Genetic improvement of oxidative stress tolerance and longevity of the entomopathogenic nematode *Heterorhabditis bacteriophora*

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Dedication

To my alpha and omega; my first and last; my beginning and end; my Abba Father

be all the glory, honor and praises.

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1. Introduction

Entomopathogenic nematodes (EPNs) of the species *Heterorhabditidis bacteriophora* (Rhabditomorpha: Strongyloidea) are biological control agents (BCA) for a variety of economically important pest (Grewal *et al.*, 2005). This species is known to be ubiquitous, to have a short generation time and exist in the soil only as the free-living third stage infective juvenile, also known as the dauer juvenile (DJ) (Johnigk & Ehlers, 1999a; Hominick, 2002). Dauer juveniles thus are obliged to survive in the soil, seek for insect hosts, penetrate their target via natural openings and deliver a few cells of the bacterial symbiont *Photorhabdus luminescens* (Enterobacteriales: Enterobacteriaceae) into the insect hemocoel. Each DJ carries about 200–2,000 *P. luminescens* cells in the anterior part of its intestine. The bacteria cause mortality to noxious insects (Ehlers, 2001). Dauer juveniles are shear-stress tolerant and can be applied with conventional spraying methods or irrigation systems (Georgis, 1990; Wright *et al.*, 2005). In addition, DJs do not pollute the environment and are specific to insects. Therefore, EPNs are safe to non-target organisms including humans, other vertebrates and plants (Ehlers, 2003).

Dauer juveniles of EPNs have been used commercially for almost two decades with a very successful record of control of insect pests. Their performance against insect pests is nevertheless limited by poor shelf-life, low persistence and sensitivity to environmental stress (Grewal *et al.*, 2002; Ehlers *et al.*, 2005; Toepfer *et al.*, 2010; Mukuka *et al.*, 2010a). Industrially, *H .bacteriphora* DJs are produced in monoxenic liquid culture in bioreactors and are stored at densities that can reach up to 500,000 DJs/ml before formulation and transport to the user (Ehlers, 2001). When stored at 7.5°C this species can be stored for 125 days with little more than 20% mortality, however, longer storage periods can result in the loss of infectivity of the DJs (Strauch *et al*., 2000). Prolonged storage in water or formulated, together with pre- and post-application stress factors (e.g. desiccation, UV-radiation, high temperatures) are

strongly reducing the DJ survival during commercial handling and in the soil after application. The improvement of the DJ longevity during storage and also after application is thus a major task for the improvement of *H. bacteriophora* as a BCA. Several niches where nematodes are applied have already been explored by e-enema GmbH, a leading producer of nematodes as biocontrol products. However, the cost of *H. bacteriophora* in large field applications (e.g, Western Corn Root, *Diabrotica virgifera virgifera*) is still high and a comprehensive research is needed in order to reduce the costs. To reduce the cost of application and be competitive with synthetic chemicals, DJs must be applied in the field at maize sowing time during spring (April) when *Diabrotica* eggs are still in the stage of diapause. Thus, the nematodes must survive and stay virulent for a period of at least 4−6 weeks until the first emergence of the host larvae (Pilz *et al.,* 2014). Currently, 2 billion nematodes are applied per ha. Should it be possible to increase the longevity of the nematodes, it might be possible to reduce the application rate to 1 billion and thus be competitive with conventional chemical treatments. Hence, DJ quality in terms of prolonged shelf-life and persistence is a target for improvement within a breeding programme. The understanding of the physiological and molecular factors underlying DJ longevity and tolerance to environmental stresses is therefore a priority task for future research approaches in EPNs (Grewal *et al.*, 2011).

The influence of single environmental stress factors in the DJ survival during storage, formulation, transportation and field application has been widely assessed (Grewal, 2000; Strauch *et al.*, 2000; 2004). Temperature for instance plays a major role in survival, infectivity, development and reproduction of EPNs (Chung *et al.*, 2010). At high temperature (>35°C) the nematode metabolism is high and the consumption of reserves like lipids increases, leading to lifespan reduction (Susurluk & Ehlers, 2008; Andalo *et al*., 2011). Concerning survival in the soil, the persistence of EPNs below 20°C was found higher than under elevated temperatures (Kurtz *et al.,* 2007). In agreement with this observation, Susurluk & Ehlers (2008) further

recorded that persistence of *H. bacteriophora* in soil samples from oil seed rape and potato fields was up to 60% lower at 25°C than at 15°C. Desiccation stress in parallel has a strong effect on DJ longevity since they cannot survive under low relative humidity conditions. Mukuka *et al.* (2010b) determined that desiccation stress tolerance varies significantly among nematode strains and species with the mean tolerated water activity (a_w) values (MW_{50}) ranging from 0.90 to 0.95 for DJs not adapted to stress and 0.67 to 0.99 for adapted DJ populations. To prolong the shelf-life of *H. bacteriophora* under storage, the metabolism of the nematodes is reduced by inducing a mild desiccation (Solomon *et al*., 2000; Strauch *et al*., 2004; Mukuka *et al.*, 2010b).

Physiologically, thermal- and desiccation stress and other environmental perturbations such as hypoxia and UV-radiation can result in a strong additional internal stress (oxidative stress) with severe detrimental consequences for the organism. This stress is caused by overproduction of reactive oxygen species (ROS) due to a metabolic disequilibrium (Bokoch & Diebold, 2002).The state of imbalance between ROS production and the capability of a biological system to detoxify the reactive intermediates can generate adverse modifications to cell components like lipids, proteins and DNA (Valko *et al*., 2006; Birben *et al.*, 2012; Dias *et al*., 2013). ROS such as superoxide anion radicals ($O_2^{\bullet-}$), nitric oxide (NO), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) are produced as by-products of cellular metabolism, mostly in the mitochondria (Riess *et al*., 2004; Barrera, 2012). Hydrogen peroxide for example, is one of the most abundant and stable ROS that plays an important role as a signaling molecule in various physiological processes. Endo- and exogenous H_2O_2 increases during environmental stresses and influence the longevity of an organism (Slesak *et al.*, 2007; Cypser & Johnson, 2002; Rhee *et al.*, 2005). Oxidative stress is thus another important factor affecting the lifespan of nematodes. Earlier reports in nematodes indicate that pre-adaptation of nematode populations with low doses of oxidative stress increases tolerance to other stresses by inducing defense genes (Butov *et al.*, 2001; Cypser & Johnson, 2002; Cypser *et al.*, 2006). On the other hand,

medium and high levels of ROS, may cause senescence and cell death, respectively (Kim & Sun, 2007). In the model nematode *Caenorhabditis elegans*, a correlation between oxidative stress tolerance and lifespan has already been reported. For example, in *C. elegans* experiments using the so-called ‘’Age’’ mutants, an increase response to several stress factors such as oxidative and heat- and UV stress was observed (Johnson *et al*., 2000). The authors suggest that the role of the ‘’Age’’ genes as a main evolutionary determinant of longevity is the capacity to respond to stress factors. Other extensive studies reported that long-lived dauer mutants exhibited increased resistance to oxidative stress and antioxidant genes were up-regulated (Larsen, 1993; Honda & Honda, 1999; Johnson *et al.,* 2002; Yanase *et al.,* 2002; Murphy *et al.,* 2003). Selected mutants of *C.elegans* that are resistant to juglone (a compound of black walnuts used as a herbicide and an intracellular ROS generator) were reported to have increased the mean and maximum lifespan (de Castro *et al.,* 2004). At 0.4 mM Paraquat (a herbicide and an intracellular ROS generator) about 10% of *age-1* mutants survived in comparison to wild type nematodes. *SGK-1* mutants exhibit 40% survival at 150 mM paraquat for 6 days in contrast to 100% mortality in wildtypes (Yanase *et al.,* 2002; Hertweck *et al.*, 2004). Deletion of hydrogen peroxide detoxifying enzymes resulted in accelerated aging in *C. elegans* (Petriv & Rachubinski, 2004; Olahova *et al.*, 2008). However, most of the existing reports have been directed to evaluate the lifespan of individuals within a life cycle (from egg to adult). How oxidative stress is directly affecting the survival of the DJ stage is still to be unraveled and not fully understood.

In *H. bacteriophora*, an existing study on the relation between DJ longevity and several environmental factors including hypoxia has already been reported (Grewal *et al*., 2002). The authors found a strong relation of longevity with heat, UV-radiation and hypoxia tolerance. However, the influence of oxidative stress in *H. bacteriophora* DJs has up to date not been studied yet.

For environmental stresses such as heat and desiccation, high heritability values (e.g. 0.48 for desiccation and 0.68 for heat stress tolerance for adapted populations) have been determined and a gain in mean tolerated temperature and water potential in DJs has been achieved through selective breeding (Strauch *et al.*, 2004; Ehlers *et al.*, 2005; Mukuka *et al.*, 2010a, b; Anbesse *et al.*, 2013a, b). Success through genetic selection is dependent on heritability (h^2) of a certain beneficial trait (Hartl & Clark, 1989). Heritability refers to the proportion of the genetically caused variance in the phenotypic variation of a population (Falconer, 1984; Strauch *et al.*, 2004). Up to date, no report exists about the variability on oxidative stress tolerance in EPNs and its heritability. Assessing the heritability of the oxidative stress tolerance in *H. bacteriophora* will answer the question whether genetic selection might be a feasible approach for improvement of longevity and stress tolerance in general.

The development of a faster and efficient improvement method will support the industrial exploitation of as the biocontrol potential of nematodes. Classical genetics serve as a powerful tool to enhance the nematode´s beneficial traits using the natural phenotypic and genetic variability as starting points. Success in the improvement of heat and desiccation stress tolerance of the *H. bacteriophora* and *Steinernema carpocapsae* was achieved through cross-breeding and successive genetic selection. For instance, Shapiro *et al.* (1997) first enhanced heat tolerance in *H. bacteriophora* through hybridization and Shapiro-Ilan et al. (2005) showed that hybridization with 2 strains of *S. carpocapsae* using controlled crosses can lead to improvement of beneficial traits. In addition, Ehlers *et al.* (2005) increased the mean tolerated temperature of a *H. bacteriophora* hybrid strain to 39.2 ºC. Subsequently, Mukuka *et al*. (2010a, c) screened 60 *H. bacteriophora* strains from different geographical locations and reported an increase to 44.0 ºC for 2 h after eleven selection steps. Concerning desiccation stress tolerance, Strauch *et al*. (2004) and Mukuka *et al.* (2010b) achieved a reduction in mean tolerated water potential (a_w-value) by exposing DJs to polyethylene glycol (PEG 600) at different concentrations and carrying out selection steps. As trait improvement was lost when nematodes

were propagated *in vivo*, Anbesse *et al*. (2013a, b) managed to stabilize the aforementioned heat and desiccation tolerance in *H. bacteriophora* by selecting tolerant homozygous inbred lines in monoxenic liquid cultures.

The exploitation of mutagenesis in breeding and genetics robustly complements the use of natural genetic variability. Mutagenesis with ethyl methanesulfonate (EMS) is a broadly used non-transgenic, non-targeted mutagenesis method that causes G/C to A/T transitions generating stop codons and synonymous substitutions (Flibotte *et al*., 2010). In *C. elegans*, mutagenesis screens using EMS have led to substantial insights into the genetic control of animal development and physiology by generating a broad plethora of phenotypes (Lehrbach *et al.*, 2017). Through EMS mutagenesis, the function of important longevity genes such as *daf-2, daf-16, age-1, fer-1, unc-31* has been determined (Friedmann & Johnson, 1988; Kenyon *et al*., 1993; Larsen *et al*., 1995; Johnson *et al*., 2002; Garigan *et al.*, 2002). Cheng *et al.* (2003) also found that *C. elegans* mutants *pre-33*, *pre-7* and *pre-1* exhibited higher resistance to phosphine and longer average lifespan in relation to wild type N2. In *Heterorhabditis* species, few studies on the use of EMS mutagenesis have been reported. For example, dumpy mutants (designated *Hdpy*- and *Rhdpy l)* were isolated from the F_2 generation of a mutagenized population in *H. bacteriophora*. The mutation was recessive with 100% penetrance and uniform expressivity (Zioni *et al.*, 1992; Rahimi *et al.*, 1993). *Heterorhabditis megidis* strain UK211 was also mutagenized using EMS to develop desiccation tolerant mutants (Sean & Ann, 1997). These mutant lines were tolerant to dehydration only at low relative humidity. In other EPN species, *S. feltiae* morphological and behavioral mutants were characterized by Tomalak (1994). Here, spontaneous mutants in a single locus (*Sfdpy-1)* were found and classified as dumpy (*dpy*). Apart from morphological changes, the mutation also affected the nematode movement activity and infectivity to insect hosts. The use of EMS to generate long-living *H. bacteriophora* lines is therefore an approach offering a very promissory application potential.

The advent of high throughput sequencing technologies has revolutionized genomic and transcriptomic approaches in biological studies. With these new techniques, enormous sequence data are now accessible not only for *C. elegans* but also for EPN species. For instance, transcriptomic data of *H. bacteriophora* GPS11 and TT01 strains have generated a total of 1,000 ESTs and 10,886 distinct EST sequences, respectively (Sandhu *et al.*, 2006, Bai *et al.*, 2007, 2009). Recently, Vadnal *et al.* (2017) profiled the transcriptional response of the same species during the early stage of infection by carrying out next generation RNA-sequencing. Additionally, Bai *et al.* (2013) reported the genome sequence draft of this species. In the close species *H. indica*, a transcriptomic insight of DJ is also available with sequences deposited in a public repository (Somvanshi *et al.*, 2016). Additional sequence data is still needed to enhance the sequence knowledge of the EPN species and relevant genes may be identified and further used as genetic markers for stress response (Somvanshi *et al.*, 2008).

Transcriptional profiling of stress tolerance in the nematodes with contrasting phenotypes have been reported. In a comparative study between *H. bacteriophora* and other EPNs, Somvanshi *et al.* (2008) assessed the gene expression of five desiccation tolerant and susceptible nematodes by real time quantitative PCR method. In their study, four genes were differentially up-regulated in the desiccation-susceptible *H. bacteriophora* TT01 strain (i.e. *aldh, nap 1, gpx* and *hsp-40*) compared to *S. carpocapsae* and *S. riobrave* (most tolerant to desiccation). Recently, Yaari *et al.* (2016) assessed the transcriptome in *Steinernema* species (*S. riobrave* and *S. feltiae*) with contrasting tolerance to heat- and desiccation stress, reporting that more genes were down-regulated in a tolerant strains, while more genes were up-regulated in the less tolerant strains. Insights on how similar or dissimilar are, a transcriptome re-modelling between oxidative stress and other environmental stresses in EPNs will complement these previous reports. Up to date, high-throughput transcriptome analysis on oxidative stress in *H. bacteriophora* DJ has not yet been reported. Thus, new transcriptome analysis will further contribute to confirm and generate more sequence information in *H.bacteriophora.*

This study mainly focused on two stream lines: (i) the use the genetic (i.e. natural and artificial) variability to extend longevity of *H. bacteriophora* by the characterization and (ii) understanding of the underlying mechanisms associated to longevity in *H. bacteriophora* DJs. A prolonged shelf-life, increased tolerance to stress and enhanced persistence in the field will lead to the generation of improved nematode products, which can be applied effectively in larger scale markets such as for the control of *D. virgifera virgifera* in maize. Reducing the application cost by lowering the current application density of $2*10^9$ ha^{-1} to $1*10^9$ ha^{-1}, will make EPNs be more competitive with the synthetic chemical products. Then, preference will be given to EPN applications in to order control insect pests as they are safe to humans and the environment.

The objectives of this study were:

- Assessment of oxidative stress tolerance and longevity of *H. bacteriophora* dauer juveniles (DJs) among wild type strains, inbred and hybrid lines with natural genetic diversity
- Assessment of DJ persistence in sand in a subset of *H. bacteriophora* strains and lines
- Investigation of a hybrid strain and EMS-mutants for oxidative stress tolerance and longevity improvement
- Investigation of the virulence and reproductive potential of the hybrid and mutant lines of *H. bacteriophora*
- Assessment of the differential gene expression of oxidative stress tolerance in *H. bacteriophora* DJs

2. Results and Discussion

2.1. Oxidative stress tolerance and longevity is correlated in *H. bacteriophora* dauer juveniles

The relation between DJ oxidative stress tolerance and longevity in *H. bacteriophora* was investigated at 25°C and 7°C. From 40 *H. bacteriophora* wild type strains, inbred and hybrid lines investigated, the mean time survived by 50% (MTS_{50}) and 10% (MTS_{10}) of the DJ populations were determined for with and without oxidative stress treatment at 25°C and 7°C. With exposure to oxidative stress at 25°C, the MTS_{50} ranged from 3 to 22 days and the MTS_{10} from 6 to 50 days (Publication 1, Fig. 1). When stored at 7°C, tolerance to oxidative stress was relatively increased for an average difference of 3 days (Publication 1, Fig. 2). For strains S-DT1 and IT4 the lowest and highest MTS_{50} of 5 and 25 days, respectively, were recorded. Concerning the MTS_{10}, a maximum of 53 days was observed at this temperature, also indicating a difference of 3 days. These results demonstrate that oxidative stress can strongly override other environmental factors (e.g. temperature). It has been described that prolonged periods of exposure to high H_2O_2 levels can incite secondary and tertiary oxidative irreversible modifications in the cell that could affect the animal lifespan (Sanz, 2016).

In parallel, DJ longevity was assessed without oxidative stress induction by storing in Ringer's solution only. At 25°C, the MTS_{50} ranged from 21 to 57 days and the MTS_{10} from 34 to 104 days (Publication 1, Fig. 3). At 7°C, a maximum MTS_{50} of 94 days and a maximum MTS_{10} of 157 days was determined (Publication 1, Fig. 4).These results demonstrate that DJ longevity was extended at 7°C rather than at 25°C indicating the influence of temperature. This result could be attributed to the DJs physiological activity, metabolic rate and lipid reserve utilization (Grewal & Peters, 2005; Susurluk & Ehlers, 2008; Andalo *et al*., 2011). A similar finding was reported by

Strauch *et al.* (2000) who measured the percentage mean survival of a hybrid strain of *H. bacteriophora* stored in a Ringer's solution and observed a higher survival of DJs stored at 7.5°C (~150 days) than at 25°C (51 days). Overall, these results evidenced a large variability in the MTS_{50} and MTS_{10} of the DJ populations for both with and without oxidative stress treatment at 25°C and 7°C; underpinning the observations of Mukuka *et al.* (2010a, b) who found high variabilities of *H. bacteriophora* strains and lines collected from different geographical regions in their tolerance to stress conditions like heat and desiccation. For all experimental conditions, the wild type strain IT4 was found to be the most tolerant to oxidative stress and long-living while the commercial line (EN01) was least tolerant to oxidative stress and short-living. This evaluation provides information for selecting potential strains to generate hybrids and inbred lines with improved longevity.

Furthermore, the correlation of DJ longevity and oxidative stress was determined within the 40 *H. bacteriophora* strains and lines in their MTS_{50} and MTS_{10} at 25°C and 7°C. Consistent with the result in the preliminary experiment using the EN01 commercial line, a significant positive correlation between DJ survival at oxidative stress (70 mM H_2O_2) and survival when stored in Ringer´s solution (without oxidative stress) was also recorded for 25°C ($MTS_{50,}$ R = 0.604; $MTS_{10,}$ R = 0.758) (Publication 1, Fig. 7A). Correspondingly, a strong positive correlation was determined for both MTS_{50} (R = 0.802) and MTS_{10} (R = 0.652) when stored at 7°C (Publication 1, Fig.7B). Between 7°C and 25°C, a positive correlation held under oxidative stress conditions for both MTS_{50} (R = 0.653) and MTS_{10} (R = 0.585) was also observed (Publication 1, Fig. 8A). Although this study first determined the relation of oxidative stress and longevity in *H. bacteriophora* DJs, this nevertheless confirms other studies in different model organisms that oxidative stress is one of the major determinants of the lifespan. As an example, a finding in *C.*

elegans was reported by Knoefler *et al*. (2012), who correlated the changes of oxidation level with lifespan and found that short-lived *daf-16* and long-lived *daf-2* mutants exhibited higher and lesser ROS, respectively, compared to the N2 strain. Thamsen (2011) in addition observed that lifespan of *C. elegans* was influenced by H_2O_2.

2.2. Oxidative stress tolerant *H. bacteriophora* strains persist longer in the soil

As a biological control agent used to control important pests, like the Western Corn Rootworm, DJs must persist without losing infectivity for a period of 4–6 weeks in the field. Therefore, this study assessed the DJ survival after application in the sand (persistence) and whether oxidative stress can be also used as a predictor. A total of 16 pre-selected *H. bacteriophora* strains, 8 with the highest MTS_{50} under oxidative stress conditions were compared against 8 with lowest MTS_{50} for persistence in sand for a period of 5 weeks at 25°C. Approximately 30–45% of the nematodes were not recovered after one day of incubation and low survival was found after 5 weeks. The MTS_{50} values were significantly different between strains and lines with S-VI-MM8 and TR4 obtaining the highest and lowest DJ survival (persistence) of 11 and 5 days, respectively. Regarding the $MTS_{10,}$ the values ranged from 10 to 20 days at 25°C (Publication 1, Fig. 5). The sudden decline in DJ density within the first day after application can be due to the inefficiency of extraction methods and has also been reported by other authors (e.g., Fan & Hominick, 1991). The low survival in the sand is presumably an effect of several environmental factors, such as temperature, soil moisture content, soil type and finally the potential of the DJs to use the energy reserves effectively (Glazer, 2002; Pilz *et al*., 2014). Interestingly, the results indicated a strong correlation between survival of nematodes under oxidative stress and DJ survival in sand in both MTS_{50} (R = 0.826) and MTS_{10} (R = 0.815) (Publication 1, Fig. 8B). Despite this low DJ survival,

the strong correlation on DJ survival between the oxidative stress assay and persistence sand bio-assay implies that oxidative stress can be used as predictor for DJ persistence.

In addition, the effect of the temperature on DJ survival in the sand was elucidated using selected inbred lines and hybrids with contrasting tolerance to oxidative stress. Higher percentages of DJ survival (52–81%) were observed at 15°C compared to 25°C (31–67%). Survival after 3 weeks of incubation at 25ºC was lower than at 15°C for all strains and lines tested (Publication 1, Fig. 6). Storage at 15°C resulted in an average increase of percentage survival ranging from 14–21% and 22–27% for inoculation densities of 6,400 and 400 DJs, respectively. This observation supports the report of Kurtz *et al.* (2007). According to these authors, the persistence of EPNs could be higher in *D. v. virgifera*–infested areas with colder spring and summers, where soil temperatures remain steadily below 20°C at depths of 15–20 cm. In colder seasons, DJs of *H. bacteriophora* can survive over 42 days in maize fields overlapping with the period of the larval development of *D. v. virgifera* (Kurtz *et al.*, 2007; Pilz *et al*., 2014). Survival and persistence of DJs were found to be also influenced by lipid reserves (Lee & Atkinson, 1976; Hass *et al*., 2002; Fitters & Griffin, 2006). Although the lipid reserves in this study were not investigated, it will be interesting to quantify and determine the optimal amount of energy reserve (lipid content) required to move and infect their target insects.

2.3. Heritability of oxidative stress tolerance is high in *H. bacteriophora*

Aside from the high variability between available strains, the major factor that could influence success in selective breeding is the heritability (h^2) of a certain beneficial trait (Hartl & Clark, 1997). This present study first determined and reported the heritability of oxidative stress tolerance and longevity in Ringer's solution of *H. bacteriophora* DJs. For both storage regimes,

25°C and 7°C, high heritability values of h^2 = 0.91 and 0.93, respectively, were recorded for oxidative stress tolerance indicating that 91–93% of the individual differences that were observed in oxidative stress tolerance can be attributed to the genotype. In contrast, low heritability values for DJ longevity stored at 25°C and 7°C of 0.11 and 0.23, respectively were assessed. Similar results were found by Reynolds and Phillips (2013) that oxidative stress resistance of *C. remanei* is a heritable trait (h^2 = 0. 95 for female; 0. 75 for male). The oxidative stress resistance was assessed by exposing the one-day-old virgin adults to 3.5 mM of H_2O_2. The same authors determined a remarkable low heritability for the lifespan using inbred lines (h^2 = 0.08 for female; 0.06 for male and 0.06 for parental offspring). Brooks and Johnson (1991) also noted that the heritability of longevity using recombinant inbred strains of *C. elegans* ranged from 0.05 to 0.36. In this present study, the low heritability of longevity in Ringer's solution may be due to the prolonged storage (~150 days) resulting in high levels of environmental variance. Past reports have demonstrated that longevity, as a life-history trait, which is more related to fitness, is expected to have a lower heritability than physiological, morphological and behavioral traits (metric traits, Gustafsson 1986; Charlesworth, 1987; Hartl & Clark, 1989; Falconer, 1989; Price & Schluter, 1991). Price and Schluter (1991) developed the causal relationship between metric traits and life-history traits to show that a life-history trait is anticipated to have a low heritability whether or not the population is at equilibrium because of the influence of environmental variations. Generally, the main advantage of the use of oxidative stress as predictor is the relatively short testing period. These results will allow us to carry out selection with a high probability of obtaining a long-living strain under control conditions (low environmental variance), which is practically hard to achieve without the oxidative stress assay.

2.4. Oxidative stress tolerance and DJ longevity can be improved by cross-breeding and mutagenesis

Genetic improvement serves as a powerful tool to increase tolerance of the EPNs to environmental perturbations (Gaugler, 1989). Similar to previous studies on the improvement of heat and desiccation tolerance in *H. bacteriophora* and cold temperature activity in *S. feltiae* through hybridization (Strauch *et al*., 2004; Ehlers *et al*., 2005; Mukuka *et al.,* 2010a, c; Anbesse *et al.,* 2013 a, b; Nimkingrat *et al*., 2013a, b), this study performed hybridization as an approach to further enhance oxidative stress tolerance and extend longevity of the DJs. Using two oxidative stress tolerant and long-living strains (AU1 and HU2) (as reported in Publication 1) as parents in the genetic cross, the resulting hybrid, hereafter referred to as AU1×HU2, acquired both higher tolerance to oxidative stress and longer survival in Ringer's solution (DJ longevity) than its parents. This finding can be likely attributed to the phenotypes associated with increased longevity and tolerance to oxidative stress damage (Larsen, 1993; Martin *et al*., 1996; Finkel & Holbrook 2000). With oxidative stress induction, the parental strains HU2 and AU1 had MTS_{50} of 10.5 and 9.9 days, respectively, whereas AU1×HU2 survived 2.6 days longer than the best parent. Consistently, AU1×HU2 had the highest MTS_{10}, which survived 3.5 days longer relative to its best parent, HU2. The commercial line EN01 was observed to be sensitive to oxidative stress (Publication 2, Fig. 2A). Concerning DJ longevity when stored in Ringer´s solution, the MTS_{50} values for AU1 and HU2 were 75 and 56.7 days, respectively, whereas AU1×HU2 survived 18 days longer than its best parent HU2 (Publication 2, Fig. 2B). Moreover, a higher MTS_{10} was also observed for AU1×HU2 (MTS_{10} = 147.3 days). This increase of tolerance to oxidative stress and longevity may be attributed to a heterosis effect (Falconer, 1984). The combination of alleles through cross-breeding provided an increased stress tolerance, which is congruent with the report

of Mukuka *et al*. (2010c), who recorded that hybridization of most heat tolerant strains of *H. bacteriophora* improved the mean tolerated temperature and likewise increased the desiccation tolerance. A similar result was also found with *S. feltiae* by Nimkingrat *et al*. (2013a, b). A hybrid strain HYB01 was more active at low temperature than the parents.

The use of mutagenesis as an alternative source of genetic variability was explored in this study. EMS mutagenesis was applied to then select mutants with improved oxidative stress tolerance and longevity. While mutations with a recessive effect generally eliminate the functions of particular genes, mutations with dominant effects can lead to acquiring novel gene activity (Muller, 1932; Waterson, 1981; Lodish *et al*., 2000). For example, Park and Horvitz (1986) reported that in *C. elegans*, the dominant mutation effects in the determined nine genes are caused by novel gene functions. In this study, EMS-mutants were created, screened and selected in order to obtain a potential line with improved traits. The non-mutagenized donor line IL3 is an inbred line derived from the commercial line EN01. This commercial line was previously characterized to have a lower tolerance to oxidative stress (as reported in Publication 1). After the first screening, M-OX1 was found to have acquired a high tolerance to oxidative stress and successively, 6 inbred lines were generated for seven generations of propagation (Publication 2, Fig.1). Each of the lines was then separately cultured under pooled conditions to produce enough DJ inbred material for the stress test in which only four lines survived and were compared with each other for oxidative stress tolerance (60 mM H_2O_2) and then also with the donor line IL3 (Publication 3, Fig. 4). The inbred line MOX1-IL6 turned out to be the most tolerant surviving 9.2 days (MTS_{50}), whereas the donor line IL3 had a MTS_{50} of 3.7 days (Publication 2, Fig. 4).

Further on, to decrease the possibility of secondary mutations inferring with the phenotypic analysis, a cross between MOX-IL6 and IL3 was performed. This hybrid hereafter referred to as

MOX-IL6×IL3 was compared to their parental lines (Publication 2, Fig. 5A). After exposure to oxidative stress (79 mM H_2O_2), MOX-IL6 survived longest with a MTS_{50} of 8.5 days followed by MOX-IL6×IL3 with a MTS_{50} of 5.2 days. Even after crossing, the progeny of MOX-IL6×IL3 still had higher tolerance to oxidative stress compared to IL3 (MTS_{50} = 2.7 days) and was found to be comparable with the commercial line EN01 (MTS_{50} = 3 days). An overall increase of tolerance to oxidative stress for 5.8 days was obtained with mutagenesis. Regarding DJ longevity when stored in Ringer´s solution, MOX-IL6 survived longer (MTS_{50} = 47.8 days) followed by MOX-IL6×IL3 and EN01 with MTS_{50} values of 37.9 and 37.7 days, respectively. IL3 had the lowest MTS_{50} of 19 days. The generated EMS-mutants MOX-IL6 and MOX-IL6×IL3 did not only confer tolerance to oxidative stress but also exhibited a long-lived phenotype. This result signifies that the particular mutation may have dominant and/or pleiotropic effects. Mutagenesis and the isolation of novel or improved phenotypes in parallel with natural genetic variability has led to genetic variations endowed with beneficial traits. In general, the progress obtained by mutagenesis is quite remarkable considering the origin of the IL3 inbred line, which originated from one of the sensitive and short-living strains (the commercial EN01). Consequently, future research should try to also apply EMS mutagenesis to superior genetic material (e.g, strain IT4). Most likely the success might even be higher.

From this research, three potential nematodes (AU1×HU2, MOX-IL6 and MOX-IL6×IL3) with increased tolerance to oxidative stress and extended lifespan were obtained, which can be utilized for future research. Genetic analysis to confirm the dominant effect and identify the mutated gene that gave rise to a phenotype with increased tolerance to oxidative stress will help to better understand the genetic background of stress related genes. Next generation sequencing technologies (NGS) now allows the development of new methods for rapid mapping and

identification of EMS-induced mutations and can supply necessary information to identify the mutation by comparing the nucleotide sequences of wild type and mutant genomes. However, genetic analysis and mapping still remains a challenge in *H. bacteriophora* research.

2.5. Fitness of *H. bacteriophora* is not compromised as a result of hybridization and mutagenesis

Trade-off effects as a result of genetic improvement of *H. bacteriophora* can be a major drawback (Mukuka *et al*., 2010d). Therefore, this study compared the fitness by assessing the virulence and reproduction potential of the hybrid and mutant lines with an improved oxidative stress tolerance. For comparison, the parental and donor lines were used. A higher virulence to *T. molitor* was obtained by AU1xHU2 (73.9%), gaining an average increase of 10% relative to its best parent (Publication 2, Fig. 3A). Moreover, both MOX-IL6×IL3 and MOX-IL6 obtained comparable mean mortality values of 54.2% and 52.2%, respectively while IL3 had a mean mortality of 43.3% only (Publication 2, Fig 6A). The commercial line EN01 obtained the lowest infectivity. This implies that creation of hybrid and mutant lines did not only increase tolerance to oxidative stress but also improved virulence. Similar results were found by Nimkingrat *et al.* (2013b), who recorded a higher virulence in *S. feltiae* hybrids, HYB13-A and HYB13-NA with LD_{50} values of 19.6 and 23.2, respectively, compared to the commercial line COM with a LD_{50} of 37.9 against *Cydia pomonella.*

For the reproductive potential, no significant differences were observed between the hybrid, parental and commercial lines (with mean number of DJ offspring ranging from 99,051 to 104, 067 per insect) (Publication 2, Fig. 3B) and also between the mutants, donor and commercial lines (with mean number of DJs offspring ranging from 75,327 to 94,487) (Publication 2, Fig.6B).

No negative effect for the reproduction was also observed. These results further underpin the report of Nimkingrat *et al.* (2013a, b) that reproduction of *S. feltaie* was not negatively affected by hybridization and selection in order to enhance activity at low temperature and tolerance to desiccation.

2.6. The transcriptome of *H. bacteriophora* indicates fine-tuning as crucial for stress response

2.6.1. Homology and differentially expressed transcripts in *H. bacteriophora*

Oxidative stress has been recently reported to have a direct influence in the DJ longevity of *H. bacteriophora* (Publication 1, Fig.7A). Therefore, understanding mechanisms by which DJ respond to oxidative stress can provide insights to improve DJ longevity of this EPN species. In this study two generated inbred lines with differing tolerance to oxidative stress, HU2-IL1 (stress-tolerant) and PT1-IL1 (stress-sensitive) was used (Publication 3, Fig. 1). A comparative transcriptomic analysis of the early stage of oxidative stress induction (4 h) in the two *H. bacteriophora* inbred lines was performed. To assess the transcriptome, MACE, a versatile RNA-seq variant was applied.

The *de novo* transcriptome assembly yielded a total of 20,022 transcripts, from which 10,290 were linked to 9,776 different UniProt accessions. After comparison with other nematodes, large fraction of transcripts (49%) observed to have no known homology in nematodes or other organisms for which sequence data are available in public repositories. The majority of the annotated transcripts were homologous with genes described for vertebrate parasitic nematodes (43%), whereas only 6% were homologous with free-living nematodes (Publication 3, Fig.2). Vandal *et al.* (2017) observed diverse degree of homologies to different nematode clades I, III, IV

and V (as reported by Blaxter *et al*., 1998) and found that under Clade V hits matched with *Ancylostoma ceylanicum* (790 genes), *Necator americanus* (706 genes), *C. elegans* (551 genes) and matched *Haemonchus contortus* (363 genes). In *Steinernema* species, Yaari *et al.* (2016) studied the transcriptome profile of heat and desiccation stress response and also observed a higher similarity to other vertebrate parasites. The authors assumed that this similarity could be attributed to the DJ survival mechanisms as well as the different representation of the gene resources for the parasitic and free-living nematodes.

Concerning differentially expressed transcripts, the up-regulated transcripts 4 h after stress induction in PT1-IL1 and HU2-IL1 were 969 and 800, respectively (Publication 3, Fig. 3A). A substantially higher number of transcripts were globally down-regulated after stress induction (Publication 3, Fig. 3B). A higher down-regulation was observed in PT1-IL1 with 5,207 transcripts whereas HU2-IL1 only had 1,844 transcripts down-regulated with 1,054 transcripts shared by both lines. Yaari *et al*. (2016) detected an inverse relation between gene expression and tolerance to desiccation and heat stress in *Steinernema* species with differing phenotype. The authors suggested that a lower level of transcriptome re-modelling on a stress-tolerant line may signify that several stress-related genes are active before the stress onset. With reference to the very large number of down-regulated transcripts in the stress-sensitive line PT1-IL1, it can be assumed that this line failed to maintain the basic transcriptome level of many genes playing an important role under high ROS conditions.

Regarding the identity of the transcripts with most contrasting differential expression in HU2-IL1 and PT1-IL1, 12 out of 40 transcripts with up-regulation expression had homology to UniProt accessions with 7 UniProt accessions described as uncharacterized proteins (Publication 3, Table 3). Similarly, 18 out of 40 transcripts with down-regulation expression had homology to

UniProt accessions with 9 accessions described as uncharacterized protein (Publication 3, Table 4). These differentially expressed uncharacterized transcripts might play an important role in oxidative stress and that inclusive research on gene functions will in due course elucidate other essential genes in response to stress.

In studies related to the expression profiling on the trait deterioration in *H. bacteriphora*, Adhikari *et al.* (2009a) found that about 10% of DGEs did not have homology to any available sequences, which indicates novel *Hb* genes. In other nematode species like *Plectus murrayi*, Adhikari *et al.* (2009b) also observed that in the subtractive library of the desiccated nematodes revealed 80 transcripts differentially expressed during desiccation stress, which 17 (21%) novel transcripts that had no similarity to known sequences in GenBank. A thorough investigation on these uncharacterized genes, which might lead to discovery of genes and functions; playing a crucial in oxidative stress response is an interesting research outlook.

2.6.2. Global transcriptome re-modelling of *H. bacteriophora* upon oxidative stress induction

Gene Ontology (GO) terms on the common and exclusive differentially expressed genes were analyzed to have a global overview of biological processes that are represented in both lines after stress induction (Publication 3, Tables 5 and 6). A total of 34 and 28 GO terms (biological processes) were commonly up-regulated and down-regulated in both lines, respectively. The most represented GO categories in the up-regulation were associated to single-organism cellular processes, single-organism metabolic processes, carbohydrate metabolic processes, regulation of biological quality, oxidation-reduction and ion-transport. Most of the repressed processes (down-regulated) were associated to oxidation reduction and response to stimulus. With the large extent

of down-regulated genes observed, the stress-sensitive PT1-IL1 line showed GO terms associated to basic metabolic processes, while the stress-tolerant HU2-IL1 line indicated GO terms associated to signaling and protein modifications. These results support the assumption that a large degree of down-regulation in this stress-sensitive line indicates a global turn-down of vital processes. Despite a high level of redundancy observed in processes associated to transcripts that are exclusively up-regulated in both lines, the genes beneath them can be totally different in both analyzed lines.

In *H. bacteriophora*, Vadnal *et al.* (2017) determined that metabolic and cellular processes, reproduction, development and growth were found to be over-represented in response to infection and Adhikari *et al.* (2009) reported that metabolic, developmental, multicellular organismal and cellular processes and growth were over-expressed during trait deterioration. In *Steinernema* species, Yaari *et al.* (2016) observed that under desiccation stress, the enriched GO terms were under alcohol metabolic processes, carbohydrate metabolic processes, organic hydroxy compound metabolic processes and lipid catabolic processes, while GO enriched terms in response to heat stress were response to stress, response to topologically incorrect protein and membrane organization.

2.6.3. Expression of oxidative stress-signaling and responsive gene

An in-depth analysis on the transcription responses from genes related to main detoxification pathways was done. The differential expression in the HU2-IL1 and PT1-IL1 lines was compared with *C. elegans* orthologous genes in the pathway model ko04212 from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Publication 3, Fig. 5) to give inclusive analysis on the transcription responses from genes related to main detoxification pathways. Nearly all the

genes in the *C. elegans* oxidative stress response pathway model were also present and identified in the *H. bacteriophora* transcriptome data. Within the *H. bacteriophora* transcriptome library, transcripts were found to have high homology with MAPKKK, SEK-1, PMK-1 and SKN-1, gluthatione cycle-related genes, oxidized and reduced gluthathione redox couple system, superoxide dismutases and catalases and ROS-scavenging genes (Publication 4, Fig. 4).

As an example, one transcript variant homologous to the Skinhead-1 gene (*SKN-1*) was identified in our transcriptome data (EN-Hb_oxid-57101). This transcript was up-regulated (log2-FC = 1.32) in the HU2-IL1 upon exposure to oxidative stress and slightly down-regulated in the PT1-IL1 (log2-FC = -0.15). Several transcripts were identified in the transcriptome data for the glutathione cycle-related genes. For example, transcript EN-HB_oxid-48672 was found to be homologous to the γ-glutamyl cyclotransferase (*GGC*) gene and was up-regulated in both, PT1-IL1 and HU2-IL1 inbred lines. For the glutamate cysteine ligase (*GCL*) gene, for which transcription is directly activated by SKN-1, a homologous transcript was identified (EN-Hb_oxid-45560) to be slightly down-regulated in both lines. Three transcripts annotated to γ-glutamyl transpeptidase (*GGT*) genes had varying expression response (with log2-FC ranging from -1.27 to 1.33). Transcript EN-Hb_oxid-58900 was annotated to the glutathione-disulfide reductase *(GSR)* gene, which was significantly up-regulated in the stress tolerant HU2-IL1 (log2-FC 1.56) and stress-sensitive lines PT1-IL1 (log2-FC = 1.62). Three transcripts were annotated to glutathione peroxidase (*GPx*) genes exhibiting only minor expression changes. Ten transcripts were annotated to proteins of the glutathione-S-transferase family (GST), showing high variability in expression levels. Within those, four transcripts were up-regulated upon oxidative stress in both nematode lines. For instance, transcript EN-Hb_oxid-53277 was significantly up-regulated in HU2-IL1 (log2-FC = 2.37) and PT1-IL1 (log2-FC= 1.71). Three transcripts were annotated to catalase

enzymes (CAT) and of those, transcript EN-Hb_oxid-56752 was significantly up-regulated in PT-IL1 (log2-FC = 2.89) and HU2-IL1 (log_2-FC = 2.13). In line with transcripts annotated to superoxide dismutase enzymes (SOD), three transcripts were found in our transcriptome database not showing large changes in expression.

In a global overview, results indicate no drastic differences in the responses of the stress-tolerant (HU2-IL1) and the stress-sensitive line (PT1-IL1). Differences were only observed in line with some transcript variants that exhibited some contrasting regulation, which may indicate that stress tolerance or sensitivity is linked to fine-tuned responses and certain transcript variants or isoforms play specific roles. According to Yan *et al.* (2017), alternative splicing and transcription (results in variations of the transcript number or the 5′/3′ terminal protein variants) could increase the functional capacity of genes and give an opportunity for gene regulation and a different function. Yadav *et al.* (2017) also observed that several isoforms were differentially regulated upon infection to *Drosophila melanogaster* with symbiotic nematodes compared to axenic nematodes. In response to drought tolerance in maize, Song *et al.* (2017) reported that differential isoform expression were found for a subset of candidate genes. In this work, the GRMZM2G140355 gene did not show significant differentially expression, however, exhibited changes in isoform expression. Thus, specific isoforms deserve focus on future research approaches.

Generally, the SKN-1 transcription factor and GSTs were found to be playing an important role in oxidative stress tolerance of nematodes. The SKN-1 transcription factor is said to orchestrate the activation of GST expression in response to oxidative stress, independent of DAF-16/FOXO transcription activity. The expression of several antioxidant enzymes, including GSTs, has been brought in connection with differences in longevity in *C. elegans* (An & Blackwell, 2003;

Tullet *et al.*, 2008, Zhou *et al.*, 2011; Back *et al.*, 2012). Several GSTs have already been identified in *C. elegans* (van Rossum *et al*., 2001), for example, Leiers *et al*. (2003) found that a decrease of the *Ce*-GST-p24 enzyme level resulted in a significant decrease in the oxidative stress resistance of nematodes. GSTs were also found to be playing a significant role in response to desiccation stress of entomopathogenic nematodes (Adhikari *et al*., 2009b; Yaari *et al*., 2016). It was reported that the desiccation process make nematodes and other anhydrobiotes more sensitive to ROS (Burnell & Tunnacliffe, 2011). This study is the first report on the expression of GST in response to stress conditions in *H. bacteriophora*.

2.6.4. MACE sequence data is confirmed by RT-qPCR expression

The validation of changes in expression observed by differential expression analysis of RNA-seq (MACE) data and RT-qPCR was done using eight selected genes. Genes were chosen based on the fold changes (FC), either positive or negative, showing significant differential expression ($P \leq 0.05$), physiological groups and putative function of the transcript (Publication 3, Table 7). A significant positive correlation between MACE and RT-qPCR expression (R = 0.66) (Publication 3, Fig. 5) and the results was confirmed by cluster analysis (Publication 3, Fig. 6).

3. Summary

Poor persistence in the soil, product shelf-life and loss of virulence during storage is a major constraint for the use as a biocontrol agent of the entomopathogenic nematode-bacterium complex *Heterorhabditis bacteriophora-Photorhabdus luminescens*. A better understanding of environmental stress factors (e.g. desiccation, high temperature, UV-radiation) limiting DJ survival and longevity can lead to the identification of better nematode strains.

A more targeted approach exploits classical and molecular genetics, making use of the enormous diversity among natural populations for improvement of nematode beneficial traits. This study therefore aimed at the genetic improvement of oxidative stress tolerance and DJ longevity of *H. bacteriophora*.

Using natural and genetic variability as starting points, a total of 40 *H. bacteriophora* wild type strains, inbred and hybrid lines of different geographic origin was assessed for their tolerance to oxidative stress and longevity at 25 and 7°C. A high variability among strains of this species was determined, presenting a good foundation for selection and hybridization of superior strains. The mean time survived by 50 % of the DJ population (MTS_{50}) ranged from 21−57 days without oxidative stress induction (Ringer's solution) and from 3−22 days under oxidative stress (70 mM H_2O_2) at 25°C. At 7°C, the MTS_{50} ranged from 5−25 days when exposed to oxidative stress, while 38−94 days was recorded without stress. Only a slight increase (3 days) was found when nematodes were stored at 7°C under oxidative stress. Under oxidative stress, lifespan was not remarkably extended as it was observed for the storage without stress. Oxidative stress tolerance was strongly positive correlated with DJ longevity. Therefore, the oxidative stress tolerance can be used as a predictor for DJ longevity, permitting a selection process within a shorter testing period. Furthermore, the heritability of the trait "survival in Ringer's solution" was lower (h^2 = 0.23 at

7°C, 0.11 at 25°C) than the heritability of "oxidative stress tolerance" (h^2 = 0.93 at 7°C; 0.91 at 25°C).

In order to search for even more tolerant and long-living strains, hybridization and mutagenesis was performed. A hybrid strain deriving from two oxidative stress tolerant and long-living parental strains (AU1 and HU2) was produced. The resulting hybrid AU1×HU2 survived 2.6 days and 18 days longer than its best parent under oxidative stress (79 mM H_2O_2) without stress, respectively. As an additional source of variability, an EMS-mutant pool (MOX-I) with high longevity was generated. One of the resulting inbred lines (MOX-IL6) survived 5.8 days and 28.4 days longer than its non-mutagenized donor line (IL3) under oxidative stress and control conditions, respectively. Even after crossing between the mutagenized inbred line and its donor line, MOX-IL×IL3 still survived 2.5 days and 18.5 days longer than the donor line under oxidative stress and control conditions, respectively. Indication for possible trade-off effects for an improved oxidative stress tolerance was assessed by monitoring virulence and reproductive potential. No trade-off effects were observed. The mean insect mortality caused by AU1×HU2 and MOX-IL6 was even 10% and 8% higher than its best parent and the donor line, respectively. No significant change was observed for the reproduction potential of the nematodes. Further monitoring of the stability of other beneficial traits.

To elucidate the underlying mechanisms by which DJs respond to oxidative stress, a comparative transcriptomic analysis on the early stage of oxidative stress induction (4 h) in two inbred lines with contrasting stress-tolerance, HU2-IL1 (stress-tolerant) and PT1-IL1 **(**stress-sensitive**)** was performed. The *de novo* transcriptome assembly produced a total of 20,022 transcripts, from which 10,290 were linked to 9,776 different UniProt accessions. A larger fraction of the annotated transcripts exhibited homology with parasitic nematodes like *Ancylostoma* rather

than with the free-living *Caenorhabditis elegans*. The number of up-regulated transcripts (log2 FC ≥ 2.0) in the stress-sensitive line PT1-IL1 (630) was higher than in the stress-tolerant line HU2-IL1 (461) under oxidative stress. The number of down-regulated transcripts in both lines were high, however, the stress-sensitive line had here a higher number (5,207 transcripts) compared to the stress-tolerant line (1,844 transcripts). It seems that the stress-sensitive line failed to maintain vital biological processes in contrast to the tolerant line. On the other hand, downregulation seems to be also a crucial factor for survival as it can be seen for the tolerant line. Both lines activated similar biological processes known to be directly involved in detoxification of reactive oxygen species (ROS). The MACE profiles were confirmed by RT-qPCR with a significant positive correlation (R = 0.66) between both techniques.

The results of this study will facilitate the selection longevity predictor genes in this EPN. Further analysis will allow the identification of molecular markers and facilitate breeding for improved lines. Nematode strains with prolonged longevity can provide a longer persistence in the soil after application and thus open a door in larger scale markets such as for the control of *D. virgifera virgifera* in maize.

4. Zusammenfassung

Der entomopathogenen Nematoden-Bakterium Komplexes *Heterorhabditis bacteriophora-Photorhabdus luminescens* wird im biologischen Pflanzenschutz eingesetzt. Eine geringe Persistenz der Nematoden im Boden, die Produkthaltbarkeit und der Verlust der Virulenz während der Lagerung beschränken den Einsatz. Ein besseres Verständnis der umweltbedingten Stressfaktoren (z. B. Austrocknung, hohe Temperatur, UV-Licht), die die Langlebigkeit der Dauerlarven (DL) limitieren, kann zur Identifikation von verbesserten Nematodenstämmen führen.

Ein zielgerichtetes Vorgehen integriert sowohl klassische als auch molekulargenetische Ansätze, um die enorme Diversität natürlicher Populationen für die Verbesserung von nützlichen Merkmalen zu nutzen. Diese Untersuchungen verfolgten das Ziel einer genetischen Verbesserung der oxidativen Stresstoleranz und damit der Langlebigkeit der DL des Nematoden *H. bacteriophora*.

Als Ausgangspunkt wurde die genetische Variabilität von 40 *H. bacteriophora* Wildtyp-Stämmen, sowie Inzucht- und Hybridlinien unterschiedlicher geographischer Herkunft genutzt. Die Stämme wurden auf Toleranz gegenüber oxidativem Stress und Langlebigkeit bei 25 und 7°C getestet. Eine hohe Variabilität wurde zwischen den Stämmen ermittelt, die eine gute Vorrausetzung für die Selektion und Hybridisierung zur genetischen Optimierung darstellt. Die mittlere Zeitspanne, die 50% der DL Population überlebten (MTS_{50}), rangierte zwischen 21–57 Tagen in Ringer-Lösung (Kontrolle) und von 3–22 Tagen unter oxidativem Stress (70 mM H_2O_2) bei 25°C. Bei 7°C rangierte der MTS_{50} zwischen 5–25 Tagen unter oxidativem Stress und zwischen 38–39 Tagen ohne oxidativen Stress (Kontrolle). Nur eine geringer Anstieg (3 Tage) wurde festgestellt, wenn die Nematoden bei 7°C unter oxidativem Stress gelagert wurden. Unter

oxidativen Stress trat somit keine Verlängerung der Lebensdauer ein, wie sie für die Lagerung ohne Stress zu beobachten war. Die Toleranz gegenüber oxidativem Stress war stark positiv korreliert mit der Langlebigkeit der DL. Aus diesem Grund kann die oxidative Stresstoleranz als Vorhersage für die Langlebigkeit verwendet werden, wodurch ein Selektionsprozess in sehr viel kürzer durchgeführt werden kann. Darüber hinaus ist die Heritabilität des Überlebens in Ringerlösung niedriger (h^2 = 0,23 bei 7°C, 0,11 bei 25°C) als die Heritabilität für oxidative Stresstoleranz (h^2 = 0,93 at 7°C; 0,91 bei 25°C).

Für die Suche nach toleranteren und langlebigen Stämmen wurde Hybridisierung und Mutagenese verwendet. Ein Hybridstamm wurde aus zwei für oxidativen Stress toleranten Stämmen (AU1 und HU2) produziert. Der resultierende Hybridstamm AU1xHU2 überlebte 2,6 länger unter oxidativem Stress (79 mM H_2O_2) bzw. 18 Tage ohne Stress. Als eine zusätzliche Quelle der Variabilität wurde eine EMS-Mutanten Pool (MOX-I) mit höher Langlebigkeit produziert. Eine daraus gezüchtete Inzuchtlinie (MOX-IL6) überlebte 5,8 Tage länger als die nicht mutagenisierte Ausgangslinie (IL3) unter oxidativem Stress (79 mM H_2O_2), bzw. 28,4 Tage länger ohne Stress. Selbst das Kreuzungsprodukt aus MOX-IL6 und IL3 überlebte 2,5 länger als die Spenderlinie, bzw. 18.5 Tage ohne Stress. Durch eine erhöhte Toleranz für oxidativen Stress hervorgerufene mögliche „trade-off“ Effekte wurden anhand der Virulenz und des Vermehrungspotentials überprüft. Dabei wurden keine negativen „trade-off“ Effekte beobachtet. Die mittlere Mortalität der Insekten verursacht durch AU1×HU2 und MOX-IL6 war sogar 10% bzw. 8% höher als bei dem besten Ausgangsstamm. Weitere Untersuchungen sind hinsichtlich der Stabilität anderer nützlicher Eigenschaften notwendig.

Eine vergleichende Transkriptomanalyse wurde während des frühen Stadiums der Induktion von oxidativem Stress (4 h) in zwei Inzuchtlinien mit kontrastierender Stresstoleranz,

HU2-IL1 (stresstolerant) und PT1-IL1 (stresssensitiv**)** durchgeführt, um die zugrundeliegenden genetischen Mechanismen zu untersuchen. Die *de novo* Transkriptomanalyse ergab insgesamt 20.022 Transkripte, von denen 10.290 mit 9.776 verschiedenen UniProt Akzessionen verknüpft werden konnten. Eine größere Fraktion der annotierten Transkripte zeigten Homologien mit parasitischen Nematoden wie *Ancylostoma*, anstelle des freilebenden Nematoden *Caenorhabditis elegans*. Die Anzahl hochregulierter Transkripte (log2 FC ≥ 2.0) in der stresssensitiven Line PT1-IL1 (630) waren unter oxidativem Stress höher als in der stresstoleranten Linie HU2-IL1 (461). Die Anzahl herunterregulierter Transkripte war in beiden Linien hoch, aber die stresssensitive Linie hatte auch hier eine höhere Anzahl (5.207) als die stresstolerante Linie (1.844). Es scheint, dass die stresssensitive Linie im Gegensatz zur toleranten Linie nicht in der Lage ist, vitale biologische Prozesse aufrecht zu erhalten. Anderseits scheint die Herunterregulierung ebenfalls ein kritischer Faktor für das Überleben zu sein, wie man an der toleranten Linie sehen kann. Beide Linien aktivieren die gleichen biologischen Prozesse, die an der Detoxifikation von sogenannten ROS (reactive oxygen species) beteiligt sind. Die MACE Profile wurde durch RT-qPCR aufgrund der signifikanten positive Korrelation (R = 0.66) bestätigt.

Die Resultate dieser Studie werden die Selektion von Prädiktorengenen für die Langlebigkeit bei EPN erleichtern. Weitere Analysen werden eine Identifizierung von molekularen Markern erlauben und die Züchtung von verbesserten Linien erleichtern. Nematodenstämme mit einer verlängerten Lebensdauer können eine längere Persistenz im Boden nach Applikation ermöglichen und so die Tür für größere Märkte wie für die Bekämpfung des Westlichen Maiswurzelbohrers *D. virgifera virgifera* öffnen.

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Annex 1: Publication 1

Phenotyping dauer juvenile oxidative stress tolerance, longevity and persistence within wild type and inbred lines of the entomopathogenic nematode *Heterorhabditis bacteriophora*

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Summary – The commercial use of the entomopathogenic nematode *Heterorhabditis bacteriophora* as a biocontrol agent against noxious insects is limited due to its relatively short shelf-life. Longevity of dauer juveniles (DJ) during storage and in transit to end users is considerably restricted by environmental stresses. As a derivative stress triggered by environmental factors, oxidative stress causes a strong internal metabolic imbalance leading to lifespan reduction. In this study, the relation between DJ oxidative stress tolerance and longevity in *H. bacteriophora* was investigated at 25 and 7°C. A strong and significant correlation between DJ oxidative stress tolerance and longevity during storage in Ringer's solution ($R = 0.802$ at 7°C; $R = 0.604$ at 25°C) was recorded. Phenotyping of these traits was performed for 40 *H. bacteriophora* wild type strain and inbred line collections. At 25°C, the mean time survived in Ringer's by 50% of the DJ (MTS_{50}) ranged from 21 to 57 days, whereas under oxidative stress, survival was from 3 to 22 days. At 7°C, a maximum MTS_{50} of 94 days was assessed when DJ were stored in Ringer's, while the maximum MTS_{50} was only 25 days with oxidative stress induction. The heritability of DJ tolerance to oxidative stress, determined by using homozygous inbred lines, is high ($h^2 > 0.9$), an indication of a high probability for successful selective breeding. In a subset of preselected *H. bacteriophora* inbred lines, DJ oxidative stress tolerance correlated with the DJ survival (persistence) after application to sand ($R = 0.719$). The study provides fundamental data required for a genetic breeding programme to produce hybrids with improved stress tolerance and prolonged shelf-life and soil persistence.

Keywords – dauer survival, environmental stress, heritability, nematode shelf-life, reactive oxygen species, storage temperature.

Entomopathogenic nematodes (EPN) of the species *Heterorhabditidis bacteriophora* (Rhabditidomorpha: Strongyloidea) are biological control agents for a variety of economically important pests (Grewal *et al.*, 2005). The species is known to be ubiquitous, to have a short generation time and to exist in the soil only as the free-living third-stage infective juvenile also known as the dauer juvenile (DJ) (Johnigk & Ehlers, 1999a; Hominick, 2002).

Heterorhabditis bacteriorophora is also used to control larvae of the western corn rootworm (WCR, *Diabrotica virgifera virgifera*) (*e.g.*, Toepfer *et al.*, 2010). DJ are applied in the field at sowing time during spring (March/April) when *Diabrotica* eggs are still in the state of diapause. The nematodes then must survive and stay virulent for a period of at least 4-6 weeks until the first emergence of the host larvae (Pilz *et al.*, 2014). Currently, 2×10^9 DJ ha^{-1} are applied. In order to compete with the use of synthetic insecticides, a reduction of the application density is required. To maintain a high level of control, DJ persistence and virulence will be improved by the application of a breeding programme.

This study investigated longevity in 40 wild type strains, inbred and hybrid lines of *H. bacteriophora* of different geographical origin. Several studies reported that long-lived dauer mutants of *Caenorhabditis elegans*

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exhibited increased resistance to oxidative stress and antioxidant genes were upregulated (Larsen, 1993; Honda & Honda, 1999; Johnson *et al.*, 2002; Yanase *et al.*, 2002; Murphy *et al.*, 2003). Consequently, we checked whether longevity and persistence correlate with oxidative stress resistance of *H. bacteriophora* DJ. The influence of temperature on the longevity of *H. bacteriophora* DJ was also assessed.

Materials and methods

Nematode strains and culture

Forty *H. bacteriophora* strains (wild type, inbred and hybrid lines) of different geographic origin (see Mukuka *et al.*, 2010a, b) were used and cultured in Nematode Growth Gelrite (NGG) (Muschiol & Transpurger, 2007; Addis *et al.*, 2016). Solid NGG was prepared containing 2.5 g l^{-1} peptone from casein, 3 g l^{-1} NaCl and 3 g l^{-1} Gellan Gum (Gelrite; SERVA). Autoclaved solutions of 1 ml of a 147 g l^{-1} of $CaCl_2 \cdot 2H_2O$, 1 ml of 246.6 g l^{-1} $MgSO_4 \cdot 7H_2O$ and 25 ml of 136 g l^{-1} KH_2PO_4 buffer at pH 6 were added after the autoclaved medium had cooled down to 55°C. Lastly, 1 ml of filter-sterilised 5 g l^{-1} cholesterol suspended in ethanol (>99%) was added. Semi-solid NGG was prepared in a similar manner but containing 2 g l^{-1} peptone from casein and 1.5 g l^{-1} gelrite. The prepared solid NGG was dispersed to Petri plates (diam. 5 cm) and the semi-solid NGG was stored at 4°C for bacterial cell dilution.

The symbiotic bacterium, *Photorhabdus luminescens*, obtained from the stock culture of e-nema GmbH, was cultured in a standard nematode liquid medium (NLM) (Ehlers *et al.*, 1998) and incubated at 180 rpm (rotation diam. 4 cm) at 25°C for 24 h. To harvest cells the culture was centrifuged at 3939 *g* for 10 min at 4°C and washed once as described by Addis *et al.* (2016). Bacterial density was counted by diluting 100 μl of the bacterial suspension with 900 μl of 10% Histofix (to retard the movement of the bacterial cells; Carl Roth), using a Thoma chamber (0.01 mm depth; Poly Optic) to determine the required cell density. Prepared solid NGG plates were coated with 2 ml of semi-solid NGG containing 20×10^9 cells ml^{-1} and incubated at 25°C for 24 h.

About 1000 DJ were inoculated on each NGG plate (diam. 5 cm) and incubated at 25°C for 6 days. Hermaphrodites that had developed to *endotokia matricida* (Johnigk & Ehlers, 1999b) containing DJ were separated from other developmental life stages through sieving (250 μm mesh), then washed and incubated at 25°C from 24 h in Ringer's solution. Emerging DJ were then harvested by separation from the maternal carcass using the above-mentioned mesh size. The collected DJ were cleaned using a cotton trap method, placed in culture flasks at 4°C and used for all assays within 1 week.

Assessment of oxidative stress tolerance

Survival of DJ was characterised by exposure to oxidative stress at 7 and 25°C with three replications and three independent experimental trials. A total of 1000 DJ in 400 μl Ringer's solution were transferred to 24-well plates followed by addition of 11 μl of 70 mM H_2O_2 to each well. DJ were examined by taking an aliquot of 10 μl every day for the first 5 days and at intervals of 3-5 days until all experimental individuals were dead. Scoring of mortality was done by addition of 0.35 M ascorbic acid (Carl Roth) and by prodding with a platinum wire. Dead and live nematodes were counted to obtain the mean time at which 50% of the population (MTS_{50}) and 10% of the population had survived (MTS_{10}). An identical set-up was carried out for treatments without oxidative stress induction and only Ringer's solution was added to each well.

Persistence of dauer juveniles in sand

Heterorhabditis bacteriophora strains with high tolerance to oxidative stress (AU1, IT6, IT4, DE6, S-VI-MM8, IL7, HU1 and HU2) and low tolerance to oxidative stress (PT1, PT4, S-HT1, S-DT1, TR4, DE2, XX2 and EN01) were used for persistence assays (Fig. 1). Survival after 1, 2, 3 and 5 weeks of incubation was assessed. Petri dishes (diam. 15 cm) were filled with sand (150 g per plate with 10% moisture). A total of 6400 DJ were inoculated in each plate and stored at 25°C. To prevent drying and maintain at least 10% sand moisture, the plates were placed inside a humidity box containing tap water in the bottom part and then sealed properly. At the end of the incubation time, the nematodes were extracted from the entire 150 g sand using a simple stirring and decanting process (Cobb, 1918). Living DJ were then counted in the solution. DJ extracted at day 1 were taken as the reference population. The percentage DJ survival after 1, 2, 3 and 5 weeks of incubation was determined and the MTS_{50} and MTS_{10} were calculated.

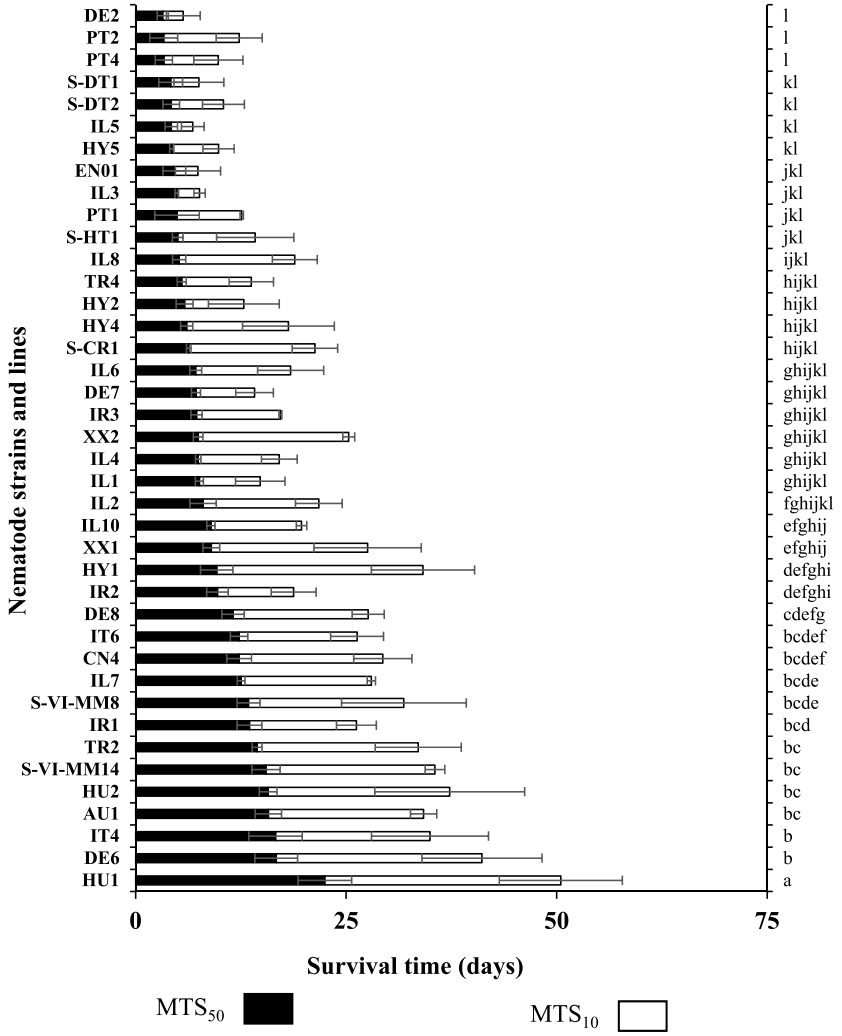

Fig. 1. Mean time survived by 50% (MTS_{50}) and 10% (MTS_{10}) of 40 *Heterorhabditis bacteriophora* strain and inbred line dauer juvenile (DJ) populations after oxidative stress (70 mM H_2O_2) induction and storage at 25°C. Error bars indicate standard deviation of three independent trials with three replications. Different letters above error bars indicate significant differences of the MTS_{50} between nematode strains and lines (Tukey's HSD test at $P \leqslant 0.0001$).

To determine whether temperature has an effect on DJ survival, selected inbred and hybrid lines (PT1-IL1, HU2-IL1, HUPT and sel-HUPT) were exposed to 25 and 15°C in the sand persistence assay. Nematodes with densities of 400 and 6400 were inoculated. After 3 weeks, the percentage DJ survival was calculated as described above.

Statistical analyses

The MTS_{50} and the MTS_{10} values were calculated by fitting the original data (percentage of active DJ and survival time) to the cumulative normal distribution (Strauch *et al.*, 2004; Ehlers *et al.*, 2005; Mukuka *et al.*, 2010a, b). This was determined by reducing the Chi^2 through comparison of the original data and the theoretical normal distribution. The mean and standard deviation of the fitted normal distribution was used to estimate the median and standard deviation of the oxidative stress tolerance and longevity in the given nematode population. MTS_{50} was used to compare and identify strains and lines while MTS_{10} (10% quantile of the normal distribution) was likewise assessed to

determine the most tolerant population which would be used for future crosses and selection experiments. Probit analysis (Finney, 1952) was used to determine MTS_{50} and MTS_{10} for persistence test in sand bio-assays. Prior to analysis of variance (ANOVA), percentages were arcsine-transformed. Treatment differences were analysed using one way ANOVA and differences between treatments were compared using posthoc Tukey's HSD test at $P \leqslant 0.05$. The relationships between oxidative stress, longevity, the influence of storage temperature and persistence were assessed using the Pearson correlation test at $P \leqslant 0.05$.

Heritability is defined as the proportion of phenotypic variance due to additive effects of genes. The heritability was calculated following the descriptions of Johnigk *et al.* (2002) and Falconer & Mackay (1996): $h^2 = \sigma_g^2/(\sigma_{g+}^2\sigma_e^2)$, where σ_g^2 is the genetically caused variance and σ_e^2 is the environmentally caused variance. Inbred lines were used to determine the heritability of oxidative stress tolerance and DJ longevity. It can be presumed that the inbred lines originating from the strain PSH were homozygous (Johnigk *et al.*, 2002; Strauch *et al.*, 2004). Thus, the observed variability of the oxidative stress tolerance in one inbred line is an effect of environmentally caused variability (σ_e^2). Differences in the mean tolerance between the inbred lines, on the other hand, can be assumed as genetically caused variance (σ_g^2). The variability within and between the inbred lines was determined based on the mean of the standard deviation in each inbred line and the standard deviation of the mean values, respectively.

Results

Oxidative stress tolerance among *H. bacteriophora* strains

Longevity of DJ was assessed with oxidative stress induction (70 mM H_2O_2 at 25 and 7°C) in a set of 40 *H. bacteriophora* strains and lines, and MTS_{50} and MTS_{10} values were determined. At 25°C, a high variability and significant differences in survival time between strains ($F = 36.62$; df = 39, 80; $P < 0.0001$) were recorded (Fig. 1). At 25°C, strain HU1 had the highest mean MTS_{50} (22 ± 3.2 days) followed by DE6 and IT4 with MTS_{50} of 17 ± 2.5 and 17 ± 3.2 days, respectively. By contrast, the strains PT4, PT2 and DE2 had the lowest mean MTS_{50} of 3 ± 1, 3 ± 1.7 and 3 ± 0.7 days, respectively. The MTS_{10} ranged from 6 to 50 days and also exhibited significant differences ($F = 12.163$; df = 39, 80; $P < 0.0001$). In agreement with the MTS_{50} values, we determined the highest MTS_{10} for the HU1 strain (50 ± 7.3 days) followed by DE6 (41 ± 7.1 days), while strain DE2 had the lowest MTS_{10} (6 ± 2 days) followed by the inbred line IL5 (7 ± 2.7 days).

Survival of DJ stored at 7°C under oxidative stress exhibited a relative increase of MTS_{50} ranging from 5 to 25 days. Significant differences ($F = 47.47$; df = 39, 80; $P < 0.0001$) were likewise observed among the treatments (Fig. 2). For this temperature regime, the strains IT4 (25 ± 2 days) and DE6 (19 ± 1.7 days) had the highest mean MTS_{50}, while the strains S-DT1, IL3 and HY4 had the lowest MTS_{50} of 5 ± 0.2, 6 ± 1.1 and 6 ± 0.5 days, respectively. The highest MTS_{10} was determined for IT4 (53 ± 9 days) followed by DE6, IL7 and IT6 with comparable respective means of 35 ± 4, 35 ± 2.1 and 35 ± 4 days. Concerning short survival time, the line IL3 had the lowest MTS_{10} (11 ± 1.6 days).

DJ longevity of *H. bacteriophora* stored in Ringer's solution

DJ longevity was also assessed without oxidative stress induction by storing DJ in Ringer's solution only. Significant differences between the MTS_{50} among strains and lines were recorded for storage at 25°C ($F = 11.17$; df = 39, 80; $P < 0.0001$) (Fig. 3) and 7°C ($F = 14.3$; df = 39, 80; $P < 0.0001$) (Fig. 4). At 25°C, the highest mean MTS_{50} was obtained with strain IT4 (57 ± 3.7 days) followed by S-VI-MM14 and AU1 with respective MTS_{50} of 51 ± 5.5 and 51 ± 5.4 days. Strain TR4, by contrast, had the lowest mean MTS_{50} of 21 ± 1.5 days followed by S-DT2 (25 ± 2.3 days), EN01 and S-HT1 (27 ± 2.7 and 27 ± 1.3 days). An average DJ longevity extension during storage at 7°C ranged from 17 to 37 days. The highest mean MTS_{50} (94 ± 12.5 days) was recorded for IT4 followed by IT6 (91 ± 5 days). S-DT1 had the lowest MTS_{50} of 38 ± 5.4 days.

Significant differences between nematode strains and lines were also observed for the mean MTS_{10} with storage at 25°C ($F = 12.163$; df = 39, 80; $P < 0.0001$) (Fig. 3) and 7°C ($F = 6.05$; df = 39, 80; $P < 0.0001$) (Fig. 4). When stored at 25°C, strain S-VI-MM14 had the highest mean MTS_{10} (104 ± 7.5 days) followed by DE6 (95 ± 13.8 days) and IT4 (93 ± 11 days), while TR4 and EN01 had the lowest MTS_{10} of 34 ± 1.8 and 41 ± 4.9 days, respectively. An average extension of the DJ longevity of 55 days was recorded for storage at 7°C. The strain CN4

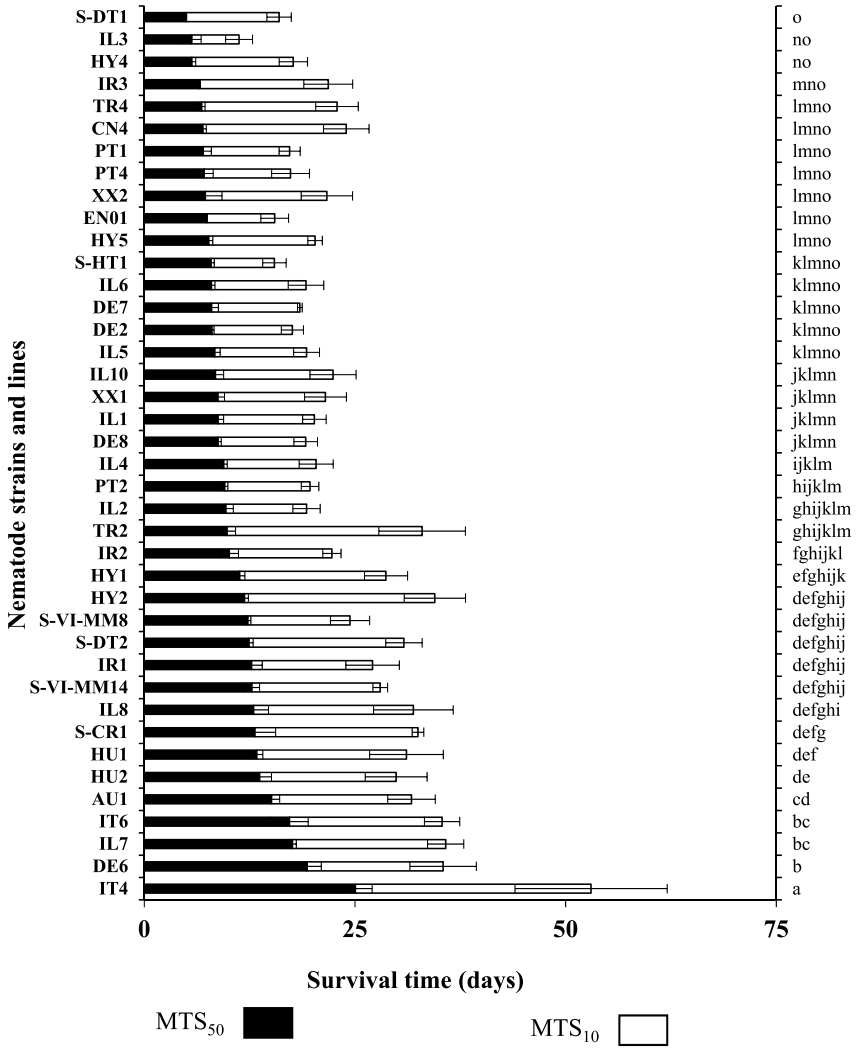

Fig. 2. Mean time survived by 50% (MTS_{50}) and 10% (MTS_{10}) of 40 *Heterorhabditis bacteriophora* strain and inbred line dauer juvenile (DJ) populations after oxidative stress (70 mM H_2O_2) induction and storage at 7°C. Error bars indicate standard deviation of three independent trials with three replications. Different letters above error bars indicate significant differences of the MTS_{50} between nematode strains and lines (Tukey's HSD test at $P \leqslant 0.0001$).

had the highest mean MTS_{10} of 157 $\pm$ 3.6 days, while strain PT1 had the shortest 89 $\pm$ 8.2 days.

Heritability of DJ longevity and oxidative stress tolerance

The heritability was determined for DJ longevity and oxidative stress tolerance of *H. bacteriophora* using inbred lines. For both storage regimes, 25 and 7°C, high heritability values of $h^2 = 0.91$ and 0.93, respectively, were recorded for oxidative stress tolerance indicating that 91-93% of the individual differences that were observed in oxidative stress tolerance can be attributed to the genotype. For DJ longevity stored in Ringer's solution, low heritability values of 0.11 at 25°C and 0.23 at 7°C were calculated, implying that genetic variance in longevity is overruled by environmental factors.

Survival estimation of nematodes in the sand

In the sand bio-assay at 25°C, nematode survival decreased with incubation time. Approximately 30-45%

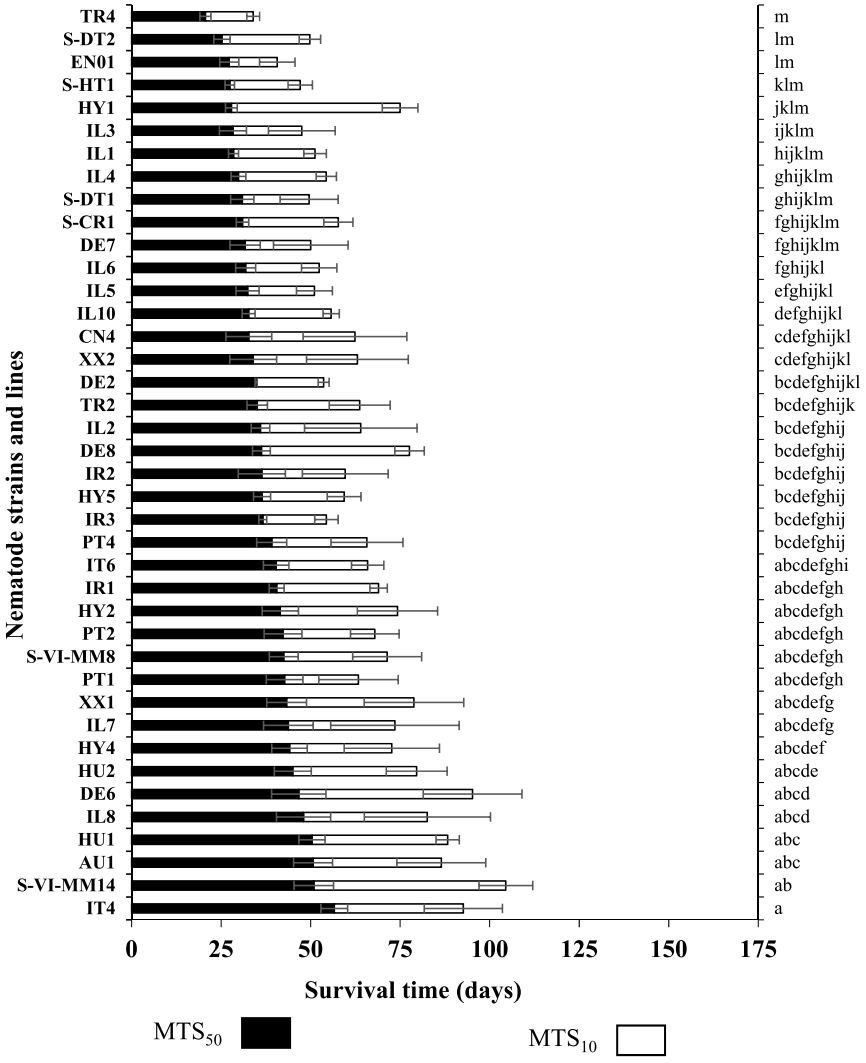

Fig. 3. Mean time survived by 50% (MTS_{50}) and 10% (MTS_{10}) of 40 *Heterorhabditis bacteriophora* strain and inbred line dauer juvenile (DJ) populations stored in Ringer's solution at 25°C. Error bars indicate standard deviation of three independent trials with three replications. Different letters above error bars indicate significant differences of the MTS_{50} between nematode strains and lines (Tukey's HSD test at $P \leqslant 0.0001$).

of the nematodes could not be recovered after 1 day of incubation. Based on the number of nematodes recovered on day 1 (as a reference population), the percentage nematode survival ranged from 12 to 65% after 1 week, 9 to 42% after 2 weeks and 1 to 10% after 3 weeks of incubation, while low survival was found after 5 weeks.

The MTS_{50} values were significantly different between strains and lines ($F = 14.33$; df = 15, 32; $P < 0.0001$) (Fig. 5). The highest persistence was obtained with S-VI-MM8 (11 ± 1.8 days), followed by HU1 (10 ± 1.2 days) and DE6 (9 ± 0.8 days). TR4 had the lowest persistence with MTS_{50} of 5 ± 0.7 days. The MTS_{10} values ranged from 10 to 20 days ($F = 23.37$; df = 15, 32; $P < 0.0001$).

Nematode persistence in sand was also assessed at 15°C. Survival after 3 weeks of incubation at 25°C was lower than at 15°C for all strains and lines tested (Fig. 6). Storage at 15°C resulted in an average increase of percentage survival ranging from 14 to 21% and

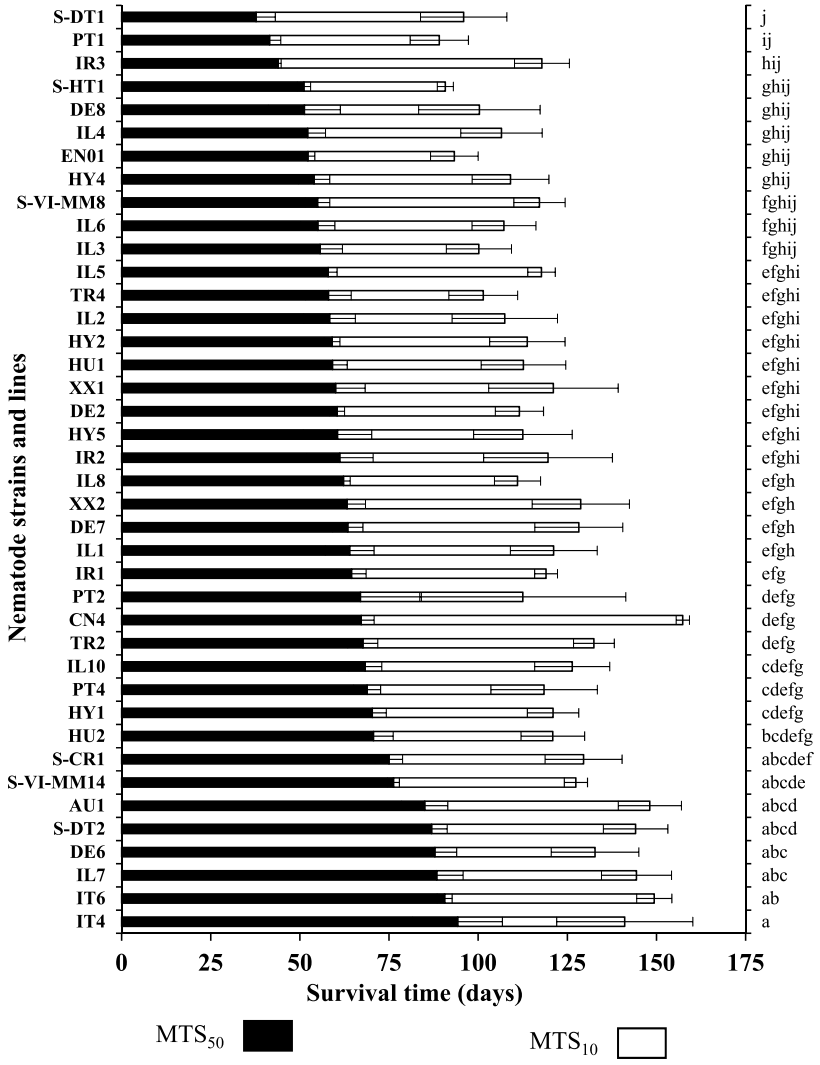

Fig. 4. Mean time survived by 50% (MTS_{50}) and 10% (MTS_{10}) of 40 *Heterorhabditis bacteriophora* strain and inbred line dauer juvenile (DJ) populations stored in Ringer's solution at 7°C. Error bars indicate SD of three independent trials with three replications. Different letters above error bars indicate significant differences (Tukey's HSD test at $P \leqslant 0.0001$).

22 to 27% for inoculation densities of 6400 and 400 DJ, respectively. The highest percentage of extracted DJ was recorded for strain sel-HUPT (69 ± 3.4% at 15°C and 42 ± 11.8% at 25°C) for 400 DJ. Starting from 6400 DJ inoculum density, strain HUPT had the highest DJ survival of 81 ± 4 at 15°C and 67 ± 1.6 at 25°C. With 400 DJ inoculum density, the lowest numbers of extracted DJ at 15°C and 25°C were recorded for strain PT1-IL1 (49 ± 2.1%) and HUPT (27 ± 6.4%), respectively.

Correlation between oxidative stress tolerance, longevity, storage temperature assay and persistence

The relationship between survival time (MTS_{50} and MTS_{10}) of *H. bacteriophora* populations with oxidative stress and without oxidative stress induction at different temperatures (25 and 7°C) and persistence in sand under laboratory conditions was determined. A significant positive correlation between DJ survival with oxidative stress

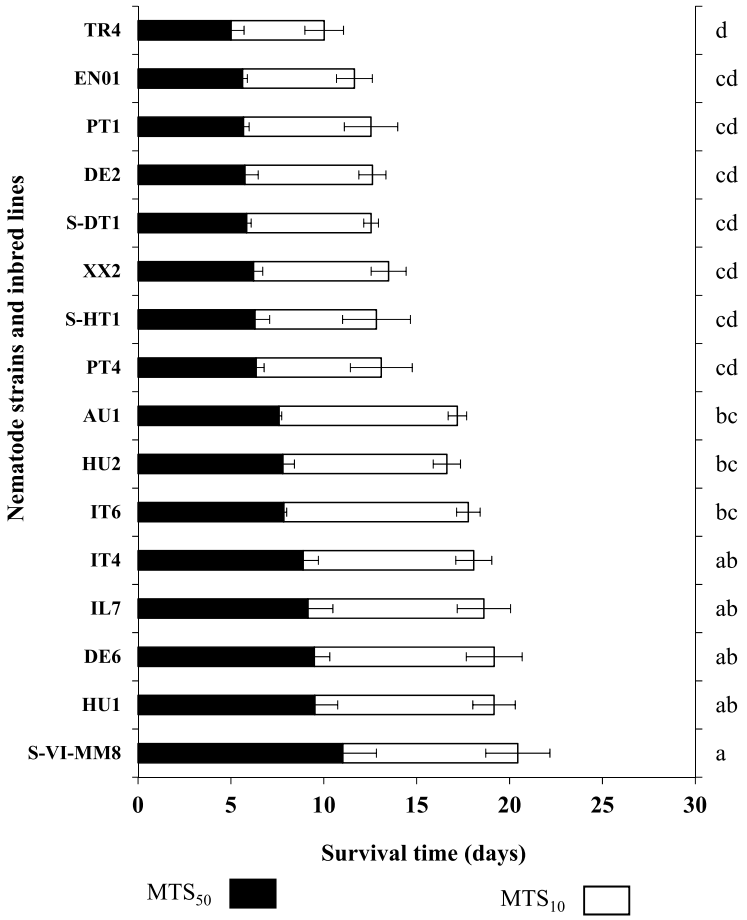

Fig. 5. Mean time survived by 50% (MTS_{50}) and 10% (MTS_{10}) of selected *Heterorhabditis bacteriophora* dauer juvenile (DJ) populations in a sand bio-assay stored at 25°C. Error bars indicate SD of two independent trials with three replications. Different letters above error bars indicate significant differences (Tukey's HSD test at $P \leqslant 0.0001$).

(70 mM H_2O_2) and survival when stored in Ringer's solution (without oxidative stress) was recorded for 25°C ($MTS_{50,}$ $R = 0.604$; $P < 0.0001$ and $MTS_{10,}$ $R = 0.758$, $P < 0.0001$) (Fig. 7A). Correspondingly, a significant positive correlation was determined for both MTS_{50} ($R = 0.802$; $P < 0.0001$) and MTS_{10} ($R = 0.652$; $P < 0.0001$) when stored at 7°C (Fig. 7B). Between two storage temperatures, a positive correlation held under oxidative stress conditions for both MTS_{50} ($R = 0.653$; $P < 0.0001$) and MTS_{10} ($R = 0.585$; $P < 0.0001$) was recorded (Fig. 8A). A strong correlation between survival of nematodes under oxidative stress and persistence of nematodes in sand for MTS_{50} ($R = 0.826$; $P = 0.001$) (Fig. 8B) and MTS_{10} ($R = 0.815$; $P < 0.0001$) was also found (Fig. 8B).

Discussion

The commercialisation of *H. bacteriophora* as a biological control agent is restricted due to its limited survival resulting in a short shelf-life for nematode products. DJ produced in bioreactors can be stored in water for a maximum of 6 weeks before formulation and an additional 6 weeks before application in the field. Desiccation and heat stresses can influence EPN longevity in formulated products during storage and transport (Strauch *et al.*, 2004; Mukuka *et al.*, 2010a, b). It is important to understand other factors involved in longevity that can be used to identify EPN strains with superior survival traits (Grewal *et al.*, 2011). In the model organism *C. elegans*, several studies demonstrated a relationship between

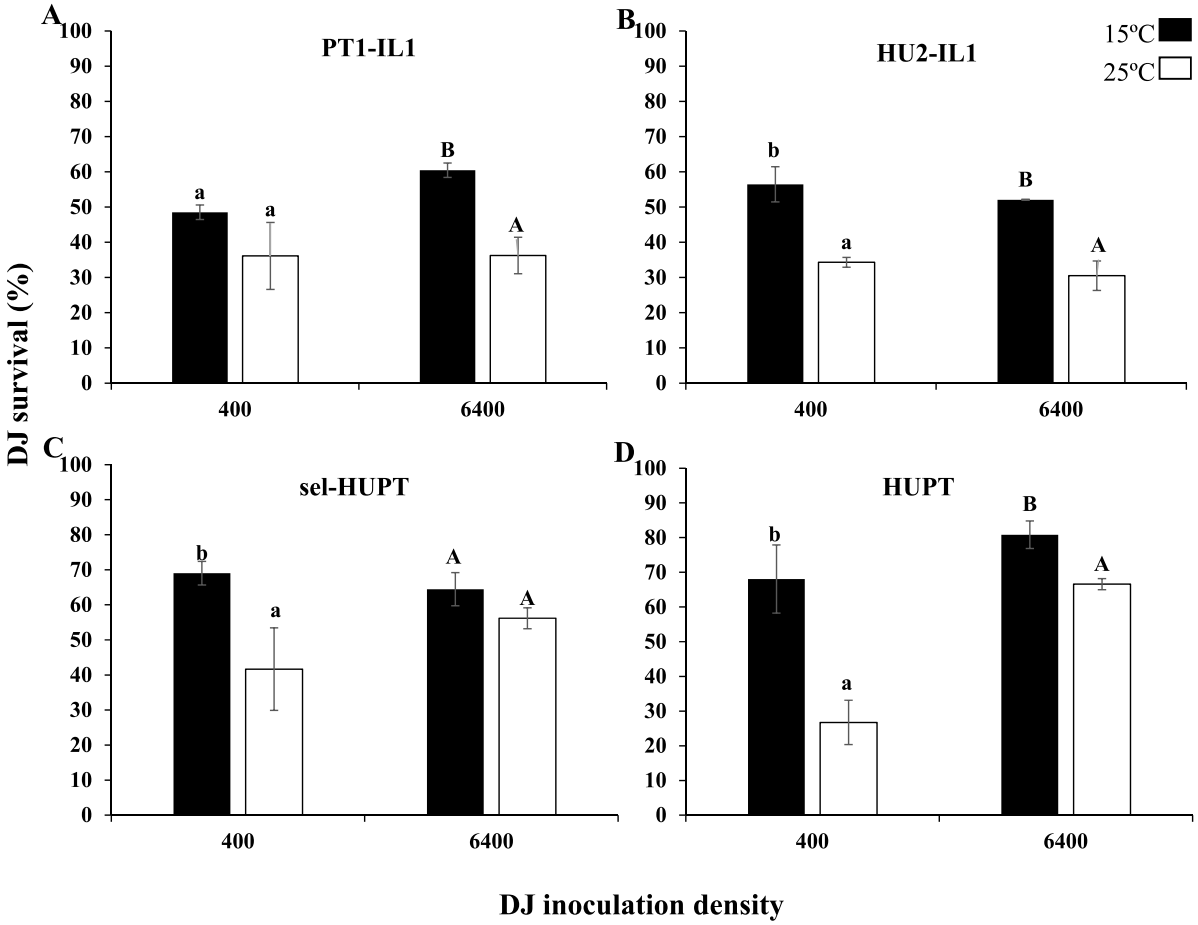

Fig. 6. Percentage dauer juvenile (DJ) survival of *Heterorhabditis bacteriophora* inbred lines (A, B) and crosses (C, D) after three weeks of incubation at 15°C and 25°C in the sand bio-assay. Nematodes were inoculated at 400 DJ and 6400 DJ per plate. Error bars indicates standard deviation of three replicates. Different letters on the error bars (lower case for '400'; upper case for '6400') indicate significant differences in DJ survival (Tukey's HSD test at $P < 0.05$).

longevity and stress resistance against *e.g.*, oxidative, heat and UV stress (Johnson *et al.*, 2001; Mukarami & Johnson, 2001; Munoz & Riddle, 2003). An earlier report on *H. bacteriophora* presented a strong association between DJ longevity and hypoxia (Grewal *et al.*, 2002). However, the influence of oxidative stress on the survival of *H. bacteriophora* DJ has not been studied until now. To correlate oxidative stress tolerance and longevity, a reliable and reproducible bio-assay was optimised by exposing the DJ of the commercial strain EN01 to hydrogen peroxide (H_2O_2) concentrations ranging from 0 to 100 mM. Subsequently, the 70 mM H_2O_2 concentration was selected as the optimal oxidative stress treatment for characterisation studies (data not shown). As a strong correlation was recorded between DJ longevity in Ringer's solution and oxidative stress resistance, we have a reliable bio-assay to determine longevity within a much shorter time. The correlation with results obtained on persistence in sand is also quite high and, consequently, the oxidative stress assay can be used to examine the potential of strains and lines to persist in the field after application.

In the present work, screening of 40 *H. bacteriophora* strains and lines has shown a large variability in the survival time with and without oxidative stress treatment at 25 and 7°C. Regardless of storage temperature, all DJ exposed to oxidative stress had substantially shorter MTS_{50} and MTS_{10} than those without stress exposure. Our screening underpins the observations of Mukuka *et al.* (2010a, b) who found high variability of *H. bacteriophora* strains and lines collected from different geograph-

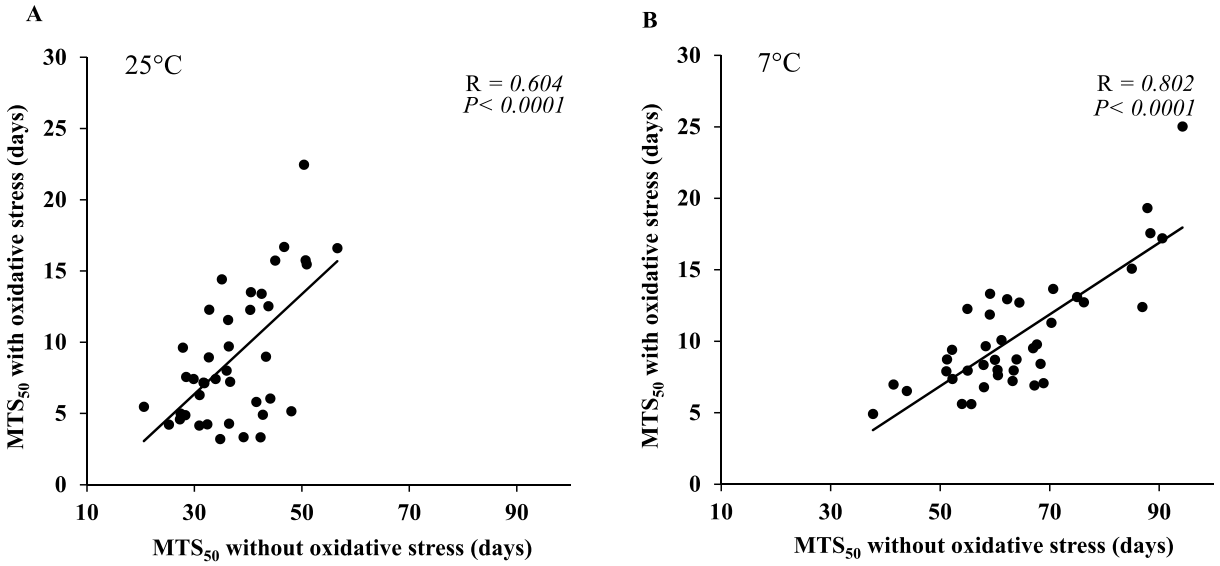

Fig. 7. Correlation between the mean time survived by 50 % (MTS_{50}) of *Heterorhabditis bacteriophora* dauer juvenile (DJ) populations exposed to oxidative stress (70 mM H_2O_2) and after storage in Ringer's solution and stored at (A) 25°C and (B) 7°C. Each data point represents an average of three replicates of three different batches (Pearson's coefficient test, $P \leqslant 0.05$).

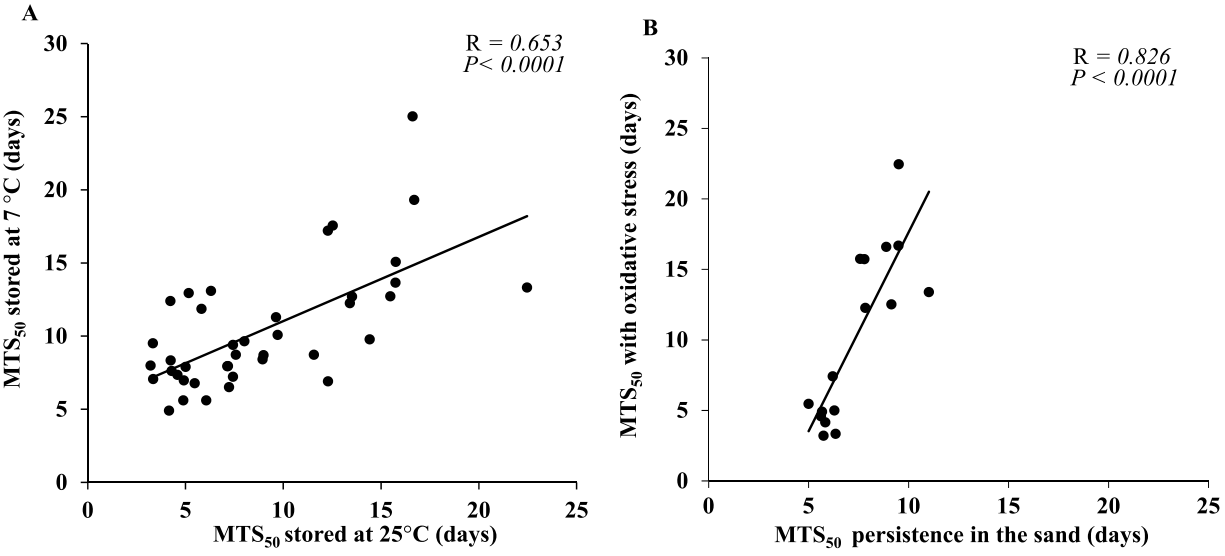

Fig. 8. Correlation between the mean time survived by 50% (MTS_{50}) of *Heterorhabditis bacteriophora* dauer juvenile (DJ) populations (A) stored at 25°C and 7°C under oxidative stress and (B) between MTS_{50} in oxidative stress assay and persistence in the sand assay. Each data point represents an average of three replicates of three different batches (for A) and two batches (for B) (Pearson's coefficient test, $P \leqslant 0.05$).

ical regions in their tolerance to stress conditions like heat and desiccation. We used mostly strains (wild types) and lines (hybrid and inbred) that had previously been characterised for heat- and desiccation-stress tolerance, as well as for virulence to hosts insects like *Melolontha melolontha* (Berner *et al.*, 2001; Strauch *et al.*, 2004; Mukuka *et al.*, 2010a, b; Anbesse *et al.*, 2013b). We particularly observed that most strains and lines were not tolerant to the same extent to all tested stress conditions. As an example, the wild type strain AU1 was highly tolerant to

oxidative stress but less tolerant to desiccation and heat stresses. The strain IT4 had the highest tolerance to oxidative stress but a very low tolerance to heat stress. A selected line, S-V1-MM14, which had been used to infect *M. melolontha* for 14 cycles, was highly tolerant to oxidative stress and moderately tolerant to heat and desiccation stresses. Thus, different responses to several stress conditions can have a different genetic background. Although some strains and lines (*i.e.*, TR4, DE2 and EN01) were consistently the least tolerant, none was observed to have acquired high tolerance to all stresses. Only the wild type strain IT4 was found to be tolerant to almost all stress conditions except for heat stress. The results on the phenotyping can now be used to choose strains/lines for genetic selection and subsequent cross-breeding programmes to produce hybrids with improved beneficial traits.

The influence of hydrogen peroxide as one of the major reactive oxygen species (ROS) in the longevity and ageing of *C. elegans* (Kumsta *et al.*, 2011; Thamsen *et al.*, 2011; Aan *et al.*, 2013) and *C. remanei* (Reynolds & Phillips, 2013) has previously been investigated. Hydrogen peroxide acts as secondary messenger, a molecule that reacts with metal ions to form harmful hydroxyl radicals (·OH) activating different signalling pathways and transcription factors that regulate gene expression and cell-cycle processes (Crichton *et al.*, 2002; Ślesak *et al.*, 2007). This molecule has been also reported to either lengthen or shorten the lifespan of the nematodes in a concentration-dependent manner. Lifespan increase is due to stress-induced hormesis following exposure to low doses of oxidative stress, whilst decrease is due to excessive production of ROS and over oxidation causing inactivation of an antioxidant enzyme, peroxiredoxin (Cypser & Johnson, 2002; Rhee *et al.*, 2005). We observed that high levels of oxidative stress reduce the mean survival time (MTS_{50}) of *H. bacteriophora* DJ. How hydrogen peroxide influences longevity of *H. bacteriophora* DJ and the underlying genetic mechanisms still need to be investigated further. Nevertheless, the present study confirms that oxidative stress is one of the major determinants of lifespan in *H. bacteriophora* DJ. Knoefler *et al.* (2012) observed similar results by exposing the *C. elegans* N2 strain and mutants like the short-living *daf-16* and the long-living *daf-2* to 6 mM of H_2O_2. The authors correlated the changes of oxidation level with lifespan and found that short-lived *daf-16* and long-lived *daf-2* mutants exhibited higher and lesser ROS, respectively, compared to the N2 strain. Thamsen (2011) also observed that lifespan of *C. elegans* was influenced by H_2O_2. In congruence with these observations, it has been reported that knock-out of H_2O_2 detoxifying enzymes causes accelerated aging in *C. elegans* (Harman, 1956; Finkel & Holbrook, 2000; Petriv & Rachubinski, 2004; Olahova *et al.*, 2008). Whether similar genetic mechanisms are found in *H. bacteriophora* is being investigated.

Nematode survival during storage and transport and in the field is also influenced by temperature. Previous research works have demonstrated the importance of temperature during storage and activity at low temperature for *H. bacteriophora* and *Steinernema feltiae* for the control of insect pests (Strauch *et al.*, 2000; Ehlers *et al.*, 2005; Nimkingrat *et al.*, 2013). In this study, DJ stored at 7°C survived longer than those stored at 25°C. The highest MTS_{50} of 94 days was recorded for storage at 7°C, whilst the highest for those stored at 25°C was 57 days. This MTS_{50} surpasses the average longevity of the commercial strain (EN01). Strauch *et al.* (2000) measured the percentage mean survival of a hybrid strain of *H. bacteriophora* stored in a Ringer's solution containing about 20 000 DJ ml^{-1} in cell culture flask with temperature ranged from 5 to 25°C. The authors observed a higher survival of DJ stored at 7.5°C (*ca* 150 days) than at 25°C (51 days) supporting our results, although we determined the MTS_{50} and $MTS_{10.}$ When formulated in clay, *H. bacteriophora* survived for only 2 weeks when stored at 25°C, whereas in wettable powder, strain EN01 has an estimated shelf-life of 0.5-1.0 month when stored at 22-25°C or even longer (2-3 months) when stored at 2-10°C (Grewal & Peters, 2005). These results can be attributed to the DJ physiological activity, metabolic rate and lipid reserve utilisation (Grewal & Peters, 2005; Sursuluk & Ehlers, 2008; Andaló *et al.*, 2011). Temperature plays a crucial role in the survival of nematodes. Under warmer conditions, for instance, energy reserves deplete much faster (Jung, 1996; Georgis & Kaya, 1998). While storage at low temperature extended the longevity of *H. bacteriophora* DJ, it did not significantly enhance the tolerance to oxidative stress. We subjected the DJ of the different strains and inbred lines to 70 mM H_2O_2 for about 70 days at 7 and 25°C until no surviving DJ were detected. We only determined a difference of 3 days between MTS_{50} under oxidative stress for both temperatures, which demonstrates that oxidative stress can strongly override other environmental factors.

As a biological control agent used to control important pests, like the western corn rootworm (*D. virgifera virgifera*), DJ must persist without losing infectivity for a period of 4-6 weeks in the field. Eight *H. bacteriophora* strains with the highest MTS_{50} under oxidative stress

conditions were compared against eight with the lowest MTS_{50} for persistence in sand for a period of 5 weeks at 25°C. The immediate decline in DJ density within the first day after application can be attributed to the inefficiency of extraction methods and has also been reported by other authors (*e.g.*, Fan & Hominick, 1991). The low survival in the sand is presumably an effect of several environmental factors, such as temperature, soil moisture content, soil type and finally the potential of the DJ to use the energy reserves effectively (Glazer, 2002; Pilz *et al.*, 2014).

Despite the low survival in the sand, we detected a strong correlation on DJ survival between the oxidative stress assay and persistence sand bio-assay, implying that oxidative stress can be used as a predictor for DJ persistence as well. Those selected strains and lines that have high and low tolerance to oxidative stress also had similar survival in the sand bio-assay. We also elucidated the effect of the temperature on the DJ persistence. Higher percentages of DJ survival (52-81%) were observed at 15°C compared to 25°C (31-67%). This observation agrees with the report of Kurtz *et al.* (2007). According to these authors, the persistence of EPNs could be higher in *D. v. virgifera*-infested areas with colder springs and summers, where soil temperatures remain below 20°C at depths of 15-20 cm. In colder seasons, DJ of *H. bacteriophora* can survive over 42 days in maize fields overlapping with the period of the larval development of *D. v. virgifera* (Kurtz *et al.*, 2007; Pilz *et al.*, 2014). DJ survival and persistence were found to be also influenced by lipid reserves (Lee & Atkinson, 1976; Hass *et al.*, 2002; Fitters & Griffin, 2006). Lipid, particularly neutral lipid, serves as the main DJ energy reserve constituting about 32-49% of the DJ body weight (Selvan *et al.*, 1993b; Lewis *et al.*, 1995; Menti *et al.*, 2003). Studies have demonstrated that depletion of lipid reserves resulted in a decline of activity as well as infectivity to host insects in most EPN species (Lewis *et al.*, 1995; Patel *et al.*, 1997). Although we did not assess the lipid reserves in this present study, it would be interesting to quantify and determine the optimal amount of energy reserve (lipid content) required by EPN to move and infect their target insects.

To improve the longevity and persistence traits through genetic selection is a major task that can be supported by data on oxidative stress tolerance of the breeding products. Besides a high variability between available strains, the major parameter that influences the success of genetic selection is heritability (Strauch *et al.*, 2004). Reynold & Patrick (2003) found that oxidative stress resistance of *C. remanei* is a heritable trait ($h^2 = 0.95$ for female, 0.75 for male). The oxidative stress resistance was assessed by exposing the 1-day-old virgin adults into 3.5 mM of H_2O_2. The same authors determined a remarkable low heritability for the lifespan using inbred lines ($h^2 = 0.08$ for female, 0.06 for male and 0.06 for parental offspring). Parallel to these results, we also determined a lower heritability for longevity ($h^2 = 0.11$ for storage at 25°C and 0.23 at 7°C), whereas it was very high for MTS_{50} under oxidative stress. Brooks & Johnson (1991) also noted that the heritability of longevity using recombinant inbred strains of *C. elegans* ranged from 0.05 to 0.36. In this study, longevity of the DJ was assessed for a period of 150 days. The prolonged storage could presumably have resulted in high levels of environmental variance, hence low heritability estimates. Earlier reports have demonstrated that longevity, as a life-history trait that is more related to fitness, is expected to have lower heritability than physiological, morphological and behavioural traits (metric traits; Gustafsson, 1986; Charlesworth, 1987; Falconer, 1989; Hartl & Clark, 1989; Price & Schluter, 1991). This is deduced in line with Fisher's fundamental theorem, which means that populations are near evolutionary equilibrium and genetic variance in total fitness is low (Fisher, 1930; Wright, 1931; Price, 1972; Charlesworth, 1987). Price & Schluter (1991) developed the causal relationship between metric traits and life-history traits to show that a life-history trait is anticipated to have a low heritability whether or not the population is at equilibrium because of the influence of environmental variations. What is generally interesting in this investigation is that oxidative stress tolerance (physiological and metric trait) had high heritability ($h^2 = 0.91$ for storage at 25°C and 0.93 at 7°C) suggesting that oxidative stress tolerance is determined mainly by the genotype. The high heritability readily permits selective breeding (Johnson & Wood, 1982). In addition, a positive correlation between oxidative stress tolerance and DJ longevity confirms that oxidative stress predicts longevity. The major advantage of the use of oxidative stress as predictor is the relatively short testing period. Hence, our results will allow us to carry out selection with a high probability of obtaining a long-living strain under control conditions (low environmental variance), which is difficult to achieve without the stress induction assay.

To improve strains and lines further through selective breeding, only the best 10% of a population should be included in a cross-breeding and selection programme and,

hence, the MTS_{10} is an excellent indicator to determine at which oxidative tolerance level the selection pressure should be set. The highest MTS_{10}, for example, was 53 days after exposure to oxidative stress indicating possible progress, which could be utilised in the future for inclusion in a breeding programme. Under all experimental conditions the commercial line (EN01) was one of the least tolerant to oxidative stress and has a short mean time survival compared to other strains and lines. This evaluation provides information for selecting potential strains to generate hybrids, inbred lines and consecutively perform genetic selection, which was found to be successful for desiccation and heat tolerance (Strauch *et al.*, 2004; Ehlers *et al.*, 2005; Mukuka *et al.*, 2010a, b; Anbesse *et al.*, 2013a, b). A hybrid strain with a higher tolerance to stress, longer shelf-life and persistence will enhance the quality of commercial nematode products and be successfully utilised in larger scale markets such as for the control of *D. virgifera virgifera* in maize. Thus, the results are worthwhile for the commercialisation of *H. bacteriophora* in integrated pest management.

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Annex 2: Publication 2

Applying inbreeding, hybridization and mutagenesis to improve oxidative stress tolerance and longevity of the entomopathogenic nematode *Heterorhabditis bacteriophora*

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Applying inbreeding, hybridization and mutagenesis to improve oxidative stress tolerance and longevity of the entomopathogenic nematode *Heterorhabditis bacteriophora*

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ABSTRACT

Poor shelf-life and sensitivity to environmental stress of entomopathogenic nematodes (EPNs) are traits, which deserve attention for improvement. Recently, a strong positive correlation between oxidative stress tolerance and longevity of *Heterorhabditis bacteriophora* dauer juveniles (DJs) has been reported. In this study, the improvement of *H. bacteriophora* DJ longevity was achieved by hybridization and mutagenesis. A hybrid pool deriving from two oxidative stress tolerant and long-living parental strains was generated. This hybrid AU1 × HU2 survived 2.6 days and 18 days longer than its best parent under oxidative stress and control conditions, respectively. In addition to the natural genetic variability, an EMS-mutant pool (M-OXI) with high longevity was generated and one of the derived mutagenized inbred lines (MOX-IL6) survived 5.8 days and 28.4 days longer than its donor line (IL3) under oxidative stress and control conditions, respectively. A genetic cross between the mutagenized inbred line and its donor line (MOX-IL × IL3) still survived 2.5 days and 18.5 days longer than the donor line under oxidative stress and control conditions, respectively. Concerning virulence and reproductive potential, trade-off effects were not observed as a result of hybridization and mutagenesis. These results underline the potential of classical genetic approaches for trait improvement in the nematode *H. bacteriophora*.

1. Introduction

The entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* Poinar (Rhabditomorpha: Strongyloidea) is an effective biological control agent (BCA) against insect pests attacking several economically important crops (Grewal et al., 2005). This species has a symbiotic association with the bacterium *Photorhabdus luminescens* (Enterobacteriales: Enterobacteriaceae), which is carried by the nematodes in the free-living, developmentally arrested dauer juvenile stage (DJ). *Heterorhabditis bacteriophora* is used commercially mainly for the control of several curculionid weevil larvae in soft fruit and ornamentals (Long et al., 2000), white grubs in turf (Koppenhöfer et al., 2015) and against the invasive maize pest *Diabrotica virgifera virgifera* (Western Corn Rootworm) (Toepfer et al., 2005).

Despite several advantages offered by this species, its use in larger scale agriculture is restricted by a limited shelf-life and high sensitivity to environmental stress conditions (Strauch et al., 2004; Mukuka et al., 2010b). DJs of *H. bacteriophora* are industrially produced in monoxenic liquid culture in bioreactors and are stored in liquid suspension at high densities for maximum 6 weeks after production (Ehlers, 2001; Strauch et al., 2000). For storage and transport to the end-users, DJs need to be formulated under moderate desiccation (Grewal and Peters, 2005). In addition to the stress to which EPNs are exposed during production and transport, post-application stress factors such as UV-radiation, high temperatures, drought and oxidative stress decrease DJ survival before they can actively find and kill their target insect hosts (Grewal et al., 2002; Strauch et al., 2004; Ehlers et al., 2005; Mukuka et al., 2010a; Sumaya et al., 2017).

The influence of stress factors on DJ survival during storage, formulation, transportation and field application has been broadly assessed (Grewal, 2000; Strauch et al., 2000, 2004). These stress factors can result in a strong physiological internal oxidative stress with severe detrimental consequences for the nematode. Oxidative stress is caused by overproduction of reactive oxygen species (ROS) due to a metabolic

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disequilibrium (Bokoch and Diebold, 2002). The state of imbalance between ROS production and the capability of a biological system to detoxify the reactive intermediates can generate adverse modifications to cell components like lipids, proteins and DNA (Valko et al., 2006; Birben et al., 2012; Dias et al., 2013). ROS such as superoxide anion radicals ($O_2^{\cdot -}$), nitric oxide (NO), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) are produced as by-products of cellular metabolism, mostly in the mitochondria (Riess et al., 2004; Barrera, 2012). Earlier reports in nematodes indicate that pre-adaptation of nematode populations with low doses of oxidative stress increases tolerance to other stresses by inducing defense genes (Butov et al., 2001; Cypser et al., 2006). On the other hand, medium and high levels of ROS, may cause senescence and cell death, respectively (Kim and Sun, 2007; Schieber and Chandel, 2014). In the model nematode *Caenorhabditis elegans,* a correlation between oxidative stress tolerance and lifespan has been reported. Johnson et al. (2000) found that the so-called *C. elegans* "Age" mutants were associated with oxidative stress along with heat- and UV stress resistance. Other studies reported that long-lived dauer mutants had increased resistance to oxidative stress as well as upregulated antioxidant genes (Larsen, 1993; Honda and Honda, 1999; Johnson et al., 2002; Yanase et al., 2002; Murphy et al., 2003). In *H. bacteriophora*, Grewal et al. (2002) found a strong correlation of DJ longevity with heat, UV-radiation and hypoxia tolerance. In addition to this finding, we recently found a strong correlation between oxidative stress tolerance and DJ longevity in *H. bacteriophora*. We then used oxidative stress tolerance assays as selector parameter for DJ longevity. The same strains that expressed increased oxidative stress resistance also elicited a high longevity and soil persistence. Thus, the phenotypic variability in oxidative stress tolerance of *H. bacteriophora* materials can be exploited for a breeding approach targeting improvement of DJ longevity, shelf-life and persistence (Sumaya et al., 2017).

Classical genetics have often been applied in beneficial trait improvement programs using the natural phenotypic and genetic variability as starting points. Cross-breeding and successive genetic selection have evidenced a significant heat and desiccation stress tolerance improvement in *H. bacteriophora* and *Steinernema carpocapsae*. For instance, Shapiro et al. (1997) first enhanced heat tolerance in *H. bacteriophora* through hybridization and Shapiro-Ilan et al. (2005) showed that hybridization with 2 strains of *S. carpocapsae* using controlled crosses can lead to improvement of beneficial traits. In addition, Ehlers et al. (2005) increased the mean tolerated temperature of a *H. bacteriophora* hybrid strain to 39.2 °C. Subsequently, Mukuka et al. (2010a, 2010c) screened 60 *H. bacteriophora* strains from different geographical locations and reported an increase to 44.0 °C after eleven selection steps. Concerning desiccation stress tolerance, Strauch et al. (2004) and Mukuka et al. (2010b) achieved a reduction in mean tolerated water potential (a_w-value) by exposing DJs to polyethylene glycol (PEG600) at different concentrations and carrying out selection steps. As trait improvement was lost when nematodes were propagated *in vivo*, Anbesse et al. (2012, 2013a, 2013b) managed to stabilize the aforementioned heat and desiccation tolerance in *H. bacteriophora* by selecting tolerant homozygous inbred lines in monoxenic liquid cultures.

Success through genetic selection is dependent on the heritability (h^2) of a certain beneficial trait (Hartl and Clark, 1997). We used the term heritability to refer to the proportion of the genetically caused variance in the phenotypic variation of a population (Falconer, 1984; Strauch et al., 2004). The heritability was calculated following the descriptions of Johnigk et al. (2002) and Falconer and Mackay (1996) ($h^2 = \sigma_g^2/(\sigma_g^2 + \sigma_e^2)$; where σ_g^2 is the genetically caused variance and σ_e^2 is the environmentally caused variance). Sumaya et al. (2017) reported a heritability of the oxidative stress tolerance of $h^2 > 0.9$ in *H. bacteriophora*. Thus it can be assumed that the probability of success of a selective breeding for improved oxidative stress tolerance in *H. bacteriophora* is high.

Induction of mutation can strongly complement the use of natural genetic variability. Mutagenesis with ethyl methanesulfonate (EMS) is a widely used, non-transgenic, non-targeted method that causes G/C to A/T transitions, generating stop codons and synonymous substitutions (Flibotte et al., 2010). In *C. elegans*, mutagenesis screens using EMS have led to substantial insights into the genetic control of animal development and physiology by generating a broad plethora of phenotypes (Lehrbach et al., 2017). In line with longevity enhancement on either the full life cycle or of the DJ stage in *C. elegans*, several attempts were made to isolate strains with increased lifespan via EMS mutagenesis. For instance, Klass (1983) started from the temperature-sensitive spermatogenesis defective line [*fer-15(b26ts)* II] and mutagenized it to screen the F_2 population for individuals with longer lifespan. Through EMS mutagenesis, the function of important longevity genes such as *daf-2*, *daf-16*, *age-1, fer-1, unc-31* have been determined (Friedman and Johnson, 1988; Kenyon et al., 1993; Larsen et al., 1995; Johnson et al., 2002; Garigan et al., 2002).

There are few previous reports on the use of EMS mutagenesis in *Heterorhabditis* species. For instance, dumpy mutants (designated *Hdpy-* and *Rhdpy l*) were isolated from the F_2 generation of a mutagenized population in *H. bacteriophora*. Phenotypically, the *dumpy* mutants were shorter than the wild- type. The mutation was completely recessive with 100% penetrance and uniform expressivity (Zioni et al., 1992; Rahimi et al., 1993). *Heterorhabditis megidis* strain UK211 was also mutagenized using EMS to develop desiccation tolerant mutants (O'Leary and Burnell, 1997). The use of EMS to generate long-living *H. bacteriophora* lines may therefore be a promising approach.

The objective of this study was to improve tolerance to oxidative stress and longevity of *H. bacteriophora* DJs. For this, a genetic cross of two oxidative stress tolerant and long-living strains were produced. Complementarily, mutagenesis was carried out as an alternative source to natural genetic variation. The phenotypes of hybrid and mutagenized strains were then screened and compared through a series of physiological assays with the parental and donor lines. Fitness of both hybrid and mutant lines were compared to the commercial line (EN01) by assessment of virulence and reproductive potential on the insect hosts *Tenebrio molitor* and *Galleria mellonella*, respectively.

2. Materials and methods

2.1. Nematode strains

Two highly oxidative stress tolerant and long-living strains of *H. bacteriophora* (AU1 and HU2) were used as parental strains in this study (Sumaya et al., 2017). For the mutagenesis experiment, the inbred line (IL3), deriving from the commercial line of *H. bacteriophora* (EN01), was used as the donor line (Fig. 1). Strain EN01 was included for comparison.

2.2. Monoxenic liquid culture

The symbiotic bacterium *P. luminescens* was isolated and cultured in Nematode Liquid Medium (NLM) and glycerol stocks were kept at −80 °C according to Ehlers et al. (1998). All studies were done with the same bacterial strain HB1.3. Monoxenic cultures were produced through egg sterilization (Lunau et al., 1993). Mass production was carried out in Erlenmeyer flasks with NLM according to Ehlers et al. (1998). *Photorhabdus luminescens* were incubated in NLM at 180 rpm and 25 °C for 24 h followed by the inoculation of approximately 4000 DJs ml^{-1}. After 15 days post DJ inoculation, the cultures were stored at 4 °C on a shaker (70 rpm, rotation diam. 2 cm). For experimental use, nematodes were harvested by centrifugation at 1500*g* for 4 min. and washed twice by adding Ringer's solution (9.0 g NaCl, 0.42 g KCl, 0.37 g $CaCl_2 \times 2\ H_2O$, 0.2 g $NaHCO_3$ dissolved in 1 liter of distilled water) to the precipitate followed by centrifugation. As reported by Strauch et al. (1994), *H. bacteriophora* can only reproduce through self-fertilization of hermaphordites in liquid culture. Therefore, after seven generations of subculturing, the nematode population consists of inbred

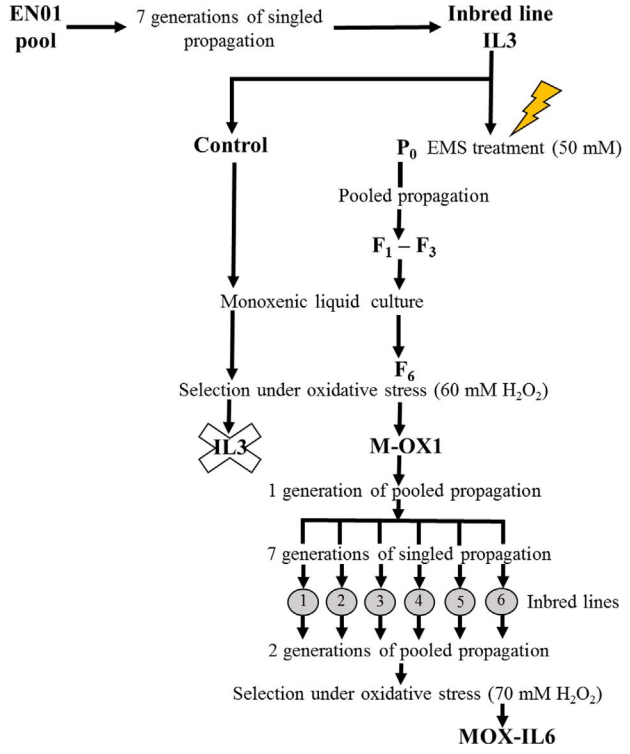

Fig. 1. Flowchart describing mutagenesis step, origin of strains and production of inbred lines by pooled or singled *in vitro* propagation or monoxenic liquid culture and expose to oxidative stress bioassay.

lines that were expected to be 95% homozygous (Hartl and Clark, 1997).

2.3. Pooled culture on solid NGG medium to produce inbred populations

To augment a small population of inbred lines, nematodes were produced on solid culture on Nematode Growth Gelrite (NGG) medium in Petri dishes (diam. 5 cm) containing 2.5 g l^{-1} peptone from casein, 3 g l^{-1} NaCl and 3 g l^{-1} Gellan Gum (Gelrite; SERVA, Heidelberg, Germany). After this sterile medium had cooled down to 55 °C, autoclaved solutions of 1 ml of 147 g l^{-1} $CaCl_2 \times 2\ H_2O$, 1 ml of 246.6 g l^{-1} $MgSO_4 \times 7\ H_2O$ and 25 ml of 136 g l^{-1} KH_2PO_4 buffer at a pH 6 were added. This medium was then supplemented with 1 ml of a filter-sterilized 5 g l^{-1} cholesterol solution in ethanol (> 99%). Then one ml of a 20×10^9 cells ml^{-1} suspension of *P. luminescens* was added to each plate. Therefore, bacteria were cultured in NLM for 24 h at 180 rpm (rotation diam. 4 cm) and cells were harvested by centrifugation at 4000*g* for 10 min. at 4 °C. The supernatant was discarded and bacterial pellets were re-suspended and washed with sterilized K-medium (containing 3.1 g l^{-1} NaCl and 2.4 g l^{-1} KCl). Centrifugation was repeated at the same speed for 5 min. Bacterial pellets were re-suspended using sterile semi-solid NGG (same as solid, but containing only 2 g l^{-1} peptone from casein and 1.5 g l^{-1} gelrite). Cell density was determined by counting samples in a Thoma chamber (0.01 mm depth; Poly Optic). After cooling of the NGG medium, it was seeded with 1 ml of 20×10^9 cells ml^{-1} of *P. luminescens* suspended in semi-solid NGG and incubated at 25 °C for 24 h. Thereafter, ~1000 DJs were inoculated into each NGG plate and incubated for 6 days at 25 °C, until DJs had developed to hermaphrodites in the stage of *endotokia matricida* (Johnigk and Ehlers, 1999), indicated by hatching of first stage juveniles (J1) in the uterus (intra-uterine juvenile hatching). These hermaphrodites were washed off from the plates and stored in Ringer's solution until DJs moved out of the maternal carcass. Subsequently, DJs were harvested, pooled and stored in Ringer's solution at 4 °C. Comparable to DJs cultured in liquid media, subculturing following this protocol on solid NGG yields DJs only resulting from self-fertilization and after approximately 7 subcultures the DJ population consists of a pool of different highly homozygous inbred lines. For mass production, they were transferred to monoxenic liquid culture.

2.4. Singled cultures in semi-solid NGG medium to produce inbred lines

In order to produce a single inbred population, single hermaphrodites were propagated each in one cell well of a 12-cell well plate (Sarstedt, Germany) filled with semi-solid NGG containing 20×10^9 cells ml^{-1} of *P. luminescens*. Single hermaphrodites were transferred from 3–4 days-old Petri dish cultures into semi-solid cultures, which were incubated at 25 °C. The hermaphrodites had ceased egg-laying and DJs develop only through *endotokia matricida*. From each hermaphrodite one DJ was then subcultured on semi-solid NGG. When this procedure is repeated for approximately 7 generations, a highly homozygous inbred line is the result (Hartl and Clark, 1997; Bai et al., 2005). One hermaphrodite yields up to 200 (Addis et al., 2016) offspring from *endotokia matricida*. In order to use large amounts of DJs of the resulting inbred line for experiments, the DJs after 7 generations of inbreeding were propagated in solid NGG Petri dish cultures and later in monoxenic liquid culture. This technique yields single, highly homozygous inbred lines.

2.5. Cross-breeding

A cross between AU1 and HU2 was carried out as described by Iraki et al. (2000). Last instar larvae of *G. mellonella* (Lepidoptera: Pyralidae) were infested with 25 DJs/insect. Five days after inoculation at 25 °C, insect cadavers were dissected to pick males and unfertilized fourth stage juvenile (J4) females (Mukuka et al., 2010c). The unfertilized J4 females (they lack eggs in the uterus and copulation plug on the vulva) were identified using a stereomicroscope (Strauch et al., 1994). Crosses were performed on the lid of 12-cell well plates containing a drop of 300 µl of solid NGG coated with a 5 µl of *P. luminescens* at a density of 20×10^9 bacterial cells ml^{-1}. Pairings were placed in a drop using one AU1 unfertilized J4 female and five HU2 males; a total of 15 replicate pairs were prepared for this crossing experiment. The same number of reciprocal crosses (unfertilized J4 female HU2 and male AU1) were carried out as described above. For virginity confirmation test, unfertilized single J4 of each strain were placed in drops without males (n = 5). The plates were incubated at 25 °C for 3–4 days until hermaphrodites had entered into *endotokia matricida*. DJs from gravid hermaphrodites were harvested and stored in Ringer's solution. The success of crosses was confirmed by the absence of progeny in virginity test drops.

To mass-produce hybrid nematodes, the resulting DJ progeny of the crosses (AU1 × HU2) were pooled and further propagated in solid NGG medium for a total of nine generations (F_9) as described in Section 2.3. A total of 72 gravid females were used to produce the next generation. Approximately 1000 gravid females were harvested from the second generation (F_2). Selfing of hermaphrodites was continued until the seventh generation (F_7). DJs collected from F_7 were pooled and propagated until the ninth generation (F_9).

2.6. Assessment of oxidative stress tolerance of the hybrid AU1 × HU2

Oxidative stress tolerance of DJs was assessed in wells of 24-cell well plates containing 1000 DJs in 400 µl of Ringer's solution and adding 12.9 µl of 79 mM H_2O_2 to each well. After incubation at 25 °C in the dark for one day, DJ survival was examined by taking an aliquot of 10 µl in each replicate. Sampling was done every day during the first five days and then at an interval of 3–5 days until all individuals had died. Scoring DJs for dead and alive individuals was done by prodding with a platinum wire. Data were used to calculate the mean time survived (MTS_{50}) by 50% of the DJ population. The MTS_{10}, referred to as

the time at which 10% of the population had survived, was also calculated. A similar set-up was performed for control conditions (i.e. without exposure to oxidative stress) by adding 12.9 µl Ringer's solution to each well. Treatment and control experiments (AU1 × HU2, AU1, HU2 and EN01) were done with three replicates and repeated three times with different nematode production batches.

2.7. Generation of EMS-mutants and mutant inbred lines

The generation of an EMS-mutant line was performed according to Brenner (1974). To obtain the inbred line IL3, the commercial hybrid EN01 was propagated over 7 generations following the protocol described in Section 2.4 and then larger quantities were produced following protocol in Section 2.3. For mutagenesis, approximately 1000 DJs of the inbred line IL3 were propagated on solid NGG medium. Three days after inoculation, early fourth stage juveniles (J4) were harvested on a 250 µm mesh sieve, washed with Ringer's solution and placed into 12 ml Ringer's solution and 64 µl ethyl methanesulfonate (EMS) (Sigma, Germany) to obtain a final concentration of 50 mM EMS (Fig. 1). A second batch of J4 was prepared as described above but not treated with EMS (control). After J4 were exposed for 4 h to EMS they were centrifuged at 500*g*, the supernatant was disposed according to the manufacturer's instruction and the J4 were washed with Ringer's solution. The washing was repeated 4 times and J4 were then placed on solid NGG and incubated at 25 °C in the dark until they reached maturity. Resulting DJ progenies of the first generation (F_1) were harvested from adult hermaphrodites and transferred to NGG medium for production of a second (F_2) and third (F_3) generation according to protocol in Section 2.3. Starting from the third generation (F_3), nematodes were transferred to monoxenic liquid cultures as described in protocol above (Section 2.2). All tools and materials that had been in contact with EMS were decontaminated in 0.1 M sodium hydroxide and 20% w/v sodium thiosulfate by soaking.

2.8. Screening for oxidative stress tolerant EMS-mutants

A total of 11,700 DJs in 3 ml Ringer's solution of each, mutagenized and IL3 donor line, were transferred to 6-cell well plates and 18.7 µl of 9.7 M H_2O_2 was added to each cell well to obtain a final H_2O_2 concentration of 60 mM. Cell well plates were then kept at 25 °C and survival was assessed (Fig. 1). These M-OX1 DJs were first reproduced according to protocol in Section 2.3 for one generation and then propagated by self-fertilization for seven generations (F_7) following the protocol described in Section 2.4 to produce six homozygous inbred lines. To obtain large numbers of DJs, each of the six inbred lines was then propagated for two generations according to protocol in Section 2.3 and then the lines were assessed for oxidative stress tolerance as described above (Section 2.6), but with an increasing H_2O_2 concentration from 60 mM to 70 mM. The most tolerant line out of six lines (MOX–IL6) was used for further experiments.

2.9. Crossing MOX-IL6 and IL3 and oxidative stress tolerance of the hybrid

In order to reduce secondary background mutations possibly inferring with the phenotypic analysis of the oxidative stress tolerance, the mutant inbred line (MOX-IL6) was crossed with the donor line (IL3) as described in Section 2.5. Oxidative stress assays were carried out following the procedure described under Section 2.6 and the tolerance of the cross MOX-IL6 × IL3 was compared with the parental lines MOX-IL6 and IL3. Treatment and control experiments (MOX-IL6 × IL3, MOX-IL6, IL3 and EN01) were replicated three times and the experiments were repeated three times with different nematode production batches.

2.10. Virulence test

The virulence of *H. bacteriophora* strains and lines was assessed using the last instar larvae of mealworms, *T. molitor* (Coleoptera: Tenebrionidae) according to Strauch et al. (2000). Forty *T. molitor* larvae were placed into Petri dishes (diam. 150 mm) with 150 g sand at 10% moisture. A total of 800 DJs (20 DJs per insect larva) in 1 ml Ringer's solution were inoculated in the middle of the plates and incubated at 25 °C. Control plates received 1 ml Ringer's solution. Mortality of mealworms was assessed after seven days. The mortality of larvae was assessed and nematode infectivity was confirmed by checking the bioluminescence of insect cadaver using a Luminometer (Lumat Berthold, Germany) and by dissecting the insect cadaver to confirm presence of nematodes. The experiment was repeated three times with three different batches of nematodes. The commercial strain (EN01) of *H. bacteriophora* was used as a standard control.

2.11. Reproduction potential

The reproductive potential of *H. bacteriophora* strains and lines was assessed using last instars of the Greater Wax Moth, *G. mellonella*, modified after Mukuka et al. (2010d). One hundred DJs of each line/strain were inoculated to a single last instar of *G. mellonella* with an average weight of 150 ± 10 mg in an Eppendorf tube containing moistened filter paper. After 5 days at 25 °C, five infected insect cadavers from a total 8 were randomly picked and kept individually in a White trap (White, 1927) in order to harvest the emerging DJs. The total number of DJs produced was recorded 14 days after infection. Three different batches of nematodes were evaluated with three replications (n = 45; n, represents the total number of *Galleria* infected with each strain/line). The total number of DJs produced per host was determined. For comparison, the commercial EN01 was included in this test.

2.12. Statistical analyses

The mean time at which 50% survived (MTS_{50}) and (MTS_{10}), the time at which 10% of the population survived was evaluated by fitting the original data (percentage of active DJs and survival time) to a cumulative normal distribution (Strauch et al., 2004). This was determined by reducing the Chi^2 through comparison of the original data and the theoretical normal distribution. The mean and standard deviation of the fitted normal distribution was used to estimate the median and standard deviation of the oxidative stress tolerance and longevity in the given nematode population. The MTS_{50} was used to compare and identify strains and lines. The MTS_{10} is an important parameter to be used as selection pressure.

The mortality data from virulence tests were Abbott corrected. The reproduction (DJ offspring) obtained for EN01 was used for comparison for both experiments in the hybrid and mutant lines. Prior to analysis of variance (ANOVA), percentages were arcsine transformed. Treatment differences were analyzed using one way ANOVA and difference between treatments were compared using posthoc Tukey's HSD and Dunnett's tests at $P \leq .05$.

3. Results

3.1. Crossing of oxidative stress tolerant inbred lines

Two *H. bacteriophora* long-living strains (AU1 and HU2) previously characterized to possess high tolerance to oxidative stress were selected as parents for the genetic cross. The cross for AU1 females and HU2 males yielded 10 out of 15 females, which successfully reproduced. In the virginity test, 4 out of 5 females had unfertilized eggs, 1 unfertilized female died during the survey. The reciprocal crosses produced 11 out of 15 fertilized females, whereas all 5 females were unfertilized in the virginity test. All 21 fertilized females developed to *endotokia matricida* and were individually transferred to one well of 12-cell well plates containing NGG medium.

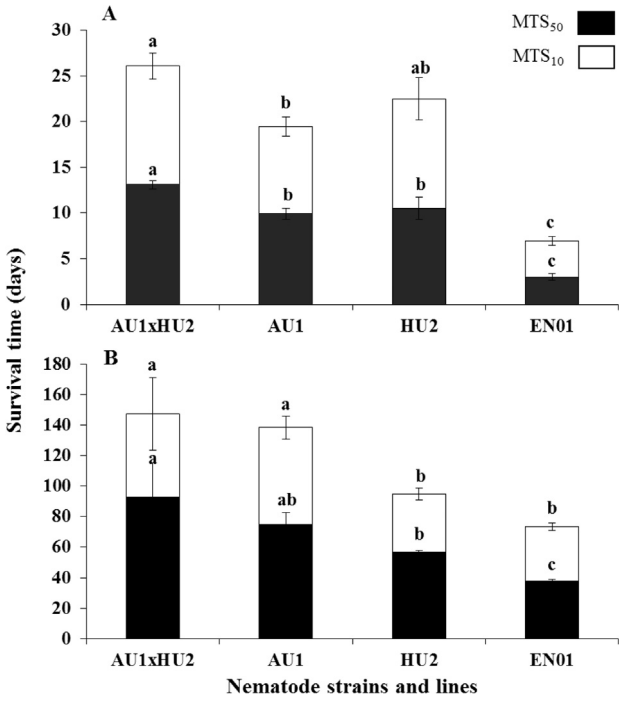

Fig. 2. Mean time survived by 50% (MTS_{50}) and 10% (MTS_{10}) of DJ populations with oxidative stress (79 mM H_2O_2) (A) and in Ringer's solution without oxidative stress (B) in *Heterorhabditis bacteriophora* hybrid, parental lines and a commercial strain (EN01) stored at 25 °C. Error bars indicate standard deviation from three independent trials with three replications. Different letters above error bars indicate significant differences between strains and lines (Tukey's HSD test at $P \leq .0001$).

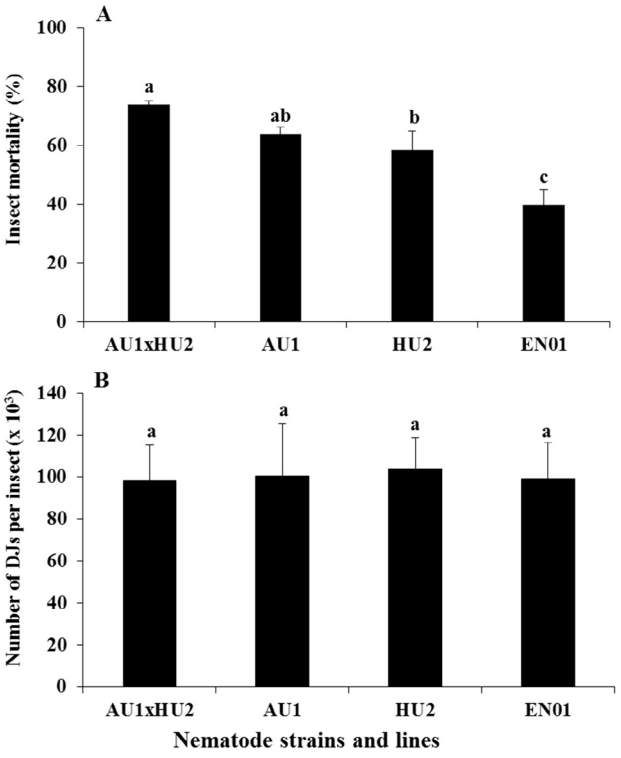

Fig. 3. Mean Abbott corrected mortality of *Heterorhabditis bacteriophora* hybrid and parental strains against *Tenebrio molitor* larvae at 25 °C one week after DJ inoculation. (A) and the mean number of DJ offspring obtained from last instar larvae of *Galleria mellonella* with *H. bacteriophora* hybrid and parental strains after exposure to 100 DJs of *H. bacteriophora* per insect larva and recorded at 14 days after DJ inoculation (B). Error bars indicate standard deviations from three independent trials with three replications. Different letters above error bars indicate significant differences between strains and lines (Tukey's HSD test, $P \leq .0001$).

3.2. Oxidative stress tolerance and longevity of the hybrid line

Stress tolerance of the F_9 hybrid pool, hereafter referred to as AU1 × HU2, and the parental lines was significantly different ($F = 95.42$; $df = 3, 8$; $P < .0001$) (Fig. 2A). The parental strains HU2 and AU1 had a MTS_{50} of 10.5 ± 1.2 days and 9.9 ± 0.6 days, respectively whereas AU1 × HU2 survived 2.6 days longer than the best parent, HU2. The commercial strain (EN01) had the shortest survival with a MTS_{50} of 3 ± 0.3 days. Significant differences were also recorded for the MTS_{10} ranging from 7 to 26 days ($F = 106.63$; $df = 3, 8$; $P < .0001$). The longest MTS_{10} value was recorded from AU1 × HU2 with 26 ± 1.4 days followed by the parental lines HU2 and AU1 with MTS_{10} values of 22.5 ± 2.3 and 19.5 ± 1 days, respectively. The commercial strain (EN01) had the shortest MTS_{10} of 7 ± 0.5 days.

Longevity of DJs when stored in Ringer's solution (control conditions) differed significantly between strains and lines ($F = 27.72$; $df = 3, 8$; $P = .0001$) (Fig. 2B). The MTS_{50} values recorded for AU1 and HU2 were 75 ± 7.6 days and 56.7 ± 3.8 day, respectively. Consistently, AU1 × HU2 survived 36 days longer compared to the parental strains. The MTS_{10} values were significantly different ranging from 73.4 to 147.3 days ($F = 27.3$; $df = 3, 8$; $P = .0001$). The longest survival was observed for AU1 × HU2 with a MTS_{10} of 147.3 ± 23.8 days, while the commercial line EN01 had a shortest MTS_{10} of 73.4 ± 2.2 days.

3.3. Virulence and reproduction of the hybrid

The mean mortality of *T. molitor* caused by *H. bacteriophora* DJs ranged from 39.7 to 73.9% with significant differences observed among nematode strains and lines ($F = 25.28$; $df = 3,8$; $P = .0001$) (Fig. 3A). A mean mortality of 2.2 ± 0.78% was recorded for controls and the mean mortality of treatments was corrected using Abbott's formula. The hybrid AU1 × HU2 had the highest virulence (73.9 ± 1.3%) followed by AU1 (63.9 ± 2.4%) and HU2 (58.3 ± 6.7%), whereas EN01 had the lowest infectivity (39.7 ± 5.1%). For the reproductive potential, no significant differences among all nematode strains and lines were observed ($F = 0.98$; $df = 3,8$; $P = .982$) with mean number of offspring ranging from 99,051 to 104, 067 (Fig. 3B).

3.4. Oxidative stress tolerance of EMS-mutant lines

Approximately 1600 young hermaphrodites survived the EMS treatment and produced approximately 126,000 DJs. This pool was successfully propagated in pooled culture on NGG for three generations and then in monoxenic liquid culture for an additional three generations. An increase in fertility (DJ production) along the first generations of the EMS-population was observed compared with the IL3 donor line. Inbred line IL3 produced 3.5 million and the F_6 mutant pool produced 7.8 million DJs. Both nematode pools were grown in parallel in the same bacterial batches and NGG plates. The total number of DJs produced in EMS-mutant pool was almost double in comparison to the donor line.

The following oxidative stress test killed almost all DJs of the donor line IL3 after 40 days, while less than 1% of the mutagenized DJs survived. This population (M-OX1) was then again successfully propagated in pooled culture on NGG and then for seven generations in singled cultures resulting in 6 inbred lines. Each of the lines were then separately cultured under pooled conditions to produce enough DJ inbred materials for the stress test. During this propagation two lines did not propagate well and were lost. The remaining four lines were compared for oxidative stress tolerance (60 mM H_2O_2) and compared with the donor line IL3 (Fig. 4). Significant differences were recorded between the lines ($F = 6.9$, $df = 4, 10$, $P = .006$) and inbred line MOX1-IL6 turned out to be the most tolerant surviving 9.2 ± 3.5 days (MTS_{50}), which was significantly different to the donor line IL3 ($F = 17.2$,

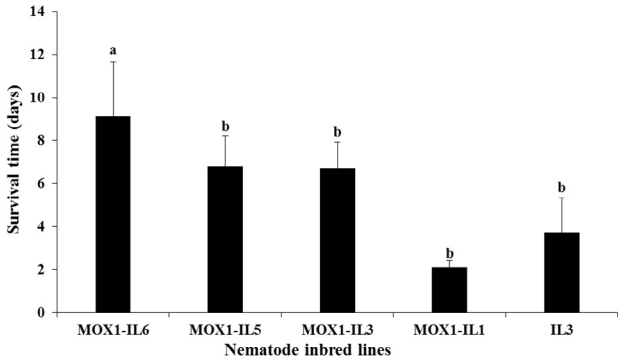

Fig. 4. Mean time survived by 50% (MTS_{50}) DJ populations after exposure to 70 mM H_2O_2 stored at 25 °C. Four single inbred lines were propagated for seven generations without selection pressure in NGG medium before oxidative stress exposure and compared with the parental line IL3. Error bars indicate standard deviations from two independent trials with three replications. Different letters above error bars indicate significant differences (Dunnett's test at $P \leq .0001$).

df = 1, 4, *P* = .014) (Fig. 4) with a MTS_{50} of only 3.7 ± 2.2 days.

3.5. Longevity of the MOX-IL6 × IL3 hybrid

To reduce the possibility of secondary mutations inferring with the phenotypic analysis, a cross between MOX-IL6 and IL3 was performed. This hybrid, hereafter referred to as MOX-IL6 × IL3 was compared to their parental lines. A significant difference in oxidative stress tolerance between the strains and lines ($F = 375.8$; $df = 3, 8$; $P < .0001$) (Fig. 5A) was recorded. MOX-IL6 survived longest with a MTS_{50} of 8.5 ± 0.2 days followed by MOX-IL6 × IL3 with a MTS_{50} of 5.2 ± 0.2 days. IL3 had the lowest survival (MTS_{50} = 2.7 ± 0.2 days) and was found to be comparable with the commercial line EN01

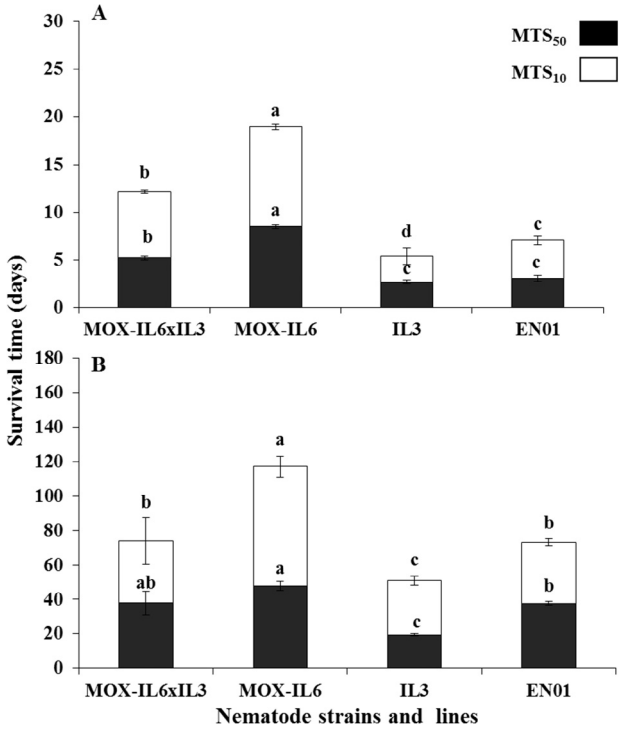

Fig. 5. Mean time survived by 50% (MTS_{50}) and 10% (MTS_{10}) of DJ populations with oxidative stress (79 mM H_2O_2) (A) or in Ringer's solution without oxidative stress (B) in *Heterorhabditis bacteriophora* mutant, donor lines and a commercial strain (EN01) stored at 25 °C. Error bars indicate standard deviations from three independent trials with three replications. Different letters above error bars indicate significant differences between strains and lines (Tukey's HSD test at $P \leq .0001$).

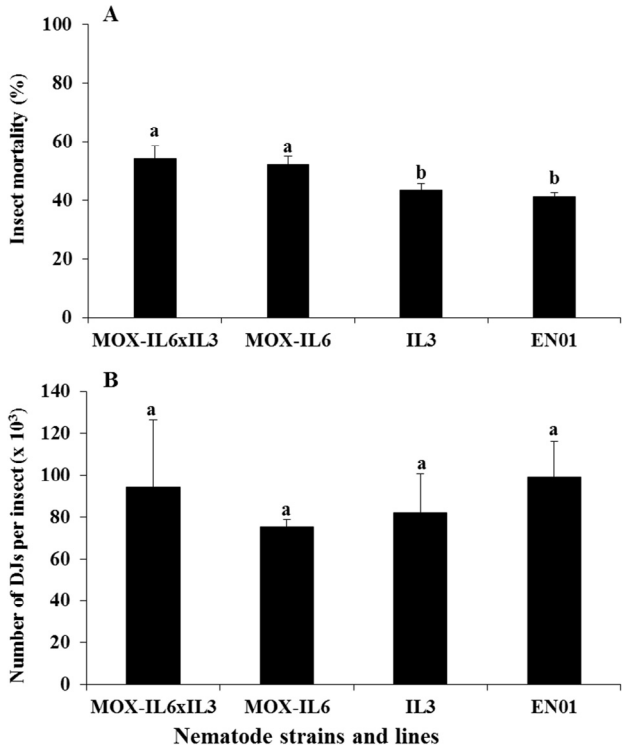

Fig. 6. Mean Abbott corrected mortality of *Heterorhabditis bacteriophora* mutant and donor lines against *Tenebrio molitor* larvae at 25 °C one week after DJ inoculation. (A) and the mean number of DJ offspring obtained from the last instar larvae of *Galleria mellonella* with *H. bacteriophora* mutant and donor lines after exposure to 100 DJs of *H. bacteriophora* per insect larva and recorded at 14 days after DJ inoculation (B). Error bars indicate standard deviations from three independent trials with three replications. Different letters above error bars indicate significant differences between strains and lines (Tukey's HSD test at $P \leq .0001$).

(MTS_{50} = 3 ± 0.3 days). An overall increase of tolerance to oxidative stress for 5.8 days was obtained with mutagenesis. Significant differences were observed between nematode strains and lines also for the MTS_{10}, ranging from 5.4 to 19 days ($F = 399.38$; $df = 3, 8$; $P < .0001$). The nematode with the highest tolerance to oxidative stress was MOX-IL6 with a MTS_{10} of 19 ± 0.3 days, while the least tolerant line was the IL3 with 5.4 ± 0.9.

Longevity of the different strains and lines in water ranged from MTS_{50} = 19.4 to 47.8 days with significant differences between nematode lines ($F = 29.62$; $df = 3, 8$; $P = .0001$) (Fig. 5B). MOX-IL6 survived longer (MTS_{50} = 47.8 ± 2.7 days) followed by MOX-IL6 × IL3 and EN01 with MTS_{50} values of 37.9 ± 6.9 and 37.7 ± 1.3 days, respectively. IL3 had the lowest MTS_{50} of 19 ± 0.6 days. Concerning MTS_{10}, MOX-IL6 had the highest survival with a MTS_{10} of 117.1 ± 6.1 days, while IL3 had the shortest survival with a MTS_{10} = 50.9 ± 2.9 days.

3.6. Virulence and reproduction of the EMS-mutant lines

The mean mortality of *T. molitor* larvae ranged from 41.4 to 54.2% (Fig. 6A). The mean mortality of the controls was 2.5 ± 0.68% and the mean mortality of the treatments was Abbott corrected. Significant differences were recorded between the mutant, donor and commercial lines ($F = 15.72$; $df = 3,8$; $P = .001$). The highest mortality was caused by MOX-IL6 × IL3 with 54.2 ± 4.3% followed by MOX-IL6 with 52.2 ± 2.9%. IL3 and EN01 reached comparable results of 43.3 ± 2.2% and 41.4 ± 1.3%, respectively. The mean number of DJs produced per *G. mellonella* larva ranged from 75,327 to 94,487 (Fig. 6B). No significant differences among all strains and lines were observed ($F = 0.83$; $df = 3,8$; $P = .52$).

4. Discussion

The exploration of robust methods in breeding and genetics could provide a huge potential for improvement of nematode beneficial traits. The screening for oxidative stress tolerance and DJ longevity among a set of 40 *H. bacteriophora* strains and lines from different geographical origin revealed a high variability among strains of this species, providing an excellent foundation for selection or hybridization of superior strains (Sumaya et al., 2017). The mean time survived by 50% (MTS_{50}) of the DJ population ranged from 21 to 57 days in water and between 3 and 22 days under oxidative stress treated with 70 mM H_2O_2 at 25 °C. A strong correlation between oxidative stress tolerance and longevity was reported. This finding implies that oxidative stress can be used as a predictor for DJ longevity, thus permitting a selection process within a shorter testing period, which is practically difficult to obtain without stress inductions, because the heritability of the trait "survival in water" was lower than the heritability of "oxidative stress tolerance". In order to search for even more tolerant and long-living strains, this study was carried out.

Genetic improvement is one of the suggested approaches to increase tolerance of the EPNs to environmental extreme conditions (Gaugler, 1989). Remarkable progress has been achieved for the improvement of heat and desiccation tolerance in *H. bacteriophora* and cold temperature activity in *Steinernema feltiae* through hybridization and successive selection (Strauch et al., 2004; Ehlers et al., 2005; Mukuka et al., 2010a, 2010c; Anbesse et al., 2013a, 2013b; Nimkingrat et al., 2013a, 2013b). In this study, hybridization was also performed to further increase oxidative stress tolerance and prolong DJ longevity. Two most oxidative stress tolerant and long-living strains (AU1 and HU2) (as reported by Sumaya et al., 2017) were used as parents in the genetic cross. The resulting hybrid (AU1 × HU2) was not only more tolerant to oxidative stress but also superior in longevity than its parents. This finding can be likely attributed to the phenotypes associated with increased longevity and tolerance to oxidative stress damage (Larsen, 1993; Martin et al., 1996; Finkel and Holbrook, 2000; Sumaya et al., 2017). An increase in survival of 2.6 days under oxidative stress and 18 days in water was recorded for AU1 × HU2, which might be the result of a heterosis effect. This combination of alleles through cross-breeding provided an increased stress tolerance, which is congruent with the report of Mukuka et al. (2010c), who recorded that hybridization of most heat tolerant strains of *H. bacteriophora* improved the mean tolerated temperature and likewise increased the desiccation tolerance. Similar result was also found with *S. feltaie* by Nimkingrat et al. (2013a, 2013b). A hybrid strain HYB01 was more active at low temperature than the parents. One might hypothesize that even wild-type populations of EPNs are highly inbred already, possible due to small amount of starter cultures when transferred to lab conditions.

As an alternative source of genetic variability, we explored the use of mutagenesis in this study. EMS mutagenesis was applied to then select mutants with improved oxidative stress tolerance and longevity. EMS mutagenesis not only produces loss-of-function mutants but also leads to the discovery of novel alleles with a high phenotype variability. These new alleles include dominant or gain-of-function mutations. While mutations with a recessive effect generally eliminate the functions of particular genes, mutations with dominant effects can lead to acquiring novel gene activity (Muller, 1932; Waterston, 1981; Lodish et al., 2000). For instance, Park and Horvitz (1986) reported that in *C. elegans*, the dominant mutation effects in the determined nine genes are caused by novel gene functions. In this study, we created, screened and selected mutants using the donor line IL3 in order to obtain a potential line with improved traits. IL3 is an inbred line derived from the commercial line EN01. We used EN01 which was previously characterized to have lower tolerance to oxidative stress (Sumaya et al., 2017).

Initially, after EMS treatment we observed an increase in fertility (DJ production) along the first EMS generations, which has not been reported before. In *C. elegans*, the effect of six mutant *daf-2* alleles on fertility was measured by Gems et al. (1998). Nematodes were raised at 15 °C and tested at 20 °C and 25.5 °C. At 20 °C, fertility was reduced to 10–45% of wild-type controls. However, congruent with the present results, at 25.5 °C, three mutant strains were as fertile as the wild-type (N2) strain or even indicated increased fertility up to 188% in comparison to the wild-type strain. We presumed that the reason for the increase in DJ production observed in the EMS population in *H. bacteriophora* could be attributed to the modifications in the ratio of intra/extra uterine eggs in the mutant hermaphrodites induced by stress. However, latter resulting inbred lines were lost because of reduced fertility. Our results suggest that the underlying mechanisms on the effect of EMS treatment on the fertility of *H. bacteriophora* would be an interesting research outlook.

In general, the survival and oxidative stress tolerance of the mutant line was lower compared to the hybrid obtained from crosses of two most tolerant and long-living wild-type strains, which had been identified by phenotyping. One could argue that phenotyping and crossing most tolerant or long-living might be more successful than application of mutagenesis. However, if we consider the origin of the IL3 inbred line originating from one of the least tolerant and long-living strains, the commercial hybrid EN01, then the progress obtained by mutagenesis is quite impressive. Consequently, future research should try to also apply EMS mutagenesis to superior genetic material. Possibly the success might even be higher.

To ensure no secondary mutations inferring phenotypic analysis of oxidative stress tolerance, the mutant MOX-IL6 was crossed with the donor line IL3. Even after crossing, the progeny of MOX-IL6 × IL3 still had higher tolerance to oxidative stress compared to IL3. Generated MOX-IL6 and MOX-IL6 × IL3 did not only confer tolerance to oxidative stress but also exhibited a long-lived phenotype. Overall, we assumed that this particular mutation may have dominant and/or pleiotropic effects. Mutagenesis and the isolation of novel or improved phenotypes in parallel with classical breeding have led to genetic variations endowed with beneficial traits. We have obtained an inbred mutant line that elicits a significant level of tolerance to oxidative stress and higher longevity.

Trade-off effects can also be a major drawback as a result of genetic improvement of *H. bacteriophora* (e.g., Mukuka et al., 2010d). Therefore, this study compared the fitness by assessing the virulence and reproduction potential of the hybrid and mutant lines in comparison with their parental and donor lines. Hybrid and mutant lines were found superior in virulence against *T. molitor* when compared to the parental, donor and commercial lines. The reproduction potential was obviously not changed. Trait stability or even improvement of other traits might not always be the result and one can by no means generalize and consider mutagenesis or hybridization to be without negative effects on other traits. All attempts of genetic improvement always need to monitor the stability of other beneficial traits.

Our results clearly document the potential of hybridization and mutagenesis for improvement of tolerance to oxidative stress and extended DJ longevity. From this research, we obtained three potential materials (AU1 × HU2, MOX-IL6 and MOX-IL6 × IL3) with increased tolerance to oxidative stress and extended lifespan, which can be used for future research. Our results are also consistent with the hypothesis that oxidative stress tolerance correlates with longevity. In this study, we have screened the fitness of our mutant and hybrid lines by assessing the virulence and reproductive potential to insect hosts and we found no trade-off effect. Genetic analysis to confirm the dominant effect and identify the mutated gene that gave rise to the phenotype with increased tolerance to oxidative stress will help to better understand the genetic background of stress related genes. Next generation sequencing technologies (NGS) now allow the development of new methods for rapid mapping and identification of EMS-induced mutations and can supply necessary information to identify the mutation by comparing the nucleotide sequences of wild-type and mutant genomes (Blumenstiel et al., 2009; Lehrbach et al., 2017). However, genetic analysis and

mapping of *H. bacteriophora* has only just started and in the future will contribute to acceleration of progress in the field of classical breeding techniques and approaches in domestication. For further improvement of strains and lines, the surviving 10% of the DJ population (MTS_{10}) can be selected and propagated for next selection cycles. Our results also suggest that the role of environmentally induced plasticity played a minor role in the observed phenotypes. With the high heritability of oxidative stress tolerance and considerable phenotypic variability the chance for improvement by selection is high (Johnson and Wood, 1982; Gaugler, 1989; Strauch et al., 2004) and this approach has proven to be successful already in the past in the improvement of *H. bacteriophora* and *S. feltiae* heat, cold and desiccation tolerance (Mukuka et al., 2010c; Nimkingrat et al., 2013a, 2013b). Genetic selection, hybridization and the application of mutagenesis can provide multiple hybrid strains with high tolerance to stress, prolonged lifespan, longer persistence in the field and even higher infectivity and one day might provide target-adapted commercial strains of high quality, which will further support the success of EPN in biological control of insect pests.

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Annex 3: Publication 3

Gene expression analysis of oxidative stress tolerance of the entomopathogenic nematode *Heterorhabditis bacteriophora*

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RESEARCH ARTICLE

Gene expression analysis of oxidative stress tolerance in the entomopathogenic nematode *Heterorhabditis bacteriophora*

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Summary–The entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* is used as a biological control agent against many insect pests. Nematode-based products contain dauer juveniles (DJ). Shelf-life of nematode-based products (DJ longevity) is limited. DJ Longevity of *H. bacteriophora* is correlated with oxidative stress tolerance. Thus, understanding mechanisms by which DJ respond to oxidative stress can provide insights to improve DJ longevity of this EPN species. In this study we carried out a comparative transcriptomic analysis on the early stage of oxidative stress induction (4 h) in two *H. bacteriophora* inbred lines with contrasting oxidative stress-tolerance, HU2-IL1 (stress-tolerant) and PT1-IL1 (stress-sensitive). To assess the transcriptome, MACE, a versatile RNA-seq variant was applied. Our *de novo* transcriptome assembly generated a total of 20,022 transcripts, from which 10,290 were linked to 9,776 different UniProt accessions. The majority of the annotated transcripts present high homology to parasitic nematode genera like *Ancylostoma*, whereas homology to the genus *Caenorhabditis* (free-living nematode) was negligible. A total of 630 transcripts were up-regulated (log2 fold-change [FC] $\geq$ 2.0) in the stress-sensitive and 461 in the stress-tolerant line. The proportion of down-regulated transcripts was higher for both lines. However, downregulation (log2 FC $\leq$ 2 .0) in the stress-sensitive line (5,207 transcripts) showed a larger extent than in the tolerant line (1,844 transcripts). This finding indicates that targeted suppression of biological processes is also a crucial factor for the survival of *H. bacteriophora* under oxidative stress. Our global view of the transcriptome re-modelling under oxidative stress suggests that the stress-sensitive line failed to maintain vital biological processes in contrast to the tolerant line. Interestingly, both lines activated similar biological processes known to be directly involved in detoxification of reactive oxygen species (ROS), indicating that expression changes of transcript isoforms is of high relevance in this context. This study will facilitate the selection of DJ longevity predictor genes in this EPN and further analysis will allow the design of molecular markers for the breeding of improved lines.

41 Keywords: *DJ survival, stress-related genes, transcriptomic analysis, RNA-seq, next*
42 *generation sequencing*

Heterorhabditis bacteriophora Poinar (Rhabditomorpha: Strongyloidea) is among the few entomopathogenic nematodes (EPN) species currently produced at industrial scale used for insect pest management (Ehlers, 2001; Lacey *et al*., 2015). From production to field application, various biotic and abiotic stress factors reduce dauer juvenile survival (DJ longevity) and virulence of this biological control agent (Glazer, 2002). It has been previously determined that environmental stresses such as heat, desiccation, UV-radiation and hypoxia influence *H. bacteriophora* survival (Grewal *et al*., 2002; Mukuka *et al*., 2010a, b). In addition, we recently reported that oxidative stress tolerance is strongly correlated with *H. bacteriophora* DJ longevity (Sumaya *et al*., 2017a). By screening 40 wild type strains and lines collected from different geographical locations, the mean survival time under control conditions and under oxidative stress conditions allowed to identify strains with enhanced DJ longevity. In a subsequent work, we demonstrated that oxidative stress tolerance can be used as selector for DJ longevity in this species (Sumaya *et al.*, 2017b). As proof of principle, an EMS-mutant inbred line obtained after oxidative selection pressure survived longer than its non-mutagenised donor inbred line under control conditions (+ 28.4 days) and under oxidative stress (+ 5.8 days). Thus, elucidating the underlying mechanism involved in oxidative stress tolerance in *H. bacteriophora* can be a tool to identify limiting factors of DJ longevity. Relevant genes may be identified and further used as genetic markers for stress response (Somvanshi *et al.,* 2008).

Physiologically, oxidative stress is caused by overproduction of reactive oxygen species (ROS) in respect to the antioxidants availability in a given organism (Bokoch & Diebold, 2000). It has been reported that high levels of ROS such as superoxide (O_2^-), hydroxyl (OH^-) radicals, and hydrogen peroxide (H_2O_2) can damage proteins, lipids and DNA. This damage disrupts cellular functions and can lead to cell death (Kim & Sun, 2007; Schieber & Chandel, 2014). In the model nematode *Caenorhabditis elegans*, the role of oxidative stress in aging has been extensively studied (Thamsen, 2011; Ortuno-Sahagun *et al.*, 2014). Many antioxidant enzymes

are identified to be involved in defense against overproduction of ROS; e.g. superoxide dismutases (SOD), catalases (CAT), glutathione peroxidases (GPx) and peroxiredoxins (Prxs) (Moreno-Arriola *et al.,* 2014). The expression of genes involved in ROS-scavenging and longevity are regulated by signaling pathways including Insulin/Insulin-like Growth Factor 1-mediated signalling (IIS) and Skinhead-1 (SKN-1) stress response pathways. The IIS pathway has been determined to influence lifespan in diverse organisms ranging from invertebrates to mammals, including humans (Kenyon, 2010). In *C. elegans*, DAF-16/FOXO (forkhead box) transcription factor of the IIS pathway is identified as one of the most important downstream lifespan-regulators (Altintas *et al.*, 2016; Braeckman & Dhondt, 2017). This transcription factor regulates many genes that influence lifespan and stress response (Henderson & Johnson, 2001; Back *et al.*, 2012; Lapierre & Hansen, 2012). In parallel, SKN-1 pathway is homolog of NRF-2 (nuclear factor E2-related factor-2) in mammals and other vertebrates that is also activated in response to oxidative stress in *C. elegans*. The SKN-1 transcription factor is also believed to play an essential role in intestinal development, metabolism, proteostasis and maintenance of homeostasis in *C. elegans* (Park *et al*., 2009; Blackwell *et al.*, 2015). In addition to ROS-scavenging enzymes, genes involved in general stress response, aging, oxidative phosphorylation, and ATP synthesis have also been identified to react upon oxidative stress. These genes include heat shock proteins, collagen-related proteins, and signaling genes such as *daf-2* and *daf-16* (Larsen, 1993; Henderson & Johnson, 2001; Shin *et al*., 2011). Thus, genes from related to diverse biological processes may be indicators of ROS-tolerance in nematodes. Transcriptional profiling of stress tolerance in nematodes with contrasting phenotypes have been reported. In *C. elegans*, for instance, a comparison of transcriptional response was investigated for *aak-2* mutants, which was considered as short-living due to a 12% reduction in their lifespan compared with wild type N2 adult worms after 3 h of oxidative stress induction (Shin *et al*., 2011). The authors found that oxidative stressed *aak-2* mutants have notably more

up-regulated genes in relation to the wild type. Heat shock proteins (*e.g. hsp-16.1*, *hsp-16.2*), short-chain acyl CoA dehydrogenases (*acdh-1*), microtubule-associated anchor proteins (*lgg-1*), lysozymes (*e.g. lys-4*), collagen genes and fatty acid desaturases (*fat*) were found to be more up-regulated in *aak-2* mutants compared to the wild type N2 strain.

In a comparative study between *H. bacteriophora* and other EPNs, Somvanshi *et al.* (2008) assessed the gene expression of five desiccation tolerant and susceptible nematodes by real time quantitative PCR. In their study, four genes were differentially up-regulated in the desiccation-susceptible *H. bacteriophora* TT01 strain (*i.e. aldh, nap 1, gpx* and *hsp-40*) compared to *Steinernema carpocapsae* and *S. riobrave* (most tolerant to desiccation). Recently, Yaari *et al.* (2016) assessed the transcriptome in *Steinernema* species (*S. riobrave* and *S. feltaie*) with contrasting tolerance to heat- and desiccation stress, reporting that more genes were down-regulated in tolerant strains while more genes were up-regulated in the less tolerant strains. Insights on how similar or dissimilar is the transcriptome re-modelling between oxidative stress and other environmental stresses in EPNs would complement these previous reports. Up to date, high-throughput transcriptome analysis on oxidative stress in *H. bacteriophora* DJ has not yet been reported. With the advent of new techniques, massive sequence data are now accessible for this species. For instance, transcriptomic data of *H. bacteriophora* GPS11 and TT01 strains generated a total of 1,000 ESTs and 10,886 distinct EST sequences, respectively (Bai *et al.*, 2009; Bai *et al*., 2007; Sandhu *et al*., 2006). Recently, Vadnal *et al.* (2017) profiled the transcriptional response of the same species during the early stage of infection by carrying out next generation RNA-sequencing. Additionally, Bai *et al*. (2013) have published a draft genome sequence. In the closely related species *H. indica*, a transcriptomic insight of DJ is also available with sequences deposited in a public repository (Somvanshi *et al.*, 2016). Thus, new transcriptome analysis will further contribute to confirm and generate more sequence information in *H. bacteriophora.*

In this present study, we compared the transcriptome of the stress-tolerant inbred line HU2-IL1 against the stress-sensitive inbred line PT1-IL1 four hours (4 h) after oxidative stress induction. The transcriptome of both lines was assessed by the RNA-seq variant known as Massive Amplification of cDNA-ends (MACE) described by Zawada *et al*. (2014). A *de novo* assembly containing more than 20,000 transcripts was generated and differential expression was calculated for each transcript in both inbred lines. The transcriptome analysis was validated by RT-qPCR quantification using a subset of selected genes in the inbred lines.

Materials and Methods

NEMATODE CULTURES

For this study all *H. bacteriophora* nematodes were cultured in Nematode Growth Gelrite (NGG) medium. Solid NGG: gelrite 3.0 g l^{-1}, peptone 2.50 g l^{-1}, 51 mM NaCl, 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 1 mM KH_2PO_4, 12 µM filter-sterilized cholesterol in 99% ethanol. Semi-solid NGG was prepared with similar content except with only 2 g l^{-1}peptone and 1.5 g l^{-1}gelrite. The prepared solid NGG was poured onto Petri plates (diam. 5cm) and the semi–solid NGG was stored at 4°C for bacterial cell dilution (Addis *et al*., 2016; Sumaya *et al*., 2017a).

The symbiotic bacterium *Photorhabdus luminescens* (strain HB1.3) was cultured in a standard Nematode Liquid Medium (NLM) (Ehlers *et al*., 1998) and incubated at 180 rpm (rotation diam. 4 cm) at 25°C for 24 h. To harvest bacterial cells, the culture was washed following the description of Addis *et al.* (2016). The prepared solid NGG plates were coated with 2 ml of semi-solid NGG containing 20×10^9 cells ml^{-1} and incubated at 25°C for 24 h. Inoculation of approximately 1,000 DJ on each NGG plate (diam. 5cm) was carried out for subsequent incubation at 25°C for 6 days. DJ harvesting and washing was followed as described

by Sumaya *et al.* (2017a). Clean and fresh DJ were stored in culture flasks and kept at 4°C until used for characterization.

GENERATION OF HU2-IL1 AND PT1-IL1 INBRED LINES AND CULTURE

Prior to the present research, forty *H. bacteriophora* wild type strains and lines from different geographic locations were assessed for their longevity and tolerance to oxidative stress (Sumaya *et al.*, 2017a). From this characterization, strains HU2 (stress-tolerant) and PT1 (stress-sensitive) were selected for further analysis. NGG cultures for each strain were started as described above and a single mature hermaphrodite in the developmental stage of *endotokia matricida* was picked and re-plated in NGG for a subsequent generation (~ 7 days). This procedure was repeated 7 times, each time picking a single mature hermaphrodite as described by Sumaya *et al.* (2017b). The resulting two HU2-IL1 and PT1-IL1 inbred lines were selected for transcriptome studies. To generate large amounts of DJ from HU2-IL1 and PT1-IL1 inbred lines, DJ were cultivated for further two generations in 9 cm NGG plates and harvested and washed as described above. Suspensions of DJ were stored in Ringer's solution (NaCl 9 g l^{-1}, KCl 4.42 g l^{-1}, $CaCl_2 \cdot 2H_2O$ 0.37 g l^{-1}, NaHCO3 0.2 g l^{-1}) and the number of DJ per ml was determined by counting three aliquots on a counting chamber. For oxidative stress assays, large amount of DJ were produced by inoculating ten NGG plates (diam. 9 cm) per line.

OXIDATIVE STRESS TOLERANCE ASSAY FOR MACE SEQUENCING

For transcriptome analysis, HU2-IL1 and PT1-ILI inbred lines were subjected to oxidative stress by exposure to 70 mM hydrogen peroxide (H_2O_2) at 25°C for four hours (4 h). Nine populations of 20,000 DJ from each line were transferred to single well positions of 6-cell well plates each with a final volume of 4 ml. Subsequently, 126 µl of 1.94 mM H_2O_2 were added to three of the populations (each with 20,000 DJ) to obtain a final concentration of 70 mM (stress induction). After 4 h of stress induction, treated DJ were vacuum-cleaned in 10 µm

sterile sieves, re-suspended in 40 µl sterile nuclease-free water, and immediately frozen in liquid nitrogen. Three untreated DJ populations were vacuum-cleaned, re-suspended in 40 µl sterile nuclease-free water and immediately frozen in liquid nitrogen. Three DJ populations per line were exposed to 70 mM H_2O_2 for a period of two weeks to confirm the differences in survival time between both lines. Therefore, 20 µl aliquots from each DJ population were taken and the number of alive and dead nematodes was recorded. This procedure was repeated every 24 h. The mean time survived by 50% of the DJ populations (MTS_{50}) for each line was calculated by Probit analysis (Finney, 1952). The complete procedure was repeated two times, starting from freshly grown nematodes. Transcriptome analysis was done separately for each treated and untreated batch.

TRANSCRIPTOME PROFILING IN RESPONSE TO OXIDATIVE STRESS

Transcriptome profiling from each line and treatment was performed using RNA-seq Massive Amplification of cDNA Ends (MACE). RNA-extraction, cDNA synthesis, amplification, sequencing and quantification was carried out by GenXpro GmbH (Germany) following the MACE protocol (Zawada *et al*., 2014). RNA was isolated from deep-frozen DJ populations using the NucleoSpin® miRNA kit (Macherel Nagel) according to the manufacturer's instructions. For preparation of MACE libraries, the poly-adenylated mRNA was isolated from >1 µg total RNA using magnetic beads (Life Technologies, GmbH) in combination with biotinylated adapters for the Illumina® Hiseq2000 system. After processing, sequenced reads were used to produce a *de novo* transcriptome assembly of the *H. bacteriophora* transcripts. Mapping of reads to the *de novo* transcriptome was performed with novoalign (http://novocraft.com). Differential expression calculation was done in the statistical programming language R (www.rproject.org/) with the DEseq package.

Advanced annotation was conducted with the software BLAST+ (https://blast.ncbi.nlm.nih.gov). Complementary protein hit information and associated Gene Ontology terms were retrieved from the protein information resource (PIR, http://pir.georgetown.edu). *P* values for Gene Ontology (GO) categories (biological processes) observed in the DJ samples were calculated using the Gene Score Re-sampling (GSR) analysis of the ErmineJ 3.0.2 software package (http://erminej.chibi.ubc.ca/).

TRANSCRIPTOME DATA FILTERING AND PATHWAY ANALYSIS

The transcriptome data were analyzed to identify transcripts that were significantly differentially expressed between inbred lines PT1-IL1 and HU2-IL1. The transcripts were filtered based on some unique parameters. Genes with differential gene expression (DGE) with *P* values of less than 0.05 and a minimum DGE log2-FC of 1.00 (2-fold) were selected for both inbred lines, PT1-IL1 and HU2-IL1. The filtered transcripts were subsequently BLASTed against UniProt and NCBI databases to identify homologies with oxidative stress response, ROS-detoxification and other stress-response genes. For the oxidative stress pathway analysis, the *C. elegans* KEGG longevity pathway (04212) was retrieved from the KEGG pathway database (http://www.genome.jp/kegg-bin/show_pathway?ko04212). Some of the genes involved in this pathway were identified in the generated transcriptome data and used as a model to construct the *H. bacteriophora* oxidative stress response pathway, which was subsequently conjugated with the gamma-glutamyl cycle (Ristoff & Larson, 2007) and other phase II detoxification systems.

To find categories that are over-represented amongst the DGEs in HU2-1l1 and PT-IL1, a GO enrichment analysis was carried out using the ErmineJ software (Lee *et al*., 2005). An over-representation analysis was performed in a set of genes selected by log2-FC. *P* values for

GO group under biological processes detected in the test samples were calculated using the GSR analysis as described above.

VALIDATION OF TRANSCRIPTOMIC DATA BY QUANTITATIVE REAL-TIME PCR

For validation with RT-qPCR, two independent oxidative assays were carried out with DJ populations of HU2-IL1 and PT1-IL1 under the conditions described above. Target genes were selected based on their log2-FC value (either positive or negative having significant differential expression, $P \leq 0.05$), physiological group and putative function of the transcript. Sequences from the transcripts meeting the selecting criteria were analyzed with the software package CLC-Bio main workbench (Qiagen, Germany) and primers were designed using Primer3 (v. 0.4.0, http://primer3.ut.ee/) online software (Table 2). Total RNA was isolated with the peqGOLD Total RNA Kit (VWR GmbH, Germany) according to manufacturer's instructions. First-strand cDNA synthesis was started with 500 ng of total RNA using the qScriptTM XLT cDNA SuperMix (Quanta BioSciences, Germany) according to the manufacturer's protocol, using oligo $(dT)_{15}$ and random hexamers as primers. RT-qPCR amplifications were carried out with 1 µl of 1/10 cDNA dilutions and 2x GoTaq® qPCR Master Mix (Promega GmbH, Germany) in 15 µl reactions. Thermal cycling was done in a StepOne Plus RT-qPCR device (Thermofisher GmbH, Germany) with the following profile: initial denaturation of 95°C for 10 min, 5 cycles of 95°C for 15 sec, 62°C for 45 sec (-0.5°C/cycle) and 40 cycles of 95°C for 15 sec and 60°C for 45 sec. A melting curve analysis was done by increasing the final amplicon temperature from 60 to 95°C, by steps of 0.3°C every 10 seconds. Expression of the target genes was normalized against the 18S rRNA gene using the primers reported by Yaari *et al*. (2016). Targets quantification was done following Pfafl (2004), using the untreated (T 0 h) samples from each line as control, according to the formula,

$$Relative\ quantity = \frac{(E_{Target})^{\ \Delta C_{p\ Target}(control-sample)}}{(E_{Ref})^{\ \Delta C_{p\ Ref}(control-sample)}}$$

where E is the calculated efficiency for the target and housekeeping (Ref) genes and C_p is the cycle at which the amplification reached the exponential phase. Significant differences between the expression levels of the different DJ populations along time were determined by one-way ANOVA and Tukey's HSD test ($P \leq 0.05$) using the XLSTAT statistical software. For Cluster analysis, log2-FC in expression were calculated using the RT-qPCR relative quantity of each gene, time point and material having the control time point (0 h) as reference. Expression values of genes and samples were hierarchically clustered and average-linked using the Cluster 3.0 software (http://www.geo.vu.nl/~huik/cluster.htm). The Treeview software was used for heat-maps visualization.

Results and Discussion

OXIDATIVE STRESS TOLERANCE OF *H. BACTERIOPHORA* CONTRASTING LINES

Dauer juveniles of the HU2-IL1 and PT1-IL1 inbred lines were exposed to 70 mM H_2O_2 and their tolerance to oxidative stress was determined by calculating mean time survived by 50% (MTS_{50}). Significant differences in MTS_{50} between two the inbred lines were observed ($F = 9.54$; $df = 1,10$; $P = 0.011$). Under oxidative stress, the lowest MTS_{50} value was determined for the inbred line PT1-IL1 (2.62 ± 0.97 days), whereas HU2-IL1 survived 2.78 days longer with a MTS_{50} of 5.4 ± 1.48 days (Fig. 1).

MACE EXPRESSION ANALYSIS

H. bacteriophora MACE libraries were sequenced via Illumina Hiseq2000 and the expression of each transcript was determined. A total of 66.6 million short reads were obtained representing 4.14 Gb of sequence data (Table 1). Our *de novo* assembly generated a total of

20,022 transcripts, from which 10,290 transcripts were linked to proteins from the UniProt database, representing 9,776 different accessions. A total of 6,456 transcripts were associated to Gene Ontology (GO) terms (http://geneontology.org). To test the direct degree of homology between our *de novo* assembly and existing *Heterorhabditis* data, we carried out a direct BLASTX comparison against the *Heterorhabditis indica* sequences reported by Somvanshi *et al.* (2016) deposited in the public repository (accession numbers: HADG01000001 - HADG01013593; European Nucleotide Archive). Only about 5% of the transcripts presented high homology. This could be attributed to the methods used - MACE is strongly representing the 3' ends of the transcripts- and to the fact that the *H. indica* DJ were not challenged by stress. Additionally, we BLASTed our *de novo* assembly against unpublished *H. bacteriophora* RNA-seq-derived data (information kindly provided by Prof. Itamar Glazer and Prof. Nelson Simoes), yielding more than 16,000 shared transcripts (information not shown). Recently, Vadnal *et al.* (2017) reported on the infection-related RNA-seq transcriptome of *H. bacteriophora* DJ. Transcript sequences (gene models or coding sequences) derived from this work are not yet deposited in public databases for full download or direct BLASTing.

Concerning sequence similarities to other nematodes, the majority of the annotated transcripts showed homology to genes from vertebrate parasitic nematodes (43%), whereas only 6% had a hit in free-living nematodes. From parasitic nematodes, the highest hit proportion was presented by nematodes of the genus *Ancylostoma* (19%), followed by *Haemonchus* and *Oesophagostomum* with 7% and 6%, respectively (Fig. 2). This degree of homology was also observed in *H. indica* by Somvanshi *et al.* (2016). According to these authors, *H. indica* DJ transcripts exhibited higher orthology with parasitic nematodes (e.g. *Ascaris, Ancylostoma* and *Haemonchus*) compared to free-living nematodes. Similarly, Vandal *et al.* (2017) observed diverse degree of homologies to different nematode clades. These authors blasted protein sequences of *H. bacteriophora* DGEs against all nematodes in Clades I, III, IV and V (as

reported by Blaxter *et al.*, 1998) and found a total of 172, 405, 263 and 802 gene hits, respectively. With a high number of hits in nematodes of the Clade V, a protein BLAST was further performed and found hits matched for *Ancylostoma ceylanicum* (790 genes), *Necator americanus* (706 genes), *C. elegans* (551 genes) and matched *Haemonchus contortus* (363 genes). In other EPN species, Yaari *et al.* (2016) studied the transcriptome profile of heat and desiccation stress response in *Steinernema* species and likewise observed a higher similarity to other vertebrate parasites. The authors presumed that this similarity could be attributed to the survival mechanisms of dauer juveniles as well as the different representation of the gene resources for the parasitic and free-living nematodes.

DIFFERENTIALLY EXPRESSED TRANSCRIPTS

Differential gene expression (DGE) for all 20,022 transcripts was calculated for each sequenced genotype taking as reference the respective control treatment. Shared proportions of up- and down-regulated transcripts in PT1-IL1 (stress-sensitive) and HU2-IL1 (stress-tolerant) sequenced MACE libraries is shown in Fig. 3. A total of 969 and 800 transcripts were up-regulated (log2-FC $\geq$ 1.0 $P \leq 0.05$) 4 h after stress induction in PT1-IL1 and HU2-IL1, respectively. From these transcripts, 339 transcripts were shared between both genotypes whereas 630 and 461 were exclusively up-regulated in PT1-IL1 and HU2-IL1, respectively. Interestingly, a substantially higher number of transcripts was globally down-regulated after stress induction. From both lines, the stress sensitive PT1-IL1 exhibited the largest degree of down-regulation. In this line 5,207 transcripts were found to be down-regulated (log2-FC $\leq$ 1.0, $P \leq 0.05$), whereas 1,844 transcripts were down-regulated in HU2-IL1. From those transcripts, 1,054 were common for both lines, whereas 790 and 4,153 were exclusively down-regulated in HU2-IL1 and PT1-IL1, respectively.

These results are contrasting with similar studies made in *C. elagans* (Shin *et al.*, 2011). The authors compared the transcriptome of the wild type N2 and *aak-2* mutants (stress-senstive)

306 in adult individuals 3h after oxidative stress induction. First, a higher number of up-regulated
307 genes than down-regulated genes were observed in stressed *aak-2* mutants and the N2 wild
308 type. This discrepancy may rely on species-related differences or in the assessed developmental
309 stage. Whereas we assessed the DJ transcriptome, Shin *et al.* (2011) evaluated the responses in
310 adult worms. In EPN species, Yaari *et al*. (2016) detected an inverse relation between gene
311 expression and tolerance to desiccation and heat stress in *Steinernema* species with contrasting
312 phenotype. The authors found that as a response to desiccation stress, a large proportion of
313 down-regulated genes were observed in the desiccation-tolerant *S. riobrave* (1,756) whereas a
314 large proportion of up-regulated genes in the desiccation-susceptible *S. feltiae* Carmiel strain
315 (SFCar) (2,067). In response to heat stress, the heat-tolerant *S. feltiae* Gvulot strain (SFG) had
316 a lesser fraction of up-regulated genes (935) while a higher proportion of up-regulated genes
317 (1567) was noted for SFCar strain. A higher down-regulation was found in SFG (2,202)
318 compared with SFCar (1,015). In our study, however, the oxidative stress-sensitive PT1-IL1
319 presented both, more up-regulated and more down-regulated genes than the oxidative stress-
320 tolerant HU2-IL1. As suggested by Yaari *et al.* (2016) a lower level of transcriptome re-
321 modelling on a stress-tolerant line may imply that several stress-related genes are active before
322 the onset of stress. With reference to the very large number of down-regulated transcripts in the
323 stress-sensitive line PT1-IL1, we can assume that this line failed to maintain the basic
324 transcriptome level of several genes that may be important for the survival under high ROS
325 conditions.

326 Concerning the identity of the transcripts with the most contrasting differential
327 expression, 12 out of 40 transcripts considered to have the most contrasting expression have
328 homology to UniProt accessions. Of these, 7 UniProt accessions are described as
329 uncharacterized proteins. From transcripts not having homology to UniProt accessions, 11 out

of 40 have high homology to *H. bacteriophora* unpublished RNA-seq *de novo* transcripts generated in parallel studies in different institutes (information not shown).

Information on the transcripts with the most contrasting up-regulation for both inbred lines is presented in Table 3. A similar scenario is observed in respect to down-regulated transcripts. Eighteen out of 40 considered transcripts had homology to UniProt accessions, of which nine accessions are described as uncharacterized protein. Regarding transcripts presenting no homology to UniProt accessions, 10 out of 40 transcripts were found in not yet published *H. bacterioiphora* RNA-seq transcripts. Information on the transcripts with the most contrasting down-regulation for both inbred lines is presented in Table 4. We construe that these significantly expressed uncharacterized transcripts might play an important role in oxidative stress and that further comprehensive research on gene functions will eventually elucidate other crucial genes involved in stress response.

In similar studies related to the expression profiling but on the trait deterioration in *H.bacteriphora*, Adhikari *et al.* (2009a) found that about 10% of DGEs did not have homology to any available sequences, which indicates novel *Hb* genes. The authors suggest that the findings might lead to discovering new genes and gene functions, genetic networks and metabolic pathways specific to *H. bacteriophora* and other EPNs. In other nematode species like *Plectus murrayi,* Adhikari *et al.* (2009b) observed that a subtractive library of the desiccated nematodes revealed 80 transcripts differentially expressed during desiccation stress, of which 17 (21%) were novel transcripts that had no similarity to known sequences in GenBank.

GLOBAL TRANSCRIPTOME RE-MODELLING OF *H. BACTERIOPHORA* UPON OXIDATIVE STRESS INDUCTION

To have a general overview of biological processes that are over- or under-represented in both lines after stress induction, we analysed the representation of Gene Ontology (GO) terms

on the common and exclusive differentially expressed genes. Considering common up-regulated genes, 34 GO terms (biological processes) were found. Among these processes we found GO categories associated to single-organism cellular process (GO:0044763), single-organism metabolic process (GO: 0044710), carbohydrate metabolic process (GO:005975), regulation of biological quality (GO:0065008), oxidation-reduction (GO:005514) and ion-transport (GO:0006811). As to GO terms associated with commonly down-regulated genes in both lines, 28 GO terms were determined. Oxidation reduction (GO:0055114) and response to stimulus (GO:0050896) were among the most repressed processes. In parallel, we analysed the GO terms associated to the line-exclusive down- and up-regulated genes. Considering the large extent of down-regulated genes we observed, the stress-tolerant HU2-IL1 line showed GO terms associated to signaling, and protein modifications, the stress-sensitive PT1-IL1 line on the other hand showed GO terms associated to basic metabolic processes. This finding supports our assumption that the large extent of down-regulated genes in this stress-sensitive line implies a general turn-down of vital processes. Regarding processes associated to transcripts that are exclusively up-regulated either in the stress-sensitive or the stress-tolerant line, we observed a high level of redundancy. This high redundancy level can rely on the fact that although GO terms may not differ, the genes beneath them can be totally different in both analysed lines. Deep details in this phenomenon will be shown in an upcoming section. An overview of the GO term representation is deposited in Tables 5 and 6.

In EPN species, Yaari *et al.* (2016) observed that under desiccation stress, the enriched GO terms in *Steinernema* species were under alcohol metabolic process, carbohydrate metabolic process, organic hydroxy compound metabolic process and lipid catabolic process while GO enriched terms in response to heat stress were response to stress, response to topologically incorrect protein and membrane organization. Additionally, Somvanshi *et al.* (2016) found that metabolic process, cellular process, localization and biological regulation

appeared to play an important role in *H. indica* infective juvenile. In *H. bacteriophora*, Vadnal *et al.* (2017) determined that metabolic and cellular processes, reproduction, development and growth were over-represented in response to infection. Similarily, Adhikari *et al.* (2009) reported that metabolic, developmental, multicellular organismal and cellular processes and growth were over-expressed during trait deterioration. In *C. elegans aak-2* mutants, Shin *et al.* (2011) reported that amongst the most highly represented biological processes in the oxidative stress were monocarboxylic acid metabolic, carboxylic acid metabolic, organic acid metabolic and fatty acid metabolic processes.

Expression of Oxidative stress-signaling and responsive genes

Subsequent to our analysis of GO terms representation, a deeper analysis on the transcription responses from genes related to main detoxification pathways was carried out. We compared the differential expression in the HU2-IL1 and PT1-IL1 lines for *C. elegans* orthologous genes comprised in the pathway model ko04212 from the Kyoto Encyclopedia of Genes and Genomes (KEGG). An overview of the expression for the pathway components is presented in Fig. 5. Almost all the genes in the *C. elegans* oxidative stress response pathway model were present and identified in the *H. bacteriophora* transcriptome data. Regarding signaling genes, one transcript variant homologous to Skinhead-1 gene (*SKN-1*) was identified in our transcriptome data (EN-Hb_oxid-57101). This transcript was slightly down-regulated in the stress-sensitive inbred line PT1-IL1 (log2-FC = -0.15) whereas it was up-regulated (log2-FC = 1.32) in the stress-tolerant inbred line HU2-IL1 upon exposure to oxidative stress. Concerning glutathione cycle-related genes, several transcripts were identified in the transcriptome data. For example, transcript EN-HB_oxid-48672 was found to be homologous to the γ-glutamyl cyclotransferase (*GGC*) gene and was up-regulated in both PT1-IL1 and HU2-IL1 lines. For the glutamate cysteine ligase (*GCL*) gene, for which transcription is directly activated by SKN-1, a homologous transcript was identified (EN-Hb_oxid-45560) to be slightly

down-regulated in both lines. Three transcripts annotated to γ-glutamyl transpeptidase (*GGT*) genes had varying expression response (with log2-FC ranging from -1.27 to 1.33). Some transcripts were annotated to genes, which are playing crucial roles in the glutathione (GSH)/glutathione disulfide (GSSG) redox couple system. Transcript EN-Hb_oxid-58900 was annotated to the glutathione-disulfide reductase *(GSR)* gene, which was significantly up-regulated in the stress tolerant HU2-IL1 (log2-FC 1.56) and stress-sensitive lines PT1-IL1 (log2-FC = 1.62). Three transcripts were annotated to glutathione peroxidase (*GPx*) genes showing only slight expression changes. Ten transcripts were annotated to proteins of the glutathione-S-transferase family (GST), showing high variability in expression levels. Within those, four transcripts were up-regulated upon oxidative stress in both nematode lines. For instance, transcript EN-Hb_oxid-53277 was significantly up-regulated in HU2-IL1 (log2-FC = 2.37) and PT1-IL1 (log2-FC= 1.71). Three transcripts were annotated catalase enzymes (CAT) and of those, transcript EN-Hb_oxid-56752 was significantly up-regulated in PT-IL1 (log2-FC = 2.89) and HU2-IL1 (log_2-FC = 2.13). In line with transcripts annotated to superoxide dismutase enzymes (SOD), three transcripts were found in our transcriptome database not showing large changes in expression.

Globally, our results show no drastic differences in the global responses of the stress-tolerant (HU2-IL1) and the stress-sensitive line (PT1-IL1). Differences are only observed in the context of some transcript variants that showed some contrasting regulation. The results may suggest that stress tolerance or sensitivity may be linked to fine-tuned responses and certain transcript variants or isoforms may play specific roles. Interestingly, Yan *et al*. (2012) reported that alternative splicing and transcription (results in variations of the transcript number or the 5′/3′ terminal protein variants) could increase the functional capacity of genes and gives an opportunity for gene regulation and another function. Yadav *et al.* (2017) observed that several isoforms were differentially regulated upon infection to *Drosophila melanogaster* with

symbiotic nematodes compared to axenic nematodes. In response to drought stress in maize, Song *et al.* (2017) reported differential isoform expression for a subset of candidate genes. In this work, the GRMZM2G140355 gene did not show significant differentially expression, however, exhibited changes in isoform expression. Thus, specific isoforms deserve focus on future research approaches.

Among the genes that displayed contrasting results we can underpin the differences observed in the SKN-1 and GST regulation. The SKN-1 transcription factor is reported to orchestrate the activation of the GST expression in response to oxidative stress, independently of the DAF-16 (a FOXO transcription factor) activity. The expression of several antioxidant enzymes, including GSTs, has been brought in connection with differences in longevity in *C. elegans* (An & Blackwell, 2003; Tullet *et al.*, 2008; Zhou *et al.*, 2011; Back *et al.*, 2012). GSTs are a family of enzymes with cellular detoxification activity and catalyze the conjugation of various electrophiles with glutathione, detoxifying both, exogenously and endogenously derived toxic compounds by producing molecules that are generally less reactive and more soluble (Leiers et al., 2003; Perrz & Wharton, 2011). Several GSTs have already been identified in *C. elegans* (van Rossum *et al*., 2001), for example, Tawe *et al.* (1998) showed that GST-p24 in *C. elegans* was up-regulated at the steady state mRNA level under oxidative stress induction. A further elucidation of the GST-p24 role in oxidative stress was investigated by Leiers *et al*. (2003) who used *Ce*-GST-p24 promoter-reporter constructs in transgenic *C. elegans* stains. The authors found that a decrease of the *Ce*-GST-p24 enzyme level resulted in a significant decrease in the oxidative stress resistance of the nematodes. GST was also found to be playing a significant role in response to desiccation stress of nematodes. It was reported that the desiccation process makes nematodes and other anhydrobiotes more sensitive to ROS (Burnell & Tunnacliffe, 2011). For instance, Adhikari *et al*., (2009) reported three transcripts encoding GST-1 were expressed in the ESTs of *P. murrayi* and one transcript was found to be up-

regulated under desiccation stress. In *Steinernema* species, GST transcripts were likewise found responding to desiccation stress (Yaari *et al*., 2016). Seven out of eight were observed to be down-regulated in *S. riobrave*. In *S. feltaie* (Carmiel strain) and *S. feltaie* (Gvulot strain), eight and three transcripts were up-regulated whereas two and six transcripts were down-regulated, respectively. In *H. bacteriophora*, there is no report on the expression of GST in response to stress conditions.

VALIDATION OF TRANSCRIPTOMIC DATA BY QUANTITATIVE REAL-TIME PCR

To validate changes in expression observed by differential expression analysis of RNA-seq (MACE) data, RT-qPCR was done on eight selected genes. DJ populations of the HU2-IL1 and PT1-IL1 inbred lines were exposed to 70 mM H_2O_2 and RNA probes were taken comparing two time points, 0 and 4 h after stress onset. Genes were chosen based on the fold changes (FC), either positive or negative showing significant differential expression ($P \leq 0.05$), physiological group and putative function of the transcript. Genes used to test the pattern by RT-qPCR are shown in Table 7. We found a significant positive correlation between MACE and RT-qPCR expression (R = 0.66; $P = 0.006$) as depicted in Fig. 5. The previous result was confirmed by cluster analysis. MACE and RT-qPCR profiles were further grouped together for both inbred lines (Fig. 6). Our results corroborate with the study of Vadnal *et al.* (2017) who validated the changes in expression of 10 genes in *H. bacteriophora* and Yaari *et al.* (2016) who validated the changes in expression of 10 genes in *S. riobrave.* Both reports found similar expression patterns between RNA-seq and RT-qPCR. Similarly, the expression pattern of 10 genes involved in the dietary restriction and TORC1 pathways were validated with a positive correlation to RNA-seq in *C. elegans* (Heintz *et al*., 2017). As also observed in the current study, the magnitude of expression between the two techniques is different. This difference may be due to differences in sensitivity and specificity of the techniques, growing conditions and culture of nematodes. Aside from this, the expression analyses done by RT-qPCR on further *H.*

bacteriophora materials with contrasting longevity is expected to be at least relatively comparable with reference genome wide transcriptome studies.

CONCLUSION

The present study yielded a total of 20,022 transcripts with a large fraction of transcripts (49%) observed to have no known homology in nematodes or other organisms for which sequence data are available in public repositories. From the annotated transcripts, a high homology to other vertebrate parasitic nematodes was identified. More genes were observed to be down-regulated in both nematode lines (HU2-IL1 and PT1-IL1) compared to up-regulation. Remarkably, a high down-regulation of genes was detected in the stress-sensitive PT1-IL1. Moreover, the global transcriptional view of re-modelling under oxidative stress revealed that no major changes on the biological processes was observed. Our results suggest that oxidative stress tolerance or sensitivity may be associated with fine-tuned responses where transcript variants or isoforms are presumed to play specific roles. We also confirmed that MACE profiles were positively correlated with RT-qPCR ($R = 0.66$, $P = 0.006$). We further suggest a thorough investigation on these uncharacterized genes, which might lead to discovery of genes and functions, playing a crucial role in oxidative stress response. Further studies will likewise facilitate the identification and design of appropriate molecular markers for breeding of EPN with improved beneficial traits for biological control of insect pests.

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507

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669 **Table 1.** Massive analysis of cDNA ends (MACE; a RNA-seq variant) libraries used for
670 transcriptome analysis of *Heterorhabdits bacteriophora* under oxidative stress.

671

Nematode	Properties	Replicates	Treatment	Total Reads (Millions)	Gigabases sequenced
HU2-IL1	Tolerant inbred line	3	Control	21.9	1.36
HU2-IL1	Tolerant inbred line	3	4 h 70 mM H_2O_2	14.6	0.91
PT1-IL1	Sensitive inbred line	3	Control	15.8	0.98
PT1-IL1	Sensitive inbred line	3	4 h 70 mM H_2O_2	14.3	0.89

672

673

Table 2. Primer sequences used for RT-qPCR and their amplification efficiency. Primer efficiencies were determined using four-point dilution series of the target DNAs.

Gene	Forward primer (5'→ 3')	Reverse primer (3'→ 5')
Glutathione-S-transferase	CCAAGGTGGCGCATAATAGC	AGGGAGAAATGGCCGGAAAT
HSP 90	AAATCGGTTGGTGTCATCGC	ACAGCAAAGCAGTCTCGAAGA
HSP DnaJ domain	TCACCCTATCTTGCTGCGAA	TGCATGTATCACCACCGCAT
Nematode cuticle collagen domain	CAAAGTTACGCATGGACCACC	CGTCCAGTTGTTCAATCATACCA
Glycosyltransferase, group 2 family	GCAAGCTTAGATTCGCCAAAGA	GGAGGTGGTGTATCTGGTTGG
Phosphoinositide phospholipase C	ATCAGATCGGAGTCATCGCTAG	GTTCTGTGGAGGTTTGTCTGC
V-type ATPase, A subunit	GCACTGGCCATATTTGAAGCA	CACAAGATTCTCCTGCCACCT
Type I phosphodiesterase	GCAGCACTTTCATCGGCAAA	TGCAGGTAAATTGGGTTCCGT
18S rRNA gene	CCTTTCGGCAATGGAAGGAC	GCATGGCCGTTCTTAGTTGG
WD domain, G-beta repeat protein	GCGTCTTGTGGCTAGCACTA	AGGGGCACTCTGGGATGTAT
Eukaryotic translation initiation factor 3	CCTCCGGAAGTGAGTAAAGACG	AAGCTCTTCTGGCTAATTCGGA

684 **Table 3.** Top 20 differentially expressed transcripts exclusively up-regulated in the HU2-IL1

685 (stress-tolerant) (A) and PT1-IL1 (stress-sensitive) (B) *Heterorhabditis bacteriophora* inbred

686 lines.

A.

Name	UniProt accession	Organism	Description	log2-FC PT1-IL1	log2-FC HU2-IL1
EN-Hb_oxid-30973	-	-	-	-0.27	4.50
EN-Hb_oxid-16700	A0A0D6LYF9	*Ancylostoma ceylanicum*	Type I phosphodiesterase	-0.86	3.59
EN-Hb_oxid-24046	A0A0D8Y9J0	*Dictyocaulus viviparus*	Uncharacterized	-0.86	3.40
EN-Hb_oxid-05478	A0A016SYN6	*Ancylostoma ceylanicum*	Uncharacterized	-0.27	2.92
EN-Hb_oxid-43049	-	-	-	-0.86	2.92
EN-Hb_oxid-02422	-	-	-	-0.44	2.60
EN-Hb_oxid-34759	-	-	-	-0.27	2.60
EN-Hb_oxid-52448	-	-	-	-0.27	2.58
EN-Hb_oxid-26048	-	-	-	-1.86	2.49
EN-Hb_oxid-34638	-	-	-	-1.27	2.46
EN-Hb_oxid-00703	-	-	-	-2.02	2.39
EN-Hb_oxid-27009	-	-	-	-2.44	2.39
EN-Hb_oxid-34294	-	-	-	-1.08	2.35
EN-Hb_oxid-38765	U6P722	*Haemonchus contortus*	Major sperm domain	-0.32	2.28
EN-Hb_oxid-46242	-	-	-	-0.86	2.28
EN-Hb_oxid-12063	-	-	-	-0.51	2.18
EN-Hb_oxid-24430	-	-	-	-0.44	2.18
EN-Hb_oxid-25465	-	-	-	-0.44	2.17
EN-Hb_oxid-47015	-	-	-	-1.18	2.17
EN-Hb_oxid-24689	-	-	-	-0.37	2.16

687

B.

Name	UniProt accession	Organism	Description	log2-FC PT1-IL1	log2-FC HU2-IL1
EN-Hb_oxid-10203	A0A016TYM8	*Ancylostoma ceylanicum*	Uncharacterized	4.22	-1.06
EN-Hb_oxid-17753	-	-	-	3.60	-0.79
EN-Hb_oxid-40813	-	-	-	3.34	-0.26
EN-Hb_oxid-13264	A0A0D8XXD9	*Dictyocaulus viviparus*	Uncharacterized	3.31	-0.16
EN-Hb_oxid-36696	-	-	-	3.15	0.00
EN-Hb_oxid-45501	-	-	-	3.15	0.00
EN-Hb_oxid-46811	-	-	-	3.08	-0.71
EN-Hb_oxid-53779	W2T9R0	*Necator americanus*	Carbamoyl-phosphate synthase	3.06	-2.85
EN-Hb_oxid-11553	-	-	-	2.85	-0.42
EN-Hb_oxid-00221	-	-	-	2.85	-0.74
EN-Hb_oxid-11290	-	-	-	2.73	-0.55
EN-Hb_oxid-40251	-	-	-	2.73	-1.49
EN-Hb_oxid-44285	-	-	-	2.65	-0.42
EN-Hb_oxid-37002	A0A0D8XYZ8	*Dictyocaulus viviparus*	Uncharacterized	2.65	-0.82
EN-Hb_oxid-48055	U6PS47	*Haemonchus contortus*	Fragile site protein Fra10Ac1	2.56	-1.70
EN-Hb_oxid-24457	-	-	-	2.52	-0.11
EN-Hb_oxid-11304	A0A016W6S8	*Ancylostoma ceylanicum*	Uncharacterized	2.49	-0.28
EN-Hb_oxid-41931	A0A016S6H6	*Ancylostoma ceylanicum*	Uncharacterized	2.47	-0.15
EN-Hb_oxid-55056	A0A0C2GAJ5	*Ancylostoma duodenale*	CHDNT domain	2.46	-0.10
EN-Hb_oxid-35666				2.46	0.00

Table 4. Top 20 differentially expressed transcripts exclusively down-regulated in the HU2-IL1 (stress-tolerant) (A) and PT1-IL1 (stress-sensitive) (B) *Heterorhabditis bacteriophora* inbred lines.

A.

Name	UniProt accession	Organism	Description	log2-FC PT1-IL1	log2-FC HU2-IL1
EN-Hb_oxid-48026	-	-	-	0.37	-4.59
EN-Hb_oxid-30210	A0A0D8XG17	*Dictyocaulus viviparus*	GAF domain	1.56	-4.25
EN-Hb_oxid-38049	-	-	-	0.00	-4.00
EN-Hb_oxid-13379	A0A016W129	*Ancylostoma ceylanicum*	Uncharacterized	0.44	-3.76
EN-Hb_oxid-33603	A0A0F5C4R7	*Pristionchus pacificus*	G protein-coupled receptor	0.00	-3.57
EN-Hb_oxid-30283	A0A0C2F5D3	*Ancylostoma duodenale*	EXS family	0.01	-3.50
EN-Hb_oxid-42750	E1GK48	*Loa loa*	Uncharacterized	0.00	-3.42
EN-Hb_oxid-55459	A0A085M8H7	*Trichuris suis*	Ribosomal protein S28e	0.46	-3.33
EN-Hb_oxid-03668	U6PHG6	*Haemonchus contortus*	Clc domain containing	1.28	-3.25
EN-Hb_oxid-20512	A0A016SSH6	*Ancylostoma ceylanicum*	Uncharacterized	0.73	-3.17
EN-Hb_oxid-51366	-	-	-	1.14	-3.12
EN-Hb_oxid-47731	-	-	-	0.46	-3.12
EN-Hb_oxid-42668	-	-	-	0.14	-3.12
EN-Hb_oxid-57433	A0A0D6LQS7	*Ancylostoma ceylanicum*	Arrestin domain	1.04	-3.06
EN-Hb_oxid-38473	W2TSD1	*Necator americanus*	Uncharacterized	0.14	-3.06
EN-Hb_oxid-03613	A0A0D8XYL3	*Dictyocaulus viviparus*	Uncharacterized	0.00	-3.06
EN-Hb_oxid-19866	-	-	-	0.15	-3.04
EN-Hb_oxid-31533	-	-	-	0.00	-2.92
EN-Hb_oxid-43405	Q6PYZ9	*Dictyocaulus viviparus*	Uncharacterized	0.82	-2.88
EN-Hb_oxid-53779	W2T9R0	*Necator americanus*	Carbamoyl-phosphate synthase	3.06	-2.85

B.

Name	UniProt accession	Organism	Description	log2-FC PT1-IL1	log2-FC HU2-IL1
EN-Hb_oxid-43321	-	-	-	-4.32	0.22
EN-Hb_oxid-08124	-	-	-	-4.18	0.00
EN-Hb_oxid-03736	A0A0D8XXI0	*Dictyocaulus viviparus*	Uncharacterized	-4.11	0.58
EN-Hb_oxid-07182	E3NT64	*Caenorhabditis remanei*	Uncharacterized	-4.03	0.23
EN-Hb_oxid-44914	-	-	-	-3.95	0.26
EN-Hb_oxid-24785	A0A0D6M0M6	*Ancylostoma ceylanicum*	Ribosomal RNA methyltransferase J	-3.95	0.20
EN-Hb_oxid-11857	-	-	-	-3.86	0.26
EN-Hb_oxid-42677	-	-	-	-3.81	0.17
EN-Hb_oxid-38062	W2TCG8	*Necator americanus*	Uncharacterized	-3.77	0.69
EN-Hb_oxid-35020	-	-	-	-3.71	0.00
EN-Hb_oxid-06734	-	-	-	-3.66	0.03
EN-Hb_oxid-53061	-	-	-	-3.45	0.58
EN-Hb_oxid-15214	-	-	-	-3.45	0.07
EN-Hb_oxid-33409	-	-	-	-3.31	0.44
EN-Hb_oxid-45586	-	-	-	-3.31	0.03
EN-Hb_oxid-14159	-	-	-	-3.23	0.29
EN-Hb_oxid-05374	-	-	-	-3.18	0.87
EN-Hb_oxid-39334	-	-	-	-3.18	0.05
EN-Hb_oxid-06596	-	-	-	-3.17	0.13
EN-Hb_oxid-52427	U6PIZ8	*Haemonchus contortus*	Uncharacterized	-3.17	0.00

730 **Table 5.** Common and exclusively enriched gene ontology (GO) terms in the differentially
731 up-regulated transcripts of *Heterorhabditis bacteriophora* HU2-IL1 (stress-tolerant) and PT1-
732 IL1 (stress-sensitive) inbred lines in response to oxidative stress (4 h, 70 mM H_2O_2).

GO number	GO description
GO terms exclusively found in: HU2-IL1 up-regulated transcripts	
GO:0008152	metabolic process
GO:0006139	nucleobase-containing compound metabolic process
GO:0006725	cellular aromatic compound metabolic process
GO:0090304	nucleic acid metabolic process
GO:0034641	cellular nitrogen compound metabolic process
GO:0044260	cellular macromolecule metabolic process
GO:0046483	heterocycle metabolic process
GO terms exclusively found in: PT1-IL1 up-regulated transcripts	
GO:0065007	biological regulation
GO:0080090	regulation of primary metabolic process
GO:0051171	regulation of nitrogen compound metabolic process
GO:0019637	organophosphate metabolic process
GO:0006082	organic acid metabolic process
GO:0019752	carboxylic acid metabolic process
GO:0043436	oxoacid metabolic process
GO terms commonly found in: PT1-IL1 and HU2-IL1 up-regulated transcripts	
GO:0044763	single-organism cellular process
GO:0044710	single-organism metabolic process
GO:0044281	small molecule metabolic process
GO:0044237	cellular metabolic process
GO:0044699	single-organism process
GO:0005975	carbohydrate metabolic process
GO:0065008	regulation of biological quality
GO:0006082	organic acid metabolic process
GO:0019752	carboxylic acid metabolic process
GO:0043436	oxoacid metabolic process
GO:0055114	oxidation-reduction process
GO:0009987	cellular process
GO:1901564	organonitrogen compound metabolic process
GO:0006811	ion transport
GO:0034641	cellular nitrogen compound metabolic process
GO:0071704	organic substance metabolic process
GO:1901135	carbohydrate derivative metabolic process
GO:0006950	response to stress
GO:0016070	RNA metabolic process

733 **Table 5. (cont.)**

GO number	GO description
GO:0044238	primary metabolic process
GO:0034654	nucleobase-containing compound biosynthetic process
GO:0044271	cellular nitrogen compound biosynthetic process
GO:0006807	nitrogen compound metabolic process
GO:0009056	catabolic process
GO:0018130	heterocycle biosynthetic process
GO:1901575	organic substance catabolic process
GO:0050789	regulation of biological process
GO:0050794	regulation of cellular process
GO:0046483	heterocycle metabolic process
GO:0044711	single-organism biosynthetic process
GO:0009058	biosynthetic process
GO:0006725	cellular aromatic compound metabolic process
GO:0019438	aromatic compound biosynthetic process
GO:0006793	phosphorus metabolic process
GO:0006796	phosphate-containing compound metabolic process

734

735

736 **Table 6.** Common and exclusively enriched gene ontology (GO) terms in the differentially
737 down-regulated transcripts from *Heterorhabditis bacteriophora* HU2-IL1 (stress-tolerant) and
738 PT1-IL1 (stress-sensitive) inbred lines in response to oxidative stress (4 h, 70 mM H_2O_2).

GO number	GO description
GO terms exclusively found in: HU2-IL1 down-regulated transcripts	
GO:0043412	macromolecule modification
GO:0065007	biological regulation
GO:0016310	Phosphorylation
GO:0050789	regulation of biological process
GO:0006464	cellular protein modification process
GO:0036211	protein modification process
GO:0006793	phosphorus metabolic process
GO:0006796	phosphate-containing compound metabolic process
GO:0050794	regulation of cellular process
GO:0009058	biosynthetic process
GO:0044267	cellular protein metabolic process
GO:0007154	cell communication
GO terms exclusively found in: PT1-IL1 down-regulated	
GO:0043170	macromolecule metabolic process
GO:0044260	cellular macromolecule metabolic process
GO:0009987	cellular process
GO:0006807	nitrogen compound metabolic process
GO:0044237	cellular metabolic process
GO:0044238	primary metabolic process
GO:0071704	organic substance metabolic process
GO:0008152	metabolic process
GO terms commonly found in: PT1-IL1 and HU2-IL1 down-regulated	
GO:0055114	oxidation-reduction process
GO:0050896	response to stimulus
GO:0044710	single-organism metabolic process
GO:0015672	monovalent inorganic cation transport
GO:0032501	multicellular organismal process
GO:0030001	metal ion transport
GO:0006812	cation transport
GO:0006811	ion transport
GO:0044711	single-organism biosynthetic process
GO:0018130	heterocycle biosynthetic process
GO:0019438	aromatic compound biosynthetic process
GO:1901362	organic cyclic compound biosynthetic process
GO:0055086	nucleobase-containing small molecule metabolic process

Table 6. (cont.)

GO number	GO description
GO:0006753	nucleoside phosphate metabolic process
GO:0019637	organophosphate metabolic process
GO:0044699	single-organism process
GO:0006810	transport
GO:0051179	localization
GO:0051234	establishment of localization
GO:0034654	nucleobase-containing compound biosynthetic process
GO:0006082	organic acid metabolic process
GO:0019752	carboxylic acid metabolic process
GO:0043436	oxoacid metabolic process
GO:0044271	cellular nitrogen compound biosynthetic process
GO:1901135	carbohydrate derivative metabolic process
GO:0044281	small molecule metabolic process
GO:0044763	single-organism cellular process
GO:1901576	organic substance biosynthetic process

739

Table 7. Selection of candidate transcripts from oxidative stress-responsive transcriptome assays after 4 h of stress induction in *Heterorhabditis bacteriophora*. Function I: Antioxidant and transferase activity; II: protein folding; III: protection and structure of the cells; IV: transport; V: catalysis; VI: hydrolysis and signal transducer; VII: ion binding and oxidoreduction; VIII: protease inhibitor; IX: uncharacterized.

Function	Unified code	Transcript annotation
I	EN-Hb_oxid-53277	Glutathione-S-transferase
	EN-Hb_oxid-47719	Glycosyltransferase, group 2 family protein
II	EN-Hb_oxid-48750	Heat shock protein 90
	EN-Hb_oxid-44284	Heat shock protein DnaJ domain containing protein
III	EN-Hb_oxid-22070	Nematode cuticle collagen domain protein
IV	EN-Hb_oxid-44233	V-type ATPase, A subunit
V	EN-Hb_oxid-16700	Type I phosphodiesterase / nucleotide pyrophosphatase
VI	EN-Hb_oxid-39769	Phosphoinositide phospholipase C

Text for Figures

Fig. 1. Mean time survived by 50% (MTS_{50}) of *Heterorhabditis bacteriophora* DJ populations from the inbred lines PT1-IL1 (stress-sensitive) and HU2-IL1 (stress-tolerant) under oxidative stress conditions. DJ were kept at 25°C in Ringer's solution and oxidative stress was induced by exposing the DJ to 70 mM H_2O_2. Mortality was assessed over time. For each line, three independent randomized replicates were evaluated. Error bars indicate standard deviation and different letters denote significant differences (Tukey's HSD test at $P \leq 0.05$).

Fig. 2. Proportions of *Heterorhabditis bacteriophora* transcripts sequenced from the lines PT1-IL1 and HU2-IL1 according to the genus or species showing the best homology hits in public repositories (UniProtKB database).

Fig. 3. Venn diagram illustrating the numbers of significant differentially expressed genes (DGEs): (A) up-regulated and (B) down-regulated transcripts for HU2-IL1 and PT1-IL1 *Heterorhabditis bacteriophora* inbred lines in response to oxidative stress (log2 fold-change [FC], $P \leq 0.05$).

Fig. 4. The main oxidative stress detoxification pathway from the model nematode *Caenorhabditis elegans* and expression of their orthologous genes in *Heterorhabditis bacteriophora*. Fold-change expression profiles of the genes are depicted for the following genotypes and conditions: (P) PT1-IL1, (H) HU2-IL1 4 h after stress induction. Red-coloured squares: up-regulation, green-coloured squares: down-regulation. High up-regulation levels are mostly observed in effector genes (e.g. Glutathione-related genes), whereas the transcript of signalling or sensing genes are not responsive to the stress.

Fig. 5. Correlation between gene expression changes determined by RT-qPCR (ordinate) and MACE (abscissa). For correlation analysis, eight transcripts were considered and data from two independent experiments were used. For RT-qPCR, the 18S rRNA gene was taken as housekeeping reference (Pearson's coefficient test, $P \leq 0.05$).

Fig. 6. Heat map and cluster analysis of gene expression for eight selected transcripts according to MACE and RT-qPCR assays 4 h after stress induction in two *Heterorhabditis bacteriophora* contrasting lines. Long-living line: HU2-IL1. Short-living line: PT1-IL1. Genes (horizontal arrangement) and expression techniques (vertical arrangement) were hierarchically clustered (average linkage) with Cluster 3.0 software. The expression of the genes measured by two techniques from same inbred line is clustered together. Up- and down-regulation are depicted in red and green, respectively (scale far right). Housekeeping gene: 18S rRNA gene. Reference sample: time 0 of respective lines.

Fig. 1

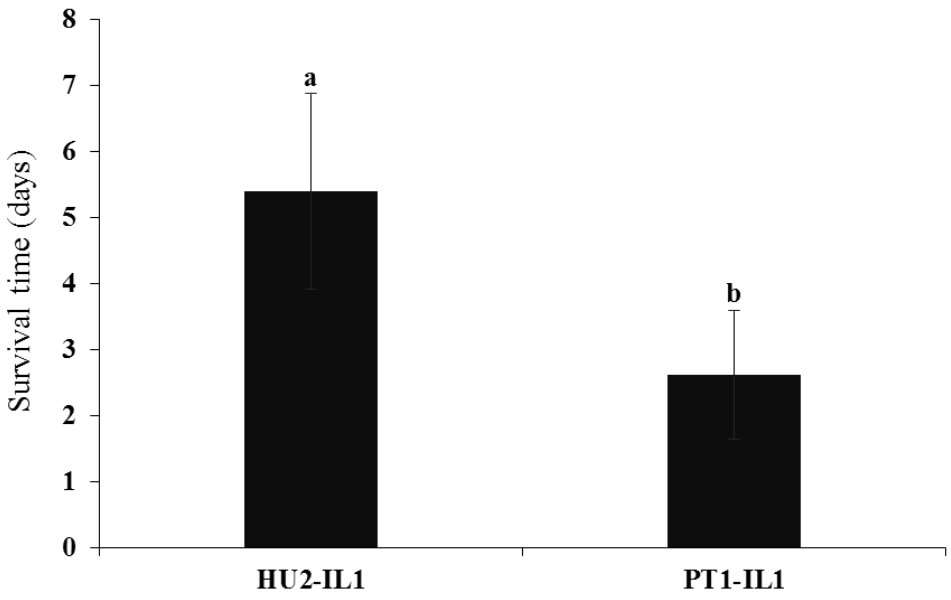
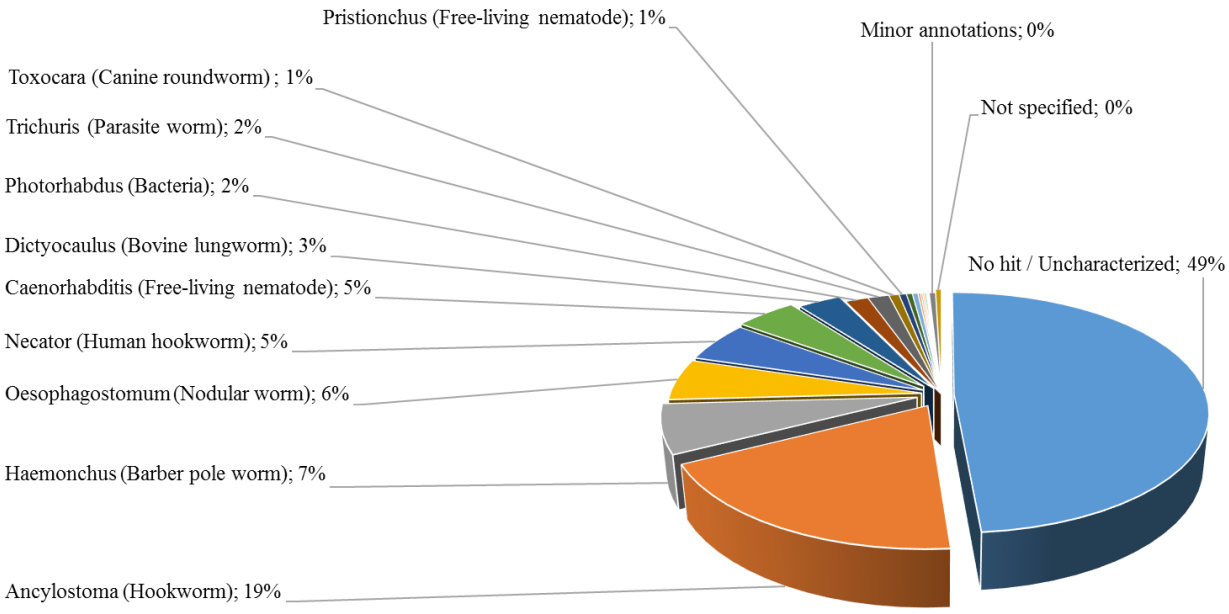


Fig. 2


Pristionchus (Free-living nematode); 1%
Minor annotations; 0%
Toxocara (Canine roundworm); 1%
Not specified; 0%
Trichuris (Parasite worm); 2%
Photorhabdus (Bacteria); 2%
Dictyocaulus (Bovine lungworm); 3%
No hit / Uncharacterized; 49%
Caenorhabditis (Free-living nematode); 5%
Necator (Human hookworm); 5%
Oesophagostomum (Nodular worm); 6%
Haemonchus (Barber pole worm); 7%
Ancylostoma (Hookworm); 19%

835 **Fig. 3**

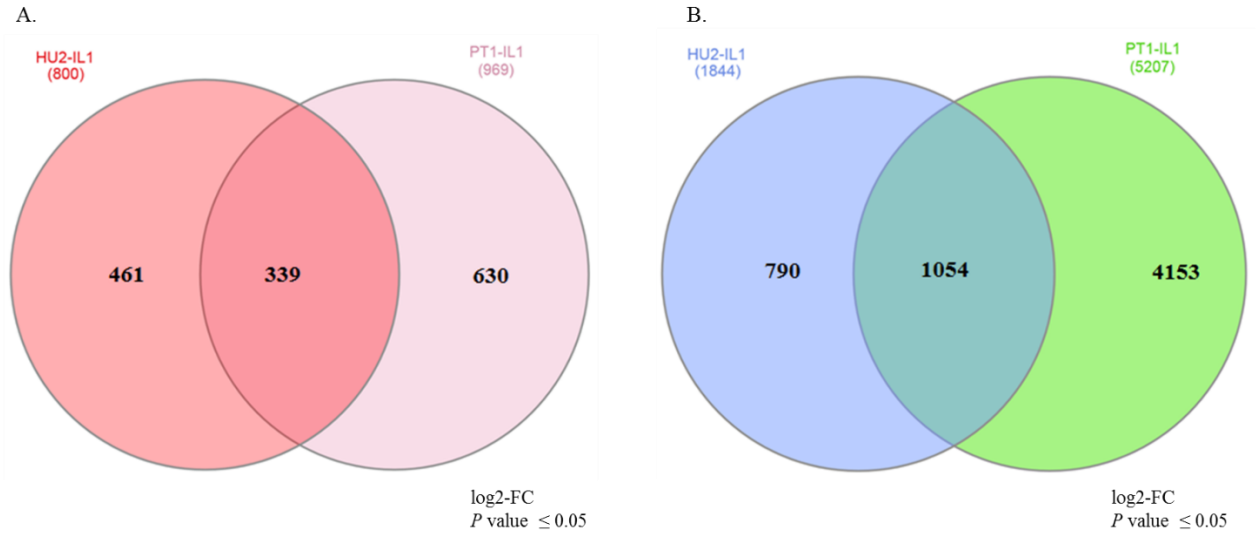


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Fig. 4

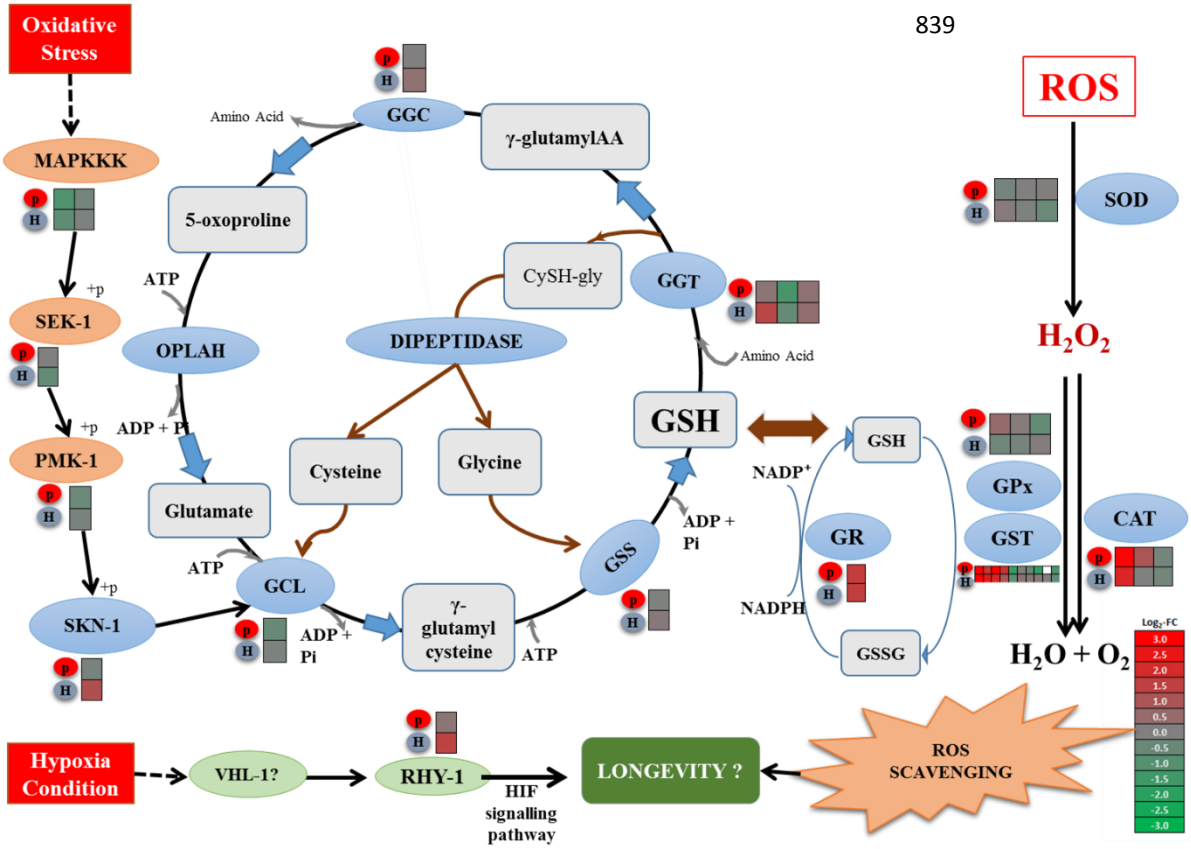

Oxidative Stress
MAPKKK
SEK-1
PMK-1
SKN-1
+p
ATP
ADP + Pi
OPLAH
5-oxoproline
Amino Acid
GGC
γ-glutamylAA
CySH-gly
GGT
DIPEPTIDASE
Cysteine
Glycine
Glutamate
GCL
γ-glutamyl cysteine
GSS
GSH
NADP+
NADPH
GR
GSSG
ADP + Pi
ROS
SOD
H2O2
GPx
GST
CAT
H2O + O2
Log2-FC
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
Hypoxia Condition
VHL-1?
RHY-1
HIF signalling pathway
LONGEVITY ?
ROS SCAVENGING

Fig. 5

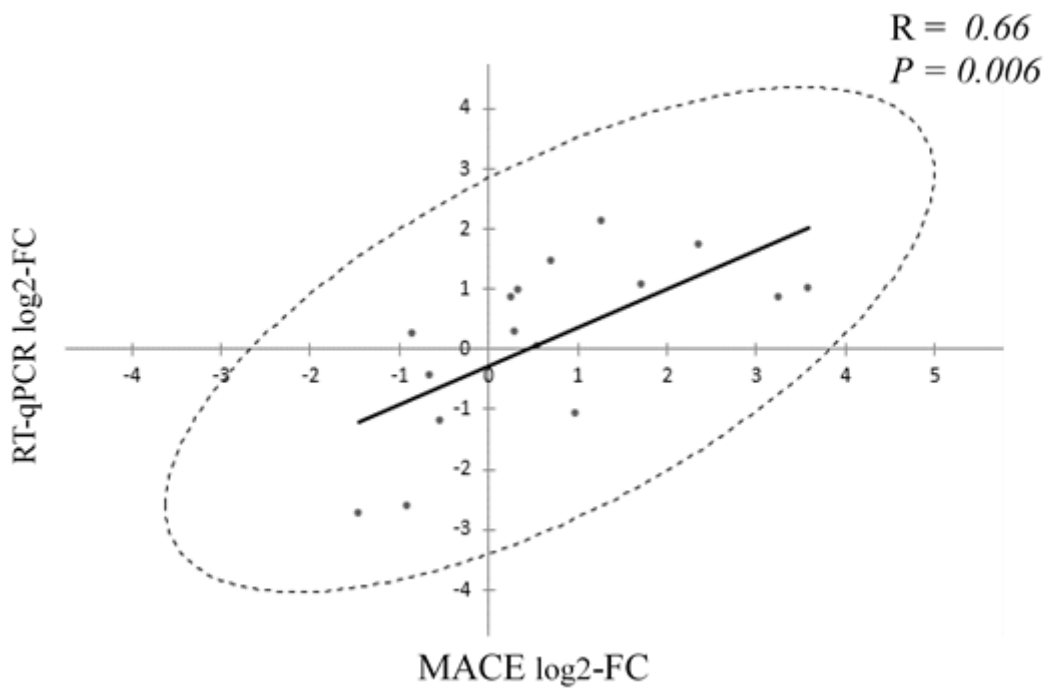
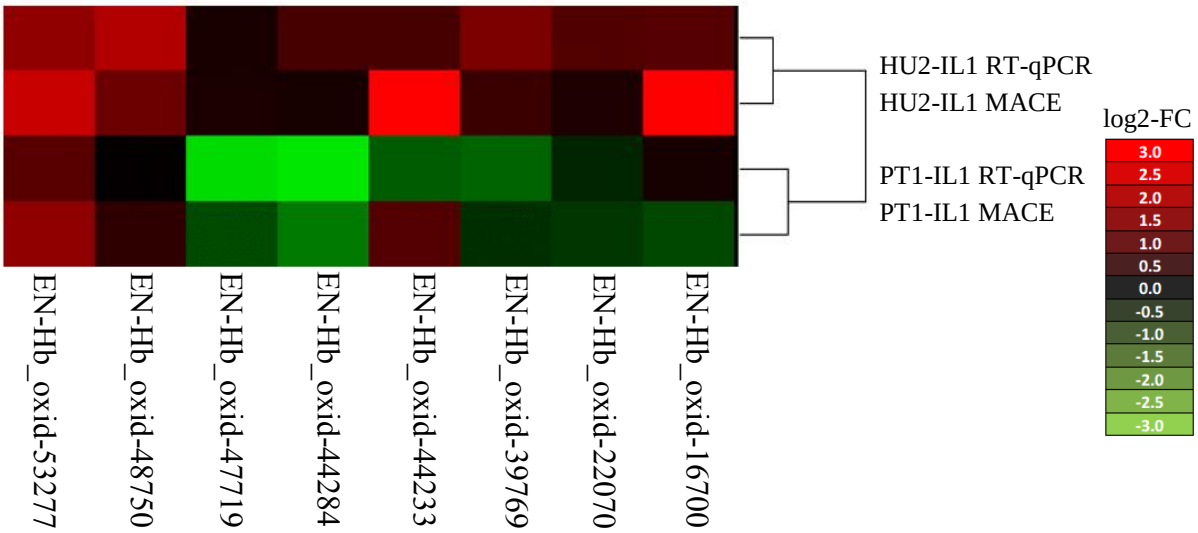

R = 0.66
P = 0.006
RT-qPCR log2-FC
MACE log2-FC
4
3
2
1
0
-1
-2
-3
-4
-4
-3
-2
-1
0
1
2
3
4
5

Fig. 6


HU2-IL1 RT-qPCR
HU2-IL1 MACE
PT1-IL1 RT-qPCR
PT1-IL1 MACE
log2-FC
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
EN-Hb_oxid-53277
EN-Hb_oxid-48750
EN-Hb_oxid-47719
EN-Hb_oxid-44284
EN-Hb_oxid-44233
EN-Hb_oxid-39769
EN-Hb_oxid-22070
EN-Hb_oxid-16700

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I have gone this far and I am of who I am today because of you all!

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Dr. Carlos Molina:	Co-supervisor, introduced required tools for molecular data analyses and editing
Dr. Olaf Strauch:	Statistics analysis and editing (publication 1)
Mrs. Verena Doerfler:	Technical assistant
Mr. Mike Barg:	Technical assistant
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