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Comparative Effectiveness of Entomopathogenic Nematodes Against the Pupae of Mustard Beetle, *Phaedon cochleariae* F. (Chrysomelidae: Coleoptera)

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Abstract.- *Steinernema carpocapsae, Steinernema feltiae* (Steinernematids), *Heterorhabditis indica* and *Heterorhabditis bacteriophora* (Steinernematids) were tested to control the pupae of *Phaedon cochleariae* under different laboratory bioassays in sterile sand. Results showed a significant difference in mortality percentage among nematode isolates. All nematode isolates were found more effective when exposure time was increased up to 4 days. *S. carpocapsae* and *S. feltiae* showed better mortality at 25°C. Both Heterorhabditids caused maximum mortality as compared to Steinernematids at 30°C. When different moisture levels were tested in the sand arena, a medium level of moisture (12%) caused maximum pupal mortality in all isolates. However, highest concentration of each isolate (200 IJs per ml) proved to be most appropriate for maximum insect death. Similarly, both Heterorhabditis nematodes killed all beetle pupae as compared to the other two Steinernematids. Infectivity test showed that *S. carpocapsae* produced the maximum number of infective juveniles in pupae at 25°C as compared to other nematodes. Infectivity of *H. indica* was better at 30°C in pupae of *Phaedon cochleariae* followed by *H. bacteriophora*, *S. carpocapsae* and *S. feltiae*. This study suggests some useful basic findings in developing biocides with suitable virulent entomopathogenic nematodes for controlling pupae of *P. cochleariae*.

Keywords: *Heterorhabditis*, *Steinernema*, biological control, biocide, biopesticide.

INTRODUCTION

The mustard beetle, *Phaedon cochleariae* Fabricius, is one of the most serious pests of cruciferous crops such as cabbage, swedes, celery, turnip, rape, cauliflower and watercress (Wilson, 1960). Both larvae and adults attack foliar and fruiting parts of the plants, make holes in the leaves and give plants a ragged look. This insect pest attacks flowers and tender buds, pods and seeds, and hence causes severe economic damage (Trought, 1965).

Adult mustard beetles are small round shiny metallic blue about 3-4 mm in length. Beetles become active mostly in the spring season and begin to feed on mustard and cabbage plants. Eggs are yellowish and laid on underside of the leaves of the crops. The larvae have black and yellow streaks and pupate in soil. The adults emerge after 8-12 days (Gladders *et al.*, 1989). Life cycle is 35-45 days at 22°C. Adults emerge from hibernation and attack the foliage of host plants. There are 2-3 generations in a season. Each female lay about 300-400 eggs over a 3 week period. Larvae are brownish-yellowish to dark grey in colour and up to 6 mm long. They pass through three instars and are fully fed in about 3 weeks. Pupation takes about 10-12 days (Jones and Jones, 1974; Hill, 1978).

Traditionally the beetle is controlled by spraying or dusting with recommended chemical pesticides. In biological control, entomopathogenic nematodes infect hundreds of different insect species of most orders and affect their hosts in different ways. The non-feeding infective third stage juveniles (IJs) of the genera *Steinernema* and *Heterorhabditis* enter their hosts through natural openings *i.e.* mouth, anus, spiracles, cuticle and penetrate into the blood circulatory system of the insect (Poinar, 1990). They release bacteria which produce toxins that kill hosts within 24-48 hours (Kaya, 1985).

Different approaches have been carried out to reduce the soil insect pest infestation with virulent isolates of entomopathogenic nematodes.

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Temperature influences nematode mobility, reproduction and development (Mason and Hominick, 1995). Mahar *et al.* (2005a) investigated the same nematode isolates against the pupae of vine weevil, *Otiorhynchus sulcatus*. Maximum infection was recorded by *S. feltiae* at 20°C than all other isolates, but *H. bacteriophora* resulted higher infection than *H. indica* and *S. carpocapsae* at 25°C. One of the fundamental steps in development of an entomopathogenic nematode for biocontrol is choosing an appropriate strain. Virulence against the target pest is a basic factor of biological control programme.

Temperature plays a major role in an insect's life cycle. Temperature interacts with other factors such as humidity, food availability and light and since temperature is easily measured and controlled, it is common practice to examine its influence upon species of economic importance (Howe, 1967). Thorough knowledge of the effects of temperature on infectivity and mortality of mustard beetle is critical for developing and optimizing control techniques.

The pupal stage is very critical stage in insect pest's life, which can be easily controlled by selective nematodes. Pupae live in soil habitat and nematodes also live in soil, and, therefore, it is easier for nematodes to infect this stage of insect life.

Very little work has been done on the control of pupae of *P. cochleariae*. We have investigated susceptibility of *P. cochleariae* pupae to four different entomopathogenic nematodes comparing mortality at different time exposures, effect of temperature, moisture, doses and infectivity in laboratory bioassays in sand. These findings could be incorporated in pest management programmes to control the pupae of *P. cochleariae*.

MATERIALS AND METHODS

Maintenance of P. cochleariae *culture*

The pupae of *P. cochleariae* were used in all the experiments. For this purpose the main culture was obtained from the Department of Horticulture (Entomology Lab.), University of Reading, UK. The culture was used throughout the experiments and reared on Chinese cabbage plants. Five weeks old plants were used for rearing the *P. cochleariae* culture. Males and females were allowed to lay fresh eggs on the leaves and fresh eggs were transferred on the fresh leaves of Chinese cabbage. Larvae were fed for 3 more weeks in an insectary at 24-26°C and 70% RH. Pupae were reared in different wooden cages (1 x 0.5 x 0.5 m in size) covered with muslin cloth. Each cage had different insect stages. The late stage larvae pupated in the soil in plastic pots (9.5 cm diameter x 9 cm depth). Pupae were collected from the soil for experimental purposes. Some pupae were left in separate cages to develop into adults in order to get next generation. The plastic pots were covered with muslin cloth until the pupae were formed, which were transferred to the pupae cage. The same age, size and weight pupae were obtained for experimental purpose.

Maintenance of nematode culture

Larvae (6th instar) of *Galleria mellonella* were also obtained from the Mealworm Company, Universal Crescent, Sheffield, UK until required for experiments. Larvae were infected with IJs of different entomopathogenic nematodes for producing fresh cultures. *S. carpocapsae* (All isolate, cultured at 25°C) was obtained from Biosys, USA. *S. feltiae* (cultured at 25°C) and *H. bacteriophora* (HW79 isolate, cultured at 28°C) were supplied by CAB Institute of Parasitology, St. Albans, UK. Whereas, *H. indica* supplied by Pakistan Nematological Research Centre, Karachi, was cultured at 28°C. *S. carpocapsae* and *S. feltiae* were stored at 7°C, while *H. indica* and *H. bacteriophora*) were stored at 15°C. Fresh IJs were used within one week of harvesting from White traps using the techniques described by Woodring and Kaya (1988).

Maintenance of Chinese cabbage plant culture

Pupae of mustard beetle were obtained from pots containing the full fed larvae near the roots of cabbage plants. *P. cochleariae* larvae and adults were reared on Chinese cabbage cv. Wong Bok in a growth room at 25°C. Fresh one month old plants were regularly supplied to the larvae and adults in different cages.

Effect of exposure time on mortality of pupae

Fine dry sand (100g) was autoclaved at

120°C (1.2 atm for 20 minutes) and placed in an aerated plastic box (110mm x 25mm). For inoculation, 12 ml suspension of freshly emerged IJs (100 IJs per ml) of each isolate were evenly distributed in sand to achieve 12% moisture (w/w). Ten pupae of mustard beetle, *P. cochleariae,* were placed on the sand and the boxes were placed in an incubator at 28°C. Water alone was used as control treatment. Replication was four fold and mortality assessment was recorded after 1, 2, 3 and 4 days. In all experiments, dead pupae were dissected in Ringers solution to confirm the presence of IJs as a cause of death.

Effect of temperatures on mortality of pupae

The same experimental procedure was used as described above. Ten pupae were placed in containers in which different isolates were applied and these were then incubated at 20, 25 and 30°C. Replication was four fold and mortality assessment was recorded after 3 days.

Effect of moisture contents on mortality of pupae

Containers prepared as above were inoculated with four nematodes and were maintained at 9, 12 or 15% moisture content. For inoculation, suspensions of freshly emerged (100 IJs per ml) were added to dry sand to achieve 9, 12 and 15 % moisture, by adding 9, 12 and 15 ml of suspension, respectively. Ten pupae were placed in each container which were then incubated at 28°C. Replication was four fold and mortality percentage was observed after 3 days.

Effect of nematode concentrations (doses) on mortality of pupae

Three nematode concentrations 50, 100 and 200 IJ per ml were prepared and put in the containers having 100g sand. Water was used as control. Moisture content was 12% and ten pupae were placed in each container and the containers were placed in an incubator at 28°C. Replication was four fold and mortality assessment was recorded after 3 days.

Infectivity test of nematodes against pupae

Infectivity of four entomopathogenic nematodes to *P. cochleariae* pupae was compared using sand-based assay (Bedding, 1984) at two different temperatures. Ten gram of autoclaved sand was infected with 100 IJs and placed in multi-well dishes with 12 cells (2.5 cm in diameter and 2.0 cm in depth). Single pupae of *P. cochleariae* were added to each well. After 3 days pupae were transferred to Petri dishes containing Ringer solution and were dissected. The total number of beetles emerging from pupae was counted. The tests were replicated 10 times

Statistical procedures

Data from all the experiments were statistically analysed using one/two-way ANOVA techniques of GENSTAT, Release 8.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Graphs were prepared with Microsoft Excel.

RESULTS

Effect of exposure time on mortality of pupae

Nematodes had a significant ($P < 0.001$) effect on the mortality of *P. cochleariae* pupae. A significant difference in mortality percentage was observed among the four nematode isolates and control. *H. indica* gave the highest mortality (59.4%). Also, mortality of *P. cochleariae* pupae increased as the time of exposure increased. The maximum average mortality (76%) of *P. cochleariae* pupae occurred after 4 days (Fig. 1A). Interaction between time and nematodes on the mortality was significant ($P < 0.001$). Maximum mortality (100%) occurred when *P. cochleariae* pupae were treated with *H. indica* within 3 days of exposure. It was closely followed by *S. carpocapsae* (95%), *H. bacteriophora* (92%) and *S. feltiae* (87.5%) after four days exposure.

Effect of temperatures on mortality of pupae

Temperature had a significant ($P < 0.05$) overall effect on mortality percentage of *P. cochleariae* pupae. The highest percentage mortality was recorded at 25°C (Fig. 1B). However, a non-significant ($P < 0.001$) difference was observed between the two Heterorhabditid isolates at three temperatures (20, 25 and 30°C), which were significantly different from Steinernematids. The response of different isolates varied ($P < 0.001$) at

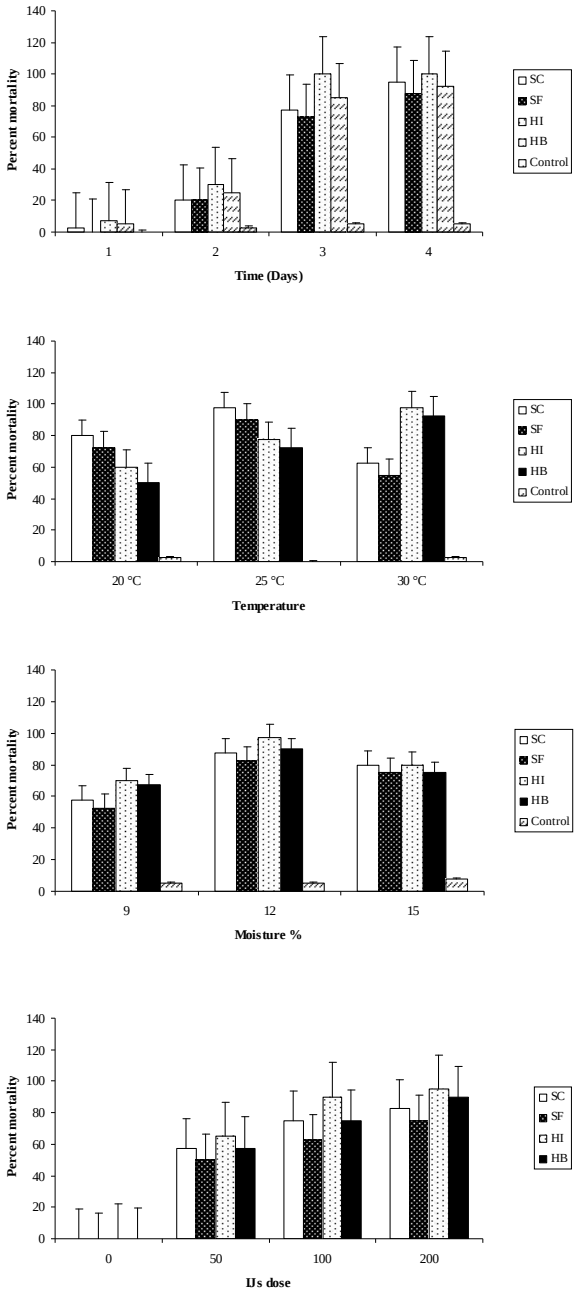

Fig. 1. The percent mortality of mustard beetle *P. cochleariae* pupae treated with four nematode isolates *S. carpocapsae, S. feltiae, H. indica, H. bacteriophora* and control at different time intervals (A), at three different temperatures (B), at three different moisture levels (C), and different doses of nematode isolates (D). Y error bars represent standard error.

each temperature. Both tropical isolates of *Heterorhabditis* performed well at 30°C and caused maximum mortality (97.5% and 92%) of beetle pupae, whereas, 25°C was suitable for the temperate zone isolates, *S. carpocapsae* (97.5%) and *S. feltiae* (90%).

Effect of moisture contents on mortality of pupae

Four nematode isolates showed a significant ($P < 0.001$) response to three moisture levels (9, 12 and 15%) when *P. cochleariae* pupae mortality was compared (Fig. 1C). All isolates showed maximum mortality at 12% moisture content. Like temperature, both Heterorhabditids isolates showed maximum mortality (97.5% and 90%) which was greater than the Steinernematids (87.5% and 82.5%) after 3 days at 12% moisture level. At high level of moisture (15%), mortality of *P. cochleariae* pupae in all treatments was found lower than that of 12% level in each nematode treatment.

Effect of nematode concentrations (doses) on mortality of pupae

Results for the effect of different doses of IJs on the mortality of *P. cochleariae* pupae following exposure of the pupae to four nematodes are shown in Figure 1D. Three nematodes concentrations (50, 100 and 200 IJs ml^{-1}) when applied showed at significant ($P < 0.001$) effect on the mortality of *P. cochleariae* pupae. The highest numbers of *P. cochleariae* pupae were found dead when treated with higher concentration of 100 IJs per ml in all isolates. Mortality of 95% and 90% was obtained when 100 IJs per ml doses of *H. indica* and *H. bacteriophora* were applied, respectively. Similarly, a dose 100 IJs per ml caused 82.5 and 75% *S. carpocapsae* and *S. feltiae* pupal death, respectively.

Infectivity test of nematodes against pupae

Temperature alone had significant effect ($P = 0.004$) on infectivity of *P. cochleariae* pupae. Temperature of 30°C gave the higher infectivity (28.8%) as compared to 25°C. Nematodes were significantly different ($P = 0.003$) in infectivity of *P. cochleariae* pupae. *H. indica* gave the highest infectivity (32.2%). *S. feltiae* gave the least infectivity (20.5%). The temperature x nematode interaction was highly significant ($P < 0.001$). *H.*

indica and *H. bacteriophora* showed similar patterns of infectivity in which both increased with increased temperature of 30°C. *S. carpocapsae* and *S. feltiae* showed a different response; their infectivity decreased with increased temperature. *H. indica* was found to be highly infective (46.2%) at 30°C as compared to 18.2% at 25°C.

Temperature showed a significant effect for all isolates when infectivity was tested. *S. carpocapsae* was found most virulent and appeared to be more infective at 25°C in *P. cochleariae*, followed by *S. feltiae*, *H. indica* and *H. bacteriophora* (Fig. 2). Number of IJs found in *P. cochleariae* pupae at 30°C showed significantly greater number of *H. indica*, followed by *H. bacteriophora*, *S. carpocapsae* and *S. feltiae*.

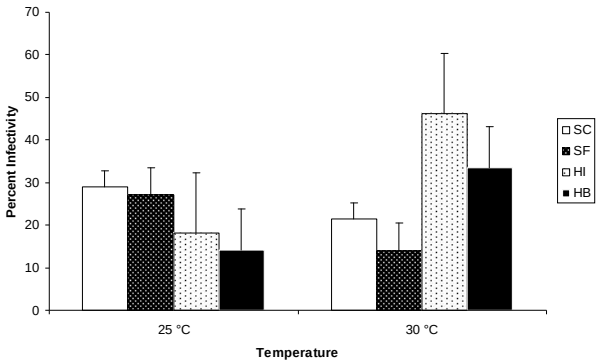

Fig. 2. The number of infective juveniles of four nematode isolates penetrated in a single pupa of mustard beetle *P. cochleariae* at 25 and 30°C temperatures. Y error bars represent standard error.

DISCUSSION

The purpose of present study was to demonstrate that it is possible to use entomopathogenic nematodes to control *P. cochleariae* pupae. A combination of right time of exposure, temperature, moisture, dose and infectivity are necessary to investigate the biological factors affecting *P. cochleariae* pupae. This could possibly lead to full scale use of nematodes as a soil treatment. Irrigation, an important source of moisture for nematode survival could also be useful when targeting the pupae of *P. cochleariae*. Pupation of this insect takes place in the soil. As demonstrated in this work penetration of the pupae by these nematodes is a possibility. This is important with respect to the use of irrigation in crops such as cabbage because it would enable the use of nematode to control the pupae in the soil. An important aspect regarding the use of Steinernematids and Heterorhabditids is their survival and dispersal after their application in the field. The infective juveniles and its mutualistic bacteria are found predominately in the upper soil profile (Womersely and Ching, 1989).

Exposure time is a major factor affecting nematode penetration into the pupae of *P. cochleariae*. The results agree with the findings of Glazer *et al.* (1991). They worked with *Steinernema* spp. and *Heterorhabditis* spp. on Egyptian cotton leaf worm. They showed that nematodes in cadavers of *Spodoptera littolaris* infected with 100 IJs of *S. carpocapsae* increased with increasing exposure time from a mean of 3.5-34.4 for a period of 3-12 hours. Susceptibility of the host insect is another important factor whether using insecticides or biological control agents. Differences in susceptibility were observed between the nematode isolates used in the experiments.

The nematode *S. carpocapsae* at 25°C and *H. indica* at 30°C demonstrated a higher mortality and infectivity in *P. cochleariae* pupae. The *S. carpocapsae* developed better than *S. feltiae* at 25°C. These findings are similar to those reported by Waturu (1991). He found higher mortality of *Phaedon* pupae within 2-3 days, after infection with 50, 100, 200 or 400 *S. carpocapsae* and *Heterorhabditis* spp. per 30 insect larvae at 25°C. Perhaps the entry through spiracles into the beetle pupae was more efficient with regard to *Steinernema* spp. than *Heterorhabditis* spp. at 25°C. Higher infection rate, mortality and production was observed in beetle pupae when treated with *H. indica* and *H. bacteriophora* than *S. carpocapsae* and *S. feltiae* at 30°C. Georgis and Hague (1988) recorded low infection of the saw fly (*Cephalcia lariciphila*) with *S. feltiae* at 25°C. They established that mortality increased with higher concentration of nematodes.

Elawad *et al.* (1996) found production, establishment, effectiveness and temperature range of a new Steinernematid nematode was similar to

Steinernema riobrave when they used *G. mellonella*. They further suggested that the recovery of these nematodes in tropical environment would be useful for biological control programmes. They could be mass-produced, formulated and used commercially for the control of various noctuid Lepidoptera and incorporated in pest management programmes. Kaya and Hara (1981) reported that beet armyworm, *Spodoptera exigua* (Hubner) and to a lesser extent cabbage looper, *Trichoplusia ni* (Hubner), as well as a number of other lepidopterous species were susceptible to *S. carpocapsae* infection.

In a sand based assay, temperature affects the infectivity (penetration ability and insect mortality) of different nematodes. Proper sand moisture conditions improved the effectiveness of nematodes against *P. cochleariae* pupae. Mahar *et al.* (2004) reported that pupae of *Plutella xylostella* were more susceptible to *S. carpocapsae* at 25°C but *S. riobrave*, *S. karii* and *S. abbasi* infected better at 30°C in sand bioassays. Mahar *et al.* (2005b) carried out investigation using the same nematode isolates against cabbage butterfly, *Pieris brassicae* pupae in sand bioassays. They compared the production and infectivity, and showed that *S. carpocapsae* penetration was better than all isolates at 25°C but *H. indica* gave higher and better penetration than all other isolates at 30°C. In another study Hudson and Nguyen (1989) observed that these species could be used against the desert locust nymphal stage to reduce the pest damage in field conditions.

Maximum insect mortality was recorded using higher concentrations of IJs (200 IJs per ml). The results of this study show that pupae are susceptible to different nematode isolates. Significantly more infective juveniles of the *H. indica* isolate penetrated and developed in pupae of *P. cochleariae* than *H. bacteriophora*, *S. carpocapsae*, and *S. feltiae*. Waturu (1991) reported that highest number of juveniles was observed in *P. cochleariae* pupae during the infectivity test when treated with *S. carpocapsae* UK strain followed by *Heterorhabditis* spp. strain M145 at 25-26°C. Our findings are also closely related to these findings.

Grewal *et al.* (1993) worked on the infectivity of *Steinernema scapterisci* to range of insect species of different orders including Lepidoptera. They reported that in a sand based assay, temperature affected the infectivity (penetration ability and insect mortality of both *S. scapterisci* and *S. carpocapsae*. *S. scapterisci* being more infective at higher temperature than *S. carpocapsae*). The optimum temperature of nematode penetration and establishment in *G. mellonella* larvae was 24°C for *S. carpocapsae* and 32°C for *S. scapterisci*. Temperature also affected the rate of *G. mellonella* mortality by nematodes, compared with *S. carpocapsae*, *S. scapterisci* killed hosts slowly (Grewal *et al.*, 1994).

In conclusion, these nematodes could be used in IPM approach to bring together all available insect pests control options. It would be necessary to study more environmental factors for improving the efficiency and persistence of these nematodes in field conditions. As most of the nematodes are exempted for pesticide regulation it seems appropriate that all isolates of entomopathogenic nematodes will have to be subjected to thorough testing under different environments before they are used as bio-pesticides. Further research work in laboratory and in field condition would therefore be necessary to investigate the possibility of targeting the other stages such as larvae and adults of mustard beetle.

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