Isolation, Identification, and Biocontrol Potential of Entomopathogenic Nematodes and Associated Bacteria against *Virachola livia* (Lepidoptera: Lycaenidae) and *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae)

Saqer S. Alotaibi 1,*, Hadeer Darwish 1, Madiha Zaynab 2, Sarah Alhaththi 3, Akram Alghamdi 4, Amal Al-Barty 4, Mohd Asif 5, Rania H. Wahdan 6, Alaa Baazeem 4 and Ahmed Noureldeen 4

1 Department of Biotechnology, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia; hadeer@tu.edu.sa
2 Shenzhen Key Laboratory of Marine Bioresource & Eco-Environmental Sciences, College of Life Sciences and Oceanography, Shenzhen University, Shenzhen 518107, China; madiha.zaynab14@gmail.com
3 Department of Chemistry, College of Science, Taif University, P.O. BOX 11099, Taif 21944, Saudi Arabia; sarah.alhaththi@tu.edu.sa
4 Department of Biology, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia; a.alghamdi@tu.edu.sa (A.A.); aalbarty@tu.edu.sa (A.A.-B.); aabazeem@tu.edu.sa (A.B.); a.noureldeen@tu.edu.sa (A.N.)
5 Regional Ayurveda Research Institute (CCRAS), Ranikhet 263645, Uttarakhand, India; asifgc2616@gmail.com
6 Agricultural Research Center, Department of Nematode Diseases Research, Plant Pathology Research Institute, Giza 12619, Egypt; d.rwahdan44@gmail.com
* Correspondence: saqer@tu.edu.sa

Simple Summary: For sustainable agriculture, there is a need to identify and evaluate more biocontrol agents, including entomopathogenic nematodes (EPNs). In this study, EPNs and their associated entomopathogenic bacteria symbionts (EPBs) were isolated and identified from 320 soil samples collected in Taif, Saudi Arabia. The biocontrol efficacy of EPNs and EPBs was also evaluated against third instar larvae of the pomegranate butterfly, *Virachola livia*, and the carob moth, *Ectomyelois ceratoniae*, two important insect pests of pomegranate, under laboratory conditions. Our results show that the EPNs *Steinernema* spp. were more virulent than *Heterorhabditis* spp. against the two pomegranate insects. In addition, the EBP *Stenotrophomonas maltophilia* CQ1, isolated from *Steinernema* spp., surpassed *Pseudomonas mosselii* SJ10, associated with *Heterorhabditis* spp., in their ability to kill *V. livia* or *E. ceratoniae* larvae. We conclude that either application of EPNs’ infective juveniles (IJs) or their associated EPBs could serve as potential biocontrol agents for *V. livia* and *E. ceratoniae*.

Abstract: *Virachola livia* (Lepidoptera: Lycaenidae) and *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae) are the key pests of pomegranates in Saudi Arabia that are managed mainly using broad-spectrum pesticides. Interactions between the entomopathogenic nematodes (EPNs) *Steinernematids*, and *Heterorhabditids*, and their entomopathogenic bacterial symbionts (EPBs) have long been considered monoxenic 2-partner associations responsible for killing insects and, therefore, are widely used in insect pest biocontrol. However, there are limited reports identifying such organisms in Taif, Saudi Arabia. The current study aimed to identify the EPNs and their associated bacteria isolated from Taif, Saudi Arabia, and evaluate their biocontrol potential on third instar larvae of *V. livia* and *E. ceratoniae* under laboratory conditions. A total of 35 EPN isolates belonging to *Steinernema* (20) and *Heterorhabditis* (15) were recovered from 320 soil samples. Twenty-six isolates of symbiotic or associated bacteria were isolated from EPNs and molecularly identified as *Xenorhabdus* (6 isolates), *Photorhabdus* (4 isolates), *Pseudomonas* (7), or *Stenotrophomonas* (9). A pathogenicity assay revealed that *Steinernema* spp. were more virulent than *Heterorhabditis* spp. against the two pomegranate insects, with LC50 values of 18.5 and 13.6 infective juveniles (IJs)/larva of *V. livia* for *Steinernema* spp. and 52 and 32.4 IJs/larva of *V. livia* for *Heterorhabditis* spp. at 48 and 72 h post-treatment, respectively. Moreover, LC50 values of 9 and 6.6 IJs/larva (Steinernema spp.) and 34.4 and 26.6 IJs/larva...
(Heterorhabditis spp.) were recorded for E. ceratoniae larvae at 48 and 72 h post-treatment. In addition, the EPB Stenotrophomonas maltophilia CQ1, isolated from Steinernema spp., surpassed Pseudomonas mosselti SJ10, associated with Heterorhabditis spp., in their ability to kill V. livia or E. ceratoniae larvae within 6 h post-application, resulting in 100% mortality in both insects after 24 and 48 h of exposure. We conclude that either application of EPNs’ IJs or their associated EPBs could serve as potential biocontrol agents for V. livia and E. ceratoniae.

**Keywords:** entomopathogenic nematodes; entomopathogenic bacteria; Virachola livia; Ectomyelois ceratoniae; biological control

---

1. **Introduction**

Nematodes that parasitise insects, known as entomopathogenic nematodes (EPNs), have been identified within 23 nematode families [1]. For several years, EPNs belonging to the Steinernematidae and Heterorhabditidae (Rhabditida) families have received the most attention as potential biocontrol agents because of their wide host spectrum, active host-seeking, easy mass production, low cost, long-term efficacy, easy application, compatibility with most chemicals, environmental safety for humans and other non-target organisms, capacity to reduce pesticide residues in food, and ability to increase activity of other natural enemies and increase biodiversity in managed ecosystems [2]. Photorhabdus and Xenorhabdus are Gram-negative bacteria of the family Enterobacteriaceae and are symbiotically associated with the entomopathogenic nematodes Heterorhabditis and Steinernema, respectively [3]. The infective juveniles (IJs) of Steinernematid and Heterorhabditid nematodes carrying symbiotic bacteria in their midguts live in the soil of diverse ecological systems [4]. These nematodes actively seek insect hosts, penetrating through an insect’s mouth, anus, or spiracles to reach the hemocoel, where symbiotic bacteria (e.g., Xenorhabdus spp. and Photorhabdus spp.) are released [5]. Subsequently, the symbiotic bacteria colonise the insect haemolymph, degrade insect tissues, and produce several immunosuppression factors, including toxin complexes, hydrolytic enzymes, and haemolytic and antimicrobial compounds that kill insect hosts, typically within 48 h [6,7]. Finally, the symbiotic bacteria replicate rapidly and cause septicaemia in insects [8]. This process converts the insect cadaver into suitable food for nematode development and reproduction. However, several studies have cast doubt on this view because some entomopathogenic pairs have been found to have attenuated virulence or to be non-virulent when injected alone into insects [9–11]. EPNs have been used against soil pests such as white grubs, root weevils, rootworms, sciarid flies, cutworms, and armyworms [12]. Satisfactory results concerning the effects of EPNs and/or EPBs in the laboratory and under field conditions have been recorded for controlling cherry fruit flies and mosquitoes as well as the pomegranate aphid, cabbage worm, and scarab beetle [13–15]. The protease inhibitor protein encoded gene from strains BJFS526 and Xbpi-1 of the symbiotic bacterium Xenorhabdus bovienii has been identified and expressed. The effects of this protein on pea aphids (Acyrthosiphon pisum) have also been investigated [16,17]. Fuchs et al. [18] reported that Xenorhabdus szenitirmai is a unique source of highly efficient antimicrobial peptides against almost all known plant pathogens. At present, EPNs have been identified in several geographical areas, including approximately 100 species of Steinernema and 26 species of Heterorhabditis [19–24]. However, the diversity and application of EPNs and their symbionts have not been thoroughly studied in several countries, including Saudi Arabia. Therefore, many species and strains of such potential control organisms must be evaluated to identify new biological control agents. In 2018, Noureldeen [25] isolated an EPN species, Steinernema sp., from the rhizosphere of pomegranate trees in Taif, Saudi Arabia and evaluated the genetic diversity among the Saudi Arabian and Egyptian EPNs’ genotypes via RAPD and ISSR markers.
Pomegranate cultivation and its potential yield and quality are affected by many insect pests. The most problematic insect pests for pomegranates are aphids, caterpillars, fruit flies, and leaf-footed bugs [26,27]. The pomegranate butterfly, *Deudorix (= Virachola) livia* (Lepidoptera: Lycaenidae), and the carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae), are major Lepidopteran insect pests of pomegranate. Their larvae feed on the seeds, causing serious damage in many countries, including Saudi Arabia [28], Iran [29], Tunisia [30], Egypt [31], Oman [32,33], and Jordan [34]. *Virachola livia* is also reported as a pest of the date palm in Morocco [35], Saudi Arabia, Egypt, and Tunisia. In Tunisia, the yearly damage rate caused by *E. ceratoniae* to pomegranate fruits ranges from 29–72% [36], whereas the economic loss caused by this pest in Iran is 30–80% [29]. Because the larval activity of both insects is unknown, commercial insecticides are not efficient against them [37]. When pomegranates are heavily treated with pesticides, the pesticide residues are mainly concentrated in the fruits. Given that these fruits are consumed raw, contamination with pesticides is undesirable [38]. The objectives of this study were to isolate and identify EPNs and their associated symbiotic bacteria from Taif, Saudi Arabia, and to evaluate the activity of EPNs/EPBs against the pomegranate butterfly (*V. livia*) and the carob moth (*E. ceratoniae*) under laboratory conditions.

2. Materials and Methods

2.1. Sampling and Isolation of Entomopathogenic Nematodes (EPNs)

Taif is a high-altitude region in Saudi Arabia, which includes high mountains, agricultural plates, and valleys, expected to be rich in EPN and EPB fauna because of an abundance of insect hosts. In this study, a total of 320 soil samples were randomly collected from 10 soil localities in the Taif governorate, either from cultivated sites with pomegranate, grapevine, mango, apple, fig, citrus, and rose crops, or uncultivated ones. At each site, 32 soil samples (500 g each) were collected using a hand shovel, placed into a plastic container, labelled with vegetation and date, and then transferred under ambient temperature to the laboratory for the isolation of EPNs. The soil collection process was performed as described by Khashaba et al. [39]. The soil samples were processed using a baiting technique [40] with *Galleria mellonella* larvae followed by a modified White trap [41] to isolate EPNs. The infective juvenile stages (IJs) of the EPNs emerged from the *G. mellonella* cadavers and moved to water. The IJs were collected daily in a culture flask, kept in distilled water, and stored at 15°C in a refrigerator. To increase the population size and confirm the pathogenicity of EPNs, a fresh *G. mellonella* larva was infected with IJs. The isolated nematodes were morphologically identified as *Steinernema* spp. or *Heterorhabditis* spp. by examining the morphometrics of the IJs and first-generation males [42].

2.2. Isolation of EPN-Associated Microbiota

Symbiotic bacteria associated with EPNs were isolated from the haemolymph of dead *G. mellonella* larvae, which had been infected with the IJs of EPNs according to the method of Poinar and Thomas [43], as modified by Vitta et al. [44]. In summary, the dead larvae of *G. mellonella* were surface sterilised via washing with absolute ethanol for 1 min and placed in a sterile Petri dish to dry. Subsequently, the 3rd segments from the heads of *G. mellonella* larvae were incised by a sterile sharp needle to create an influx of the haemolymph containing symbiotic bacteria. A sterile loop was used to distribute and streak the haemolymph samples on NBTA medium (nutrient agar with 0.004% triphenyl tetrazolium chloride and 0.025% bromothymol blue) and incubated at 28°C for 48 h [14]. Bacteria were routinely grown every 24 h until pure isolated colonies were obtained. A single colony of each isolate of symbiotic bacteria was inoculated in 5 mL of Luria–Bertani (LB) broth and incubated with shaking at 220 rpm overnight at 28°C. For the bioassay experiments, the isolated bacterial cultures in LB broth were diluted in sterile distilled water to an OD$_{600}$ of 0.1 with a spectrophotometer. Finally, the resulting bacterial cell suspension concentration was adjusted to $1 \times 10^8$ colony-forming units (CFU) per mL. The bacteria were then stored at −80°C with 20% glycerol (v/v) for further study.
2.3. Identification of EPN-Associated Bacteria

In order to characterize the isolates, we selected only Isolate4 and Isolate16 for further molecular characterization. The genomic DNA of isolated bacteria was extracted from bacterial pellets using a bacteria genomic DNA miniprep kit (QIAprep Spin Miniprep Kit). The genomic DNA of bacteria was stored at −20 °C for further use in PCR. PCR-based analysis and 16S rRNA gene sequencing were performed to identify bacterial species [45] using the inter-universal primers 785F (GGATTAGATACCCTGGTA) and 907R (CCGT-CAATCMTTTRAGTTT).

2.4. Molecular Characterisation through Phylogenetic Tree Analysis

The 16Sr RNA sequence of each isolate was blast against the NCBI “16S ribosomal RNA sequence (Bacteria and Archaea)” database, and the top five targets were collected for further phylogenetic tree analysis. All 16S sequences were aligned using the MUSCLE program with 50 iterations. The aligned sequences were displayed in a CLC viewer. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura–Nei model [46]. The tree with the highest log likelihood (-1426.34) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with a superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 242 positions in the final dataset. Evolutionary analyses were conducted using MEGA7 [47] with 1000 bootstrap values.

2.5. Insects

Following methods by Alotaibi et al. [48], pomegranate butterfly (V. livia) and carob moth (E. ceratoniae) larvae were collected from commercial pomegranate orchards located in Taif, Saudi Arabia, and then reared on a wheat bran diet (300 g of wheat bran, 50 g of sugar, 120 mL of water, 130 mL of glycerol, and 9 g of yeast), whereas adults were fed with a 10% honey–water solution. The rearing room was adjusted to 25 ± 1 °C, a relative humidity of 60% and a light–dark cycle of 16 L:8 D.

2.6. Bioassays

2.6.1. Pathogenicity of EPNs

Five third instar larvae of the pomegranate butterfly or carob moth were placed individually in 5-cm-diam. Plates lined with a Whatman’s No. 2 filter paper. One millilitre of EPN suspension from each species (Steinernema spp. and Heterorhabditis spp.) was added directly by micropipette at rates of 160, 80, 40, 20, 10, and 5 IJs per insect larva. Five grams of pomegranate seeds were provided as food for the larvae. In the control treatment, 1 mL of distilled water was added to the plate. The plates were then covered and incubated in a controlled growth chamber under the same conditions described above. Subsequently, V. livia and E. ceratoniae larval mortalities were monitored daily for up to 4 days, and the dead larvae were dissected under the stereomicroscope to confirm the infections. Each treatment was replicated 5 times, and the entire experiment was conducted twice.

2.6.2. Pathogenicity of EPBs

In this experiment, two isolates of bacterial associations were selected for use in the bioassay. The bacteria, including Pseudomonas mosselii SJ10 associated with Heterorhabditis spp. and Stenotrophomonas maltophilia strain CQ1 associated with Steinernema spp., were used to determine the oral toxicity against V. livia and E. ceratoniae larvae. In summary, 5 g of pomegranate seeds were immersed in 2 mL of each bacterial suspension at concentrations of $1 \times 10^8$, $1 \times 10^6$, $1 \times 10^4$, and $1 \times 10^2$ CFU/mL for 30 sec. The treated seeds were then
picked up and placed in a plastic plate (5 cm) lined with filter paper (Whatman number 2). Then, 5 V. livia or E. ceratoniae larvae were put into the plastic plate, which was then covered and incubated as previously described. Equal proportions of distilled water were used as controls. Finally, the mortality rates of V. livia and E. ceratoniae larvae were recorded for 6, 12, 24, and 72 h following treatment. Each bioassay was repeated twice in five replicates, each on different dates.

2.7. Statistical Analysis
The data obtained are expressed in terms of mean ± standard error (M ± SE). Percente larval mortality was analysed using 2-way analysis of variance (ANOVA), with IJ rate or CFU concentration and exposure duration as independent factors, followed by Duncan’s multiple range test. All mortality data analyses were conducted using the Costat program (Version 6.45). Furthermore, data for LC_{50} and LC_{90} values were subjected to chi-square tests for both EPNs and EPBs using SPSS Version 23 (p < 0.05), in which p-values less than 0.05 indicate statistical significance.

3. Results
3.1. Isolation of Entomopathogenic Nematodes from Soil Samples
Out of the 10 soil sites from Taif Province, Saudi Arabia, seven tested positive for EPNs (Table 1). The positive sites were Thomala, Al-Haweyia, Bani-Malik, Al-Shafa, Taif University, Alsail Alkabir, and Garoah. Soil samples recovered from the Thomala region revealed the largest number of EPNs (10), followed by Al-Haweyia (7) and Al-Shafa (6). Of the 32 soil samples collected from Bani-Malik, five EPN isolates were recorded, whereas Taif University gardens revealed the presence of three EPN isolates. Moreover, two EPNs were present in the soil of two sites: Alsail Alkabir and Garoah (Table 1). Data presented in Table 2 show that out of the eight vegetation types examined, six were positive for Steinernema and five for Heterorhabditis isolates. Among the 320 soil samples, 35 (10.9%) were positive for Steinernema or Heterorhabditis (Table 1). This yielded 15 isolates (4.69%) of Heterorhabditis and 20 isolates (6.25%) of Steinernema. Most EPNs were isolated from the soil of the citrus rhizosphere (13 isolates), followed by the pomegranate (9 isolates) and the grapevine (7 isolates), with occurrence rates of 32.5%, 22.5% and 17.5%, respectively. Two EPN isolates were found in the rhizosphere soil of fig and mango vegetation, with occurrence rates of 5% each, whereas only one EPN isolate was recorded from rose and apple crop soil. Furthermore, none of the EPNs were isolated from the uncultivated soil (Table 2).

3.2. Isolation and Identification of EPBs through 16S rRNA Gene Sequencing
Based on colony morphology on NBTA agar, 26 isolates of EPN-associated bacteria were identified as Xenorhabdus (6 isolates), Photorhabdus (4 isolates), Pseudomonas (7) or Stenotrophomonas (9). The molecular characterization of Isolate4 and Isolate16 also validated our morphological identification as Pseudomonas sp. and Xenorhabdus sp. For instance, the Isolate4 showed a 99.419% identity match with Pseudomonas soli strain F-279 (NR_134794.1), Pseudomonas mosselii strain CFML 90-83 (NR_024924.1) and Pseudomonas entomophila L48 (NR_115336.1) (Figure 1). Similarly, Isolate16 showed identity (99% similarity) with Xenorhabdus sp. The phylogenetic tree analysis also confirmed that Isolate4 belonged to Pseudomonas sp., and Isolate16 belonged to Xenorhabdus sp.
Table 1. Characterization of the vegetation and the sampling sites.

| Location        | Vegetation                        | Entomopathogenic Nematodes |
|-----------------|-----------------------------------|-----------------------------|
| Thomala (32) *  | Citrus (6) *, Grapevine (7), Pomegranate (10), Uncultivated (9) | + (10) **                  |
| Al-Haweyia (32) | Fig (8), Mango (7), Citrus (10), Grapevine (7)                  | + (7)                       |
| Al-Mathnah (32) | Grapevine (13), Fig (9), Pomegranate (10)                       | -                           |
| Liyah (32)      | Mango (13), Apple (12), Uncultivated (7)                        | -                           |
| Bani-Malik (32) | Citrus (10), Grapevine (7), Pomegranate (10), Uncultivated (5) | + (5)                       |
| Al-Hada (32)    | Grapevine (6), Uncultivated (9), Roses (17)                     | -                           |
| Al-Shafa (32)   | Citrus (9), Uncultivated (6), Apple (17)                        | + (6)                       |
| Taif University (32) | Pomegranate (5), Citrus (5), Roses (19), Uncultivated (3) | + (3)                       |
| Alsail Alkabir (32) | Apple (11), Pomegranate (5), Fig (11), Roses (4), Uncultivated (1) | + (2)                       |
| Garoah (32)     | Fig (12), Mango (20)                                               | + (2)                       |

* Numbers between parentheses are the number of collected samples from each site and vegetation. ** Number between parentheses indicates the number of EPNs isolates recovered from each positive site.

Table 2. Occurrence of EPNs isolated from the rhizosphere of vegetation at Taif governorate, Saudi Arabia.

| Vegetation         | EPNs                  | Steinernema sp. | Heterorhabditis sp. | Total of EPNs Isolates | Frequency of Occurrence % |
|--------------------|-----------------------|-----------------|---------------------|------------------------|----------------------------|
| Citrus (40)        | + (7) *               | + (6)           |                     | 2 (13)                 | 32.5                       |
| Pomegranate (40)   | + (5)                 | + (4)           |                     | 2 (9)                  | 22.5                       |
| Grapevine (40)     | + (4)                 | + (3)           |                     | 2 (7)                  | 17.5                       |
| Fig (40)           | + (2)                 | -               |                     | 1 (2)                  | 5                          |
| Apple (40)         | -                     | + (1)           |                     | 1 (1)                  | 2.5                        |
| Mango (40)         | + (1)                 | + (1)           |                     | 2 (2)                  | 5                          |
| Roses (40)         | + (1)                 | -               |                     | 1 (1)                  | 2.5                        |
| Uncultivated (40)  | -                     | -               |                     | 0                      | 0                          |
| Total of positive vegetation | 6 (20)              | 5 (15)         | 11 (35)             |                         | 10.94                      |

* Numbers between parentheses are the number of positive samples.
3.3. Pathogenicity Assays

3.3.1. Virulence of EPNs

The insecticidal activity of the isolated EPNs and their associated bacteria against insect hosts was further evaluated to determine their capacity to be used as biological control agents. The data in Figure 2, A and B show that both *Steinernema* spp. and *Heterorhabditis* spp. had a highly significant effect on the mortality of *V. livia* larvae (*p* < 0.05). The results show that both nematode species increased mortality of *V. livia* larvae (*p* < 0.05) when compared to the control treatment, which recorded zero mortality at all exposure times. The *Steinernema* spp. isolate induced 100% mortality, and the *Heterorhabditis* spp. isolate induced 88% mortality at 160 IJs/larva and 96 h post-treatment. The results also show a direct, significant relationship between the mortality rates and both IJ concentration and exposure time (*p* < 0.05). Thus, as the IJ concentration and exposure time increased, the mortality rate increased. The overall mortality of *V. livia* larvae after treatment with 5 to 160 IJs of *Steinernema* spp./larva ranged between 8% and 100% (Figure 2A), whereas it ranged from 0% to 88% (Figure 2B) for *Heterorhabditis* spp.

The data in Figure 3, A and B show that third instar *E. ceratoniae* larvae were highly susceptible (*p* < 0.05) to both *Steinernema* spp. and *Heterorhabditis* spp.; they exhibited 100% mortality at 96 h post treatment. According to the results, *Steinernema* spp. surpassed *Heterorhabditis* spp. in inducing mortality in *E. ceratoniae* larvae. *Steinernema* spp. induced 100% larval mortality from 48 to 96 h exposure, compared with 88%, 96% and 100% mortality induced by *Heterorhabditis* spp. at 160 IJs/larva and the same exposure times, respectively. Compared to the control (0 mortality), an increase in larval mortality with the increase of IJ concentration and exposure time was also observed (Figure 3). At 24 h post treatment, *Steinernema* spp. isolates caused larval mortality rates ranging from 16% to 88% (Figure 3A). *Heterorhabditis* spp. did not cause larval mortality at a concentration...
of 5 IJs/larva, and exhibited virulence (8% mortality) up to 72% when applied at a range between 10 and 160 IJs/larva (Figure 3B).

As shown in Table 3, the data indicate that *Steinernema* spp. was significantly more efficient against *V. livia* larvae than *Heterorhabditis* spp. under laboratory conditions. The *Steinernema* spp. isolate exhibited lower LC$_{50}$ and LC$_{90}$ values of 43 and 352.8 IJs/larva, respectively, at 24 h; 18.5 and 208.1 IJs/larva, respectively, at 48 h; 13.6 and 97.9 IJs/larva, respectively, at 72 h; and 12.9 and 85.5 IJs/larva, respectively, at 96 h. However, for the *Heterorhabditis* spp. isolate, the LC$_{50}$ and LC$_{90}$ values were 118 and 586.2 IJs/larva, respectively, at 24 h; 52 and 359.6 IJs/larva, respectively, at 48 h; and 32.4 and 243.9 IJs/larva, respectively, at 72 h. At 96 h, it exhibited LC$_{50}$ and LC$_{90}$ values of 28.3 and 226 IJs/larva, respectively (Table 3).

Likewise, the data in Table 4 reveal that the *Steinernema* spp. isolate was more effective against *E. ceratoniae* than the *Heterorhabditis* spp. isolate, with LC$_{50}$ values at 24, 48, 72, and 96 h after treatment of 28.1, 9, 6.6, and 5.7 IJs/larva, respectively, for the former and 68.3, 34.4, 26.6, and 20.2 IJs/larva, respectively, for the latter. Similar results were obtained for the LC$_{90}$ values, which were significantly lower for *Steinernema* spp. than for *Heterorhabditis* spp. Furthermore, the data in Table 4 show that the highest degree of homogeneity in the *E. ceratoniae* larval response was observed after exposure to the *Heterorhabditis* spp. isolate, with a slope value of 0.119 at 72 h post-treatment. In contrast, the other tested IJ levels exhibited low slope values (Table 4), which indicates heterogeneity in the response of *E. ceratoniae* larvae to these levels.
Figure 3. Mortality percentage (mean ± SE) of the carob moth, *Ectomyelois ceratoniae*, exposed to different levels (5, 10, 20, 40, 80, and 160 IJ/Larva) of *Steinernema* spp. (A) and *Heterorhabditis* spp. (B) at different exposure times (24, 48, 72 and 96 h). Bars annotated with the same letter are not significantly different ($p < 0.05$, based on Duncan test).

Table 3. Pathogenicity of two EPNs against the pomegranate butterfly, *Virachola livia*.

| EPNs          | Exposure Time (h) | LC$_{50}$ IJ/Larva (95% LCL–UCL) | LC$_{90}$ IJ/Larva (95% LCL–UCL) | Slope ± SE | Intercept | $X^2$ | p-Value |
|---------------|-------------------|----------------------------------|----------------------------------|------------|-----------|-------|---------|
| **Steinernema**<br>spp. | 24                | 43 (29.8–66.3)                  | 352.8 (179.6–1254.9)             | 0.11 ± 0.02 | 4.94      | 0.74  | 0.007   |
|               | 48                | 18.5 (11.2–27.9)                 | 208.1 (106.3–793.4)              | 0.095 ± 0.019 | 9.4      | 1.56  | 0.008   |
|               | 72                | 13.6 (8.6–19.3)                  | 97.9 (59.6–238)                  | 0.103 ± 0.021 | 10.6    | 3.47  | 0.007   |
|               | 96                | 12.9 (8.2–18.2)                  | 85.5 (53.5–194.2)                | 0.101 ± 0.024 | 11.01   | 2.70  | 0.014   |
| **Heterorhabditis**<br>spp. | 24                | 118 (82.8–210.7)                 | 586.2 (295.2–2421.9)             | 0.092 ± 0.012 | 0.49    | 1.58  | 0.002   |
|               | 48                | 52 (37–79.9)                     | 359.6 (189.7–1177.3)             | 0.108 ± 0.022 | 3.8      | 3.87  | 0.008   |
|               | 72                | 32.4 (22.7–47.4)                 | 243.9 (134.3–720.1)              | 0.109 ± 0.024 | 6.1      | 1.75  | 0.011   |
|               | 96                | 28.3 (19.4–41.3)                 | 226 (124.2–674.6)                | 0.105 ± 0.025 | 7        | 0.80  | 0.014   |

LC$_{50}$—lethal concentration that kills 50% of insects; LC$_{90}$—lethal concentration that kills 90% of insects; LCL—lower confidence limit; UCL—upper confidence limit; $X^2$—chi-square value; SE—standard error; p-value—probability.
3.3.2. Virulence of EPN-Associated Bacteria

The data in Table 5 represent the toxicity of two species of bacteria—*Pseudomonas mosselii* SJ10 (associated with the EPN *Heterorhabditis* spp.) and *Stenotrophomonas maltophilia* CQ1 (associated with *Steinernema* spp.)—against *V. livia* larvae under laboratory conditions. Both EPB species significantly affected larval mortality (*p* < 0.05); however, percent larval mortality caused by *S. maltophilia* CQ1 (78.5%) was significantly higher than that caused by *P. mosselii* SJ10 (66%). The percentage of larval mortality increased significantly with bacterial cell concentration and exposure duration (*p* < 0.05). The interactive effect of EPB species, cell concentrations, and exposure time on larval infection was not significant (*p* = 0.9742). The highest mortality percentage (100%) was recorded in the plates where the larvae were exposed to *S. maltophilia* CQ1 at the rate of 10^8 CFU/mL distilled water after 24 and 48 h of application, and the lowest (32%) was recorded when the larvae were exposed to 10^2 CFU/mL of *P. mosselii* SJ10 6 h post-treatment (Table 5).

**Table 4.** Pathogenicity of two EPNs against the carob moth, *Ectomyelois ceratoniae*.

| EPNs          | Exposure Time (h) | LC50 IJ/Larva (95% LCL–UCL) | LC90 IJ/Larva (95% LCL–UCL) | Slope ± SE | Intercept | X^2     | p-Value |
|---------------|-------------------|----------------------------|----------------------------|------------|-----------|---------|---------|
| *Steinernema* | 24                | 28.1 (19.4–40.8)           | 217.5 (120.8–628.7)         | 0.11 ± 0.023 | 6.7       | 0.64    | 0.009   |
| *spp.*       | 48                | 9 (4.3–14.1)               | 97.7 (54.9–311.2)           | 0.085 ± 0.015 | 12.9     | 4.17    | 0.005   |
|              | 72                | 6.6 (3.1–10.2)             | 51.4 (32.2–123.8)           | 0.077 ± 0.021 | 14.9     | 5.87    | 0.021   |
|              | 96                | 5.7 (2.3–9.1)              | 47 (29.3–116)               | 0.07 ± 0.02  | 15.8     | 5.4     | 0.026   |

**Table 5.** Larvicidal activity of two bacterial species cells on the pomegranate butterfly, *Virachola livia*, under laboratory conditions.

| Bacterial Species | Concentration (CFU/mL) | 6 h | 12 h | 24 h | 48 h | Bacterial Species Means |
|-------------------|------------------------|-----|------|------|------|-------------------------|
| *Pseudomonas mosselii* | 10^2                   | 48 ± 4.9 i | 48 ± 4.9 i | 56 ± 7.5 ghi | 64 ± 7.5 efg | 66 b |
|                   | 10^4                   | 52 ± 4.9 hi | 60 ± 9 fgh | 68 ± 4.9 ef | 80 ± 0 cd |                   |
|                   | 10^6                   | 60 ± 6.3 fgh | 72 ± 4.9 de | 80 ± 0 cd | 84 ± 4 bc |                   |
|                   | 10^8                   | 64 ± 9.8 efg | 68 ± 4.9 ef | 80 ± 6.3 cd | 88 ± 4.9 bc |                   |
| *Stenotrophomonas maltophilia* | 10^2                   | 48 ± 4.9 i | 52 ± 4.9 hi | 56 ± 7.5 ghi | 72 ± 4.9 de | 78.5 a |
|                   | 10^4                   | 72 ± 4.9 de | 80 ± 0 cd | 84 ± 4 bc | 88 ± 4.9 bc |                   |
|                   | 10^6                   | 72 ± 4.9 de | 80 ± 0 cd | 88 ± 4.9 bc | 92 ± 4.9 ab |                   |
|                   | 10^8                   | 84 ± 4 bc | 88 ± 4.9 bc | 100 ± 0 a | 100 ± 0 a |                   |
| Control           | 0 ± 0 k                | 0 ± 0 k | 0 ± 0 k | 0 ± 0 k | 0 ± 0 k | 0 c |

* Exposure Time Means | 40.3 d | 45.7 c | 51 b | 55.7 a |

* Each treatment in this experiment was represented by five replicates, each with five larvae insects. ** Numbers in each column indicate mortality ± standard error. Means with different letters within the same column or row differ significantly (*p* < 0.05 using Duncan’s Multiple Range Test).
The data in Table 6 show the toxicity levels of the EPBs. *S. maltophilia* CQ1 was more effective than *P. mosselii* SJ10. After 6 and 12h of exposure against *V. livia* larvae, *S. maltophilia* CQ1 exhibited LC$_{50}$ values of $1.26 \times 10^2$ and $5.01 \times 10^1$ CFU/mL, respectively, whereas *P. mosselii* SJ10 exhibited LC$_{50}$ values of $1.26 \times 10^4$ and $7.94 \times 10^2$, respectively. However, at 48 h of exposure, no significant difference was detected between the LC$_{50}$ values of these bacterial species. *Stenotrophomonas maltophilia* CQ1 exhibited LC$_{50}$ value of 6.31 CFU/mL compared with 10 CFU/mL for *P. mosselii* SJ10. It is also clear that both EPBs exhibited high slope values (more than 2), which indicates homogeneity in the response of *V. livia* larvae to these bacteria (Table 6).

**Table 6.** Lethal concentrations of *Pseudomonas mosselii* and *Stenotrophomonas maltophilia* on the pomegranate butterfly, *Virachola livia*, under laboratory conditions.

| Bacterial Species | Exposure Time (h) | LC$_{50}$ CFU/mL (95% LCL–UCL) | LC$_{90}$ CFU/mL (95% LCL–UCL) | Slope ± SE | Intercept | $X^2$ | $p$-Value |
|-------------------|-----------------|-------------------------------|-------------------------------|------------|-----------|------|--------|
| *Pseudomonas mosselii* |
| 6                 | 4.1 (1.7–7.2)   | 15 (13–17.9)                 | 2.0 ± 0.42                    | 2.6        | 0.123     | 0.019 |
| 12                | 2.9 (0.5–4.4)   | 13.3 (10.4–15.6)             | 2.1 ± 0.61                    | 4.2        | 0.087     | 0.044 |
| 24                | 1.5 (0–2.8)     | 11.3 (7.7–13.2)              | 2.3 ± 0.74                    | 5          | 0.202     | 0.052 |
| 48                | 1 (0–2.2)       | 9.4 (5.4–11.5)               | 2.5 ± 0.88                    | 6          | 0.056     | 0.069 |
| *Stenotrophomonas maltophilia* |
| 6                 | 2.1 (0.3–3.2)   | 13.4 (7.5–15.8)              | 2.4 ± 0.65                    | 4.2        | 0.612     | 0.034 |
| 12                | 1.7 (0.3–2.7)   | 9.1 (5.8–14)                 | 2.6 ± 0.75                    | 4.8        | 0.689     | 0.043 |
| 24                | 1.3 (0.2–2.1)   | 5 (3.6–8.4)                  | 2.8 ± 0.87                    | 6          | 2.04      | 0.051 |
| 48                | 0.8 (0–1.7)     | 3.9 (1.9–6.5)                | 2.7 ± 1.1                     | 7.4        | 1.49      | 0.089 |

LC$_{50}$—lethal concentration that kills 50% of insects; LC$_{90}$—lethal concentration that kills 90% of insects; LCL—lower confidence limit; UCL—upper confidence limit; $X^2$—chi-square value; SE—standard error; $p$-value—probability. * Each figure is represented as a power of 10.

Similarly, as shown in Tables 7 and 8, both *S. maltophilia* CQ1 and *P. mosselii* SJ10 bacteria successfully induced mortality in *E. ceratoniae* larvae ($p < 0.05$). The data also indicate that the mortality rate had a direct relationship with the exposure time and bacterial CFU concentration ($p < 0.05$). The regression analysis of the data shows that the mortality of *E. ceratoniae* larvae significantly increased with increasing bacterial concentration ($R^2 = 0.9743$; $p < 0.05$). Maximum (58.7%) and minimum (38.7%) mortality rates were achieved when the larvae were treated with $10^8$ and $10^2$ CFU/mL, respectively. *Stenotrophomonas maltophilia* CQ1 was more virulent than *P. mosselii* SJ10; it induced 82.3% mortality in third instar *E. ceratoniae* larvae compared with 71.3% for *P. mosselii* SJ10. The maximum larval mortality rate was caused by *S. maltophilia* CQ1 at a concentration of $10^8$ CFU/mL (100%) after 24 and 48 h of application, whereas the minimum was recorded in the larvae treated with the isolate *P. mosselii* SJ10 at $10^2$ CFU/mL (40%) after 6 h (Table 7). The calculated LC$_{50}$ and LC$_{90}$ values obtained with *S. maltophilia* CQ1 at 24 and 48 h after treatment against *E. ceratoniae* were 9.33 and $1 \times 10^4$ CFU/mL and 4.79 and $1 \times 10^3$ CFU/mL, respectively, whereas they were 15.8 and $2.51 \times 10^5$ CFU/mL and 10 and $7.94 \times 10^5$ CFU/mL, respectively, for *P. mosselii* SJ10 (Table 8). Furthermore, the highest degree of homogeneity for the larvae of *E. ceratoniae* was observed under exposure to *S. maltophilia* CQ1, with a slope value of 2.8.
**Table 7.** Larvicidal activity of two bacterial species cells on the carob moth, *Ectomyelois ceratoniae*, under laboratory conditions.

| Bacterial Species | Concentration (CFU/mL) | **Mortality %**       | Bacterial Species Means |
|-------------------|------------------------|-----------------------|-------------------------|
|                   |                        | 6 h | 12 h | 24 h | 48 h |                        |
| *Pseudomonas mosselii* | 10⁵ | **40 ± 6.3** h | 52 ± 4.9 g | 60 ± 6.3 fg | 68 ± 4.9 ef | 71.3 b |
|                   | 10⁴ | 60 ± 0 fg | 68 ± 4.9 ef | 76 ± 4 de | 84 ± 4 cd |                        |
|                   | 10³ | 60 ± 6.3 fg | 76 ± 4 de | 84 ± 4 cd | 92 ± 4.9 abc |                        |
| *Stenotrophomonas maltophilia* | 10⁵ | 68 ± 8 ef | 76 ± 4 de | 84 ± 4 cd | 92 ± 4.9 abc | 82.3 a |
|                   | 10⁴ | 76 ± 4 de | 84 ± 4 cd | 92 ± 4.9 abc | 96 ± 4 ab |                        |
|                   | 10³ | 88 ± 4.9 bc | 96 ± 4 ab | 100 ± 0 a | 100 ± 0 a |                        |
| Control                | 0 ± 0 i | 0 ± 0 i | 0 ± 0 i | 0 ± 0 i | 0 ± 0 i | 0 c                    |
| Exposure Time Means   | 43.3 d | 49.3 c | 53.7 b | 58.3 a |                        |

* Each treatment in this experiment was represented by five replicates, each with five larvae insects. ** Numbers in each column indicate mortality ± standard error. Means with different letters within the same column or row differ significantly (*p* < 0.05 using Duncan’s Multiple Range Test).

**Table 8.** Lethal concentrations of *Pseudomonas mosselii* and *Stenotrophomonas maltophilia* on the carob moth, *Ectomyelois ceratoniae*, under laboratory conditions.

| Bacterial Species | Exposure Time (h) | LC₅₀ (95% LCL–UCL) CFU/mL | LC₉₀ (95% LCL–UCL) CFU/mL | Slope ± SE | Intercept | X² | p-Value |
|-------------------|-------------------|--------------------------|--------------------------|------------|-----------|----|--------|
| *Pseudomonas mosselii* | 6      | 2.6 (0–4.3)             | 13.4 (11.6–15.6)         | 2.1 ± 0.56 | 3.8       | 0.087 | 0.036 |
|                   | 12     | 1.7 (0–3.1)             | 11.3 (8.9–14.2)          | 2.2 ± 0.72 | 4.8       | 0.180 | 0.055 |
|                   | 24     | 1.2 (0–2.4)             | 9.4 (6.2–13.5)           | 2.4 ± 0.83 | 5.6       | 0.170 | 0.063 |
|                   | 48     | 1 (0.01–2)              | 5.9 (3.9–8.1)            | 2.6 ± 0.94 | 6.4       | 0.172 | 0.070 |
| *Stenotrophomonas maltophilia* | 6      | 1.8 (0.2–2.8)          | 10.5 (6.4–12.8)          | 2.5 ± 0.70 | 4.6       | 0.687 | 0.038 |
|                   | 12     | 1.7 (0.5–2.5)           | 6.2 (4.5–9.3)            | 2.8 ± 0.77 | 5         | 1.22  | 0.038 |
|                   | 24     | 0.97 (0.02–1.8)         | 4 (2.6–6.3)              | 2.7 ± 1    | 7         | 1.33  | 0.076 |
|                   | 48     | 0.68 (0.1–1.5)          | 3 (0.6–5)                | 2.7 ± 1.2  | 8         | 0.697 | 0.106 |

LC₅₀—lethal concentration that kills 50% of insects; LC₉₀—lethal concentration that kills 90% of insects; LCL—lower confidence limit; UCL—upper confidence limit; X²—chi-square value; SE—standard error; *p*-value—probability. * Each figure is represented as a power of 10.

### 4. Discussion

The present investigation showed a 10.94% rate of recovery of EPNs in the soil samples from the Taif region of Saudi Arabia. These results may be attributed to soil parameters (e.g., temperature, pH, and moisture), which are important for EPN survival and infectivity. The isolated EPNs were only identified to the genus level in the present study; thus, future research should identify them to species level.

Our findings are in accordance with those reported by Noureldeen [25], who recorded the occurrence of one EPN species, *Steinernema* sp., in the rhizosphere of pomegranate trees in Taif, Saudi Arabia. Likewise, EPNs have been identified in several geographical areas including approximately 100 species of *Steinernema* and 26 species of *Heterorhabditis* [22,24]. Of the symbiotic bacteria, the morphologically identified *Xenorhabdus* species (isolate16) was molecularly characterized as *Xenorhabdus budapestensis* or *Xenorhabdus szentirmaii*, whereas the isolate4 was molecularly identified as *Pseudomonas fulva*. In addition, in the present study, we found that the bacteria *S. maltophilia* CQ1 was associated with *Steinernema* spp., whiles the bacteria *P. mosselii* SJ10 was symbiotically associated with *Heterorhabditis*.
spp., which are reported for the first time in Saudi Arabia. These results are consistent with those previously recorded by Ogier et al. [49], who mentioned that the association between *Steinernema* and *Xenorhabdus* was never monoxenic. Therefore, the bacterial community associated with laboratory-reared IJs from the *Steinernema carpocapsae*, *S. feltiae*, *S. glaseri*, and *S. weiseri* EPN species consisted of several Proteobacteria. The authors also reported that the laboratory-reared IJs of *S. carpocapsae* had a bacterial community composed of the core symbiont *Xenorhabdus nematophila*, together with a frequently associated microbiota (FAM) consisting of about a dozen Proteobacteria (*Pseudomonas*, *Stenotrophomonas*, *Alcaligenes*, *Achromobacter*, *Pseudochrobactrum*, *Ochrobactrum*, *Brevundimonas*, *Deftia*, etc.) [49].

Two species, *Pseudomonas protegens* and *P. chlororaphis*—the FAM members potentially involved in the parasitic lifecycle of *Steinernema*—displayed entomopathogenic properties suggestive of a role in *Steinernema* virulence and involvement in the *Steinernema* pathobiome. Indeed, in our study, the isolated bacteria were frequently detected in the haemolymph of insects infected with *Steinernema* and *Heterorhabditis*. It is generally assumed that non-symbiotic bacteria randomly “hitchhiked” in the IJ vectors via the cuticle or intercuticular space are introduced into the insect haemocoel during IJ penetration [50]. For example, *Stenotrophomonas*, *Ochrobactrum*, and *Pseudomonas* have often been identified in the soil-dwelling *Caenorhabditis elegans* nematodes [51]. Likewise, Proteobacteria is the most abundant phylum in bacterial communities associated with plant roots [52], as well as in plant-covered soils, such as the rhizosphere [53].

Regarding their insecticidal activity, our investigations show that *Steinernema* spp. were more effective than *Heterorhabditis* spp. against *V. livia* and *E. ceratoniae* larvae under laboratory conditions. These results are consistent with those of Memari et al. [54], who observed that mortality rates of *E. ceratoniae* larvae in laboratory tests corresponded to LC<sub>50</sub> values of 2.02 IJ/larva for *S. feltiae* and 2.05 IJ/larva for *S. carpocapsae*. On the contrary, *H. bacteriophora* showed low virulence against the larvae, with an LC<sub>50</sub> of 426.92 IJ/larva [54]. Our findings also show that *E. ceratoniae* larvae have higher susceptibility to both EPN species tested than *V. livia*, which may be due to the strong virulence of EPNs on members of the Pyralidae family such as the wax moth (*G. mellonella*) larvae. Furthermore, because *E. ceratoniae* overwinters throughout the autumn and winter seasons as larvae within infested fruit that drop on the soil, we hypothesise that it is possible to use EPNs for their control. Although most of the Steinernematids have ambush behaviours, the success of our tested species could be attributed to the mobile behaviours of *V. livia* and *E. ceratoniae* larvae, which may increase the distribution patterns of *Steinernema* spp. and can thus, increase the pest’s mortality [55]. For instance, Christos et al. [56] also reported the potential of three concentrations (100, 300 and 900 IJs/larva) of *S. feltiae* for the control of the Mediterranean flour moth, *Ephestia kuehniella* (Lepidoptera: Pyralidae), in stored wheat.

The larvicidal activity of the two EPN-associated bacterial species was also evaluated against the two pomegranate pests in the current study. It is clear that the bacteria *S. maltophilia* CQ1 and *P. mosselii* SJ10 have the capacity to control *V. livia* and *E. ceratoniae* larvae. Our data also revealed that *S. maltophilia* CQ1 was more effective than *P. mosselii* SJ10 against both *V. livia* and *E. ceratoniae* larvae; however, *E. ceratoniae* was more susceptible. The higher lethality of *S. maltophilia* CQ1 (that is associated with *Steinernema* spp.) in comparison to *P. mosselii* SJ10 (that is associated with *Heterorhabditis* spp.) correlates with the greater lethality of *Steinernema* spp. over *Heterorhabditis* spp. on *V. livia* and *E. ceratoniae* larvae. These results are consistent with those of Alotaibi et al. [48], who recorded that mortality of *E. ceratoniae* larvae caused by two *Xenorhabdus* bacterial species was significantly higher than that of *Photorhabdus* species. In contrast, a recent study by Elbrense et al. [15] showed that *H. bacteriophora* and its symbiont, *Photorhabdus* sp., were more virulent than *S. riobravis* and its symbiont, *Xenorhabdus* sp. against *Pieris rapae* and *Pentodon algerinus*. Our results are also consistent with those of Jabeen et al. [57], who quantified bacterial chitinase production by *S. maltophilia* and their termiticidal activity. Moreover, this study concurred with Amer et al. [58], who stated that *S. maltophilia* shows promising antagonistic activity against a panel of multidrug-resistant bacteria and fungi. Similarly, Berg [59] re-
ported that *S. maltophilia* inhibited the growth of phytopathogen *Rhizoctonia solani*, possibly because of antibiosis and the production of some lytic enzymes that act against pathogenic fungi. Subsequent studies have revealed that the metabolic diversity of *S. maltophilia* is responsible for the production of novel bioactive compounds, including agents that can be used in biocontrol against microorganisms and insects [60].

5. Conclusions

In this study, EPNs and their associated bacteria were isolated and identified from Taif, Saudi Arabia, and their biocontrol efficacy was evaluated against the third instar larvae of *V. livia* and *E. ceratoniae*, two important insect pests of pomegranate. *Steinernema* spp. were more virulent than *Heterorhabditis* spp. against the two pomegranate insects. In addition, the bacteria *Stenotrophomonas maltophilia* CQ1, isolated from *Steinernema* spp., surpassed those of the bacteria *Pseudomonas mosselii* SJ10, associated with *Heterorhabditis* spp., in their ability to kill *V. livia* or *E. ceratoniae* larvae. We conclude that either application of either EPNs’ IJs or their associated EPBs could serve as potential biocontrol agents for *V. livia* and *E. ceratoniae* for sustainable agriculture. These studies were conducted under laboratory conditions; thus, future studies need to validate these results under field conditions.

**Author Contributions:** Conceptualisation, S.S.A. and A.N.; data curation, H.D., S.A. and A.N.; formal analysis, H.D., A.N. and A.A.-B.; investigation, S.S.A., A.A., A.N.; methodology, H.D., A.N. and A.A.-B.; project administration, S.S.A.; resources, S.S.A., S.A. and A.N.; validation, H.D., R.H.W. and M.Z.; visualisation, A.N.; writing—original draft, S.S.A. and A.N.; writing—review and editing, S.S.A., A.N., A.B. and M.A. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Deputyship for Research and Innovation, Ministry of Education in Saudi Arabia through the project number 1-441-117.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** The authors extend their appreciation to the Deputyship for Research and Innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number 1-441-117.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. Koppenhofer, A.M. Nematodes. In *Field Manual of Techniques in Invertebrate Pathology: Application and Evaluation of Pathogens for Control of Insects and Other Invertebrate Pests*, 3rd ed.; Lacey, L.A., Kaya, H.K., Eds.; Springer: Dordrecht, The Netherlands, 2007; pp. 249–264.

2. Kaya, H.K. Soil ecology. In *Entomopathogenic Nematodes in Biological Control*; Gaugler, R., Kaya, H.K., Eds.; CRC: Boca Raton, FL, USA, 1990; pp. 93–115.

3. Boemare, N.E.; Akhurst, R.J.; Mourant, R.G. DNA relatedness between Xenorhabdus spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer Xenorhabdus luminescens to a new genus, Photorhabdus gen. nov. *Int. J. Syst. Bacteriol.* 1993, 43, 249–255. [CrossRef]

4. Dillman, A.R.; Guillermin, M.L.; Lee, J.H.; Kim, B.; Sternberg, P.W.; Hallem, E.A. Olfaction shapes host–parasite interactions in parasitic nematodes. *Proc. Natl. Acad. Sci. USA* 2012, 109, E2324–E2333. [CrossRef] [PubMed]

5. Salvadori, J.D.M.; Defferrari, M.S.; Ligabue-Braun, R.; Lau, E.Y.; Salvadori, J.R.; Carlini, C.R. Characterization of entomopathogenic nematodes and symbiotic bacteria active against *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and contribution of bacterial urease to the insecticidal effect. *Biol. Control.* 2012, 63, 253–263. [CrossRef]

6. Fang, X.; Li, Z.; Wang, Y.; Zhang, X. In vitro and in vivo antimicrobial activity of *Xenorhabdus hovisii* YL002 against *Phytophthora capsici* and *Botrytis cinerea*. *J. Appl. Microbiol.* 2011, 111, 145–154. [CrossRef]

7. Shi, D.; An, R.; Zhang, W.; Zhang, G.; Yu, Z. Sterilene derivatives from *Photorhabdus temperata* SN259 and their antifungal activities against phytopathogenic fungi. *J. Agric. Food Chem.* 2017, 65, 60–65. [CrossRef]

8. Forst, S.; Dowds, B.; Boemare, N.; Stackebrandt, E. *Xenorhabdus* and *Photorhabdus* spp. Bugs that kill bugs. *Annu. Rev. Microbiol.* 1997, 51, 47–72. [CrossRef]
9. Bisch, G.; Pages, S.; McMullen, J.G.; Stock, S.P.; Duvidic, B.; Givaudan, A.; Gaudriault, S; Mauer, T.J.; Ogier, J.C.; Ensign, J.C.; et al. The insect pathogenic bacterium Xenorhabdus bovienii has attenuated virulence in multiple insect model hosts yet encodes a potent mossotoxicidal toxin. BMC Genom. 2017, 18, 927. [CrossRef]

10. Kim, I.-H.; Aryal, S.K.; Aghai, D.T.; Cavanova-Torres, A.M.; Hillman, K.; Kozuch, M.P.; Mans, E.J.; Mauer, T.J.; Ogier, J.C.; Ensign, J.C.; et al. The insect pathogenic bacterium Xenorhabdus innexi has attenuated virulence in multiple insect model hosts yet encodes a potent mossotoxicidal toxin. BMC Genom. 2017, 18, 927. [CrossRef]

11. McMullen, J.G.; McQuade, R.; Ogier, J.C.; Pages, S.; Gaudriault, S.; Stock, S.P. Variable virulence phenotype of Xenorhabdus bovienii (γ-Proteobacteria: Enterobacteriaceae) in the absence of their vector hosts. Microbiolology 2017, 163, 510–522. [CrossRef]

12. Hazr, S.; Yaya, H.K.; Stock, S.P.; Keskin, N. Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) for biological control of soil pests. Turk. J. Biol. 2003, 27, 181–202.

13. Herz, A.; Koppler, K.; Vogt, H.; Elias, E.; Katz, P.; Peters, A. Biological control of the cherry fruit fly, Rhagoletis cerasi L. (Diptera, Tephritidae) by use of entomopathogenic nematodes: First experiences towards practical implementation. In Proceedings of the 12th International Conference on Cultivation Technique and Phytopathological Problems in Organic Fruit Growing (Eco-fruit), Weinsberg, Germany, 18–20 February 2006; pp. 67–72.

14. Yooyangket, T.; Muangpat, P.; Polseela, R.; Tandhavanant, S.; Thanwisai, A.; Vitta, A. Identification of entomopathogenic nematodes and symbiotic bacteria from Nam Nao National Park in Thailand and larvicidal activity of symbiotic bacteria against Aedes aegypti and Aedes albopictus. PLoS ONE 2008, 13, e0195681. [CrossRef] [PubMed]

15. Elbrenese, H.; Elmasry, A.M.; Seleman, M.F.; Al-Harbi, M.S.; Abd El-Raheem, A.M. Can symbiotic bacteria (Xenorhabdus and Photorhabdus) be more efficient than their entomopathogenic nematodes against Pieris rapae and Pseudotodon algerinus larvae? Biology 2021, 10, 999. [CrossRef] [PubMed]

16. Zeng, F.; Xue, R.; Zhang, H.; Jiang, T. A new gene from Xenorhabdus bovienii and its encoded protease inhibitor protein against Acyrthosiphon pisum. Pest. Manag. Sci. 2012, 68, 1345–1351. [CrossRef] [PubMed]

17. Jin, D.; Zeng, F.; Dong, S.; Zhang, H.; Stok, E. Effects of a protease inhibitor protein from Xenorhabdus bovienii on physiology of pea aphid (Acyrthosiphon pisum). Pestic. Biochem. Physiol. 2014, 108, 86–91. [CrossRef]

18. Fuchs, S.W.; Grundmann, F.; Kurz, M.; Kaiser, M.; Bode, H.B. Fabclavines: Bioactive peptide–polyketide–polymysino hybrids from Xenorhabdus. Chembiochem 2014, 15, 512–516. [CrossRef]

19. Mraček, Z.; Liu, Q.Z.; Nguyen, K.B. Steinernema xueshanense n. sp. (Rhabditidida, Steinernematidae), a new species of entomopathogenic nematode from the province of Yunnan, southeast Tibetan Mts., China. J. Invertebr. Pathol. 2009, 102, 69–78. [CrossRef]

20. Li, X.Y.; Liu, Q.; Nermut, J.; Puža, V.; Mraček, Z. Heterorhabditis beicheriana n. sp. (Nematoda: Heterorhabditidae), a new entomopathogenic nematode from the Shunyi district of Beijing, China. Zootaxa 2012, 3569, 25–40.

21. Cimen, H.; Lee, M.M.; Hatting, J.; Hazr, S.; Stock, S.P. Steinernema innovationi n. sp. (Panagrolaimomorpha: Steinernematidae), a new entomopathogenic nematode species from South Africa. J. Helminthol. 2014, 3, 1–4.

22. Phan, K.L.; Mraček, Z.; Puža, V.; Nermut, J.; Jarosova, A. Steinernema huense n. sp., a new entomopathogenic nematode (Nematoda: Steinernematidae) from Vietnam. Nematology 2014, 16, 761–775. [CrossRef]

23. Malan, A.P.; Knoetze, R.; Tiedt, L. Heterorhabditis noenieputensis n. sp. (Rhabditidida: Heterorhabditidae), a new entomopathogenic nematode from South Africa. J. Helminthol. 2014, 88, 139–151. [CrossRef]

24. de Bride, A.L.; Rosa, J.M.O.; de Oliveira, C.M.G.; de Castro e Castro, B.M.; Serrao, J.D.; Zununcio, J.C.; Leite, L.G.; Wilcken, S.R.S. Entomopathogenic nematodes in agricultural areas in Brazil. Sci. Rep. 2017, 7, 45254. [CrossRef] [PubMed]

25. Novelldeen, A.H. Validation of DNA fingerprinting techniques in characterization of entomopathogenic nematodes. J. Entomol. Zool. Stud. 2018, 6, 226–231.

26. Day, K.R.; Wilkins, E.D. Commercial pomegranate (Punica granatum L.) production in California. Acta Hort. 2011, 890, 275–286. [CrossRef]

27. Graham, M. Insect Larvae Associated with Dropped Pomegranate Fruits in an Organic Orchard in Tunisia. J. Entomol. Nematol. 2015, 7, 5–10.

28. El-Hawagry, M.S.; Sharaf, M.R.; Al Dhafer, H.M.; Fadil, H.H.; Aldawood, A.S. Addenda to the insect fauna of Al-Baha Province, Kingdom of Saudi Arabia with zoogeographical notes. J. Nat. Hist. 2016, 50, 1209–1236. [CrossRef]

29. Hoseini, S.A.; Goldansaz, S.H.; Sadeghhasani, S.; Mousavi, S.G. A field screening of 10 high yield pomegranate cultivars for resistance to the carob moth, Ectomyelois ceratoniae, in the climate condition of Karaj, Alborz, Iran. In Proceedings of the 21st Iranian Plant Protection Congress, Urmia, Iran., 23–26 August 2014.

30. Ksentini, I.; Jardak, T.; Zeghal, N. First report on Virachola ilivia Klug. (Lepidoptera: Lycanidinae) and its effects on different pomegranate varieties in Tunisia. Bull. OEPP 2011, 41, 178–182. [CrossRef]

31. Sayed, A.A.; Temez, S.A.; Lysandrou, M. The use of different insect control regimes using three green chemicals to combat Virachola ilivia on date palm fruit in Egypt. Acta Hortic. 2010, 882, 471–479. [CrossRef]

32. Abbas, M.S.T.; Razvi, S.A.; Shidi, R.H.; Al-Khatry, S.A. Role of egg parasitoids for controlling the pomegranate butterfly, Virachola ilivia Klug (Lycanidinae: Lepidoptera) in Sultanate of Oman. J. Biol. Pest Contr. 2008, 18, 43–46.

33. Kinawy, M.M.; Al-Waili, H.M.; Elmandhari, A.M. Review of the successful classical biological control programs in Sultanate of Oman. J. Biol. Pest Contr. 2008, 18, 1–10.
34. Obeidat, W.; Akkawi, M. Bionomics and control of pomegranate butterfly *Virachola (Deudorix) livia* (Klug) (Lepidoptera: Lycaenidae) in Northern Jordan. *Diras. Agri. Sci.* **2002**, *29*, 1–12.

35. Sedra, M.H. *Le Palmier Dattier Base de la Mise en Valeur des Oasis au Maroc, Techniques Phoénicoles et Création d’Oasis*; INRA-Editions: Rabat, Morocco, 2003.

36. Dhouibi, M.H. *Potential of three entomopathogenic bacterial isolates for management of the carob moth,* *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae). *Egypt. J. Biol. Pest Control* **2020**, *30*, 55. [CrossRef]

37. Shakeri, M. *Bionomics and control of pomegranate butterfly Manduca sexta* (Lepidoptera: Sphingidae) in Northern Jordan. *Diras. Agri. Sci.* **2002**, *29*, 1–12. [CrossRef]

38. Pertot, I.; Caffi, T.; Rossi, V.; Mugnai, L.; Hoffmann, C.; Grando, M.S.; Gary, C.; Lafond, D.; Duso, C.; Thiery, D.; et al. A critical review of plant protection tools for reducing pesticide use on grapevine and new perspectives for the implementation of IPM in viticulture. *Crop Protect.* **2017**, *97*, 79–84. [CrossRef]

39. Athanassiou, C.G.; Palyvos, N.E.; Kakouli-Duarte, T. Insecticidal effect of *Xenorhabdus nematophila* Poinar and Thomas (Achromobacteraceae: Eubacteriales) against *Periplaneta americana* (L.) (Insecta: Blattodea: Blattidae). *Appl. Environ. Microbiol.* **2014**, 80, 4277–4285. [CrossRef] [PubMed]

40. Atta, W.; El-Mekhalafy, S.A.; El-Sayed, M. *In Vitro Activity of Neoaplectana caudata* (Diptera: Calliphoridae) against *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae). *Agriculture* **2021**, *11*, 1256. [CrossRef]

41. Kaya, H.K.; Koppenhoffer, A.M. Effects of microbial and other antagonistic organism and competition on entomopathogenic nematodes. *Biocontrol. Sci. Technol.* **1996**, *6*, 357–372. [CrossRef]

42. Ahmed, N.M.; Ali, H.; Maamoun, M.; Younis, T.; Al-Sawaf, A. Potential of bacterial chitinolytic, *Stenotrophomonas maltophilia* in biological control of termites. *Egypt J. Biol. Pest Cont.* **2018**, *28*, 86. [CrossRef]

43. Amer, A.; Hamdy, B.; Mahmoud, D.; Elanany, M.; Rady, M.; Alahmadi, T.; Alharbi, S.; Al Ashaal, S. Antagonistic activity of bacteria isolated from the *Periplaneta americana* L. gut against some multidrug-resistant human pathogens. *Antibiotics* **2021**, *10*, 294. [CrossRef]
59. Berg, R.D. The indigenous gastrointestinal microflora. *Trends Microbiol.* **1996**, *4*, 430–435. [CrossRef]

60. Ribitsch, D.; Heumann, S.; Karl, W.; Gerlach, J.; Leber, R.; Birner-Gruenberger, R.; Gruber, K.; Eiteljoerg, I.; Remler, P.; Siegert, P.; et al. Extracellular serine proteases from *Stenotrophomonas maltophilia*: Screening, isolation and heterologous expression in *E. coli*. *J. Biotechnol.* **2012**, *157*, 140–147. [CrossRef] [PubMed]