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Evolution of the Larvicidal Effects of Secondary Metabolites Produced by *Bacillus subtilis* (Ehrenberg, 1835) Cohn, 1872 Wild Type UTB1 and Mutant M419 against *Plutella xylostella* (L., 1758) (Lep.: Plutellidae)

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ABSTRACT

The effects of *Bacillus subtilis*, wild type bacterium UTB1 and mutant M419, on two larval instar groups of the diamondback moth, *Plutella xylostella* L. (Lep.: Plutellidae) until three days after treatment have been investigated. Larval mortality rate increased with increasing the concentration and over the days after treatment. Increasing the larval instar resulted in elevating the LC_{50} value for both types of biosurfactants. In terms of lethality, there was no significant difference between the two bacteria in the final mortality rate after three days.

Keywords: Plutella xylostella, biosurfactant, *Bacillus subtilis*, larvicidal effect, biology, *Brassicaceae.*

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INTRODUCTION

At present, food security and the sustainable development of agriculture are among the most important national priorities in Iran. However, the agricultural sector faces significant problems and challenges to achieve these objectives. The most significant challenges in this area include living and non-living environmental stresses such as pests and diseases, drought, salinity, high production costs, low quality of crops, environmental pollution caused by the usage of pesticides and chemical fertilizers and the high amount of agricultural wastes (Nematollahi & Bagheri, 2006).

The Brassicaceae family includes 10 tribes, 350 genera, and about 400 species. The most important member of this family is *Brassica oleracea* (cabbage), which has about 20 different varieties (Nematollahi & Bagheri, 2006). Also, there is a wide range of ornamental plants in this family which have many applications in food, industrial and medicinal areas (Avato & Argentieri, 2015).

One of key pests of the *Brassicaceae* family in Iran is the diamondback moth with the scientific name of *Plutella xylostella* L. This pest which belongs to the Lepidoptera order and the Plutellidae family causes most of the damage to *Brassicaceae* crops (Nematollahi & Bagheri, 2006). Feeding these species' larvae causes cabbage crop damage. Although the pest's larvae are very small in size, they can completely remove the leaf tissue except for veins. This is especially destructive to seedlings which may cause inflorescences cutting-off on cabbage, cauliflower, and broccoli (Knodel & Gaehiarachchi, 2008).

In many countries, the diamondback moth has become resistant to many artificial insecticides. Also, this moth has shown resistance to DDT and continued success of the wide utilization of *Bacillus thuringiensis* (Bt) is threatened by the development of resistance in this pest (Tabashnik, 1994; Sarfaraz & Keddie, 2005). Given that in the past decade chemical insecticides and chemical fertilizers were removed from the market in modern agriculture due to their hazardous effects on the natural environment, the biological control agents including effective microorganisms and bacterial products have recently attracted attention, instead of chemicals (Ghribi et al, 2012a).

Among the beneficial bacteria, the species of the genus *Bacillus* are known as biological pesticides to protect plants. *Bacillus* is a large and diverse genus of about 50 species from a family called Bacillaceae. The bacterium *Bacillus subtilis* was named in 1972 by Ferdinal Cohn. These bacteria are rod-shaped, gram-positive, obligate aerobic or facultative anaerobic and catalase-positive. Also, they produce endospores that can tolerate adverse environmental conditions. *Bacillus subtilis* bacteria are considered saprophytes in soil and vegetables (Alcazar-Fouli, Mellado, Alastruey-Izquierdo, Cuenca-Estrella, & Rodriguez-Tudela, 2008).

The advantage of *Bacillus* species is the production of a wide range of secondary metabolites with antimicrobial and pesticide activity including biosurfactant compounds (Ghribi et al, 2012; Kim, Ryu, Kim, & Chi, 2010). Biosurfactants have no toxic or allergic effects on humans or animals and can replace chemical pesticides (Quentin, Besson, Peypoux, & Michel, 1982).

Among these compounds, amphiphilic cyclic lipopeptides are of particular importance which are divided into three families, depending on their amino acid structure: iturins, fengycins, and surfactins (Ongena & Jacques, 2008).

Biosurfactant lipopeptides produced by *B. subtilis* are known as biocontrol compounds for disease reduction and plant pest control. *B. subtilis* UTB1, a biocontrol bacterium isolated from Iranian pistachio nuts, had shown antagonistic activity against aflatoxin-producing *Aspergillus flavus* R5 (Farzaneh & Nikkhah, 2012). This strain produces lipopeptide compounds iturins, fengycins and surfactins but the amount of these lipopeptides is not considerable (Afsharmanesh, Ahmadzadeh, Javan-Nikkhah, & Behboudi, 2014). Mutant *B. subtilis* M419 was created by random mutagenesis using gamma irradiation on strain UTB1. Production of lipopeptides surfactin, fengycin and iturin families increased in M419 (Afsharmanesh et al, 2014). M419 has shown inhibitory and mortality effects against both *Aspergillus flavus* as a fungal species and *Papillio demoleus* L. as an insect species (Afsharmanesh et al, 2014; Osouli & Afsharmanesh, 2016) which can make it an appropriate candidate for environmentally friendly bio pesticide and commercial product.

Considering the growing population of the diamondback moth and the ineffectiveness of numerous insecticides available, the present study attempts to provide a solution as one of the safe biological control methods. In this study, we assessed larvicidal effect of lipopeptides of strain *B. subtilis* UTB1 and *B. subtilis* mutant M419 on mortality of *P. xylostella* and compared them together. Mutant M419 bacterium produces more biosurfactant lipopeptides than its wild type UTB1; so, if they have at least the same effect in terms of larval mortality, using mutant M419 is recommended to produce high volumes of biosurfactants.

MATERIALS AND METHODS

Collecting the diamondback moths

After analyzing *Brassicae oleracea* cabbage field of Alborz province (35.9960° N, 50.9289° E), infested cabbage with different biological stages of the diamondback moth, were collected. The collected samples were placed in plastic containers and transferred to the laboratory.

Insect laboratory rearing

The walk-in incubator by 3×4×3 m dimensions was used to rear insects at 26±2°C under 16:8 hours photoperiod and 65±5% relative humidity. Fifty couples of males and female adults were confined in the fiber glass oviposition cages with the dimensions 50×50×50 cm. A square hole was designed inside each wall of the cages and covered with mesh cloths for ventilation. The cages contained 10% sucrose solution to feed the adult insects and red cabbage for egg-laying (because of color contrast with the white eggs). Red cabbages including eggs were collected daily from the cages and transferred to plastic containers (30×25×20 cm) with some holes (covered by mesh clothes) in their walls for ventilation. Hatched larvae were kept in these containers until

the last growth stage and fresh cabbage put inside the container as their food every other day. The last-age larvae were transferred to the petri dishes and conveyed to the oviposition cage after entering the pupal stage. Voucher specimens of *P. xylostella* from this study were deposited in the insect collection of department of entomology, Tarbiat Modares University, Tehran, Iran.

Providing and storage of bacterial isolates

The antagonist bacterium *B. subtilis* UTB1 which were (isolated from pistachio green skin), was received from Department of Plant Protection, University of Tehran, Tehran, Iran.

The mutant bacterium *B. subtilis* M419 obtained by gamma irradiation (2 KGy dose) from the parental bacterium *B. subtilis* UTB1, in Nuclear Agriculture Research School at Alborz Health and Agricultural Research Center. The research conducted by Afsharmanesh et al (2014) showed that the biological control of mutant M419 against *A. flavus* was significantly increased compared to wild type strain UTB1. For long-term storage of bacterial strains, a 16-hour culture medium of bacteria in the nutrient broth (NB) was mixed with 30% sterile glycerol in Eppendorf vials and, then transferred to a freezer at -70°C.

Cultivation conditions of bacterial isolates

At first, a colony of UTB1 and M419 bacteria were grown in 5 mL of NB medium at 30°C in a shaker incubator and then inoculated into 50 ml of medium for producing cyclic lipopeptides called MOLP (optimal medium for lipopeptides production) which were grown for three days in a shaker incubator at 30°C (Ahimou, Jacques, & Deleu, 2000). Then, the supernatants were separated by centrifugation at 10,000xg and 4°C for 15 minutes.

Extraction of cyclic lipopeptides from bacterial isolates

Cyclic lipopeptides were extracted from the supernatants of UTB1 and mutant M419 in three steps by adding a quarter (vol) of normal butanol solvent and vigorous shaking for 60 min on a shaker in the first step and 15 minutes in the second and third steps based on the method described by Yazgan, Ozcengiz, & Marahiel (2001). After each step, the organic phase was separated from the inorganic phase by centrifugation at 10000xg for two minutes, and butanol layers were collected in a separate container. The butanol layer was completely evaporated under laboratory fume hood in room temperature. The remaining residues were collected, and their weights calculated. Then, different concentrations of the extracted lipopeptides were prepared, and their effects on the mortality of *P. xylostella* larvae were investigated.

Bioassay experiment

To study the biological factors (developmental period of each immature stage, the mortality of each stage, total number of eggs laid, percentage of eggs hatching), the eggs with the age interval of 0-24 hours were taken from adult insects. For this purpose,

each pair was placed in small cylindrical containers (15×7 cm) covered with meshes and the pest-free red cabbage leaves were supplied for 24 hours for oviposition of females. The experiments were performed on different instars of the diamondback moth larvae which were kept hungry for 5 hours. To perform these experiments, due to the results of previous studies which show that larvae with instar (1 - 2) and (3 - 4) are different in terms of resistance to control methods, the experiments performed in two stages for young and more developed larvae. For this purpose, pre-tests were designed with doses of 1000, 1500, 2000, 2500 and 3000 mg/l. According to the results of initial experiments, the main experiments were designed in the dose range of 1500 to 3500 µg/ml and 3500 to 9500 with logarithmic intervals of doses for young and more developed larvae, respectively. Thus, the young larvae were placed in Petri dishes on leaves that were immersed in previously determined concentrations (2850, 2300, 1850, 1500 and 3500 µg/ml) from UTB1 and M419 biosurfactants and dried in ambient air. After 24, 48, and 72 hours, larval mortality was recorded daily in each petri dish.

In the next step, the old larvae were tested. The same experiment was performed with more developed larvae at concentration of 3500, 4500, 6000, 7500 and 9500 µg/ml) of UTB1 and M419 biosurfactants. This experiment was conducted in triplicate with 50 larvae for each replication.

Data analysis

In this study, the mortality doses (LC_{50} and LC_{90}) were calculated for each experiment by probit analysis using SPSS18 software. The results of the mortality rate of two different bacteria (UTB1 and M419) were measured by split plots based on the randomized complete block design with three replications. The normality and homoscedasticity assumptions were carried out using SPSS 18. The mean values were compared by Tukey's test. All the graphs were drawn using Microsoft Excel version 2019.

RESULTS

Larvicidal effect of *B. subtilis* UTB1 and M419 biosurfactants against two larval age groups of *P. xylostella*

The results showed that LC_{50} of UTB1 and M419 bacteria for larvae instar 1-2 of the diamondback moth at the end of initial 3 days after the exposure were 2390.303 and 2607.124 µg/ml, respectively. Also, LC_{50} of these bacteria was calculated on larvae instar 3-4 which was measured as 5764.964 and 6149.872 µg/ml for UTB1 and M419 bacteria at the end of third day after the treatment, respectively. The LC_{90} of bacteria UTB1 and M419 were also calculated for both larval age groups. The obtained values were 4706.520 µg/ml at lower ages and 13412.563 µg/ml at higher ages for UTB1, and 5747.513 µg/ml at lower ages and 15048.042 µg/ml at higher ages for M419, respectively (Table 1).

According to these results, LC_{50} and LC_{90} values for both bacteria increased with increasing larval age. Also, these values significantly decreased over post-exposure days.

Table 1. LC_{50} and LC_{90} of *Bacillus subtilis* UTB1 and M419 biosurfactant, against two larval age groups of *Plutella xylostella*, 1, 2 and 3 days after contact.

Lethal concentration LC (µg/ml)	Larval instar	Bacillus subtilis	Days after contact		
			1st	2nd	3rd
50	1-2	UTB1	4260.618	2627.884	2390.303
		M419	4343.449	2993.162	2607.124
	3-4	UTB1	9719.804	7020.316	5764.964
		M419	10429.007	7366.478	6149.872
90	1-2	UTB1	13150.643	5624.671	4706.520
		M419	8697.090	6151.343	5747.513
	3-4	UTB1	22247.278	16884.868	13412.563
		M419	20775.247	16370.502	15048.042

LT_{50} value for different applied concentration of UTB1 and M419 biosurfactants on two larval age groups of *P. xylostella*

Table 2 shows LT_{50} value for each applied concentration of UTB1 and M419 biosurfactants on the diamondback moth larvae aged 1-2th and 3-4th instars. This value for younger larvae exposed to UTB1 was measured as 45.239 and 1.201 days at 1500 and 3500 µg/ml (lowest and highest applied doses) and as 11.057 and 1.581 days for M419, respectively. Measured LT_{50} in the concentrations of 3500 and 9500 µg/ml (lowest and highest doses applied) on older larvae was 12.350 and 1.096 days for UTB1 and 20.481 and 1.143 for M419. The results indicated that the required time causing a 50% mortality rate in larvae of both age groups are significantly reduced with increasing doses. The results also revealed that the larval mortality of first group, at two doses of 2850 and 3500 µg/ml for UTB1 and only at dose of 3500 µg/ml for M419 reached 50% up to 3 days after exposure and this value reach 50% at the doses of 6000, 7500 and 9500 µg/ml for UTB1 and 7500 and 9500 µg/ml for M419 on older larval group, up to 3 days after exposure. The feeding ability of larvae decreased with increasing doses and the change in the body color was also evident.

Table 2. LT_{50} value for different applied concentration of UTB1 and M419 biosurfactants on two larval age groups of *Plutella xylostella.*

Larval instar	Bacillus subtilis	LT_{50} for each concentration (days)				
1-2	Concentration (µg/ml)	1500	1850	2300	2850	3500
	UTB1	45.239	15.644	3.113	2.611	1.201
	M419	11.057	7.386	4.234	4.006	1.581
3-4	Concentration (µg/ml)	3500	4500	6000	7500	9500
	UTB1	12.350	5.192	2.847	2.132	1.096
	M419	20.481	4.794	4.041	2.488	1.143

Dose-response lines for the larval mortality of *P. xylostella*

The effect of *B. subtilis* UTB1 and M419 against the diamondback moth larvae in two different age groups, on days 1, 2, and 3 after treatment are shown in Figs. 1, 2,

3 and 4, as the response of probit dose line. The estimated dose line for each day is drawn, and the observation points are placed around this line. Based on the results, the larvicidal effect of biosurfactant of these bacteria increased by increasing the doses and during the days after treatment.

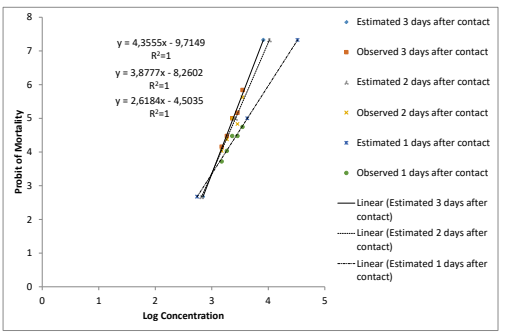

Fig. 1. Dose-response line for the younger larval mortality (1 and 2 instars) of *Plutella xylostella* after applying UTB1 biosurfactant.

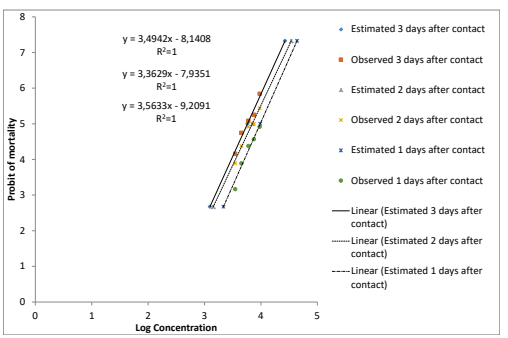

Fig. 2. Dose-response line for the older larval mortality (3 and 4 instars) of *Plutella xylostella* after applying UTB1 biosurfactant.

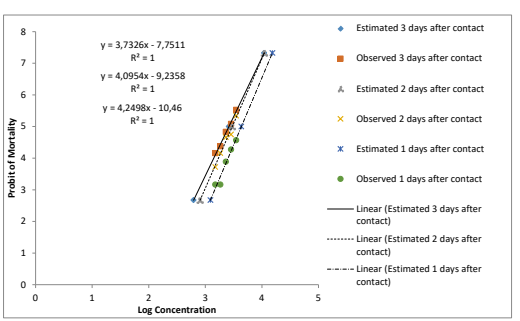

Fig. 3. Dose-response line for the younger larval mortality (1 and 2 instars) of *Plutella xylostella* after applying M419 biosurfactant.

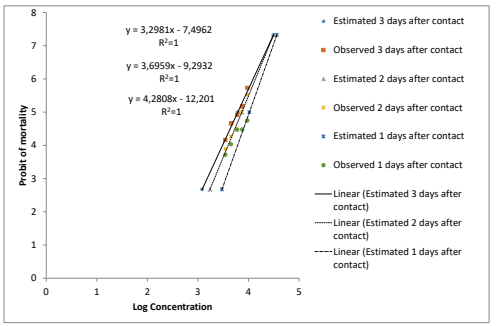

Fig. 4. Dose-response line for the older larval mortality (3 and 4 instars) of *Plutella xylostella* after applying M419 biosurfactant.

Analyzing variance of the diamondback moth larval mortality rate after applying different doses of UTB1 and M419 bacteria

Table 3 and 4 show the results of the analysis of variance of different doses of UTB1 and M419 bacteria on the diamondback moth larvae instars 1-2 and 3-4, 3 days after exposure, respectively. Based on the results of the analysis of variance, the effects of doses of UTB1 and M419 on the mortality rate of both larval instar groups were significant, but the effects of bacterial type and the interaction between dose and bacterial type were not significantly different.

Table 3. Analyzing variance of the mortality rate of the diamondback moth larvae instar 1-2 after application of different doses of UTB1 and M419 biosurfactants.

Source	Degree of freedom	Mean Square
Replication	2	243.333
Concentration	4	3046.667**
Error	8	64.167
Bacterial type	1	213.333
Concentration × Bacterial type	4	13.333
Error	10	73.333
Total	30	

**Significant at 1% probability level (P≤0.01, F=41.545)

Table 4. Analyzing variance of the mortality rate of the diamondback moth larvae instar 3-4 after applying different doses of UTB1 and M419 biosurfactants.

Source	Degree of freedom	Mean Square
Replication	2	163.333
Concentration	4	3158.333**
Error	8	88.333
Bacterial type	1	83.333
Concentration × Bacterial type	4	8.333
Error	10	43.333
Total	30	

**Significant at 1% probability level (P≤0.01, F=72.885)

DISCUSSION

Plutella xylostella larvae attack the leaves of cruciferous vegetables and cause severe damage. This pest has shown significant resistance to almost every synthetic insecticide applied in the field. In the present research, the effects of the biosurfactant secreted by *B. subtilis* UTB1 and M419 strains, as an ecofriendly method for crop protection against the cabbage moth were investigated in comparison with each other. UTB1 strain was isolated from Iranian pistachios and has the ability to secrete lipopeptide compounds. M419 mutant strain has the ability to produce more amounts of biosurfactants compared to UTB1 and in this regard is more suitable for commercial products.

In the present study, the effects of different doses of biosurfactant of UTB1 and M419 *B. subtilis* bacteria in the range of 1500-9500 µg/ml on the mortality rate of the diamondback moth larvae of different instars were measured to estimate the minimum suitable dose for controlling of this insect, for the first time. LC_{50} and LC_{90} values were measured after 1, 2 and 3 days of exposure. The results showed that the mortality rate of larvae increased by increasing doses and days after exposure of both UTB1 and M419 bacteria. The tolerance to bacterial biosurfactant doses increased by larval instars. Based on the results, there was no significant difference between UTB1 and M419 bacteria in causing final larval mortality after 3 days.

Several published studies have described mortality effects of *B. subtilis* biosurfactant on lepidopteran larvae. Osouli and Afsharmanesh (2016) measured the effect of biosurfactant of *B. subtilis* mutant M419 on the mortality rate of *Papilio demoleus* L. (Lep.: Papilionidae) larvae in the first and second instars using leaf immersion in doses of 450, 900, 1800 and 3600 mg/l in the laboratory. LC_{50} and LC_{90} values were measured after four days of exposure, and the results showed that the mortality rate of larvae increased by increasing doses of bacterial biosurfactants and also over days after exposure. The crude biosurfactant retained the larvicidal activity even when autoclaved at 121ºC for 15 min. M419 mutant could significantly inhibit the growth of *Aspergillus favus* in cross-culture experiments.

Ghribi et al (2012a) investigated the toxic effects of biosurfactant *B. subtilis* SPB_1 against *Spodoptera littoralis* (Boisduval) (Lep.: Noctuidae) neonate larvae. Lipopeptide biosurfactant displayed toxicity with an LC_{50} of 251 ng/cm^2 with 95% confidence limits of (195-307 ng/cm^2). The treatment effect with different concentrations of SPB1 biosurfactants was manifested in reducing adult emergence and prolongation of the maturity period. Larval survival rate and success in entering the pupal stage increased by decreasing the concentration; meanwhile, no mortality was observed in control treatment (buffer solution).

The insecticidal activity of lipopeptid biosurfactant of *B. subtilis* SPB1against *Ectomyelois ceratoniae* (Zeller) (Lep.: Pyralidae), was investigated by Mnif, Elleuch, Chaabouni, & Ghribi (2013). The LC_{50} and LC_{90} values after six days of contact were measured as 152.3 and 641 µg/g, respectively. The mean lethal dose measured on four and five days after treatment was much higher than that obtained after six days of treatment, indicating the effect of time on mortality and their results represented a strong positive correlation between the applied doses and larval mortality.

There are reports on larvicidal effects of biosurfactant extracted by *B. subtilis* strains on mosquito species. The study on the effects of *B. subtilis* metabolites on mosquito larvae showed that *B. subtilis* isolated from soil specimens killed larvae of the *Aedes aegypti* (Lineaus) (Dip: Culicidae). The results indicated that the mortality rate was dose-dependent for all instars of larvae. The LC_{50} and LC_{90} values were determined as 1.73 and 3.71 µg/ml, respectively after 24 h of contact indicated that secondary metabolites of *B. subtilis* had high larvicidal activity against *A. aegypti* and larval survival was significantly reduced at all examined concentrations. A significant reduction in the activities of acetylcholinesterase, α-carboxylesterase, and acid phosphatases were recorded in larvae exposed to the metabolite (Revathi et al, 2013).

In the study by Das & Mukherjee (2006), larvicidal potency of cyclic lipopeptides (CLPs) secreted by two *B. subtilis* DM-03 and DM-04 strains against third instar larvae of *Culex quinquefasciatus* Say (Dip: Culicidae) were recorded. LC_{50} of the crude CLPs were determined as 120.0 ± 5.0 and 300.0 ± 8.0 mg/l respectively, post 24 h of treatment.

In the present study, feeding ability of larvae decreased with increasing doses and reached zero three days after treatment and the change in the body color was evident. These effects indicated the deteriorative effect of bacterial metabolites on the midgut and digestive cells of the larvae, which caused the mature larvae to be unable to enter the pupal stage due to loss of feeding ability and they remained in the larval stage without feeding until they died.

Studies on the histopatological effects of *B. subtilis* biosurfactants on the insect midgut cells revealed the lethal changes at the cellular level. Vesicale formation in the apical of cells, lysis vacuolization of columnar cells and destruction of epithelial cell and their boundaries in the midgut larvae of *E. ceratoniae* (Mnif et al, 2013) and *S. littoralis* (Ghribi et al, 2012a) were represented. Also, according to Ghribi, Elleuch, Abdelkefi-Mesrati, Boukadi, & Ellouze-Chaabouni (2012b), the tested dose level of *B. subtilis* SPB1 caused strong histopathological disturbances in the midgut of *Ephestia kuehniella* Zeller (Lep: Pyralidae). The most frequents of which were recorded as cell vacuolization, microvilli damage and epithelium cell content passing into the midgut lumen.

Despite increasing mortality over the days after treatment in our study, the mortality rate of larvae instar 1-2, after three days of treatment with biosurfactants UTB1 and M419 in concentrations less than 2300 and 3500 µg/l, still did not reach 50%. Also, the mortality rate for larvae instar 3-4 of this insect, for two bacteria UTB1 and M419, at doses lower than 2300 and 2850 µg/l, respectively could not create 50% mortality either. In general, the results indicated that the impact rate of UTB1 bacteria was higher than M419, and at similar doses, a shorter time was required to cause 50% mortality in the larval population of both groups.

In the research by Osouli & Afsharmanesh (2016), the effect of *B. subtilis* biosurfactant M419 was calculated after 2, 3 and 4 days of the exposure to *P. demoleus* L. As the dose increased, the mortality rate increased, and LT_{50} for doses of 1800 and 3600 mg/L was 2.48 and 2.841 days, respectively. Also, the mortality rate of larvae after four days at concentrations of 4500 and 900 did not reach 50%.

CONCLUSION

The biosurfactants of UTB1 bacteria and mutant M419 could produce larvicidal effects at appropriate concentrations. Larval mortality rate increased with increasing the concentration and over the days after treatment. Increasing the larval instar resulted in elevating the LC_{50} value for both types' biosurfactants, which indicated higher resistance in older larvae. In terms of lethality, there was no significant difference between the two bacteria in the final mortality rate after three days. However, the speed of biosurfactant effect of UTB1 was higher than that of M419 at similar doses, resulting in more days after treatment to cause 50% mortality in larvae of the same instar. Assuming that larvae's lethality three days after treatment is considered as an acceptable result, mutant M419 can be used on a large scale due to higher biosurfactant production. According to these results, accurate analysis of the chemical components of the metabolite secreted by the bacterium M419 compared to UTB1 and evaluating their effects on more pest species seems to be necessary.

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