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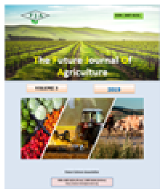

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IMPACT OF PLANT GROWTH PROMOTING RHIZOBACTERIA AS BIO-CONTROL AGENTS ON CITRUS NEMATODE, *TYLENCHULUS SEMIPENETRANS* INFECTING BALADY ORANGE (*CITRUS SINENSIS* L.) AND IMPROVING ITS PRODUCTIVITY

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ABSTRACT: Three bacterial species of plant growth promoting rhizobacteria (PGPR) namely *Paenibacillus polymyxa*, *Methylobacterium mesophilicum* and *Methylobacterium radiotolerans* were tested alone or combined with humic acid as bio-control agents against the citrus nematode *Tylenchulus semipenetrans* under laboratory and field conditions. Results cleared that; all tested PGPR species produced IAA, HCN, ammonia, chitinase and protease enzymes and also solubilized phosphate in laboratory. *P. polymyxa* emphasized the superiority among other species in all PGPR properties except for phosphate solubilization, whereas *M. radiotolerans* showed highest amount of phosphorus solubilized in culture media. On the other hand, the results of the nematode survey conducted on orange, *Citrus sinensis* L. cv Balady grown in different localities of Ismailia and Sharkia Governorates during season 2019, revealed the presence of seven genera and species of plant-parasitic nematodes. Among which, *T. semipenetrans* occurred in all examined samples (100% frequency of occurrence) with a relatively high population density of 2330 and 2640 infected juveniles (J_2) /250 g soil in Ismailia and Sharkia Governorates, respectively. Field experiments were conducted in two different locations, at Ismailia and Sharkia Governorates to assess the effectiveness of PGPR strains alone or combined to reduce the numbers of *T. semipenetrans* during season 2019. It was found that, all treatments caused significant ($P \leq 0.05$) reduction in *T. semipenetrans* population, compared to control treatment. The nematicide, Nemathorin® 10% G followed by *P. polymyxa* (20 L.fed^{-1}) + foliar spraying of *M. mesophilicm* (5 L.fed^{-1}) gave the highest efficacy in controlling the citrus nematode. Percentage reduction in numbers of J_2/250g soil and adult females/1g roots for these treatments in Ismailia Governorate were 91% (85.7%) and 91.4% (89.5%), respectively. While the parallel values in Sharkia Governorate were 90% (87%) and 94% (90%), respectively. The combination of *P. polymyxa* and humic acid (20 L.fed^{-1}) with foliar spraying of *M. mesophilicm* (5 L.fed^{-1}) gained the third position. All treatments increased the fruit yield compared to control treatment. The highest percentages of increase were determined with Nemathorin (160% and 206%) followed by *P. polymyxa* + Humic acid (20 L.fed^{-1}) + foliar spraying of *M. mesophilicum* (155.7% and 193%) and *M. radiotolerans* + *P. polymyxa* + foliar spraying of *M. mesophilicum* (153% and182%) in Ismailia, and Sharkia Governorates respectively.

Key words: Citrus nematodes, *Tylenchulus semipenetrans*, Plant-Growth Promoting Rhizobacteria (PGPR), *Paenibacillus polymyxa*, *Methylobacterium mesophilicum*, *Methylobacterium radiotolerans*, humic acid, biocontrol agents.

INTRODUCTION

Citrus is one of the most important economic cro ps in many mediterranean, and subtropical countries. It is infected by many species of plant-parasitic nematodes that cause damage to the trees i.e., *Tylenchulus semipenetrans*, *Helicotylenchus* spp., *Hoplolaimus* spp., and *Tylenchorhynchus* spp. Among which the citrus nematode, *T. Semipenetrans* has spread throughout the world's citrus-growing regions and causes significant reduction in fruit yield and weight. Yield losses due to *T. semipenetrans* was found to be in the range of 10% to 30%, depending on the level of infestation **(Duncan, 2005)**.

There are different management strategies of plant-parasitic nematodes via resistant cultivars, chemical control, biological control, and other cultural practices (**Abd-Elgawad, 2013**). However, the nematicides have been applied widely to control plant-parasitic nematodes with fast–acting and considerable results but, they are unfriendly methods, costly and produce environmental hazards. For instances, aldicarb residues exceeded the reference doses in orange fruits at Sharkia governorate, Egypt (**Tchounwou *et al*., 2002**). Moreover, growing public concerns about the overuse of synthetic chemical pesticides, have prompted scientists, to concentrate a large number of their studies on the biological control agents as a safe alternative to these chemical substances. For example, the assessment of soil amended with biological control agents and/or compost for control the citrus nematode, *T. semipenetrans* on sour orange **(El-Mohamedy *et al*., 2016**) and on Volkamer lime **(Hammam *et al*. 2016**).

Application of certain soil bacteria as seed or root inoculants to improve plant growth and productivity are known as plant-growth promoting rhizobacteria PGPR **(Khan *et al.*, 2012).** Well known PGPRs include *Bacillus*, *Azospirillium*, *Azotobacter*, *Burkholderia*, *Pseudomonas*, *Rhizobium*, *Serratia* and several novel PGPRs like *Methylobacterium*, *Azoarcus*, *Exiguobacterium*,, *Paenibacillus* and *Pantoea* etc**., (Hemlata *et al.*, 2015).** According to their mode of action they can be classified as biofertilizers and biopesticides **(Martinez et al., 2010).** Thus, PGPR have grabbed the scientists' attention to be used as an alternative eco-friendly biological control agents. PGPR operate through either direct or indirect mechanisms or a combination of both. Direct mechanisms of plant growth promotion include the secretion of plant growth promoting metabolites like indole acetic acid (IAA), cytokinins, gibberellins, etc. and facilitating the uptake of essential nutrients (N, P, Fe, Zn, etc.) from the atmospheric air and soil. Indirect promotion of the plant growth occurs when PGPR lessen or prevent the deleterious effect of phytopathogenic organisms by the production of antibiotics, siderophores, hydrogen cyanide (HCN), etc. **(Ashraf *et al.*, 2013)**. It is also been proven that some strains of these bacteria have main role in reduction of the crop damage caused by nematodes **(Zeinat *et al*., 2010 and Anwar-uI-Haq *et al.*, 2011).**

Paenibacillus polymyxa is an agriculturally important microbe widely studied for its plant growth-promoting and biocontrol abilities *,as* fixing atmospheric nitrogen, solubilizing phosphate, and producing phytohormones **(Padda *et al.*, 2017).**Also, the secondary metabolites of certain *Bacillus* spp. are responsible for their nematocidal activity **(Zhang, *et al*., 2016),** such as production of ammonia and hydrogen cyanide HCN **(Jha *et al*., 2015).**

Methylobacterium spp are known as Pink Pigmented Facultatively Methylotrophic (PPFM) species have benefit effects on plants by several ways, where they have been found in the phyllosphere region and acts synergistically, utilizing methanol from leaves as the sole source of carbon and energy and improves plant growth by production of phytohormones auxins and cytokinins and also produce siderophore which enhanced the plant growth and acted as biocontrol agent **(Omer *et al*. 2004** and **Madhaiyan *et al*.2005)**.

The role of PPFM-produced cytokinin in the stimulation and translocation of minerals and organic compounds in leaves, as well as the potential of plant leaves and roots to utilize siderophores produced by *Methylobacterium mesophilicum* have also been discussed **(Ivanova *et al.*, 2000** and **Orf *et al.*, 2005)**. Methylobacteria are also considered as plant-growth promotion bacteria by several mechanisms include phytohormones production (IAA), nitrogen metabolism and N_2-fixing. Moreover, they play important role as bio-agents by suppressing plant pathogens in the rhizospher via several mechanisms such as ISR, lytic enzymes production, siderophores production and phosphate solubilization activity **(Ardanov *et al*., 2012).**

Humic acid which is known for major organic constituents of humus, has an effect on alleviating damages of plant roots infected with root-knot namatodes **(Kesba and Hossam, 2012).** According to research done by **Kim (2016)**, application of humic acid treatments on infected plant roots with root-knot nematodes can reduce the contents of lipid peroxdaise and H_2O_2 in plant roots by improving the activities of antioxidants enzymes. Thus, humic acid is considered as good resource to strengthen plant roots and inhibit nematode activities at the same time.

The objective of this study is to determine the effect of citrus root inoculation with three species of PGPR as biocontrol agent alone or combined with humic acid on the citrus nematode *T. semipenetrans* performance and reproduction under field conditions.

MATERIALS AND METHODS

Plant growth promoting rhizobacteria (PGPR): Two bacterial species of PPFMs namely *Methylobacterium mesophilicum* and *Methylobacterium radiotolerans* and *Paenibacillus polymyxa* were used as bioagents, were kindly provided from Department of Microbiology, Soils, Water and Environment Research Institute (SWERI), Agricultural Research Center (ARC), Giza, Egypt.

Culture media: Nutrient agar medium was used for cultivation and maintenance of *Paenibacillus polymyxa* **(Dowson, 1957),** whereas, Methanol Mineral Salts (MMS) agar medium was used for cultivation and maintenance of *Methylobacterium* species. **(Holland and Polacco, 1992).**

Organic inoculum: Humic acid was extracted from compost, provided from Department of Microbiology, Soils, Water and Environment Research Institute (SWERI), Agricultural Research Center (ARC), Giza, Egypt, using alkali/acid fractionation procedure as proceeded by **Valdrighi *et al.* (1996).** Some physical, chemical and biological properties of the used compost was presented in (Table 1).

Table 1. Some physical, chemical and biological properties of the used compost

Property	Value
Bulk density (kg/m^3)	4777.2
pH (1:10)	7.53
E.C (dS/m at 25ºC)	4.26
Organic matter %	42.68
Total nitrogen %	1.18
C/N Ratio	20.97
Total phosphorus %	0.49
Total potassium %	1.37
Dehydrogenases (μg TPF/g dry weight/day)	296.53
Seed germination test of cress seeds (%)	93

The compost was soaked in 0.1 N KOH extractant at ratio 1:10 w/v for overnight at room temperature. The insoluble humin fraction was separated by filtration to obtain the filtrate of fulvic and humic acid. The filtrate was then acidified to be pH 2.0 using 6.0 N H_2SO_4 and maintained at room temperature for overnight in order to precipitate humic acid. Finally, humic resuspended and neutralized to pH 6.5 using 0.1 N KOH.

Assayment of PGPR activities *in vitro*

Indole acetic acid production: Qualitative analysis of indole-3-acetic acid (IAA) was performed by the method of described by **Brick *et al*. (1991)**. The tested rhizobacteria were grown on Luria-Bertani agar medium amended with L-tryptophan **(Bakker and Schippers, 1987).**

Hydrogen cyanide production: Luria-Bertani agar medium supplemented with glycine was used to detect the production of HCN by the rhizobacteria under investigation **(Bakker and Schippers, 1987).**

Ammonia formation: Fresh bacterial cultures were tested for the ammonia production by using Nessler's reagent due to **Bakker and Schippers (1987).**

Quantitative estimation of phosphate solubilization: Quantitative measurement of phosphorus released by tested rhizobacteria was done by growing the bacteria in Pikovskaya broth medium **(Pikovskaya, 1948)**, the available phosphorus in medium was measured according to **Watanabe and Oslen (1965).**

Production of chitinase and protease enzymes: Chitinolytic and proteolytic activities of the tested rhizobacteria were indicated by the ability of those bacteria to form clear zone on agar media. Proteolytic agar medium containing 1% glucose and 1% skimmed milk **(Dunne *et al.*, 1997)**, was used for protease production, while chitinolytic agar medium used for chitinase production was done according to **Strzelezyk *et al.* (1990)**.

Soil used: The field experiments were conducted in two different locations, at the experimental farm of El-Kassasin Horticultural Research Station, Ismailia Governorate. ARC, Egypt, and in Arab Company for Agricultural Projects, Wadi El-Molak, Sharkia Governorate, Egypt in season 2019.

The physical, chemical and biological properties of the two samples of soils used were conducted according to **Jackson (1973)** and are shown in Table (2).

Survey study: The present survey was carried out in selected randomly orchards of 10 to 15 years old orange, *Citrus sinensis* L. cv Balady grown in different localities of Ismailia and Sharkia Governorates during the season 2019. Samples were taken from these orchards at different times (February, March, and April). The soil samples were collected to a depth of 20-30cm from the rhizosphere around the feeder roots of the trees. Each sample was made of 5 sub samples which were mixed together to form a composite sample of about 0.5 kg. The collected samples were sent in labeled polyethylene bags to the laboratory and kept in refrigerator at 5 °C until nematode extraction.

Nematode extraction, counting and identification: Each soil sample was carefully mixed and an aliquot sample of 250 g was processed for nematode extraction using sieving and Baermann plates technique according to the method described by **Ayoub (1980)**, and counted under a stereomicroscope**.** Nematodes were identified to the generic level based on the morphology of adult females and juvenile forms according to **Mai and Lyon (1975).** For each genus, population density (P.D.) per 250 g soil and frequency of occurrence% (F.O. %) were calculated according to **Norton (1978**).

Table 2. The physical, chemical and biological properties of the used soils

El-Kassasin, Ismailia Governorate		Wadi El-Molak, Al Sharkia Governorate	
Soil characters	**Value**	**Soil characters**	**Value**
Particle size distribution %:		**Particle size distribution %:**	
Sand	90.51	Sand	90.21
Silt	2.24	Silt	2.14
Clay	7.25	Clay	7.15
Texture grade	Sandy	Texture grade	Sandy
Chemical characters:		**Chemical characters:**	
pH	7.21	pH	7.20
E.C (ds m^{-1} 25ºC)	0.18	E.C (ds m^{-1} 25ºC)	0.18
Soluble cations (meq/L):		**Soluble cations (meq/L):**	
Ca^{++}	0.52	Ca^{++}	0.53
Mg^{++}	0.26	Mg^{++}	0.27
Na^{+}	0.19	Na^{+}	0.19
K^{+}	0.07	K^{+}	0.09
Soluble anions (meq/L):		**Soluble anions (meq/L):**	
CO_3^{--}	--	CO_3^{--}	--
HCO_3^{-}	0.12	HCO_3^{-}	0.13
CL^{-}	0.26	CL^{-}	0.25
SO_4^{--}	0.64	SO_4^{--}	0.66
***Dehydrogenase enzyme**	344.19	***Dehydrogenase enzyme**	334.22

*DHG enzyme activity was expressed as µg TPF/100g dry soil/24h.

Field experiment

Evaluation of PGPR as plant growth promoting and biocontrol agents *in vivo*

Two field experiments were conducted in the experimental farm of El-Kassasin Horticultural Research Station, Ismailia Governorate, and in Arab Company for Agricultural Projects, Wadi El-Molak, Sharkia Governorate during the period from February to November 2019 to determine the effectiveness of biocontrol agents namely *P. polymyxa, M. radiotolerans and M.mesophilicum* against *T. semipenetrans* in Balady orange orchards. The trees were grafted on sour orange rootstock (*Citrus aurantium*), nearly uniform in vigor, size, age (14 years), and spaced at 3 X 7 m apart (175 trees / Fed.). The surface drip irrigation system was used in the two orchards.

The applied treatments were used as follows:

1- Inoculation with *M. radiotolerans* alone at rate of 10L.Fed $^{-1}$(T_1).

2- Inoculation with *P. polymyxa* alone at rate of 10L.Fed $^{-1}$(T_2).

3- Addition of Humic acid alone at rate of 10L.Fed $^{-1}$(T_3).

4- Inoculation with *M. radiotolerans* + *P. polymyxa* (1:1 vol/vol) at rate of 10L.Fed $^{-1}$(T_4).

5- Inoculation with *M. radiotolerans* + Humic acid (1:1 vol/vol) at rate of 10L.Fed $^{-1}$(T_5).

6- Inoculation with *P. polymyxa* + Humic acid (1:1 vol/vol) at rate of 10L.Fed $^{-1}$(T_6).

7- Inoculation with *M. radiotolerans* +*P. polymyxa* + Humic acid (1:1:1 vol/vol) at rate of 10L.Fed $^{-1}$(T_7).

8- Inoculation with *M. radiotolerans* alone at rate of 20L.Fed $^{-1}$(T_8).

9- Inoculation with *P. polymyxa* alone at rate of 20L.Fed $^{-1}$(T_9).

10- Addition of Humic acid alone at rate of 20L.Fed $^{-1}$(T_{10}).

11- Inoculation with *M. radiotolerans* + *P. polymyxa* (1:1 vol/vol) at rate of 20L.Fed $^{-1}$(T_{11}).

12- Inoculation with *M. radiotolerans* + Humic acid at rate of 20L.Fed $^{-1}$(T_{12}).

13- Inoculation with *P. polymyxa* + Humic acid *M. radiotolerans* at rate of 20L.Fed $^{-1}$(T_{13}).

14- Inoculation with *M.radiotolerans* +*P. polymyxa* + Humic acid (1:1:1 vol/vol) at rate of 20L.Fed $^{-1}$(T_{14}).

15-Additon of Nemathorin®10% G at rate of 12.5 kg.Fed^{-1}(T_{15}).

16- Control (left without any amendment or chemical) (T_{16}).

Foliar spraying (F) with *M.mesophilicum* at rate of 5L.Fed^{-1} was applied with all biocontrol agents treatments at flowering stage one time to induce systemic resistance and improve productivity of citrus trees. Before the application of biocontrol agents, the nematode population was assessed. Nematodes were extracted from 250gm soil, according to sieving and modified Baermann technique. Roots were stained with acid fuchsin in lactic acid **(Byrd *et al*., 1983)** and counted for females/1g root. The nematode population was recorded in soil and roots after biocontrol agents were applied. Reduction percentages (Red.%) of number of female /1g root, and second stage juveniles (J_2) in soil were counted in comparison with a control treatment. The bioagents were applied every 15 days at four times, from 1st March to 15th April 2019. The treatments were added individually or combined at two rates, i.e., 10L.Fed^{-1} and 20L.Fed^{-1} for each or mixed treatment. Five trees were treated with the nematicide Nemathorin®10% G at the recommended rate of 12.5 kg.Fed^{-1}. Another five trees were kept as a control (without any treatment or nemiticide). During October 2019, the harvest of each tree was picked separately to obtain the exact number of oranges from each tree individually. The average weight of 10 randomly selected mature fruits has been calculated for each tree (g). Increasing percentages (%) of fruit yield/tree were counted in comparison with a control treatment.

Statistical analysis: All experiments were performed twice in a completely randomized design with 5replicates in each treatment. Data were subjected to analysis of variance (ANOVA) using MSTAT-C program version 2.10 **(Anonymous, 1991)**. Means were compared by Duncan's multiple range test at $P \leq 0.05$ probability **(Duncan,1955).**

RESULTS AND DISCUSSION

In vitro assessment of rhizobacteria as bioagents and growth promoters

Mechanisms of PGPR in nematode suppression

Different mode of actions had exhibited by the rhizobacteria in the rhizosphere to suppress plant parasitic nematodes. The mechanism of nematode suppression can be divided into two categories (i) direct antagonism: such as producing enzymes (chitinase and protease enzymes) and other metabolic products such as HCN and ammonia(.ii)-indirect antagonism :effect either by plant growth promotion *via* production of regulating hormones (IAA) and support the plant with minerals *via* phosphate solubilization or by inducing systemic resistance.

Indole acetic acid formation:

In vitro plant growth promoting hormones IAA produced by tested PGPR is depicted in Table (3), in which all PGPRs studied produced IAA, but they greatly fluctuated in color intensity apparent on L.B. medium amended with tryptophan. The highest color intensity was obtained with *P. polymyxa* as compared to the other two bacteria. In this respect, many investigators evidenced the ability of various PGPRs for producing indole compounds, particularly in the presence of tryptophan as essential for indole secretion **(Ahmed and Hasnain 2010).** In this concern, **Saraf *et al*. (2010)** stated that, Plant growth promotion by *Methylobacterium* include synthesis of the major plant hormones IAA. Also, **Madhaiyan *et al*. (2006)** reported that the *Methylobacterium* inoculation lead to increase IAA accumulation in plants that leads to induce plant growth and development. High proportion of rhizo-microorganisms including *P.polymyxa* are capable to produce plant growth hormone (IAA), which act as root growth stimulator and increase the root branching and surface area as well**(Badawi *et al*., 2011 & El-Sayed *et al*., 2016).**

HCN and ammonia secretion

Also results in Table (3) showed the potential role in production of HCN and ammonia. Therefore, the ability of tested rhizobacteria to produce cyanide and ammonia was done through qualitative screening through color intensity. The visual inspection of the plates appeared that all rhizobacterial assayed has cyanogenic potential as the result of changing the color of indicator paper. However, color intensity was gradually different amongst the tested microorganisms, in which *P. polymyxa* may considered as strong HCN producer followed by *M. radiotoleran*s while, *M.mesophilicum* showed lowest color intensity.

Same trend was observed in the production of ammonia by tested PGPR. *P. polymyxa* had greater ability in ammonia production which scored (+++) followed by *M. radiotolerans* (++) *and M. mesophilicum* (+).

The antagonistic effect of tested PGPR may have occurred through their production of nematoxic chemicals such as HCN and NH_3, which are toxic to nematodes **(Abdel-Baset and El-Egami, 2019).** This is in agreement with **Khan *et al*. (2012) & Siddiqui and Ehtshamul Haque (2001)** who reported that these metabolites may affect egg hatching and nematode parasitism.

Phosphate solubilization

The ability of the tested bacteria used in the study to solubilize phosphate in the cultures is shown in Table (3). The greatest solubilization in culture broth media was induced by *M. radiotolerans* followed by *P. polymyxa*, *and M. mesophilicum* in which the amounts of phosphorus released by *M. radiotolerans, P. polymyxa and M. mesophilicum* were (82.21,77.26 and 73.50), respectively. **Goldstein *et al*. (2003)** proposed direct oxidation of glucose to gluconic acid (GA) as a major mechanism for mineral phosphate solubilization (MPS) in Gram-negative bacteria. *Methylobacterium* spp. is influencing the growth promotion through producing gluconic acid and solubilizing insoluble phosphates, to combat phytopathogens and induce systemic resistance that induce plant growth **(Jha *et al.*, 2010).**

Large amounts of solubilized phosphorus in the soil may have an inhibitory effect on the nematode. **(Khan *et al.*, 2012).** Phosphate –solubilizing microorganisms are the most important and may prove efficient biocontrol agents of plant nematodes. The Phosphate Solubilizing Microorganisms (PSMs) may suppress rhizospheric nematode population by promoting host growth through solubilizing the minerals in soil **(Siddiqui and Shaukat, 2004)**, inducing systemic resistance and/or producing nematoxic metabolites **(Khan *et al.*, 2009).** In this connection, **Pandy *et al.* (2008)** considered *P. polymyxa* as the promoting phosphate- solubilizer inhabiting agricultural soils and possess potential of nematode antagonism.

Table 3. *In vitro* evaluation of the rhizobacteria for exerting plant growth promotion and biocontrol properties.

PGPR-property		***P.polymyxa***	*M.mesophilicum*	*M.radiotolerans*
IAA	Color intensity	+++	+	++
HCN	Color intensity	+++	+	++
Ammonia formation	Color intensity	+++	+	++
Phsphate solubilization	Quantity(ppm)	77.26	73.50	82.21
Chitinase production	Zone diameter(cm)	3.2	1.9	2.5
Protease production	Zone diameter(cm)	3.0	1.5	1.9

+ Low ++ Moderate +++ high

Detection for lytic enzymes formation

Also, data in Table (3) exhibited the ability of tested rhizobacteria to produce lytic enzymes represented in chitinase and protease enzymes. In which all bacteria under investigation have the ability to form clear zone around the grown colonies. Results revealed that *P. polymyxa, M. mesophilicum* and *M. radiotolerans* exhibited a chitinolytic and proteolytic activity. The three tested microorganisms formed clear zone with different diameter 3.2, 1.9 and 2.5 cm for *P. polymyxa*, *M. mesophilicum* and *M. radiotolerans*, respectively. However, *P. polymyxa* showed higher proteolytic activity as exerted 3.0 cm diameter of inhibition, while *M. mesophilicum and M. radiotolerans* formed clear zones with diameter 1.5 and 1.9 cm respectively. The results are in agreement with **Mena and Pimentel (2002)** who reported reduction in the Tricodorid nematode population in potato due to plant treatment with *B. mycoides* and *Pseudomonas* sp. Chitinase also considered as defense enzyme that initiate the induction of resistance by producing phytoalexine and phenolic compounds **(Pokhare *et al.*, 2012, Mhatre *et al.*, 2017 and Viswanathan *et al.*, 2003).**

Also, **Meena *et al*. (2012)** reported that the highest enzymatic activity with lowest nematode population in tomato when treated with PGPR.

Since different structural proteins are present in different life stage of nematodes, where nematode eggs are composed of chitin/ protein complex. Thus, protease enzyme considered as nematicidal activity of biocontrol agents. In which protease can hydrolyses peptide bonds and N-acetyl-D-glucosamine polysaccharide chain, such as those found in nematode eggs composition **(Castaneda-Alvarez & Abaillay, 2016).**

Frequency of occurrence and population density of nematode genera or species infested Balady orange orchards in Ismailia and Sharkia Governorates

The occurrence and population density of plant-parasitic nematodes infested Balady orange orchards in Ismailia and Sharkia Governorates are listed in Table (4).

Data showed that, the highest occurrence was recorded with the citrus nematode, *T. semipenetrans*.Since, the percentage of occurrence of in the surveyed orchards was 100%. While, the second widely distributed genus is the dagger nematode *Xiphinema* spp., with the occurrence of 15% and 7% in Ismailia, and Sharkia Governorates, respectively. In the meantime, the lowest distributed genus in Sharkia Governorate was the stubby root nematode, *Trichodorus* spp., with 2% frequency of occurrence. While, the reniform nematode, *Rotylenchulus reniformis* was the lowest distributed species in Ismailia Governorate, with the occurrence of 3%. On the other hand, a relatively higher value of population density was detected with *T. semipenetrans* in Ismailia and Sharkia Governorates (2380 and 2649 juveniles per 250 g soil, respectively).

Table 4. Population density (P.D.) and frequency of occurrence (F.O.) % of nematode genera and species infected Balady orange in Ismailia and Sharkia Governorates during season, 2019

Nematode genera or species	Ismailia Governorate		Sharkia Governorate	
	P.D.	F.O. (%)	P.D.	F.O. (%)
Helicotylenchus spp.	60	12	40	5
Hoplolaimus spp.	20	7	20	4
T. semipenetrans	2380	100	2640	100
Longidorus spp.	24	10	-	-
Trichodorus spp.	40	6	20	2
Xiphinema spp.	80	15	52	7
Rotylenchulus reniformis	20	3	-	-

Population Density (P.D.) = Total numbers of individuals of each genus per 250g soil/ Number of samples containing this genus.

Frequency of Occurrence % (F.O. %) = Number of samples containing a certain genus/ total number of collecting samples X 100

The survey results confirmed by **Abd-Elgawad (1992), Ibrahim (1994)**, **Radwan and Fatima (2003), and Baklawa (2004)** who mentioned that *T. semipenetrans* was the most common nematode associated with citrus root seedlings and had the highest distribution on collecting samples from several nurseries in Kalubiya, Sharkiya, Ismailia and Behira Governorates in Egypt. Furthermore, **El-Banhawy *et al.* (2006)** reported that citrus nematode, *T. semipenetrans* was recorded throughout, citrus fruit trees in the Nile Delta and Middle Egypt.

Efficacy of bio -agents on *T. semipenetrans* infected Balady orange trees in Ismailia Governorate

Two bio- agents (*M. radiotolerans* and *P.polymyxa*) and humic acid, alone and in combination at two different rates in the presence of foliar spraying with *M. mesophilicum* were studied against citrus nematode, *T. semipenetrans* under field conditions. Data in Table (5) reveled that all treatments caused significant ($P \leq 0.05$) reduction in *T. semipenetrans* population, compared to control treatment. Two months after the fourth application (June 2019) of treatments, the nematicide, Nemathorin® 10% G (T_{15}) recorded the highest percentage of reduction in number of second stage juveniles (J_2) /250g soil (85.0%), and number of adult females/1g roots (89.6%), respectively. The populations of *T. semipenetrans* were obviously decreased with increasing bio-agents rates. The combination treatment of *P. polymyxa*+ Humic acid at the rate 20L.Fed^{-1} in the presence of foliar spraying of *M. mesophilicum* (T_{13}) recorded the highest percentage of reduction in the number of J_2/250g soil (68%).

Meanwhile, the highest percentage of reduction in the number of adult females/1g roots was 88% with the combination treatment of *M. radiotolerans* + *P. polymyxa* at the rate 20L. fed^{-1} in the presence of foliar spraying with *M. mesophilicum* (T_{11}). While, the humic acid at rate of 10 L.fed^{-1}in the presence of foliar spraying (T_3) was relatively the least effective treatment in percentage of reduction in the number of J_2/250g soil and the number of adult females/1g roots with values 34%, and 51.7% respectively.

On the other hand, five months after the fourth application (September, 2019), the nematicide, Nemathorin® 10% G (T_{15}) had the highest efficacy in controlling the citrus nematode population. Since, the highest values of percentage reduction in number of J_2/250g soil and number of adult females/1g roots were 91% and91.4% respectively, followed by the application of *P. polymyxa* at a rate of 20 L. fed^{-1} + foliar spraying of *M. mesophilicm* at rate of 5L. fed^{-1} (T_9) with the parallel values of 85.7%, and 89.5%, respectively. The combination of *P. polymyxa* with humic acid at a rate of 20L.fed^{-1} with foliar spraying of *M. mesophilicm* at a rate of 5L.fed^{-1} (T_{13}) was the third effective treatment in reducing the population of citrus nematode in soil and root with 83%, and 88% respectively.

Table 5. Efficacy of biocontrol agents and humic acid alone and in combination, at two different rates with foliar spraying of *Methylobacterium mesophilicm* against citrus nematode, *T. semipenetrans* on Balady orange trees in Ismailia Governorate under field conditions

Treatments	15th June 2019				15th September 2019			
	Nematode population/ 250g soil	Percent of decrease %	Adult female population/ g of root	Percent of decrease %	Nematode population/ 250g soil	Percent of decrease %	Adult female population/ g of root	Percent of decrease %
T_1) *M. radiotolerans* (R1) + F	1240c	46	20c	58.5	740 cd	73.5	14.6 b	72.7
T_2) *P. polymyxa* (R1) + F	1172.3c	49	16.6c	65.6	680 de	75.7	10.6 de	80
T_3) Humic acid (R1) + F	1500 b	34	23.3b	51.7	1006.6 b	64	15 b	72
T_4) T_1 +T_2 + F	1046.3 d	54.5	16.6 d	65.6	720 cd	74	7.6 fgh	85.8
T_5) T_1 +T_3 + F	1032.3 d	55	14de	71	673.3de	75.9	10.3 ef	80.7
T_6) T_2 + T_3 + F	933.3 ef	59	13ef	73	580 efg	79	9.6 efg	82
T_7) T_1+ T_2+T_3 + F	1020de	55	14.3de	70	800 c	71	14.6 b	72.7
T_8) *M. radiotolerans* (R2) + F	1020 de	55	10.6 fg	78	480 gh	82.8	7 ghi	86.9
T_9) *P. polymyxa* (R2) + F	900f	60.8	9.3gh	80.7	400 h	85.7	5.6 hi	89.5
T_{10}) Humic acid (R2) + F	1212.3c	47	10 d	79	666.6 def	76	13.3 bcd	75
T_{11}) T_8+ T_9 + F	900f	60.8	5.6 ij	88	580 efg	79	14 bc	73.8
T_{12}) T_8+ T_{10} + F	900.3f	60.8	8.3 ghi	82.8	553.3 fg	80	7.3 ghi	86
T_{13}) T_9+ T_{10} + F	726.6g	68	8.6gh	82	466.6 gh	83	6.3 hi	88
T_{14}) T_8+ T_9+T_{10} + F	766.6 g	66.6	7.3 hij	84.8	580 efg	79	11.3 cde	79
T_{15}) Nemathorin® 10% G	346.6 h	85	5 j	89.6	240 i	91	4.6 i	91.4
T_{16}) Control	2300 a	---	48.3 a	--	2800a	--	53.6 a	--

(R1): at rate10L.Fed. $^{-1}$ (R2): at rate20L.Fed. $^{-1}$ (F) Foliar spraying(F) with *M.mesophilicum* at rate 5L.Fed^{-1}

*Different letter(s) indicate significant differences among treatments within the same column according to Duncan's multiple range test ($P \leq 0.05$).

Efficacy of bio- agents on citrus nematode, *T. semipenetrans* infecting Balady orange trees in Sharkia Governorate.

Two bio- agents, namely *M. radiotolerans* and *P.polymyxa* and humic acid, alone and in combination treatments at two different rates in the presence of foliar spraying of *M. mesophilicum* were studied against citrus nematode, *T. semipenetrans* under field conditions. Data in Table (6) reveled that all treatments caused significant ($P \leq 0.05$) reduction in *T. semipenetrans* numbers, compared to control treatment. Two months after the fourth application of these treatments (June, 2019), the nematicide, Nemathorin® 10% G (T_{15}) recorded the highest percentage of reduction in number of J_2/250g soil, and number of adult females/1g roots with values 90%, and 92% respectively. The application of *P. polymyxa* at a rate of 20 L. fed^{-1} + foliar spraying of *M. mesophilicm* at rate of 5L. fed^{-1} (T_9), and the combination of *P. polymyxa*+ Humic acid+ *M. radiotolerans* at the rate 20L.Fed^{-1} in the presence of foliar spraying of *M. Mesophilicum* (T_{14}), recorded the same results in percentage of reduction in the number of J_2/250g soil (76%), and the number of adult females/1g roots was (87%).

Five months after the fourth application (September 2019), Nemathorin® 10% G (T_{15}) showed the highest efficacy in controlling the citrus nematode as indicated by the highest percentage reduction in the number of J_2/250g soil (90%), and the number of adult females/1g roots (94%), followed by the application of The application of *P. polymyxa* at a rate of 20 L. fed^{-1} + foliar spraying of *M. mesophilicm* at rate of 5L. fed^{-1} (T_9)with percentage reduction in the number of J_2/250g soil (87%), and the number of adult females/1g roots (90%). While, the third most effective treatment in percentage reduction in the number of J_2/250g soil, and the number of adult females/1g roots was the combination of *P. polymyxa*+ Humic acid at rate of20 L. fed^{-1} + foliar spraying of *M. mesophilicm* at rate of 5L.fed^{-1} (T_{13}) with values of 85% and 89% respectively.

Table 6. Efficacy of biocontrol agents and humic acid alone and in combination, at two different rates with foliar spraying of *Methylobacterium mesophilicm* against citrus nematode, *T. semipenetrans* on Balady orange trees in Sharkia Governorate under field conditions

Treatments	15th June 2019				15th September 2019			
	Nematode population/ 250g soil	Percent of decrease %	Adult female population/ g of root	Percent of decrease %	Nematode population/ 250g soil	Percent of decrease %	Adult female population/ g of root	Percent of decrease %
T_1) *M. radiotolerans* (R1) + F	1153.3cd	60	22.3b	58	606.6cd	81	14.6b	74
T_2) *P. polymyxa* (R1) + F	1133.3cd	60	20.3b	61	623.3cd	80	10.6cd	81
T_3) Humic acid (R1) + F	1413.3b	51	20.3b	61	1006.6b	68	15b	73
T_4) T_1 +T_2 + F	1153.3cd	60	12.6def	76	720bcd	77	7.6efg	87
T_5) T_1 +T_3 + F	1160cd	60	12efg	77	673.3bcd	79	10.3de	82
T_6) T_2 + T_3 + F	1073.3de	63	15.6cd	70	580cd	82	9.6def	83
T_7) T_1+ T_2+T_3 + F	1220c	57	16.6c	68	800 bc	75	14.6b	74
T_8) *M. radiotolerans* (R2) + F	820gh	71	10.6fgh	80	680bcd	79	10.6cd	81
T_9) *P. polymyxa* (R2) + F	693.3h	76	7ijk	87	400d	87	5.6gh	90
T_{10}) Humic acid (R2) + F	1213.3c	58	15cde	71	530cd	83	13.3bc	76
T_{11}) T_8+ T_9 + F	960 ef	67	9ghi	83	620cd	80	8.6 def	84
T_{12}) T_8+ T_{10} + F	766.6gh	73	9ghi	83	573.3cd	82	7.3fg	87
T_{13}) T_9+ T_{10} + F	833.3fg	71	9.3ghi	82	480cd	85	6.3g	89
T_{14}) T_8+ T_9+T_{10} + F	700 h	76	7ijk	87	480cd	85	7fg	88
T_{15}) Nemathorin® 10% G	286.6i	90	4.3k	92	320e	90	3.3 h	94
T_{16}) Control	2866.6	--	52.6	--	3166.6	--	56.3	--

(R1): at rate10L.Fed. $^{-1}$ (R2): at rate20L.Fed. $^{-1}$ (F) Foliar spraying(F) with *M.mesophilicum* at rate 5L.Fed^{-1}

*Different letter(s) indicate significant differences among treatments within the same column according to Duncan's multiple range test ($P \leq 0.05$).

Management of plant-parasitic nematodes are difficult in particular under field conditions due to the optimal growing conditions in citrus orchards (soil moisture and temperature) most of the year (**Díez and Dusenbery, 1989**). Regardless of their fast- acting, nematicides may be harmful to both human and environment. The bio-agents or soil amendments are alternative methods in controlling plant parasitic nematodes.Since,the results of this study demonstrated a major advanced in controlling of the citrus nematode, *T. semipenetrans* with all the tested treatments under field conditions. It may be due to bacterial products like antibiotics and secondary metabolites such as protease, HCN and chitinase production. The toxic effect of HCN on the nematodes is consistent with the loss of mitochondrial function through the inhibition of cytochrome c oxidase **(Zdor, 2015)**,or the sequestration of Fe from the host cells due to the formation of FeCN **(Rijavec and Lapanje, 2016).**

The current study proved that *P. polymyxa* has the ability to produce chitinase enzyme. The production of lytic enzymes may be playing an important role in the reduction of *T. semipenetrans* populations. Many investigators studied the effect of chitinase production in reducing the nematode population, **Jung *et al*. (2002)** determined that *Paenibacillus illinoisensis* KJA-424 a chitinase-producing bacterium causes *M. incognita* egg shell lysis, particularly in the early juvenile stage, resulting in egg hatching inhibition and/or egg kill. Chitinolytic activity lysis plays a role in regulating root-knot nematode *M. incognita*.

Jha *et al*. (2015) illustrated that *Methylobacterium* induce systemic resistance by production of proteins like phenylalanine ammonia lyase, peroxidase, chitinase and β-1, 3-glucanase and phenolic compounds. On the other hand, the microbial protease may contribute to infection of hosts by degrading the host's protective barriers (**Ahman *et al*., 2002and Huang *et al*., 2004**). It has also been shown that bacterial proteases can degrade and digest nematode cuticle or even kill hosts. The extracellular protease from nematophagous fungi inhibits nematode infection by degrading cuticle proteins (**Morton *et al*., 2004**). The results were further supported by the findings of **Denizci *et al*. (2004)** and **Lian *at al*. (2007**) who reported that antagonism of *Bacillus* species related to the production of protease.

Using low-molecular-weight organic acids is another strategy to manage plant-parasitic nematodes (**Browning *et al*., 2006**). Many organic acids produced during the decomposition of organic matter exhibit nematicidal activity (**Mian & Rodriguez-Kabana 1982and Renco *et al*., 2012**). Humic acid typically contains hetero cyclic compounds with carboxylic, phenol and carbonyl functional groups. Because of the nature of their functional groups, it is possible that humic acids may have lethal effects on nematodes too. Many investigators reported the role of humic acid against root-knot nematode, **Saravanapriya & Subramanian (2007)** reported that soil amended with humic acid significantly decreased the number of galls, and egg masses/plant and soil populations of *M.incognita* in tomato. Similarly, **Seenivasan & Senthilnathan (2017**) showed that hatching of *M. incognita* eggs was inhibited byb50%–100% following incubation in 0.08%-2.0% humic acid *in vitro*. Exposure of juveniles (J_2) of *M. incognita* to different humic acid concentrations significantly affected the mobility of J_2 *in vitro*, also humic acid improves growth of the banana.

In the current study, the combination of *P. polymyxa* with humic acid was more effective in reducing the population of citrus nematode in soil and roots, this may be due to the addition of humic acid to soil stimulates the growth of rhizosphere bacteria and actinomycetes (**Vallini *et al*., 1993).** As well**, Fallah *et al*. (2012)** reported that humic acid improved the growth of *Azotobacter chroococcum* and *A. vinelandii*.

Effect of biocontrol agents and humic acid on Balady orange fruit yield under field conditions in Ismailia and Sharkia Governorates

Effect of the two biocontrol agents, (*M. radiotolerans* + *P. polymyxa*), and humic acid alone, and in combination, at two rates+foliar spraying of *M.mesophilicum* was evaluated on Balady orange fruit yield under field conditions in Ismailai and Sharkia Governorates. Results in Table (7) revealed to all treatments increased the fruit yield compared to control treatment. The nematicide Nemathorin®10% G (T_{15}) gave the highest fruit yield/ tree as well as the average of fruit weight (g), followed by the combined treatment of *P.polymyxa*+ Humic acid at rate of 20L.Fed^{-1}+ foliar spraying of *M.mesophilicum* (T_{13}). The highest percentage increase in fruit yield were (160% and206%), (155.7% and 193%), and (153% and 182%) with Nemathorin® 10% G (T_{15}), treatment *P.polymyxa* + Humic acid at rate of 20L.Fed^{-1} + foliar spraying of of *M. mesophilicum* (T_{13}), treatment and *M. radiotolerans* + *P. polymyxa* + foliar spraying of *M. mesophilicum* treatment (T_{11}), respectively in Ismailai, and Sharkia Governorates respectively.

In this respect **Shehata *et al.* (2006)** reported that, foliar application of PPFM in the presence of specific rhizobial inoculation scored significant increases in economic turnover of chickpea in the range of 21-32% as compared to N-fertilization. **Madhiayan *et al.* (2006)** also stated that, application of 30% of methanol or PPFMs as a foliar spray significantly increased plant height, plant dry weight, leaf area, boll number and boll dry weight leading to an increase of seed cotton yield over control. Foliar applications with 20% of methanol with PPFMs 3or 4 spraying times gave the significantly higher values of cotton growth and yield parameters **(Al-Mohamed *et al*., 2009).**

The benefits of humate application to plants, including increases in soil water retention, growth of beneficial soil micro-organisms (especially if exposed to contaminant toxicity), root respiration enzyme activity, root growth, and plant yield (**Ouni *et al*., 2014**)**.** In addition, humic acid increases root growth up to 53% and facilitates more efficient nutrient absorption, it is obvious that the application of humic acid results in higher root growth and nutrient uptake in grape (**Kesba and Hossam, 2012**).

Table 7. Effect biocontrol agents alone and in combination with humic acid with foliar spraying of *M. mesophilicm* on fruit yield of Balady orange infected with citrus nematode, *T. semipenetrans* under field conditions

Treatments	Fruit yield, in Ismailia Governorate, season 2019			Fruit yield, in Sharkia Governorate, season 2019		
	Number of fruit /tree	Fruit weight (g)	Percent increase in fruit yield%	Number of fruit /tree	Fruit weight (g)	Percent increase in fruit yield%
T_1) *M. radiotolerans* (R1) + F	324.3k	164d	15.5	384.3i	163.3d	49
T_2) *P. polymyxa* (R1) + F	355.6 j	175 abcd	26.7	392.3i	185ab	52
T_3) Humic acid (R1) + F	411.3i	171 abcd	46.5	434.6h	188.3a	69
T_4) T_1 +T_2 + F	481g	166.6 cd	71.4	491g	177.3 abc	91
T_5) T_1 +T_3 + F	442 h	175 abcd	57.5	465.3g	178.3abc	81
T_6) T_2 + T_3 + F	488.6 g	169.6 bcd	74	532f	169.6cd	107
T_7) T_1+ T_2+T_3 + F	526.3 f	171.3 abcd	87.5	536.3f	185.6ab	108
T_8) *M. radiotolerans* (R2) + F	629.3 d	180.3abc	124	652.6d	1843ab	154
T_9) *P. polymyxa* (R2) + F	697 b	180 abc	148	697c	180abc	171
T_{10}) Humic acid (R2) + F	576.3 e	167 cd	105	576.3e	169.3cd	124
T_{11}) T_8+ T_9 + F	711.3 ab	174.3 abcd	153	724.3bc	183abc	182
T_{12}) T_8+ T_{10} + F	659.6 c	172.6 abcd	135	659.6d	172.6bcd	156
T_{13}) T_9+ T_{10} + F	717.6 ab	185a	155.7	754.6b	175.6abcd	193
T_{14}) T_8+ T_9+T_{10} + F	632.6 d	182.3 ab	125	646d	173.6bcd	151
T_{15}) Nemathorin® 10% G	731 a	185 a	160	787.6a	184.3ab	206
T_{16}) Control	280.6 l	137.3 e	--	257.3j	147.3e	--

(R1): at rate10L.Fed. $^{-1}$ (R2): at rate20L.Fed. $^{-1}$ (F) Foliar spraying(F) with *M. mesophilicum* at rate 5L.Fed^{-1}
*Different letter(s) indicate significant differences among treatments within the same column according to Duncan's multiple range test ($P \leq 0.05$).

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