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Effect of soil salinity on entomopathogenic nematode survival and behaviour

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Summary – Soil salinity, measured as electroconductivity (dS m^{-1}), is a major problem in crop production, including areas where entomopathogenic nematodes (EPN) are applied as biological control agents. EPN species, primarily *Heterorhabditis*, have been isolated from coastal areas and agricultural soils with high salinity (>4.0 dS m^{-1}). Given the aqueous nature of their environment, soil salinity may play an important role in EPN movement and host finding. We assessed the survival of *Steinernema riobrave*, *S. glaseri*, *Heterorhabditis indica*, *H. sonorensis* and *H. bacteriophora* exposed to saline soils within the range found in agricultural soils. Survival and infectivity were generally unaffected by salinities ranging from 0 to 50 dS m^{-1} (50 dS m^{-1} is similar to the salinity of seawater). Salinity had been shown to negatively impact foraging in *S. riobrave* so additional experiments analysing the behaviour and attraction to host cues by *S. riobrave* and *H. indica* were conducted. Agar-based behavioural assays revealed species-specific responses to salinity. At the higher salinity levels (30 and 50 dS m^{-1}) movement of *H. indica* decreased, the path taken was more circuitous and individuals did not move toward a host. There was a strong antagonistic effect on *H. indica* motility and host-finding behaviour. No significant differences were observed for *S. riobrave* exposed to any of these salinity levels. However, under simulated field conditions, high saline conditions (30 and 50 dS m^{-1}) reduced the distance both *H. indica* and *S. riobrave* travelled toward a host. Both species are used for biological control of weevil pests in orchards where salinities have been recorded up to 20 dS m^{-1}. Field efficacy of EPN applied for biological control in saline soils may be improved by timing applications to avoid late season build-up of salts in irrigated crops and applying the appropriate EPN species.

Keywords – agriculture, foraging, nematode behaviour, salt, soil characteristics.

Dissolved solids, especially salts, accumulate in soil where irrigated and fertilised crops are grown. Soil salinity is consequently a major problem for crop production, especially in arid and semi-arid production regions (Thurston *et al.*, 1994). Salinity is measured as electroconductivity (EC, expressed in dS m^{-1}) where a value of 0 dS m^{-1} is neutral and a value of 50 dS m^{-1} is similar to that of seawater. Soils are considered 'saline' when they exceed threshold values >4.0 dS m^{-1}, although some crops are sensitive to salt concentrations that measure >1.0 dS m^{-1}. The soil-dwelling microinvertebrate community is also sensitive to soil salinity, and the composition of that community is influenced by this characteristic. For example, several species of free-living nematodes have been isolated in numerous extreme habitats, including Antarctic Dry Valleys and the Mojave Desert (Freckman, 1982; Freckman & Virginia, 1997) where soil moisture, extreme temperatures and/or saline soil prohibits survival of other less tolerant species. Free-living nematodes in Antarctica are not found where salinity exceeds 4 dS m^{-1} (Nkem *et al.*, 2006). Entomopathogenic nematodes (EPN) have only one free-living soil stage, which may have characteristics evolved for survival in harsh environmental conditions. They have been isolated from a wide range of natural and managed habitats including woodlands, grasslands, natural pastures, coastal dunes and citrus and row crops (Griffin *et al.*, 1994a; Stock *et al.*, 1999, 2008; Kaspi *et al.*, 2010), demonstrating diverse environmental tolerances.

Entomopathogenic nematodes in the families Heterorhabditidae and Steinernematidae (Nematoda: Rhabditida) are obligate parasites of insects that move through the soil on the water film in the pore space between soil particles. Differences in environmental tolerances, behaviour and ecology among species of EPN are likely to affect their success in saline *versus* non-saline condi-

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tions. The third-stage infective juvenile (IJ) is the only free-living stage and is the only stage that exists outside the host, and thus is most likely to encounter high-salinity condition. IJ locate hosts by following gradients of temperature, CO_2 and other host volatiles, faeces, indirect chemical cues from plants due to host feeding, electromagnetic fields and vibration (Byers & Poinar, 1982; Lewis *et al.*, 1993; Torr *et al.*, 2004; Rasmann *et al.*, 2005; Shapiro-Ilan *et al.*, 2009). IJ have species-specific foraging strategies ranging from 'cruiser' to 'ambushing'. Cruiser nematodes move through the substrate to locate hosts and are most efficient at infecting sedentary larvae belowground. Ambushing nematodes employ a sit-and-wait strategy and are usually associated with insects that move on the substrate surface (Lewis *et al.*, 2006). Once a host is located, the IJ enter the host haemocoel *via* natural openings after which they release symbiotic bacteria (*Xenorhabdus* spp. from *Steinermena* spp. and *Photorhabdus* spp. from *Heterorhabditis* spp.) causing host death usually within 48 h. The bacteria assist in overcoming the host immune response and provide nutrients necessary for nematode development. Nematodes complete 2-3 generations within a host, with tens to hundreds of thousands of new IJ emerging after host resources are depleted.

Given the aqueous nature of their environment, soil salinity may play an important role in EPN movement and host finding. The proximity of Heterorhabditidae populations to coastal environments (Hara *et al.*, 1991) has lead to the hypothesis that they can be transported by seawater (Griffin *et al.*, 1994b) and may survive well in saline environments. However, long term exposure to salt solutions (seawater, NaCl, KCl, CaCl) negatively impacted some *Heterorhaditis* spp. but did not inhibit survival of *Steinernema glaseri* (Griffin *et al.*, 1994b; Thurston *et al.*, 1994). At high concentrations, *S. glaseri* showed sensitivity to all salts, whereas *Heterorhabditis bacteriophora* was only sensitive to NaCl. Specifically, increasing salinity (NaCl) strongly decreased movement (Thurston *et al.*, 1994). In a separate study, the foraging ability of *S. riobrave* was negatively related to the level of soil salinity (Kaspi *et al.*, 2010). While it can be concluded that salinity impacts nematode fitness, there are species-specific differences and the underlying mechanisms are unknown. The objective of this study is to quantify how salinity influences EPN behaviour so that biological control programmes incorporating applications of EPN for control of root-feeding insects may take the species-specific effects of soil salinity into account.

Materials and methods

Aqueous salt solutions (1, 2, 5, 15, 20, 50 $\pm$ 0.25 dS m^{-1}) were made by adding the appropriate amount of Instant Ocean (Aquarium Systems, Mentor, OH, USA) to 1.0 l deionised water (dH_2O), after which the solution was boiled and allowed to cool to ensure complete dissolution of salts. Instant Ocean is designed to be similar to the salts found in seawater and at 35 ppt has a composition of chloride 19 290, sodium 10 780, sulphate 2660, magnesium 1320, potassium 420, calcium 400, carbonate/bicarbonate 200, bromide 56, strontium 8.8, boron 5.6, fluoride 1.0, lithium 0.3 and iodide 0.24 ppm. Aqueous salt solutions were measured as dS m^{-1} using a Hanna Instruments HI255 electroconductivity (EC) probe. To create 'saline soils', sand was washed to remove dissolved solids and baked at 100°C to dry. Two sand types were used; a course grit sand with particle sizes 21.7 (100 mm), 34.7 (0.50 mm), 25.0 (0.250 mm), 14.2 (0.106 mm), 2.0 (0.053 mm) and 2.4 (<0.053 mm) was sieved through a 250 μm screen and then mixed 1 : 4 with 250 grit sand. The sand mixture was moistened to 10% (v/v) with an aqueous salt solution (0, 1, 2, 5, 15, 30, 50 dS m^{-1}) to create the corresponding 'saline soil'.

Nematodes

Steinernema riobrave (strain 355), *S. glaseri* (NC strain), *H. indica*, *H. bacteriophora* and *H. sonorensis* for testing under the different salinity conditions were cultured in last instars of the greater wax moth, *Galleria mellonella* (Kaya & Stock, 1997). Infective juveniles were stored in dH_2O at 20°C for not more than 14 days before testing. *Heterorhabditis sonorensis* was isolated by Kaspi *et al.* (2010) from a citrus orchard in Blythe, CA, USA; the soil EC measured 17.95 dS m^{-1}, pH 7.8 and soil composition of 84.5 : 7.5 : 8.3 (sand : silt : clay). For all experiments, a block represents a temporally separate replicate of the bioassay, using different nematode stock solutions (separate *G. mellonella* infections).

Survival and infectivity bioassay

Survival of all EPN species was assayed by counting live IJ after exposure to soils with different salinities (0, 1, 2, 5, 15, 30, 50 dS m^{-1}). The survival arena consisted of 50 ml centrifuge tubes (Sarstedt, Nümbrecht, Germany) filled with 45 ml of lightly packed sand. Five hundred IJ were added to each tube in 400 μl dH_2O. After 3 days, nematodes were extracted by washing the sand

and separating nematodes using a 400 μm mesh screen. Nematodes were rinsed from the screen and stored in *ca* 40 μl dH_2O until live and dead nematodes were counted.

Infectivity of the recovered IJ following exposure to the salt solutions was determined by adding 20 IJ to a 90 mm Petri plate lined with moistened filter paper (Fisherbrand, Fisher Scientific, Pittsburgh, PA, USA) containing ten *G. mellonella* (Thurston *et al.*, 1994). *Galleria mellonella* used in all bioassays were rinsed in dH_2O prior to use. Three days after exposure began, the filter paper was changed to remove any IJ that had not entered a host. Larvae were checked daily for 10 days for signs of infection. Once a larva showed signs of nematode infection, it was recorded and removed from the arena and the total number of infected *G. mellonella* was summed per treatment.

The study was designed with two blocks, each containing five replicates of each soil salinity level and run for each nematode species. Survival data were analysed using linear regression while infectivity was analysed for species-specific differences with a one-way ANOVA and Tukey's HSD for mean's comparison. To meet assumptions of normality, survival data were square root transformed. There was not a significant block effect in either assay and data were pooled for analysis.

Motility assay

Behavioural responses of *H. indica* and *S. riobrave* to salinity were measured on 2% agar plates made with a selected salinity solution (0, 5, 15, 30, 50 dS m^{-1}) in lieu of dH_2O. Each Petri plate (100 mm × 15 mm; Star Dish, Phoenix Biomedical, Mississauga, ON, Canada) was filled with 50 ml of 'saline' agar. This behavioural assay was conducted with an individual *S. riobrave* or *H. indica* IJ on a 90 mm Petri dish of selected salinity (0, 5, 30, 50 dS m^{-1}). To observe individual responses under saline conditions, velocity (cm s^{-1}), path meander (degrees cm^{-1}) and distance moved (cm) were measured. Meander measures the level of nematode turning/curling during the 3-min observation period. In conditions with exposure to potentially toxic compounds, nematode turning/curling may increase (Spence & Lewis, 2010). Each IJ was transferred to the Petri dish and recorded using a digital microscope imager (Celestron, Torrance, CA, USA) mounted on a dissecting microscope for 3 min or until the IJ left the field of view (3.95 mm × 2.95 mm). The digital video was analysed using behavioural software (EthoVision®). No significant differences existed between the two trials, so the data for each response variable were pooled within each species. The rank-transformed data were analysed using one-way ANOVA, followed by Tukey's HSD for means comparison.

Attraction to host cue

Movement of *H. indica* and *S. riobrave* towards a host cue was measured using a modified Petri dish bioassay. Petri dishes were prepared with 2% agar with salinity solutions (0, 5, 15, 30, 50 dS m^{-1}), as above. Petri dish lids were modified to accommodate the narrow end of one 1.0 ml pipette tip at the perimeter of the lid. Inside the pipette tip were two *G. mellonella* larvae acting as a host cue, the tip was plugged with steel wool and the open end sealed with parafilm. The pipette tip was then inserted through the lid 5 mm from the rim, suspending it 2.0 mm above the agar and sealing it with clay. A concentration gradient of CO_2 was allowed to form for 1 h within the Petri dish. After the hour, *ca* 150 IJ were collected by vacuum filtration and placed on the surface of the agar *via* a hole in the lid centre, which was then sealed with tape. Nematode response to the host cue was measured after another hour by counting the number of IJ moving toward *G. mellonella* or away. Response was quantified as the number of IJ in two 5 mm circles, positive (encircling *G. mellonella* cue in pipette tip) or negative (opposite edge of Petri dish, away from *G. mellonella*). The total number of IJ was then counted on the agar plate.

The response of IJ to a host cue ('Attraction Index') while in a saline environment was measured as the proportion of positive IJ, corrected for negative movement:

$$((\text{No. of IJ in positive circle}) - (\text{No. of IJ in negative circle})) \times (\text{total IJ in Petri dish})^{-1}.$$

There was no significant block effect and data from the two blocks were pooled for analysis. Data for each species were rank-transformed and analysed using one-way ANOVA, followed by Tukey's HSD for mean comparison.

Foraging behaviour

The ability of EPNs to locate their host through soil was tested using a column bioassay (Fig. 1). PVC columns 10.0 cm in length were divided into four 2.5 cm sections and filled with a selected saline soil (0, 5, 30, 50 dS m^{-1}). At the bottom of the column, a 2.0 cm ring with <20 μm screen (Small Parts, Lexington, KY, USA) glued to the

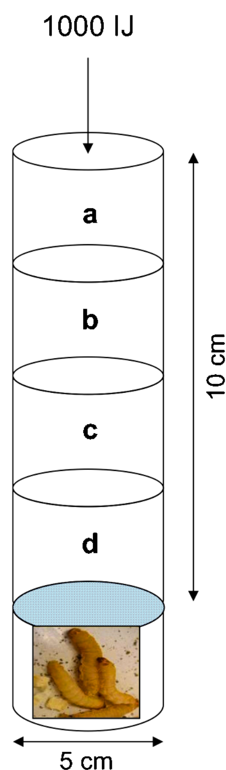

Fig. 1. *Schematic of foraging efficacy column bioassay. This figure is published in colour in the online version of this journal, which can be accessed* via *http://www.brill.nl/nemy*

top prevented the entrance of IJ from the arena sections above. Two *G. mellonella* larvae were placed inside this final section, providing host cues to the foraging IJ. While the IJ were able to move down through the column sections to approach the hosts, the screen prevented host penetration. A Petri plate lid to decrease desiccation topped the columns. One thousand IJ were added to the top of the column and after 3 days the column sections were separated. Nematodes were extracted by washing the sand and separating nematodes using a 400 μm mesh screen. Nematodes were rinsed from the screen and stored in dH_2O until counting. The number of live nematodes in each section was counted and an index was calculated to determine the average distance in the column travelled per individual nematode:

$$(1.25a + 3.75b + 6.25c + 8.75d) \times N^{-1}.$$

The values a, b, *etc*. represent the number of nematodes in a given section, the constants (*i.e.*, 1.25, 3.75, *etc*.) indicate the distance from the top of the column to the midpoint of the section, and N is the total live nematodes per column. This provides a weighted average of the distance travelled by nematodes within the column, with greater values indicating increasing proximity to the hosts. Two or three blocks were conducted for each nematode species each with five replicates per block. Because there was no significant block effect, data from the two blocks were pooled for analysis. The data did not conform to homogeneity of variance; therefore, one-way analysis of variance (ANOVA) was performed on the rank-transformed data followed by Tukey's HSD for mean comparison.

The statistical software program JMP® was used for all statistical analyses (Version 5.1, SAS Institute, Cary, NC, USA).

Results

Survival and infection

Increasing salinity did not reduce survival (Fig. 2). Salinity did significantly increase survival for *H. bacteriophora* ($r^2 = 0.27$, $P < 0.01$), *H. sonorensis* ($r^2 = 0.09$, $P = 0.01$) and *S. glaseri* ($r^2 = 0.06$, $P = 0.03$, linear regression equation shown in figure). There was no significant effect of salinity on survival of *H. indica* ($r^2 = 0.03$, $P = 0.16$) or *S. riobrave* ($r^2 = 0.01$, $P = 0.53$). Infectivity of each nematode species was evaluated against *G. mellonella* larvae at a rate of 2 : 1 nematodes per larvae at each salinity concentration. Only *S. glaseri* and *H. indica* were significantly different from each other ($F_{3,335} = 2.71$, $P = 0.03$) with higher infectivity observed with *S. glaseri* and reduced infectivity with *H. indica*.

Motility assay

Two EPN species, *H. indica* and *S. riobrave*, were selected for further analysis. These species were chosen because they are commercially available and recommended for control of the citrus root weevil (*Diaprepes abbreviatus*) in California citrus orchards. At the higher EC levels, *H. indica* IJ velocity ($F_{3,27} = 8.80$, $P < 0.003$) and distance moved ($F_{3,27} = 3.71$, $P < 0.023$) decreased while path meander increased ($F_{3,27} = 7.74$, $P < 0.001$) relative to the control (0 dS m^{-1}) (Fig. 3). No significant differences were observed in the velocity, distanced moved, or meander of *S. riobrave* IJ exposed to any of these salinity levels (Fig. 3).

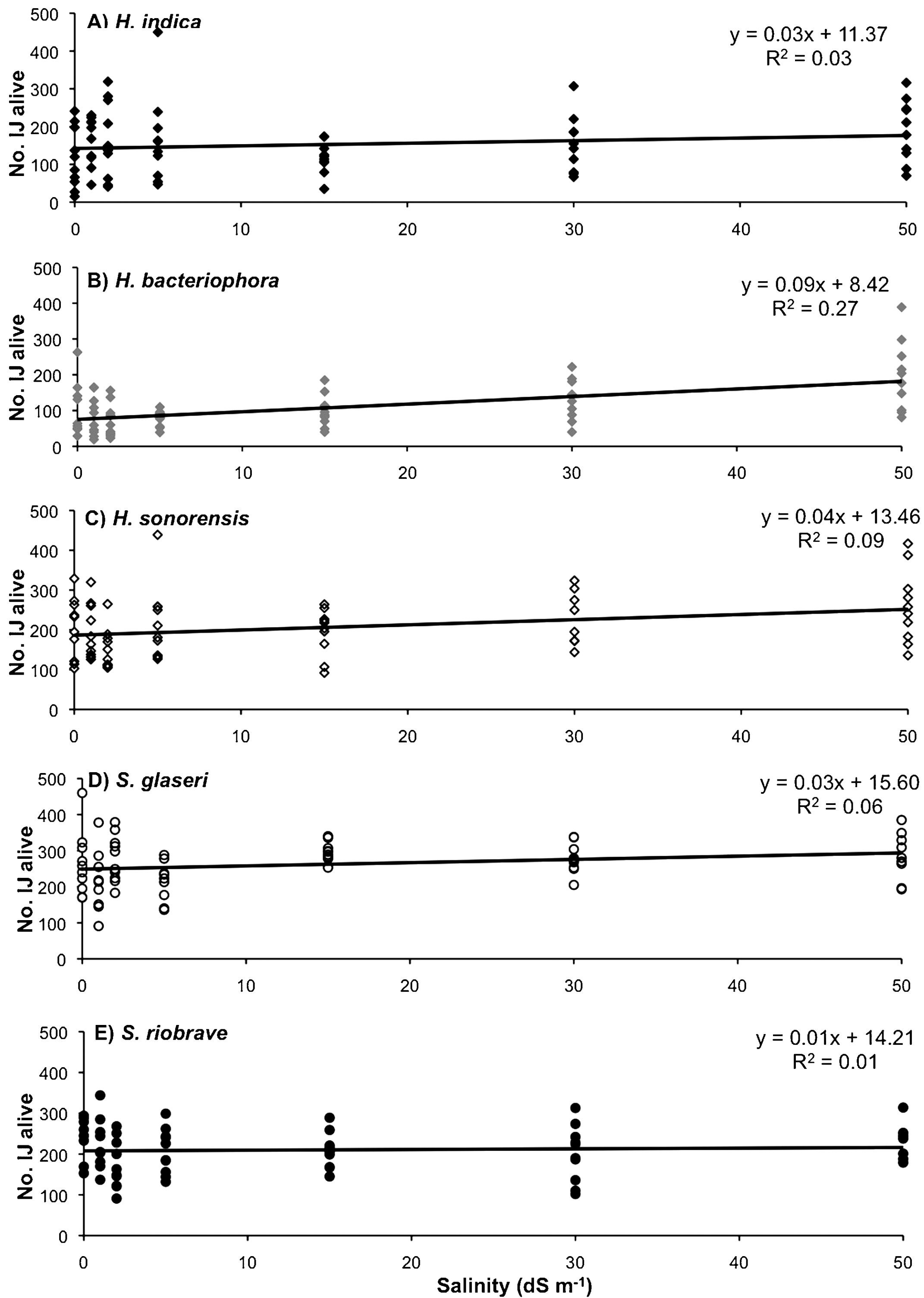

Fig. 2. *Survival of entomopathogenic nematodes under increasing saline conditions. A:* Heterorhabditis indica*; B:* H. bacteriophora*; C:* H. sonorensis*; D:* Steinernema glaseri*; E:* S. riobrave*.*

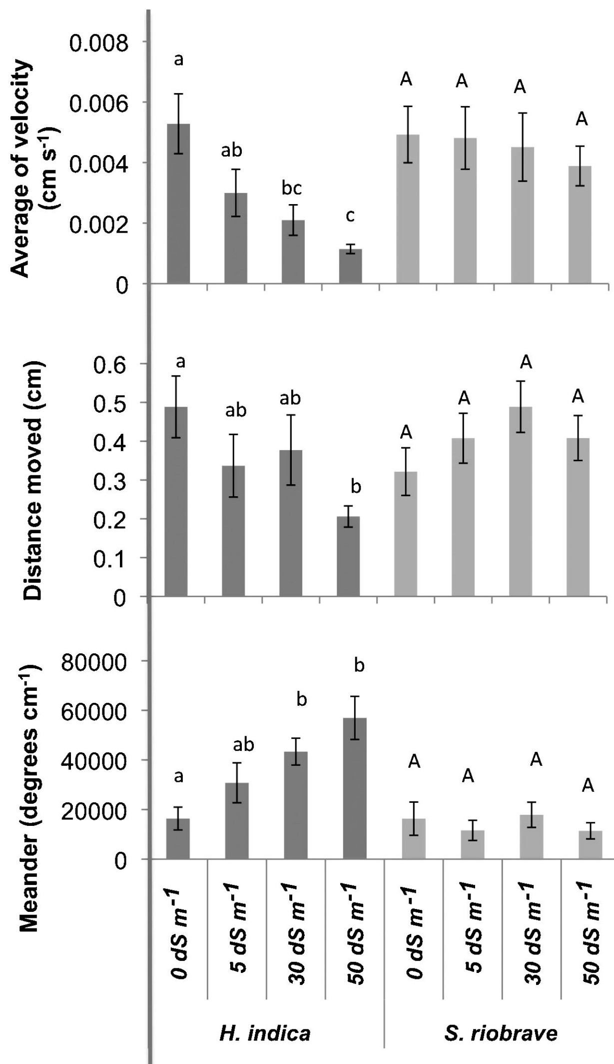

Fig. 3. *Individual behavioural response (mean ± SEM) of* Heterorhabditis indica *and* Steinernema riobrave *during exposure to increasing salinity levels in a motility bioassay. Means with the same letter are not significantly different.*

ATTRACTION TO A HOST CUE

Short-term exposure of *H. indica* and *S. riobrave* to salinity influenced their attraction to a host cue, but the response was species-specific (Fig. 4). All levels of salinity significantly lowered the attraction index of *H. indica* ($F_{4,55} = 53.26$, $P < 0.0001$), with 30 and 50 dS m^{-1} treatments having almost no nematode movement toward the host cue. Attraction to a host cue by *S. riobrave* was not influenced at any of the salinity values ($F_{4,55} = 2.06$, $P = 0.10$) and a response was still observed at salinity levels similar to seawater (50 dS m^{-1}).

FORAGING BEHAVIOUR

Increased salinity (EC) significantly affected the distance moved by *H. indica* and *S. riobrave*. The effect of salinity on foraging behaviour in a 3-D matrix was measured in a soil column as the average distance travelled by a nematode toward a host. Distance travelled by each nematode species decreased under increasing saline conditions (Fig. 5). The dH_2O control did not differ significantly from low saline conditions (5 dS m^{-1}). High saline conditions (30 and 50 dS m^{-1}) reduced the distance *H. indica* and *S. riobrave* travelled within the column (*H. indica*: $F_{3,36} = 25.65$, $P < 0.0001$; *S. riobrave*: $F_{3,55} = 36.08$, $P < 0.0001$).

Discussion

Soil samples taken from multiple locations in California commercial citrus operations have levels of salinity ranging from EC values 0 to 51.78 dS m^{-1} (Kaspi *et al.*, 2010). Of these sites, 25% had saline soils, which emphasizes the importance of identifying how salinity impacts biological control agents such as EPN. Isolation of the EPN species *H. sonorensis* from soils with salinity as high as 18 dS m^{-1} in citrus orchards (Kaspi *et al.*, 2010) suggests that some EPN species may have a higher tolerance for saline conditions than other free-living nematodes. Antarctic nematodes were not found in saline soils exceeding 4 dS m^{-1} (Nkem *et al.*, 2006).

Entomopathogenic nematodes are used as biological control agents against a wide range of soil-dwelling insects, including *D. abbreviatus*, a pest of citrus (Jetter & Godfrey, 2009). EPN species *H. indica* and *S. riobrave* are recommended for control of *D. abbreviatus* larvae in citrus and ornamentals (McCoy *et al.*, 2000). Our laboratory experiments demonstrate that increasing salinity concentrations did not reduce survival of EPN. Infectivity was likewise not reduced by increasing salinity. However, in our study, the nematodes were rinsed and stored in dH_2O for 1 week prior to *G. mellonella* inoculation, during which time IJ can recover from the effects of salinity (Griffin *et al.*, 1994b). Long-term exposure to seawater (without a recovery period) decreases infectivity of *H. bacteriophora* (Griffin *et al.*, 1994b), only at 50 dS m^{-1} EC (data unpubl.).

Although survival and infectivity were generally unaffected by high salinity conditions, high levels of salinity inhibited movement towards a host cue (*G. mellonella*) for *H. indica* but not for *S. riobrave*. This could be caused by

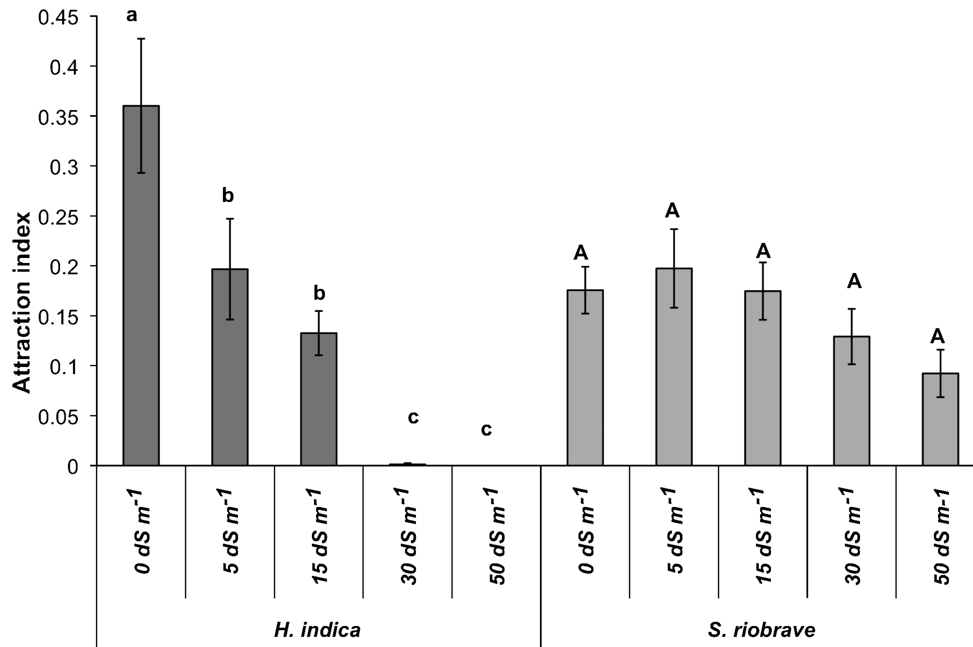

Fig. 4. *Mean ± SEM attraction index of* Heterorhabditis indica *and* Steinernema riobrave *responding to a host cue under saline conditions in Petri dish bioassay. Means with the same letter are not significantly different.*

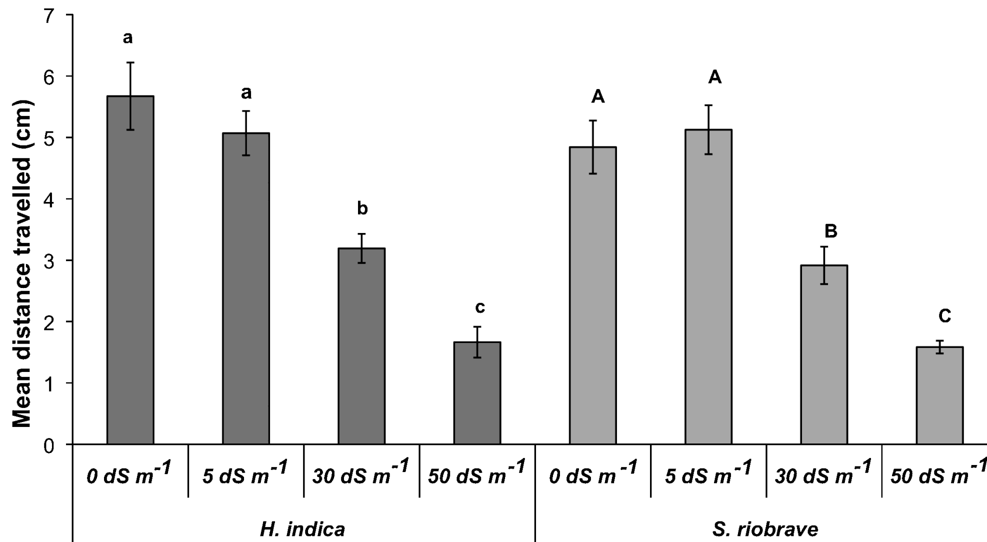

Fig. 5. *Mean ± SEM distance travelled by* Heterorhabditis indica *and* Steinernema riobrave *under saline conditions in a 10 cm sand column towards a host. Means with the same letter are not significantly different.*

disruption of the response to orientation cues or the inhibition of movement of any kind. In absence of a host cue, velocity and distance travelled decreased, while meander of *H. indica* increased, suggesting that *H. indica* cannot move as quickly and mostly in a circular pattern (meander). The same result was not observed for *S. riobrave*, which indicates species-specific differences in the response to environmental conditions. Both EPN species responded similarly in a soil column where foraging distance of *H. indica* and *S. riobrave* was signifi-

cantly shorter through sand moistened with high levels of salt (30 and 50 dS m^{-1}). A reduction in foraging distance is consistent with results in mineral soils for *S. riobrave* (Kaspi *et al.*, 2010). Infectivity of *S. glaseri* (30 dS m^{-1}) and *H. bacteriophora* (16 dS m^{-1}) was not decreased by moderate soil salinities (Thurston *et al.*, 1994). However, the mermithid nematode, *Romanomermis culicovorax*, had decreased survival and infectivity at 4.8 dS m^{-1} (Brown & Platzer, 1978).

Few studies have evaluated the effect of salinity within the range found in agricultural soils, as represented here. In our study, differences in response of *S. riobrave* in the foraging distance versus the behavioural bioassays suggest that in an applied setting (which the column bioassay more closely mimics), foraging of *S. riobrave* is inhibited by high levels of salinity. This could be due in a large part to the longer exposure during the foraging efficacy bioassay. Integration of these nematode species into commercial applications is possible for control of soil dwelling pests such as *D. abbreviates*; however, a good match between soil salinity and biological control agent is necessary and can be determined by soil testing prior to application.

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