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Evaluation of Actinomycetes Isolates as A Biocontrol Agent Against *Spodoptera littoralis* (Boisd.) and *Fusarium oxysporum*

Abeer M. Mohammad
Faculty of Science & Arts – Al-Dayer , Jazan University
Plant Protection Research Institute, Agric. Ares. Center

ABSTRACT

According to soil isolationtechniques, 23 isolates of actinomycetes were obtainedfrom different soil samples in KSA and Egypt. All isolates were examined for their biological activity against both, the pest*Spodopteralittoralis* (Boisd.),also the plant pathogen *Fusariumoxysporum*. Results illustrated that, three isolates, named DI,DV and FII exhibited high efficacy against the 4th instar of *S. littoralis*, with mortality percentage 70, 80and 60%respectively. Also, other stages'development of *S. littoralis*were affected. Meanwhile, metabolites of 20 isolates showed antagonistic activity against *F.oxysporum*, including the 3 isolates which showed efficacy against the insect pest, the % of inhibitionranged from67 – 49 %.These 3 isolates were belong to the species *Stryptomyceslavendulae*and*Str. clavuligerus*.

Keywords: Actinomycetes, biocontrol, Spodopteralittoralis and Fusariumoxysporum

INTRODUCTION

Pesticides are designed basically to kill, and because their non-specific mode of action to one organism, they often kill or harm non-target organisms even humans. Also, excessive use of fungicides has led to deterioration ofpublic health, environmental pollution and appearance of pathogen resistance. (Harpreet and Parihar, 2010).Because ofthis problem a serious efforts is needed to find an alternative method for controlling insect pests and plant pathogens (Canaday, 1995). Many groups of microorganisms such fungi, bacteria and viruses have been reported as biocontrol agents. Actinomycetes are aerobic,gram+vebacteria which widely spread in nature, have been well known for the production of secondarymetabolites (Lo *et al.*, 2002). Recently, this organismplays an important role in insect and plant pathogencontrol,hence they produce metabolites active against many insects and fungi. (Hussain*et al.*, 2002).Chitinase is an enzyme used by insects for degradation the structural polysaccharide "chitin" during molting stages.This enzyme is very important in biological control of insects (Reguera&Leschine,2001), and plant pathogenic fungi (El-Tarabily, 2003).Actinomycetesare also produce secondarymetabolites like hydrocyanicacid (HCN) and indole acetic acid (IAA)(Gopalakrishnan*et al.*, 2011).

So, the main purpose of this research is to isolate local isolates of actinomycetes from soil, later these isolates will be tested for their biological activity against both *Spodopteralittoralis*larvae and the plant pathogen *Fusariumoxysporum*.

MATERIALS AND METHODS

Isolation of actinomycetes from soil.

Soil samplefor isolation, were collected randomly from different localities in Egypt's Governorates and farms of Agricultural Research Center, Abo-Areesh&Fayfaa farms, Jazan, KSA. Samples were then packed and storedat 15°C. Samples were then dried for 1 week prior isolationto decrease gram negative population.Starch casein agar (SCA) were used for isolation. Onegram of each sample were mixed with 10 ml of sterile distilled water, 100µl ofeach sample were spread onto fresh SCAplates and kept forincubation at 28° C for 7 days. Colonies ofactinomycetes were selected by using sterile toothpick then placed on SCAplates and incubated to get pure colonies.(Sinha, *et al.*, 2014).

Fungi isolation and growth conditions

The pathogen of vascular wiltdisease (*F.oxysporum*) was isolated from diseased vegetables by direct plating method, maintained as pure culture on potato dextrose agar medium at 25 °C for a week and later stored at -20 °C in glycerol.Vicente *et al.*, 2014.

Media used for isolation.

Starch- casein agar(SCA). For actinomycetes growth;soluble starch 1%, casein 0.03%, K-NO_3 0.2%, NaCl 0.2%, K_2HPO_4 0.005%, $CaCO_3$0.002%, $FeSO_4 \cdot 7H_2O$ 0.001%, and agar 2.0%).

Potato dextrose Agar (PDA) media; for fungal isolation and growth.

Identification of actinomycetes.

Isolates' identificationwas done according to spore chain morphology, color of mycelium, production of pigments, colonyhardness, odor(earthy/geosmin smell) and gram staining (Gram +ve). Further investigations were done for the isolates to be characterized by physiological and biochemicalproperties. Bergey's Manual of Determinative Baacteriology (1984).

Determination of chitinase producing isolates.

A preliminaryexperiment was carried out to determine chitinase production by isolated strains, using colloidal chitin agar and observed for a clear zone around colonies suggesting production of the enzyme. (Hsu &Lockhood, 1975).

Secondary metabolites extraction

According to the initial observation ofchitin degradation,the selected strainswere cultured in broth medium andfurther inoculated (10%) into fermentation medium (glucose 1%, peptone 2% and pH 7.2) then kept for fermentation at 28° C for 7 days. Then theculture was centrifuged at 10,000 rpm for ten min.The cell-free supernatant obtained wasadded to equal volume of ethylacetate and shook for 2 hours. The layer containingthemetabolites wasseparated. Ethylacetatewasevaporated and the obtained residueafter evaporation was weighed and used for its

antagonistic effectagainst*F. oxysporum*.(Sinha, *et al*., 2014).

Antifungalactivity

Dual culture technique was done to screen the ability of actinomycetes isolates to inhibit plant pathogenic fungus *F.oxysporum*, growth. Fifteen mm disc of a pure culture of *F.oxysporum* was placed at the centre of Petri dishes ofdifferent agar plates inoculated withactinomycetes isolates and incubated for 7 days at 28 ± 2°C. The antagonistic efficacy was measuredby the colony growth of the pathogen (radius growth towards each strain) and the growthinhibition (%) was estimated incomparison with control plates.Three replications weremaintained for each treatment. (Harpreet & Parihar , 2010).

(%) inhibition = (R - r) / R × 100;

Where r: the radial growth of thefungal colony (mm) against to theantagonistic strain, R: the radial growthof pathogen (mm) in control.

Maintenance of *S. littoralis*

Larvae of cotton leaf worm, *S.littoralis*, were reared in lab, on castor leaves for many generations, at 25 ± 2 °C, as described by El-Defrawi*et al*., 1964.

Insecticidal and metamorphic effects of the isolates

Supernatants of the actinomycete isolates were used to estimate their activity against 4th instar larvae of *S.littoralis*with leaf- dipping technique (Makkar& El Mandarawy, 1996) under their optimumnatural conditions. For testing the effectiveness of the isolates, fresh castor leaves, were washed with tap water followed by rinsing in sterile water and left fordrying for 5 min. Plant leaves were dipped in isolates supernatants containing their metabolites. The tested plant leaves were left for drying for 5 min and then placedinto glass jars. Ten larvae (L2) were put in one jar then the jars were placed atroom condition and kept at 27 °C ± 2 °C for 7 days. Three replicates were done for each treatment. In control treatment plant leaves were dipped in distilled water only. Then, mortalitywas recorded daily.Pupal duration and % adult emergence were also estimated.

RESULTS

Isolation of microorganisms:

Actinomycetes isolates

Of all the samples collected, twenty-three actinomycete isolates, were obtained according to soil isolation techniques (Table 1). Nine isolates were obtained from soil in KSA and 14 isolates from Egyptian soil. The isolates were isolated and identified due to morphological, physiological and biochemical characters. Then categorized in Table (1) according to colony color, place of isolation and biological activity against both*S. littoralis* and *F. oxysporum*.Colony color ranged from white, grey and brown. Identification was done according to Bergey's Manual of Determinative Baacteriology, which illustratedthat ,the active isolates were belonging to *Stryptomyceslavendulae*and*Str. clavuligerus*. Fig.($1_{a,b}$) showed that the morphology character of vegetative cells and spore chains of actinomycetes isolate (DI and FII).

Table (1):Actinomecetes isolates: colony color, place of isolation and biological activity

Isolate	location	Colony color	% mortality *S. littoralis*	% inhibition of *F. oxysporum*
DI	Dakahliya Governorate	whitish	70	67
DII	---	Grey	10	51
DIII	---	Dark grey	0	60
DIV	---	Dark grey	0	56
DV	---	Grey	80	62
KSI	Kafr-Shiekh Governorate	Brownish	0	62
KSII	---	Yellowish	0	65
KSIII	---	Dark brown	0	60
KSIV	---	Grey	0	56
KSV	---	Dark brown	0	58
GI	Giza Gov.	Yellowish white	0	49
GII	---	Grey	0	53
GIII	---	Grey	0	51
AbI	Abo Areesh	Brownish	0	58
AbII	---	Dark brownish	0	60
AbIII	---	Grey	0	58
FI	Fayfaa	whitish	0	56
FII	---	Yellowish white	60	60
FIII	---	Grey	0	58
FIV	---	Dark brownish	0	56

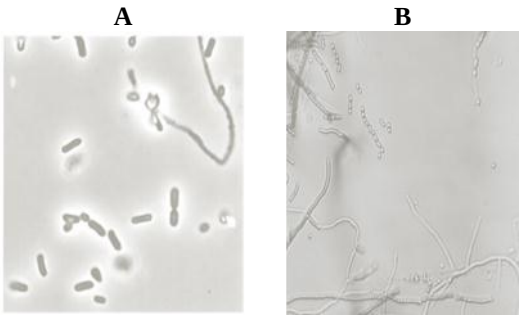

Fig.(1): Vegetative and spore chains of the isolates; A) DI ;B) FII

Fungal pathogen

Fusariumoxysporum, causes serious problems all over the world, for many field crops. For isolation, diseased tomato plants were tested for vascular wilt symptoms' characteristics and then the isolation procedures were done for the fungus. The lesion root areas with brownish rot showed existence of fungi which were isolated according toVicente *et al*., 2014, then identified as *F.oxysporum*. The isolation was done on PAD media. Then the fungi was examined microscopically. Fig.(2) illustrated the morphology of macroconidia and microconidia (asexual spores) of *F.oxysporum*.

Fig.(2): Macroconidia and Microconidia of *Fusariumoxysporum*

Enzymatic screening and antifungal activity

Enzymatic tests were done to determine the actinomycetes' ability to act as a degrader of organic compounds (chitin) when used in agricultural applications. *In vitro*,tests using the dual culture technique to examine the potential of actinomycetes isolates as bio-control agents, were done against the fungal pathogen *F.oxysporum*.The test organism were isolated from the diseased tomato plants.Results revealed that,from all the 23isolates, there are 20 able to inhibit the growth of the pathogen.The antagonistsisolated bacteria grew faster than the fungus, limiting the growth of the pathogen in varying, which appeared in various diameters. Looking at the growth diameters, it is clearly appeared, as indicated in Table (1) and Fig. (3), the percentage of inhibition ranged from 67 – 49 %. There was significant differences ($P< 0.05$) in percentage inhibition of radial growth of pathogen by all the isolates (Table 2). Inhibition were clearly observed by all treatments. (Fig. 3). Also, the metabolite extracts of the isolates were used to examine their antifungal activity against the pathogen. Metabolic extracts showed the ability to inhibit the pathogenic fungal growth.

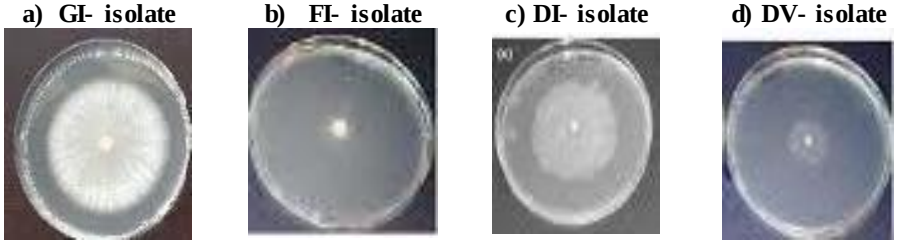

Fig. (3) Antagonistic reaction against *Fusariumoxysporum* growth.

Table (2): Radial growth (mm) and inhibition (%) of pathogen in dual culture test

Isolate	*Radial growth of fungus (mm)	% growth inhibition
DI	1.4[d]	67
DII	2.1[a]	51
DIII	1.7 [b]	60
DIV	1.9[b]	56
DV	1.6[c]	62
KSI	1.6[c]	62
KSII	1.5[c]	65
KSIII	1.7 [b]	60
KSIV	1.9[b]	56
KSV	1.8[b]	58
GI	2.2[a]	49
GII	2.0 [b]	53
GIII	2.1[a]	51
AbI	1.8[b]	58
AbII	1.7 [b]	60
AbIII	1.8[b]	58
FI	1.9[b]	56
FII	1.7 [b]	60
FIII	1.8[b]	58
FIV	1.9[b]	56

R = 4.3 mm; * = Means of three replicates; Means followed by different letters differ significantly at P = 0.05

Insecticidal activity

In order to study the efficiencyof theactinomycetes isolates against the insect pest *S.littoralis*, the secondary metabolites were introduced to the larval food. Data of this experiment are presented in Tables (1 &3). Three isolates represented high efficacy against the larvae.DI isolate caused 80 % mortality in tested larval group, and DV isolate caused 80 % while FII caused 60%. Also, the next stages of the survived larvae are affected, larval duration changed, the pupalduration and adult emergence changed, consequently. Prolongation of larval stageswere observed, also pupal duration. Not all pupae survived, succeed to transform to adult stages (moth).

Table (3): Stages' development of *S. littoralis* exposed to actinomycetes secondary metabolites.

Isolate	% Mortality	Pupalduration(days±SD)	% Adult emergence
DI	70b	9.69±0.84ns	38
DV	80a	8.05±1.2	21
FII	60c	9.08±0.56ns	40

Means±SD followed by ns; not significantly different (p>0.05).

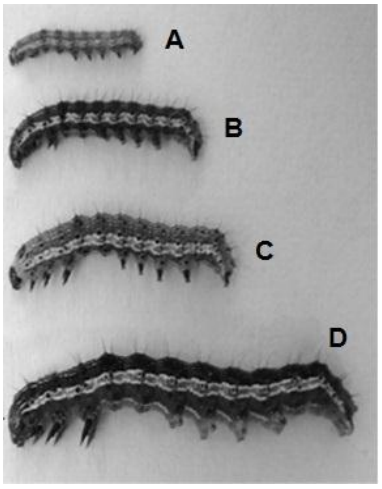

Fig. (4): Effects of different secondary metabolites (isolates) on larval developments (6th instar): a) DV-isolate; b) DI-isolate; c)FII-isolate and d) control.

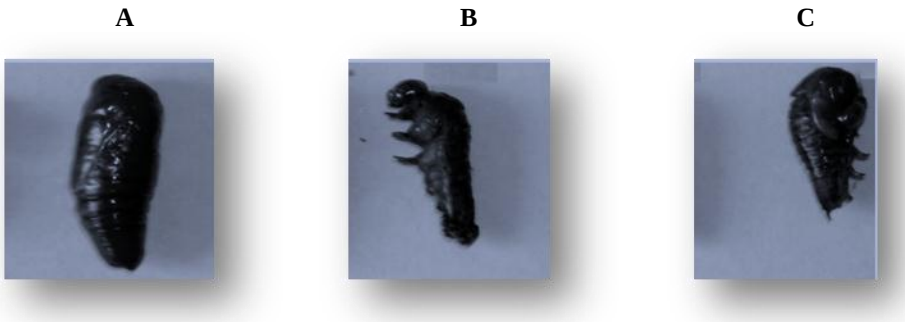

Fig. (5):Metamorphic effects of the isolates, a) control treatment; b) FII-isolatec) DV- isolate

DISCUSSION

Insect pests and fungal pathogens pose serious problems worldwide for economically important plants.*S.littoralis* considered as one of the major economic pests all over the world, infesting more than 40 plant families. Also the plant pathogen, *F.oxysporum*, which cause vascular wilt of many crops, occurs in most major crops production regions worldwide.

And because of the hazard effects of insecticides and fungicides, which cause toxicity for human and other organisms, an alternative methods environmentally friendly must be used. The microorganisms considered as a powerful agent could be used for biological control. Since then , there were many trials for using and developing microorganisms in this field. Recently, studies on chitinolytic microorganisms have introduced an increase of knowledge including their role in inhibition of growth of fungal plant pathogen and insect pests.

Actinomycetes which secret metabolites (enzymes and antibiotics), have both mycolyticand insecticidal activity. This suggests that, it can be possible for using as biological control agent.Harpreet&Parihar (2010) isolated actinomycetes from soil and proved that, the isolates were effective against the test fungi, including *Fusarium*sps. Similar results have been recorded by many authors such, Kharmna*et al*., 2009, who reported that, the crude extractsof antifungal compounds was active against test fungi.

Last decades actinomycetes have proved, by many authors, to be a powerful source for biological control of insect pests, where it is producing metabolites have insecticidal efficacy. Bream*et al*., 2001, mention the possibility of using actinomycetes metabolites for controlling *S.littoralis*. While, GadElHak*et al*., 2005 & El-Khawagh*et al*., 2011, made investigation confirmed the ability of actinomycetes for controlling Drosophila and mosquitoe larvae.

CONCLUSION AND FUTURE PROSPECTS

Secondary metabolites of the fermentation media from isolated actinomycetes were toxic to both , the plant pathogen *F. oxysporum* and the larvae of *S. lituralis*.Therefore, these metabolites show considerable potential for sharing in pest management procedures, asnew biopesticidal formulation. Also it can be used as a environmentally friendly fungicides.

In the further studies into the use of the actinomycetes for agricultural industry will be done to benefit from these important microbes for agricultural development. Many insect pests and plant pathogens may be controlled by using these organisms safely.

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تقييم عزلات محلية من البكتيريا الخيطية كعامل مكافحة حيوية ضد دودة ورق القطن *Spodoptera littoralis* و الممرض الفطري *Fusarium oxysporum*

عبير محمود محمد
كلية العلوم و الآداب . الداير. جامعة جازان
معهد بحوث وقاية النباتات - مركز البحوث الزراعية

تعتبر الكائنات الدقيقة عامل قوي من الممكن استخدامه في مجال المكافحة الحيوية. و قد جرت محاولات عديدة لاستخدامها و تطويرها لهذا الغرض. لذلك يهدف هذا البحث لعزل عدة عزلات محلية للبكتيريا الخيطية (الاكتينوميسيتات)، من التربة و تجربتها حيويا ضد كل من دودة ورق القطن*Spodopteralittoralis* وكذلك الممرض الفطري *Fusariumoxysporum*. تبعا للطرق المتبعة للعزل من التربة ، تم عزل ٢٣ عزلة بكتيرية من البكتيريا الخيطية من التربة الزراعية بمصر و بالمملكة العربية السعودية. ثم تم اختبار الفعالية الحيوية للعزلات ضد كل من يرقات العمر الرابع للآفة *S. littoralis* و كذلك الممرض الفطري *F.oxysporum* المسبب المرضي لمرض الذبول الوعائي الفيوزارمي. و قد أظهرت النتائج أن هناك ثلاث عزلات (DI, DV and FII) ذات فعالية ضد يرقات العمر الرابع لدودة ورق القطن بنسبة وفيات ٧٠ ، ٨٠ ، ٦٠ % على التوالي. كذلك فقد ظهرت فعالية على الأطوار الأخرى في صورة تشوهات و فشل في التعذر و خروج الفراش. بينما أثبتت النتائج أن ٢٠ عزلة من الكل (بما فيها الثلاث الفعالة ضد الآفة) ذات فعالية في تثبيط النمو للمرض الفطري بنسب تفاوتت من ٤٩ – ٦٧ %. كانت العزلات الثلاث تنتمي للأنواع البكتيرية *Stryptomyceslavendulae*، *Str. clavuligerus.*