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Microbial induction of resistance in tomato against root-knot nematode Meloidogyne javanica with biocontrol agents

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Abstract

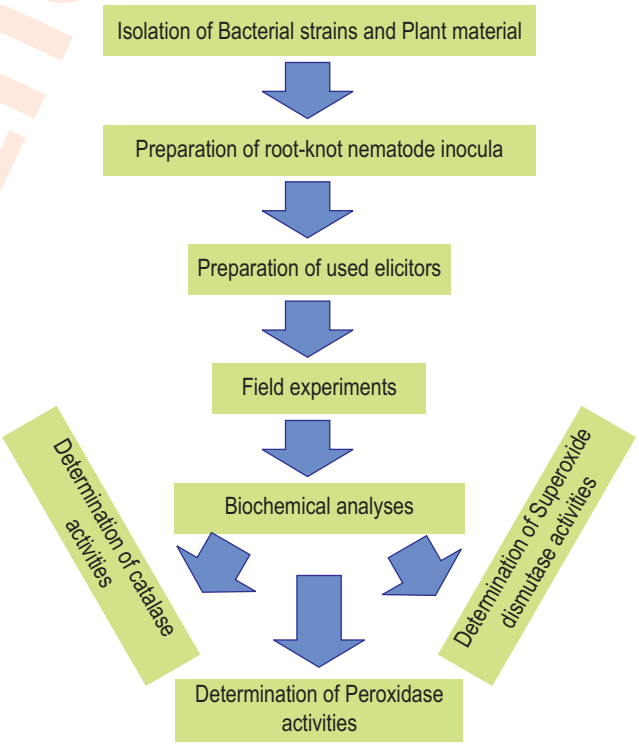
Aim: To investigate the effect of some bacterial and algal strains on induction of resistance against root-knot nematode (*Meloidogyne javanica*) in tomato (*Solanum lycopersicum* L.) roots.

Methodology: Application of *Bacillus subtilis, Serratia marcescens,* and *Spirulina platensis* on root area of nematode infected tomato plant, the number invading nematode, *M. javanica*, galls and egg masses in tomato roots were determined.

Results: The reduction percentage (94.97%) of developmental stages of root-knot nematode was highest in *S. platensis* treatment, among the biological agents and compared to 97.48 % in chemical pesticide Vydate treatment. The highest reduction percentage (90.43%) in female numbers was recorded with *B. subtilis*. All tested bioagents significantly increased vegetative weight of tomato plants. *B. subtilis, S. marcescens* and *S. platensis* increased the activity of peroxidase, polyphenol oxidase, superoxide dismutase and catalase in tomato plants infected with *M. javanica*.

Interpretation: Biological control of nematodes using alga and bacteria that could potentially enhance plant health, productivity and promotes its growth.

Key words: *Bacillus subtilis,* Biological control, Root-knot nematode, *Serratia marcescens, Spirulina platensis,*



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Introduction

Root-knot nematodes, *Meloidogyne* spp. are considered the most serious pathogens to be causing 5% loss to all economic crops of the world (Jones *et al*., 2013). 10% of all nematode species are plant parasites, 50% are free living species that feed on other organisms in marine water, 25% of nematodes inhabit freshwater or soil, and 15% are zoo parasitic (Adam *et al*., 2014). Approximately, two thousand plant species have been registered as host to root-knot nematodes, and most of cultivated crops are attacked by at least one root-knot nematode specie (Abd-Elgawad and Askary, 2018). Root knot nematode is one of the major pathogens of tomato worldwide, causing stunt in growth and decrease in fruit production (Sikora and Fernandez, 2005). The use of chemical nematicides has been the main agents to control plant parasitic nematodes over the past five decades (Dela *et al*., 2014). However, due to the adverse environmental impacts associated with application of chemicals in agriculture directed the researchers to use safe methods for biological control of parasitic nematode (Dela *et al*., 2014; Abd-Elgawad *et al.,* 2010; Gawade *et al.,* 2017).

Several biological control strategies, such as host plant resistance, rotation with non-hosts, drainage and avoidance, destruction of residual crop roots, and wise use of nematicides, are considered as effective means for controlling root knot nematode (Abd-Elgawad *et al.,* 2010; Elhady *et al.,* 2017).

Induced resistance is accomplished by the inoculating plant with a mild virulent or non-pathogenic isolate (Topalović, and Heuer, 2019). Plant growth promoting rhizobacteria (PGPR) is considered as an alternative method to biological control and promoted plant growth (Cao *et al.,* 2015; Cordovez *et al.,* 2018). The potentiality of PGPR in agriculture is steadily increased as it offers an attractive way to replace the use of chemical fertilizers, pesticides and other supplements. Growth promoting substances are likely to be produced in large quantities by these rhizosphere microorganisms that indirectly influence the overall morphology and health of the plants.

Rhizobacteria eliminate parasitic nematodes through induction of plant systemic resistance (Hasky-Gunther *et al*., 1998), change nematode behavior (Bhattacharyya and Jha, 2012) and activating plant growth (El-Nagdiand Youssef, 2004; Karlidag *et al*., 2007). Additionally, cyanobacteria or blue-green algae produce large number of compounds with various biochemical activities. Holajjer *et al*. (2013) demonstrated that were used as test plants, cyanobacterial extracts inhibit hatching and induce immobility and mortality in plant parasite nematodes; moreover, its use in soil may decrease nematode infection and increase plant productivity.

In view of the above, the present study was undertaken to investigate the effect of bio-control agents like *Bacillus subtilis, Serratia marcescens,* and *Spirulina platensis* on, *Meloidogyne* spp., a root knot nematode infecting tomato roots.

Materials and Methods

Bacteria and algal strains source, preparation, inducers and elicitors : Two bacterial strains, *Bacillus subtilis*, *Serratia marcescens* and cyanobacterial strain, *Spirulina platensis* were selected as bio-control agents and were obtained from Agricultural Gene Bank, Ain Shams University, Egypt and cultured on Zarrouk medium (Nikoo, 2014). Bacterial strains were isolated the rhizosphere healthy tomato plants Menoufia University Farm and identified using 16S rDNA according to Hassan and Ismail, (2014) (Data not show). Cyanobacterial strain was also obtained from the Agricultural Gene Bank, Ain Shams University, Egypt and cultured on Zarrouk medium (Nikoo, 2014). Vydate 24% L.; methyl 2-(dimethylamino)-N-(methyl carbamoyloxy)-2-oxoethanimidothioate (DuPont™ Vydate®) a nematicide and insecticide of group 1A organocarbamates was used chemical treatment. Four-week-old tomato seedlings (*Solanum lycopersicum*. cv. Beto) were

The culture of *Meloidogyne javanica* was prepared from a single egg mass of adult female and identified by the morphological characteristics of female perineal pattern as described by Taylor and Sasser (1978). Eggs of nematodes were extracted from identified tomato roots using 0.5% sodium hypochlorite (Hussey and Barker, 1973). Roots were cut into small pieces and macerated for 10 sec with a electric blender, shaken for three min to release eggs from the gelatinous matrix. Eggs were collected on the sieve (400 mesh) and washed several times with tap water to remove the residual of NaOCl, and transferred to a flask in tap water for counting. Root-knot nematode was applied by adding aqueous suspension of approximately 2000 eggs around the root zone.

Greenhouse experiment: Five tomato seedlings were planted in a 20 cm diameter plastic pots filled with autoclaved sandy loam soil (1:1). Biotic elicitors (*Bacillus subtilis*, *Serratia marcescens*, and *Spirulina platensis*) (5 ml of 10^9 CFU ml^{-1}) were added at the time of planting. All pots were inoculated with nematode by adding aqueous suspension of approximately 2000 eggs around the root zone and left until the end of experiments. Control was treated only with 2000 eggs of *M. javanica*, furthermore five tomato pots without nematode were used for more confirmation. Five replicates of each treatment were prepared. Experiment was designed in a complete randomized block and kept in the greenhouse at 25± 5ºC, pots were irrigated, and fertilized when required. Seventy days after nematode inoculation , tomato plants were carefully uprooted . Roots were washed by water and stained in phloxine B stain ; Root knot nematode galls and egg masses were extracted and counted per gram root weight. Reduction percentage of root knot nematode stages , galls and egg masses was calculated.

Nematode stages extraction and counting: Egg masses of *M. javanica* were stained by dipping the root system in phloxine B solution (0.15 g l^{-1} tap water) for 20 min (Daykin and Hussey, 1985). Phloxine B primarily stains the gelatinous egg-mass and naked viable eggs were counted. Roots from each replicate were placed in hot acid fuchsin lactophenol, at least for 24 hr, then transferred to Petri dish and examined under dissecting microscope for counting the developing stages and females of *M. javanica*. Soil sample of 200 cm^3 of each treatment were processed to nematode extraction in modified Baermann funnels for 72 hrs. Second stage juveniles of *M. javanica* were counted using Hawksely counting slide under dissecting stereomicroscope. The length and weight of dry shoots and roots each plant were measured from each treatment.

Biochemical analyses: Terminal buds in addition to first and second young leaves were used for estimation of peroxidase, polyphenol oxidase, superoxide dismutase and catalase activities. In this regard, 2 g of plant materials were homogenized with 10 ml of phosphate buffer (pH 6.8), then centrifuge at 2°C for 20 min at 20000 rpm in a refrigerated centrifuge. The clear supernatant (containing enzymes) was taken as the source of enzymes (Misra and Fridovich, 1972).

Peroxidase activity was assayed by using solution containing 5.8 ml of 50 mM phosphate buffer (pH 7), 0.2 ml of enzyme extract and 2 ml of 20 mMH_2O_2 after adding 2 ml of 20 mM pyrogallol, the rate of increase in absorbance as pyrogallol was determined spectrophotometrically within 60 sec at 470 nm and 25ºC (Bergmeyer, 1974). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the conversion of one micromole of H_2O_2 per minute at 25ºC (Kong *et al*., 1999).

Polyphenoloxidase activity was assayed using 125 µmol. of phosphate buffer (pH 6.8), 100 µmol pyrogallol and 2 ml of enzyme extract according to Kar and Mishra (1976). Boiled enzyme extract was used as blank sample, the color was measured at 430 nm and enzyme activity was measured as change in the optical density g^{-1} f. wt. hr^{-1}.

The activity of superoxide dismutase was measured by the inhibition degree of auto-oxidation of pyrogallol according to Marklund and Marklund (1974). A unit of enzyme activity is the amount of enzyme that results in 50% inhibition of auto-oxidation rate of pyrogallol at 25ºC (Kong *et al*., 1999).

Catalase activity was assayed according to Chen *et al*. (2000). Catalase activity is equal to the change in hate of H_2O_2 absorbance in 60 sec with a UV- spectrophotometer at 250 nm. A unit of enzyme activity is equal to the amount of enzyme that reduces 50% of H_2O_2 in 60 sec at 25 ºC (Kong *et al.,* 1999).

To assay the enzyme, volume at zero time was considered as blank and the activity of enzyme was calculated by the equation: (Δ × T v × 60 min) / (t × v × f.wt.), where Δ is the absorbance of the sample after incubation minus that at zero time; T v is the total volume of filtrate; t is the time (min.) of incubation with substrate and v is the volume of filtrate used for incubation (Fick and Qualset, 1975).

Statistical analysis: The difference between means of studied treatments were tested by ANOVA at 5 % probability level. A post hoc (Duncan 's Multiple Range and LSD) Tests was applied using CoStat software program (Version 6.400).

Results and Discussion

Root-knot nematodes are dangerous pest for many economic crops. (Abd-Elgawad and Askary, 2018). Biological control of plant parasitic nematodes is the best choice for human health and safe environment (Gawade *et al.,* 2017). Nematophagous fungi and bacteria are widely used among the nematode-antagonistic organisms (Askary and Martinelli, 2015); where they include tolerant genera to sun heat, desiccation and are not influenced by the regular use of agricultural chemicals (Adam *et al*., 2014; Stevens and Lewis 2017; Topalović and Heuer, 2019). In the present study, application of bio-control agents, *B. subtilis, S. marcescens* and *S. platensis* significantly decreased the number of *M. javanica* and galls in tomato roots (Table 1). Statistical analysis showed significant differences in gall numbers between all treatments (5-48 galls) as compared to the control (87 galls). Reduction percentages in root gall numbers were highest (94.25%) in Vydate treatment, followed by 79.31 % in *B. subtilis* and 68.97 % in *S. platensis* treatment, while it was only 44.83 % in *S. marcescens* treatment, respectively. The number of egg masses significantly varied among all treatments (1-15) compared to control (50). The highest percentage was recorded in Vydate pesticide (98 %), followed by 84 % in *B. subtilis,* 76% in *S. platensis* and 70% in *S. marcescens* treatments. There are different modes of action of bacteria against plant parasitic nematodes. The microbial control of nematode plant parasites include gelatinase, protease and chitinase activities that affect nematodes (Bahloul, 2013). Some nematophagous mycelial endospore-forming bacteria of genus *Bacillus* have protease genes and can infect nematodes by adhering their spores to nematode cuticle (Tian et al., 2007). *B. subtilis* has genes producing antibiotics such as surfactin and iturin, however, *S. marcescens* produces toxic metabolites that affect parasitic nematodes (Abd-Elgawad and Askary, 2018). *Bacillus* is considered as plant growth promoting rhizobacteria and microbial control agent for nematodes (Dong and Zhang, 2006).

The root gall index was highest with *S. marcescens* followed by in *B. subtilis* and *S. platensis* treatments as compared to the control. On the other side, egg mass index was 3.0 with *S. marcescens* and *S. platensis* and 2.0 with *B. subtilis* treatments as compared to the control (4.0). The effect of *B. subtilis*, *S.*

Table 1 : Effect of *B. subtilis, S. marcescens*, and *S. platensis* on galls and egg-masses of *M. javanica* infecting tomato plants under greenhouse conditions

Treatments	Average number of galls g^{-1} roots	Reduction %	Root gall index	Average number of egg mass g^{-1} roots	Reduction %	Egg mass index*
B. subtilis	18.0 [d]	79.31	3	8.0 [c]	84.0	2
S. marcescens	48.0 [b]	44.83	4	15.0 [b]	70.0	3
S. platensis	29.0 [c]	68.97	3	12.0 [bc]	76.0	3
Vydate 24%L	5.0 [e]	94.25	2	1.0 [d]	98.0	1
Control (Nematode only)	87.0 [a]	-	5	50.0 [a]	-	4
LSD5%	9.4	-	-	5.0	-	-

*where 0 = no galls or egg masses; 1 = 1 to 2; 2 = 3 to 10; 3 = 11 to 30; 4 = 31 to 100; and 5 = more than 100 galls or egg masses (Taylor and Sasser, 1978)

Table 2 : Effect of *B. subtilis, S. marcescens,* and *S. platensis* on different stages of *M. javanica* infecting tomato plants under greenhouse conditions

Treatments	Second stage juveniles 250 g^{-1} soil		Developmental stages g^{-1} root		Females g^{-1} root	
	Average number	Reduction %	Average numbers	Reduction %	Average numbers	Reduction %
B. subtilis	89.0 [d]	80.31	10.0 [c]	93.71	9.0 [c]	90.43
S.marcescens	188.0 [b]	58.41	12.0 [b]	92.45	17.0 [b]	81.91
S. platensis	142.0 [c]	68.58	8.0 [bc]	94.97	14.0 [bc]	85.11
Vydate 24%L	25.0 [e]	94.47	4.0 [d]	97.48	2.0 [d]	97.87
Control (Nematode only)	452.0 [a]	-	159.0 [a]	-	94.0 [a]	-
LSD 5%	27.1	-	2.4	-	5.8	-

marcescens and *S. platensis* on different stages of *M. javanica* infecting tomato plants under greenhouse conditions is shown in Table 2. As compared to control, the number of second stage juveniles were as follows: 188 individuals in *S. marcescens,* 142 individuals in *S. platensis* and 89 individuals in *B. subtilis* treatments respectively.

Percentage of second stage juveniles of root knot nematode was highly reduced (80.31%) in *B. subtilis*, 68.58 % in *S. platensis,* 58.41 % in *S. marcescens* and 94.47 % in Vydate pesticide treatments. The number of developmental stages of root knot nematode significantly varied among tested materials and control; the lowest number of individuals was recorded in *S. platensis* (8.0), followed by *B. subtilis* (10.0) and *S. marcescens* (12.0) treatments as compared to control (150.0). The highest reduction percentage of developmental stages of root knot nematode was recorded in *S. platensis* (94.97%), followed by *B. subtilis* 93.71 %, *S. marcescens* 92.45 % and Vydate pesticide 97.48 % treatments. This could be due to the toxic metabolites produced by the bacteria (Abd-Elgawad and Askary, 2018) or endospores that attach to the cuticle of second stage juvenile (Tian *et al.,* 2007), however, vydate acts as cholinesterase inhibitor for nematodes (Kesba and Al-Shalaby, 2008).

The number of root knot nematode females per gram root differed significantly between different materials and control. It was 17 females in *S. marcescens*, 14 females in *S. platensis* and 9 females in *B. subtilis* treatments as compared to 94 females in control. The highest reduction percentage in female number was recorded in *B. subtilis* (90.43 %), followed by *S. platensis* (85.11%), *S. marcescens* treatments (81.91%). The effect of *B. subtilis, S. marcescens,* and *S. platensis* treatments on vegetative weights of infected tomato plants showed significant differences in measurements between tested materials and control with or without nematodes (Table 3). The lowest decrease in percentage of shoot weight was recorded in *S. platensis* (14.2 %), followed by *S. marcescens* (25.8 %) and *B. subtilis* treatments (36.4 %) as compared to control (51.9 %) with nematode only. Regarding to root weight, the lowest decrease percentage was recorded in *S. platensis* (12.7 %), followed by *S. marcescens* (22.8 %) and *B. subtilis* (25.5 %) treatments as compared to the control (44.9 %) with nematode only. On the other side control without nematode recorded the highest dry shoot and root weights as it is not infected and with better nutrient intake leading to healthy growth.

The results of the present study on the effect of some bacterial strains and cyanobacteria on the number of *M. javanica* juveniles, their developmental stages and galls in tomato roots is in accordance with the reports Saad *et al*. (2010) who used biological control agents like *Pseudomonas fluorescens*, *Trichoderma harzianum* and their mixture. Mahfouz *et al*. (2010) used carbofuran, *S. marcescens* and three *T. harzianum* isolates; for controlling the population density of *M. incognita* infecting

Table 3 : Effect of *B. subtilis, S. marcescens* and *S. platensis* on shoot and root weights of tomato plants infected with *M. javanica* under greenhouse conditions

Treatment	Shoot dry weight (g)	Percent decrease	Root dry weight (g)	Percent decrease
B. subtilis	54.0 [d]	36.4	40.8 [c]	25.5
S.marcescens	63.0 [c]	25.8	42.3 [c]	22.8
S. platensis	72.8 [b]	14.2	47.8 [bc]	12.7
Vydate 24%L	78.8 [ab]	7.1	50.1 [ab]	8.6
Control (Nematode only)	40.8 [e]	51.9	30.2 [d]	44.9
Check (No nematode)	84.8 [a]	-	54.8 [a]	-
LSD 5%	6.4	-	6.8	-

Table 4 : Effect of *B. subtilis, S. marcescens* and *S. platensis* on the enzyme activity (unit g^{-1} f.wt. hr^{-1}) of peroxidase, polyphenoloxidase, superoxide dismutase and catalase in tomato plants infected with *M. javanica* under greenhouse conditions

Treatment	Peroxidase	Polyphenoloxidase	Superoxide-dismutase	Catalase
B. subtilis	5.24 [a]	1.46b [c]	2.81 [c]	1.21 [ab]
S. marcescens	5.17 [a]	1.78 [a]	2.92 [bc]	0.98 [bc]
S. platensis	4.03 [b]	1.67 [ab]	3.22 [ab]	0.87 [c]
Vydate 24%L	3.42 [c]	1.31 [cd]	3.36 [a]	1.43 [a]
Control (Nematode only)	3.49 [c]	1.09 [d]	2.77 [c]	0.81 [c]
Check (No nematode)	1.57 [d]	0.73 [e]	0.81 [d]	0.12 [d]
LSD 5%	0.24	0.25	0.32	0.23

All values are means of unit g^{-1} fresh leaves per hour

tomatoes. All the treatments enhanced the plant growth of tomato and showed indirect effect on the length and weight of root and shoot systems, and significantly decreased the nematode population.

The efficiency of antagonistic effect of *bacterial strains*, *B. subtilis, S. marcescens* and *Cyanobacterial strain S. platensis* in reducing of root galls and egg masses (Cao *et al*., 2015; Sharaf *et al.*, 2016b), and number of second juveniles in soil , females and developmental stages (Sharaf *et al* . 2016 a) of root - knot nematode , M. javanica infecting tomato roots have been reported. Some studies used coated crops seeds with bio-control agents like that used in the present study but it is expensive and not commercial option (Askary and Martinelli, 2015; Elhady *et al*., 2017), although it allows targeted delivery and potentially enhances rhizosphere colonization. Microbial seed treatment is used for controlling diseases and insects, and also for managing nematodes (Glare *et al.* 2012; Stevens and Lewis, 2017).

Statistical analysis of the obtained results of the inductive effects of *B. subtilis, S. marcescens* and *S. platensis* on the activity of peroxidase, polyphenoloxidase, superoxide dismutase and catalase in tomato plants, infected with *M. javanica* (Table 4) revealed significant differences between the values of these enzymes in the treated plants and control. Peroxidase enzyme displayed the highest increase percentage (233.76 %) in *B. subtilis* treatment, followed by 229.3 % in *S. marcescens*, and 156.69 % in *S. platensis* treatments compared to 122.29 % in control. The highest percentage for polyphenol oxidase enzyme was 143.84 % in *S. marcescens* treatment, followed by 128.77 % in *S. platensis*, and 156.69 % in *B. subtilis* treatment compared to 49.32 % in control with nematode only. Superoxide dismutase activity was highest *S. marcescens* treatment (620.49 %), followed by *B. subtilis* 246.91 %, and *S. platensis* (160 %) treatment as compared to control 241.98 %. The highest increase in catalase enzyme activity was *B. subtilis* (908.33 %) treatment, followed by *S. marcescens* (716.67 %) and *S. platensis* (625 %) treatment as compared to control (575 %) with nematode only; however the lowest enzyme activities were found in control without nematode as there was no initiation or induction of oxidase enzymes due to lack of infection.

It was noticed that all tested bioagents significantly increased enzyme activities which were toxic for root-knot nematodes, as inducers to activate the resistance of tomato plants against the invading root-knot nematode. In addition, it was found that *S. marcescens* occupied the first rank in inducing plants to face pests, followed by *B. subtilis* and *S. platensis.* Sahebani and Hadavi (2009); Bhattacharyya and Jha (2012); and Elhady *et al*. (2017) obtained similar results for b-aminobutyric acid (BABA), salicylic acid (Hadis *et al*., 2014) and *Pseudomonas fluorescens* on the activity of superoxide dismutase, guaiacol peroxidase, and catalase in tomato roots infected with *M. javanica*. Plant-mediated systemic resistance

against *M. javanica* in tomato by increasing the activity of their scavenging antioxidant enzymes, especially catalase using chemical and biological elicitors was carried out by Fatemeh *et al*. (2014) and against *M. incognita* using ten bacterial strains by Anter *et al*. (2014).

The results of this study suggests that use of chemicals for controlling parasitic nematodes should be replaced by environmental, safe bio-agents like: bacterial strains, *Serratia marcescens* and *B. subtilis* or algal strain, *Spirulina platensis* strain. Additionally, enzyme activities play a vital role as bio-inducers to activate resistance in tomato plants against invading nematodes.

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