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

Nanette Hope Sumaya[a,b], Riddhi Gohil[b], Christopher Okolo[c], Temesgen Addis[a], Verena Doerfler[a], Ralf-Udo Ehlers[a,b,c,*], Carlos Molina[a]

[a] e–nema, GmbH, Klausdorfer Str. 28-36, 24223 Schwentinental, Germany
[b] Faculty of Agricultural and Nutritional Sciences, Christian-Albrechts-University Kiel, Hermann-Rodewald-Str. 4, 24118 Kiel, Germany
[c] Department of Biology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium

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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

* Corresponding author at: e–nema, GmbH, Klausdorfer Str. 28-36, 24223 Schwentinental, Germany.
E-mail address: ehlers@e-nema.de (R.-U. Ehlers).

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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