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Morpho-molecular identification and management of *Erysiphe heraclei* causing dill powdery mildew using a biocide, essential oils, and organic acids

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Abstract

This study aimed to evaluate the efficacy of Bio-Cure F, essential oils of thyme and clove, ascorbic and salicylic acids in controlling dill powdery mildew under greenhouse conditions. The causal pathogen of powdery mildew was isolated from infected dill plants showing symptoms of the disease during a survey in Fayoum governorate, Egypt, in 2022. *Erysiphe heraclei* was morphologically characterized using a light microscope as the causal pathogen of powdery mildew in infected dill plants. The nucleotide sequence of the internal transcribed spacer (ITS) of the causal pathogen DNA was deposited in GenBank (Accession No. OP999071.1), which showed 98-100% similarity with accession numbers of *E. heraclei* isolates obtained from the NCBI database. Under greenhouse conditions, dill plants were sprayed with biocide (Bio-Cure F), elicitors (ascorbic and salicylic acids), and essential oil emulsions of clove and thyme three times after inoculation with *E. heraclei*. The results showed that Bio-Cure F was the treatment that showed the highest efficacy in reducing both disease severity and incidence percentage, followed by thyme essential oil. The highest percentages of increase in dill plant height (cm) as well as branches/plant were obtained by Bio-Cure F treatment compared to the control, followed by salicylic acid treatment. The results suggest that Bio-Cure F, salicylic acid, and thyme oil emulsion could be used to control dill powdery mildew.

Keywords: dill, *Erysiphe heraclei*, powdery mildew, Bio-Cur F, thyme essential oil, salicylic acid.

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1. Introduction

The dill plant (*Anethum graveolens* L.) is a medicinal and aromatic plant that has a prominent place in flavorings and belongs to the Apiaceae (Umbelliferea) family. All plant green foliage may be used to make salads, soups, pickles, and fish meals. Dill plants in different regions are affected by a significant foliar disease known as powdery mildew. It is caused by *E. heraclei* and results in significant damage to the fruits and foliage. The disease was recorded on dill for the first time in Egypt by El-Sayed Ziedan (2010), who revealed that the symptoms developed as white irregular areas on leaves, fruits, and stem inflorescences of dill. At all stages of growth, from transplanting to harvesting, the dill plant exhibits symptoms of powdery mildew, as reported by Bubici (2015). In spite of the high efficiency of chemical fungicides, repeated use of these chemicals has resulted in numerous problems, including toxicity residues in foods, environmental pollution, and resistance development in pathogen populations (Yoon et al., 2013). Consequently, agro-scientists are constantly searching for safer ways to manage plant diseases. Recently, alternative approaches to plant pathogen control have been developed, including bioagents, essential oil emulsions, and organic acids (Pereira et al., 2011). Different *Trichoderma* species are considered effective in controlling powdery mildew in many plants. Deore and Sawant (2000) reported that *Trichoderma* is highly efficient in managing guar powdery mildew. Awad et al. (2012) found that Bio-zeid (*Trichoderma album* 25 × 10^6 spores/g) is the most effective biocide that significantly reduced conidial production and disease severity percentages of cucumber powdery mildew compared to control. Abdel-Kader et al. (2013) reported that the application of *Bacillus subtilis* or *Trichoderma harzianum* resulted in a significant reduction in the powdery mildew incidence of pepper, cantaloupe, and cucumber in comparison with the application of the other tested bioagents and control. Essential oils have been found to be effective for plant disease management (Lo Cantore et al., 2009; Groot et al., 2004). According to Saltos-Rezabala et al. (2022), thyme essential oil strongly suppressed conidial germination and *Alternaria linariae* growth, the pathogen responsible for tomato early blight disease. The antimicrobial effects of bioactive compounds found in thyme essential oil have been demonstrated in opposition to several plant pathogens (Mugao et al., 2021; Doost et al., 2020). Thyme essential oil, which has multiple mechanisms against plant pathogens, is increasingly becoming popular in the search for fungicidal alternatives (Jamiołkowska, 2020). Salicylic acid is a chemical that can stimulate plant resistance, reducing the disease incidence (Oostendorp et al., 2001). Zhang et al. (2001) found that salicylic acid is significant in protecting certain crops from late leaf spot. Salicylic acid affects several molecular and biochemical processes related to resistance stimulation against plant diseases (Hammerschmidt & Smith-Becker, 1999). The current study aimed to evaluate the effectiveness of fungicidal alternatives, including biocide, essential oil emulsions, and organic acids, in controlling dill powdery mildew under greenhouse conditions. Additionally, the study aimed to identify the pathogen responsible for dill powdery mildew disease using morphological and molecular methods.

2. Materials and methods

2.1 Disease survey

Survey of dill powdery mildew disease was carried out during 2022 growing season in six regions which covered the most dill widespread cultivated areas in Fayoum governorate, Egypt. These areas are located at Tamiya, Abshaway, Etsa, Sinnoures, Youssef El-Seddik and El-Fayoum center. In order to illustrate the spread of this disease during the growing season, the survey was conducted during February and March 2022. The obtained survey data was arranged as percentages of disease severity and incidence. The percentages of disease incidence were calculated using the formula described by El-Helaly et al. (1970) and Ahmed (2013) as follows:

$$\text{Disease incidence (\%)} = (\text{Number of infected plants}/\text{Total plant numbers}) \times 100$$

The severity of the disease was calculated based on the equation and the scale (of 0 to 4) described by Yan et al. (2002) and Parkunan et al. (2013), where 0 = no disease, 1 = 1-25% area covered with lesions/ plant, 2 = 26-50% area covered with lesions/ plant, 3 = 51-75% area covered with lesions/ plant, 4 = 76-100% area covered with lesions/plant. The disease severity percentages were calculated as follows:

$$\text{Disease severity (\%)} = [\sum (n \times c)] / (N \times C) \times 100$$

Where: n= Number of infected plants, c= Category number, N= Total number of examined plants and C= The highest category number of infections.

2.2 Isolation and identification of the causal agent based on microscopic examination

Dill plants infected with powdery mildew were sampled during the survey of disease in February 2022. To identify the causal fungus of powdery mildew, epidermal strips (bearing the fungal conidiophores and conidia) from the diseased dill plants were used for microscopic preparations on glass slide, then examined using light microscope. Pathogen was identified according to morphological characteristics according to Soylu and Soylu (2003) and confirmed by the staff member of Mycological Center of Assiut University, Egypt.

2.3 DNA extraction and PCR amplification

The fungal conidia of powdery mildew were gathered in a 1.5 ml microcentrifuge tube, and the method of Walsh et al. (1991) was used to extract DNA. The primer pairs used for amplification were ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White et al., 1990). The total volume of PCR mixtures was 20 µl including 2 µl of 10x buffer, 2 µl of dNTPs, 1 µl of each primer, 2 µl of DNA template (50–100 ng), 0.3 µl Taq DNA polymerase and the remaining volume amount of double-distilled water. A thermal cycler was used for the PCR amplification with conditions as follows: Initiation for 5 min at 95 °C followed by 11 cycles included denaturation for 10 s at 94 °C, annealing for 10 s at 65 °C, extension for 30 s at 72 °C followed by 30 cycles included denaturation for 10 s at 94 °C, annealing for 10 s at 55 °C, extension for 30 s at 72 °C and then, a final extension cycle at 72 °C for 5 min.

2.4 Sequencing and phylogenetic analysis

Sequencing of amplified PCR products was carried out at Eurofins Genomics Company (Germany). As with the PCR amplification, the same primers were employed for sequencing. Data acquired from the nucleotide sequence was deposited in GenBank. A BLAST analysis was performed at the NCBI (http://ncbi.nlm.nih.gov/BLAST) to get homologous sequences. MEGA11 (Molecular Evolutionary Genetics Analysis Version 11) software was used for constructing the phylogenetic tree from the isolate of the current study and the other isolates which were obtained from NCBI database.

2.5 Greenhouse Experiments

2.5.1 Fungal inoculum preparation

Inoculum of *E. heraclei* was gained from dill plants showing symptoms of powdery mildew from Abshaway, Fayoum, Egypt and conserved on healthy dill plants in the greenhouse for further investigation. Young, virulent conidia were used for the inoculation, which formed within 24 h after removing the old conidia from the leaves by agitating. Inoculation was carried out by gently dusting powdery mildew infected plants above the healthy plants. Inoculated plants were then covered with plastic bags, kept at 25±2°C for 24 hours with enough moisture, and then checked daily for disease progress.

2.5.2 The efficiency of some alternative control methods for controlling dill powdery mildew under greenhouse conditions

All experiments were carried out in the greenhouse of Ornamental, Medicinal and Aromatic Plants Diseases Research Department, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt, at 25±2°C and 60-70% R.H. Seeds of dill (*Anethum graveolens* L.) were kindly obtained from Medicinal and Aromatic Plants Department, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt. Plastic pots (25 cm in diameter) containing sandy loam soil were planted with ten seeds. Essential oil emulsions of thyme or clove (prepared at Ornamental, Medicinal and Aromatic Plants Diseases Research Department, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt**)** at a rate of 2 ml/l (Hassanin et al., 2017), ascorbic or salicylic acids [Sigma Company] at a rate of 0.5 g/l (Halawa et al., 2018) and Bio-Cure F 1.15% WP (*Trichoderma viride* 1 × 10^6 cfu/g, M/S.T. Stanes Company Limit-India) at a rate of 6 g/l (Hassanin et al., 2020) were examined in this experiment. Plants were sprayed with the tested treatments three times after inoculation with the conidial spores of the causal fungus. The first spray was given 48 h. after inoculation, second and third sprays were given 7 days intervals. Plants were sprayed with water served as an untreated control. For each treatment or control, three replicates were employed. The percentages of disease severity and incidence were calculated 7 days after every spray according to the formulas mentioned before. Additionally, the height of the plants and their number of branches were under investigation, and the percentage of increases in these parameters were calculated as follows:

$$\text{Percentage of increase (\%)} = [(A - B) / B] \times 100,$$

Where, A = Value of treatment and B = Value of control.

2.6 Statistical analysis

In this factorial experiment, three replicates were used in an overall random design according to Snedecor and Cochran (1989). The L.S.D. test was used at 0.05 for the statistical analysis using MS-TATEC software.

3. Results

3.1 Disease symptoms of dill powdery mildew

Symptoms of powdery mildew were observed on dill plants in several fields of Fayoum governorate during the 2022 growing season in February and March. Symptoms began as white powdery areas on the lower parts of plant, became combined and increase in size to cover the stems and all leaves (Figure 1).

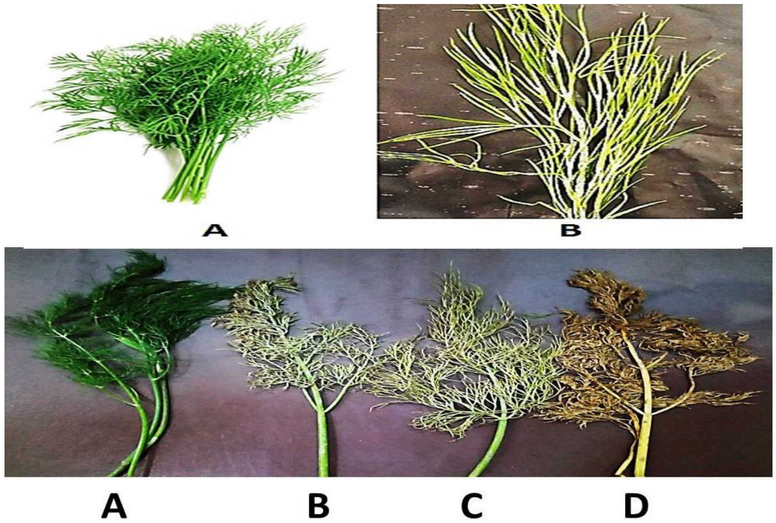

Figure 1: Symptoms of dill powdery mildew. A is a healthy plant. B, C and D are naturally infected plants.

3.2 Disease survey

A disease survey was conducted in February and March 2022 to investigate the spread of powdery mildew in dill. The greatest percentages of disease severity and incidence were observed in March in all survey regions. Youssef El-Seddik and Etsa had the greatest average percentages of disease incidence during February and March (85.9% and 81.5%, respectively), as well as the highest mean percentages of disease severity for the season (65.5% and 59.3%, respectively). Sinnoures had the lowest incidence of disease (60.5%), while Fayoum had the lowest severity of disease (40.2%). The mean disease incidence in Fayoum governorate was 74.0%, and the mean disease severity was 50.6% (Table 1).

3.3 Identification of the causal pathogen of powdery mildew using microscopic examination

In symptomatic dill plants, *E. heraclei* was examined via microscopy. *Erysiphe heraclei* conidiophores were upright, short, unbranched, and cylindrical to oval, and varied in length from 25 to 38 µm and width from 9 to 13 µm (Figure 2). Cylindrical foot cells measuring (7.50- 9 × 21-26 µm) followed by one or two cells that are somewhat smaller (7-8.50 × 13-15.50 µm). Conidia were created individually and ranged in shape from cylindrical to oblong elliptical, measuring 27 to 42 µm in length and 12 to 16 µm in width, with an average of 34.50 × 14 µm. It had no fibrosin bodies and formed a lobed appressorium (Polygoni type) at the extremities of their germ tubes, which might be extremely short or very long. Cleistothecia measures were 108-122 (115 µm) × 85-114 (99.50 µm), which appeared as dark brown to black spots on leaves, inflorescences and stems with a circular shape. Appendages were brown to black with forked tips and measured 87-225 (156) µm in length and 3-5 (4) µm in width.

Table 1: Incidence and severity of dill powdery mildew in Fayoum governorate, Egypt, during the growing season 2022.

Regions surveyed	Disease Incidence (%)		Mean	Disease Severity (%)		Mean
	February	March		February	March	
Tamiya	62.7	81.3	72.0	31.3	65.9	48.6
Abshaway	69.7	88.0	78.9	31.4	67.7	49.6
Etsa	73.0	90.0	81.5	44.6	74.0	59.3
Sinnoures	55.0	66.0	60.5	25.2	55.4	40.3
Youssef El-Seddik	76.7	95.0	85.9	53.9	77.0	65.5
El-Fayoum	58.0	71.7	64.9	27.6	52.7	40.2
Mean	65.9	82.0	74.0	35.7	65.5	50.6
L.S.D. at 5%	1.7	0.9	-	1.4	1.0	-

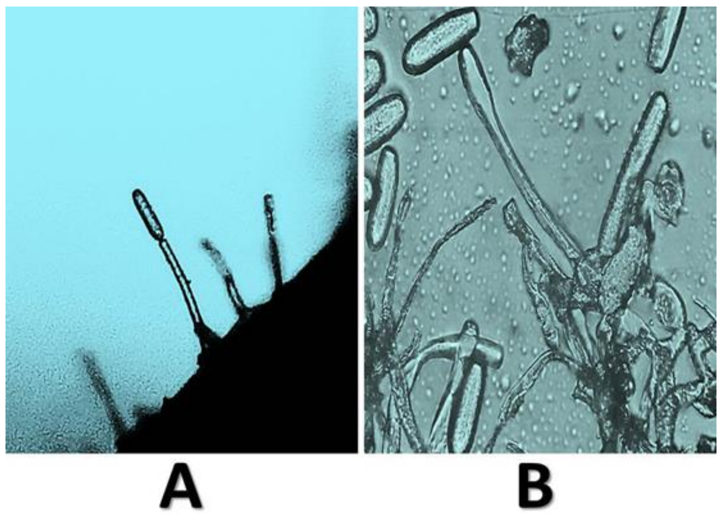

Figure 2: Conidia and conidiophores of *Erysiphe heraclei*. A is a conidium carried on conidiophore emerging from dill leaf. B is mycelium and conidia of *E. heraclei*.

3.4 Molecular Characterization and phylogenetic tree

After PCR amplification of DNA extracted from fungal conidia, the PCR product (621 bp) was sequenced and submitted in GenBank with accession number (OP999071.1). The isolate sequence of the current study showed 98-100% sequence homology with sequences of *E. heraclei* isolates obtained from GenBank database with accession numbers; (KR269918.1), (KP055630.1), (OM856023.1), (AB104513.1), (OM856020.1), (LC270862.1), (KY073878.1), (MT703849.1), (AB000942.1) and (LC009917.1), after being analyzed with BLAST. The phylogenetic tree, as shown in Figure (3), was then constructed using MEGA 11 software.

3.5 Efficacy of alternative control methods for dill powdery mildew in greenhouse environments

Table (2) shows that a higher reduction in incidence and severity of disease (%) compared with the control was obtained from Bio-Cure F treatment (90 and 95.1%, respectively), followed by thyme essential oil emulsion (82.5 and 87.4 %, respectively). On contrast, treatment with ascorbic acid was the least successful in decreasing percentages of incidence and severity of disease (67.5 and 72.7%, respectively).

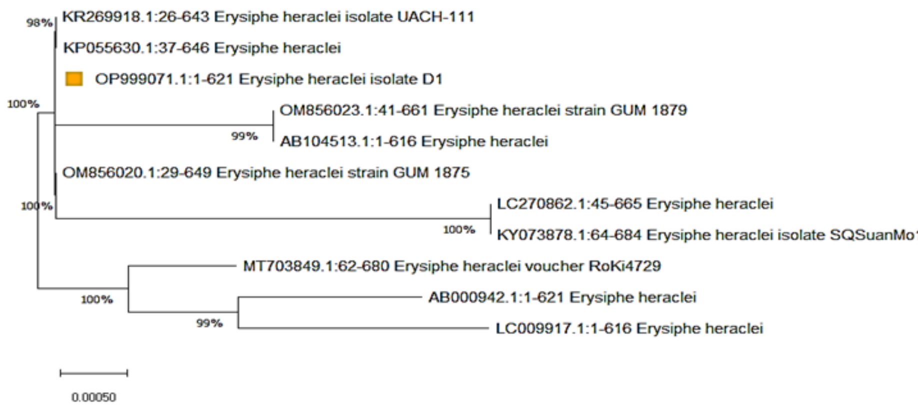

Figure 3: A phylogenetic tree generated depending on the sequences of ITS rDNA regions of *E. heraclei* isolate from the current study and other isolates obtained from NCBI database. The tree was constructed through MEGA11 software.

Table 2: Impact of tested treatments on dill powdery mildew incidence and severity in greenhouse through the 2022/2023 cultivation season.

Treatments	Disease Incidence (%)	Reduction (%)	Disease Severity (%)	Reduction (%)
Bio-cur F	10	90	2.8	95.1
Thyme essential oil emulsion	17.5	82.5	7.2	87.4
Clove essential oil emulsion	20	80	8.3	85.5
Salicylic acid	25	75	12.6	77.9
Ascorbic acid	32.5	67.5	15.6	72.7
Control	100	-	57.1	-
L.S.D. at 5%		-	1. 84	-

As shown in Table (3), Bio-Cure F treatment resulted in the greatest increase in dill plant height (60.54%) and the number of branches (121.09%), in comparison with the control. Salicylic acid application followed, with increases of 46.26% and 94.79%, respectively. Treatment with clove oil emulsion resulted in the lowest increase in plant height (30.72%), while ascorbic acid treatment resulted in the lowest increase in number of branches per plant (52.61%).

Table 3: Impact of tested treatments on dill plant height and branching in greenhouse through the 2022/2023 cultivation season.

Treatments	Plant height (cm)	Increase (%)	Number of branches/Plant	Increase (%)
Bio-cur F	86.16	60.54	9.33	121.09
Thyme oil emulsion	77.16	43.77	8.00	89.57
Clove oil emulsion	70.16	30.72	7.22	71.09
Salicylic acid	78.50	46.26	8.22	94.79
Ascorbic acid	73.22	36.43	6.44	52.61
Control	53.67		4.22	
L.S.D. at 5%	2.86		0.80	

4. Discussion

In the present study, morphology and molecular analysis were used to identify the powdery mildew causal agent, and the efficacy of some fungicidal alternatives was also evaluated against powdery mildew of dill in addition to their impacts on plant growth measurements under greenhouse conditions. Infected dill plants with powdery mildew were obtained during the survey of disease in February 2022. The onset of the symptoms began at vegetative stages, which progressively increased through pre-maturity and fruiting stages. These findings are in agreement with those reported by Soylu and Soylu (2003), Cho et al. (2012) and Bubici (2015). The survey trials which were done in different regions of Fayoum governorate during February and March indicated that, powdery mildew in March recorded the highest mean disease incidence and severity compared with February, particularly in Youssef El-Seddik and Etsa. These findings highlighted that, the appearance and severity of powdery mildew infection on dill were greatly impacted by climatic changes as relative humidity and temperature in 2022. According to Kolte (1985), cool temperatures and low relative humidity through crop development favored inoculum accumulation, resulting in severe epidemics of powdery mildew on sunflowers. A study by Aust and Hoyningen-Huene (1986) found that powdery mildew is more likely to become severe on crops when the nights are cool, and the weather is dry. Based on the characteristics of the anamorph and teleomorph phases, via light microscope, *E. heraclei* was identified as the causal agent of dill powdery mildew. These results are in accordance with those mentioned by El-Sayed Ziedan (2010), Cho et al. (2012) and Bubici (2015). Braun (1995) found that the morphological characterization of powdery mildews was mostly dependent on teleomorphs, like the number of asci and the shape of the appendages on the cleistotheium. Additionally, more than twelve morphological features, including conidia, conidiophores, houstoria, appressoria, fibrosin bodies, and mycelium, are present in the powdery mildew's anamorphic stage (Boesewinkel, 1980). Cunnington et al. (2003) reported that using of molecular characterization, particularly the ITS region, offers encouraging results for identifying the species of several powdery mildews. The obtained results revealed that the isolate of the current study (Accession No. OP999071.1) showed 98-100% similarity with accession numbers of *E. heraclei* isolates obtained from GenBank database; (KR269918.1), (KP055630.1), (OM856023.1), (AB104513.1), (OM856020.1), (LC270862.1), (KY073878.1), (MT703849.1), (AB000942.1) and (LC009917.1). Under greenhouse condition, dill plants were sprayed with clove or thyme essential oils emulsions, elicitors: ascorbic or salicylic acids and biocide: Bio-Cure F, three times after inoculation with *E. heraclei*. The obtained results showed that the higher decrease in disease severity and incidence percentages was achieved by using Bio-Cure F, followed by thyme essential oil. The ability of the biocide to effectively reduce disease incidence and severity in dill plants may be attributed to the production of growth regulators that encourage the plant to tolerate infection (Naglot et al., 2015; Kumar et al., 2012). Pokhrel et al. (2022) mentioned that using of *Trichoderma* spp. or its released metabolites make it as biological control agents to manage plant diseases caused by pathogenic fungi. *Trichoderma* spp. are bioagents that attack plant pathogens through some mechanisms, including an effective enzymatic system, the production of antibiotics, and competition for nutrients (Shoresh et al., 2010). *Trichoderma* spp. were capable of producing enzymes that lysed cell walls as well as secondary metabolites that were effective against plant pathogens, as mentioned by Rahman et al. (2009). Zimand et al. (1996) reported that *Trichoderma* spp. suppresses some fungal enzymes like pectinases, which are essential for the fungus to penetrate the leaf surface. The effect of thyme essential oil emulsion against powdery mildew on dill plants maybe as a result of some active ingredients in the oil that have an antifungal impact. The antifungal properties of thyme essential oil are due to the presence of Thymol, p-cymene and borneol as reported by Hammad and Hassanin (2022). Hassanin et al. (2017**)** reported that because thyme volatile oil emulsion contains active ingredients, such as thymol, p-cymene and Borneol, it has the best antifungal impact at the lowest concentrations. Ni et al. (2021) mentioned that thyme essential oil can damage proteins of the cell membrane and genetic material by inhibiting gene expression, as well as damage cell walls. Also, cellular defenses such as peroxidase activity can be stimulated indirectly against pathogens by the bioactive compounds found in thyme essential oil, as reported by Jamiołkowska (2020). Also, the results of present work showed that the highest percentages of increasing in dill plant height (cm) as well as branches/plant were obtained by Bio-cur F treatment compared with the control, followed by the treatment of salicylic acid. When a plant becomes infected with a pathogen, an induced plant resistance or acquired resistance is created. This resistance can protect the plant against further infections and has a positive impact on the plant growth. These findings are in accordance with those reported by Abdel-Kader et al. (2013) and Mergawy (2016). Spletzer and Enyedi (1999**)** reported that the addition of 200 mM salicylic acid to tomato plants could induce the expression of the pathogenesis-related IB gene and stimulate systemic acquired resistance. Salicylic acid is a compound generated from plants and related to plant defense towards pathogens through the activation of systematic acquired resistance (Ryals et al., 1994) and stimulation of antioxidant enzymes in plants (Janda et al., 1999). According to Hamada and Hashem (2003), wheat grains soaked in salicylic acid before planting resulted in significant decreases in the mean disease values that were caused by *Fusarium oxysporum*, *F. oxysporum*, or *Bipolaris sorokiniana*. Plant growth promotion and induction of resistance to plant pathogens were also reported as benefits of salicylic acid (Vidhyasekaran, 1990; Nickell, 1983).

5. Conclusion

During the 2022 season, *Erysiphe heraclei* was characterized as the causal agent of powdery mildew in dill plants obtained from Fayoum governorate, through morphological and molecular characterization. The efficacy of several fungicidal alternatives, including Bio-Cure F (*Trichoderma viride*), essential oil emusions (clove and thyme), ascorbic and salicylic acid, was evaluated against the powdery mildew of dill. The results showed that Bio-Cur F, emulsion of thyme volatile oil, and salicylic acid were the most effective applications for managing dill powdery mildew and improving plant growth under greenhouse conditions. These findings suggest that these alternatives have the potential to be used as fungicides.

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