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Isolation, identification and efficacy of three bacterial isolates against the red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae)

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Abstract

Background: The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), overruns date palm ranches in most Oases of Saudi Arabia and causes massive economic losses. Integrated pest management (IPM) of the RPW by biocontrol agents of bacterial isolates was the primary aim of the present study.

Results: Thirty-seven bacterial isolates were isolated from the larvae of *R. ferrugineus*, which were collected from infested *Phoenix dactylifera* trees growing in different parts of Saudi Arabia. 16S rDNA sequencing showed that the isolated bacteria were: *Serratia marcescens, Klebsiella pneumoniae* and *Bacillus thuringiensis*. The potential of these isolates was tested against *R. ferrugineus* in the laboratory. The use of these three isolates showed significant mortality percentages against *R. ferrugineus*. The highest reduction in mortality was recorded at the concentration of 1×10^8 CFU/ml; *B. thuringiensis* isolate displayed the highest potency in mortal percentage rate (100%), 4 days post-treatment, followed by *S. marcescens* isolate after 5 days, and then *K. pneumoniae* isolate after 6 days.

Conclusions: These results suggest that the use of these bacterial isolates was very useful in in vitro experiments, and it may consider those bacterial strains of interest as a potential biocontrol agent of *R. ferrugineus*.

Keywords: Biological control, Red palm weevil, Entomopathogenic, Integrated pest management (IMP)

Background

The date palm (*Phoenix dactylifera* L. (Arecales: Arecaceae)) is a crucial agricultural crop as well as the core of oasis farming practices in date palm-producing countries. About 5000 (Bashah 1996) or 3000 (Zaid and de Wet 2002) date palm cultivars cultivated worldwide but might be some of their synonyms of one another in various places with a different name. In Saudi Arabia, date palm (*Phoenix dactylifera* L.) is an economic crop and has an ancient history (Chao and Krueger 2007), and about 450 cultivars widely known as the best date-producing were estimated (Bashah 1996), and the date palm production is ca. 1,539,756 tons with a harvested area around 117,881 ha (FAO 2019). The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), is a vital insect pest in many parts of Saudi Arabia. It was recorded in 1987 in Katif, Saudi Arabia. It is thought that it was brought in ornamental palms. It is now distributed in most oases (Ferry et al. 2016).

It causes great damages to palm trees, where its larvae spend their whole life inside the trunk (Asiry et al. 2018). Kaakeh (2005) found that the heavily infested date palm trees with *R. ferrugineus* die within a couple of weeks post-infestation as later instars of these weevils move

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inside the trunk of the infested palms through feeding and form tunnels with a diameter of 2–3 cm and up to 40 cm length within 36 h. The controlling methods of this pest mainly depend on insecticides; however, the use of insecticides as a control method can cause environmental, social and economic problems. It is essential to avoid using insecticides to control the pests (Shahina et al. 2009). Other methods are integrated pest management tactics, e.g., cultural, chemical and mechanical control practices, and pheromone traps are used to control RPW (Murphy and Briscoe 1999).

Biological control is another choice method to enhance management options against the weevil. Entomopathogenic bacteria (EPB) are also a matter of discussion for the proper management of harmful insect pests. These bacteria mostly belong to families: Enterobacteriaceae, Streptococcaceae, Pseudomonadaceae and Bacillaceae (Mazza et al. 2011). *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) (subspecies: *kurstaki*) has been isolated from the larvae of red palm weevils and is being implemented for its appropriate control (Alfazairy 2004). The efficacy of some bacteria, e.g., *B. thuringiensis, Bacillus amyloliquefaciens* Priest (Bacillales: Bacillaceae), *Bacillus laterosporus* Laubach (Bacillales: Bacillaceae), *Pseudomonas aeruginosa* (Schröter) Migula (Pseudomonadales: Pseudomonadaceae), was assayed in the laboratory against the weevil of *R. ferrugineus* (Francesca et al. 2015). Francesca et al. (2015) reported that a lethal strain of *B. thuringiensis* was used against *R. ferrugineus* by the applications in the field. It causes 70–85% mortality rate, and they declared that it is the most appropriate and significant management tool to be implemented in RPW integrated management technique.

The aims of the study were to isolate and identify the bacterial community associated with RPW larvae, and to assay the most potent ones on the mortality of *R. ferrugineus* under laboratory conditions as a bioagent candidate for field control.

Methods

Rhynchophorus ferrugineus collection and laboratory rearing

The laboratory study was carried out in the Department of Plant Protection, University of King Abdulaziz, Saudi Arabia, during 2020. The larvae of *R. ferrugineus* were collected from naturally infested palms at Al Koutha village, the Hail region, Saudi Arabia. Collected larvae were kept separately in plastic jars and brought to the laboratory. A colony was established in the laboratory in plastic boxes ($30 \times 60 \times 60$ cm) having a lid covered with mesh wire gauze (60 mesh size, 10 cm diameter) in the middle for aeration, and the rearing technique was completed following the method of El-Zoghby and Abdel-Hameid (2018). The rearing conditions were maintained at 25 ± 2 °C, 65 ± 5% RH and 12L/12D photoperiods.

Isolation of bacteria bioagent

To isolate the associated bacteria, larvae of RPW were collected from different locations. Larvae were surface-sterilized with sodium hypochlorite for 2 min and then washed several times with distilled water (Poinar and Thomas 1978). Using a glass tissue grinder, the bodies of RPW larvae were homogenized in nutritional broth (NB) and filtered. The sample extracts (0.1 ml) were then plated on a nutrient agar medium (NA) in Petri dishes (9 cm) for 3 days at 30 °C. The isolated bacteria were identified according to their molecular characteristics. Pure cultures of bacterial colonies were prepared and stored in a slant of NA media at 4 °C.

Primary screening of 37 bacterial isolates on the larvae of *R. ferrugineus*

A single concentration bioassay was used to investigate the pathogenicity of the bacterial isolates on *R. ferrugineus* larvae. Purified 37 bacterial isolates were cultured in nutrient broth for 18 h at 37 °C and then agitated at 200 rpm/min overnight; bacterial density was adjusted to 10^6 CFU (Emine et al. 2014). For pathogenicity tests, the second larval instar of *R. ferrugineus* was chosen. For each bacterium, three replicates were used. Each replicate comprised 18 larvae of RPW, under controlled laboratory conditions. Following inoculation, the number of dead and alive larvae was counted daily for 8 days.

Molecular identification of the bacterial isolates

The bacterial isolates were grown on a flask having NB medium and incubated at 37 °C for 16 h with shaking at 200 rpm. Ten milliliters of NB medium was centrifuged at 5000 rpm for 5 min to precipitate bacterial pellets for DNA extraction.

DNA extraction

Three bacterial isolates (No. 2001, 2011 and 2030), which showed the highest mortality percentages against larvae of *R. ferrugineus,* were selected for molecular identification using 16S-rRNA gene sequences. Bacterial isolates were cultured in sterile test tubes containing 10 ml of nutrient broth medium (NB) (Zimbro et al. 2009). Bacterial cultures were incubated at 37 ± 1 °C for 48 h. DNA extraction was carried out using the Patho Gene-spin DNA/RNA Extraction Kit provided by the Intron Biotechnology Company, Korea. DNA samples were then sent to SolGent Company, Daejeon, South Korea, for polymerase chain reaction (PCR) and gene sequencing. Amplification of the 16S rRNA gene was performed with 27F and 1492R universal primers.

Bioassay of the tested bacterial isolates at different concentrations against *R. ferrugineus*

This experiment was carried out with the most effective bacterial isolates on larvae of *R. ferrugineus* according to their mortality percentages in the preliminary test. The used bacterial concentrations were 1×10^4, 1×10^6 and 1×10^8 CFU/ml. Three replicates were used for each concentration, and each replicate comprised 18 larvae, the methods of treatment as in the tests mentioned previously (Emine et al. 2014). The number of dead and alive larvae was recorded daily for 8 days following inoculation.

Data analysis

Statistical analyses were executed with SPSS 21.0 for Windows (SPSS Inc., Chicago, IL, USA) with a significance level of 0.05. The mortalities of the treated larvae were presented as percentages; besides, if control mortality was zero, no correction for mortality was calculated. The time–mortality response data were evaluated by a probit model. The median and 90 lethal times (LT_{50} and LT_{90}) and 95% confidence intervals (CIs) were then estimated. The LT_{50} and LT_{90} values were statistically compared for significance by CIs overlapping (Robertson and Preisler 1992).

Results

Identification of three bacteria isolates

Based on the antagonistic activity and mortal activity, potential bioagent isolates were further classified by PCR amplification of internal transcribed spacer (ITS) regions with universal primer ITS5 and ITS4 and Sanger sequencing of PCR products. The obtained PCR products were approximately 560–680 bp in length. Sequences were submitted to NCBI database under accession numbers of MW740163.1, MW740162.1 and MW740161.1. Comparison of obtained ITS sequences to NCBI database entries by basic local alignment search tool (BLAST) would show the bacterial isolates fall into the following species: *Klebsiella pneumoniae* (Schroeter) Trevisan (Enterobacterales: Enterobacteriaceae) (MW740163.1, Fig. 1A), *Serratia marcescens* Bizio (Enterobacterales: Yersiniaceae) (MW740162.1, Fig. 1B), and *Bacillus thuringiensis* (MW740161.1, Fig. 1C). These obtained sequences for the three tested species were compared as each obtained isolate separately with other isolates from the same genus as indicated in Fig. 1. MN326591.1 Massilia varians strain PA10 was used as out-group species for the three isolates. The phylogenetic tree contained *Serratia marcescens* indicated that this isolate is a different clade with other collected sequences from the same genus. The same result was obtained for the studied *Klebsiella pneumonia* isolate. Meanwhile, *Bacillus thuringiensis* strain KNKI-2011 was different than all collected sequences, except of *B. thuringiensis* (LC178545.1) that was in the same clade with it.

Mortality of second instar RPW larvae treated with tested bacterial isolates

Mortality percentages of RPW larvae exposed to the three bacterial isolates (*K. pneumoniae*, *S. marcescens* and *B. thuringiensis*) as a bait treatment at three different concentrations (1×10^4, 1×10^6 and 1×10^8 CFU/ml) were compared (Table 1 and Fig. 2). When RPW larvae were tested, the concentration of 1×10^4 CFU/ml showed a slow death effect on the larvae by day passing. The mortality percentage was 92. 59, 77.78 and 62.96% for *K. pneumoniae, S. marcescens* and *B. thuringiensis*, respectively, after 8 days of treatment. At the concentration 1×10^6 CFU/ml, the mortality percentage increased rapidly to reach 100% 6 days post-treatment for the three tested isolates—a day in advance for *S. marcescens* isolate. At the concentration 1×10^8 CFU/ml, *B. thuringiensis* isolate displayed the highest potency in mortal percentage rate (100%), 4 days post-treatment, followed by *S. marcescens* isolate after 5 days, and then *K. pneumoniae* isolate after 6 days.

In general, *K. pneumoniae* isolate gave the highest mortality percentage (92.59%) at the concentration of 1×10^4 CFU/ml after 8 days of treatment. At the concentration of 1×10^6 CFU/ml, *S. marcescens* isolate gave 100% mortality 5 days post-treatment—a 1 day in advance of *B. thuringiensis* and *K. pneumoniae*, whereas *B. thuringiensis* gave the best effect (100% mortality) at the concentration of 1×10^8 CFU/ml after 4 days of treatment.

The time, which is required to kill 50 or 90% of treated larvae (LT_{50} or LT_{90}), is shown in Table 2. At the concentration of 1×10^4 CFU/ml, the results revealed that the respect LT_{50} and LT_{90} values were calculated for *S. marcescens* (5.25 and 15.62 days), *K. pneumoniae* (6.25 and 8.00 days) and *B. thuringiensis* isolates (6.39 and 21.38 days). At the concentration of 1×10^6 CFU/ml, the LT_{50} and LT_{90} values had the same trend, and the isolates differ from each other by a few hours (less than half day at maximum) to reach either LT_{50} or LT_{90}. At the concentration of 1×10^8 CFU/ml, the three bacterial isolates showed the same way to achieve the LT_{50} or the LT_{90} as in concentration 1×10^6 CFU/ml with also half a day lag maximum among them. Ultimately, according to the mortality percentages, *B. thuringiensis* and *S. marcescens* isolates were the fastest to attain 100% mortality one day before *K. pneumoniae*; on the other hand, *K. pneumoniae* isolate succeeded to give an LT_{50} few hours before the other two isolates.

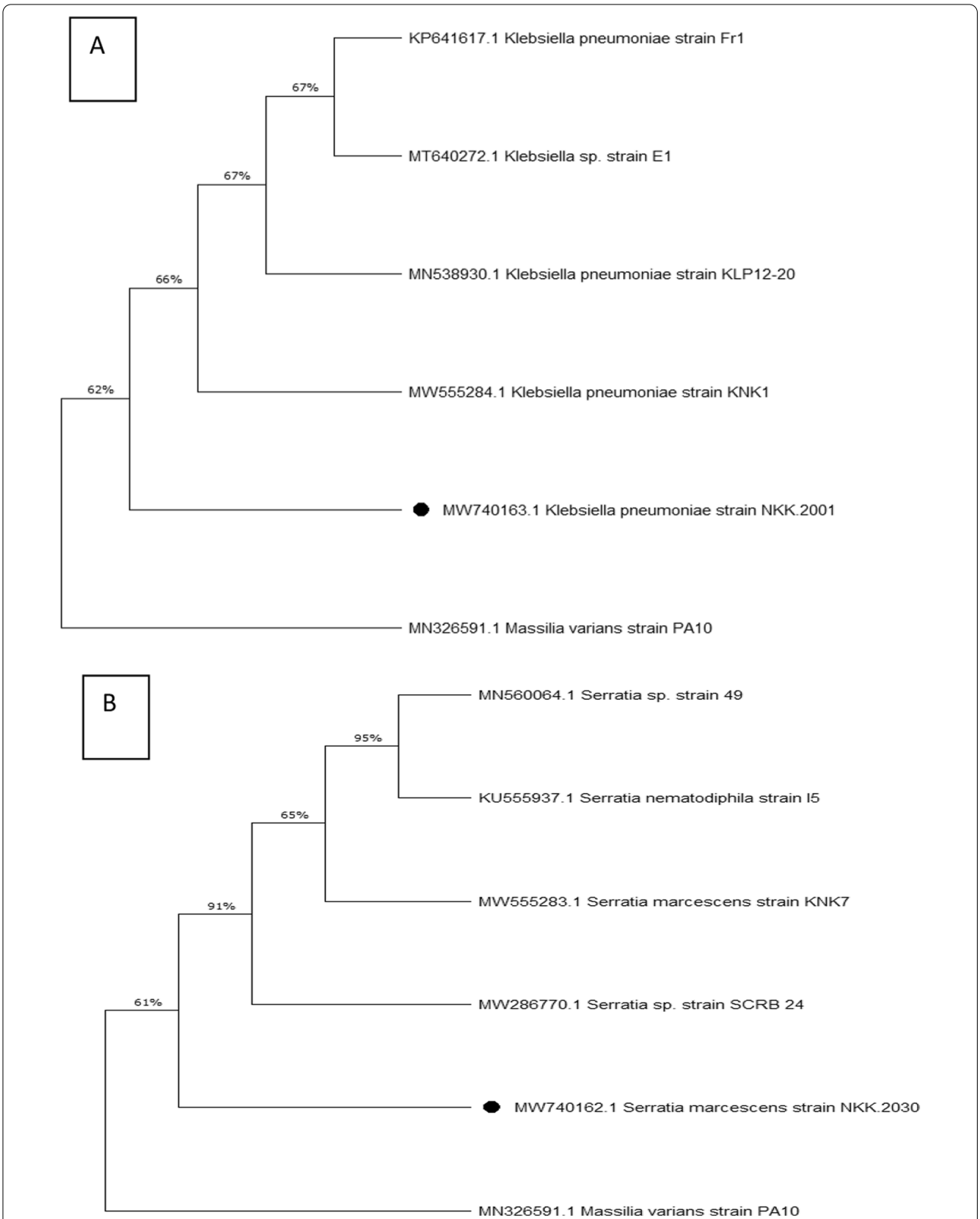

Fig. 1 Maximum likelihood phylogenetic tree-based ribosomal region 16s-rRNA and bootstrap support values > 60 (BS) are given at the nodes (BS) on 16s-rRNA sequences of rDNA of *Klebsiella pneumoniae* isolate (MW740163.1, Fig. **A**), *Serratia marcescens* isolate (MW740162.1, Fig. **B**), and *Bacillus thuringiensis* isolate (MT487672.1, Fig. **C**) aligned with closely related sequences accessed from the GenBank. The marker reflects the relative phylogenetic distance measurement

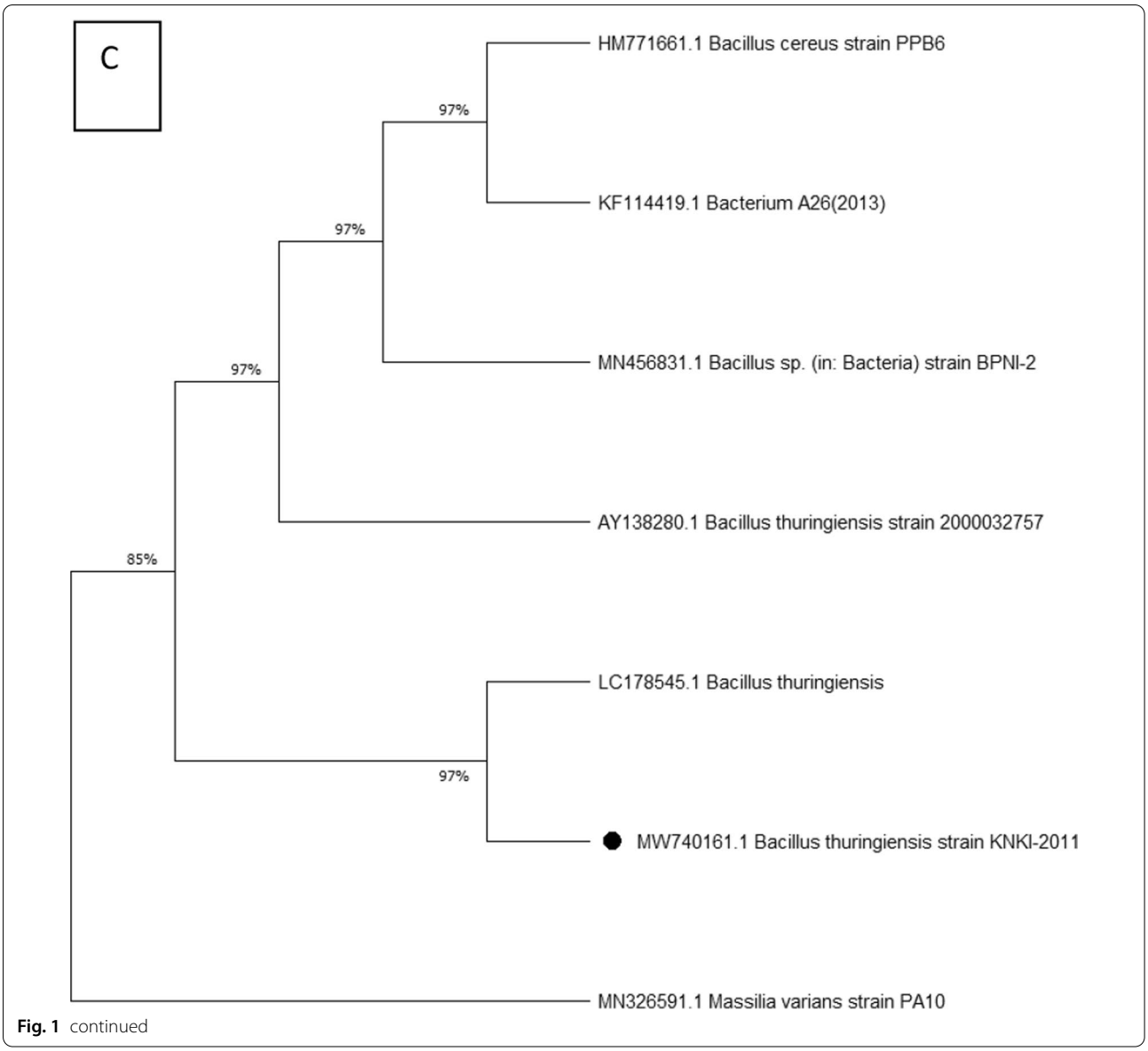

Fig. 1 continued

Discussion

Many physical and chemical ways that have been applied to control the dispersal of the RPW have been not quite successful; this might be due to that the larvae, which mainly cause the damage, exist in the trunk that gives it protection. Biocontrol agents are useful for controlling pest populations, but rarely eliminate them. Even when they are efficient under laboratory conditions, several biological control agents do not give sufficient control of red palm weevil in the field (El-Sufty et al. 2007). Yet, the scientists still look up for biological enemies as an essential step to build up IPM tactics against *R. ferrugineus* because of the lack of an active and eco-friendly management method safe for human wellness. The potency of EPB has been researched since the previous century when the ability of some *Bacillus* species was found in regulating serious insect pests (Ruiu et al. 2013). The incidence of various bacteria on RPW has been described by several researchers, e.g., Alfazairy (2004). Three isolates were identified as *K. pneumoniae*, *S. marcescens and B. thuringiensis* out of 37 isolates in this study. In agreement with the results, Alanazi (2019) identified some bacterial isolates from *R. ferrugineus* mainly as *Proteus mirabilis* Hauser (Enterobacterales: Enterobacteriaceae), *Klebsiella pneumoniae*, *Serratia marcescens*, *Staphylococcus sciuri* Kloos et al. (Bacillales: Staphylococcaceae) and *Providencia rettgeri* Rettger (Enterobacterales: Morganellaceae).

Table 1 Larval mortality percentages of three bacterial isolates against the second instar larvae of red palm weevil, *Rhynchophorus ferrugineus*, under controlled laboratory conditions

Conc. (CFU/ml)	Isolates	Days after treatment						
		2	3	4	5	6	7	8
		Larval mortality (%)						
1×10^4	*K. pneumoniae*	25.93	29.63	22.22	37.04	44.44	66.67	92.59
	S. marcescens	18.52	22.22	33.33	44.44	51.85	62.96	77.78
	B. thuringiensis	14.81	18.52	25.93	40.74	44.44	55.56	62.96
1×10^6	*K. pneumoniae*	44.44	66.67	74.07	88.89	100.00	100.00	100.00
	S. marcescens	22.22	40.74	85.19	100.00	100.00	100.00	100.00
	B. thuringiensis	22.22	51.85	77.78	96.30	100.00	100.00	100.00
1×10^8	*K. pneumoniae*	48.15	66.67	74.07	92.59	100.00	100.00	100.00
	S. marcescens	40.74	40.74	96.30	100.00	100.00	100.00	100.00
	B. thuringiensis	25.93	40.74	100.00	100.00	100.00	100.00	100.00

Few studies have been done to assay the biocontrol capability of bacteria against RPW. In this study, three isolates (*K. pneumoniae*, *S. marcescens and B. thuringiensis*) out of 37 isolates collected from *R. ferrugineus* were tested against the second instar RPW larvae as a potential biocontrol agent against this pest. The obtained results showed that the median lethal time for the three bacterial isolates ranged from 5.30 to 6.40 days for the concentration of 1×10^4 CFU/ml and 2.35 and 2.83 days and 2.28 and 2.67 days for the concentrations of 1×10^6 and 1×10^8 CFU/ml, respectively. In 1995, *Pseudomonas aeruginosa* was isolated and its biocontrol ability was examined by three various treatment techniques as follows: (1) injecting *P. aeruginosa* into the larvae, (2) forced feeding and (3) immersing the larvae into the suspension. The results were non-satisfactory and revealed that the lethal median time for small larvae was 6 days for injection and nearly 8 days for forced feeding and wading (Banerjee and Dangar 1995); these results are in accordance with our results due to the first concentration. Also, another researcher isolated yeast from the hemolymph of *R. ferrugineus* and reported 4 days to kill 50% larvae of RPW (Dangar and Banerjee 1993), which is almost similar to the obtained results for the last two concentrations. In addition, Pu et al. (2017) found that the LT_{50} values of the second instar RPW larvae decreased when the concentration of *B. thuringiensis* increased.

Our results revealed that the larval mortality percentages varied from bacterial isolate to another. Only *B. thuringiensis* isolate could attain 100% mortality, 4 days post-treatment at the concentration of 1×10^8 CFU/ml. Somehow, the obtained results disagree with that *B. thuringiensis* has several different cry toxin gene forms, and each form usually acts independently on orders of insects (Crickmore et al. 1998). Salama et al. (2004) isolated three strains of *Bacillus* from date palm and identified them as *Bacillus sphaericus* Meyer and Neide (Bacillales: Bacillaceae) (strain 73), *B. megaterium* de Bary (Bacillales: Bacillaceae) (strain 15), and *B. laterosporus* (strain 27). The three strains caused 40–60% mortality of the second instar RPW larvae under laboratory conditions, and the most active culture was *B. sphaericus* (strain 73). Also, *B. thuringiensis* subsp. *kurstaki* was first isolated from dead RPW and caused as high as 70% mortality for the larval stage under laboratory conditions (Alfazairy 2004). Meanwhile, Sivasupramaniam et al. (2007) stated that the RPW showed various vulnerabilities to *B. thuringiensis*. Also, the growth of the *B. subtilis* (Ehrenberg) Cohn (Bacillales: Bacillaceae) and *B. thuringiensis* was constrained due to the polarity of red palm weevil adult or large larvae extracts (El-Sufty et al. 2007). In addition, the suppression of larval growth and interaction with hemocytes were noticed for the RPW which provides evidence that *Bt* could propagate in the hemolymph when eaten by the larvae (Manachini et al. 2011a, b). In the same trend, Raio et al. (2016) examined 35 bacterial strains isolated from larvae and pupae of *R. ferrugineus* gathered from various Italian localities. Also, it was found that *B. thuringiensis* strains, *Providencia rettgeri,* and *S. marcescens* might be regarded as a possible biocontrol agent of RPW. Also, Pu and Hou (2016) found that *S. marcescens* had higher toxicity (> 50%) than other tested strains. The virulence of *K. pneumonia* subsp. *pneumonia* was followed to *S. marcescens* at 44.82%. The toxicity of *S. sciuri* was only 38.45%, and *Proteus* spp. had the lowest insecticidal activity when tested on the fourth instar RPW larvae. In addition, *B. thuringiensis* was able to cause 100% mortality after 4 days of treating the second instar RPW larvae, in the present study, where their corrected mortality increased significantly with concentration increased to

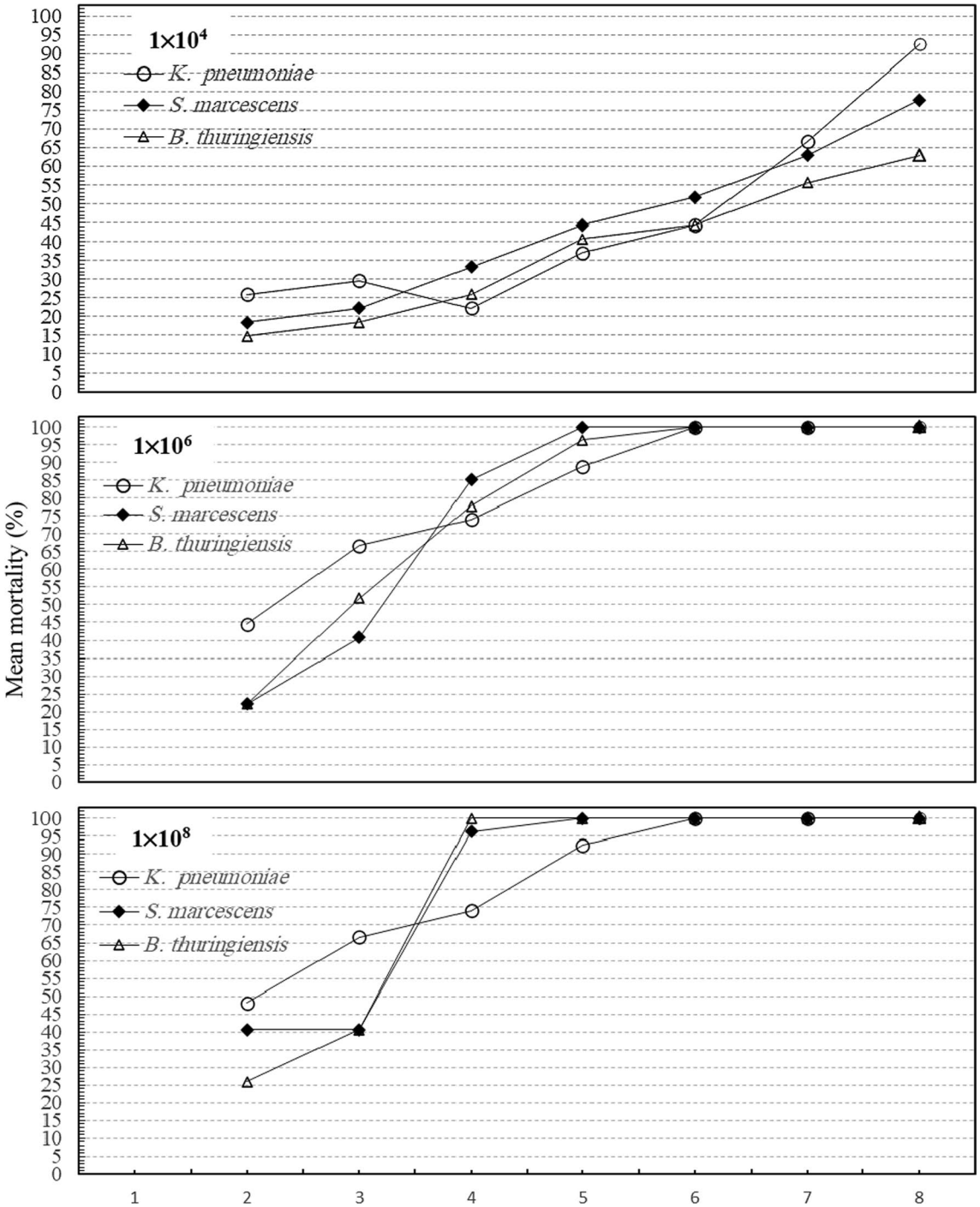

Fig. 2 Mean mortal changes of the red palm weevil, *Rhynchophorus* ferrugineus, second larval instar exposed to different bacterial isolate concentrations in relation to time under controlled laboratory conditions

be 16.97–94.32% after 15 days (Pu et al. 2017). *S. marcescens* gave a maximum of 60% larval mortality (Pu and Hou 2016), while in the present study, the mortality percentages increased as the concentration increases to reach 44.44% at the concentration of 1×10^4 CFU/ml and 100% mortality at 1×10^6 CFU/ml and 1×10^8 CFU/ml concentrations after 5 days.

Compared to the outcomes from earlier experiments, the present results confirmed that the pathogenicity of

Table 2 The LT_{50} and LT_{90} of second instar *Rhynchophorus ferrugineus* larvae exposed to three different concentrations of three tested bacterial isolates under controlled laboratory conditions

Conc. (CFU/ml)	Isolates	Slope	LT_{50} (days)	Confidence intervals (95%)		LT_{90} (days)	Confidence intervals (95%)		Intercept (*a*)	χ^2
				Lower	Upper		Lower	Upper		
1×10^4	*K. pneumoniae*	11.95	6.25	3.50	6.89	8.00	7.17	24.80	− 9.49	2.59
	S. marcescens	2.71	5.25	4.53	6.25	15.62	11.23	29.99	− 1.95	9.04
	B. thuringiensis	2.44	6.39	5.78	7.26	21.38	16.15	32.90	− 1.97	4.19
1×10^6	*K. pneumoniae*	4.34	2.35	1.67	2.83	4.64	3.87	6.27	− 1.61	18.16
	S. marcescens	7.23	2.83	2.39	3.23	4.26	3.69	5.50	− 3.21	22.22
	B. thuringiensis	6.42	2.81	2.53	3.07	4.45	4.03	5.11	− 2.88 ±	8.66
1×10^8	*K. pneumoniae*	4.33	2.28	1.54	2.78	4.50	3.74	6.24	− 1.54	19.84
	S. marcescens	6.32	2.51	1.47	3.19	4.00	3.16	8.60	− 2.52	55.34
	B. thuringiensis	7.95	2.67	2.01	3.26	3.88	3.20	6.62	− 3.40	45.47

Overlapping confidence intervals mean insignificant difference

the *B. thuringiensis* isolate would seem to be satisfactory and is, consequently, possibly helpful in biological control programs against RPW larvae. Despite the results of the other two isolates, *S. marcescens* and *K. pneumoniae* which appear appropriate in controlling RPW, the *B. thuringiensis* isolate has the priority to be chosen due to its accessibility, high efficiency and safety to humans.

In the earlier period, control endeavors by *Bt* failed mainly because of applying inadequate concentrations and/or variations in the potency of the formulations used. The outcomes of this work have displayed that, apart from suggesting the concentration that would be applied against a pest species, data on the pesticide tag should contain the exposure time needed to attain the requisite level for suppressing the target pest. Such data would allow farmers to choose the period in-between applications. Also, enhancing the application system of *B. thuringiensis* might raise the mortality rates of RPW.

Conclusions

Bioassays of *B. thuringiensis* were conducted under controlled laboratory conditions in this work. The results of different evaluations revealed that the larval mortality percentages varied according to the bacterial isolates and the concentrations reaching 100% mortality by *S. marcescens* after 5 days of treatment. Hence, further studies are required to be centered on the pertinence of the laboratory results to manage *B. thuringiensis* under field environments and to explore the insecticidal range and the application technology in the field to create a robust basis for future improvement and utilization.

Abbreviations

IMP: Integrated pest management; PCR: Polymerase chain reaction; ITS: Internal transcribed spacer; ANOVA: Analysis of variance; RPW: Red palm weevil.

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Author contributions

NMA contributed to conceptualization, methodology, formal analysis, and writing—original draft. KAA performed supervision and review and editing. KAMA-E helped in conceptualization, formal analysis, and review and editing. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable. This manuscript is in accordance with the guide for authors available on the journal's Web site. Also, this work has not been published previously and is approved by all authors and host authorities.

Consent for publication

Not applicable.

Competing interests

No potential competing interests was reported by the authors.

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