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RESEARCH ARTICLE

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Rosmarinus officinalis essential oil as an effective antifungal and herbicidal agent

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Abstract

In order to reduce the use of chemical pesticides, great interest has been focused on environment-friendly biological control agents and botanicals that preserve biodiversity. In this context, our study aimed to assess the antifungal and herbicidal activities of *Rosmarinus officinalis* essential oil (EO) to find an alternative to synthetic pesticides. The chemical composition of *R. officinalis* essential oil was determined by gaz chromatography-mass spectrometry analysis (GC-MS). Results showed that *R. officinallis* EO was rich in monoterpenes and the major constituents were 1,8-cineole (54.6%), camphor (12.27%) and α-pinene (7.09%). However, under laboratory conditions, two tests were carried out. The first one consisted on the study of EO antifungal activity using ELISA microplates and the second one consisted on evaluating the effect of EO on seedling growth of weeds. It was confirmed that this EO significantly inhibits spore germination of *Fusarium oxysporum, Fusarium culmorum, Penicillium italicum* and at 6 mM, the percentage of inhibition reached 100% on *Fusarium oxysporum*. Indeed, EO slows down seedling growth of *Trifolium incarnatum*, *Silybum marianum*, and *Phalaris minor*. In fact, EO at 5 mM completely inhibits seed germination. On the other hand, another experiment was carried out to evaluate the herbicidal activity by spraying EO on weeds. This showed that a novel herbicide formulation was set up for the first time to improve the activity of *R. officinalis* EO on post-emergence. Overall, *R. officinalis* EO can be suggested as a potential eco-friendly pesticide and suitable source of natural compounds potentially usable as natural pesticides.

Additional keywords: biological control; 1,8 cineole, fungicidal activity; bio-herbicidal activity; formulation.

Abbreviations used: EO (essential oil); PDA (potato dextrose agar); PDB (potato dextrose broth).

Authors' contributions: SBK, MH, RK and HJ conceived and designed the research. SBK conducted the experiments. SBK and CB analyzed the data. SBK, IBR, CDC and MLF wrote the manuscript. All authors commented, discussed, and approved the manuscript.

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Introduction

Agricultural production has always been threatened by the presence of plant pathogens such as fungi, bacteria, and viruses (Kordali *et al.*, 2016). Moreover, weeds are another major issue. In fact, they compete with crops for resources (water, nutrients, light) and cause huge economic losses, up to 34% in major crops (Araniti *et al.*, 2015). Every year, approximately 2.5 million tons of pesticides are used on crops worldwide to fight plant diseases (Koul *et al.*, 2008) with consequences on human health, soils and the environment (groundwater contamination and development of weed resistance). This intensive use has been recognized as one of the main drivers of biodiversity losses (Schütte *et al.*, 2017).

In the last few decades, there has been growing interest in investigating eco-friendly alternatives, in particular essential oil (EO)-based methods in order to curtail pesticide use because pesticides cause extensive damage to agricultural and natural systems (Ben Ghnaya *et al.*, 2013). Moreover, the use of EOs obtained through a cheap production process can reduce the frequent applications of certain synthetic pesticides that have deleterious effects on the environment and human health (Pavela & Benelli, 2016). But EOs

can also present a low health risk during application. One of the great challenges for further research is to design an efficient stabilization process so as to apply EOs in fields. In the same vein, several studies have pointed out that EOs may have not only antifungal activity (Koul *et al.*, 2008; Tian *et al.*, 2012; Ahluwalia *et al.*, 2014; Hmiri *et al.*, 2015; Boubaker *et al.*, 2016) but also the ability to inhibit weed seedling growth (Uremis *et al.*, 2009; Poonpaiboonpipat *et al.*, 2013). In the Mediterranean region and especially in Tunisia, the most widespread botanical family is Lamiaceae, which has antimicrobial properties (Pintore *et al.*, 2002). Among these aromatic plants, the most interesting species is *Rosmarinus officinalis* (*R. officinalis*) which is known for its antifungal activity (Angioni *et al.*, 2004; Giamperi *et al.*, 2011; Hmiri *et al.*, 2015) and its richness in EOs characterized by the predominance of monoterpenes – mostly 1,8 cineole –, camphor, and α-pinene (Zaouali *et al.*, 2010). Hence, the main aims of this study were (1) to assess the antifungal activity of *R. officinalis* EO against three potential plant-pathogenic fungi, (2) to evaluate its herbicidal activity on three weed species for the first time, and then (3) to formulate a bioherbicide in order to enhance its efficiency and stability.

Material and methods

Plant material and essential oil extraction

In March 2014, *R. officinalis* plants, which belong to the *Lamiacae* family, were collected at the flowering stage in a naturally diversified mountain of the Seliana region in the northeast of Tunisia (36°06'47.9"N 9°35'30.0"E). The plants were identified by the botanist of the Biotechnology Center of Borj-Cedria (CBBC). All selected plants were shade-dried for 15 days at 30°C. One hundred grams of dried leaves and flowers were chopped and subjected to hydrodistillation using a Clevenger-type apparatus for 2 h (Ben Jemia *et al.*, 2015). The essential oil was stored at 4°C in amber vials.

GC–MS analysis

The EOs were analyzed by a gas chromatography-mass spectrometry analyzer (Hewlett Packard HP5890 series II, USA) equipped with an HP-5 column coated with 5% phenyl methyl siloxane (30 m × 250 µm × 0.25 µm). The carrier gas was helium, at a pressure of 1 ml/min. The mass spectrometer (Agilent Technologies, USA) ionized the compounds at an electron impact of 70 eV prior to identification. The program was the following one: 40 °C for 1 min, then a 4 °C/min increase up to 100 °C, 100 °C for 5 min, followed by a 6 °C/min increase up to 200 °C, then 200 °C for 5 min, and finally a 15 °C/min increase up to 250 °C. The total running time for each sample was 46 min. The components were identified by comparison with the W9N11.L library and calculated retention indexes relatively to C_8-C_{24} n-alkanes injected in the HP 5MS column. The relative area percentages of the different EO constituents were calculated from the peak areas of the total ions.

Formulation

A formulation was used to mix the EOs in water and facilitate the penetration of active molecules through the epicuticular waxes. It contained amphiphilic substances to render interactions between polar and non-polar parts possible. The compounds of the formulation were chosen to allow better stability, efficacy, and a small droplet size. The detailed composition of the formulation is presented in Table 1.

Preparation of the culture media

Potato dextrose agar (PDA) was used to grow the fungal pathogens in Petri dishes, while potato dextrose broth (PDB) and tomato juice (V8) were used for growth in ELISA microplates.

Fungal strains and preparation of the inoculum

The fungal species *Fusarium oxysporum* (MUCL 38936), *Fusarium culmorum* (MUCL28166) and *Penicillium italicum* (MUCL 15608) were obtained from the BCCM/MUCL Agro-food & Environmental Fungal Collection (Louvain La Neuve, Belgium). They were cultured on PDA and incubated at 20°C under a 16h L: 8h D photoperiod.

A spore suspension was made by adding 10 mL of sterile distilled water to 0.05% Tween 20 on the surface of a 14-day-old fungal colony. The surface was gently

Table 1. Composition of the formulated natural herbicide based on the use of *Rosmarinus officinalis* essential oil.

Composition	% Content
Essential oil	3.4
Hazelnut vegetable oil	3.4
Tween 20	0.7
Span 80	0.3
Atplus UEP-100	0.25
Ethanol	0.5
Water	91.45
Total	100

scratched to suspend the spores in the liquid. The spore suspension was filtered through a sterilized double layer of fine cloth to remove mycelial fragments. The spore concentration was adjusted to 10^6 spores/mL with a Bürker haemocytometer.

Evaluation of the antifungal activity

The antifungal activity of the EO was evaluated using ELISA microplates with a randomized block design, as described by Kaddes *et al.* (2016). The growth of each pathogen was monitored in a volume of 200 µL containing diluted (3.10^{-2} v/v) PDB medium for *P. italicum* and *F. oxysporum*, and V8 medium for *F. culmorum*, the inoculum, and the EO at 1, 3, and 6 mM. The optical density of each well was measured at a wavelength of 630 nm every 24 h for 120 h, using a spectrophotometer for ELISA plates. Eight replications were conducted for each concentration, and tween 20 at 1% v/v was used as a negative control. The inhibition percentages were then calculated using the following equation:

$$\% \text{ inhibition} = \frac{\text{AV}(ODX'(\text{t}=0) - ODX'(\text{t}=120\text{h})) - \text{AV}(ODHx(\text{t}=0) - ODHx(\text{t}=120\text{h}))}{\text{AV}(ODX'(\text{t}=0) - ODX'(\text{t}=120\text{h}))}$$

where AV is the average value, *ODX*'(t=0) is the optical density of the pathogen growth control just after inoculation, *ODX*'(t=120) is the optical density of the pathogen growth control after 120 h, *ODHx* (t=0) is the optical density of the pathogen in association with the EO just after inoculation, and *ODHx* (t=120h) is the optical density of the pathogen in association with the EO after 120 h.

Seed germination bioassay

Seeds of *Phalaris minor* were collected in Tunisia from wheat fields. However, seeds of *Trifolium incarnatum* and *Silybum marianum* were obtained from ECOSEM industry in Belgium. They were sterilized using 5% sodium hypochlorite for 2 min. Filter papers were placed in 11-cm-diameter Petri dishes and moistened with 2 mL of Tween 1% solution (which did not interfere with the different assays) for the seedling control, or with EO solutions at 0.625, 1.25, 2.5, and 5 mM for the treated seedlings. Ten seeds of *T. incarnatum, S. marianum* or *P. minor* were then placed immediately in Petri dishes, and three replicates were prepared for each EO concentration. All Petri dishes were randomly placed in a growth chamber at a temperature of 23±1°C, in the dark. The number of germinated seedlings was counted, and their hypocotyls and root lengths were measured after 7 days (Amri *et al.*, 2012; Ben Ghnaya *et al.*, 2013). In order to know if *R. officinalis* EO had only slowed down germination or completely inhibited it, a supplementary test was carried out. It consisted in transferring the treated seeds from filter paper moistened with EO at 5 mM to agar solution, to check if germination might continue/resume or not. But no seed had germinated after 5 days.

Post-emergence activity of the essential oil

Another experiment was performed to study the effect of EO on 2-3-week-old *T. incarnatum, S. marianum*, and *P. minor* plantlets under controlled conditions (natural photoperiod supplemented with artificial light if needed, with 20 ± 3°C according to the sunlight. The relative humidity was 60 ± 3%). Only *P. minor* seeds were sown in boxes, whereas *T. incarnatum* and *S. marianum* seeds were sown in pots. The weed seeds were sown in 11-cm-diameter pots, and the plants were watered every day. Once the weeds reached the 2-3-leaf stage, several solutions were sprayed. They consisted of 10 mL of *R. officinalis* EO at 7.5, 20, and 34 mM, formulated *R. officinalis* EO at 34 mM, adjuvants alone (as negative controls), distilled water, and a commercial biological herbicide containing 34 mM of pelargonic acid (as a positive control). Three replications were conducted for each treatment, in a completely randomized manner. Seven days after spraying, the treated weed plants were examined to assess wilting, necrosis, and chlorosis. The percentage of efficacy was calculated following the equation :

$$\text{Percentage of efficacy (\%)} = {}^{N}/_{T} * 100$$

where *N* refers to the number of necrotic or withered leaves, and *T* represents the total number of leaves.

Statistical analysis

Pre-emergence and post emergence tests were conducted using a randomized block design with 3 replications. Statistical analyses were performed with Minitab 17 Statistical Software (Minitab Inc., State College, PA, USA). Results were examined statistically using one-way analysis of variance (ANOVA) followed by Tukey's multiple range tests. The differences between individual means were considered significant if $p<0.05$.

Results

Chemical composition of *R. officinalis* essential oil

The EO obtained by hydrodistillation of dried *R. officinalis* flowers and leaves had a clear green color

and emitted a pungent smell. The extraction yield was *ca.* 1.2% (w/v). The EO components identified by gas chromatography/mass spectrometry (GC/MS) are listed in Table 2. This process identified 98.71% of the compounds present in the EO. The *R. officinalis* EO was characterized by the predominance of the monoterpene class, among which 1,8 cineole, camphor, and α-pinene were the most present. This class was followed by ketones and alcohols, while esters and sesquiterpenes were found in minor quantities.

Antifungal activity of *R. officinalis* essential oil

In a dose-response bioassay, our results showed that this EO had an interesting potential at different concentrations (1, 3, and 6 mM). A rise in EO concentration increased spore germination inhibition of plant pathogens after 5 days of incubation. At the lowest EO dose, *P. italicum* was less sensitive than *F. culmorum* and *F. oxysporum*. In fact, that concentration was the least effective one. Furthermore, at 6 mM, the inhibition percentages of spore germination were very high, *i.e.* 85.99%, 100%, and 95.40% for *F. culmorum, F. oxysporum*, and *P. italicum*, respectively (Fig. 1).

Table 2. Chemical constituents of the essential oil extracted from *Rosmarinus officinalis* dried leaves and flowers.

Compounds	RI[a]	RI[b]	(%)[c]
Monoterpene hydrocarbons			17.09
α-Thujene	928	910–935	0.31
α-Pinene	931	921–944	7.09
Camphene	950	936–965	3.09
β-Pinene	980	962–987	3.81
Myrcene	993	975–991	0.44
Phellandrene	1005	990–1009	0.10
γ-3 carene	1011	997–1027	0.27
p-Cymene	1026	1004–1029	1.39
γ-Terpinene	1062	1049–1069	0.41
α-Terpinene	1012	1154–1195	0.18
Oxygenated monoterpenes			80.19
Camphor	1143	1481–1537	12.27
1,8 cineole	1033	1021–1044	54.60
Borneol	1165	1653–1728	9.66
Terpinen 4 ol	1178	1165–1189	0.90
Terpineol	1189	1178–1203	2.76
Esters			0.72
Bornyl-acetate	1286	1264–1297	0.72
Sesquiterpenes			0.71
β-Caryophyllene	1421	1384–1430	0.62
α-Humulene	1455	1430–1466	0.04
γ Cadinene	1525	1498–1531	0.05

[a]Calculated retention indexes relatively to C_8-C_{24} n-alkanes injected in the HP 5MS column. [b]Retention indexes relatively to C_8-C_{24} n-alkanes injected in the HP 5MS column, based on Babushok *et al*. (2011dimethyl silicone with 5% phenyl groups (slightly polar). [c]Relative quantifications were calculated by dividing the peak area of each compound by the total area of each chromatogram.

Herbicidal activity of *R. officinalis* essential oil under laboratory conditions

The application of EO at 5 mM completely inhibited seed germination of three weeds (*T. incarnatum, S. marianum* and *P. minor*) (Table 3). Moreover, EO at 1.25 and 2.5 mM caused significant delays in shoot and root growth of the same weeds after 7 days as compared to the control. As far as germination is concerned, *T. incarnatum* proved more resistant than *S. marianum* and *P. minor* and exhibited no response at the lowest EO concentration. By contrast, the EO had strong effects on the seedling growth of these weeds, even at low concentrations.

Herbicidal activity of *R. officinalis* essential oil under greenhouse conditions

Studies in which EOs are applied in post-emergence conditions are scarce. For this reason, *R. officinalis* EO was sprayed on 2-3-week-old weed plants in another set of experiments to determine its post-emergence herbicidal activity. The treatment using 7.5 mM EO showed weed resistance and no visual damage. At 20 mM, the EO caused a few symptoms of injuries on *T. incarnatum* and *P. minor* (Table 4). However, at 34 mM, the EO caused more visible injuries ranging from wilting after 1 day and chlorosis after 3 days on *T. incarnatum*. Its herbicidal activity reached up to 45%. *S. marianum* was consistently more resistant than *T. incarnatum* and *P. minor* at all concentrations. Pelargonic acid (used as positive control at 3.4%) completely punctured *T. incarnatum* and stopped *P. minor* and *S. marianum* growth. We also used the same EO in a formulated version to enhance the distribution, the coverage, and the penetration of the active molecules. It presented a high herbicidal activity, higher than the non-formulated EO, which reached 71.33% against *T. incarnatum*. Six hours after spraying the formulated EO, *T. incarnatum* and *P. minor* leaves were already wilting.

Discussion

Our results show that *R. officinalis* EO is an interesting antifungal and herbicidal agent from which

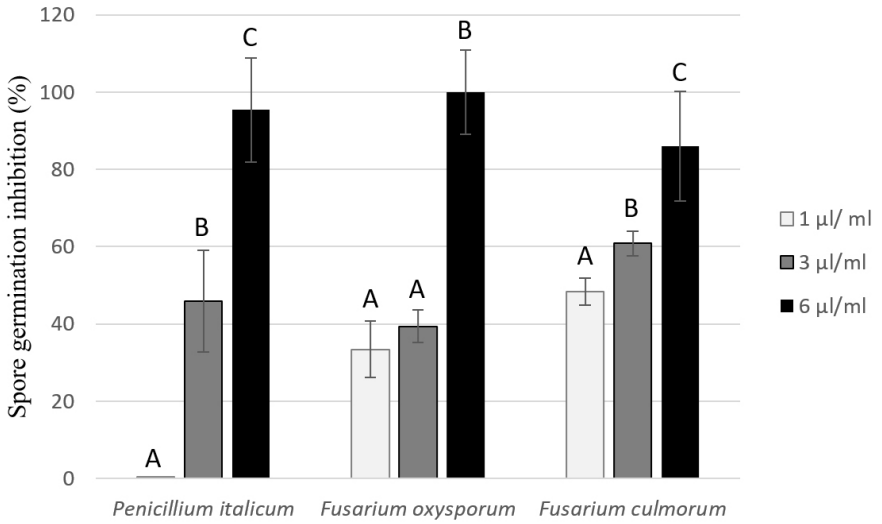

Figure 1. Fungicidal activity of *Rosmarinus officinalis* essential oil against three plant pathogens (*Fusarium oxysporum, Fusarium culmorum*, and *Penicillium italicum*) after 120 h. Different letters mean significantly different results with the same strain (p<0.05, Tukey's statistical test).

Table 3. Inhibitory effects of *Rosmarinus officinalis* essential oil extracted from leaves and flowers at the vegetative stage on the germination and seedling growth of *Trifolium incarnatum, Silybum marianum*, and *Phalaris minor* after 7 days.

Weeds	Dose (mM)	Germination (%)	Root length (cm)	Shoot length (cm)
T. incarnatum	Control	100.0±0.00 ^{A}	4.33±0.11 ^{A}	3.95±0.16 ^{A}
	0.625	100.0±0.00 ^{A}	0.26±0.04 ^{B}	0.85±0.01 ^{B}
	1.25	100.0±0.00 ^{A}	0.26±0.02 ^{B}	0.63±0.01 ^{B}
	2.5	100.0±0.00 ^{A}	0.15±0.01 ^{BC}	0.31±0.02 ^{C}
	5	0.0±0.0 ^{B}	0.0±0.0 ^{C}	0.0±0.0 ^{D}
S. marianum	Control	86.67±8.89 ^{A}	1.75±0.05 ^{A}	2.69±0.09 ^{A}
	0.625	80.00±13.33 ^{A}	1.73±0.05 ^{A}	2.78±0.09 ^{A}
	1.25	70.00±6.66 ^{AB}	1.10±0.04 ^{B}	1.03±0.08 ^{B}
	2.5	43.33±4.44 ^{B}	0.70±0.007 ^{C}	0.75±0.01 ^{C}
	5	0.0±0.0 ^{C}	0.0±0.0 ^{D}	0.0±0.0 ^{D}
P. minor	Control	86.66±4.44 ^{A}	2.5±0.17 ^{A}	5.11±0.11 ^{A}
	0.625	56.66±4.44 ^{B}	2.24±0.04 ^{A}	4.72 ±0.10 ^{A}
	1.25	36.66±4.44 ^{C}	1.79±0.03 ^{B}	3.82±0.12 ^{B}
	2.5	16.66±4.44 ^{D}	0.9±0.07 ^{C}	2.22±0.19 ^{C}
	5	0.0±0.0 ^{E}	0.0±0.0 ^{D}	0.0±0.0 ^{D}

Means followed by different capital letters in each column are significantly different (p<0.05, Tukey's statistical test).

a more environment-friendly alternative to chemical herbicides might be derived. The antifungal and herbicidal activities of EOs have been widely reported in recent years (Pintore *et al.*, 2002; Salamci *et al.*, 2007 Tian *et al.*, 2012; Amri *et al.*, 2012; Kaur *et al.*, 2012; Ben Ghnaya *et al.*, 2013; Ahluwalia *et al.*, 2014; Bouabidi *et al.*, 2015; Hmiri *et al.*, 2015; Alipour & Saharkhiz, 2016; Synowiec *et al.*, 2017), but to our knowledge, only a few studies have focused on their effect on post emergence when sprayed on weeds (Hazrati *et al.*, 2017). *R. officinalis* is largely used in traditional medicine (Pintore *et al.*, 2002; Ben Jemia *et al.*, 2015) and widely known for its antimicrobial and antioxidant activities (Bozin *et al.*, 2007; Celiktas *et al.*, 2007; Zaouali *et al.*, 2010), but the present study unveils its herbicidal effect in pre-emergence and post-emergence for the first time. On the other hand, GC-MS analysis of our *R. officinalis* EO extracted from dried leaves and flowers identified 19 compounds dominated by oxygenated monoterpenes including 1,8 cineole, camphor, and borneol. These results are in agreement with Zaouali *et al.* (2010), who showed that these three major components are also predominant in the Tunisian *R. officinalis* EO. However, their per-

Table 4. Herbicidal activity of *Rosmarinus officinalis* essential oil (EO) extracted from leaves and flowers at the vegetative stage on weeds under greenhouse conditions.

Treatment	Dose (%)	*Trifolium incarnatum*	*Silybum marianum*	*Phalaris minor*
Negative control	-	0.0±0.0 ^{E}	0.0±0.0 ^{C}	0.0±0.0 ^{D}
EO-free formulation	-	0.0±0.0 ^{E}	0.0±0.0 ^{C}	0.0±0.0 ^{D}
EO	0.75	0.0±0.0 ^{E}	0.0±0.0 ^{C}	0.0±0.0 ^{D}
	2	24±2.66 ^{D}	0.0±0.0 ^{C}	27.33±4.44 ^{C}
	3.4	45±2.0 ^{C}	0.0±0.0 ^{C}	34.33±2.88 ^{C}
Formulated EO	3.4	71.33±2.44 ^{B}	18±4.66 ^{B}	46.33±2.22 ^{B}
Formulated pelargonic acid	3.4	100±0.0 ^{A}	100±0.0 ^{A}	100±0.0 ^{A}

Means followed by different letters in each column are significantly different (p<0.05, Tukey's statistical test).

centages varied between 26.0-51.2%, 4.9-29.7% and 3.3-10%, respectively. These differences in chemical composition could be related to environmental factors (the climate, the season, the soil), the genetic diversity of the species, and the geographic conditions (Ben Ghnaya *et al.*, 2013). Interestingly, the monoterpenes identified as main constituents in our EO have been described as powerful inhibitors of the seed germination and growth of several plant species (De Martino *et al.*, 2010; Barton *et al.*, 2014). These compounds also showed antifungal activity (Ben Ghnaya *et al.*, 2013; Ahluwalia *et al.*, 2014; Marei & Abdegaleil, 2018).

In addition, EOs from plants of the *Lamiaceae* family, and among them *R. officinalis*, are known for their antimicrobial activity (Hendel *et al.*, 2016). In our study, *R. officinalis* significantly inhibited the spore germination of *P. italicum, F. oxysporum*, and *F. culmorum*. *F. oxysporum* and *F. culmorum* have been widely documented as the most important plant pests; they cause substantial economic losses worldwide (Hollingsworth & Motteberg, 2008). *R. officinalis* EO from Greece caused a dose-dependent inhibition of the mycelial growth of five fungi (*Sclerotinia sclerotiorum, Phytophthora nicotianae, Sclerotium cepivorum, F. oxysporum*, and *Fusarium proliferatum*) (Pitarokili *et al.*, 2002). In addition, Sardinian *R. officinalis* EO (450 and 900 µL/mL) showed a weak activity against all tested fungi (*Botrytis cinerea, F. oxysporum lycopersici, Fusarium graminearum, F. culmorum, and Rhizoctonia solani*). On the other hand, these EOs present multiple mechanisms of action due to a large number of active compounds that reduces the development of fungal resistance. For instance, a recent study confirmed that 1,8 cineole alone had a low antifungal power but showed an important synergistic effect with α-pinene (Hmiri *et al.*, 2015). These two compounds were identified in our EO. In the same line, other reports suggested that 1,8 cineole combined with terpinen-4-ol, also the major component of *Melaleuca alternifolia* EO, had a significant synergistic effect on the hyphal morphology of *B. cinerea* and its ultrastructure as compared to the treatment using either component alone. In fact, 1,8 cineole can penetrate the cell and damage cellular organelles without affecting membrane permeability. On the other hand, terpinen-4-ol destroys membrane integrity and increases permeability, resulting in ion leakage and membrane dysfunctioning. Several studies reported that EOs could cause structural and functional damage by disrupting the membrane permeability and the osmotic balance of the cell (Yu *et al.*, 2015). Other studies have shown that they can acidify the external medium and decrease ATPase and dehydrogenase activities in *Aspergillus flavus* cells (Tian *et al.*, 2012). Furthermore, EO from seeds of *Anethum graveolens* showed fungicidal activity against *Sclerotinia sclerotiorum* by inhibiting mycelial growth and sclerotial germination. This effect is the consequence of the inhibition of ergosterol synthesis, malate dehydrogenase, and succinate dehydrogenase (Ma *et al.*, 2015).

In parallel, to our knowledge, no study had yet tackled the herbicidal activity of *R. officinalis* EO. In fact, our experiments highlighted the outstanding inhibition of three different weeds after treatment with our EO. This was seen on the percentage of germination, root growth, and hypocotyl length. In fact, 100% inhibition of germination and seedling growth was observed with our EO at 5 mM. In this context, Poonpaiboonpipat *et al.* (2013) showed that at 1 µL and 2 µL/Petri dish of *Cymbopogon citratus* EO, there was no significant effect on shoot or root length, but seedling length was shorter at 4 and 8 µL/Petri dish. The strong phytotoxic activity was due to the presence of oxygenated monoterpenes, which is quite similar to that of Tunisian *Eucalyptus erthrocorys*

EO, renowned for its overwhelming phytotoxic effect (Ben Ghnaya *et al.*, 2013). In this context, among 12 EOs tested on weeds, caraway, thyme, peppermint, and sage oils were classified as the most phytotoxic ones owing to the existence of oxygenated monoterpenes in a 64.1–93.3% range (Synowiec *et al.*, 2017). In line with this, among six monoterpenes tested by Gouda *et al.* (2016), 1,8 cineole and (S)-limonene were showed to inhibit *Echinochloa crus-galli* shoot growth. The major components of EOs are very important for their biological activity, but even the minor ones could have significant synergistic effects (Synowiec *et al.*, 2017). Many other individual compounds identified in *R. officinalis*, such as α-terpineol, citronellal, citronellol, and α-pinene, have been confirmed to have phytotoxic activity (Zhang *et al.*, 2014). In contrast, among 25 EOs, only those containing volatile phenolic compounds such as thymol, carvacrol, eugenol, alcohols or ketones, showed strong phytotoxic effect on different weed seeds, even though the mode of action of all these compounds has not yet been detailed and a number of effects and hypotheses have been reported by many authors. Several authors assume that EOs act by causing biochemical and physiological changes in seedling growth (De Martino *et al.*, 2010). For instance, *Cymbopogon citrates* EOs notably slowed down α-amylase activity in *E. crus-galli* seeds (Poonpaiboonpipat *et al.*, 2013). Another clear example is *Artemisia* sp. EO: it induced reactive oxygen species production, which in turn caused damage resulting in lipid peroxidation, decreased membrane fluidity, and finally increased membrane leakiness and inactivated receptors, enzymes and ion channels (Kaur *et al.*, 2012). Moreover, 1,8 cineole inhibited root growth and stopped DNA synthesis through several steps (Koitabashi *et al.*, 1997).

We applied *R. officinalis* EO not only in pre-emergence tests but also for the first time in post-emergence tests, by spraying it on weeds under greenhouse conditions. Based on the visual damage induced by this EO on weeds three days after spraying, herbicidal properties were noticed. Necrosis and wilting leaves were observed at a concentration of *R. officinalis* EO starting from 20 mM. Similar results showed that the spraying of *Cymbopogon citratus* EO from 1.25 mM to 10 mM on *E. crus-galli* leaves caused wilting, and the leaves exhibited desiccation symptoms. In addition, this EO decreased the chlorophyll a, b and carotenoid contents, and caused electrolyte leakage, indicating membrane disruption and loss of integrity (Poonpaiboonpipat *et al.*, 2013). Monoterpenes, which are present at 80.19% in our *R. officinalis* EO, may affect plant photosynthesis, energy metabolism, and the biosynthesis of secondary metabolites such as phenolic compounds (Gouda *et al.*, 2016). In addition, it has been confirmed that the penetration of monoterpenes through the cell wall and cell membrane can cause cellular potassium leakage that inhibits glucose-dependent respiration. A recent study showed that the spraying of a nano-emulsion of *Satureja hortensis* EO reduced the weed chlorophyll content, and increased electrolyte leakage and cell membrane disruption (Hazrati *et al.*, 2017).

We investigated a formulation of *R. officinalis* EO as a bioherbicide for the first time, based on the following observations: (1) as *R. officinalis* EO is lipophilic, it does not dissolve well in water; (2) in the same line, the reported herbicidal effect of *Satureja hortensis* EO in the absence of tween 20 was lower on control weeds; and (3) EOs contain terpenoids that are volatile, thermolabile, and may be easily oxidized and hydrolyzed (Pavela *et al.*, 2016). For these reasons, we used an emulsifier providing better stability, efficacy and persistence for the formulation. An ionic surfactant reduced the effective concentration of eucalypt oil for a high herbicidal activity against *P. minor* (Batish *et al.*, 2007). Based on that, a recent study showed that a formulation containing palm oil, tween 20 and span 80 improved the herbicidal activity of metabolites from *Phoma* sp. (Todero *et al.*, 2018).

To our knowledge, this is the first report that links the chemical composition of Tunisian *R. officinalis* EO to its fungicidal and bio-herbicidal effects on plant pathogens and weeds, respectively. Moreover, the formulation of the bio-herbicide based on Tunisian *R. officinalis* EO was attempted in this work for the first time. Hence, this work opens new perspectives on the application of Tunisian *R. officinalis* EOs as a novel biocontrol strategy against harmful plant pathogens and weeds. It also paves the way for new strategies and pathways for the biopesticide industry to create alternative chemical pesticides designed to be less harmful to the environment and human health than current ones. For agronomic applications, we found that *R. officinalis* EO could be used as a biofungicide at low concentrations between 1 mM and 6 mM without any phytotoxic effect in post-emergence tests. At concentrations higher than 20 mM, this EO can be used as a post-emergence bioherbicide. According to our preliminary results, the use of EOs in the formulation of bioherbicides can offer new prospects for the sustainable production and practical use of EOs. To go further in the experiments, it could be really interesting to determine the modes of action of *R. officinalis* EO on weeds and fungi and try to improve the effectiveness and stability of the bioherbicidal *R. officinalis* EO formulation.

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