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Effect of Treatment Time on Biocontrol Efficacy of *Bacillus amyloliquefaciens*, *Lysinibacillus sphaericus* and their Fusants against Root knot Nematode *Meloidogyne incognita* Infecting Tomato Plants

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ABSTRACT

A pot experiment was conducted to evaluate the nematicidal activity of *Bacillus amyloliquefaciens*, *Lysinibacillus sphaericus* and four protoplast fusants (Bas3, 6-2, 8 and 11) as soil treatment with bacterial suspensions at three times (simultaneously, one week before and one week after) against the root knot nematode *Meloidogyne incognita* infecting tomato cv. Super Strain B . Results demonstrated that, all treatments showed significant ($P \leq 0.05$) reduction in root galls and egg masses on root system, as well as, juvenile's numbers in the soil. Treated soil with bacterial suspensions simultaneously and or before nematode inoculation were more effective than after nematode inoculation. The fusants exhibited nematicidal effect more than their wild types. Bas 8 provided the maximum reduction in *M. incognita* J_2 in soil, root galls and eggmasses in root system by 100%, 93.7% and 100%, respectively, when it was applied simultaneously with nematode inoculation and recorded the highest increase in shoot and root lengths and fresh weight by 61.94%, 60% 171.46% and 228%, respectively over control when it was applied one week before nematode inoculation. While Bas 6-2 recorded the maximum increase in shoot dry weight 144.85% over control when it was applied one week before nematode inoculation.

***Key words*:** *Bacillus amyloliquefaciens*, *Lysinibacillus sphaericus,* Protoplast fusants, *Meloidogyne incognita*, Tomato, Biocontrol, Time of treatment

Introduction

Plant parasitic nematodes especially which belong to *Meloidogyne* spp. are recognized worldwide as one of the major damaging of vegetable cultivation mainly in light soil and warm regions. Infected plants showed distinguished root galls formation that affect both water and food absorption (Metwally *et al*., 2015) resulting in stunting, wilting, poor plant growth and significant yield losses. Moreover, they are able to interact with other pathogens to form complex disease syndromes (Agrios, 2005). A number of methods for the management of the root-knot nematode has been tried with different levels of successes such as chemical control, organic amendments, resistant varieties, soil solarization and biological control (Terefe *et al*., 2009). Chemical nematicides are the most effective method but their negative impact on human health and environment as well as ineffectiveness after prolonged use and the recent drive to produce food free from chemicals residues have led to innovate safe and ecofreindly control method. Biological control using antagonistic microorganisms especially rhizosphere bacteria which have been reported to be effective against nematodes reproduction and improve plant growth (Becker *et al*., 1988 and Tian *et al*., 2007) by different properties that act directly against nematodes viability, including toxin production, metabolic by-products, antibiotics, siderophores and production of damaging enzymes and nutrient competition (Dong and Zhang 2006, Padgham and Sikora, 2007). Rhizobacteria stimulate plant growth by production of plant growth hormones, nitrogen fixing ability, enhancing mineral availability in soil (Khan *et al*., 2011). To overcome the inconsistent results of the biological control under field conditions, few studies have been attempted to utilize the protoplast fusion technique as helpful tools for developing more powerful bacterial strains

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that combine all the desired properties in one organism. Yari *et al.,* (2002) reported that the concentration of δ-endotoxin of *B. thuringiensis* fusion was 1.48 times more toxic than the wild type. El-Hamshary *et al.,* (2006) found that, the fusant strain between *Pseudomonas fluorescens* and *P. aeruginosa* was more effective than their parental strains in reducing different nematode parameters as well as enhanced plant growth. Zaied *et al*., (2009) reported that fusants between *Serratia and Pseudomonas* induced high mortality levels against *M. incognita* when compared with the parental strains under laboratory conditions, and all the obtained fusants controlled nematodes and protected cucumber plants from nematode infection causing yield losses. Application time of biocontrol agents is one of the most essential factors influencing the effectiveness of bacterial antagonists to plant parasitic nematodes. Mahdy, (2002) reported that *B. cereus* applied 10 days before nematode inoculation caused significant reductions in root galling and number of galls. Lee and Kim, (2016) found that the application of *B. pumilus* with the same time of nematode inoculation proved to be more effective than application 2 days after inoculation with nematode.

The aim of this study is to evaluate the nematicidal potency of *B. amyloliquefaciens* and *L. sphaericus* and four fusants against *M. incognita* infecting tomato cv. Super Strain B using soil treatment at three times (simultaneously, one week before and one week after under greenhouse conditions.

Materials and Methods

This experiment was carried out to evaluate the different application time of *B. amyloliquefaciens* and *L. sphaericus* as parental strains and four fusants, Bas 3, 6-2, 8 and 11 were prepared according to (Hopwood *et al*., 1981) to affect *M. incognita* reproduction and improve tomato plants growth under greenhouse conditions at three times of soil treatment (simultaneously, one week before - and one week after nematode inoculation). One month-old tomato seedlings cv. Super Strain B were transplanted in 25 cm diam. pot filled with about one kg autoclaved soil 1:1 w/w sandy clay soil. Four days later, soil was treated with 5 ml cell suspension ($2x10^6$ cfu/ml) from the aforementioned bacterial strains and their fusants cultured in Lauria-Bartani (LB) medium (Davis *et al*., 1980) at the times mentioned above. Tomato seedlings were inoculated with 2000 J_2 of *M. incognita* from pure culture pipette in three holes around the roots. Plants received only 2000 J_2 of *M. incognita* in 5ml distilled water /pot was used as the control. Each treatment was replicated four times. Pots were arranged in a randomized complete block design under greenhouse conditions at 28°C±2. All plants were watered after nematode inoculation and thereafter, whenever needed. The experiment was terminated 60 days after nematode inoculation; plants were uprooted and were thoroughly washed free of soil and blotted with tissue paper. Shoot and root fresh weights and lengths, total number of galls and egg masses were recorded. *M. incognita* J_2s were extracted from 200g soil by sieving and decanting techniques and counted under a light microscope. Tomato fresh shoots were kept in oven at 70°C for 24 hours and the dried shoot weight was measured. Percentage decrease or increase in each parameter was calculated with respect to untreated control. The data were subjected to the analysis of variance and means were compared according to Duncan Multiple Range Test.

Results

The nematicidal effect of *B. amyloliquefaciens*, *L. sphaericus* and four fusants viz., Bas 3, 6-2, 8 and 11 on the root knot nematode, *M. incognita* infesting tomato plant cv. Super Strain B as soil drench at three times of application (simultaneously one week before - and one week after nematode inoculation) was recorded in Table 1. The obtained data showed that all treatments had the potentiality to reduce the root–knot nematode infectivity and reproduction, to a great extend as compared to untreated control and significantly $P \leq 0.05$ suppressed J_2 in soil, root galls and egg masses /root system as compared to untreated control. The percentages reduction, however,

varied among treatments according to bacterial strains and fusants and the times of application. The fusants were more effective than its wild types (Table 1). At the end of the experiment, the greatest reduction recorded in J_2s in soil was 100% when the fusants were applied one week before - and/or simultaneously with nematode inoculation. When the fusants Bas 6-2, 8 and 11 were drenched one week after nematode inoculation, the percentages reduction in *M. incognita* J_2 were 86.21, 81.03 and 78.45%, respectively as compared to untreated control. The parents were more effective in reducing *M. incogmita* J_2 in soil when they were applied simultaneously than one week before nematode inoculation (Table 1). The numbers of galls and eggmasses on root system of tomato plants were significantly $P \leq 0.05$ reduced due to all treatments as compared to untreated control (Table 1). Bas 8 proved to be the most effective treatment which minified the root galls by 95.35% when it drenched one week after nematode inoculation as compared to untreated control (Table 1). No eggmasses were detected in plants treated with Bas 3 when it applied one week after - and before nematode inoculation. Bas 6-2 resulted in 100% reduction in eggmasses when it added simultaneously with nematode inoculation and Bas 3 induced 100% reduction in eggmasses when soil was drenched simultaneously and one week before nematode infestation. As shown in Table 1, the parents were more effective in affecting nematode parameters when it was applied simultaneously more than when applied one week before - or after nematode inoculation.

Table 1: Effect of *Bacillus amyloliquefaciens* and *Lysinibacillus sphaericus* parentals and their fusants on *Meloidogyne incognita* reproduction on tomato cv. Super Strain B under greenhouse conditions.

Treatments and time of application	No. of J_2 in 200g soil	% Red.	No. of galls/root system	% Red.	No. of eggmasses/ root system	% Red.
Mi one week before *Ba*	43^{b}*	62.93	21^{bc}	51.16	11^{b}	42.11
Mi simultaneously *Ba*	0^{d}	100	12^{cd}	72.09	2^{cd}	89.47
Mi one week after *Ba*	7^{cd}	93.97	31^{b}	7.91	5c	73.68
Mi one week before *Ls*	20^{cd}	82.76	22^{bc}	48.84	14^{b}	26.32
Mi simultaneously Ls	0^{d}	100	15^{cd}	65.12	4^{cd}	78.95
Mi one week after Ls	6^{cd}	94.83	30^{b}	30.23	11^{b}	42.11
Mi one week before Bas 3	0^{d}	100	9^{de}	79.07	0^{d}	100
Mi simultaneously Bas 3	0^{d}	100	9^{de}	79.07	2^{cd}	89.47
Mi one week after Bas3	0^{d}	100	5^{ef}	88.37	0^{d}	100
Mi one week before Bas 6-2	16^{cd}	86.21	13^{cd}	69.77	2^{cd}	89.47
Mi simultaneously Bas 6-2	0^{d}	100	3^{f}	93.02	0^{d}	100
Mi one week after Bas 6-2	0^{d}	100	13^{cd}	69.77	3^{cd}	84.21
Mi one week before Bas 8	22^{cd}	81.03	17^{cd}	60.47	3^{cd}	84.21
Mi simultaneously with Bas 8	0^{d}	100	7^{de}	83.72	0_{d}	100
Mi one week after Bas 8	0^{d}	100	2^{f}	95.35	0_{d}	100
Mi one week before Bas 11	25^{bc}	78.45	20^{c}	53.49	4^{cd}	78.95
Mi simultaneously Bas 11	0^{d}	100	6^{ef}	86.05	2^{cd}	89.47
Mi one week after Bas 11	0^{d}	100	5^{ef}	88.37	1^{d}	94.74
Control	116^{a}	-------	43^{a}	------	19^{a}	--------

**Means followed by the same letter(s) are not significantly different according to Duncan's Multiple Range Test. Mi= Meloidogyne incognita, Ba= Bacillus amyloliquefaciens, Ls= Lysinibacillus sphaericus, % Red.= % Reduction.*

Data represented in (Table 2) showed the influence of the wild types and their fusants on growth parameters of tomato plant. Data revealed that the most used treatments recorded significant increase in shoot and root lengths and weights when compared to untreated control. The best performing conditions are those when bacteria were added one week before nematode inoculation followed by simultaneously or one week after nematode incoulation. *B. amyloliquefaciens* was more effective than *L. sphaericus* in improving plant growth parameters. Data represented in (Table 2) indicated the positive performance of the fusants in improving

tomato plant growth parameters in the most treatments than its parents. The application of Bas 8 one week before nematode inoculation achieved the great increase in shoot and root lengths and fresh weight by 61.95%, 171.46%, 60% and 228% respectively, as compared to untreated control. The highly increase was (144.85%) over untreated control in shoot dry weight as compared to control was obtained from the treatment of Bas 6-2 one week before nematode inoculation.

Table 2: Effect of *Bacillus amyloliquefaciens* and *Lysinibacillus sphaericus* parental strains and their fusants on plant growth of tomato cv. Super Strain B infected with *Meloidogyne incognita* under greenhouse conditions.

Treatments and times of application	Shoot length (cm)	% Inc.	Shoot fresh weight (g)	% Inc.	Shoot dry weight (g)	% Inc.	Root length (cm)	% Inc.	Root fresh weight (g)	% Inc.
Mi one week before *Ba*	38.00^{bc}*	34.51	6.90^{cd}	74.24	2.09^{a}	26.67	16.25^{bc}	8.33	2.76^{ef}	--
Mi simultaneously *Ba*	37.75^{f}	33.63	4.25^{gh}	7.32	2.59^{ab}	56.97	12.00^{de}	--	2.67^{ef}	--
Mi one week after *Ba*	38.25^{bc}	35.40	7.75^{bc}	95.71	2.89^{ab}	75.15	18.25^{b}	21.67	6.21^{bc}	107
Mi one week before *Ls*	31.25^{de}	10.62	3.90 ^{h}	--	2.15^{c}	30.30	16.00^{bc}	6.67	2.26^{ef}	--
Mi simultaneously Ls	28.00^{f}	--	4.45^{fg}	12.37	1.92^{c}	16.36	13.00^{cd}	--	2.83^{ef}	--
Mi one week after Ls	36.75^{bc}	30.09	6.16^{de}	55.56	2.72^{ab}	64.85	17.00^{bc}	13.33	6.7^{b}	123.33
Mi one week before Bas 3	33.25^{bc}	17.70	6.13^{de}	54.80	3.09^{ab}	87.27	9.00^{f}	--	3.31^{de}	10.33
Mi simultaneously Bas 3	31.5^{cd}	11.5	3.98^{gh}	0.51	2.26^{bc}	36.97	11.25^{ef}	--	1.94^{f}	--
Mi one week after Bas3	38.5^{bc}	36.28	9.06b	128.79	2.18^{c}	32.12	16.50^{bc}	10	4.31^{cd}	43.67
Mi one week before Bas 6-2	38.00^{bc}	34.51	7.10^{cd}	79.29	2.87 ^{a}	73.93	15.25^{bc}	1.67	6.84^{b}	128.00
Mi simultaneously Bas 6-2	40.00^{ab}	41.59	7.52^{bc}	89.90	3.01^{ab}	82.42	15.25^{bc}	1.67	2.53^{ef}	--
Mi one week after Bas 6-2	34.25^{bc}	21.24	7.81^{bc}	97.22	4.04^{ab}	144.85	16.50^{bc}	10	5.1^{bc}	70.00
Mi one week before Bas 8	31.20^{de}	10.44	8.53^{bc}	115.40	2.85^{ab}	72.73	16.25^{bc}	8.33	6.32^{bc}	110.67
Mi simultaneously Bas 8	33.00^{bc}	16.81	5.68^{ef}	47.98	2.82^{ab}	70.91	13.25^{cd}	--	1.92^{f}	--
Mi one week after Bas 8	45.75^{a}	61.95	10.75^{a}	171.46	3.16^{ab}	91.52	24.00^{a}	60	9.84^{a}	228.00
Mi one week before Bas 11	38.00^{bc}	34.51	7.07^{cd}	78.54	2.27^{c}	37.58	16.75^{bc}	11.67	5.78^{bc}	92.67
Mi simultaneously Bas 11	30.75^{ef}	8.85	6.03^{de}	52.27	2.22^{c}	34.55	15.25^{bc}	2.20	2.31^{ef}	--
Mi one week after Bas 11	35.75^{bc}	26.55	8.94^{b}	125.76	3.69^{ab}	123.64	22.00^{a}	46.67	6.17^{bc}	105.67
Untreated control	28.25^{f}		3.96^{gh}		1.65^{c}		15^{bc}		3de	

**Means followed by the same letter(s) are not significantly different according to Duncan's Multiple Range Test. Mi= Meloidogyne incognita, Ba= Bacillus amyloliquefaciens, Ls= Lysinibacillus sphaericus, % Red.= % Reduction.*

Discussion

The rhizosphere supports large microbial populations capable of exerting beneficial, neutral or detrimental effects on plant growth. Rhizobacteria (PGPR) are defined as free-living soil rhizosphere bacteria that, under certain conditions, are beneficial for plants. Several mechanisms are attributed to the suppression of plant parasitic nematodes due to the application of the rhizobacteria *i.e.* production of the antagonistic substances, e.g. hydrogen cyanide, proteases, chitinases, antibiotics and competition for iron and space or indirectly by inducing plant resistance to pathogens and influencing on plant growth directly by fixing the atmospheric nitrogen, solubilizing of minerals such as phosphorus, production of siderophores that dissolve the iron, or producing of plant growth hormones that regulate plant growth at different steps of development (Kamel, 2009). Some rhizobacteria (*Bacillus* spp.) have been found to produce lipopeptides, surfactins, bacillomycin D, and fengycins, which are secondary metabolites mainly with inhabitant pathogen activity (Chen *et al.*, 2006). The present study demonstrated that treatment of *Bacillus amyloliquefaciens* and *Lysinibacillus sphaericus* and their fusants induced 100% reduction in J_2s in soil, when they were applied simultaneously or one week before nematode incoulation, this is in conformity with finding of Burkett- Cadena *et al*., 2008 who found that *B. amyloliquefaciens* FZB42 reduced nematode eggs in roots, juveniles in soil, and plant galls on tomato. Moussa and Zawam, (2010) reported that *Bacillus amyloliquefaciens*, *Brevibacterium otitidis* and *Sanguibacter inulinus* inhibited the egg-masses hatching of *M. incognita in vitro* and exhibited strong nematicidal activity by reducing the second stage juveniles

(J_2s) of nematode in tomato plants under greenhouse conditions. Chowdhury *et al*, (2015) cited that the analysis of the whole *B. amyloliquefaciens* subsp. *plantarum* genome revealed an impressive capability to produce different secondary metabolites suppressing harmful microbes and nematodes inhabiting the plant rhizosphere and enhancing yield of crop plants. Therefore it was used commercially as biofertilizer and biocontrol agent in agriculture. The present results revealed that treatment of rhizobacteria prior to nematode infection gave the best reduction in nematode parameters in agreement with Mahdy, (2002) who found that ten days period was sufficient for the rhizobacteria to establish and produce metabolites that affect nematode infectivity by the induced systemic resistance, as reported by Li *et al.,* (2015) who detected that a production of free salicylic acid (SA) and expression of one pathogenesis-related (PR) gene PR-1 in cucumber leaves were markedly elevated after treating with *B. amyloliquefaciens*, suggesting that SA-mediated defense response was stimulated. Also, Hasky-Gunther, *et al.,* (1998) and Garcia, (2007) refer the suppressing effect of *L. sphaericus* on *M. incognita* infecting tomato to its ability to induce systemic resistance. These may explain the absence of eggmasses in some plants treated with bacterial strains despite the occurrence of root galls i.e. the larvae could not complete its life cycle due the defense response from the bacterial metabolites. Data in Table 2 showed the potentiality of the evaluated the wild types and fusants suspensions to increase tomato plant growth parameters. It is well known that rhizobacteria that colonize plant roots like *B. amyloliquefaciens* and *L. sphaericus* promote plant growth directly or indirectly, as reported by (Maneu, 2015). *B. amyloliquefaciens* can synthesize plant growth promoting substances such as gibberellins and indole-acetic acid resulted in improving plant growth. Idriss *et al*., (2002) suggested that the extracellular phytase activity of *B. amyloliquefaciens* stimulate the plant growth parameters in tomato and resulted in increased yield which are in accordance with our resulting where *B. amyloliquefaciens* treatments were more potent in improving tomato plant growth than *L. sphaericus* treatments. Moreover, Zakry *et al.,* (2012) found that inoculation of young immature oil palm with *B. sphaericus* UPMB-10 significantly increased the N and dry matter yields of the palm leaflets and rachis which could be a new and important source of nitrogen biofertilizer. Our results in Tables 1 and 2 demonstrated that, the fusants were more effective than their parents in suppressing nematode reproduction and improving plant growth parameters, similar results were obtained by Yari *et al.,* (2002), El-Hamshary *et al.,* (2006) and Zaied *et al*., (2009) who indicated that protoplast fusion technique is a promising biotechnological approach to improve the potentiality of bacterial strains to control plant parasitic nematode and improve plant growth by increasing production of toxic compounds and plant growth hormones which provide an effective policy for the biological control of nematodes and produce food free from chemicals residues.

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