

Article

Comparative In Vitro Antifungal Activity of Azoxystrobin–Difenoconazole and Essential Oils Against *Passalora fulva*, the Causal Agent of Tomato Leaf Mold

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Abstract

Tomato leaf mold, caused by *Passalora fulva*, is one of the most destructive fungal diseases affecting greenhouse tomato production and can cause substantial yield losses under conditions of high relative humidity and moderate temperatures that favor pathogen infection and disease development. This study evaluated the in vitro antifungal activity of a commercial fungicide formulation, Priori Top® (azoxystrobin + difenoconazole), and three essential oils derived from *Eucalyptus globulus*, *Artemisia herba-alba*, and *Mentha pulegium* against two monoconidial isolates of *P. fulva*. Unlike previous studies, which have mainly focused on mycelial growth, the present work simultaneously evaluated the effects of the tested products on mycelial growth, sporulation, and conidial germination, providing a comprehensive assessment of antifungal efficacy across three key developmental stages of the pathogen. Antifungal activity was quantified by estimating IC₅₀ and IC₉₀ values using Probit regression analysis. The azoxystrobin–difenoconazole formulation consistently exhibited the highest antifungal activity across all evaluated parameters and showed the lowest estimated IC₅₀ and IC₉₀ values for both fungal isolates. Among the tested essential oils, *Artemisia herba-alba* exhibited the greatest antifungal activity, reducing mycelial growth by 83% and 78% at 500 ppm for isolates 1 and 2, respectively, and consistently outperforming *Mentha pulegium* and *Eucalyptus globulus*. Differences in sensitivity were observed between the two fungal isolates, indicating intraspecific variability in their response to the tested treatments. These findings demonstrate the strong antifungal efficacy of the azoxystrobin–difenoconazole formulation against *P. fulva* and identify *A. herba-alba* essential oil as the most promising botanical treatment for integrated management of tomato leaf mold. Further greenhouse and field studies are required to validate these findings, optimize formulation strategies, and evaluate the practical application of *A. herba-alba* essential oil under commercial production conditions.



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Keywords: *Passalora fulva*; tomato leaf mold; essential oils; *Artemisia herba-alba*; azoxystrobin; difenoconazole; antifungal activity; integrated disease management; sustainable agriculture

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most widely cultivated vegetable crops worldwide and represents a major component of protected horticulture. Intensive greenhouse tomato production systems provide highly favorable conditions for the development and spread of fungal diseases because of high planting densities, continuous cropping,

restricted air circulation, and prolonged periods of high relative humidity, which create an ideal microclimate for pathogen infection and epidemic development [1–4]. Among the most destructive foliar diseases of greenhouse-grown tomatoes is tomato leaf mold, caused by *Passalora fulva* (Cooke) U. Braun & Crous (syn. *Cladosporium fulvum*), a highly specialized biotrophic pathogen capable of causing severe yield and fruit quality losses under warm and humid greenhouse conditions [5–9]. The disease is characterized by chlorotic lesions on the adaxial leaf surface and olive-green sporulation on the abaxial surface, leading to progressive defoliation, reduced photosynthetic activity, and significant reductions in marketable yield [5,6].

Management of tomato leaf mold relies mainly on the application of synthetic fungicides, particularly quinone outside inhibitors (QoIs) and demethylation inhibitors (DMIs), including azoxystrobin and difenoconazole [10,11]. Although these fungicides remain highly effective, their repeated and intensive use has increased selection pressure on pathogen populations, resulting in the emergence of fungicide-resistant isolates and reducing the long-term sustainability of chemical control [10–16]. Recent studies have documented the evolution of resistance to several fungicide classes in *P. fulva* and emphasized the urgent need for integrated disease management strategies combining conventional fungicides with alternative control approaches [14–16].

In recent years, increasing scientific interest has been directed toward plant-derived bioactive products as sustainable alternatives to synthetic fungicides. Among these, essential oils have attracted considerable attention because of their broad-spectrum antimicrobial activity, rapid biodegradability, low environmental persistence, and the diversity of their bioactive constituents [17–22]. Numerous studies have demonstrated that essential oils simultaneously disrupt fungal membrane integrity, alter cellular permeability, inhibit enzymatic activity, induce oxidative stress, and interfere with mitochondrial metabolism, thereby suppressing fungal growth and development [19–21]. Essential oils extracted from aromatic and medicinal plants, particularly species of *Artemisia*, *Eucalyptus*, and *Mentha*, have shown promising antifungal activity against a wide range of phytopathogenic fungi, highlighting their potential as complementary tools for sustainable disease management [22–32].

Despite the growing body of literature on the antifungal properties of essential oils, information regarding their activity against *Passalora fulva* remains scarce. Moreover, comparative evaluations of botanical products and conventional fungicides across multiple developmental stages of this pathogen are still limited, restricting our understanding of their relative efficacy and their potential integration into disease management strategies [7,16,22].

Therefore, the present study aimed to comparatively evaluate the *in vitro* antifungal activity of three essential oils (*Eucalyptus globulus*, *Artemisia herba-alba*, and *Mentha pulegium*) and a commercial fungicide formulation containing azoxystrobin and difenoconazole against two monoconidial isolates of *P. fulva*. Unlike previous studies, which have mainly focused on radial mycelial growth, this work simultaneously assessed the effects of the tested products on mycelial growth, sporulation, and conidial germination, thereby providing a comprehensive evaluation of antifungal efficacy across three key developmental stages of the pathogen. In addition, dose–response relationships were quantified using Probit regression analysis, allowing the estimation of IC₅₀ and IC₉₀ values to compare the relative antifungal potency of the tested treatments. The results provide new insights into the potential use of essential oils, particularly *Artemisia herba-alba*, as complementary components of integrated management strategies for tomato leaf mold.

2. Materials and Methods

2.1. Sampling

Tomato leaves (*Solanum lycopersicum* L. cv. 'Angelle') exhibiting typical symptoms of tomato leaf mold, including chlorotic lesions on the adaxial leaf surface and olive-green sporulation on the abaxial surface, were collected on 17 May 2023 from a commercial tomato greenhouse located in the Chtouka-Aït Baha production area (Souss-Massa region, Agadir, Morocco), one of the largest greenhouse tomato-producing regions in Morocco [1,2]. The tomato crop had been established in August 2022 and was cultivated under conventional greenhouse production practices.

To obtain a representative sample of the pathogen population, approximately 20–30 symptomatic leaves were randomly collected from naturally infected plants distributed throughout the greenhouse. Immediately after collection, each leaf was placed in a sterile polyethylene bag, transported to the laboratory in insulated containers to preserve tissue integrity and pathogen viability, and stored at 4 °C before being processed within 24 h [33–36].

All laboratory experiments, including fungal isolation, morphological characterization, and antifungal susceptibility assays, were conducted at the Laboratory of Biotechnology, Conservation and Valorization of Bioresources (LBCVB), Faculty of Sciences Dhar El Mahraz, Sidi Mohamed Ben Abdellah University, Fez, Morocco.

2.2. Isolation of the Pathogenic Fungus

Fungal isolation was carried out from symptomatic tomato leaves following standard phytopathological procedures for the isolation of foliar fungal pathogens, with minor modifications [37–41]. Small leaf fragments (approximately 5 × 5 mm) were excised from the advancing margin between healthy and necrotic tissues, where fungal colonization is generally most active.

To eliminate epiphytic microorganisms, the leaf fragments were surface-sterilized by immersion in 1% (v/v) sodium hypochlorite (NaOCl) for 1 min, rinsed three times with sterile distilled water, and dried on sterile filter paper under aseptic conditions in a laminar-flow cabinet [42,43].

The surface-sterilized leaf fragments were aseptically transferred onto Potato Dextrose Agar (PDA; Biokar Diagnostics, Beauvais, France) supplemented with streptomycin sulfate (50 mg L⁻¹) to suppress bacterial contamination. Plates were incubated at 24 ± 1 °C in darkness for 7 days, conditions reported to favor the isolation and growth of *Passalora fulva* and related cercosporoid fungi [39,44–47].

Actively growing hyphal tips emerging from infected tissues were transferred onto fresh PDA plates. A second hyphal-tip transfer was performed after 10 days to obtain pure cultures. Pure cultures were maintained on PDA at 24 ± 1 °C and periodically subcultured until use for monoconidial isolation, morphological characterization, and antifungal susceptibility assays.

2.3. Obtaining Monoconidial Cultures

To obtain genetically homogeneous fungal cultures, monoconidial isolates were established from purified *Passalora fulva* cultures following a modified single-spore isolation procedure widely used for phytopathogenic fungi [41–43]. Conidia were harvested from 10-day-old PDA cultures by adding 10 mL of sterile distilled water to each Petri dish and gently scraping the colony surface with a sterile glass spreader to release the conidia into suspension.

The resulting conidial suspension was filtered through two layers of sterile cheesecloth to remove mycelial fragments and obtain a uniform conidial suspension. Serial tenfold

dilutions (10^{-1} – 10^{-6}) were prepared in sterile distilled water, and 0.5 mL aliquots were evenly spread onto PDA plates. Plates were incubated at 24 ± 1 °C in darkness for 10–15 days, until colonies originating from individual conidia became visible.

Well-separated colonies arising from single conidia were selected under a stereomicroscope and aseptically transferred onto fresh PDA plates. Each monoconidial isolate was subcultured twice to ensure culture purity before being maintained as working cultures on PDA at 24 ± 1 °C for subsequent morphological identification and antifungal susceptibility assays. All manipulations were performed under aseptic conditions in a laminar-flow cabinet to minimize the risk of contamination throughout the isolation procedure.

2.4. Morphological Characterization of Isolates

Purified monoconidial isolates were morphologically characterized after incubation on Potato Dextrose Agar (PDA) at 24 ± 1 °C for 15 days under dark conditions. Macroscopic characteristics, including colony diameter, colour, texture, colony margin characteristics, surface topography, aerial mycelium development, zonation, and reverse pigmentation, were recorded.

Microscopic observations were performed using a light microscope (Olympus BX43, Olympus Corporation, Tokyo, Japan) at 400× magnification. Fungal structures were mounted in lactophenol cotton blue, and at least 30 conidia and 20 conidiophores were examined for each isolate. The morphology and dimensions of hyphae, conidiophores, conidiogenous cells, and conidia, including their size, shape, pigmentation, septation, branching pattern, and arrangement, were evaluated.

Species identification was based on the diagnostic morphological characteristics currently accepted for *Passalora fulva*, including pigmented septate hyphae, fasciculate and branched conidiophores, sympodially proliferating conidiogenous cells bearing conspicuous conidial scars, and cylindrical to ellipsoid conidia produced in short branched chains. Particular attention was paid to the morphology of the conidiogenous loci and conidial hila, which represent important taxonomic characters for distinguishing *Passalora* species from other cercosporoid fungi. Colony morphology and microscopic features were compared with the taxonomic revisions of Braun, Crous and co-workers, together with recent taxonomic treatments of cercosporoid fungi and *Passalora* species [44–47]. Because the objective of the present study was the comparative evaluation of antifungal activity rather than fungal taxonomy, species identification was based on detailed morphological criteria. Molecular identification and pathogenicity tests were therefore beyond the scope of the present study.

Two monoconidial isolates consistently displaying the diagnostic macroscopic and microscopic characteristics of *P. fulva* were selected for subsequent antifungal susceptibility assays. Although slight differences in colony morphology and pigmentation were observed between the isolates, these differences did not affect the diagnostic characteristics used for species identification.

2.5. Plant Material, Essential Oil Extraction and Fungicide Formulation

Three aromatic plant species—namely, *Eucalyptus globulus* Labill., *Artemisia herba-alba* Asso, and *Mentha pulegium* L.—were selected based on their documented antifungal activity and their widespread use in Moroccan traditional medicine [23–30]. The aerial parts (leaves, stems, and flowers) were collected during April 2023 from the Ain Taoudate area (Fez–Meknes region, Morocco). Plant materials were botanically identified using standard floristic keys and taxonomic descriptions, and the nomenclature follows the World Flora Online (WFO) and Plants of the World Online (POWO) databases [48,49].

The plant material was air-dried in the shade at room temperature (22–25 °C) until a constant dry weight was obtained. Essential oils were extracted by hydrodistillation using a Clevenger-type apparatus in accordance with the European Pharmacopoeia (11th Edition) [50]. Briefly, 250 g of dried plant material were immersed in 1600 mL of distilled water in a 4 L round-bottom flask and hydrodistilled for 3 h after the onset of boiling. The essential oils were collected, separated from the aqueous phase using a separatory funnel, dried over anhydrous sodium sulfate, transferred into airtight sterile amber glass vials, and stored at 4 °C until further use [37,38,51].

A commercial fungicide formulation containing azoxystrobin (200 g L⁻¹) and difenoconazole (125 g L⁻¹) was used as the synthetic reference treatment because this combination is widely employed for the management of foliar fungal diseases in greenhouse tomato production, combining two fungicide classes with complementary modes of action; namely, quinone outside inhibitors (QoIs) and demethylation inhibitors (DMIs) [10–13,52–61].

Immediately before each experiment, stock emulsions of the essential oils were prepared in sterile distilled water containing 0.1% (v/v) Tween 80 as a non-ionic emulsifying agent to ensure homogeneous dispersion of the oils in the culture medium. Control treatments contained the same concentration of Tween 80 without essential oil to verify that Tween 80 alone did not affect fungal growth [62–68].

2.6. Mycelial Growth Inhibition Assay

The antifungal activity of the essential oils and the commercial azoxystrobin–difenoconazole formulation against *Passalora fulva* was evaluated in vitro using the poisoned food technique, a widely used method for assessing the effects of antifungal compounds on fungal radial growth [51–53].

Appropriate volumes of each stock emulsion or fungicide solution were incorporated into molten Potato Dextrose Agar (PDA) cooled to approximately 45 °C to obtain the desired final concentrations before the medium solidified. Control plates consisted of PDA supplemented with 0.1% (v/v) Tween 80, corresponding to the highest surfactant concentration used in the treatments.

A 4 mm-diameter mycelial plug was aseptically excised from the actively growing margin of a 10-day-old colony and placed at the centre of each amended PDA plate with the mycelial surface facing downward. Each treatment–concentration combination was evaluated using four independent replicates for each of the two monoconidial *P. fulva* isolates.

The plates were incubated at 25 ± 1 °C in darkness for 7 days. Colony diameter was measured along two perpendicular axes, and the mean value was calculated for each replicate.

The percentage of mycelial growth inhibition (MGI) was calculated according to the following equation:

$$\text{MGI}(\%) = \frac{D_c - D_t}{D_c} \times 100$$

where

D_c = mean colony diameter of the untreated control;

D_t = mean colony diameter of the treated colony.

The inhibition data were subsequently used to establish dose–response relationships and to estimate the IC₅₀ and IC₉₀ values by Probit regression analysis.

2.7. Sporulation Inhibition Assay

The effect of the tested treatments on the sporulation of *Passalora fulva* was evaluated after completion of the mycelial growth inhibition assay following standard haemocytometric procedures for fungal spore quantification [52–54]. After 7 days of incubation, 10 mL of sterile distilled water were added to each Petri dish, and the colony surface was

gently scraped with a sterile glass spreader to release the conidia into suspension. The resulting conidial suspension was homogenized by gentle agitation to ensure uniform spore distribution.

Conidial concentration was determined using a Thoma haemocytometer under a light microscope (Olympus BX43, Olympus Corporation, Tokyo, Japan). For each replicate, conidia were counted in four large haemocytometer squares following the standard counting procedure, and three independent counts were averaged to obtain the final conidial concentration. Conidial concentration (conidia mL⁻¹) was calculated according to the standard haemocytometer formula.

Sporulation inhibition (SI) was expressed as the percentage reduction in conidial production relative to the untreated control using the following equation:

$$SI(\%) = \frac{N_c - N_t}{N_c} \times 100$$

where

N_c = mean conidial concentration of the untreated control;

N_t = mean conidial concentration of the treated culture.

The inhibition data were subsequently used to establish dose–response relationships and estimate the corresponding IC₅₀ and IC₉₀ values by Probit regression analysis.

2.8. Conidial Germination Assay

The inhibitory effects of the tested essential oils and the commercial azoxystrobin–difenoconazole formulation on conidial germination of *Passalora fulva* were evaluated using PDA amended with the different concentrations of each treatment [52–54].

Conidial suspensions were prepared from 14-day-old cultures by adding 10 mL of sterile distilled water to each Petri dish and gently scraping the colony surface with a sterile glass spreader. The resulting conidial suspension was homogenized, and a 10⁻³ dilution was prepared with sterile distilled water. Preliminary assays indicated that this dilution provided an appropriate conidial density for microscopic evaluation.

Aliquots (100 µL) of the diluted suspension were uniformly spread onto PDA plates containing the different treatment concentrations. Control plates consisted of PDA supplemented with 0.1% (*v/v*) Tween 80, corresponding to the highest surfactant concentration used in the treatments. Each treatment–concentration combination was evaluated using four independent replicates for each of the two monoconidial isolates.

The inoculated plates were incubated at 25 ± 1 °C in darkness for 24 h. Conidial germination was assessed by examining at least 100 randomly selected conidia per replicate under a light microscope (Olympus BX43, Olympus Corporation, Tokyo, Japan) at 400× magnification. A conidium was considered germinated when the germ tube length was equal to or greater than the smallest conidial diameter, according to the widely accepted criterion used in fungal germination studies [53,69–71].

Conidial germination inhibition (CGI) was calculated using the following equation:

$$CGI(\%) = \frac{G_c - G_t}{G_c} \times 100$$

where

G_c = percentage of germinated conidia in the untreated control;

G_t = percentage of germinated conidia in the treated cultures.

2.9. Dose–Response Analysis and Determination of IC₅₀ and IC₉₀ Values

The antifungal efficacy of the tested essential oils and the commercial azoxystrobin–difenoconazole formulation was quantified by estimating the concentrations required to inhibit 50% (IC₅₀) and 90% (IC₉₀) of mycelial growth, sporulation, and conidial germination. Dose–response relationships were established separately for each fungal isolate, treatment, and biological parameter using Probit regression analysis, a widely accepted approach for estimating effective inhibitory concentrations in fungicide sensitivity and toxicological studies [55–57].

For each treatment, the observed inhibition percentages were transformed into Probit units, whereas the corresponding concentrations were converted to their decimal logarithms (log₁₀). Linear regression equations were fitted according to the following model:

$$Y = a \log_{10}(X) + b$$

where

Y = Probit-transformed inhibition response;

X = treatment concentration (ppm);

a = regression slope;

b = regression intercept.

The fitted regression equations were subsequently used to estimate the IC₅₀ and IC₉₀ values from the corresponding Probit responses. The goodness-of-fit of each regression equation was evaluated using the coefficient of determination (R^2), and the regression parameters were calculated independently for each treatment and fungal isolate.

When the inhibition response did not reach 50% or 90% within the tested concentration range, the corresponding IC₅₀ or IC₉₀ value was considered to be greater than the highest concentration tested.

2.10. Statistical Analysis

All experiments were conducted using a completely randomized design (CRD) with four independent replicates per treatment. Results are presented as mean $\pm$ standard deviation (SD).

Statistical analyses were performed using Minitab Statistical Software, Release 21 (Minitab LLC, State College, PA, USA). The effects of fungal isolate, treatment, concentration, and their interactions on mycelial growth inhibition, sporulation inhibition, and conidial germination inhibition were evaluated using a three-way analysis of variance (ANOVA), with isolate, treatment, and concentration considered as fixed factors [57–59].

Prior to ANOVA, the assumptions of normality and homogeneity of variances were assessed by examining residual plots, normal probability plots, and residual-versus-fitted plots generated by the software. Statistical significance was considered at $p < 0.05$. When significant differences were detected, treatment means were compared using Tukey's honestly significant difference (HSD) test at the 5% significance level [59].

Dose–response relationships were analysed independently for each fungal isolate and biological parameter using Probit regression analysis. The fitted regression equations were used to estimate the IC₅₀ and IC₉₀ values, and the goodness-of-fit of each regression model was evaluated using the coefficient of determination (R^2) [55–57].

3. Results

3.1. Morphological Characterization of *Passalora fulva* Isolates

Two monoconidial isolates of *Passalora fulva* were successfully isolated from symptomatic tomato leaves collected in commercial greenhouses in the Souss-Massa region.

After 15 days of incubation on PDA at 24 ± 1 °C, both isolates developed slow-growing, olive-green to greyish colonies characteristic of *P. fulva*, although noticeable differences in colony morphology were observed (Figure 1). Isolate 1 exhibited a sectorial growth pattern with turquoise-green pigmentation and irregular colony margins, whereas isolate 2 formed a predominantly flat colony with a pronounced central elevation, resulting in a crateriform colony morphology and displaying a greyish-green pigmentation.

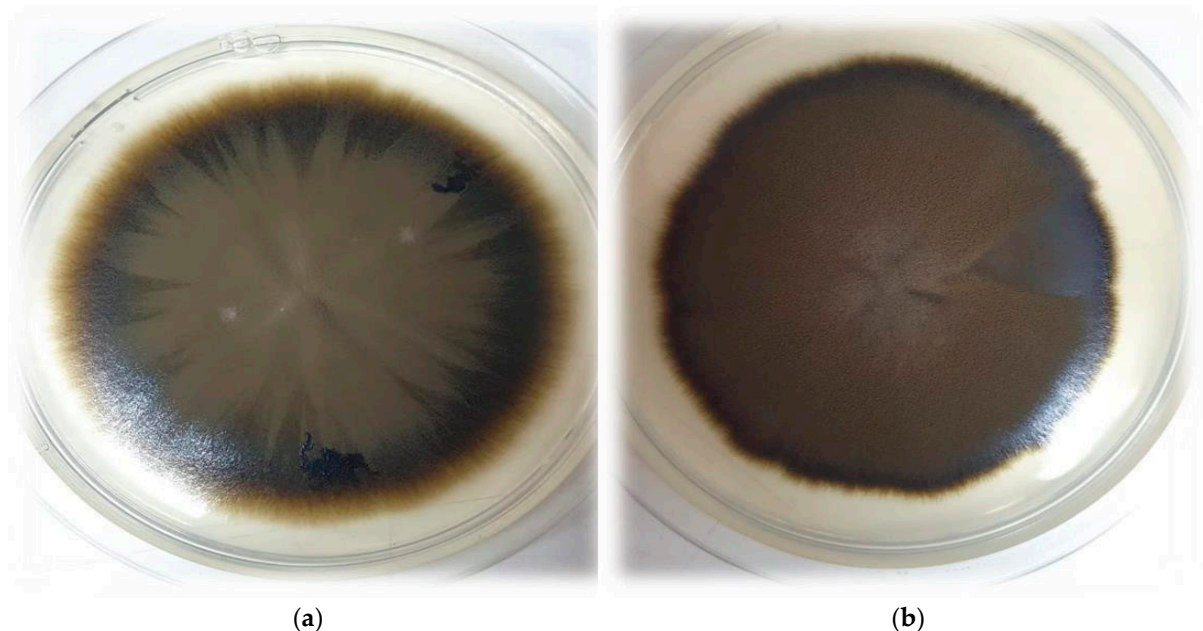


Figure 1. Macroscopic morphology of *Passalora fulva* isolates: (a) isolate 1 showing sector-like growth pattern; (b) isolate 2 showing a flat colony with central elevation.

Microscopic examination confirmed that both isolates shared the diagnostic morphological characteristics of *P. fulva*, including pigmented septate hyphae, branched conidiophores with sympodially proliferating conidiogenous cells, and cylindrical to ellipsoid conidia produced in short branched chains (Figure 1). These observations were consistent with the current taxonomic descriptions of *Passalora fulva* and related *Passalora* species [44–47], thereby confirming the morphological identity of the isolates used throughout the antifungal assays.

The observed differences in colony morphology suggest the existence of phenotypic variability within *P. fulva* populations. Similar intraspecific variation has previously been reported and may reflect underlying genetic diversity, adaptation to resistant tomato cultivars, and environmental conditions [7,72–74]. Despite these phenotypic differences, both isolates exhibited the diagnostic characteristics of *P. fulva* and were therefore considered suitable for the comparative evaluation of antifungal susceptibility.

3.2. Effect of Treatments on Mycelial Growth of *Passalora fulva*

The mycelial growth of both *Passalora fulva* isolates was progressively inhibited as treatment concentration increased (Figures 2 and 3), although the magnitude of inhibition differed significantly among the tested products. Factorial ANOVA confirmed that treatment, concentration, and their interaction significantly affected mycelial growth inhibition ($p < 0.001$), whereas the isolate effect was comparatively smaller (Table 1).

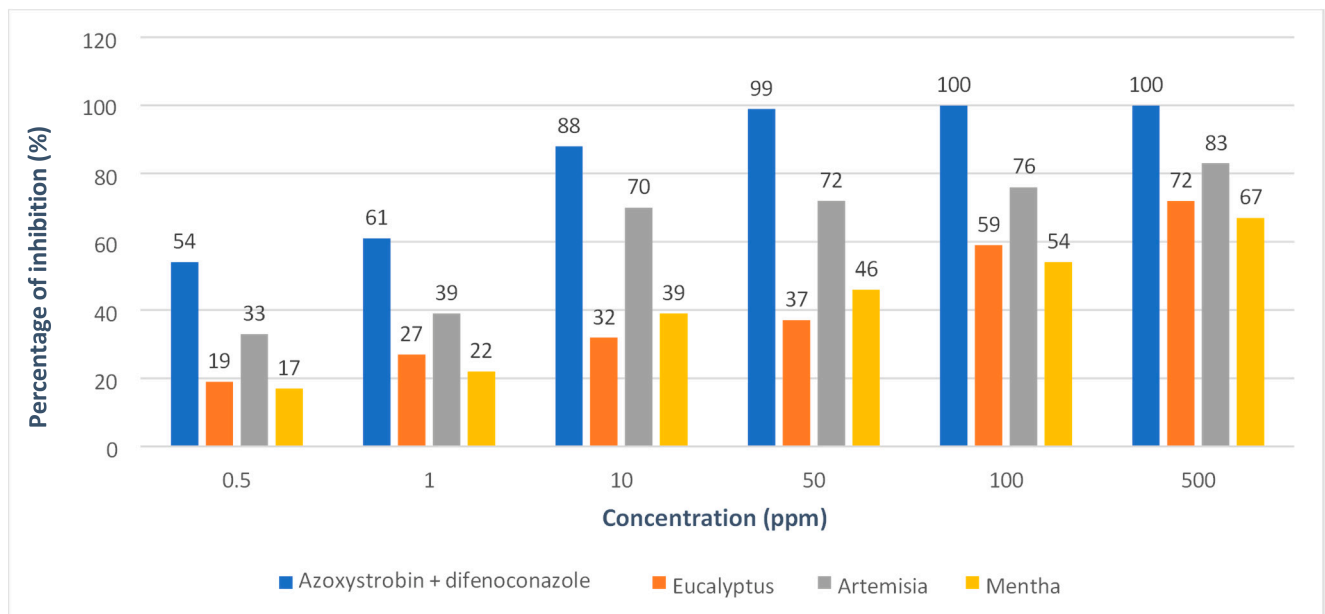


Figure 2. Percentage inhibition of mycelial growth of *P. fulva* isolate 1 on PDA medium amended with the four tested products.

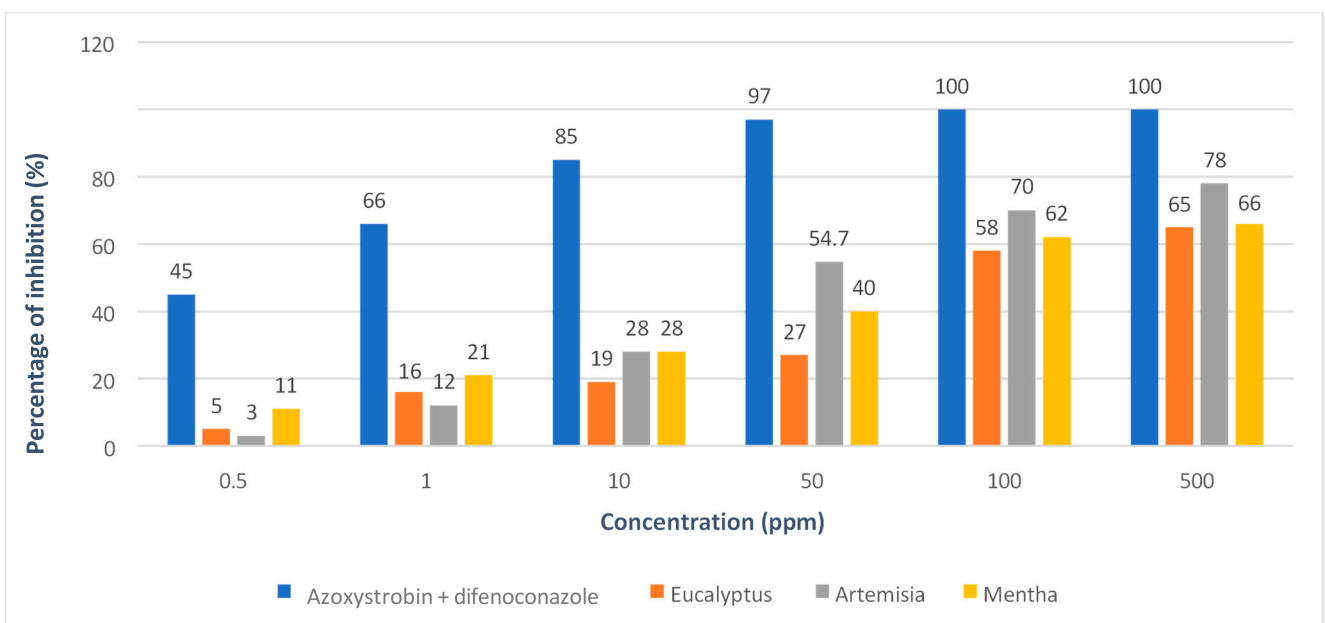


Figure 3. Percentage inhibition of mycelial growth of *P. fulva* isolate 2 on PDA medium amended with the four tested products.

The azoxystrobin–difenoconazole formulation exhibited the highest antifungal activity, resulting in complete inhibition of mycelial growth at concentrations ≥ 100 ppm in both isolates. This superior efficacy was further confirmed by the lowest estimated IC_{50} and IC_{90} values among all tested treatments, indicating a high sensitivity of *P. fulva* to the fungicide mixture.

Among the essential oils, *Artemisia herba-alba* was the most effective botanical treatment, producing a marked reduction in colony growth and considerably lower IC_{50} values than *Mentha pulegium* and *Eucalyptus globulus*. *Mentha pulegium* exhibited intermediate antifungal activity, whereas *Eucalyptus globulus* was consistently the least effective treatment, with appreciable fungal growth remaining even at the highest concentration tested.

Table 1. IC₅₀ and IC₉₀ values (ppm) of the four tested products for the inhibition of mycelial growth.

Product	Concentrations Corresponding to Isolate 1 (ppm)		Concentrations Corresponding to Isolate 2 (ppm)	
	IC ₅₀	IC ₉₀	IC ₅₀	IC ₉₀
Azoxystrobin + Difenoconazole	0.489	6.756	0.018	1.442
Essential oil of <i>Eucalyptus globulus</i> sp.	55.79	4.70×10^4	157.64	2.12×10^4
Essential oil of <i>Artemisia herba-alba</i> sp.	2.86	1.59×10^3	40.82	1.23×10^3
Essential oil of <i>Mentha pulegium</i> sp.	58.76	4.39×10^4	74.38	1.97×10^4

Overall, the antifungal efficacy of the tested treatments followed the order: azoxystrobin + difenoconazole > *Artemisia herba-alba* > *Mentha pulegium* > *Eucalyptus globulus*.

Although both isolates showed the same ranking of treatment efficacy, isolate 2 was slightly more sensitive to the commercial fungicide, whereas isolate 1 exhibited greater sensitivity to the essential oils, particularly *A. herba-alba*, indicating moderate intraspecific variability in antifungal susceptibility.

3.3. Effect of Different Treatments on Conidial Sporulation of *Passalora fulva*

All treatments reduced conidial sporulation of both *Passalora fulva* isolates in a concentration-dependent manner (Figures 4 and 5). Factorial ANOVA revealed significant effects of treatment, concentration, and their interaction on sporulation inhibition ($p < 0.001$), whereas isolate effects were comparatively less pronounced.

The azoxystrobin–difenoconazole mixture exhibited the highest inhibitory activity, completely suppressing sporulation at concentrations ≥ 100 ppm in both isolates. This superior efficacy was confirmed by the lowest IC₅₀ and IC₉₀ values obtained from the Probit analysis (Table 2), indicating high sensitivity of *P. fulva* to the fungicide mixture.

Among the essential oils, *Artemisia herba-alba* consistently showed the greatest inhibitory effect on conidial production, followed by *Mentha pulegium*, whereas *Eucalyptus globulus* exhibited the weakest activity throughout the tested concentration range. Although all essential oils produced a progressive reduction in sporulation with increasing concentration, none achieved complete inhibition at the maximum concentration tested.

The two isolates displayed similar response patterns, with isolate 2 being slightly less sensitive than isolate 1 at intermediate concentrations. Nevertheless, the ranking of treatment efficacy remained identical for both isolates:

Azoxystrobin + difenoconazole > *Artemisia herba-alba* > *Mentha pulegium* > *Eucalyptus globulus*.

This ranking was further supported by the dose–response analysis (Table 2), which showed markedly lower IC₅₀ and IC₉₀ values for the azoxystrobin–difenoconazole formulation than for the essential oils. Among the botanical treatments, *A. herba-alba* exhibited the lowest inhibitory concentrations, confirming its superior antifungal activity against conidial sporulation.

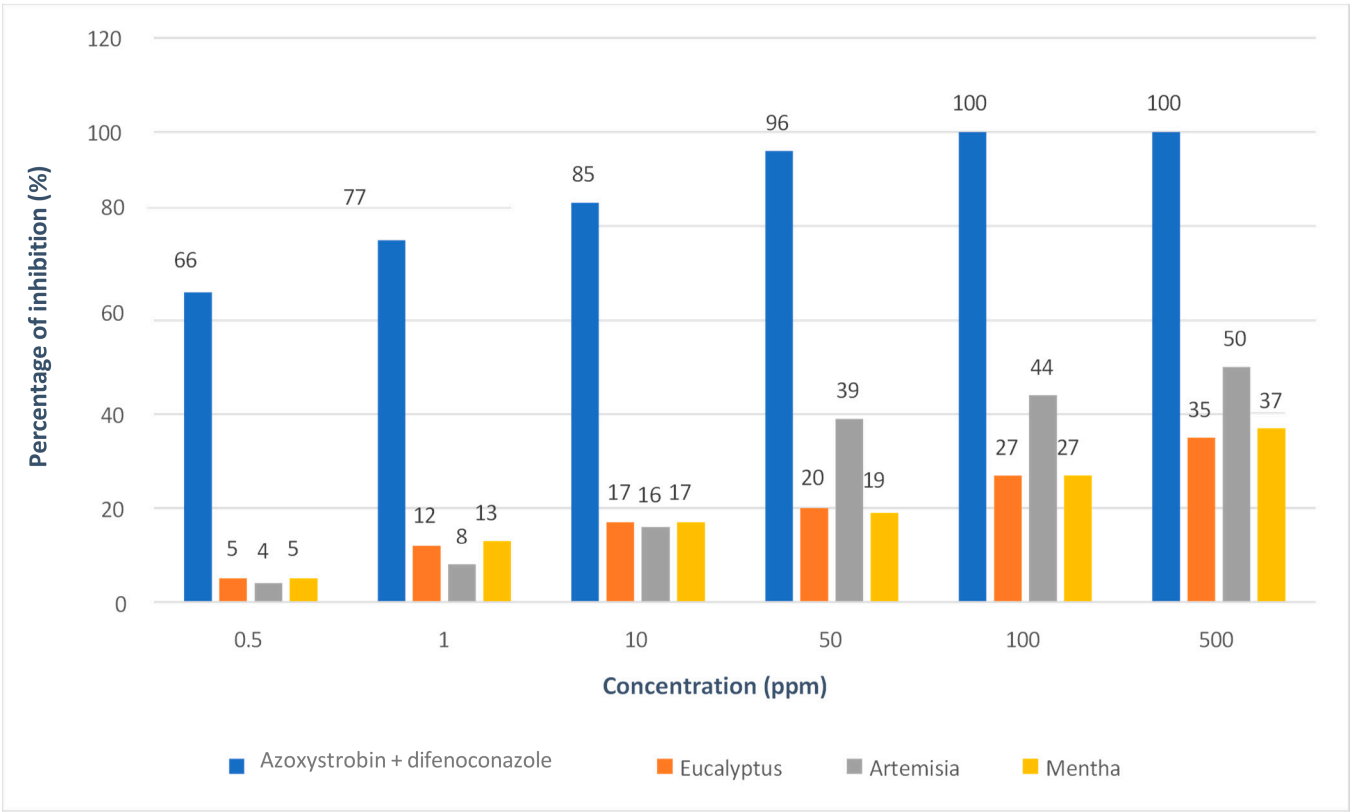


Figure 4. Percentage inhibition of sporulation of *P. fulva* isolate 1 on PDA medium amended with the four tested products.

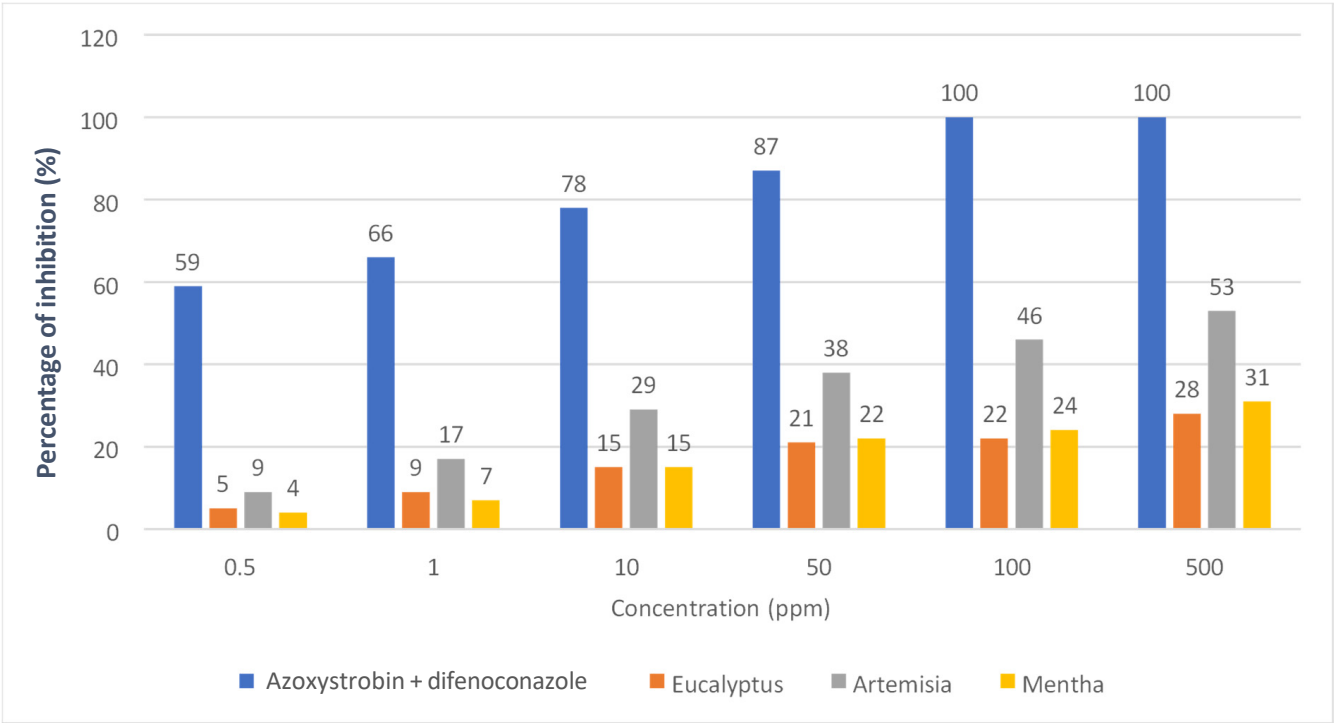


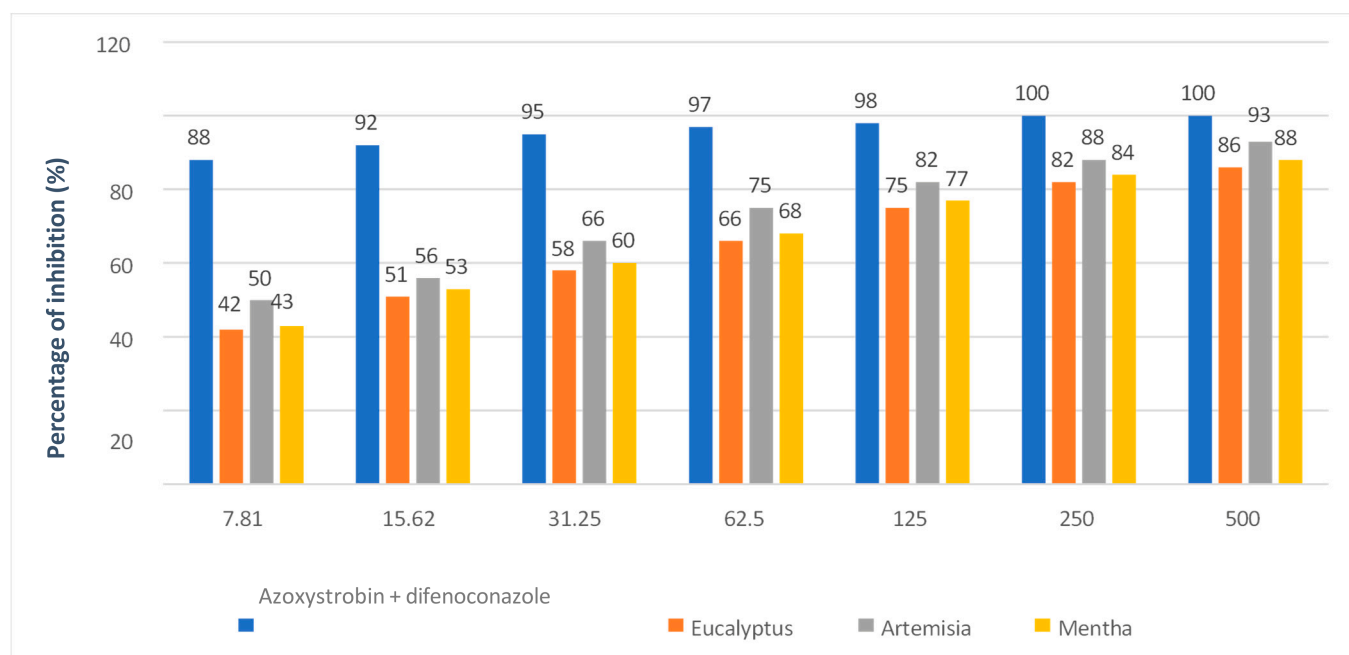
Figure 5. Percentage inhibition of sporulation of *P. fulva* isolate 2 on PDA medium amended with the four tested products.

Table 2. IC₅₀ and IC₉₀ values of the three tested products for the inhibition of sporulation.

Product	Corresponding Concentrations for Isolate 1 (ppm)		Corresponding Concentrations for Isolate 2 (ppm)	
	IC ₅₀	IC ₉₀	IC ₅₀	IC ₉₀
Azoxystrobin + Difenoconazole	0.243	5.617	0.526	10.211
Essential oil of <i>Eucalyptus globulus</i> sp.	5.93×10^4	2.08×10^7	1.92×10^4	1.37×10^8
Essential oil of <i>Artemisia herba-alba</i> sp.	274.179	3.48×10^4	227.485	1.58×10^5
Essential oil of <i>Mentha pulegium</i> sp.	5.36×10^3	1.81×10^7	6.07×10^3	9.07×10^6

3.4. Effect of Different Treatments on Conidial Germination of *Passalora fulva*

Conidial germination of both *Passalora fulva* isolates was progressively inhibited as treatment concentration increased (Figures 6 and 7). Three-way ANOVA confirmed that treatment, concentration, and their interaction significantly affected conidial germination inhibition ($p < 0.001$), whereas the isolate effect was comparatively smaller.

**Figure 6.** Percentage inhibition of spore germination of *P. fulva* isolate 1 on PDA medium amended with the four tested products.

The azoxystrobin–difenoconazole formulation exhibited the greatest inhibitory activity, resulting in almost complete inhibition at intermediate concentrations and complete suppression of conidial germination at concentrations ≥ 250 ppm in both isolates. This superior efficacy was further confirmed by the lowest estimated IC₅₀ and IC₉₀ values obtained by Probit regression analysis (Table 3), indicating a high sensitivity of *P. fulva* conidia to the fungicide formulation.

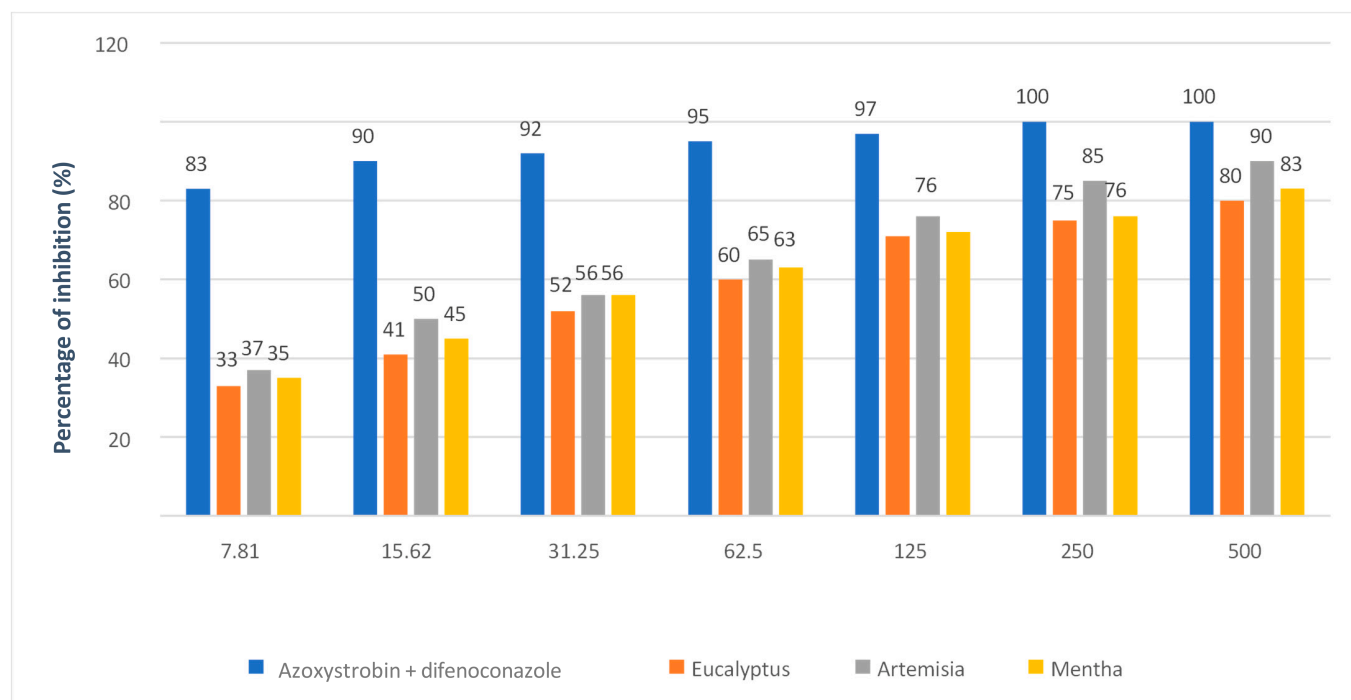


Figure 7. Percentage inhibition of spore germination of *P. fulva* isolate 2 on PDA medium amended with the four tested products.

Table 3. IC₅₀ and IC₉₀ values of the four tested products for the inhibition of spore germination.

Product	Corresponding Concentrations for Isolate 1 (ppm)		Corresponding Concentrations for Isolate 2 (ppm)	
	IC ₅₀	IC ₉₀	IC ₅₀	IC ₉₀
Azoxystrobin + Difenoconazole	0.935	12.924	1.823	19.564
Essential oil of <i>Eucalyptus globulus</i> sp.	15.084	905.302	29.221	1956.137
Essential oil of <i>Artemisia herba-alba</i> sp.	9.431	324.056	18.717	528.441
Essential oil of <i>Mentha pulegium</i> sp.	13.429	664.844	23.007	1343.837

Among the essential oils, *Artemisia herba-alba* was the most effective botanical treatment, followed by *Mentha pulegium*, whereas *Eucalyptus globulus* exhibited the lowest antifungal activity throughout the tested concentration range. Although all essential oils displayed clear concentration-dependent inhibition, none achieved complete suppression of conidial germination at the highest concentration evaluated.

Dose–response analysis further supported these observations (Table 3). The azoxystrobin–difenoconazole formulation exhibited the lowest estimated IC₅₀ and IC₉₀ values, whereas *A. herba-alba* showed the greatest antifungal potency among the botanical treatments, followed by *M. pulegium* and *E. globulus*. Probit regression analysis demonstrated strong linear relationships between the logarithm of treatment concentration and the inhibition response for all treatments, with steeper regression slopes for the fungicide formulation and *A. herba-alba*, indicating greater biological potency.

Overall, the inhibitory activity of the tested treatments against conidial germination followed the order azoxystrobin + difenoconazole > *Artemisia herba-alba* > *Mentha pulegium* > *Eucalyptus globulus*.

Although both isolates responded similarly to the tested treatments, isolate 2 consistently exhibited slightly higher estimated IC₅₀ and IC₉₀ values than isolate 1, indicating slightly lower sensitivity while maintaining the same overall concentration–response pattern.

3.5. Analysis of Variance Across Different Assays

3.5.1. Analysis of Variance for Mycelial Growth Inhibition

A three-way analysis of variance (ANOVA) was conducted to evaluate the effects of isolate, treatment, concentration, and their interactions on the inhibition of *Passalora fulva* mycelial growth (Table 4). All main factors significantly affected mycelial growth inhibition ($p < 0.001$). Among the main factors, concentration had the greatest influence, followed by treatment, indicating that antifungal activity was strongly dose-dependent and differed markedly among the tested products.

Table 4. Three-way ANOVA for the effects of isolate, treatment, and concentration on the inhibition of mycelial growth of *Passalora fulva*.

Source of Variation	df	F-Value	p-Value	Significance
Isolate	1	185.86	<0.001	***
Treatment	3	918.78	<0.001	***
Concentration	6	1289.26	<0.001	***
Isolate × Treatment	3	42.94	<0.001	***
Isolate × Concentration	6	23.00	<0.001	***
Treatment × Concentration	18	35.67	<0.001	***
Isolate × Treatment × Concentration	18	5.93	<0.001	***
Error	168	—	—	—

Significance codes: *** $p < 0.001$; — = not applicable.

Significant treatment × concentration, isolate × treatment, and isolate × concentration interactions ($p < 0.001$) indicated that the response of *P. fulva* depended on both the treatment applied and its concentration. The significant three-way interaction further indicated that the concentration-dependent response differed slightly between the two isolates according to the treatment evaluated.

Overall, the ANOVA demonstrated that mycelial growth inhibition was primarily determined by treatment concentration, while treatment type and isolate also contributed significantly to the observed variability. These results support the dose-dependent antifungal activity of the tested products and the subsequent dose–response analyses. Furthermore, they indicate that antifungal efficacy depended not only on treatment potency but also, to a lesser extent, on isolate-specific susceptibility.

3.5.2. Analysis of Variance for Sporulation Inhibition

A three-way analysis of variance (ANOVA) was conducted to assess the effects of isolate, treatment, concentration, and their interactions on the inhibition of *Passalora fulva* sporulation (Table 5). Treatment and concentration had highly significant effects on sporulation inhibition ($p < 0.001$), indicating that conidial production was strongly influenced by both the nature of the antifungal treatment and its concentration.

In contrast, the isolate effect was not significant, indicating that both *P. fulva* isolates responded similarly with respect to conidial sporulation. Among the interaction terms, only the treatment × concentration interaction was significant ($p < 0.001$), indicating that the effect of increasing concentration depended on the treatment applied. Neither the isolate × concentration interaction nor the three-way interaction was statistically significant, indicating similar concentration–response patterns for both isolates regardless of the treatment applied.

Table 5. Three-way ANOVA for the effects of isolate, treatment, and concentration on sporulation inhibition of *Passalora fulva*.

Source of Variation	df	F-Value	p-Value	Significance
Isolate	1	1.19	0.276	ns
Treatment	3	549.22	<0.001	***
Concentration	6	144.52	<0.001	***
Isolate × Treatment	3	3.13	0.027	*
Isolate × Concentration	6	0.21	0.975	ns
Treatment × Concentration	18	17.00	<0.001	***
Isolate × Treatment × Concentration	18	0.62	0.883	ns
Error	168	—	—	—

Significance codes: *** $p < 0.001$; * $p < 0.05$; ns = not significant; — = not applicable.

Overall, the ANOVA demonstrated that sporulation inhibition was primarily determined by treatment type and concentration, whereas isolate identity had little influence on the observed variability. These findings indicate that differences in sporulation inhibition were mainly attributable to treatment efficacy rather than isolate-specific variability.

3.5.3. Analysis of Variance for Conidial Germination Inhibition

A three-way analysis of variance (ANOVA) was conducted to evaluate the effects of isolate, treatment, concentration, and their interactions on the inhibition of *Passalora fulva* conidial germination (Table 6). All main factors significantly influenced conidial germination inhibition ($p < 0.001$). Among the main factors, concentration had the greatest influence, followed by treatment, indicating that antifungal activity was strongly dose-dependent and differed markedly among the tested products.

Table 6. Three-way ANOVA for the effects of isolate, treatment, and concentration on conidial germination inhibition of *Passalora fulva*.

Source of Variation	df	F-Value	p-Value	Significance
Isolate	1	185.67	<0.001	***
Treatment	3	1629.20	<0.001	***
Concentration	7	3796.55	<0.001	***
Isolate × Treatment	3	8.79	<0.001	***
Isolate × Concentration	7	5.87	<0.001	***
Treatment × Concentration	21	70.74	<0.001	***
Isolate × Treatment × Concentration	21	1.25	0.215	ns
Error	192	—	—	—

Significance codes: *** $p < 0.001$; ns = not significant; — = not applicable.

Significant treatment × concentration, isolate × treatment, and isolate × concentration interactions ($p < 0.001$) indicated that the inhibitory response depended on both the treatment applied and its concentration. In contrast, the three-way interaction was not statistically significant, indicating that the concentration–response pattern of each treatment was generally consistent across the two isolates.

Overall, the ANOVA demonstrated that conidial germination inhibition was primarily determined by treatment concentration and treatment type, whereas isolate-related differences contributed to a lesser extent to the observed variability. These results further support the reproducibility of the inhibitory effects of the tested treatments against both *P. fulva* isolates. These findings indicate that the inhibitory effects on conidial germination were highly reproducible across both fungal isolates despite minor differences in susceptibility.

4. Discussion

The present study provides a comprehensive evaluation of the antifungal activity of a commercial azoxystrobin–difenoconazole formulation and three essential oils (*Artemisia herba-alba*, *Mentha pulegium*, and *Eucalyptus globulus*) against two monoconidial isolates of *Passalora fulva*. Unlike previous studies, which have generally focused on radial mycelial growth or evaluated botanical extracts against other phytopathogenic fungi, this work simultaneously assessed mycelial growth, sporulation, and conidial germination, thereby providing a broader assessment of antifungal efficacy and its relevance for integrated management of tomato leaf mold [7,20,72,75]. Across all biological parameters, the azoxystrobin–difenoconazole formulation consistently exhibited the greatest antifungal activity, producing the lowest IC₅₀ and IC₉₀ values and the highest inhibition percentages. These findings are consistent with previous studies reporting the high efficacy of QoI–DMI mixtures against foliar fungal pathogens of tomato and other economically important crops [10–16,76]. Moreover, the factorial ANOVA identified treatment and concentration as the principal sources of variation, whereas isolate effects were comparatively limited, indicating that antifungal efficacy was primarily determined by treatment potency rather than isolate identity. The agreement among ANOVA, Probit regression, and IC₅₀/IC₉₀ estimates further supports the robustness of the experimental approach.

The superior efficacy of the azoxystrobin–difenoconazole mixture can be explained by the complementary modes of action of its two active ingredients. Azoxystrobin, a quinone outside inhibitor (QoI), disrupts mitochondrial respiration by blocking electron transfer at the cytochrome bc₁ complex, whereas difenoconazole, a demethylation inhibitor (DMI), inhibits sterol 14 α -demethylase (CYP51), thereby impairing ergosterol biosynthesis and fungal membrane formation. Similar synergistic effects between QoI and DMI fungicides have been widely reported against several phytopathogenic fungi, including *Alternaria*, *Botrytis*, *Fusarium*, and *Colletotrichum*, supporting the broad-spectrum efficacy of this fungicide combination [11,14–16,77,78]. The simultaneous disruption of energy production and membrane integrity therefore likely explains the consistently high inhibition of mycelial growth, sporulation, and conidial germination observed in the present study.

Antifungal activity increased significantly with treatment concentration, demonstrating a clear dose–response relationship across all biological parameters. This trend, confirmed by factorial ANOVA and Probit regression analyses, indicates that the inhibitory response of *P. fulva* was strongly concentration dependent. The low IC₅₀ and IC₉₀ values obtained for the azoxystrobin–difenoconazole formulation further confirmed its high antifungal potency, in agreement with previous studies reporting that QoI–DMI mixtures provide superior efficacy and broader activity than single-site fungicides against several phytopathogenic fungi [15,16,57,76–78].

Among the tested essential oils, *Artemisia herba-alba* consistently exhibited the greatest antifungal activity, producing the lowest IC₅₀ and IC₉₀ values and the highest inhibition of mycelial growth, sporulation, and conidial germination. Similar results have been reported for *Artemisia* species against *Fusarium oxysporum*, *Alternaria alternata*, *Botrytis cinerea*, and *Colletotrichum* spp., where inhibition levels ranging from 60 to more than 90% have been attributed to the abundance of oxygenated monoterpenes and sesquiterpenes [23–30,79]. Although no previous study has specifically evaluated *A. herba-alba* against *P. fulva*, the present results extend the known antifungal spectrum of this species and identify it as a promising candidate for tomato leaf mold management.

The superior activity of *A. herba-alba* is likely associated with its phytochemical profile, particularly its high content of camphor, α - and β -thujone, borneol, chrysanthenone, and 1,8-cineole [24,26–30,80]. These metabolites have been shown to disrupt plasma membrane integrity, impair mitochondrial respiration, interfere with ergosterol biosyn-

thesis, and induce oxidative stress, resulting in inhibition of fungal growth and reproduction [19–21,33–36,81]. Because these compounds act on multiple cellular targets, essential oils may exert a lower selection pressure for resistance than conventional site-specific fungicides, although this hypothesis requires further experimental confirmation.

In contrast, *Mentha pulegium* and *Eucalyptus globulus* displayed comparatively lower antifungal activity throughout the study. Similar differences among essential oils have been widely attributed to qualitative and quantitative variations in their chemical composition, which are influenced by genotype, geographical origin, environmental conditions, phenological stage, and extraction method [23,25,27,29,82]. Consequently, differences in antifungal efficacy reported among independent studies should be interpreted in light of this natural phytochemical variability. Future studies integrating GC–MS profiling with bioassays would facilitate the identification of the metabolites responsible for antifungal activity and improve the standardization and reproducibility of botanical fungicides [15,83,84].

The statistical analyses reinforced the biological interpretation of the results by revealing significant treatment $\times$ concentration interactions for mycelial growth, sporulation, and conidial germination, confirming distinct dose–response patterns among the tested products [55–59]. The consistency between factorial ANOVA, Probit regression, and IC_{50}/IC_{90} estimates provides robust evidence for the superior efficacy of the azoxystrobin–difenoconazole formulation and the comparatively greater activity of *Artemisia herba-alba*.

A major strength of this study is the simultaneous evaluation of three key developmental stages of *P. fulva*. Unlike studies limited to radial mycelial growth, this approach demonstrated that antifungal efficacy varied according to the fungal developmental stage, with conidial germination being the most sensitive and sporulation the least sensitive process. Similar stage-dependent responses have been reported for several phytopathogenic fungi, emphasizing that antifungal compounds may differ considerably in their activity depending on the biological process targeted [54,81,85,86].

The high susceptibility of conidial germination observed in the present study is consistent with previous reports showing that fungal conidia are generally more vulnerable than mature mycelia because germination requires rapid activation of respiration, membrane biosynthesis, and germ tube formation [69–71,87]. Consequently, disruption of mitochondrial respiration and ergosterol biosynthesis by azoxystrobin and difenoconazole, together with membrane damage and oxidative stress induced by essential oil constituents, can effectively prevent the establishment of infection [11,19–21,33–36].

In contrast, sporulation required higher concentrations to achieve comparable inhibition, suggesting that fungal reproductive processes are more resilient than early germination events. Similar observations have been reported for other phytopathogenic fungi exposed to plant-derived essential oils, reinforcing the importance of evaluating multiple developmental stages when screening antifungal compounds for integrated disease management [54,79–81,88].

The IC_{50} and IC_{90} values provided a robust measure of antifungal potency by integrating the entire dose–response relationship. Their consistently lower values for the azoxystrobin–difenoconazole formulation confirmed its superior efficacy, whereas *Artemisia herba-alba* exhibited the greatest antifungal activity among the botanical treatments across all developmental stages. These findings were further supported by the Probit regression analyses, which showed steeper dose–response slopes for the fungicide mixture than for the essential oils, reflecting its higher biological potency [55–57,76,78].

Although both *P. fulva* isolates exhibited the same overall ranking of treatment efficacy, differences in sensitivity were observed for mycelial growth and conidial germination, whereas sporulation was only marginally affected by isolate identity. Similar intraspecific variability has been reported in *P. fulva* populations and has been associated with genetic

diversity, adaptation to tomato resistance genes, and long-term fungicide selection pressure [7,72–75]. Nevertheless, treatment $\times$ concentration interactions accounted for a much larger proportion of the observed variability than isolate identity, indicating that antifungal efficacy depended primarily on treatment potency. The consistent activity of *A. herba-alba* against both isolates further suggests that its antifungal effect is relatively stable despite the natural variability of *P. fulva* populations.

Collectively, these findings demonstrate that evaluating multiple developmental stages and fungal isolates provides a more comprehensive assessment of antifungal efficacy than assays based solely on radial mycelial growth. Although the azoxystrobin–difenoconazole formulation remained the most effective treatment, *Artemisia herba-alba* consistently ranked as the most active botanical product, showing broad-spectrum inhibition of mycelial growth, sporulation, and conidial germination. These results support its potential as a complementary component of integrated disease management (IDM) programmes aimed at reducing reliance on site-specific fungicides such as QoIs and DMIs, thereby helping to mitigate the risk of resistance development [9–16,76–78].

The superior antifungal activity of *Artemisia herba-alba* is most likely associated with its phytochemical composition, particularly its high content of oxygenated monoterpenes such as camphor, α -/ β -thujone, borneol, chrysanthenone, and 1,8-cineole [24,26–30,80]. These compounds are known to disrupt membrane integrity, impair mitochondrial respiration and ergosterol biosynthesis, and induce oxidative stress, thereby inhibiting fungal growth through multiple cellular targets [19–21,33–36,81]. Such a multitarget mode of action may reduce the likelihood of rapid resistance development compared with site-specific fungicides. In contrast, the comparatively lower activity of *Mentha pulegium* and *Eucalyptus globulus* probably reflects differences in their phytochemical composition, which is influenced by genotype, environmental conditions, phenological stage, and extraction procedures [23,25,27,29,82]. These findings highlight the importance of detailed chemical characterization, particularly by GC–MS, to identify the metabolites responsible for antifungal activity and facilitate comparisons among studies [15,83].

Although *A. herba-alba* showed promising antifungal activity, the present findings should be interpreted cautiously because all experiments were performed under controlled in vitro conditions. Environmental factors, together with the volatility and limited stability of essential oils, may substantially reduce their efficacy under greenhouse or field conditions [84–87]. Future research should therefore combine GC–MS profiling with greenhouse and field validation, optimize formulation technologies such as nanoemulsions or microencapsulation to improve stability and persistence, and evaluate combinations of essential oils with reduced fungicide doses. Such approaches could enhance the practical application of botanical products while reducing fungicide inputs and selection pressure within integrated disease management (IDM) programmes [15,88–91].

The present study provides robust evidence of the superior antifungal efficacy of the azoxystrobin–difenoconazole formulation against *Passalora fulva* while identifying *Artemisia herba-alba* as the most promising botanical treatment. These findings support the potential integration of plant-derived products into sustainable tomato leaf mold management programmes as complementary tools to reduce reliance on conventional fungicides. Future research should focus on the chemical characterization of *A. herba-alba* by GC–MS, optimization of formulation technologies, evaluation of synergistic combinations with reduced fungicide doses, and validation under greenhouse and field conditions to confirm its practical applicability [15,83–93].

5. Conclusions

To our knowledge, this study provides the first comprehensive comparative evaluation of the antifungal activity of a commercial azoxystrobin–difenoconazole formulation and three plant-derived essential oils against *Passalora fulva* by simultaneously assessing three key stages of the pathogen life cycle: mycelial growth, sporulation, and conidial germination. This integrated approach provides a broader assessment of antifungal efficacy than conventional evaluations based solely on mycelial growth.

Among the tested treatments, the azoxystrobin–difenoconazole formulation consistently exhibited the highest antifungal efficacy, with the lowest estimated IC₅₀ and IC₉₀ values and the highest inhibition rates across all biological parameters. Among the botanical products, *Artemisia herba-alba* was the most effective essential oil, significantly inhibiting fungal growth, sporulation, and conidial germination, although at higher concentrations than the synthetic fungicide. The inclusion of two monoconidial isolates also demonstrated moderate intraspecific variability in antifungal sensitivity, highlighting the importance of considering pathogen diversity when evaluating novel disease management strategies.

Although the experiments were conducted under controlled in vitro conditions, the results identify *Artemisia herba-alba* as a promising source of natural antifungal compounds that could complement conventional fungicides within integrated disease management programmes, thereby reducing fungicide inputs and limiting selection pressure for fungicide resistance. Future research should prioritize GC–MS characterization of its bioactive constituents, optimization of formulation technologies, evaluation of synergistic combinations with reduced fungicide doses, and validation under greenhouse and field conditions. Such investigations will be essential for determining the practical feasibility of integrating plant-derived antifungal products into sustainable management programmes for tomato leaf mold.

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Abbreviations

The following abbreviations are used in this manuscript:

IC ₅₀	Half-maximal inhibitory concentration
IC ₉₀	Ninety percent inhibitory concentration
PDA	Potato Dextrose Agar
ANOVA	Analysis of Variance
Ppm	Parts per million
Df	Colony diameter in treated plates

Dt	Colony diameter in control plates
IS	Inhibition of sporulation
NT	Number of spores per cm ² in the control
NF	Number of spores per cm ² in treated samples

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