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Effective and Promising Strategy in Management of Tomato Root-Knot Nematodes by *Trichoderma harzianum* and Arbuscular Mycorrhizae

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Abstract: The ecosystem is considerably affected due to the extensive use of chemical pesticides and fertilizers. As an alternative strategy, this study aimed to assess the biocontrol potential of the bioagents arbuscular mycorrhizal fungi and plant growth-promoting *Trichoderma harzianum* MZ025966 against tomato root-knot nematodes (*Meloidogyne javanica*). *T. harzianum* showed a great potentiality to produce indole acetic acid (IAA) (12.11 ± 2.12 µg/mL) and exhibited a noticeable activity of ammonification. Furthermore, *T. harzianum* revealed protease and lipase enzymatic activity of 28.36 ± 2.82 U/mL and 12.30 ± 0.31 U/mL, respectively, which may illustrate the control mechanism of nematode eggs and juveniles. As in mycorrhizal and/or *T. harzianum* inoculated tomato plants, the penetration rates of nematodes, as well as the number of juveniles, females, egg mass, and galls were significantly reduced. The lowest number of juveniles was observed in the case of either single mycorrhizal inoculation (45%) or in combination with *T. harzianum* (55%). The enzymatic activity of glutathione peroxidase and catalase was enhanced in tomato plants inoculated with the bioagents to overcome the negative impact of nematode parasitism. Our results proved that the application of biocontrol agents not only reduced the nematode population and penetration rate but also improved the plant growth, increased the nutritional elemental content and stimulated the plant's systematic resistance.

Keywords: biocontrol agent; plant systematic resistance; arbuscular mycorrhizal fungi; *Trichoderma harzianum*; *Meloidogyne javanica*

1. Introduction

Nematodes, or roundworms, the most abundant animals in the world, constitute the phylum Nematoda. Nematodes occur as parasites in plants, animals, or as free-living forms in different habitats (marine environment, freshwater, and soils). Root-knot nematodes (genus *Meloidogyne*) (RKN) are endoparasites of roots and have been assessed to cause around USD 173 billion of annual damages to crops planted worldwide [1]. Nematode larvae infect the plant root system and develop root-knot galls that exhaust the plant's nutrients and photosynthate. Plant yields are reduced by nematode infection, while infection may be lethal in young plants [2]. Furthermore, nematodes are likely to be undervalued, because farmers are often oblivious of their presence because the plant symptoms caused by nematode infection are oftentimes non-specific, so it is difficult to attribute losses in crop to nematode infection [3]. Meanwhile, further losses might be correlated to the quality of

food and morphological imperfections associated with nematode infection symptoms [4]. Nowadays, there is an increasing requirement for agricultural products from the growing population [5], and this can be met by optimizing the productivity potential and by controlling the crop losses caused by the plant-parasitic nematode [6]. Chemical nematicides are used to control nematode infection in agriculture, but they have adverse and toxic effects on human health, bioflora, animals, and are constituted of contaminants for the environment [7]. Hence, the need to use an effective and ecofriendly strategy for nematode management is increased and intensified with the reduction of the use of pesticides due to EU regulations (EC No1107/2009).

Nematode biological control occurs by reducing nematode infection and/or regulation of its populations through the activity of organisms that are antagonistic to them [8]. These organisms can interact with nematodes directly through antibiosis and competition for space or nutrients or interact indirectly with plant pathogenic nematodes by inducing resistance in the host plant [8,9]. Plant-growth-promoting fungi (PGPF) can act as efficient and ecofriendly nematode biocontrol, as well as biofertilizers for plant growth and yield improvement. It was reported that *Trichoderma* and mycorrhizal fungi are the most significant PGPF that were studied and used as bioagents against root-knot nematode as resistance inducers [8].

Arbuscular mycorrhizal fungi (AMF) are obligate root symbionts of, primarily, vascular plants. AMF are mainly used as biofertilizers, which can form symbiotic interactions with 90% of crop plants [10]. The biocontrol impact of mycorrhizal fungi has been observed against a wide range of phytopathogenic microorganisms [11,12]. Nematode infection decreased in mycorrhizal plants, and it is not clear if the reason for this reduction was due to systematic plant resistance or a direct effect [8], but the direct mechanisms used by AMF against nematode population or infection are not yet distinguished as they act only through the host plant (an indirect mechanism). Mycorrhizal fungi improve plant resistance through many activities such as the increase in plant nutrient uptake, the alteration in root morphology and structure, and the alteration of the rhizosphere interaction that makes the host plants more competitive for space and nutrients than other plants [13]. Moreover, mycorrhizal fungi induce systematic resistance (ISR) in plants against infection by pathogens and/or pests [14,15]. Mycorrhizal fungi stimulate the production of antioxidant enzymes, accumulation of non-enzymatic compounds inside cells of host plants, reduction in malondialdehyde production, the activation of enzyme-encoded genes involved in the biosynthesis of lignin, and in the shikimate pathway which produces forebears of different aromatic secondary metabolites against nematodes [16–19].

Trichoderma species were recognized as widely applicable microorganisms in agricultural technology as a biological control agent for many plant pathogens that inhabit plant-soil ecosystems. Furthermore, *Trichoderma* showed many unique properties including the ability to colonize plant roots [20], being easily culturable and propagated, improving plant growth and disease resistance [21], and improving nutrient utilization efficacy [22] and, consequently, improving its efficiency and applicability in agriculture and sustainable cropping systems. Interestingly, *Trichoderma* spp. were investigated by many authors for their potentiality in controlling plant pathogenic nematodes [23]; however, there is little information concerning the mechanisms of *Trichoderma*–nematode plant interactions.

So, the main aim of the current study was to estimate the potential application of microbial bioagents (arbuscular mycorrhizal fungi and *T*. *harzianum*) as biocontrol agents and biofertilizers to manage tomato root-knot nematodes (*Meloidogyne javanica*). Here, we studied the possible mechanisms that occur between the two bioagents and the host plant and subsequent root-knot nematode infection.

2. Materials and Methods

2.1. Nematode Inoculum

The *Meloidogyne javanica* nematode population was isolated from tomato plants (*Lycopersicon esculentum* L.) grown in an open field and maintained on susceptible host

tomato plants in the greenhouse. For nematode inoculum preparation, the tomato root system was separated from the whole plant and washed with water to remove soil adhesion. Egg masses of *M. javanica* were recovered from galls of the infected tomato roots and stirred for 4 min in 0.5% NaOCl [24]. Nematode egg suspensions were sieved through a 500 mesh sieve (25 μm) and collected in sterilized water. The second stage juveniles (J2) were obtained from the incubated eggs (after 3–5 days) using a modified Baermann funnel method [25]. *M. javanica* inoculum was used for the pot experiment consisting of 3000 ± 5 J2 dispensed in 50 mL of water.

2.2. Arbuscular Mycorrhizal Inoculum

The propagules of AMF were recovered from tomato rhizosphere soils by the wet sieve method [26]. The identification of AM fungal spores, recovered from rhizospheric soil, was performed by picking up the AMF spores from the filter paper with the help of syringe or fine point camel brush and mounting them on a glass slide with a drop of polyvinyl lactophenol (PVL) and a cover slip, then they were examined using a light microscope at $40\times$. Subsequently, the recovered spores were identified with the help of a taxonomic manual [27]. The most abundant mycorrhizal species were *Acaulospora bireticulata* F.M. Rothwell & Trappe, *Entrophospora infrequens* (Hall) Ames & Schneid, *Funneliformis geosporum* (Nicolson & Gerd.) Walker & Schüßler, *Funneliformis mosseae* (Nicolson & Gerd.) Walker & Schüßler, and *Gigaspora margarita* Becker & Hall.

The inoculum of mycorrhizal fungi used in the greenhouse experiments (most common mycorrhizal species from tomato plants rhizosphere) was bulked up in pot sterilized soil and grown with maize (*Zea mays* L.) as the trap culture. After complete life cycles of AMF (about 2 months), the maize roots were separated from the whole plants. Root segments were mixed with the soil of the experimental pots. The mycorrhizal fungal inoculum (100 g) contained mycorrhizal hyphae, colonized root segments, and 10 ± 2 spores/g soil was used as a bio-inoculating agent for tomato plants. In the non-mycorrhizal pots, an equivalent amount (100 g soil) of sterilized soil was added. The soil was sterilized to kill any spores of microorganisms and nematodes.

2.3. Trichoderma harzianum Inoculum

T. harzianum AUMC14897 was obtained from the center of Prof. A. H. Moubasher for mycological sciences (AUMMC), Assiut University. The species identification was confirmed by sequence analysis of the regions of ITS1 & ITS4 of rDNA and the sequence was deposited in the GenBank under accession No. MZ025966.

Assay for Plant Growth-Promoting and Enzymatic Activities of *T. harzianum*

The bioagent *T. harzianum* was screened for plant growth-promoting trials. Indole acetic acid (IAA) production by the fungus was determined using a modified method by Glickmann and Dessaux [28]. Ammonium production was assayed through the cultivation of the fungus on peptone water medium for 4 days at 28 ± 2 °C. After the incubation period, 0.5 mL of Nessler's reagent was added and the obtained color (from faint yellow to deep yellow or brownish) indicated ammonia production. Proteases and lipases activity was assayed in vitro in a liquid medium [29]. The fungus was grown on potato dextrose agar (PDA) at 28 °C for 7 days until sporulation. *T. harzianum* spores were collected in sterile distilled water to form a homogenous spore suspension, and then a 10 mL spore suspension was used as starter inoculum for liquid PD medium. After 7 days, the suspension cultures, maintained in an incubator with agitation (150 rpm), were used to inoculate the tomato seedlings as bioagents [30]. The spore suspension concentration was adjusted to 2×10^6 spores/mL.

2.4. Raising of Test Plants

Tomato seeds (*Solanum lycopersicum* L.), cultivar Dareen F1 were used in the greenhouse pot experiment. The seeds were soaked in 70% ethanol as surface sterilization for

2 min, and then the seeds were rinsed several times with sterile H_2O and planted in plastic cell plug trays (4×4 cm) packed with vermiculite. The trays were kept under greenhouse conditions for 3 weeks and irrigated regularly.

2.5. Experimental Setup

The pot experiment was laid out to evaluate the effects of AMF and *T. harzianum* on tomato growth and root-knot nematode infection in the greenhouse at the Department of Plant Pathology, Faculty of Agriculture, Assiut University, Assiut, Egypt, using a completely randomized design (CRD). Tomato seedlings were planted in pots containing a 5 kg mixture of loam and sandy soil (1:1, *w/w*). The properties of the tested soil were determined according to Jackson [31]. Five tomato seedlings were planted in each pot and they were thinned to three per pot after 6 days of planting. Pots were irrigated regularly, and plants were grown under natural conditions of light, temperature (averaged 32/20 °C day/night), and 85% relative humidity.

The experiment consisted of eight treatments, (1) control of healthy plants (plants without treatments); (2) plants inoculated with mycorrhizal fungi (MY); (3) plants inoculated with *T. harzianum* (TH); (4) plants inoculated with both mycorrhizal fungi and *T. harzianum* (MY + TH); (5) plants treated with J2 nematodes (NE); (6) MY + NE; (7) TH + NE and (8) MY + TH + NE. The mycorrhizal and *T. harzianum* inocula were added to each pot and completely mixed with the soil surface during planting. The nematode inoculum was added around the root zone one week after planting. Each treatment was performed in five replicates. Plants were harvested 50 days after planting, comparable to the late vegetative stage of tomato growth.

2.5.1. Plant Analysis

Morphological Analysis of Tomato Plants

The effects of mycorrhizal fungi and *T. harzianum* on overall tomato growth, root length (cm), shoot length (cm), plant dry weight (g/plant), and leaf area (cm^2) were estimated in 50-day-old treated and untreated tomato plants with root-knot nematodes. Tomato shoots and roots were separated, and the length was measured. The dry weight of the tomato plants was determined after drying the plant for 2 days at 70 °C. Tomato leaf area (cm^2) was measured from leaf images taken with a standard scanner using the ImageJ program (https://imagej.nih.gov/ij/, accessed on 15 November 2021). The leaves were taken from the same leaf pair on the treated plants.

Determination of Tomato Shoot Nutrients' Content

Shoots of tomato plants were ground and then acid-digested (2:1 HNO_3:$HClO_4$) for P, K, and Ca concentrations using ICP (inductively coupled plasma) atomic emission spectrometry. As well, for N shoot content, samples were digested with H_2SO_4 following the Kjeldahl method.

Physiological Analysis of Tomato Plants

A. Photosynthetic pigments
The chlorophyll a, chlorophyll b, and carotenoid contents of the tomato plants were estimated by spectrophotometric method, elicited in 95% ethyl alcohol, and calculated as mg/g fresh weight [32].

B. Reactive oxygen species (ROS)
The hydrogen peroxide content (H_2O_2) in the leaves of treated and untreated tomato plants was spectrophotometrically estimated as described by Sellers [33] with minor modification. The molar absorptivity of 935 L mol/cm was used in the calculation of H_2O_2 as μmole/g FW.

C. Lipid peroxidation content
Lipid peroxidation (malondialdehyde (MDA)) was detected in tomato leaves using the thiobarbituric acid reaction by monitoring malondialdehyde formation as explained by

Hodges et al. [34]. The level of lipid peroxidation was expressed as nmol/g FW of MDA formed using an extinction coefficient of 155 mM/cm.

D. Antioxidant enzymes

Fresh tomato leaves (0.5 g) were ground to a fine powder in liquid N_2, and then homogenized in 5 mL of 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediamine tetra-acetic acid, disodium salt (Na_2-EDTA), and 0.1 g polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 18,000 rpm for 10 min at 4 °C and the supernatants were collected and used for the assays of catalase (CAT; EC 1.11.1.6) according to the modified method of Aebi [35] and guaiacol peroxidase (GPX; EC 1.11.1.7) according to the method of Tatiana et al. [36]. The specific activity was expressed as units/mg protein. Protein concentrations in the enzyme extract were determined by the method of Lowry et al. [37].

E. Total phenolic content

Total phenolic content was calculated based on the Sampietro et al. [38] method using the Folin–Ciocalteu reagent on the leaves' methanolic extract, and the data was expressed as mg/gm FW using gallic acid as a standard curve.

F. Lignin content

Lignin content in a tomato root was determined according to a modified method of Dence [39].

G. Assay of hydrolytic enzymes in plant roots

One gram of a tomato plant's roots from each treatment was ground in 10 mL phosphate buffer, and then the root extracts containing crude enzymes were centrifuged at 10,000 rpm for 3 min. Protease and lipase enzyme-specific activities were assayed on the tomato root extract supernatant as described in our published paper [29].

2.5.2. Nematode Infection Parameters

The root-knot nematodes' penetration rates were determined in treated tomato plant's roots at 0, 5, 10, 15, and 20 days after *M. javanica* inoculation. Fifty days after nematode inoculation, the plants were uprooted and the soil adhering to their roots was removed (100 g/pot) by agitation in water. The population density of J2 in the soil, the number of galls, females, and egg masses were determined in root samples stained with acid fuchsin lactophenol. Counting was done with the aid of a dissecting microscope and a hand tally counter.

Nematode penetration (NP) (%) = (No. of nematode juveniles in each treatment/No. of nematode juveniles added) × 100

The reproduction factor (RF) = Pf/Pi,

where Pf is the No. of nematode juveniles at 50 days and Pi is the No. of nematode juveniles used in soil infestation.

Nematode reduction (%) = (C1 − C2/C1) × 100,

where C1 = No. of nematode juveniles in control and C2 = No. of nematode juveniles in treatments

2.5.3. Evaluation of Mycorrhizal Root Colonization

The mycorrhizal colonization of the tomato plant's roots was determined 50 days after inoculation with *Acaulospora bireticulata*, *Entrophospora infrequens*, *Funneliformis geosporum*, *Funneliformis mosseae*, and *Gigaspora margarita*. Twenty root segments (2 cm) were cleared with KOH (10% *w/v*) at 60 °C for 10 min and then stained using Trypan blue (0.5% *w/v*). The mycorrhizal tomato root colonization was evaluated by Phillips and Hayman [40]. The mycorrhizal spore count (spore density) was determined in 10 g dry soil by soil sieving and counting under a stereomicroscope at 40×.

2.6. Statistical Analysis

The data were analyzed by one-way ANOVA using the software program SPSS (SPSS Inc., Chicago, IL, USA) Version 19.0. All treatments were carried out in 5 replicates ($n = 5$).

Means were compared by Duncan's multiple range tests and statistical significance was conducted ($p < 0.05$).

3. Results

3.1. Assay for Plant Growth-Promoting and Hydrolytic Enzyme Activities of the Bioagent T. harzianum In Vitro

The biocontrol agent *T. harzianum* exhibited high potentiality for indole acetic acid production, recording 12.11 ± 2.12 μg/mL, as well as obvious ammonification activity, where orange precipitate formed after adding Nessler's reagent to the fungal supernatant (Table 1). The data shown in Table 1 revealed that *T. harzianum* exhibited a potential activity for hydrolytic enzyme production. The results exhibited that in the liquid medium, the protease and lipase activity produced by *T. harzianum* were 28.36 ± 2.82 and 12.30 ± 0.31 U/mL, respectively. Whereas the protease and lipase specific activities retained by *T. harzianum* were estimated to be 20.40 ± 5.35 and 9.50 ± 1.45 U/mg protein, respectively (Table 1).

Table 1. Plant growth-promoting and hydrolytic enzyme activity of *T. harzianum*.

Assay	Value
Plant growth-promoting activities	
Indole acetic acid (μg/mL)	12.11 ± 2.12
Ammonia production	+++
Hydrolytic enzyme activities	
Lipase activity (U/mL)	12.30 ± 0.31
Lipase specific activity (U/mg protein)	9.50 ± 1.45
Protease activity (U/mL)	28.36 ± 2.82
Protease specific activity (U/mg protein)	20.40 ± 5.35

+++ = strong positive reaction.

3.2. Effect of the Bioagents (Mycorrhizal fungi and T. harzianum) on Tomato Plant Growth In Vivo

The physicochemical properties of the soil used in the pot experiment were analyzed and the data is presented in Table 2. The cation and anion of soil soluble salts are also presented in Table 2. The organic matter of the tested soil was 0.73%, the available phosphorus value was 7.3 (mg/kg), and the total nitrogen content was 12.3 (mg/kg).

Table 2. Physicochemical parameters of soil used in pot experiment.

| Parameter	Value
$EC_{1:5}$ (dS/m)	3.79
$pH_{1:2:5}$	7.60
OM%	0.73
Available P (mg/kg)	7.3
Total N (mg/kg)	12.3
Cations and anions (mg/100 g)	
Ca^{++}	1.25
Mg^{++}	2.75
Na^{+}	1.12
K^{+}	0.16
Cl^{-}	1.87
HCO_3	1.3

The growth parameters (length, dry weight, and leaf area) of tomato plants infected with *M. javanica* were determined at the end of the experiment (Table 3). The results showed that mycorrhizal fungi, singly or in combination with *T. harzianum*, significantly improved the dry weight, and the root and shoot length of the treated plants. They increased the shoot length by 42% from the control and increased the dry weight by 131% from the control. Meanwhile, the infection with *M. javanica* decreased the shoot length of the plants by 11.9% from the control. The reduction of the dry weight of the infected plants was visible but not

significant. A gradual and significant ($p < 0.05$) increase in leaf area of the plants inoculated with the mycorrhizal fungi and/or *T. harzianum* was observed (Figure 1 & Table 3). We identified that inoculation with MY + TH exercised a synergistic effect on plant biological traits. Following dual inoculation, the plants registered higher values of root length, shoot length, dry weight, and leaf area. The nematode-infected plants recorded the lowest value of leaf area (59.9 cm^2), while the mycorrhizal and/or *T. harzianum* inoculations minimized the negative effects of NE parasitism on the leaf area.

Table 3. Effect of mycorrhizal and/or *T. harzianum* inoculations on 50-day-old tomato plants' growth infected with *M. javanica*.

Treatment	Root Length (cm)	Shoot Length (cm)	Dry Weight (g)	Leaf Area (cm^2)
Control	13.67 ± 1.52 [a]	50.87 ± 1.94 [b]	2.77 ± 0.24 [a]	63.07 ± 0.97 [b]
MY	24.17 ± 1.90 [c]	60.70 ± 2.42 [c]	4.07 ± 1.16 [b,c]	91.47 ± 0.60 [d]
TH	16.50 ± 1.32 [a]	52.87 ± 2.01 [b]	3.16 ± 0.38 [a,b]	84.19 ± 0.58 [c]
MY + TH	25.20 ± 1.70 [c]	72.67 ± 3.13 [e]	6.40 ± 0.31 [d]	94.17 ± 0.32 [e]
NE	13.77 ± 1.36 [a]	44.80 ± 1.67 [a]	2.31 ± 0.16 [a]	59.93 ± 0.36 [a]
MY + NE	19.67 ± 1.52 [b]	61.63 ± 3.27 [c]	4.47 ± 0.76 [c]	92.83 ± 0.32 [e]
TH + NE	16.60 ± 1.63 [a]	51.23 ± 1.88 [b]	3.33 ± 0.50 [a,b]	82.54 ± 0.25 [c]
MY + TH + NE	21.40 ± 1.76 [b]	66.50 ± 2.50 [d]	5.17 ± 0.76 [c]	90.77 ± 0.73 [d]

Values followed by the same letter(s) in the same column are not significantly different at $p < 0.05$ using Duncan's multiple range test.
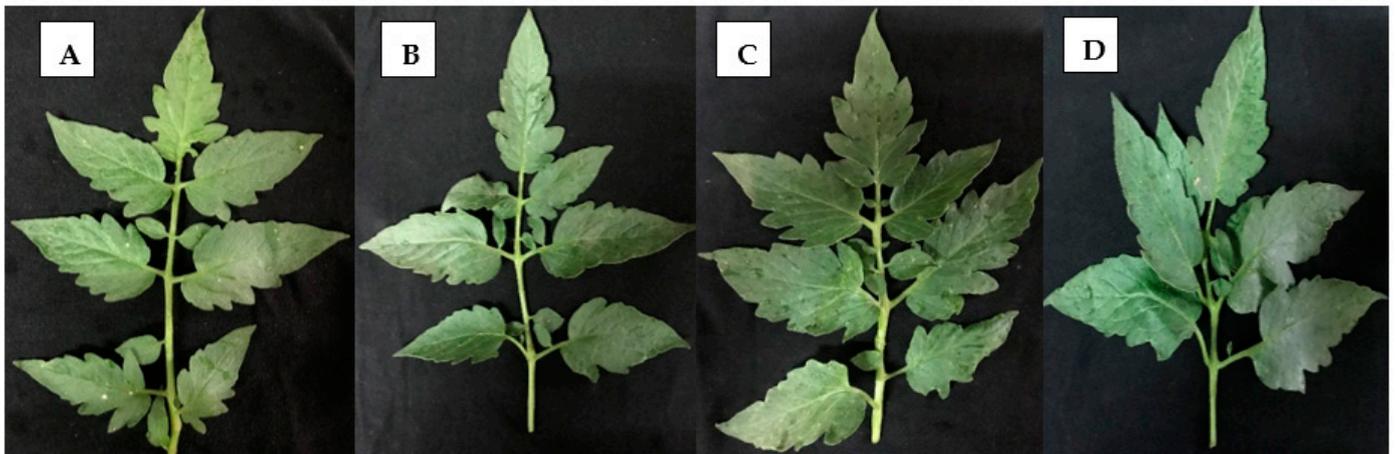
Figure 1. Morphology of tomato plants' leaves inoculated with the biocontrol agents and infected with the root-knot nematode. (**A**) Healthy tomato plant; (**B**) Nematode-infected tomato plant; (**C**) Infected plant inoculated with mycorrhizal fungi; (**D**) Infected plant inoculated with mycorrhizal fungi and *T. harzianum*.

3.3. Tomato Plants' Shoot Nutrients Composition

Tomato plants' shoot N, P, K, and Ca content as a function of nematode infection and bioagent inoculation is shown in Table 4. Overall, the mycorrhizal fungi and/or *T. harzianum* significantly increased shoot nutrient content (N, P, K, Ca), even with *M. javanica* infection. While the nematode infection alone significantly ($p < 0.05$) reduced the nutrient content to the lowest value among the treatments. Furthermore, the P content in healthy and infected plants' shoot was higher with dual inoculation, recording a 33.83%, 41.68% increase from the control, respectively. K content recorded the highest value in the case of *T. harzianum* and/or mycorrhizal inoculation; whereas nematode infection did not affect K shoot content.

Table 4. Shoot N, P, K, Ca content (mg/g DW) of tomato plants infected with *M. javanica* and inoculated with mycorrhizae and/or *T. harzianum*.

Treatment	N	P	K	Ca
Control	13.50 ± 0.30 [b]	9.43 ± 0.13 [b]	5.57 ± 0.40 [a]	3.38 ± 0.14 [b]
MY	14.45 ± 0.17 [c,d,e]	10.68 ± 0.18 [d]	7.17 ± 0.25 [b]	3.85 ± 0.27 [c,d]
TH	14.10 ± 0.20 [c]	10.07 ± 0.19 [c]	10.12 ± 0.26 [d]	3.46 ± 0.05 [b]
MY + TH	14.57 ± 0.25 [d,e]	12.62 ± 0.16 [f]	10.25 ± 0.28 [d]	4.47 ± 0.15 [e]
NE	12.97 ± 0.26 [a]	6.64 ± 0.41 [a]	5.53 ± 0.68 [a]	2.90 ± 0.15 [a]
MY + NE	14.75 ± 0.13 [e]	13.18 ± 0.26 [g]	7.27 ± 0.25 [b]	3.70 ± 0.10 [b]
TH + NE	14.30 ± 0.17 [c,d]	12.13 ± 0.21 [e]	9.13 ± 0.40 [c]	4.13 ± 0.32 [d,e]
MY + TH + NE	15.37 ± 0.32 [f]	13.36 ± 0.15 [g]	10.77 ± 0.21 [d]	4.43 ± 0.23 [e]

Values followed by the same letter(s) in the same column are not significantly different at $p < 0.05$ using Duncan's multiple range test.

3.4. Physiological Analysis of Tomato Plants

3.4.1. Photosynthetic Pigments

Nematode infection significantly decreased the content of total chlorophyll and carotenoids; we recorded the lowest value (5.80 mg/g FW) in the infected non-inoculated plants (Table 5). While their content improved significantly with the application of the bioagents. Compared to relevant non-inoculated tomato plants and those with *T. harzianum* inoculation, mycorrhizal inoculation significantly improved plant pigments content, and the dual inoculated plants had higher photosynthetic pigments contents (7.20 mg/g FW). Leaf photosynthetic pigments' content was increased in nematode infected tomato plants that were also inoculated with the bioagents as compared with infected plants alone, thus indicating simultaneous improvement in the photosynthetic process.

Table 5. Effect of mycorrhizal and/or *T. harzianum* inoculations on photosynthetic pigments (mg/g fresh weight) in tomato plants infected with *M. javanica*.

Treatment	Chlorophyll a	Chlorophyll b	Carotenoids
Control	8.40 ± 0.13 [a]	6.01 ± 0.15 [a]	3.98 ± 0.15 [b]
MY	8.80 ± 0.09 [c]	7.49 ± 0.68 [b]	4.82 ± 0.17 [e]
TH	8.55 ± 0.07 [a,b]	6.37 ± 0.11 [a]	4.41 ± 0.13 [c]
MY + TH	9.15 ± 0.53 [d]	7.52 ± 0.35 [b]	4.94 ± 0.14 [f]
NE	7.92 ± 0.32 [a,b]	5.94 ± 0.34 [a]	3.55 ± 0.16 [a]
MY + NE	8.77 ± 0.14 [c]	6.14 ± 0.24 [a]	4.46 ± 0.12 [c,d]
TH + NE	8.80 ± 0.32 [c]	6.02 ± 0.05 [a]	4.05 ± 0.16 [b]
MY + TH + NE	9.31 ± 0.16 [d]	6.31 ± 0.10 [a]	4.66 ± 0.14 [d,e]

Values followed by the same letter(s) in the same column are not significantly different at $p < 0.05$ using Duncan's multiple range test.

3.4.2. Reactive Oxygen Species (ROS)

Plants infected with *M. javanica* (NE) stimulated the oxidative burst in the infected tomato plants recording an increase of H_2O_2 content, which is an oxidative stress index (Figure 2A). The highest value of H_2O_2 occurred in the infected and non-inoculated plants that was 2-fold from the control. The reduction in H_2O_2 content was significant in the infected tomato plants inoculated with the two bioagents, recording the highest reduction in the infected dual inoculated plants (−49%).
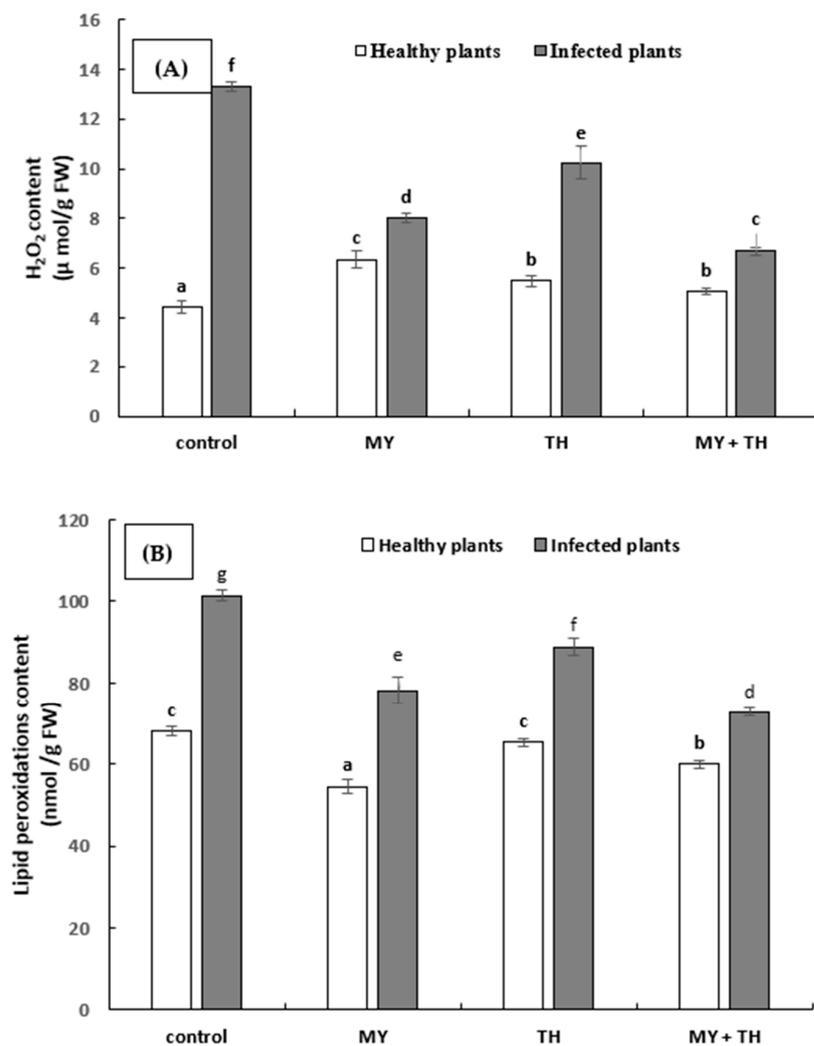
Figure 2. (**A**) Hydrogen peroxide (H_2O_2) and (**B**) lipid peroxidation (malondialdehyde (MDA)) contents as oxidative stress indicators of healthy and infected tomato plants inoculated with mycorrhizal fungi (MY), *T. harzianum* (TH), and AMF + TH. Vertical bars indicate ± SE (*n* = 5); columns followed by different letters are significantly different at *p* < 0.05.

3.4.3. Lipid Peroxidation Content

The malondialdehyde (MDA) content in tomato plants indicates the lipid peroxidation process. Figure 2B shows that the MDA content of the tomato plants infected with *M. javanica* was noticeably increased compared with the inoculated plants with bioagents, which lowered the MDA content in plants. The lowest MDA content was detected in non-mycorrhizal-treated plants (54.5 nmol/g FW) and the highest content was in the infected non-inoculated plants (101.54 nmol/g FW).

3.4.4. Antioxidant Enzymes

The antioxidant enzyme activity in tomato leaves is presented in Figure 3. Data showed catalase (CAT) and glutathione peroxidase (GPX) enzyme activity in the infected tomato plants and the plants inoculated with mycorrhizae and/or *T. harzianum*. There was a reduction in CAT activity in the nematode-infected non-inoculated plants compared with the infected and inoculated plants. Meanwhile, CAT activity significantly increased by dual inoculation; we recorded the highest value in the infected and dual inoculated plants (131.57% increase from the control). The glutathione peroxidase (GPX) enzyme content in the tomato plants infected with NE significantly increased because of the bioagents' inoculation. The infected and non-inoculated plants had no significant effect on GPX

content as compared with the healthy non-inoculated plants. The highest value of GPX was recorded in the infected and dual inoculated plants (133.59 % increase from the control).
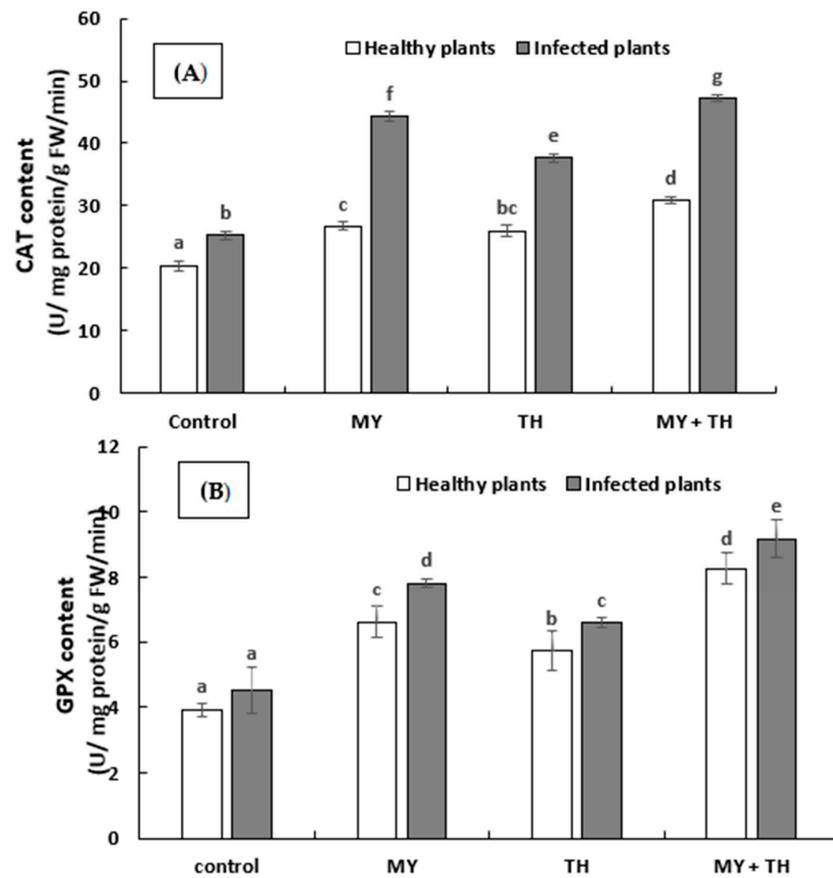
Figure 3. Antioxidant enzyme content: (**A**) Catalase (CAT) and (**B**) glutathione peroxidase (GPX) of healthy and infected tomato plants inoculated with mycorrhizal fungi (MY), *T. harzianum* (TH), and AMF + TH. Vertical bars indicate $\pm$ SE (n = 5); columns followed by different letters are significantly different at $p < 0.05$.

### 3.4.5. Total Phenolic and Lignin Contents

The bioagents (mycorrhizae and *T. harzianum*) stimulated the production of total phenolics in tomato plant cells compared with the infected non-inoculated plants (Figure 4A). The highest phenolic content was recorded in the infected dual inoculated plants (1.02 mg/g FW), followed by the mycorrhizal–infected plants (0.92 mg/g FW). Lignin content as a secondary metabolite is presented in Figure 4B. Lignin biosynthesis significantly increased in infected and non-inoculated tomato plants, where we recorded the highest value (130% from the control). Furthermore, lignin content was reduced significantly in dual inoculated plants.
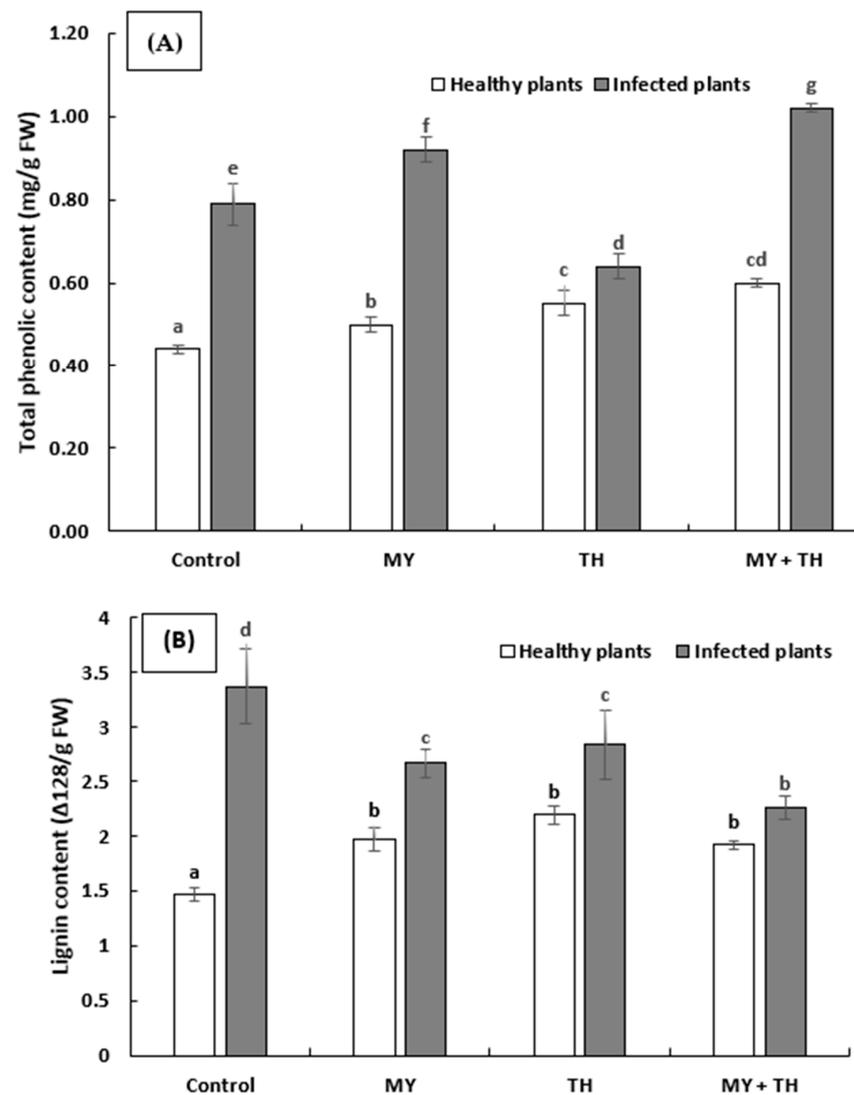
Figure 4. (**A**) Total phenolic content and (**B**) lignin content of healthy and infected tomato plants inoculated with mycorrhizal fungi (MY), *T. harzianum* (TH), and AMF + TH. Vertical bars indicate $\pm$ SE (*n* = 5); columns followed by different letters are significantly different at *p* < 0.05.

3.4.6. Hydrolytic Enzyme Activity in Tomato Plants' Roots

The obtained data illustrated that the lowest hydrolytic enzyme activity was recorded in healthy tomato plants compared with the infected plants. Meanwhile, the bioagents, applied individually or in combination, exhibited a significant increase (*p* < 0.05) in the hydrolytic enzyme activity of tomato root extracts in the infected plants compared with the healthy plants (Figure 5). Similarly, root extracts of the infected and inoculated tomato plants revealed the highest protease and lipase enzyme activity with both bioagents, arbuscular mycorrhizal fungi and *T. harzianum*.
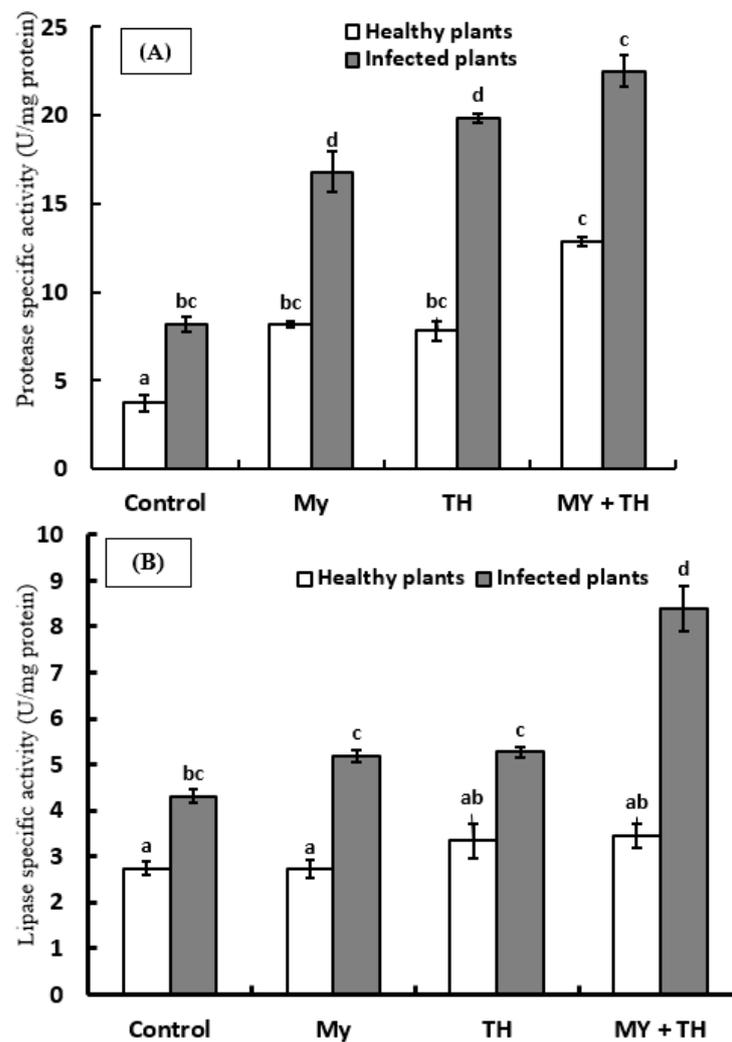
Figure 5. Hydrolytic enzyme activity in tomato plants' roots: (**A**) Protease and (**B**) lipase in healthy and infected tomato plants' roots inoculated with mycorrhizal fungi (MY), *T. harzianum* (TH), and AMF + TH. Vertical bars indicate $\pm$ SE (*n* = 5); columns followed by different letters are significantly different at *p* < 0.05.

3.5. Nematode Infection

Five days after nematode inoculation, the penetration rates (ratio of the number of penetrated nematodes to the number of added nematodes) were determined in all treatments (Figure 6). The penetration rate of nematodes in all treatments improved over time, with the highest improvement (6.2–9.03%) towards the end of the 20 days. The penetration rate in the infected and non-inoculated control was 9% 20 days after nematode inoculation, while it reached 6% in the case of the dual bioagents' treatment. Table 6 illustrates the potential of the two bioagents to reduce the populations of nematodes. By applying mycorrhizal fungi and/or *T. harzianum*, the number of larvae, females, egg masses, and galls was significantly reduced. The highest juveniles' number was detected in the infected and non-inoculated tomato plants, while the lowest number of J2s was recorded in the case of MY and MY + TH, corresponding to 45% and 55% reduction from the control, respectively (Figure 7). The number of females and egg masses produced by *M. javanica* was high in the infected and non-inoculated tomato plants (infected control) (Table 6). It was found that the number of females and egg masses in the inoculated and infected plants was significantly lower than that of the infected control, and the dual inoculation achieved the greatest reduction in numbers. Compared with the infected control, mycorrhizal fungi and *T. harzianum* caused a 68% reduction in the number of

females and a 43% reduction in egg masses (Figure 7). In pots infected with NE, more galls on the tomato plants' roots were found on non-inoculated plants (Table 6), of which the highest number of galls (105/root) was recorded. Application of mycorrhizal fungi alone or combined with *T. harzianum* reduced the formation of galls by 40.63% and 52.38% from the infected control, respectively. The reproduction factor (RF) was calculated for all treatments and was significantly different. As the highest reproduction factor (RF = 3.24) was recorded in the infected control. The dual inoculation showed the lowest RF (1.46) followed by the single inoculation.
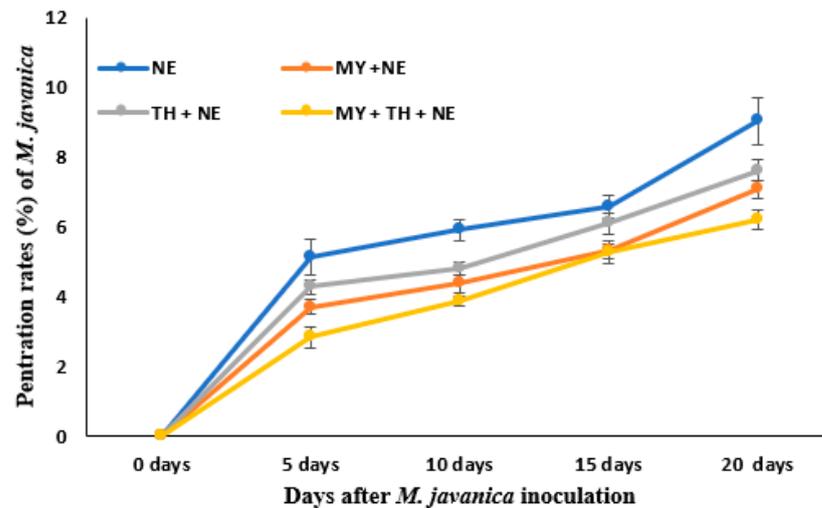
Figure 6. Root-knot nematode penetration rates (%) in tomato plants' infected roots as affected by inoculation with mycorrhizal fungi (MY), *T. harzianum* (TH), and AMF + TH. Vertical bars indicate ± SE (*n* = 5).
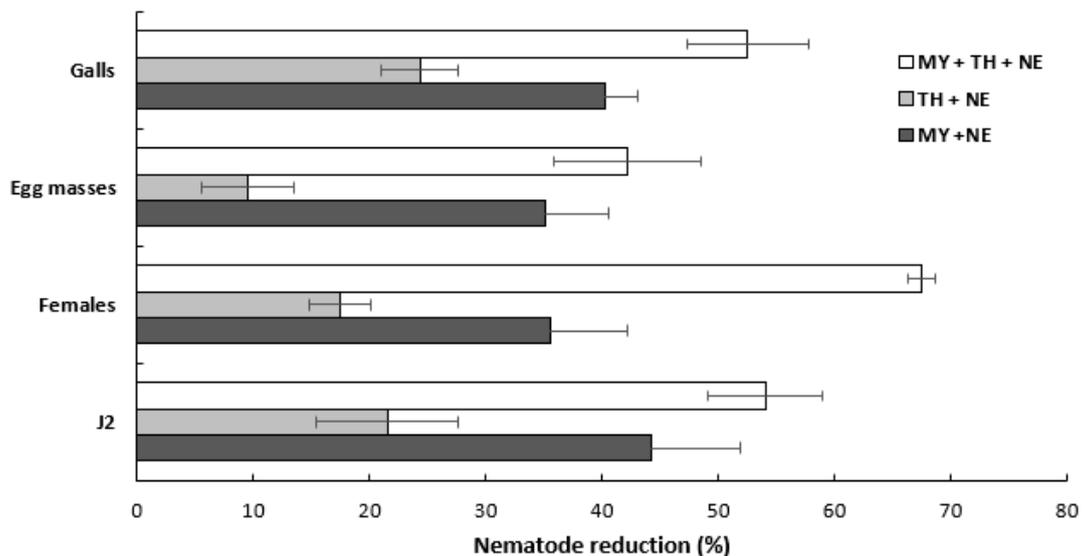
Figure 7. Percentage of juvenile, female, egg masse, and gall reduction in tomato infected roots as affected by inoculation with mycorrhizal fungi (MY), *T. harzianum* (TH), and AMF + TH. Vertical bars indicate ± SE (*n* = 5).

Table 6. Effect of the biocontrol agents alone or in combination on the population of nematodes.

Treatment	J2/100 g Soil	Females/Root	Egg Masses/Root	Galls/Root	RF
NE	242.67 ± 9.69 [c]	35.67 ± 6.03 [c]	142.80 ± 11.37 [b]	105.00 ± 8.00 [c]	3.24 ± 0.39 [c]
MY + NE	133.00 ± 13.46 [a]	23.00 ± 4.00 [b]	91.67 ± 7.02 [a]	62.33 ± 4.51 [a]	1.78 ± 0.20 [a]
TH + NE	193.33 ± 14.59 [b]	29.33 ± 4.04 [b,c]	128.33 ± 7.25 [b]	79.33 ± 8.50 [b]	2.58 ± 0.33 [b]
MY + TH + NE	109.00 ± 10.58 [a]	11.33 ± 3.21 [a]	81.67 ± 9.07 [a]	50.00 ± 6.56 [a]	1.46 ± 0.14 [a]

Values followed by the same letter(s) in the same column are not significantly different at $p < 0.05$ using Duncan's multiple range test. RF is the nematode reproduction factor.

3.6. Mycorrhizal Colonization

The mycorrhizal colonization of the tomato plants' roots was determined with the application of *T. harzianum* in the presence or absence of *M. javanica* (Table 7). There was a significant difference in root colonization between healthy and infected tomato plants, with mycorrhizal colonization being higher in the infected tomato plants. The tomato plants' roots inoculated with the mycorrhizal fungi were occupied by intra-, inter-, and extra-radical hyphae, arum-type of arbuscules, and vesicles (Figure 8). Meanwhile, the intra- and inter-radical hyphae grew from cell to cell within the intercellular space, penetrating the cell walls and forming arbuscules (Figure 8). The hyphal colonization of tomato plants inoculated with a mixture from *Acaulospora bireticulata*, *Entrophospora infrequens*, *Funneliformis geosporum*, *Funneliformis mosseae*, and *Gigaspora margarita* ranged from 70–95%, and the highest value was recorded in the tomato plants inoculated with the mycorrhizal fungi. Arbuscular root colonization of the tomato plants was positively affected by nematode infection, of which the highest colonization was reported in the infected mycorrhizal-inoculated plants. The vesicle colonization was significantly influenced by nematode infection, ranging from 45–70%, of which the lowest value occurred in infected–mycorrhizal tomato plants (30.76% decrease from healthy mycorrhizal plants). The numbers of glomerospores in the tomato plants were significantly increased by the nematode infection (Table 7). The highest mycorrhizal spore density was recorded in the rhizosphere soil of dual inoculated plants.

Table 7. Percentage (%) of root colonization (hyphal, arbuscular, vesicle colonization) of 50-day-old tomato plants inoculated with mycorrhizae and/or *T. harzianum* and infected with *M. javanica*.

Treatment	Hyphal Colonization	Arbuscular Colonization	Vesicle Colonization	Mycorrhizal Spore Density
MY	73.3 [a,B]	50.0 [a,A]	65.0 [b,B]	55.7 [a]
MY + NE	91.7 [b,C]	70.0 [b,B]	50.0 [a,A]	64.2 [a,b]
MY + TH	70.0 [a,B]	48.3 [a,A]	70.0 [b,B]	66.6 [a,b]
MY + TH + NE	95.0 [b,C]	78.3 [b,B]	45.0 [a,A]	73.3 [c]

Means within each column followed by the same lowercase letter (for each parameter) and means within each row followed by the same uppercase (for each treatment in colonization parameter) are not significantly different at $p < 0.05$ using Duncan's multiple range test.
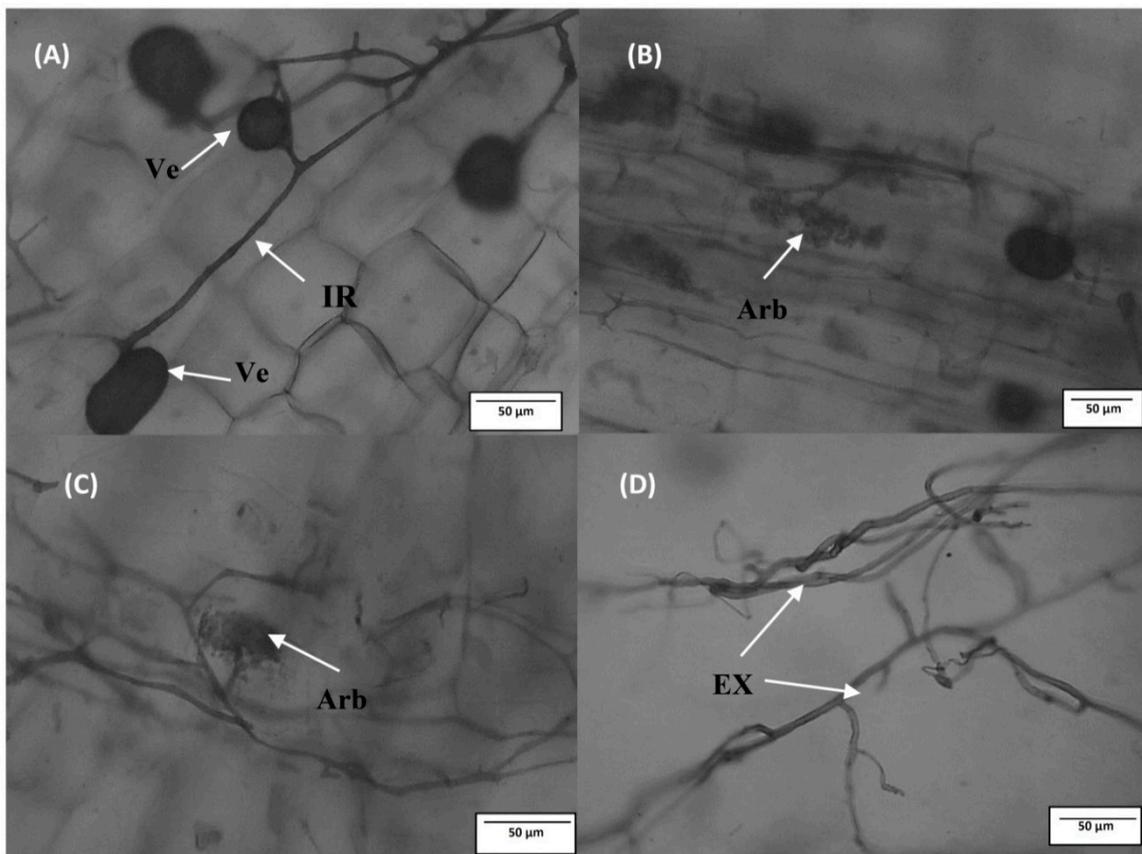
Figure 8. Root colonization patterns of 50-day-old tomato plants inoculated with dual inoculations under nematode infection: (**A**) vesicles (Ve) and intra-radical hyphae (IR); (**B**,**C**) arum-type of arbuscules (Arb); (**D**) extra-radical hyphae (EX).

4. Discussion

Plants inoculated with beneficial, indigenous microbes have been recommended for increasing the sustainability of plant yield and protecting plants against stress [41]. Furthermore, the interaction between these beneficial microorganisms and the plant rhizosphere enhances plant growth, nutrient acquisition, yield, and soil energy conversion. Mycorrhizal fungi and *T. harzianum* are well-known as natural, biosafe biocontrol agents of nematode infections. Root-knot nematodes sorely affect tomato plants' growth and yield all over the world [42,43]. Our study reported that mycorrhizal fungi and *T. harzianum* can limit the growth and reproduction of the nematodes and reduce their parasitism in tomato plants' roots.

In the current study, the biocontrol agent *T. harzianum* showed high potentiality to produce plant growth-promoting compounds, as well as a potential for encouraging hydrolytic enzyme production. Nieto-Jacobo et al. [44] stated that *Trichoderma* species are soil-borne fungi that are commonly employed for various plant health benefits, due to their potentiality for improved plant growth, abiotic stress tolerance, and disease resistance. The capability of various *Trichoderma* species to produce the plant phytohormone auxins (indole-3-acetic acid) that is determined by the presence of the main precursor L-tryptophan, which is produced as plant exudates [12,45]. Indole acetic acid (IAA) production by *Trichoderma* explained their efficiency in promoting plant root growth that could change root architecture, leading to increased root mass and subsequently increased the root area for beneficial microbial colonization and enhanced nutrient uptake [46]. Furthermore, ammonia production by soil microbes showed important properties in stimulating nitrogen assimilation and plant development [12,47]. Interestingly, Zhang et al. [7] reported that the hydrolytic enzyme activity of *Trichoderma* species may explain the direct mechanism

of *Trichoderma*–nematode interaction through the first step of *Trichoderma* parasitism on nematodes by hydrolyzing the nematode eggs and the second-stage juveniles. So, for disturbing nematode eggs and juveniles and consequently controlling the plant pathogenic nematodes, a combination of lytic enzymes including proteases, chitinases, and lipases are required [48].

In the present study, dual inoculation with mycorrhizal fungi and *T. harzianum* improved the nutrient acquisition and growth of tomato plants. The use of dual inoculation favored improvement in the shoot length and dry weight more than the single inoculation. In the same context as our finding, dual inoculation with *Glomus mosseae* and plant growth-promoting rhizobacteria enhanced tomato plant growth more than single inoculation [49]. In our study, the interaction between tomato plants and nematode pathogens led to a morphological and physiochemical change in the tomato host. *M. javanica* infection significantly diminished the tomato plants' growth and shoot nutrient composition, while the bioagents' application reduced the harmful effect of the nematode infection. The biocontrol efficiency of mycorrhizal fungi against nematodes has been recorded previously in tomato plants [50]. *Rhizophagus intraradices* and *Funneliformis mosseae* decreased nematode tomato root penetration by *Nacobbus aberrans* [51]. Mycorrhizal fungi can improve the vitality of the host plants by enhancing nutrient uptake, mainly phosphorus, nitrogen, and potassium, and increase water-use efficiency. Improved branching and growth of mycorrhizal roots may increase the plants' tolerance towards NE infection and balances the inhibited root branching caused by nematodes [52]. Meanwhile, mycorrhizal bioprotective effects were reported to be systemically induced as they act through the host plant [8]. Consequently, in the current study, the enhancement effects on plant growth following inoculation with *T. harzianum*, were also explained by improvement of the plant's nutritional status. In addition, mycorrhizal fungi and *T. harzianum* effectively take possession of the rhizosphere and inhibit nematode populations within the phytobiome, hence, increasing every aspect of tomato growth.

Our results proved that photosynthetic pigments increased in the infected tomato plants that were also inoculated with the bioagents, thus indicating simultaneous improvement in the photosynthetic process. Mycorrhizal fungi being natural root symbionts, support essential inorganic nutrients uptake to host plants, thereby causing mycorrhizal plants to have higher chlorophyll content and higher photosynthetic activity. While in the infected and non-inoculated plants, photosynthetic pigments were significantly reduced, as previously reported [53]. The impairment of the chlorophyll and carotenoids mainly happened due to the suppression of enzymatic activities implicated in the violaxanthin cycle, which disturbed the photosynthetic apparatus stabilization [54].

The reactive oxygen species (ROS) contents are stimulated during environmental stresses, which could cause damage to plant tissues, deterioration of lipid and protein, DNA mutation, the disordering of cell organelle, and decay of the photosynthetic apparatus [55]. Plant oxidation, non-enzymatic and enzymatic, has been reported as plant oxidative response systems that interfered with the aerobic process to minimize the oxidative cleavage due to more ROS production. Catalase (CAT) and guaiacol peroxidase (GPX) were reported as protective oxidative enzymes in plants. In our study, NE stimulated the oxidative burst in the infected and non-inoculated plants, recording the highest value in H_2O_2 and MDA content. Similar to other pathogen stress, nematode infection produces excessive free radicles such as H_2O_2, which accumulates during hypertrophy and cell death [8]. Mycorrhizal plant resistance against nematodes occurs through improvement in the systematic defensive capacity of roots due to the activation of the genes that encoded chitinases, pathogenesis-related proteins, enzymes implicated in the detoxification of ROS, enzymes implicated in lignin biosynthesis, and in the pathway of the shikimate, which, in turn, develop precursors of different aromatic secondary metabolites against the infection of nematodes [16,19]. Our results demonstrated that CAT and GPX enzyme activity was enhanced in infected dual inoculated tomato plants to eliminate the toxicity of H_2O_2. Similar to our results, the systematic resistance developed by mycorrhizal fungi to

reduce nematode infection occurred through the activity of phenolics and plant defense enzymes with the reduction in MDA and H_2O_2 contents in tomato plants inoculated with *Rhizophagus irregularis* and enhanced plant growth [17].

In the present study, lignin content of the infected tomato plants was significantly increased. Infection and reproduction by NE are influenced by changes in lignin composition or content. Nematodes create their constant feeding places inside the vascular cylinder, which is the main location of the roots' secondary wall formation and lignification [56]. The change in lignin content is an important factor that explains the structural differences in root cell walls: if its content increased, it implies that root cell walls lignify, thicken, and create barriers to defend against the invasion of the pathogen. The inoculated plants with the bioagents compared with infected tomato plants showed a significant reduction in the lignin content due to decreased nematode infection and gall numbers.

Furthermore, the current study revealed that the hydrolytic enzyme activity in tomato plant roots was significantly enhanced in the infected plants inoculated with the bioagents. Zhang et al. [7] stated that the potential effect of bioagents may be due to the direct parasitism mechanisms that have lethal impacts on the nematode eggs and J2 activities. Consequently, an increase of extracellular hydrolytic enzyme activity was reported, which allowed a direct effect on nematode eggshells and penetration of the eggs, leading to a decrease in the number of nematode eggs capable of hatching and, thus, the number of infective juveniles (J2) [7]. Therefore, the bioagents (MY and TH) could be applied as a biocontrol agent for the management of nematode infections in economic crop plants.

In the present study, the simultaneous tomato root inoculation by MY and TH significantly reduced the nematode penetration rates and populations, indicative of indirect and direct biocontrol activity against NE as previously reported [43]. The effect of mycorrhizal and dual inoculations was significantly pronounced on juveniles, the abundant and important life stage in nematode inoculum. These results may be related to difficult nematode migration towards the roots because the bioagents occupied the rhizosphere and therefore caused difficulty in finding feeding space, so a reduction in nematode stage developments and gall formation occurred. Compared with other nematode populations, juveniles were the most active and greatest food seekers, which resulted in a higher root penetration rate [57]. The direct mechanism of nematode population reduction by *T. harzianum* inoculation occurred by fungal hyphae penetrating the egg mass matrix of nematodes and reducing nematode hatching [58], or by the production of secondary fungal toxic metabolites, which prevented nematode penetration and infection [59].

Arbuscular root colonization of tomato plants was positively affected by nematode infection, as the highest colonization was reported in infected mycorrhizal plants. Furthermore, the mycorrhizal spore density increased under nematode infection. Space competition between glomerospores and endoparasitic nematodes could be related to increasing mycorrhizal colonization due to competing for space and photosynthates. This may occur in plants under stress. Competition for space indicates higher mycorrhizal colonization and might lead to a higher level of mycorrhizal mediated biocontrol [60]. Mycorrhizal colonization is characterized by the presence of a high amount of arbuscules, which seems to be a requirement for biocontrol.

5. Conclusions

The biological approaches for plant-parasitic-nematodes constitute an effective alternative to the conventional toxic chemical nematicides. The use of different species of mycorrhizal fungi, in addition to *T. harzianum*, aims at ecofriendly protection, increased soil fertility, and improved plant growth and, thus, has an advantage to sustainable agriculture. *T. harzianum* exhibited a high potentiality to produce plant growth promoting compounds (indole acetic acid and ammonia). As well, *T. harzianum* revealed varied enzymatic activities (protease and lipase) that may illustrate their hydrolytic abilities against nematode eggs and juveniles. Furthermore, in bioagent inoculated tomato plants, the penetration rates of nematodes, juveniles and females numbers, egg and gall mass were reduced. Furthermore,

glutathione peroxidase and catalase enzymatic activities were enhanced. So, the applied bioagents revealed various effective strategies for the management of nematodes, including the production of secondary metabolites, hydrolytic enzymes, as well as providing greater nutrient and water uptake to the host plant, altering root morphology and rhizospheric interactions and consequently competing for colonization/infection sites or photosynthates. Moreover, the biocontrol agents stimulate induced systematic resistance in plants against infection by pathogen and/or pests. In summary, the use of biocontrol agents in combination is a promising biocontrol tactic in agriculture against plant-parasitic nematodes that may enhance the feasibility and commercial application of ecofriendly biofertilizers in agriculture cropping systems.

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Conflicts of Interest: The authors declare no conflict of interest.

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