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Isolation and characterization of *Pasteuria* parasitizing root-knot nematode, *Meloidogyne incognita*, from black pepper fields in India

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Abstract

Root-knot nematode (RKN), *Meloidogyne incognita*, is one of the most lingering and difficult to manage pest of black pepper in India. The options for controlling RKN are becoming increasingly limited due to the potential risk involved in environmental and health hazards. Biological control using *Pasteuria* is one of the most effective and efficient ways of nematode management. *Pasteuria* spp. are obligate parasites of plant-parasitic nematodes and completely inhibit their fecundity. There is also a tremendous opportunity for the discovery of native strains adapted to local environmental conditions and nematode species. Therefore, in the present study, efforts were made to isolate the native strain of *Pasteuria* from the fields of black pepper. Random sampling was done from black pepper-growing areas of Kerala and Karnataka states of India. Out of 39 samples, *Pasteuria* was found in 8 samples from the fields of ICAR-IISR, Kozhikode, Kerala, India. The host range study revealed that the identified *Pasteuria* strain was very specific to *M. incognita* and completed its life cycle in RKN. Infected females laid no eggs or egg masses; thus, *Pasteuria* prohibited the total fecundity of the nematodes. The *Pasteuria* strain was named as IISR-MiP for it was found in the fields of ICAR-IISR and its specificity towards *M. incognita*. The average size of the identified *Pasteuria* strain IISR-MiP endospore was 2.75 μm. Light as well as scanning electron micrographs revealed 3 types of endospore attachments viz., conventional, inverted, and sideways. Further, it was found that endospores attached to the nematode cuticle in the maximum number in a conventional type of attachment (87.62%), followed by inverted (6.55%) and sideways attachments (5.82%). The inverted and sideways attachments were unique to the biology of *Meloidogyne-Pasteuria* interactions, indicating the presence of collagen-like fibres on the entire surface of *Pasteuria* endospores. *Pasteuria* strain IISR-MiP had the potential biocontrol capabilities and provided an opportunity for its evaluation against *M. incognita* on black pepper under field

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conditions.

Keywords: Black pepper, *Meloidogyne incognita*, *Pasteuria*, Endospore attachment, Biological control

Background

India is one of the major producers, consumers, and exporters of black pepper in the world (Thangaselvabal et al. 2008). Its production is threatened by several biotic and abiotic stresses. Among the biotic stresses, plant-parasitic nematodes (PPNs) are one of the major limiting factors and are responsible for the yield losses of up to 15–35% (Abd-Elgawad and Askary 2015). Among the PPNs, root-knot nematode, *Meloidogyne* spp., is one of the major hurdles due to its damage-causing potential (Ravindra et al. 2014). The root-knot nematode is an obligate endoparasite that spends its entire life inside the plant roots. After entry inside the plant roots, root-knot nematode (RKN) induces the formation of "giant cells" in the vascular tissues (Jones and Goto 2011). The feeding by RKN makes disturbances in water and nutrient uptake by the plant roots, moreover, the giant cells are metabolically active cells that act as nutrient sink for the fulfilment of increasing nutritional demands of RKN females for their reproduction (Mhatre et al. 2015). Each female can deposit about 200–500 eggs and its life cycle is completed in 24–30 days. Thus, it can complete many life cycles within a season resulting in the build-up of a huge population that cause a substantial impact on the quality and quantity of the final product.

Various synthetic chemicals have been used for the control of several PPNs, but due to serious non-target effects and environmental hazards, most of the pesticides have been withdrawn from the market, the latest being carbofuran. Hence, there is an instant need to adopt an alternative, economical, and eco-friendly strategy for nematode management that can be easily accepted by the farmers. Biological control offers all these merits along with safer crop protection to overcome the nematodes stress (Mhatre et al. 2019). Among the various biocontrol agents, *Pasteuria* spp. are one of the most promising bacterial bioagents for many nematode species as they have the potential to completely suppress the nematode reproduction by acting as an ovarian parasite (Mankau 1980 and Sayre 1980).

Pasteuria is a gram-positive, dichotomously branched, endospore-forming bacterial parasite of a wide range of invertebrates originally observed parasitizing water flea, *Daphnia* spp. (Metchnikoff, 1888). Till date, 6 species of *Pasteuria* parasitizing PPNs (Mohan et al. 2012) and one species parasitizing bacterivorous nematode have been identified (Giblin-Davis et al. 2003). *Pasteuria* became the most promising biocontrol agent that also led to the concept of "nematode suppressive soils" (Davies et al. 1990; Trudgill et al. 2000 and Botelho et al. 2019). The success of any biocontrol agent depends on its adaptability to the particular climatic conditions. There is also a tremendous opportunity for the discovery of new *Pasteuria* strains adapted to local environmental conditions and targeted nematodes.

Therefore, in the present study, efforts were made to isolate the native strains of *Pasteuria* against *M. incognita* infecting black pepper from 2 south Indian states viz., Kerala, and Karnataka.

Materials and methods

Culturing and identification of root-knot nematodes infecting black pepper

The root-knot nematode population used in the present study was collected from black pepper root galls and pure culture was build-up using a single egg mass inoculated on tomato plants (*Solanum lycopersicum* L.) in the net house of ICAR-Indian Institute of Spices Research, Calicut, Kerala, India (11°17′53″ N; 75°50′26″ E). Infected roots were washed carefully and egg masses were hand-picked and kept for hatching in a Petri dish at 28 °C for 24–48 h (Whitehead and Hemming 1965). Freshly hatched second-stage juveniles (J2s) were used for further experiments.

Morphologically, the RKN was identified based on the perineal pattern (Mulvey et al. 1975). Further, the identity was confirmed by PCR, using primers targeting the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). The genomic DNA was extracted from females, using the protocol given by Joyce et al. (1994) with some modification. The PCR reaction was performed for amplification of the complete ITS, using forward primer TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and the reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) (Joyce et al. 1994). The amplification profile was carried out using a BioRad thermo-cycler, which was preheated at 94 °C for 5 min, followed by 35 cycles of 92 °C for 1 min, 60 °C for 30 s and 72 °C for 1 min, followed by the final extension of 72 °C for 10 min. Qiagen Gel Purification Kit was used to purify the amplified product. Further, the DNA fragments were subjected to sequencing by Sanger's method (Eurofins Genomic India Pvt. Ltd., Bengaluru, India).

Sampling and extraction of *Pasteuria*

Random sampling was done and soil samples were collected from 39 black pepper-growing fields of northern Kerala viz., Wayanad (21 samples), Kasaragod (5 samples), ICAR-IISR, Kozhikode (9 samples), and from southern Karnataka, i.e. ICAR-IISR Regional Station, Appangala (4 samples). The soil samples were collected from the black pepper rhizosphere. *Pasteuria* spores from soil were extracted according to the technique described by Hatz and Dickson (1992) with few modifications. Approximately, 1000 freshly hatched J2 of *M. incognita* were added to 10 g of soil in a Petri dish (9-cm diameter) and incubated at 28 °C for 24 h. After this, the J2s were extracted from soil using Cobb's method (Townshend, 1962), followed by modified Baermann's funnel technique, where after washing, sieving and decanting, each sample was placed on the wire gauge lined with double-layered tissue paper. The entire setup was incubated at 28 °C for 24–48 h. The extracted nematodes were observed for endospore attachment under a Leica DM5000 B microscope (Leica Microsystems, Germany).

Host range study

To study the host range of the identified strain of *Pasteuria* (IISR-MiP), the endospore attachment assay was carried out, using 6 commonly observed nematode species/genera viz., *M. incognita*, *Radopholus similis*, *Pratylenchus sp*., *Helicotylenchus* sp., *Tylenchorhynchus* sp., and *Hoplolaimus* sp. from the black pepper rhizosphere. The procedure described by Hewlett and Dickson (1993), with few modifications, was followed where 1 ml suspension of *Pasteuria* spores (1×10^4 ml^{-1}) was mixed by 40 freshly collected J2s of above mentioned nematodes and the same was centrifuged at 6000×*g* for 3–4 min. After 2 h, from each sample, about 10 juveniles were randomly selected and observed for endospore attachment.

Scanning electron microscopy (SEM) studies

For studying the in-detailed orientations of attached endospores, the SEM was performed with IISR-MiP infected J2s of *M. incognita*. The infected juveniles were fixed in 2–4% glutaraldehyde buffered with 0.1 M phosphate buffer at pH 7.2 for 12 h at 4–6 °C. Subsequently, the samples were post-fixed with 2% osmium tetroxide solution for 4–6 h at 25 °C, dehydrated with graded series of ethanol (consisting of 40, 50, 60, 80, and 90% and absolute ethanol) and allowed for critical point drying with liquid CO_2. The dried samples were mounted on SEM stubs, finally coated with gold-palladium in a sputter coater, and observed under the scanning electron microscope (Tescan Vega 3).

Spore attachment study

To study the differences in spore attachments, the above-described procedure was followed (Hewlett and Dickson 1993). A total of 10 infected juveniles were heat-killed at 55 °C for 60 s and fixed in a double strengthen TAF solution (Seinhorst 1959) and mounted on slides by the wax-ring method (Hooper 1986). Spores attached in a different type of orientation were counted under the Leica DM5000 B microscope (Leica Microsystems, Germany).

Results and discussion

Based on the perineal pattern, RKN was identified as *M. incognita* (Fig. 1a). The results were validated by PCR and the primer pair targeting the ITS region of rDNA yielded a single fragment of approximately 800

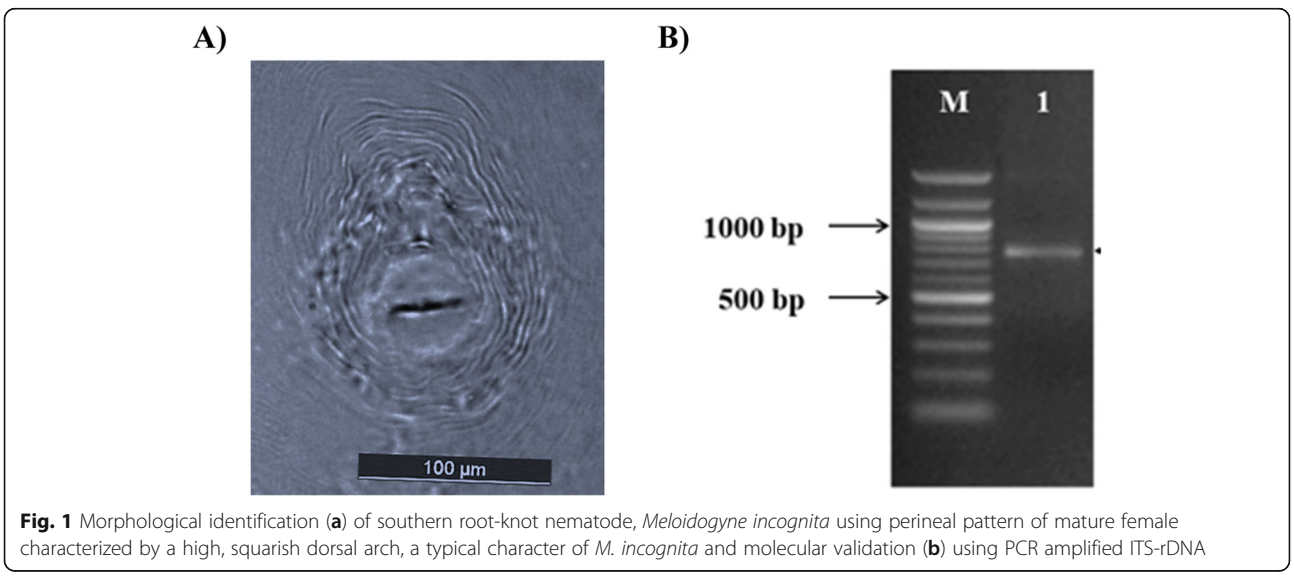

Fig. 1 Morphological identification (**a**) of southern root-knot nematode, *Meloidogyne incognita* using perineal pattern of mature female characterized by a high, squarish dorsal arch, a typical character of *M. incognita* and molecular validation (**b**) using PCR amplified ITS-rDNA

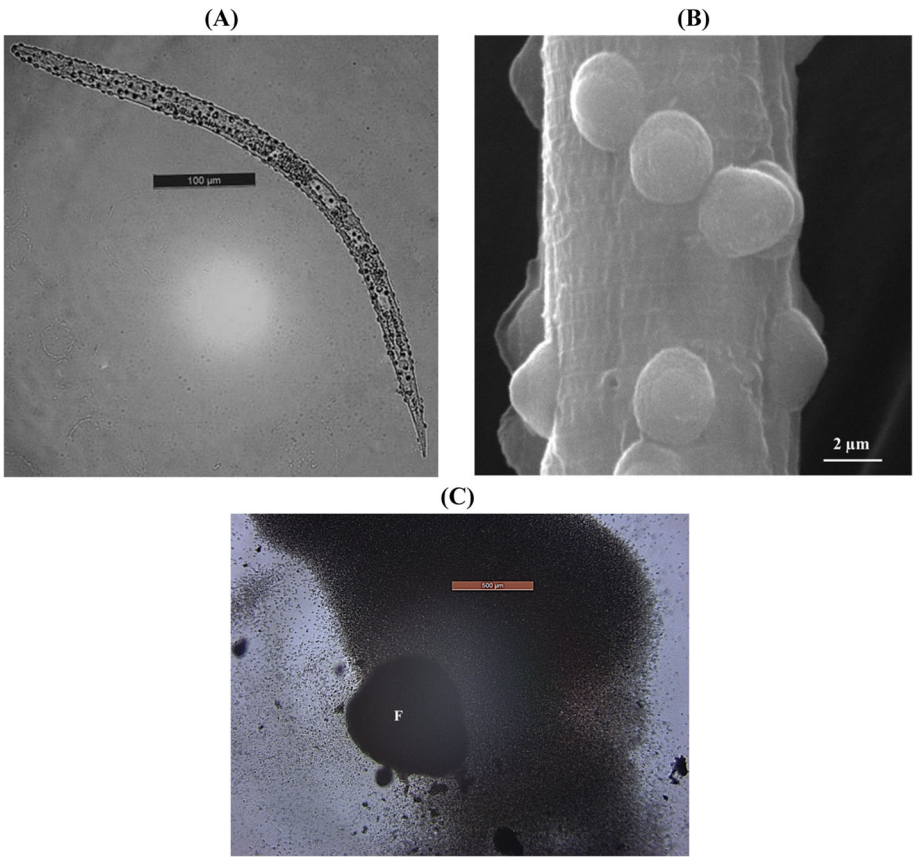

Fig. 2 *Pasteuria* attachment and reproduction on *Meloidogyne incognita*. Light micrograph (**a**) and scanning electron micrograph (**b**) of *Pasteuria* endospore attachment to the cuticle of second-stage juvenile of *Meloidogyne incognita*. Light micrograph of crushed infected root-knot nematode female with released *Pasteuria* endospores (**c**)

base pairs (Fig. 1b). The sequence was deposited in the GenBank database (accession no. MG194429.1). The sequence revealed 99.56% similarity with *M. incognita* isolate from China (MH 665425; MH113859; MH113858; MH113856; KC464469) and the USA (KP901063).

When juveniles were released in the moist soil, the samples having *Pasteuria*, yielded infected worms (Fig. 2a), whereas samples devoid of *Pasteuria* had normal healthy worms without any attached endospores. Out of 39 samples, the 8 samples from IISR-Kozhikode, Kerala were observed with the presence of *Pasteuria* spores and yielded *Pasteuria*-infected worms. These infected worms were further inoculated onto the tomato seedlings for the multiplication of *Pasteuria* spores. After 30 days of inoculation, the *Pasteuria* spores were obtained from RKN females. The infected females were observed devoid of eggs, this showed the potential of the *Pasteuria* to suppress the 100% fecundity of *M. incognita* (Fig. 2c). The same results were obtained in earlier studies where different strains of *Pasteuria* were found to suppress the total fecundity of their respective host nematode species (Mankau 1980; Sayre 1980; Mohan et al. 2012 and Phani and Rao 2018). *Pasteuria* spp. are the obligate parasites of PPNs (Davies 2009 and Srivastava et al. 2019), their multiplication in the host nematodes play a major role in their persistence in the field, it would not only result in mortality of the nematodes but its recycling ability can also manage the further build-up of nematode population (Bird and Brisbane 1988). Since RKN can complete multiple generations in a year, the reproduction ability of IISR-MiP observed in the present study able to tackle the succeeding generations of RKN in the field conditions.

The results of the host range study revealed that the *Pasteuria* spores attached only to the J2s of *M. incognita* whereas other nematode species (*R. similis*, *Pratylenchus* spp., *Helicotylenchus* spp., *Tylenchorhynchus* spp., and *Hoplolaimus* spp.) were found free of *Pasteuria*. These observations showed the specificity of *Pasteuria* strain only to the *M. incognita*. This RKN-specific strain was isolated from ICAR-IISR, Kerala, and therefore was

designated as the IISR strain of *M. incognita-Pasteuria* (IISR-MiP). Obtained results of host specificity of IISR-MiP are in agreement with Atibalentja et al. (2004), where endospores of the North American strain of *Pasteuria* were found genus-specific as these attached to *H. lespedezae*, *H. schachtii*, and *H. trifolii* and not to the other species from different genus, i.e. *M. arenaria*, *Tylenchorhynchus nudus*, and *Labronema* sp. The specificity in the attachment has been reported due to the differences in collagen-binding domains of different nematodes, which inhibit endospore attachment with a non-host nematode (Mohan et al. 2001). However, the cross-infectional attachments were also observed with some of the strains of *Pasteuria*, e.g. *Pasteuria* originally identified from *H. cajani* were also found to attach with *H. glycines*, *H. trifolii*, *H. schachtii*, *Globodera pallida*, *G. rostohiensis*, and *Rotylenchulus reniformis* (Sharma and Davies 1996).

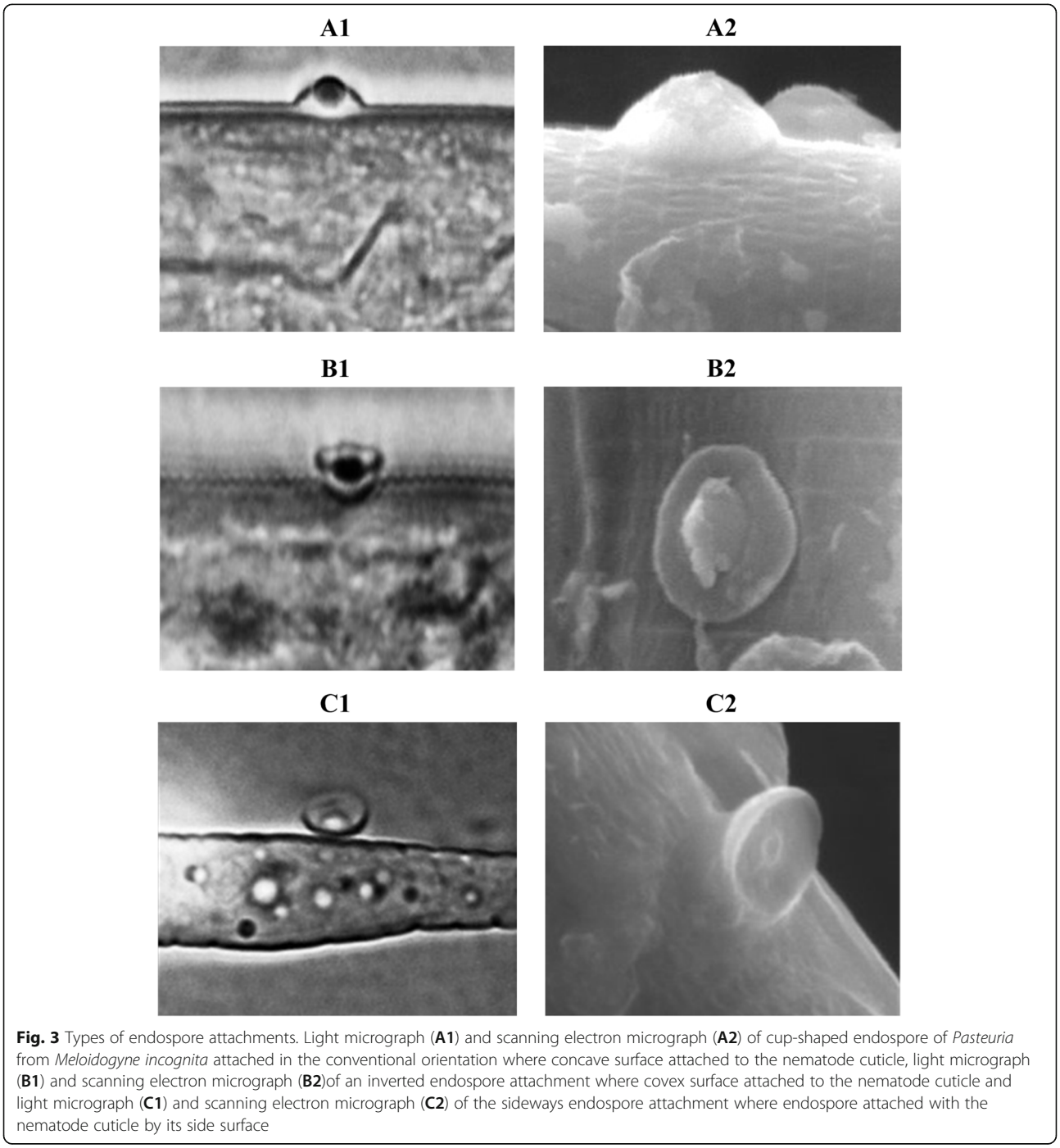

Fig. 3 Types of endospore attachments. Light micrograph (**A1**) and scanning electron micrograph (**A2**) of cup-shaped endospore of *Pasteuria* from *Meloidogyne incognita* attached in the conventional orientation where concave surface attached to the nematode cuticle, light micrograph (**B1**) and scanning electron micrograph (**B2**)of an inverted endospore attachment where covex surface attached to the nematode cuticle and light micrograph (**C1**) and scanning electron micrograph (**C2**) of the sideways endospore attachment where endospore attached with the nematode cuticle by its side surface

Interestingly, in the present study, the light micrograph observations revealed 3 different types of endospore attachments viz., conventional, inverted, and sideways, and the same was evident in SEM study (Fig. 3). The endospores of IISR-MiP attached randomly to the entire body length of juveniles from head to tail and the average diameter of the attached IISR-MiP endospores was 2.75 μm (Fig. 2b). The spore size of the IISR-MiP strain was smaller than the original description of *Meloidogyne-Pasteuria* endospore, where the size of the spores was reported to be 4.0 μm (Sayre and Starr 1985). This difference in the size of IISR-MiP could be due to the differences in the geographical origin of these 2 strains. Ratnasoma and Gowen (1991) reported that the highest spore attachment was associated with the size of spores and nematode species. Moreover, the size of the IISR-MiP was found similar to the *Pasteuria* from *H. cajani* cultured in the laboratory conditions (Sharma and Davies 1996).

The results of the differential spore attachment study showed that a total of 87.62% of the IISR-MiP endospores were attached in a conventional manner where the spores were oriented in such a way that, the concave surface was in contact with the nematode cuticle (Fig. 3A1, A2). Some spores showed an inverted attachment (6.55%), where spore attached to nematode cuticle by their convex surface (Fig. 3B1, B2), whereas very few spores showed a sideways type of attachment (5.82%), in which spores attached with the nematode cuticle by its side surface (Fig. 3C1, C2) (Table 1). The conventional type of attachment is the most common type of attachment observed with all the species of *Pasteuria* strains (Sayre and Starr 1985; Sharma and Davies 1996 and Mohan et al. 2012), the inverted type of attachment was observed only with the *Pasteuria* from *H. cajani* (Mohan et al. 2012), while with *Meloidogyne-Pasteuria*, it is the first report of this type of attachment. However, the sideways spore attachment was not reported with any strain of *Pasteuria* and to our knowledge, this is the first report of the sideways type of endospore attachment.

The collagen-like fibres from the dorsal surface of *Pasteuria* endospore and receptor(s) from the nematode cuticle have been reported for successful spore attachment (Davies and Opperman. 2006; Davies et al. 2008; Davies 2009 and Mouton et al. 2009). Recently, Orr et al. (2018) and Srivastava et al. (2019) identified 17 genes from 5 different phylogenetic clusters, encoding collagen-like fibres from *Pasteuria penetrans* and suggested that these genes are an important source of genetic diversity in *Pasteuria* and involved in the determination of attachment specificity. Davies (2009) reported that collagen-like fibres were found in greater density on the concave surface than the convex surface of the endospore. However, the results of the present study demonstrated that the attachment sites were present on the whole body of *Pasteuria* endospore and the differences in the attachments were due to the difference in the density and spatial distribution of the collagen-like fibres on endospores. In addition to this, from the present study, it could be concluded that the identified strain of *Pasteuria* was highly pathogenic to the *M. incognita*, suggesting that the soil application of this bacteria could effectively manage the infection of root-knot nematode on black pepper plants.

Table 1 Comparison of types of IISR-MiP endospore attachments (mean ± SE) with the cuticle of second-stage juveniles of *Meloidogyne incognita* (n = 10)

Type of spore attachment	Spore attachment	
	Numbers	Percentage
Conventional	146.90 ± 20.03 (119–175)	87.62
Inverted	11.10 ± 3.07 (7–17)	6.55
Sideways	9.80 ± 1.87 (6–12)	5.82

Conclusion

The inverted and sideways endospore attachments observed in the present study were unique to the biology of *Meloidogyne-Pasteuria* indicating the presence of collagen-like fibres on the entire surface of *Pasteuria* endospores. A newly identified *Pasteuria* strain IISR-MiP is a potential biocontrol agent of *M. incognita* as it did not allowed egg production by *M. incognita* females. But before including this strain in integrated nematode management programme of *M. incognita*, further experiments are required to know the real potential of this indigenous *Pasteuria* strain in an open field conditions.

Abbreviations

ICAR: Indian Council of Agricultural Research; PPNs: Plant-parasitic nematodes; RKN: Root-knot nematode; J2s: Second-stage juveniles; ITS: Internal transcribed spacer; rDNA: Ribosomal deoxyribonucleic acid; PCR: Polymerase chain reaction; SEM: Scanning electron microscope; IISR: Indian Institute of Spices Research; MiP: *Pasteuria* from *Meloidogyne incognita*; CO_2: Carbon dioxide

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Authors' contributions

PHM: Research concept and design, collection and/or assembly of data, data analysis and interpretation, writing the article, and critical revision of the article. SJE: Research concept and design, and data analysis and interpretation. GC: Scanning electron microscope analysis and critical revision of the article. RP: Data analysis and interpretation, and critical revision of the article. AVN: Collection and/or assembly of data and writing the article. ST: Scanning electron microscope analysis and critical revision of the article. MG: Research concept and design. The authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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