

IDENTIFICATION, INSECTICIDAL EFFECT AND ANTIBIOSIS STUDIES OF SOME BACTERIA ISOLATED FROM NATURALLY INFECTED *Pectinophora gossypiella* (SAUND.) COLLECTED FROM COTTON FIELDS IN EGYPT.



Abou-zeid N. M.¹; Kreema A. El-Lebody²; Noher A. Mahmoud¹ and Manal A. ElSharkawy²

1. Microorganisms unit, Plant Pathology Research Institute, Agriculture Research Centre Giza Egypt.

2. Bollworm Research Division, Plant Protection Institute, Agriculture Research Centre, Giza, Egypt.

ABSTRACT

One of the important Integrated Pest Management Strategies (IPM) against economic pests in Egypt is the use of biological control methods. The use of entomopathogenic bacteria is one of these methods. The authors achieved four isolates of bacteria from the naturally infected *P. gossypiella* in cotton fields at Assuit and Kafr El-Sheikh Governorates, Egypt. Isolates were characterized based on morphological properties. Morphological analyses were carried out based on Bergey's Manual of Systematic Bacteriology. The identification of the isolated bacteria was confirmed by using Biolog-System as, *Staphylococcus sciuri*, *Micrococcus luteus*, *Brevibacterium lines* & *Brevibacterium casei*. In addition, their larvicidal effect was evaluated. The results revealed that, *S. sciuri* and *M. luteus* were the most effective against 1st instar larvae of PBW. Furthermore, their latent effect caused the lowest pupation % resulted from treated PBW larvae. *S. sciuri* was the most promising bacterial isolate to be developed as a biological control agent against *P.gossypiella*.

The antagonists of bacterial strain against the mycelial growth of the pathogenic fungi (*Rhizoctonia solani*, *Fusarium solani* and *Macrophomia phaseolina*), was experimentally tested to evaluate growth reduction of all pathogenic fungi.

Keywords: Pink Bollworm, PBW, *Pectinophora gossypiella*, Identification, Insecticide effect, Antibiosis isolated bacteria, *Staphylococcus sciuri*, *Micrococcus luteus*, *Brevibacterium lines*, fungi, *Brevibacterium casei* (*Rhizoctonia solani*, *Fusarium solani*, *Macrophomia phaseolina*).

INTRODUCTION

Pectinophora gossypiella (Saund.) (Gelchiidae: Lepidoptera) is worldwide distributed and considered one of the serious pests of cotton. Although, various cultural, chemical and biological methods are being used to control this pest, its damage still continues effectively all over the world.

However, the use of biological control methods offers a plentitude of approaches for a highly selective and environmentally sound control of insect pests. These include the application of beneficial arthropods, entomopathogenic, micro-organisms (including fungi, bacteria and viruses), and natural substances and botanicals for pest control. And they are of great importance for Integrated Pest Management Strategies (IPM) (Johannes, 2013). In this regard, many investigation were carried out to isolate

entomopathogenic bacteria from either healthy or moribund insect pests e.g., *Brevibacterium lines* and *Staphylococcus sciuri* have been isolated from health *Xylosandrus germanus* (Blandford) (Coleoptera: Curculionidae) collected in hazelnut orchards in Turkey (Ahmet and Hatice, 2013). While, *Micrococcus luteus* isolated from *Euproctis chrysorrhoea* L. (Lepidoptera: Lymantriidae) as well as from Asian live longhorned beetle, *Anaplophora glabripennis* (Motschulsky), larvae and adults (Mustafa *et al.*, 2000 and John *et al.*, 2013). Ahmet and Hatice (2013) reported that, several species of bacterial isolates related to genera *Brevibacterium* & *Staphylococcus* and others are used in biological control as an antifungal and insecticidal against agricultural pests.

Also, the study on the bacterial community of PBW may give a better chance to find significant microbial control agent against this pest. In this regard, many bacterial strains representing several genera were isolated from infected active and / or resting PBW larvae (Abul-Nasr *et al.*, 1978; Khoja *et al.*, 2006 and Reda *et al.*, 2013). Furthermore, the symbiont, *Enterobacter gergoviae*, isolated from the gut of the pink bollworm (PBW), has been transformed to express Cyt1A, a cytolytic protein toxin lethal to mosquito and black fly larvae, as a model system. These transgenic bacteria might be used to spread genes encoding insecticidal proteins to populations of agricultural insects or as replacement for chemical insecticides (Lyudmila *et al.*, 2002).

The present study aimed to isolate, identify and determine the potency of virulent bacteria of infected *P. gossypiella* larvae. The study was extended to investigate the effect of these isolates on the immature stages of PBW resulted from treated newly hatched larvae and try to find a safe bio-control agent against this pest in IPM program.

MATERIALS AND METHODS

Obtaining of the infected PBW larvae:

Healthy PBW full grown larvae were excluded from green cotton bolls collected from various locations of Kafr El-sheikh and Assiat Governorates, Egypt during season 2014. The larvae were kept individually into sterilized glass tubes (2x7cm) capped by cotton wool to prevent any contamination, and incubated under room conditions. These larvae were examined weekly. The sick larvae which show symptoms of entomopathogenic bacterial infection were separated and examined daily. As soon as the death of moribund larvae (Fig1-4) they were kept in a refrigerator at 5 °C until isolation of bacterial community of PBW larvae.

Isolation and identification of bacteria of infected PBW larvae:

The plate count method was used for isolation and identification of bacteria from dead PBW larvae using various assays (Ahmet and Hatice 2013). The surface of larvae specimens were sterilized with 70% ethanol (Pidiyar *et al.*, 2004) in a sterile hood. The sick larvae were homogenized in 9 ml of sterile distilled water by using a glass tissue grinder.

Serial dilutions from 10^{-1} to 10^{-6} were prepared from the suspension by using sterile distilled water as dilution to study the microorganisms. One ml of both dilutions (10^{-4} and 10^{-6}) were used for the isolation of bacteria on Petri

plates containing nutrient agar medium (Thomas *et al.*, 2012), for isolation of bacteria 0.1 ml of each desired dilution was placed and streaked on the surface of each plate using special sterile glass rod. The plates were incubated at (28 ± 2 °C) and (37 ± 2 °C) for 2-7 days until developing of single colonies. Different separated bacterial colonies were picked up, then transferred on a new medium, and re-incubated again.

The bacterial isolates were purified by employing single colony isolation. Bacteria strains were purified by using the streak technique and selected based on their colors and colony morphologies. Morphological analyses were carried based on Bergey's Manual of Systematic Bacteriology (Schreckenberger *et al.*, 2007 and Logan *et al.*, 2009).

The resulted bacterial isolates were maintained on nutrient agar and PDA slant media, respectively and kept in a refrigerator at 5 °C till achieving identification. The identification of the isolated micro-organisms was confirmed by using Biolog-System technique which belonged to identification of microorganisms unit, Plant Pathology Research Institute, A.R.C., Giza, to differentiation between them by using of carbon and amino acid compounds.

Larvicidal effects of bacterial isolates against *P. gossypiella*:

Insect used:

Newly hatched larvae of *P. gossypiella* were obtained from a colony maintained in the laboratory for several generations at (27 ± 1 °C and 75 ± 5 % R.H). Larvae were reared on a modified artificial diet as described previously by Rashad and Amar, (1985).

larvicidal studies:

The stock suspension of 4 bacterial isolates (contain 1×10^8 spore /ml) related to three genera of bacteria recorded for the first time associated with *P. gossypiella*, were evaluated against newly hatched larvae of PBW as follows: a volume of each suspension of each bacterial isolate was homogeneity mixed with artificial diet (without the antimicrobial agents) to obtain the tested concentration (1×10^8 spore/ml / 1gm diet / isolated bacterium). After preparation of treatments, each one was individually folded into 4 Petri dishes (9 cm in diameter). Fifty neonate larvae of pink bollworm susceptible laboratory strain were added to each Petri-dish. A control experiment was done, but Petri-dishes were prepared with diet mixed with double distilled water. Larvae of all treatments were allowed to feed on the treated diet for one day then alive larvae were transferred individually to glass tubes (2×7 cm) containing untreated artificial diet. Tubes were plugged with cotton wool and incubated at (26 ± 1 °C and 70 ± 5 % R.H). Percentages of mortalities of 1st and 4th larval instars were estimated.

In vitro Antibiosis Studies

An antagonistic effect of Bacterial isolates (Brevibacterium lines, Staphylococcus sciuri, Micrococcus luteus and Brevibacterium casei), were tested against different pathogenic fungi (Rhizoctonia solani, Fusarium solani and Macrophomia phaseolina), isolated from rotted cotton using PDA medium.

Disks (5mm-diam.) taken from seven day old culture of pathogenic fungi, were transferred onto potato dextrose agar PDA plates (9cm). The antagonistic tested bacteria were individually streaked in opposite direction of the pathogenic fungi. Three Petri-dishes were used for each isolate and the same number was kept as control with pathogen alone. The inoculated plates were incubated at (26-28°C) until the growth completely covered the plate surface in control treatment.

The interaction between pathogenic fungi and bacteria isolates were recorded to determine the more effective antagonistic isolates. The reduction percent in mycelia growth of pathogenic fungi was calculated using the formula as follows:

$$\text{Reduction \%} = \frac{C-T}{C} \times 100$$

Where:

C = fungal growth of pathogenic fungi in control treatment.

T = fungal growth of pathogenic fungi in presence of antagonism.

RESULTS AND DISCUSSION

The authors achieved four isolates of Bacteria from the infested *P. gossypiella* larvae related to three genera of bacteria. Photography was taken for diseased larvae of each bacterium which die due to the infection (Figs. 1-4); as well as healthy one (Fig.A). Isolates were characterized based on morphological properties. Morphological analyses were carried out based on Bergey's Manual of Systematic Bacteriology. The identification of the isolated bacteria was confirmed by using Biolog-System as, *Staphylococcus sciuri* (samples of Assuit), *Micrococcus luteus*, *Brevibacterium lines*, and *Brevibacterium casei*. In addition, the larvicidal potency of these bacterial isolates was determined against *P. gossypiella* larvae.

In present study, the isolated bacteria from *P. gossypiella* were found to be different from previous studies performed by Abul-Nasr *et al.* (1978); Khoja *et al.*, (2006) and Reda *et al.* (2013). They isolated 13 bacteria from *P.gossypiella*, including, *Bacillus thuringiensis*, *B. t. var. thuringiensis*, *B. t. var. finitimus*, *B. cereus*, *B. polymyxa*, *B. subtilis*, *Cellulomonas flavigen*, *Pseudomonas viridiflava*, *P. pyrrocinia*, *P. aeruginosa*, *Serratia grimesii*, *Serratia marcescens* and *S. rubidaea*. The probability for this difference is conceptualized to the geographical local position.

On the other hand, the bacteria isolated recently from *P. gossypiella* had been isolated from various insect species before. In this regard, entomopathogenic bacteria were isolated from either healthy or moribund insect pests e.g., *Brevibacterium lines*, *Brevibacterium casei* and *Staphylococcus sciuri* had been isolated from health *Xylosandrus germanus* (Blandford) (Coleoptera: Curculionidae) collected in hazelnut orchards in Turkey (Ahmet and Hatice ,2013). While, *Micrococcus luteus* isolated from *Euproctis chrysorrhoea* L. (Lepidoptera: Lymantriidae) as well as from Asian

live longhorned beetle, *Anaplophora glabripennis* (Motschulsky), larvae and adults (Mustafa *et al.*,2000 & John *et al.*,2013).

Larvaicidal effects of Isolates:

These isolates were evaluated against 1st and 4th instars larvae of PBW as well as their latent effects on larval stage and pupation % under laboratory conditions.

Data in Table (1) indicated that the mortality % obtained from all isolates ranged between (22% to 67.3%) against the treated PBW 1st instar larvae within 2 days after application of 1x10⁸ cfu /mL bacterial concentration at 27°C under the laboratory conditions. While, the accumulated percent mortalities in 4th instar larvae were (31% to 88.7%). In comparison the mortality % in control were 4% and 6% of 1st and 4th instars larvae, respectively. In addition, the obtained accumulated mortality of larvae from all treatments ranged between (47 to 90%). The corresponding accumulated mortality in control was 8%. In sequence the pupation % of all treatments ranged between (10% - 53%) compared to 92% in the control. Present findings indicate that these bacterial isolates appear to be a good candidate to be utilized as promising biocontrol agents for use against PBW *P. gossypiella*. In this regard, Ahmet and Hatice (2013), reported that, several species of bacterial isolates related to genera *Brevibacterium* and *Staphylococcus* and others are used in biological control as an antifungal and insecticidal against agricultural pests.

Table (1): Effect of isolates against treated *P.gossypiella*.

Instar / stage	Days after treatment	Mortality % in control and treated PBW				
		control	<i>Staphylococcus sciuri</i>	<i>Micrococcus luteus</i>	<i>Brevibacterium lines</i>	<i>Brevibacterium casei</i>
1 st instar	2	4	67.3	45	22	23.4
4 th instar	11	6	88.7	74	31	39
Larval stage	21	8	90	84	80	47
Pupation %						
Pupal stage	21	92	10	16	20	53

In this respect, this results demonstrating that *Brevibacterium lines*, *Staphylococcus sciuri*; *Micrococcus luteus* and *Brevibacterium casei* are recorded for the first time as pathogenic to *P. gossypiella* is considered the first record.

Comparison of the insecticidal effects of isolates against PBW:

1-*Staphylococcus sciuri*:

The body of naturally infected PBW larvae with *Staphylococcus sciuri* is shrunk with dark brown color (Fig.1). In this respect, Abul-Nasr *et.al* (1978) found that two pathogenic bacterial strains, *Bacillus thuringiensis* var. *finitimus* and *B. cereus*, were isolated from sick or dead resting larvae of *P. gossypiella* carrying dermal brown lesions.

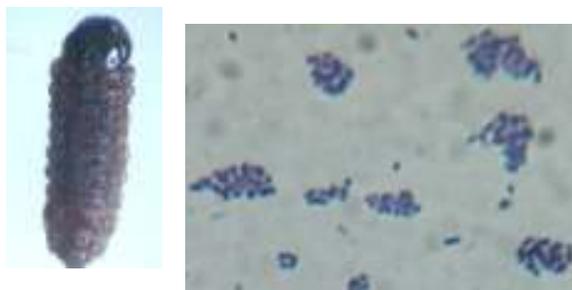


Figure (1): Naturally infected PBW with *Staphylococcus sciuri*

As shown in Table (1) *Staphylococcus sciuri* caused the highest mortality % (67.3%) & (90%) either in PBW 1st larval instar or in all larval stage (accumulated larval mortality). Also, it caused the lowest percent of PBW pupation (10%). Upon, these results *Staphylococcus sciuri* is considered the most effective against PBW because it achieved the control of protective type. However, Ardahan *et al.* (2013) reported that, *Staphylococcus* species is one of the most well-known bacterial pathogens of insects. Furthermore, Pascal *et al.* (2011) found that, *Staphylococcus sciuri*, acts as a kairomone enhancing the efficiency of aphid natural enemies.

On other hand, Hauschild and Schwarz (2003) indicated that, Twenty-nine *Staphylococcus sciuri* strains were isolated from free-living insectivores and rodents. Also, *Staphylococcus sciuri* may be human pathogen. Jerry *et al.* (2010) reported that, *Staphylococcus sciuri* has been associated mostly with wound infections although it can be isolated from other infections such as urinary tract infections and endocarditic.

In conclusion, *Staphylococcus sciuri* may have potential for future development as microbial control agents against *P. gossypiella*, but, the healthy alerts and indications must be followed at the use of this bacterium.

2-Micrococcus luteus:

As shown in Fig. (2) the infected PBW with this bacterium exhibited following symptoms:

The color of dead PBW body did not significantly differ from that of the healthy larvae. The whole body was soft and the internal organs were broken down and liquefied.



Figure (A): Healthy PBW larvae

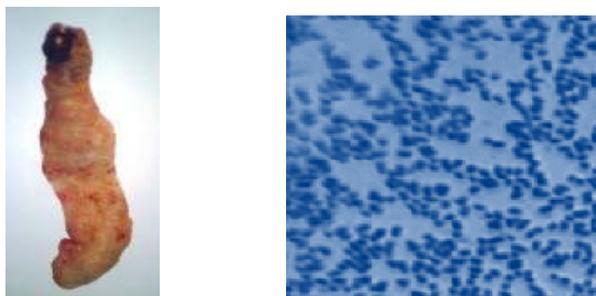


Figure (2): Naturally infected PBW with *Micrococcus luteus*

This isolate got the second position of efficiency against *P. gossypiella*. It caused 45%, 74% & 84% mortality in the 1st and 4th larval instars and the whole larval stage. Only 16% of PBW larvae reached the pupal stage. The present study indicates that, this isolate appears to be a good bio-control agent against *P. gossypiella*. While, Mustafa *et al.* (2000) isolated *Micrococcus luteus* from *Euproctis chrysorrhoea* L. (Lepidoptera: Lymantriidae) and found that, the insecticidal effect % of this isolate is 15% against *Euproctis chrysorrhoea* L. However, *Micrococcus luteus* (Schroeter) can be a nosocomial human pathogen (Peces *et al.* 1997 (c.f. John *et al.*, 2013))

3-Brevibacterium linens and Brevibacterim casei:

The infected PBW with *Brevibacterium linens* appears curved with yellow thorax and dark brown body. Also, there was a yellow liquid under the abdomen portion (Fig.3). While, the *Brevibacterim casei* infection caused infelation of abdomen and the body color became light brown. (Fig.4)

Both of *Brevibacterium linens* (samples of Assuit) and *Brevibacterim casei* (samples of Kafr El- sheik) are not effective against *P. gossypiella*. They caused (22% & 23.4%) and (31% & 39%) mortality in the 1st and 4th larval instars, respectively. In contrary, *Brevibacterium linens* was more effective (80%) against the larval stage where only 20% of treated larvae reach pupal stage. The corresponding latent insecticidal effects of *Brevibacterim casei* were 47% and 53%, in larval and pupal stages, respectively.

However, Homopteran insects contain bacteria in their cells and tissues known as "secondary symbionts," *Brevibacterim casei* which under special environmental circumstances act against their host insects (Mazen *et al.*, 2010). But, Ahmet and Hatice (2013) reported that, several species of bacterial isolates related to genera

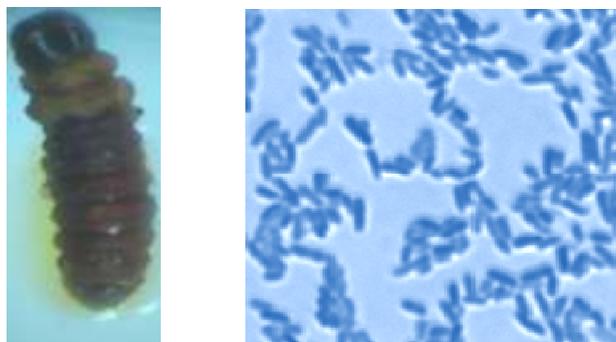


Figure (3): Naturally infected PBW with *Brevibacterium linens*

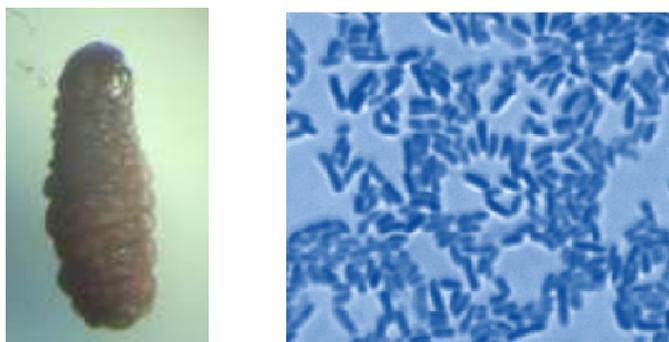


Figure (4): Naturally infected PBW with *Brevibacterium casei*

Brevibacterium and others are used in biological control as an antifungal and insecticidal against agricultural pest. Because of the weakness effect of *Brevibacterium linens* and *Brevibacterim casei* against the 1st instar of *P. gossypiella*, makes it worse in control of this pest.

The present study concluded that, *Staphyloccus sciuri* and *Micrococcus luteus* isolates may have potential for future development as microbial control agents against *P. gossypiella*. But, the healthy alerts and indications must be followed at the use of these bacteria. Also, further studies should include field efficacy of these isolates.

Antibiosis studies *in vitro*

The antagonistic effect of bacterial isolates on the growth of pathogenic fungi :

This experiment was carried out to evaluate the effect of different antagonistic bacterial strains (*Brevibacterium lines*, *Staphyloccus sciuri*, *Micrococcus luteus* and *Brevibacterium casei*), against the mycelial growth of the pathogenic fungi (*Rhizoctonia solani*, *Fusarium solani* and *Macrophomia phaseolina*), isolated from rotted cotton.

Results in Table (2) indicated that the tested antagonist's strains show no difference between them in their antagonistic activity against all of the

tested pathogenic fungi .The growth reduction recorded 0.0 % of both fungi as the control.

Table (2): The antagonistic effect of 4 bacterial strains against three pathogenic fungi (*Rhizoctonia solani*, *Fusarium solani* and *Macrophomia phaseolina*), *In vitro*.

Bacterial isolates	<i>Rhizoctonia solani</i>		<i>Fusarium solani</i>		<i>Macrophomia phaseolina</i>	
	Linear growth	Reduction %	Linear growth	Reduction %	Linear growth	Reduction %
<i>Brevibacterium lines</i>	9.0	0.0	9.0	0.0	9.0	0.0
<i>Staphylococcus sciuri</i>	9.0	0.0	9.0	0.0	9.0	0.0
<i>Micrococcus luteus</i>	9.0	0.0	9.0	0.0	9.0	0.0
<i>Brevibacterium casei</i>	9.0	0.0	9.0	0.0	9.0	0.0
Control	9.0	0.0	9.0	0.0	9.0	0.0

The antagonistic bacterial strains were no specific against all of the tested pathogenic fungi. These results are in harmony with the findings of Yuliar, *et al* (2013), where they verified the positive and the negative role of 14 bacterial strains in inhibition ability to both of *R. solani* and *F. oxysporum* growth in PDA media. The selected strains showed that their ability to suppress of *R. solani* growth were in range of 16-60% in PDA media. Five strains did not suppress *R.solani*.

Nine of the selected strains inhibited *F. oxysporum* growth in the range of 10-47% in PDA media and five strains did not suppress *F. oxysporum* in PDA media media.

Some possibilities of fungal pathogens suppression mechanisms by bio - control agent had been investigated by some author within series of action including antibiosis work (Souto *et al.*,2004; Yuliar *et al.*, 2011); as lytic enzymes activities such as chitinases and proteases production (Huang *et al.*2005) ; competition of iron through the production of siderophore and caused the induction of systematic resistance and ability producing antibiosis of iturin and surfactin, as well as due to enzymatic chitinase action (De Boer *et al.*, 2003).

Antibiosis mechanism could be due to their extracellular metabolites production excreted by endophytic bacteria which acted fungal cell membrane damage. In the laboratory test for antagonism through this observation, media used causing dissimilarity of positive and negative result of antagonism affect for some certain endophytic strain, while the quantity result differences also occur within the same strain. The range of inhibition ability in different media showed different effect to inhibition percentage. The result of the present study confirms previous reports of antibiotic polypeptide production by bio - control investigation had been worked by Shoda (2000) and Akpa *et al.* (2001). Also, Raaijmaker (2002) indicated that suppression of fungal pathogen *R. solani* and *F. oxysporum* by some certain endophytic bacterial strains are because of antibiosis production of bioactive iturin and surfactin, and also caused by chitinase enzyme production.

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تعريف بعض العزلات البكتيرية من يرقات دودة اللوز القرنفلية المصابة طبيعياً في بعض حقول القطن في مصر وتقييم فعاليتها معملياً ضد الحشرة و أعفان جذور القطن.

ناجى محمد أبو زيد^١ , كريمة عبد الرحمن اللبودى^٢ , نهير عبد النظير محمود^١ و منال عبد المحسن الشرقاوى^٢

- ١- مركز البحوث الزراعية – معهد أمراض النبات
٢- مركز البحوث الزراعية – معهد وقاية النباتات

دودة اللوز القرنفلية وأعفان جذور القطن من أخطر الآفات التي تصيب القطن في مصر و العالم. البكتريا الممرضة للحشرات من أهم عناصر مكافحة المتكاملة ضد هذه الآفة. الهدف من الدراسة الحالية عزل وتعريف البكتريا الممرضة طبيعياً لهذه الآفة في حقول القطن وكذلك تقييم تأثيرها ضد دودة اللوز القرنفلية وأعفان جذور القطن. أوضحت النتائج أن دودة اللوز القرنفلية تصاب طبيعياً ببكتريا *Staphylococcus sciuri*, *Micrococcus luteus* *Brevibacterium lines*, and *Brevibacterium casei*. و أثبتت التقييم المعملى أن معاملة الفقس الحديث بكلاً من *Staphylococcus sciuri* و *Micrococcus luteus* سبب أعلى نسب موت من يرقات الفقس الحديث (٦٧.٣% و ٤٥%) ثم أقل نسب عذارى ناتجة ١٠% و ١٦% على التوالي. على الجانب الآخر ليس لهذه العزلات تأثير على نمو فطريات وأعفان جذور القطن.