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Full Length Research Paper

Biocontrol potential of a *Pseudomonas aeruginosa* strain against *Bactrocera oleae*

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A formulation was developed from the metabolite(s) of *Pseudomonas aeruginosa* (IL5) and tested against 3rd instar larvae of the olive fruit fly *Bactrocera oleae*, the most serious pest on olive cultivations in the world. In this study, we have isolated 115 bacterial strains from various ecological niches from the soil, water, dead insects and infested olives and tested their ability to protect the olive fruits against this pest. The results show a high mortality at pH = 6 observed 72 h later. The LC50 dosage for larvae of this insect species was 24.0784 µl/ml. Our results conclude that this bacterial strain may be used as natural biocides.

Key words: *Bactrocera oleae,* larvae, *Pseudomonas aeruginosa,* bio-control.

INTRODUCTION

The olive fruit fly, *Bactrocera oleae* (Rossi) (formerly *Dacus oleae*), is a serious pest of olives in most of the countries around the Mediterranean region where over 98% of the world's olives are produced. The female lays eggs in green olive fruits, and larval development is completed within the fruits. The third instars tunnels to the fruit surface and scrapes away pulp below the skin, leaving a transparent "window" through which it exits as a larva or adult (Vossen et al., 2006). This parasitic infestation causes extensive damage to the olive fruit and significantly deteriorates by pre and post harvest damage due to the attack of insects, which strongly alters the quality of olives (Mraicha et al., 2010). The damage caused by these insects' results in about 10 to 30% loss of the olive crop even with the yearly routine pesticide treatments to control the olive fruits fly populations (Economopoulos, 2002; Economopoulos et al., 1982; Michelakis, 1990). In the absence of treatment and under optimum climate conditions the pest could infect up to 100% of the olive fruits (Athar, 2005). The predominant method to control the olive fly population has been the use of traditional chemical insecticides. However, continued use of insecticides has caused enormous problems such as; environmental pollution, emergence of pesticide resistant insect populations as well as rising prices of new chemical pesticides, which have stimulated the search for new eco-friendly vector control tools (Mittal, 2003). As an alternative to chemical control or as part of integrated pest management (IPM) programs, there is a resurgence of interest in the use of microbial insecticides for the biological control of insect pests (Mahmoud, 2009). Use of microorganisms or their products as bio-control agents of insects, and more specifically against *B. oleae* will allow us to fight against this insect.

Among the Pseudomonas, *P. aeruginosa* is well known for its capability to produce rhamnolipid biosurfactant with potential surface-active properties when grown on different carbon substrates. This particular organism produces two types of glycolipids both containing rhamnose as the carbohydrate moiety (Desai and Banat, 1997). One of the best products that was recently developed is GLUTICID, (Company trademark), a biological compound

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which is an antifungal product constituted by antimicrobial metabolites such as siderophore pyoverdine and salicylic acid produced by *P. aeruginosa* PSS. This product has been very effective against *Paeronospora tabacina* in tobacco culture, *Alternation solani* in tomato and *Pseudoperonospora cubensis* in cucumber (Fallahzadeh et al., 2010). Other studies have shown that *P*. aeruginosa oxyR mutant has already revealed its ability to kill the insect *D*. melanogaster (Lau et al., 2003). Additional studies have shown a Positive Correlation between Virulence of *P. aeruginosa* in insects like *Galleria mellonella* (Bulla et al., 1975; George et al., 2000).

Shanmugaiah et al. (2009) purified and characterized an antimicrobial compound produced by *P. aeruginosa*, and evaluated its activity against rice pathogens, *Rhizoctonia solani* and *Xanthomonas oryzae pv. Oryzae*. Toxins from these bacteria are able to inhibit the radial growth of *Macrophomina phaseolina, Fusarium solani* and *Fusarium oxysporum* by producing a zone of inhibition of 2. 6 and 10 mm respectively (Farrukh et al., 2007). Moreover *P. aeruginosa* has been used in agriculture as a biological agent and has provided substantial control on a variety of soil-borne plant pathogens including *Macrophomina phaseolina*, *Botrytis cinerea*, *Rhizoctonia solani*, *Colletotrichum truncatum*, *Pythium*, *Fusarium. Pseudomonas aeruginosa* also acts as a bio-control instrument against *Ganoderma boninense*, the causative agent of Basal Stem Rot (BSR) of oil palm (Badri and Sariah, 2009).

The aim of this study was to screen, identify, and characterize pathogenic bacteria isolated from various biotopes (soils, waters, dead insects and infested olives…) and evaluate their toxicities against *B. oleae*'s larvae in order to use them in different bio-control strategies.

MATERIALS AND METHODS

Isolation of bacterial strains

Bacteria screened and studied in this work were isolated from different biotopes (soils, water, dead insects and infested olives). Soil sample preparations were prepared and performed as described by Geetha et al. (2007), with minor modifications. In brief, 1 g of soil was weighed, transferred to a vial containing 10 ml of sterile water, and kept on a rotary shaker (Edmund Buhler GmbH, Germany) at 150 rpm for 30 min, to dislodge bacterial cells from the soil particles. Water samples of 50 ml each were collected from Ain chkaf and Mon Fleuri River using sterile Pasteur pipette in sterile vials that were fitted with screw caps. Sterilization of the vials was performed by autoclaving at 12°C for 15 min prior to sampling. To get samples with minimal effect of ultraviolet (UV) light, soil and water samples were collected from about 2 to 3 cm below the surface of the habitat. Samples were transported to the Microbiology laboratory (Fez, Morocco) and analyzed within 2 h. The infested fruit of olive were collected from olive orchards in Fez region. Samples were crushed in 1 ml of liquid and sterile LB medium. All sample supernatants were diluted 10-fold and 0.1 ml was spread on pre-solidified LB agar. The suspension drop in each Petri dish (9 cm diameter) was spread evenly using a flame-sterilized glass rod. The Petri dishes were incubated at 37°C for 2 days and bacterial colonies on the medium were individually identified by appearance, color and smell. Bacterial cells of selected colonies were transferred onto LB slants in glass tubes using a sterilized inoculation loop. The stock cultures were labeled, incubated at 37°C for 48 h and stored at 4°C until use and a replicate was stored in sterile 20% glycerol solution at -80°C.

Collecting of *Bactrocera oleae* larvae

The *Bactrocera oleae* adults used in this work were reared in our laboratory as previously described by El Haidani et al. (2008). In brief, the reproductive cycle was accomplished by placing fresh olives inside cages thus allowing the females to lay eggs. To avoid contamination by fungi, freshly harvested green olives of intermediate ripening were chosen. The infested fruits were retrieved every 24 h, incubated at 25°C and replaced by new ones. The infested olives were then placed in 5 liter jars in which small openings were drilled allowing the transfer of larvae from one jar to another for easy recovery.

Screening for *Bactrocera oleae* larvicidal and pupicidal activity

Overnight cultures of the bacterial strain tested was spread over an LB medium and incubated at 37°C for 24 h. Third instars larvae from 20 *B. Oleae* were recovered from olive fruits and incubated in a synthetic medium (Tsitsipis, 1977) for 24 h at 25°C and 65 to 70% relative humidity. In our laboratory, we showed that the transition in the synthetic medium increases the yield of larvae (unpublished data). The *B. oleae* larvae were then transferred onto LB plates with the bacterial strain and incubated under the same conditions. Appropriate controls without the addition of the strain culture were maintained. After 48 h of exposure, mortality was scored by counting the number of dead larvae and determining the dead larvae/total larvae rate present in the respective cups (El Haidani et al., 2008). A strain isolate was considered potent if it had caused more than 80% mortality of the tested larvae or pupae. The potential bacterial cultures were further screened to find out whether the bacterial cells or their metabolites exhibited insecticidal activity. These tests were performed five times. *Escherichia coli* (DH5α) was used as a negative bacterial strain.

PCR amplification and sequencing of 16S rRNA gene

The 16S rRNA gene, was PCR-amplified using the primers, fD1 (5'AGAGTTTGATCCTGGCTCAG3') and Rs16 (5'TACGGCTACCTTGTTACGACTT3') (Weisberg et al., 1991) Genomic DNA was extracted from bacterial strains using thermal shock (Sambrook et al., 1989). The PCR mixture contained 1.5 mM $MgCl_2$, 200 µM of each dNTP's (Promega, Madison, USA), 1 µM of each primer (Metabion, Bangalore, India), 4 µl of Taq buffer (5X) and 1 unit of Taq polymerase (GoTaq Gold, Promega, and Madison USA). To this mixture, 2 µl of the DNA template was added. In the control tube 2 µl of ultrapure water was added instead of DNA. Total reaction volume was 20 µl. The reaction was amplified in a Thermal Cycler (TECHNE, UK). The PCR conditions were: denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min. A final extension step at 72°C for 10 min was also included. The size of the PCR products was determined by agarose gel electrophoresis using appropriate size markers. DNA sequencing was performed using ABI 3130; Applied Biosystems according to the manufacturer instructions. Sequence similarity searches were performed using the online sequence analysis resources BLAST.

Growth conditions and isolation of crude insecticidal toxins (CIT)

The isolated strain was grown under suitable conditions for growth and sporulation as follows: tubes containing 5 ml LB medium were inoculated with a loopful of culture from the agar slant. The tubes were incubated on a rotary shaker overnight (Edmund Buhler GmbH, Germany) at 37°C and 250 rpm. A 1 ml inoculum from this culture was added to a flask containing 100 ml LB medium. The flask was incubated on a shaker for 48 h as mentioned above. Thereafter, 100 ml of bacterial culture containing 3.03 x 10^9 UFC.ml^{-1} was centrifuged at 6,000 rpm for 30 min. The proteins in the supernatant were precipitated using ammonium sulfate (75% of saturation). The mixture was left stirring overnight at 4°C to obtain the maximum precipitate. This precipitate was collected by centrifugation at 10,000 rpm for 10 min. The obtained precipitate was re-dissolved in 1 ml of 20 mM Tris–Cl buffer, pH 8.0, 7.0, 6.0, 5.0, and it was dialyzed against the same buffer. The metabolite was finally stored at 20°C for further use. Dilutions of the protein were prepared as follows: Ci (undiluted), Ci/2, Ci/3, Ci/4, Ci/5, Ci/6, Ci/7, Ci/8, Ci/9 and Ci/10.

Metabolite activity of *P. aeruginosa* strain

Bioassays were conducted in a Petri dish containing 20 third instar larvae of *Bactrocera oleae* in 200 µl of each dilution of the metabolite preparation. Negative controls were included in the study containing 200 µl of 20 mM Tris–Cl buffer. After incubation at room temperature for 48 h (25°C), the numbers of dead larvae were counted. The mortality rate was scored by counting the number of dead larvae present in the Petri dish divided by the total number of larvae deposited. The experiment was conducted in four replicates.

Influence of temperature and pH on larvicidal efficacy

For assessing heat resistance of CIT, 200 µl of CIT was heated at 100°C for 15 min following which the larvicidal efficacy (post 48 h) of heat exposed CIT was tested. Effect of pH on the larvicidal efficacy of CIT was assessed by maintaining the pH of this metabolite at 6.0, 7.0 and 8.0 and then determining the post 48 h LC 50 and LC 90 value of CIT at each pH (Table 3). There were three replicates for each experiment and a control was run in parallel.

Statistical analysis

The probit regression and mortality percentages of all developmental stages for each bacterial treatment were analyzed using one way ANOVA, using Minitab16 statistical software (Fez, Morocco). Values of $p < 0.05$ were considered significant.

RESULTS

Isolation and screening of bacterial strains

Based on phenotypic criteria, a total of 114 bacteria were isolated and selected randomly from soil, water, dead insects and infested olive niches; studied and screened, then tested for their insecticidal activity (Table 1). The results indicated that 80% showed zero mortality, whereas only 20% of strains showed variable mortality between low and medium. There was only one strain named IL 5 among 114 that showed strong activity. Compared to the control, the IL 5 strain had a strong cumulative larvicidal and pupicidal activity corresponding to 80.82% (+/- 1%) mortality rate (Table 2). This strain was mainly isolated from dead insect sample.

Molecular characterization

Comparison with Gen-Bank database (EMBL) of the 16S rRNA gene sequences of isolate, chosen according to their source, phenotypic characteristics and biocontrol efficiency, indicated that the IL5 strain exhibited a strong homology percentage to the 16S rRNA genes of *P. aeruginosa.* The corresponding positions of these identified bacteria in their phylogenetic trees further indicated that they are a new bacterial strain (Figure 1). Evolutionary distances were calculated using the method of Jukes and Cantor. The branch length is proportional to the number of substitutions per site.

Determination of larvicidal efficacy of *P. aeruginosa* CIT

The bacterial biomass and CITs produced by *P. aeruginosa* during different stages of growth and the efficacy of their metabolites against *B. oleae* larvae are presented in Figure 2. Maximum biomass obtained after 48 h of growth was 3.03.x 10^9 UFC/ml. The metabolite extract exhibited larvicidal activities to third instar of *B. oleae* at various pH levels. The LC50 and LC90 values at pH 6 were 24.0784 µl/ ml and 47.3440 µl/ ml, pH 7 were 26.7486 and 52.5306 µl/ml, pH 8 were 27.3905 and 824.7422 µl/ml respectively (Table 3). The larvae were most susceptible at pH 6 than the other pH. The values were significant with $p < 0.05$. Moreover, our results showed that the CITs from *P. aeruginosa* strain were highly sensitive to temperature. The larvicidal potency is lost after heating at 100°C (Figure 3).

DISCUSSION

The exotoxins of microbial origin, including *Pseudomonas* species, are also known to be toxic to larvae of mosquitoes as well as lepidopteran insects (Murty et al., 1994). Depending upon the nature of the toxin(s), their action on different species and stages of insects might vary. Some exotoxins, such as those of *P. aeruginosa* (Schroeter) Migula have been noted to be absorbed through the cuticle of insects and act on the haemolymph proteins (Kucera and Lysenko, 1971). To our knowledge, this study is the first and foremost attempt to identify and describe that *P. aeruginosa* strain confers an efficient protection against *B. oleae*. Among the 115 tested strains, preliminary investigations showed that one

Table 1. Entomopathogenic Activity against *B. oleae* of 114 isolates. The GPS locations are given: l (latitude), L (longitude), S N (satellite number), and P (precision). + + + + corresponds to 100% mortality, + + -- corresponds to a mortality between 25 and 50%, + --- for a mortality between 5 and 25% and ---- for absence of mortality

Source of biotop	GPS location	Number of strains	Entomopathogenic activity
Faculty of sciences and technics (soil)	l: N 34° 0.0165 L: W 4° 9.3212 S N: 4 P: 6.75 m	1 5 14	++-- +--- ----
Mont fleuri (soil)	l: N 34° 0.7614 L: W 4°59.0959 S N: 5 P: 28 m	2 14	+--- ----
Ain chkaf (soil)	l: N 33° 58.4211 L: W 4° 1.2502 S N: 4 P: 6.25 m	3 11	++-- ----
Mont fleuri (water)	l: N 34° 0.7614 L: W 4° 59.0959 S N: 5 P: 28 m	1 18	++-- ----
Ain chkaf (water)	l: N 33° 58.7271 L: W 4° 1.1448 S N: 4 P: 5.5 m	1 23	+--- ----
Dead insects	l: N 33° 58.4211 L: W 4° 1.2502 S N: 4 P: 6.25 m	1(IL5) 8 12	**++++** ++-- ----
Escherichia coli			----

Table 2. Test of bacterial strain *Pseudomonas aeruginosa* pathogenesis on *B. oleae* development

Bacteria strain	Source	Total larvea	Dead larvae		Dead pupae		Total lethality		Emerged adults	
			Number	%	Number	%	Number	%	Number	%
P. aeruginosa	Dead insect	219	84	38.35	93	42.46	177	80.82	42	19.17
Control *E. coli DH5α*		100	1	1.00	3	3.00	04	4.00	96	96.00

bacterial strain showed an important rate of mortality against the larvae of *B. oleae* (80.82%) (Table 2). The 16S rRNA homology provided the phylogenetic position of that strain, indicating that it is a new and distinct bacterial strains for biocontrol purpose and named it, *P. aeruginosa* IL5.

As shown in Figure 2, crude CITs from *P. aerugenosa* strain exhibited a dose dependent larvicidal activity against third instar B. oleae larvae. However, the CITs from *P. aeruginosa* (IL5) showed significantly higher ($p < 0.05$) larvicidal potency with LC50 = 24.0784 µl/ml and LC90 = 47.3440 µl/ml (Table 3). The larvicidal potency of *P. aeruginosa* was due to the presence of quantitatively as well as qualitatively different proportions of bio-surfactants in the crude glycolipids (CILs). Rhamnolpids are the best studied glycolipids in which one or two molecules of rhamnose are linked to one or two molecules of b-hydroxydecanoic acid (Desai and Banat, 1997). Production of rhamnose-containing glycolipids was first described in *P. aeruginosa* (Jarvis and Johnson, 1949).

Environmental factors and growth conditions such as pH, and temperature affect the production of biosurfactants through their effects on cellular growth or activity. The pH of the medium plays an important role in sophorolipid production by *T. bombicola* spell genus

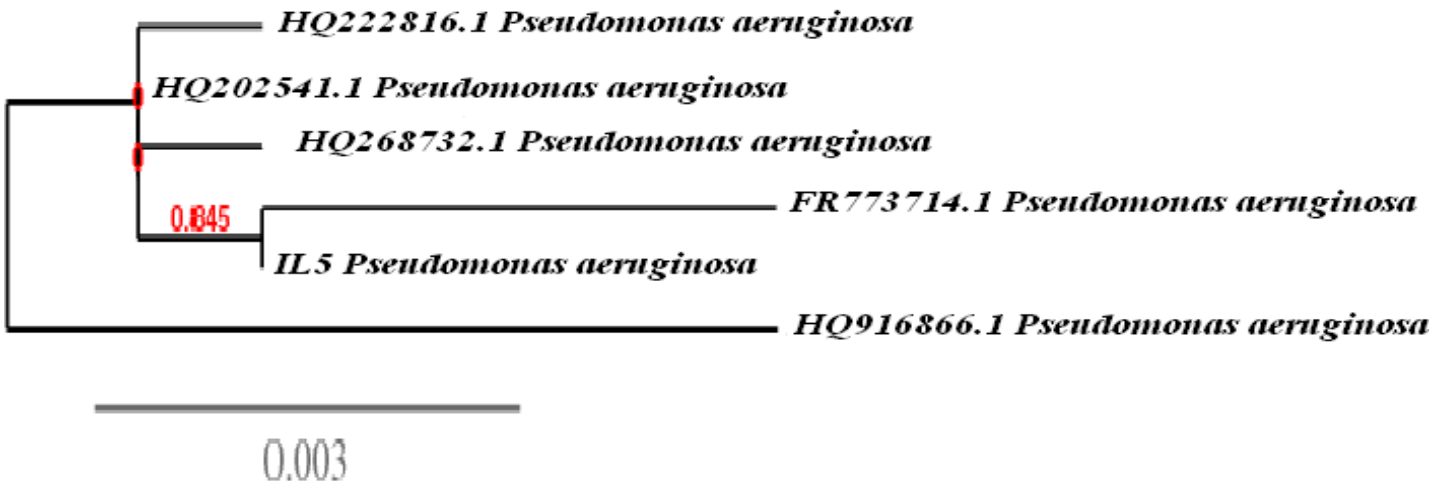

Figure 1. Phylogenetic relationships based on 16S gene analysis of bacterial strain IL5.
Evolutionary distances were calculated using the method of Jukes and Cantor. The branch length is proportional to the number of substitutions per site.

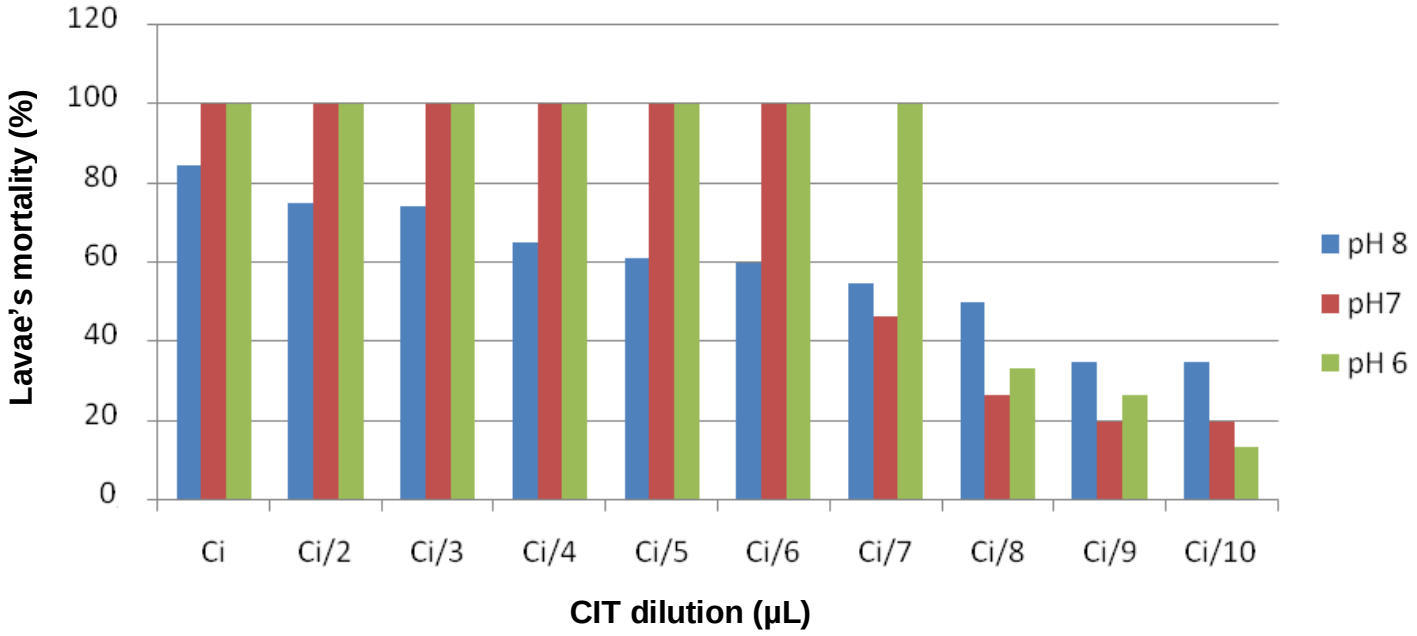

Figure 2. Toxicity of the formulation of protein metabolite (CIT) of *Pseudomonas aeruginosa* against III-instar larvae of *B. oleae* at different pHs and various doses.
Ci: Initial concentration of toxin (CIT). Ci/2 → Ci/10: CIT dilution.

Table 3. Effect of pH on the larvicidal efficacy of CIT from *P. aeruginosa.*

Coefficients of probit regression equation* Y= a+b.X			LC50 (µl.ml^{-1}) (95% FL)	LC90 (µl.ml^{-1}) (95% FL)
pH	a	b		
6.0	1.314	-0.098	24.0786	47. 3440
7.0	1.315	-0.109	26.7486	52.5306
8.0	0.887	-0.053	27.3905	824.7422

*Y=Probit mortality; X = dose in µl toxin ml^{-1}.

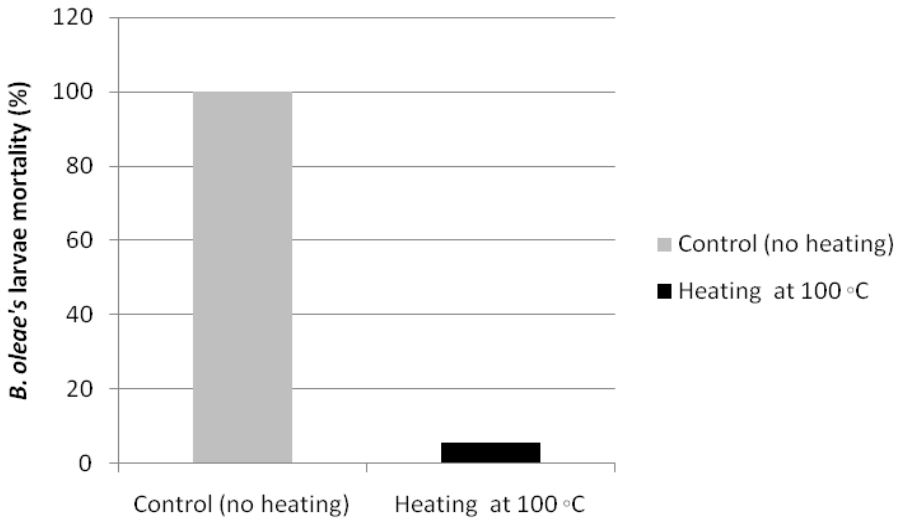

Figure 3. Effect of heat on larvicidal CIT activity

(Gobbert et al., 1984). In the present study, there was significant ($p < 0.05$) decrease in the larvicidal potency (LC50) of *P. aeruginosa* CITs at pH 8.0, as compared to pH 6.0 (Table 3). The optimum pH at which *P. aeruginosa* CITs exhibited maximum larvicidal efficacy was at pH 6 (Figure 2). However rhamnolipid production in *Pseudomonas* spp. was at its maximum at a pH range from 6 to 6.5 and decreased sharply above pH 7 (Guerra-Santos et al., 1984).

It is reported in Syldatk study on the *Pseudomonas* sp. strain DSM-2874, that temperature causes alteration in the composition of the biosurfactant produced. Heat treatment of some biosurfactants caused no appreciable change in biosurfactant properties such as the lowering of surface tension and interfacial tension and the emulsification efficiency, all of which remained stable after autoclaving at 120°C for 15 min (Syldatk et al., 1985). Additionally, in our study, after heating the CIT of *P. aeruginosa* strains at 100°C, the larvicidal potency was lost (Table 2), indicating the protein nature of CITs.

In conclusion, the CITs secreted by *P. aeruginosa* strain have shown larvicidal activity against third instars *B. oleae's* larvae. These properties can be exploited for the formulation of a safer, novel biopesticide for effective control of insect larvae. Further studies to assess the larvicial activities of purified CITs and their mode of action are in progress.

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