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Effective Influence of Essential Oils and Microelements against *Sclerotinia sclerotiorum*

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Abstract: *Sclerotinia sclerotiorum* is a destructive pathogen of several economically important crops, including soybean, pea, bean, canola and sunflower. This pathogen exhibits little host singularity and has a host range that includes more than 400 primarily dicotyledonous plant species. In this study, the inhibitory effects of 5 essential oils and 4 microelements against *S. sclerotiorum* were examined. Cinnamon, clove and mint oils completely inhibited the *in vivo* mycelial growth of the fungus at all concentrations. In addition, iron exhibited a marked antimicrobial effect against *S. sclerotiorum*. A soil application containing cinnamon oil significantly reduced the incidence of disease caused by *S. sclerotiorum,* producing 75% plant survival compared to the control. Calcium also significantly reduced the disease incidence that giving 80% living plants.

Key words: Cinnamon oil, white rot, beans, iron

INTRODUCTION

The use of plants and their products for the treatment of a variety of fungal infections is an old practice. This is because many plants contain a variety of compounds that have promising potentials for antimicrobial activity. The antimicrobial activity of medicinal and aromatic plants have been known and described for several centuries (Shamsuddeen and Sheshe, 2014). The oil possesses antiemetic, analgesic, antibacterial, antifungal and anticarcinogenic properties (Chami *et al*., 2005; Miyazawa and Hisama, 2001; Ogata *et al*., 2000).

The disease sometimes known as "White rot" is caused by *S. sclerotiorum* (Lib.) de Bary and is important because it affects over 450 species and subspecies of plants, including a wide range of economically important crops worldwide, especially in protected agricultural areas and in glasshouse crops (Bolton *et al*., 2006; Elgorban *et al*., 2013). In high-humidity environments, the fungus is among the most severe known, due to its influence on the leaves, stems, flowers and fruits (Zhou and Boland, 1998; Elgorban *et al*., 2013). *Sclerotinia sclerotiorum* produces sclerotia, which are structures for overwintering. The sclerotia can survive in the soil for many years and, when the environmental conditions are suitable, they germinate either carpogenically, producing apothecia that release ascospores or myceliogenically, giving rise to infective hyphae (Coley-Smith and Cooke, 1971). Different strategies have been developed to use soil borne pathogens to decrease the survival of resting fungal structures, such as the sclerotia. Agrochemicals can inhibit infections by ascospores, although, due to the difficultly of achieving spray penetration of the crop canopy, disease can still occur. Once the fungus has become established in the soil, steam sterilization and/or fumigation with methyl bromide can be used to enucleate the sclerotia. The rising cost of agrochemicals and steam sterilization, the development of fungicidal pathogen isolates, governmental restrictions on the use of fumigants due to environmental concerns about the regular use of fungicides and the difficulty of finding suitable rotation crops that will suppress pathogen propagules, have led to the search for an efficient alternative to fungicides for the suppression of *S. sclerotiorum* (Zhou and Boland, 1998; Soylu *et al*., 2007; Zhenhua *et al*., 2013). Consequently, concern in secondary metabolites from plant extracts and fundamentally essential oils as antifungal agents for use in many fields such as pharmacological applications, crop protection and food preservation and have increased through the last decade (Isman, 2000; Burt, 2004). Moreover, the rapid rise in request for organically produced fruit and vegetables will increase the request for

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natural pesticides such as essential oils. Newly, several studies on utilizing essential oils as the antifungal activities against fungal pathogens have been stated (Kalemba and Kunicka, 2003; Soylu *et al*., 2007). However, very few studies have concentrated on the antifungal activities of essential oils to manage this pathogen (Soylu *et al*., 2005, 2007; Zhenhua *et al*., 2013). Though there have been numerous reports on the antifungal activities of essential oil *in vitro* conditions, there is no research on the antifungal activity of the essential oil to soil borne fungal pathogen *in vivo* conditions. In the study, we assessed *in vitro* and *in vivo* antifungal effects of the essential oils against *S. sclerotiorum*. Also, antifungal activity of microelements against the pathogen was evaluated.

MATERIALSANDMETHODS

In vitro

The pathogen: The *S. sclerotiorum* used in our study was isolated from sclerotia on beans plants, collected from Ismailia governorate, Egypt. Sclerotia were surface disinfected and plated on Potato Dextrose Agar (PDA) amended with 50 μg mL from streptomycin sulfate and rifampicin 50 μg. The Petri dishes were incubated at 20±2°C for 6 days to allow hyphae to grow. Fungal isolate was re-inoculated on to beans seedling and found to be highly pathogenic. Stock cultures were maintained on PDA and kept at 4°C.

Plant material and isolation of essential oils: For the extraction of essential oils, five plant species were used: Cinnamon bark (*Cinnamomum zeylanicum*), cumin seeds (*Cuminum cyminum*), mint leaves (*Mentha* spp.), garlic cloves (*Allium sativum*) and clove seeds (*Syzygium aromaticum*). Air-dried plant materials (250 g) were placed in a 5l round-bottom distillation flask and 3l double distilled water added. The essential oils were obtained by steam distillation for 3 h using Clevenger-type apparatus, according to European Pharmacopoeia method (Council of Europe, 2005). The oils were separated, dried over anhydrous sodium sulfate and stored in an amber bottle at 4°C until used.

Salient features of the plants used: Cumin (*Cuminum cyminum* L.) is an aromatic plant included in the Apiaceae family and is used to flavor foods, added to fragrances and used in medical preparations (Iacobellis *et al*., 2005). Cumin also helps to enhance immunity. With its abundance of vitamin C, A and essential oils, cumin increases your ability to fight infections. Cumin also contains dietary fiber and has stimulating, anti-microbial and anti-fungal properties.

Cinnamon (*Cinnamomum zeylanicum* Blume) is an evergreen tropical tree, belonging to the Lauraceae family. Cinnamon barks and leaves are widely used as spice and flavoring agent in foods and for various applications in medicine (Schmidt *et al*., 2008). The essential oil from its bark is rich in trans-cinnamaldehyde with antimicrobial effects against animal and plant pathogens, food poisoning and spoilage bacteria and fungi (Faix *et al*., 2009).

The genus *Mentha* (Lamiaceae) consists of 19 species distributed in the old and new world. Mint species are famous all over the world for their essential oils. The aromatic leaves of mint are used fresh and dried as flavorings or spices in a wide variety of foods (Grisi *et al*., 2006).

Garlic cloves (*Allium sativum* L.) is an herb plant, belongs to the Liliaceae family comprising onions, leeks, shallots, asparagus etc. Garlic requires a sunny spot and rich soil (Johnson *et al*., 2013). Garlic oil is an effective antibiotic, anti-viral, anti-fungal agent which could be used to prevent nausea, diarrhea, ease coughs, even treatment in conditions such as malaria and cholera probably an immune system enhancement, some studies have found lower rates of certain types of cancer in people (Bhuiyan *et al*., 2010).

Syzygium caryophyllatum (L.) Alston (syn. *Syzygium aromaticum* (L.) Merr and Perry commonly called clove, which belongs to the family Myrtaceae, is an important aromatic spice. Clove is commercially cultivated in India, Madagascar, Sri Lanka, Indonesia and the south of China (Bhuiyan *et al*., 2010). The high levels of eugenol contained in clove essential oil responsible for strong antimicrobial activity.

Inhibitory effect of essential oils on the radial growth of *S. sclerotiorum*: The antifungal activities of five essential oils previously mentioned against *S. sclerotiorum* were evaluated. Potato Dextrose Agar (PDA) was autoclaved, then cooled to about 45°C. The essential oils were mixed with sterile PDA to obtain final concentrations 0, 10, 100, 250 and 500 ppm. Tween 80 at 0.01% was used as a surfactant to disperse the oil in PDA. The PDA was poured into each petri plates. Mycelial disks of 5 mm diameter, cut out from the periphery of 7-day-old cultures of *S. sclerotiorum*, were aseptically inoculated upside down on the PDA. Four replicates were used per treatment. All plates were incubated at 25±2°C and observations were recorded on day 7. The percentages of Mycelial Growth Inhibition (MGI) were recorded using the formula:

$$MGI(\%) = \frac{dc - dt}{dc} \times 100$$

where, dc and dt represent the mycelial growth diameter in control and treated petri plates, respectively.

Inhibitory effect of microelements on the radial growth of *S. sclerotiorum*: The effect of different concentrations (0, 100, 250, 500 and 1000 ppm) from Fe (Ferric-EDTA (13.2% Fe)), Mn (Mn-EDTA (13% Mn)), Zn (Zn-EDTA (16% Zn)) and Ca (Ca-EDTA (12% Ca)) were studied against *S. sclerotiorum*. These elements were separately mixed in PDA medium before solidification and then poured into petri plates (9 cm). Four plates for each concentration were used. All plates were inoculated with 5 mm fungus disc. Petri plates were incubated at 25±2°C. The mycelial growth of the fungus was measured when the full growth of the fungus was observed in control treatment. The percentages of mycelial growth inhibition were recorded as mentioned previously.

Statistical analysis: Data was analyzed using one-way analysis of variance (ANOVA) followed by Duncan comparison test to estimate statistical differences between means at p = 0.05. The LC50 values of five essential oils were calculated by probit analysis (Finney, 1974) using SAS 9.1 and POLO-PC programs.

In vivo

Inhibitory effect of essential oils on the disease incidence caused by *S. sclerotiorum*: This test was done to evaluate the antifungal activity of three essential oils; cinnamon, clove and mint, previously tested in laboratory against *S. sclerotiorum* as soil drenching. Pots (25×30×25 cm) were filled with autoclaved sandy loam soil (50% s and +50% loam, about 5 kg pot^{-1}), then artificially infested with inoculum of *S. sclerotiorum*. *Sclerotinia* inoculum was prepared in grinded maize and perlite for the soil incorporation method. The 100 g of grinded maize: perlite (15:85 w:w) moistened with 20% distilled water was placed in 250 mL conical flasks and autoclaved. Flasks were then inoculated with 5 discs (5 mm) from *S. sclerotiorum*, then incubated at 25±2°C in the dark for 3 weeks. The amount of inoculum in the maize: perlite was determined using a dilution technique described by Whipps *et al.* (1989). Inoculum was added to pots at rate 1 g kg^{-1} soil (about 1.25×10^7 CFU g^{-1}). Pots were watered immediately after inoculation. After 7 days, five seeds of bean variety paulista were planted in each pot. Each pot considered as one replicate and each treatment consisted of four replicates. Control treatments were done by sowing surface sterilized seeds in non-infested soil (as absolute control) and by sowing surface sterilized seeds in infested soil without inoculation with essential oils. After 7 days from sowing, soil drenching with suspension of essential oils at 10 ppm concentrate. Numbers of survival plants were recorded after 15, 30, 45 and 60 days.

Inhibitory effect of microelements on the disease incidence caused by *S. sclerotiorum*: The test was done to investigation the effect of four tested microelements; Fe, Mn, Zn and Ca) on *S. sclerotiorum*. The microelements were applied as soil drenching (100 ppm and 20 mL pot^{-1}). Control treatments were as mentioned before. Data collection was recorded as mentioned before in the other greenhouse test.

RESULTS

Inhibitory effect of essential oils on the radial growth of *S. sclerotiorum*: The antifungal activity of essential oils against the mycelial growth of *S. sclerotiorum* is presented in Table 1. It was noticed that out of five essential oils tested, mint oil (67.1%) showed maximum inhibitory effect against the mycelial growth of *S. sclerotiorum* followed cinnamon (57.0%) and clove oil (51.1%) at 1 ppm concentration when compared to untreated control. On the other hand, cinnamon, mint and clove oil completely inhibited the mycelial growth of the pathogen at 10 ppm concentration. Otherwise, cumin and garlic oil completely suppressed the mycelial growth of the fungus at 500 ppm concentration. The Minimum Inhibitory Concentrations (MIC) value was lowest at cinnamon and clove oil treatment with 2 ppm concentration while MIC was 200 ppm in case of cumin

Table 1: Effect of some essential oils on radial growth of *Sclerotinia sclerotiorum*

	Concentration (ppm)													
	0		1		10		100		500					
Oils	RG	Inh. (%)	RG	Inh. (%)	RG	Inh. (%)	RG	Inh. (%)	RG	Inh. (%)	LC_{50} (ppm L^{-1} air)	MIC (ppm)	95% confidence limits	Slope±SE
Cinnamon	84.5^{a}	0.0	36.3^{c}	57.0	0.0^{e}	100.0	0.0^{e}	100.0	0.0^{e}	100	-	2	-	-
Cumin	84.5^{a}	0.0	81.8^{a}	3.2	75.8^{a}	10.3	67.0^{a}	20.7	0.0^{e}	100	110.7	200	997.8-3305.4	1.36±0.016
Mint	84.5^{a}	0.0	27.8^{d}	67.1	0.0^{e}	100.0	0.0^{e}	100.0	0.0^{e}	100	-	5	-	-
Garlic	84.5^{a}	0.0	82.5^{a}	2.4	52.5^{b}	37.9	46.3^{b}	45.2	0.0^{e}	100	39.1	200	638.2-2244.5	1.1±0.008
Clove	84.5^{a}	0.0	41.0^{b}	51.5	0.0^{e}	100.0	0.0^{e}	100.0	0.0^{e}	100	-	2	-	-
LSD	0.00	-	3.15	-	2.89	-	3.24	-	0.00	-	-	-	-	-

Inh. (%): Inhibition %, RG: Radial growth, Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (p = 0.05)

Table 2: Effect of some microelements on radial growth of *Sclerotinia sclerotiorum*

	Concentration (ppm)									
	0		10		100		500		1000	
Elements	RG	Inh. (%)	RG	Inh. (%)	RG	Inh. (%)	RG	Inh. (%)	RG	Inh. (%)
Fe	84.5^{a}	0.0	84.5^{a}	0.0	84.5^{a}	0.0	7.5^{d}	91.1	0.0^{d}	100.0
Zn	84.5^{a}	0.0	84.5^{a}	0.0	84.5^{a}	0.0	42.5^{b}	49.7	11.8^{b}	86.0
Mn	84.5^{a}	0.0	44.3^{b}	47.6	40.3^{b}	52.3	27.8^{c}	67.1	8.3^{c}	90.2
Ca	84.5^{a}	0.0	84.5^{a}	0.0	84.5^{a}	0.0	84.5^{a}	0.0	35.8^{a}	57.6
LSD	0.00	-	4.58	-	5.23	-	5.55	-	6.12	-

Inh. (%): Inhibition %, RG: Radial growth, Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (p = 0.05)

Table 3: Effect of some essential oils on white rot disease incidence of bean caused by *Sclerotinia sclerotiorum*

	Days											
	15			30			45			60		
Treatments	No.	Mo. (%)	Sur. (%)	No.	Mo. (%)	Sur. (%)	No.	Mo. (%)	Sur. (%)	No.	Mo. (%)	Sur. (%)
Non-infested	5.00^{a}	0.0	100.0	5.00^{a}	0.0	100.0	5.00^{a}	0.0	100.0	5.00^{a}	0.0	100.0
Infested	3.25^{b}	35.0	65.0	3.00^{b}	5.0	60.0	3.00^{b}	0.0	60.0	2.75^{b}	5.0	55.0
Clove oil	3.50^{b}	30.0	70.0	3.50^{b}	0.0	70.0	3.25^{b}	5.0	65.0	3.00^{b}	5.0	60.0
Mint oil	3.75^{ab}	25.0	75.0	3.75^{ab}	0.0	75.0	3.50^{b}	5.0	70.0	3.00^{b}	10.0	60.0
Cinnamon oil	4.25^{ab}	15.0	85.0	4.25^{ab}	0.0	85.0	4.00^{ab}	5.0	80.0	3.75^{b}	5.0	75.0
LSD	1.31	-	-	2.26	-	-	1.08	-	-	1.07	-	-

No.: No. of living plants, Mo. (%): Mortality percentage, Sur. (%): Survival plants %, Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (p = 0.05)

Table 4: Effect of some microelements on white rot disease incidence of bean caused by *Sclerotinia sclerotiorum*

	Days											
	15			30			45			60		
Treatments	No.	Mo. (%)	Sur. (%)	No.	Mo. (%)	Sur. (%)	No.	Mo. (%)	Sur. (%)	No.	Mo. (%)	Sur. (%)
Non-infested	5.00^{a}	0.0	100.0	5.00^{a}	0.0	100.0	5.00^{a}	0.0	100.0	5.00^{a}	0.0	100.0
Infested	3.25^{b}	35.0	65.0	3.00^{b}	5.0	60.0	3.00^{b}	0.0	60.0	2.75^{c}	5.0	55.0
Zn	3.75^{ab}	25.0	75.0	3.75^{ab}	0.0	75.0	3.50^{b}	5.0	70.0	3.25^{bc}	5.0	65.0
Mn	3.75^{ab}	25.0	75.0	3.75^{ab}	0.0	75.0	3.75^{ab}	5.0	70.0	3.25^{bc}	5.0	65.0
Fe	4.00^{ab}	20.0	80.0	4.00^{ab}	0.0	80.0	3.75^{ab}	5.0	75.0	3.50^{bc}	5.0	70.0
Ca	4.25^{ab}	15.0	85.0	4.25^{ab}	0.0	85.0	4.25^{ab}	5.0	85.0	4.00^{ab}	5.0	80.0
LSD	1.36	-	-	1.32	-	-	1.29	-	-	1.07	-	-

No.: No. of living plants, Mo. (%): Mortality percentage, Sur. (%): Survival plants %, Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (p = 0.05)

and garlic oil (Table 1). Also, The LC_{50} values, the concentration causing 50% inhibition of radial growth of mycelium, were 110.7 and 39.1 ppm in case of cumin and garlic oil at oregano treatment followed by thyme treatment.

Inhibitory effect of microelements on the radial growth of *S. sclerotiorum*: Results in Table 2 illustrated all microelements were found to be significantly active against *S. sclerotiorum*. Iron completely inhibited the mycelial growth of the pathogen at 1000 ppm concentrate when compared with control. This was followed by manganese (Mn) that reduced the mycelial growth of the fungus with 47.6, 52.3, 67.7 and 90.2% at 10, 100, 500 and 1000 ppm, respectively. Also, zinc (Zn) significantly suppressed the mycelial growth of the pathogen that produced 86.0% reduction at 1000 ppm concentration. On the other hand, calcium (Ca) gave a moderate reduction by 57.6% at 100 ppm concentrate when compared with control.

Inhibitory effect of essential oils on the disease incidence caused by *S. sclerotiorum*: After 60 day, cinnamon oil produced the best result for reducing the white rot disease incidence that produced 75% survival plants when compared to controls (Table 3). Even as, mint and clove oil gave a slight reduction in the disease incidence with 60% survival plants in both oils when compared with controls (55% living plants in case of untreated control and 100% living plants in case of absolute).

Inhibitory effect of microelements on the disease incidence caused by *S. sclerotiorum*: Data in Table 4 demonstrated that calcium was the most effective element

for suppressing the white rot disease incidence with 80% survival plants after 60 day when compared with untreated control (55%) and absolute control which produced 100% survival plants. Whereas, Fe, Mn and Zn gave moderate reduction in the disease incidence giving 70, 65 and 65% survival plants, respectively when compared to controls.

DISCUSSION

Considering the need for alternative ecofriendly approach to manage the plant pathogenic fungi, it was believed to be worthwhile to study the antifungal activity of essential oils and microelements. In the present study, the mycelial growth of *S. sclerotiorum* was completely inhibited by cinnamon, clove and mint oil. This result is in agreement with the findings of Lee *et al*. (2005), Fu *et al*. (2007), Aminifard and Mohammadi (2013) and Zhenhua *et al*. (2013). The antimicrobial activity of cinnamon and clove oils may be attributed to the presence of a phenolic OH group and an aromatic nucleus such as cinnamaldehyde, ethyl cinnamate, eugenol and α-pinene (Friedman *et al*., 2002; Pasay *et al*., 2010) that is known to be reactive and to form hydrogen bonds with active sites of target enzymes (Farag *et al*., 1989). As defined earlier, the hydroxyl group is important for the activities of some antimicrobial compounds; these activities are enhanced by the presence of a-b double bonds (Ultee *et al*., 2002). Based on the results obtained in this investigation, essential oils should be considered as alternative natural fungicides.

It was concluded that the essential oil of *Mentha* spp. possesses considerable antimicrobial potential and may be used as a natural fungicide (Sokovic *et al*., 2009). Aqil *et al*. (2000) evaluated mint oil for their antifungal activity against *Aspergillus niger*, *Alternaria alternata* and *Fusarium chlamydosporum* via the agar well diffusion method. The authors found that this essential oil can be exploited as antifungal agents in the management of infectious plant diseases and post-harvest spoilage of crops (Aqil *et al*., 2000). This high antifungal activity of mint oil may be attributed to compounds that include tomenthol, menthyl acetate, menthone, carvone and menthone (Iscan *et al*., 2002; Moghtader, 2013).

In this study, it was found that iron significantly suppressed the mycelial growth of *S. sclerotiorum*. This result is in agreement with that of Lahoz *et al*. (2008) who stated that the free iron inhibited the mycelial growth of *Aspergillus niger*, *Sclerotium rolfsii*, *Rhizoctonia solani* and *Phoma exigua*. Furthermore, Brown and Swinburne (1982) reported that iron sulfate suppressed the aggressiveness of *B. cinerea* and inhibited the formation of leaf necrosis induced by this fungus on *Vicia faba* (Vedie and Le Normand, 1984). Our results indicate that iron may be able to control this fungus. This fact can be attributed to two general possibilities that should be verified in the future. First, the discrepancy could be linked to different absorption capacities of the various fungi. In fact, fungi exhibit varying degrees of different strategies to absorb iron, including the heat sink transfer of specific materials and binding to secreted siderophores (De Luca and Wood, 2000; Haas, 2003; Philpott, 2006).

A number of reports have shown that such microelements cause systemic protection, most likely through encouraged changes in the host metabolism. The reports also showed that Mn can control a number of diseases, since Mn plays an important role in lignin biosynthesis, phenol biosynthesis and photosynthesis; the reduction of several diseases is most often attributed to improved nutrition that boosts host defenses or the direct inhibition of fungal growth and activity (Yousef *et al*., 2013). In most cases, the minerals work by directly suppressing the propagules' potential, improving host tolerance or both (Von Broembsen and Deacon, 1997; Yousef *et al*., 2013).

CONCLUSION

This research confirms the antifungal effects of essential oils and microelements both *in vitro* and *in vivo* on white rot disease. Therefore these essential oils and microelements could be alternative methods to fungicides to manage plant pathogenic fungi on bean.

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