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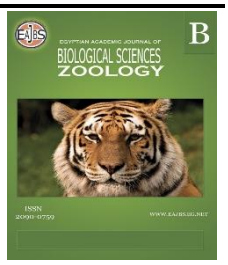

Induced Cucumber Plant Resistance to *Meloidogyne incognita* By Certain Biotic and Abiotic Inducers in Relation to Some Biochemical.

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ABSTRACT

This study was conducted to evaluate the role of certain abiotic and biotic inducers in inducing cucumber resistance against the root-knot nematode, *Meloidogyne incognita*, under greenhouse conditions. The abiotic inducers comprised acetylsalicylic acid (ASA) and Indole acetic acid (IAA) while the biotic inducer was *Bacillus pumilus* (B.P.). The results revealed that, among all treatments, the double combinations of IAA before B.P. by 3 days as well as ASA before B.P.by 3 days showed the best treatments to increase plant resistance against *Meloidogyne incognita* infection and improved plant vigor based on fresh and dry weights of shoots and roots compared to using them individually and control inoculated non-treated plants. A generalized increase was observed in the peroxidase activities and in proline amino acids and total phenols due to the application of the investigated inducer. The degree of increase differed according to treatment and biochemical aspects. The overproduction of these biochemical aspects may play a part in the induction of plant defenses against nematode infestation. In conclusion, the development of safety control measures by inducing plant resistance will protect a wide range of plant crops against nematode threats and can be used in integrated pest management programs.

INTRODUCTION

Cucumber (*Cucumis sativus L.*) is a vegetable crop produced on a large scale. However, its production is seriously threatened by root-knot nematodes. *Meloidogyne spp.* (Sikora and Fernández, 2005). In greenhouse production, the damage caused by root-knot nematodes becomes even more serious. Four *Meloidogyne* species were frequently found worldwide, and *M. incognita* is the most dominant species in the temperate zone. Recently, inducing resistance in plants to control plant parasitic nematodes has interestingly increased to avoid the environmental problems caused by nematicides. Induced resistance is a term used to describe the phenomenon whereby plants express elevated resistance to pathogenic attack after treatment with certain biotic and/or abiotic activators. Many activators were identified, however, relatively few, have been commercialized and therefore induced resistance remains an under-utilized resource in crop protection. Such resistance is based on the stimulation of defense mechanisms by metabolic changes that enable the plants to

defend themselves more efficiently (Steiner and Schonbeck, 1995). These defense mechanisms can be triggered by incompatible pathogens or chemical inducers (Oka and Cohen, 2001). The enhancement in resistance to an extrinsic stimulus without a known alteration of the genome is called induced resistance.

In the present work, the potential to exploit induced resistance to control *M. incognita* was investigated in cucumber cv. Madinah. This study includes: 1- evaluation of the role of IAA, ASA and B.P. as inducers against root-knot nematode, *M. incognita* under greenhouse conditions, 2- and determination of chemical changes that play a role in plant resistance towards nematode viz; peroxidase, total phenols and proline in plants inoculated with *M. incognita* and treated with single or mixed abiotic or biotic inducers compared to inoculated non-treated plant.

MATERIALS AND METHODS

Preparation of Root-Knot Nematode Inoculum:

The pure culture of *M. incognita* was propagated in the screen-house on cucumber plants (*cucumis sativus*) in sterile sandy clay soil. Newly hatched second-stage juveniles (J2) from galled roots were extracted using a mist chamber. The IJ2 were collected and refrigerated for the experimental use. Perineal patterns of adult females from cucumber plant roots were used occasionally to confirm the nematode species (Taylor and Sasser, 1978).

Preparation of *Bacillus pumilus*:

Bacillus pumilus was secured from the Plant Pathology Department, National Research Center, Dokki, Cairo, for inoculum preparation of these bacteria. They were separately inoculated in nutrient sucrose (2%) broth medium (beef extract 3g, peptone 5g, glucose 10g) in 1 L of distilled water, and pH was adjusted at 7.4±0.2. The bacterial culture was incubated at 28°C for 48h. Then, the bacterial inocula were adjusted to a 10^8 colony-forming unit (CFU)/ml by turbidity method (Baird *et al.*, 2000). Bacterial inocula were applied as a mixture of bacterial cells and cultural filtrate (Abd-El-Khair *et al.*, 2019).

Preparation of Abiotic Chemicals:

Inducers were obtained from Scientific East Group Company, Dokki, Egypt. Each Solution of Indole Acetic Acid (IAA) and Acetyl Salicylic Acid (ASA) was prepared by dissolving the adjusted weights of each material in distilled water to prepare 5000 PPM of concentration.

Planting and Experimental Treatments:

Three-week-old cucumber cv. Madinah seedlings were used. The seedlings were transplanted in a 15 cm diameter pot filled with 2.5 kg sterilized sandy clay soil (1:1 w/w). Plants were fertilized weakly with N-P-K as recommended in cucumber culture and watered as needed. One month later, seedlings were treated with *Bacillus pomilus* culture solution at a concentration of (10^8 CFU/ml), IAA concentration of 5000 PPM and ASA concentration of 5000 PPM. The experimental treatments were designed as follows:

1- healthy plants (without Mi).
2- incubated plant (Control) infected with 1000 Mi.
3- *B.pumilus* and after 3 days 1000 Mi were added.
4- IAA and after 3 days 1000 Mi were added.
5- ASA and after 3 days 1000 Mi were added.
6- (*B.pumilus* + IAA) and after 3 days 1000 Mi were added.
7- (*B.pumilus* + ASA) and after 3 days 1000 Mi were added.
8- *B.pumilus* and after 3 days IAA were added then 1000 Mi after 3 days.
9- *B.pumilus* and after 3 days ASA were added then 1000 Mi after 3 days.
10- IAA and after 3 days *B.pumilus* were added then 1000 Mi after 3 days.

11- ASA and after 3 days *B.pumilus* were added then 1000 Mi after 3 days.

Two weeks after nematode inoculation replicates of treated plants were uprooted to analyze some biochemical changes according to Dixon (2001). After 45 days from nematode inoculation, plants were uprooted and the number of galls, egg masses, and nematode developmental stages in roots were counted. The percentage reduction of the final nematode population and the plant growth parameters were recorded.

Biochemical Determination:

Certain biochemical agents that may have a role in inducing plant resistance to nematode infestation were investigated after 14 days of treatment as follows:

1- Determination of Proline Amino Acid Concentration:

Proline concentration was determined using a ninhydrin colorimetric method of Troll and Lindsley (1955) as modified by Petters *et al.* (1997). Frozen tissues of roots were ground using mortar and pestle and homogenized with 100 mM sodium phosphate buffer pH 6. The extraction ratio was 5 ml for each gram of plant tissue. Afterwards, the homogenate was centrifuged for 10 min at 6000 rpm then 200 µl of the extract was reacted with 1 ml of ninhydrin solution (2.5 g dissolved in 100 ml of orthophosphoric acid, acetic acid, and water with ratios of 15:60:25, V: V: V, respectively) for 1 h in boiling water. Thereafter, the developed color was extracted with 1 ml of toluene and vigorously vortexed for 15 s. The toluene phase was used to measure the color at 515 nm using a spectrophotometer (UV-Vi's spectrophotometer UV 9100 B, LabTech). The proline concentration was calculated from the standard curve of L-proline. Proline concentration was expressed as µg proline. g-1 Fresh weight (FW).

2- Determination of Total Phenols Concentration:

Determination of total soluble phenols was performed using the method described by Shahidi and Naczk (1995). A known fresh weight of root tissue (0.5 g) was homogenized in 5 ml ethanol 80% and kept in a dark bottle for 24 h at 0˚ C. The samples were re-extracted 3 times then the clarified extract was completed to 15 ml with ethanol 80%. Then 1 ml of the extract was mixed with 0.5 ml Folin-Ciocalteu reagent in a test tube and thoroughly shaken. After 3 min, 1 ml of Na2CO3 (20 %) was added to the mixture then the volume was completed to 10 ml with distilled water. The reaction was allowed to proceed for 1 h. Then, the absorbance was recorded at 725 nm using a spectrophotometer (UV-Vi's spectrophotometer UV 9100 B, LabTech). The concentration of total soluble phenols was calculated using the standard curve of catechol. Total phenol concentration was expressed as µg equivalents of catechol per g FW of the sample.

3- **Determination of Guaiacol Peroxidase (G-POD):**

Root tissues (0.5 g) were homogenized in a chilled sodium phosphate buffer (100 mM, pH=7) containing 1% (w/v) polyvinylpyrrolidone (PVP) and 0.1 mM EDTA. The extraction ratio was 4 ml extraction buffer for each gram of plant tissues. The homogenate was centrifuged at 6000 rpm at 4º C for 15 min. The supernatant was used for the measurement of guaiacol peroxidase (G-POD) activity according to Gaspar *et al.* (1982).

RESULTS AND DISCUSSION

Data in Table (1) illustrates the effect of different biotic and abiotic agents in inducing resistance in cucumber plants against root-knot nematode, *M .incognita* compared to inoculated non-treated control treatment. It is clear that all applications significantly ($p \leq 0.05$) decreased the root-knot disease incidence as measured by numbers of final nematode population, root galls and egg masses/pot. However, the lowest final population and percentage reduction in *M. incognita* compared to control inoculated non-treated plants were 1734 nematode (37%) and 2011 nematode (45%) recorded in treatments of IAA before B.P. by 3 days and ASA before B.P.by 3 days; respectively.

With regard to the plant growth response of infected cucumbers treated with biotic and abiotic agents, data in Table (2) revealed that, applied materials improved growth parameters on the basis of fresh and dry weights of shoots and roots of cucumber. Moreover, the highest value increase in total fresh weights was obtained in all treatments compared with the inoculated non-treated control. It is noteworthy that, both treatments of IAA before B.P. by 3 days and ASA before B.P. by 3 days supported an appreciable improvement in plant vigor and an obvious nematode control as shown in Tables 1&2.

It could be conculcated here that the investigated abiotic and biotic treatment induced the plant resistance to nematode infection due to its action or increasing lignin formation at plant cell walls of infection sites due to its role in plant metabolism modification. Thus, these sites become physically harder to penetrate by nematodes and render the treated roots temporarily less attractive to those infected by them. The present results agree with Pankaj *et al.* (2005), who found that salicylic acid decreased gall index and improved cowpea plant growth. Also, our results partially agree with Nandi *et al.* (2003a) who found that SA reduced nematode infestation and promoted plant growth, by enhancing the synthesis of pathogen-related protein.

The reduction in nematodes obtained in treated plants may be explained by the fact that, the antibiotic agents in the tissues of host plants induced accumulation of phytoalexins. In addition, it decreased the total content of free sterols and changed their composition, producing adverse effects on nematodes, mainly activation of chitinase, glucanase, lipoxygenase, peroxidase, phenylalanine ammonyalase, stimulated the generation of reactive oxygen species and increases the metabolic effects of phenolic compounds. This mixture of elicitors caused a more significant immunostimulation effect in plants (Benhamou *et al.*, 1994 and Aboud *et al.*, 2002).

Table 1: Nematodes parameters affected by certain abiotic and biotic inducers to induced resistance to *Meloidogyne incognita* in cucumber cv. Madinah.

Treatments	Nematode Parameters (Average Number/pot/plant)				
	Pf	**Rr**	**%Rr**	**No. of Galls**	**No. of Egg mass**
Incubated plant (control)	4452 a	4.4 a	100%	39 a	20 b
B. P. → Mi	2118 cd	2.1 cd	48%	28 c	23 a
IAA → Mi	2024 de	2 de	45%	35 b	10 de
ASA → Mi	2432 b	2.4 b	54%	38 a	9 e
B. P. + IAA → Mi	2214 bcd	2.2 bcd	50%	25 d	11 d
B. P. + ASA → Mi	2040 d	2 d	45%	25 d	11 d
B. P. → IAA → Mi	2482 b	2.5 b	57%	30 c	16 c
B. P. → ASA → Mi	2360 bc	2.36 bc	54%	30 c	17 c
IAA → B. P. → Mi	1734 e	1.7 e	37%	22 e	9 e
ASA → B. P. → Mi	2011 de	2 de	45%	25 d	11 d
LSD	339.7	0.3		2.15	1.33

LSD = least significant difference at $P \leq 0.05$. + = at the same time, → = after 3 days, B. P. = *Bacillus pumilus*, IAA= indole acetic acid, ASA= acetyl salicylic acid, Rr= rate of nematode reproduction=Pf/Pi, Pi= initial nematode population, Pf= final nematode population, Mi= 1000 IJ2 of *Meloidogyne incognita*.

Data in (Table 3) indicated that treatments with indole acetic acid (IAA) or with acetylsalicylic acid (ASA) followed by *bacillus pomilus* (B.P.) after 3 days significantly increased the specific activity of peroxidase (POD) in treated roots than its activity in root of control plants infected with nematodes by about 3.3 times and by about 1.4 times than control plants without infection (healthy plant). In general, all the other treatments showed a significant decrease in peroxidase activity than control healthy plants and an insignificant increase in control with infection, except treatments B.P. followed by IAA and B.P. followed by ASA which recorded an insignificant decrease in enzyme activity. It could be

concluded that the activity of peroxidase enzymes plays a main role in inducing resistance to nematode attack. Our results of nematode infection parameters confirmed this consolation, thus the treatment with IAA or ASA followed by B.P. showed the lowest infected parameter i.e., number of galls, number of egg mass and the relative percent reproduction. Also, the results of plant parameters of total fresh weight, shoot fresh and dry weight, number of leaves and shoot lengths for the nematode infection confirmed our consult that these two treatments showed the highest significant parameter than other treatment and control samples. Peroxidase is the key enzyme required for lignin synthesis and plays an important role in the mechanism of resistance in the host plants against pest infection. The peroxidase activity of the host plant is likely to change more or less depending upon the plant's defense against the plant's parasitic nematodes.

Table 2: Plant parameters affected by certain abiotic and biotic inducers to induced resistance to *Meloidogyne incognita* in cucumber cv. Madinah.

Treatments	Plant parameters					
	Number of leaves	**Shoot length**	**Shoot fresh weight (g)**	**Shoot dry weight (g)**	**Root weight (g)**	**Total fresh weight (g)**
Healthy plant	40 d	80 e	48 c	11 bc	22 c	70 d
Incubated plant (control)	39 d	70 f	45 d	11 bc	18 e	63 e
B. P. → Mi	33 e	88 d	45 d	10 cd	28 a	73 bcd
IAA → Mi	33 e	67 f	36 e	9 d	26 b	62 e
ASA → Mi	35 e	90 d	35 e	9 d	25 b	60 e
B. P. + IAA → Mi	44 c	90 d	55 b	13 a	17 ef	72 cd
B. P. + ASA → Mi	48 b	102 bc	58 a	13 a	20 d	75 bc
B. P. → IAA → Mi	50 b	101 c	55 b	12 ab	16 f	71 d
B. P. → ASA → Mi	50 b	105 b	58 a	13 a	18 e	76 b
IAA → B. P. → Mi	60 a	110 a	58 a	13 a	22 c	80 a
ASA → B. P. → Mi	61.8 a	112 a	58 a	12 ab	22 c	80 a
LSD	2.12	3.33	2.35	1.46	1.80	3.44

LSD = least significant difference at P ≤ 0.05. + = at the same time, → = after 3 days, B. P. = *Bacillus pumilus*, IAA= indole acetic acid, ASA= acetyl salicylic acid, Mi= 1000 IJ2 of *Meloidogyne incognita*.

Table 3: Biochemical changes associated with induction of cucumber plant resistance to root-knot nematode.

Treatments	total phenol (µg/g FW)	Proline conc. (ug/g)	Peroxidase sp. Activity (IU/mg protein)
Healthy plant	74.28 defg	18.14 f	38.77 b
Control with Mi	185.83 a	41.86 cd	15.03 de
B. P. → Mi	143.01 b	41 cde	14.3 de
IAA → Mi	94.32 cdef	34.09 de	42.57 b
ASA → Mi	118.01 bc	31.93 de	14.6 de
B. P. + IAA → Mi	102.64 cde	32.29 de	18.46 d
B. P. + ASA → Mi	64.84 fg	62.13 a	27.72 c
B. P. → IAA → Mi	67.22 efg	49 bc	11.64 e
B. P. → ASA → Mi	123.53 bc	29.87 ef	12.63 e
IAA → B. P. → Mi	48.63 g	31.05 de	54.75 a
ASA → B. P. → Mi	108.86 bcd	57.31 ab	53.12 a
LSD	37.8	11.74	5.8

LSD = least significant difference at P ≤ 0.05, B. p.= *Bacillus pomilus*, IAA= indole acetic acid, ASA= acetylsalicylic acid, + = at the same time, → = after 3 days and Mi = *Meloidogyne incognita*.

In this respect, Siddiqui and Husain (1992) reported that peroxidase is known to catalyze several reactions including those involved in the metabolism of phenols and indoles. Peroxidase plays an integral part in the biosynthesis of plant cell wall components,

including lignin, suberin, and cross-linked extension (Lamport, 1986). Lignification and wall thickening are well-known as plant defense responses to pathogens (Gaspar *et al.* 1982). Increasing activity of peroxidase was associated with induced systemic resistance in tobacco to a variety of pathogens (Lagrimini and Rothstein, 1987). Also, Melillo *et al.* (1992) reported that high levels of root enzymes especially peroxidase (PO) are considered a part of the general activation of cell metabolism, which takes the form of de novo synthesis of enzymatic proteins with peroxidase activity. The peroxidases localized near the infected tissues catalyze the formation of suberin, which aids in the defense of the plant by forming a barrier that blocks the pathogen.

Mostafa *et al.* (2014). Found that application of commercial product to Bio-arc (commercial formulation of *Bacillus megaterium*) + nemastrol (20 ml + 0.25 ml) to leaves of Sugar-beet increased the activities of peroxidase, polyphenol oxidase much greatest the control. Subsequently, an Induction in systemic resistance against root-knot nematode occurred.

Data in (Table 3) also revealed that treatment of the cucumber root with B.P. + ASA exhibited the highest significant induction of proline amino acid concentration than the other treatment and control samples. The treatment with ASA followed by B.P. recorded the 2nd highest proline concentration category with an insignificant difference between them. After that, the treatment with B.P. followed by IAA recorded an insignificant increase of proline content in the infected control sample. These results could be confirmed by our data of nematode infection parameters and infected cucumber plant parameters, that the treatments by IAA followed by B.P. and IAA alone with insignificant difference between them. B.P. mixed with ASA and ASA followed by B.P., these inducers recorded the best protective effect against root-knot nematode infestation. This could be conculcated that proline amino acid may have a role in inducing resistance of cucumber root infestation by root-knot nematode.

That inducers ASA mixed or followed by B.P. and B.P. followed by IAA caused stress on treated cucumber roots resulting in an overproduction of proline in the plant. The overproduction of proline enhanced stress tolerance in plants. Thus, Hayat *et al.* (2012) reported that proline imparts stress tolerance by maintaining cell turgor or osmotic balance, stabilizing the membrane thereby preventing electrolyte leakage and bringing the concentration of reactive oxygen species (ROS) within normal ranges, thus preventing oxidative burst in plants. In this respect, Raj *et al.* (2003) stated that the amino acid proline was evaluated for its efficiency in eliciting resistance in pearl millet *penisetum glaucum* against downy mildew disease caused by *sclerospra graminicola* under greenhouse and field conditions.

The obtained data in (Table 3) Also revealed that the treatment with inducers B.P. alone, followed by B.P., ASA alone and followed by B.P., and IAA mixed with B.P. insignificant differences between them in inoculated roots of cucumber plant compared with all the other inducer treatments or healthy plant.

However, the phenol contents for the nematode-infested plants (control) showed a significant increase in phenols than healthy plants and all the investigated treatments. This finding revealed that the induction of phenol occurred after treatment of cucumber roots with the effectiveness inducers mainly (ASA and B.P.), resulting in induced resistance of roots to infection root-knot nematode. There are several authors confirmed that the accumulation of phenols has a defensive role against root pests. Sorial *et al.* (2020) reported that ethylene inducers increased the total phenols in pepper plants more than the nematode-infested plants. Also, Nayak (2015) conculcated that an increasing trend was observed in phenolic contents in the brinjal plant roots of both healthy and resistant cultivars. Baker and Hewedy (2018) stated that salicylic acid foliar and root application decreased nematode

infection criteria and increased total phenol, Also, Yang *et al.* (2023), recorded that total phenolic and lignin contents were higher in resistance cultivars of sweet potato plants.

In general, it could be conculcated from the biochemical studies that treatments with ASA alone or B.P. after 3 days of ASA and IAA mixed with B.P. application were the most effective inducers for cucumber plant resistance against root-knot nematode. These inducers activated the plant's own genetical programmed defense pathways, resulting in changes in the synthesized of inducible defense compounds that resist the effects of subsequent biotic attack, as reported by Agrawal *et al.* (1999), Eyles *et al.* (2010) and Chavan *et al.* (2022).

In this respect, Chavan *et al.* (2022) reported that exogenous treatments with the oxidized form of ASA induced systemic resistance in rice against root-knot nematode through the production of ROS and activation of SA pathway, which inducted several genes related to plant stress responses, immunity, antioxidant activity and secondary metabolism already at 1 day after treatments happened. Also, Walters *et al.* (2013) stated that reduction in infection criteria due to SA application attributed to its role as a signaling molecule involved in both reactions at the induction of systemic resistance, Maher *et al.* (2011) concluded that, exogenous application of SA might influence the status of glutathione (GSH) however, GSH activates SA alters the expression of defense genes to modulate plant resistance against pathogens in tomato plants, Taher and Ami (2022) reported that SA foliar and root application of cucumber plants decreased infection criteria and increased total phenol, peroxidase, and phenol oxidase activities which have a role in the induction of plant resistance to the nematode, Molinri (2007) stated that SA is a physiological inhibitor of catalase enzyme, it was an elicitor of resistance in tomato attacked by root-knot nematode.

The effectiveness of *Bacillus sp.* As a biotic inducer to cucumber resistance against root-knot nematode was confirmed by Kloepper *et al.* (2004) who reported that, elicitation of induced systemic resistance (ISR) in plants by *Bacillus sutilis, B. pasteurii, B. cereus, B. pomilus,* has been recorded in green house or field trials on tomato, pepper, cucumber. Gattomi *et al.* (2023) stated that after one week of treatment the *Bacillus spp.* stimulated a SA-responsive defense-related gene. The long-term systemic response to *Bacillus spp.* indicated SA that also plays a role in defense conferred by these bacteria. On the other hand, the application of SA and *pseudomonas flurescens* inducer proved to be active in the induction of induced plant resistance to nematode infestation. thus, our results for the application of *Bacillus pomilus* with SA were confirmed by Nikoo *et al.* (2014) induced the removal of high concentrations of toxic ROS via an increase in the activity of their scavenging antioxidant enzymes, especially that catalase enzymes for stimulating plant defense reaction in moderately resistant tomato challenged with *M. javanica.*

Finally, this study will help aid in implementing the biotic and abiotic inducer for induction of plant resistance to root-knot nematodes as a tool in sustainable pest management.

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