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ISOLATION, IDENTIFICATION, AND EVALUATION OF SOME FUNGICIDES FOR CONTROLLING PURPLE BLOTCH OF ONION AND GARLIC IN MINIA GOVERNORATE, EGYPT

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ABSTRACT

Onion and garlic are the oldest vegetables plant species of the Genus *Allium.* Purple blotch and Stemphylium blight of onion and garlic are important foliar diseases which are induced by the fungi *Alternaria porri* and *Stemphylium versicarium.* The survey was conducted in major onion and garlic cultivation villages of three districts in Minia Governorate during winter of 2020 – 2021 season. The survey revealed prevalence of purple blotch and Stemphylium blight in all locations under study. Direct microscopic examination of infected samples proved that either *Alternaria porii, Stemphylium* sp. or both together associated with the purple blotch disease lesions. Thirty-one isolates of *Stemphylium* sp. and *Alternaria porri* were obtained and the identification of the most aggressive isolates of *Alternaria* and *Stemphylium;* OAp1and OSv5, respectively, were performed using PCR technique. The pathogenicity test indicated that all tested isolates of both pathogens (*S. vesicarium* and *A. porri)* infected onion and garlic leaves causing different degrees of purple blotch and blight symptoms. The evaluation of nine fungicides against the growth of isolates OAP1 and OSV5 of *Alternaria porii, Stemphylium vesicarium,* respectively, under laboratory experiment revealed that the growth of *S. vesicarium* was completely inhibited at 200 ppm Ridomil Gold Plus, while Dithane M-45 and Pronto completely inhibited the growth of *A. porri* and *S. vesicarium* at 400 ppm.

Key words *:* Onion, garlic, *Alternaria porri, Stemphylium versicarium,* fungicides,

INTRODUCTION

Both the onion (*Allium cepa* L.) and the garlic (*Allium sativum* L.) are important vegetable bulb crops that are members of the *Alliaceae* family's subfamily *Allioideae* (order *Asparagales*) **(A.P.G. 2003).** In at least 175 nations, onions have been grown for approximately 5000 years. According to the Papyrus Ebers, which is based on ancient Egyptian texts and knowledge, the spherical bulb was viewed by the ancient Egyptians as a representation of the universe. Leek played a significant part in the ancient Egyptian civilizations, according to **Pareek *et al.* (2017).** Onions are often kept as annuals and harvested during their first growing season, despite the fact that they are typically biannual or perennial plants.

Other species of the *Allium* genus, including the Egyptian onion (*Allium proliferum*), Canada onion (*Allium canadense*), and the Japanese bunching onion (*A. fistulosum*), are also grown for food **(Fritsch and Nikolai, 2002).** The Chinese onion, garlic, scallion, leek, and chive are among its near relatives **(Block, 2010).** An *Allium* species of bulbous flowering plant, garlic belongs to the same family as onions. Its near cousins include the Welsh onion, Chinese onion, shallot, onion, and leek (**Block, 2010).** Welsh onion and Chinese onion **(Annon. 2010).** It has a long history of human consumption and use, dating back several thousand years, and is a native of Central Asia and northeastern Iran (**Vavilov, 1951, McNeal *et al*., 2002 and Block, 2010).** Due to its strong flavour, garlic is frequently used as a flavoring or condiment around the world. It was also employed as a food flavoring as a traditional remedy by the ancient Egyptians (**Annon.; 2018 and Annon. 2016**).

Due to their low calories content and presence of several vitamins, minerals, and potent plant chemicals that have been demonstrated to support health in a variety of ways, onions and garlic may have numerous favorable benefits on a number of different areas of health. Onions have adequate levels of proteins, carbohydrates, and vitamins C and B complex (including B9, folate, and B6, pyridoxine), all of which are crucial for the production of red blood cells, human metabolism, and nerve function. They also have a distinctively pungent flavor and some therapeutic benefits.

An excellent source of antioxidants is onions. In actuality, they include more than 25 different types of flavonoid antioxidants (**Slimestad *et al.*, 2007**). The distinct flavonoid plant pigments known as anthocyanins are what give red onions, in particular, their vibrant colour. Heart disease is less likely in people who consume anthocyanins. The nine most impressive health advantages of onions are: packed in nutrients, good for the heart, and full of antioxidants. Contain cancer-fighting compounds, aid in blood sugar regulation, and possibly increase bone density. possess antimicrobial qualities and may improve digestive health. Garlic has no discernible nutritional value in a normal serving size of 1-3 cloves (3-9 g), with the amount of all key nutrients being less than 10% of the Daily Value (DV) **(Annon., 2014**), including vitamins B6, thiamin, and folate, vitamin C, and the dietary

minerals manganese, iron, calcium, phosphorus and zinc.

It has been determined that a number of variables contribute to the low productivity of onions worldwide. Diseases like purple blotch, downy mildew, Stemphylium blight, base rot, and storage rots, as well as the lack of cultivars resistant to biotic and abiotic pressures, are the main causes. **Schwartz and Mohan (2008)** described more than sixty diseases that affect onion and garlic plants at various stages of their lives. Of these, 40 were fungi-related, 14 were bacterial-related, one was yeast-related, six were caused by nematodes, three were viruses, and one was caused by phytoplasma-like organisms.

In addition to the biggest yearly production and storage losses, the majority of fungal infections are significant throughout the world's onion and garlic-producing regions and can result in significant crop losses.

Alternaria porri and *Stemphylium versicarium* can infect onion leaves causing purple leaf blotch (PLB) and Stemphylium leaf blight (SLB). The PLB is thought to be a complex produced by both pathogens because the symptoms are similar to those of Stemphylium leaf blight, which is brought on by *S. vesicarium* (Wallr.) Simmons **(Suheri & Price, 2000).** However, these diseases affect seed crops more severely than bulb crops **(Gupta & Pathak, 1988 and Tomaz & Lima, 1988).** often resulting in a 100% loss of seed yield **(Schwartz, 2004).** *Allium* spp. are regarded as having major diseases, particularly in warm, humid settings, such as purple leaf blotch and Stemphylium blight of onion and garlic **(Maude, 1990 and Miller & Lacy, 1995).** which causing up to 60% damage on garlic in India **(Bisht & Agrawal, 1993).** and 59% losses in onion bulb yield **(Gupta & Pathak, 1988).**

This investigation aimed to isolate, identify the pathogen(s) associated with onion and garlic leaf blotch and blight symptoms, and to study the effectiveness of some commercial fungicides on the growth of the isolated pathogens under laboratory conditions.

MATERIALS AND METHODS

1- Survey for the Incidence and severity of onion and garlic purple blotch and Stemphylium blight in Minia Governorate, Egypt.

Onion and garlic purple blotch and Stemphylium blight survey was carried out in three districts belonging to Minia Governorate; Al-Edwa (in Northern West; 28^{o} 42’43” N 30^{o} 45’0’E High 40 m), Beni Mazar (in the North, 28°30′N 30°48′E, Hight 43 m) and Abou Qurqas (in the Southern of the Governorate, 27°55′51″N 30°50′11″E, Hight 54 m). These districts are scattered in different geographical locations and climatic conditions. Survey was conducted during the period between November 2020 and February 2021, in major onion and garlic growing villages, two villages were chosen from each district (Table 3). Based on the information, in each village, two onion fields and two garlic fields were surveyed at random. In each field, 100 plants were selected at five locations, four corners of the field and one at the center to record the percentages of purple

blotch and plants suffered from blight incidence and severity. Per cent of disease incidence was calculated by using the following formula:

$$\text{Per cent disease Incidence (PDI)} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

The disease index, as described by **Islam *et al.* (2020)** was used to classify the disease severity in this study. However, the leaves (200 or 175 leaves/ of onion or garlic, respectively/ plot) infection was rated on a scale of 0 to 5 categories as follows:

0 = no infection (leaves are completely healthy), 1= a few white spots towards the tip covering less than 10% of leaf area, 2 = several dark purplish brown patches covering up to 20% of leaf area, 3 = several patches with paler outer zone covering up to 40% of leaf area, 4 = Leaf streaks covering up to 75% of the leaf area, and leaf breaking from the centre, and 5= Leaf breaking from the base, and leaf drying completely. Disease severity index (DSI, %) of purple blotch or Stemphylium blight was estimated using the following formula (**Liu *et al.*, (1995):**

$$\text{Disease severity index (\%)} = \frac{\Sigma\,(n \times v)}{ZN} \times 100$$

Where:

n = Number of leaves in each category, v = Numerical value of each category, z = Numerical value of highest category, and N = Total number of leaves in the sample.

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2- Collection of diseased leaf samples

Natural infected leaves and floral stalks samples of onion and garlic showing typical symptoms of purple blotch and Stemphylium blight diseases were collected from commercial farms in previous mentioned districts for isolation and identification the associated pathogen(s).

3- Isolation and identification of the pathogen(s):

The diseased leaves were detached from the plants grown in the farmer's field using sterilized scissors and put into brown paper envelopes and taken to the laboratory, Department of Plant Pathology, Faculty of Agricultural, Minia University, for isolation of the pathogen (s).

The pathogen linked to the purple blotch disease lesions was found through direct investigation. A portion of the infected lesion was placed on a slide, gently cleaned with sterile distilled water, two drops of clear lactophenol were applied, and the lesion was then viewed under a microscope to observe the conidia and conidiophore of the causal agent (**Suheri *et al.*, 1997).**

The detached leaves were washed with tap water, then surface sterilized by immersing it in aqueous sodium hypochlorite (5%) for 5 min and 70% ethanol for 0.5 min, thrice rinsed with sterile distilled water. Infected lesions showing typical purple blotch and Stemphylium blight symptoms were cut into small pieces (about 1-1.5 cm^2 each). Five pieces were aseptically deposited on a Petri dish (9 cm diameter) containing 20 ml Potato Dextrose Agar (PDA) medium **(Suheri & Price, 2000).** Three replicates were used for each sample. The plates were incubated in the dark at 20±2 °C for 5–6 days. The emerged hypha was purified using single spore and hyphal tip techniques, by inoculating in Petri dishes containing PDA medium.

Identification of the growing fungal isolates of the pathogens was carried out based on the macro- and microscopic characteristics **(Ellis, 1971).** Small bits of the emerged hypha were taken and slide will be prepared by lactophenol. The slide was examined under a microscope for identification of the pathogens. The identification of the most two aggressive isolates was performed using PCR techniques.

Molecular identification of fungal isolates:

Two isolates of fungi, *Alternaria porri* (OAp1) and *Stemphylium vesicarium* (OSv5), which showed high onion and garlic purple blotch virulence values, were chosen for molecular identification upon their high virulence. The fungal isolates were grown in sterile Petri plates containing autoclaved potato dextrose agar (PDA) medium and incubated for 7 days at 22 ±2°C (**Pitt and Hocking, 2009**)**.** The cultures were sent to the Microbial Molecular Biology Lab., Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, Egypt, for DNA extraction and to complete the steps of identification as follows:

5-1- DNA preparation

Genomic DNA was extracted from pure cultures of the fungus isolates using a DNeasy plant extraction kit (Qiagen, CA, USA) according to the manufacturer's instructions.

1. PCR Reactions:

The PCR amplification was performed in a total volume of 50 ul, containing 25µl Master Mix, 2µl primer F and 2µl primer R (10pcmol each all primers), 3 µl template DNA (10ng) and 15 µl dH_2O, according to **(White *et al*., 1990) (Table 1).**

2. Thermo-cycling PCR program

PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 35 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 1 min, an annealing step at 55ºC for 1 min, and an elongation step at 72ºC for 1,30 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle.

3. Detection of the PCR Products

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts. A 100bp DNA ladder was used as a molecular size standard. PCR products were visualized on UV light and photographed using a Gel Documentation System **(BIO-RAD 2000).**

4. Purification of PCR Products

Amplified products for all PCR were purified using EZ-10 spin column PCR products purification PCR reaction mixture was transferred to 1.5 ml microfuge tube and three volumes was added of binding buffer 1 after that the mixture solution was transferred to the EZ-10 column and let it stand at room temperature for 2 minutes after that centrifuge, 750 ul of wash solution was added to the column and centrifuge at 10.000rpm for two minutes, repeated washing, 10.000 rpm was spine for an additional minute to remove any residual

wash solution. The column was transferred into a clean 1.5 ml microfuge tube and added 50 ul of elution buffer, incubated at room temperature for 2 minutes and when store purified DNA at -20 °C.

5. ITS sequencing analysis

The sequencing of the product PCR was carried out in an automatic sequencer ABI PRISM 3730XL Analyzer using Big Dye TM Terminator Cycle Sequencing Kits following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using Rbcl Forward primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were Resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Microgen Company).

5-1- Computational analysis (BLASTn) ITS.

The sequences were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) Sequences were aligned using Align Sequences Nucleotide BLAST (Figure 1).

The purely identified cultures of the isolated fungi were transferred to PDA slants and kept in a refrigerator at 4 °C for further studies.

4- Pathogenicity test and disease assessment:

Pathogenicity tests of all isolates (21 isolates of *Stemphylium* sp. and 10 isolates of *A. porri*) were carried out during 2020/2021 winter growing season under greenhouse conditions in experimental field of Plant Pathology Department, Faculty of Agriculture, Minia University, Egypt.

Surface disinfected onion sets (Giza 6 white cv.) and garlic cloves (Lady Ba cv.), purchased from commercial farms, were sown in sterilized clay pots (30 cm in diameter) containing autoclaved Nile clay (about 4 Kg/pot) soil. Sterilization of Pots and soil was carried out (two weeks before sowing) by dipping the pots in formalin solution (5%) for about 5 minutes then aerated for 15 days before being used. Soil was autoclaved at 121^{o}C for two hours, then aerated for 15 days before being sowed at 1st December, 2020. Surface seed (sets of onion or cloves of garlic) sterilization was performed through dipping in sodium hypochlorite solution (2%) for 2 minutes followed by washing in several changes of sterilized water.

Five onion sets or garlic cloves were sown in each pot and immediately after emergence, seedlings were thinned to three/pot. Plants were inoculated thirty days later. Three pots were used for each isolate.

The tested isolates' inocula were made by letting the isolates grow for 10 days at 25°C on PDA medium in Petri dishes, 9 cm diameter. The mycelial growth was carefully scraped off of each plate at the conclusion of the incubation period using a sterile needle. The ensuing conidial suspension from each isolate was then used for infection. Tween-80 (2 -3 drops) were supplemented to the suspension as a dispersion agent. Two completely grown

leaves in physiological maturity stage of each plant, were chosen in random, were inoculated, 30 days after sowing, by spraying the conidial suspension ($3x10^6$ conidia/ml). Plants were covered with polyethylene bags for 48h. to maintain high humidity, then the bags were removed, and the plants were kept in normal condition. Disease incidence (DI) and disease severity (DS) percentages were recorded 15 days after inoculation **(Hussein *et al*. 2007).**

Disease assessments:

Twenty days after inoculation, the disease incidence (DI, %) and disease severity (DS, %) were determined as described before in survey experiment.

Depending on the results of pathogenicity test, isolates OAp1 and OSv5 of *Alternaria porri* and *Stemphylium vesicarium* respectively, were PCR identified as described before.

Re-isolation from the artificially diseased leaves and pods was carried out and the resultant fungi were compared with the original cultures.

3- Evaluation of some fungicides against *A. porri* and *S. vesicarium*, producing onion purple blotch, *in vitro*

This experiment was conducted during January 2022 at the Laboratory of Plant Pathology Department of the Faculty of Agriculture, Minia University. The study was designed in a completely randomized design with nine compounds and four replications. Amistar Top (29.6%), Azoxystrobin (23% SC), Collis (30% SC), Dithane M-45 (80%WP), Luna Experience 40% SC, Miracle 10% EC., Myclobutanil, Pronto (32% SC), and Ridomil Gold Plus Gold (68%WG) (Table 2) were evaluated for their effects against isolates OAp1 and OSv5 of *A. porri* and *S. vesicarium* respectively, *in vitro* using poisoned food technique (**Dahal and Shrestha, 2018).** Seven concentrations, 0, 50, 100, 200, 400, 600 and 1000 ppm, of each fungicide were tested. The fungicides were prepared in a previously calculated volume of autoclaved Czapek-Dox agar medium. The tested concentrations were added to the medium directly before solidifying and poured into the plates for measuring their fungicidal value through the inhibition of fungal mycelial growth. All plates were incubated at 25±2°C. The inhibitory effect of tested fungicides was estimated by measuring the linear growth obtained on treated and untreated media. The rate of inhibition (%) was calculated using the following formula:

$$\text{Growth inhibition (\%)} = \frac{\text{Growth in control} - \text{growth in treatment}}{\text{Growth in control}} \times 100$$

Statistical analysis:

All experiments were set up in a complete randomized design. Two-way ANOVA was used to analyze differences between antagonistic inhibitor effect and linear growth of pathogenic fungi *in vitro.* Data of all experiments were analyzed by analysis of variance (ANOVA) using the General Linear Models procedure of CoStat. Significance between means was tested by "F" test and the value of LSD (p=0.05) was calculated **(Winer, 1971).**

RESULTS

1. Survey for the Incidence and severity of purple blotch and Stemphylium blight of onion and garlic in Minia Governorate, Egypt.

A roving survey was carried out for recording the incidence and severity of purple blotch and Stemphylium blight disease of onion and garlic during winter of 2020 – 2021 season in three major onion growing districts of Northern, middle and southern Minia Governorate, *viz.*, Al-Edwa (El-Misid and El-Atef), Beni Mazar (Sholkam and El-Grnous) and Abou Qurqas (Nazlet- Asmant and Com al- Zuhair). Plants were at physiological maturity stage of the growth and the data pertaining to survey work is presented in Table 3.

The survey revealed that prevalence of purple blotch and Stemphylium blight in all locations under study and disease severity ranged from 56.72-8.28 on onion and between 55.53 and 7.06 on garlic per cent disease incidence and disease index (PDI) were 30.64 and 1.85% on onion and 39.12 and1.81% on garlic in different villages of the districts surveyed. The highest severity (30.64 and 39.12 PDS on onion and garlic, respectively) of purple blotch and Stemphylium blight were noticed in fields of El-Atef village in Al-Edwa district whereas the least severity (1.85 and 1.81% DS) of the disease was recorded at Com al- Zuhair village in Abou Qurqas district. The maximum average severity was on Giza 6 (30.64%) and on Giza red (5.70%) of onion cvs. and on Garlic China (55.53%) and on Lady By (7.37%) garlic cvs. The highest disease index per cent was recorded in El-Edwa district (30.07% and 29.68% on onion and garlic, respectively, followed by Beni Mazar (17.91 and 19.73% on onion and garlic, PDI). The lowest disease severity of 10.66 -2.33 on onion and 12.04- 1,97 per cent disease index on garlic was recorded in Abou Qurqas district (Table 2).

The Purple blotch of onion and garlic was severe in Al-Edwa district compared to Abou Qurqas district. This could be because of favorable environmental conditions and initial inoculum prevailed in this Al-Edwa might have helped in the rapid development of the disease in winter.

4- Isolation, purification and identification of PLB and Stemphylium blight pathogens

Samples of naturally infected leaves of onion and garlic showing typical symptoms of purple blotches and Stemphylium blight were collected from different areas in Minia Governorate were used to isolate the associated pathogen(s).

Direct microscopic examination of infected samples proved that either *Alternaria porii, Stemphylium* sp. or both together associated with the purple blotch disease lesions.

Twenty-one fungal isolates of *Stemphylium* sp. as well as ten isolates of *Alternaria porri* (Table 4) were purified and identified macroscopically and light microscopy depending on their morphological and cultural characters.

Pathogenicity tests

Pathogenicity tests of 21 isolates of *S. vesicarium* and ten isolates of *A. porri* were carried out using Giza 20 onion cultivar and Lady Ba garlic cv. under greenhouse conditions. Data pointed to the isolates OSV1- OSV 15 and isolates GSV1 – GSV6 of *S. vesicarium* were isolated from infected onion and garlic, respectively, whereas isolates OAP1- OAP7and isolates GAP1- GAP3 of *A. porri* were isolated from infected onion and garlic, respectively. Results in **Table 3** indicated that all tested isolates of both pathogens (*S. vesicarium* and *A. porri)* infected onion and garlic leaves causing blight and purple blotch symptoms at different degrees. Both isolates of *A. porri* OAP1and OAP2 exhibited the highest virulence degrees on onion at the rate of 100 and 94.4% (DI%) and 75.56 and 61.11% DS, respectively. Isolates GAP12 and GAP1 caused the highest percentages of disease incidence (72.22 and 66.67) and disease severity (44.44 and 42.44) on garlic. While isolates OSV5, OSV2, OSV6, OSV11, and OSV15 of *S. vesicarium* isolated from onion diseased leaves caused between 50 and 44% of disease incidence and 28.89 and 25.56% disease severity on onion and isolates GSV2, GSV1 caused 33.33%DI and 13.33 and 11.11% Ds, respectively. Data showed that *Alternaria porii* were highly aggressive than *Stemphyllim* isolates which came in the second rank of moderate virulence.

Data also showed three isolates OAP1, OAP2 and OAP3 of *Alternaria* were very high aggressive on onion, causing 100-77.76% DI and 75-55% DS, following by four isolates, OAP5, OAP6, GAP1and GAP3 which were moderate inducing 50- 30% Di and 28.89 -20.0 Ds%, whereas isolates OAP4, OAP7 and GAP2 were the lowest aggressive ones inducing between 27.78 and 16.67% DI and 17,78 – 6.67 DS%. Fourteen isolates of *Stemphylium* showed moderate disease incidence (between 50 -30%) and disease severity (between 28.89 and 15.56%). The remaining isolates (7 isolates) of *Stemphylium* were low aggressive inducing 27.78 – 16.67 DI% and 13.33 - 6.67% DS%. On garlic, isolates of *Alternaria* GAP1, GAP2, OAP1 and OAP5 causing 72.22 – 50% DI and 44.44 – 28.89% DS, whereas isolates GAP3, OAP3, OAP4 and OAP5 considered moderate causing 28.89% - 27.78, while isolates OAP6 and OAP7 were the lowest aggressive ones (causing less than 10% DS%),

The BLAST results showed that:

Sequence alignments of the fungal isolate no 1 (OAp1): Showed identities 98.91 with *Alternaria porri* isolates (Fig. 2). Our isolate registered under accession no. SUB12232226 *Alternaria* "OP740798"

Sequence alignments of the fungal isolate no 2 (OSv5): Showed identities 100% with *Stemphylium vesicarium* isolate UKPg (GenBank accession No. MN328404.1). Our isolate (OSv5) registered under accession no. SUB12232298 Stemphylium OP745414 as *Stemphylium vesicarium* (Fig. 3).

Sample _1 (isolate OAp1):

GAGTGTAGCTTTGCCTGCTATCTCT
TACCCATGTCTTTTGAGTACCTTCG
TTTCCTCGGCGGGTCCGCCCGCCG
ATTGGACACATTTAAACCCTTTGT
AGTTGCAATCAGCGTCTGAAAAAC
TTTAATAGTTACAACTTTCAACAA

CGGATCTCTTGGTTCTGGCATCGA
TGAAGAACGCAGCGAAATGCGAT
AAGTAGTGTGAATTGCAGAATTCA
GTGAATCATCGAGTCTTTGAACGC
ACATTGCGCCCCCTGGTATTCCGG
GGGGCATGCCTGTCCGAGCGTCAT
TTGTACCTTCAAGCTTTGCTTGGTG
TTGGGTGTTTGTCTCGCCTCTGCGC
GCAGACTCGCCTCAAAACAATTGG
CAGCCGGCGTATTGATTTCGGAGC
GCAGTACATCTCGCGCTTTGCACT
CATAACGACGACGTCCAAAAAGT
ACATTTTTTACACTCTTGACCTCGG
A

Notes: Sample OAp 1 showed 98.91% identity with several strains of *Alternaria porri*

Sample_2 (isolate OSv5):

TTGGTCATTTAGAGGAAGTAAAAG
TCGTAACAAGGTCTCCGTTGGTGA
ACCAGCGGAGGGATCATTACCAG
AGTGCCCTAGGCTCTCCAACCCAT
TGTGAACATACCTATCGTTCCCTC
GGCGGGCTCAGCGCGCGGTGCCTC
CGGGCTCCGGGCGTCCGCCGGGGA
CAACCAAACTCCGATTTTATTGCG
AATATCTGAGGGGCGAAAGCCTG
AAAACAAAATGAATCAAAACTTTC
AACAACGGATCTCTTGGTTCTGGC
ATCGATGAAG*AACGCAGCGAAATG*
CGATAAGTAATGTGAATTGCAGAATT
*CAGTGAATCATCGA*ATCTTTGAACG
CACATTGCGCCCGCCGGCACTCTA
AAGGGCATGCCTGTCCGAGCGTCA
TTTCAACCCTCAAGCTTTGCTTGGT
GTTGGGCGTCTTTGTCTCTCACGA
GACTCGCCTTAAAATGATTGGCAG
CCGACCTACTGGTTTCGGAGCGCA
GCACAATTCTTGCACTTTGAATCA
GCCTTGGTTGAGCATCCATCAAGA
CCACATTTTTTTCAACTTTTGACCT
CGGATCAGGTAGGGATACCCGTCT
AGAACTTAAGCATATCAATAAGCG
AGAAGAAC

2- Effect of fungicides on the onion purple blotch pathogens *in vitro*:

Nine commercial fungicides were evaluated against the growth of isolates OAP1 and OSV5 of *A. porri* and *S. vesicarium*, respectively, in laboratory conditions and the percentages of inhibition over control were calculated. Results revealed that all the fungicides significantly minimized the fungal growth in comparison to control. Among the different treatments, 100% growth inhibition was recorded at 200 ppm Ridomil Gold Plus for *S. vesicarium* and for the two fungi tested at 400 ppm by Dithane M-45 and Pronto (Figures 4 and 5). Amistar 29.6% inhibited the growth of *S vesicarium* at 600 ppm. At the same concentration (600 ppm), Rent 80% WG, Collis, Luna Experience, and Myclobutanil completely inhibited the growth of the two fungi tested. More than 60% of growth inhibition of *A. porii* and *S vesicarium* was recorded by Collis, Luna Experience, Miracle and Myclobutanil at 400 ppm, but it was at 200 ppm for Dithane M-45. Minimal inhibition at 1000 ppm was observed by Miracle while, it reduced the fungal growth by more than 70 % at 600 ppm. Figures (4 and 5) showed that the compounds Dithane M-45, Pronto and Ridomil Gold Plus were the more active compounds against both pathogens reducing the mycelial growth more than 60% and at the minimal concentrations (200 and 400 ppm).

DISCUSSION

Onion (*Allium cepa* L.) and garlic (*A. sativum* L.) are two of the most important crops grown throughout the world. Onions and garlic suffer from many diseases caused by fungi, bacteria, viruses, nematodes and abiotic factors. Among them fungal diseases, purple blotch and stemphylium blight disease caused by *Alternaria porri* (Ellis) Ciferri and *Stemphylium vesicarium* (Wallr.) E.G. Simmons has remained a major concern in agriculture for both farmers and research fraternity as it severely damages the crops and drastically reduces the yield. There are the most serious and devastating diseases of *Allium* spp. (onions, garlic, shallots, leeks, scallions, and chives) limiting the quality and quantity of both bulbs and seeds. Our observations during winter of 2020 – 2021 season was proved that the symptoms of this disease have been showed in all major onion and garlic cultivation villages in Al-Edwa (El-Misid and El-Atef villages), Beni Mazar (Sholkam and El-Grnous villages) and Abou Qurqas (Nazlet- Asmant and Com al- Zuhair villages) districts, Minia Governorate. The highest severity of purple blotch and Stemphylium blight were recorded in fields of Al-Edwa district (El-Atef village) in Northern-West of the Governorate, whereas the least one was recorded at Abou Qurqas district (Com al-Zuhair village) in South of the Governorate. Onion Giza 6 cv. and garlic China cvs. showed higher disease severity than onion Giza red and Garlic Lady By cvs. Pathogenicity tests of twenty-one isolates of *S. vesicarium* and ten isolates of *A. porri* which were isolated from naturally infected onion and garlic plants, was revealed that all tested isolates of both pathogens (*S. vesicarium* and *A. porri)* infected Giza 20 onion cultivar and Lady Ba garlic cv leaves causing blight and purple blotch symptoms at different degrees. Both isolates OAP1and OAP2 of *A. porri*, which were isolated from natural infected onion leaves, were very high aggressive and exhibited the highest virulence degrees on onion whereas isolates GAP12 and GAP1, which were isolated from natural infected garlic leaves, caused the highest percentages of disease incidence on garlic. While isolates OSV5, OSV2, OSV6, OSV11 and OSV15 of *S. vesicarium* isolated from onion diseased leaves caused moderate disease incidence and severity on onion, followed by isolates GSV2 and GSV1. Isolates of *Alternaria porii* were highly aggressive than *Stemphyllim* isolates which came in the second rank as moderate virulence. Three isolates of *Alternaria porri*, OAP1, OAP2 and OAP3, were high aggressive on onion, causing 100-77.76% DI and 75-55% DS, followed by four isolates, OAP5, OAP6, GAP1and GAP3 were moderate inducing 50- 30% Di and 28.89 -20.0 Ds%, whereas isolates OAP4, OAP7 and GAP2 were the lowest aggressive ones, inducing 16.67-27.78% DI and 17.78 – 6.67 DS%. At the same time, fourteen isolates of *Stemphylium* showed moderate disease incidence (between 30-50%) and disease severity (15.56 - 28.89 %). The remaining isolates (7 isolates) of *Stemphylium* were low aggressive, inducing 16.67 - 27.78 DI% and 13.33 - 6.67% DS. On garlic, isolates of *Alternaria* GAP1, GAP2, OAP1 and OAP5 caused 50.0-72.22% DI and 28.89-44.44% DS, whereas isolates GAP3, OAP3, OAP4 and OAP5

considered moderate causing 27.78-28.89%, while isolates OAP6 and OAP7 were the lowest aggressive ones (causing less than 10% DS%), whereas isolates of *Stemphylium*, in general, were moderate and week virulent ones inducing 2.22-16.67% DS%. The identification of two more aggressive isolates, i.e. OAP1 and OSV5 of *Alternaria* and *Stemphylium*, respectively, were confirmed by using PCR technique and identified as *A. porri* and *S. vesicarium.* These results are agreed with several collaborates. Abdel-Rahim ***et al* (2017)** concluded that onion purple blotch symptoms at Assiut Governorate, Egypt, caused by *A. porri* and/or *S. vesicarium* and the synergistic effect caused by association between *Alternaria porri* and *Stemphylium vesicarium*.

Alternaria porri and *S. vesicarium* are fungi of Dothideomycetes class, order Pleosporales and family Pleosporaceae **(DAR *et al.*, 2020).** Stemphylium leaf blight is reported as an important disease affecting onion (*Allium cepa* L.), garlic (*A. sativum* L.), leek (*A. porrum* L.), shallot (*A. cepa* L. var. *aggregatum*), asparagus (*Asparagus officinalis* L.), European pear (*Pyrus communis* L.), lucerne (*Medicago sativa* L.), mango (*Mangifera indica* L.), tomato (*Solanum lycopersicum* L.), radish (*Raphanus sativus* L.), sunflower (*Helianthus annuus* L.), parsley (*Petroselinum crispum* (Mill. Fuss), and soybean (*Glycine max* (L.) Merr.) in different worldwide regions **(Hay *et al.*, 2021).** The disease is caused by *Stemphylium vesicarium* (Wallr.) E.G. Simmons (teleomorph: *Pleospora herbarum* [Pers.] Rabenh., syn. *P. allii*). The two mentioned pathogens survive on infected plant debris and resumes growth during favorable weather conditions in spring. They then produce spores that spread to nearby plants by the wind. The most severe onion disease, purple blotch, also known as leaf blotch and caused by *Alternaria porri*, is described as hurting both bulb and seed development by breaking flowering stalks **(Ahmed and Hossain, 1985 and Munoz *et al.*, 1984).** Purple blotch and Stemphylium blight on onion and garlic is distributed throughout many parts of Africa, the USA, Canada, the West Indies, India, Western Europe, South America and many other parts over around the world **(Sherf and MacNab, 1986). In 1967, Boelema and Ehlers** diagnosed a disease in south Africa, on the leaves of onion, as caused by *A. porri*. Whereas in previous years the disease occurred on the stems of the seed crop only, in the autumn of 1967 it was found on the leaves of small seedlings and older plants where it had never been troublesome before. Nowadays, *Stemphylium vesicarium*, the cause of Stemphylium blight of onion, is also indirectly responsible for purple onion blotch. The condition referred to as purple blotch complex because *Alternaria porri* and *Stemphylium vesicarium* both contribute to the development of purple blotches. **Sarnobat *et al.* (2020)** reported that this disease drastically reduces onion productivity, quality and yield.

The synthesis of host-specific or non-specific toxins by *Alternaria* spp. is associated with their pathogenicity and its potential to cause disease symptoms. These poisons are primarily secondary metabolites, which cause leaf necrosis to destroy sensitive cultivars **(Mamgain *et***

***al*. 2013).** Additionally, anthraquinones such as erythroglaucin have been purified from *A. porri* **(Horiuchi *et al.*, 2003 and Montemurro & Visconti, 1992 and Andersen *et al.*, 2008). Horiuchi *et al.*, 2003** found**,** from the culture medium of *Alternaria porri*, a zinniol-related chemical compound called purritoxine sulfonic acid with an isoindoline skeleton.

The evaluation of nine fungicides against the growth of isolates OAP1 and OSV5 of *A. porri* and *S. vesicarium*, respectively, under laboratory conditions revealed that all the fungicides tested significantly minimized the fungal growth in comparison to control. Among the different treatments, the growth of *S. vesicarium* was completely inhibited at 200 ppm Ridomil Gold Plus, while Dithane M-45 and Pronto completely inhibited the growth of two tested pathogens at 400 ppm. At 600 ppm, fungicides Amistar29.6%, Azoxystrobin 23% SC, Collis, Luna Experience, and Myclobutanil complete stopped the growth of one and/or two isolates. Dithane M-45, Pronto and Ridomil Gold Plus were the more active compounds against both pathogens, reducing the mycelial growth more than 60% and at the minimal concentrations (200 and 400 ppm). The same results were obtained by many researchers who reported that fungal pathogens are mostly controlled by chemical compounds **(Mathur and Sharma, 2006, and Meena and Verma, 2017).**

Several studies indicated that available fungicides may have low potentiality to manage onion purple blotch disease because they have not been evaluated to control *A. porri* and *S. vesicarium* as collective causative agent **(Uddin *et al*.,2006 and Abdel- Hafez *et al*., 2015**). **Abdel-Hafez *et a*l. (2015)** reported that Ridomil Gold Plus (0.2 %) is more effective on *S. vesicariumtha* and *A. porri* causing reduction in the disease to 6.8 and 34.7 %, respectively. Hence, the treatment of purple blotch, as a disease caused by *A. porri* and *S. vesicarium*, with effective chemicals not only becomes more economical but also environmentally safer than using nonspecific fungicides. Dithane M-45 and Rovral 50 WP were recorded as the best fungicides to control onion purple blotch, which scored the maximum thousand seed weight and yield, whereas the lowest seed weight and yield was observed in control treatment, which was statistically similar to that of Bavistin 50 WP, Tilt 250 EC and Ridomil Gold Plus MZ-72 **(Uddin *et al.*, 2006).**

CONCLUSION:

The current study was carried out during winter of 2020 – 2021 season and concludes that purple blotch and Stemphylium blight was prevalent in all onion or garlic cultivated areas, under study, in Minia Governorate. Thirty-one isolates of *Alternaria* and *Stemphylium* were purified, and their pathogenicity ranged between 16.67-100% DI and 6.67- 75.56%DS% on onion, and between 0.0 – 72.22 DI% and 0.00 - 44.44%DS%. On garlic. Isolates of *A. porri (*SUB12232226 *Alternaria* "OP740798") and *S. vesicarium* (SUB12232298 Stemphylium OP745414) were the most aggressive isolates on onion and garlic. Fungicides Amistar Top 29.6%, **Rent 80% WG,** Collis 30% SC, Dithane M-45 WP, Luna Experience 40% SC, Miracle 10% EC.,

Myclobutanil 20 EW, Pronto 32% SC and Ridomil Gold Plus WP were decreased the growth of both pathogens *in vitro* The percent of inhibition ranged between 44.29% and 69.68%.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

Table 1): Primer code, RNA sequence and product size

Primer Code	Sequence	Product Size
(ITS-1) F	5′- GCATCGATGAAGAACGCAGC -3′	650bp
(ITS-4) R	5′- TCCTCCGCTTATTGATATGC-3′	

Table 2: Trade name, active ingredient and manufacturer of the used fungicides

Trade name	Active ingredient (IUPAC name)	Manufacturer
Amistar Top 29.6%	Azoxystrobin 18.2% Sc + difenoconazole 11.4%	Agrosiaa, Syngenta India Ltd
Rent 80% WG	**Azoxystrobin 22.8%+ Dimethomorph 57.2%**	**Shaanxi Tunpsion Biological Technology Co., Ltd- China**
Collis 30% SC	Kresoxim-methyle 10% + Boskalis 20%	Basf SE, Ludwigshafen, Germany
Dithane M-45 WP	Manganese ethylenebis (Dithiocarbamate)	Sumitomo ChemicaL Turkey Kimya SAN. ve Tic. A.Ş.
Luna Experience 40% SC	Fluopyram 17.7%+ Tebuconazole17.7% w/w SC	Bayer, Crop Science, Germany,
Miracle 10% EC.	250 g / L Tebuconazole	Hektaş Co. Ltd., Çankaya/Ankara, Istanbul.
Myclobutanil 20 EW	Myclobutanil: alpha-butyl-alpha-(chlorophenyl)-1H-1,2,4, triazole-1-propanenitrile 19.7% other ingredients*: 80.3%	Control Solutions, Inc Genoa Red Bluff, Pasadena, TX 77507
Pronto 32 % SC	Azoxystrobin 12 % + Tebuconazole 20%	AAKO, Holland Bridge Trade Company, Egypt
Ridomil Gold Plus WP	Minfanoxam +Copper hydroxide	Agrochem, Egypt

Table 2: Survey for purple blotch and Stemphylium blight of onion and garlic in different districts of Minia Governorate.

District	Village	Onion Giza 6(*)		Onion Giza red		Grilic China		Grilic lady Ba	
		%, DI	%, DS	%, DI	%, DS	%, DI	%, DS	%, DI	%, DS
El-Edwa	El-Misid	54.33	30.64	20.78	5.70	53.25	29.68	15.54	7.37
	El-Atef	56.72	29.51	20.84	5.63	55.53	30.12	16.03	6.12
	Mean	55.52	30.07	20.81	5.66	54.39	29.90	15.78	6.75
Beni-Mazar	Sholkam	46.35	19.04	18.35	4.855	42.685	20.4	11.55	3.49
	El-Grnous	38.58	16.79	16.60	4.64	41.58	19.06	11.09	3.68
	Mean	42.46	17.91	17.48	4.75	42.13	19.73	11.32	3.59
Abo-Qurqas	Nazlet-Asmant	32.65	11.57	11.73	2.81	32.94	12.41	8.42	2.13
	Com al-Zuhair	27.05	9.76	8.28	1.85	30.34	11.68	7.06	1.81
	Mean	29.85	10.66	10.00	2.33	31.64	12.04	7.74	1.97

(*)Each reading is an average of 200 onion or 175 garlic plants.
%, DI= % of infected Plants %, DS= % of disease severity

Table 3. Local, source and % disease incidence and severity of 27 pathogenic isolates (*S. vesicarium* and *A. porri*) for onion (Giza 20 cv) and garlic (Lady Ba cv)

Isolate, ID	Host source	DI, % on		DS, % on	
		Onion	Garlic	Onion	Garlic
OSV1	onion	38.89	16.67	20.00	6.67
OSV2	onion	44.44	16.67	28.89	8.89
OSV3	onion	38.89	11.11	22.22	8.89
OSV4	onion	38.89	16.67	24.44	11.11
OSV5	onion	50.00	16.67	26.67	12.22
OSV6	onion	44.44	11.11	26.67	8.89
OSV7	onion	27.78	0.00	10.00	0.00
OSV8	onion	33.33	16.67	15.56	11.11
OSV9	onion	27.78	22.22	13.33	11.11
OSV10	onion	27.78	5.56	11.11	3.33
OSV11	onion	44.44	16.67	17.78	3.33
OSV12	onion	38.89	22.22	25.56	15.56
OSV13	onion	33.33	11.11	20.00	4.44
OSV14	onion	33.33	11.11	20.00	6.67
OSV15	onion	44.44	5.56	22.22	3.33
GSV1	Garlic	33.33	33.33	12.22	12.22
GSV2	Garlic	33.33	33.33	16.67	16.67
GSV3	Garlic	27.78	27.78	11.11	11.11
GSV4	Garlic	27.78	27.78	13.33	13.33
GSV5	Garlic	16.67	16.67	6.67	10.00
GSV6	Garlic	22.22	22.22	10.00	10.00
OAP1	onion	100.00	50.00	75.56	43.33
OAP2	onion	94.44	27.78	61.11	23.33
OAP3	onion	77.78	38.89	55.56	28.89
OAP4	onion	27.78	38.89	17.78	27.78
OAP5	onion	50.00	50.00	28.89	28.89
OAP6	onion	38.89	11.11	21.11	6.67
OAP7	onion	22.22	11.11	11.11	4.44
GAP1	Garlic	38.89	66.67	20.00	42.22
GAP2	Garlic	16.67	72.22	6.67	44.44
GAP3	Garlic	38.89	38.89	25.56	28.89

*) Each figure represents a sample average of 18 leaves (two leaves X three plants X three pots).
Degree of disease severity: High (more than 50%)., M: Moderate degree of disease severity (26–50 %).
L: Low degree of disease severity (12.5–25 %) and W: Weak degree of disease severity (less than 12.5%).

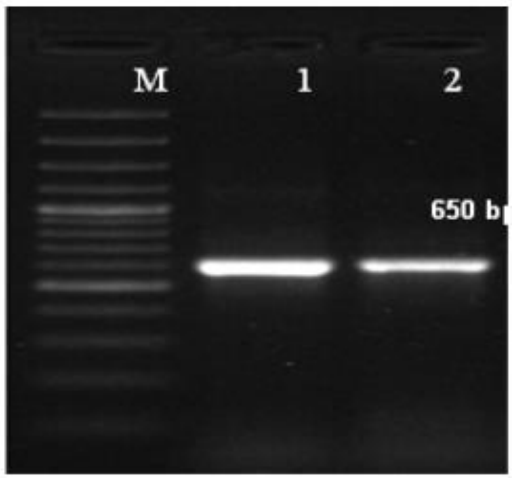


Figure (1): PCR amplification of DNA extracted from the two fungal isolates: Lane M, 100 bp DNA ladder (ferments).
Lane 1 , 2 a 650 bp ITS rDNA region for the two isolates.

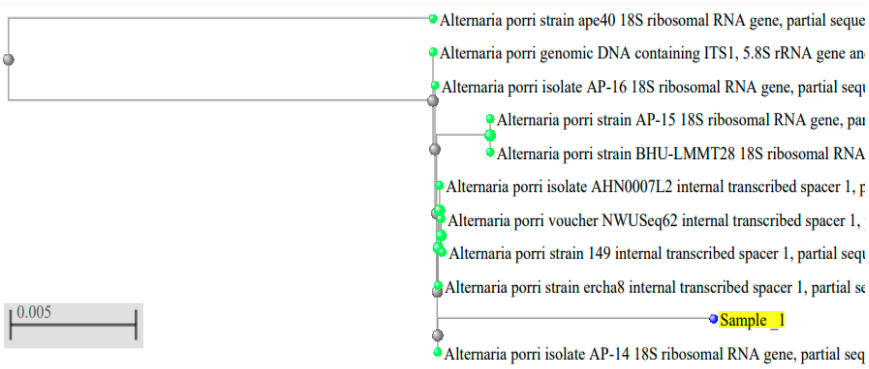


Figure (2): Phylogenetic tree based on ITS sequences of rDNA of the fungal sample isolated in the present study (OAp 1) aligned with closely related strains accessed from the GenBank.

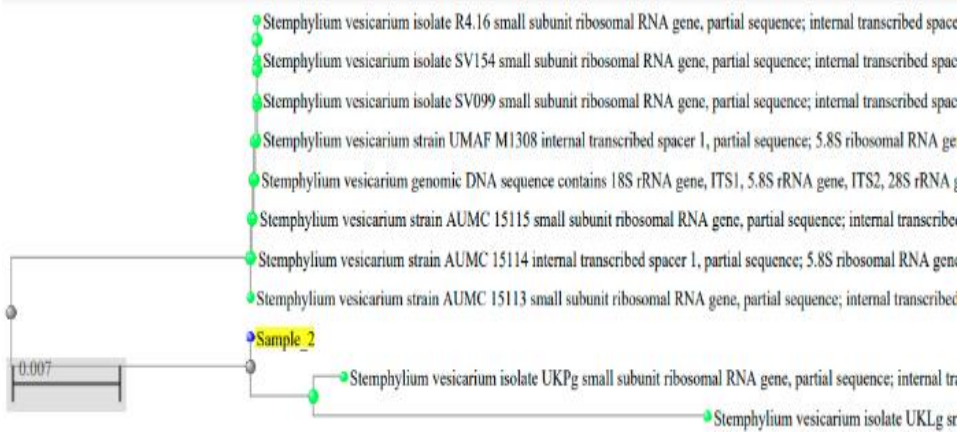
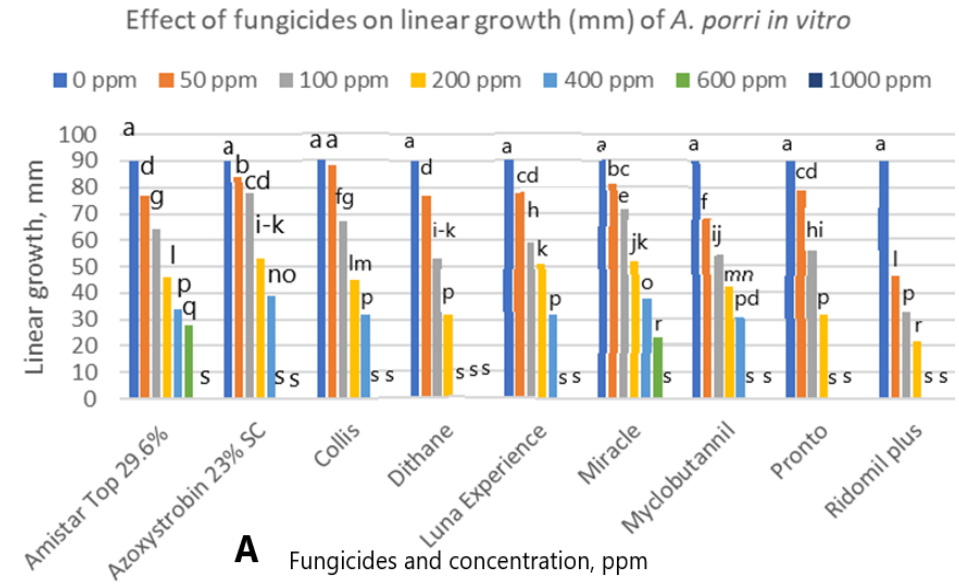


Figure (3): Phylogenetic tree based on ITS sequences of rDNA of the fungal sample isolated in the present study (OSv5) aligned with closely related strains accessed from the GenBank.



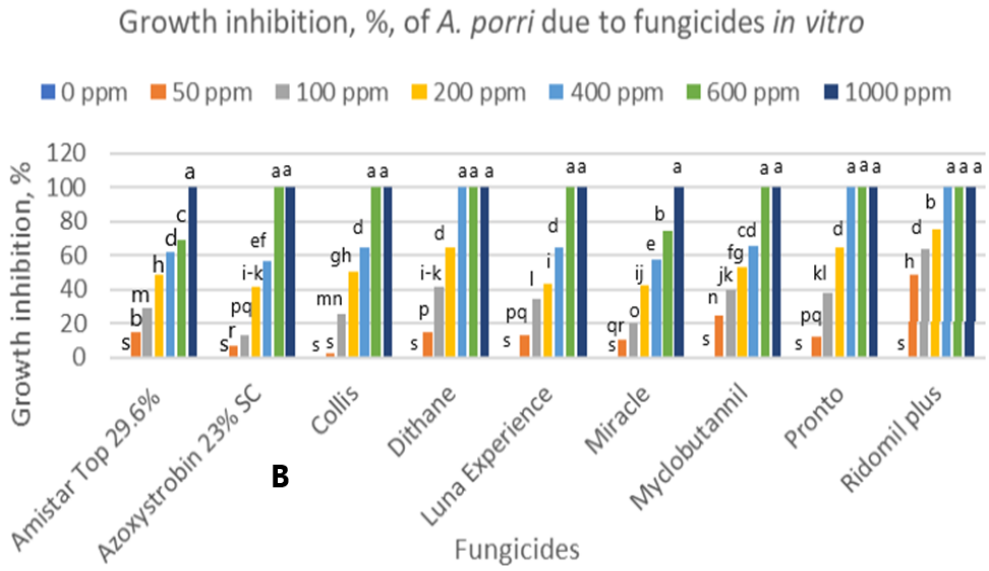


Figure 4 (A, and B): Effect of fungicides on linear growth (A) and percent of growth inhibition (B) of *Alternaria porri in vitro*

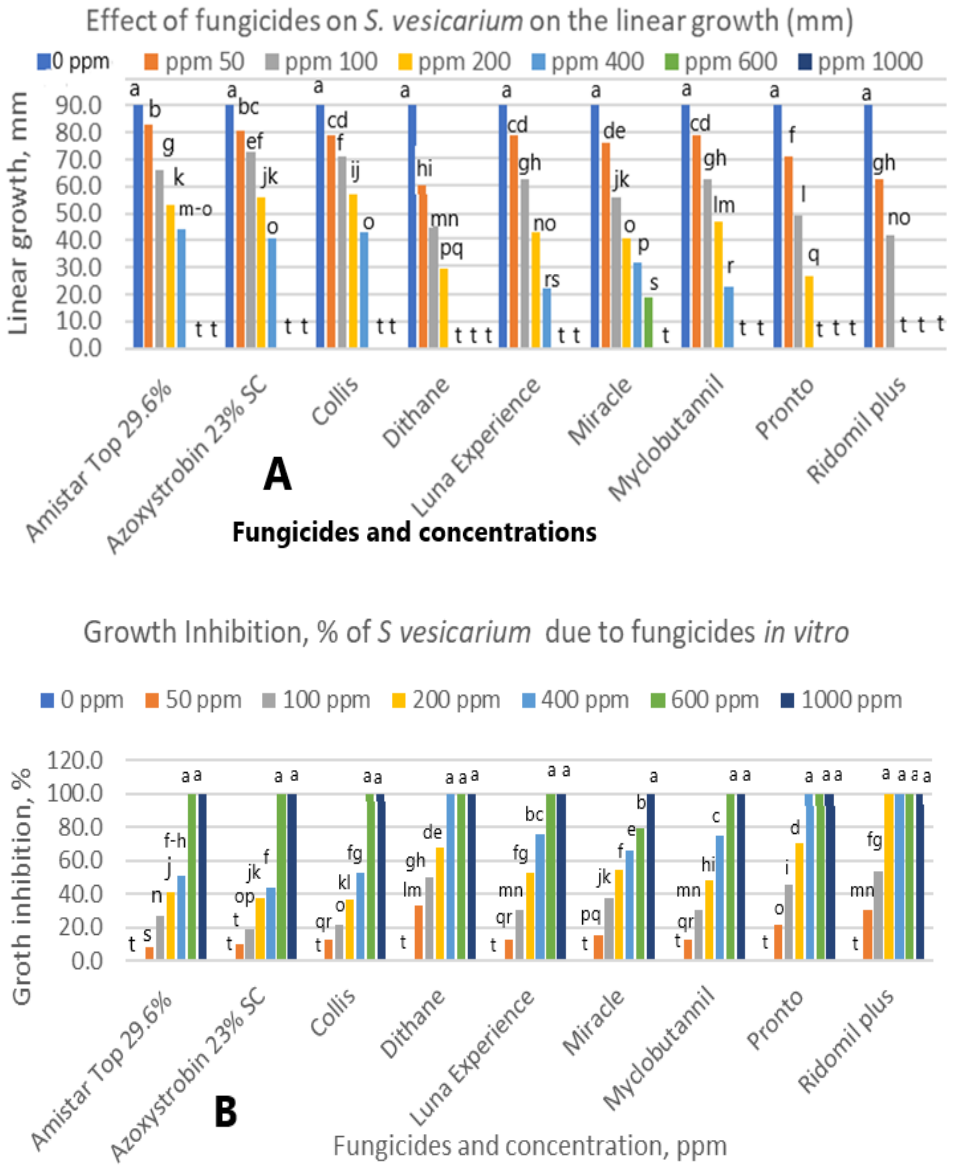


Figure 5 (A, B): Effect of fungicides on linear growth of *Stemphylium vesicarium* and percent of growth inhibition

(1) Data presented the average of four replicates

(2) Values followed by the same letter(s) within each column don't differ significantly.

LG = Linear growth (mm), Conc. = concentration (ppm),

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الملخص العربي

عزل وتشخيص وتقييم بعض المبيدات لمقاومة مسببات مرض اللطعة الأرجوانية في البصل والثوم في محافظة المنيا – مصر

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يعتبر كل من البصل والثوم من أقدم المحاصيل التي زرعت في غالبية دول العالم. مرض اللطعة الارجوانية ولفحة الاستمفليوم في البصل والثوم من الأمراض التي تصيب المجموع الخضري ويتسببان عن الإصابة بالفطرين Alternaria porri وStemphylium versicarium. خلال شتاء موسم 2020-2021 تم إجراء حصر للمرض في ثلاث مراكز بمحافظة المنيا مشهورة بزراعة البصل والثوم وقد ثبت انتشار المرض في جميع حقول الدراسة. وقد بين الفحص الميكروسكوبى للعينات النباتية المصابة تواجد كل من الفطرين المسببين للمرض، وتم عزل وتنقية واحد وثلاثون عزلة لهذين الفطرين. أظهر اختبار القدرة المرضية لهذه العزلات انها جميعا قادرة على احداث المرض وإظهار أعراض اللطعة الأرجوانية ولفحة الإستمفليوم على كل من البصل والثوم ولكن بدرجات مختلفة، وأكد تعريف العزلتين الأشد فراسة وقدرة مرضية OSv5 OAp1 بواسطة تقنية ال PCRانهما للفطرين Alternaria porri وStemphylium versicarium المسببان للمرض. كما تم تقييم تسع مبيدات فطرية في دراسة معملية لتقدير قدرتها على تثبيط نمو العزلتين تحت الدراسة وقد سبب المركب ريدوميل جولد بلس تثبيط كامل لنمو الفطر Stemphylium versicarium عند تركيز 200 جزء/مليون، بينما ثبطا كل من المركبين دايثين ام-45 وبرونتــو نمو الفطرين تحت الدراسة تثبيطا تاما عند تركيز 400 جزء/ مليون