Identification and establishment of a culture method for the spiral nematode *Helicotylenchus microlobus* from tomato in China

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Abstract

Background: Spiral nematodes of the genus *Helicotylenchus* are globally distributed, and contain various species complexes that normally exhibit similar diagnostic characteristics. Some studies on the parasitism of tomato by spiral nematodes include only census data, or morphological data and lack detailed molecular data in China. At present, carrot disks have been successfully applied in the culture of plant-parasitic nematodes, but carrot disks are not suitable for rearing all migratory plant-parasitic nematodes, and only a small number of plant-parasitic nematodes can be cultured. However, the establishment of a culture method for *Helicotylenchus* has not been reported.

Results: Spiral nematodes were recovered from tomato roots and soil samples, and the purified spiral nematode LQ-1 population was identified based on morphological and molecular biological technology. Both the morphological characteristics and molecular analysis of the internal transcribed spacer (ITS) and D2-D3 expansion region of 28S ribosomal RNA sequences revealed that the species of the spiral nematode LQ-1 population was *Helicotylenchus microlobus*. Phylogenetic analysis with the rRNA sequences of the ITS and 28S D2-D3 regions was consistent with molecular identification, suggesting that the spiral nematode LQ-1 population formed a highly supported clade with other *H. microlobus* populations. Additionally, a method for culture of *H. microlobus* on carrot disks was established, and the effect of temperature on the reproduction rate (Rr) of this nematode was investigated. The optimum temperature for *H. microlobus* culture on carrot disks was 27.5 °C, and the Rr reached 406 after 90 days of inoculation with 30 females.

Conclusions: To our knowledge, this is the first systematic description of *H. microlobus* from tomato in China. This study also demonstrated for the first time that the carrot disk method is suitable for the culture of *H. microlobus*. This study lays a foundation for other related research on *H. microlobus*, and has significance for the study of spiral nematodes.

Background

Tomato (*Solanum lycopersicum* L.) is the most economically important member of the family Solanaceae and is cultivated worldwide. It is one of the most important vegetable crops in China and is rich in minerals, vitamins, antioxidants and other micronutrients [1]. China has the largest area of tomato cultivation in the world, and Henan Province is the dominant production area for tomato cultivation. Tomatoes are susceptible to damage from pests (insects, mites, nematodes, and so on) as well as fungi, viruses, or bacteria [2]. Nematode diseases are the major factor limiting tomato production worldwide and cause severe economic losses.

The genus *Helicotylenchus* Steiner 1945 is one of the ten most important plant parasitic nematodes in the world, and these nematodes are commonly referred to as spiral nematodes because of their coiled habitus mortis [3]. Within the genus *Helicotylenchus*, more than 230 nominal species have been described worldwide [4, 5]. Spiral nematodes are ectoparasites or semi-endoparasites that may occur in large

numbers and feed on the roots of a variety of plants, causing plant growth reduction [6]. Many of the described species have not received widespread attention as serious plant pathogens, because there are no available data showing that they damage and inhibit plant growth [4]. However, plant growth inhibition has been consistently associated with several cosmopolitan species that have also increased secondary infections of fungal pathogens [7], such as, *H. dihystra*, *H. digonicus*, *H. multicinctus*, *H. pseudorobustus* and *H. varicaudatus* [8-10]. At present, 50 valid species are listed as spiral nematodes in China, and these species are parasitic on a large variety of plants including ornamental plants, fruit trees, cucumber, rice, grape and megranate [11-14]. However, only Qi et al. reported that *H. dihystra* parasitized tomato in Shandong Province, China [15]. It was also reported that *H. dihystra* was isolated from the rhizosphere soil of tomato in Shanxi Province, China [16]. At present, there are still no reports of *H. microlobus* on the roots of plants in China. However, *H. microlobus* is widely distributed abroad. Subbotin et al. recorded that *H. microlobus* was collected in California, Illinois, and Iowa, in the United States of America and in several European countries, including Spain, Italy and Russia [4]; Yan et al reported the spiral nematode *H. microlobus* infecting soybean in North Dakota [17]. Abraham and Dong also found *H. microlobus* in Turfgrass in Korea [18]. In addition, the culture of plant-parasitic nematodes is very important for the study of other aspects of these nematodes. Researchers have explored sterile culture methods for plant-parasitic nematodes, and several methods have been reported over the years [19-21]. At present, carrot disks have been successfully applied in the culture of plant-parasitic nematodes, but carrot disks are not suitable for rearing all migratory plant-parasitic nematodes, and only a small number of plant-parasitic nematodes can be cultured [21]. However, the establishment of a culture method for *Helicotylenchus* has not been reported. In 2019, soil samples were collected from the rhizosphere of plants in a tomato field near Zhuma village in Tongxu County of Kaifeng city, Henan Province, China. Spiral nematodes were separated from the samples and one individual female nematode was selected for propagation on carrot disks at 25 °C by parthenogenesis. In this study, the purified spiral nematode LQ-1 populations were identified based on morphological and molecular biological technology, a culture method for spiral nematodes was established, and the reproduction of *H. microlobus* was detected on carrot disks. To our knowledge, this is the first report of *H. microlobus* from tomato in China using morphological and molecular biological technology. It also represents the establishment of an artificial culture method for *H. microlobus*. The purpose of this study was to identify a species of spiral nematode from tomato in China and to establish an artificial culture method for *H. microlobus*, which would be very useful for other related biological studies of *H. microlobus*.

Methods

Nematode collection

In August 2019, five samples of roots and corresponding rhizosphere soils were collected from a tomato (cv. Maohong 801) fields near Zhuma village in Tongxu County of Kaifeng city, Henan Province, China. Each sample consisted of at least five sampling points from patches of poor growth. Samples were placed in plastic bags and stored at 16 to 18 °C [21]. Nematodes were extracted using the modified

Baermann funnel method for 2 days [38], by which time 100 g of tomato soil and root samples were extracted from each sample.

Nematode culturing and experiments

Carrot disks were prepared in the following way. The surface of a carrot was sterilized with 95% ethanol. The external tissues were removed with a sterile knife, and then the carrot was cut into disks (approximately 6 mm thick). Each carrot disk was placed in a petri dish, in an incubator maintained at 25 °C approximately 15 days prior to use. One individual female nematode was selected and sterilized with 0.3% streptomycin sulfate, and then transferred to carrot disks at 25 °C in the dark for propagation. After that, the purified spiral nematode LQ-1 population cultured on carrot disks were used for morphological and molecular analysis.

Morphological identification

Nematodes were heat-killed, fixed in FG solution (formalin: glycerin: water=10:1:89) [39], and processed to glycerin by the formalin glycerin method [40]. Photomicrographs and morphometric data of the specimen LQ-1 population were obtained using a Nikon Eclipse Ti-S microscope (Japan). Images of key morphological features were processed using Photoshop CS5. The De Man formula was used for measurements. All measurements are expressed in micrometers (µm), unless otherwise stated.

Molecular characterization and phylogenetic relationships

DNA from one individual spiral nematode was extracted using proteinase K-based lysis [41]. The rRNA-internal transcribed spacer (ITS) region and the D2-D3 region of the 28S rRNA gene were amplified with primers TW81/AB28 (5'-GTTTCCGTAGGTGAACCTGC-3'/5'-ATATGCTTAAGTTCAGCGGGT-3') [42] and primers D2A-D3B (5'-ACAAGTACCGTGAGGGAAAGTTG-3'/5'-TCGGAAGGAACCAGCTACTA-3') [43], respectively. The processes of PCR amplification, cloning and were carried out according to the method of Wang et al [44]. and sequencing was performed by Sangon Biotech Co. Ltd. (Shanghai, PR China). The newly obtained DNA sequences were submitted to the GenBank database.

The obtained ITS region and D2-D3 region of the 28S sequences were subjected to multiple alignment using Clustal W in MEGA 5.0 [45] with other spiral nematode species sequences published in the NCBI GenBank database. Outgroup taxa were selected based on a previous study [20]. Sequence datasets were analysed with Bayesian inference (BI) using MrBayes 3.2.6 [46] under the best-fit model of GTR+G+I, according to Akaike Information Criteria [47]. BI analysis for each gene was run with a random starting tree and four Markov chains for 1×10^6 generations. The Markov chains were sampled at intervals of 100 generations. After discarding burns in samples, the remaining samples were used to generate a 50% majority rule consensus tree. Posterior probabilities (pp) are given on appropriate clades.

Reproduction tests

Using the carrot disk method, experiments were conducted to determine the reproductive potential of *H. microlobus* females and the optimum temperature for culturing *H. microlobus* on carrot disks. In the experiment, 30 females were surface sterilized with 0.3% streptomycin sulfate and then transferred to one carrot disk in a petri dish. These petri dishes were sealed with parafilm and incubated at 25, 27.5, and 30 °C in the dark. The number of nematodes and reproduction rate ($R_r = \text{final number of nematodes} / \text{initial number of nematodes}$) on each carrot disk were determined at 90 days after inoculation.

A maceration method was used to collect nematodes [21]: the carrot tissues were placed in sterile water and macerated in a blender, and to isolate nematodes from carrot tissue, the suspension was then passed through 0.250-mm and 0.150-mm-pore sieves. The nematodes were collected through the sieves into a beaker, and carrot tissues were discarded. Then, the nematode suspension in the beaker was left to settle for at least 4 h, and the supernatant was removed. The total nematode number was observed under a stereomicroscope to assess whether *H. microlobus* reproduction had occurred, and the R_r (final number of nematodes/initial number of nematodes) was determined. There were five replicates for each experiment, and each experiment was conducted twice.

Results

Morphology of the spiral nematodes

The morphology of the spiral nematode LQ-1 population collected from tomato was photographed (Fig. 1). The morphometric data from the LQ-1 population closely resembled *H. microlobus* as described previously [22] (Table 1).

Female spiral nematodes had a spiral body shape after being heat-killed (Fig. 1 A). The lip region was hemispherical, composed of 4-5 annuli (Fig. 1 B, C, D). The stylet was robust, with hemispherical knobs that varied little in shape, and was approximately 2-3 μm high and 4–6 μm wide (Fig. 1 B, C, D). An excretory pore was immediately posterior to the hemizonid (Fig. 1 E). Lateral fields with four longitudinal lines occurred, not areolated in the tail region (Fig. 1 K). Pharyngeal glands overlapped the intestine ventrally (Fig. 1 F). Inner lateral field incisures in the tail region were mostly fused distally into a Y-shaped configuration (Fig. 1 H, I, J). Mean anal body ped configuration. Mean of anal body diameter ranged from 12.7 to 14.4 (Fig. 1 H, I, J). The tail was generally longer than the anal body diameter, bearing 6-13 ventral annuli and ending in a pronounced ventral projection, usually rounded terminally, without a mucro (Fig. 1 J). The distal half of the tail was either without annulation or indistinctly annulated with marked dorsally convex-conoids (Fig. 1) (Table. 1).

Male spiral nematodes were not found.

Molecular characterization and phylogenetic relationships of *H. microlobus*

The primers TW81/AB28 and D2A/D3B were used to amplify the ITS and D2-D3 regions, respectively, of the 28S rRNA gene sequences of the spiral nematode LQ-1 population. The amplified PCR products were

1105 bp and 787 bp in length, respectively (Fig. 2). The obtained ITS sequences and the D2-D3 region of 28S rRNA sequences in this study were deposited in the NCBI GenBank database for a BLAST search. The ITS rRNA gene sequences obtained in this study (GenBank accession No. MZ2708013) showed 99.7% similarity with *H. microlobus* sequences available in GenBank (KM506859). The obtained D2-D3 region of the 28S rRNA gene sequences of the spiral nematode (MZ2707554) showed 100% similarity with *H. microlobus* sequences available in GenBank (MN764328).

Phylogenetic analysis within the genus *Helicotylenchus* based on ITS rRNA gene sequences was performed and indicated 28 ingroups and one outgroup taxon (Fig. 3). The phylogenetic tree showed that the newly obtained sequence for *H. microlobus* (MZ2708013) was clearly different from other *Helicotylenchus* spp. ITS rRNA gene sequences formed a 100% supported clade with *H. microlobus*. Phylogenetic analysis within the genus *Helicotylenchus* based on the D2-D3 region of the 28S rRNA gene indicated 45 ingroups and one outgroup taxon. This phylogenetic tree indicated that the newly obtained sequence for *H. microlobus* (MZ2707554) was clearly different from other *Helicotylenchus* spp. 28S rRNA gene sequences in GenBank formed a highly supported clade with *H. microlobus* (100%) (Fig. 4). The above results confirmed that the obtained spiral nematode LQ-1 population in this study was *H. microlobus*.

Effects of temperature on the Rr of *H. microlobus*

At 90 days after inoculation with 30 females, reproduction occurred on each carrot disk, and the Rr reached 232, 406, and 53 at 25, 27.5 and 30 °C, respectively (Table 2). The nematode Rr at 27.5 °C was significantly higher than that at 25 and 30 °C ($P < 0.05$). In addition, when incubated at 25 and 27.5 °C for 90 days, spiral nematodes gathered on the surface of the Petri dish, and the number of nematodes reached 6947(35.9% females, 34.4% juveniles, 29.7% eggs and no males) and 12190(34.0% females, 29.9% juveniles, 36.1% eggs and no males), respectively (Fig. 5) (Table. 2). At 90 days after inoculation with 30 females, the carrot disks presented obvious infection symptoms and had turned a brown or dark-brown colour. Nematode swarms could be observed on the bottoms of the petri dishes (Fig. 2). These results demonstrate for the first time that the carrot disk method is suitable for the culture of the spiral nematode *H. microlobus*.

Discussion

Plant parasitic nematodes are some of the most important pathogens of tomato. *Meloidogyne*, *Helicotylenchus*, *Pratylenchus*, *Tylenchorhynchus* and *Aphelenchoides* can all infect tomato roots and cause severe economic losses [23, 24]. Spiral nematodes of the genus *Helicotylenchus* have been reported in the rhizosphere soil of tomato in China and abroad. For example, in Turkey, *H. digonicus*, *H. tunisiensis* and *H. varicaudatus* have been extracted from tomato rhizosphere soils [24]. Kim et al. (2014) reported that *H. dihystra* can suppress the growth of tomato [25]. *H. thornei* n. sp. is also described from soil around the roots of tomato in Ludhiana, India [26]. *H. dihystra* was identified in the rhizosphere soil of tomato in Shandong and Shanxi provinces in China [15, 16]. However, these studies on the parasitism

of tomato by spiral nematodes include only census data, or morphological data and lack detailed molecular data.

In this study, morphological identification of the spiral nematode LQ-1 population collected from tomato rhizosphere soils and roots in Henan province, China, was carried out. We found that the main morphological characteristics of the LQ-1 population were basically consistent with *H. microlobus* as described previously, with only slight differences in some measurements. Compared with the data of Mwamula et al. (2020), the L, b, c and max body diameter values for female nematodes were relatively smaller [22]; the pharynx length and m value of measurements were relatively larger. The reason for differences may be related to underlying genetic variation and geographical position. In addition, spiral nematodes are a genera of plant parasitic nematodes known to contain various species complexes that normally exhibit similar diagnostic characteristics [27]. However, species delineation within the genus is not always reliable due to enormous intraspecific variability in characters apparently influenced by environmental conditions [28]. This adds to taxonomic problems and leads to some misidentifications within the genus, due to lack of consensus among different taxonomists on the validity of some species [29, 4]. Therefore, to facilitate species identification, a molecular approach is needed. In recent years, sequences of nuclear ribosomal RNA genes have been used for reconstruction of phylogenetic relationships and molecular characterization within *Helicotylenchus* [30]. This study revealed that the species of the spiral nematode extracted from the rhizosphere of tomato in Kaifeng city, Henan Province, was *H. microlobus*. The Bayesian tree showed that the results of the phylogenetic analysis of the ITS rRNA gene and 28S rRNA gene D2-D3 region were consistent, and clearly separated *H. microlobus* from its sister *Helicotylenchus* species. This is the first report of *H. microlobus* from tomato in China using morphological and molecular biological technology.

Obtaining a large number of plant-parasitic nematodes is very important because many types of studies can be performed with these nematodes, such as pathogenicity tests and biological and genetic studies [21]. For a long time, researchers have been looking for methods to culture plant-parasitic nematodes using plant tissues [19-21]. Some culture methods for plant-parasitic nematodes have been successfully employed, but there have been great differences in culture techniques. For example, *Aphelenchoides besseyi*, *Ditylenchus destructor* and *Bursaphelenchus xylophilus* can be cultured on certain fungi [31-33]. *A. ritzemabosi* and *D. dipsaci* can reproduce rapidly on alfalfa tissue [19]. The carrot callus method is suitable for culturing *A. ritzemabosi*, *A. besseyi*, *R. similis*, and most species of *Pratylenchus* [21, 34, 35]. Until now, successful mass culture of *Helicotylenchus* has not been reported. Sterile carrot disks are usually regarded as a relatively low-cost, straightforward and effective method for culturing migratory endoparasitic nematodes that results in greater nematode multiplication compared with other methods [37]. However, our study is the first to establish an artificial culture method for *H. microlobus*. The carrot disk method produces large numbers of nematodes quickly and requires less time and labour. Once the culture technique is established, growth can be subdivided into many carrot disks, which can be kept for at least two months and contain many live nematodes. Our results indicated that *H. microlobus* had the highest reproduction rate on carrot disks at 27.5 °C. We deem that the optimum temperature for culturing *H. microlobus* on carrot disks is 27.5 °C. Therefore, we recommend carrot disks as a suitable method for

culturing *H. microlobus*. These results lay the foundation for other related biological studies of *H. microlobus*.

Conclusion

In our study, both morphological and molecular analyses revealed that the species of the spiral nematode LQ-1 population was *Helicotylenchus microlobus*. This is the first report of *H. microlobus* from tomato in China using morphological and molecular biological technology. We established a method for culture of *H. microlobus* on carrot disks, and the optimum temperature for *H. microlobus* culture on carrot disks was 27.5 °C, and the Rr reached 406 after 90 days of inoculation with 30 females. This study also demonstrated for the first time that the carrot disk method is suitable for the culture of *H. microlobus*.

Declarations

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Authors' contributions

YH Xia, K Wang and Y Li conceived and designed the study, and YH Xia, J Li and FF Xu performed the experiments, YH Xia, B Lei, Y Li and HL Li analyzed the data and YH Xia, K Wang and Y Li wrote the manuscript. All authors approved the final version of the manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are available in the GenBank repository (<https://www.ncbi.nlm.nih.gov/>). The newly obtained rRNA sequences in this study were deposited in the NCBI GenBank (<https://submit.ncbi.nlm.nih.gov/subs/genbank/>) database for a BLAST search (accession no. MZ2708013 and MZ2707554).

Ethics approval and consent to participate

We collected samples of roots and corresponding rhizosphere soils in areas where no specific permits were required. The land used as the collection area is neither privately owned nor protected in any way, and the field studies did not involve endangered or protected species. Procedures for field sample collection were explained to village authorities and their verbal agreement was obtained before collected samples.

Consent for publication

Not applicable

Conflict of interests

The authors of this work declare that there is no conflict of interest.

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Tables

Table 1 Morphometrics of females of *Helicotylenchus microlobus*

Character	LQ-1	Mwamula, et al. (2020)
n	18	12
L	679.7±36.2(623.1-741.7)	732.5±31.1(680.0-792.9)
a	26.8±0.7(25.6-28.1)	25.6±0.9(24.4-26.9)
b	5.3±0.6(4.6-6.3)	5.9±0.3(5.4-6.4)
b'	4.7±0.4(4.2-5.5)	5.0±0.2(4.6-5.3)
c	33.1±2.8(28.2-38.3)	37.7±3.1(31.9-42.9)
c'	1.5±0.2(1.3-1.8)	1.2±0.1(1.1-1.3)
V	62.3±2.3(58.3-68.7)	60.5±1.1(58.6-62)
O	41.3±2.4(34.5-47.8)	43.2±3.2(38.5-48.2)
m	48.8±3.6(44.8-53.4)	43.7±1.6(41.9-47.3)
Stylet length	25.6±1.0(25.3-28.8)	25.2±0.8(24.1-26.6)
Conus length	13.0±0.8(11.8-14.6)	11.0±0.6(10.1-11.9)
Stylet knob width	5.1±0.3(4.4-5.8)	—
Stylet knob height	2.4±0.3(1.8-2.9)	—
DGO from stylet base	10.9±0.8(9.3-12.5)	10.9±0.8(9.7-12.0)
Anterior to median bulb valve	79.3±2.3(74.4-88.2)	81.5±3.2(9.7-12.0)
Anterior end to excretory pore	110.0±5.0(103.6-126.7)	80.9±3.1(76.6-86.6)
Pharynx length	144.3±6.0(134.5-155.6)	123.6±3.9(117.6-128.3)
Max body diam.	25.4±1.5 (23.7-28.7)	28.6±0.8(27.3-29.6)
Vulval body diam.	23.5±1.9(20.2-27.5)	—
Anal body diam.	14.3±1.2(11.5-16.4)	16.0±1.0(14.6-17.9)
Tail length	20.7±1.5(18.2-23.3)	19.5±1.8(16.9-23.2)
No. of tail annuli	10.5±0.7(9.0-12.0)	9.7±0.5(9.0-10.0)
Vulva to annus distance	235.4±22.1(195.1-281.3)	—
Lateral field width	6.5±0.2(6.1-7.0)	7.0±0.6(5.9-8.1)
Lip width	7.2±0.7(6.3-8.4)	6.9±0.4(6.3-7.8)
Lip height	4.0±0.6(3.5-5.7)	4.1±0.3(3.7-4.6)

Note: All measurements are in μm and in the form of mean $\pm$ SD (range). n: Number of specimens measured; L: Body length; a: Body length/greatest body width; b: Body length/length from the lips to the junction of esophageal gland and intestine; b': Body length/ length from the lips to esophageal gland end; c: Body length/ tail length; c': Tail length/tail diameter at anus; V: Distance of vulva from the lips $\times$ 100/body length; o: DGO from stylet base $\times$ 100/Stylet length; m: Conus length $\times$ 100/Stylet length; DGO: Distance between dorsal esophageal gland opening and stylet knobs.

Table. 2 Effect of temperature (Tm) on the reproduction of *Helicotylenchus microlobus* on carrot callus 90 days after inoculation with 30 females.

Tm (°C)	Females	Juveniles	Eggs	<i>Pf</i>	<i>Rr = Pf/Pi</i>
25	2496 $\pm$ 283.4 b	2390 $\pm$ 376.1 b	2061 $\pm$ 400.8 b	6947 $\pm$ 718.4 b	232 $\pm$ 23.9 b
27.5	4141 $\pm$ 459 a	3646 $\pm$ 410 a	4403 $\pm$ 510.1 a	12190 $\pm$ 881.9 a	406 $\pm$ 29.4 a
30	514 $\pm$ 95.6 c	499 $\pm$ 65.2 c	590 $\pm$ 113.8 c	1603 $\pm$ 236.9 c	53 $\pm$ 7.9 c

Different letters in columns indicate significant differences ($P < 0.05$) according to Duncan’s multiple range test. Values represent the mean $\pm$ standard error. *Pf* = final number of nematodes, including eggs and vermiform stages. Reproduction rates of nematodes ($Rr = Pf/\text{initial number of nematodes } (Pi)$)

Figures

Figure 1

Light micrographs of *Helicotylenchus microlobus* from tomato in Henan Province of China. Females (A - L): A, Entire body. B-D, lip region. E, Anterior region. F, The junction of genital gland and intestine. G-J, tail region. K, Lateral line. L, Ovary region. Scale bars: 50 μm (A) and 20 μm (B-L).

Figure 2

PCR amplification of the D2-D3 region of 28S rRNA and the ITS rRNA gene of *Helicotylenchus microlobus*. M: DL2000 Marker. 1: 28S. 2: ITS.

Figure 3

Bayesian tree of *Helicotylenchus* as inferred from ITS rRNA gene sequences under GTR+I+G model. Posterior probabilities more than 50% are given for appropriate clades. Newly obtained sequence is indicated in bold font.

Figure 4

Bayesian tree of *Helicotylenchus* as inferred from the D2-D3 region of 28S rRNA gene sequences under GTR+I+G model. Posterior probabilities more than 50% are given for appropriate clades. Newly obtained sequence is indicated in bold font.

Figure 5

The carrot disks presented infection symptoms and nematode swarms at 25, 27.5 and 30°C after 90 days. A: blank control without nematodes; B-D: the carrot disks presented infection symptoms at 25°C, 27.5°C and 30°C, respectively; E: nematode swarms on the bottoms of the petri dishes; F-H: microscopic images of nematode swarms; e=egg; n=nematode; Scale bars: F: 400 µm; G: 200 µm; H: 25 µm.

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