Plant Archives Volume 20 No. 2, 2020 pp. 9555-9560 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

FIELD EVALUATION OF SOME GENETICALLY IMPROVED RHIZOBACTERIA FOR CONTROLLING *MELOIDOGYNE INCOGNITA* INFESTING TOMATO PLANTS

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Abstract

Root knot nematode *Meloidogyne incognita* is one of the major constraints of tomato production worldwide. Chemical nematicides are effective in suppressing root knot nematode disease but due to their adverse effects to human health and environment; biological control using antagonist's microorganisms is gaining popularity as alternative control method. Scientists count on the biotechnological approaches *viz*., protoplast fusion or gene cloning techniques to maximize the biocontrol potency of such bioagents by increasing the production of antibiotics, toxins and enzymes. A field experiment was carried out to evaluate the nematicidal and plant growth promoting potencies of the protoplast fusant strain F71/4 built from A*noxybacillus flavithermus* and *Bacillus pumilus* against *M. incognita* infesting tomato plant cv Alisa by drenching the soil once and twice times with 50ml suspension (2×10^7 cfu/ml) from the two parents singly or combined and the fusant strain F71/4. The nematicide Vydate® was included for comparison. Results revealed that all treatments significantly reduced nematode population and improved tomato plants parameters and yield production with varying degrees. Vydate® was the highly effective in suppressing nematode population. Drenched soil twice was more effective than once. The fusant strain F71/4 showed superiority than all other biopreparations in reducing nematode population. Drenched soil twice with the fusant strain F71/4 found to be comparable to that of Vydate® in suppressing *M. incognita* population. Furthermore, all treatments improved significantly tomato plant growth (plant height, fresh and dry shoot weights). *B. pumilus* twice induce the maximum plant height 94.44%, while the fusant strain F71/4 twice recorded the greater fresh and dry shoot weights and the highly percentage increase in tomato yield production by 67.56, 156.52 and 140% respectively, as compared to control.

Conclusion: Protoplast fusion technique appears to be a useful tool for combining desirable traits and improved the nematicidal and plant growth potencies of such bacterial strains. The fusant strain F71/4 is a good candidate to control *M. incognita* and improve tomato yield production under field conditions.

Key words: *Meloidogyne incognita*, biological control, rhizobacteria, fusant strain, tomato.

Introduction

In Egypt, Tomato (*Solanum lycopersicum* L.) is one of the most popular vegetable crops grown next to potato, is being grown in about 440 million hectares with an annual production of 7.7 million tones. Tomato crop is subjected to many pathogens. Among them, root-knot nematode caused by *Meloidogyne incognita* is the major constraint (Ibrahim, 2011). They cause more than 27% yield losses worldwide and the damage is much higher in seedling stage, light soil and tropical and subtropical climate (Mc Clure, 1977; Kaur *et al.,* 2011; Kiewnick and Sikora 2006; Khan *et al*., 2008). *Meloidogyne incognita* is sedentary endoparasite, once the infective stage (J_2) penetrate the roots migrate to the vascular cylinder, induce severe root galling and affect water and nutrients uptake leading to stunted growth and consequently reduce the market value of the fruits. Moreover, root knot nematodes act as a predisposing agent for the entry of bacteria and fungi resulting in disease complex and leads to resistance break down in infected plants (Jatala *et al.,* 1975; Munif *et al*., 2013).

Traditional nematicides are effective in suppressing root knot nematode but have a negative impact on human health; environment; non-target soil organisms and the ineffectiveness after prolonged use. These prompt nematologists to find efficient safer and ecofriendly

alternative control methods (Talavera *et al*., 2012). Biological control using antagonist's microorganisms *i.e.* fungi and bacteria provides efficient control with no hazard to soil, environment, human health and does not allow the nematodes to develop into new races or biotypes (Noling and Becker, 1994; Weller 1988). Among the biological control agents that have been assessed against plant parasitic nematodes are the antagonistic rhizobacteria *Pseudomonas* spp. *Bacillus* spp. and *Serratia* spp. are the most studied genera. They exhibit a wide range of suppressive activities such as alteration of root exudates and the consequent limitation of nematode penetration in the roots, reduction of juvenile hatching, production of enzymes and toxic compounds and the induction of systemic resistance in the host plant ((Lian *et al*., 2007; Oostendorp and Sikora, 1990). In addition, they promote plant growth by facilitating the uptake of certain nutrients from the environment and release some stimulatory metabolites, such as auxins and gibberellins (Glick, 1995; Gutierrez-Manero 2001).

To maximize the biocontrol potency of such bio agents scientists count on the biotechnological approaches *viz.*, protoplast fusion or gene cloning techniques to combine all the desired properties in one organism or and increase the production of such toxins or enzymes. Yari *et al.,* (2002) found that *Bacillus thuringiensis* spp. (H14) fusants have 1.48 times more δ-endotoxins than their parents. Under greenhouse conditions El-Hamshary *et al*., (2006) found that the fusant strain (Psa::Psf), between *P. fluorescens* and *P. aeruginosa*, proved to be more effective than its parental strain in reducing different nematode parameters as well as enhanced sunflower plant (*Helianthus annus*) growth either as soil drench or seed soaking. Zaied *et al*., 2009 reported that protoplast fusion between *Serratia* and *Pseudomonas* strains produce antibiotic, chitinoltic enzymes, chitinases and bacteriocin more than their parent which resulted in high mortality levels in nematodes if compared with the parental strains. Abdel Salam *et al*., 2018 reported that the fusant from *Bacillus amyloliquefaciens* subsp. plantarum SA5 and *Lysinibacillus sphaericus* Amira strain exhibit highly biocontrol potency against *M. incognita* than their parents by increasing production of chitinase.

In previous study Elkelany 2017 carreid out a protoplast fusion between the thermophilic bacterial strain *Anoxybacillus flavitherma* and *Bacillus pumilus*. From twenty-one fusants the F71/4 isolate showed high nematicidal activity towards *M. incognita in vitro* and in pot experiment.

Considering the potential advantages obtained from the previous study the aim of this work is to verify the highly biocontrol potency of the fusant F71/4 against root knot nematode *M. incognita* infesting tomato *cv* Alisa under field conditions.

Materials and Methods

A field experiment was carried out during tomato growing season to evaluate the efficacy of the biopreparations from *A. flavitherma*, *B. pumilus* and the obtained fusant F71/4 in comparison with the chemical nematicides Vydate® and untreated control treatments against root knot nematode disease in soil naturally infested with *M. incognita* located in Mansouria village, Giza governorate, Egypt. Soil is typically sandyloam. The maximum and minimum temperature during the growing season varied between 20 to 26°C and 33 to 37°C, respectively. A paired-plot design arranged in randomized complete blocks was used. One pair of each plot received one dose from each treatment at planting time and the other treated twice by one-month interval from the first. Plots were divided into rows each of 8m long and 50cm width and 50cm between each plant. One-month-old tomato seedlings (*cv.* Alisa) were transplanted in rows. The plants were subjected to the following treatments by drenching the soil with 50ml contain ~2×10^7 cfu/ml from *A. flavithermus*, *B. pumilus*, *A. flavithermus* +*B.pumilus* fusant isolate F71/4, Vydate® and control untreated. Vydate® was added as recommended for tomato production by the Egyptian Ministry of Agricultural, plant treated with *A. flavithermus* + *B. pumilus* drenched with 25 ml from each, control untreated seedlings received an equal volume of water.

Preparation of bacterial inoculation

Pure culture of each bacterial cells strains table 1 were maintained in Luria-Bertani broth amended with 20% glycerol (Fisher Scientific) and stored at -80C the bacterial cells were streaked onto LB agar and a single colony was inoculated into LB broth (100ml in 250 ml Elementry flask) with constant shaking at 150 rpm for 48hr at 30C. Bacterial cells were suspended in sterilized distilled water and the concentration was ~2x10^7cfu/ml.

Recording Data

Table 1: Bacterial Strains origins and references.

Bacterial isolates	Strains	Origin	Reference
Anoxybacillus flavitherma	BTN7B	Microbial Genetics Dept., NRC	Elkylany 2017
Bacillus pumilus	I1	Microbial Genetics Dept., NRC	Elkylany 2017
Fusant isolate F71/4	F71/4	Plant Pathology Dep. NRC, Egypt	Elkylany 2017

The initial population densities of *M. incognita* were determined prior to planting time from 250 g soil from each treatment according to (Barker 1985). At harvest time three months after transplanting, final count of *M. incognita* J_2 in soil was determined from each treatment as previously described and expressed as J_2 per 250g soil. Numbers of J_2, root galls and eggmasses/roots as well as numbers of eggs/eggmass were recorded and the percentages nematode reduction in soil were determined according to Henderson and Tilton formula (Puntener, 1981).

$$\text{Nematode reduction (\%)} = \left[1-\left(\frac{PTA}{PTB}\times\frac{PCB}{PCA}\right)\right]\times 100$$

Where :

PTA = Population in the treated plot after application,

PTB = Population in the treated plot before application,

PCB = Population in the check plot before application and

PCA = Population in the check plot after application.

Data on tomato plant growth were recorded as percentage increase in plant height, fresh and dry shoot weights and tomato yield production.

Data were subjected to analysis of variance using Assistat program (Snedecor and Cochran, 1980) and means were compared using Multiple Range Test at $P = 0.05$ Duncan's 1955

Results

Field experiment was carried out to evaluate the bio-control efficiency and plant growth promotion of the fusant F71/4 in comparison with their parents *A. flavithermus* and *B. pumilus*singly and or in combination as well as the nematicide Vydate® against root knot nematode *M. incognita* infesting tomato *cv.* Alisa by drenching the soil once and or twice times with 50 ml cell suspension from each strain containing 2×10^7cfu/ml. Results showed that all treatments significantly reduced *M. incognita* population in soil and roots and enhanced plant growth and tomato yield production compared to untreated control table 2. As expected, traditional nematicide Vydate® showed the higher nematicidal actions than the biopreparations. The fusant F71/4 was the most effective biopreparation in suppressing *M. incognita* population and surpass its parents singly or in combination. Drenching soil twice was more effective than once. The efficiency of the fusant F71/4 twice was found to be comparable to that of Vydate®. The recorded percentage reductions in *M. incognita* population were 57.87, 63.43, 70.77, 72.87 and 24.94% reduction in J_2 in soil and roots, galls and eggmasses in roots and eggs/eggmass, respectively, as compared to control table 2. Results showed that *A. flavithermus* was more effective against *M .incognita* larvae than *B. pumilus* while in contrary *B. pumilus* was more effective in reducing root galls and eggmasses than *A. flavithermus* table 2.

Table 1: Effects of the biopreparations from*Anoxybacillusflavithermus, Bacillus pumilus* and the Fusant 71/4 on *Meloidogyne incognita*reproduction on tomato cv.Alisa under field conditions.

Treatments	No. of *M. incognita* larvae /250 g soil			J_2 in roots	% Red.	No. of galls/ /root system	% Red.	No. of eggmasses/ root system	% Red.	Eggs /Egg-mass	% Red.
	Initial population	Final population	% Red.								
Anoxybacillus flavithermus one dose	189ab	404b	36.80	234e	52.72	218b	37.54	191b	39.75	404bc	13.11
*Anoxybacillusflavithermus*two doses	197ab	328f	50.77	212ef	57.17	187cd	46.42	159cd	49.84	395bc	15.05
*Bacillus pumilus*one dose	183bc	398bc	35.70	406b	17.97	201bc	42.41	163c	48.58	424b	8.81
*Bacillus pumilus*Two doses	198a	350e	47.74	262d	47.07	193cd	44.70	149cd	53.00	403bc	13.33
Anoxybacillus flavithermus + *Bacillus pumilus* one dose	182c	390c	36.64	313c	36.76	175d	49.86	146d	53.94	414b	10.96
Anoxybacillus flavithermus + *Bacillus pumilus* two doses	185ab	363d	41.99	289c	41.61	122ef	65.04	78fg	75.39	400bc	13.97
Fusant 71/4 one dose	187ab	291g	53.99	202fg	59.19	141e	59.60	103e	67.51	362d	22.15
Fusant 71/4 two doses	192ab	274h	57.87	181g	63.43	102fg	70.77	86f	72.87	349d	24.94
Vydate one dose	199a	161j	76.08	193fg	61.01	108f	69.05	91ef	71.29	399bc	14.19
Vydate two doses	186ab	98j	84.42	146h	70.50	79g	77.36	69g	78.23	372cd	20.0
Control	191ab	646a	----	495a	----	349a	----	317a	----	465a	----

Each value represents mean of ten replicates. Means followed by the same letter(s) within a column are not significantly ($P \leq 0.05$) different according to Duncan's Multiple range test. No. = Numbers % Red. = % Reduction.

It is evident that all treatments showed significant increase in plant height, fresh and dry shoot weights and drenched soil twice were more valuable than once table 3. Amongst the various treatments evaluated, maximum plant height was observed in plants treated with *B. pumilus* 94.44% followed by fusant F71/4 87.5%, while Vydate® resulted in only 45.83% increase as compared to control. Drenched the soil twice with the fusant F71/4 exhibit the maximum increase in shoot fresh and dry weights 67.56 and 156.52% respectively as compared to control. The improvement in plant growth parameters were more pronounced in the biopreparation treatments than the nematicide.

All the treatments significantly increased the tomato crop yields compared with untreated control. The highest percentage increase (140.81%) was achieved in the soil drenched twice with the fusant F71/4 followed by the combined bioagents twice (97.79%) then *B. pumilus* twice (92.75%) as compared to control table 3.

Discussion

Root- knot nematodes are the most prevalent and very important group of plant parasitic nematodesoccurring all over the world. They reduce the market value of infected plants by depriving their nutrients. As recorded in the present study chemical nematicides are the most effective in suppression nematode population but their persistence poses ecological problems El-Sherbiny *et al*., 2007; Hadad and Al- Hashmi 2012; Khalil *et al*., 2012. Therefore, biological management using antagonistic microorganisms has become one of the most promising alternatives to nematicides.

The present investigation revealed that the rhizobacterial strains A. *flavithermus*, *B. pumilus* and the fusant isolate F71/4 possessed nematicidal activities against *M. incognita* by reducing *M. incognita* J_2, root galls, eggmasses and eggs/eggmass. These results documented the findings of Gokte and Swarup. 1988; Ahmadian 2007; Lee *et al.,* 2016 and El-Nagdi *et al*., 2018 who reported that *B. pumilus* reduced the number of galls and eggs of *M. javanica* on roots by secrete protease and two chitinases which inhibit *M. incognita* egg hatch and destroyed the eggshell also Mercer *et al*., 1992 and Woo-Jin *et al*., 2002 found that chitinase interfered with the hatching of *Meloidogyne* sp. eggs resulting in the early emergence of juveniles that were less able to survive in soil and complete the life cycle and initiate root galls and eggmasses. Moreover, Jeong *et al*., 2014 found that endophyte *Bacillus pumilus* INR7, triggering induced systemic resistance in field crops While *A. flavithermus* is a heat tolerant bacterium and recognized for its capability to function at the extreme environmental condition that grew in the range of 30-70°C and produced a subtilisin-like extracellular protease which directly affect nematodes infective stages and cause significant damage to their cuticle Yadav *et al*., 2018.

Our results consistent with the results obtained by Elkylany 2017 who found that the fusant strain F71/4 was more effective than its parents in controlling root

Table 3: Effects of the biopreparations from*Anoxybacillusflavithermus, Bacillus pumilus* and the Fusant 71/4 on plant growth parameters and yield production of tomato cv. Alisa under field conditions.

Treatments	Plant height	% Incr.	Shoot fresh weight(g)	% Incr.	Shoot dry weight (g)	% Incr.	Yield kg/	% Incr.
Anoxybacillus flavithermus one dose	88f	22.22	143e	28.82	68e	47.82	157.6e	39.22
*Anoxybacillus flavithermus*two doses	93ef	29.16	165bc	48.64	73e	58.69	180.9cd	59.80
*Bacillus pumilus*one dose	121bc	68.05	159cd	30.18	89d	93.47	163.4e	44.34
*Bacillus pumilus*Two doses	140a	94.44	181ab	63.06	115ab	150.0	218.2b	92.75
Anoxybacillus flavithermus + *Bacillus pumilus* one dose	93ef	29.16	155cd	39.63	92cd	100.0	171.5de	51.50
Anoxybacillus flavithermus + *Bacillus pumilus* two doses	105de	45.83	168ab	51.35	102bc	121.73	223.8b	97.79
Fusant 71/4 one dose	119cd	65.27	171ab	54.05	105ab	128.26	189.6c	67.49
Fusant 71/4 two doses	135ab	87.50	186a	67.56	118a	156.52	272.6a	140.81
Vydate one dose	99ef	37.50	149de	34.23	88d	91.30	168.5de	48.85
Vydate two doses	105de	45.83	158cd	42.34	96cd	52.08	211.6b	86.92
Control	72g	----	111f	----	46f	----	113.2f	----

Each value represents mean of ten replicates.

Means followed by the same letter(s) within a column are not significantly ($P \leq 0.05$) different according to Duncan's Multiple range test. No. = Numbers % Inc. = % Increase

knot nematodes and improve plant growth parameters under laboratory and greenhouse evaluations. The fusant gave better results than the parents singly or in combination this related to their capability to produce antibiotic, toxins and enzymes more than the wild types which in turn resulted in high mortality levels in nematodes population. These are in adjustable conformity with the findings of Yari *et al*., 2002 that the protoplast fusion in *Bacillus thuringiensis* spp (H14) produce δ-endotoxins 1.48 times more than their parents and El-Hamshary *et al.*, 2006 that the intrageneric fusants between *Pseudomonas fluorescens* and *P. aeruginosa* were more effective than its parental strains in reducing nematode population and enhancing plant growth also, Zaied *et al*., 2009 reported that the intergeneric fusants between *Serratia* and *Pseudomonas* induced high mortality levels in *M. incognita* and Abdel-Salan *et al.,* 2018 cited that the intergeneric protoplast fusion between *B. amyloliquefaciens* and *L. sphaericus* Amira strain enhanced the production of chitinase which increased the percentage mortality of *M. incognita* J_2.

Furthermore, as shown in table 3 all treatments significantly ($P \leq 0.05$) increased plant growth parameters (plant height, fresh and dry shoot weights) and tomato yield production as compared to control. Drenched soil twice with *B. pumilus* performed the highly increase in plant height, whilst the fusant F71/4 twice exert the highly increase in shoot fresh and dry weights and tomato production. These results are in accordance with the work by Gutierrez-Maneroa *et al*., 2008 who reported that *B. pumilus* can produce auxins and gebbrellin which are responsible in elongation of stem tissues and promote the plant growth. Amar *et al*., 2013; Hafeez *et al*., 2006 suggested that *Bacillus pumilus* produce high amount of IAA, siderophores and solubilised phosphate which increase plant biomass and total N and P in wheat.

In conclusion

Chemical nematicides like Vydate® are efficient in controlling nematodes but their persistence poses ecological problems. Biological control using antagonistic microorganisms is suggested to be a safer and efficient solution. The fusion bacterial strains were more effective in suppressing nematode population and improve plant growth than their parents. The fusant F71/4 from *A. flavitherma* and *B. pumilus*is a good candidate for preparing bioformula due to their ability to form endospores that allow them to survive for extended periods and its capability to function at the extreme environmental condition with secretion of chitinase, protease, auxins and gibberellin as biocontrol and plant growth promoting substances. Improved bacterial strain F71/4 could replace to some extent the chemical nematicides.

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