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Research Article

Effect of *Meloidogyne incognita* on the growth, physiology and expression of *ME-1* gene and pathogenesis related proteins in *Phaseolus vulgaris*

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Abstract

Two experiments were designated to study the effect of root-knot nematode (*Meloidogyne incognita*) infection on the growth characteristics of *Phaseolus vulgaris* and response of plants towards the nematode. With an increase in initial inoculum levels of *M. incognita*, the growth and physiological parameters of *P. vulgaris* were decreased. In response of nematode infection, the expression of nematode resistance gene *ME-1* and pathogenesis related (PR) proteins PR1, PR2, PR3 and PR5 were induced in the roots of *P. vulgaris*. These PR proteins are used as a markers for salicylic acid and jasmonic acid. During root-knot nematode infection the higher inoculum level caused up regulation of *ME-1,* PR1, PR2, PR3 and PR5 in the affected root tissue of *P. vulgaris*. From the results it was revealed that high population of root-knot nematode reduced plant growth and triggered the plants to defend themselves by inducing the nematode resistance gene *ME-1* and pathogenesis related protein (PRs). From the findings of the experiments it may be suggested that root-knot nematode adversely affected the plant growth and also regulated the production of *ME-1* and PR proteins in nematode infected *P. vulgaris* plants.

Keywords: Expression Pattern; *Meloidogyne incognita;* Nematode Resistance Gene *ME-1;* PR Proteins; *Phaseolus vulgaris;* Plant Response

Introduction

Red kidney bean (*Phaseolus vulgaris*) an important food component in the developing countries, comprising 86% of the worldwide production of the bean crops is cultivated at small scale by the farmers who have been using its traditional cultivars for a long time. The crop is affected by the several pathogens causing the significant economic losses.

Plant parasitic nematodes are the major pathogens of both temperate and tropical agriculture crops, which have a global economic effect of more than US$ 100 billions each year [1]. Among plant parasitic diseases root-knot disease is caused by *Meloidogyne* spp. of which *Meloidogyne incognita* is the most destructing species that results in huge economic losses. These nematodes invade and colonize host plant roots subvert the host machinery to their own benefit and overcome host defenses [2]. Feeding-site formation enables the parasite to withdraw large amounts of nutrients from the plant vascular system. Many morphological and physiological changes that occur during the formation of feeding sites are reflected by altered gene expression in the host [3,4]. In response to pathogen attack, the plants express both basal and inducible defense mechanisms. The basal responses are mediated by salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), which induce race specific resistance [5].

In *P. vulgaris,* the root-knot nematode resistance gene is *ME-1* which confers resistance to *M. incognita*, *M. javanica* and *M. arenaria* [6]. In plant nematode interactions the closely related nucleotide binding (NB) and leucin-rich repeat (LRR) resistance genes are effective. The *ME-1* gene triggers a localized tissue necrosis, the

hypersensitive response (HR), on penetration of the nematodes into the root. Although *ME-1* is the only cloned insect R-gene, there is evidence that similar genes, which recognize specific effectors to mediate resistance against piercing-sucking insects, do exist in several plant species [7]. Recognition of elicitors derived from the invading pathogens triggers the defense responses.

Synthesis of PR proteins serves as a marker for disease resistance. Their occurrence in a wide variety of plant species, of both mono- and dicotyledons, suggests that they play an important role in plant disease resistance. These proteins restrict pathogen development and its spread in the plant, and have been reported to be induced in 17 families in response of pathogenic fungi, bacteria, viruses, and nematode [8]. The PR proteins are also induced in specific plant organs or tissues in response to environmental stimuli such as cold stress and wounding.

The potential change in expression of *ME-1*, PR1, PR2, PR3 and PR5 in the root of *M. incognita* infected *P. vulgaris* is unknown. The aim of this study was to examine the effects of *M. incognita* on the growth and physiology of *P. vulgaris* and to analyze the expression pattern of the gene *ME-1* and four pathogenesis related proteins in *P. vulgaris* (PR1, PR2, PR3, and PR5) on infection in *P. vulgaris* by the root-knot nematode (*M. incognita*).

Material and Methods

Preparation of inoculum

Meloidogyne incognita (Kofoid and White) Chitwood was selected as test pathogen. To perform experiment during the period of research, pure culture of *M. incognita* was maintained on egg-plant (*Solanum melongena* L.) roots in the glass house by using single egg mass. The egg masses from the galled root of egg plant were picked with the help of sterilized forceps and rinsed thrice in distilled water. The eggs in the egg masses were allowed to hatch at 28 ± 2°C under aseptic conditions in a sieve lined with tissue paper and kept in a petri-dish containing sufficient amount of sterilized distilled water. The second-stage juveniles were collected in distilled water and counted with the help of counting dish.

Inoculation of nematode

The seeds of *P. vulgaris* were surface sterilized with 0.1% sodium hypochlorite (NaOCl) for 2 minutes and washed thrice in sterilized distilled water. The seeds were sown in pots filled with sterilized soil, and allowed to germinate. Three-leaf stage seedlings were inoculated by making holes 5-7 cm depth around the plant within the radius of two centimeters. The second-stage juveniles at the rate of 50, 500, 1,000, 2, 000 and 5,000 per 10 ml of water were pipetted into the soil through the holes. The holes were plugged with the sterilized soil soon after inoculation. Each treatment was replicated five times and the pots were arranged in complete randomized block design in the glasshouse. Uninoculated set of plants served as control. Biochemical parameters were taken after 15 days of inoculation. Total chlorophyll content, leaf nitrate reductase activity, shoot N content and Leaf protein content were measured by the methods given by Arnon [9], Jaworski [10], Lindner [11] and Lowry *et al.,* [12] respectively. For growth analysis the plants were harvested after 60 days of inoculation.

The results were analysed statistically by the analysis of variance followed by least significant difference (LSD) at $p \leq 0.05$, and Duncan's multiple test for testing significant differences by using SPSS (16.0).

For qRT analysis the seeds were allowed to germinate in plastic pots filled with sterilized soil maintained in growth chamber at 25 ± 2°C with a photoperiod of 16 h and dark period of 8h for 3 weeks. After one week of germination seedlings were inoculated with second-stage juveniles of root-knot nematode at the rate of 50, 500, 1,000, 2,000 and 5,000 J_2 per seedling. The plants were harvested after two week of inoculation and stored in deep freezer at -80°C for subsequent RNA extraction and qRT- PCR analysis.

RNA isolation and qRT- PCR

For gene expression analysis, total RNA was isolated from frozen tissue samples using the RNeasy kit according to the manufacturer's protocol (Qiagen, Valencia, CA). The RNA quantity and quality were analyzed before proceeding to cDNA synthesis. Initially the single strand of cDNA synthesized from 2 µg of total RNA in 20 µL of reaction volume using high-capacity c DNA Reverse Transcription kit (Applied Biosystems, USA). qRT-PCR was carried out from 1:50 diluted cDNA samples using SYBR-Green PCR master mix in 384-well optical reaction plate in Applied Biosystems 7500 Fast Real Time PCR System as per manufacturer's protocol (Applied Biosystems, USA). *EF1BB* was used as endogenous control for all experiments, and gene expression calculation was done by ΔΔCT method [13]. The primers were generated from unique region of the genes using PRIMER EXPRESS version (Applied Biosystems, USA) with default parameters.

Results

Plant growth

Different inoculums levels of *Meloidogyne incognita* had different effects of on the growth of *Phaseolus vulgaris* as is evidence from the result (Figure 1a and 1b). At lower initial inoculum levels the extent of reduction in plant growth was less but at higher inoculum levels, reduction in plant growth was remarkable. In comparison to control and healthy plants the plant length and the plant weight both were found decreased at lower as well as higher inoculum levels of *M. incognita*. At lowest inoculum level ($50J_2$), reduction was low and non-significant as compared to control. At the next inoculum level (500 J_2), the reduction in plant growth, over the control was significant. The greatest reduction in the plant length and weight were observed with the higher number of second-stage juveniles (5,000 J_2) (Figure 2 a, b) With progressive increase in inoculum levels a progressive decrease in plant growth was observed (Table 1).

The yield in terms of the number of pods per plant was reduced when the plants were infected with nematode. Reduction in number of pods per plant was non-significant at the lowest inoculum level (50 J_2) when compared with the control. At higher inoculum levels, on comparing with the control, reduction in the yield were significant. The number of pods per plant were significantly lower on comparing with one inoculum level with the next higher inoculum level (Figure 2c, Table 1). At the highest inoculum level the extent of reduction was highest (Figure 1b). From the data it is clear that at lower as well as higher inoculum levels reduction in yield occurred, when compared with the control or with one another. All these unusual phenomena resulted in transport of metabolites in low amount towards the growing parts specifically developing reproductive parts. Due to insufficient supply of photosynthates, the number of pods and seed were decreased.

The root-knot nematode, on infecting a plant damages the root and causes the formation of galls in the root. Galling was observed on the root of all the infected plants of *P. vulgaris*. The number of galls on the infected root of *P. vulgaris* was increased when the number of second-stage juveniles was increased (Figure 2e, Table 1). The number of galls was maximum in T5 inoculated with 5,000 J_2 of *M. incognita* (Figure 1c). Increased in number of egg masses was from T1 to T5. increased in the number of galls was significant in the treatment T3 to T5 and maximum increase was exhibited by the treatment T5 inoculated with the highest number of juveniles (Figure 2d, Table 1).

Figure 1: Effect of *Meloidogyne incognita* infection on the growth of *Phaseolus vulgaris.* The nematode treatment was significantly perturbed the shoot growth (a, b) reduced pod number and root growth (b) in a dose-dependent manner. The gall formation in the roots of infected plants (c); only T5 sample is shown.

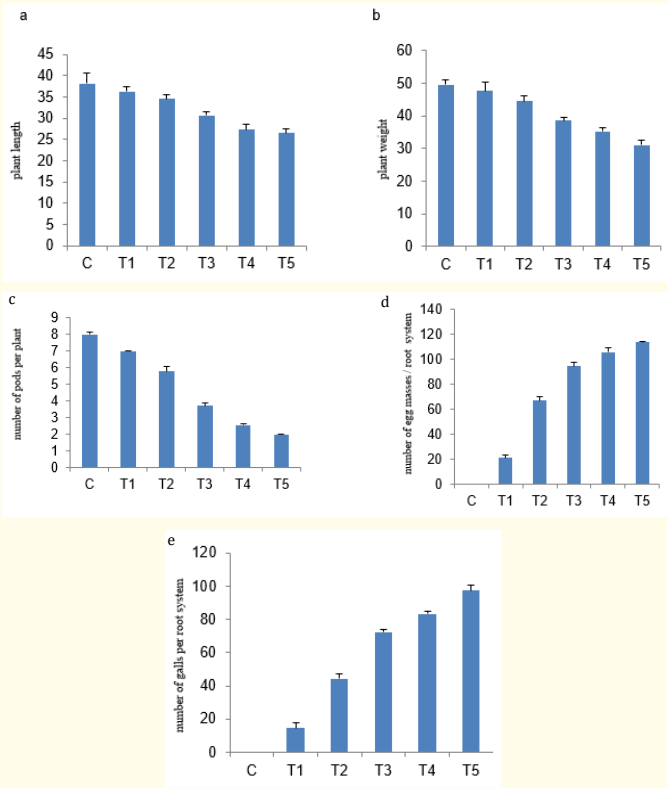

Figure 2: Effect of *Meloidogyne incognita* infection on the growth parameters.

The plant length (a), plant weight (b), pod number (c), egg mass number in roots (d) and number of galls per plant (e) were estimated to analyze the impact of nematode infection *on P. vulgaris.* Graphs represent the average of each parameter while the error bars represent the mean ± SD of the replicates and different letters above the bars indicate significant difference (P≤ 0.05, analysis of varience) by Duncan's multiple test.

Treatments	Plant length (cm)	Plant weight (g)	Number of pods/plant	Number of egg masses/root system	Number of galls/root system
C	38.18 c	49.62 e	8.61 c	0.00 ± 0.00	0.00 ± 0.00
T1	38.00 c	48.63 e	8.00 c	21.86 e	14.38 e
T2	37.31 c	46.12 d	7.75 c	67.26 d	44.20 d
T3	34.67 b	41.52 c	5.67 b	94.32 c	72.40 c
T4	32.00 a	39.20 b	4.93 a	105.51 b	83.32 b
T5	31.50 a	36.83 a	4.20 a	113.60 a	97.50 a
LSD= (0.05)	1.77	2.40	0.92	16.55	13.85

Table 1: Effect of different inoculum level of root-knot nematode on the growth of *P. vulgaris*

Each value is a mean of five replicates, Means in each column followed by the same letter (s) did not differ at p≤ 0.05 according to Duncan multiple-range test.

Effect of root knot nematode on the plant physiology

The root-knot nematode on infection causes changes in metabolic reaction of the host plant. The parameters such as chlorophyll content, NRA, protein, and nitrogen contents were analyzed in nematode infected plant. In nematode infected plants the total chlorophyll contents were decreased. At lower inoculum level, the effect on chlorophyll content was less but at higher inoculum level it was more drastic. Highest reduction was recorded in T5 plants inoculated with highest number of juveniles (Figure 3a, Table 2). The amount of chlorophyll a and chlorophyll b decreased at all the initial inoculum levels. The changes in nutrient concentration, after root-knot nematode infection, altered host metabolic pathways. The data revealed that decreasing trends in nitrate reductase activity in the leaves of inoculated plants with increasing inoculum level of *M. incognita*. Maximum reduction in NRA in leaves was recorded in T5 plants inoculated with highest nematode population (Figure 3b, Table 2). The shoot nitrogen content decreased with increase in initial number of juveniles from treatments T1 to T5, over the healthy control. The treatment T5 exhibited highest reduction at the highest number of second stage juveniles (5,000) per plant (Figure 3c). The root-knot nematode caused the reduction in the leaf protein content (Figure 3d, Table 2).

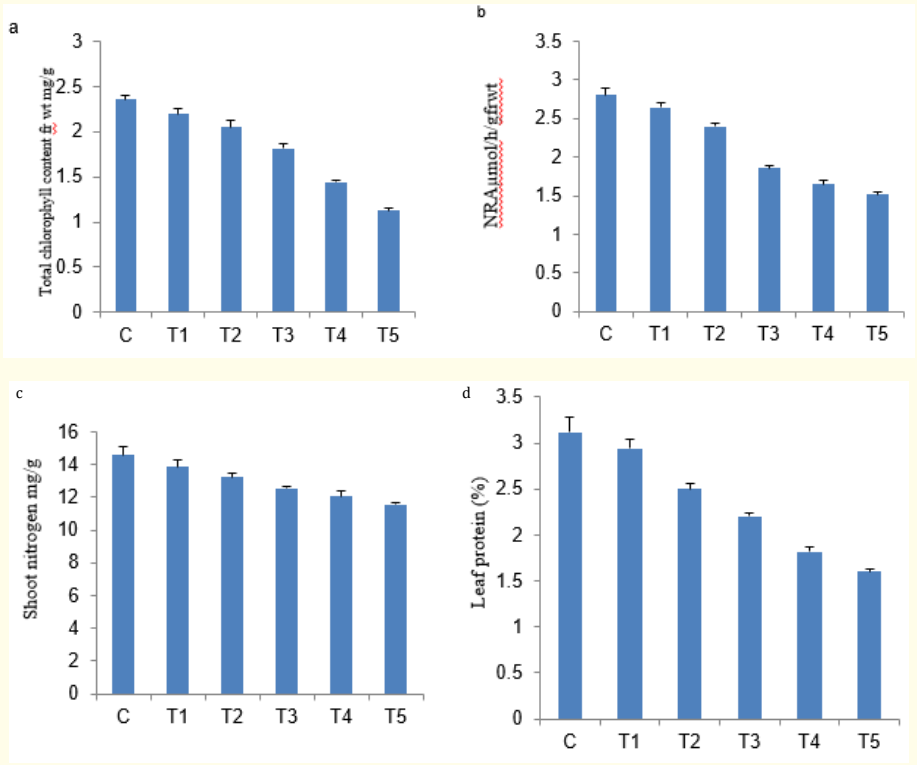

Figure 3: Effect of *Meloidogyne incognita* infection on biochemical parameters.

The total chlorophyll content (a), nitrate reductase activity (b), shoot nitrogen content (c) and protein content in leaves (d) were estimated to analyze the impact of nematode infection *on P. vulgaris.* Graphs represent the average of each parameter while the error bars represent the mean ± SD of the replicates, different letters above the bars indicate significant difference (P≤ 0.05, analysis of varience) by Duncan's multiple test.

Treatments	Total chlorophyll mg/g	NRA µ mol/h/g/ fr wt	Shoot nitrogen mg/g	Leaf protein (%)
C	2.359 d	2.80 d	14.60 e	3.12 f
T1	2.284 d	2.64 c	13.86 d	2.94 e
T2	2.268 c	2.59 c	12.85 c	2.50 d
T3	2.068 b	2.13 b	12.53 c	2.20 c
T4	2.008 b	2.07 b	12.06 b	1.82 b
T5	1.923 a	1.93 a	11.54 a	1.40 a
LSD= (0.05)	0.169	0.19	0.41	0.23

Table 2: Effect of different inoculum levels on the physiology of rajma plant.

Each value is a mean of five replicates, Means in each column followed by the same letter (s) did not differ at p≤0.05 according to Duncan multiple-range test.

Gene name	Locus ID	Forward primer	Reverse primer
EF1BB	Phvul.009G167000	CATTGAGGAAAGACTCACAGTTGAG	GGCCGCAATGTCACAACTC
ME1	Phvul.011G014300	CTTTGGTCTTTGAGTGGCTTGA	AGCGTGTCATACATTTGTTTCGA
PR1	Phvul.006G196900	AGAATCGCGTGCCAGCTT	CAGTTAACTCAGCGATAAAACACATTT
PR2	Phvul.009G256400	GCAAAGATTGTTGTTGATGAGTTCA	GGTTCCAACCTTACATGTCACTCTTA
PR3	Phvul.009G116600	GAAGGAAGGGAAACGGAAAGA	TTCCTCCAACTAACAGCATCCA
PR5	Phvul.001G005000	TGCCACCACGCCACTTC	TGATCGAAACCACCTTCGTACA

Table 3: The locus ID and primers of genes analyzed in this study.

Expression of *Me-1* and PRs genes in the root of *P. vulgaris* infected with different inoculum level of *M. incognita*

The qRT-PCR analysis (Figure 4B) revealed that expression of the gene *Me-1* was down regulated in T1 and T2 treatments, when compared with the control. The expression was enhanced in the treatments T3 and T4 over the treatment T2 but it was lower than the control. In the treatment T5, expression of *Me-1* gene was upgraded to a very high level when compared with the control and with the other treatments.

Expression of the gene PR1, as is evident from Figure 4C was higher in all the treatments from T1 to T5 when compared with the control. In T1 (50 J_2) and T2 (500 J_2) plants, the enhancement in gene regulation was slightly higher than the control. In comparison to the control, T1 and T2 the gene expression was found upgraded in the treatments T3, T4, and T5.

The gene PR2, was expressed in all the treatments from T1 to T5 as is evident from the Figure 4D. There was gradual up regulation in the gene expression from T1 to T4, when compared with the control. Highest up regulation of the gene PR2 was observed in the treatments T4 (2,000 J_2), followed by the T3 (1,000 J_2) when compared with control and the other treatments. In the treatment T5 (5,000 J_2), expression of PR2 gene was down regulated as compared to control and the other treatments.

The Figure 4E revealed that in the treatments T1 and T2 (50 J_2 and 500 J_2 respectively), expression of the gene PR3 was down regulated over the control. Expression of the gene PR3, was highly up regulated in the treatments T3 (1,000 J_2) and T5 (5,000 J_2) when compared with T1, T2, T4 and the control. The enhancement in the gene regulation was higher in the treatment T4 (2,000 J_2) than the control but lower than the T3 and the T5 plants.

Expression of the gene PR5 was down regulated in the treatments T1, T2, T3 and T4 when compared with the control. The differences in regulation, in comparison to the control, were greater in T1 and T2 and smaller in T3 and T4, with least difference in T3 plants. In the treatment T5 (5,000 J_2), expression of PR5 was highest, on comparing with the control and the other treatments (Figure 4F).

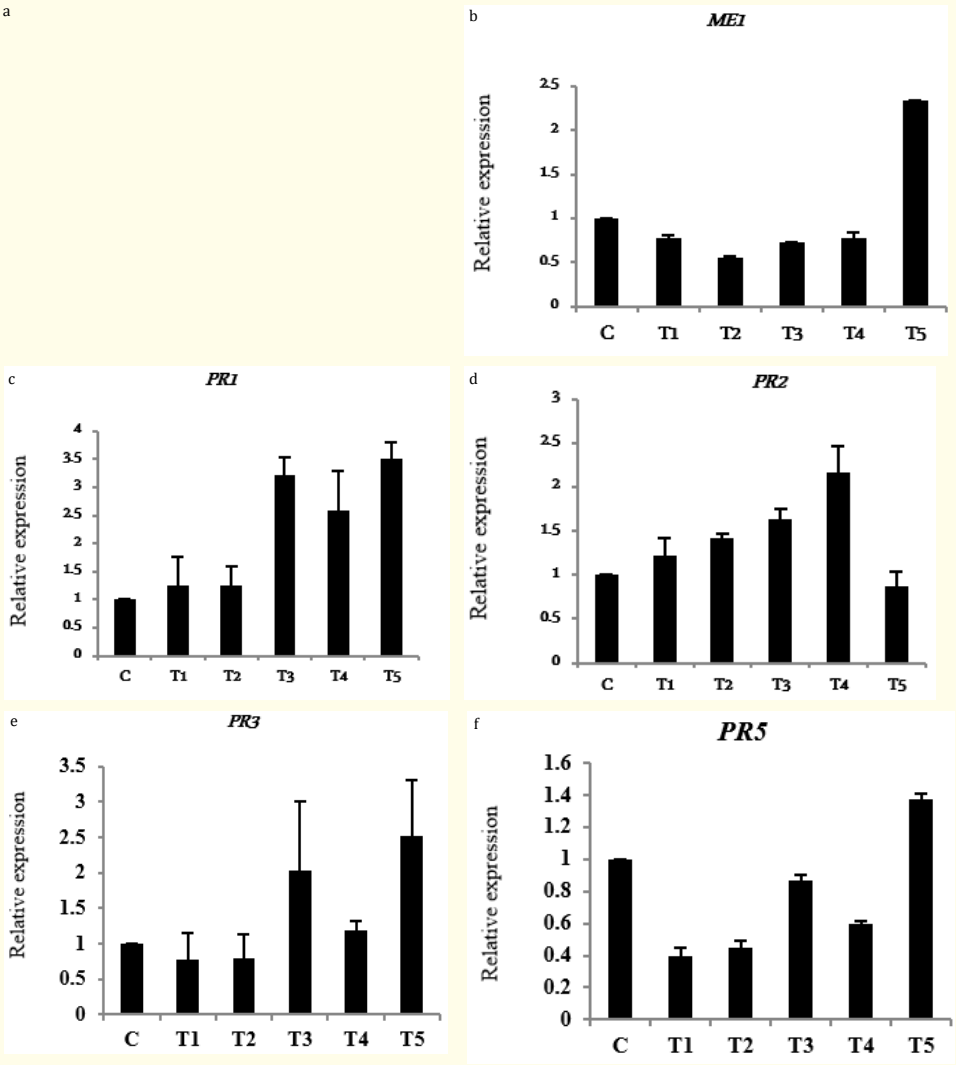

Figure 4: The expression of *ME*1 and *PR* genes in response to nematode treatment.

Expression of *Me-1* and PRs gene of *Phaseolus vulgaris* was evaluated at different inoculums levels of *Meloidogyne incognita* (a). epression of Me-1 gene (b), expression of PR1 (c), expression of PR2 (d), expression of PR3 (e), and expression of PR5 (f). The degree of expression of the gene in control plants was taken as control to compare expression at different inoculums levels. The *EF1BB* gene was used as endogenous control. In the figures, graph represent the average while the error bars represent the mean ± SD of replicates.

Discussion and Conclusion

The root-knot nematodes are sedentary endoparasites which secrete effector molecules into selected host root cells in response of which certain cells get transformed into metabolically active feeding sites which are critical for the survival of the nematode [14]. From our results it is evident that the plant growth was retarded at different inoculum levels of the root-knot nematode, in terms of plant length and plant weight. The yield was found reduced after nematode infection. Azmi [15] recorded significant reduction in shoot weight at 1,000 J_2 and above in subabool (*Leucaena leucocephala*). Raut and Sethi [16]; Jagdale *et al.* [17]; Mishra and Singh [18]: Ali [19] and Chahal and Chahal [20] reported adverse effects of *Meloidogyne incognita* on the growth characteristics of soybean, betel vine, jute, cardamom and mung bean, respectively. In this study the amount of chlorophyll, NRA, decreased at different inoculum levels of the root-knot nematode, maximum reduction in chlorophyll and NRA was occurred in the plants with higher nematode population. Maximum reduction in all biochemical parameters was recorded in the treatment T5. Leaf pigments are sensitive to nematode infection which result in the loss of photosynthetic pigments [21]. *Meloidogyne* spp. reduced total chlorophyll in French bean and rice and horse gram *Macrotyloma uniflorum* [22-24]. The leaf protein content, in *P. vulgaris* were decreased with increase in inoculum levels, with maximum reduction in T5 (5,000 J_2) plants. Reduction in protein content with an increase in inoculum levels indicated that the developing nematode continuously withdrew large amount of nutrients from the plant through the giant cells [25]. Nitrogen is required for cellular synthesis of enzymes, proteins, chlorophyll, DNA and RNA, and is therefore important in plant growth and the production of food [26]. In this study significant reduction in shoot nitrogen content was also noticed with increase in initial inoculum level. With the increase in nematode population there was a corresponding decrease in the chlorophyll content, number of nodules, nitrogen content of shoot and protein content of grain of *Phaseolus aureus* [27] and papaya, *Carica papaya* [28]. Deleterious effects of different inoculum densities of different *Meloidogyne* species have been observed by different workers on different hosts [29-31]. On *P. vulgaris* more number of galls and egg masses were observed at highest inoculums level 5,000 J_2. Higher galling might be due to the fact that at higher inoculum levels more number of juveniles reach at the feeding sites and searched new locations to induce infection and result in increased number of galling and egg mass production. Increase in gall number on increasing inoculum level have been found in several plants [32,33].

After establishment of permanent feeding sites, the nematode represses or controls the defense response of the host plant [34]. The defense responses may include the production of jasmonic acid (JA) and salicylic acid (SA); hypersensitive responses; cell wall strengthening; production of pathogenesis related protein (PRs); and other cellular defense responses. The gene *ME-1* is a type of nematode resistance (R) gene in *P. vulgaris* and responsible for recognition of specific elicitors produced by the root-knot nematode, conferring resistance to the plant. Besides conferring resistance to root-knot nematodes, the *Mi* gene also conferred resistance to some isolates of potato aphid (*Macrosiphum euphorbiae*) and sweet potato whitefly (*Bemisia tabaci*) [35,36].

The data revealed that expression of the gene *Me-1* was down-regulated in *P. vulgaris* treated with the lower inoculums level of *Meloidogyne incognita* than uninoculated control plants. From findings of this experiment it might be inferred that the lower nematode population suppressed the signal mechanism related defense against the root-knot nematode. This view was supported by the work carried out by Li *et al.,* [37] who proposed loss of signaling of defense activation system. At the highest level of nematode inoculum, the expression level of the gene *Me-1* was increased enormously indicating that in response of attack by higher number of nematodes, induced resistance mechanism and activated expression of the gene *ME-1* to a higher degree. From this finding it might be inferred that higher level of nematode population triggered defense system of *P. vulgaris* and induced the gene *ME-1* to be expressed at an enhanced rate. The result of this experiment are similar to those are carried out by Ogallo and McClure [38] who reported that the intensity of induced resistance was initially increased with increasing population density of advance inoculum to about 5,000 J_2 per potted plant.

From the analysis of qRT-PCR it was found that *M. incognita* caused elicitation of both SA-dependent and JA dependent genes in infected root of *P. vulgaris* as is evident from the increased level of transcript of PRs genes. Pathogenesis related proteins (PR) are induced systemically by the interaction of a pathogen with its host [39]. At lower levels of inoculum in the treatments T1 (50, J_2) and

T2 (500, J_2), the expression of PR1 and PR2 was slightly enhanced, however, with an increase in inoculum levels, the expression level was considerably increased as is evident from the data of T3 (1,000 J_2) and the T4 (2,000 J_2) plants. The enhanced rate of expression of PR1 with increased inoculum level indicated inducement of SA pathway, leading to the induction of PRs protein, after the nematode attack. Increase in PR-1 protein suggested that there might be an increase in the level of salicylic acid. Our data revealed that higher expression of PR2 indicating that higher inoculum levels of root-knot nematode triggered the plant to enhance concentration of PR protein leading to activating of defense response. Higher level of expression of PR1, PR3, PR5 was observed in the plants inoculated with 5,000 J_2 of *M. incognita*. The PR3 is associated with chitinase activity; and increased activity of chitinase in rice infected with rice root-knot nematode *M. graminicola* induced the defense responses in plant [40]. Application of SAR elicitors to tomato plants has been shown to activate *PR-1* and *PR-2*, and to reduce plant susceptibility to root-knot nematode infection [41]. Sanz-Alferez *et al.,* [41] observed the down regulation of PR genes in tomato plant infected with *M. incognita.* During interaction of soybean roots and RKN, several genes encoding several PR proteins were altered at the time their expression [42]. From these finding it might be expected that higher nematode population induced resistance in plants by activating higher level of the expression of PR1, PR2 and PR3 that triggered the defense responses after the attack by the nematode. Our results are supported by the findings of Hamamouch, *et al.,* [43] who observed expression of PR proteins in *Arabidopsis* roots treated with the root-knot nematode and cyst nematode at different time intervals, and also found that the expression level of PR proteins was higher in treated roots at 9 dpi which declined at 14 dpi indicating that root-knot nematode infection induced both SA- and JA dependent SAR in roots of infected plants. Benjamin *et al.,* [44] demonstrated up- regulation of the PR1, PR2 and PR5 in an incompatible interaction with the soybean cyst nematode in soybean plants. The *Hero* mediated incompatible response to potato cyst nematode showed up-regulation of PR1 and PR5 in the roots of tomato [45].

All of these data indicated that there was a higher production of transcripts of genes *ME-1, PR1, PR2, PR3* and *PR5* encoding proteins involved in the plant defense response in the root of *P. vulgaris* inoculated with higher inoculum levels (1,000 J_2, 2,000 J_2 and 5,000 J_2) of the root-knot nematode. The transcripts of these genes were lower or the genes were down-regulated in the treatments which were inoculated with lower inoculum levels of *M. incognita* leading to a weaker defense response against root-knot nematode in the roots of *P. vulgaris*. The PR proteins are present in plant in trace amounts but are produced in much greater concentration following pathogen attack or stress [46-50].

From our findings it can be suggested that with increased inoculum level, the nematode reduced the growth and physiological parameters, however, after the attack of nematode, the plant defended by expressing the defense related genes; the expression of these genes is dependent on the severity of the attack. From our result it might be suggested that *M. incognita* had adverse effects on the growth of *P. vulgaris*. This nematode affects the plant growth by interfering the photosynthesis, protein synthesis and nitrogen metabolism. Upon perceiving this signal, plants increase the expression of *ME-1*, *PR1, PR2, PR3* and *PR5* genes in the infected root of *P. vulgaris* in a dose-dependent manner. So it might be concluded that in plant-nematode interactions, the intensity of induced resistance in plant increased with increasing the population density of root-knot nematode resulting higher level of expression of resistance genes and PR proteins.

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