Fungicidal Potential of Essential Oils in Organic Corn Grains during Storage

Jianmei Yu*, Esther Iwayemi, Ivana Pedroso, and Nona Mikiashvili

Abstract

Mold and mycotoxin contamination of cereal grains not only affect human and animal health but also have a significant economic impact on cereal growers and food processors due to grain loss and product recall. Synthetic fumigant is usually used to control mold growth during grain storage, but it is not permitted for organic grains. Therefore, the objective of this study was to investigate the antifungal potential of some essential oils (EOs) for organic corn grain protection at storage temperature 25 and 35°C and water activities 0.85 and 0.9 by a simulated fumigation method. A 4x2x2 experimental design was used. The three factors were the types of EOs (cinnamon, clove, oregano and thyme oils), storage temperature (25 and 35°C) and water activity (a_w) (0.85 and 0.9). Organic corn grains were weighed into a set of glass jars and adjusted to the desired water activity (a_w). EOs were diluted to 10% using 10% DMSO, 0.5 mL diluted EO was added to a cotton ball taped to the lid. The jars were capped immediately and stored at the desired temperature for 7-35 days. Samples treated with fungicide Pyraclostrobin and 10% DMSO were used as positive control and negative control, respectively. Mold growth was monitored every 7 days by photographing and aflatoxins in the samples were determined by ultra-high-performance-liquid-chromatography (UPLC). The study found that the mold growth and aflatoxin contents of corn grains were affected by the type of EO, storage temperature and moisture or water activity. At 25°C and a_w=0.85, the negative control was molded at day 28, while others were not molded; aflatoxin B1 and B2 were almost unchanged in positive control and cinnamon oil treated samples; aflatoxin G1 and G2 were below the detection limit in positive-control and EO-treated samples. At 35°C, the negative-control was molded in 7 days at a_w=0.9 and 14 days at a_w=0.85, while samples treated with fungicide and cinnamon oil were not molded until day 28 and 35, respectively. The lowest aflatoxin B1 concentration was detected in the samples treated with cinnamon oil, oregano oil and positive control. The results indicated that cinnamon oil and oregano oil at a concentration 0.4 mL/100g corn grains have great potential to replace toxic fumigants to protect organic grains from mold and mycotoxin contamination during storage.

Keywords: Essential oils; Mold growth inhibition; Organic corn grains; Storage

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1. Introduction

Mold and mycotoxin contamination of cereal grains not only affect human and animal health but also have a significant economic impact on cereal growers and food processors due to grain loss and product recall (CAST, 1989; Kumar et al., 2021). Mycotoxin contamination of various crops is considered an unavoidable and unpredictable problem, posing a difficult challenge to food and feed safety, food security and international trade (Alshannaq et al., 2017). Aflatoxins including Aflatoxin B1, B2, G1 and G2 are considered the most toxic among mycotoxins identified so far and are classified as Group I human carcinogens (IARC, 2012). They are produced by pathogenic molds *Aspergillus flavus* and *A. parasiticus* (Pitt et al., 2013). Toxic fumigant is usually used to control mold growth during grain storage, which is harmful to operator and environment, and is not permitted for organic grains (Pimentel, 2005; Özkara et al., 2016). Therefore, there is a need to explore safer fungicide alternatives for organic grain protection. As popular flavor additives in cosmetic and food products, essential oils (EOs) have an inhibitory effect on pathogenic microorganisms (Wan et al., 2020). However, the research in the use of EOs to control molds and mycotoxins in the stored cereal grains is limited. More studies are needed. Therefore, the objective of this study was to investigate the antifungal potential of some essential oils (EOs) for organic corn grain protection at storage temperature 25 and 35 °C and water activities 0.85 and 0.9 by a simulated fumigation method.

2. Methodology

2.1 Materials

Organic corn grains (11.35 kg/bag) were purchased from Great River Milling Company (Fountain City, WI, USA). Four EOs including cinnamon oil, clove oil, oregano oil and thyme oil, and fungicide Pyraclostrobin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aflatoxin standard mix which contains Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), Aflatoxin G1 (AFG1) and Aflatoxin G2 (AFG2) was also purchased from Sigma-Aldrich. Dimethyl Sulfoxide (DMSO), HPLC grade methanol and acetonitrile, and analytical grade trifluoroacetic acid were purchased from Fisher Scientific (Suwanee, GA, USA).

2.2 Experiment Design and Treatment

A simulated fumigation method was used to test the antifungal potential of EOs in stored organic corn grains using a 4x2x2 experimental design. The three factors were the type of EO (cinnamon, clove, oregano and thyme oils), storage temperature (25 and 35 ºC) and water activity (a_w) (0.85 and 0.9). The a_w was measured using an AquaLab 4TE Water Activity Meter (Meter Group, Pullman, MA, USA). Organic corn grains (25g) were weighed into a set of 60 ml glass containers and adjusted to the desired a_w (0.85 and 0.9) by adding a pre-calibrated amount of water. EOs were diluted to 10% using 10% DMSO and 0.5 mL diluted EO was added to a cotton ball taped to the lid. This resulted in an EO concentration of 0.4 mL/100g corn grains. The selection of this concentration is based on the work of Eesiah et al. (2022) in which EO concentration of 0.4

mL/100g corn resulted in more about 90% maize weevil mortality. The container was capped immediately and stored at desired temperature for 7, 14, 21, 28 and 35 days. Fungicide Pyraclostrobin and 10% DMSO were used as positive and negative controls, respectively. Samples were taken weekly and the mold growth status was recorded by taking photos of corn grains. The samples were then used for aflatoxins quantification.

2.3 Aflatoxin Extraction, Purification and Quantification

Aflatoxins in samples were extracted using 80% methanol. Briefly, 25 g of organic corn grains sample was blended with 100 mL of 80% methanol, then centrifuged at 3,000g for 20 min. The supernatants were collected and purified with solid phase extraction (SPE) columns. The purified extracts were dried under nitrogen gas, reconstituted in 50% acetonitrile in deionized water, and then derivatized using trifluoroacetc acid (TFA). The aflatoxin concentrations in the derivatized extracts were determined by an ACQUITY UPLC system (Water, Milford, MA, USA) using external standard. Briefly, 10 µL of derivatized aflatoxin extract was injected into the UPLC system coupled with an ACQUITY UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm) and a fluorescence detector. The injected sample was eluted with isocratic mode using a mobile phase consisting of water: methanol:acetonitrile = 64:18:18 at a flow rate of 0.2 mL/min. Four standard curves were developed using aflatoxin standard mix (Sigma-Aldrich, St. Louis, MO, USA) under the same chromatography conditions for calculating the concentrations of aflatoxin B1, B2, G1 and G2. The limits of detections of AFB1, AFB2, AFG1 and AFG2 were 0.15, 0.045, 0.15 and 0.045 ng/mL, and the average recoveries of these toxins were 96.1, 95.8, 88.4 and 85.1%, respectively.

2.4. Determination of fungicidal activities of essential oils

For the antifungal potential of each EO on molds grown on corn grains during storage. The naturally molded corn grains (25g) were mixed with 100 mL of phosphate buffer in an autoclavable bag and blended in a Stomacher (Seward Ltd, Bohemia, New York, USA). The liquid was filtered using a cell strainer (70 µm). The filtrate was inoculated on Rose Bangel Agar (RBA) plates, which is a selective medium for mold growth, and incubated at room temperature (23 ºC) for 7 days. The mold colonies on RBA plates were washed using phosphate buffer and filtered using a cell strainer. The filtrate containing mold spores was stored in a sterile bottle at 4 ºC.

The fungicidal activity of each essential oil was determined according to the method of Hasheminejad et al. (2019) with some minor modifications. Briefly, the mold spores (10 µl) was placed at the center of potato dextrose agar (PDA) plate containing 0, 0.1, 0.2, 0.4, 0.6, and 0.8 mg/ml of EO (6 replications at each concentration). The plates were incubated at 23 ºC for 7 days. The diameters of mold colonies were measured and the total fungicidal activities of each EO at different concentrations were expressed as antifungal index (AI) which were calculated using the equation below:

$$AI\ (\%) = (D_0 - D_E)\ x100/D_0$$

Where D_0 is the colony diameter on the PDA plate without EO, D_E is the diameter of mold colony on the PDA plate containing EO.

2.4 Data analysis: The simulated fumigation experiment of corn grains was conducted without replication, but the analysis of aflatoxins were conducted twice for each sample. Antifungal activity test on PDA plates for each EO at each concentration was repeated 6 times, and the AI values of different EOs at same concentration were compared by post-hoc Tukey test at 5% significance level.

3. Results

3.1 Mold growth in organic corn grains during storage

The growth of mold in organic corn grains was affected by storage temperature, the water activity of grains and the type of EOs (Figure 1). At 25 ºC, negative control was molded at week 4, others were not molded until week 5 at water activity (a_w) 0.85 and 0.9. At 35 ºC and a_w 0.85, samples without any treatment (negative control) were molded after 2 weeks of storage but samples treated with commercial fungicide and EOs were not molded until week 5. At 35 ºC and a_w 0.9, the negative controls were molded within one week, while samples treated with fungicide showed mold at week 2, samples treated with oregano oil exhibited mold at week 3, and all samples were molded at week 5 with cinnamon oil treated sample being the lest molded. Comparing Fig. 1A, 1B, 1C and 1D, we conclude that grain water activity had a larger effect on mold growth than temperature.

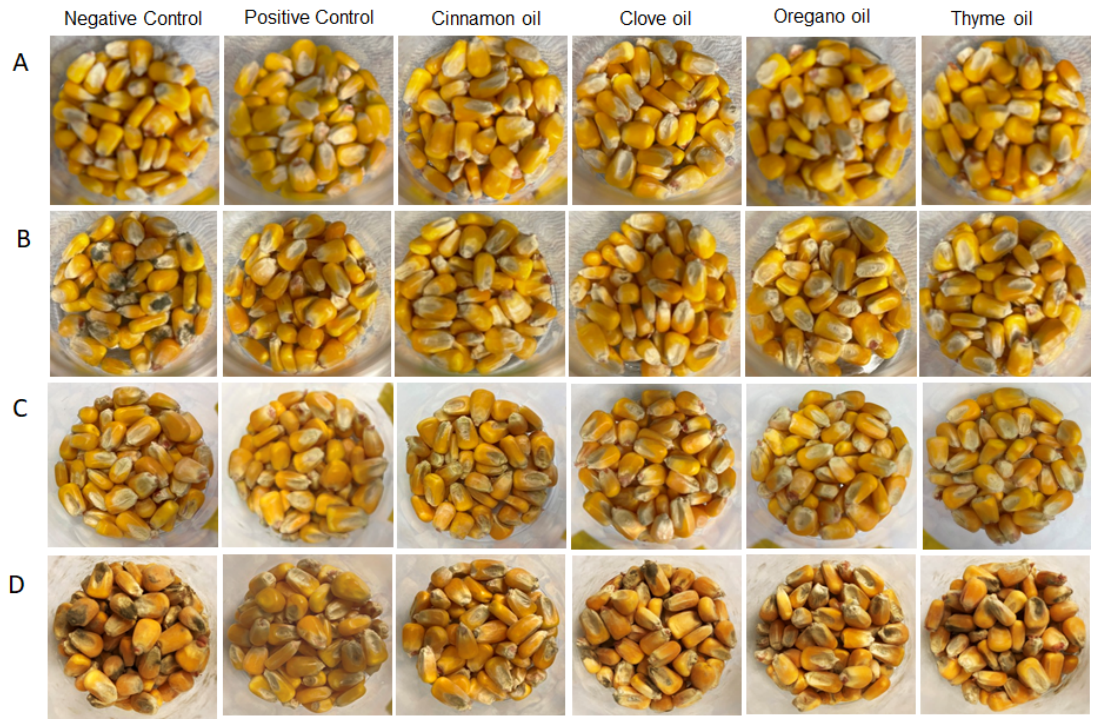

Fig. 1 Mold growth in organic corn grains at day 35 of storage without and with EOs at (A) 25 ºC, a_w 0.85, (B) 25 ºC, a_w 0.9, (C) 35 ºC, a_w 0.85, and (D) 35 ºC, a_w=0.9. (Negative control: 10% DMSO, Positive control: fungicide Pyraclostrobin at concentration of 5mg/kg corn).

3.2 Aflatoxin contents of corn grains stored at different conditions for different time

At 25 ºC and a_w 0.85, AFB2 was not detected in all samples, AFB1 was not detected until day 21 and its concentration was lower than 0.5 ng/g corn except the sample treated with clove oil which showed much higher AFB1 level in day 28 of the storage, while samples treated with other EOs showed lower AFB1 content that negative control (Fig.2A). Aflatoxin G1 and G2 levels in all EO treated samples were below 0.2 ng/g corn except the sample treated with clove oil which showed extremely high AFG1 at day 28. Similar results were obtained for samples stored at 25 ºC and a_w 0.9, but AFB1 contents in all samples increased compared to samples stored at a_w 0.85 particularly, samples treated with oregano and thyme oils having higher AFB1 than negative control (Fig. 2B). The clove oil treated samples taken at day 28 showed highest AFB1 (3.20 ng/kg grains) and AFG1 (11 ng/kg grains). Data presented in Fig. 2 indicate that cinnamon oil effectively inhibited AFB1 formation among all tested EOs.. In addition, the higher a_w might favor the formation of Aflatoxin B1, and G2 at 25 ºC. The extreme high AFB1 and AFG1 in clove oil treated sample at day 28 might be caused by the heterogeneous distribution of mold spores and aflatoxins in the bulk corn grains which makes sampling a major source of error in the accurate assessment of aflatoxin levels in food (Donnelly et al., 2022).

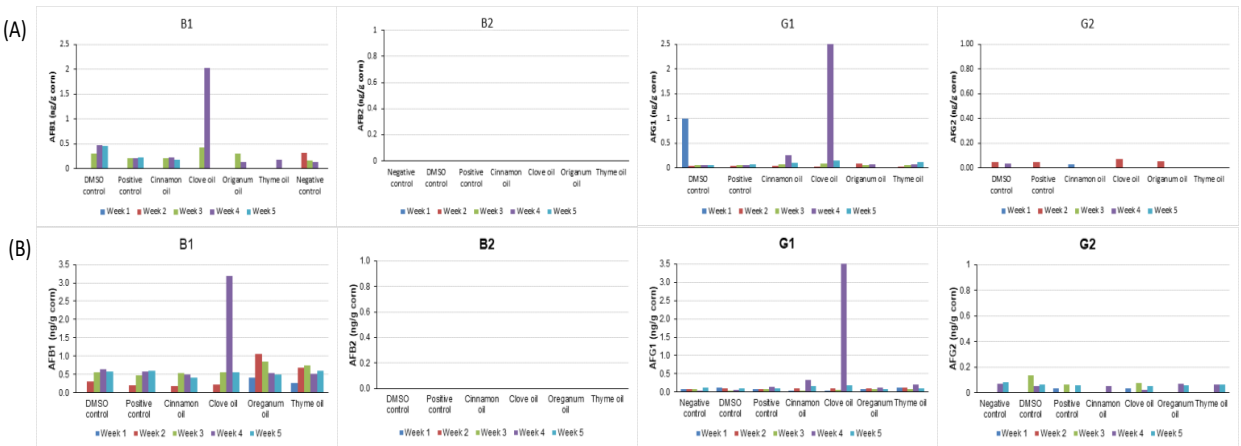

Fig. 2 Aflatoxin contents of organic corn grains stored at 25 ºC for 1-5 weeks. (A): a_w=0.85, (B): a_w=0.9

At 35 ºC, most samples stored at a_w 0.85 showed higher aflatoxin contents than samples stored at a_w 0.9, particularly, AFB1, although corn kernels were less molded at a_w 0.85 (Fig. 3). At a_w 0.85, cinnamon oil treated samples had the lowest AFB1, AFB1, AFG1 and AFG2 contents. The sample treated with thyme oil for 14 days showed the highest AFB2, while the sample treated with clove oil for 21 days showed the highest AFB1, AFB2 and AFG1 (Fig. 3A). This could be caused by the

uneven distribution of mold spores and aflatoxins in the bulk corn grains where experimental samples were taken from. At a_w 0.9, all EO treated samples except that treated with clove oil showed very low AFB1 and AFG2 over a 35-day storage period with slight AFB1 increase in week 4 and 5 (Fig. 3B). The sample treated with clove oil for 4 weeks exhibited highest AFG1 and AFG2 content. The data in the Fig.3 suggest that cinnamon oil, oregano oil and thyme oil may be better EOs to control aflatoxin production at high temperature.. Further, at higher temperature, higher moisture favor the mold growth, while lower water activity may favor the production of aflatoxins in corn grains. This supports the previously reported findings that the optimal temperatures and water activities for mold growth and mycotoxin production can be different (Daou et al., 2021), and hot weather and drought stress promote the production of the aflatoxin by *A. flavus* and *A. parasiticus* (Herrman, 2002).

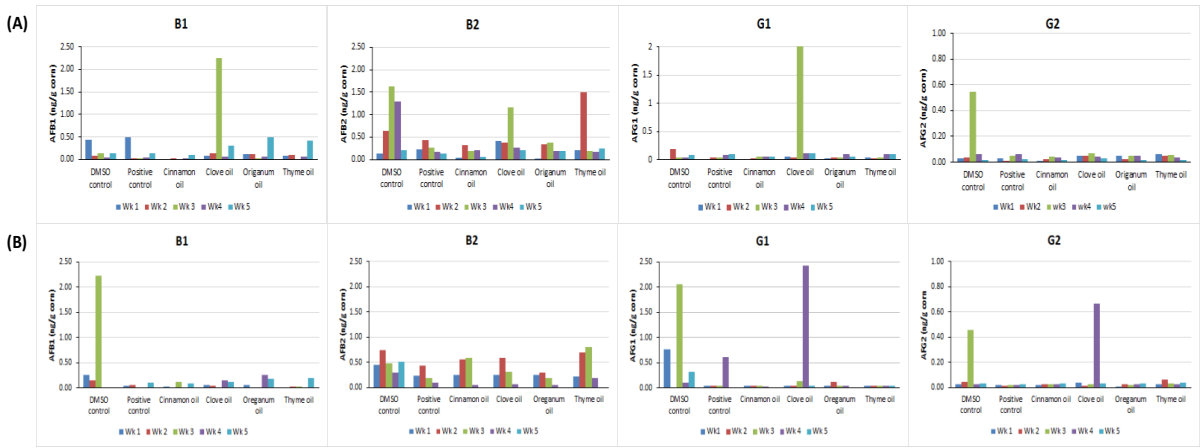

Fig. 3 Aflatoxin contents of organic corn grains stored at 35 ºC for 1-5 weeks. (A) a_w = 0.85, (B) a_w = 0.9

3.3 Fungicidal activities of essential oils

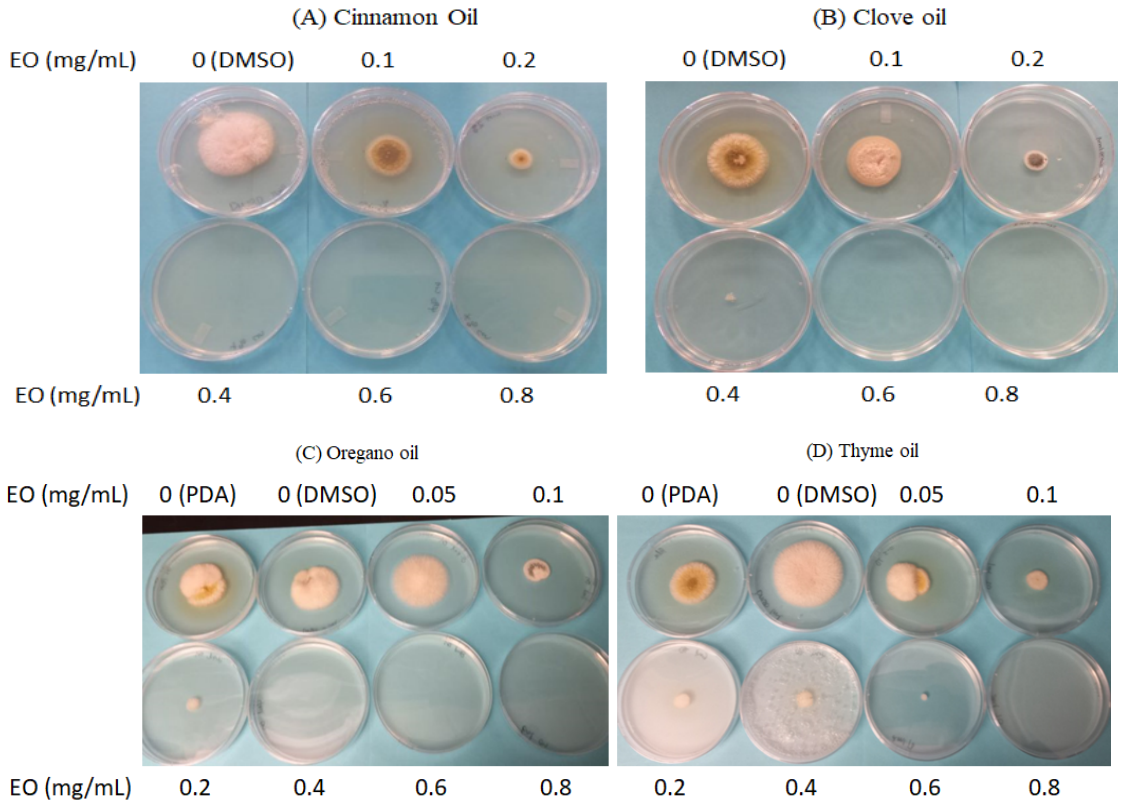

Figure 4. Inhibition of EOs on total mold growth on PDA plates

Figure 4 shows that all tested EOs inhibited the growth of fungi isolated from molded organic corn grains on PDA plates. The mold growth was completely inhibited by cinnamon oil and oregano oil at 0.4 mg/mL, clove oil at 0.6 mg/mL and thyme oil at 0.8 mg/mL. At same concentration, the cinnamon and oregano oils also had significantly higher antifungal index (AI) than clove and thyme oil ($P<0.05$) (Table 1). The data are consistent with the observations shown in Figure 1. The EOs tested in this study exhibited higher antifungal potential compared to those tested in other studies (Nguefack et al., 2004; Gemeda et al., 2014).

Table 1 Total Antifungal Index (AI) of different essential oils against the growth of molds isolated from molded organic corn grains at ambient temperature

	EO Concentration (mg/mL)					
Type of EO	**0.05**	**0.1**	**0.2**	**0.4**	**0.6**	**0.8**
Clove oil	13.90 ± 2.52^{d}	18.37 ± 3.13^{c}	65.60 ± 7.06^{b}	87.51 ± 5.19^{b}	100.00 ± 0.00^{a}	100.00±0.00
Cinnamon oil	33.05 ± 2.00^{a}	42.09 ± 3.60^{b}	77.12 ± 2.80^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00±0.00
Oregano oil	20.91 ± 6.00^{bc}	38.56 ± 18.40^{b}	73.56 ± 16.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00±0.00
Thyme oil	25.97 ± 4.53^{b}	61.36 ± 5.15^{a}	66.68 ± 1.16^{b}	69.08 ± 6.94^{c}	87.26 ± 1.50^{b}	100.00±0.00

Note: At same EO concentration (same column), data with different superscripts are significantly different at 5% significance level (n=6).

Conclusion

The four EOs tested in this study are classified as Generally Recognized As Safe (GRAS) by US Food and Drug Administration. This study indicate that all the tested GRAS EOs inhibited mold growth in organic corn grains and in the cultural medium but not all EOs inhibit the production of aflatoxins. Among these EOs, cinnamon oil and oregano oil have great potential to serve as fumigants to protect organic grains from mold and mycotoxin contamination during storage. However, this study only tested one EO concentration which is relatively high compared to positive control, and only aflatoxins were analyzed. More studies are needed to optimize the effective EO concentration, to understand the effects of EO treatment of corn grains on the production of other mycotoxins and the inhibitory effects of EOs on specific mold species.

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