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Ammonia concentration at emergence and its effects on the recovery of different species of entomopathogenic nematodes

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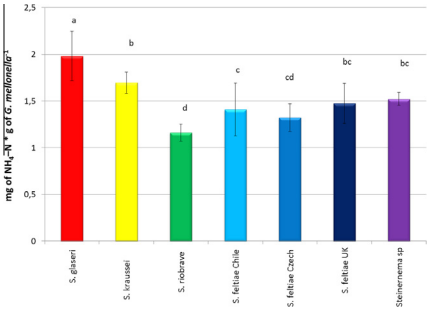
HIGHLIGHTS

- *Steinernema* spp. showed different ammonia triggering point values.
- Ammonia regulates the emergence of new infective juveniles.
- Ammonia concentration impairs the recovery process of infective juveniles.
- Large amounts of ammonia increase the mortality among infective juveniles.

GRAPHICAL ABSTRACT



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ABSTRACT

The life cycle of entomopathogenic nematodes (EPN) occurs inside an insect cadaver and an accumulation of ammonia initiates as a consequence of the nematodes defecation. This accumulation reduces the food resources quality and creates a detrimental environment for nematodes. When a given ammonia concentration is reached, the nematodes start their emergence process, searching for a new host. In the present work, this parameter, ammonia triggering point (ATP) was measured in 7 *Steinernema* species/strains. The effect of different ammonia concentrations on the recovery process and their consequences in the nematodes survival were also investigated. The results indicate that ATP varies among nematode species; *Steinernema glaseri* showed the highest ATP of the evaluated species (1.98 ± 2.6 mg of NH_4–N * g of *Galleria mellonella*$^{-1}$); whereas *Steinernema riobrave* presented the lowest ATP (1.16 ± 0.1 mg of NH_4–N * g of *G. mellonella*$^{-1}$). On the other hand, the nematode emergence could be a repulsive response when ATP is reached. As the ammonia concentration increased the recovery percentage of *Steinernema feltiae* (Chile strain) dropped gradually from 79.4 ± 11.9% in the control treatment to 0% when 1 mg of NH_4–N * ml of bacterial broth^{-1} was added. It is possible, that emergence process could be a repulsive response of the nematodes due to ammonia concentration when is reaching the ATP. The role of ammonia inside the insect cadavers, might suggests connections with some stages of the EPN life cycle.

1. Introduction

Entomopathogenic nematodes (EPN) are soil dwelling organisms which have been use as biopesticides for many years due to their ability to kill insects. Their life cycle occurs inside the insect cadaver and after 1 or 2 generations, juveniles of the third stage

Abbreviations: EPN, entomopathogenic nematodes; IJ, infective juvenile; ATP, ammonia triggering point.

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become infective juveniles (IJ), emerge from the cadaver and search for a new host (Kaya and Gaugler, 1993). Nevertheless, many aspects that regulate the life-cycle processes of the EPN are unknown.

What stimulates EPN to emerge from host cadavers has generally been related to a shortage of food resources (food quantity hypothesis); however, O'Leary et al. (1998) postulated an alternative hypothesis linking emergence to the accumulation of metabolites from the breakdown of the insect's tissues (food quality hypothesis). Recently, the role of ammonia, produced by nematode defecation, in triggering the emergence process of *Steinernema feltiae* has been studied (San-Blas et al., 2008) and results support the idea that the increase of ammonia concentration inside the insect host can stimulate the emergence process.

Ammonia has been proved to be responsible for many effects on nematodes: some plant parasitic nematodes are very sensitive to low concentrations of this compound and die (Rodriguez-Kabana, 1986; Oka and Pivonia, 2004; Oka et al., 2006). On the other hand, different attraction-repulsion effects produced by ammonia have been reported as a response of EPN for seeking a new host (Schmidt and All, 1979; Pye and Burman, 1981; Grewal et al., 1993).

Ammonia is a common excretory product of nematodes. Between 40 and 90% of non-proteic nitrogen is excreted as ammonia probably, because this compound is highly water soluble and is therefore less energy demanding to excrete than other forms of nitrogenous waste, such as urea or uric acid (Wright and Perry, 2002). Nonetheless, ammonia is also toxic and must be excreted to avoid its detrimental effects on organisms.

Once the insect has been invaded by EPN, the cadaver becomes an ammonia reservoir because adult and juvenile nematodes defecate inside. The cadaver of a waxmoth (*Galleria mellonella*) larva might be filled by several thousand nematodes (Zervos et al., 1991; Wang and Bedding, 1996; Elawad et al., 2001). Thus, if this number of nematodes are eating and defecating in such a confined space, and if we assume that some nematode species (i.e., *Caenorhabditis elegans*) can defecate every 45 s (Bayles, 2005), the concentration of waste-products will substantially increase and the nutritional status of the cadaver will deteriorate. It is therefore possible that ammonia concentrations could be linked to various processes related to a nematode's life cycle, other than just emergence, e.g., recovery.

The recovery process is the ability of the IJ to restore their development following diapause, once conditions become suitable. Stimuli responsible for this transformation in species of *Steinernema* have been found within the insect hemolymph (Hirao and Ehlers, 2009), recovery-inducing factors from insect cells (Han et al., 2000; Kikuta et al., 2009), cell density and phase variant of their symbiotic bacteria (Hirao and Ehlers, 2009). Due to the fact that EPN recovery occurs inside the insect host and also EPN possess attraction–repulsion behaviour when ammonia is present in their environment, it is plausible that ammonia could also interfere with the recovery process. The objectives of this works were: to determine differences in the ammonia triggering point (ATP) amongst *Steinernema* species and, to evaluate the effect of ammonia concentration on the IJ recovery process.

2. Materials and methods

2.1. Nematode culture

The species *S. feltiae* (wild strains from UK, Czech Republic and Chile), *Steinernema kraussei*, *Steinernema riobrave*, *Steinernema glaseri* and *Steinernema* sp. (isolated in Venezuela and from the bicornutum group) (Rhabditida: Steinernematidae) were cultured in *G. mellonella* larvae (Lepidoptera: Pyralidae) following Dutky et al. (1964). The *G. mellonella* larvae were bred in the laboratory (using honey combs as a feed resource). The infected larvae were incubated at 20 °C. The IJ were collected using adapted White traps (White, 1927) and used in the experiments within 2 weeks.

2.2. Bacteria source

The EPN symbiotic bacteria, *Xenorhabdus* sp. (Enterobacteriales: Enterobacteriaceae), used for the experiments, was isolated from *G. mellonella* larvae which had been exposed to the nematode species 48 h before. *G. mellonella* cadavers were surface sterilized with 70% methanol for 10 min; under sterile conditions they were then dissected and their contents plated onto a selective media of nutrient bromothymol agar (NBTA) (3.3% nutrient agar, 0.025% bromothymol blue and 0.004% 2,3,5- triphenyltetrazolium chloride). The plates were incubated for 48 h at 35 °C and stored until experimentation (no longer than 2 days).

2.3. Determination of ammonia triggering point of different Steinernema species

Ten *G. mellonella* larvae were placed in 24-well plates (one larvae in one chamber) filled with silver sand, then exposed to 20 IJ (1 well plate was prepared for each species). After 48 h, cadavers were washed in tap water and changed to 9-well plates filled with double strength agar (one cadaver in one chamber). The cadavers were checked every 24 h, under the stereomicroscope, for IJ emergence. When the first IJ were detected emerging from the cadavers, determination of the ammonia concentration was done, using the methodology proposed by Dorich and Nelson (1983) for soil samples and modified by San-Blas et al. (2008) for insect samples. The quantity of ammonia was determined by a direct colorimetric measurement (at 636 nm) of ammonium in 2 M potassium chloride extracts from infected *G. mellonella* larvae. The infected larvae were weighed and their body contents were exposed by dissection; 2 M KCl was added at a rate of 5 ml 2 M KCl * g *G. mellonella*$^{-1}$. After 1 h incubation (30 °C), 0.5 ml of the resulting suspension was extracted and centrifuged at 6000 rpm for 10 min. An aliquot of 0.020 ml of the centrifuged sample was used for ammonia determination by adding 2 ml of phenol–nitroprusside reagent followed by 4 ml of buffered hypochlorite reagent (Dorich and Nelson, 1983). The results were presented as mg of NH_4^+–N g of *G. mellonella*$^{-1}$.

The experiment was done 3 times. ANOVA was used to analyze the mean of the results, with a significance level of 0.05, and the results are presented as the mean ± standard error of the mean.

2.4. Effect of ammonia concentration on juvenile recovery and mortality

A series of 5 flasks containing 100 ml of bacterial symbiont suspension (grown in nutrient broth) was prepared for each EPN species, 48 h prior to the experiment, at 35 °C. A stock solution of ammonia hydroxide (30% of NH_4OH) was made at 0.1 M and aliquots were added into the flasks (0, 2.225, 5.558, 11.11 and 22,234 ml to have a final flask gradient equivalent to 0, 0.1, 0.25, 0.5 or 1 mg of NH_4–N * ml of bacterial broth^{-1}). The resulting bacteria+ammonia suspensions were poured into wells of a 96 well plate (0.1 ml per well). Ten IJ were placed into each well with their respective bacteria+ammonia broth, then placed in the incubator at 25 °C for 48 h. The nematodes were observed directly under a light microscope (400X) to assess the number of recovered nematodes (i.e., mouths open and moulted to J4-stage), and the nematode mortality rate (probing them with a needle). The experiment was replicated 5 times and repeated 3 times. Data of recovery and mortality were percentages and angular