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Combined releases of soil predatory mites and provisioning of free-living nematodes for the biological control of root-knot nematodes on 'Micro Tom tomato'

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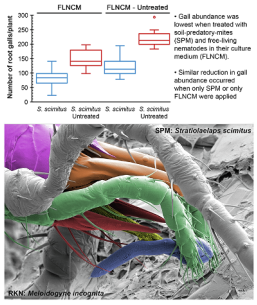
GRAPHICAL ABSTRACT



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ABSTRACT

Soil predatory mites feed on a diverse diet making them excellent candidates for conservation biocontrol. Free-living nematodes (FLN) are commonly found in soils and serve as prey for many acarine predators. Our goal was to determine whether conservation biological control of plant-parasitic nematodes by predators could be enhanced by provisioning FLN with their culture medium (FLNCM) under semi-field conditions. We conducted two experiments on dwarf tomato plants, the first until the beginning of flowering and the second until harvest. The treatments evaluated were with and without: 1) the root-knot nematode *Meloidogyne incognita*, 2) the predator *Stratiolaelaps scimitus*, and 3) the FLN *Rhabditella axei* in its culture medium. In both experiments, gall abundance was lowest in the combined treatment of FLN and predators. Similar reduction in gall abundance occurred when only predators or only FLNCM was added to the soil mix. Additionally, in the FLNCM treatment, foliar macronutrients N and K were significantly higher than the negative control. Our original aim was to use FLN as a supplementary food source for predators in conservation IPM. Based on the significant reduction in gall numbers, and the increase in foliar macronutrients, it is clear that the FLNCM treatment played additional roles.

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Finally, for demonstration, the predation of *M. incognita* was visualized in high resolution imaging using a low-temperature-scanning electron microscope. Accordingly, we recommend that future research focus on identifying soil amendments that will foster the establishment of beneficial microbiota, FLN and soil predators for the conservation biological control of soil pests.

1. Introduction

Phytoparasitic nematodes are important soil pests of agricultural crops, inflicting substantial economic loss (Agrios, 1997). Root-knot nematodes (RKN; *Meloidogyne* spp.) are biotrophic endoparasitic nematodes that attack a wide range of plants, including important agricultural crops (Perry et al., 2009). RKN spend most of their life cycle as sessile life stages inside the root with the motile second-stage juveniles (J2) exposed to predation. Laboratory bioassays with soil mites of different taxonomic groups have demonstrated predation potential on RKN and other plant-parasitic nematodes. *Pergalumna* sp. (Oribatida) has been reported to consume 18 J2 of *Meloidogyne javanica* (Treub) and 42 adults and J2 of *Pratylenchus coffeae* (Zimmermann) per day (Oliveira et al., 2007). *Protogamasellus mica* (Athias-Henriot), a very small predatory mite belonging to Mesostigmata, preyed on 40 J2 of *M. javanica* and 50 specimens of *Pratylenchus zeae* Graham per day (Stirling et al., 2017). Studies reporting on the invasion of greenhouse pot cultures of *Tylenchulus semipenetrans* Cobb and *Meloidogyne incognita* (Kofoid & White) by mites belonging to Prostigmata and Mesostigmata are additional indications that predatory mites play a role in plant-parasitic nematode control (Walter and Kaplan, 1991; Walter et al., 1993).

While plant-parasitic nematodes receive scientific attention due to the economic losses they inflict on crops, they are actually far less abundant in soils than free-living nematodes (FLN). This is true for agricultural soils and especially so in natural ecosystems. FLN provide important eco-services such as increased nutrient availability and enhanced colonization of plant growth promoting rhizobacteria (de Vries et al., 2013; Holajjer et al., 2016; Knox et al., 2004; Neher, 2010).

Predation of FLN has been reported for numerous mite species from different families, mostly belonging to the suborder Mesostigmata (Castilho et al., 2009; Epsky et al., 1988; Walter, 1987; Walter, 1988; Walter and Ikonen, 1989; Walter and Stirling, 2018). FLN are high quality food due to essential nutrients such as omega 3 fatty acids (Menzel et al., 2018). Interestingly, in several demographic studies on the potential of novel acarine biocontrol agents, FLN seemed to be more suitable as prey than the intended target pests. For example, the fecundity of *Lasioseius floridensis* Berlese (Mesostigmata: Blattisociidae) was five-fold higher when fed the FLN *Rhabditella axei* (Cobbold) than when it was fed the broad mite *Polyphagotarsonemus latus* (Banks) (Britto et al., 2012). Similarly, fecundity of *Cosmolaelaps jaboticabalensis* Moreira, Klompen and Moraes (Mesostigmata: Laelapidae) (Moreira et al., 2015) and *Macrocheles embersoni* Azevedo, Berto and Castilho (Mesostigmata: Macrochelidae) (Azevedo et al., 2018) was significantly higher when fed *R. axei* compared to their designated pests, the western flower thrips (WFT) *Frankliniella occidentalis* (Pergande) and the stable fly *Stomoxys calcitrans* (L.), respectively. Immatures of *Parasitus bituberosus* Karg successfully completed development when fed *R. axei*, but were unable to do so when only fed WFT. However, the fecundity of *P. bituberosus* was higher when fed both diets in comparison to each provisioned alone (Rueda-Ramírez et al., 2019).

Provisioning supplemental food to boost predator abundance and maintain predator populations during low prey abundance periods has been reported in numerous studies. For example, pollen applied with blowers (Pijnakker et al., 2016) and released from hedge rows (Duso et al., 2004) or cover crops (Maoz et al., 2011; Smith and Papacek, 1991; Warburg et al., 2019) improved the conservation of phytoseiid (Mesostigmata: Phytoseiidae) mites in biological control scenarios. Likewise, sachets containing species of the mite cohort Astigmatina (OConnor, 2009) and their respective diet have been developed as open rearing units for several phytoseiid species to support the control of greenhouse pests (Calvo et al., 2015; Sampson, 1998; Shipp and Wang, 2003). Astigmatina have also been used as alternative prey to conserve populations of *Macrocheles robustulus* (Berlese), enhancing the biological control of sciarid flies (Grosman et al., 2011), and for *Cosmolaelaps* sp. for the control of prepupae and pupae of the WFT (Munoz Cardenas, 2017). In our recent case study on conservation biological control of the house fly *Musca domestica* L. with the predatory mite *M. embersoni*, the provisioning of *R. axei*, in its culture media of decomposing bean, significantly enhanced fly control, whereas the release of the predator without the FLN did not (Azevedo et al., 2019). This was attributed to a significantly and substantially higher abundance of *M. embersoni* (three fold) in the FLN provisioned treatment. Interestingly, the fecundity of the predator, determined in small arena experiments, was markedly higher when fed nematodes reared on a culture media of decomposing bean, containing a diverse microbial community, compared to when it was reared on a single species of yeasts.

In the present study our objective was to determine whether this FLN in its culture media could be used to improve the efficacy of the generalist predator *Stratiolaelaps scimitus* (Womersley) in the conservation biological control of the economically important RKN *M. incognita*. With this aim, we conducted two experiments on dwarf tomato plants, the first until the beginning of flowering and the second until harvest. To visualize the act of predation we performed high resolution imaging of *S. scimitus* feeding on *M. incognita* and an additional plant-parasitic nematode of economic importance, the soybean cyst nematode *Heterodera glycines* Ichinohe.

2. Methods & materials

2.1. Experiments 1 & 2 on Micro Tom tomato

2.1.1. Sources of nematodes

In both experiments 1 and 2, the FLN and the plant-parasitic nematodes used were respectively *R. axei* and *M. incognita*. The first nematode was collected from a cow pasture on Escola Superior de Agricultura “Luiz de Queiroz” campus (ESALQ; Piracicaba, São Paulo state), by placing pieces of green bean pods (*Phaseolus vulgaris* L.) as bait in the soil. Ten days later, pieces colonized by *R. axei* and microbes were removed and transferred to a container partially filled with water. The colony was maintained by addition of cut fresh green bean pods and water three times a week (Azevedo et al., 2019). Species identity of *R. axei* was confirmed by L. Carta. Specimens of *M. incognita* were obtained from colonies regularly maintained on tomato plants at the Department of Plant Pathology and Nematology. To obtain eggs and J2 of RKN, infected roots were harvested and egg extraction was performed with 0.05% (v/v) sodium hypochlorite (NaOCl), followed by sucrose flotation, as described by Hussey and Baker (1973). To determine the volume that contained 2,000 mixed eggs and J2, density was assessed in four samples of 5 μl each. Accordingly, the determined volume was added to each pot.

2.1.2. Experimental setups

The experiments were conducted in a walk-in screenhouse of Department of Entomology and Acarology, ESALQ, under uncontrolled ambient conditions. Three main effects, each with negative controls, were evaluated: *S. scimitus* (10 gravid female individuals/pot, released every five days; negative control); FLN *R. axei* (10,000 FLN in 2 mL of decomposing bean medium/pot, provisioned three times a week;

negative control); *M. incognita* (one time inoculation of 2,000 mixed eggs and J2 per pot; negative control) All eight (2*2*2) combined treatments were replicated 12 times in a random block design. The experimental unit consisted of a plastic pot (300 mL) with one wild type 'Micro Tom' tomato plant, known to be semi-resistant to *Meloidogyne* species. One 16-day-old seedling per pot was transplanted into a soil mix containing peat, biochar, pine bark (Basaplant, Artur Nogueira, São Paulo) and vermiculite (expanded type B), mixed with 8 g of NPK 10.10.10 and 4 g of limestone per L of substrate. One plant of each of the combined treatments was placed on an upside-down tray (in total 8 plants), constituting a block, and all bocks were placed side by side onto a screenhouse bench. Plants were watered daily, from the top of the pot, with a hose at minimal pressure. To establish FLN and predators prior to RKN exposure, releases began one day after transplanting and 5 days before RKN inoculation.

Experiment 1 was designed to assess the effects of treatments only on vegetative growth; hence, it ended when plants began to flower, thirty days after RKN inoculation. In this case, no pesticide application was considered necessary. The experiment was conducted from mid-October to mid-December 2018, when average temperature was 22.9 ± 4.2 °C.

Experiment 2 was designed to assess the effects on yield. Thus, the experiment was longer, including two harvests. In addition, RKN infestation level (4,000 RKN mixed eggs and J2 per pot) was double the dose of the first experiment. Also, due to the extended duration of the experiment, insecticide and fungicide applications were done to control whiteflies, spider mites and powdery mildew (see Table 1 for target pests, application dates, active ingredients, dose and spray volume). To avoid direct negative effects of the pesticides to soil fauna, pot soil was covered with aluminum foil before each spray and removed a day later. The experiment was conducted from early March to late June 2019, when average temperature was 21.7 ± 4.8 °C. Bi-weekly releases of *S. scimitus* were conducted until the end of May (four weeks before the experiment ended) but the provisioning of *R. axei* with its culture medium continued until the end of the experiment. Note, although conducted in different months and at ambient conditions, the two experiments were performed under very similar climatic conditions (see temperatures above), ideal for tomato plant growth.

2.1.3. Effect on plant parameters and soil nutrients-Experiment 1

Relative water content (RWC) of leaves was adapted from Barrs and Weatherley (1962). Five leaf discs (1 cm^2) from each of eight plants of each treatment were collected and put in 1.5 mL microtubes with 1 mL of distilled water. The microtubes were weighed before and after the addition of the leaf discs to determine their fresh weight (FW). The discs were kept in distilled water for 3–4 h to hydrate to full turgidity, then excess water was carefully wicked off the leaf discs with filter paper for the turgid weight (TW) determination. Leaf discs were then oven dried at 70 °C for 72 h and weighed to obtain the dry weight (DW). RWC was determined using the equation: RWC (%) = [(FW - DW) / (TW - DW)] × 100

Total plant height, height of the plant to the primary shoot (height to insertion of the first inflorescence) and to the main stem, and lengths of third, fourth and fifth internodes were measured. All leaves of each plant were scanned, and the total area was determined using ImageJ software (Rasband, 2018). Internode length and diameter were determined using a digital caliper (Western Ferramentas, São Paulo, São Paulo state). The above ground parts of each plant were then gathered into a paper bag for dry weight measurement. The root system was carefully separated from the potting mix, washed and imaged with a Lumix Panasonic DMC-FZ300. Root area was calculated with ImageJ software (Rasband, 2018) using the color threshold function and subsequently measured with the "measure" tool.

To assess the effect of provisioning FLN on soil nutrients, only two treatments were compared, with and without FLN, both with *S. scimitus* and RKN. The soil assessments of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), aluminum (Al) and sulfate (S-SO_4) concentrations as well as potential acidity (H + Al) were carried out according to Silva (1999), whereas organic matter and total nitrogen (total N) were determined as described by Raij et al. (2001).

2.1.4. Effect on RKN galls and final number of predatory mites-Experiments 1 & 2

Roots were cut in sections, RKN galls were counted under a dissecting microscope and roots from each plant were gathered into a paper bag for dry weight measurement. To evaluate the final abundance of mites (at the end of the experiment), the potting mix of each pot was collected in a cylinder (10 cm diameter × 6 cm high), the bottom was covered with a coarse 2 mm mesh window screen serving as a sieve to catch the potting mix but allowing free movement of mites. For mite extraction, these sieves were kept in modified Berlese funnels for 10 days until the soil mix was completely dry. To prevent escape and contamination between treatments, the outer rims of the funnels, above and below, were coated with a thin layer of petroleum jelly. *Stratiolaelaps scimitus* were subsequently counted under a dissecting microscope in 70% ethanol.

2.1.5. Effect on leaf nutrient analysis, yield and fruit quality-Experiment 2

In experiment 2, leaf nutrient analysis was performed for all treatments. Mature leaves were dried in an oven with forced circulation at 60 °C until weight stabilization, and then crushed. Given the minimum requirement of 1 g of leaf per sample for the analysis to be performed, pot foliage from pairs of blocks were combined according to the treatment (blocks 1 + 2, 3 + 4, etc.) yielding 6 replicates. The evaluated nutrients (following Malavolta et al., (1987) were N, P, K, S, boron (B), calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn) and zinc (Zn).

The first harvest was performed at the beginning of June, three months from the initiation of the experiment, picking only mature red fruits. The second and final harvest was conducted three weeks later when the experiment ended. At each harvest, the number of fruits, fruit weight and total soluble solid content (Brix), determined with a digital refractometer (PR-101, Atago, Tokyo, Japan) from at least 5 fruits per plant, were evaluated. Yield data were analyzed for each harvest separately and as sums for total yield.

2.2. Statistical analysis

Three-way (2 × 2 × 2) analysis of variance (ANOVA) with blocks were used to determine the effects of predator <*S. scimitus*> (2 levels), FLN <*R. axei*> (2 levels) and root-knot nematodes <*M. incognita*> (2 levels) on plant parameters (Relative water content, leaf dry weight,

Table 1
Target pests, application dates, active ingredients, amount and volume of pesticides applied in experiment 2.

Target pest	Date	Active ingredient	Amount	Volume
Whiteflies	01/04/2019	Thiamethoxam	0.4 g	2 l
Whiteflies	14/04/2019	*Beauveria bassiana* (8 X 10^9 CFU/g)	1.5 g	2 l
Spider mites	27/04/2019	Abamectin	1.5 mL	2 l
Sphaerotheca fuliginea	03/06/2019	Sulfur	4 g	2 l

root dry weight, shoot/root dry weight ratio, total height, primary shoot, main shoot, internode length 3, 4 and 5, internode diameter 3, 4 and 5, leaf area, root area) for the first experiment and leaf nutrient analysis (N, P, K, Ca, Mg, S, B, Cu, Mn, Fe and Zn) for the second experiment. Cook's distance was used to detect outliers on the variables leaf dry weight, root dry weight, shoot/root dry weight ratio, total height and internode diameter 3, while the variable main shoot was transformed with logarithm. Outliers were deleted after calculation of Cook's distance and after corroborating that the data influenced the measures of position and dispersion of the data set. In some cases, the outliers corresponded to the same repetition for different variables (McDonald, 2002), which was an indicator that that repetition could have had an external influence. Additionally, logarithmic transformation was performed to meet the assumptions of normal distributions and homoscedasticity.

One-way analysis of variance (ANOVA) with blocks were used to determine effects of FLN <*R. axei*> (2 levels) on the soil nutrients (Organic matter, N, P, K, Ca, Mg, S, Al, H + Al, Sum of Base (SB), cation-exchange capacity (CEC), base Saturation (V), aluminum saturation (m)) for the first experiment.

Two-way (2 × 2) analysis of variance (ANOVA) with blocks were used to determine the effects of predator <*S. scimitus*> (2 levels) and FLN <*R. axei*> (2 levels) on galls/plant for both experiments.

Due to the absence of mites in the pots without the release of predators in the first experiment, a two-way analysis (2 × 2) analysis of variance (ANOVA) with blocks were used to determine the effects of FLN <*R. axei*> (2 levels) and root-knot nematodes <*M. incognita*> (2 levels) on predatory mite abundance. However, due to the migration of mites to pots without the release of the predators in the second experiment, three-way (2 × 2 × 2) analysis of variance (ANOVA) with blocks were used to determine the effects of predator <*S. scimitus*> (2 levels), FLN <*R. axei*> (2 levels) and root-knot nematodes <*M. incognita*> (2 levels) on predatory mite abundance. Cook's distance was used to detect outliers on the predatory mite abundance for the second experiment in order to meet the assumptions of normal distributions and homoscedasticity.

All the statistical analyses were performed with the software R (version 3.5.2, 2019; packages lme4, car, MASS, ExpDes.pt and hnp).

2.3. Scanning electron microscopy of nematode predation by Stratiolaelaps scimitus

To visualize predation, predatory mites were cryoimmobilized while actively feeding on nematodes prior to examination by low-temperature-scanning electron microscopy (LTSEM). Towards this aim, a container of *S. scimitus* was purchased from IPM Laboratories Inc (Locke, NY). Before imaging, adults and deutonymphs were starved for 3–4 days, but were provided with water twice a day. *Meloidogyne incognita* and *H. glycines* were grown in the greenhouse at the USDA Beltsville Agricultural Research Center and cleaned according to methods in Wen et al. (2019). Immediately prior to imaging, one of the nematode species were suspended in 10 µl water and dropped onto filter paper, glued onto a standard brass plate for LTSEM imaging. Mites were observed under a stereomicroscope until they were seen feeding on the nematodes, at which point they were conductively frozen by quickly placing the brass plate onto the surface of a pre-cooled (-196 °C) brass bar whose lower half was submerged in liquid nitrogen. After 20–30 s, the brass plate containing the frozen sample was transferred to the Quorum PP2000 cryo transfer system (Quorum Technologies, East Sussex, UK) attached to an S-4700 field emission scanning electron microscope (Hitachi High Technologies America, Inc., Dallas, TX). The specimens were freeze-etched inside the cryotransfer system to remove any surface contamination (condensed water vapor) by raising the temperature of the stage to −90 °C for 10–15 min. Following etching, the temperature inside the chamber was lowered below −130 °C, and the specimens were coated with a 10 nm layer of platinum using a magnetron sputter head equipped with a platinum target. The specimens were transferred to a pre-cooled (-130 °C) cryostage in the SEM for observation. An accelerating voltage of 5 kV was used to view the specimens. Images were captured using a 4pi Analysis System (Durham, NC).

3. Results

3.1. Effects of S. scimitus, R. axei and M. incognita on plant fitness and nutrients

In the first experiment no significant effects were found for relative water content, plant height, internode length and diameter, leaf area and dry weight of leaves and roots. Nor was there any substantial effect on yield parameters in the second experiment. The only significant plant parameter effect recorded was in the first experiment where *S. scimitus* had a positive effect on root area compared to its negative control (29.8 ± 1.0 vs. 26.6 ± 0.8 cm^2; $F_{1,47}$ = 7.5, P = 0.0075).

In the soil analysis of the first experiment, mean K level was substantially and significantly ($F_{1,9}$ = 16.4, P = 0.0029) lower (151 mg/dm^3) in the treatments provisioned with *R. axei* (415 mg/dm^3), compared with the negative control (566 mg/dm^3) (Table 2). The only other very significant positive effect by *R. axei* was on the nutrient magnesium ($F_{1,9}$ = 24.0, P = 0.0009). In the second experiment, significantly lower levels of P (7.0 ± 0.3 vs. 8.8 ± 0.3 g/kg; $F_{1,23}$ = 21.7, P less than 0.0001) and K (23.0 ± 1.1 vs. 28.3 ± 1.8 g/kg; $F_{1,22}$ = 8.5, P = 0.0062) were observed in leaves of plants infested with *M. incognita* than in the control treatments. Conversely, significantly higher levels of N (22.9 ± 0.5 vs. 21.3 ± 0.4 g/kg; $F_{1,23}$ = 6.1, P = 0.0189) and K (28.7 ± 1.8 vs. 22.6 ± 0.9 g/kg; $F_{1,22}$ = 11.0, P = 0.0022) were observed on plants receiving periodic applications of *R. axei*. Releases of *S. scimitus* did not affect levels of leaf nutrients. When considering the combined treatments of *R. axei* and *M. incognita*, N and K foliar levels were highest in plants provisioned with *R. axei* + negative control of *M. incognita* (Fig. 1a, 1c). N was lowest in the negative control of *R. axei* regardless of *M. incognita* inoculation. P was lowest in the plants inoculated with *M. incognita* with no effect of *R. axei* provisioning (Fig. 1b). In contrast, no clear trends on the effects of *M. incognita* and *R. axei* on microelement levels were observed.

Table 2
Effects of the free-living nematode (FLN) *Rhabditella axei*, provisioned three times a week with its microbial community (administered in its culture medium composed of decaying bean) on soil nutrients, 5 weeks post inoculation of *Meloidogyne incognita,* experiment 1. Different lower case letters following nutrient values between columns (in the same row) indicate significant differences with P values less than 0.05.

	Treatment Control	*Rhabditella axei*
Organic matter (Carbon) (g.dm^{-3})	110.7 ± 4.7 a	121.7 ± 6 a
N (mg/kg)	1081.5 ± 61.2 a	1064 ± 60.4 a
P (mg/dm^3)	592.6 ± 47.5 a	582.6 ± 31.3 a
K (mg/dm^3)	566.4 ± 30.5 a	414.7 ± 42.7b
Ca (cmol/dm^3)	14.4 ± 0.3 a	14.9 ± 0.5 a
Mg (cmol/dm^3)	3.4 ± 0.1 a	4 ± 0.1b
S (mg/dm^3)	120 ± 8.8 a	131.2 ± 12.5 a
Al (cmol/dm^3)	0.1 ± 0 a	0.1 ± 0 a
H + Al (cmol/dm^3)	4.8 ± 0.2 a	5.2 ± 0.2 a
Sum of Base (SB) (cmol/dm^3)	19.3 ± 0.4 a	19.9 ± 0.6 a
Cation-exchange capacity (CEC) (cmolc/dm^3)	24 ± 0.5 a	25.1 ± 0.5 a
Base Saturation (V) (%)	80.1 ± 0.7 a	79.3 ± 1.2 a
Aluminum saturation (m) (%)	0.6 ± 0.1 a	0.4 ± 0.1 a

Note: The two treatments compared were with and without *R. axei*, with the releases of *Stratiolaelaps scimitus* and with the inoculation of *Meloidogyne incognita*.

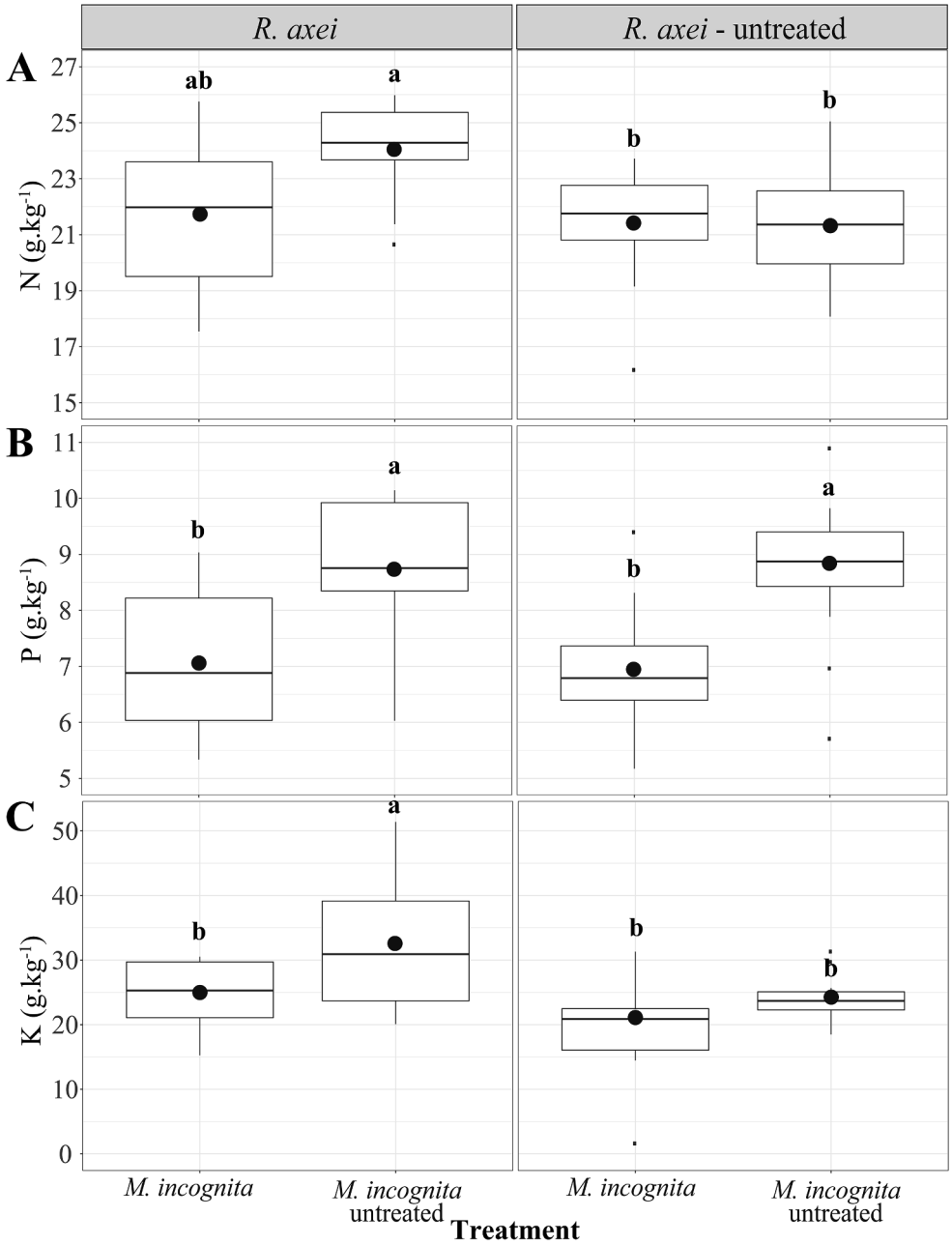

Fig. 1. Effects of the free-living nematode *Rhabditella axei*, provisioned three times a week with its microbial community (administered in its culture medium composed of decaying bean) and the root-knot nematode *Meloidogyne incognita* inoculated once, on leaf macro nutrients of Micro Tom dwarfed tomato, 15 weeks post RKN inoculation: (A) N ($g.kg^{-1}$); (B) P ($g.kg^{-1}$); (C) K ($g.kg^{-1}$). Different lower case letters above box plots, within the same macro nutrient, indicate significant differences between combined treatments with P values less than 0.05. Circles indicate means.

3.2. Effects of S. scimitus and R. axei on number of galls

No galls were found where RKN was not inoculated. While galls were only discernable under the stereo microscope in the first experiment, in the second they were clearly visible to the naked eye, in which RKN were inoculated at a much higher rate. In both experiments, the number of galls per plant was lowest in the combined treatment of *S. scimitus* and *R. axei* (7 and 85 galls/plant, respectively in experiment 1 and 2) and highest (48 and 222 galls/plant) without predators or FLNs (Fig. 2a, 2b). In the first experiment the effects of *S. scimitus* ($F_{1,19}$ = 15.4, P = 0.0004) and *R. axei* were very significant ($F_{1,19}$ = 18.6, P = 0.0001) while the interaction was not ($F_{3,9}$ = 0.6, P = 0.40). Only releasing *S. scimitus* was similar to the non-treated control, whereas the sole provisioning of FLN differed significantly from the latter. In the second experiment, the effects of *S. scimitus* ($F_{1,23}$ = 126.6, P less than 0.0001) and *R. axei* ($F_{1,23}$ = 55.9, P less than 0.0001) were even more significant, as well as their interaction ($F_{3,11}$ = 7.0, P less than 0.0125). Releasing only predators without FLN or provisioning only FLN without predators had similar effects on the number of galls per pot, both differing from the combined treatment of FLN and predators and the non-treated control.

3.3. Effects of M. incognita and R. axei on S. scimitus final abundance

In the first experiment *S. scimitus* was only recovered (at the end of the experiment) from pots into which they had been released (Fig. 3a). *Rhabditella axei* significantly ($F_{1,23}$ = 12.2, P = 0.0014) enhanced the abundance of *S. scimitus* whereas *M. incognita* did not. In contrast, in the

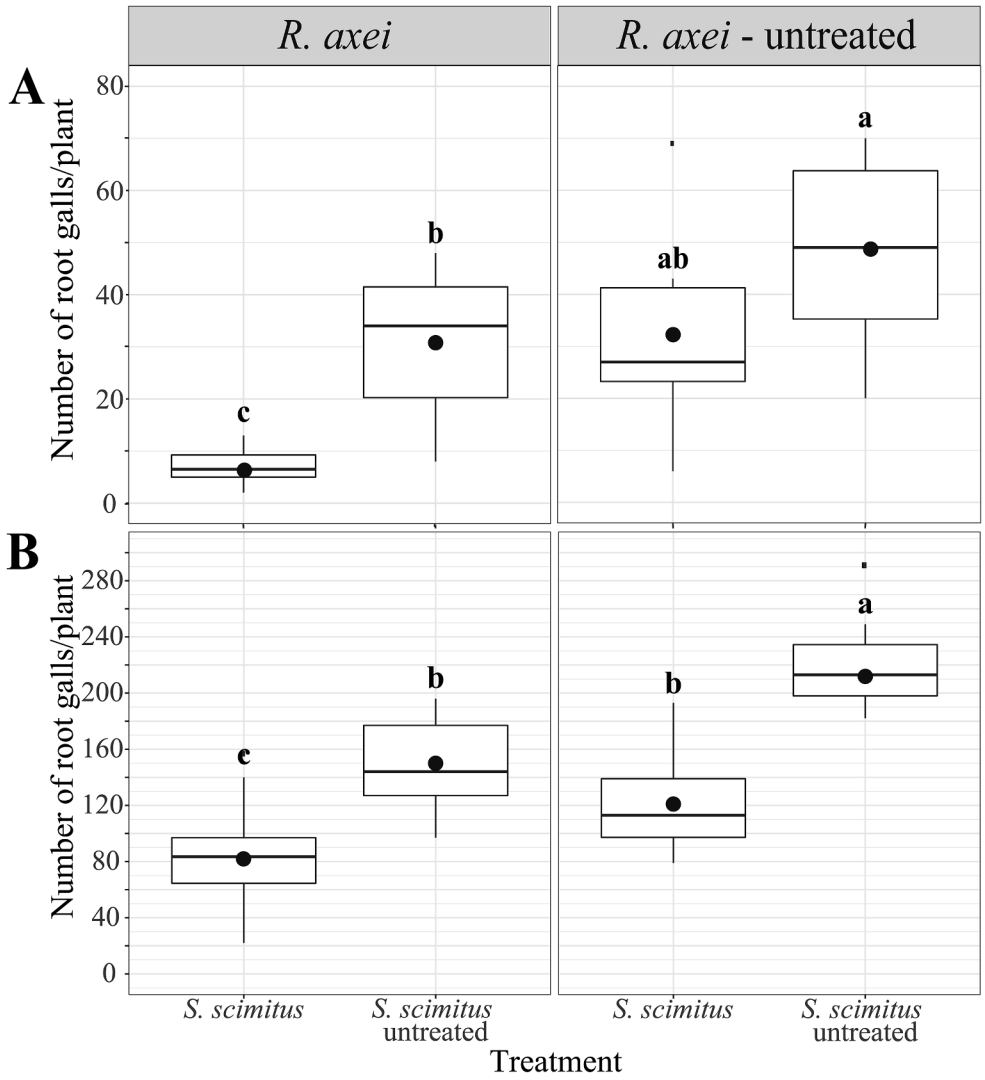

Fig. 2. Effect of the predatory mite *Stratiolaelaps scimitus* released bi-weekly and the free living nematode *Rhabditella axei*, provisioned three times a week with its microbial community (administered in its culture medium composed of decaying bean) on gall abundance caused by the root knot nematode (RKN) *Meloidogyne incognita*. (A) Experiment 1, five weeks post RKN inoculation. (B) Experiment 2, 15 weeks post RKN inoculation. Different lower case letters above box plots, within the same experiment, indicate significant differences between combined treatments with P values less than 0.05. Circles indicate means.

second experiment *S. scimitus* were also found (at the end of the experiment) in the treatments where it was not released (Fig. 3b). *Stratiolaelaps scimitus* ($F_{1,45}$ = 8.5, P = 0.0047), *R. axei* ($F_{1,44}$ = 11.1, P = 0.0014) and *M. incognita* ($F_{1,44}$ = 8.5, P = 0.0020) all significantly affected *S. scimitus* abundance. Additionally, the interaction between *S. scimitus* and *R. axei* was significant ($F_{3,21}$ = 7.1, P = 0.0097). As could be expected, *R. axei* and *M. incognita,* both serving as prey, enhanced abundance of *S. scimitus*. In contrast, the releases of *S. scimitus* had a negative effect on predator abundance.

In both experiments, despite bi-weekly releases of 10 mites per pot, the number of *S. scimitus* determined at the end of the experiment from all treatments never exceeded 21 mites/pot (Fig. 3).

3.4. *Stratiolaelaps scimitus predation of plant-parasitic nematodes*

LTSEM imaging of *S. scimitus* predation of the plant-parasitic nematodes *M. incognita* and *H. glycines* are presented in Figs. 4 and 5, respectively. RKN were picked up from the substrate and held between the palps as the labrum, corniculi and chelicera were extended to begin feeding on the nematodes (Fig. 4). The nematode feeding stylet was observed fully extended as the nematode was squeezed by the movement of the palps of the mite (Fig. 4d). During predation episodes of *S. scimitus* multiple J2s of the soybean cyst nematode were consumed (Fig. 5).

4. Discussion

To determine the effects of *S. scimitus* and *R. axei* on RKN, we used the dwarfed 'Micro Tom' tomato cultivar. These plants are very compact and quite uniform, allowing the eight combined treatments to be replicated in 12 blocks in a reduced space. The relative tolerance of this tomato variety to nematodes (Ejima et al., 2011) may explain why we saw no effects on plant fitness, yield or fruit quality, except for the effect on root area in the first experiment. Nevertheless, with high doses of eggs and motile J2 (2,000 and 4,000/pot in the first and second experiments, respectively) we were successful in obtaining a substantial number of galls where *M. incognita* were inoculated and no galls where they were not added. Significantly lower levels of the leaf macronutrients P and K in the second experiment were additional indicators that plants were negatively impacted by the *M. incognita* treatment. Similar reports on the negative effects of *Meloidogyne* species on macronutrient levels have been reported for bean (Melakeberhan et al.,

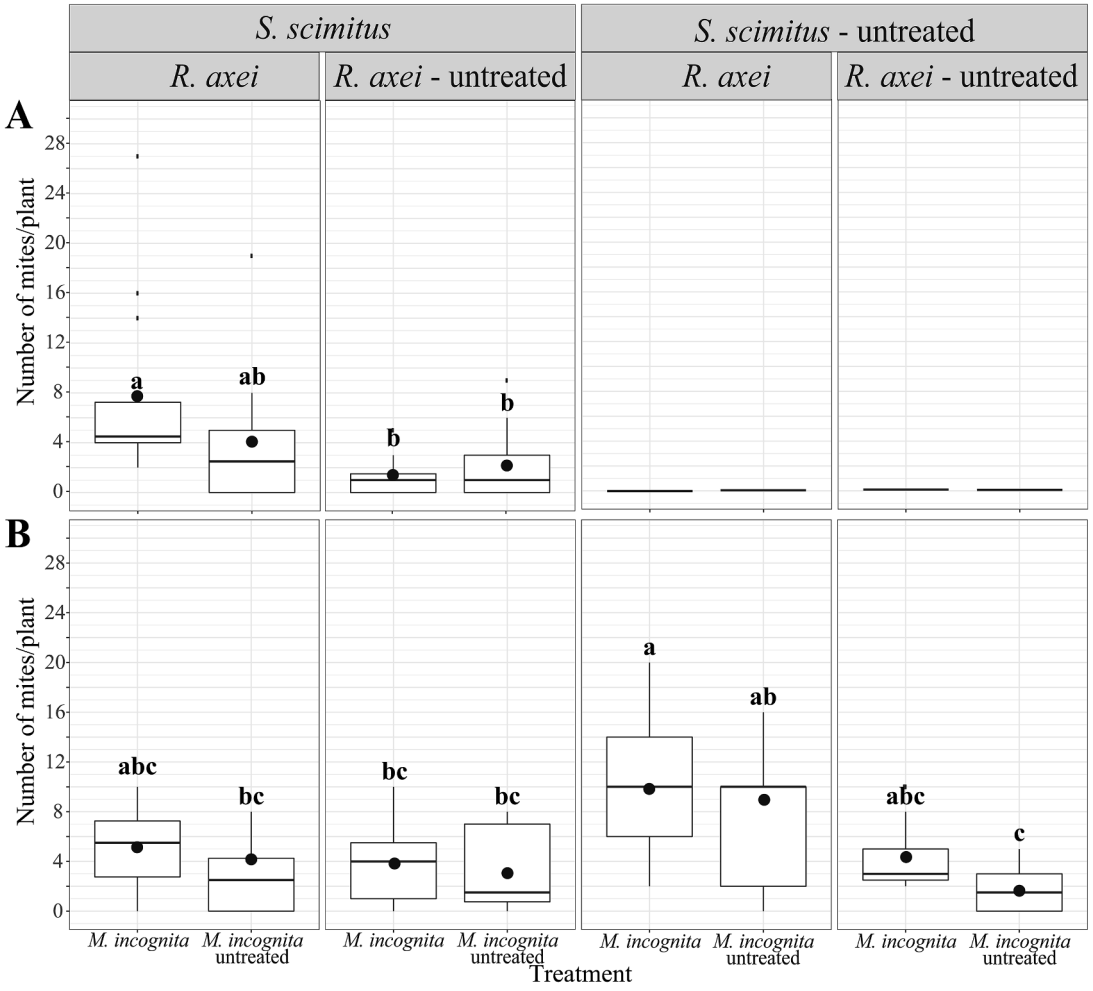

Fig. 3. Effect of the predatory mite *Stratiolaelaps scimitus* released bi-weekly, the free living nematode *Rhabditella axei*, provisioned three times a week with its microbial community (administered in its culture medium composed of decaying bean) and the root knot nematode (RKN) *Meloidogyne incognita* inoculated once, on final *S. scimitus* abundance (at the end of the experiment). (A) Experiment 1, five weeks post RKN inoculation. (B) Experiment 2, 15 weeks post RKN inoculation. Different lower case letters above box plots, within the same experiment, indicate significant differences between combined treatments with P values less than 0.05. Circles indicate means.

1987), tomato (Spiegel et al., 1982) and banana (Devrajan et al., 2003).

In the soil nutrient analysis in the first experiment, the lack of significant differences between treatments with and without FLN for levels of N or P, despite the provisioning of 10,000 FLN in 2 mL of decomposing bean medium/pot three times a week, could be due to the consumption of those nutrients within the FLN culturing medium by the bacterial community there found. Interestingly, the level of potassium in the soil treated with FLN culturing medium had 27% less potassium compared to the negative control. In comparison, in the leaf nutrient analysis in the second experiment, the K level was 26% higher in the FLN culturing medium treatment. Both results, one complementing the other, suggested that K solubilizing rhizobacteria (Parmar and Sindhu, 2013) were active in the culturing medium. The positive effect of *R. axei* provisioning on foliar N was anticipated as FLN are known to contribute to N mineralization (Buchan et al., 2013; Ferris et al., 2004; Neher, 2010).

Previously, we demonstrated that diet supplementation with FLNCM enhanced the predation efficacy of *M. embersoni,* where the housefly was used as a model prey, simulating a pest (Azevedo et al., 2019). What is novel in the present study is that this approach proved effective for RKN, a cosmopolitan pest of economic importance, and that only provisioning FLNCM had an effect similar to only releasing predators on reducing gall abundance. Thus, demonstrating that *S. scimitus* predation was not the sole factor responsible for reducing the number of galls caused by RKN, and that *R. axei* in its culture medium is not only serving as alternative food for *S. scimitus*. In a review on the effects of plant amendments on plant-parasitic nematodes, Thoden et al. (2011) suggested that boosting bacterial populations and subsequent bacterial-feeding nematode numbers could stimulate crop growth, rendering plants less susceptible to plant-parasitic nematodes. Another interesting result in the first experiment was the positive effect of *S. scimitus* on root area. This could be the result of increased soil aeration due to predator activity in the soil mix.

In the first and second experiment, sequential releases of 10 *S. scimitus*/pot were conducted every five days (in total 50 and 120 mites/pot in experiments 1 and 2), yet only a mean of 7 and 10 mites (in the best treatments), respectively, were recovered in the Berlese extractions (at the end of the experiment). This result differs remarkably from our previous study where the single release of six females of *M. embersoni*, with the provisioning of house fly eggs and *R. axei* in a closed ventilated container, developed in four weeks to a mean population of 1,143 mites (extracted with the same Berlese funnels) (Azevedo et al., 2019). While it is clear that S. *scimitus* and *M. embersoni* belong to different families and have very different biological attributes, it seems evident that the open potted plant system vs. closed arenas is at least partially responsible for these differences in predator abundance. Additionally, the potted plant was exposed to daily irrigation applied to the top of the substrate surface, potentially creating a flushing effect, thereby

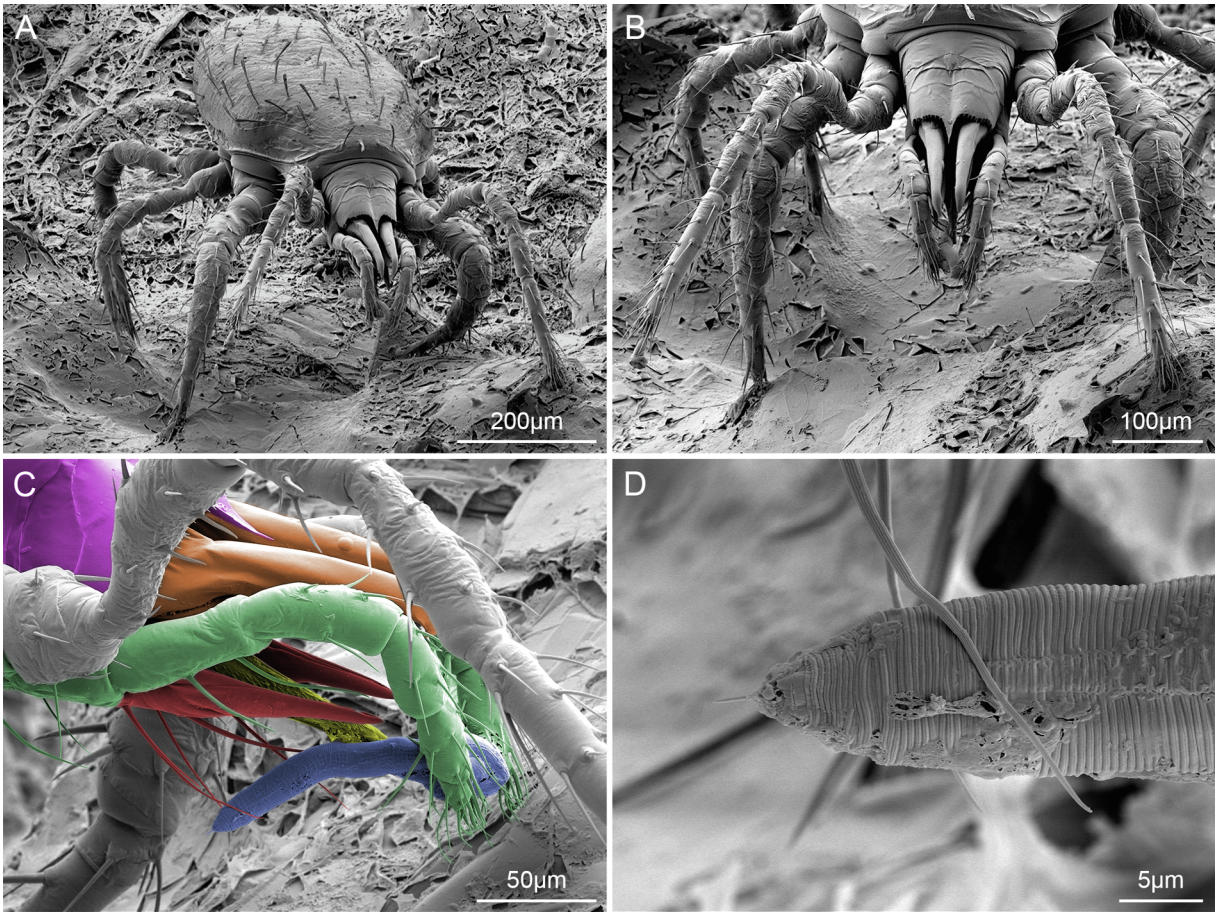

Fig. 4. Low temperature scanning electron microscope (LTSEM) image of the predatory mite *Stratiolaelaps scimitus* feeding on the plant-parasitic root knot nematode *Meloidogyne incognita*.(A, B) *S. scimitus* holding *M. incognita* within the palps as the labrum, corniculi and chelicera are extended to begin feeding on the nematodes. (C) Colorized for clarity: nematode (blue), palps (green), labrum (yellow), corniculus (red), chelicera (orange), subcapitulum (purple). (D) The nematode feeding stylet fully extended as the nematode is squeezed by the palps.

facilitating the outbound predator movement from their release sites. However, it should be noted that this flushing effect is likely an artifact of the potted plant experimental setup, as in real soils, the mites will remain in the upper strata, avoiding submersion. Not anticipated was the highest abundance of *S. scimitus* at the end of the second experiment in pots in which the predator was not released. Two factors could have contributed to this result: 1) A buildup of FLN in the combined treatment of FLN and no releases of predators, allowing for the migrating predators to establish and reach higher abundances. 2) Continued releases of *S. scimitus* had a negative impact on its own abundance, because of cannibalism of immatures by released adult female predators (Berndt et al., 2003), episodes of which we observed repeatedly when handling the commercially produced *S. scimitus* prior to SEM imaging. This raises the question of whether single, multiple or sequential releases of predators should be applied when provisioning with FLN, a question also applicable when conducting releases in greenhouses.

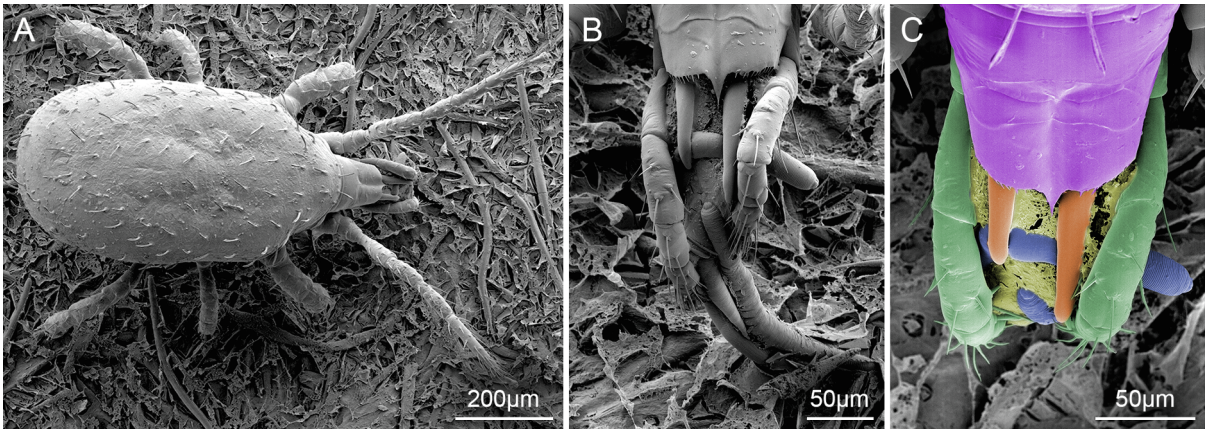

Fig. 5. Low temperature scanning electron microscope (LTSEM) image of the predatory mite *Stratiolaelaps scimitus* feeding on the plant-parasitic soybean cyst nematode *Heterodera glycines*. (A, B) Multiple juveniles of *H. glycines* could be picked up and quickly consumed at once. (C) Colorized for clarity: nematode (blue), palps (green), gut contents of the nematode (yellow), chelicera (orange), subcapitulum (purple).

Additionally, it seems evident that most of the RKN predation was performed by the *S. scimitus* released in the first month of both experiments, because after the J2 colonize the roots, the RKN are protected from *S. scimitus* predation.

Molecular tools have been used in gut analysis to detect and demonstrate nematode predation in Mesostigmata and Oribatida (Heidemann et al., 2011; Read et al., 2006). In the present study, using high resolution LTSEM imaging, we complemented these studies by capturing *S. scimitus* preying on *M. incognita* and *H. glycines*. We believe this to be a significant and important step forward in convincing the biocontrol community at large of the potential of using predatory mites for the conservation biological control of plant-parasitic nematodes.

5. Conclusion

Our original aim in this study was to determine whether the provisioning of the FLN *R. axei,* as supplementary food, would increase the performance of *S. scimitus*. While this clearly occurred, we also found that the standalone FLNCM treatment reduced RKN gall abundance and enhanced foliar nutrient uptake. Based on the literature, FLNs are known to be involved in N mineralization, whereas the substantial effect on foliar K is more likely to be related to rhizobacteria in the culturing medium. In an ongoing study (M.F.P. Moreira unpublished) aimed at characterizing the rhizobacteria in the culturing medium (the same medium used in the present study), species of *Pseudomonas*, *Bacillus* and *Enterobacter* were found. K solubilizing bacteria have been identified from these three genera (Teotia et al., 2016), and one very effective species (Prajapati and Modi, 2012) recently found in that medium is *Enterobacter hormaechei.* The effect of FLNCM on K is extremely interesting as it is involved in many aspects related to plant resistance to pathogens and pests (Wang et al., 2013). Species of *Bacillus* and *Lysinibacillus* another genus of the Bacillaceae family also found in the FLNCM (M.F.P. Moreira, unpublished) produced nematicides toxic to *Meloidogyne* species (Oka et al., 1993; Yang et al., 2012).

Our study emphasizes the importance of an integrated approach for conserving and augmenting elements in the soil food web for plant-parasitic nematode control. We provisioned FLN in their culture media, serving as a food source for soil predatory mites. In agro-ecosystems, soil amendments promote the activity of beneficial soil microbiota (Mazzola and Freilich, 2017), enhance the abundance of FLN and reduce plant-parasitic nematodes (Marahatta et al., 2012). Future interdisciplinary research should focus on identifying soil amendments for specific crops, soils and climates that will foster the establishment and conservation of beneficial microbiota, FLN and soil predators for the conservation biological control of soil pests.

CRediT authorship contribution statement

L.H. Azevedo: Funding acquisition, Conceptualization, Writing - review & editing, Supervision. **M.F.P. Moreira:** Methodology, Conceptualization, Writing - review & editing. **G.G. Pereira:** Methodology, Conceptualization, Writing - review & editing. **V. Borges:** Methodology, Conceptualization, Writing - review & editing. **G.J. Moraes:** Project administration, Conceptualization, Writing - review & editing. **M.M. Inomoto:** Methodology, Conceptualization, Writing - review & editing. **M.H. Vicente:** Methodology, Conceptualization, Writing - review & editing. **M. Desiqueira:** Methodology, Conceptualization, Writing - review & editing. **L.E.P. Peres:** Methodology, Conceptualization, Writing - review & editing. **D. Rueda-Ramírez:** Formal analysis, Conceptualization, Writing - review & editing. **L. Carta:** Methodology, Conceptualization, Writing - review & editing. **S.L.F. Meyer:** Methodology, Conceptualization, Writing - review & editing. **J. Mowery:** Methodology, Conceptualization, Writing - review & editing. **G. Bauchan:** Methodology, Conceptualization, Writing - review & editing. **R. Ochoa:** Methodology, Conceptualization, Writing - review & editing. **E. Palevsky:** Project administration, Funding acquisition, Writing - original draft, Conceptualization, Writing - review & editing.

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