EVALUATION OF CRUDE ENZYME PRODUCED BY *BACILLUS SUBTILIS* SY134D CULTURE AS A BIOCONTROL AGENT AGAINST *DACTYLOPIUS OPUNTIAE* (DACTYLOPIIDAE: HEMIPTERA) ON CACTUS PEAR

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ABSTRACT

The cochineal, *Dactylopius opuntiae*, is a key pest on cactus pear, *Opuntia ficus-indica,* plants in Syria. The objective of this study was to analyze the efficacy of crude enzyme solution produced from the local strain *Bacillus subtilis* SY134D in controlling of *D. opuntiae* at different life cycle stages under laboratory conditions. The crude enzyme solution showed a significant insecticidal activity against *D. opuntiae*. Results showed that the crude enzyme concentrations of 35%, 65% and 100% were significantly effective against cochineal infestation and the 100% concentration was the most effective one. The wax covering nymphs was strongly degraded after applying the crude enzyme. To our knowledge, this is the first study that provides information about the use a crude enzyme solution for controlling *D. opuntiae* infestation which could be considered as a promising biocontrol agent for controlling this pest in the field in an environmentally friendly manner.

Keywords: Biocontrol, *Bacillus subtilis*, *Dactylopius opuntiae*

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INTRODUCTION

Cactus crops are gaining increasing interest across the globe, in particular cactus pear (*Opuntia ficus-indica*), because of its drought resistance, salinity tolerance, and the ability to grow in harsh conditions (Fidwy *et al*., 2016). In Syria, as in Jordan and Lebanon, cactus pear has long been cultivated, planted extensively in the rural areas of Damascus mostly for fruit production. It is noticed that, in the last two years of the case of Syria, the interest has increased in cactus pear in relatively stable parts of Syria, such as, Homs, Sweida and the coastal areas due to increasing demands for fruit products (Inglese *et al.,* 2017). Cochineal, *Dactylopius opuntiae* (Cockerell) (Hemiptera:Coccoidea: Dactylopiidae) has already becoming the most important pest of cactus pear in Syria. The cochineal *D. opuntiae* infestation was noticed for the first time in southern Lebanon in 2012, after that in Palestine in 2016, and in the south of Syria in 2016 (Moussa *et al*., 2017; Spodek *et al*., 2014; Basheer *et al*., 2016). Accordingly, during the past few years, the widespread *D. opuntiae* infestation on cactus pear farms caused severe injuries on plant cladodes (Basheer *et al*., 2016). Thus, these damages reducing the fruit yield that constitute additional income sources for farmers in this regions (Moussa *et al*. 2017). However, more than a century has passed since the first registration of *D. opuntiae*) in Mexico in 1896 by Cockerell (De Lotto, 1974). Up to date, there is no method available to control this pest including either chemical insecticides or botanical insecticides (Bouharroud *et al.,* 2018; Fitiwy *et al*., 2016). Bio-pesticides provide an alternative to synthetic pesticides because of their advantages, environmentally friendly pest management tools and low toxicity to human (Al-qwabah *et al*., 2018). Many biocontrol agents with specific fungal and insect targets have been reported and the results demonstrated the importance of enzymes application to control various insects and diseases. Chitinase produced from *Bacillus subtilis* and *Bacillus atrophaeus* (Chandrasekaran *et al*., 2012; Al-qwabah *et al*., 2018) has been used against *Spodoptera litura* Fab and *Drosophila melanogaster* larvae, respectively. However, the objective of the present study was to evaluate the efficacy of the crude enzyme solution produced from the local strain of *B. subtilis* SY134D as a biocontrol agent against *D. opuntiae on O. ficus-indica* (L.) under laboratory conditions.

Materials and methods

The collection of cactus cladodes infected by cochineal

Cactus cladodes were collected randomly from one of the cochineal infested cactus field in Qatana city (30 Km southwest of Damascus). 36 infested cactus cladodes were chosen and selected to have the same size, form and infestation ratio. The cactus cladodes were then divided into three groups, each group was subdivided

into four sections. Three cactus cladodes in each section were attached to the shelves (100 x 50 x 50 cm) with humidity (70%), temperature (25°C) and light (12:12). Stereo microscope inspection was carried out to count nymphs and adults for the female insects before and after any treatment for all experiments according to Zhang, 2017. All treatments replicated three times.

The crude enzyme production

Bacterial strain, *B. subtillis* SY134D used in this work, was isolated in our laboratory from Syrian soil (Bakri *et al*., 2012). This strain was used for the production of crude enzyme under solid state fermentation (SSF) using wheat bran as substrate. Enzyme production was carried out in trays containing 50g of wheat bran and nutrients. The fermentation medium consisted of 1g/L K2HPO4; 3g/L of NaCl; 0.3g/L of MgSO4.7H2O; and 3g/L yeast extract and 5g/L peptone, as a nitrogen source. Trays were removed from the incubator 3days after cultivation. Then, the culture medium was transferred to a flask to which 25 ml of distilled water containing 0.1% Triton X-100 was added and the resulting mixture was stirred for 90 min on a magnetic stirrer. The supernatant was collected as crude enzyme extract by centrifugation. The crude enzymes solution extracted was analyzed for enzymatic activity and was then stored at -20°C until used.

Activity of crude enzymes

Eight enzymatic assays were done to determine the enzymes activity included in the crude enzyme. These enzymes were: lipase, chitinase, carboxymethyl-cellulase, filter-paperase, pectinase, phytase, xylanase and amylase. Lipase activity was determined titirmetrically on the basis of olive oil hydrolysis (Macedo *et al*., 1997). The reaction mixture contained 5 mL olive oil emulsion substrate, 1 mL of crude enzyme and 4 ml buffer. The reaction mixture was incubated at 50°C for 20 min in a water bath with a shaking speed of 150 rpm. 10 ml ethanol acetone mixture (1:1) was added to stop the reaction. Liberated fatty acids were titrated with 0.05 mol/L NaOH. A lipase unit was defined as the amount that release one µmol fatty acid per min. Chitinolytic activity was determined by the estimating the released reduced sugars from the chitin as described by Jabeen & Qazi (2014). One unit of chitinolytic activity was described as a 1 µmol of liberation of N-acetylglucosamine per minute. Xylanase activity was determined as described by Bailey *et al*. (1992) by using 1% birchwood xylan as the substrate. The reducing sugars produced were determined according to the dinitrosalicylic acid (DNS) procedure by using xylose as the standard (Miller, 1959). One unit (U) of enzyme activity is defined as the amount of enzyme releasing 1 µmol xylose per ml per minute. On the other hand, carboxymethylcellulase activity was assayed similarly as xylanase activity, wherein 1% of carboxymethylcellulose solution (sodium salt, ultra-low viscosity) was used. DNS method was used to assay the reducing sugars released as well while filter paperase activity (FPA) was assayed

according to the method recommended by Ghose (1987). One international unit of FPA is the amount of enzyme which forms 1µmol glucose (reducing sugar as glucose) per min during the hydrolysis reaction. Amylase activity was determined as described by Okolo *et al*. (1995). One unit amylase is defined as the amount of enzyme releasing 1 µmol glucose equivalent per minute under the assay conditions. Polygalacturonase activity was determined according to Marcia *et al*., (1999). One unit of enzymatic activity was defined as 1 µmol of galacturonic acid release per minute. Phytase enzyme activity was determined as described by Heinonen *et al*., (1981), by measuring the amount of liberated inorganic phosphate. One unit (U) of phytase is defined as the amount of enzyme releasing 1 µmol of inorganic phosphorus per ml per minute.

2-4 Plant toxicity

The toxicity testing was carried out according to the European and Mediterranean Plant Protection Organization, 2014. First group of cladodes cactus cleaned from cochineal infesting by sterile water. After air dry for about 15 minutes, the cladodes were treated by three crude enzyme concentrations (35%, 65%, 100 %), and water as a control. Thus, the plant toxicity was studied using a scale of 0-5 for plant plate coloring as following: 0 No symptoms, 1: less than 10% cladode coloring, 2: 10 to 25% cladode coloring, 3: 26 to 50% cladode coloration. 4: from 51% to 75% cladodes coloration and 5: full cladodes colorization.

2-5 Effects of crude enzyme concentrations and pesticides on *D. opuntiae*

The second group of cladodes infested by cochineal was treated by three crude enzyme concentrations (35%, 65%, 100 %), and water as a control. While, the cladodes of the third group were treated by three different pesticides (Table 1). The fourth group was treated as the same group 2 followed by three times of spray.

2-6 Data collection

All data were collected using stereo microscope. Four samples spot of 10 cm size for each treatment were selected randomly to detect the number of cochineal nymphs and adults of. The corrected efficacy (%) and the corrected nymphs and adults mortality (%) were calculated (Paramasivam, M& Selvi, C., 2017)

$$\text{Corrected efficacy \%} = \frac{(1 - \text{N in Co before treatment} * \text{N in T after treatment}) * 100}{\text{N in Co after treatment} * \text{N in T before treatment}}$$

Where: N = Insect population, T = treated, Co = control

$$\text{Corrected \%} = \frac{\text{(Mortality \% in treated plot - Mortality \% in control plot)} * 100}{100 - \text{Mortality \% in control plot}}$$

2-7 Statistical analysis

All statistical analyses were performed using STATISTIC program version 6 (Statsoft, Inc. 2003) at 5% level (P= 0.05). Data were

subjected to analysis of variance (ANOVA; Tukey's HSD test) for the determination of differences in means between treatments at each concentration. The percentages analyzed by applying normal approximation test (analysis of proportions). Significance of liner relationship was tested by t-tests.

Results and Discussion

Results

Enzymes activity and Plant toxicity

The activity values of eight enzymes including the crude enzyme solution produced from the local strain *Bacillus subtilis* SY134D are shown in table 2. The enzyme activity were as the following: lipase (4.38 IU/g), filter-paperase (4.9 IU/g), phytase (15.05 IU/g), carboxymethyl-cellulase (38.71 IU/g), chitinase (76.72 IU/g), pectinase (79.1 IU/g), xylanase (595 IU/g) and amylase (1505 IU/g). It is noteworthy that no plant toxicity on cactus cladodes was observed when concentrations of the crude enzyme produced by *B. subtilis* SY 134D strain was applied.

The effect of the crude enzyme and various pesticides by one spray on nymph and adult females of *D. opuntiae*

Figure 2 demonstrated the effectiveness percentage of three crude enzyme concentrations (35 %, 65% and 100%) and three pesticides on *Opuntia ficus-indica* cladodes which were infested by adults and nymphs of *D. opuntiae* for three days. Differences in the percentage of effectiveness on nymphs and adults were highly significantly for each concentration of crude enzyme comparing with the control. On the contrary, there was no significant effect between concentrations of crude enzyme for nymphs, but for adults there were significant between the values (df = 17, f-value=3125.6, p<.0001; df = 17, f-value=234.3, p<.0001, respectively). The mortality percentage for adults was significantly lower comparing with nymphs (df =71, t-value =5.06, p<0.05). There was no significant in the effectiveness percentage within days for adults and nymphs, and the same cases were observed between three pesticides and control.

The effect of crude enzyme by three times of spray on nymphs and adult females of *D. opuntiae*

The insecticides were excluded in this test because neither one presented more than 20% effectiveness against the adult females and nymphs of *D. opuntiae* (Fig 1). Table 3 showed that the effectiveness percentage of crude enzyme concentrations when adults and nymphs exposed to three times consecutively. The effectiveness percentage on nymphs and adults were increased significantly with spraying times at 65% and 35% crude enzyme concentrations (df = 10, f-value=148.281, p<.0001; df = 10, f-value=212.682, p<.0001, respectively). While, there were not significantly in the effectiveness percentage between treatments for all spraying times at concentrations 100% either in adults or nymphs. Also, the

effectiveness percentage for adults was significantly lower comparing with nymphs (df=8, t-value =2.49, p<0.05).

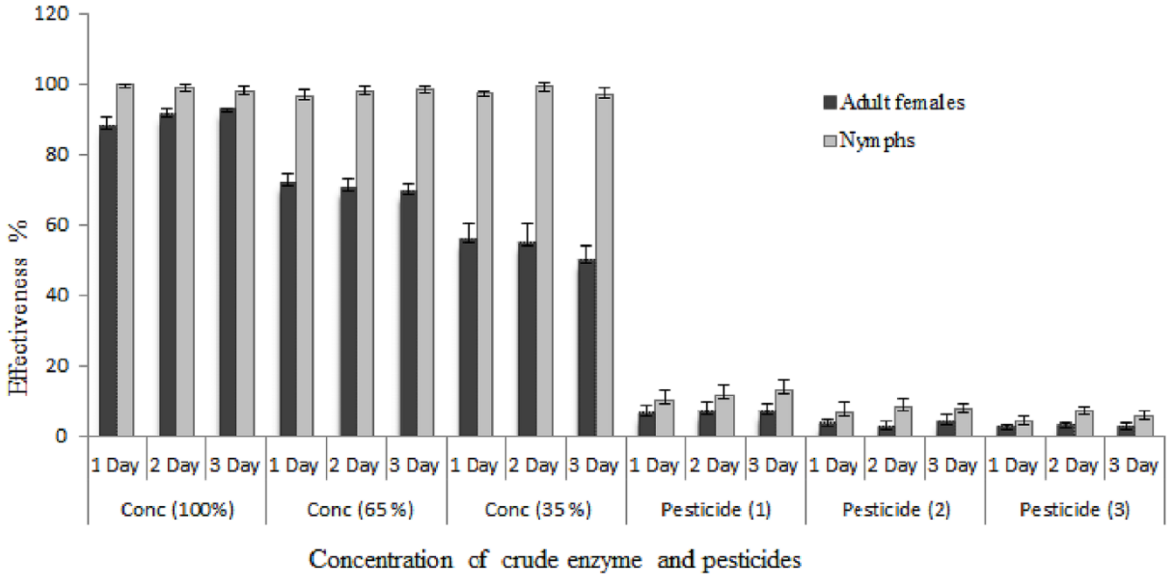

Fig 1. Effects of crude enzyme concentrations and pesticides on the effectiveness percentage on *Dactylopiys*

Table 1. List of insecticides with their compound and rate of application

Name of insecticide	Compound	Rate of application/ 100 L water
LENTREK48%	CHLORPEREFOS	150 ml
BYDOR 70%	IMIDACLOPRAD	20 g
AGRI THOAT 40 EC	DIMETHOATE	150 ml

Table 2. Enzymes activities including in crude enzyme solution produced by *Bacillus subtilis* SY134D

Enzyme	IU/g
Lipase	4.38
Chitinase	76.72
Carboxy methyl-cellulase	38.71
Filter-paperase	4.9
Pectinase	79.1
Phytase	15.05
Xylanase	595
Amylase	1505

Table 3. Effects of spray numbers of crude enzyme concentrations on the effectiveness percentage on nymph and adult females of *Dactylopiys opuntiae*

Concentrations	Number of spraying	Effectiveness of nymphs %	Effectiveness of adult females %
35%	Spr1	58.3±6.6 C	49.1±4.8 D
	Spr2	72.6±5.5 B	66±2.4 C
	Spr3	98.07±1.9 A	91.2±2.4 A
65%	Spr1	69.9±2.8 C	68.5±2.4 C
	Spr2	88.5±2.1 B	89.2±2.6 B
	Spr3	100±0 A	100±0 A
100%	Spr1	95.8±1.6 A	84.7±0.8 A
	Spr2	98.3±0.9 A	98.3±0.9 A
	Spr3	100±0 A	100±0 A

Percentages within a column between applied concentrations followed by a different letter are significantly different at P< 0.05 (Analysis of proportion).

Discussion

The cochineal scale insect, *Dactylopius opuntiae* (Cockerell), reduces the plants useful-life and affects production of their cladodes and fruit. Many studies were conducted for controlling *D. opuntiae* and deferent kinds of methods were evaluated, but none of them were effective against this pest which constitutes a risk for environment and human health (Bouharroud *et al*., 2018). For this reason, other management strategies are required. This study presents an investigation on the effects of crude enzymes solution as a new controlling agent that could be more effectively against this pest and environment friendly in the absence of natural enemies. However, the current study is the first that applied the crude enzyme solution produced from *B. subtilis* SY134D, which has contains many enzymes activity on cactus pear plant. Janusz *et al*., 2017, reported that the enzymes produced from microorganisms may play a role in the degradation of plant tissues. Our results confirmed that applying the crude enzyme produced by *B. subtilis* SY134D strain at high concentration on the cladodes of cactus pear did not show any plant toxicity to the plants. In the other hand, the cochineal insects protect themselves by covering their bodies with wax against the extreme weather conditions, natural enemies and pesticides (Esalat-Nejad *et al* ., 2013). This wax material is secreted by integument glands. The wax production increases until the nymph ages reach to the mature female stage (Meinwald *et al*., 1975). Our results revealed that the cochineal wax was degraded immediately in a few minutes after spraying of the crude enzyme produced by *B. subtilis* SY134D strain. Thus, our results strongly suggested that the enzymes activity available in the crude enzymes solution lead to the death of nymphs and mature females of *D. opuntiae* after degradation the wax. Moreover, the mature females insects treatment required higher enzymes concentration than the nymphs, may be due to the large amount of wax covering females bodies in comparison with nymphs. This wax degradation was almost complete when using the crude enzyme at 100% concentration. Also, when the crude enzyme activity was increased by using three spraying with 35% and 65% concentrations on cactus cladodes infested by cochineal, the treatment effective was increased (Table 3). The use of biological control agents for the plant pathogens management is considered as a safer and sustainable strategy for safe and profitable agricultural productivity. Therefore, previous studies have been reported that *Bacillus* strains play a fundamental role in the biopesticides field. Many *Bacillus* species have proved to be effective against a broad range of plant

pathogens. The potential biocontrol of *Bacillus* species in relation with their antagonizing attributes against plant pathogens. These attributes include production of lipopeptides, antibiotics and enzymes (Shafi *et al*., 2017). *Bacillus* species are capable to produce enzymes like chitinase and b-1,3 -glucanase and other hydrolytic enzymes which having a very strong lytic activity (Schisler *et al*., 2004; Chandrasekaran *et al*., 2012; Ghafil, 2013;Suci *et al*., 2018). Thus, they are used as a biopesticides against plant diseases and insects. The strain of *B. subtilis* SY134D used in this work is a good producer chitinase and lipase and other six hydrolytic enzymes have been detected in the crude enzyme solution. These hydrolytic enzymes could be the responsible for the observed insecticidal effect. It is known that, the peritrophic matrix and the exoskeleton of insects were composed in majority from chitin (Zhu *et al*., 2016). Thus, based on these facts the death of nymphs and adults mature could be attributed to insects wax hemolysis by the lipase and then chitin degradation by chitinase. These results are in agreement with the results found by Salunkhe *et al*., 2013 by using the *Bacillus* strains to degrade the *Maconellicoccus hirsutus* wax. Chitinases have also been demonstrated to affect the insect growth by decompose chitin, which ultimately leads to insect death (Veliz *et al*., 2017). Al-qwabah *et al*., 2018 have reported the potential of *Bacillus atrophaeus* A7 crude chitinase against Drosophila melanogaster larvae.

Conclusion

This study provides the first report about using the crude enzyme solution produced by *B. subtilis* SY134D strain against cochineal on *Opuntia ficus-indica* plant. The significant effectiveness of crude enzyme on the life table of cochineal infestation suggests the benefit using of this crude enzyme as a biopesticide agent against cochineal. It could be applied with in integration with other methods in pest management programs in the future. Also, for simulate all the conditions that exist in the field, further work and research must be taken to confirm these laboratory results under field conditions.

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