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Induced resistance in tomato plants against root knot nematode using biotic and abiotic inducers

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Abstract

A greenhouse experiment was conducted in the experimental farm station of plant pathology research institute during 2015 season at three times of application of biotic and a biotic elicitors, to evaluate the efficient antagonistic bacterial strains *Bacillus subtilis*, *Serratia marcescens*, Cyanobacterial strain *Spirulina platensis* and silver nanoparticles (AgNPs) against root-knot nematode *Meloidogyne incognita* infecting tomato plants. Nematode reproduction, Plant growth characters, endogenous hormones, anti-nematocidal protein markers as response to induction of SAR in leaves was recorded. Data recorded that using *Bacillus subtilis* at the same application time of *M. incognita* recorded 86% and 88% reduction of root galls and egg masses respectively. All tested elicitors appeared significant reduction in nematode reproduction. Also data revealed that treated tomato plants with tested elicitors recorded more significant values of growth characters. This was the fact when being compared with those of non-treated plants. Moreover, data revealed that contents of both GA and IAA were markedly decreased in infected plants than that of healthy ones. At the same time, marked increases in the contents of JA and ABA were observed in infected plants as being compared with healthy ones. On the other hand, results showed different responses as regards the determined hormones due to the application of different used elicitors. On the other hand, the results appeared that, a new pattern of proteins were produced, as well as, different increasing in the density of bands according to the type of used elicitors and also to the time of application.

Keywords: Cyanobacterial, *Bacillus subtilis*, silver nanoparticles- nematodes

Introduction

Plant-parasitic nematodes are microscopic obligate biotrophic pathogens that feed on plant roots. They cause severe damage to a wide variety of crops and lead to significant yield losses of approximately 78 billion dollar worldwide annually (Caillaud *et al*., 2008).

Abou-Aly *et al*. (2015) reported that root-knot nematodes are serious pathogens that severe damage to major crops. The tomato (*Lycopersicon esculentum* L.) is an important vegetable crop across the world. The fruits of tomato are popular throughout the world and are used in all kinds of vegetable and also are eaten as raw salad. Ripe tomato fruit has high nutritive value being a good source of vitamin A, B, C and minerals (USDA, 2005).

Biological control employs natural enemies of pests or pathogens to eradicate or control their population. This

can involve the introduction of exotic species, or it can be a matter of harnessing whatever form of biological control exists naturally in the ecosystem. The induction of plant resistance using non-pathogenic or incompatible microorganisms is also a form of biological control (Schouten *et al*., 2004). Plant treatments with various biotic and abiotic agents can induce resistance against subsequent pathogen attack (Walters *et al*., 2005). Induced resistance is a physiological "state of enhanced defensive capacity" elicited by specific environmental stimuli (Van Loon *et al*., 1998). This enhanced state of resistance is effective against a broad range of pathogens and parasites, including fungi, bacteria, viruses, nematodes parasitic plants and even insect herbivores (Vallad and Goodman, 2004). The establishment of SAR is associated with the accumulation of pathogenesis-related (PR) proteins, salicylic acid (SA) and jasmonic acid (JA) throughout the plant (Vallad and Goodman, 2004). Nanoparticles usually have better or different qualities than the bulk material of the same element. In the case of silver nanoparticles the antibacterial effect is greatly enhanced and because of their tiny size. Nanoparticles have immense surface area relative to volume. Therefore minuscule amounts of Silver Nanoparticles can lend antimicrobial effects to hundreds of square meters of its host material (Theivasanthi and Alagar, 2011).

This investigation aimed to study the positive performance of biological agents and silver nanoparticles against root- knot nematodes, (*M. incognita*), which considered among the most difficult crop pests to control. Furthermore, to evaluate the effect of tested elicitors as alternative and safety method in Integrated Pest Management (IPM) programs to management the root-knot nematodes. Moreover, to study the impact of used elicitors on plant growth.

Materials and Methods

Plant material:

Four weeks -Tomato seedlings (*Solanum Lycopersicon* L. cv. Castlerock II PVP) were obtained from agricultural research center (ARC), ministry of agriculture, Giza, Egypt.

Root-knot nematodes (*Meloidogyne incognita*):

The nematode population used in this research originated from green house culture maintained at the plant pathology research, were *Meloidogyne incognita* chitwood was reared in a green house on tomato plants. Eggs of *M. incognita* were extracted from roots in 0.5 % sodium hypochlorite (Hussey and Barker, 1973) and caught on a 25 µm sieve. Second stage Juveniles (J2) were hatched from these eggs on Baermann funnels and only (J2) less than 2 days old were used for experimentation.

Source and application methods of inducers:

Tow bacterial strains namely *Bacillus subtilis*, and *Serratia marcescens* which recorded high values for nematicidal activities as well as maximum hydrolysis zone values of gelatinase, protease and chitinase (Bahloul, 2013) were selected. Bacterial inocula were prepared using poly broth medium (Bourgouin *et al*., 1984). *B. subtilis* strain was isolated from Egyptian Soils and identified by Bio-log Technique at Biofertelizer production unit, Soil, Water and Environment Research institute, Agricultural Research Center (ARC), Giza, Egypt. The concentration of *B. subtilis* in suspension was counted by most probable number (MPN). *Serratia marcescens* strain isolated from Egyptian Soils. It was produced by Soils, Water and Environ. Res. Inst. Agriculture research center, and distributed on a commercial scale (trade name, Nemaless). The concentration of *B. subtilis* or *S. marcescens* in suspension was counted by most probable number (MPN).The inocula suspension was approximately adjusted to 10^9 CFU/ml culture (colony forming unit).

Algal strain source and growth conditions:

Cyanobacterial strain *Spirulina platensis* was obtained from the Microbiology Department; Soils, Water and Environment Research Institute (SWERI), Agricultural Research, Center (ARC), Giza, Egypt. The algae was grown on Zarrouk medium (Zarrouk, 1966) and was incubated in growth chamber under continuous illumination (2000 lux) at 35˚C± 2˚C for 30 days. The inoculum suspension was approximately adjusted to 10^9 CFU/ml culture.

Silver nanoparticles (AgNPs):

Biosynthesis of silver nanoparticles (AgNPs) of the *Streptomyces cyanoalbus*, (100 ppm) was carried out according to method described by (Kalishwaralal *et al*., 2008; El-Batal *et al*., 2013) with slight modification.

Greenhouse experiment:

Tomato seedlings were transplanted to 20 cm diameter plastic pots filled with autoclaved sandy loam soil (1:1,V:V) each pot contained one tomato plant. Biotic and a biotic elicitors (*Bacillus subtilis*, *Serratia marcescens*, *Spirulina platensis* and silver nanoparticles (AgNPs) were added at three times. The first treatment was applied one week before inoculation with 2000 second stage juveniles of *M. incognita*, the second treatment was applied at the same time of inoculation with 2000 second stage juveniles of *M. incognita* and the third treatment was applied one week after inoculation with 2000 second stage juveniles of *M. incognita*. Five plastic pots with tomato seedlings inoculated with 2000 second stage juveniles of *M. incognita* and left untreated with elicitors. Also five plastic pots with tomato seedlings each left untreated with elicitors and uninoculated with nematode that served as control. Each treatment was replicated five times and all treatments were arranged in a complete randomized block design. Pots were kept in the greenhouse at 25±5 receiving water and ordinary nutrient solution as required. Sixty days after the nematode inoculation, tomato plants were carefully uprooted and then weights and lengths of shoots and roots for each treatment were determined. Roots were washed free of soil and root knot nematode galls and egg masses were counted per one gram roots stained in phloxine B. Reduction percentages of root knot nematode galls and egg masses numbers were counted in comparison with nematode only.

Determination of hormonal contents:

Determination of endogenous hormones (gibberellins, indol acetic acid, abscisic acid, and jasmonic acid) in leaves of treated plants as well as the control was carried out as described by (Lee *et al*., 1989).

Electrophoresis analysis of protein by SDS- PAGE:

SDS-PAGE was used to detect the induction of systemic resistance (ISR) in tomato plants against *M. incognita* via quantitative and qualitative determination of the total proteins. This method was done according to Laemmli (1970) as modified by Studier (1973).

Statistical analyses:

Experimental data were subjected to one way analysis of variance (ANOVA) and the differences between means were separated by the least significant difference (L.S.D) at 5% level of probability using M-state software (Snedecor and Cochran, 1982).

Results

1-Effect of tested elicitors on galls and egg masses numbers in infested tomato plants with *M. incognita*:

Obtained data presented in table (1) indicated that treatment of tomato plants with tested elicitors significantly decreased galls number and egg masses number of nematode. Regarding the effect of different used elicitors, the obtained results in table (1) revealed that the average numbers of galls and egg masses in 1g of roots were decreased according to the application of *S. platensis* one week after the inoculation of *M. incognita* recording 19 and 2 as average numbers of galls and egg masses respectively. While, the application of *B. subtilis* at the same time of *M. incognita* inoculation recording 20 and 6 as average numbers of galls and egg masses respectively. Also, results in table (1) showed that the application of *S. platensis* one week before, at the same time and one week after the inoculation of *M. incognita* recording 3 root gall index in all time of application.

Table (1): Impact of biotic and abiotic elicitors, time of application on galls and egg-mases of *Meloidogyne incognita* infecting tomato plants under greenhouse conditions.

Treatment			Average number of galls/1(g) of root	Red (%)	RGI	Average number of egg masses/1(g) of root	Red (%)	EI
Time of application	Material	Dose/pot						
One week before infection	*B. subtilis*	2 ml	56.00 d	61.64	4	13 gh	74	3
	S. marcescens	2 ml	97.00 b	33.56	4	22 de	56	3
	S. platensis	4 ml	27.00 ef	81.51	3	10 hi	80	2
	AgNPs	2 ml	78.00 c	46.58	4	15 fgh	70	3
At the same time of infection	*B. subtilis*	2 ml	20.00 f	86.30	3	6 ij	88	2
	S. marcescens	2 ml	43.00 de	70.55	4	17 efg	66	3
	S. platensis	4 ml	27.00 ef	81.51	3	12 ghi	76	3
	AgNPs	2 ml	110.00 b	24.66	5	35 b	30	4
One week After infection	*B. subtilis*	2 ml	55.00 d	62.33	4	20 ef	60	3
	S. marcescens	2 ml	80.00 c	45.21	4	28 cd	44	3
	S. platensis	4 ml	19.00 f	86.99	3	2 j	96	1
	AgNPs	2 ml	102.00 b	30.14	5	30 bc	40	3
Nematode only			146.00 a	0.00	5	50 a	0.00	4
LSD 5%			16.569			4.6		

***Root gall index (RGI) or egg-masses index (EI) was determined according to the scale given by Taylor& Sasser (1978) as follows : 0= no galls or egg masses, 1= 1-2 galls or egg masses , 2= 3-10 galls egg masses, 3= 11-30 galls or egg masses, 4= 31-100 galls or egg masses and 5= more than 100 galls or egg masses.**

Results illustrated in Fig. (1) showed that using *B. subtilis* at the same time of application of *M. incognita* recorded 86.30% reduction of root galls. While, using *S. platensis* one week after or one week before the inoculation of *M. incognita* recorded 86.99% and 81.51% reduction in galls number respectively. Also, application of *B. subtilis* one week before the inoculation of *M. incognita* recorded 61.64% reduction of root galls.

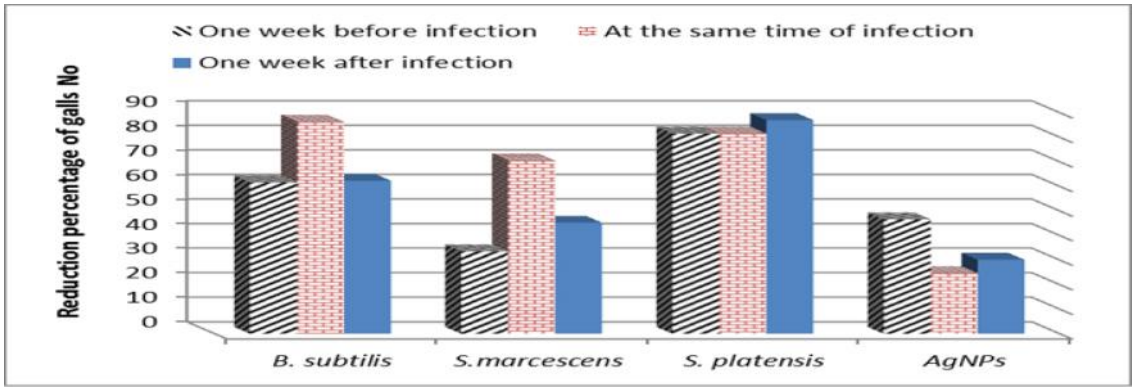

Fig (1): Impact of biotic and a biotic elicitors on percentage reduction of root galls.

Data in fig (2) showed that the application of *B. subtilis* at the same time or one week after inoculation of *M. incognita recorded* 88% and 60% reduction of egg masses number respectively. While, application of *S. platensis* one week before the inoculation of *M. incognita* recorded 80% reduction in egg masses number.

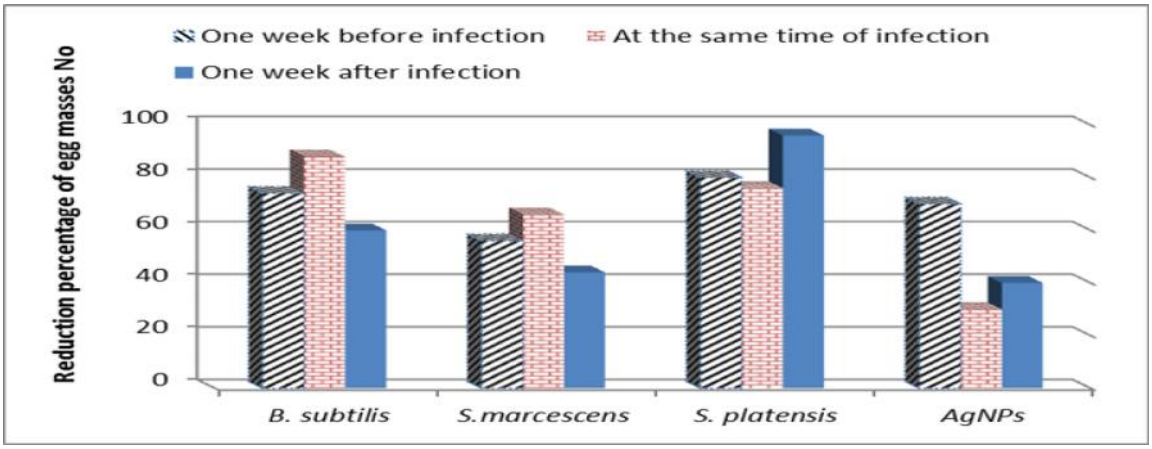

Fig (2): Effect of biotic and a biotic elicitors on percentage reduction of egg masses.

2-Vegetative growth parameters:

2-1-Shoot length's:

Data in table (2) indicated that the length's of shoots in nematode-infected plants were significantly decreased than that of non-infected ones (healthy plants). On the other hand, treatment with tested elicitors resulted in different responses as regards the shoot lengths of nematode-infected plants. These responses were varied according to the type of used elicitor and also to the time of application as follows:

Results of elicitors that applied one week before infection noticed that (Table 2) nematode-infected plants pretreated with *B. subtilis* gave the most potent effect as regard the plant height (23.66 cm) in comparison with plants treated with *S. platensis* (23 cm)then followed by *S. marcescens* (22.5 cm) and (AgNPs) (21.33 cm), respectively. On the other hand, results of elicitors that applied at the same time of infection (Table 2) showed that application of AgNPs was more effective in increasing the plant height. Also, significant increases in the plant height of nematode-infected plants were resulted in response to the treated with *S. platensis*. However, treatment with either *B. subtilis* or *S. marcescens* was insignificantly affect the length of shoot in nematode-infected plants as being compared with corresponding controls. While, at one week after infection the obtained results (Table 2) revealed that treatment with *B. subtilis* resulted in significant increase in shoot length of nematode-infected plants (29.33 cm) followed by treated with *S. platensis* which caused significant increase in shoot length (26.50 cm). However, application of either *S. marcescens* or (AgNPs) resulted in insignificant changes as regard the shoot length of nematode-infected plants.

2-2-Root length's:

Results showed in table (2) revealed that root's length of tomato plants were decreased significantly in response to the infection with nematode. However, the results of other treatments were demonstrated as follow:

One week before infection treatment with *B. subtilis* resulted in increasing significantly the root length of nematode-infected plants. This was the fact when being compared those of non-treated plants. Treatment with *S. marcescens*, *S. platensis* and/or AgNPs were generally increased the root's length of nematode-infected plants. These increases were found to be statistically insignificant. Also, at the same time infection the obtained results (Table2) showed that the use of AgNPs was more effective in increasing the root length of nematode-infected plants where it highly significantly increased than that of control ones. Also, significant increases were resulted as regards the lengths of tomato roots due to the treatments with either *S. platensis*, *B. subtilis* or *S. marcescens*. While, one week after infection Results in table (2) illustrate that application of *S. marcescens*, *B. subtilis* and *S. platensis* were effective in increasing significantly the root lengths of nematode-infected plants. However, the use of AgNPs was found to be less effective in increasing the root length when being compared with other used elicitors.

2-3-Number of leaflets/plant:

Results in table (2) showed that the plants infected with nematode recorded significant decrease in the number of leaflets/plant as being compared with healthy ones. Regarding the effect of different used elicitors the obtained results revealed that the numbers of leaflets/plant were significantly increased in tomato-infected plants due to the treatment with different elicitors (*B. subtilis*, *S. marcescens*, *S. platensis* and AgNPs). This was valid in different periods of treatment (before, at the same time and post infection.

2-4-Fresh weight of shoots & roots:

As regards the fresh weight of tomato shoots and roots, results shown in table (2) revealed that nematode infection caused significant decrease a being compared with healthy plants. However, the application of elicitors resulted in different responses as regards the shoot and root fresh weight. These different responses were clearly demonstrated as follows:

One week before infection the results in table (2) showed that treatment with *B. subtilis* was more effective in increasing significantly the fresh weight of shoots and roots in infected tomato plants. Treatments with *S. marcescens* in significantly affect the fresh weight of shoots and roots. However, application of *S. platensis* and AgNPs markedly increased the fresh weight of shoot. Also, at the same time infection Treatment with different elicitors (*B. subtilis*, *S. marcescens*, *S. platensis* or AgNPs) all significantly increased the fresh weights of shoots and roots of tomato plants infected with nematode (Table 2). While, one week after infection Treatment with either Bacillus or *S. platensis* was effective in increasing significantly the fresh weights of shoots and roots of nematode-infected plants when being compared with healthy ones. However, application of either *S. marcescens* or AgNPs was no effective in overcoming the adverse effect of nematode as regards the fresh weight of shoots and roots of tomato plants. This was the case when application was done during the infection (Table 2).

Table (2): Effect of some elicitors, time of application and their interactions on shoot length, root length and leaflets number of tomato plants, infected with *M. incognita* under greenhouse conditions.

Treatment			Shoot		Root		Number of leaflets/plant
Time of application	Material	Dose/pot	length (cm)	fresh weight(g)	Length (cm)	fresh weight(g)	
One week before infection	*B. subtilis*	2 ml	23.66 ab	4.23 ab	14.66 cdef	2.33 cde	55.00 b
	S. marcescens	2 ml	22.50 abc	2.76 bc	11.33 ef	1.66 de	49.66cde
	S. platensis	4 ml	23.00 abc	3.46 abc	11.33 ef	1.30 e	48.66 ef
	AgNPs	2 ml	21.33 abc	3.16 bc	11.66 ef	1.66 de	51.00 cd
At the same time of infection	*B. subtilis*	2 ml	19.66 bc	4.16 ab	15.66 bcde	2.40 cde	49.33 de
	S. marcescens	2 ml	20.66 abc	3.96 ab	15.00 cde	2.73 bcde	47.33 fg
	S. platensis	4 ml	23.33 abc	4.50 ab	16.66 bcde	2.16 cde	44.33 h
	AgNPs	2 ml	24.33 ab	4.30 ab	18.00 abcd	2.23 cde	44.33 h
One week After infection	*B. subtilis*	2 ml	29.33 a	5.13 a	21.66 ab	5.30 a	51.33 c
	S. marcescens	2 ml	19.66 bc	2.63 bc	23.33 a	4.53 ab	48.33efg
	S. platensis	4 ml	26.50 ab	4.26 ab	20.33 abc	3.43 abcd	46.66 g
	AgNPs	2 ml	18.66 bc	3.53 abc	13.66 def	3.96 abc	48.00efg
Nematode only			14.66 c	2.00 c	8.33 f	1.50 de	37.33 i
Control			19.33 bc	3.06 bc	10.33 f	1.80 e	60.66 a
L.S.D			8.852	1.8878	6.493	2.009	1.982

3-Endogenous hormones:

It was noticed that, contents of both GA and IAA were markedly decreased in infected plants than that of healthy ones. At the same time, marked increases in the contents of JA and ABA were observed in infected plants as being compared with healthy ones. On the other hand, the obtained results (Table 3) showed different responses as regards the determined hormones due to the application of different used elicitors. These results can be demonstrated as follows:

One week before infection the obtained results (Table 3) revealed that contents of GA as well as IAA were generally increased due to the application of different used elicitors. Contents of JA were slightly affected in response to treatment with *B. subtilis*, *S. marcescens* and *S. platensis*. While, AgNPs caused marked decrease in the contents of JA as being compared with controls. Regarding the contents of ABA, all the applied elicitors, with one exception, markedly decreased it than that of corresponding controls. The exceptional case was represented by marked increase in ABA contents due to the treatment with AgNPs. Also at the same time infection each of *B. subtilis*, *S. marcescens*, *S. platensis* as well as AgNPs caused marked increases in the contents of GA and IAA in nematode-infected plants. Contents of JA and ABA were markedly decreased in response to the application of all aforementioned elicitors. While, one week after infection the results shown in table (3) revealed that application of either *B. subtilis* or *S. platensis* was more effective in increasing contents of GA and IAA and decreasing the contents of both JA and ABA. Treatment with either *S. marcescens* or AgNPs was slightly affecting the measured hormones as being compared with the other elicitors used.

Table (3): Effect of some elicitors, time of application and their interactions on endogenous hormones (mg/100 g. Fr.Wt.) of tomato plants, infected with *M. incognita* under greenhouse conditions.

Treatment			Endogenous hormones (mg/100 g. Fr.Wt.)			
Time of application	Material	Dose/pot	GA	IAA	JA	ABA
One week before infection	*B. subtilis*	2 ml	3.69	4.01	3.17	2.12
	S. marcescens	2 ml	3.43	3.69	3.31	2.42
	S. platensis	4 ml	3.67	3.27	3.05	2.31
	AgNPs	2 ml	3.15	3.01	2.31	4.79
At the same time of infection	*B. subtilis*	2 ml	3.12	2.97	2.06	3.01
	S. marcescens	2 ml	2.94	2.56	2.51	3.11
	S. platensis	4 ml	3.20	2.33	2.42	3.02
	AgNPs	2 ml	3.01	2.85	2.73	2.91
One week After infection	*B. subtilis*	2 ml	2.99	3.66	2.16	3.02
	S. marcescens	2 ml	2.79	3.01	2.67	3.31
	S. platensis	4 ml	3.01	3.71	2.15	3.01
	AgNPs	2 ml	2.94	2.77	2.84	3.65
Nematode only			3.07	1.84	3.01	4.27
Control			3.19	2.93	2.16	3.41

(1) GA=Gibberellins.
(2) IAA=Indol acetic acid.
(3) JA=Jasmonic acid.
(4) ABA=Abscisic acid.

4- Biochemical marker as indicators for ISR:

Results shown in tables (4, 5, and 6) revealed that the tomato plants treated with tested elicitors and infected with *M. incognita* showed variation in number, molecular weight and density of protein bands due to the application of different used elicitors and also to the time of application as follows:

One week before infection the variability analysis among tested inducers appeared 46 protein bands. It was found that AgNPs gave (10 protein bands) related to *B. subtilis* (9 protein bands) and *S. marcescens* (9 protein bands) and *S. platensis* (5 protein bands), respectively, and Nematode only (7 protein bands), as well as control gave (6 protein bands). The molecular weight of polypeptides were determined related to

protein markers ranged from 129.625 to 12.875 KDa. The most prominent specific polypeptide alteration (polymorphic bands) ranged molecular weight from 25.729 to 18.11 KDa with percentage 10.8%. These bands may be related to tested elicitors. The prominent polypeptide bands in all inducers (monomorphic or common polypeptide) were ranged molecular weight from 19.710, to 12.875 kDa with percentag11e 65.2%. These bands may be related to tomato plant. The unique (polypeptide markers) were appeared in tomato plant infected with *M. incognita* plants treated with biotic inducers ranged from 126.951 to 19.873 kDa with percentage 23.9%. These bands may be related to polypeptide markers (Table 4). Also, at the same time infection results in table (5) showed that application of tested elicitors on the challenged plants with *M. incognita*, it was found that *B. subtilis, S. marcescens* , *S. platensis* gave highest number of bands(7,7 and 7 bands), respectively followed by AgNPs (3 bands), respectively **(**Fig. 3). Also, it was found that Nematode only gave (7 protein bands), as well as control gave (7 protein bands). The molecular weight of polypeptides ware determined related to protein markers ranged from 66.766 to 12.981 KDa. The most prominent specific polypeptide alteration (polymorphic bands) ranged molecular weight from 66.766 to 13.746 KDa with percentage 63.158%. These bands may be related to tested elicitors. The prominent polypeptide bands in all inducers (monomorphic or common polypeptide) were ranged molecular weight from 17.832, to 12.981 kDa with percentage 15.87%. These bands may be related to tomato plant. The unique (polypeptide markers) were appeared in tomato plant infected with *M. javanica* plants treated with biotic inducers ranged from 15.386 to 13.646 kDa with percentage 5.263%. These bands may be related to polypeptide markers (Table 5). While, one week after infection the obtained results (Table 6) that the Tomato plants treated with tested elicitors and inoculated with *M. incognita* showed variation in number, molecular weight and density of protein bands. The variability analysis among three inducers appeared 38 protein bands. It was found that *S. platensis* gave (12 protein bands) related to *S. marcescens* (10 protein bands), AgNPs (10 protein bands) and *B. subtilis* (9), respectively, and nematode only (10 protein bands), as well as free nematode gave (8 protein bands). The molecular weight of polypeptides ware determined related to protein markers ranged from 105.699 to 12.346 KDa. The most prominent specific polypeptide alteration (polymorphic bands) ranged molecular weight from 105.699 to 12.346 KDa with percentage 15.25%. These bands may be related to tested elicitors.

Table (4): Protein bands in leaf of tomato plants infected with nematode treated with inducers (one week before infection) using SDS-PAGE.

MWt	*B. subtilis*	*S. marcescens*	*S. platensis*	AgNPs	Nematode only	Control	Polymorphism
126.951	-	-	-	-	+	-	**Unique**
93.237	-	+	-	-	-	-	**Unique**
90.300	-	-	-	+	-	-	**Unique**
66.019	-	-	-	+	-	-	**Unique**
47.478	-	-	-	+	-	-	**Unique**
36.073	+	-	-	-	-	-	**Unique**
33.310	-	+	-	-	-	-	**Unique**
27.407	+	-	-	-	-	-	**Unique**
26.157	-	+	-	-	-	-	**Unique**
25.729	-	-	-	-	+	+	**Polymorphic**
24.895	-	-	-	+	-	-	**Unique**
19.873	+	-	-	-	-	-	**Unique**
19.710	+	+	+	+	+	+	**Monomorphic**
18.811	+	+	-	+	-	-	**Polymorphic**
18.201	+	+	+	+	+	+	**Monomorphic**
15.865	+	+	+	+	+	+	**Monomorphic**
14.529	+	+	+	+	+	+	**Monomorphic**
12.875	+	+	+	+	+	+	**Monomorphic**
Total bands	**9**	**9**	**5**	**10**	**7**	**6**	**46**

Table (5): Protein bands in leaf of tomato plants infected with nematode treated with inducers (at the same timeinfection) using SDS-PAGE.

MWt	*B. subtilis*	*S. marcescens*	*S. platensis*	AgNPs	Nematode only	Control	Polymorphism
66.766	-	-	-	-	+	+	**Polymorphic**
19.221	+	+	+	-	-	-	**Polymorphic**
17.832	+	+	+	+	+	+	**Monomorphic**
16.752	+	+	+	-	+	+	**Polymorphic**
15.464	+	+	+	-	+	-	**Polymorphic**
15.386	-	-	-	-	-	+	**Unique**
14.563	+	+	+	-	+	+	**Polymorphic**
13.749	+	+	+	-	+	+	**Polymorphic**
13.646	-	-	-	+	-	-	**Unique**
12.981	+	+	+	+	+	+	**Monomorphic**
Total bands	**7**	**7**	**7**	**3**	**7**	**7**	**38**

The prominent polypeptide bands in all inducers (monomorphic or common polypeptide) were ranged molecular weight from 78.902, to 15.037 kDa with percentage 81.85 %. These bands may be related to tomato plant. The unique (polypeptide markers) were appeared in tomato plant infected with *M. javanica* plants treated with biotic inducers ranged from 89.768 to 14.149 kDa with percentage 1.69 %. These bands may be related to polypeptide markers (Table 6).

Table (6): Protein bands in leaf of tomato plants infected with nematode treated with inducers (one week after infection) using SDS-PAGE.

MWt	*B. subtilis*	*S. marcescens*	*S. platensis*	AgNPs	Nematode only	Control	Polymorphism
105.669	-	+	+	+	+	-	**Polymorphic**
89.768	-	-	-	-	+	-	**Unique**
78.902	+	+	+	+	+	+	**Monomorphic**
67.520	-	-	+	+	+	-	**Polymorphic**
48.255	+	+	+	+	+	+	**Monomorphic**
38.573	+	+	+	+	+	+	**Monomorphic**
32.928	+	+	+	+	+	+	**Monomorphic**
30.387	+	+	+	+	+	+	**Monomorphic**
27.770	+	+	+	+	+	+	**Monomorphic**
23.419	-	-	+	-	-	-	**Unique**
20.286	+	+	+	+	+	+	**Monomorphic**
15.037	+	+	+	+	+	+	**Monomorphic**
14.149	-	-	-	-	+	-	**Unique**
12.346	+	+	+	-	-	-	**Polymorphic**
Total bands	**9**	**10**	**12**	**10**	**10**	**8**	**59**

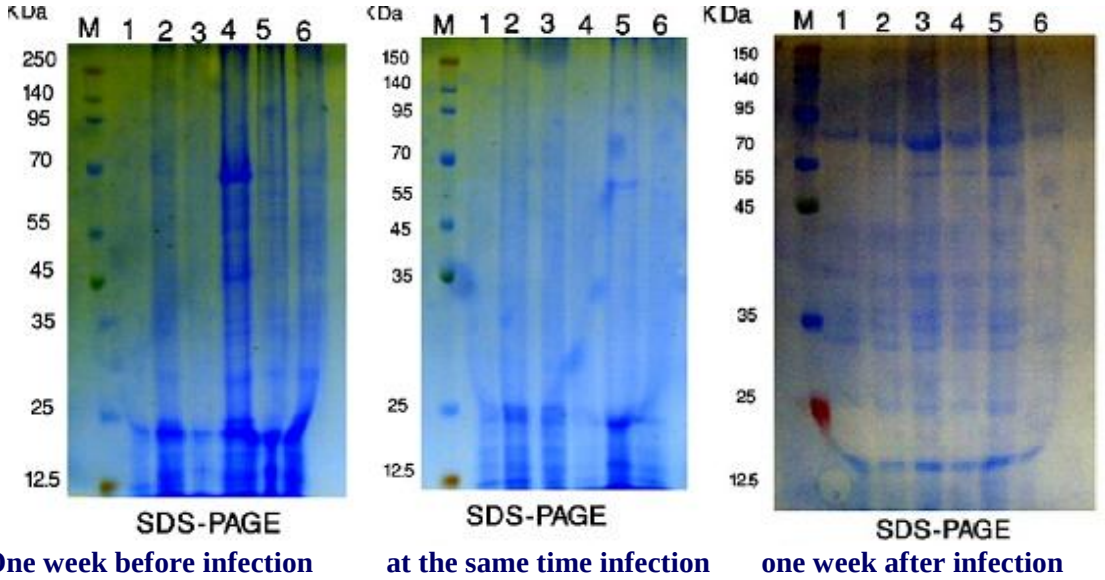

Fig (1): Protein fractions of tomato plants treated with some elicitors at (pre, during and post infection) using SDS-PAGE. M: Marker. Monomorphic (Common polypeptide). Polymorphic (Specific polypeptide) Unique (Polypeptide marker) or (genetic marker).-=Absence of band and + = presence of band

Discussion

The objectives of this study were induction of physiological immunity in tomato plants against root-knot nematode (*M. incognita*) infection. No strategies are currently available to completely protect these plants against Plant-parasitic nematodes. Biological control is considered as new efficient method that becomes widely used for controlling plant parasitic nematodes, as aim to decrease the extent of environment degradation and the effect of the excessive toxic nematicides. By direct inoculation with a constant number of nematode larval juveniles, the results showed different abilities of PGPR (*B. subtilis* & *S. marcescens*), cyanpbacterial strain (*S. platensis*) and silver nanoparticle AgNPs according to the type of used elicitors and also the time of application in controlling the plant parasite nematodes Meloidogyne, which infect tomato.

Obtained data showed that using *B. subtilis* at the same time infection with the nematode *M. incognita* recorded 86.30% and 88% reduction of root galls and egg masses. This result is in agreement with those of Khan *et al.* (2007) found that soil infestation with the nematode *M. incognita* caused severe galling on roots of mungbean. Also, they reported that the application of *Pseudomonas fluorescens* or *B. subtilis* suppressed the gall formation, reproduction and population of *M. incognita*. Khalil *et al.* (2012) recorded some treatments such as abamectin, azadirachtin 0.15%, azadirachtin 0.03%, *B. subtilis*, *Pseudomonas fluorescens*, *Paecilomyces lilacinus* and oxamyl as effective agents against root-knot nematode (*Meloidogyne incognita*) on the tomato cv. super strain B. This strain was the most investigated that obviously reduced root galls and egg masses on root system, and juvenile numbers in the soil and remarkably increased tomato plant growth characters.

It has been suggested induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated (Kuc, 1995). The obtained results showed that a retarded growth in nematode-infected plants. Plant height, fresh and dry weights of both shoots and roots and the number of leaflets/plant were significantly decreased due to nematode infection. Which has been confirmed by (Kankam *et al.*, 2015; ElSayedI *et al.*, 2014; Radwan *et al.*, 2009) they reported that nematode-infested plants become stunted due to root dysfunction, reduction of rooting volume and efficient utilization of water and nutrients. The first criterion to judge the occurrence of ISR in nematode-infested plants treated with tested elicitors, more significant values of growth characters. PGPR plays an important role in enhancing plant growth through a wide variety of mechanisms. PGPR are not only associated with the root to exert beneficial effects on plant development but also have positive effects on controlling phytopathogenic microorganisms (Son *et al.*, 2014). In our results, Cleary that effect of used elicitors on growth characters of infested tomato plants with root-knot nematode *M. incognita* it is presented in table (2)

The obtained data showed different responses. These responses were varied according to the type of used elicitors and also to the time of application.

The mechanisms of PGPR include regulating hormonal and nutritional balance, inducing resistance against plant pathogens, and solubilizing nutrients for easy uptake by plants. In addition, PGPR show synergistic and antagonistic interactions with microorganisms within the rhizosphere and beyond in bulk soil, which indirectly boosts plant growth rate (Vejan *et al.,* 2016). Some PGPR have nematicidal potentials and can confer protection against root-knot nematode infections (Köber *et al*., 2013). *Bacillus spp.* associated with entomopathogenic nematodes may be used as biocontrol agents against postharvest fungal disease (Han, 2006). Therefore, in addition to the plant growth-promoting effects of *Bacillus*, its interactions with nematodes require further research. The ability to form endospores allows PGPR, especially *Bacillus spp*. and *Pseodomonas spp*., to survive in a wide range of environmental conditions, thus facilitating the effective formulation of biofertilizer (Perez-Garcia *et al.,* 2011). Also, our result agreed with Aballay *et al*. (2013) and Ashoub and Amara (2010) they found that three strains, of *Bacillus megaterium, Pseudomonas fluorescens* and *Serratia marcescens* treatments significantly increased all growth parameters in the presence or absence of the pathogen and confirmed that *Serratia marcescens* and *Pseudomonas fluorescens* were potent as bio-control agents for root-knot nematodes. A further three strains, of *Bacillus megaterium*, *P. agglomerans* and *Pseudomonas savastanoi*, significantly increased plant growth. Under greenhouse conditions, cell suspensions of different *Pseudomonas fluorescens* strains have been found to be effective in suppressing populations of *Meloidogyne incognita* (Ashoub and Amara, 2010).

These findings are in agreement with those given by Khalil *et al*. (2012) and Hashem and Abo-Elyousr (2011) they found that yield and growth parameters of plants treated with *P. fluorescens, B. subtilis* and Trichoderma showed a significant increase. Also, Abou-Aly *et al.* (2015) reported that soil infestation with root-knot nematode significantly decreased the growth characters of tomato as compared with control treatment. Generally, growth characters of tomato significantly increased when plants were treated with chemical nematicide, nemaless and bioagents (*B. subtilis* B38, *Ps. fluorescens* B103 and *S. marcescens*) compared to infested treatment.

In our results, Cleary that, application of cyanobacteria (*S. Platensis*) on infected–tomato plants exhibited antagonistic action and affected on all growth parameters. *S. Platensis* have been known to possess nematicidal properties that may be release in soil. In addition, cyanobacteria are produce compounds, such as Asetamide, hexamethyl, methoxy phenyl, phenol, and others, which are normally toxic to the root-knot nematode. Likewise, the cyanobacteria such as *S.platensis,* Microcystis, Anabaena, Nostoc and Oscillatoria produce a great variety of secondary metabolites (Moore 1996; Namikoshi and Rinehart, 1996; Gerwick *et al.,* 2001), such as nitrogen-containing compounds, polyketides, lipopeptides, cyclic peptides. Similarly, Magda *et al.* (2015) reported that *S. platensis* caused the highest records of shoot length, shoot diameter, lateral shoots number, leaves number, leaf dry weight and leaves contents of chlorophyll and carbohydrates of peach seedlings under the two fungus of root rot disease.

Biosynthesis of silver nanoparticles (AgNPs), a new class of material with remarkably different physicochemical and biological characteristics from convenient silver-containing substances, has been shown to have antibacterial, antifungal, antiviral and nematicidal effects and it can reduce damage and losses caused by diseases (Choi *et al*., 2009; Eo and Lee, 2009).

On the contrary, results of the present work showed that nematode-infected plants treated with AgNPs generally significantly improved plant growth, as shown by an increase of plant height, fresh and dry weights of both shoots and roots and the number of leaflet/plant. Our results are in accordance with those reported by Ma *et al*. (2010) and Shah and Belozerova (2009) they reported that it has great influence on plant growth and development such as germination, root-shoot ratio, seedling growth, root growth, root elongation, and senescence inhibition .Recently; AgNPs increased plants growth profile (shoot and root length, leaf area) and biochemical attributes (chlorophyll, carbohydrate and protein contents, antioxidant enzymes) of *Brassica juncea*, common bean and corn (Salama, 2012; Sharma *et al.,* 2012).

Phytohormones play a direct or indirect role in modulating plant cell growth at nematode feeding sites (Goellner *et al*., 2001). Recent morphological and biochemical research have revealed that local phytohormone levels and pathways are altered in nematode-parasitized roots. Our results demonstrated that, application of used elicitors on plants at pre

infection with nematodes revealed that contents of GA as well as IAA were generally increased due to application of different used elicitors compared with those of nematode infected plants. Plant growth regulators, also termed plant exogenous hormones, are synthetic substances that are similar to natural plant hormones. They are used to regulate the growth of plants and are important measures for boosting agricultural production. One of the terms for the prominent modes of action for growth promotion by PGPR is phytostimulator, or plant growth regulator. This is defined as microorganisms that have the ability to produce or alter the concentration of growth regulators such as IAA, GA, cytokinins, and ethylene. The mechanism that is being projected is the production of phytohormones (plant hormones) such as auxins, cytokinins, and GA. In this respect (El-Tayeb *et al*. 2006; Gravel *et al*., 2007; Mandal *et al*., 2009; Sofy *et al.,* 2014) they reported that, Indole acetic acid (IAA) is endogenous plant growth regulator and play an important role in many physiological processes under different biotic and abiotic stresses. Exogenous application of GA and IAA has been demonstrated to enhance plant resistance against pathogens by acting as potent inducer of systemic resistance (Ueno *et al.*, 2011). The plant growth promoting rhizobacteria have been reported to improve plant growth either through direct stimulation by the synthesis of phytohormones (Xie *et al*., 1996) or by decreasing the effect of pathogens (Weller *et al.,* 2002). PGPR releases antimicrobial factors including lytic enzymes which lead to the accumulation of phenolics (Meena *et al*., 2000; Sofy *et al.,* 2014) by secretion of indole acetic acid that induced phenol metabolism in plants (Shabaev *et al*., 1999). The use of PGPR for inducing systemic resistance against phytonematodes has been well documented (Patricia *et al*., 2009; Sofy *et al.,* 2014). In addition to some species of Pseudomonas, Bacillus reported to induce systemic resistance in plants against invading pathogens and antagonists to root-knot nematodes of *Meloidogyne spp.* (Kloepper and Ryu, 2006). Endogenous JA is a key signal, involved in the activation of plant defense responses to fungal, bacterial, viral, nematode attacks. Because inoculation with *B. subtilis*, *S. marcescens* and *S. platensis* (especially pre-infection) on the challenged plants with nematode , caused a significant increase in JA level in plant leaves (Table 4), it is reasonable to assume that several plant hormones either individually or in combinations modulate the complex processes involved in plant defense signaling pathways. The first hormones to be marked as central players in defense against plant pathogens were salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Glazebrook, 2005; Sofy *et al.,* 2014), with roles more recently attributed to abscisic acid (ABA), gibberellins, and auxin (Kazan *et al.*, 2009). Studies have demonstrated that JA is the major defense-signaling molecule associated with the wound response (Dong, 1998).The natural resistance of plants to pathogens and parasitic weeds is based on the combined effects induced mechanisms among which the systemic acquired resistance (SAR) that controlled by signaling pathway and depends on endogenous accumulation of salicylic acid and jasmonic acid (JA) (Yang *et al*., 2010). ABA promotes resistance in some plant–pathogen interactions, whereas it increases susceptibility in others (Forcat *et al*., 2008). Regarding the contents of ABA our results demonstrated that application of AgNPs at different time of infection was represented by marked increase. These findings are in agreement with those given by Ton *et al.* (2005) who mentioned that ABA also plays a major role in signaling due to biotic and abiotic stress, but disease resistance may differ with the type of pathogen and timing of applications. Recently, abscisic acid (ABA), auxins and gibberellins emerged as forces on the battle field as well. In many cases, these hormones interact antagonistically or synergistically with the SA-JA-ET backbone of the plant immune signaling network, thereby redirecting its defense output (Ton *et al.*, 2009; De Vleesschauwer *et al.,* 2010; Jiang *et al.,* 2010).

Quantitative proteins of induced tomato plants infected with nematode were determined using SDS-PAGE, our results indicated that, a new pattern of proteins were produced, as well as, different increasing in the density of bands according to the type of used elicitors and also to the time of application. It has been suggested that, the induced proteins may help to limit spread or multiplication of pathogen (Chen *et al.,* 2006; El-Dougdoug *et al.*, 2014; Sofy *et al.,* 2014). The early accumulation of chitinase is associated with induced systemic resistance. Chitin is major cell wall components of many fungi and bacteria. Chitinase can hydrolyze cell walls of the pathogen (Hassan, 2004). Chitinase may be involved in the defense of plants against fungi, bacteria and nematode by their action on the cell walls of invading. Several investigators have indicated that induced resistance in plants was associated with a great increase in chitinase activities. Our results showed that presence of molecular weight,27, 32 KD (PR3 –Chitinase) was found in all plants treated with biotic inducers (either pr-infection or post-infection) these induced proteins have been defined as

pathogenesis related proteins, they implicated in plant defense because of their anti-pathogenic activities (Van-Loon *et al*., 1994). Application of tested elicitors at pre- infection showed that The unique (polypeptide markers) were appeared in tomato nematode –infected plants treated with tested elicitors ranged from 89.768 to 14.149 kDa with percentage 1.69% Also The unique (polypeptide markers) were appeared in tomato plant infected with *M. incognita* and treated with biotic inducers ranged from 89.768 to 14.149 kDa with percentage 1.69%. These bands may be related to polypeptide markers. Furthermore, new protein with molecular weight 93, 33, 26, KD (PR2-B1,3-glucanase) was found in plants treated with *S. marcescens*. These induced proteins have been defined as pathogenesis related proteins, they implicated in plant defense because of their anti-pathogenic activities (Van-Loon *et al*., 1994). The continuous accumulations of newly-induced proteins may help in the suppression of plant parasitic disease (root-knot nematode) activity in tomato using biotic or a biotic agents; the reverse is not true, since the presence of a non-significant amount of induced proteins is a necessary condition to the observed systemic infection. Based on current knowledge of the biochemistry of resistance, it can be concluded that SAR results from the expression of several parameters, including changes in cell wall composition and *de novo* synthesis of phytoalexins and PR (pathogenesis related) proteins. Moreover, the local *de novo* synthesis of phytoalexins is often related to the induced resistance stage (Walter *et al*., 2007). Recently, Protein content, chitinase and peroxidase activities in leaves of tomato plants was significantly increased by inoculation of tomato plants with bioagents (*Serratia marcescens*, and Trichoderma harzianum) compared with nematicide may play either a direct or indirect role in the suppression of root-knot nematode *M. incognita* (Abd-Elgawad and Kabeil, 2010).

Finally, it could be concluded that the results from this study indicated that using of both antagonistic microorganisms (*B. subtilis*, *S. marcescens*, *S. platensis*) and AgNPs achieved a highly activity against the root-knot nematode, in addition gave increasing in plant growth. Therefore, the results imply that it should focus on using biological agents and nanotechnology as a safety method for human and environment to management the root-knot nematode in Egypt.

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