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Bioinsecticide Entomopathogenic Nematodes as Biological Control Agent for Sustainable Agriculture

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ABSTRACT

Entomopathogenic nematodes (EPNs) of the genera *Heterorhabditis* and *Steinernema* are available in the market for use as pest control agents. They are symbiotically associated with bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively. Mainly, media development of EPNs as biological control agents is directed towards cost reduction, and it is possible for a variety of protein sources to be metabolised by the bacteria for optimal conditions for nematode reproduction. The aim of this research is to examine the LC_{50} with leaf disc assays at concentrations of 0.01, 0.10, 1, 10, 100 and 1000 ppm using TUREK® (*Bt* var *aizawai*), BITE® (*Bt* var *aizawai*) and THURICIDE® (*Bt* var *kurstaki*) on larvae of *P. xylostella* (n=180). The results from the field trial clearly indicated that the biocontrol agent *B. thuringiensis* (*Bt*) is superior to the chemical insecticide. *B. thuringiensis* is accepted as safe, readily mass produced, highly susceptible and easily formulated and applied as biological control agents for sustainable agriculture. Recent scientific progress has been helpful in providing better understanding of the biological and technical parameters that influence the process, thus enabling transfer of knowledge and application to industry. As a consequence, costs for nematode-based products can be significantly reduced.

Keywords: Biological control, economic sustainable development, entomopathogenic nematodes, mass production, symbiotic bacteria

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INTRODUCTION

Among antagonist insects that are found in the phylum nematoda, only the species within the genera *Steinernema* and

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Heterorhabditis (Rhabditida) are known to play a significant role as biocontrol agents for plants. More than 30 species of what are known as entomopathogenic nematodes (EPNs) have been analysed and it is believed there are many more species to be discovered (Hominick et al., 1997). EPNs that are closely related to *Caenothabditis elegans* is the current model organism used to analyse animal growth and genetics (Riddle et al., 1997) and is genome sequence has just been completed. Unique to EPNs is their close symbiotic relationship with bacteria of the genera *Xenorhabdus* and *Photorhabdus.* Techniques have been developed to use them as biological control agents in industry scale bioreactors and many small- and medium-size enterprises (SMEs) have started to produce and market EPNs.

Today in Indonesia, *Heterorhabditis* spp. and *Steinernema* spp. are the species applied in biocontrol of white grubs (*Lepidiota stigma*) and caterpillars (*Spodoptera litura, Spodoptera exigua)*. Production costs for these nematodes have dropped mainly due to enhancement of production process stability and significant increase of yields. In the following sections of this paper the biological control and technical factors influencing the success of biotechnical production systems in SMEs in Indonesia will be discussed.

Not many strains of symbiotic bacteria have been analysed and studied in detail. Forst and Nealson (1996) explained their molecular biology, and this drew significant attention to symbionts as their marketability was revealed. It has also been shown that symbiotic bacteria in insecticidal metabolites become active on ingestion by insects, causing symptoms in the gut analogue of *Bacillus thuringiensis* δ-endotoxin (Blackburn et al., 1998). They are a good alternative to *B. thuringiensis* (*Bt*) toxin genes for expression in transgenic plants (Guo et al., 1999).

The type of symbionts in both genera are varied. It is believed that there are two extreme phases, the primary phase and the secondary phase (Akhurst, 1980). Additional phases have been shown by Gerritsen and Smits (1997). The main phase is differentiated from the *dauer juveniles* (DJ) or infected insects, while the next phase appears either after or before subculturing, as the nematodes emigrate from the cadaver (Grunder, 1997). The secondary phase is not retained by the DJs of *H. bacteriophora* (Han & Ehlers, 2001). Krasomil-Osterfeld (1995) induced the secondary phase by cultivating a primary form of the species under stress conditions.

Subcultures that are placed under long stress conditions result in stable secondary-phase cultures. Although several metabolic functions are lost by the next form, for example, production of protease, lipase, intracellular crystalline proteins, antibiotics and pigments (Boemare & Akhurst, 1988), the negative impact is that secondary-phase bacteria can have a major and detrimental impact on nematode growth

and yields (Völgyi et al., 2001). Bintrim and Ensign (1998) believed that one major ingredient for nematode nutrition is crystalline inclusion protein. However, Hussein and Ehlers (2001) pointed out that this is not the only essential nutritional factor yielded in the primary phase. All necessary steps must be taken to stop phase variation.

Phase changes are usually avoided by minimising stress caused by lack of oxygen, high temperature and deviation from the optimal osmotic strength of medium during bacterial inoculum production, inoculation and pre-culture. The mechanisms during transition between phases are yet unresolved, although genetic variation has been excluded (LeClerc & Boemare, 1991; Akhurst et al., 1992; Wang & Dowds, 1993).

EPNs provide several benefits that qualify them as commercially valuable biocontrol agents. They have a high level of effectiveness as they often exceed the control outcomes achieved using chemical compounds. Unlike chemicals, which should not be displaced by water in the soil and which decay within a few days, EPNs are mobile and persistent. The use of EPNs is considered safe for both the user as well as the environment. They have little detrimental effects on non-target insect populations and neither the nematodes nor their bacterial associates cause any detrimental effects to mammals or plants (Bathon, 1996; Ehlers & Hokannen, 1996).

In most countries, EPNs are not required to be registered, and this allows small- and medium-sized enterprises to increase the development of nematode-based plant protection products. Furthermore, EPNs can be placed in safe storage for quite a long time (several months), and this makes them marketable. *Dauer juveniles* (DJs) are considered resistant to shear forces. Hence, they can be applied using conventional spraying equipment. As the control potential of EPNs is not limited by customary agrochemicals, they can be integrated into standard chemical control practice. Nowadays, nematodes are mainly used in environments that do not tolerate chemical compounds i.e. in soil, insect galleries or in environments developed to resist insecticides.

This study laid the basis for *in-vitro* production. The research was mostly focussed on the potential biocontrol of *Steinernema carpocapsae* (all strains) and TUREK® (*Bacillus thuringiensis* [*Bt*] var. *aizawai*) on *Plutella xylostella* and *Crocidolomia binotalis* in cabbage crops at Bromo Mountain, Probolinggo region, East Java, Indonesia. Only the presence of symbiotic bacteria in monoxenic cultures produced suitable conditions for nematode reproduction with high numbers of offspring. Uneven distribution of nematodes in the medium prevented systematic sampling; thus, improvement of the technique used is needed. The exploitation of the potential of EPNs in this study for plant protection required the development of liquid culture technology.

MATERIALS AND METHODS

Evaluation of Diamont Black Moth (DBM) Resistance to *Bacillus thuringiensis* (*Bt*)

EPNs were grown on Petri dishes using different agar media (House, Welch, & Cleugh, 1965; Wouts, 1981; Dunphy & Webster, 1989). The tests were done to determine the LC_{50} with leaf disc assays at concentrations of 0.01, 0.10, 1, 10, 100 and 1000 ppm using TUREK® (*Bt* var *aizawai*), BITE® (*Bt* var *aizawai*) and THURICIDE® (*Bt* var *kurstaki*) on larvae of *P. xylostella* (n=180). The insects were collected from four regions in East Java, Malang, Probolinggo, Jember and Bondowoso, and maintained in the laboratory.

Testing Combination of Biocontrol Agents

The formulations provided by Chirstian Albrecht-University zu Kiel, Germany were field-tested in cabbage crops cultured at Bromo Mountain, Probolinggo (East Java, Indonesia). The biocontrol agents used was *S. carpocapsae* (all strains) at concentration of 500.000 IJ/m^2 in the following formulations to control *P. xylostella* and *C. binotalis* larvae: K=EPNs in water, F1=EPNs in water with wetting agent, F2=EPNs in the BeXaRi formulation (0.3% Bevaloid, 0.3% xanthan, 0.3% Rimulgan), F3=BeXaRi supplemented with the wetting agent Agristic (0.025% alcarylpolyglykol-ether). EPNs were sprayed on Days 14 and 28 after planting of the cabbage crop. All applications were sprayed at 4 pm with a knapsack sprayer of volume 15 L. The crop was planted with 50 x 60 cm space for each plant. Each experimental plot contained 100 plants with a number of live (Diamont Black Moth) DBM larvae in each block. The population of *P. xylostella* larvae from 10 cabbage plants that had been sampled randomly from each plot was counted.

Field Testing of EPNS and Novel Formulation

Field tests were done to evaluate the potential biocontrol of *Steinernema carpocapsae* (all strains) and TUREK® (*Bt* var. *aizawai)* on *P. xylostella* and *C. binotalis* in cabbage crops on Bromo Mountain, Probolinggo region, East Java, Indonesia. The EPNs used were *S. carpocapsae* (all strains) produced in liquid culture in China. The field trial was conducted from March to September, 2002 with *S. carpocapsae* (all strains) and TUREK® (*Bt* var. *aizawai*) to control *P. xylostella* and *C. binotalis*. The wetting agents used were Agristic®, Alkilarilpoliglikol eter (0.025% L^{-1}). Concentration used for *Bt* was 1 gL^{-1} and *S. carpocapsae* 0.5 million m^{-2}. All applications were conducted at 4 pm with a knapsack sprayer of volume 15 L after 14 days of planting cabbage crops. Three different treatments were used: W (wetting agent with water), BtW (*Bt* with wetting agents) and Bt (*Bt* with water). The EPN treatments were conducted as W (wetting agents and water), NW (nematodes with water) and NfW (nematodes with wetting agents).

Field Testing of Biological Control Agents *Bacillus thuringiensis* (*Bt*)

Field trials were done to evaluate the control potential of *Steinernema carpocapsae* (all strains) against *P. xylostella* and *C. binotalis* in the cabbage crops. Cabbage crops were planted in January 2003. Trials were performed at Ijen Mountain, Bondowoso, East Java, Indonesia, which is approximately 60 km away from Jember. The EPN material of *S. carpocapsae* (all strain) was produced in solid media according to the Bedding method at Jember University, Indonesia with liquid culture from China. The design of the field trial followed the rules of the Random Complete Block Design (RCBD) with the following four treatments: PO: control with water only; PN: 500.000 IJ m^{-2} *S. carpocapsae* sprayed with wetting agent (AGRISTIC®); PP: 500 g L^{-1} profenofos (CURACRON®); and PI: *Bt var. aizawai* (TUREX®) 1 gL^{-1} with wetting agent (AGRISTIC®). Four plots for each treatment of size 5 x 6 m were planted with approximately 100 plants in each plot with 50 cm between each plant and 60 cm between the rows. *S. carpocapsae* treatment was applied every two weeks, while *B. thuringiensis* and insecticide was applied every week. *S. carpocapsae* was applied on Days 12, 26, 40, 54, 68 and 82 of the cabbage crops and *B. thuringiensis* was applied on days of nematode application and also on Days 19, 33, 47, 61, 75 and 89. All applications were sprayed at 4 pm using a knapsack sprayer of volume 15 L.

RESULTS AND DISCUSSION

Testing Combination of Biocontrol Agents

Diamont Black Moth (DBM) resistance to *Bacillus thuringiensis* (*Bt*) was evaluated. The results of using LC_{50} of TUREK® (*Bt.* var. *aizawai*) on *P. xylostella* are presented in Table 1.

Table 1
The ratio resistance of LC_{50} of TUREK® (*Bt.* var. *aizawai*) *on P. xylostella tested in different East Java region*

East Java Region	LC_{50}	Ratio Resistance
Jember	2.12 ppm	1
Malang	6.17 ppm	6.77
Bondowoso	22.85 ppm	10.78
Probolinggo	18.18 ppm	18.18

A low concentration of 2.12 ppm of LC_{50} was used in Jember region producing a ratio resistance of 1, while in Malang region 6.77 ppm was used and an rr of 6.77 was obtained, in Probolinggo region 18.18 ppm was used and an rr of 18.18 was obtained and in Bondowoso, 22.85 ppm was used and an rr of 10.78 was obtained. *P. xylostella* larvae from Jember region was more susceptible to *Bt* than DBM from

the other three regions in East Java, namely Malang, Probolinggo and Bondowoso. The lowest LC_{50} was recorded for TUREK® (*Bt var. aizawai*) on *P. xylostella* population from Jember region (rr=1). The highest LC_{50} was concluded for TUREK® (*Bt var. aizawai*) on *P. xylostella* population from Probolinggo region (rr=18.18). The results indicated that TUREK® (*Bt* var. *aizawai*) and BITE® were more toxic than THURICIDE® (*Bt* var. *kurstaki*) on *P. xylostella* larvae. *P. xylostella* from Jember region was highly resistant (1,342.17 ppm for THURICIDE®) with a ratio resistance of about 75.50.

The best control was achieved with the BeXaRi formulation supplemented with Agristic. All treatments reduced the number of larvae compared with the control. The results showed that the wetting agents did not affect the virulence of *B. thuringiensis* (var. *aiziwai*) and *Steinernema carpocapsae* (all strains) as biocontrol of *P. xylostella* and *C. binotalis* after 52 days. It was obvious that the *Bt* treatment best reduced the population of DBM, followed by the nematode treatment and then the insecticide treatment. Thus, all the treatments kept the population at a lower density than in the untreated control.

The population of *C. binotalis* in the control was quite high (up to 59 per plant) between Days 26 and 75 and dropped to fewer than 10 after this period. Again, compared with the control, all the treatments were able to reduce the number of these insects. The most effective treatment was the insecticide treatment followed by the *Bt* treatment. The effect of using *S. carpocapsae* was much less compared with using the other treatments. However, it significantly reduced the population compared with the control. It looked like pesticide resistance had not yet developed in the population of *C. binotalis*. We concluded that this insect might be less susceptible to EPNs than *P. xylostella*.

Development of Intregrated Biological Pest Management in Indonesia

The results from the field trial clearly indicated that the biocontrol agent *B. thuringiensis* (*Bt*) was superior to the chemical insecticide. The results from the trials using the entomopathogenic nematode *S. carpocapsae* had not reached comparable results. This might be due to the limited survival of EPN_S on the foliage. Biocontrol using EPNs was able to effectively control *P. xylostella* larvae in the field and thus, represents a potential control measure for cabbage growers. As resistance was developed against the chemical insecticide, the same can be expected for *Bt* if applied 12 times in one cropping season. Alternative control measures to prevent the development of resistance are therefore urgently needed. It should be mentioned that bacterial diseases can significantly decrease yields and quality of cabbage. For that reason, resistance-inducing and plant-growth promoting microorganisms should be tested on their effect of the bacterial diseases.

The application of agents like *Bacillus subtilis*, *Pseudomonas fluorescens* or *Trichoderma harzianum* may be either by seed treatment of by spraying. Yields measured in mean weight of 10 cabbage heads were also recorded as well as damage caused by the bacteria *Xanthomonas campestris pv. Campestris*, *Erwinia carotovora pv carotovora* and by *P. xylostella*. The highest yields and lowest damage levels were obtained using the *Bt* treatment. The only treatment resulting in a significant increase in yields was the *Bt* treatment. Diseases in all the treatments were lower than in the controls. Damage by *P. xylostella* was significantly reduced by all the treatments. Compared with the other treatments, *S. carpocapsae* was less effective (1.75% crop damage). A strategic approach can be alternating the application of *B. thuringiensis* and *S. carpocapsae* to effectively limit outbreaks that exceed the economic threshold population of DBM larvae (currently at three larvae per plant).

Additionally, different *Bt* subspecies may be alternated. During the first project periods we observed resistance development against the subspecies *kurstaki* (trade name Thuricide) that could be reduced with the use of *B. thuringiensis* (*Bt* var. *aizawai*) in the products TUREX and BITE. A possible strategy could be weekly applications twice a month *Bt* (1 gL^{-1}) alliterating the two subspecies and the other two applications with *S. carpocapsae* 500,000 IJ m^{-2}. Both biocontrol agents should be applied in the evening to avoid damage by UV light. The treatments with *B. thuringiensis* and *S. carpocapsae* had a positive effect on the parasitisation of *Diadegma* spp. and other invertebrate antagonists, and this supports the effect of the biocontrol agents. *B. thuringiensis* can also be used as a biological control agent of *Crocidolomia binotalis*.

CONCLUSION

It can be concluded that there remains major problems related to EPN liquid culture mass production that have not been completely solved. Physiological parameters that allow one DJ to respond to a bacterial food signal and another to remain in DJ stage were considered unidentified in this study. Another source of process instability was the results of the phase transition of the bacteria. Further investigation is needed for both fields with the aims of improving process stability and increasing outputs. The close relationship of EPNs to the model nematode *C. elegans* and the sequencing project on *P. luminescens* may hopefully yield some background information about the nematode-bacterium complex metabolism that is believed to be valuable for developing process technology.

Comparing the nematode process with the cultivation of *Escherichia coli* or other microorganisms is limited because there remains very little knowledge of nematode cultivation. Additional research into symbiosis and its genetic background should identify the essential growth factors provided by bacteria and elucidate the functions of phase transition. Recently, the use of EPNs has been expanded to outdoor

environments such as strawberry patches and turf grass. Furthermore, EPNs can control many pests existing in vegetables and fruit.

Nevertheless, potential markets are likely to demand nematode products only when the products drop in price. Although the price has been cut by half following the introduction of liquid culture technology, it is still considered too high to permit application among low-value crops. The continuous scale-up of bioreactor volumes will bring further reduction in production costs. However, this development must be accompanied by further progress in improving process stability and downstream processing, extending EPN shelf life and improving transport logistics. If this can be achieved, EPNs will further substitute insecticides and contribute to the stabilisation of agriculture environments and crop yields.

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