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Detection of root-knot nematode disease in cucumber under protected cultivation in Kumaon region

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The increasing adoption of protected cultivation in India has led to significant challenges, one of the most serious being the infestation of root-knot nematodes (RKNs), particularly *Meloidogyne* spp., which threaten sustainable vegetable production. Despite its importance, limited attention has been given to the identification and characterization of RKNs under protected cultivation in hilly regions. Addressing this gap, the present study was undertaken to investigate the RKN populations affecting cucumber crops grown under polyhouse conditions in the Kumaon hills of Uttarakhand, a part of the Indian Himalayan region. A field survey was conducted during 2021–2022 in farmers' polyhouses growing cucumbers. Random sampling was employed by dividing each polyhouse into four units to assess spatial variability in nematode distribution. Integrative taxonomic methods were used, combining symptomatology, morphometric analysis, and molecular identification. Morphological characterization was performed on males, second-stage juveniles (J2), and adult females, with detailed morphometric measurements at various stages. Molecular identification involved amplification and sequencing of the rDNA ITS region. Phylogenetic analysis revealed that the ITS sequence obtained formed a distinct clade, closely related to the *Meloidogyne incognita* isolate OP030611.1. This study provides the first authentic characterization and report of *M. incognita* infecting cucumber under protected cultivation in the hilly terrain of Uttarakhand. The findings offer valuable insights into the nematode's occurrence and distribution in the mid-Indian Himalayan region. The study highlights the need for regular surveillance and monitoring of RKN populations to inform integrated nematode management strategies and ensure the sustainability of cucumber production under protected conditions.

Keywords: Himalayan region, Integrative taxonomy, ITS, *Meloidogyne*, Polyhouse, Sequencing

Root-knot nematodes (RKN), particularly *Meloidogyne incognita*, are economically significant and extensively studied pests, causing substantial damage to plant roots by inducing characteristic swollen galls. This infestation results in altered root structure, hampered physiological processes, reduced water and nutrient uptake, impaired growth, and increased susceptibility to secondary infections[1]. Understanding the occurrence, distribution, and impact of *Meloidogyne* spp. on various crops is crucial for effective nematode management in field[2].

Accurate species identification is crucial for the success of management initiatives, and molecular tools offer advantages over traditional methods. Traditional morphological methods for nematode surveys face challenges due to conserved morphology and lack of specific life stages. Advancements in molecular biology, DNA sequencing, and molecular marker technology offer faster and more sensitive identification of species at various life stages[3,4].

Back in the 1990s, in the hilly districts of Uttar Pradesh state, over 50% of vegetable fields and more than 40% of vegetable crop root samples were found to be infested by RKNs[5]. Previous surveys of the Kumaon region detected the presence of RKN in vegetable crops like tomato, brinjal, and okra[6]. However, limited literature exists on nematode infestation in cucumbers in the Kumaon region, highlighting a significant knowledge gap. The present study addresses this gap, providing authentic documentation of *M. incognita* infesting cucumbers in the Kumaon region. This is the first authentic report of *M. incognita* infestation in cucumber grown under protected cultivation and emphasizes the importance of developing site-specific and sustainable management strategies to mitigate nematode damage and enhance cucumber production. Monitoring and implementing control measures are vital to protect crops and reduce economic losses in the region. The study presents the detailed information on species

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prevalence, population dynamics, and distribution, forming the basis for targeted and sustainable nematode management strategies.

Materials and Methods

Study area and sample collection

A survey was conducted during the years 2021-23 at the farmers' polyhouse growing cucumbers, located at N 29°35'00" E 79°52'44" in the Kumaon region of Uttarakhand. The geographical coordinates of the study site were accurately recorded using the 'Examobile' application on a GPS-enabled smart Android device. The random sampling technique employed divided the entire polyhouse into four units to study spatial variation. The farmer had been regularly cultivating cucumber crops using recommended practices. The cucumber plants are displaying symptoms of yellowing and stunting. A total of five soil cores, each weighing 50 grams, were collected from each unit, amounting to 250 grams. Uprooted cucumber plants with adhering soil samples were gathered for analysis. The confirmation of infestation was based on observable above-ground symptoms, including marginal yellowing of leaves, stunting of foliar parts, and the presence of characteristic knots on roots, which strongly indicated root-knot nematode (RKN) infestation. Subsequently, the infected roots were carefully collected in polythene bags, labelled, and transported to the laboratory. For identification purposes, different life stages (J3, J4, female, and male) were directly obtained by dissecting galled roots with the aid of a needle and forceps. Additionally, egg masses, extracted from the dissection of galled roots, were placed on a modified Baermann funnel[7] to collect juveniles for further examination and analysis. The study on characterization, morphometry, and molecular confirmation was carried out at ICAR–Vivekananda Parvatiya Krishi Anusandhan Sansthan (VPKAS), Almora.

Morphological identification

Harvested eggs were placed on tissue paper over a wire mesh in a petri dish and incubated at 25±2°C for 3 days. The hatched juveniles were fixed in triethanolamine-formalin (TAF) solution and processed in Seinhorst I and II solutions to enhance visibility[8]. After fixation, each juvenile was individually mounted on glass slides and labeled. Slides were examined under a light microscope (Olympus, Japan), and morphometric measurements, including body length, width, stylet length, tail length, spicule length, and dimensions of females and eggs, were recorded using measurement software (Olympus, Japan). This process aimed to analyze the key morphological variables of RKNs efficiently.

Molecular analysis

For molecular diagnosis, genomic DNA extraction from juveniles utilized Holterman lysis buffer[9]. In this process, 4–6 juveniles were combined with 25 µl of sterile water and 25 µl of lysis buffer, followed by crushing with a sterile micro-pestle. Lysates were incubated at 56ºC for 3 hours and 95ºC for 5 minutes. After centrifugation, lysates were stored at -20ºC. PCR was performed using the ribosomal DNA (rDNA) internal transcribed spacers (ITS) marker *V5367/26S*[4]. The PCR cycles are programmed as initial denaturation at 94°C (5 minutes), followed by 35 cycles of denaturation at 94°C (30 seconds), annealing at 72°C (30 seconds), and extension at 72°C (30 seconds), followed by a final extension cycle of 72°C (4 minutes). PCR products were resolved on 2% agarose gel (Himedia, India) and visualized in a UV transilluminator, revealing a specific product of ~600 bp judged against a 100 bp DNA ladder.

Sequencing and phylogenetic analysis

After amplifying the DNA via PCR, the PCR product was purified using a PCR purification kit (Qiagen, Germany). First, the PCR product was loaded onto the spin column of the kit and centrifuged at 8000-12000 × g for 1 minute to allow binding. Following this, a wash buffer was added to remove contaminants, and the column was centrifuged again at the same speed for 1 minute. This washing step was repeated as per the kit's instructions. Finally, the purified DNA was eluted by placing the column in a new microcentrifuge tube, adding the elution buffer, and centrifuging at 8000-12000 × g for 1 minute to collect the purified DNA. Sequencing was performed using the outsourced Sanger platform (Applied Biosystems 3730 XL DNA Analyzer). The obtained reads were subjected to BLASTn analysis, revealing a significant similarity to *Meloidogyne incognita*. The resulting sequence was submitted to NCBI with the accession no. OR880059. For the construction of the phylogenetic tree, *Globodera rostochiensis* (accession no. LC030413.1) was selected as the outgroup. Using MEGA 10 software, multiple sequence alignment and the phylogenetic tree for 14 *M. incognita* populations were generated[10]. The evolutionary history was deduced through the 'Maximum Likelihood Method' and 'Tamura-Nei model'[11], using a bootstrap consensus of 1000 replicates[12]. Initial tree(s) for the heuristic search were obtained automatically by applying the 'Neighbour joining' and 'BioNJ' algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value.

Assessment of nematode population density and statistical analysis

Soil sampling in a cucumber polyhouse involved systematic collection from each row to ensure representative coverage. Using a spade, samples were gathered from various locations to account for potential nematode distribution variability. Nematode counts were recorded at three different depths (0-10 cm, 10-20 cm, and 20-30 cm) across four rows 40 cm apart. Sub-samples (50 grams each) were used for nematode extraction via the modified Baermann funnel method[7]. Soil was placed in a mesh bag, suspended in a funnel with distilled water, allowing nematodes to migrate. After a set period, harvested nematodes were filtered through a sieve. Microscopic examination, focusing on morphological characteristics, aided in preliminary identification. Nematode counts were recorded for each sample, and population density was expressed as nematodes per 250 cm³ of soil.Descriptive statistics (mean and standard deviation) were calculated for nematode population densities across the four soil samples. Statistical significance was determined using one-way ANOVA to assess differences in nematode populations among the samples.

Results and Discussion

Morphology and symptomatology

Meloidogyne incognita (RKN) exhibits a global distribution and infects a wide range of hosts and stands as one of the most destructive species in tropical and warmer regions across the globe. RKN infestation and significant damage were identified in the Kumaon region under polyhouse-grown cucumber (Fig. 1a-b). The infected plants were exhibiting stunted growth, leaf marginal yellowing, and the formation of typical root knots (Fig. 1b-d). Morphological examinations confirmed the presence of *M. incognita*, with observations of juvenile nematodes at stained J3 and J4 stages, along with distinctive adult females recovered from galled tissues (Fig. 1e-i). The findings are consistent and supported by prior research on RKN morphological characteristics[13-15]. In fact, in addition to visible symptoms upon RKN infestation, it also disrupts the physiological processes within cucumber plants, thereby compromising overall health and productivity[16]. We also observed the yield loss in the polyhouse, which can be attributed to perturbed growth and loss of chlorophyll, but the physiological and biochemical processes are a matter of research upon RKN infestation. The earlier studies on surveys conducted in eight hilly districts of Uttarakhand state

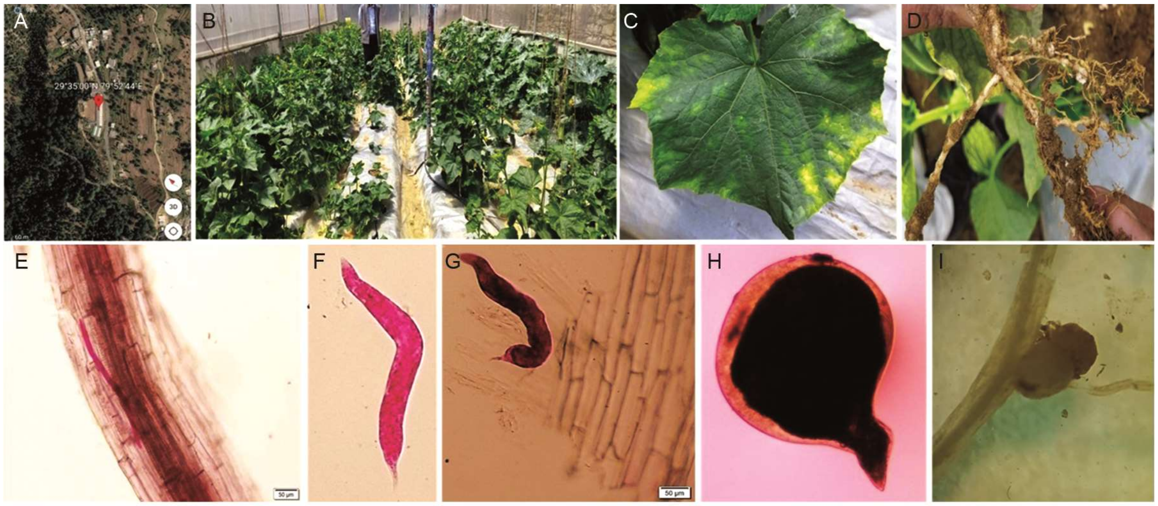

Fig. 1 — Sampling site, morphological and symptomatic analysis of root-knot nematode in cucumber crop. (A) Geographic coordinates of sampling location (N 29°35'00" E 79°52'44"); (B) Polyhouse with damaged cucumber plants; (C) Leave yellowing in Cucumber; (D) Root showing typical knots; (E) Juveniles in root tissue; (F) Stained J3 stage; (G) Stained J4 stage; (H) Adult female; and (I) Individual egg mass on root surface.

(earlier in Uttar Pradesh) revealed widespread infestations of root-knot nematodes in vegetable fields, with over 50% of fields affected[5]. Eggplant and okra were the most affected crops, followed by tomato, cucumber, and pepper[5]. *M. incognita* was identified as the dominant nematode species, with variations in the prevalence of different races observed. Effective management strategies are urgently needed to mitigate the impact of nematode infestations on agricultural productivity in region 7.

Morphometric measurements on second-stage juveniles (J2), adult males, and adult females revealed standardized characteristics in J2 and substantial morphological variations in adult males, highlighting sexual maturity features (Table 1). Egg measurements provided insights into *M. incognita* egg characteristics, and adult females exhibited significant morphological diversity. These results are consistent with established reports on *M. incognita*[17-19].

Molecular confirmation of *Meloidogyne incognita*

Molecular confirmation of *M. incognita* was done through rDNA ITS marker amplification, sequencing, and multiple sequence alignment. The rDNA ITS marker *V5367/26S* amplified a specific product of ~600 bp (Fig. 2). This PCR product was eluted and sequenced to reveal the species identity and variation. A total of 14 *M. incognita* sequences from the NCBI database and our study RKN sequence were compared for determination of genetic variation and unique/conserved regions identification. The present study RKN sequence is submitted to NCBI with 'accession no. OR880059'. Multiple sequence alignment showed sequence similarity of 96.40% with the GenBank accession OP030611.1 of *M. incognita* (Fig. 3). A phylogenetic tree based on the ITS region was reconstructed using 14 sequences of *M. incognita*, with *Globodera rostochiensis* (GenBank accession no. LC030413.1) serving as the outgroup (Fig. 4). The *M. incognita* population under study formed a well-supported clade with other |*M. incognita* isolates. Notably, the ITS sequence from the present study constituted a single clade with the Indian isolate KP179226.1 (https://www.ncbi.nlm.nih.gov/nuccore/KP179226.1/). This clustering pattern suggests a close genetic relationship between the *M. incognita* population in the Kumaon region and other geographically distinct *M. incognita* isolates, particularly with the Indian counterpart. The formation of a well-supported group in the phylogenetic tree further strengthens the molecular evidence supporting the classification of the studied nematode population as *M. incognita*.The high query coverage with accession OQ632601.1 in the BLAST analysis, along with the phylogenetic clustering with other *M. incognita* isolates, affirms the robustness of the molecular identification. The observed genetic proximity to the Indian isolate KP179226.1 suggests a shared genetic background among *M. incognita* populations across different regions. The genetic diversity is present within *M. incognita*, as reflected in the BLAST results and the phylogenetic tree (Figs. 3 & 4), and emphasizes the need for a holistic

Table 1 — Morphometric data of 2nd stage juvenile (J2), adult male, and adult female of *Meloidogyne incognita* isolate from the present study

Measurement of juvenile n=20	Present study
Reported place	Almora
Host plant	Cucumber
Body length	334.75± 1.95
Max. Body width	14.39±0.59
Stylet length	13.83±0.40
Tail length	21.65±0.72
Measurement of Males (n = 10)	
Body length	1842.92±2.98
Stylet length	23.68±0.24
Body width	35.01±0.70
Spicule length	11.99±0.33
Measurement of Egg (n=20)	
Length (L)	89.761±1.42
Width(W)	36.44±0.76
Measurement of female (n=20)	
Length (L)	821.16±821.16
Width (W)	529.11±529.11
Stylet length	15.99±15.99

Note: All the measurements represented are in µm in pattern of means ±SD. 'n' refers to number of specimens examined.

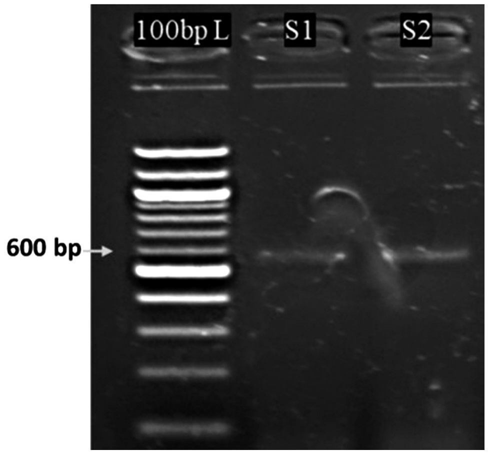

Fig. 2 — Thegel picture of amplification product of rDNA ITS marker *V5367/26S*. Abbrev.: 100 bp ladder DNA (first lane); S1: Sample 1(second lane); S2:Sample 2 (third lane)

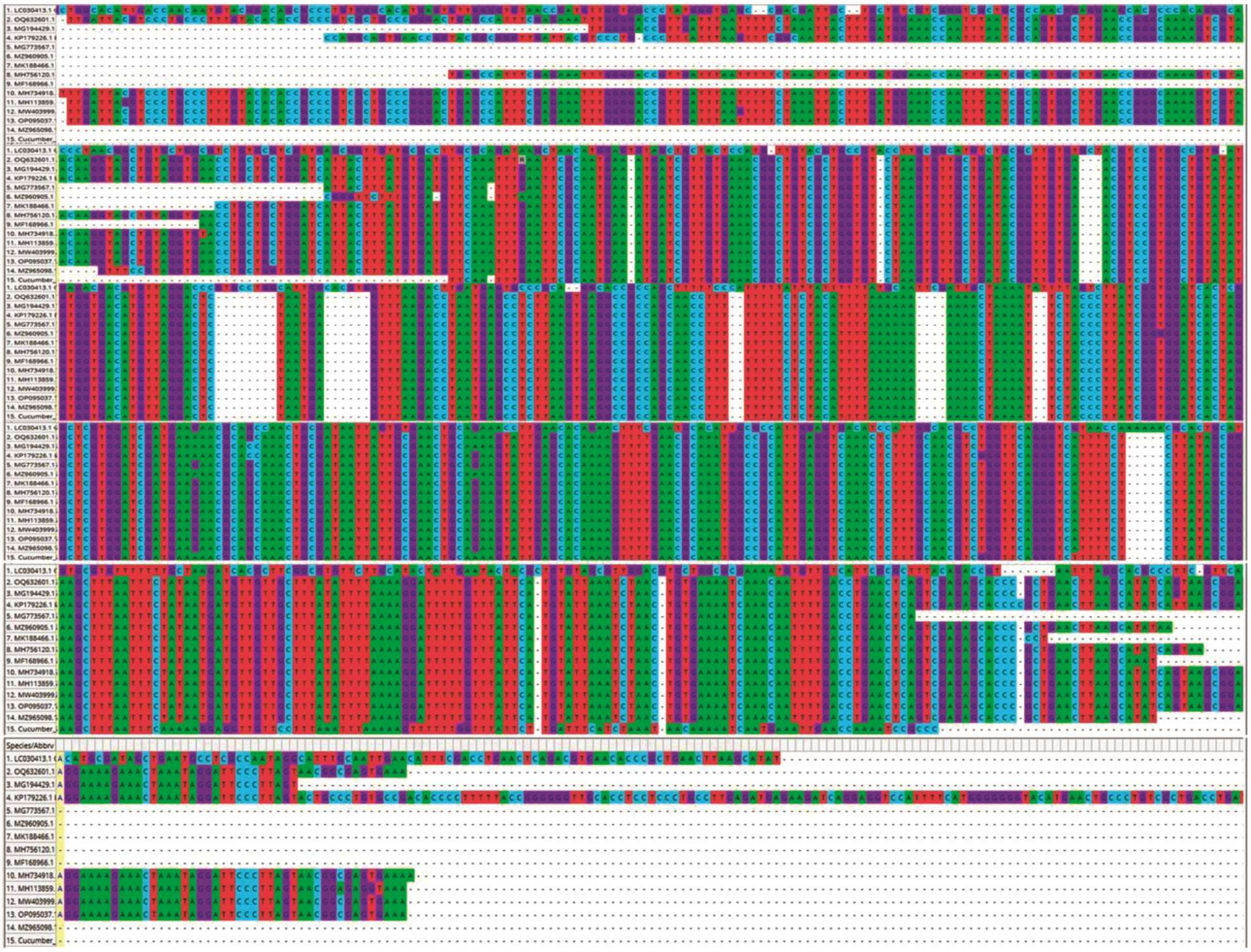

Fig. 3 — Multiple sequence alignment of various populations of *Meloidogyne incognita.* Comparison between total 14M. Incognita sequences from the NCBI database and one from the current study is shown here. *Globodera rostochiensis* (as outgroup, line 1), Indian isolate KP179226.1 (line 4) and our sequence (line 15)

approach in characterizing nematode populations. The analysis of DNA sequences revealed generally consistent phylogenetic relationships, with the sequences being grouped into distinct clades.

Thus, the molecular findings support and complement morphometric analyses and emphasize the comprehensive approach for nematode characterization[3]. Genome sequencing and molecular markers have been crucial in diversity analysis and species characterization[3,4,9]. Identification of *M. incognita* was feasible through DNA sequencing, as the ITS genes demonstrated a high level of conservation compared to other closely related RKNs, as indicated by both BLAST searches and phylogenetic trees. The observed sequence similarities, albeit not 100 percent identical, align with previously documented genetic variations within *M. incognita* isolates. The disparities in sequence similarity may be indicative of genetic diversity within *M. incognita* populations, reinforcing the need for molecular tools in addition to morphometric analyses for accurate species identification[3]. Thus, integrating molecular analyses, such as sequencing and phylogenetic reconstruction, with traditional morphometric studies provides a comprehensive understanding of the species and aids in the development of targeted management strategies.

Spatial distribution of the RKN in the polyhouse

Determining the critical nematode population density is crucial at the time of planting. Information on nematode density in soil facilitates the establishment of predictive relationships between pre-planting nematode populations and the subsequent yield loss[20,21].

Therefore, we also investigated the spatial distribution of RKN in a protected environment.

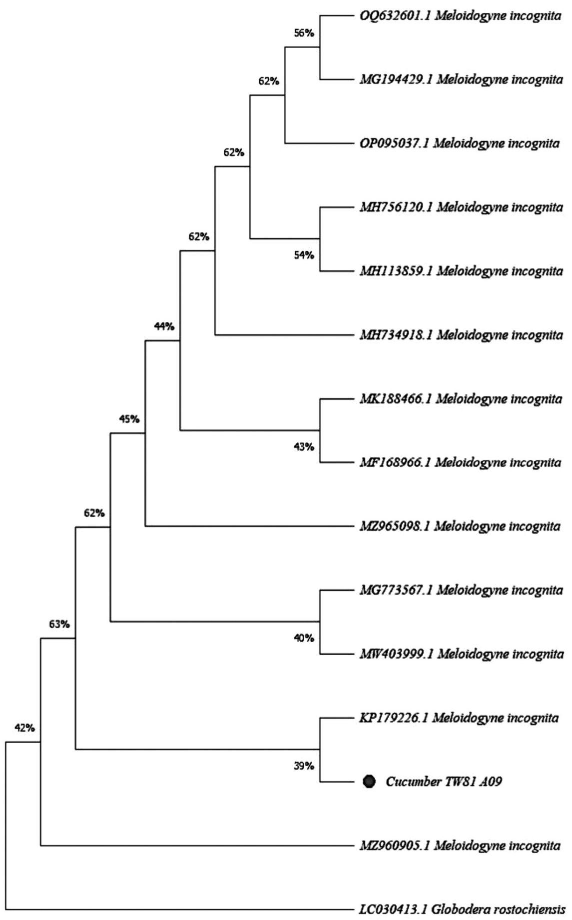

Fig. 4 — The evolutionary history of *Meloidogyne incognita* inferred by 'Maximum Likelihood Method' and 'Tamura-Nei Model'. The tree with the highest log likelihood (-3274.36) is presented here. The highlighted clade shows the sequences of present study and their close relatives. A bootstrap consensus tree was generated from 1000 replicates

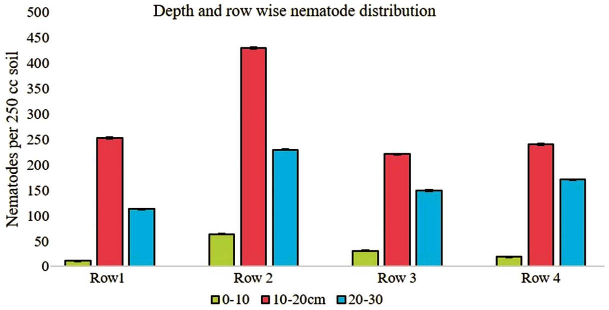

Fig. 5 — Spatial distribution of root-knot nematodes in a polyhouse grown cucumber. Nematode counts were recorded at three different depths across four rows (40 cm apart)

Nematode counts were recorded at three different depths across four rows. Statistical analysis and interpretation were conducted to understand the variations in nematode abundance among rows and depths. A one-way ANOVA was performed to assess the significance of differences in RKN population counts among the rows at each depth interval. The analysis revealed a significant effect of row on nematode counts at all depths ($p < 0.05$). Post-hoc tests indicated specific depth intervals and rows where significant differences existed. Row 2 consistently exhibited the highest nematode counts, while Row 1 consistently showed the lowest counts. The significant variation in RKN population counts among rows suggests that the spatial distribution of nematodes is not uniform within the polyhouse. Row 2 consistently showed higher nematode counts (Fig. 5), indicating a potential hotspot for nematode activity. This may be influenced by factors such as soil conditions, plant root distribution, or other environmental variables specific to nematode dispersal in Row 2. The higher nematode counts in Row 2 raise questions about potential management strategies to mitigate nematode damage in this specific row. Precision treatment or targeted interventions in Row 2 may be considered to optimize nematode control efforts and improve overall crop health in the polyhouse. The observed differences in nematode counts across depth intervals further emphasize the need for a comprehensive understanding of nematode ecology in polyhouses. Factors like root depth and soil characteristics may contribute to the observed patterns. Under controlled environments with consistently favorable conditions of temperature, humidity, and host presence, there is a substantial increase in the population of RKN, reaching levels as much as 12-15 times higher than those observed under typical field conditions[22]. Therefore, rows indicating a low population may experience an increase in the future, contingent upon the availability of favorable conditions such as temperature, humidity, and the presence of suitable hosts.

Conclusion

This study presents an analysis of *Meloidogyne incognita* (root-knot nematode, RKN) affecting cucumber plants grown under polyhouse conditions in the Kumaon region. The findings reveal significant insights into the morphological characteristics, molecular identification, and spatial distribution of *M. incognita* populations. The RKN infestation led to notable symptoms such as stunted growth, leaf marginal yellowing, and typical root knot formation. Morphological examinations confirmed the presence of *M. incognita,* with distinct juvenile and adult stages observed. rDNA ITS sequencing and phylogenetic analysis confirmed the identity of *M. incognita*, showing a high sequence similarity with previously reported isolates, particularly the Indian isolate KP179226.1. The study revealed significant variations in nematode abundance across different rows and depths within the polyhouse, with Row 2 consistently exhibiting the highest nematode counts. The determination of critical nematode population density at planting time is essential for predicting yield loss and developing effective management strategies. Targeted interventions in specific areas with high nematode activity, such as Row 2, can optimize nematode control efforts and improve overall crop health and productivity. Future research is needed to investigate the physiological and biochemical processes affected by RKN infestation in cucumber plants. Continued molecular characterization of *M. incognita* populations from diverse geographic locations will help elucidate genetic diversity and adaptation mechanisms. This can aid in developing robust diagnostic tools and resistant crop varieties. Developing and implementing integrated pest management (IPM) strategies that integrate biological, chemical, and cultural control methods will be crucial for sustainable management of RKN in polyhouse environments. Extension programs focused on educating farmers about nematode identification, symptoms, and management practices are essential.

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Conflict of interest

All authors declare no conflict of interest.

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