Isolation of Entomopathogenic Nematode-Bacteria Complex with a Potential for Use as a Biological Insecticide in Nigeria

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ABSTRACT

The larvae of G. mellonella obtained from honey combs were successfully grown to adult on newly developed artificial medium in the laboratory. Generations of the moth were grown and maintained on the artificial medium. The larvae were used as bait to trap entomopathogenic nematodes from the soil. The nematodes isolated were identified as Steinernema species based on their morphology. The pathogenicity of the nematodes was tested after storing them in water for three and six months respectively. It was observed that the ability of the nematodes to kill the larvae reduced with increase in the period of storage. Nematodes stored for six months killed only 20% of larvae treated while that stored for three months killed 40% of larvae treated. Death of the larvae occurred within 24 hours for freshly harvested infective juvenile nematodes while it took 72 hours for nematodes stored for three months and 168 hours (7days) for those stored for 6 months. The nematode-bacteria complex isolated has a potential for use as a biological insecticide.

Keywords:

INTRODUCTION

Chemical insecticides are used widely by farmers in Nigeria to control insect pests of agricultural importance both on the fields as well as while in storage. The chemical insecticides have a very wide range of action killing both pests and beneficial insects such as predators, parasitoids and pollinators. They also contaminate the environment in addition to causing human health problems and pest resistance and resurgence. Apart from this, they also have residual effect in the soil and have been reported to cause extensive pollution of ground water aquifers. Biological control of insect pest helps to reduce the dependence on chemicals and re-establishing the balance between pests and their natural enemies in the environment. Bacteria, fungi, ricketsia and viruses have been incorporated in integrated pest management systems. Commercial controls of insects using bacteria have advanced over the years. The most important species of bacteria employed in insect control are the spore forming bacilli. Insects of the group diptera, lepidoptera and coleoptera have been successfully controlled by the use of *Bacillus thuringiensis* and *B.shpaericus* (Burges 1982; Aronson, Beckman and Dunn, 1986, Owuama 2001). These bacteria produce a

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ISSN: 2141-2731

crystal protein (_" endo-toxin) during sporulation. Susceptible insect hosts are killed after ingesting the toxin (Knowles *et al*., 1989; Owuama 2001). These bacteria cause passive infection hence their use as biological insecticides are greatly reduced. A more potent group of bacteria *Xenorhabdus* and *Photorhabdus* species kill their insect hosts within 24-48hrs using an active infection. These bacteria are carried into their susceptible hosts by their entomopathogenic nematode symbionts (Gaugler and Kaya 1990; Bedding, Akhurst and Kaya, 1993; Owuama 2001). An understanding of the life cycle of the nematode-bacteria complex will help us understand the mode of action of the complex as a biological insecticide. The infective juveniles (third-stage nematodes) are ingested by the host and enter the hemocoel by penetrating directly through the mid gut.

The nematode may also enter the host through the anus, spiracles and penetrate the tracheae (Shapiro-llan and Gaugler, 2002, Bedding, Akhurst and Kaya, 1993). In the host hemocoeal, the nematodes release their bacterial symbionts which multiply and kill the host by septicemia within 24-48 hrs (Akhurst, 1982; Akhurst and Boemare, 1990; Morgan *et al*., 1997). The nematodes feed on the *Xenorhabdus* or *Photorhabdus* bacteria and the host tissues. The nematodes develop rapidly to the adult stage, mate and produce eggs. As soluble nutrients in the cadavers are depleted, the progeny of the second and third generations develop into infective juveniles, which exit from the cadavers carrying the bacterial symbiont to seek new hosts (Poinar, 1990; Bedding, Akhurst and Kaya, 1993). In the laboratory, infective juveniles exit from the host in 8-14 days after infection. This natural migration of infective juveniles away from the carcass to search for new hosts in the environment has also made the use of these nematode-bacteria complex a very successful and reliable bioinsecticide. Nematode-bacteria complex have a number of advantages for use as bio-insecticides. They have a wide host range hence can be used to control numerous insect pests such as *Manduca sexta* (Ribeiro *et al*., 2003), *Pieris brassicae* (Sergent *et al*., 2003), South Ameriacan leaf miner, *Liriomyza huidobrensis* (Willliam and Walters, 2000), and the Oriental fruit moth, *Grapholita molesta* (Ekaterina *et al*., 2006). The bacterial symbionts (*Xenorhabdus* or *Photorhabdus* species) of the nematodes produce toxic proteases and enzymes which are used to kill their insect host within 24 hours. The nematodes have an efficient host seeking ability. Both the nematodes and the bacteria can be grown on artificial media thus encouraging commercial production. Infective stage of nematodes is durable, can live without nourishment and withstand wide temperature ranges.

The nematode-bacteria complex has the advantage that the susceptible hosts apparently have no natural or acquired resistance to *Xenorhabdus* or *Photorhabdus* species. Neither the nematodes nor their symbiotic bacteria are pathogenic to humans (Shapiro-llan and Gaugler, 2002). A number of products based on nematode-bacteria complex are available commercially in parts of Europe, America and Africa and have been used successfully to control insect pests of agricultural importance (Shapiro-llan and Gaugler 2002; Nguyen et al 2006; Grewal and Peters, 2005). Farmers in Nigeria depend largely on chemical insecticides for pest control in spite of the dangers they pose on human and animal health as well as on the environment. This research is therefore aimed at isolating nematode-bacteria complex with a potential for use as a biological insecticide.

MATERIALS AND METHOD

Identification of Susceptible Insect Hosts: Honey combs were obtained from bee keepers in Girei local government area of Adamawa State. The honey combs were kept in aerated wide mouthed plastic jugs (10 liters) and kept in a dark cupboard at room temperature (28-34ÚC). The lid of the plastic jug was perforated to allow for air ventilation. A piece of cotton net was placed over the mouth of the jug before covering with the perforated lid. This was done to prevent the escape of the larvae from the container. The combs were examined for emergence of *Galleria mellonella* larvae (Greater wax moth) over a period of two weeks (Dewey 1992).

Rearing of Larvae: The larvae obtained from the honey combs were allowed to develop into adult moths in the aerated plastic jugs. Adult moths emerged and were maintained on a modification of the artificial medium described by Pinyon *et al*., (2000). The medium comprised wheat meal (200g), Cerelac a high protein baby cereal (Nestle) (250g), glycerol (150ml), honey (150ml), Kunimed yeast (2g), and the contents of ten multivitamin capsules (Supradyn, Roche). The ingredients were mixed properly and used to sustain various stages of development of the insect.

Isolation and Storage of Nematodes: The nematodes were isolated from soil samples by a modification of the insect baiting technique (Mracek, 1980; Kaya and Stock, 1997; Mracek and Becvar, 2000). Five last instar larvae of *G.mellonella* were placed in a 250ml conical flask containing damp soil obtained from cultivated farmland in the University. The flask was covered with cotton net and turned upside down and kept in the dark at room temperature (28-34ÚC), for 7 to 9 days. The soil was examined daily for dead larvae which were removed and placed in a modified White trap (White, 1927) for subsequent emergence of infective juvenile nematodes. The nematodes obtained were stored in water temporary at room temperature and used to infect fresh larvae of *G. mellonella* in a Petri dish. The Petri dish was lined with Whatman filter paper and infected with infective juvenile nematodes using a 5ml pipette. A control experiment comprising of another Petri dish containing fresh live larvae of *G. mellonella* and sterile distilled water was also set up. The plates were incubated at room temperature in a dark cupboard for two days.

Identification of Nematode Genera: The nematodes isolated were identified using physical characteristics of nematode infected insect (Kaya and Stock, 1997). They reported that larvae infected by heterorhabditid nematodes usually turned brick red in colour while larvae infected with steinernematid nematodes turned brownish-yellow in colour. Light microscope with photographic attachment (Olympus) was used to identify the nematodes morphologically and photographs of heat fixed and live specimens were taken using the ×400 magnification (Dix *et al*., 1992; Kaya and Stock, 1997; Luc *et al*., 2000).

Identification of Bacterial Symbionts: The nematodes isolated from soil were used to infect fresh *Galleria mellonella* larvae. Dead larvae were removed and stored for seven days to allow for the development of infective juveniles. The larvae were then sterilized by

immersing them in 70% ethanol solution in a petri dish. The larvae were removed and placed in another Petri dish and a strile needle was used to rupture them (Mahar *et al*., 2004). The infective juveniles where rinsed in a test tube containing ringer solution and were later ruptured in fresh ringer solution in a test tube using a glass rod. It was then allowed to stand until it sedimented. Excess ringer solution was decanted and the sediment streaked onto nutrient agar bromthymol blue (37g nutrient agar, 25mg bromothymol blue powder and 1000ml distilled water), nutrient agar, and MacConKey agar plates. The plates were sealed with masking tape and incubated at room temperature (28-34ÚC) in the dark for 48 hours (Akhurt, 1982; Molina-Ochoa *et al*., 2000).

Test for Pathogenicity: The nematode-bacteria complex stored in water for three and six months respectively was used to infect a total of fifteen larvae of *G. mellonella*. A 5ml Pasteur pipette was used to transfer the nematode-bacteria complex to a Petri dish lined with wet filter paper. The dish was kept in a dark cupboard at room temperature and observed at 24 hour interval for the death of *G.mellonella* larvae (Maher *et al*., 2004). A control was set up using a Petri dish with distilled water only with larvae of *G. mellonella* placed in it.

RESULTS AND DISCUSSION

Identification of *Galleria Mellonella:* The larvae of *G. mellonella* (greater wax moth) emerged from honey combs about two weeks after they were placed in the aerated jugs. The emerging larvae were about 0.1 cm in length and whitish in colour. They turned light grey as they grew older. The larvae grew to about 2.0cm in length after 14days before pupating. The larvae usually shied away from light and left a tread of silk along their trail as they crawled from one place to another. Two weeks after emergence from the eggs the larvae created boat shaped cocoons into which they pupated. Adult moths emerged about two after pupating. The whole life cycle took about one month to complete in the honey comb. Adult female moths are different from the male moths. Adult females were observed to be bigger than the male moths. The female moths were dark grey in colour while the male moths were light grey in colour. The wings of the moths folded like a roof over their body. The male moths have a scalloped front wing margin while that of the female is smooth. The adult moths have a pair of antennae and three pairs of walking legs. These observations are consistent with those of (Dewey, 1992).

Maintenance of *G. Mellonella* on Artificial Medium: Adult moths were handpicked from the honey comb and placed on the artificial medium. The female moths laid eggs on a piece of crumbled paper placed in the container. However some of the moth laid their eggs on the cotton net used to cover the mouth of the jug. The moths died a few days after egg laying. The larvae emerged from the eggs and continued feeding on the artificial medium. As the larvae burrowed through the medium, they spun silk threads within which they usually rested. The silk spun by the larvae formed a network within the medium and seemed to bind the medium together into a firm consistency which resembled their original

honey comb home. When the larvae reached prepupa stage, some crawled away from the medium and spun boat shaped cocoons in which they pupated. The external features developed and imago moths emerged.

Isolation and Identification of Nematodes: Nematodes were obtained from only three of the fifty larvae used as bait. A suspension of the nematodes was used to infect fresh larvae of *G. mellonella* in a Petri dish. All larvae in Petri dish containing nematode suspension died within two days but all larvae in the control plate (without nematode suspension) pupated within the same period (table 1). A parasitized larva opened three days after infection revealed infective juvenile nematodes at various stages of development. Both live and heat fixed nematodes were observed under a light microscope with photographic attachment. Third stage infective juvenile nematodes have bodies that smoothly taper towards both ends. The tail tapers to a finely pointed terminus (plates 1and 2). Nematodes stained with methylene blue on a glass slide revealed a second stage cuticle of an infective juvenile hanging loosely from the body which is a characteristic of *Steinernema* species (plate 3). The infective juveniles usually coiled up when they are resting and unfolded gradually when disturbed (plate 2). First generation males of *Steinernema* species have their body slightly swollen in the middle and heat killed specimens of the first generation male of *Steinernema* species assumed a C-shape or a J-shape (plate 4). Adult female *Steinernema* species have a blunt posterior end (plate 5) while that of *Heterorhabditid* species is known to have a pointed posterior end.

Identification of Bacterial Symbiont: The bacterial colonies obtained from ruptured nematodes and placed on nutrient agar, showed a characteristic swarming ability of *Xenorhabdus* species. Colonies on nutrient agar impregnated with bromothymol blue adsorbed the dye from the medium and turned blue. Colonies grown on Mackonkay agar appeared green. Gram stain revealed gram-negative rods and biochemical tests revealed that the organism was catalase negative, citrate negative, indole negative and methyl red negative.

Pathogenicity Testing

Nematode-bacteria complex: After three and six months of storage in water at room temperature, the pathogenicity of the nematode-bacteria complex was tested. Fresh larvae of *G. mellonella* were treated with freshly obtained and stored nematode-bacteria complex. During the first 24 hours of treatment with freshly harvested nematode-bacteria, most of the larvae died pilling up on each other in the middle of the Petri dish (plate 6). During the next 24 hours, two more larvae died and four pupated. Two weeks later three adult moths emerged and one of the pupa was not viable (table 1). Larvae treated with nematode-bacteria complex suspension (stored for three months) however, exhibited different characteristics. About fifteen larvae were treated with three-month old nematode-bacteria complex. Two larvae died each day for three consecutive days. About nine of the larvae pupated but only five adult moths emerged. Four of the pupae were not viable (table 1, plates 7 and 8). All larvae in the control plate pupated and adult moths emerged.

Larvae treated with nematode-bacteria complex suspension (stored for six months) showed a reduced pathogenicity as none of the larvae died until one week after treatment with the nematodes. Only three of the larvae died, twelve pupated, seven emerged and five were not viable (table 1). The nematodes were isolated from cultivated maize and cowpea field at the Modibbo Adamawa University of Science and Technology, Yola, Adamawa State (a North-Eastern State of Nigeria). They were identified as Steinernema species based on morphological characteristics as described by (Dix et al., 1992; Kaya and Stock, 1997 and Luc et al., 2000). The percentage recovery of enthomopathogenic nematodes from the fifty larvae used as bait was 6%. This could be due to low adaptation to newly introduced host. This finding is consistent with the report of Fan and Hominick (1991), where they recorded low infectivity of G. mellonella used as bait for nematodes in the soil.

In this study it was observed that nematodes stored in water have a reduced infectivity compared to freshly harvested nematodes. This may be due to the storage conditions such as temperature and aeration as well as medium of storage (Shapiro-Ilan and Gaugler, 2002). For example (Shapiro-Ilan and Gaugler, 2002) showed that the shelf life of the nematode-bacteria can be improved by reducing their metabolism and immobilizing them through refrigeration and partial desiccation which we could not try due to limitations in the facilities available. Table 1 clearly shows stored nematode -bacteria complex have a reduced pathogenicity compared to freshly harvested organisms which killed most of the larvae within 48 hours. Apparently, the storage affects the pathogenic determinants. The dead larvae pilled up on top of each other (plate 6). The reason for the dead larvae sticking together is not clear but may suggest exudation of chemical attractants apparently distress signals/sos chemicals by the dying larvae that attracts others towards it. Nematodes stored for three months killed the larvae slowly and only a few of the larvae (20%) were killed and most pupated (80%). But of the number that pupated only (33%) were viable.

The inability of the pupae to develop to imago may be a reflection of a decline in pathogenicity as the stored complex is yet to re-establish in the host. However, nematodes stored for six months in water took one week to kill only three out of the fifteen larvae (20%) treated showing a further reduction in pathogenicity with age of storage. This indicates that pathogenicity decreases with increase in storage period in water. This result is consistent with the report of Shapiro-Ilan and Gaugler (2002), that there is a reduction in the ability of nematodes stored in water to infect insect hosts compared to freshly harvested nematodes. The isolated bacterial symbiont was identified as Xenorhabdus species based on its colonial characteristics on nutrient agar and their ability to adsorb the dye, bromothymol blue on nutrient agar bromothymol blue plate (Akhurst 1982). The organism was also found to be gram-negative rods, catalase negative, citrate negative, indole negative and methyl red negative. These are consistent with the characteristics of Xenorhabdus spp. (Akhurst 1982; Mahar et al., 2004). From the foregoing therefore, the nematode-bacteria complex isolated and studied has the potential for use as a biological insecticide. It is therefore important to identify these organisms at their species level. It is also important to search other fields for the presence of new nematode-bacteria complexes and also study their potential to control insect pests. This research is the first time in Nigeria that nematode-bacteria complex will

be isolated. The organisms were used to kill larvae of G. mellonella in the laboratory thereby establishing its potential for development of a biological insecticide based on nematode-bacteria complex.

Plate 1. (Top left): infective juvenile nematode assume J-shape when resting. (Bottom right): Infective juvenile in motion.

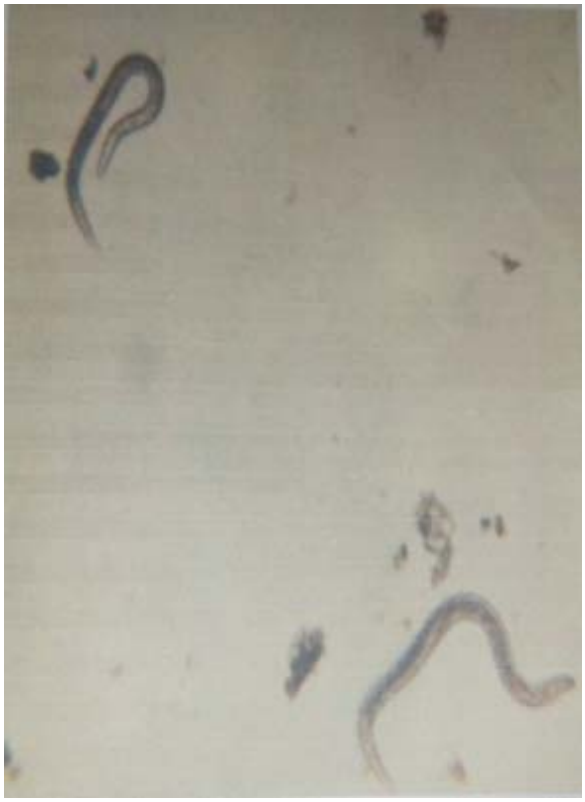

Plate 2. (Top left): Infective juvenile coils up when resting. (Bottom right): Infective juvenile unfold when disturbed.

Plate 3. Slide of an infective juvenile stained with methylene blue. The arrow shows the loosely hanging sheath of the third stage infective juvenile, a characteristic of *Steinernema* species

Plate 4. Slide of heat fixed first generation male of *Steinernema* species stained with methylene blue. The arrows show its swollen middle which is a characteristic of *Steinernema* species

Plate 5. Adult female of *Steinernema* species. A shows the anterior end and B shows the posterior end

Plate 6: Larvae of G. mellonella treated with freshly harvested nematodes. Dead larvae pile up in the middle of the Petri-dish while live larvae roam the edge

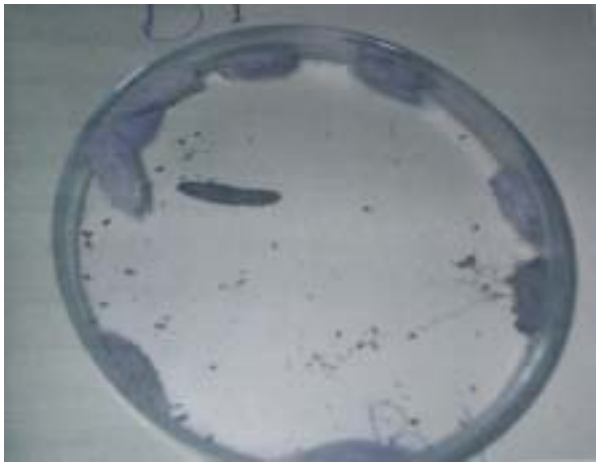

Plate 7: Larvae of G. mellonella infected with nematodes stored for three months. Larvae pupated at the edge of the Petri-dish and dead larva at the middle.

Plate 8: Dead larvae transferred from plate 7 and placed on a petri-dish lined with wet filter for emergence of infective juveniles.

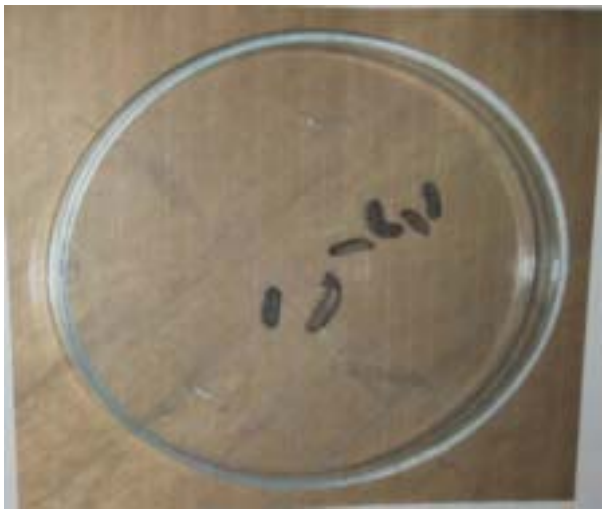

Plate 9: Nematode infected larvae pupated but no adult moth emerged.

Table 1: Effect of fresh and stored nematodes on G. mellonella larvae over a period of 7 days

Larvae	**mortality/day**				**pupae**	**non viable**	**adult moths**	**total**
No. of days	1	2	3	7				
Distilled water	-	-	-	-	15	-	15	15
Fresh IJs	9	2	-	-	4	1	3	15
Stored IJs (3 months)	2	2	2	-	9	4	5	15
Stored IJs (6 months)	-	-	-	3	12	8	4	15

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ISSN: 2141-2731

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