Control of the Western Corn Rootworm in maize with
_Heterorhabditis bacteriophora_: Influence of field persistence
and infectivity

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Control of the Western Corn Rootworm in maize with *Heterorhabditis bacteriophora*: Influence of field persistence and infectivity

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**Summary** – *Heterorhabditis bacteriophora* is used for the control of the Western Corn Rootworm, *Diabrotica virgifera virgifera* LeConte. High virulence is a desired characteristic of entomopathogenic nematodes to reduce insect pest populations but it can vary between strains of the same species. The virulence of forty-two strains and one strain pool of *H. bacteriophora* was evaluated against *T. molitor*. The concentration at which 50% (LC$_{50}$) of *T. molitor* larvae were infected ranged between 1.4 to 30.5 DJs per insect. Five strains out of the forty-two were selected for comparison of virulence between *D. v. virgifera* and *T. molitor*. Strains were more effective against *T. molitor* than in *D. v. virgifera* and no correlation for virulence was found between insects. In the field, nematode applications are made during spring together with sowing when eggs of *D. v. virgifera* are still in diapause. Thus, nematodes must persist in soil until larvae hatch but also need to stay infective for a minimum of six weeks until most larvae have hatched. To assess persistence, a commercial strain, an inbred line produced from the commercial strain and a pool of 33 strains were inoculated to potted maize plants and the presence of nematodes was assessed by addition of *Galleria mellonella* larvae. All strains were able to remain infective and to persist in soil over one hundred days. Nematodes recovered from the pots after 103 days were again inoculated into pots and persistence was compared with a pooled population as used in the first experiment. Persistence did not differ significantly between selected and the other
strains. In the first experiment alternating high and low percentage of infested insect over time were detected for all strains. In the second experiment this pattern was only present in results obtained with the commercial line and the derived inbred line. The results document the potential of breeding programs to select for improved virulence and persistence in order to enhance the control of *D. v. virgifera* and other target pests.

**Keywords:** *Diabrotica virgifera virgifera*, virulence, biological control, entomopathogenic nematodes, EPN.

The Western Corn Rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) is an economically important pest in North America and Europe (Toepfer *et al.*, 2010). The WCR attacks maize plants during its three larval stages feeding on maize roots, which can ultimately lead to plant lodging (Moeser & Hibbard, 2005). The adults feed on silk of the plant causing reduced fertilization, also resulting in yield losses (Chiang, 1973). It is hypothesized that the pest has its origin in Mexico and its fast expansion has been due to the increase of maize cultivations and the decreasing crop rotation with non-host plants (Steffey *et al.*, 1999). Since its introduction into Serbia in 1992 this invasive pest is rapidly dispersing over Europe and it is expected that, in the future, the WCR will be present in all maize cultivars all over Eurasia (Kuhlmann & van der Burgt, 1998).

Different methods are used to control the WCR. Rotation of maize with non-host crops is a successful management strategy of the WCR as the species overwinters in the egg stage usually on the fields where maize has been grown (Branson & Ortman, 1970). However, this option is not the most economic possibility. Also, a decrease in pest suppression by cop rotation was reported due to an adaptation of the insect’s egg laying behavior. Increase in oviposition in
soybean, pumpkin, sunflowers, sugar beet and other non-host plants jeopardize the effect of crop rotation (Levine et al., 2002; Mabry et al., 2004; Moeser & Vidal, 2004; Foltin & Robier, 2014). Insect resistant varieties of maize, like Bt maize, which produces toxins derived from the bacterium *Bacillus thuringiensis*, work against several pest insects providing promising control (Huang et al., 2005; Hutchison et al., 2010). However, due to the weak genetic basis of pyramid Bt toxins against Coleoptera (Cry3 or Cry34/35), a fast increase in resistance and adaptability of WCR has been reported in field conditions (van Rensburg, 2007; Tabashnik et al., 2008; Zhang et al., 2011; Gassmann et al., 2014; Tabashnik & Carrière, 2015). Another control option is seed coating with Neonicotinoide insecticides (Noleppa & Hahn, 2013). Several studies, however, affirm that the residuals of the systemic neonicotinoids are founded in water, soil, sowing dust, pollen, nectar, etc. and can have negative effects on non-target pollinating insects (FERA, 2013; van der Sluijs et al., 2013). Insecticides have often been applied as a fast method of control over pest insects, but the development of resistance to these insecticides, issues related to human safety and the concerns about the environment push the research to the development of new management practices (Levine & Oloumisadeghi, 1991). Among biological control approaches, the use of entomopathogenic nematodes (EPNs) has been quite successful (Toepfer et al., 2010; Ehlers pers. communication). EPNs have a wide host range including larvae of the WCR (Jackson, 1996; Toepfer et al., 2005), they actively search for a host and are safe to humans and the environment (Ehlers, 2003).

Among the species of EPNs, Steinernematidae and Heterorhabditidae are the most studied for their application in biological control (Blaxter et al., 1998; Stock, 2005). *Heterorhabditis bacteriophora* is the species which has been widely tested under field conditions with high control results (Gaugler & Kaya, 1990; Toepfer et al., 2010) The symbiotic relation of *H.*
bacteriophora with the bacterium Photorhabdus luminescens provides the nematode with a pathogenic partner killing the host insects (Simoes et al., 1992). H. bacteriophora is known to be a highly virulent species against the three larval stages of D. v. virgifera (Kurtz et al., 2009). This nematode has been found not only attacking the larval stage but also the pupal stage (Jackson, 1996; Kurtz et al., 2009). In a study by Toepfer et al. (2005) about virulence of different EPN species, H. bacteriophora was the most promising candidate out of 8 tested EPN species to control D. v. virgifera. At an application rate of 2 x 10⁹ nematodes per ha H. bacteriophora provide equally or often better control than currently available pyrethroid granular insecticides applied at 15 kg per ha (Ehlers, personal communication). However, the use of H. bacteriophora still is more costly than the use of e.g. granular pyrethroides. Therefore research is focusing on the selection of more virulent and better persisting nematodes in order to be able to reduce the application rate and be more competitive with synthetic pesticides (Ehlers, personal communication).

For studies on nematode virulence under laboratory condition larvae of the mealworm Tenebrio molitor are widely used as bait for diverse bioassays to test the quality of EPNs and other insect parasitic organisms (Zimmermann, 1986; Bharadwaj & Stafford, 2011). Should the virulence against D. v. virgifera correlate with the virulence of T. molitor, selection for more virulent nematodes would be easier, as T. molitor is easier to rear and to handle than WCR. It was therefore investigated, whether results in virulence match between the two insect species.

In order to obtain an effective control of D. v. virgifera it is necessary that H. bacteriophora is not only virulent but also persistent in soil. In field, the application of the nematodes is done by injecting the nematodes together with the maize seeds when sowing with the cultivator. This application method provides enough water for the nematodes to establish in the rhizosphere and
needs a minimum of nematode material because of the highly targeted application technique (Toepfer et al., 2010). However, by that time of sowing, the eggs of the WCR are still in diapause and larval emergence starts at 4-6 weeks after maize seeds have been planted in the field (Chiang, 1973). A comparative study on the time of nematode application by Kurtz et al. (2007) has proven that there are no differences in field efficacy whether nematodes are apply during sowing or during larval emergence 6-8 weeks later. Application at sowing in March/April, however, requires that DJs of H. bacteriophora must persist for at least a period of time until the eggs of D. v. virgifera hatch. The persistence depends on factors such as soil type and amount of nematodes injected (Pilz et al., 2014). Nowadays it is known that H. bacteriophora can remain in field over 42 days and it can persist in maize fields during the period of the juvenile development of D. v. virgifera (Kurtz et al., 2007; Pilz et al., 2014), but the number of nematodes decrease in the course of time (Pilz et al., 2014). Should one be able to select a nematode strain/line with longer persistence, it might also be possible to use less nematode and be more competitive, likewise when using more virulent nematodes. Therefore, the work also targeted at investigating the persistence of nematodes in the maize environment.

In order to improve the knowledge on the control of D. v. virgifera with the nematode H. bacteriophora, this study had as objectives (1) the assessment of the virulence of 42 strains of the species H. bacteriophora by calculation of the Lethal Concentration (LC$_{50}$), (2) the comparison of the infectivity against T. molitor and D. v. virgifera and (3) the assessment of persistence in soil of the different strains and the selection of the most persistent strains for further studies.
Materials and methods

Nematode culture and storage

All nematodes used in this study were provided by e-nema GmbH, Raisdorf. Descriptions of the strains of *Heterorhabditis bacteriophora* used in this study are summarized in Table 1. The nematodes were reared at 25°C on last instars of the wax moth *Galleria mellonella* as described by Kaya and Stock (1997). The freshly emerged DJs were stored in Ringers (9.0 g NaCl, 0.42 g KCl, 0.37 g CaCl₂ × 2H₂O, 0.2 g NaHCO₃ dissolved in 1 l of distilled water) solution at 5°C before use.

Insects

Larvae of *Diabrotica virgifera virgifera* were provided by BTL Bio-Test Labor GmbH Sagerheide. *Tenebrio molitor* and *Galleria mellonella* larvae were obtained from Futterinsektenfarm Schulz, Eschach-Holzhausen.
Table 1. Strains of *Heterorhabditis bacteriophora* used in the study and Megamix strain components (From 1 to 33).

<table>
<thead>
<tr>
<th>No.</th>
<th>Strain designation</th>
<th>Line/Activity</th>
<th>Geographical origin</th>
<th>Source / Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DE2</td>
<td>Wild type</td>
<td>Kaiserstuhl, Germany</td>
<td>H. Bathon</td>
</tr>
<tr>
<td>2</td>
<td>S-HT1</td>
<td>Heat tolerance</td>
<td></td>
<td>J. Mukaka</td>
</tr>
<tr>
<td>3</td>
<td>IR1</td>
<td>Wild type</td>
<td>Karaj, Iran</td>
<td>J. Karimi</td>
</tr>
<tr>
<td>4</td>
<td>IR2</td>
<td>Wild type</td>
<td>Karaj, Iran</td>
<td>J. Karimi</td>
</tr>
<tr>
<td>5</td>
<td>IR3</td>
<td>Wild type</td>
<td>Karaj, Iran</td>
<td>J. Karimi</td>
</tr>
<tr>
<td>6</td>
<td>IL1</td>
<td>Inbred line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>IL2</td>
<td>Inbred line</td>
<td></td>
<td>Strauch et al. 2004</td>
</tr>
<tr>
<td>8</td>
<td>IL3</td>
<td>Inbred line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>EN01</td>
<td>Commercial strain</td>
<td>Schwentinental, Germany</td>
<td>E-nema</td>
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<td>DE7</td>
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<td></td>
<td></td>
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<tr>
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<td>IL4</td>
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<td></td>
<td>Strauch et al. 2004</td>
</tr>
<tr>
<td>12</td>
<td>DE8</td>
<td>Wild type</td>
<td></td>
<td></td>
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<tr>
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<td>IL5</td>
<td>Inbred line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>S-CR1</td>
<td>Cold resistant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>HY1</td>
<td>Hybrid</td>
<td>Aadin, Turkey</td>
<td>Selcuk</td>
</tr>
<tr>
<td>16</td>
<td>HY2</td>
<td>Hybrid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>TR2</td>
<td>Wild type</td>
<td>Aadin, Turkey</td>
<td>Strauch et al. 2004</td>
</tr>
<tr>
<td>18</td>
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<td>Inbred line</td>
<td>Bari, Italy</td>
<td>K. Desté</td>
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<tr>
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<td>IT6</td>
<td>Wild type</td>
<td></td>
<td></td>
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<td>20</td>
<td>S-VI-MM1</td>
<td>Selected for virulence on <em>M. melolontha</em> in 8 selection rounds</td>
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<td>S-VI-MM14</td>
<td>Selected for virulence on <em>M. melolontha</em> in 14 selection rounds</td>
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<td>A. Fodor</td>
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<td>A. Fodor</td>
</tr>
<tr>
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<td>Selcuk</td>
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<td>S-DT1</td>
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<td>S-DT2</td>
<td>Desiccation tolerant</td>
<td></td>
<td>Strauch et al. 2004</td>
</tr>
<tr>
<td>31</td>
<td>IL8</td>
<td>Inbred line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>HY3</td>
<td>Hybrid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>XX2</td>
<td>Wild type</td>
<td></td>
<td></td>
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<tr>
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<td>PT1</td>
<td>Wild type</td>
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<td>D. Toubarros</td>
</tr>
<tr>
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<td>PT2</td>
<td>Wild type</td>
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<td>D. Toubarros</td>
</tr>
<tr>
<td>36</td>
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<td>Wild type</td>
<td>Azores, Portugal</td>
<td>D. Toubarros</td>
</tr>
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<td>D. Toubarros</td>
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<td>S-IL-OX1</td>
<td>Inbred line selected for oxidative stress resistance</td>
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<td>EMS-Mutant selected for heat tolerance</td>
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<td>42</td>
<td>IL10</td>
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<td>Strauch et al. 2004</td>
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</table>
SCREENING FOR VIRULENCE IN *H. BACTERIOPHORA* ON *TENEBRIO MOLITOR*

Forty larvae of *Tenebrio molitor* were placed into Petri dishes (150 mm) filled with 150 g of sand adjusted to 8.5 % water content. Forty-two individual strains and one pool of 33 strains (Table 1), called Megamix were used for the tests. The Megamix consisted of a nematode pool with equal amounts of the different strains. Nematode concentrations of 80, 200, 400, 800 and 2000 DJs in 1 ml of Ringers solution were inoculated in the middle of the plates and incubated at 25°C for 7 days. Control plates received 1 ml Ringers solution. Four replicates were done for each nematode concentration. After seven days, the Petri dishes were observed and sampled for larvae and mortality was determined. Infection by nematodes was checked with the luminometer (LUMAT LB 9501 Berthold Germany). Due to the release and propagation of the symbiotic bacteria, cadavers are bioluminescent. The mean mortality by nematode infection of *Tenebrio molitor* was compared among 42 strains.

COMPARISON OF VIRULENCE OF *HETERORHABDITIS BACTERIOPHORA* AGAINST *TENEBRIO MOLITOR* AND *DIABROTICA VIRGIFERA VIRGIFERA*

Petri dishes (150 mm) were supplied with a one week-old germinated maize seedling each (cv Ronaldinho) as food source for the *D. v. virgifera* larvae. The maize seedling was also placed into dishes inoculated with *T. molitor*. The plates were filled with 150 g of sand adjusted to 8.5 % water content and subsequently twenty third instar larvae of *D. v. virgifera* were placed in Petri dishes. The nematodes used in this experiment were selected from a first screening on virulence against *T. molitor* (strains DE2, IR2, EN01, IL4 and S-VI-MM14). Nematode concentrations of 100, 200, 400, 800, 1600 and 3200 DJs in 1 ml of Ringers solution were inoculated in the middle of the plates. For *T. molitor* the same method was followed but the nematode concentrations were
adjusted to 20, 40, 100, 200, 400 and 1000 DJs in 1 ml of Ringers solution. Control plates received 1 ml of Ringers solution. Three replicates were done for each nematode concentration. Plates were incubated at 25°C for 7 days. Then dead and living larvae were counted. In case that not all larvae were found in the sand, the sand was washed and the water was decanted in a flat recipient to recover the remaining larvae. Infection was checked by presence of luminescence in the cadavers of both insect larvae with the luminometer. The mean mortality by infection of *T. molitor* and *D. v. virgifera* was compared among the five strains and correlated between both insects.

**SCREENING FOR PERSISTENCE OF VIRULENCE IN HETERORHABDITIS BACTERIOPHORA**

Two liter pots were filled with a mixture of 75% potting soil (Einheitserde Type VM) and 25% sand and adjusted to 65 % water content. Three maize seeds (cv Ronaldinho) were sown and pressed three cm deep into the soil. Densities of 20 thousand DJs per pot were inoculated in three inoculation holes. The holes were closed after inoculation. Twenty one pots were used for each of the four treatments: EN01 (commercial strain), IL3 (inbred line produced from EN01 strain), Megamix (strain pool) and Control (no nematodes). Maize plants were thinned after ten days. Three pots were randomly chosen for each of the four treatments at each collecting date. The maize plants were cut and ten last instar larvae of *Galleria mellonella* were collocated in three cm holes into the soil to bait persisting nematodes. Then, the pots were filled to the top with potting soil, carefully taped and incubated at 25°C for seven days. Baiting was done at 1, 41, 46, 77, 89, 96 and 103 days after the set-up of the pots in order to explore the dynamics in the persistence of infective *H. bacteriophora* over time. Dead and alive larvae of *Galleria mellonella* were counted and the percentage of infected cadavers was measured with the luminometer.
Nematodes of all three variables from the last collection on day 103 were recovered from infected larvae to start a new population for selection on persistence. The selected EN01 (commercial strain), selected IL3 (inbred line produced from EN01 strain) and selected Megamix (strains pool from first selection round) were compared with a Megamix again produced by mixing of all 33 *H. bacteriophora* strains and a control (without nematodes). The same methodology used for the set-up of the first experiment was followed for the second. Baiting was done after 21, 42, 63, 84, 105 and 126 days.

**DATA ANALYSIS**

The percentage of dead insects recorded as dead and luminescent was used for the calculations of the Lethal Concentration (LC$_{50}$) for the virulence by the formula: mortality = a (1 - ln(-bx)) + c. The data were compared with the saturation curve through minimizing the chi-square ($\chi^2$) fitting to a value nearest to 0. The values obtained were analyzed for normality with the Shapiro-Wilkinson test (p>0.05). In case of not-normal-distribution, log transformation was used. For normal distributed data ANOVA and Tukey’s test for multiple comparisons were done. For non-normal distributed data the Kruskal-Wallis test with post hoc Conover-Iman test for multiple comparisons were used. The Bonferroni test was performed for correction of significant differences. All statistical analyses were conducted using XLSTAT (version 2013). To fit the error bars for percentages in persistence graphics (Fig. 4) the following formula was used:

$$SD = \sqrt{\frac{Infected \ rate \times \ Non - infected \times Total \ insects \ per \ treatment}{Total \ insects \ per \ treatment}}$$
Results

**Virulence of H. bacteriophora strains on Tenebrio molitor**

All 42 strains and the pooled strains (Megamix) were able to infect *Tenebrio molitor*. Significant differences were found among the LC$_{50}$ data on *T. molitor* ($K = 120.55$; $df = 42$; $p < 0.0001$). The LC$_{50}$ of the strains of *H. bacteriophora* ranged from 1.4 to 30.5 nematodes per insect (Fig. 1). PT1, from Portugal, was the most virulent strain against *T. molitor* larvae with a LC$_{50}$ of $1.4 \pm 0.33$, followed by the Hungarian HU2 = $1.8 \pm 0.23$ and the strain from Iran, IR2 $2.3 \pm 0.51$. S-IL-OXI was the least virulent strain with an LC$_{50}$ of $30.6 \pm 6.30$ DJ$s$ per insect, followed by the Chinese CN4 = $22.8 \pm 3.48$ and the mutant strain M-HT = $19.9 \pm 3.01$. Furthermore, wild type strains were significantly more infective compared to inbred lines, whereas hybrids were intermediate with no significant difference ($K = 17.01$; $df = 2$; $p \leq 0.0001$).
Figure 1. LC$_{50}$ of 42 strains and one strain pool (Megamix) of *Heterorhabditis bacteriophora* against *Tenebrio molitor* larvae assessed after one week of exposure at 25°C to infective dauer juveniles. Error bars indicate standard deviation of four replicates. Different letters on the error bars indicate significant differences (Conover-Iman test with Bonferroni correction, p < 0.0001)
**Virulence of *H. Bacteriophora* against *Diabrotica virgifera virgifera* and *Tenebrio molitor***

The strains DE2, IR2, EN01, IL4 and S-VI-MM14 were selected to be compared for virulence against *T. molitor* and *D. v. virgifera*. Significant differences were found among the LC$_{50}$ data between *D. v. virgifera* and *T. molitor* ($F = 5.035; df = 9; \ p < 0.001$) (Fig. 2) only for strain DE2 against *T. molitor* when compared to results obtained with IR2 and IL4 against *D. v. virgifera*. The IR2 strain had an extraordinary high LC$_{50}$ against *D. v. virgifera*. The LC$_{50}$ was ranging from 19.3 to 112.2 in *D. v. virgifera* and from 6.3 to 18 in *T. molitor*.

![Figure 2](image-url)

**Figure 2.** LC$_{50}$ of the strains DE2, IR2, EN01, IL4 and S-VI-MM14 of *Heterorhabditis bacteriophora* against *D. virgifera virgifera* and *Tenebrio molitor* larvae after one week of exposure at 25°C to infective dauer juveniles. Error bars indicate standard deviation of three replicates. Different letters on the error bars indicate significant differences (Turkey’s HSD test, $p < 0.05$)
The strain DE2 displayed the lowest LC$_{50}$ value for both insect hosts, 19.3 ± 5.9 for *D. v. virgifera* and 6.3 ± 2.1 for *T. molitor*. In *D. v. virgifera*, DE2 was followed by the commercial strain EN01 36.9 ± 4.0, the selected strain S-VI-MM14 37.7 ± 15.9, the inbred line IL4 52.6 ± 16.4 and the Iranian IR2 112.2 ± 18.7. In *T. molitor*, the second best was also EN01 15.8 ± 8.3, followed by IL4 16.5 ± 9.2, IR2 17.0 ± 17.0 and S-VI-MM14 18.0 ± 6.8.

No positive correlation of the nematode virulence between the LC$_{50}$ against *Tenebrio molitor* and *Diabrotica virgifera virgifera* was found ($R^2=0.0121$; $p>0.05$) (Fig. 3).

![Figure 3](image-url). Correlation between LC$_{50}$ of different *Heterorhabditis bacteriophora* strains against *Tenebrio molitor* and *Diabrotica virgifera virgifera*. 

$y = 0.6552x + 68.339$

$R^2 = 0.0121$

$P = 0.644$
PERSISTENCE OF *HETERORHABDITIS BACTERIOPHORA* IN SOIL

All treatments in the first experiment persisted and remained infective in the pots for a period of 103 days (Fig. 5A). Persistence was measured as percentage infected baiting insects (*Galleria mellonella*). Results obtained with the commercial strain EN01 were significantly variable over the period of baiting ($K = 13.621; \text{df} = 6; p = 0.034$). The highest infectivity was recorded at the first day of baiting and the lowest after 41 days. The inbred line IL3 significantly more often infected baiting insects in the soil on days 1, 77, 96 and 103 compared to days 41, 46 and 89 ($K = 16.468; \text{df} = 6; p = 0.011$). The Megamix followed a similar pattern as IL3. A high number of dead insects was recovered at the same days with the only exception of day 77 ($F = 5.675; \text{df} = 6; p = 0.004$). When all data obtained for the three strains were analyzed in one evaluation, significant differences were recorded for persistence among days of observations ($K = 46.960; \text{df} = 20; p < 0.001$), but no significant differences were found between treatments ($K = 2.206; \text{df} = 2; p = 0.332$). IL3 presented the higher percentage of infected baited insects among time with 45%, followed by Megamix with 41% and EN01 with 39% (Table 2).

In the second experiment, comparing the EN01, IL3 and Megamix, which had been harvested on day 103 from the first experiment (selection for persistence), with the unselected Megamix, all strains remained infective in the pots until 126 days after application (Fig. 5B). Persistence measured as percentage infected insects for EN01 was significantly higher at day 21 and 103 and less infective at day 84 ($F = 5.603; \text{df} = 5; p = 0.008$). IL3 infected significantly more insects at the two first baiting points (21 and 41) and less after 84 and 126 days ($K = 13.417; \text{df} = 5; p = 0.020$). For the Megamix no significant differences between baiting points
were assessed (K = 4.778; df = 5; p = 0.444) and also for the selected Megamix population no significant differences were recorded (K = 4.503; df = 5; p = 0.479).

Furthermore, when data were pooled, significant differences were also found in the second experiment between strains (K = 46.548; df = 23; p = 0.003). The comparison between treatments using Megamix and Megamix Selected with EN01 and IL3 indicated significant difference between the four strains (K = 22.854; df = 3; P = 0.0001). Percentage infected insects for Megamix and Megamix always surpassed 50% with less variable changes over time (Table 3). EN01 had 49% of infected insect pest, followed by IL3 with 44%.
Figure 4. Persistence measured as percentage of infected baiting insects (*Galleria mellonella*) for EN01 (A1), IL3 (A2) and Megamix (A3) in first experiment and results from second experiment, using dauer juveniles harvested from baiting during the first experiment on day 103 for EN01 (B1), IL3 (B2), Megamix (B3) and a Megamix Selected (B4), using dauer juveniles as inoculated in the first experiment. Error bars indicate standard deviation of three replicate pots. Different letters on the error bars indicate significant differences (Kruskal-Wallis test with Conover-Iman test for non-normal distributed data and ANOVA with Turkey HSD test for normal distribution of data).
Table 2. Mean percentage infected insects, standard deviation and ranges from treatments of first persistence experiment with strains EN01, IL3 and a pooled population of 33 strains (Megamix)

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>EN01</th>
<th>IL3</th>
<th>Megamix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>30</td>
<td>45</td>
<td>41</td>
</tr>
<tr>
<td>SD</td>
<td>10.55</td>
<td>9.66</td>
<td>12.81</td>
</tr>
<tr>
<td>Range</td>
<td>3 to 80</td>
<td>3 to 83</td>
<td>3 to 70</td>
</tr>
</tbody>
</table>

Table 3. Mean percentage infected insects, standard deviation and ranges from treatments of second persistence experiment with strains EN01, IL3 and a pooled population of 33 strains (Megamix), inoculated with nematodes harvested from infected insect recovered at day 103 during the first experiment and a Megamix, prepared from 33 strains like prepared for the 1 experiment.

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>EN01</th>
<th>IL3</th>
<th>Megamix</th>
<th>Megamix Selected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>49</td>
<td>44</td>
<td>82</td>
<td>89</td>
</tr>
<tr>
<td>SD</td>
<td>14.47</td>
<td>11.30</td>
<td>16.39</td>
<td>9</td>
</tr>
<tr>
<td>Range</td>
<td>0 to 80</td>
<td>3 to 90</td>
<td>77 to 100</td>
<td>80 to 100</td>
</tr>
</tbody>
</table>

Discussion

Nematode virulence is a widely studied subject (Morris et al., 1990; Glazer, 1992; Caroli et al., 1996; Ricci et al., 1996) and it is defined as the relative capacity to cause damage in a host (Casadevall & Pirofski, 1999). Numerous studies with different insect host have demonstrated high variability in EPNs virulence (Poinar, 1979; Caroli et al., 1996). The variation in virulence
of nematode strains to diverse host can be attributed to the ability of the DJs to locate and invade the hosts and overcome the immune system and propagate inside the insect (Bedding & Molyneux, 1982; Glazer, 1992; Caroli et al., 1996). This study reports, to our knowledge for the first time, results of differences in virulence between a large number (42) of *H. bacteriophora* strains. Other studies had been done comparing few strains or comparing species of *Heterorhabditis* spp. against *Steinernema* spp. (Caroli et al., 1996; Ricci et al., 1996; Goude & Shapiro-Ilan, 2003; Kurtz et al., 2007; Barbosa-Negrisoli et al., 2013). Only strains of the species *H. bacteriophora* were chosen. Toepfer et al. (2005) worked with several concentrations in small-volume arenas testing 8 nematode species on larvae of *D. v. virgifera*. *H. bacteriophora* was the most effective at a concentrations 16 DJs cm$^{-2}$. The recommended application rate is 50 DJs cm$^{-2}$. Other results confirmed the high virulence of this species in the control of *D. v. virgifera* (Kurtz et al., 2009). *H. bacteriophora* is distributed worldwide (Hominick, 2002), it is easy to produce *in vitro* (Johnigk & Ehlers, 1999), it has a short generation time (Lunau et al., 1993) and production of inbred line populations is possible, enabling maintenance of improvements of beneficial trait obtained by selective breeding approaches (Strauch et al., 1994).

Although inside the strain pool, Megamix, the most virulent strains were included, it was not among the best performers. This may be due to the fierce intraspecific competition of the strains for an insect host, but also competition within the host for successful establishment. That was also indicated by the observation that many dead insects were colored red, indicating a successful establishment of *H. bacteriophora*, but they were not luminescent, so they were excluded from the LC$_{50}$ evaluation.

Barbosa-Negrisoli et al. (2013) reported that *H. bacteriophora* (strains RS57, RS33, RS56, RS72 and RS88) were more virulent than *Steinernema* spp. on *T. molitor* larvae. The LC$_{50}$
of the *H. bacteriophora* strains varied between the 20 and 50 DJs per insect. In this investigation the LC$_{50}$ was much lower and the maximum value was smaller than 40 nematodes per insect. According to Athanassiou *et al.* (2010) the variability in LC$_{50}$ can be explained by the target insect species, the concentration and the temperature. Trdan *et al.* (2006) concluded that for nematode efficacy the concentrations are a less important factor. Pilz *et al.* (2014) run a test with *H. bacteriophora* strains and infection of 50% on 40 *T. molitor* larvae were recorded and considered as the threshold level to use them for field experiments. Based on this assumption, only the CN4 and the S-IL-IXO do not reach the threshold level.

In studies about *D. v. virgifera* different authors considered that the origin of a strain, its host specificity, host finding strategy, the survival ability, movement, persistence in soil and the environmental conditions in the soil are characteristics that may influence the efficacy of the entomopathogenic nematodes (Jackson & Brooks, 1995; Goude & Shapiro-Ilan, 2003; Toepfer *et al.*, 2005) In this investigation strain DE2 had the lowest LC$_{50}$ (19.3).

The study could not test all strains also against WCR, because the availability of this insect was limited. Soonly five strains were tested on *D. v. virgifera* and the results compared to results obtained with *T. molitor*. The comparison between *Diabrotica virgifera virgifera* and *Tenebrio molitor* revealed that the virulence was not correlated. *T. molitor* is widely used as bait insect for quality bioassays on EPNs (Zimmermann, 1986; Bharadwaj & Stafford, 2011). The possibility to use this insect instead of the target host WCR would be an important improvement in monitoring virulence, however, as no correlation was recorded, this possibility is excluded.

Another observation indicates that the virulence tests are not only influenced by insect species but may also be influenced by the number of insects used in an assay. In the assessment
of the LC\textsubscript{50} using only \textit{T. molitor}, 40 insects per concentration were used, whereas in the comparison with WCR 20 were used in the bioassays. The resulting LC\textsubscript{50} of, for instance, DE2 was 2.8 DJs in the assessment using 40 insects, but in the test with 20 insects it was 6.3 DJs and IR2 was 2.3 and 17 DJs, respectively. Peters and Piening (unpubl.) suggested that aggregation might influence the results of bioassays. Nematodes tend to aggregate in already infected hosts. However, this would result in a higher LC\textsubscript{50} at higher host density. A repellent effect was also considered to exist by the authors. The emission of a signal from the symbiotic bacteria of \textit{H. bacteriophora} (\textit{Photorhabdus luminescens}) and nematode in infected insect hosts might repel secondary invasion into infected insect host and thus promoting primary invasion into non-infected hosts. Whether such an effect might serve to explain the differences in LC\textsubscript{50} needs further investigation.

Sufficient persistence in EPNs is an important required character for control of \textit{D. v. virgifera} (Kurtz \textit{et al.}, 2007), because DJs are applied already with sowing, approximately 4-6 weeks before occurrence of the pest insect larvae. In this study, persistence of \textit{H. bacteriophora} was investigated and the impact on one selection step tested. Nematodes in this study persisted for up to 103 days in the first and 126 days in the second experiment, but with variable numbers of infected insects. One would expect that the number of DJs in the soil declines over time and that the percentage of infested insects thus will also decrease. However, number of infected insects declined already after 41 days in EN01 and later the number increased again to latter decrease again. In the first experiment this pattern was observed for all three strains. This effect could have been due to differences in attractiveness of the insects. Such a “U-shape curve” was observed also by Griffin (1996). She found variability in the infectivity over storage time at different temperatures. However, this observation was made only in the first but not in the second
experiment. Pilz et al. (2014) investigated persistence in the field and only observed a decline in percentage infested insects. Toledo et al. (2014) observed the same that percentages of infective insects decreased over time accompanied by a gradual loss of the nematode infectivity. In this study, DJs of all strains not only persisted in soil for more than 100 days but also remained highly virulent until the last sampling point. This confirms that an early application of nematodes is justified and the remaining DJs in the soil until the hatch of *D. v. virgifera* larvae. Diverse factors have been suggested as limiting the DJs performance in field conditions as the soil type, soil temperature, soil moisture, occurrence of natural antagonists, intensive soil tillage practices and lack of host for propagation (Kurtz et al., 2007; Susurluk & Ehlers, 2008; Kurtz et al., 2009). The study of Koppenhöfer and Fuzy (2007) reported recovery of nematodes at soil water potential from -10 kPa to -3,000 kPa over time. In the same study, *H. bacteriophora* showed alternating slight increases and decreases in infectivity depending on soil moisture. In the reported experiments, the influencing factors were restricted in soil type, lack of host for propagation and the confinement of the environment in all experimental pots, thus much of possible influences were excluded. However, as results reflect there are still a high number of possible influencing factors. E.g., the Megamix in the first experiment reached a mean percentage of infested insect of 41 % and in the second 82 %. These pooled populations were produced in *G. mellonella*, for the first experiment strains were coming mostly from liquid nitrogen storage and for the second they had been propagated consecutively in the wax moth larvae. Possibly, results in the second experiment were better, because nematodes had been propagated several times within a short period before use.

The question is, whether the selection step has increased the potential to persist. The mean percentage for EN01 increase from 30 to 49 % and the Megamix from 41 to 89 %. Considering
these results, one could conclude that the selection was successful. Only the IL3 strain remained almost constant in mean percentage of infested insects. As this is an inbred line of strain EN01 its genetic plasticity is less pronounced and thus inbred lines are less receptive to genetic selection approaches. This is also reflected in the standard deviation which is lower in the inbred line than in EN01 and Megamix except in the selected Megamix.

In conclusion, all strains used in this study were infective to *T. molitor* and five of them were infective to *D. v. virgifera*. A positive correlation in virulence between both insect hosts could not be detected. In an attempt to select for higher persistence strains, the commercial strain EN01, the inbred line IL3 produced from the commercial strain and the Megamix selected and unselected were screened and persistence in soil was recorded by measuring larval infection over time in two selection rounds. All strains were able to remain infective and to persist in soil over hundred days. In the first experiment alternating high and low infectivity rates over time were detected for all strains. In the second experiment, this infectivity pattern was only present on the commercial strain and the derived inbred line. The results document the potential of selecting programs for higher virulence and persistence to enhance the control of *D. v. virgifera* and other target pests.

More research is needed on the Megamix to determine if it is a good candidate for improved virulence and persistence. Factors affecting virulence and persistence in soil on *H. bacteriophora* could bring valuable information to improve the selection assay. Success of genetic selection can be jeopardized by variability of the bioassay results. Less variability and reproducibility is a pre-requisite for successful improvement by genetic selection. Further studies and a continuation of selection with *H. bacteriophora* are needed to further enhance management of *D. v. virgifera* in the field.
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