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Antifungal activity of oregano essential oil and thymol against some fungi isolated from corn grains

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ABSTRACT: The development of fungi in stored grains present a health risk to customers due to their ability to produce and accumulate mycotoxins. Then, chemical antifungal agents are applied to grains to reduce their contamination by molds. The aim of the present study was to evaluate the antifungal potency of oregano essential oil and thymol on some species of fungi involved in corn spoilage. Molds were isolated, and then identified by their macroscopic and microscopic characteristics after cultivation on standard media. The antifungal activity of oregano essential oil and tymol was conducted by broth dilution method. The mold species isolated were *Aspergillus niger*, *Aspergillus flavus*, *Penicillium sp.*, *Fusarium sp.,* and *Mucor sp*. Oregano essential oil and thymol demonstrate an antifungal effect against all these isolates. These compounds may be useful as alternative in limiting or preventing the development of harmful fungi and mycotoxins in food.

KEYWORDS: Fungi, corn, oregano essential oil, thymol, antifungal activity.

1 INTRODUCTION

Corn grains provide an excellent substrate for the growth of molds and subsequent synthesis of mycotoxins [1], [2]. The most frequent fungal contaminants belonged to *Penicillium*, *Aspergillus* and *Fusarium*. The presence of these molds is also a health risk to consumers due to their ability to produce and accumulate mycotoxins [3], [4], [5]. It is therefore necessary to use antifungal agents to reduce this fungal contamination. Frequent use of these antifungal agents led to the emergence of resistant strains to these agents, as well as the presence of toxic residues in grains [6], [7], [8]. Therefore, the search for alternative drugs with low resistance substantial attention has focused on natural products with antifungal properties.

Several studies have shown that essential oils (EOs) have an inhibitory effect on the growth of many fungi that contaminate several foodstuffs [9], [10].

The objective of our study was twofold: Firstly, it attempts to isolate and identify some molds that contaminated corn grains. Secondly, it purports to evaluate the antifungal effect of oregano EO and thymol against some fungi species isolated from corn.

2 MATERIAL AND METHODS

2.1 MATERIAL

The maize used in this study is a local variety destined to animal alimentation. It was provided by Alf Sahel-Fes, Morocco.

2.2 FUNGAL ISOLATION

Sample of 10 g of corn grains were milled and homogenized with 90mL sterile distilled water for 30 min on horizontal shaker. Serial ten-fold dilutions were prepared by transferring 1mL aliquots of the initial dilution into succeeding dilution bottles containing 9 mL of sterile distilled water. One-milliliter aliquots of these dilutions were plated on Malt Extract Agar (MEA, Difco) with 0.01% of chloramphenicol to avoid bacterial growth. The Petri dishes were incubated at 25°C for 6 to 10 days [11]. Then, the relative density (RD) of each species was calculated as described previously [12]:

RD (%) = Number of isolates of species or genus $\times$ 100 / Total number of samples

2.3 PURIFICATION AND IDENTIFICATION OF FUNGI

Individual colonies with a visual difference in morphology from the mother culture were subcultured on fresh MEA plates for obtaining pure culture of each isolates. The Petri dishes were incubated at 25°C during 7 days. We continue to sub-culture until we are certain we have a pure fungal culture. Then, purified fungal colonies were transferred onto different culture media, including: Czapek Yeast Autolysate agar (CYA) and Malt Extract agar (MEA). Parallel sets of colonies were cultivated at 25°C, 5°C and 37°C. After 7 to 10 days of incubation, the isolated fungi were identified using the taxonomic key of Pitt and Hocking [13] based on the microscopic and macroscopic characteristics.

2.4 INOCULUM PREPARATION

The suspension of spores was prepared by growing each isolate on MEA at 25°C until sporulation. Some spores were then detached, and transferred in a tube containing sterile NaCl 0.9%. This suspension was counted in Malassez counting chamber, and diluted to obtain a final inoculum of approximately 10^6 spores/ml.

2.5 ANTIFUNGAL AGENTS

The products tested in this work were carvacrol (Fluka, Steinhein, Germany) and oregano EO (Oreganum compactum) containing 35% of carvacrol and 25% of thymol provided by the society of "Méditerranéenne des Aromes". Carvacrol and oregano EO were dispersed in 0.2% sterile agar [14]. The range of concentrations tested was from 0.01% to 0.4%.

2.6 DETERMINATION OF MINIMAL INHIBITORY CONCENTRATION (MIC) AND MINIMAL FUNGICIDAL CONCENTRATION (MFC)

The MIC and MFC of oregano EO and carvacrol against all fungi isolates were evaluated by broth dilution method. This was conducted in triplicate in a liquid medium (malt extract) that contained different concentration of these antifungal agent in contact with inoculum suspension according to the method described previously [14] and incubated at 25°C for 6 days. The MIC was defined as the lowest antifungal agent concentration at which there was a visual absence of growth compared to that produced by the growth control tube [15]. In order to evaluate the MFC, fractions of 20 µl from the tubes showing no growth were aseptically transferred onto new tubes containing 980 µl of sterile liquid medium. The tubes were incubated at 25°C until growth was visible in the growth control. The MFC was defined as the lowest antifungal agent concentration at which there was a visual absence of growth [16].

3 RESULTS

3.1 IDENTIFICATION OF THE FUNGI ISOLATED FROM CORN GRAINS

Isolated fungi belonged to genera *Aspergillus*, *Penicillium*, *Fusarium* and *mucor*. Relative densities of different fungal genera based on total fungal isolates were *Aspergillus*, 45%; *Penicillium*, 10%; *Fusarium*, 8%; and *Mucor*, 12 %. The remaining 25% are represented by other genera unknown that we have not insisted to identify, as the most popular molds are already there (figure 1 and 2). Relative density among species was maximum for *A. flavus* and *A. niger*, followed by *Mucor* sp. *Penicillium* sp., and *Fusarium* sp.

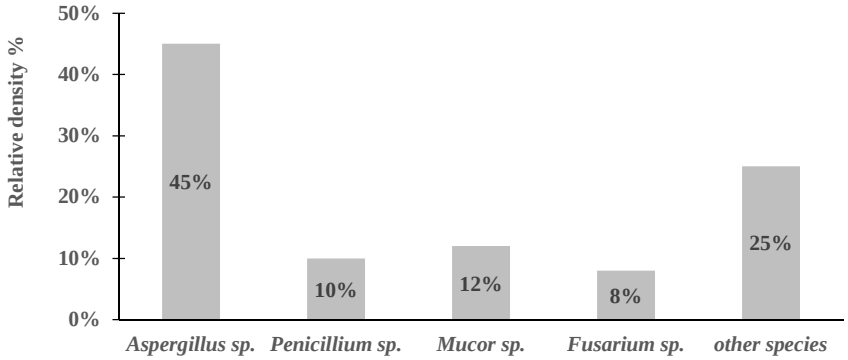

Fig. 1. Relative density of different fungi isolated from corn grains.

Fig. 2. Photographs of plates of pure culture of some fungi isolated from corn grains. (MEA at 25°C)

3.2 ANTIFUNGAL EFFECT OF OREGANO ESSENTIAL OIL AND THYMOL AGAINST FUNGI ISOLATED FROM CORN GRAINS

Table 1. Present the results of MIC and MFC of oregano EO and thymol against some fungi isolates from corn grains. Oregano EO and thymol inhibit the growth of all the tested fungi with some differences of MIC and MFC values. Among the fungi, *Mucor* sp. proved to be the most sensitive with similar MIC and CMF values (0.0125 % for oregano EO and 0.01 % for thymol), followed by *Fusarium* sp. (MIC=MFC: 0.025 % for oregano EO and 0.01% for thymol). Concerning *Aspergillus* sp., the values of MIC and MFC of thymol were between 0.016 and 0.05%, while the values of MIC and MFC of oregano EO were between 0.025 and 0.1%. However, compared to the others species, *Penicillium* sp. seems to be less sensitive, with MIC and MFC values between 0.1 and 0.2% for oregano EO. However, thymol exhibited a strong antifungal effect on *Penicillium* species with low MIC and MFC values between 0.01 and 0.1%.

Table 1. MIC and MFC of oregano essential oil and thymol against some fungal species isolated from corn grains.

	Oregano EO (%)		Thymol (%)	
Isolated fungi	**MIC**	**MFC**	**MIC**	**MFC**
***Mucor* sp.**	**0.0125**	**0.0125**	**0.01**	**0.01**
***Fusarium* sp.**	**0.025**	**0.025**	**0.01**	**0.01**
Aspergillus flavus	**0.025**	**0.1**	**0.016**	**0.05**
Aspergillus niger	**0.05**	**0.05**	**0.025**	**0.025**
***Penicillium* sp.**	**0.1**	**0.1**	**0.01**	**0.01**
Penicillium. citrinum	**0.2**	**0.2**	**0.05**	**0.1**

4 DISCUSSION

In this work, we tried to isolate some fungi that naturally contaminate corn grain. As a result, several molds were found, including *Aspergillus*, *Penicillium*, *Fusarium* and *Mucor*. They are generally known as storage fungi [17], [18]. There are many reports indicating that these fungi species are harmful for human and animal health. In the one hand, these molds reduce the food's nutritive value, and on the other hand, they are expected to secrete mycotoxins that may cause many different adverse health effects because they pass through the food chain [3], [19]. It is therefore necessary to use antifungal agents to reduce this mycoflora. Storage fungi are commonly controlled by synthetic compounds to increase the shelf life of food, among them fungicides are used to prevent the growth of microorganisms that may cause spoilage or food poisoning. However, a major concern is the emergence of fungal strains resistant to these antifungal agents [6], [8] and the presence of residues of these agents in the food [7]. Hence, natural alternatives are needed to substitute the evidence of the use of these chemical preservatives agents. In this perspective, we evaluated the *in vitro* inhibitory effect of oregano EO and thymol on the growth of some fungi isolated from naturally contaminated corn kernels. The oregano EO and thymol were chosen in this study since they have proven to be the most effective in other tests [20]. The results of the present work showed also that oregano EO and thymol have an antifungal activity on all fungi isolated from corn kernels with occasionally differences of MIC and MFC values. These differences may be explained either by the strains sensitivity vis-a-vis the tested agents, or by the nature of the antifungal agent. The genera *Mucor* seems to be the most sensitive to thymol and oregano EO followed by *Aspergillus* and *Fusarium*. However, *Penicillium* appears less sensitive with MIC and MFC values slightly higher. In addition, thymol had better effect than oregano EO. These results are in agreement with other workers [21], which showed that oregano EO inhibited the growth rate and spore germination of spoilage fungi such as *A. niger*, *A. ochraceus* and *A. flavus*. In the same way, some investigators have reported that oregano EO has an important antifungal effect against many *Aspergillus* species including *A. flavus* [22], MIC and MFC values obtained by these authors are proximate to ours. These results are also in agreement with other finding that showed an inhibitory effect of eugenol and thymol on the growth of *Penicillium citrinum* as well as the production of citrinin [23]. A recent study have reported that volatile phase of combinations of thyme oil and clove oil showed good potential in the inhibition of growth of *Penicillium* sp. [24]. Other workers have shown the efficacy of five phenolic compounds, namely eugenol, thymol, methyl cinnamate, linalool and 1,8- cineole against some phyto-pathogenic fungi, these authors have proved that thymol is the most effective on the all studied fungi [25]. Our finding are also in agreement with those, which demonstrated that oregano EO was more effective than the others EOs tested on postharvest fungi such as *A. flavus* and *Penicillium* sp. [26]. They have also described that the main compound in oregano EO, thymol, was found to be among the best oils to fight storage fungi and they suggest therefore that oregano EO can be used as natural alternative to control fungi that produce mycotoxin in maize.

5 CONCLUSION

Essential oils and some of their phenolic compounds, such as oregano and thymol, could be safely applied during the storage of cereals and foodstuffs as a natural alternative to chemical fungicides commonly used. Supplementary investigations on maize grain might be assessed to study the effectiveness of these natural agents to prevent and to stop the developpement of mold on maize grain.

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