Influence of pre-plant densities of *Meloidogyne incognita* on growth and root infestation of spinach (*Spinacia oleracea* L.) (Amaranthaceae) – an important dimension towards enhancing crop production

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Abstract

Vegetables represent a main source of micro-nutrients which can improve the health status of malnourished poor in the world. Spinach (*Spinacia oleracea* L.) is a popular leafy vegetable in many countries which is rich with several important micro-nutrients. Thus, consuming Spinach helps to overcome micro-nutrient deficiencies. Pests and pathogens act as major yield constraints in food production. Root-knot nematodes, *Meloidogyne* species, constitute a large group of highly destructive plant pests. Spinach is found to be highly susceptible for these nematode attacks. Though agricultural production has largely benefited from modern technologies and innovations, some important dimensions which can minimize the yield losses have been neglected by most of the growers. Pre-plant or initial nematode density in soil is a crucial biotic factor which is directly responsible for crop losses. Hence, information on pre-plant nematode densities and the corresponding damage is of vital importance to develop successful control procedures to enhance crop production. In the present study, effect of seven initial densities of *M. incognita*, i.e., 156, 312, 625, 1250, 2,500, 5,000 and 10,000 infective juveniles (IJs)/plant (equivalent to 1000cm^3 soil) on the growth and root infestation on potted spinach plants was determined in a screen house. In order to ensure a high accuracy, root infestation was ascertained by the number of galls formed, the percentage galled-length of feeder roots and galled-feeder roots, and egg production, per plant. Fifty days post-inoculation, shoot length and weight, and root length were suppressed at the lowest IJs density. However, the pathogenic effect was pronounced at the highest density at which 43%, 46% and 45% reduction in shoot length and weight, and root length, respectively, was recorded. The highest reduction in root weight (26%) was detected at the second highest density. The Number of galls and percentage galled-length of feeder roots/per plant showed significant progressive increase across the increasing IJs density with the highest mean value of 432.3 and 54%, respectively. The two shoot growth parameters and root length showed significant inverse relationship with the increasing gall formation. Moreover, the shoot and root length were shown to be mutually dependent on each other. Suppression of shoot growth of spinach greatly affects the grower's economy. Hence, control measures are essentially needed to ensure a better production of spinach via reducing the pre-plant density below the level of 0.156 IJs/cm^3.

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Introduction

Spinach (*Spinacia oleracea* L.) (Amaranthaceae) is considered as a nutritionally powerful food which is a good source of dietary potassium, magnesium and of iron as well as several vitamins. It is largely cultivated in the southern wet zone of Sri Lanka on a commercial scale as well as in back-yard gardens. It has been reported that root-knot nematodes, *Meloidogyne* spp. are one of the most common and damaging nematodes on spinach in several countries, limiting production (Potter & Olthof, 1993; Castillo & Jiménez-Díaz, 2003). *Meloidogyne* spp. is among the top major pathogens affecting the global agricultural industry (Sasser & Freckman, 1987; Eisenback & Triantaphyllou, 1991). They cause serious losses in vegetable cultivation, particularly in tropical and sub-tropical regions where environmental conditions favour their growth, survival and distribution. Sikora and Fernandez (2005) reported more than 30% of yield losses in highly susceptible vegetable crops. The life cycle of *Meloidogyne* nematodes constitutes six developmental stages, egg, four juvenile stages and the adult. Second-stage juveniles, hereafter named as infective juveniles (IJs), initiate infestation, via entering the root systems in soil. Once inside the root tissues, they establish permanent feeding sites called giant cells (Hussey & Grundler, 1998), leading to characteristic gall formation. As a result, water and nutrient uptake of the plants is disrupted which ultimately affect the growth and yield (Williamson & Hussey, 1996). However, infestation at the young stage is found to be lethal to host plants. Worldwide, four species, i.e., *Meloidogyne javanica* (Treub), *M. arenaria* (Nealare), *M. incognita* (Kofoid et White) and *M. hapla* (Chitwood) are of great agronomic importance and these species account for at least 90% of total damage caused by this nematode group (Castagnone-Sereno, 2002). *Meloidogyne* species, *M. incognita, M. javanica* and *M. arenaria* are commonly found in tropics, whereas *M. hapla* is more prevalent in temperate countries or at higher altitudes in warmer countries (Eisenback & Triantaphyllou, 1991). In Sri Lanka, altogether six *Meloidogyne* species has been recorded among which *M. incognita* Kofoid and White (Nematoda: Tylenchidae) is predominant, causing severe damage in vegetable cultivation (Ekanayake & Toida 1997; Ekanayake, 2001; Premachandra et al., 2011). It has been reported that *Meloidogyne* spp. are one of the most common and damaging nematodes on spinach in several countries limiting production (Potter & Olthof, 1993; Castillo & Jiménez-Díaz, 2003). In Sri Lanka too, spinach is a highly susceptible crop for *Meloidogyne* nematodes, in particular, *M. incognita* (Ekanayake et al., 1988; Premachandra et al., 2006).

Once *Meloidogyne* nematodes invade a crop field, their control is challenging because of their short generation time, high reproductive capacity and difficulties in identification due to the insidious nature. The damage caused by these nematodes depends on the root penetration of IJs and subsequent development and reproduction within the root tissues (Shahab & Sharma, 2011). Since IJs invade roots in soil, pre-plant density of IJs in soil is the most crucial factor determining the extent of damage (Patel et al., 1996; Khan et al., 2006; Greco & Di Vito 2009; Pang et al., 2009). Low to high densities of IJs in soil cause differential effects on host plants. Previous reports indicated that low IJs densities enhanced the growth and yield (Madamba et al., 1965; Olthof & Potter, 1972), caused serious damage (Barker & Olthof, 1976) and/or had zero effect (Madamba et al., 1965). However, some researchers stated that the growth of a plant is inversely proportional to the initial population density of *Meloidogyne* species (Kinloch, 1982) implying that high densities cause severe growth impairment, high yield reductions or sometimes death of the host plants (Griffin, 1981; Fortnum et al., 1991; Mekete, et al., 2003). However, a mere presence of IJs in soil does not cause crop damage and yield reductions as the IJs density does not reach minimal or threshold density (Schomarker & Been, 2006). It has been reported that the threshold density differs with nematodes species/strain, host plant species/cultivar and the environmental conditions (Barker & Olthof, 1976). When the nematode population exceeds the economic threshold, their control is a difficult task. Hence, information on effects of different initial densities of root-knot nematodes on the growth and infestation of economically important crops are vital to predict crop losses, establish damage thresholds and design effective nematode management programs (Ferris, 1978; Korayem, 2006). In addition, information on the response of plants to different pre-plant IJs provides powerful clues to develop nematode resistant plants and thus serve as cornerstones in resistance breeding programs.

Despite its great importance, information on the degree of damage caused by *Meloidogyne* nematodes on spinach at different pre-plant nematode densities is lacking. This paper elucidates the effect of seven initial densities of IJs of *M. incognita* on the growth and severity of infestation on spinach with the aim of adopting successful management strategies to ensure better production.

Materials and methods

The trials were conducted in a screen house at the Department of Zoology, University of Ruhuna, Matara in southern Sri Lanka. *Meloidogyne incognita* cultures were maintained on spinach plants in outdoor plastic pots (84 cm diameter; 20 cm height) containing steam-sterilized soil (sand 85%, clay 1.28%, silt 11.3% and organic matter 2.8%). The initial inoculum was derived from a single egg mass of *M. incognita*, collected from field-grown spinach plants. Mature egg masses were obtained by teasing off the knotted roots of two month-old plants. Subsequently, the egg masses were set to hatch in glass cavity blocks containing sterile distilled water (SDW) at the ambient temperature, 30° C ±2. Inoculum was prepared by concentrating 24-72 h-old IJs using a mesh sieve with 25 µm pore size. The seeds of spinach cv. "Yodha" obtained from the Department of Agriculture, Matara, Sri Lanka) were sown in plastic pots (capacity 1000 cm^3) containing 1000 cm^3 of steam- sterilized soil (sand 85%, clay 1.28%, silt 11.3% and organic matter 2.8%) and compost mixture (3:1). The pots were arranged in a completely randomised design with five replicates each on benches of the screen house. The seedlings were allowed to grow for four weeks. The temperature prevailed in the screen house during the study period ranged from 28-30° C. At four weeks post-germination, plants were inoculated with IJs at the densities of 156, 312, 625, 1250, 2500, 5000 and 10000 IJs per plant (i.e., per pot) in 15 ml SDW. The plants received only 15 SDW served as untreated controls. At fifty days post-inoculation, the plants were uprooted and the root system was washed gently with tap water to remove adhering soil. Subsequently, length and weight, of both shoots and roots, total number of feeder roots over galled-lateral roots, were recorded. In addition, number of galls and egg masses per root system was recorded. The number of egg-masses per root system was counted on stained roots with Phloxine B. Moreover, the length of all feeder roots and the galled-length of these roots were recorded per plant. After summing the total length and galled-length of the feeder roots separately, an overall percentage of galled-length feeder roots were calculated per plant. Effect on growth by *M. incognita* was evaluated based on the shoot and root length, and shoot and root weight while the damage severity was estimated using percentage infested feeder roots and galled-length of feeder roots and, total number of galls and egg masses, per plant.

Statistical analysis

After confirming the homogeneity of variance between the repeated trails using Brown and Forsythe's test (SAS institute, 1999), the data were combined for the further analysis. The data on shoot length and weight, and root length and weight, the number of galls and egg masses per root system were subjected to $\log_{10}$ transformation while percentage galled-length and infested feeder roots plant with arcsin square root transformed, prior to the analysis. The differences in shoot and root growth parameters between the non-inoculated and inoculated plants were compared using Dunnett's test (SAS institute, 1999). In addition, all the growth and infestation parameters with respect to different IJs densities were compared using one way ANOVA. When the analysis of variance yielded significant F values, means were compared using Tukey's range test. Linear regression analysis was performed to determine the relationship between the growth parameters and gall formation as well as shoot and root growth parameters (SAS institute, 1999). In all analysis, a 0.05 alpha level of significance was used.

Results

At 50-day post-inoculation, shoot length and weight as well as root length of inoculated spinach plants were significantly lower (P<0.0001) than those of non-inoculated plants at all the inoculum levels tested (Table 1). However, a significant reduction (P<0.0001) in root weight was observed above 156 IJs/plant. Percentage reduction in all growth parameters of inoculated spinach plants increased

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Figure 1a: Appearance of shoot of spinach plant at the highest nematode density, 10000 IJs/plant

Figure 1b: Appearance of shoot of spinach plant at the zero nematode density

Table 1: Growth parameters of four-week old spinach plants at 50 days post-inoculation with different pre-plant densities of *Meloidogyne incognita* juveniles

Inoculum level (IJs/pot)	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Root weight (g)
0	33.62 a	52.27 a	12.70 a	15.79 a
156	31.03 b	43.76 b	11.53 b	14.97 ab
312	30.84 b	42.16 b	11.49 b	14.21 bc
625	28.14 c	41.05 bc	11.05 b	13.42 cd
1250	25.74 d	38.62 cd	9.80 c	12.59 de
2500	24.70 d	36.57 d	9.23 c	11.95 e
5000	20.87 e	31.38 e	7.98 d	11.71 e
10000	18.70 f	28.12 f	7.04 e	12.47 e

Means followed by the same letter in columns indicate no significant differences (P=0.05, Tukey's Honest significant test, after log transformation: SAS Institute 1999).

progressively with the increasing IJs density level. In comparison to non-inoculated plants, the highest reduction in shoot length and weight was recorded as 43% and 46%, respectively, while root length and weight was reduced by 45% and 25%, respectively. Significant differences among the different Ijs densities were detected with respect to all growth parameters tested (shoot length F=103.09; shoot weight F=8.72; root length F=85.77; root weight F=29.13, df=6,63, P<0.0001). There was no significant decrease in shoot length and root weight at the two lower densities, i.e., 156 and 312 IJs/plant. In addition, shoot weight and root length did not show significant decrease from 156-625 IJs/plant (Table 1). In contrast, at the three higher IJs densities, i.e., 2500, 5000 and 10000 IJs/plant, a significant and progressive reduction in shoot length and weight, as well as root length was observed. Root weight showed a non-significant increase at the highest density, i.e., 10000 IJs/plant, compared to

2500 and 5000 IJs/plant. At the highest density, all the leaves of spinach plant turned into yellow in colour (Figure 1a and1b).

Total number of galls and percentage galled-length of feeder roots showed a significant and progressive increase (galls: F=757.64, df=6,63, P < 0.0001; galled-length F=168.18, df = 6,63, P < 0.0001) with the increasing IJs density (Table 2). The maximum mean number of galls/plant was 432 (±2.17) (Figure 2a and 2b) while the highest galled-length was found as 54% (±1.11)/plant. One Hundred percent of the feeder roots had galls above the IJs density of 1250 IJs/plant. Total number of egg masses on *M. incognita* per plant was significantly and progressively increased (P<0.0001) up to the density level of 5000 IJs/plant, and further increase in IJs density resulted in significant reduction in egg production (Table 2).

Figure 2a: Appearance of root system of spinach plant at the highest nematode density, 10000 IJs/plant

Figure 2b: Appearance of root system of spinach plant at the zero nematode density

Table 2: Root infestation of spinach plants at 50 days post-inoculation with different pre-plant densities of *Meloidogyne incognita*

Inoculum level IJs/pot	Total number of galls/root system	Percentage galled- length of feeder roots	Percentage galled-feeder roots	Total number of egg masses/root system
156	221.20 a	13.71 a	89.18 a	77.00 a
312	249.10 b	18.90 b	94.30 a	88.60 b
625	344.90 c	28.01 c	99.58 b	115.70 c
1250	361.70 d	34.68 d	100.00 b	133.40 d
2500	394.10 e	41.68 e	100.00 b	161.10 e
5000	414.60 f	47.24 f	100.00 b	181.90 g
10000	432.30 g	53.98 g	100.00 b	171.70 f

Means followed by the same letter in columns indicate no significant differences (P=0.05, Tukey's Honest significant test, after log transformation: SAS Institute 1999).

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Regression analysis showed an inverse linear relationship between gall count and shoot length (F=161.98, R^2=0.70, P<0.0001), shoot weight (F = 107.60, R^2 = 0.61, P<0.0001) and root length (F = 125.31, R^2 = 0.65, P<0.0001). Similar trend was found with the percentage galled-length (shoot length F=247.11, R^2=0.78, P<0.0001; shoot weight F=174.76, R^2=0.72, P<0.0001; root length F=190.95, R^2=0.73, P<0.0001). In addition, shoot growth showed a strong linear relationship (shoot length F=552.89, R^2 = 0.89, P<0.0001; shoot weight F= 263.48, R^2 = 0.79, P < 0.0001) with the root length.

Discussion

The damage caused by root-knot nematodes, *Meloidogyne* species, on various crops depends on species or physiological race and in particular, the magnitude of nematode population densities in soil at the time of planting (Sasanelli, 1994). In order to guarantee a better crop production, plant growers essentially need information on the pre-planting (initial) nematode population densities in soil prior to establishing their crops. Additionally, the response of crops for varying initial IJs densities of *Meloidogyne* nematodes is of crucial importance to apply control measures in advance to minimize the yield losses. The findings of this study showed that the variation in growth and root infestation of spinach plants in response to series of initial population densities of *M. incognita*. The response of growth to different nematode inocula was determined based on four parameters, i.e., shoot and root length and, shoot and root weight. More often root infestation caused by *Meloidogyne* nematodes is assessed in terms of gall index which is a measure of number of galls per root system. However, such a parameter does not provide accurate estimate mainly because of the size of galls varies with the existing nematode densities in the root tissue the plant species that they infested (Eisenback and Triantaphyllou, 1991). Previous investigations reported that the galls produced by *M. incognita* on spinach roots varied in size (Vito et al., 2004). Moreover, Azam et al. (2011) reported that the size of the galls formed by *M. incognita* at lower densities (500 IJs/plant) was smaller than those produced at higher densities, i.e., 1000 and 3000 IJs/plant, on tomato. As such, in the present study, in addition to the gall count, proportion of galled-length of feeder roots and infested feeder roots per plant were determined with respect to each of the seven IJs densities. The findings of this study clearly indicated that *M. incognita* was highly pathogenic to spinach resulting suppression of shoot and root growth even at the lowest IJs density i.e., 156 IJs/plant, i.e., 0.156 IJs/cm^3 soil (Table 1). However, the pathogenic effect was more pronounced (44 %-46 % growth reduction) at the highest IJs density for all the growth parameters, except the root weight. Declined shoot growth greatly affect the marketable value of spinach as it is a leafy vegetable. The reduction in root weight was lower compared to other three growth parameters. The slight elevation of the root weight at the highest IJs density might be caused by the severe root galling making the roots bulky (Barker & Olthof, 1976; Charegani et al., 2012). However, it is clear, that the length of the root is curtailed by 9-46 % implying the retardation of root growth by *M. incognita*. Similar to our findings, previous investigations revealed that *M. incognita* and *M. hapla* significantly reduced the growth of spinach plants (Potter & Olthof, 1974; Pankaj et al., 2001; Vito et al., 2004). Vito et al. (2004) indicated that the tolerant limit of fresh top weight in spinach (cv. Symphony) for *M. incognita* was 0·25 IJs/cm^3 which was bit higher (in this study 0.156 IJs/cm^3) compared to the present study. In addition, the same authors reported that an initial IJs density higher than 32 IJs/cm^3 soil, was lethal to spinach (cv. Symphony). However, such a high IJs density was not included in our trials. The variations in growth could most probably be caused by species/cultivar differences of a particular crop, virulence of species/strains as well as climatic conditions. The three parameters used to evaluate the root infestation clearly showed the severity infestation over different IJs densities of *M. incognita*. In agreement of the findings reported by previous researchers, gall count was found to be increased significantly with the increasing inoculum level (Kankam & Adomako, 2014; Zahid et al., 2001; Mekete et al., 2003). The galled-length of the feeder roots also showed the similar trend (Table 2). At the highest inouclum level, gall number per root system was two-fold from the lowest whereas overall galled-length of feeder roots was four-fold compared to the lowest level implying gall number should not be a sole parameter to estimate the root infestation. Infestation of all the feeder roots of the root system beyond the density level of 1250 IJs/plant confirms the severity of infestation and thus it also serves as a good indicator of root damage. Severe root infestation can partly be caused the reduction of the root length which interrupted the functioning of the root system. The decreased shoot length and weight as well as root length with increasing gall number and galled-length of feeder roots indicated that the reduced top growth could most probably be associated with gall formation. Anwar and Din (1986) reported that *Meloidogyne* infestations lead to decrease the uptake of water and nutrients which in turn cause suppression of the top growth. In addition, our findings revealed that shoot and root length was mutually dependent on each other confirming the reduced top growth with the deceasing root growth (Anwar & Van Gundy, 1993). Apart from that, exploitation of nutrients by the female nematodes in galled-roots could also be caused shoot growth impairment. Moreover, Taylor and Sasser (1978) revealed Meloidogyne infestations induce increased protein synthesis in galls and disruption of growth regulators between the roots and stems resulting in reduced growth. The declined egg production at the highest IJs density might be due to the destruction of tissues (Ferris, 1985). Moreover, Olthof and Potter (1977) reported increased root weight at higher density levels discouraged the nematode reproduction. In agreement with the findings of this study, previous researchers reported lower reproduction potential at higher inoculation levels in spinach infested by *M. incognita* (Vito et al., 2004).

Conclusions

M. incognita caused damaged on spinach plants even at a density level as low as of 0.156 IJs/cm^3 in soil, which might be induced by the warm climatic conditions prevailed in southern Sri Lanka, cultivar susceptibility or high virulence of the nematodes species or their interactive effects. It is of great importance to take steps for not allowing these nematodes to enter the crop as it can cause adverse effects at a low density level. The response of root weight to different nematode densities showed a substantial deviation, compared to other growth parameters. Thus, root weight was not seemed to be a good parameter for such evaluations. However, the two infestation parameters, i.e., proportion of galled-length of feeder roots and infested feeder roots per plant, showed the actual root damage which did not express by the gall number. Altogether, the three infestation parameters clearly reflected the severe root damage which caused the top growth impairment. In addition, continuous monitoring of soil is highly recommended for the presence of IJs and it is essential to keep the IJs density below 0.156 IJs/cm^3 soil. Moreover, in order to have estimates of pre-plant IJs densities, it is of great importance to develop accurate sampling techniques. Further trials are needed for the confirmation of these findings under field conditions.

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Conflict of Interests

The authors hereby declare that there is no conflict of interests.

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