Management of Meloidogyne javanica with biological pesticides and oils in a lettuce field

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HIGHLIGHTS
• Oils and biological agents keep the nematodes population suitably low, reducing farmers’ losses.
• The use of oils and biological agents for managing nematodes does not cause significant damage to the soil microbiota.
• The sequence of lettuce crops causes significant losses to the farmer when effective methods for managing nematodes are not used.

ABSTRACT: Lettuce (Lactuca sativa L.) is a vegetable crop used worldwide in salads. Among the factors limiting its production are the root-knot nematodes, Meloidogyne spp. Since synthetic nematicides cannot be used in lettuce, alternative ways to manage these pathogens are needed. Four consecutive experiments were carried out in an area cultivated with lettuce cv. Regina 2000 and naturally infested with M. javanica. We tested Pochonia chlamydosporia, Trichoderma harzianum, neem oil, castor oil, and nematicide terbufos and calculated the Relative Efficiency of the treatments. The sequence of four crops led to a considerable increase in the population of M. javanica in lettuce when no management method was applied, which made the growth of the crop impracticable after the third crop cycle. The Relative Efficiency was variable in the first three cycles and none of the treatments had an efficiency over 70%; however, in the last cycle, all the treatments had a Relative Efficiency over 77%, and treatments neem oil, castor oil, and terbufos were the most efficient, with Relative Efficiency 93.3, 92 and 94%, respectively.

Keywords: damages, root knot nematodes, Lactuca sativa.

Cite as

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INTRODUCTION
Lettuce (Lactuca sativa L.) is the most consumed leafy vegetable in Brazil and it can be grown throughout the whole year[1]. However, the susceptibility of lettuce to diseases is a limiting factor in its production[2] and root-knot nematodes, Meloidogyne Goeldi, are the major pathogens problems in this vegetable grown under tropical conditions[3]. Ferraz et al.[4] indicate several management measures for Meloidogyne spp., and point out the fundamental importance of knowing the right time to implement them. Among these measures, the use of castor oil (Rinicis communis L.) and neem (Azadirachta indica A. Juss.) has been investigated by many scientists around the world, with promising results[5-6].
Biological control of phytonematodes has also been extensively studied in recent decades and, in this context, it is important to note that fungi, such as *Pochonia chlamydosporia* Zare & Gans (syn. *Verticillium chlamydosporium* Goddard) and *Trichoderma* spp., are already produced on a commercial scale. Soil microorganisms play a fundamental role in the genesis of the soil and also act as regulators of nutrients by decomposing organic matter and cycling elements, acting therefore as a source and drain of nutrients for plant growth. Although alternative measures for managing phytonematodes, such as the use of plant oils and biological control, have been widely studied in the past few years, there is still the need to study how these measures affect soil microorganisms.

That being said, the aim of the present research was to compare the efficiency of essential and fixed oils and biological control in reducing the population of *Meloidogyne javanica* (Treub) Chitwood in a lettuce crop field and to study the impact of these management measures on the microbiota of the soil.

**MATERIAL AND METHODS**

Four consecutive experiments were carried out in a lettuce field cv. Regina 2000, and naturally infested with *M. javanica* in Iúna, ES, Brazil. The following treatments were tested: Pc - biological pesticide containing the biological agent *Pochonia chlamydosporia* (2 kg/ha); Th – biological pesticide containing the biological agent *Trichoderma harzianum* (1 L/ha); Neem – pesticide based on neem oil, at a concentration of 0.45% (or 4.8% of a.i.), with a total of 100 L of solution/ha; Cas - castor oil, using cold extraction at 1% of concentration; Terb - nematicide Terbufos (20 kg/ha) and Cont - control.

The experiments were carried out in randomized blocks, with arrangement 6 (treatments) × 4 (replications). The blocks consisted of beds of 15.0 × 1.5 m, each one being cultivated with approximately 210 plants of lettuce, planted in rows. Each bed/block was divided into six areas (plots) of 2.5 × 1.5 m, randomized and labeled for the treatments, which were kept at the same plots until the end of the experiment. The treatments were applied in the hole dug for the seedling before the beginning of each of the four lettuce crop cycles.

The identification of the species of *Meloidogyne* present in the naturally infested area was made by using the electrophoresis technique of esterase isoenzyme. For that, before the experiment 30 plants with root galls were collected randomly and 10 females were taken from each plant for identification, resulting in a total of 300 analyzed females. The initial nematode population in the soil was determined by Jenkins’ technique and expressed in number of eggs + second stage juveniles (J2) in 100 cc of soil. To this end, 5 soil samples were collected at depths of 0 to 20 cm, randomly, in each of the plots before each treatment. These samples were mixed and homogenized, forming 6 composite samples from 30 single samples per block, which were taken to the laboratory for analysis.

To evaluate the effect of each treatment on the microbiological activity in the soil (MA) the fluorescein diacetate hydrolysis method (FDA), described by Schuner & Rosswall, was used. This method consists of quantifying the fluorescein diacetate hydrolysis in samples containing organic matter.

Calculations of Relative Efficiency of the treatments were made by using formula 1:

\[
RE = \left(1 - \frac{P}{P_C}\right) \times 100
\]

Where: P - incidence or severity of the disease in each treatment evaluated; T - incidence or severity of the disease in Control.

Castor oil was extracted from castor beans (*Ricinus communis* L.) variety IAC 80. For this, the seeds were washed in water and dried in an oven at 40 °C for 24 h. The oil extraction was performed by using the cold pressing method. For this, 1 kg of seeds were placed in a press and then filtered. After extraction, 480 g of oil were obtained. Subsequently, the oil was stored in a sealed glass container, labeled and kept in a room at 25 ± 2 °C and photoperiod of 12 h until the execution of the experiments.

The soil temperature throughout the four experimental periods was monitored by using two thermometers introduced into the soil at 20 cm depth and equidistant in the experimental area, and the temperatures were taken daily at 7 a.m. and 3 p.m., with the mean being calculated for each experimental period. The air temperature and mean precipitation were monitored in the experiment region and provided by the Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural of Espírito Santo - Incaper.

Before collecting the plants, the total chlorophyll content of leaves was evaluated in 5 plants, by using the handset PlantPen NDVI 300® (Photon System Instruments Inc.). At the end of each lettuce crop cycle 5 plants were collected from the center of each plot to avoid edge effects for a total of 20 plants/treatment.
Subsequently, we evaluated the dry matter of aerial parts; number of leaves; stem diameter measured with a digital caliper; number of galls per plant; and the final population of *M. javanica* in the soil, represented by the number of eggs + juveniles per plant, using the method proposed by Hussey and Barker and modified by Boneti & Ferraz\[13\], which consists in triturate the infected roots in a blender with 5% of sodium hypochlorite solution, then collect all the content on 80 (for coarse particles) and 400 (for nematodes eggs and juveniles) Mesh sieves. The content retained in the 400 Mesh sieve was collected in Becker glass and analyzed in Peters chamber under a stereoscopic microscope, and for each sample three counts of eggs and juveniles were made and the mean between them was calculated.

The means of the dry mass of aerial parts, total chlorophyll content of leaves, stem diameter, number of leaves, number of galls per plant, initial nematode population, final population of nematodes and microbial activity were subjected to analysis of variance (ANOVA) and, when significant differences in F test were detected, the Duncan test was used at a level of 5% of significance, by using the R software\[14\].

**RESULTS AND DISCUSSION**

The species present in the experimental area was *M. javanica*, one of the most common species of root-knot nematodes in agricultural areas in Brazil\[15\]. Table 1 shows the experimental periods, the air and soil temperatures and the mean precipitation during the crop cycles.

The initial population of nematodes were low, possible due to the fact that the soil had been fallow for six months before the installation of the experiments, and the mean between the plots did not show statistic differences, thus the general mean of second stage juveniles in 100 mL of soil in the experimental area was 6.3. Finding root-knot nematodes at low populations, even after fallow is common because these pathogens can survive in a dormant state or as parasites of other plants, as highlighted by Costa et al.\[16\], especially in the case of *M. javanica*, a polyphagous species with a wide host range\[3\].

For the analysis of soil microbial activity, there was no effect of treatments during the first three lettuce crop cycles. However, in the last assessment, made after the fourth crop cycle, microbial activity was higher for treatments Pc and Th compared with treatments Neem, Cas and Terb (Figure 1). It is known that some pesticides, like metalaxyl and fenarimol fungicide, can negatively affect the soil microorganisms, as evaluated by Silva et al.\[17\]. In order to protect the agroecosystem, it is important to determine whether nematode management options have adverse effects on microflora and microfauna\[18\]. Although azadirachtin is the most studied substance present in neem plants, other compounds of this plant also show effects on microorganisms, including on plant pathogens\[19\]. This is a characteristic of the plant that makes it not very specific or selective in the control of microorganisms. This may explain the negative impact of the treatment Neem on soil microbial activity in the fourth lettuce crop cycle (Figure 1). The higher microbiological activity in the soil after the fourth crop cycle for treatments Pc and Th, probably occurred due to the accumulation of these microorganisms in the soil throughout each lettuce crop cycle, since in each cycle a new application of antagonists was made, and also due to the fact that these antagonistic organisms have good saprophytic survival capacity in the soil, independent of the presence of nematodes\[20\].

During the experimental period, there was no incidence of leaf or root diseases or insect attack, apart from the nematodes. Therefore, except for the third crop cycle, when there was a slight negative

<table>
<thead>
<tr>
<th>Crop cycles</th>
<th>Experimental period</th>
<th>Duration of the experimental period (days)</th>
<th>Maximum and minimum T of the soil (°C)</th>
<th>Maximum and minimum T of the air (°C)</th>
<th>Average precipitation (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>04/26/2013 – 06/10/2013</td>
<td>46</td>
<td>18.5 to 22</td>
<td>20 to 29</td>
<td>68.2</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>07/06/2013 – 08/21/2013</td>
<td>47</td>
<td>14 to 19</td>
<td>15 to 27</td>
<td>8.2</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>11/01/2013 – 12/20/2013</td>
<td>50</td>
<td>19 to 23</td>
<td>18 to 26</td>
<td>342.5</td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt;</td>
<td>02/04/2014 – 03/20/2014</td>
<td>45</td>
<td>20 to 23</td>
<td>23 to 30</td>
<td>22.1</td>
</tr>
</tbody>
</table>
impact on plant growth due to the high precipitation index (Table 1), damage caused to plants was caused by the parasitism of *M. javanica*.

The Relative Efficiency was variable and none of the treatments had efficiency higher than 70%, except in the fourth cycle when it was higher than 77% for all the treatments (Table 2). It is noticeable that, numerically, an increase in the population of *M. javanica* occurs in the second lettuce crop cycle compared to the first, which was to be expected, since the nematode had contact with the susceptible crop over a longer period of time and that allowed its population to increase. After the third crop cycle it is possible to see that the consecutive lettuce crop without application of any treatment for the control of nematode (Cont) promotes a final population increase of the pathogen compared to the plants that received treatments, and from this cycle on, generally, the number of nematodes tends to increase more and more.

Considering the first three crop cycles, the third presented the highest population of nematodes in plants that received no treatment (control); 7,650 individuals and 18 galls (Figure 2). The concept of economic damage threshold is specific to each crop and it is based on disease intensity, in which the benefit of control is equal to the cost of control. In the same area where this research was carried out, Rabello reported that the economic damage threshold to the lettuce crop cv. Vitória de Santo Antão was 10,151 nematodes/plant or 109 galls/plant. Therefore, the nematode population was not sufficient to cause significant damage to the crop in this study until the third crop cycle.
Table 2. Relative efficiency (%) of six treatments applied in four consecutive cycles of lettuce cv. Regina 2000 (C1, C2, C3 and C4) for management of Meloidogyne javanica in a field naturally infested by the pathogen in Ituã, ES.

<table>
<thead>
<tr>
<th>Treatments*</th>
<th>Final population of nematodes C1</th>
<th>Relative Efficiency 1 (%)</th>
<th>Final population of nematodes C2</th>
<th>Relative Efficiency 2 (%)</th>
<th>Final population of nematodes C3</th>
<th>Relative Efficiency 3 (%)</th>
<th>Final population of nematodes C4</th>
<th>Relative Efficiency 4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pc</td>
<td>1,300</td>
<td>33.33</td>
<td>3,875</td>
<td>25.48</td>
<td>2,975</td>
<td>61.11</td>
<td>3,050</td>
<td>77.81</td>
</tr>
<tr>
<td>Th</td>
<td>1,450</td>
<td>25.64</td>
<td>6,540</td>
<td>0</td>
<td>2,750</td>
<td>64.05</td>
<td>2,025</td>
<td>85.27</td>
</tr>
<tr>
<td>Neem</td>
<td>750</td>
<td>61.53</td>
<td>2,750</td>
<td>47.11</td>
<td>5,425</td>
<td>29.08</td>
<td>925</td>
<td>93.27</td>
</tr>
<tr>
<td>Cas</td>
<td>2,150</td>
<td>0</td>
<td>4,662</td>
<td>10.34</td>
<td>6,350</td>
<td>16.99</td>
<td>1,100</td>
<td>92.00</td>
</tr>
<tr>
<td>Terb</td>
<td>650</td>
<td>66.66</td>
<td>3,950</td>
<td>24.03</td>
<td>4,425</td>
<td>42.15</td>
<td>825</td>
<td>94.00</td>
</tr>
<tr>
<td>Cont</td>
<td>1,950</td>
<td>0</td>
<td>5,200</td>
<td>0</td>
<td>7,650</td>
<td>0</td>
<td>13,750</td>
<td>0</td>
</tr>
</tbody>
</table>

*Treatments: Pc – biological pesticide consisting of the biological agent Pochonia chlamydosporia; Th – biological pesticide consisting of the biological agent Trichoderma harzianum; Neem – pesticide based on neem oil, at a concentration of 0.45% (or 4.8% of a.i.); Cas – castor oil, using cold extraction at 1% of concentration; Terb – nematicide Terbufos (20 kg/ha) and Cont – control.

Figure 2. Final Population of nematodes per plant and Number of galls of each lettuce crop cycle cv. Regina 2000 in the region of Ituã, ES, in soil naturally infested with M. javanica. Pc – biological pesticide consisting of the biological agent Pochonia chlamydosporia; Th – biological pesticide consisting of the biological agent Trichoderma harzianum; Neem – pesticide based on neem oil, at a concentration of 0.45% (or 4.8% of a.i.); Cas – castor oil, using cold extraction at 1% of concentration; Terb – nematicide Terbufos (20 kg/ha) and Cont – control. Means followed by the same letter do not differ between each other by Duncan test at 5% significance level.

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In the fourth crop cycle (Figure 2) it was noted that in the control (Cont) the number of galls per plant and final population of nematodes were considerably higher than the treatments and the control in the previous cycles, which was favored by the consecutive crops and the temperatures, higher than in previous cycles, which is favorable to the reproduction of *M. javanica*.[23]. In this cycle, all treatments were efficient in the management of *M. javanica*. Th, Neem, Cas, and Terb were equally effective in reducing the number of galls per plant and Neem, Cas and Terb were the treatments that most reduced final population of nematodes (Figure 2).

Taylor & Sasser[24] reported that over time an increase in the population of plant nematodes is expected, since these pathogens multiply in a logarithmic scale in the presence of a host plant. Thus, after four lettuce crop cycles, it was expected that the level of inoculum and *M. javanica* would gradually increase in the field. It is important to note that this increase was significantly higher in the control. Rabello[25] noticed that, after three sequential crops, the lettuce crop parasitized by *M. javanica* showed noticeable damage due to growing populations of the nematode, but these were still at a low level, not making the field unviable and not reducing farmers’ income considerably. However, in the fourth crop cycle, only 35% of the plants was marketable, which caused serious losses to farmers.

In the fourth crop cycle the antagonists *P. chlamydosporia* and *T. harzianum* showed Relative Efficiency of 77.8 and 85.3%, respectively (Table 2). Dallemole-Giaretta et al.[26] evaluated the management of *M. javanica* by applying coffee chaff colonized or not by *P. chlamydosporia*, and concluded that the incorporation of the material colonized by the fungus into the soil reduced the number of galls per plant and final population of nematodes by up to 46.6 and 71.7%, respectively. In this study, *P. chlamydosporia* reduced the number of galls per plant and final population of nematodes by 154% and 350.8%, respectively, in the fourth crop cycle, compared to the control. In another study, the isolate Pc-10 of *P. chlamydosporia* caused a reduction in the number of galls per plant and final population of nematodes of *M. javanica*.[27] Lopes et al.[27] also observed the reduction in final population of this nematode in different experiments in which four isolates of fungus were used.

Bourne[28] reported that *P. chlamydosporia* grows on organic matter present in soil, which makes it independent of the presence of nematodes for nutrition. Added to these advantages, the fungus produces chlamydospores, storage structures of nutritional reserves and survival,[29] which probably ensured the increase in the activity of this antagonist over four lettuce crop cycles in this study (Figure 1) and the consequent reduction in the population of *M. javanica*.

In the fourth crop cycle, the reduction in number of galls per plant and final population of *M. javanica* exercised by *Trichoderma* sp. was 250% and 579%, respectively, compared to control. Two mechanisms are used by this antagonist fungus in reducing the population of phytonematodes, which are: direct parasitism on eggs and juveniles through increased chitinase and proteases activity, which is indicative of the ability to infect eggs and induction of host defense mechanisms.[30]

The Relative Efficiency of the pesticides based on neem oil and castor oil was 93.3 and 92%, respectively, only lower than the synthetic nematicide (94%) (Table 2). In plants treated with neem oil a reduction of 460% and 1386.4% was noticed for number of galls per plant and final population of nematodes, respectively, compared to untreated plants (control) in the last lettuce crop cycle (Figure 2). Several authors have demonstrated the effectiveness of neem extracts and oils in the management of *Meloidogyne* spp.[30] The neem effect against nematodes is probably due to the presence of several chemical substances such as azadirachtin, nimbin, solanin, and others. Products based on neem are desirable because they do not affect spiders and adults of several species of beneficial insects. It is possible that, due to its relative selectivity, these products can be recommended in many integrated management programs since they will probably not pollute the environment[30].

The fixed castor oil promoted reduction of number of galls per plant and final population of nematodes up to 600% and 1150%, respectively, in the fourth lettuce crop cycle (Figure 2). The oil extracted from castor beans has ricin, an alkaloid responsible for activity against phytonematodes. The use of oil, cake, dried leaves and stems from castor beans has been related to a decreased population of nematodes as well as the best development of plants.[31] Indeed, in studies done by Akhtar & Mahmood[32], it was proved that the incorporation of fresh castor leaves into the ground caused the same effect as that observed by the nematicide carbofuran in reducing the population of *M. incognita*.

There was no effect of the treatments on the Chlorophyll content at any cycle, what was expected, since *M. javanica* is a root parasite and do not affect directly the lettuce leaves, which is the case of *Septoria lactucae* Pass. and *Cercospora longissima* Cugini. Some types of stress, such as those caused by leaf diseases, are capable of causing significant changes in the photochemical process of plants, because of the lack of intermediaries required in order to follow the chain of events, or the degradation or disorganization of the photosynthetic apparatus. The results of this paper are corroborated by
Bispo[33], who did not observe significant differences in chlorophyll content in guava plants parasitized or not with *M. mayaguensis*.

The treatments did not influence the variables related to the plant growth in the first three crop cycles. Stem diameter was between 15 and 17 mm for Th and Cont, respectively, in the first cycle, 22 and 26 (Cas and Neem) in the second, and 9 and 10 (Cas and Neem) in the third cycle. Number of leaves varied from 45 for Th and Terb to 50 for Cont in the first cycle, 57 to 63 (Cas and Th) in the second, and 54 (Cont and Neem) to 56 (Th) in the third. The variation presented in dry mass of aerial parts was 16 (Cont and Neem) and 20 (Th and Terb) in the first crop cycle, 23 (Cas) and 27 (Pc) in the second, and 19 (Th) and 24 (Th and Neem).

In the third crop cycle (Table 1) temperatures and precipitation index were higher compared to previous crop cycles. Nevertheless, there was no prejudice to the development of lettuce. The lack of effect of treatments on the growth characteristics of plants until the third lettuce crop cycle can be explained by the low population of nematodes in the area because it remained fallow for six months prior to the experiment.

In the fourth cycle, the variables number of leaves and stem diameter were lower in the control compared to all other treatments (Figure 3). A higher number of leaves was noticed in plants treated with nematicide (Terb) compared to Pc, Th, Neem and Cas, whereas, for these four treatments, the averages of number of leaves were equal. The dry mass of aerial parts was lower in the control compared to Neem and Terb. Pc, Th, and Neem were as effective as the nematicide (Terb), not allowing reduction of dry mass of aerial parts (Figure 3).

Lower growth of plants parasitized by root-knot nematodes has been observed by other authors such as Santos et al.[21], who noticed that the stem diameter of lettuce plants cv. Elisa, when they are sown directly in a field infested by *M. javanica*, can be reduced by 18%. The parasitism of the plants by nematodes is characterized by the creation of permanent feeding sites on root tissues, consisting of giant cells in the cortex, endodermis, pericycle and parenchyma[34]. These feeding sites become drains for photoassimilates, and thus impair plant growth and development. Moreover, mechanical damage caused by nematodes when they penetrate and move in plant tissues causes blocking of vascular tissues in the feeding sites, limiting the translocation of water and nutrients, suppressing plant growth and

![Figure 3. Plant evaluations carried out in the fourth lettuce crop cycle cv. Regina 2000 in the region of Iúna, ES, Brazil in soil naturally infested with *M. javanica*. Pc – biological pesticide consisting of the biological agent *Pochonia chlamydosporia*; Th – biological pesticide consisting of the biological agent *Trichoderma harzianum*; Neem – pesticide based on neem oil, at a concentration of 0.45% (or 4.8% of a.i.); Cas – castor oil, using cold extraction at 1% of concentration; Terb – nematicide Terbufos (20 kg/ha) and Cont – control. Means followed by the same letter do not differ between each other by Duncan test at 5% significance level.](http://dx.doi.org/10.4322/nematoda.01515)
crop yield[15], these physiological and mechanical damage lead to reduction plant growth[15], which may explain the decline in variables evaluated in the lettuce crop in this study in the fourth crop cycle.

CONCLUSIONS

We concluded from this work that the sequence of four crops provided a considerable increase in the population of *M. javanica* in lettuce when no management method was used, which impaired the growth of this crop after the third crop cycle.

The Relative Efficiency of antagonists and oils was higher in the fourth cycle, since these treatments showed Relative Efficiency varying between 77.81 and 93.27%.

Treatments did not affect soil microbiota in the first three lettuce crop cycles. However, in the fourth crop cycle, microbiological activity was higher for plants treated with *P. chlamydosporia* and *T. harzianum*. Even if the microbiological activity of the soil had been negatively affected by neem and castor oils, it is important to highlight their efficiency in the management of *M. javanica*, since these treatments significantly reduced the final population of nematodes and ensured greater crop yield.

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