Journal of Sohag Agriscience (JSAS) 2024, 9(1): 28-35

ISSN 2305-1088
https://jsasj.journals.ekb.eg
JSAS 2024; 9(1): 28-35

Received: 22-08-2023
Accepted: 10-01-2024

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Controlling Tomato *Fusarium* Welt Disease via *Streptomyces rochei* in Actinophage Resistant Forms

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Abstract

This study aimed to control tomato welt disease caused by *Fusarium oxysporum* using *Streptomyces rochei* which known to have high antagonistic activity against the plant pathogenic fungi. Since presence of actinophages in the soil can reduce the density of such antagonistic microorganism (*i.e. S. rochei*) it was of a particular interest to prepare S. *rochei* inoculum in actinophage resistant forms. Therefore, a spontaneous phage resistant mutant of *S. rochei* was successfully isolated. Moreover, *S. rochei* inoculum was prepared in alginate immobilized form. The obtained results indicated that tomato plants that were inoculated with *S. rochei* free cells and *Fusarium oxysporum* showed moderate resistance to welt disease. The percentage of infection increased when treated with *S. rochei* free cells and actinophages. Immobilized cells of *S. rochei* significantly decreased the disease severity of infected plants, even in presence of actinophages. Furthermore, inoculation with actinophage-resistant mutant of *S. rochei* in presence of actinophage can protect plants and reduce welt disease symptoms. Results in this study revealed that the depressive effect of the actinophages can be avoided by application of *S. rochei* inoculum in the form of alginate immobilized cells. Moreover, isolation of phage resistant mutants of such desired bacteria can be used as well to avoid the phage attack. Therefore, application of immobilized cells or phage resistant mutants of these desired bacteria as a biological control agent against pathogenic fungi is highly recommended to avoid the phage attack and to promote the efficiencies and maintenance of this microorganism in the soil.

Keywords: Actinophage, Welt Disease, Tomato.

INTRODUCTION

Many chemical fungicides can effectively inhibit or kill plant fungal pathogens, but their excessive use would not only lead to environmental pollution and human health hazards, but also induce the resistance or reduce the susceptibility of pathogenic fungi. (Pieterse *et al*., 2016). The utilization of microorganisms and their metabolites is a promising and environment-friendly alternative to the effective prevention and control of plant diseases. Due to the extensive production of secondary metabolites, *Streptomyces* species have attracted much attention to the biological control of soil pathogens (Sun *et al*., 2016). Viaene *et al.* (2016) reported that various *Streptomyces* strains that have been described as efficient PGPR that can stimulate plant growth. Inoculation of plants with several *Streptomyces* strains resulted in an increase in plant biomass (Lin and Xu 2013; Palaniyandi *et al*., 2014; Cordovez *et al*., 2015). Bacteriophages are of widespread occurrence and are usually readily isolated from areas, which contain the appropriate host bacteria. These viruses are likely to have a significant role in the ecology of their hosts specially those of economically importance in agricultural purposes.

Upon the above-mentioned information, the presence of actinophages may affect the density and activity of *Streptomyces* in the soil and hence the antagonistic activity against pathogenic fungi can be affected as well. Therefore, this investigation aimed to use *streptomyces rochei* as biocontrolling agent for tomato welt disease caused by *Fusarium oxysporum*. Because the presence of actinophages can reduce the density of streptomyces *rochei*, this study aimed also to protect *streptomyces rochei* aginst phage attack via isolation of phage resistant mutant and preparation of *streptomyces rochei* inoculum in alginate immobilized form.

MATERIALS AND METHODS

1- The used microorganisms

a- Streptomyces:

Streptomyces rochei which was previously isolated from rhizosphere soil of tomato plants growing in Sohg Governorate and identified via 16S rRNA (Hammad *et al.,* 2023) was used in this study.

b- ***Fusarium oxysporum*:**

Identified fungal isolate of *Fusarium oxysporum* was kindly supplied by Department of Plant Pathology, Faculty of Agriculture, Sohag University.

c- Actinophages:

Two phage isolates of head-and-tail type specific to *Streptomyces rochei* belong to family *Siphoviridae* and family *podoviridae* which were isolated and characterized previously by Hammad *et al.* (2023) were used in this study.

2- Preparation of high titer phage suspension: Agar double layer plates method described by Maniatis *et al.* (1982) was used to prepare the high titer phage suspension for the bacteriophage as described by Hammad and Dora (1993). Titer of the prepared phage suspension was estimated using the method described by Kiraly *et al.* (1970) and expressed as plaque forming unit (pfu)/ml.

3- Isolation of *Streptomyces rochei mutant* resistant to phage attack the method described by Adams (1966) was used for isolation of phage resistant mutants of *Streptomyces rochei*. Five hundred µl of *Streptomyces rochei* liquid culture (10^8 cells/ml) were mixed with five hundred µl of phage lysate (10^{10} pfu/ml) in an Eppendorf tube. The tube was incubated for 5 min at 30°C to ensure that all *Streptomyces rochei* cells which can adsorb phages were infected. One hundred µl of the adsorption mixture was placed on the surface of a plate containing ISSA medium (Isenberg, 2004) and spread uniformly with a glass rod until all the liquid had been adsorbed by agar. After incubation at 30°C for 48-72 h the phage resistant mutants were observed as single colonies on the agar surface. These colonies were picked and transferred onto slant surface of ISSA medium (Isenberg, 2004) in test tubes and maintained at 4°C.

4- Inocula preparation of *Streptomyces rochei* wild type and phage resistant mutant:

Streptomyces rochei wild type and the isolated phage resistant mutant were grown in Erlenmayer flasks each containing 100 ml of broth ISSA Medium (Isenberg, 2004) / flask and incubated in a shaker at 30°C for 96 h. (giving 33-45 $x10^8$ cell/ml). These liquid cultures were used as inocula.

5- Sodium alginate-immobilized *Streptomyces rochei* cells inoculum: One hundred ml of a sterile solution of sodium alginate (2% w/v) was mixed with equal volume of *Streptomyces rochei* liquid culture. The mixture was added dropwise into 200 ml of 2% $CaCl_2$ sterile solution using a sterile Pasteur pipette. Beads of approximately 2 mm in diameter were formed and were hardened in $CaCl_2$ solution for 2 h before washing. The beads were then washed with sterilized water and stored at 4 ºC. All steps were carried out under aseptic conditions.

6- The antagonistic activity of *Streptomyces rochei* against *Fusarium oxysporium*:

Antagonistic activaty of, *Streptomyces rochei* against *Fusarium oxysporium* was studied. A plate containg PDA medium was prepared. *Streptomyces rochei* was streaked at one peripheral side of the prepared plate. At the same time, one disc (5mm in diameter) of *Fusarium oxysporium* was placed in the middle of each plate. A control plate without *Streptomyces rochei* was prepared containing one disc (5mm in diameter) of the *Fusarium oxysporium* in the middle of each plate. Plates were incubated at 28ºC until the fungal growth covered the plate surfaces of control plate (9.0 cm in diameter). Inhibition zone was observed. The percentage of mycelial growth inhibition was calculated based on the following equation:

Mycelial growth inhibition % =

[A — B/A] x 100

Where:

A = Length of the control hyphal growth.

B = Length of the hyphal growth in plates containing *Streptomyces rochei*.

7- Protection of tomato plants from *Fusarium* welt disease using different forms of *Streptomyces:*

In a pot experiment, the different forms of *Streptomyces* (*i.e.* Free, immobilized cells, and a phage resistant mutant) were used to protect tomato plants from *Fusarium* welt disease in presence and absence of specific actinophages.

Fired clay pots containing 5 kg soil/pot were prepared. The pots were planted with 5 tomato seedlings. Three replicates for every treatment were involved.

All the pots were inoculated with *Fusarium oxysporium* and subjected to the following treatments:

- Inoculation with *Streptomyces* free cells.
- Inoculation with *Streptomyces* immobilized cells.
- Inoculation with *Streptomyces* phage resistant mutants.
- Inoculation with *Streptomyces* free cells and actinophage suspension.
- Inoculation with *Streptomyces* immobilized cells and actinophage suspension.
- Inoculation with mutant of *Streptomyces* resistant to actinophage and actinophage suspension.
- Uninoculated plants as a control were involved.

In the treatments inoculated with free cells of either the wild type or mutant of *Streptomyces*, 5 ml of the prepared liquid cultures inocula were added to each pot. In case of inoculation with the immobilized cells, a calculated weight of beads containing the same number of bacterial cells (in the 5 ml of free cells inoculum) was added to each pot. For inoculation with phages, 5 ml of the high titre phage suspension were added to each pot. Symptoms were measured using disease severity scale (Table 1) (Lebeda and Buczkowski ,1986 modified by Popoola *et al*., 2015).

8- Statistical analysis:

Data were statistically analyzed according to Steel and Torrie (1980).

Table (1): Disease severity scale for tomato *Fusarium wilt*

Disease Severity Score	Symptom Description	Range of Disease Severity Score	Inference
1	Symptomless, stems and leaves free of any visual symptoms	0.00–1.44	Immune (I)
2	Very limited wilting, 5% leaves yellowed and wilted	1.45–2.44	Resistant(R)
3	Limited wilting, 6–10% leaves yellowed and wilted	2.45–3.44	Moderately Resistant (MR)
4	Moderate wilting, 11%–20% leaves yellowed and wilted	3.45–4.44	Moderately Susceptible (MS)
5	Severe wilting, 21%–50% leaves yellowed and wilted	4.45–5.44	Susceptible (S)
6	Very severe wilting, above 50% leaves yellowed and wilted	Above 5.45	Highly Susceptible (HS)

RESULTS AND DISCUSSION

***Streptomyces rochei*:**

Using microscopic examination *Streptomyces rochei* was found to be filamentous gram-positive, forming extensively branched substrate and aerial mycelia. The aerial mycelia forms chains of spores at maturity (Figure 1).

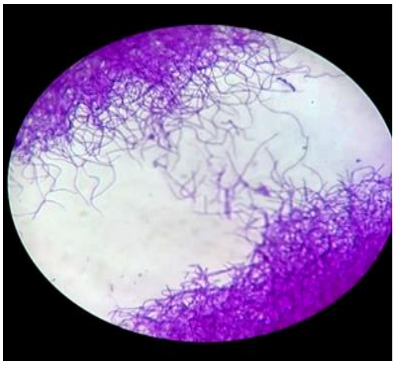

Figure (1): Light micrograph of Gram-stained *Streptomyces rochei*, isolated from Sohag soil.

Korayem *et al* (2015) isolated two streptomyces isolates from Sohag Governorate soil. Moreover, Hasani *et al*. (2014) reported that the Gram-positive filamentous bacteria known as *Streptomycetes* are found in various types of soils, including composts, water, and plants.

The antagonistic activity of *Streptomyces rochei* against *Fusarium oxysporium*:

As shown in Figure (2) *Streptomyces rochei* was found to have a high antagonistic activity against *Fusarium oxysporium.* The percentage of fungal growth inhibition was calculated to be 53.7%. Such result may indicate that *Streptomyces rochei* synthesize secondary metabolites like antibiotics which have inhibitory effect on *Fusarium oxysporium*. Chaiharn *et al.* (2018) studied the antagonistic activity of 150 isolates of Streptomyces against *F. oxysporum* and found that only 14.6 % of 150 actinomycetes strains were positive for antifungal activity, with the percentage of mycelia inhibition of the active strain ranged from 21.8% to 27.0%. Hasani *et al*. (2014) reported that *Streptomycetes* are most known for their ability to synthesize secondary metabolites like antibiotics. They produce more than two-thirds of the naturally derived antibiotics that are clinically helpful (such as neomycin and chloramphenicol). Taddei *et al.* (2006) stated that, among the *Streptomyctaceae* family, the genus *Streptomyces* has the largest number of species and varieties. Their morphology, physiology, and metabolic activities differ substantially, and they produce the majority of known antibiotics.

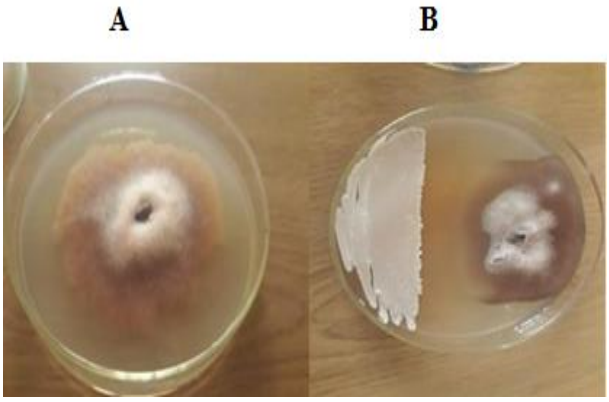

Figure (2): Antagonistic activity of Streptomyces *rochei* against Fusarium *oxysporum* (B) it is clearly seen as compared to the control plate (A) containing Fusarium *oxysporum* alone.

High titer actinophage suspension:

Five hundred ml of high titer phage suspension were prepared for each of the two actinophage isolates using agar double layer plates method as described by Maniatis *et al.* (1982) to be used in this study. The titer of the prepared phage suspensions was estimated. The obtained results as revealed that the titers ranged from $1.27x10^9$ pfu/ml to $3.8x10^9$ pfu/ml for phages No. 1 and No. 2, respectively. Such high concentrations of phages were not surprising, since a single plaque of 2mm in diameter may contain between 10^7 and 10^9 recoverable phage particles (Gunsalus and Stanier, 1960 and Adams, 1966).

***Streptomyces rochei mutant* resistant to phage attack:**

A spontaneous phage resistant mutant was successfully isolated for *S. rochei* according to Adams (1966) and Hammad and Ali (1999).

Using the spot test technique, susceptibility of the isolated mutant to actinophages was tested. As shown in Figure (3) no lyses was detected on the plate seeded with the mutant and spotted with the isolated actinophage. Whereas, lyses of the wild type can be clearly seen. *i.e.* the isolated mutant exhibited high resistance to phages. Such results may indicate that exposing susceptible bacteria (wild type) to virulent phages may led to development of actinophage resistant mutant (Defives, *et al*., 1996 and Coakley *et al*., 1977).

Since, the phage resistant mutant of *Streptomyces rochei* exhibited high resistant to actinophages, it was of a particular interest to study its efficiencie in controlling wilt disease caused by *Fusarium oxysporum*in in presence and absence of actinophages.

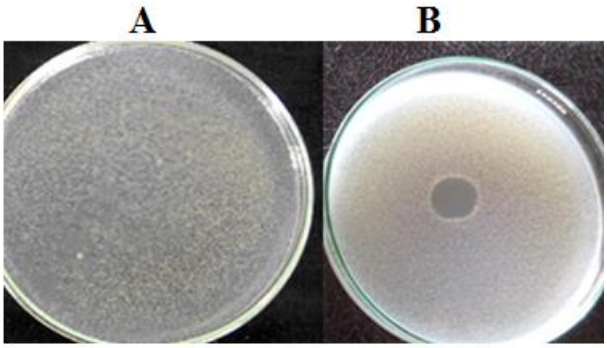

Figure (2): Lawns of *S.rochei* phage-resistant mutant (A) and the wild type (B) spotted with the actinophage lysate.

Immobilization of *S. rochei*:

Alginate immobilized bacteria have been successfully used for industrial purposes (Zayed and Winter, 1995) and in agriculture (Sougonfara *et al.*, 1989) for promoting the biological activities of certain bacteria. In addition, Hammad (1998) and Zayed (1998) reported that the immobilization system provided high protection for *Azotobacter* and *Bacillus megaterium* against phage attack and increased their biological activities. Therefore, the tested *streptomyces rochei* was used in immobilized form as inoculum for tomato plants in presence of their phages to find out how is it possible for the immobilization system to provide protection for the immobilized bacteria against their phages.

Figure (3): Alginate beads contain *S. rochei* to be used as phage resistant inoculum.

Controlling tomato welt disease caused by *Fusarium oxysporum* :

As shown in Table (2), welt disease severity was measured after four weeks of treatments. Tomato plants that were inoculated with *S. rochei*'s free cells and *Fusarium oxysporum* showed moderate resistance to welt disease. The percentage of infection was greatly increased when treated with *S. rochei's* free cells and actinophages; such observation may indicate that actinophages reduced density of *S. rochei* and then negatively affected its antagonistic activity against *F. oxysporum*. Immobilized cells of *S. rochei* significantly decreased the disease severity of infected plants, even in presence of actinophages. This finding indicates that immobilized cells can protect *S. rochei* from phage attack. Furthermore, inoculation with actinophage-resistant mutants can protect plants and reduce welt disease symptoms, whereas, presence of actinophages had no effect because the *S. rochei* is resistant to actinophage. Similarly, alginate immobilized bacteria have been successfully used for industrial purposes (Zayed and Winter, 1995) and in agriculture (Sougonfara *et al.*, 1989) for promoting the biological activities of certain bacteria. In addition, Hammad (1998) and Zayed (1998) reported that the immobilization system provided high protection for *Azotobacter* and *Bacillus megaterium* against phage attack and increased their biological activities.

Table (2): Disease severity and resistance status on tomato seedlings four weeks after *Fusarium* pathogen inoculation.

Treatments	Disease Severity Score	Inference
S. rochei's free cells	2.65	Moderately resistant
S. rochei's free cells + actinophages	6.25	Highly susceptible
S. rochei's immobilized cells	1.85	Resistant
S. rochei's immobilized cells + actinophage	1.85	Resistant
S. rochei's phage resistant mutants.	2.15	Resistant
*S. rochei's*actinophage resistant mutants + actinophage	2.05	Resistant
Fusarium oxysporum (control)	6.50	Highly susceptible
LSD (0.05)	1.55	

Generally, on the basis of the obtained results it can be concluded that, the presence of actinophages specific to *S. rochei* in the soil is one of the most important environmental factors affecting the activity and maintenance of such desired bacteria. Presence of bacteriophages reduced the densities of the applied bacterial inocula and consequently the desired biological activities of these bacteria negatively affected. Therefore, the depressive effect of actinophages can be avoided by application of immobilized cells or phage resistant mutants of these desired bacteria as a biological control against pathogenic fungi is highly recommended to avoid the phage attack and to promote the efficiencies and maintenance of these microorganisms in the soil.

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الملخص العربى

مقاومة مرض ذبول الطماطم الفطري باستخدام *Streptomyces rochei* المقاومة للاصابة بالأكتينوفاج

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هدفت هذه الدراسة إلى مقاومة مرض ذبول الطماطم الناجم عن *Fusarium oxysporum* باستخدام *Streptomyces rochei* المعروفة بفعاليتها المضادة للفطريات الممرضة للنبات . نظرًا لان وجود الفاجات المتخصصة على *S. rochei* في التربة يمكن أن يقلل من كثافة هذه الكائنات الحية الدقيقة المضادة (*S. rochei*)، فقد كان من الضروري تحضير لقاح من *S. rochei* في أشكال مقاومة للفاجات .لذلك تم عزل طفرة تلقائية من *S. rochei* مقاومة للفاجات علاوة على ذلك ، تم تحضير لقاح *S. rochei* في شكل مثبت على الالجينات. أشارت النتائج المتحصل عليها إلى أن نباتات الطماطم التي تم تلقيحها بالخلايا الحرة من *S. rochei* و *Fusarium oxysporum* أظهرت مقاومة لمرض الذبول . زادت نسبة الإصابة عند التلقيح بالخلايا الحرة من *S. rochei* والفاجات المتخصصة عليها . ادى التلقيح بخلايا *S. rochei* المثبتة على الالجينات الى خفض نسبة الاصابة بالذبول بشكل ملحوظ ، حتى في وجود الفاجات .علاوة على ذلك ، فإن التلقيح بطفرة *S. rochei* المقاومة للفاجات وفى وجود الفاجات ادى الى تقليل أعراض مرض الذبول .أظهرت النتائج في هذه الدراسة أنه يمكن تجنب التأثير الضار للفاجات عن طريق استخدام لقاح *S. rochei* في شكل خلايا مثبتة على الالجينات. كما يمكن أيضًا استخدام طفرات مقاومة للفاجات لهذه البكتيريا المرغوبة لتجنب الاصابة بالفاجات .لذلك ، يوصى باستخدام الخلايا المثبتة أو الطفرات المقاومة للفاجات لهذه البكتيريا المرغوبة للمقاومة الحيوية ضد الفطريات المسببة لامراض النبات .