Acta Phytopathologica et Entomologica Hungarica 56 (2021) 2, 111–131
DOI: 10.1556/038.2021.00126

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Effects of *Meloidogyne incognita, Pseudomonas syringae* pv. *pisi* and *Rhizobium leguminosarum* inoculated alone, simultaneously, and sequentially, on the growth and biochemical parameters of pea (*Pisum sativum*) in three soil types

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RESEARCH ARTICLE

Received: July 10, 2021 • Revised manuscript received: August 2, 2021 • Accepted: October 13, 2021
Published online: November 10, 2021
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ABSTRACT

Effects of *Meloidogyne incognita*, *Pseudomonas syringae* pv. *pisi* and *Rhizobium leguminosarum* were studied on growth and biochemical parameters of pea (*Pisum sativum* L.) in three soil types. Plants grown in 20% fly ash attained higher plant growth, chlorophyll and carotenoid followed by loam soil and 20% sand. Inoculation of *R. leguminosarum* resulted in increased plant growth, nodulation, chlorophyll and carotenoid over control. Root nodulation and proline contents were high in plants grown in 20% sand and least in 20% fly ash. Inoculation of *M. incognita* prior to *P. syringae* pv. *pisi* resulted in a greater reduction in plant growth, nodulation, chlorophyll and carotenoid content and least where *P. syringae* pv. *pisi* was inoculated prior to *M. incognita*. Inoculation of pathogens increased proline contents. Galling and population of *M. incognita* was high in 20% sand followed by loam soil and fly ash amended soil. *P. syringae* pv. *pisi* and *R. leguminosarum* had adverse effect on galling and nematode population. The principal component analysis identifies interaction of pathogens and showed segregation of various treatments in the plots.

KEYWORDS

bacterial blight, fly ash, loam, *Pisum sativum*, root nodule bacterium

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INTRODUCTION

Pea (*Pisum sativum* L.), is an herbaceous annual plant that belongs to Fabaceae family. It is a cool season crop and grown in many parts of the world. Peas are used as a fresh or canned vegetable, animal fodder and consumed as split dry pulse or dal. It contains fiber, protein, starch, trace elements, and many phytochemical substances (Rungruangmaitree and Jiraungkoorskul, 2017). Pea has the ability to fix atmospheric nitrogen with their root nodule bacteria and its seeds are high in fibers, vitamins and important minerals (Harmankaya et al., 2010).

Pests and pathogens are generally constraints in the successful cultivation of pea. *Pseudomonas syringae* pv. *pisi* is an important pathogen responsible for bacterial blight disease of pea (Schmit et al., 1992). Infection of this bacterium causes water-soaked spots which may coalesce into larger lesions. Symptoms may present on all aerial plant parts, but are most characteristic on stems and leaves (Martín-Sanz et al., 2013). Severe infection may cause plants to wither and die. However, *Meloidogyne incognita* is important plant parasitic nematode that parasitize pea and cause root galling (Gill, 1989). In India, *M. incognita* is a serious pathogen of pea and problem for its successful cultivation. Crop losses to pea attributed to root-knot nematodes range between 20 and 50% (Reddy, 1985; Upadhyay and Dwivedi, 1987; Sharma, 1989). Plant roots infected with *M. incognita* show poor growth, symptoms of chlorosis and root galling.

Rhizobium Frank is Gram negative soil bacterium and forms its association with roots of legumes. Symbiotic process between host plants and *Rhizobium* sp. fix atmospheric nitrogen. Inoculation of *Rhizobium* sp. has positive effect on growth attributes, yield components and quality of pea. Presence of *Rhizobium* sp. may also be useful in reducing damage by plant pathogens (Siddiqui and Mahmood, 2001). Application of rhizobial isolates significantly suppress various plant diseases (Parveen et al., 2019).

Soil biology is a major component and contributes significantly to the quality and productivity of pea cultivation. The major activities of soil microorganisms include decomposition of organic matter, nutrient mineralization, crop pest's suppression and its protection. Fly ash is a residue of burning of coal/lignite in thermal power plant has traditionally been considered as a waste product. The high concentration of elements (K, Na, Zn, Ca, Mg and Fe) of fly ash increases the yield of many agricultural crops (Basu et al., 2009). Addition of 25% fly ash in soil causes a significant increase in the growth, chlorophyll and carotenoid contents of eggplant over plants grown without fly ash (Khan and Siddiqui, 2017). Similarly, carrot grown in sand mix soil showed a significant increase in root dry weight, chlorophyll and carotenoid contents (Ahmad and Siddiqui, 2017).

More than one pathogen is sometimes involved in plant diseases are commonly termed as a "complex". Such diseases involve a wide range of microbial interactions and can increase disease severity. Therefore, different pathogens may lead to more severe disease symptoms often than expected (Begon et al., 2006). The plant is infected with one pathogen its response to other pathogen is altered and these alterations exert significant influences upon disease development, etiology of pathogens involved (Siddiqui et al., 2012). Such interactions in plants are crucial for the understanding of microbial pathogenesis, evolution and for effective disease control strategies.

During the course of survey of pea fields of Aligarh district U.P., India we found simultaneous and concomitant occurrence of *M. incognita* (Kofoid and White) Chitwood and *P. syringae* pv. *pisi* (Sackett) Young, Dye and Wilkie in the pea fields. Plant roots with *M. incognita* had galling while blight symptoms were found in plants with *P. syringae* pv. *pisi.* Presently, not much information is available related to the association of *M. incognita* and *P. syringae* pv. *pisi* on pea. Therefore, an experiment was performed to find out the interactions of *M. incognita, P. syringae* pv. *pisi* and *R. leguminosarum* in three soil types on pea.

MATERIAL AND METHODS

Soil preparation

The physico-chemical characters of the soils used in the treatments were determined before sowing seeds (Table 1). Loam soil was obtained from a field belonging to Department of Botany, Aligarh Muslim University, Aligarh, India, and sand from a nearby river. Sand: loam soil was mixed in 20:80 (v/v) and autoclaved at 137.9 kPa for 20 min. Similarly, fly ash was dried in the sun for 10 days while field soil which was collected and autoclaved at 137.9 kPa for 20 min. Later, fly ash and loam soil were mixed in 20:80 ratio (v/v). Clay pots (15 cm dia.) were filled with 1.0 kg soil (20% fly ash with 80% loam soil, 20% sand with 80% loam soil and 100% loam soil) with 3 types separately. Soil samples were passed through a 2 mm sieve before analyses and the following properties were determined: porosity and water holding capacity by hydrometry; pH, conductivity and cation exchange capacity (CEC) using soil: distilled water in pH and conductivity meters; and sulphur content determined. Organic carbon was estimated following Nelson and Sommers (1972), zinc, manganese, copper, and iron were determined following Chopra and Kanwar (1982); nitrogen was determined with the Kjeldahl digestion (Nelson and Sommers, 1972); and phosphorus by phosphomolybdic blue colorimetry (Jackson, 1958).

Table 1. Physico-chemical characteristics of soil mixes

Characteristics	Types of soil			L.S.D. $P = 0.05$
	Loam	20% sand	20% fly ash	
pH	5.9c	6.9a	6.4b	0.35
EC (mmho·cm^{-1})	0.997b	0.921c	1.142a	0.62
Water holding capacity (%)	47.2b	36.8c	50.1a	3.1
Organic carbon (%)	0.52a	0.40b	0.43b	0.04
N (kg·ha^{-1})	201.4a	172.2c	181.3b	0.42
P (kg·ha^{-1})	18.0a	16.4b	15.1c	0.6
K (kg·ha^{-1})	220.05b	165.9c	328.16a	8.2
S (mg·kg^{-1})	10.86b	10.12c	12.88a	0.5
Zn (mg·kg^{-1})	5.82a	3.51c	3.84b	0.22
Fe (mg·kg^{-1})	4.80a	4.20c	4.37b	0.13
Mn (mg·kg^{-1})	2.71c	2.80b	2.81a	0.07
Cu (mg·kg^{-1})	0.42b	0.34c	0.51a	0.5

Values within one within characteristic followed by same letter are not significantly different at $P = 0.05$

Sowing and maintenance of test plants

Pea seeds (cv. BK-10) were dipped in 0.1% NaOCl solution for 1 min for surface sterilization and later washed three times with sterile distilled water. Five seeds were sown in clay pots (15 cm dia.) having 1 kg sterilized soil. Plants were thinned to 1/pot and seedling was inoculated with pathogens one week after emergence as shown in Table 3. Pots were kept at 20°C on a glass-house bench. Each of these pots was watered with 100 mL tap water daily.

Inoculum of the root-knot nematode

Pea roots were used for the collection of *M. incognita* egg masses. *M. incognita* was multiplied on the roots of *Solanum melongena* L. from an egg mass. Eggplant roots having high infection of *M. incognita* were used for hand-picking of large numbers of egg masses with sterilized forceps. The egg masses were washed with sterilized distilled water and placed in small sieves (1-mm pore) with crossed layers of tissue paper. Sieve was placed in a petri plate having sufficient distilled water so that egg masses may remain in contact with water. These petri-plates having sieves were kept in an incubator at 25 ± 1 °C to obtain the required *M. incognita* J_2 for inoculation. The hatched J_2 were collected after every 24 h from the petri plates. Fresh water was poured in each petri-plate and the process for collection of hatched juveniles was repeated. Mean of 5 counts was taken to determine the density of J_2 in the suspension. Each mL of nematode suspension may contain 200 ± 5 J_2 was so adjusted by adding or decanting water. Freshly hatched 2000 J_2 (10 mL suspension) was poured in the rhizosphere of around a pea seedling as per the treatments in each pot.

Inoculum of the pathogenic bacterium

For obtaining sufficient inoculum, nutrient agar medium was used for the culture of *P. syringae* pv. *pisi*. A pure colony of *P. syringae* pv. *pisi* was streaked under aseptic condition on sterilized petri dishes with nutrient agar medium and placed for 24 h at 30 ± 1 °C in incubator. Single colony from freshly cultured plate of *P. syringae* pv. *pisi* was separately inoculated into nutrient broth flasks and incubated for 72 h at 30 ± 1 °C. Cell density of *P. syringae* pv. *pisi* was estimated by Sharma (2005), its each mL contains 1.2×10^5 colony-forming units (CFU).

Inoculum of the symbiotic bacterium

Charcoal culture of *R. leguminosarum* (pea strain, 100 g) was dissolved in 1 liter sterilized distilled water. Thinning of seedlings was done before inoculation. One g inoculum (10 mL suspension) was used for inoculation of each seedling per pot.

Inoculation techniques

For inoculations of *M. incognita*, *P. syringae* pv. *pisi* and *R. leguminosarum* sterilized forceps were used carefully to remove soil around the roots by avoiding damage to roots. The inoculum suspensions were poured in the rhizosphere of around each seedling and soil was placed again. In control treatments, sterile water of equal volume was poured around the seedling in the same way.

Inoculation of pea

The experiment was performed in a Completely Randomized Design (CRD) in three soil types i.e. loam soil, 20% fly ash mixed soil and 20% sand mixed soil. Pathogens i.e. *M. incognita* and *P. syringae* pv. *pisi* were inoculated in 6 combinations i. e. 1. Control (C); 2. *M. incognita* (M); 3. *P. syringae* pv *pisi* (P); 4. *M. incognita* inoculated simultaneously with *P. syringae* pv. *pisi* (M+P); 5. *M. incognita* inoculated 20 days prior to *P. syringae* pv. *pisi* (M→P); 6. *P. syringae* pv. *pisi* inoculated 20 days prior to *M. incognita* (P→M) ($6 \times 3 = 18$ treatments). These 18 treatments were tested without *R. leguminosarum* and with *R. leguminosarum* ($18 \times 2 = 36$ treatments). Each treatment had 5 replications i.e. $36 \times 5 = 180$ pots. Experiment was performed in two years i.e. 2018 and 2019, and pooled data of both years are presented.

Observations

Ninety days after pathogen inoculation harvesting was done. Observation data were recorded on plant length, plant fresh weight, plant dry weights, chlorophyll, carotenoid, proline, bacterial blight index, galling and nematode population. For recording plant length, length was measured in cm from the first leaf to end of the root. Before weighing, the extra water of plants was blotted with blotting sheets for recording fresh weight. The shoots and roots were separated with a knife above the base of the root. Shoots and roots were placed in an incubator at 60 °C for 2–3 days before weighing for dry weights. Well-mixed soil of each treatment (250 g subsample) was processed for isolation of nematodes using Cobb's sieving and decanting technique and Baermann funnel extraction (Southey, 1986). Nematode suspension was collected after 24 h and counting of nematodes was done under binocular microscope from 1 mL of suspension from each sample. The mean of 5 counts were taken to calculate the nematodes population per kg soil. Root tissues (1 g subsample) were homogenized in a Waring blender. Numbers of J_2, eggs and females inside the roots were counted from the suspension obtained from roots. Numbers of nematodes present in 1 g of root were multiplied by the weight of root in order to calculate total nematodes present in the root.

Disease index

Scoring of disease severity was done on the observations of disease symptoms on leaves (Nesha and Siddiqui, 2013). Rating was done for recording blight disease index on a scale 0–5 where 0 = No disease and 5 = Severe blight.

Chlorophyll and carotenoid estimation

Estimation of chlorophyll and carotenoid was done by Mackinney (1941). One g freshly cut leaves was ground in 20 cm^3 of 80% acetone using a mortar and pestle. The mixture was centrifuged at 5,000 rpm for 5 min and supernatant was collected in 100 cm^3 volumetric flask. Washing of residue was done thrice with 80% acetone, collected in the same volumetric flask and volume was made with 80% acetone up to mark. Spectrophotometer Shimadzu UV-1700, Tokyo, Japan was used to read absorbance at 645 and 663 nm for chlorophyll and 480 and 510 nm for carotenoid against the blank (80% acetone).

Proline estimation

Method suggested by Bates et al. (1973) was used for proline content estimation in fresh leaves. Leaf sample (300 mg) was homogenized in 3 mL (3% sulphosalicylic acid). The filtrate was reacted with ninhydrin and glacial acetic acid (1 mL each) for 1 h in a test tube at 100 °C in a warm water bath. The sample was transferred to ice bath, mixture was extracted with toluene and was read at 520 nm using L-proline as a standard.

Statistical analysis

The data obtained were analyzed using 3-way ANOVA i.e. *R. leguminosarum* × Types of soil × Pathogen. DNMRT (Duncan's New Multiple Range Test) was used to denote significant differences between treatments. Graphs of nematode population and number of galls/per root system were prepared using Sigma Plot™ and error bars represent standard error. The principal components analysis (PCA) was used to determine the total variability of data using Origin Pro 2021.

RESULTS

Three-way analysis of variance (ANOVA) revealed that effect of soil types, pathogen, and *R. leguminosarum* and their interactions on plant length, plant fresh weight, shoot dry weight, root dry weight and chlorophyll were significant at $P = 0.05$ (ANOVA not shown). Similarly, effect of soil types, pathogen, and *R. leguminosarum* and their interactions on root nodulation and no. of galls per root system were significant at $P = 0.05$ except interaction of *R. leguminosarum* × Soil types × Pathogens. Individual effect of soil types, pathogen, and *R. leguminosarum* were significant on nematode population and proline contents at $P = 0.05$ while effect of soil types, pathogen, and *R. leguminosarum* and interactions of *R. leguminosarum* × pathogens were significant on carotenoid contents at $P = 0.05$ (ANOVA not shown).

Inoculation of *R. leguminosarum* resulted in increased plant growth parameters (Plant growth, plant fresh weight, shoot and root dry weights) nodulation, chlorophyll and carotenoid contents over uninoculated control (Table 2). Inoculation of *R. leguminosarum* resulted in non-significant effect on proline content over uninoculated control. Plants grown in 20% fly ash amended soil attained higher plant growth, chlorophyll and carotenoid contents followed by loam soil and 20% sand amended soil. However, root nodulation and proline contents were high in 20% sand amended soil followed by loam soil and 20% fly ash amended soil. Inoculation of *M. incognita* caused a greater reduction in plant growth parameters, nodulation, chlorophyll and carotenoid contents than by *P. syringae* pv. *pisi*. Inoculation of pathogens alone and in combination increased proline contents over uninoculated control. Inoculation of *M. incognita* prior to *P. syringae* pv. *pisi* caused highest reduction in plant growth parameters, nodulation, chlorophyll and carotenoid contents followed by simultaneous inoculation of both pathogens and inoculation of *P. syringae* pv. *pisi* prior to *M. incognita*. Inoculation of *M. incognita* 20 days prior to *P. syringae* pv. *pisi* resulted in highest increase in proline contents (Table 2).

Table 2. Effect of types of soil, pathogens and *R. leguminosarum* on the growth, chlorophyll, carotenoid and proline contents of pea

Treatments	Plant length (cm)	Plant fresh weight (g)	Soot dry weight (g)	Root dry weight (g)	No. of nodules/root system	Chlorophyll content (mg/g FW)	Carotenoid content (mg/g FW)	Proline content (µmol/g FW)
Rhizobium								
Control	42.00b	4.43b	1.35b	0.08b	3.17b	0.388b	0.047b	0.170a
R. leguminosarum	55.92a	7.11a	1.78a	0.12a	22.50a	0.507a	0.058a	0.168a
L.S.D. *P* = 0.05	0.50	0.10	0.01	0.005	0.49	0.009	0.002	0.006
Soil type								
Loam soil	46.81b	5.36b	1.58b	0.10b	13.25b	0.471b	0.053b	0.171b
20% Sand	44.53c	4.81c	1.12c	0.07c	16.08a	0.387c	0.047c	0.184a
20% Fly ash	55.53a	7.14a	1.99a	0.13a	9.17c	0.484a	0.058a	0.151c
L.S.D. *P* = 0.05	0.62	0.13	0.01	0.006	0.61	0.012	0.002	0.007
Pathogens								
Control	65.55a	8.24a	2.17a	0.16a	19.67a	0.619a	0.079a	0.079e
M. incognita (M)	52.73c	6.12c	1.70c	0.10c	11.67c	0.477c	0.055c	0.152d
P. syringae pv. *pisi* (P)	55.06b	6.60b	1.81b	0.12b	13.50b	0.506b	0.059b	0.143d
M+P	40.13e	4.61e	1.25e	0.07e	11.17d	0.363e	0.041e	0.211b
M→P	36.98f	3.92f	0.99f	0.06f	9.33e	0.328f	0.035f	0.242a
P→M	43.30d	5.12d	1.47d	0.08d	11.67d	0.392d	0.045d	0.186c
L.S.D. *P* = 0.05	0.88	0.18	0.02	0.008	0.86	0.017	0.003	0.010

Data were analyzed by least square mean (LSM); values within a column and one type of treatment followed by the different letter are significantly different at $P = 0.05$ by DNMRT (Duncan's New Multiple Range Test); → = second inoculation of pathogen 20 days after first inoculation; + = simultaneous inoculation.

Effect on plant growth

Inoculation of *M. incognita* and *P. syringae* pv. *pisi* resulted in a significant reduction in plant growth parameters both in *R. leguminosarum* inoculated and uninoculated plants in all the three soil types (Table 3). Inoculation of *M. incognita* 20 days prior to *P. syringae* pv. *pisi* caused a higher reduction in plant growth followed by simultaneous inoculation of both pathogens and inoculation of *P. syringae* pv. *pisi* prior to *M. incognita* in all the three soil types. Inoculation of *R. leguminosarum* increased plant growth in all the three soils types in both pathogens inoculated and uninoculated plants. Plant growth was better in fly ash amended soil followed by loam soil and sand amended soil (Table 3).

Root nodulation

Nodulation was very poor in plants without *R. leguminosarum* (Table 3) while plants with *R. leguminosarum* has high root nodulation. Highest nodulation was observed in plant with *R. leguminosarum* grown with 20% sand mix soil and least in fly ash mixed soil. Nodulation caused by *R. leguminosarum* was decreased in plants with pathogens. Highest reduction in root nodulation caused by *R. leguminosarum* was observed where *M. incognita* was inoculated prior to *P. syringae* pv. *pisi* followed by simultaneous inoculation of both pathogens and inoculation of *P. syringae* pv. *pisi* prior to *M. incognita* (Table 3).

Effect on chlorophyll and carotenoid content

Chlorophyll and carotenoid were found greater in plants grown in 20% fly ash amended soil followed by loam soil and 20% sand amended soil (Table 4). Inoculation of *R. leguminosarum* increased chlorophyll and carotenoid contents over plants without *R. leguminosarum.* However, inoculation of *M. incognita* or *P. syringae* pv. *pisi* caused significant reduction in chlorophyll and carotenoid contents over control. Combined, pre and post inoculations of *M. incognita* and *P. syringae* pv. *pisi* caused a greater reduction in chlorophyll and carotenoid content than inoculation of either of them singly (Table 4).

Effect on proline content

Inoculation of *M. incognita*/*P. syringae* pv. *pisi* caused increase in proline content over uninoculated plants (Table 4). Proline content was found greater in plants with *M. incognita* plus *P. syringae* pv. *pisi* than caused by them alone. Plants grown in 20% sand amended soil had greater proline contents followed by loam soil and 20% fly ash amended soil. Inoculation of *R. leguminosarum* in plants with pathogens and soil types had no significant effect on proline contents (Table 4).

Effects on root galling and nematode multiplication

Root galling and multiplication of *M. incognita* was high when alone (Figs 1 and 2). Galling and multiplication of *M. incognita* was high in 20% sand amended soil followed by loam soil and least in fly ash amended soil. *P. syringae* pv. *pisi*/*R. leguminosarum* had adverse effect on galling and nematode multiplication while inoculation of both together had greater adverse effect than by them alone. Inoculation of *P. syringae* pv. *pisi* prior to *M. incognita* had greater adverse effect on galling and nematode multiplication followed by simultaneous inoculation of both pathogens and inoculation of *M. incognita* prior to *P. syringae* pv. *pisi* (Figs 1 and 2).

Table 3. Effect of interactions *Meloidogyne incognita* and *Pseudomonas syringae* pv. *pisi* on the growth attributes and nodulation of pea in the presence and absence of *Rhizobium leguminosarum* in three types of soil

	Soil type	Pathogens	Plant length (cm)	Plant fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)	No. of Nodules/root system
Without *R. legumnosarum*	loam soil	C	56.78fg	6.68ef	1.96h	0.13de	6l
		M	45.46l	4.60lm	1.57k	0.09ghi	3m
		P	47.23kl	5.12jk	1.66j	0.10fgh	4lm
		M+P	32.74q	3.42q	1.14op	0.05klm	4lm
		M→P	30.41r	2.86s	0.72s	0.04lm	2m
		P→M	36.23p	3.94nop	1.43l	0.07ijk	2m
	20% sand amended soil	C	49.12jk	5.61hi	1.44l	0.09ghi	6l
		M	39.28o	4.16mno	0.98qr	0.06jkl	3m
		P	42.54m	4.54lm	1.19o	0.07ijk	4lm
		M+P	32.61q	2.92rs	0.78s	0.04lm	3m
		M→P	28.64r	2.38t	0.63t	0.03m	2m
		P→M	36.17p	3.37qr	0.91r	0.05klm	3m
	20% fly ash amended soil	C	61.52e	7.15e	2.23d	0.16bc	4lm
		M	50.13j	5.34ij	1.86i	0.10fgh	2m
		P	52.36i	5.87gh	1.98gh	0.12ef	2m
		M+P	38.32op	3.97nop	1.30n	0.07ijk	2m
		M→P	36.24p	3.56pq	0.98qr	0.06jkl	2m
		P→M	40.16no	4.22mn	1.58k	0.08hij	3m
With *R. leguminosarum*	loam soil	C	76.46b	9.93b	2.42c	0.17bc	36b
		M	63.17de	7.16e	2.04fg	0.12ef	21gh
		P	64.16d	7.84d	2.06f	0.13de	23fg
		M+P	36.42p	4.28mn	1.34mn	0.09ghi	20h
		M→P	33.28q	3.68opq	1.02q	0.08hij	17i
		P→M	39.32o	4.82kl	1.65j	0.11efg	21gh
	20% sand amended soil	C	69.62c	7.98d	1.80i	0.12ef	40a
		M	53.14hi	5.63hi	1.30n	0.08hij	28d
		P	56.23g	6.28fg	1.39lm	0.09ghi	31c
		M+P	42.04mn	4.99jkl	1.02q	0.05klm	25ef
		M→P	38.16op	4.15mno	0.94r	0.04lm	22gh

(continued)

Table 3. Continued

Soil type	Pathogens	Plant length (cm)	Plant fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)	No. of Nodules/root system
	P→M	46.82l	5.72hi	1.10p	0.07ijk	26de
20% fly ash amended soil	C	79.80a	12.10a	3.16a	0.28a	26de
	M	65.17d	9.85b	2.42c	0.15cd	13jk
	P	67.86c	9.97b	2.60b	0.18b	17i
	M+P	58.62f	8.09d	1.94h	0.11efg	13jk
	M→P	55.12gh	6.88e	1.62jk	0.10fgh	11k
	P→M	61.10e	8.67c	2.16e	0.12ef	15ij
L.S.D. $P = 0.05$ *R. leguminosarum* × Soil Types		0.88	0.18	0.02	0.008	0.86
L.S.D. $P = 0.05$ *R. leguminosarum* × Pathogens		1.24	0.26	0.03	0.012	1.22
L.S.D. $P = 0.05$ Soil types × Pathogens		1.53	0.32	0.04	0.015	1.49
L.S.D. $P = 0.05$ *R. leguminosarum* × Soil types × Pathogens		**2.16**	**0.45**	**0.06**	**0.02**	**2.11**

Values in a column followed by the same letter are not significantly different at $P \leq 0.05$ using Ducan's New Multiple Range Test (DNMRT); M = *M. incognita*; P = *P. syringae* pv. *pisi*; C = Control; → = second inoculation of pathogen 20 days after first inoculation; + = simultaneous inoculation.

Table 4. Effect of interactions *M. incognita* and *P. syringae* pv. *pisi* on the chlorophyll, carotenoid, proline contents and disease index of pea in the presence and absence of *R. leguminosarum* in three types of soil

Rhizobium	Soil type	Pathogens	Chlorophyll content (mg/g FW)	Carotenoids content (mg/g FW)	Proline content (μmol/g FW)	Blight index
Without *R. leguminosarum*	loam soil	C	0.521e	0.073cde	0.082m	–
		M	0.416ghijk	0.046jklmn	0.145hij	–
		P	0.452fg	0.050ijkl	0.140ij	3
		M+P	0.348mnop	0.038mnopq	0.207cde	5
		M→P	0.322p	0.034opq	0.234bc	5
		P→M	0.369klmnop	0.042klmnop	0.192efg	5
	20% sand amended soil	C	0.478ef	0.068defg	0.089lm	–
		M	0.364lmnop	0.042klmnop	0.161hi	–
		P	0.392jklm	0.046jklmn	0.148hij	3
		M+P	0.246q	0.032pq	0.232bc	5
		M→P	0.225q	0.029q	0.265a	5
		P→M	0.268q	0.037nopq	0.214cde	5
	20% fly ash amended soil	C	0.602cd	0.078bcd	0.074m	–
		M	0.441fghi	0.052hijk	0.151hij	–
		P	0.483ef	0.058ghi	0.144hij	3
		M+P	0.351mnop	0.043jklmnop	0.198def	5
		M→P	0.328op	0.034opq	0.208cde	5
		P→M	0.372klmno	0.049ijklm	0.174fgh	5
With *R. leguminosarum*	loam soil	C	0.724b	0.086ab	0.086lm	–
		M	0.636c	0.065efg	0.162hi	–
		P	0.640c	0.068defg	0.156hij	3
		M+P	0.402hijkl	0.045jklmno	0.210cde	5
		M→P	0.374klmno	0.039lmnopq	0.251ab	5
		P→M	0.445fgh	0.049ijklm	0.192efg	5
	20% sand amended soil	C	0.615c	0.079bc	0.079m	–
		M	0.436fghij	0.058ghi	0.165ghi	–
		P	0.472f	0.061fgh	0.158hij	3
		M+P	0.393ijklm	0.038mnopq	0.223bcd	5
		M→P	0.342nop	0.032pq	0.272a	5

(continued)

Table 4. Continued

Rhizobium	Soil type	Pathogens	Chlorophyll content (mg/g FW)	Carotenoids content (mg/g FW)	Proline content (µmol/g FW)	Blight index
		P→M	0.418ghijk	0.041klmnop	0.199def	5
	20% fly ash amended soil	C	0.776a	0.089a	0.062m	–
		M	0.569d	0.069cdef	0.128jk	–
		P	0.594cd	0.072cde	0.112kl	3
		M+P	0.437fghij	0.049ijklm	0.198def	5
		M→P	0.378klmn	0.044jklmno	0.220cde	5
		P→M	0.478ef	0.054hij	0.143ij	5
L.S.D. $P = 0.05$ *R. leguminosarum* × Soil Types			0.017	0.003	0.010	–
L.S.D. $P = 0.05$ *R. leguminosarum* × Pathogens			0.024	0.005	0.015	–
L.S.D. $P = 0.05$ Soil types × Pathogens			0.029	0.006	0.018	–
L.S.D. $P = 0.05$ *R. leguminosarum* × Soil types × Pathogens			**0.042**	**0.009**	**0.025**	–

Values in a column followed by the same letter are not significantly different at $P \leq 0.05$ using Ducan's New Multiple Range Test (DNMRT); M = *M. incognita*; P = *P. syringae* pv. *pisi*; C = Control; → = second inoculation of pathogen 20 days after first inoculation; + = simultaneous inoculation.

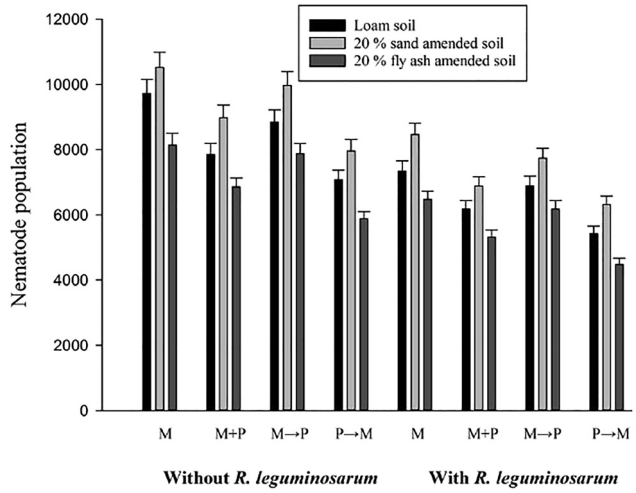

Fig. 1. Effect of *P. syringae* pv. *pisi* (P), *R. leguminosarum* and soil types on the population of *M. incognita* (M); + = Simultaneous inoculation; → = Inoculated 20 days after first inoculation

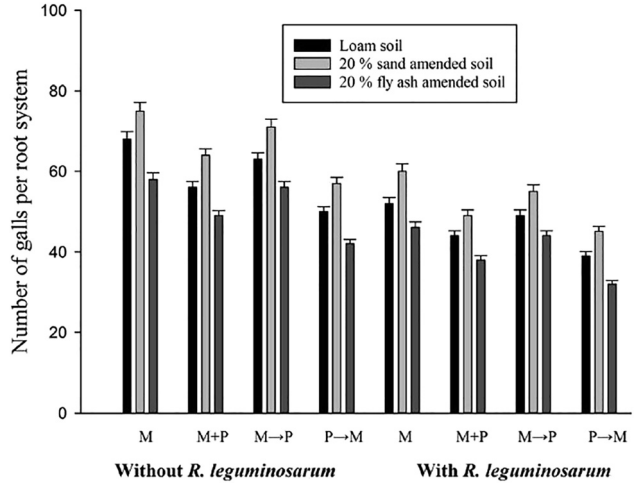

Fig. 2. Effect of *P. syringae* pv. *pisi* (P), *R. leguminosarum* and soil types on the galling of *M. incognita* (M); + = Simultaneous inoculation; → = Inoculated 20 days after first inoculation

Bacterial blight indices

Bacterial blight indices were 3 in plants with *P. syringae* pv. *pisi* in both with and without *R. leguminosarum* and grown in either soil types (Table 4). Similarly, bacterial blight indices were 5 in plants inoculated simultaneously or sequentially with both pathogens in either soil types both in the presence and absence of *R. leguminosarum* (Table 4).

Principal component analysis (PCA)

Interaction of test pathogens on various studied attributes were also analyzed through principal component analysis (PCA). Principal components showed 80.81% of data variability (PC1 = 70.01%; PC2 = 10.80%) (Fig. 3). Plant length, plant fresh weight, shoot and root dry weight, chlorophyll and carotenoid (clustered together) were positively correlated with root nodulation. Plant length, plant fresh weight, shoot and root dry weight, chlorophyll and carotenoids were negatively correlated with proline content, root galling and nematode population (Fig. 3). The PCA clearly identified the interaction of the test pathogens under study with various studied attributes as revealed from the segregation of different treatments in the plots. Treatments where *R. leguminosarum* was inoculated with 20% sand or loam soil were placed in the upper 2 plots while treatments without *R. leguminosarum* were placed in the lower 2 plots. Inoculation of *M. incognita* prior to *P. syringae* pv. *pisi* with *R. leguminosarum* with 20% sand was placed on the top left side of the plot. Similarly, treatment where *M. incognita* was inoculated prior to *P. syringae* pv. *pisi* with *R. leguminosarum* with 20% fly ash was placed on the right side of the plot near the center. Treatments where two pathogens were inoculated simultaneously or sequentially in 20% fly ash were generally cluster at the right side of lower plot (Fig. 3).

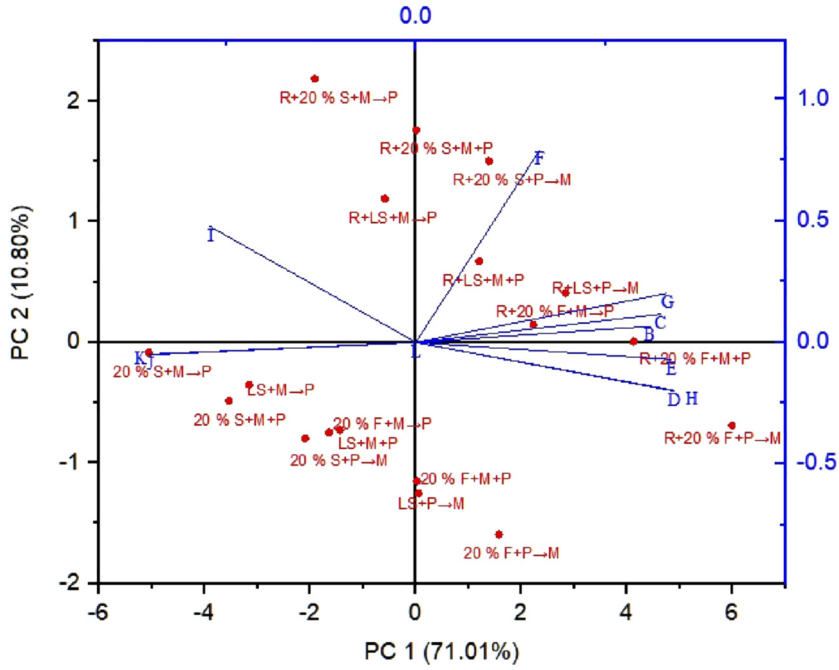

Fig. 3. Principal component analysis (PCA) showing the effect of interaction *M. incognita* and *P. syringae* pv. *pisi* in the presence and absence of *R. leguminosarum* in three types of soil. LS = Loam soil, 20% S = 20% Sand, 20% F = 20% Fly ash, M = *M. incognita*, P = *P. syringae* pv. *pisi*, and M+P = MP, R = *R. leguminosaram*, B = Plant lengh, C = Plant fresh weigh, D = Shoot dry weight, E = Root dry weight, F = Nodulation, G = Chloropyll, H = Carotenoid, I = Proline, J = root galling K = Nematode population

DISCUSSION

Inoculation of *M. incognita* or *P. syringae* pv. *pisi* resulted in reduced growth, chlorophyll and carotenoid content. Necrotic spots and streaks on above-ground parts of pea was caused by *P. syringae* pv. *pisi* were observed as reported earlier (Hagedorn, 1991). Severe infections on peduncles, flowers and pods of pea may result in withering, death of infected part or necrosis which reduce the assimilating surface. Therefore, reduced plant growth, chlorophyll and carotenoid of pea. However, parasitism by *M. incognita* involves the establishment of permanent feeding sites, the giant cells in the root cortex, endodermis, pericycle, and vascular parenchyma (Vovlas et al., 2005). Decrease in growth, chlorophyll and carotenoid content of pea were observed with parasitism of *M. incognita* on pea.

Soil characteristics play an important role in a plant's ability to grow to their potential. Pea prefers nutrient rich soil, preferably loam soil (Dhall, 2017). Fly ash is considered as a good soil and source of secondary plant nutrients as well as micronutrients and increase in porosity and water holding capacity (Panda and Biswal, 2018). Growth, chlorophyll and carotenoid contents of pea in 20% fly ash amended soil was good due to the availability of a greater amount of utilizable plant nutrients as revealed by the chemical analysis of the soil. A greater amount of potassium, sulphur, manganese and copper etc. were present in the fly ash amended soil were absorbed by the roots and utilized by the plant for proper growth of pea. The nutrients from fly ash have been reported to be beneficial for the plant growth and yield of rice (Sarangi et al., 1997), wheat, chickpea (Dubey et al., 1982) and tomato (Khan and Khan, 1996). The soil analysis of 20% sand mix soil indicated less nutrients. Therefore, pea grew in sand amended soil has least plant growth, chlorophyll and carotenoid contents. Plant grown in loam soil had intermediate growth because of availability of moderate amount of nutrients present.

Greater availability of nutrients in fly ash-amended soil enabled the plants to grow better and inhibit invasion of *M. incognita*. Higher uptake of boron and potassium etc. helped the plants in building natural defense against the nematode (Francois, 1984; Khan et al., 1997). Therefore, galling and nematode multiplication was reduced in fly ash amended soil than in sand amended soil. The substantial decline in the galling and nematode population indicates that the fly ash caused direct inhibitory effect on the survival and multiplication of *M. incognita*. Amendment of 20% fly ash had no apparent effect on the bacterial blight disease indices of pea. Moreover, there was no substantial decline in the soil population of *P. syringae* pv. *pisi* in fly ash amended soil indicates that the fly ash had no direct inhibitory effect on the survival and multiplication of the bacterial pathogen in the soil.

Multiplication and galling of *M. incognita* is often greater in sandy soils where nematodes can move more freely (Ravichandra, 2014). Adequate soil moisture is essential for free movement of nematodes. In the present study, plant growth and chlorophyll and carotenoid contents were affected by nematodes in soil mixes (Trudgill and Phillips, 1997). Mechanisms by which soil suppresses pathogens, although not well understood, can involve biotic (soil microbiome) and/or abiotic factors (soil physicochemical properties) and may also vary with the pathogen. Fluctuations in soil moisture content might confound the principles and relationships between plants and soil types and may influence microbial communities including bacteria (Garbeva et al., 2004). Different types of soil mixes are found in different localities and have important role in plant growth. Similarly, different pathogens prefer different types of soil for their proper multiplication and survival. Interaction studies of different pathogens, soil

mixes and different crops may provide a better understanding on their effects on their growth and productivity.

Interaction of *M. incognita* and *P. syringae* pv. *pisi* on pea causes a disease complex under field conditions. Inoculations of *M. incognita* and *P. syringae* pv. *pisi* in combinations caused greater damage to pea than by individual inoculation. Interactions between these pathogens may have both direct and indirect effects on disease severity. The direct effect includes physical interactions of pathogens in the rhizosphere and occupancy of same infection site inside the root. The occupancy of same infection site generally had antagonistic effect on pathogen multiplication (Siddiqui et al., 2012). Indirect effects of interactions are generally via plant response, such as breaking of disease resistance, and modification of host substrate which had synergistic effects on disease severity.

M. incognita generally causes physical damage to roots that can allow secondary infection by other pathogens (Sitaramaiah and Pathak, 1993). *Meloidogyne* spp. wound roots allowing other pathogens to become established (Siddiqui et al., 2012). Synergistic effects of nematode and bacterial interactions have also been reported (Rubio-Cabetas et al., 2001; Mallesh et al., 2009). Inoculation of nematodes with *P. syringae* caused greater damage to plant growth than caused by them alone. Nematodes aggravated disease by allowing bacterial pathogen to enter the plant as these are less adapted for penetrating the host's epidermis (Back et al., 2002). Modifications in the host substrate due to nematode infestation by creation of an infection court are one way modifies host to enhance infection by additional pathogens. Changes in biochemistry of the host are probably the most important factors favoring disease complexes involving nematodes (Slack, 1963).

Results also showed that *P. syringae* pv. *pisi* adversely affected multiplication of *M. incognita*. Our *in vitro* study also suggests that *P. syringae* pv. *pisi* had adverse effect on hatching of *M. incognita* and their penetration into roots. Adverse effect of bacterium inoculation on nematode multiplication as observed in the present findings is in conformity with our *in vitro* study. The contents of giant cells degenerated following bacterial invasion, leaving virtually empty cells resulting into the death of root-knot nematodes. Similarly to our finding, Swain et al. (1987) observed inhibitory effect of bacterium on *M. incognita*. Inoculation of *M. incognita* alone produces more galls and egg-masses compared to its association with *P. syringae* pv. *pisi*. Probably, establishment of bacterial pathogen induces certain changes in root system which are not favorable for nematodes (Hazarika, 2003; Hussain and Bora, 2009).

Rhizobium–legume symbioses are important due to their ability to fix atmospheric nitrogen. Inoculation of *R. leguminosarum* increased plant growth and reduced galling and nematode multiplication. Rhizobial strains have the biocontrol properties and can lead to potential disease control (Gopalakrishnan et al., 2015). The mechanisms of biocontrol by rhizobia include, competition for nutrients (Arora et al., 2001), production of antibiotics (Bardin et al., 2004; Chandra et al., 2007; Deshwal et al., 2003a), production of enzymes to degrade cell walls (Ozkoc and Deliveli, 2001) and production of siderophores (Carson et al., 2000; Deshwal et al., 2003b). Rhizobial strains also compete for nutrients by displacing the pathogens. *Rhizobium* spp. induced changes in seed proteome and metabolome by *Rhizobium* sp. enhanced resistance against plant pathogens (Sistani et al., 2017).

The infection of test pathogens ultimately led reduced nitrogen fixation by nodules due to reduced production and supply of carbohydrates (Minchin and Pate, 1973; Chahal et al., 1983).

Conspicuously large and pink-coloured nodules were found in plants with *R. leguminosarum* (without *M. incognita* or *P. syringae* pv. *pisi*) whereas nodules were of brownish coloured in plants infected with *M. incognita* or *P. syringae* pv. *pisi*. The numbers of nodules per plant were significantly reduced by the infection of *M. incognita* or *P. syringae* pv. *pisi* also reported earlier (Chahal et al., 1985). Moreover, nodule development is adversely affected by secretions by nematodes (Barker et al., 1972) and by the competition between juveniles of nematodes and root nodules for food and space (Malek and Jenkins, 1964). Disturbed functioning of nodules was observed by invading pathogens which alter host nutrition (Doney et al., 1970), and also reduce bacteroids content of nodules (Ali et al., 1981).

Proline is a multi-functional amino acid which plays an important role in plant defense (Cecchini et al., 2011; Senthil-Kumar and Mysore, 2012). Increased proline content in plants after infection occurs in response to plant defense (Fabro et al., 2004; Verslues and Sharma, 2010). Therefore, increase in proline contents with *M. incognita* or *P. syringae* pv. *pisi* inoculation was observed. Simultaneous or sequential inoculation of *M. incognita* and *P. syringae* pv. *pisi* resulted in higher increase in proline contents than single inoculation of pathogen. It is possible due to greater increase in plant resistance. Moreover, no increase in proline content was observed after *R. leguminosarum* inoculation probably due non-increase in plant resistance. Higher proline contents in plants grown in sand amended soil with poor growth indicate plant was under abiotic stress while least proline contents in plant with fly ash amended soil had better plant growth indicate better nutrient availability with least abiotic stress.

PCA revealed interaction of test pathogens under study. The PCA results are in agreements with the criteria of Sneath and Sokal (1973), who showed that data should represent at least 70% of total data variability (Fig. 3). The positive correlations were observed in plant length, plant fresh weight, plant dry weight, chlorophyll and carotenoids contents were negatively correlated with proline content, root galling and nematode population. Segregation of different treatments in the plots clearly identified the interactions of the test pathogens on various attributes. The plots also characterized the inoculation of pathogens into distinct groups (Fig. 3).

CONCLUSIONS

Soil amendment with 20% fly ash was better for plant growth, chlorophyll and carotenoid content while root nodulation and proline content were high in plants grown in 20% sand amended soil. Inoculation of *M. incognita* prior to *P. syringae* pv. *pisi* increased disease severity by predisposing plants to bacterial pathogen. Soil amended with 20% sand was better for multiplication of *M. incognita* and root galling. Both *P. syringae* pv. *pisi* and *R. leguminosarum* had adverse effect on galling and nematode multiplication.

CONFLICT OF INTEREST

No conflict of interest has been declared by authors.

ACKNOWLEDGEMENTS

First author is thankful to Aligarh Muslim University, Aligarh, UP, India and University Grants Commission, New Delhi, India for the award of University Fellowship to carry out this work.

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