Short Research Article

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

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 pest as biocontrol agents.

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-symbiotic bacteria (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]. Entomopathogenic nematodes (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 activities in the use of nematode for controlling insect pests or soil plant pathogens was emphasized [6], as well as in Japan, where the development of commercial nematodes was available. 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 entomopathogenic nematodes out of the 23 species obtained from soil samples collected from different regions of China. The bacterial strain names were given according to the entomopathogenic nematode 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 wereobtained.

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.

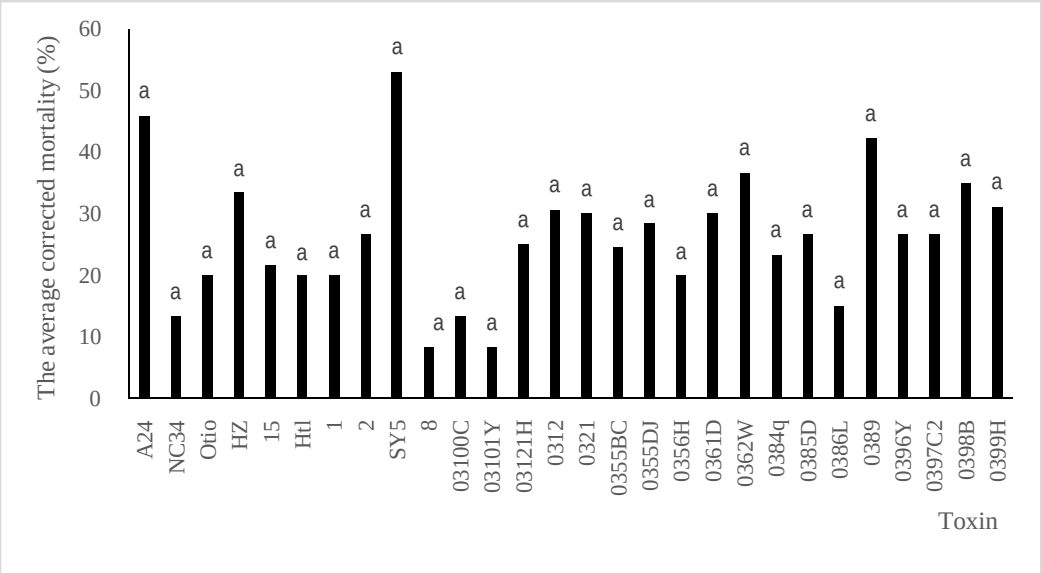
The toxin (50 μg /ml)) and cooled PDA agar were mixed as 1ml: 25 ml in 9 cm Petri dishes. The control dishes were 1 ml distill water and 25 ml cooled PDA agar. The plant pathogenic fungi plug (5 mm, kindly provided by Shenyang Chemical Engineering Research College.) was added to the center of the dish after the mixed PDA solid. All dishes were incubated at 28℃ 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*



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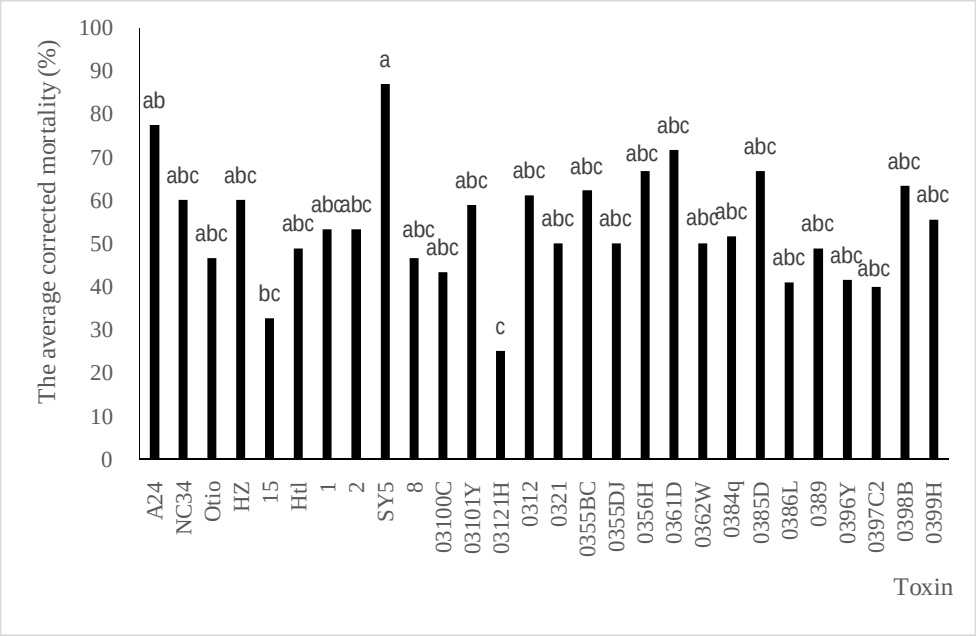

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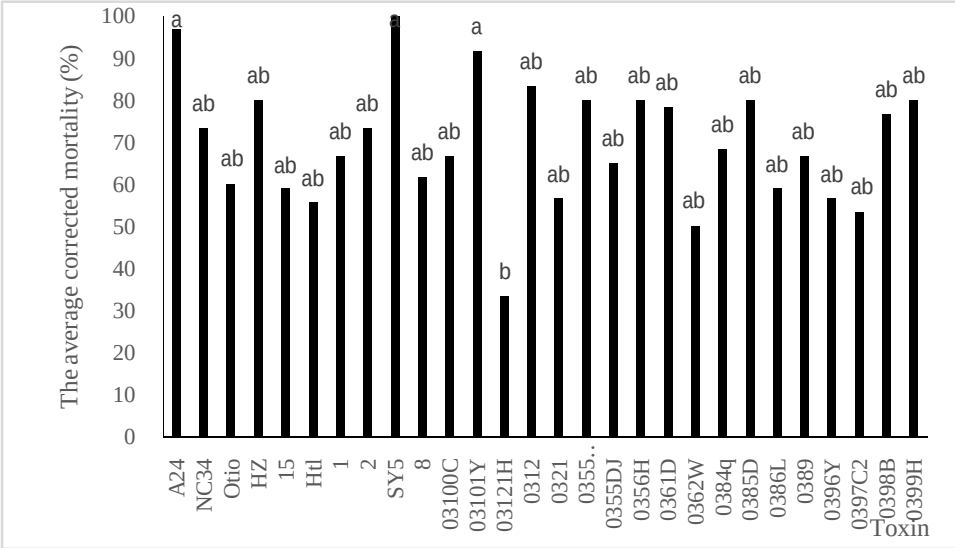

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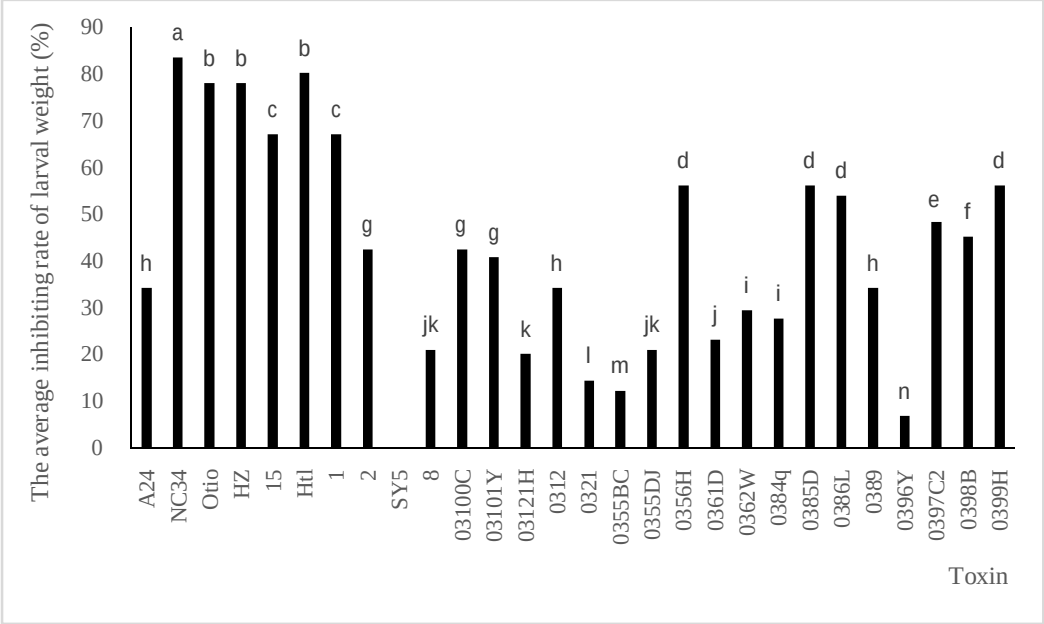


D

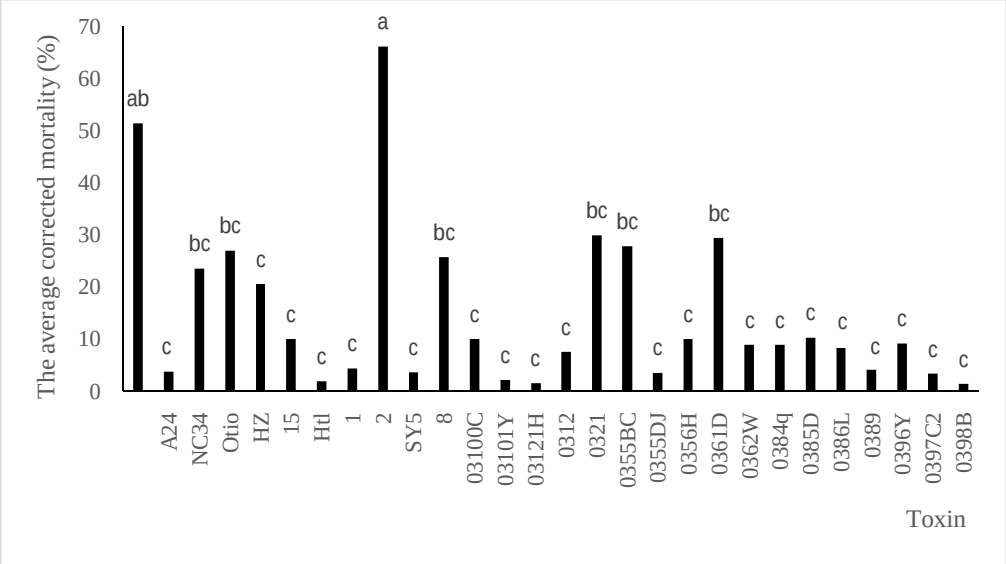
Fig.1 Insecticidal activity of toxins against *P. xylostella*: A) The average corrected mortality of 3d; B) The average corrected mortality of 4d; C) The average corrected mortality of 5d; D) The average inhibiting rate of larval weight

The 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*



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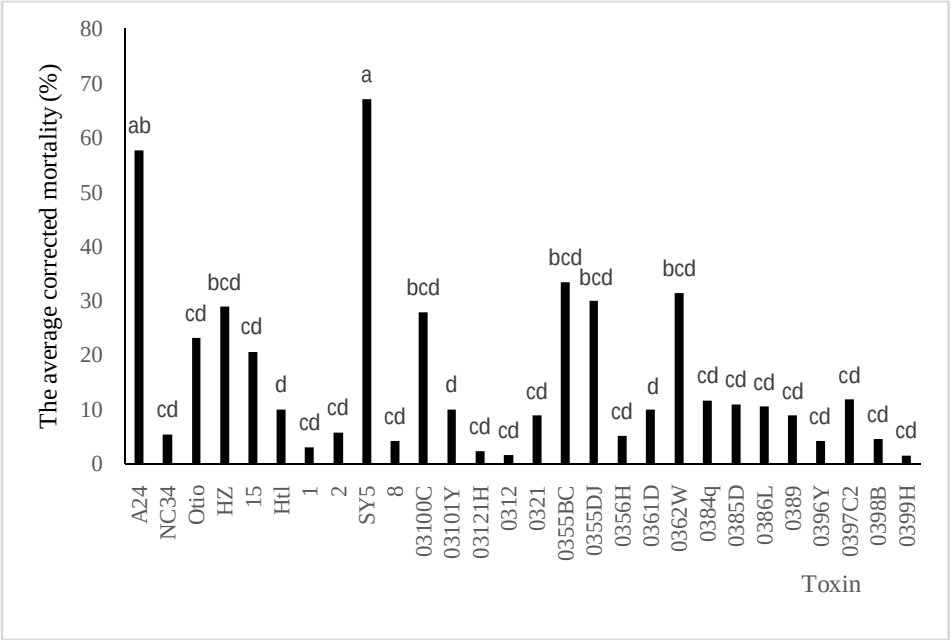
The average corrected mortality (%)
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03101Y
03121H
0312
0321
0355BC
0355DJ
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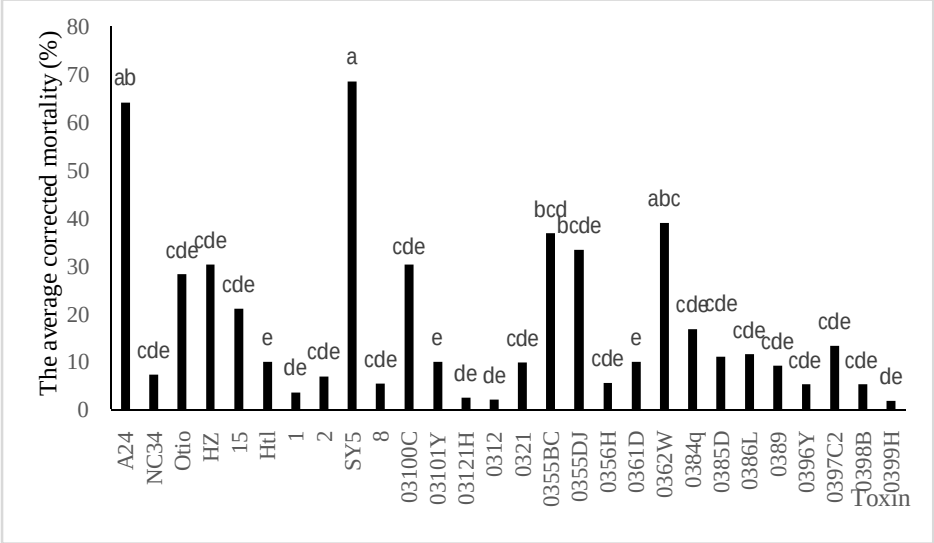
The average corrected mortality (%)
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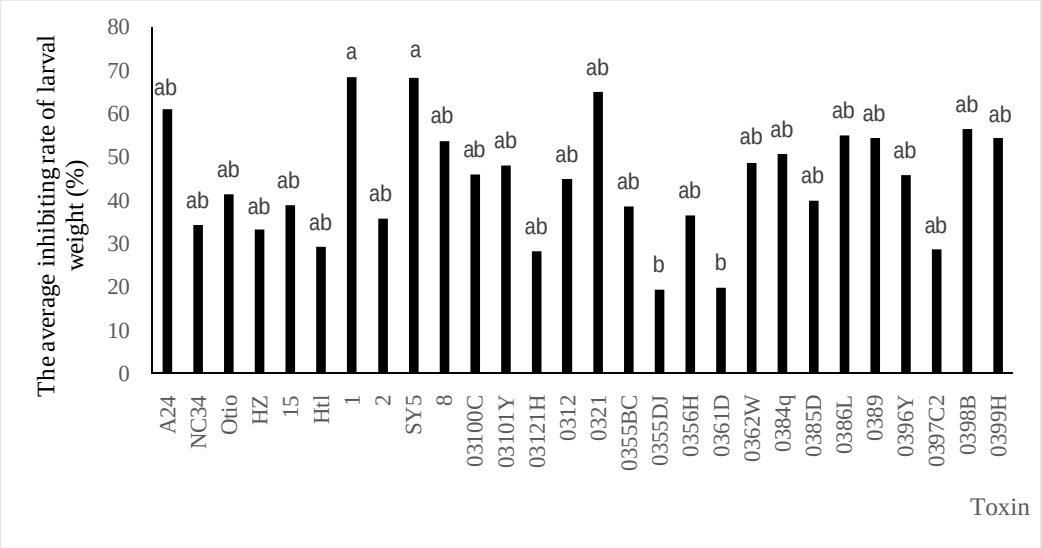

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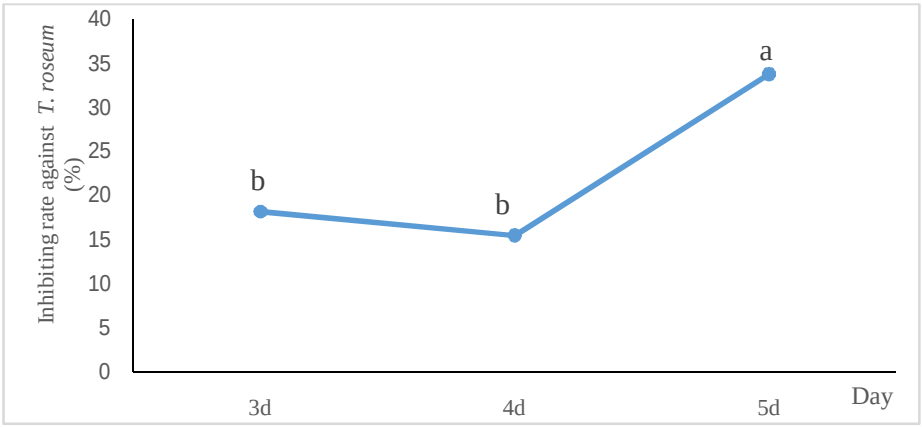
Fig.2 Insecticidal activity of toxins against *L. exigua*: A) The average corrected mortality of 3d; B) The average corrected mortality of 4d; C) The average corrected mortality of 5d; D) The average inhibiting rate of larval weight

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.

3.3 Antifungal activities of the toxin to *T. roseum* and *F. oxysporum*



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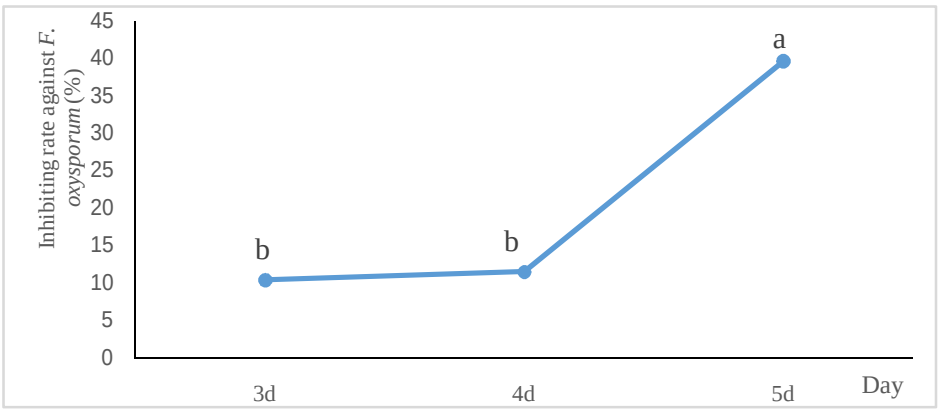

B

Fig3. 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*

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 entomopathogenic nematodes [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.

ACKNOWLEDGMENT

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REFERENCES

1. Ren YF, Gao P, Zhu F. Meteorological grade prediction of rice blast incidence in Jiangsu Province. Jiangsu Agricultural Science. 2016; 44(8): 151-154.
2. Liu AX, Zhang H, Ji CQ. Research on supporting technologies for green prevention and control of watermelon fusarium wilt. Agricultural Staff; 2017(13): 13.
3. Monika G, Benjarong KT. First detection of *Trichothecium roseum* causing leaf spots on tomato in Germany. Plant Dis. 2022; doi: 10.1094/PDIS-07-22-1588-PDN. Online ahead of print.
4. Owen H, James CF, Alexi KD, Nicholas SD, Md Emran A. *Fusarium oxysporum* f. sp. niveum molecular diagnostics past, present and future. Int J Mol Sci. 2021; 22(18): 9735.
5. Sun R, Wu H, Gong Q, Zhang Y, Sheng X. Key technologies for controlling several fruit tree pests using entomopathogenic nematodes. Deciduous Fruit Trees. 2018; 50(5): 40-41.
6. Tomar P, Thakur N, Yadav A. Endosymbiotic microbes from entomopathogenic nematode (EPNs) and their applications as biocontrol agents for agro-environmental sustainability. Egy. J. Biological Pest Control. 2022; 32: 80-98.

7. Kaya H, Aguillera M, Alumai A, Choo H, Torre M, Fodor A, Ganguly S, Hazir S, Lakatos T, Pye A, Wilson M, Yamanaka S, Yang H, Ehler R. Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. Biological Control, 2006; 38: 134–155.
8. Qiu X, Han R. General situation of entomopathogenic nematodes resources and advances in classification technology. Acta Entomology. 2007; 3: 286-296.
9. Wu W, Yin J, Cao Y. Research and application status of entomopathogenic nematodes in my country. Chinese Journal of Biological Control. 2014; 30(6): 817-822.
10. Jouzani GS, Valijanian E, Sharafi R. *Bacillus thuringiensis*: a successful insecticide with new environmental features and tidings. Appl Microbiol Biotechnol. 2017; 101(7): 2691-2711.
11. Melo AL, Soccol VT, Soccol CR. *Bacillus thuringiensis*: mechanism of action, resistance, and new applications: a review. Crit Rev Biotechnol. 2016; 36(2):317-26.
12. Sun G. American Mycogen company found BT strain with nematicidal activity. Biotechnology Bulletin. 1990; 7:13-14.
13. Yang XF, Qiu DW, Zhang YL, Zeng HM, Liu Z, Yuan JJ, Yang HW. A toxin protein from *Xenorhabdus nematophila* var. *pekingensis* and insecticidal activity against larvae of *Helicoverpa armigera*. Biocontrol Sci. Techn. 2009; 19: 943-955.
14. Yang XF, Qiu DW, Yang HW, Liu Z, Zeng HM, Yuan JJ. Antifungal activity of xenocoumacin 1 from *Xenorhabdus nematophila* var. *pekingensis* against *phytophthora infestans*. World J. Microb. Biot. 2011; 27: 523-528.
15. Selvan S, Gaugler R, Campbell JF. Efficacy of entomopathogenic nematode strains against *Popillia Japonica* (Coleoptera: Scarabaeidae) larvae. Journal of Economic Entomology. 1993; 86: 353-359.
16. Sergeant M, Baxter L, Jarrett P, Shaw E, Ousley M, Winstanley C, Morgan JAW, Identification, typing, and insecticidal activity of *Xenorhabdus* isolates from entomopathogenic nematodes in United Kingdom soil and characterization of the

xpt toxin loci. Applied and Environmental Microbiology. 2006; 72: 5895-5907.

17. Furgani G, Böszörményi E, Fodor A, Máthé-Fodor A, Forst S, Hogan JS, Katona Z, Klein MG, Stackebrandt E, Szentirmai A, Sztaricskai F, Wolf SL. *Xenorhabdus* antibiotics: a comparative analysis and potential utility for controlling mastitis caused by bacteria. Journal Applied Microbiology. 2008; 104: 745-758.
18. Waterfield NR, Ciche T, Clarke D, *Photorhabdus* and a host of hosts. Annual Review of Microbiology. 2009; 63: 557-574.
19. Ogier JC, Pages S, Frayssinet M. Entomopathogenic nematode-associated microbiota: from monoxenic paradigm to pathobiome. Microbiome. 2020; 8(1): 25.
20. Cong B, Liu W, Yang H. History, current situation and prospect of research and utilization of entomopathogenic nematodes. Journal of Shenyang Agricultural University. 1999; 3: 343-353.
21. Kaya HK. Entomophogenic nematodes. Ann Rev Entomol. 1993; 38: 181-206.
22. Yu Z. Insect resistance mechanism of insect resistance gene and its application status and prospect. Bulletin of Biology. 2000; 35(7): 8-10.
23. Han R, Ehlers RU. Pathogenicity, development, and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic in vivo conditions. Journal of Invertebrate Pathology. 2000; 75(1): 55-58.
24. Poinar GO. Taxonomy and biology of Steinernmatidae and Heterorhabditidae. In: Gaugler R., Kaya H. Entomopathogenic nematodes in biological control. Boca Raton, CRC Press, 1990; 23-61.
25. Ji GH, Qian XJ, Xing YF, Liu CZ. Changes of the activities of protective enzymes in *Pieris rapae* infected by *Steinernema feltiae*. Plant Protection. 2009; 35(4): 66-69.
26. Zhang SJ, Qian XJ, Li CJ, Pan FJ, Xu YL. Pathogenicity of entomopathogenic nematode on Xestia c-nigrum in Soybean Field. Soybean Science. 2013; 32(1): 63-67.
27. Qian XJ, Gu LN, Xing YF, Liu CZ. Occurrence of entomopathogenic nematodes in Gansu Province. Acta Agrestia Sinica. 2014; 22(3): 593-599.

28. Bowen DJ, Ensigh JC. Purification and characterization of a high molecular weight insecticidal protein complex produced by the entomopathogenic bacterium *Photorhabdus luminnescens.* Applied and Environmental Microbiology. 1998; 8: 3029-3035.
29. Bowen D, Rocheleau TA, Blackburn M, Andreev O, Golubeva E, Bhartia R, ffrench-Constant RH. Insecticidal toxins from the bacterium *Photorhabdus luminescens.* Science. 1998; 280: 2129-2132.