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The Efficiency of *Streptomyces antibiotics* as a Biocontrol Agent for Damping Off Disease of Sunflower (*Helianthus annuus*)

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ABSTRACT

In pot experiments, a local isolate of the Actinomycete Streptomyces was used as a biocontrol agent against Macrophomina phaseolina, the causative agent of damping-off in sunflowers (Helianthus annuus L.). Seeds and soil treatments with S. antibiotics resulted in a significant decrease in the percentage of infected plants. Disease severity of damping off of the seedlings reached (31%), As compared to untreated seeds planted in soil contaminated with M.phaseolina, which was(40.12%) and (0.40) respectively, However, seed treatment with the biocontrol agent was superior to soil treatments in causing more reduction in both percent infection and disease severity of seedlings damping off reached (20.63%) and (0.21) respectively, and also improving growth characters of the plants. The ability of S. antibiotics to produce the hormone (IAA) was confirmed, and IAA was separated and purified by TLC. Quantitative determination by HPLC showed that the IAA concentration was 7.59 mg/L.

Keywords: *Streptomyces antibiotics, Macrophomina phaseolina*, Biocontrol, *Helianthus annuus*

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كفاة البكتريا *Streptomyces antibiotics* في المكافحة الحيوية لمرض سقوط بادرات وتعفن جذور زهرة الشمس *Helianthus annuus*

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قسم علوم الحياة، كلية التربية، جامعة الموصل، الموصل، العراق

الملخص

تم استعمال العزلة المحلية من البكتريا الخيطية *Streptomyces antibiotics* لغرض المكافحة الحيوية للفطر *Macrophomina phaseolina* المسبب لمرض سقوط بادرات نبات زهرة الشمس، فقد أدت معاملة التربة وبذور نبات زهرة الشمس بهذه البكتريا الى تقليل نسبة وشدة الاصابة بموت البادرات المتسبب عن الفطر *M.phaseolina* قبل وبعد ظهورها فوق سطح التربة. في تجارب السنادين. ادت معاملات التربة بعزلة البكتريا الى خفض معنوي في نسبة الاصابة وشدتها بلغت (31%) مقارنة بالبذور غير المعاملة بالبكتريا والمزروعة في الترب الملوثة بالفطر *M. phaseolina* اذ بلغت (40.12) (0.40). الا ان معاملات البذور الملقحة بعزلة البكتريا تفوقت على معاملات التربة بها إذ اعطت أقل نسبة إصابة بموت البادرات الكلي وشدتها بلغت (20.63%) و (0.21) على التوالي وكذلك في تحسين معايير النمو في النبات. في اختبار قدرة البكتريا الخيطية *S. antibiotics* على إنتاج هرمون اندول حامض الخليك أظهرت النتائج قدرة العزلة على إنتاج هرمون أندول حامض الخليك (IAA) عن طريق الكشف عنه وفصله وتنقيته باستخدام (TLC) وأشارت نتائج التقدير الكمي بتقنية كروماتوكرافيا السائل عالي الاداء High-Performance Liquid Chromatography (HPLC) الى انتاج العزلة للهرمون (IAA) بمقدار (7.59) ملغم/التر.

INTRODUCTION

Actinomycetes are among the most promising candidates for biological control and have received considerable attention in future studies. Many attempts have been made to develop biological control and root bacterial preparations for commercial use. [1]. Although Streptomyces are used in biological control, studies confirm that they promote plant growth. [2] Rhizobacteria have not been sufficiently studied as plant growth promoters. Rhizobacteria (PGPR). The synergy between plants and bacteria can enhance control of pathogenic organisms through improved methods.[3, 4]. Streptomyces are among the most delicate soil organisms, yet they are highly efficient at colonizing plant root systems and can withstand unfavorable growing conditions because they form spores.[5]. The Gram stain readily stains actinomycetes, a group of bacteria, especially species of the genus Streptomyces, that are widely distributed in nature and are also known as saprophytic soil bacteria. [6]. Microphomina phaseollina causes damping-off disease in sunflowers, which poses a high risk to the plant, and can persist in the soil on infected plant remains from season to season. [7].

To improve plant growth, about 80% of the bacteria in the rhizosphere secrete the growth regulator Indole Acetic Acid (IAA), such as Streptomyces olivaceaviridis, S.rochei, S.rimosus, and S.antibiotics [8].

The study aimed to control the fungi that cause damping-off and charcoal rot of sunflower (Helianthus annuus) roots and to improve growth properties by locally isolating Streptomyces antibiotics.

MATERIALS AND METHODS

Isolation of Actinomycetes:

Actinomycetes isolation Agar medium was prepared according to the instructions of the company and meal (22)gm from the medium in (1) liter of distilled water, then the pH was controlled at (8.1) and sterilized in an Autoclave. In the isolation method, the dilution plate technique was used on soil samples containing CaCO3, and the plates were incubated at 30 °C for 4 days. After that, the extracted soil is made using Ringer's solution, and the diluted samples are mixed with Actinomycetes Agar on an Actinomycetes Agar plate and incubated at 28 °C ±1 for (7-14) days [2, 9].

In a separate study, Streptomyces antibiotics isolated from the rhizosphere of sunflower plants were selected from isolates exhibiting the highest inhibition of M. phaseolina growth (inhibition zone of 30 mm), identified on a starch medium in an antagonism test, and subsequently used in the current study.

Depending on the Agar well diffusion method [2]. The Streptomyces antibiotic isolates were transferred to a well on a potato and sucrose medium (PDA) containing Macrophomina phaseolina, then incubated at 28 °C ±1 °C for 28 hours, and the results were recorded by measuring the growth-free zone. [7, 8].

Isolation of *Macrophomina phaseolina:*

The fungus causing damping off and charcoal rot of the roots of the sunflower plant was isolated by [10]and then the developing fungal growths were examined. The purification of the growing fungus was performed on a slant. Then, the diameter was defined by following the taxonomic properties indicated in [11]. An antagonism ability test was performed to prove the definitive relationship between the pathogenic fungus and the host plant. [12].

Greenhouse experiments:

Soil sterilization

The soil was sterilized with formaldehyde at a concentration (of 10%) after that the soil was covered and left for (15) days and then exposed to air for (7) days, taking into account stirring it every day to get rid of formaldehyde residues, the soil was distributed into anvils of (9) kg per anvil, and the compound fertilizer NPK was added at a rate of (5) g / anvil.

Preparation of soil inoculation:

Millet seeds were used to obtain M. phaseolina inoculation. This method was done according to [13]. The surface layer was contaminated with the fungus M.phaseolina, and (30 g) of millet seeds contaminated with fungus were mixed well with sterile soil and sprayed with water**.**

Preparation of spore suspension for the actinomycetes *S. antibiotics* :

To treat the seeds of the sunflower plant with a suspension of spores of Streptomyces, a suspension of spores of the actinomycete Streptomyces was prepared according to [14], and the seeds were immersed in the spore suspension and placed in the vibrating incubator for an hour with the addition of molasses by (5%), then removed from the incubator and left in laboratory conditions for (20) hours before planting, while the control treatment immersed its seeds in sterile distilled water and after (3 days) of soil treatment with the pathogenic fungus M.phaseolina Seeds were planted at the rate of (20) seeds per anvil capacity of (9) kg soil and three replication for each treatment and the germination rate was calculated after a week of planting. Soil treatments were also carried out according to the modified method [15]. Method by adding a spore suspension of Streptomyces at a rate of (7.5) ml to the soil per anvil, taking the vaccine from a 10-day farm with a concentration of (5×410) ml spores before planting. The soil was treated with Streptomyces suspension by adding (7.5) ml of bacterial suspension per anvil one month after planting the seeds. The number of plants in each anvil was reduced to (3) plants only after (45) days of planting. The wirehouse is prepared and Affiliated with the Department of Biology / College of Education, University of Mosul. The following Treatments were

carried out, in which the biological control agent S. antibiotics was used to treat sunflower seeds of the variety.

The results were calculated by measuring the incidence of seedling damping-off before emergence and after a week of planting, and by measuring the final incidence of damping-off after emergence and the severity of infection after two weeks of planting. The severity of the injury was calculated by following the McKinney equation of (0-3) degrees. [16, 17].

The results were analyzed statistically using a complete random design (CRD) in SAS, and the averages were tested using Duncan's Multiple Range Test.

Detection of Indoleacetic Acid hormone (IAA)

The IAA was detected and purified from the extract by ethyl acetate extraction. Using TLC panels, the spots were shown by spraying the board with Salkowski reagents, as pink indicates the production of (IAA). At the same time, brown refers to the production of (IAA) when spraying the panel with the Dragendroff reagent and showing the results of quantitative determination of the hormone (IAA) using HPLC. A yeast and malt extract medium was prepared to determine whether the selected isolate could produce IAA. [18, 19].

Extraction and purification of IAA by Thin Layer Chromatography (TLC)

IAA was extracted from the Streptomyces culture using the method described by [18]. After evaporation, the residue on the wall was dissolved in methanol and then applied to the TLC plate using a hexane:Ethyl acetate (20:80, v/v) solvent system. Before the solution reached the end of the plate, the plate was removed and left to air-dry. One of the panels was sprayed with a Salkowski reagent to determine the IAA. Salkowsky reagent and the other with the Dragendorff reagent [19].

Separation (IAA) using High-Performance Liquid Chromatography (HPLC)

After extracting IAA by thin-layer chromatography, it was scraped from the TLC plate and dissolved in methanol. IAA aggregates were separated using the HPLC type SHIMADZU type C18 Column, Acetonitrile: Acetic Acid, Water, and in proportions: (35:65:1), and the speed of flow (1) ml/minute, and wavelength 280 nanometers. [19, 20] in the lab of the biology department of Mosul University.

RESULTS AND DISCUSSION

Isolation *of M. phaseolina* :

The fungus was isolated from the roots and stems of the infected sunflower seedlings. After purifying the fungus, a person following the taxonomic properties indicated by[13] As its colonies are fast-growing, the fungal yarn is initially transparent to white, then turns black (Figure 1). This transformation is central to the inclusion of the entire colony. Growths appear with a highly fluffy appearance above the colony. Upon microscopic examination, the stone bodies are spherical, and symptoms are characterized by wilting, a sudden apex of the plants, followed by drying and blackening, with blackening of the stem beginning at the bottom and progressing to the top. As the infection progresses, the roots turn reddish-brown; the affected plants dry out, and the roots become dark gray, approaching black.

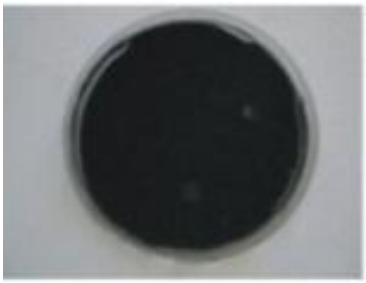

Fig. 1: Show *Microphamina phasolina*

The Streptomyces isolate was identified and showed a gray color in the medium (Figure 2-A). The microscope showed filaments divided by septa Figure (2-B). Figure 3 shows the antagonistic activity of Streptomyces antibiotics against Microphomina phasolina using the agar well diffusion method.

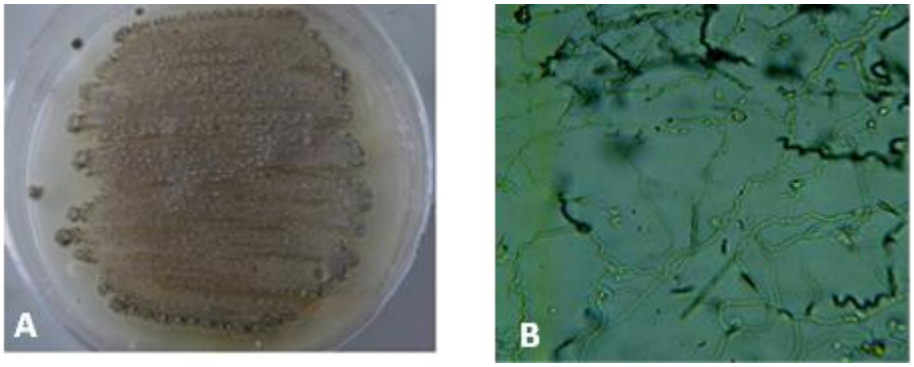

Fig. 2: A- Show an isolate of Streptomyces antibiotic iv plate. B-aireal Hypha of S. antibiotic under microscope (40x).

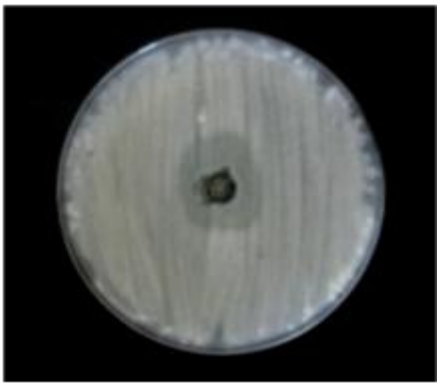

Fig. 3: Shows the antagonism ability of *Streptomyces antibiotics* to *Microphomina phasolina* by agar well diffusion method

Effect of soil and seed treatments with S. *antibiotics,* Figure 4, on the percentage of infection before and after emergence, and its severity with the fungus *M.phaseolina.*

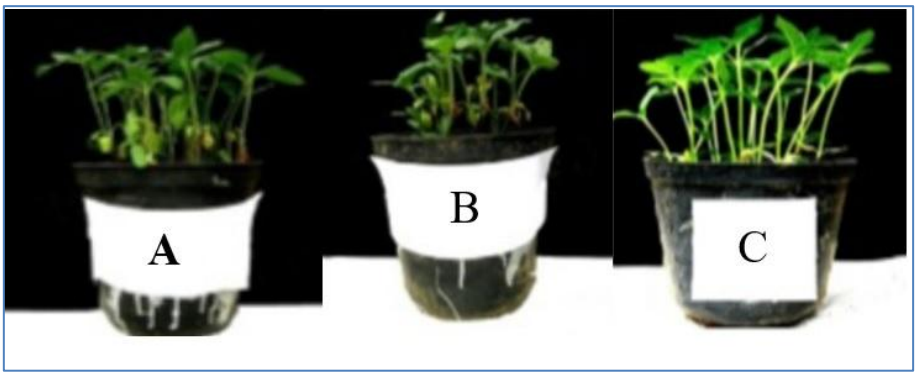

Fig. 4: shows (A) control, (B) *Macrophomina phasolina*, (C) *M. phasolina* + *Streptomyces antibiotic*s

The results of Table 1. show that the soil and seed treatments treated by S. antibiotics showed a significant reduction in the percentage of infection of sunflower seedlings before and after their appearance above the soil surface and the severity of their illness with the disease, and with a significant difference from the rest of the soil treatments contaminated with M.phaseolina and the treatments of seeds inoculated with bacterial isolation outperformed the soil treatments treated with M.phaseolina, as it gave the lowest incidence of total seedling damping off and severity (20.63), (0.21) M.phaseolina compared to untreated seeds grown in soils treated with fungi, which amounted to (40.12), (0.40) respectively. No percentage of infection was observed in sterile soils not treated with pathogenic fungi and planted with treated or untreated seeds with bacterial isolation, as the incidence rate was (0.00).

Table 1: Effect of Treatments *with S.antibiotics* on Seed germination and Seedling damping off and Severity of Sunflower Plants Growing in Soil treated with *M.* *phaseolina.*

#	Treatments	Impression Percentage			Severity of injury
		Total seedling damping off	After the appearance	Before appearing	
1	Untreated seeds in sterile soil (control)	0.00 D	0.00 D	0.00 C	0.00 D
2	Soil contaminated with the fungus *M. phaseolina*	40.12 A	23.33 A	18.33 A	0.40 A
3	Sterile soil treated with *S.antibiotics*	0.00 D	0.00 D	00.0 C	0.00 D
4	soil treated with S.*antibiotics* and *M. phaseolina*	31.0 B	16.66 B	13.33 B	0.31 B
5	seeds treated with *S.antibiotics*	0.00 D	0.00 D	0.00C	0.00 D
6	*S.antibiotics treated seeds planted* in contaminated soil *M.phaseolina*	20.63C	13.33 C	8.33 A	0.21 C

*Similar letters indicate that there are no significant differences between treatments in one column at the probability level (0.05) according to the Dunkin' multi-range test

Effect of soil and seed treatments by *S.antibiotics* isolation on the growing qualities of sunflower plant in soils treated with *M. phaseolina.*

The results of Table(2) indicate that the soil treatments contaminated with the fungus *M.phaseolina* led to a significant reduction in plant specifications and a significant difference from non-contaminated soils, and pollination of soil and seeds with bacterial isolation led to an improvement in the studied plant growth standards The treatment of

seeds treated with bacteria and planted in non-treated soil (control) gave the highest specifications for plant growth amounting to (48.9) cm (6.6) cm, (0.822) g for each of the seedling height, root length and dry weight of the plant respectively compared to seeds that are not pollinated with bacteria. They reached (38.0) cm (4.0) m (0.652) g, respectively.

These findings are corroborated by[21], who reported that treating tomato seeds with Streptomyces isolates and then planting them in soil contaminated with F. increased the efficiency of these isolates in combating the pathogen. They noted that the wet and dry weights of seedlings transferred to contaminated soil and treated with bacteria were higher than those treated with bacteria alone.

Table 2: Effect of *S.antibiotics Isolated Soil and Seed* Treatments on Growing Characteristics of Sunflower Plants Developing in Soils Contaminated with *M. Phaseolina*

#	Treatments	Seedling dry Weight (gm)	Root length (cm)	Seedling height (cm)
1	Non-treated sterile soil (control)	0.652 C	4.0 C	38.00 B
2	Soil treated with *M. phaseolina*	0.195 E	2.5 D	24.8 D
3	Soil treated with *S.antibiotics*	0.786 B	5.0 B	47.66 A
4	S.antibiotics *treated soil* contaminated with *M.phaseolina*	0.475 D	3.3 CD	32.76 C
5	*S.antibiotics treated seeds* planted in sterile soil	0.822 A	6.6 A	48.93 A
6	*S.antibiotics treated seeds* planted in soil contaminated with the fungus *M.phaseolina*	0.496 D	3.5 C	34.26 BC

* Similar letters indicate that there are no significant differences between the coefficients in one column at the probability level (0.05) according to Dunkin' multi-range test

This may be due to a greater number of Streptomyces forming around the roots of seedlings when planted in sterile soil. Their transfer to soil contaminated with fungus led to greater antagonism, which may have degraded fungal cell walls, making fungal cell components a nutrient for Streptomyces and the plant, thereby increasing the wet and dry weights of the treated plants.[22]. Mentioned the Production of Streptomyces species toxic substances (toxins), which cause the cessation of vegetative growth and the death of fungal spores of pathogenic fungi in a direct way. He also pointed out that Streptomyces can act as plant growth inducers and root settlement even at concentrations of 1-10 units[23] . The bacterium S. antibiotics have used it. As a biological control agent, it demonstrated a superior ability to reduce the pathogenicity of F. solani in both sensitive and resistant soybean varieties, reducing the incidence rate from 94.39% to 22.6%, respectively.

One of the mechanisms of biological control of pathogenic fungi is related to the formation of the fungal cell wall from chitin and β-glucan, as the enzymes Chitinase B 1-3β-glucanase, which are biological control agents, including actinomycetes, secrete, are responsible for the decomposition and destruction of the fungal cell wall. [23, 24].

It is beneficial to use several plants growth-promoting rhizobacteria (PGPR) isolates on certain crops. PGPR can affect plant growth in two ways, directly or indirectly, as indirect stimulating effects occur when PGPR works to reduce or prevent the harmful effects of one or more harmful microorganisms, and this is mainly through biological control or in contrast to pathogens endemic in the soil, especially settlement or Antibiotic biosynthesis [25]Secondary metabolites

can prevent the invasion of secondary pathogens. The direct method occurs by stimulating plant growth effects by PGPR when plants are equipped with compounds that are synthesized by bacteria that work to absorb the plant soil nutrients and potentially by fixing nitrogen, synthesizing chelating substances and plant hormones, and dissolving mineral substances to make them available for absorption by the plant The manufacture of IAA is believed to be a characteristic of the root environment and the symbiotic bacteria of the plant, which stimulate and facilitate plant growth. [26, 27].

Indole Acetic Acid (IAA) Purification

S. antibiotics have shown a significant ability to produce IAAs, with several studies indicating the ability of species belonging to the genus *Streptomyces sp* found in the root periphery of the plant to produce IAA[20]As Figures 1,2 the HPLC analysis of the production of (IAA) by S. antibiotics reached (7.59) mg/L using yeast and barley extract medium (YME), and with the addition of tryptophan, with an incubation period of (96) hours.

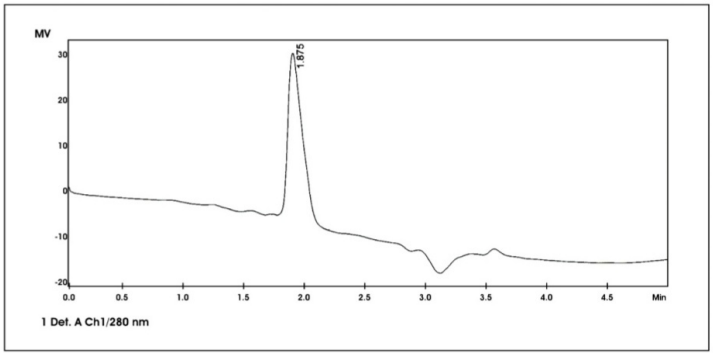

Fig. 1: Standard curve of IAA

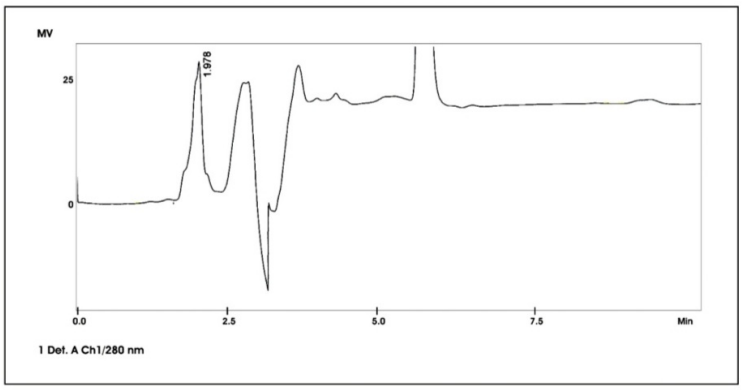

Fig. 2: Production of IAA by *S. antibiotics* using HPLC

[29] found that *S.aldolase* mediated by the enzyme tryptophan-2-monooxygenase, which in turn converts to IAA mediated by the enzyme IAM-hydrolase [28, 29].

It starts with the production of (IAA) after (24) hours of incubation and reaches the maximum production after (96) hours of incubation and indicated that the maximum production is during the stationary phase The production of (IAA) by this isolation reached (34) µg/ml ' Under optimal conditions, IAA is synthesized through the Indol-3-acetamide (IAM) pathway. In this pathway, tryptophan is converted to indole-3-acetic acid

[30] found that the species S. olivaceus, S. coelicolor, and S. purpurascens produced IAA at concentrations of 28.4, 21.8, 14.2, and 15.5 µg/ml, respectively.[28]mentioned that IAA produced by soil microorganisms is enhanced by tryptophan found in root secretions or cells [28]. S.viridis, isolated from the root periphery of the Cymbagon

citrebus plant, has shown its ability to produce IAA at high levels. [8] pointed out that the genus *Streptomyces* isolated from the root periphery of the tomato plant can produce IAA, which stimulates plant growth by increasing the dry weight of the root.

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Author contribution: Authors contributed equally to the study.

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