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Original Article

Isolation and Molecular Identification of *Serratia Nematodiphila* associated with Red Palm Weevil, *Rhynchophorus* Olivier (Coleoptera: *Curculionidae*) as bio-insecticide in Egypt

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Abstract

The red palm weevil (RPW), *Rhynchophorus ferrugineus* Olivier (Coleoptera: *Curculionidae*) is a major date palm pest. In this study, we aimed to isolate and identify the *Serratia nematodiphila* from RPW as potential biocontrol agents. We isolated the bacteria from infected RPW larvae and adults and identified using colony morphology characteristics, biochemical tests, and PCR followed by 16S rRNA sequencing. This is the first study reporting the *Serratia nematodiphila* as an extracellular symbiont of RPW from Egypt. The potential of this bacteria to be used as biocontrol agent was conducted by a screening bioassay through its effect on RPW eggs. The study noted that treated eggs were unable to hatch and not turned red in color, indicating the potential of this bacteria to be used as bio-pesticide. These results presented novel insights into the microbiome of RPW and suggest the potential of *Serratia nematodiphila* as a biocontrol agent for RPW management. Moreover, further studies are required to explore the mechanism and potential of these bacteria in field applications. Nevertheless, this study provides a promising direction for the development of sustainable and environmentally friendly RPW management strategies.

Keywords: Red Palm Weevils (RPW), *Rhynchophorus ferrugineus*, Coleoptera, *Curculionidae, Serratia nematodiphila*, Biocontrol agent

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Introduction

The date palm, *Phoenix dactylifera* L. belong to *Arecaeae* family is a fruit crop of tropical regions. Its cultivation has spread all over the world which is also known as oldest fruit tree planted in Middle Eastern countries, especially in the Arab Gulf states and Egypt. The Arab countries dominate the world's date production, while Egypt is the country that majorly producing dates worldwide. Egypt produces more than one million metric tons of fresh dates annually which accounts almost 18 % of all dates produced globally and 23 % of Arab production, with 1.590.414 million tons of the total estimated global production of nine million tons in 2017 (El-Sharabasy and Rizk, 2019).
Date palms are susceptible to many destructive pests, which negatively affect production as well as quality (Alatawi et al., 2018).
The RPW, *Rhynchophorus ferrugineus* (Olivier), is an important date palm pest around the world (Al-Ayedh, 2020; Munawar et al., 2020). Since mid-1980s, RPW known to be disseminated at large geographical area including Asia, America, Africa and Europe (EPPO, 2020). From its origin, it spread widely through the trade of palm trees and offshoots from infected areas. The larvae is the main destruction stage. It drills into palm trees and eats succulents, remain hidden for most of its life cycle. In 2009, the expected losses in Gulf Cooperation Council countries of the Middle East were estimated to be 5.18 to 25.92 million USD due to the severe infestation of date palms at a rate of 1.0% to 5.0% respectively (El-Sabea et al., 2009). The Food and Agriculture Organization of United Nations classified *R. ferrugineus* as a class-1 pest in the Middle East and North Africa (MENA) regions (El-Shafie and Faleiro, 2020) and A2 pest in the Europe (Faleiro et al., 2019). It poses a significant problem to the livelihood of date palm producers in rural areas of MENA**.**
There are various strategies that can be applied to control red palm weevil (RPW), including chemical, cultural, biological, and physical control methods. The chemical control comprises the use of insecticides, such as chlorpyrifos and deltamethrin, to kill RPW larvae and adults. However, cultural control involves various implementing measures such as pruning, sanitation, and trapping to reduce RPW infestations while physical prevention consists of practicing the physical barriers, for example sticky bands, to restrict RPW from climbing up the palm trees. The biological way of control uses the natural enemies, such as predators and parasites, to control RPW population. The use of all these control methods called as integrated pest management (IPM) which is a comprehensive approach to manage RPW populations sustainably. The selection of an appropriate control strategy depends on various factors, such as severity of RPW infestation, local environment, and economics.
Biological control is known as one of the best strategies in combating the red palm weevil because its less expensive, ecofriendly, minimum legal and public health concerns (Al-Dosary et al., 2016; Ramzan et al., 2023). As the biological control uses the vital live enemies which can be pathogens, predators, and parasites such as bacteria and fungi (Zhu et al., 2010; Pu and Hou, 2016; Abd-Elfatah et al., 2023). It provides advantageous conditions for the environment and economy since yield loss may be reduced without causing unfavorable natural effects, which can happen when pesticides are used (Bianchi et al., 2006). Entomopathogenic bacteria belong to the major microbial families which are *Bacillaceae*, *Streptococcaceae, Micrococcaceae*, *Enterobacteriaceae*, and *Pseudomonadaceae* (Kaya et al., 1993; Mehmood and Ashraf, 2023). Previously, *B. thuringiensis* subspecies kurstaki was isolated from RPW larvae in Egypt (Alfazariy, 2004), *Serratia marcescens* and *Serratia nematodiphila* in China (Zhang et al., 2009; Pu and Hou, 2016) was used to successfully control the RPW under laboratory settings. This is the first study isolated and identified the *Serratia nematodiphila* symbiotically associated with RPW, *Rhynchophorus ferrugineus* (Olivier) from Egypt and were assessed for bio-insecticide activity against *Rhynchophorus ferrugineus*.

Material and Methods

Red palm weevil collection and laboratory culture
RPW larvae, pupae, and adults were collected from infested date palms (*Phoenix dactylifera* L.) located in various areas of Qena governorate, namely, South Valley Agricultural farm, Qeft, and Qus, from April 2020 to August 2021. Adult RPW were reared in plastic boxes (20×15×10 cm) fitted with a perforated cover for ventilation. It was provided with pieces of peeled Sugarcane reeds for feeding and laying eggs to obtain the eggs and the following stages, such as larvae and pupae. The rearing was carried out under laboratory conditions at 25 °C and 65% HR. The larvae were provided with reed sticks that were

hollowed out for feeding, and it was possible to get the pupae, which were placed in the same boxes as before until the adult weevil emergence.

Isolation of bacteria

The bacteria were isolated from already collected RPW pupae from different geographical location. Two knots were taken from the bacteria inside the cocoon, and they were dissolved in about 5 ml of sterile distilled water. The amount of a knot was taken from it using a sterile inoculation loop, and streaked on nutrient agar plates (10 g peptone: 3 g meat extract, 5g NaCl, 15 g agar and 1000 ml distilled, pH 7.3). The plates were incubated in an incubator at a temperature of 29°C for 48 hours. Different single isolated colonies were selected and re-cultured for further purification and to observe colony characteristics following the same incubation conditions. They were re-cultured again to ensure the purity of the bacterial cultures. The resultant growth of different colonies was observed for individual characteristics such as colony texture, color, size, and transparency. The identified strains were stored first at 4ºC and then at -80 ºC in 25% glycerol for further processing.

16S ribosomal DNA (rDNA) based molecular identification

Extraction of bacterial DNA

The bacterial DNA was extracted in Microbial Genetics Department, Biotechnology Research Institute, National Research Centre, Cairo, Egypt from the fresh overnight grown bacterial culture using Qiagen genomic DNA kit (Qiagen, Shanghai, China) as per guidelines of manufacturer. The extracted DNA was stored at -20 ºC.

Identification by PCR amplification of the 16S rDNA gene

All extracted DNA was subjected to PCR amplification of 16S rDNA gene and later on sequencing. The PCR amplification of 16S rDNA gene was done using the universal primer as described earlier 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CTACGGCTACCTTGTTACGA-3'). The PCR solution (50µL) consists of 4 µL DNA as template, 2 µL of each 10mM forward and reverse primers, 25 µL of Master Mix and 17 µL of nuclease free water. The PCR were run under following conditions; first denaturation at 94 ºC for 3 minutes along with 35 cycles of denaturation at 94 ºC for 30 sec, annealing at 55 ºC for 30 sec, and initial extension at 72 ºC for 1 min with a final extension at 74°C for 10 min and stored at 4 ºC for infinite time. The PCR product yielded were analyzed on 1.0% (wt./vol) agarose gel after staining with ethidium bromide.

Agarose gel electrophoresis, purification, and sequencing

Agarose powder was dissolved in 1X TBE buffer at 1% and boiled in hot air oven for few minutes, then the ethidium bromide, gel staining dye was mixed in pre-heated gel solution when the temperature reaches approximately to 55°C. Then the gel was poured in the plastic tray and the comb was placed immediately and removed after solidification. Then the gel was placed in 1X TBE electrophoresis buffer filled in electrophoresis apparatus. The whole PCR product were loaded in each well and compared with 5 µl of 2500 bp DNA ladder. Later, the PCR product was extracted and purified from agarose gel using Wizard Genomic DNA Purification Kit (Promega, USA). The purified PCR product were sent for sequencing by HVD Life Science, Germany.

Phylogenetic analysis

Chromatograms were edited and trimmed using Bio Edit software. Edited sequences were aligned using Clustal X of Clustal W packages. The 16S rDNA sequence obtained were compared with highly similar publicly available sequences in NCBI database. All of the sequences were aligned and phylogenetic tree was constructed by using neighbor-joining (NJ) method in MEGA 11 (Kumar et al., 2018).

Bio-assay to check the insecticide activity on RPW eggs

The bacterial culture was mixed in sterile water and diluted into three different concentrations; 1×10^6, 1.5×10^6, 1×10^7 CFU/mL, which was further used in the whole bioassay. Sterile water was used as control group and the three bacterial concentrations were used as three treatments on RPW eggs. A total of 30 eggs were evaluated for each treatment and repeated thrice. Treatments entailed uniformly dosing 10 mL of the solution under test over the dish and its contents before adding 10 nondestructive, freshly laid eggs to a Petri dish (90 mm) with cotton on the bottom. Normally, it takes 3-5 days for RPW eggs to hatch, we counted the number of hatching larvae every day up to the seventh day. The larvae that had just hatched were swiftly

transferred to other petri dish to protect the other eggs from harm. The eggs were deemed non-viable if they did not hatch after seven days. By comparing the quantity of viable eggs before and after the administration of treatments, egg hatching rates were calculated.

Statistical analysis

The statistical analyses were performed using SPSS computer program (SPSS Inc., Chicago, IL, USA) at $\alpha=0.05$ (level of significance). The data of the egg hatching rate, adjusted mortality, and boring rate at the various dosages were compared using one-way ANOVA. Tukey's honestly significant difference (HSD) test was applied to compared the means within and between the groups.

Results

Isolation and colony characteristics of *Serratia nematodiphila*

Firstly, RPW larvae were detected by properties such as viscous layer on their surface and foul smelling. The surface was disinfected and washed with sterile Normal saline (0.9%) and isolation of bacteria was done on nutrient agar. The bacteria showed creamy color colonies, with smooth texture, bulging shape, irregular margins and transparent which are indicative of particular bacteria.

16S rDNA gene-based identification and sequence analysis

The 16S rDNA gene was amplified by PCR to identify the bacterial isolates. The PCR product was pictured on 1% agarose gel at almost 1400-1500 bp length. The amplified gene was sequenced using Sanger sequencing technique and it showed 100% similarity to already submitted sequence in NCBI Genbank under the accession number, NR_044385.1. The bacterial sequence was blasted in NCBI blast to query the related sequences in NCBI database to identify clonal relationship (Table 1). Chromatograms were edited and trimmed using Bio Edit software (Fig. 1). The phylogenetic tree was constructed using Neighbor-Joining method in MEGA-11 by aligning the sequences through Clustal W (Fig. 2). The phylogenetic tree showed that the isolated strain was belonging to different clade as compared to similar species under the same genus but there was a high degree of similarity in their genomes (≥95%). Apart from this, the sequence was also greatly similar, 95.6% to *Pectobacterium spp.*, *Coronobacter dublinensis subspp. Lausanensis*, *Citrobacter murliniae*, and *Enterobacter Kobei* strains.

Table 1. BLAST result of 16S rDNA gene sequence of *Serratia nematodiphila*

Bacterial Strains	Max Score	Total Score	Query Cover	E value	Percent Identity	Accession
Serratia nematodiphila	1589	1589	90%	0.0	98.03%	NR_044385.1
Serratia marcescens subsp. marcescens, JCM 1239	1587	1587	90%	0.0	97.93%	NR_113236.1
Serratia marcescens subsp. marcescens, DSM 30121	1578	1578	90%	0.0	97.82%	NR_041980.1
Serratia surfactantfaciens, YD25	1567	1567	90%	0.0	97.60%	NR_169468.1
Serratia ureilytica, NiVa 51	1533	1533	90%	0.0	96.94%	NR_042356.1
Serratia entomophilia, DSM 12358	1478	1478	90%	0.0	95.86%	NR_025338.1
Serratia ficaria strain DSM 4569 16S ribosomal RNA, partial sequence	1472	1472	90%	0.0	95.75%	NR_041979.1
Serratia ficaria, NBRC 102596	1469	1469	90%	0.0	95.64%	NR_114155.1
Serratia ficaria, JCM1241	1467	1467	90%	0.0	95.64%	NR_112005.1
Pectobacterium aroidearum, SCRI 109	1467	1467	90%	0.0	95.63%	NR_159926.1
Serratia odorifera, PADG 1073	1461	1461	90%	0.0	95.52%	NR_037110.1

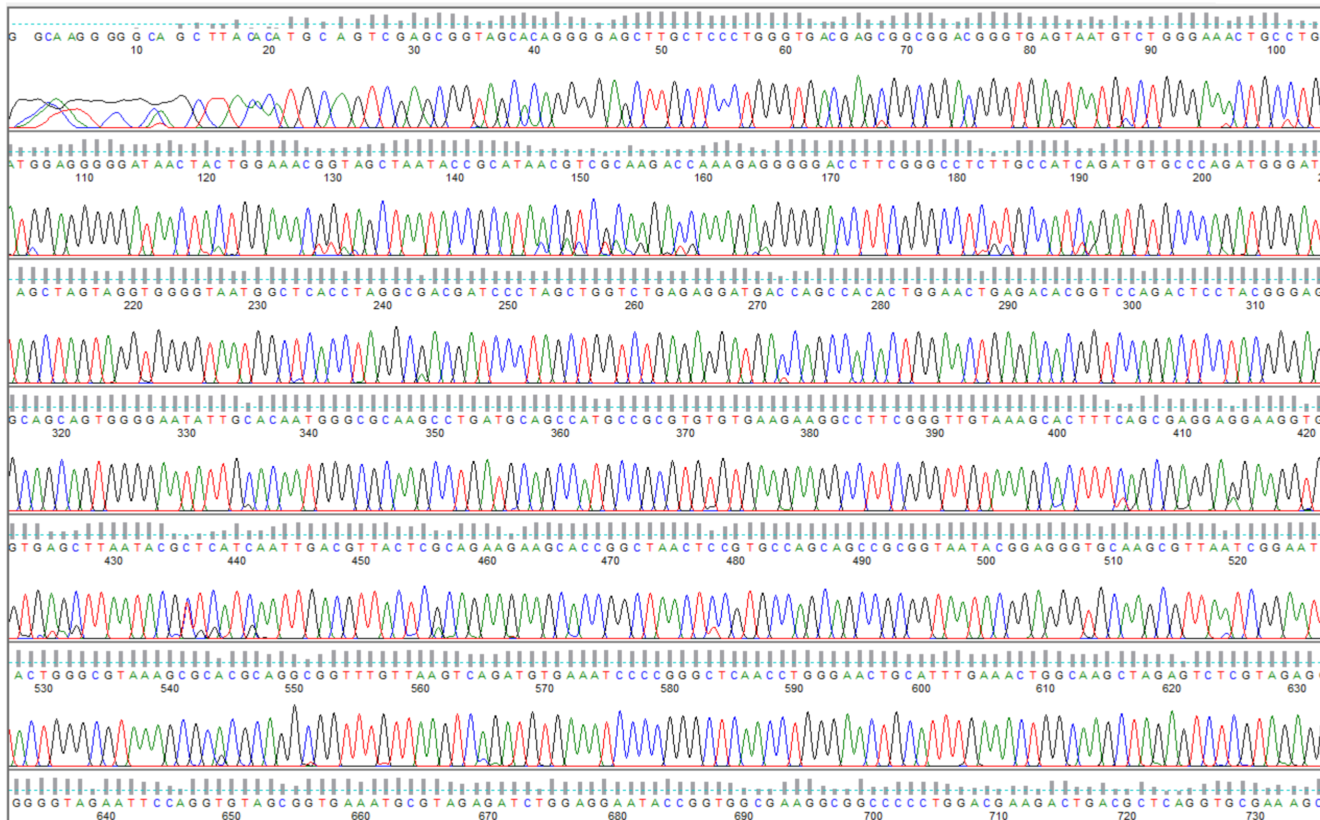

Figure-1. Chromatogram of the 16S rDNA gene sequence of isolated *Serratia nematodiphila*

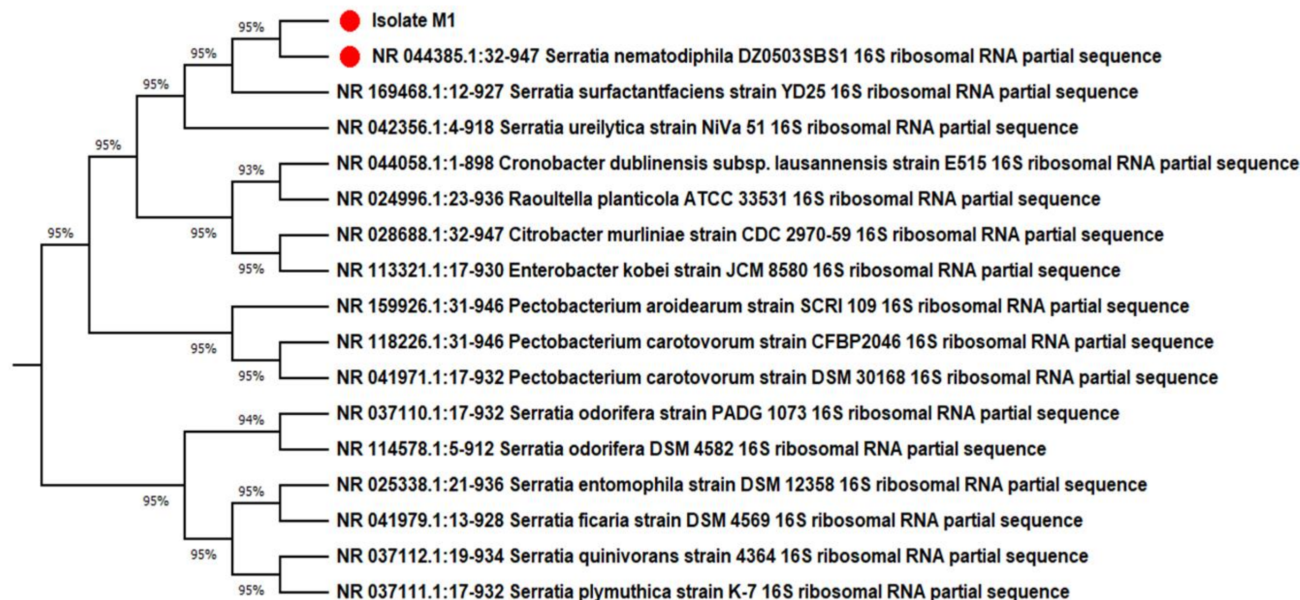

Figure-2. Phylogenetic tree based on partial 16S rDNA sequences retrieved from NCBI database, showing the association between *Serratia nematodiphila* and other species.

Bioassay to check the insecticide activity

The effect of *Serratia nematodiphila* on RPW eggs was observed, as soon as the bacterial concentrations applied, red color appeared on eggs after 3 days (Fig. 3b). After application of different bacterial isolate concentrations sprayed on RPW eggs, hatching rates were significantly affected ($F_{3,8}$ =161.222, *P*= 0.000, Table 2). As the bacterial concentration increased, there was a tendency for fewer eggs to hatch (Table 3). The egg's average hatching rate were ranged between 3.3 and 8.67 which was considered as significant difference ($p<0.05$). The eggs with the highest response rate to effectively dying were with 1×10^7 CFU/mL concentration. In contrast, there was a noticeable difference between the other various effective concentrations (Fig. 4A). Similar pattern was observed against larvae (Fig. 4B).

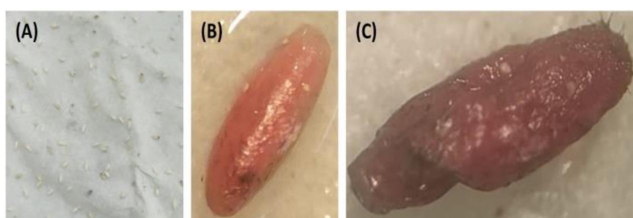

Figure-3. RPW eggs (A) Control group 0X, (B) treated eggs after 3 days 40X, (C) treated eggs after 5 days 40X.

Table-2. One-Way ANOVA test results

Treatments	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	120.917	3	40.306	161.222	0.000
Within Groups	2.000	8	0.250	-	-
Total	122.917	11	-	-	-

Determination of LC_{50} and LC_{90} of different biological agents against RPW eggs and larvae

The results of present study revealed the susceptibility of different biological agents to red palm weevil eggs and larvae, emphasizing key parameters such as LC_{50}, Index, Resistance Ratio (RR), Slope with uncertainty, and LC_{90}. The LC_{50} of *Serratia nematodiphila* egg showed that it was more susceptible to the treatment compared to other microorganisms listed in Table 3. This suggests that *Serratia nematodiphila* may have fewer defense mechanisms or a higher inherent vulnerability to the treatment compared to the other species. On the other hand, *Aspergillus flavus*, noted to have a more robust defense mechanism, a slower metabolic rate, or a lower uptake of the treatment. The other species, *Metarhizium anisopliae*, *Beauveria bassiana*, and *Trichoderma harzianum* exhibited varying degrees of susceptibility between these two species. Overall, the study provides insight into the varying responses of different species to the treatment (Table 3).

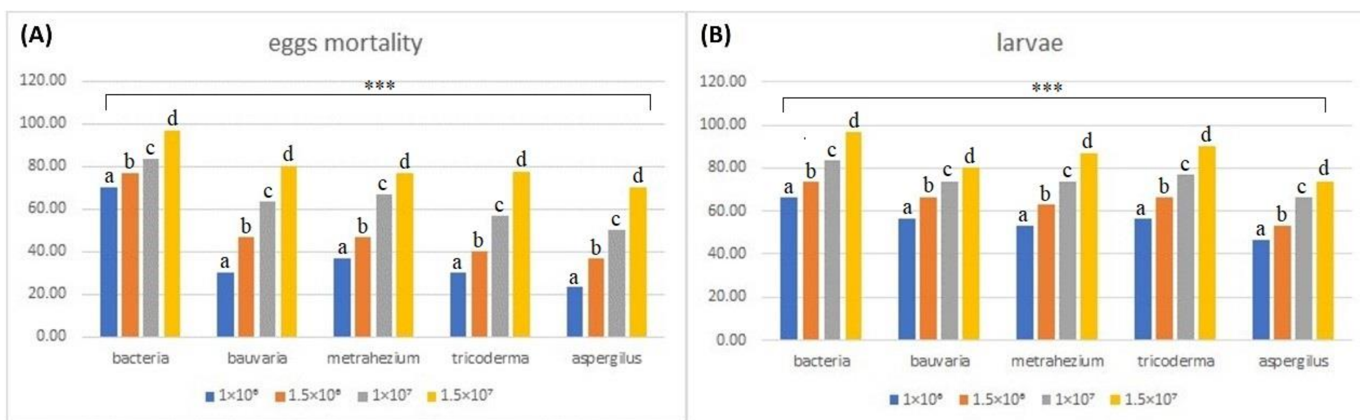

Figure-4. Effect of different concentrations of different biological agents on the (A) eggs and (B) larvae of RPW; Different superscripts on bar graphs indicate significant difference (*p<0.05*) within the group while asterisk sign (***) indicate significant difference between the groups.

Table- 3. LC_{50} and LC_{90} of different biological agents against RPW eggs

Biological agents	LC_{50}	Index	RR	Slope	Slope +/-	LC	LC_{90}
Serratia nematodiphila	253770	100	1	0.722	0.159	2.54E+05	1.51E+07
Metarhizium anisopliae	3266100	7.77	12.87	0.859	0.146	3.27E+06	1.01E+08
Beauveria bassiana	3931000	6.456	15.49	1.052	0.149	3.93E+06	6.50E+07
Trichoderma harzianum	4937200	5.14	19.455	0.953	0.149	4.94E+06	1.09E+08
Aspergillus flavus	5138300	4.939	20.248	1.207	0.155	5.14E+06	5.92E+07

LC_{50}= LC denotes lethal concentration of any drug or treatment which will results in 50% death of specie/insect/animal while LC_{90} indicates 90% death rate. RR denotes the resistance ratio which indicate the resistance exhibited by microorganism to a specific drug or biological agent.

Table-4. LC_{50} and LC_{90} of different biological agents against RPW larvae

Biological agents	LC_{50}	Index	RR	Slope +/-	LC_{90}
Metarhizium anisopliae	330350	119.261	0.838	0.491±0.147	1.35E+08
Serratia nematodiphila	393980	100	1	0.796±0.157	1.60E+07
Trichoderma harzianum	774210	50.888	1.965	0.76±0.149	3.77E+07
Aspergillus flavus	1826800	21.567	4.637	0.569±0.142	3.26E+08
Beauveria bassiana	2973800	13.248	7.548	0.337±0.155	1.89E+10

LC_{50}= LC denotes lethal concentration of any drug or treatment which will results in 50% death of specie/insect/animal while LC_{90} indicates 90% death rate. RR denotes the resistance ratio which indicate the resistance exhibited by microorganism to a specific drug or biological agent.

Notably, *Metarhizium anisopliae* exhibited a significantly lower LC_{50} (330350) with a high Index (119.261), suggesting superior efficacy. *Trichoderma harzianum* showed a moderate Index (50.888) and RR of 1.965, indicating reasonable effectiveness. *Aspergillus flavus* and *Beauveria bassiana* demonstrated higher LC_{50} values and substantial RR, implying reduced potency against red palm weevil larvae (Table 4). These findings underscore the importance of assessing multiple parameters for a comprehensive evaluation of biological agents in pest management strategies.

Discussion

Previously, a great attention has been applied to control agriculture pests using various microbes such as fungi (Zhong and Chen, 2012; Buentello-Wong et al., 2015). Few researches have been conducted to evaluate the role of bacterial agents as biocontrol against RPW. To our knowledge, this is the first study which confirm the presence of the external symbiotic bacteria, *Serratia nematodiphila* specie associated with RPW. The genus *Serratia* includes almost 10 species and they are well known as red-pigmented bacteria because of a chemical called as Prodigiosin. Some of its species have a potential entomopathogenic role in control of pests such as *Serratia marcescens* (Singh et al., 2008). Moreover, traditional biocontrol microbes include and limited to Metarhizium, Bacillus, Beauveria, nematodes and polyhedrosis viruses. The bacterial strain, *Serratia nematodiphila* will provide a new research horizon to be used as biocontrol agent against RPW in Egypt to eradicate *Rhynchophorus ferrugineus* (RPW).

The bacterial strain was isolated and identified successfully through microbiological and molecular techniques such as PCR and sequencing. The phylogenetic tree showed a close relationship with other Serratia species as shown in Fig 2. Moreover, the bioassay demonstrated the effect of this bacterial strain at different concentrations on the hatching of RPW eggs. Our findings demonstrated that the uppermost concentration (1×10^7 CFU/mL) decreased the hatching of eggs by 13.3%. As compared to the findings of previous researches, our findings proved that the *Serratia nematodiphila* can control the hatching the eggs which will avoid the date palm infestation from RPW larvae. Most previous studies concentrated on isolation, identification, but few studies focused on *Serratia* entomopathogenic effect on insects such as *S. marcescens* cell-free culture supernatant effected anti-feeding activity significantly and mortality to *P. Blanchard* (Pineda-Castellanos et al., 2015). *Serratia marcescens* causes the insect death. Previously, it was confirmed that *S. marcescens*

had significant insecticidal effect on the larvae of *S. exigua* and causes high mortality (Konecka et al., 2019). Another study conducted by isolated *S. marcescens* and *Serratia nematodiphila* from mangoes also present its anti-fungal activity (Trejo-López et al., 2022) and its reported previously that Serratia species produce a chemical compound known as prodigiosin which has good antimicrobial activity (Darshan and Manonmani, 2015; Lapenda et al., 2015).

In present study, five different microbial species were tested for their entomopathogenic effect on RPW eggs hatching rate and larvae death. It was noted that *Serratia nematodiphila* exhibited higher antimicrobial activity in terms of eggs hatching and larvae deaths as compared to other microbial species. Among the other microbial species, *Beauveria bassiana* exhibited higher antimicrobial activity as compared to *Metarhizium anisopliae* > *Trichoderma harzianum* >*Aspergillus flavus* on the hatchability of eggs. These results are in consistent with the findings of El Husseini (2019) that *Beauveria bassiana* causes the high mortality of RPW at different stages of life cycle. Another study conducted by Cito et al. (2014) studied the biochemical and antimicrobial activities of two *Metarhizium* strains, *Metarhizium anisopliae* and *Metarhizium pingshaense*. Both of the strain showed entomopathogenic activity by producing toxins and degrading proteases. Similarly, the pathogenic activity of *Trichoderma harzianum* as biocontrol agent has also been studied against other pathogens (Alahmadi et al., 2012; Sundaravadivelan and Padmanabhan, 2014) but not too much studies are reported. While in case of larvae deaths, the antimicrobial efficacy was in following order; *Trichoderma harzianum* > *Metarhizium anisopliae* > *Beauveria bassiana* > *Aspergillus flavus*. Among all these species, *Aspergillus flavus* showed lowest antimicrobial activity. *Aspergillus flavus* can also be the potential candidate for biological control of RPW as studied previously in Hail, Northern Saudi Arabia (Alanazi et al., 2020). These findings suggest *Serratia nematodiphila* as potential candidate for biological control of RPW in Egypt.

However, the bioassay was conducted in strictly controlled laboratory conditions. The variation between current study findings may be due to different bacterial strains, different environmental conditions, and restricted research on current bacterial strains worldwide. Therefore, more studies are needed evaluate the bio-insecticidal efficacy of *S. nematodiphila* in field trials in addition to investigate the toxicity and applications to develop a solid basis for future utilization and development at large scale. Another concern which need to be studied in future is to evaluate the toxicity effect on non-target species such as humans and animals. It is of much concern to check their effects on non-target species to develop highly specific, less toxic and efficient microbial agents not only to control RPW but also other invasive species such as coleopteran pests.

A limitation of the current study is that the bioassays demonstrating insecticidal efficacy of *Serratia nematodiphila* were conducted under controlled laboratory conditions. While the results are promising, further studies are needed to evaluate the biocontrol potential of this bacterium under real-world field conditions against RPW infestations in date palm plantations. Additional factors like environmental influences, application methods, and persistence in the field may impact the insecticidal activity observed in this lab investigation. Field trials will be important to validate the potential of *Serratia nematodiphila* as a viable biopesticide for managing RPW in agriculture. This could be highlighted as an area for future investigation. Identification of bacterial diversity that are pathogenic to insects can lead to development of new biocontrol agents and our research reports highlighted the role of newly identified bacteria against RPW with high pesticide activity which make it a as future candidate for further research as a new bio-insecticide and recommends field studies.

Conclusion

The protracted utilization of chemical pesticides is discouraged due to its potential harm to beneficial insects, contribution to environmental pollution, and the development of resistance in *R. ferrugineus* populations, rendering pest management more challenging. Consequently, there is an urgent need for alternative and integrated pest management approaches that effectively addresses the *R. ferrugineus* infestation along with environmental safety. Such approaches may encompass biological control strategies, such as the deployment of natural predators or the utilization of pheromone traps to disrupt the mating patterns. Moreover, raising awareness among the garden managers and general population regarding the significance of early detection and preventative measures is pivotal in curtailing the further spread of this invasive species.

Further investigation is necessary to better comprehend the extent of its impact.

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Contribution of Authors

Ali MA: Conceptualization, methodology and original draft preparation
Mahmoud MAB: Project administration, data curation and software input
Shoaib M, Bhutta ZA & Rajeh N: Visualization, writing, reviewing and editing of manuscript
Ali NM & Asfour HZ: Formal analysis, writing, reviewing and editing of manuscript
Ali N & Eletmany MR: Funding acquisition, writing, reviewing and editing of manuscript

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