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BIOCHEMICAL CHANGES IN THE LEAVES OF SPINACH (*SPINACIA OLERACEA*) AT DIFFERENT INOCULUM LEVELS OF *MELOIDOGYNE INCOGNITA* IN GREENHOUSE EXPERIMENTS

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ABSTRACT

Root knot nematodes are sedentary parasites that form galls in plant roots thereby reducing the water and nutrient uptake to the plant, impeding the growth, hindering the fruit production, and cause yellowing of leaves and wilting. In this study, an attempt to understand the effect of increasing *Meloidogyne incognita* infestation on various biochemical parameters in spinach was made. Biochemical analysis of spinach plants with varied nematode inoculum was carried out in plastic pots. Four weeks after planting, freshly hatched second-stage juveniles (J2s) were used as inoculation at the rate of 500, 1000, 2500 and 5000 J2s per pot. Uninoculated plants served as control. Each experiment was replicated three times. After 45 days of inoculation, the plants were pulled out of the pots and analysed for changes in the following biochemical parameters: chlorophyll content, protein content, total phenolic content and defense enzymes (peroxidases and polyphenol oxidases). Plants inoculated with 5000 J2s showed significantly increased levels of peroxidase and polyphenol oxidase activity as compared to the control. Chlorophyll and protein content decreased while the total phenolic content was found to increase with increasing inoculum level. All these parameters showed little to no difference in plants inoculated with 500 J2s as compared to controls.

Keywords: Nematode Inoculum, Peroxidase Activity, Polyphenol Oxidase, Spinach, Total Phenolic Content.

INTRODUCTION

Spinach (*Spinacia oleracea*) is a dioecious annual herb that is a member of family Amaranthacea along with chard, quinoa and beets. It is a common vegetable with edible leaves that can be eaten raw, boiled or baked into various dishes. It is a good

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source of vitamin K, iron, calcium, and phosphorus. It also contains folate, vitamin C, vitamin A and antioxidants like beta-carotene (Morelock and Correll, 2008).

The most significant economic barriers to the production of spinach are viral and fungal diseases (Correll et al., 1994). However, it has been observed that a number of plant-parasitic nematodes also harm the crop. Root-lesion nematodes (*Pratylenchus penetrans*), cyst (*Heterodera schachtii*), and root-knot nematode (RKN) species (*Meloidogyne incognita, M. hapla, M. arenaria, M. javanica*) are common nematode species causing diseases in spinach (Di Vito et al., 2004).

RKNs are obligate biotrophic and polyphagus plant parasites belonging to the genus *Meloidogyne* and order Tylenchida that pose a serious and growing threat to the agriculture. Since they have a wide range of hosts, these nematodes are regarded as economically the most important group of plant parasitic nematodes (Jones and Goto, 2011). Their wide host ranges contribute to the enhancement of their impact on economically important plants. According to estimates, more than 2000 plant species are susceptible to infection by most common *Meloidogyne* species (Trudgill and Blok, 2001).

RKNs have complex interactions with their host and cause morphological and physiological changes in them (Williamson and Gleason, 2003). Due to RKN infection in plants, leaf area, rate of photosynthesis, quantity of chlorophyll pigment, carbohydrate, protein, phenolic and oil contents are altered. Plant defense against pathogens involves accumulation of enzymes polyphenol oxidase (PPO), peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD) which help in antioxidant mechanism and the formation of a physical barrier which prevents further penetration of the tissue by a nematode by the process of lignification and suberization (Zacheo et al., 1997). The nematode infection can cause alteration in these plant enzymes which induces resistance in plants.

The production of spinach is reported to be limited by different *Meloidogyne* spp., which are among the most harmful and prevalent nematodes (Premachandra and Gowen, 2015; Murungi et al., 2018; Basyony et al., 2020). Several studies have observed a reduction in spinach yield with an increase in inoculum densities of RKNs (Premachandra and Gowen, 2015; Taning et al., 2022). To develop effective management strategies and predict potential crop damage, it's important to study how different initial densities of *Meloidogyne* affect the growth and biochemistry of spinach plants. Furthermore, understanding how spinach plants respond to these nematodes can offer valuable information for creating resistant cultivars, which could be used in breeding programs.

This study is aimed to evaluate the impact of *M. incognita* inoculum levels on the biochemical parameters of spinach leaves. Specifically, we investigated changes in chlorophyll content, peroxidase activity, polyphenol oxidase activity, protein content, and total phenolic content, in order to better understand the mechanisms underlying plant-nematode interaction.

MATERIAL AND METHODS

Species identification and collection of *M. incognita*

For the present experiment, initial population of *M. incognita* was obtained from the infected brinjal plants collected from nearby areas of Panjab University, Chandigarh. Plants with galled roots were taken to the laboratory. Females were collected from the galls for species identification and egg masses were collected to get infective second-stage juveniles (J2s). Identification of species was done using perineal pattern as well as molecular methods.

Perineal Pattern: Perineal pattern was prepared by manually isolating a female from the galls. It was then taken in 45% lactic acid drop on a glass slide and split into two halves using a surgical blade. The posterior half retaining perineal cuticular pattern was cleared using a brush to get rid of the tissues stuck to the portion. In the glycerol medium, the female cuticle was eventually trimmed into a square shape containing the specified perineal pattern. Each pattern was then mounted in anhydrous glycerine on glass slide and observed under light microscope.

SCAR PCR: For molecular identification using SCAR markers, DNA was extracted from 50-80 *Meloidogyne* females using Qiagen DNAeasy Blood and Tissue kit. DNA quantification was done using Nanodrop Spectrometer. The genomic DNA was further used for PCR amplification using the SCAR primers MIF/MIR (F: GTGAGGATTCAGCTCCCCAG; R: ACGAGGAACATACTTCTCCGTCC) (Meng et al., 2004) to identify the nematode species. PCR product was analysed using Agarose Gel Electrophoresis.

A pure population of *M. incognita* was maintained in tomato plants (Pusa Ruby) in a greenhouse in Department of Zoology, Panjab University, Chandigarh. For collection of *M. incognita* second-stage juveniles (J2s), plants were uprooted and egg masses were picked manually. These egg masses were kept in a petri plate for hatching. After hatching J2s were collected and a suspension was made containing 2000 J2s in 3 mL distilled water.

Experimental Setup

Spinach seeds (All Green variety) were sown in plastic pots kept in greenhouse. Four weeks after germination, the roots of spinach were inoculated using freshly hatched J2s at the rate of 500, 1000, 2500 and 5000 J2s of *M. incognita* per pot. Uninoculated plants served as control. Each experiment was replicated three times. 45 days after inoculation (dai), the plants were uprooted and number of galls and egg masses were observed. These plants were analysed for changes in the biochemical parameters (Fig. 1).

Figure 1. Spinach plants grown in plastic pots in greenhouse for biochemical analyses

Chlorophyll Estimation of Leaf

Chlorophyll estimation was carried out according to the protocol given by Arnon (1949) with slight modifications. For extraction of chlorophyll, 100 mg of spinach leaf was cut and homogenized in 10mL of 80% acetone. It was then centrifuged at 13000 rpm for 10 minutes. Absorption of the extract was recorded at 645 nm and 663 nm using a spectrophotometer (JUVS-55 double beam spectrophotometer, Jenway).

$$\text{Chlorophyll a (mg/g fresh weight of leaf)} = (12.7\ A_{663} - 2.69\ A_{645}) \times \frac{V}{1000 \times W}$$

$$\text{Chlorophyll b (mg/g fresh weight of leaf)} = (22.9\ A_{645} - 4.68\ A_{663}) \times \frac{V}{1000 \times W}$$

Total Chlorophyll (mg/g fresh weight of leaf) = Chlorophyll a + Chlorophyll b

Where V= total volume of solution, W= weight of plant tissue used in extraction, A_{663}= absorbance at 663 nm and A_{645}= absorbance at 645 nm

Preparation of Enzyme Extracts

One g leaf sample was homogenized using 5 mL of 100 mM sodium phosphate buffer (pH 6.5) with pestle and mortar in ice. It was then filtered using muslin cloth and underwent centrifugation for 15 minutes at 5000 rpm and 4˚C. Supernatant was collected and used as enzyme extract for peroxidase and polyphenol oxidase assays.

Determination of Peroxidase (POX) Activity in Leaf

POX activity was estimated using the method described by Srivastava (1987). In the sample cuvette, 1.5 mL pyrogallol (50 mM) was taken and 500 µL enzyme extract was added to it. The reaction was started by adding 500 µL of 1% H_2O_2. The reaction was read at 420 nm in a spectrophotometer and changes in absorbance at 30s intervals were recorded. The PO activity was expressed as changes in absorbance min^{-1} g^{-1} of tissue on fresh weight basis.

Determination of Polyphenol Oxidase (PPO) in Leaf

PPO activity was estimated with the help of the procedure given by Mayer et al. (1966) with some modification. In both the sample and reference cuvettes, 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5) and 1 mL of enzyme extract were added. The reaction was initiated by adding 500 µL of 0.01 M Catechol in sample cuvette. The reaction was read at 495 nm in a spectrophotometer and changes in absorbance at 30s intervals were recorded. PPO activity was expressed as change in the absorbance of the reaction mixture min^{-1} g^{-1} of tissue on fresh weight basis.

Determination of Total Protein Content

Bradford (1976) method was used to estimate the total protein content. Protein extract was prepared by homogenizing 1 g of leaf in ten millilitres of 0.1 M sodium acetate buffer (pH 4.7). It was then centrifuged for 15 min at 5000 rpm and 4^{o}C. The supernatant was used for the determination of soluble protein. Standard curve was prepared using bovine serum albumin as the protein standard. The absorbance was read at 595 nm in a spectrophotometer. The protein content was expressed as mg albumin equivalent of soluble protein per g on fresh weight basis.

Determination of Total Phenolic Content

Total phenolic content in the leaves of spinach plant infected with *M. incognita* was determined using Folin-Ciocalteu reagent. Method given by Ainsworth and Gillespie (2007) was used with slight modifications. Plant extract was prepared by homogenizing 1 g of leaf sample in 10 mL of 80% ethanol. It was then centrifuged at 10,000 rpm for 20 minutes. Supernatant was collected and the extraction was repeated with the residue using 5 mL of 80% ethanol. Catechol was used as a standard to prepare a calibration curve. 0.5 mL of the leaf extract was taken in a test tube and 2 mL of the Folin-Ciocalteu reagent (1:10 v/v with water) was added to it. After 3 minutes, 4 mL of 7.5% sodium carbonate solution was added to the reaction mixture. It was allowed to stand at room temperature for 30 minutes. Absorbance was measured at 765nm using JUVS-55 double beam spectrophotometer (Jenway). Total phenolic content was estimated from the linear equation of catechol standard curve. It was expressed as mg/g catechol equivalent of fresh weight.

Statistical Analysis

Data were subjected to Analysis of Variance (ANOVA) using SPSS software and comparisons of means were done using Least Significant Difference (LSD) at 5% significance level.

RESULTS AND DISCUSSION

Species identification

Perineal pattern showed high squarish dorsal arch with wavy striations, indistinct lateral lines (Fig. 2a) which were very similar to those of *M. incognita* (Eisenback et

al., 1985). Amplification with the SCAR primers MIF/MIR yielded a fragment of ~960 bp which is characteristic for ***M. incognita*** (Fig. 2b). To further confirm these results, sequencing of the PCR product was done and the sequence was analysed using NCBI BLAST. The sequence showed 95-99% similarity to the *M. incognita* SCAR marker OP26-01_{1200} sequences. The sequence has been submitted to NCBI GenBank database with accession number **MN444135.**

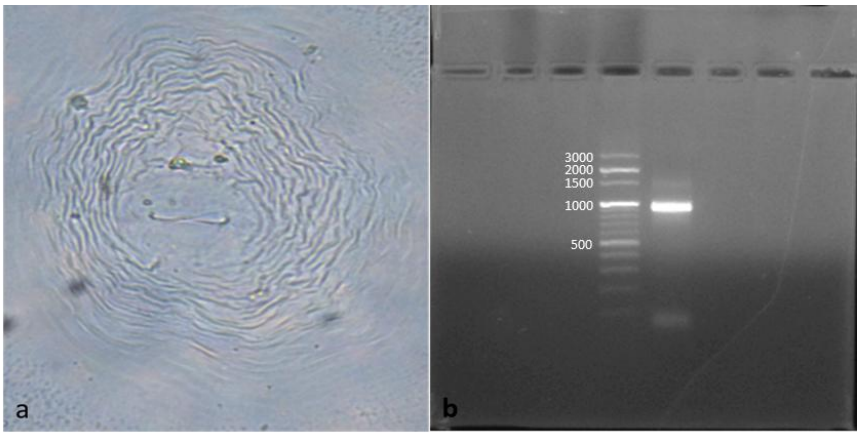

Figure 2. (a) Perineal pattern of *M. incognita* female. (b) Agarose Gel Electrophoresis results showing PCR product of ~ 960 bp

Number of Galls and Egg Masses

Spinach plant uprooted after 45 dai showed root galls and egg masses (Fig. 3). The number of galls per plant increased from 28 in 500 J2 inoculation level (gall index = 3) to 85.67 in 5000 J2 inoculation level (gall index = 4). However, no significant difference was found in number of egg masses/plant. It increased from 5 in 500 J2 inoculation level to 6.3 in 5000 J2 inoculation level.

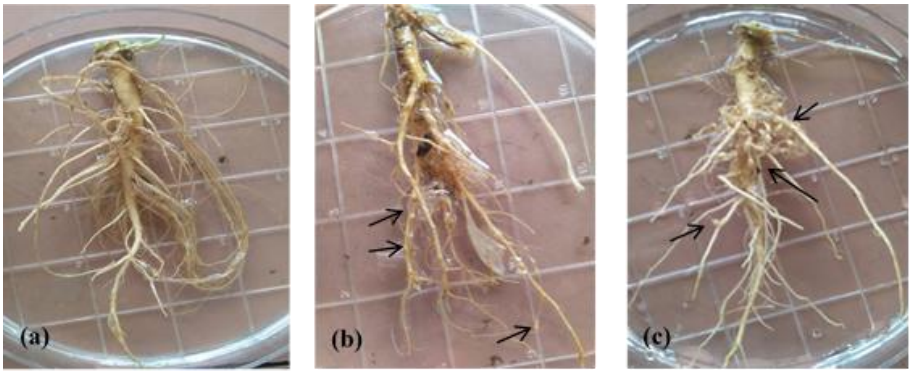

Figure 3. Spinach roots showing galls due to varying RKN infection. (a) Uninoculated, (b) 1000J2s, (c) 5000 J2s. Arrows indicate the galls

Chlorophyll Content

Chlorophyll content in plants decreased progressively with increasing J2 level (Fig. 4a). Plants with 5000 J2s showed a significant decrease in total chlorophyll content (1.69 mg/g fwt.) as compared to the control (2.50 mg/g fwt.) (Table 1). Reduction in chlorophyll content due to increasing *Meloidogyne* infection has been observed in various other plants like mungbean (Ahmed et al., 2009), zucchini (López-Gómez et al., 2015), carnation (Meena et al., 2016), and babchi (Danish et al., 2018). Swain and Prasad (1988), Perveen et al. (2006) and Abbasi and Hisamuddin (2014) also reported the reduction in chlorophyll content of the *Oryza sativa*, *Mentha arvensis* and *Vigna radiata* plants infected by RKNs. Reduced chlorophyll content with increasing infestation can be attributed to the sensitivity of pigment composition to biotic stress and impaired photosynthesis due to nematode infection (Banora and Almaghrabi, 2019).

Table 1. Effect of different inoculum levels of *M. incognita* on the chlorophyll content present in the spinach leaves at 45 dai

Group (No. of J2/pot)	Chlorophyll a (mg/g fwt. of leaf)	Chlorophyll b (mg/g fwt. of leaf)	Total Chlorophyll (mg/g fwt. of leaf)
0	1.89 ± 0.07^{a}	0.61 ± 0.05^{a}	2.50 ± 0.12^{a}
500	1.84 ± 0.08^{a}	0.58 ± 0.04^{a}	2.43 ± 0.10^{a}
1000	1.49 ± 0.17^{b}	0.46 ± 0.07^{b}	1.95 ± 0.24^{b}
2500	1.37 ± 0.20^{b}	0.37 ± 0.07^{b}	1.74 ± 0.27^{b}
5000	1.31 ± 0.13^{b}	0.37 ± 0.07^{b}	1.69 ± 0.19^{b}

Values are the mean of 3 replicates± Standard Deviation (SD) and those followed by same letter do not differ significantly by LSD at 5% significance level. $F_{(chl\ a)} = 11.18$; $P_{(chl\ a)} = 0.001$; $F_{(chl\ b)} = 10.454$; $P_{(chl\ b)} = 0.001$and $F_{(total\ chl)} = 11.345$; $P_{(total\ chl)} < 0.001$

POX and PPO Activity

Plants infected with different number of J2s showed an increase in POX and PPO activity with increasing inoculum levels (Fig. 4b and 4c). Plants inoculated with 5000 J2s showed a significantly higher level of peroxidase activity (13.67 ΔA_{420} $min^{-1}g^{-1}$fwt.) as compared to the control (8.15 ΔA_{420} $min^{-1}g^{-1}$fwt.) (Table 2). Plants inoculated with 5000 J2s showed a significantly higher level of PPO activity (0.54 ΔA_{495} $min^{-1}g^{-1}$fwt.) as compared to the control (0.19 ΔA_{495} $min^{-1}g^{-1}$fwt.) (Table 2). Lobna et al. (2017) reported the enhanced activities of peroxidase and PPO enzymes after *M. javanica* infection and these were found more in the resistant cultivar when compared to the susceptible one. Mohanty et al. (1995) observed higher activities of peroxidase and catalase enzymes in the inoculated samples than the normal in two

varieties of brinjal due to *M. incognita* infection. Higher accumulation of PPO and peroxidases were recorded in rice roots inoculated with bacteria *Pseudomonas fluorescens* and *M. graminicola* (Anita and Samiyappan, 2012). Peroxidases and polyphenol oxidase are among the different defense enzymes whose products confer resistance to the plants against pathogens. Increase in these enzymes help in lignification and suberization as a response to the infection (Langcake and Wickins, 1975). Increase in lignin synthesis thus helps in providing structural rigidity and lowers the penetration of nematodes in plants (Khanna et al., 2019). Polyphenol oxidase enzyme oxidises the phenols to quinones which cause browning of plant tissue as well as act as antimicrobial and nematicidal compounds. They provide resistance to the plant but also affect its edibility (Ahuja and Ahuja, 1980).

Table 2. Effect of different inoculum levels of *M. incognita* on the POX and PPO activity present in the spinach leaves at 45 dai

Group (No. of J2/pot)	POX Activity (ΔA_{420} $min^{-1}g^{-1}$fwt.)	PPO Activity (ΔA_{495} $min^{-1}g^{-1}$fwt.)
0	8.15 ± 0.9^{c}	0.19 ± 0.07^{b}
500	10.02 ± 2.18^{bc}	0.24 ± 0.05^{b}
1000	11.00 ± 0.94^{b}	0.32 ± 0.06^{b}
2500	13.30 ± 1.32^{ab}	0.47 ± 0.08^{a}
5000	13.67 ± 1.14^{a}	0.54 ± 0.09^{a}

Values are the mean of 3 replicates± SD and those followed by same letter do not differ significantly by LSD at 5% significance level. F_{POX}=8.353; P_{POX}=0.003 and F_{PPO}=12.507; P_{PPO}<0.001

Total Protein Content

Total protein content in plants did not vary in a particular trend with increasing J2 level (Fig. 4d). Plants with 5000 J2s showed a significant decrease in protein content (1.87 mg/g fwt.) as compared to the control (2.58 mg/g fwt.). However, plants inoculated with 1000 and 2500 J2s did not vary significantly in protein content from those with 5000 J2s. Plants that were inoculated with 500 J2s showed higher protein content than the control while those with 2500 J2s had more protein than 1000 J2 inoculum level (Table 3). Ahmed et al. (2009) also recorded a decline in protein content in the leaves of mungbean infected with *M. javanica* at 30 days after exposure suggesting the response by the plants to withstand the adverse effects of infection. However, our results are in contradiction to the observations of Meena et al. (2016) and Pandey et al. (2016) who observed an increase in the amount of total protein in the black gram cultivars and carnation plants due to *M. incognita* infection.

Total Phenolic Content

Phenol content in plants increased progressively with increasing J2 level except that 500 J2 level plants showed lower phenolic content than control (Fig. 4e). Plants with 5000 J2s showed a significant increase in phenolic content (2.39 mg/g fwt.) as compared to the control (0.887 mg/g fwt.). However, plants inoculated with 1000 and 2500 J2s did not vary significantly in phenolic content from control (Table 3). Increase in total phenol contents in okra, mung bean, different brinjal varieties and babchi due to RKN infection has been reported by Agarwal et al. (1985), Ahmed et al. (2009), Danish et al. (2018) and Nayak (2015). This increase in phenolic content can be explained as a resistance strategy of the plant to create lignins by breakdown of bound phenols or shifting of phenols to alternative pathways (Nayak, 2015).

Table 3. Effect of different inoculum levels of *M. incognita* on the total soluble protein and total phenolic content present in the spinach leaves at 45 dai.

Group (No. of J2/pot)	Total protein (mg/g fwt.)	Total phenol (mg/gfwt.)
0	2.58 ± 0.04^{ab}	0.887 ± 0.45^{bc}
500	2.62 ± 0.44^{a}	0.650 ± 0.51^{c}
1000	2.06 ± 0.20^{c}	1.269 ± 0.29^{bc}
2500	2.16 ± 0.32^{bc}	1.738 ± 0.60^{ab}
5000	1.87 ± 0.37^{c}	2.390 ± 0.53^{a}

Values are the mean of 3 replicates ± SD and those followed by same letter do not differ significantly by LSD at 5% significance level. $F_{total\ protein}$=5.131; $P_{total\ protein}$ =0.016 and $F_{total\ phenol}$ =6.146; $P_{total\ phenol}$ =0.009

All the levels of nematode inoculum densities we studied altered the physiological and biochemical parameters in the All-Green variety of spinach. Moreover, the gall index was found near 3 and 4 in all inoculum levels which indicates its high susceptibility to *M. incognita*. Decline in chlorophyll levels impair the photosynthesis in the plant thus causing lower yields as well as affecting color, nutritional quality and shelf life of spinach leaves. Since spinach is used for its leaves, increase in amounts of phenols and quinones due to enhanced PPO activity at higher inoculum densities reduces its edibility as well as economic value. Excessive phenols can lead to bitterness of spinach and quinones not only cause browning of leaves but also lead to degradation of other nutrients like vitamins and antioxidants.

The results of our study thus suggest that managing nematode densities in spinach fields is important for maintaining its quality and yield. Higher nematode densities not only affect the growth of the spinach plants but also reduces its nutrition value as well as impact the edibility of the leaves. Therefore, in order to manage the nematode

in the fields and the damage caused by them, some strategies should be undertaken such as use of organic amendments, biocontrol agents, soil solarisation, crop rotation along with the use of resistant varieties and destroying stubble after harvesting (Kaur et al., 2015; Hasan et al., 2021; Taning et al., 2022).

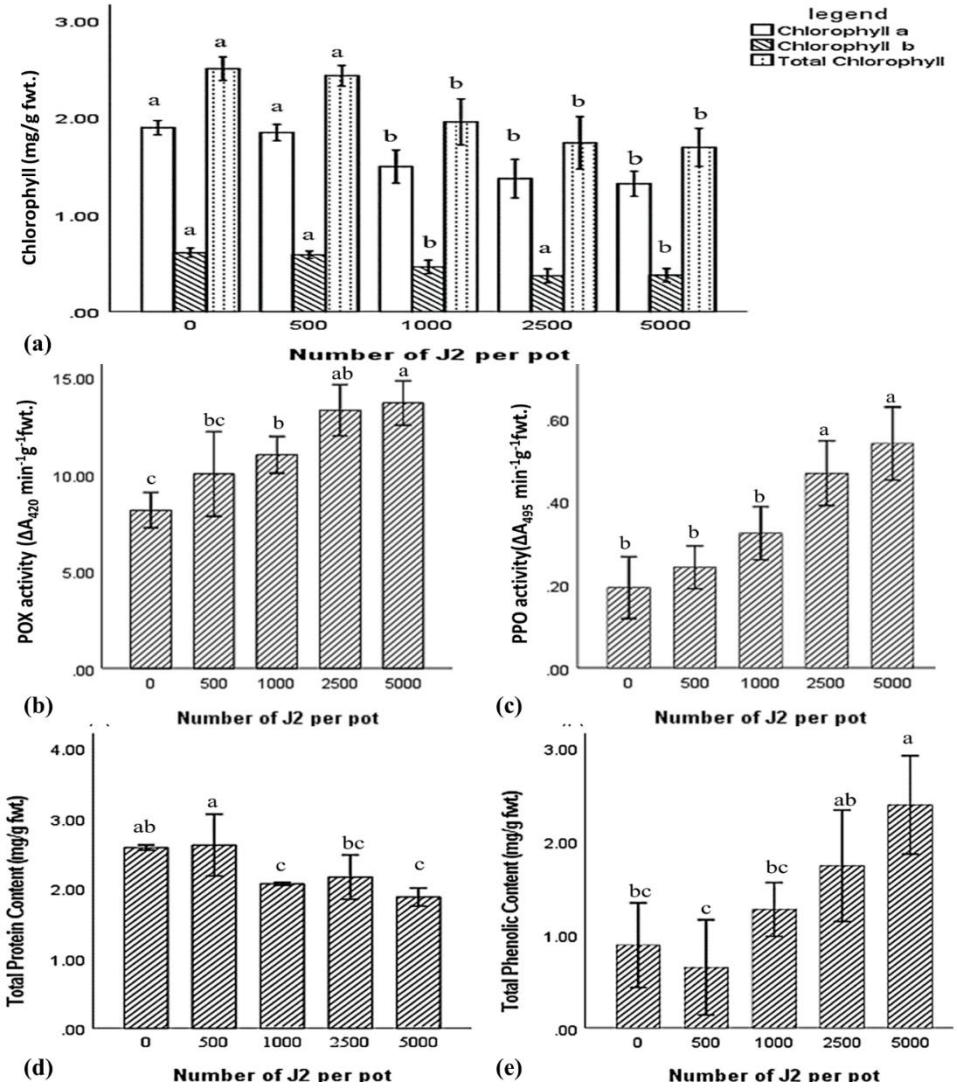

Figure 4. Changes in (a) chlorophyll content, (b) peroxidase activity, (c) polyphenol oxidase activity, (d) total protein content and (e) phenolic content in spinach leaves at 45 dai and at different inoculum levels of *M. incognita*. Bars represent the mean of 3 replicates and error bars represent SD.

CONCLUSION

From the results of the present study, it can be concluded that All Green variety of spinach is highly susceptible to *M. incognita* and the nematode can pose a serious threat to the crop. Higher population densities of *M. incognita* lead to lower chlorophyll content and a rise in activity of antioxidant enzymes like peroxidase, polyphenol oxidase and phenolic content. The increase in antioxidant enzymes is indicative of plant's defense response against the nematode. Thus, biotic stress in spinach can cause impaired photosynthesis and oxidative stress. The information obtained from this study will help in better understanding the response of spinach to RKNs and the results can suggest some biochemical markers for developing resistant cultivars of spinach.

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