ORIGINAL ARTICLE

Composition of essential oil of Moroccan *Dysphania ambrosioides* and its antimicrobial activity against bacterial and fungal phytopathogens

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Abstract

This study aimed to characterize the essential oil (EO) of *Dysphania ambrosioides* grown in Morocco and to evaluate its antimicrobial activity against fungal (*Verticillium dahlia, Fusarium oxysporum* f. sp. *melonis* and *Fusarium culmorum*) and bacterial (*Agrobacterium tumefaciens, Pseudomonas syringae* pv. *tabaci, P. syringae* pv. *syringae* and *Erwinia amlovora*) phytopathogens. Essential oil was extracted using the steam distillation method in Clevenger-type apparatus and analyzed by gas chromatography-mass spectrometry (GC/MS). Its effect was studied using tests of inhibition zones in vitro and pathogenicity trials *in planta*. Results showed that EO of the Moroccan *D. ambrosioides* belongs to the ascaridole chemotype and exhibits elevated antifungal activity in vitro. When used at 500 μg ml^{-1}, inhibition of radial growth of *Fusarium oxysporum* f. sp *melonis*, *Fusarium culmorum* and *Verticillium dahilae* ranged from 78% to 90%, while it was extremely reduced with purified ascaridole, suggesting that its main antifungal activity relies on constituents other than ascaridole. However, EO and purified ascaridole also exhibited great potential for in vitro antibacterial activity against *Pseudomonas syringae* pv. *syringae, Pseudomonas syringae* pv. *tabaci, Erwina amylovora* and moderate activity against *Agrobacterium tumefaciens*. Furthermore, pathogenicity trials *in planta* revealed that EO provided elevated protection against wild fire disease caused by *Pseudomonas syringae* pv. *tabaci* on *Nicotiana benthamiana* when used at 100 μg ml^{-1}. It also protected *Solanum lycopersicum* plants against crown gall disease, however higher concentrations were needed. These results suggest that the EO from *D. ambrosioides* can be used as an antimicrobial agent to protect crops against fungal and bacterial disease.

Keywords Ascaridole · Phytopathogenic fungi and bacteria · Essential oil

Introduction

Plant-derived secondary metabolites are largely substituting synthetic antimicrobial compounds (Cowan 1999). Among them, essential oils (EO) present in specialized cells or glands of some plants deserve particular attention. They are considered as complex mixtures of monoterpenes, sesquiterpenes and their oxygenated derivatives that can be isolated from the whole plant or from organs by different methods such as steam distillation (Dhifi et al. 2016).

The genus *Dysphania* (Amaranthaceae) (Mosyakin and Clemants 2002) includes more than 47 species that are distributed in hot sub-tropical and sub-temperate regions (Perth 2013). *Dysphania ambrosioides* is extensively dispersed throughout Morocco and is used in traditional medicine against different diseases such as oral abscesses, ulcers and

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gastrointestinal disorders (Bellakhdar 1997). The chemical composition of *D. ambrosioides* essential oil depends on its geographical origin (Al-Kaf et al. 2016). However, all the EO studied had quite diverse compositions and high content of monoterpenes compounds, mainly α-terpinene, *p*-cymene, ascaridole, thymol, carvacrol and isoascaridole. The EO of *D. ambrosioides* also possesses antibacterial, antifungal, antioxidant, insecticidal, allelopathic and anthelmintic activities (Chekem et al. 2010; Chu et al. 2011; Jardim et al. 2008; Jiménez-Osornio et al. 1996; Ketzis et al. 2002; Prassard et al. 2010; Santiago et al. 2016).

Nowadays, phytopathogenic microorganisms are responsible for huge economic losses in agriculture. Various agrochemical products have been developed and used to overcome persistent attack of plants by these pathogens. However, because of their toxicity to humans and of their negative environmental effects on soil organisms and plant pollinators, development of natural products, such as EO, to control plant pathogenic diseases is gaining more interests. Thus, the EO of *Oreganum onites*, *Lavandula stoechas*, *Foeniculum vulgare*, *Laurus nobilis* and *Myrtus cummunis* has a strong inhibitory activity of mycelial growth and spore germination of *Fusarium oxysporum* f. sp. *radices-cucumericum* (Soylu and Incekara 2017). *Syzygium aromaticum* EO inhibits mycelial growth and conidia germination of *Fusarium oxysporum* f. sp. *lycopersici* in vitro and reduces development of symptoms of fusarium wilt in *Solanum lycopersicum*. The essential oils of *Zataria multiflora* and *Ruta montana* also inhibit the mycelial growth of *Alternaria alternata* and *Botrytis cinerea,* (Mahmoudi et al. 2012; Hammami et al. 2015). In addition, extracts of *Origanum* are effective in inhibiting the in vitro growth of several phytopathogenic bacteria such as *Xanthomonas axonopodis* pv. *vesicatoria* and are used as seed disinfectants or as soil biofumigants (Vasinauskiene et al. 2006; Altundag and Aslim 2011; Dadasoglu et al. 2011; Alves et al. 2014).

The objectives of this work were: i) to analyze the chemical composition of the essential oil constituent of *D. ambrosioides* grown in Morocco, ii) to evaluate its effect in vitro on some important phytopathogenic bacteria and fungi and to identify the bioactive molecule behind the possible activities of EO using a purified fraction of its major constituent, ascaridole and iii) to determine whether the EO can be used to protect plants preventively against wild fire and crown gall diseases caused by *Pseudomonas syringae* pv. *tabaci* and *Agrobacterium tumefaciens*, respectively.

Materials and methods

Plant materials

Dysphania ambrosioides was collected in February 2015 from Sidibennour, at 70 km on the South of El Jadida (Morocco).

The plant was identified by Professor M. Fennane from the Scientific Institute (Rabat, Morocco). A voucher specimen was deposited in the botanic department of the national herbarium RAB of the Scientific Institute under the accession number RAB102638.

Nicotiana benthamiana and *Solanum lycopersicum* seeds of the variety Campbell 33 were disinfected with 5% sodium hypochlorite for three min and rinsed three times with sterile water. Then they were dried and germinated in half Murashige and Skoog medium. After germination, seedlings were transferred to pots containing a sterile mixture of peat and sand (75%: 25%) at a depth of 2 cm. Forty-five day old seedlings were used for bacterial inoculation.

Essential oil isolation and gas chromatography - mass spectrometry analysis (GC-MS)

Batches of 500 g of fresh aerial parts (stems, leaves and fruits) were submitted to hydrodistillation, under laboratory conditions, for 3 h using a Clevenger apparatus. The yellowish oils obtained with yields of 0.7% (w / w) on a fresh-weight basis, were dried over anhydrous sodium sulphate, and stored in airtight glass vials, at 4 °C until used.

GC-MS analyses were performed on a Hewlett-Packard 5973–6890 system operating in EI mode (70 eV) equipped with a split/splitless injector (220 °C), a split ratio 1/10, using 2 different columns: a fused silica HP-5 MS capillary column (30 m × 0.25 mm (i.d.), film thickness: 0.25 μm) and a HP-Innowax capillary column (30 m × 0.25 mm (i.d.), film thickness: 0.50 μm). The temperature program for the HP-5 MS column was from 60 °C (5 min) to 280 °C at a rate of 4 °C/min and for the HP-Innowax column from 60 °C to 260 °C at a rate of 3 °C/min. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The injection volume of the sample was 2 μl. Retention indices for all compounds were determined according to the Van der Dool approach (Van der Dool and Kratz 1963), using n-alkanes as standards. The identification of the volatile compounds (Table 1) was based on comparison of their mass spectra with those of Wiley and NBS Libraries and those described by Adams (2001) as well as by comparison of their retention indices with literature data (Massada 1976). Optical rotation was measured on a Perkin Elmer 341 Polarimeter.

Isolation of ascaridole from essential oil

The essential oil (5.33 g) was subjected to column chromatography on silica gel 60 (70–230 mesh, Fluka). A stepwise gradient of hexane–diethylether was used to give nine fractions. Fraction II (hexane–diethylether (96:4), 1.33 g) gave pure ascaridole (Fig. 1). The other fractions were mixtures.

Table 1   Chemical composition of *Dysphania ambrosioides* essential oil

Compounds	RI[a]	Percentage
δ-3-Carene	1009	0.2
α-Terpinene	1018	13.3
p-Cymene	1029	18.2
Limonene	1033	0.7
γ-Terpinene	1058	0.4
p-Cymen-8-ol	1184	0.6
Ascaridole	1239	34
cis-Piperitone oxide	1256	0.8
trans-Piperitone oxide	1261	1.4
trans-Ascaridolglycol	1272	4.5
Thymol	1298	6.4
Carvacrol	1304	6.1
Isoascaridole	1307	5.3
β-Ionone	1487	0.7
Total identified		92.6

[a] RI: retention indices calculated against C9-C24 *n*-alkanes on the HP-5 column

^{1}H and ^{13}C-NMR analysis of ascaridole

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 spectrometer, operating at 300.14 MHz for ^{1}H and 75.47 MHz for ^{13}C. ^{1}H chemical shifts were referred to the residual chloroform signal (7.26 ppm); ^{13}C NMR chemical shifts were referred to the central peak of CDCl$_3$ (77.16 ppm). Ascaridole: Yellow oil; represents
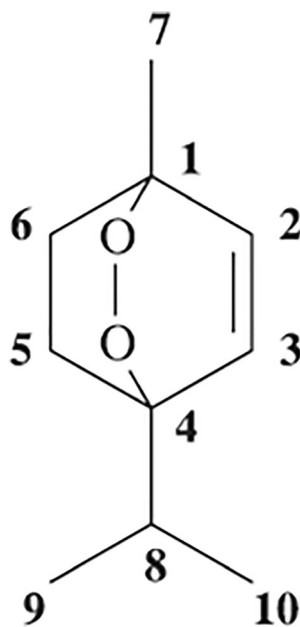
Fig. 1 Structure of ascaridole purified from the essential oil of *Dysphania ambrosioides*

24.95% of the essential oil; ^{1}H and ^{13}C-NMR data are shown in Table 2.

Fungal, bacterial material and culture media

Two strains (SH and SJ) of *Verticillium dahliae*, the causal agent of Verticillium wilt disease were isolated from olive tree in the Souss region (Morocco) (Cherrab et al. 2002; Smaili et al. 2017). *Fusarium oxysporum* fsp. *Melonis,* the causal agent of fusarium wilt was isolated from melon field in Doukkala region. *Fusarium culmorum*, the causal agent of root rot was isolated from durum wheat in Settat region in Morocco (Smaili et al. 2017). All the fungal strains were cultured on potato dextrose agar (PDA) plates and incubated at 26 °C in the dark.

Four strains (Ach5, A281, C58 and EHA105) of *Agrobacterium tumefaciens,* the causal agent of crown gall disease, were cultured on Luria Bretani (LB) medium supplemented with agar at 28 °C during 24 h (Hood et al. 1986). The strains CFBP2106 of *Pseudomonas syringae* pv. *tabaci* causing wild fire disease on tobacco and the strain CFBP692 of *P. syringae* pv. *syringae* causing bacterial canker of plum trees were from the "Collection Francaise des Bactéries Phytopathogènes, INRA, Angers, France". *Erwinia amlovora* was isolated from fire blight diseased trees in Morocco (Smaili et al. 2017). They were cultured on King B (KB) medium (King et al. 1954) overnight at 28 °C.

In vitro antifungal growth assessment

EO was dissolved in 0.05% Tween 80 according to Chutia et al. (2009) and added at the concentrations of 50, 100, 200, 300, 400 and 500 μg ml^{-1} to the PDA medium. The isolated ascaridole was dissolved in 0.05% Tween 80 and added at the concentration of 500 μg ml^{-1}. The amended media was poured into petri dishes and inoculated using 5-mm plugs of

Table 2 ^{1}H and ^{13}C-NMR data of ascaridole (300 MHz, in CDCl$_3$)

	δ ^{1}H NMR (ppm)	δ ^{13}C NMR (ppm)
1	–	74,305
2	6,38 (d; J = 8.4 Hz)	132,999
3	6,46 (d; J = 8.4 Hz)	136.358
4	–	79,730
5	1,95–2,1 (m)	25,579
6	1,95–2,1 (m)	29,486
7	1,33 (s)	21,360
8	1,88 (m)	32,099
9	0,96 (d; J = 7,2 Hz)	17,12*
10	0,96 (d; J = 7,2 Hz)	17,20*

δ: values in ppm and coupling constants J (in parentheses) in Hz; *: Values can be interchanged

agar and mycelium taken from actively growing cultures of fungus on PDA. Petri plates were incubated in the dark at 26 °C. The mycelia growth was estimated by measuring two orthogonal diameters of each colony after 5 days of incubation. Mycelial growth was compared with growth on PDA amended with 0.05% Tween 80 and the percentage of inhibition growth was expressed relative to the control. Three replicates were used.

The effect of EO on sporulation was evaluated according to Mouria et al. (2014). After ten days of incubation, 4 mycelial discs of 5 mm diameter were taken from the edge of each petri dish on the same line. They were introduced into a test tube containing 1 ml of sterile DW. After mechanical homogenization, conidia were counted using Malassez cell under light microscope. The experiment was repeated three times.

The regression line between colony diameter of the pathogen or the number of conidia and Log concentration of products was determined to calculate IC50 values.

In vitro antibacterial growth assessment

Antibacterial activity of the EO and ascaridole was investigated by the agar disc diffusion method as described in Smaili et al. (2017). Bacteria that were grown overnight were diluted to concentration of 3×10^8 CFU ml^{-1}. Sterile cellulose discs of 6 mm diameter were impregnated with 0.05% Tween 80 or with the EO or ascaridole (100 and 1000 μg ml^{-1} after solubilization in 0.05% Tween 80) and placed on LB or KB solid medium previously amended with $3 \ 10^8$ CFU ml^{-1} of bacteria. 0.05% Tween 80 was used as negative control while 50 μg ml^{-1} of chloramphenicol was used as positive control. The inoculated plates were incubated at 28 °C for 24 h. The antimicrobial activity was evaluated by measuring the clearance zone around the discs which indicates a positive antibacterial activity of the respective products. All experiments were done in triplicate.

Plant treatments, inoculations and disease assessments

EO was tested in the greenhouse as preventive treatment on *N. benthamiana* against *P. syringae* pv. *tabacci* and on *S. lycopersicum* seedlings against *A. tumefaciens*. The attached leaves of forty five days-old plants *N. benthamiana* were sprayed to runoff with 30 mL of 100 or 1000 μg ml^{-1} of the EO or with DW supplemented with 0.05% Tween 80 as negative control. Two sprays were performed at 7 days and 3 days before inoculation with 100 μl of bacterial suspension of the strain CFBP2106 of *P. syringae* pv. *tabaci* at concentration of $3. 10^8$ CFU ml^{-1}. Young leaves of *N. benthamiana* were inoculated by infiltration using a syringe. Symptoms were recorded 5 days after inoculation. The diameter of necrosis was measured as described in Smaili et al. (2018). For each treatment, the average diameter was calculated from of

21 leaf panels taken from three young leaves belonging to seven biolgical replicates.

To induce resistance against *A. tumefaciens* stems of forty five day-old *S. lycopersicum* seedlings were sprayed to runoff with 30 ml of 100 or 1000 μg ml^{-1} of the EO or with DW supplemented with 0.05% Tween 80 as negative control. Two sprays were performed at 7 days and 3 days before inoculation with the strain C58 of *A. tumefaciens*. Inoculation was performed as described in Faize et al. (2012). Over-night grown bacteria were pelleted and resuspended in 5 mM sodium citrate pH 5.5 supplemented with 2% sucrose and 0.1 mM acetosyringone. Stems were wounded at four sites using scalpel (1 cm length) and filled with 10 μl of bacterial suspension at 3×10^8 CFU ml^{-1}. Inoculated plants were kept in the greenhouse and the diameter of developed tumors was recorded every week during 8 weeks from 12 sites belonging to three biological replicates.

Quantification of bacterial growth *in planta*

To determine the bacterial population *in planta*, discs of leaves (1 cm in diameter) previously infiltrated with100 μl of *P. syringae* pv. *tabaci* adjusted to 3×10^8 CFU ml^{-1} were taken at 5 dpi. They were homogenized in 50 mM phosphate buffer, pH 7.2, then serially diluted and plated on KB agar plates. Visible colonies were counted from three biological replicates after 48 h of incubation at 28 °C and values were expressed as log CFU cm^{-2}. The experiment was repeated two times and data of typical experiment were shown.

Statistical analysis

In vitro antifungal and antibacterial activity data are mean and confidence intervals from three replicates. They were subjected to one-way analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test ($P < 0.05$). For disease assessments, the experimental design was a completely randomized block. The experimental units were seven plants (21 replicates) for *P. syringae* pv. *tabaci* inoculation and three plants (12 replicates) for *A. tumefaciens* inoculation. Data of disease assessment were subjected to ANOVA, and means were compared using Tukey's HSD test ($P < 0.05$). Experiments were repeated two times and data of typical experiment was reported.

Results

Chemical characterization of the essential oil of *D. ambrosioides*

Hydrodistillation of the fresh aerial tissues of *D. ambrosioides* offered pale yellow essential oil in yield of 0.70% (*w/w*)

calculated on a fresh weight basis. The composition of the oil was determined by GC-MS and fourteen constituents were identified (Table 1) representing 92.6% of the oil. Oxygenated monoterpenoids (59.1%) and monoterpene hydrocarbons (32.8%) represent the main classes of volatile compounds. Ascaridole (34%), α-terpinene (13.3%), p-cymene (18.2%), thymol (6.4%) and carvacrol (6.1%) were the predominant components.

## In vitro antifungal activity of the essential oil and AS

The effect of EO and AS was assessed on mycelial growth and conidia production of *F. oxysporum* f. sp. *melonis* (FOM), *F. culmorum* (FC) and of the strains SH and SJ of *V. dahliae* (Fig. 2). EO was tested at concentrations of 50, 100, 200, 300, 400 and 500 µg ml^{-1}, however for clarity only data for 400 and 500 µg ml^{-1} were presented. Regression lines revealed IC50 values of 123 µg ml^{-1} for FOM. More than 70% and 90% of radial growth inhibition of FOM was achieved with 400 and 500 µg ml^{-1} of EO, respectively (Fig. 2a). However,

inhibition dropped to less than 10% when using 500 µg ml^{-1} of the purified AS.

Similar tendency was observed against FC (Fig. 2b). However, IC50 values averaged 228 µg ml^{-1}. Once more, the maximum of activity obtained with 500 µg ml^{-1} of EO was almost completely annihilated when using the purified AS.

Comparable trends were also obtained with the strains SH and SJ of *V. dahliae* (Fig. 2c, d) and IC50 values were 176 µg ml^{-1} and 451 µg ml^{-1}, respectively. The activity was also drastically reduced with 500 µg ml^{-1} of AS.

The effect of EO was also assessed on conidia production of FOM, FC and of the strains SH and SJ of *V. dahliae* (Fig. 3). Three hundred and 400 µg ml^{-1} of EO reduced conidia formation by 50 and 70%, respectively (Fig. 3a) and IC50 values averaged 278 µg ml^{-1}. Against FC, reductions were relatively lowers (Fig. 3b) (IC50 values of 430 µg ml^{-1}). A significant inhibition of conidia formation was obtained against the two strains of *V. dahliae*, and IC50 values were 188 and 298 for the strain SJ and SH, respectively (Fig. 3c, d).
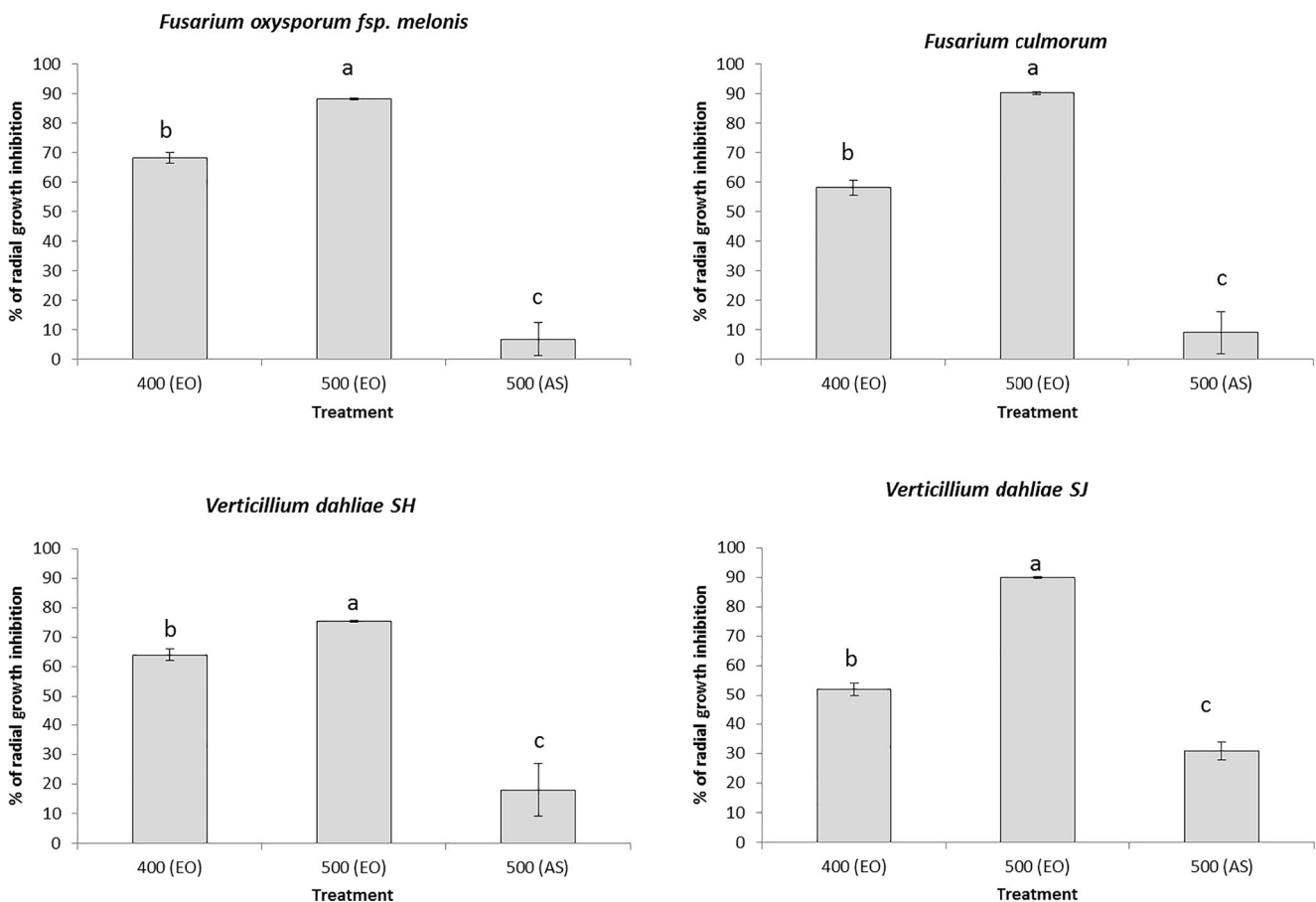
Fig. 2 Effect of the essential oil of *Dysphania ambrosioides* and the purified ascaridole on the in vitro inhibition of radial growth of the phypathogenic fungi (**a**) *Fusarium oxysporum* f. sp *melonis*, (**b**) *Fusarium culmurum* and the strains (**c**) SH or (**d**) SJ of *Verticillium dahliae*. Essential oil (EO) was added at the concentrations of 400 and 500 µg ml^{-1} to the PDA, while the ascaridole (AS) was added at 500 µg ml^{-1}. Mycelial growth was compared with growth on PDA amended with 0.05% Tween 80 and the percentage of inhibition growth was expressed relative to the control. Data are means ± confidence intervals (95%) from three replicates and values with different letters are significantly different according to Tukey's HSD test

In vitro antibacterial activity of the essential oil and ascaridole

The in vitro antibacterial activity of different concentrations of EO and ascaridole (AS) was assayed against the most important genera of bacterial phytopathogens (*Pseudomonas*, *Erwinia* and *Agrobacterium*) using the disc diffusion method and measuring inhibition zone diameters. EO was effective against *P. syringae* pv. *tabaci*, *P. syringae* pv. *syringae* and *E. amylovora* when it was used at 1000 μg ml^{-1} with inhibition zones ranging from 14 to 17 mm (Fig. 4). Similar levels of antibacterial activity were obtained with the positive control (chloramphenicol) against *P. syringae* pv. *tabaci*. AS also exhibited significant antibacterial activity when used at 1000 μg ml^{-1} however this activity was slightly lower than that obtained with the whole EO.

EO and AS exhibited a lower degree of bacterial growth inhibition against *A. tumefaciens* and no significant differences in susceptibility was found between the four strains (Fig. 5). Even at the highest concentration, inhibition averaged 12 mm and was significantly lower than that observed with chloramphenicol.

## In planta antibacterial activity of the essential oil

The preventive effect of EO on wild fire disease development was tested on *N. benthamiana* after treatment of leaves with 100 or 1000 μg ml^{-1} of EO or with DW supplemented with 0.05% of Tween 80 as negative control. Disease assessment was carried out based on measurement of diameter of necrosis at 5 dpi (Fig. 6a). The diameter of lesion in leaves of the control inoculated with *P. syringae* pv. *tabaci* was significantly higher than in those pre-treated with the EO, suggesting that EO are able to protect *N. benthamiana* plants from wild fire disease. However, protection was significantly higher with the lowest concentration of EO (100% of protected leaves) than with the highest concentration (30% of protected leaves).

Bacterial growth was also quantified from inoculated plants in order to confirm disease reduction (Fig. 6b). When compared to the control, bacterial populations were
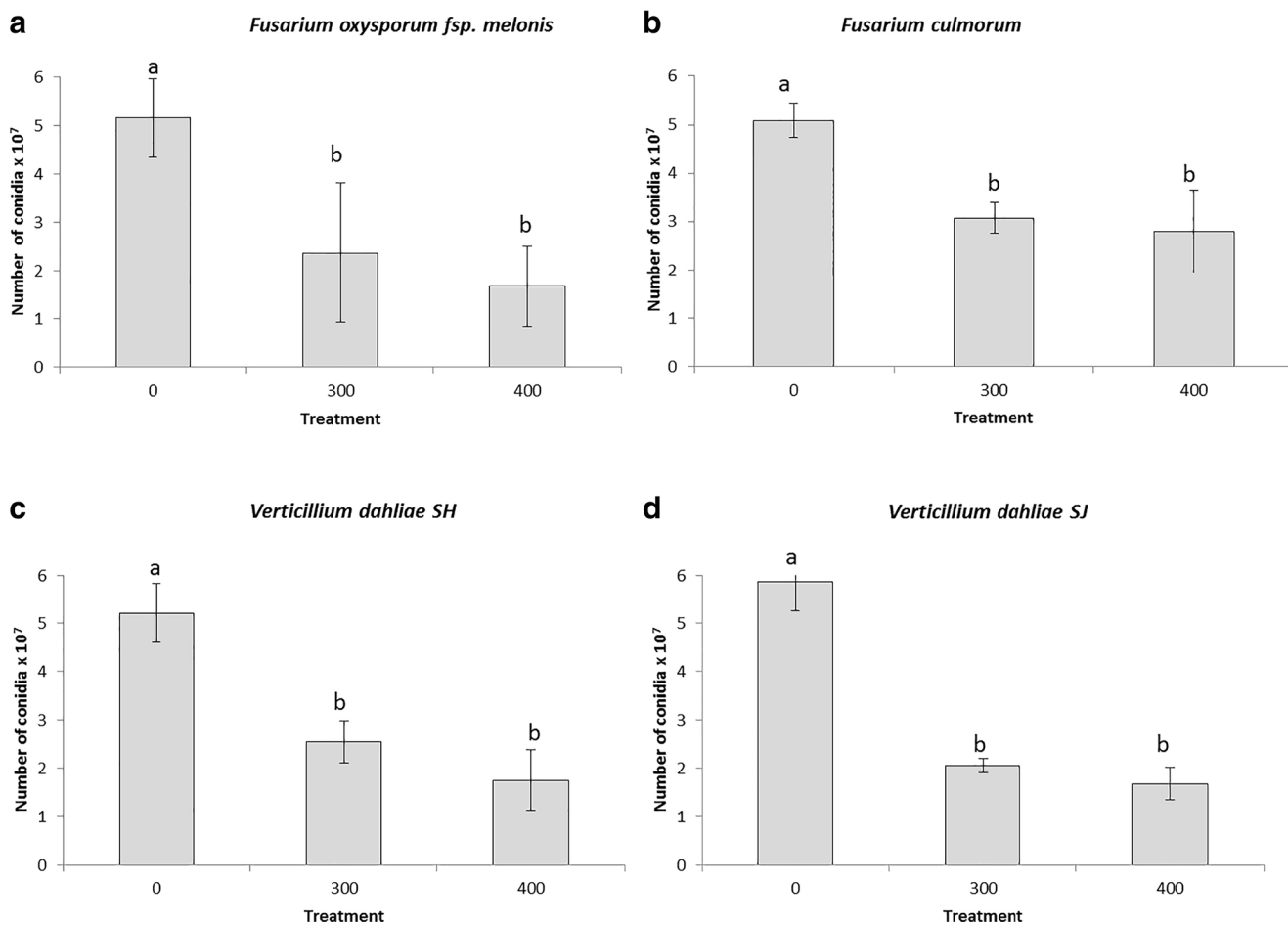
Fig. 3 Effect of the essential oil of *Dysphania ambrosioides* on conidia formation in the phypathogenic fungi (**a**) *Fusarium oxysporum* f. sp *melonis*, (**b**) *Fusarium culmurum* and the strains (**c**) SH or (**d**) SJ of *Verticillium dahliae*. Essential oil (EO) was added at the concentrations of 400 and 500 μg ml^{-1} to the PDA. Data are means ± confidence intervals (95%) from three replicates and values with different letters are significantly different according to Tukey's HSD test

a

b

c
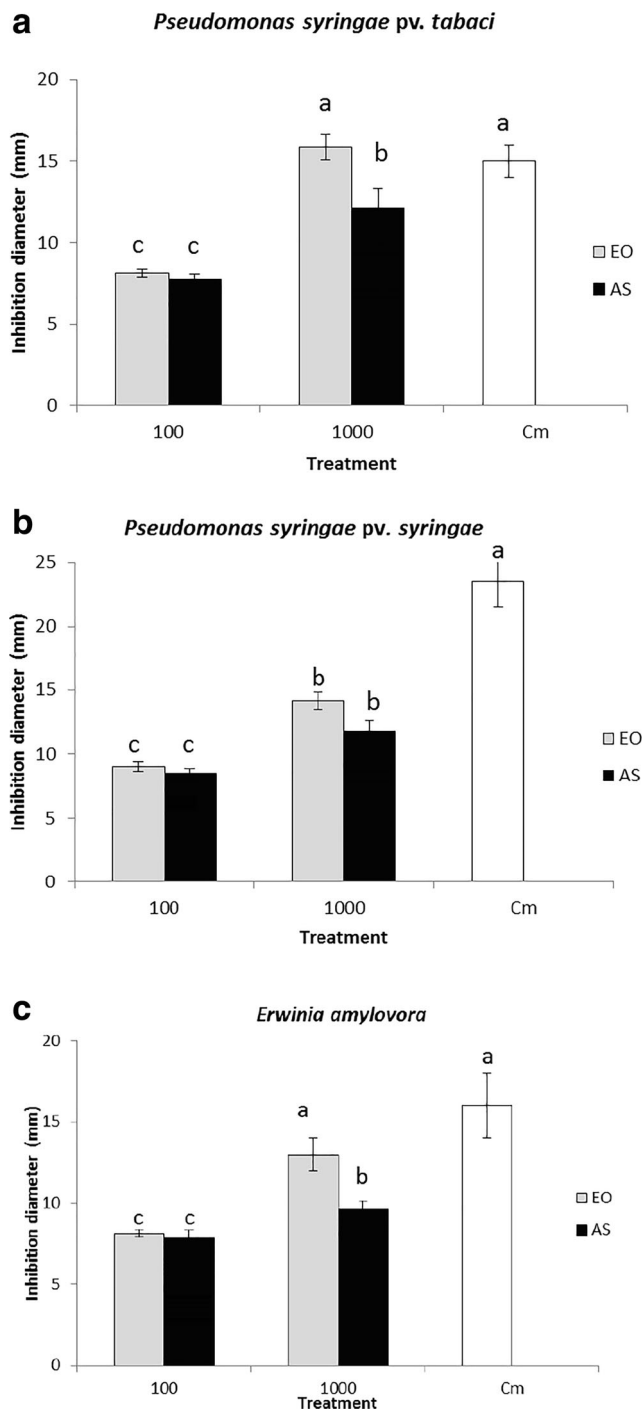
Fig. 4 Effect of the essential oil of *Dysphania ambrosioides* and the purified ascaridiole on the in vitro inhibition of the growth of (**a**) *Pseudomonas syringae* pv. *tabaci*, (**b**) *Pseudomonas syringae* pv. *syringae* and (**c**) *Erwinia amylovora*.. Sterile discs of 6 mm diameter impregnated with 100 or 1000 μg ml^{-1} of the essential oil (EO) or ascaridole (AS) were placed on KB solid medium amended with 3 × 10^8 CFU ml^{-1} of bacteria. 0.05% Tween 80 was used as negative control (0 μg ml^{-1}) while 50 μg ml^{-1} of chloramphenicol was used as positive control. The inoculated plates were incubated at 28 °C for 24 h. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone. Data are means ± confidence intervals (95%) from three replicates and values with different letters are significantly different according to Tukey's HSD test

significantly reduced with EO and growth was reduced by 0.6 logarithmic units with the highest concentration and by 1 logarithmic unit with the lowest concentration.

An illustration of protection conferred by the EO is shown in Fig. 6c.

The effect of EO was also studied on *S. lycopersicum* stems against crown gall disease after treatment with 100 or 1000 μg ml^{-1} of EO or with DW supplemented with 0.05% of Tween 80 as negative control. Disease assessment was followed during eight weeks based on measurement of tumor diameter (Fig. 7a). In the control, tumor diameter increased gradually to reach 2 cm eight weeks after inoculation. In plants pre-treated with EO increases were significantly lower during the whole duration of the experiment, reaching at the end of the experiment 1.5 cm in diameter with 100 μg ml^{-1} and 1 cm with 1000 μg ml^{-1}.

Tumor development in protected plants and in the control is shown in Fig. 7b.

Discussion

Various reports described the effects of plant extracts and essential oils on bacterial and fungal phytopathogens, however to our knowledge none of them focused on the antiphytopathogenic potential of the essential oil from the Moroccan *D. ambrosioides*. The geographical origin and the existence of chemotypes within this species are factors that can directly influence the chemical composition of the EO (Chekem et al. 2010). However, despite this variability, the EO consists mainly of mono and sesquiterpenes (Kliks 1985; Cruz et al. 2007). The Moroccan *D. ambrosioides* EO can be classified as ascaridole chemotype since its chemical composition is somewhat similar to those described in previous reports on fresh oil from Cuba with 35.1% of ascaridole, 20.7% of α-terpinene and 21.3% of p- cymene (Monzote et al. 2011) and from Yemen with 54.2% of ascaridole, 27.7% of isoascaridole and 8.1% of p-cymene (Al-Kaf et al. 2016). Other chemotypes have been also reported: including α-terpinene from Benin (Alitonou et al. 2012), Nigeria (Kasali et al. 2006) and India (Gupta et al. 2002); p-cymene from Cameroon (Tapondjou et al. 2002); limonene from Spain (De Pascual et al. 1980) and carvacrol from Cuba (Monzote et al. 2009).

The EO of *D. ambroisides* revealed a remarkable antifungal effect against *F. oxysporum* f. sp *melonis*, *F. culmorum* and *V. dahliae* when used at 500 μg ml^{-1} with a radial growth inhibition percentage ranging from 75 to 90%. The EO had also a strong detrimental effect on sporulation of all the tested fungal plant pathogens, which may result from a reduction in mycelial growth and from disturbance by the EO of the signal transduction pathways occurring during the transition from the vegetative to reproductive phases (Tzortzakis and

a

b

c

d
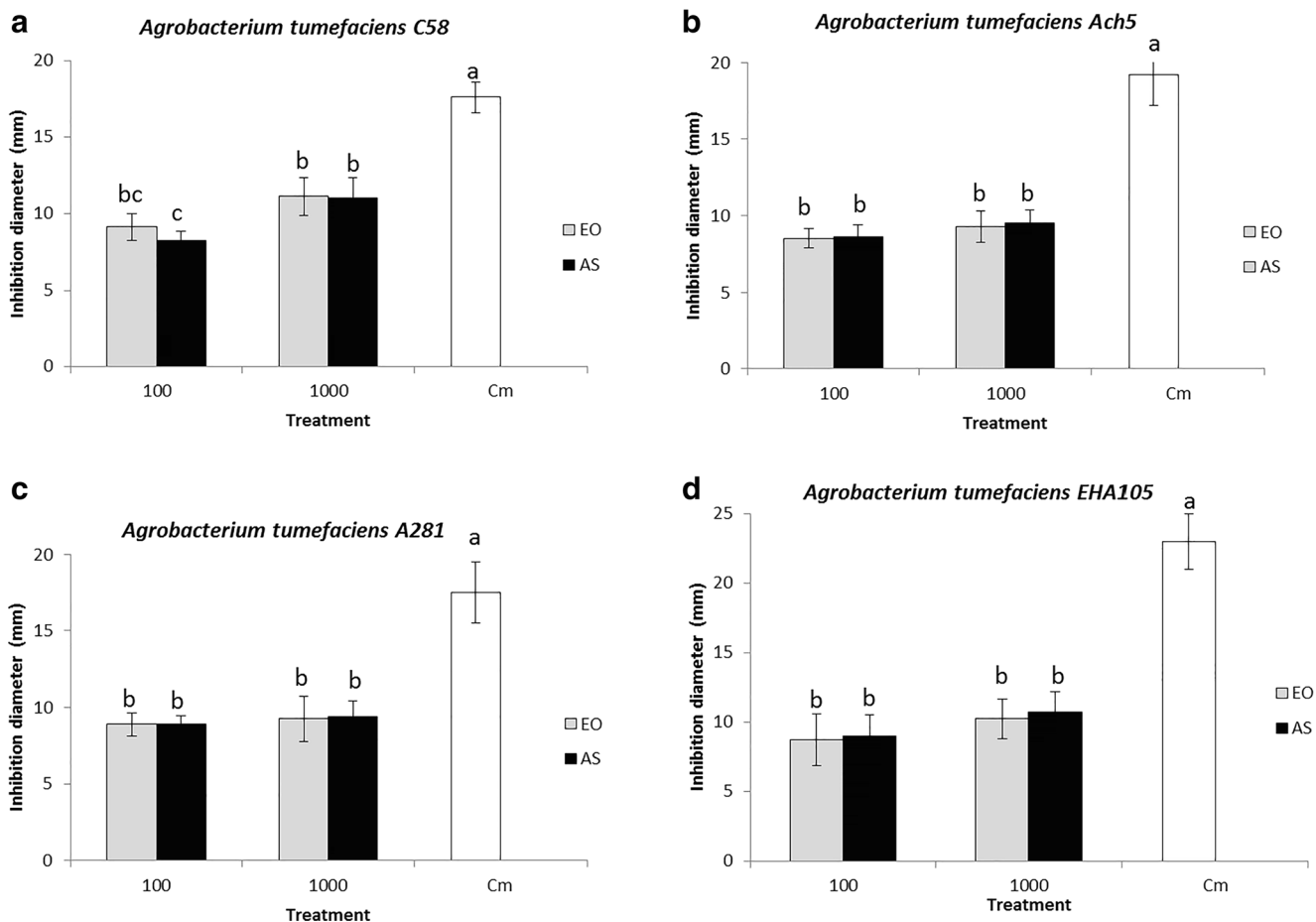
Fig. 5 Effect of the essential oil of *Dysphania ambrosioides* and the purified ascaridole on the in vitro inhibition of the growth of *Agrobacterium tumefaciens* strains (**a**) C58, (**b**) A281, (**c**) Ach5 and (**d**) EHA105. Sterile discs of 6 mm diameter impregnated with 100 or 1000 μg ml^{-1} of the essential oil (EO) or ascaridole (AS) were placed on LB solid medium amended with 3×10^8 CFU ml^{-1} of bacteria. 0.05%

Tween 80 was used as negative control (0 μg ml^{-1}) while 50 μg ml^{-1} of chloramphenicol was used as positive control. The inoculated plates were incubated at 28 °C for 24 h. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone. Data are means ± confidence intervals (95%) from three replicates and values with different letters are significantly different according to Tukey's HSD test

Economakis 2007). The antifungal activity of EO might be due to their highly lipophilic nature and low molecular weight of components that are capable of inactivating fungal enzymes and disrupting the cell membrane, causing cell death or inhibiting the sporulation (Hu et al. 2017). Several studies revealed the antifungal activity of EO, however higher concentrations were needed to achieve the same level of inhibition observed here. For instance, the leaf essential oil of *R. montana* inhibited radial growth and sporulation of several phytopathogenic fungus including *F. oxysporum* and *V. dahliae* when used at concentrations two to three times higher than those used in our study (Hammami et al. 2015). The antifungal activity of EO of *D. ambrosioides* was reported to inhibit the growth of dermatophytes (Kishore et al. 1999). However, relatively few studies have been conducted on the effects on phytopathogenic fungi. Jardim et al. (2008) showed that higher concentrations of the EO were needed to achieve complete inhibition of some post-harvest deteriorating fungi such as *Aspergillus*, and *Colletotrichum*. In addition, a fraction

consisting of ascaridole and *p*-cymene, which completely inhibited their growth lead the authors to suggest that ascaridole was the principal fungitoxic components of the Brazilian EO. Nevertheless, our results disagree with this statement since the purified fraction of ascaridole was unable to significantly inhibit the growth of the four phytopathogens studied here and that the antifungal activity of EO extracted from the Moroccan chemotype may rely on the *p*-cymene rather than on the ascaridole alone. It should be emphasized that a synergism between the components of the EO must be taken into account in assigning the antifungal activity.

The essential oil from the Moroccan *D. ambroiside* revealed to be effective in vitro against the phytopathogenic bacteria *P. syringae* pv. *tabaci*, *P. syringae* pv. *syringae* and *E. amylovora* while moderate activity was observed against *A. tumefaciens*. Such a differential ability to directly inhibit bacterial phytopathogens was already reported with essential oil from different plants. For instance, while only moderate

activities were observed against the phytopathogenic *A. tumefaciens* using EO from *Eucalyptus cinerea* (Kahla et al. 2017). Oregano was found to be highly effective in inhibiting the in vitro growth of *P. syringae* pv. *syringae*, *P. syringae* pv. *S. lycopersicum*, *Pseudomonas marginalis* pv. *marginalis*, *Xanthomonas campestris* pv. *vesicatoria* and *Pectobacterium carotovorum* (Vasinauskiene et al. 2006). Even though contrasting results were reported regarding the antibacterial activity of EO of *D. ambroiside* from different geographical origins against human pathogens, to the best of our knowledge, this is the first report of inhibition of bacterial phytopathogens. For instance, the EO from the Nigerian *D. ambroiside* belonging to the α-terpinene chemotype did not display any antibacterial activity against either Gram-positive bacteria *Bacillus cereus* or *Staphylococcus aureus*, or the Gram-negative bacterium *Escherichia coli* (Owolabi et al. 2009). At the opposite, the EO from the Egyptian *D. ambroisides*, which is rich in both *o*-cymene and α-terpinene exhibited a noticeable antibacterial activity against *Bacillus subtilis* and *E. coli* (Harraz et al. 2014; Santiago et al. 2016). The antibacterial activity exhibited by the EO from the Moroccan *D. ambroisides*, relies mainly on its main constituent since ascaridole was able to inhibit significantly the growth of the studied phytopathgenic bacteria. These findings are thus a hint that ascaridole may be an interesting novel candidate drug against phytopathogenic bacteria.

Fig. 6 Effect of the essential oil (EO) of *Dysphania ambrosioides* on the development of wild fire disease caused by *Pseudomonas syringae* pv. *tabaci* on *Nicotiana benthamiana*. **a** Diameter lesion, (**b**) growth of bacterial populations *in planta*, (**c**) photo showing disease symptoms in the control and treated plants. The aerial parts of *N. benthamiana* were sprayed to runoff with 100 or 1000 μg ml^{-1} of EO two times at 7 and 3 days before infiltration with 100 μl of suspension of *P. syringae* pv. *tabaci* adjusted at $3\ 10^8$ CFU ml^{-1}. 0.05% Tween 80 was used as control. Diameter lesion was recorded at 5 days after inoculation from of 21 leaf panels taken from three young leaves belonging to seven plants. Bacterial growth was recorded at 5 days after inoculation from three leaf discs belonging to three different plants. Data are means ± confidence intervals (95%) and values with different letters are significantly different according to Tukey's HSD test. Experiments were repeated two times and data of typical experiment were shown
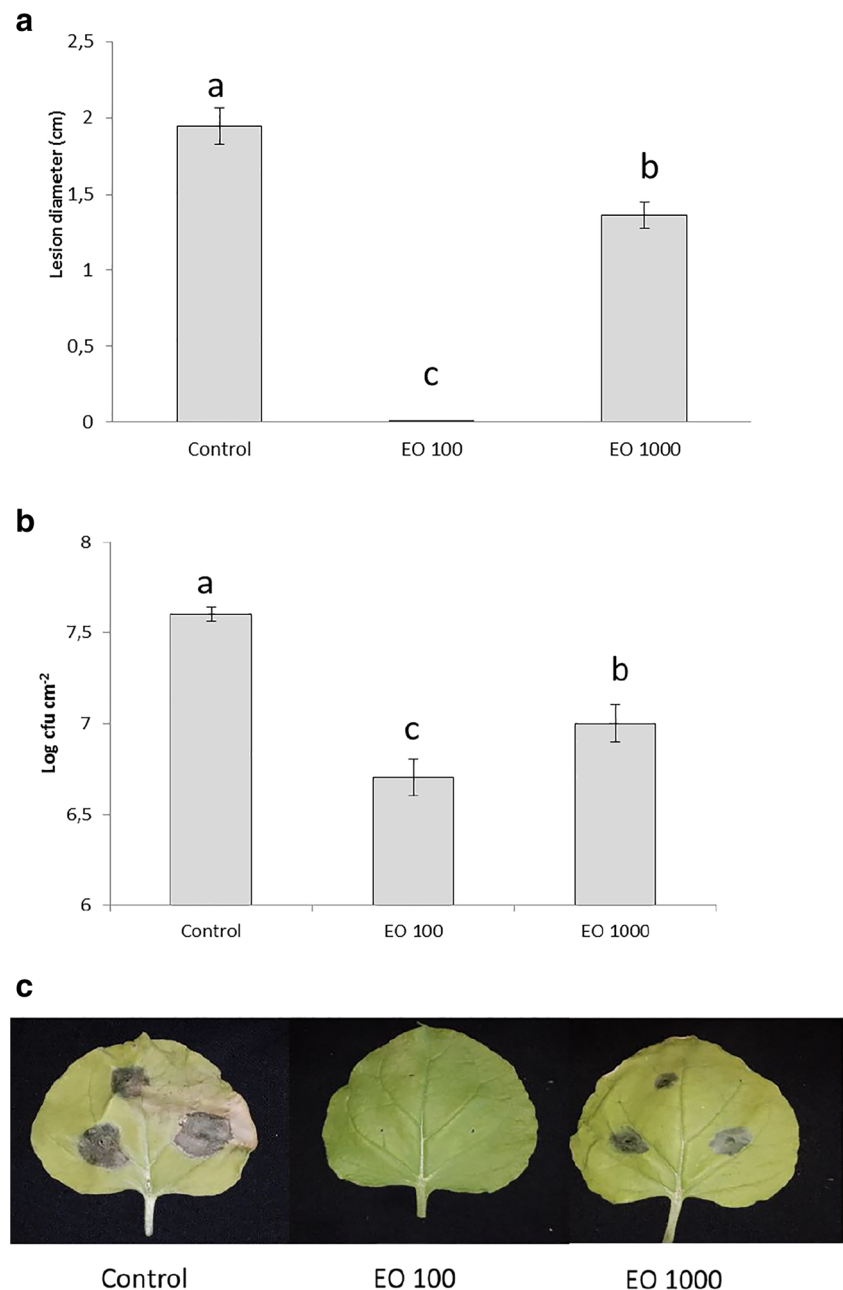
Fig. 7 Effect of the essential oil (EO) of *Dysphania ambrosioides* on the development of crown gall disease caused by *Agrobacterium tumefaciens* on tomato plants. **a** Evolution of tumor diameter (**b**) photo showing disease symptoms in the control and treated plants. Tomato stems were sprayed to runoff with 100 or 1000 µg ml^{-1} of EO two times at 7 and 3 days before inoculation with 10 µl of suspension of the strain C58 of *A. tumefaciens* adjusted at 3 × 10^8 CFU ml^{-1}. 0.05% Tween 80 was used as control. Tumor diameter lesion was recorded during eight weeks and photo was taken at the end of the experiment. Data are means ± confidence intervals (95%) from 12 replicates belonging to three different plants and values with different letters are significantly different according to Tukey's HSD test. Experiments were repeated two times and data of typical experiment were shown
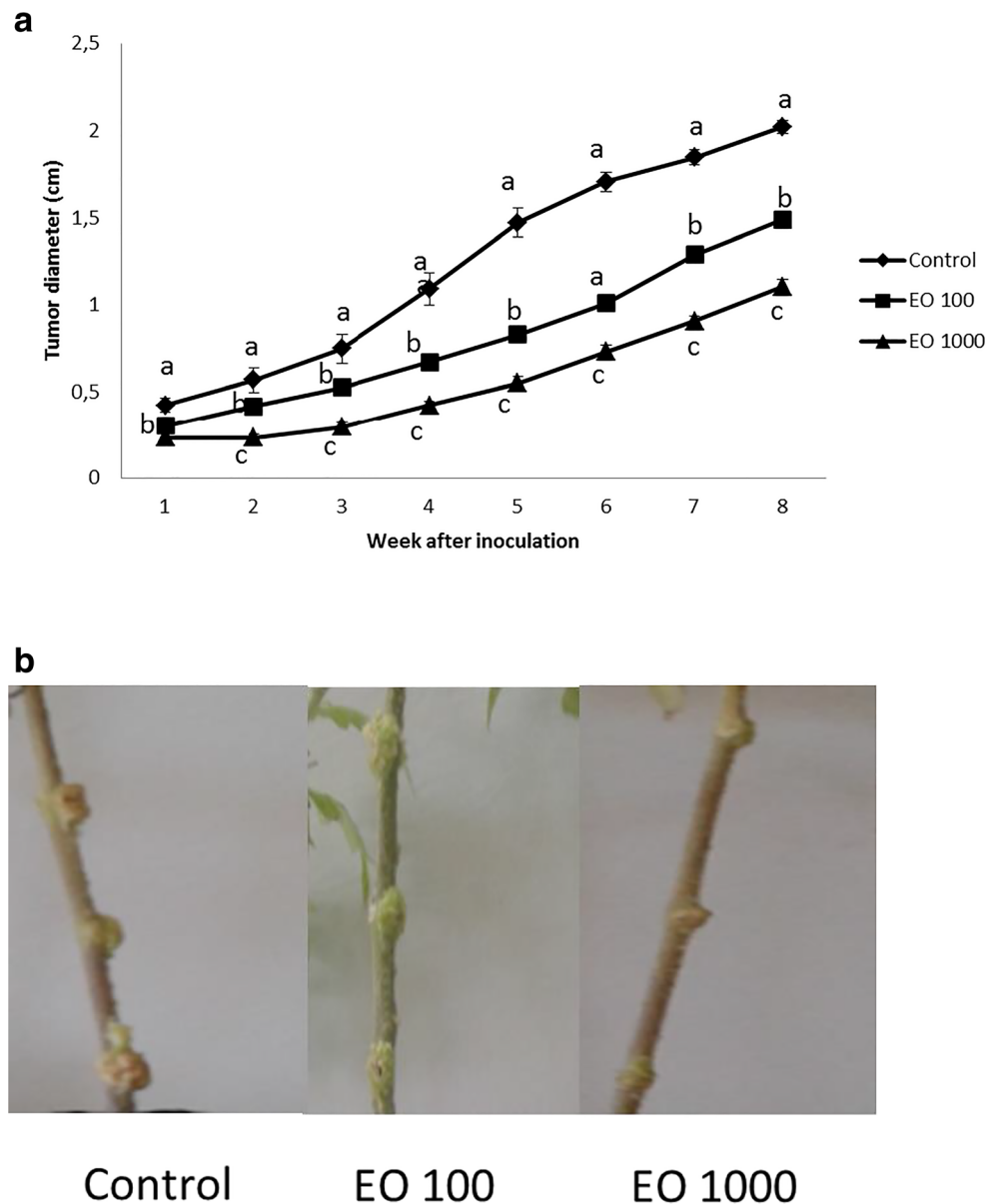
Although in vitro test is an important step in the selection of EO with antibacterial potential against plant pathogens, *in planta* investigations are necessary to confirm their efficacy shown in vitro or to elucidate their possible different modes of action. The EO from the Moroccan *D. ambroisides* was able to prevent wild fire disease development caused by *P. syringae* pv. *tabaci* on *N. benthamiana*. With the exception of their use as disinfectants or soil biofumigants to control phytopathogenic bacteria, only few studies have focused on the treatment of aerial parts of plants (Dadasoglu et al. 2011; Alves et al. 2014). For instance, the EO of *Cleistocalyx operculatus* or *Metasequoia glyptostroboides* completely suppressed bacterial spot disease caused by *X. campestris* pv. *vesicatoria* when applied to the aerial parts of oriental melon plants (Bajpai et al. 2010a, b). However, concentrations ranging from 1000 to 2000 µg ml^{-1} were necessary to achieve complete protection. In our study, concentration as low as 100 µg ml^{-1} was sufficient to suppress completely disease development, while concentration 10 times higher resulted in lower efficacy. This could be explained by the phytotoxicity of EO and the high sensitivity of leaf tissues. Phytotoxicity is a limiting factor that prevents the use and commercialization of EO. Therefore, reducing its concentrations may be a good solution to this problem of phytotoxicity. However, this is not always possible because for other plant diseases high concentrations are needed to achieve good protection. Indeed, the EO from *D. ambroisides* was also able to reduce crown gall disease on *S. lycopersicum* plants nevertheless the best results were achieved with the highest concentration. These results are in agreements with those carried out in bitter almond plants,

which revealed that the EO from *R. montana* was poorly effective at reducing gall formation induced by *A. tumefaciens* (Hammami et al. 2015).

Taken together, these data suggest that the in vitro antifungal activity of EO is supported by the occurrence of synergy between its components, which are usually less effective than the whole EO. Our results also indicate that this EO was successful in controlling wild fire and crown gall diseases *in planta*. However, since our trial is too small, further experiments with other bacterial and fungal pathosystems are needed to demonstrate its real possibility to use for disease management in the field. The preventive potential of EO from *D. ambroisides* is possibly due to the presence of compounds present in smaller amounts that may indirectly contribute to controlling diseases, by activating plant defense responses. Further research should be conducted in order to quantify plant defense-related genes and enzymes. More studies related to toxicity and environmental issues are necessary to overcome the barriers of the use of EO of *D. ambroisides* in plant disease control. Phytotoxicity tests will serve as a basis to determine the concentrations that can be applied to plants without harming them. Volatility, short shelf life and non-persistence in the soil and fresh water for disbursing it freely in environment should be included in future studies.

Compliance with ethical standards

Conflict of interest Manal Zefzoufi declares that she has no conflict of interest, Amal Smaili declares that she has no conflict of interest, Rabiaa Fdil declares that she has no conflict of interest, Lalla Aicha Rifai declares that she has no conflict of interest, Lydia Faize declares that she has no conflict of interest, Tayeb Koussa declares that he has no conflict of interest, Kacem Makroum declares that he has no conflict of interest, Abdelkader Ben Ali declares that he has no conflict of interest, Mohamed Tabyaoui declares that he has no conflict of interest, Abdelkarim Mouzdahir declares that he has no conflict of interest, Khadija Sraidi declares that she has no conflict of interest, Mohamed Faize declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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