https://doi.org/10.17221/134/2018-PPS

Efficacy of essential oils of various aromatic plants in the biocontrol of Fusarium wilt and inducing systemic resistance in chickpea seedlings

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Citation: Moutassem D., Belabid L., Bellik Y., Ziouche S., Baali F. (2019): Efficacy of essential oils of various aromatic plants in the biocontrol of Fusarium wilt and inducing systemic resistance in chickpea seedlings. Plant Protect. Sci., 55: 202–217.

Abstract: This study evaluated the antifungal activity of essential oils (EOs) against *Fusarium oxysporum* f.sp. *ciceris* Padwick (FOC) and their effects in inducing systemic resistance in chickpeas. The EOs chemical composition was analysed by GC-MS. Carvacrol, geraniol and 1,8-cineole were the major constituents in thyme, lemongrass and laurel EOs. The latter showed effective antifungal activity against FOC. *In vivo* tests revealed that thyme and lemongrass EOs were also very effective in reducing the severity in chickpeas and reached an inhibition rate of 99.3 and 88.9%, respectively. Chickpea resistance is mostly attributed to phenolic compounds accumulation. The highest content of polyphenols was obtained with thyme (22.7 to 57.5 mg/g). The studied EOs could be used as promising antifungal agents in preventing the occurrence of Fusarium wilt in chickpeas.

Keywords: plant disease; phytochemicals; antifungal activity; *Fusarium oxysporum* f.sp. *ciceris*; polyphenols; flavonoids

Fusarium wilt disease caused by the fungus *Fusarium oxysporum* f.sp. *ciceris* Padwick (FOC) is one of the most frequent diseases resulting in severe economic losses of chickpea production around the world. The annual yield losses, caused by the disease are estimated at 10–15% (Jiménez-Fernández *et al*. 2013). However, the disease can completely destroy the crop under favourable conditions (Jiménez-Díaz *et al*. 2015). Fusarium wilt is a monocyclic epidemic caused by the primary inoculum present in the soil (Jiménez-Díaz & Jiménez-Gasco 2011) and control of the disease should focus on eliminating or reducing the quantity and/or effectiveness of the initial inoculum (Jiménez-Díaz *et al*. 2015).

Chemical synthetic fungicides are the most frequently used agents for the management of phytopathogenic fungi. However, the emergence of chemical-resistant strains of fungi and the undesirable effects on soil health, humans and non-targeted organisms in the environment has confounded the current use of these chemicals, leading to the search for alternative and safe remedies (Sharma *et al*. 2017).

Recently, researchers have shown that biological control is of primary interest in the management of plant pathogens. More importantly, it has been pointed out that the use of plant bioactive products is considered as a more effective method for the sustainable control of plant diseases. It is well established

Supported by the Ministry of Higher Education and Scientific Research of Algeria, Projects No. D04N01UN340120140012 and No. D01N01UN340120180001.

https://doi.org/10.17221/134/2018-PPS

that medicinal and aromatic plants are potential sources of microbiocide compounds attributed to secondary metabolites which have the advantage of being part of a wide variety of chemical structures and possessing a very wide range of biological activity (Cowan 1999). These compounds have target specificity, are biodegradable to nontoxic products, and are potentially appropriate for integrated use (Harish *et al*. 2008). Bioactive compounds from plant origins can be effectively utilised against soilborne pathogens or as an antifungal agent in a safe manner (Belabid *et al*. 2010; Soylu *et al*. 2010). In modern times, the use of essential oils (EOs) and their derivative compounds have been of great interest due to their antimicrobial activity against a wide variety of plant pathogens (Gahukar 2018). These properties are commonly attributed to the presence of active components, such as thymol, carvacrol, and linalool (Ahmad *et al*. 2011; Herman *et al*. 2016). Moreover, various studies have shown that treatment with EOs might induce plant systemic resistance by triggering a variety of substances responsible for plant defence mechanisms such as peroxidases and phenolic compounds, known to be implicated in cell wall strengthening and lignification against phytopathogens in natural conditions (Ben-Jabeur *et al*. 2015).

There are many scientific reports on the *in vitro* and *in vivo* antimicrobial properties of plant EOs. However, there is a real scarcity of available literature on the application and the mechanism action of plant EOs regarding FOC induced vascular wilt disease in chickpeas. Therefore, the aim of this study was to investigate the fungicidal effectiveness of EOs from six different aromatics plants in managing Fusarium wilt disease caused by FOC *in vitro* and *in vivo* conditions. This work particularly investigates the effect of EOs on decreasing the disease severity of Fusarium wilt in chickpeas and their role in inducing systemic resistance by triggering phenolic and flavonoid compounds.

MATERIAL AND METHODS

Biological material

Six aromatics plants species were studied: thyme (*Thymus pallescens* Noë), wormwood (*Artemisia herba-alba* Asso), laurel (*Laurus nobilis* Linnaeus), pine (*Pinus halepensis* Miller), lemongrass (*Cymbopogon citratus* (de Candolle ex Nees) Stapf), and the Peruvian peppertree (*Schinus molle* Linnaeus). The plants were harvested during the flowering season from different locations of Mascara, M'sila and Bordj Bou Arréridj in north Algeria.

Fusarium oxysporum von Schlechtendal isolate was obtained from the infected part of a chickpea plant collected in the region of Mascara, north-western Algeria. Prior to the experiment the strain was tested to confirm its pathogenicity inoculated sensible variety ILC 482.

Isolation and analysis of the essential oils

The aerial parts of each plant were dried at room temperature for 3 days. The EOs were extracted by a hydrodistillation process using a Clevenger-type apparatus (Glassco, India). The extracted oils were dried over anhydrous sodium sulphate, and stored at 4°C until used for gas chromatography-electron ionisation mass spectrometry (GC-MS) analysis and biological activity tests.

Gas chromatography analysis was accomplished with a Hewlett Packard 5975B apparatus system using a VF WAX and HP-5 capillary column (60 m × 0.25 mm × 0.5 µm film thickness). The column temperature programme was 60°C for 6 min, with a ramp of 2°C/min toward 250°C and held at 250°C for 20 minutes. Diluted samples (1/10 hexane, v/v) of 1 µl were injected by splitting, and the split ratio was 1 : 25. Injection was performed at 250°C. A flow rate of 0.5 ml/min carrier gas (He) was used. Flame ionisation detection was performed at 320°C. The relative proportions of the EO constituents were the percentages obtained by FID peak-area normalisation. Gas chromatography-electron ionisation mass spectrometry analysis was performed with a Hewlett-Packard computerised system comprised of a 6890 gas chromatograph coupled to a 5973A mass spectrometer using the non-polar column HP5MS (60 m × 0.25 mm × 0.5 µm film thickness). The conditions for the GC-MS spectra were: He was used (23 psi/SM–30 psi/FID) as the carrier gas at flow rate of 0.8 ml/min; split mode (1 : 25); 1 µl (1/10 in hexane, v/v) as the injected volume; and 250°C as injection temperature. The oven temperature programme is described above for the GC analysis. The mass range varied from m/z 30 to 350 amu (atomic mass units). The oil constituents were identified by the combined search of the retention times according to the Pyrenessences laboratory library and mass spectra NKS library, 75 000 entries. The percentages are

https://doi.org/10.17221/134/2018-PPS

calculated from the peak areas given by the GC/FID, without use of a correction factor. Identification of the constituents was based on the comparison of the retention times with those of the authentic samples, compared their linear retention index relative to the series of n-hydrocarbons and computer matching against a commercial (NIST 98 and ADAMS) and home-made library mass spectra, built up from pure substances and components of known oils and MS literature data (Tuberoso *et al*. 2007).

In vitro effect of the EOs on the FOC mycelial growth, sporulation and spore germination

Effect of the EOs on the radial growth. The effect of the EOs on the radial growth of the FOC was carried out by using the poisoned food technique as described by Soylu *et al*. (2006). Tween 80 was used at 3%. Briefly, a volume of 3 ml of the Tween 80 solution was added to 97 ml of sterile distilled water. The solution was stirred for 30 min and conserved at 4°C. The EOs were dissolved in Tween 80 (3%) and immediately added to the PDA displayed onto the Petri dishes (90 mm) at a temperature of 40–45°C. The tested concentrations were 0.1, 0.5, 1, 2.5, and 5 µl/ml. The controls received the same quantity of Tween 80 (3%) mixed with PDA. After adding these various dilutions and their solidification on Petri dishes (15 ml/dish), mycelia discs of 5 mm from the edge of the 8-day old FOC were placed at the centre of each Petri plate and incubated at 25 ± 3°C for 8 days. The mean radial mycelial growth of the FOC was determined by measuring the diameter of the colony in two directions at right angles 8 days after inoculation. Four replicate plates were tested for each concentration. The percentage of mycelial growth inhibition (MGI) was calculated by using Eq. 1:

$$\mathrm{MGI}(\%) = \frac{(d_c - d_t)}{d_c} \times 100 \quad (1)$$

where: d_c, d_t – mycelial growth diameter in the control and treated Petri plates, respectively

The experiments were conducted twice. The fungistatic-fungicidal nature of the EOs was tested by observing the revival growth of the inhibited mycelial disc following its transfer to the non-treated PDA.

Effect of the EOs on the spore production. The assessment of the sporulation was realised from plates used to study the mycelia growth according to the technique described by Bouzidi and Mederbal (2016). Five discs of 5 mm diameter were introduced in a tube containing 5 ml of distilled water. The spore suspension was then agitated using a vortex. After counting the complete number of the spores using a Malassez cell, the values were expressed as a number of spores per unit of area (mm^2). The percentage of the inhibition of the sporulation (SI) was calculated by Eq. 2:

$$\mathrm{SI}(\%) = \frac{(N_t - N_c)}{N_t} \times 100 \quad (2)$$

where: N_c, N_t – mean number of the spores estimated for the control and in the presence of the EO, respectively

These experiments were repeated four times.

Effect of the EOs on the conidial germination. A modified protocol of Feng and Zheng (2007) was developed and a spore germination assay was performed in a potato dextrose broth (PDB). The EOs were mixed in Tween 80 (3%) then added to the PDB and poured into 20 ml glass tubes. At the same time, 100 µl aliquots of a pathogen spore suspension (10^6 spores/ml) of FOC were added to each tube. The percentage of spore germination was estimated under an optical microscope. The percent inhibition was calculated according to Abbott's formula (Eq. 3):

$$\mathrm{MGI}(\%) = \frac{(G_c - G_t)}{G_c} \times 100 \quad (3)$$

where: G_c, G_t – mean number of germinated conidia in the control and treated Petri plates, respectively

Four replicates were conducted for each treatment and a minimum of 100 spores were counted in each replicate. The experiments were conducted twice.

In vivo effect of the EOs on the disease development

Preparation of the FOC inoculum. The preparation of the FOC inoculum was carried out according to the technique of Nene and Haware (1980). In 3 kg plastic bags containing sieved sand and corn flour moistened with distilled water, according to the proportion of 9/1/2 (w/w/v), respectively. The mixture was sterilised three times in an autoclave at 121°C for 30 min and then seeded with 50 explants of 8 mm diameter taken from a 15-day FOC culture. The incubation was carried out for 21 days at 25 ±

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3°C. Agitation of the bags every 3 days was carried out in order to allow for the homogeneous colonisation of the medium by the fungus. The obtained inoculum was then incorporated into pots containing the sterilised culture substrate at the rate of 100 g of inoculum per 1 kg of substrate which is composed of a mixture of sand, soil and potting soil in the proportions of 1/1/1 (v/v/v) sterilised at 121°C for 24 hours.

Preparation of the EOs and the soil treatment. The EOs at 0.5, 2.5 and 5 µl/ml were prepared by mixing 0.5, 2.5 and 5 ml of each EO with 1 l of sterile distilled water and Tween 80 (3%), respectively (Belabid *et al*. 2010). After stirring, this formulation was introduced into a spray device producing fine droplets. The soil already infected with FOC was pulverised by the mixture three successive times. Two control treatments were used: (*i*) negative control pots uninoculated with FOC and treated with sterile distilled water, (*ii*) positive control pots inoculated with FOC and treated with aqueous Tween 80 (3%).

Seed plantation. Chickpea line (Guab 5) seeds were surface disinfected using sodium hypochlorite (2%) for 3 min, rinsed in sterile water, and germinated (10 seed/disc) for 8 days in a Petri disc containing 2 sterilised filter papers. The Petri disc containing the seeds were then placed under laboratory conditions (25 ± 3°C). The two-week-old roots of chickpea seedlings grown on peat were immersed in the EOs in order to ensure better application and carefully transferred into the soil treated with the EO formulation (Ben-Jabeur *et al*. 2015).

Disease assessment. The severity of Fusarium wilt was assessed at 2-day intervals according to the technique described by Trapero-Casas and Jiménez-Díaz (1985). The disease incidence (DI) was assessed by counting the number of plants showing symptoms. The severity of Fusarium wilt was assessed on a scale of 0 to 4 according to the percentage of foliage with yellowing or necrosis (0 = 0%, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant). Incidence symptoms (0 or 1) and severity data (rated from 0 to 4) were used to calculate the disease index intensity (DIS) using Eq. 4:

$$\mathrm{DIS} = \frac{I \times S}{4} \tag{4}$$

where: I – disease incidence; S – disease severity

Additionally, for each treatment, the area under the disease progress curve (AUDPC) was estimated by calculating the AUDPC value (Eq. 5):

$$\mathrm{AUDPC} = \sum_{i+1}^{n} \left[\left(x_i + x_{i+1} \right) / 2 \right] \left(t_{i+1} - t_i \right) \tag{5}$$

where: x_i, x_{i+1} – disease severity index at time t_i, t_{i+1}, resp.

Determination of the total phenolic and flavonoid contents

The total phenolic content was determined using the Folin-Ciocalteu procedure as described by Ardestani and Yazdanparast (2007). Briefly, to prepare a host plant extract, 10 ml of 80% methanol was added to 250 mg of the dried-milled chickpea then shaken slowly. The obtained solution was filtered and 0.5 ml of the methanolic extract was mixed with 2.5 ml of Folin-Ciocalteu's reagent (1 : 10 diluted with distilled water) and 2 ml of a 7.5% Na_2CO_3 solution in a tube test and shaken well. The mixture was maintained at 30°C in a hot water bath for 90 minutes. The absorbance of the mixture was measured at 765 nm using a spectrophotometer. The total polyphenolic content was expressed as mg gallic acid equivalents/g of the dried extract. All the measurements were replicated four times.

The aluminium chloride colorimetric method was used for the determination of the total flavonoids (Chua *et al*. 2011). A volume of 1 ml of the extract was added to 2 ml of the methanolic solution containing $AlCl_3$ (2%). After 15 min of incubation, the mixture turned pink and the absorbance was measured at 430 nm. The total flavonoids content was given in mg quercetin equivalent/g of the extract. All the measurements were replicated four times.

Statistical analysis

The statistical analysis was performed by one and two-way ANOVA and the correlation test using STATISTICA software (Version 8.0, 2008). The mycelial growth, spore production and germination mean, *in vivo* tests of each treatment were compared by the Tukey test with a probability level of $P < 0.05$.

RESULTS

Chemical composition. The chemical composition of the EOs of the selected aromatic plants and the percentages of the various compounds are listed in Table 1. More than 76 compounds were identified. The

https://doi.org/10.17221/134/2018-PPS

Table 1. The chemical composition of the six essential oils as determined by gas chromatography-electron ionisation mass spectrometry analysis

	RT (min)	*Thymus pallescens* Noë	*Cymbopogon citratus* (de Candolle ex Nees) Stapf	*Artemisia herba-alba* Asso	*Laurus nobilis* Linnaeus	*Schinus molle* Linnaeus	*Pinus halepensis* Miller
Organic compound							
α-Pinene	7.3	1.13	2.12	0.92	3.38	3.88	12.47
Camphene	8.5	0.19	7.89	2.7	0.50	t	0.20
Camphor	8.5	0.42	0.12	24.6	0.13	t	0.10
Sabinene	10.3	t	0.14	0.28	4.96	–	4.29
Δ3-Carene	11.5	t		–	0.12	t	–
α-Phellandrene	12.2	t	t	0.16	t	16.27	0.22
α-Terpinene	12.8	0.86	t	0.30	0.38	t	0.43
β-Myrcene	12.0	0.58	0.6	0.10	0.40	1.76	12.51
Limonene	13.8	0.30	7.9	t	1.22	7.85	0.67
1,8-Cineole	14.2	0.34	t	4.48	40.77	0.10	t
β-Phellandrene	14.4	0.10	0.32	0.40		6.19	0.66
γ-Terpinene	16.3	8.47	t	0.67	0.67	t	–
p-Cymene	17.6	4.23	0.19	0.97	0.35	5.58	0.23
Terpinolene	18.2	t	0.5	t	0.23	–	6.12
α-Gurjunene	33.5	t			t	0.98	–
α-Thuyone	26.5	0.11	–	3.78	t		t
Isochrysantenone	27.6	0.12	–	4.34	t		–
β-Thuyone	27.7	0.10		3.98	t		t
Chrysanthenol isomere	28.3	–	–	1.32	–	–	–
Citronellal	30.3	–	2.37	–	–	–	–
Chrysanthenone	31.7	–	–	14.06	t	–	–
Linalol	34.6	3.06	0.70	0.31	16.87	0.12	0.28
Pinocarvone	35.5	0.07		2.07	–	–	–
Bornyl acetate	36.5	–	0.71	0.18	0.30	t	t
β-Elemene	37.2	–	1.07	–	0.22	1.26	–
β-Caryophyllene	37.5	2.02	1.86	0.12	0.19	1.66	28.43
Terpinene-4-ol	37.8	0.45	–	1.02	2.85	t	1.16
Aromadendrene	38.2	0.55	–			0.21	–
Citronellyl acetate	41.6	–	0.98	–	–	t	–
α-Humulene	41.9	0.11	0.3	t	t	0.94	4.99
γ-Muurolene	43.2	0.14	0.17	t	–	0.64	0.11
Ledene	43.5	0.43	–			0.22	–
α-Terpineol	43.6	t	2.21	0.20	2.29	0.18	0.27
Terpenyl acetate	43.7	–	0.29	–	7.53	–	–
Borneol	43.8	0.35	4.72	1.22	0.30		t
Germacrene D	44.5	t	0.97	1.29	t	5.03	0.45
Geranial	45.6	–	0.99	–		–	–
Bicyclogermacrene	45.7	0.1	2.6		0.09	2.78	t
α-Muurolene	46.5	t	0.42	–	t	1.46	0.73
δ-Cadinene	47.2	0.25	4.1	0.1	0.16	8.39	2.22

https://doi.org/10.17221/134/2018-PPS

Table 1. to be continued

	RT (min)	*Thymus pallescens* Noë	*Cymbopogon citratus* (de Candolle ex Nees) Stapf	*Artemisia herba-alba* Asso	*Laurus nobilis* Linnaeus	*Schinus molle* Linnaeus	*Pinus halepensis* Miller
Organic compound							
Geranyl acetate	47.3	–	3.42	–	–	–	–
Citronellol	47.7	–	2.83	–		–	–
Germacrene B	51.2	–		–		1.2	–
p-Cymene-8-ol	52.1	0.12		0.17		0.13	–
Geraniol	52.4	–	21.86	–	0.11	–	0.28
Sesquiterpenol	56.9	t	–	t	0.2	2.2	0.13
Caryophyllene oxyde	59.5	0.19	0.32	0.18	0.34	0.28	1.28
Valerate de 2-phenylethyle	60.1	–	–	–	–	–	5.44
Chrysanthenone isomere	60.6	–		4.99		–	–
E-Methyleugenol	60.8	–	0.19		4.5		–
Methylisoeugenol	60.9	–	7.5	–	t	–	t
Elemol	64.6	–	1.37		t	3.23	0.12
Spathulenol	66.7	0.24	–	0.57	0.35	2.71	–
Eugenol	68.8	0.27	t	0.16	0.82	–	–
γ-Eudesmol	68.9	–	0.24		–	1.02	t
Cembrene *Mw* = 272	69.6	–	–		–	–	1.25
Thymol	69.6	16.24	–	0.27	–	–	–
α-Muurolol	69.7			–	t	1.41	–
Sesquiterpene epoxide	70.2	t	t	t	0.35	1.78	t
α-Eudesmol	71.2	–	0.34	t	t	1.76	t
Carvacrol	71.7	54.09	t	0.46	t	0.50	–
β-Eudesmol	71.8	–	0.32	–	t	2.62	t
α-Cadinol	71.9	–	0.37	–	0.12	1.53	t
Cycloalkyl acide *Mw* = 168	78.0	–	–	2.35	–	–	–
Thunbergol	87.5		–	–	–	–	1.88
Total (%)		96.73	79.95	80.08	94.48	86.55	89.11
Organic compounds according to the functional groups							
Aliphatic alcohol		3.81	26.07		17.69	0.23	0.62
Terpene alcohol		1.10	9.70	10.59	6.70	1.39	1.66
Ketone terpene		1.17	–	60.86	–	0.18	0.25
Monoterpene		16.47	20.68	6.39	15.38	36.68	42.39
Oxyde			2.95	4.81	41.62	2.31	1.63
Phenol		70.69		–		0.50	
Phenol ether			9.03	–	5.61		
Sesquiterpene		4.37	13.81	2.22	1.31	29.81	38.27
Cyclic sesquiterpene			3.46		1.61	21.28	1.80

Mw – molecular weight; RT – retention time; t – trace

https://doi.org/10.17221/134/2018-PPS

results revealed that phenols were the most important portion of the EO of *T. pallescens* (70.69%), while, monoterpenes were the main fraction of *S. molle* and *P. halepensis* EOs with percentages approximately of 36.68 and 42.39%, respectively. Aliphatic alcohols, terpenic ketones and oxides were the most important fractions of the EOs of *C. citratus*, *A. herba-alba* and *L. nobilis* with percentages of 26.07, 60.86, and 41.62%, respectively. The chemical profile of *T. pallescens* EO revealed more than 30 compounds, with carvacrol (54.09%), thymol (16.24%), γ-terpinene (8.47%), *p*-cymene (4.23%), and linalol (3.06%) being the major molecules, while *C. citratus* EO was found to contain 36 compounds, with the highest content being geraniol (21.86%), limonene (7.9%), camphene (7.89%), methylisoeugenol (7.5%), borenol (4.72%), citronellol (2.38%), and citronellal (2.37%). Regarding the EO of *S. molle*, among the 33 identified constituents, α-phellandrene was the most abundant (16.27%) along with γ-terpinene (8.47%), δ-cadinene (8.39%), limonene (7.85%), germacrene D (5.03%), *p*-cymene (5.58%), and α-terpinene (3.88%). The chemical analysis of *L. nobilis* EO revealed more than 30 compounds representing 99.4% of the total oil, and the major constituent was 1,8-cineole (40.77%) followed by linalol (16.87%), geranyl acetate (7.53%), sabinene (4.96%), *E*-methyleugenol (4.5%), β-pinene (2.88%), and α-terpineol (2.29%). A total of 33 compounds were identified in the *A. herba-alba* EO and the main constituents were camphor (24.6%), chrysanthenone (14.6%), chrysanthenoneisomere (4.99%), 1,8-cineole (4.48%), α-thuyone (3.78%), β-thuyone (3.98%), and camphene (2.7%). The *P. halepensis* EO contained 27 compounds including β-caryophyllene (28.43%) as a major compound followed by β-myrcene (12.51%), β-pinene (12.47%), terpinolene (6.12%), α-humulene (4.99%), and sabinene (4.29%).

In vitro antifungal activity of the EOs. The *in vitro* antifungal activity of the EOs was investigated quantitatively against the FOC. The effect of the various concentrations of the studied EOs on the mycelium growth, sporulation as well as spore germination of the FOC are presented in Table 2. We noticed significant differences in terms of antifungal activity among the tested concentrations ($P \leq 0.05$). In addition, each EO exhibited different degrees of antifungal activity.

The results obtained by the incorporation method showed a significant reduction of mycelial growth of the FOC (Figure 1). It was found that the rate of reduction gradually increased by increasing the concentration of the EOs. The *T. pallescens* and *C. citratus* EOs were the most effective in inhibiting the FOC mycelial growth. These two EOs showed inhibitory effects with all the tested concentrations. The range of mycelial growth inhibition by the *T. pallescens* EO was between 86.51 and 100%. At a concentration of 0.5 µl/ml, the EO completely inhibited the mycelial growth of the FOC. The *C. citratus* EO showed pronounced inhibition activity on the FOC mycelial growth. The total inhibitory effects of mycelial growth were observed with 2.5 and 5 µl/ml. As for *C. citratus*, the *A. herba-alba* EO also exhibited an inhibitory effect on the mycelial growth and completely inhibited the FOC at the concentrations of 2.5 and 5 µl/ml. The *S. molle* and *L. nobilis* EOs were less effective against FOC at low concentrations (3.11 and 14.80%). However, these same oils were more effective at 2.5 and 5 µl/ml with inhibitory zones ranging from 70.59–75.18% for *S. molle* and from 80.36–100% for *L. nobilis*. In this study, *P. halepensis* showed the lowest antifungal activity with percentages ranging from 7.19–39.44%.

Transfer experiments were performed to provide some distinction between the fungistatic and fungicidal EOs on the FOC. The results showed that *T. pallescens* and *C. citratus* exhibited fungicidal activity.

The results revealed variable inhibitory effects of the studied EOs on the FOC sporulation according to the plant species and the tested concentration. The best inhibitory effect was obtained with the EOs of *T. pallescens* and *C. citratus*. FOC sporulation was completely inhibited by *T. pallescens* at the concentrations of 5, 2.5, 1 and 0.5 µl/ml. 0.1 µl/ml also inhibited FOC sporulation at 83.42%. The EO of *C. citratus* significantly reduced the FOC sporulation with all the tested concentrations and the rate of inhibition ranged between 30.39 and 100%. As well, the spore production was completely inhibited by the *A. herba-alba* EO at the concentrations of 2.5 and 5 µl/ml. Furthermore, strong inhibitions of the spore production were obtained with the EOs of *L. nobilis* and *S. molle* (100 and 84.65% with 5 µl/ml, respectively). The lowest inhibition of the FOC sporulation was noted with the *P. halepensis* EO.

The inhibitory effects of the EOs were also evaluated against the FOC spore germination. Microscopic observations of the spores after exposure to different concentrations of the EOs showed some morphological abnormalities, after 48 h of treatment. The visible loss of cytoplasm content, bursting, the total or partial inhibition of the germination and lysis of the germinate tubes were noticed (Figure 2). A broad variation in the germination inhibition of the investigated EOs was observed. The results given in Table 2 represent the

https://doi.org/10.17221/134/2018-PPS

Table 2. The effect of the different concentrations of the essential oils on the mycelium growth inhibition (MGI), sporulation (SI) and spore germination (SGI) of the *Fusarium oxysporum* f.sp. *ciceris* Padwick

Species	Concentration (µl/ml)	MGI (%)	SI (%)	SGI (%)
Thymus pallescens Noë	0.1	86.51 ± 1.71^{b}	83.42 ± 1.54^{b}	87.93 ± 0.60^{bcd}
	0.5	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
	1	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
	2.5	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
	5	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
Cymbopogon citratus (de Candolle ex Nees) Stapf	0.1	17.50 ± 1.07^{ij}	30.39 ± 4.88^{efg}	83.16 ± 0.66^{bcde}
	0.5	38.57 ± 6.34^{fgh}	58.37 ± 4.78^{cd}	90.50 ± 0.90^{abc}
	1	92.50 ± 1.97^{ab}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
	2.5	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
	5	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
Schinus molle Linnaeus	0.1	14.80 ± 3.14^{ij}	14.63 ± 2.18^{hi}	32.39 ± 3.26^{i}
	0.5	14.90 ± 2.81^{ij}	35.92 ± 3.83^{ef}	61.74 ± 5.22^{hij}
	1	62.96 ± 2.27^{de}	59.98 ± 0.61^{cd}	76.46 ± 4.44^{defg}
	2.5	70.59 ± 0.82^{cde}	69.56 ± 2.64^{c}	80.64 ± 3.03^{cdef}
	5	75.18 ± 2.84^{cd}	84.65 ± 1.57^{b}	83.00 ± 2.92^{bcde}
Laurus nobilis Linnaeus	0.1	3.14 ± 0.62^{j}	21.02 ± 1.06^{gh}	36.02 ± 3.02^{i}
	0.5	11.92 ± 1.60^{ij}	41.55 ± 3.57^{e}	54.25 ± 2.57^{ij}
	1	21.36 ± 5.42^{hi}	55.13 ± 0.73^{d}	94.61 ± 1.97^{ab}
	2.5	80.26 ± 4.13^{bc}	70.51 ± 2.55^{c}	100.00 ± 0.00^{a}
	5	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
Artemisia herba-alba Asso	0.1	1.25 ± 1.25^{j}	4.25 ± 1.82^{i}	12.81 ± 1.73^{m}
	0.5	11.28 ± 1.61^{ij}	20.02 ± 1.82^{gh}	19.76 ± 2.85^{m}
	1	53.75 ± 10.23^{ef}	40.49 ± 3.02e	36.61 ± 1.94^{l}
	2.5	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	70.03 ± 0.68^{fgh}
	5	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	72.93 ± 0.70^{efgh}
Pinus halepensis Miller	0.1	7.19 ± 1.21^{ij}	11.51 ± 2.35^{hi}	11.54 ± 4.41^{m}
	0.5	22.31 ± 4.65^{ghi}	27.55 ± 3.42^{fg}	41.36 ± 1.63^{l}
	1	36.63 ± 5.16^{fgh}	29.74 ± 3.21^{efg}	53.63 ± 2.62^{j}
	2.5	38.76 ± 2.41^{g}	36.38 ± 2.72^{ef}	65.85 ± 0.70^{ghi}
	5	39.44 ± 2.05^{fg}	58.69 ± 1.14^{dc}	66.60 ± 0.95^{hg}

The values represent the mean of the 4 replicates ± standard error; data marked by the different letters in the columns indicate a significant difference at $P = 0.05$

percent of inhibition of the FOC spore germination. Here too, this inhibitory effect varied according to the plant species and the concentration of the tested EOs. As for the mycelium growth and sporulation, the *T. pallescens* and *C. citratus* EOs showed the highest inhibition of the spore germination of the FOC. *T. pallescens* caused 100% of inhibition with concentrations of 5, 2.5, 1, 0.5 µl/ml and 87.93% of inhibition with 0.1 µl/ml, while *C. citratus* completely (100%) inhibited the spore germination with concentrations of 5, 2.5 and 1 µl/ml. The *L. nobilis* and *S. molle* EOs also exhibited a potent inhibitory effect on the spore germination of the FOC (36.02–100% and 32.39–83%, respectively). *P. halepensis* and *A. herba-alba* presented the lowest level of inhibition regarding the FOC spore germination.

In vivo antifungal activity of the EOs. The effect of the various EOs on the severity of *Fusarium* Link after 4 weeks of treatment is reported in Figure 3. The inoculated, non-treated, and control chickpea plants showed symptoms of Fusarium wilt from 13 days of sowing (Figure 4). The symptoms of the

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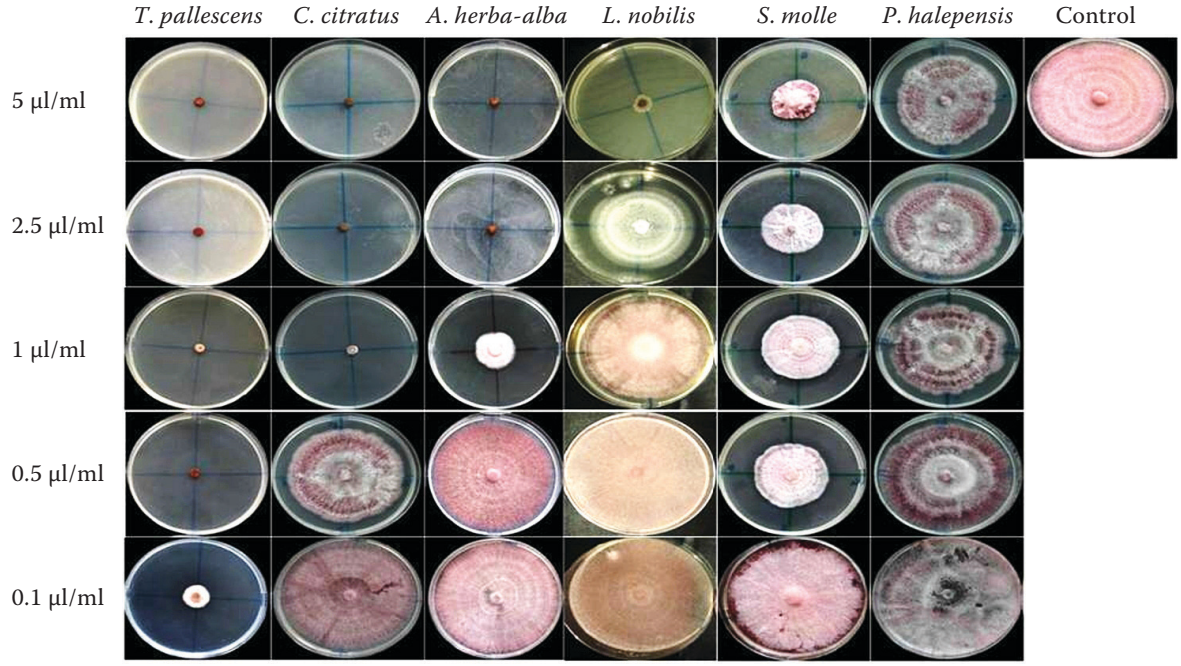

Figure 1. The effects of the different concentrations of the plant essential oils on the mycelial growth of the *Fusarium oxysporum* f.sp. *ciceris* Padwick

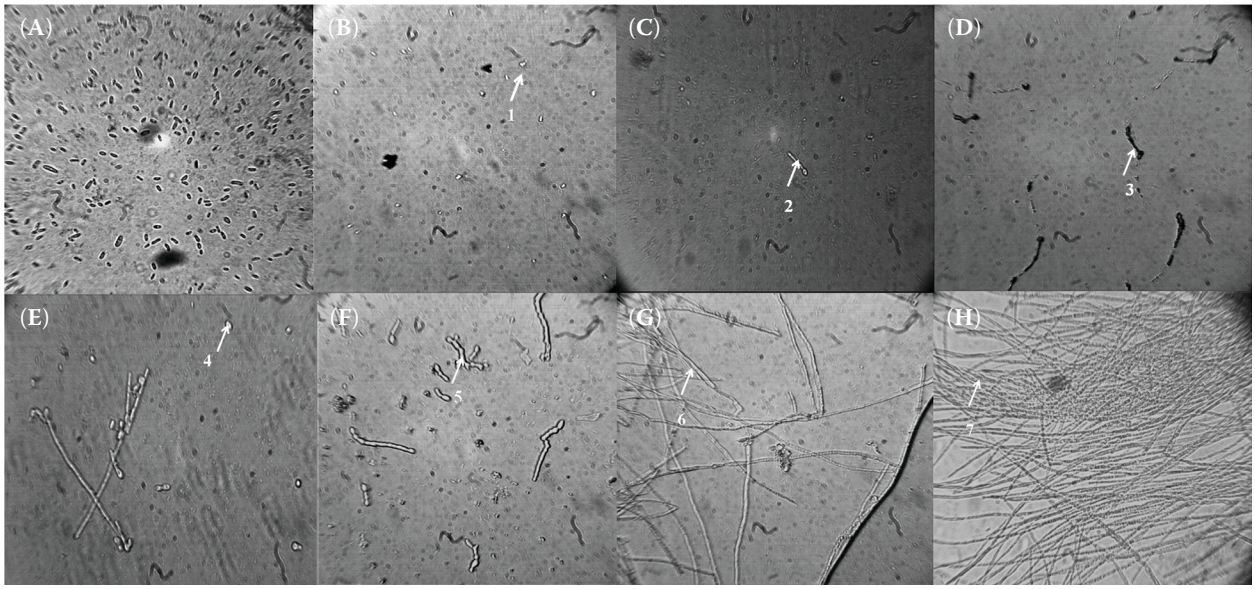

Figure 2. The morphological abnormalities caused by the essential oils (EOs) on the *Fusarium oxysporum* f.sp. *ciceris* Padwick (FOC) spore germination at the end of the experiment: the spore FOC suspension before treatment (**A**), the suspension treated with the different EOs (**B–G**), the control not treated (**H**)

1 – reduction of the cytoplasm content; 2 – tube germinated initiation; 3 – lysis of the germinated tubes; 4 – inhibition of the germination; 5, 6 – massive germination; 7 – mycelium initiation

disease were evident from 20 days in the cultures pre-treated with the *P. halepensis* and *A. herba-alba* EOs. In the seedlings treated with the *L. nobilis* and *T. pallescens* EOs, the symptoms occurred from 25 and 32 days of sowing, respectively.

Plants treated with the EOs presented AUDPC values ranging from 15 to 1 680, whereas, the untreated control presented an AUDPC value of 2 045, being significantly higher than all the treated chickpea cultures. As for the *in vitro* study, the *T. pallescens*

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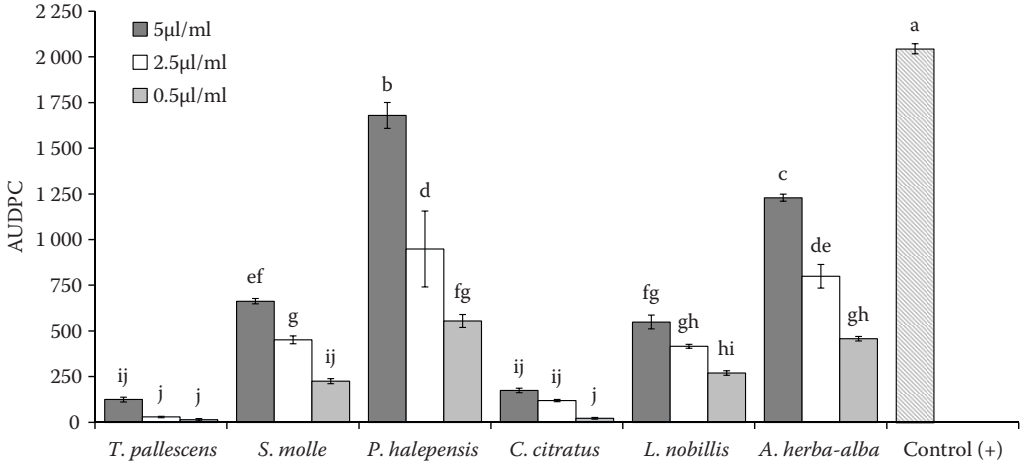

Figure 3. The severity of Fusarium wilt in the chickpea line treated with the different concentrations of the various essential oils (aromatics plants species: *Thymus pallescens* Noë, *Schinus molle* Linnaeus, *Pinus halepensis* Miller, *Cymbopogon citratus* (de Candolle ex Nees) Stapf, *Laurus nobilis* Linnaeus, *Artemisia herba-alba* Asso)

AUDPC – area under the disease progress curve; the values represent the mean of the 4 replicates ± standard error; data marked by the different letters in the columns indicate a significant difference at $P = 0.05$

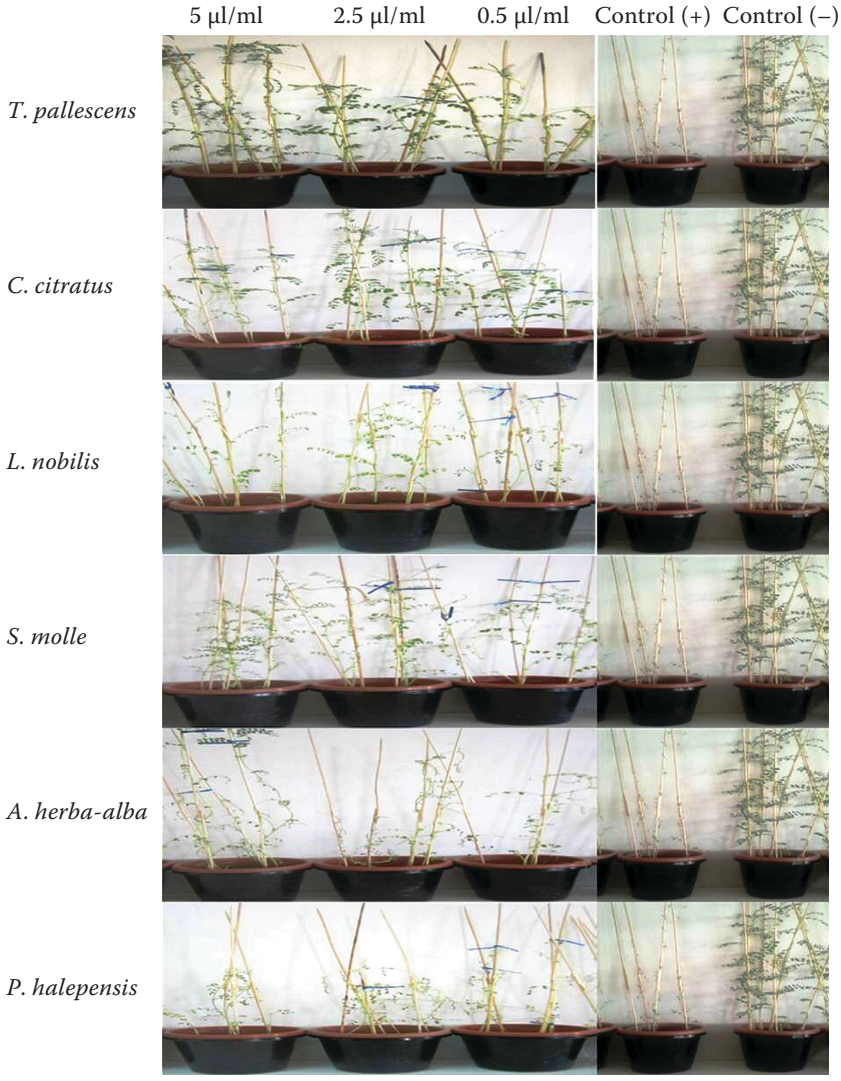

Figure 4. The effect of the essential oils on the disease severity at the end of the experiment

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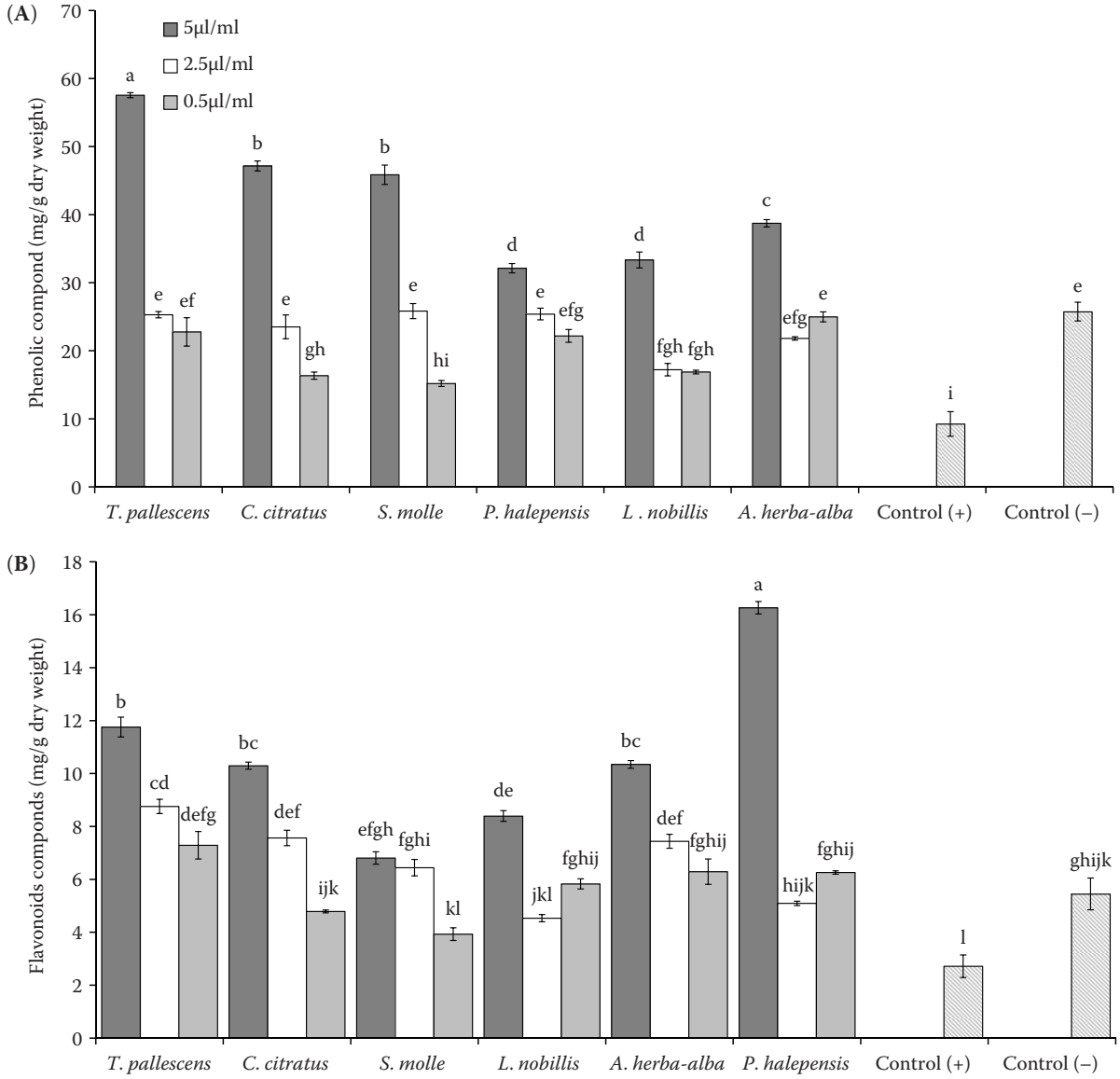

Figure 5. The levels of the polyphenols (**A**) and flavonoids (**B**) in the seedlings treated with the essential oils of six plants (*Thymus pallescens* Noë, *Cymbopogon citratus* (de Candolle ex Nees) Stapf, *Schinus molle* Linnaeus, *Laurus nobilis* Linnaeus, *Artemisia herba-alba* Asso, *Pinus halepensis* Miller)

The values represent the mean of the 4 replicates ± standard error; data marked by the different letters in the columns indicate a significant difference at $P = 0.05$

EO was the most effective against wilt disease and significantly reduced the AUDPC values ($P \leq 0.05$). The concentrations of 2.5 and 5 µl/ml of the *T. pallescens* EO showed an AUDPC of 30 and 15 which correspond to 98.53 and 99.27% of the disease reduction. The *C. citratus*, *L. nobilis* and *S. molle* EOs were also very effective against the wilt disease. The mean values of the AUDPC were 22.5, 270 and 225.5 at high concentration (5 µl/ml) representing 98.90, 86.81 and 88.97% of the reduction in the disease severity. The treatment of chickpea seedlings with the *P. halepensis* and *A. herba-alba* EOs showed the lowest effect against Fusarium wilt.

The total phenolic and flavonoid contents. To explore the potential effect of the EOs in inducing a plant defence system when applied in a more preserved way, the phenolic and flavonoid contents were measured in the chickpea seedlings treated with different EOs. The levels of phenolic and flavonoid compounds are illustrated in Figure 5.

Plants treated with the EOs induced the production of the phenolic compound in a dose dependent manner,

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ranging between 15.19 to 57.56 mg/g. The positive and negative controls presented average levels of phenolic and flavonoid compounds of 9.25 and 25.75 mg/g, respectively. The data showed that the highest levels of polyphenols were recorded in the seedlings treated with the *T. pallescens* (22.77–57.56 mg/g), *C. citratus* (16.3–47.16 mg/g) and *S. molle* (15.19–45.86 mg/g) EOs. In terms of the flavonoids, plants treated with various EOs showed values ranging between 3.93 to 16.26 mg/g which are significantly higher when compared to the positive and negative controls (2.72. and 5.45 mg/g, respectively). Here, the highest levels of flavonoids were recorded in the seedlings treated with the *P. halepensis* (6.25–16.26 mg/g) and *T. pallescens* (7.29–11.75 mg/g) EOs.

DISCUSSION

EOs have a very large variety of action since they inhibit both the growth of bacteria, yeasts and fungi. Their antimicrobial activity is mainly a function of their chemical composition, and in particular of the nature of their major volatile compounds. In this study, the chemical composition and antifungal activity of six plant EOs against the FOC were studied. According to the results, the major constituents of the EOs of *T. pallescens*, *C. citratus*, *S. molle*, *L. nobilis*, *A. herba-alba* and *P. halepensis* were carvacrol (54.09%), geraniol (21.86%), α-phellandrene (16.27%), 1,8-cineole (40.77%), camphor (24.6%), β-caryophyllene (28.43%), respectively. The differences in the chemical composition of the studied EOs could be attributed to numerous factors including the geographical area, variety, the age of the plant, the nutritional status of the plants, the method of drying, the method of extraction of the oil and other factors (Curado *et al.* 2006). Several authors have reported carvacrol as the dominant constituent of the EO of *T. pallescens* which is in good agreement to our results (Hazzit *et al.* 2009). Similarly, the chemical profile of the *S. molle* EO corroborates several other studies in which, α-phellandrene was detected as the main phytoconstituent (Martins *et al.* 2014). Likewise, 1,8-cineole was identified as the most important compound of *L. nobilis*; this is in accordance to some other previous reports (Santamarina *et al.* 2016). Some of the analysed EOs presented slight differences to those reported in the literature. Bassolé *et al.* (2011) reported that geranial and neral were the major compounds of the *C. citratus* EO. Otherwise, Belhattab *et al.* (2012) revealed that camphor, α-thujone and chrysanthenone were the main compounds of *A. herba-alba*, but, in this study, α-thujone was not detected. Also, the chemical composition of the *P. halepensis* EO was different to that obtained by Djerrad *et al.* (2015) where α-pinene was detected at a high concentration.

Our results demonstrated that the EOs inhibited the mycelial growth, sporulation and conidial germination of the FOC and reduced the severity of Fusarium wilt of the chickpea under the controlled conditions according to the plant species and the tested concentration. The antifungal activity and the differences observed in the effectiveness of the tested EOs might be attributed to the various bioactive compounds and their variability according to the plant species. The antifungal effect of the EOs can be variable and closely associated to the activity of the main components, which in turn is caused by the different interactions between the EO compounds (Herman *et al.* 2016). Some researchers found that phenols (1,8 cineole, carvacrol, octanol, etc.), alcohols (α-terpineol, terpinen-4-ol, linalol), aldehydes, ketones (camphor, etc.), α-pinene, β-pinene, *p*-cymene are chemical compounds with a wide spectrum of biological effects (Herman *et al.* 2016; Sharma *et al.* 2017). It can be assumed that the tested plant EOs are rich sources of these compounds. Krishna Kishore *et al.* (2007) demonstrated the antifungal properties of these components regarding *Rhizoctonia solani* J.G. Kühn, *F. oxysporum*, *Penicillium digitatum* (Persoon) Saccardo, *Aspergillus niger* van Tieghem, *Alternaria alternate* (Fries) von Keissler, *Aspergillus flavus* Link, *Cochliobolus lunatus* R.R. Nelson & Haasis, *Fusarium moniliforme* J. Sheld., *Fusarium pallidoroseum* (Cooke) Saccardo, *Phoma sorghina* Saccardo, *Cercospora arachidicola* Hori, *Phaeoisariopsis personata* (Berkeley & M.A. Curtis) Arx, and *Puccinia arachidis* Spegazzini. EOs affect the microbial cells by various mechanisms, such as attacking the cell membrane, destroying the enzyme systems, compromising the genetic material, the disturbance of the proton motive force, electron flow, active transport and coagulation of the cell contents (Oussalah *et al.* 2006). Since, EOs are hydrophobic, they can enter in the phospholipid bilayer of the cell wall and that of the mitochondria distorting the structure and making them more susceptible to cell leakage (Badawy & Abdelgaleil 2014). The mechanisms of the action of carvacrol and thymol have been shown to originate from the inhibitory

https://doi.org/10.17221/134/2018-PPS

consequence of ergosterol biosynthesis and cause the disruption of cell membrane integrity (Moleyar & Narasimham 1986). Carvacrol and 1,8-cineole caused severe modification in the cell wall constitution, degradation of the plasma membrane, shrinkage of the cells, condensation of the cytoplasmic content, leakage of the intracellular material, and cell collapse (De Sousa *et al*. 2015). Thymol has been revealed to cause alteration of the cellular membrane, inhibition of the ATPase activity, and release of intracellular ATP and other constituents (Oussalah *et al*. 2006).

The antifungal activity of the EOs may not be easily correlated with any individual constituent, but with a mixture of compounds present in this oil (Krishna Kishore *et al*. 2007). The synergistic effects of carvacrol with linalool, eugenol and thymol have been subsequently established, and it might be due to the fact that thymol and carvacrol disintegrated the external membrane, making it easier for eugenol to enter the cytoplasm and combine with the proteins (Bassolé *et al*. 2011).

The obtained results showed important morphological abnormalities presented by the visible loss of the cytoplasm content, fragmentation, bursting, the total or partial inhibition of the germination and lysis of the germinate tubes. These results are in line with those obtained by Cordova-Albores *et al*. (2015) who reported that application of the *Jatropha curcas* Linnaeus EO and its derivatives caused morphological and cell damage in the mycelium and conidia of *Fusarium oxysporum* f.sp. *gladioli* (Massey) W.C. Snyder & H.N. Hansen. Although the mode of inhibitory action of the EOs against the germination still remains unclear. Possible action mechanisms by which conidia germination may be reduced or totally inhibited have been proposed. Several authors reported that EOs inhibited cell division and induced structural breaks and decomposition in the roots (De Martino *et al*. 2009). Moreover, the impacts of the EOs on the spore germination may be attributed to denaturation of the enzymatic system responsible for the conidia germination or interfering with the amino acid and/or inhibition of the transduction activity of the signals involved in the germination process (Tian *et al*. 2012).

In this study, the EO of *T. pallescens* was the most effective against the FOC mycelium growth, sporulation and spore germination. It is characterised by the relatively high content of carvacrol, thymol and *p*-cymene; which could be related to these major components. Hazzit *et al*. (2009) demonstrated that the EO isolated from *T. pallescens* collected from different regions of Algeria showed the highest antimicrobial activity against *Candida albicans* (C.P. Robin) Berkhout, *Staphylococcus aureus* Rosenbach, *Salmonella* sp., and *Bacillus cereus* Frankland & Frankland. The effects of the *C. citratus* EO and its principal components, geraniol, limonene and camphene, on the growth of some plant pathogenic fungi species such as *R. solani*, *F. oxysporum*, *P. digitatum*, *Mucor* sp., *Rhizopus stolonifer* Vuillemin and *A. niger* have been previously reported (Moleyar & Narasimham 1986). Our results corroborate those obtained by Singh *et al*. (2010) who demonstrated that at 750 ppm, the EO of *C. citratus* completely inhibited the growth of *Aspergillus fumigatus* Fresenius, *A. niger*, *Aspergillus terreus* Thom, *A. alternate*, *Cladosporium herbarum* (Persoon) Link, *C. lunatus*, *F. oxysporum* and *Penicillium italicum* Wehmer. The antifungal activity of the *L. nobilis* EO has been extensively investigated also (Santamarina *et al*. 2016). In our study, 1,8-cineole, linalool, terpineol acetate, and methyl eugenol were the most abundant components. These compounds have previously been reported to have antimicrobial activity against a variety of plant pathogenic fungi and bacteria (Moleyar & Narasimham 1986; Herman *et al*. 2016). The *P. halepensis* EO showed important antifungal activity against *Fusarium avenaceum* Fries, *Fusarium culmorum* (Wm.G. Smith) Saccardo, *F. oxysporum*, *Fusarium subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas, *Fusarium verticillioides* (Saccardo) Nirenberg, *Fusarium nygamai* L.W. Burgess & Trimboli, *Rhizoctonia* sp., *Microdochium nivale* (Fr.) Samuels & I.C. Hallett, *Alternaria* sp., and *Bipolaris sorokiniana* (Saccardo) Shoemaker, which could be attributed to the high presence of β-caryophyllene, β-myrcene, and β-pinene (Amri *et al*. 2013). Tantaoui-Elaraki *et al*. (1993) mentioned that spore germination, mycelial elongation and sporulation of their fungal asexual reproduction (*Zygorrhynchus* sp., *A. niger* and *P. italicum*) were significantly affected by the EO of *A. herba-alba*, and the mycelium growth was the most susceptible, followed by spore germination and spore production. The antifungal activity of *A. herba-alba* is due to the bioactive compounds such as camphor, chrysanthenone, chrysanthenone isomere and 1,8-cineole. In our study, *S. molle* showed a significant inhibition of the mycelium growth, spore production and germination of the FOC. Several authors reported the antimicrobial activity of the EO of *S. molle* (López-Meneses *et al*. 2015). In general, there was a relationship between

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the antifungal activity and the major components including α-phellandrene, γ-terpinene, δ-cadinene, and limonene.

Our results demonstrated that the EOs of six plants exhibited antifungal activity *in vivo* and reduced the severity of Fusarium wilt of chickpeas under controlled conditions. The efficacy of the EOs *in vivo* could be attributed to the reduction of the soil fungal populations and the induction of the host resistance. Belabid *et al.* (2010) reported that treatment with the EOs formulation significantly reduced the soil population densities of *Fusarium oxysporum* f.sp. *lentis* W.L. Gordon and, consequently, the diseases incidence under laboratory conditions. The level of protection of the tested EOs against *Botrytis cineria* Persoon varied according to the applied dose and the type of preventive and curative treatment (Soylu *et al.* 2010). The effect of the EOs on the disease could be directly linked to the intensive accumulation of other secondary metabolites such as phenolic and flavonoid compounds in the infected plant (Ben-Jabeur *et al.* 2015). It is probable that these compounds play an important role in the establishment of defence mechanisms (Clérivet *et al.* 1996). It has been reported that phenolic compounds, identified to be involved in cell membrane lignification and could explain the decrease in the Fusarium wilt severity by limiting pathogen diffusion (Jin *et al.* 2011). The obtained results are also in accordance with the observations of Jin *et al.* (2011) who found that EO treatments significantly enhanced the level of total phenolic and flavonoid contents of raspberries. The authors reported that EO components such as carvacrol and cinnamic acid could induce constitutive increases in antioxidant activity in plant tissues which in turn reduce the physiological deterioration and improve the resistance of tissue against pathogen invasion and decrease the spoilage of berry fruits. Ben-Jabeur *et al.* (2015) concluded that the protective effect against Fusarium wilt of tomatoes could be due to the induction of a resistance, as such thyme oil induced phenolic and peroxidase accumulation in the roots and leaves 3-days after treatment.

In this study, EOs from six aromatic plants were evaluated regarding their capacity to control chickpeas against Fusarium wilt. The results clearly showed that the EOs may afford *in vitro* and *in vivo* antifungal activity against the FOC agent and can be used as a promising biofungicide for future chickpea Fusarium wilt disease management. Suppression of the mycelial growth, spore production and germination by EO treatments could make a major contribution to limit the spread of the pathogen by lowering the spore load in the soil. The efficacy of the EOs *in vivo* conditions may result from the combination of direct antifungal activity and the elicitation of defence responses in the host plant.

Acknowledgements. This work was supported by the Laboratory of Characterization and Valorization of Natural Resources, the Faculty of Nature and Life Sciences, Mohamed El Bachir El Ibrahimi University.

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Received: September 10, 2018
Accepted: March 24, 2019