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Use of Some Essential Oils as Natural Fungicides for *Alternaria Radicina* Controlling and Improving Anise (*Pimpenella anisum* L.) productivity.

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THE ALTERNARIA leaf spot fungus cause a serious problem for anise (*Pimpenella anisum*) cultivation in Egypt. The aim of this study was to examine the possibility of using some essential oils as alternatives to synthetic fungicides inhibiting *Alternaria radicina* growth. The effect of those essential oils (EOs) on growth and productivity of anise was also investigated in two successive seasons of 2010/2011& 2011/2012. *In vitro* experiments were carried out using six essential oils of thyme, caraway, clove, mint, rosemary and lemongrass with three concentrations of 1, 5 and 10 µl ml^{-1}. Complete inhibition of growth of the pathogen was obtained by lemongrass oil at 5µl ml^{-1} .Oils of thyme and caraway were inhibitory at relatively higher concentrations (10µl ml^{-1}). Oils of lemongrass, caraway and thyme, gave pronounced protection of anise plants against invasion of leaf spot pathogen. The maximum reduction of disease incidence (DI) and severity (DS) of leaf spot along the growth period in both growing seasons was observed by lemongrass at both concentrations (5 and 10 µl ml^{-1}), and by caraway and thymes oils at 10 µl ml^{-1} concentrations.

Vegetative growth of anise *i.e.* plant height, number of branches and number of leaves/plant and yield components *i.e.* fruits and essential oil yield were responded positively to different essential oils application. Significant increases in the photosynthetic pigments of anise leaves were determined as a result of different essential oils application. Lemongrass essential oil at 10 µl ml^{-1} increased seed yield per plant with 77.38 % over control plants followed by, thyme oil at 10 µl ml^{-1} reordering 66.85 % increment (average of two seasons) while, caraway oil at 10 µl ml^{-1} ranked thirdly. The maximum reduction of disease incidence and severity of leaf spot along the growth period was observed by lemongrass at both concentrations (5 and 10 µl ml^{-1}) followed by caraway and thymes oils.

This study has demonstrated that the EOs are promising antifungal agents, which could be used widely as natural fungicides in the protection of anise against *Alternaria radicina* and improving plant growth and productivity.

Keywords: Anise, *Alternaria radicina,* Essential oils, Growth, yield and Biological control

Anise (*Pimpinella anisum* L.) is an annual plant belongs to the family *Apiaceae* (*Umbelliferae*). It is native to eastern Mediterranean, western Asian, and North Africa regions, the Middle East, Egypt, Mexico and Spain (Salehi Surmaghi, 2010). The anise seeds have essential oil as an active substance. Anethole is the most important constituent of anise oil used in pharmaceutical, food, perfumery and flavoring industry (Ozkan and Chalchat, 2006).

Aniseeds could cause gastric protection, muscle relaxant, and affect digestive system. In diabetic patients, it has hypoglycemic and hypolipidemic effects and reduces lipid peroxidation. Furthermore, aniseeds showed anticonvulsant effect, reduced morphine dependence and induced conditioned place aversion in mice. It is also has beneficial effects on dysmenorrhea and menopausal hot flashes in women (Shojaii and Fard, 2012).

Leaf blight caused by *Alternaria radicina* of anise is a serious disease commonly observed in Egypt (Ghoneem, 2004). The pathogen caused a complete death of anise seedlings one month from sowing date. The fungus was also reported to infect and transmitted by other apiaceous seeds including carrot, celery, dill, fennel, parsley and parsnip causing black root rot, damping-off of seedlings and a foliar blight (Marthe *et al*. 2003). These fungi may produce toxins and enzymes which inhibit seed germination. Simmons (1992) reported that *Alternaria* species produced a unique group of mycotoxins including alternariol, alternuenes, altertoxins, tennuazoic acid and AAL toxins. Many other *Alternaria* species produced toxins that diffuse into host tissues ahead of the fungus (Rotem, 1998). It was also reported that polygalacturinase (PG) could produced by *A. radicina* at a higher percentage (Galal *et al*., 2000).

A common solution to control these pathogenic fungi is the use of synthetic chemical fungicides, however, their use often cause other problems. These problems include threatening human health and the environment by supporting the emergence of resistant pathogens and by leaving pesticide deposits on food (Motallebi *et al.*, 2013 and Zhang *et al.*, 2013). Recently, natural alternatives such as essential oils and /or their basic component (monoterpenoids) and other plant extracts were assessed and proved promising effects in controlling plant diseases (Vokou *et al.*, 2003 and Li *et al.*, 2014). Essential oils play an important role in the protection of plants as antimicrobial, insecticides and against herbivores by reducing their appetite for such plants (Combrinck *et al*., 2011).

Recently, Ghoneem *et al.* (2012) indicated that using essential oils of caraway and clove improved plant growth of faba bean in terms of plant height and number of branches and leaves per plant . Also application of those oils increased yield as them were effective in controlling chocolate spot disease. El-Said *et al.* (2008) indicated that soaking carob seeds in essential oils improved germination behavior and seedling growth.

The objective of the performed research work was to investigate the antifungal activity of the six essential oils *in vitro* on growth of *A. radicina* as

well as evaluate the protective effects of the most effective ones against the fungal invasion of anise plants under greenhouse conditions. In addition, to investigate the effect of those effective essential oils on growth and productivity of anise.

Material and Methods

Two plastic pots experiments were carried out during two successive seasons of 2010/2011 and 2011/2012, at Mansoura Horticulture Research Station, HRI, ARC, Giza, Egypt. The pots (40 cm in diameter with drainage holes) were filled with clean air dry soil. The experiment was designed in a complete randomized blocks design and arranged in seven different groups with five replicates and three pots for each.

Homogenous lot of anise local variety seeds was obtained from Department of Medicinal and Aromatic Plants, Horticulture Research Institute. Seeds were soaked in water for 12 hours. After soaking, thirty seeds were planted in each pot. After 6 weeks from sowing, the plants were thinned to leave only 5 uniform plants per pot. Fertilization was done as recommended by the ministry of agriculture, Egypt. Irrigation was conducted whenever required throughout the experimental period.

Ninety-day-old anise plants were evenly sprayed with suspensions of essential oils in 1% Tween 20 and subjected to one of the following treatments , 1) lemongrass oil at 5 µl ml^{-1}, 2) lemongrass oil at 10 µl ml^{-1} , 3) thyme oil at 5 µl ml^{-1} 4) thyme oil at 10 µl ml^{-1} , 5) caraway oil at 5 µl ml^{-1} , 6) caraway oil at 10 µl ml^{-1} , or 7) control.

The tested oils were applied as a prophylactic application at 2 h before the pathogen inoculation. The plants were challenge inoculated with conidial suspension of *A. radicina* (conidia at 8×10^4 ml^{-1}) in 0.01% Tween 20 applied as a uniform foliar spray using a hand atomizer.

After 105 days from sowing, both of growth parameters per plant (height, numbers of branches and leaves) were examined in samples of anise, in both seasons. At the same growth period in the two seasons, Photosynthetic pigments (chlorophyll (Chl) a, b and total Chl) were determined in the blade of the third leaf of plant tip (terminal leaflet) (Mackinney, 1941). At harvest, umbels number $plant^{-1}$, seeds weight $plant^{-1}$ and seed index (weight of 1000-seed) and oil content (mg 100 g^{-1} seeds) were recorded.

Isolation of Alternaria radicina

Diseased anise leaves and petioles were collected from different growing fields in the Egyptian governorates including Dakahlia, Faiyum and Minya. Anise leaves with necrotic spots were placed in 10 cm Petri dishes on moist filter paper and incubated for 2-3 days at 25 ºC under cool white fluorescence light

with alternating cycle of 12h light /12h darkness to promote fungal growth and sporulation. Single spore isolation of *Alternaria radicina* was grown for 7-10 days on Potato Dextrose Agar (PDA, Difco, Kansas City, Mo, USA), Oat agar (OA) or water agar (WA) in an incubator at 25°C. After incubation, cultures were examined for colony colour margin, colony texture and the development of pigments or crystals in the agar medium. The average spore size was calculated by measuring 100 conidia from 10-day-old OA cultures at 400× on a command pound microscope. Stock cultures obtained from single spore were maintained on Potato Carrot Agar (PCA) and kept at 4°C and sub-cultured monthly. Fungal species were identified according to Pryor and Gilbertson (2002). Isolate 7D-1 of *A. radicina* that was most pathogenic out of eight isolates obtained from highly infected anise leaves in south of Egypt was selected for experiments.

Extraction of essential oils (EOs)

Essential oils were extracted from different parts of several plants, i.e. seeds of *Carum carvi*, dry leaves at flowering stage of *Thymus vulgaris*, *Mentha spicata*, *Rosmarinus officinalis Cymbopogon citratus*, and dried flower buds of *Syzygium aromaticum*. Tested Eos were obtained through hydro distillation for 150min of Plant materials using Clevenger apparatus according to the method described by Egyptian Pharmacopoeia (1984). The extracted essential oils in pure form were stored at 4°C in a clean amber glass bottle until used.

Antifungal activity of the essential oils tested in vitro

Antifungal activities of the five essential oils extracted were evaluated. The EOs extracts were added to conical flasks containing sterilized PDA before solidification to obtain the proposed concentrations of 1, 5 and 10 µl ml^{-1}. 20 ml of amended media were poured into 9 cm diameter Petri dishes, and another set of untreated PDA plates was used as control. For each treatment, 3 replicates (plates) were used. All plates were inoculated individually with 0.5 cm diameter discs of the tested *A. radicina* cultures and then incubated in the dark at 25±2°C, until the control plates reached full growth. BELLIS 38% WG (50 g/100L) as a fungicide (BASF of the Chemical Company) was used for comparison.

Determination of in vivo disease control

The most potent plant EOs was selected from the *in vitro* test [1) lemongrass oil at 5 µl ml^{-1}, 2) lemongrass oil at 10 µl ml^{-1} , 3) thyme oil 5, at µl ml^{-1} 4) thyme oil at 10 µl ml^{-1} , 5) caraway oil at 5 µl ml^{-1} , 6) caraway oil at 10 µl ml^{-1}, or 7)] distilled water as control and evaluated for control of Alternaria leaf spot disease in a controlled environment. Ninety-day-old anise plants of cv. Local Varity, which are highly susceptible to late leaf spot, were used in the experiment. The seedlings were grown in a potting mixture of sandy loam soil in a greenhouse at 28 ± 3ºC. Physical and chemical characteristics of the used soil (Jackson, 1973) are presented in Table 1. Plants in 25-cm-diameter pots (five per pot) were evenly sprayed with suspensions of essential oils in 1% Tween 20 at concentrations of 5 and 10µl ml^{-1}. Plants treated with 1% Tween 20 and Bellis 38% WG (BASF

Chemical Company, Egypt) at 50 g 100 liter^{-1} served as untreated control and fungicide control, respectively. The test oils were applied as a prophylactic application at 2 h before the pathogen inoculation. The plants were challenge inoculated with conidial suspension of *A. radicina* (conidia at 8×10^4 ml^{-1}) in 0.01% Tween 20 applied as a uniform foliar spray using a hand atomizer. Inoculated plants were kept under greenhouse conditions (day temperature 31±2°C, night temperature 15.6±3°C and 16 h photoperiod) for 10 days after inoculation. Severity of Alternaria leaf spot in different treatments was measured and scored for the disease incidence (DI) as well as for disease severity (DS) of the infected leaves. Leaves were labeled serially and evaluated for *Alternaria* leaf blight symptoms using a symptom index key. Values for individual leaves were summed and averaged to derive DS for each plant. An index key designed by Strandbeerg (1988) for *Alternaria* leaf blight on carrots was adapted with modification in percentage of leaf area damaged on anise plants (Fig. 1). In this index, the infected leaves were categorized according to the following disease 0-4 symptom scale, 0= healthy plant, 1=1-9%, 2=10-19%, 3=20-39% and 4= ≥40 of leaf area damaged. To rate the damaged leaf areas in all tests, Xerographic copies were made of the indexed different infected leaves. Estimation of disease severity (DS) was carried out by comparing the copies to the assigned damage scale, then the averages of damage index were calculated. Two pots of each treatment were considered as one replication and the experiment was conducted three times with three replications of each treatment. All pots were arranged in a complete randomized design.

TABLE 1. Physical and chemical characteristics of the soil used in the pathogenicity test.

Physical characteristics		**Chemical characteristics**	
Texture	loam	$CaCo_3$ (%)	4.52
Sand (%)	41.6	Organic matter (%)	0.94
Clay (%)	26.35	N (mg. kg^{-1})	46.9
Silt (%)	32.05	P (mg. kg-1)	4.15
Electrical conductivity (dS.m^{-1})	1.13	K (mg. kg-1)	278.5
pH (1:2.5 soil : water)	7.92	Exchangeable sodium percentage (%)	52.5
Anion exchange capacity (meq 100 g^{-1} soil)		Cation exchange capacity (meq 100 g^{-1} soil)	
CO_3^{--}	0.0	Ca^{2+}	1.19
HCO_3^-	0.98	Mg^{2+}	0.73
CL^-	3.55	K^+	0.09
SO_4^{--}	1.26	Na^+	3.78

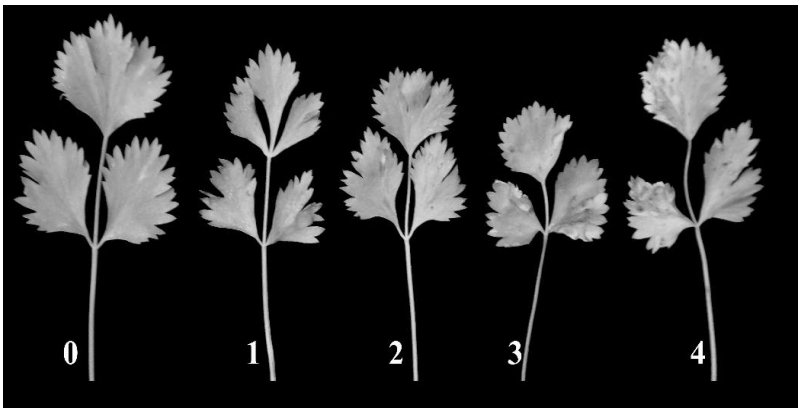

Fig.1. Disease severity index of anise leaves infected with *Alternariaradicina*, using 0-4 symptom scale, in which, 0=healthy plant, 1 = 1-9%, 2 = 10-19%, 3 = 20-39% and 4 ≥ 40% of leaf area damaged.

Host range studies

Ten plant species [anise (*Pimpinella anisum* L), caraway (*Carum carvii* L.), coriander (*Coriandrum sativum* L.), fennel (*Foeniculum vulgare* Mill.), cumin (*Cuminum cyminum* L.), parsley (*Petroselinum crispum* Mill.), dill (*Anethum graveolens* L.), celery (*Apium graveolens* L.), khella (*Ammi visnaga*) and carrot (*Daucus carota* L.)] of the family *Apiaceae* were used to study the host range of *A. radicina*. The previous method of inoculum preparation and inoculation was used. The same disease damage index key was also used to determine disease severity.

Extraction of anise essential oil

Samples of anise taken from the dried and cleaned seeds were ground up. For each treatment, three 50g subsamples were subjected to hydro distillation for 150 min in order to obtain the yield of essential oil in Clevenger apparatus according to the method described by Egyptian Pharmacopoeia (1984).

Statistical analysis

The obtained data were statistically analyzed as a complete randomized block design (SAS, 1996) for the comparison among the treatment means, Duncan's new multiple range test was used at 5%.

Results

Host rang study

Among tested *Apiaceae* plants, the host range test proved the ability of *Alternaria radicina* to infect anise, carrot, celery, cumin and parsley causing leaf spot and foliar blight symptoms. The host range of *A. radicina* is limited primarily to anise.

Antifungal activity of essential oils (in vitro study)

The antifungal activity of six plant essential oils against growth of *A. radicina* was evaluated. This survey is to select the most efficient oil in prevention or at least reducing the growth of pathogen. Results showed that plant essential oils of lemongrass completely inhibited the growth of *A. radicina* at 5µl m^{-1} whereas, oils of caraway and thyme at 10µl m^{-1} exhibited fungicidal activity against growth of leaf spot fungus (Fig. 2). At 10µl m^{-1}, mint oil showed strong inhibition rates on mycelial growth (72.6%). On the other hand, clove and rosemary essential oils had the lowest inhibition rates (52 and 13.4%, respectively) on mycelial growth of *A. radicina* as compared to check and fungicide treatments (Table 2).

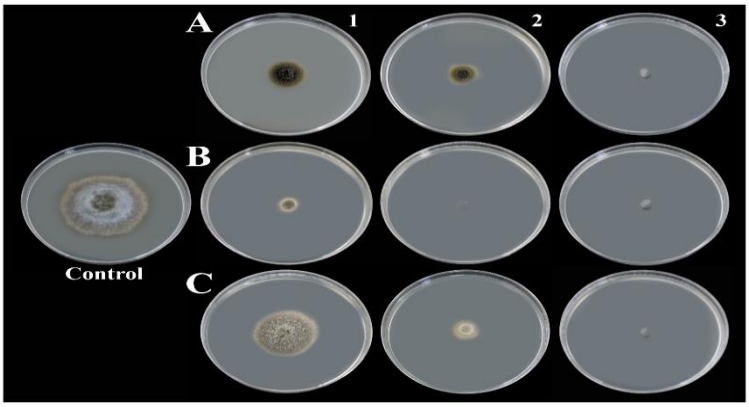

Fig. 2. Mycelial growth of *Alternaria radicina* submitted to the oils of A) caraway, B) lemongrass and C) thyme at concentrations of 0 (control), 1 (1), 5 (2) and 10 (3) µl ml^{-1}.

TABLE 2. Growth (cm) of *A. radicina* as affected by different essential oil concentrations.

Essential oil		Essential Oil concentration (µl ml^{-1})					
		1		5		10	
Scientific name	English name	Linear growth (cm)	Reduction %	Linear growth (cm)	Reduction %	Linear growth (cm)	Reduction %
Carum carvi	Caraway	2.52 f	49.6	1.58 g	68.4	0.0 i	100
Thymus vulgaris	Thyme	4.13 c	17.4	1.70 g	66	0.0 i	100
Syzygium aromaticum	Clove	3.65 d	26.6	3.67 d	27	2.40 f	52
Cymbopogon citratus	Limon grass	0.83 h	83.4	0.0 i	100	0.0 i	100
Mentha arvensis	Mint	3.17 e	36.6	2.67 f	46.6	1.37 g	72.6
Rosmarinus officinalis	Rosemary	4.95 a	1	4.33 ab	13.4	4.33 bc	20
Bellis 38% WG		0.0 i	100	-	-	-	-
Check		5.0 a					

*Different letters within a column indicates significant difference at $P \leq 0.05$ (Duncan test).

Greenhouse evaluation of essential oils

Incidence (DI) and severity (DS) of leaf spot disease on anise plant

Based on the *in vitro* results, lemongrass, thyme and caraway were selected for further investigation. The ability of the selected oils to alleviate leaf spot disease was evaluated under greenhouse conditions. As can be seen from the results Table 3 most of the tested oils reduced DI and DS in both growing season, irrespective to fungicide treatment. At 90 days, lemongrass, thyme and caraway oils at 10µl ml^{-1} as well as fungicide applications showed the highest reduction in disease parameters in comparison with all other treatments in both seasons. Lemongrass and thymes oils at 10µl ml^{-1} concentration as compared fungicide and check treatment showed the maximum reduction of DI of leaf spot along the growth period in both growing seasons.

TABLE 3. Effect of essential oils on Alternaria leaf spot under greenhouse conditions.

Essential oils	1st season		2nd season	
	DI	DS	DI	DS
Control (water)	0.0 d	0.0 e	0.0 d	0.0 e
Control (only pathogen)	65.5 a	2.73 a	82.55 a	2.85 a
BELLIS 38% WG	3.75 d	0.075 d	6.0 e	0.05 e
Lemon grass 5 µl ml^{-1}	32.11 c	0.76 c	27.84 d	0.81 c
Lemon grass 10 µl ml^{-1}	5.0 d	0.15 cd	6.25 e	0.28 de
Thyme 5 µl ml^{-1}	48.93 b	1.78 c	51.43 c	1.73 b
Thyme 10 µl ml^{-1}	3.13 d	0.063 d	5.63 e	0.19 de
Caraway 5 µl ml^{-1}	64.28 ab	2.61 a	69.0 b	2.77 a
Caraway 10 µl ml^{-1}	31.75 c	0.72 c	25.0 d	0.62 cd

*Different letters within a column indicates significant difference at $P \leq 0.05$ (Duncan test).

Field evaluation of essential oils foliar application

Effect of essential oils on the growth characters

Data in Table 4 indicate that, plant growth characters such as plant height (cm), leaf number ($plant^{-1}$), branch number ($plant^{-1}$), umbel number ($plant^{-1}$), herb fresh weight (g $plant^{-1}$) and herb dry weight (g $plant^{-1}$) of anise, were significantly increased by different essential oils treatments. Thus, the various growth characters in general significantly increased under the various essential oils treatments and was lemongrass essential oil (10 µl ml^{-1}) the most effective treatment in enhancing plant growth followed by thyme essential oil (10 µl ml^{-1}) compared with caraway oil and infected plants in the two seasons of the study. The results of growth characters agree with those obtained by (Vokou *et al*., 2003 and El-Said *et al*., 2008).

Effect of essential oils on chlorophyll A, B and Total chlorophyll

Significant increases in the photosynthetic pigments of anise leaves were detected as a result to different essential oils foliar application during the two seasons were indicated in Table 5. Spraying anise plants with lemongrass essential oil at 10 µl ml^{-1} recorded the highest content of Chl a (0.456 and 0.462

mg/g), Chl b (0.321 and 0.330 mg/g) and total Chls (0.777 and0.792 mg / g) at the first and second seasons, respectively. While the infected plants gave the lowest values of Chl a (0.396 and 0.393 mg/g), Chl b (0.255 and 0.257 mg/g) and total Chls (0.651 and 0.650 mg /g) in both seasons respectively.

TABLE 4. Effect of essential oil on vegetative growth of anise after 105 days from sowing.

Treatment	Plant height		Branches No. Plant^{-1}		Leaves No. Plant^{-1}		Umbles No. plant^{-1}	
	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
Control (only pathogen)	34.30 d	35.17 e	3.33 e	3.67 d	6.00 d	6.33 e	8.00 f	10.67 e
Lemon grass 5 µl ml^{-1}	39.93 ab	41.00 bc	7.67 c	9.00 b	7.67 c	8.33 d	21.33 c	23.0 c
Lemon grass 10 µl ml^{-1}	41.33 a	42.73 a	10.67 a	11.67 a	12.67 a	14.33 a	28.33 a	30.0 a
Thyme 5 µl ml^{-1}	38.80 bc	40.10 d	6.00 d	6.67 b	6.67 cd	7.33 de	18.67 d	19.67 d
Thyme 10 µl ml^{-1}	40.60 a	41.27 b	9.67 b	10.33 ab	10.33 b	13.33 b	26.67 a	28.33 a
Caraway 5 µl ml^{-1}	37.97 c	39.53 d	5.33 d	6.00 c	6.33 d	6.67 e	16.33 e	18.33 d
Caraway 10 µl ml^{-1}	40.17 ab	40.33 cd	9.00 b	9.67 b	9.67 b	10.67 c	23.67 b	25.67 b

*Different letters within a column indicates significant difference at $P \leq 0.05$ (Duncan test).

TABLE 5. Chlorophyll of anise plants as response to essential oils spray after 60 days from sowing.

Treatment	Photosynthetic pigments					
	Chl a		Chl b		Total Chls.	
	1st season	2nd season	1st season	2nd season	1st season	2nd season
Control (only pathogen)	0.396 g	0.393 g	0.255 g	0.257 g	0.651 g	0.650 g
Lemon grass 5 µl ml^{-1}	0.427 d	0.430 d	0.286 d	0.290 d	0.713 d	0.720 d
Lemon grass 10 µl ml^{-1}	0.456 a	0.462 a	0.321 a	0.330 a	0.777 a	0.792 a
Thyme 5 µl ml^{-1}	0.419 e	0.421 e	0.275 e	0.278 e	0.694 e	0.699 e
Thyme 10 µl ml^{-1}	0.445 b	0.449 b	0.310 c	0.316 b	0.755 b	0.765 b
Caraway 5 µl ml^{-1}	0.409 f	0.411 f	0.267 f	0.269 f	0.676 f	0.680 f
Caraway 10 µl ml^{-1}	0.438 c	0.441 c	0.293 c	0.298 c	0.731 c	0.739 c

*Different letters within a column indicates significant difference at $P \leq 0.05$ (Duncan test).

Effect of essential oils on the seed yield /plant, 1000 seed weight and essential oil content

Data presented in Table 6 showed that lemongrass essential oil at 10 µl ml^{-1} increased seed yield per plant with 77 .38 % over control plants followed by, thyme oil at 10 µl ml^{-1} reordering 66.85 % increment (average of two seasons)

while, caraway oil at 10 µl ml^{-1} ranked thirdly (mean of the two seasons). Also increased at all essential oils spraying treatments. The highest accumulation of essential oil was recorded at the highest lemongrass essential oil concentration 10 µl ml^{-1} (4.97 and 5 ml/100g seeds) while the lowest accumulation of essential oil was recorded in infected plants (3.20 and 3.29 ml /100g seeds) in the two seasons, respectively.

TABLE 6. Effect of essential oils spraying on anise yield and its attributes.

Treatment	Yield attributes					
	Seed weight Plant^{-1}		Seed index (1000 seeds wt)		Oil content ml/100g seeds	
	1st season	2nd season	1st season	2nd season	1st season	2nd season
Control (only pathogen)	4.08 f	4.18 g	1.21 d	1.27 f	3.20 f	3.29 f
Lemon grass 5 µl ml^{-1}	6.07 c	6.16 d	1.86 b	1.90 d	4.85 cd	4.87 c
Lemon grass 10 µl ml^{-1}	7.22 a	7.43 a	2.05 a	2.10 a	4.97 a	5.0 a
Thyme 5 µl ml^{-1}	5.22 d	5.32 e	1.83 bc	1.88 d	4.81 de	4.84 d
Thyme 10 µl ml^{-1}	6.84 b	6.94 b	1.95 a	2.0 b	4.92 ab	4.94 a
Caraway 5 µl ml^{-1}	4.92 e	5.03 f	1.78 c	1.83 e	4.77 e	4.80 e
Caraway 10 µl ml^{-1}	6.23 c	6.54 c	1.91 a	1.95 c	4.89 bc	4.91 b

*Different letters within a column indicates significant difference at $P \leq 0.05$ (Duncan test).

Discussion

Essential oils are a rich source of broad-spectrum antifungal plant derived metabolites that inhibit both fungal growth and production of toxic metabolites (Bakkali *et al.*, 2008). This study evaluated the broad spectrum antifungal activity of selected essential oils and their components and their use as fungicides for management of Alternaria leaf spot disease of anise. Our results indicated that all tested essential oils showed an antifungal activity against growth of *A. radicina* with 100% reduction at 5µl ml^{-1} of lemongrass and 10µl ml^{-1} of caraway and thyme essential oils. This is in agreement with that of Plotto *et al*. (2003) who reported vapors of lemongrass and thyme oils, and their respective major components showed completely growth inhibition of *Botrytis cinerea* and *Alternaria arborescens*. In addition, Arrebola *et al*. (2010) recorded that thyme and lemongrass oils showed over 50% and 25% inhibition of radial mycelial growth, respectively. The *in vitro* efficacy of lemongrass, thymus and caraway oils was reported against different pathogens (Tzortzakis & Economakis, 2007, Al-Askar & Rashad, 2010, Arslan & Dervis, 2010, Abdel-Kader *et al*., 2011, Combrinck *et al*., 2011 and Abd-Alla *et al*., 2011).

The antifungal activity was strongly associated with monoterpenic phenols, especially citral, thymol, carvacrol and carvone, in the three selected oils

(Bakkali *et al*., 2008). Citral (3,7-dimethyl-2,6-octadienal) is the major component of lemongrass oil, present at levels of, approximately, 65-85%. Citral is the name given to a natural mixture of two isomeric acyclic monoterpene aldehydes: geranial (*trans*-citral, citral A) and neral (*cis*-citral, citral B). In addition to citral, the lemongrass oil consists of small quantities of geraniol, geranylacetate and monoterpene olefins, such as myrcene (Silva *et al*., 2008 and Al Yousef, 2013). Literature points that citral acts as a fungicidal agent because it is able to form a charge transfer complex with an electron donor of fungal cells, resulting in fungal death (Kurita *et al*., 1981). In this respect, citral, Methyl anthranate and Nerol as some fractions of citrus essential oil caused complete inhibition of the linear growth of *Giotrichum candidum*, *Penicillium digitatum* and *P. italicum* as causal agents of fruit citrus diseases (El-Mohamedy *et al.,* 2002). Citral has the ability to complete inhibition the growth of *Alternaria alternata*, *Aspergillus flavus*, *Curvularia lunata*, *Fusarium moniliforme*, *F. pallidoroseum* and *Phoma sorghina* in paper disc agar diffusion assays. Using citral as low as 0.01% (vol/vol) was reported to inhibit the spore germination of *Cercospora arachidicola*, *Phaeoisariopsis personata*, and *Puccinia arachidis* by >90% *in vitro* (Kishore *et al*., 2007).

In vitro experiments, a complete reduction in linear growth of *Alternaria tenuis* and *Cercospora beticola* was obtained with citral, methyl anthranate and nerol at concentration of 5.0 ml/l (Fatouh *et al*., 2011). Recently, it was demonstrated that citral has the ability to destroy the integrity of the cell membrane, releasing the cellular components of *Geotrichum citri-aurantii* (Zhou *et al*., 2014) and dramatically inhibiting the mycelial growth of *Penicillium italicum* through a mechanism of cell membrane damage, compromising its integrity and permeability (Tao *et al.,* 2014). Following exposure to citral, the hyphal cell surface of *Magnaporthe grisea* became wrinkled with folds and cell breakage that were observed under scanning electron microscopy. There was damage to hyphal cell walls and membrane structures, loss of villous-like material outside of the cell wall, thinning of the cell wall, and discontinuities formed in the cell membrane following treatment based on transmission electron microscopy. This increase in chitinase activity both supports the morphological changes seen in the hyphae, and suggests a mechanism of action (Li *et al*., 2014).

Natural isopropyl cresols, thymol (2-isopropyl-5-methylphenol) and carvacrol (5-isopropyl-2-methylphenol) produced mainly from thyme are credited with a series of pharmacological properties, including antimicrobial and antifungal effects (Ahmad *et al*., 2011). The two compounds showed a complete growth inhibition of *Botrytis cinerea*, *Alternaria arborescens* and *Rhizopus stolonifer* (Plotto *et al*., 2003). Abdolahi *et al.* (2010) reported that thyme essential oils exhibited strong antifungal activity against *Botrytis cinerea* and *Mucor piriformis*. Combrinck *et al.* (2011) reported thyme as the best inhibitor oil against common postharvest pathogens of fruits. The oil was most effective against *Lasiodiplodia theobromae*, isolated from mango, and caused total inhibition of the pathogen at a concentration of 200 µl/l. Moreover, Vitoratos *et al.* (2013) reported that thyme essential oils exhibited strong

antifungal activity against *Penicillium italicum* and *P. digitatum* growth. Thymol and carvacrol affect the surface electrostatics of the cell membrane and membrane integrity (Lambert *et al*., 2001 and Sánchez *et al*., 2004), as well as, damaging the cell wall, cell membrane and cellular organelles (Rasooli and Owlia, 2005).

Caraway fruits contain 1-6% of essential oils consisting of about 30 compounds, from which carvone and limonene account form the main portion, about 95% (Sedláková *et al*., 2003). It is a common terpenoid that is produced by over 70 different plants (Burdock, 1995). Carvone exists as two enantiomers, (R)-(-)-carvone which has a spearmint aroma and (S)-(+)-carvone which has a caraway aroma, carvone can be used as antimicrobial agents and as a potato sprout inhibitor, the oxygenated monoterpenes play the major role in this respect (Carson and Riley 1995). In the same time, do not show negative effects on plant quality (Hartmans *et al*., 1995). The investigation done by Hartmans *et al*. (1995) showed high activity of caraway oil against fungi occurred during potato storage: *Fusarium sulphureum*, *Phoma exigua* var. foveata, *Helminthosporium solani*. Simic *et al*. (2008) observed similar effect of antifungal activity of caraway oil against *Cladosporium cladosporioides*, *Fulvia fulvium*, *Alternaria alternata*, *Phoma macdonaldii*, *Phomopsis helianthi* at concentration 2.5 mg ml^{-1}.

The host range study of *A. radicina* is limited primarily to anise, carrot, celery, cumin and parsley causing leaf spot and foliar blight symptoms. *A. radicina* also has been reported to cause a foliar blight of parsley and a stalk and root rot of celery (Tahvonen, 1978 and Wearing, 1980). Our results are agreement with finding of Pryor and Gilbertson (2002) who reported higher pathogenic effect of *A. radicina* on carrot and parsley seedlings, while it was weakly or moderately pathogenic on celery, cilantro, and fennel seedlings and weakly or not pathogenic on caraway, dill and parsnip seedlings. This may be back to the specificity of the pathogen to anise and infected Apiaceous plants.

In a controlled environment, all the essential oils tested were effective against Alternaria leaf spot *in vivo* when applied at least 2 h before pathogen inoculation. The effectiveness of essential oils in reducing the severity of the foliar disease could be due largely to the volatile nature of essential oils. It is known that germination and penetration of *Alternaria* conidia on the leaf surface of Apiaceous family like carrot requires 8 to 56 h (Strandberg, 1988**)**. The majority of the essential oils are more fungistatic than fungicidal (Kishore and Pande, 2004.) and thus, their persistence on the phylloplane determines their *in vivo* efficacy. Our results indicate that it is practical to use essential oils especially lemongrass oil as foliar sprays for control of Alternaria leaf spot.

It has been established that some plant species respond to monoterpenes (Godard *et al*., 2008). Additionally, the components of essential oils from many species have been demonstrated to act as allelochemicals that suppress the emergence and growth of nearby plants.

A possible interference of monoterpenes on phytohormones is suggested by the hormesis-type dose responses of some monoterpenes, i.e., they may act as stimulators of growth responses at lower concentrations and inhibitors at higher concentrations (Stebbing , 1982). The mechanisms of these effects are not known, but it is possible that they may interfere with the actions of phytohormones, considering the roles of hormones, such as abscisic acid (ABA), ethylene, gibberellins (GA), indole-3-acetic acid (IAA), on seed germination and other important plant physiological processes (Nambara and Marion-Poll, 2003 and Koomneef *et al.*, 2002).

Increasing photosynthetic pigments by foliar application of different essential oils is expected to increase carbohydrate content in plant tissues. Carbohydrates are the main repository of photosynthetic energy, they comprise structurally polysaccharides of pectin that consider a barrier against plant pathogens invasion and phenolic compounds are associated with structural carbohydrates, which play a major and important role in plant defense (Hahlbrock and Scheel, 1989). In addition, the enhancement in chlorophyll content is resulting from stimulation pigment synthesis, which is an important element in essential oil biosynthesis (Cseke *et al*., 2006) and increasing the efficacy of photosynthetic apparatus with better potential for resistance as well as decreasing photophosphorylation rate, which occurred after infection (Amaresh and Bhatt, 1988).

Yield loss from leaf spot has been attributed to the reduced umbels number and subsequent reduction in weight of seeds per plant from pathogen infection (Ghoneem, 2004). Yield increases given by EOs applications came mainly from increases in the weight of individual seeds, those yield increases given from better umbels retention (Table 6).

In this respect the previous works were done by Vokou *et al.* (2003) and El-Said *et al*. (2008) who indicated that essential oils significantly increased seedlings growth parameters of carob (tip length, root length, number of leaves and dry weight) during the two seasons. In addition, our work results were in agreement with those obtained by Ghoneem *et al.* (2012) on faba bean. There are few reports, however, in the literature that describe the possible interaction of monoterpenes with phytohormones. The *in vivo* effects of exogenous monoterpenes on seed/seedlings will depend not only on the physico-chemical characteristics of each compound but also on their rates of diffusion across plant cell membranes, seed coat morphologies, possible chemical transformations of the compounds within the cells, and the effective concentrations that are reached in the intra- cellular compartments (Ishii-Iwamoto *et al*., 2012).

Conclusion

Considering the reduction in the mycelial growth *in vitro* and incidence of disease symptoms on treated anise plants, we concluded that essential oils of lemongrass, thyme and caraway could be used as possible natural fungicides

alternative to synthetic fungicides against leaf spot fungus. Moreover, the foliar application of those oils improved plant growth, yield and essential oil content of anise plants and could be recommended as growth enhancement treatments.

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استخدام بعض الزيوت العطرية كمبيدات فطرية طبيعية لمقاومة فطر *Alternaria radicina* وتحسين إنتاجية الينسون (*Pimpenella anisum* L.)

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من المشاكل التي تواجه زراعة الينسون في مصر، هي الضرر الناتج عن إصابته بفطر التبقع الألترنارى. وتهدف هذه الدراسة الى إستخدام الزيوت العطرية كأحد البدائل لاستخدام المبيدات الكيماوية في مقاومة مرض التبقع الألترنارى الناتج عن الفطر *Alternaria radicina* وكذلك دراسة تأثير هذه الزيوت على نمو وانتاجية نبات الينسون خلال موسمين متتاليين من٢٠١٠/٢٠١١و٢٠١١/٢٠١٢. أجريت سلسلة من التجارب الأولية في المعمل باستخدام ست(٦) من الزيوت العطرية وتشمل الزعتر، الكراوية، القرنفل، النعناع، حصاللبان وحشيشة الليمون بثلاثة تركيزات 1 و٥ و١٠ ميكرولترمل$^{-1}$ وذلك لدراسة قدرتها على تثبيط نمو فطر *A. radicina*. وقد لوحظ وجود تثبيط كامل لنمو فطر *A. radicina* باستخدام زيت حشيشة الليمون بتركيز ٥ ميكرولترمل$^{-1}$، بينما كان لزيوت الزعتر، والكراوية نفس القدرة التثبيطية ولكن بالتركيز الأعلى (١٠ميكرولترمل$^{-1}$). وقد أظهر كل من زيت حشيشة الليمون والكراوية والزعتر كفاءة عالية في مواجهة غزو المسبب المرضى، حيث لوحظ انخفاض معنوي كبير في تواجد (DI) وشدة إصابة (DS) نباتات الينسون بالمرض خلال مراحل النمو المختلفة في كلا موسمي النمو وذلك مع استخدام زيت حشيشة الليمون بكلا تركيزية (٥ و١٠ ميكرو لترمل$^{-1}$) وزيتي الكراوية والزعتر بتركيز ١٠ ميكرولترمل$^{-1}$. كما أظهر استخدام الزيوت العطرية تأثير معنوي إيجابي على النمو الخضري لنباتات الينسون من حيث ارتفاع النبات، وعدد الأفرع وعدد أوراق النبات$^{-1}$ وكذلك على المحصول من حيث محصول البذور والزيت. كما أدى استخدام معاملات الزيوت السابقة الى حدوث زيادة معنوية في محتوى نباتات الينسون من صبغات البناء الضوئي. وقد أظهرت المعاملة بزيت حشيشة الليمون بتركيز ١٠ ميكرولترمل$^{-1}$ زيادة في محصول البذور للنبات تصل الى ٧٧,٣٨% مقارنة بالكونترول، يليها زيت الزعتر بتركيز ١٠ ميكرولترمل$^{-1}$ حيث سجل ٦٦,٨٥٪ زيادة (متوسط موسمين)، بينما جاء زيت الكراوية بتركيز ١٠ ميكرولترمل$^{-1}$ في المرتبة الثالثة. وتظهر هذه الدراسة الأهمية التطبيقية لاستخدام الزيوت العطرية وخاصة زيت حشيشة الليمون رشا على المجموع الخضري في مقاومة مرض التبقع الألترنارى. وعلاوة على ذلك، فإن رش المجموع الخضري أدى الى تحسين صفات النمو والإنتاجية في نبات الينسون.

الكلمات الرئيسية : اليانسون، *Alternaria radicina*، والزيوت العطرية، والنمو والمحصول، والمكافحة البيولوجية.