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Activity of five natural essential oils against fungal contamination in fresh and stored maize grains

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Abstract Thyme, citral, methyl anthranilate, rosemary and clove essential oils were tested for their antifungal activity to investigate the possibility of their use for maize crop treatment to control seed-borne fungi associated to maize grains as *Fusarium verticillioides*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus, Talaromyces verruculosus* and other species. The efficacy of the five essential oils against natural fungal mycoflora developed on maize crop under naturally infected field conditions at two experiment trials at three storage intervals on two different media. The percentage of seed germination, the percentage of infection and isolation frequency were reported. The five Eos had a significant inhibitory effect against maize grain germination and fungal frequency. Thyme had the least impact on the germination of maize grains, while clove had an obvious effect in reducing the total frequency of fungal isolates associated to maize grains. The five Eos had an obvious inhibitory activity against the frequency of fungal isolates associated with maize grains. These findings imply that EOs, particularly thyme and clove oils, are effective at controlling maize seed-borne fungi.

Keywords: Bio-fungicides, Maize crop, Essential oils, Seed-borne fungi

Introduction

According to Golob *et al*. (2004) and Kyenpia *et al*. (2009), corn, also known as maize (*Zea mays*), is an annual grass in the family *Poaceae* and is the third most widely farmed cereal in the world. It is also the second most important cereal grain grown in Egypt (Anonymous, 2018). With a total production of 1.09 billion metric tonnes in 2018/2019, maize is a major source of income for farmers and is regarded as a staple food crop for many people, especially in underdeveloped nations (Olga and Tibor, 2015; Shahbandeh, 2020).

One of the main challenges towards the production of both high-quality and abundant crops is disease management (Haggag, 2020). An essential method for diseases to spread widely and a means to ensure their survival in nature is the link between seeds and pathogens (Ahmad *et al*., 2016; Özer and Coşkuntuna, 2016). Seedborne pathogens have the potential

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to significantly reduce agricultural yields and cost growers their incomes (Chang *et al*., 2020; Gaur *et al*., 2020; Zhang *et al*., 2020; Zhao *et al*., 2020). Sowing healthy seeds is crucial to enhancing crop yields and food production because about 90% of the world's food crops are grown from seed (Abdulsalaam and Shenge, 2011). The goal of integrated pest management approaches is to offer seedborne disease management options that are both commercially viable and environment friendly (Perczak *et al*., 2020). These tactics are required to reduce the potential pathogen inoculum on seeds, and they rely on management tools that are either already available to farmers or will be in the near future (Thomas-Sharma *et al*., 2017).

Essential oils (EOs) have been shown to be effective *in vitro* against bacterial and fungal plant diseases in a number of investigations (Moumni *et al*., 2021; Al-Ansary *et al*., 2022; Al-Ansary, 2023). Few studies, however, Della Pepa *et al*. (2019) and Riccioni *et al*. (2019) have concentrated on the *in vivo* effects of essential oils against seedborne fungus.

Many investigations have indicated that EOs have antifungal activities that are effective against phytopathogenic fungi. The fungicidal assays of EOs employ a variety of techniques that can be evaluated *in vivo* or *in vitro*. The data are often presented as zone of inhibition (ZOI), minimal inhibitory concentration (MIC), half maximum inhibitory concentration (IC50), and minimal fungicidal concentration (MFC) (Shuping and Eloff, 2017).

The present study was conducted to evaluate the bio-fungicides activity of five tested essential oils against the natural occurrence of fungal contamination in fresh and stored treated maize grains.

Materials and methods

Essential oils

The ready-to-use five essential oils Thyme, Methyl anthranilate, Citral, Rosemary and Clove were obtained from the Pressing and Extraction of Natural Oils Unit, National Research Centre.

Preharvest foliar spraying treatments

The efficacies of liquid formulation of safe techniques against natural infection of maize crop were conducted during season 2020 under natural infection conditions. Two experiments were performed at Qalyubia governorate; El Qanater El Khayrya and Toukh. The field experiments were arranged in a randomized complete block design with five replications for each treatment.

Spraying treatments were started after about 45 days after planting (two weeks before silking stage) then repeated three times with two weeks intervals. After the end of the growing season, the cobs were collected, dried, and then stored for six months. The percentage of seed germination, the percentage of infection and the percentage of isolation frequency were calculated at zero time, after three months and after six months.

Percentage of grain germination

The percentage (%) of the grain germination was calculated by using the following formula according to Tsedaley and Adugna (2016).

G % = Number of germinated grains / Total number of plated grains ×100 where: G= Grain germination

Total frequency of fungal isolates

Fungal infection associated with maize grains, defined as the percent of fungi-yielding grains was calculated and expressed as percent by using the following formula according to Tsedaley and Adugna (2016).

Infection (%) = number of infected grain/ total number of grain x 100

Frequency of fungal isolates associated with grains

The frequency of the inhibition of each fungus isolated from maize grains was calculated and expressed as percent by using the following formula according to Tsedaley and Adugna (2016).

Fungal isolates Frequency (%) = Number of occurrence of fungus species/ total number of isolated fungi x 100.

Statistical analysis

Data were statistically computed and presented as means and standard errors using (SAS, 2006). Comparisons were made using Duncan's Multiple Range Test (Duncan, 1955).

Results

Evaluation of some essential oils in El Qanater El Khayrya and Toukh

The effect of thyme, methyl anthranilate, citral, rosemary and clove on percentage of germinated grains, and fungal total frequency associated with maize grains, and the total frequency of associated fungi isolated from maize grains were determined in two experiment sites under naturally infected field conditions at the early stage of cob storage on two different media (0,3 and 6 months).

Percentage of grain germination

Results revealed that thyme, methyl anthranilate and rosemary increased significantly the percent of germinated grains (100.0%) compared to untreated maize crop (control) 89.83%, in El Qanater El Khayrya, while in Toukh, all essential oils influenced significantly the percent of germinated grains ranging 91.20 – 100.0% in compared with control (90.17%). Thyme and methyl anthranilate were the most significant treatments as they increased percentage of grain germination to 100.0%, in addition to rosemary (100%) as seen in Table 1.

Total frequency of fungal isolates

The fungal isolation on potato dextrose agar medium showed that all of essential oils influenced significantly the frequency of fungal isolates grains ranging 29.67 – 40.0% in compared with control (100.0%). Thyme was the most significant treatment as it reduced the frequency of fungal isolates to (29.67%) in comparison with the control followed by rosemary (30.0%). On the other hand, results of the fungal isolation on malt salt agar medium indicated that all of the essential oils decreased significantly the percent of germinated grains (10.0 – 50.33%) compared to untreated maize crop (control) 96.33%, as same as the experiment in the first site. Thyme was the most effective essential oils as it reduced the frequency of fungal isolates associated with maize grains significantly to 10.0%, while methyl anthranilate (50.33%) was the least effective treatment if compared to the control in El Qanater El Khayrya.

In Toukh, results of isolation on potato dextrose agar medium indicated that all of the essential oils used significantly reduced the percent of fungal isolates associated with treated maize grains (25.67 – 60.0%) compared to untreated maize crop (control) 100.0%. Thyme was the most effective treatment in reducing the frequency of fungal isolates significantly with the value 25.67%, while methyl anthranilate showed least significant efficacy (60.0%) in comparison with the control. On the other hand, frequency of isolates which isolated on malt sat agar medium were reduced with all of the essential oils used significantly (16.0 – 43.67%) compared to untreated maize crop (control) 74.0%, the most reduction in percentage of fungal isolates was when citral (16.0%) applied if compared with control, followed by thyme (20.0%). Clove was the less significant treatment (43.67%) according to Table 1.

Table 1. Heatmap showing percentage of infected germinated and infected maize seeds (at zero time) as affected with pre-harvest treatments application under field conditions in El Qanater El Khayrya and Toukh, Qalyubia Governorate

	El Qanater El Khayrya				Toukh				
	PDA		MSA		PDA		MSA		
	Germinated seeds %	Infected seeds %	Germinated seeds %	Infected seeds %	Germinated seeds %	Infected seeds %	Germinated seeds %	Infected seeds %	Effective concentration (ppm)
Thyme	100.0	29.7	0.0	10.0	100.0	25.7	0.0	20.0	0
Methyl anthranilate	100.0	34.3	0.0	50.3	100.0	60.0	0.0	26.0	10
Citral	90.2	32.2	0.0	33.1	91.2	42.0	0.0	16.0	20
Rosemary	100.0	30.0	0.0	36.0	95.2	37.8	0.0	38.0	30
Clove	90.3	40.0	0.0	46.0	93.7	40.0	0.0	43.7	40
Control	89.3	100.0	0.0	96.1	89.3	100.0	0.0	74.0	50
									60
									70
									80
									90
									100

Frequency of fungal isolates associated with grains

The frequency of most identified species in the control sample in El Qanater El Khayrya which isolated on potato dextrose agar medium, were *Fusarium verticillioides* (66.8%), followed by *Talaromyces verruculosus* (12.7%), *Aspergillus flavus* (6.7%) *Aspergillus niger* (7.1%), *Fusarium* spp. (4.4%), and other species (2.3%) were represented by frequency values smaller than two species reported previously (Table 2). While the frequency of isolated fungi associated with treated maize grains which isolated on malt salt agar medium were as follows: *F. verticillioides* (80.0%), *A. flavus* (13.3%) and *T. verruculosus* (6.7%) (Table 3).

Thyme was the most effective inhibitor; it significantly inhibited the frequency of *A. flavus* (16.4%), on the other hand methyl anthranilate was the best treatment for *F. verticillioides* frequency reduction (24.7%) at zero time of maize grains storage in El Qanater El Khayrya. In Toukh, citral was more successful in completely inhibited the frequency of *A. flavus,* while thyme inhibited significantly the frequency of *F. verticillioides* (45.17%) (Table 2).

In El Qanater El Khayrya, frequency of isolated fungi associated with treated maize grains which isolated on malt salt agar medium were as follows: F. verticillioides (80.0%), A. flavus (13.1%) and T. verruculosus (6.9%) as recorded in Table 2. In Toukh and on malt salt agar medium, frequency of isolated fungi differed as F. verticillioides (55.4%), A. niger (16.0%), T. verruculosus (11.7%), and A. flavus (9.1%), while Aspergillus terreus (2.2%) and other species (3.0%) were noticed with values of frequency smaller than previous genera reported in Table 3.

The results indicated that foliar application of thyme and clove was significantly affected ($P < 0.001$) in completely inhibiting the frequency of both A. flavus and F. verticillioides when compared with the control (untreated maize crop grains) at zero time of maize grains storage in El Qanater El Khayrya (Table. 3). While in Toukh, citral was more successful in the complete inhibition of the frequency of A. flavus and thyme was the most effective essential oil in reducing the frequency of F. verticillioides (45.17%) (Table3).

After three months of storage

Percentage of grain germination

Results revealed that thyme was the most effective essential oil as it increased significantly the percent of germinated grains (100.0, 100.0%) compared to untreated maize crop (control) 96.70 and 96.70% respectively, while methyl anthranilate had not significantly affected on the grain germination with value 95.57%. On the other hand, citral and clove (90.00, 91.00%) were decreased significantly in percentage of grain germination in El Qanater El Khayrya (Table 4). Methyl anthranilate and citral were increased significantly the grain germination percentage (99.33, 99.3% respectively) in Toukh (Table 4). Rosemary was the treatment with the most significant effect on decreasing the percentage of grain germination in El Qanater El Khayrya (68.90%) and Toukh (93.67%).

Total frequency of fungal isolates

The fungal isolation on potato dextrose agar medium showed that all of essential oils influenced significantly the percent of frequency of fungal isolates ranging 38.90 – 96.0% as compared with control (100.0%). Rosemary was the most significant treatments as it reduced the frequency of fungal isolates to (38.90%) in comparison with the control, while there were no significant differences between thyme, methyl anthranilate and citral as they were the least effective treatments in fungal isolates frequency reduction (96.0%). On the other hand, results of the fungal isolation on malt salt agar medium indicated that all the essential oils decreased significantly the percent of germinated grains (10.0 – 94.23%) compared to untreated maize crop (control) 100.0%, clove was the most effective essential oils as it reduced the frequency of fungal isolates associated with maize grains significantly to 10.0%. As for thyme, methyl anthranilate and citral showed the same less significant efficiency (92.0, 94.23, 94.23% respectively) in El Qanater El Khayrya.

Table 2. Heatmap showing frequency of isolated fungi of maize seeds (at zero time) on PDA as affected with treatments as preharvest applications under field conditions in El Qanater El Khayrya and Toukh Qalyubia Governorate

	Frequency % of isolated fungi on PDA																Effective concentration (ppm)
	El Qanater El Khayrya								Toukh								
	A. flavus	*A. niger*	*A. terreus*	*F. verticillioides*	*Fusarium sp.*	*T. verruculosus*	*Alternaria sp.*	Other	*A. flavus*	*A. niger*	*A. terreus*	*F. verticillioides*	*Fusarium sp.*	*T. verruculosus*	*Alternaria sp.*	Other	
Thyme	16.4	16.4	0.0	50.7	0.0	16.5	0.0	0.0	11.0	16.4	0.0	45.2	0.0	11.0	30.0	3.0	0.0
Methyl anthranilate	72.5	2.8	0.0	24.7	0.0	0.0	0.0	0.0	6.6	2.8	0.0	83.7	0.0	6.3	0.0	3.0	10.0
Citral	18.1	9.1	0.0	53.4	0.0	18.1	0.0	1.2	0.0	9.1	0.0	89.8	0.0	10.0	0.0	0.0	20.0
Rosemary	10.3	11.4	0.0	60.8	1.2	14.2	0.0	2.1	8.1	11.4	0.0	63.7	0.0	9.8	15.5	2.7	30.0
Clove	12.3	10.2	0.0	58.7	0.0	17.3	0.0	1.5	7.4	10.2	0.0	75.6	0.0	8.4	6.3	2.4	40.0
Control	6.7	7.1	0.0	66.8	4.4	12.7	0.0	2.3	9.1	15.6	2.2	54.0	0.0	11.7	4.4	3.0	50.0
																	60.0
																	70.0
																	80.0
																	90.0
																	100.0

Table 3. Heatmap showing frequency of isolated fungi of maize seeds (at zero time) on MSA as affected with treatments as preharvest applications under field conditions in El Qanater El Khayrya and Toukh Qalyubia Governorate

	Frequency % of isolated fungi on MSA																
	El Qanater El Khayrya								Toukh								
	A. flavus	*A. niger*	*A. terreus*	*F. verticillioides*	*Fusarium sp.*	*T. verruculosus*	*Alternaria sp.*	Other	*A. flavus*	*A. niger*	*A. terreus*	*F. verticillioides*	*Fusarium sp.*	*T. verruculosus*	*Alternaria sp.*	Other	Effective concentration (ppm)
Thyme	0.0	0.0	0.0	0.0	0.0	97.3	0.0	2.8	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0
Methyl anthranilate	11.1	9.5	0.0	58.5	0.0	7.5	11.1	2.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0
Citral	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	20.0
Rosemary	6.0	0.0	0.0	52.3	0.0	10.0	29.8	2.0	10.0	14.8	0.0	69.8	0.0	4.0	0.0	1.5	30.0
Clove	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	50.0	0.0	0.0	0.0	0.0	50.0	0.0	0.0	40.0
Control	13.3	0.0	0.0	80.0	0.0	6.7	0.0	0.0	6.8	9.5	0.0	67.9	0.0	12.8	0.0	3.0	50.0
																	60.0
																	70.0
																	80.0
																	90.0
																	100.0

In Toukh, results of isolation on potato dextrose agar medium indicated that all of the essential oils were significantly reduced the percent of fungal isolates associated with treated maize grains (13.30 – 23.97%) as compared to untreated maize crop (control) 100.0% (Table 4). Citral, thyme and rosemary were the most effective treatments in reducing the frequency of fungal isolates significantly with the values of 13.30, 13.53 and 13.53%. Methyl anthranilate showed least significant efficacy (23.97%) in comparison with the control. On the other hand, frequency of isolates which isolated on malt sat agar medium were reduced with all of the essential oils significantly (3.30 – 16.13%) compared to untreated maize crop (control) 48.90%. The most reduction in percentage of fungal isolates was when clove (3.30%) applied as compared with control. Methyl anthranilate was the less significant treatment (16.13%).

Table 4. Heatmap showing percentage of infected germinated and infected maize seeds (after three months) as affected with pre-harvest treatments application under field conditions in El Qanater El Khayrya and Toukh, Qalyubia Governorate

	El Qanater El Khayrya				**Toukh**			
	PDA		**MSA**		**PDA**		**MSA**	
	Germinated seeds %	**Infected seeds %**	**Germinated seeds**	**Infected seeds %**	**Germinated seeds %**	**Infected seeds %**	**Germinated seeds %**	**Infected seeds %**
Thyme	100.0	96.0	0.0	92.0	100.0	13.5	0.0	10.0
Methyl anthranilate	95.6	96.0	0.0	94.2	99.3	24.0	0.0	16.1
Citral	90.0	96.0	0.0	94.2	99.3	13.3	0.0	10.0
Rosemary	68.9	38.9	0.0	13.3	93.7	13.5	0.0	5.4
Clove	91.0	61.1	0.0	10.0	98.0	23.0	0.0	3.3
Control	96.7	100.0	0.0	100.0	96.7	100.0	0.0	48.9

Effective concentratio (ppm)
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
100.0

Table 5. Heatmap showing frequency of isolated fungi of maize seeds (after three months) on PDA as affected with treatments as preharvest applications under field conditions in El Qanater El Khayrya and Toukh Qalyubia Governorate

	Frequency % of isolated fungi on PDA															
	El Qanater El Khayrya								**Toukh**							
	A. flavus	***A. niger***	***A. terreus***	***F. verticillioides***	***Fusarium sp.***	***T. verruculosus***	***Alternaria sp.***	**Other**	***A. flavus***	***A. niger***	***A. terreus***	***F. verticillioides***	***Fusarium sp.***	***T. verruculosus***	***Alternaria sp.***	**Other**
Thyme	77.0	12.9	0.0	7.1	0.0	0.0	0.0	3.0	0.0	50.0	0.0	0.0	0.0	46.7	0.0	3.0
Methyl anthranilate	70.7	11.7	0.0	14.7	0.0	0.0	0.0	2.9	0.0	0.0	0.0	64.5	0.0	32.8	0.0	2.8
Citral	13.6	81.0	0.0	5.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	50.0	0.0	50.0	0.0	0.0
Rosemary	50.0	50.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
Clove	44.6	22.3	0.0	33.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	97.0	0.0	3.0
Control	46.7	8.1	0.0	34.0	0.0	8.1	0.0	3.0	6.5	6.5	0.0	74.0	0.0	0.0	10.0	3.0

Effective concentration (ppm)
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
100.0

Table 6. Heatmap showing frequency of isolated fungi of maize seeds (after three months) on MSA as affected with treatments as preharvest applications under field conditions in El Qanater El Khayrya and Toukh Qalyubia Governorate

	Frequency % of isolated fungi on MSA															
	El Qanater El Khayrya								**Toukh**							
	A. flavus	***A. niger***	***A. terreus***	***F. verticillioides***	***Fusarium sp.***	***T. verruculosus***	***Alternaria sp.***	**Other**	***A. flavus***	***A. niger***	***A. terreus***	***F. verticillioides***	***Fusarium sp.***	***T. verruculosus***	***Alternaria sp.***	**Other**
Thyme	37.6	55.0	0.0	7.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
Methyl anthranilate	89.6	0.0	0.0	0.0	0.0	10.4	0.0	0.0	19.8	0.0	0.0	0.0	0.0	80.3	0.0	0.0
Citral	6.1	87.0	0.0	0.0	0.0	6.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	97.0	0.0	3.0
Rosemary	25.0	75.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
Clove	22.2	77.9	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Control	56.4	10.0	0.0	33.6	0.0	0.0	0.0	0.0	9.2	16.0	2.2	55.4	0.0	14.2	0.0	3.0

Effective concentration (ppm)
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
100.0

Frequency of fungal isolates associated with grains

The frequency of most identified species in the control sample in El Qanater El Khayrya which isolated on PDA were *A. flavus* (46.7%), *F. verticillioides* (34.0%), *A. niger* and *T. verruculosus* frequency of occurrence of 8.1% (Table 5). While in Toukh, the frequency of isolated fungi associated with treated maize grains which isolated on PDA were *F. verticillioides* (74.0%), *Alternaria* sp. (10.0%), *A. flavus* (6.5%), *A. niger* (6.5%) and other species (3.0%) were recorded with frequency values smaller than previous species (Table 6).

As comparison to control group without EOs treatment which showed significantly inhibition in the frequency of *A. flavus* (13.6%) when treated with citral, and *F. verticillioides* (0.0%) treated with rosemary in El Qanater El Khayry. While in Toukh, all essential oils were significantly affected in the complete inhibition of the frequency of *A. flavus,* while clove was the effective treatment for the inhibition of *F. verticillioides* (Table 5).

In El Qanater El Khayrya the frequency of isolated fungi associated with treated maize grains which isolated on MSA were as follows: *A. flavus* (56.4%), *F. verticillioides* (33.6%) and *A. niger* (10.0%) (Table5). While in Toukh and on MSA, frequency of isolated fungi differed as seen in F. *verticillioides* (55.4%), *A. niger* (16.0%), *T. verruculosus* (11.7%), and *A. flavus* (9.2%), *A. terreus* (2.2%) and other species (3.0%) (Table 6).

Among the plants treated with essential oils showed that only citral was the most efficient treatment in reducing the frequency of A. flavus, while all the essential oils were effective in inhibiting the frequency of F. verticillioides as compared to the control in El Qanater El Khayrya (Table 6). On the other hand, thyme, citral and rosemary were the best treatments in inhibiting frequency of *A. flavus* and *F. verticillioides* in Toukh.

After six months of storage

Percentage of grain germination

Results in El Qanater El Khayrya revealed that thyme and methyl anthranilate increased significantly in percentage of germinated grains (100.0%) as compared to untreated maize crop (control) 96.57%, while rosemary (72%) had significantly affected on reducing the percentage of grain germination (Table 7). While in Toukh, thyme and citral were influenced significantly in percentage of germinated grains with value 100.0, 99.33% as compared with control (98.90%) (Table 7).

Total frequency of fungal isolates

The fungal isolation on PDA showed that all essential oils influenced significantly the frequency of fungal isolates ranging 53.30 – 95.0% (100.0%) as seen in Table 7. Rosemary was the most significant treatments as it reduced the frequency of fungal isolates to (53.30%) in comparison with the control, while methyl anthranilate was the least effective treatment in fungal isolates frequency reduction (95.0%). On the other hand, results of the fungal isolation on MSA indicated that all the essential oils decreased significantly the percentage of germinated grains (20.33 – 89.0%) as compared to untreated maize crop (control) 96.70%, except citral (98.90%). The clove was the most effective essential oils as it reduced the frequency of fungal isolates associated with maize grains significantly to 20.33%. As for methyl anthranilate and thyme showed the same less significant efficiency (87.80, 89.0% respectively) in El Qanater El Khayrya.

Table 7. Heatmap showing percentage of infected germinated and infected maize seeds (after six months) as affected with pre-harvest treatments application under field conditions in El Qanater El Khayrya and Toukh, Qalyubia Governorate

	El Qanater El Khayrya				Toukh			
	PDA		MSA		PDA		MSA	
	Germinated seeds %	Infected seeds %	Germinated seeds %	Infected seeds %	Germinated seeds %	Infected seeds %	Germinated seeds %	Infected seeds %
Thyme	100.0	100.0	0.0	89.0	100.0	40.0	0.0	18.8
Methyl anthranilate	100.0	95.0	0.0	87.8	98.9	56.1	0.0	26.0
Citral	92.0	99.0	0.0	98.9	99.3	30.0	0.0	18.0
Rosemary	72.0	53.3	0.0	23.3	96.7	55.8	0.0	16.6
Clove	90.0	70.8	0.0	20.3	96.8	28.3	0.0	5.8
Control	96.6	100.0	0.0	96.7	98.9	93.3	0.0	43.3

Effective concentration (ppm)
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
100.0

In Toukh, results of isolation on PDA indicated that all the essential oils were significantly reduced the percentage of fungal isolates associated with treated maize grains (28.33 – 56.13%) as compared to untreated maize crop (control) 93.30% (Table 7). Both clove and citral were the most effective treatments in reducing the frequency of fungal isolates significantly with the values of 28.33 and 30.0%. Rosemary and methyl anthranilate were the least significant treatments (55.80, 56.13%). On the other hand, frequency of isolates which isolated on MSA were reduced with all the essential oils were significantly (5.80 – 26.0%) compared to untreated maize crop (control) of 43.30%. The most reduction in percentage of fungal isolates was shown when clove (5.80%) was applied as compared with control. Methyl anthranilate was the less significant treatment (26.0%).

Frequency of fungal isolates associated with grains

The frequency of most identified genera in the control sample in El Qanater El Khayrya which isolated on PDA were *A. flavus* (44.3%), *F. verticillioides* (34.4%), *A. flavus* (16.3%), *T. verruculosus* (6.1%) and *A. niger* (4.6%) which were represented by frequency values smaller than two genera reported previously in Figure 8. While in Toukh and on PDA, *F. verticillioides* (41.7%) and *A. flavus* (31.6), *A. niger* (14.7%) and *T. verruculosus* (12.2%) were recorded in Table 9.

Thyme, methyl anthranilate, rosemary and clove were the most effective inhibitors. They were completely inhibited the frequency of *F. verticillioides* frequency after six months of maize grains storage in El Qanater El Khayrya (Table 8). In Toukh, thyme was more successful in completely inhibiting the frequency of *A. flavus,* and *F. verticillioides.*

In El Qanater El Khayrya, the frequency of isolated fungi associated with treated maize grains which isolated on MSA were as follows: *A. flavus* (75.8%), *A. niger* (9.1%) and *T. verruculosus* (5.1%) as shown in Table 8. While In Toukh and on MSA, frequency of isolated fungi differed as *A. niger* and *T. verruculosus* (40.0%) which isolated with the higher frequency, while *A. niger* (13.3%) and *A. flavus* (6.7%) were noticed with values of frequency smaller than previous species. The results indicated that foliar application of citral was significantly effective ($P < 0.001$) when compared with the control (untreated maize crop grains) against frequency of *A. flavus* at zero time of maize grains storage in El Qanater El Khayrya. While in Toukh, Thyme, methyl and citral were more successful in inhibiting the frequency of *A. flavus* (Table 9).

Table 8. Heatmap showing frequency of isolated fungi of maize seeds (after six months) on PDA as affected with treatments as preharvest applications under field conditions in El Qanater El Khayrya and Toukh Qalyubia Governorate

	Frequency % of isolated fungi on PDA															
	El Qanater El Khayrya								**Toukh**							
	A. flavus	***A. niger***	***A. terreus***	***F. verticillioides***	***Fusarium sp.***	***T. verruculosus***	***Alternaria sp.***	**Other**	***A. flavus***	***A. niger***	***A. terreus***	***F. verticillioides***	***Fusarium sp.***	***T. verruculosus***	***Alternaria sp.***	**Other**
Thyme	72.7	27.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	83.5	0.0	0.0	0.0	16.6	0.0	0.0
Methyl anthranilate	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	53.0	35.0	0.0	0.0	0.0	11.8	0.0	0.0
Citral	67.8	13.0	0.0	9.6	0.0	9.6	0.0	0.0	0.0	44.4	0.0	55.6	0.0	0.0	0.0	0.0
Rosemary	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	60.0	40.0	0.0	0.0	0.0	0.0	0.0	0.0
Clove	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	75.0	0.0	0.0	12.5	0.0	12.5	0.0	0.0
Control	44.3	4.6	0.0	34.4	0.0	16.3	0.0	0.0	31.6	14.7	0.0	41.7	0.0	12.2	0.0	0.0

Effective concentration (ppm)
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
100.0

Table 9. Heatmap showing frequency of isolated fungi of maize seeds (after six months) on MSA as affected with treatments as preharvest applications under field conditions in El Qanater El Khayrya and Toukh Qalyubia Governorate

	Frequency % of isolated fungi on MSA															
	El Qanater El Khayrya								**Toukh**							
	A. flavus	***A. niger***	***A. terreus***	***F. verticillioides***	***Fusarium sp.***	***T. verruculosus***	***Alternaria sp.***	**Other**	***A. flavus***	***A. niger***	***A. terreus***	***F. verticillioides***	***Fusarium sp.***	***T. verruculosus***	***Alternaria sp.***	**Other**
Thyme	77.7	18.7	0.0	0.0	0.0	3.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
Methyl anthranilate	77.2	19.1	0.0	0.0	0.0	3.8	0.0	0.0	0.0	50.0	0.0	0.0	0.0	50.0	0.0	0.0
Citral	70.0	30.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0
Rosemary	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	40.0	60.0	0.0	0.0	0.0	0.0	0.0	0.0
Clove	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Control	75.8	9.1	0.0	0.0	0.0	5.1	0.0	0.0	6.7	13.3	40.0	0.0	0.0	40.0	0.0	0.0

Effective concentration (ppm)
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
100.0

Discussion

The key management method for the development of healthy plant populations and for good harvest yields is typically recommended to use of pathogen-free seeds (Thomas-Sharma *et al*., 2017; Gaur *et al*., 2020). For the control of seed-borne diseases, integrated pest management techniques can offer more economically viable and environmentally responsible alternatives. These methods rely on management tools that have already accessible to farmers or may be used soon (Thomas-Sharma *et al*., 2017). The efficiency of foliar applications with essential oils against maize seed-borne fungi is still unknown. In the present study, five essential oils were shown to be effective against the associated seedborne fungi when applied to maize crop that was naturally infected.

Our result showed that grains of maize crop treated with tested essential oils were significantly affected the percentage of germinated grains. Thyme had the least impact on the germination of maize grains, while clove had the biggest impact. The use of EOs had an impact on grain germination, entirely inhibiting it or greatly reducing germination rate. The frequency of associated fungi isolated from maize grains or the frequency of all fungal isolates, on the other hand, is greatly influenced by EO concentrations. These findings agreed with those reported by Karaca *et al*. (2017) as it revealed that the oils had different impacts on the germination rates of wheat seeds. The most effective oils were clove and oregano, which completely prevented seed germination (Karaca *et al*., 2017). This finding was consistent with prior work that showed EOs greatly hindered kernel germination. The largest effect on the inhibition of total frequency of fungal isolates was confirmed at by clove EOs, either in volatile or carrier contact assay (Al-Ansary *et al.,* 2022). They significantly and unacceptably decreased the germination of treated kernels (<60%) (Karaca *et al*., 2017). Van der Wolf *et al*. (2008) demonstrated that thyme, oregano, cinnamon, and clove essential oils were significantly reduced the growth of fungi on cabbage seeds. Here, all the treated maize crop's grains exhibited a typical germination rate of 70–100%. It demonstrated that none of these five essential oils are phytotoxic to the germination of maize grains. Additionally, Orzali *et al*. (2020) revealed that the germination rates of tomato seeds were unaffected by an essential oil of *Origanum vulgare*. Additionally, while the essential oils considerably reduced the contamination of the grains in this instance. It did not significantly decrease in germination. For these reasons, this essential oil was tested the germination of the maize crop grains and the disease incidence for maize crop in the last phases of the previous study (Al-Ansary *et al*., 2022).

Clove was the most significant treatment as it reduced the frequency of fungal isolates to in both trials along the storage period. Wang *et al*. (2019) revealed that the complex essential oils (0.02%) significantly inhibited the total fungal counts and against fungi in stored maize. The oils had different impacts on the fungal load of wheat seeds. The most effective oil was clove which completely prevented fungal growth on wheat seeds

(Karaca *et al*., 2017). The largest dose of mint oil was completely prevented fungal growth and seed germination in wheat seeds, but it had no effect on fungi that were presented in the seeds (Karaca *et al*., 2017). According to Somda *et al*. (2007) who stated that *C. citratus* at a concentration of 6% was successful prevented *F. moniliforme* and other diseases from infecting sorghum seeds. Other researchers' work is shown that using essential oils can significantly slow the growth of infections belonging to the *Fusarium* sp. (Perczak *et al*., 2019). Significantly reducing the growth of the examined pathogens was made possible by clove essential oil.

All the tested essential oils revealed that thyme, citral, methyl anthranilate, rosemary and clove were significantly effective inhibitors as they were successfully inhibited the frequency of both *Aspergillus flavus* and *Fusarium verticillioides.* These results were in the same trend with the result illustrated by Wang *et al*. (2019) that toxic species of *Aspergillus* was inhibited because of the complex essential oils. This supported the conclusions of research conducted by us and other researchers (Velluti *et al*., 2003; Marín *et al*., 2004; Bluma and Etcheverry, 2008; Cvek *et al*., 2010, Passone *et al*., 2012; Yuan *et al*., 2013; Wang *et al*., 2019). Numerous earlier research, using various laboratory media and plant materials revealed that antifungal activities of essential oils toward *Fusarium* spp. (Matusinsky *et al*., 2015; Kumar *et al*., 2016; Ferreira *et al*., 2018). Essential oils had significant ability to suppress the growth of *Fusarium* infection on maize seeds according to Perczak *et al*. (2019).

Moghtader *et al*. (2011) found that rosemary essential oil had significant antifungal activity on *A. flavus*. Since the monoterpens combination had been shown to be antifungal effects (Okamura *et al*., 1994), the antifungal activities of rosemary essential oil can be attributed to it. Oluwatuyi *et al*. (2004) and Peng *et al*. (2005) who reported similar outcomes. The primary ingredients of rosemary extract and essential oil are differed. The primary components of rosemary essential oil were identified by GC-MS analysis as being eucalyptol (1,8-cineole) (24.02%), pinene (20.06%) and isoborneol (15.84%) (Ceylan *et al*., 2022). The chemical composition of essential oils which determined their effects is controlled by a variety of variables, including plant genotype, geographic location, environmental factors, and agronomic circumstances (Yeşil Çeliktaş *et al*., 2007). The antimicrobial properties of essential oils in this context determined the compounds' content, structure and functional groups (Omidbeygi *et al*., 2007). The biological effects of rosemary include antifungal, antimicrobial, antioxidant and antiviral properties. According to recently report, *R. officinalis* greatly reduced the growth of *F. verticillioides* (da Silva Bomfim *et al*., 2015). Another investigation was confirmed *R. officinalis* prevented *F. verticillioides* from growing and producing conidia (Achimón *et al*., 2021). Waithaka *et al*. (2007) stated that the inhibition of the fungal pathogens by EOs obtained from rosemary and eucalyptus disagree with a previous study by Vignesh *et al*. (2016).

According to Brügger *et al*. (2017) reportet their commercial *C. citratus* essential oil is also included significant levels of nonan-4-ol (6.5%)

and camphene (5.2%), in addition to the high proportion of citral. A Brazilian commercial *C. citratus* essential oil revealed a unique composition that was high in nonterpenes and the amount of citral was less than 37% (Macedo *et al*., 2019). Other fungi, such as *Aspergillus flavus*, had been shown to be resistant to the antifungal effects of *C. citratus* essential oil. Citral, geraniol, and myrcene are only a few of the components that contribute to this activity (Sonker *et al*., 2014; Supardan *et al*., 2019). The mycelial growth of *Fusarium oxysporum, Colletotrichum gloeosporioides, Bipolaris* sp and *A. alternata* can be effectively inhibited by citral and geranol according to several research (Kishore *et al*., 2007; Dalcin *et al*., 2017). These *C. citratus* essential oil's main constituents are shown to be antioxidant and antibacterial properties (Farias *et al*., 2019). Additionally, Kurita *et al*. (1981) explained that citral's ability to take electrons from the fungal cell via charge transfer with an electron donor in the cell that causes the fungi to die.

In conclusion, our study is clearly demonstrated that essential oils of thyme, methyl anthranilate, citral, rosemary, and clove showed antifungal activity against fungal isolates associated with maize grains and can be used as potential bio-fungicides to prevent and control spread of seedborne diseases in commercially produced seeds, instead of synthetic fungicides. Therefore, moving forward with developing strategies for an integrated pest control program is made possible by the findings of this study on the maize seedborne fungi.

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