Fungicidal effect of locally formulated thymol against some plant pathogenic fungi.

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Abstract: Fungicidal effect of thymol (crystal) formulated as Oil in water emulsion (EW) 10 % was assessed against *Rhyzoctonia solani* and *Fusarium moniliforme* (soil borne fungi) compared with fungicide Uniform 390 SE (mefenoxam + azoxystrobin) as well as against *Alternaria solani* (air borne fungus) compared with fungicide Score 250 EC (difenoconazole) using the poisoned food (PF) technique. Thymol EW10%, uniform 390 SE and score 250EC tested at concentrations 6.25, 12.5, 25, 50, 100 and 200 ppm. The tested fungi were inoculated with 5 mm mycelial plugs from 7 days old cultures and incubated at 25 ± 2ºC. The growth of fungal species was recorded then percentage of inhibition was calculated.

Results indicated that the complete inhibition of the mycelial growth of *R. solani* was at 100 ppm with thymol and uniform. EC_{90} values for *R. solani* with thymol EW 10% and Uniform SE 390 were 32.27and 39.29ppm respectively. Also, the EC_{90} values for *F. moniliforme* with thymol and Uniform were recorded at164.1 and 147.8 ppm respectively. While the EC_{90} values for *A. solani* with thymol and Score EC 250 were 52.73and 28.90ppm respectively.

Mycelial growth was considerably reduced by increasing the concentration of thymol. Also the results showed that thymol formulated as 10% EW was more efficient than Uniform against *R. solani* and *F. moniliforme* but less than Score against A. *solani*. Generally *R. solani* was the most sensitive fungus and *F. monliforme* was the less sensitive fungus to thymol.

Keywords: Essential oil, fungicide, EW formulation, thymol.

1.Introduction

Fungi are considered the most important plant pathogen and *Rhizoctonia solani* is a soil borne fungal pathogen causing damping off, root rot and aerial blights of economically important crops, forest trees, ornamentals and turf grasses, as well as decay of post-harvest products.

Soil borne fungal pathogens, including *R. solani* have been traditionally controlled using chemicals, some of which deplete natural resources, inconsistent in efficacy and toxic to the environment. Thus, eco-friendly management using safer chemicals are being increasingly sought to control plant diseases **Kamlesh *et al.*, (2017).**

A. solani and *A. alternate* are the causal agents of early blight, and then are important foliar pathogens of potato worldwide. Susceptibility to infection is primarily determined by the age of the host plant; *A. solani* causes collar rot in tomato seedlings and early blight in mature tomato and potato (**Rotem 1998**).

Many *F. moniliforme* cultures were isolated from corn and rice as human food and animal feeds. Microconidia of this species were formed in a chain and the sexual stage was *Gibberrella moniliforme* (Sheld.) Syn. Commonly, *F. moniliforme* was mostly associated with moldy corn in the country and it was isolated from rice, corn and other cereal grains **Saremi *et al.*, (2011)**.

Corn and rice as other major crops were infested by fusarium species which showed sever damages. These two main crops are the key food for humans and animals so low production due to disease effect have economical problem for producers. Therefore it is necessary to recognize the pathogen and find the best managing way for their control in farming areas. **Singleton, *et al.*, (1992).**

Ear rot of maize is caused by many fungi among which *F. moniliforme* which is the most important. Infestation by *F. moniliforme* occurred several effects such as loss of production in the field, post-harvest losses, mycotoxins in grains and in the subsequent crop, loss of germination, seedling blight, stalk and ear rot disease **Tagne, *et al.*, (2013).**

Management of fungal diseases with conventional fungicides has been erratic due to high genetic variability of pathogens. Also, persistence of many fungicides following use has resulted in pathogen resistance to several fungicides, pollution of the environment, and dangerous accumulation of toxic chemicals in the food chain. Plants are inexhaustible reservoirs of secondary metabolites, some of which are antimicrobial in nature and are believed to function as a natural defense mechanism against pests and pathogens. **Kamlesh (2017).**

Thymol dissipates from soil within 5 days of treatment, also degrades faster; those features make it an ideal fumigant to control soil borne pathogens **Hu, D. and Coats, J. (2008).**

The present study investigated the antifungal effectiveness of thymol formulated as Oil in Water Emulsion (EW) comparing with standards of chemical fungicides against the common soil borne plant pathogenic fungus *R. solani*, and *F. moniliforme,* and air borne fungus *A. solani* with respect to in vitro growth inhibition.

2.Materials and Methods

2.1. Chemicals:

2.1.1. Chemical fungicides.

2.1.1.1. Uniform 390SE (mefenoxam + azoxystrobin).

2.1.1.2.Score 250EC (difenoconazole).

Supplied by Syngenta agro Swiss.

2.1.2. Locally formulated thymol 10EW. It was prepared by the same method mentioned by **Eskander (2018)**

Thymol (crystal) extra pure ($C_{10}H_{14}O$) supplied by EL-Gomhoria Co., Cairo, Egypt.

2.1.3. Surface active agents:

2.1.3.1. Sodium dodecyl sulfate,tween 20 and tween 80 were supplied by EL-Gomhoria Co., Cairo, Egypt.

2.1.3.2. Poly ethylene glycol 600 di-oleate, produced by the National Co., for yeast and detergent, Alexandria, Egypt.

2.1.4.Solvents: Xylene (Dimethyl benzene), acetone and N,N-dimethyl formamide (DMF), Supplied by EL-Nasr pharmaceutical, chemicals co.

2.2.Determination of the physico- chemical properties for the constituents of formulation:

Active ingredient:

2.2.1.Solubility (w/v): Solubility was determined by weighting 0.5 gram from thymol (crystal) and determined the volume for complete solubility from each solvent, distilled water, xylene, acetone and dimethyl formamide (DMF). The % solubility was calculated according to this equation.

% solubility= weight of material/ solvent or water volume x 100.

2.2.2.Free acidity or alkalinity: it was determined according to **CIPAC (2002).**

Surface active agents:

2.2.2.1. Free acidity or alkalinity: it was determined according to **WHO specifications (1979)**

2.2.2.2.Hydrophilic- lipophilic Balance (HLB): the solubility of a surfactant in water can use a guide in approximating their HLB and their usefulness **Lynch and Griffin, (1974)**

2.2.2.3.Critical micelle concentration (CMC):

The concentration of surfactant which no decrease in surface tension obtained by increasing the surfactant concentration. It was determined according to **Osipow, (1964)** Surface tension was measured by Du-Nouy tensiometer for solutions containing 0.1- 1 % surfactant according to **ASTM- 1331**.(2001)

2.3. Preparation of thymol as Oil in water Emulsion (EW) 10%.

Several trials were conducted to prepare thymol as oil in water emulsion (EW) according to **Salvica *et al.*, (2012),** emulation stability ,foam and free acidity or alkalinity were studied before and after storage at 54 ± 2ºC for three days to determine the successful formula according to **CIPAC (2002).**

2.4. Determination the physico- chemical properties of the prepared EW formulation.

The following parameters, emulsion stability, foam, free acidity and alkalinity, stability at 0 ºC. (Cold storage) and Stability at elevated temperature 54 ± 2 ºC (accelerated storage) were measured according to **CIPAC (2002).**

2.5. Determination the physico- chemical properties of the spray solution at the expected application rate (0.5%) of formulated thymol 10% EW.

2.5.1. Surface tension: it was determined as mentioned before.

2.5.2. Viscosity: determined according to **ASTM (2005).**

2.5.3. pH value: it was determined by Cole – Parmer pH/ Conductivity meter 1484-44 according t0 the method of **Dobrat and Martijn, (2002)**

2.5.4.Electrical conductivity: it was determined according to of **Dobrat and Martijn, (2002)**

2.6. Fungal cultures

Phytopathogenic fungi including *A. solani, F. moniliforme* and *R. solani* were supplied from the Department of Fungicides, Bactericides and Nematicides Central Agricultural Pesticides Laboratory.

2.6.1. *In-vitro* antifungal assay:

The antifungal activity against the tested pathogens was determined by the poisoned food (PF) technique as mentioned by **(Grover & Moore, 1962).**

In PF technique 15 ml of Potato Dextrose Agar (PDA) was poured into sterilized Petri dishes (9cm in diameter) and measured the amount of compounds (thyme oil, uniform fungicide and score fungicide) which was added separately to get the required concentrations, 6.25, 12.5, 25, 50, 100 and 200 ppm sterile molten PDA (**Feng & Zheng, 2007**). The tested fungi were inoculated with 5 mm mycelial plugs from 7-days old cultures and incubated at 25 ± 2ºC. Other plates containing free media treatment. Four replicates were used for each treatment. The growth of fungal species was recorded after 3 days for *R. solani* and one week for *F. moniliforme* and *Al. solani* of incubation. Results expressed as percentage inhibition and as effective concentration EC_{50} and EC_{90}.

2.7. Statistical analysis:

Inhibition percentages were corrected using **Abbott s formula (1925),** and the concentration inhibition regression lines were drawn according to the method of **Finney (1952).**

3.RESULTS AND DISCUSSION

3.1. Formulation Part:

3.1.1. Physico- chemical properties of formulation constituents:

3.1.1.1. Active ingredient:

Data in Table (1) indicated that thymol crystals non soluble in water, while, it soluble in xylene, acetone and dimethyl formamide. It has low acidity % 0.098, and low melting point (50 ºC) so it suitable for EW formulation.

Table (1): The physico- chemical properties of the active ingredient (thymol).

% Solubility at 25 ºC (w/v.) in				Free acidity % as H_2SO_4	Melting point ºC
Water	acetone	Xylene	DMF		
N.S	142.85	100	125	0.098	50

3.1.1.2. Surface active agent:

Data in Table (2) showed that sodium dodecyl sulfate soluble in water but it was non soluble in acetone and xylene. PEG 600DO soluble in acetone and xylene, while give emulsion in water, (tween 80 and tween20 soluble in water, acetone and xylene. Surfac-

Table (2): the physico- chemical properties of the suggested surface active agent.

Surfactant	% Solubility at 25 ºC (w/v)			HLB	Free		CMC at 25 ºC %(w/v)	Surface tension at (dyne/cm) 0.5%
	water	acetone	Xylene		Acidity % as H_2SO_4	Alkalinity % as NaOH		
Sodium dodecyl sulfate	36	N/S	N/S	18	-	0.027	0.3	29
PEG 600 di-oleate	Emulsion	24	23.9	10- 12	-	0.3	0.9	39.1
Tween 80	26	58.7	56.3	15	0.06	-	0.5	46.3
Tween 20	42	67.1	61.4	16	0.42	-	0.2	41.2

tant soluble in water such as tween 80 and tween 20 should be used as dispersing agent, while those give emulsion in water and soluble in acetone and xylene should be used as emulsifiers for emulsifiable concentrates.

SDS, tween 80 and tween 20 has high HLB value so it suitable to be used as dispersing and suspending agent, while PEG 600DO had HLB value (10 - 12) suitable to be used as emulsifying agent. Also SDS and PEG 600DO were alkaline and the alkalinity %as NaOH were 0.027 and 0.3 respectively. While tween 80 and tween 20 showed slightly acidic.

PEG 600DO had the height CMC value, followed by tween 80,SDS and tween 20 their CMC values were 0.9, 0.5 ,0.3 and 0.2 %(w/v) respectively. It could be stated that local surfactant proved effectiveness in stabilizing the formed emulsions and diminishing the high surface tension of water **(Eskander, 2008)** SDS was the best one in reducing surface tension of water followed by PEG 600 DO, tween 20 and tween 80.

3.1.2. Preparation of thymol (crystal) as Oil in water Emulsion (EW) 10 %.

The oil-in-water emulsion (EW) was prepared by progressively adding oil phase in water phase under stirring. The oil phase was prepared by dissolving the active ingredients in suitable solvent.

Data in Table (3) indicated that, the prepared EW formulation passed successfully through emulation stability test with hard and soft water at the concentration 5%(v/v), where no oily or cream separation formed, also passed form cold storage 0 ºC± 2 for 24hours, the foam formed was less than WHO specifications. No observable changes in emulsion stability; foam and free acidity of prepared (EW) formulation after accelerated storage at 54 ± 2 ºC for three days only slightly decrease in acidity.

Data in Table (4) indicated that, spray solution of thymol prepared as 10%EW formulation had low surface tension value 32.47 dyne/cm than water and high viscosity value 1.94 cm poise, while it has acidic pH value 5.99. These results agreeable with **Ryckaert *et al.*, (2007)** who indicated that, the reduction in surface tension of spray solution cause a good wettability, spreading and depositing of the particles of the solution on the treated surfaces. Also **(Tawfik and EL-Sisi 1987)** indicated that increase of electrical conductivity and decrease in pH values of pesticidal spray solution would lead to deionization of pesticide and increase its deposit and penetration in the tested surface then will increase the pesticidal efficiency.

3.2. Fungicidal effect:

The antifungal activity of the thymol10%EW against the tested pathogens at different concentrations i.e. 6.25, 12.5, 25, 50, 100 and 200ppm are shown in Tables 5, 6 and 7. Mycelial growth was considerably reduced by increasing the concentration of the thymol 10% EW

Data in Table (5) showed that the thymol 10% EW is a most inhibitory to the mycelial growth of *R. solani* at concentrations (6.25, 12.5, 25 and 50ppm) compared to the fungicide Uniform, since the percent of inhibition was 34.44, 66.66, 86.66 and 93.33 for thymol 10% EW and it was 31.11, 63.33, 84.44 and 91.11for Uniform fungicide.

Complete inhibition of the mycelial growth of *R. solani* was recorded at concentrations 100 and 200ppm for thymol and Uniform fungicide. The EC_{50} values with thymol and Uniform fungicide were 8.77 and 9.67 ppm respectively.

Data also showed that the inhibition of mycelial growth of *R. solani* increased by increasing concentrations of both thymol 10%EW and uniform fungicide. These results in agreement with **Kamlesh *et al.*, (2017)** who reported that, amongst the chemical tested, thymol acetate, thymol benzylether, and thymol showed the highest antifungal activities, demonstrating complete (i.e., 100%) inhibition of fungal growth of *R. solani* at the lowest tested concentration (0.075%) of the chemicals

Data in Table (6) Indicated that the mycelial growth of *F. moniliforme* was differently inhibited on treated medium with thymol 10%EW or Uniform fungicide at different concentrations (6.25: 200ppm).

Table (3): the physico- chemical properties of the prepared Oil in water emulsion (EW).

Type of water	Emulsion stability (ml.cream sep.)	Foam (cm)	Free acidity % as H_2SO_4	Cold storage at 0ºC± 2 for 24hours	Accelerated storage at 54 ± 2ºC for 3 days		
					Emulsion stability (mL.cream sep.)	Foam (cm)	Free acidity % as H_2SO_4
Hard water	0.0	6	0.277	passed	0.0	7	
Soft water	0.0	4			0.0	5	0.19

Table (4): the physico- chemical properties of the prepared EW 10% spray solution at 0.5% concentration

Materials	Surface tension (dyne/cm.) at 25 °C	pH value	Conductivity μ mhos	Viscosity (cm poise)
EW 10%	32.47	5.99	93.4	1.94
Water	72	7.8	396	1.0

Complete inhibition occurred with high concentration (200ppm) for both thymol 10%EW and Uniform fungicide. The inhibition percentages of the thymol 10%EW were 7.77, 21.11, 34.44, 57.77 and 72.22 while the inhibition percentages for Uniform fungicide were 12.22, 24.44, 48.88, 64.44 and 74.44 at concentrations 6.25, 12.5, 25, 50 and 100ppm respectively. The EC_{50} values of thymol 10%EW and uniform fungicide were recorded 36.7 and 29.16 ppm respectively.

Lopez-meneses *et al.,* (2015) reported that, thymus EO at concentrations of 1000 and 2500 μL L–1 totally inhibited the growth of *F. moniliforme* and *Aspergillus parasiticus*, respectively. Eucalyptus and thymus EO significantly reduced spore germination of *A. parasiticus*. Inhibition of spore germination of *F. moniliforme* was 84.6, 34.0, and 30.6% when exposed to eucalyptus, pirul, and thymus EO, respectively. Thymus and eucalyptus EO reduced aflatoxin (4%) and fumonisin (31%) production, respectively. Spore viability was affected when oils concentration increased.

The effect of thymol 10% EW and Score fungicide at their tested concentrations on the linear growth of *A. solani* is shown in Table (7). Results in table (7) stated that, both thymol and Score fungicide reduced linear growth of *A. solani* on PDA medium treated with 100ppm of thymol or Score fungicide, the percentages of inhibition recorded 96.66 and 100 respectively.

A. solani was sensitive to score fungicide at different tested concentrations compared to thymol. Complete inhibition in the linear growth of *A. solani* occurred at concentration 200ppm of thymol 10%EW but the complete inhibition occurred at concentration 100ppm of Score fungicide. The EC_{50} values of thymol 10 %EW and Score fungicide were 14.00and 8.91 ppm respectively.

Ragab, *et al.,* (2016) reported that, fungal mycelial growth of tomato pathogenic fungus *A. solani* decreased significantly as the concentrations of essential oils were increased, to reach the fungal growth's minimum at the highest concentration used. Pepper mint, lemongrass, thyme and sweet basil showed highly effect that they caused complete inhibition to fungal growth at concentration of 2%.

Results obtained about the fungicidal effect of thymol (crystal) prepared as 10 % EW against soil borne and air borne fungus are in consistent with **Lopez-meneses, *et al.,* (2015), Feng and Zheng (2007), Kamlesh *et al.,* (2017) and Ragab *et al.,* (2017)** finding about the fungicidal effect of essential oils against different types of fungus.

The antimicrobial or antifungal activity of essential oil might be caused by the properties of terpenes/terpenoids that due to their highly lipophilic nature and low molecular weight are capable of disrupting the cell membrane, causing cell death or inhibiting the sporulation and germination of food spoilage fungi. **Nazzaro *et al.,* (2017).**

Conclusion

Laboratory fungicidal tests against some fungus infested many crops: soil and air borne fungus indicated that the locally prepared thymol exhibited high fungicidal activity against soil and air borne fungus since it gave 100% growth inhibition of fungus at concentration 200ppm as the same as tested recommended fungicides :Uniform and Score . These results gave a prediction of possibility of using this locally prepared and safe thymol as alternative for conventional fungicides.

Recommendations

Since locally prepared thymol 10%EW showed high fungicidal activity equal to the two recommended fungicide: Uniform and Score especially at concentration 200 ppm. Consequently, further field trials must carry out to evaluate the efficacy of thymol 10% EW as a fungicide under field condition.

Table (5): The inhibition percent, EC_{50}, EC_{90} and slope values for *R. solani* treated with thymol EW 10% and Uniform fungicide.

Rhizoctonia solani								
Conc. ppm	**Thymol EW 10%**				**Uniform 390 SE**			
	%of inhibition	**EC_{50} (ppm)**	**EC_{90} (ppm)**	**Slope**	**%of inhibition**	**EC_{50} (ppm)**	**EC_{90} (ppm)**	**Slope**
200	100	8.77	32.27	2.265± 0.2176	100	9.67	39.29	2.1048± 0.1992
100	100				100			
50	93.33				91.11			
25	86.66				84.44			
12.5	66.66				63.33			
6.25	34.44				31.11			
0	0				0			

Table (6): The inhibition percent, EC_{50}, EC_{90} and slope values for *F. moniliforme* treated with thymol EW 10% and Uniform fungicide.

Fusarium moniliforme								
Conc. ppm	**Thymol EW 10%**				**Uniform 390 SE**			
	%of inhibition	**EC_{50} (ppm)**	**EC_{90} (ppm)**	**Slope**	**%of inhibition**	**EC_{50} (ppm)**	**EC_{90} (ppm)**	**Slope**
200	100	36.7	164.1	1.969± 0.1370	100	29.16	147.8	1.8179± 0.1286
100	72.22				74.44			
50	57.77				64.44			
25	34.44				48.88			
12.5	21.11				24.44			
6.25	7.77				12.22			
0	0				0			

Table (7): The inhibition percent, EC_{50}, EC_{90} and slope values for *A. solani* treated with thymol EW 10% and Score fungicide.

Alternaria solani								
Conc. ppm	**Thymol EW 10 %**				**Score 250 EC**			
	%of inhibition	**EC_{50} (ppm)**	**EC_{90} (ppm)**	**Slope**	**% of inhibition**	**EC_{50} (ppm)**	**EC_{90} (ppm)**	**Slope**
200	100	14.00	52.73	2.2257± 0.1854	100	8.91	28.90	2.5083± 0.2601
100	96.66				100			
50	87.77				95.55			
25	73.33				91.11			
12.5	48.88				61.11			
6.25	18.88				35.55			
0	0				0			

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التاثير الابادي للثيمول المجهز محليا علي بعض الفطريات الممرضة للنبات

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الملخص العربى:

تم دراسة الاثر الابادي للثيمول الذى تم تحضيرة علي صورة مستحلب زيت في ماء(10%) باستخدام بعض المواد النشطة سطحيا. وقد تم قياس الخواص الطبيعية للزيت قبل وبعد التجهيز (اختبار الذوبان , الحموضة والقلوية , ثبات الاستحلاب, التخزين البارد والساخن........) التى اكدت نجاح المستحضر وقابليتة للتطبيق .

اجريت الاختبارات الحيوية لتوضيح الاثر الابادى لهذا المستحضر وكذلك التركيز المثبط لنصف النمو الميسيليومى للفطريات (ريزوكتونيا سولاني, فيوزاريم مونيلوفورم و الترناريا سولانى) حيث استخدم المستحضر فى سلسلة من التركيزات 6.25 – 12.5 – 25 – 50 – 100 - 200 جزء في المليون واستخدمت مبيدات فطرية تجارية (يونيفورم و سكور) للمقارنة وبنفس التركيزات في بيئة بطاطس ديكستروز اجار ضد الفطريات موضع الدراسة مع وجود اطباق بترى تحتوى على بيئة بدون اضافة اى مبيد للمقارنة.

وقد اظهرت النتائج ان مستحضر الثيمول 10% (زيت \ ماء) لة فاعلية ضد الفطريات المختبرة تعادل المبيدات الفطرية الكيميائية وان فطر ريزوكتونيا سولانى كان اكثر استجابة للمستحضر (ثيمول 10% زيت /ماء) بينما اظهر الفطر فيوزاريم مونيلوفورم اقل استجابة لمستحضر الثيمول