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Suppression and management of *Meloidogyne incognita* in soil using *Trichoderma harzianum* NFCF160 and *Trichoderma virens* Isf-77

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ABSTRACT

Aims: *Meloidogyne incognita* adversely affects numerous crop plants worldwide. Therefore, the modern world has been moving towards biocontrol methods to prevent nematode attacks. This study was aimed to (i) investigate the potential use of *Trichoderma harzianum* NFCF160 and *T. virens* Isf-77 in managing *M. incognita* in soil and (ii) identify trapping mechanisms employed by both *Trichoderma* strains to suppress *M. incognita*.
Methodology and results: Three weeks old, *Basella alba* L. plants were subjected to five different treatments. The above and below ground growth parameters and the galling indices of these plants were measured every four weeks for three sampling times. Trapping mechanisms employed by *Trichoderma* strains were examined following plate assays. Plants treated with *T. harzianum* NFCF160 and *T. virens* Isf-77 had significantly higher values for the total number of leaves (34 ± 2.84) and (27 ± 2.61), fresh weight of the shoot (81 ± 9.51 g) and (91 ± 9.70 g), dry weight of the shoot (71 ± 5.24 g) and (62 ± 5.81 g), respectively eight weeks after inoculation of *M. incognita*. Significantly low galling indices (2 and 2) were recorded in *B. alba* treated with *Trichoderma* strains. Both *Trichoderma* strains exhibited various nematode-trapping mechanisms, such as non-constricting rings and adhesive spores.
Conclusion, significance and impact of study: This investigation highlighted the potential of both *Trichoderma* strains as biocontrol agents to control *M. incognita* effect in sustainable agriculture.

Keywords: Basella alba, biocontrol, *Meloidogyne incognita*, *Trichoderma harzianum* NFCF160, *Trichoderma virens* Isf-77

INTRODUCTION

Root-knot nematodes (*Meloidogyne incognita*, Family – Heteroderidae) are widely spread plant pathogens that affect crops and ornamental plants worldwide. They are ranked top on the list of animate pathogens affecting the production of economically important plants. Sri Lanka and many Asian countries have economies that rely predominantly on agriculture. Thereby, pathogenic threats to commercial crops can bring about substantial economic losses to a country's economy. In Sri Lanka, there are several reported incidences of *M. incognita* attacks on different commercially essential crops. Specifically, ornamental plants, leafy vegetables and yams are at the highest risk (Premachandra, 2007). The most prominent and direct disease symptom of plants attacked by *M. incognita* includes the development of root galls (in various sizes) in the roots of the infected plant. The common indirect symptoms observed in plants infected by *M. incognita* include stunted growth and reduction in leaf number. The tendency of wilting is high in infected plants on exposure to warm weather (Agrios, 2005).

The frequent remediation technique employed is the usage of chemical nematicides. These chemical nematicides have been able to bring about economic benefits to the crop through the reduction in yield loss and an improved economic gross margin (Perry and Moens, 2006). Apart from the beneficial effects of the nematicides, there are many other adverse effects of using chemical nematicides in the environment. It has been found that acute exposure to pesticides is associated with adverse health effects such as cancer, respiratory diseases, Parkinson's disease and death (Van Maele-Fabry *et al.*, 2012). Agricultural workers in Sri Lanka often experience adverse health conditions caused by acute or chronic exposure to pesticides owing to their widespread use (Schreinemachers and Tipraqsa, 2012).

Considering the adverse effects of chemical nematicides on human health and the environment, the usage of biological control methods to control pests has gained the attention of scientists. One of the most

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common techniques thus used is applying nematophagous fungi (Zhang and Hyde, 2014). Research suggests that these fungal species are also equipped to enhance plants growth, including leafy vegetables (Hewavitharana and Kannangara, 2019). Relevant literature suggests that *Trichoderma* has significant potential in controlling plant-parasitic nematodes (Al-Hazmi and TariqJaveed, 2016) through physical trapping and infecting mechanisms like the production of constricting rings, adhesive knobs and also secreting nematicidal metabolites. Hence, the present study was carried out to investigate the potential of using *T. harzianum* NFCF160 and *T. virens* Isf-77 to control *M. incognita* (root-knot nematode). In this investigation, *Trichoderma* strains were selected explicitly as biocontrol agents of *M. incognita*, concerning the previous records (Al-Hazmi and TariqJaveed, 2016; Hewavitharana and Kannangara, 2019) on the growth enhancement of selected crops and ornamental plants by *Trichoderma* containing organic amendments. *Basella alba* (spinach) plants were selected as the host plants since their disease manifestations are prominent compared to the other host plants of *M. incognita*. At the beginning of the present investigation, it was assumed that *T. harzianum* NFCF160 and *T. virens* Isf-77 enhance the growth and development of *B. alba* plants by controlling the development of *M. incognita* through the employment of various trapping and infective mechanisms.

MATERIALS AND METHODS

The research was carried out at the Department of Plant and Molecular Biology, University of Kelaniya, Sri Lanka during the period from March 2021 to January 2022.The main objective of this research was to determine the potential of *T. harzianum* NFCF160 and *T. virens* Isf-77 in controlling the root-knot nematodes *M. incognita*. Moreover, the research aimed to identify the trapping mechanisms employed by both these fungal species in controlling the nematodes. In order to investigate these, the following procedure was followed.

Fungal inoculum preparation

Pure cultures of *T. harzianum* NFCF160 (KT852821.1) and *T. virens* Isf-77 (KP985643.1) were obtained from the Department of Plant and Molecular Biology culture collection. These *Trichoderma* strains, which were maintained at 4 °C, were isolated from decomposing plant litter materials from the Sinharaja tropical rainforest, Haggala montane forest and Kithulgala rain forest of Sri Lanka in previous research (Hewavitharana and Kannangara, 2019). The conidial suspension (108 mL) of *T. harzianum* NFCF160 with a density of 8.10×10^6 conidia/mL and the conidial suspension (108 mL) of *T. virens* Isf-77 with a density of 5.75×10^6 conidia/mL were prepared by flooding the Petri dishes with the relevant *Trichoderma* strains with sterilized distilled water (9 mL) separately. The suspensions were stored in the refrigerator at 4 °C until the potting mixtures were amended.

Solar sterilization of soil and its analysis for the presence of nematodes

Garden topsoil was collected randomly from an area with spinach plants devoid of root-knot nematodes in the Gampaha District, Sri Lanka. The soil was mixed thoroughly and solar sterilized (temperature at 5 °C to 55 °C and solar radiation intensity 500-650 W/m^2) for four days. The absence of nematodes in soil was further verified by following the Baermann funnel method (Taylor, 1971).

Basella alba seed collection and maintenance of seedlings

Healthy *B. alba* seeds were obtained from the Department of Agriculture, Sri Lanka and were planted in nursery trays containing solar sterilized (the temperature at 5 °C to 55 °C and solar radiation intensity 500-650 W/m^2) soil. The seedlings were maintained for three weeks.

Preparation of potting medium and plantation of *Basella alba* seedlings

Solar sterilized soil was placed separately in equal amounts (800.00 g) in 90 polythene bags (15 cm by 30 cm). The conidial suspension (10.0 mL) of *T. harzianum* NFCF160 was added to 18 bags. Similarly, for another 18 bags, *T. virens* Isf-77 conidial suspension (10.0 mL) was added. Three weeks old healthy *B. alba* seedlings were planted in polythene bags containing *Trichoderma* amended and non-amended soils. The bags were maintained in a nursery in the Gampaha district with a homogenous environment (temperature; 31 ± 2.5 °C and humidity; 71 ± 3.5%) and were watered (100 mL per bag at one time) daily at frequent time intervals (two times per day; 7.00 am and 6.00 pm).

Isolation of eggs and larvae of *Meloidogyne incognita*

Eggs and larvae of *M. incognita* were isolated from the galls in the root system of nematode infected *B. alba* plants using the method described by Hussey and Barker (1973). The density of the prepared suspension was 44 second-stage juveniles per mL.

Inoculation of *M. incognita*

Four weeks after transplanting the *B. alba* seedlings in the polythene bags, the prepared nematode suspension was inoculated into the respective potting mixtures. The different types of treatments used in the experiment were soil, soil + nematodes, soil + nematodes + nematicides (carbofuran, 62.5 ug/g of soil), soil + nematodes + *T. harzianum* NFCF160 and soil + nematodes + *T. virens* Isf-77.

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Disease assessment

Six replicate samples of plants from each treatment were uprooted four-weeks after the inoculation of nematodes. The plants' growth parameters (total number of leaves, shoot fresh weight and shoot dry weights) were measured. The galling indices were calculated based on the five-scale system of the galling index developed by Pakeerathan and Mikunthan (2009). This was repeated for another two sampling times at four-week intervals.

The presence of *T. harzianum* NFCF160 and *T. virens* Isf-77 in the potting mixture were verified at the end of each sampling time. 5 mg of soil from five samples each from *T. harzianum* NFCF160 and *T. virens* Isf-77 treated potting mixtures were placed in Petri dishes containing PDA. 0.5 mL of sterilized distilled water was added to each petri dish and soil was spread throughout the plate using a sterile spreader. The plates were then incubated at room temperature (30 °C) for five days and fungi growing from the soil were isolated into pure cultures. The existence of amended *Trichoderma* strains in the potting mixtures were confirmed by observing the growth of particular *Trichoderma* colonies in higher frequencies (more than ten colonies per plate).

Observation of the nematode-trapping mechanisms of the fungi

The nematode-trapping mechanisms were observed by introducing nematode suspension (1.00 mL) into Petri dishes containing pure cultures of *T. harzianum* NFCF160 and *T. virens* Isf-77. The trapping mechanisms employed by both fungal species were observed under the low, mid and high power of the phase-contrast microscope (Model-CX41RF, Philippines) after one day of fungal incubation.

Statistical analysis

The growth parameters and galling indices data were analyzed statistically using MINITAB (version 18) statistical analysis software. Data were subjected to a Two-way Analysis of Variance (ANOVA) and the means were compared using Tukey's pairwise comparison.

RESULTS AND DISCUSSION

B. alba plants treated with *T. harzianum* NFCF160 and *T. virens* Isf-77 showed significantly higher growth (as discussed below) than the controls (plants treated with the nematicide and the untreated plants infected with the nematodes).

Total number of leaves

The results obtained for the total number of leaves are shown in Figure 1. *Basella alba* plants treated with *T. harzianum* NFCF160 had the significantly highest number of leaves (34 ± 2.84) compared to the plants infected only with *M. incognita* (21 ± 2.76). During the third sampling time (twelve weeks), plants treated with *T. virens* Isf-77 showed the highest number of leaves (21.17 ± 4.92) compared to the plants inoculated only with the nematodes (8.27 ± 10.68). This may be because *T. harzianum* NFCF160 controlled the growth and development of *M. incognita* in the plant roots and thereby, the plant was not deprived of its necessary nutrients. As a result, the leaves showed comparatively higher growth than the plants infected only with the nematodes. The results obtained for the total number of leaves in the plants treated with nematicide, carbofuran and the plants treated with *Trichoderma* strains revealed no significant differences between the two treatments. This observation highlighted that the *Trichoderma* strains (*T. harzianum* NFCF160 and *T. virens* Isf-77) used in the present study were as effective as the commercial nematicide carbofuran in controlling root-knot nematodes.

Fresh weight and dry weight of the shoots

As per Figure 2a the fresh weight of the shoots showed significant differences within the treatments. At the second sampling time (eight weeks after inoculation of *nematodes*), plants treated only with *M. incognita* had the significantly lowest fresh weight (50 ± 6.37 g) compared to the plants treated with nematicides (97 ± 9.70 g), *T. harzianum* NFCF160 (81 ± 9.51 g) and *Trichoderma virens* Isf-77 (91 ± 9.70 g). Similarly, during the third sampling time (twelve weeks after inoculation of *M. incognita*), plants infected only with the nematodes had a significantly lower fresh weight (44.55 ± 4.00 g) than the plants treated with other treatments. Apart from that, the plants treated with *Trichoderma* had significantly higher fresh weights than the plants grown without any control treatment and nematodes (Figure 2a). The reason for this may be the ability of *Trichoderma* to increase the growth and development of the plant. This is evident from the research done by Stewart and Hill (2014), where they have mentioned that *Trichoderma* can enhance the growth and development of the plants through various growth promoters such as indole-3-acetic acid, gibberellins, brassinosteroids, ethylene, jasmonate, salicylic acid, strigolactones and cytokinins. Saba *et al.* (2012) mentioned that colonization of *Trichoderma* in the roots of the plants enhances the root growth and development as a bio controller suppressing the growth and development of many plant parasites by the production of extracellular beta-(1,3)-glucanases, chitinases, lipases and proteases which degrade the cell walls of these pathogens. Apart from that, the long-lasting colonization of root surfaces and penetration into the epidermis by *Trichoderma* prevent the entry of many plant pathogens into the root system. It has increased the plant's overall productivity, simultaneously improving the uptake and the use of nutrients. This is in line with the present research where the plants treated with *Trichoderma* showed higher shoot weights. Similarly, Figure 2b shows that the variation of the dry weight was almost similar to the variation observed for the fresh weight of the plants. During both second and third sampling times, the plants infected with *M. incognita* displayed

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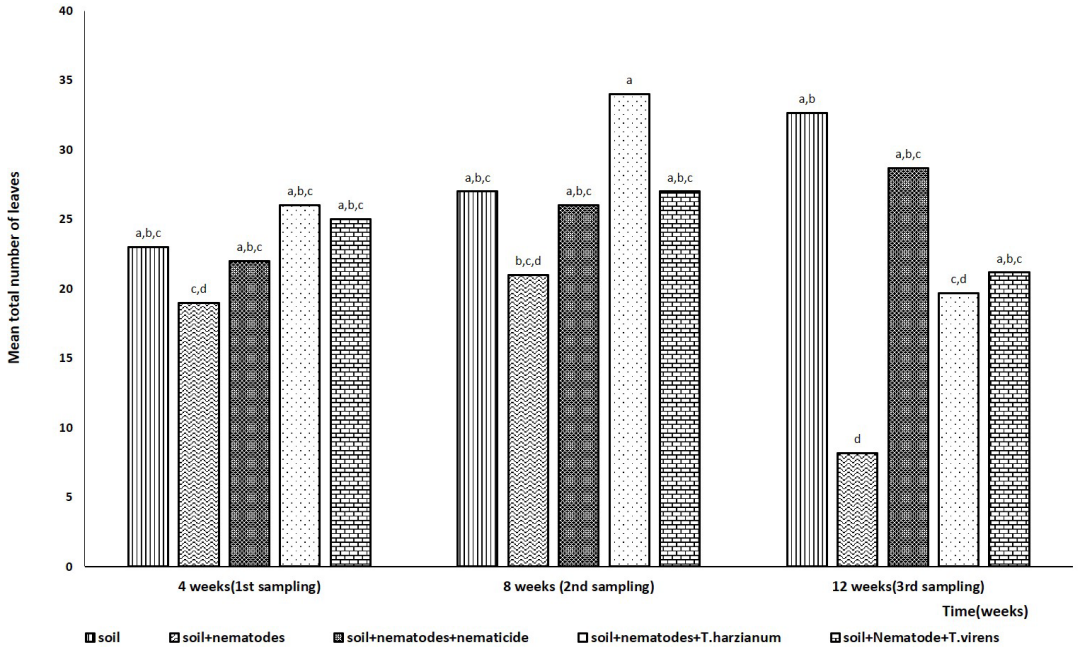

Figure 1: Total number of leaves of differently treated *B. alba* L. plants. Each data represents the mean of six replicates. Means sharing a common letter(s) are not significantly different by Tukey's multiple comparison test at $p<0.05$.

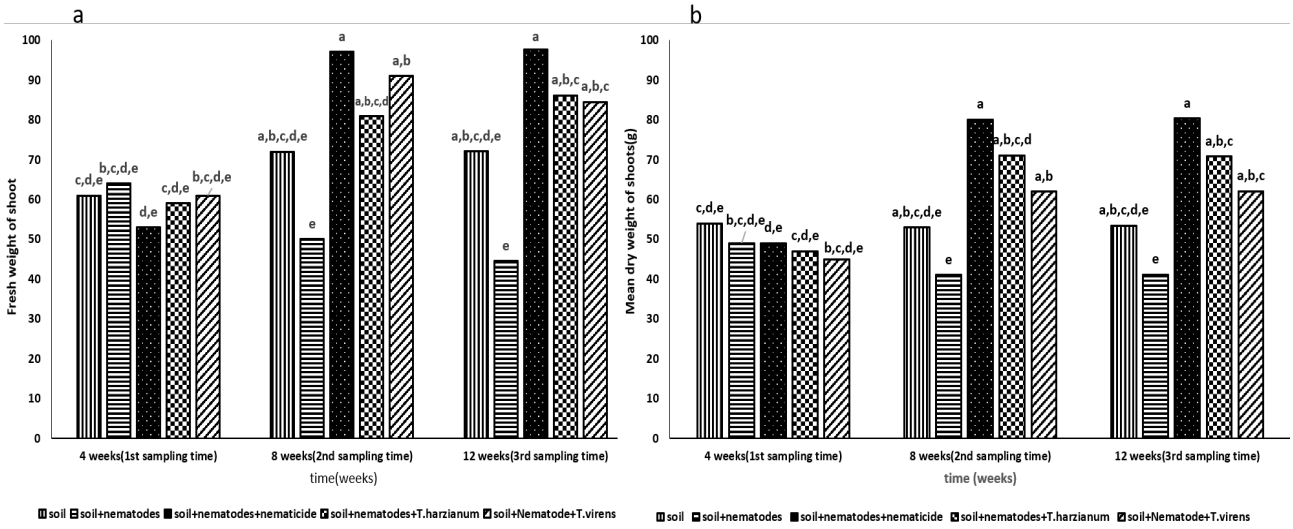

Figure 2: Effect of *T. harzianum* NFCF160 and *T. virens* Isf-77 on (a) Mean fresh weight of *B. alba* L. plants and (b) Mean dry weight of *B. alba* plants. Each data represents the mean of six replicates. Means sharing a common letter(s) are not significantly different by Tukey's multiple comparison test at $p<0.05$.

significantly lower dry weights for shoots, as evident in Figure 2b. This is due to the interruptions caused by *M. incognita* in the vascular system of *B. alba* plants which affect especially the photosynthesis and mineral uptake of plants (Agrios, 2005). A variation in the dry weight similar to the present research was observed by Khan *et al.* (2018). As per Figures 2a and 2b, it was evident that the used *Trichoderma* strains (*T. harzianum* NFCF160 and *T. virens* Isf-77) were similarly effective compared to the effectiveness of the nematicides in controlling introduced nematodes.

Number of galls and galling index

One of the most striking and characteristic below-ground features seen in the plants infected by *M. incognita* is the

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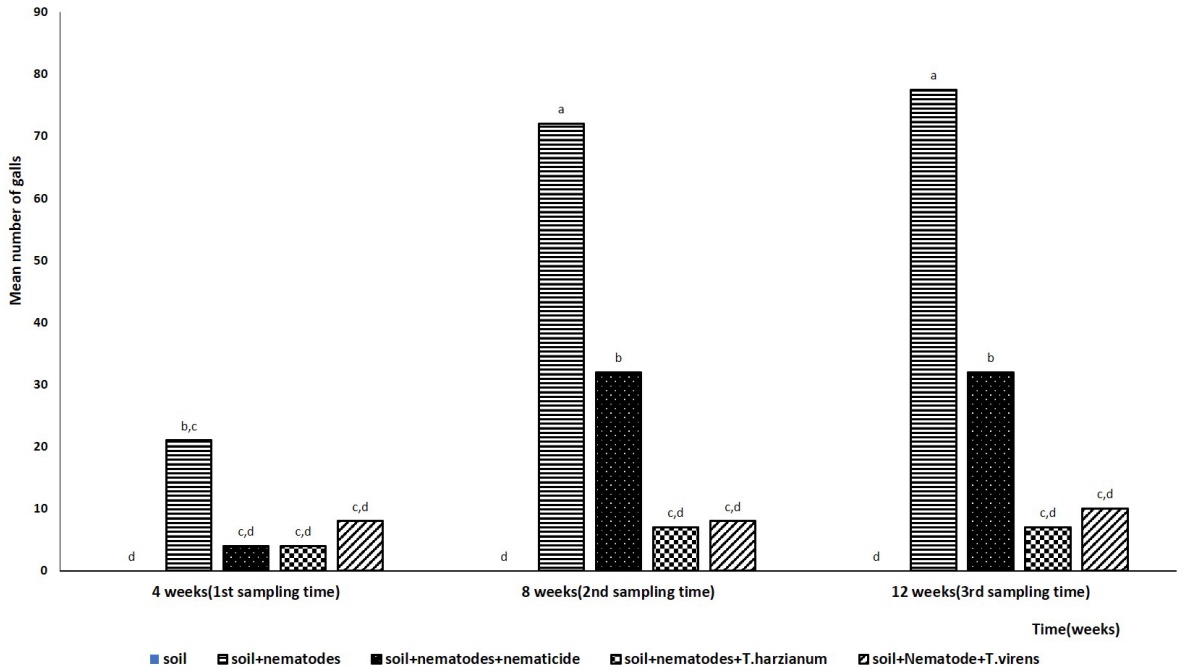

Figure 3: Effect of *T. harzianum* NFCF160 and *T. virens* Isf-77 on mean number of galls of *B. alba* plants. Each data represents the mean of six replicates. Means sharing a common letter(s) are not significantly different by Tukey's multiple comparison test at $p<0.05$.

Table 1: The galling indices of *B. alba* plants.

Sampling time (weeks)	Soil	Soil + nematodes	Soil + nematodes + nematicide	Soil + nematodes + *T. harzianum* NFCF160	Soil + nematodes + *T. virens* Isf-77
Four	0	3	2	2	2
Eight	0	4	4	2	2
Twelve	0	4	4	2	2

No galls, immune=0; 1-2 galls, resistant=1; 3-10 galls, resistant=2; 11-30 galls, moderately susceptible=3; 31-100 galls, highly susceptible=4; More than 100 galls, highly susceptible=5.

formation of root galls. According to Agrios (2005), galls usually contain about 3 to 6 giant cells formed due to the toxic substances and effector molecules contained in the saliva of the nematodes. These effector molecules in movement through plant tissues establish and maintain the feeding structures (Mitchum *et al*., 2013). Swellings of the root result in cell enlargement and division of the cells surrounding the giant cells. This may also be due to the enlargement of the feeding nematode. In line with the above facts in the present study, the formation of root galls was observed in the plants infected with the nematodes. During the second sampling time, the plants infected only with the nematodes had a significantly higher number of galls (72 ± 6.97) and the highest galling index value of 4 compared to other plants subjected to other treatments (Figure 3 and Table 1).

Similarly, during the third sampling time, plants treated only with the nematodes showed a significantly higher value for the number of galls (77.5 ± 3.32) and the highest galling index value (4) compared to the plants treated with other treatments (Figure 3 and Table 1). This highlights the fact that the *Trichoderma* can reduce the nematode infection, thereby reducing the number of galls. This is in line with the research findings of Khan *et al.* (2018). Al-Hazmi and TariqJaveed (2016) concluded that there was a significant reduction in the number of galls in plants treated with *T. harzianum* and *T. viride* than in the plants treated with nematodes alone. The number of galls observed in the *B. alba* plants subjected to different treatments are shown in Figure 3 is in agreement with their observations. Another important observation made in the present investigation was that the plants grown in soil without nematodes or other control treatment had no galls in their roots. This highlights the fact that the soil used for the experiment was properly solar sterilized and is devoid of any nematodes. Furthermore, this highlights that the nematodes in the infected roots have entered the plants only through the nematode suspension that was introduc-

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Table 2: Types of trapping mechanisms present in *T. harzianum* NFCF160 and *T. virens* Isf-77.

Fungal species	Non constricting rings	Adhesive spores	Adhesive knobs
T. harzianum NFCF160	√	√	√
T. virens Isf-77	√	√	√

√ - Represents the presence of trapping mechanisms in each fungal species.

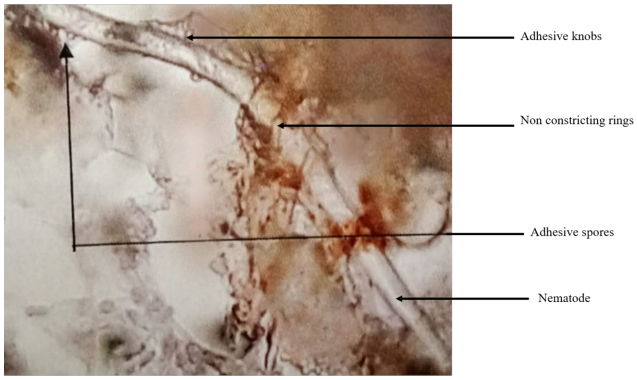

Figure 4: Non constricting rings, adhesive knobs and adhesive spores formed by *T. hazianum* NFCF160 (10×40).

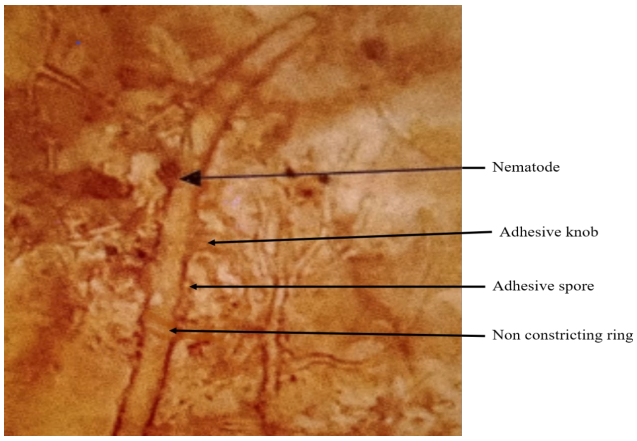

Figure 5: Non constricting rings, adhesive knobs and adhesive spores formed by *T. virens* Isf-77 (10×40).

-ed to the respective soil medium. The next interesting thing observed in the present study was, compared to the number of galls in the root system of the plants treated with nematicides (second and third sampling time), the galls observed in the plants treated with two *Trichoderma* strains were significantly lower. This highlights that the *Trichoderma* strains used in the present investigation were more effective than the used commercial nematicides, carbofuran, in suppressing the gall formation by controlling the population of nematodes. Taking all these findings into account, it can be concluded that *T. harzianum* NFCF160 and *T. virens* Isf-77 are capable of managing the root-knot nematode, *M. incognita* in potting media.

Nematode trapping and infective mechanisms employed by *Trichoderma*

Apart from the disease assessment, the various trapping mechanisms employed by the two *Trichoderma* species were also evaluated in the present study. As mentioned in Su *et al*. (2017), the trapping devices differ from the typical vegetative hyphae in two different ways and one such structural difference is the presence of numerous organelles named dense bodies in the trapping devices but not in typical hyphae. Another characteristic feature of these traps is the presence of extensive layers of extracellular polymers that are important in adhesion to the surface of the nematodes. However, the significance of the dense bodies is unknown. According to the observations of the present study, it was evident that the *Trichoderma* use different trapping mechanisms like non-constricting rings (Figure 4 and Figure 5) and adhesive knobs to trap the nematodes meanwhile producing adhesive spores as infective mechanisms (Table 2). Further, it was observed that all the trapped nematodes by *Trichoderma* strains get degraded within five to seven days.

Formation of non-constricting rings

Non-constricting rings are formed when the rigid lateral branches form the vegetative hyphae, which curve to form a thick three-celled ring. These non-constricting rings are usually found alongside adhesive knobs. The non-constricting rings of the nematophagous fungus capture the nematode with the support of an adhesive reagent that covers the ring surface and then the fungus absorbs the nourishment from the nematodes via degrading them by respective enzymes (Kendrick, 2017). Non-constricting rings are commonly seen in the fungal species *Monascoporium candidum* (Zhang and Hyde, 2014). In the present investigation, these non-constricting rings were frequently observed in both *T. harzianum* NFCF160 and *T. virens* Isf-77 (Figure 4 and Figure 5). Since these rings have not consisted with three enlarged cells as in constricting rings, their identity was confirmed as non-constricting rings.

Formation of adhesive knobs

Yang *et al.* (2007) indicated that the adhesive knob is a morphologically distinct spherical or a sub-spherical

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adhesive cell. The adhesive knobs can be either sessile on the hypha or found at the apex of a slender, erect and non-adhesive stalk composed of one to three cells. These adhesive knobs are characteristic of fungal species like *Monascoporium ellipsosporum* and *M. haptotylum* (Su *et al*., 2017). Adhesive knobs capture the nematodes by the adhesive layer present on the surface of the knobs. As discussed above, the captured nematodes are then degraded by the enzymes secreted by the nematophagous fungus. The formation of adhesive knobs to trap the introduced nematodes was observed in both *Trichoderma* in the present study.

Formation of adhesive spores and adhesive networks

The sticky spores of both *T. harzianum* NFCF160 and *T. virens* Isf-77 get adhered to the surface of the body of the nematode. After two or three days, the nematodes get infected through the germination of these spores. According to the literature, adhesive networks are the most commonly found trapping mechanism in nematophagous fungi (Zhang and Hyde, 2014; Su *et al*., 2017). The adhesive networks are formed when vegetative hyphae curve to form loops. These adhesive networks similarly capture the nematodes to the non-constricting rings. They capture the nematode through the adhesive reagents that cover the surface of the adhesive network (Su *et al*., 2017). These trapping devices are found in species like *Arthrobytrys* spp. (Zhang and Hyde, 2014). However, in the present study, these adhesive network systems were observed neither in *T. harzianum* NFCF160 nor *T. virens* Isf-77.

From the observations of the present investigation, it was evident that *T. harzianum* NFCF160 and *T. virens* Isf-77 manage and suppress the spread of nematodes by employing the trapping as mentioned above and infective mechanisms (production of non-constricting rings, adhesive knobs and adhesive spores). Eventually, these fungi would parasitize on the nematode, finally leading them to death, as mentioned by Herrera-Estrella *et al.* (2016). Therefore, these two fungal species can be used as effective biocontrol agents in sustainable agriculture. When applying this fungal consortium as a bionematicide, many field experiments should be conducted to evaluate the effect of environmental factors on the activity of fungal species in the nematode controlling. As per the present results, while controlling the *M. incognita*, these two fungal species enhanced the growth of a particular crop. It is an added advantage for the farmers who will use this as a bionematicide.

CONCLUSION

The observation of fewer galls and the comparatively high shoot weights shown by the respective *B. alba* plants from the present study revealed that both *Trichoderma* strains have vast potential in managing the *M. incognita* infestations in *B. alba* plants. The current study results highlighted that both *Trichoderma* strains (*T. hazianum* NFCF160 and *T. virens* Isf-77) have used efficient mechanisms like the non-constricting ring and adhesive spores to trap and infect *M. incognita*. Further research and development are recommended for the commercial application of these microorganisms (*Trichoderma*) as biocontrol agents in the future.

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CONFLICT OF INTERESTS

The authors do not have any conflicts of interest.

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AUTHOR CONTRIBUTION STATEMENT

Nithini Rajakaruna: Experiment performance, data collection and analysis, preparation of the first draft of the manuscript; Lanka Undugoda: Data analysis, preparation of the first draft of the manuscript; Sagarika Kannangara: Conceptualization of the study, experimental design, article review; Krishanthi Abeywickrama: Contribution towards improving the experimental design, final review of the article.

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