DESICCATION AND OXIDATIVE STRESS TOLERANCE AS MEASURES TO EVALUATE LONGEVITY IN ENTOMOPATHOGENIC NEMATODES

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SUMMARY

Attempts at improving the performance of the entomopathogenic nematodes, *Heterorhabditis bacteriophora* for the bio-control of insect pests, are focused on the free-living forms, the infective juveniles (IJs). This stage is a non-feeding and developmentally arrested form in which the nematodes remain until environmental conditions become favourable. This study evaluated the IJ longevity of two *Heterorhabditis bacteriophora* inbred lines, *H. bact* IL-1 and *H. bact* IL-2, by subjecting the IJs to desiccation and oxidative stress conditions. Desiccation stress was induced on the IJs after exposing batches of the IJs to 7 concentrations of PEG 600. Tolerance to desiccation was expressed as water activity (a_w), which is a measure of the relative proportion of unbound water available to the IJs for survival. Oxidative stress was induced on the IJs after exposing batches of the IJs to 60 mM of Hydrogen Peroxide (H_2O_2). The longevity of IJs from both inbred lines was compared to their tolerance to oxidative stress and desiccation. This study showed that tolerance to high oxidative stress correlates with tolerance to low water activity, which indicates the survival and extent of longevity in IJs of *H. bacteriophora*. This offers a firm support and validates the use of either oxidative stress or desiccation tolerance as a functional trait to correlate variations in environmental stress tolerance with the genes modulating longevity in *H. bacteriophora* IJs.

Keywords: Longevity, *Heterorhabditis bacteriophora,* desiccation, oxidative stress tolerance, bio-control agents.

The entomopathogenic nematodes (EPNs) of the genera *Heterorhabditis* and *Steinernema* are exploited for their effects on insect pests due to their interaction with the enteric bacteria symbionts *Photorhabdus* and *Xenorhabdus* species, respectively (10). This nematode-bacteria association presents a strong alternative to the use of chemical insecticides in the control of crop insect pests which, offers long-term goals in yield increase and reduction of crop production costs. However, the effective use of EPNs as bio-control agents is still limited by environmental factors and industrial production conditions. To resolve this, the genetic variability among various strains/species needs to be explored to understand the molecular basis of important bio-control traits such as longevity in storage (shelf life), tolerance to stress, virulence on pest, and persistence on field after applications (11, 16).

Improving the performance and effective use of EPNs is channelled to the third larval stage referred as infective juveniles (IJs). This is a non-feeding and developmentally arrested stage induced mainly by food shortage or overcrowding and remains in this form until the IJs find and invade the larva of a suitable insect most of which are pests to crops. In the biology of EPNs, the early developmental stages of the juveniles (J1 and J2), respond to signals using sensory neurons to evaluate the environment. When unfavourable conditions are sensed, mechanisms are activated that prepare the juveniles for an enduring phase called dauer period (9). These mechanisms include accumulating trehalose for water to maintain cell membrane integrity, accumulation of fatty tissues and cessation of feeding. The final stage of this development is the search for new hosts by the IJs (3, 5).

The IJs are mass produced in bioreactors under liquid growth conditions and stored at high densities of about 500,000 IJs/ml (7). In this process, the IJs experience an increase in fat reserve metabolism and are exposed to overcrowding and hypoxic conditions that can lead to the accumulation of reactive oxygen moieties and reduction of its lifespan due to oxidative stress. Therefore, prolonged storage under suboptimal conditions can impact negatively on the longevity of the IJs (15, 18).

This study aims at evaluating the direct influence of desiccation and oxidative stress on *Heterorhabditis bacteriophora* IJs and how both stress factors correlate with IJs longevity.

MATERIALS AND METHODS

Nematode strains and culture

For this study, two pre-selected *H. bacteriophora* inbred lines of different origin were used. Both inbred lines were cultured and maintained *in vitro* on nematode growth gelrite (NGG) media (1.5 g gelrite, 1.25 g peptone, 1.5 NaCl, 500 µl $CaCl_2 \times 2H_2O$, 500 µl $MgSO_4 \times 7H_2O$, 12.5 ml KH_2PO_4 and 500 µl Cholesterol dissolved in 486 ml of mineral water) pre-inoculated with a lawn of the symbiotic bacteria *Photorhabdus luminescens*. *In vivo* culture of the lines was performed in the last instar larval stage of the Great Wax Moth *Galleria mellonella* as described by (6). For physiological tests, *in vitro* propagation of the inbred lines was done in 4 different NGG batches, while *in vivo* propagation was carried out using 5 *G. mellonella* larvae. To avoid batch-derived effects, all growth batches were pooled with respect to the mode of propagation.

Assessment of desiccation tolerance in *H. bacteriophora* inbred lines

To assess tolerance to desiccation, IJs were exposed to batches 7 concentrations of PEG 600. as described by Anbesse *et al* (2) Mukuka *et al* (12). Tolerance to desiccation was expressed as water activity (a_w), which is a measure of the relative proportion of unbound water available to the IJs for survival. Fresh batches of IJs, pooled from four different growth media, were used for the desiccation test in 24-cell well plates. The IJs were kept for 72 h in adaptation solution of 40.3% PEG 600, (a_w of 0.96), prepared from a concentrated PEG 600 stock solution (Carl Roth, Karlsruhe, Germany). To minimize evaporation, the 24-cell well plates were sealed with Parafilm (Pechiney "M" Plastic Packaging, Chicago, USA). After adaptation, batches of 1500 IJs in 3 replicates were exposed to 7 PEG 600 concentrations for 24 h: 20% (a_w 0.98), 30% (a_w 0.97), 40.3% (a_w 0.96), 50% (a_w 0.93), 60% (a_w 0.89), 70% (a_w 0.83), and 80% (a_w 0.74). For control test, IJs were transferred to Ringer's solution (9.0 g NaCl, 0.42 g KCl, 0.37 g $CaCl_2 \times 2H_2O$, 0.2 g $NaHCO_3$ dissolved in 1 L of distilled water) after the adaptation period (a_w 0.99). After the treatment, the mortality of IJs was assessed in the 3 replicates by counting number of active and inactive nematodes using a counting chamber. Percentage IJ mortality in different replicates was used to calculate the mean water activity (MW) tolerated by 50% of the population (MW_{50}) and the MW tolerated by the most tolerant 10% of the IJs population (MW_{10}).

The MW values were determined from a normal distribution graph. To achieve this, the experimental data (percentage IJs mortality per treatment) were fitted to a cumulative normal distribution curve. The mean and the standard deviation from the fitted normal distribution were used to estimate the mean and standard deviation of the water activities by reducing the χ^2 while comparing the experimental data to the expected

normal distribution values. Significant differences and statistical ranking between the MW of the strains were detected by *t*-test and R HSD test package (14).

Assessment of oxidative stress tolerance in *H. bacteriophora* inbred lines

To assess tolerance to oxidative stress of both inbred lines, batches of IJs were stored in 400 ml of Ringer's solution containing 60 mM of H_2O_2. Each treatment was carried out in replicate in a 24-cell plates while batches for control treatment were left at 25°C in cell wells without H_2O_2. To assess the IJs mortality over time, 50 µl aliquots from each experimental replicate were counted in a counting chamber. The percentage IJ mortality was used to determine differences in the mean survival time of 50% of the population (LT_{50}) and the survival time of the most tolerant 10% of the IJ population (LT_{10}) for each strain. The determination of the LT values followed the same procedure as that described for the desiccation test.

RESULTS

Desiccation tolerance for infective juveniles of two *H. bacteriophora* inbred lines

A comparative experiment was performed between *H. bact* IL-1 and *H. bact* IL-2 inbred lines to assess their desiccation tolerance by exposing the IJs to PEG 600 solutions of varying water activities.

The mean water activity tolerated by 50% of the IJ population (MW_{50}) and a_w of the 10% most tolerant IJs (MW_{10}) were derived from a fitted cumulative normal distribution curve (Fig. 1). For both inbred lines, active nematodes were only recorded at a_w values above 0.75 after adaption to desiccation condition for 24 h at aw of 0.96. The MW_{50} for *H. bact* IL-1 was 0.91 ± 0.01 compared to 0.96 ± 0.01 for *H. bact* IL-2 IJs. Similarly, *H. bact* IL-1 and *H. bact* IL-2 showed MW_{10} of 0.83 ± 0.01 and 0.87 ± 0.01 respectively (Fig. 2). The contrasting differences in desiccation tolerance between IJs of the two inbred lines were statistically significant for both population sizes ($t_{df} = 5 = 2.92$, P = 0.008). Thus, *H. bact* IL-1 was determined to have a higher desiccation tolerance compared to *H. bact* IL-2.

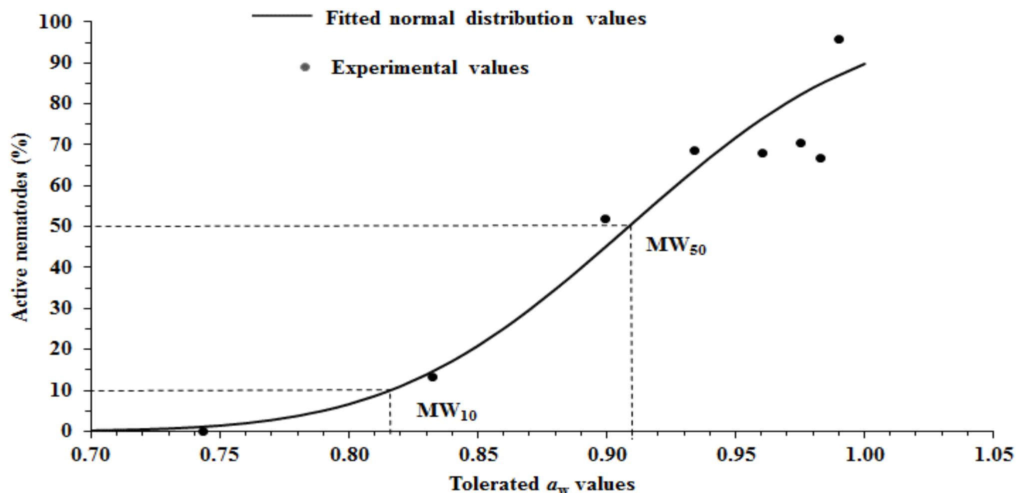

Figure 1: Percentage of active *Heterorhabditis bacteriophora* infective juveniles after exposure to different water activities (a_w) and fitted to a cumulative normal distribution. The number of active nematodes at each a_w was used to calculate the mean water activity (MW_{50}) and the water activity tolerated by 10% of the population (MW_{10}). For both IJ populations, no active IJs were found under a_w of 0.75.

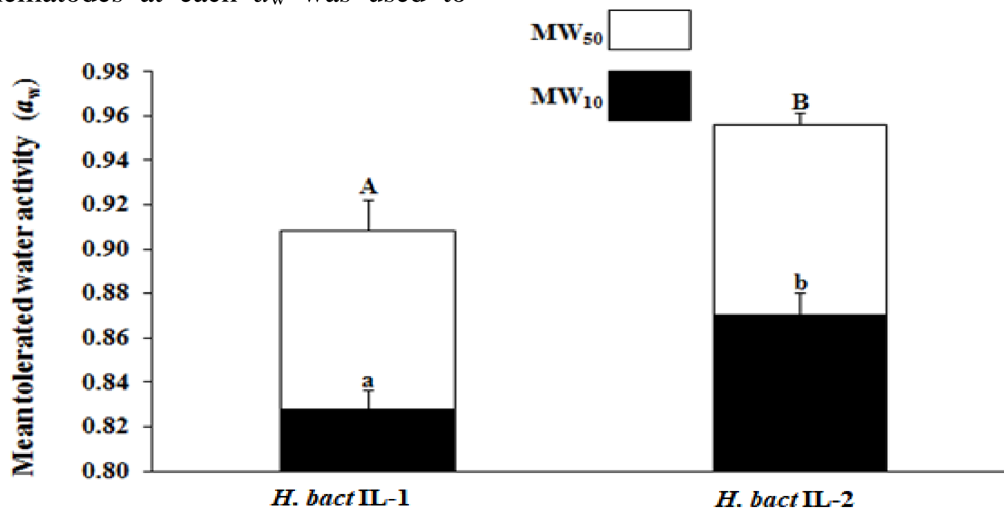

Figure 2: Differences in the desiccation tolerance of the *Heterorhabditis bacteriophora* inbred lines, *H. bact* IL-1 and *H. bact* IL-2. The mean water activity tolerated by 50% of the population for each strain (MW_{50}) are expressed in the white bars while the mean water activity of the 10% most tolerant IJs in each strain (MW_{10}) are expressed in black bars. Mortality of IJs was assessed after 24 h of exposure to different a_w. Error bars indicate standard deviation and different small or capital letters statistically significant differences. Significant differences were based on *t*-test (P = 0.008).

Oxidative stress tolerance for two *H. bacteriophora* inbred lines

The longevity of the infective juveniles of *H. bact* IL-1 and *H. bact* IL-2 inbred lines was assessed for the time taken to reach 50% mortality (LT_{50}) and the time to reach 10% survival (LT_{10}) by the most tolerant individuals in each IJ population after exposure to both oxidative stress (60 mM H_2O_2) and control conditions. The LT_{50} and LT_{10} of the IJs were calculated as described for the desiccation tolerance assay. Under control conditions, the mean LT_{50} of *H. H. bact* IL-1 IJs was 38.58 ± 13.19 days and LT_{10} of 68.82 ± 24.28 days. For *H. bact* IL-2, the LT_{50} and LT_{10} was determined at 26.46 ± 3.66 days and 47.98 ± 6.89 days respectively. A significant difference was detected in the IJs survival time between both inbred lines held under control conditions (LT_{50}: $t_{df = 5} = 2.92$, P = 0.028; LT_{10}: $t_{df = 5} = 2.92$, P = 0.048). Whereas, after exposure to oxidative stress conditions, *H. bact* IL-1 inbred lines IJs had a LT_{50} and LT_{10} of 6.28 ± 1.46 days and 14.00 ± 1.52 days, respectively, *H. bact* IL-2 had a LT_{50} and LT_{10} of 2.69 ± 0.13 and 4.87 ± 0.24, respectively. The difference in the IJ survival time under oxidative stress conditions was also statistically significant (LT_{50}: $t_{df = 5} = 2.92$, P = 0.032; LT_{10}: $t_{df = 5} = 2.92$, P = 0.011). In both control and treatment conditions, the oxidative stress tolerance of *H. bact* IL-1 was significantly superior to *H. bact* IL-2 (Fig. 3).

A Pearson correlation test was conducted to determine the association between of IJ longevity without stress factor and their survival under oxidative stress. A significantly positive correlation was detected for IJ survival in both conditions (LT_{50}, R=0.96; LT_{10}, R = 0.98, P < 0.01, Fig. 4). This reveals that oxidative stress tolerance can serve as an effective measure of longevity in IJs of *H. bacteriophora* inbred lines. Comparing the performances of the two inbred lines

for both desiccation and oxidative stress tolerances, a significantly negative correlation was observed (R = - 0.93, *P* = 0.047). Thus, the tolerance of the IJs to low water activity correlates with high oxidative stress tolerance and enhanced longevity of *H. bacteriophora* (Fig. 5).

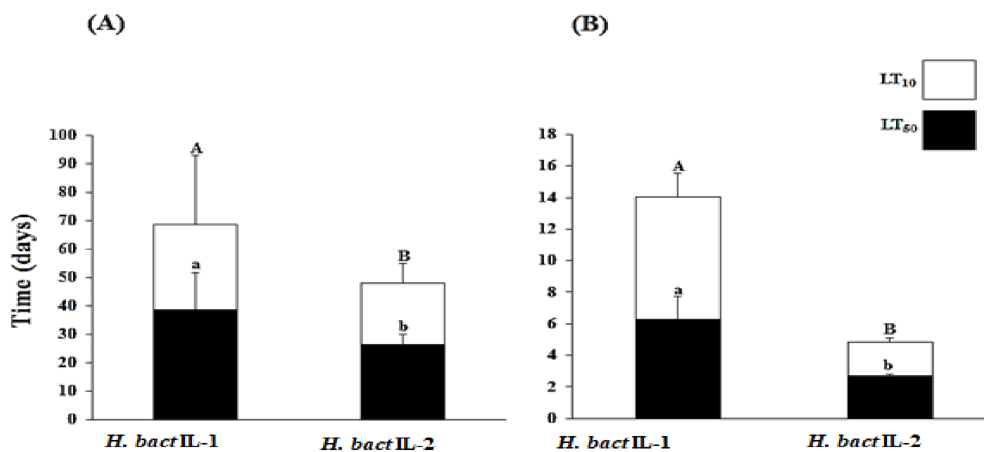

Figure 3: Survival time of the inbred lines, *H. bact* IL-1 and *H. bact* IL-2., under control (A) and oxidative stress (B) conditions. IJs were kept at 25°C in Ringer's solution under control conditions while oxidative stress was induced by exposing IJs to 60 mM of H_2O_2. Mortality was assessed over time, in 3 replicates. Average time taken to achieve 50% (LT_{50}) and 10% (LT_{10}) survival in each strain was recorded (white and black bars, respectively). Error bars on each bar indicate standard deviation. Different small or capital letters indicate statistically significant differences based on the *t*-test. **(A)**: (LT_{50}: P = 0.028; LT_{10}: P = 0.048), **(B)**: (LT_{50}: P = 0.032; LT_{10}: P = 0.011).

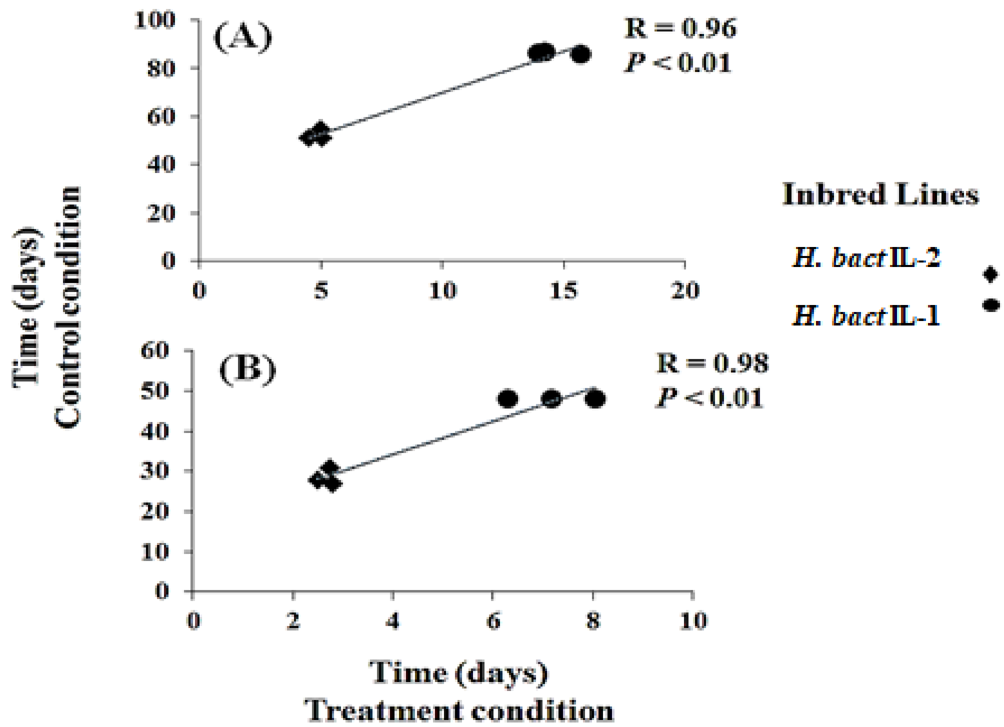

Figure 4: Pearson correlation between IJ survival time with and without exposure to oxidative stress of two *Heterorhabditis bacteriophora* inbred lines. LT_{50} **(A)** and LT_{10} **(B)**. Each data point represents one replicate, df = 4.

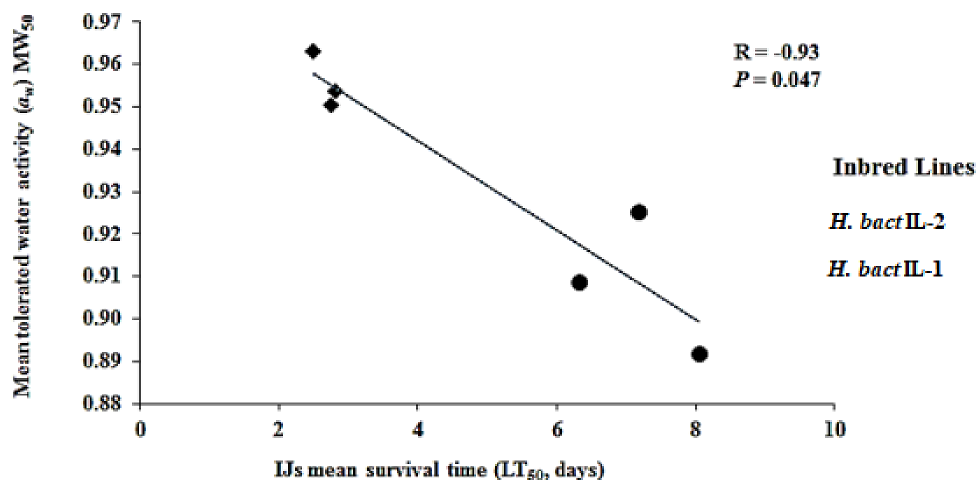

Figure 5: Pearson correlation between IJ survival time after exposure to oxidative stress (using 60 mM of H_2O_2) and mean tolerated water activity of two *Heterorhabditis bacteriophora* inbred lines (*H. bact* IL-1 and *H. bact* IL-2). The infective juveniles of both inbred lines were, under both conditions, held at 25°C. Each data point represents one replicate, df = 4.

DISCUSSION

Longevity of IJs in storage is an important factor in the effective use of *Heterorhabditis bacteriophora* for biological control of insect pests. To improve this trait, researches are targeted towards enhancing IJs tolerance to environmental stress mainly through series of selection processes following adaptation phases (1, 12, 13). This strategy is often not sustainable due to environmental influences and instability of the improved traits after removal of selection pressure (1). For example, Anbesse *et al* (2) reported that after 15 cycles of *in vitro* culturing to maintain heat tolerance in inbred lines of *H. bacteriophora* under selection pressure, trait deterioration resulted when selection pressure was removed. Therefore, it is imperative to target at the genetic components controlling traits of interest by marker-assisted selection.

The finding from the present study shows that tolerance to high oxidative stress correlates significantly with tolerance to low desiccation conditions and both correlates with longevity in *H. bacteriophora*. Thus, this offers a firm support that validates the subsequent use of

oxidative stress tolerance in further assays as a functional trait to correlate phenotypic variances in environmental stress tolerance with the genes modulating longevity in *H. bacteriophora* IJs. The influence of oxidative stress on survival in nematodes has been reported extensively in *C. elegans* (10, 17) and only recently replicated in *H. bacteriophora* (18). In *C. elegans*, the Insulin/IGF-1 pathway has been linked to influence the expression of genes modulating longevity and survival of dauer juveniles to oxidative stress (8). Following the discovery of the same pathway in *H. bacteriophora*, it is expected that similar genes modulating longevity should play a role in the oxidative stress tolerance in *H. bacteriophora*. Similarly, studies on paraquat resistant mutants of *H. megidis* reveal that IJs resistant to ROS produced by paraquat show an increase in the levels of catalase, superoxide dismutase and enzymes which provides protection from oxidative damages and this was linked to increased IJs longevity (4). The extension of IJs longevity reported in paraquat resistant mutants of *H. megidis* supports the theory in the current study that increased resistance to 60 mM of H_2O_2 leads to increased longevity in *H. bacteriophora* IJs.

All efforts to improve shelf life of *H. bacteriophora* and enhance their use in bio-control can only be transferred to commercial scale for pest control in plant production if it does not come at the detriment of other important traits such as infectivity and virulence. This will provide a platform for the genetic improvement of other EPN products by marker-assisted selection.

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