Journal of Applied Sciences

ISSN 1812-5654

science alert

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OPEN ACCESS Journal of Applied Sciences

ISSN 1812-5654
DOI: 10.3923/jas.2023.60.70

Research Article
Applications of *Pseudomonas* spp., as a Biopesticide to Control *Dactylopius opuntiae* (Cockerell) (Hemiptera: Dactylopiidae)

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Abstract

Background and Objective: *Dactylopius opuntiae* (Cockerell) is a major pest of *Opuntia* spp., cactus worldwide. This study was conducted to evaluate the efficacy of six *Pseudomonas* spp., isolates in managing this pest. **Materials and Methods:** The cochineal adult female was used for the isolation of the isolates. In the laboratory bioassays, insecticidal and repellent activities were evaluated. The insecticidal activity test evaluated four concentrations of bacterial suspensions: 0 (control), 10^3, 10^6, 10^8 and 10^{10} CFU mL^{-1} against first instar nymphs and adult females of the cochineal, whereas only 10^{10} CFU mL^{-1} concentration of three isolates (*EL01SB*, *EL02SB* and *EL03SB*) that exhibited the highest insecticidal activity was used for the repellent bioassay. **Results:** The *EL01SB*, *EL02SB* and *EL03SB* isolates at 10^{10} CFU mL^{-1} were the most virulent, causing the highest mortality in nymphs and adult females (92%, 88% and 76%, respectively (nymphs) and 87%, 83% and 71%, respectively (adult females)) after 6 days of exposure. Repellent activity was the highest with *EL01SB* isolate which exhibited a repellency index value of 75.1% at 24 DAT. Under greenhouse conditions, only the three isolates (*EL01SB*, *EL02SB* and *EL03SB*) were tested and isolates *EL01SB* (79.8% mortality) and *EL02SB* (64.7% mortality) at 10^{10} CFU mL^{-1} showed significantly higher mortality and high potential for degradation of the protective wax. Regarding the functional mechanism of action, all three isolates produced chitinase, cellulase and protease and only *EL01SB* was found to be able to fix atmospheric nitrogen. **Conclusion:** The results of this study indicated that isolate *EL01SB* could be included in the integrated pest management approach against *D. opuntiae*.

Key words: *Opuntia* cactus, *Dactylopius opuntiae*, IPM, repellent activity, *Pseudomonas* spp.

Citation: El Aalaoui, M. and M. Sbaghi, 2023. Applications of *Pseudomonas* spp., as a biopesticide to control *Dactylopius opuntiae* (Cockerell) (Hemiptera: Dactylopiidae). J. Appl. Sci., 23: 60-70.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Scale pests are considered one of the most notable threats affecting agriculture due to their dual role as pests and vectors of plant pathogens[1]. The *Opuntia* spp., cochineal *Dactylopius opuntiae* (Cockerell) (Hemiptera: Dactylopiidae) is a specific insect that can reduce yields and completely destroy plants of more than 16 *Opuntia* spp., species[2]. The scale insect (*D. opuntiae*) tends to create white colonies of various sizes that sometimes completely cover the cladode[3]. As a result, the fruits drop and the infested cladodes dry out and eventually fall off as well[4]. Indeed, *Opuntia* cactus plants colonized by the cochineal show patches of chlorotic necrosis on their cladodes and heavy infestations lead to very quick desiccation of entire cactus plants[5]. Such situations considerably favor the colonization of weakened plants by other pathogens, other than *D. opuntiae*, leading to the death of the cactus plants[6]. *Dactylopius opuntiae* is considered to be a very harmful pest because of its waxy covering on their dorsal side that protects them from insecticides, a high reproduction rate (150-400 eggs per female) and a very rapid dissemination ability by natural supports (wind, rain)[7,8]. Several studies conducted on its biology and life cycle both in laboratory and field conditions, they reported that under laboratory conditions, the complete life cycle of *D. opuntiae* lasts 77 and 43 days, respectively for females and males with a sex ratio of 1:1[9,10], while other works noted that the life cycle duration in real environment was 55-140 days for females and 35-52 days for males depending on the time of the year and climatic conditions[4,11]. Different Integrated Pest Management (IPM) approaches that includes several techniques based on mechanical, physical, biological, chemical and other methods have been developed worldwide to control *D. opuntiae*[2,12]. Also, instead of chemicals, the use of microbial biopesticides that affect the insect's chitin-protein complex can be used in ecological pest control.

Bacterial organisms, such as *Pseudomonas entomophila*, *Streptomyces* spp., *Xenorhabdus* spp., *Yersinia entomophaga, Burkholderia* spp., *Chromobacterium* spp., *Bacillus* spp. and *Saccharopolyspora* spp., have all gained commercial interest in the production of many biopesticides[13,14]. Pseudomonas-derived biopesticides have been shown to be effective in controlling many important agricultural pests, including the two-spotted spider mite (*Tetranychus urticae* Koch)[15]. *Pseudomonas fluorescens* produces chitinases that effectively control parasites by hydrolyzing their chitinous exoskeleton[16]. Also, certain bacteria that produce chitinase and protease enzymes with activity similar to the chemical compounds that break down chitin and protein molecules in fungal cell walls and insect exoskeletons are considered more promising. In addition, the ability of these bacteria to degrade wax may disrupt the cottony filaments that protect scale insects[17]. Therefore, the objective of this study was to isolate bacteria associated with the cactus cochineal *D. opuntiae*, determine their possible functions and evaluate their efficacy in controlling this cochineal in laboratory and under greenhouse conditions.

MATERIALS AND METHODS

Collection and rearing of cactus cochineal: *Dactylopius opuntiae* individuals were collected from the infested *Opuntia* spp., cactus plantation in the locality of Zemamra in the Casablanca-Settat Region (33°15'N, 8°30'W), Morocco. Field-collected cochineals were mass reared on *Opuntia ficus-indica* (L.) Miller cladodes disinfected with 5% sodium chlorite under laboratory conditions at 26±2°C, 60±10% relative humidity and a 12:12 hr L:D regime to avoid the influence of host plants on gut bacterial diversity[18,19]. Laboratory-reared scale insects were used for subsequent experiments.

Isolation of cactus cochineal associated *Pseudomonas* spp.: Mass-cultured *D. opuntiae* adult females were dewaxed with a paintbrush, superficially sterilized with 75% ethanol for 5 sec and washed three times with saline water. The specimens were then air-dried and placed in Petri dishes (9 cm diameter) containing King B culture medium[20] amended with cycloheximide (100 μg mL^{-1}) at the rate of one adult female per Petri dish. Twenty replicates were made and all were incubated for 48 hrs at 30°C and checked every 24 hrs for new colonies. Colonies were differentiated based on morphology (size and color) and biochemical characteristics such as pyoverdin production, Gram staining, motility, catalase and oxidase testing, arginine dihydrolase, sugar fermentation and then, a single isolate representative of each morphotype was subcultured, streak purified and stored at -80°C in 40% glycerol[19,21].

Laboratory trials: Bioassays were conducted to evaluate the lethal effect of 6 *Pseudomonas* spp., bacteria on nymphs and adult females of *D. opuntiae*. The trials were conducted in Petri dishes (14.5 cm diameter), contained pieces of *O. ficus-indica* cladodes (60 cm^2) in the center. Ten nymphs ("Trial 1") and ten adult females ("Trial 2") were transferred to each Petri dish. In both trials, each *Pseudomonas* spp., isolates tested was applied at the rates of 10^3, 10^6, 10^8 and 10^{10} CFU mL^{-1}. The experiment design was completely randomized with 5 replicates. Control Petri dishes received only tap water. The concentrations tested in this study were obtained using the

method described by Qessaoui *et al.*[15]. Briefly, an aliquot of each bacterial isolate was mixed with 100 mL of nutrient broth containing 0.1% Tween 80 and incubated in an orbital shaker incubator at 28°C and 150 rpm for 24 hrs. The mixture was then centrifuged at 10,000 rpm and the concentration of bacteria was determined using a spectrophotometer (Optizen 3220UV/VIS double beam, Mecasys, South Korea) at 640 nm. Mortality rates were recorded at 1, 3 and 6 days after treatment (DAT). The accuracy of the results was verified by repeating all experiments twice. The three *Pseudomonas* spp., isolates showing higher mortality rate were selected for further repellent activity and greenhouse studies.

Repellent activity: The repellent activity of three *Pseudomonas* spp., isolates (*EL01SB*, *EL02SB* and *EL03SB*) that showed the highest insecticidal activity in laboratory bioassays was tested using free choice test. Healthy *O. ficus indica* cuttings, of not more than 140 cm^2 surface were planted in plastic buckets (14.5 cm diameter by 13 cm height) with a mixture of soil (1/3 w/w), sand (1/3 w/w) and peat (1/3 w/w). These cuttings were allowed to develop until three young cladode stage in the greenhouse (11 length×7 width×3 height m) at 30°C±2 and then transferred to the laboratory. The selected bacterial suspensions were sprayed as a mist over the plant using a Potter spray tower (Burkard Scientifc Ltd., Uxbridge, UK) at a rate of 10^{10} CFU mL^{-1}. The control plants were sprayed with sterile distilled water. After spraying, *Opuntia* cactus plants were allowed to dry for 6 hrs under laboratory conditions (26±2°C, 60±10% RH and a 12:12 hr L:D regime) and then placed inside cages (80-80-80 cm) consisting of a wooden frame covered with mesh fabric to allow ventilation, with each cage including all the treatments tested (each cage representing a replicate). Two hundred first instar nymphs of *D. opuntiae* were introduced into Petri dishes (9 cm diameter) that were placed inside the cages and equidistant from the plants. This system allowed nymphs to move freely to the control or treated plants. Ten replicates were used and all experiments were repeated three times. Migration of *D. opuntiae* nymphs to the treated and control cactus plants was determined at 12, 24 and 48 hrs after release. The repulsion index was calculated using Pascual-Villalobos and Robledo[22] formula:

$$RI = \frac{C - T}{C + T} \times 100$$

Where:

RI = Repellency index
C = Number of *D. opuntiae* nymphs in the control plant
T = Number of *D. opuntiae* nymphs in the treated plant

Greenhouse trials: *Pseudomonas* spp., isolates (*EL01SB*, *EL02SB* and *EL03SB*) that provided the highest mortality against both nymphs and adult females of *D. opuntiae* in laboratory bioassays were selected for greenhouse tests. Two concentrations (10^{10} and 10^8 CFU mL^{-1}) were prepared for each isolate. *Opuntia ficus indica* plants were grown in plastic pots (27 cm diameter by 24 cm height) with a mixture of soil (1/3 w/w), sand (1/3 w/w) and peat (1/3 w/w) in a greenhouse (30±2°C under natural light). When the cactus plants were at the 3-cladode stage, 20 first instar nymphs of *D. opuntiae* were released on the plants and all were sprayed with the tested treatments using a Potter spray tower. A tap water spray only was used as the control treatment. The plants were covered with a mesh screen to limit the movement of the nymphs. There were seven treatments (five plants were treated by each treatment). Five plants per treatment were considered replicate and four replicates were conducted for all treatments, arranged in a randomized complete block design. The experiment was repeated twice at different times. First instar nymphs mortality was recorded at 3 DAT.

Mechanism of action of *Opuntia* cactus cochineal associated bacteria

Chitinase production: The ability of the three *Opuntia* cactus-associated bacteria to produce chitinase was determined using the method described by Cattelan *et al.*[23]. Each of the isolates was plated on chitin medium and incubated at 28°C for 72 hrs. A clear area surrounding the colony indicates solubilization of chitin by chitinase-producing bacteria.

Cellulase production: Qualitative cellulase production was assessed using M9 medium agar amended with 1.2 g L^{-1} yeast extract and 10 g L^{-1} cellulose according to the method described by Miller *et al.*[24]. A clear halo around the bacteria colony after 8 days of incubation at 28°C was reported as a successful response for cellulase production.

Protease production: Protease production by the isolates was determined on skim milk agar using the method described by Jha *et al.*[25]. The tested *Pseudomonas* isolates were inoculated onto the medium and incubated for 48 hrs at 28°C. The light area around the colony indicates protease production.

Fixation of N_2: The capacity of the isolates to fix atmospheric nitrogen was determined by inoculating each isolate on a sterile Agar Nourished medium. The medium was then incubated at 30°C for 24 hrs. The growth of the organism indicates that the isolate may have the ability to fix nitrogen[26].

Acetone production: Acetone production was revealed by Voges Proskauer (VP) reagent on Clark and Lubs medium according to the method described by Cherif-Silini *et al.*[27]. The medium was inoculated with 100 µl of the bacterial culture. After incubation at 30°C for 24 hrs, the reagents VPI and VPII were added and the presence of red color indicates acetone production.

Statistical analysis: Insect numbers and Abbot corrected mortality data[28] were subjected to Analysis of Variance (ANOVA) followed by Turkey's LSD Test. The LC50 values were calculated using Probit regression analysis. But before the analysis, LC_{50} values were predicted from the probit lines. Finney's method[29] was used to determine the lethal time (LT_{50}) from the probit analyses. Survival analysis using the Kaplan-Meier method was used to describe both the mean survival time and the median lethal time (LT_{50}) (the number of days until 50% of the insects were dead, for each treatment). All statistical analyses were performed using IBM SPSS 23.0 software (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Isolation and characterization of the isolates: A total of 6 *Pseudomonas* spp., isolates were isolated on *Opuntia* cactus cochineal specimens using King B culture medium and evaluated against *D. opuntiae* nymphs and adult females (*EL01SB*, *EL02SB*, *EL03SB*, *EL04SB*, *EL05SB* and *EL06SB*). *Pseudomonas* isolates produced mucoid, creamy white, irregular colonies. All isolates were Gram-negative, testing positive for motility, catalase, oxidase and arginine and leven production, *EL01SB*, *EL01SB* and *EL01SB* were testing positive for glucose and sucrose fermentation and only *EL01SB* produced a fluorescence emission with a diffusible yellow-green pigment that fluoresces under ultraviolet light at 360 nm (Table 1).

Laboratory trials: The six *Pseudomonas* spp., isolates evaluated provided significant percentage mortality against first instar nymphs (Table 2) and adult females (Table 3) of *D. opuntiae* with significant differences in their pathogenicity. Mortality of the cochineal was calculated from 1 day to 6 hrs at 1day time interval. *Dactylopius opuntiae* first instar nymphs mortality was significantly higher in the treatment with the cochineal associated *Pseudomonas* spp., bacteria *EL01SB* at 10^{10} (92%) and 10^{8} (79%) CFU mL^{-1} and *EL02SB* at 10^{10} CFU mL^{-1} (88%) followed by *EL03SB* at 10^{10} CFU mL^{-1} (76%) after 6 days of bacteria isolates application. For adult females, the highest mortality rates were achieved 6 days after the application of *EL01SB* (87%) and *EL02SB* (83%) at 10^{10} CFU mL^{-1}. For both first instar nymphs and adult females, the lowest mortality rate was achieved by the control treatment (tap water) (0%), 8 days after application. The pathogenicity of all isolates tested increased significantly with increasing concentration and exposure period ($p<0.0001$). The entomopathogenic (EP) bacteria tested reduced the movement of the insects before killing them and changed the color of the cadavers which became dark brown instead of bright red.

Also immediately after treatment, the females become smaller in size and smoother and the waxy filaments begin to dry out and disappear. Probit analysis results showed that *EL01SB* was the most toxic with LC_{50} values of 1.2 (nymph) and 7.7 (adult female) CFU mL^{-1} followed by *EL02SB* (239.0 (nymph) and 1984.4 (adult female) CFU mL^{-1}) and *EL03SB* (251.9 (nymph) and 6762.1 (adult female) CFU mL^{-1}), while *EL06SB* had the lowest LC_{50} values (112371.0 (nymph) and 5632229.9 (adult female) CFU mL^{-1}) 6 days after application (Table 4).

The mean mortality of *D. opuntiae* was significantly ($p \leq 0.001$) affected by exposure time and applied concentrations of the tested EP *Pseudomonas* spp., isolates, with insects exposed during the experimental period (6 days) to the highest concentration (10^{10} CFU mL^{-1}) showing a significantly higher mortality rate that exceeded 50% (Table 5 and 6). For the first instar nymph, the mean survival time value was highest in *EL06SB* and *EL05SB* treatments at the lowest concentration 10^{3} CFU mL^{-1} (4.9 d) and was lowest in *EL01SB* treatment at the highest concentration 10^{10} CFU mL^{-1} (4.0 d) (Table 5). The same trend was obtained for an adult female,

Table 1: Biochemical characteristics of *Pseudomonas* spp., isolates selected

Pseudomonas spp.	Gram	Mt	Ca	Ox	Arg	L	GluFr	SucFr	Fl
EL01SB	-	+	+	+	+	+	+	+	+
EL02SB	-	+	+	+	+	+	+	+	-
EL03SB	-	+	+	+	+	+	+	+	-
EL04SB	-	+	+	+	+	+	-	-	-
EL05SB	-	+	+	+	+	+	-	-	-
EL06SB	-	+	+	+	+	+	-	-	-

Mt: Motility, Ca: Catalase, Ox: Oxidase, Arg: Arginine, L: Leven, frGlu: Glucose fermentation, frSuc: Sucrose fermentation and Fl: Fluorescence

Table 2: Effects of different inoculum concentrations of *Pseudomonas* spp., isolates on percent mortality of *Dactylopius opuntiae*, first instar nymphs

		Time (Days)			
Pseudomonas spp.	Concentrations (CFU mL^{-1})	1	3	6	p-value
EL01SB	10^3	38.0±6.4^{ghi}	54.0±6.8^{defgh}	67.0±9.0^{cdefgh}	p<0.0001
	10^6	43.0±5.6^{efgh}	59.0±3.6^{cdefg}	72.0±8.4^{cdef}	p<0.0001
	10^8	56.0±4.8^{abc}	70.0±4.0^{abc}	79.0±7.4^{abc}	p<0.0001
	10^{10}	65.0±5.0^{a}	80.0±6.0^{a}	92.0±8.0^{a}	p<0.0001
EL02SB	10^3	38.0±4.8^{ghi}	51.0±5.4^{fghij}	58.0±6.4^{ghijk}	p<0.0001
	10^6	42.0±8.0^{efghi}	53.0±7.0^{efghi}	61.0±7.2^{fghij}	p<0.0001
	10^8	49.0±1.8^{bcdef}	64.0±6.0^{bcde}	75.0±10.0^{bcde}	p<0.0001
	10^{10}	57.0±4.2^{ab}	74.0±4.8^{ab}	88.0±6.4^{ab}	p<0.0001
EL03SB	10^3	36.0±4.8^{hi}	47.0±4.2^{hij}	54.0±4.8^{hijk}	p<0.0001
	10^6	37.0±4.2^{hi}	50.0±4.0^{ghij}	59.0±7.2^{fghijk}	p<0.0001
	10^8	43.3±6.0^{defgh}	60.0±6.0^{cdefg}	69.0±7.2^{cdefg}	p<0.0001
	10^{10}	53.0±4.2^{bcd}	69.0±5.4^{abc}	76.0±6.8^{bcd}	p<0.0001
EL04SB	10^3	33.0±5.6^{i}	45.0±5.0^{hij}	51.0±7.2^{ijk}	p<0.0001
	10^6	36.0±4.8^{hi}	50.0±6.0^{ghij}	57.0±7.0^{ghijk}	p<0.0001
	10^8	41.0±1.8^{fghi}	59.0±3.6^{cdefg}	64.0±6.0^{defghi}	p<0.0001
	10^{10}	51.0±3.6^{bcde}	67.0±5.6^{bc}	72.0±5.2^{cdef}	p<0.0001
EL05SB	10^3	33.0±5.6^{i}	42.0±4.8^{ij}	49.0±5.4^{ijk}	p<0.0001
	10^6	35.0±5.0^{hi}	47.0±4.2^{hij}	55.0±5.0^{hijk}	p<0.0001
	10^8	39.0±3.6^{ghi}	55.0±7.0^{defgh}	62.0±6.4^{efghij}	p<0.0001
	10^{10}	50.0±4.0^{bcdef}	65.0±8.0^{bcd}	70.0±4.0^{cdefg}	p<0.0001
EL06SB	10^3	33.0±5.6^{i}	40.0±4.0^{j}	46.0±4.8^{k}	p<0.0001
	10^6	35.0±5.0^{hi}	46.0±4.8^{hij}	50.0±4.0^{ijk}	p<0.0001
	10^8	37.0±5.6^{hi}	51.0±5.6^{fghij}	57.0±6.2^{ghijk}	p<0.0001
	10^{10}	47.0±4.8^{cdefg}	62.0±6.4^{cdef}	67.0±7.0^{cdefgh}	p<0.0001
Control		0.0±0.0^{j}	0.0±0.0^{k}	0.0±0.0^{l}	-
Statistical analysis		F = 21.4	F = 48.5	F = 45.1	
		p<0.0001	p<0.0001	p<0.0001	

Column means followed by the same lowercase letters are not statistically different according to Tukey's LSD Test at α = 0.05

Table 3: Effects of different inoculum concentrations of *Pseudomonas* spp., isolates on percent mortality of *Dactylopius opuntiae*, adult female

		Time (Days)			
Pseudomonas spp.	Concentrations (CFU mL^{-1})	1	3	6	p-value
EL01SB	10^3	33.0±4.2^{ijkl}	49.0±3.6^{fghi}	62.0±5.2^{defg}	p<0.0001
	10^6	38.0±3.2^{fghij}	54.0±4.8^{defgh}	67.0±5.6^{cde}	p<0.0001
	10^8	51.0±3.6^{bc}	65.0±6.0^{abc}	74.0±7.2^{bc}	p<0.0001
	10^{10}	60.0±4.0^{a}	75.0±5.0^{a}	87.0±4.2^{a}	p<0.0001
EL02SB	10^3	33.0±4.2^{ijkl}	46.0±4.8^{hij}	53.0±4.2^{ghijk}	p<0.0001
	10^6	37.0±4.2^{ghijk}	48.0±4.8^{ghi}	56.0±4.8^{fghi}	p<0.0001
	10^8	44.0±4.8^{cdefg}	59.0±3.6^{bcdef}	70.0±6.0^{cd}	p<0.0001
	10^{10}	52.0±3.2^{b}	69.0±3.6^{ab}	83.0±4.2^{ab}	p<0.0001
EL03SB	10^3	31.0±1.8^{jkl}	42.0±3.2^{ijk}	49.0±1.8^{ijkl}	p<0.0001
	10^6	32.0±3.2^{ijkl}	45.0±5.0^{hijk}	54.0±4.8^{ghij}	p<0.0001
	10^8	39.0±5.4^{efghi}	55.0±6.0^{cdefgh}	64.0±4.8^{def}	p<0.0001
	10^{10}	48.0±3.2^{bcd}	64.0±6.0^{bcd}	71.0±5.4^{cd}	p<0.0001
EL04SB	10^3	28.0±3.2^{l}	40.0±0.0^{ijk}	46.0±4.8^{jkl}	p<0.0001
	10^6	31.0±1.8^{jkl}	45.0±5.0^{hijk}	52.0±3.2^{hijk}	p<0.0001
	10^8	36.0±4.8^{hijk}	54.0±6.0^{defgh}	59.0±1.8^{efgh}	p<0.0001
	10^{10}	46.0±4.8^{bcde}	62.0±4.8^{bcd}	67.0±7.0^{cde}	p<0.0001
EL05SB	10^3	28.0±3.2^{l}	37.0±4.2^{jk}	44.0±4.8^{kl}	p<0.0001
	10^6	30.0±0.0^{kl}	42.0±4.8^{ijk}	50.0±2.0^{hijkl}	p<0.0001
	10^8	34.0±4.8^{ijkl}	50.0±8.0^{efghi}	57.0±4.2^{fghi}	p<0.0001
	10^{10}	45.0±5.0^{bcdef}	60.0±6.0^{bcde}	65.0±6.0^{cdef}	p<0.0001
EL06SB	10^3	28.0±3.2^{l}	35.0±5.0^{k}	41.0±1.8^{l}	p<0.0001
	10^6	30.0±0.0^{kl}	41.0±5.4^{ijk}	45.0±6.0^{jkl}	p<0.0001
	10^8	32.0±3.2^{ijkl}	46.0±6.0^{hij}	52.0±4.8^{hijk}	p<0.0001
	10^{10}	42.0±4.8^{defgh}	57.0±5.6^{cdefg}	62.0±4.8^{defg}	p<0.0001
Control		0.0±0.0 m	0.0±0.0 l	0.0±0.0 m	-
Statistical analysis		F = 61.2	F = 57.7	F = 82.9	
		p<0.0001	p<0.0001	p<0.0001	

Column means followed by the same lowercase letters are not statistically different according to Tukey's LSD Test at α = 0.05

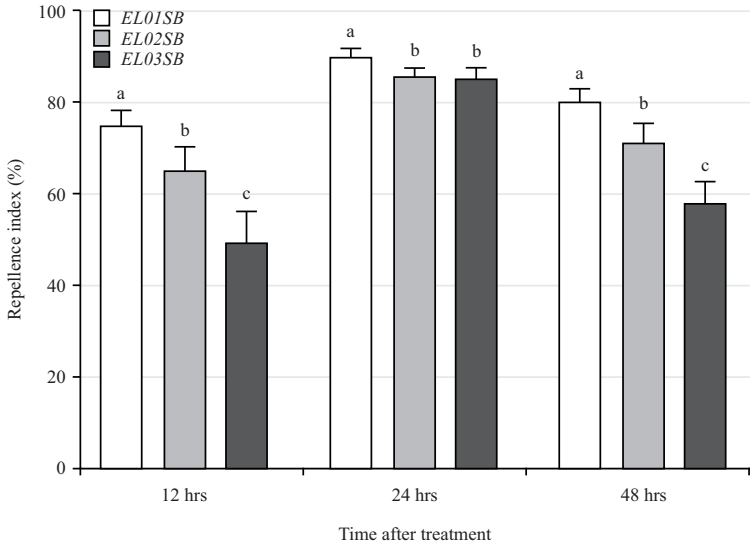

Fig. 1: Repulsive effect of *Pseudomonas EL01SB*, *EL02SB* and *EL03SB* isolates on *D. opuntiae* first instar nymphs and the repellence index (%) followed by the same letters do not differ at $p \leq 0.0001$ according Tukey's LSD Test

Table 4: Median lethal concentration LC_{50} (CFU mL^{-1}) of *D. opuntiae* treated by *Pseudomonas* spp., isolates (ANOVA, $\alpha = 0.05$)

Pseudomonas spp.	Cochineal stage	DAT	Slope±SE	LC%50	Chi-test (χ2) Sig	df	p-value
EL01SB	Nymph	1	4.1±0.03	4220775.4	7.3	38	p<0.0001
		3	4.1±0.03	519.2	10.7	38	p<0.0001
		6	4.3±0.03	1.2	27.6	38	p<0.0001
	Adult female	1	4.1±0.03	$7.8x10^{7}$	5.5	38	p<0.0001
		3	4.0±0.03	8275.5	6.7	38	p<0.0001
		6	4.0±0.03	7.7	12.2	38	p<0.0001
EL02SB	Nymph	1	2.8±0.03	$1.1x10^{8}$	6.3	38	p<0.0001
		3	3.5±0.03	4413	9.5	38	p<0.0001
		6	4.9±0.03	239.0	21.3	38	p<0.0001
	Adult female	1	2.8±0.03	$7.1x10^{9}$	3.9	38	p<0.0001
		3	3.5±0.03	124989.9	6.7	38	p<0.0001
		6	4.7±0.03	1984.4	9.9	38	p<0.0001
EL03SB	Nymph	1	2.5±0.03	$6.9x10^{9}$	5.7	38	p<0.0001
		3	3.3±0.03	51494.9	8.4	38	p<0.0001
		6	3.5±0.03	251.9	11.2	38	p<0.0001
	Adult female	1	2.6±0.03	$6.0x10^{11}$	5.0	38	p<0.0001
		3	3.3±0.03	1892008.6	6.5	38	p<0.0001
		6	3.4±0.03	6762.1	5.3	38	p<0.0001
EL04SB	Nymph	1	2.6±0.03	$4.2x10^{10}$	5.3	38	p<0.0001
		3	3.3±0.03	124471.2	7.0	38	p<0.0001
		6	3.2±0.03	1327.0	10.3	38	p<0.0001
	Adult female	1	2.7±0.03	$2.8x10^{12}$	4.1	38	p<0.0001
		3	3.3±0.03	4707939.8	4.8	38	p<0.0001
		6	3.1±0.03	54925.6	5.0	38	p<0.0001
EL05SB	Nymph	1	2.4±0.03	$2.2x10^{11}$	7.1	38	p<0.0001
		3	3.4±0.03	1134879.7	9.4	38	p<0.0001
		6	3.1±0.03	5741.4	8.5	38	p<0.0001
	Adult female	1	2.5±0.03	$1.9x10^{13}$	4.2	38	p<0.0001
		3	3.4±0.03	$4.0x10^{7}$	8.7	38	p<0.0001
		6	3.1±0.03	254659.9	4.9	38	p<0.0001
EL06SB	Nymph	1	1.9±0.03	$1.3x10^{13}$	7.4	38	p<0.0001
		3	3.1±0.03	6835370.6	8.4	38	p<0.0001
		6	3.1±0.03	112371.0	8.7	38	p<0.0001
	Adult female	1	2.0±0.03	$2.9x10^{15}$	4.1	38	p<0.0001
		3	3.1±0.03	$3.1x10^{8}$	7.2	38	p<0.0001
		6	3.1±0.03	5632229.9	6.0	38	p<0.0001

DAT: Day after the treatment

Table 5: Mortality (%), mean survival time and LT_{50} (days) of *D. opuntiae* first instar nymphs-treated *Pseudomonas* spp., isolates

Pseudomonas spp.	Concentrations (CFU mL^{-1})	Mortality (%)[a]	Mean survival time±SE[b]	LT_{50} (95% CI)	N[c]
EL01SB	10^3	53.0	4.7±0.1	6.0	100
	10^6	58.0	4.5±0.1	6.0	100
	10^8	68.3	4.2±0.1	6.0	100
	10^{10}	79.0	4.0±0.1	3.0	100
EL02SB	10^3	49.0	4.7±0.1	6.0	100
	10^6	52.0	4.6±0.1	6.0	100
	10^8	63.0	4.4±0.1	6.0	100
	10^{10}	73.0	4.2±0.1	6.0	100
EL03SB	10^3	46.7	4.7±0.1	6.0	100
	10^6	49.7	4.7±0.1	6.0	100
	10^8	60.7	4.4±0.1	6.0	100
	10^{10}	67.0	4.2±0.1	6.0	100
EL04SB	10^3	43.0	4.8±0.1	6.0	100
	10^6	47.7	4.7±0.1	6.0	100
	10^8	55.7	4.5±0.1	6.0	100
	10^{10}	64.3	4.3±0.1	6.0	100
EL05SB	10^3	41.3	4.9±0.1	6.0	100
	10^6	45.7	4.8±0.1	6.0	100
	10^8	52.0	4.6±0.1	6.0	100
	10^{10}	61.7	4.4±0.1	6.0	100
EL06SB	10^3	39.7	4.9±0.1	6.0	100
	10^6	43.7	4.8±0.1	6.0	100
	10^8	48.3	4.7±0.1	6.0	100
	10^{10}	58.7	4.4±0.1	6.0	100

[a]Abbott-corrected percentage mortality of *D. opuntiae* first instar nymphs at the end of experiment, [b]The mean survival time and its standard error and [c]Total number of scale insects in bioassay

since the highest value of mean survival time (5.1 days) was recorded in *EL06SB* at 10^3 CFU mL^{-1} and the lowest value (4.1) was observed in *EL01SB* at 10^{10} CFU mL^{-1}. A similar value (4.9) was recorded in *EL06SB* at 10^8 and 10^6 CFU mL^{-1}, *EL05SB* at 10^6 CFU mL^{-1} and *EL03SB* at 10^8 and 10^6 CFU mL^{-1} treatments (Table 6). For both first instar nymph and adult female, the lowest LT_{50} value (3 days) was found in *EL01SB* at 10^{10} CFU mL^{-1} and there was not a significant difference among the estimated LT_{50} values (6 days) of the other EP *Pseudomonas* spp., isolates tested.

Repellent activity: All three *Pseudomonas* isolates assessed repelled *D. opuntiae* nymphs 12, 24 and 48 hrs after application. The repulsion index (RI) of the three isolates tested at 10^{10} CFU mL^{-1} was presented in Fig. 1. The results showed that all isolates tested had a higher repellent effect 24 hrs after application. *Pseudomonas EL01SB* isolate provided the highest repellent effect at 12 hrs (75.1%), 24 hrs (89.9%) and 48 hrs (71.1%) after application, while *Pseudomonas EL01SB* had a low repellent effect on *D. opuntiae* nymphs with RI ranging from 49.4 to 85.3%. No significant difference in RI values was recorded between *Pseudomonas EL02SB* and *EL03SB* 24 hrs after application ($F = 29.8$, $df = 2$ and $p<0.0001$).

Greenhouse trials: The results obtained from the greenhouse bioassay experiment were presented in Fig. 2. Mortality of first instar nymph was calculated 3 days after application of the EP *Pseudomonas* spp., isolates tested. First instar nymphs mortality was significantly higher in the treatment with *D. opuntiae* associated *Pseudomonas* spp., isolate *EL01SB* at 10^{10} CFU mL^{-1} (79.8%) followed by *EL01SB* at 10^8 CFU mL^{-1} (64.8%) and *EL02SB* at 10^{10} CFU mL^{-1} (64.7%) ($F = 97.5$, $df = 5$ and $p<0.0001$). Before applying the three EP treatments, *D. opuntiae* nymphs were very mobile and moved their legs, but when applying the selected isolates, they began to tremble, especially when applying isolates *EL01SB* and *EL02SB*.

Mechanisms of action: To determine the most potential functional and nutritional mechanisms of action that might be involved in the mortality and repellency of *D. opuntiae*, the three *Pseudomonas* isolates that provided the highest mortality in nymphs and adult female of the cochineal were characterized biochemically *in vitro* for chitinase, cellulase, protease activities, N_2 fixation and acetone production. Results showed that all three the isolates produced chitinase, cellulase and protease, only *EL01SB* was found to be able to fix atmospheric nitrogen and no acetone production was recorded for *EL03SB* (Table 7).

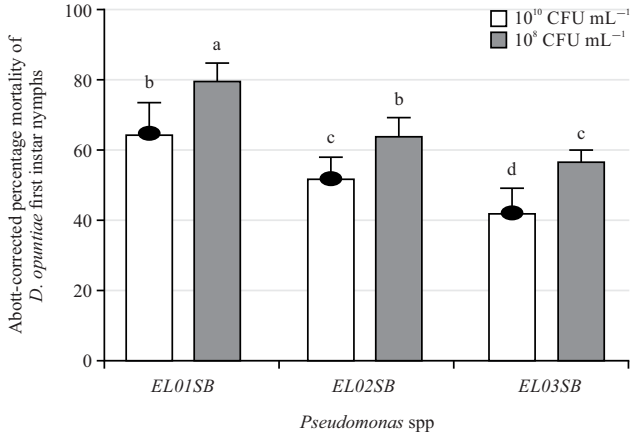

Fig. 2: Abbott-corrected percentage mortality of *Dactylopius opuntiae* first instar nymphs at 6 days after application of *Pseudomonas EL01SB*, *EL02SB* and *EL03SB* isolates under greenhouse conditions

Table 6: Mortality (%), mean survival time and LT_{50} (days) of *D. opuntiae* adult females-treated *Pseudomonas* spp., isolates

Pseudomonas spp.	Concentrations (CFU mL^{-1})	Mortality (%)[a]	Mean survival time±SE[b]	LT_{50} (95% CI)	N[c]
EL01SB	10^3	48.0	4.8±0.1	6.0	100
	10^6	53.0	4.7±0.1	6.0	100
	10^8	63.3	4.3±0.1	6.0	100
	10^{10}	74.0	4.1±0.1	3.0	100
EL02SB	10^3	44.0	4.8±0.1	6.0	100
	10^6	47.0	4.8±0.1	6.0	100
	10^8	57.7	4.5±0.1	6.0	100
	10^{10}	68.0	4.3±0.1	6.0	100
EL03SB	10^3	40.7	4.9±0.1	6.0	100
	10^6	43.7	4.9±0.1	6.0	100
	10^8	52.7	4.6±0.1	6.0	100
	10^{10}	61.0	4.4±0.1	6.0	100
EL04SB	10^3	38.0	5.0±0.1	6.0	100
	10^6	42.7	4.9±0.1	6.0	100
	10^8	49.7	4.7±0.1	6.0	100
	10^{10}	58.3	4.4±0.1	6.0	100
EL05SB	10^3	36.3	5.0±0.1	6.0	100
	10^6	40.7	4.9±0.1	6.0	100
	10^8	47.0	4.8±0.1	6.0	100
	10^{10}	56.7	4.4±0.1	6.0	100
EL06SB	10^3	34.7	5.1±0.1	6.0	100
	10^6	38.7	4.9±0.1	6.0	100
	10^8	43.3	4.9±0.1	6.0	100
	10^{10}	53.7	4.6±0.1	6.0	100

[a]Abbott-corrected percentage mortality of *D. opuntiae* first instar nymphs at the end of experiment, [b]The mean survival time and its standard error and [c]Total number of scale insects in bioassay

Table 7: Functional significance of the *Pseudomonas* spp., isolates selected

Pseudomonas spp.	Chitinase production	N_2 Fixation	Cellulase production	Protease production	Acetone production
EL01SB	+	+	+	+	+
EL02SB	+	-	+	+	+
EL03SB	+	-	+	+	-

(-) isolates with no enzymatic activity and (+) isolates with enzymatic activity

DISCUSSION

The present study investigated the isolation of *Pseudomonas* spp., associated with the specific *Opuntia* cactus cochineal, *D. opuntiae* and their evaluation as biological control agents against this very damaging scale pest of cactus cultivation worldwide. In a similar way, the *Pseudomonas* genera has previously been isolated from many scale insects species such as *Rhizoecus amorphophalli* (Bertram) (Hemiptera: Pseudococcidae)[30] and *Paracoccus*

marginatus Williams & Granara de Willink (Hemiptera: Pseudococcidae)[19]. *Serratia plymuthica*, *Staphylococcus cohnii*, *Enterobacter agglomerans* and *Bacillus* sp., were also isolated from the mealybug *Pseudococcus longispinus* (Targioni Tozzetti) (Hemiptera: Pseudococcidae)[31]. In general, the relationship between microorganisms and insects ranges from symbiosis and mutualism to parasitism[19,32] and some microorganisms found associated with insects are able to produce toxins that alter tissues causing death of the infected pests[19]. In this context, the results of the present study indicated that *Pseudomonas* isolates, especially *EL01SB*, *EL02SB*, *EL03SB*, have high biological control efficiency against *D. opuntiae*. These EP bacteria, at the maximum dose tested (10^{10} CFU mL^{-1}), caused the highest mortality in nymphs and adult females (92%, 88% and 76%, respectively (nymphs) and 87%, 83% and 71%, respectively (adult female)) after 6 days of exposure, resulting in LT_{50} values of 3.0, 6.0 and 6.0 days, respectively. *Dactylopius opuntiae* females are covered with white flocculent wax and have a rigid body, which protects them from direct contact with the EP application compared to the nymphal stages. This explains the higher percentage of mortality observed in first-instar nymphs (LC_{50} values ranged from 1.2-1.3$\times10^{13}$ CFU mL^{-1}) compared to adult females (LC_{50} values ranged from 7.7-2.9$\times10^{15}$ CFU mL^{-1}) in this study. The genus *Pseudomonas* was reported to have biocontrol potential for many pest species attacking several economically important crops such as *T. urticae*[15], the red spider mite (*Oligonychus coffeae*)[16] and the leaf-folding insect, *Cnaphalocrocis medinalis*[33]. The current investigation provides additional evidence that *Pseudomonas* spp., cause increased mortality of the major *Opuntia* spp., cactus pest, *D. opuntiae* and have the potential as EP agents for the control of this harmful scale pest. Enzymes produced by the cochineal-associated bacteria may act as a virulence factor by degrading the waxy filaments that cover the body of the cochineal and could also be essential for survival inside the host as well as neutralizing its defense system[34]. All the tested isolates were found positive for catalase and oxidase production.

Selection of EPs for use as a biological control agent in the greenhouse should be based on their high pathogenicity under laboratory conditions and their ability to reproduce and multiply on the target pest[35]. Thus, three isolates *EL01SB*, *EL02SB* and *EL03SB* that showed the highest mortality rates against the scale insect and the ability to degrade and break down the protective waxy filaments covering the insect body were selected for greenhouse tests. The results of the greenhouse tests showed that the application of *Pseudomonas* spp., isolate *EL01SB* at 10^{10} CFU mL^{-1} was effective in hemolyzing the covering wax and caused significantly higher mortality in first instar nymphs of *D. opuntiae* (79.8%), followed by *EL02SB* at 10^{10} CFU mL^{-1} (64.7%). This may be explained by the ability of these EP bacterial isolates to enter the scale insect body through the cochineal mouthparts and suppress its immunity as well as survive in the digestive tract and alter the metabolism of the insect by disrupting the peritrophic membrane[19]. Caldas *et al*.[36] reported that *Xenorhabdus nematophila* used protease enzymes to suppress insects immune system. Also, the efficiency of these isolates in hemolyzing the wax and killing the cochineal could be explained by their ability to produce enzymes with high hemolytic activity, especially chitinase, cellulase and protease. Qessaoui *et al*.[15] reported that *pseudomonas* spp., cause mortality in the red spider *T. urticae* due to high hemolytic activity, which involves proteins, such as chitinase, protease and cellulase. Similarly, *Pseudomonas entomophila* provides higher mortality to *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) due to its strong chitinase and lipase activities[37]. The wax-degrading ability of some bacteria, including *P. aeruginosa* and *Bacillus subtilis*, adversely affects the development, reproduction and weight of *Maconellicoccus hirsutus* (Green) (Hemiptera: Pseudococcidae) females[17]. Brillard *et al*.[38] reported that hemolysin enzymes produced by EP bacteria target cell membranes and lead to cell rupture. This could also play a role in the pathogenicity of *Pseudomonas* species. On *D. opuntiae*, particularly by reducing the scale wax content and given the greater mortality caused by *EL01SBA* and *EL02SB* isolates. In addition, EP bacteria employ different cell membrane surface-associated metabolites that facilitate their adherence to the host surface and help in establishing efficient colonization[37]. If applicable to the isolates of *Pseudomonas* tested in this study, the adhesion to the host surface could enhance the incursion of chitinases, proteases and cellulases through the wax covering the body of *D. opuntiae*, which can lead to rapid death of the scale pest, since reducing the wax content of the cochineal pests is the most important tool for controlling them. Insect-associated bacteria demonstrate a nutritional relationship with their insect partners[39]. In the present study, the *Pseudomonas* isolate *EL01SB* that exhibited the highest percentage mortality of *D. opuntiae*, was the isolate that exhibited chitinase, cellulase and protease activities as well as nitrogen fixation, acetone production and also wax degrading ability. These characteristics, in addition to those mentioned above, could give the isolate an advantage in its competition and adaptation to various ecosystems. Furthermore, the highest repellent effect exhibited by *Pseudomonas* isolates *EL01SB* and *EL02SB* in this study can possibly be explained by the

production of volatile metabolites such as acetone that kept *D. opuntiae* nymphs away from treated cladodes. Qessaoui *et al.*[15] reported that *Pseudomonas* isolates kept mites from treated tomato leaves through the production of secondary metabolites, including volatile metabolites.

CONCLUSION

The current study showed that all six *Pseudomonas* isolates tested provided significant percentage mortality and high repellency effect against *D. opuntiae*. *Pseudomonas* isolates *EL01SB* and *EL02SB* were the most potential candidates for the management of *D. opuntiae* and could be included in an integrated pest management approach to control this major pest of *Opuntiae* cactus worldwide. Future research is needed to evaluate the performance of these *Pseudomonas* isolates under field conditions and to assess lethal, sub-lethal and repellent effects to control other key pests of cactus and other crops of economic importance. In addition, the molecular characterization of these isolates is important and should be studied before their adoption in the field.

SIGNIFICANCE STATEMENT

The speed and rapidity with which *D. opuntiae* spreads and destroys cactus plantations in Morocco and worldwide, required extensive studies for the development of an effective and sustainable management approach against this scale pest. This work aims to evaluate the effects of six isolates of *Pseudomonas* spp., in the management of this insect pest. Isolate *EL01SB* reduced the survival rate of the cochineal and showed great potential for degrading the protective wax. Thus, our results indicate that *Pseudomonas* spp. (*EL01SB*) could be included in the integrated pest management approach against *D. opuntiae*.

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