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RESEARCH

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Isolation of *Pasteuria penetrans*, an obligate hyper-parasite, infecting root knot nematode, *Meloidogyne* spp. from the rhizosphere of pulses in India

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Abstract

Background *Pasteuria penetrans* is a mycelial, endospore forming, obligate, bacterial parasite that has shown enormous potential for biological control of root-knot nematode, *Meloidogyne* spp. In the present study, the effort has been made to isolate effective and high temperature tolerant indigenous strains from pulse rhizosphere in India.

Results Surveys were carried out in four different agro-climatic zones and collected 106 root-knot nematode infected soil and plant samples from pulses rhizosphere. Of these, we observed *Pasteuria* spore attachment on root knot nematode juveniles from seven samples: three from Varanasi, two from Salem and one each from Deoria and Kushinagar. The pure cultures of these seven isolates (named as IIPR-Pp-1 to IIPR-Pp-7 from single infected female, respectively) were developed in 7 cm pot and soil-less CYG medium. The nematode host species was identified as *Meloidogyne incognita* by perineal pattern and confirmed by sequencing of ITS region. The host range study with *M. incognita* and *Heterodera cajani* showed the spore attachment was specific to *M. incognita* revealed that the species of the parasite was *Pasteuria penetrans*. SEM study on morphometrics of *Pasteuria* spores belong to two groups. In vitro assay on endospore attachment assay with pre-treated endospores at 20, 30, 40, 50, 60 and 70 °C revealed that maximum number of endospore attachment of all isolates was observed at 30 °C followed by 40 °C, and the least number of them was observed at 60 °C and above. The isolate Pp3 exhibited maximum number of spore attachments among all isolates at 30 °C treatment (14) and 40 °C treatment (10.67). Endospore attachment assay with pre-treated J2 at 20, 25, 30, 35, 40 and 45 °C revealed that maximum number of attachment of all isolates was observed at 30 °C treatment, and no attachment was observed at 40 °C and above.

Conclusion *Pasteuria penetrans* isolate IIPR-Pp3 exhibited high temperature tolerance and can be used further to develop *Pasteuria*-based biopesticide for management of root-knot nematode, *M. incognita* in pulses.

Keywords *Pasteuria penetrans*, Host range, Endospore attachment, SEM morphometrics

Background

Pasteuria penetrans (Thorne) Sayre and Starr is an obligate, mycelial forming, endospore forming, gram negative hyper parasitic bacterium of the nematode genus *Meloidogyne* and has been receiving specific attention both for its host specificity and biocontrol potential (Aurelio Ciancio 2018). The endospore of the bacterium is present in soil attached to the cuticle surface of the moving

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second stage juveniles. The endospore starts to enter into nematode body once juvenile starts feeding on selected cell in the vascular region of the root. The nematode body content is consumed by the growing bacterium, and the fecundity is completely destroyed, thus arresting the next generation (Phani and Rao 2018). There are other four species which are parasites specific to particular genus viz., *Pasteuria thornei* parasitizes root-lesion nematode, *Pratylenchus thornei*, *Pasteuria nishizawae* parasitizes cyst nematode, *Heterodera* and *Globodera*, and *Pasteuria usage* parasitizes sting nematode, *Belonolaimus longicaudatus*. The bacterium is widely distributed and was reported from 20 countries (Devi 2023). The obligatory nature of the bacterium and host specificity are two important factors impeding commercial exploitation of this highly specialized bacterium (Orr et al. 2020). *Pasteuria* Bioscience Inc., an incubation center at MIT developed a proprietary *Pasteuria* manufacturing process based on submerged (liquid fermentation) fermentation method. Syngenta launched biocontrol products based on *Pasteuria nishizawae*—Pn, CLARIVA™ pn and CLARIVA™ Elite Beans as seed treatment nematicide for the management of soybean cyst nematode, *H. glycines* in USA in 2014 and now available for use in Brazil and Canada. Similarly, *P. penetrans* is also having similar potential of huge commercial exploitation, and the diversity in India and the potential of the bacterium is remain unexplored, so this study aimed to isolate *P. penetrans* from pulse rhizosphere which is a very important step to find out highly temperature tolerant and virulent native strains or biotypes for root-knot nematode, *Meloidogyne* spp. management.

Methods

Collection of soil samples

Surveys were carried out in different agro climatic zones of pulse growing regions, and nematode infected soil and plant samples from pulses rhizosphere were collected based on random sampling method. At each site, 200–300 g of soil and root samples from rhizosphere were collected and packed well with proper labeling. Information on crop history, soil type and other relevant data was also collected for further reference. The samples were stored at 4 °C in refrigerator for further processing.

Pasteuria penetrans isolation

The root-knot nematode infected soils were filled in 20 cm diameter pots and planted with susceptible host plant for nematode multiplication, this will act as a mother culture. The infected roots were used for isolation of *P. penetrans* by direct observation method. The infected roots were chopped and kept in Petri dish-wire mesh-tissue paper apparatus for nematode extraction from infected roots. After 24 h, the juveniles in the suspension were examined individual by individual under LEICA MZ125 stereoscopic microscope for spore attachments. The spore encumbered juveniles were immediately inoculated along with healthy juveniles on susceptible host plant grown in 7.5 cm pots for spore multiplication.

Pure culture

Pure population of P. penetrans

CYG germination pouch method was used for infecting of *Pasteuria* spore attached nematode juveniles for isolation of spore filled adult female on green gram roots. Individual female filled with spores was crushed, and all spores were collected in 1.5 ml centrifuge tube. Freshly, hatched healthy juveniles (2500/tube) were added to each tube. The suspension was mixed through centrifugation at 10,000 g for 3 min. The juveniles were observed under microscope for spore attachment. These spore attached juveniles were inoculated along with healthy juveniles on susceptible host plant of 5 days old grown in CYG germination pouch or 2 to 3 leaf stages grown in 7.5 cm pots. After 30 days of inoculation, the infected females were removed from roots and used for further studies.

Host range study

To study the host range of the identified strain of *Pasteuria,* the endospore attachment assay was carried out, using 2 commonly observed nematode species/genera viz., *M. incognita*, *Heterodera cajani* infecting pulses. The procedure described by Hewlett and Dickson (1993), with few modifications was followed, spore suspension of *Pasteuria penetrans* at 1.0×10^6/ml of distilled water was prepared for each strain. A total of 0.5 ml of suspension were placed into five 1.5 ml Eppendorf tubes per strain. After that, a total of 250 µl of nematode suspension at10000 J_2/ml of distilled water was added to each tube. Tubes were centrifuged at 8000g for 3 min and randomly 15 juveniles were selected for spore attachment observation.

Microscopy

Light microscopy

Second-stage juveniles of *M. incognita* with attached *Pasteuria* endospores were mounted in distilled water on a glass slide, and spores were observed at 400, 600 and 1000X magnification to confirm endospore attachment on cuticle surface. The image of juveniles and spore attachments was taken with microscope attached imaging system "Leica Application Suite version 4.13.0 [build:167]".

Scanning electron microscopy (SEM)

The endospore attachment and orientations were studied in-detail with the help of SEM. The endospore attached juveniles were fixed in 2% glutaraldehyde (buffered with 0.1 M phosphate buffer at pH 7.2 for 12 h) at 4 °C. After that, the samples were fixed again with 2% osmium tetroxide solution for 4 to 6 h at 25 °C (post-fixation). Then, the samples were dehydrated with series of graded concentration of ethanol (consisting of 40, 50, 60, 80, and 90% and absolute ethanol) and further processed for critical point drying with liquid CO_2. The dried samples were mounted on SEM stubs, coated with gold–palladium in a sputter coater and observed under the scanning electron microscope (Tescan Vega 3) (Mhatre et al. 2020). The morphometrics (spore length and width) of each isolates were carried out using the software imageJ.

Morphological characterization: Perineal pattern

Perineal patterns of adult females were prepared according to procedure given by Hasan and Abood (2018). Adult females were removed gently from root tissues with forceps and needle and the posterior end was excised and cleared the debris of body contents in a solution of 45% lactic acid. The perineal pattern region was trimmed and transferred to a drop of glycerin for observations under microscope. The perineal pattern was then compared to the description given by Hunt and Handoo (2009).

Molecular characterization

From infected roots, pure culture from ten (10) egg mass of *M. incognita* was developed based on perineal pattern. The genomic DNA from adult female from the populations of *M. incognita* was extracted through Worm Lysis Buffer (WLB) method described by Williams et al. (1992) with slight modification. The ITS region was amplified with suitable primers, and the purified PCR products were sequenced and analyzed with NCBI Blast. The species of all the sequences was identified as *M. incognita*. The sequences were aligned through CLUSTAL W, trimmed, submitted and received accession numbers from NCBI.

Effect of temperature on endospore attachment

Treatment of endospores

Concentration of endospore suspension of all *Pasteuria* isolates was adjusted to 2×10^6 endospores/100µl of distilled water and suspension of 0.5 ml were placed in 2 ml centrifuge tube and treated at 20, 30, 40, 50, 60 and 70 °C for 5 h/day in water bath for one week. A total of 250 µl of nematode suspension containing 2000 J_2/ml were added to each tube, and endospore attachment study was carried out with three replications.

Treatment of juveniles

One and half ml polypropylene centrifuge tubes containing 1000 juveniles of *M. incognita* in 0.5 ml of water were treated at 25, 30, 35, 40, 45, or 50 °C. for 10 min in water bath. After incubation, a total of 250 µl of a water suspension of *P. penetrans* at 2×10^6 endospores/100 µl were added to each tube and endospore attachment study was carried out with three replications.

Endospore attachment assay

In vitro assay was carried out to study the effect of different temperature on endospore attachment on second stage juveniles (J_2) of *M. incognita* as described by Hewlett and Dickson (1993).

Data analysis

The experimental data on endospore attachment assay were analyzed using Minitab USA software [Minitab® 21.1 (64-bit)]. Analysis of variance (ANOVA) was performed, and grouping was done through Tukey pairwise comparison with 95% confidence interval. The experimental data on endospore morphometrics were analyzed using OPSTAT software developed by Sheoran et al. (1998).

Results

Collection of soil samples

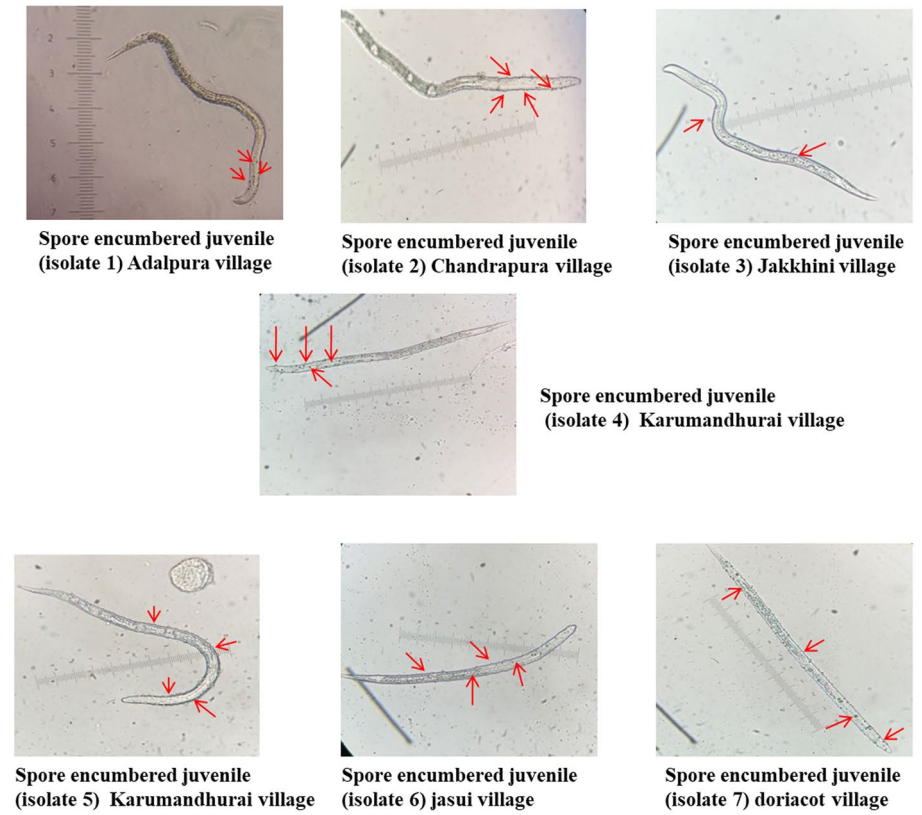
Totally, 106 nematodes infected soil and plant samples from pulses rhizosphere in four different agroclimatic zones of India, viz., eastern plains (Varanasi, Uttar Pradesh), central plains (Sitapur, Uttar Pradesh), north-eastern plains (Deoria & Kushinagar, Uttar Pradesh) and southern plateau and hill zone (Salem, Tamil Nadu) were used. Out of the 106 samples processed and observed, three samples from Varanasi, two samples from Salem, one sample each from Deoria and Kushinagar were having bacterial attachments on juveniles (Fig. 1).

Isolation of *Pasteuria* spp. and development of pure culture

These infected juveniles from seven samples were immediately inoculated separately on susceptible plant host for host-parasite life cycle completion, which is necessary for bacterial spore multiplication and further the infected female were harvested from the roots, and the pure culture was developed using CYG germination pouch (Fig. 2).

Identification of species

The endospores from all the isolates were allowed to get attached specifically on second stage juveniles of *M. incognita* and *H. cajani*, and the results showed that endospores were attached to only on *M. incognita* but



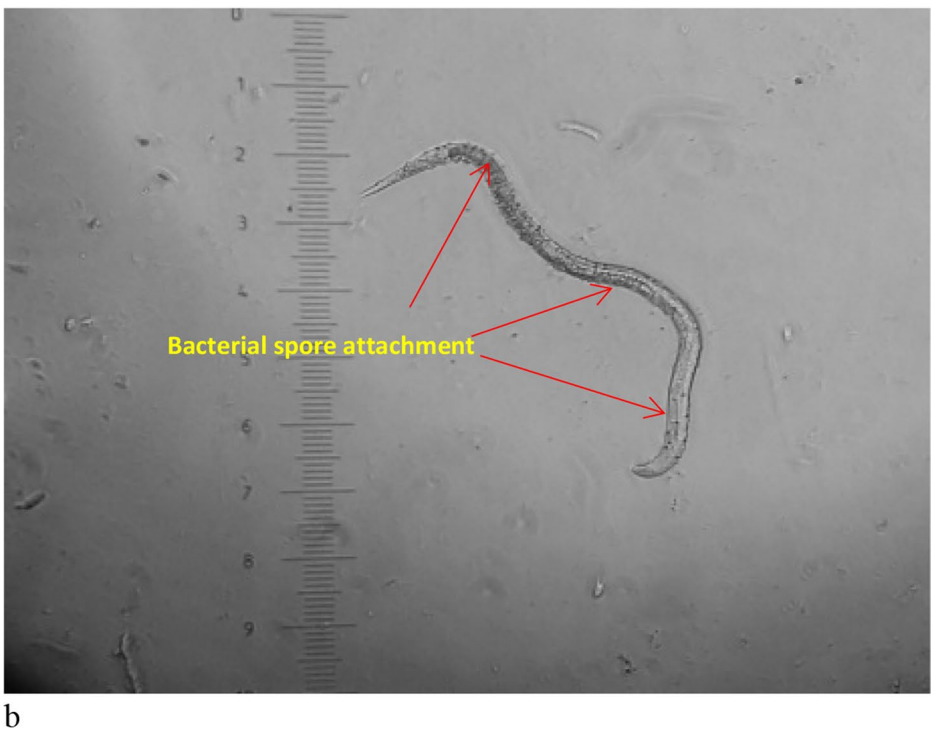


Fig.1 Isolation of *Pasteuria penetrans* **a** Isolates from different agroclimatic zones **b** individual juvenile encumbered with *Pasteuria* spores

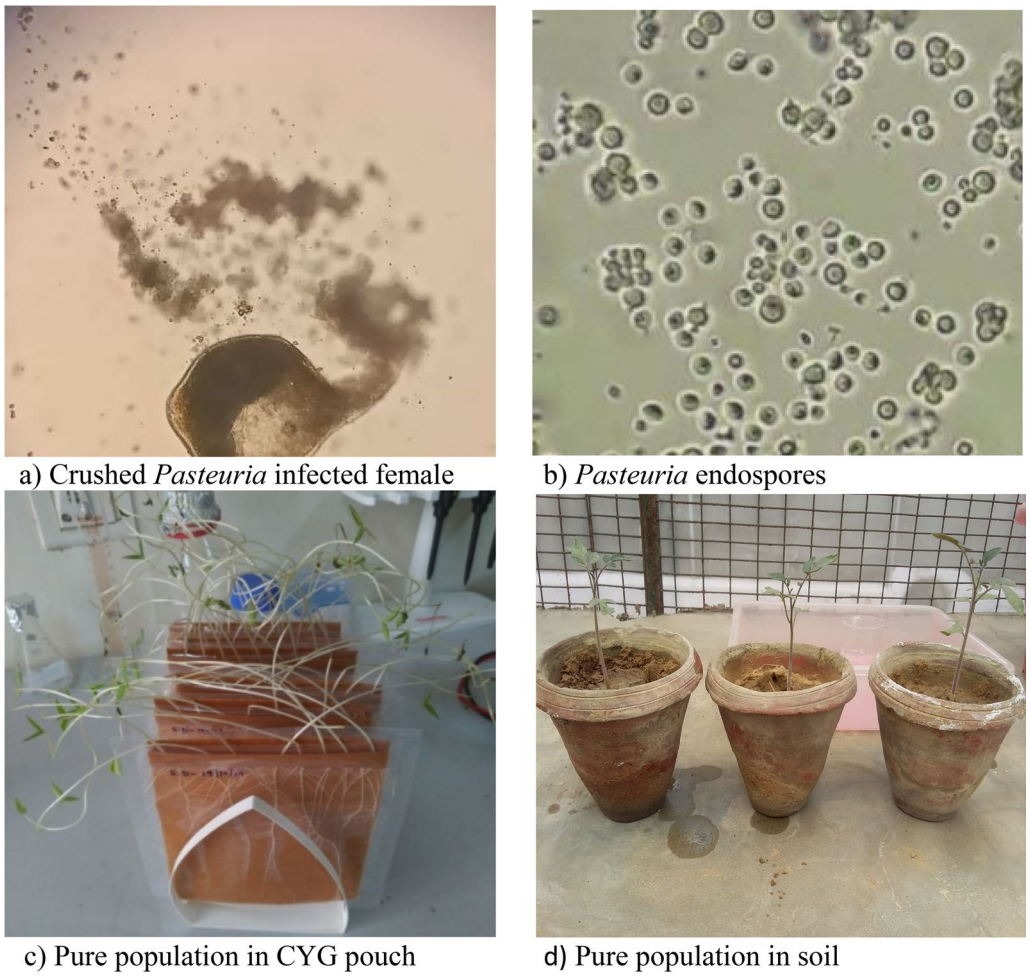


Fig. 2 Development of pure population from single infected female

Fig. 3 Host range study **a** spore attachment on *Meloidogyne incognita* **b** spore not attached on *Heterodera cajani*

not on *H. cajani*; this indicates that all the isolates were belonging to *P. penetrans* (Fig. 3).

Endospore morphometrics

It was observed through Scanning Electron Microscopy that the spores were very diverse in their size and shape, which grouped into two categories. The highest length was observed in isolate IIPR-Pp6 (4.34 µm), and the lowest length was observed in isolate IIPR-Pp7 (3.53 µm). The highest width was observed in isolates IIPR-Pp3 (3.25 µm) and the lowest width was observed in isolate IIPR-Pp7 (2.75 µm) (Table 1, Figs. 4, 5).

Table 1 Morphometrics of *Pasteuria penetrans*

Pasteuria penetrans isolates	Length (µm)	Width (µm)
IIPR-Pp-1	3.79[a]	2.95[a]
IIPR-Pp-2	4.04[abc]	2.96[a]
IIPR-Pp-3	4.32[cd]	3.25[a]
IIPR-Pp-4	3.7[ab]	2.38[a]
IIPR-Pp-5	4.27[cd]	2.93[a]
IIPR-Pp-6	4.34[cd]	3.03[a]
IIPR-Pp-7	3.79[ab]	2.75[a]
C.D	0.39	0.31
SE(m)	0.136	0.108
SE(d)	0.193	0.152
C.V	7.616	8.328

Note: the values are the average of seven replications, and the analysis on ANOVA was carried out by OPSTAT software

[a, b, c] indicates statistical grouping and the mean value that do not share a letter are statistically different from each other

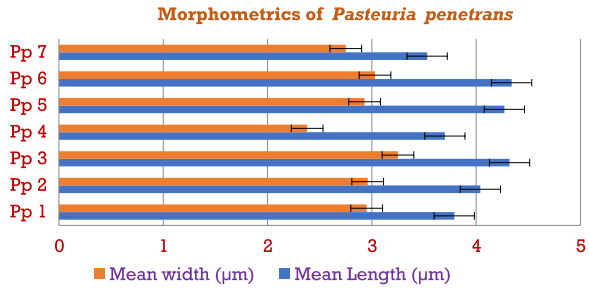


Fig. 5 Morphometrics of *Pasteuria penetrans* endospores of various isolates

Identification of *Meloidogyne* spp.

The species of root-knot nematode was identified through perineal pattern. The typical trapezoidal dorsal arch and spherical low arch were clearly observed (Fig. 6a). PCR reaction of genomic DNA from adult female for ITS region yielded 800bp product (Fig. 6b). Sequence blast with NCBI revealed that species belongs to *M. incognita* with 99% similarity, the sequences were submitted, and NCBI accession number was obtained (Table 2).

Identification of temperature tolerant strain

Based on the results obtained from endospore attachment assay experiment, it was clearly evident that endospore attachment was influenced by temperature. Pre-treated endospores at 20, 30, 40, 50, 60 and 70 °C revealed that maximum number of endospore attachments of all isolates was observed at 30 °C, followed by 40 °C. The least number of endospore attachments was observed at 60 °C and above. The isolate IIPR-Pp3

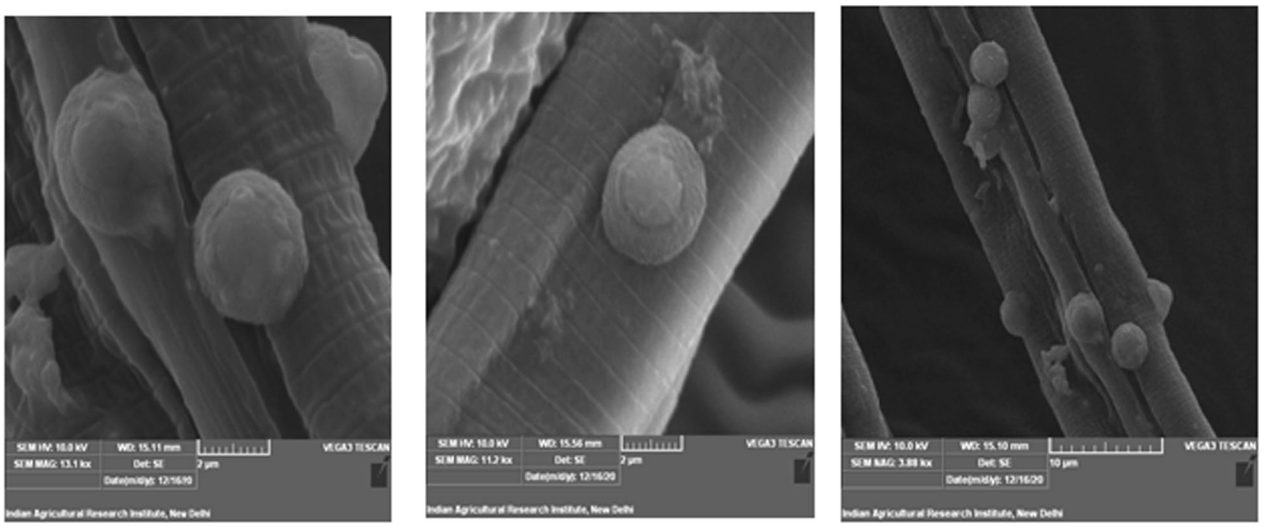
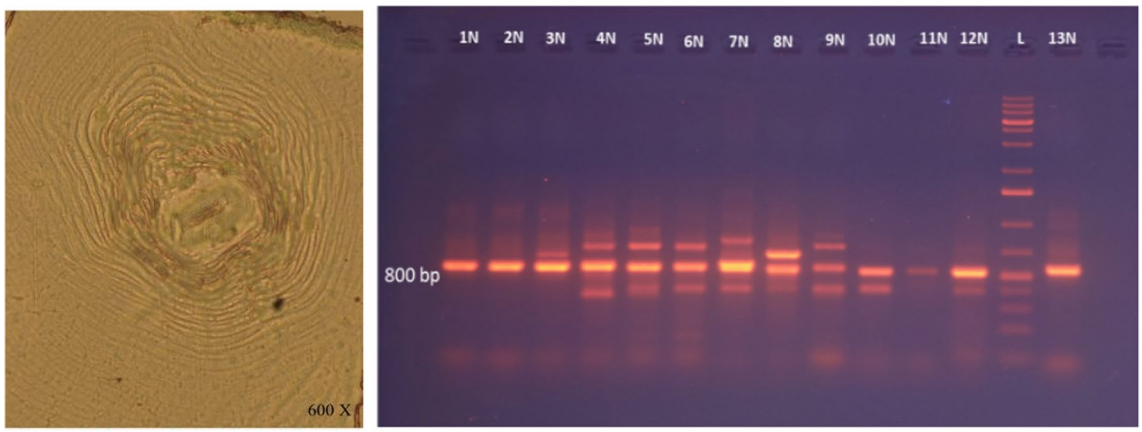


Fig. 4 SEM observation on *Pasteuria penetrans*



a b L: 1000kb ladder; N1 to N13 Nematode DNA samples

Fig. 6 **a** perineal pattern of *Meloidogyne incognita* **b** PCR product of ITS region of genomic DNA (Primer: Forward: ITS1, Reverse: ITS4)

Table 2 List of *Meloidogyne incognita* strains sequences submitted and received NCBI accession number

Organism	Strains	Sequence	Accession
Meloidogyne incognita	IIPR-Mi-1	ITS1-5.8S-ITS2	MZ960901
M. incognita	IIPR-Mi-2	ITS1-5.8S-ITS2	MZ960902
M. incognita	IIPR-Mi-3	ITS1-5.8S-ITS2	MZ960903
M. incognita	IIPR-Mi-4	ITS1-5.8S-ITS2	MZ960904
M. incognita	IIPR-Mi-5	ITS1-5.8S-ITS2	MZ960905
M. incognita	IIPR-Mi-6	ITS1-5.8S-ITS2	MZ960906

exhibited maximum number of spores' attachments among all isolates at 30 °C treatment (14) and 40 °C treatment (10.67) (Table 3, Fig. 7). Endospore attachment assay with Pre-treated J_2 at 20, 25, 30, 35, 40 and 45 °C revealed that maximum number of endospore attachment of all isolates was observed at 30 °C treatment and no attachment was observed at 40 °C and above (Table 4, Fig. 7).

Discussion

Plant-parasitic nematodes cause economic yield losses of more than US$157 billion worldwide (Singh et al. 2014). Chemical fumigants have been the primary means of control measures, but due to their harmful effects (Rani et al. 2021), new effective biopesticides are being developed and used (Singh et al. 2022). *P. penetrans* is an endospore forming obligate bacterial parasite of *Meloidogyne* spp. and has the ability to arrest entire generation of reproduction and make the soil suppressive for root-knot nematode (Devi 2023). In India, with the huge agro-ecological diversity, existence of tremendous diversity in virulence and specificity of *P. penetrans* is possible, and it is the need of the hour to explore it for root-knot nematode management. The frequency of observation of *Pasteuria* spores from the nematode infected samples revealed that

Table 3 Effect of pre-treated endospore on attachment

Strains	Temperature treatment to endospores (°C)					
	20	30	40	50	60	70
Pp1	6.00^{a}	10.00^{ab}	9.00^{a}	5.00^{ab}	2.00^{b}	0
Pp2	7.67^{a}	11.67^{ab}	8.00^{a}	2.67^{b}	2.33^{b}	0
Pp3	8.00^{a}	14.00^{a}	10.67^{a}	6.67^{a}	6.00^{a}	0
Pp4	9.00^{a}	8.67^{b}	8.33^{a}	3.67^{ab}	1.67^{b}	0
Pp5	7.33^{a}	12.00^{ab}	9.67^{a}	3.33^{b}	2.00^{b}	0
Pp6	7.33^{a}	9.00^{ab}	9.67^{a}	3.33^{b}	1.67^{b}	0
Pp7	6.67^{a}	9.67^{ab}	9.00^{a}	5.00^{ab}	3.00^{b}	0
Pooled StDev	1.48	1.75	1.58	1.03	0.80	
P (=0.05)	0.265	0.028	0.582	0.007	0.001	

Note: The value of no of attached *Pasteuria* endospore is the mean of three replications. Different case letters in a column represents statistically differences of mean according to Tukey Pairwise Comparisons (Grouping Information using the Tukey Method and 95% Confidence)

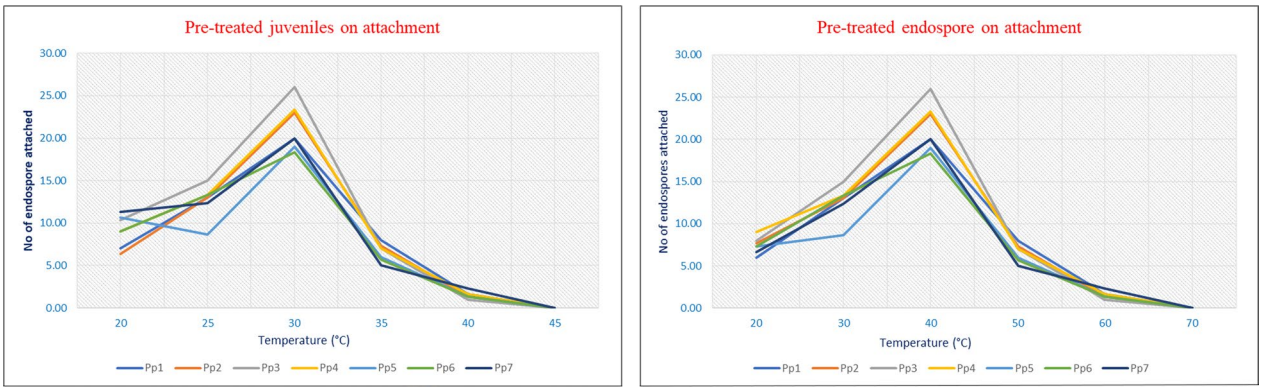


Fig. 7 effect of temperature on endospore attachment

Table 4 Effect of pre-treated juveniles on attachment

Strains	Temperature treatment to juveniles (°C)					
	20	25	30	35	40	45
Pp1	7.00^{bc}	13.00^{a}	20.00^{ab}	8.00^{a}	0	0
Pp2	6.33^{c}	13.00^{a}	23.00^{ab}	7.33^{a}	0	0
Pp3	10.33^{ab}	15.00^{a}	26.00^{a}	7.00^{a}	0	0
Pp4	9.00a^{bc}	13.33^{a}	23.33^{ab}	7.00^{a}	0	0
Pp5	10.67^{ab}	8.67^{a}	19.00^{b}	6.00^{a}	0	0
Pp6	9.00^{abc}	13.33^{a}	18.33^{b}	5.67^{a}	0	0
Pp7	11.33^{a}	12.33^{a}	20.00^{ab}	5.00^{a}	0	0
Pooled StDev	1.21	2.89	2.17	1.40		
P (=0.05)	0.002	0.376	0.010	0.240		

Note: The value of no of attached *Pasteuria* endospore is the mean of three replications. Different case letters in a column represents statistically differences of mean according to Tukey Pairwise Comparisons (Grouping Information using the Tukey Method and 95% Confidence)

not all the juveniles contained spores' attachment, which meant their distribution was widespread, and there was no specific hotspot/specific place for its distribution.

Host specificity was the second most important characteristic features of *Pasteuria* after obligate parasitism. The attachment of endospores only on *M. incognita* was not on *H. cajani* indicated that the species belonged to *P. penetrans* and specific effector-receptor interaction may be the reason for host-specificity. Attachment of endospore to the nematode cuticle was the determinative step for host specificity. The surface coat of plant parasitic nematodes possessed many biomolecules which is important for host-parasite interaction. Phani et al. (2017) revealed that fatty acid- and retinol-binding protein, *Mi-far-1* (573 bp) plays a key role in endospore attachment to the cuticle of *M. incognita*. Another important polypeptide molecule, mucins, known for their role in host-parasite interaction, was identified as a key determinant of endospore attachment to the cuticle of *M. incognita* (Phani et al. 2018). The ultrastructure and function of *Pasteuria* endospore viz., exosporium and fibrils on the outer surface and biomolecules associated with surface of nematode cuticle and their possible role in bacteria-nematode interaction were studied. Furthermore, the collagen fibrils present on the surface of the endospore and the microvilli presents on the surface of the infective juvenile and interact through Velcro-like attachment mechanism may play significant role in specificity in attachment (Davies et al. 2023). The study on gain of function of Pasteuria collagen and its role in host specificity was difficult because of its obligate parasitism, and it was demonstrated that *Pasteuria* collagen was similar to *Bacillus thuringiensis* collagen which is easily culturable and can be used as a gain of function platform/model (Srivastava et al. 2022).

It was observed in the present study that endospores varied in size and shape, which statistically belonged to two categories. Mohan et al. (2012) observed that *Pasteuria* spp. was not only showed host specificity to the species but also showed specificity to their native populations. Liu et al. (2019) explained that *P. penetrans* exhibited the ability to adhere and infected only a particular

population of root knot nematodes, i.e., very narrow host range will be a major practical constrain, and it is required to identify a population with wide host range for successful management of rapidly evolving heterogenous populations in the field. So, it is very important to understand the mechanism of host-specificity in the bacterium-nematode interaction, in order to identify effective populations of *Pasteuria* to control specific nematode races and hopefully identify bacterial strains with a wide host range. Aurelio Ciancio (2018) observed that there was a link between spore size and thickness of nematode cuticle. It was further correlated this link with Haldane's law of host and parasites during co evolution. The variations indicated that the possibility of presence of biotypes of the species. Whether there was any host specificity at *Pasteuria* biotype-*Meloidogyne* race level needs to be investigated.

Temperature plays an important role in spore attachment to cuticle; the study showed that pre-treated spores at even 50°C was able to get attachment on juvenile cuticle confirmed the previous reports of high temperature tolerance of *Pasteuria* endospores. The pre-treated juveniles that supported endospore attachment up to 40 °C only indicated that endospore was more tolerant to temperature than juveniles. Dutta and Phani (2023) observed that solubilization or denaturation of protein recognition sites or ligands on the nematode cuticle surface occurred due to high temperatures and hampers attachment of endospores. These proteins recognize certain carbohydrates and are thought to be responsible for endospore attachment because they bind to N-acetylglucosamine on the surface of endospores. Simon Gowen (2020) explained that the biocontrol efficiency of *P. penetrans* depended on the temperature tolerance and sufficient concentrations of viable spores in the soil, the probability of contact with the nematode juvenile and the host-specificity of the native population of *P. penetrans*. Also, it was observed that commercial success will depend on finding and developing native populations of *P. penetrans* with a broad spectrum of pathogenicity.

Accurate identification of *Meloidogyne* species was crucial to understanding the host-parasite interaction between *Pasteuria-Meloidogyne*. Confirmation of six root-knot nematode populations from different locations identified as *M. incognita* based on perineal pattern and through DNA sequences on ribosomal DNA 18S-ITS-5.8S region indicated that *M. incognita* was more widespread and harboring *Pasteuria* spores in these locations.

Conclusion

Seven isolates of *Pasteuria penetrans* were isolated (IIPR-Pp1 to IIPR-Pp7) from four different agro-climatic regions of India. The endospores of all the isolates were capable of arresting egg production in adult females of root-knot nematode, *M. incognita* and the strain IIPR-Pp3 was expressing high temperature tolerance. The strain can be further evaluated in field conditions and utilized for nematode management in pulses. Further, profiling of host specificity between *M. incognita* races and *P. penetrans* isolates can be studied for identification of race specific bacterial strains.

Abbreviations

ICAR	Indian Council of Agricultural Research
IIPR	Indian Institute of Pulses Research
Pp	*Pasteuria penetrans*
ITS	Internal Transcribed Spacer
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
rDNA	Ribosomal DNA
CO_2	Carbon-di-oxide
NCBI	The National Center for Biotechnology Information

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Author contributions

RJ was contributed to conceptualization, design of the work, manuscript drafting, data analysis. JD was contributed to carrying out all the experiments and data collection. BS was contributed to assistance in identification of pasteuria penetrans.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors give their consent to publish the submitted manuscript as "Original paper" in EJBPC.

Competing interests

The authors whose names are listed immediately below certify that they have NO affiliation with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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