Rammali S *et al.* (2023)
Notulae Scientia Biologicae
Volume 15, Issue 4, Article number 11574
DOI:10.15835/nsb15411574
Research Article

Insecticidal potential of *Streptomyces* sp. dichloromethane extracts against the cactus cochineal *Dactylopius opuntiae* (Cockerell)

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Abstract

Destructive damages caused by *Dactylopius opuntiae* (Cockerell) to cactus worldwide require an ecofriendly IPM approach. *Streptomyces* sp. produce wide range of biologically active secondary metabolites that can be an interesting alternative to chemical insecticides for pest control, as they are less toxic and easily biodegradable. The efficacy of dichloromethane extracts of four Moroccan *Streptomyces* sp. strains: *Streptomyces bellus*- E23-2, *Streptomyces galilaeus*- E23-9, *Streptomyces africanus*- E23-3, and *Streptomyces bellus*- E25-12 (applied at 11, 13, 15, 17 and 20 mg mL^{-1}) against *D. opuntiae* nymphs and adult females was evaluated under laboratory and greenhouse conditions. Results showed that *Streptomyces bellus*- E23-2 and *Streptomyces galilaeus*- E23-9 dichloromethane extracts applied at 20 mg mL^{-1} were more effective, causing higher mortality against nymphs (92% and 91%, respectively) and adult females (90% and 95%, respectively) after 8 days of exposure, resulting in an LT_{50} value of 3.0 days (nymph), and 3.0 and 6.0 days (adult female), respectively. *Streptomyces bellus*- E25-12 extract had the lowest mortalities [88% (nymph) and 68% (adult female)]. In greenhouse experiment, the highest first instar nymph mortality was achieved by *Streptomyces bellus*- E23-2 (55.5%) and *Streptomyces galilaeus*- E23-9 (50.5%) dichloromethane extracts at 20 mg mL^{-1}. The metabolites found in dichloromethane extracts of *Streptomyces bellus*- E23-2 and *Streptomyces galilaeus*- E23-9 show considerable potential to be used in the development of new biopesticide formulations for use in integrated pest management programs against *D. opuntiae*.

Keywords: *Dactylopius opuntiae*; dichloromethane extract; EP bacteria; IPM; *Streptomyces* sp.

Received: 25 May 2023. Received in revised form: 18 Oct 2023. Accepted: 08 Nov 2023. Published online: 15 Nov 2023.
From Volume 13, Issue 1, 2021, Notulae Scientia Biologicae journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

Introduction

Dactylopius opuntiae (Cockerell), is one of the most important Hemipteran scale pests of *Opuntiae* sp. cactus worldwide. It feeds on more than 16 cactus species including *Opuntia engelmannii* Salm-Dyck ex Engelm., 1850 var., *Opuntia ficus-indica* (L.) Mill., 1768, *Opuntia fuliginosa* Griffiths 1908, *Opuntia stricta* (Haw.) Haw., 1812, and others (Mazzeo *et al*., 2019). The cochineal nymphs and adults tend to form colonies of varying size on cladodes, fruits and stems that in some cases are completely covered by the insect (Cruz-Rodrílguez *et al*., 2016). Therefore, the fruits fall off and the infested cladodes desiccate and eventually fall off (El Aalaoui *et al*., 2019). Whereas, heavy infestations result in very fast desiccation of entire cactus plants (Rocha, 2012). Such situations considerably favor the colonization of the weakened cactus plants by other pathogens, except *D. opuntiae*, which leads to the death of the infested cactus plant (Lopes and Emepa-Pb, 2010). A total of 100,000 ha of cactus, estimated at $25 million has been destroyed in Brazil by *D. opuntiae* where it was introduced in 2009 (Lopes *et al*., 2009). Also, in Mexico, the native country of *D. opuntiae*, the damages caused by this pest are severe, resulting in premature fall of fruits and young edible cladodes of *Opuntia*, and consequently, a decrease in yields and an increase in production costs for Mexican cactus crops (Portillo and Vigueras, 2006). In Morocco, since its appearance in 2014, the cactus cochineal has engendered considerable damage in several cactus-growing regions where tens of thousands of hectares of cactus are totally destroyed, causing huge socio-economic and environmental losses (Sbaghi *et al*., 2019). The damage caused by the cochineal leading the local authorities to adopt an emergency intervention, uprooting and incinerating more than 400 ha of plantations in the Doukkala zone (El Aalaoui *et al*., 2019).

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* (Torres and Giorgi, 2018; Mazzeo *et al*., 2019).

Recently, the use of biological control as part of IPM approach against *D. opuntiae* has attracted the interest of researchers as it is an environmentally friendly and safe control strategy. Thus, hundreds of research projects have been carried out in the last four years in several countries worldwide in order to find effective biopesticides against this scale insect (Vigueras *et al*., 2009; Perez-Ramírez *et al*., 2014; Ramdani *et al*., 2020), but the results are still very moderate. Therefore, there is a need for further research on new bioactive molecules that have higher specificity, low relative cost and more environmental friendliness to be used for the control of *D. opuntiae*. Among the various sources of bio molecules actives, microorganisms play a key role as they provide an unlimited reservoir of new secondary metabolites (Gopalakrishnan *et al*., 2016) . More than 80,000 microbial natural products have been identified, of which fungi are the main contributors (40%), followed by an almost equal contribution from Actinobacteria and single-celled bacteria (Gopalakrishnan *et al*., 2016). Whereas, among Actinobacteria, the genus *Streptomyces* alone (10,400 products) is the main contributor (Bérdy, 2012). The secondary metabolite derived from *Streptomyces* sp. has the advantage of exhibiting lower phytotoxic activity compared to fungal metabolites (Bérdy, 2005). *Streptomyces* sp. are filamentous soil bacteria that produce a wide range of bioactive molecules (Singh *et al*., 2011; Rammali *et al*., 2022). *Streptomyces* sp. is highly valued for their ability to produce chitinase and protease enzymes; these enzymes have been found to possess activities similar to chemical compounds that can disrupt the chitin and protein molecules in the peritrophic membrane of insects (Singh *et al*., 2011). Various studies have highlighted the crucial role that certain secondary metabolites produced by *Streptomyces* sp. play in managing agricultural pests of significant importance, such as: *Spodopetra littoralis* (Biosduval, 1883) (Bream *et al*., 2001) (Lepidoptera: Noctuidae), *Musca domestica* (Li, 1758) (Diptera: Muscidae) (Hussain *et al*., 2002), *Culex quinquefasciatus* (Say, 1823) (Diptera: Culicidae) (Sundarapandian *et al*., 2002), *Drosophila melanogaster* (Meigen, 1830) (Diptera: Drosophilidae) (Al-Kaabi, 2005), *Helicoverpa armigera* (Hubner, 1808) (Lepidoptera: Noctuidae) (Osman

and Mohamed, 2008), *Anopheles mosquito* larvae (Theobald, 1901) (Diptera: Culicidae) (Dhanasekaran *et al*., 2010), *Spodopetra litura* (Fabricius, 1775) (Lepidoptera: Noctuidae) (Kaur *et al*., 2014), *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) (Soliman *et al*., 2021), and *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Yponomeutidae) (Kim *et al*., 2022). Additionally, by disabling the chitin contained in the peritrophic membrane, the fungal and bacterial chitinase enzyme has been utilized to suppress bollworm *Helicoverpa armigera* (Hübner) and stem borer [*Eldana saccharina* (Walker, 1865) (Lepidoptera: Pyralidae), *Sesamia calamistis* (Hampson, 1910) (Lepidoptera; Noctuidae), and *Chilo partellus* (swinhoe, 1885) (Lepidoptera: Pyralidae)] larvae (Binod *et al*., 2007; Okongo *et al*., 2019).

The main ways in which bacteria infect insects are through ingestion and the digestive tract, and less often through the egg, integument and trachea (Cokola, 2019). They can also penetrate into the insect via parasitoids and predators (Tanada and Kaya, 1993). Most bacteria found in insects are isolated from the digestive tract (Cokola, 2019). However, few bacteria are relatively pathogenic to insect hosts and have received particular attention because of their potential to control crop pests (Cokola, 2019). In the digestive tract, the bacteria produce enzymes (e.g. proteinase, chitinase and lecithinase) that act on midgut cells and allow the bacteria to enter the haemocoel (Tanada and Kaya, 1993). Bacteria that enter the hemocoel of the host insect cause septicaemia and in sometimes the death of the infected insect (Sabbahi and Hock, 2022).

Streptomyces sp. are characterized by their ability to produce toxic proteins, that can be harmful to insects (Ganesan *et al*., 2018). There is a lack of published information regarding the use of *Streptomyces* sp. as biocontrol agents against *Dactylopius* sp. Previously, we investigated the antimicrobial and antioxidant activities of sixteen *Streptomyces* sp. strains isolated from an extremely cold and microbiologically unexploited region of Morocco (Fez-Meknes region, Morocco) (Rammali *et al*., 2022). Thus, in this study, we evaluated the efficacy of the four most active strains in controlling nymphs and adult females of *D. opuntiae* under laboratory and semi-field conditions.

Materials and Methods

The cochineal rearing

The colony of *D. opuntiae* was developed from adult females collected from cactus plantations located in the locality of Zemamra, Casablanca-Settat region (33°15' N, 8°30' W), Morocco as described by EL Aalaoui *et al*. (2019). The collected individuals were placed on one-year-old *Opuntia ficus-indica* (L.) cladodes, weighing approximately 3 to 4 kg. The entire setup was placed inside cages (80-80-80 cm) made of wooden frames covered with insect-proof netting to allow for ventilation. These cages were kept under controlled conditions of 26± 2 °C, 60± 10% relative humidity, and a 12:12 hour L:D light cycle.

Actinomycete used

The strains *Streptomyces bellus*- E23-2 (NCBI GenBank Acc. No: OM883988), *Streptomyces galilaeus*- E23-9 (NCBI GenBank Acc. No: OM883992), *Streptomyces africanus*- E23-3 (NCBI GenBank Acc. No: OM883989), and *Streptomyces bellus*- E25-12 (NCBI GenBank Acc. No: OM883998), which were previously reported to exhibit antimicrobial and antioxidant activities (Rammali *et al*., 2022), were subsequently utilized in the present study.

Production and extraction of bio molecules actives

The method described by Aouiche *et al*. (2014) with some modifications was adopted for production and extraction of bioactive metabolites. Briefly, the four *Streptomyces* sp. strains tested were subjected to fermentation and then extraction of secondary metabolites using dichloromethane. Erlenmeyer flasks (500

mL) containing 100 mL of ISP2 culture medium were inoculated with each selected active strain of *Streptomyces* sp. and incubated under constant agitation at 150 rpm under controlled conditions (28 °C). *Streptomyces* sp. cultures were centrifuged at 10,000 g for 20 minutes to remove the mycelia mass. A volume of supernatants was collected and mixed vigorously in a separating funnel with the same volume of dichloromethane. The organic extracts obtained were then evaporated at 45 °C to remove the dichloromethane, and the resulting residue was used for insect treatment. Finally, the dry organic extracts obtained as well as the residual aqueous phases were stored at 4 °C for further use.

Laboratory trials

Bioassays were conducted to evaluate the pathogenic effect of dichloromethane extracts of the four *Streptomyces* sp. strains tested on nymphs and adult females of *D. opuntiae*. The trials were performed in Petri dishes (14.5 cm diameter), contained pieces of *O. ficus-indica* cladodes (60 cm^2) in the center. Ten *D. opuntiae* nymphs ("Trial 1") and ten adult females ("Trial 2") were transferred to each Petri dish. In both trials, each *Streptomyces* sp. strain tested was applied at the rates of 11, 14, 15, 17, and 20 mg mL^{-1}. Petri dishes were arranged in a completely randomized design with 5 replicates. Control Petri dishes received only tap water. Tap water was used as the control in all bioassays instead of dichloromethane to mimic the insect's natural environment and eliminate the possibility of any confounding effect of the solvent. Moreover, the minimum concentration of dichloromethane for insecticide activity was reported to be 500 mg mL^{-1} (Pantoja-Pulido *et al*., 2020). Mortality percentage was recorded at 1, 3, and 8 days after treatment (DAT). To ensure the reproducibility of results, all experiments were independently repeated twice over time. All the laboratory experiments were conducted under similar conditions (26 ±2 °C, 60 ±10% RH, and 12 hours of photoperiod).

Greenhouse trials

Based on the laboratory results, the dichloromethane extracts of the two strains of *Streptomyces* sp. (*Streptomyces bellus*- E23-2 and *Streptomyces galilaeus*- E23-9) that generated the highest mortality against both nymphs and adult females of *D. opuntiae* were selected for the greenhouse tests. Two concentrations (17 and 20 mg mL^{-1}) were prepared for each strain. *Opuntia ficus-indica* cactus plants were grown in a greenhouse (26 ± 2 °C under natural light). When the cactus plants were in the 3-5 cladode stage, 20 first instar nymphs of *D. opuntiae* were released on the plants and all were sprayed with the test treatments using a laboratory sprayer to ensure complete coverage. The control treatment was a tap water spray only. The plants were covered with a plastic pot (with a wire mesh top) to limit the movement of the nymphs. There were five treatments (five plants were treated by each treatment). Five plants per treatment were considered as replicate, and four replicates were conducted for all treatments, arranged in a randomized complete block design. The experiment was repeated twice at different times. Nymphal mortality was recorded at 10 DAT.

Statistical analysis

Mortality percentage data were corrected using Abbott's formula (Abbott 1987) and then were subjected to analysis of variance (ANOVA), followed by Turkey's LSD test. LC_{50} values were calculated using Probit regression analysis. Prior to analysis, LC_{50} values were predicted from the probit lines. Finney's method (Finney 1971) was used to determine the lethal time (LT_{50}) of the probit analyses. The Kaplan-Meier survival analysis 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 was dead, for each treatment). All statistical analyses were performed using IBM SPSS 23.0 software (SPSS Inc., Chicago, Illinois, USA).

Results

Laboratory trials

Dichloromethane extracts of four Moroccan strains of *Streptomyces* sp: *Streptomyces bellus*-E23-2, *Streptomyces galilaeus*-E23-9, *Streptomyces africanus*-E23-3 and *Streptomyces bellus*-E25-12 were evaluated for their pathogenicity against nymphs and adult females of *D. opuntiae* based on their infectivity rates. The *Streptomyces* sp. extracts assayed at different concentrations were significantly different in their pathogenicity against both first instar nymphs (Table 1) and adult females of the cochineal (Table 2). The greatest 8-day first instar nymph cumulative mortality was achieved by *Streptomyces bellus*- E23-2 applied at 17 and 20 mg mL^{-1} (91 and 92%, respectively), *Streptomyces galilaeus*- E23-9 applied at 17 and 20 mg mL^{-1} (83 and 91%, respectively), *Streptomyces africanus*- E23-3 applied at 17 and 20 mg mL^{-1} (79 and 88%, respectively), and *Streptomyces bellus*- E25-12 applied at 20 mg mL^{-1} (88%) dichloromethane extracts.

At 8 days after treatment, the greatest adult female mortality was achieved by the *Streptomyces bellus*-E23-2 applied at 17 and 20 mg mL^{-1} (87 and 90%, respectively), and *Streptomyces galilaeus*- E23-9 applied at 20 mg mL^{-1} (85%) dichloromethane extracts. At the end of the experiment, the cumulative mortality of adult female for *Streptomyces africanus*- E23-3 and *Streptomyces bellus*- E25-12 extracts at high concentration (20 mg mL^{-1}) reached 76 and 68%, respectively. The lowest percentage of first instar nymph and adult female mortality at 8 days after treatment was achieved by the control treatment (tap water) (0%). The pathogenic efficacy of all *Streptomyces* sp. strains tested against *D. opuntiae* first instar nymph and adult female increased significantly as the exposure period increased (Tables 1 and 2).

Table 1. Effects of different concentrations of four Streptomyces sp strains dichloromethane extracts on the percentage mortality of *Dactylopius opuntiae*, first instar nymphs

Streptomyces sp	Concentrations (mg mL^{-1})	Time (Days)			P value
		1	3	8	
Streptomyces bellus-E23-2	11	26.0±7.0 hijk	36.0±9.7 ijk	47.0±10.6 hij	P < 0.0001
	13	35.0±7.1 fghi	52.0±7.9 fgh	61.0±7.4 efg	P < 0.0001
	15	50.0±11.5 cde	65.0±9.7 def	76.0±7.0 bcd	P < 0.0001
	17	59.0±12.9 abc	86.0±8.4 ab	91.0±5.7 a	P < 0.0001
	20	70.0±11.5 a	91.0±8.8 a	92.0±4.2 a	P < 0.0001
Streptomyces galilaeus- E23-9	11	23.0±6.7 ijk	31.0±5.7 jk	42.0±6.3 ijk	P < 0.0001
	13	28.0±6.3 ghijk	47.0±8.2 ghi	53.0±9.5 fghi	P < 0.0001
	15	42.0±7.9 defg	52.0±10.3 fgh	66.0±7.0 def	P < 0.0001
	17	52.0±9.2 bcd	71.0±8.8 bcde	83.0±12.5 abc	P < 0.0001
	20	65.0±12.7 ab	81.0±7.4 abc	91.0±3.2 a	P < 0.0001
Streptomyces africanus- E23-3	11	19.0±7.4 jk	27.0±8.2 jk	36.0±7.0 jk	P < 0.0001
	13	24.0±5.2 ijk	41.0±8.8 hij	49.0±7.4 ghij	P < 0.0001
	15	36.0±7.0 efghi	49.0±12.0 ghi	58.0±6.3 fgh	P < 0.0001
	17	49.0±7.4 cdef	67.0±11.6 cdef	79.0±11.0 abcd	P < 0.0001
	20	61.0±9.9 abc	78.0±9.2 abcd	88.0±4.2 ab	P < 0.0001
Streptomyces bellus-E25-12	11	14.0±7.0 kl	25.0±10.8 k	29.0±7.4 k	P < 0.0001
	13	16.0±7.0 jk	38.0±11.4 hijk	41.0±5.7 ijk	P < 0.0001
	15	29.0±7.4 ghij	42.0±10.3 hij	50.0±10.5 ghi	P < 0.0001
	17	40.0±12.3 defgh	60.0±10.5 efg	72.0±17.5 cde	P < 0.0001
	20	51.0±9.9 bcd	66.0±13.5 cdef	88.0±6.3 ab	P < 0.0001
Control (tap water)		0.0 l	0.0 l	0.0 l	-
Statistical analysis		F= 45.48 P < 0.0001	F= 57.88 P < 0.0001	F= 88.05 P < 0.0001	

Means within a column followed by the same letters are not significantly different according to Tukey's LSD test at α = 0.05 following ANOVA.

Table 2. Effects of different concentrations of four *Streptomyces* sp strains dichloromethane extracts on the percentage mortality of *Dactylopius opuntiae*, adult female

Streptomyces sp	Concentrations (mg mL^{-1})	Time (days)			P value
		1	3	8	
Streptomyces bellus- E23-2	11	18.0±7.9 ghijk	27.0±8.2 jklm	36.0±12.6 hij	P < 0.0001
	13	27.0±8.2 fgh	43.0±9.5 fghi	54.0±9.7 fg	P < 0.0001
	15	41.0±8.8 cde	58.0±9.2 cde	70.0±6.7 cd	P < 0.0001
	17	54.0±11.7 abc	75.0±11.8 ab	87.0±11.6 ab	P < 0.0001
	20	61.0±8.8 a	83.0±9.5 a	90.0±8.2 a	P < 0.0001
Streptomyces galilaeus- E23-9	11	15.0±5.3 hijk	23.0±6.7 lm	34.0±9.7	P < 0.0001
	13	21.0±5.7 ghij	39.0±8.8 ghij	46.0±10.7 ijk	P < 0.0001
	15	35.0±9.7 ef	46.0±12.6 efg	57.0±6.7 defg	P < 0.0001
	17	43.0±8.2 bcde	62.0±6.3 bcd	75.0±12.7 bc	P < 0.0001
	20	56.0±14.3 ab	68.0±10.3 bc	85.0±7.1 ab	P < 0.0001
Streptomyces africanus- E23-3	11	11.0±3.2 jkl	21.0±3.2 lm	29.0±7.4 jk	P < 0.0001
	13	13.0±4.8 ijkl	32.0±9.2 hijkl	40.0±8.2 hij	P < 0.0001
	15	26.0±8.4 fghi	38.0±11.4 ghijk	49.0±7.4 gh	P < 0.0001
	17	39.0±7.4 def	54.0±7.0 def	67.0±9.5 cdef	P < 0.0001
	20	51.0±11.0 abcd	65.0±7.1 bcd	76.0±7.0 bc	P < 0.0001
Streptomyces bellus- E25-12	11	5.0±7.1 kl	14.0±5.2 m	21.0±5.7 k	P < 0.0001
	13	7.0±6.7 kl	25.0±8.5 klm	30.0±6.7 jk	P < 0.0001
	15	17.0±4.8 ghijk	30.0±10.5 ijkl	40.0±6.7 hij	P < 0.0001
	17	30.0±9.4 efg	44.0±7.0 fgh	56.0±9.7 efg	P < 0.0001
	20	38.0±12.3 def	54.0±8.4 def	68.0±6.3 cde	P < 0.0001
Control (tap water)		0.0±0.0 l	0.0±0.0 n	0.0±0.0 l	P < 0.0001
Statistical analysis		F= 45.48 P < 0.0001	F= 61.26 P < 0.0001	F= 77.30 P < 0.0001	

Means within a column followed by the same letters are not significantly different according to Tukey's LSD test at α = 0.05 following ANOVA.

The sensitivity of *D. opuntiae* to a particular tested *Streptomyces* sp. species at a specific concentration is the key element affecting LC_{50} values. Table 3 shows the doses required vs. exposure times to cause 50% mortality in first instar nymphs and adult females of *D. opuntiae* when the four *Streptomyces* sp. strains dichloromethane extracts tested were applied. The Probit analysis used to analyze the mortality results showed that *Streptomyces bellus*- E23-2 extract had the lowest median lethal concentration values, whereas *Streptomyces bellus*- E23-2 extract had the highest ones (Table 3).

Table 3. Median lethal concentration LC50 (mg mL-1) of *D. opuntiae* treated by four *Streptomyces* sp strains dichloromethane extracts (ANOVA, $\alpha = 0.05$)

Streptomyces sp	Cochineal stage	DAT	Slope ± SE	LC_{50}%	Chi-test (χ^2) Sig	df	P-value
Streptomyces bellus- E23-2	Nymph	1	4.6±0.7	15.3	21.3	48	P < 0.0001
		3	6.9±0.7	12.6	26.0	48	P < 0.0001
		6	6.3±0.8	11.4	16.9	48	P < 0.0001
	Adult female	1	4.8±0.7	16.9	18.6	48	P < 0.0001
		3	6.3±0.7	13.8	22.7	48	P < 0.0001
		6	6.8±0.7	12.4	30.5	48	P < 0.0001
Streptomyces galilaeus- E23-9	Nymph	1	4.5±0.7	16.6	16.7	48	P < 0.0001
		3	5.3±0.7	13.8	16.0	48	P < 0.0001
		6	6.1±0.7	12.3	19.1	48	P < 0.0001
	Adult female	1	4.7±0.7	18.5	18.0	48	P < 0.0001
		3	4.7±0.7	15.4	18.5	48	P < 0.0001
		6	5.7±0.7	13.3	22.0	48	P < 0.0001
Streptomyces africanus- E23-3	Nymph	1	4.7±0.7	17.5	13.2	48	P < 0.0001
		3	5.4±0.7	14.5	22.0	48	P < 0.0001
		6	6.0±0.7	13.0	15.8	48	P < 0.0001
	Adult female	1	5.3±0.7	19.7	14.2	48	P < 0.0001
		3	4.6±0.7	16.6	13.5	48	P < 0.0001
		6	5.0±0.7	14.5	14.1	48	P < 0.0001
Streptomyces bellus- E25-12	Nymph	1	4.7±0.7	19.7	20.4	48	P < 0.0001
		3	4.2±0.7	15.7	27.7	48	P < 0.0001
		6	6.5±0.7	14.0	28.7	48	P < 0.0001
	Adult female	1	5.7±0.8	22.1	29.1	48	P < 0.0001
		3	4.5±0.7	18.9	15.2	48	P < 0.0001
		6	5.1±0.7	16.3	11.1	48	P < 0.0001

DAT: Day after the treatment.

One-way ANOVA analysis shows that total mean mortality was significantly ($P \leq 0.001$) affected by exposure of first instar nymphs (Table 4) and adult females (Table 5) of *D. opuntiae* to different concentrations of the four *Streptomyces* sp. strains dichloromethane extracts tested. Insects exposed during the period from 1 to 8 days after treatment to the highest concentration (20 mg mL^{-1}), showed a significantly higher mortality rate.

The mean survival time of first instar nymphs (Table 4) and adult females (Table 5) of *D. opuntiae*, treated with dichloromethane extracts of four *Streptomyces* sp. strains tested at different concentrations (11-20 mg mL^{-1}), ranged from a minimum of 3.8 days (nymph) and 4.0 days (female) in the extract of *Streptomyces bellus*-E23-2 at 20 mg mL^{-1}, to a maximum of 5.4 days (nymph) and 5.7 days (female) in the extract of *Streptomyces bellus*-E25-12 at 11 mg mL^{-1}. In terms of median survival time (LT_{50}), the lowest value of LT_{50} (3 days) was observed in the extracts of *Streptomyces bellus*-E23-2, *Streptomyces galilaeus*-E23-9, and *Streptomyces africanus*-E23-3 at 20 mg mL^{-1} for nymphs (Table 4) and only in the extract of *Streptomyces bellus*-E23-2 at 20 mg mL^{-1} for adult females (Table 5). The highest LT_{50} value (6 days) was recorded in the other treatments tested.

Table 4. Mortality (%), mean survival time, and LT_{50} (days) of *D. opuntiae* first instar nymphs-treated four *Streptomyces* sp. strains dichloromethane extracts

Streptomyces sp	Concentrations (mg mL-[1])	Mortality (%)[a]	Mean survival time ± SE[b]	LT_{50} (95% CI)	Nc
Streptomyces bellus-E23-2	11	36.3	5.1±0.1	6.0±0.3	100
	13	50.7	4.7±0.1	6.0±0.3	100
	15	63.7	4.4±0.1	6.0±0.2	100
	17	78.7	4.0±0.1	3.0±0.2	100
	20	84.3	3.8±0.1	3.0±0.2	100
Streptomyces galilaeus- E23-9	11	32.0	5.2±0.1	6.0±0.3	100
	13	42.7	4.9±0.1	6.0±0.3	100
	15	53.3	4.6±0.1	6.0±0.2	100
	17	68.7	4.3±0.1	6.0±0.1	100
	20	79.0	4.0±0.1	3.0±0.2	100
Streptomyces africanus- E23-3	11	27.3	5.3±0.1	6.0±0.4	100
	13	38.0	5.0±0.1	6.0±0.3	100
	15	47.7	4.8±0.1	6.0±0.3	100
	17	65.0	4.3±0.1	6.0±0.2	100
	20	75.7	4.1±0.1	3.0±0.2	100
Streptomyces bellus-E25-12	11	22.7	5.4±0.1	6.0±0.5	100
	13	31.7	5.2±0.1	6.0±0.4	100
	15	40.3	4.9±0.1	6.0±0.3	100
	17	57.3	4.6±0.1	6.0±0.2	100
	20	68.3	4.3±0.1	6.0±0.1	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

[c]Total number of scale insects in bioassay.

Table 5. Mortality (%), mean survival time, and LT_{50} (days) of *D. opuntiae* adut females-treated four *Streptomyces* sp strains dichloromethane extracts

Streptomyces sp	Concentrations (mg mL^{-1})	Mortality (%)[a]	Mean survival time ± SE[b]	LT_{50} (95% CI)	Nc
Streptomyces bellus- E23-2	11	27.0	5.3±0.1	6.0±0.4	100
	13	41.3	5.0±0.1	6.0±0.3	100
	15	56.3	4.6±0.1	6.0±0.2	100
	17	72.0	4.2±0.1	6.0±0.1	100
	20	78.0	4.0±0.1	3.0±0.2	100
Streptomyces galilaeus- E23-9	11	24.0	5.4±0.1	6.0±0.4	100
	13	35.3	5.1±0.1	6.0±0.4	100
	15	46.0	4.8±0.1	6.0±0.3	100
	17	60.0	4.5±0.1	6.0±0.2	100
	20	69.7	4.2±0.2	6.0±0.1	100
Streptomyces africanus- E23-3	11	20.3	5.5±0.1	6.0±0.5	100
	13	28.3	5.3±0.1	6.0±0.4	100
	15	37.7	5.0±0.1	6.0±0.3	100
	17	53.3	4.6±0.1	6.0±0.2	100
	20	64.7	4.3±0.1	6.0±0.2	100
Streptomyces bellus- E25-12	11	13.0	5.7±0.1	6.0±0.5	100
	13	20.7	5.5±0.1	6.0±0.5	100
	15	29.0	5.3±0.1	6.0±0.4	100
	17	43.3	4.9±0.1	6.0±0.3	100
	20	53.3	4.7±0.1	6.0±0.2	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;
[c]Total number of scale insects in bioassay.

A color change in EP bacteria-invaded insects is typically reported for many insect hosts. In this study, the scale pests became dark brown when infected by the four *Streptomyces* strains tested (Figure 1).

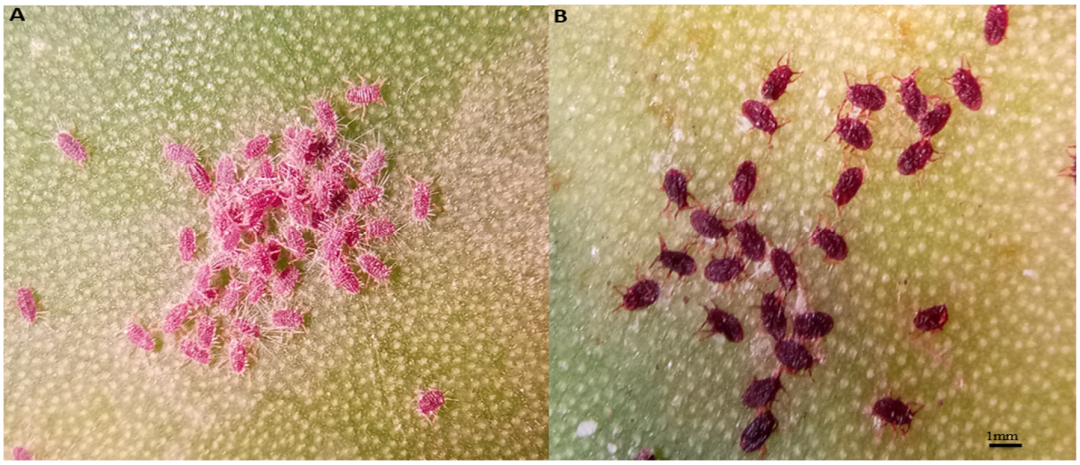

Figure 1. (**A**) Dead *D. opuntiae* first instar nymphs treated Streptomyces sp. dichloromethane extract, (**B**) *D. opuntiae* first instar nymphs treated tap water

Greenhouse trials

The dichloromethane extracts of *Streptomyces* sp. strains treatments tested were significantly different in their contact toxicity against first instar nymphs of *D. opuntiae* (Figure 2). The greatest ten days first instar nymph abott-corrected mortality was achieved by *Streptomyces bellus*- E23-2 (55.5%) and *Streptomyces*

galilaeus- E23-9 (50.5%) dichloromethane extracts at 20 mg mL^{-1}. The lowest percentage of nymph mortality was achieved at 10 days after treatment with the lowest concentration (17 mg mL^{-1}) for the both *Streptomyces* sp. treatments tested.

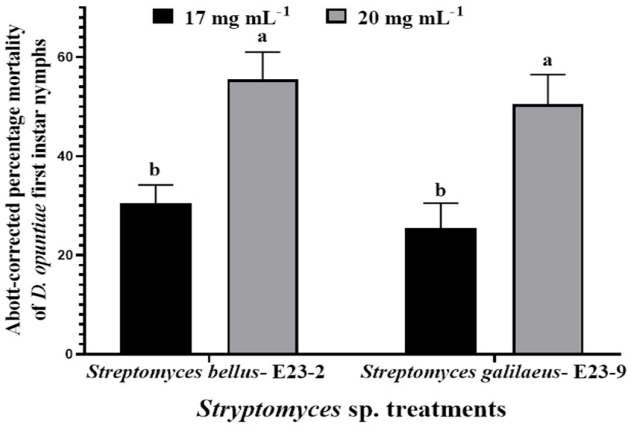

Figure 2. Abbott-corrected percentage mortality of *D. opuntiae* first instar nymphs at 10 days after application of Streptomyces sp. dichloromethane extracts under greenhouse conditions.

Discussion

Several micro-organisms, including bacteria, nematodes, fungi, viruses and protozoa species have been employed as entomopathogens (EPs) for pest control in many IPM programs worldwide (Mazzeo *et al*., 2019). More than 22,500 active biomolecules are derived from micro-organisms and *Actinobacteria*, in particular *Streptomyces* sp. have provided a wide variety of biologically active substances of considerable importance in agriculture and, to this day, continue to be systematically screened for new bioactive compounds. In the current study, the insecticidal potential of dichloromethane extracts of four *Streptomyces* sp. strains isolated from soil in an extremely cold and microbiologically unexploited region of Morocco (Fez-Meknes region) and identified on the basis of spore and colony morphology as well as 16 rRNA gene sequencing (Rammali *et al*., 2022), were evaluated against first instar nymphs and adult females of *D. opuntiae* under laboratory and greenhouse conditions by considering concentrations and time required for the *Streptomyces* sp. strains extracts to kill the host. Dichloromethane was chosen as a solvent in this study due to its ability to effectively extract biologically active compounds from *Streptomyces* sp. strains, which produce a wide range of such compounds (Jinfeng *et al*., 2017). It is a low toxicity, non-polar solvent that is easy to remove from the final product due to its high volatility (Li *et al*., 2018). Dichloromethane's ability to extract a wide range of compounds, including those with low polarity and lipophilic compounds, which may have insecticidal activity (Fonseca *et al*., 2015), makes it an ideal solvent for this study. Our results showed that *Streptomyces bellus*- E23-2 *Streptomyces galilaeus*- E23-9 dichloromethane extracts were the most active against *D. opuntiae*. These metabolites at the maximum dose tested (20 mg mL^{-1}) caused the highest mortality of nymphs and adult females after 8 days of exposure, resulting in LC_{50} values of 11.4 and 12.3 mg mL^{-1} (nymph) and 12.4 and 13, 3 mg mL^{-1} (young female), respectively, whereas the *Streptomyces bellus*- E23-2 dichloromethane extract had the lowest mortalities. The same trend was observed for LT_{50}, as the lowest value, 3 days, was recorded in the *Streptomyces bellus*- E23-2, *Streptomyces galilaeus*- E23-9, and *Streptomyces africanus*- E23-3 extracts at 20 mg mL^{-1} in the case of nymphs and only in the *Streptomyces bellus*- E23-2 extract in the case of adult females, while the highest value, 6 days, was recorded

in the other treatments tested. A similar study shows that crude ethanolic extracts of *Streptomyce*s sp. had anti-insect efficacy in terms of larval mortality, with 100% mortality at the concentration 24 mg mL^{-1} against *Sitophilus oryzae* (Linnaeus) in a dose-dependent manner (Rishikesh *et al*., 2013). Also, *Streptomyces* sp. AP-123 polyketide metabolite showed larvicidal activity against *H. armigera* and *S. litura* at 1000 ppm, with 68.41% and 60.02% mortality, respectively (Arasu *et al*., 2012). These differences in pathogenicity of *Streptomyces* sp. may be due to several factors and understanding the dosage for host (insect pest) infection, host range, dynamics of EP-insect-plant interactions, and the ecobiology of the insect host and EP are the most important ones for effective use of EP-based biopesticides for pest control in forestry, agriculture, and urban areas (Lacey *et al*., 2015).

Hemalatha *et al*. (2018) investigated the effectiveness of toxins from the bacteria *Xenorhabdus nematophilus* in controlling striped mealybugs, and according to their findings, the toxins enter the mealybug during piercing and sucking of plant nutrients. The toxins from the bacteria could have damaged the peritrophic membrane of the host insect gut and led to reduced nutrient absorption (Krishnamoorthy *et al*., 2020). This could probably be the reason for the mortality of *D. opuntiae* in the present study.

In this study, all the four *Streptomyces* sp. strains dichloromethane extracts tested were found to be pathogenic to both nymphs and adult females of *D. opuntiae* and their efficacy increased with increasing exposure time and concentration. Rahoo *et al*. (2017) reported that the control of any pest selected by EP-based biopesticides is related to the concentration of EP metabolites, as higher concentrations increase the chances of infections and, consequently, mortality rates.

The effects of abiotic factors (climate change) and cultural practices on EPs bacteria extracts in the delivery of multiple agricultural systems need to be studied for their optimal use as biopesticides under semi-field and field conditions. In addition, robust technologies are needed to improve the production and persistence of EPs bacteria extracts under unfavorable environmental conditions. Many studies have demonstrated the effectiveness and persistence of several EPs bacteria under real conditions (Grzywacz *et al*., 2014; Sabbahi and Hock, 2022).

In the greenhouse experiments, *Streptomyces bellus*- E23-2 and *Streptomyces galilaeus*- E23-9 dichloromethane extracts applied at 20 mg mL^{-1} were effective in reducing the numbers of first instar nymphs of the cochineal. There were also small but significant reductions of *D. opuntiae* first instar nymphs in the same treatments applied at 17 mg mL^{-1}. Several studies have reported the sensitivity of several pest species to EPs bacteria derived biomolecules. the metabolite 12-epi-Hapalindole J isonitrile, derived from a soil bacterium ("*Cyanobacterium terium*"), induced 100% larval death in the dipteran *Chironomus riparius* (Meigen) at a concentration of 26 μM within 48 hours of exposure (Becher *et al*., 2007). Toxins from *Paenibacillus bacilluries* and *Paenibacillus popilliae* (milky spores) are used against larvae of the Japanese beetle, *Popillia japonica* Newman (cause of milky disease) (Shanovich *et al*., 2019). Also, biopesticides derived from *Chromobacterium subtsugae* and *Burkholderia rinojensis* have been shown to have an insecticidal effect against *Leptinotarsa decemlineata* (Say) and other insect pests (Córdova-Kreylos *et al*., 2013). *Pseudomonas chlororaphis* is a potent microbial biocontrol agent that produces insecticidal proteins (Péchy-Tarr *et al*., 2008) which directly suppress populations of the hornworm, *Manduca sexta* (Linnaeus, 1763) (Lepidoptera: Sphingidae) (Anderson and Kim, 2018). In addition, *P. fluorescens* has been shown to cause significant mortality and growth inhibition of both the tobacco cutworm, *S. litura*, and the cotton bollworm, *H. armigera* (Gopalakrishnan *et al*., 2011; Sahayaraj *et al*., 2018). No previous research has yet been conducted on the control of *D. opuntiae* with EP bacteria. Depending environmental conditions, targeted pest and crop, EP bacteria derived biomolecules actives have good compatibility and can be used in different ways, alone, in rotation, or in combination with other IPM strategies such as other EPs, beneficial insects (e.g. predators and parasitoids), clones resistant, and chemical insecticides in order to obtain significant results (Mazzeo *et al*., 2019). Moreover, the integration of EPs bacteria into IPM programs requires the precise identification of the selected microbial EPs agents and

sufficient knowledge of the bioecology and behavior of the inoculum and its effects on non-target insects (predators, pollinators, parasitoids). This challenge is compounded by the difficulties in detecting and monitoring microbial agents released into the field (Wang *et al*., 2016). To address this challenge, there is a need to develop molecular tools capable of distinguishing isolates while simultaneously screening large quantities of samples and detecting the EPs in infected hosts.

Several studies have investigated the effects of Streptomyces metabolites on non-target insects' behavior and reproduction. Ganesan *et al*. (2018) found that Streptomyces metabolites are highly toxic to mosquitoes but have low toxicity to non-target organisms. These metabolites are host-specific and do not have any detrimental effect on non-target organisms or the surrounding environment (Schneider *et al*. 2004). Secondary metabolites produced by various *Streptomyces species*, including blasticidin-S, kusagamycin, streptomycin, oxytetracycline, validamycin, polyoxins, natamycin, actinovate, mycostop, abamectin/avermectins, emamectin benzoate, polynactins, and milbemycin, have found commercial applications as crop protection agents due to their efficacy (Aggarwal *et al*., 2016). These microbial secondary metabolites are known for their high specificity, making them safer options for beneficial insects, mammals, and humans (Gopalakrishnan *et al*., 2020). As a result, Streptomyces-derived compounds offer a promising alternative for the management of insect pests and plant pathogens affecting agriculturally important crops, with reduced non-target impact (Aggarwal *et al*., 2016). Streptomyces secondary metabolites are considered a significant new-generation pesticide by IPM practitioners due to their specific toxicity and favorable non target organism and ecological profile (Schneider *et al*., 2004). In addition, the use of Streptomyces secondary metabolites has gained public acceptance due to its minimal impact on ecosystems and humans, as well as other non-target organisms (Chen *et al*., 2023). Microbial insecticides are highly valuable because of their extremely low toxicity to non-target animals and humans (Dhanasekaran and Thangaraj, 2014). In addition, the highest insecticidal activity exhibited by the *Streptomyces bellus*-E23-2 strain may have been caused by the synergistic effect of the secondary metabolites present in this strain dichloromethane extract.

Conclusions

The present work represents the first study regarding the possibility of biological control of the cactus cochineal *D. opuntiae* (Moroccan biotype) using dichloromethane extracts of new *Streptomyces* sp. strains isolated from soil in an extremely cold region of Morocco. Our results indicate that *Streptomyces bellus*- E23-2 and *Streptomyces galilaeus*- E23-9 strains applied dichloromethane extracts at 17 and 20 mg mL^{-1} showed strong insecticidal and toxic activities against *D. opuntiae* under both laboratory and greenhouse conditions and can be used for the development of new insecticide formulations as an alternative to toxic chemicals for controlling the scale insect under field conditions. Further studies are needed to evaluate the effect of other ecological factors, only some addressed in this study, on the efficacy and persistence of these bioactive metabolites under field conditions as well as their lethal and side effects on non-target organisms (predators, parasitoids and pollinators). In addition, future research to gain a better understanding of EP-*D. opuntiae* interactions in the cactus-plant system as well as the optimization of the production and application of *Streptomyces* sp. derived bioactive substances under field conditions are planned.

Authors' Contributions

Conceptualization: ME and SR; Data curation: ME and SR; Formal analysis: ME and SR; Funding acquisition: ME, SR, MS, AA, KD, BB and AK; Investigation: ME, SR, MS, AA, KD, BB, and AK; Methodology: ME and SR; Project administration: SR and BB; Resources: SR and BB; Software: ME and SR;

Supervision: SR, BB, and AK ; Validation: ME, SR BB and AK; Visualization: ME, SR BB and AK ; Writing - original draft: ME and SR; Writing - review and editing: ME and SR. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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