# Effect of Six Bacterial Strains on the Activity of Second Instar Larvae and Egg Hatching of *Meloidogyne incognita*

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**Abstract** [**Objectives**] The paper was to screen effective biocontrol strains against *Meloidogyne incognita*. [**Methods**] The effect of six bacterial strains sourced from the research group's strain library on the activity of second instar larvae of *M. incognita*, as well as on egg hatching, was evaluated. [**Results**] The treatment of fermentation supernatant derived from the X-2 strain exhibited a pronounced lethal effect on *M. incognita*, with a corrected mortality rate reaching 97% within 72 h. Additionally, this treatment significantly inhibited egg hatching, achieving an inhibition rate of 94.69% at a 20-fold dilution. The strain was identified as *Bacillus velezensis*, belonging to the genus *Bacillus*, and was designated as RKN1111. [**Conclusions**] This study presents alternative strains and a theoretical framework for the biological control of *M. incognita*.

**Key words** *Meloidogyne incognita*; Bacterial strain; Second instar larvae; Egg hatching

## 1 Introduction

Root-knot nematodes are a group of phytopathogenic nematodes that adversely affect crop growth and, in severe instances, can result in the death of the entire plant. These nematodes are prevalent globally and possess a broad host range, infesting over 3 000 species of plants, including garden flowers and field crops[1-3]. Nearly 100 species of root-knot nematodes have been documented, with the predominant species identified as *Meloidogyne arenaria* (Neal) Chitwood, *M. incognita* (Kofoid & White) Chitwood, *M. hapla* Chitwood, and *M. javanica* (Treub) Chitwood[4]. *M. incognita* is the most prevalent species, primarily impacting vegetable crops belonging to the Solanaceae and Cucurbitaceae families[5-7]. The current management of root-knot nematodes predominantly relies on chemical treatments[8]. However, these chemicals pose significant environmental pollution risks, rendering them unsafe for both humans and animals during application. Furthermore, their use is highly likely to contribute to soil degradation and a reduction in biodiversity. Consequently, biological control has garnered increasing attention and has emerged as a focal point in the research on the management of root-knot nematodes. The exploration and development of novel biocontrol agents represent a significant scientific and technological challenge that must be addressed in agricultural production.

Bacteria play a significant role as biocontrol agents. Presently, several bacterial strains have been documented for their efficacy in controlling root-knot nematodes, including *Bacillus subtilis*, *B. cereus*, *B. aryabhattai*, *Pseudomonas putida*, and *P. fluorescens*[9-12]. While these strains demonstrate varying degrees of effectiveness in managing root-knot nematodes, there are instances where the control efficacy of individual strains is inconsistent. Consequently, the continued screening and identification of effective biocontrol strains against root-knot nematodes is of considerable importance for the advancement of biocontrol agents.

This study investigated the effect of six bacterial strains on the activity of second instar larvae and egg hatching of *M. incognita*, with the objective of identifying effective biocontrol agents for this nematode. The findings of this research will contribute to the identification of alternative bacterial strains and provide a theoretical foundation for the effective management of root-knot nematodes.

## 2 Materials and methods

**2.1 Preparation of strain fermentation supernatant** Single colonies of each bacterium were isolated using an inoculating loop and transferred to LB culture medium. The cultures were then incubated at 28 ℃, 180 r/min for a duration of 2 d. Following the preparation of the fermentation broth for each of the six bacterial strains, namely X-1, X-2, X-3, X-4, X-5, and X-6, the fermentation supernatant for each strain was collected through centrifugation at 4 ℃, 6 000 r/min for a duration of 15 min to eliminate the cellular biomass. Each fermentation supernatant was diluted in ratios of 1∶10, 1∶20, and 1∶50, and these dilutions were utilized for *in vitro* experimental testing.

**2.2 Preparation of *M. incognita* suspension** A potted plant that had been inoculated with nematodes was taken, and the walls of the pot were gently pinched to dislodge the soil and plant from the plastic container. The plant, with its roots intact, was removed using gentle movements, and the root surface was subsequently rinsed with ultrapure water. Oocysts located on the roots were found and subsequently collected using sterilized forceps under a stereomicroscope. 2% Sodium hypochlorite solution was pre-

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pared to immerse the collected oocysts, which were agitated for 3 min to facilitate the rupture of the follicle walls. Following this, the oocysts were rinsed with purified water. The washed oocysts were then placed on a 500-mesh sieve and incubated under the following conditions: 28 ℃, in darkness, for a duration of 3 – 4 d, in order to obtain a suspension of second instar larvae.

**2.3 Preparation of egg suspension of *M. incognita*** A potted plant that had been inoculated with nematodes was taken, and the walls of the pot were gently pinched to dislodge the soil and plant from the plastic container. The plant, with its roots intact, was removed using gentle movements, and the root surface was subsequently rinsed with ultrapure water. The roots were subsequently cut into pieces measuring 2 – 4 cm and placed into the beaker using scissors. The sodium hypochlorite solution was prepared by dissolving 10 mL sodium hypochlorite in 90 mL of water, and subsequently introduced into a beaker containing the cut roots, ensuring that the solution thoroughly permeated the roots. The beaker was shaken thoroughly for a duration of 3 min to facilitate the release of nematode eggs. The contents of the shaken beaker were subsequently poured evenly into a series of sieves (80, 140, 180, 200, and 500 mesh, arranged from top to bottom) and rinsed with ultrapure water 5 – 7 times. This procedure was conducted to ensure the collection of an adequate number of nematode eggs while effectively removing any excess sodium hypochlorite. The 500 mesh sieve was rinsed using a wash bottle, and a beaker was employed to collect the rinse solution. A volume of 20 μL of the rinsing solution was transferred with a 100 μL pipette into a dry and clean Petri dish to create a water droplet. This droplet was then placed under a microscope for observation and concentration calculation. Subsequently, a specific volume of sterile water was added to produce an egg suspension containing approximately 1 000 eggs/mL.

**2.4 Effect of fermentation supernatant on the activity of second instar larvae of *M. incognita*** In a 48-well plate, 200 μL of a prepared suspension containing second instar larvae (approximately 100 individuals of *M. incognita* per well) was sequentially added to each well. Subsequently, equal volumes of various concentrations of fermentation supernatant were individually introduced into the wells. The experimental treatments were conducted in triplicate, with sterile water serving as a blank control. All samples were incubated in the dark at a temperature of 26 ℃ within an incubator. The number of deceased second instar larvae was counted every 12 h under a stereomicroscope utilizing the posture method. In this method, nematodes exhibiting a flexed and mobile body were classified as alive, while those that remained rigid after 24 h of water resuscitation were deemed deceased. Subsequently, the mortality and corrected mortality rates of the second instar larvae were computed.

Mortality rate (%) = (Number of deceased nematodes/Total number of nematodes surveyed) × 100% (1)

Corrected mortality rate (%) = (Mortality rate in treatment group – Mortality rate in control group)/(100 – Mortality rate in control group) × 100% (2)

**2.5 Effect of fermentation supernatant on egg hatching of *M. incognita*** 1 mL of both egg suspension and fermentation supernatant was introduced into 24-well plates. The control group consisted of sterile water combined with eggs, with a total of 100 eggs per well, and each treatment was replicated three times. The 24-well plates were incubated at 28 ℃ for a duration of 3 – 4 d. The number of hatching eggs of *M. incognita* was observed using a stereomicroscope, and subsequently, the egg hatching rate and relative inhibition rate were calculated.

Egg hatching rate (%) = Number of eggs hatched/Number of eggs supplied × 100% (3)

Relative inhibition rate (%) = (Egg hatching rate in control group – Egg hatching rate in treatment group)/Egg hatching rate in control group × 100% (4)

**2.6 Data processing** Statistical data were collected utilizing Microsoft Office Excel software, analyzed through one-way ANOVA with IBM SPSS Statistics Version 27.0, and assessed for significance employing the Student – Newman – Keuls (S-N-K) method ($P < 0.05$).

## 3 Results and analysis

**3.1 Effect of fermentation supernatant on the activity of second instar larvae of *M. incognita*** As illustrated in Fig. 1, among the six bacterial strains examined, the fermentation broth derived from strain X-2 exhibited the most significant impact on the activity of second instar larvae of *M. incognita*. The corrected mortality rates were recorded at 95% and 97% following treatment with 10-fold and 20-fold dilutions of the fermentation broth for a duration of 72 h, respectively. The fermentation broth derived from strain X-6 exhibited minimal impact on the activity of second instar larvae of *M. incognita*, resulting in a corrected mortality rate of merely 28.23% when subjected to a 10-fold dilution over a treatment period of 60 h. Under conditions of equal treatment duration, the corrected mortality rates of fermentation supernatant stock solutions diluted at various multiples exhibited significant differences. Conversely, when the dilution multiples were held constant, the corrected mortality rates observed at different treatment durations were significantly different. The mortality rate of second instar larvae of *M. incognita* exhibited a positive correlation with the concentration of the fermentation broth. Additionally, the mortality rate of these larvae also increased with prolonged treatment duration.

**3.2 Effect of fermentation supernatant on egg hatching of *M. incognita*** As illustrated in Table 1, the hatching rates of *M. incognita* eggs exhibited a range from high to low, specifically 10-fold, 20-fold, and 50-fold, in response to different concentrations of bacterial fermentation supernatant. This trend suggested that increased concentrations of the bacterial fermentation solution had a more significant inhibitory effect on egg hatching. X-2 exhibited the highest inhibition rate of 99.12% against the egg hatching of *M. incognita* at a 10-fold dilution, followed by X-5 (93.40%), X-1 (91.15%), X-3 (89.38%), X-4 (86.75%), and X-6 (59.58%).

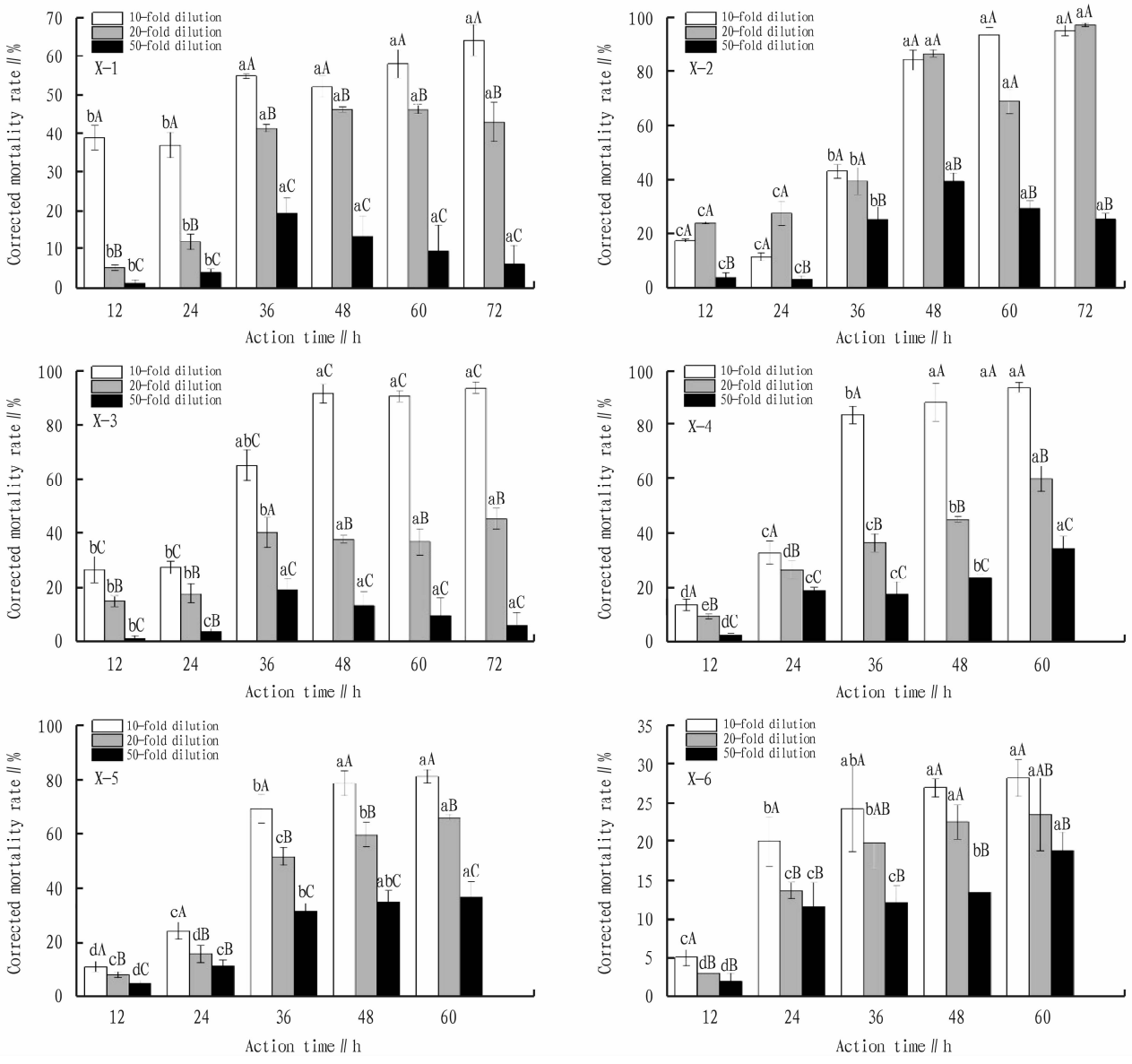


**Fig. 1 Corrected mortality of second instar larvae of *Meloidogyne incognita* by the fermentation supernatant of six bacterial strains**

**Table 1 Effect of fermentation supernatant on egg hatching of *Meloidogyne incognita***

| No. of strain | Inhibition rate of egg hatching // % | | |
|---|---|---|---|
| | 10-fold | 20-fold | 50-fold |
| X-1 | 91.15 ± 1.32 Ac | 52.21 ± 2.47 Bd | 53.10 ± 1.80 Ba |
| X-2 | 99.12 ± 0.5 Aa | 94.69 ± 1.29 Ba | 50.44 ± 4.97 Cb |
| X-3 | 89.38 ± 0.82 Ac | 61.06 ± 3.37 Bc | 45.13 ± 6.24 Cc |
| X-4 | 86.75 ± 0.84 Ad | 61.05 ± 3.47 Bc | 19.85 ± 4.04 Ce |
| X-5 | 93.40 ± 1.41 Ab | 69.28 ± 8.67 Bb | 36.75 ± 3.06 Cd |
| X-6 | 59.58 ± 1.84 Ae | 30.18 ± 2.20 Be | 11.78 ± 5.24 Cf |

**NOTE** Different capital letters denote statistically significant differences in the same row ($P < 0.05$), while different lowercase letters indicate statistically significant differences in the same column ($P < 0.05$).

## 4 Discussion

In recent years, there has been a growing emphasis on environmentally sustainable plant protection and the development and utilization of microbial resources. Consequently, biocontrol microbial preparations have emerged as a significant method for controlling root-knot nematodes and have become a focal point of research. The use of microbial nematicides for the management of root-knot nematodes has been increasingly documented in recent years. Sun Yanfang *et al.* [13] conducted a study to analyze the effect of *B. thuringiensis* on the biological activity of *M. incognita*. Similarly, Ding *et al.* [14] reported that the bacterial strain PFMP-5 exhibited a significant inhibitory effect on *M. incognita*. Zhao *et al.* [15] investigated the virulence of *Bacillus* fermentation broth against tomato root-knot nematode. Furthermore, Yao *et al.* [16] demonstrated that the metabolites of *Penicillium*-producing strains were effective in the biological defense against southern root-knot nematode disease. Zhu *et al.* [17] explored the effect of *Bacillus* on both the biological activity of tomato root-knot nematode and the

growth of tomato plants. Lastly, Sun Ke *et al.* [18] screened for biocontrol *Bacillus* species against burdock root-knot nematode through *in vitro* bioassays of fermentation broth and field trials.

In this study, the fermentation supernatants of six distinct bacterial strains were individually assessed to evaluate their *in vitro* biological activity against *M. incognita*. Additionally, the biological effectiveness of a single bacterial strain against *M. incognita* was initially elucidated. After screening, it was found that among the six bacterial strains, the treatment of the fermentation supernatant of strain X-2 had the most obvious lethal effect on *M. incognita* and increased with time, with the corrected mortality rate reaching 97% at 72 h. The treatment of *M. incognita* eggs also produced the effect of preventing the nematode from hatching. The strain had been identified as *B. velezensis*, belonging to the genus *Bacillus*, and was designated as RKN1111. It has been deposited in the Centre for General Microbiology under the China Microbial Strain Preservation and Management Committee. In this experiment, the inhibitory effect of the fermentation broth derived from this strain on the activity of second instar larvae and the egg hatching of *M. incognita* was preliminarily assessed. However, the specific mode of action remains unclear. Further investigation into the mechanism of action will be conducted in subsequent studies to provide alternative strains and theoretical references for the research and development of biocontrol agents targeting *M. incognita*.

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(From page 10)


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