Biological Control of Some Species of Land Snails Infesting Citrus Trees

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Abstract: The microorganisms which present on shells, gastropods, and juveniles at *Monacha cartusiana* snails were studied. The result recorded that 7 genera of fungi (*Acremonium, Aspergillus, Penicillium, Blastomyces, Fusarium, Trichoderma* and *Candida*) and 5 genera of bacteria (*B. thuringiensis (Bt), E. coli, Pseudomonas, Klebsilla and Shigella*) were isolated from the snails. Post-treatment in laboratory 3 x 10^5 spores/ml of *Aspergillus flavus* causing highest mortality 30% to *M. cartusiana* snails. *B. thuringiensis* causing 100% mortality to *M. cartusiana* in laboratory treatment. Using the morphological and biochemical tests *B. thuringiensis* was identified as *B. thuringiensis* subsp. Kurstaki (ES^+, Sa^+, Le^+, Su^-) on whole isolate. The analysis of nucleotide sequence of 16s rDNA confirmed that the 8 tested local *Bt* isolates was belonged to *B. thuringiensis***.** Parasporal bodies of *B. thuringiensis* isolate had biological activity when assayed against *M. cartusiana* snails. The protein composition of parasporal bodies 140 Kilo Dalton (KDa). Parasporal bodies caused mortality reached 100% to *M. cartusiana* snails by concentration 7.8 mg/ml. Field application of *B. thuringiensis* as toxic spray on citrus trees of infected parts with adults of *M. cartusiana* snails by using 2 x 10^3 cfu/ml showed that mortality reached 89% of snails within 21 day.
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1. Introduction

Land snails are dangerous agricultural pests which classified under phylum Mollusca, Class Gastropoda., these snails have increased rapidly causing economic damage in the field crops, vegetables as well as horticultural crops. Moreover they are fungal feeders, consume a variety of fungi as *Agaricus bisporus* and *Pleurotus ostreatus* (Puslednik, 2002).

In Egypt, land snails have been increased and distributed rapidly in most Governorates. They caused considerable damage especially in most areas where they found the optimum conditions for survival and dispersion (El-Okda, 1981). In addition they have a role as intermediate hosts for many parasitic diseases which infected human, animals and birds. Some of them have been identified as disseminators of the spores of phytopathogenic fungi.

Bacillus thuringiensis is an entomopathogenic bacterium which forms a parasporal crystal during sporulation. *B. thuringiensis* is used commercially as an insecticide, and is marketed under several trade names as Dipel.

B. thuringiensis and *Bacillus cereus* are very closely related species. The species have been compared by several methods including biochemical clustering studies, comparing the utilization of sugars, DNA homology, and agglutination testing to determine the degree of relatedness. From the results of DNA homology studies, *B. thuringiensis* is considered a variant of *B. cereus* (Berkley and Good Fellow, 1968).

The spores of *B. thuringiensis* and *B. cereus* were different. *B. cereus* spores are more resistant to many adverse chemical and environmental conditions such as extreme temperature, ultraviolet light, desiccation, pH, and enzyme action (Kornberg *et al*., 1968). Because of its lack of resistance, *B. thuringiensis* is unable to maintain its dormant state at effective insecticidal levels in the field.

The aim of the present study was to isolation of microorganisms associated with *M. cartusiana* snails with screening for their antimicrobial activities, then selected and identified the most potent isolates. Furthermore, the bactericidal substance was extracted and purified from the most potent isolates.

2. Material and Methods

Survey of land snails

Survey of different land snails was carried out to study the distribution and population density of land snail species on citrus trees in 6 districts, Sharkia Governorate. The survey occurred monthly starting from January 2011 to December 2011. (Fakos, El-Salhia, Abohamad, Aboukabeer, Meniet – Elkamh and Belbies).

Collection of snails

The adult snails of *M. cartusiana* snails and juveniles were collected from citrus trees in Fakos,

El-Salhia, Abohamad, Meniet Elkamh and Belbies districts, Sharkia Governorate. These snails were kept in a muslin bags, transferred to the laboratory and provided with fresh cabbage leaves.

Culture media

The media used during this study were prepared according to Gams et al. (1998).

Survey of fungi and bacteria associated with snails

Bacteria and Fungi were isolated from these snails using dilution method (Johnson *et al.,* 1959) on nutrient agar, Sabouraud's glucose agar medium (SABG) and Czapek's-yeast agar medium (CZYA) or potato dextrose agar media (PDA), respectively.

Isolation from egg, juvenile and adult snails according to (Baker *et al.,* 1991)

Identification of the microbial isolates

Bacterial and Fungal isolates were identified according to Bergey's manual of determinative bacteriology (Holt *et al*., 1984). Isolation of *B. thuringiensis* Bioassay according to (Padua *et al*., 1980). Identification of *B. thuringiensis* isolates and causing death of *M. cartusiana* according to (Lecadet *et al*., 1999).

Identification of B. thuringiensis isolates by 16S rDNA

The DNA selected bacteria was extracted using GenElute™ Genomic DNA Kit, sigma Aldrich according to Birnboim (1983). To extract the DNA, the test *Bt* strain were cultured in nutrient broth over night at 37°C. Cells were harvested by centrifugation at 4000 xg for 15 min, washed 3 times with sterile distilled water centrifugation, and finally, the supernatant was discarded. An aliquot of 100 ul of cell-pellet was processed for cell lysis with lysis buffer, and protease K at 65 °C for 10 min, thereafter heating at 95 °C for 5 min to denature the proteases and stop the reaction. The treated samples were then processed using ROCHE MagNA Pure automated DNA isolation system according to the manufacturer's instructions (MagNA Pure LC DNA Isolation Kit III, Bacteria & Fungi). PCR amplification of 16S rDNA gene from tested *Bt* isolates was performed using the universal primers:

Forward (518F); 5'-CCAGCAGCCGCGGTAATACG-3', reverse (800R); 5'-TACCAG- GGTATCTAATCC-3'. Thirty five amplification cycles performed at 94°C for 45 s, 55°C for 60 s and 72°C for 60 s. DNA amplicons of about 1500 bp was obtained, purified, and sequenced (Macrogen, Seoul, Korea). Sequences were searched against the database (www.ncbi.nlm.com) using BLAST tool with a similarity cut off of 99.5% to identify the bacterium based on sequence similarity. The multiple alignments and phylogenetic analysis were carried out to discriminate and compare those sequences among each other and to determine the evolutionary DNA relatedness and the genetic distance.

The pathogenic ability of isolated bacteria and fungi

The isolated bacteria and fungi were investigated for their pathogenicity in apparently healthy snails of *M. cartusiana* according to Hogg (2005).

Inclusion protein preparation

Sporulated cultures were washed three times with distilled water before solubilization and used as crude parasporal inclusions. Parasporal inclusions were purified from sporulated cultures as described by (Saitoh *et al*., 1998). The crude or purified inclusions were suspended in 50 mmol l^{-1} Na_2CO3 (pH 10)+10 mmol l^{-1} DTT (dithiothreitol) + 1 mmol l^{-1} EDTA for 60 min at 37 °C. After solubilization of the inclusions, the solution was added with phenylmethylsulphonyl fluoride (Wako Pure Chemical, Tokyo, Japan) to stop the proteolytic reaction. The mixture was then centrifuged at 20 g for 5 min at 4 °C. Protein concentration of the supernatant fluid was determined by the method of Lowry *et al*. (1951).

Biological control

Due to the highest death percent which recorded by *B. thuringiensis* against *M. cartusiana* snails in the laboratory it was tested against the same snails by different concentration in the laboratory (*in vitro*) and in the field (*in vivo*) according to Ghamry (1997) and Lokma and Harpy (1999) with some modification. Field experiment (*In vivo* treatment) according to Hashem *et al.* (1992). Isolation and solubilization of parasporal bodies isolate of *B. thuringiensis* according to Ang and Nickerson (1978).

3.Results

Survey and infestation levels of land snails to determine land snail species, occurred in citrus trees in 6 districts, Sharkia Governorate. The obtained data in Table (1) clear that two species of terrestrial snails belonging to order: Stylommatophora were found in the different districts, these species were the *Monacha cartusiana* and *Succinia putris*. All districts were infested by *M. cartusiana* snail only, but *S. putris* appeared only (one snail) in Fakos. It is clear that *M. cartusiana* was infested most citrus trees and *S. putris* appeared one time on lemon trees at Fakos district.

The microorganism recovered in this study belonging to seven fungi and five bacteria as shown in tables (2 &3).

The highest mortality rate (30%) occurred to *M. cartusiana* by Aspergillus (Table 4). *B. thuringiensis*, *E. coli*, *Klebsilla, Pseudomonas* and *Shigella* show

the highest mortality rate 100% occurred to *M. cartusiana* by *B. thuringiensis* (Table 5).

B. thuringiensis grown on three media produced flat, dry, white colonies with uneven borders. *B. thuringiensis* (Bt) is gram positive soil-dwelling, spore-forming rod shaped bacteria. It produces a spherical and bipyramidal shaped crystal from its crystal protein (Cry proteins).

Most strains produce spherical and bipyramidal crystals. Only a low percentage of strain (16%) formed atypical crystals often heterogeneous in size and shape. The protein profiles of spherical and bipyramidal crystals consists of poorly defined component which could be a source of novel insecticidal prosperities (Fig. 1).

Using the biochemical typing method, all the *B. thuringiensis* strains isolated were divided into four biochemical types. Based on biochemical typing, *B. thuringiensis* isolates found that it BT Subsp. Kurstaki (Es^+ Sa^+, Le^+ and Su^-) of the whole isolates.

Extraction of total DNA from *B.t* strain for PCR analysis a total of 8 randomly selected (Bt) isolates representative for the different assigned morphology; biochemical type and abundant crystal shape morphology were selected for this experiment. The analysis of PCR products by agarose gel electrophoresis revealed amplified target bands ~1550 bp (Fig. 2).

Isolate sequence

Seq16 sequence exported from seq16-Sequencer4a

TTAAAAAAAAGTGGGGGGGGGGCAA
TCAGCAGGCGAGCGATGGGTTAGAGT
TGCTCTTATGAAGCTAGCGTCGGAAT
AAGACCAGGAGCGTGTCCCTGCAATG
ATACTGGACCACTCTCAGAACAGTAA
CTATATGGGTACCACTTAAGCTGAAG
AATCCTTCATATTAATGCTACATTGAC
ATCTGAGACTCGTGCCGGGAGTGTCT
CGAGTCTTCTACCGCTATTGTCGTTTT
TCTGTACGGTGTATAGTCGCCCCTAAG
GGATCTGACCAGAGTTTTTCTGTTGGG
CACGACAGCACGGAGAGTCGCTGTTT
AATGATG

The BLAST analysis of the nucleotide sequence returned confident results and confirmed that the 8 tested local (Bt)isolates was belonged to *B. thuringiensis* as in Table (6).

Parasporal bodies of (Bt) isolate had biological activities when assayed against snails. The estimated concentration of parasporal bodies killed 50% of *M. cartusiana* for toxins of (Bt)isolate was 3.9 mg/ml (Table 7). The protein compositions of the parasporal bodies in (Bt)isolate were 140 kilo Dalton (KDa) (Fig.3).

Efficacy of different concentrations *in vitro* of (Bt)isolates as toxic of juveniles and adult stage of *M. cartusiana* revealed that none of the tested *B. thuringiensis* exhibit any bacteriological activity one day post treatment. Mortality percentage increased gradually to reaches in maximum after 21 days where it gave highest effect 100% (Table 8).

Field application of *B. thuringiensis* as toxic spray on adults of *M. cartusiana* snails at different period showed that initial bactericidal was 12.5% after 7 day post treatment and highest gave mortality percentages 89% after day 21 post treatment (Table 9).

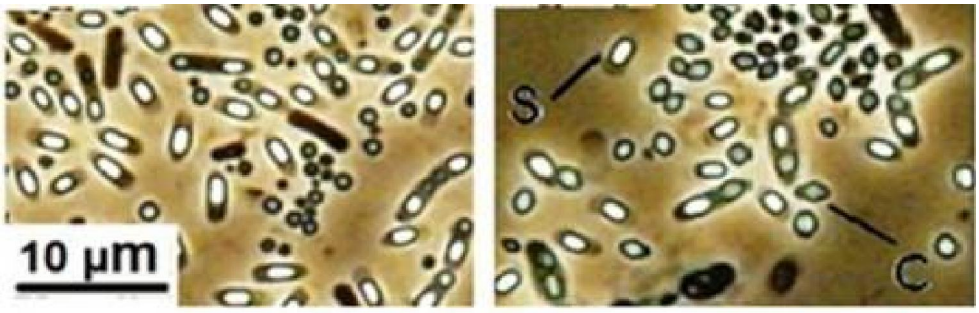

Fig. 1. Phase contrast micrographs of BT isolates showing spore (S) and crystal shapes ©: isolates number 1 (spherical), 2 (bipyramidal)

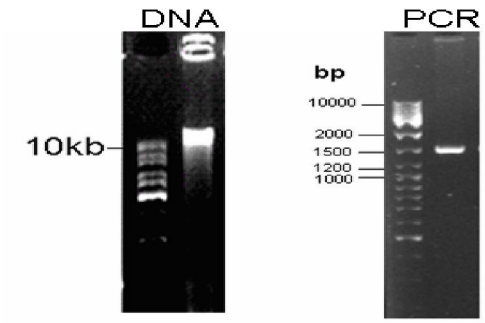

Fig. 2. Analysis of PCR products by agarose gel electrophoresis amplified target band ~ 1550bp.

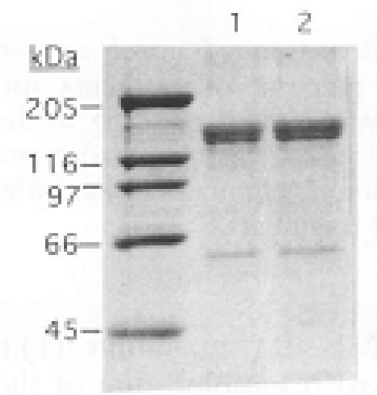

Fig. 3. Cry protein profiles of *B.t* isolates on SDS- 7.5% PAGE (lane 1 and 2).

Table 1. Survey and ingestion of land snails at districts of sharkia governorate from january 2011 to december 2011

Districts	Species	Mean number of snails sand	Host citrus trees and level infestation
Abohamad	*M. cartusiana*	39	Abussrh +++ Yousefi +
El-Salhia	*M. cartusiana*	19	Baladi ++ limon +
Meniet Elkamh	*M. cartusiana*	15	Abussrh ++ Balady +
Fakos	*M. cartusiana* - S.putris	13	Lemon ++ Balady +
Belbas	*M. cartusiana*	7	Yousefi +++
Abokabier	*M. cartusiana*	9	Lemon ++ Balady ++

+++ = heavy infestation ++ = moderate infestation + = low infestation

Table 2. The isolated fungi from shell, gastropod, juvenile and eggs of *m. Cartusiana* snails

Genus	Shell	Gastropod	Juveniles	Eggs
Acremonium	-	+	+	-
Aspergillus	-	+	+	-
Penicillum	-	+	-	+
Blastomyces	-	-	-	+
Fusarium	-	-	+	-
Trichoderma	-	+	-	-
Candida	-	+	+	-

Table 3. The isolated bacteria from shell, gastropod, juvenile and eggs of *m. Cartusiana* snails

Genus	Shell	Gastropod	Juveniles	Eggs
Bacillus thuringiensis	-	+	+	+
E. coli	-	+	-	-
Klebsilla	-	+	+	-
Pseudomonas	-	-	-	+
Shegella	-	+	+	-

Table 4. Effect of the isolated fungi on mortality of juveniles and adult of *m. Cartusiana* snails

Fungi isolates	Juveniles		Adult stage	
	No.	%	No.	%
Acremonium	2	20	2	20
Aspergillus	3	30	3	30
Penicillum	0	0	0	0
Blastomyces	1	10	1	10
Fusarium	0	0	0	0
Trichoderma	0	0	0	0
Candida	0	0	0	0

Table 5. Effect of the isolated bacteria on mortality of juveniles and adult of *M. Cartusiana* snails

Bacteria isolates	Juveniles		Adult stage	
	No.	%	No.	%
Bacillus thuringiensis	10	100	10	100
E. coli	5	50	5	50
Klebsilla	1	10	0	0
Pseudomonas	0	0	0	0
Shigella	0	0	0	0

Table 6. Sequences similarity of the isolate sequenced 16s rDNA genes from *B.t* isolates.

Accession	Description	E value	Max ident
GU936826.1	Uncultured Bacillus sp. clone A8DMCS05 16S ribosomal RNA gene, partial sequence	6e-06	90%
EU201189.1	*Bacillus cereus* strain DS-4 16S ribosomal RNA gene, partial sequence	6e-06	90%
JQ289048.1	*Bacillus thuringiensis* strain SP-17-SP-15 16S ribosomal RNA gene, partial sequence	7e-05	86%
JQ289052.1	*Bacillus thuringiensis* strain SP20R-SU7 16S ribosomal RNA gene, partial sequence	2e-04	88%
JQ289046.1	*Bacillus thuringiensis* strain SP15F-Sp-20 16S ribosomal RNA gene, partial sequence	2e-04	88%
JN887351.1	*Bacillus cereus* strain CB4 16S ribosomal RNA gene, partial sequence	2e-04	88%
JN698960.1	*Bacillus cereus* strain AIMST 9MPE1 16S ribosomal RNA gene, partial sequence	2e-04	88%

Table 7. Effect of different concentrations of parasporal bodies on mortality of juveniles and adult of *M. Cartusiana* snails

Concentration of (140 KDa)	Juveniles		Adult stage	
	No.	% of mortality	No.	% of mortality
1.8 mg/ml	1	10	1	10
3.9 mg/ml	6	60	5	50
7.8 mg/ml	10	100	10	100
11.8 mg/ml	10	100	10	100

Table 8. Efficacy of different concentration in vitro of *B.t* isolate on juveniles and adult stage of *M. Cartusiana.*

Concentrations of BT	Reduction percentage juveniles				Reduction percentage adult			
	1 day	7 days	14 days	21 days	1 day	7 days	14 days	21 days
2 x 10^2 cfu/ml	0	4	4	10	0	3	20	30
2 x 10^3 cfu/ml	0	20	30	100	0	30	50	100
2 x 10^5 cfu/ml	0	30	50	100	0	40	60	100

Table 9. Effect of *B.t* isolate isolated on *M. Cartusiana* snails under field condition at sharkia governorate

Concentration of BT	Reduction percentage after treatment (days)			
	7 day	14 days	21 days	28 days
2 x 10^2 cfu/ml	2.8	8.2	20.1	20.1
2 x 10^3 cfu/ml	12.5	56	89.2	89.5
2 x 10^5 cfu/ml	14.2	62	89.5	89.5

4. Discussion

Land snails have become increasingly important as crop pests in agriculture and vector. Land snails that cause plant damage have been the subject of intensive study and control measures.

Firstly, the ecological studies were occurred because it the most important point for control this pest. These studies were occurred in Fakos, El-Salhia, Abohammad, Meniet El-Kamh and Belies districts, Sharkia Governorate, Egypt. It including of the two main parts which is survey and the population dynamics of the land snails in this districts. Survey showed that *M. cartusiana* was the only snails in Abohammad, El-Salhia, Meniet El-Kamh, Aboukabeer and Belies, while *M. cartusiana* and *S. purist* were the snails in Fakos districts species infested citrus trees at these districts where it infested host, with few, moderate and high members. *M. cartusiana* the major snail which infested different citrus trees in six different districts, this record by Arafa (1997).

The obtained results showed that 12 genera (7 fungi and 5 bacteria) were isolated from egg, juvenile, and adult of *M. cartusiana* snails. Fungi were the dominant contaminated microorganisms isolated from eggs, juveniles and adult. These results were agree with those obtained by Ghareb, (2007) who investigated twenty seven genera (24 fungi and 3 bacteria) isolated from eggs, juveniles and adult of Cochlicella acuta, Theba pisana land snails, 3 fungi isolated from juveniles surface were active against tested snails. These result are in agree with those obtained by Acremonium, Blastomyces representing the most potent fungi isolates were selected for identification.

Bacillus thuringiensis was found effective against *M. cartusiana* snails, the mortality rate reached 100% according to variety of bacteria and the method of pathogen application and spraying method was found highly effective application.

Isolation and identification of *B. thuringiensis* have been found to colonize many different habitat (Heimpel, 1967; Goldberg and Margalit, 1977 and Meadows *et al*., 1992) but its normal habitat is the soil (Dulmage and Aizawa, 1982).

Using the biochemical typing method, all *B. thuringiensis* isolates were typing as *B. thuringiensis* Kurstaki. This system is based on the biochemical tests which have been identified by De Barjac (1981) and have been used for *B. thuringiensis* classification in many investigation (Dow and Lone 1999).

Several researchers (Joung and Cote, 2002; Soufiane and Cote, 2009; Poornima *et al*., 2010) have used 16S rDNA gene analysis as a molecular identification tool for *Bt*, while Soufiane and Cote (2009) not only used this tool for the identification of Bt species but also claimed its ability to discriminate Bt different H serotypes. In the present study, 16S rDNA gene analysis proved useful in the identity of the tested local *Bt* isolates and the two *Bt* reference strains as belonged to *B. thuringiensis*. Hence, it unambiguously confirmed the biochemical phenotypic identity.

In our results *B. thuringiensis* isolates we containing either Cryl A(a), Cryl A(b) and Cryl (C) gene profile exhibited conferred efficacy against snailcidal agreed with result of Visser *et al*. (1990) were discovered the Cryl (c)-type genes insecticidal activity against spodoptera exigua.

This work we developed assays for testing potential snailcidal properties of *Bt*. Snailcidal *Bt* toxin expressed transgenically in appropriate root and stem tissue, might provide an effective strategy to control plant-parasitic, a major class of agriculture pest that cause billion of dollars in crop damage per year in United States alone (Sasser and Freckman, 1987). These results indicate that Cryl A could be useful for managing *M. cartusiana* snails.

Conclusion

In this study the microorganisms associated with *M. cartusiana* snails were isolated and screened for their antimicrobial activities. *B.t* was the most potent isolates. A new parasporal bodies isolated from *B.t* were 140 kilo Dalton (KDa) had biological activities against *M. cartusiana* snails.

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Disclosure Statement

No competing financial interests exist.

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