J. BIOL. ENVIRON. SCI.,
2008, 2(6), 65-71

Comparison of Some Characterizations of Recovered from Soil and Newly Fermented Entomopathogenic Nematode, *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae)

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ABSTRACT

Entomopathogenic nematodes (EPNs) in the families Heterorhabditidae and Steinernematidae have considerable potential as biological control agents of soil-inhabiting insect pests. One of the most important factors for sustainable biological control is their successful establishment in the soil, infectivity against pest insects, reproductive potential and persistence in released areas. To determine the differences of infectivity, reproductive potential and persistence between recovered from the field one year after release and newly fermented (one-week-old) *Heterorhabditis bacteriophora* in a bioreactor, the two type populations of *H. bacteriophora* were compared each other on these capabilities. Prior to study, the fermented nematodes were reproduced once in *Galleria mellonella* larvae in order to achieve equal conditions with recovered populations. The results showed that infectivity and reproduction potentials of recovered populations were significantly higher than newly fermented or laboratory populations of the nematode. However, differences of persistence of both populations were not significant. This is the first report that quality control of some biological abilities of *H. bacteriophora* after one year in field.

Key Words: Fermentation, *Heterorhabditis bacteriophora*, infectivity, persistence, quality control, recovery, reproduction.

INTRODUCTION

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* symbiotically associated with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively, are safe antagonists as a commercial bioinsecticide for many economically important insect pests in ornamentals, vegetables, fruit and turf (Ehlers and Peters 1995; Ehlers and Hokkanen 1996; Georgis et al 2006). Governmental agencies today demand environmentally safe insecticides with low toxicity and short-term persistence, low mobility in the soil to prevent ground-water contamination and limited effects on non-target organisms. These prerequisites have reduced the change of getting new insecticidal compounds registered for soil application and older compounds have been banned. EPNs can alternatively be used to control soil insects and even have substantial advantages over chemical compounds, such as: No toxicity, sustainable and environmentally friendly. In commercial production process of EPNs, the appropriate symbiotic bacteria were introduced into the sterile medium with sterile first-stage juveniles of the nematode to establish the so-called monoxenic systems (Lunau et al 1993; Gaugler and Han 2001; Ehlers and Shapiro-Ilan 2004). Efficacy of applied EPNs relates closely to the used strains or species and environmental and technical conditions, that no two situations are identical or comparable. Factors of major importance are age and lipid reserves of infective juveniles (IJs). These characteristics directly influence the ability of nematodes to survive a period of time without a host and their ability to find and infect a host (Womersley 1993). Many studies mainly in the laboratories have tested the persistence of EPNs under various conditions in sterile soil. The data generally indicated a survival of weeks rather than months and a gradual decline in the numbers of living nematodes recovered. In some cases, their infectivity potential, however, does not follow the same pattern because at least some nematode individuals are capable of entering a quiescent state and later become active again when conditions permit host-finding activity (Fan and Hominick 1991; Womersley 1993). Behavioral adaptations and phases of anhydrobiosis or quiescence (Womersley 1993) will influence the pattern of survive. Nematodes developed strategies to survive adverse environmental conditions. In a dormant stage, the quiescent their metabolism is lowered and a longer persistence is possible. However, nematodes in this condition are not pathogen and only if the environmental conditions are favorable, they retain active and are able to penetrate into insects. The quiescent stage can be induced by extreme temperatures, oxygen deficiency and high salt content (Glazer 2002). After application nematodes rapidly disappear (Molyneux 1985; Kung et al 1991). But after the rate of population decline slows down.

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The aim of the present study is to compare reproduction capability, pathogenicity and persistence of isolated *H. bacteriophora* one year after application, and newly fermented or fresh *in-vitro* produced *H. bacteriophora* at variable temperatures and doses.

MATERIALS AND METHODS

Nematode

Entomopathogenic nematode, *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) strain en1 (Nematop®) were used in the experiments. The nematode was supplied by the company e-nema GmbH (Raisdorf, Germany) and had been cultured according to Ehlers et al (2000), Ehlers et al (1998) and Ehlers (2001) in a bioreactor. Only IJs younger than 1 week old were selected for the experiment. Two types of *H. bacteriophora* were used in the present experiments. One of both was fresh fermented in a bioreactor and the other was isolated one year after one-week-old fermented *H. bacteriophora* had been applied into soil. Newly *in-vitro* produced *H. bacteriophora* were propagated once in *G. melonella* larvae in order to achieve equal conditions with isolated *H. bacteriophora* from soil, and then emerged IJs were used in the study.

Test insects

The mealworm larvae, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) were used to determine the occurrence and pathogenicity of *H. bacteriophora* in infectivity experiment. The larvae were supplied by the company Morio Zoobedarf (Röttingen, Germany). The greater wax moth, *Galleria mellonella* L. (Lepidoptera: Galleriidae), was reared on a mixture of 900 g of liquid honey, 900 g of glycerin, 200 g of bees wax, 400 g of yeast flakes and 1300 g of whole meal at 25°C. The insect culture was reared in 1500 ml volume glass containers (11 cm diameter and 15 cm height) at 30-32°C on an artificial medium according to Wiesner (1993). Last instar larvae of the insect were used to recover IJs from soil and to detect reproduction abilitiy of *H. bacteriophora.*

Preparation of the experimental plots

A field that had not been cultivated for 3 years was selected for the experiments at the Lindhof, the experimental agriculture station of the Faculty of Agriculture and Nutritional Sciences, University of Kiel, located 26 km northern of Kiel, Germany. The treatments were arranged in an adjacent plot (10 x 80 m) in the field. The farm is following organic agriculture practice since 1997. The plot was kept fallow during the experiment. The application was performed during overcast sky. Precipitation, soil temperature and humidity in the field, and air tempereaturs were measured weekly and recorded during the study.

Nematode application

In field trials, one-week-old fermented *H. bacteriophora* in a bioreactor were sprayed using a concentration of 5 x 10^5 IJs m^{-2} in 250 ml water with an experimental plot application system (Pneumatic Sprayer Type PSG, Schachtner, Ludwigsburg, Germany) into soil in June. For spraying of EPN in laboratory experiment into experimental pots (15x15x15 cm), a 500 ml volume hand sprayer was used, which was equipped with a 0.5 mm diameter nozzle (Susurluk and Ehlers 2008).

Soil sampling and recovery of nematode

Soil samples were collected prior to and after the nematode application. The aim of soil sampling before application was detection of endemic *H. bacteriophora* in experimental plot. The sampling in order to detect endemic species was replicated three times in one month. Each sample contained approximately 40 g soil from an area of 3 cm². The samples were placed in a plastic bag and transported to the laboratory. Fourty samples per plot with 2 meter intervals were taken from the plot, and then were kept at 4°C until analyzed. Each soil sample was involved 2 last instar *G. mellonella* larvae and incubated at 25ºC for 3 days. After this period, infected larvae were transferred into White trap and then recovered IJs were stored at 4 ºC until using (Susurluk 2008).

***Confirmation of recovered* H. bacteriophora**

The identification of recovered nematode isolates was made by PCR-RFLP of the ITS region (Internal Transcribed Spacer) of the ribosomal DNA. The amplification of the ITS region was carried out in a reaction volume of 100 µl for each strain, containing 75.6 µl of H_2O, 2 µl of dNTPs (2mM), 10 µl of 10 × PCR-Buffer, 1 µl of Primer Forward (200 µM), 1 µl of Primer Reverse (200µM), 0.4 µl of Taq polymerase (5U/µl)

and 10 µl of purified DNA. The primers 18S (5`-TTGATTACGTCCCTGCCCTTT-3`) and 26S (5`-TTTCACTC GCCGTTACTAAG G-3`) were used as forward and reverse primers, respectively (Vrain et al. 1992).

The digestions with different restriction enzymes to produce RFLPs (Restriction Fragment Length Polymorphism) was carried at 37 °C for 3 hours in the thermal cycler using the corresponding restriction buffers with approximately 200 µl of a PCR product. Nine different enzymes were used: Alu I, Hae III, Hind III, Dde I, Hha I, Hinf I, Hpa II, Rsa I and Sau 3Al (Amersham Biosciences, Freiburg, Germany). Each cap containing 1 µl enzyme, 3 µl its buffer and 4 µl aqua dest, except for Hind III, which was used at 0.5 µl Hind III, 3 µl of its buffer and 4.5 µl aqua dest and Hinf I at 1.25 µl enzyme, 3 µl of its buffer and 3.75 µl aqua dest (Reid and Hominick 1998).

Persistence assay

Recovered and *in-vitro* produced *H. bacteriophora* were applied at a concentration of 50 IJs cm^{-2} in pots of 12 x 12 x 12 cm filled with soil. Prior to the experiment, the soil had been baited to assure that no indigenous nematode population was present. Sterile water was added to the soil to achieve water content of 10% (w/v). The soil moisture was checked with electronic moister analyzer (Sartorius MA 40, Göttingen, Germany). An amount of 7200 IJs were applied per pot and incubated at 16 ºC for 12 weeks. For each type of *H. bacteriophora*, 20 pots were used. Every 2 weeks one soil sample of approximately 3 cm^2 and 40 g was taken from each pot with a soil borer (2 cm diameter and 12 cm height). Two *G. mellonella* larvae were added to the each sample to bait the nematodes. Baiting was repeated three times with 3-day intervals. The infected larvae were dissected and the number of IJs per insect was counted. Dead *G. mellonella* were washed in Ringer´s solution in order to remove IJs on the surface of the insect and the cuticle of the larva was removed with the help of a scissors and pins. The haemolymph was homogenized by consecutive passages of the haemolymph together with 2 ml Ringer`s solution through a Pasteur pipette. The suspension was then poured into a 10 ml glass tube and centrifuged at 2000 rpm for 2 min. After centrifugation, the supernatant was carefully removed and the sediment containing the nematodes was diluted by adding Ringer´s solution at a ratio of 1:5. All IJs were counted in counting cell-wells under the microscope (Susurluk 2005). This experiment was repeated two times.

Bioassay to test the infectivity at different temperatures

Concentrations of 5, 10, 20, 30, 60 and 120 IJs/*T. molitor* larva were tested at temperatures of 12, 18 and 24 °C. The bioassay was carried out in small boxes (5 x 5 x 5 cm) filled with moist silver sand (10 % w/v) of 0.1-0.5 mm particle size, which had been sterilised at 80°C for 12 h, and with 10 last instars of *T. molitor*. After application of *H. bacteriophora*, the boxes were incubated at above described temperatures. Five days after the incubation, mortality of the insect was assessed. The experiment was replicated 10 times.

Reproductive potential at different temperatures and doses

In order to evaluate whether the reproductive potential of the nematode was affected between the types of *H. bacteriophora*, the number of nematode offspring per insect was evaluated. The experiment was carried out as described in the infectivity bioassays using the concentrations of 10, 50 and 100 IJs per *G. mellonella* larva. In this assay, *G. mellonella* larvae were used with a weight between 145 and 188 mg. Each concentration of the IJs was tested on 10 *G. mellonella* larvae. After 48 h incubation at 25 °C, the infected cadavers, recognised their red colour, were removed from the sand, rinsed, transferred to water traps and incubated in the dark at 25 °C. All emerging IJs from a single larva were recovered over a period of 10 days and stored in a 50 ml flask (Boff et al 2000). The content of each flask (nematode suspension from individual cadavers) was mixed thoroughly using air bubbles. Eight samples of 10 µl from each suspension were examined under a stereomicroscope and the total number of IJs per cadaver was calculated (Susurluk 2006).

Statistical analyses

In the infectivity experiment, first, total insect mortality data were corrected for control mortality using Abbott`s formal (Abbott 1925). Means of reproductive capability, infectivity and persistence of recovered and fresh *in-vitro* produced *H. bacteriophora* were analyzed by analysis of variance ANOVA (breakdown one way Anova) and followed by a Least Significant Difference (LSD) test. The minimum level of significance was taken as $p<0.05$ (Statistica 1991).

RESULTS

In order to investigate whether the fitness of the released fermented nematodes will change within one year after application in the field, IJs infectivity, reproduction capacity and the potential to persistence were compared between a *H. bacteriophora* population recovered from plots where the strain had been released in June, and a population taken from *in vitro* mass production provided by e-nema GmbH. Prior to comparison between the samples of the different sources of the nematode, the fermented nematodes one-week-old were reproduced once in *G. mellonella* larvae.

First result of the study was that an endemic population of EPNs was not detected in the experimental plots. But, nevertheless, recovered *H. bacteriophora* in the plots was identified by using of PCR-RFLP method. According to the result of the molecular identification, it was confirmed that recovered *H. bacteriophora* population was the released *H. bacteriophora*. Moreover, no insect pest was fixed in the plots during the experiment.

One year after application, it was found in 3 plots that 50 of 240 *G. mellonella* larvae were infected with the released *H. bacteriophora*.

The mean annual precipitation is 670 mm and the mean temperature is 8.5ºC. Measured soil temperatures were from 0 to 19°C, humidity of the soil varied 8 and 90% in 15 cm, air temperatures were between -10 and 35°C.

Differences in infectivity of *H. bacteriophora* originating from *in-vitro* cultured and recovered nematodes can be detected particularly when low nematode concentrations are used against *T. molitor* larvae. At low temperature the difference in infectivity is none statistically different. Significant differences were detected at a concentration of < 120 IJs at 18°C and at < 60 IJs per larva at 24°C (Figure 1). Thus, recovered nematodes were more effective than those, which had been continuously produced, in *in vitro* culture (F = 70.9, df = 5, 54, p<0.00001 for the dose of 5 IJs; F = 117.3, df = 5, 54, p<0.00001 for the dose of 10 IJs and F = 188.1, df = 5, 54, p<0.001 for the dose of 30 IJs). In control treatments, mortality was between 0 and 8%.

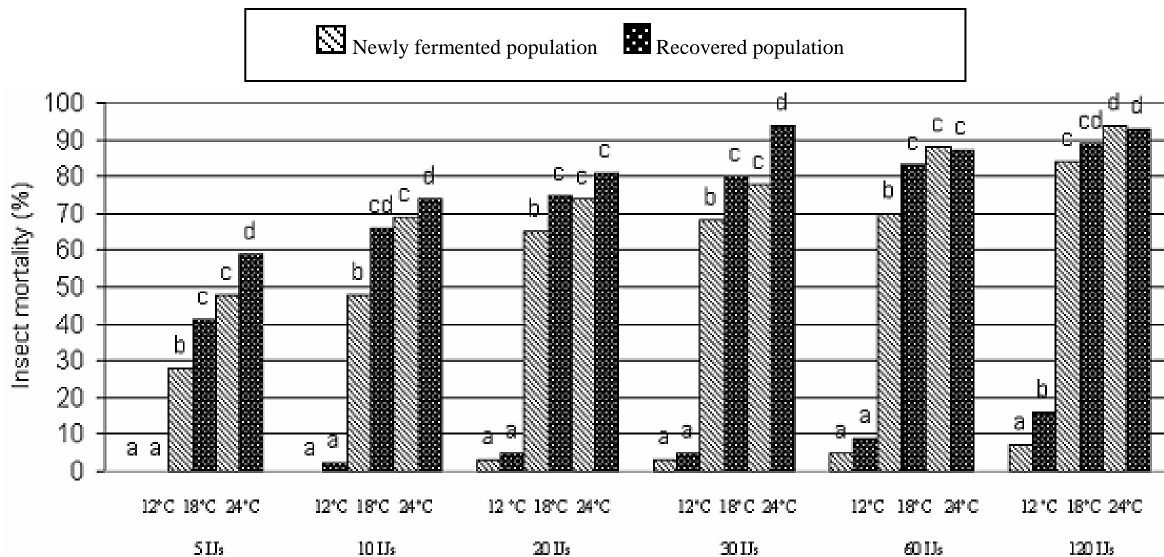

Figure 1. Mortality of *T. molitor* larvae (%) exposed for 5 days to one-week-old fermented population and recovered *H. bacteriophora* populations at doses of 5, 10, 20, 30, 60 and 120 IJs at 12, 18 and 24 °C. Columns with the same letter are not statistically different for one dose. The data were analysed for variance (P<0.05) and subjected to the least significant-different test (LSD) for testing pair wise differences between treatments.

Results on the reproduction of IJs are presented in Figure 2. IJs of the recovered *H. bacteriophora* was more productive (F = 31.4; df = 5, 54; p<0.00001) except that the differences at a dose of 50 IJs were not statistically different between the two groups.

The results in Figure 3 on the persistence are presented, indicating that the number of recovered *H. bacteriophora* IJs was usually higher in tests with the recovered population. However, both types of *H. bacteriophora* persisted in the soil until 12 weeks. The tests were replicated until no IJs were detected.

Except for the 4th and 12th weeks, statistically differences between the two populations were detected (F = 117.7; df = 13, 266; p = 0.001).

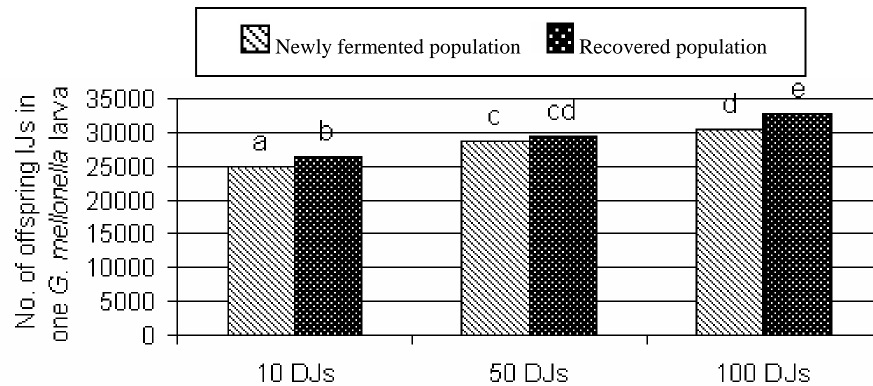

Figure 2. Number of IJs produced from one *G. mellonella* last instar larva inoculated with one-week-old fermented and recovered *H. bacteriophora* populations at different doses. Data followed by the same letters are not significantly different from each other at $p<0.05$. The data were analyzed for variance ($P<0.05$) and then subjected to the least significant-different test (LSD) for testing pair wise differences between treatments.

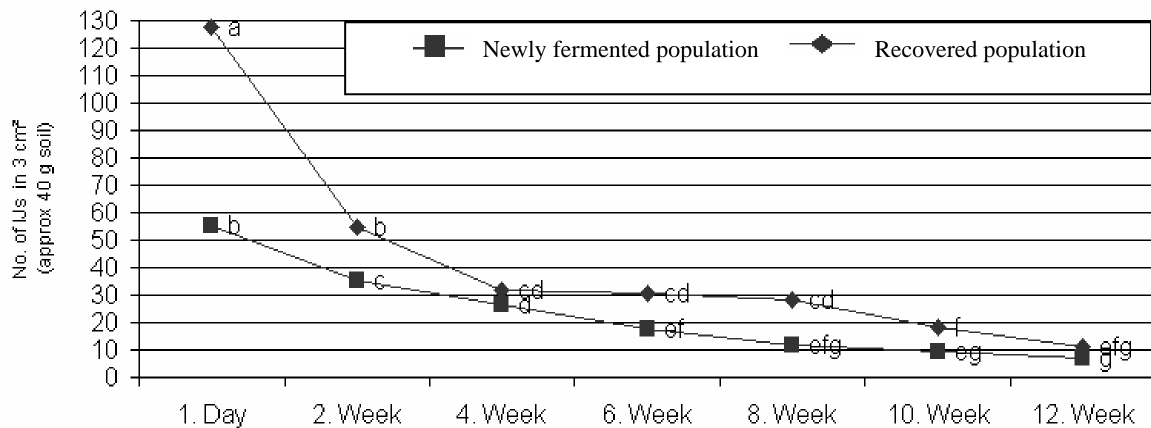

Figure 3. Numbers of isolated IJs of one-week-old fermented and the recovered *H. bacteriophora* populations from the soil which had been stored at 16 °C. Data points with the same letters are not significantly different from each other at $p<0.05$. The data were analysed for variance ($P<0.05$) and then subjected to the least significant-different test (LSD) for testing pair wise differences between treatments.

DISCUSSION

Quality control is one of the key issues in the recent industrial development. With living biological products, which are intrinsically variable in performance, quality control will never provide the same product stability as with technical products. Quality of EPNs can be defined as their performance against target pests, persistence and reproductive potentials in practice.

Once EPNs have established in the soil, their persistence will depend on favourable environmental conditions. Environmental conditions can have a significant influence upon survival rates. Humidity can be excluded as an influencing factor on persistence as soils in the experimental region provided enough humidity throughout the investigation period. Temperatures above 40 °C and below 8 °C are lethal for most EPNs (Griffin 1993; Grewal et al 1994). In the range of 15-25°C, higher temperatures increase the rate of metabolism and shorten the life span. Extremely high temperatures are rare in the soil environment, but temperatures below 8°C are common in Northern Germany. Therefore, released *H. bacteriophora* can survive during one year after application. Moreover, Susurluk and Ehlers (2008) also found that the longest field persistence of 23 months was recorded after application to beans for *H. bacteriophora* in the same location.

Boff et al. (2000) studied on a reproduction examination of *H. megidis* (strain NLH-E87.3) at following doses: 10, 30, 100, 300, 1000 and 3000 IJs. Results indicated that total production of the species nearly 28000 and 30000 IJs per larva at the doses of 10 and 100 IJs, respectively. However, in the present study, the highest reproduction was observed 30000 and 33000 IJs/larva in *in-vitro* produced and recovered *H. bacteriophora*, respectively, at a dose of 100 IJs per larva. Moreover, the present result was accordance with Selvan et al (1993) who observing that the production of IJs of *H. bacteriophora* increased with increasing the initial density up to approximately 100 IJs/larva.

The present study showed that significant differences were recorded between *in-vitro* produced and recovered populations of one year after application of one-week-old fermented *H. bacteriophora* in infectivity, reproduction potential and persistence. It might be concluded that the *in-vitro* population (laboratory population) has genetically deteriorated. However, the origin of the recovered population is population of the *in-vitro* produced. If the laboratory generation had decreased in its control potential then this change has happened within the year following the release of the recovered population. The detrimental genetic changes can be prevented by creating inbred lines (groups that are the same genetically). Another possibility is that the higher fitness of the recovered population is a result of naturel selection into soil. Johnigk et al (2002) and Strauch et al (2004) demonstrated that beneficial trait of the strain used in this study can be improved by genetic selection. Moreover, Strauch et al (2004) reported that a hybrid strain 30 homozygous of *H. bacteriophora* inbred lines was propagated in liquid culture several times; the percentage of recovered IJ and the final IJ yields were recorded. The laboratory strain was not exposed to environmental stress, whereas the released population had to adapt to natural conditions. If this is what happened with the released population, this is rather good news, as it indicated that the genetic plasticity is sensible to changing selective factors.

ACKNOWLEDGEMENTS

Thanks are due to e-nema GmbH for a scholarship to the first author. We also thank Johanna Schmidt, Doris Ziermann, Michael Wingen, Helga Ladehoff, Xiaoli Yi and Nicola Benecke for technical support.

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