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Synergistic Effect of Bioagents and Antioxidants Against Root-Knot Nematode, *Meloidogyne incognita* on Sunflower

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ABSTRACT

Root-knot nematodes are globally considered one of the most common phytonematodes infecting sunflower crops. In this context, current study, treatments were designed to enhancing the effect of biological control against *Meloidogyne incognita* by *Trichoderma harzianum* and *Bacillus subtilis* alone or in combination with antioxidant; ascorbic and salicylic acids. All treatments significantly suppressed root-knot nematode compared to control. Generally, the results indicated that the treatments of mixtures of antioxidants + bioagents showed a better response in suppression of root-knot nematode than the bioagents alone, as mixture of *T. harzianum* + *B. subtilis* gave the highest reduction in nematode population (72.3% and 80.0%).While the best synergistic effect was noticed by combination between *T. harzianum* and salicylic acid (74.9% and 84.9%) during the growing seasons 2018 and 2019,respectively.This positively reflected on the plant health through induction of defense related components (phenolic content and oxidative enzymes).

Keywords: Bioagents; Antioxidants; Root-knot nematodes; Sunflower.

INTRODUCTION

Sunflower, *Helianthus annuus* L. is considered one of the oilseed crops, It was ranked third as an oil crop globally produced (Pilorge, 2020 and Khan et al., 2015). In Egypt, the cultivated area of sunflowers reached 10,000 hectares, with total productivity 19,000 tons (USDA, 2019). Among the wide range of pests and pathogens that affect sunflowers, phytonematodes represent one of the most important obstacles to sunflower production (Fourie et al., 2010). *Meloidogyne incognita* represents the predominant root-knot nematode species in leguminous and oilseed crops (Devappa, 1996; Fourie et al., 2017).

In Egypt, root-knot nematode, *Meloidogyne incognita* (Kofoid and White,1919) Chitwood, 1949 is the most deleterious phytonematodes contributing to stunting, wilting, poor plant growth and significant yield losses (Ibrahim and Mokbel, 2009; El-Sagheer,2019).Bio-control of soil borne pathogens as phytonematodes by antagonistic microorganisms has been regarded as a more acceptable alternative to existing chemical methods to decrease the populations of phytonematodes (Askary and Martinelli, 2015).

Bacillus is a genus of gram-positive, rod-shaped bacteria, a member of the phylum Firmicutes, producing numerous bioactive ingredients with an expansive range of activities towards pathogens or stimulating induced resistance (Mazzuchelli et al., 2020). Among the promising bacteria as a bio-agent to control of soil pathogens, *Bacillus subtilis* represents one of the most studied (Bettiol and Morandi, 2009).This bacterium is local natural in soil in Egypt (Abd-Elsalam and El-Hanafy, 2009), produces enzymes, plant hormones and antibiotics which provide benefits to plants, and improve their growth (Silveira, 2001). In addition to the beneficial effects on the crop yield, *B. subtilis* was able to control *M. incognita* in vegetable crops through a wide spectrum of effects that bacterium has in suppressing plant nematodes and was mentioned in previous studies (Araujo, 2008; de Araújo et al., 2011; Higaki and Araujo, 2012; Vlamakis et al., 2013; Rao et al., 2014) which nominated it as one of the most important control factors that its effects must be expanded in the suppression of plant nematodes (Bettiol and Morandi, 2009).

The filamentous fungi, *Trichoderma* spp., one of a hyphomycetes fungal species have been used and applied in recent decades for phytonematodes biocontrol (Kerry, 2000). Through an interaction of many mechanisms, *Trichoderma* spp. have antagonist effects on phytonematodes including; (a) direct mycoparasitism which involves the production of cell-wall-degrading enzymes (Lorito et al., 2010), (b) producing chitinase into the culture or rhizoshere which might help in the inhibition of egg hatching. (Sharon et al., 2001), (c) the conidia of *Trichoderma* parasitize on nematode cuticle or/and egg shell (Sharon et al., 2007), (d) help plants in tolerance against stress condition by enhancing the root development through promoting to solubilization of inorganic nutrients (Sharma and Rakesh, 2009), (e) producing a variety of metabolites (mycotoxins) which inhibit egg-hatching and juveniles development (Naserinasab et al., 2011).

The interaction between several factors within the biological control strategy represents an effective approach to reduce nematode damage to plants (Sikora, 1992; Kerry, 2000). In this context, using some organic acids (ascorbic and salicylic acids) on plant growth and yield of sunflower as well as their effects on reproduction and development of many phytonematodes were studied (Seo and Kim, 2014; Kim et al., 2018). Ascorbic acid is a non-enzymatic antioxidant ($C_6H_8O_6$) which plays an important role in the activation of biological defense mechanisms of plants to pathogens such as root-knot nematodes. (Arrigoni et al., 1979). (Al-Sayed and Thomason 1988) found that solutions of ascorbic acid suppressed the numbers of second stage juveniles, females, egg masses, and root galls of *M. incognita* root-knot nematode on other hosts. Ascorbic acid gave the best nematode control and plant growth. Mohamed (2005) examined different concentration levels of ascorbic acid (2500, 5000 and 10.000 ppm.) for controlling *M. incognita* on sunflower. Where he stated that all the tested concentrations reduced nematode build up relative to control. A significant reduction in nematode population and counts of egg masses / root and improved growth parameters in plant were obtained by 1000 ppm concentration.

Salicylic acid is known as a key endogenous signal molecule in the induction of defense mechanisms that mediates defense gene expression and disease resistance in many dicotyledonous species (Renault et al., 1996). Exogenous application of SA and functionally related analogues, including (C_6H_4 (OH) COOH) has been shown to induce resistance to nematode pathogens in both dicotyledonous and monocotyledonous plants (Hammerschmidt and Smith-Becker, 1999 and Klessig et al., 2000). Nandi et al. (2002) who reported that the application of salicylic acid significantly reduced the number of nematode second stage juveniles, other

developmental stages and consequently lowered reproduction factor of *M. incognita* on cowpea plants. The mode of action of salicylic acid i.e. could enhance defense mechanisms in the plant tissue (Sirohi and Rohatgi, 2006) and increase resistance against *Meloidogyne* spp. (Nandi et al., 2002; Canet et al., 2010; Osman et al., 2012).

So, the current study aims to study the synergistic or combination suppression effects of bioagents (*Trichoderma harzianum* and *Bacillus subtilis*) and antioxidants (ascorbic and salicylic acids) as synergistic biological control strategy against root-knot nematode, *M. incognita* on sunflower.

MATERIALS AND METHODS

Preparation of *Meloidogyne incognita*

Egg masses were collected from roots of coleus plants (*Coleus blumei* L.) heavily infected with *M. incognita* grown in horticultural nursery and propagated on coleus plants as highly susceptible host for 3 months under greenhouse conditions at Nematology Research Unit, Plant Pathology Research Institute, Giza, Egypt. A sodium hypochlorite (NaOCl) extraction technique (Hussey and Barker, 1973) was undertaken in order to collect eggs of *M. incognita*. Infected roots were washed to free of soil and cut into 2-3 cm segments. Root segments were, then shaked in 200ml of 1.0% NaOCl solution for 1-2 minutes. NaOCl solution was quickly passed through a 60-mesh sieve nested over a 400-mesh sieve to collect free eggs. A 400-mesh sieve with eggs was quickly placed under a stream of tap water to remove residual NaOCl and eggs were collected. The remaining roots were rinsed with water to remove additional eggs which were collected.

Propagation of bioagents

Both isolates of bioagents provided by Central Lab. of Organic Agriculture, Agricultural Research Center, Egypt. *Bacillus subtilis* isolate was grown in liquid nutrient glucose medium (NGM) developed by Dowson (1957) for 2 days at 25ºC. and prepared at concentration 20 x 10^7 CFU/ ml. *Trichoderma harzianum* isolate was grown on liquid gliotoxin fermentation medium (GFM) developed by Brain and Hemming (1945) for 11 days under complete darkness condition at 25ºC to stimulate toxin production. Then, was prepared as suspension at concentration of 30 x 10^6 CFU / ml. To increase adhesive capacity and improve distribution of bioagent on the surface of treated seeds, suspensions were mixed with 5 % Arabic gum and 0.5 % potassium soap.

Preparation of mixture of bioagents

To increase the efficacy of the different single bioagents on nematode control, combinations of the two used bioagents were prepared; each mixture was prepared by mixing the two antagonists at the rate 1:1.

Preparation of antioxidants

Two different organic acids as antioxidants (ascorbic and salicylic acids) were formulated singly and/or combined. Solution of each organic acid was prepared by dissolving in 2 g/ L water. Seeds of sunflowers cv. 162 were separately dipped in each of these solutions for 30 minutes.

Preparation of mixture of bioagents with antioxidants

The two bioagents were mixed separately either with ascorbic acid or salicylic acid at the rate 1:1 (v: v),immediately before the treatment at the same time without direct mixing, to compare the effect of these mixtures with the effect of either single bioagent or single antioxidant. The treatments of tested materials were as follows:1. Salicylic acid; 2. Ascorbic acid; 3. Salicylic acid + Ascorbic acid; 4. *B. subtilis*; 5.

T.harzianum; 6. .*B.subtilis* + *T.harzianum*; *7. B.subtilis* + Salicylic acid; 8. *B.subtilis* + Ascorbic acid; 9.*T.harzianum* + Ascorbic acid; 10. *T. harzianum* + Salicylic acid and 11.Control.

Laboratory Experiments

Evaluation of *Trichoderma harzianum* and *Bacillus subtilis* alone or combination with ascorbic and salicylic acids on egg hatching and mortality of *M. incognita*.

Eggs hatching test

In vitro studies, ten egg masses in medium size (Al-Sayed and Thomason 1988), similar age and undifferentiated, were immersed in 5 ml of each single treatment on tissue paper in petri dishes, and covered to avoid evaporation and placed at room temperature (26±2°C) on laboratory bench. Each treatment was replicated three times, egg masses in equal volumes of distilled water served as control. The number of emerged second-stage juveniles as the percentages of hatched eggs inhibition were calculated at 24, 48 and 72 hours (Southey,1986). Egg masses hatching inhibition percentage and mean number of juvenile hatching per egg mass were calculated using the following formula:

$$\text{Mean number of non hatched eggs per egg mass} = \left(\frac{\text{Number of non hatched eggs in treatment}}{\text{Initial no. egg masses}}\right)$$

$$\text{Hatching inhibition \%} = \left(\frac{\text{Number of non hatched eggs in treatment} - \text{Number of non hatched eggs in control}}{\text{Total number of eggs}}\right) \text{x100}$$

Mortality test

Egg masses were picked from the galled sunflower roots and incubated in sterile distilled water at room conditions at 26+2°C for 48 hours (Lee and Atkinson 1976). Hatched second stage juveniles that had passed through the tissue paper into the petri dish were counted and concentrated until reached 1 ml of distilled water. Approximately 100 second stage juveniles (J_2) were used for each of treatments including the control. Population density of second stage juveniles (J_2) in stock suspension (100 J_2 /1ml distilled water) was considered as mean population number from 3 times of 1 ml of stock suspension. 1 ml of this juveniles suspension poured in screw-capped test tubes which contained 5 ml of different concentrations of tested materials timely prepared and incubated in dark at 26±2°C for four days and the numbers of dead juveniles were counted at 24, 48 and 72 hours (Demeure and Freckman 1981), using nematode counting slide in accordance with the Hussey and Barker (1973) technique modified by Boneti and Ferraz (1981). Each treatment was replicated three times, distilled water served as control. The corrected mortality percentages were calculated according to the Abbott's formula (1925). The J_2 mortality (%) were assessed as compared to the control according to the following equation; juvenile mortality %= $[C_1\text{-}C_2/C_1]$ x100 Where C_1 is the number of dead nematode juveniles in treatment and C_2 is the number of dead nematode juveniles in control.

Nematode inoculum preparation

Females and egg masses of *Meloidogyne incognita* were isolated from infected sunflower roots. Cultures of this nematode species were established from single egg-masses of adult females previously identified by the morphological characteristics of the female perineal patterns (Taylor and Sasser, 1978) and reared on Rutgers sunflower plants in a greenhouse. The root-knot nematode eggs were extracted from

infected sunflower roots using sodium hypochlorite (NaOCl) solution as described by Hussey and Barker (1973).

Field Experiment design

Evaluation of *T. harzianum* and *B. subtilis* alone or combination with ascorbic and salicylic acids on sunflower infected with *M. incognita* under field conditions.

In order to study the effects of two antioxidants (ascorbic acid and salicylic acid) as well as the two bioagents (*T. harzianum* and *B. subtilis*) singly and/or mixed on controlling root-knot nematode , *M. incognita* infecting sunflower and their effect on plant growth parameters of sunflower, a field experiment was carried out in Agricultural Research Center, Giza governorate over the course of two successive seasons (2018/2019). The experiment covered a total area of 150 m^2, in a randomized complete block design with four replicates per treatment. Each plot consisted of two rows, 30 cm wide and 3m long. Sunflower seeds cv. 162 were dipped tested materials for 30 minutes and kept in cheese cloths for 3 days before sowing. Treated sunflowers seeds were transplanted in infected plots at the rate of 5 seeds/ plot. Untreated sunflower seeds cv.162 were used as control. All agricultural practices were carried out according to the recommendations of the Ministry of Agriculture, Egypt.

Sixty plots for each experimental location were planted with (2-3 seeds/hill) of sunflower. One week later, plants were treated with treatments as soil drench, the antioxidants solutions were prepared at a level of 2000 ppm. Through eleven treatments (as previously mentioned), suspension of each treatment was prepared at a level of 0.1 L /10 L distilled water each. Plants were foliar sprayed by treatments at 2 times, with 14 days interval. Four plots were freed of any treatment served as control. Plants were harvested, 45 days after treating, and roots were washed to free from adhering soil. Data dealing with fresh shoot and root weights, dry shoot weight, shoot and root lengths, were recorded. Second stage juveniles (J_2s) were extracted from soil using sieving and modified Baermann technique (Goodey, 1957). Roots were stained in 0.01 acid fuchsin (Byrd et al., 1983) and examined for the developmental stages, females, galls, and egg-masses under stereomicroscope. Root galling or egg masses were rated on a scale of 0-5 where 0= no galls or egg masses, 1= 1-2 galls or egg masses, 2= 3-10 galls or egg masses, 3= 11-30 galls or egg masses, 4= 31-100 galls or egg masses, 5= more than 100 galls or egg masses per root system (Taylor and Sasser, 1978).

Chemical analysis

Phenolic contents estimation

Free, conjugated and total phenols were determined by mixing 1 ml of the sample extract with 0.25 ml HCL and boiled in a water bath for 10 minutes, then left to cool. One ml of the reagent folin-Denis and 6 ml Na_2Co_3, were added. The mixture was completed to final volume (10 ml) using distilled water. Color optical density of the reacted mixture was measured on absorbance spectrophotometer Miltonroy Spectronic 601 at 520 nm (Snell and Snell, 1953). Phenol content was determined as mg/g fresh weight/min.

Enzymes assay

Enzyme extracts were prepared following the method described by Maxwell and Bateman (1967). Dry root tissues (0.5 g) of each treatment were ground in 3 ml Na-

phosphate buffer at pH 6.8 in a mortar and then, centrifuged at 1.500 g / 20 min at 6 °C. The resultant supernatant fluids were processed for enzyme assays.

Polyphenol oxidase was assayed using photochemical method as described by Coseteng and Lee (1987). The reacted mixture was added as the following sequences: 2.7 ml potassium phosphate buffer 90.05M, pH 6.2, 0.25 ml of 0.25 M catechol, and 0.05 ml of enzyme extract. The increasing in absorbance at 420 nm was measured. One unit of enzyme activity is defined as the amount of the enzyme that causes an increase of 0.001 absorbance units per minute at 25°C.

Peroxidase was assayed using photochemical method as described by Amako et al. (1994). The reacted mixture was added as the following sequences, 1500 ml phosphate buffer, 480 ml hydrogen peroxidase, 1000 ml pyrogallol, and 20 ml sample extract. The increasing in the absorbance at 430 nm was recorded against blank with phosphate buffer instead of enzyme extract. One unit of enzyme activity was defined as the amount of the enzyme, which changing the optical density at 430 nm per min. at 25°C under standard assay conditions. Specific activity was expressed in units as mg/g/fresh weight/min

The activity of catalase was determined as described by Aebi (1974). Enzyme extract (0.1ml) was added to 2.9 ml of a reacted mixture containing 0.3M. H_2O_2 5% and 0.5M sodium phosphate buffer (pH 7.6). The activity of catalase was measured by monitoring the reduction in the absorbance at 240 nm as a result of H_2O_2 consumption. Catalase activity was expressed in unit as mg/g/fresh weight/min. One unit of enzyme activity was defined as the decomposition of 1µmol of H_2O_2 per min.

Data Analysis

The experiments were repeated twice (in two seasons) to confirm the results. Data obtained were subjected to statistical analysis of variance (ANOVA) for completely randomized design and randomized complete block design. The means were compared according to Duncan's multiple range tests at $P \leq 0.05$ (Duncan, 1955).

RESULTS

Evaluation of *Trichoderma harzianum* and *Bacillus subtilis* alone or in combination with ascorbic and salicylic acids on egg hatching and mortality of *Meloidogyne incognita in vitro*.

Data in Table (1) represented the effect of the ascorbic and salicylic acids as well as the *T. harzianum* and *B. subtilis* on egg hatching inhibition and juveniles mortality of *M. incognita*. All the tested treatments were found to cause significant inhibition in hatching and juveniles mortality percent to various extents. The dual treatment of Ascorbic acid + Salicylic acid sustained the highest and the most significant percentage of juvenile mortality (96.7 %) followed by the combination of *T. harzianum* + Salicylic acid (64.7%). However, the least percentage of mortalty was recorded with Ascorbic acid (21.0%) followed by *B. subtilis* (38.0%).

The previously mentioned treatments at three tested exposure periods showed nematicidal activity against egg hatching. Meanwhile, the dual treatment of *T. harzianum* + Salicylic acid achieved the highest percentage of egg hatching after 72 h (100 %), then the combination of *T. harzianum* + *B. subtilis* (97.0 %). The least one, Ascorbic acid recorded 36.0% after 72 h.

Table 1: Evaluation of *Trichoderma harzianum* and *Bacillus subtilis* alone or in combination with ascorbic and salicylic acids on egg hatching inhibition and mortality of *Meloidogyne incognita* after intervals (hrs.) *in vitro*

Treatments	Mortality %			Egg hatching inhibition %
	24hr	48hr	72hr	
Ascorbic acid	16.0	27.0	36.0	21.0
Salicylic acid	29.7	37.4	48.7	45.4
Ascorbic acid + Salicylic acid	33.0	52.0	84.0	96.7
B. subtilis	28.0	55.0	63.4	38.0
T. harzianum	30.4	47.0	68.0	44.0
B. subtilis + *T. harzianum*	34.7	69.4	97.0	52.7
B. subtilis + Ascorbic acid	13.4	37.0	44.7	37.4
B. subtilis + Salicylic acid	20.0	37.4	52.7	44.4
T. harzianum +Ascorbic acid	28.0	52.0	75.4	45.4
T. harzianum + Salicylic acid	34.0	75.0	100.0	64.7
Nematode alone (control)	0.0	0.0i	0.0	16.0

Each value represents the mean of four replicates.

Evaluation of *Trichoderma harzianum* and *Bacillus subtilis* alone or in combination with ascorbic and salicylic acids on *Meloidogyne incognita* criteria on sunflower plants under field conditions

Influence of *T. harzianum* and *B. subtilis* alone or in combination with ascorbic and salicylic acids against root- knot nematode, *M. incognita* infecting sunflower (cv. 162) was evaluated under open field conditions. Data presented in Table (2) revealed that the nematode criteria; numbers of root galls, developmental stages/root, number of nematodes in soil, number of eggs/egg mass, final population, and rate of nematode reproduction were significantly ($P<0.05$) reduced and nematode reduction was significantly increased ($P\leq0.05$) as compared with control. In addition, data showed that *M. incognita* population density whether in soil or roots was significantly suppressed with reproduction factor (Rf) ranging from 0.09 to 0.79 and 0.15 to 0.56 compared to nematode alone (Rf = 1.54 and 1.41) for the first and second seasons, respectively. Among screened treatments, *T. harzianum* combined with salicylic exhibited the greatest reduction (74.89 and 84.9%) followed by *T. harzianum* combined with *B. subtilis* (72.3 and 80.0%) and the dual treatment of two antioxidants, Ascorbic acid and Salicylic acid caused 70.0 and 76.1% for the first and second seasons, respectively. However, the least reductions in total nematode populations were recorded with *B. subtilis* + Ascorbic acid (48.5%) and Salicylic acid (59.6%) for the first and second seasons, respectively. Also, root galling of *M. incognita* was significantly reduced by all the tested treatments with root gall index (RGI) ranging from 3.0 to 3.5 and 0.66 to 3.0, for the first and second seasons, respectively. Similarly, nematode fecundity was significantly affected by certain treatments with egg masses index (EI) ranging from 2.0 to 3.3 and 0.0 to 3.0 for the first and second seasons, respectively. The highest percentages of reduction in root galling occurred by *T. harzianum* combined with Salicylic acid (65.2 and 65.5%) and *T. harzianum* combined with *B. subtilis* (60.9 and 71.0%) in root galling and *T. harzianum* combined with Ascorbic acid (64.3 and 76.0%) in egg masses reduction compared to control for the first and second seasons, respectively.

Table 2: Evaluation of *Trichoderma harzianum* and *Bacillus subtilis* alone or in combination with ascorbic and salicylic acids on *Meloidogyne incognita* criteria on sunflower plants under field conditions during two growing seasons, 2018 and 2019.

Treatments	Criteria of root-knot nematode											
	Soil	Root										
	2nd juveniles	Developstages	Females	Final population (Pf)	% Reduction	Reproduction factor	No. of galls	Red. %	Root gall index	No. of egg masses	Red. %	Egg masses index
First season (2018)												
Ascorbic acid	498.0de	2.7ce	17.0cd	517.7	66.4	0.52	30.0 b	34.8	3.5	27.0 a	3.6	3.3
Salicylic acid	451.0ef	4.0bc	19.0bc	474.0	69.2	0.47	30.0 b	34.8	3.0	25.0 ab	10.7	3.0
Ascorbic acid + Salicylic acid	448.0ef	3.0be	11.0f	462.0	70.0	0.46	29.0 bc	37.0	3.0	25.0 ab	10.7	3.0
B. subtilis	714.0bc	3.3be	13.0ef	730.3	52.6	0.73	24.0 bd	47.8	3.3	21.0 bc	25.0	3.0
T. harzianum	552.0d	4.0bc	10.0f	566.0	63.2	0.57	20.0 df	56.5	3.0	18.0 ce	35.7	3.0
B.subtilis + *T. harzianum*	414.0fg	2.7ce	10.0f	426.7	72.3	0.43	18.0 df	60.9	3.0	16.0 cf	42.9	3.0
B. subtilis + Ascorbic acid	767.0b	4.3ab	21.0b	792.3	48.5	0.79	23.0 ce	50.0	3.8	19.0 bd	32.1	2. 5
B. subtilis + Salicylic acid	646.0c	2.3de	16.0cd	664.3	56.8	0.66	23.0 ce	50.0	3.0	15.0 cf	46.4	2.8
T. harzianum +Ascorbic acid	509.0de	3.0be	12. 0f	524.0	66.0	0.52	22.0 cf	52.2	3.0	10.0 fi	64.3	2.0
T.harzianum + Salicylic acid	367.0gi	3.7bd	16.0de	386.7	74.9	0.39	16.0 fg	65.2	3.0	13.0 dg	53.6	2. 5
Nematode alone	1502.0a	5.3b	33.0a	1540.3	0.0	1.54	46.0 a	0.0	4.0	28.0 a	0.0	3.3
Second season (2019)												
Ascorbic acid	399.0 d-g	6.0ab	29.0 b	447.0	67.8	0.45	21.0bc	41.0	3.0	13.0c	48.0	3.0
Salicylic acid	517.0 cd	3.0dg	27.0 bc	560.0	59.6	0.56	18.0cd	49.1	3.0	13.0c	48.0	3.0
Ascorbic acid + Salicylic acid	299.0 dh	4.0be	21.0 ce	331.0	76.1	0.33	12.0f	65.5	3.0	7.0de	73.2	2.0
B. subtilis	476.0 c-f	2.0eh	18.0 df	505.0	63.6	0.51	14.0ef	60.1	3.0	9.0de	64.0	2.3
T. harzianum	399.0 dg	8.0a	21.0 ce	436.0	68.6	0.44	12.0f	65.5	3.0	8.0de	68.0	2.3
B.subtilis+*T. harzianum*	253.0 eh	4.0be	15.0dh	278.0	80.0	0.28	10.0f	71.0	2.0	6.0ef	76.0	2.0
B. subtilis + Ascorbic acid	238.0 fh	6.0ab	21.0ce	283.0	80.0	0.28	23.0b	35.5	3.0	18.0 b	29.2	3.0
B. subtilis + Salicylic acid	395.0 dg	2.0eh	19.0de	426.0	69.3	0.43	17.0de	51.9	3.0	10.0d	61.2	2.0
T.harzianum +Ascorbic acid	391.0 dg	4.0be	11.0fi	412.0	70.3	0.41	12.0f	51.9	3.0	6.0de	76.0	2.0
T.harzianum+ Salicylic acid	176.0 gi	5.0bd	20.0de	210.0	84.9	0.21	17.0de	65.5	3.00	9.0de	64.0	2.3
Nematode alone (Control)	1309.0 a	6.0ab	47.0 a	1387.0	0.0	1.41	36.7a	0.0	4.00	25.0a	0.0	3.0

Each value represents the mean of four replicates. Initial population = 1000 second stage juveniles of *M.incognita*. Means in each column followed by the same letter(s) did not significantly different at (P≤0.05) according to Duncan multiple range test - .

Reproduction factor (Rf) = Final population /Initial population.Nematode final population (Pf) = [Egg masses no. /root × Eggs no. / Egg masses] + [Developmental stages/root] + [Second stage juveniles /soil] + [Adult females/root]. Root gall index (RGI) or egg masses index (EI) was determined according to the scale given by Taylor and Sasser (1978).

Evaluation of *Trichoderma harzianum* and *Bacillus subtilis* alone or combination with ascorbic and salicylic acids on vegetative characteristics of sunflower infected with *Meloidogyne incognita* under field conditions

Effects of *T. harzianum* and *B. subtilis* alone or combination with ascorbic and salicylic acids on vegetative characteristics of sunflower (cv. 162) infected with *M. incognita* under field conditions, were estimated under open field conditions. Data Tables (3 and 4) revealed that, all of the tested treatments recorded significant increases of plant growth characteristics ($P \leq 0.05$) and decreased the negative effects of nematodes on plants compared control.

Table 3: Evaluation of *Trichoderma harzianum* and *Bacillus subtilis* alone or in combination with ascorbic and salicylic acids on of growth parameters of sunflower infected with *Meloidogyne incognita* under field conditions.

Treatments	Increase% in growth parameters					
	Shoot increase%		Root increase%		Increase in Total fresh weight %	Increase in Shoot dry weight %
	Length	Weight	Length	Weight		
Ascorbic acid	18.17	38.140	63.8	62.62	28.7	55.7
Salicylic acid	23.61	45.161	25.8	7.01	40.3	65.3
Ascorbic acid+ Salicylic acid	26.50	51.992	12.5	57.01	48.7	75.4
B. subtilis	18.75	23.150	3.3	42.06	21.0	25.7
T. harzianum	65.28	60.152	2.5	43.93	55.8	63.5
B. subtilis + *T. harzianum*	45.49	37.761	37.5	60.75	35.4	46.1
B. subtilis + Ascorbic acid	12.62	44.972	43.8	53.27	41.9	61.7
B. subtilis + Salicylic acid	37.38	53.131	35.4	51.40	49.5	63.5
T. harzianum + Ascorbic acid	15.74	38.140	40.0	49.53	35.4	35.9
T. harzianum + Salicylic acid	46.76	68.311	25.4	43.93	63.5	99.4

Each value represents mean of four replicates. The increase was calculated as a percentage of the control.

Table 4: Evaluation of *Trichoderma harzianum* and *Bacillus subtilis* alone or in combination with ascorbic and salicylic acids on growth parameters of sunflower infected with *Meloidogyne incognita* under field conditions during growing season, 2019.

Treatments	Increase% in growth parameters					
	Shoot increase%		Root increase %		Increase in Total fresh weight %	Increase in Shoot dry weight %
	Length	Weight	Length	Weight		
Ascorbic acid	3.34	45.06	44.75	152.54	45.7	53.0
Salicylic acid	11.08	47.33	24.66	58.47	45.8	84.6
Ascorbic acid + Salicylic acid	28.84	52.67	18.27	114.41	34.4	91.3
B. subtilis	8.54	24.90	0.46	60.17	24.4	41.6
T. harzianum	34.45	58.64	10.51	65.25	56.8	69.7
B. subtilis + *T. harzianum*	34.58	46.50	30.59	143.22	46.9	56.3
B. subtilis + Ascorbic acid	8.01	50.00	41.09	107.63	49.5	63.4
B. subtilis + Salicylic acid	31.38	60.91	28.31	85.59	59.5	81.6
T. harzianum +Ascorbic acid	14.55	44.86	36.71	80.51	43.9	77.3
T. harzianum + Salicylic acid	53.40	63.17	22.84	68.64	61.3	130.7

Each value represents mean of four replicates. The increase was calculated as a percentage of the control.

The obtained results indicated that the treatments, *T. harzianum* as well as *B. subtilis* combined with salicylic acid showed remarkable improvement in total plant fresh weight as well as dry shoot weight of infected sunflower. However, the dual treatment of *T. harzianum* + salicylic acid appeared to be the most effective in increasing total plant fresh weight (63.5 and 61.3%) and shoot dry weight (99.4 and 119.0%) compared to control during the two growing seasons 2018 and 2019, respectively. Using *B. subtilis* alone, the lowest values in fresh and dry shoot weights were noticed

Evaluation of *Trichoderma harzianum* and *Bacillus subtilis* alone or in combination with ascorbic and salicylic acids on phenolic contents and oxidative enzymes of sunflower infected with *Meloidogyne incognita* under field conditions.

As a clear indicator of plant response to previous treatments under nematode invasion, data indicated that phenolic contents were significantly affected by the tested bioagents and antioxidants on sunflower during the two growing seasons, 2018 and 2019. Results in Table (5) indicated that, phenolic contents including the free, conjugated, and total phenols were noticeably higher in some treated plants than the control during the two growing seasons, 2018 and 2019. The highest total phenolic contents were induced by combination of *T. harzianum* + salicylic acid treatment, which recorded 12.49 and 12.81 in the two seasons, respectively. This is consistent with the results of vegetative growth and nematode criteria.

Concerning formation of oxidative enzymes in sunflower, the obtained data in Table (6) indicated that the lowest values of oxidative enzyme were detected in control. Generally, the two antioxidants and the two bioagents either individually or combined succeeded to increase enzymes activity, In this respect, the highest activities in all determined enzymes were induced by mixture of *T. harzianum* + salicylic acids compared to other treatments during the two growing seasons 2018 and 2019. When bioagents were evaluated regarding oxidative enzyme. *T. harzianum* showed the highest effect than *B. subtilis* on peroxidase (PO), catalase and polyphenoloxidase (PPO) during the two growing seasons 2018 and 2019.

DISCUSSION

Generally, the average rate of nematode suppression found in this study was consistent with previous studies (Molinari and Miacola, 1997; Henkle-Dührsen and Kampkötter, 2001; Mahgoob and Zaghlool, 2002; Goswami and Singh, 2004). Application of *T. harzianum* revealed the most significant biocontrol activity against root-knot nematode *M. incognita in vitro* and *in vivo*. Toward improving the biocontrol strategy, we studied its effect on root-knot nematode under field conditions of Egypt. In our results, *T. harzianum* and *B. subtilis* as bioagents represented the most effective treatments for suppression of nematode *in vitro* and *in vivo* either alone or in combination with antioxidants. These results agreed with those obtained by Spiegel and Chet (1998) who reported that reducing egg production of the root-knot nematode occurred by soil treatments with *T. harzianum*. Also, Goswami et al. (2008) mentioned that this effect may be due to fewer nematodes were able to penetrate the roots in treated soil by *T. harzianum*. This was noted by Khan and Haque (2011) who suggested the ability of *T. harzianum* effects may be occurred also through antibiosis production (Nielson et al., 1998), siedorphore production (Glick, 1995), production of phytohormones and induced systemic resistance (Kloepper et al., 1992) or other compounds with some nematicidal proprieties (Marek and Skorupska, 2001; Leonetti et al., 2017).

Table 5: Evaluation of *Trichoderma harzianum* and *Bacillus subtilis* alone or in combination with ascorbic and salicylic acids on phenolic content of sunflower infected with *Meloidogyne incognita* under field conditions during the two growing seasons 2018 and 2019.

Treatments	Phenol components (mg/g fresh weight/min)					
	First season (2018)			Second season (2019)		
	Total phenols	Conjugated phenols	Free phenols	Total phenols	Conjugated phenols	Free phenol
Ascorbic acid	5.98g	2.67h	3.32ab	5.10h	3.35fg	1.74 de
Salicylic acid	8.78e	6.37d	2.41efg	5.68gh	3.12fgh	2.56abc
Ascorbic acid + Salicylic acid	10.78cd	8.22c	2.56ab	10.41de	8.00c	2.42bc
B. subtilis	6.63ef	4.45f	2.18fg	6.77g	4.63f	2.03d
T. harzianum	10.75cd	8.10cd	2.65cde	8.93f	6.62de	2.31bcd
B. subtilis + *T. harzianum*	12.69c	9.69bc	3.00c	13.40b	10.87b	2.54abc
B. subtilis + Ascorbic acid	9.00d	5.98de	3.02c	9.39e	6.94d	2.44c
B. subtilis + Salicylic acid	8.94de	5.15def	3.80a	9.04ef	6.34def	2.69ab
T. harzianum +Ascorbic acid	12.49b	9.82b	2.68cd	12.81c	10.44bc	2.37bcd
T. harzianum + Salicylic acid	15.69a	12.56a	3.13bc	15.12a	12.32a	2.80a
Control	4.49h	3.00gh	1.48g	3. 63e	2.24 h	1.39e

-Means in each column followed by the same letter(s) did not differ at $P \leq 0.05$ according to Duncan`s multiple range test.
- Each value represents the mean of four replicates.

Table 6: Evaluation of *Trichoderma harzianum* and *Bacillus subtilis* alone or in combination with ascorbic and salicylic acids on oxidative enzymes of sunflower infected with *Meloidogyne incognita* under field conditions during the two growing seasons 2018 and 2019.

Treatments	The oxidative enzymes (mg/g fresh weight/min)					
	First season (2018)			Second season (2019)		
	Peroxidase	Catalase	Polyphenol oxidase	Peroxidase	Catalase	Polyphenol oxidase
Ascorbic acid	0.693de	1.15a	0.025c	0.030cd	1.380a	0.84g
Salicylic acid	1.196cd	1.18a	0.027c	0.030cd	1.416a	1.44bc
Ascorbic acid + Salicylic acid	1.250c	1.28a	0.046j	0.062bc	1.536a	1.50b
B. subtilis	0.583def	1.23a	0.053ab	0.064bc	1.476a	0.69h
T. harzianum	1.340bc	1.24a	0.058ab	0.060bc	1.488a	1.61ef
B. subtilis + *T. harzianum*	1.626ab	1.31a	0.075ab	0.090b	1.572a	1.95e
B. subtilis + Ascorbic acid	1.173cd	1.27a	0.046b	0.060bc	1.524a	1.41bc
B. subtilis + Salicylic acid	1.043d	1.29a	0.047b	0.056bc	1.548a	1.26c
T. harzianum + Ascorbic acid	1.612ab	1.28a	0.056ab	0.060bc	1.536a	1.94e
T. harzianum + Salicylic acid	1.711a	1.33a	0.082a	0.094a	1.596a	2.05a
Control	0.263f	1.05a	0.001d	0.000d	1.260a	0.32d
L.S.D.	0.981	0.920	0.101	0.120	1.104	1.177

Means in each column followed by the same letter(s) did not differ at $P \leq 0.05$ according to Duncan`s multiple range test.
Each value represents the mean of four replicates.

According to Lindberg (1981), *Bacillus* effects may be due to the nematocidal volatile products produced by this bacterium and characterized to include mainly the benzene acetaldehyde, 2-nonanone, decanal, 2-undecanone and dimethyl di-sulphide, which were active against *M. incognita* juveniles. The mechanisms by which reduction on nematode population occurred might be due to premature egg hatching and reduction in viability and mortality of juveniles which induced by non-cellular extract (filtrate) and toxic metabolites like bacillo-peptidase, subtillin and a lactamase from *Bacillus* spp. El-Sherif et al. (1994) reported that the cultures of bacteria inhibited hatching of *M. incognita* and were toxic to juveniles. Niknam and Dhawan, (2002) evaluated *B. subtilis* for its efficacy against *M. incognita* which inhibited hatching and caused mortality of immature nematodes by their cell free culture filtrates. Nagesh et al. (2005) confirmed the *B. subtilis* which produced antibiotics, and 2, 3-DHBG. This antagonist acts through antibiosis, secretion of volatile toxic metabolites, destructive enzymes, and competition for space and nutrition. Numerous antifungal metabolites are produced by bacteria that act against nematodes both *in vitro* and *in vivo* (Farkas and Kiraaly, 1962). These include bacillomycin, iturin, surfactin, mycosubtilin, bacilysin, fengymycin, mycobacillin, ammonia, butyrol actones, 2, 4- diacetyl phloroglucinol, kanosamine, oligomycin, oomycin, phenaz ine-1-carboxylic acid, pyoluterin, pyrrolnitrin, viscosinamide, xanthobaccin and zwittermycin (Mahadtanapuk et al., 2007). Ruiz et al., (2014) suggested that the cell-free culture filtrate of *B. subtilis* might contain toxic metabolites against *M. incognita*. Zaghloul et al. (2015) reported the high production of certain enzymes i.e. protease, chitinase and gelatinase by *B. subtilis* which suppressed nematodes. Chitinases from micro-organisms is a potential weapon for the control of root-knot nematodes, (the chitinolytic bacteria), because the nematode egg shell cuticle is containing of a chitin layer.

On other hand, the combination of bioagents + antioxidants (amino acids) enhanced the bioagents to reduce the ability of nematodes to develop, which were in line with Tanda et al.(1989) who reported that inhibitory effect on egg hatching and juvenile mortality in root-knot nematode, *M. incognita* on sesame root exudates *in vitro* due to their containment of amino acids as antioxidants. A similar was founded by Lee et al. (2014) and Cronin et al., 1997) where noted that with increasing concentration of acids, the egg hatching proportionally decreased. Some previous studies explained the action mode of antioxidants affecting nematodes and their reflection on the general health of plants (Ryan, 2000; Gupta et al., 2017; Labudda, 2018). The effect of antioxidants on *M. incognita* may be due to direct toxicity or the nematodes (Perry and Clarke, 1981); affected by the pH and/or osmotic pressure of the solution. (Ahmed and Khan, 1964) or may interfere with essential pathways of intracellular metabolism and enzymes in nematodes (Crow et al., 2009) or as Cooper et al. (2005) who added that the activation of signaling compounds of induced resistance promoted expression of plant defenses such as proteinase inhibitors and polyphenol oxidase, as well as volatile organic compounds that attract parasitoids of herbivores (Ryan, 2000).

CONCLUSION

The combination of bioagents, *T. harzianum* and *B. subtilis* with antioxidants, ascorbic and salicylic acids which evaluated in current study under field conditions, showed the feasibility of integration of biological control agents in the management of nematodes in sunflower crop. This synergistic method can reduce the main problems

encountered in the nematodes control and reduce environmental impacts from chemical control.

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الملخص العربي

تأثير التكامل بين عوامل المكافحة الحيوية ومضادات الأكسدة في الحد من الاضرار التي تسببها نيماتودا تعقد الجذور لنباتات عباد الشمس

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تعتبر نيماتودا تعقد الجذور واحدة من أكثر أنواع النيماتودا النباتية شيوعًا و التي تصيب محصول عباد الشمس فى مصر. و في هذا السياق ، اجريت الدراسة الحالية لمعرفة كيفية تعزيز تأثير المكافحة الحيوية للنوع *Meloidogyne incognita* بواسطة *Trichoderma harzianum* و *Bacillus subtilis* بمفردهما أو بالاشتراك مع مضادات الأكسدة (حمضى الأسكوربيك والسلسليك) كاستراتيجية محسنة للمكافحة الحيوية. بشكل عام. وقد أظهرت النتائج قدرة هذه الاستراتيجية على تثبيط أعداد النيماتودا مقارنةً بالكنترول ، حيث اوضحت النتائج ان الدمج بين *T. harzianum* و *B. subtilis* سجل أعلى انخفاض معنويا في تعداد النيماتودا (72.3٪ و 80.0٪على التوالى). بينما لوحظ أفضل تأثير تآزري عند الجمع بين *T. harzianum* وحمض السلسليك (74.9٪ و 84.9٪). كما انعكس هذا التأثير إيجابيا على نمو النبات من خلال حث انتاج المركبات المناعية للنبات (المحتوى الفينولي والإنزيمات المؤكسدة).