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RESEARCH ARTICLE

Isolation, identification and biocontrol treatments of *Alternaria. Alternata* of *Vicia faba*

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Abstract

Vicia faba, also referred to as the broad bean, is a plant that is extensively grown for human food. It can be eaten both raw and cooked. Alternaria leaf spot is caused by the alternate occurring late in the growing season, which can severely impact yield. In this experiment six isolates of *A. alternata* were isolated from diseased natural fields of *Vicia faba* plants from different areas in the Qena governorate, identified according to their morphological characters as A. *alternata.* The effect of biological agents *Trichoderma harzianum* and *Bacillus subtitles* and natural plants extract garlic (*Allium sativum)* and neem (*Azadirachta indica)*, used in the concentration of 5% 10% and 15%, respectively were examined in the laboratory for their antagonistic effect against *A. alternata*. *Trichoderma harzianum* had a 71% inhibition percent for the pathogenic linear growth and *Bacillus subtitles* had 58%. Natural plants extract garlic and neem were more effective and could suppress 90 and 100% respectively. Natural plants extract were more effective in reduce linear growth of *A. alternate* isolated from *vicia faba* compared with *Trichoderma harzianum* and *Bacillus subtitles*.

Keywords: *Alternaria alternata*; Garlic extract; Leaf spot; Neem extract; *Vicia Faba*.

1. Introduction

Fava bean is considered one of the most important legume crops in Egypt and the worldwide. It is grown mainly for its green pods and dried seeds, which are rich in protein, vitamins, carbohydrates, dietary fibers, minerals and secondary metabolites such as phenolics. Thus, it is an important component of human nutritional consumption (Randhir *et al.*, 2002; Sahile *et al.*, 2011). Furthermore, *vicia faba* improves the environment soil fertility by fixing atmospheric nitrogen, reducing costs and minimizing environmental impact. Therefore, increasing the plant crop production is one of the major targets of Egypt Agriculture policy (Mahmoud *et al.*, 2004; Bendahmane *et al.*, 2012). Faba bean is suffering from many destructive diseases. It is attacked by more than 100 pathogens in the Mediterranean region. Diseases can inflict great losses in faba bean production. Alternaria leaf spot disease is predominant on faba bean during the last years due to global climate change, especially in Egypt (Reis *et al.*, 2007; Juroszek, 2011). Biological control is considered an important approach for controlling many fungal plant pathogens and exploration for new biological agents is increasing as potential biological control antagonists. (Porras *et al.*,2008; Deshmukh *et al.*, 2010; Ryota *et* al., 2010; Gveroska and Jovancev, 2011). Trichoderma spp., and Bacillus spp were most promising and effective

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biocontrol agent tested against Alternaria leaf spot (Gan *et al.,* 2006, Boubekeur *et al.,* 2012; Tran, 2010). Medicinal plants have attracted researchers to evaluate their antimicrobial activities against economically important plant pathogens. During last decades several types of research have been conducted on plant extracts and oils to find out such alternatives and valuable results have been achieved (Ayoub and Niazi, 2001; Bowers and Locke, 2000; Suprapta and Kalimi, 2009; Singh, 2004). the southern upper –Egypt with high temperature such leaf spot disease incidence is unusual and an integrity control method is under investigated. This study's objective was to isolate, identify, and investigation the impact of employing the bioagents *Trichoderma harzianum* and *Bacillus subtilis* as well as plant extracts from neem and garlic for control *A. alternata* in fava bean under laboratory conditions.

2. Material and methods

2.1. Isolation and Identification of pathogenic fungi

Symptoms of naturally infected plants were briefly described and photographed. Infected faba bean plants collected from different fields of Qena Governorate. Affected parts of the infected leaves were cut into small pieces (3 mm in length), surface sterilized with 5% of sodium hypochlorite, washed several times with distilled water and then dried. The fixed leaves pieces were transferred into potato dextrose agar (PDA) medium supplemented with penicillin (20 µL/mL) and incubated at 25 ± 1°C, then examined daily for fungal growth. The fungal colonies were purified using single spore or hyphal tip isolation technique suggested by (Booth. 1985) and identified based on cultural and microscopic morphological characters according to the key given by Barnett and Hunter (1977)

2.2. Pathogenicity test

The experiment was conducted to determine the pathogenicity of the six *A. alternata* isolates on local fava bean verities (Giza843). Broad bean plants were grown in a glasshouse throughout the investigation. Seeds were sown in 20-cm-diameter plastic pots (five seed per pot) containing 5 kg of commercial garden soil. A spore suspension of the isolates, obtained by flooding with sterile water and rubbing 7- to 10-day-old cultures grown on PDA agar medium, was used as inoculum. Harvested spores were filtered through double layers of cheese cloth, and the spore concentration of the resultant suspension was adjusted to 5 ×lo ^{5} spores/ml by hemocytometer, plants leaves were sprayed and watered as needed. Inoculated leaves were kept in moist plastic boxes and incubated in dark at 25°C. Untreated control plants were sprayed only with sterilized water. Disease assessment was recorded 15 days after the microbial spraying using the disease scale from 1;5, where 1 = No lesion; 2, lesion on less than 50% ;3, lesion on 50 to 75%; 4, lesion on more than 75%., 5 majority of the leaf was necrotic and drop.

2.3. Antagonistic Fungi source

Biocontrol agents *Trichoderm harzianum* and *Bacillus.subtilis* were kindly provided by Plant Botany Department, South Valley University, Faculty of Agriculture. The bioagents fungal plates and bacteria were grown in special media and incubated at 25+2°C for further studies.

2.4. Effect of antagonists on Alternaria alternate linear growth:

An equal disc (5mm in diameter) of *Alternaria alternata* was inoculated in one side of 9 cm PDA plates another side inoculated with (5mm in diameter) of *Trichoderma harzianum*. For *Bacillus. subtilis,* Petri dishes were inoculated with 5 mm disc of A. alternate and anther side 2 streaks for bacteria. The control plates were inoculated with the pathogen *Alternaria alternate* only. Five replicates were used for each treatment. The percent inhibition of A. alternate was calculated by adopting the following formula. Percent inhibition%= Radial growth in control- Radial growth of treatment ÷ Radial growth in control (C) × 100

2.5. Plant extracts preparation

Plants extracted methods were according to methods mention by (Zaker and Mosallanejad, 2010) with some modification as follows: Matured leaves of neem (*Azadirachta indica*) and garlic (*Allium sativum*) were thoroughly washed in running water and kept to dry. Fifty gm of dry leaves were ground by a blender with 50 ml of 50% methanol (99.5%) and homogenized for 20 min with the help of a homogenizer. Mixtures were then centrifuged for 10 min to obtain clear extracts. The methanol was completely removed from the clear solutions using a rotary evaporator. Final extracts were passed through 0.2 µ seitz filters to remove any unwanted bacteria and were used as 100% pure extracts.

2.6. Evaluation of plant extracts antagonistic in vitro

According to (Schmitz, 1930) Poisoned food technique was used to evaluate the effect of plant extracts on mycelial growth of *A. alternata*. One hundred milliliter of PDA were prepared in 200 mL, then Erlenmeyer flasks sterilized for 20 min and kept under sterilized hood to cool up to 60°C. Exact amounts of pure extracts were then added to each flask and shake gently to prepare PDA containing 5, 10 and 15% of extracts respectively. Petri dishes were poured with PDA containing known percentage of extracts. Discs of *A. alternata* about 5 cm were kept in the center of each Petri dish. Petri dishes were incubated at 25-27°C for 7 days and then the smallest and largest diameters of mycelial growth of each petri dish were measured and recorded daily. Five petri dishes were used for each treatment. A plate only with PDA and fungal disc was considered as control and the diameter of growth of fungus in this plate was used as a control for the calculation of percent inhibition of test fungus. Inhibition percentage: The inhibition percentage was calculated measuring the radial growth of the fungus grown on control and amended plates, using the following formula (Harlapur et al., 2007): P% % = 100 × (C – T) / C

Where, P% = inhibition percentage of pathogen growth, C = average radial growth in control plates and T = average radial growth in plates amended with seaweed extract.

2.7. Statistical analysis

The experimental design was completely randomized, consisting of five replicates for each treatment. The experiment was repeated at least twice and treatment means obtained were separated using a Duncan's multiple range tests (Duncan's test P> 0.05) (Gomez and Gomez., 1984).

3. Results and discussion

3.1. The Natural symptoms

The disease was noted in natural fields. The natural symptoms on leaves were slight brown lesion, water soaked, circular to irregular. Plants leaves had coalescing necrosis surrounded by yellowing. Finally, the leaves became blighted from the margin to the center and most of the diseased plants defoliated. These lesions have also appeared on stems and pods, and plants defoliated completely fig. (1).

3.2. Isolation, morphology and identification of the causal fungus

Fungal isolates were purified and identified according to their morphological characteristics (fig.2). An Alternaria species was the most frequent of fungal isolates growing from the lesions on fava bean leaves. On PDA plates, *Alternaria alternate* fugal developed aerial hyphae on grayish white colonies, which later turned olive-green to black on PDA medium figure (3), In PDA culture grows as long chains with dark brown conidiophores, producing asexual spores known as conidiospores (conidia).

Figure1. symptoms of leaf spot by *A.alternate* under natural field

Figure 2. *Alternaria alternata* under light microscopy the conidia are ovoid or ellipsoidal with a cylindrical beak. The spores are pale brown, smooth-walled

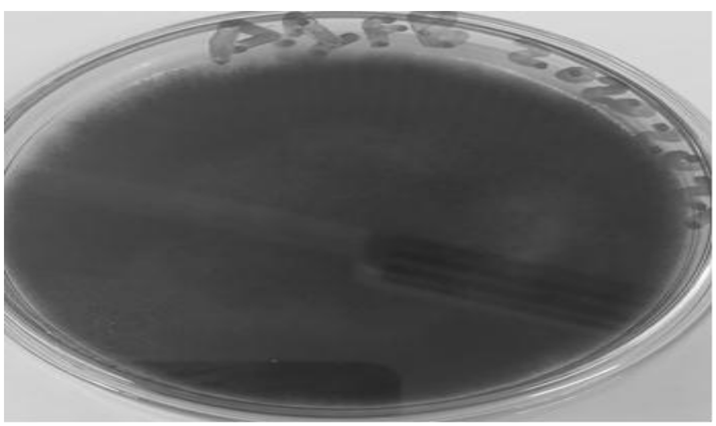


Figure 3. Colony of *A. alternata* growing on PDA agar for 7 days at 28°C.

The conidia grow on the conidiophore. The spores are large and appear dark. They also have short beaks and fine long septate. Spores are pale brown, smooth-walled Fig (2). This is believed to be the first report of *A. alternata* causing leaf spots in fava bean plants under Qena governorate Upper Egypt. The same symptoms and fungus description was reported previously by (Akhtar *et al.,* 2004; Nivedha *et al.,* 2019).

3.3. Pathogenicity test

All six tested isolates were pathogenic to broad bean leaves, Leaves with severe coalescing of necrotic spots leads to drooping and withering of the entire plant. There were no symptoms on plants treated with sterile distilled water figure (5). Figure, (4) indicated a significant difference between the isolates in disease incidence. Isolate A1 isolated from Abo-Tesht (isolate A1) was the most aggressive one recording the maximum values of disease incidence 88%, therefore this isolate chosen for in vitro test.

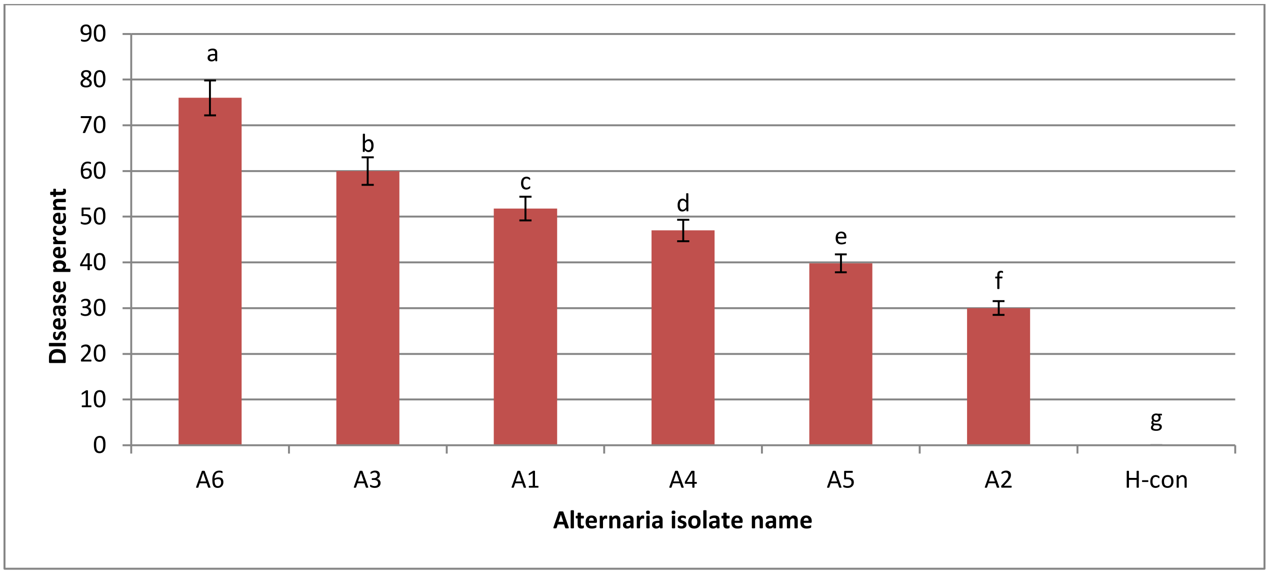


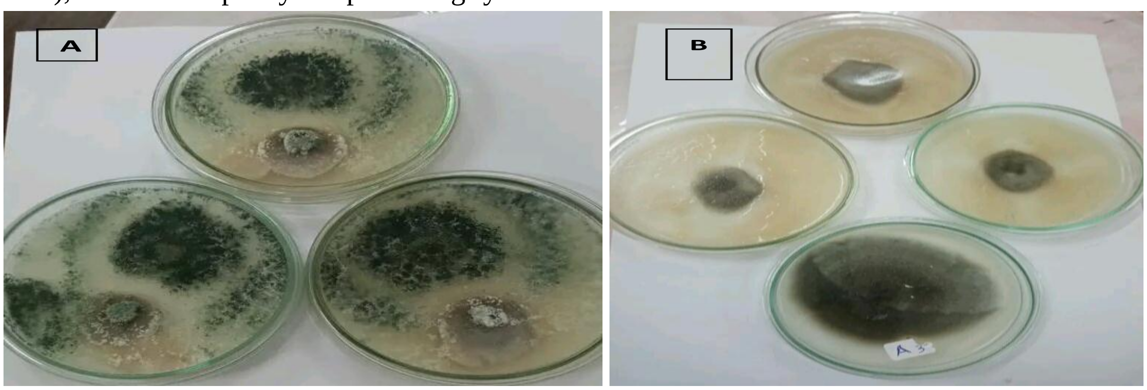
Figure 4. Pathogenicity tests of fungal isolates on *vicia faba* plants. The values in the column followed by the same letter are not significantly different according to Duncan's at P<0.05.

A. Infected plants B. Control plants

Figure 5. symptoms of pathogenicity test under greenhouse. Isolate A1 isolated from Abo-Tesht was most virulent.

3.3.1. Effect of different biological antagonists on Alternaria alternate linear growth

Five days incubation with *T. harzianum* and *B. subtilis* in petri dishes. All the tested bio-microorganisms showed clear significant effect compared with control treatment. Reduction percentage was differed from *T. harzianum* to *B. subtilis*. Maximum mycelial growth reduction was by *T. harzianum* (71 %) and B. subtilis (58 %). The results were in accordance with Kayim *et al.* (2018) who tested five *Trichoderma harzianum* isolates (T1, T2, T3, T4 and T5) among them Trichoderma isolates (T2 and T3) were found the most effective against *A. alternata* causing Alternaria leaf spot disease of broad bean. It is well established in the literature that several species of the genus Trichoderma can suppress important plant diseases. The potential of these fungi as biocontrol agents, has been related to mycoparasitism of phytopathogens, their ability to compete for space and nutrients (Sánchez *et al.,* 2007), and their capacity for producing lytic enzymes such as quitinases and β-1, 3-glucanases (El Komy *et al.,* 2015). Bacterial strains from the order Bacillales have been particularly useful against plant pathogens (Guevara-Avendaño *et al.,* 2018; Burkett-Cadena *et al.,* 2019; Johnson and Dunlap, 2019) and are frequently used for these applications (Dunlap, 2019). This biocontrol activity has been attributed to the ability of the bacteria to produce antibiotic compounds and to efficiently compete for space and nutrients in the rhizosphere (Zhao *et al.,* 2013). Chowdappa *et al.* (2013) reported that strains of *T. harzianum* and *B. subtilis*, in vitro, inhibited the growth of mycelium from *A. solani* and *P. infestans*.



A.alternatea + *T. harzianum* *A. alternatea* + *B. subtilis*

Figure 6. in vitro antagonistic effect of *A. alternatea* +*T. harzianum and A. alternatea* +*B. subtilis*

3.3.2. Effect of plants extracts on A. alternate linear growth

The results showed natural extracts from garlic and neem could reduce *Alternaria. alternata* linear growth in PDA medium and there are significant differences between neem and garlic extract concentration figures (7&8). Garlic extract was more effective on pathogenic linear growth. Furthermore, when concentration of extracts increases the percent of inhibition also increased. The highest inhibition percentage arrived to 100% on garlic extract at 15% concentration. Whereases, neem extract at 15% was (73%). The results were agreed with Nashwa *et al.* (2012) mentioned that plant extracts of, *Ocimum basilicum*, *Azadirachta indica*, *Eucalyptus chamadulonsis*, *Datura* stramonium, *Nerium oleander*, and Allium sativum, caused a significant reduction in the linear growth of *A. solani*. This reduction was gradually increased by increasing the concentration of extracts in the growth medium). Lima *et al*. (2016) reported that garlic extract and the orange essential oil showed the potential to control *A.dauci* and *A.alternata*, at lower concentrations. (Nivedha *et al.,* 2019) tested twenty-four plant extracts and four oils for antifungal activity against *A.alternata in vitro* condition. Among the tested plants *Allium sativum* (5%) recorded the highest reduction of the mycelial growth of 100% followed by leaf extract (10%) of Datura metel (68.44%) and oil (3%) and *Azadirachta indica* was (59.88%). The medicinal plants used in this study are widely known and have been used in many countries. Several authors have studied their active substances and biological activities in several methods (Jesonbabu *et al.*, 2012; Phachonpai *et al.*, 2012; Rajeshkumar and Sundararaman, 2012; Madduluri *et al.*, 2013; Rai *et al.*, 2013). Mechanisms of disease suppression by plant extracts products have suggested that the active principles present in plant extracts may either act on the pathogen directly (Amadioha, 2000) or induce systemic resistance in host plants resulting in a reduction of the disease development (Kagale *et al.*, 2004). Finally, plant extracts and oils have potential effect to be developed as potent fungicides.

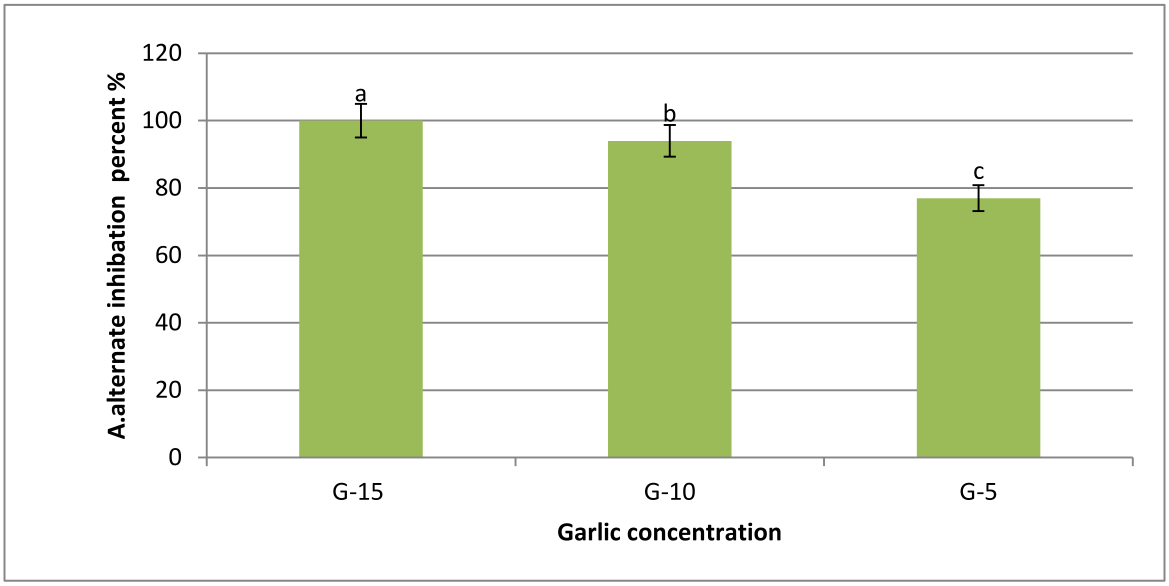


Figure 7. Effect of different concentration of garlic extracts on A. alternate linear growth. The values in the column followed by the same letter are not significantly different according to Duncan's at P<0.05.

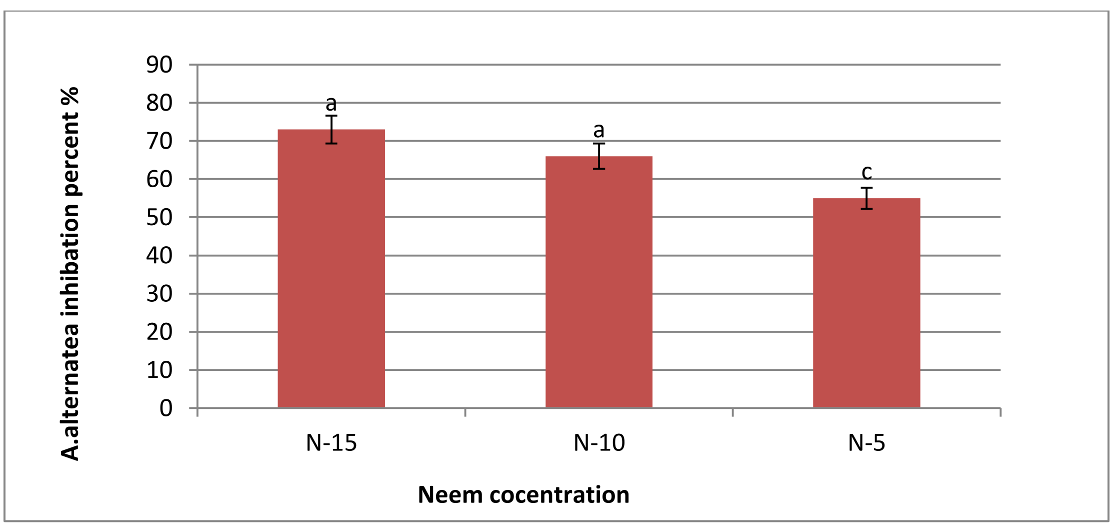


Figure 8. Effect of different concentration of (*Azadirachta indica)* neem extracts on A. alternate linear growth. The values in the column followed by the same letter are not significantly different according to Duncan's at P<0.05

4. Conclusion

It could be concluded that natural plants extract garlic and neem were more effective and could suppress 90 and 100% respectively. Natural plants extract were more effective in reduce linear growth of *A. alternate* isolated from *vicia faba* compared with *Trichoderma harzianum* and *Bacillus subtitles*.

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Data Availability Statement
Data presented in this study are available on fair request from the respective author.
Ethics Approval and Consent to Participate
Not applicable
Consent for Publication
Not applicable.
Conflicts of Interest
The authors disclosed no conflict of interest starting from the conduct of the study, data analysis, and writing until the publication of this research work

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