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Research Article

Efficacy of formulation of nematode antagonistic bacterium, *Pasteuria penetrans* (Thorne) Sayre and Starr, 1985 against root-knot nematode, *Meloidogyne incognita*

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ABSTRACT: Root-knot nematodes, *Meloidogyne* spp. are extremely polyphagous pests of both tropical and subtropical crops that cause a great reduction in crop yields and quality. In vegetable production, 10–30% yield loss is caused by root-knot nematode infestation. *Pasteuria penetrans* is a hyperparasitic bacterium of *M. incognita* that has a high degree of biocontrol potential. Though, the application of *P. penetrans* in the form of root powder had been tested by a few scientists in the nurseries, it is highly impractical in the main fields. Commercial formulation of this bacterium is not available in India till date. Based on these facts, four different formulations of *P. penetrans viz.,* kaolinite clay, Emulsifiable Concentrate (EC), wettable talc powder and sodium alginate beads were prepared and tested for its parasitization potential. Among the four, EC formulation showed the highest parasitization potential of 84.6% with a spore load of 11.2/J2 (second stage juvenile). These formulations were stored under room temperature (27±1ºC) to record spore viability. The observation showed that the spores were viable upto 60 days with the highest parasitization of 84.7% in EC formulation. An *in-vitro* test was carried out in tomato plants to document the infection in *M. incognita* by endospores released from the formulations. The observation also showed that the spores released from the formulations were multiplied in the female body. Two cell stage and four cell stages of spores were observed in J3 and pre-adult stages of the nematode. The results of this study showed that the EC formulation is highly suitable for field application.

KEY WORDS: Formulation*, Meloidogyne incognita, Pasteuria penetrans,* Parasitization*,* Shelf-life

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INTRODUCTION

Root-knot nematode, *Meloidogyne* sp. is the most prevalent nematode, causing heavy damage to many crops. The host range of this nematode includes more than 3000 plant species (Abad *et al.,* 2003). The genus *Meloidogyne* has more than 95 described species and common species are *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla.* In world crop production, 10-30% yield loss is caused by *Meloidogyne* spp. every year (Manjunatha, 2017). Chemicals are used for controlling root-knot nematode but non selective application leads to the revival of sedentary endoparasitic nematodes (Degenkolb and Vilcinskas, 2016). Hence, there is a need to find out other strategies for control of root-knot nematodes in an effective and eco-friendly manner. Non-chemical management approaches include mainly crop rotation and biological control agents (Stirling, 1980).

Pasteuria penetrans (Thorne) is an endospore forming bacterium and it was first described as *P. romosa* a parasite of water fleas, *Daphina magna* (Skerman *et al*., 1980). Later, Cobb (1906) described *Pasteuria* infection on nematode, *Dorylaimus bulfiferous*. It was renamed as *Duboscqia penetrans* (Thorne, 1940) and further as *Bacillus penetrans* (Mankau, 1975). Later it was renamed as *Pasteuria penetrans* (Sayre and Starr, 1985) which is highly host specific in nature and shows promise in controlling the root-knot nematodes *Meloidogyne* spp. (Stirling, 1984; Chen *et al*., 1996). It is an obligate, gram-positive bacterium and has direct inhibitory effect on root-knot nematodes (*Meloidogyne* spp). Mukhtar *et al*. (2013) reported that *P. penetrans* was more effective compared to other bioagents such as *Pochonia chlamydosporia, Purpureocillium lilacinum* and *Trichoderma harzianum.* Potential of *P. penetrans* in biological control of phytopathogenic nematodes were studied by several authors (Chen and Dickson, 1998; Swarnakumari and Sivakumar, 2005). Ayanaba (1993) invented the slow-release bio-degradable granules containing endospores of *P. penetrans* in order to suppress nematode reproduction. Experiments

conducted by Swarnakumari *et al*. (2019) with sodium alginate beads entrapped endospores of *P. penetrans* against *M. incognita* in tomato showed that the entry of juveniles in root system was reduced by this formulation. But these beads took more time for degradation. Hence, other methods were tried to identify a potential formulation. The methodology adopted and results obtained are described in this paper.

MATERIALS AND METHODS

Pure culture maintenance of *P. penetrans* on root-knot nematode, *M. incognita*

Pure culture of *P. penetrans* was maintained in tomato (var. PKM-1) plants. Egg masses of *M. incognita* were collected from the infected field and incubated at room temperature (27± 2 °C) in water for hatching. After egg hatching, endospores were added to the suspension containing second stage juveniles (J2) of *M. incognita* and incubated for 3 days for spore attachment (Hussey and Barkar, 1973; Regina *et al*., 1999). Then, the endospore attached J2 were inoculated into the tomato plants and maintained in the sterile pot mixture (Sand: Red soil: Sand - 1:2:2). These plants were pulled out 30 days after inoculation to confirm the multiplication of *P. penetrans*.

Developing different formulations of *Pasteuria penetrans*

Clay (kaolinite) based bio-degradable formulation

Endospores were collected in Eppendorf tubes containing sterile water (2 ml) from the *P. penetrans* infested females by crushing them manually. Kaolinite clay (2 g) was added to 2 ml of endospores suspension ($1x10^6$ spores). Then, to this suspension one pellet of sodium hydroxide (0.13 g) was mixed. After thorough mixing, small beads were prepared manually (Molina *et al.,* 2018). The individual beads weighed 0.5 ± 0.01g.

Developing water soluble emulsifiable concentrate (EC) formulation

Endospores were collected as described in the preparation of clay formulation. EC formulation was prepared by using lecithin (emulsifier), Triton-X (surfactant), sterile water and vegetable oil. Sterile water (19 ml) was heated using hot plate at 50ºC. Lecithin (1 ml) was added to the hot water and mixed thoroughly. Vegetable oil (4ml) and Triton-X (4 ml) were added to this mixture and heated at 50ºC for 3 minutes. (Zhou *et al.*, 2010). Endospore suspension (1ml) was added to this mixture and stored in a glass vial at room temperature (27±1ºC).

Wettable talc powder formulation

Wettable talc powder was prepared using talc powder (300 mesh size). 20 ml of sterile water containing endospores was added to 70g of talc powder and mixed thoroughly and then 0.5g of carboxy methyl cellulose was added to this mixture (Vidhyasekaran and Muthamilan, 1995; Pahari *et al*., 2017). This was dried under shade to remove excess water.

Assessing the degradation of formulations in soil

Plastic cups were filled with red sandy loam soil (80 g) and vermicompost (20 g). Then, the endospores entrapped formulations of kaolinite clay (1 bead), EC (12 ml), wettable talc powder (0.5 g), sodium alginate beads (1 bead) were inoculated into the cups. These cups were incubated with 100 juveniles (100 J2/cup) of root-knot nematode, *M. incognita* at room temperature (27±1 ºC). Time taken for the degradation of these formulations and number of J2 encumbered endospores were recorded. A completely randomized block design (CRBD) with five replicates for each treatment was adopted.

Shelf-life of endospores entrapped formulation

Endospores entrapped kaolinite clay, EC, wettable talc powder, sodium alginate beads and root powder formulations were packed in zip-lock cover and in glass vials and maintained at room temperature (27±1 ºC). Observations on viability of endospores and condition of the formulations were recorded 30 and 60 Days after inoculation (DAI). Completely randomized block design was adopted with four replications for each treatment.

Effect of different formulations of *P. penetrans* on penetration of J2 in tomato

Plastic cups were filled with red sandy loam soil (100 g). The formulations of kaolinite clay, EC, wettable talc powder, sodium alginate beads were inoculated into the soil and tomato seedlings (var. PKM-1) were transplanted 7 DAI. Juveniles (J2s) of *M. incognita* were inoculated to each cup at the rate of 100 / cup. On 7^{th} day, tomato plants from each treatment were removed and observed for penetration using acid fuschin staining. Completely randomized block design was adopted with four replications.

Statistical analysis

The data generated by these experiments were analyzed using ANOVA DMRT (Duncan's Multiple Range Test) at 1% level using AGRES statistical software (Panse and Sukhatme, 1954).

RESULTS AND DISCUSSION

Properties of the formulations

The physical characters of the formulations showed that they were stable and retained the original properties upto 2 months. Kaolinite clay (Fig. 1a.) formulation was white in colour, spherical in shape with 32±0.5 mm circumference, coarse surface, odourless and insoluble in water as mentioned by Jamo and Abdu (2014). The weight of each bead was

Table 1. Assessing the degradation of different formulations containing endospores of *Pasteuria penetrans*

Formulations	% parasitization(1)	Average number of spores/J2(2)
T_1- Kaolinite clay	81.6b (64.61)	9.2 (0.96)
T_2 -Emulsifiable concentrate	84.6b (67.21)	11.2 (1.04)
T_3- Wettable talc Powder	81.2b (64.3)	9.8 (0.98)
T_4- Sodium alginate beads	82.4b (65.20)	10.6 (1.02)
Sed	0.695	0.038
CD (p=0.01)	2.03	NS
CV (%)	1.68	6.08

Figures in parentheses are Arcsine (1) transformed values and log transformed values (2).

Table 2. Assessment of shelf life of different formulations entrapped with endospores of *Pasteuria penetrans*

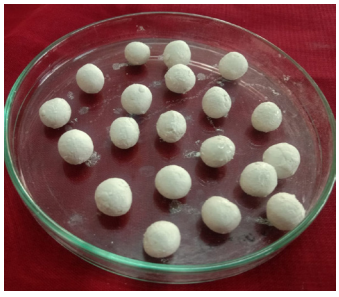
Formulations	% of parasitization(1)		Average number of spores/J2 (2)	
	30th day	60th day	30th day	60th day
T_1- Kaolinite clay	82.5b (65.27)	80.75b (63.9)	8.75 (0.94)	8.5 (0.92)
T_2- Emulsifiable concentrate	84.75b (67.01)	84.75b (67.01)	11.26 (1.04)	10.25 (1.0)
T_3- Wettable talc powder	81.25b (64.34)	81.5b (64.53)	9.0 (0.95)	9.5 (0.97)
T_4- Sodium alginate beads	82.75bb (65.34)	82.25bb (65.09)	10.5 (1.01)	9.0 (0.91)
T_5- Root powder	83.5bb (66.04)	83.5bb (66.04)	9.75 (1.23)	9.5 (0.97)
Sed	0.59	0.67	0.03	0.38
CD (p=0.01)	1.75	1.90	NS	NS
CV (%)	1.28	1.47	5.44	5.65

Figures in parenthesis are Arcsine (1) transformed values and log transformed values (2).

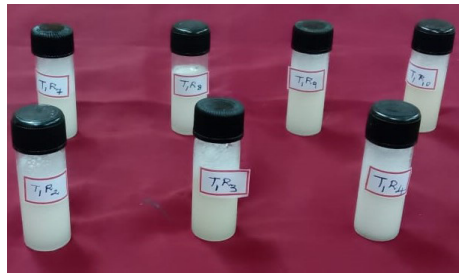
Table 3. Effect of different formulations of *Pasteuria penetrans* on penetration of J2 in tomato plants

Treatments	Average number of J2 penetrate/ each replications on 7th day
T_{1-} Kaolinite clay	6.75 (2.54)
T_2-Emulsifiable concentrate	8.5 (2.9)
T_3- Wettable talc powder	6.5 (2.53)
T_4- Sodium alginate beads	7.5 (2.72)
T_5- Control	9.25 (3.03)
Sed	0.22
CD(p=0.01)	NS
CV%	11.73

Figures in parantheses are square root transformed values at 1% level by DMRT.



1(a)

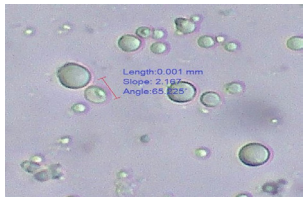


1(b)

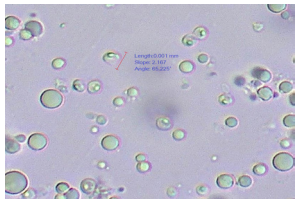
1(c)

1(d)

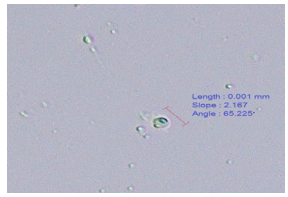
Fig. 1. Different formulations of *P. penetrans*.
1(a) kaolinite clay formulation; 1(b) EC formulation; 1(c) Wettable -powder formulation; 1(d) Sodium alginate beads formulation.



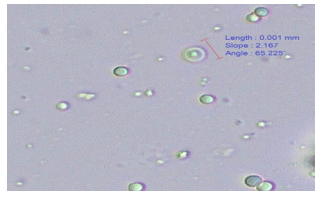
Kaolinite clay formulation



EC formulation



Wettable powder formulation



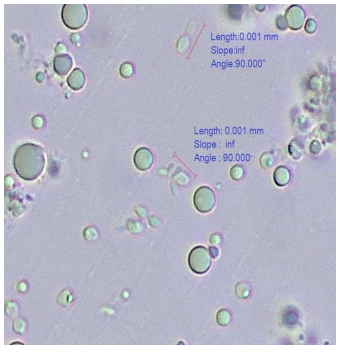
Sodium alginate beads formulation

Fig. 2. Viability of endospores in formulations of *P. penetrans*. (40 x).

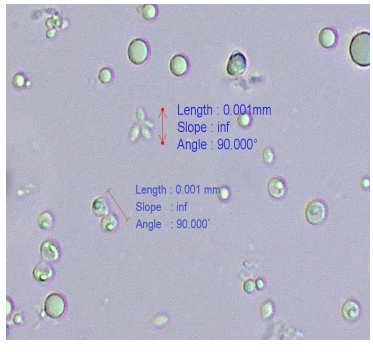
Galled roots observed on 7th day

Galled roots observed on 12th day

Fig. 3. Effect of different formulations of *P. penetrans* on penetration of J2 in tomato Gall formation observed on 7th day and 12th day.



Endospores in third stage juvenile (J3) of *M. incognita*



Endospores in pre- adult stage of *M. incognita*

Fig. 4. Two cell stages and four cell stage of *P. penetrans* endospores. (40 x).

0.5±0.01g and contained a spore load of $1x10^6$/bead. It acted as an inert carrier material for many bacterial biocontrol agents (Karise *et al.,* 2014; Jayasudha *et al*., 2017). The Emulsifiable Concentrate (EC) formulation was milky white in colour, more viscous than water with vegetable oil odour and soluble in water (Fig. 1b.). Raut (2012) stated that the lecithin acts as an emulsifier with other surfactants and increases the efficacy of formulation. The EC formulation contained 2 $x10^6$ spores in 25 ml. The wettable talc powder formulation (Fig. 1c) was dull white in colour, odourless and coarse in nature with a spore load of $1x10^6$ per gram. Sodium alginate beads were dull white in colour, odourless with smooth surface but sticky in nature (Fig. 1d). These beads were insoluble in water but soluble in sodium carbonate (Na_2CO_3) solution at 2%. The weight of each bead was 0.75 g and contained the spore load of $1x10^6$ per bead. Bashan *et al*. (2002) developed alginate microbeads as inoculant carriers for plant growth promoting bacteria, *Azospirillum brasilense* and weight of each bead was 100 – 200 µg containing 10^4 to 10^6 CFU/g released from each microbead.

Degradation of *P. penetrans* formulations

Degradation of formulations was recorded after specified time intervals. In the combination of red sandy loam soil and vermicompost, kaolinite clay and sodium alginate beads took 3 days and 7 days for the complete degradation respectively, whereas EC formulation took only one day for complete degradation. Parasitization potential of each formulation was observed on 7th day (Table 1) in which EC formulation showed the highest potential of 84.6% with spore load of 11.2/J2 of *M. incognita* followed by sodium alginate beads and clay formulation which was highly significant from other formulations. The efficacy of *P. penetrans* combined with various oil seed cakes (Neem, castor, Mustard and *Citrollus*) were evaluated by Chaudhary and Kaul (2013) for the management of root-knot nematode, *M. incognita* in chilli and pepper. The castor oil seed cake had the highest parasitization potential of 75.86% compared to others.

Shelf-life assessment

The viability of endospores in each formulation was observed on 30 DAI and 60 DAI (Table 2). There was no change in the condition of formulations for 2 months (60 days) and the endospores were observed in all subsequent formulations in phase contrast bright field microscope (iScope – Euromex) (Fig. 2). There were no significant differences recorded in the spore attachment on J2 between the formulations at 30th day and 60th day. The EC formulations showed the highest parasitization potential of 84.75% with the viable spore load of 11.2/J2 and 10.2/J2 on 30 and 60 DAI, respectively. Yanez-Mendizabal *et al*. (2012) prepared the formulation of *Bacillus subtilis* by spray drying and maintained for 6 months at two different temperatures of 4±1 and 20±1ºC and reported that there were no significant differences in the CFU/g in both the temperatures and 10^6 CFU/g was observed initially and slightly reduced at the 6 months after storing.

Penetration study

Penetration of second stage juveniles of *M. incognita* in tomato were examined and observed for average number of J2 penetrated per plant on 7th day in tomato roots after inoculation of different formulations of *P. penetrans* (Table 3). The penetration and galled roots were observed on 7th day and 12th day (Fig. 3). Two cell stage and four cell stages of endospores were observed in phase contrast bright field microscope (iScope – Euromex) in all formulations (Fig. 4) from third stage juvenile (J3) and pre-adult stages of root knot nematode, *M. incognita*. Stephen *et al*. (1985) demonstrated the development of *P. penetrans* in *M. incognita* juveniles after penetration into the roots which is in agreement with the current observations. Results of these experiments confirmed that the EC formulation recorded rapid degradation and high parasitization potential. This formulation is also water soluble and found to be effective against root-knot nematode, *M. incognita*.

CONCLUSION

Pasteuria penetrans is found to be an efficient biocontrol agent against root-knot nematode, *M. incognita*. The results of the current study showed that the formulations of *Pasteuria penetrans* were able to degrade in soil and the endospores were viable and their bio-efficacy was proved by the penetration study. Among all the formulations of *Pasteuria penetrans*, emulsifiable concentrate (EC) showed the highest parasitization potential followed by sodium alginate beads and the highest spore attachment. Hence, EC formulation may be recommended for main field application for the management of *M. incognita*.

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