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Evaluation of the efficacy of some organic substances to induce resistance in cucumber against root knot nematode, *Meloidogyne* spp.

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Abstract

The study aimed to evaluate the impact of certain biofertilizers on some growth parameters, total chlorophyll content and the concentrations of enzymes phenylalanine ammonia -lyase **(**PAL) and peroxidase (POX) pre and post infestation of cucumber with root-knot nematodes (*Meloidogyne* spp.). The results demonstrate that supplementing substances before or after nematode infection resulted in enhanced growth parameters of cucumbers, including shoot growth, fresh and dry weight of root, total chlorophyll content and the concentrations of the enzymes PAL and POX, with significant differences compared to non-infected plants. Among those that showed a positive effect were ascorbic acid, K_2HPO_4, liquid earthworms, crushed fertilizer and compost. Among the test biofertilizers evaluated, the liquid vermicompost proved to be the standout performer compared to the other treatments, demonstrating the most substantial growth improvements such as the highest increase in both shoot fresh weight (44.67 g per plant) and dry weight (5.93 g per plant). Additionally, this treatment resulted in a remarkable 83 % increase in total chlorophyll content and a significant increase in PAL and POX enzyme activity (18.25 and 25.29 IU g^{-1} respectively).

Keywords

Biofertilizers; earthworm; cucumber; nematode infestation; growth enhancement

Introduction

Vegetables are an important and essential component of the human diet worldwide. They are grown in greenhouses and open fields and include several families, the most important of which is the Cucurbitaceae family, that includes about 96 genera and about 1000 species (1). Cucumber is one of the most important crops of the Cucurbitaceae. Its fruits are used for direct consumption or indirectly in the processing industries, such as in pickles (2). The global production of cucumbers in 2019 was estimated at 87.8 million tons (3). The production of cucumber by all of its varieties in 2022 in Iraq reached 196 thousand tons, reflecting an increase of 5.6 % compared to the previous year's production, which was 185.5 thousand tons (4). Cucumbers are infected with various insect pests and diseases, with powdery and downy mildew root rot being the most significant diseases. The root-knot nematode, *Meloidogyne* spp., is known to be the most dangerous among plant-parasitic nematodes due to its global distribution and wide host range. It causes a significant reduction in the quantity and quality of cucumber production, greatly affecting the marketability of these crops (5).

Environmental conditions in greenhouses are favorable for the spread of pests and diseases, unlike open-field cultivation, which is less susceptible to pests (6). Among the various soil-borne pests, root-knot nematode (RKN) (*Meloidogyne* spp.) has become a major limiting factor in crop production in greenhouses. RKN are pathogens that cause significant yield losses, reduced crop productivity and damage to economically important crops (7). RKN is classified as one of the most important plant-parasitic pathogens, causing devastating crop losses annually, resulting in significant damage and production losses in many seasonal plants, especially vegetable crops (8). The root knots that appear after crop infection reduce water and nutrient uptake, leading to wilting, mineral deficiency, reduced plant growth, yield reduction and plant biomass (9).

The proper management and control of plant diseases is one of the main objectives that must be given attention in order to preserve the quality and abundance of food produced by farmers. Farmers have always relied on chemical pesticides, which are considered a traditional method of controlling these pathogens (10, 11). The use of biological agents to stimulate plant resistance has a positive effect on reducing the root-knot index caused by *M. javanica* and the severity of infection in plants. Among these factors include bacteria such as *Glomus* spp. and *Pseudomonas* spp. (12). The treatment of fig seedlings with *Hirsutella* spp. and Verox prompted remarkable increment in leaf chlorophyll content, foliage fresh weight, root fresh weight, foliage dry weight and root dry weight as obtained 37.17, 44.67, 60.63, 16.90 and 17.2 g respectively in seedlings treated with *Hirsutella* spp., and 35.83, 44.33, 52.50, 14.63 and 19.20 g respectively in seedlings treated with Verox, compared to the control which resulted in 30.90, 28.57, 39.00, 11.63 and 12.07 g respectively (13). In assessing the adequacy of natural pesticides, Biocont-T and *Paecilomyces lilaccinus* as well as the compound pesticide Rygbe, against root-knot nematodes (*Meloidogyne* spp.), the results demonstrated beneficial outcomes of all factors in lessening the quantity of eggs and juveniles compared with the control treatment (14). However, the repeated and indiscriminate use of these pesticides has led to many problems, such as disrupting the ecological balance, effects on non-target microorganisms and weakening their role in natural control, poisoning non-target organisms and the emergence of resistance to pesticides (15). The long-term use of these pesticides has led to the ban or restriction of many of them around the world. This has led to the need to find safer and more environmentally friendly methods to control insect pests. One of the most promising alternatives is the use of biocontrol agents, which are environmentally friendly and effective against pathogens, especially RKN (16). Another important strategy used against pests and pathogens is induced resistance, which is achieved by controlling disease-related proteins (17).

There are chemical pesticides against RKN (*Meloidogyne* spp.), which are characterized by their slow decomposition and high toxicity to humans and non-target organisms. This study aimed to find out environmental friendly alternatives to these pesticides (chemical and biological).

Materials and Methods

The current study was conducted in the greenhouses and laboratories of the Plant Protection Department, College of Agricultural Engineering Sciences, University of Baghdad, Iraq.

Preparation of the root-knot nematode (Meloidogyne spp.) inoculum

Samples used in the experiments were collected from the roots of cucumber infected with RKN (*Meloidogyne* spp.) from one of the greenhouses in Salah al-Din Governorate, Sharqat district, after observing symptoms of infection on them, such as yellowing and wilting of the shoot parts as well as dwarfing and knots on the root system. The samples were placed in polyethylene bags and transferred to the Nematology Laboratory, Plant Protection Research Department, Agricultural Research Directorate, Abu Ghraib. A standard method was followed to extract the root-knot nematode (*Meloidogyne* spp.) inoculum (18).

Preparation of chemical and biological materials

Ascorbic acid

1.761 mg of ascorbic acid was dissolved in 200 mL distilled water with continuous stirring until dissolved to obtain a concentration of 50 mM.

Preparation of Dipotassium hydrogen phosphate (K_2HPO_4) salt

Dipotassium hydrogen phosphate (K_2HPO_4) salt was purchased from Thomas Baker (chemicals) Pvt. Ltd. India. A stock solution of K_2HPO_4 salt was prepared by dissolving 1.761 mg of K_2HPO_4 salt in 200 mL distilled water with continuous stirring until dissolved to obtain a concentration of 50 mM.

Preparation of dry worm powder from cultured earthworms (dry vermicompost)

Earthworms were collected from a farm established in January 2022. After raising by culturing the worms, they were collected from the farm and transferred to the laboratory to use in the experiments. After cleaning the worms and washed them with distilled water to remove impurities and mud accumulated on their body surfaces. Then, it was placed in a dish to dry and ground. The required weight in the experiment was 0.07 g.

Preparation of the aqueous extract of dry earthworm powder from cultured earthworms (liquid vermicompost)

0.7 g of the previously prepared dry worm powder was taken and placed in a glass flask. The volume was adjusted to 100 mL with distilled water and then placed on a magnetic stirrer for 24 h to homogenize the solution. Then, it was filtered using 2 layers of muslin cloth and the extract was taken in plastic tubes and centrifuged at a speed of

3000 rpm for 10 min to obtain a supernatant. The supernatant was then stored in a bottle until used in the experiments (19, 20).

Commercial compost

The commercial powder used was obtained from a company called "Baghdad's Banks" and a dry weight of 0.07 g was taken to use in the experiments.

Diagnosis of earthworms used in this study

They were morphologically diagnosed at the Natural History Research Center and Museum/University of Baghdad by Dr. Nibras Fadel Jijan (Department of Life Sciences/College of Sciences/Al-Mustansiriya University) using the taxonomic key as *Aporrectodea tuberculata*.

To study the effect of ascorbic acid, K_2HPO_4, dry and liquid vermicompost and compost on some growth parameters

This experiment used 3 kg capacity Styrofoam cups containing soil that had been previously autoclaved to ensure aseptic condition. The soil was mixed with sterilized agricultural peat moss in a ratio of 2:1 and used as a medium for growing the seedlings. Cucumber seedlings with 3-4 true leaves were planted in the cups and left for five days under greenhouse conditions to allow the plants to acclimatize and recover their vitality after being transferred to a new environment. The seedlings were treated with RKN inoculum in 2 ways: the inoculum was added three days before and after the addition of chemical and biological elicitors. These two groups of plants were treated with the addition method as follows:

Group 1: Addition of the inoculum containing eggs and second-stage juveniles (J2) (after contamination)

Five longitudinal holes were made 2 cm away from the plant stem and 3 cm deep (21). 1500 ± 50 eggs and juveniles (J2) were added per plant. Three days later, the chemical factors (acid and salt) and the biological factors (dry and liquid vermicompost and dry compost) prepared in advance were added. Only the effective concentration of these factors was used for this study (22).

The experiment included the following treatments:

- Sterilized soil + nematode inoculum + ascorbic acid
- Sterilized soil + nematode inoculum + Dipotassium hydrogen phosphate
- Sterilized soil + nematode inoculum + liquid vermicompost
- Sterilized soil + nematode inoculum + dry vermicompost
- Sterilized soil + nematode inoculum + compost (dry)
- Sterilized soil + distilled water

Group 2: Addition of the experiment factors (before contamination)

The chemical factors (acid and salt) and the biological factors (dry and liquid vermicompost and dry compost) prepared in advance were added 3 days before the addition of the inoculum at a rate of 1500 ± 50 eggs and juveniles per plant. Only the effective concentration of these factors was used.

The experiment included the following treatments:

- Sterilized soil + ascorbic acid + nematode inoculum
- Sterilized soil + Dipotassium hydrogen phosphate + nematode inoculum
- Sterilized soil + liquid vermicompost + nematode inoculum
- Sterilized soil + dry vermicompost + nematode inoculum
- Sterilized soil + compost (dry) + nematode inoculum
- Sterilized soil + distilled water

For further analysis, the third leaf from the crown area was taken from 4 plants in each replicate and 4 replicates per treatment were used for this study (23).

Evaluation of the effect of concentrations of salt, acid, dry and liquid vermicompost and compost on the activity of POX and PAL enzymes

The activity of the inducible enzymes POX and PAL was estimated after 3, 9 and 15 days of the treatments in the Plant Diseases Laboratory of the Ministry of Agriculture/ Department of Crop Protection. The leaves were collected in polyethylene bags and the name of the treatments and codes were labeled on them. The leaves were washed with tap water to remove the soil and then with sterile distilled water. Then they were placed in a cool, dark box.

Peroxidase enzyme activity (POX)

One gram of the stimulated plants was crushed with 2 mL of sodium phosphate buffer (0.01 M, pH 6.5) at 4 °C. The mixture was filtered through 4 layers of cheesecloth and then the filtrate was centrifuged at 6000 rpm for 20 min 4 °C. The supernatant was used as the enzyme source. The activity of the POX enzyme was determined using 100 µL of the enzyme extract with 1.5 mL of 0.05 M pyrogallol (24). To start the reaction, 100 µL of 1 % hydrogen peroxide (v/v) was added and the absorbance was recorded every 30 s for 300 s at 420 nm using a spectrophotometer. This is not complete. Along with this please add the final equation to calculate the enzyme activity.

$$\text{Change in absorbance} = \Delta A / \Delta T / \text{g fresh weight}$$

Where, ΔA - change in absorbance and ΔT - change in time (minutes).

Phenylalanine ammonia-lyase enzyme activity (PAL)

1g of the plant was squashed with 5 mL of sodium phosphate buffer (0.1 M, pH 7) containing 0.1 g polyvinyl pyrrolidone (PVP). The concentrate was separated through two layers of cheesecloth and the filtrate was centrifuged at 20000 rpm for 20 min. The supernatant, which was the source of the enzyme, was taken and 0.4 mL of it was blended with 0.5 mL of sodium borate (0.1 M, pH 8.8) and 0.5 mL of L-phenylalanine at a concentration of 12 mM for 30 min at 30 °C. The absorbance was measured at 290 nm using a spectrophotometer. 0.4 mL of the protein mixed with 1 mL of the borate was used as the blank. The activity of the enzyme was assessed by estimating how much cinnamic corrosive formed (nM $min^{-1} g^{-1}$)using an extinction coefficient of 9630 $M^{-1} cm^{-1}$(25).

Statistical analysis

The greenhouse experiment was conducted with 3 replicates, each with 2 plants, for the induction measurement experiment. The data were analyzed statistically using a Randomized Complete Block Design (RCBD) using GenStat software. The significant differences between the means were compared using the Least Significant Difference (LSD) test at a significance level of ($p \leq 0.05$).

Results and Discussion

Effect of test factors adding before the infection with RKN (*Meloidogyne* spp.)

The results in Table 1 showed that adding the factors at the effective concentration to cucumber-planted in soil before infection with RKN resulted in an increase in some growth parameters of cucumbers, including shoot weight, root weight, total chlorophyll content (%) and PAL (%) and POX (%) enzymes, with significant differences ($p < 0.05$) compared to the infected control and the non-infected plant.

The test treatments resulted in an increase in some growth parameters, such as an increase in the fresh weight of the shoot system in cucumber in all treatments, with a significant difference compared to the fresh weight of the shoot system in the non-infected plant (21.33 g per plant). However, it was found that the liquid vermicompost treatment gave the highest values in terms of growth parameters (fresh weight of the shoot system 74.72 g per plant, dry weight 6.50 g per plant) and total chlorophyll content (86.00 %) and enzyme concentration of PAL and POX (19.82 % and 25.29 % respectively) in cucumber leaves, with significant differences ($p < 0.05$) compared to all other treatments. On the other hand, the ascorbic acid treatment gave the least increase in some growth parameters in terms of shoot and root weight (dry and fresh).

Effect of test factors adding after the infection with RKN (*Meloidogyne* spp.) on some growth parameters

The results in Table 2 indicate that adding the factors at the effective concentration to cucumber-planted in soil after the infection with RKN resulted in an increase in some growth parameters of cucumbers, including shoot weight, root weight, total chlorophyll content (%), PAL (%) and POX (%) enzymes, with significant differences ($p < 0.05$) compared to the infected control and the non-

Table 1. The effect of adding the test factors 3 days before infection with the root-knot nematode (*Meloidogyne* spp.) on some growth parameters of cucumbers, total chlorophyll content and the concentration of PAL and POX enzymes.

Treatment	Shoot system g $plant^{-1}$		Root system g $plant^{-1}$		Total chlorophyll (%)	PAL enzyme (%)	POX enzyme (%)
	Fresh weight	Dry weight	Fresh weight	Dry weight			
T1	28.83	2.77	3.60	0.36	74.44	15.42	18.49
T2	34.17	3.37	3.80	0.45	82.89	17.21	19.78
T3	47.27	6.50	5.70	0.94	86.00	19.82	25.29
T4	41.47	5.57	3.93	0.81	85.89	19.10	23.34
T5	37.43	3.60	3.80	0.43	79.44	11.58	16.92
T6	9.20	0.97	2.90	0.20	36.67	6.27	9.37
T7	21.33	1.87	2.53	0.38	60.08	7.23	13.61
L.S.D (0.05)	2.45**	0.43**	0.61**	0.06**	2.69**	0.70**	1.88**

T1: Ascorbic acid, **T2**: K_2HPO_4, **T3**: Aqueous extract of dry earthworm powder, **T4**: Dry earthworm powder, **T5**: Compost, **T6**: Control, **T7**: Non infected plant.

Table 2. Effect of adding the test factors 3 days after the infection with RKN (*Meloidogyne* spp.) on the growth parameters of cucumbers, total chlorophyll content n and the concentration of PAL and POX enzymes.

Treatment	Shoot system g $plant^{-1}$		Root system g $plant^{-1}$		Total chlorophyll (%)	PAL enzyme (%)	POX enzyme (%)
	Fresh weight	Dry weight	Fresh weight	Dry weight			
T1	24.80	2.50	2.53	0.31	66.23	13.69	15.93
T2	29.27	2.87	3.07	0.36	72.00	16.37	18.99
T3	44.67	5.93	4.67	0.85	83.00	18.24	22.08
T4	38.56	5.20	3.93	0.79	81.31	16.50	21.83
T5	34.87	3.38	3.37	0.43	77.11	11.24	15.64
T6	9.20	0.97	0.91	0.20	36.67	6.31	9.37
T7	21.33	1.87	2.53	0.25	60.08	7.23	13.61
L.S.D (0.05)	1.98**	0.20**	0.42**	0.04**	3.50**	0.49**	1.37**

T1: Ascorbic acid, **T2**: K_2HPO_4, **T3**: Aqueous extract of dry earthworm powder, **T4**: Dry earthworm powder, **T5**: Compost, **T6**: Control, **T7**: Non infected plant.

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The test treatments resulted in an increase in some growth parameters, such as an increase in the fresh weight of the shoot system in cucumber in all treatments, with a significant difference compared to the fresh weight of the shoot system in the non-infected plant (21.33 g per plant). However, it was found that the liquid vermicompost treatment gave the highest values in terms of growth parameters (fresh weight of the shoot system 44.67 g per plant, dry weight 5.93 g per plant) and total chlorophyll content (83.00 %) and enzyme concentration of PAL and POX (18.24 % and 22.08 % respectively) in cucumber leaves, with significant differences ($p < 0.05$) compared to all other treatments. On the other hand, the ascorbic acid treatment gave the least increase in some growth parameters in terms of root and shoot weight (dry and fresh).

The results of the pot experiments showed that infection of cucumber with RKN resulted in a decrease in some growth parameters of cucumber, with significant differences compared to the non-infected plant and the test treatments of ascorbic acid, K_2HPO_4, liquid and dry vermicompost and compost. This can be explained by the weakness of plants due to nutrient deficiency, which results in weak plants that are more susceptible to nematode infection and weaken plant growth (26). Nematode infection causes a reduction in root and plant growth and thus reduces the absorption of nutrients and water. On the other hand, the addition of organic fertilizers and compost improves soil quality and increases the availability of nutrients, nitrogen and organic acids, which increases plant growth (27). Vermicomposting is the best and cheapest way to reduce costs and environmental pollution (28). Earthworms act as mechanical mixers to mix and blend all soil components to increase soil fertility and enrich it with humus, macro and micronutrients and beneficial microorganisms to nourish and improve plants (29).

The increase in growth parameters of cucumbers treated with liquid and dry vermicompost can be attributed to the fact that these fertilizers are rich in humic acid and the availability of microorganisms that fix nitrogen and produce growth-promoting hormones. A study mentioned that vermicompost is a complex of earthworms and the microorganisms associated with them or living inside the worm cavity, growth-promoting bacteria and nitrogen-fixing bacteria and these help to fix atmospheric nitrogen in the soil and produce growth hormones and inhibit the growth of plant pathogens (30). The increase in fresh and dry weight of the shoot system and dry weight of cucumbers treated with vermicompost can also be explained by the availability of plant hormones in the vermicompost and the activity of the accompanying microorganisms (31). The high concentration of vermicompost also increases nutrients in the soil (32). Another study indicated that adding organic matter to the soil stimulates plant roots to release compounds that are antagonistic to nematodes that parasitize plants (33). A study conducted to assess the effects of different formulations of organic emulsion (Appetizer) and Nanoscale NPK fertilizer combined with urea fertilizer on the growth and yield of yellow corn varieties, significant differences were observed among the different fertilizer treatments (34). The Nanoscale fertilizer treatment achieved the highest average in most growth parameters, including the number of days from planting to harvest, plant height, leaf number and chlorophyll content (35). In another study, the results showed wheat's response to potassium fertilization and foliar application of iron and manganese. Treatment with K 120 and foliar application of Mn 25 with Fe 100 twice during the elongation and heading stages resulted in the highest grain yield of 6240 kg/ha. This was associated with the highest nitrogen concentration of 3.39 %, phosphorus concentration of 0.36 % and potassium concentration of 2.68 %, which correlated with the best N, P and K indices of 0.1, 0.34 and 0.41 respectively. The total cumulative index for NPK indicators amounted to 1. Another study also proved that vermicompost can change the primary and secondary products of plant metabolites and increase its resistance to nematodes (36). The results are consistent with a study, which found that adding vermicompost produced from many biowastes to soil planted with tomato plants, increased plant nutrients in the soil, increased tomato plant growth and resistance to RKN and reduced infection (37). The addition of vermicompost also led to growth and an increase in the parameters of tomato and okra plants (38). The presence of humic acids in vermicompost increases root growth and number. It was found that treating sunflower seeds with ascorbic acid improves plant growth and increases the number of leaves, dry weight, and chlorophyll content (39).

A study stated that biofertilizers containing nitrogen-fixing bacteria have the ability to reduce nematode infestation (40). It was found that vermicompost, potassium fertilizers and plant leaf residues increased tomato plant growth and reduced the number of knots on plant roots (41).

Another study also proved that adding organic matter to the soil increases the presence of fungi and bacteria that are pathogenic to nematodes (42). The results indicated that adding compost produced from cork reduces the number of nematodes in the soil, the rate of infection with RKN and the number of knots on tomato roots (43). It was found that adding compost made from some plants or animal residues reduces the number and percentage of infection with RKN (*Meloidogyne javanica*) in sunflower plants. Our results are consistent with another study that some phosphatic fertilizers reduce the incidence of nematodes pathogenic to plant (*Colletotrichum gloeosporioides*) (44). It was mentioned that adding potassium phosphate fertilizers and phosphonate salts gives plants required internal resistance against plant pathogens. It was also found that phosphatic fertilizers reduce the incidence of RKN (45). A group of authors demonstrated that folic acid and magnesium oxide had a significant effect against root-knot nematodes (*Meloidogyne* spp) (46, 47). Treatment with different concentrations of FA and MgO (1000, 2000, 3000 ppm) in the laboratory led to a significant decrease in egg hatching and juveniles' viability after 3 and 7 days compared to the control treatment.

In another study evaluating the efficacy of some organic stimulants, such as humic acid, against root-knot nematodes, the results showed that it had a significant lethal effect on juveniles, which was correlated with increasing concentrations compared to the control treatment. It also led to an increase in growth parameters compared to the control treatment, including an increase in fresh root weight (48). It also proved that phosphatic fertilizers increase shoot growth and fresh weight and reduce the incidence of RKN in tomatoes. The results are consistent with another study (49). The results indicated that combining potassium application through both soil incorporation and foliar spraying yielded the best outcomes and exhibited significant superiority over individual applications in tomato plants. The percentage increase varied across comparisons and nutrient elements (Mg, Ca, K, P, N) within the ranges of 32-55 %, 28-136 %, 19-32 %, 5-21 % and 30-94 % respectively. A study showed that adding orange peel residues (rich in ascorbic acid) reduced the incidence of RKN, the number of eggs produced and the second-generation juveniles in the soil surrounding the roots of tomato plants and it increased the plant growth (50). A study reported that dipotassium hydrogen phosphate (K_2HPO_4) achieved complete inhibition, reaching 100 % of fungal growth at a concentration of 200 mM (51).

Conclusion

This study effectively assessed the efficacy of different biofertilizers in enhancing the resistance of cucumber to RKN. Supplementation with biofertilizers, both before and after nematode infestation, significantly improved various growth parameters compared to infected and noninfected plants. Notably, ascorbic acid, K_2HPO_4, dry and liquid vermicompost fertilizers and compost offered notable benefits. However, liquid vermicompost emerged as the most successful intervention, significantly increasing shoot fresh and dry weight, chlorophyll content and PAL and POX enzyme activity. These findings suggest that liquid vermicompost holds promise as a sustainable and effective strategy for mitigating nematode damage and enhancing cucumber growth. Further research could explore the mechanisms underlying this effect and optimize application strategies for wider agricultural applications.

Authors' contributions

Methodology: STAY; Software: STAY and HKAG; Writing, review and editing: HKAG; Supervision and project administration: STAY. All authors have read and agreed to the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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