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Research Article

Piriformospora indica modifies cucumber's tolerance to *Meloidogyne incognita* by regulating various agro-physiological traits, antioxidant enzymes, and abscisic acid pathway genes

Mohamed M. EL-MOGY[1,2*], Mohamed A.M. ATIA[3a], Nour El-Houda A. REYAD[4], Mohamed ABDELSATTAR[5], Dina S.S. IBRAHIM[6], Abdel Wahab M. MAHMOUD[7], Suzy M. ABDELAZIZ[8], Emad A. ABDELDAYM[2b*], Mohamed E. ABDELAZIZ[2,9]

[1]*King Faisal University, College of Agricultural and Food Sciences, Department of Arid Land Agriculture, Al-Ahsa 31982, Saudi Arabia; elmogy@kfu.edu.eg (*corresponding author)*
[2]*Cairo University, Faculty of Agriculture, Vegetable Crops Department, Giza 12613, Egypt; emad.Abdeldaym@agr.cu.edu.eg (*corresponding author); mohamed.ewis@agr.cu.edu.eg*
[3]*Molecular Genetic and Genome Mapping Laboratory, Genome Mapping Department, Agriculture Genetic Engineering Research Institute (AGERI), Agriculture Research Centre (ARC), Giza 12619, Egypt; matia@ageri.sci.eg*
[4]*Cairo University, Faculty of Agriculture, Plant Pathology Department, Giza 12613, Egypt nourelhoudareyad53@gmail.com*
[5]*Plant Molecular Biology Department, Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza 12619, Egypt; mteima@ageri.sci.eg*
[6]*Nematology Department, Plant Pathology Institute, Agricultural Research Center (ARC), 12619 Giza, Egypt; Dina.Serage@acr.sci.eg*
[7]*Cairo University, Faculty of Agriculture, Department of Agricultural Botany, Plant Physiology Division, Giza 12613, Egypt; mohamed.mahmoud@agr.cu.edu.eg*
[8]*Agricultural Research Centre, Horticulture Research Institute, Cross Pollinated Vegetable Research Department, 12611, Egypt; Drsuzy.abdelaziz@arc.sci.eg*
[9]*The National Research and Development Center for Sustainable Agriculture (Estidamah), Riyadh Techno Valley, Riyadh, 11422, Saudi Arabia*

Abstract

Root knot nematode (RKN), *Meloidogyne incognita*, is considered a major soil-borne pathogen that can cause severe yield losses for vegetables and diverse crops. Usually, reducing of *M. incognita* damage is mainly relies on the application of nematicides and good agricultural practices. However, the use of synthetic nematicides is restricted due to concerns about their impact on the environment and human health. As a result, the use of alternative strategies is becoming necessary to combat RKN resistance. This study evaluates the antagonistic impact of the root mutualistic fungus *Piriformospora indica* on *M. incognita*. It also assesses its influence on the nutritional status, photo-synthesis, antioxidant enzyme activity, endogenous abscisic acid (ABA) levels, and selected ABA related-responsive genes in cucumber plants. Roots of cucumber seedlings were inoculated with *P. indica* and the second-stage juveniles (1000 J2 per plant). The results demonstrated that *P. indica* significantly reduced *M. incognita* invasion in roots, resulting in a 24% reduction in root galling and

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42.6% decline in final population. Inoculating plants with both *P. indica* and RKN increased performance of root fresh and dry weight, as well as improved photochemical efficiency of PSII (Fv/Fm), photosystem II efficiency (PSII), catalase (CAT), peroxidase (POD), and superoxide dis-mutase (SOD). Furthermore, *P. indica* colonization, either alone or in combination with *M. incognita*, significantly improved number of fruits per plant, average fruit weight, the plant's marketable yield, and leaf nutrient content (N, P, K and Mg), Moreover, there was an increase in IAA content combined with a decrease in ABA content in roots of dual inoculation plants, if compared to *M. incognita* infested plants. The highest ABA content was recorded in the root of RKN-cucumber plants. The decline in ABA content due to *P. indica* treatment was consistent with the modulation of ABA pathway genes, specifically PP2C, PYL1, RK2,1, and RK2,2. The mixed of *P. indica* and *M. incognita* led to a decrease in the expression of PP2C, PLY1, RK2,1, and RK2,2 in comparison to the control group. These results indicate that *P. indica* application could help reduce the negative effects of RKN on important crops.

Keywords: *Cucumis sativus*; marketable yield; nutrient uptake; phytohormones; root knot nematode

Introduction

Root-knot nematode (RKN), *Meloidogyne incognita*, is an important soil-borne pathogen that inflicts substantial damage on vegetable crops in both greenhouse and open field (Buttar *et al*., 2023). Cucumber is a significant economic crop cultivated in greenhouses and is vulnerable to root-knot nematodes (Fassuliotis 1979). Root-knot nematodes are mostly caused by four Meloidogyne species: *M. incognita, M. arenaria, M. javanica,* and *M. hapla* (Fassuliotis, 1982).

M. incognita induces a wide variety of symptoms in cucumber plants, including stunting, wilting, yellowing, and reduced yields (Atia *et al*., 2020). Furthermore, RKN makes plants more sensitive to other biotic stresses, such as pathogenic fungi, bacteria, and viruses (You *et al.,* 2023). With over 100 species in the genus *Meloidogyne*, *M. incognita* is a highly invasive species that infest crops of high economic value in at least 143 countries (Onkendi *et al*., 2014; Bebber *et al.,* 2014). According to Sasser and Freckman (2013), plant-parasitic nematodes cause economic losses annually estimated at 12.3% in 40 main crops worldwide; the losses are increased by 14.6% and 8.8% in developing and developed countries, respectively. Furthermore, these types of nematodes decrease the world's agricultural output by up to 10%, causing annual losses reaching more than US$125 billion.

Management of root-knot nematodes is a main challenge for vegetable growers world-wide (Uddin *et al*., 2023). Many reports indicated that applying chemical nematicides is effective in managing RKNs, but also toxic to environmental and human health (Atia *et al*., 2020). Furthermore, environmentally friendly farming strategies have been studied in relation to root-knot nematodes (Atia *et al*., 2020). Moreover, the use of biological control agents was found to enhance plant resistance to root-knot nematode in cucumber (Dutta and Thakur, 2017; Atia *et al*., 2020; You *et al.,* 2023).

Mycorrhizal and endo-phytic fungi, as filamentous fungi, is considered a better strategy to control RKN (Burns *et al*., 2023). This filamentous fungus can reduce the damage produced by plant-pathogenic nematodes by parasitism, antibiosis, paralysis, and the secretion of lytic enzymes. They prevent damage through space and resource competition, increasing nutrient intake and water absorption by the economic plant, modifying root architecture, and influencing rhizosphere interactions to promote plant growth (Poveda *et al*., 2020). Furthermore, these fungi can enhance plant defense mechanisms against nematodes by boosting hormone-mediated pathways such as Indole-3-acetic acid, cytokinin, salicylic acid, jasmonic acid, and strigolactones (Poveda *et al*., 2020). Additionally, the modification of the transport of chemical defense constituents through

the plant or the metabolism of plant secondary metabolic compounds and various enzymes can also contribute to improving plant defenses (Burns *et al.,* 2023; Malviya *et al*., 2023).

Piriformospora indica, a member of the Sebacinales order in the Basidiomycota phylum, is a beneficial root endophytic fungus that has been extensively researched for its positive effects on plant development performance (Saleem *et al*., 2021). *P. indica* is a priming beneficial fungus that improves the agro-physiological attributes of various plant species under both normal and stressful conditions (Roylawar *et al*. 2023). Daneshkhah *et al*. (2013) found that the presence of the endophytic fungus *P. indica*, along with fungal exudates and cell wall extracts, had a substantial impact on the vitality, infectivity, development, and reproduction of *Heterodera schachtii* in *Arabidopsis* roots. In addition, Jha *et al.* (2021) indicated that *P. indica* enhances root growth and root hair formation, stimulating the expression of genes involved in nutrient uptake, and converting unavailable nutrients into plant-available forms. *P. indica* enhances the nutritional condition of plants and boosts the resilience of host plants against various stressors (Saleem *et al*., 2021). The ability of *P. Indica* to stimulate the synthesis of various compounds that support plants in their defence against harmful microorganisms is significant (Li *et al*., 2023). This study aims to investigate the bio-protective abilities of the endophytic fungus *P. indica* in controlling the root-knot nematodes *M. incognita* by analyzing their impact on agro-physiological traits, yield, abscisic acid (ABA) levels, and specific naturally sensitive genes.

Materials and Methods

Experimental design

As previously reported by Atia *et al*. (2020), two pot experiments were conducted in a fiberglass greenhouse at Faculty of agriculture, Cairo university. Cucumber plants (*Cucumis sativus* cv. 'Hesham') were used as host for *M. incognita* infestation. The experiment was arranged in four treatments, 1) Negative control: Untreated plants (Ctr), 2) RKN alone (N), 3) *P. indica* alone (P) and, 4) *P. indica* and RKN (PN) combination. Seedlings were inoculated with *P. indica* two hours before transplanting. An extra dose of *P. indica* suspension was added to each seedling at 7-, 15-, and 21-days' post transplantation. Cucumber was set in polyethylene pots (20 cm in diameter and 35 cm in depth) filled with growing medium (sand, peat moss, and vermiculite, in a 1:1:1 ratio). Two weeks post planting, seedlings in the N and PN treatments were inoculated with second-stage juveniles (J2) at a rate of 1000 J2/pot. All plants were irrigated twice a week and fertilized once with NPK fertilizers. At the end of the experiment, both N and PN plants were harvested for nematological analysis.

Piriformospora indica propagation and inoculation

The mutualistic fungus *Piriformospora indica* (accession DSM 11827) was kindly provided by Prof. Hribet Hirt, King abdulla university for science and technology (Kaust), KSA. Stock was propagated on solid KM medium (Hill and Kafer, stock was incubated at 28 °C±1, in the dark, for 2 weeks in a growing enrichment medium consists of 4 g yeast extract, 10 g malt extract, 4 g glucose and 20 g agar (Pridham *et al*., 1957). Three fungal plugs were introduced into 150 ml of the liquid culture medium and then cultured for 14 days at 28 °C while being agitated at 150 rpm on a rotary shaker. The mycelium of the mature fungus was harvested, centrifuged, and rinsed three times with distilled water. A 2% *P. indica* spore suspension (1.7 x 10^5 spores/ml) was achieved by mixing 20 grams of *P. indica* mycelium and spores with 1000 ml of sterilized water (Abdelaziz *et al*., 2021). Ten millilitres inoculum per plant was added directly around the root zone, while water was used as a mock. Number of spores were counted using 0.05% trypanblue, and the number of spores were counted using hemocytometer under light microscopy at 20× magnification (Jiang *et al*., 2020).

Meloidogyne incognita isolation and preparation

Meloidogyne incognita was extracted from infected cucumber roots by immersing in 2.0% Sodium hypochlorite (NaOCl) solution for 5 min (Hussey and Barker, 1973). Next, distribute them on a 30 µm nylon sieve placed on a Petri dish with distilled water. The second-stage juveniles (J2) hatched from the eggs were gathered daily, preserved at 15 °C, and utilized within a three-day period. The quantity of J2 in 1 ml was determined using a dissecting microscope and the concentration was adjusted to 1000 J2/ml by diluting the suspension with purified water.

Bioassay of Meloidogyne incognita

The density of *M. incognita* population was assessed at 40 days' post *P. indica* inoculation. Cucumber roots were removed and cleaned to eliminate dirt particles. Atia *et al*. (2020) published the measurement of soil larva density (Juvenile2, J2), developmental stage, number of egg mass, and number of females in the root as their initial research findings. The number of galls per root was rated on a 0–5 scale (0 = 0–10% galled roots; 1 = 11–20%; 2 = 21–50%; 3 = 51–80%; 4 = 81–90%; 5 = 91–100%), as described by Barker (1985). The final population (FP) of RKN was quantified by summation of eggs, egg mass, and J2. The reproduction factor (Rf) was calculated by dividing the final population (FP) and initial populations (IP) as following formula (Ferris, 1985):

$$RF = \frac{FP}{IP} \qquad (1)$$

where, RF= reproduction factor, FP = final population and IP = initial population of the nematode.

Anatomy analysis

Paraffin sections of cucumber roots were produced according to the methodology of Wang *et al*. (2018) to study cellular alterations caused by *M. incognita* infection. Root samples were gathered four days after inoculation, preserved in FAA, passed through a succession of ethanol (10-100%) and chloroform (10-100%), and then enclosed in paraffin. The specimens were sliced into slices that were 8 micrometers thick, dyed with hematoxylin and eosin, sealed with gum, and examined using a fully automated upright fluorescence microscope (OLYMPUS BX63).

Determination of cucumber root biomass and yield

Ten plants from each treatment were selected to determine fresh roots weigh, using digital balance. For root dry weight, samples were kept in a forced air-drying oven at 105 °C for 3 days until the weight was constant. The marketable fruits were harvested and weighed until the end of the experiment.

Physiological analysis

Chlorophyll fluorometer (Opti-Sciences Inc, New Hampshire, US) was used to determine the maximum photochemical efficiency of PSII (Fv/Fm), and effective PSII quantum yield (PSII). Chlorophyll fluorescence parameters were performed after 40 days of the RKN infestation and all the parameters were recorded four times. The fully expanded leaves of four plants were selected randomly and placed in the dark for 20 min. Minimal fluorescence (F0) and maximum fluorescence (Fm) were recorded using a low-intensity red light and a saturating light pulse (600 mmol m^{-2} s^{-1} light). Under 12,000 mmol m^{-2} s^{-1} light, the previous parameters were measured [Minimal fluorescence (F0) and maximal fluorescence (Fm)]. The Maximal variable fluorescence (Fv=Fm–F0), photochemical efficiency of PSII (Fv/Fm), and photosystem II efficiency (PSII) for adapted leaves were computed.

Leaf nutrient content

The nutrient content of the cucumber leaves was evaluated by digesting 0.2 g of air-dried leaf material with a mixture of sulfuric and perchloric acids. The mixture was heated at 50 °C for 10 minutes, then 0.5 ml of

perchloric acid was added and the heating continued until a clear solution was obtained (Piper, 1950). The total Kjeldahl nitrogen content of the dried material was determined using the modified micro-Kjeldahl procedure (A.O.A.C.1990). Phosphorus was assessed calorimetrically using the chlorostannous molybdophosphoric blue color method (Jackson, 1973). The potassium levels were measured using a flame photometer device (CORNING M 410, Germany). Calcium (Ca) and magnesium (Mg) levels were analyzed using atomic absorption spectroscopy utilizing air-acetylene fuel (Pye Unicam, SP-1900, US).

Quantification of antioxidant enzymes activity

Five hundred mg of cucumber plants enlarged fourth leaves were powdered in liquid nitrogen and combined with 5 ml of potassium phosphate buffer (100 mM, pH 7.0). After centrifugation with cooling, the supernatants were utilized to evaluate the activity of antioxidant enzymes. Peroxidase activity (POD; EC 1.11.1.7) was assessed following the protocol outlined by Quessada and Macheix in 1984. Superoxide dismutase activity was determined following the procedures described by Dhindsa *et al.* (1981), whereas catalase activity was measured using the Aebi method (1984).

Quantification of IAA and ABA in roots

The frozen dried cucumber roots (equivalent to 5 g of fresh weight) were ground to a fine powder. The powder was washed three times with 80% v/v methanol (15 ml per g of fresh weight) and butylated hydroxytoluene at 4 °C in the dark. The extract was centrifuged at 4000 rpm, and the resulting liquid at the top was moved to flasks covered with aluminum foil. The residue was removed two more times. The aqueous extract was neutralized to pH 8.6 and then subjected to three extractions with pure ethyl acetate in equal volumes. The alkaline ethyl acetate extract was dehydrated using anhydrous sodium sulphate and then filtered. The filtrate was evaporated under a vacuum at 35 °C until dry, then reconstituted in 1 ml of 100% methanol. The leftover liquid extract was made acidic (pH 2.6) and then extracted with ethyl acetate as previously explained. The methanol extract was methylated according to Fales and Jaouni (1973) for the determination of abscisic acid (ABA) and indole-acetic acid (IAA). The endogenous phytohormones were quantified using a Varian 610 Series gas chromatograph equipped with a flame ionization detector according to the method described by Vogel (1975). The phytohormones were fractionated on a coiled glass column (1.5 m x 4 mm) packed with 1% OV-Gases flow rates of 30, 30, and 330 ml/min for nitrogen, hydrogen, and air, respectively. The peaks were identified and quantified using external authentic hormones and a Microsoft program to calculate the concentrations of the identified peaks.

RNAs Isolation and Quantitative PCR Analysis

Thirty-five days after infestation with the RKN, roots were collected from three replicates per treatment and preserved in liquid nitrogen. RNA was extracted using Trizol reagent and treated with DNase I. cDNA was synthesized using the SuperScript™ II reverse transcriptase according to the manufacturer's instructions. Gene-specific primers for PP2C, PYL1, RK2.1, and RK2.2 related to the ABA pathway, together with Actin as a housekeeping gene, were utilized as shown in Table 1. A qPCR analysis was conducted using a Mx3000P QPCR equipment from Agilent Technologies with a total reaction volume of 15 µL, following the protocol outlined by Beaubois *et al*. (2007). Three biological replicates, each consisting of 3 plants per treatment, were assessed to calculate the mean and standard deviation values.

Table 1. List of primer sequences used for qPCR (Pu *et al*., 2014)

Gene	Amplicon size (bp)	Primers (5' – 3')
Actin	122	TCCACGAGACTACCTACAACTC
		GCTCATACGGTCAGCGAT
PLY1	239	TTTGGAGATGGACAGGCAGGAG
		AAGCATACACCACCATGGACAAAAC
PP2C	201	TTATGGAGACTGATGCAGCTTTTGC
		ATCTCCTATGGCACGTGTAACTCCG
RK2,1	200	TCGCAACCTTCTTTCTCGC
		ATTTCCTCAACGCTCTGTG
RK2,2	209	ATTTGCGACTTCGGCTATTC
		TAAGCTCCAACCAGCATCAC

Statistical analysis

A combined analysis was undertaken on the findings from the two growth seasons using analysis of variance (ANOVA) due to their similarities. The data acquired were compared using Duncan's test (P <0.05) in Statistica 7 software (version 2004). The data are presented as mean values with standard error (SE). An online tool called RSplot was used to conduct heatmap correlation and Pearson analysis for data analysis and visualization.

Results

Impact of P. indica colonization on root biomass and M. incognita population

Colonizing cucumber plants with *P. indica* treatment (P) increased the fresh and dry weights of cucumber roots by 61.8% and 9.4%, respectively, compared to the control group (Ctr). In the case of dual inoculation by *P. indica* and *M. incognita* (PN), the fresh and dry weights of cucumber roots rose by 183.6% and 110.4% compared to the control group and by 20.9% and 44.3% compared to the *M. incognita* treatment (Table 2). Furthermore, PN treatment significantly reduced the final density of the *M. incognita* population in cucumber roots, exhibiting a reproduction factor (RF) of 0.72601 compared to the *M. incognita* treatment, which displayed an RF of 1.44003. A similar trend was noticed regarding root galling (RGI), where PN treatment resulting in considerable reductions in gall index (24% reduction) and the final population (FP) of *M. incognita* (42.6% reduction) ($p \geq 0.05$) compared with *M. incognita* treatment.

Table 2. Effect of *P. indica* colonization on root fresh and dry weight, root gall index (RGI), final population (FP) and reproduction factor (FP/IP)

Parameters	Ctr	N	P	PN
Root fresh weight (g)	5.5±0.21 d	12.9±1.12 b	8.9±0.76 c	15.6±1.01 a
Root dry weight (g)	0.96±0.039 d	1.40±0.11 b	1.05±0.068 c	2.02±0.092 a
Root gall index (RGI)	nd	5.00±0.100 a	nd	3.80±0.45 b
Final population (FP)	nd	1440.03±72.00 a	nd	826.01±27.12 b
Reproduction factor (FP/IP)	nd	1.44003±31.00 a	nd	0.72601±0.02 b

Ctr= control, P = *P. indica*, N = *M. incognita,* PN = dual inoculation of *P. indica* + *M. incognita*. Means with the different letters indicate significant differences according to Duncan's test ($p < 0.05$). ± value presented standard error, Initial population (IP) = 1000 larva, nd= non-detected.

Anatomy analysis

The treated and non-treated root tissue sections were microscopically examined and illustrated (Figure 1A-D). The *M. incognita* induced pronounced alterations in cells of the cortical and stellar regions in cucumber roots. In the positive control (*M. incognita* only), giant cells were found to be prolonged in vascular parenchyma cells with diverse shapes, from circular to irregular. Clusters of giant cells, each comprising three to four cells, were observed to occupy a significant portion of the vascular tissues. This led to significant disruption of the epidermal and cortical tissues, which were observed to be greatly separated from one another, along with the existence of several gaps within the cortical layer (Figure 1B). This also coincided with a decrease in the dimensions of the xylem components (Figure 1C).

Conversely, within root tissues co-inoculated with *P. indica,* giant cells that were poorly formed and ranged from two to five in number were detected in the stele. Notably, the dimensions of the xylem cells in this instance were increased relative to the positive control (Figure 1D). While the negative control, which was devoid of root-knot nematode (*M. incognita*) and *P. indica*, displayed normal cellular architecture (Figure 1A).

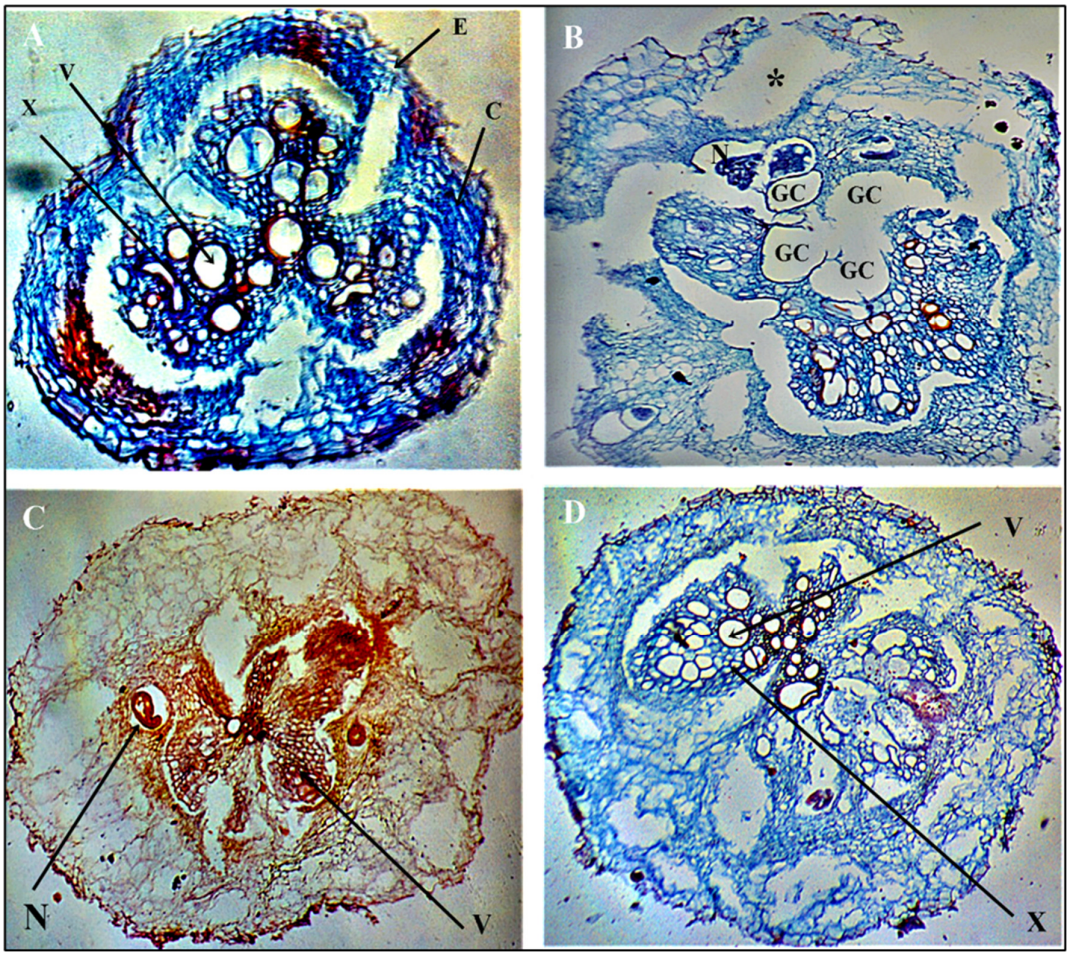


Figure 1. Microphotographs of cross sections through the cucumber roots, 40 days' post *M. incognita* infestation. Scale bars = 500 µm. (A) Negative control. (B and C) Positive control. (D) Plants inoculated by *M. incognita* + *P. indica*. Abbreviations: GC— giant cells; N—Adult female nematode; E—epidermis; C—cortical cells; v—vessels; and x—xylem. * Show the presence of gaps within the cortical layer

Impact of P. indica colonization on chlorophyll fluorescence and antioxidant enzymes

Piriformospora indica colonized cucumber plants significantly affected chlorophyll fluorescence measurements (PSII and Fv/Fm), and antioxidant enzymes (CAT, POD and SOD) of cucumber leaves. Dual inoculation with *P. indica* (P) and *M. incognita* (PN) improved the Fv/Fm and PSII by 16.1% and 17.2%, respectively, compared to *M. incognita* treatment (N), as shown in Figures 2 (A and B). A similar pattern was observed with antioxidant enzymes (Figure 2 C, D and E). Under *M. incognita* infestation, it was observed that *P. indica* (NP) treatment colonization led to a more pronounced increase ($p \geq 0.05$) in levels of antioxidant enzymes than in the negative and positive control. Furthermore, no significant differences were found between untreated plants (Ctr) and plants inoculated with *P. indica* (P) in PSII, Fv/Fm, CAT, POD and SOD.

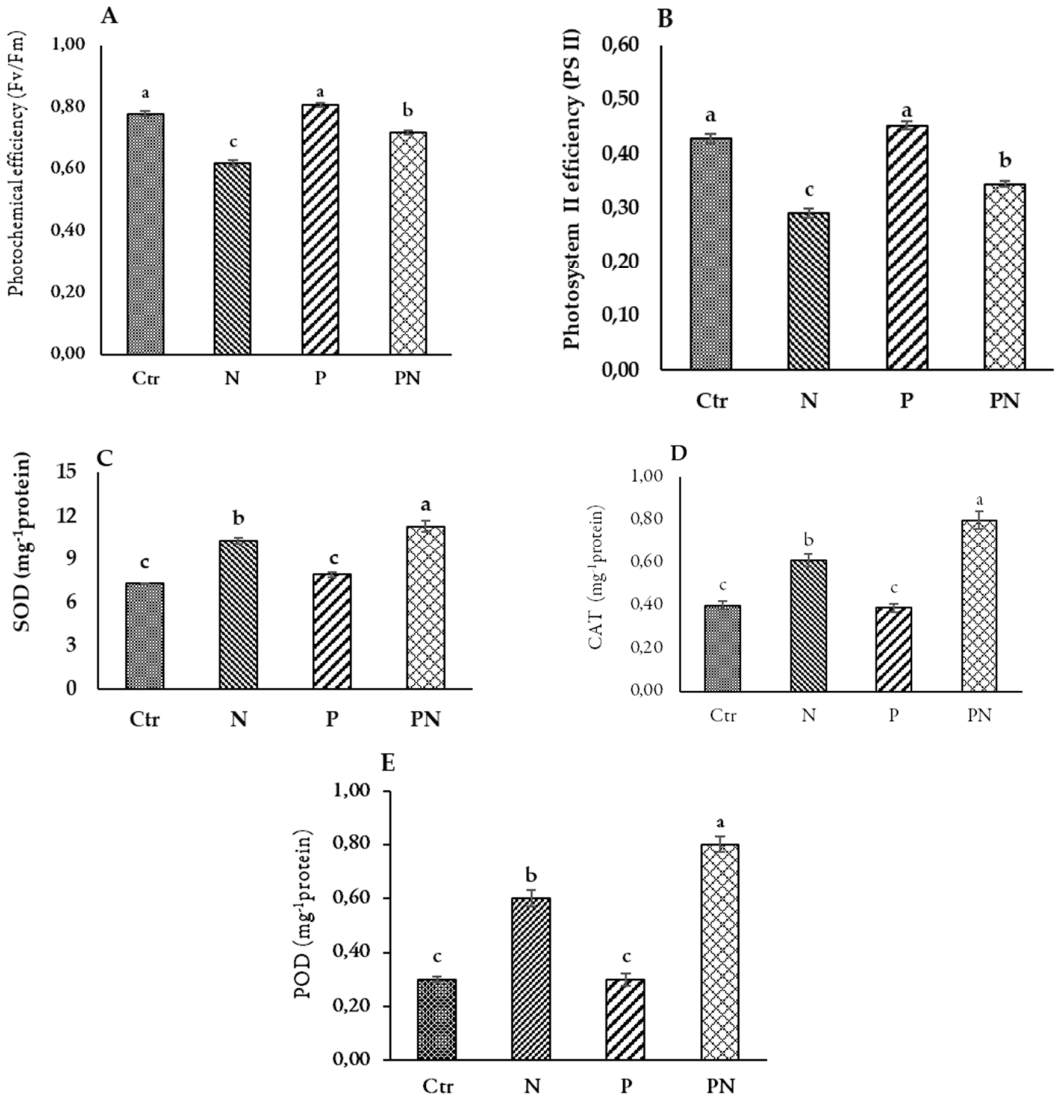


Figure 2. Impact of *P. indica* and *M. incognita* on photosynthesis II efficiency (PSII - A), photochemical efficiency of PSII (Fv/Fm - B), superoxide dismutase (SOD – C), catalase (CAT- D) and peroxidase (POD - E) of cucumber leaves

Treatments were organized as follow: Ctr = control, P = *P. indica*, N = *M. incognita,* PN = dual inoculation of *P. indica* and *M. incognita*. Columns with different letters indicate significant differences according to Duncan's test ($p < 0.05$), The vertical bar indicates standard error.

Impact of P. indica colonization on marketable yield and its components

Our results revealed that *M. incognita* (N) led to a notable decrease in marketable yield, fruit weight, and fruit number of cucumber plants, as presented in Table 3. *Piriformospora indica* (P) improved these traits by 36.8%, 17.3%, and 17.6%, respectively, compared to the control plants (negative control) and by 444.2%, 91.3%, and 185.7%, respectively, compared to the M. incognita treatment (positive control). In the case of dual inoculation with *P. indica* and *M. incognita* (NP), marketable yield, fruit weight, and fruit number decreased by 52%, 20.1%, and 40%, respectively, compared to the negative control, but increased by 90.7%, 30.3%, and 45.7%, respectively, compared to the positive control.

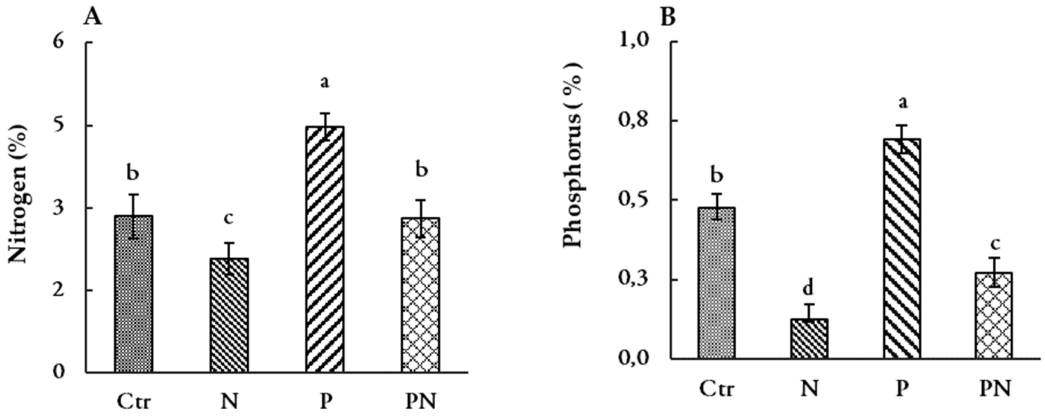
Table 3. Impact of *P. indica* and *M. incognita* on makeable yield per plant, fruit number and fruit weight, 40 days' post *M. incognita* inoculation

Treatment	Marketable yield per plant (kg)	Average fruit weight (g)	Number of fruits per plant
Ctr	1.71±0.21 b	100.30±2.72 b	17±1.12 a
N	0.43±0.13 d	61.50± 1.29 d	7±0.56 d
P	2.34±0.24 a	117.68±3.90 a	20±1.82 a
PN	0.82 ± 0.14 c	80.15±1.98 c	10.2±0.84 c

Treatments were organized as follow: Ctr = control, P = *P. indica*, N = *M. incognita,* PN = dual inoculation of *P. indica* and *M. incognita*. Means with the different letters indicate significant differences according to Duncan's test ($p < 0.05$). ± value indicates to standard error.

Impact of P. indica colonization on leaf nutrient content

P. indica enhanced the accumulation of endogenous nutrients in leaf tissues *M. incognita* infestation (PN) and non-infested (P) conditions (Figure 3A-F). Elevated mineral content was determined in leaves of cucumber colonized with *P. indica* compared to non-colonized plants (Ctr). Without *M. incognita*, the presence of *P. indica* led to increase in N, P, K, Ca and Mg by 56.8%, 46.8%, 32.7%, 26.3% Ca, 16.6%, respectively, compared to control plants (negative control). On the other hand, *M. incognita* (N) led to a notable decrease in leaf concentrations of N, P, K, Ca, and Mg by 27.0%, 72.3%, 63.3%, 10.7%, and 62.8%, respectively, as compared to the control group. The nutrient levels increased by 35.1%, 108%, 105%, 49.3%, and 127.6% in plants inoculated with both *P. indica* and *M. incognita* (NP) compared to the positive control (*M. incognita* alone).



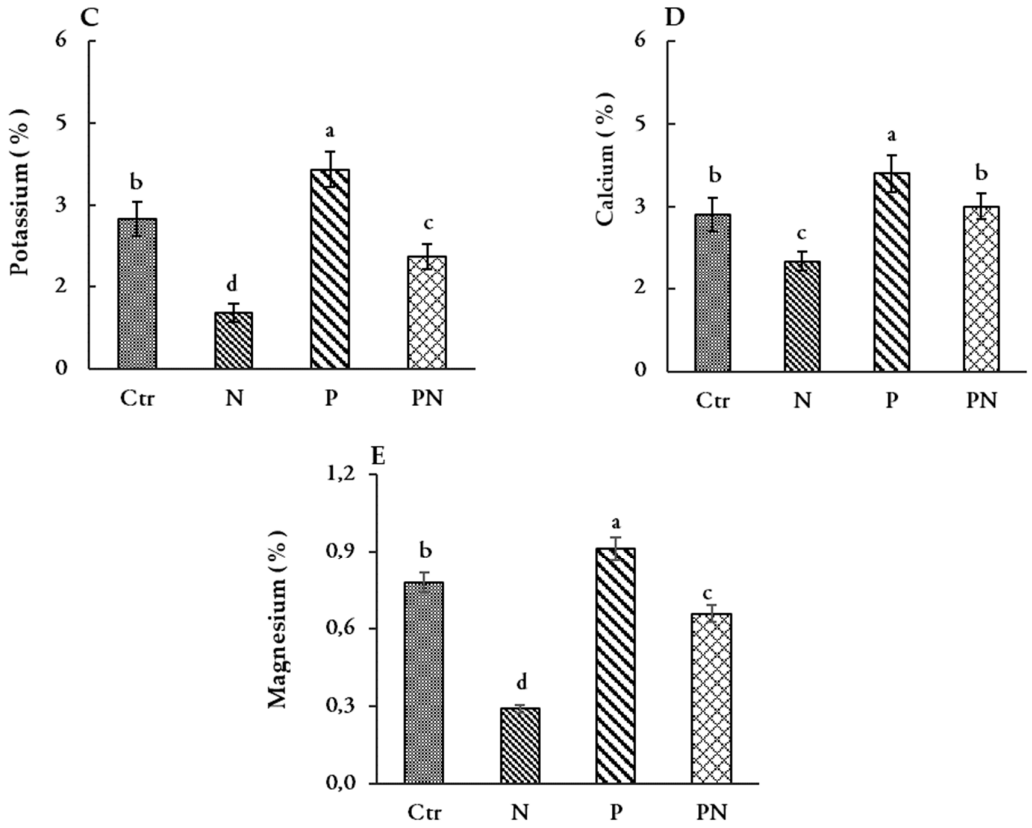


Figure 3. Impact of *P. indica* and *M. incognita* on nutrient content of cucumber leaves at 40 days post RKN inoculation

Treatments were organized as follow: Ctr = control, P = *P. indica*, N = *M. incognita,* PN = dual inoculation of *P. indica* and *M. incognita*. Columns with different letters indicate significant differences according to Duncan's test ($p < 0.05$), The vertical bar indicates standard error.

Impact of P. indica colonization on IAA and ABA accumulation in cucumber roots

Piriformospora indica colonization altered IAA and ABA in the roots of cucumber plants (Figure 4 A and B), while infested roots with the *M. incognita* (N) showed a significant reduction in the endogenous IAA content compared to all the other treatments. On the other hand, *P. indica* colonization improved the IAA content in roots by 461.5% under non-infested (P) and by 138.5% compared to the *M. incognita* alone treatment" after "under infested conditions (PN) (Figure 4A).

On the contrary, the maximum ABA level was obtained from plants infested by *M. incognita* alone (N) if compared to the other treatments (Figure 4 B). Further, *P. indica* + *M. incognita* (NP) treatment caused a reduction in root ABA concentration under infested conditions by 25.3% compared to *M. incognita* alone (Figure 4B). In addition, fungal inoculation did not show any significant changes in ABA concentration in roots under uninfected conditions compared to control plants.

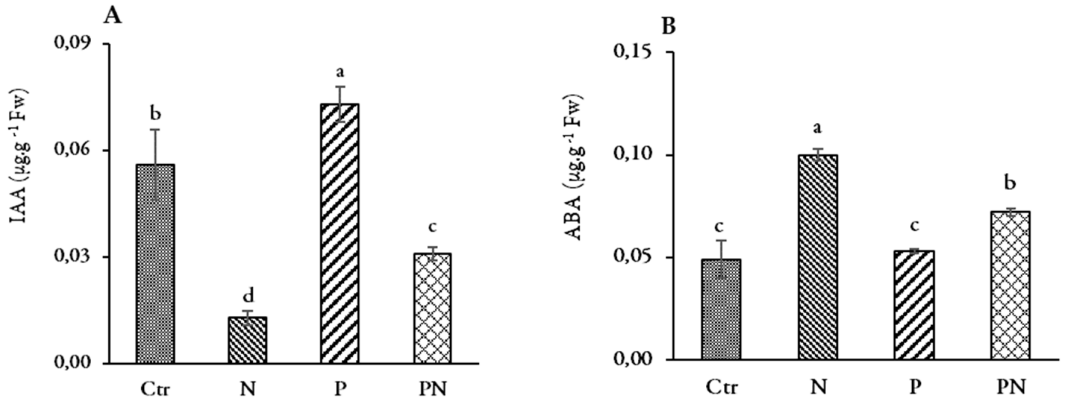
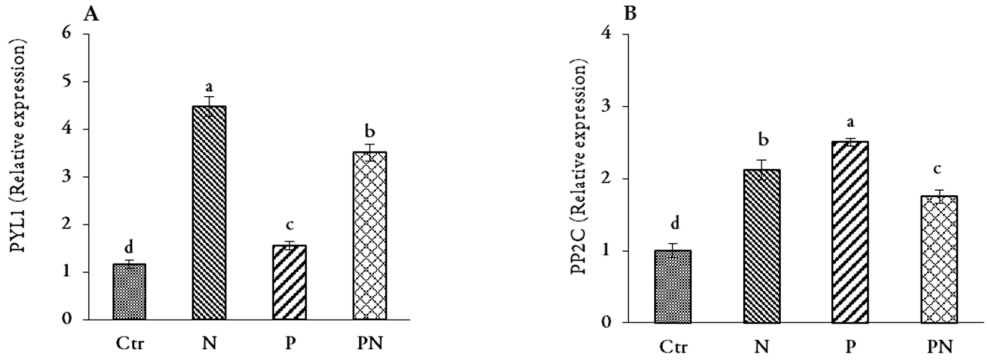


Figure 4. Impact of *P. indica* and *M. incognita* on endogenous IAA content (A) and ABA content of cucumber roots at 40 days' post RKN inoculation

Treatments were organized as follow: Ctr = control, P = *P. indica*, N = *M. incognita,* PN = dual inoculation of *P. indica* and *M. incognita*. Columns with different letters indicate significant differences according to Duncan's test ($p < 0.05$), The vertical bar indicates standard error.

Effect of P. indica colonization on expression levels of ABA related genes

Piriformospora indica inoculation showed significantly higher records regarding expression levels of PYL1, PP2C, RK2.1, and RK2.2 genes in cucumber roots (2.5-fold for PP2C, 3.8-fold for RK2.1, and 4.1-fold for RK2.2) compared to control (Ctr). While infested plants with *M. incognita* (N) showed a significant increase in the expression of PYL1, RK2.1, and RK2.2 genes compared to other treatments (4.5-fold for PYL1, 5.4-fold for RK2.1, and 5.2-fold for RK2.2) compared to control (Ctr). Interestingly dual inoculated plants (PN) significantly mitigated/downregulated tested genes compared to plants infested with (N) alone (1.8-fold for PP2C, 3.5-fold for PYL1, 3.1-fold for RK2.1, and 4.5-fold for RK2.2), as shown Figure 5.



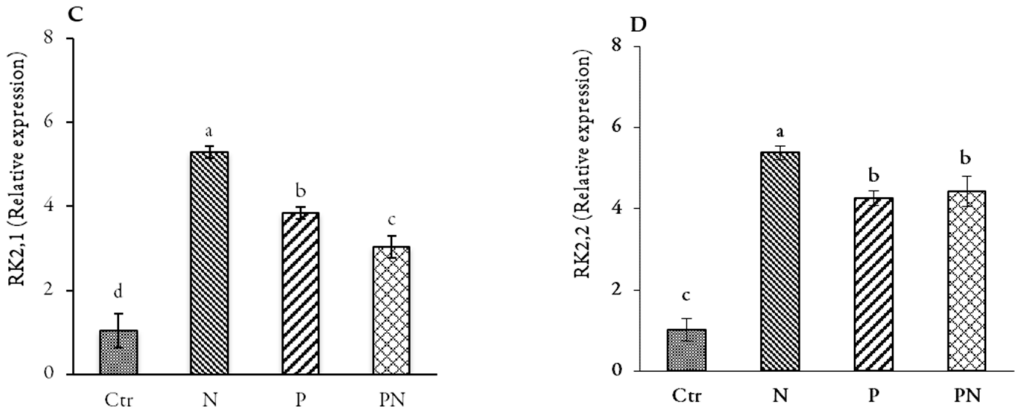


Figure 5. Relative gene expression of PLY1 (A), PP2C (B), RK2,1 (C) and RK2,2 (D) in roots of the *P. indica* colonized and non-colonized cucumber plants at 40 days' post *M. incognita* infestation

Treatments were organized as follow: Ctr = control, P = *P. indica*, N = *M. incognita,* PN = dual inoculation of *P. indica* and *M. incognita*. Columns with different letters indicate significant differences according to Duncan's test ($p < 0.05$), The vertical bar indicates standard error.

Correlation study

Pearson's analysis and heatmap correlation present the variations in Agro-physiological and biochemical properties of cucumber plants inoculated with *P. indica* and *M. incognita* (Figures 6 and 7). The heatmap is based on the 21 measurements, evidently classified into two group (A and B), while the (P) and (PN) treatments were placed under group (A). Meanwhile, control (Ctr) and (N) treatments are inserted in group (B). The heatmap correlation indicates that *P. indica* colonization treatment has a more positive impact on antioxidant enzymes (CAT, POD, and SOD), and root fresh weight under (N) infestation condition. Furthermore, *P. indica* has clear positive effects on PSII, Fv/Fm, nutrient accumulation in plant leaves (N, K, Mg, and Ca) and marketable yield, under normal condition. In contrast, *M. incognita* (N) has clear negative impacts on above parameters.

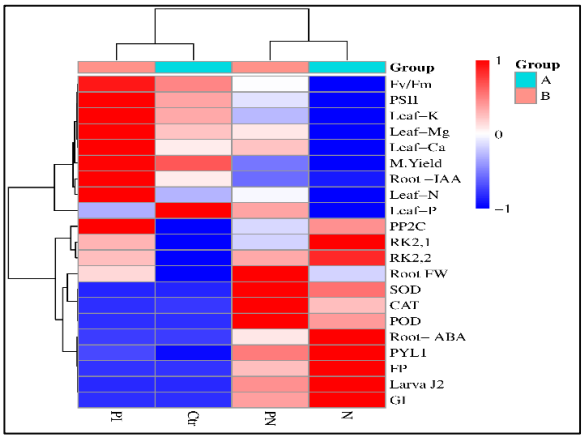


Figure 6. Heatmap correlation between Agro-physiochemical properties and gene expression of cucumber plants infested by *Meloidogyne incognita* and treated *P. indica*

Abbreviations*:* PI. *P. indica*; Ctr, control; PN, *P. indica* and Nematode; N, nematode *Meloidogyne incognita*; Root FW, root fresh weight; ABA, abscisic acid; IAA, indole-3-acetic acid; PSII, photosystem II efficiency; Fv/Fm, photochemical efficiency of PSII; CAT, catalase; SOD, superoxide dismutase; POD, Peroxidase; GI, gall index; FP, final population; Larava J2, soil larva J2; ABA genes (PP2C, RK2,2, RK2,1, PYL1); and M. yield, marketable yield. The red color shows a positive effect, and the blue color displays a negative effect.

Correspondingly, Pearson's correlation analysis was used to assess the positive and negative correlations between the studied parameters (Figure 7). This correlation analysis revealed that marketable yield positively correlated with root fresh weight (Root-FW), root IAA content, leaf nutrient concentrations (N, K. Mg and Ca), and photosynthesis parameters (PSII, and Fv/Fm). On the contrary, marketable yield is negatively correlated with to antioxidant enzymes (CAT, POD and SOD), final population of *M. incognita*, and root gall index.

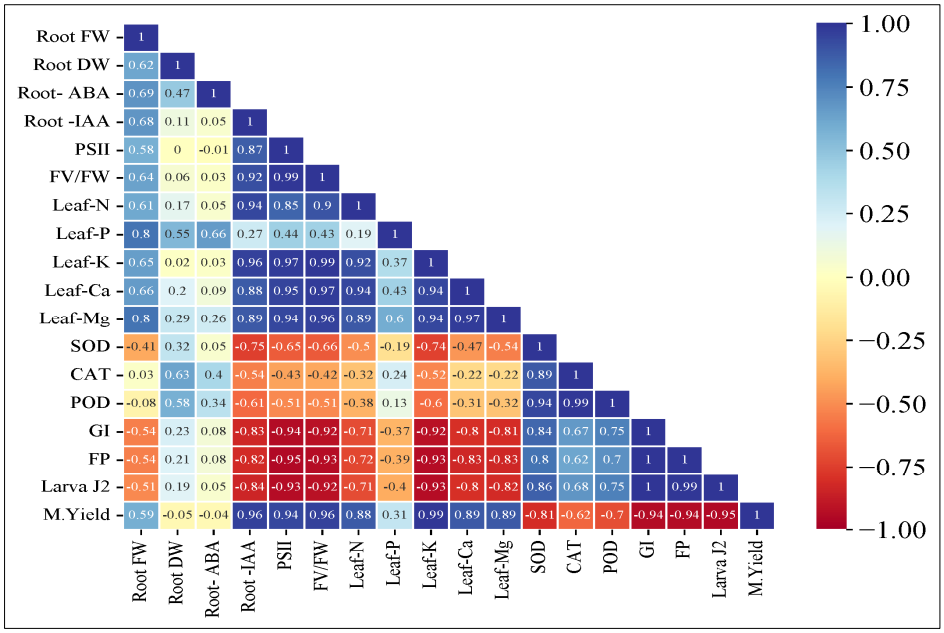


Figure 7. Pearson's correlation analysis between morphological and physiochemical properties of cucumber plants infested by *P. indica* and *Meloidogyne incognita*

Abbreviations: Root FW, root fresh weight; Root DW, root dry weight; ABA, abscisic acid; IAA, indole-3-acetic acid; PSII, photosystem II efficiency; Fv/Fm, Chlorophyll florescence; CAT, catalase; SOD, superoxide dismutase; POD, Peroxidase; GI, gall index; FP, final population; and M. yield, marketable yield. A positive correlation (blue colour), and negative relationships (red colour).

Discussion

Employing biocontrol agents presents as a viable alternative to the application of chemically synthesized nematicides causing adverse environmental impacts (Seo *et al*., 2019). Within biocontrol methodologies, the utilization of endophyte fungi, such as *P. indica*, has proved to inhabit the intercellular spaces of plant tissues asymptomatically and have garnered attention for their contribution to sustainable agricultural practices (Xu *et al*., 2018). Their beneficial attributes encompass the facilitation of plant growth, augmentation of yield, and amplification of plant resilience to both living (biotic) and non-living (abiotic) stressors (Xu *et al*., 2018).

Recent reports indicate that plants colonized with *P. indica* exhibit resilience against soil borne disease and environmental stresses through modulation of plant hormones, antioxidant enzymes, chlorophyll formation, nutrient uptake, improved photosynthesis, and related genes (Atia *et al*., 2020; Abdelaziz *et al*., 2021). There is limited research available on the inhibitory impact of *P. indica* on root knot nematode and its effects on the growth characteristics and yield of vegetables when infested with RKN. The previous study demonstrated that colonization cucumber with *P. indica* led to a considerable decrease in nematological parameters (number of egg/ egg mass, egg masses, and soil Juvenile 2), while also enhancing plant growth

performance, endogenous salicylic acid levels, and the expression of their responsive genes (Atia *et al*., 2020). The present study examines the regulatory influence of *P. indica* on photosynthetic efficiency (PSII and Fv/Fm), antioxidant enzymes, nutritional absorption, plant hormones (IAA and ABA), and ABA-responsive genes.

The study demonstrates that the presence of *P. indica* increased the fresh and dried biomasses of cucumber roots in both non-infested and infested situations (Table 2). Pearson's correlation analysis indicated a positive association between root IAA content and root fresh weight, as shown in Figure 7. The rise in root biomasses may be attributed to the elevated levels of IAA content, which plays a crucial role in cell division, elongation, and carbohydrate storage (Fu *et al*., 2015). According to Lee *et al*. (2011), *P. indica* significantly enhanced the expression of genes related to carbohydrate accumulation, phytohormone signaling, and root development. The rise in root biomasses in plants affected by *M. incognita* alone may be attributed to extensive gall formation (Oclarit and Cumagun, 2009).

Meloidogyne incognita infestation revealed a significant reduction in root gall index (GI), final nematode population (FP) and reproduction factor (RF) of *M. incognita* when plants were colonized with *P. indica* (NP) as compared to non-colonized. Whereas the reduction in aforementioned nematological parameters (GI, FP and RF) in colonized plants is probably not only associated with physical barriers formed by fungal hyphae but correlated to the release of nematicidal complexes that considerably reduced egg hatching and J2 mortality (Daneshkhah *et al.,* 2013). Research has shown that fungal endophytes can alter the chemical composition of root exudates or induce plants to produce more compounds or phytohormones that deter or disrupt nematode infestation (Le *et al*., 2009). The study by Dababat and Sikora (2007) found that the root exudates of *Solanum lycopersicum* plants had a strong repellant impact on *M. incognita* when combined with beneficial endophytic *Fusarium oxysporum*. Further studies reported that the *P. indica* stimulated the production of phytohormones such as, jasmonic acid (JA), ethylene (ET), gibberellin (GA), salicylic acid (SA), and abscisic acid (ABA) (Li *et al*., 2023). Phytohormones have significant functions in plant defense against root knot nematode (*M. incognita*) and cyst nematode (Le *et al*., 2009; Atia *et al*., 2020).

Furthermore, the anatomy analysis of non-colonized roots infested with *M. incognita* showed a larger giant cells, higher compressed cells and greater disruption of the xylem vessels and cortex layers of roots than colonized plants by *P. indica* alone (Figure 1A-D). The disruption in root cells causes a reduction in water absorption, and nutrients uptake consequently decreases the photosynthetic efficiency, yield quantity and quality (Saleh *et al*., 2014). Furthermore, a negative correlation was found between gall index (GI) and leaf nutrient content (N, K, Ca and Mg), as presented in Figure 7.

On the contrary, better nutrient accumulation (such as N, P, K, Mg, and Ca) was observed in leaf tissues of plants treated with *P. indica* than in untreated plants under both non-infested and infested conditions (Figures 3 and 6). Li *et al*. (2023) confirmed that *P. indica* is endophyte fungus which is commonly engaged for different plant health benefits due to its ability to enhance plant growth, abiotic stress tolerance, and disease resistance. Additionally, the efficiency of *P. indica* is not only to protect the plant roots from damage caused by soil bone disease but also to increase the root auxin content. This phytohormone induces plant root growth that might modify root architecture, leading to improved root length and subsequently augmenting the root area for useful microbial colonization and improved nutrient uptake (Khoso *et al.,* 2024)

The study shows that *P. indica* reduced the harm caused by *M. incognita* on the photosynthetic system, leading to an improvement in the photochemical efficiency of PSII (Fv/Fm) and Photosynthesis II efficiency (PS II) as shown in Figure 2 (A and B). The improvement of fluorescence characteristics has been reported to be similar when *P. indica* colonization occurs under both biotic and abiotic stressors (Shahabivand *et al*., 2017; Ghorbani *et al*., 2018, Li *et al*., 2020). Shahabivand *et al*. (2017) confirmed that *P. indica* stimulated the chlorophyll information and photosynthesis system under soybean cyst nematode by increasing electron transfer and enhancing the plant's capability for using light energy (Lee *et al*., 2019). Enhancing the

fluorescence characteristics demonstrates how *P. indica* enhances the efficiency of the photosynthetic system in cucumber plants when they are infested with RKN.

Plant parasitic nematodes induce oxidative damage and interfere with the physiological functions of infested plants by generating high levels of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) at the location of worm invasion (Baker and Orlandi, 1995). In addition, the inhibition of ROS-scavenging mechanisms can initiate various metabolic processes leading to cell death and tissue necrosis at the point of entry of the invading juveniles (Molinari and Leonetti, 2023). Plants have evolved enzymatic and non-enzymatic ways to protect themselves from oxidative damage. Important enzymes that scavenge reactive oxygen species (ROS), including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX), are essential for neutralizing superoxide radicals and hydrogen peroxide (Mittler *et al*., 2002).

The SOD is known as the primary defence against ROS-induced oxidative damage in most living cells, catalysing the removal of $\bullet O_2^-$ by converting it into O_2 and H_2O_2 (Sahu *et al*. 2022). The H_2O_2 produced must be eliminated by conversion to H_2O in subsequent reactions (Talaat, 2015). CAT is involved in various plant physiological responses during vegetative and reproductive stages (Yang *et al*., 2019; Zhang *et al*., 2020), converting H_2O_2 into water and O_2. Meanwhile, POD plays a crucial role in plant physiology, including strengthening the cell wall and acting as a physical barrier to pathogen entry at the point of interaction (Sahu *et al*., 2019; Dos Santos and Franco, 2022). It scavenges H_2O_2 using reductants in the extracellular space (Apel and Hirt, 2004; Talaat, 2015).

The results of the present study show an increase in SOD, CAT, and POD activities in leaves of cucumber exposed to RKN infection either alone (N) or in combination with *P. indica* (NP). While, increasing activity of antioxidant enzymes in colonized plants higher than non- colonized plants, under RKN infestation conditions (Figure 2 C, D, and E). Likewise, Pearson's correlation showed that root gall index (GI) positively correlated with leaf SOD, CAT and POD (Figure 7). This raising antioxidant enzymes possibly indicating either increased ROS production or the manifestation of an antioxidant defence. These results align with a study by Danish *et al*. (2021), whom recorded a significant increase in enzyme activity in *Trachyspermum ammi* plants infected with *M. incognita*. Additionally, RKN-stressed plants treated with *P. indica* exhibited the highest activity levels of antioxidant enzymes (Figure 2 C, D, and E). These results of the current study were consistent with findings reported by Wu *et al*. (2022) who reported that the activity of SOD, CAT, and APX and their relative genes increased in plants exposed to various stresses (Zhang *et al.,* 2018; Tsai *et al*., 2020; Zhang *et al*., 2022; Boorboori *et al.,* 2022). *P. indica* not only boosts the activities of antioxidant enzymes but also plays a crucial role in managing the balance of essential plant phytohormones such as indole-3-acetic acid (IAA), and abscisic acid (ABA). The regulation of these hormones is vital to plants' defensive mechanisms and their interaction with pathogens, which is significant for resistance to various stresses caused by living organisms (Li *et al*. 2023). IAA is fundamental for cell division, growth, and differentiation (Mwange *et al.,* 2003), and essential for initializing and developing sites within the plant that parasites like nematodes feed on (Karczmarek *et al*., 2004). ABA interplays with the biosynthesis and function of IAA, whereas GA3 is important for the growth and maturation of nematode feeding structures (Klink *et al*., 2007; Kyndt *et al*., 2012; Kammerhofer *et al*., 2015).

In this research, cucumber plants infected with root-knot nematodes (RKN) showed a steady decrease in IAA levels, while ABA levels significantly surged (Figure 6 A-D). This finding concurs with earlier observations by Kyndt *et al*. (2017), who reported an increased levels of ABA upon RKN exposure in plants. In contrast, plants treated with *P. indica* exhibited a protective effect against the destructive influence of *M. incognita*. Remarkably, *P. indica*-treated plants under RKN stress managed to retain higher IAA levels and lower ABA levels (Figure 4 A and B) if compared to non-colonized plants. This is in harmony with Abdelaziz *et al.* (2021). where colonization by *P. indica* led to increase the accumulation of IAA and decrease the

concentration of ABA in cucumber leaves under water stress. The adjustments in the levels of these endogenous phytohormones suggest how *P. indica* might govern the defence against RKNs. For instance, elevated IAA levels can trigger the opening of stomata and counteract the stomatal closing caused by ABA (Levitt *et al*., 1987). Additionally, some investigators reported that, under stress conditions, auxin drives a wide range of physiological processes, including the stomatal closure, the production of aquaporin, the positioning of lateral roots (Naser and Shani, 2016), and the increase the concentration of osmoregulation substances (such as soluble sugar and proline) (Shiraz *et al*., 2020; Mir *et al*., 2020). Furthermore, auxin contributes to the stress tolerance of plants through the scavenging of ROS and protection of the photosystem II (PSII) from damage (Gong *et al*., 2014; Kaya *et al*., 2010). In addition, the ABA increased under water stress which caused by RKN infestation due to root damage and blocking water absorption (Dababat and Sikora, 2007). ABA stimulates water flow in root cells indicating to ABA modulates turgor by reducing transpiration and increasing water influx into roots (Glinka and Reinhold, 1971). The findings of the present study confirmed that colonization with *P. indica* reduced ABA content in the plant roots more than non-colonized cucumber plants. This indicates that *P. indica* has the ability to alleviate the damage caused by *M. incognita* and improve water use efficiency in infested plants. Given the intricate interplay of phytohormones and their capacity to interconnect, they are prime agents in orchestrating plant defence strategies against the root-knot nematode *M. incognita* (Dababat and Sikora, 2007; Le *et al*., 2009; Li *et al*., 2023).

Additionally, the results of this study clearly demonstrated that the application of *P. indicia* induces the upregulation of PP2C, PYL1, RK2.1, and RK2.2 genes in cucumber roots (Figure 5), indicating the activation of ABA signalling pathways. PP2C is known to be a key player in ABA signalling, serving as a molecular switch that regulates plant resistance against various stresses and developmental cues (Yang *et al*., 2018). When ABA levels are high, ABA binds to PYR/PYL/RCAR receptors, forming complexes with PP2Cs, leading to their inactivation. This inactivation allows SnRK2 to remain active, resulting in enhanced plant resistance (Park *et al.,* 2009). In the positive control group, where plants were solely inoculated/infested with PN, the downregulation of PP2C expression suggests that *P. indica* may manipulate the ABA signalling pathway to mitigate the negative impact of the nematode infestation. By upregulating PP2C, PYL1, RK2.1, and RK2.2, nematodes potentially stimulate the production of ABA, because of plant resistance induction (Ma *et al*., 2009; García-Andrade *et al.,* 2020). Interestingly, in the simultaneous inoculation group with both N and *P. indica*, the significant decrease in PP2C, PYL1, RK2.1, and RK2.2 expression suggests that *P. indica* may counteract the negative effects of nematode infestation on ABA signalling. This could potentially reactivate the ABA signalling pathway, resulting in enhanced plant resistance against nematode. The results of current study are in line with those obtained by García-Andrade *et al*. (2020), who found that SnRK2s kinases were actively engaged in activating resistance towards *Plectosphaerella cucumerina*. These findings reinforce the existing knowledge that ABA signalling plays a crucial role in plant immune responses and stress tolerance. However, the precise mechanisms by which ABA influences immune responses in different plant-pathogen interactions remain incompletely understood.

In the end, to understand well the role of *P. indica* in reducing *M. incognita*, decreasing root ABA content and its relative genes, improving the enzymatic antioxidants, enhancing leaf photosynthesis II efficiency (PSII), leaf photochemical efficiency of PSII (Fv/Fm), increasing yield and its components. The results of the greenhouse experiment are summarized in a schematic diagram (Figure 8) including all studied measurements in this research. The diagram indicates the actions of *P. indica* colonization and its contributions to alleviating the *M. incognita* damages and improving the growth and productivity of cucumber plants.

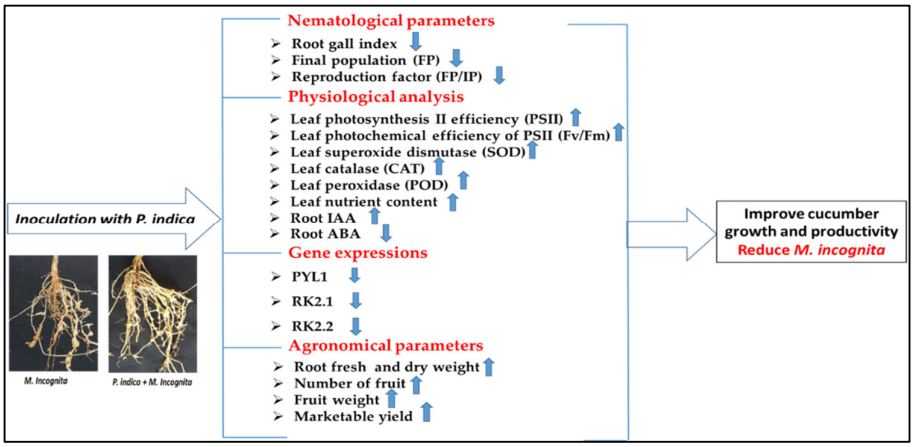


Figure 8. Schematic illustration clarifies the effect of *P. indica* colonization on *M. incognita* population measurements and agro-physiological parameters of cucumber plants

Conclusions

Piriformospora indica shows promising potential as a biocontrol agent against the root knot nematode (RKN), *Meloidogyne incognita*, in cucumber plants. Colonization by *P. indica* resulted in a significant reduction in RKN damage, improved growth parameters, and mitigated the negative effects of RKN on photosynthesis in cucumber plants. Furthermore, *P. indica* colonization led to a decrease in nematode infection and an increase in nutrient and phytohormone levels in cucumber plant. The study revealed that *P. indica* modulates abscisic acid (ABA) levels in cucumber roots, which antagonizes RKN and alleviates the severity of damage. The downregulation of ABA concentration in cucumber roots correlated with the upregulation of genes involved in the ABA pathway. The presence of *P. indica* in RKN-infested cucumber plants resulted in altered expression of specific genes related to ABA signaling. These findings suggest that *P. indica* has the potential to be developed as an effective biocontrol agent against RKN in cucumber plants. However, further research is needed to validate these results in commercial field conditions to elucidate the underlying market potential of *P. indica*-mediated RKN resistance.

Authors' Contributions

Conceptualization: EAA, MEA, and MAMA; Data curation: NEAR, MA, DSSI, and AWMM; Formal analysis: MME and SMA; Funding acquisition: MME, EAA, and MAMA; Investigation: MME, NEAR, DSSI, EAA, and MEA; Methodology: SMA, EAA, MEA, MAMA; Project administration: MME, MA, and AWMM; Resources: AWMM, MAMA, and EAA; Software: MME, SMA, and DSSI; Supervision: MEA, EAA, and DSSI; Validation: MAMA and MA; Visualization: MME and MEA; Writing - original draft: MME, MAMA, NEAR, MA, DSSI, AWMM, SMA, EAA, and MEA; Writing - review and editing: NEAR, EAA, EAA, and SMA. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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