Monoxenic Culture of a New Thai Strain of Entomopathogenic Nematodes (*Steinernema* sp. KB Strain) on Artificial Media

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Abstract

A new Thai strain of entomopathogenic nematodes, *Steinernema* sp. KB strain, was cultured monoxenically with associated bacteria, *Xenorhabdus* spp., of the nematode. The experiment was done to compare the growth of the nematode on five modified artificial agar media and one lipid agar medium. All modified media contained 10 g/liter of either soya milk (SM), wheat flour (WF), soybean flour (SF), corn syrup (CS) or corn flour (CF), 5 g/liter of yeast extract, 1 ml of sunflower oil and 10 g/liter of agar. The maximum yield of 33.1 x 10^6 and 30.8 x 10^6 IJs per one liter was obtained from SM and SF media while CS, WF and CF yielded 8.6 x 10^6, 5.3 x 10^6 and 1.2 x 10^5 IJs per one liter respectively. Although the modified artificial media are inferior to the lipid agar medium in the number of harvested nematodes which was 60.8 x 10^6 IJs per one liter, the cost of nematode production by these modified media is 4.7 times less than that of the lipid agar medium.

Introduction

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* have long been recognized as effective insect control agents and have attracted considerable interest as a bio-pesticide. The nematodes have been proved to be potential control agents of a number of insect pests and consist of an infective juvenile stage which penetrates a host either through body openings, e.g. mouth, anus and spiracle or interskeletal membranes [1].

The symbiotic bacteria, *Xenorhabdus* and *Photorhabdus* species, held in the foregut of infective juveniles, are excreted into the host's hemocoel. These bacteria cause a lethal septicemia which kills the insect host within 48 hours and establishes suitable conditions for the reproduction of the nematodes [2]. The nematodes feed on the proliferating bacteria and the decomposing insect cadavers. Maturation of the nematodes occurs inside the cadaver. Male and female nematodes develop, mate, and

produce eggs, and these develop into a new generation of infective juvenile which exit the host in search of further insect hosts [3].

Entomopathogenic nematodes are tested for pro-duction, field application and safety standards. A number of commercial enterprises worldwide are now producing entomopathogenic nema-todes [4]. Steinernematid and Heterorhabditid can be cultured in either a host insect or on synthetic diet [5]. The advent of artificial media incorporating the nematodes'symbiotic bacteria was a major step toward commercial production of these nematodes for insect control [6].

Mass production of entomopathogenic nema-todes has evolved from the first large scale *in vitro* solid medium production by Glaser *et al.* [7]. He was the first to develop an axenic culture process for *S. glaseri* and later *S. carpocapsae*. Dutky *et al.* [5] refer to the "nematode-bacterium complex" which they maintained on peptone-glucose agar and pork kidney and found that a medium with the bacterial associate could support the reproduction of *S.carpo-capsae* DD136 strain. Bedding [8] developed a semi-solid culture method whereby nematodes were reared on an animal protein and lipid-based medium coated onto a polyether polyurethane sponge matrix to give a large surface area for passive aeration. This medium-coated matrix was inoculated with the symbiotic bacteria several days prior to the addition of the nematode inoculum.

Monoxenic mass culture techniques with the goal of producing, cost effectively, large numbers of infective nematodes have been used for several years. However, the effects of dietary components on nematode culture under monoxenic conditions are insufficiently known to enable optimum culture of the nematodes. Dunphy and Webster [9] documented the effects of nutritional components, lipids, nitrogen source, carbohydrates, vitamins and salts of the medium for maximum production of *S. carpocapsae* DD136 and *H. heliothidis.*

Development of production technology, distri-bution, and sales of entomopathogenic nematode-containing products is occurring now at a rapid pace. In many countries, commercial production has proved successful in insect control.

In Thailand, the first indigenous strain of ento-mopathogenic nematode was recovered from Kanchanaburi province in 1996 [10]. The nematode was shown to be a new species of *Steinernema* by morphological and molecular characteristics (Tangchitsomkid, unpublished). Tangchitsomkid and Sontirat [11] documented the pathogenicity test on larvae of the greater wax moth (*Galleria mellonella*) which yielded 100 % mortality in 22 hours at 30 and 35 ^{o}C. The efficacy of this nematode was also evaluated with positive results obtained for *Spodoptera litura, S. exigua, Plutella xylostella* and *Henedecasis duplifacialis.* The mortality of the insects within 24 hours was 55.6, 60.0, 88.9 and 100 %, respectively. The Thai strain nematode could serve as a resource for use in the tropics because it could be more effective controlling native insect pests and also present less risk to non target organisms, compared with exotic isolates.

The present study examines the effect of selected nutritional components of the artificial agar media on the growth and reproduction of *Steinernema* sp. Thai strain. The data are used to formulate media appropriate for the pilot scale mass production of Thai strain nematodes.

Materials and Methods

Nematode inoculum

The nematode was obtained by exposing the larvae of the greater wax moth (*Galleria mellonella*) to 5,000 infective juveniles of *Steinernema* sp. Thai strain in 9 cm Petri dishes lined with two filter papers (Whatman No.2). Twenty wax moths were released in each Petri dish and all dishes were maintained in an incubator at 30 ^{o}C. Five days after exposure, the cadavers were transferred to the White's water trap. Five days later, infective-stage juveniles were extracted from the cadavers into the water trap. The infective juveniles were surface sterilized with 0.1 % hyamine for 15

minutes and gravity washed for three times with distilled water by using a high speed micro-centrifuge at 8,000 rpm/min.

Bacterial inoculum

The primary form of *Xenorhabdus* sp. was isolated from *Steinernema* sp. Thai strain reared continuously *in vivo* at the Nematology Section, Plant Pathology and Microbiology Division, Department of Agriculture.

To isolate the bacteria, infective-stage juvenile nematodes were rinsed with distilled water, transferred to a solution composed of 0.1 % thimerosal and 0.6 % streptomycin sulphate and maintained for 12 hours under aseptic conditions. The nematodes were then rinsed three times with sterile water and transferred, as a suspension in distilled water, to a sterile tissue grinder and crushed. An aliquot of the resulting suspension, consisting of nematode tissues and fluids and bacterial cells, was pipetted into 9 cm Petri dishes containing T7-TTC medium. The Petri dishes were maintained in an incubator at 28°C. After 48 hours, single colonies of bacteria showing any morphological differences such as size, shape, growth rate, or color were subcultured by streaking them onto a nutrient agar and left at 28°C for approximately 48 hours. Single colonies were streaked one more time onto nutrient agar to insure purity. These colonies were transferred to glycerol medium in 2 ml vials and stored at -70 °C.

Culture media

All modified media contained 10 g of either soya milk (SM), wheat flour (WF), soybean flour (SF), corn flour (CS) or corn flour (CF) and were mixed with 5 g yeast extract, 1 ml sunflower oil, 10 g agar and 1 liter distilled water. The lipid agar was prepared according to Dunphy and Webster [9] : 10 g corn syrup, 5 g yeast extract, 25 g nutrient agar, 2.5 ml cod liver oil, 2 g $MgCl_2$ and 1 liter distilled water.

The media were autoclaved at 121°C for 15 min and then poured aseptically into 9 cm Petri dishes.

Experiment procedures

The bacterial colonies were removed from storage and subcultured to 125 ml Erlenmeyer flasks filled with 20 ml nutrient broth under continuous agitation for 24 hours. Each culture dish was inoculated with 1 ml of broth. The Petri dishes were incubated at 30°C for 24 hours and then inoculated with 5,000 infective juveniles per dish. All cultures were incubated at 30°C for 10 days.

Harvesting

All nematode cultures were harvested 10 days post inoculation by flooding the Petri dishes with 10 ml of distilled water for 10 min to dislodge juvenile nematodes from the agar-bacterial matrix. The total collective number of nematodes per dish was estimated based upon the average yield of nematodes in three samples of suspension per dish. Each culture was replicated five times.

Results and Discussion

Yields were estimated by counting infective juveniles in five samples from modified media compared with lipid agar.

The number of infective juveniles produced in 20 g medium of SM, WF, SF, CS and CF is shown in Table 1. The yield of infective juveniles was very high in media containing soybean. Dunphy and Webster [9] reported that yeast extracts are a nitrogen source which stimulate the reproduction of nematode. The major components of SM and SF are proteins and lipids which are necessary for the production of nematode progeny. Soya milk and soybean flour (concentration of 10 g per liter) were the supplemented media, both of these media differently supported greater nematode reproduction than did the wheat or corn medium.

The bacterial symbionts are essential for successful mass culture of the nematodes. These bacteria proliferate on artificial media and the nematodes feed on the bacterial cells and other

Table 1. Number of infective juveniles (IJ) of a new Thai strain of entomopathogenic nematodes (*Steinernema* sp. KB strain) after 10 days on 20 g of different media.

Media	No.of IJs/dish (Mean ± SE) (N = 5)
SM	661,400 ± 66,725
WF	106,200 ± 14,469
SF	615,000 ± 89,259
CS	173,000 ± 25,052
CF	2,400 ± 1,200

necessary nutrients to reproduce large numbers of offsprings. A new Thai strain, *Steinernema* sp. KB strain can be grown and reproduced in proteins and lipids from soybean. However, the yield of nematodes in lipid agar was higher than in modified media (Figure 1). The lipid agar is a complex medium of lipid (cod liver oil), nitrogen (yeast extract), carbohydrate (corn syrup) and salt ($MgCl_2$). The nutritional components are screened for their ability to support monoxenic nematode culture.

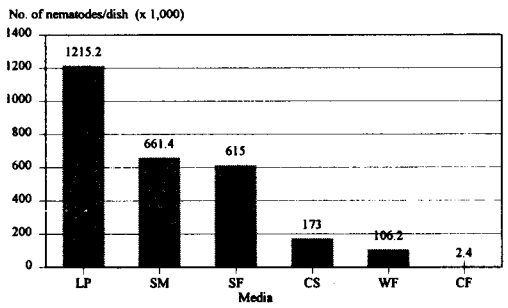

Figure 1. Comparison of modified media (SM, SF, CS, WF and CF) with lipid agar medium (LP) in the number of infective juveniles (*Steinernema* sp. KB strain) produced.

Throughout the study, as exemplified by the data from the modified media, monoxenic cultures of a new Thai strain of entomopathogenic nematodes exhibited qualitative and quantitative differences in their responses to many of the modified media. This may reflect species differences in nematode and/or bacteria metabolism and differences in the nematode-bacterium metabolic association.

The cost of the production of nematodes on larvae of wax moth (*Galleria mellonella*), or on dog biscuit, is $US 1 per million nematodes [12]. This experiment demonstrated that the cost of nematode production using SM, WF, SF, CS and CF media was less than 41 baht per 50 dishes (1 liter) which was 4.7 times lower than that of lipid agar. The total nematode yield was $33x10^6$ and $31x10^6$ in SM and SF, respectively. (Table 2). Consequently, the nematodes can be produced in very large numbers and at low cost.

Table 2. Comparison of total nematode yield per 50 dishes (1 liter) and the cost of production between modified media and lipid agar.

Media	Total nematode yield/liter (50 dishes)	Cost per liter (Baht)
SM	33.1×10^6	40.90
SF	30.8×10^6	39.95
CS	8.6×10^6	40.40
WF	5.3×10^6	39.95
CF	1.2×10^5	39.98
LP	60.8×10^6	186.04

The monoxenic agar culture described here establishes a baseline of media for *Steinernema* KB strain and other steinernematid nematodes. The data arising from this research can be used as a guideline to obtain more reliable pro-ductions and greater yields of all

stages of the Thai strain of entomopathogenic nematodes in laboratory culture *in vitro* and is a step towards improved mass industrial production.

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