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Management of Alternaria leaf blight in tomato plants by mentha essential oil

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Abstract: The essential oil obtained by the hydro-distillation of the leaves of *Mentha arvensis* Linnaeus was evaluated for its antifungal activity against the causal agent of the *Alternaria* blight of tomatoes, i.e., *Alternaria alternata* (Fries) Keissler. The antifungal activity of the mentha essential oil was assessed both *in vitro* and *in vivo*. The chemical composition of the mentha oil was also identified by GCMS analysis. The *in vitro* test revealed that the maximum inhibition in the mycelial growth (93.6%) and conidia germination (90.6%) was at the highest concentration (40 µL/mL), furthermore, it was found that the inhibition of the mycelial growth and conidia germination was dose dependent. The *in vivo* test proved that the application of the mentha essential oil (40 µL/mL) significantly increased the plant height (84.6%), fresh weight (81.5%) and dry weight (80.0%) when compared to the untreated tomato plants. The disease incidence was 3.5 in the untreated plants, while it was 0.93 for the mentha essential oil treated plants and was 0.08 in the carbendazim treated plants. The GC-MS analysis of the mentha essential oil identified 18 compounds in total, among which the percentage of menthol was the highest (69.2%). The mentha essential oil was successful in managing the *Alternaria* leaf blight in the tomato plants. Therefore, it can be explored further for the development of a natural fungicide.

Keywords: plant pathogenic fungi; eco-friendly; *Solanum lycopersicum;* plant growth; *Alternaria alternata*; *Mentha arvensis*

Alternaria spp. not only causes direct damage to fruits, plants and vegetables by infection, but also indirectly spoils the food by producing a mycotoxin (Agrios 2005). *Alternaria* leaf blight, caused by *Alternaria alternata* (Fries) Keissler possesses a great threat to agricultural plants (Mehmood et al. 2014). The disease on the leaf appears as leaf spots and blight. *Alternaria* is considered to be necrotrophic since it kills the host cells at an early plant stage. The toxic substances produced by the fungus causes necrotic lesions (Chung 2012). Alternaria blight brings morphological and physiological changes in the plant that leads to tissue necrosis and foliar lesions and senescence, thus reducing the food production (Agrios 2005). The tomato (*Solanum lycopersicum* Linnaeus) is an important horticultural crop grown and consumed throughout the world, it is attacked by various pathogens including *A. alternata*. To control the Alternaria infection, the use of a fungicide is a must besides other cultural practices (Akhtar et al. 1994). A synthetic fungicide creates various environmental problems such as environmental pollution, human health hazard and pathogen resistance (Yang et al. 2011). To overcome these issues, scientists are extensively exploring for environmentally-friendly chemicals that can control the plant pathogenic fungi. Essential oils from plants are among the products that have been explored to replace chemical fungicides. *Mentha* sp. has been reported for possessing an antimicrobial activity (Tiwari 2016;

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Franca 2018), however, the potential of the *Mentha arvensis* Linnaeus mentha essential oil to manage *A. alternata* is unexplored, thus, the study was aimed at assessing the potential of the mentha essential oil in managing the Alternaria leaf blight of tomatoes (*S. lycopersicum*.) caused by *A. alternata in vitro* and in pot conditions (*in vivo*).

MATERIAL AND METHODS

Isolation and identification of the fungi. Leaves of tomato plants (*S. lycopersicum*) showing symptoms of *Alternaria* leaf blight were cut into small pieces and surface sterilised with a 1% sodium hypochlorite solution for 1 minute. The sterilised samples were placed on a potato dextrose agar (PDA) medium and incubated at 25 ± 1 °C till the fungal growth was visible. The fungi were purified and identified by studying the macroscopic and microscopic structure of the isolated fungi (Simmons 1992). The Indian type collection centre confirmed the identity of the isolated fungus as *A. alternata* (ITCC 10.637.17). The pure culture of the fungus (*A. alternata*) was sub-cultured on the PDA and stored at 4 °C.

Isolation of the essential oil. The isolation of the essential oil from the leaves of *M. arvensis* was carried out following the same protocol as mentioned by Bokhari et al. (2016). Briefly, 100 g of fresh leaves of *M. arvensis* were hydro-distilled in a Clevenger-type apparatus for 4 hours (Clevenger 1928). The oil was dried over anhydrous sodium sulfate. The oil (mentha essential oil) was stored in a glass amber vial at 4 °C until analysed.

Effect of the mentha essential oil on the mycelial growth. To determine the reduction in the mycelial growth of *A. alternata* by the mentha oil, the poison food technique was employed (Sharma & Triparthi 2006). Tween 20 (Himedia, India) (0.5%) was added to the mentha essential oil to prepare an emulsion. The mentha essential oil emulsion was added in a molten sterile PDA and was poured into petri plates. The final concentration of the oil in the PDA was 40, 20, 10, 5 and 2.5 µL/mL. After solidification of the PDA, the plates were inoculated with a mycelial plug (5 mm) of 5 days old *A. alternata*. The mycelia plug of the tested fungus was placed upside down into the centre of the PDA plates. The control plates were treated in the same manner except that the mentha essential oil was replaced with the sterile distilled water and carbendazim (0.2%) in a negative and positive control, respectively. The inoculated plates were incubated at 25 ± 1 °C for 5 days. There were four replicates for each treatment and the experiment was repeated twice. The diameter of the fungal growth was measured and the inhibition of the mycelial growth was calculated by the following formula:

$$\mathrm{FI}(\%) = \frac{F_c - F_T}{F_c} \times 100 \quad (1)$$

where: FI – the fungal mycelium inhibition (%), F_C – diameter of the fungal colony in the control plate; F_T – the diameter of the fungal colony in the treated plate (Singh & Tripathi 1999).

Effect of the mentha essential oil on the conidia germination. The effect of the mentha essential oil on the conidia germination of *A. alternata* was determined as mentioned by Feng and Zheng (2007) with a slight modification. Different oil concentrations of (40, 20, 10, 5 and 2.5 µL/mL) were obtained by serial dilution. Tween-20 (0.5%, v : v) was also added into the tubes to enhance the dispersion of the oil. After that, 100 µL of conidia suspension (1×10^6 conidia/mL) was added into each tube. The tubes containing Carbendazim (0.2 %, v : v) and distilled water in place of the mentha essential oil acted as the positive and negative control, respectively. There were three replicates for each treatment and the experiment was repeated twice. After 3 days of incubation at 25 ± 1 °C, 100 µL of aliquot was examined under a light microscope to observe the conidia germination. The number of conidia germinated/mL and the percentage of the inhibition were calculated.

Determination of the bio efficacy of the mentha essential oil against *A. alternata in vivo*. A pot trial was conducted to determine the bio efficacy of the mentha essential oil against *A. alternata* (Ara et al. 2012). The tomato seedlings (4 weeks old) raised in a sterile soil were transplanted in sterile pots (one seedling per pot) containing a sterile peat moss soil and sand (5 : 1). After a day, an aliquot of 5 mL conidia suspension (1×10^6 conidia/mL) was sprayed evenly over the leaves of the tomato plant. After a day of incubation, the leaves of the tomato plants were sprayed with 5 mL of the mentha essential oil containing Tween 20 (0.5%) as an emulsifier and diluted with sterile distilled water (40 µL/mL). The inoculated untreated control received the same amount of treatment excluding the mentha oil, while the uninoculated control was devoid of the *A. alternata*

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inoculation. In a same manner, a set of replicates was sprayed with carbendazim (0.2%). For each treatment set, there were four replicates. The plants were watered with sterile distilled water as and when required. The experiment was terminated after a month, the plants were uprooted, and the data, such as plant height, plant fresh weight, were recorded. The uprooted plants were evaluated on a scale of 0 to 4 for the disease index where: 0 = symptomless, 1 = up to 25% of the plant with small lesions, 2 = 25 to 50% of the plant with large lesions, 3 = 50 to 75% of the plant blighted and 4 = 75 to 100% of the plant blighted (Biermann & Lindermann 1981). The plant dry weight was also recorded after drying the plants at 105 °C for 30 min and, after that, 70 °C for 24 hours.

GC-MS analysis. For the gas chromatography (GC) analysis, a gas chromatograph instrument (GC 5890II series, Hewlett Packard, USA) with a flame ionisation detector (FID) was used. The split/splitless injector (split ratio of 1 : 50) and an HP-ChemStation (version B.04.03) GC data system was used, equipped with CP-Sil 8 CB column (30 m × 0.25 mm id, a film thickness of 0.25 µm) (Agilent Technologies, USA). First, the oven temperature was increased to 70 °C for 4 min and then held at 220 °C for 5 min. The temperature of the injector port, ionisation chamber and FID was 210, 230 and 250 °C, respectively. Helium was the carrier gas (1 L/min). The percentage composition of the essential oil was calculated from the peak areas using the normalisation method. The gas chromatography – mass spectrometry (GC-MS) was carried out on a Clarus 500 (Perkin Elmer, USA) gas chromatography coupled with a mass spectrometer (MS) (Clarus 500, USA), equipped with a CP-Sil 8 CB column (30 m × 0.32 mm id, a 0.25 µm film thickness). The oven temperature for the column was identical to the GC analysis. For homogeneity, the peaks were identified by studying the mass chromatograms of the fragmentation pattern of the compound obtained by the mass spectrometry analysis and also by using the peak purity function of the MSD software. The oil constituents were identified by comparisons of their retention indices and their mass spectra with the authentic standards mass spectra interpretation and comparison with the National Institute of Standards and Technology (NIST) libraries (Alshaikh & Perveen 2017).

Statistical analysis. The statistical analysis to find the differences among treatments was performed by a one-way ANOVA using XLSTAT (version 2019). The significance of difference among the means was carried out using Tukey's HSD tests at $P = 0.05$ and the results were expressed as a mean ± SD.

RESULTS

To determine the effect of the essential oil of *M. arvensis* on the mycelial growth and conidia germination of *A. alternata,* five different concentrations (40, 20, 10, 5 and 2.5 µL/mL) of the mentha essential oil were selected. The results indicate that the mentha essential oil significantly ($P < 0.05$) rendered the mycelial growth and was dose dependent ($R^2 = 0.977$) (Figure 1). The maximum mycelial growth inhibition by the mentha essential oil was 93.6% at the concentration of 40 µL/mL, whereas carbendazim showed 100% mycelial inhibition at the recommended dose. Similar results were observed on the conidia germination (Figure 1). The mentha essential oil exhibits a significant difference in the activity over the dose ($P < 0.05$). The maximum reduction in the conidia germination of 90.6% was recorded at 40 µL/mL and was dose dependent ($R^2 = 0.961$).

The inhibitory effect of the essential oil of *M. arvensis* against *A. alternata* was assessed in pots. It was observed that the untreated plants were stunted with the disease symptoms apparent on the tomato

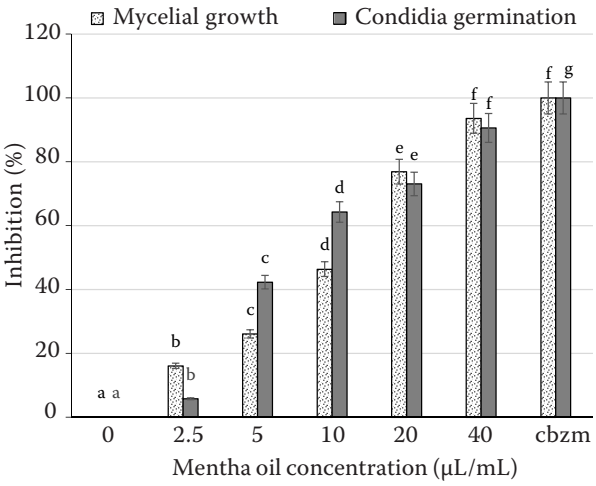

Figure 1. The effect of the different concentrations of the mentha essential oil on the mycelial growth and conidia germination of *Alternaria alternata* (Fries) Keissler

cbzm – carbendazim; the value represents the mean of the 4 replicates ± the standard deviation; the data marked by the different letters in the columns are significantly different ($P \leq 0.05$) according Tukey's HSD tests

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leaves while the mentha essential oil treated plants had less symptoms of *A. alternata* when compared to the untreated plants. The data represented in Figure 2 shows that the application of the mentha essential oil significantly improved ($P < 0.05$) the plant height, fresh and dry weight when compared to the untreated tomato plants. The reduction in the untreated tomato plant height, fresh and dry weight was 65%, 51% and 48% when compared to the control tomato plants while the percent reduction in the same parameters in the mentha essential oil treated plant was 10% (plant height), 12% (fresh weight) and 13% (dry weight) when compared to control plants. Therefore, it can be concluded that the treatment with the mentha essential oil improved the plant height, plant fresh and dry weight by 84.6, 81.5, and 80.0%, respectively when compared to the untreated plants. Whereas the carbendazim treatment improved the plant height, plant fresh and dry weight by 96.9% when compared to the untreated plants. It has been observed that the application of the mentha essential oil reduced the disease incidence significantly ($P < 0.05$). The disease incidence in the untreated plants was 3.5, while it was

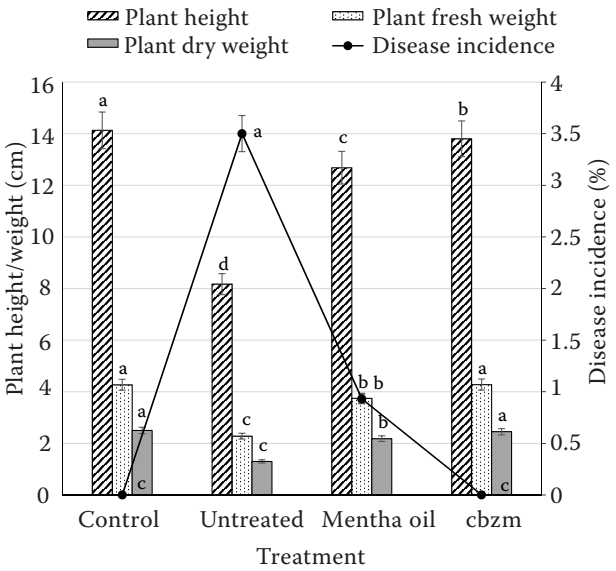

Figure 2. The effect of the mentha essential oil (40 µL/mL) on the disease development and growth of the tomato plant infected with *Alternaria alternata* (Fries) Keissler in the pot condition (*in vivo*)

cbzm – carbendazim; the value represents the mean of the 3 replicates ± the standard deviation. The data marked by the different letters in the columns are significantly different ($P \leq 0.05$) according to Tukey's HSD tests. Disease incidence: 0 – symptom less; 1 – up to 25% plant with small lesions; 2 – 25 to 50% plant with large lesions; 3 – 50 to 75% plant blighted; 4 – 75 to100% plant blighted

Table 1. The chemical composition of the mentha essential oil hydro-distilled from the fresh leaves of *Mentha arvensis* L.

No.	Compound	RI	%
1	α-pinene	936	0.5
3	myrcene	989	0.7
4	limonene	1 038	1.9
5	octanal	1 065	0.6
6	isopulegol	1 165	1.3
7	menthone	1 172	7.9
8	isomenthone	1 185	6.7
9	menthol	1 191	69.2
10	α-terpineol	1 201	0.6
11	*cis*-tarveol	1 204	1.6
12	myrtenal	1 210	0.2
13	piperitone	1 251	1.0
14	menthyl acetate	1 278	3.8
15	β-caryophyllene	1 417	0.7
16	isomenthol	1 449	0.5
17	β-farnesene	1 455	0.8
18	δ-cadinene	1 517	0.2

RI – retention index

0.93 in the mentha essential oil treated plants and was lowest in the carbendazim treated plants (0.08).

The mentha essential oil obtained by the hydro-distillation of the leaves of *M. arvensis* was slightly yellow in colour and the oil yield was 0.69%. The GC-MS analysis identified 18 compounds representing 98.2% of the total oil (Table 1). Among all the compounds detected, the menthol percentage was highest (69.2%); the other major constituents were menthone (7.9%), isomenthone (6.7%), methyl acetate (3.8%) and limonene (1.9%) carveol (1.6%), isopulegol (1.3%), piperitone (1.0%).

DISCUSSION

Mentha essential oil showed the potential to inhibit *A. alternata* by rendering both the mycelial growth and conidia germination. Reports are available on the inhibitory effect of essential oils on pathogenic fungi (Isman 2000; Kumar & Kudachikar 2017; Madjouko et al. 2019). The complete inhibition of *A. alternata* with 1000 mg/L of essential oils of *Piper hispidinervum* (C. DC.) and *Citrus reticulate* (0.2 mL/100mL) has been reported (Nascimento et al. 2008; Chutia et al. 2009). The essential oil of *M. spicata* inhibited the mycelial growth of *Aspergillus niger* van Tieghem (92.93%) at the concentration of 40 µL/mL (Jha

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& Regmi 2018). Mona et al. (2016) reported the absolute inhibition of the *Alternaria solani* (Ell. & Mart.) L.R. Jones and Grout mycelial growth by peppermint, lemongrass, thyme and sweet basil oils at a concentration of 2%. The current study observed that the effect of the mentha essential oil on *A. alternata* was dose dependent, thus, it reflects that a complete mycelial inhibition can be achieved at a higher concentration of the mentha essential oil. Kuinkel et al. (2016) observed the absolute inhibition of *Glomerella cingulata* (Stoneman) Spaulding & Schrenk with the 40% essential oil of *Thymus linearis* Benth and *M. arvensis*. Recently, Franca et al. (2018) reported the mycelial growth inhibition of *A. alternata* by a peppermint essential oil that was dose dependent. Pérez-González et al. (2016) also reported similar results while treating *A. alternata* with different concentrations of oregano oil. The tested concentration of the mentha essential oil exhibited satisfactory results in reducing the Alternaria blight of tomato plants. A greenhouse study reported that the treatment of oregano oil (1%) was at par with the chemical fungicide tested to treat tomato plants infected with *A. alternata* (Pérez-González et al. 2016).

Reports from different regions of the world have identified the main constituents of the essential oil of *M. arvensis* as menthol (Pino et al. 1996; Singh et al. 2005; Bokhari et al. 2016). Besides that, menthone, methyl acetate, isomenthone, limonene has been reported as the dominant constituents in the essential oil of *M. arvensis* (Singh et al. 2005; Bokhari et al. 2016). The GC-MS analysis of the essential oil of *M. arvensis* shows the presence of similar compounds, however, variations in the percentage of these compounds has been observed which could be due to the variation in the cultivars or cultivation conditions (Singh et al. 2005).

The antifungal activity of the mentha essential oil was probably due to the synergistic effect of the main constituents of the oil (Tian et al. 2012). The most abundant compound detected in the mentha essential oil was menthol, which is an aromatic oxygenated monoterpene, the other chemical constituents that were identified were menthone, isomenthone, menthyl acetate, limonene, β-pinene which also have been reported for their antimicrobial activity (Bokahri et al. 2016). Iscan et al. (2002) carried the bio-autography of the oil obtained from *Mentha piperita* Linnaeus and found that menthol was responsible for the antimicrobial activity. Edris and Farrag (2003) observed that the vapours of menthone and menthol of a hydro-distilled peppermint essential oil alone and in combination were able to reduce the growth of certain fungi.

The present study reflects the significant potential of the mentha essential oil in managing the Alternaria tomato blight caused by *A. alternata*. *M. arvensis* has been known for several medicinal properties that include anti-allergic and anti-inflammatory activities (Thawkar 2016). Therefore, the lower health risk along with being economical and eco-friendly makes the mentha essential oil a suitable ingredient for the development of a natural fungicide, which can reduce the use of chemical fungicides. The study suggests that there is a requirement of determining the active components of the essential oil, further field trials are needed to assess the actual potential of the mentha essential oil. Alternaria is a ubiquitous pathogen, thus, this study can help in utilising mentha essential oil in managing the pre- and post-harvest diseases caused by *Alternaria* to important agricultural products.

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