

## ASEAN ECONOMIC BIOPESTICIDE: PRODUCTION OF BIOPESTICIDE ENTOMOPATHOGENIC NEMATODES FOR BIOLOGICAL CONTROL INSECT PESTS FOR ORGANIC FARMING

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### ABSTRACT

Entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema* are commercially used to control pest insects. They are symbiotically associated with bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively, which are the major source for the nematodes. The biology of the nematode-bacterium complex is described, a historical review of the development of *in vitro* cultivation techniques is given and the current use in agriculture is summarised. Media development is mainly directed towards cost reduction, as the bacteria are able to metabolise a variety of protein sources to provide optimal conditions for nematode reproduction. The process technology is described, discussing the influence of bioreactor design and process parameters required to obtain high nematode yields. As two organisms are grown in one vessel and one of them is a multicellular organism, the population dynamics and symbiotic interactions need to be understood in order to improve process management. Major problems can originate from the delayed slow development of the nematode inoculum and from phase variants of the symbiotic bacteria that have negative effects on nematode development and reproduction. Recent scientific progress has helped to understand the biological and technical parameters that influence the process, thus enabling transfer to an industrial scale. As a consequence, costs for nematode-based products could be significantly reduced.

**Key words:** ASEAN economic, biopesticide, biological entomopathogenic nematodes, biological control, organic farming.

### Entomopathogenic nematodes as Future Biological control insect pest

Many insect antagonists are found within the phylum nematoda, but only species within the genera *Steinernema* and *Heterorhabditis* (Rhabditida) have gained major importance as biocontrol agents in plant protection. Today, more than 30 species of these so-called entomopathogenic nematodes (EPN) have been described and many more will follow (Hominick et al. 1997). EPN are closely related to *Caenorhabditis elegans*, which is the current model organism for studying animal development and genetics (Riddle et al. 1997) and whose genome sequence has recently been completed. Unique to EPN is their close symbiotic association with bacteria of the genera *Xenorhabdus* and *Photorhabdus*.

Only a few strains of the symbiotic bacteria have been described and studied in detail. Their molecular biology has been described by Forst and Nealson (1996). Since then, the symbionts have gained considerable attention, due to commercial interest in insecticidal metabolites active on ingestion by the insect and causing symptoms in the gut similar to the *Bacillus thuringiensis*  $\delta$ -endotoxin (Blackburn et al. 1998). The genes represent a possible alternative to *B. thuringiensis* toxin genes for expression in transgenic plants (Guo et al. 1999). Typical for symbionts of both genera is the phenomenon of phase variation, the two extremes of which are the primary and the secondary phase (Akhurst 1980). Intermediate phases have been reported (Gerritsen and Smits 1997). The primary phase is isolated from the DJ or infected insects, whereas the secondary phase occurs either after *in vitro* subculturing or *in vivo*, when the nematodes emigrate from the cadaver (Grunder 1997). The secondary phase is not retained by the DJ of *H. bacteriophora* (Han and Ehlers 2001). Krasomil-Osterfeld (1995) induced the secondary phase by cultivating a primary form under stress conditions, for instance in media with low osmotic strength, when the bacteria were subcultured under standard conditions, they reverted to the primary phase. Prolonged subculture under stress conditions produced stable secondary phase cultures. Despite the loss of several metabolic functions by the secondary form, like production of protease, lipase, intracellular crystalline proteins, antibiotics and pigments (Boemare and Akhurst 1988), the major drawback is that secondary phase bacteria can have a significant and detrimental effect on nematode development and yields (Ehlers et al. 1990; Völgyi et al. 1998; Han and Ehlers 2001). The crystalline inclusion protein is of major importance for nematode nutrition (Bintrim and Ensign 1998). However, it is not the only essential nutritional factor provided by the primary phase (Hussein and Ehlers 2001). All measures should therefore be taken to avoid the phase variation. In general, the phase shift can be prevented by carefully reducing stresses (lack of oxygen, high temperature, deviation from optimal osmotic strength of medium) during bacterial inoculum production, inoculation and the pre-culture. The mechanisms causing the phase transition are yet unresolved, although genetic variation has been excluded (LeClerc and Boemare 1991; Akhurst et al. 1992; Wang and Dowds 1993).

### Commercial use Entomopathogenic nematodes in biocontrol

EPN have several advantages that qualify them as commercially valuable biocontrol agents. They are highly effective and often surpass the control results achieved with chemical compounds. In contrast to chemicals, which should not be displaced by water in the soil and have to decay within a few days, EPN are mobile and persistent. The recycle inside the host insect (Fig. 1), thus causing long-term, sustainable effects on the pest populations (Peters 1996). The use of EPN is safe for both the user and the environment. They have little detrimental effects on non-target insect populations and neither the nematodes nor their bacterial associates cause any detrimental effect to mammals or plants (Bathon 1996; Ehlers and Hokannen 1996). In almost all countries, EPN are exempted from registration requirements, which enables small and medium-sized enterprises to develop nematode-based plant protection products. EPN can be stored for some months, which facilitates the marketing of nematode-based products. DJ are resistant to shear forces and can thus be applied with conventional spraying equipment. As the control potential of EPN is not limited by customary agrochemicals, they can be integrated into standard chemical control practice. Today, nematodes are mainly used in environments where chemical compounds fail, i.e. in the soil, in the galleries of boring insects, or in cases where resistance to insecticides has developed (Table 1).

Table 1: Commercial use of entomopathogenic nematodes. Applications against pest insect marked <sup>m</sup> are currently under development for market introduction

Common name	Scientific name	Order	Culture
Fungus gnats	<i>Lycoriella solani</i> , <i>L. auripila</i> , <i>Lycoriella</i> spp	Diptera	Mushrooms
Fungus gnats	<i>Bradysia coprophila</i> , <i>Bradysia</i> spp	Diptera	Ornamentals
Cabbage root fly <sup>m</sup>		Diptera	Cabbage
March flies	<b><i>Delia radicum</i></b>	Diptera	Turf
House fly <sup>m</sup>	<i>Bibio hortulans</i>	Diptera	Stables
Leafminer	<i>Musca domestica</i>	Diptera	Ornamentals, vegetables
Black vine weevil	<i>Liriomyza</i> spp	Coleoptera	Strawberry, ornamentals
Strawberry weevil		Coleoptera	Strawberry, cranberry
Hop weevil <sup>m</sup>	<b><i>Otiorynchus sulcatus</i></b>	Coleoptera	Hop
Sugarbeet weevil	<i>O. ovatus</i>	Coleoptera	Sugarbeet
Citrus root weevil	<i>O. ligustici</i>	Coleoptera	Citrus
White grubs	<i>Temnorhinus mendicus</i>	Coleoptera	Turf
Peanut white grub <sup>m</sup>	<i>Diaprepes abbreviatus</i>	Coleoptera	Sweet potatoes, peanuts
Cutworms	<i>Popillia japonica</i> , <i>anomala</i> spp, <i>Phyllopertha horticola</i> , etc.	Lepidoptera	Various
Banana moth		Lepidoptera	Ornamentals
Ghost moth	<b><i>Maladera matrida</i></b>	Lepidoptera	Chives, ornamentals
Cockroach <sup>m</sup>	<i>Agrotis ipsilon</i> , etc.	Blattaria	Household
Mole cricket		Orthoptera	Turf
Western flower thrips <sup>m</sup>	<b><i>Opogona sacchari</i></b>	Tysanoptera	Ornamentals, vegetables
Cat flea	<i>Hepialus</i> spp <i>Periplaneta americana</i> , <i>Blatta</i> spp <i>Scapteriscus</i> spp	Siphonptera	House gardens
	<b><i>Frankliniella occidentalis</i></b>		
	<b><i>Ctenocephalides felis</i></b>		

### Management of nematode population dynamics in liquid culture

It can be expected that a certain medium has a fixed potential for a defined nematode yield. However, yields in the same medium can vary considerably (Ehlers et al. 1998; Strauch and Ehlers 2000). The reason why the population dynamics are so important becomes apparent when data obtained from the commercial production are analysed. In Fig. 4, the relationship between hermaphrodite density and DJ yields is presented for *H. bacteriophora* (Peters and Ehlers, unpublished results). The same medium was used for all processes. Maximum yields were obtained with approximately 4,000 hermaphrodites/ml counted on day 3 after nematode inoculation. Consequently, an inoculation of >4,000 DJ/ml should result in maximum yields. This hermaphrodite density, however, cannot be obtained by defining the DJ inoculation density, because DJ recovery is highly variable in liquid culture. Whereas almost 100% of the DJ recover within 1 day after having entered the haemocoel of an insect, liquid media lack any kind of food signal that could trigger recovery. Fortunately, the symbiotic bacteria produce such food signals and they therefore enable the production of EPN in vitro, through pre-culturing the symbiotic bacteria. However, the bacterial food signals cause 18-90% of the DJ to recover within a period of several days (Strauch and Ehlers 1998). The variable hermaphrodite density recorded 3 days after DJ inoculation (Fig. 4) is a result of variable recovery. The main reason for unstable DJ yields is the unpredictable, unsynchronised and low DJ recovery in vitro cultures. It prevents the population management required to maximise yields and to shorten the process time and makes additional scale-up steps necessary.

### Evaluation of Diamont Black Moth resistance to *Bacillus thuringiensis*

The tests were done with leaf disc assays at concentrations of 0.01, 0.10, 1, 10, 100, and 1000 ppm. on larvae of *Plutella xylostella* (n = 180). The insects were collected from four regions in East Java (Malang, Probolinggo, Jember and Bondowoso) and maintained in the lab. The results showed that TUREK® (Bt var. *aizawai*) and BITE® (Bt. var. *aizawai*) were more toxic than THURICIDE® (Bt var. *kurstaki*) on *P. xylostella* larvae, the LC<sub>50</sub> of TUREK® on *P. xylostella* from Jember regions is very low 2.12 ppm. (rr = 1), Malang region 6.77 ppm. (rr = 6.77), Probolinggo region 18.18 ppm. (rr = 18.18) and Bondowoso was 22.85 ppm. (rr = 10.78). *P. xylostella* larvae from Jember region is more susceptible to Bt than DBM from other regions in East Java, like Malang, Probolinggo, and Bondowoso. *P. xylostella* from Jember region was highly resistant (1,342.17 ppm for THURICIDE®) with a resistance ration (rr) of 75.50. The lowest LC<sub>50</sub> was recorded with Bt var. *aizawai* with the *P. xylostella* population from Jember and Malang regions (ratio resistance = 1).

### Testing combination of biocontrol agents

The formulations provided by CAU were field tested in cabbage crop cultured at Bromo Mountain, Probolinggo (East Java, Indonesia). The biocontrol agents used was *S. carpocapsae* (All strain) at concentration of 500.000 IJ/m<sup>2</sup> in the following formulations to control of *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% alcarylpoliglykol-ether). EPN were sprayed on days 14 and 28 after planting of the cabbage crop. All application were sprayed at 16:00 in the evening with a knapsack sprayer of 15 liter volume. The crop was planted with 50 x 60 cm space for each plant. Each experimental plot contain 100 plants. The number of alive DBM larvae in each block was. Population of *P. xylostella* larvae were counted from 10 cabbage plants, which had been sampled randomly from each plot. The result show that best control results were achieved with the BeXaRi formulation supplemented with Agristic.

### Field testing of EPN and Novel Formulation

Field test was done to evaluated the potential biocontrol of *Steinernema carpocapsae* (All strain) and *Bacillus thuringiensis* var. *aizawai* (TUREK®) on *P. xylostella* and *C. binotalis* in cabbage crop on Bromo Mountain, Probolinggo region, East Java, Indonesian. The EPN used were *S. carpocapsae* (All) produced in liquid culture in China (Partner 2). The field trial was conducted from March until September 2002 with *S. carpocapsae* (All) and *B. thuringiensis* (var. *aizawai*) to control *P. xylostella* and *C. binotalis* with three different treatment were W: Wetting agent with water, BtW: Bt with Wetting agents, and Bt: Bt with water and also for EPN treatment was; W: Wetting agents and water, NW: Nematodes with water and NfW: Nematodes with Wetting agents. The Wetting agents was used Agristic®, Alkilarilpoliglikol eter (0,025%/liter). Concentration was used for Bt 1 gram/liter and *S. carpocapsae* 0.5 Million/m<sup>2</sup>. All application was conducted at 16.<sup>00</sup> evening with knapsack sprayer volume 15 liter after 14 days cabbage crop planting. Result show that wetting agents was not affected to the virulence of *Bacillus thuringiensis* (var. *aizawai*) and *Steinernema carpocapsae* (All) as biocontrol of *P. xylostella* and *C. binotalis* after 52 days.

### Field testing of biological control agents *Bacillus thuringiensis* (Bt.)

The field trials were done to evaluated the control potential of *Steinernema carpocapsae* (All strain) against *P. xylostella* and *C. binotalis* in the cabbage crop. Trails were performed at Ijen Mountain, Bondowoso, East Java, Indonesian, which is approximately 60 km away from Jember. The EPN material of *S. carpocapsae* (All) was produced in solid media according to the Bedding method at UNEJ, Indonesia and liquid culture from China. The design of the field trial followed rules of the Random Complete Block Design (RCBD) with the following four treatments; **PO**: control with water only; **PN**: 500.000 IJ/m<sup>2</sup> *S. carpocapsae* sprayed with wetting agent (AGRISTIC®); **PP**: 500 gram/l profenofos (CURACRON®); and **PI**: *B. thuringiensis* (var. *aizawai*) (TUREX®) 1 gram/l with wetting agent (AGRISTIC®). Four plots for each treatment of the 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. Cabbage had been planted during January 2003. *S. carpocapsae* treatment was applied every two weeks and *B. thuringiensis* and the insecticide every week. *S. carpocapsae* was applied on days 12, 26, 40, 54, 68, and 82 days cabbage crop age and *B. thuringiensis* was applied at days of nematode application and also on days 19, 33, 47, 61, 75, and 89. All application were sprayed at 16:00 in the evening with knapsack sprayers of 15 liter volume.

All treatments reduced the number of larvae compared to the control. When summarizing all larvae found during the first and second generation and calculating a mean density at the days of counting, it is obvious that Bt treatment best reduced the population of DBM compared, followed by the nematode treatment and than the insecticide treatment. Thus all 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 day 26 and 75 and dropped to less than 10 after this period. Again, compared to the control, all treatments were able to reduce the number of these insect. Most effective was the treatment with the insecticide followed by the Bt treatment. The effect of *S. carpocapsae* was much less compared to the other treatments. However, it significantly reduced the population compared to the control. It looks like pesticide resistance is not yet developing in the population *C. binotalis*. We can also conclude that this insect might be less susceptible to EPN than *P. xylostella*.

## Development of Intregrated Biological Pest Management in Indonesian

The results from the field trial clearly indicate that the biocontrol agent *B. thuringiensis* is superior to the chemical insecticide. The results with entomopathogenic nematodes, *S. carpocapsae* have not reach comparable results. This might be due to the limited survival of EPN on the foliage. The biocontrol with EPN were able to effectively control *P. xylostella* larvae in the field and thus represent potential control measures for cabbage growers. As resistance has already developed against the chemical insecticide and 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* could be either by seed treatment of by spraying. Yields measured in mean weight of 10 cabbage heads was also recorded as well as damage caused by bacteria, *Xanthomonas campestris* pv. *campestris* and *Erwinia carotovora* pv. *carotovora* and by *P. xylostella*. Highest yields and lowest damage levels were obtained with the Bt treatment. The only treatment resulting in a significant increase in yields is the Bt treatment. The diseases in all treatments were lower than in the controls. Damage by *P. xylostella* was significantly reduced by all treatments. Compared with the other treatments, *S. carpocapsae* was less effective (1.75% crops damage). The strategy approach can be the alternating application of *B. thuringiensis* and *S. carpocapsae* to effectively limit outbreaks, which exceed the economic threshold population of the DBM larvae (currently at 3 larvae per plant).

This concept could also alternate different Bt subspecies. During the first project periods we observed resistance development against the subspecies *kurstaki* (trade name Thuricide), which could be reduced by the use of *B. thuringiensis* (var. *aizawai*) in the products TUREX and BITE. A possible strategy could be weakly applications of two times per month Bt (1 gram/liter) alliterating the two subspecies and the other two applications with *S. carpocapsae* 500.000 IJ/m<sup>2</sup>). Both biocontrol agents should be applied in the evening to avoid the damage by UV light. Treatments with *B. thuringiensis* and *S. carpocapsae* have a possitive effect on the parasitation of *Diadegma* spp. and other invertebrate antagonists, which will support the effect of the biocontrol agents. *B. thuringiensis* can also be used as biological control agents of *Crociodolomia binotalis*.

## Conclusions

Major problems related to EPN liquid culture mass production remain unsolved. Physiological parameters that cause one DJ to respond to the bacterial food signal and another to remain in the DJ stage remain unknown. Another source of process instability results from the phase transition of the bacteria. Both fields deserve further investigation, in order to enhance process stability and increase yields. Comparing the nematode process with the cultivation of *Escherichia coli* or other microorganisms, very little is known about nematode cultivation. The close relation of EPN to the model nematode *C. elegans* and the sequencing project on *P. luminescens* will hopefully yield some background information about the metabolism of the nematode-bacterium complex which will be valuable for improving process technology. Additional research on the symbiosis and its genetic background should identify the essential growth factors provided by the bacterium and elucidate the function of the phase transition.

At this moment, EPN are taking the step towards outdoor environments (turf grass and strawberries). In vegetable and fruit, many pests exist that can be controlled by EPN. However, these potential markets will only demand nematode products when these are available at a lower price. Although the price has been cut by half following the introduction of liquid culture technology, it is still considerably too high to permit any application on low-value crops. The continuous scale-up of bioreactor volumes will bring further reductions in the production costs. However, this development must be accompanied by further progress in improving process stability and downstream processing, in measures to extend EPN shelf life and in 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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