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ESSENTIAL OILS ANTIMICROBIAL ACTIVITY IN LIMITING THE DEVELOPMENT OF MAIN SPOILAGE FUNGI ASSOCIATED WITH STORED CEREALS

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ABSTRACT

Fungal pathogens can significantly reduce the potential yield and seed quality of cereal crops, the major risk is contamination with toxic fungal secondary metabolites known as mycotoxins, which have negative consequences in human and animal health.

The influence of different concentrations of the oregano, basil and thyme essential oils on the development of the mycelium of *Fusarium*, *Aspergillus*, *Penicilium* species was analyzed and action of inhibiting the capacity of molds' occurrence and development at stored wheat and maize. The chemical composition of the all essential oils was determined by gas chromatography/mass spectrometry analysis. Oregano and basil essential oils inhibit completely development of *Fusarium* spp., *Penicillium* sp. and *Aspergillus* sp. pathogens at 2000-5000 ppm concentration exerted a fungitoxic effect. However, thyme oil in 10-100 µl/ml concentration was the strongest in fungicidal activity of the tested *Fusarium, Penicilium, Aspergillus,* species.

In conclusion, all essential oils used in this study could be suitable for applications in the food industry to control molds and improve the safety of stored grain.

Keywords: thyme oil, basil oil, wheat, corn, molds.

INTRODUCTION

The use of plant extracts as an alternative to synthetic chemicals for the control of phytopathogenic fungi with mycotoxigenic action has gained popularity as a result of the increased awareness of the polluting, residual, carcinogenic and phytotoxic effect of many chemical based fungicides. Worldwide, at the level of the scientific community, a series of environmentally friendly bioactive plant principles have been discovered and a multitude of researches have been carried out on the potential of essential oils and mixtures of volatile organic compounds, as biopesticides and alternative bio-fumigant products for use them in sustainable agriculture.

Essential oils are volatile substances commonly produced by different part of plants (stems, bark, leaves, roots, flowers, fruits) to provide a competitive advantage for food resources and protection from pests. These, have in their composition, large amounts of terpenoids, occurring in the form of sesquiterpenes and for the most part in the form of monoterpenes as well as aromatic and aliphatic compounds (Grzanka et al., 2021).

Essential oils are biologically active compounds due to their antimicrobial, antimycotoxigenic, antioxidant, antiparasitic and bioregulatory properties, being recognized as having good fungi-toxic activity (Saleem et al., 2014; Chaieb et al., 2018; Mutlu-Ingok et al., 2020). The bioactive compounds and derivatives of essential oils are considered eco-efficient alternatives to control diseases (Matusinsky et al., 2015; Strelková et al., 2021) and pests (Tripathi et al., 2009; Saleem et al., 2014) of cultivated plants and their rapid degradability in the environment develops specificity and favors beneficial organisms.

Essential oils are plant protection products of natural origin and some experiments

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indicated possibility of their use as fungicides (Nguefack et al., 2008; Amini et al., 2012; Kocić-Tanackov et al., 2012). Pérez et al. (2010) analyzed the activity of essential oils as a biorational alternative to control phytopathogenic fungi and coleopteran insects in stored grains.

The essential oils of oregano and thyme were used as fumigants and were applied against the growth of mycelia and spores of *Aspergillus flavus*, *Apergillus niger* as well as against the natural microflora of wheat grains (Paster et al., 1995). They also emphasized the fungitoxicity of these essential oils against fungi that attack stored grains and the possibility of using them as an alternative to the chemical treatments used to preserve stored wheat.

The *in vitro* antimicrobial activity of essential oils (EOs) has been studied against a number of microorganisms, usually using direct-contact antimicrobial assays, such as different types of diffusion or dilution methods, as reviewed by some literatures (Janisiewicz and Korsten, 2002; Tripathi and Dubey, 2004; Holley and Patel, 2005).

The aim of this study was to analyze the chemical composition of oregano, basil, thyme essential oils and examine the antifungal and phytotoxic effect on the main moulds genera that contaminates stored cereal grains.

MATERIAL AND METHODS

Essential oils

The essential oil of oregano, thyme and basil were purchased from Solaris Society and stored at +4°C in a refrigerator until analysis. The essential oils used in the experiments were subjected to Gas Chromatography-Mass Spectroscopy (GC-MS) analysis in order to determine the percentage of the main volatile compounds. The analysis was carried out using 7000 Triple Quad GC/MS Agilent. A DB-WAX capillary column of 30 m x 0.25 mm and 0.25 in film thickness was used. Helium was used as the carrier gas with a flow rate of 1.4 ml min^{-1}. The column was temperature programmed as follows: 2 min at 70°C; then raises with 10°C/min to 220°C and held for 10 min. The injector and detector temperatures were to 220 and 250°C, respectively. Injection was carried out automatic mode. Peak areas and retention times were measured by Electronic Integration. Chemical compounds of the essential oils were identified by matching mass spectra with mass spectral libraries (NIST08). The concentration of the identified compound was calculated based on the normalization of the relative peak area (%).

Microorganisms

The biological material used consisted in fungal strains from the RDIPP microorganisms' collection, isolated from wheat and maize caryopses samples, collected from a private storage unit located in Călăraşi County. The fungal strains were isolated by classic technique using Ulster method (Hulea, 1969; Raicu and Baciu, 1978) and successive sub-culturing in Petri dishes of 10 mm diameter with potato-dextrose-agar growth medium, incubated at 24ºC (Radu et al., 2011).

The strains were classified as belonging to *Fusarium, Aspergillus,* and *Penicillium* genera based on macroscopic and microscopic observations compared with Leslie and Summerell's Fusarium Laboratory Manual and identified at species level by PCR ITS RFLP molecular technique (Zaharia et al., 2022). Stock cultures of fungal strains were grown on potato-dextrose-agar medium at 26°C for 7 days before the experiment.

The pathogenic fungi used in the experiments consisted of a series of isolate, respectively, *Fusarium graminearum* - FS2, *Fusarium tricinctum* - FS1, *Aspergillus* sp. - Asp. Vers., *Penicillium expansum* - Pen 2, *Penicillium* spp. - Pen 1, P,iz.m., *Aspergillus flavus* - P3, *Aspergillus niger* - A.nig, isolated from wheat and maize seeds.

In order to highlight the antimicrobial activity of the selected essential oils and its efficacy we used the disk volatilisation method. After solidification of PDA medium, Petri plates were kept in an inverted position. Inside each lid of the Petri dishes, a sterile 30 mm filter paper was placed in the centre and fixed there with a small amount of agarized medium. The filter paper was soaked with different concentrations of volatile essential oil using a micropipette. In parallel, the Petri dishes were inoculated with fungal mycelium of 5 mm diameter from each fungal strain. The Petri plates were sealed with parafilm and incubated for 7 days, in thermostatic chamber set at the optimal growth temperature of each fungus. Observations and measurements regarding the growth and development of the colonies were performed 3 and 7 days after the treatment. All treatment were performed in three replicates.

RESULTS AND DISCUSSION

The chemical composition of the three essential oils analysed by GC/MS is presented in Figure 1, Figure 2 and Figure 3. Compound identification was obtained by comparing retention times and mass spectra with those of the NIST database.

As shown in figure below, the compounds of oregano essential oil analysed had a high percentage of carvacol - 69.6%, followed by 15.73% o-cymene, 4.86% caryophyllene, 2.12% terpinene; 1.13% limonene and 1.21% α-pinene.

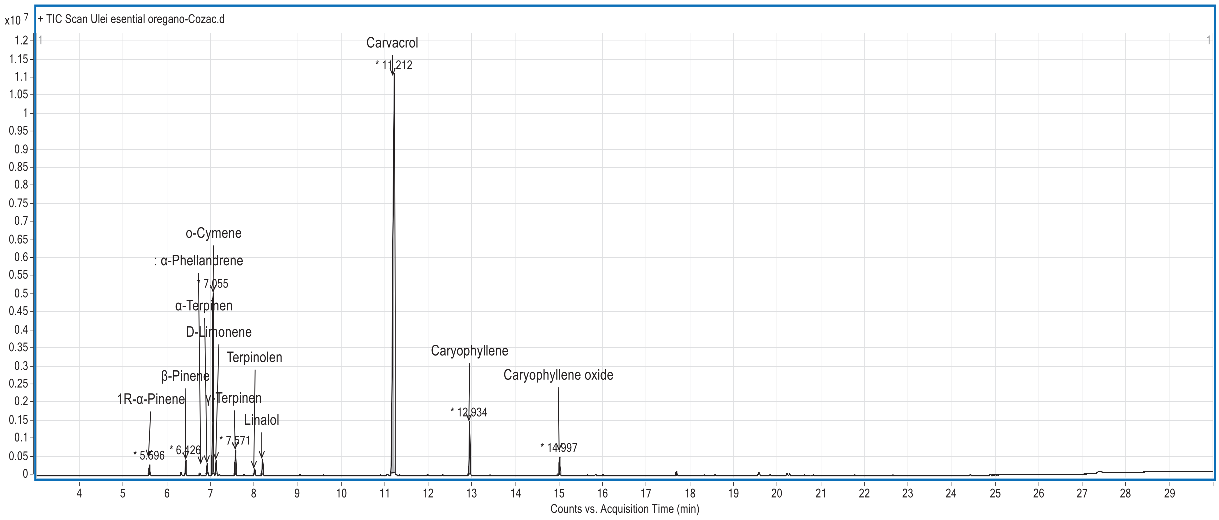

Figure 1. Oregano essential oil major compounds

The predominant component of basil essential oil analyzed as can be seen in Figure 2, was Estragole - 65.18%, followed by 26.98% Linalool, 2.38% Euclyptol and 2.06% Bergamot.

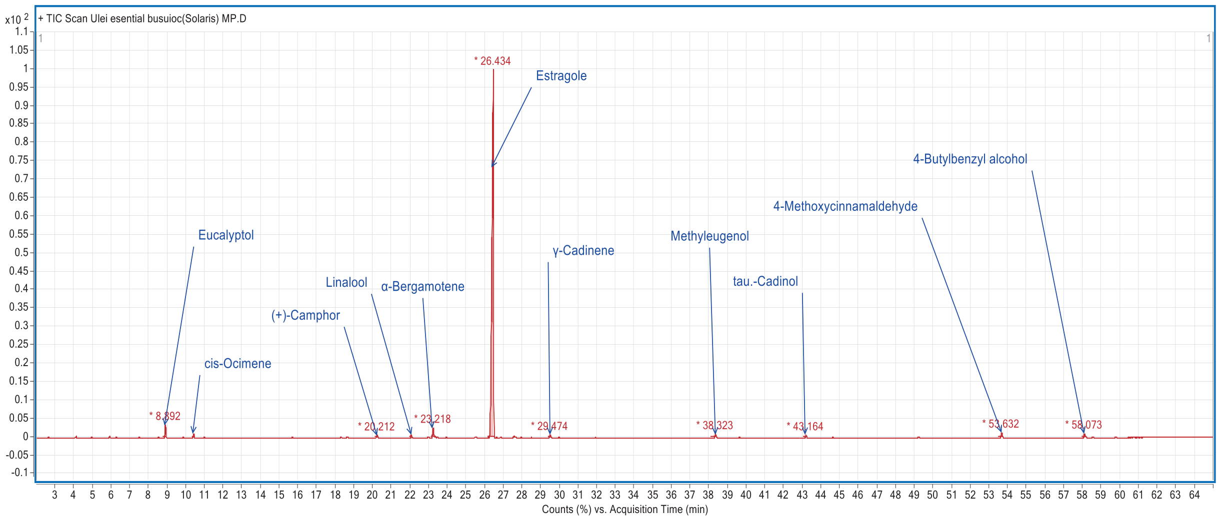

Figure 2. Basil essential oil major compounds

The main compounds of thyme essential oil analyzed as can be seen from the chromatogram (Figure 3) were Thymol 42.5%, followed by o-Cymene 18.11%, γ-Terpinene 9.18%, β-Caryophyllene 7.95% and Limonene 6.95%. This results are accordance with those obtained byother authors (Nikolic et al., 2014; Matusinsky et al., 2015).

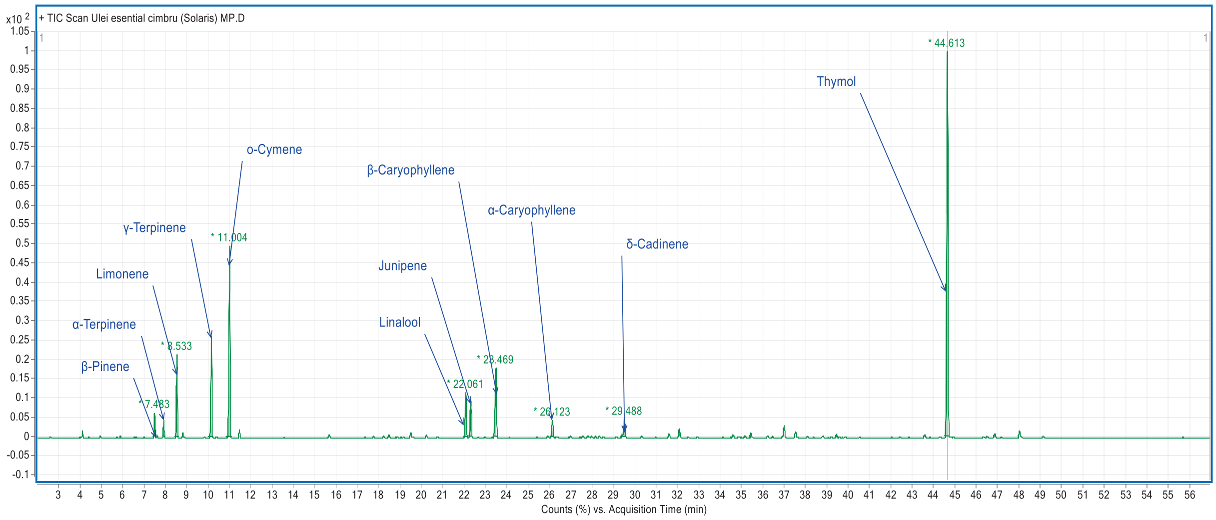

Figure 3. Thyme essential oil major compounds (Solaris, Romania)

All the EOs showed different inhibitory effects on growth of isolate storage fungi studied. However, the extent of inhibition was widely dependent upon the composition and the concentration of EOs.

The inhibitory effect of the basil essential oil was studied to *Fusarium* sp. FS1 and *Penicillium* sp. - Pen 1. The tested doses for essential oil were 50, 500, 1000, 2000, 3000 and 5000 ppm, and the control variant was treated with sterile distilled water. As can be seen in the Figure 4 below, both fungal isolates were susceptible to volatile oil vapours.

Figure 4. Penicillium sp. inhibited by different basil EO concentrations compared with negative control

Regarding the efficacy of basil oil on *Penicillium* sp. it was recorded that in the dose range 50 ppm - 1000 ppm (Figure 5) basil oil had a fungistatic effect, the fungus showed weak growth in the presence of oil vapours, depigmented colonies and very few fructifications compared to the untreated control. Starting from the value of 2000 ppm, basil oil exerted a fungi-toxic effect, totally suppressing the growth and development of the fungus.

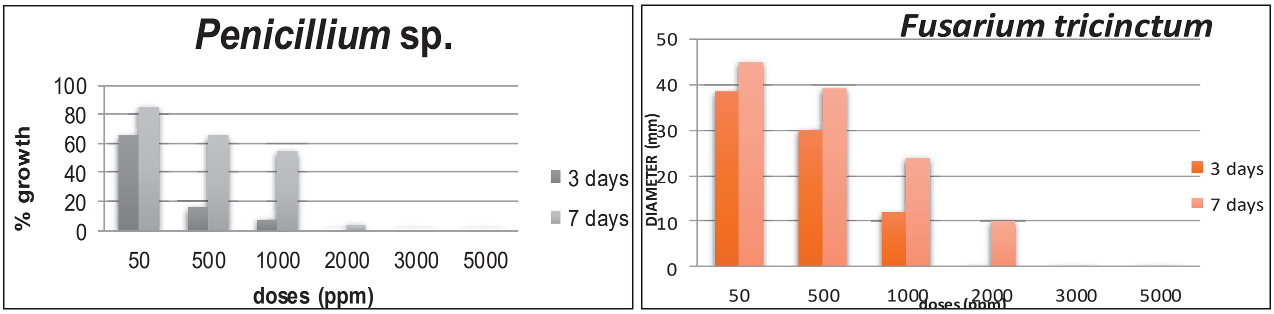

Figure 5. Effect of basil EO at different concentration on mycelial growth of *Penicillium* sp. and *Fusarium* sp.

In a previous study, essential oils obtained from oregano, thyme, fennel, rosemary and lavender plants were found to inhibit the growth of *Phytophthora infestans* (Soylu et al., 2006).

Sumalan's et al. (2013) experiments demonstrated that essential oils could be recommended as natural preservatives for stored cereals. They showed that the inhibitory effect of essential oils from *Melissa officinalis, Salvia officinalis, Coriandrum sativum, Thymus vulgaris, Mentha piperita* against natural mycoflora on stored seed was dose dependent.

The *Fusarium* species showed similar behaviour in the presence of basil essential oil, it had a growth with good vegetative mass but weak sporulation, in the range of 50 ppm and 1000 ppm, followed by a sudden decline starting with the concentration of 2000 ppm. The results obtained are similar to those obtained by Dambolena et al. (2010) who evaluated the antifungal activity of oils from different varieties of basil against *Fusarium* spp. It was also found that the degree of inhibition depends on the humidity of the cereal grains and the type of strain analysed.

In the presence of the oregano essential oil applied in doses of 50, 500, 1000, 2000, 3000 and 5000 ppm, the *Fusarium* and *Aspergillus* strains were inhibited completely even by the lowest treatment dose, the results being similar to Perczak et al. (2019) who reported the strong antifungal effect of oregano oil against phytopathogenic fungi *F. culmorum* and *F. graminearum* in wheat grains under laboratory conditions. Also, some authors, Paster et al. (1995) emphasized the fungitoxicity of oregano essential oil against *Aspergillus flavus, Apergillus niger* fungi that attack stored grains and the possibility of its use as an alternative to chemical treatments used to preserve stored wheat.

Essential oils of thyme are generally strongly associated with their content in monoterpene phenols, thymol, carvacrol, cymene, eugenol, estragole, and limonene. Both thymol and cymene and carvacrol are present in thyme essential oil and are characterized by strong antimicrobial activity (Bouzidi et al., 2013). Antifungal activity of thyme essential oil has been demonstrated against different fungi such as *Botrytis cinerea*, *Aspergillus* spp. (Paster et al., 1995), *Fusarium* spp. (Kumar et al., 2016).

The results obtained from our experiments show that thyme essential oil partially inhibited the mycelial growth of both *Penicilium*, *Fusarium* and *Aspergillus* species at concentrations of 1 µl/ml, the diameters of the colonies developed with this dose were lower than those of the control. However, thyme essential oil at 10 and 100 µl/ml concentrations totally inhibits growth of all tested fungi.

Consequently, the increase in the concentration of thyme essential oil leads to the increase in the speed of inhibition of the pathogenic fungi development. This aspect is also supported by Rasooli et al. (2006), whose results demonstrated that the application of thyme oil in increasing concentrations leads to the degradation of the cytoplasm and the thickening of the cell wall of pathogenic fungi. The antifungal effect of thyme essential oil can be explained by the structural and functional damage of the fungal cells that can lead to changes in the integrity of the hyphae and the disaggregation of the permeability of the plasma membrane and the osmotic balance of the cell.

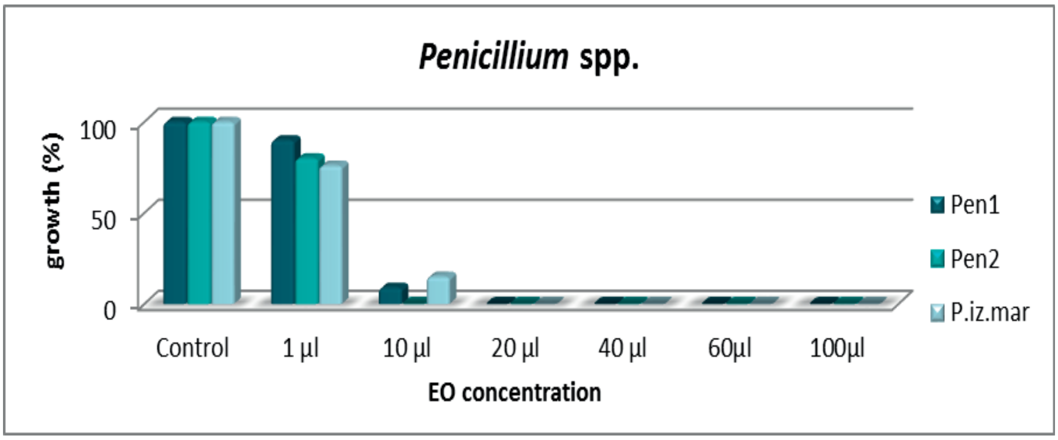

Figure 6. Effect of basil EO at different concentration on mycelial growth of *Penicillium* sp. and *Fusarium* sp.

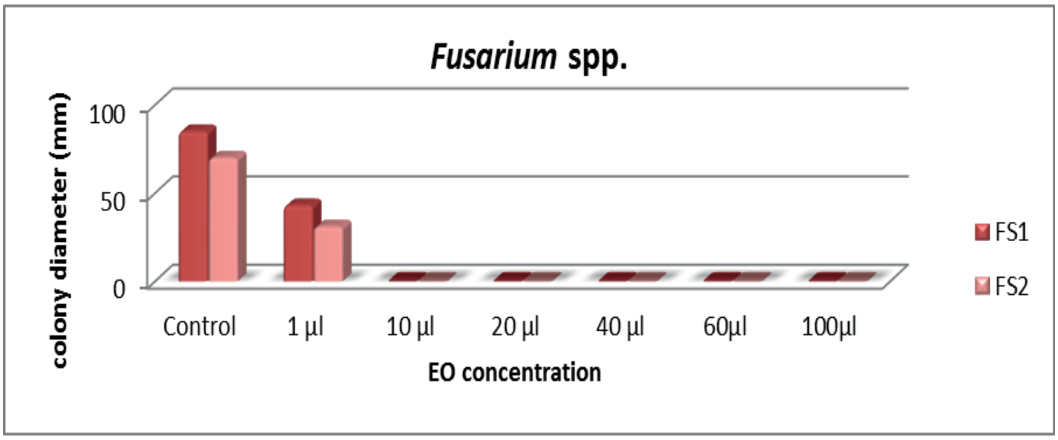

Figure 7. Effect of thyme EO at different concentration on mycelial growth of *Fusarium* sp.

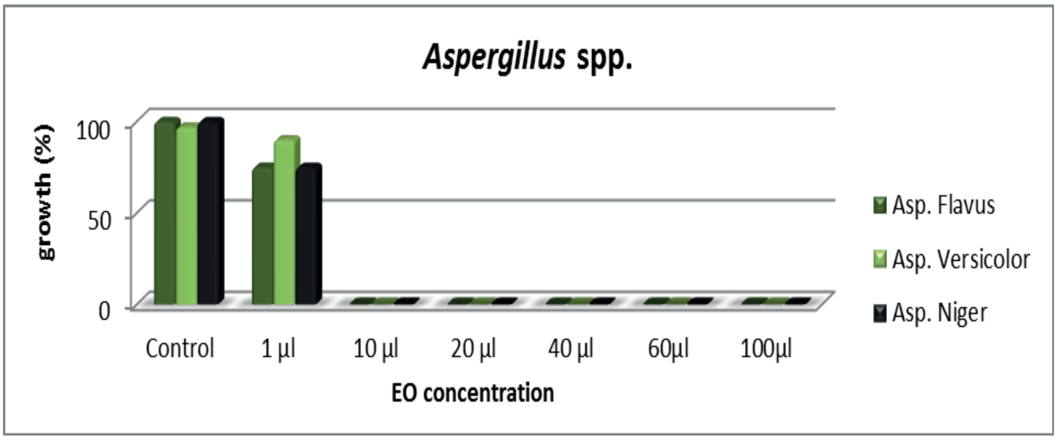

Figure 8. Effect of thyme EO at different concentration on mycelial growth of *Aspergillus* spp.

The results obtained are in accordance with those obtained by Paster et al. (1995) who showed that thyme oil in concentrations of 10, 15 and 20 µl/ml significantly inhibited the contamination with molds of the stored grains compared to the untreated control. The studies of researchers Anžlovar et al. (2017) showed a broad spectrum of phytotoxicity of thyme essential oil in vitro against pathogenic fungi of the genus *Fusarium* sp. and *Aspergilus* spp. in wheat during storage. The results of Matusinsky et al. (2015) show however, that thyme oil in a low concentration of 1 µl/ml led to a 98% inhibition of the growth of different strains of *Fusarium* spp.

CONCLUSIONS

Among the constituents of oregano essential oil, the carvacol, o-cymene, cariofilen, terpinen, limonene și α-pinen compounds have shown a stronger antifungal activity.

Also the compounds involved in the antimycotic action of basil essential oil used in these studies are represented by estragole, linalool, eucalyptol and bergamoten.

Thymol, o-cymene, γ-terpinene, β-caryophyllene and limonene were found to be as predominant constituents of thyme essential oil.

Total inhibition of all tested pathogenic fungi by thyme essential oil was observed especially at 10-100 µl/ml concentration, however at 1 µl/ml concentration inhibited partially mycelial growth.

Oregano and basil essential oils can significantly reduce the development of pathogens of the *Fusarium* spp., *Penicillium* sp. and *Aspergillus* sp. at 50-1000 ppm concentration exerted a fungistatic effect but in concentration 2000-5000 ppm a fungitoxic effect was observed, the growth of the isolates being completely inhibited.

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