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Thyme Oil Nanoemulsion: Antifungal Activity Against *Sclerotinia sclerotiorum* and Phytotoxicity on Fennel Plant

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ABSTRACT

During this study, the bioactive components of thyme essential oil were analyzed using gas chromatography, and the results were as follows: thymol, 1,8-cineole, ρ-cymene and α-terpinene are the main components. The Particle size of thyme essential oil nanoemulsion was evaluated using ultrasound. The results showed that the PS after 30 min of sonication was about 36.65 nm, and Transmission electron microscopy analysis showed a spherical shape. Laboratory investigations showed that nanoemulsions at different concentrations (1000-5000 ppm) were able to inhibit the growth of *Sclerotinia sclerotiorum* fungi. At a concentration of 3000 ppm, the nanoemulsion completely inhibited fungal growth. Treating fennel seeds with TEON, TEOE and TEOS had little effect on germination inhibition, with nanoemulsion being the most effective in promoting germination, while nanoemulsion was the best in terms of improving seed germination. As a result of treatment with nanoemulsions, the damping-off rates were significantly reduced, and plant growth was improved.
Keywords: Fennel, Thyme, Sclerotinia, Emulsion, Suspension.

INTRODUCTION

Fennel (*Foeniculum vulgare*) is an important medicinal herb in Egypt. the White rot in fennel, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary causing economic losses in the growth and yield of plants (Hilal *et al.*, 2007 & Choi *et al.*, 2016). *S. sclerotiorum* is one of the most common and successful plant pathogens. The fungal mycelium grows within and between cells during primery infection, which occurs in late winter or early spring. Patches of dead plant components increase and consolidate during the spring, causing significant stand losses (Bolton *et al.*, 2006). Fungicides are effective at controlling rot diseases; however, they have a severe impact on human health and the environment. Long-term intensive use of fungicides in open fields has resulted in the formation of fungicide-resistant populations. In general, the widespread use of fungicides has numerous detrimental environmental consequences that cannot be overlooked (Barnet and Hollomon, 1998; Hilal *et al.*, 2007; Feng and Zheng, 2007). As a result, various attempts have been made to use natural fungicidal alternatives to control plant diseases. In this regard, thyme essential oil has been shown to exhibit antifungal properties against certain fungi (Zengin and Baysal, 2015; Attia *et al.*, 2023). Emulsion of thyme essential oil is a kinetically stable system formed by dispersing one liquid into another, where each liquid is immiscible or poorly miscible in the other (Purwanti *et al.*, 2015). The particle size differentiates oil emulsion to nanoemulsion, when the size of the oil particles gets smaller, the emulsion's stability improves dramatically (Anton and Vandamme, 2011; Hassanin *et al.,* 2017). Nanomaterials (1-100 nm) have recently been produced and have the potential for more effective and safer fungicide administration through improved active ingredient distribution and less environmental drift (Gogoi *et al.,* 2009; Hassanin *et al.,* 2017; Hammad and Hassanin 2022). Thyme oil antifungal activity against Fusarium, Sclerotinia, Aspergillus, Alternaria, Penicillium, *Rhizopus* sp. and Cladosporium was attributed primarily to p-cymene, 1,8-cineole, and other thymol components (Šegvic Klaric *et al.,* 2007; Hassanin *et al.,* 2017; Hammad and Hassanin 2022). In this study, Thyme oil was chosen as a natural biocide for the synthesis of emulsion, nanoemulsion, and suspension in water using a non-ionic surfactant. *In vitro* and *in vivo* antifungal activities of thyme oil (emusion, nanoemulsion, and suspension) were investigated.

MATERIALS AND METHODS

Biochemical analysis of thyme essential oil using Gas Chromatography:

The biochemical constituents of the thyme essential oil emulsion tested was determined at the Laboratories of the National Research Centre (NRC), Giza, Egypt; using Gas Chromatography (GC) technique, as described by Elhalawany *et al.,* 2019. In this method, GC was performed using a Ds Chrome 6200 Gas Chromatograph. The machine was composed of a flame ionization detector; a 5% phenyl polysillphenylene - siloxane (30 m × 0.25 mm IDo × 0.25 µm film thickness), and Column: BPX-5. The temperature program ranged from 70 to 200 °C at an average of 10 °C/ min., while the used temperature for the detector was 280 °C. Nitrogen flowed at the rate of 30 ml/ min.; hydrogen at 30 ml/ min., and air at 300 ml/ min.

Preparation of emulsions and suspension of thyme oil:

Thyme essential oil was purchased from Medicinal Plants Research Department, Hort. Res. Inst., Agric. Res. Center., Giza, Egypt. Thyme essential oil nanoemulsion (TEON) is prepared as follows: Ten milliliters of thyme oil and five milliliters of the nonionic surfactant (Tween 80) were carefully mixed together until a homogenous mixture formed. Then, to help spread and completely absorb the thyme oil, water (85 ml) was added to make the final mixture 100 ml. The mixture was then swirled for 30 minutes using a magnetic stirrer. The mixture was separated into two portions and sonicated for 15 and 60 minutes at 350 W using an Ultrasonicator (Bande-lin SONOPULS HD 2200, Germany). After 30 days of storage at room temperature (27 C), the particle size of 10% TEON for each amount was measured using a hydrodynamic light scattering analyzer (DLS). Thyme essential oil emulsion (TEOE) was created without sonication, whereas thyme essential oil suspension (TEOS) was prepared as follows: Ten ml of oil and five ml of the nonionic surfactant (Tween 80) were carefully mixed together until a homogenous mixture formed. Then, 85 ml of water was added to make the final mixture 100 ml.

Droplet size of nanoemulsion:

Droplet size measurement of TEON was performed at room temperature using dynamic light scattering studies with Zeta Nano ZS (Malvern Instruments, UK). 30µl of the nanoemulsion was diluted with 3 ml of water at 25 C before to testing. Particle size (PS) data were reported as the mean of three different batches of nanoemulsions' Z-averages. The Nanotechnology Laboratory, Regional Center for Food and Feed, ARC, Giza, Egypt, carried out this study.

Transmission electron microscopy (TEM):

Twenty microliters of diluted samples were deposited on a film-coated 200-mesh copper specimen grid for 10 minutes before being removed with filter paper. After that, the grid was stained with one drop of 3% phosphotungstic acid and left to dry for three minutes. After drying, the coated grid was inspected using a TEM microscope (Tecnai G20, Super twin, double tilt, FEI, The Netherlands). The samples were examined while working at 200 kV.

Effect of TEON, TEOE and TEOS on growth of *S. sclerotiorum*:

The paper disc method published by Šegvic Klaric *et al.*, (2007) was used to investigate the efficacy of TEON, TEOE and TEOS in inhibiting fungal growth. Three PDA medium-containing plates were infected with *S. sclerotiorum* discs (5 mm in diameter). The inoculated Petri dishes were inverted, and filter paper discs (5 mm) soaked with various concentrations (0, 1000, 3000, 5000 ppm) of the volatile oil preparations were placed in the center of the plate lids and incubated at 18 C. According to the formula proposed by Topps and Wain (1957), percentages of fungal growth inhibition were computed when the fungal growth of the control plates (without treatments) entirely filled the plates as follows:

$$\% \text{ Inhibition} = \frac{A - B}{A} \times 100$$

A= The linear growth in control treatment.
B= The linear growth of treated fungus.

Effect of TEON, TEOE and TEOS on seed germination:

Sets of 300 fennel seeds were soaked for 15 minutes in each oil formulation (TEON, TEOE, & TEOS) at the rates (0, 1000, 3000, 5000 ppm). Seeds were placed in Petri-dishes on layers of moistened blotters at a rate of (100) seeds/dish after being treated with each concentration. Dishes were then incubated for 15 days in a tightly controlled environment (27 C) with alternate cycles of 12 hours light and 12 hours dark. Finally, the percentage of germination for each treatment was calculated as follows:

$$\% \text{ Germination} = \frac{\text{No. of germinated seeds}}{\text{Total number of experimented seeds}} \times 100$$

Greenhouse studies:

All greenhouse studies used 25 cm diameter pots filled with soil (1 sand: 1 clay, w/w). Formalin-disinfected soils were infested with *S. sclerotiorum* at a rate of 3% soil weight. Each treatment received three replications. To compare the inhibitory activity of the fungicide, TEON (3000 ppm), TEOE & TEOS (5000 ppm), and the systemic fungicide Switch 62.5% WG (a-Cyprodinil b-Fludioxonil) (2 g/L water) suspension was applied. Before planting in the contaminated soil, seeds were soaked for 20 minutes in the previously produced emulsions and fungicide or in water alone as a control. There were three pots in each treatment and control. Sixty fennel seeds (20 seeds per pot) were distributed to each treatment. After 15 and 45 days, the percentages of pre- and post-emergence damping-off were reported. 90 days after sowing, plant height and root length of healthy surviving plants were measured.

RESULTS AND DISCUSSION

Chemical composition of the thyme oil:

Using GC technique, the results presented in Table (1) show that the major components of thyme essential oil were Thymol (27.945 %); 1.8-cineole (16.34 %), ρ-cymene (15.744 %) and α-terpinene (10.983 %). On the other hand, little amounts of β-caryophyllene (1.628%) and α-pinene (1.356 %) were detected. These results are somewhat in agreement with those of Grigore *et al.*, 2010, who performed a quantitative analysis of thyme essential oil using GC and a qualitative analysis using high performance thin layer chromatography (HPTLC). The results reported that essential oil of thyme contained thymol and p- cymene in high quantities.

Table 1. Chemical composition of thyme oil detected using GC.

Component	Area %
α-terpinene	10.983
α-pinene	1.356
Broneol	4.179
Camphene	2.187
Myrcene	4.682
Thymol	27.945
ρ-cymene	15.744
Limonene	5.729
1.8-cineole	16.34
γ-terpineol	4.184
β-caryophyllene	1.628
Sabinene	3.122
Total	98.079

Effect of sonication on droplet size of oil:

The influence of sonication on the droplet size of TEON was investigated. (Fig.1). After 30 days of storage at room temperature, it demonstrated the stability of TEON generated by ultrasonication for 30 minutes at 350 W. Sonication has a significant impact on droplet size. The relative increase in sonication duration could be attributed to an increase in shear pressures applied to the droplets, causing greater droplet fragmentation. It is recommended that the droplet size decreases as the sonication time increases. Figure (1) represents a PS created by ultrasonication for 30 minutes. It was small (about 36.65 nm) and had a high polydispersity index (PDI) of 244. Surfactant ability and performance may impact droplet size reduction. In an oil-in-water emulsion, stirring is known to reduce droplet size (Sajjadi *et al.*, 2002). In the presence of double bonds in the nonpolar chain of nonionic surfactants, Dai *et al.* (1997) examined the creation of nanoemulsions with smaller droplet sizes. The findings confirmed recent findings (Shahavi *et al.*, 2015; Doghish *et al.*, 2023).

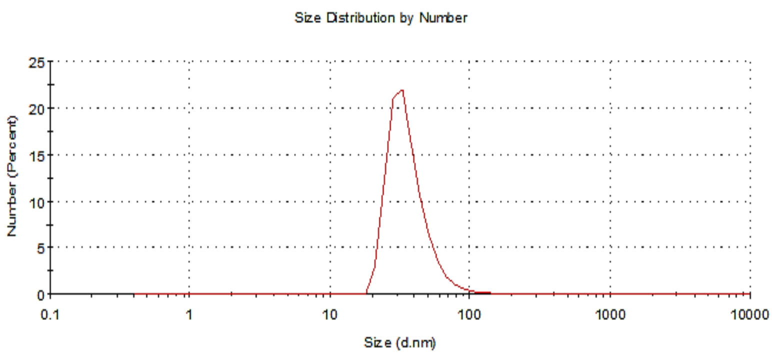

Fig. 1. Effect of sonication on particle size of TEON prepared by ultrasonication method for 30 min. (peak at 36.65 nm) & (PDI= 244).

Transmission electron microscopy (TEM):

The real size and shape of the TEON are revealed by TEM analysis; the droplets in the nanoemulsion appear black. The TEON was round in form and relatively homogeneous, with dimensions ranging from 29.1 nm to 51.3 nm (Fig.2). The droplet size correlated well with the results of dynamic light scattering droplet size study (Abd-Elsalam and Khokhlov, 2015; Doghish *et al.*, 2023).

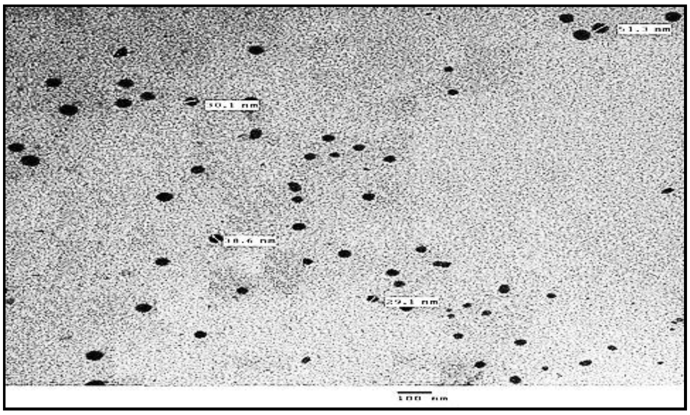

Fig.2. The result of sonication on the TEM of TEON generated by ultrasonication for 30 minutes (with sizes ranging from 29.1 nm to 51.3 nm).

Effect of thyme oil on fungal growth of *S. sclerotiorum*:

According to the data in Table 2, TEON was associated with a decrease in linear fungal growth where fully stopped. At a concentration of 3000 ppm, mycelial growth occurs. In contrast, TEOE was always associated with a reduction in linear fungal growth. The diameters of mycelial growth, however, were 9.0 (control), 2.3, 1.7 and 0.5 cm at concentrations of 0 (control), 1000, 3000 and 5000 ppm, respectively. Also, decreased the fungal growth with TEOS, 9.9, 3.1, 2.2 and 0.9 at the concentrations of 0 (control), 1000, 3000 and 5000 ppm, respectively. The size of the nanoemulsion particles is what distinguishes it from emulsion. The emulsion's stability increases considerably when the size of the oil particles is lowered (Anton and Vandamme, 2011). Nanoparticles (100 nm particle size) are being developed and offer the possibility to administer fungicides, insecticides, herbicides, and fertilizers more efficiently and safely due to greater active component delivery and reduced environmental drift (Gogoi *et al.*, 2009). Zedan *et al.*, 1994 reported on the antifungal harmful effects of many essential oils, including the one under investigation. According to Šegvic Klaric *et al.*, 2007, thymol, 1,8-cineole, and p-cymene discovered in thyme oil are responsible for its antifungal effect. However, thyme essential oil action could be attributed to chitin penetration of the cell wall, which disrupts the lipoprotein cytoplasmic membrane, allowing cytoplasm to escape (Zambonelli *et al.*, 1996).

Table 2. The *in vitro* effect of concentrations of TEON, TEOE & TEOS on linear growth of *S. sclerotiorum*.

Treatments	**Linear growth (cm) at concentration of:**			
	0 ppm	1000 ppm	3000 ppm	5000 ppm
(TEON)	9.0	0.8	0.0	0.0
(TEOE)	9.0	2.3	1.7	0.5
(TEOS)	9.0	3.1	2.2	0.9
L.S.D. at 5% for:	**Treatment (A)= 0.92**	**Concentrations (B)= 1.0**	**A × B= 1.3**	

Effect of thyme oil on seed germination:

The results demonstrated the influence of TEON, TEOE, and TEOS on germination of fennel seeds when employed as a soaking treatment for a specific period of time (Table 3). TEON treatment resulted in 100% germination of treated seeds at doses of 1000, 3000, and 5000 ppm. In the instance of TEOE, germination (%) was gradually enhanced by increasing concentration to achieve 50.0%, 66.7%, and 70.0% at the concentrations of 1000, 3000 and 5000 ppm, respectively. On the other hand, the effect of TEOS on germination of fennel seeds was the least effective in germination increasing. The results of our study can be interpreted in terms of the various types of terpenoids found in plant essential oils and their potential impacts on seed germination. Terpenoids, particularly sesquiterpenes, are said to be a class of chemicals with varying biological activity. These compounds revealed a unique structure-activity relationship at low concentrations (Beekman *et al.*, 1997; Hassanin *et al.*, 2017).

Table 3. Effect of TEON, TEOE & TEOS on percentage of seeds germination.

Treatments	% Concentrations			
	0 ppm	1000 ppm	3000 ppm	5000 ppm
TEON	100	100	100	100
TEOE	100	50.0	66.7	70.0
TEOS	100	44.0	54.0	68.7
L.S.D. at 5% for:	Treatment (A)= 10.3	Concentrations (B)= 9.4		A × B= 11.03

Greenhouse tests:

According to the data in Table (4), when compared to the controls, all of the tested treatments substantially lowered the percentages of pre- and post-emergence damping-off caused by *S. sclerotiorum* and significantly enhanced plant survival. TEON, on the other hand, was the most effective treatment in terms of lowering pre- and post-emergence damping-off percentages (8.3% and 13.3%, respectively). As a consequence, it outperformed the control in terms of seedling survival (78.4%). All treatments examined enhanced plant growth parameters such as plant height (cm) and root length (cm) when compared to the control (no treatment), according to the data (Table 4). There were substantial differences between these treatments and the controls. The best seed soaking treatment overall was TEON, which resulted in higher plant growth parameters (plant height: 30.0 cm & root length: 17.3 cm) than the other treatments, followed by TEOE and Switch fungicide. In comparison to the control, TEOS had the least influence on plant height (27.0 cm) and root length (15.5 cm). The small size of nanoemulsion particles may contribute to its antifungal efficacy (Hassanin *et al.*, 2017). Increasing in plant growth metrics may be owing to biochemical changes in stem base tissues, or they may be related to their efficacy in minimizing or avoiding disease infection and development. Peroxidase enzyme activity, growth hormones, and phenol chemicals all rise as a result of this shift. Hassanin (2013 & 2017), Hammad and Hassanin (2022), and Zedan *et al.* (2011) all observed quite comparable findings on numerous crops cultivated in naturally or purposefully polluted soil.

Table 4. Effect of (TEON, TEOE and TEOS) and the fungicide Switch 62.5%WG on damping-off incidence % and growth of fennel Plants, grown in artificially infested soil, Under greenhouse conditions.

Treatments	Pre-emergence (%)	Post-emergence (%)	Survivals (%)	Plant height (cm)	Root length (cm)
(TEON)	8.3	13.3	78.4	30.0	17.3
(TEOE)	11.7	20.0	68.3	29.2	17.0
(TEOS)	23.3	26.7	50.0	27.0	15.5
Switch	18.3	20.0	61.7	28.3	16.4
Control	38.3	41.7	20.0	21.0	11.2
L.S.D. at 5%	13.4	10.3	19.7	6.5	6.1

Conclusions:

The present investigation evaluated efficacy of TEON, TEOE, & TEOS as antifungal and management Sclerotinia rot disease of fennel compared with fungicide (Switch 62.5% WG). TEON and TEOE were the most effective treatments tested since they gave significant decreases in disease incidence. These results indicate TEON, TEOE possesses strong antifungal properties and also, the possibility of using as an alternative fungicide agent against Sclerotinia rot disease to fennel plants.

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