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Research Paper

Essential oil from fennel seeds (*Foeniculum vulgare*) reduces Fusarium wilt of tomato (*Solanum lycopersicon*)

Fatma KALLELI[1,2], Ghassen ABID[3], Ibtissem BEN SALEM[4], Naïma BOUGHALLEB-M'HAMDI[4], Mahmoud M'HAMDI[1,2,*]

[1] *Department of Horticultural Sciences and Vegetable Crops, High Institute of Agronomy of Chott Mariem, University of Sousse, 4042 Sousse, Tunisia*
[2] *Horticultural Science Laboratory,National Agronomic Institute of Tunisia. University of Carthage, Tunisia*
[3] *University of Tunis El Manar, Center of Biotechnology of Borj Cedria, Laboratory of Legumes, BP 901, 2050 Hammam-Lif, Tunisia*
[4] *Department of Biological Sciences and Plant Protection, High Institute of Agronomy of Chott Mariem, University of Sousse, UR13AGR03, 4042 Sousse, Tunisia*
* Corresponding author: address: mhamdimahmoud@yahoo.fr

Summary. Natural products have been considered a viable alternatives for managing plant diseases. This study investigated *in vitro* and *in planta* antifungal activity of an essential oil from fennel seeds against *Fusarium oxysporum* f. sp. *lycopersici* (FOL). The essential oil inhibited FOL *in vitro* mycelium growth by up to 83% and sporulation by up to 97%. Protective effects against *Fusarium* wilt were observed *in planta,* especially when the oil was applied curatively at a concentration of 500µl mL^{-1}. The fennel essential oil reduced disease severity from 98% in untreated FOL-inoculated plants to 57% in plants inoculated with FOL and treated with the oil at 8 weeks after of inoculation. GC-MS spectrometry analyses showed that the major chemical components in the essential oil were *trans*-anethole (78%), fenchone (11%), estragole (5%) and limonene (4%). Applications of the essential oil resulted in increased soluble sugars, total phenolic and total flavonoid contents in leaves compared with untreated inoculated (control) plants. The defence-related genes, such as those encoding pathogenesis-related (*SlPR1*) proteins, *SlWRKY*, thaumatin-like protein (*SlTLP*), lipoxygenase (*SlLOX*), ethylene response factor (*SlERF*) and chitinase (*SlChi*), were differentially expressed. This study has indicated that essential oil from fennel seeds has potential as a control agent against *Fusarium* wilt of tomato.

Keywords. Antifungal activity, defence related genes, plant disease, phytochemicals, RT-qPCR.

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INTRODUCTION

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL) is one of the most aggressive and destructive soil-borne diseases affecting tomato. FOL infects host roots and colonizes the xylem vessels, causing wilting, stunting and death of plants, and results in significant economic losses

(Salim *et al*., 2016). Integrated management approaches against FOL, such as cultural practices, soil disinfestation, crop rotations, resistant cultivars, and chemical treatments, were reported to be inefficient in reducing the incidence and severity of the disease, due to the production of resistant spores by the pathogen (chlamydospores) and the emergence of new races (Amini and Sidovich, 2010). The use of synthetic fungicides may also lead to adverse effects on human health and the environment, due to their high toxicity, slow degradation and bioaccumulation (Mohammadi and Aminifard 2013). Therefore, development of new "green" technologies for plant disease management based on biostimulant and biocontrol tools, could be reliable for reducing the amount of chemical residues in the environment (Dihazi *et al*., 2011). In plants, infection with microorganisms induces secondary metabolic pathways. The production of secondary metabolites, including antifeedants, phytoanticipins and phytoalexins, enhances plant defence systems (Pino *et al*., 2013). Medicinal and aromatic plants are important rich sources of plant secondary metabolites, which exhibit antifungal activity against numerous phytopathogenic fungi, and may induce systemic acquired resistance processes (Pusztahelyi *et al*., 2015). These bioactive products are biodegradable, sustainable for integrated disease management, and are generally less toxic than synthetic products towards nontarget species.

Essential oils are complex organic substances, biosynthesized through secondary metabolic pathways of plants, which play important roles as signaling molecules in plant defence against bio-aggressors including pest insects, fungi, bacteria and viruses (Rehmana *et al,*. 2016). According to Zake (2016), essential oils from aromatic and medicinal plants exhibited *in vitro* and *in vivo* activity against plant pathogenic fungi, and can be used as bio-fungicide products. Essential oils have been used in various contexts and were proposed as possible tools for safe and sustainable alternative agriculture. This is because they possess various agronomic activities, including antimicrobial, antiviral, antifungal, insecticidal and herbicidal, they have better biodegradability compared to conventional synthetic fungicides (Arshad *et al*., 2014).

Several studies have demonstrated that plant hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) could play crucial roles in plant defence signalling (Yang *et al.,* 2012). Several families of defence-response (DR) genes, including plant transcription factors and signalling molecules such as peroxidase, PR1, PR10, chitinase, osmotin, thionin, SAR8.2 and defensin, were shown to be associated with plant responses to diverse biotic stresses (Lee and Hwang, 2005).

Fennel (*Foeniculum vulgare* Mill), belonging to the *Umbelliferae*, is a perennial or annual herbaceous plant that grows in several regions of the world (Ozcan *et al.,* 2006). Roby *et al.* (2013) reported that essential oils from fennel showed different degrees of antimicrobial and antioxidant activities depending on the applied doses. Essential oil from fennel was reported to possess antifungal activity by reducing the mycelium growth and germination of *Sclerotinia sclerotiorum* (Soylu *et al*., 2007). The objectives of the present study were to (i) evaluate the effectiveness of essential oil from local fennel seeds for inhibition of hyphal growth of *Fusarium oxysporum* f. sp. *lycopersici, in vitro*; (ii) reduce the *in planta* severity of *Fusarium* wilt of tomato; and (iii) determine the expression of defence-related genes in tomato**.**

MATERIALS AND METHODS

Plant material

Fully ripened fennel seeds used in this study were collected from cultivated plants from the experimental station of the Higher Agronomic Institute of Chott Mariem, Sousse, Tunisia. Collected seeds were dried at room temperature and stored in polyethylene bags at 4°C until use.

Isolation of essential oil

The essential oil was obtained from fennel seeds by hydro-distillation using a Clevenger apparatus, according to Bettaieb *et al.* (2011). The obtained oil was dried over anhydrous sodium sulphate and stored at 4°C after filtration, until used in tests and analyses.

Identification and quantification of the oil components

Analyses of the essential oil were performed using an Agilent 7890A gas chromatograph (GC) coupled to an Agilent 5972C mass spectroscopy detector with electron impact ionization (70 eV). A HP-5 MS capillary column (30 m × 0.25 mm, coated with 5% phenyl methyl silicone, 95% dimethylpolysiloxane, 0.25 µm film thickness; Agilent Technologies) was used to separate compounds. Components were identified based on the comparison of their mass spectra with Wiley Registry 9th Edition/ NIST 2011 mass spectral library, and from comparison

of their retention indices either with those of authentic compounds or with literature values. Relative percentage amounts of the identified compounds were obtained from the electronic integration of the peak areas without the use of the correction factor.

In vitro *antifungal effects of fennel essential oil*

Fungal culture. Isolate FO14 of *Fusarium oxysporum* f. sp. *lycopersici*, obtained from tomato, was tested for its pathogenicity. The isolate was identified morphologically (using microscopy) according to Leslie and Summerell (2006), and molecularly by sequencing the 18S rDNA using ITS1/ITS4 primers (White *et al*., 1990). The pure culture was maintained on potato dextrose agar (PDA) and stored in glycerol at -20°C until required.

Mycelium growth inhibition. The fennel essential oil was incorporated into molten PDA at desired final concentrations, and well mixed. Different concentrations of essential oil were prepared by dissolving the required amounts in sterile PDA amended with Tween 20 (0.1%, v/v) to obtain the desired concentrations (C0, negative control; C1, 15.6 µL mL^{-1}; C2, 31.3; C3, 62.5; C4, 125; C5, 250 or C6, 500 µL mL^{-1}). The medium was then poured into Petri dishes. After overnight pre-incubation, the centre of each plate was inoculated with a mycelium plug (0.7 mm diam.) from a 5-day-old culture, and the plates were then incubated at 28°C. Mycelium growth was assessed daily by measuring the horizontal and vertical diameters of the colony in each plate, and antifungal activity was assessed 6 d post-inoculation (DPI) according to Soylu *et al*. (2007). For each essential oil concentration treatment, five plates were used. The experiment was conducted twice independently. The percentage of mycelium growth inhibition (MGI) was determined according to the formula:

$$MGI\ (\%) = ((dc-dt)/dc) \times 100;$$

where dc was the diameter of the control (C0) colony, and dt was the diameter of the essential oil treated colony.

Sporulation test. Assessment of sporulation was carried out according to the method of Kanoun *et al*. (2014). Each entire Petri dish (15-d after inoculation) containing the fungus was washed with 10 mL of sterile distilled water to release all the conidia. Subsequently, a pooled conidial suspension was obtained by mixing all five plates (50 mL). The number of conidia (conidia mL^{-1}) for each treatment (three replicates) was counted with a Malassez cell using a light microscope.

In planta *antifungal effects of fennel essential oil*

Fungal inoculum. Inocula of FOL were prepared by culturing the isolate in potato dextrose broth (PDB) amended with streptomycin sulfate, and with agitation at 25 ± 2°C for 5 d. Conidia were counted using a Malassez cell and microscopy, and appropriate dilutions were made to adjust the inoculum to a concentration of 10^6 conidia mL^{-1} with sterile distilled water.

Host plant material, growth conditions and treatments. Five-week-old seedlings of tomato 'Murano' (susceptible to FOL) were transplanted into 20 cm diam. plastic pots (one plant/pot), which were arranged in a randomized complete block design with three replicates per treatment. The seedlings were then grown in a greenhouse at 30/25°C (day/night) and 14 h photoperiod, with relative humidity of 70 ± 10%.

All the tomato seedlings were drench-inoculated with 1 mL of FOL conidium suspension (10^6 mL^{-1}) around the seedling rhizospheres. For a preventive treatment, 50 mL of fennel essential oil at 500 µL mL^{-1} was applied as a soil drench, 1 week before inoculation. The effects of a curative treatment were also evaluated, with an application of the essential oil preparation 2 weeks after inoculation. Plants were irrigated with distilled water served as untreated experimental controls. All combinations of treatments are listed in Table 1 and the experiment was repeated twice.

Table 1. Experimental treatments applied to tomato seedlings.

T1	Plants non inoculated and non-treated.
T2	Plants non-inoculated and treated with fungicide: Tachigaren fungicide was applied by soil drench two weeks after inoculation with FOL at a dose of 0.25 mL/plant.
T3	Plants non-inoculated and treated with essential oil by soil drench application.
T4	Plants were drench-inoculated with 1 mL of conidium suspension of FOL (10^6 conidia. mL^{-1}) around the tomato rhizosphere.
T5	Plants inoculated with FOL and treated with fungicide by soil drench application.
T6	Plants inoculated with FOL and treated with essential oil by soil drench application 1 week before inoculation.
T7	Plants inoculated with FOL and treated with essential oil by soil drench application 2 weeks after inoculation.

Disease severity assessments

The severity of Fusarium wilt was estimated according the scale of Song *et al.* (2004), where: 1 = healthy, no sign of symptoms; 2 = slight symptoms, mainly on lower leaves (about 25% of full scale); 3 = advanced symptoms (≅50%); 4 = extensive symptoms (≅75%); and 5 = entire plant affected (dead plant). Four plants were randomly chosen from each treatment in each experiment (eight in total). Disease severity (%) was calculated from the disease rating using the following formula:

Disease severity (%) = (∑ scale number of infected plants) * 100 / (greatest scale * total number of plants).

The disease severity assessments were carried out 60 d after plant inoculation. Re-isolation of the pathogen was accomplished at the end of the assay to confirm Koch's postulates.

Biochemical analyses

Preparation of tomato leaf extracts. For each treatment, tomato leaves from five plants were collected 3, 5 or 7 weeks after inoculation in the greenhouse experiment. Sample extracts were obtained following Mau *et al.* (2001) with slight modifications. To prepare the extracts, 2.5 g of dry leaf powder was extracted with 25 mL of methanol (60%) solvent. Each mixture was then magnetically stirred for 30 min and the extracts were then kept at 4°C for 24h in darkness. The mixtures were then filtered through a Whatman No. 4 filter paper and evaporated to dryness under vacuum. The extracts thus obtained were stored at 4°C for further use.

Determination of total phenolic contents. The total phenolic content of the tomato leaf extracts was determined using Folin-Ciocalteu reagent, according to Falleh *et al.* (2008). Absorbance was determined against a blank at 760 nm using UV-visible spectroscopy. The content of total phenolic compound in each extract was expressed as mg of gallic acid equivalents per g fresh weight (mg GAE g^{-1} FW) from a calibration curve with gallic acid. All determinations were carried out in triplicate. Gallic acid was used as the reference standard for plotting the calibration curve.

Determination of total flavonoid contents. The total flavonoid content in methanolic extracts of leaves was determined with a colorimetric assay, using a method described by Faudale *et al.* (2008). Briefly, 1 mL of methanolic extract from each sample was mixed with 5 mL of distilled water, followed by addition of 0.3 mL of a 5% (w/v) $NaNO_2$ solution. After 6 min, 0.6 mL of $AlCl_3$ (10% m/v) was added. After 5 min of incubation at room temperature, 2 mL of NaOH (1 M) was added. The final volume was adjusted to 10 mL with the addition of distilled water. The mixture was homogenized and the absorbance was determined at 510 nm. Total flavonoid content was measured using a quercetin calibration curve, and was expressed as mg of quercetin equivalents (QE) per g of dry weight (mg QE g^{-1} FW). All samples were analyzed in three replications.

Determination of soluble sugars. The soluble sugars were estimated using the colorimetric method described by Dubois *et al.* (1956). For each sample, 100 mg of dried leaf material was homogenized with 3 mL of 80% ethanol (v/v) and then mixed with concentrated sulfuric acid and 5% phenol. The mixture was kept for 1 h and then the absorbance at 490 nm was determined with a spectrophotometer. Glucose was used as standard. Contents of soluble sugars were expressed as mg g^{-1} FW. Analyses were carried out in triplicate.

Expression of defence-related genes

RNA isolation and cDNA synthesis. For quantitative real-time PCR, tomato leaf tissues (from treatments T1, T2, T3, T4, T5 and T7) were collected at 0, 6, 12, 24, 48 or 72 h post treatment (HPT). Two hundred milligrams of each sample was ground to a fine powder using liquid nitrogen and transferred into a 2 mL centrifuge tube for total RNA extraction following the protocol described by Chang *et al.* (1993). The quantity and quality of total RNA were assessed on a NanoDrop Spectrophotometer and by electrophoresis in a 1.2% agarose gel. Genomic DNA contamination was removed by treating the samples with 1 µL of DNase I, RNase-free (5 U μL^{-1}) (Biomatik) at 37°C for 30 min. First-strand cDNA was synthesized from 5 µg of total RNA using 200 U Turbo-I reverse transcriptase (Biomatik) according to the manufacturers' instructions.

Quantitative RT-PCR (RT-qPCR) analysis. Gene-specific primer pairs for *SlChi*, *SlERF*, *SlPR1*, *SlLOX*, *SlTLP* and *SlWRKY* genes used in this study were designed using the Primer3 Input (version 0.4.0) software (Rozen and Skaletsky 2000) (http://frodo.wi.mit.edu/primer3/), with default criteria of the software and with amplified products ranging from 80 to 150 bp and Tm approx. 60°C. Actin was used as a control to normalize the samples. The primer sequences are listed in Table 2. RT-qPCR was performed in a 7300 Real-Time PCR System (Applied Biosystems) using the Maxima SYBR Green/ROX qPCR Master Mix (2X) kit (Biomatik). Each 20 µL reaction mix contained 10 µL Maxima SYBR Green/ROX qPCR Master Mix (2X), 1 µL of each primer at 10 µM (Table 2), 6 µL dd H_2O and 2 µL cDNA (50 ng). The reactions were performed in trip-

Table 2. Sequences of the primers used for RT-qPCR analysis.

Gene name	GenBank accession ID	Forward and reverse primer 5'-3'	Amplicon size (bp)
SlChi	U30465	Forward: 5'-ATCGGCACACGATGTCATTA-3' Reverse: 5'-TGAACCGCTGTTACATTCCA -3'	127
SlERF	NM_001247384	Forward: 5'-CCCAATTTCCCGTTACTTCA-3' Reverse: 5'-GCCTTCTCCTTACCCCTCTG-3'	149
SlPR1	NM_001247429	Forward: 5'-ACGTCTTGGTTGTGCTAGGG -3' Reverse: 5'-TCAAAAGCCGGTTGATTTTC -3'	126
SlLOX	XR_183132	Forward: 5'-TGGGATTAAACTGCCAGACC-3' Reverse: 5'-GGCATCGGAAATTTGAGAAA-3'	114
SlWRKY	XM_004232149	Forward: 5'-TCTCGATCTGACCAGGTTCC-3' Reverse: 5'-TTGCCGTCTTCGTTCTCTTT-3'	139
SlTLP	XM_004235762	Forward: 5'-CCATCTTTGCTTCCCACATT-3' Reverse: 5'-ATCGGTTTACCTGCACTTGG-3'	100
SlActin	U60480	Forward: 5'-AGGCACACAGGTGTTATGGT-3' Reverse: 5'-AGCAACTCGAAGCTCATTGT-3'	177

licate for each sample with the following settings: initial denaturation at 95°C for 5 min followed by 40 cycles at 95°C for 30 s and 60°C for 1 min. The specificity of the PCR amplification was verified with a melt curve analysis (from 55°C to 94°C) following the final cycle of the PCR. All reactions were performed in triplicate. The relative expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method, as described by Schmittgen and Livak (2008).

Statistical analyses

Data were subjected to an analysis of variance (ANOVA), and means and standard errors were calculated. All parameters were subjected to a one-way-analysis ($P<0.001$) and compared using Tukey's test at 5% of probability. The statistical analyses were performed using Statistical Package for the Social Sciences software (SPSS; version 20).

RESULTS

Chemical composition of the essential oil

The yield of hydro-distilled essential oil from fennel seeds was found to be 0.54% (w/w). The chemical composition of the oil is given in Table 3. GC-MS analysis 18 compounds in different seed tissues. These compounds were assigned to three classes: phenylpropanoids (83%), oxygenated monoterpenes (11%) and monoterpene hydrocarbons (6%). This oil was characterized by a high content of the *trans*-anethole (78%), L-fenchone (11%), estragole (5%) and limonene (4%).

In vitro *antifungal effects of fennel seed essential oil*

Mycelium growth. The *in vitro* antifungal activity of the essential oil against FOL was recorded 6 DPI (Figure 1). Greatest inhibition of mycelium growth by the oil was at 500 µL mL^{-1}, where mean inhibition was 83%. At 250 µL mL^{-1} the oil also reduced the mycelial growth of FOL by 44%.

Sporulation test. The presence of essential oil in PDA reduced FOL conidium production compared to the experimental controls without essential oil. Conidium production decreased with increasing oil concentrations, and the greatest highest reduction (97%) was recorded at 500 µL mL^{-1} (Figure 2).

In planta *effects of fennel seed essential oil*

Disease severity. As the infections progressed, disease severity increased ($P < 0.05$) for the untreated tomato plants, reaching severity greater than 90%. Disease severity was least for tomato plants treated with fungicides preventively or curatively. Application of the essential oil also provided some protection against Fusarium wilt when the oil was applied curatively (Figure 3). At the end of the assay, Koch's postulates were confirmed by re-isolating FOL from roots, collars and leaves of inoculated plants.

Total phenolic contents of leaves. The total phenolic content in the tomato leaves showed differences depending on treatments (Figure 4). This significantly greater in treatments T2, T3, T4, T5, T6 and T7 compared with T1, at 21, 28 or 35 DPI. Phenolic content was greatest in plants inoculated and treated with essential oil (T6 and

Table 3. Chemical composition of the essential oil extracted from fennel seeds, determined using GC-MS.

Compounds	RI[a]	RI[b]	Identification[c]	Percentage
α-pinene	1032	934	RI, MS	0.43 ± 0.02
Camphene	1086	951	RI, Co-GC	0.10 ± 0.02
Sabinene	1132	975	RI, MS, Co-GC	0.16 ± 0.02
β-pinene	1123	980	RI, Co-GC	0.08 ± 0.02
β-myrcene	1166	991	MS	0.26 ± 0.02
I-phellandrene	1118	980	RI, MS, Co-GC	0.10 ± 0.02
p-cymene	1280	1026	RI, MS, Co-GC	0.09 ± 0.03
Limonene	1206	1030	MS	3.88 ± 0.47
trans-β-ocimene	1266	1050	RI, MS, Co-GC	0.29 ± 0.03
γ-terpinene	1255	1062	RI, MS, Co-GC	0.12 ± 0.03
L-fenchone	1402	1072	RI, MS, Co-GC	10.64 ± 0.03
Camphor	1532	1143	RI, MS, Co-GC	0.25 ± 0.04
Terpinen-4-ol	1611	1178	RI, MS, Co-GC	0.09 ± 0.04
p-anisic aldehyde	1976	1281	RI, MS, Co-GC	0.16 ± 0.05
Cis-anethole	1655	1252	RI, MS	0.25 ± 0.05
Estragole	1688	1196	RI, MS, Co-GC	4.67 ± 0.04
trans-anethole	1654	1297	RI, MS, Co-GC	78.26 ± 0.01
α-fenchyl acetate	1643	1220	RI, MS	0.18 ± 0.04
Class of compounds (%)				
Monoterpene hydrocarbons				5.50
Oxygenated monoterpenes				11.32
Phenylpropanoids				83.18
Total identified				100

Values are means of three replicates ± SD. [a] Retention index on an apolar column (HP-5). [b] Retention index on a polar column (HP-Innowax). [c] RI, identification by retention index relative to C8-C22 n-alkanes on the (HP-Innowax); MS, identification by mass spectrometry; Co-GC, identification by co-injection with authentic compound.

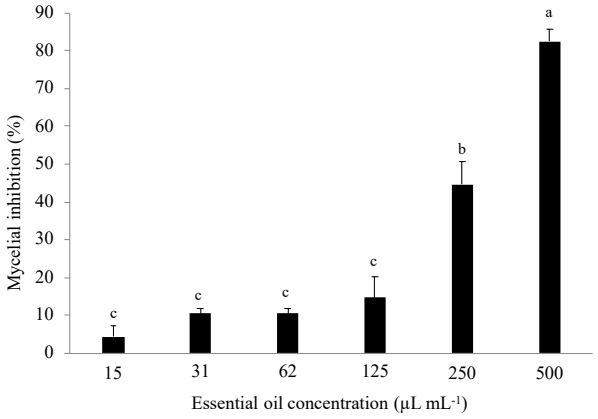

Figure 1. Mean *in vitro* growth reduction (%) of *Fusarium oxysporum* f. sp. *lycopersici* mycelium when exposed to different concentrations of fennel essential oil in PDA medium. Each value represents the mean of three replicates (two Petri plates per replicate). Values accompanied by different letters differ significantly ($P \leq 0.05$) according to Tukey's test.

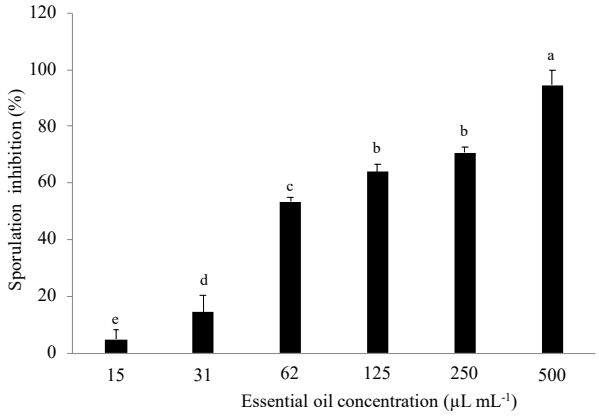

Figure 2. Mean inhibition of *Fusarium oxysporum* f. sp. *lycopersici* sporulation *in vitro* (%) when exposed to different concentrations of fennel essential oil in PDA medium. Each value is the mean of three replicates. Values accompanied by different letters differ significantly ($P \leq 0.05$), according to Tukey's test.

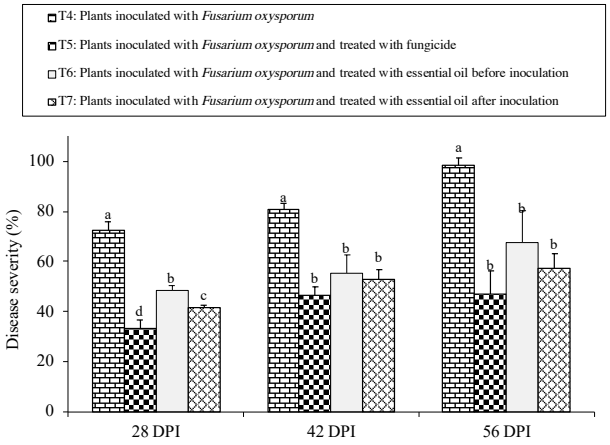

Figure 3. Mean Fusarium wilt severity for non-inoculated, inoculated and treated tomato plants at 28, 42 or 56 d post inoculation (DPI). Each value is the mean of the three replicates, and error bars represent ±SD. Values for each time accompanied by different letters differ significantly ($P \leq 0.05$), according to Tukey's test.

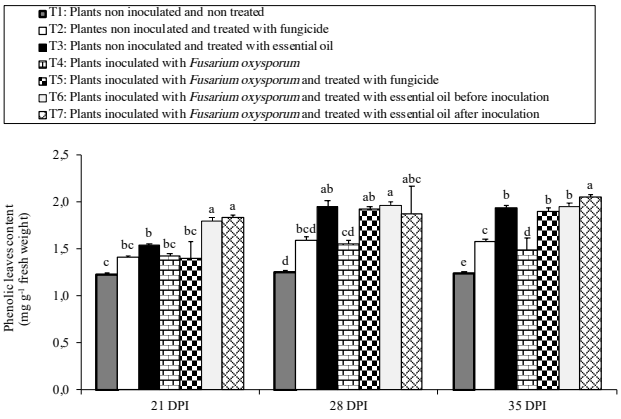

Figure 4. Mean amounts of total phenol in tomato leaf samples, from different treatments of FOL inoculation and essential oil application, at 21, 28 or 35 d post inoculation (DPI). Each value is the mean of the three replicates, and error bars represent ±SD. Values for each time accompanied by different letters differ significantly ($P \leq 0.05$), according to Tukey's test.

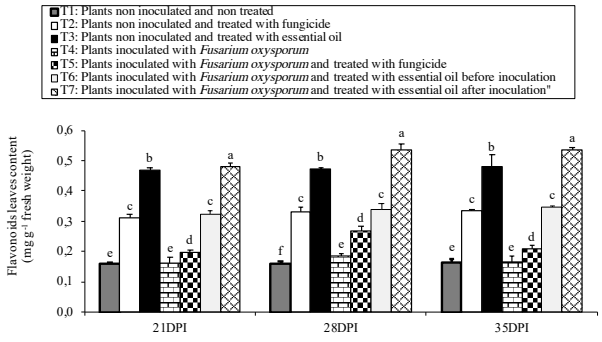

Figure 5. Mean amounts of total flavonoid in tomato leaf samples from different treatments of FOL inoculation and essential oil application, at 21, 28 or 35 d post inoculation (DPI). Each value is the mean of the three replicates, and error bars represent ±SD. Values for each time accompanied by different letters differ significantly ($P \leq 0.05$), according to Tukey's test.

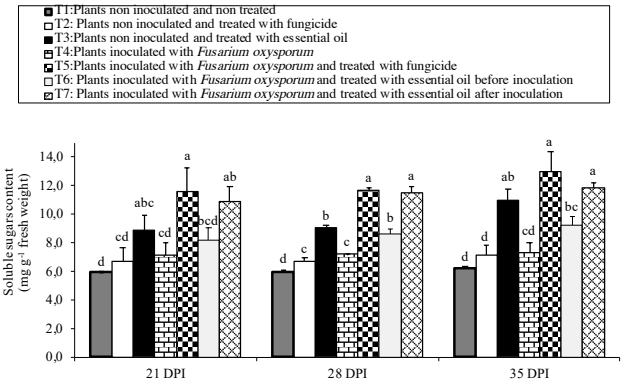

Figure 6. Mean amounts of soluble sugars in tomato leaf samples from different treatments of FOL inoculation and essential oil application, at 21, 28 or 35 d post inoculation (DPI). Each value is the mean of the three replicates, and error bars represent ±SD. Values for each time accompanied by different letters differ significantly ($P \leq 0.05$), according to Tukey's test.

T7), while fungicide did not significantly affect the phenol content in non-infected plants (T2). At 28 or 35 DPI, the least total phenolic contents were observed in non-inoculated and non-oil treated plants (T1). These results indicated that preventive and curative applications of the essential oil could be responsible for the increases of total phenolic contents in tomato leaves infected with FOL.

Total flavonoid contents

Total flavonoid contents (TFC) varied among the treatments (Figure 5). These were significantly greater in T2, T3, T6 and T7 compared to other treatments at 21, 28 or 35 DPI.

Soluble sugars contents

The greatest soluble sugars contents were observed in tomato plants treated with fungicide and in those that received a curative treatment with the essential oil (Figure 6). The least sugars content was in the experimental control plants.

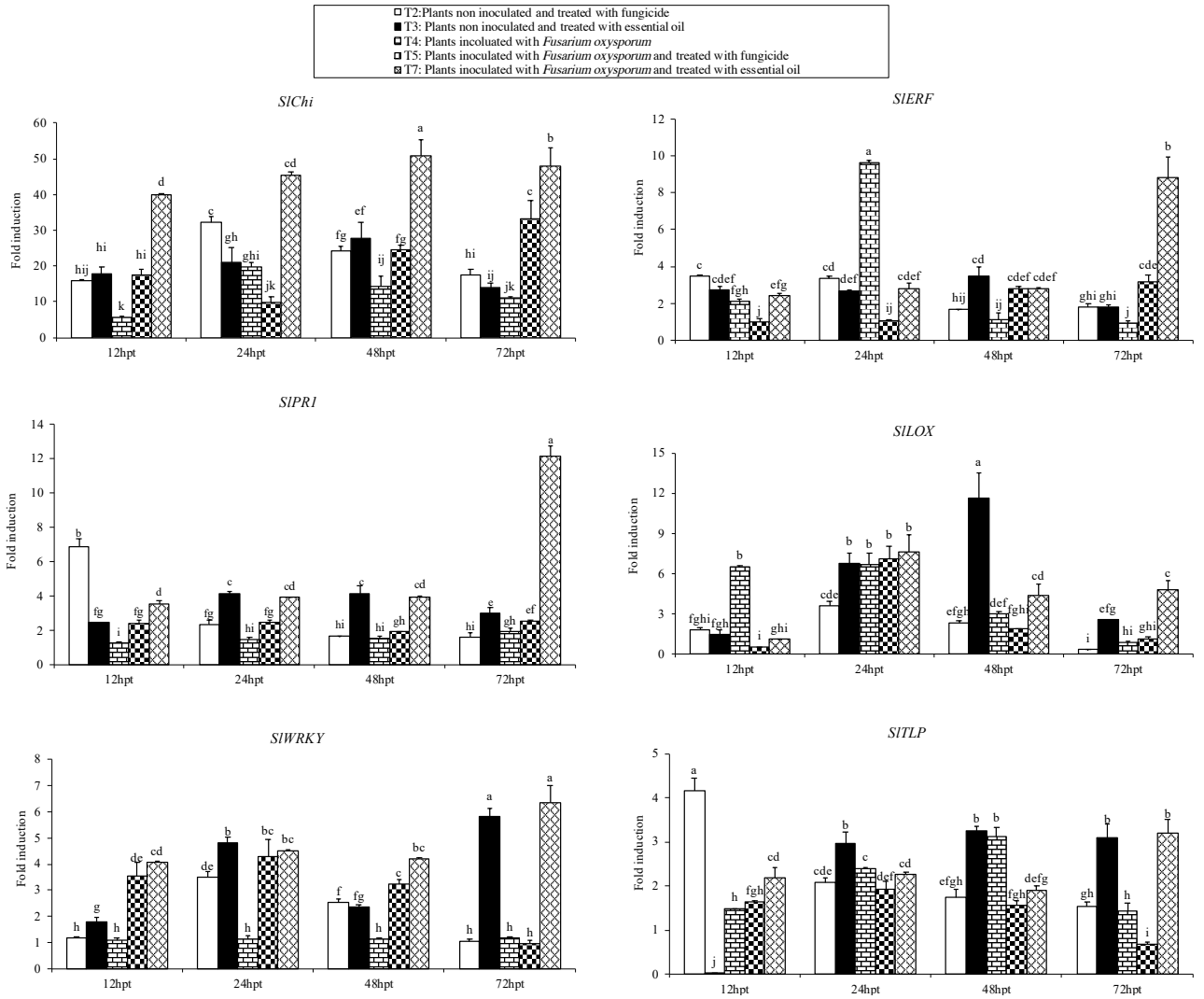

Figure 7. Fold changes in accumulation of the genes *SlChi, SlERF, SlPR1, SlLOX, SlWRKY* and *SlTLP* in tomato leaf samples, for different treatments relative to nil treatment (controls), and at different period (h post treatment; hpt). Values are the means (± SD) of three repeated experiments. Different letters above columns for all sampling stages indicate significant differences ($P \leq 0.05$), according to Tukey's test.

Expression profiles of defence genes in tomato leaf tissues. To confirm whether tomato plant defence mechanisms would be affected by treatments with the essential oil, expression of defence genes, including *SlChi, SlERF, SlPR1, SlLOX, SlWRKY* and *SlTLP* were quantified in leaf tissue at 0, 12, 24, 48 and 72 HPT, using RT-qPCR (Figure 7). Generally, from all treatments, the selected genes were upregulated compared to the experimental control. *SlChi* and *SlWRKY* were highly induced after curative treatment with the essential oil compared to the control. However, after fungicide treatment *SlChi* and *SlWRKY* showed the least expression. *SlERF* displayed high expression after fungicide treatment at 24 HPT, and after curative treatment with the essential oil at 72 HPT. The least expression of *SlERF* was measured in inoculated plants treated with fungicide at 12 and 24 HPT. At 12 HPT, FOL induced a 6.5-fold increase in expression of the *SlLOX* gene compared to the non-inoculated controls (plants treated with water), while no significant upregulation of *SlLOX* expression was observed in plants inoculated and treated with essential oil or fungicide. At 24 HPT, *SlLOX* was similarly induced in all treated plants compared to non-infected plants treated with fungicide. *SlLOX* transcript accumulation was significantly increased at 48 HPT, with respect to the other treatments, for plants treated with the essential oil. In the infected plants treated with the essential oil, *SlLOX* expression at 72 HPT was less than that at the

other time points after treatment. Expression of *SlPR1* decreased from 12 to 72 HPT in the fungicide treated plants, but high expression levels of this gene were measured only at 72 HPT in the inoculated and essential oil treated plants. However, in untreated and FOL-infected plants, levels of *SlPR1* gene transcription remained similar at all time points. The thaumatin-like protein (*SlTLP*) was up-regulated at 24, 48 and 72 HPT in the non-inoculated and essential oil treated plants. *SlTLP* expression was maximum at 12 HPT, followed by decreases at later stages in non-inoculated plants and treated with fungicide. There were no significant *SlTLP* expression differences at 12 or 24 HPT in the FOL inoculated and essential oil treated plants. However, *SlTLP* transcript at 48 and 72 HPT was significantly greater than at the other time points.

DISCUSSION

This study was within the scope of evaluating Tunisian medicinal and aromatic plants for discovery of new bioactive natural products that could be used against fungal diseases such Fusarium wilt of tomato caused by FOL. Based the results obtained, the essential oil from fennel seeds, which was rich in *trans*-anethole, exhibited good antimicrobial activity against FOL, as shown by the results for MGI and disease severity. Other compounds, such as α-pinene, sabinene, camphor, limonene and β-myrcene are present at concentrations usually less than 1%. According to Anwar *et al.* (2009), the major constituents of the fennel seeds essential oil tested were *trans*-anethole (70%), fenchone (10%), estragole (5%) and limonene (5%). Mimica-Dukic *et al.* (2003) also observed that the major compounds identified in the essential oil from fennel seeds were *trans*-anethole (74%, fenchone (11%), estragole (5%), and limonene (3%), which were constituents similar to those determined in the present study. In the Podgorica region of central south Montenegro, the essential oil composition assessed by Damjanović *et al.* (2005) was *trans*-anethole (62%), fenchone (20%), estragole (5%) and limonene (3.%) as the major components. Bernath *et al.* (1996) indicated that anethole (40-70%), fenchone (1-20%) and estragole (2-9%) were the most abundant compounds in fennel seed essential oil. *Trans*-anethole, the dominant component in fennel seeds essential oil, was also found in essential oils from other plants, and has been reported to possess insecticidal, larvicidal, and antimicrobial activities (Zoubiri *et al.*, 2014).

The results of the present study indicated that essential oil from fennel seeds inhibited mycelium growth of FOL *in vitro* at 500 µL mL^{-1}. Inhibition of mycelium growth increased from 5 to 80% with increasing concentration of the essential oil. The essential oil showed significant activity against FOL sporulation. Recently, Bomfim Costa *et al.* (2015) concluded that the essential oil from *Ocimum selloi* and its major constituents (phenylpropanoids) including methyl chavicol, were efficient inhibitors of mycelium growth and conidium germination of *Moniliophthora perniciosa*. Similar results were obtained with essential oil from *Ocimum basilicum* which reduced mycelium growth of *Botrytis fabae* (Oxenahm *et al.*, 2005). In the present study, the essential oil showed antifungal activity against FOL, which could be attributed to the presence of phenylpropanoids such as *trans*-anethole. Huang *et al.* (2010) reported that antifungal activity of the essential oil from *Illicium verum* was attributed to the high content of *trans*-anethole. In contrast, the essential oil from fennel seeds has shown low antifungal activities *in vitro* and *in vivo* against cucumber root and stem rot caused by *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (Soylu and Incekara 2017).

In the present study, the activity of fennel seed essential oil was also investigated *in planta* under greenhouse conditions. Curative and protective treatments with the oil were effective in reducing FOL infection, especially when essential oil was applied after inoculation. The least disease severity was observed at 28 DPI. These data suggest that all treatments exerted their greatest effects on early fungal invasion.

Fennel seed essential oil significantly increased total phenolic content in inoculated and non-inoculated tomato plants (treatments T3, T6 and T7) at 21, 28 or 35 DPI compared to T1, T2, T4 and T5. Similar results were reported by Akladious *et al.* (2015), Rajeswari (2014), Benhamou *et al.* (2000) and Pearce *et al.* (1998). These reports suggested that phenolic compounds may impede pathogen infection by increasing the mechanical strength of host cell walls resulting in the inhibition of pathogen invasion. Flavonoids are the polyphenolic compounds produced by plants for overcoming the oxidative stress in cells (Panche *et al.*, 2016). Our results showed that flavonoid content only increased in plants inoculated and treated with the essential oil (curative treatment) in comparison with either untreated inoculated plants or control plants. The increased content of phenol in tomato plants inoculated and treated with the essential oil may be due to induction of systemic resistance in the host plants (Rajeswari, 2014).

Total soluble sugar contents were increased in all treated plants at all time points (21, 28 or 35 DPI) compared to controls. The greatest level was recorded in

plants inoculated and treated with fungicide, followed by those treated with essential oil (curative treatment). These results are similar to those from other research. Nath *et al.* (2015) indicated that sugars enhanced the oxidative burst at early stages of infection, increasing lignification of host cell walls, stimulating synthesis of flavonoids, phenolics and phytoalexins, which suppress the pectolytic and cellulolytic enzymes that are essential for pathogenesis and induction of PR proteins.

In order to investigate the molecular mechanisms involved in fennel seed essential oil-induced resistance in tomato, the expression of the six defence-related genes, *SlChi, SlERF, SlPR1, SlLOX, SlWRKY*and *SlTLP*, was analyzed in treated tomato at different time points (12, 24, 48 or 72 HPT). After these time periods, the essential oil increased chitinase (*SlChi*) gene expression in infected tomato plants compared to the controls, while much lower levels have often been observed in other treatments. This could be related to the early response of tomato, which counteracts the penetration by FOL mycelium (Banani *et al*., 2018). Ahmed *et al.* (2012) reported that plant chitinases target fungus cell wall components as substrate that limits the invasion and growth of pathogens into their hosts. In sugar beet (Nielsen *et al.,* 1993), wheat (Anguelova *et al.,* 2001) and tomato (Lawrence *et al.,* 2000), high expression and strong induction of chitinase against phytopathogen systems were reported in resistant compared to susceptible varieties. Transgenic tobacco plants over-expressing the bean (*Phaseolus vulgaris*) chitinase gene showed increased resistance to *Rhizoctonia solani* infection and delayed development of disease symptoms (Broglie *et al.,* 1991). Transgenic grapevine plants overexpressing the rice chitinase gene had enhanced resistance against powdery mildew caused by *Uncinula necator* (Yamamoto *et al*., 2000). Early and high expression of *SlChi* in FOL-infected and essential oil treated tomato plants may affect the cell walls of germinating FOL conidia, releasing elicitors leading to the expression of host PR-genes and disease resistance.

The PR1 gene has been frequently used as a marker for salicylic acid-mediated disease resistance (Breen *et al*., 2017). Transgenic plants overexpressing *PR-1* exhibit enhanced resistance to numerous potential phytopathogens, including bacteria and fungi. Overexpression of pepper *PR-1* (*CABPR1*) in tobacco plants enhanced tolerance to *Phytophthora nicotianae*, *Ralstonia solanacearum* and *Pseudomonas syringae* pv. *tabaci* (Sarowar *et al.,* 2005). Transgenic tobacco plants overexpressing *PR*-1 showed increased tolerance to *Peronospora tabacina* and *Phytophthora nicotianae var. parasitica*, by exhibiting reduced disease symptoms (Alexander *et al*., 1993). Accumulation of *PR-1* transcripts was reported in broad bean plants inoculated with *Puccinia striiformis* f. sp. *tritici,* the causal agent of wheat stripe rust (Cheng *et al.,* 2012). In the present study, the greatest expression of *SlPR1* was found at 72 HPT in tomato plants treated with essential oil. We initially suggested that induction of *SlPR1* is characteristic of Systemic Acquired Resistance (SAR) in tomato. Our results for *SlPR1*gene expression in tomato leaves following treatment with the fennel seed essential oil (elicitor) support the hypothesis that induction of the SAR pathway (Bonasera *et al*., 2006) may be responsible for the phenotypic increase in tomato resistance to FOL.

High transcriptional levels of *PR-1*, *PR-2* and *PR-5* genes were reported in transgenic *Arabidopsis* plants expressing *AtWRKY18* or *AtWRKY70* (Li *et al.,* 2004). These data suggested that some *WRKY* genes could play a role in plant immunity and act as activators of fungal elicitor-induced genes (*PRs*) in plants. As for *SlPR1* expression, *SlWRKY* was differentially expressed, and the greatest level of *SlWRKY* was measured in the present study at 72 HPT, in tomato plants inoculated and treated with the essential oil. It is possible to hypothesize that *SlWRKY* might induce the expression of genes containing *W-box* in their promoters, including *SlPR1*. Similar observations were reported by Aamir *et al.* (2018). In root and leaf tissues of tomato infected with FOL, increased expression was found for *SolyWRKY4*, *SolyWRKY33*, *SolyWRKY37* at 24 and 48 HPT.

Based on amino acid composition, structure and biochemical function, PRs can be classified into 17 families. Among them, Thaumatin-like proteins (TLPs) are the products of a large and complex gene family involved in host defence. An lovar and Dermastia (2003) reported that specific TLPs have been shown to protect plants against osmotic stress and pathogen attack. Over-expression of the rice thaumatin-like protein (*Ostlp*) gene in transgenic cassava conferred enhanced tolerance to *Colletotrichum gloeosporioides* (Ojola *et al*., 2018). Transgenic wheat overexpressing *tlp-1* showed reductions in Fusarium head blight severity (Mackintosh *et al.,* 2007). *Brassica oleracea* TLPs (*BoTLP*5, 8 and 12) responded positively after *Pectobacterium carotovorum* subsp. *carotovorum* infection, and Ahmed *et al.* (2013) suggested these TLPs were useful resources for biotic stress resistance. Jiao *et al.* (2018) suggested that a Thaumatin-like protein from banana (*BanTLP*) inhibited *in vitro* conidium germination of *Penicillium expansum*. In the present study, *SlTLP* was differentially expressed after different treatments. This suggests that *SlTLP* may be responsive to FOL attack and that essential oil from fennel seeds may have acted as an elicitor.

Expression of *SlERF* was induced by inoculation with FOL at 24 HPT, and after treatment with the fennel essential oil at 72 HPT in inoculated tomato plants. At

24 and 72 HPT the high expression of *SlERF* could activate the expression of several ethylene-inducible genes, including *PR* genes through binding specifically the *GCC* box sequence of these gene families (Ohme-Takagi and Shinshi 1995). Previous investigations showed that increased expression of Ethylene-responsive element binding factor (ERF) genes in transgenic *Arabidopsis* or tobacco plants induced expression of various PR genes involved in increased resistance to microbial pathogens (Yi *et al.,* 2004; Zuo *et al*., 2007). Overexpression of *ERF1* conferred enhanced resistance to *Botrytis cinerea* and *Fusarium oxysporum* in transgenic *Arabidopsis* lines (Berrocal-Lobo *et al*., 2002).

Porta and Rocha-Sosa (2002) suggested that lipoxygenase (*LOX*) may be responsible for the synthesis of chemical compounds with plant defence signalling functions against biotic stresses. Wilson *et al.* (2001) also found that resistance to fungal pathogens in maize seeds was positively correlated with *LOX* transcript levels. *Botrytis cinerea* strongly induced the expression of *VvLOXC* and *VvLOXO* in *Vigna vinifera* (Podolyan *et al*., 2010). Transcriptome analyses by Wang *et al.* (2016) showed differential expression of several *LOX* genes after *Aspergillus flavus* infection. In the present study, expression of *SlLOX* steadily increased after FOL inoculation at 24 HPI, which suggested that *SlLOX* gene expression is related to the response to FOL infection. The greatest RNA level was observed at 48 HPI in tomato plants treated with the fennel essential oil. Expression patterns of the *SlLOX* gene differed significantly between treatments.

CONCLUSIONS

An essential oil from fennel seeds, applied at a concentration of 500 µL mL^{-1} had antifungal activities against FOL *in vitro* and *in planta* on tomato plants. The expression profile of the genes *SlChi, SlERF, SlPR1, SlLOX, SlWRKY*and *SlTLP* indicated that these genes may play crucial roles in the mechanism of resistance, whereby the essential oil reduced Fusarium wilt in tomato. Although further research is required to validate and fully understand of its mode of action, the essential oil from fennel seeds has promise as a promising biofungicide against FOL.

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