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Record of *Alternaria tenuissima* as a causal pathogen of Leaf Spots in Chard Plant in Basrah, Iraq, and It's *In vitro* Management

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Abstract

The chard plant is an important vegetable crop, thus this study aimed to isolate and identify *Alternaria tenuissima* and efficiency evaluation of *Pseudomonas fluorescens, Trichoderma viride, Trichoderma harzianum, and Paecilomyces fumosoroseus* suppression against *A. tenuissima. T. harzianum* demonstrated its ability to inhibit the *A.tenuissima* by 63.8%. Also, it reduced the leaf spot number to 0.39 cm/leaf compared to the control that scored 2.19 cm/leaf. The *T. harzianum* increased the leaves and root weight, followed by *P fluorescens* treatment. This is considered the first recorded in Basra, Iraq.

Key words:*Alternaria tenuissma, Trichoderma harzianum* ,Chard plant ,leaf spot disease

I. INTRODUCTION

Chard, scientifically referred to as *Beta vulgaris* L. var. cicla, goes by the common name of leaf beet in English. Notably, the larger-leaved varieties of this vegetable are recognized as Swiss chard. This versatile vegetable is a vital component of various culinary traditions and offers a essential nutrients that contribute to human well-being. Swiss chard, or simply chard, is celebrated for its nutritional richness. It serves as an excellent source of vital minerals like iron, calcium, magnesium, and sulfur, all of which are crucial for maintaining optimal health. In addition to these essential minerals, chard boasts an array of vitamins, including vitamin C, various B vitamins, and vitamin A. This impressive nutritional profile makes chard a valuable addition to a balanced diet. BVr contains secondary metabolites, called betalains, which are used as natural dyes in food industry and show anticancer activity.(Ninfali and Angelino, 2013)

Chard leaves also contain Phytopigments that protects the human body against cancer and improve immune system(Fiedor and Burda,2019). The crop is affected by many agricultural pests, such as insects and diseases like fungal, viral, nematodes, and parasitic flowering plants. However, leaf spot caused by *Alternaria* is a common

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disease on this crop. Chemical pesticides reduce crop infection with many agricultural pests and diseases but affect our environment. They are hazardous (Khan *et.al*,2018). *A.tenuissima* recored in Malaysia causing leaf spot on eggplant(Nasehi,*et.al*.,2012)

In addition, many of them have lost their effectiveness due to development of new strains of pathogens that resist these chemicals fungicide was dangerous on health (Lerox,*et.al*.,2002) As a result of these problems, much attention has been directed toward biological factors (*Pseudomonas fluorescens*, *Bacillus* spp, and *Trichoderma* spp .)in resistance to Plant diseases are often caused by pathogens, and the soil is a complex ecosystem teeming with diverse microorganisms that interact with one another. These intricate relationships have been harnessed in innovative ways to combat plant pathogens. Among these relationships, mechanisms of antagonism, parasitism, and competition were found between fungi (Prasad ,*et.al*.,2013).

Bacteria *P.fluorescens* have several mechanisms through which they influence plant pathogens Such as hydrogen cyanide (Voisard et al., 1989) and Siderophore and Pterines, Phenazines, and Pyrroles are considered antibiotics produced by Bacteria (Pyrrolnitrin (Prn) and Pyoluteorin (PLT) such as *P. fluorescens*(Thomashow and Weller,1996).

Phyenazin Carboxylic acid (PCA), Diacetylphloroglycinol (DAPG), and Phyenazin Carboxylic acid (PCA) are the primary interface in the field of biological resistance research, and it has recently been used to describe the chlorination of the genes responsible for the biosynthetic of these compounds (Dwivedi and Johri 2003). At the same time, *Trichoderma* spp. has diverse mechanisms, Such as competition for the site, food, biological antagonism, and enzymatic activity, which affect the plant pathogens (Benhamou, 1993, De meyer,*et.al*., 1998, Barakat et al., 2007). The study was aimed to identify the leaf spot pathogen of swiss chard, and the biological agent evaluation used in controlling it in the laboratory.

II. MATERIALS AND METHODS

1-Isolation and identification of the fungus *A.tenuissima*

Samples of chard exhibiting symptoms of the fungus were collected from the area between healthy tissue and the brown spots on chard leaves. To ensure cleanliness, the leaves were carefully washed under running tap water to remove any dust particles and subsequently allowed to air dry for a period.Then cut up leaves into pieces of 0.5-1 cm and disinfected with sodium hypochlorite at a concentration of 3% for 1 minute, that it was washed with distilled water to remove traces of the solution and dried on filter paper Whattman_NO.l was then transplanted into a petri dish with a diameter of 9 cm containing potato dextrose agar PDA. The dishes were incubated at 25°C for seven days, and the growth of the isolates was observed and purified on the morphology base. Characterization of *A.tenuissima* isolated from leaves depending on the external appearance of the colony, such as color, shape, colony

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diameter, and height using culture media (PDA, PCA), and microscopic characteristics such as size, shape, structure, spores, and other structures according to taxonomic bases. Existing reports in(Ellis, 1971; Woudenberg et al., 2013and Sun,et al., 2023).

2-Molecular analysis

The DNA of the isolated fungi was extracted using a gSYNCTM kit ,and the primers F:TCCGTAGGTGAACCTGCGG: R:TCCTCCGCTTATTGATATGC were used to amplify the ITS1-ITS4 gene region. After the amplification process, the electrophoresis technique was used on an agarose gel. The gel was examined with a gel documentation apparatus to determine the success of the DNA amplification process (Tarini et .al,2010). Then, 20 µl of the amplification product for each isolate was sent to the Korean company Macrogen to determine the sequences of nitrogenous bases in the genes used and then match them with the National Center for Biotechnology Information NCBI and record them.

3-Fungal inoculum development:

Prepare the inoculum of the biological agents separately, using an appropriate amount of the seeds of local millet (*miliaceum panicum*) were washed to remove dust and dirt. After that, it was soaked for six hours, then the water was removed by placing it on blotter paper. The seeds were distributed in 250 ml glass flasks at a 50 g/ beaker rate. Their nozzles were blocked with cotton plugs. Then the seeds were sterilized with an autoclave. The temperature is 121°C, and the pressure is 15 pounds/in for one hour (Dewan,1989). The flasks containing sterilized millet seeds were inoculated with fungi that were meant to be tested individually, with five discs of 0.5 cm diameter from the fungus on the nutrient medium. The beakers were placed in the incubator at a temperature of 25-+2°C for five weeks, shaking the beaker every 2-3 days To ensure that the inoculum was distributed to all seeds.

4- The antagonistic of biological agents against *A.tenuissima in vitro*

The antagonistic biological agents were tested against *A. tenuissima*, at the laboratory of Senior higher studies in the Department of Plant Protection / College of Agriculture, following the method of dual culture technique. PDA culture medium was poured into Petri dishes with a diameter of 9 cm and leave for solidification. The plates were divided into two equal parts by drawing a line at the bottom of the plate. Inoculate the center of the first half with a disk of diameter 0.4 cm. Poison was taken from the edge of the colony of the pathogenic fungus half. The *A.tenuissima* second was inoculated with a disc with a diameter of 0.5 cm from the edge of its colony, one of the fungi used in Biological control. Three dishes were used for each treatment, while the comparison treatment was vaccinated Dishes with the pathogenic fungus *A.tenuissima* Alone (Ghisalberti *et.al*.,1990)

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5-Pots Experiment:

In this experiment, a mixture of soil and peatmoss was used in a ratio of 1:3 w/w. The soil was sterilized using a commercial formalin solution by preparing a solution of (1:50 formalin/water). Use the solution in a ratio of 3 liters of formalin solution / m^3 of soil and after ten Days of sterilization were placed in 1 kg plastic containers, in equal quantities, then Distributed into treatments in three replicates (three pots), the soil was inoculated with the agent's biogenic *P.fluorescens*, *T.viride* and *T. harzianum* and the biological agent *Pacillomyces* sp. grown on millet by 1%Weight/weight. The soil was moistened and left for four days after covering it with nylon bags. The chard seeds were sown in each pot, and ten seeds were planted on the date of 10/3/2013. After the plants grew, they were inoculated with the pathogenic fungus, then the number of spots was calculated—the fresh and dry weight of both shoots and roots.

III. RESULTS AND DISCUSSION

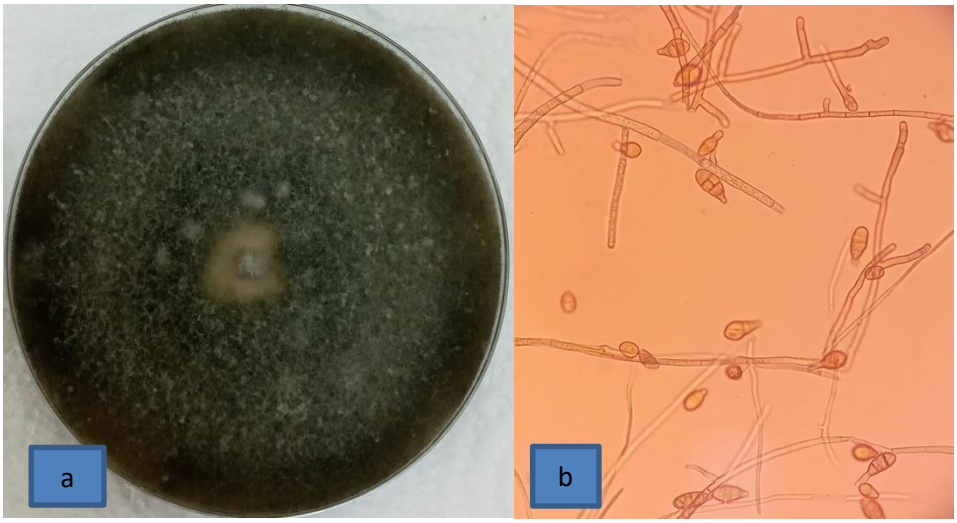

Figure.1.a. *A.tenuissima* growth in PDA :b. conidia of *A.tenuissima*

1.Morphological identification

In Fig.1.a, the growth of *A.tenuissima* in PDA shows the dark green color, and the growth covers the whole Petri dish. Spores were septate with small cylinder shape.

2. Screening of antagonists against *A.tenuissima* under in vitro

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The antagonistic of biological agents against the pathogenic *A.tenuissima* on PDA. The results of Table (1) indicated the superiority of the biological fungus *T. harzianum* that inhibition pathogen, with a percentage of inhibition of 63.8%, was followed by the bioagent *T. viride*. The percentage reached 55.5%, while bacteria had a minor effect on inhibiting pathogenic fungi, reaching 33.3 %. This result agreed with what was preached by Al-Saadoun (2011), as we showed that bioagent *T. harzianum* could inhibit the growth of the pathogen *A.tenuissima* in the middle. The inhibition rate reached 73.3%. The anti-fungal ability may be due to its possession.

Several mechanisms affect the growth of pathogenic fungi, such as the production of degrading enzymes on the cell walls of pathogenic fungi, such as chitinase enzymes and B1,3-glucanase,NA Gase,chitinase,acid phosphatase and alginate lyase(Qualhato,*et.al.*,2013)

Table. 1: Effect of biological control agents in growth inhibition *A.tenuissima in vitro*

Treatments	Inhibition %
T . harzianum	63.8
T. viride	55.5%
P. fumosoroseus	35.3%
P. flourecense	33.3%

3. Effect of biological control agents on Chard leaf spot caused by *A.tenuissima*.

The results of the pot experiment are shown in Table (2) in plants inoculated with fungi
that the treatment of the biological agent *T. harzianum* led to a reduction in the number of spots. The leaves used in the comparison containing the pathogenic fungus only, which amounted to 2.19 spots/leaf to 0.39 spots/leaf in the treatment of the biological fungi and the pathogenic fungus, while it was Bacteria have less effect on the pathogen, as the number of spots in it is 1.62 spots/leaf. It may be due to The possession of a biological factor by one or more mechanisms such as competition for food and space, Fungal parasitism, or the production of enzymes and antibiotics, which all work on the Inhibition of pathogenic fungi (Barakat et al., 2007).

Table.2. Effect of biological control agents in plant infestation of chard plant with *Alternaria* leaf spot

Treatments	No. of spots(spot/leaf)*
T. harzianum+ A. tenuissima	**0.39**

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T. viride+ A. tenuissima	**0.62**
P.flourescens+ A. tenuissima	**1.62**
P. fumosoroseus+ A. tenuissima	**1.32**
Control	**2.19**

*all number was mean of 3 replicates

Table (3) shows the best treatments that led to the reduction of pathogenic, thus Increasing the fresh and dry weight of shoots and roots treatment *A.tenuissima +T. harzianum*, the fresh weight of the two groups reached ~~50.1~~1.50 and ~~80.0~~0.80 g/plant, respectively, and the dry weight was ~~26.0~~0.26 and ~~12.0~~0.12 g/plant, respectively, compared to the control treatment containing pathogenic fungi only for fresh weight. The dry weight of both groups is ~~80.0~~0.08, ~~23.0~~0.23, ~~05.0~~0.50, and ~~04.0~~0.40 g/plant.

Table.3. Effect of biological factors of fresh and dry weight for plants

treatments	**Fresh plant weight***	**Fresh root weight**	**Dry plant weight**	**Dry root weight**
T. harzianum+ A. tenuissima	**1.50**	**0.80**	**0.26**	**0.12**
T. viride+ A. tenuissima	**1.40**	**0.38**	**0.14**	**0.08**
P.flourescens+ A. tenuissima	**1.69**	**0.43**	**0.22**	**0.13**
P.fumosoroseus + A. tenuissima	**1.20**	**0.38**	**0.23**	**0.12**
Control	**0.80**	**0.23**	**0.05**	**0.04**
R.L.S.D 0.05	**1**	**0.13**	**0.15**	**0.07**

As for the ability of the biological factor *T. harzianum* to encourage growth, it may be attributed to the fungus's ability to increase the readiness of some nutrients in the soil, such as phosphorous, potassium, iron, zinc, and copper, and then increase the plant's content of these The elements (Singh and Islam, 2010), or perhaps attributable to the secretion of the biological factor Auxin-like plant growth regulators such as IAA auxins and GA3 gibberellins act in

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concert with mechanisms Others include increasing the absorption and readiness of nutrients for the plant, as it stimulates the growth regulator GA3 Cell growth and expansion due to increased hydrolyzed starch and other multiply sugars And increasing the softness of the cell walls, and then the expansion of cells in the internodes of some plants, as well as Stimulating IAA production, increasing its formation rate and decreasing its breakdown rate (Hasan,2010).

IV. CONCLUSION

In this study , *A. tenuissima* was isolated from the symptoms of swisschard leaf spot in Basra , Iraq .Microscopic characteristics was closed to the features mentioned by (woundenburg et al 2013 ,Khan *et.al*.,2020). DNA sequence data was applied by using Internal Transcribed spacer (ITS) region of the ribosomal DNA (r DNA), both the sequenced data and the blast (software) revealed 98.71% identity with *A. tenuissima* (Genbank accession No. OP 048982.1).This study gave a clear indication that *A . tenuissima* caused leaf spot diseased on chard based on pathogenicity experiment ,these result are in agreement with another studies that showed *A.tenuissima* causing *Alternaria* Leaf Spot on Sugar Beet (*Beta vulgaris*) in Minnesota, U.S.A.(khan et.al ,2020).And leaf spot in date palm (Manea et.al ,2023).

Pot experiments showed that the treatment of the biological agent Trichoderma harzianum led to reduce the number of spot on leaves compare with pathogenic treatment only.Pot experiment also showed that the treatment of biological agent *Trichoderma harzianum* led to reduce the number of spots on leave, and it was the best treatment which increasing of fresh and dry weight of both shoot and vegetative system, the reason for this may be due to Some species of Trichoderma have a significant impact on the agricultural environment, making them unique for their use in agriculture. they colonize plant roots without clear negative reactions (Lopez– Bucio et al., 2015) . *Trichoderma* species have the ability to enhance plant grow by stimulating the plants mechanical defense against pathogens (Harman et al 2004).

V. REFERENCES

1. Barakat,M.R.;AL-Mahareeq F.; Ali-Shtayeh,M.and AL-Masri ,M.(2007).Biological Control of Rhizoctonia solani by Indigenous *Trichoderma* spp. Isolates from Palestine. Hebron University Research Journal.Vol.(3), No.(1), pp.(1 – 15).
2. Benhamou,N.and Chet,I.(1993).Hyphal interactions between *Trichoderma harzianum* and *Rhizoctonia solani*:Ultrastructure and gold cytochemistryofthemy .coparasitic process. Phytopathology 83:1062-107. DOI:10.1094/PHYTO-83-1062.

3. De Meyer, G., Bigirimana, J., Elad, Y. et al. Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. European Journal of Plant Pathology 104, 279–286 (1998) . https://doi.org/10.1023/A:1008628806616.
4. Dewan,M.M.(1989).Identity and frequency of occurrence of fungi in root s of wheat and ryegrass and their effect on _take-all of whaet and host growth. .Ph.D.Thesis. University WesternAustralia,210pp doi: 10.33899/magrj.2010.36223.
5. Dwivedi, D., & Johri, B. N . (2003) . Antifungals from fluorescent pseudomonads: Biosynthesis and regulation. Current Science, 85(12), 1693–1703. http://www.jstor.org/stable/24109974.
6. Ellis, M.B. (1971) Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England, 608.
7. Fernández, R. L., Rivera, M. C., Varsallona, B., & Wright, E. R. (2015). Disease prevalence and symptoms caused by Alternaria tenuissima and Pestalotiopsis guepinii on blueberry in Entre Ríos and Buenos Aires, Argentina. *American Journal of Plant Sciences*, *6*(19), 3082.
8. Ghisalberti, E. L., Narbey, M. J., Dewan, M. M., & Sivasithamparam, K. (1990). Variability among strains of *Trichoderma harzianum* in their ability to reduce take-all and to produce pyrones. Plant and Soil. https://doi.org/10.1007/bf00012323.
9. Harman, G. E.; Howell, C. R.; Viterbo, A.; Chet, I. and Lorito, M. (2004). *Trichoderma* species-opportunistic, avirulent plant symbionts. Nature Reviews Microbiology, 2: 43-56.
10. Hassan Taha, K. (2010). New biotype of *Trichoderma* spp effective in production of some plant growth regulators. Mesopotamia Journal of Agriculture, 38(0), 75-82. https://doi.org/10.1016/j.fitote.2013.06.004
11. Khan, M. F. RHaque, .; M. E. ; Bloomquist,M.; Bhuiyan, M. Z. R. ; Brueggeman,R.; Zhong,S.; Sharma Poudel,R.; Gross,T.; Hakk,P.; Leng,Y. and Y. Liu.(2020). First Report of Alternaria Leaf Spot Caused by *Alternaria tenuissima* on Sugar Beet (*Beta vulgaris*) in Minnesota, U.S.A. Plant Disease .104:2, 580-580. https://doi.org/10.5197/j.2044-0588.2011.023.036.
12. Khan, M.F.R., Windels, C.E. & Bradley, C.A. (2018).Comparison of Cercospora and bacterial leaf spots on sugar beet North Dakota State University Extension Service. https://www.ag.ndsu.edu/publications/crops/comparison-of-cercospora- and-bacterial-leafspots-on-sugar-beet.
13. Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach,J., Gredt, M.Chapeland, F. (2002).Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. Pest Management Science, 58: 876-888.
14. Leslie,J.F;and B.A.summerell(2006).The Fusarium Laboratory manual.388pp. ISBN-13: 978-0-8138-1919-8.

15. López-Bucio, J., Pelagio-Flores, R., & Herrera-Estrella, A. (2015). *Trichoderma* asbiostimulant: exploiting the multilevel properties of a plant beneficial fungus. Scientia horticulturae, 196, 109-123. https://doi.org/10.1016/j.scienta.2015.08.043
16. Manea, Alaa O., Mohammed A. Fayyadh, and Yehya A. Salih. "Efficiency Evaluation of Silver Nanoparticles in the Controlling of the Fungi Associated with the Date Palm Offshoots." Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences 60.3 (2023).
17. Nasehi, A.; Kadir, J. B.; Abidin, M. A. Zainal; Wong, M. Y.; Mahmodi, F. (2012). "First Report of *Alternaria tenuissima* Causing Leaf Spot on Eggplant in Malaysia". Plant Disease. 96 (8): 1226. https://doi.org/10.1094/PDIS-03-12-0237-PDN
18. Ninfali, P., & Angelino, D. (2013). Nutritional and functional potential of Beta vulgaris cicla and rubra. *Fitoterapia*, *89*, 188-199.. https://doi.org/10.1016/j.fitote.2013.06.004.
19. Prasad, R., Kamal, S., Sharma, P. K., Oelmüller, R., & Varma, A. (2013). Root endophyte Piriformospora indica DSM 11827 alters plant morphology, enhances biomass and antioxidant activity of medicinal plant Bacopa monniera. Journal of basic microbiology, 53(12), 1016-1024.
20. Qualhato, T.F.; Lopes, F.A.C.; Steindorff; A.S. Brandão,R.S.; Jesuino,R.S.A and Ulhoa,C.J. (2013). Mycoparasitism studies of *Trichoderma* species against three phytopathogenic fungi: evaluation of antagonism and hydrolytic enzyme production. Biotechnol Lett 35, 1461–1468 https://doi.org/10.1007/s10529-013-1225-3.
21. Singh,A. and Islam,M.N.(2010). In vitro evaluation of *Trichoderma* spp. Against Phytophthora nicotianae. Int. J. Expt. Agric. 1(1):20-25.
22. Sun X, Wang C, Gao X, Wu X, Fu Y.(2023). Characterization of *Alternaria* Species Associated with Black Spot of Strawberry in Dandong, China. Agronomy.; 13(4):1014. https://doi.org/10.3390/agronomy13041014.
23. Tarini, N.M.A.; Wahid, M.H.; Ibrahim, F.; Yasmon, A.; Djauzi, S.(2010). Development of multiplex-PCR assay for rapid detection of Candida spp. Med. J. Indones. 19, 83–87.
24. Thomashow L .S. and Weller D. M.(1996). Current concepts in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites. In: Stacey G, Keen N T, editors. Plant-microbe interactions. Vol. 1. New York, N.Y: Chapman and Hall;. pp. 187–235.
25. Voisard,C;Kell,C;Hass, D.And Defago,G;(1989).Cyanide production by *pseudomonas fluorescence* helps suppers black rot of tobacco under gnotobiotic conditions .EMBO .J;8:351-35. DOI: 10.1002/j.1460-2075.1989.tb03384.x.
26. Woudenberg, J. H. C., Groenewald, J. Z., Binder, M., & Crous, P. W. (2013). Alternaria redefined. *Studies in mycology*, *75*(1), 171-212., https://doi.org/10.3114/sim0015

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