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Morphological and molecular identification of Diaporthe eres, causing foliar blight on Chrysanthemum morifolium in Egypt: Integrated assessment of pathogen characterization, host genetic diversity and fungicide efficacy

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Abstract

Background

Chrysanthemum morifolium is considered a vital export crop in Egypt, but its production is severely constrained by fungal foliar blights. While Alternaria species are known and well-addressed pathogens, identification of other fungal agents and their economical impact on crops remain poorly characterized in Egypt, leading to ineffective disease management.

Methods

Fungal agents from symptomatic chrysanthemum plants' leaves, collected from Menofia governorate, were purified and identified, both morphologically and molecularly (ITS sequencing). Pathogenicity was confirmed via Koch's postulates. In vitro efficacy of five fungicides (Difinoconazole, Chlorothalonil, Azoxystrobin, Propamocarb and Thiophanate-methyl; at three concentrations: 1000–3000ppm) was assessed using poisoned food technique. Start Codon Targeted (SCoT) oligonucleotides were used on genetic matter (DNA) extracted from infected chrusanthemum plants to distinguish genetic diversity within forementioned fungal agents.

Results

Current study provides the first conclusive report of *Diaporthe eres* as a high virulence pathogen of chrysanthemum in Egypt, alongside infections by *Alternaria sp*., confirmed by pathogenicity tests. Fungicide screening revealed superior efficacy of Difinoconazole, achieving up to 90% mycelial growth inhibition, against all pathogens. In contrast, Thiophanate-methyl was largely ineffective, indicating probable resistance due to the pathogen's genetic background. SCoT analysis revealed genetic distinctness of *D. eres* from *Alternaria* isolates as well as moderate polymorphism (29.59%), providing insights into pathogen's population structure.

Conclusion

The ineffectiveness of certain fungicides, due to emergence of *D. eres*, signifies a major shift chrysanthemums disease profile in Egyptian. Our findings advocate for an integrated disease management strategy that prioritizes application of an effective fungicide, Difinoconazole, combined with within rotation program. We highly recommend prohibiting distribution and application of ineffective chemicals, to reduce further emergence of pathogen mutants that overcome promising effective fungicides. Finally, pathogens' and plants' molecular characterization leverages genetic of great importance that can be utilized in developinmg disease-resisatnt cultivars.

1. Introduction

Cut flowers are a cornerstone of Egypt's agricultural economy, contributing significantly to export revenues and rural livelihoods. Among these cut flowers, *Chrysanthemum morifolium* Ramat (chrysanthemum), a member of the Asteraceae family that stands out as one of the most economically important ornamental plants globally. Revered in East Asian cultures as a symbol of vitality and longevity, chrysanthemums are equally prized in international markets as cut flowers and potted plants [1, 2]. It is highly valued as both a potted plant and a cut flower in marketing international. The demand for chrysanthemum flowers is consistently high, especially from European markets in winter months and during the year in our country. However, high-quality cut flowers production, year-round, in open-field environments considered a challenge. In India, several factors, contributing to this challenge, have been identified. Significant factors

include a) diseases such as *Alternaria* leaf blight, Septoria leaf spot, rust, wilt, and bacterial blight; b) availability of varieties with genetic background (genes) conferring resistance/tolerance to both biotic and abiotic stresses. Egypt's strategic geographic position, neighboring European markets' high demand, enables year-round chrysanthemum production, particularly during winter months. However, achieving consistent, high-quality yields remains a challenge due to biotic stresses, especially fungal diseases, which devastate crops, reduce marketability and threaten the livelihoods of smallholder farmers. Leaf blight symptoms caused by *Alternaria chrysanthemi* [3] are considered destructive and lead to significant losses in both field and market conditions. Necrotic lesions, chlorosis, and flower deformation in cut flowers, resulted from *Alternaria* cause yield loss by 30–50% under pathogen-favorable conditions [4]. Lack of commercial cultivars resistant/tolerant to biotic/abiotic stresses further complicates this issue, forcing farmers to rely heavily on chemical controls. Although fungicides like mancozeb and carbendazim are widely used, prolonged application has led to alarming levels of pathogen tolerance, rendering commercial fungicides increasingly ineffective [5]. Mutations in pathogens' genetic background and the emergence of novel pathogens, such as *Diaporthe* species; which are destructive in crops like hazelnuts and cherries, chrysanthemums are believed to be related to extensive fungicides utilization over long-term [6].

The economic remifications stemming from fungal diseases are profound. In India, where chrysanthemum cultivation faces similar challenges as Egypt, Annual losses due to decreased exports and deteriorating quality of cut flowers were estimated at approximately $12 million [7]. In Egypt, the absence of region-specific data on pathogen diversity and commercial fungicide efficacy contributes to the wide-spread of mutated fungal isolates that are resistant to fungicides; which in return compounds economic losses, leaving farmers without tailored solutions. Overcoming these gaps requires dual approach; 1) Identifying sustainable alternatives to failing fungicides through systematic evaluation of new chemical and biological agents and; 2) Developing resistant cultivars either via traditional and modern breeding programs or even utilizing genetic engineering techniques to assess and harness genetic diversity.

Advanced molecular marker technologies offer promising avenues for plant breeding programs. While techniques like Random Amplified Polymorphic DNA and Inter Simple Sequence Repeats have been used to scrutinize for genetic diversity, Start Codon Targeted (SCoT) markers have emerged as a superior tool, due to their reproducibility, high polymorphism and direct association with functional gene regions [8, 9]. The later primers target conserved sequences flanking the start codon (ATG), enabling precise characterization of genetic variations, linked to stress tolerance and disease resistance [10]. This technology has already revolutionized crop improvement in species like wheat and rice, but remains underutilized in ornamental species like chrysanthemums.

The current study aims to:

1. Reporting the first incidence of *Diaporthe eres* causing foliar blight on Egyptian chrysanthemums. Pathogenicity analysis, followed by morphological and molecular characteristics were utilized to confirm our findings.
2. Evaluation of five fungicides' efficacy (including Difinoconazole and Azoxystrobin) at three concentrations to identify sustainable alternatives to chemicals that are no longer effictive, due to pathogens' genetic background.
3. Utilizing SCoT markers to assess genetic diversity among genetic material (DNA) isolated from chrysanthemum plants, infected with fungl isolates under investigation, providing a foundation that might help in predicting candidate resistance genes for further molecular breeding programs.

Integration of pathogen characterization, fungicide effectiveness along with pathogens' characterization at molecular level, this manuscript offers better understanding of the relationship between chrysanthemum, leaf-spot fungi and fungicides; which would help mitigating economic losses via reducing reliance on chemical controls. As a result, safeguarding Egypt's position in the global floriculture market would be achievable within the next few seasons.

2. Materials and methods

2.1 Isolation and identification of fungal strains

Fungal isolates were obtained from *Chrysanthemum morifolium* plants, exhibiting symptoms of foliar blight including leaf spots, necrosis and chlorosis. Samples representing infected leaf tissues were collected, individually, from commercial greenhouses in Menofia governorate, Egypt. Surface debris were removed from samples by thorough washing under running tap water, followed by surface sterilization with 10% sodium hypochlorite (NaOCl) solution for 2 minutes. Samples were then rinsed thrice with sterile distilled water, to get rid of NaOCl residues; air-dried, on sterile filter paper. Small fragments of each sample (approximately 5 × 5 mm) were cut from the margins of sympiotic lesions, then placed on potato dextrose agar plates. Cultured plates were incubated at 26 ± 2°C for 72 hours, to allow optimal fungal-growth conditions. Pure cultures of pathogenic fungi were obtained utilizing the hyphal tip technique; where individual hyphal tips were transferred to fresh PDA plates. Purified fungal colonies were preserved on PDA slants and kept at 4°C for further experiments.

Morphological keys for *Alternaria* [3] and *Diaporthe* [11] were used for preliminary identification. Further morphological determination of fungal colonies was conducted for characteristics such as colony color, texture, growth rate, as well as presence of reproductive structures. Microscopic examination was performed using a compound microscope (Olympus CX21 Brightfield) at 400x magnification power. Conidial morphology, including shape, size and septation, was recorded.

2.2 Pathogenicity test

To confirm fungal isolates pathogenicity, the four criteria described in Koch's postulates were performed. Healthy *Chrysanthemum morifolium* seedlings were transferred from greenhouses and grown (upon roots formation) in pots (20 cm diameter; four plants per pot), five pots per fungal isolate (in duplicates) to ensure reproducibility.

Conidial suspensions were prepared by flooding 14-day-old fungal cultured plates with sterile distilled water and gently scraping agar surface with a sterile swab. The thorough suspension was filtered using sterile cheesecloth, to remove hyphal fragments and debris. Conidial concentration was adjusted to 1×10^6 conidia/mL, utilizing hemocytometer. Finally, Tween 80 (0.05% w/v) was added as a surfactant.

Conidial suspension of each isolate was sprayed on healthy chrysanthemum plants leaves using an atomizer designated for each fungal isolate to prevent contamination. Inoculated plants were covered with plastic bags to maintain optimal humidity for disease development. Disease symptoms were assessed 10 days after inoculation and the percentage of disease occurrence was observaed. Reisolation of the fungi from symptomatic tissues confirmed their pathogenic nature.

2.3 Molecular identification

Genomic DNA was extracted from pure cultures of the fungal isolates using the DNeasy® Plant Extraction Kit (Qiagen, Germany) following the manufacturer's instructions. The internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) was amplified using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') in accordance with [12].

PCR amplification was performed in a 50 µL reaction mixture containing 25 µL of Master Mix (Sigma-Aldrich), 3 µL of each primer (10 pmol/µL), 3 µL of template DNA (10 ng/µL), and 16 µL of sterile distilled water. The thermal cycling conditions were as follows: initial denaturation at 94°C for 5 minutes; 40 cycles of denaturation at 94°C for 40 seconds, annealing at 50°C for 40 seconds, and extension at 72°C for 1 minute; and a final extension at 72°C for 7 minutes.

The PCR products were resolved by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.5 µg/mL) and visualized under UV light using a Gel Documentation System (Bio-Rad 2000). The amplified products were purified using the EZ-10 Spin Column PCR Product Purification Kit (BioBasic, Canada) and sequenced using an ABI PRISM 3730XL Analyzer (Macrogen, Korea).

The obtained sequences were analyzed using the BLASTn tool available at NCBI database (http://www.ncbi.nlm.nih.gov/BLAST) and aligned with reference sequences using MEGA-X software [13]. Phylogenetic trees were constructed using the neighbor-joining method with 1000 bootstrap replicates.

2.4 Scanning Electron Microscopy (SEM)

Morphological characterization of *Diaporthe eres* isolate was conducted using SEM. Small agar blocks (approx. 5 × 5 mm) bearing mycelia and spores from the margins of 14-day-old cultures grown on PDA at 26 ± 2°C were aseptically excised. Samples were mounted directly onto aluminum stubs using double-sided adhesive carbon tape. To ensure electrical conductivity, the mounted samples were then coated with a thin layer of gold-palladium using an S150A sputter coater (Quorum technologies, Lewes, U.K.). Examination was conducted utilizing Quanta FEG-250 environmental SEM operated at an accelerated voltage of 10 to 15 kV, under high-vacuum condition.

2.5 SCoT polymorphism analysis

To assess fungal genetic diversity, SCoT polymorphism analysis was performed. Ten SCoT primers (Table 1) were used to amplify DNA fragments isolated from *Chrysanthemum morifolium* plants (infected with fungal isolates under investigation). The PCR reaction mixture (20 µL) contained 10 µL of Master Mix (Sigma-Aldrich), 2.5 µL of primer (10 pmol), 2.5 µL of template DNA (10 ng), and 5 µL of sterile distilled water [14].

Table 1
SCoT primers used to detect polymorphism

Primer Name	Sequence
SCoT-1	5'-ACGACATGGCGACCACGC-3'
SCoT-2	5'-ACCATGGCTACCACCGGC-3'
SCoT-3	5'-ACGACATGGCGACCCACA-3'
SCoT-4	5'-ACCATGGCTACCACCGCA-3'
SCoT-5	5'-CAATGGCTACCACTAGCG-3'
SCoT-6	5'-CAATGGCTACCACTACAG-3'
SCoT-7	5'-ACAATGGCTACCACTGAC-3'
SCoT-8	5'-ACAATGGCTACCACTGCC-3'
SCoT-9	5'-ACAATGGCTACCACCAGC-3'
SCoT-10	5'-ACAATGGCTACCACTACC-3'

Table 2
Amplicon size; Number. of monomorphic and polymorphic amplicons and; percentage of polymorphism yielded per SCoT oligonucleotide designed for *Chrysanthemum morifolium* accessions.

Primer code	Monomorphic bands	Polymorphic w/o unique bands	Unique bands	Polymorphic with unique bands	Total # of amplified bands	Polymorphism percentage	Amplicon size range (bp)	
SCoT-1	6	1	1	2	8	25%	640	200
SCoT-2	7	1	0	1	8	12.5%	940	260
SCoT-3	3	2	0	2	5	40%	950	190
SCoT-4	3	3	2	5	8	62.5%	500	150
SCoT-5	7	2	3	5	12	41.7%	1300	220
SCoT-6	9	0	0	0	9	0%	770	190
SCoT-7	6	0	0	0	6	0%	1000	300
SCoT-8	6	0	0	0	6	0%	950	290
SCoT-9	4	2	1	3	7	42.9%	780	170
SCoT-10	11	0	0	0	11	0%	1000	250
Total	**62**	**11**	**7**	**18**	**80**			
Average	**6.2**	**1.1**	**0.7**	**1.8**	**8**	**22.5%**		
Amplicon size							**Max. 1300**	**Min. 150**

Thermal cycling conditions were as follows: initial denaturation at 94°C for 5 minutes; 40 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for 50 seconds, and extension at 72°C for 1 minute; and a final extension at 72°C for 7 min. The amplified products were resolved on a 1.5% agarose gel, and the banding patterns were scored as present (1) or absent (0).

Data were analyzed using PAST software [15]. A binary matrix was constructed, and genetic similarity coefficients were calculated using Dice's coefficient. A dendrogram was generated using the unweighted pair group method with arithmetic averages (UPGMA).

2.6 *In Vitro* antifungal assay

Antifungal activity of five fungicides, Difinoconazole (Curve 25%); Chlorothalonil (Brado 72%); Azoxystrobin (Kafrostar 25%); Propamocarb Hydrochloride (Zeus 72.2%) and; Thiophanate-methyl (Actamyl 70%) was evaluated using the poisoned food technique described by [16].

The forementioned fungicides were added, individually, to molten PDA at concentrations of 1000, 2000, and 3000 ppm. The medium was then poured into 9 cm Petri dishes and allowed to solidify. Mycelial plugs (5 mm diameter) from 7-day-old fungal cultures were placed at the center of the treated plates. Control plates contained PDA without fungicides. Treatment and control plates were incubated at 28°C for 10 days and the radial growth of the fungal colonies was measured on day 10.

Percentage of mycelial growth inhibition was calculated using the following equation:

$$\textbf{Inhibition rate } (\%) = \frac{\mathbf{C} - \mathbf{T}}{\mathbf{C}} \times 100$$

where C and T represent inibition area in the control and treatment plates, respectively.

3. Results and Discussion

3.1 Fungal isolation and microscopic determination

Our study successfully identified two distinct fungal pathogens responsible for foliar blight on *Chrysanthemum morifolium* in Egypt. A total of nine isolates were recovered from symptomatic leaf tissues, and morphological analyses confirmed five, out of the nine isolates, as *Alternaria sp.* and the remaining four isolates were identified as *Diaporthe sp.* (Fig. 1). Colonies of *Diaporthe sp.* on PDA, exhibited white aerial mycelia that later turned gray; incontrast, *Alternaria sp.* produced olivaceous-green colonies with concentric rings. Microscopic analysis revealed alpha conidia formation in *Ascomycete fungus* (*Diaprothe*), while *Alternaria sp.* produced muriform conidia preserving longitudinal and transverse septa.

3.2 Pathogenicity test

Pathogenicity tests, following Koch's postulates, demonstrated that both species are highly virulent, with ascomycete fungus isolates causing a 100% disease incidence and *Alternaria* isolates causing a 94.4% incidence within 10 days post-inoculation (Fig. 2). Our findings mark the first report of another ascomycete fungus, causing disease on chrysanthemum in Egypt; expanding its known host-range as well as geographic distribution [17].

3.3 Molecular identification

Two *Alternaria sp.* isolates (1 & 7) and one *Diaporthe* isolate (6) were chosen for molecular identification (Fig. 3A). Three fungal isolates were selected for sequencing, targeting internal transcribed spacer 1; partial sequence of the 5.8S ribosomal RNA gene; the complete sequence of internal transcribed spacer 2 and; partial sequence of the large subunit ribosomal RNA gene. Resulted sequences were analyzed using Sanger dideoxy sequencing technology and deposited to GenBank database under accession numbers PP388215 (*A. alternata*, isolate 1), PP388217 (*A. alternata*, isolate 7) and PP388218 (*Diaporthe eres*).

Phylogenetic analysis revealed that strains clustered closely with *Diaporthe sp* and *Alternaria alternata*, showing over 99% similarity to reference sequences available at GenBank database. Analysis of the phylogenetic tree (Fig. 3C), illustrating the relationships among investigated strains and related taxa, revealed that all fungal isolates in the current study belong to genera *Alternaria* and *Diaporthe*. This is considered a confirmation of the first report of *D. eres* infecting chrysanthemums in Egypt. These results confirm the presence of *D*. *eres* as a causal agent of foliar blight in *Chrysanthemum morifolium* in Egypt, highlighting its potential impact on local cultivation practices [18].

ITS sequencing confirmed the identity of isolates: **Alternaria 1** (GenBank PP388215) clustered with *A. alternata* strains from Argentina; **Alternaria 7** (GenBank PP388217) showed 99% similarity to *A. tenuissima*, suggesting cryptic diversity within the *Alternaria* complex (Woudenberg et al., 2015) And; **Diaporthe eres** (GenBank PP388218) annotated to *D. eres* clade, alongside Chinese strains (Fig. 3B).

The phylogenetic divergence of *Alternaria* 7 highlights the need for multi-locus sequencing (e.g., *tef1*, *gapdh*) to resolve species boundaries in *Alternaria* [6].

3.4 SEM analysis

SEM data provided high-resolution morphological characterization of *Diaporthe eres* isolate (PP388218), confirming key taxonomic features observed under light microscopy (Fig. 4A-C). Light micrographs showed septate hyphae, the development of globose pycnidia and the production of fusiform, aseptate alpha conidia.

SEM analysis further elucidated these structures in greater details. The fungus exhibited a network of septate hyphae with a smooth surface and irregular branching (Fig. 4D & 4E). Fungal reproductive structures included ostiolate pycnidia (Fig. 4F). Crucially, two conidial types were clearly observed: alpha conidia were fusiform to ellipsoidal, aseptate, and hyaline (Fig. 4G & 4H). Additionally, less abundant beta conidia were filiform and hamate (hook-shaped) (Fig. 4I).

The forementioned morphological characteristics, especially the presence of alpha and beta conidia, produced within pycnidia align definitively with the taxonomic description of *Diaporthe eres* [11, 18]. Therefore, SEM analysis revealed robust morphological validation that corroborates the molecular identification (ITS sequence PP388218), conclusively confirming the identity of the pathogen responsible for foliar blight in Egyptian chrysanthemums.

3.5 Genetic diversity via SCoT markers

SCoT primers are designed to target the conserved short region surrounding the start codon (ATG), the beginning of most genes. This means the markers generated are directly related to functional genes and transcribed regions of the genome and the utility of SCoT markers stems from their well-known characteristics: rich genetic content and high polymorphism. Since SCoT primers are linked to functional genes and corresponding traits, the amplicons can be converted to gene targeted marker systems [19]. Descriptive data of bands' sizes, numbers as well as number of monomorphic, polymorphic bands and percentage of polymorphism obtained per SCoT-primer, are shown in **Table (2)**. SCoT-primers, shown in Fig. 5, were used to amplify DNA fragments, extracted from infected *Chrysanthemum morifolium* plants, and resulted in the amplification of eighty bands, with an average of eight bands per primer, ranging from ~ 150 to ~ 1300 base pairs. Out of the aforementioned eighty bands, twenty-three bands were polymorphic across all cultivars, with an average of 2.3 bands per primer. The average percentage of polymorphism was 29.59%. It is worth mentioning that SCoT-05 oligonucleotide yielded the greatest number of bands, twelve, SCoT loci. In contrast, SCoT-03 showed the least polymorphic differences among plants (five bands), while SCoT-02 yielded the lowest polymorphism percentage (12.50%). Such differences in polymorphic bands are of great importance in the identification of *Chrysanthemum morifolium* plants, based on treatments under investigations and DNA utilization [20]. In addition, several unique bands were detected among the tested primers. One unique band was generated in PCR product, resulted from *Chrysanthemum* DNA and SCoT-01 primer. Another unique band was observed in PCR product generated from *Chrysanthemum* DNA and SCoT-09 primer. In contrast, two unique bands resulted from utilizing SCoT-04 primer, and three unique bands were detected in PCR product from *Chrysanthemum* DNA and SCoT-05 primer. These unique bands may serve as potential molecular markers for the identification and discrimination of *Chrysanthemum* accessions harboring disease resistance features.

3.6 Dendrogram resulted from SCoT assay

The dendrogram generated by UPGMA cluster analysis based on Nei and Kumar coefficient shows the clustering of data from *Chrysanthemum morifolium* plants into four groups (representing 4 treatments; Fig. 6). Investigations are divided into two groups at distance 0.92. The first group contained plants from treatment number 2 only. The second group is divided into two sub-groups at distance 0.94. The first sub-group is separated into two sub-subgroups at distance 0.95; one includes plants from treatment 1 (only), while the other sub-subgroup consists of plants associated with treatments 3 and 4. Since SCoT oligonucleotides have great potential in cultivar identification and can reproducibly amplify polymorphic bands in plants that are closely related, selection of molecular markers used in identification is a crucial step.

Polymorphic bands, from four treatments of *Chrysanthemum morifolium* plants, generated by SCoT primers were utilized to construct a dendogram (Fig. 6), which illustrates high genetic relatedness with similarity coefficients' ranging from 0.92 to 0.97. Maximum similarity, 0.97, was observed between plants from treatments 3 and 4, indicatind close genetic homology between these two groups of plants. In contrast, minimum similarity, 0.92, was observed between plants from treatments 2 and 4, referrig to higher genetic divergence, compared with similarity coefficients values within plants representing treatments 1, 3 and 4. Similarity coefficients revealed that plants associated with treatments 3 and 4 fall under the same cluster. While plants in treatment 2 has shown a distal relationship, plants from treatment 1 showed a relationship with plants associated with treatments 3 and 4. [20, 21] have reported similar clustering patterns between closely related chrysanthemum species, where high similarity coefficients were found to be associated with common genetic origin.

The high similarity coefficients' values observed among *Chrysanthemum morifolium* plants under investigation indicate a narrow genetic base among them. Our results were found to be consistent with previous research that concluded high genetic similarity among cultivated Chrysanthemum varieties, due to intensive selection and breeding from closely related parental line [20]. Moreover, molecular markers used in previous research revealed high levels of genetic similarity, ranging between 0.85 and 0.98, within ornamental plant cultivars [21].

Since this study revealed that data from plants represent treatments 3 and 4 fall within the same group, it highly suggests that these groups are believed to share a common ancestral origin or similar breeding lineage. Previous phylogenetic studies have shown a comparable clustering behavior among genetically related cultivars, which indicated that closely related genotypes tend to cluster together within the same branch of the phylogenetic tree [22, 23].

3.7 *In vitro* Antifungal Activity of Fungicides

In vitro efficacy of the five fungicides investigated in this study, with distinct modes of actions, was quantitatively assessed against primary pathogens *A. alternata* (isolates number 1 & 7) and *D. eres*. Radial growth (in mm) measurements and percentage of inhibition were used to evaluate fungicides performance across three concentrations, 1000:, 2000 and; 3000 ppm. Statistical analysis (ANOVA, Tukey's HSD, $p \leq 0.05$) confirmed significant differential efficacy among treatments, compared to control.

3.7.1 *Alternaria* 1

Difinoconazole exhibited dose-dependent inhibition, reducing radial growth from 14.3 mm (1000 ppm) to 9.0 mm (3000 ppm), achieving 90% inhibition at the highest concentration (Table 3). This aligns with its systemic mode of action as a demethylation inhibitor (DMI), disrupting ergosterol biosynthesis [24]. Thiophanate-methyl showed minimal efficacy, with radial growth decreasing only from 88 mm (1000 ppm) to 76 mm (3000 ppm), corresponding to 15.9% inhibition (Fig. 7). This likely reflects resistance development in *Alternaria* populations due to prolonged benzimidazole use [25]. Propamocarbwas limited activity (27.78% inhibition at 3000 ppm) suggests its primary utility lies in oomycete control [26].

Table 3

In vitro antifungal activity of fungicides under investigation against *Alternaria* spp. and *Diaporthe eres*.

Fungi	*Fungicide*	Concentration (ppm)		
		1000 ppm	***2000 ppm***	***3000 ppm***
Alternaria 1	**Difinoconazol**	84.07^{a}±0.26	85.93^{a}±0.26	90.00^{a}±0.00
	Chlorothalonil	49.63^{b}±1.59	51.48^{b}±0.26	60.37^{b}±0.69
	Azoxystrobin	27.41^{c}±0.52	30.37^{c}±0.69	30.37^{c}±0.52
	Propamocarb	18.52^{c}±0.26	23.70^{c}±0.52	27.78^{c}±0.45
	Thiophanat	2.22^{d}±0.91	13.33^{c}±0.91	15.19^{d}±0.69
	Control	0.00^{d}±0.00	0.00^{d}±0.00	0.00^{e}±0.00
	p value	**0.000**	**0.000**	**0.000**
	Significance	***	***	***
Diaporthe	**Difinoconazol**	88.89^{a}±0.00	90.00^{a}±0.00	90.00^{a}±0.00
	Chlorothalonil	90.00^{a}±0.00	90.00^{a}±0.00	90.00^{a}±0.00
	Azoxystrobin	42.59^{b}±0.26	44.81^{b}±0.26	49.63^{c}±0.26
	Propamocarb	28.15^{c}±0.52	37.41^{c}±0.94	46.30^{c}±0.69
	Thiophanat	38.15^{b}±1.46	46.30^{b}±0.26	59.63^{b}±0.69
	Control	0.00^{e}±0.00	0.00^{e} ±0.00	0.00^{d}±0.00
	p value	**0.000**	**0.000**	**0.000**
	Significance	***	***	***
Alternaria 7	**Difinoconazol**	85.93^{a}±0.53	87.41^{a} ±0.26	89.26^{a}±0.52
	Chlorothalonil	50.74^{b}±0.26	53.70^{b}±0.52	57.04^{b}±0.26
	Azoxystrobin	37.41^{c}±0.52	42.96^{c}±0.26	48.15^{b}±0.52
	Propamocarb	25.19^{c}±0.69	31.48^{d}±0.69	38.15^{c} ±0.94
	Thiophanat	0.74^{e}±0.52	11.48^{e}±0.94	38.52^{c}±0.69
	Control	0.00^{e} ±0.00	0.00^{f} ±0.00	0.00^{e} ±0.00
	p value	**0.000**	**0.000**	**0.000**
	Significance	***	***	***
p value		**0.000**	**0.000**	**0.000**

Values represent mean percentage inhibition of radial growth (± standard error). Different letters within a column for each fungus indicate significant differences, according to Tukey's HSD test ($p < 0.05$).

Fungi	*Fungicide*	Concentration (ppm)		
		1000 ppm	*2000 ppm*	*3000 ppm*
Significance (B*C*D)		***	***	***

Values represent mean percentage inhibition of radial growth (± standard error). Different letters within a column for each fungus indicate significant differences, according to Tukey's HSD test ($p < 0.05$).

3.7.2 *Alternaria* 7

Difinoconazole exhibited consistently effective, with inhibition increasing from 85.93% (1000 ppm) to 89.26% (3000 ppm), wherase azoxystrobin moderate inhibition (48.15% at 3000 ppm) likely stems from its role as a quinone-outside inhibitor (QoI), targeting mitochondrial respiration [27]. Thiophanate-methyl was low inhibition (38.15% at 3000 ppm) underscores reduced sensitivity in this isolate, possibly due to mutations in β-tubulin genes [28].

3.7.3 *Diaporthe eres*

Difinoconazole was achieved 90.0%, inhibition at 2000 and 3000 ppm while 88.89% at 1000 ppm,which confirming its broad-spectrum efficacy. Azoxystrobin was exhibited moderate inhibition (49.63% at 3000 ppm) reflects QoI fungicides' variable activity against Ascomycetes. Propamocarb was limited inhibition (46.30% at 3000 ppm) highlights its unsuitability for *Diaporthe* management.

Results demonstrate that Difinoconazole is highly effective in inhibiting *Alternaria* and *D*. *eres* growth, as evidenced by its low radial growth measurements and high percentage inhibition values, across all cultivated plates containing various fungicide concentrations; reaching up to 90%. This aligns with previous studies that have highlighted Difinoconazole's broad-spectrum activity against plant pathogenic fungi due to its mode of action as a demethylation inhibitor in fungal sterol biosynthesis [29]. Chlorothalonil and Azoxystrobin also showed moderate efficacy, particularly at higher concentrations, but their performance was inferior to Difinoconazole. Chlorothalonil's contact mode of action may explain its relatively lower efficacy, compared to systemic fungicides like Difinoconazole [5]. In contrast, Thiophanate exhibited the least effectiveness among the tested fungicides, particularly against *D*. *eres*, where it roughly inhibited 36 to 42% of fungal growth at higher concentrations. This could be attributed to potential genetic resistance development in the pathogen, or its limited spectrum of activity against certain fungal pathogens [4].

Our findings suggest that Difinoconazole is a promising candidate for managing leaf spot disease in Chrysanthemum plants caused by *Alternaria spp*. and *D*. *eres*. However, integrating Difinoconazole into a broader disease management strategy, that includes cultural practices and rotation with other fungicides, is recommended to minimize resistance development in the pathogens' genomes. Our results concluded that; Difinoconazole demonstrated High efficacy against *Alternaria spp*. and *D*. *eres*.; making it a valuable tool for managing leaf spot disease in *Chrysanthemum* plants.

Difinoconazole's superior performance correlates with its systemic mode of action as a demethylation inhibitor (DMI), disrupting ergosterol biosynthesis in fungal cell membranes [29]. In contrast, we believe that thiophanate-methyl's limited efficacy against *D*. *eres* (42% inhibition) was due to mutated strains in pathogens, similar to reported incident in *Alternaria* populations, exposed to benzimidazoles [25]. Azoxystrobin, a quinone-outside inhibitor (QoI), showed moderate activity against *D. eres* (56.7%), likely due to its broad-spectrum activity against Ascomycetes [27].

3.8 Implications for disease management

The outstanding efficacy of Difinoconazole against *Alternaria* spp. and *Diaporthe eres* ranks it as crucial first-line fungicide for managing foliar blight in Egyptian chrysanthemums. However, its systemic mode of action as a demethylation inhibitor (DMI) necessitates resistance mitigation strategies, such as rotation with non-DMI fungicides, like Chlorothalonil and integration of cultural practices (e.g., crop rotation & field sanitation) to reduce mutation rate, and

as a consequence, delay evolution of resistant fungal populations [30]. Azoxystrobin, a quinone-outside inhibitor (QoI), offers moderate suppression of *Alternaria* (30–48% inhibition) therefore, it could be strategically deployed in rotations to reduce selection pressure on DMIs, though its variable efficacy against *Diaporthe eres* (49.63% inhibition) underscores the need for complementary approaches. The limited effectiveness of Thiophanate-methyl in controlling *D. eres* (38–60% inhibition) and *Alternaria 7* (38.52%) highlights the declining utility of benzimidazoles in Egyptian fields and is likely due to widespread resistance linked to β-tubulin mutations [25]. Similarly, Propamocarb's rapid activity against *D. eres* (46.30% inhibition) suggests it is better suited for managing oomycete pathogens, compared to Ascomycete-dominated blight. The low genetic diversity observed among Egyptian isolates via SCoT markers (29.59% polymorphism) further emphasizes the risk of clonal pathogen expansion, which could accelerate resistance development if fungicides are overused. To address this, broader regional surveys to map pathogen diversity and field trials to validate fungicide efficacy under real-world conditions are urgently needed [6]. Collectively, these findings advocate for an integrated disease management (IDM) framework that prioritizes Difinoconazole as a cornerstone while incorporating Azoxystrobin rotations, resistance monitoring, and modern breeding programs for resistant cultivars to sustainably safeguard Egypt's chrysanthemum industry.

4. Conclusion

The currecnt study establishes *Diaporthe eres* as first-reported and highly virulent pathogen causing foliar blight on chrysanthemums in Egypt. It is worth mentioning that such threat is compounded by co-occurance with *Alternaria alternata*. *In vitro* assays delineated a clear efficacy hierarchy, identifying the demethylation inhibitor, Difinoconazole, as the most potent fungicide. In contrast, benzimidazole Thiophanate-methyl was the least fungicide in prohibiting fungal growth. SCoT marker analysis provided critical genetic insights for pathogen differentiation and future breeding. These findings mandate an immediate shift to an Integrated Disease Management strategy, pivoting on strategic utilization of Difinoconazole and the exclusion of ineffective benzimidazoles, to sustainably protect an important cut-flowers supplier, Egypt, for the European, and other countries, markets.

Declarations

Author contributions: M.F.H. and M.M.A.: conceptualization and methodology. M.F.H., M.M.A., Abdelrahman A. A. and R.I.E.: investigation and writing- original draft preparation. A.M.A. and N.M.S.: data curation. A.A.A. and M.F.H.: preparing supplementary file, reviewing and editing. E.M.E. and Abdelrahman A.A.: validation. A.E.E.: formal analysis (statistical analysis).

Availability of data and materials

The sequences generated and analyzed during the current study are available in the NCBI GenBank repository. ITS sequences for *Alternaria alternata* isolates (1 & 7) and *Diaporthe eres* have been assigned accession numbers PP388215, PP388217, and PP388218, respectively. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Not applicable. This study did not involve human participants, human data, or animals.

Consent for publication

Not applicable.

References

1. Barreda VD, Palazzesi L, Tellería MC, Olivero EB, Raine JI, Forest F. Early evolution of the angiosperm clade Asteraceae in the Cretaceous of Antarctica. Proc Natl Acad Sci U S A. 2015;112:10989–94. https://doi.org/10.1073/pnas.1423653112.
2. Liu PL, Wan Q, Guo YP, Yang J, Rao GY. Phylogeny of the Genus Chrysanthemum L.: Evidence from Single-Copy Nuclear Gene and Chloroplast DNA Sequences. PLoS ONE. 2012;7. https://doi.org/10.1371/journal.pone.0048970.
3. Simmons E, Alternaria N. 2007. https://scholar.google.com/scholar?hl=en&as_sdt=0%2C5&q=Alternaria%3A+An+identification+manual.+CBS+Fungal+Biodiversity+Centre&btnG=. Accessed 23 Feb 2025.
4. Islam T, Danishuddin, Tamanna NT, Matin MN, Barai HR, Haque MA. Resistance Mechanisms of Plant Pathogenic Fungi to Fungicide, Environmental Impacts of Fungicides, and Sustainable Solutions. Plants. 2024;13:1–27. https://doi.org/10.3390/plants13192737.
5. Ishieze PU, Amuji CF, Ugwuoke KI, Baiyeri PK, Eze MO. Comparative Efficacy of Systemic and Combination Fungicides for the Control of Alternaria Leaf Spot of Cabbage. Appl Microbiol. 2023;3:906–14. https://doi.org/10.3390/applmicrobiol3030062.
6. Long H, Zhang Q, Hao YY, Shao XQ, Wei XX, Hyde KD, et al. Diaporthe species in south-western China. MycoKeys. 2019;57:113. https://doi.org/10.3897/MYCOKEYS.57.35448.
7. Adolf KM, Ali MK. Phytopathological studies on Alternaria blight of Chrysanthemum (Chrysanthemum indicum L). Int J Agric Technol. 2023;19:877–98.
8. Collard BCY, Mackill DJ. Start codon targeted (SCoT) polymorphism: a simple, novel DNA marker technique for generating gene-targeted markers in plants. Plant Mol Biol Rep. 2009;27:86–93.

9. Feng S, Zhu Y, Yu C, Jiao K, Jiang M, Lu J, et al. Development of species-specific SCAR markers, based on a SCoT analysis, to authenticate Physalis (Solanaceae) species. Front Genet. 2018;9:192.
10. Tyagi R, Sharma V, Sureja AK, Das Munshi A, Arya L, Saha D, et al. Genetic diversity and population structure detection in sponge gourd (Luffa cylindrica) using ISSR, SCoT and morphological markers. Physiol Mol Biol Plants. 2020;26:119–31.
11. Gomes RR, Glienke C, Videira SIR, Lombard L, Groenewald JZ, Crous PW. Diaporthe: a genus of endophytic, saprobic and plant pathogenic fungi. Persoonia-Molecular Phylogeny Evol Fungi. 2013;31:1–41.
12. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc a Guid to methods Appl. 1990;18:315–22.
13. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35:1547–9.
14. Ibrahim SD, Abd El-Hakim AF, Ali HE, Abd El-Maksoud RM. Genetic differentiation using ISSR, SCoT and DNA Barcoding for Quinoa genotypes. Arab J Biotech. 2019;22:103–18.
15. Hammer Ø, Harper DAT. Past: paleontological statistics software package for educaton and data anlysis. Palaeontol Electron. 2001;4:1.
16. Seliem MK, Taha NA, El-Feky NI, Abdelaal K, El-Ramady H, El-Mahrouk ME, et al. Evaluation of five Chrysanthemum morifolium cultivars against leaf blight disease caused by Alternaria alternata at rooting and seedling growth stages. Plants. 2024;13:252.
17. Udayanga D, Castlebury LA, Rossman AY, Hyde KD. Species limits in Diaporthe: molecular re-assessment of D. citri, D. cytosporella, D. foeniculina and D. rudis. Persoonia-Molecular Phylogeny Evol Fungi. 2014;32:83–101.
18. Udayanga D, Castlebury LA, Rossman AY, Chukeatirote E, Hyde KD. Insights into the genus Diaporthe: phylogenetic species delimitation in the D. eres species complex. Fungal Divers. 2014;67:203–29. https://doi.org/10.1007/S13225-014-0297-2/FIGURES/8.
19. Samarina LS, Malyarovskaya VI, Reim S, Yakushina LG, Koninskaya NG, Klemeshova KV, et al. Transferability of ISSR, SCoT and SSR markers for Chrysanthemum× Morifolium Ramat and genetic relationships among commercial Russian cultivars. Plants. 2021;10:1302.
20. Feng SG, He RF, Jiang MY, Lu JJ, Shen XX, Liu JJ, et al. Genetic diversity and relationships of medicinal Chrysanthemum morifolium revealed by start codon targeted (SCoT) markers. Sci Hortic (Amsterdam). 2016;201:118–23. https://doi.org/10.1016/J.SCIENTA.2016.01.042.
21. Zhang Y, Wang C, Ma HZ, Dai SL. Assessing the genetic diversity of Chrysanthemum cultivars with microsatellites. J Am Soc Hortic Sci. 2013;138:479–86. https://doi.org/10.21273/jashs.138.6.479.
22. Rohlf FJ. Numerical Taxonomy and Multivariate Analysis System, Version 2.2. New York, USA: Applied Biostatistics Inc. Setauket; 2000.
23. Nei M, Kumar S. Molecular Evolution and Phylogenetics. New York, USA: Oxford University Press; 2002. https://doi.org/10.1177/1748895810383807.
24. Yin Y, Miao J, Shao W, Liu X, Zhao Y, Ma Z. Fungicide Resistance: Progress in Understanding Mechanism, Monitoring, and Management. Phytopathology. 2023;113:707–18. https://doi.org/10.1094/PHYTO-10-22-0370-KD/ASSET/IMAGES/LARGE/PHYTO-10-22-0370-KDT2-1683780647472.JPEG.
25. Tashiro N, Ide Y, Noguchi M, Watanabe H, Nita M, Tashiro N et al. Emergence of Benzimidazole- and Strobilurin-Quinone Outside Inhibitor-Resistant Strains of Colletotrichum gloeosporioides sensu lato, the Causal Fungus of Japanese Pear Anthracnose, and Alternative Fungicides to Resistant Strains. Plant Dis - Curr Threat Manag Trends. 2019. https://doi.org/10.5772/INTECHOPEN.90018

26. Cohen Y, Coffey MD. Systemic Fungicides and the Control of Oomycetes. Annu Rev Phytopathol. 1986;24:311–38. https://doi.org/10.1146/ANNUREV.PY.24.090186.001523.
27. Fernández-Ortuño D, Torés JA, De Vicente A, Pérez-García A. Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. Res Fernández-Ortuño, JA Torés, A Vicente, A Pérez-GarcíaInternational Microbiol 2008•researchgate.net. 2014. https://doi.org/10.2436/20.1501.01.38
28. Avenot H, Sellam A, … GK-, 2008 undefined. Characterization of Mutations in the Iron-Sulphur Subunit of Succinate Dehydrogenase Correlating with Boscalid Resistance in Alternaria alternata from California. Am Phytopath Soc Avenot, A Sellam, G Karaoglanidis, TJ MichailidesPhytopathology, 2008•Am Phytopath Soc. 2008;98:736–42. https://doi.org/10.1094/PHYTO-98-6-0736.
29. Martinez-Prada I, Kortekamp A. Effect of sterol biosynthesis inhibitors and azole-type inducers on growth and development of Plasmopara viticola on grapevine. Vitis - J Grapevine Res. 2015;54:91–6.
30. Gisi U. Resistance to carboxylic acid amide (CAA) fungicides and anti-resistance strategies. Fungic Resist Crop Prot Risk Manag. 2011;96–103. https://doi.org/10.1079/9781845939052.0096.

Figures

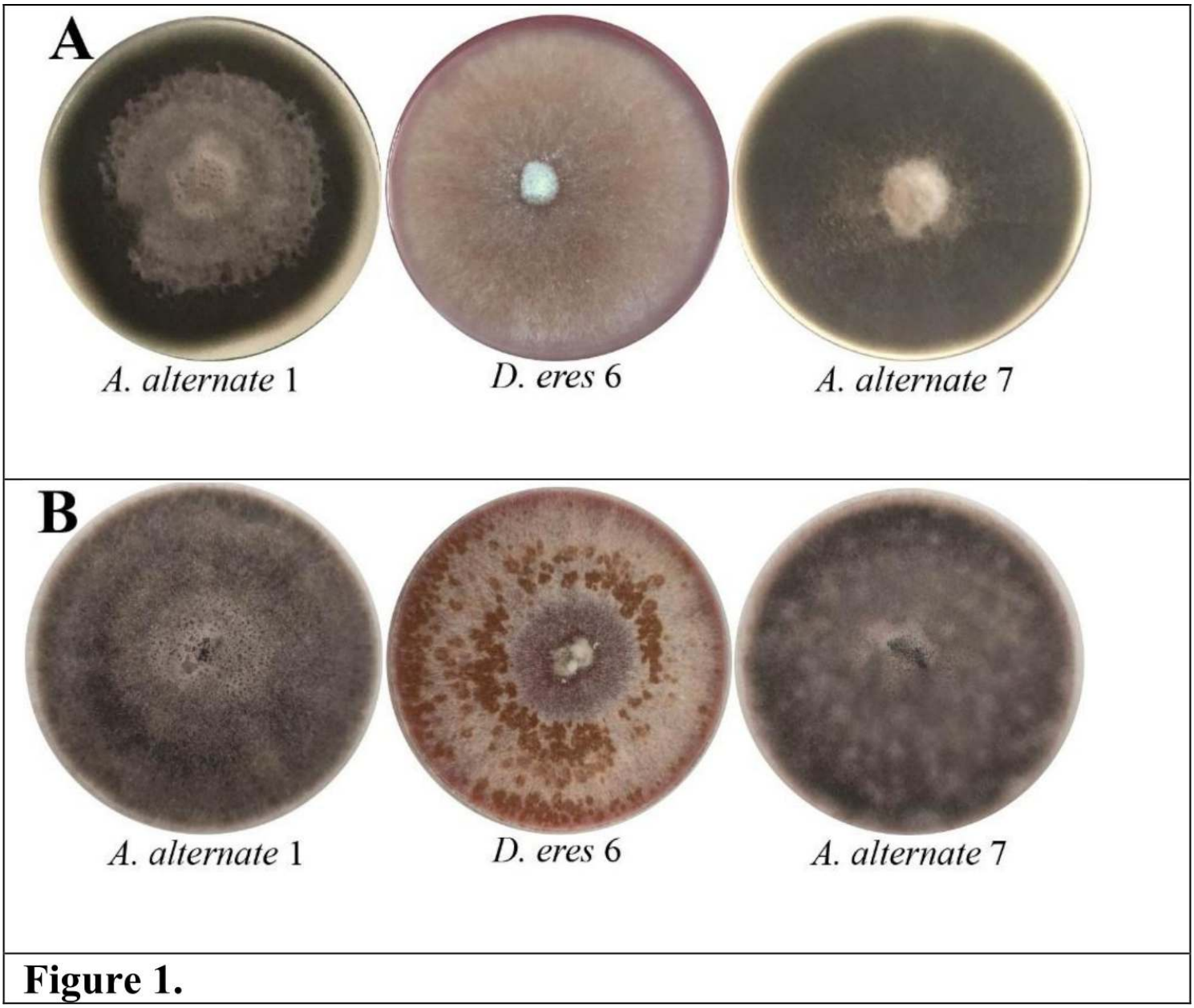

Figure 1

Fungal cultural morphology on PDA. (**A**) 7-day-old cultures. (**B**) 14-day-old cultures. Isolates 1 & 7: *Alternaria sp*.; Isolate 6: *Diaporthe sp*.

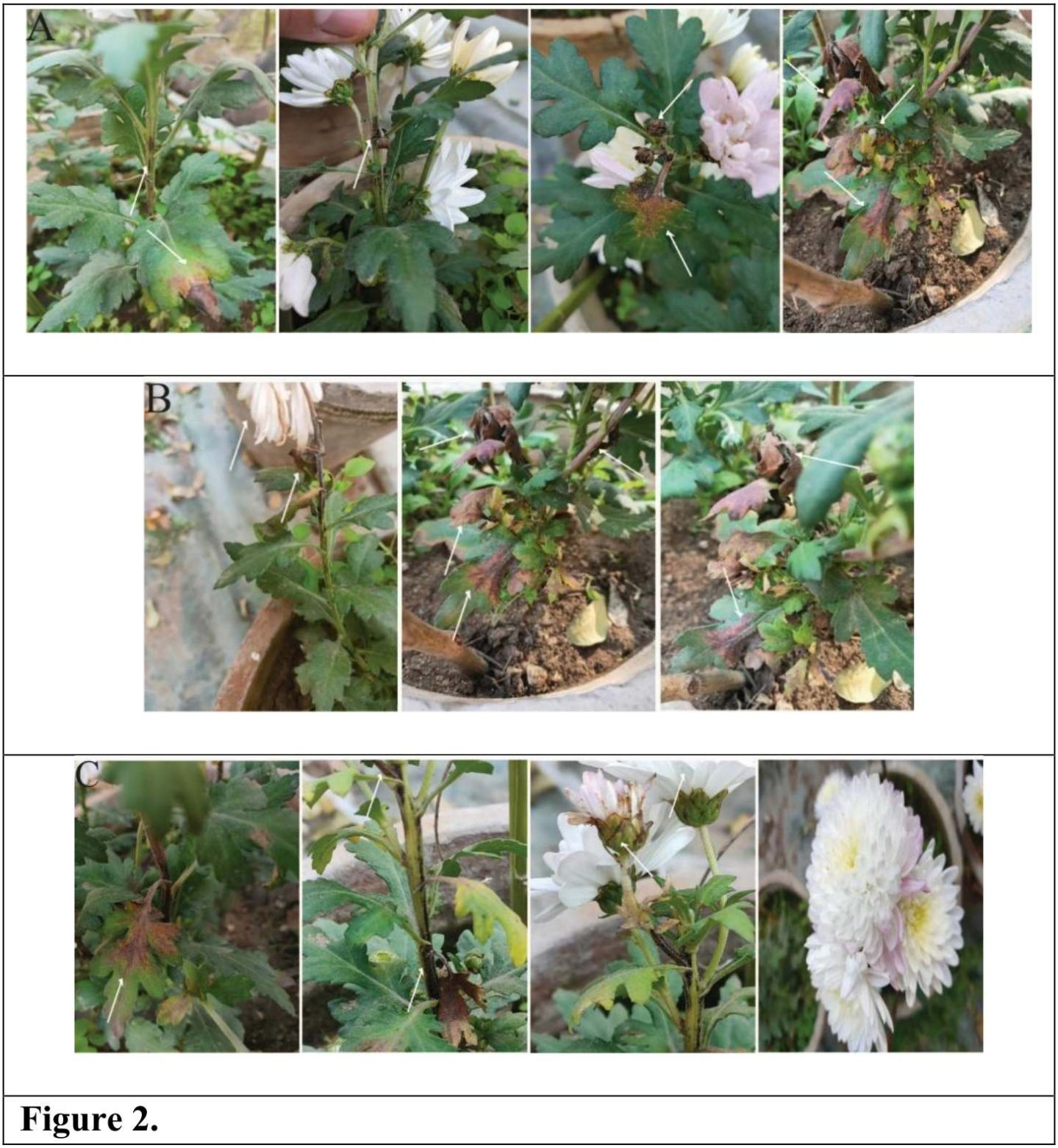

Figure 2.

Figure 2

Confirmation of pathogenicity test on chrysanthemum plant. (**A**) *Alternaria* sp. (isolate 1). (**B**) *Diaporthe sp.* (isolate 6). (**C**) *Alternaria sp*. (isolate 7). Symptoms recorded 10 days post-inoculation.

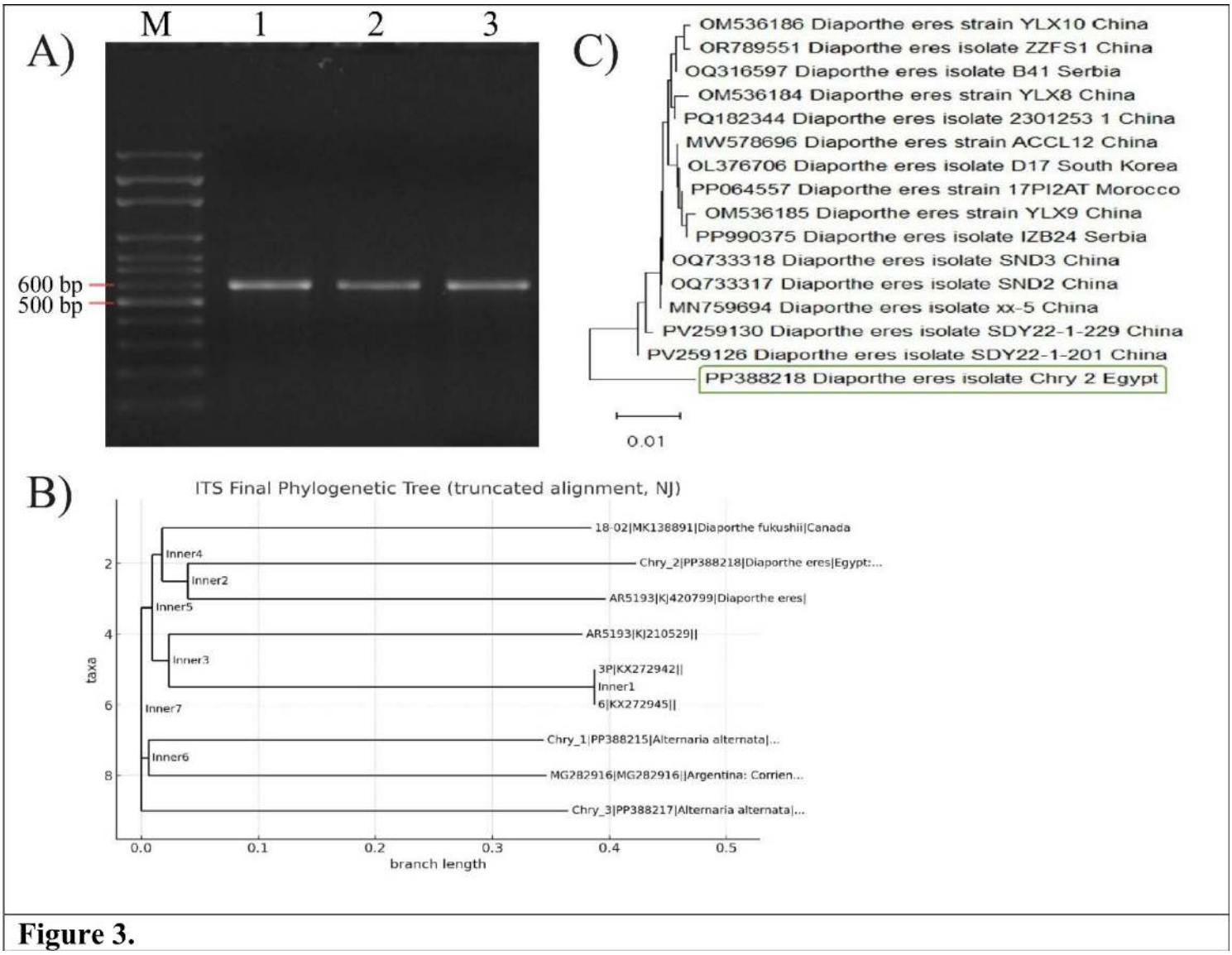

Figure 3.

Figure 3

Molecular identification of fungal isolates from infected chrysanthemum plants' DNA. (**A**) PCR amplification of the ITS region. Lane M: DNA ladder; Lane 1: *Alternaria alternata* (isolate 1); Lane 2: *Diaporthe eres* (isolate 6); Lane 3: *Alternaria alternata* (isolate 7). (**B**) Phylogenetic tree, based on ITS sequences showing Egyptian *Alternaria alternata* isolates' position (**C**) Phylogenetic tree based on ITS sequences showing Egyptian *D. eres* isolate position **PP388218** within *D. eres* clade.

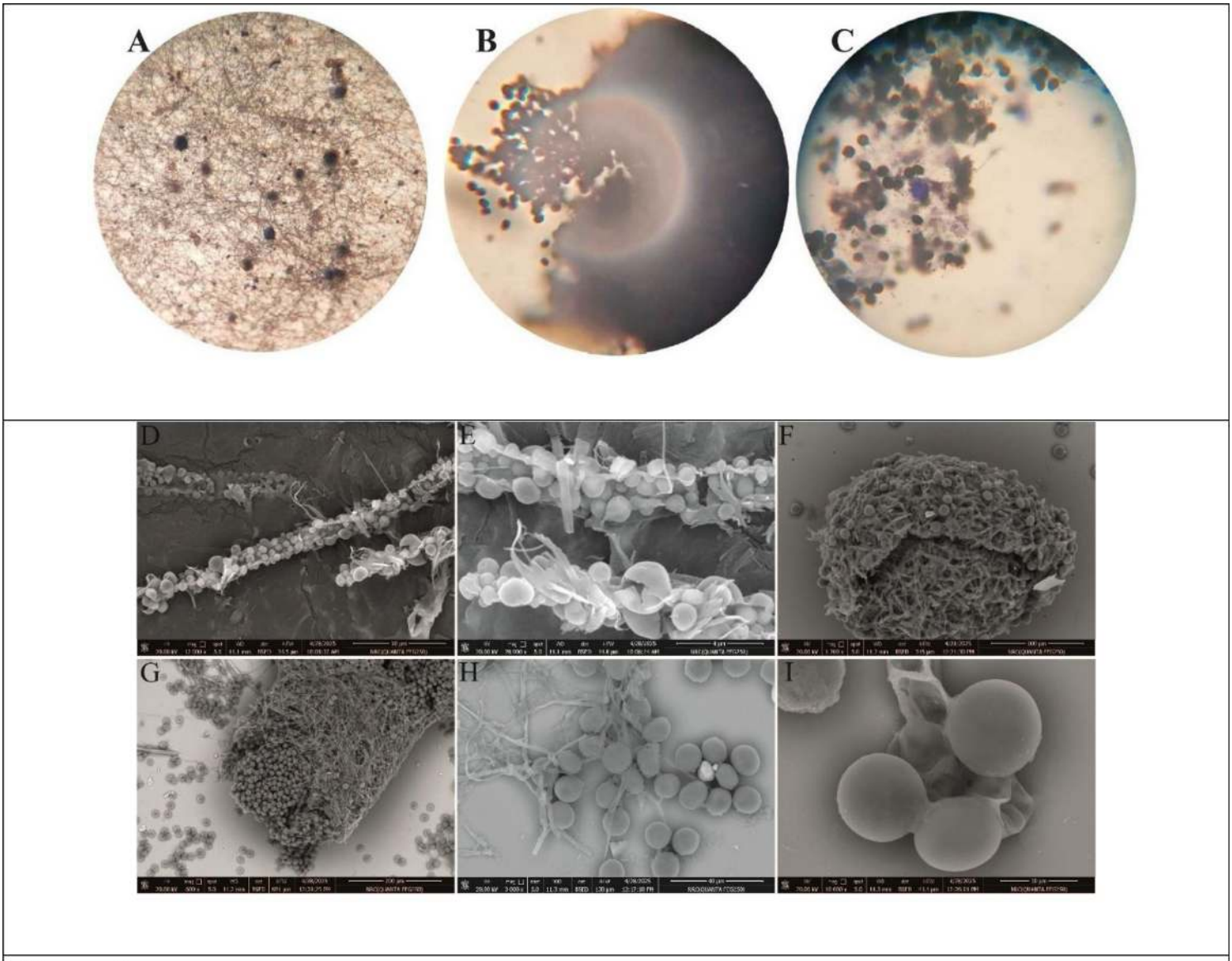

Figure 4.

Figure 4

Morphological features of *D. eres*(isolate PP388218) from chrysanthemum. (A-C) Light micrographs: (A) Mycelial growth on PDA at 14 days, (B) Pycnidium, (C) Alpha conidia. (D-I) Scanning electron micrographs: (D & E) Septate hyphae, (F) Pycnidium with ostiole, (G & H) Fusiform alpha conidia and (I) Filiform beta conidia.

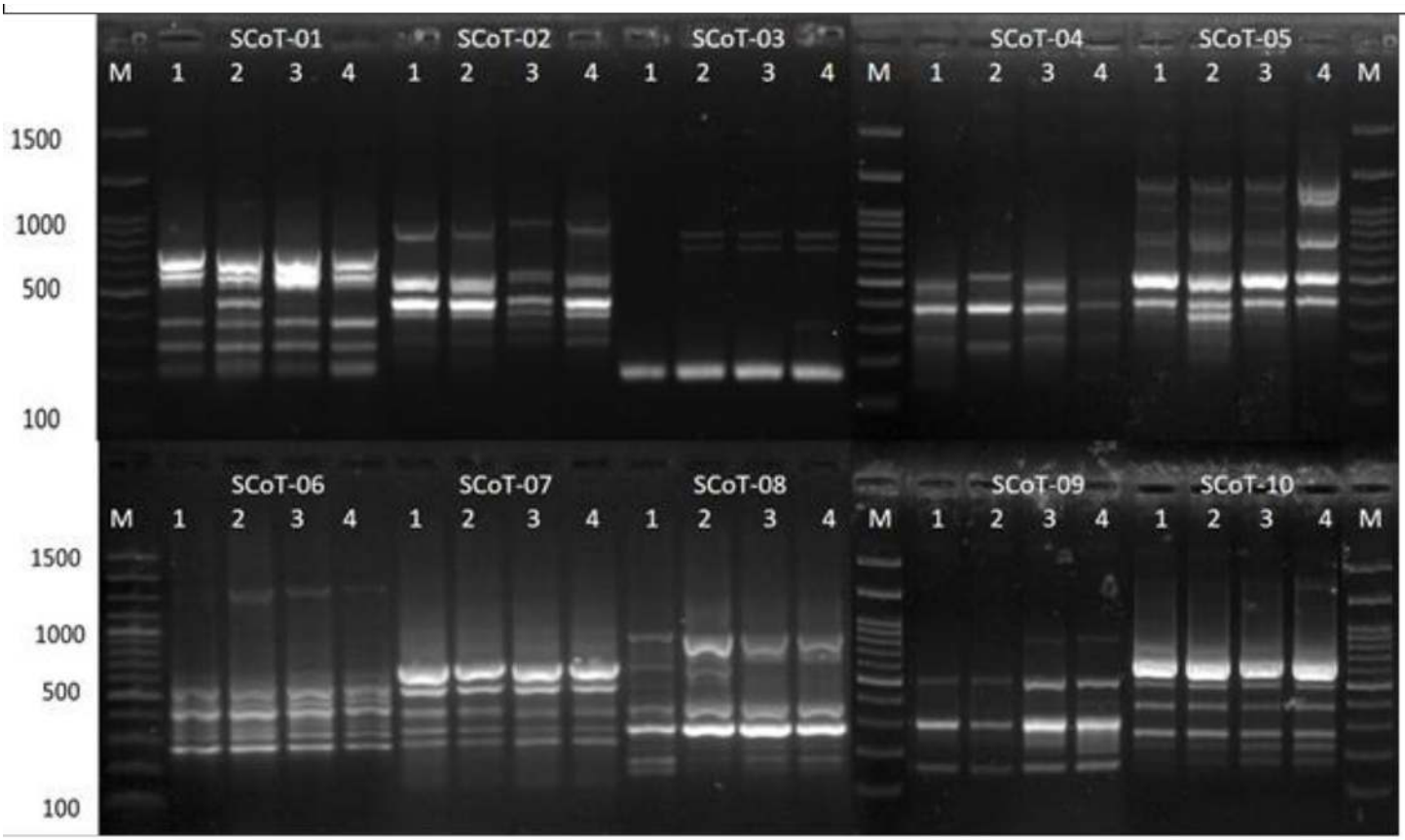

Figure 5.

Figure 5

Banding profile of SCoT oligonucleotides designed for *Chrysanthemum morifolium*. 1 kb DNA marker was loaded at the left lane of each gel; **1**: Control; **2**: *Alternaria* 1; **3**: *Diaporthe*; **4**: *Alternaria*7.

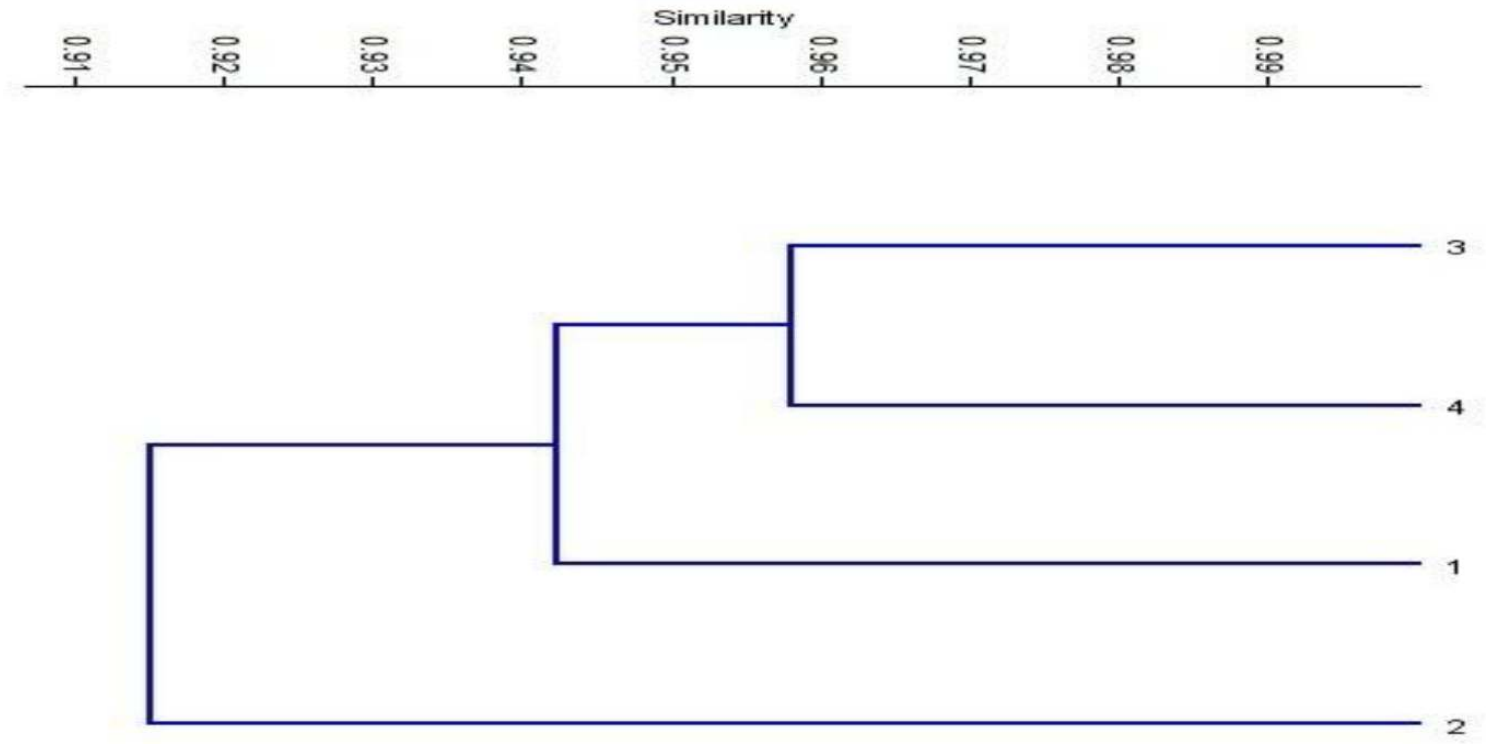

Figure 6

Phenogram obtained by UPGMA cluster analysis based on Nei and Kumar coefficient generated by SCoT-Markers.

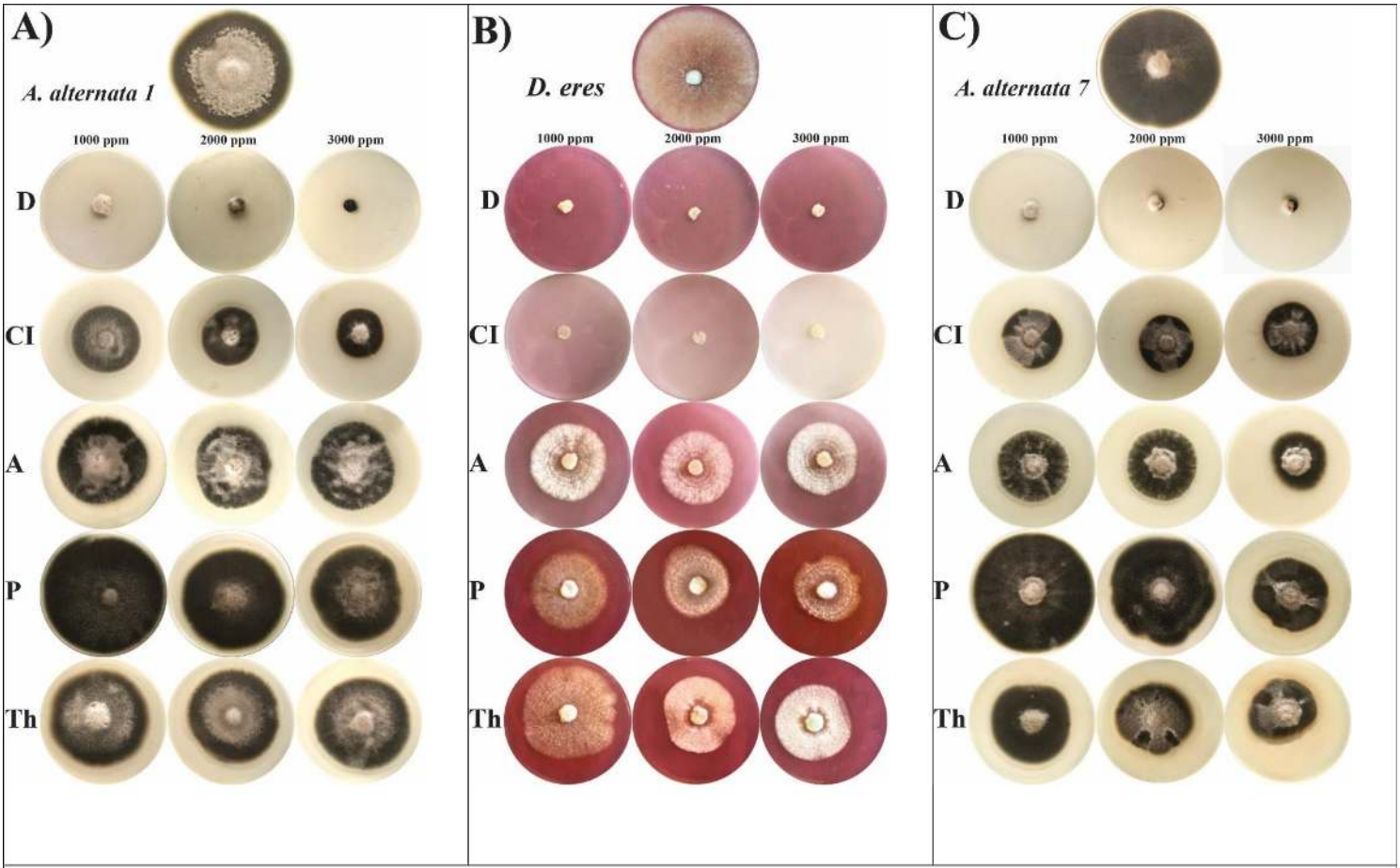

Figure 7.

Figure 7

Poisoned food assay showing the effect of various fungicides on mycelial growth in: A- *Alternaria* 1; B- *Diaporthe eres*and; C- *Alternaria* 7.

Supplementary Files

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