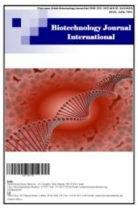

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Laboratory Evaluation of the Insecticidal Toxins from Entomopathogenic Nematode Symbiotic Bacteria to Control Vegetable Diseases and Pests

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aims: Entomopathogenic nematode (EPN) and its symbiotic bacterium are used worldwide as microbial control agents. Toxins from EPN symbiotic bacteria were isolated and provided basis for using this potential resource as biocontrol agent against vegetable diseases and pests.
Study Design: The toxins were extracted from 28 strains of bacteria associated with entomopathogenic nematodes. The insecticidal activity and antibiotic activity against vegetable diseases and pests were determined through bioassay.
Place and Duration of Study: College of Bioscience and Biotechnology, between May 2020 and September 2021.
Methodology: The toxins were extracted by $(NH_4)_2SO_4$ precipitation method. The insecticidal activities and antibiotic activities were evaluated using bioassay in the laboratory.
Results: The toxins of the symbiotic bacteria associated with EPN had certain insecticidal activities on the first instar larvae of *Plutella xylostella* and *Laphygma exigua*, and strain SY5 showed the most obvious antifungal activities against *Trichothecium roseum* and *Fusarium oxysporum*.
Conclusions: The toxins of the EPN symbiotic bacteria SY5 had good insecticidal activity and antibiotic activity. Therefore, it has the potential for use against vegetable diseases and pests as biocontrol agents.

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Keywords: *Entomopathogenic nematodes; symbiotic bacteria; toxins; vegetable diseases and pests; bioassay.*

1. INRTODUCTION

Vegetables are one of the essential foods in people's daily diet, but the production of vegetables will be greatly reduced by plant diseases and pests. *Plutella xylostella*, *Laphygma exigua*, *Trichothecium roseum* and *Fusarium oxysporum* are main pests and diseases of vegetables. "The diamondback moth, *P. xylostella* (Lepidoptera: plutellidae), is one of the most serious pests of cultivated Brassicaceae (such as cabbage, radish, and rapeseed) worldwide. *Laphygma exigua* (Lepidoptera: Noctuidae) is polyphagous, migratory, gluttonous, cosmopolitan, and intermittent pests, which damage cabbage, tomato, pepper, eggplant, cucumber and other vegetables and plants" [1-3]. "Fungal diseases are considered a great problem in vegetable production. *Trichothecium roseum* is the causal for many diseases of tomatos, and *F. oxysporum* is a worldwide distributed soil borne pathogenic fungus, which can cause fusarium wilt of melons, solanaceae, bananas, cotton, legumes and other plants" [4,5]. It is necessary to control insect pests and fungal plant disease in vegetable production.

"Application of entomopathogenic nematode (EPNs) with their bacterial endosymbionts become a prime approach in the biocontrol sector as an ecologically safer tool in a sustainable agriculture perspective as well as in integrated pest management" [6]. "Since the early 1970s, there has been a tremendous research and commercial interest in entomopathogenic nematodes and their associated bacteria" [7]. "In the 1990s and twentieth century's vast studies on EPNs were carried out, and it was reported that EPNs were distributed worldwide" [6]. (EPNs) exist widely in the soil. They are non-toxic and harmless to plants, humans, animals and the environment [8]. **"**EPN (genera Steinernema and Heterorhabditis) kill insects with the aid of a mutualistic association with symbiotic bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp. for Steinernematidae and Heterorhabditidae, respectively). *Xenorhabdus* and *Photorhabdus* bacteria secrete a wide variety of substances into the culture medium including toxins, lipases, proteases, antibiotics and lipopolysaccharides. EPN and its symbiotic bacterium are used worldwide as microbial control agents in agriculture" [9-12]. "Entomopathogenic nematode-bacterium complex research is being conducted in many parts of the world. Many countries and regions working on these important biological control agents of soil pests. In Central America, initial attempts to control insect pests and mass production research are reported" [7]. "In North America and Europe, emphasis on the status of commercially available nematodes was placed. In China, Korea, and India, research in the use of nematode for controlling insect pests or soil plant pathogens was emphasized , as well as in Japan, where the development of commercial nematodes was available" [6]. Overall, the intensity of research varies by country or regions. In most cases, the research in developing countries shows that the emphasis is to demonstrate the usefulness of the entomopathogenic nematodes or their symbiotic bacteria against various pests. In this study, the toxins were extracted from entomopathogenic nematode symbiotic bacteria. The insecticidal activities and antifungal activities to vegetable diseases and pests were determined through bioassay. The result will be helpful for the development of new microbial insecticides, and will provide new ways and methods for biological control of vegetable against pests and fungal-plant diseases.

2. MATERIALS AND METHODS

2.1 Materials

The EPNs were provided by Pest Biological Control Laboratory, Shenyang Agricultural University. Twenty-eight strains of EPN symbiotic bacteria were isolated from 5 species of EPNs out of the 23 species obtained from soil samples collected from different regions of China. The bacterial strain names were given according to the EPN hosts coded in our lab.

2.2 Production of Bacterial Cell and the Crude Extract

A single colony was inoculated into nutrient broth (18 g nutrient broth in 500 ml distilled water) in a flask and placed in a shaking incubator at 160 rpm for 40 h at 27°C. The concentration of bacterial cells in the broth suspension was determined on a spectrophotometer at 600nmwavelength. After cultivation, the supernatant of the bacterial strains was collected by centrifugation (3400 g, 4°C, 15min) and then $(NH_4)_2SO_4$ was added to give 85% final content. Through dialyzing and frost-desiccation, the insecticidal toxins were obtained.

2.3 Bioassay

We used the larvae of *P. xylostella* and *L. exigua*, which were kindly provided by Pest Biological Control Laboratory, Shenyang Agricultural University, to detect the oral insecticidal activity of the toxins. The toxins were mixed into artificial diet at 50 μg/g of diet as the test sample. Only distilled water was given as control samples. The mixed diet (0.2g) and larvae were placed individually into 5 ml clear plastic airtight pots. Each batch had 20 larvae, and three batches were repeated. Survival and weight of larvae were recorded at 3rd, 4th, 5th day. The corrected mortality and the inhibiting rate of larval weight were calculated. The corrected mortality =100 × (treatments mortality – contrast mortality) / (1- contrast weight). The inhibiting rate of larval weight = 100 × (contrast weight – treatments weight) / contrast weight.

The toxin (50 μg /ml)) and cooled PDA agar were mixed as 1ml: 25 ml in 9 cm Petri dishes. The control dishes were 1 ml distill water and 25 ml cooled PDA agar. The plant pathogenic fungi plug (5 mm, kindly provided by Shenyang Chemical Engineering Research College.) was added to the center of the dish after the mixed PDA solid. All dishes were incubated at 28°C and each sample had three replicates. The zone of inhibition (the diameter of contrast and treatment) was observed and measured by the cross method at 3rd, 4th, and 5th days. The inhibiting rate of plant pathogenic fungi was calculated by the formula (The inhibiting rate against plant pathogenic fungi = 100 × (the diameter of contrast –the diameter of treatments)/contrast diameter).

2.4 Statistical Analysis

SPSS 23.0 software (one-way ANOVA) was used to analyze the data. The data on the corrected mortality, the inhibiting rate of larval weight and the inhibiting rate were analyzed by repeated measures ANOVA. The differences between treatments were determined using contrasts. The differences between treatments were analyzed by Duncan. All comparisons were considered as significance at $p<0.05$.

3. RESULTS AND ANALYSIS

3.1 Insecticidal Activity of Toxins against *P. xylostella*

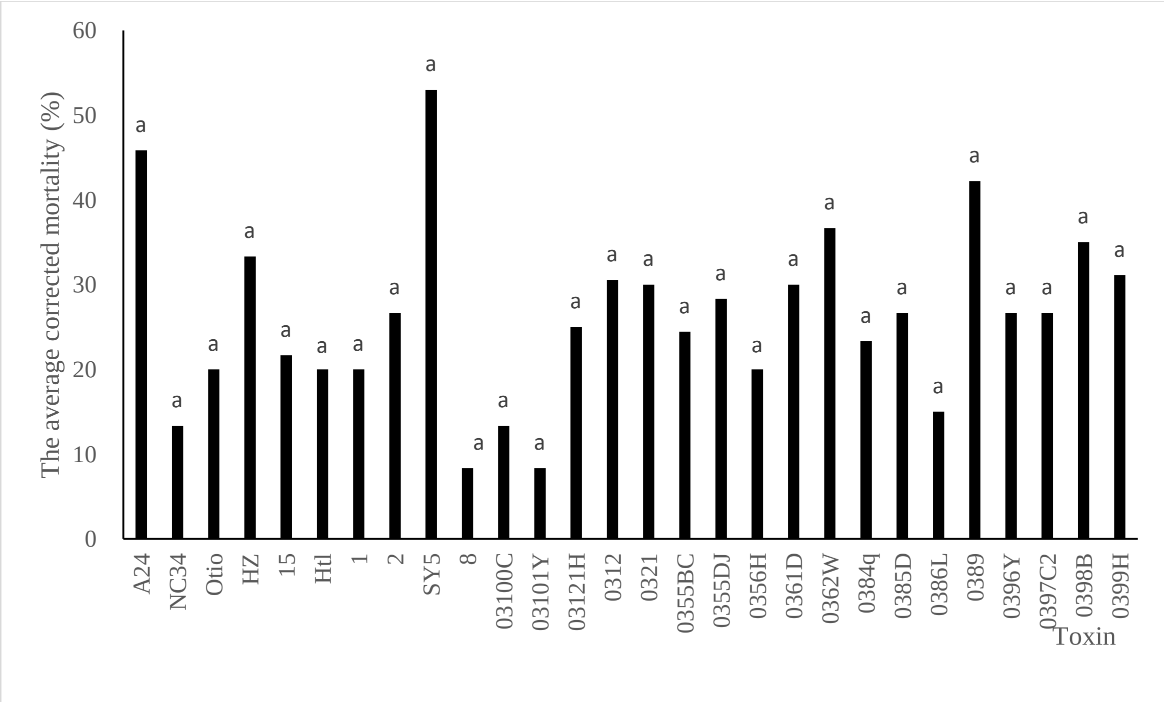
The reference EPN symbiotic bacterial toxin (*X. nematophila*, *X. poinarrii*, *X. bovienii*, *P. temperatae* and *P.luminescens*) exhibited highly insecticidal activities [13-18]. Bioassay results showed that the toxin of *X. nematophila* A24 had the highest oral insecticidal activity among the reference strains. The average of corrected mortality at 3rd, 4th, and 5th day and the average inhibiting rate of larval weight to *P. xylostella* were 45.83% (Fig. 1A), 77.50% (Fig. 1B), 96.67% (Fig. 1C) and 34.12% (Fig. 1D).

These toxins were extracted from 23 EPN symbiotic bacterial isolates which were gathered in different vegetation from different regions of China. Bioassay results indicated that all these bacterial strains had oral insecticidal activity to *P. xylostella*. The insecticidal activity of all toxin had no significant difference on the 3rd day (Fig. 1A), while it had significant difference on the 4th day and 5th day. Among these strains, the toxin of SY5 showed the highest oral insecticidal activity to *P. xylostella*, with 100% of the average corrected mortality at 5th day.

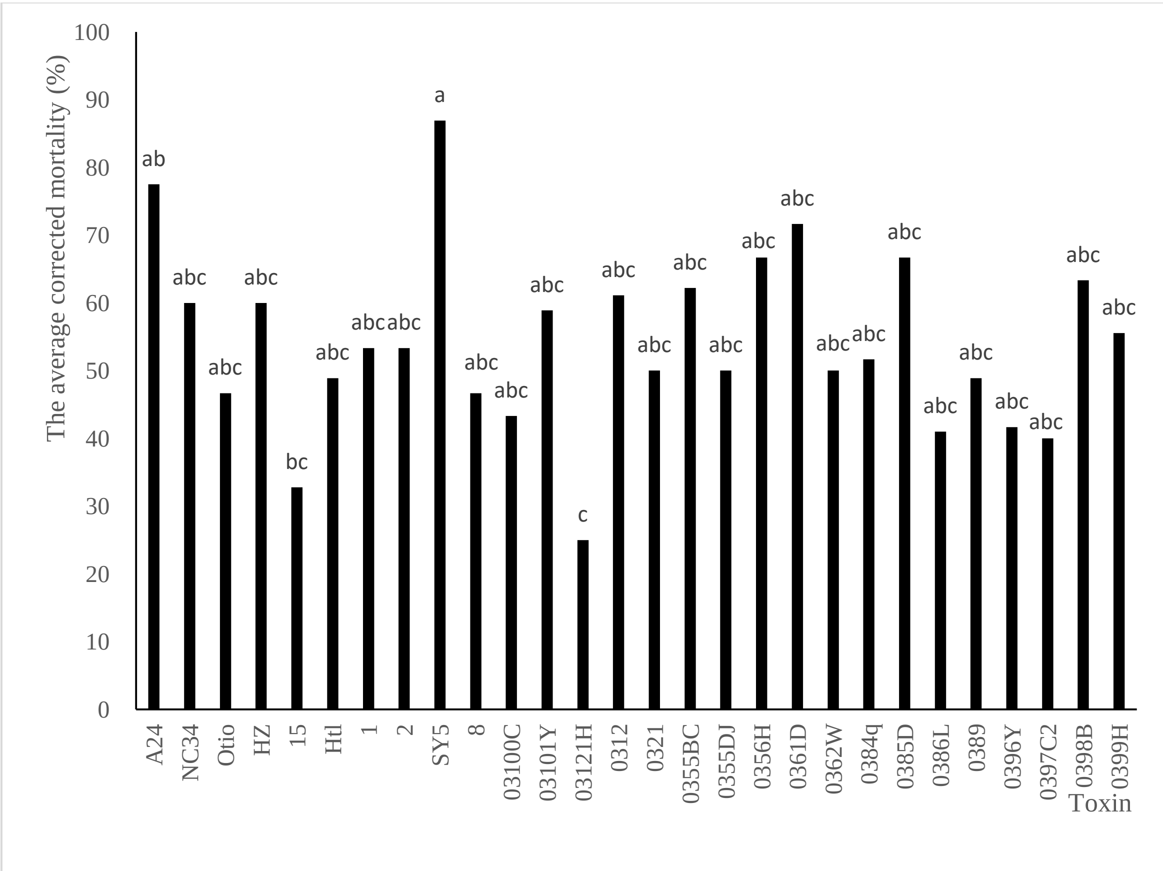
3.2 Insecticidal Activity of Toxins against *L. exigua*

At 3rd day, 4th day and 5th day, SY5 had the most obvious insecticidal activity among the 28 strains of symbiotic bacteria toxin (Fig. 2). The average corrected mortality rates were 66.14% (Fig. 2A), 67.03% (Fig. 2B) and 68.52% (Fig. 2C), respectively. The average corrected mortality to *L. exigua* of SY5 toxin was not significantly different from the broth of strains A24 and 0362W, but its average inhibiting rate of larval weight was more than these two strains (Fig. 2D).

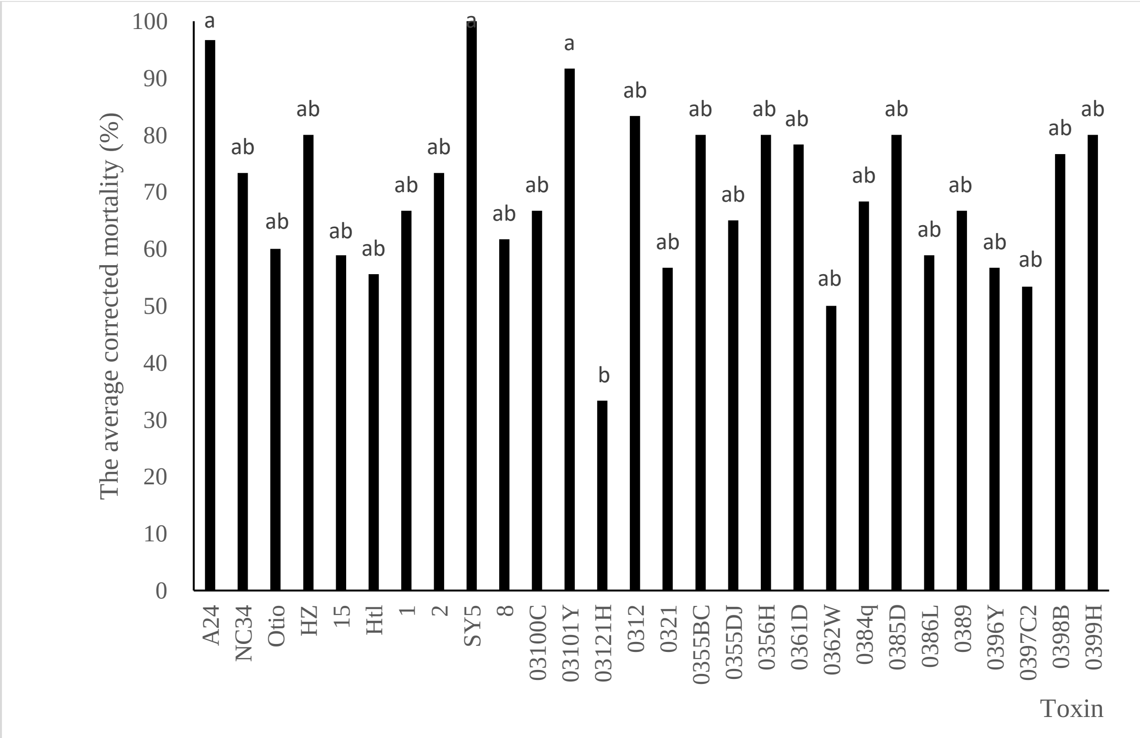
Taken together, the toxin of strain SY5 had the highest virulence to *P. xylostella* and *L. exigua*. Therefore, SY5 was selected as the most highly virulent symbiotic bacteria for further study.


The average corrected mortality (%)
Toxin
A24
NC34
Otio
HZ
15
Htl
1
2
SY5
8
03100C
03101Y
03121H
0312
0321
0355BC
0355DJ
0356H
0361D
0362W
0384q
0385D
0386L
0389
0396Y
0397C2
0398B
0399H

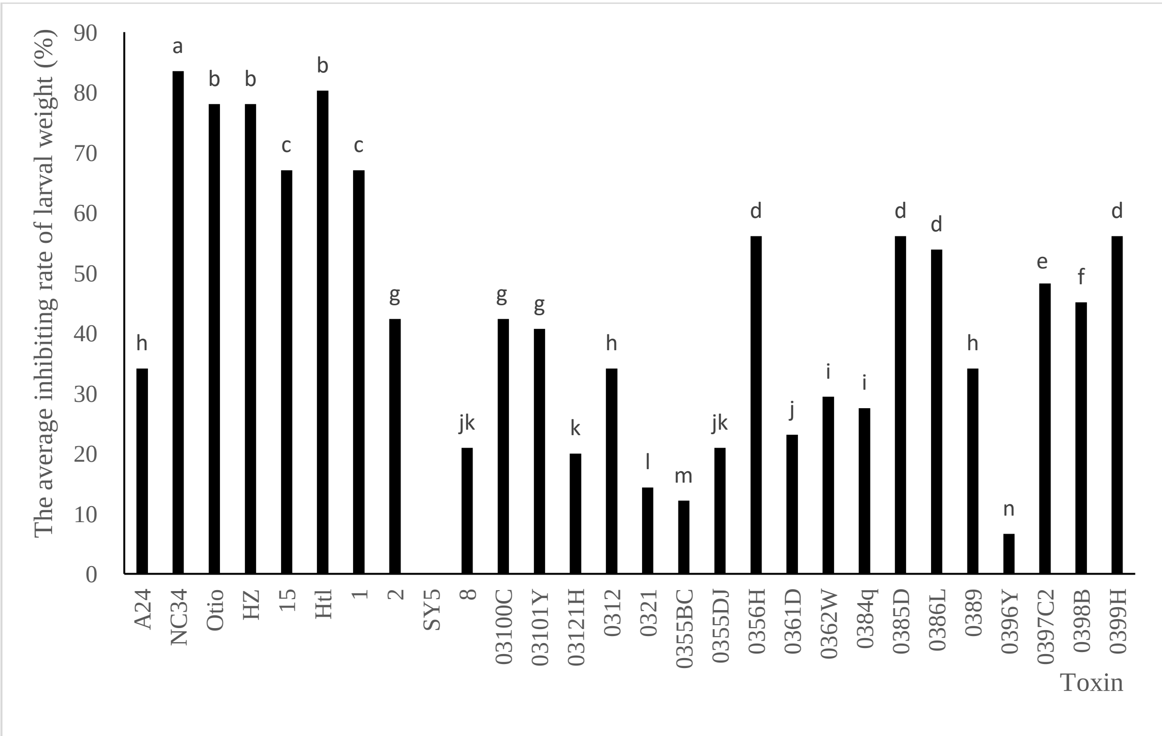
A


The average corrected mortality (%)
Toxin
A24
NC34
Otio
HZ
15
Htl
1
2
SY5
8
03100C
03101Y
03121H
0312
0321
0355BC
0355DJ
0356H
0361D
0362W
0384q
0385D
0386L
0389
0396Y
0397C2
0398B
0399H

B

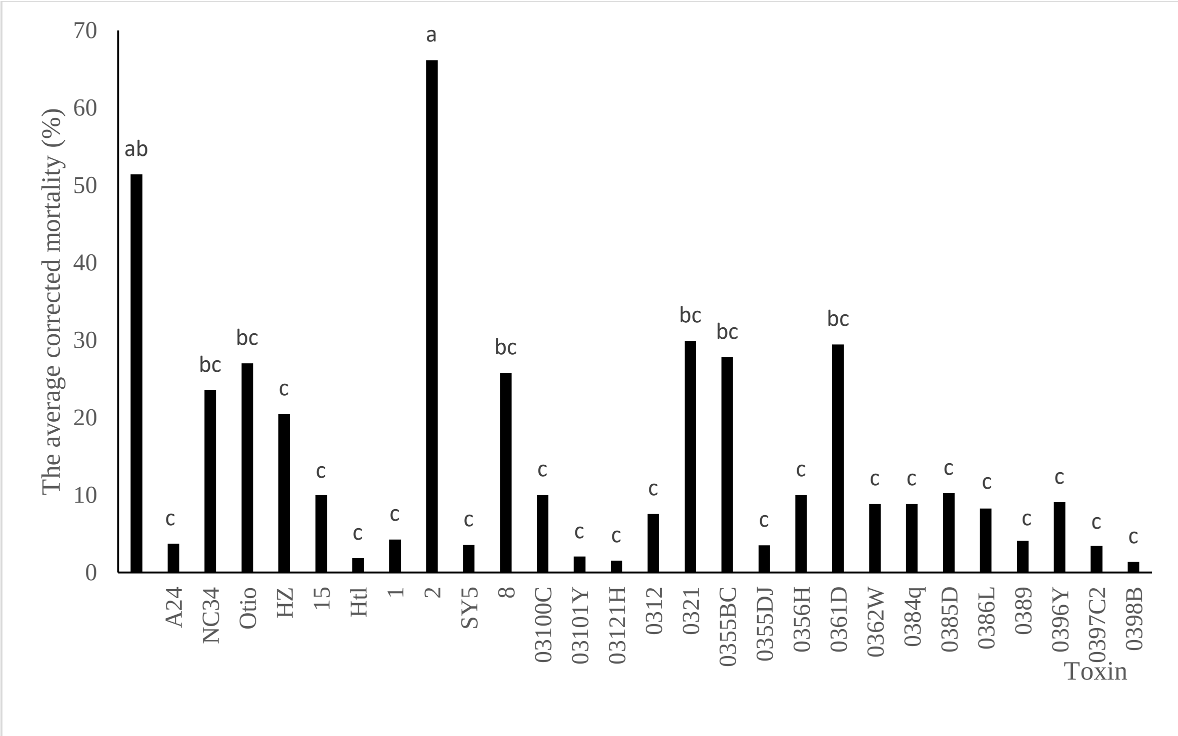


C

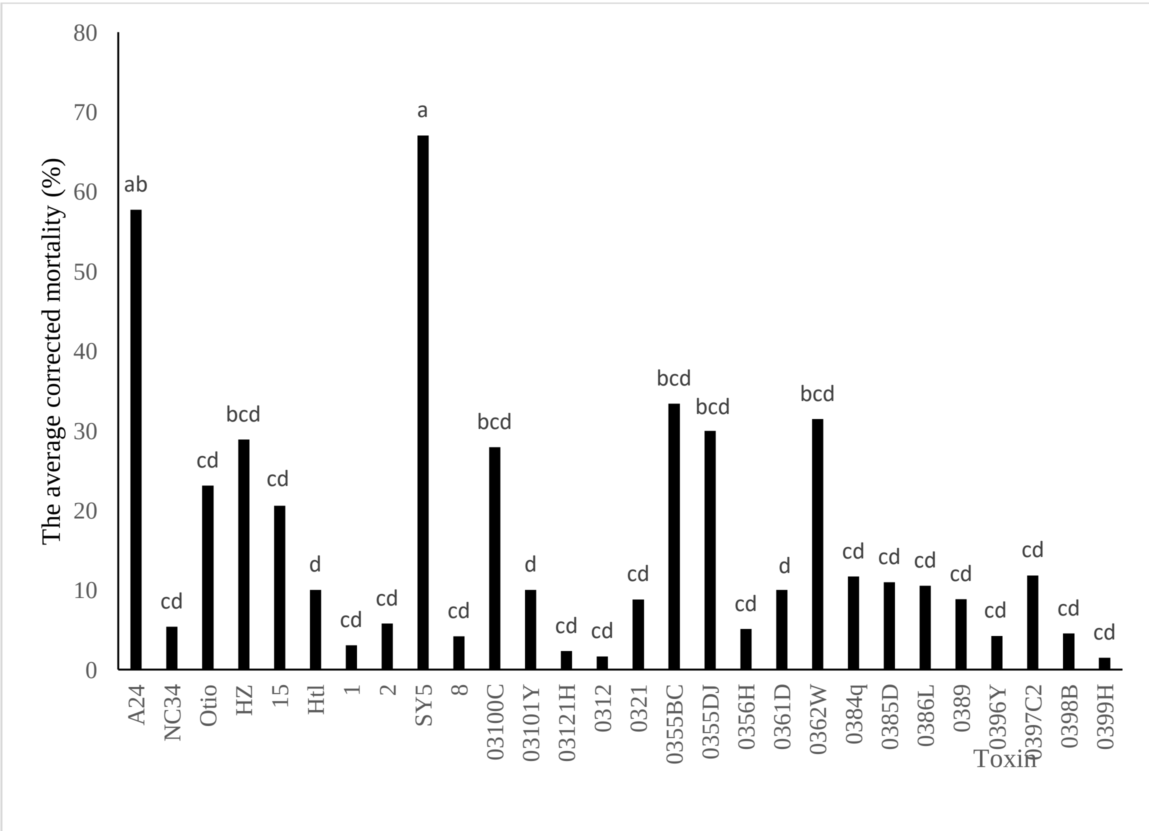


D

Fig. 1. Insecticidal activity of toxins against *P. xylostella*: A) The average corrected mortality of 3d; B) The average corrected mortality of 4d; C) The average corrected mortality of 5d; D) The average inhibiting rate of larval weight


The average corrected mortality (%)
Toxin
A24
NC34
Otio
HZ
15
Htl
1
2
SY5
8
03100C
03101Y
03121H
0312
0321
0355BC
0355DJ
0356H
0361D
0362W
0384q
0385D
0386L
0389
0396Y
0397C2
0398B

A


The average corrected mortality (%)
Toxin
A24
NC34
Otio
HZ
15
Htl
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SY5
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03100C
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03121H
0312
0321
0355BC
0355DJ
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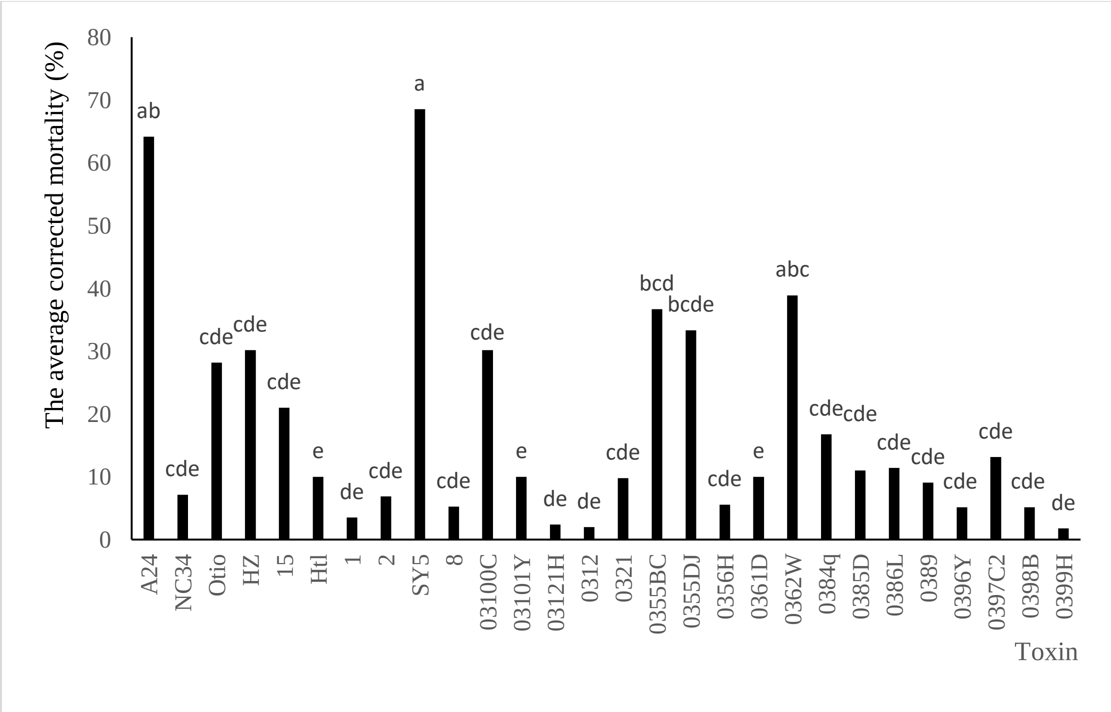
B



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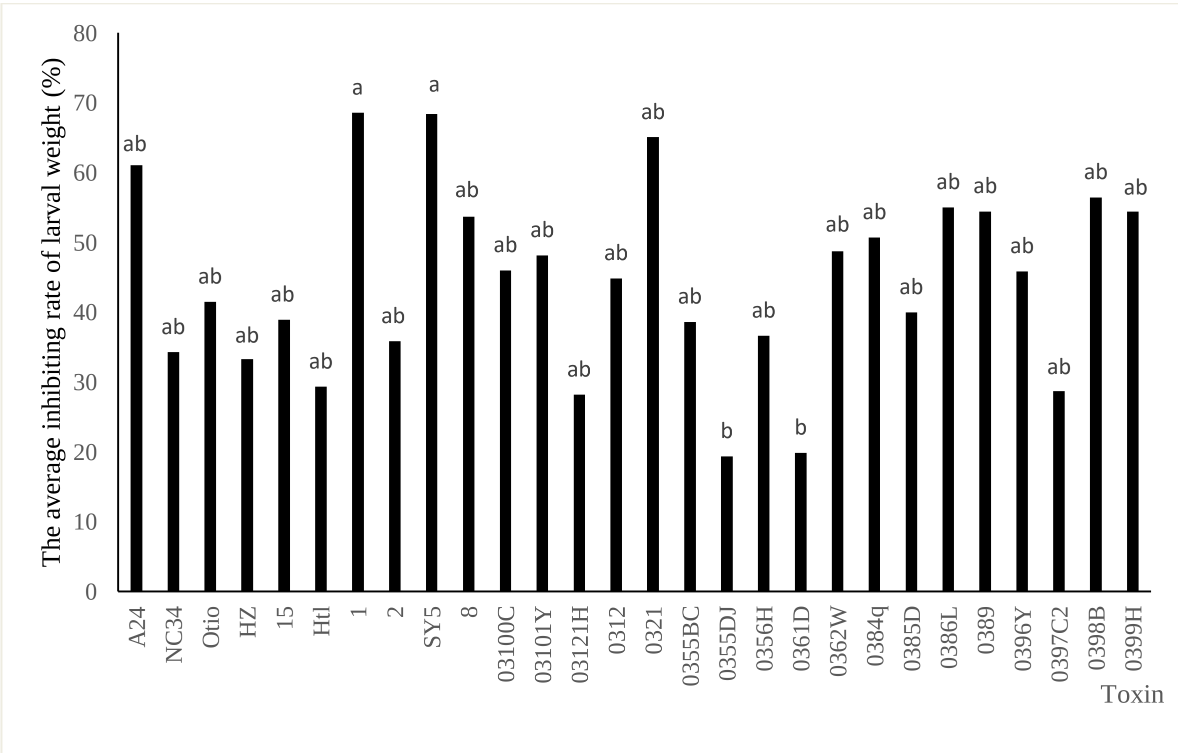
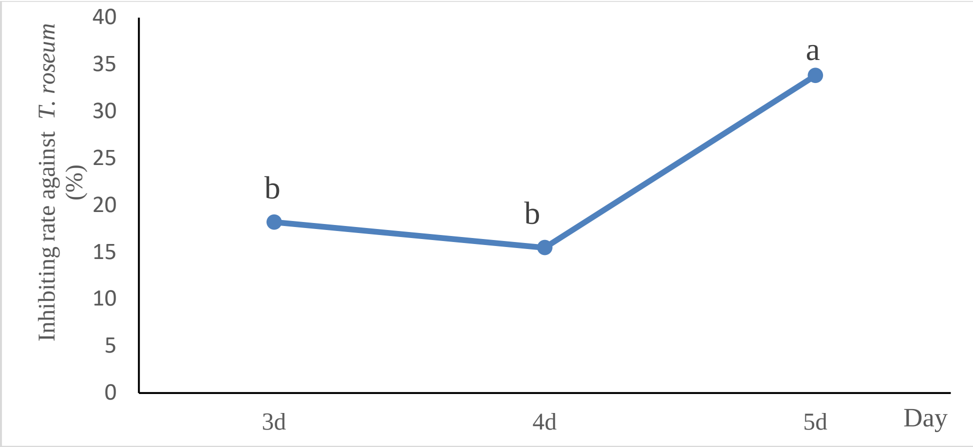
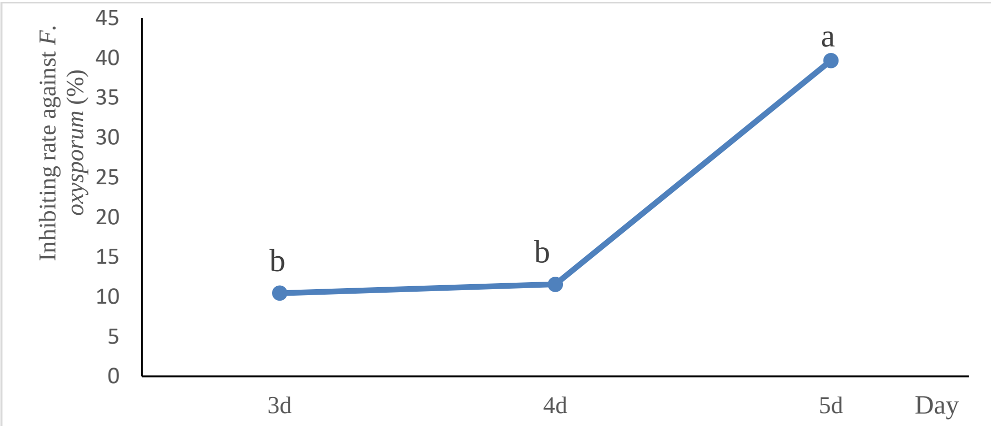


D

Fig. 2. Insecticidal activity of toxins against *L. exigua*: A) The average corrected mortality of 3d; B) The average corrected mortality of 4d; C) The average corrected mortality of 5d; D) The average inhibiting rate of larval weight



A



B

Fig. 3. Antifungal activities of the toxin to *T. roseum* and *F. oxysporum*: A) The inhibiting rate against *T. roseum*; B) The inhibiting rate against *F. oxysporum*

3.3 Antifungal Activities of the Toxin to *T. roseum* and *F. oxysporum*

Our results showed that the toxin of the symbiotic bacteria SY5 had antifungal activity against *T. roseum* and *F. oxysporum*. As shown in Fig. 3, the antifungal activities increased over time. The inhibiting rate against *T. roseum* and *F. oxysporum* at 5th day were 33.82% and 39.66%, which significantly raised comparism to the inhibiting rate of 3d and 4d.

4. DISCUSSION

Green food and biological pesticides are the priority development agenda in agricultural production, and the research and development of biological control, ecological control and other alternative control technologies are the main way to achieve this agenda. *Bacillus thuringiensis* (Bt) is the most wildly used biocontrol bacteria. With the extensive use of Bt products, the resistance of agricultural pests (such as *P. xylostella*) is becoming more and more obvious [19]. So, it is necessary to find some new biocontrol resource for the control and management of pests and plant diseases.

As an important biological control resource, EPN have been used to control a variety of agricultural, forestry, grassland, flower, and sanitary pests such as grubs, leeks, and cutworms [19-21]. EPNs and their symbiotic bacteria have a wide range of activity against parasitic pests, and can produce different types of insecticidal toxins. Studying on such bacteria and their insecticidal substances are helpful for developing new microbial insecticides,

insecticides toxins and genes. "In different strain types and species, the antibiotic production of *Xenorhabdus* and *Photorhabdus* are different qualitatively and quantitatively" [22-26]. In this study, the toxin was the extracellular protein of native isolated *Xenorhabdus* and *Photorhabdus*. Our result showed that the insecticidal activities of toxin were tested differently among different strains. The toxin of SY5 showed higher larval mortality than the other stains. The insecticidal activities of symbiotic bacteria SY5 toxin against the two pest were different, which may be due to the different ability of different insects to respond to the toxins. The strain also showed good antifungal activity against two vegetable disease, *T. roseum* and *F. oxysporum*.

There have been many reports on the toxins and genes of the symbiotic bacteria of EPN [27-29]. The strains used in this experiment were all collected and isolated in China. Insecticidal activity substances are separated, purified and identified in order to discover new insecticidal substances and insecticidal genes. The results will provide new materials for the development of new microbial insecticides, insecticidal genes and new materials for the biological control of vegetable pests.

5. CONCLUSION

In this study, the toxins from 28 strains of the symbiotic bacteria were extracted. The highly virulent strains SY5 was screened through bioassay. This strain had the highest insecticidal activity against *P. xylostella* and *L. exigua*, and good antifungal activity against *T. roseum* and *F. oxysporum*. The study provided an alternative resource for controlling pests and diseases of vegetables. The results of the present study will be helpful for the development of new microbial insecticides, insecticides toxins and will provide new ways and methods for vegetable pest and fungal plant disease biological control.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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