Effect of temperature and desiccation stress on infectivity of stress tolerant hybrid strains of *Heterorhabditis bacteriophora*

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Summary. The effect of stress exposure on the infectivity of heat and desiccation tolerant hybrid strains of the entomopathogenic nematode *Heterorhabditis bacteriophora* was assessed against last instars of *Galleria mellonella*. Nematode dauer juveniles (DJs) were exposed to desiccation stress at a water activity (a_w-value) of 0.85 for 24 h or a temperature treatment at 40°C or 0°C for 24 h prior to inoculation of 5 DJs per insect. Hybrid strains resulting from crosses of the three very best heat or desiccation tolerant strains and crosses of heat with desiccation tolerant strains were compared with a commercial strain of *H. bacteriophora*. Exposure to desiccation stress caused significant reduction of the infectivity in all strains not surpassing 25% mortality, except one that was not affected and achieved 37.5% mortality. Infectivity of untreated DJs of desiccation tolerant hybrids differed significantly with a mean infectivity of 54%, ranging from 33.8% to 89.6%. The mean mortality of heat tolerant hybrids was significantly higher (78.2%). The commercial and two other hybrids were not affected by the heat treatment. The lowest mortality after the low temperature treatment was recorded for the commercial strain. The reduction in infectivity after cold temperature treatment was not much different from the effect recorded after heat treatment and results were not consistent between strains. Consequently, we conclude that the infectivity of heat tolerant strains is not necessarily affected by low temperature stress. Monitoring of beneficial traits like infectivity is essential during attempts to improve genetically other traits by crossing tolerant strains or selective breeding.

Key words: adaptation, entomopathogenic nematodes, *Galleria mellonella, Heterorhabditis bacteriophora*, mortality, stress tolerant hybrids, temperature, water activity.

Entomopathogenic nematodes (EPNs) of the family Heterorhabditidae (Rhabditidomorpha: Strongyloidea) are lethal insect antagonists of many important insect pests in fruits, vegetables and ornamentals (Grewal *et al.*, 2005). *Heterorhabditis bacteriophora* Poinar 1976 is found on all continents except Antarctica (Stock & Hunt, 2005) and is one of the best studied species among EPNs. It is safe for non-targets and the environment (Ehlers, 2003), can be produced in large scale liquid culture (Ehlers, 2001) and has recently been sequenced (Ciche, 2007). *Heterorhabditis bacteriophora* is symbiotically associated with the bacterium *Photorhabdus luminescens* Poinar and Thomas 1979, a Gram-negative gamma proteobacterium belonging to the family Enterobacteriaceae (Ehlers *et al.*, 1988). The infective dauer juveniles (DJs) of this worm carry cells of their symbiont in the intestine (Ciche *et al.*, 2006). After invasion of an insect host the bacteria are released into the haemocoel. *Photorhabdus luminescens* kills the host and in the cadaver produces suitable conditions for reproduction of the nematode, which is unable to develop in the absence of its symbiont (Han & Ehlers, 2000).

Although already successfully used in insect control, beneficial traits of this biocontrol agent can certainly be improved by domestication. For instance, the tolerance to environmental stress like heat and desiccation is rather limited in *H. bacteriophora* (Grewal *et al.*, 1994; Glazer, 2002). Improvement of such beneficial traits of EPN by

selective breeding was first proposed by Gaugler (1986). The heritability of the traits heat and desiccation tolerance is relatively high in *H. bacteriophora* (Glazer *et al.*, 1991; Strauch *et al.*, 2004; Ehlers *et al.*, 2005), making selective breeding a feasible approach for genetic improvement of these traits. Reasonable progress was already obtained by Strauch *et al.* (2004) for improvement of desiccation tolerance and by Ehlers *et al.* (2005) in heat tolerance through genetic selection. Mukuka *et al.* (2010a, b) obtained similar tolerance when screening among natural populations of *H. bacteriophora* for heat and desiccation tolerant strains. In a next step the three most tolerant strains were crossed and the resulting hybrids provided additional progress in stress tolerance (Mukuka *et al.*, 2010c). The mean heat tolerance of a strain was assessed on a temperature gradient (Ehlers *et al.*, 2005), the mean desiccation tolerance in hypertonic solutions of decreasing water activity (Strauch *et al.*, 2004). Only the most tolerant 10% of each *H. bacteriophora* strains were used for production of the hybrids (Mukuka *et al.*, 2010c). Hybridization of these tolerant strains resulted in several hybrids with increased stress tolerance (Table 1). The maximum mean tolerated temperature (survival of 50% of the DJ population after heat treatment) reached 42°C and the desiccation tolerance decreased to a minimum mean tolerated water activity (a_w) of 0.65 (Mukuka *et al.*, 2010c).

Heat and cold shock induce the synthesis of heat shock proteins and trehalose in *H. bacteriophora* (Jagdale *et al.*, 2005); adaptation to desiccation results in accumulation of glycogen in *Heterorhabditis* spp. (O`Leary *et al.*, 2001). These treatments facilitate the biochemical adaptation necessary for enhanced stress tolerance (Glazer, 2002). Therefore, the screening among strains and characterisation of the mean stress tolerance always assessed tolerance with and without adaptation to the relevant stress factor.

The objective of this study was to evaluate whether the infectivity of the hybrid *H. bacteriophora* strains to the insect *Galleria mellonella* L. (Lepidoptera, Pyralidae) is affected by exposure to heat or desiccation stress and to compare the results with a commercial strain.

Table 1. Description of hybrid strains, their temperature and desiccation tolerance given as mean tolerated temperature (°C) and water activity (a_w) after adaptation (A) and without prior adaptation (NA) to stress conditions according to Mukuka *et al.*(2010c).

Strain designation	Description	Temperature tolerance		Desiccation tolerance	
		A	NA	A	NA
EN 01	Commercial hybrid strain	38.2	36.5	0.985	0.951
HA1	Hybrid of 2 strains with high heat tolerance after adaptation	-	41.3	-	-
HA2	HA1 crossed with another strain with high heat tolerance after adaptation	-	39.2	-	-
HH1	Hybrid of 2 strains with high heat tolerance without adaptation	39.2	-	-	-
HH2	HH1 crossed with another strain with high heat tolerance without adaptation	42.1	-	-	-
H3	Hybrid of HA2 and HH2	40.0	39.2	-	-
DA1	Hybrid of 2 strains with high desiccation tolerance after adaptation	-	-	0.745	-
DA2	DA1 crossed with another strain with high desiccation tolerance after adaptation	-	-	0.733	-
DD1	Hybrid of 2 strains with high desiccation tolerance without adaptation	-	-		0.766
DD2	DD1 crossed with another strain with high desiccation tolerance without adaptation	-	-		0.755
D3	Hybrid of DA2 and DD2	-	-	0.755	0.792
HD4	Hybrid of H3 and D3	41.2	39.9	0.672	0.697

MATERIAL AND METHODS

The nematodes strains used in the study were hybrid strains produced by crossing three most stress tolerant strains resulting from a screening for heat (Mukuka *et al.*, 2010a) and desiccation tolerance (Mukuka *et al.*, 2010b) among natural populations and inbred lines and then crossing the obtained hybrids with each other (Mukuka *et al.*, 2010c) (Table 1). The commercial strain is a hybrid provided by the biotechnology company e-nema GmbH (Schwentinental, Germany). All strains (Table 1) were cultured in *G. mellonella* according to Kaya & Stock (1997). All DJs were stored in Ringer´s solution at 15°C and used within 1 week after harvest from the insect cadavers.

Prior to testing the infectivity against *G. mellonella*, DJs of the hybrid strains were exposed to stress conditions and compared with untreated DJs. Before exposure to desiccation stress, 6,000 DJs of each desiccation tolerant hybrid strain were adapted to desiccation by transfer to 5 ml 39.5% (w/w) polyethylene-glycol 600 (PEG) for 72 h, corresponding to an a_w-value of 0.96 (Strauch *et al.*, 2004). They were then treated with 62% PEG (a_w-value of 0.85) for 24 h and next kept in Ringer´s solution for 24 h for rehydration. Untreated DJs were kept in Ringer´s solution (a_w-value of 1.00). All treatments were done at 25°C.

For heat exposure 5,000 DJs of each heat tolerant

hybrid strain were placed in Ringer´s solution in an incubator at 40°C or in a freezer at 0°C for 2 h and then left at 25°C to recover for 1 h. Untreated DJs were kept at 25 °C.

For testing the infectivity against last instar *G. mellonella* the five-on-one sand bioassay according to Peters (2005) was used. DJs from untreated controls and those surviving the stress exposure were hand-picked with a pipette and 5 DJs were placed into each cell (16 mm diam.) of a 24 cell-well plate (Iwaki 24 well, Asahi Techno Glass, Tokyo, Japan) containing 1g of sterile sand (10% water w/w). In each cell-well one last instar *G. mellonella* was added. Control plates contained insects without nematodes. The plates were wrapped with Parafilm and then incubated in a dark room at 25°C. The number of dead insect larvae in each plate was recorded after 72 h and the percentage mortality was determined. Insect mortality was expressed as percentage of the total number of insects tested in each replicate (n=24). The experiment was repeated three times with different batches of DJs of each hybrid strain.

Percentage mortality data were *arcsin* transformed and analysed by ANOVA and differences between treatments were compared using Tukey's HSD test ($P \leq 0.05$). The mortality data were corrected for control mortality following Abbott (1925). To compare the infectivity of desiccation with heat tolerant hybrids in the untreated control, percent mortality data were *arcsin* transformed and means were compared by Student´s *t*-Test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Exposure to desiccation stress caused significant reduction of the infectivity in all strains (F=5.09; df=6, 20; p=0.006), except in DD2, which decreased from 42.2% to 37.5% (Fig. 1). Probably, hybrid strain DD2 needs lower a_w-values to cause an effect on infectivity since this strain had a mean tolerated water activity a_w=0.755 (Table 1). Infectivity of the other strains did not surpass 25%. Infectivity of untreated DJs differed significantly among the strains, ranging from 33.8% (strain DD1) to 89.6 (strain HD4) (F=22.07; df = 6, 34; $p \leq 0.0001$). Three desiccation tolerant strains were less and three more infective than the commercial strain EN01 (54%).

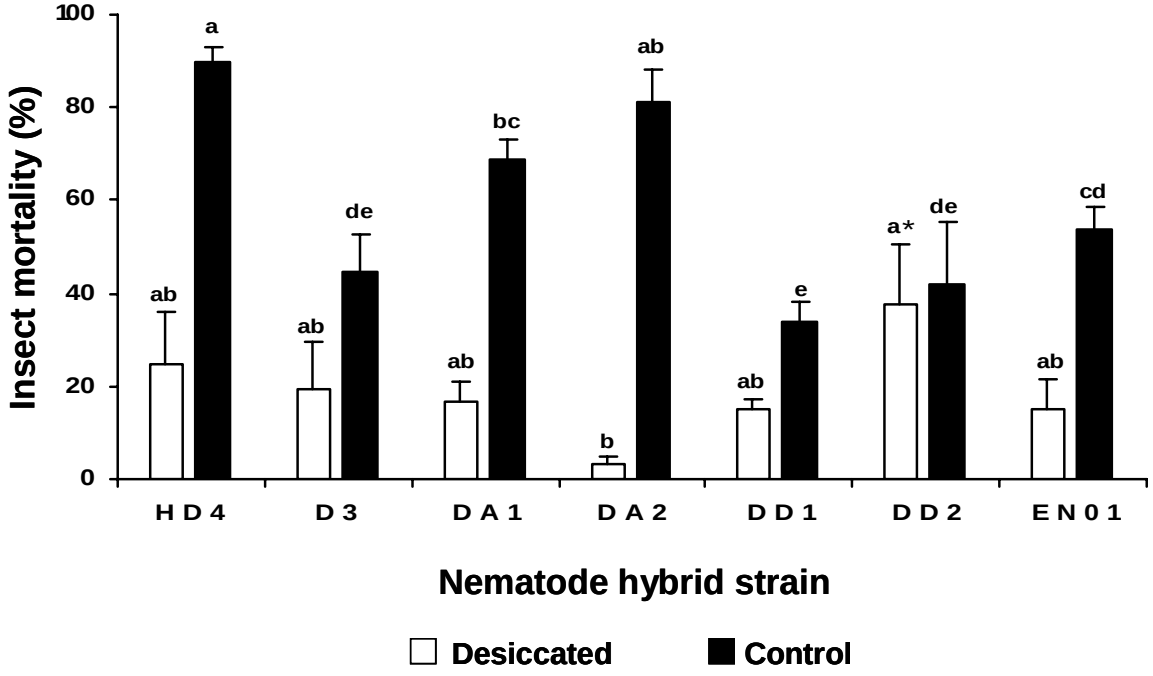

Fig. 1. Mean Abbott-corrected mortality of last instar *Galleria mellonella* after exposure to 5 DJs of different *Heterorhabditis bacteriophora* hybrid strains and the commercial hybrid (EN 01) for 72 h at 25°C. Prior to inoculation in the assays, DJs had been adapted to desiccation stress in PEG solution of a water activity of a_w=0.96 for 72 h, exposed to stress at an a_w=0.85 for 24 h and then rehydrated in Ringer´s solution for 24 h (white bars). Control DJs were untreated (black bars). Each column represents a mean mortality of three replicates with 24 insects each. Different letters on the error bars indicate significant differences within equal treatments according to ANOVA HSD test ($p < 0.05$). The * indicates no significant decrease in infectivity.

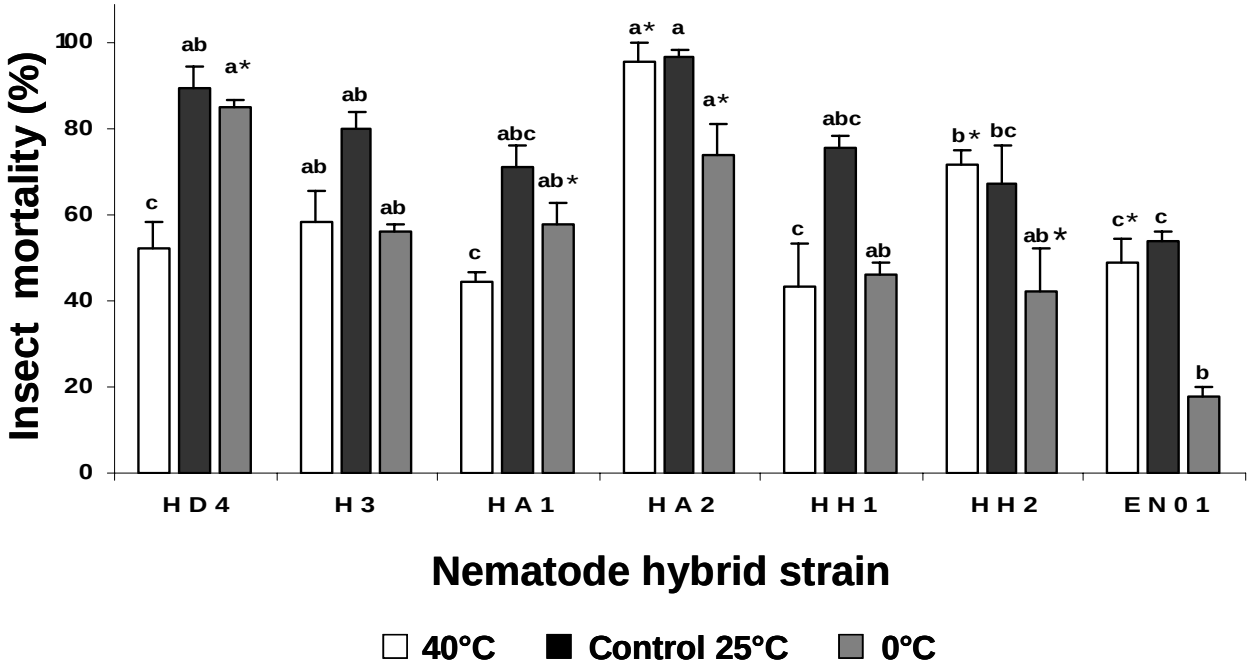

Fig. 2 Mean Abbott-corrected mortality of last instar *Galleria mellonella* after exposure to 5 DJs of different *Heterorhabditis bacteriophora* hybrid strains and the commercial hybrid (EN 01) for 72 h at 25°C. Prior to inoculation in the assays, DJs were exposed to either 40 °C (white bars) or 0 °C (grey bars) for 24 h and then left to recover for 1 h at 25°C. Control DJs were kept at 25°C (black bars). Each column represents a mean mortality of three replicates with 24 insects each. Different letters on the error bars indicate significant differences within equal treatments according to ANOVA HSD test ($p < 0.05$). The * indicates no significant change in infectivity compared to the control.

The heat treatment at 40°C significantly reduced the infectivity in four hybrid *H. bacteriophora* strains HD4, H3, HA1 and HH1 (F= 24.8; df = 6, 20; $p \leq 0.0001$). In strain HA2 (96% to 95.4%) (F=0.89; df=1, 7; p=0.78) and EN01 the reduction was not significant (F=1.15; df=1, 7; p=0.33), like in HH2 with an increase in infectivity (67% to 71.5%) (Fig. 2). It cannot be excluded that the high temperature treatment might also have affected the infectivity of the symbiotic bacteria in some of the hybrid strains.

The cold treatment at 0°C reduced the infectivity in all strains and the reduction was significant in EN01, HH1 and H3 (F= 3.7; df = 6, 20; $p \leq 0.02$). The lowest infectivity after cold treatment was recorded for the commercial strain (EN01=18.4%).

The reduction in infectivity was highest after exposure to desiccation stress (40.2%) followed by cold treatment (22.2%) and then heat treatment (17.2%) (F= 17.86; df= 2, 20; $p \leq 0.0001$).

Comparing the results obtained with untreated DJs of each strain in the controls (kept at 25°C) the highest mortality was caused by HA2 (96%), which was significantly different to the commercial strain (54%) and HH2 (67%) (F=9.8; df=6, 34; $p \leq 0.0001$) (Fig. 2). The commercial strain was the least infective. However, the comparison of desiccation tolerant hybrids with the commercial strain reveals that the commercial strain ranges in the middle. The mean infectivity of untreated control DJs of the hybrids resulting from crosses of desiccation tolerant strains (Fig. 1) was 54% with a significant difference (78.2%) to the heat tolerant strains (Fig. 2) (F=4.48; df=24; $p \leq 0.0002$). Thus, the use of more heat tolerant strains will not necessarily result in reduced infectivity of the DJs. These results contradict results reported by Grewal *et al.* (2000), who found that infectivity of *S. carpocapsae*, *S. riobrave* and *S. feltiae* were not influenced by exposure to desiccation stress.

One could also suggest that more heat tolerant strains might loose in infectivity after cold treatment. However, the data do not show any indication for such an assumption. The results underline the importance of monitoring beneficial traits like infectivity during attempts to improve heat or desiccation tolerance by crossing tolerant strains or genetic selection.

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Mukuka, J., Strauch, O., Hisham Al Zainab, M., Ehlers, R.-U. Воздействие температуры и вызванного иссушением стресса на инвазионную способность устойчивых к высушиванию штаммов *Heterorhabditis bacteriophora*.

Резюме. Исследовано воздействие стрессовых состояний, вызванных изменениями температуры и влажности на способность личинок гибридных штаммов энтомопатогенной нематоды *Heterorhabditis bacteriophora*, устойчивых к высушиванию, заражать гусениц последнего возраста *Galleria mellonella*. Стрессовые воздействия на инвазионных личинок (ИЛ) состояли в подсушивании в течение 24 часов (значение a_w = 0.85), а также содержании при 40°C или 0°C на протяжении 24 часов перед инокуляцией пяти ИЛ в насекомое. Гибридные штаммы, полученные в результате скрещивания трех начальных штаммов с наилучшими показателями устойчивости в высушиванию и повышенной температуре, сравнивали с коммерческими штаммами *H. bacteriophora*. Подсушивание приводило к существенному сокращению инвазионности для всех штаммов, которая, в таких случаях не превышала 25%. Лишь один штамм показал смертность насекомых на уровне 37,5%. Инвазионная способность ИЛ не подвергнутых стрессовым воздействиям существенно различалась между штаммами, в среднем составляя 54% (33,8% - 89,6%). Средняя смертность насекомых при инокуляции их штаммами устойчивыми к повышенным температурам была достоверно выше (78,2%). Воздействие повышенными температурами не вызывало изменения инвазионной способности у одного коммерческого и двух гибридных штаммов. Самая низкая инвазионность при воздействии пониженной температуры была отмечена для одного из коммерческих штаммов. Воздействие низкой температуры не отличалось достоверно от воздействия высокой температуры. При этом характер воздействия существенно различался между отдельными штаммами. Предполагается, что инвазионная способность штаммов, устойчивых к повышенным температурам, не меняется существенно при воздействии пониженных температур. Постоянный контроль за сохранением таких важных полезных свойств штаммов, как инвазионная способность, необходима на всем протяжении процесса генетического улучшения природных штаммов, как за счет скрещивания, так и в процессе селекции.