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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(6): 4134-4137
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www.thepharmajournal.com
Received: 16-03-2023
Accepted: 19-04-2023

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Integration of *Pasteuria penetrans* with organic amendments for the management of root knot nematode, *Meloidogyne javanica* in tomato nursery

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Abstract
Leachates of four organic amendments were collected and tested *in vitro*, for their effect on the encumbrance of endospore of *Pasteuria penetrans* to second stage juvenile (J_2) of *Meloidogyne javanica*. Among the tested organic amendments, higher attachment of endospores was observed in leachates collected from mustard cake and minimum was observed from farmyard manure. Then, application of *P. penetrans* at different spore load ($1x10^3$, $1x10^4$ and $1x10^5$ spores/g soil) in combination with mustard cake at the rate of 10 g/kg soil was tested for the management of *M. javanica* in tomato nursery. All the three doses of *P. penetrans* along with mustard cake improved plant growth parameters. Maximum seedling height and seedling weight were observed in treatment where *P. penetrans* at the rate of $1x10^5$ spores/g soil was applied. The number of galls per root system and reproduction potential of root-knot nematode decreased with the increase in the dose of bacterial parasite. Minimum number of galls, eggs per root system and final nematode population were observed, where parasitic preparation of *P. penetrans* was applied at dose of $1x10^5$ spores/g soil along with mustard cake.

Keywords: Organic amendments, leachates, *Pasteuria penetrans*, *Meloidogyne javanica*

Introduction
Haryana, a north-western state situated between 27° 39/ to 30° 50/ North latitude and 74° 27/-8"to 77° 365" East longitude is an agriculturally important state. Haryana shares its boundary with Himachal Pradesh in the north, Uttarakhand in north-east, Rajasthan in south and south-east, Uttar Pradesh and Delhi in east and Punjab and Chandigarh in north-west. The state being near capital, occupies an important position in respect of supply of agricultural and horticultural produce Delhi and other neighboring areas. Vegetables are important constituent of human diet and provide necessary ingredients for good health. Among vegetables, tomato is the second most commonly grown vegetable crop in world after potato. In India, tomato is grown in 778 thousand ha area with production of 19397 thousand MT (nhb.gov.in). Haryana is on 11th positions in tomato production and produces approximately 3.11% of the total production of India. Haryana's average area is approximately 29000 ha with an average tomato yield of 20 -23 t/ha (https://agricoop.gov.in). Root-knot nematode (*Meloidogyne* spp.) is among the most economically damaging category of plant parasitic nematodes on vegetables crops particularly; tomato, brinjal and okra are the most preferred hosts and suffer enormous losses due to root-knot nematodes (Radwan *et al.*, 2012) [16], causing 21.3% crop losses amounting to Rs. 102,039.79 million (1.58 billion USD) annually (Kumar *et al.*, 2020) [9]. They are distributed globally and are obligate parasites of roots of thousands of plant species. Raising a vegetable nursery in own field is a common practice in vegetable cultivation in India. Such nursery sites usually harbor root-knot nematode populations and serve to disseminate nematode through infected seedlings to main field. *P. penetrans* is an endospores forming gram-positive bacteria that infects one of the most destructive parasite of agricultural crops, root-knot nematode (Tian *et al.*, 2007; Davies, 2009; Timper *et al.*, 2016; Ciancio, 2018; Mohan *et al.*, 2020) [23, 4, 24, 13]. The spores of this obligate bacteria *P. penetrans* (Lopes *et al.*, 2019) [12] attach to the second stage juvenile's cuticle (Davies *et al.*, 2011) [5] which is ample to initiate infection, without interfering with the ability of nematodes to invade roots (Rao *et al.*, 1997) [17]. The nematode reproduction was blocked after the endospore-encumbered J_2 entered roots and the bacterium developed in the nematode pseudocoel (Bird, 1986; Davies *et al.*, 2011; Vagelas *et al.*, 2012; Lopes *et al.*, 2018) [1, 5, 11].

Endospore dormancy in soil and resistance to temperature are the key traits attributing to success in suppressing root-knot nematodes on both annual and perennial crops under various ecological conditions (Freitas *et al.*, 1997; Cho *et al.*, 2013) [6, 2]. Secondly, the addition of organic amendments in the soil for the management of plant parasitic nematode changes the chemical structure of soil environment. Soil amendments having high nitrogen and carbon ratios have been reported to exhibit nematicidal and fungicidal activity mainly through the release of ammonia from the amendments during their decomposition in the soil or through increased populations of antagonistic microorganism (Rodriguez-Kabana, 1986; Spiegel *et al.*, 1987; Oka *et al.*, 1993; Panpatte *et al.*, 2021) [20, 22, 14, 15].

Therefore, nursery soil treatment with *P. penetrans* in combination with organic amendments is very helpful in treating small areas with the limited quantity of bacteria and mustard cake. This management strategy offers initial but vital protection to seedlings against nematodes.

Material and Methods

The experiments were carried out in laboratory and screen house, Department of Nematology, CCS Haryana Agricultural University, Hisar. The materials and methods used for different experiments are as follows:

Collection of organic amendment leachates

Organic amendments *i.e.* neem, mustard, castor cakes and FYM at rate of 10 g/kg soil were added to earthen pots (15cm) of 1 kg soil capacity. Pots were watered daily to the saturated point of soil and given usual care. Plastic pots were placed below the earthen pots. Seven days decomposition period was given to organic amendments. The leachates were collected in beaker when fresh water was applied to the pots. Neem cake, castor cake, mustard cake and farmyard manure were procured from market and used in experiments.

Preparation of pure nematode inoculum

Pure culture of root-knot nematode, *Melodogyne javanica* was raised in screen house in earthen pots containing steam sterilized sandy soil. Four week old healthy seedlings of tomato were procured from the university nursery situated at gate number four and transplanted in the earthen pots. Perineal pattern of root knot female was cut and their respective egg masses were labeled. After proper identification of *M. javanica* second stage juveniles (J_2) were inoculated around the root of tomato seedlings in pots. The culture was allowed to multiply for 2-3 generations and was further sub cultured as and when required. General green house care was given to the plants.

Preparation and estimation of bacterium inoculums

P. penetrans infected root-knot females were dissected out from the tomato plant roots under stereomicroscope. Alternatively, the infected roots were dipped in tap water and left at room temperature for 2-3 days, after which the roots were put on a 20 mesh (840 µm aperture) sieve placed over 100 mesh (150 µm aperture) sieve. A strong jet of water from the top disintegrates the cortex portion of the roots leaving the stelar region intact, thus separating the females from the roots. The content of the 100 mesh sieve was collected in a beaker and examined under incident light in a stereo-zoom microscope. The infected females appear opaque and dark in color and were picked with the help of micropipette (1 ml) the tip of which was cut with the help of sharp blade to allow the suction of root-knot females. The infected females were collected in distilled water in a cavity block and kept in glass vials in refrigerator till further use. Infected females were transferred to an Eppendorf tube (2 ml) containing small amount of distilled water just enough to submerge the nematodes. Using the blunt end of a plastic rod, the females were crushed mechanically to release the endospores in water. This action was repeated several times to ensure that all the females have been completely crushed. The suspension was passed through a 500 mesh sieve. More water was added to Eppendorf tube and the suspension passed through the sieve. Finally the endospore suspension (filtrate) was collected in a clean beaker. The endospore count was taken with the help of a haemacytometer at 400x under a compound microscope.

Results

Table 1: Effect of different organic amendments on adherence of *Pasteuria penetrans* endospores to second stage juveniles of *Meloidogyne javanica*

Organic Amendments (OA)	No. of endospores attached per Juvenile after		Mean
	24 h	48 h	
Neem cake @ 10 g/kg soil	10.2	13.7	12.0
Castor cake @ 10 g/kg soil	9.7	14.5	12.1
Mustard cake @ 10 g/kg soil	11.2	15.2	13.2
FYM @ 10 g/kg soil	8.7	12.2	10.5
Water (Control)	16.0	19.2	17.6
Soil leachate (Control)	19.2	22.0	20.6
Mean (Time)	12.5	16.1	

CD (P=0.05%): Organic amendments = 1.24, Duration = 0.72, OA x Time = NS

Table 2: Effect of different doses of *Pasteuria penetrans* in combination with mustard cake on growth parameters of tomato against root-knot nematode (*Meloidogyne javanica*)

Treatment	Seedling height (cm)	Weight of ten seedlings (g)
Mustard cake @ 10 g/kg soil + *Pasteuria penetrans* @ $1x10^3$ spores/g soil	16.3	70.8
Mustard cake @ 10 g/kg soil + *Pasteuria penetrans* @ $1x10^4$ spores/g soil	23.1	96.3
Mustard cake @ 10 g/kg soil + *Pasteuria penetrans* @ $1x10^5$ spores/g soil	23.6	101.6
Carbofuran @ 1 kg a.i./ha	19.9	94.8
Untreated control	13.6	59.7
C.D. at 5%	1.19	12.8

Table 3: Effect of different doses of *Pasteuria penetrans* in combination with mustard cake on reproduction parameters of root-knot nematode (*Meloidogyne javanica*) on tomato

Treatment	No of galls per root system	No of eggs per root system	Final nematode population per 200 cc soil
Mustard cake @ 10 g/kg soil + *P. penetrans* @ $1x10^3$ spores/g soil	39.7 (6.4)	1909.7 (43.7)	245.7 (15.6)
Mustard cake @ 10 g/kg soil + *P. penetrans* @ $1x10^4$ spores/g soil	41.3 (6.5)	1012.3 (31.8)	53.0 (7.3)
Mustard cake @ 10 g/kg soil + *P. penetrans* @ $1x10^5$ spores/g soil	30.0 (5.6)	488.3 (22.0)	37.3 (6.2)
Carbofuran @ 1 kg a.i./ha	31.3 (5.7)	1454.0 (37.9)	160.6 (12.7)
Untreated control	51.7 (7.25	6452.3 (80.1)	329.0 (18.1)
C.D. at 5%	(0.62)	(7.37)	(2.46)

Fig. in parentheses are √n transformed values

Results

The adherence of endospores of *P. penetrans* to second stage juveniles of *M. javanica* varied in the leachates collected from pots applied with different organic amendments added at the rate of 10 g/kg soil. Maximum and significantly higher spore adherence (20.6) was observed in soil leachates, where no organic amendment was added, while minimum spore encumbrance (10.5) was observed in leachates collected from the pots where farmyard manure was added. Significantly higher spore adherence was observed after 48 hours of exposure of juveniles to suspension as compared to 24 hours. However, the number of spores adhered (more than 10.5) to second stage juveniles were sufficient to parasitize the root-knot nematode. The interaction between organic amendments and duration of exposure was found non-significant (Table 1)

Second attempt was made to standardize the dose of bacterial parasite, *P. penetrans* along with mustard cake which was found better in above experiment. Three doses of *P. penetrans i.e.* $1x10^3$, $1x10^4$and $1x10^5$ spores/g soil were applied in tomato nursery along with mustard cake @ 10 g/kg soil, which was best among the organic amendments in above experiment. It is observed from the data in Table 2 that all the doses of *P. penetrans* along with mustard cake improved plant growth parameters (seedling height and weight of tenseedlings) in tomato nursery. Maximum seedling height (23.6 cm) and seedling weight (101.6 g/10seedlings) were observed in treatment where *P. penetrans* at the rate of $1x10^5$ spores/g soil was applied which was statistically at par to that of *P. penetrans* at the rate of $1x10^4$ spores/g soil followed by carbofuran application at the rate of 1 kg a.i./ha. Data in Table 3 depicted number of galls per root system and reproduction potential of root-knot nematode decreased with the increase in the dose of bacterial parasite. Minimum number of galls per root system (30.0), number of eggs per root system (488.3) and final nematode population (37.3 J_2/200cc soil) were observed where parasitic preparation of *P. penetrans* was applied at dose of $1x10^5$ spores/g soil along with mustard cake followed by $1x10^4$ spores/g soil. All the treatments reduced the number of galls and reproduction potential of root-knot nematode as compared to the untreated control.

Discussion

The chemical moieties on the surface of *P. penetrans* endospores and corresponding moieties on the surface of nematode cuticle govern endospore attachment (Spiegel *et al.*, 1996) [21]. The results of above experiment indicated that the adherence of *P. penetrans* endospores to second stage juveniles of *M. javanica* varied with the application of different organic amendments. Leachates were collected after application of organic amendments to the soil. Maximum spore adherence was observed in soil leachate. Thus, the soil leachate alone (without organic amendment) did not influence the spore attachment indicating that water along with soil chemical does not influence the attachment of endospores to juveniles. Among the tested organic amendments, in mustard and neem cake, the attachment of endospores to juveniles was higher than that of other organic amendments. Minimum spore encumbrance was observed in leachate collected from the pots where farmyard manure was applied, however, the number of spores adhered to second stage juveniles were sufficient to parasitize the root-knot nematode. However, Rocha *et al.* (2004) [19] studied the effect of root exudates of seven plant species and recorded a variable response. The incubation of J_2 for 12 hours in root exudates (except tomato) reduced the number of *P. penetrans* endospore attachment to second stage juveniles. Their study revealed adverse impact of root exudates not only on endospore adhesion but also on subsequent production of infected females. Liu *et al.* (2017) [10] studied the influence of root exudates and soil on attachment of *Pasteuria penetrans* to *Meloidogyne arenaria* and observed that the increase in incubation period, the spore encumbrance increased as in the present study, maximum encumbrance was recorded after 24 hours of exposure. The efficacy of *P. penetrans* along with mustard cake was found best in one experiment and then other experiment was carried out for the standardization of doses of *P. penetrans* along with most effective organic amendment *i.e.* mustard cake at the rate of 10 g per kg soil in nursery. Raising a vegetable nursery in own field is a common practice in vegetable cultivation in India. Such nursery sites usually harbor root-knot nematode populations and serve to disseminate nematode through infected seedlings to main field.

Therefore, nursery soil treatment with *P. penetrans* helps in treating small areas with the limited quantity of *P. penetrans* to large areas (which is currently not possible) through infected seedlings, and offers initial but vital protection to seedlings against nematodes. It is worth noting that *P. penetrans* dose $1x10^4$ spores/g soil along with mustard cake at the rate of 10 g per kg soil were comparable to recommended dose of carbofuran. Higher dose of *P. penetrans* was better than that of carbofuran as final eggs and juvenile population were drastically reduced. Though *P. penetrans* undoubtedly suppressed the nematode population as revealed by reduced galling and final eggs and J_2 population, the organic amendment similarly also imparted manorial and nematode suppression effect which boosted seedling health and provided tolerance to the plant against nematodes. Ravichandra *et al.* (2008) conducted a field trial to evaluate the efficacy of *P. penetrans*, neemark 0.03 EC and carbofuran 3G individually in the nursery for the management of *Meloidogyne incognita* infesting tomato cv. Pusa Ruby. They reported that *P. penetrans* at the rate of $1x10^9$ spores/m^2 was found to be effective in reducing nematode population in the main field over inoculated check. Besides, improving plant growth and nutritional status of fruits, plants were also observed with highest yield compared to inoculated control. Walia and Dalal (1994) [27] recorded 18-20 per cent increase in tomato yield by treatment of *M. javanica* infested nursery soil with *P. penetrans*. Application of endospores in root powder preparation was very effective in reducing the nematode damage to tomato. Addition of spore concentration of $1x10^4$/g nematode infested soil resulted in 30 percent parasitization of *M. javanica* females and it increased to 67 per cent at $1x10^5$ spore density (Walia, 1994) [26]. The population densities of *P. penetrans* increase gradually over time and build up to levels that may keep the nematode population below damaging levels in long run. Thus, even if large-scale production of inoculums of *P. penetrans* proves to be difficult or too costly, the use of this antagonist in integrated nematode management system is feasible.

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