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Vapor Phase Application of *Thymus vulgaris* Essential Oil to Control the Biodeteriogenic Fungus *Alternaria alternata*

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Featured Application

The obtained results on the application of thyme essential oil in the vapor phase provide useful indication for the control of biodeteriogenic filamentous fungi in indoor air such as archives and libraries. Tests on the model organism, *A. alternata*, involve exposure to EO vapors for more than 40 days, allowing us to define the duration of the growth inhibition supplying a practical indication for the EO treatment in a real contaminated library.

Abstract

In the present work, the antimicrobial efficacy of *Thymus vulgaris* essential oil (EO) was investigated on *Alternaria alternata* strain BNR; a paper biodeteriogen was used as a model for a contaminated library. The influence of EO volume and diffusion modality, treatment duration, and inoculum age was evaluated in the vapor phase. In Petri dish screening, the influence of different EO volumes (5, 7.5, and 10 µL) on the microbial growth lag phase was investigated, and the growth inhibition period was established. The most effective treatment (10 µL EO) was then scaled up in a glass airtight container of 2650 cm^3; a cold diffusion method was applied in order to quickly reach the maximum concentration of active compounds in the vapor phase. These tests demonstrated that EO efficacy is affected by the inoculum age and the contact time, and that the treatment should be performed as early as is feasible. A mycostatic effect was confirmed to be proportional to the utilized EO volume and independent from the treatment method. The information obtained in the present work will be applied to the set-up of an EO treatment in a library characterized by different levels of air contamination.

Keywords: essential oil; MID and MAM; cold diffusion method; natural biocide

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1. Introduction

The antimicrobial and antioxidant properties of essential oils (EOs) and their hydrolates (Hys) have been recognized for more than 60 years, and have been applied in different fields such as cosmetic, food flavors, cooking, therapeutic, perfumery, and active packaging [1–4]. Extensive reviews were published on EOs and Hys biological properties and potential applications [5–7]. In recent years, several scientific papers faced the problem of biodeteriogenic microorganisms, both at the laboratory scale [8,9] and in real cultural heritage areas [10]. In these concerns, EOs were applied in the vapor phase for two hours in an unventilated indoor space, and the microbial contamination was monitored after a maximum of 24 h. Moreover, as reviewed by [8], treating paper items with oil in the vapor phase allows us to avoid significant damage in terms of color alterations or pH acidification.

The antimicrobial effect of EOs in the vapor phase can be expressed by means of the minimum inhibitory dose (MID), that is, the minimum dose in the gaseous phase able to inhibit microbial growth [11]. MID, a parallel method to the minimum inhibitory concentration (MIC) for EO in liquid phase, must be evaluated in a sealed container at a controlled temperature since it depends on the amount of the volatile components in the vapor phase. A slow evaporation rate can be obtained by placing the pure EO in a (glass) container, while a more rapid evaporation rate can be obtained by using different methods: through a filter paper impregnated with pure or diluted EO (e.g., in ethyl acetate or Dimethyl Sulphoxide (DMSO)) or by spraying small droplets that immediately turn into vapor [12]. Since heating the EOs can destroy or modify some of their components and thus can affect their antimicrobial efficacy, temperature control is a fundamental issue [13].

Bozin et al. [14] investigated the antimicrobial activity of *Ocimum basilicum*, *Origanum vulgare*, and *Thymus vulgaris* EOs against 13 bacterial strains using the agar diffusion method, and against 6 fungal strains using the microdilution method. All of the tested EOs exhibited a low minimum fungicidal concentration (MFC) for some fungal strains, which was lower than the value obtained for bifonazole, a broad-spectrum antifungal drug; thyme and oregano EOs showed higher antimicrobial activity, probably due to the similar chemical profiles in which phenolic monoterpenes are the major components. In particular, carvacrol and thymol showed an antimicrobial effect against both Gram-positive and Gram-negative bacteria and fungi, and they also exhibit antibiofilm activity.

Puškárová et al. [15] investigated the antimicrobial activity of six EOs, containing different concentrations of phenolic components, against pathogenic and environmental bacterial strains and five different fungi, representative of microbial contaminants in the indoor air. Among the tested EOs, oregano, thyme, clove, and arborvitae showed very strong antibacterial activity against all tested strains. The EOs exhibited fungistatic and fungicidal activity depending on the applied method; in particular, their application in the vapor phase showed fungicidal activity. This result agrees with the growing evidence that EOs in the vapor phase are more effective against fungi [16,17]. The results confirmed those reported by Tullio et al. [18], which was related to the in vitro activity of some EOs against clinical and environmental fungal strains. The MIC was determined by a microdilution method and by a vapor contact assay. Results indicated that the EO activity depended on the experimental assay used and the EO inhibition in the vapor phase being higher than that of the liquid one. Also, in this case, thyme red and clove showed the widest spectrum of activity against all tested fungi.

Šĕgvić-Klarić et al. [19] studied the antifungal activity of the thyme EO and pure thymol on different genera of molds isolated from damp dwellings: *Aspergillus*, *Penicillum*, *Alternaria*, and *Trichoderma*. The antifungal activity was determined by the dilution method and the vapor phase assay. The results confirmed that the EO vapor phase exhibited long-lasting suppressive activity on fungal growth and that thyme EO possesses a wide range spectrum of fungicidal activity, as was also reported by Pietrzak et al. [20]. Zabka et al. [21] tested the antifungal activity of 20 EOs against *Alternaria alternata*, *Stachybotrys chartarum*, *Cladosporium cladosporioides* and *Aspergillus niger*. The results obtained evidenced that *Origanum vulgare*, *Pimenta racemosa*, and *Thymus vulgaris*, EOs with a high content of thymol, carvacrol, or eugenol, had the highest antifungal effects and the lowest MIC values. Regarding the toxicity of *O. vulgare* and *T. vulgaris* EOs, Ahmad et al. [22], by means of the in vitro haemolytic assay, demonstrated that thymol and carvacrol are significantly less cytotoxic than conventional antifungals. Moreover, they are quickly metabolized and excreted in human and animals, thus providing a very promising "safe" alternative in the field of antifungal applications. Over the past 20 years, different EOs and their main constituents were investigated as antimicrobial agents for control of biodeteriogenic microorganisms

in archives and libraries; in particular, the research focused on the antifungal activity in vapor phase, evaluated at laboratory scale by means of micro-atmosphere method (MAM). Rakotonirainy et al. [23] studied the antifungal activity of nine EOs and five of their main constituents against a mixture of fungal strains isolated from historical archives. The MAM, in Petri dishes, was applied as a preliminary screening to obtain the Minimum Fungicidal Concentration (MFC) value; after that, the experiment was carried out in a test chamber with a larger volume. Paper sheets, inoculated with the fungi, were inserted in a book and the EOs were arranged at the bottom of the chamber for 21 days, in controlled conditions of temperature and humidity. In the last case, the linalool oil had only a fungistatic effect rather than a fungicidal one as observed in Petri dishes. Among the different tested EOs, tea tree and lavender ones and their main components, eugenol and linalool, were particularly active in inhibiting fungal strain growth.

In the work of Lavin et al. [24], MAM was used to evaluate the biocidal activity of *O. vulgare* and *T. vulgaris* EOs against *Scopulariopsis* sp. and *Fusarium* sp., isolated from documentary heritage. The EOs tested showed a high level of activity against both fungal isolates and a low environmental impact. Pietrzak et al. [20] also underlined that EO treatments of archival materials must ensure safety of treated goods and personnel along with treatment efficacy. The use of oregano EOs' volatile compounds against oil painting biodeteriogens [25] was proposed as innovative and eco-friendly cleaning method.

The aim of the present work was to gather information about the possibility to apply red thyme EO in a library, mainly contaminated by filamentous fungi. To this end, the fungus *Alternaria alternata,* used as a model of paper biodeteriogens, was treated with *Thymus vulgaris* oil in vapor phase. The MAM in Petri dishes was used as preliminary screening, the treatment was then scaled up in an airtight container to test the influence of different evaporation rate on MID value.

2. Materials and Methods

2.1. The Microorganism and Media

Alternaria alternata BNR strain, a biodeteriogen filamentous fungus, was tested. It was maintained at +4 °C on Malt Extract agar, MEA, (20 g/L Malt extract cod. 1.05391, 2 g/L Neutralised bacteriological peptone cod. 90765, 20 g/L D(+) Glucose cod. 49139, 2% *v*/*v* Agar cod. 05040) or on Czapeck, CZ, agar (3 g/L $NaNO_3$ cod. S5022, 1 g/L K_2HPO_4 cod. 60353, 0.5 g/L $MgSO_4$ cod. M2643, 0.5 g/L KCl cod. P5405, 0.01 g/L $FeSO_4$ * 7 H_2O cod. F8263, 30 g/L Glucose, 2% *v*/*v* Agar) plates. MEA and CZ were also utilized for the fungus cultivation at 25 or 30 °C, as specified in Table 1 (see Section 2.3). Moreover, depending on the purpose of the test, *A. alternata* was cultivated on sterilized squared paper sheet (3.5 × 3.5 cm or 7 × 7 cm for 5.5 and 9 cm Ø Petri dishes, respectively; paper was sterilized in autoclave at 121 °C, pressure 2 atm for 20 min) laid down on CZ or CZ prepared without Glucose (CZ_{mod}). All the utilized reagents were of analytical grade and were provided by MERCK KGaA (Darmstadt, Germany).

2.2. Micro-Atmosphere Method (MAM) in Petri Dishes with Thyme EO

Thyme EO (*Thymus vulgaris*, cod. OE0970), commercially available, was purchased from Erboristeria Magentina-Witt Italia Spa Company, Poirino, Italy. The company provided the following information: the EO chemotype is thymol and the main components are Thymol (54.1%), *p*-Cymene (23%), α-Terpinene (1.1%), γ-Terpinene (4.8%).

Thyme EO was applied pure or diluted using Dimethyl Sulfoxide, DMSO cod. D4540. The Micro-atmosphere Method (MAM) was carried out into 15 mm high Polystyrene, PS, Petri dishes with 90 mm diameter, filled with 20 mL of MEA; the agarized medium was 5 mm high, giving a free head space of 63.62 cm^3. *A. alternata* was inoculated in the middle

of the dish by means of a mycelial plug (Ø 8 mm), cut from the edge of a 7-day old colony, grown at 30 °C on MEA. After 16 h of incubation, which allowed the mycelium to adhere to the agar surface, the EO, pure or DMSO diluted, was poured onto a sterile filter paper disk (Ø 1 cm, Whatman n°1) attached in the middle of the dish lid. Disks without EO, soaked with water or DMSO, were used for control samples. Filter disks were sterilized in autoclave; this sterilization method was also utilized for all the glassware.

Different EO concentrations in the vapor phase were obtained soaking the paper disk with pure or DMSO diluted EO. More precisely 10, 7.5, or 5 µL of pure EO or 10 µL of 50% (5 µL of pure EO and 5 µL DMSO) or 75% (7.5 µL of pure EO and 2.5 µL of DMSO) were applied. Each sample and control were prepared in triplicate. All the described operations were carried out in sterile conditions. Petri dishes, closed with Parafilm and enveloped with transparent cellophane film to limit the loss of EO vapors, were incubated at 30 °C. To check if the EO tested concentrations were mycostatic or mycocidal, after different incubation times (6 h, 1 day, 2, and 7 days), Petri lids were substituted with new ones without the soaked paper and the incubation was extended till to 42 days. Details about this test are listed in Table 1.

2.3. Scale-Up and Optimization of the EO Treatment in GAC

The treatment in Petri dishes (head space 63.62 cm^3) with 10 µL thyme EO (calculated vapor phase concentration $1.57 \cdot 10^{-4}$) was scaled up in glass airtight containers (head space 2650 cm^3) (see Table 1). The concentration of the EO in the free space may be simply calculated dividing the total EO volume by the volume of the free space assuming that the whole amount of EO is vaporized during the test. This hypothesis may be verified considering the vapor pressure of the main EO components. At 25 °C, the vapor pressure of the pure components are the followings: $4.47 \cdot 10^{-5}$ bar for Thymol, $4.77 \cdot 10^{-4}$ bar for *p*-Cymene, $2.57 \cdot 10^{-5}$ bar for α-Terpinene and $7.432 \cdot 10^{-5}$ bar for γ-Terpinene [26–29].

Assuming an ideal behavior, it is possible to calculate the vapor pressure of each component in the solution equal to the product of the pure component vapor pressure and its molar fraction and this value results higher than the EO partial pressure in the free volume during the vaporization process.

The containers are characterized by a shrinkage (see Figure A1) which allows to fit a plastic grid for Petri dish location. When 9 cm Ø Petri dishes were used, each GAC contained one dish, and both the control and EO treated samples were triplicated. Using 5.5 cm Ø Petri dishes it was possible to insert the three replicates (for the control and for the EO treated samples) in the same GAC.

The container size allowed to carry out the EO dispersion in three different modalities. In the first one, the EO, 210 or 420 µL (calculated vapor phase concentrations $0.79 \cdot 10^{-4}$ and $1.58 \cdot 10^{-4}$, respectively), was poured into a small glass beaker placed on the plastic grid. This experiment was carried out utilizing 90 mm diameter MEA Petri dishes, without the lid, inoculating *A. alternata* in the middle of the dish by means of a mycelial plug (Ø 8 mm). A control glass airtight container was set-up without EO, and all the containers were maintained at 25 °C (see Figure A1A).

In the second group of experiments the EO dispersion was realized by sticking a filter paper disk, 30 mm Ø, by means of bi-adhesive tape, on the top lid of the container; the disk was then soaked with 210 µL of EO. One or three Petri dishes, inoculated with *A. alternata* (inoculum age of 72 h), were positioned in GAC before the beginning of the treatment (see Figure A1B).

In the third set of experiments each glass container was saturated with the EO vapors using a waterless dispersion device (see Figure A1C); in this case 10 µL of EO (calculated vapor phase concentrations $0.377 \cdot 10^{-5}$) were poured onto a non-woven fabric (TNT) gauze

fixed inside the device and the dispersion was accelerated by the forced convection of air, result of a fan placed behind the gauze adjustment. During the saturation, the switched-on device was placed inside the closed glass container. After 30 min of dispersion, the device was quickly removed and Petri dishes with *A. alternata* (inoculum age of 24, 72, and 96 h) were immediately placed onto the plastic grid. These three methods aimed to get different oil dispersion rates: in the first one the rate of dispersion in the container was the lowest, while in the second it was accelerated thanks to the higher contact area between the oil and the environment, being the highest rate that obtained with the third method, thanks to the turbulence obtained through the fan. This set of experiments was carried out utilizing 55 mm diameter CZ_{mod} Petri dishes. CZ has been chosen as synthetic medium to have the possibility to replace the carbon source (i.e., paper sheet instead of glucose). In this case, *A. alternata* was inoculated in the middle of the dish by means of 30 µL of a spore suspension (optical density at 650 nm, OD_{650} 0.8–1.0), obtained from a 7-day old colony, grown at 25 °C on CZ.

The details of all the experiments, carried out in the presence of EO, are summarized in Table 1 and referred to the specific Section 3.

Table 1. Details of the experiments in the presence of thyme EO.

Section	Diffusion Method	Media	Petri Ø (cm)	Temperature (°C)	EO Amount	Disk Removal Time	Duration (Days)	Inoculum Type/Age (h)
Section 3.1.	MAM *	MEA	9	30	5, 7.5, 10 µL 50, 75% *v*/*v* in DMSO	7 days	42	M 72
Section 3.2.	MAM *	MEA	9	30	10 µL	6 h 1, 2, 7 days	42	M 72
Section 3.3.	GAC, Beaker **	MEA	9	25	420, 210 µL	-	42	M 72
Section 3.5.	GAC, paper disk *	CZ_{mod}	5.5	25	210 µL	-	21	M 72
Section 3.5.	GAC, Device **	CZ_{mod}	5.5	25	10 µL	-	8	S 24, 72, 96
Section 3.5.	GAC, Device **	CZ_{mod}	5.5	25	10 µL	-	24	S 72

* Vapor contact; ** Vaporisation. GAC = glass airtight container; M = mycelial plug; S = spore suspension.

2.4. Data Collection and Analysis

Throughout the experiments, every 24 h or for longer time intervals, fungal colonies were visually inspected and photographed; moreover, for each sample, two perpendicular diameters were measured, by means of a caliper, and averaged. Fungal colony growth rate was calculated according to (Equation (1)), where d_1 and d_2 are the averaged diameters, measured at two consecutive incubation times, that is t_1 and t_2:

$$\text{Growth rate} = (d_2 - d_1)/(t_2 - t_1) \quad (1)$$

Known the average diameter of the control, dC, and that of the treated samples, dS, at a certain treatment time, the Minimum Growth Inhibition (MGI%) was calculated according to Equation (2):

$$MGI\% = (dC - dS)/dC \times 100 \quad (2)$$

2.5. Statistical Analysis

The reported data are means of three biological replicates with their standard deviation (SD), shown in graphs as error bars (only for the treatment with EO diluted with DMSO, 4 biological replicates were prepared).

Three technical replicates (n = 9) were carried out for each EO volume and treatment time.

Differences between two given groups were tested for statistical significance using Student's *t*-test ($p < 0.05$), while those between more than two groups were evaluated by one way analysis of variance (ANOVA) followed by the Bonferroni test. Differences with $p < 0.05$ are considered to be statistically significant; "ns" is reported for $p > 0.05$, "*" for $p < 0.05$, "**" for $p < 0.01$, "***" for $p < 0.001$, and "****" for $p < 0.0001$.

3. Results

3.1. Effect of Thyme EO in Vapor Phase on A. alternata

Initially, the efficacy of thyme EO on the fungus *A. alternata* BNR strain was evaluated in Petri dishes with Malt extract agar by means of MAM. In Figure 1A, the mean diameter of the colonies was reported and the behavior obtained in tests carried out with EO, pure or diluted DMSO, were compared. The paper disk, soaked with thyme EO, was removed after 7 days of incubation to test the mycostatic or mycocidal effect: the plates treated with 5 µL EO and 50% EO in DMSO were both inhibited for only 3 days. For 7.5 µL EO and 75% in DMSO, a longer inhibition was observed (10 and 9 days, respectively). Finally, in the test with 10 µL EO, *A. alternata* growth was inhibited for the longest period (16 days). For all the tests, the inhibition percentage values (MGI%) of mycelial growth were calculated up to 15 days of culture, when the control (pure EO) reached the edge of the Petri dishes. As it is possible to observe in Figure 1B, colonies treated with 10 and 7.5 µL of pure EO showed the highest MGI%, with comparable values and a similar behavior until the 10th day. At the 8th day of incubation, corresponding to the paper disc removal, MGI% were 83.2 ± 1.4 and 82.11 ± 1.5, for 10 and 7.5 µL, respectively; the difference was not statistically significant (for the Student's *t*-test, *p* value is equal to 0.20, indicated in the graphic as "ns"). At 15th day, the MGI% were reliable different: 84 ± 1.8 and 88 ± 1.8 for 7.5 and 10 µL EO (for the Student's *t*-test, *p* is equal to $6.28E^{-6}$, indicated in the graphic as "****"). Concerning the other conditions tested, the inhibition time was reduced until the 7th–8th day of incubation.

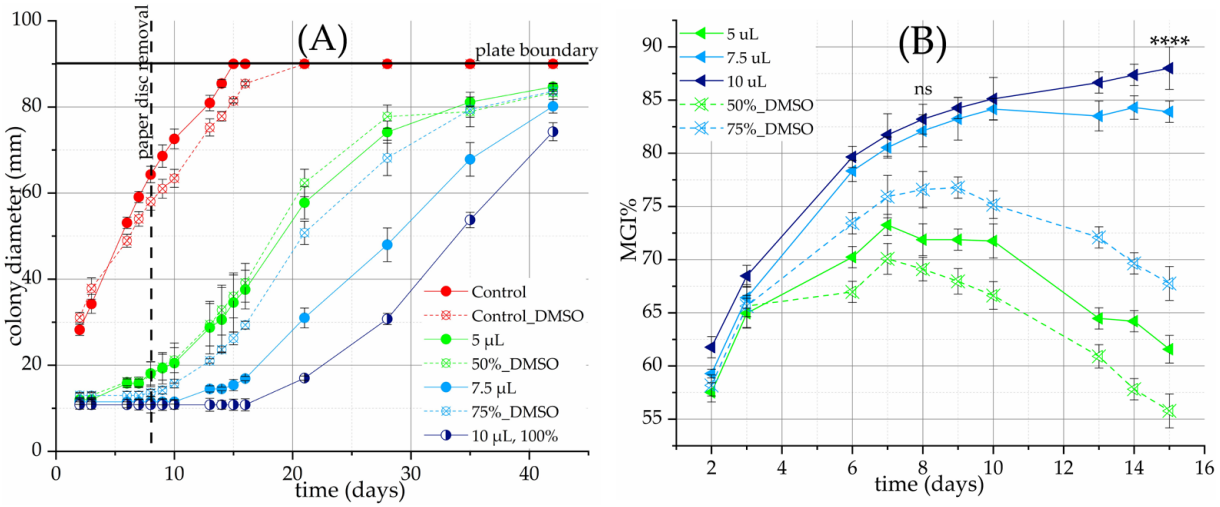

Figure 1. (**A**) MAM in presence of *A. alternata*: colony diameter, measured at different incubation times. Dashed line= time of paper disk removal. (**B**) MGI% values, for EO pure or diluted in DMSO, in the first 15 days of incubation (ns, $p = 0.20$; **** $p = 6.28 \times 10^{-6}$).

In Figure 2, the growth rate of *A. alternata* was reported. In the two controls, without and with DMSO, the maximum values were obtained at the 8th and 10th days of incubation (4.8 ± 0.16 and 5.5 ± 0.20 mm/day, respectively). In the presence of EOs, pure or diluted in DMSO, in all the samples the maximum growth rate diminished and was reached later, between the 21st and the 35th days of incubation. The growth rate values were in the range 3.8 ± 0.17–4.25 ± 0.23 with differences not statistically significant. As an example at 21 days of incubation *p* value for pure (5 µL) and DMSO diluted EO (50%) was equal to 0.18, indicated with "ns" in the graphic (Student's *t*-test). Comparing the obtained values with those of the corresponding controls, an inhibition of about 30% was calculated for all the pure EO volumes. As regards 50% and 75% EO in DMSO, lower inhibition percentages were obtained (10% and 20%, respectively). Since the low miscibility of the EO in DMSO influenced the active compounds volatilization, see Figure A2b, following experiments were carried out with pure EO.

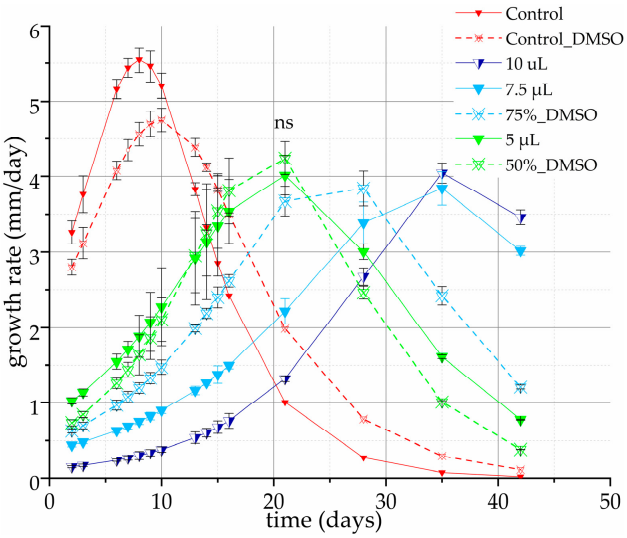

Figure 2. MAM in presence of *A. alternata*: mycelial growth rate (at 21 days: $p = 0.18$, "ns").

3.2. Effect of Thyme EO on A. alternata at Different Exposure Times

Considering the results obtained in terms of MGI% and growth rate, the effect of exposure time on *A. alternata* inhibition was tested in the presence of pure EO (10 µL). A MAM test, prolonged for 42 days, was performed removing the paper disk from Petri dishes at different incubation times (6 h-0.25 day, and 1, 2, and 7 days).

The growth curve of *A. alternata* exposed to EO for 6 h (0.25 day) showed a similar behavior with that of the control, in both cases a lag-phase was not present. Concerning the maximum growth rate, a lower value (4.3 ± 0.16 mm/day) was obtained for the 6 h exposure to EO respect to the control (5.8 ± 0.19 mm/day). On the contrary, at the higher exposure times, an EO effect on lag-phase duration was clearly observed: for 1 and 2 days of exposure, the lag-phase lasted 3 days, while prolonging the treatment (7 days), the fungal growth was inhibited for the longest time (14 days) (Figure 3A). The maximum growth rate was in the range between 3.8 ± 1.65 and 4.2 ± 0.22 and the values, obtained at different EO exposure times (1, 2, and 7 days), were not statistically different: as an example, a *p* value equal to 0.16 indicated as "ns" was obtained at 21st day for exposure time of 1 and 2 days (Student's *t*-test).

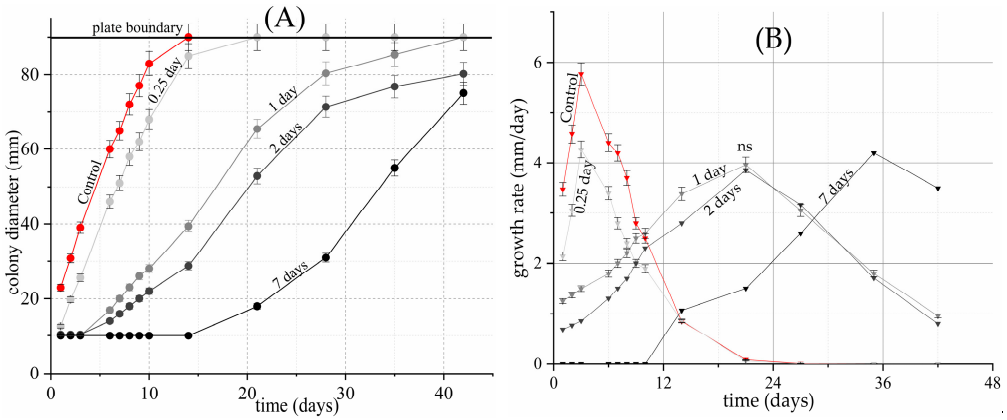

Figure 3. (**A**) Mean colony diameter of *A. alternata*; the time of disc removal is reported on the corresponding line. (**B**) Mycelial growth rate (ns, $p = 0.16$).

Images of *A. alternata* colonies, at the different EO exposure times, along with the control, are reported in Figure 4. An inhibition of conidiogenesis, for exposure time from 1 to 7 days, was observed at 14, 21 and 42 days of incubation.

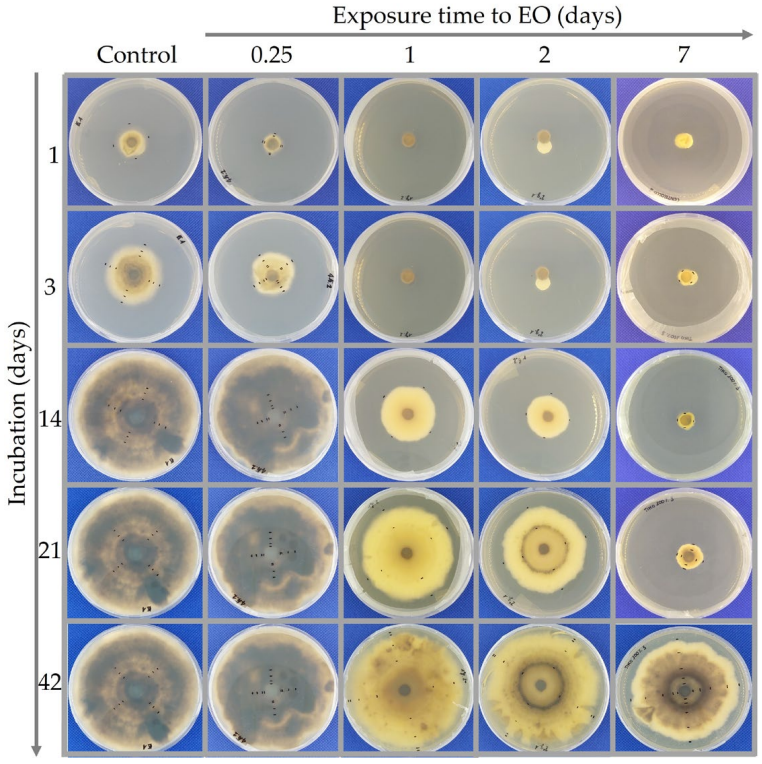

Figure 4. Thyme EO effect on *A. alternata* colonies at different exposure times.

3.3. Scale-Up of the EO Treatment in Airtight Glass Container

The results obtained in Petri dishes with MEA medium were used for the scale-up of the EO treatment in a glass airtight container (GAC) applying an EO vaporization method at a slower rate (see Section 2.3). Two different volumes were tested: 420 µL were selected to obtain in the GAC vapor phase the same EO concentration as that of the Petri dishes (10 µL EO), and 210 µL aimed to verify the effect of the EO volume. The obtained results were reported in Figure 5.

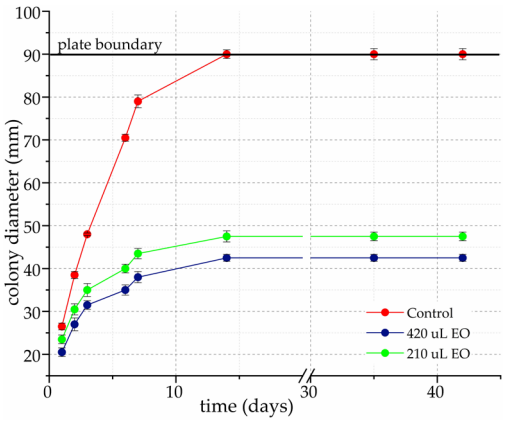

Figure 5. Vaporization of different EO volumes on *A. alternata* colonies in GAC: mean colony diameters.

In the first 3 days of incubation, the mean diameter of the colonies, treated with the two different EO volumes, were comparable (33 ± 1.2 mm) and lower than that of the control (50 ± 0.8 mm). After 14 days, the mean diameter reached a constant value of about 42 ± 0.8 mm for 420 µL EO and 47 ± 1 mm for 210 µL EO, and a total growth inhibition was observed until the 42nd day (six week) of incubation. The corresponding inhibition percentage was equal to 45% and 52%, respectively.

3.4. The Approach to a Real Case Study

To adopt *A. alternata* as a model microorganism for paper biodeteriogens, it was necessary to confirm the EO effect in experimental conditions that simulate a real case study (i.e., the treatment of *A. alternata* in a library). To this end, the growth of *A. alternata* was verified on CZ medium, with or without a paper sheet as carbon source.

Three different CZ media were tested: CZ with Glucose, CZ with Glucose and paper, and CZ_{mod} with the paper as the only carbon source. In Figure 6, the mean colony diameter of *A. alternata* on CZ with or without paper sheet and CZ_{mod} are reported. The growth rates on the three different media are very similar until the 7th day of incubation (for the Student's *t*-test, $p = 0.11$, "ns" in the graphic) After that, the mean diameter on CZ medium without paper sheet was slightly higher than that on CZ with paper, the colony reached a final medium diameter of 8.8 ± 0.24 and 8.3 ± 0.26 cm, respectively. The difference had a statistical significance with a $p = 0.037$ ("*" in the graphic; Student's *t*-test). On the contrary, in CZ_{mod} medium, the mycelium reached the edge of the paper sheet and there it stopped the growth at 7 days of incubation; at the 21st day, the statistical difference with CZ with Glucose and paper was characterized by a $p = 1.08 \times 10^{-6}$, indicated with "****" in the graphic (Student's *t*-test).

3.5. Optimization of the EO Treatment

The inhibition percentage, obtained in GAC on MEA, was higher for 420 µL EO, nevertheless the duration of inhibition was comparable for the two tested EO volumes, until 42 days. For this reason, in the second set of tests we applied the lower EO volume (210 µL). One or three Petri dishes (55 mm Ø) with CZ_{mod} medium were positioned in GAC before the EO volatilization as in MAM test. The growth of *A. alternata* was inhibited starting from the 1st day of treatment, correlated to the lower time necessary to reach the maximum EO concentration in the vapor phase (Figure 7A). In both the treated containers, the maximum mean diameter value, reached at the 3rd day, was very similar (i.e., 1.62 ± 0.23 and 1.56 ± 0.13 cm for 1 and three colonies, respectively); in the same day, the diameter of the control was 2.76 ± 0.41 cm and, at the 6th day, it reached the plate boundary.

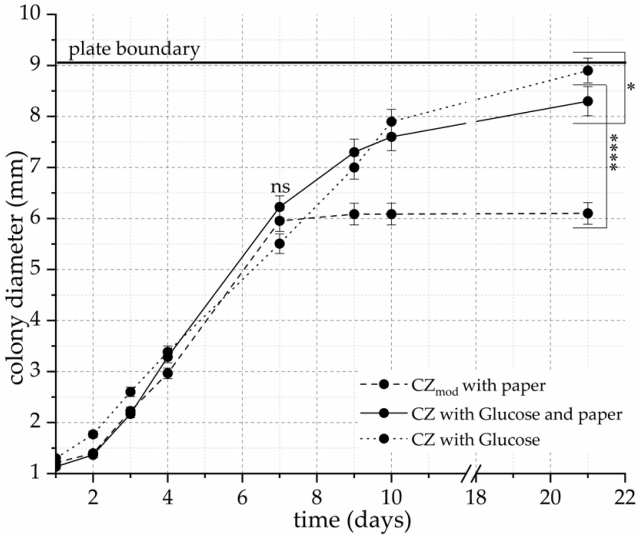

Figure 6. *A. alternata* growth on different CZ media (at the 7th day ns, $p = 0.11$; at the 21st day "*" $p = 0.037$ and "****" $p = 1.08 \times 10^{-6}$).

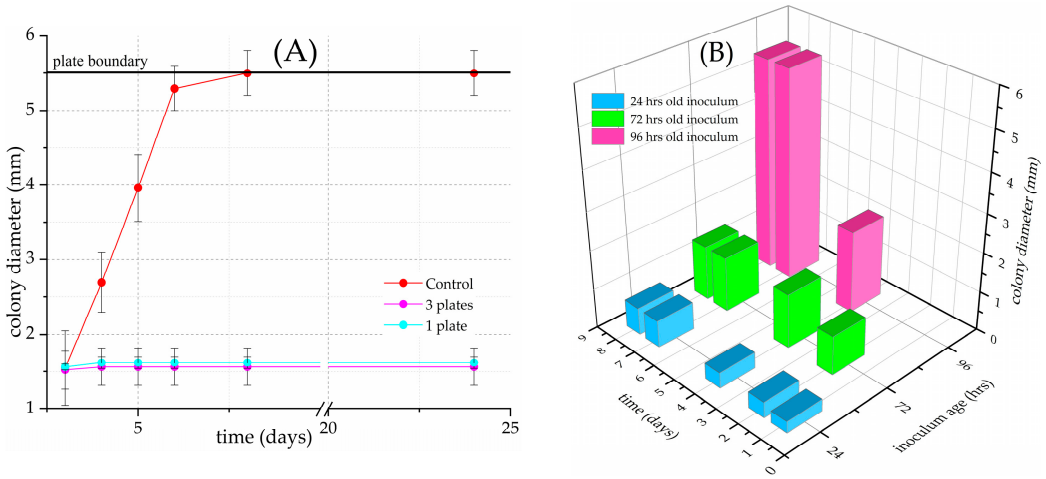

Figure 7. (**A**) Disk volatilization test in airtight container. (**B**) Cold diffusion test in airtight container.

In the third set of experiments, pure EO was diffused as microparticles and inocula of different ages, 24, 72 and 96 h were tested.

The results, reported in Figure 7B, clearly show that the EO treatment has a different efficacy depending on the inoculum age: more precisely, a higher inhibition was observed in the case of the 24- and 72- hours inoculum. In these samples, the mean diameter value, after 7 days of incubation, was 0.68 and 1 cm, respectively. In the case of 96 h inoculum, the EO inhibition was lower, in fact the colony diameter reached the edge of the paper sheet at the 7th day of incubation.

In a further test, which was extended up to 24 days, a 72 h inoculum was used (see Figure A2). Comparing the behavior of EO treated samples with that of the *A. alternata* control, it is possible to highlight that, while the control reached the edge of the paper (4.6 cm) after 2 days of incubation, on the contrary, the EO treated sample was inhibited for all the incubation periods.

4. Discussion

In the present work, thyme EO has been applied on *A. alternata* classified among the "indoor fungi" able to produce toxic and/or allergenic secondary metabolites on the spore surface [30]. Among the different EO, known for their antifungal activity, *Thymus vulgaris* has been selected considering its several effects, both at the level of cell membrane/wall and within the microbial cell [31]. Moreover, thymol, *p*-cymene, and γ-terpinene, main compounds present in thyme EO, are Generally Recognized as Safe (GRAS) [32]. In scientific literature, different methods (i.e., direct contact and vapor phase) have been applied to evaluate the antimicrobial effect of thyme EO on *A. alternata* as reported below.

In the work of Zabka et al. [21], thyme EO inhibition on mycelial radial growth of *A. alternata* was tested by the agar dilution method: MIC50 and MIC100 values were 0.077 and 0.146 µL/mL, respectively. Perina et al. [33] reported that thyme EO from leaves and thymol had MIC of 500 and 250 µg mL^{-1}, respectively, lower than the value (1250 µg mL^{-1}) obtained with a commercial fungicide. A MIC of 26.7 µg/l and a 20% reduction in fungal biomass were obtained for *A. alternata* exposed to vapors of commercial thyme EO (72 h, at 25 °C) [32]. In the work of Feng et al. [34], the mycelial growth inhibition was proportional to thyme EO concentration: 48.0% and 100%, were obtained with 1 and 5 µL.

In the present study, the effect of thyme EO on *A. alternata* growth was evaluated with MAM in Petri dishes. Results clearly showed the influence of applied EO volume on the lag-phase duration, see Figure 1A. A linear relationship (R-Square = 0,987) was observed between the pure EO volume applied and the inhibition period of *A. alternata* growth, see Figure A2a. Otherwise, for the diluted EO, the influence of DMSO on oil evaporation was observed (see Figure A2b), as previously reported by Delespaul et al. [35]. Also, for MGI% (see Figure 1B), the inhibition was proportional to EO concentration, according to results of Feng et al. [34]. The low influence on the maximum growth rate value (see Figure 2) indicates a mycostatic effect of EO, depending on the applied volume. For a given EO volume (10 µL), the exposure time delays the achievement of the maximum growth rate (see Figure 3B) and influences the conidiogenesis. After 6 h (0.25 day) exposure, it is possible to observe the conidiogenesis at the same time of the control (see Figure 4), while prolonging the exposure for at least 1 day, an inhibition of conidiogenesis is evident, as previously reported for the mycotoxigenic *A. flavus* [36–38]. Concerning the EO effect on metabolism, in [39] many targets and mechanisms are reported such as cell wall damage or changes in hyphal morphology which even led to the inhibition of conidiogenesis.

The scale-up of the process in GAC (EO volumes of 210 and 420 µL) proved that the evaporation rate influences the starting point of the inhibition; in fact, in tests with MEA medium, at low evaporation rate (Figure 5), 14 days were necessary to reach the MID of the EO active components in the vapor phase, independently from the EO volume applied, after that the fungal growth was inhibited till to 42 days.

Once the EO treatment was set-up in GAC with CZ_{mod} medium, with the lowest EO volume (210 µL) and applying the disc volatilization method, one day was enough to inhibit the *A. alternata* growth, independently from the number of treated colonies. In this case, the higher contact area between the oil and the environment reduced the time necessary to reach the MID in the vapor phase (see Figure 7A).

In the work of Diaz-Alonso [10], a fast vaporization method of thyme EO in indoor air was used, the effectiveness was monitored for 1 day reporting a 48.7% reduction in fungal colonies. In the present work, in view of an EO treatment in a contaminated library, the effect of a longer exposure time was tested on *A. alternata* and monitored for more than 20 days to establish the duration of the inhibition.

To simulate a contaminated library, thyme EO was diffused in GAC as microparticles using a fast vaporization method on *A. alternata* colonies of different ages, demonstrating

that the efficacy of the EO depends on the colony age (see Figure 7B) and consequently that the antimicrobial treatment should be done as earlier as is feasible. It was proved that, in GAC, a low EO volume (10 µL) coupled with a short vaporization time (30 min), was sufficient to inhibit the growth for 24 days, as long as the system guarantees a prolonged exposure to EO vapor phase (see Figure A3). Therefore, a fast volatilization method permits to reduce the EO amount maintaining the growth inhibition for a long time.

Considering that the aim of the EO treatment is to inhibit as longer as possible the fungal growth, its duration has a key role along with the contact time. In this perspective, the feasibility of the treatment to a real case appears less challenging on the condition that a fast vaporization method (e.g., cold diffusion) coupled with a suitable treatment time and EO concentration are applied. As an example, considering a room with a free volume of 50 m^3, to obtain the EO mycostatic concentration applied in GAC (10 µL fast dispersed), 190 mL would be necessary. Nevertheless, using the appropriate contact time, a lower EO volume could be likewise effective.

5. Conclusions

In the present work, the antimicrobial efficacy of red thyme EO, in vapor phase, was investigated on *Alternaria alternata.* The obtained results confirmed the possibility to apply this paper biodeteriogen as a model organism for treatments in a contaminated library.

The results obtained at laboratory scale, in MAM and in GAC, proved the key role of the EO concentration, the diffusion modality and the exposure time. The strong influence of the EO treatment on the lag phase of the *A. alternata* growth curve suggest a mycostatic effect of EO proportional to the applied volume. Regard the EO dispersion, a cold EO diffusion method has to be adopted to quickly reach the MID in the vapor phase to obtain an instantaneous growth inhibition.

Considering that MAM tests are usually carried out for short time periods with short exposure times to EO, not necessarily they give information that can be reproduced in real conditions; therefore, it is mandatory to pay attention in transferring MID values, obtained in MAM, in the scale up of the process.

For this reason, in the present work, a GAC test was proposed to achieve information useful for the set-up of an EO treatment in an indoor environment (e.g., a library) applying reduced EO concentrations for prolonged time periods.

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Abbreviations

The following abbreviations are used in this manuscript:

CZ	Czapeck
CZ_{mod}	Modified Czapeck
DMSO	Dimethyl Sulfoxide
EO	Essential Oil
GAC	Glass airtight container
Hys	Hydrolates
M	Mycelial plug
MAM	Micro-atmosphere method
MEA	Malt extract agar
MFC	Minimum fungicidal concentration
MGI%	Minimum Growth Inhibition
MIC	Minimum inhibitory concentration
MID	Minimum inhibitory dose
OD_{650}	Optical density at 650 nm
PS	Polystyrene
S	Spore suspension
SD	Standard deviation
TNT	Nonwoven fabric

Appendix A

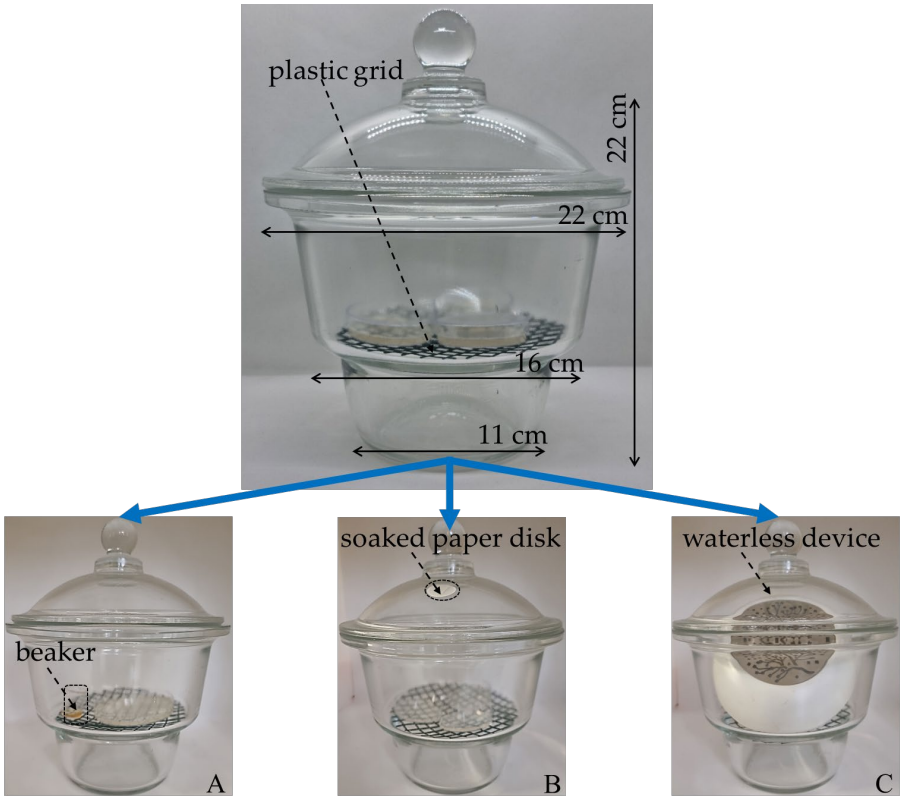

Figure A1. Glass airtight container (GAC). (**A**) EO contained in a glass beaker, (**B**) EO dispersion with a filter paper disk, (**C**) Saturation with EO vapors with a waterless device.

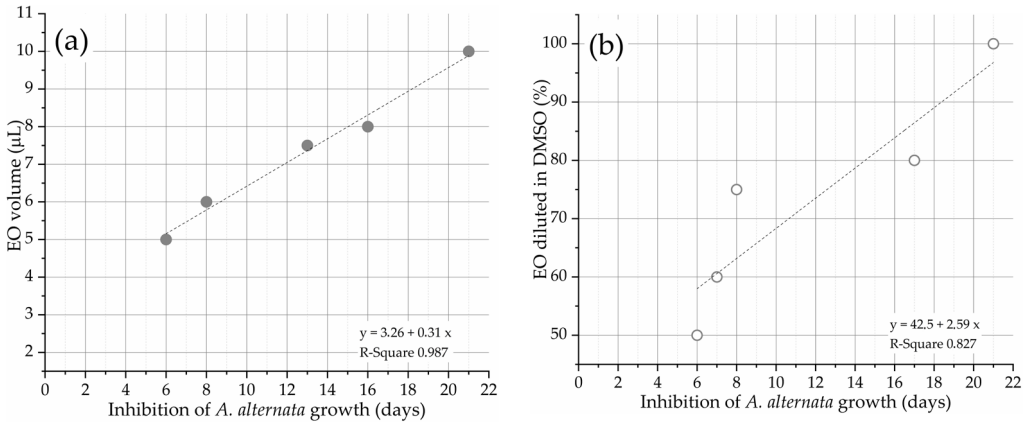

Figure A2. Linear relationship between the EO volume (**a**) or the % of EO diluted in DMSO (**b**) applied and the inhibition period of *A. alternata* growth.

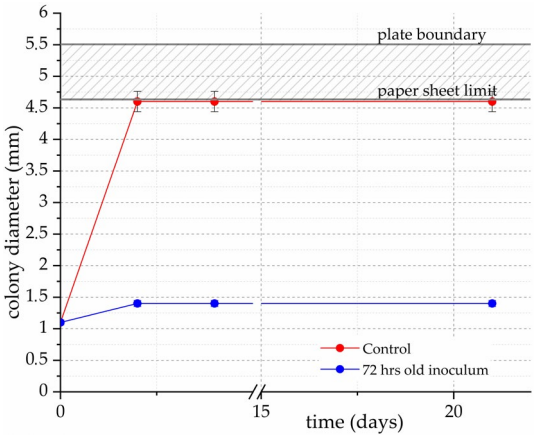

Figure A3. Cold diffusion test in airtight container, 72 h old inoculum.

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