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EFFECT OF SPORE ATTACHMENT METHODS AND LEVELS ON INVASION AND PARASITISM OF ROOT-KNOT NEMATODE *MELOIDOGYNE JAVANICA*

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ABSTRACT

Spore attachment is the first stage in the life cycle of *Pasteuria penetrans*. Therefore, the present studies were planned to find out whether an attachment level which can provide better reduction in root-knot disease. There was a significant difference (P<0.01) regarding numbers of eggmasses produced by females of *Meloidogyne javanica* in the treatments. Higher numbers of eggmasses (237) were recorded in the treatment where unencumbered J2s were inoculated after spinning followed by the treatment where J2s were inoculated without spores (225) but this difference was not significant. Both spore attachment techniques revealed their influence on eggmass production as fewer eggmasses were recorded in the treatment where encumbered J2s were inoculated compared to controls. Significantly lesser numbers of eggmasses (123) were recorded where J2s were encumbered by exposing them to a spore suspension in plastic trays than by the centrifuge treatment (173). Root galling differed significantly (P<0.01) among treatments showing higher root galling in control treatment (5.2). Lesser root galling was observed where J2s were encumbered in the normal manner by exposing nematodes to spore suspension (3.6) while greater root galling (4.0) was observed in the treatment where J2s were encumbered after centrifugation. Similarly, there was a significant difference (P<0.01) among females parasitized by *P. penetrans* showing a higher level of infectivity (13.8) in the treatment where J2s were encumbered by the normal attachment method as compared to the centrifugation method (11.6). There was no significant difference (P>0.05) in fresh root weight of plants among treatments. There was no significant difference (P>0.05) in number of eggs/eggmass produced by females of *M. javanica* between the treatments.

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INTRODUCTION

Spore attachment is the first stage in the life cycle of *Pasteuria penetrans* (*Pp*). The development of *Pasteuria* begins with the germination of the endospore following attachment to the cuticle of nematode and infection occurs after the spore carrying second stage juveniles (J2) have entered the plant roots. The parasitized nematodes are able to develop into females but are incapable of reproduction. The bodies of fully developed infested females become filled with endospores of the bacterium which are eventually released into soil upon host disintegration. The parasite's development appears to be synchronised with that of its host, destroying the reproductive capacity and causing minimum damage to feeding, moulting and normal growth of the nematode (Bird, 1986; Bird and Brisbane, 1988; Bird et al., 1990).

Different populations of the bacterium seem to be highly specific not only to particular genera (Starr and Sayre, 1988) but also to different populations of the same species and variability may also exist within populations of the bacterium (Channer and Gowen, 1992; Davies et al., 1988; Spaull, 1984; Stirling, 1985; Vouyoukalou and Gowen, 1995). Not all *Meloidogyne* spp. or populations are equally susceptible to *P. penetrans*. Both *Meloidogyne* and *Pasteuria* are heterogenic in respect of spore attachment. Different populations of nematodes within a single species exhibited different affinities to spores of *Pasteuria*. Successful deployment may require careful matching of available isolates with the nematode host. Infection of the nematode with *Pp* is dependent on many variables of which the number of spores attached to J2s is considered to be important. However, Stirling (1984) reported that attachment of more than 5 spores/J2 was required to ensure 90% infection. Davies et al. (1988) reported that attachment of more than 11 spores/J2 reduced invasion. Generally infection of females increases with an increase in spores attachment levels (Giannakou, 1998; Giannakou et al., 1997; Rao et al., 1997; Tzortzakakis et al., 1995; Vouyoukalou and Gowen, 1995). Therefore obtaining high spore attachment on second stage juveniles could be an effective strategy in the use of *Pp* for the biological control of nematodes. Attachment of endospores to nematodes can be improved approximately 5 to 6 times for each 10- fold increase in number of endospores in the suspension by the centrifugation method (Hewlett and Dickson, 1993). Therefore centrifugation provides a more reliable means of studying the host ranges of *Pp* isolates and even this method is more effective in those nematodes, which produce fewer spores in cadavers.

Although centrifugation increases the level of spore attachment, the question arises whether this forceful attachment acquired by spinning has any influence on *P. penetrans* efficiency and ultimately on the development of root-knot disease. To answer these questions the present studies were carried out.

Similarly, the spore attachment techniques have revealed their influence on management of root-knot nematode as centrifugation accelerated attachment rate and uniformity of spores but conventional attachment proved better in suppressing nematodes and increased infection of *M. javanica* females. But what is an ideal spore attachment level which can reduce nematode populations as well as increasing bacterium inoculum by increasing numbers of infected females without affecting invasion of nematodes into roots. To find out an attachment level which can provide better reduction in root-knot disease, the present studies were planned.

MATERIALS AND METHODS

The root-knot nematode, *Meloidogyne javanica* used in the studies 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 was used in the experiments.

Effect of spore attachment methods on invasion and parasitism of *M. javanica*

Two spore attachment methods were used for spore encumbering J2s of *M. javanica.* In the first case, the endospore-nematode-water suspension was incubated at 28°C to get desired attachment level (6-12 spores/J2). In the second case, the endospore- nematode -water suspension was spun in a centrifuge. Twenty thousand J2s (2-4 days old) of *M. javanica* were added to a spore suspension of *Pp*3 (3.85×10^3/ml) in plastic trays (10 × 20 cm). The nematodes and endospores suspension was incubated at 28°C to get the desired level of attachment (6-12 spores/J2). This occurred in about 4 hours. The same numbers of unencumbered J2s were kept in water in an incubator at 28°C to serve as control. After obtaining the desired attachment level, the suspension was sieved through a 20 μm sieve to separate the encumbered J2s. The final volume was made up to 25 ml containing 3×10^3 J2s/ml. In the second case, an 8 ml suspension was placed in each of 16 tubes (10 ml) containing 2000 J2s (2-4 days old) of *M. javanica* and *Pp*3 spores (3.85×10^3/ml). These tubes were then placed in a centrifuge and balanced to avoid any loss of suspension during spinning.

The centrifuge (FISONS MSE CANTAUR 2) was run for 2 minutes at 3000 rpm. After spinning, the suspension was sieved through 20 μm sieve to separate the encumbered J2s from the spore suspension and collected in a beaker. The same numbers of unencumbered J2s were spun for the same time to serve as a control. Six-week old tomato plants growing in 1l pots were inoculated with 1500 encumbered J2s/pot. Two control treatments, one for spun encumbered J2s and the other for normally encumbered J2s were used. Therefore four treatments replicated fivefold in a completely randomised design were grown in a growth room (24 ± 6°C) for 6 weeks. Plants were harvested and data regarding fresh root weight, production of eggmasses, eggs/eggmass, root galling, total number of females and infection of females by *Pasteuria* were recorded.

Effect of spore attachment levels on invasion and infection of *M. javanica*

Two groups of 2×10^4 J2s (2-4 days old) of *M. javanica* were introduced to spore suspension of *Pp*3 (3.85×10^3 spores/ml) in plastic trays (10 × 20cm) and incubated at 28°C to have two levels of spore attachment (4-9 spores/J2 and 10-15 spores/J2), this was contrived by using different quantities of spore suspension. The encumbered J2s were separated from the suspensions by sieving through 20 μm sieve and a spore count was made on 40 J2s before inoculation. The group which had 4-9 spores/J2 was kept in water for the same time till the second attachment level 10-15 spores/J2 was achieved. Inoculations were made @ 1500 encumbered J2s/plant with a mean attachment level of 5.8 and 12.9 respectively. Unencumbered J2s were inoculated at the same density on plants to serve as controls. Inoculated plants were kept in a growth room under the same conditions as described above. Harvesting was made after 6 weeks, plants were washed and data regarding fresh root weight, production of eggmasses, eggs/eggmass, root galling, total number of females and infection of females by *Pasteuria* were recorded.

RESULTS

Effect of spore attachment methods on invasion and parasitism of *M. javanica*

There was a significant difference (P<0.01) regarding numbers of egg masses produced by females of *M. javanica* in the treatments (Table 1). Higher numbers of eggmasses (237) were recorded in the treatment where unencumbered J2s were inoculated after spinning followed by the treatment where J2s were inoculated without spores (225) but this difference was not significant. Both spore attachment techniques revealed their influence on eggmass production as fewer eggmasses were recorded in the treatment where encumbered J2s were inoculated compared to controls. Significantly lesser numbers of eggmasses (123) were recorded where J2s were encumbered by exposing them to a spore suspension in plastic trays than by the centrifuge treatment (173). Root galling differed significantly (P<0.01) among treatments showing higher root galling in control treatment (5.2). Lesser root galling was observed where J2s were encumbered in the normal manner by exposing nematodes to spore suspension (3.6) while greater root galling (4.0) was observed in the treatment where J2s were encumbered after centrifugation. The fact that there were similar numbers of females in the root systems of the different treatments suggests that there was no significant difference in invasion rate (P>0.05) among treatments.

Table 1: Effect of *Pasteuria penetrans* spores attached by spinning and normal attachment method on number of egg masses, root galling and total females of *Meloidogyne javanica* after 750 - degree days.

Treatments	Egg masses/plant	Root galling (0-10)	Total females
Pasteuria (spinning)	173	4.0	395
Control (spinning)	237	5.2	426
Pasteuria (normal attachment)	123	3.6	382
Control (normal attachment)	225	5.2	410
SED	15.84	0.46	22.68
F test	P<0.01	P<0.01	P>0.05

Data are means of 5 replicates.

There was a significant difference (P<0.01) (Table 2) among females parasitized by *P. penetrans* showing a higher level of infectivity (13.8) in the treatment where J2s were encumbered by the normal attachment method as compared to the centrifugation method (11.6). There was no significant difference (P>0.05) in fresh root weight of plants among treatments. There was no significant difference (P>0.05) in number of eggs/eggmass produced by females of *M. javanica* between the treatments.

Effect of spore attachment levels on invasion and infection of *M. javanica*

Both spore attachment levels reduced significantly root galling (P<0.01), numbers of eggmasses (P<0.01) and numbers of females (P<0.05) as compared to untreated control (Table 3). Lesser numbers of eggmasses (320), root galling (4.0) and females (464) were recorded in the treatment where J2s were encumbered by the higher attachment level (10-15 spores/J2) compared to the lower attachment level (4-9 spores/J2).

Table 2: Effect of *Pasteuria penetrans* spores attached by spinning and normal attachment methods on number of egg/eggmass, fresh root weight and infectivity of females of *Meloidogyne javanica* after 750 degree-days.

Treatments	Fresh root wt (g)	Eggs/egg mass	Infected females (out of 20)
Pasteuria (spinning)	5.34	386	12.2
Control (spinning)	5.21	403	_
Pasteuria (normal attachment)	5.48	383	13.8
Control (normal attachment)	5.23	399	_
SED	0.29	22.04	0.28
F test	>0.05	P>0.05	P<0.01

Data are means of 5 replicates.

Table 3: Effect of spore attachment levels of *Pasteuria penetrans* on numbers of eggmasses, root galling and total females of *Meloidogyne javanica* after 750 degree days.

Treatments	Egg masses/plant	Root galling (0-10)	Total females
M. javanica + *Pp*3 (4 - 9spores/J2)	369	4.2	490
M. javanica + *Pp*3 (10 - 15 sp/J2)	320	4.0	464
Control (*M. javanica*)	513	6.8	516
SED	14.01	0.34	14.65
P value	<0.01	<0.01	<0.05

Data are means of 5 replicates.

There was no statistically significant difference between high and low attachment levels in case of fresh root weight of plants (P>0.05) and numbers of eggs/eggmass in the treatments (P>0.05). Higher numbers of eggs/eggmass (474) were found in the control and the difference in infection on females of nematode by *P. penetrans* was non-significant (P>0.05) (Table 4).

DISCUSSION

Pasteuria penetrans was found effective in reducing eggmasses and gall infestation. Inoculations with juveniles without spores resulted in higher numbers of eggmasses and galling. Although spinning of nematodes by centrifugation improved the speed and uniformity of attachment of endospores to the cuticles of J2s, the subsequent levels of infection were lower than in the non-spinning treatment. The variance recorded for centrifugation was lower (3.66) compared to the variance value calculated for conventional spore attachment (8.24). This shows the significance of spinning that resulted in uniform spore attachment, when attachment on 25 J2s/treatment was recorded before inoculations. Higher numbers of eggmasses and gall infestation were observed in the treatment where centrifuged J2s were inoculated to plants.

Table 4: Effect of spore attachment levels of *Pasteuria penetrans* on fresh root weight, numbers of eggs/eggmass and infection of females of *Meloidogyne javanica* after 750 degree days.

Treatments	Fresh root weight (g)	Eggs/egg mass	Infected females (out of 20)
M. javanica + *Pp*3 (4 - 9spores/J2)	5.50	405	13.6
M. javanica + *Pp*3 (10 - 15 sp/J2)	5.47	422	12.4
Control (*M. javanica*)	5.36	474	_
SED	0.14	27.9	0.78
P value	>0.05	<0.1	>0.05

Data are means of 5 replicates.

Furthermore, fewer females were found parasitized as compared to normal attachment treatment. Although higher invasion was observed where the spinning of nematodes was done, less infection and parasitism of females resulted in this treatment. Why should spinning result in poor infection and parasitism? This may have been because the spores were less well bound to the cuticles and detached in the period between inoculations of the plants and invasion as detachment of spores has been reported (De silva, 1992; De Silva and Gowen, 1994; De Silva et al., 1996; De Silva and Gowen, 1990; Ratnasoma et al., 1991). Also, if spores were less well bound they may have been less capable of germinating / penetrating the cuticles or more non-viable spores may have been attached when spun. Lower infectivity despite higher attachment has been reported by some workers (Espanol et al., 1997; Tzortzakakis et al., 1997; Tzortzakakis et al., 1995; Tzortzakakis and Gowen, 1994a, b) suggesting the incompatibility between the spore isolate and the nematode population or adhesion may be independent of infection. The failure of *P. penetrans* to infect nematodes has been reported to be due not only to poor recognition of the spore surface but also to some degree of resistance to germination, penetration or even mycelial development in the developing nematode (De silva, 1992; De Silva and Gowen, 1990).

It is interesting that a quite high level of spore attachment may still leave many nematodes uninfected even if root galling and egg production are significantly reduced. Furthermore, it is clear that a *Pasteuria* isolate that attaches at higher rates does not always guarantee greater reduction of egg laying. The reasons could be high variance in spore attachment or spore detachment during movement of juveniles in soil. It is not clear what is the minimum numbers of spores which have to be attached in order to ensure good infectivity. The contradictory results reported by the previous workers (Davies et al., 1988; Giannakou, 1998; Rao et al., 1997; Stirling, 1981) might be due to the nematode population or isolates of *Pasteuria* used. Also, individuals within a single nematode species or a mixture of species may resist spore attachment and infection (Tzortzakakis et al., 1997; Tzortzakakis and Gowen, 1994a, b). Some of these spores may not be viable or able to germinate at that time. Also some of them might not be compatible with the population of root-knot used while others are. Davies et al. (1988) also found significant difference between the numbers of females that developed from juveniles encumbered with different *Pasteuria* isolates at a similar level of attachment. If we compare the mean numbers of eggmasses in both attachment levels, the higher attachment level affects not only the eggmass productivity but also the invasion rate. The low parasitism recorded supports the suggestion that invasion might have been influenced by a higher spore burden. From a practical point of view, attachment, even without subsequent infection, is useful in that juveniles encumbered with many spores are less capable of invading roots (Brown and Nordmeyer, 1985; Brown and Smart Jr, 1985; Davies et al., 1988; Stirling, 1981). The processes which influence and decrease the invasion rate when many spores are attached to the cuticle of juveniles are unknown.

AUTHOR'S CONTRIBUTION

MS and SRG designed the study, MS performed the experiments, collected and analyzed the data, BP provided technical assistance, MS wrote the manuscript and BP and SRG proofread the paper.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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