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EVALUATION OF THE INHIBITORY EFFECT OF ESSENTIAL OILS AGAINST *Fusarium sporotrichioides* ISOLATED FOR THE FIRST TIME FROM STRAWBERRY PLANTS IN ROMANIA

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Abstract

This study evaluated the in vitro inhibitory effects of essential oils (EO) on Fusarium sporotrichioides isolated for the first time from strawberry plants in Romania. Oregano and cinnamon EO exhibited strong and consistent inhibitory activity, achieving complete fungal inhibition at both high (500 and 1000 µl/l) and moderate (125 µl/l for oregano and 250 µl/l for cinnamon) dosed. Clove EO revealed to be effective only in higher doses (500 and 1000 µl/l) and showed reduced efficacy at lower dosage (35-125 µl/l) compared to oregano and cinnamon EO. In contrast, lemon, mint, and tea tree EO demonstrated limited and inconsistent fungal inhibition. Lemon EO intensified mycelial pigmentation without affecting the growth. Mint EO reduced the mycelial density but did not hinder radial expansion, while tea tree oil had minimal inhibitory impact, with only 17.96% inhibition at 1000 µl/l after 6 days of incubation. These findings highlight the potential use of some specific EO to control F. sporotrichioides, as oregano and cinnamon EO showed high fungal inhibitory efficacy, even at lower doses.

Key words*: biocontrol, essential oils, fungal inhibition, Fusarium sporotrichioides, strawberry.*

INTRODUCTION

Strawberry production in Romania has seen rapid growth, driven by favorable climate and soil conditions across various regions. These conditions make strawberries a highly adaptable crop, suitable for a variety of soil types and microclimates. Romania has considerable potential for high strawberry productivity, with average yields often exceeding 20 tons per hectare (Soare & Chiurciu, 2021). Projections suggest further growth, with an estimated output of approximately 25,270 metric tons by 2026, fueled by expanding cultivated areas and advancements in agricultural practices (ReportLinker, n.d.). However, despite these favorable prospects, strawberry cultivation is increasingly threatened by *Fusarium* wilt, primarily caused by *Fusarium oxysporum* f. sp. *fragariae* (Fof), which leads to significant economic losses globally, affecting both yield and quality (Fang et al., 2012; Koike & Gordon, 2015).

The management of *Fusarium* wilt presents a significant challenge due to the soil-borne nature of the pathogen and the restrictions on chemical fumigant use (Koike & Gordon, 2015). Although some chemical fungicides, such as tebuconazole, have been employed, the increasing issue of reduced sensitivity among certain *Fusarium* spp. strains has limited their effectiveness (Cho & Kwak, 2022). Recent studies have evaluated the efficacy of hydrogen peroxide and azoxystrobin treatments in controlling *Fusarium* spp. in strawberry crops, highlighting their potential role in disease management (Țane Lumînare & Cristea, 2024). However, the long-term effectiveness of such treatments and their impact on pathogen resistance remain concerns, emphasizing the need for alternative, sustainable disease management strategies.

Biological control agents, including essential oils (EOs), have gained increasing attention as eco-friendly alternatives to synthetic fungicides. Previous studies have demonstrated the

antifungal properties of EOs, including those of cinnamon, oregano, and thyme, which inhibit the growth of *Fusarium oxysporum* in a dose-dependent manner (Park et al., 2017; Massoud et al., 2020; Stan Tudora et al., 2022). Other plant-derived EOs have also shown strong antifungal activity against various *Fusarium* species, supporting their potential use in disease management. For example, *Mentha arvensis* EO has been shown to inhibit the growth of *F. proliferatum* and *F. verticillioides* (Kumar et al., 2016), whereas savory and thyme EOs have demonstrated significant antifungal activity against *Fusarium oxysporum* f. sp. *basilici* (Lopez-Reyes et al., 2016).

The use of plant-based biopesticides and natural extracts in plant protection has also been explored, with research highlighting their antimicrobial and phytostimulatory effects (Ichim et al., 2017; Perișoara et al., 2022). For instance, extracts from *Tagetes erecta* have been reported to exhibit synergistic antipathogenic effects in the presence of rhizobacteria (Perișoara et al., 2022), while fenugreek extracts have been studied for their influence on seed germination and plant health (Perișoara et al., 2023). Additionally, biofungicidal prototypes have shown promising *in vitro* antifungal activity against *Fusarium* spp. (Cristea et al., 2024). These findings support the increasing interest in developing plant-based alternatives to chemical treatments.

Furthermore, EOs from *Bursera morelensis* and *Lippia graveolens*, particularly rich in carvacrol, have shown potent antifungal effects on *Fusarium* species by affecting hyphal morphology, spore production, and germination (Medina-Romero et al., 2022). The efficacy of EOs has been demonstrated in multiple studies, with cumin, basil, and geranium EOs significantly reducing root rot caused by various *Fusarium* species in cumin plants under both greenhouse and field conditions (Hashem et al., 2010). However, in certain cases, such as with basil seeds, pathogen control provided by heat treatments combined with biocontrol agents and chemical fungicides did not perform as well as chemical fungicides (Lopez-Reyes et al., 2016). The antifungal activity of EOs is largely attributed to their chemical composition, particularly the presence of oxygenated compounds, which disrupt fungal cell functions (Kumar et al., 2014; Roselló et al., 2015). Despite their promising potential, the effectiveness of EOs can vary depending on the targeted *Fusarium* species and the specific bioactive constituents of the oils used. This variability makes EOs a promising alternative to chemical pesticides for controlling *Fusarium* infections in crops (Samie, 2012; Fontana et al., 2020; Radice et al., 2022).

Although the antifungal potential of EOs is well documented, limited research exists regarding their effects on *F. sporotrichioides*, particularly in strawberry plants. This study aims to address this gap by evaluating the inhibitory effects of selected EOs on *F. sporotrichioides* isolated for the first time from strawberry plants grown in Romania. By exploring these eco-friendly alternatives, we aim to provide valuable insights into their potential role in integrated disease management strategies for strawberry cultivation. Our findings could provide a foundation for sustainable solutions to combat *Fusarium*-related diseases, reduce dependence on chemical treatments, and minimize environmental impacts.

MATERIALS AND METHODS

Pathogen isolation and identification

Strawberry plant samples of the Alba cultivar, expressing stunting and leaf wilting symptoms, were collected from a commercial strawberry plantation in Giurgiu County. The sampling focused on crown areas exhibiting visible symptoms of fusariosis to ensure that the material was representative. The samples were handled under sterile conditions to prevent contamination and promptly transported to the laboratory for subsequent analysis. Disinfection involved a four-step protocol: (1) washing with sterile distilled water for 1 min to remove surface debris, (2) immersion in 70% ethanol for 1 min to eliminate surface contaminants, (3) treatment with 4% sodium hypochlorite for 2 min for thorough disinfection, and (4) three subsequent rinses with sterile distilled water, each lasting 1 min. The disinfected material was dried in a laminar flow hood to prevent additional contamination. Using a sterile scalpel, the plant material was cut into small fragments (approximately 0.5 cm), which were then placed in sterile Petri dishes containing

potato dextrose agar (PDA) medium. The plates were incubated at 25°C in darkness, for five days, to promote fungal growth. After incubation, the individual fungal colonies were transferred to fresh PDA medium to obtain pure cultures. The initial identification of fungal isolates was based on macroscopic colony characteristics such as color, texture, and growth pattern, and microscopic examination of reproductive structures using an optical microscope (Nikon Eclipse 2000 LED MV R, Nikon Corporation, Tokyo, Japan). To confirm species identity, DNA sequencing was carried out, targeting the ITS1-5.8S-ITS2 region. Fungal DNA was extracted from fresh pure culture using the ZR Fungal/ Bacterial MiniPrep™ commercial kit (ZymoResearch, SUA). The ITS-5.8S region was amplified using the primers set ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The PCR was performed in 50 µl reaction volume, containing 1X Green Buffer with magnesium chloride, 0.2 mM dNTPs, 0.5 µM of each primers, 0.2 U of DreamTaq DNA Polymerase (Thermo-Scientific, Baltics, UAB, Vilnius, Lithuania), and 10 ng of template DNA, in sterile MilliQ water (Mohamed et al., 2024). The PCR program included an initial step of 4 min at 94°C for DNA denaturation, followed by 35 cycles of denaturation at 94°C 1 min, primers' annealing at 45°C for 1 min, and elongation at 72°C for 2 min, followed by a step of 10 min at 72°C for final elongation 10 min. The amplified product was verified through agarose gel electrophoresis, and the PCR products was sent for purification and DNA sequencing at CeMIA (Cellular and Molecular Immunological Applications, Greece). The resulting forward and reverse sequences were assembled with BioEdit software and submitted to the NBLAST program to be compared to similar DNA sequences found in the National Center for Biotechnology Information (NCBI) database. The ITS-5.8S partial sequence of the identified fungal isolate was then submitted to NCBI GenBank.

Evaluation of essential oils antifungal activity

To determine essential oils (Eos) antifungal activity against *Fusarium* sp., six EOs were tested, oregano, clove, mint, cinnamon, tea tree, and lemon. These EOs were purchased from ELLEMENTAL, a certified supplier, that ensure their high quality and purity. Stock solutions of each EO were prepared by mixing the oils with Tween 80 as an emulsifier in a 2:1 (v/v) ratio to enhance dispersion in the culture medium. Six concentrations (35, 65, 125, 250, 500, and 1000 µl/l) were prepared for each EO, and *in vitro* treatments were conducted in triplicate, to ensure reliable results. The emulsified oil mixtures were then incorporated into a molten PDA medium before solidification to achieve uniform distribution. The media were aseptically purred in 9 cm diameter sterile Petri plates. PDA medium was used as control. Mycelial discs, 0.5 cm diameter, were aseptically cut from the edge of 7-day-old *Fusarium* colonies and placed in the center of Petri dishes. The plates were incubated at 25°C, and colony diameters were measured in two perpendicular directions after 3, 6, 8, 11, and 15 days post-inoculation. The average diameter was used to calculate mycelial growth inhibition (MGI) using the formula: $MGI\ (\%) = (C - T)/C \times 100$, where C is the mean colony diameter in the control group and T is the mean colony diameter in the treatment group (Riccioni & Orzali, 2011). The IC50 and IC90 values, representing the concentrations required to inhibit 50% and 90% of mycelial growth, respectively, were calculated using regression analysis.

Data Analysis

Statistical analyses were conducted using the R software to compare the antifungal effects of EOs and their concentrations over time. ANOVA tests were used to determine whether there were significant differences, and Tukey's HSD tests were used to pinpoint where these differences occurred. A significance level of $p < 0.05$ was used for all analyses.

RESULTS AND DISCUSSIONS

Fungal macroscopic appearance

Symptomatic strawberry plants revealed a fungal pathogenic attack. The isolated phytopathogen was identified as *Fusarium* sp., based on macroscopic morphological appearance. Isolated colonies exhibited rapid growth on PDA medium, reaching a diameter of 7-9 cm within approximately 7 days at 25°C. In the early growth stages, the mycelium was dense and

white, with a slight violet hue. As the colonies matured, the color transitioned to pink and reddish-brown, becoming more pronounced in the central region. The texture of the colonies was fluffy, and the mycelial filaments extended radially, forming a uniform and well-defined pattern. Upon maturation, the color intensified, and the pigment diffused into the culture medium, creating a pinkish or orange tint in the basal layer. The colony margins remained well-defined, with a compact and consistent structure across the surface of the medium.

Microscopic characteristics

Microscopic examination confirmed the presence of characteristic fusiform macroconidia with 3 to 5 septa and curved apical cells, along with pyriform microconidia and globose chlamydospores. The macroconidia were generally grouped in sporodochia, whereas the chlamydospores were observed either solitarily or in chains, displaying thick walls. These features align with previous descriptions and ensure accurate identification of *Fusarium* sp., showing high morphological correspondence to *F. sporotrichioides*, as described in the literature (Ivanová et al., 2016; Abdelmagid et al., 2021).

Molecular Identification

The isolated strain, encoded FusCL, was identified at species level based on DNA sequence analysis of the ITS1-5.8S-ITS2 region. The internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence of this strain have 530 bp in length, and are available on NCBI under the GenBank no. PQ675606.1. Molecular comparison of the FusCL strain with other highly similar DNA sequences from NCBI database revealed 100% identity with *F. sporotrichioides* ATCC MYA-3963 (accession number FJ614637.1) with 98% query coverage. This genetic identification supports the morphological and microscopic observations, confirming that the isolated strain belongs to *Fusarium sporotrichioides* species.

Fungal inhibition using EOs

Tested EOs demonstrated varying degrees of inhibitory effects on *Fusarium sporotrichioides* FusCL, with notable differences based on the oils type, concentration and exposure time (Figure 1).

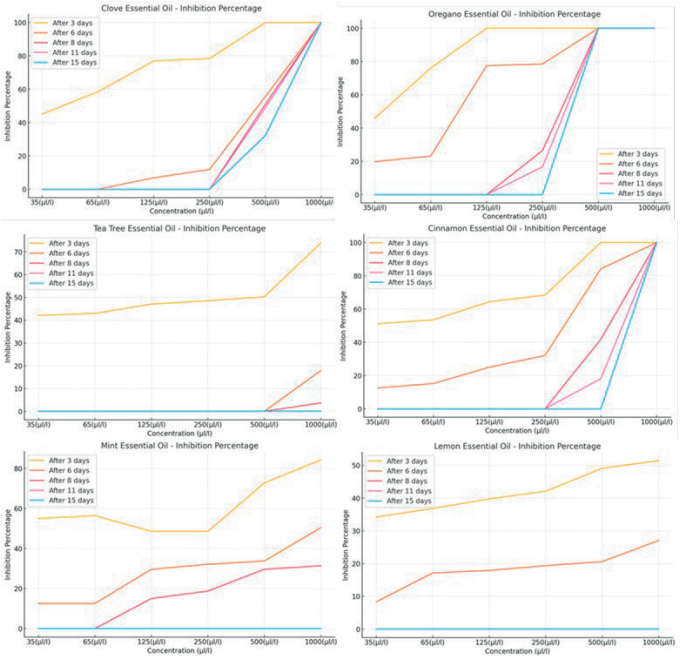

Figure 1. Inhibition percentages of *Fusarium sporotrichioides* induced by different concentrations of essential oils over time

Oregano EO exhibited the most consistent and potent inhibitory effects. At concentrations of 125 µl/l and higher, complete inhibition (100%) was observed as early as day 3, and this effect was maintained through day 15 at the highest concentrations (500 µl/l and 1000 µl/l). In contrast, lower concentrations (35 and 65 µl/l) showed a gradual decline in inhibition over time, with no effect observed by day 15. Lemon EO had a moderate initial inhibitory effect, with percentages ranging from 34.21% to 51.46% on day 3. However, its efficacy diminished rapidly, and no inhibition was observed beyond day 6, regardless of concentration. Cinnamon EO displayed strong inhibition at high concentrations, achieving complete inhibition (100%) at 500 µL/l and 1000 µL/l by day 3, and sustained this effect at 1000 µL/l through day 15. Lower concentrations exhibited a gradual reduction in inhibition, with no effect observed after day 8 at 35 µl/l and 65 µl/l. Mint EO initially showed significant inhibition, particularly at 500 µl/l and 1000 µl/l, with percentages of 72.81% and 84.21%, respectively, on day 3. However, the inhibitory effect was inconsistent, and all concentrations lost their efficacy by day 11. Tea tree EO exhibited the lowest overall efficacy, achieving a maximum inhibition of 73.98% at 1000 µl/l after day 3. The effect decreased rapidly, with minimal inhibition observed by day 8, and no inhibition detected by day 11 or beyond. Clove EO demonstrated a significant inhibitory effect, achieving complete inhibition (100%) at 1000 µl/l after 6 days, and sustained this effect through day 15. At 500 µL/l, inhibition remained substantial, but gradually decreased over time. The lower concentrations exhibited limited and short-lived effects.

IC50 and IC90 values

Oregano EO demonstrated the highest efficacy, with IC50 values ranging from 309.31 µl/l to 449.80 µl/l and IC90 values between 312.77 µl/l and 453.13 µl/l. An intermediate efficacy was obtained with the cinnamon EO, which exhibited IC50 values ranging from 426.96 µl/l to 667.13 µl/l and IC90 values between 430.42 µl/l and 670.33 µl/l. Clove EO showed the lowest efficacy among the three, with IC50 values ranging from 403.49 µl/l to 501.49 µl/l and IC90 values between 406.95 µl/l and 505.90 µl/l (Figure 2).

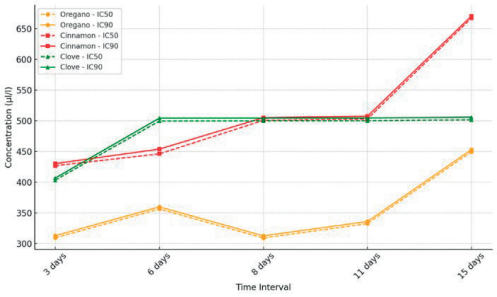

Figure 2. Concentrations required to inhibit 50% (IC50) and 90% (IC90) of mycelial growth with oregano, cinnamon, and clove essential oils over time

The other essential oils tested (lemon, mint, and tea tree) did not exhibit sufficiently high inhibition percentages to allow for the calculation of these two indicators.

Statistical analysis: ANOVA and Post-Hoc Tests

During the first three days of the experiment, the clove, mint, cinnamon, and oregano EOs exhibited significant differences in inhibiting mycelial growth ($p < 0.001$), indicating a rapid and potent antifungal effect. A direct relationship between concentration and inhibition efficacy was observed ($p < 0.001$), underscoring the importance of an adequate dose to maximize the effect. By day 6, the differences among the EOs remained significant ($p < 0.001$), confirming the short-term consistency of their effects.

On day 8, the differences between the EOs were no longer significant ($p = 0.068$), suggesting a stabilization of the individual antifungal effects. However, concentration continued to have a significant influence ($p = 0.003$), emphasizing the critical role of dosage in maintaining long-term efficacy. Throughout the entire observation period, oregano and clove EOs maintained a pronounced antifungal effect, particularly at higher concentrations. By days 11 and 15, differences between oils became nonsignificant ($p = 0.091$ and $p = 0.182$, respectively), but the significant impact of concentration persisted ($p = 0.002$ and $p = 0.003$), highlighting the importance of dosage as a critical factor in the effective management of the pathogen.

The ANOVA and post-hoc Tukey HSD tests confirmed that, although the differences among EOs were more pronounced in the early days, the long-term efficacy was significant for oregano and clove EOs. This finding underscores the necessity of an appropriate dosing strategy to sustain durable and effective antifungal control against *F. sporotrichioides*. The results of the statistical analysis are summarized in Table 1.

EOs effects on *F. sporotrichioides* mycelium

The experiments revealed distinct effects of the EOs on the fungal color and density of the mycelium, which varied depending on the EOs concentration used (Figure 3).

Cinnamon and oregano EOs caused noticeable depigmentation of the mycelium, indicating significant metabolic stress, particularly at higher concentrations. This depigmentation effect was observed across all concentrations of clove and tea tree EOs used. However, unlike the tea tree EO, which did not inhibit mycelial growth, the clove EO exhibited a pronounced inhibition of growth at higher concentrations.

Another important observation was related to the mint EO, where the pink coloration of the mycelium persisted, but the density significantly decreased when increasing the EO concentration. This suggests a partial inhibition of hyphal formation, though radial expansion of the mycelium remained unaffected. Conversely, the lemon EO intensified the pink coloration but neither inhibited mycelial growth nor affected its density.

These observations highlight the complex interactions between the bioactive compounds present in the EOs and the fungal metabolism, influencing both the metabolic processes and the physical structure of the mycelium.

Table 1. Summary of statistical analysis results (ANOVA and Post-Hoc Tests)

Incubation time	EOs showing significant differences	Concentrations with significant differences	EOs	Concentrations	Significance
			p-Values ANOVA		
3 days	Clove, Mint, Cinnamon, Oregano	All concentrations are significant ($p < 0.001$)	< 0.001	< 0.001	***
6 days	Clove, Mint, Cinnamon, Oregano	All concentrations are significant ($p < 0.001$)	< 0.001	< 0.001	***
8 days	No significant differences between the EOs	Concentrations are significant ($p = 0.003$)	0.068	0.003	*(concentrations)
11 days	No significant differences between the EOs	Concentrations are significant ($p = 0.002$)	0.091	0.002	**(concentrations)
15 days	No significant differences between the EOs	Concentrations are significant ($p = 0.003$)	0.182	0.003	* (concentrations)

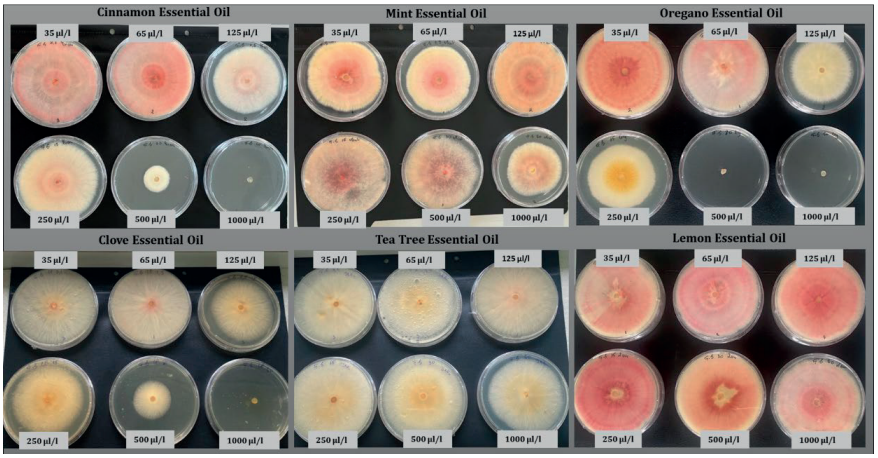

Figure 3. EOs effects in different concentrations on the growth of *F. sporotrichioides*

Our study evaluated the inhibitory effects of various EOs on *F. sporotrichioides*, isolated for the first time from strawberry plants in Romania. Tested EOs revealed significant variation in antifungal efficacy depending on the EO type, concentration, and exposure duration.

Oregano EO demonstrated the most consistent inhibitory effect, with complete inhibition (100%) of the fungal growth observed at concentrations as low as 125 µl/l by day 3, and up to day 15 at higher concentrations (500 µl/l and 1000 µl/l). These results are in accordance to with those of Střelková et al. (2021), who reported the potent antifungal activity of oregano EO against various *Fusarium* spp. Previous studies by Peschiutta et al. (2016) and Harčárová et al. (2021) attributed this efficacy to the high concentrations of carvacrol and thymol, which disrupt fungal cell membranes. This consistency across studies supports oregano EO as a promising natural antifungal agent.

Clove EO also exhibited substantial antifungal effects, with complete fungal inhibition at 1000 µl/l sustained for 15 days. Similarly, Sharma et al. (2016) and Irshad et al. (2023) reported clove EO with significant antifungal activity, emphasizing the role of eugenol in distorting fungal spores and disrupting cell membranes (Rana et al., 2011). The high eugenol content of clove oil makes it an effective alternative to synthetic fungicides for controlling *Fusarium* infections.

Cinnamon EO showed strong inhibition at high concentrations, achieving complete inhibition at 500 µl/l and 1000 µl/l by day 3, with sustained effects at 1000 µl/l through day 15. Roselló et al. (2018) and Lee et al. (2020) also highlighted the efficacy of cinnamon EO against *Fusarium* species, which is attributed to the ability of cinnamaldehyde to disrupt fungal cell walls and interfere with tubulin polymerization (Shahina et al., 2018). Our findings reinforce the potent antifungal potential of cinnamon EO and suggest its practical application in integrated disease-management strategies.

Mint EO initially exhibited significant inhibitory effects, achieving 72.81% and 84.21% inhibition at 500 µl/l and 1000 µl/l, respectively, on day 3. However, this efficacy diminished over time, with no inhibition observed on day 11. Similar observations were made by Sreenivasa et al. (2011) and Rachitha et al. (2017), who found that peppermint EO antifungal activity decreases as volatile components dissipate. The morphological changes in the hyphae observed in other studies (Rachitha et al., 2017) may partially explain the transient nature of mint oil's efficacy.

Tea tree EO exhibited moderate initial antifungal activity, with inhibition of 73.98% at 1000 µl/l on day 3, but the effect declined rapidly, with no inhibition detected on day 11. Previous studies, such as those by Sahab et al. (2014) and Paramalingam et al. (2023), demonstrated the antifungal properties of tea tree EO against various *Fusarium* spp., although its efficacy can vary based on fungal strain and environmental conditions. The rapid evaporation and degradation of tea tree EO likely contribute to the limited duration of its inhibitory effect.

Lemon EO showed the weakest overall efficacy, with an initial moderate inhibitory effect (34.21% to 51.46% on day 3) that quickly diminished, with no inhibition observed beyond day 6. Homa et al. (2015) and Palfi et al. (2019) similarly reported that lemon EO has a lower antifungal effectiveness than other tested EOs. The relatively weak potency of lemon oil may limit its use as a standalone treatment; however, it could have potential in combination strategies.

This study presents the first report of *F. sporotrichioides* on strawberry plants in Romania. Although this pathogen is primarily known to infect cereals such as wheat and corn (Beccari et al., 2018; Xue et al., 2019; Wang et al., 2020), research has demonstrated its ability to infect other plants, including soybean, peach, and pine (Ivanová et al., 2016; Khan et al., 2022; Xu et al., 2024). The significance of *F. sporotrichioides* identification on strawberries is highly valuable due to its potential production of harmful mycotoxins, such as T-2 and HT-2 (Meneely et al., 2023; Svoboda et al., 2024), which have implications on food safety. Future research should focus on testing fungal pathogenicity of *F. sporotrichioides* on strawberry plants to better understand the impact of the attack and to assess the risk of mycotoxin contamination.

Our findings emphasize the potential of EOs, particularly oregano, clove, and cinnamon, as natural antifungal agents against *F. sporotrichioides*. These EOs could offer

sustainable alternatives to chemical fungicides. However, further studies are needed to optimize their application methods and evaluate their efficacy under field conditions.

CONCLUSIONS

This study marks the first report of *Fusarium sporotrichioides* on strawberry plants in Romania, confirmed through both microbiologic and molecular analyses. These findings are significant, given the pathogen's known ability to produce harmful mycotoxins and their potential impact on food safety.

This study also explored the antifungal potential of various EOs, such as cinnamon, oregano, and clove, demonstrating the strong inhibitory effects against *F. sporotrichioides*, particularly at concentrations of 500 and 1000 µl/l. These EOs not only inhibited the radial growth of fungal colonies, but also disrupted the mycelial structure, suggesting a significant impact on the physiological processes of the pathogen. In contrast, lemon, mint, and tea tree EOs exhibited lower and inconsistent efficacies, most probably imposing higher doses to achieve substantial effects.

Overall, this study provides foundational insights into the presence and potential threat of *F. sporotrichioides* on strawberry crops and highlights the potential of EOs as eco-friendly biocontrol alternatives. However, further research, including field trials, is essential to validate these findings and fully understand their implications for sustainable disease management. The integration of natural antifungal solutions such as essential oils could play a crucial role in advancing sustainable agricultural practices and enhancing plant health management.

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