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Brevibacillus laterosporus as a Natural Biological Control Agent of Soil-Dwelling Nematodes

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Abstract: The nematocidal potential of an entomopathogenic strain of *Brevibacillus laterosporus* previously known for its antimicrobial properties was assessed on the free-living nematode microworm *Panagrellus redivivus* and the root-knot nematode *Meloidogyne incognita*. Laboratory bioassays showed significant nematocidal properties of the culture supernatant of the bacterium, achieving over 90% mortality of both microworms and *M. incognita* juveniles when the supernatant was collected at the sporulation phase, which related to the progressive production and release of virulence factors and toxins in the culture medium at this stage of bacterial growth. A protein fraction obtained by precipitation from the bacterial culture supernatant was found to be very active against nematodes with a concentration-dependent effect and an LC_{50} value of 0.4 µg/µL on *M. incognita*. Bacterial preparations based on either spores or a culture supernatant proved to be effective in reducing *M. incognita* density in treated compared with untreated soil, which makes the use of *B. laterosporus* as a biological control agent of soil-dwelling nematode pests particularly promising.

Keywords: biocontrol; BCA; pest management; toxins; virulence factors; biopesticide

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1. Introduction

Brevibacillus laterosporus Laubach is a ubiquitous bacterial species, whose spores, typically associated with a canoe-shaped parasporal body, are frequently isolated from the soil, where it interacts with a wide variety of living organisms, taking part in the biogeochemical cycles [1]. As a component of the rhizosphere microbial community, it promotes plant growth, competing for space and nutrients with phytopathogens [2]. In addition to this indirect protective action, *B. laterosporus* shows high antimicrobial potential due to the production of several compounds, including peptides and antibiotics [3,4]. The biological control properties of this bacterial species are also expressed against invertebrates, such as insects, nematodes, and mollusks [5]. Recent studies on its genome revealed the presence of several traits associated with such potential, with the involvement of protein toxins, enzymes (e.g., proteases, chitinases), and various polyketides and nonribosomal peptides [6]. Nematocidal properties have preliminarily emerged in some studies, highlighting a likely prominent role of cuticle-degrading proteases, which, however, would not be a prerogative of *B. laterosporus*, with these gene traits being shared with several *Bacillus* species [7]. The latter include the most studied bacterial entomopathogen, *Bacillus thuringiensis* Berliner, whose nematocidal action appears instead to be mainly due to the Cry proteins it produces [8]. The pore-forming mechanism of action of these toxins involves highly specific binding to receptors of the intestinal epithelial cell membrane, which would support a common evolution of this bacterium and nematodes. According to different routes of interaction of *B. thuringiensis* with nematodes, even if its ecology is still not well understood, the nematode was proposed to be an alternative dominant host [9]. Similarly, other bacterial species were found to show pathogenic behavior against soil nematodes, which points to their potential exploitation in pest management [10]. The nematocidal action of specific *B. laterosporus* strains has been reported, emphasizing the role of extracellular proteases, although a more complex mechanism of action with the involvement of

several yet unidentified virulence factors has been proposed [11]. On the other hand, most works have been conducted on free-living nematode species, while to determine the actual potential against plant parasitic nematodes would be very important for the purposes of their employment as biological control agents (BCAs) [12].

Soil-borne nematode pests affecting agricultural crops are responsible for significant economic losses, and their management, historically based on the heavy use of chemicals, is of vital importance to protect agricultural production [13]. In this scenario, the development of eco-friendly biocontrol strategies, such as the application of effective entomopathogenic-bacteria-derived products, is a priority [10]. A major and very common nematode species of worldwide importance is the root-knot nematode *Meloidogyne incognita* (Kofoid and White). This polyphagous species typically causes the formation of irregular galls on the roots of attacked plants, causing significant damage to crops [13].

The aim of this study was primarily to assess the nematocidal potential of an entomopathogenic strain of *B. laterosporus*. For this purpose, preliminary bioassays were conducted on the free-living nematode microworm *Panagrellus redivivus* (Linnaeus) Goodey, followed by experiments with *M. incognita*.

2. Materials and Methods

2.1. Bacterial Preparations and Analyses

The bioinsecticidal *Brevibacillus laterosporus* strain F5 maintained in the collection of the University of Sassari and originally isolated from a honeybee body was used in this study [14]. Bacterial cultures were routinely conducted in Luria Bertani (LB) broth at 30 °C, shaking at 180 rpm, and harvested by centrifugation at 15,000× *g* at 4 °C for 15 min. The sporulation medium T3 was used to obtain synchronized bacterial growth [15] in order to collect the culture supernatant at different stages of growth (exponential, stationary, sporulation). The stage of growth was routinely checked under a phase-contrast microscope. For soil experiments, pure spore suspensions were dehydrated under a laminar flow hood, made into powder using a grinder, and quantified by CFU counts. Fresh culture supernatants, after being sterilized by Minisart® filters (pore size = 0.2 μm), were directly used in bioassays or subjected to protein extraction by ammonium sulfate precipitation, followed by dialyses against phosphate-buffered saline (PBS), as described elsewhere [16]. The protein profile of this fraction extracted from a sporulated culture supernatant was determined by *liquid chromatography–mass spectrometry* (LC–MS/MS) in a previous work in which its antimicrobial properties were investigated (Table S1) [16].

The protein concentration of the preparations used in nematode bioassays was determined through the Bradford dye-binding method employing the Bio-Rad Protein Assay.

2.2. In Vitro Bioassays with Panagrellus redivivus

These bioassays had the purpose of evaluating in vitro the bionematocidal activity of different bacterial preparations against *P. redivivus* in order to identify the most active bacterial fractions. The nematocidal activity of the whole bacterial culture was determined in a preliminary experiment, which was followed by more focused bioassays employing the culture supernatant collected at different stages of growth. The pure protein fraction extracted from the culture supernatant at the sporulation phase was also assayed at the following concentrations: 0.1 μg, 0.5 μg, and 1 μg/μL.

Microworms (*P. redivivus*) of mixed sexes were provided by the rearing facility of the Department of Agricultural Sciences of the University of Sassari (Italy), where they were maintained on a rearing substrate containing wheat bran, water, and brewer's yeast.

Lethal effects were determined by dose-response bioassays using 96-well polystyrene microplates filled with different bacterial preparations (200 μL/well) or just sterile water (control) [11]. The bioassay design included four replicates per treatment represented by 4 wells, each containing 20 nematodes. Plates were covered to avoid liquid evaporation and maintained in an incubator at 27 °C. Nematode mortality was checked daily for 48 h

under a stereo microscope, considering dead the nematodes that did not move even after being touched with a needle. Each experiment was repeated three times.

2.3. Experiments with M. incognita

These experiments had the purpose of assessing the efficacy against root-knot nematodes of the bacterial fractions and the protein extracts selected in previous bioassays with microworms.

Second-stage juveniles (J2s) of *M. incognita* were obtained from egg masses collected from the roots (galls) of tomato plants (*Solanum lycopersicum* L.) used to maintain nematode rearing under controlled conditions.

A first experiment was conducted in vitro using 96-well microplates according to the same experimental design and procedures previously described for *P. redivivus* [11]. These bioassays assessed the nematocidal potential of both the culture supernatant collected at different stages of growth and its protein precipitate at the sporulation phase at progressive concentrations in the range of 0.1 and 1 µg/µL to determine the median lethal concentration (LC_{50}). This experiment was repeated three times.

A second experiment was carried out to evaluate the potential of *B. laterosporus* to act in the natural nematode environment (i.e., the soil). For this purpose, bacterial spore powder was mixed with sterile medium texture soil to a final concentration of 10^9 spores/g soil. Similarly, a filter-sterilized culture supernatant of sporulated *B. laterosporus* (5 mL) was mixed with soil (10 g). Controls that received no treatment or treated with sterile water at the same dose as the culture supernatant were included. The experimental unit was represented by a 25 mL glass vial containing 10 g soil in which 200 nematode juveniles were placed. Nematodes were maintained in an incubator at 27 °C and 70% RH, and their recovery number from treated and control samples was determined after 7 days by collecting active specimens through a 38 µm sieve placed on a Baermann funnel [17]. Each treatment had five replicates, and the whole experiment was repeated twice.

2.4. Statistical Analyses

Data for statistics were processed using the R software, version 4.2.0 [18].

Direct comparison between the treated and control group in the preliminary experiment with *P. redivivus* and the whole bacterial culture was based on *t*-test.

Data on nematode percentage mortality and recovery number in different experiments with *P. redivivus* and *M. incognita* were analyzed by one-way ANOVA (factor: treatment), followed by Duncan's new multiple range test (DMRT) for post hoc comparison of means.

The relationship between nematode mortality and protein extract concentration was analyzed by linear regression analyses, while probit regression was used to calculate the median lethal concentration (LC_{50}).

3. Results

3.1. Bioassays with Panagrellus redivivus

Treatment of *P. redivivus* with the whole *B. laterosporus* sporulated culture in preliminary experiments determined a highly significant mortality level (92.9%) compared with the control (1.2%) ($t = 5.6937$; $df = 13$; $p < 0.001$).

Significant lethal effects were associated with the culture supernatant of *B. laterosporus*, albeit with different levels of effectiveness, depending on the stage of bacterial growth at which it was collected (Table 1). The highest mortality percentage after 48 h exposure was caused by the culture supernatant harvested at the sporulation stage (>90%), followed by the stationary (58%) and the exponential (28%) phases ($F_{3,44} = 218.47$; $p < 0.001$).

The protein extract obtained from the culture supernatant of the bacterium collected at the sporulation phase showed significant mortality of microworms ($F_{3,44} = 363.08$, $p < 0.001$). The lethal effects were concentration dependent, and the average percentage mortality after 48 h exceeded 90% at a concentration of 1 µg/µL (Figure 1).

Table 1. Mortality (mean ± sd) of *Panagrellus redivivus* after exposure for 48 h to *Brevibacillus laterosporus* culture supernatant collected at different bacterial growth stages.

Bacterial Growth Phase	Mortality [1] (%)
Control	2.1 ± 3.3 a
Exponential	28.3 ± 15.7 b
Stationary	58.3 ± 7.5 c
Sporulation	94.6 ± 5.8 d

[1] Means followed by different letters are significantly different (one-way ANOVA, followed by DMRT test, $p < 0.001$).

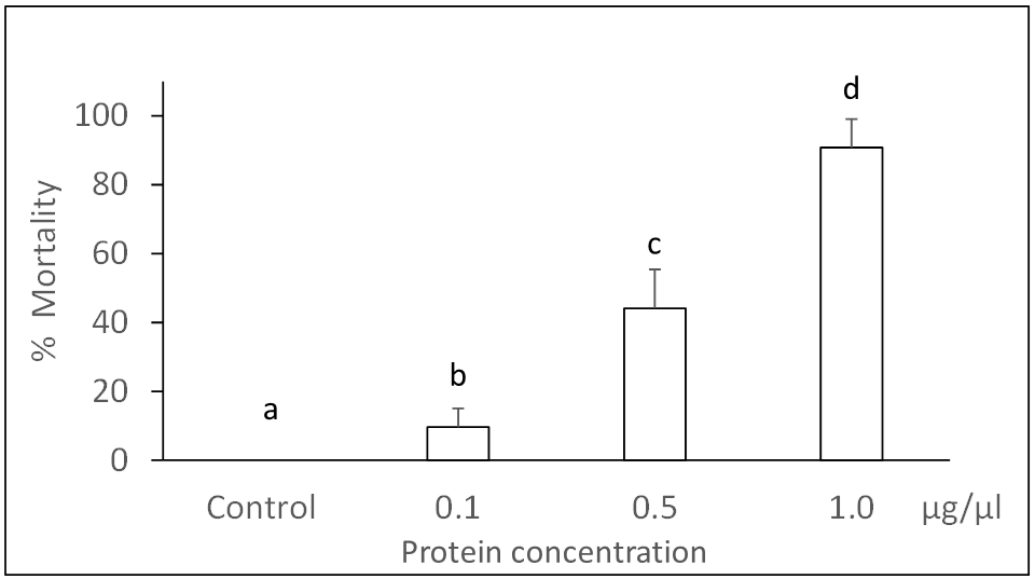

Figure 1. Mortality percentage (mean ± sd) of *Panagrellus redivivus* exposed for 48 h to different concentrations of the protein extract from the culture supernatant of *Brevibacillus laterosporus*. Different letters above bars indicate significantly different means (one-way ANOVA, followed by DMRT test, $p < 0.001$).

3.2. Experiments with Meloidogyne incognita

Second-stage juveniles of the root-knot nematode appeared to be highly susceptible to the culture supernatant of *B. laterosporus*, especially when it was collected at the sporulation phase of bacterial cultures ($F_{3,44} = 633.35$; $p < 0.0001$). In the latter phase, the supernatant determined more than 90% mortality, which remained around 50% when treatments were made with the culture supernatant collected at the stationary phase (Figure 2).

This nematode species survival was also affected by the protein extract from the supernatant of the bacterial culture at the sporulation phase with a concentration-dependent effect (Figure 3). Based on linear regression analyses, a significant correlation between concentration and mortality was observed (adjusted $R^2 = 0.84$; $F = 510.60$; $p < 0.0001$). According to probit analysis, LC_{50} (CI) values for the protein extract were 0.42 (0.32–0.49) µg/µL (slope = 1.70 ± 0.28; $\chi 2 = 13.78$; df = 94).

In the second experiment with *M. incognita*, nematode recovery 7 days after inoculation from soil treated with *B. laterosporus* spores (10^9/g) or the culture supernatant (0.5 mL/g) was significantly affected by treatments compared with water and untreated controls ($F_{3,36} = 79.26$; $p < 0.0001$). In more detail, spores determined a decrease in a percentage recovery of around 50% in respect to the untreated control, while a reduction of around 80% was caused by the culture supernatant treatment compared with the water control (Figure 4).

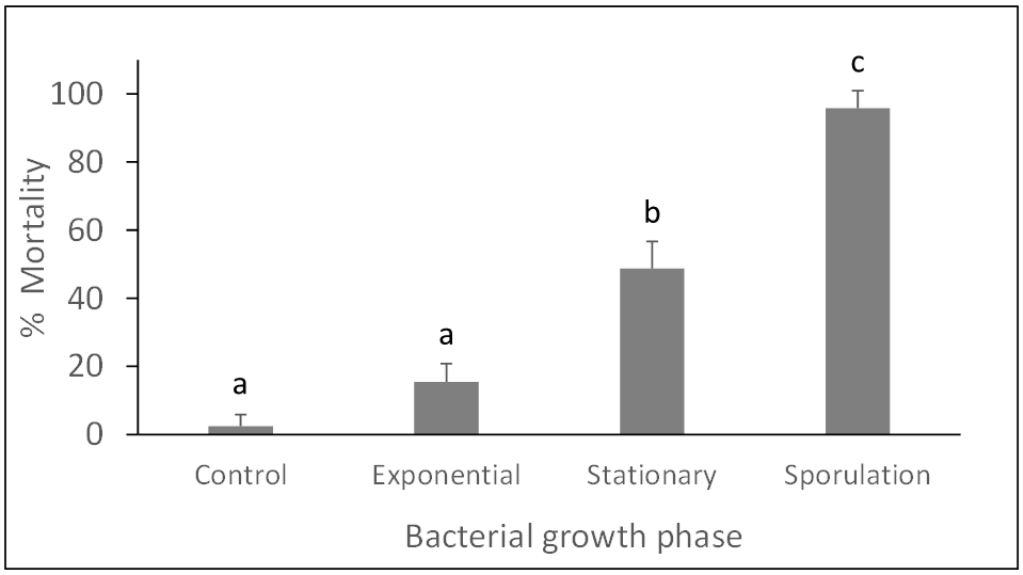

Figure 2. Mortality percentage (mean ± sd) of *Meloidogyne incognita* exposed for 48 h to the culture supernatant of *Brevibacillus laterosporus* at different growth phases. Different letters above bars indicate significantly different means (one-way ANOVA, followed by DMRT test, $p < 0.001$).

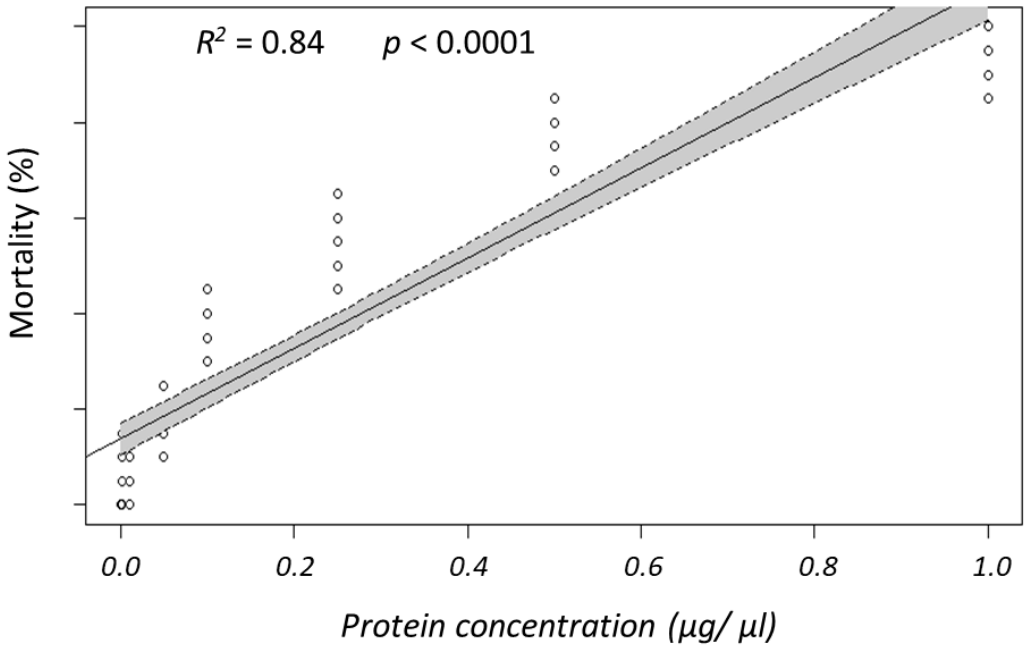

Figure 3. Relationship between *Meloidogyne incognita* mortality and concentration of the protein extract from the *Brevibacillus laterosporus* culture supernatant at the sporulation phase. Shaded areas in the linear regression plot represent 95% confidence intervals.

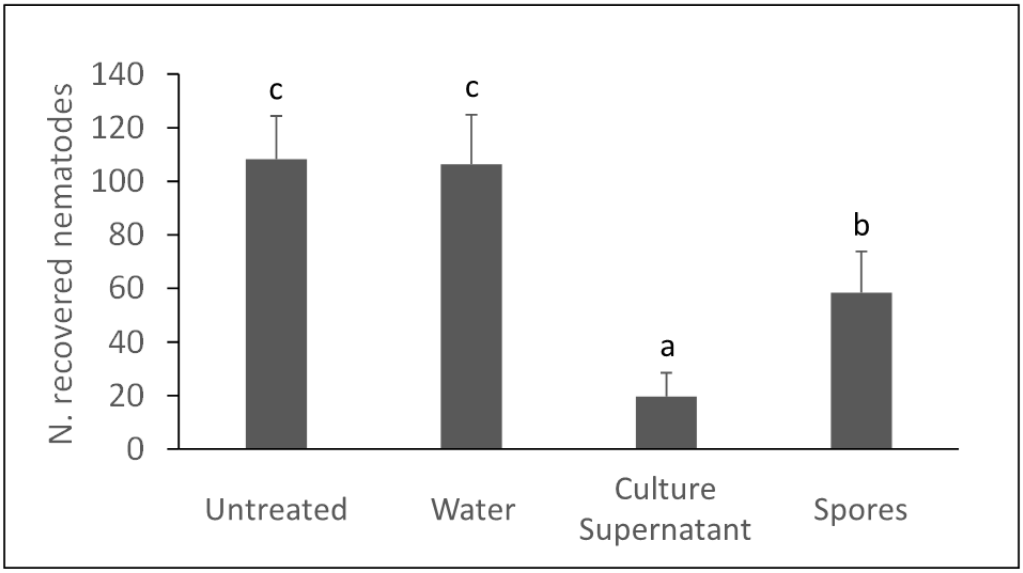

Figure 4. Number (mean ± sd) of *Meloidogyne incognita* individuals recovered from soil samples treated with different *Brevibacillus laterosporus* preparations and inoculated with 200 nematode juveniles. Different letters above bars indicate significantly different means (one-way ANOVA, followed by DMRT test, $p < 0.001$).

4. Discussion

Brevibacillus laterosporus is a ubiquitous bacterial species frequently isolated from different types of soil [1]. While the ecological role of this species has yet to be fully understood, there is significant evidence of its ability to contribute to fertility and to support plant health [19–21]. In particular, the potential of different strains of this species as antagonists of phytopathogens or as bioinsecticides was explored [2,22]. Some studies using specific bacterial strains have also shown a promising nematocidal effect [23]. Accordingly, our experiments with the free-living nematode *P. redivivus* and the crop pest *M. incognita* proved a high susceptibility of these species to the bacterium. The nematocidal effect was associated with the culture supernatant, and a higher mortality was achieved when the latter was collected at the sporulation phase. This finding is in line with previous knowledge on *B. laterosporus*, which is known to produce and release in the culture medium, especially during this growth phase, several compounds, including proteins, small peptides, and antibiotics [15,24]. The insecticidal and antimicrobial activities of the bacterial strain employed in our study, originally isolated from a honeybee body [14,16], were previously reported. These bioactivities were observed to be associated with different bacterial fractions. In particular, high insecticidal properties were associated with the live spores [15], while high antimicrobial power was attributed to the same protein fraction that we tested in vitro on nematodes [16]. According to these studies, the action of bacterial bioactive compounds was concentration dependent, which corroborates our observations on nematodes. Previous investigations reported the potential of the *B. laterosporus* culture supernatant against some nematode species, including *Panagrellus* and *Meloidogyne* species [25,26]. Among proteins that were found to be implicated in the nematocidal action, extracellular proteases were found to be major virulence factors, allowing the enzymatic degradation of the nematode cuticle [11]. Consistently, the protein extract that caused a high nematocidal activity in our experiments contained proteases and chitinases, even though they were not the main component. On the other hand, a specific production of these enzymes by the bacterial cell is expected to be stimulated in the presence of the host, which may support their greater implication when living cells/spores would come into contact with nematodes in the soil. According to a more complex mechanism of action, our study highlights other *B. laterosporus* virulence factors possibly involved in the nematocidal effects. The most abundant component of the bioactive protein mixture was the 5.7 kDa antimicrobial

peptide laterosporulin, whose toxicity potential, although previously highlighted, was not reported for nematodes [27]. Our study, for the first time, highlights the importance that this small peptide might also have toward these targets. However, it is more likely that the nematocidal action is the result of a combined effect of multiple virulence factors that, in addition to the above proteolytic enzymes, would include other bioactive extracellular components that we identified in the nematocidal extract, among these, a lectin domain protein and a 60 kDa chaperonin and several putative uncharacterized proteins whose functional properties still need to be specifically evaluated [16].

Interestingly, the culture supernatant of *B. laterosporus* was active against *M. incognita* juveniles in our soil experiments, which looks very promising in the prospect of applying it directly against this nematode species in its natural environment. Additionally, a significant nematode control potential in the soil was also found to be associated with living bacterial spores. The spores of *B. laterosporus* are characterized by a typical spore coat-canoe-shaped parasporal body (SC-CSPB) complex, associated with insecticidal activity due to the presence of certain protein virulence factors [15]. In addition, spore germination would trigger a pathogenic process that brings into play numerous other virulence factors potentially leading the nematode to death, as observed against other invertebrate pests [14,28,29]. From an ecological point of view, the spore represents the most frequent form in which this bacterial species is found in soil [1]. Therefore, it is not unlikely that this species has coevolved in the same environment as soil nematodes, corroborating the same hypothesis already made for *B. thuringiensis*, that is, that in nature, the nematode may represent an alternative host [9].

According to the results of our study, *B. laterosporus* bioactive strains and their virulence factors represent valuable resources to be exploited for nematode pest management. From a practical point of view, their application in the soil appears to be very promising. However, field trials involving appropriately developed *B. laterosporus*–based formulations are needed for a full evaluation of their actual pest biocontainment potential, including safety assessment for nontarget organisms, under the complex conditions characterizing the soil ecosystem.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12112686/s1. Table S1: Selection of proteins identified in the *Brevibacillus laterosporus* culture supernatant collected at the sporulation phase and related to potential nematocidal activity [16].

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