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Suppression of Seeds Mycoflora Involved on Cotton Seedling Damping-Off Disease by Chitinolytic Actinomycetes

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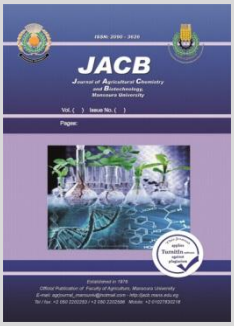

ABSTRACT

Actinobacteria as biological pesticides are considered an economical and safe method, especially for improving plant growth. In this study, five actinobacterial isolates were obtained from farming soil samples and evaluated for their biocontrol capacity against five or more fungal isolates that cause seedling damping-off disease in cotton. From non-sterilized cotton seeds, the frequency (%) of isolated fungi was as follows: *Rhizoctonia solani* (25.12%), *Fusarium* spp. (24.82%), and *Macrophomina phaseolina* (22.12%) were the most dominant fungi. Other fungi were found at lower frequencies, including *Penicillium* sp. (5.44%) and *Aspergillus* sp. (5.12%). When Koch's postulates were applied, the fungal isolates caused the same symptoms of pre- and post-emergence damping-off, as well as reduced seedling survival. *R. solani*, *Fusarium* spp., and *M. phaseolina* exhibited high virulence in cotton seedlings, while *Penicillium* sp. and *Aspergillus* sp. were considered moderately virulent. Several morphologically distinct actinomycete isolates were screened for chitinase production. The actinomycete isolate that produced the highest chitinase activity, indicated by the formation of a clear zone, was identified. This isolate was found to match *Streptomyces gelaticus* with 99.73% similarity. Antagonistic tests showed that this *Streptomyces gelaticus* strain inhibited all fungal pathogens. The highest inhibition zones were observed against *Rhizoctonia solani*, *Fusarium* spp., *Penicillium* sp., and *Aspergillus* sp., while *Macrophomina phaseolina* exhibited the lowest inhibition. In conclusion, actinomycetes, particularly *Streptomyces* spp., hold great potential as biocontrol agents for sustainable agriculture and the management of plant diseases.

***Keywords*:** Actinomycetes, Chitinolytic, Seed-borne fungi

INTRODUCTION

Seeds containing fungi consist of both field and storage fungi. Field fungi typically invade cotton seeds before harvest, while storage fungi grow on seeds during storage (Amer 1986). Under Egyptian conditions, there are several fungi with seeds of cotton contained such as spp. of *Aspergillus*, *Fusarium*, *Penicillum* and others as well as *Rhizoctonia solani* (Amer 1986). The main of these organisms include fungi contributing to the seedling disease complex of plant as *Gossypium* spp. (Davis *et al.* 1981). Disease symptoms include seedling root rot and pre- and post-emergence damping-off, which individually or in combination lead to stand reduction and decreased seedling vigor, ultimately delaying growth and maturity (Bastson and Trevathan 1988). Non-sterilized seeds of cotton were contains seed-borne fungi. The observed fungi were *Aspergillus* spp., *Penicillium* spp., *Alternaria* spp., *Fusarium* spp. and *Rhizopus* spp. (Abd-Elsalam *et al*. 2023) and others fungi were unknown.

Soil microorganisms include a large group of Actinobacteria. These prokaryotes are known for producing secondary metabolites and actinomicrobial compounds that contribute to the biological control of plant pathogens and promote plant growth as plant growth-promoting rhizobacteria (PGPR).(Franco-Correa *et al.* 2010 & Afify and Ashour 2024). Plant protection has been reviewed by Krishnaraj *et al.* (2014) & Ashour and Afify (1999) who reported that the important role of Actinomycetes. The antagonistic compounds are antifungal, antibacterial compounds and extra cellular enzymes (Afify 2024). These compounds are considerd as activity of Streptomyces (Srividhya *et al*. 2012). Also, Silva *et al.* (2022) reported that Actinomycetes are important microbial communities in the soils with increasing agricultural applications, especially in the biological control of insect-pest and plant disease. To develope plant growth Actinonycetes are producing antifungal as biocontrol agents against fungal pathogens. The main group of Actinobacteria are Streptomycetes, they are found in the soil microorganisms in the rhizosphere of plants with soil microflora such as bacteria and fungi. (Ashour and Afify 1999 & Chakraborty *et al*. 2023). Many antifungal compounds have been produced by the genus Streptomyces, which is considered a promoter of plant growth and a suppressor of plant diseases. (Afify and Ashour 2018 & Jose *et al*. 2019). Chitinase and others lytic enzymes have been produced by all potential actinomycetes isolates (Budi *et al*. 2022). These Actinomycetes are known as biocontrol agents directly and indirectly (Chakraborty 2019 & Afify and Ashour 2024).

Herein, we report an investigation in which pathogenic fungal isolates were isolated and re-isolated to apply Koch's postulates and identify these fungi in the seeds of an Egyptian cotton cultivar to evaluate their effects on cotton seedling damping-off disease.Additionally, this study focused on isolating actinomycetes from soil capable of producing chitinase. The selected isolate was identified and tested for its ability to suppress the growth of cotton seed-associated fungal isolates.

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MATERIALS AND METHODS

Isolation of phytopathogenic fungi

Naturally infected plants seedlings showing damping-off symptoms were collected from samples of cotton seedlings (cultivar Giza 86) with typical damping-off symptoms and the samples were collected from different regions at Kafr El-sheikh Governorate. For isolation plate containing PDA medium were prepared to place small pieces of pathogens from infected roots, after that incubated at 20ºC for only week (Sneh *et al.* 1991). For isolation and purification were used hyphal tip and single spore techniques. By using binocular microscope or light microscope for examination each colony. Identification was done according to Booth (1971) & Domsch *et al.* (1980) and some isolates were identified to species level verified by Mycological Centre, Assiut University (Omar *et al.* 2015). Frequency as the percentage of seedlings for each fungus isolates were recorded. The isolates were cultured and maintained on PDA slants for further studies.

Pathogenicity test

Pure fungal isolates were tested for their pathogenicity on pots (20cm) trails filled with clay soil (pH7.5 clay 62.1 %, E.C. 1.4 mmhos/cm) with four replications. Before sowing soil infestation adding as 10 ml of each fungal culture conaining $5x10^6$/ml to 1kg of soil and mixed thoroughly. Seeds of the tested cultivar (Giza 86) were sown at the rate of 10 seeds /pot. Uninoculated soil was served as control. Damping-off disease was observed and calaulated as pre- and post- emergence after 15 and 45 days, respectively.

From diseased cotton seedlings, the pathogenic fungal isolates were re-isolated to meet koch postulates.

Assay seedlings disease variables of cotton

According to Klich (1986) soil was infested individually with each of the tested fungi. Ten non sterilized seeds for each pot were planted on glasshouse and recorded the following parameters:

1- Survival%
2- Pre and post-emergence damping-off

Isolation of Actinomycetes

Actinomycetes colonies were isolated from farming soils samples described by Oskay *et al.* (2004). Colonies of Actinomycetes were cultured on Starch Nitrate agar (ISP2) and Yeast-Malt Extract agar (YEME) plate (Shirling and Gottlieb 1996) and incubated at 28-30ºC for week. After incubation period the colonies of Actinomycates are characterized on culture medium as sharp round edges are maintaned on slants for used.

Chitinase production

Chitinase production by isolates of actinomycetes were determined using media supplemented with 0.1% colloidal chitin (Vyas and Deshpande 1989). Each plate was inoculated with a old isolates of actinomycetes on a Yeast-Malt Extract (YEME), after incubation time clear zone were seen and measured in (mm) around the colonies as an indicator of chitinase production (Duncan *et al.* 2006).

Identification of chitinolytic actinomycete isolate

Determination of the spore-bearing hyphae and spore chains morphology were done by cover slip culture technique (Kawato and Shinobu 1959) or by direct microscopically examination to the surface of the culture on the growth plates.

Detection of antagonists between actinomycete isolate and pathogenic fungal isolates

Actinomycete isolate shown to be potent antagonists test to some isolated pathogenic fungi *in vitro* using Potato Dextrose Agar (PDA) medium. The medium were inoculated in Petri dishes containing (10 ml) with l disks (7-mm-diam.) of each fungal pathogens as old culture which placed at the periphery of the plate. The chitinlytic bacterium was streaked at the center of each plate by a loop of bacterial culture broth. Antagonistic effect detected by observation inhibation zone was done. Petri dishes inoculated with all tested fungal pathogens were served as control.

Statistical analysis of data

ANOVA were used to analyse the obtained data and to evaluate significant differences between the treatments.The differences of P=0.05 were considered as siginificant. Data from results were obtained to analyze according to Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Fungi isolated

Pathogenic fungi were isolated and identified by team work of Plant Pathol. Lab., ARC, Giza, Egypt, during working in the Department programs**.** The mean percentage of pathogenic fungi isolated (Table1) showed that *Rhizoctonia solani* (25.12%), *Fusarium* spp. (24.82%), *Macrophomina phaseolina* (22.12%) were the most dominant fungi and other fungi few occurred at frequencies as *Penicillium* sp. (5.44%), *Aspergillus* sp. (5.12%) isolated from the non sterilized cotton seeds.

A major seedling problem in cotton is damping-off disease caused by *R. solani* (Lifshitz *et al*. 1984). Under favorable conditions, *Rhizoctonia solani* and *Fusarium* spp. attack the plant at seedling stage causing great economic losses (Nyvall 1981). *Fusarium* species are one from several important pathogens in the aetiology of cotton damping-off in Egypt. Genus Fusarium and others fungi are recorded as a causers of disease in soil borne fungi (Abd-ELSalam *et al*. 2006). Phytopathogenic fungi infections, such as those caused by *Rhizoctonia solani*, result in significant yield losses in several economically important crops (Ashour and Afify 2024b).

Table 1. Frequencies (%) of isolated pathogenic fungi from cotton seeds of cultivar Giza 86

Fungi	Frequencies (%) of fungi isolated
Rhizoctonia solani	25.12
Fusarium spp.	24.82
Macrophomina phaseolina	22.12
Penicillium sp.	5.44
Aspergillus sp.	5.12
Unknown of fungal genera	17.38

Pathogenicity tests

Fungal isolates were tested for their pathogenic capability against cotton cv. Giza 86. Data presented in Table (2) indicate that all tested fungal isolates were able to cause symptoms of pre-emergence and post-emeregence damping-off of cotton seedlings. Data also indicate that *R.solani*, *Fusarium* spp. and *M. phaseolina* showed high virulance to cotton seedlings, while isolates *Penicillium* sp. and *Aspergillus* sp. were consider as middle virulent.

Moreover, the tested fungal isolates varied regarding their virulence. Variation in virulence of the pathogenic isolates on cotton plants may be due to the presence of differences among the fungal isolates. These results are also in harmony with the results of William and Asher (1996).

Table 2. Pathogenicity test of fungal isolates pathogens on cotton cv. Giza 86

Fungal isolates	Damping-off (%)		Healthy survivals
	Pre-emergence	Post-emergence	
Rhizoctonia solani	60	20	20
Fusarium spp.	40	29	31
Macrophomina phaseolina	41	24	35
Penicillium sp.	24	16	60
Aspergillus sp.	22	10	68
Control	0	0	100
L.S.D. 5%	5.22	4.26	6.15

Isolation of Actinomycetes

Five morphologically Actinomycetes were isolated from farming soil. The most highest isolate of Actinomycetes which produced chitinase showed by clear zone formed was chosen. When five Actinomycetes isolates compared according to calculate by the mean values of clear zone diameter (mm) for each isolate. A2 isolate was ranked as the most high chitinolytic actinomycete producer (Table 3) and was taken for identification.

Table 3. Screening of Actinomycetes isolates for chitinase enzyme production

Isolates code	colony diameter (mm)	clear zone diameter (mm)
A1	11.05	25.13
A2	11.93	26.76
A3	8.22	13.80
A4	6.30	11.33
A5	5.00	9.16

Taxonomic of the most potent actinomycete isolate (A2) producing-chitinase

The cultural, morphological and biochemical characters of actinomycete isolate (A2) were presented in Table (4). Actinomycete isolate (A2) has aerial mycelium with spore chains. They grow as fungi with hyphae and characterisitically by " earthy odor of soil (Sprusansky *et al.* 2005). Colony of actinomycetes contain two types of mycelia, each mycelium have especial role. For growth, substrate mycelium (vegetative) plays a role in absorbing nutrients (Hastuti 2014). Liu *et al.* (2016 recorded that the genus *Streptomyces* has long chains of spores and commonly referred to as polyspores form chains.

Table 4. Morphological characteristics of actinmycete isolate (A2)

Parameters	Actinomycete isolate (A2)
Aerial mycelium	+
Spore chain	+
Gram stain	+
Growth of colony and mycelium on Yeast-Malt Extract medium:	
Colony color	Brown
Pigment production to media	-
Under colony	Brown
Growth of colony and mycelium on Starch Nitrate agar medium:	
Colony color	Grey
Pigment production to media	Yellow
Under colony	Yellow

Note:
+ Presence of parameter ; - absence of parameter

In addition, aerial mycelium was covered *Streptomyces* colonies. Colony of actinomycete isolate A2 was produced pigments. These pigments were grey and yellow on ISP 2 and YSA media. *Streptomyces* grow as filamentous mycelia in the soil. The spores germinate into a new mycelium as genus Streptomyces. Genus Streptomyces belongs to family Streptomycetaceae (Koepff *et al.* 2018). Actinomycetes (Actinobacteria) are Gram-positive bacteria that grow in a variety of environments and have a filamentous shape similar to fungi. The morphologically of actinomycetes distinguish by forming a layer of hyphae that carry chains of spores in aerial mycelium (Alenazi *et al.* 2023).

Molecular identification of selected actinomycetes

The GenBank database indicated that actinomycete isolate have similarities with *Streptomyces gelaticus* (99.73%), based by Osman *et al.* (2020). This strain known as a biocontrol agent in suppressing the growth of many pathogenic fungi in plants.

Growth inhibition of fungal pathogens isolates by chitinolytic actinomycete isolate A2

Isolate of actinomycete (A2) tested by antagonism technique showed inhibition of all fungal pathogens isolated. The inhibition zone recorded with *Rhizoctonia solani*, *Fusarium* spp., *Macrophomina phaseolina, Penicillium* sp. and *Aspergillus* sp. The highest inhibition zone was shown with fungal pathogens *Rhizoctonia solani*, *Fusarium* spp., *Penicillium* sp. and *Aspergillus* sp. While the lowest inhibition was *Macrophomina phaseolina* (Table 5). Bacteria as Actinomycetes group are characterized as biocontrol agents against several soil-borne pathogens because they are able to suppress the growth of pathogens by produce bioactive compounds (Adegboye and Babalola 2012). In the antagonistic test reported by Lim *et al.* (2018), many species from genus Streptomyces produced antifungal materials that played an important role to inhibit the growth of phytopathogens. Actinomycetes are able to inhibit the growth of several soil-borne pathogens such as *F. oxysporum, R. solani* and *A. flvus* (Aouar *et al.* 2020). Also, Actinomycetes are important microbial communities in the soils according to Silva *et al.* (2022) who reported that for increasing agricultural applications, especially in the biological control of insect-pest and plant disease. The bacterial isolates exhibited multiple antifungal substances for example hydrolytic enzyme chitinase (Ashour and Afify 2024a)

Table 5. Rate of growth inhibition fungal pathogens isolates by actinomycete isolate A2

Fungal isolates	Rate of growth inhibition by actinomycete isolate A2
Rhizoctonia solani	++
Fusarium spp.	++
Macrophomina phaseolina	+
Penicillium sp.	++
Aspergillus sp.	++
Control (only fungus)	Full growth
Control (only actinomycete isolate A2)	Normal growth

Note:
++, Inhibition of pathogen by over actinomycete isolate A2
+, Inhibition of pathogen

CONCLUSION

Five fungal isolates involved on cotton seedling damping-off disease. Isolated fungi from seeds of cotton were *Rhizoctonia solani*, *Fusarium* spp., *Macrophomina*

phaseolina were the most dominant fungi and *Penicillium* sp., *Aspergillus* sp. were a few occurred. When application koch postulates, fungal isolates recorded symptoms of pre- and post-emeregence damping-off as well as seedling survival of cotton seedlings. Therefore, we reported that the most highest chitinolytic actinomycete as *St. gelaticus* A2 and the experiment could be considered to use as a biocontrol agent for fungal pathogens isolated.

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تثبيط الفطريات الموجودة على البذور المسببة لمرض موت بادرات القطن بواسطة الأكتينوميستات المحللة للكيتين

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[٢]قسم الميكروبيولوجيا الزراعية- كلية الزراعة- جامعة المنصوره- المنصوره- مصر

الملخص

تستخدم الأكتينوميستات كطرق إقتصادية وآمنه فى المقاومة الحيوية وتحسين نمو النبات. لهذا فقد تم الحصول على خمسة من عزلات الأكتينوميستات النقية من الأراضى الزراعية وذلك لتقييم قدرتها فى مقاومة حوالى أكثر من خمسة أجناس من الفطريات المسببة لأمراض سقوط البادرات فى القطن. هذه الأجناس الخمسة من فطريات مسببات مرض سقوط البادرات تم عزلها وتعريفها عند إجراء التقدير النوعى والكمى للفطريات على سطح بذور أحد أصناف القطن المصرية (جيزة ٨٦) وهنا أظهرت النتائج أن أكثر الفطريات تكرارا هى ريزكتونيا سولانى (٢٥,١٢٪) يليها أنواع الفيوزاريوم (٢٤,٨٢٪) ثم الماكروفومينا فاصولينا (٢٢,١٢٪) بينما كانت أقل النسب توزيعا هى أنواع البنيسليوم (٥,٤٤٪) وأنواع الأسبراجلس (٥,١٢٪). وبتطبيق فروض كوخ لهذه العزلات الفطرية وذلك بإعادة عدوى بذور نفس الصنف من القطن للتأكد من ظهور نفس الأعراض على البادرات وتقدير نسب الإنبات بعد ١٥ يوم وبعد ٤٥ يوم وكذلك تقدير عدد البادرات الباقية على قيد الحياة. فقد أظهرت عزلات الريزوكتونيا سولانى وأنواع الفيوزاريوم والماكروفومينا فاصولينا أعلى نسب فى الإصابة بينما سجلت أنواع البنيسليوم والأسبراجلس نسب متوسطة .وبناءا على ذلك فقد تم عزل الأكتينوميستات من التربة الزراعية وبالتالى الحصول على أنواع لأجناس مختلفة أختبرت فى قدرتها على تحليل الكيتين (المكون الرئيسى لجدر خلايا الفطريات) وذلك بإجراء إختبار إنتاج إنزيم الكيتينيز بتنميتها فى بيئة تحتوى على الكيتين وأظهرت عزلات الأكتينوميستات قدرتها على تحليل الكيتين وظهور المناطق الرائقة حول النمو دليل التحلل للكيتين وإنتاج الكيتينيز ولكن بدرجات متفاوتة لعزلات الأكتينوميستات. تم إختيار أعلى العزلات فى قدرتها على إنتاج الكيتينيز (A2) لتعريفها بطرق التعريف القياسية المختلفة وبلغت نسبة التشابه فى التعريف (٩٩,٧٣٪) وتنتمى للإستربتوميسس جيلاتكيس بعدها تم إجراء إختبار قدرة هذه السلالة من الإسستربتوميسس على التضاد فى الأطباق معمليا للفطريات الممرضة وأظهرت النتائج أعلى مناطق تثبيط نمو للفطريات وكانت أعلاها مع ريزوكتونيا سولانى وأنواع الفيوزاريوم والبنيسليوم والأسبراجلس بينما أقل تثبيط كان مع فطر الماكروفومينا فاصولينا. وبهذا نستطيع القول أن أجناس الأكتينوميستات ذات تأثير عالى فى تحسين مستقبل الزراعة فى مصر.