

Antagonistic potential of *Pasteuria penetrans* based oil dispersion formulation against *Meloidogyne incognita* in medicinal coleus, *Coleus forskohlii*

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Abstract

Medicinal coleus (*Coleus forskohlii*) is an important herbal plant of Indian Medicine. Root-knot nematode, *Meloidogyne incognita*, is the predominant pest of medicinal coleus that causes yield reduction upto 72 per cent. Nematode affects the medicinal property of the tubers by producing root-knot galls on them. Chemical nematicides are not suitable as the molecule, forskolin is extracted from the tubers. Biological control is the only suitable solution to prevent pesticide residual issues. *Pasteuria penetrans* is a potential antagonistic bacterium of *M. incognita*. The current investigation, an oil dispersion formulation (ODF) of *P. penetrans* was developed and tested for its effectiveness against *M. incognita*. The formulation was water soluble and the spores were viable at 60 days of incubation that was confirmed through juvenile parasitization. Various concentrations of this formulation were prepared and screened against *M. incognita* infection in *C. forskohlii* under glasshouse conditions. Soil drenching of the formulation at the rate of 400 µL in 1000 mL of water recorded 86 per cent reduction in egg mass production. *P. penetrans* endospores were recovered from treated plants that confirms the efficiency of the formulation. Based on this result, field trials were conducted to evaluate the efficacy of ODF for two seasons. The results showed that the application of ODF @ 4 L/ha in 200L of water significantly reduced nematode infection to a tune of 74.9–84.8% and 61–66.5% reduction in egg mass production and gall index. The same formulation promoted tuber yield, that was 16.7–21.5 per cent increase over control.

Introduction

Root-knot nematode (*Meloidogyne* sp.) is one of the major plant parasitic nematodes and the prevalent species are *Meloidogyne incognita*, *M. javanica*, *M. arenaria* and *M. hapla* (Jones et al., 2013). Root-knot nematode causes 10–30% yield loss in global crop production per year (Manjunatha et al., 2017) and creates great reduction in crop yield and quality. Medicinal coleus infected by the root knot nematode, *Meloidogyne* spp. Affected crops show slow or stunted growth, yellowing of leaves, wilting of the plant despite of adequate soil water content and finally leading to death of individual plants. Severely infected seedlings produce a fewer roots and usually die rapidly. Heavy infection of older plants cause the plant to wilt unexpectedly and die off early (Singh et al., 2011). Chemical control of the root-knot nematode is effective, but at the same time indiscriminate treatment causes resurgence of sedentary endoparasitic nematodes, which leads to nematicide resistance and has detrimental effects on the environment (Degenkolb and Vilcinskis, 2016). These factors necessitate in finding other strategies to control the root-knot nematode in a more effective and eco-friendly manner. Non-chemical approaches include crop rotation and exploitation of biological control agents (Stirling, 1991) in which biocontrol agents play a significant role in the management of root-knot nematode. Fungal bioagents *Pochonia chlamydosporia* and *Purpureocillium lilacinum*, as well as bacterial biocontrol agents viz., *Bacillus* spp. and *Pasteuria penetrans* are commonly available in nature. *P. penetrans* (Thorne) is an obligate, gram positive, endospore forming bacterium. *P. penetrans* is highly host specific and found to be effective in controlling the root-knot nematodes, *Meloidogyne* spp. (Chen et al., 1996). Since 1991, many scientists have attempted to culture *P. penetrans* in axenic culture media and many of the methods were found to be ineffective. However, Hewlett et al. (2004) was the first group of scientists to successfully culture *P.*

penetrans under *in vitro*, which was indeed a breakthrough in this field. Moreover, commercial formulation of *P. penetrans* is still unavailable in India and may not reach the farmers in the near future. There is no a viable formulation of *P. penetrans* is available till today. This paper describes a method for preparation of oil dispersion formulation and its effect against *M. incognita* in medicinal coleus, *Coleus forskhlii*.

Medicinal coleus, *Coleus forskohlii* (Wild.) Briq. is one of the important herbal plants of Indian medicine and has the native of India (Valdes *et al.*, 1987). It belongs to the family Lamiaceae. It is widely grown in India and other countries like Arabia, Brazil, Egypt, Ethiopia, Pakistan, Sri Lanka, and Tropical East Africa. Medicinal coleus is succumbed to root-knot nematode, *Meloidogyne* spp infection. Four different types of *Meloidogyne* species are were reported from *C. forskohlii* viz., *M. javanica*, *M. incognita*, *M. hapla* and *M. arenaria*. In which the most predominant species *M. incognita* causes a yield reduction of upto 86 per cent (Senthamarai *et al.*, 2006), while severe loosens also occur in *C. forskohlii* because of *M. arenaria* infestations (Bhandari *et al.*, 2007). Interaction between *M. phaseolina* and *M. incognita* was studied by Senthamarai *et al.* (2008) and they have reported that this complex resulted in cent percent wilting of plants. With this background, a field study was undertaken to manage nematode infection using the oil dispersion formulation of the biocontrol agent, *P. penetrans*.

Materials and Methods

Inoculum preparation of *P. penetrans* on root-knot nematode, *M. incognita*

Root-knot nematode, *M. incognita* culture was collected from the infected tomato field (Variety PKM 1) in Madhampatti village, Coimbatore (Longitude-76°51'38.05"E, Latitude – 10°58'24.1"N). The species identity was confirmed through posterior cuticular pattern. Egg masses of *M. incognita* were collected from the galled roots and incubated at room temperature ($28 \pm 2^{\circ}\text{C}$) in tap water for hatching. Endospores of *P. penetrans* were collected from adult females maintained in tomato plants under glasshouse condition at the Department of Nematology, TNAU, Coimbatore. After egg hatching, the second stage juveniles (J2) were incubated with these endospores at the rate of 1×10^6 spores/ 100 J2 in 50 ml water. The spore attachment was confirmed under research microscope (Leica-020-518500). These endospores attached J2 were inoculated to tomato plants (Variety PKM 1) maintained in pots filled with sterile pot mixture (Red earth: Sand: FYM at the ratio of 2:1:1) under glasshouse for further studies.

Developing oil OD formulation of *P. penetrans*

Endospores were collected in microfuge tubes containing sterile water (2 mL) from the *P. penetrans* infested females by crushing them manually. The spore load was assessed using Haemocytometer. Water soluble formulation was prepared by using soy-lecithin (emulsifier), Triton-X (surfactant), sterile water and sunflower oil. Sterile water (19 ml) was heated using hot plate at 50°C . Soy-lecithin (1 mL)

was added to hot water and mixed thoroughly. Sunflower oil (4 mL) and Triton-X (4 mL) were added to this mixture and heated at 50 °C for 3 minutes (Zhou et al., 2010). Then, 1 mL of endospore suspension containing 1×10^6 spores / mL was added to the mixture and stored in the glass vial at room temperature (28 ± 2 °C).

Assessing the spore release from OD formulation of *P. penetrans*

Plastic cups were filled with red sandy loam soil (80 g) and vermicompost (20 g). Endospores entrapped OD formulation was mixed into the soil at the rate of 1 ml / cup. These cups were inoculated with juveniles of root-knot nematode, *M. incognita* at the rate of 100J₂/ cup and incubated at room temperature (28 ± 2 °C). Parasitization of J₂ was recorded. Same observation was recorded at 30 and 60 days after incubation to confirm the shelf life.

Effect formulation of *P. penetrans* on penetration of J₂ in tomato

Plastic cups were filled with red sandy loam soil (100 g). The formulations were inoculated into the soil and tomato seedlings were transplanted 7 DAI. Second stage juveniles (J₂) of *M. incognita* were inoculated to each cup at the rate of 100 J₂/ cup. Nematode penetration was observed by staining the roots using acid fuchsin.

Bioefficacy study of oil OD formulation against *M. incognita* in medicinal coleus, *Coleus forskohlii*

Pot culture experiment

Medicinal coleus (variety Co 1) cuttings were transplanted to 5 kg sterile pot mixture (Red earth: Sand: FYM at the ratio of 2:1:1). Various concentrations viz., 1, 2, 3, 4 and 5 per cent of OD formulation containing endospores of *P. penetrans* were prepared using sterile distilled water. The formulation was applied 15 days after planting (DAT). A positive check with carbofuran 3 G was maintained along with a untreated control for comparison. Five days after application of the formulation, juveniles of *M. incognita* were inoculated at the rate of 100 J₂ / pot. Each treatment was replicated three times. The whole plant was uprooted and observations on nematode infestation and plant biometric characters were recorded 60 days after planting.

Field trial

Based on the results of pot culture experiment, a field trial was conducted to test the efficacy of OD formulation under field condition for two seasons. *M. incognita* infested field was selected for the study. Nematode population was assessed using Cobb's wet sieving (Cobb, 1918) and modified Baermann's method (Schindler, 1961). Three treatments were maintained with 7 replications. T1 - *P. penetrans* OD

formulation ($(1 \times 10^6$ endospores / ml) was applied at the rate of 2 L / ha. T2 - Carbofuran 3G @1 kg a.i / ha (33.3 kg / ha) was applied as a positive check and T3 - untreated control without any treatment. Each treatment was replicated seven times. Trial was conducted for two seasons. Observations on nematode infection in soil and roots were recorded. Number of females filled with *P. penetrans* endospores and plant biometric observations on shoot length, root length, weight and number of tubers were also recorded.

Statistical analysis

The data obtained from above mentioned experiments were analysed using ANOVA DMRT (Duncan's Multiple Range Test) at 1% level (Panse and Sukhatme, 1978) using AGRES statistical software.

Results

Assessing the spore release from OD formulation of *P. penetrans* in soil

The observation showed that the endospores were release from the OD formulation within a day and the highest parasitization potential of 85 per cent with spore load of 11.2 / J_2 of *M. incognita* was recorded. Viability of endospores in the formulation was observed on 30 DAI and 60 DAI. There was no change in the condition of formulations for 2 months (60 days) of storing and the endospores were observed under phase contrast bright field microscope (iScope – Euromex). The parasitization potential was 84.7% with a spore load of 10.2 spores/ J_2 on 60th day of storage.

Penetration of J_2 of *M. incognita* into the tomato root was examined and observed for average number of J_2 penetrated per plant on 7th day after inoculation of OD formulation of *P. penetrans*. The formation of minutes galls on roots was observed. Development of J_3 and pre-adult stages of *M. incognita* were observed on 12th day. Formation of four-cell stage and two-cell stages of endospores in third stage juvenile (J_3) and pre-adult stages of *M. incognita* were recorded.

Pot culture condition

The results showed that the soil application of *P. penetrans* OD formulation recorded a significant reduction in nematode infection invariable of concentrations (Table 1).. Among various concentrations tested highest reduction in root galling and egg mass production was recorded at 400 μ L of formulation with the lowest number of root-knot galls that was reflected as 60 per cent and 66.6 per cent reduction over control respectively. The same treatment reduced nematode infection in soil upto 59.4 per cent over control. The second best treatment was soil application of OD formulation at the rate of 300 μ L per pot. Matured endospores of *P. penetrans* were observed in adult female nematodes. The highest number of *P. penetrans* infected females were recorded in plants received 400 μ L formulation. There was a negative correlation was existed between the concentration of OD formulation and soil nematode population ($r = - 93.6$).

Table 1
Efficacy of *P. penetrans* OD formulation in medicinal coleus, *C. forskholii* under glasshouse condition (pot culture)

Treatments	Number of galls/ 5g of root	Number of egg masses/ 5g of root	Number of J2 in soil (100 cc)	Number of infested females/ 5g of root
T1 - 100 µl <i>Pp</i> OD formulation / Litre of water / pot	17.7 ^b (4.16)	21.30 ^c (4.54)	98.33 ^b (9.90)	8.30 ^b (2.88)
T2–200 µl <i>P. penetrans</i> OD formulation / pot	15.66 ^b (3.95)	17.0 ^{bc} (4.31)	98.0 ^{ab} (9.28)	5.36 ^c (2.37)
T3–300 µl <i>P. penetrans</i> OD formulation / pot	14.66 ^{ab} (3.82)	15.72 ^{ab} (4.46)	89.33 ^{ab} (9.87)	7.66 ^b (2.76)
T4–400 µl <i>P. penetrans</i> OD formulation / pot	12.65 ^a (3.55)	13.46 ^a (4.19)	77.15 ^a (8.77)	10.0 ^a (3.15)
T5 - Carbofuran @ 3 g /pot	22.65 ^c (4.74)	34.66 ^d (5.79)	167.66 ^c (12.93)	-
T6 – Control (Untreated)	31.66 ^d (5.6)	40.33 ^e (6.34)	190.33 ^c (13.79)	-
CD (p = 0.05)	0.39	0.21	1.82	0.23
*Figures in parentheses are square root transformed values. In a column, means followed by a common alphabet are not				

significantly different from each other at 5% level by DMRT.

Field trial

Field trials were conducted in nematode sick plot for two seasons. The results on nematode infection and yield showed that the soil application of OD formulation of *P. penetrans* reduced nematode population significantly with an improved yield when compared to control.

Season I

Soil application of OD formulation of *P. penetrans* resulted in higher shoot length and shoot weight of treated plants with 45.16 cm and 60.33g respectively while it was only 38.16cm and 46.36g in untreated control. Similarly, plants received carbofuran 3G treatment was also inferior when compared to *P. penetrans* treatment. Root length and root weight were also higher in the plants received *P. penetrans* soil drenching with 23.87 cm and 30.85g respectively (Table 2). The lowest plant growth parameters

were observed in the plants uprooted from untreated control plots. Yield parameters such as number tubers per plant, weight of tubers and yield was also influenced by nematode suppression that was achieved by the application of *P. penetrans* OD formulation with a lowest root gall index (RKI) of 2.0 (Table 3). Highest root gall index was observed in plants that was maintained as untreated control. The relationship between nematode reproductive index and yield was ascertained by analysis ($r = -0.98$) (Fig. 4). The tuber yield was increased by 21.5 per cent by the application of *P. penetrans* OD formulation when compared to untreated control.

Table 2

Effect of OD formulation of *P. penetrans* on growth of medicinal coleus, *C. forskholii* under field condition (Season I)

Treatments	Shoot length(cm)	Shoot weight (g)	Root length (cm)	Root weight (g)	Numbers of tubers	Weight of tubers (g)	Yield (tn / ha)
T1 - <i>P. penetrans</i> OD formulation @ 2 L / ha	45.16	60.33 ^a	23.83 ^a	40.92	6.50 ^a	39.95 ^a	18.1
T2 - Carbofuran 3G 1kg a.i / ha (33.3 kg / ha)	41.30	42.54 ^c	19.33 ^a	30.85	4.30 ^b	17.00 ^b	16.6
T3 – Control (Untreated)	38.16	46.36 ^b	10.16 ^b	29.30	3.50 ^c	9.91 ^c	14.2
CD (p = 0.05)	NS	0.02	0.13	NS	0.18	0.16	2.82

*Figures in parentheses are log transformed values. In a column, means followed by a common alphabet are not significantly different from each other at 5% level by DMRT.

Table 3
Effect of OD formulation of *P. penetrans* against *M. incognita* infestation in medicinal coleus, *C. forskholii* under field conditions (Season I)

Treatments		Initial nematode population	Final nematode population at harvest	Number of galls / 5g of root	RKI	Number of egg masses / 5g of root	Rf value	Number of infested females / 5g of root
T1	<i>P. penetrans</i> OD formulation @ 2 L / ha	272.5 (16.5)	171.2 (13.0)	15.16 ^a (3.86)	2.0	8.83 ^a (3.23)	0.63	8.6 (1.5 X 10 ⁶ /female)
T2	Carbofuran 3G 1kg a.i / ha (33.3 kg / ha)	281.2 (16.6)	223.2 (14.9)	21.50 ^b (4.59)	2.5	15.83 ^b (3.55)	0.80	-
T3	Control (Untreated)	275.4 (16.8)	366.8 (19.2)	38.83 ^c (6.2)	5.0	35.16 ^c (5.82)	1.33	-
CD (p = 0.05)		NS	0.43	0.72		0.99		-

*Figures in parentheses are square root transformed values. In a column, means followed by a common alphabet are not significantly different from each other at 5% level by DMRT.

Season II

Results obtained from season II had a similar trend as that of season I. Plant growth parameters were also significantly higher in the plants received OD formulation of *P. penetrans* which showed a root length of 25 cm with 42.8g of tuber weight that were better than the untreated control (Table 4). Similarly yield was also 16.7 per cent higher when compared to untreated control. Application of OD formulation reduced soil and root nematode infection to a tune of 55.6 and 67 per cent over untreated control. Same treatment also showed reduction in egg mass production (Table 5). A negative correlation existed between nematode reproductive index and yield ($r = -0.99$) (Fig. 5).

Table 4

Bioefficacy of oil OD formulation of *P. penetrans* on growth of medicinal coleus, *C. forskholii* under field condition (Season II)

Treatments	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Root weight (g)	Numbers of tubers	Weight of tubers (g)	Yield (tn / ha)
T1- <i>P. penetrans</i> OD formulation @ 2 L / ha	49.3	64.8 ^a	25.0 ^a	42.8 ^a	8.0 ^a (0.78)	18.1 ^a	19.1 ^a
T2 - Carbofuran 3G 1kg a.i / ha (33.3 kg / ha)	43.2	49.2 ^c	20.3 ^a	34.3 ^b	4.0 ^b (0.43)	14.6 ^b	17.3 ^b
T3 Control (Untreated)	39.2	44.0 ^b (1.65)	12.2 ^b	32.0 ^c	3.0 ^c (0.15)	12.2 ^c	15.9 ^c
CD (p = 0.05)	1.71	2.21	8.25	1.25	0.24	1.52	1.25

Figures in parentheses are log transformed values. In a column, means followed by a common alphabet are not significantly different from each other at 5% level by DMRT.

Table 5

Effect of OD formulation of *P. penetrans* against *M. incognita* infestation in medicinal coleus, *C. forskholii* under field conditions (Season II)

Treatments	Initial nematode population	Final nematode population at harvest	Number of galls / 5g of root	RKI	Number of egg masses / 5g of root	Rf value	Number of infested females / 5g of root
T1 - <i>P. penetrans</i> OD formulation @ 2 L / ha	272.5 (12.6)	159.5 ^a (2.23)	11.3 ^a (3.7)	2.0	6.83 ^a (2.61)	0.58	10.2 (1.5 X 10 ⁶ spores / female)
T2 - Carbofuran 3G @ 1kg a.i / ha (33.3 kg / ha)	281.2 (14.7)	271.3 ^b (2.35)	18.2 ^b (4.26)	2.3	16.83 ^b (4.10)	0.96	-
T3 - Control (Untreated)	275.4 (18.9)	359.4 ^c (2.56)	33.8 ^c (4.82)	4.4	45.17 ^c (6.72)	1.31	-
CD (p = 0.05)	NS	0.30	0.21		0.62		-

*Figures in parentheses are log transformed values. In a column, means followed by a common alphabet are not significantly different from each other at 5% level by DMRT.

Discussion

The results of the above investigation confirmed that the oil dispersion formulation of *P. penetrans* is very effective in suppressing nematode infection. The probable reasons for nematode reduction are discussed below.

The OD formulation very effective due to the fact that the oil OD formulation only took one day to degrade completely in soil and had a faster mode of action in soil than other formulations. This was in accordance with Weibelzahl-Fulton et al. (1996) who tested the efficacy of *P. penetrans* against *M. incognita* and *M. javanica* in tobacco plant. The biocontrol potential of *P. penetrans* proved by several authors (Mukhtar et al., 2013; Kamran et al., 2014; Kumari, 2017) in various crops.

Tzortzakakis et al. (1997) conducted the interaction study between *P. penetrans* and *M. incognita* under pot culture condition. The spore load was sufficient in females and successfully controlled the *M. incognita*. Chaudhary and Kaul (2013) evaluated the efficacy of *P. penetrans* and various oil seed cakes in the management of *M. incognita* in chilli and pepper (*Capsicum annum*). Combination of castor oil cake and *P. penetrans* showed greater reduction in galling index (84.75%) and final population of *M. incognita* was also reduced (85.74%) over control than other treatments.

The yield parameters were higher in OD formulation treated plants that is supported by the findings of These observations were similar with the efficacy test of *P. penetrans* on *M. incognita* reproduction in tomato which was documented by Kamran et al. (2014). Vagelas and Leontopoulos (2022) reported that mobility of nematodes was arrested and capability to locate host was affected when the nematodes encumbered with spores of *P. penetrans* and failure of reproductive organs in adult nematodes which lead to reduce production of next generation of nematode.

Tuber yield was higher in OD treated plants compared to untreated plants that was attributed by the suppression of nematode population. These observations were is in accordance with the findings of Jena et al. (2017) who has reported that the combined application of *P. penetrans*, *Glomus fasciculatum* and *T. asperellum* decreased the population of root-knot nematode and increased the growth of black pepper when compared to carbofuran 3G.

In conclusion, the findings of above study demonstrated that the oil OD formulation of *Pasteuria penetrans* is considered to be an efficient formulation to contain nematode infestation in medicinal coleus. The endopores of this bacterium multiplies in nematode female body that releases into the soil. These spores restrict the development next generation of nematodes. Hence, application of this bacterium could protect in future crop from nematode attack.

Declarations

Authorship contribution

Swarnakumari N. She has standardized the protocol for preparation of OD formulation of *Pasteuria penetrans*. She has executed the field trials and completed the observations related to nematode infection process.

Geethalakshmi. I. She was involved in recording all plant biometric characters such as shoot weight, length and tuber yield.

Srishalini. S. She has conducted pot culture experiment. She has maintained the pure culture of *P. penetrans* in the nematode host, *Meloidogyne incognita* (race 3).

Jothi. G. She was involved in drafting of the manuscript and data analysis.

Author Contribution

The corresponding author has designed the whole research work. She also standardised the protocol for the preparation of formulation. She has assessed the nematode infection level also.

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Figures

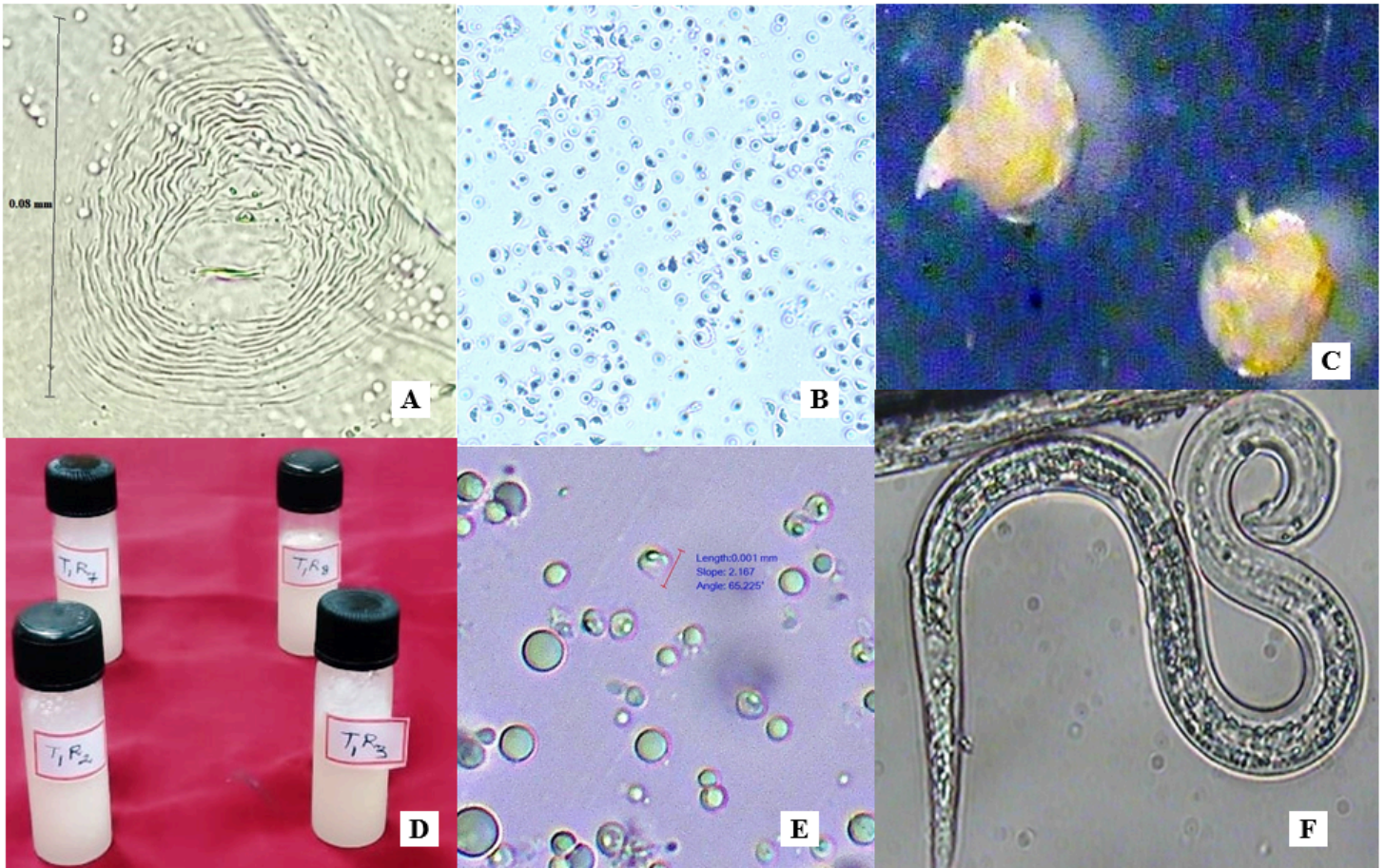


Figure 1

A. Posterior cuticular pattern of *Meloidogyne incognita* female nematode; **B** - Endospores of *P. penetrans*; **C** - OD formulation of *P. penetrans*; **D** - Endospores trapped in the formulation; **E**- Endospores infested female; nematodes; **F**- Attachment of endospores on J2



Figure 2

Effect of OD formulation of *P. penetrans* against *M. incognita* infection in medicinal coleus; A - Untreated; B - Treated

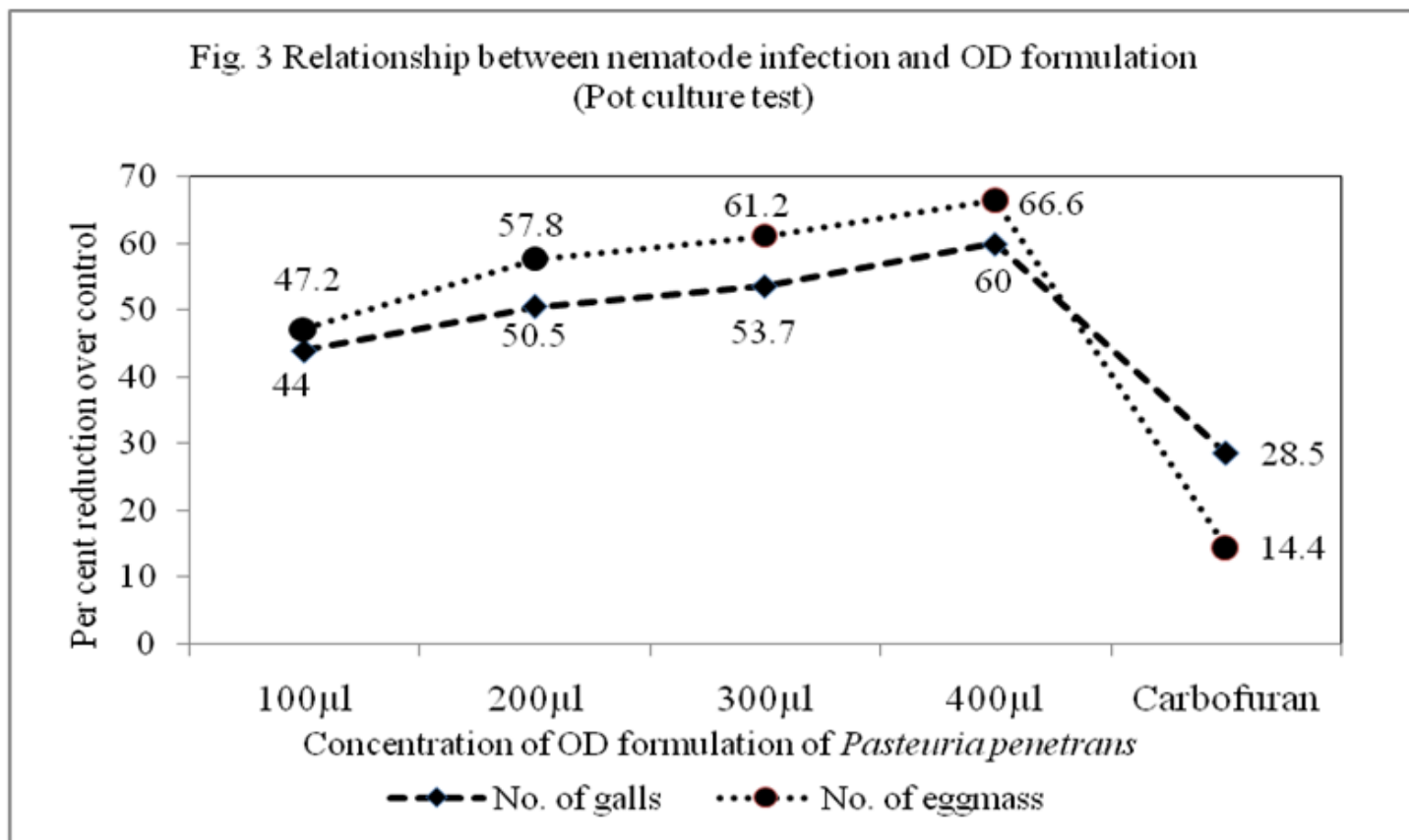


Figure 3

See image above for figure legend

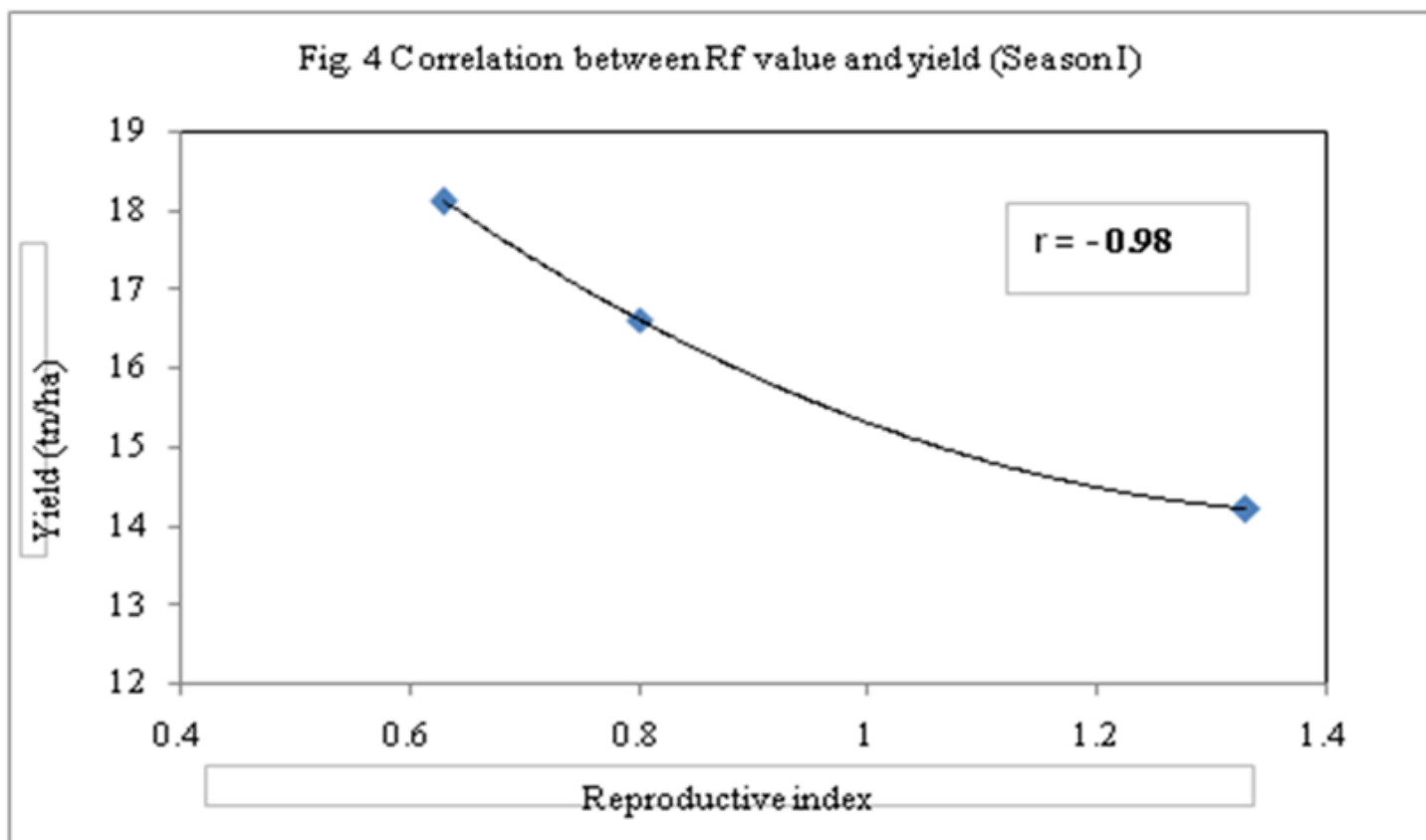


Figure 4

See image above for figure legend

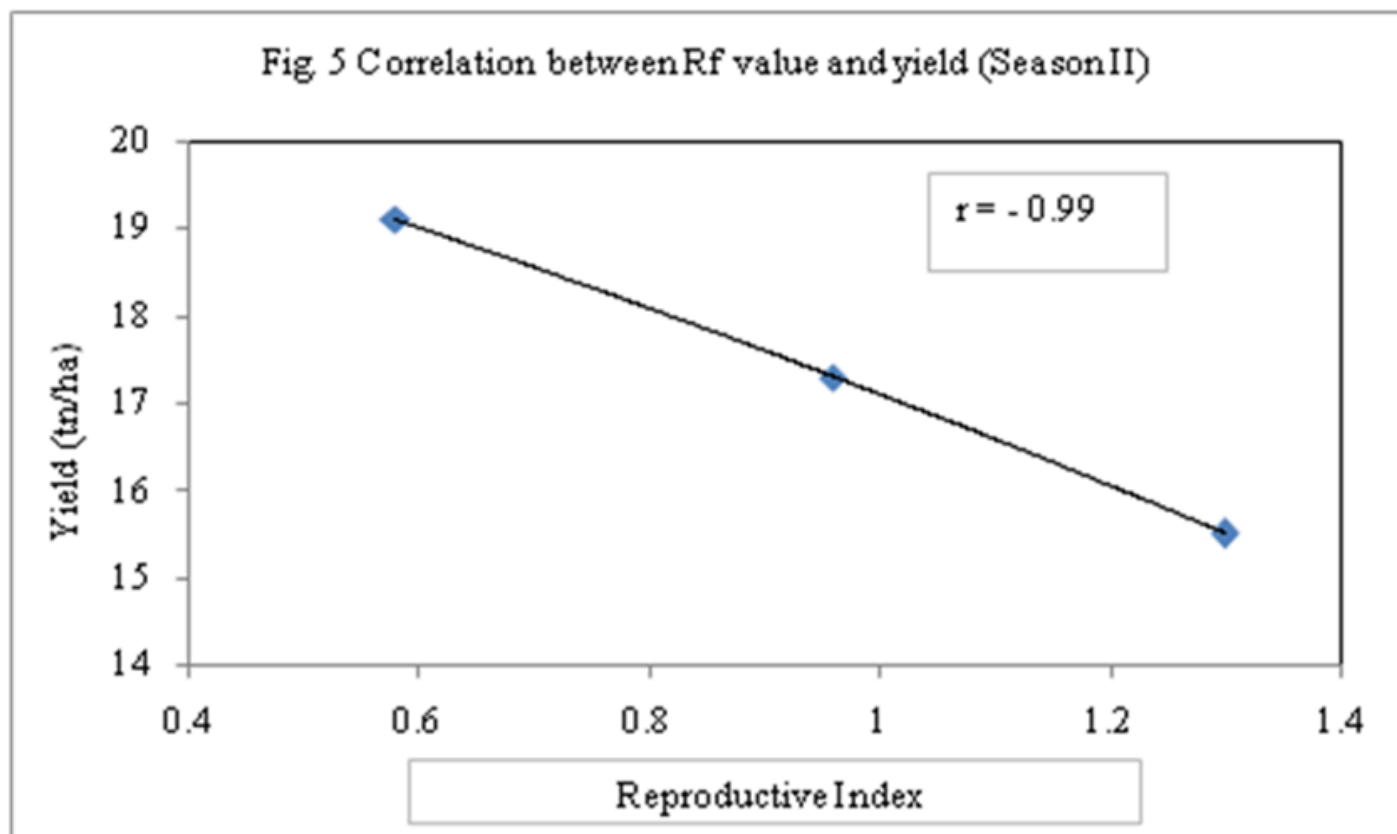


Figure 5

See image above for figure legend