International Journal of Agricultural Technology 2022Vol. 18(5):1897-1916
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Evaluation of antifungal activity of some natural essential oils against fungal pathogens associated with maize grains

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Al-Ansary, N. A., Haggag, W. M. and Ali, M. K. (2022). Evaluation of antifungal activity of some natural essential oils against fungal pathogens associated with maize grains. International Journal of Agricultural Technology 18(5):1897-1916.

Abstract Mycotoxin-producing moulds which considered as common maize grains contaminants are presented in the genera *Fusarium*, *Aspergillus* and *Penicillium*. One of the natural and safe ways to protect grains from mould contamination is the use of essential oils. The antifungal activity of thyme, citral, methyl anthranilate, rosemary and clove, essential oils (EOs) against *Fusarium. moniliforme*, *Aspergillus niger*, *Penicillium* sp., *Aspergillus flavus* and *Aspergillus terreus* was evaluated in direct contact assay. All EOs showed significant impact on mycelial growth and spore germination inhibition. Thyme showed the best inhibitory effect while, methyl anthranilate and rosemary were the most effective EOs for spore germination inhibition. The effect of EOs added to grains in different application methods (volatile and carrier contact assays) were evaluated. In volatile assay, frequency of *F. moniliforme* and *A. niger* isolated from maize grains was greatly inhibited by rosemary, *P.* sp. was most affected by methyl anthranilate, and *A. flavus* growth reduced by thyme. On the other hand, frequency of *F. moniliforme* and *Penicillium.* sp. were greatly inhibited by the clove at EC50 =4.5009 and EC90 =6.9351mg/mL, while *A. niger* was most affected by citral as well as rosemary and methyl anthranilate leaded to the major frequency reduction of *A. flavus*. Our results cleared that vapor contact assay had stronger antifungal activity as a post-harvest treatment than carrier contact assay. These results suggested that fungi can be controlled by EOs, especially clove and thyme oils.

Keywords: Alternative control; Essential oil; Inhibitory effect; Maize grains

Introduction

Corn, commonly known as maize (*Zea mays*), an annual grass in the family *Poaceae*, is the third most extensively farmed cereal in the world (Golob *et al*., 2004, Kyenpia *et al*., 2009) at the same time it is the second most significant cereal grain cop in Egypt (Anonymous, 2018). Maize is a main source of farmers' income and due to its important role in grain production, it considered a staple food crop for many people, mainly in developing countries (Olga and Tibor, 2015), with a total production of 1.09 billion metric tons achieved in 2018/2019 (Shahbandeh, 2020).

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The most three common fungal pathogens that are found in the stored grains of corn are *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. (Ortiz *et al*., 2010, Castlellarie *et al*., 2010) and they are known to produce toxins that threatens human's health and can lead to death (Vess *et al*., 2007, Castlellarie *et al*., 2010). In field, when the relative humidity is high, between 90 - 95%, and the seed moisture content is between 20 - 25%, these pathogens can occur in the seeds even before storage (Santos *et al*., 2016, Pushpavathi *et al*., 2017). Thus, pathogens occur on the surface or inside the seeds should be identified and controlled (Karaca *et al*., 2017).

Due to the impacts of fungicides on the environment and human health, alternative chemicals or management strategies have become more necessity. Use of more safe chemicals, especially in organic agriculture, is crucial. Suzuki *et al*., 2015 stated that plant extracts and essential oils (EOs) considered safer natural fungicides, that known to have antimicrobial properties and used in controlling plant diseases (Kavitha and Satish, 2016, Gormez *et al*., 2016, Al-Dhabi *et al*., 2018).

EOs are oily liquids, aromatic, complex, volatile, and derived from various plant parts as seeds, stems, bark, leaves, roots, buds ,flowers and fruits, belongs to various families such as *Alliaceae*, *Apiaceae, Pinaceae*, *Lamiaceae*, *Rutaceae*, and *Lauraceae* (Shannon *et al*., 2011, Solorzano-Santos and Miranda-Novales 2012, Calo *et al*., 2015). Recently, and due to that natural antimicrobials EOs have known as generally recognized as safe (GRAS) status, the importance of food preservation EOs has increased also. Essential oils can be extracted by stream or hydrodistillation from plants. The fungicidal activity of essential oils depends on the used concentrations (Al-Dhabi *et al*., 2019), which in turn affects the inhibition of mycelial growth (Kumar *et al*., 2014, Elgorban *et al*., 2015, Perczak *et al.*, 2016). In addition, spore production inhibition by essential oils could significantly contribute in limiting the spread of the pathogen, by reducing the spore loads in the storage environment and on surfaces and so decreasing toxin development (Tzortzakis and Economakis, 2007). Essential oils of plant origin have been found to prolong the shelf lives and improve the quality of the products. The antifungal potential of EOs against seed-borne pathogens has been subject by numerous investigations in the recent years *i.e*. *Aspergillus* and *Fusarium* genera, mainly on cereal matrices such as rice, oat, maize or wheat (Prakash *et al*., 2015, Kumar *et al*., 2016, Santamarina *et al*., 2016, Boukaew *et al*., 2017; Bozik *et al.*, 2017, Spadaro *et al*., 2017) and on their potentials on targeted cells with direct contact assays.

The fungicidal assays of EOs include different methods with either *in vitro* as fumigant via their vapor phase or coated onto carrier make them more effective than conventional dipping procedures or *in vivo* assessments as foliar spraying (Moodley, 2015, Chang *et al*., 2022). Since the EOs vapor phase may be used in lower concentrations than its liquid one and has no impact on the flavor or aroma of product, the vapor phase of essential oils presents the most practical choices for the prevention of post-harvest diseases (Naeini *et al*., 2010).

Thus, the study aimed to systematically screen some locally available essential oils, as natural products for their suitability and efficacies as biofungicides within two application methods for the control of these pathogens as a single component control measure.

Material and methods

Plant material

Twenty five samples (1 kg each) of white maize grains were collected randomly from different locations in Cairo, Giza, Qalyubia, Fayoum and Sharqia governorates, Egypt. Each sample was placed in dry sterile containers.

Fungal strains

Five plant pathogenic fungi, *Fusarium. moniliforme*, *Aspergillus niger*, *Penicillium* sp., *Aspergillus flavus* and *Aspergillus terreus* were isolated from maize grains in which fifty grains (10 grains per plate) were surface sterilized by dipping in 1 % aqueous sodium hypochloride solution for 1 min, followed by three successive rinses in sterile distilled water plated on Potato Dextrose Agar (PDA, Difico) for 7 day at 25±2 ℃ (Dhingra and Sinclair, 1985). The cultures were single-spored and the cultures were transferred onto PDA slants for identification. The isolated fungi were identified according to colony morphology and microscopic examination according to the keys of (Thom and Raper, 1945; Gilman, 1957; Nelson *et al*., 1983; Barnett and Hunter, 1986 and Leslie and Summerell, 2006).

Essential oils

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

Direct contact assay

Antifungal activity was studied by using an in vitro direct contact assay (Cakir *et al*., 2004, Gong *et al*., 2009).

Mycelial growth inhibition

Mycelial radial growth inhibition assay was conducted according to (Quiroga *et al*., 2001, Cakir *et al*., 2004, Song *et al*., 2004, Gong *et al*., 2009). The percentage (%) of mycelial growth inhibition was determined as [(Mc – Mt)/Mc] ×100, where Mc average of five replicates of fungal mycelial growth measured on control agar medium, and Mt is an average of five replicates of fungal mycelial growth measured on treated agar medium with the essential oils.

Inhibitory activity on spore germination

Spore germination inhibition assay was conducted according to (Liu *et al*., 2009, Wang *et al*., 2010). The percentage (%) of the spore germination inhibition was determined as [(Gc – Gt)/Gc] ×100, where Gc is an average of five replicates of germinated spore in the control, and Gt is an average of five replicates of germinated spores in the treated ones.

Application methods

Two application methods *i.e.* Volatile assay and Carrier contact assay were tested with the essential oils at different concentrations and evaluated for their capability to suppress fungal infection of maize grains during storage.

Volatile assay

The method of Paster *et al*. (1995) and Bill *et al*. (2015) was modified. The effect of volatile components in the oil was tested using a 12 L translucent plastic container (20 x 20 x 30 cm) by placing each essential oil in a Petri plate (9-cm in diameter) in bottom with different concentrations. Maize grains (0.5k) were surface disinfected by dipping in Ethanol 70% for 1 minute then air dried and placed above in container for 30 days. Plastic container treated without oil was used as the control. In this assay, the grains were not in direct contact with the essential oils and any effect could be due to the volatile components. Five replicates were used for each treatment.

Carrier contact assay

Carrier contact assay was done with modification from Wang *et al*. (2019). Wheat bran as carrier was autoclaved at 120 ^{o} C for 60 min. Each tested oil was added individually to sterilize wheat bran at the rate of 1:2 (Essential oil:Wheat bran V:W), then mixed thoroughly to even ensure equal distribution of mixed carriers. The prepared mixture was added at disinfected maize grains (at rate 20 g/Kg) by dipping in Ethanol 70% for 1 minute then air dried and stored for 30 days. Five replicates were used for each treatment.

Frequency of fungal isolates associated with grains

The frequency of the inhibition of each fungus isolated from maize grains was calculated.

Statistical analysis

The median effective inhibitory concentrations (EC_{50} and EC_{90}) against tested fungi were calculated using coefficient equation between propit of means of inhibition (%) and log of concentrations used according to (Ramadan *et al*., 2007).

Results

Direct contact assay

Results revealed that all tested essential oils significantly reduced linear growth and spore germination of all tested fungi as seen in Figures 1 and 2.

Mycelial growth inhibition

The EC_{50} and EC_{90} values of the essential oil against mycelial growth of five tested pathogenic fungi are shown in Figure 1. The essential oils had an obvious inhibitory activity against all test fungi, but the best treatment with the lowest effective concentration used and significantly reduced the mycelial growth was Thyme, as its EC_{50} values ranged from 0.3933 mg/mL to 0.9940 mg/mL and EC_{90} values ranged from 1.6074 mg/mL to 2.0446 mg/mL, while EC_{50} values of Citral ranged from 0.8817 mg/mL to 1.6068 mg/mL and EC_{90} values ranged from 1.6074 mg/mL to 2.0792 mg/mL, EC_{50} values of Methyl anthranilate ranged from 0.8952 mg/mL to 0.9529 mg/mL and EC_{90} values ranged from 1.6189 mg/mL to 1.9378 mg/mL, EC_{50} values of Rosemary ranged from 1.0113 mg/mL to 1.3536 mg/mL and EC_{90} values ranged from 1.9236 mg/mL to 2.3632 mg/mL and EC_{50} values of Clove ranged from 0.7346 mg/mL to 1.5385 mg/mL and EC_{90} values ranged from 3.9030 mg/mL to 6.7499 mg/mL (Figure 1). This indicated that all the essential oils had a broad spectrum of activity against all tested pathogenic fungi. Among them, *F. moniliforme, A. niger, Penicillium.* sp., *A. flavus* and *A. terreus* were the relatively sensitive fungi. According to figure 1, the most effective essential oil observed for *F. moniliforme* and *A. terreus* growth inhibition was Methyl anthranilate, where EC_{50} (0.8952 , 0.8964 mg/mL) and EC_{90} (1.6189 , 1.9262 mg/mL) respectively. While the mycelial growth of *A. niger* and *A. flavus* were most affected by Thyme, where EC_{50} (0.8704 , 0.3933 mg/mL) and EC_{90} (1.6074 , 1.7791 mg/mL) respectively. Citral leaded to the major inhibitory on *Penicillium*. sp. in which EC_{50} (0.8817 mg/mL) and EC_{90} (1.6068 mg/mL).

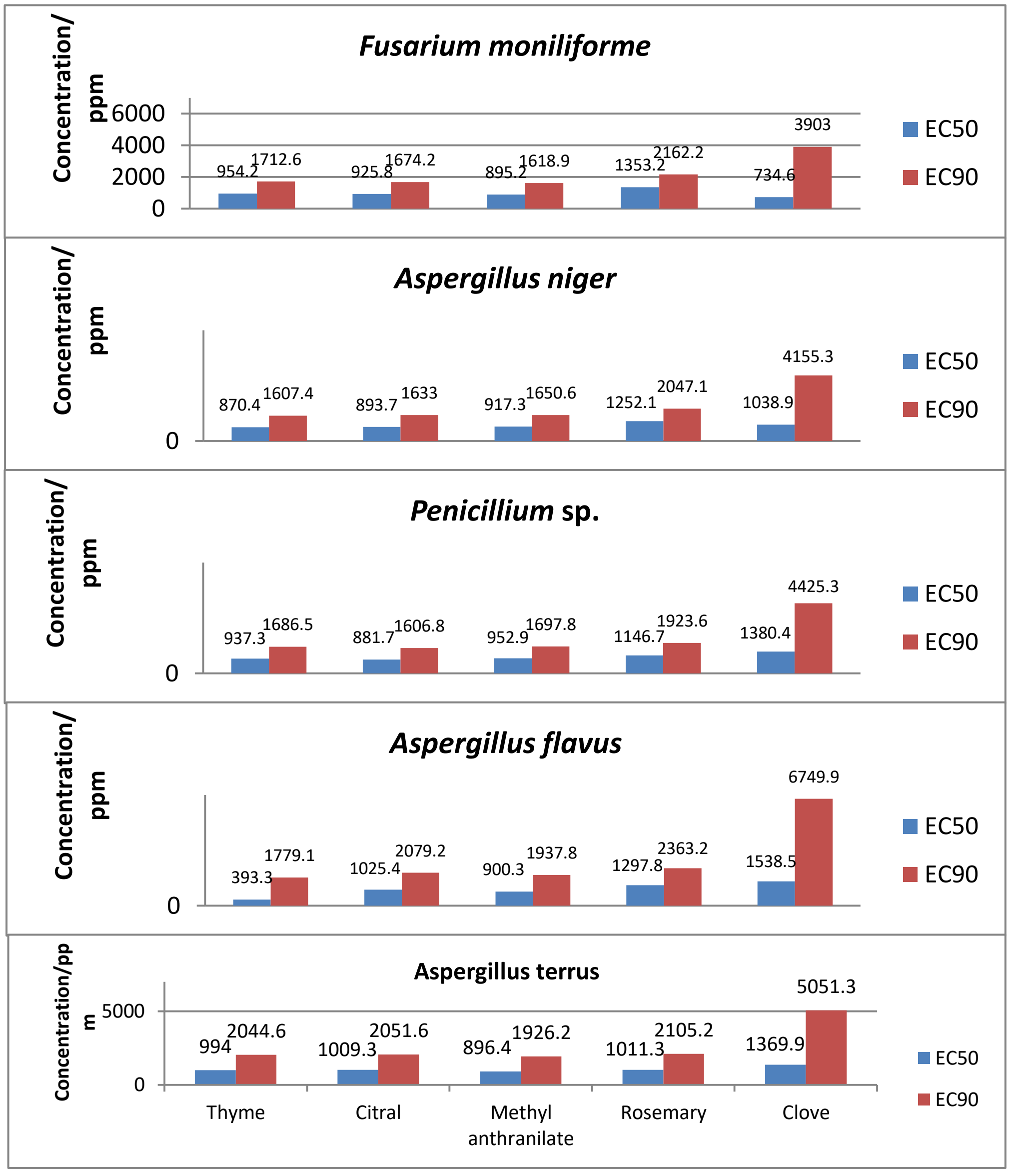

Figure 1. Effect of different concentrations of some essential oils on mycelial growth of fungal isolates associated with maize grains

Inhibitory activity on spore germination

The inhibitory activity of the essential oils on the spore germination of tested fungi was determined. Although the essential oils had an inhibitory activity on all test fungal spore germination, Methyl anthranilate and Rosemary were the most effective treatments with the least effective concentration used for spore germination inhibition, as there EC_{50} values ranged from 0.5904, 0.5777 mg/mL to 0.7454, 0.8175 mg/mL and EC_{90} values ranged from 1.0105, 1.0757 mg/mL to 1.2784, 1.3609 mg/mL respectively, while EC_{50} values of Thyme ranged from 0.7454 mg/mL to 1.2028 mg/mL and EC_{90} values ranged from 1.2784 mg/mL to 1.7678 mg/mL, EC_{50} values of Citral ranged from 0.7502 mg/mL to 0.8127 mg/mL and EC_{90} values ranged from 1.2840 mg/mL to 1.3555 mg/mL and EC_{50} values of Clove ranged from 1.1411 mg/mL to 1.4888 mg/mL and EC_{90} values ranged from 1.8286 mg/mL to 2.2661 mg/mL (Figure 2). This indicated that all the essential oils had a broad spectrum of activity against all tested pathogenic fungi. According to figure 2, the most effective essential oil observed for *F. moniliforme*, *A. niger* and *A. flavus* spore germination inhibition was Rosemary, where EC_{50} (0.6572 , 0.5777 , 0.6818 mg/mL) and EC_{90} (1.1739 , 1.0757 , 1.2034 mg/mL) respectively. While the spore germination of *Penicillium* sp. and *A. terreus* were most affected by Methyl anthranilate, where EC_{50} (0.5904 , 0.6818 mg/mL) and EC_{90} (1.0105, 1.2034 mg/mL).

Application methods

Although the both application methods , Volatile and Carrier contact assay that were tested with the five essential oils proved to be effective to suppress maize grains fungal infection during storage, but EC_{50} and EC_{90} values clearly clarified that Volatile assay much better than Carrier contact assay (*i.e*. less concentrations, ease of application).

Volatile assay

Frequency of fungal isolates associated with grains

Results indicated that all essential oils reduced the total frequency of associated fungi isolated from maize grains (Figure 3). After grain exposure of the volatile components of essential oils diffused from the filter paper, the fungal growth of *F. moniliforme* and *A. niger* isolated from maize grains was greatly frequency inhibited by the least concentrations of Rosemary than the other used essential oils, in which the values of EC_{50} and EC_{90} (3.9317, 6.4059 mg/mL , $Y = 3.2082x - 5.0792$, $R^2 = 0.8668$, where Y is the probit of means of the inhibition, X is concentration logarithm of the essential oils, EC_{50} : effective concentration at 50 percent, EC_{90} : effective concentration at 90 percent and R^2 is coefficient of determination) and EC_{50} and EC_{90} (1.3858, 3.4728 mg/mL) , Y = (3.2082x - 5.0792), R^2 = (0.8668) respectively. While the frequency of *P.* sp. was most affected by Methyl anthranilate whereas EC_{50} and EC_{90} (1.5796, 3.7503 mg/mL), Y = (3.4087x - 5.9029), R^2 = (0.8668). Thyme leaded to the major reduction of *A. flavus* frequency in which EC_{50} and EC_{90} (0.9010, 1.9170 mg/mL), Y = (3.9033x - 6.5331), R^2 = (0.9237).

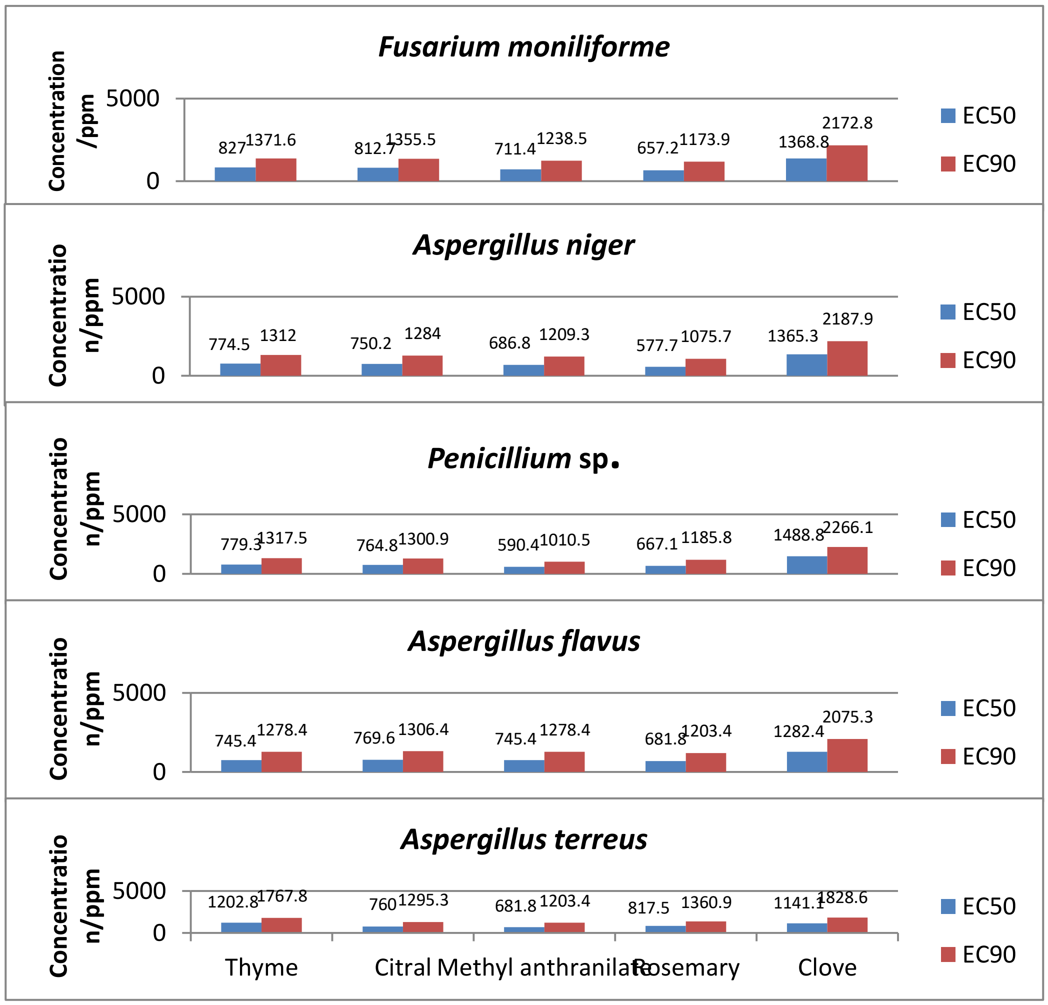

Figure 2. Effect of different concentrations of some essential oils on spore germination of fungal isolates associated with maize grains

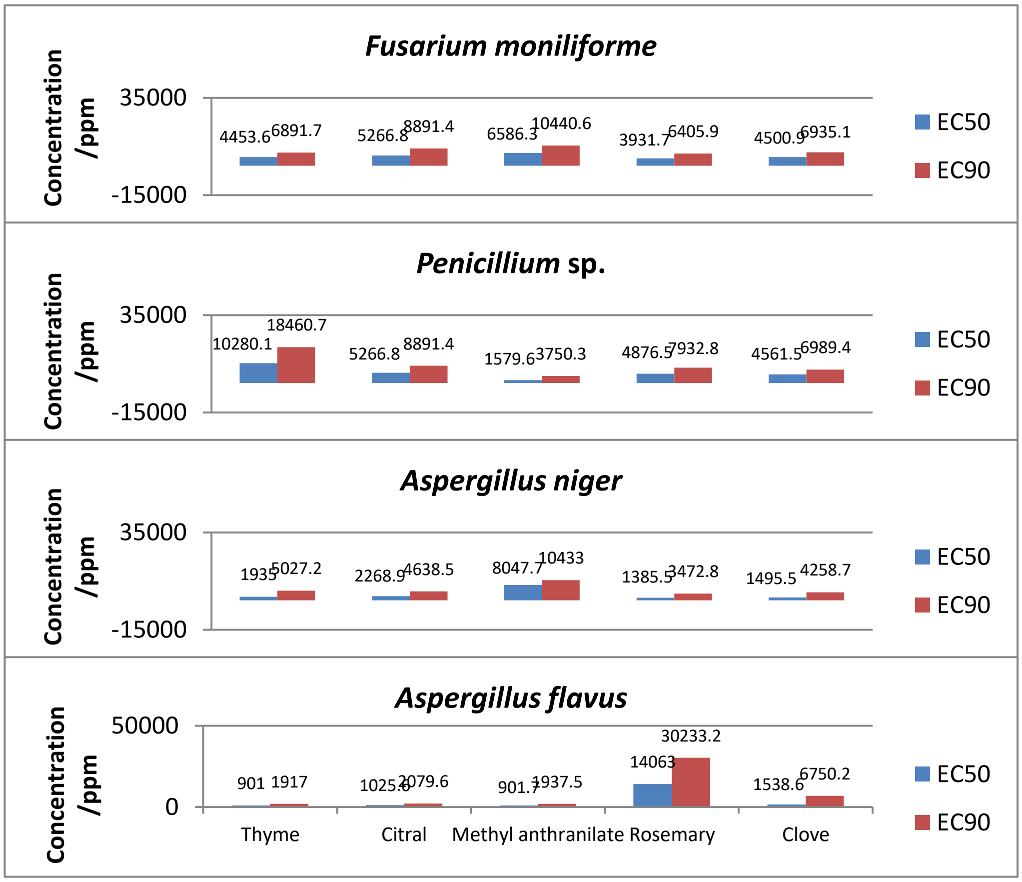

Figure 3. Effect of different concentrations of some essential oils volatile contact assay on frequency of fungal isolates associated with maize grains

Carrier contact assay

Frequency of fungal isolates associated with grains

Results indicated that all essential oils reduced the total frequency of associated fungi isolated from maize grains (Figure 4). After grain direct contact with the carrier of essential oils, the frequency of *F. moniliforme* and *Penicillium* sp. isolated from maize grains were greatly inhibited by the least concentrations of Clove EC_{50} (4.5009 , 0.0146 mg/mL) , EC_{90} (6.9351 , 0.0262 mg/mL), Y = (5.9944x - 17.511 , 5.6812x - 15.238) and R^2 = (0.7646 , 0.8668) respectively. While the frequency of *A. niger* was most affected by Citral as well as Rosemary whereas EC_{50} and EC_{90} (1.8178, 4.0725 mg/mL) for both oils, Y = (9.3039x - 30.925 , 3.6538x - 6.9097), R^2 = (0.8668) for both oils. Methyl anthranilate leaded to the major frequency reduction of *A. flavus* frequency in which EC_{50} and EC_{90} (3.6485, 6.7173 mg/mL), Y = (3.9233x - 10.823), R^2 = (0.9786).

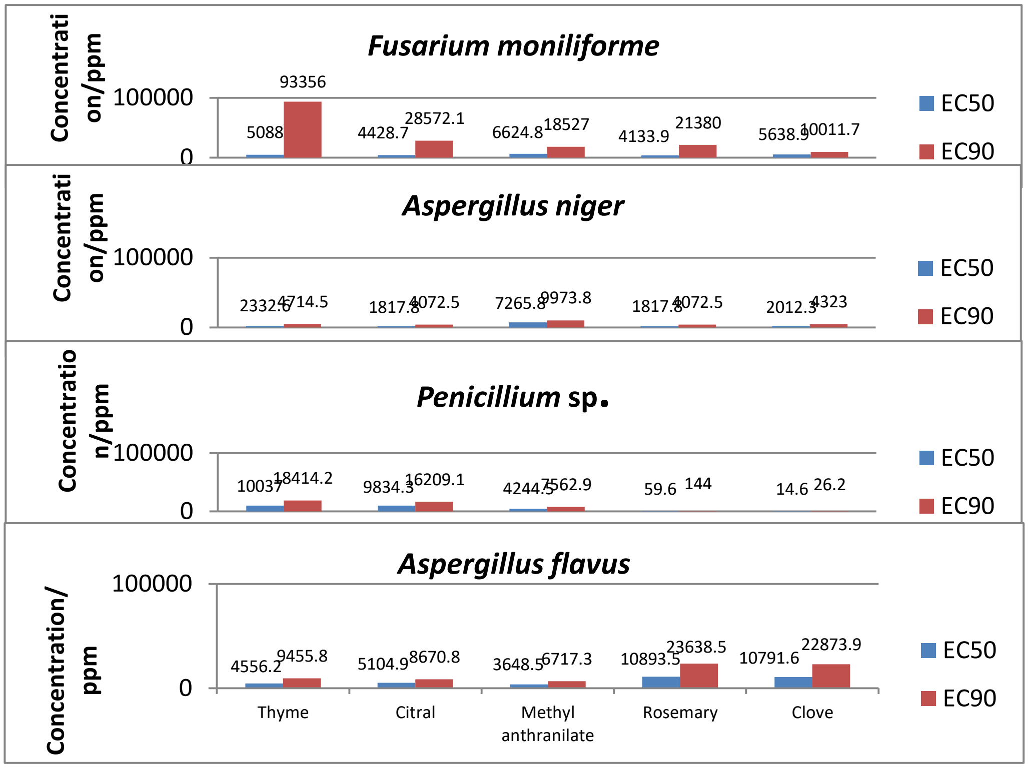

Figure 4. Effect of different concentrations of carrier contact assay with some essential oils on frequency of fungal isolates associated with maize grains

Discussion

The utilisation of novel techniques with a natural or "green" character is currently popular; for example, the application of essential oils represent good candidates in controlling of toxigenic fungi and subsequent mycotoxins in stored grains and in food industries as alternatives to chemical fungicides (Velluti *et al*., 2003, Bluma and Etcheverry, 2008, Čvek *et al*., 2010, Passone *et al*., 2012, Yuan *et al*., 2013, Vergis *et al*., 2015, Sawinska *et al*., 2020).

The present study showed that the five EOs tested had significant inhibitory effect on growth rate, and spore germination of the fungi isolated from maize grain. The best treatment which significantly reduced the mycelial growth was thyme, on the other hand rosemary were the most effective treatments with the least effective concentration used for spore germination inhibition. The statistical analysis demonstrated that the effectiveness of these EOs was influenced by changes in their concentrations.

EOs can comprise more than 60 individual components (Russo *et al*., 1998, Anthony 2003, Tzortzakis and Economakis 2007, Mart ńez, 2012, Daniel *et al*., 2015). Up to 85% of the EO can be mainly composed of major components, while the remaining 15% is simply a trace amount.Some researchers reported that there is a relationship between the chemical structures of the most abundant compounds in the EOs and the antimicrobial activity. The secondary components have an important and crucial role in the antimicrobial activity, possibly by a synergistic effect between the other components (Burt, 2004). Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antimicrobial activity is not attributable to one specific mechanism but to the existence of several targets in the cell (Skandamis and Nychas, 2000, Carson *et al*., 2002, Lopez-Reyes *et al*., 2013).

This finding was similar to those reported by Sinha *et al*. (1993) and Thanaboripat *et al*. (2004) as they demonstrated that the *A. flavus* growth in maize grain with various concentrations inhibited with citronella EO. In the same trend, earlier results showed that thyme oil clove, anise, and other EOs directly inhibits pathogen growth, spore germination and aflatoxin production by affecting the active sites of enzymes and cellular metabolism (Ozcan and Boyraz, 2000, Bluma *et al*., 2009, Arrebola *et al*., 2010, Božik *et al*., 2017). The mycelium treated with thyme oil showed alteration in the morphology of the hyphae, which appeared collapsed and also caused a reduction in condition. In the same trend (Sahab *et al*., 2014, Perczak *et al*., 2019) reported that the EOs affected the growth of *F. culmorum* on sterile wheat grains. 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). Soliman and Badeaa (2002) demonstrated that there is inhibitory effect on *Aspergillus flavus, A. parasiticus, A. ochraceus*, and *F. verticilloides* of thyme EOs.

The effect of clove EO and its main components eugenol, on the growth of fungi and their production of mycotoxins for some toxin-producing fungi such as *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. has been studied by Farag *et al*. (1989), Sinha *et al*. (1993), Velluti *et al*. (2003). Studies have shown that this component has the ability to inhibit both growth and/or mycotoxin production.

Citral, geraniol and citronellol showed the highest antifungal activities among terpenoids (Viollon and Chaumont, 1994). Baratta *et al*. (1998) and Pawar and Thacker (2006) examined essential oil from different commercial plants including *Citrus limon* (Lemon), *Cymbopogon citratus* (Lemon grass) and *Rosmarinus officinalis* (Rosemary). Studies have shown that all essential oils tested have the ability to inhibit the growth of *A. niger*. The previous results also showed that sage had a weak activity, on the contrary, thyme, oregano and salty ones showed effective activity against all types of mold fungi tested (Ozcan and Boyraz, 2000).

Khan and Ahmad (2011) have highlighted the multiple sites of action of eight EOs in fungal cells, includes harm to the cytoplasm, cell membranes, and cell walls of *Aspergillus fumigatus* and *Trichophyton rubrum*. The authors also demonstrated that the test oils had the ability to inhibit the activities of the enzymes elastase and keratinase. The lipophilic properties of oils are believed to aid in penetration and passage across cell membranes as well as the extent of polysaccharide accumulation under conditions of water stress, which may cause plasma rupture of fungal cells and inhibition of their growth (Ultee *et al*., 2002).

Bluma and Etcheverry (2008) revealed that α-terpinolene (73.8%) and α-terperpine (15.3%) are the main components present in boldo, while peperitenone oxide (48.6%) and limonene (24.5%) are the main phytochemicals of poleo. Eugenol (75.4%) and α-cariofilene (14.7%) were the main components of clove. Studies were conducted on the main active ingredient EO, in cloves to determine the its antimicrobial effect. The effects of clove EO and its principal component, eugenol, on growth and mycotoxin production by some toxigenic fungal genera such as *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. have been reported by Amiri *et al*. (2008), Nesci *et al*. (2011). Nguefack *et al*. (2008) clarified that similarity between the antifungal activity of EOs from *T. vulgaris* and *O. gratissimum* and their superiority to *C. citratus* could be attributed to their contents of known antimicrobial compounds. Nguefack *et al*. (2007) have also demonstrated that the EO from *C. citratus* contained mostly citral. A similar conclusion was made by Tassou *et al*. (2000) reported that the terpenes containing alcoholic compounds are more effective against micro-organisms than the ones having aldehydic function. This could explain the lesser control effect recorded with the EO from *C. citratus* which contained mainly aldehydic terpenes as compared to those from *O. gratissimum* and *T. vulgaris* which are rich in terpenic compounds with alcoholic function.

Our study has clearly confirmed that essential oils should find practical application (volatile and carrier contact assay) as inhibitors of mould growth, especially because they are generally regarded as safe. They could be applied to stored grains to prevent fungal growth. Our results are an agreement with the results of other researchers, who stated that vapor contact assay had stronger antifungal activity as a post-harvest treatment than carrier contact assay. Such a strong effect has previously been obtained with EOs towards pathogens reported by Chang *et al*. (2022). Although, the tested EO in this work, in the contact assay showed higher inhibition than the volatile and carrier contact assay.

One is its bioactivity in the vapor phase, a characteristic which makes them attractive as fumigants for stored product protection such as maize grains (Tripati and Dubey, 2004 , Čvek *et al*., 2010). On the one hand, Bluma *et al*. (2009) demonstrated that the vapor generated by poleo oil significantly reduced growth of *Aspergillus* section Flavi (Bluma and Etcheverry, 2008). On the other hand, Nesci *et al*. (2011) observed that 3000 μL/g of thyme oil couldn't of reduce the counts *Aspergillus* section Flavi in stored peanut.

The EO carrier coating leads to the disinfection of the artificial-inoculated seeds and the treatment showed protective effects against bacterial infections as an alternative control measurement for seed protection (Yilmaz *et al*., 2021). Also, a film coating pesticide is an eco-friendly alternative and economically available option (Jacob *et al*., 2009). This technique allows the coating of seeds with pesticides, alternative matters (Keawkham *et al*., 2014), and EOs (Zeng *et al*., 2010). The technique could increase the performance of organic pesticides and also decreases the costs due to the consumption of toxic chemicals causing environmental pollution. Somda *et al*. (2007) reported that *C. citratus* at a concentration of 6% was effective in controlling the seed-borne infection of *F. moniliforme* and other pathogens in sorghum seed. Research developed by other scientists also indicates that the use of essential oils can significantly reduce the development of pathogens of the *Fusarium* genus (Perczak *et al*., 2019). Clove essential oil significantly contributed to the inhibition of the development of the studied analyzed pathogens.

In conclusion, our study has clearly confirmed that EOs treatments showed a significant impact on the fungal frequency and growth of maize grains caused by seed-borne fungi. The significant practical application as inhibitors for mould growth is fumigation. Taken together, Thyme and rosemary EOs are promising as antifungal biofungicides. Finally, the results of the current study will provide an alternative strategy to using EOs for stored maize grains protection against grains fungal contamination.

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(Received: 6 July 2022, accepted: 30 August 2022)