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A Realistic Appraisal of Methods to Enhance Desiccation Tolerance of Entomopathogenic Nematodes

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Abstract: Understanding the desiccation survival attributes of infective juveniles of entomopathogenic nematodes (EPN) of the genera *Steinernema* and *Heterorhabditis*, is central to evaluating the reality of enhancing the shelf-life and field persistence of commercial formulations. Early work on the structural and physiological aspects of desiccation survival focused on the role of the molted cuticle in controlling the rate of water loss and the importance of energy reserves, particularly neutral lipids. The accumulation of trehalose was also found to enhance desiccation survival. Isolation of natural populations that can survive harsh environments, such as deserts, indicated that some populations have enhanced abilities to survive desiccation. However, survival abilities of EPN are limited compared with those of some species of plant-parasitic nematodes inhabiting aerial parts of plants. Research on EPN stress tolerance has expanded on two main lines: *i*) to select strains of species, currently in use commercially, which have increased tolerance to environmental extremes; and *ii*) to utilize molecular information, including expressed sequence tags and genome sequence data, to determine the underlying genetic factors that control longevity and stress tolerance of EPN. However, given the inherent limitations of EPN survival ability, it is likely that improved formulation will be the major factor to enhance EPN longevity and, perhaps, increase the range of applications.

Key words: biocontrol, bioinsecticides, dauer, desiccation, *Heterorhabditis*, longevity, *Steinernema*.

Nematodes need at least a film of fluid for active existence, but the success of nematodes in colonizing all environmental niches is, in part, due to the ability of some of the life cycle stages to survive adverse environmental conditions, including dehydration (Perry, 2011). The ability of different species to tolerate adverse conditions varies enormously and one of the key factors implicit in desiccation survival is the ability to control the rate of water loss. The majority of soil-dwelling nematodes show little intrinsic ability to control water loss, being dependent on the environmental conditions of high relative humidity within soil pores to slow down or prevent water loss. In general, nematode anhydrobiotes can be grouped into those that rely on environmental factors to control water loss and those that have intrinsic abilities to control water loss. Perry and Moens (2011) termed these groups **external dehydration strategists** and **innate dehydration strategists**, respectively. Womersley (1987) had previously called these groups slow- and fast-dehydration strategists, respectively, but these terms are misleading; both groups require controlled drying in order to survive, the first group to prolong the time to lethal low water content and the second group to enable biochemical changes to take place to facilitate long term survival. Control of the rate of drying is the first phase; successful entry into long-term anhydrobiosis depends on subsequent biochemical and molecular adaptations.

In some species of nematodes, there is a specialized survival or dauer stage of the life cycle. The term dauer describes an alternative developmental stage enabling nematodes to survive adverse environmental conditions. The dauer phenomenon appears to be widespread in free-living nematodes and dauers are also present in parasitic species of nematodes, such as the pine wilt nematode, *Bursaphelenchus xylophilus*. There has been extensive research on the regulation of dauer development in *Caenorhabditis elegans* and it is evident that dauers are morphologically and physiologically specialised survival forms and these attributes make them effective dispersal forms. Entomopathogenic nematodes (EPN), *Steinernema* and *Heterorhabditis* spp., have a dauer stage, which is termed the dauer juvenile or infective juvenile (IJ).

In this short review, we shall examine the survival attributes of EPN and discuss whether various ways of selecting for, or enhancing survival traits, specifically desiccation tolerance, have a realistic chance of improving commercial formulations. Improved formulations are needed not only to increase storage longevity, but also to facilitate the use of EPN in field application.

Structural Adaptations

Some species of nematodes retain the molted cuticles as sheaths to aid survival. For example, early work showed that the infective third-stage juvenile of the animal-parasitic nematode, *Haemonchus contortus*, retains the molted cuticle of the previous stage as a sheath. The ensheathed juvenile survives desiccation better than the exsheathed form because, when exposed to desiccation, the sheath dries first and becomes increasingly impermeable, thus slowing down the rate of water loss of the enclosed juvenile and enabling it to survive until it is ingested, when exsheathment occurs in the rumen of the host (Ellenby, 1968). The retention of molted cuticles is found in other species of nematodes with a soil-dwelling stage. Second-stage juveniles of the sedentary plant semi-endoparasite, *Rotylenchulus reniformis*, hatch in the soil and molt to the adult without feeding, resulting in a decrease in body volume.

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The young adults are enclosed in all three molted cuticles, retained as sheaths from the previous stages, and remain inactive in dry soil until favourable wet conditions return. Wet conditions facilitate movement and exsheathment, after which the adult locates a host root and starts to feed. The exsheathed adults survived drying poorly compared with ensheathed adults and, as with *H. contortus*, the sheaths aided desiccation survival by slowing the rate of drying of the enclosed individual (Gaur and Perry, 1991).

The IJ of both genera of EPN have molted cuticles retained as sheaths. However, there are differences between the two genera. The sheath of *Steinernema* spp. fits very loosely and is readily lost during movement through the soil; it is unlikely to aid in desiccation survival of the IJ (Patel et al., 1997). By contrast, the sheath of *Heterorhabditis* spp. is closely associated with the nematode's body. The sheath surrounding the IJ of *Heterorhabditis megidis*, slows down the rate of drying of the enclosed juvenile (Menti et al., 1997) enabling it to survive better than the exsheathed IJ. However, the reduced rate of water loss only assisted individuals of *R. reniformis* and *H. megidis* to survive for periods over which water loss was controlled. Thus, *R. reniformis* and *H. megidis* are examples of nematodes that show little intrinsic ability for anhydrobiotic survival; control of water loss merely prolongs the time taken for the nematode's water content to reach lethal low levels. It has also been pointed out by Timper et al. (1991) that the presence of a sheath may afford protection against antagonistic organisms, such as pathogenic fungi, and may not necessarily indicate a role in desiccation survival.

Behavioural and Physiological Adaptations

Coiling and clumping are behavioural responses that are frequently associated with anhydrobiosis, particularly with innate dehydration strategists, such as *Ditylenchus dipsaci* (Perry, 1999). Both responses reduce the rate of water loss and clumping additionally affords protection to the individuals situated in the center of the mass. However, as pointed out by Glazer (2002), coiling has not been consistently observed in EPN exposed to desiccation and clumping appears not to occur under natural conditions. In his useful summary of research on the desiccation tolerance of EPN, Glazer (2002) notes that on exposed surfaces EPN can survive no longer than several hours. Their survival is improved when in soil, or in slow drying model substrates, or when subjected to a slow drying regime of exposure to high humidity before progression to lower humidity (Womersley and Higa, 1998). However, it must be stressed that even when dried under these conditions EPN do not demonstrate the astonishing survival abilities of *D. dipsaci* or *Anguina tritici* (Perry and Moens, 2011). For example, fourth-stage juveniles (J4) of *D. dipsaci* have remained viable in dry plant material for 23 years; even individual J4 dried on a glass slide at 0% relative humidity can survive for days. This survival is linked to an intrinsic property of the cuticle to reduce the rate of water loss; the importance of water loss control in relation to the structural and biochemical integrity of desiccated nematodes is discussed in detail by Perry and Moens (2011) and Burnell and Tunnacliffe (2011). The remarkable ability of J4 of *D. dipsaci* to withstand extreme environments is further demonstrated by the revival of 30% of individuals after exposure to vacuum desiccation of 800 Pa for 1.5h (Perry, 1977). EPN do not begin to approach this amazing survival ability!

Adaptation to local environmental conditions occurs in nematodes, and there is evidence of increased tolerance to extreme conditions in some populations. For example, desiccation tolerant strains of EPN have been isolated from a semi-arid region in Israel (Glazer et al., 1996; Solomon et al., 1999). Mutant forms of *H. megidis* have also been isolated with enhanced desiccation tolerance to low humidity (O'Leary and Burnell, 1997). The surface of the sheaths of these mutant lines was more negatively charged than that of the wild-type, and O'Leary et al. (1998) suggested that the presence of a strongly ionized or polar layer on the surface could facilitate the maintenance of a film of water over the cuticle; removal of this outer layer resulted in loss of the mutant phenotype. However, it is clear from these studies that the improvement in survival is only slight and, as it is improvement from a low base line, survival of these populations is still unremarkable.

The ability of nematodes to survive one stress is often associated with survival of another; for example tolerance of osmotic stress is frequently associated with the ability to survive severe desiccation (Perry 2011). Yan et al. (2011) found that partial induction of anhydrobiosis in certain strains of *S. carpocapsae* greatly improved heat tolerance, which may facilitate improved storage.

Biochemical Factors Involved in Survival

The biochemical correlates of desiccation survival in nematodes have been reviewed by Barrett (2011). In relation to EPN, research has focused on lipids (mainly neutral lipids, including triglycerides) and trehalose content (Wright and Perry, 2002). Lipids are clearly important in survival as they are the main food source for the non-feeding dispersal stage, and research has examined the infectivity in relation to lipid reserves. In general, the decline in infectivity of EPN is correlated with a decline in lipid reserves. Manipulation of dietary lipid content and culture temperature can improve viability and infectivity during storage (Abu Hatab et al., 1998; Abu Hatab and Gaugler, 1997, 1999) and this information has been used to improve longevity of commercial formulations.

Trehalose has long been implicated in desiccation survival. Some nematode anhydrobiotes, such as *D.*

dipsaci J4 and second-satge juveniles of *A. tritici* sequester trehalose, which has frequently been suggested as a desiccation protectant because of its role in preserving membrane stability, preventing protein denaturation and acting as a free-radical scavenging agent (Womersley et al., 1998; Glazer, 2002; Adhikari and Adams, 2011). In EPN, trehalose accumulation was noted in *Steinernema feltiae* that were dried slowly at high relative humidity (Solomon et al., 1999). However, there are contradictory reports about the importance of trehalose (Burnell and Tunnacliffe, 2011). Synthesizing trehalose during dehydration may indicate preliminary preparation for a period in the dry state, but it does not necessarily mean that survival during subsequent severe desiccation is assured.

From the information discussed above on attributes that facilitate desiccation survival of nematodes, it is apparent that EPN do not have the intrinsic ability to withstand desiccation that is required for effective deployment as a bioinsecticide for use in non-cryptic environments. EPN are external dehydration strategists. Therefore, current work has focused on two lines of research: *i*) breeding strains with better desiccation and heat tolerance; and *ii*) molecular studies to improve survival. It is important to examine both of these approaches to determine if, realistically, they are likely to progress improvement in longevity of commercial formulations.

Strain Selection and Breeding

The successful integration of EPN into insect pest management made necessary several biological and technical modifications of production, harvest and storage procedures to transform them into successful plant protection products (Ehlers, 2001). One of the major challenges was the stabilization of nematode survival and quality during storage and transportation (Ehlers, 2007). As a response to starvation EPN produce IJ. Adapted to long-term survival and resistant to shear forces, the use of IJ made possible the development of transportable products that could be applied with conventional spraying technology. However, the shelf life of EPN products is limited. Longevity of nematodes can be enhanced by transfer of the IJ into a quiescent state, in which they use less energy and are more resistant to environmental extremes. Quiescence can be induced by exposing IJ to moderate desiccation, which transfers nematodes into a weak anhydrobiotic state. However, as indicated in the preceding sections, the desiccation tolerance of EPN is not well developed (Glazer, 2002). Consequently, the availability of nematode strains or species with a higher desiccation tolerance would mean important progress on the road to longer shelf life of nematode products, which is needed to approach larger agricultural markets.

First attempts to stabilize EPN products used absorptive materials as formulation additives to produce desiccation conditions, which reduce nematode movement and metabolism, thus enhancing survival (Grewal and Peters, 2005). Compounds like poly-acrylate, alginate, attapulgite, vermiculite and other clay products have been used to produce conditions that can induce quiescence and thus reduce IJ metabolism. Further progress is still possible, but the potential is constrained by the biological limits of the desiccation tolerance of EPN. Distributing and applying entomopathogenic nematodes in their infected insect hosts may help protect the nematodes from environmental extremes, such as desiccation. Shapiro-Ilan et al. (2001) investigated this method with *Galleria mellonella*, and subsequently (Shapiro-Ilan et al., 2008) used the yellow mealworm, *Tenebrio molitor*, whose harder cuticle can resist rupture and prevent cadavers from sticking together. More recently, Shapiro-Ilan et al. (2010) have investigated enclosing the infected host in masking tape as a way of improving ease of handling. Successful commercialization of this cadaver application approach will depend on whether it can be mass produced and is economically feasible.

Another approach is the search for tolerance among different populations or species and to apply cross-breeding and genetic selection to enhance desiccation tolerance. The ability to survive desiccation stress can be highly variable between individuals of a nematode population and there is no correlation between survival ability and the locality from where the nematode population was obtained. However, stress adaptation can be one factor influencing desiccation tolerance. Exposure of IJ to moderate stress conditions enables them to synthesize molecules, like trehalose, for protection of their membranes during water loss (Womersley, 1990). As a result IJ become more tolerant to higher desiccation stress levels. The ability to produce such protective molecules is genetically defined. The phenotype is thus a result of environmental and genetic factors. Should the desiccation tolerance be influenced mainly by genetic characters, the probability for success of a genetic selection program can be quite high. Genetic selection has been very successful for many domesticated animals and plants and thus should also be a promising approach for enhancement of desiccation tolerance of EPN.

Three factors influence the breeding success: heritability, selection pressure, and variability. The heritability reflects all genetic contributions to the phenotype of a nematode population and hence is a pre-requisite for success of genetic selection. The heritability (h^2) of the desiccation tolerance was first assessed by Glazer et al. (1991) for *H. bacteriophora* (strain HP88) at $h^2 = 0.11$. Adaptation to stress conditions was not taken into account. Strauch et al. (2004) reported a much higher heritability for a hybrid strain of *H. bacteriophora* of $h^2 = 0.48$ after adapting IJ to desiccation and $h^2 = 0.46$ when exposing them directly to the desiccation stress. They

used different concentrations of a dehydrating polymer solution of polyethylene glycol 600 to modify the water activity (a_w - value). The water activity indicates the relative proportion of unbound water in a sample. The lowest mean tolerated a_w-value of 0.85 was achieved with an adaptation phase of 72 h at an a_w-value of 0.96. Improvement of the desiccation tolerance by breeding was only obtained when the adaptation process was included in the selection process, which was related to a higher phenotypical variance in the populations after adaptation. A total of eight selection and breeding steps were carried out. Without previous adaptation the mean of the tolerated a_w-value remained almost constant between 0.94 and 0.93. By contrast, when adapting IJs prior to the exposure to desiccation stress, the tolerable a_w-values dropped continuously from 0.89 to 0.81.

In order to enhance further tolerance against desiccation stress, as a next step, Mukuka et al. (2010a) tried to increase the genetic pool for genes supporting desiccation tolerance by screening for tolerance among natural populations of *H. bacteriophora*. Mean tolerated a_w –values among these strains ranged from 0.99 to 0.67 for adapted and from 0.9 to 0.95 for non-adapted nematode populations (Mukuka et al., 2010a). Neither Mukuka et al., (2010a) nor Strauch et al. (2004) found any correlation between tolerance before and after adaptation, which indicates that different genes are involved in tolerance with and without adaptation. Of the most tolerant three strains, Mukuka et al (2010b) then picked 10% of those individuals which had tolerated the lowest a_w–values and used them for cross breeding. As the cross-breeding already included a selection step (use of 10% most tolerant individuals), the tolerance of the hybrid was further enhanced and the subsequent selection process did not result in any further significant improvements in tolerance.

Stability of selected traits can be a major drawback for a breeding and selection program. Increase in desiccation tolerance can also compromise the fitness and infectivity of EPN. Hybrids with increased desiccation tolerance were usually lower in infectivity, a possible result of a trade-off effect of selection for desiccation tolerance. Hybrid strains selected for enhanced tolerance after an adaptation to stress were generally better in fitness compared with those for which adaptation prior to stress exposure was excluded (Mukuka et al., 2010c).

In general, screening among natural populations for high tolerance to desiccation is a feasible approach and cross-breeding and genetic selection can further improve tolerance. However, when formulating *H. bacteriophora* at lower a_w–values one must consider that exposure to desiccation stress can lower infectivity (Mukuka et al., 2010d). Also, the stability of the selection progress has to be closely monitored and investigations to stabilize desiccation tolerance among selected populations are currently under way (Anbesse and Ehlers, unpublished).

Screening and selection is possible also with other EPN. In a recent study (Salame et al., 2010) a heterogeneous population of the EPN *S. feltiae* was bred for desiccation tolerance (both rapid and slow). The selection for tolerance of rapid desiccation was done by exposing IJs to ambient conditions (22-25°C; 50-65% RH) for 100 min. A survival rate of 80-90% was reached after ten selection cycles. To select for tolerance of slow desiccation, the IJs were exposed to 97% RH for 72 h, followed by further exposure to 85% RH for an additional 72 h. A high survival rate (> 85%) was observed after 20 selection cycles. In order to avoid desiccation stress in the field the population was also selected for enhanced host-seeking ability by forcing IJs to move through a sand column to reach larvae of last-instar *G. mellonella*. After 25 selection cycles, the majority (>75%) of these nematodes were found at the bottom of the column, near the insects. No reduction in fitness was detected in the selected populations. Nevertheless, the nematode population selected for enhanced host seeking displayed significantly higher infectivity than the foundation population. The population selected for slow desiccation was more tolerant of heat stress than the foundation population.

In summary, there is potential for genetic improvement of EPN through selective breeding. Considerably more progress would be possible by using nematode species that can already tolerate desiccation stress, such as *S. carpocapsae* or *S. abbasi*. However, the priority for commercial use is efficacy. Consequently, traits related to this character are essential and more important than enhanced desiccation tolerance. Efficacy is immediately impaired by water activity below 0.97, whether a tolerant or less tolerant nematode strain/species is used, as IJ stop moving. Whether screening among tolerant strains/species or improving through selective breeding, the traits related to efficacy must always be closely monitored or selected for as well.

Molecular Approaches

Future work may focus on genetic transformation of entomopathogenic nematodes to improve their stress tolerance (Burnell and Dowds, 1996; Burnell and Tunnacliffe, 2011; Grewal et al., 2011; Yaari et al., 2011). A transgenic approach was used by Gaugler et al. (1997) to introduce a heat-shock protein gene, *hsp70*A, from *C. elegans* into *H. bacteriophora* to enhance thermotolerance. If trehalose is implicated in the survival of species and/or strains of EPN, then the use of genes for enzymes involved in the synthesis of trehalose, such as *tps* 1 coding for trehalose-6-phosphate synthase, may cause trehalose overproduction and enhanced survival (Vellai et al., 1999). Fodor et al. (2010) isolated a *tps-1* gene from yeast (*Saccharomyces cerevisiae*) and transformed *H. bacteriophora* showed increased osmotolerance.

There are few studies on the anhydrobiotic genes of nematodes in general and EPN in particular. Burnell and Tunnacliffe (2011) concluded that the available information indicates that dehydration does not result in marked upregulation of many of the classical protein homeostasis genes. In *S. feltiae*, genes encoding ubiquitin and Hsp40 analogues were induced by dehydration among 81 genes identified (Gal et al., 2003) but in *S. carpocapsae*, which is less tolerant of desiccation, these or other protein homeostasis genes were not found in a set of 41 dehydration-induced ESTs (Tyson et al., 2007). In five entomopathogenic nematodes with varying tolerance of desiccation, four genes associated with stress tolerance showed the greatest degree of upregulation in the least desiccation-resistant nematode species (Somvanshi et al., 2008). The genes may already be at a high, 'stress-ready' level, or other mechanisms are responsible for maintenance of proteome functionality in the more tolerant species (Somvanshi et al., 2008; Burnell and Tunnacliffe, 2011).

Conclusions

It is clear that EPN, in general, do not have the intrinsic abilities required for routine, effective field use as bioinsecticides. The various methods outlined above to enhance desiccation survival will only confer marginal improvements, and do not offer the promise of more widespread use of EPN. This short review has focused only on desiccation survival; similar limitations of EPN are found with heat tolerance, an attribute that is important during shipment and deployment of control products. Selective breeding is the most promising approach to enhance specific survival attributes, like desiccation and heat tolerance, and, when combined with appropriate formulations, is likely to result in improved storage longevity and improvement of current commercial products for existing usage.

The use of molecular information for genetic engineering of EPN is challenging. It will be possible to determine genes from the whole genome that are being expressed, in order to detect those that are involved in desiccation tolerance. This would enable identification of the proteins involved in desiccation survival, the 'desiccome'. Progress in these areas is vital to improve our understanding of desiccation tolerance in general, and may specifically result in identifying new ways of improving longevity and survival of commercial formulations of EPN. However, at present, it is unlikely that a genetically engineered EPN strain would meet public acceptance as a control agent. Regulatory and public acceptance may only be achieved in the long term.

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