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Pseudomonas fluorescens as an efficient entomopathogenic bacterium against *Spodoptera littoralis* (Nocotiudiae: Lepidoptera)

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Abstract

The object of this study is to evaluate the toxicity as well as the latent efficiency of the *Pseudomonas fluorescens* as bio-control agent for controlling *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). Different method techniques namely, spraying, immersion and dipping were used in this study. Four concentrations *P. fluorescens* were used for each method, the immersion method was the most effective treatment Lc_{50} was 46.93% followed bythe spraying technique 62.04% and the last one was dipping method was 81.26% against the 4th larval stage the toxicity index was can be arranged in the following descending order immersion, spraying and dipping technique since the values were 100, 75.64 and 57.75%, respectively. The latent effect of LC_{50} for *P. fluorescens* was studied on some biological aspects against 4th instar of *S. littoralis*. The *P. fluorescens* increased the duration of 4th larval stage till pupation compared with control the pupation% decreased in all methods of treatment. Mode of action of *P. fluorescens* was able to produce chitinase and protease these enzymes play a biological role in controlling *S. littoralis*. 40˚c and pH 7 were optimum physical factors for *P. fluorescens* was increase the activity of protease and chitinase.

Introduction

In Egypt, filed and vegetable crops are considered of great economic importance for local consumption and exportation. The Egyptian cotton leaf worm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) was and still now considered one of the most serious phytophagous pests of Egyptian cotton. Larvae attack other wide range of field and horticulture crops such as vegetables (Kandil *et al*., 2003). Intensive use of broad spectrum chemical insecticides for controlling *S. littoralis* usually leads to adverse effects on target organisms and development of high levels of resistance to human health hazards.

Thus, in order to offer our growers alternatives to harmful synthetic chemical pesticides, it is imperative to explore eco-friendly pest management strategies utilizing biological control agents that could provide similar efficacy against this pest.

Entomopathogenic bacteria have stimulated to explore of new possibilities and alternative safer methods for controlling pests. One of the most promising lives of investigation is integrated pest management, which included also the alternative methods of using biocontrol agents for controlling the pests on the different crops. It was necessary and needed to seek some environment friendly compounds for field crop protection such as bacteria.

One of the alternative managements is using *Pseudomonas fluorescens* as microbial bio-control agent for controlling insects and mites by hydrolyzing chitinous exoskeleton (Kramer and Muthukrishnan, 1997). Bacterial spraying was significantly more effective than dipping the spray application demonstrated 100% efficacy (Aksoy *et al*., 2008). Microbial insecticides such as *Pseudomonas* have been reported to provide inadequate control of *S. littoralis* and prime candidates for use in integrated pest management program (IPM). They have high pathogenicity for target pests. Safe of most non-target organisms, and have good integration with other pest control methods (Ibrahim *et al*., 2010).

Therefore, the aim of current study was to investigate the effect of *P. fluorescens* on cotton leaf worm.

Materials and methods

1. Isolation of *Pseudomonas fluorescens*:

The 10 cm rhizhosphere soil particles loosely adhering to the roots were gently teased out and the roots were cut into small pieces and mixed well. The soil thus obtained was shaken with 90 ml of sterile distilled water for 10-20 min to obtain standard soil suspension. Isolation of Pseudomonas was made by following the serial dilutions and pour plate method using the specific King's B medium (King *et al.*, 1954). One ml of soil to suspension from aliquot dilutions was aseptically added to sterile Petri plates containing twenty ml of sterile medium and incubated at 28+2 °C for 48 hrs. After incubation, well separated individual colonies with yellow green and blue white pigments were marked and detected by viewing under UV light. The individual colonies were picked up with sterile loop and transferred to fresh King's B slants and the pure cultures so obtained were stored in the refrigerator at 4°C for further use. Morphological characterization of pure cultures of the selected isolates was streaked on King's B agar petri plates separately for colony development. The individual colonies were examined for shape, size, structure of colonies and pigmentation.

2. Pathogenicity of *Pseudomonas fluorescens* on blood agar:

Mix 100 ml of nutrient agar and 5 ml of fresh human blood and put in sterilized petri dishes and culture *P. fluorescens* and incubate. At 37˚c for 3 days. pH

3. Stock culture of *Spodoptera littoralis*:

The stock culture of susceptible Egyptian cotton leafworm, *S. littoralis* was obtained from plant protection institute (Sharkia branch) and reared on castor leaves (*Ricinus communis* **L.**) at laboratory conditions of 27 +2 °C and 70+ 5 RH.%. Egg masses were placed on leaves of castor been in 450 gm. cylindrical glass jars. The jars were covered with muslin cloth and fastened with rubber bands. First instar larvae hatched within 3-4 days. The newly hatched larvae were transferred into 2Kg capacity rearing jars bottomed with cheats of towel paper to absorb excess humidity. Castor leaves were provided daily to the larvae in sufficient amounts. The accumulated feces and debris were cleaned out daily. When larvae were in the sixth instar, moist sawdust was placed on the bottom of rearing jars so as to provide a pupation side. Pupae were eventually collected and placed in clean jars and adults emerged within 10-15 days. Newly emerged moths were sexed and kept in mating cages. Each cage was provided with 15% sugar

solution on a wad of cotton wool, which was changed daily to avoid fermentation and the growth of microorganisms. As an oviposition site, fresh green leaves of Tafla, *Nerium oleander* **L.** were daily introduced into the breeding cages. Newly laid egg masses were collected daily and transferred into the rearing jars (Five egg masses per jar) (El-Defrawi *et al*., 1964).

4. Bioassay tests:

The effect of *P. fluorescens* was evaluated against 4th larval instar *S. littoralis* adopting three different methods i.e., spraying, dipping and immersion methods of bacterial suspension.

5. Method technique:

5.1. Leaf dipping technique:

The leaf dipping technique was applied to test the effect of *P. fluorescens* on 4th instar total number of 60 divided into 6 replicates each replicate containing 10 larvae then put in castor bean leaves .For treatment Castor bean leaves were dipped in each concentration, 2.3×10^{5} & 2.3×10^{6} & 2.3×10^{7} and 2.3×10^{8}spore/ml for 30 seconds and then left to dry in the room the 4th instar larvae were confined with the treated leaves in glass jars covered with muslin for four days. The treated leaves were then removed and fresh untreated leaves were provided for other days. Bioassay included untreated check in which leaves were dipped in water only. The mortality % was calculated after 1, 2, 3 and 4 days of treatment and Lc_{50} spore/ml values were calculated.

5.2. Insect spray technique:

The same number of 4th from cotton leafworm sprayed with the same concentrations which used at the above technique then the larvae put on castor leaf bean and then sprayed with above concentration that used in leaf dipping technique and then Lc_{50} was calculated. The treated leaves were then removed and fresh untreated leaves were provided for other days.

5.3. Larvae immersion technique:

Fourth instar of *S. littorals* were immersed in each concentration for 15 seconds and then transferred and confined daily with fresh untreated castor bean leaves in glass jars covered with muslin. For each experiment mentioned previously three replicates (Each of 20 larvae) were tested for each concentration. Daily inspection was carried out for all treatments and mortality percentages were recorded for fourth days after treatment. The slop and Lc_{50} values were estimates. Toxicity index % was calculated by dividing the recommended filed rate in ppm by Lc_{50} values of each test by Finney (1971).

Toxicity index was determined according to Sun (1950) as follows:

$$\text{Toxicity index \%} = \frac{\text{Lc50 of the most effective method}}{\text{Lc50 of the other compound method}} \times 100$$

6. Latent effect of Lc_{50} for *Pseudomonas fluorescens* of some biological aspects of *Spodoptera littoralis*:

Forty larvae of 4th from *S. littoralis* put individually on castor leaf bean leaves and treated with LC_{50} for three methods technique which explained later. Survival larvae from all previous treatments were reared on castor leaf bean leaves and compared with larvae reared on untreated leaves (Control) at 25± 2˚C and 65±5% RH. the following observations were preformed: Larval duration, pupation %, adult emergency% weight of pupae and failure of pupation%.

7. Effect of Lc_{50} for *Pseudomonas fluorescens* on some biological aspects of *Spodoptera littoralis*:

The value of Lc_{50} for above methods where calculated. Ten healthy 4th instar larvae with four replicates were subjected to their methods. The alive larvae were transferred to untreated leave of castor bean leaves in clean jars and let to feed and calculated the population, weight pupation and adult emersion%.

8. Effect of *Pseudomonas fluorescens* on enzyme activity of *Spodoptera littoralis*:

8.1. Enzyme preparation:

Larvae were treated with different concentrations; 100,75 and 50%, 50 mg/ml from each concentration 2.3×10^{5} & 2.3×10^{6} and 2.3×10^{7} and 2.3×10^{8}spore/ml were taken and repeated 3 times and homogenized in distilled water (50 mg/ml) using chilled glass Teflon homogenizer. Homogenates were centrifuged at 5000rpm for 20 min at 5ºC in a refrigerated centrifuge. The deposits were discarded and the supernatants were kept in a deep freezer at -20 ºC till use.

8.2. Method of chitinase activity:

The reaction mixture according to Ishaaya and Casida (1974).

8.3. Method of protease activity:

The proteolytic activity was determined according to Birk *et al*. (1962).

9. Evaluation of the physical factors affecting lipase and chitinase activity:

To determine the optimum temperature for lipase and chitinase production, 50 ml of basal medium was inoculated with one ml of 10^6 spores/ml was incubated for 96 hrs. at three different temperatures (37,40 and 45 ºC) in an orbital shaker at 150 rpm. To evaluate the best pH for lipase and chitinase production, the basal media described above was buffered with 0.1 ml sodium phosphate to obtain pH of 5.0,6.0,7.0 and 8.0 whereas none buffered basal medium was used as control. Fifty milliliters of the sterile growth medium were inoculated for 96 hrs. in an orbital shaker at 150 rpm and 28 ºC. Incubation period was screened for the tested fungi and its relation to enzyme activities.

10. Statistical analysis:

The data obtained were subjected to statistical analysis using **Duncan (1955)** multiple range tests to determine the significance of differences between mean values of the treatment.

Result and discussion

1. Susceptibility of the 4th larval of *Spodoptera littoralis* to *Pseudomonas fluorescens*:

The susceptibility of the 4th larval instars of the laboratory strain *S. littorals* to *P. fluorescens* was evaluated by using different techniques i.e., Spraying, dipping and immersion method of technique. The highest efficiency of *P. fluorescens* was attended 96 hrs., and data in Table (1) illustrated that the immersion technique was the most effective treatment, LC_{50} was 1.92×10^{6} spore/ml followed by spray technique LC_{50} was 2.13×10^{6} spore/ml and the last one was a dipping technique where the LC_{50} was 2.57×10^{7} spore/ml. The toxicity index% can be arranged in the following descending order immersion, spraying and dipping technique since the values were 100, 90.14 and 7.47%, respectively. Line slope values for three methods technique were 1.9, 1.23 and 1.02 for immersion, dipping and spray technique. This indicated that these techniques behave similar against the 4th instar larvae of *S. littoralis*.

This result agrees with, Khidr *et al.,* (2006) who stated that the immersion technique for controlling *S. littoralis* with bacteria *Saccharopolyspora spinosa* was more effective compared with other techniques used for controlling *S. littoralis.* Bakr *et al*. (2015) who stated that *Bacillus thuringinsis* (Bt) was toxic for 4th of *S. littoralis.*

Table (1): Susceptibility of the 4th larval instars of laboratory strain of the cotton leaf worm, *Spodoptera littoralis* exposed three methods formulation for 4 days.

Method Technique	LC spore/ml	Toxicity index %	Slope	Confidence limit	
				Upper spore/ml	Lower spore/ml
Spraying	2.13×10^{6}	90.14	1.02	2.49×10^{6}	1.98×10^{6}
Dipping	2.57×10^{7}	7.47	1.23	3.21×10^{7}	2.49×10^{7}
Immersion	1.92×10^{6}	100	1.90	2.21×10^{6}	1.66×10^{6}

2. Latent effect of LC_{50} for *Pseudomonas fluorescens* of some biological aspects of *Spodoptera littoralis*:

Data in Table (2) *P. fluorescens* with three techniques increasing the duration of larval stage when 4th was treated and the pupation was 72.22, 80 and 69.56% for spray, dipping and immersion techniques with *P. fluorescens*, respectively while was 95% for control. The failure pupation was increased in technique which caused the last pupation percent to be 30.43% in the immersion technique and other familiar pupation were 27.77 and 20% for the spray and dipping techniques, respectively. The weight of pupa was effective significantly with all different technique which decreased the weight of pupa compared with control, weight of pupa was 210.36, 270.59 and 255.18 mg. for immersion, dipping and spray technique which was 315.14 mg. in control. The obtained results were in a harmony with those observed also by Tolba (2006) referred that chitinase producing bacteria X S. marcescens caused significant physiological and morphological effects on pupal and adult stages where it caused-2-significant increase in the proportion of pupal. A similar result was recorded when were larvae of *S. littoralis* treated with Bt (El-Aasar *et al*., 2016) and Abdel-Hai (2001) reported that treated larvae of *S. littoralis* with bio-insecticides caused harmful effects on some biological aspects.

Table (2): Latent effect of LC_{50} for *Pseudomonas fluorescens* with three methods on some biological aspects of *Spodoptera littoralis* under laboratory condition.

Method technique	Duration of larval stage (In days)	Pupation%	Failure of pupation %	Weight of pupa (mg)	Adult emersion%
Spraying	12.79±0.12 ab	72.22	27.77	255.18±8.56c	69.23
Dipping	10.81± 0.11 b	80	20	270.59±11.44b	81.25
Immersion	13.07±0.16 a	69.56	30.43	210.36±6.57d	62.5
Control	10.19± 0.13 C	95	5	315.14±12.95 a	94.73

Means in columns followed by the same latter are not significantly different at p5% according to Duncan's multiple range test (Duncan, 1955).

3. Evaluation of the physical factors affected protease and chitinase activity:

These enzymes can be regulated depending on nutrient conditions these enzymes are pointed out as important in the infection processes, since they hydrolyze polymer protein and chitin, the major components of the insect's cuticle. Incubation temperatures varied in their effect on growth, protease and chitinase activity production, as shown in Table (3), growth of highest value for protease activity 82.71 u/ml at 30˚C while growth of highest value for chitinase activity was 76.59 u/ml for the same degree of temperature. The same action appeared at a higher temperature value 30˚C where the biomass was 23.19 and 45.21 u/ml for protease and chitinase activity, respectively.

Table (3): Protease and chitinase activity were produced by *Pseudomonas fluorescens* isolate as influenced different temperature.

Temp.˚C	Protease		Chitinase	
	Biomass mg/ml	Activity u/ml	Biomass mg/ml	Activity u/ml
37	9.51	49.52	20.03	62.31
40	23.19	82.71	45.21	76.59
45	15.27	64.76	31.15	45.15

Deferent pH values varied in their effect on the biomass, protease and chitinase production by *P. fluorescens* (Table 4) the optimum pH value was 7 that gave the highest biomass 19.51 mg/ml for protease and its activity 79 U/ml, for chitinase the biomass was 17.71 mg/ml and activity 86.19 u/ml.

Table (4): Effect of pH values on protease and chitinase activity were produced by *Pseudomonas fluorescens.*

pH values	Protease		Chitinase	
	Biomass mg/ml	Activity u/ml	Biomass mg/ml	Activity u/ml
5	10.12	62	12	81.15
6	19.51	79	17.71	86.19
7	9.30	71	6.5	60.13
8	12.15	50.11	2.11	49.32

There were significant between physical factors and it's affected on protease and chitinase activity. 30˚c and 6 pH value was optimum temperature and pH value for protease and chitinase activity. The mode of action of bacteria was able to produce the chitinase and protease enzyme these enzymes play a biological role in controlling cotton leafworm.

4. Enzyme activity of *Pseudomonas fluorescens* in different concentrations:

4.1. Chitinase activity, the change in the sensitivity of chitinase revealed its activity to the bacteria, *P. fluorescens* used (Table 5). Figure (1) postulates that treatment with any of tested concentrations caused variable levels of sensitivity towards the enzyme. All concentrations of *P. fluorescens* increased the activity of chitinase, the maximum of which was 30.17 u /ml for $2.4x10^8$ while it was 19.22 and 10.75 u/ml for $1x10^7$ and $1x10^6$, respectively. The activity of chitinase in untreated (Control) was 1.68 u/ ml.

4.2. Protease activity, as for the protease activity, which hydrolyzes protein caused available levels of activity. The activity was marked in $2.4x10^8$ spore/ml for *P. fluorescens* 52.13 u /ml while other conc. followed by 40.11 and 29.11 u/ml for $1x10^7$ and $1x10^6$, respectively. While in control it was 6.35 u/ ml.

Table (5): Enzyme activity of *Pseudomonas fluorescens* in different concentrations.

Concentrations Spore/ml	Protease u/ml	Chitinase u/ml
2.4×10^8	52.13	30.17
2.4×10^7	40.11	19.22
2.4×10^6	29.11	10.75
2.4×10^5	11.23	3.59
Control	6.35	1.68

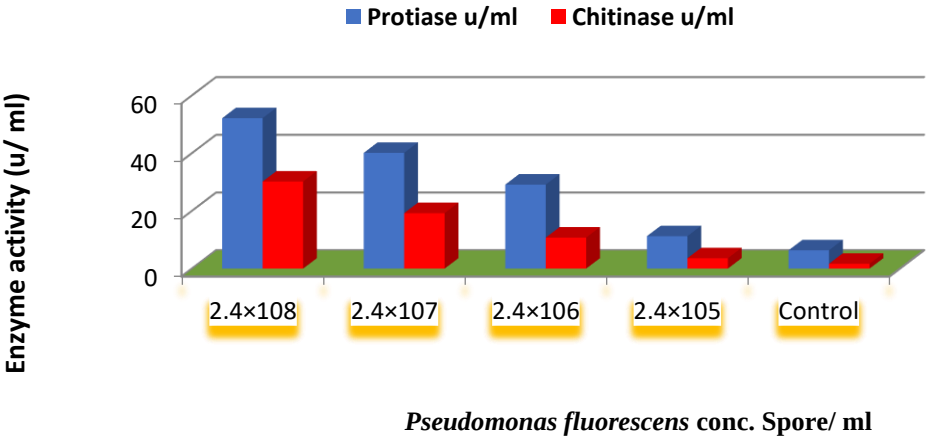

Figure (1): Effect of *Pseudomonas fluorescens* on enzyme activity of *Spodoptera littoralis*.

The present study has underlined the significance of the entomopathogenic bacteria, *P. fluorescens* in controlling *S. littoralis* which using under laboratory conditions. *P. fluorescens* had the most effect on *S. littoralis* in addition to affecting some biological aspects of worm. *P. fluorescens* affected non enzyme activity of worm where, which increased chitins and protease activities. 40˚C and 7 pH were the optimum physical factors for enzyme activity.

References

Abdel-Hai, F. (2001): Effect of some chemical additives on the efficacy of bioinsecticides against cotton leafworm, *Spodoptera littoralis* (Boisd.). PhD. Thesis. Fac. Agric., Azhar University.

Aksoy, H. M.; Ozman-Sullivan, S. K.; Ocal, H.; Celik, N. and Sullivan, G. T. (2008): The effects of *Pseudomonas putida* biotype B on *Tetranychus urticae* (Acari: Tetranychidae). Experimental and Applied Acarology, 46(1-4): 223-230.

Bakr, R. F.A.; Al-Yousef, A. F. and Hassan, S.H. (2015): Toxicological effect of the botanical extract oil seeds *Ricinus communis* and their biochemical activity on the cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). Egypt. Acad. J. Biology. Sci., 7 (1): 99-111.

Birk, Y.; Harpaz, I.; Ihasaya, I. and Bondi, A. (1962): Studies on the proteolytic activity of the beetles *Tenebrio* and *Tribolium*. J. Insect Physiol., 8:417-429.

Duncan, D.B. (1955): Multiple range and multiple F. tests. Biometrics., 11:1-41.

El-Aasar, A.A.; Abdo El-Mahasen, M.M.; Dahi, H.F. and Amin, H. S. (2016): Biochemical effects of some insect growth regulator and bio insecticides against (Boisd.) (Lepidoptera: Noctuidae). J. of Bioc. and Appl. Res., 2(8): 587- 594.

El-Defrawi, M.E.; Tappazada, A.; Mansour, N. and Zeid, M. (1964): Toxicological studies on Egyptian cotton leafworm, *Spodoptera littoralis* and susceptibility of different larval instar to insecticides. J. Econ. Entomol., 57(94):591-593.

Finney, D.J. **(1971):** Probit analysis. Astatistical treatment of the sigmoid response curve. 7th Ed., Cambridge Univ. Press, England.

Ibrahim, M. A. G.; Natalya, J.M. and Lee, B. (2010): Bt. Agenomics and proteomics perspective. Bioengineering Bugs,1 (1):31-51.

Ishaaya, I. and Casida, J. E. (1974): Dietary TH- 6040 alters composition and enzyme activity of housefly larval cuticle. Pestic. Biochem. Physiol., 4: 484-490.

Kandil, M. A.; Abdel-Aziz, N. F. and Sammour, E. A. (2003): Comparative toxicity of chlorfluazron and lufenron against cotton leafworm, *Spodoptera littoralis* (**Biosd**). Egypt. J. Agric. Res., 2: 645-661.

Khidr, A.A.; Mohamed, H.A. and Ahmed, M.A. (2006): Evaluating the toxic and latent efficacy of spinosad against the cotton leafworm, *Spodoptera littoralis*. Journal of Plant Protection and Pathology, 31(10):6725-6733.

King, E. D.; Ward, M. K. and Raney, D. E. (1954): Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med., 44: 301- 307.

Kramer, K.J. and Muthukrishnan, S. (1997): Insect chitinases: molecular biology and potential use as

biopesticides. Insect Biochem. Mol., 27:887-900.

Sun, Y. P. (1950): Toxicity index on improved method of comparing the relative toxicity of insecticides. J. Econ. Entomol., 43(1): 45 – 53.

Tolba, H.I. (2006): Biochemical studies on serratia marcescens for controlling the black the black cutworm, *Agrotis ipsilon* Huf. PhD. Thesis, Faculty of Agriculture Cairo University.