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EVALUATION OF EFFICACY OF *PASTEURIA PENETRANS* ALONE AND IN COMBINATION WITH *VERTICILLIUM CHLAMYDOSPORIUM* AGAINST *MELOIDOGYNE JAVANICA*

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ABSTRACT

The potential control of *Meloidogyne javanica* using *Pasteuria penetrans* (*Pp*) alone and in combination with *Verticillium chlamydosporium* (*Vc*) was tested in earthen pots following a cropping sequence "tomato-tomato-tomato" over three crop cycles. After the final harvesting, analysis of variance showed significant effect of treatments (P<0.01) regarding number of eggmasses, galls and nematode female populations. Similarly, significant effect of treatments (P<0.01) was also recorded in case of infected nematode females with *Pasteuria* and number of eggs/eggmass while no significant effect (P >0.0 5) was observed in case of endospore production. Higher numbers of eggmasses (360) and root galling (6.2) was observed where biocontrol agents were absent (control). The treatments showed 46.58, 58.85 and 33.13 percent reduction in number of galls, eggmasses and nematodes in *Pp* alone and 43.34, 55.21 and 30.09 percent reductions in *Vc+Pp* treatments respectively. Numbers of females infected with the endospores of *P. penetrans* were recorded higher in *Pp*-3 alone treatment (13.2) followed by *Vc+Pp* combined treatment (13.0) and maize rotated treatment (10.4) respectively. Significantly lesser number of eggmasses, galls and nematodes were recorded in pots where tomato was rotated with maize (treatment 3) compared with control. Thus rotation prevented the buildup of nematode population and resulted in a 72% decrease in numbers of eggmasses, 38% in root galling and 46% regarding female populations over the control after the final harvest. Maximum colony forming units of *V. chlamydosporium* per gram of soil were recorded after its addition to the soil. The fungus established in the soil during the first crop and soil colonization of the fungus was also observed after final crop.

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INTRODUCTION

Root knot nematodes (*Meloidogyne* spp.) are considered to be the most pathogenic plant parasitic nematodes (Castagnone-Sereno et al., 2013; Jones et al., 2013; Mukhtar and Kayani, 2019, 2020; Tariq-Khan et al., 2020; Trudgill and Blok, 2001) and application of chemicals for the control of these nematodes is a common practice. However, environmental problems

associated with the use of nematacides (Thomason, 1987) have resulted in search for alternative nematode management tactics (Kerry, 1990). The biological control of nematodes with microbial agents is an alternative management approach to avoid the notorious impacts of chemicals on vegetable crop production systems and health of living beings in ecological niche (Kiewnick and Sikora, 2006). Among different biological methods, many antagonistic bacteria and fungi are being investigated and have been proved to become a potential biocontrol agent for the control of soil borne root pathogenic nematodes (Khan et al., 2008). Successful management of root-knot nematodes requires the combined effects of different strategies; thus, biological control agents are more likely to be successful when they are used in conjugation with crops that are less favorable hosts to root-knot nematodes and with other physical or cultural control practices such as solarization, fallowing and even nematicides (Bhuiyan et al., 2018; d'Errico et al., 2019; Nazir et al., 2019).

One of the good attributes of *Pasteuria penetrans* is its compatibility with other management practices used for suppressing root-knot nematodes (Dube and Smart Jr, 1987; Eddaoudi and Bourijate, 1998; Tzortzakakis and Gowen, 1994; Walker and Wachtel, 1988). It can be deployed as a part of integrated management of plant parasitic nematodes. Similarly, *Verticillium chlamydosporium* (*Pochonia chlamydosporia*) is a widespread fungus that parasitizes females and eggs of cyst and root-knot nematodes (Kerry et al., 1982). Both the antagonists have potential as biological control agents for *Meloidogyne* spp. when thoroughly incorporated through the soil (Daudi et al., 1990; De-Leij et al., 1992; Gowen and Ahmed, 1990; Madulu et al., 1994; Mukhtar et al., 2003; Rehman et al., 2009). Therefore, integration of *P. penetrans* and *V. chlamydosporium* could be an effective strategy as *Pasteuria* reduces total number of egg producing females while *Verticillium* is more effective when eggs are produced and exposed to fungus colonizing the rhizosphere. The introduction of *P. penetrans* into soil to reduce root invasion and egg production, together with *V. chlamydosporium* as a rhizosphere colonizer and facultative egg parasite, may improve nematode control compared with either of the organism alone.

Crop rotation with poor hosts helps in reducing nematode population. It helps in integration of biocontrol agents by suppressing nematode population and ultimately increases the efficiency of biocontrol agents. Corn, a poor host to root-knot nematodes (Alam et al., 1977) was used as rotation and proved effective in suppressing root-knot nematode population and lowering nematode infection (Huang, 1984; Sundaresh and Setty, 1977).

The present study deals with the integration of *P. penetrans* with fungal biocontrol agent *V. chlamydosporium* to ascertain the effects of the antagonists to suppress root-knot nematodes in soil and rotation of a poor host like maize to see additive effects of both biocontrol agents in reducing root -knot disease.

MATERIALS AND METHODS

The population of *M. javanica* used in the experiment was already maintained on tomato in the glasshouse of the Department of Agriculture, University of Reading, UK. An isolate of *Pasteuria penetrans* designated as *Pp*-3 derived from *M. javanica* originating from South Africa, and the fungal isolate *Verticillium chlamydosporium* (*Vc*-l0) obtained from Rothamsted, England were used in the experiment.

Multiplication of Fungal inoculum

The fungal inoculum was multiplied on barley by the method described by Bourne et al. (1994). Dried milled barley was washed over a 53 μm aperture sieve and mixed 1:1(V/V) with coarse sand and left to dry until moist and easily friable. About 130-150 ml of the medium, in a 250 ml flask was autoclaved (30 min, 15 psi, 120°C), cooled, shaken and inoculated with 3-5 plugs (7 mm) of *V. chlamydosporium* on corn meal agar. After 3 weeks of incubation at 25°C, the colonized sand bran was washed through 250 μm, 53 μm and 10 μm aperture sieves with a fine water spray to remove the sand and bran and the fungal propagules were collected on 10 μm aperture sieve. The deposit was further washed to remove conidia and small hyphal fragments leaving mainly chlamydospores and blotted dry to remove extra moisture. Chlamydospores were scraped off and thoroughly mixed with fine sand (40-100 mesh; 1:10 w/w) which acted as inert carrier. A 1 g sub-sample of the inoculum was shaken in 9 ml of water and the number of chlamydospores per gram sand was then estimated using a haemocytometer. Inoculum was applied @ 5000 chlamydospores/L pot in treatment 2. Initial presence of the fungus was monitored just after adding the inoculum to the soil.

Multiplication of bacterial inoculum

The bacterial inoculum was multiplied on *M. javanica* growing on tomato in the greenhouse by the method of

Stirling and Wachtel (1980). Twenty plastic pots (1 L) were filled with John Inns compost as potting material. Four treatments with five replicates were arranged in a Completely Randomized Design and kept in a growth room at temperature ranging from 10 to 30°C. The treatments were given in Table 1.

Table 1: Description of treatments.

Treatment	First cycle	Second cycle	Third cycle
T1	*Pp* (Tomato)	*Pp* (tomato)	*Pp* (tomato)
T2	*Pp* + *Vc* (Tomato)	*Pp* + *Vc* (Tomato)	*Pp* + *Vc* Tomato)
T3	*Pp* (tomato)	*Pp* (maize)	*Pp* (tomato)
T4	Control (tomato)	Control (tomato)	Control (tomato)

Fungal inoculum was applied @ 5000 chlamydospores/L pot in all replications of treatment 2. Initial presence of the fungus was monitored just after adding the inoculum to the soil. Tomato plants cv Tiny Tim (40 days old) were transplanted singly into pots. After two weeks of transplanting, 1500 J2s of *M. javanica* encumbered with spores of *Pp*-3 with a mean of 6.8 spores/J2 were inoculated per plant in treatments 1 to 3. Similarly, J2s without spores were inoculated @ 1500/plant as control in treatment no. 4. After six weeks, harvesting was made and plants were washed and fresh root weight, galling intensity and numbers of egg masses were counted. The fungal growth was monitored by taking a 1 g soil sample from each replicate of treatment 3. Roots were dried, chopped, macerated and incorporated back into pots. Tomato plants (40 days old) were transplanted into pots in all treatments except treatment 3 where it was replaced by maize as a rotation of "poor" host. During the second crop cycle, Phostrogen (14:4.4:22.4, NPK respectively) was sprayed to provide nutrients to plants. After six weeks, harvesting was done and data were recorded as described above. In the third crop cycle, tomato plants (6 weeks old) were transplanted in all treatments. After the final harvest, data for eggmasses, root galling, final female population, endospores per female, eggs/eggmass and infectivity of females with *Pasteuria* were recorded.

RESULTS

After the final harvesting, analysis of variance showed significant effect of treatments (P<0.01) regarding number of eggmasses, galls and nematode female populations (Table 2). Similarly, significant effect of treatments (P<0.01) was also recorded in case of infected nematode females with *Pasteuria* and number of eggs/eggmass while no significant effect (P >0.0 5) was observed in case of endospore production (Table 3). Higher numbers of eggmasses (360) and root galling (6.2) was observed where biocontrol agents were absent (control). The treatments showed 46.58, 58.85 and 33.13 percent reduction in number of galls, eggmasses and nematodes in *Pp* alone and 43.34, 55.21 and 30.09 percent reductions in *Vc+Pp* treatments respectively (Table 2). Numbers of females infected with the endospores of *P. penetrans* were recorded higher in *Pp*-3 alone treatment (13.2) followed by *Vc+Pp* combined treatment (13.0) and maize rotated treatment (10.4) respectively (Table 3). Significantly lesser number of eggmasses, galls and nematodes were recorded in pots where tomato was rotated with maize (treatment 3) compared with control. Thus rotation prevented the buildup of nematode population and resulted in a 72% decrease in numbers of eggmasses, 38% in root galling and 46% regarding female populations over the control after the final harvest (Table 2, 3).

Table 2. Effect of *P. penetrans* alone and in combination with *V. chlamydosporium* on numbers of eggmasses, root galling and total females of *M. javanica* (third harvest) on tomato plants.

Treatments	Eggmasses/plant	Total females /plant	Galling/plant (0-10)
Pp-3	220	286	4.4
Pp-3 + *Vc*	164	251	4.0
Pp-3 (rotation)	100	196	3.8
Control	360	368	6.2
S.E.D	15.44	18.97	0.34
F-test	P<0.01	P<0.01	P<0.01

Data are means of 5 replicates

Table 3. Effect of *P. penetrans* alone and in combination with *V. chlamydosporium* on numbers of eggs/eggmass, endospores/female and infection of females of *M. javanica* (third harvest).

Treatments	Endospores/female (x10^3)	Infected females (out of 20)	Eggs/eggmass
Pp-3	413	13.8	406
Pp-3 + Vc	421	13.0	365
Pp-3 (rotation)	425	10.4	395
Control	-	-	446
S.E.D	23.1	0.57	27.5
P value	>0.05	<0.01	<0.1

Data are means of 5 replicates.

Data regarding soil colonization by fungus was recorded and maximum colony forming units of *V. chlamydosporium* per gram of soil were recorded after its addition to the soil (Table 4). The fungus established in the soil during the first crop and soil colonization of the fungus was also observed after final crop (Table 4).

DISCUSSION

Pasteuria alone proved effective in suppressing root-knot nematode infection over three crop cycles. This is due to reduced root penetration by the spore-encumbered juveniles and/or failure to form egg masses by the infected females. Many researchers have reported that movement and mobility of juveniles were reduced and their ability to locate host roots was affected when juveniles were encumbered with endospores (Ahmad and Mukhtar, 2007a, 2007b; Mukhtar and Ahmed, 2000). Reduced motility probably leads to high mortality of J2s in soil because movement is apparently essential to escape predators and unfavorable conditions (Chen et al., 1996; Mukhtar et al., 1999; Mukhtar et al., 2000). Since the reproductive system fails to develop in the infected females of root-knot nematodes, such nematodes do not lay eggs. This leads to marked reductions in the secondary infection by the second or subsequent generation juveniles (Mukhtar et al., 2002a; Mukhtar et al., 2002b; Mukhtar et al., 2013; Mukhtar et al., 2005). Overall, the nematode populations are considerably suppressed which may be beneficial to the ensuing crops. Percentage of infected females examined after third cycle showed that maximum number of females were recorded in *Pp* alone treatment.

Table 4: Number of colony forming units (CFU) of *V. chlamydosporium* recorded over crop cycles (1g soil).

Observations	Colony forming units
Initial inoculum	8,050 (8.97)
First crop cycle	42,375 (10.5)
Second crop cycle	85,675 (11.3)
Third crop cycle	67,400 (11.0)
SED	2729 (0.21)

Data are means of 5 replicates (data within brackets represent log x transformations).

Integration of *V. chlamydosporium* with *P. penetrans* proved effective in reducing root-knot disease compared with un-amended control. *V. chlamydosporium* complemented the bacterial pathogenic potential in suppressing root-knot nematode and ultimately resulted in reduction of eggmasses, galls, and nematode female populations over three crop cycles. These findings are in agreement with those of De-Leij et al. (1992) who observed that *P. penetrans* and *V. chlamydosporium* significantly reduced *M. incognita* populations. Data regarding soil colonization by fungus were recorded and maximum colony forming units of *V. chlamydosporium* per gram of soil were recorded after its addition to the soil (Table 4). The fungus established in the soil during the first crop and soil colonization of the fungus was also observed after final crop. These results are contradictory to those of Mukhtar et al. (2003) who reported that fungus did not colonize soil and roots of subsequent crops of the sequence. The contradictory results might be due to different conditions under which experiments were conducted. Similar experiments conducted under green house and field conditions gave different results.

Rotation of poor host (maize) suppressed significantly the multiplication of root-knot nematode which resulted in reducing numbers of eggmasses and root galling. The growth of a poor plant host in nematode infested soil for one cropping period significantly decreased the damage on a following susceptible crop, although the nematode population was not eliminated. Eggs may have survived in a diapaused stage during the growth of resistant maize, and juveniles hatched when tomato plants were planted.

P. penetrans showed tremendous potential in controlling root-knot nematodes over 3 crop cycles. Re-incorporation of roots containing *P. penetrans* spores proved effective in suppressing the root knot nematode. It seems likely that spores of the parasite build up in the soil when the roots are incorporated in soil (Daudi et al., 1990; Gowen and Ahmed, 1990). Infected females inside the roots may also degrade and release spores in soil during plant growth, thus making the soil suppressive to nematodes.

Reduction in eggmasses, root galling and final nematode populations recorded in *P. penetrans* and *V. chlamydosporium* treatment suggest if these bio-control agents parasitize different stages of the nematode life cycle and they might have a complementary effect in suppressing the *Meloidogyne* populations. Both the organisms reduced reproductive potential of the nematode. *V. chlamydosporium* was the most effective after the first harvest when most eggmasses were produced in the rhizosphere and the fungus could colonize the eggmasses. Later when eggmasses were produced within galls and were protected from the fungus, *P. penetrans* spores liberated from disintegrating females might have produced a degree of secondary infection. These findings are in agreement with De-Leij et al. (1992) who observed that *P. penetrans* and *V. chlamydosporium* significantly reduced the population of root-knot nematode, *M. incognita*. There was no statistically significant difference among females of nematodes parasitized by *P. penetrans* in the treatment where both biocontrol agents were applied. This shows that *V. chlamydosporium* does not affect the host-parasite relationship of nematode and bacterium.

V. chlamydosporium has shown tremendous potential to establish in soil over crop cycles. It has a good attribute of a successful biocontrol agent for soil borne pathogens as it is able to grow, survive and proliferate. Being a facultative parasite, it can grow even in the absence of the nematode host. The successful establishment of *V. chlamydosporium* from application of chlamydospores without an energy source simplifies its use in microplot experiments. However, the fungal growth reduced after third cycle as its growth declines rapidly after three months in the soil due to absence of energy source (Kerry et al., 1993).

Lesser eggmasses and root galling found in the maize rotation treatment reflects that during the second crop cycle (in the presence of maize host), nematodes could not invade the roots and moved in soil to locate penetration sites. J2s might have acquired spores and in the third crop cycle, invaded tomato roots, thus ultimately resulting in fewer eggmasses and root galling. When a nematode population is reduced by checking multiplication by growing a poor host, the small numbers of nematodes found might acquire more spores and this will help in reducing invasion due to high spore burden (Brown and Smart, 1985; Davies et al., 1988; Davies et al., 1991; Stirling, 1981). Thus rotation helps *P. penetrans* to suppress root-knot populations more effectively in subsequent crop cycles. The lower level of parasitism recorded in the third treatment indicated that the nematode-resistant host did not favor *Pasteuria* development in the soil. The effect of a nematode-resistant host was to prevent the increase of the parasite and this resulted in poor multiplication of *Pasteuria* as the bacterium is density dependent (Chen et al., 1996).

It is concluded from the present study that *Pasteuria* was been found effective when integrated with *V. chlamydosporium* or rotation and should be used as a part of an integrated approach in the management of root-knot nematodes.

AUTHOR CONTRIBUTION

MS and SRG designed the study, MS executed the experiment, collected data, and wrote the manuscript, SRG supervised the work, BP helped in technical work and analysis of data, SRG and BP edited the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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