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Phytopathological studies on Alternaria blight of Chrysanthemum (*Chrysanthemum indicum* L.)

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Abstract Chrysanthemum plant is considered one of the ornamental plants and cut flowers at the level of local and export production, recently. Alternaria leaf spot and blight disease are considered one of the most important diseases, which it has negatively effects on flower yield under greenhouse conditions with climate changes. This study confirmed the possibility of using plant extracts and bio-agents as a single component in an integrated control program to control Alternaria leaf spot and blight disease in chrysanthemum plants in Egypt. The disease was inventoried to the three regions of Heliopolis, (HelCa), in Darwa (MGQa) and Ezbat Al-Ahaly (MGQa) at El Qanater El Khaireya, which the symptoms were found in the form of brown spots and blight on the leaves and rotting of the flowers, also disease incidence (%) were 87.15, 61.44 and 38.72%, respectively. A total of 97 fungal isolates of *Alternaria* spp. were isolated. *In vitro*, the pathogenicity was tested by detached leaf inoculation using a single droplet technique over a period of 7-14 days, where the frequency of the high virulent isolates was (19.6%). The molecular identification was carried out using rDNA ITS for five highest pathogenic isolates, which the result was *Alternaria alternata*, where the process of cultural and morphological identification of MGQa–FYhd2(1-3) which the highest pathogenic isolate was completed. This isolate was tested with 10 different plant extracts that were extracted by methanolic extraction. Clude extract was the best result where EC_{50} was 502.4 ppm and EC_{90} was 281.6 ppm while the cinnamon extract expressed the EC_{50} value of 1969.2 ppm and EC_{90} was 10356.1 ppm. The bio-agents tesing showed the best treatments which inhibition of mycelial growth of the tested pathogen were *Trichoderma harzianum* (71.39%), and followed by *Bacillus subtilis* (69.41%).

Keywords: Chrysanthemum, *Alternaria alternata*, Methanolic plant extracts, Biological control, Climate changes

Introduction

Chrysanthemum flower is known as the "Autumn (Queen) Flower" and "Queen of the East" especially in East Asia (China and Japan) (Dana and Lerner, 1996; Saicharan *et al.*, 2017 and Shahrajabian *et al.*, 2019).

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Chrysanthemum is considered the second ornamental plant in the world after the rose plant (Kalia, 2015). In Egypt, Chrysanthemum is popular ornamental plant and cut flower, which it is widely cultivated in many areas. The importance of chrysanthemum plants summed up in several points include medical, environmental, ornamental importance, and pest control (Singh and Chettiri, 2013; Stanciu *et al.,* 2017; Suriaman *et al.,* 2018 and Sharajabian *et al.,* 2020).

Alternaria leaf spot and blight disease, one of the most common and devastating diseases of greenhouse-grown crops especially in the case of climate changes (Pugliese *et al.,* 2012a, b; Das *et al.,* 2016 and Fagodiya *et al.,* 2022), is estimated to cause a greater economic loss of ornamentals than any other disease (Crous *et al.,* 2000; Arun Kumar, 2008 and Dom ńguez-Serrano *et al.,* 2016). The symptoms resulting from the disease are represented in the form of the appearance of brown spots on all parts of the foliar system, and with the increase in humidity and dense cultivation, the spots increase in size and severity, which leads to the emergence of blight symptoms. It is also represented on the flowers, which also lead to their death and rotting (Nagrale *et al.,* 2013 and Zhu *et al.,* 2019).

The only way to identify pathogenic isolates is done by the pathogenicity assay (Gat *et al.,* 2012). The disease severity is more pronounced with humid weather and with a lack of nutrients in the leaves (Nagrale, 2007 and Nagrale *et al.,* 2012), in addition to the necrotrophic behavior of *Alternaria alternata* for the pathogenesis (Matic *et al.,* 2020).

Cultural and morphological characteristics are within any culture, there are a lot of differences in conidial morphology related to shape, size, color, septation and ornamentation that depend on conidial age (Simmons, 2007). Further, there are several small-spored *Alternaria* spp. with morphological characteristics that overlap to *A. alternata* (Woudenberg *et al.,* 2013; Lawrence *et al.,* 2016 and Elfar *et al.,* 2018). It was better to rely on molecular characteristics and phylogenetic analysis of *Alternaria*, which it has many gene regions like internal transcribed spacer of ribosomal DNA (rDNA ITS) sequences amplified by primers (ITS1/ITS4). It can differentiate between large and small-spored *Alternaria* (Zhao *et al.,* 2018 and Ma *et al.,* 2021).

In recent years, treatment with fungicides is one of the most negatively affecting plants of cut flowers, as in the chrysanthemum plant. Therefore, safe alternatives were resorted to become that treatment with several bioagents such as *Trichoderma harzianum* and *Bacillus subtilis* had positive effects on the plant and reduced the disease (Madalagi *et al.,* 2014), several strains of these antagonists also have an effective effect in controlling the disease on akarkara, asparagus, cabbage and marigold (Rajput *et al.,* 2013; Thakur and Harsh, 2014;

Priyanka *et al.*, 2018; Abdel-Rahman, 2019 and Pun *et al.*, 2020), as well as the use of plant extracts had an effective role in combating the disease, as in the extract of cinnamon and cloves (Shaban, 2014; Yeole *et al.*, 2014; Abo Zaid, 2020; Gupta and Prakash, 2021; Lengai *et al.*, 2021 and Ansary *et al.*, 2022).

The objectives of this study aimed to isolate and identify the causal pathogens of *Alternaria* leaf spot and blight of chrysanthemum and to study the efficacy of different plant extracts, essential oils, and bio-agents against the pathogen.

Material and methods

Nurseries inventory of leaf spot and blight disease

A nursery inventory of leaf spot and blight disease on chrysanthemum plants was carried out at several locations in Heliopolis (Cairo), Darwa at El Qanater El Khaireya (Qalyubia) and Ezbat Al-Ahaly at El Qanater El Khaireya (Qalyubia), one of the major chrysanthemum-producing in Egypt (September – November 2018). Three nurseries were randomly selected and inventoried (Zhu *et al.*, 2019). In each nursery, 100 plants in each of four corners were randomly selected and disease incidence was recorded using the formula by (Teng and James, 2002).

$$\text{Disease Incidence (DI) \%} = \frac{The\ number\ of\ infected\ plants}{The\ number\ of\ total\ plants\ in\ the\ sample} x100$$

Isolation of associated fungi with leaf spot and blight disease

The infected parts (leaves and peduncles) of chrysanthemum plant samples were washed thoroughly with tap water, cut into small pieces and surface-sterilized in 1 % sodium hypochlorite solution for 2 min, and then washed in sterile distilled water for 4 min. small pieces were dried between layers of sterile tissue paper and directly plated to Water Agar medium (WA) in Petri dishes. While flowers were directly plated to Water Agar medium (WA) in Petri dishes. Penicillin-G antibiotic was added in the medium at concentration of 50µg ml-1 to avoid the bacterial contamination. Plates were incubated in the dark at 25±1 ˚C for 2-4 days. Hyphal tips of the developed fungal colonies were transferred to fresh Potato Dextrose Agar (PDA) medium in Petri dishes, and the plates were incubated at 25±1 ˚C for 10-14 days (Dhingra and Sinclair, 1995 and El-Far *et al.*, 2010). Pure cultures of each obtained fungal isolate were maintained on PDA slants and kept in a refrigerator at 4 ˚C for further studies.

Pathogenicity assays in vitro

Pathogenicity of 97 fungal isolates, obtained from Chrysanthemum infected plants were evaluated on Chrysanthemum cultivar Chrystal Fresh White during the period of February - March 2020.

Inoculum preparation

Inocula of 97 fungal isolates were separately prepared from cultures grown on PDA in plates 9 cm in diameter incubated at 25 ± 1^{o}C under 12/12 hours of alternating dark/light conditions for 7-10 days. The developed cultures were flooded using 50 ml sterilized water. The growth of mycelia mats and spores was gently rubbed using smooth brush to remove fungal growth from medium surface. Prepared suspension was received in sterilized flasks 100 ml in capacity. The obtained suspension of propagules and large particles were blended and filtered through sterilized cheesecloth. The obtained fungal suspensions were adjusted to 10^4 cfu/ml using sterilized water with the aid of hemocytometer technique. Tween 20 drops of 0.5 ml-1/L were added for these suspensions. The prepared suspensions were used for inoculating chrysanthemum detached leaves *in vitro* (Sabry, 2015). For evaluation effect of methanolic plant extracts and bioagents, the mycelial disc of the fungus was aseptically cut by a sterilized cork borer (5mm) from the margin of 7-10 days old culture. A single disc was placed in the center of each plate. The disc was kept in such a manner that the mycelial portion of the disc touched the surface of the medium. The inoculated plates were incubated for 7 days at 25 ± 1 ˚C for further growth (Abo Zaid, 2020).

Detached leaf inoculation using single droplet technique in vitro

The transplants of Chrysanthemum cultivar Chrystal Fresh White were grown under greenhouse conditions of Plant Pathology Department, Faculty of Agriculture, Ain Shams University. The healthy detached leaves were taken from 60 days old from transplants and transferred to 9 cm petri dished with moisten filter paper. The lower surface of the leaves was inoculated with three drops from previously prepared the inocula of 97 tested fungal isolates, each droplet contained 30 µl of propagules suspension adjusted to 10^4 cfu/ml, after cracking the bristles in the place of inoculation using a camel hairbrush. Other healthy leaves were inoculated with the same volume of distilled water as control. Six replicates were carried out for each isolate. The inoculated leaves were incubated in a growth chamber at 25 ± 1^{o}C. The inoculated leaves were observed daily for 7-14 days after inoculation for the appearance of symptoms.

Disease severity was determined and calculated depending on disease index (0-5) (Table 1 and Figure 1) used for assessment of severity Alternaria leaf spot and blight (modified scale of disease severity from Arun Kumar, 2008) (Reni *et al.*, 2007 and Sabry, 2015).

Table 1. Modified disease rating scales, corresponding disease severity index, description of the symptoms and assigned phenotypic class for Alternaria Leaf Spot and Blight of Chrysanthemum

Disease Scale	Percent Disease Index	Description of The Symptoms	Assigned Phenotypic Class
0	No Infection	No Disease Symptoms.	**Immune**
1	1-10	A few spots towards tip.	**Resistant**
2	10-20	Several dark brown patches.	**Tolerant**
3	20-40	Several patches with paler outer zone, in addition to a partial death of some of the veins of the leaf.	**Modernity Susceptible**
4	40-75	Leaf blight or breaking of the leaves form center, In addition to a total death in the veins of the leaf.	**Susceptible**
5	75-100	Complete drying of the leaves or breaking of the leaves form center.	**Highly Susceptible**

Figure 1. Disease index (0-5) used for assessment of severity of Alternaria Leaf Spot and Blight of Chrysanthemum

Cultural and morphological characterization

Identification of the obtained fungi were done which based on culture characteristic and microscopic features according to the keys described by Ellis (1971 and 1976); and Simmons (2007) for *Alternaria* species using slide culture technique (Woudenberg *et al.*, 2013 and Lawrence *et al.*, 2016).

PCR amplification, DNA sequencing and phylogenetic analysis

The international transcribed spacer (ITS) region of the nuclear rDNA region; including ITS1, ITS4 and the 5.8S ribosomal gene; was amplified with the polymerase chain reaction (PCR) assay using the primer pair ITS1 (5'TCCGTAGGTGAACCTGCGG3') ITS4 (5'TCCTCCGCTTATTGATATGC3') (White *et al.,* 1990 and Bensassi *et al.,* 2009). For DNA amplification and sequencing, this experiment was carried out through the laboratories complex of the Faculty of Agriculture, Cairo University. The amplified rDNAs were sequenced and identified using the BLAST alignment software of GenBank database.

Management of Alternaria leaf spot and blight disease by using methanolic plant extracts and bioagents in vitro

Assay of antifungal evaluation of methanolic plant extracts

Plant materials: Plant extracts of ten plant species were used Basil (*Ocimum basilicum* L.) leaves, Rosemary (*Salvia Rosmarinus* S.) leaves, Thyme (*Thymus vulgaris* L.) leaves, Henna (*Lawsonia inermis* L.) leaves, Lemon Grass (*Cymbopogon citratus* DC.) leaves, Black Pepper (*Piper nigrum* L.) Peppercorn, Cinnamon (*Cinnamomum verum* J.) Inner bark, Clove (*Syzygium aromaticum* LM.) Flower buds, Garlic (*Allium sativum* L.) Clove and Ginger (*Zingiber officinale* R.) Rhizomes, which it purchased from Haraz Co. Ltd. (Cairo, Egypt).

Preparation of methanolic plant extracts: Dry materials were separately micronized with a hammer mill into a fine powder. The known weight of the powder of plant materials was added to pure methanol (99.9%) in the ratio of 1:10 (w/v) and then transferred to shaker for 24 h. The pulverized mass of a plant materials was then filtered using Whitman's No.2 filter paper and then centrifuged at 5000 rpm for 20 min under 4 ℃. Methanol filtrates were evaporated using a rotary evaporator, and the remaining small amounts of the alcoholic solvents were evaporated in the oven at 40 ℃. The remaining contents was collected in sterilized brown bottles and kept in refrigerator 4 ℃ and later used to test their efficacy on the growth inhibition of the fungal pathogen. (Zaker and Mosallanejad, 2010; Chethana *et al.,* 2012; Abo Zaid, 2020 and Mugao *et al.,* 2020).

Antifungal evaluation of methanolic plant extracts on mycelial growth of A. alternata under laboratory conditions: Poisoned food technique (Mohana and Raveesha, 2007; Zaker and Mosallanejad, 2010; Gurjar *et al.,* 2012; Balamurugan, 2014 and, Ramaiah and Garampalli, 2015) was used for evaluating the effect of methanolic plant extracts on mycelial growth of

A. alternata. Petri dishes were prepared with PDA incorporated with the concentrations of methanolic plant extracts at 250, 500, 1000, 1500, and 2000 ppm. Five replicate dishes were used for each treatment. These plates were inoculated at 25 ℃ for 8 days old culture of pathogens and plates without plant extract served as control. Linear growth of the fungus was measured after 8 days and the percentage inhibition in growth was calculated. Different control measures were tested *in vitro* to assess their efficiency to control Alternaria leaf spot and blight of chrysanthemum to predict which treatments. A mathematical model to correlate the concentration of tested elements of investigated control measures with its efficacy to suppress *A. alternata* radial growth *in vitro* were developed. That model was used to calculate the Half maximal effective concentration (EC_{50}), and 90% effective concentration (EC_{90}) for each plant extract. Comparison among treatments and concentrations according to their EC_{50} and EC_{90} were determined precisely for the most effective treatment and its concentration (Al-Essawy *et al.,* 2018 and Ding *et al.,* 2019).

Assay of antagonism with bioagents in vitro

Two antagonistic fungi (*Trichoderma harzianum* and *Chaetomium globosum*) and three antagonistic bacteria (*Pseudomonas putida, Pseudomonas fluorescens and Bacillus subtilis*) were kindly provided by Dr. Huda Zakaria and Dr. Nevein A. Shehata, (ARC, Giza, Egypt). Interactions between pathogenic fungal isolate and antagonistic fungi and bacteria were assayed using dual-culture technique (Skidmore and Dickinson, 1976; Abo-Elyousr *et al.,* 2014 and Adolf, 2016) on plates of PDA) medium.

Assay of antagonistic fungi

A 10 mm disk of each tested fungal culture was placed onto PDA apart from 10 mm from the edge of the Petri dish. Another disk of the same diameter of each pathogenic fungus was placed on the opposite side of the PDA Petri dish at the same distance. The control treatment was transferred with a culture disk of either a pathogenic or tested isolate alone at the same conditions. The experiments were arranged in a completely randomized design, with three replicates per isolate. All transferred plates were incubated at 25±2 ˚C; and the fungal growth diameter away from and toward the tested agent was measured after the pathogenic fungal growth in the control treatment had reached the edge of the Petri dish. The antagonistic activity was assessed after 5-7 days of incubation by measuring the radial growth of the pathogenic fungal colony (Rajput *et al.,* 2013).

Assay of antagonistic bacteria

One ml of each bacterial isolates was grown for 48 h on King's medium B or NA medium. A streak of bacterial growth culture was placed on PDA dishes, then it was incubated at 28˚C for 24 h. Interactions between pathogenic fungal isolates and tested bacterial isolate were assayed using Dual-culture technique on PDA medium. A bacterial growth culture was streaked onto PDA, 10 mm from the edge of the Petri dish. Another disk of the same diameter of each pathogenic fungus culture was placed on the opposite side of the dish at the same distance. The control plates were transferred with a culture disk of pathogenic fungus only. Three replicate plates for each isolate were done. All transferred Petri dishes were incubated at 25±2 ˚C and the fungal growth diameter away from and toward the tested bacterial isolate was measured after the pathogenic fungal growth in the control treatment had reached the edge of the Petri dish. The antagonistic activity was assessed after 5-7 days of incubation by measuring the radial growth of the pathogenic fungi colony (Adolf, 2016).

Assessment of anhibition activity

Percentages of inhibition of mycelial growth (PIMG) of the pathogenic fungi in dual plates were calculated using the formula by (Vincent, 1947; Harlapur *et al.,* 2007; Sirirat *et al.,* 2009 and Adolf, 2016):

PIMG = (A1–A2) / A1 x 100

Where,

A1: Diameter of mycelium growth of pathogenic fungus in control.

A2: Diameter of mycelium growth of pathogenic fungus in treatment.

Statistical analysis

The effective concentrations (EC_{50} and EC_{90}) against tested fungi were calculated by coefficient equation between propit of means of inhibition (%) and log of concentrations used according to (Ramadan *et al.,* 2007 and Ansary *et al.,* 2022). Also, experiments with bio-agents were performed in triplicate. Duncan Multiple Range Test was used to evaluate the significant differences between treatments ($P \leq 0.05$). ANOVA analysis was done with the SPSS software Version 22.0, 2013 (Steel *et al.,* 1997).

Results

Nurseries inventory of leaf spot and blight disease

Leaf spot and blight symptoms were observed in all nurseries as follows: on leaves and peduncles showed appearance of small brown spots fuses together, covering most of the surfaces of the affected leaves, giving the appearance of blight on the leaves, which leads to dryness, wilting, drooping and death of the affected leaves. While on flowers showed reddish-brown spots appeared on the petals of small flowers, it turned into large-sized dead spots, which leaded to the death of flower petals, gray growths appeared on flower petals, which leaded to flower rot (Figue 2). Disease incidence (%) was 87.15% in Heliopolis (HelCa), in Darwa at El Qanater El Khaireya (MGQa) was 61.44% and in EzbatAl-Ahaly at El Qanater El Khaireya (MYQa) was 38.72% (Table 2).

Figure 2. Natural symptoms of Leaf Spot and Blight of Chrysanthemum cultivars collected from different nurseries in Egypt (September – November 2018), which on leaves (A1-3) and peduncles (A4) showing appearance of small brown spots fuses together, covering most of the surfaces of the affected leaves, giving the appearance of blight on the leaves. While on flowers (A5) showing reddish-brown spots appear on the petals of small flowers, turning into large-sized dead spots, which leading to the death of flower petals, gray growths appear on flower petals, which leading to flower rot

Isolation of associated fungi with leaf spot and blight disease

A total of 97 fungal isolates were isolated from infected flowers, leaves, and peduncles of chrysanthemum plants, collected from nurseries in two locations in Egypt (Table 1). Samples from Heliopolis (HelCa) yielded 28 fungal isolates of *Alternaria* spp., while samples from Darwa at El Qanater El Khaireya (MGQa) yielded 38 fungal isolates and in Ezbat Al-Ahaly at El Qanater El Khaireya (MYQa) yielded 32 fungal isolates of *Alternaria* spp.

Table 2. Frequency of *Alternaria* spp. from different Chrysanthemum plants with number of isolates, frequency, location, season, cultivar, isolation origins and mean of disease incidence in location (%)

Number of Isolates	Frequency (%)*	Location	Season	Cultivar	Isolation Origins	Mean of Disease Incidence in Location (%)**
11	11.3	Heliopolis (HelCa)	September 2018	Moonbeam	Flower	
7	7.2				Leaves	
9	9.3				Peduncle	87.15%
15	15.5	Darwa at El Qanater El Khaireya (MGQa)	October 2018	Chelsey	Flower	
9	9.3				Leaves	
14	14.4			Moira	Leaves	61.44%
1	1	Ezbat Al-Ahaly at El Qanater El Khaireya (MYQa)	November 2018	Yoko Ono	Flower	
4	4.1				Leaves	
4	4.1			Chiffon	Flower	
9	9.3				Leaves	
4	4.1			Jolie Rose	Flower	
5	5.2				Leaves	
5	5.2			Branroyal Purple	Leaves	38.72%

(*) Frequency of *Alternaria* spp.
(**) Disease incidence of total chrysanthemum infected plants in location.

Pathogenicity assays in vitro

All tested *Alternaria* isolates (97) produced light brown few spots to complete brown drying of the leaves or breaking of the leaves depending on disease index (0-5) used for assessment of severity Alternaria leaf spot and blight after 14 days, like naturally infected leaves under nursery conditions. No symptoms of leaf spot and bight were observed on control leaves. The severity of the infection ranged from 3.33 to 43% after 7 days, as the severity of the infection increased after 14 days and ranged from 3.33 to 60% (Table 3 and Figure 3).

Table 3. Frequency of *Alternaria* spp. isolates after 7 and 14 days of pathogenicity assays according to virulence degree and percent disease index (%) *in vitro*

Virulence Degree	Percent Disease Index (%)	Number of Isolates		Frequency (%)*	
		After 7 Days	After 14 Days	After 7 Days	After 14 Days
Low	1-20	8	7	8.2	7.2
Moderate	20-40	83	71	85.6	73.2
High	40-75	6	19	6.2	19.6

(*) Frequency of *Alternaria* spp.

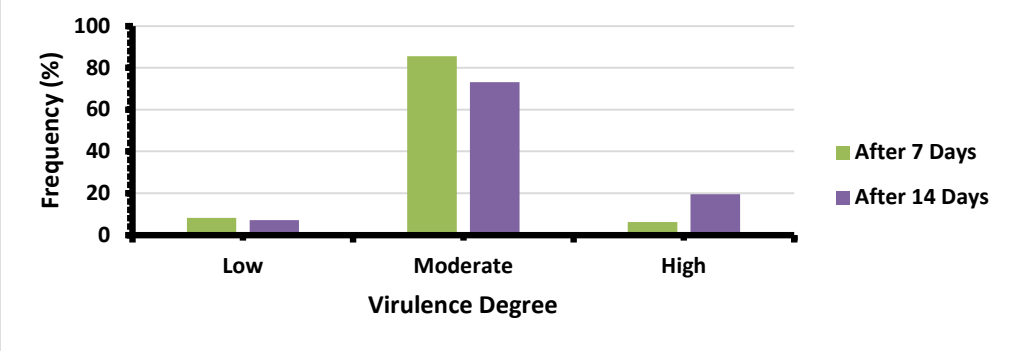

Figure 3. Frequency of *Alternaria* spp. isolates after 7 and 14 days of pathogenicity assays according to virulence degree and percent disease index (%) *in vitro*

Cultural and morphological characterization

The characteristic and microscopic features of *Alternaria alternata* isolate MGQa–FYhd2(1-3) showed the highest pathogenic isolate which colony growth was medium (70 -80 mm) / 7-10 days, colony color was olivaceous (olive green), colony appearance was fluffy, growth rate was moderate, colony shape and margin was circular and smooth, sporulation was excellent, zonation was absent (Figure 4a).

While microscopic features showed straight primary conidiophores, long, simple, with one apical conidiogenous locus. Conidia are obclavate, long ellipsoid, moderate in size, septate. The conidium body can narrow gradually into a tapered beak or secondary conidiophore. Secondary conidiophores can be formed apically or laterally with one or a few conidiogenous loci (Figure 4b).

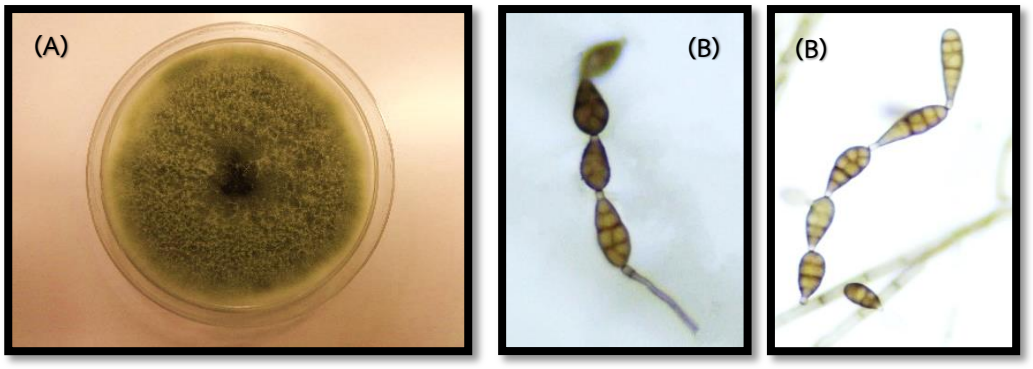

Figure 4. Cultural and morphological characteristics of MGQa–FYhd2(1-3) isolated from chrysanthemum plants. **(A)** Colony appearance of *A. alternata*, cultured on PDA plates for 7 days at 25±1°C, **(B)** Sporulation pattern and conidia of *A. alternata* using slide culture technique for 7 days at 25±1°C

DNA sequencing and phylogenetic analysis

Five fungal isolates of *Alternaria* spp. with the highest pathogenic isolates of 50 to 60% in pathogenicity assay under laboratory conditions were proved by molecular identification process. Phylogentic analysis based on the concatenated sequences of rDNA ITS (ITS1/ITS4) was delimited three main clusters (Figure 5a). Phylogenetic tree was obtained by maximum-likelihood analysis of internal transcribed spacer 1 (ITS1) and 4 (ITS4) sequence. Sequences of the rDNA ITS, showed 100% identity to the isolate of *Alternaria*

alternata (Figure 5b) as Isolates: HelCa – FWhd1(2-1), HelCa – FWhd2(1-1), MGQa – FYhd2(1-3), MGQa – LMhd1(1-2), and MYQa – FLMhd5(5-3)). Perfect similarity 99.9 - 100% among all clusters was obtained.

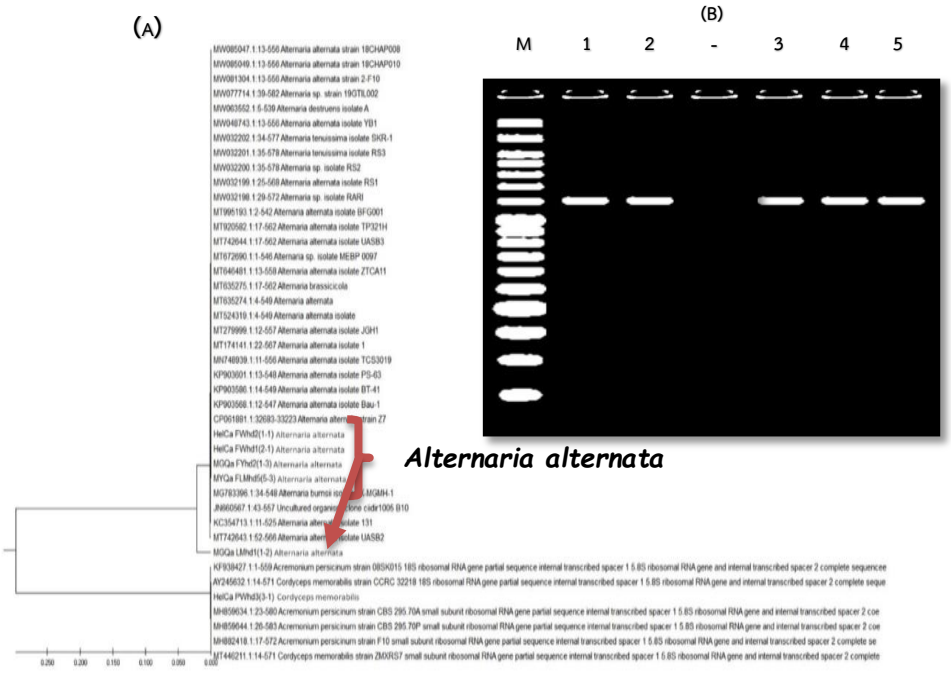

Figure 5. (A) Phylogenetic tree obtained by maximum-likelihood analysis of internal transcribed spacer 1 (ITS1) and 4 (ITS4) sequence, Sequences data for reference isolates were obtained from GenBank. The tree was rooted using sequences of *A. alternata* and (B) Ethidium bromide-stained agarose gel showing genomic DNA of *A. alternata* isolates from pathogenicity assay that were amplified using primes (ITS1) and (ITS4), which M: Molecular marker 1 Kb: 1: HelCa – FWhd1(2-1), 2: HelCa – FWhd2(1-1), 3: MGQa – FYhd2(1-3), 4: MGQa – LMhd1 (1-2), 5: MYQa – FLMhd5(5-3)

Management of Alternaria leaf spot and blight disease by using methanolic plant extracts and bioagents in vitro

Poisoned food technique with methanolic plant extracts

Result showed ten different methanolic plant extracts testing with isolate of *A. alternata* (MGQa–FYhd2(1-3)), where the mycelial growth rate was estimated with different concentrations at 250, 500, 1000, 1500, and 2000 ppm for each extract every 2 days for 8 days, where the percentage of inhibition efficiency for those extracts was calculated (Table 4). Through these different readings, it was calculated EC_{50}, EC_{90} (Figure 6) and coefficient of

determination (R^2) relationship between log of concentrations and probit of efficacy of inhibition. After calculating EC_{50}, EC_{90} and R^2, the best treatments were clove extract where EC_{50} was 502.4 ppm and EC_{90} was 281.6 ppm and R^2 value was 0.8816, while the cinnamon extract showed the EC_{50} of 1969.2 ppm, and EC_{90} was 10356.1ppm which and R^2 value was 0.6608.

Table 4. Inhibition of mycelial growth (EC_{50}) values of ten methanolic plant extracts with tween 80 against *A. alternata* (isolate MGQa – FYhd2(1-3)) *in vitro*

Treatment	EC_{50} (ppm)*	$Y = \beta_0 + \beta_1 X$**	Coefficient of Determination (R^2)
Rosemary (*Salvia rosmarinus*)	2441.2	Y = 2.3072+0.7949X	0.8756
Garlic (*Allium sativum*)	0.8	Y = 4.9353-0.7045X	0.5783
Henna (*Lawsonia inermis*)	146453.6	Y = 1.1929+0.7370X	0.9554
Basil (*Ocimum basilicum*)	8154.6	Y = -0.1333+1.3124X	0.9661
Thyme (*Thymus vulgaris*)	26485	Y = 1.7858+0.7267X	0.0548
Ginger (*Zingiber officinale*)	3952.8	Y = 1.6038+0.9442X	0.9799
Black Pepper (*Piper nigrum*)	1607.7	Y = 3.3504+0.5145X	0.9339
Cinnamon (*Cinnamomum verum*)	**1969.2**	**Y = -0.8494+1.7756X**	**0.6608**
Clove (*Syzygium aromaticum*)	**502.5**	**Y = -6.7761+4.3598X**	**0.8816**
Lemon Grass (*Cymbopogon citratus*)	3081058.9	Y = 3.0683+0.2977X	0.1680

(*) EC_{50}: Half maximal effective concentration; EC_{90}: effective concentration at 90 %.
(**) Y: Probit of means the inhibition, and X: Log of means the concentration of the test plant extract.

Antagonistic effect of five bio-agents

Result showed five different bioagents testing with isolate of *A. alternata* MGQa–FYhd2(1-3) in percentages of inhibition of mycelial growth (PIMG) for those bioagents (Figure 7). The best treatments showed significantly effective in inhibiting the growth of the tested pathogen were *Trichoderma harzianum* (71.39%) and followed by *Bacillus subtilis* (69.41%).

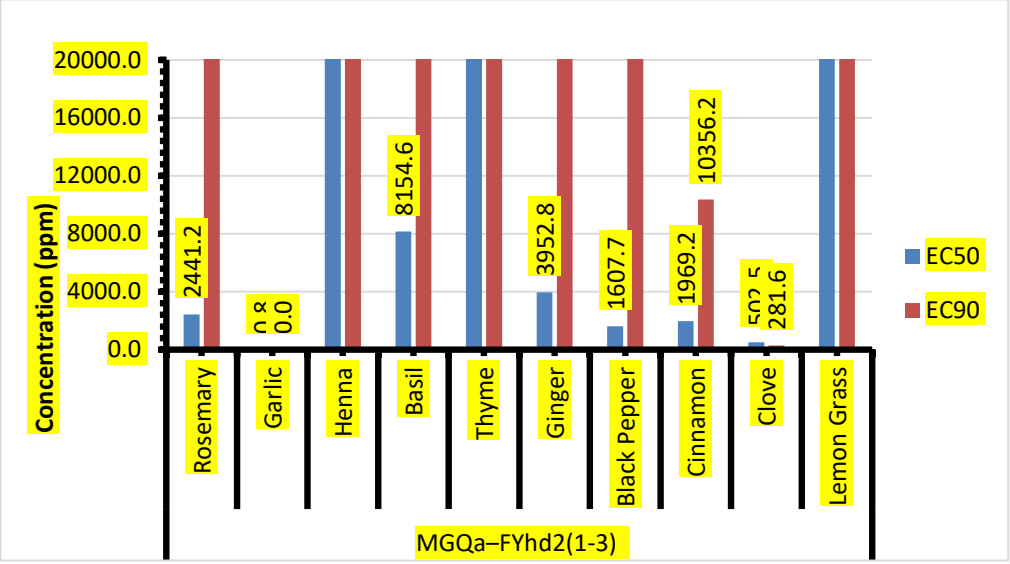

Figure 6. EC_{50} and EC_{90} values of ten methanolic plant extracts with tween 80 against *A. alternata* (isolate MGQa – FYhd2(1-3)) *in vitro*

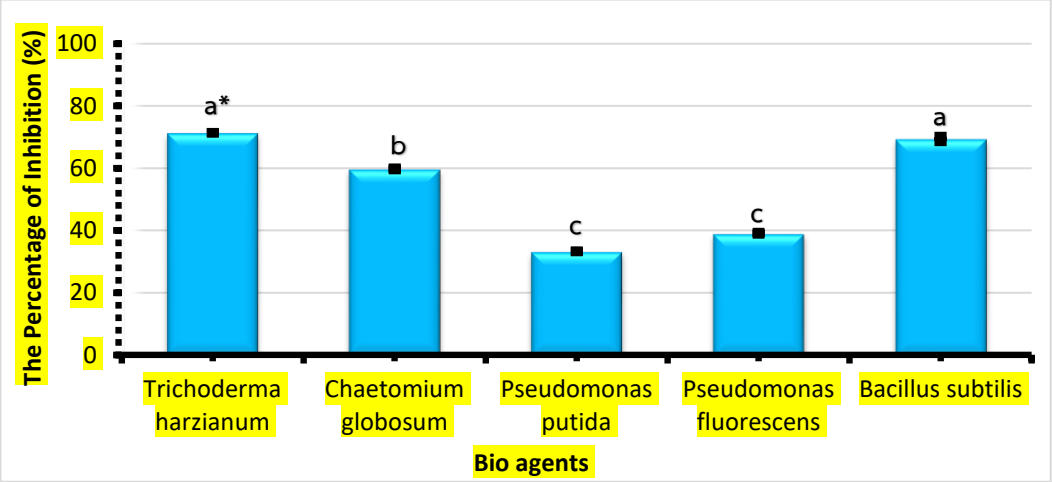

Figure 7. Antagonistic effect of five bio-agents against linear mycelial growth of *A. alternata* isolate MGQa – FYhd2(1-3) cultured on PDA at 25±1°C for 8 days *in vitro*

Discussion

In this study, the disease was inventoried to three different regions of Heliopolis, Darwa (MGQa) and Ezbat Al-Ahaly (MGQa) at El Qanater El Khaireya, in Egypt, which disease incidence (%) was varied. According to what

was stated in previous studies, yield losses were recorded because of infection with the disease (Cotty, 1987 and Zhu *et al.,* 2019). Due to potassium deficiency in leaf at the time of in infection by *Alternaria* spp. in pre-mature on infected plants, cooler temperature and high humidity may cause spread and increase in severity of infection, which leads to yield losses (Zhao *et al.,* 2013).

Through the process of isolation and purification in the laboratory, 97 different isolates of *Alternaria* spp were isolated. These results confirm findings of Arun Kumar (2008); Dom ńguez-Serrano *et al.* (2016) and Kumar *et al.* (2021), whereas highest frequency of *Alternaria* spp were detected and are common pathogens in Chrysanthemum nurseries.

In vitro, the pathogenicity experiment was conducted, where disease severity on leaves varied to 3-4 different degrees. These results prove that *Alternaria* spp. were significantly the most destructive one. Thus, it can affect typical blight symptoms addition to chlorosis according to Wang *et al.* (2007). There are several reports of *Alternaria* spp. causing foliar diseases many crops such as Chrysanthemum (Luo *et al.,* 2018), Pistachio (Pryor and Michailides, 2002) and Watermelon (Ma *et al.,* 2021).

After the completion of the pathogenicity experiment *in vitro*, the highest pathogenic isolate was identified culturally and morphologically according to different identification keys. All results showed morpho-cultural variability among *A. alternata*, causing chrysanthemum leaf spot and blight, where it agreed with many researchers Pryor and Michailides (2002), Nagrale *et al.* (2013), Shamala and Janardhana (2015), Elfar *et al.* (2018), Banne *et al.* (2021) and Ma *et al.* (2021), who they studied *A. alternata* on the chrysanthemum plants and many different hosts like gerbera, marigold, watermelon, apple and pistachio under different laboratory conditions such as culture parameters of medium, pH effect, nitrogen and carbon sources, temperature, light source, photoperiod, and light intensity.

Molecular identification of five highest pathogenic isolates resulting from the pathogenicity experiment was carried out, based on rDNA ITS using the primer pair ITS1/ITS4. These results confirm findings of Pryor and Michailides (2002); Elfar *et al.* (2018) and Ma *et al.* (2021), whereas the MP analysis of rDNA ITS sequence allowed us to clearly delimit the small-spores species of *Alternaria* within sections. But it was not informative enough to separate the species within each section. Where it was observed that he relied on ITS region, PCR test could not differentiate between *A. alternata* and *A. tenuissima*. So, analysis of the DNA sequence of *HIS3* is highly recommended in the future, especially to recognize between small-spored *Alternaria* species.

MGQa–FYhd2(1-3) isolate was tested with ten different methanolic plant extracts. The best treatments based on the calculation EC_{50}, and EC_{90} were

clove, followed by cinnamon. These results were consistent with many researchers such as Gurjar *et al.* (2012); Gupta and Prakash (2021); Lengai *et al.* (2021) and Carmello *et al.* (2022). Methanolic extraction works on many active substances such as terpenoids, saponins, tannins and flavones which have an effective effect in antagonizing various fungi, like *Alternaria alternata*. Plant extracts play an important role in combating various fungi such as clove, which it contains on many active substances e.g., kaempferol and vanilic acid, also the major bioactive constituents in clove belong to the group of secondary metabolites such as tannins, alkaloids and phenols (Pandey and Singh, 2011 and Bhowmik *et al.*, 2012), which it works on the complete inhibition of the fungus because of the ability to break down the cell wall components of the fungus and impede the growth and development of the pathogenic fungus. Also, cinnamon contained many active substances e.g., cinnamaldehyde, eugenol, p-hydroxybenzoic acid, p-coumaric acid and pyrogallol against various fungi (Barros *et al.*, 2013; Figueiredo *et al.*, 2017 and Carmello and Cardoso, 2018), which it effects on decreasing the conidia number according to Wang *et al.* (2022), it is also working on substrate deprivation, membrane disruption, complex with cell wall, bind to adhesins and inactivate enzymes as mentioned by Gurjar *et al.* (2012).

Also, as for bio-agents, the best treatments based on the calculation of the percentages of inhibition of mycelial growth were *Trichoderma harzianum*, followed by *Bacillus subtilis*. These results agree with those obtained by several researchers (Rajput *et al.*, 2013; Thakur and Harsh, 2014; Sabry, 2015; Priyanka *et al.*, 2018; Abdel-Rahman, 2019 and Pun *et al.*, 2020) that *Trichoderma* spp. and *Bacillus subtilis* had the ability to antagonism various pathogenic fungi. Different mechanisms of *Trichoderma* spp. are known and include mycoparasitism, antibiotics and degrading enzymes production have been detected (Haran *et al.*, 1996 and Sharma *et al.*, 2009). Also, the ability of *Bacillus subtilis* to suppress plant pathogenic fungi may be due to the production of antimicrobial peptide substances such as (subtilin, bacilliysin, mycobacilliysin and iturin). It also affects hyphal swelling, distortion, and cytoplasm aggregation (Sariah, 1994 and Rahman *et al.*, 2007).

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