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Biocontrol Effect of *Bacillus subtilis* against *Cnaphalocrocis medinalis* (Guenèe) (Lepidoptera: Pyralidae): A Sustainable Approach to Rice Pest Management

Muthusamy Janaki [1], Pavana K. Sivadasan Unni [1], Vethamonickam Stanley-Raja [1], Sengottayan Senthil-Nathan [1,*], Bader O. Almutairi [2] and Ahmed Abdel-Megeed [3]

[1] Sri Paramakalyani Centre for Excellence in Environmental Sciences, Manonmaniam Sundaranar University, Alwarkurichi, Tirunelveli 627 412, Tamil-Nadu, India; pavananair@gmail.com (P.K.S.U.); stanleyrajamsu@gmail.com (V.S.-R.)
[2] Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Riyadh, Saudi Arabia; bomotairi@ksu.edu.sa
[3] Department of Plant protection, Faculty of Agriculture Saba Basha, Alexandria University, Alexandria 5452022, Egypt; ahmedabdelfattah@alexu.edu.eg
* Correspondence: senthil@msuniv.ac.in

Abstract: Agricultural pests can be effectively controlled using microbes, providing an eco-friendly alternative to available synthetic pesticides. Suitable entomopathogenic bacterial strains were collected from agricultural fields and evaluated for their insecticidal potential against *Cnaphalocrocis medinalis*. In the four tested entomopathogenic bacteria (W1, Yc1, S1, EB01), the larval mortality ranged from 38 to 74%. Among these isolates, *Bacillus subtilis* (EB01) induced the highest mortality (74%). In greenhouse conditions, the tests confirm that the results were dosage-dependent: *B. subtilis* infection considerably delayed the overall development period, reduced pupal conversion, and decreased adult emergence with induced morphological deformities. Larvae fed *B. subtilis*-treated leaves initiate bacterial infection and broadly damage the midgut tissue, including the epithelial and peritrophic layers. The bacterial growth in the *C. medinalis* hemolymph considerably increases the activity of enzymes like α and β esterase (85.14 and 44% at 96 h) compared to the control. The isolate *B. subtilis*-treated diet significantly reduced the larval digestive α and β galactosidase enzyme activity (88.17 and 91.88% at 96 h). Furthermore, germination bioassay with strain EB01 in rice varieties (TN1 and ASD16) significantly increased both varieties' germination and biomass index. This study shows that the *B. subtilis* EB01 strain potentially inhibited the biological activity of *C. medinalis* and improved the rice seeds' germination index. It can be a potential biocontrol agent in sustainable pest-management strategies.

Keywords: entomopathogenic bacteria; insecticidal activity; leaf folder; enzyme analysis; seed emergence; paddy development

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1. Introduction

Oryza sativa L. is a stable food for more than 3.5 billion people globally, particularly in Asia, where 90% of people consume rice due to its high-energy constituents [1]. However, in commercial large-scale production, rice quality and productivity are adversely affected by more pests. Different stages of pests damage the rice crops through their survival and developmental activity [2,3]. The yellow stem borer, plant hoppers, rice leaf folder, rice ear bug, etc., are potential threats that damage paddy fields [4]. Among these pests, *Cnaphalocrocis medinalis* (Guenee) Lepidoptera: Pyralidae (rice leaf folder) is a predominant foliage feeder, one of the essential pests causing agricultural loss and affecting the overall rice ecosystem in India. It is vital in pest management, since they scrap the leaf chlorophyll, leading to considerable yield losses and other saprophytic infections [5]. *Cnaphalocrocis medinalis* larvae longitudinally fold the leaves by connecting the leaf margins and scraping the

mesophyll tissue within the leaves, reducing the photosynthesis rate and leading to defects in plants' vigor and development [6]. An increasing population of rice leaf folders could complicate rice cultivation and increase the usage of synthetic pesticides [7]. Synthetic pesticides widely contribute to the commercial production of crops by inhibiting economically harmful pests, which are the leading input in recent agriculture practices. Synthetic pesticides are cheap, fast-acting, readily available for farmers, and effective against various pests [8]. For the control of the rice leaf folder, several pesticides from various synthetic categories are used [9]. Conventional synthetic pesticides against *C. medinalis* include cypermethrin, monocrotophos, fipronil, imidacloprid, and dimethoate [9,10]. However, frequent usage of these insecticides also directly and indirectly impacts the environment, non-targeted organisms, and human health [11]. In the current situation, some insects are developing resistance against synthetic insecticides, which is a significant challenge [12]. In such circumstances, the efficacy of biopesticide usage has emerged as a sustainable alternative source for controlling harmful pests. The revolution of biopesticides has focused on target pests along with agricultural production without damaging plant development [13]. Microbial biopesticides can be an effective, eco-friendly method for sustainable agriculture. Unlike chemical pesticides, the microbial biopesticides are specific in action, low cast, and are environmentally sustainable without residual effects. However, the time of application, maintenance, and temperature are the few drawbacks of biopesticides [14].

Some reports have found that some bacterial species isolated from various insects have impacts on harmful pests, and they are possible sources of entomopathogenic agents [15,16]. Entomopathogens, including *Bacillus* and *Pseudomonas* spp. are widely distributed in the environment and have virulence effects against major pests [17,18]. Entomopathogens such as *B. thuringiensis* can produce toxins, which target the lepidopteran larval species and are commercially exploited for pest control [19]. Similarly, some entomopathogens from *Pseudomonas*, *Enterobacter*, and *Bacillus* spp. produce a variety of toxins that suppress the metabolic activity of insects and induce mortality in lepidopterans, coleopterans, hymenopterans, and dipterans [20]. The present work was carried out with the aim of evaluating the insecticidal potential of entomopathogenic bacteria against *C. medinalis.*

2. Materials and Methods

2.1. Sample Collection

A field survey was conducted in the paddy fields in and around Alwarkurichi, Tenkasi district, to collect the infected and dead *C. medinalis* larvae with morphological abnormalities. Infected insects were transferred immediately to the laboratory, surface sterilized with 70% ethanol for 15 min, flamed, and air-dried in the laminar airflow chamber for 2 min. The outer layers of the larvae were removed without damage to the inner area using sterile scissors and needles. The remaining larval portion was homogenized with a phosphate-buffered solution, and 1 mL of filtered suspension was poured into Luria–Bertani (LB) agar plates. The plates were incubated at 28 ± 2 °C for 24 h. Bacterial colonies were selected based on the color and morphological appearances and it was subculturing several times to obtain purified culture. After purifications, four different bacterial colonies were selected for the pathogenicity test and denoted as W1, Yc1, S1, and EB01. The bacterial strains were maintained in LB plates and broth.

2.2. Cnaphalocrocis medinalis Culture

Cnaphalocrocis medinalis larvae were collected from the paddy fields around Tirunelveli and Tenkasi, Tamil Nadu, India. In greenhouse conditions, the rice plants were grown in earthenware pots (10 plants for each pot) for insect rearing. The collected larvae were reared in potted rice plants enclosed with mesh sleeves and maintained at 28 ± 2 °C in a 14 h dark:10 h light cycle with 80% humidity. Adults were maintained in an oviposition cage with potted plants, and the moths were fed with sucrose solution (10%). The complete life cycle of *C. medinalis* culture was maintained by following the method of Senthil-Nathan et al. [21].

2.3. Screening Bioassay

Preculture of bacterial strains (W1, Yc1, S1, and EB01) of 1 mL was inoculated into fresh LB broth and incubated at 28 ± 2 °C for two days. After incubation, each bacterial culture was centrifuged at 5000 rpm at 4 °C for 20 min. The obtained pellet was resuspended in sterile distilled water after being washed with it. The bacterial density was concentrated at 3×10^9 cfu/mL (approximately) using a hemocytometer [22]. To determine the insecticidal potential of bacterial strains, second-stage larvae (10 larvae per treatment) were taken into a laboratory to be cultured. In a greenhouse condition, the rice plants were grown in earthenware pots (10 plants for each pot) for the treated larval culture. The pathogenicity was confirmed by dipping the *C. medinalis* larvae into the 20 mL bacterial suspension from each isolate for 2 s, and the remaining suspension was sprayed into potted rice plants. Then, the bacterial-treated larvae were transferred into the same potted rice plants and were covered with mesh net sleeves. Sterile water was used for the control treatment. Treatments were maintained at 28 ± 2 °C and 80% humidity. Larval mortality was recorded daily for ten days. The bacterial strain EB01, which exhibits severe infection in *C. medinalis* larvae and has the highest mortality rate, was selected for further analysis and molecularly identified using 16s rRNA gene sequencing.

2.4. Preparation of EB01 Bacterial Suspension

The bacterial strain EB01 was prepared in two ways (active form and cell-free extract). For the live bacterial cells, four different concentrations, i.e., 1.5×10^3 cfu/mL, 2×10^5 cfu/mL, 3.5×10^7 cfu/mL, and 5×10^9 cfu/mL, were prepared. For the cell-free extracts, 1 mL of preculture EB01 strain was added to the 100 mL LB broth and incubated for seven days [23]. Simultaneously, control treatments were prepared with uninoculated broth. After centrifugation, the obtained supernatant was lyophilized and extracted using methanol (100%). The dried extracts were redissolved with methanol and concentrated with 0.1 g/mL and 1 g/mL.

2.5. Concentration-Dependent Mortality and Development Bioassay

In total, eight treatments were carried out in this experiment: T1 = 1.5×10^3 cfu/mL, T2 = 2×10^5 cfu/mL, T3 = 3.5×10^7 cfu/mL, T4 = 5×10^9 cfu/mL, T5 = 0.1 g/mL, T6 = 1 g/mL, T7 = uninoculated broth with methanol (control), and T8 = sterile water (control). Two-day-old second-stage *C. medinalis* larvae were allowed to starve for 1 h for analysis of their food intake and treatment response. Eighty-day-old rice plants were separately treated with 20 mL of different concentrations of bacterial dilution or cell-free extracts using a sprayer (Kisan Kraft—PS8000, Bangalore, India). Control treatment plants were treated with water or methanol. After 2 h, 20 larvae were transferred into treated potted rice plants covered with mesh net sleeves for each treatment. All the treatments were replicated five times. After treatment, the dead larvae were analyzed for morphological changes due to bacterial infection. Larval mortality was recorded every day. Larval duration, pupal conversion, pupal deformities, pupal duration, adult emergence, and adult deformities were recorded. Afterwards, the experiment was conducted with the surviving moths. Five treatments were carried out in this part: T (moths emerged from bacterial treatments), T5 (from cell-free extracts, 0.1 g/mL), T6 (from cell-free extracts, 1 g/mL), T7 (uninoculated broth with methanol), and T8 (sterile water), and the moths were transferred into an oviposition cage to be reared with normal moths of the opposite sex, and fed with 10% sucrose solution. Potted rice plants were placed in every treatment cage. The adult longevity, total number of eggs laid by female moths, and hatchability were recorded.

2.6. Enzyme Activity

The effect of bacterial EB01 infection on detoxification enzymes of *C. medinalis* was assessed by treating the third-stage larvae (12 days old) of the insect with a 5×10^9 cfu/mL bacterial concentration for 24, 48, 72, and 96 h. For the enzyme extraction, the treated larvae were randomly selected and were homogenized in ice with phosphate buffer (0.1 M)

containing EDTA (1 mM), DTT (1 mM), PTU (1 mM), PMSF (1 mM), and glycerol (20%). The homogenate mixture was centrifuged for 10 min at 15,000 rpm at 4 °C [24]. To measure α and β carboxylesterase activity, 20 µL extract was added to the 500 µL 0.1 M phosphate potassium buffer (0.3 mM α or β-naphthyl acetate and 1% acetone). Fast blue B (0.3%) and SDS (3.3%) were added to the reaction mixture. The reaction mixture was incubated at 29 ± 1 °C for 20 min and centrifuged. The obtained supernatant absorbance was recorded at 590 nm, generated as 1 µmol of α or β-naphthol per minute [25].

The effect of bacterial EB01 infection on digestive enzymes of *C. medinalis* was assessed by treating the fourth-stage larvae (14 days old) of the insect with 5×10^9 cfu/mL bacterial concentration for 24, 48, 72, and 96 h. Larvae were treated with sterile water for the control. Larval guts were homogenized, and 20 µL of enzyme extract was added with 50 µL of 5 mM p-nitrophenyl-α-D-galactopyranoside or p-nitrophenyl-β-D-galactopyranoside and 200 µL phosphate buffer. The reaction mixture was incubated for 10 min at 37 °C [26]. The reaction was terminated with 160 µL of 1 M sodium carbonate [27]. The absorbance was recorded at 450 nm.

2.7. Histological Analysis

The effect of EB01 infection on *C. medinalis* larvae was investigated via histology of the midgut tissue. The control and increased bacterial concentration-treated larvae were taken from the culture (10 days old from the post-treatment). The larvae were dissected aseptically, and the extracted guts were kept in 5% formalin [28]. The guts were washed with sterile water several times, and dehydration of tissue was performed using alcohol concentrations from 50 to 100%. Paraffin wax was used for tissue fixation, and a microtome (Leica, Nussloch, Germany) made tiny blocks from the embedded wax. These tiny sections were placed in a slide coated with 1% Mayer's egg albumin and kept on a hot plate at 40 °C. The slides were dewaxed using Xylene for 5 min and rehydrated with 100, 90, 80, 70, and 60% ethanol concentrations. Hematoxylin and eosin were used to stain. Then, the slides were rinsed once with 100% alcohol and twice with Xylene [29]. The observation was made using a microscope, (Nikon, Tokyo, Japan), and images were captured by connecting the microscope to the computer.

2.8. In Vitro Seed Treatments

The susceptible and moderately resistant rice varieties TN1 and ASD16 were chosen for this study, and whether the entomopathogenic bacterial strain EB01 could have any adverse effect on rice plants was analyzed. Seeds were surface-sterilized with sodium hypochlorite solution (2%), washed with sterile distilled water several times, and dried with filter paper. The bacterial culture was prepared as described above and concentrated at 1.5×10^3 cfu/mL, 2×10^5 cfu/mL, 3.5×10^7 cfu/mL, and 5×10^9 cfu/mL. For control treatments, sterile distilled water was used. Twenty seeds were taken for each experiment and were soaked with the respective bacterial concentrations for 24 h. Seed emergence was analyzed using the filter paper method for seven days [30].

2.9. In Vivo Seed Treatment under Greenhouse

Before planting, silt loam soil was autoclaved for 25 min and used to fill the pots for the treatments. Rice seeds TN1 and ASD16 were treated with separate bacterial concentrations (1.5×10^3 cfu/mL, 2×10^5 cfu/mL, 3.5×10^7 cfu/mL, and 5×10^9 cfu/mL) and sown in 1 L pots in a greenhouse (5 seeds per treatment). Seeds treated with sterile distilled water were used as a control. All the tests were performed with five replications. Rice plants were maintained for 30 days with water as needed. After 30 days, all the plants were removed from the pots and cleaned with water. The plant height and fresh weight were measured. Healthy and affected leaves were also recorded for every tested plant.

2.10. Statistical Analysis

The experiments, including larval mortality, larval duration, pupal duration, pupal deformities, adult deformities, adult emergence, and enzyme analysis, were replicated five times. The mean values were represented by comparing differences in treatments using the Tukey's family error test ($p < 0.05$) using the Minitab 17.1.0 software package.

3. Results

3.1. Insecticidal Bioassay

Effective bacterial strains were screened using mortality bioassay compared to the control; all the tested strains caused mortality (Figure 1). The EB01 strain induced a higher mortality rate among the isolates (i.e., 74%) and a morphological change in the larvae. As per the molecular analysis, the EB01 bacterial strain was identified as *Bacillus subtilis*, and its GenBank accession number is OQ071610.

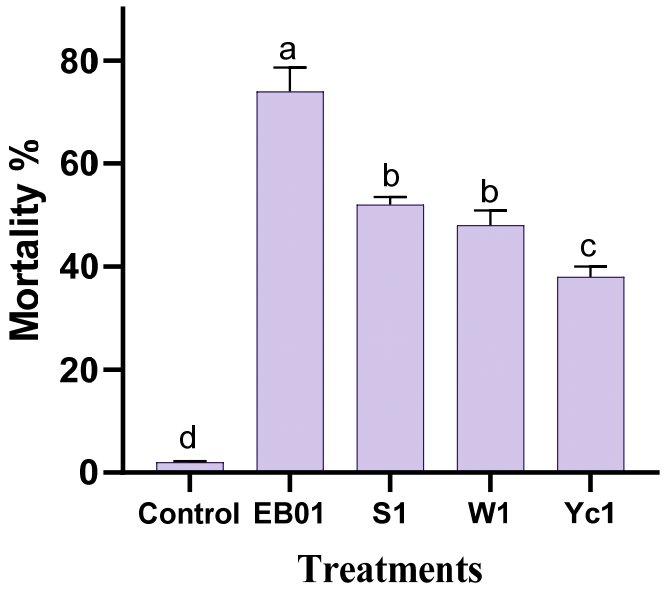

Figure 1. Mortality percentage (%) of 2nd-stage *C. medinalis* larvae treated with bacterial isolates EB01, S1, W1, and Yc1 at 3×10^9 cfu/mL concentration. Bars indicate the mean ± SE. Different letters above the bars represent significant differences at Tukey's test $p < 0.05$.

3.2. Concentration-Dependent Bioassay

The concentration response bioassay results showed that *B. subtilis* culture and its methanol extract induced toxic effects for various biological parameters of *C. medinalis* when ingested orally. The larval mortality ranged from 52 to 78% while the larvae were treated with active cell culture (T1–T4 treatments) ($p < 0.0001$). It was a concentration-dependent effect. With respect to the 3% control group, the cell-free extracts (treatments T4 and T5) also induced larval mortality in 17 and 32% (Figure 2a). During T1 treatments, the larval mortality started after 5 days and continued until the 15th day. At T4, the larvae mortality started after two days of treatments and continued until the seventh day. Compared to healthy larvae (Figure 3a), the *B. subtilis* culture-infected larvae became sluggish, stopped feeding, and their bodies ultimately turned yellow and black, leading to death (Figure 3c–f). Some larvae from the higher concentration (T4 treatment) changed their pupal stage within three days and died (Figure 3b). In the methanol-extract treatments (T5 and T6), the mortality started after eight days of treatments and continued throughout the entire stage. The growth of *B. subtilis* was observed in the infectious larvae hemolymph of treated *C. medinalis* larvae. In contrast, no growth of *B. subtilis* was detected in the extracts and control groups larvae.

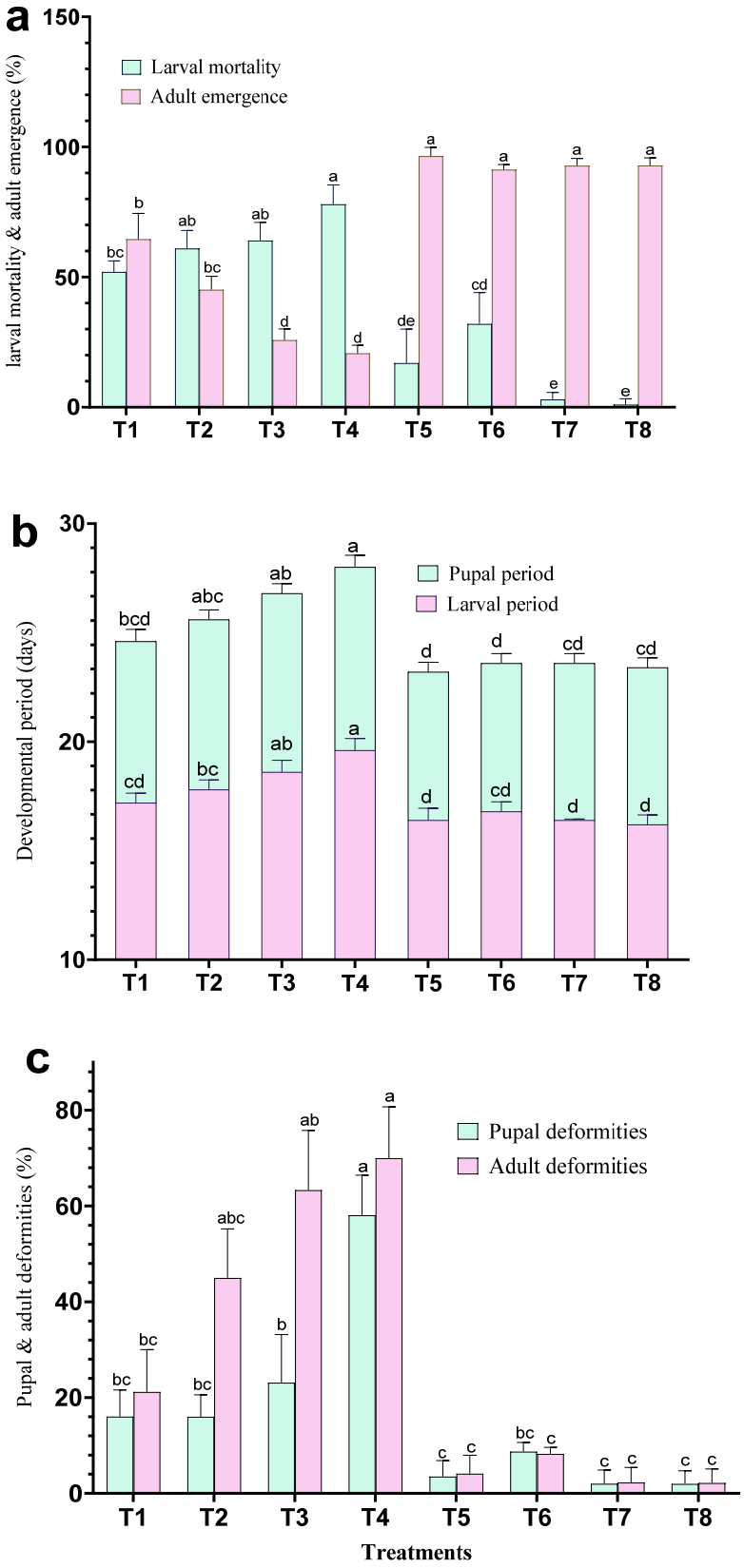

Figure 2. Treatments: T1 = 1.5 × 10^3 cfu/mL, T2 = 2 × 10^5 cfu/mL, T3 = 3.5 × 10^7 cfu/mL, T4 = 5 × 10^9 cfu/mL, T5 = 0.1 g/mL, T6 = 1 g/mL, T7 = methanol, and T8 = sterile water. Effect of *B. subtilis* on various biological parameters of *C. medinalis*. (**a**) percentage of larval mortality and adult emergence, (**b**) larval and pupal developmental duration (days), and (**c**) percentage of pupal and adult deformities. Bars indicate the mean ± SE. Different letters above the bars represent significant differences at Tukey's test $p < 0.05$.

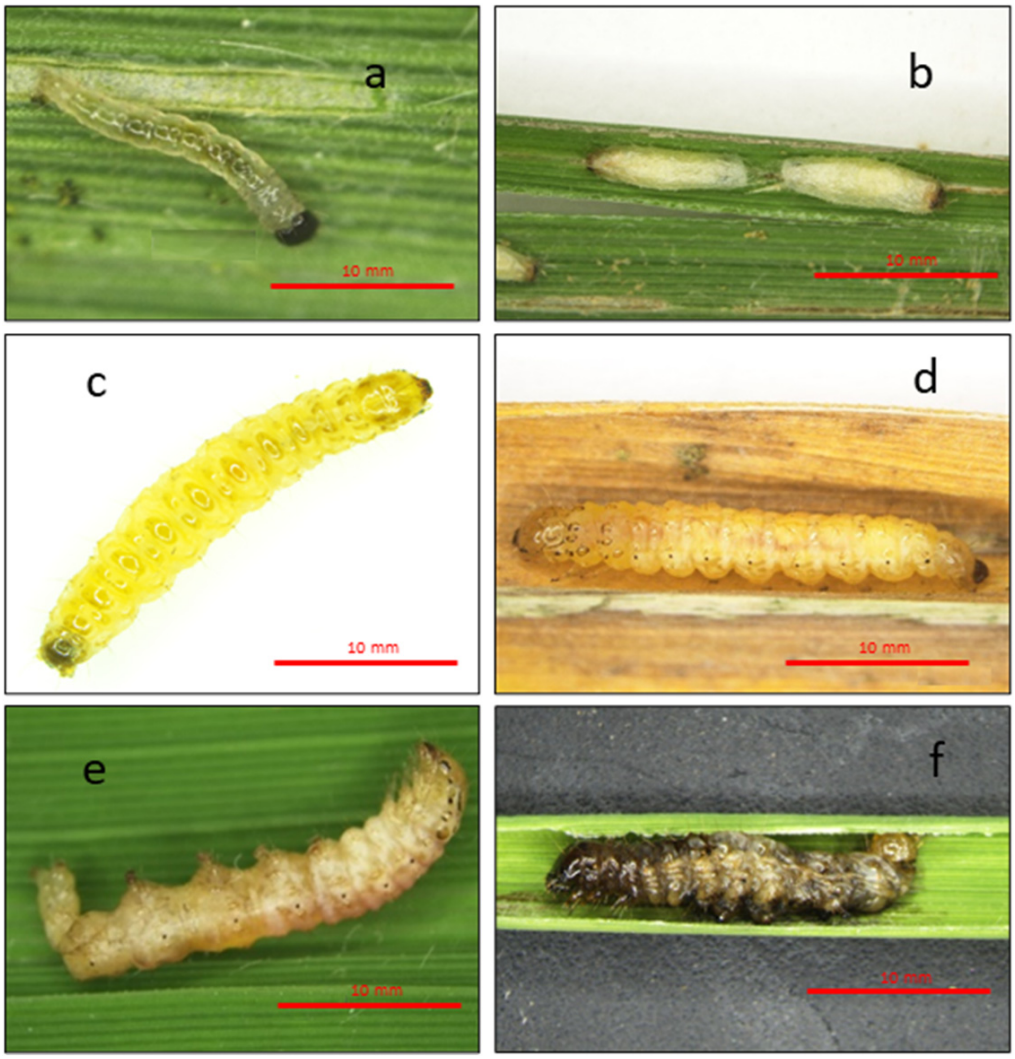

Figure 3. (**a**) Control, (**b**) cocoon formation at 2nd stage after 48 h post-treatment, (**c**) bacterial growth inside body turns yellowish after 48 h, (**d**) increased culture growth inside the body at 72 h, (**e**) the insect body swells and dies at 72 h, and (**f**) the insect body turns black and bacterial cells ooze out from the body. Scale bar—1 cm.

Oral ingestion of *B. subtilis* significantly influenced the development of *C. medinalis* larvae. Except for the bacterial extract treatments (T5 and T6), the culture of *B. subtilis* extended the larval period significantly by 1 to 3.4 days with respect to the control ($p < 0.0001$).

A significant effect was also observed during the pupal period. At the highest concentration (T4 treatment), few pupal deformities, i.e., larval–pupal intermediates, pupal–adult intermediates, and blackish body, were observed, followed by T3 and T2 treatments the notable changes were recorded (Figure 4a–e). In *B. subtilis* culture treatments (T3 and T4), only a few adults emerged, i.e., 20 and 25%, and most of the emerged adults were found to have deformities, with darkened bodies. With respect to the control groups, adults who emerged from *B. subtilis* culture treatments were found to be less active and had decreases in reproductive potential. The bacterial toxicity also induced the deformed morphological appearances of adults such as underdeveloped wings and crumpled body shapes (Figure 4f). The adverse effect of *B. subtilis* highly influenced the egg-laying capacity and larval hatchability (Figure 5) of *C. medinalis*. A significant impact was also noticed in the adult longevity that emerged from *B. subtilis* treatments, as shown in Figure 5 ($p < 0.0001$).

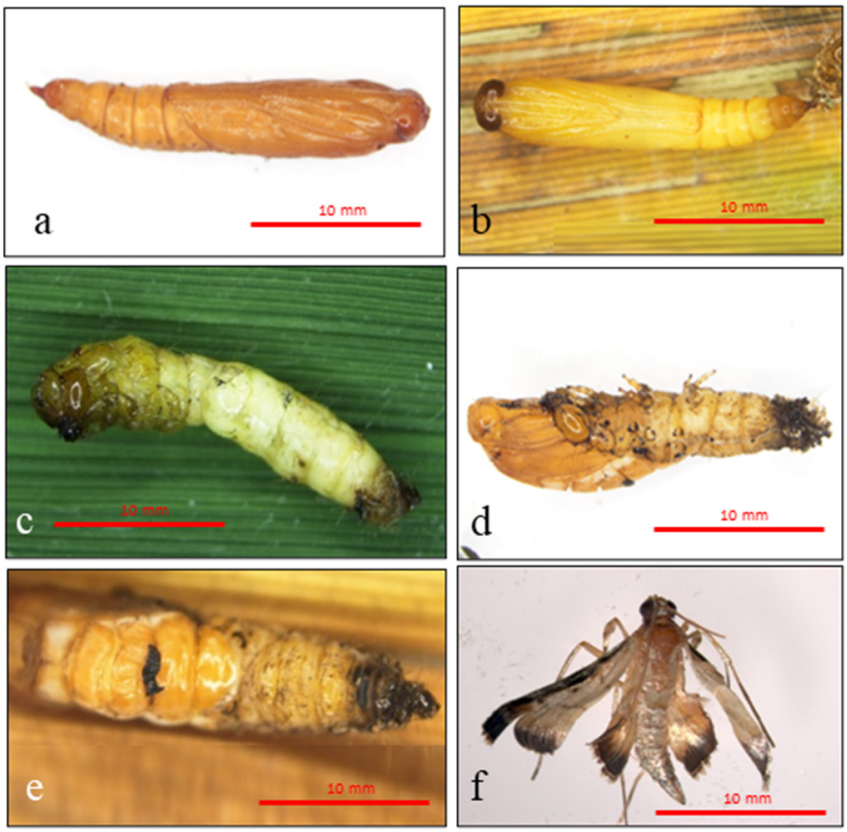

Figure 4. (**a**) Control pupae, (**b**) bacteria-infected, dead, undeveloped pupae, (**c**) underdeveloped pupae, (**d**) partially developed dead pupae, (**e**) infected unconverted larvae, and (**f**) adult deformity. Scale bar—1 cm.

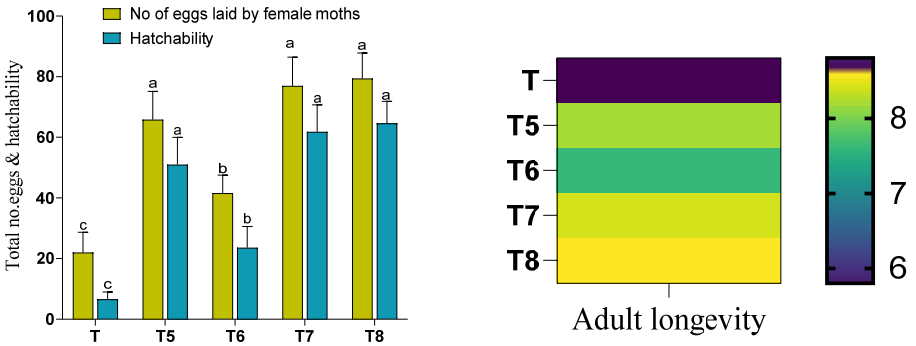

Figure 5. Effect of *B. subtilis* on reproductive potential and adult longevity of *C. medinalis* (number of eggs laid by females and total number of hatches). Bars indicate the mean ± SE. Different letters above the bars represent significant differences at Tukey's test $p < 0.05$.

3.3. Enzyme Activity

The adverse impact of *B. subtilis* on the activity of the α and β esterase activity of *C. medinalis* larvae was evident during their analysis. When *C. medinalis* larvae feed on *B. subtilis*-treated rice plants, there was a significant rise in α and β esterase 46.15 and 48.19%, 24 h; 33.87 and 27.18%, 48 h; 41.33 and 44.35%, 72 h; and 85.14 and 44.07%, 96 h, respectively, compared to control (Figure 6a,b) The effect of *B. subtilis* on the α and β galactosidases of *C. medinalis* was detected based on the reduction in activity when compared to the control. A significant drop was recorded, which showed a decreased level of 53.78 and 65.39%, 48 h; 88.17 and 91.88%, 96 h (Figure 6c,d).

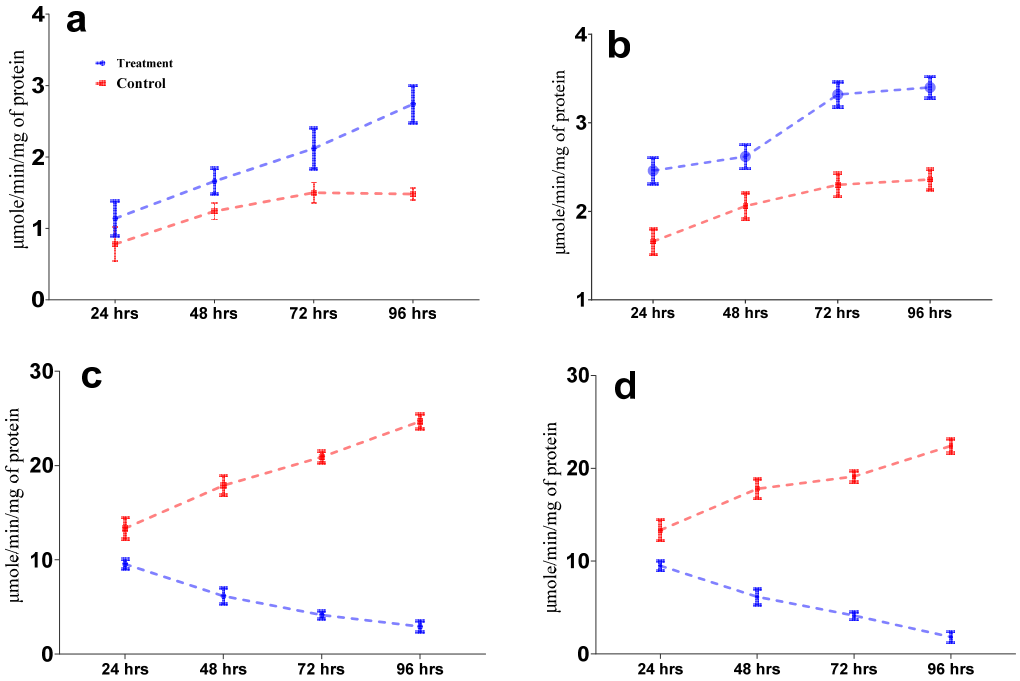

Figure 6. Effect of *B. subtilis* on the activity of (**a**) α carboxylesterase, (**b**) β carboxylesterase, (**c**) α galactosidase, and (**d**) β galactosidase enzymes of 3rd- (**a**,**b**) and 4th-stage (**c**,**d**) *C. medinalis* larvae at 5×10^9 cfu/mL. Bars indicate the mean ± SE.

3.4. Histological Analysis

We observed extensive histological alterations of midgut tissue in *B. subtilis* exposed to *C. medinalis* larvae. Infection due to *B. subtilis* disrupted the basement membrane, peritrophic, epithelial, and muscle layer of the midgut tissue of *C. medinalis*. The histology also showed that the epithelial layer was disturbed and detached from the larval midgut basement membrane (Figure 7). Control *C. medinalis* larvae displayed an excellent organization of muscle and epithelial cells.

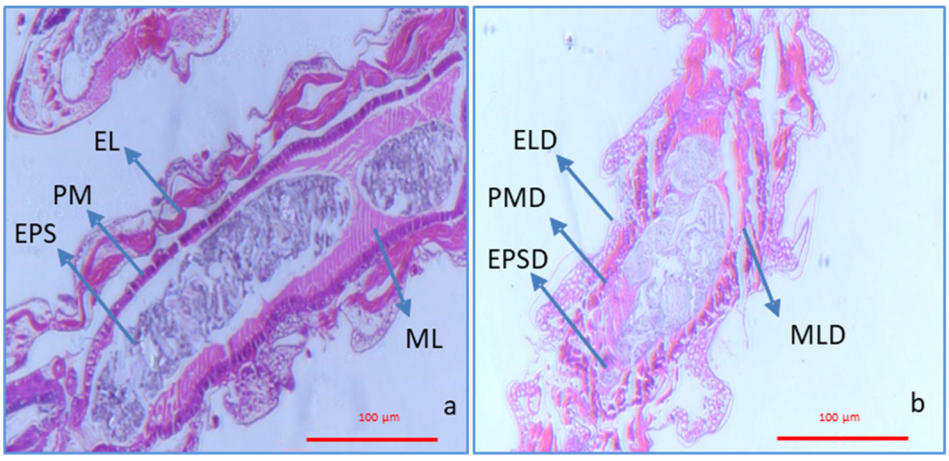

Figure 7. Longitudinal section through (**a**) midgut of fourth stage of *C. medinalis* larvae fed on control leaves showing intact peritrophic membrane (PM), endo peritrophic space (EPS), epithelial layer (EL), and muscle layer (ML). (**b**) Midgut of the larvae on treated leaves with *B. subtilis* shows disruption in the peritrophic membrane (PMD), endo peritrophic space (EPSD), epithelial layer (ELD) and muscle layer (MLD).

3.5. Plant–Bacteria Interactions

The seed treatment of *B. subtilis* (1.5×10^3 cfu/mL, 2×10^5 cfu/mL, 3.5×10^7 cfu/mL, and 5×10^9 cfu/mL) in both TN1 and ASD16 varieties of rice seeds had a positive effect on the germination rates and plant growth ($p < 0.0001$). The germination results showed a variation based on the bacterial concentrations; the germination percentages ranged from 77 to 88%, TN1; 78 to 91%, ASD16 (Figure 8a). We observed that plants grown using *B. subtilis* treatments had no adverse effects on plant biomass in both TN1 and ASD16 varieties. In the TN1 variety, *B. subtilis*-treated plants significantly increased in height from 24.96 to 30.24 cm (Figure 8b) and biomass from 237 to 264 mg. In the ASD16 variety, *B. subtilis*-treated seeds increased the plant height from 25.98 to 29.94 cm, biomass from 227 to 282 mg (Figure 8c) with respect to control plants. In both varieties, *B. subtilis*-treated seeds were germinated and grown well without any disease symptoms or stunned growth ($p < 0.0001$). The plants developed using *B. subtilis* treatment showed a healthy shoot nature compared to the control (Figure 8d).

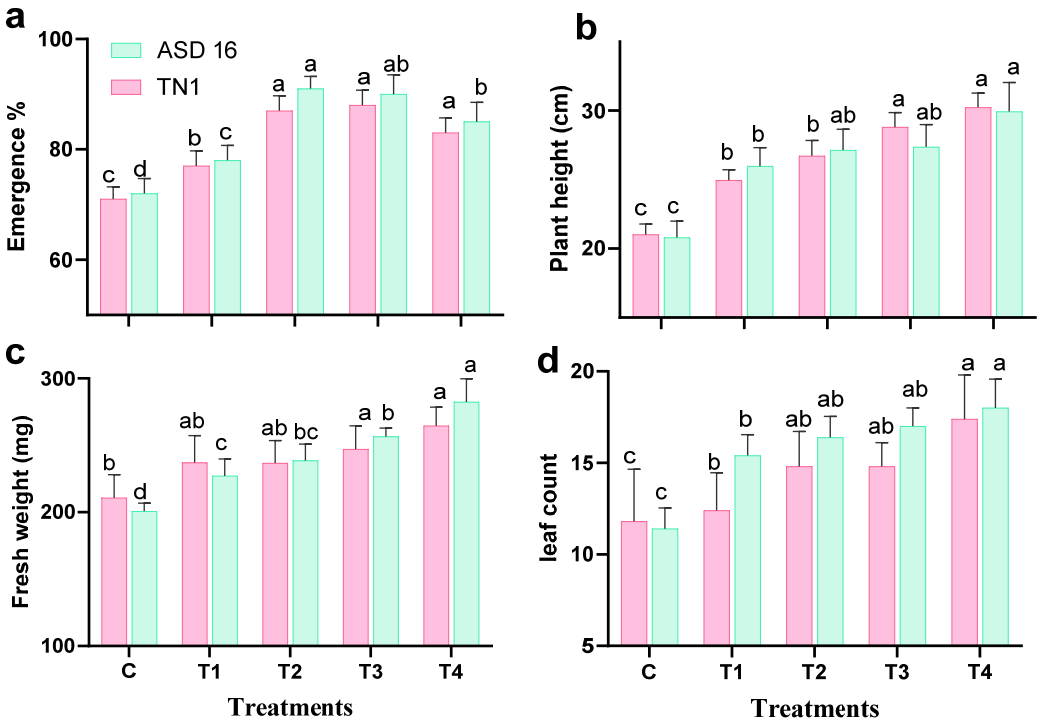

Figure 8. Efficacy of *B. subtilis* on the TN1 and ASD16 rice plants (**a**) emergence %, (**b**) plant height, (**c**) fresh weight, and (**d**) leaf count. Bars indicate the mean ± SE. Different letters above the bars represent significant differences at Tukey's test $p < 0.05$.

4. Discussion

There is a developing tendency to identify active pathogenic and efficient microbial biocontrol agents to manage effective and eco-friendly systems for controlling harmful agricultural pests. Hence, due to the demand to search for a novel biocontrol agent other than synthetic pesticides, entomopathogenic bacterial isolates from naturally infected *C. medinalis* larvae were screened for their insecticidal potential against *C. medinalis* management. Among the tested entomopathogenic bacterial strains, *B. subtilis* EB01 (OQ071610) was found to be more pathogenic, causing 74% mortality to *C. medinalis* in in vitro studies. Similarly, El-Salam et al. [31] demonstrated that the insecticidal effect of soil isolate *B. subtilis* NRC313 induced the mortality on *S. littoralis* larvae. Rizwan et al. [32] reported that entomopathogenic fungi, *B. bassiana*, controlled the growth of *C. medinalis*, causing a 74% mortality rate. Another effort of *B. subtilis* cell-free extracts also induced 32% larval mortality. The insecticidal action was slightly associated with the culture supernatant,

thus including the significant efficacy of possible soluble metabolites produced by *B. subtilis*. Larvae of *C. medinalis* infected with *B. subtilis* showed infectious symptoms such as lethargy, cessation of feeding, turning black with a flaccid body, less movement activity, decreased larval development, and, ultimately, death. A similar trend has been previously reported in many pests such as *Spodoptera litura*, *Spodoptera exigua*, and *Zophobas morio* due to infection of *Pseudomonas* sp., *B. thuringiensis*, and *Pseudomonas aeruginosa* [18,33,34]. However, Senthil-Nathan et al. [28] demonstrated that the bacterial *Btk*-toxin induced a high percentage of mortality in *C. medinalis* larvae.

The presence of bacterial proliferation in the hemolymph of *C. medinalis* larvae treated with *B. subtilis* suggests that larvae death occurs due to septicemia caused by the secretion of bacterial toxin compounds or other virulence enzymes into the hemocoel that targets the midgut epithelial barrier, breaks the body cavity, and suppresses the immune response of pests. Similarly, Sarkhandia et al. [18] reported that the mortality in *S. litura* was due to *Pseudomonas* sp. bacterial growth in hemocoel. The high proliferation of some entomopathogens in the hemolymph causes necrosis by releasing bacterial toxins [35]. Other researchers also documented mortality in *S. litura*, *Spodoptera frugiperda*, *Helicoverpa armigera*, *Plutella xylostella*, and *Delia radicum* due to the continuous proliferation of *Photorhabdus akhurstii* and *Pseudomonas protegens*.

In previous reports, several researchers found some entomopathogens from different species, such as *Bacillus popilliae*, *Bacillus lentimorbus*, *Bacillus sphaericus*, *Pseudomonas taiwanesis*, *Pseudomonas entomophila*, *Pseudomonas cedrina*, *Klebsiella pneumoniae*, *Pseudomonas paralactis*, and *Pseudomonas aeruginosa,* exhibit insecticidal activity against various pests like *Culex quinquefasciatus*, *P. xylostella*, *Drosophila melanogaster*, *Spodoptera exigua*, *S. litura*, and *Galleria mellonella* [36–40]. Some bacterial toxins like Fit toxin, exotoxin, rhizotoxins, and ExoS released from *Pseudomonas fluorescens*, *P. aeruginosa* and *P. taiwanesis* were associated with various pests causing pathogenicity that leads to death and sepsis [41–43]. Bacterial strains *P. fluorescens* and *P. protegens* contribute to the toxicity of *Drosophila* and *G. mellonella* by producing hydrolytic enzymes like chitinases, phospholipases, and proteases [44,45].

In addition to affecting mortality, treating larvae with *B. subtilis* also extended the larval developmental period and pupal period, decreased pupal conversion, and facilitated adult emergence. *B. subtilis* treatments induced deformities in pupae and adults. Delayed development of *C. medinalis* was an adverse effect of *B. subtilis*. A similar trend has been documented previously in *S. litura*, *Delia radicum*, and *H. armigera*, which emerged from *Bacillus vallismortis*, *Enterobacter cloacae*, *P. paralactis*, and *B. thuringiensis* treatments; the adults were morphologically deformed with underdeveloped and crumbled wings [14,40,46]. Similarly, several reports proved that some larval species in *H. armigera*, *D. melanogaster,* and *S. litura* have exhibited delayed development and decreases in adult emergence after being exposed to *Serratia marcescens*, *P. fluorescens*, and *B. thuringiensis* [47–49].

Our studies displayed a significant increase in the activity of α and β esterase after exposure to *B. subtilis* treatments. Other researchers also found similar enhancement in α and β esterase activity in *S. litura* after exposure to λ-cyhalothrin and insecticides [24,50]. In contrast, a significant decrease was observed in the activity of α and β galactosidases due to infection of *B. subtilis*. The suppression of digestive enzyme activity in the *B. subtilis*-treated pests may be due to effects on the efficiency of digestion [51]. A similar report documented reduced digestive enzymes in *C. medinalis*, *Diatraea saccharalis*, and *H. cunea* due to infection with *Photorhabdus temperata* and *B. thuringiensis* [52–54]. The efficiency of digestion is correlated with the histopathological impact of *C. medinalis* gut. The midgut is the major site for the digestive enzyme synthesis and secretion. In our study, histological analysis demonstrated extensive damage in midgut epithelial cells of *C. medinalis* larvae due to infection with *B. subtilis*. A bacterial toxin from *B. thuringiensis* altered the midgut epithelial cells in *S. litura* and *A. gemmatalis* [55–57]. Similar damage in the midgut tissues has been documented in *S. litura*, *S. frugiperda*, and *H. armigera* after exposure to *K. pneumoniae*, *P. akhurstii*, and *P. paralactis* [29,39].

This study aimed to develop a potential biocontrol agent without affecting crop development. The results of the plant growth promotion study show that the treatments using *B. subtilis* have increased the seed germination percentage and plant biomass in both varieties without affecting the plants' shoots and roots. In previous studies, the novel bacterial strain *B. subtilis*, isolated from soil, improved the rice plant development [58]. Javed et al. [59] reported that the bacterial entomopathogen of *Brevibacillus laterosporus* increased rice development and induced systemic resistance against *Cymothoa exigua*. Similarly, Ullah et al. [60] demonstrated that the entomopathogenic bacteria *P. temperata* promotes rice plant growth by activating gibberellins. Several studies have shown the role of entomopathogens of *Serratia marcescens* in regulating plant growth along with resistance against pests in rice [61,62].

For future implications of *B. subtilis* in integrated pest-management practices, there is a need to standardize the mass production techniques of *B. subtilis* isolate to make it cost-effective so that farmers can easily use it and, further, to evaluate its efficacy. It can be used in combination with other botanical or other control agents so as to provide effective agricultural pest control in integrated pest-management programs.

5. Conclusions

The entomopathogenic bacteria *B. subtilis* active cell culture and extract are both virulent in *C. medinalis* larvae, and this can be useful for *C. medinalis* control. Based on the analysis of *B. subtilis* culture and extract, the active cells were found to induce more pathogenicity in *C. medinalis* larvae. From the above study, we conclude that *B. subtilis* active cells are a good potential biocontrol agent for *C. medinalis* population control. In future, in order to develop good potential microbial biopesticides, the *B. subtilis* isolate will be further validated in a field evaluation.

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