

3.1 Comparative effect of the EOs on fungal growth inhibition

Comparing the results of the EOs activity on fungal growth inhibition at 1000µL/L revealed that all EOs were able to inhibit fungal growth ranging from 12.6% to 100% (Figure 1). The thyme essential oil (TEO) had the highest level of inhibition among the EOs tested against both fungi. Also, the lowest inhibitory effect was related to *M. piperita* and *J. sabina* against *F. culmorum* and *F. pseudograminearum*, respectively. Therefore, the TEO was selected to use in subsequent assessments.

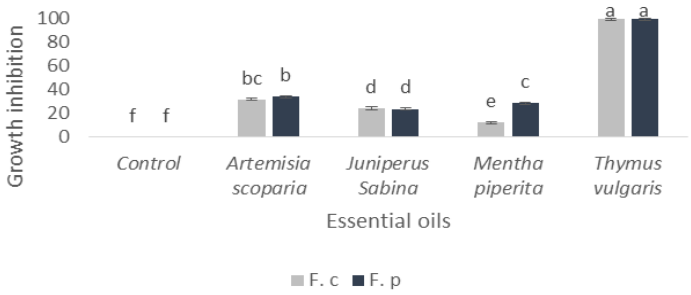

Figure 1. Comparison of antifungal effect of various plant essential oils at 1000 µL/L concentration on mycelial growth of *Fusarium culmorum* and *F. pseudograminearum.* Different letters indicate significant differences according to the Tukey's test at P=0.05. The bars indicate standard errors (SE)

3.2 Effect of the TEO on fungal growth inhibition using PF and VA assay

Effects of different concentrations of the TEO on fungal growth were studied in agar medium using the PF and VA assays. All concentrations of the TEO showed significant inhibitory effect on fungal growth. As shown in the PF assay, the TEO had a complete inhibitory effect on fungal growth at concentrations of ≥ 200µL/L for *F. pseudograminearum* and ≥ 250µL/L against *F. culmorum* (Table 2). In the VA assay, inhibitory effect of the TEO on mycelial growth was greater than the PF assay at the lowest concentration tested (Tables 3).

Table 2. Effect of different concentrations of the thyme essential oil (µL/L) on mycelial growth of *Fusarium culmorum (F.c.)* and *F. pseudograminearum (F. p.) in the poisoned food (PF) assay*

	Ctrl	100	150	200	250	300	500	750	1000
F. c.	0e	68.6^{d}	91^{c}	96^{ab}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
F. p.	0e	89^{c}	95^{b}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}

Values represent the mean of growth inhibition percentages. Values with the same letter do not differ significantly at P = 0.05 according to the Tukey's test. Ctrl: control

Table 3. Effect of different concentrations of the thyme essential oil (µL/L) on mycelial growth of *Fusarium culmorum (F.c.) and F. pseudograminearum (F. p.)* in the volatile activity (VA) assay.

	Ctrl	100	150	200	250	300	500	750	1000
F. c.	0^{e}	84^{d}	92^{c}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
F. p.	0^{e}	92.3^{bc}	95^{b}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}

Values represent the mean of growth inhibition percentages. Values with the same letter do not differ significantly at P = 0.05 according to the Tukey's test. Ctrl: control

3.3 GC-MS analysis and determination of MIC, MFC and IC_{50} values

In total, 20 components were identified in the TEO (Table 4). As the results showed, the major compounds of the thyme chemotype used in this study were carvacrol (67.1%), followed by Cymene (13.43%) and Gamma-terpenine (9.59%). Other components were present in amounts less than 2% (Table 4).

The MIC values for the TEO were 200 and 250µL/L for *F. pseudograminearum* and *F. culmorum*, respectively, in which no mycelial growth was observed in these concentrations. Investigating fungicidal activity of the TEO on the tested fungi revealed that the MFC values were 300µL/L for *F. pseudograminearum* and 350µL/L for *F. culmorum*, respectively. The IC_{50} values of the TEO were 88.5µL/L for *F. culmorum* and 71.2µL/L for *F. pseudograminearum*, which means that a higher amount of the TEO was required to inhibit 50% growth of *F. culmorum* compared to *F. pseudograminearum* (Table 5). The MIC, MFC, and IC_{50} values were also determined using the main ingredients of the TEO, including cymene, carvacrol, and gamma-terpinene. The obtained data revealed that the mentioned compounds had higher antifungal effect compared to the TEO. Cymene showed the highest level of antifungal effect against both pathogenic fungi, followed by carvacrol and gamma-terpinene, as the values obtained for both fungi by using cymene were lower than the values of two other compounds used in this assay (Table 5).

Table 4. Chemical composition of the essential oil obtained from the leaves of *Thymus vulgaris*. * RT: Retention time

No.	Compounds	RT*	%
1	Butanoic acid, 2-methyl. Methyl ester	2.22	0.2
2	Trans, 3-caren-2-ol	5.10	0.45
3	Camphene	5.92	0.29
4	2-octen-1-ol	7.20	0.43
5	Sabinene	7.60	0.62
6	p-cymene	8.94	1.02
7	Cymene	9.49	**13.43**
8	Limonene	9.61	0.17
9	Eucaliptol	9.73	0.65
10	Gamma-terpinene	11.29	**9.59**
11	1-3-8-p-methateriene	11.80	0.24
12	3-cerene	13.64	1.72
13	Isoborneol	17.44	0.85
14	1-3-8-p-methaterinen	17.96	0.65
15	α-terpineol	19.19	0.12
16	o-methylthymol	20.86	0.49
17	Methyl thymol ether	21.33	0.42
18	Carvacrol	24.91	**67.1**
19	Caryophyllene	30.60	1.17
20	Caryophyllen oxide	38.32	0.39

Table 5. Minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and inhibitory concentration 50 (IC_{50}) values (in µL/L) of the thyme essential oil (TEO) and its main constituents against *F. pseudograminearum and F. culmorum*

	F. culmorum			*F. pseudograminearum*		
	MIC	MFC	IC_{50}	MIC	MFC	IC_{50}
TEO	250	350	88.5	200	300	71.2
Carvacrol	150	200	51.2	140	270	48.5
Cymene	210	180	45.8	100	160	40.2
Gamma-Terpinene	120	300	80.2	170	290	66.4

3.4 Effect of the TEO on production and germination of fungal spores

The effect of different concentrations of the TEO on spore germination of two fungal isolates were shown in Table 5. The TEO was more effective in inhibiting spore germination of *F. pseudograminearum* compared to *F. culmorum*. Spore germination was completely inhibited at 250µL/L concentration of the oil in both fungi. Furthermore, in cases where germination was observed, the length of germ tube was reduced compared to the control in a dose-dependent manner (Figure 2).

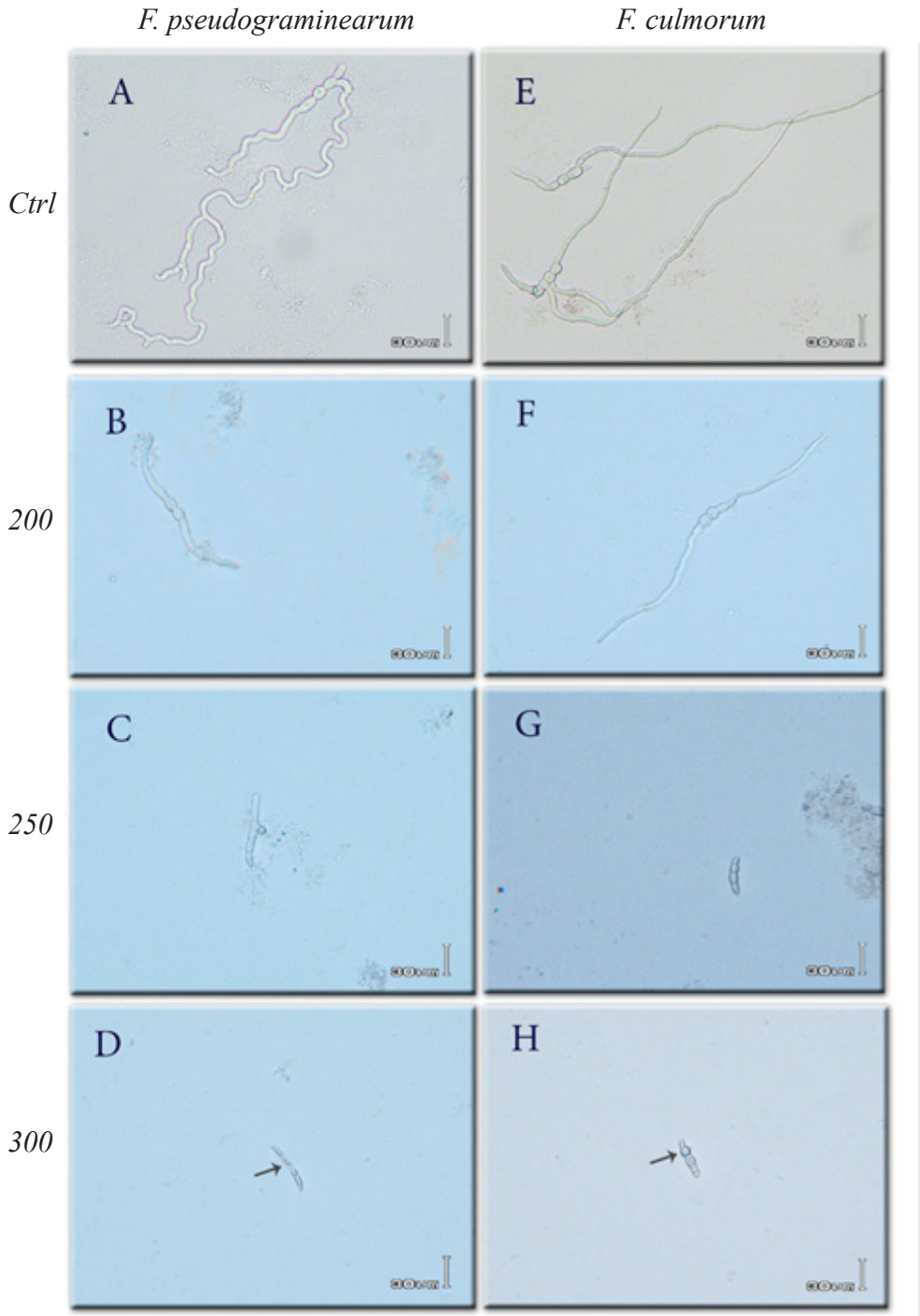

Figure 2. Effect of different concentrations (µL/L) of thyme essential oil on spore germination of *Fusarium pseudograminearum* (A-D) and *F. culmorum* (E-H). Arrows indicate morphological changes in macroconidia at concentration of 300µl/L in both fungi

Effect of the TEO on sporulation and spore germination of *Fusarium* isolates revealed that spore production and germination were significantly inhibited for both fungi in a dose-dependent manner. Spore germination was completely inhibited in both fungal species at 250µL/L concentrations of the TEO (Table 6). Whereas, spore production was completely inhibited at 150 and 200µL/L concentrations of the TEO for *F. pseudograminearum* and *F. culmorum*, respectively (Table 7).

Table 6. Inhibitory effect of different concentrations (µL/L) of the thyme essential oil on spore germination of *Fusarium pseudograminearum (F. p.)* and *F. culmorum (F. c.)*

	Ctrl	100	150	200	250	300
F. p.	0^{g}	60^{d}	88^{c}	91^{b}	100^{a}	100^{a}
F. c.	2^{g}	3.3^{g}	8^{f}	22^{e}	100^{a}	100^{a}

Values represent the mean of spore germination inhibition percentages. Values with the same letter do not differ significantly at P = 0.05 according to the Tukey's test. Ctrl: control

Table 7. Effect of different concentrations (µL/L) of the thyme essential oil on spore production of *Fusarium pseudograminearum (F. p) and F. culmorum (F. c)*

	Ctrl	100	150	200
F. p	9.5^{a}	1^{b}	0^{c}	NA^{*}
F. c	24^{a}	5^{b}	1^{c}	0^{d}

Values represent the number of conidia ($\times 10^{5}$) per mL. Values in each row with the same letter do not differ significantly at P = 0.05 according to the Tukey's test. Ctrl: control.
*** Not applicable because of complete inhibition of mycelial growth.**

3.5 Effects of the TEO on hyphal structure

Microscopic observations of vegetative growth of *F. pseudograminearum* and *F. culmorum* on PDA amended with different concentrations of the TEO showed degenerative changes in fungal hyphae. Controls grown on PDA (without the TEO) exhibited normal and smooth surfaced hyphae, but the hyphae grown in presence of the TEO were degraded, with large vesicles in their cells and these hyphal cells were shriveled. Additionally, under treatment of the TEO, fungal cell wall disruption and leakage of hyphal contents or necrosis were observed (Figure 3).

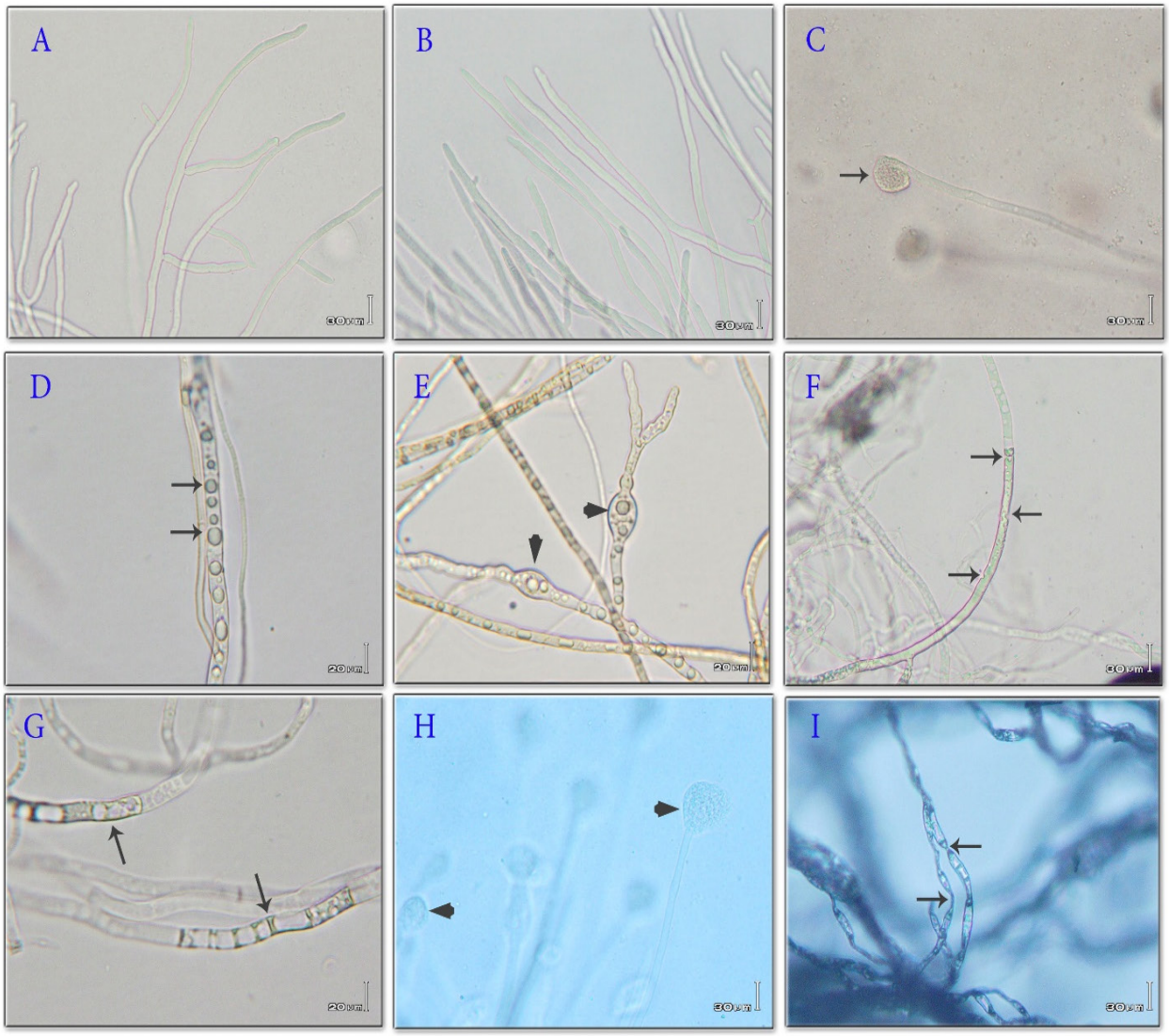

Figure 3. **Optical microscope investigation of vegetative growth of *Fusarium pseudograminearum and F. culmorum* under the thyme oil treatment at IC_{50} concentrations, (A and B) control, (C) hyphal leakage, (D) large vesicle in the cells, (E) swollen cells, (F) cell wall disruption, (G) hyphal necrosis, (H) hyphal leakage, and (I) shriveled hyphal cells**

3.6 Effect of the TEO on wheat seed conservation

The TEO was able to significantly reduce the percentage of infected wheat seeds ($P \leq 0.05$) compared to the control (Figure 4). The highest inhibtion of fungal infection was observed at 500µl/L concentration of the TEO with values of 90 and 100% for *F. culmorum* and *F. pseudograminearum*, respectively. On the other hand, no significant negative effect was observed on the germination of seeds treated with the TEO (Figure 5).

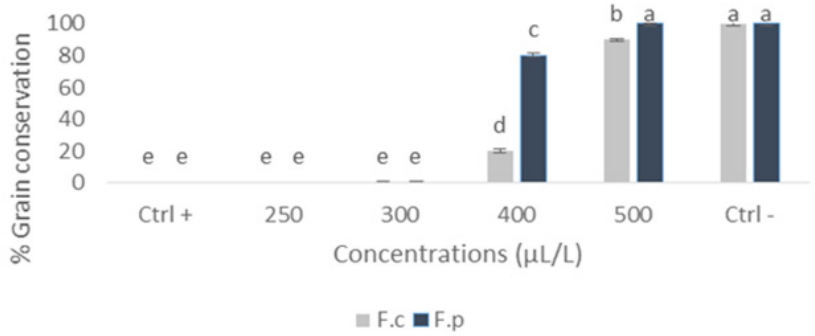

Figure 4. Effect of thyme essential oil (TEO) on wheat seed conservation against *Fusarium culmorum* (F.c) and *F. pseudograminearum* (F. p). Different letters indicate significant differences according to the Tukey's test at P = 0.05. Ctrl+: positive control (inoculated with the pathogens without the TEO treatment), Ctrl-: negative control (no fungal inoculation and no TEO treatment). The bars indicate standard errors (SE)

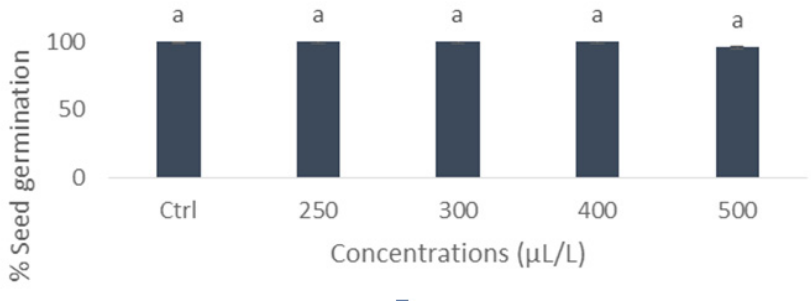

Figure 5. Effect of thyme essential oil on wheat seed germination. Different letters indicate significant differences according to the Tukey's test at P = 0.05. The bars indicate standard errors (SE)

3.7 Mycotoxin analysis by HPLC

Detecting mycotoxins producted by *Fusarium* isolates *in vitro* revealed that treating both fungal pathogens with the TEO and its main ingredients at very low concentration (0.01 IC50) without any visible effect on the hyphal growth, considerably reduced production of fungal mycotoxins such as DON, NIV, and ZEA, compared to the control (Table 8).

Table 8. Effect of the thyme essential oil (TEO) and its main ingredients at 0.01 IC50 concentration on production of mycotoxins by *Fusarium pseudograminearum and F. culmorum.*

	F. pseudograminearum			*F. culmorum*		
	DON	**NIV**	**ZEA**	**DON**	**NIV**	**ZEA**
Ctrl	60	ND	3221	1233	245	179
TEO	42	ND	2775	877	151	143
Carvacrol	33	ND	1689	752	79	96
Cymene	20	ND	1743	655	72	52
Gamma-Terpinene	21	ND	1875	672	51	67

The values presented in the table are µg kg−1 toxin production, in vitro. DON, deoxynivalenol; NIV, nivalenol; ZEA, zearalenone; ND, not detected.

3.9 Effect of the TEO on disease progress *in vivo*

Investigating the TEO effect on disease development caused by the fungal pathogens *in vivo* revealed reduction in the infection of root and crown in the wheat plants treated with the TEO compared to the controls (Table 9, Figure 6).

Table 9. Effect of different concentrations (µL/L) of the thyme essential oil (TEO) and the MIC concentration of the main components of the TEO on development of the disease caused by *Fusarium pseudograminearum and F. culmorum*

	F. pseudograminearum		*F. culmorum*	
	DI	**DSI**	**DI**	**DSI**
Ctrl	60.2 ± 1.2 [a]	78.3 ± 0.3 [a]	50.5 ± 1.4 [a]	65.5 ± 0.1 [a]
TEO 150	41.1 ± 0.8 [b]	55.5 ± 0.5 [b]	30.2 ± 0.2 [b]	48.1 ± 0.5 [b]
TEO 200	43.7 ± 0.2 [b]	50.1 ± 1.1 [b]	22.2 ± 0.1 [c]	39.6 ± 0.7 [b]
TEO 250	27.2 ± 0.9 [c]	41.2 ± 0.7 [c]	16.1 ± 0.8 [d]	20.1 ± 0.4 [c]
Carvacrol	21.4 ± 0.1 [cd]	40.8 ± 0.5 [c]	11.5 ± 0.5 [d]	12.5 ± 0.1 [d]
Cymene	12.5 ± 0.2 [d]	29.4 ± 0.7 [d]	7.4 ± 0.2 [e]	10.9 ± 0.5 [d]
Gamma-Terpinene	25.4 ± 0.7 [c]	39.5 ± 0.4 [c]	13.9 ± 0.1 [d]	12.9 ± 0.7 [d]

Values represent the mean of disease incidence (DI) and disease severity index (DSI). Values in each column with the same letter do not differ significantly at P = 0.05 according to the Tukey's test. Ctrl: control

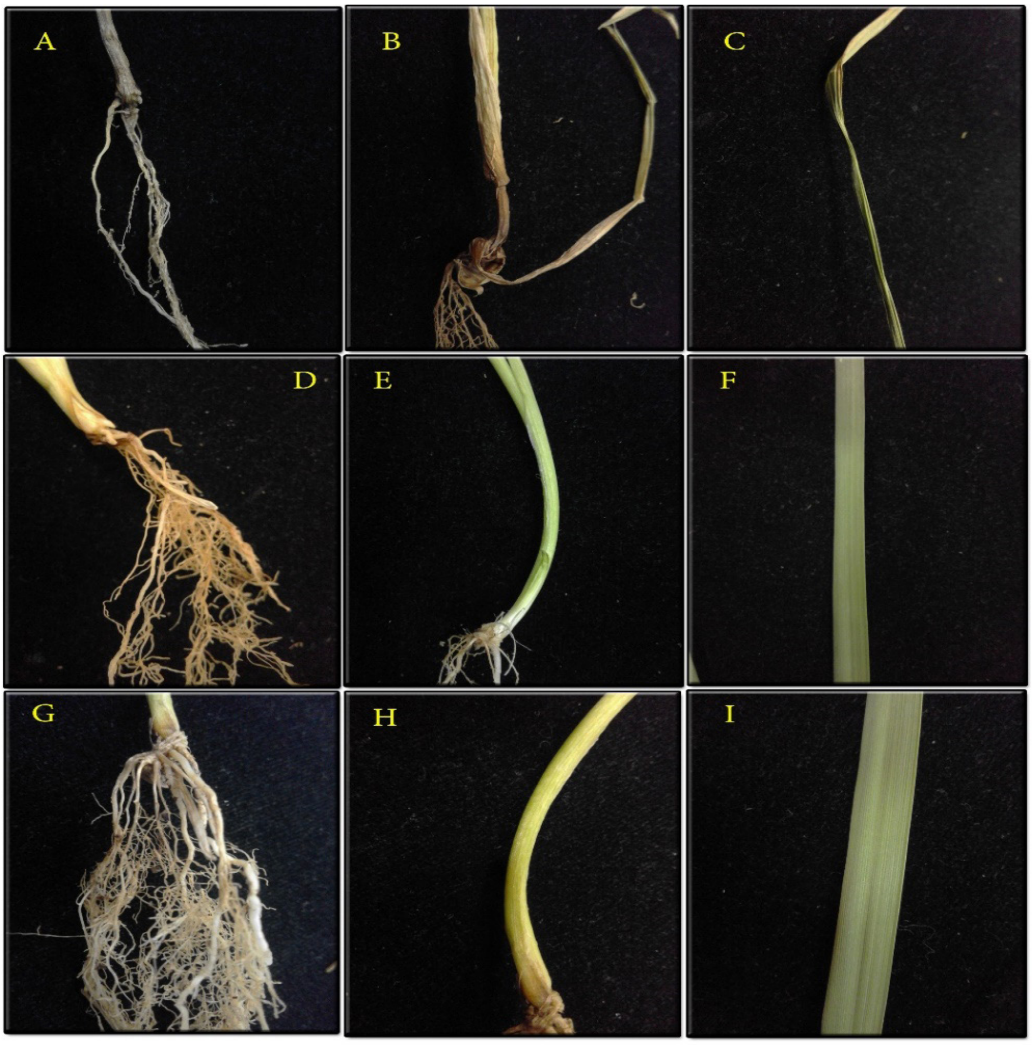

Figure 6. Effect of thyme essential oil (TEO) at 250µL/L concentration on development of the disease caused by *Fusarium* isolates on wheat. (A-C) root, crown and leaf of positive control plants (inoculated with *F. pseudograminearum* without the TEO treatment), (D-F) root, crown and leaf of negative control plants (healthy plants, without fungal inoculation and the TEO treatment), (G-I) root, crown and leaf of the TEO treated and inoculated plants.

As shown in Table 9 and Figure 6, disease incidence caused by the pathogens was not similar; since *F. pseudograminearum* was more potent for pathogenesis on wheat compared to *F. culmorum*. However, the TEO could significantly reduce disease incidence of both fungi. In the case of *F. pseudograminearum*, disease incidence percentage

was reduced by 18.9, 16.3 and 32.8% under treatment with 150, 200 and 250µL/L concentrations of the TEO, respectively, compraed to the control. For *F. culmorum*, reduction of disease incidence percentages were 20, 27.8 and 33.9% at concentrations of 150, 200 and 250µL/L of the TEO, respectively, compared to the control. The highest reduction of disease incidence was due to application of the TEO at 250µL/L concentration. Investigating effect of the main components of the TEO revealed that cymene had the highest effect in decreasing both disease incidence (DI) and disease severity index (DSI) on wheat plants inoculated by Fusarium species (Table 9). No sign of phytotoxicity was found on the tested plants at any concentration of the TEO and its main ingredients in this study.

4. Discussion

The widespread use of synthetic fungicides has significant disadvantages, including concern about fungicide residues on food, increase in agricultural costs, and harmful effects to human health and environment. Public awareness of these drawbacks has increased the interest in finding safe alternative protectants to be replaced with currently used chemical fungicides. Essential oils and their major components can be effectively used as alternative ways in management of plant diseases, since these compounds are 'eco-friendly' and because of their low-toxicity [27], and wide public acceptance [40, 41].

In this study, we investigated antifungal potential of the essential oils obtained from four different plant species on two important and phytopathogenic *Fusarium* species on wheat plants, including *F. pseudograminearum* and *F. culmorum*. Both fungi are the causal agents of "root and crown rot" and "head blight" on wheat. *In vitro* tests revealed that the TEO had the best potential to inhibit the growth of both fungi with slight differences in a dose dependent manner, compared to the other EOs tested. Investigating fungistatic and fungicidal effect of the TEO revealed that the TEO had fungicidal ability against both fungi. The TEO also showed strong inhibition on sporulation of both fungi, which could cause limitation on the spread of these pathogens by reducing the amount of spores as fungal inoculum. This effect might be due to the effects of volatile components of the essential oil on development of fungal mycelia (as the site of spore production) and/or the perception/transduction of signals involved in the switch from vegetative to reproductive phase [34]. Moreover, the other reason for inhibition of spore production could be mycelial destruction or fungal growth inhibition [42].

Spore germination inhibition was the other antifungal effect of the TEO. The results showed that spore germination in both fungi was completely inhibited by the TEO at concentrations higher than 250µl/L. In lower concentrations of the oil, the length of germ tube was significantly reduced. These findings suggest that in cases where secondary infection is dependent on the spore production and germination, the secondary infection will be significantly reduced by application of the TEO. Similar to our findings, Vitoratos et al. [43] reported significant inhibitory effects of the TEO on sporulation and vegetative growth of *Penicillium italicum* and *P. digitatum*, as the postharvest pathogens of various fruits and vegetables. Especially, *P. italicum* did not show any spore germination in presence of the TEO at concentration of 0.5µL/mL [43].

Microscopic observations of vegetative growth of *F. culmorum* and *F. pseudograminearum* treated with the TEO revealed clear changes in hyphal morphology. Degraded and shriveled hyphae with large vesicles were commonly observed in the hyphae treated with the TEO compared to the normal and smooth hyphae in control plates. The data obtained by microscopic analysis are in agreement with several former studies in which application of the EOs of medicinal plants led to morphological and ultrastructure changes in the fungal hyphae [25, 44, 45].

The *in vitro* experiment on the ability of TEO on wheat seed protection against fungal infection showed satisfactory antifungal efficacy, while no significant negative effect on seed germination was observed, promising the ability of the TEO as a post-harvest bio-fungicide for stored seeds. Seed coating with biostimulants and bioprotectors, such as various EOs, is among the major promising approaches in crop protection to increase plant tolerance/resistance to biotic and abiotic stresses. In a recent study carried out by Ben-Jabeur et al. [46], the effects of coating durum wheat seeds using the TEO on enhancing seed germination, seedling growth, and induction of drought resistance were evaluated. Their findings revealed that seed coating with the TEO can be highly effective in improving plant's water and nutrient status, which leads to growth promotion and to enhance drought resistance [46].

Various chemotypes have been described for *Thymus vulgaris* on the basis of the essential oil compositions, including (1) Borneol; (2) Linalool; (3) Geraniol; (4) Thymol; (5) Carvacrol and (6) Sabinene hydrate [47]. In the present study, GC-MS analysis results revealed that the thyme essential oil we used, was rich in carvacrol with the amount of 67.1%, which is in accordance with the finding of Kaloustian et al. [48], which showed 42% carvacrol in their "Carvacrol chemotypes" thyme. Various researchers represented that although all the chemotypes of *T. vulgaris* are antimicrobial, the highest levels of antimicrobial activities have been found in thymol and carvacrol chemotypes, which are rich in phenolic terpenoids [48]. In confirmation, many studies revealed that carvacrol has a wide spectral antimicrobial activities, such as antifungal, antibacterial and antioxidant activity [49, 50, 51].

HPLC analysis revealed that treating both *F. pseudograminearum* and *F. culmorum* with the TEO and its main ingredients (cymene, carvacrol, and gamma-terpinene) at very low concentrations without any direct effect on the hyphal growth, considerably reduced production of fungal mycotoxins such as DON, NIV, and ZEA. These mycotoxins are known as the major virulence factors of *Fusarium* spp. pathogenic on cereals [52] and also have harmful effects on the health of consumers [53, 54]. Reducing the levels of these hazardous fungal metabolites is not possible by other control strategies, such as application of chemical fungicides. Therefore, using environmentally friendly natural compounds, such as the TEO and its ingredients, can be a novel and effective method to manage the destructive wheat diseases caused by *Fusarium* spp. and also to decrease the levels of mycotoxins produced by these fungi in crop tissues.

In accordance to our study, various researchers have demonstrated the effect of numerous EOs obtained from medicinal and aromatic plants on *Fusarium* species. For example, Roselló et al. [26] assessed the antifungal effect of bay leaf, cinnamon, clove and oregano essential oils on *F. culmorum* and reported satisfactory antifungal effects of oregano oil on this fungus. In another study, Bouajaj et al. [55] examined the antifungal ability of *Ruta chalepensis* against *F. culmorum* and *F. pseudograminearum* and reported 100% inhibition for *F. culmorum* and antifungal activity against *F. pseudograminearum*. Investigating the effect of *Melaleuca alternifolia* essential oil on *F. culmorum* and *F. graminearum* revealed significant antifungal activity of this oil on both fungi [20]. In addition, other researchers have performed different studies on the antimicrobial properties of the TEO on different micro-organisms including *Aspergillus parasiticus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Staphylococcus aureus* [56, 57], *Escherchia coli*, *Klebsiella pneumonia*, *Enterococcus faecalis*, *Streptococcus pyogenes* [58], *Rhizoctonia solani*, *Macrophomina phaseolina* [25], *Aspergillus niger* [37], and *Penicillium* spp. [43], all indicating satisfactory and significant antimicrobial effects of the Thyme oil on various microorganisms studied, which is in accordance with our data. Sensitivity of numerous microorganisms towards TEO suggests that the mode of action does not seem to be very specific. To our knowledge, no scientific concerns about negative side effects of the TEO on non-target organisms are reported, so far. Only there are some concerns about negative side effects of using TEO in humans in some websites. Therefore, it seems to be necessary to investigate possible side effects of the thyme oil on various organisms.

In the *in vivo* experiments, which were performed to assess effect of the TEO on progress of the disease caused by both fungal isolates on wheat plants, the obtained results revealed that the TEO was capable of suppressing disease development. To justify the disease suppression by plant products, it is suggested that their main active components may act on the pathogen directly [59] or they might be involved in reducing disease progress by activating plant defense responses, which is necessary to be investigated in the future researches. Effects of the TEO for controlling tomato late blight, caused by *Phytophthora infestans*, under field conditions has been investigated by El-Mahamedy and Abd-El-Latif [60], who demonstrated moderate effect of the TEO on controlling late blight disease in tomato fields. But to our knowledge, there is no report on the effect of the TEO for protecting any plant species against diseases caused by *Fusarium* spp. in the field conditions. Therefore, our knowledge on influence of the TEO and its major ingredients in protecting wheat plants against the pathogens in greenhouse and field conditions might be helpful to design novel and effective disease management strategies.

In overall, the present study clearly indicates that the EO obtained from the thyme plant has strong antifungal properties against both *F. pseudograminearum* and *F. culmorum,* as destructive wheat pathogens. The use of essential oils as antifungal agents might be suitable for applications in disease management strategies and also in the food industries, which needs to be accurately tested according to the same standards applied for other antifungal chemicals. These natural plant products and their active components could be successfully used instead of synthetic chemicals, leading to have a clean world without hazardous toxic compounds.

Acknowledgements

We thank Ferdowsi University of Mashhad, Iran, for financial support of this research with the project number 3/40744 approved on 27/4/2016. The authors have no conflict of interest to declare.

References

[1] Tadesse W., Amri A., Ogbonnaya F.C., Sanchez-Garcia M., Sohail Q., and Baum M. Wheat: Genetic and Genomic Resources for Grain Cereals Improvement, Academic Press, San Diego; 2016.

[2] Braun H.J., Atlin G., Payne T. Multi-location testing as a tool to identify plant response to global climate change. *Climate Change and Crop Production.* 2010; 1: 115-138.

[3] Dixon J., Braun H., Crouch J. Overview: transitioning wheat research to serve the future needs of the developing world: Wheat facts and futures, D. F: CIMMYT, Mexico; 2009.
[4] Soliman K., and Badeaa R. Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. *Food and Chemical Toxicology.* 2002; 40(11): 1669-1675.
[5] Mitter V., Zhang M.C., Liu C.J., Ghosh R., Ghosh M., and Chakraborty S. A high-throughput glasshouse bioassay to detect crown rot resistance in wheat germplasm. *Plant Pathology*. 2006; 55(3): 433-441.
[6] Pestka, J. J. Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. *Archives of toxicology.* 2010; 84(9): 663-679.
[7] Smiley R.W., and Patterson L-M. Pathogenic fungi associated with *Fusarium* foot rot of winter wheat in semiarid Pacific Northwest USA. *Plant Disease.* 1996; 80: 944-949.
[8] Burgess L.W., Backhouse D., Summerell B.A., Swan L.J. *Crown rot of wheat*: *Fusarium*. APS Press, USA; 2001.
[9] Akinsanmi OA., Mitter V., Simpfendorfer S., Backhouse D., Chakraborty S. Identity and pathogenicity of *Fusarium* spp. isolated from wheat fields in Queensland and northern New South Wales. *Australian Journal of Agricultural Research.* 2004; 55(1): 97-107.
[10] Aoki T., O'Donnell K. Morphological and molecular characterization of *Fusarium pseudograminearum* sp. nov., formerly recognized as the Group 1 population of *F. graminearum*. *Mycologia.* 1999; 91: 597-609.
[11] Shaner G. *Epidemiology of Fusarium head blight of small grain cereals in North America: Fusarium head blight of wheat and barley.* APS Press, USA; 2003.
[12] McMullen M., Halley S., Schatz B., Meyer S., Jordahl J., and Ransom J. Integrated strategies for *Fusarium* head blight management in the United States. *Cereal Research Communications.* 2008; 36(supplement 6): 563-568.
[13] Trail F. For blighted waves of grain: *Fusarium graminearum* in the postgenomics era. *Plant Physiology.* 2009; 149(1): 103-110.
[14] Khan N., Schisler D., Boehm M., Lipps P., and Slininger P. Field testing of antagonists of *Fusarium* head blight incited by *Gibberella zeae*. *Biological Control.* 2004; 29(2): 245-255.
[15] Chakraborty S., Liu C., Mitter V., Scott J., Akinsanmi O., Ali S., Simpfendorfer S. Pathogen population structure and epidemiology are keys to wheat crown rot and *Fusarium* head blight management. *Australasian Plant Pathology.* 2006; 35(6): 643-655.
[16] Palazzini J.M., Torres A.M., and Chulze S.N. Biological control of *Fusarium* head blight of wheat: from selection to formulation *Fusarium* Head Blight in Latin America, Springer; 2013.
[17] Jochum C., Osborne L., and Yuen G. *Fusarium* head blight biological control with *Lysobacter enzymogenes* strain C3. *Biological Control.* 2006; 39(3): 336-344.
[18] Khan M.R., and Doohan F.M. Bacterium-mediated control of *Fusarium* head blight disease of wheat and barley and associated mycotoxin contamination of grain. *Biological Control.* 2009; 48(1): 42-47.
[19] Khaledi N., Taheri P., and Falahati Rastegar M. Identification, virulence factors characterization, pathogenicity and aggressiveness analysis of *Fusarium* spp., causing wheat head blight in Iran. *European Journal of Plant Pathology.* 2017; 147: 897-918.
[20] Terzi V., Morcia C., Faccioli P., Vale G., Tacconi G., and Malnati M. In vitro antifungal activity of the tea tree (*Melaleuca alternifolia*) essential oil and its major components against plant pathogens. *Letters in Applied Microbiology.* 2007; 44(6): 613-618.
[21] Soltani Nejad, M., Soltanzadeh, M., & Shahidi Bonjar, G. H. Application of Soil□borne Actinomycetes for Biological Control against Fusarium Wilt of Chickpea (*Cicer arietinum*) caused by *Fusarium solani* fsp *pisi*. *Journal of Phytopathology.* 2016; 164(11-12): 967-978.
[22] Leite S.E., Montenegro S.T.L., and de Oliveira I.E. Sensitivity of spoiling and pathogen food related bacteria to *Origanum vulgare* L. (Lamiaceae) essential oil. *Brazilian Journal of Microbiology.* 2006; 37: 527-532.
[23] Daferera D.J., Ziogas B.N., Polissiou M.G. GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on *Penicillium digitatum*. *Journal of Agricultural and Food Chemistry.* 2000; 48(6): 2576-2581.
[24] Sumalan R.M., Alexa E., and Poiana M.A. Assessment of inhibitory potential of essential oils on natural mycoflora and *Fusarium* mycotoxins production in wheat. *Chemistry Central Journal.* 2013;7(1), 32, doi: 10.1186/1752-153X-7-32.
[25] Khaledi N., Taheri P., and Tarighi S. Antifungal activity of various essential oils against *Rhizoctonia solani* and *Macrophomina phaseolina* as major bean pathogens. *Journal of Applied Microbiology.* 2015; 118(3): 704-717.
[26] Roselló J., Sempere F., Sanz-Berzosa I., Chiralt A., and Santamarina M.P. Antifungal activity and potential use of essential oils against *Fusarium culmorum* and *Fusarium verticillioides*. *Journal of Essential Oil Bearing Plants.* 2015; 18(2): 359-367.
[27] Isman, M. B. Plant essential oils for pest and disease management. *Crop Protection.* 2000; 19(8-10): 603-608.
[28] Tripathi, P., Dubey, N., & Shukla, A. Use of some essential oils as post-harvest botanical fungicides in the

management of grey mould of grapes caused by Botrytis cinerea. *World Journal of Microbiology and Biotechnology.* 2008; 24(1): 39-46.
[29] Dehghanpour-Farashah S., Taheri P., Falahati-Rastegar M. Identification and pathogenicity of Fusarium spp., the causal agent of wheat crown and root rot in Iran. *Journal of Plant Pathology*. 2019; doi: 10.1007/s42161-019-00400-9.
[30] Feng W., and Zheng X. Essential oils to control *Alternaria alternata* in vitro and in vivo. *Food Control.* 2007; 18(9): 1126-1130.
[31] Albuquerque C. C. d., Camara T. R., Mariano R. d. L. R., Willadino L., Marcelino Júnior C., Ulisses C. Antimicrobial action of the essential oil of *Lippia gracilis* Schauer. *Brazilian Archives of Biology and Technology.* 2006; 49(4): 527-535.
[32] Thompson D.P. Fungitoxic Activity of Essential Oil Components on Food Storage Fungi. *Mycologia.* 1989; 81(1):151-153, doi: 10.2307/3759462.
[33] Singh G., Kapoor I.P.S., Singh P., de Heluani C.S., de Lampasona M.P., and Catalan C.A.N. Chemistry, antioxidant and antimicrobial investigations on essential oil and oleoresins of *Zingiber officinale*. *Food and Chemical Toxicology.* 2008; 46(10): 3295-3302.
[34] Tzortzakis N.G., and Economakis C.D. Antifungal activity of lemongrass (*Cympopogon citratus* L.) essential oil against key postharvest pathogens. *Innovative Food Science & Emerging Technologies* 2007; 8(2): 253-258.
[35] Sharma A., Rajendran S., Srivastava A., Sharma S., and Kundu B. Antifungal activities of selected essential oils against *Fusarium oxysporum* f. sp. *lycopersici* 1322, with emphasis on *Syzygium aromaticum* essential oil. *Journal of Bioscience and Bioengineering.* 2017; 123(3): 308-313.
[36] Anžlovar S., Likar M., Dolenc Koce J. Antifungal potential of thyme essential oil as a preservative for storage of wheat seeds. *Acta Botanica Croatica.* 2017; 76(1): 64-71.
[37] Kumar P., Mishra S., Kumar A., Kumar S., and Prasad C.S. *In vivo* and *in vitro* control activity of plant essential oils against three strains of *Aspergillus niger*. *Environmental Science and Pollution Research.* 2017; 24(27): 21948-21959.
[38] Amadioha A. C. Controlling rice blast in vitro and in vivo with extracts of *Azadirachta indica*. *Crop Protection.* 2000; 19(5): 287-290.
[39] Taheri P., and Tarighi S. Riboflavin induces resistance in rice against *Rhizoctonia solani* via jasmonate-mediated priming of phenylpropanoid pathway. *Journal of Plant Physiology.* 2010; 167(3): 201-208.
[40] Kalemba D., and Kunicka A. Antibacterial and antifungal properties of essential oils. *Current medicinal chemistry.* 2003; 10(10): 813-829.
[41] Burt S. Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology.* 2004; 94(3): 223-253.
[42] Tantaoui-Elaraki A., Ferhout H., and Errifi A. Inhibition of the fungal asexual reproduction stages by three Moroccan essential oils. *Journal of Essential Oil Research.* 1993; 5(5): 535-545.
[43] Vitoratos A., Bilalis D., Karkanis A., Efthimiadou A. Antifungal activity of plant essential oils against *Botrytis cinerea*, *Penicillium italicum* and *Penicillium digitatum. Notulae Botanicae Horti Agrobotanici Cluj-Napoca.* 2013: 41(1): 86-92.
[44] Romagnoli, C., Bruni, R., Andreotti, E., Rai, M.K., Vicentini, C.B. and Mares, D. Chemical characterization and antifungal activity of essential oil of capitula from wild Indian Tagetespatula L. Protoplasma. 2005; 225: 57-65.
[45] Soylu, E.M., Kurt, S. and Soylu, S. In vitro and in vivo antifungal activities of the essential oils of various plants against tomato grey mould disease agent *Botrytis cinerea*. *Int J Food Microbiol.* 2010; 143: 183-189.
[46] Ben-Jabeur M., Kthiri Z., Harbaoui K., Belguesmi K., Serret M. D., Araus J. L. and Hamada W. Seed coating with thyme essential oil or *Paraburkholderia phytofirmans* PsJN strain: conferring Septoria leaf blotch resistance and promotion of yield and grain isotopic composition in wheat. *Agronomy.* 2019; 586, doi:10.3390/agronomy9100586.
[47] Satyal P., Murray B. L., McFeeters R. L. and Setzer W. N. Essential oil characterization of *Thymus vulgaris* from various geographical locations. *Foods.* 2016; 70, doi:10.3390/foods5040070.
[48] Kaloustian J., Abou L., Mikail C., Amiot M. J. and Portugal H. Southern French thyme oils: chromatographic study of chemotypes. *Journal of Science of Food and Agriculture.* 2005; 85: 2437-2444.
[49] Chami, N., Chami, F., Bennis, S., Trouillas, J., & Remmal, A. Antifungal treatment with carvacrol and eugenol of oral candidiasis in immunosuppressed rats. *Brazilian Journal of Infectious Diseases*. 2004; 8(3): 217-226.
[50] Koparal, A. T., & Zeytinoğlu, M. Effects of carvacrol on a human non-small cell lung cancer (NSCLC) cell line, A549 Animal Cell Technology: Basic & Applied Aspects, Springer; 2003. p. 207-211.
[51] Xu, J., Zhou, F., Ji, B. P., Pei, R. S., & Xu, N. The antibacterial mechanism of carvacrol and thymol against Escherichia coli. *Letters in applied microbiology*. 2008; 47(3): 174-179.
[52] Taheri P. Cereal diseases caused by Fusarium graminearum: from biology of the pathogen to oxidative burst-related host defense responses. *European Journal of Plant Pathology.* 2018; 152: 1-20.

[53] Ismaiel, A. A., and Papenbrock, J. Mycotoxins: producing fungi and mechanisms of phytotoxicity. *Agriculture*. 2015; 5(3): 492–537.
[54] Sudakin, D. L. Trichothecenes in the environment: relevance to human health. *Toxicology Letters*. 2003; 143(2): 97-107.
[55] Bouajaj S., Romane A., Benyamna A., Amri I., Hanana M., Hamrouni L., Romdhane M. Essential oil composition, phytotoxic and antifungal activities of Ruta chalepensis L. leaves from high atlas mountains (Morocco). *Natural Product Research.* 2014; 28(21): 1910-1914.
[56] Rota M.C., Herrera A., Martínez R.M., Sotomayor J.A., and Jordán M.J. Antimicrobial activity and chemical composition of Thymus vulgaris, Thymus zygis and Thymus hyemalis essential oils. *Food Control.* 2008; 19(7): 681-687.
[57] Benmoussa H., Elfalleh W., Farhat A., Bachoual R., Nasfi Z., Romdhane M. Effect of extraction methods on kinetic, chemical composition and antibacterial activities of Tunisian Thymus vulgaris. L. essential oil. *Separation Science and Technology.* 2016; 51(13): 2145-2152.
[58] Nezhadali A., Nabavi M., Rajabian M., Akbarpour M., Pourali P., and Amini F. Chemical variation of leaf essential oil at different stages of plant growth and in vitro antibacterial activity of Thymus vulgaris Lamiaceae, from Iran. *Beni-Suef University Journal of Basic and Applied Sciences.* 2014; 3(2): 87-92.
[59] Baraka M., Omar S., El-Barougy E., Zian A. Controlling seedling damping-off, root rot and wilt diseases of lupine (*Lupinus albus* L.). *Agricultural Research Journal Suez Canal University.* 2006; 6: 57-68.
[60] El-Mohamedy R. S. R. and Abd-El-latif F. M. Field application of humic acid and thyme essential oil for controlling late blight disease of tomato plants under field conditions. *Asian Journal of Plant Pathology*. 2015; 9(4): 167-174.